A genetic approach to prenatal glucocorticoid programming

Christian Topp Abrahamsen

Doctor of Philosophy

The University of Edinburgh

2007

Declaration

I hereby declare that this thesis has been composed entirely by myself and that the work presented here is the result of my own independent investigation, except where otherwise acknowledged in the text. This work has not been submitted for any other degree or professional qualification.

Christian Abrahamsen

Acknowledgements

A great number of individuals have provided their expert assistance during the course of my studies and I will be forever indebted to them. Firstly, I would like to thank my supervisors Megan Holmes and Chris Kenyon for their patience, guidance and encouragement during the last three and a half years. Furthermore, I express my gratitude to Jonathan Seckl and members of the Endocrinology Unit for their instruction, advice and support which has been instrumental in successful experimental design and implementation. Individuals are too many to name, but include: Roderick Carter, Karen French, Atsuko Kimura, Dawn Livingstone, Janet Man, Nicholas Morton, Julie Nixon, Rachel McDonnell, Lynne Ramage, Paula Stevenson and Joyce Yau. I acknowledge the staff at the BRFs including Keith Chalmers, William Mungal, Sharon Rossiter and Laraine Wells and their proficient technical skill and effort. I would also like to thank the programming group (Annick DeVries, Amanda Drake, Rebecca Reynolds, Moffat Nyirenda and more recently Caitlin Wyrwoll) for their often stimulating and enthusiastic discussions surrounding the phenomenon. Additionally, I extend my thanks to those whose friendship I have gained during my time in Edinburgh and acknowledge the game of golf, which has provided me with indispensable time away from the rigours of PhD science, taken me to the far reaches of Scotland and enabled me to fall in love with the country and its people.

Finally, I wish to express my sincere gratitude to Anne and Kaj Abrahamsen and Jane Pendry, whose unconditional love and support have helped me to persevere through the emotional roller-coaster that is academic research.

Support for this thesis was obtained through a Medical Research Council (MRC) PhD studentship.

Abstract

Overwhelming evidence from clinical epidemiology and experimental studies indicates that the prenatal environment is fundamental in determining developmental trajectories and lifelong physiology. In particular, the *in utero* glucocorticoid (GC) milieu has been identified as a key regulator of organ and system development. Excess GC exposure during this period can have detrimental effects in offspring and alter the sensitivity to later pathophysiology, including psychological disorders, metabolic syndrome, type 2 diabetes, obesity and hypertension, a phenomenon described as 'programming'. 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) rapidly inactivates GCs within the placenta and other fetal tissues, hence serving as a 'barrier' to limit exposure of the fetus to maternal GCs, ensuring an appropriate fetal milieu for normal development.

The synthetic GC, dexamethasone (DEX) is thought to bypass the 11β-HSD2 'barrier' due to poor substrate specificity. Prenatal administration in rats has been shown to programme low birth weight and anxiety, associated with hypothalamic pituitary adrenal (HPA) axis hyperactivity and the decreased hippocampal gene expression of corticosteroid receptors, an effect thought to underlie the observed behavioural phenotype. Studies described here extend previous findings and show that prenatal DEX programmes stress-potentiatiated anxiety.

Similar to most paradigms investigating prenatal GC programming, a significant limitation of our rodent model is the unavoidable maternal manipulation. In contrast, we have used a second model of GC programming which involves crossing mice heterozygous for 11β-HSD2-null allele, generating offspring of three genotypes in the same litter (11β-HSD2+/+, +/- and -/-), allowing direct study of excessive fetal exposure to GCs, independent of altered maternal physiology and/or behaviour, thus providing a unique programming model. In our studies, 11β-HSD2 null mutants displayed reduced birthweight, increased anxiety-related behaviour and mild cognitive deficits, despite normal circulating

corticosterone and limbic expression of HPA axis-associated genes. However, dysregulated expression of these candidate genes during the early postnatal period was evident, indicative of an altered developmental trajectory. In addition to programmed effects on the brain and behaviour, 11β-HSD2 null mice exhibit reduced fat deposition, acute weight maintenance deficits and spontaneous hypoactivity despite evidence of normal glucose homeostasis, including normal glucose tolerance and the unaltered hepatic mRNA expression and activity of phospholenolpyruvate carboxykinase. These metabolic findings emphasize the crucial interactions of early-life programming effects of GCs with the adult GC environment.

In conclusion, we have identified 11β-HSD2 knockout mice as a valuable tool with which to elucidate enzyme function and highlight its importance as a feto-placental 'barrier' to GCs, despite the confounding influence of altered GC metabolism and enzyme deficiency from mineralocorticoid target tissues. Our novel findings extend knowledge of prenatal GC programming, a rapidly developing axiom.

Contents

List of figures

CHAPTER 1 - INTRODUCTION

CHAPTER 2 - MATERIALS AND METHODS

CHAPTER 3 - PRENATAL DEX EXPOSURE EFFECTS ON THE HPA AXIS, BRAIN AND ANXIETY-RELATED BEHAVIOUR

CHAPTER 4 - COGNITIVE AND ANXIETY-RELATED BEHAVIOUR IN HETEROZYGOUS-BRED 11β-HSD2 KNOCKOUT MICE

CHAPTER 5 - HPA AXIS IN 11β-HSD2 KNOCKOUT MICE IN ADULTHOOD AND THE PRE-WEANING PERIOD

CHAPTER 6 - METABOLIC PHENOTYPE IN HETEROZYGOUS-BRED 11β-HSD2 KNOCKOUT MICE

CHAPTER 7 - GENERAL DISCUSSION

List of Tables

CHAPTER 1 - INTRODUCTION

CHAPTER 2 - MATERIALS AND METHODS

CHAPTER 3 - PRENATAL DEX EXPOSURE EFFECTS ON THE HPA AXIS, BRAIN AND ANXIETY-RELATED BEHAVIOUR

CHAPTER 4 - COGNITIVE AND ANXIETY-RELATED BEHAVIOUR IN HETEROZYGOUS-BRED 11β-HSD2 KNOCKOUT MICE

CHAPTER 5 - HPA AXIS IN 11β-HSD2 KNOCKOUT MICE IN ADULTHOOD AND THE PRE-WEANING PERIOD

CHAPTER 6 - METABOLIC PHENOTYPE IN HETEROZYGOUS-BRED 11β-HSD2 KNOCKOUT MICE

CHAPTER 7 - DISCUSSION

List of Abbreviations

11-DHC	11-Dehydrocorticosterone
11β-HSD(1/2)	11β-Hydroxysteroid Dehydrogenase (type 1 or 2)
5-HIAA	5-Hydroxyindoleacetic Acid
5-HT	5-Hydroxytryptamine (Serotonin)
ACh	Acetylcholine
ACTH	Adrenocorticotropic Hormone
AME	Apparent Mineralocorticoid Excess
ANOVA	Analysis of Variance
aP2	Adipocyte Fatty Acid Binding Protein
AVP	Arginine Vasopressin
BDNF	Brain-Derived Neurotrophic Factor
BET	Betamethasone
BlA	Basolateral Amygdala
BP	Blood Pressure
BRF	Biological Research Facility
BSA	Bovine Serum Albumin
CBG	Corticosteroid-binding Globulin
CBX	Carbenoxolone
CeA	Central Amygdala
CORT	Corticosterone
Cp	Crossing Point
CRH	Corticotrophin Releasing Hormone
DA	Dopamine
DEPC	Diethylpyrocarbonate
DEX	Dexamethasone
dGDP	Deoxyguanosine Diphosphate
DNA	Deoxyribonucleic Acid

DTT	Dithiothreitol
E	Embryonic Day
EDTA	Ethylenediaminetetraacetic Acid
EPM	Elevated Plus Maze
Fkbp5	FK506 binding protein 51
GC	Glucocorticoid
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HFD	High-Fat Diet
HPA	Hypothalamic-Pituitary Adrenal
icv	Intracerebroventricular
IGF1	Insulin-like Growth Factor 1
IUGR	Intrauterine Growth Restriction
LF	Little France
LI	Latent Inhibition
MeA	Medial Amygdala
MOPS	3-(N-morpholino)propanesulfonic Acid
MR	Mineralocorticoid Receptor
NA	Noradrenaline
NADH	Reduced Nicotinamide Adenine Dinucleotide
NMDA	*N*-methyl-D-aspartic Acid
OF	Open Field
P	Postnatal Day
PA	Passive Avoidance
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PEPCK	Phosphoenolpyruvate Carboxykinase
POMC	Proopiomelanocortin

PPI	Prepulse Inhibition
PS	Prenatal Stress
PVN	Paraventricular Nucleus
RAAS	Renin Angiotensin Aldosterone System
RM	Repeated Measures
RNA	Ribonucleic Acid
Rnasin	Rnase Inhibitor
SDS	Sodium Dodecyl Sulfate
Sgk1	Serum/Glucocorticoid-Regulated Kinase 1
SNK	Student-Newman Keuls
SNS	Sympathetic Nervous System
SPA	Scintillation Proximity Assay
SSC	Saline Sodium Citrate
SSRI	Selective Serotonin Reuptake Inhibitor
TE	Tris EDTA Buffer
VEH	Vehicle
WGH	Western General Hospital

CHAPTER 1
INTRODUCTION

During the last few decades, substantial evidence has emerged from both clinical and experimental studies that the fetal and neonatal environments can have a significant impact upon lifelong physiology. The glucocorticoid (GC) stress hormones play an important role in developmental maturation and under normal conditions are tightly regulated. Overexposure to GCs both *in utero* and during the neonatal period may alter the ontogenetic trajectory with lifelong effects on offspring physiology. In this chapter we shall start by introducing GCs: pre-receptor metabolism, receptor-mediated effects and overall functions. We will then describe the phenomenon of prenatal programming, the involvement of GCs and animal modelling through maternal manipulation.

1.1 Stress and glucocorticoids

It is of primary importance that an organism is able to mount an appropriate response to a changing external or physiological environment. The mechanism by which this occurs has been referred to as allostasis, defined as the achievement of stability through change (McEwen, 1998; McEwen and Wingfield, 2003). A central feature in allostasis is the classically termed stress response, which involves activation of both the sympathetic nervous system (SNS) and the hypothalamic pituitary adrenal (HPA) axis. These play integral roles in the adaptive restoration of homeostasis. SNS activation and subsequent release of catecholamines mediates many of the rapid 'fight or flight' responses to noxious stimuli including increased cardiovascular tone, mobilisation of energy stores and immune activation. GCs are released as the end result of HPA axis activation and play a lead role in the management of whole body physiology, through a diverse set of actions on a wide variety of target organs and cells. These hormones are involved in the regulation of developmental, metabolic, immunological, reproductive, cardiovascular, cognitive and emotional functions by affecting cells in the liver, kidney, thymus, gonads, blood, vasculature, heart and brain.

The nature of these GC actions can be several. Permissive actions are present before the appearance of stressful stimuli and prime the organism to respond. Stimulatory and suppressive actions occur following stress exposure and mediate the response to stimuli, but also dampen the extended response to prevent over-shooting. Preparative actions do

not participate in an acute stress response, but modulate the response to future stressors, and may be mediating or suppressive in character (Sapolsky et al., 2000). It is the integration and symphony of these actions that permit an organism to mount an appropriate reaction to, sufficiently recover from, and crucially prepare itself for any further insult, thereby facilitating homeostasis. Whilst allostasis is a critical physiological mechanism to appropriately respond to acute stimuli, when insults are excessive in intensity, duration and/or frequency, an allostatic overload is placed on the body. Under these circumstances the adaptive response may be detrimental to homeostasis and predispose an individual to pathophysiology (McEwen, 2004), through dysregulation of the HPA axis.

1.2 The hypothalamic pituitary adrenal (HPA) axis

GCs are the final effectors of the hypothalamic pituitary adrenal (HPA) axis which is a highly auto-regulated system mediating the normal diurnal rhymicity and stress-induced elevations (Figure 1.1). The paraventricular nucleus (PVN) integrates signals from extrahypothalamic sites to synthesise corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) in parvocellular neurones. These peptides travel along axon projections to the external layer of the median eminence where they are released into the hypophyseal circulation. Following travel to the anterior lobe of the pituitary the peptides bind to the CRH_1 and V_{1b} receptors on corticotrophs, respectively. CRH and AVP act synergistically to stimulate the proteolytic cleavage of proopiomelanocortin (POMC) into adrenocorticotropic hormone (ACTH) which is then released into the general circulation. ACTH binds to receptors located in the zona fasiculata of the adrenal cortex to stimulate the secretion of species-specific GCs (cortisol in humans and primates; corticosterone (CORT) in rodents). The HPA axis provides an elegant example of an intricately regulated negative feedback system. GCs released into the bloodstream bind to corticosteroid receptors within the PVN and pituitary to downregulate further synthesis and release of CRH and ACTH. The hippocampus, due to abundant corticosteroid receptor expression provides extrahypothalamic negative feedback to the PVN. Additionally, ACTH receptors are also present in the PVN, which bind the hormone ligand to further downregulate release of CRH and AVP

(reviewed in Aguilera, 1994). In addition to their role as HPA axis secretagogues, CRH and AVP can act as extrahypothalamic neuropeptides where they modulate a wide array of behavioural responses to stress (reviewed in Carrasco and Van de Kar, 2003).

1.3 Corticosteroid receptors

GCs mediate the majority of actions through their binding to two intracellular receptors. The type I, or mineralocorticoid receptor (MR) binds both aldosterone and the species-specific corticosteroid with similar affinity (Kd ≈ 0.5nM). The type II, or glucocorticoid receptor (GR) binds corticosteroids although with 10-fold lower affinity than MR (Kd ≈ 5.0 nM), although aldosterone is not a natural ligand for the GR (reviewed in Meijer and de Kloet, 1998). This subtype dichotomy in binding affinity is of particular importance in GC action under different physiological conditions. Under normal conditions with low basal circulating corticosteroid levels the higher affinity MR is partially ligand-bound and activated, whilst the low affinity GR is largely unoccupied. However, in situations of elevated GC levels seen at the diurnal peak and during stress GR will be occupied and activated, in addition to MR. The MR is therefore thought to be responsible for the 'tonic' corticosteroid effects, akin to the permissive actions previously described. GR appears to be more involved in mediating the 'phasic' activity of corticosteroids, similar to the stimulatory, suppressive and preparatory actions. The relative distribution of the two receptor subtypes within individual cells is an important determinant of the net result of GC action, an integral feature of GC hormone function (de Kloet et al., 1998).

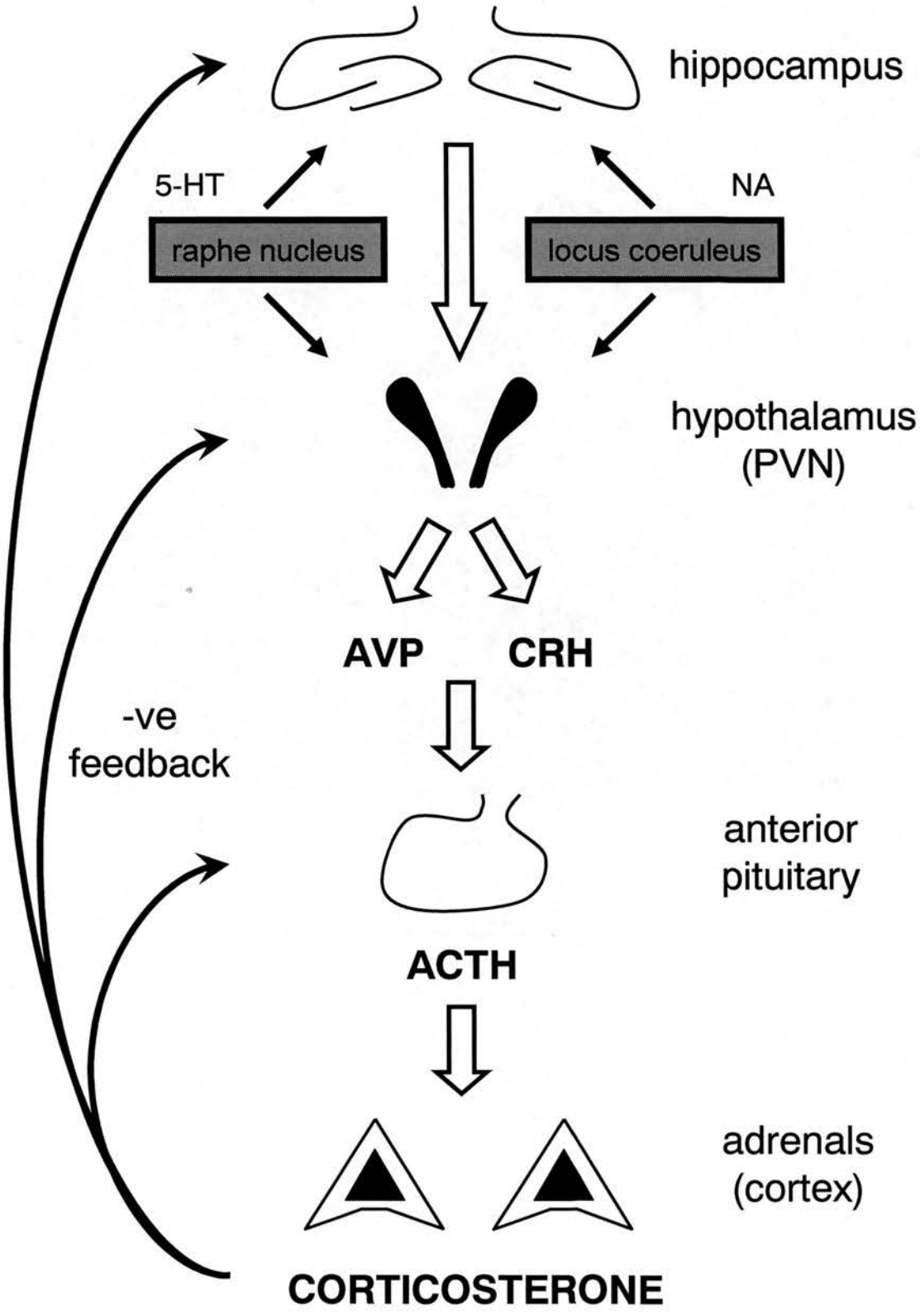


Figure 1.1 The HPA axis in rodents
5-HT = serotonin; NA = noradrenaline; PVN = paraventricular nucleus; CRH = corticotrophin releasing hormone; AVP = arginine vasopressin; ACTH = adrenocorticotrophic hormone

The corticosteroid receptors belong to a family of nuclear steroid hormone receptors which predominantly function as ligand-activated transcription factors. In the absence of ligand, these proteins are localised to the cytoplasm complexed with chaperones, including the heat shock protein 90 (Hsp90) and FK506 binding protein 51 (Fkbp5) (reviewed in Pratt and Toft, 1997). Ligand binding precipitates complex dissociation and translocation of the bound receptor to the nucleus. Following dimerization the receptor-ligand complex attaches to palindromic DNA sequences known as glucocorticoid response elements (GREs) located in the regulatory regions of target genes. Depending on the specific gene, binding to the GRE enhances or represses transcription (reviewed in Schoneveld et al., 2004). Another molecular mechanism of GR-mediated transcriptional control has been identified. Activation of GR has been observed to initiate protein-protein interactions with a number of other transcription factors, including nuclear factor-kappaB, activator protein-1 (reviewed in de Bosscher et al., 2003) and Stat5 (Stocklin et al., 1996). No direct protein-protein interactions have thus far been observed between the MR and other transcription factors. In addition to the influence of GCs on transcriptional activity, non-genomic actions have also been reported which can modulate ion permeability, as well as the release of neurohormones and neurotransmitters (reviewed in McEwen, 1991). This 'fast' feedback was initially identified in the pituitary and hypothalamus (Jones et al., 1977) and evidence suggests that these GC effects may be mediated by membrane-bound corticosteroid receptors (reviewed in Dallman, 2005). It has become clear from increasing evidence that complex reciprocal interactions exist between GCs and neurotransmitter systems including the dopaminergic, noradrenergic and serotonergic systems (reviewed in Chaouloff, 2000; Carrasco and Van de Kar, 2003; Van Craenenbroeck et al., 2005).

1.3.1 Corticosteroid receptor expression

The GR is ubiquitously expressed throughout peripheral tissues and the brain. In contrast, the MR has a more limited distribution. Peripherally, the MR has been localised to epithelial tissue including the kidney (Funder et al., 1972; Farman et al., 1991), colon (Pressley and Funder, 1975; Rafestin-Oblin et al., 1984), lung (Hirasawa et al., 1997) and skin (Kenouch et al., 1994), mineralocorticoid target tissues protected by virtue of pre-receptor inactivation of GCs (reviewed in Funder, 2005). Expression of

MR is also evident in non-epithelial tissues including the heart (Pearce and Funder, 1987; Lombes et al., 1995), vasculature (Lombes et al., 1992), mononuclear lymphocytes (Armanini et al., 1985) and the brain. In the brain, MR expression is restricted to limbic regions and discrete areas involved in salt appetite and water balance (Ahima et al., 1991; Herman, 1993; Pietranera et al., 2001). MR and GR are abundantly expressed in the hippocampus in all species studied including the rat (Herman et al., 1989; van Eekelen et al., 1988), mouse (Patacchioli et al., 1990; Kretz et al., 2001), hamster (Sutanto et al., 1988), tree shew (Meyer et al., 1998), non-human primate (Johnson et al., 1996; Patel et al., 2000; Pryce et al., 2005) and human (Seckl et al., 1991). In this structure, the receptors play a role in the modulation of neuronal excitability, the HPA axis, memory and emotion (reviewed in de Kloet et al., 2005). Genetic polymorphisms of GR have been identified in the human population. These have been associated with altered GC sensitivity and changes in body composition and metabolic parameters, but also cognition and the development of depression (DeRijk et al., 2002; Bray and Cotton, 2003; van Rossum and Lamberts, 2004; 2006). Similarly, human polymorphisms of MR been linked to altered stress responsiveness and hypertension (DeRijk et al., 2006). As a consequence of the ubiquitous expression of the corticosteroid receptors, circulating GCs can have a widespread effect on whole body physiology highlighted by the pathophysiology as a result of these polymorphisms.

1.3.2 Corticosteroid receptor mutant and transgenic studies

1.3.2.1 Glucocorticoid receptor

The generation of transgenic mice with global, regional or tissue-specific modulation of functional corticosteroid receptor expression has permitted more intricate analysis of their subtype-specific roles (reviewed in Wintermantel et al., 2005). Mice lacking functional GR expression died shortly after birth from respiratory failure (Cole et al., 1995; Finotto et al., 1999). These newborn mice had a reduced capacity to upregulate gluconeogenic enzymes in the liver and displayed adrenal hypertrophy, elevated CORT and ACTH, presumably due to impaired central feedback (Cole et al., 1995). Mice with the disrupted ability to dimerise GR (GR^{dim}) displayed full survival indicating critical GC actions in the lung are mediated independently of GRE binding (Reichardt et al.,

1998a; 1998b). In adulthood, GR^{dim} were also impaired in hepatic gluconeogenic enzyme induction, suggesting that dimerisation is however involved in GR signalling within the liver. GR function within the liver was investigated in transgenic mice with a hepatocyte-specific GR mutation and found them to be growth retarded at 4 weeks of age (Tronche et al., 2004). The mice had normal glucose levels although were hypoglycaemic following starvation as a result of impaired phosphoenolpyruvate carboxykinase (PEPCK) induction. In addition, hyperglycaemia resulting from streptozocin-induced diabetes was prevented in these mutant mice (Opherk et al., 2004). These studies indicate a key role for GR-mediated glucose homeostasis within the liver through induction of PEPCK and gluconeogenesis.

Transgenic mice expressing GR antisense were found to have significantly attenuated central GR signalling (Pepin et al., 1992). These mice have been well-studied with consequences of reduced GR signalling observed in their HPA axis activity, behaviour and energy balance (Muller et al., 2002; Barden, 2004). In brief, GR antisense-expressing mice displayed a reduction in negative feedback and HPA axis hyperresponsiveness (Stec et al., 1994; Montkowski et al., 1995; Barden et al., 1997; Karanth et al., 1997), decreased anxiety-related behaviour (Strohle et al., 1998) and cognitive deficits (Montkowski et al., 1995; Steckler et al., 1999) in association with reduced long-term potentiation (Steckler et al., 2001). Of interest was the observation that despite elevated CORT levels the GR antisense-expressing mice were found to be less anxious. Similar results were observed following CNS-specific GR deletion, which resulted in elevated HPA axis activity, reduced anxiety and altered fat deposition similar to that observed in the human Cushing's syndrome (Tronche et al., 1999). Time-specific disruption of forebrain GR in early adulthood exacerbated HPA axis hyperactivity and impaired feedback, and decreased anxiety- and depressive-like behaviours (Boyle et al., 2005; 2006).

Ubiquitous GR overexpression lead to a reduction of basal CORT levels and a blunted stress response, in addition to a suppression of inflammatory actions (Reichardt et al., 2000). Forebrain-specific GR overexpression resulted in heightened anxiety and depressive-like behaviours, despite normal basal and stress HPA axis activity (Wei et

al., 2004). The studies in GR transgenic mice provide evidence that central GR activation is a key mediator of anxiety and depression, whilst GR-mediated hippocampal signalling is an important feature of HPA axis negative feedback.

1.3.2.2 Mineralocorticoid receptor

Mutant mice null for MR die shortly after birth with symptoms resembling pseudoaldosteronism (Berger et al., 1998), although they could be rescued with salt administration (Bleich et al., 1999; Berger et al., 2000) and GC-mediated GR activation (Schulz-Baldes et al., 2001). The salt-rescued MR null mice displayed an upregulation in the HPA axis activity and initial experiments suggested the animals displayed increased anxiety (Gass et al., 2001). Forebrain-specific MR inactivation resulted in impaired learning in the water maze and hyperactivity towards a novel object, but no change in anxiety-like behaviour. Hippocampal GR mRNA was elevated in these mice, but basal and stress CORT levels were normal (Berger et al., 2006). Findings in these animals further support the importance of MR in HPA axis regulation, and anxiety-related and cognitive behaviour.

Transgenic mice overexpressing human MR exhibited abnormal morphology in the kidney and heart, despite normal blood pressure (Le Menuet et al., 2001). These mice were also found to display abnormalities in the electrolyte balance following sodium and potassium-modified diet administration (Le Menuet et al., 2004). Cardiac overexpression of MR resulted in ion channel remodelling, the precipitation of ventricular arrhythmias and sudden death, which could be blocked by the MR antagonist spironolactone (Ouvrard-Pascaud et al., 2005). Conditional antisense-mediated knockdown of MR within the heart also produced detrimental effects, precipitating reversible cardiac fibrosis and failure (Beggah et al., 2002). Investigation of these tissue-specific transgenic mice has provided strong evidence that MR is a vital component of normal cardiac physiology.

Investigations in mice with functional manipulation of corticosteroid receptor expression have provided further evidence of their importance in lung maturation, HPA axis regulation, memory, emotion, energy homeostasis, renal and cardiac function.

1.4 Pre-receptor modulation of GC signalling

In addition to the control of GC release from the adrenal cortex, a number of other pre-receptor mechanisms may modulate ligand activation of the corticosteroid receptors. Corticosteroid-binding globulin (CBG) in the circulation binds corticosteroids with high affinity to regulate the tissue availability of free ligand (Breuner and Orchinik, 2002). Furthermore, 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes metabolise GCs and regulate the intracellular hormone levels. These enzymes modulate the amount of ligand accessible to the corticosteroid receptors and thereby subsequent GC action. The existence of corticosteroid interconversion was first demonstrated by Amelung et al. (1953). Dehydrogenase activity was then observed within the placenta and kidney, whilst reductase activity was observed in the liver. The existence of two distinct 11β-HSD types was later postulated (Monder and Shackleton, 1984), which was supported by the finding of lower cortisol/cortisone ratios from renal venous, compared to hepatic venous human blood (Walker et al., 1993). Purification and cloning of 11β-HSD confirmed the presence of two isozymes (Agarwal et al., 1994), catalysing opposite reactions, at least *in vivo*, to interconvert active and inactive (11-dehydrocorticosterone (11-DHC) in rats and mice; cortisone in humans) GCs (Figure 2.1).

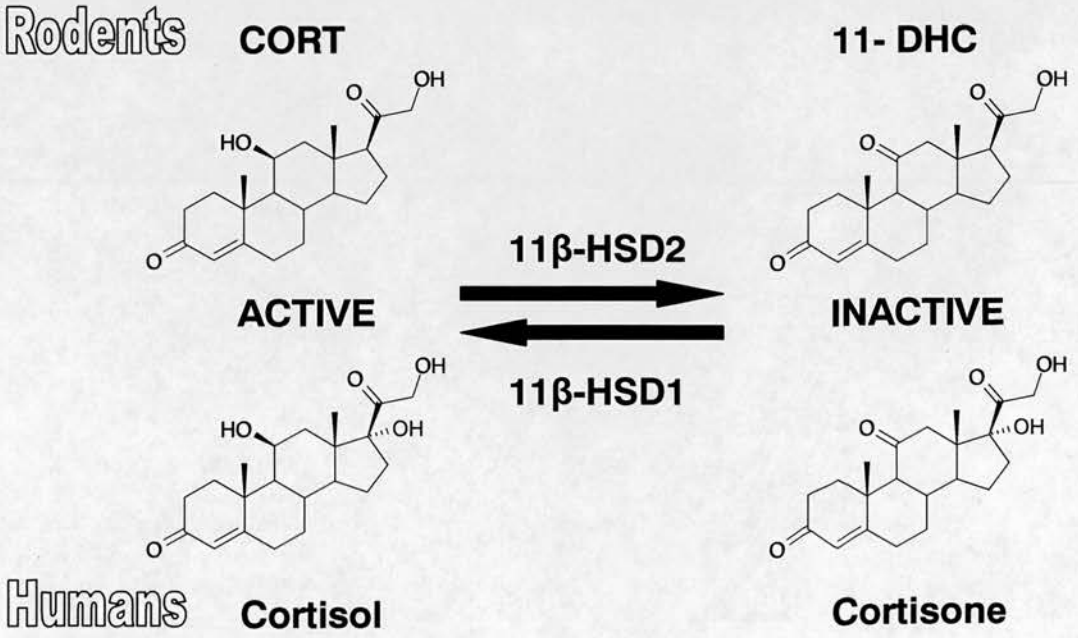


Figure 1.2 Species-specific interconversion of corticosteroids by the 11β-hydroxysteroid dehydrogenase enzymes
CORT = corticosterone; 11-DHC = 11-dehydrocorticosterone

1.4.1 11β-HSD2

1.4.1.1 Expression in the adult

11β-HSD2 was originally purified and cloned from the sheep kidney (Agarwal et al., 1994), although has since been cloned from other species including the human (Albiston et al., 1994), rat (Zhou et al., 1995) and mouse (Condon et al., 1997). Localised to the endoplasmic reticulum, with a cytosolic active site and co-factor binding domain, the enzyme displays unidirectional, NAD-dependent dehydrogenase activity, converting corticosteroids to their inactive 11-keto derivatives. 11β-HSD2 binds its substrate (cortisol in humans; CORT in rodents) with approximately 100-fold higher affinity than the type 1 enzyme suggesting it may play a more dominant role in CORT metabolism in locations where the two enzymes are co-expressed (reviewed in Draper and Stewart, 2005). In contrast to the widespread distribution of the type 1 enzyme, abundant expression of 11β-HSD2 is limited to mineralocorticoid target tissues including the kidney (Roland et al., 1995a), colon (Whorwood et al., 1994; 1995), salivary glands (Roland and Funder, 1996) and skin (Kenouch et al., 1994). The enzyme was found to co-localise with MR in renal collecting ducts and distal tubules (Roland et al., 1995a). Significant expression has also been detected in the adrenal gland (Shimojo et al., 1996) and vasculature (Christy et al., 2003; Hadoke et al., 2006b). In the rat brain 11β-HSD2 mRNA expression was identified in ventromedial nucleus of the hypothalamus, amygdala, locus coeruleus and nucleus tractus solitarius (NTS) (Roland et al., 1995b; Robson et al., 1998), areas identified to be involved in aldosterone actions on BP and salt appetite (reviewed in Gomez-Sanchez and Gomez-Sanchez, 2003). The distribution within the murine brain appeared to be even more limited, as expression was only observed within the NTS (Holmes and Seckl, 2006), consistent with a decreased aldosterone dependence on salt regulation within this species compared to the rat (Rowland and Fregly, 1988). CORT has a high affinity for 11β-HSD2 and coupled with a high capacity for its inactivation the enzyme provides an effective 'barrier' to intracellular corticosteroid activity at the receptor level. Therefore, despite the 100-fold lower circulating aldosterone levels, 11β-HSD2 confers mineralocorticoid-selectivity in tissues where the enzyme and MR are co-expressed.

1.4.1.2 Apparent mineralocorticoid excess (AME)

A distinct subset of patients has been described that possess hypertension associated with apparent mineralocorticoid excess (AME). This clinical syndrome has proven rare with only approximately 100 cases reported worldwide (Draper and Stewart, 2005). Patients present as children or young adults with severe hypertension and an underlying phenotype of hypokalaemia, suppressed renin activity and an extended half-life of cortisol (White et al., 1997). Initially, elevated mineralocorticoid action was thought to be a determining factor as the MR antagonist, spironolactone, or a low salt diet rescued the observed hypertension. However, circulating aldosterone levels were found to be normal in patients, promoting defective peripheral cortisol metabolism as an alternative hypothesis to explain the clinical symptoms (Ulick et al., 1979; Stewart et al., 1988). At present, over 33 different mutations in the 11β-HSD2 gene have been identified giving rise to partial or total attenuation of enzyme activity. In all cases, identified mutations have been found on both alleles indicating the disorder to be autosomal recessive (Draper and Stewart, 2005). In addition to severe hypertension, patients have displayed clinical features of intrauterine growth restriction (IUGR), short stature, thirst, polyuria and altered postnatal growth (Draper and Stewart, 2005).

1.4.1.3 Expression in the placenta and fetus

In addition to expression within adult mineralocorticoid target tissues, 11β-HSD2 is highly expressed in fetal tissues and the placenta. This enzyme was found to be abundant in placental trophoblast cells in all species studied, including the rat (Waddell et al., 1998), mouse (Brown et al., 1996b), baboon (Pepe et al., 1999) and human (Brown et al., 1993; Krozowski et al., 1995; Stewart et al., 1995; Sun et al., 1997). Expression of this corticosteroid inactivating enzyme within this major site of maternal-fetal transfer provides a 'barrier' to maternal GCs gaining access to the fetus, strictly regulating the fetal hormone environment during development. The functional significance of placental 11β-HSD2 is highlighted by the observation that the tissue is the most abundant source of the enzyme (Shams et al., 1998). A further layer of regulation exists in developing fetal tissues that express 11β-HSD2. This internal 'sieve' may function to provide intricate self-control, permitting the cell to fine-tune its own transient GC requirements. During embryonic and early post-natal life the

expression of GC metabolising enzymes and the corticosteroid receptors is delicately balanced to provide an optimal internal GC environment for development, which shall be described in more detail later in this chapter (Section 1.5).

1.4.1.4 11β-HSD2 null mutant and transgenic mice

The role of the 11β-HSD2 in adult physiology has been investigated through null deletion of the enzyme (see Chapter 2, page 62). These mice display a similar phenotype to the human syndrome of AME, confirming impaired 11β-HSD2 function in the underlying pathological mechanism. On an outbred MF1 background, approximately one-half of 11β-HSD2 null mutants die within the first 48 hrs of birth (Kotelevtsev et al., 1999). Mice that survived until adulthood displayed electrolyte imbalance (hypokalemia and hypochloremia), hypotonic polyuria and marked hypertension. Furthermore, an altered renal histology was seen, with hyperplasia and hypertrophy of the distal nephron in line with persistent MR activation (Kotelevtsev et al., 1999). Transfer of the targeted mutation to the C57BL/6J genetic background resulted in similar electrolyte imbalance, hypotonic polyuria, although with less severe hypertension and an absence of neonatal mortality (Bailey et al., unpublished observations). The contribution of renal and a vascular dysfunction to the etiology of the disease was investigated. Impaired sodium excretion, through increased activity of the epithelial sodium channel (ENaC) was evident during the first few months of life, but by 120 days of age this normalised. In contrast, hypertension was observed at all timepoints, even though mice remained volume contracted throughout (Holmes et al., 2006a; Bailey et al., unpublished observations). Whilst vascular responsiveness to vasoactive peptides was unaltered in 11β-HSD2 null mutants, circulating catecholamines were elevated and the hypertensive phenotype was acutely rescued with α1-adrenoreceptor blockade (Bailey et al., unpublished observations). These findings suggest that the hypertensive phenotype may shift from a renal to vascular condition due to the enhanced activity of the SNS possibly through maintained hypernatremia. The consequence of enzyme loss on the vasculature may be strain-dependent though, as earlier studies in 11β-HSD2 null mutants on a MF1 background reported impaired vascular responsiveness to vasoactive stimuli compared to wild-type controls (Hadoke et al., 2001). However, the 'illicit' MR activation due to 11β-HSD2 loss from

endothelium was not deemed responsible for the observed findings (Christy et al., 2003). It is thought that this altered vascular structure may underlie the sporadic sudden death observed in the 11β-HSD2 null mice on the MF1 background, particularly within females during pregnancy, through rupture of the aortic arch (Paterson et al., 2005).

AME appears to be a dominant feature of the 11β-HSD2 mutant mice although the severity seen was found to differ between strains, being less apparent but still present in the C57BL/6J. However, this global deletion of 11β-HSD2 will also remove the presence of the enzyme from the placental and fetal tissues, where it is thought to function as a key regulator of the fetal GC milieu during prenatal life. The 11β-HSD2 null mutant mice will therefore also exhibit consequences of prenatal GC programming, which shall be discussed later (Section 1.6.3).

Tissue-specific 11β-HSD2 expression, often in sites where the enzyme is not normally localised has been employed as a tool to not only elucidate the specific role of the enzyme in these tissues, but also to artificially downregulate GC action. Adipocyte-specific expression of the human 11β-HSD2 was generated in mice by utilising the aP2 promoter (aP2-HSD2). These transgenic mice were resistant to high-fat diet (HFD)-induced weight gain, as a result of impaired adipose deposition (Kershaw et al., 2005). This protection was associated with decreased food intake, increased energy expenditure, improved insulin sensitivity and glucose tolerance (Kershaw et al., 2005). These observations are broadly similar to those seen in the 11β-HSD1 null knockout (Morton et al., 2001; Morton et al., 2004b), and further implicate GC-mediated effects within adipose tissue in the pathological mechanism of the metabolic syndrome. 11β-HSD2 expression within the heart is generally below the level of detection (Condon et al., 1997; Moore et al., 2000), despite the presence of MR in this tissue (Lombes et al., 1995). Transgenic expression of 11β-HSD2 within cardiomyocytes precipitated marked cardiac hypertrophy and fibrosis, resulting in heart failure and premature death (Qin et al., 2003). The involvement of MR was confirmed by the attenuation of pathology by MR, but not GR blockade. The importance of GC-mediated actions in bone formation has been investigated using two separate transgenic mouse lines overexpressing 11β-HSD2 in osteoblast cells. One line displayed alterations in bone volume and

architecture (Sher et al., 2004). The other was normal under basal conditions, although displayed protection from exogenous high CORT-mediated detrimental effects (O'Brien et al., 2004). These investigators explored other cells within bone and found that osteoclast-specific 11β-HSD2 expression elicited similar effects on GC responsiveness (Jia et al., 2006). These findings indicate that a balanced intracellular GC level is important in regulating proper bone formation.

1.4.2 11β-HSD1

11β-HSD1 was observed to be bidirectional *in vitro* when originally purified and cloned from liver (Lakshmi and Monder, 1985; 1988; Agarwal et al., 1989), although more recently was found to act predominantly, if not exclusively, as a reductase when cloned into mammalian COS-7 cells (Low et al., 1994), in rat hepatocytes (Jamieson et al., 1995), in cultured hippocampal cells (Rajan et al., 1996) and *in vivo* (Jamieson et al., 2000). The physiological unidirectionality is believed to be a functional consequence of the intracellular localisation of the catalytic domain within the lumen of the endoplasmic reticulum and co-factor availability. In the reductase reaction with NADPH as a co-factor, 11β-HSD1 acts to convert 11-DHC to CORT (cortisone to cortisol in humans) and regenerate the active hormone. The enzyme is thus thought to provide intracellular amplification of GC action. It has been identified as the sole 11β-reductase *in vivo*, as 11-DHC infusion in adrenalectomized 11β-HSD1 null mice did not result in 11-DHC conversion and CORT generation (Kotelevtsev et al., 1997). The enzyme is broadly distributed, frequently co-localised with GR (Whorwood et al., 1992). Appreciable expression has been observed in the liver, adipose, skeletal and vascular smooth muscle, and the brain (Monder and Lakshmi, 1990; Rajan et al., 1995). Centrally, 11β-HSD1 has been localized to a number of regions including the cortex, hippocampus, hypothalamus, amygdala, cerebellum and pituitary (Moisan et al., 1990; Lakshmi et al., 1991; Roland et al., 1995a).

Polymorphisms of the 11β-HSD1 gene have been identified in the human population and have been associated with Alzheimer's disease (de Quervain et al., 2004), HPA axis regulation (Gambineri et al., 2006) and type 2 diabetes, although not obesity (Draper et

al., 2002; Nair et al., 2004). These findings point to a role for 11β-HSD1 in modulating cognition, stress reactivity and glucose homeostasis.

1.4.2.1 11β-HSD1 null mutant mice

A transgenic approach has been elegantly employed to permit more specific elucidation of 11β-HSD1 enzyme function. Mice null for 11β-HSD1 alleles were generated by replacement of exons 3 and 4 with a neomycin resistance cassette by homologous recombination in a 129 embryoic stem cell to generate chimeras (Kotelevtsev et al., 1997). 129 chimeras were crossed with the robust outbred MF1 strain to generate 11β-HSD1 null mutant which displayed approximately 2-fold elevation of circulating ACTH and CORT at the diurnal nadir and altered circadian CORT rhymicity, associated with adrenal hyperplasia (Harris et al., 2001). An elevated and prolonged stress response was evident, despite unaltered hippocampal corticosteroid receptor mRNA expression (Harris et al., 2001). Despite evident HPA axis hyperactivity 11β-HSD1 null mice displayed attenuated age-related cognitive decline, allied with lower intrahippocampal CORT (Yau et al., 2001).

The metabolic phenotype of 11β-HSD1 null mice has been investigated, and enzyme loss has resulted in generally protective effects. 11β-HSD1 knockout mice had an increased hepatic and adipose insulin sensitivity, improved glucose tolerance and a favourable lipoprotein profile (Morton et al., 2001; 2004a). Furthermore, these mice were more resistant to HFD-induced weight gain, even despite hyperphagia (Morton et al., 2004a) and displayed reduced hyperglycaemia following a HFD or novelty stress (Kotelevtsev et al., 1997). In addition, 11β-HSD1 knockouts had attenuated induction of the key gluconeogenic enzymes, PEPCK and glucose-6-phosphatase within the liver following starvation (Kotelevtsev et al., 1997). The HFD-diet induced hyperphagia, detected in 11β-HSD1 null mice, has been further explored. 11β-HSD1 null mice displayed an enhance ability to transiently elevate the orexigenic agouti gene related peptide within the arcuate nucleus in response to HFD, an effect associated with reduced opioid inhibitory tone (Densmore et al., 2006). This suggests that altered signalling within central appetite-regulating regions may play a mechanistic role in the metabolic consequence of 11β-HSD1 enzyme deletion.

While 11β-HSD1 null mice have a normal birthweight, postnatal development and growth trajectory (Kotelevtsev et al., 1997), they exhibited impaired lung morphology with decreased surfactant protein, consistent with reduced GR-mediated development (Hundertmark et al., 2002). In obstetric practice the role for GR activation in lung maturation is exploited by administration of steroids to hasten development in pre-term infants (Seckl and Meaney, 2004). Bone formation has also been studied in response to 11β-HSD1 null deletion. Although there were no significant changes in bone mass, adipocytes were absent suggestive of an altered differentiation pattern (Justesen et al., 2004).

1.4.2.2 11β-HSD1 transgenic mice

11β-HSD1 overexpression has been studied in a number of tissues including the liver, adipose and forebrain. 11β-HSD1 expression under the control of the enhancer-promoter region of the adipocyte fatty acid binding protein (aP2) gene (aP2-HSD1) (Masuzaki et al., 2001) led to a 2- to 3-fold increase in mRNA expression specifically within adipose tissue. The modest increase in CORT regeneration precipitated a marked metabolic phenotype of obesity, hyperlipidemia, hyperphagia despite hyperleptinemia, glucose intolerance and insulin-resistant diabetes (Masuzaki et al., 2001) as well as hypertension (Masuzaki et al., 2003). The increased blood pressure (BP) is thought to be driven by a chronically activated renin-angiotensin aldosterone system (RAAS), with increased angiotensinogen mRNA expression within adipocytes and elevated circulating renin and angiotensin II (Masuzaki et al., 2003). Despite normal circulating CORT, a three-fold elevation within the portal blood was measured in aP2-HSD1 mice (Masuzaki et al., 2001), suggesting that the metabolic effects may be, at least partially, mediated through elevated intra-hepatic GC action. To address this possibility, transgenic mice overexpressing 11β-HSD1 in the liver were generated using an HSD1 transgene driven by the apoE gene promoter (apoE-HSD1) (Paterson et al., 2004). These mice were found to exhibit features of the metabolic syndrome (insulin resistance, fatty liver, dyslipidemia and hypertension) but without obesity (Paterson et al., 2004). The hypertension was found to be correlated with hepatic angiotensinogen mRNA expression indicating a mechanistic involvement of the RAAS (Paterson et al.,

2004). Recently an involvement of intrahepatic GC metabolism has been implicated in HPA axis regulation. The intercrossing of 11β-HSD1 knockout (on a C57BL/6J background) and apoE-HSD1 transgenic mice completely rescued (Paterson et al., 2007) the HPA axis dysregulation previously observed (Harris et al., 2001). The central expression of corticosteroid receptors has yet to be analysed, but these surprising findings indicate a role for hepatic GC-sensitive processes in the regulation of the HPA axis, possibly through CORT regeneration and/or feedback communication.

It is clear from studies in mice with varying 11β-HSD1 activity, that the enzyme plays a critical role in the amplification of GC signalling in several metabolic and central processes including body composition, energy homeostasis, blood pressure regulation, the stress response and behaviour.

1.5 Development of the HPA axis

1.5.1 Prenatal Ontogeny

Intricate control and regulation of the appropriate GC environment is of vital importance in maintaining normal health. Dysregulation can have detrimental consequences and precipitate a broad range of pathophysiology. At no point during an organism's lifetime is the optimum GC milieu as critical as during development, including both *in utero* and during the early postnatal period. The specific actions of GCs during perinatal life, as indeed in adulthood, are determined by a blend of factors including the free circulating levels, pre-receptor metabolism and expression of the intracellular corticosteroid receptors. A complex balance of these components exists during early life with distinct species-specific ontogenic patterns.

1.5.1.1 Placental Ontogeny

In the baboon, placental 11β-HSD1 expression declined relative to 11β-HSD2 as gestation progressed, reflecting reduced cortisol transfer to the fetus as full term approaches (Pepe et al., 2001). The human placenta displayed increased 11β-HSD2 activity as pregnancy progressed (Shams et al., 1998) and a similar ratio of type 2 to type 1 expression at term (Pepe et al., 2001). This suggests that an equivalent placental GC metabolism may exist in primate species, where fetal cortisol exposure decreases towards the end of pregnancy.

1.5.1.1.1 Differences between rodent species

The prenatal regulation of the HPA axis is markedly different in rodents, perhaps not surprising considering the different developmental stages at birth. Rodents are born less mature than primates (Pryce et al., 2002), and the early postnatal period is thought to be a more critical period of growth in these species. The placental labyrinthe zone is primarily responsible for fetal hormone transfer (Cross, 2000) and is a major site of 11β-HSD2 expression in rodents (Brown et al., 1996b; Burton et al., 1996; Thompson et al., 2002). In both rats and mice, 11β-HSD2 rapidly decreased whilst 11β-HSD1 increased towards term (Brown et al., 1996b; Burton et al., 1996; Thompson et al., 2002; Speirs et al., 2004), consistent with the progressively increased fetal CORT

exposure observed in rats during the final trimester (Diaz et al., 1998). The basal zone is the main site of placental steroid and peptide hormone synthesis, although no expression of 11β-HSD2 was detected in this region of the mouse placenta (Thompson et al., 2002). However, in rats 11β-HSD2 mRNA expression was detected in the basal zone, although, in contrast to that seen in the labyrinthe zone, 11β-HSD2 mRNA increased during gestation, whilst 11β-HSD1 declined towards term (Burton et al., 1996). In both species, GR expression was stably expressed within both zones throughout gestation (Waddell et al., 1998; Speirs et al., 2004) suggesting an importance of local GC action in maintaining placental function. In the rat, maternal GC levels remained low during pregnancy, and close to term the HPA axis was hyporesponsive to stress (Neumann et al., 1998), coupled with altered feedforward regulation (Johnstone et al., 2000). However, in stark contrast mice exhibited a progressive surge of CORT towards term and became unable to elicit a further elevation following stress exposure (Douglas et al., 2003; Holmes et al., 2003). The availability of CBG was also significantly increased, although magnitude of the rise was approximately 10-fold less indicating that the free CORT levels were significantly elevated towards term (Douglas et al., 2003). Both species experienced rapid decline of placental 11β-HSD2 expression from the labyrinthe zone (Brown et al., 1996b; Burton et al., 1996; Thompson et al., 2002) during the final trimester, suggesting similar regulation of the feto-placental corticosteroid hormone barrier. These observations indicate that the mouse has a greater demand for GC to prepare the dam for birth and/or facilitate late fetal development. This may be important in the consideration of fetal programming across these species.

1.5.1.2 Fetal ontogeny

A dynamic ontogeny of the HPA axis has also been observed in fetal tissues. The expression of GR exhibited tissue-specific differences in time-course and abundance. Although generally increasing during embryonic life, GR mRNA displayed only temporary expression in certain sites, including the pancreas (Speirs et al., 2004), which may underlie the critical periods of vunerability to GC excess in different tissues. 11β-HSD1 mRNA expression is evident from E15 in various fetal tissues, including the liver, lung, thymus and brain regions (Speirs et al., 2004). MR expression was observed

from E13.5 in tissues including the pituitary, heart, muscle and later the gut, kidney, thymus, discrete areas of lung and several brain regions including hippocampus and hypothalamus (Brown et al., 1996b; Kretz et al., 2001). 11β-HSD2 expression was observed in many fetal tissues during early and midgestation, although rapidly decreased from E13 to E15 when distribution became limited to that observed in adult mice (Brown et al., 1996b; Condon et al., 1997). A similar distribution of 11β-HSD2, GR and MR mRNA has been observed early in human fetal life (Condon et al., 1998) suggesting that similar mechanisms of HPA axis development and regulation of fetal GC signalling may occur across species.

In the rodent brain, 11β-HSD2 mRNA and activity were dramatically silenced during the third trimester except in the thalamus and cerebellum (Brown et al., 1996b; Diaz et al., 1998), regions that have a greater reliance on postnatal maturation (Robson et al., 1998). In contrast, 11β-HSD1 was below the level of detection before E15.5, but progressively increased in transcript abundance during late gestation throughout the brain (Diaz et al., 1998). In the rat, GR mRNA was first detected in discrete regions on E12.5, but became more widely and robustly expressed during the final week. MR mRNA was initially seen on E15.5 and similarly developed until term (Diaz et al., 1998; Speirs et al., 2004). The increased expression of 11β-HSD1, GR and MR combined with 11β-HSD2 silencing in late gestation will encourage key GC-dependent maturational events. In particular appreciable expression of these genes is detected in the hippocampus towards the end of gestation (Diaz et al., 1998). When compared to the rat, mice display a similar MR ontogeny within the hippocampus. However, in contrast GR mRNA expression is absent from the antenatal murine hippocampus, the onset of which is delayed until the few days following birth (Schmidt et al., 2003; Speirs et al., 2004; Noorlander et al., 2006). Therefore, within this structure GC actions during fetal life will be exclusively MR-mediated, a potentially important difference between these rodent species.

1.5.2 Postnatal ontogeny

During the early postnatal period both mice and rats were found to exhibit a stress hyporesponsive period characterised by very low basal CORT levels and an inability of

mild stressors to induce an elevation in circulating ACTH and CORT. In the rat this lasted from about postnatal day (P) 4 to P14 (Levine, 1994; Galeeva et al., 2006), whilst in the mouse it was observed from just after birth (P1) to P12 (Schmidt et al., 2003). GR blockade disinhibited the hypoactivity, implicating enhanced GR-mediated negative feedback in the mouse (Schmidt et al., 2005). During the stress hyporesponsive period, the level of both free and total corticosterone were found to be maintained at low levels in the rat (Henning, 1978) and displayed a similar proportional increase around P14, suggesting that CBG also displayed a similar developmental trajectory during the pre-weaning period.

The postnatal ontogeny of hippocampal corticosteroid receptors has been studied in both rats and mice. In the rat a high density of MR mRNA expression was observed within all hippocampal subfields at P2 and remained so through the early postnatal period and into adulthood. GR mRNA expression increased during the first two weeks before receding to stable levels, as observed in the adult, by weaning (van Eekelen et al., 1991). In the mouse, hippocampal GR mRNA expression has been found to increase from its initial appearance following birth, primarily in the CA1 subfield, to reach adult levels by the end of the second week (Schmidt et al., 2003; Noorlander et al., 2006). MR displayed a subfield-specific mRNA expression pattern, but generally decreased during the first week when levels recovered to those observed in the adult by the third week (Schmidt et al., 2003; Noorlander et al., 2006).

Although subtle differences in postnatal development of the HPA axis are seen, the major species difference in HPA axis ontogeny between the mouse and rat is apparent in the final prenatal week. Whilst 11β-HSD2 expression is silenced in a similar time-specific manner in both species, CORT levels in the pregnant mouse multiply towards term, in contrast to the HPA axis hypoactivity observed in rats. Central GR mRNA expression is not evident in the prenatal mouse indicating GC actions on the fetal brain are mediated predominantly, if not solely through the MR. These findings imply that the optimum developmental GC milieu required for late fetal development in the mouse is much higher than the rat, although the absence of GR in the brain indicates that it may be peripheral rather than central maturation that is impacted.

1.6 Prenatal Programming

1.6.1 The phenomenon in humans

Epidemiological evidence in humans indicated that low birth weight, independent of a genetic component, was associated with a raised prevalence of cardiovascular disease, obesity, insulin resistance/type II diabetes mellitus (Barker et al., 1990; 1991; 1993; Hales et al., 1991) promoting the Barker hypothesis of the fetal origins of adult disease (Barker, 1992). This phenomenon has also been described as environmental or developmental plasticity, although we shall henceforth refer to it as programming. In humans, maternal stress has been found to lower offspring birth weight (Mutale et al., 1991; Edwards et al., 1994; Oyemade et al., 1994). Antenatal GCs, administered to promote fetal lung maturation, have been found to induce a similar birth weight reduction (French et al., 1999; Bloom et al., 2001), implicating these hormones in the mechanism of growth restriction following prenatal stress (PS). The majority of clinical studies have been retrospective, they show that PS is associated with dysregulation of HPA axis function and increased susceptibility towards a range of psychological disorders including anxiety, depression and schizophrenia (reviewed in Weinstock, 2001; Van den Bergh et al., 2005).

1.6.2 Prenatal manipulation in experimental animals

A substantial body of research has been conducted in experimental animals. Programming has been investigated in several experimental species including the rat, mouse, guinea pig, sheep and non-human primate (Nathanielsz, 2006). The prenatal component of programming has been studied experimentally through maternal manipulation including stress, exogenous GC administration and dietary alteration and a common feature of these prenatal treatments has been intrauterine growth restriction (IUGR) with the consequence of reduced birth weight. GCs are thought to play an essential role in programming through prenatal manipulations. With regard to this thesis we shall focus on programming mediated through the altered prenatal environment, rather than early postnatal life. Additionally we shall limit our discussion mainly to studies performed in rats and mice, in order to introduce the work conducted in our investigations. However, we acknowledge that important findings have also

arisen from studies conducted in other species including the guinea pig, sheep and non-human primate.

1.6.2.1 Prenatal stress exposure

Prenatal stress (PS) exposure through maternal manipulation has been widely studied. Many variations in paradigm have been employed including the nature, timing, duration and frequency of stressors applied during the prenatal period. The consequence of These stressors have been found to elicit a stress response and consequent elevation of CORT levels in the pregnant dam, thought to be the primary determinant of prenatal effects on the developing fetus. The most common stressor used has been maternal restraint, most often applied for three 45-min periods daily during the final week of gestation, although studies have also been performed with different timing, daily frequency and duration. Stressors and administration paradigms that have been studied, and the primary findings are shown in Table 1.1.

1.6.2.2 Prenatal exogenous GC administration

The synthetic GC dexamethasone (DEX) has been most widely used in studies exploring prenatal GC programming. DEX rapidly passes into the fetal circulation due to its poor substrate specificity for the placental 11β-HSD2 enzymatic barrier (Brown et al., 1993) although it also impacts maternal physiology. The synthetic GC betamethasone (BET) and CORT have also been administered by several groups. The route of GC administration and dose has differed depending on laboratory (Table 1.2). In our laboratory in Edinburgh DEX has been administered at a dose of 0.1 mg/kg/day during the final trimester through subcutaneous injection, comparing these offspring with those receiving vehicle (VEH) injections, thus correcting for the influence of handling and injection stress. Other groups have similarly administered GCs through injection although doses have varied, whilst CORT pellets and DEX osmotic minipumps have also been utilised. In contrast, DEX has also been administered through drinking water (Brabham et al., 2000; Hauser et al., 2006). Thus comparisons between various GC treatment paradigms are sometimes difficult as route-, dose- and time-dependent differences may exist.

Species	Nature of stressor	Timing	Daily Freq	Duration (Mins)	Reference(s)	Primary Findings
Rat	Restraint	E14-21	3x	60	Rimondini et al., 2003	↑ stress-potentiated anxiety
		E11-22	3x	45	Morley-Fletcher et al., 2003b	↑ CORT stress response
					Laviola et al., 2004	↑ anxiety-like behaviour
		E14-20	3x	45	Frye and Orecki, 2002a; 2002b	↓ sexual performance
					Frye and Wawrzycki, 2003	↑ depressive-like behaviour
					Zimmerberg and Blaskey, 1998	↑ anxiety-like behaviour
		E14-21	3x	45	Maccari et al., 1995	↓ hippocampal GR/MR mRNA
					Koehl et al., 1997; 1999	HPA axis phase shift
					Vallee et al., 1997; 1999	↑ anxiety-like behaviour
						↓ cognitive ability
					Morley-Fletcher et al., 2003a	↑ depressive-like behaviour
					Fumagalli et al., 2004	↓ cortex/striatum BDNF
					Bowman et al., 2004	↑ anxiety-like behaviour
						↓ cognitive ability
						Alterations in central NA and DA systems
					Poltyrev and Weinstock, 2004	↑ anxiety-like behaviour
					Louvart et al., 2005	
					Van den Hove et al., 2005	
					Zagron and Weinstock, 2006	
					Barros et al., 2006b	
					Poltyrev et al., 2005	↑ anxiolytic drug response
					Viltart et al., 2006	dysregulated regional brain activation
		E15-20	3x	45	Lemaire et al., 2000; 2006	↓ hippocampal neurogenesis
		E15-21	3x	45	Igosheva et al., 2004	↑ stress-induced blood pressure
		E15-19	3x	30	Szuran et al., 1991; 1994; 2000	↓ cognitive ability
		E15-22	3x	30	Lehmann et al., 2000	↓ fear-related cognitive ability
		E14-21	2x	60	Rao et al., 1999	sleep disturbance
		E17-20	1x	90	Gue et al., 2004	↓ cognitive ability
					Meunier et al., 2004	
		E10-20	1x	60	Smith et al., 2004	↑ depressive-like behaviour
						↑ CORT stress response
		E15-21	1x	60	Reznikov et al., 2001; 2003; 2004; 2005	alterations in central NA and DA systems
		E18-22	1x	60	Gerardin et al., 2005	↓ sexual performance
						alterations in central NA and DA systems
		E15-17	1x	30 or 240	Fujioka et al., 2001; 2006	↑ anxiety-like behaviour
		E15-19	1x	20	McCormick et al., 1995	↑ CORT stress response
	Variable	E14-22	-	-	Koenig et al., 2005	↑ schizophrenia-like behaviour
					Kinnunen et al., 2003	↑ CORT stress response
		E15-20	-	-	Murmu et al., 2006	↓ dendritic arborisation/synaptic loss
	Handling and injection	E14-21	1x	-	Cratty et al., 1995	↑ CRH in amygdala
					Ward et al., 2000	↑ stimulated CRH release in amygdala
					White and Birkle, 2001	↓ function response to 5-HT_{1A} blockade
					Griffin, III et al., 2003	↑ anxiety-like behaviour
					Dickerson et al., 2005	

Table 1.1 Experimental models of prenatal stress reported in the literature
5-HT = serotonin; BDNF = brain-derived neurotropic factor; CORT = corticosterone; DA = dopamine; E = embryonic day; GR = glucocorticoid receptor; HPA = hypothalamic pituitary adrenal; MR = mineralocorticoid receptor; NA = noradrenaline

Species	Nature of stressor	Timing	Daily Freq	Duration (Mins)	Reference(s)	Primary Findings
Rat	Crowding and injection	E15-21	1x	-	Hayashi et al., 1998	↓ cognitive ability with ↓ synaptic density altered hippocampal 5-HT turnover
		E16-21	1x	-	Peters, 1982; 1984; 1986a; 1986b; 1988b; 1990	↑ anxiety-like behaviour altered central NA and 5-HT
	Injection and forced swim	E5-birth	1x	15	Drago et al., 1999	↑ depressive-like behaviour ↓ fear-related cognitive ability
	Noise and forced swim	E10-18	1x	15	Nishio et al., 2001	↓ cognitive ability
	Noise and light	Whole	3x week	-	Poltyrev et al., 1996 Weinstock et al., 1998	↑ anxiety-like behaviour ↑ CORT stress response
		Whole	Random	-	Fride et al., 1988 Weinstock et al., 1988; 1992	↑ anxiety-like behaviour ↑ CORT stress response ↑ plasma catecholamines
	Footshock	Whole	Every other day	-	Estanislau and Morato, 2005; 2006	↑ anxiety-like behaviour ↑ stress-potentiated anxiety ↓ cognitive ability
	Cat presence	E10, E14 and E19	2x or 10x	15	Lordi et al., 1997; 2000 Patin et al., 2002; 2005	↓ cognitive ability ↑ anxiety-like behaviour ↓ maternal behaviour
Mouse	Restraint	E14-20	3x	45	Meek et al., 2000; 2001; 2006	↓ cognitive ability ↓ maternal behaviour ↓ sexual performance
					Ishiwata et al., 2005	↓ cognitive ability ↓ hippocampal 5-HT (transient) ↓ hippocampal spine densities
					Tamura et al., 2005	↓ neurogenesis factors
		E15-21	3x	30	Sternberg and Ridgway, 2003	↑ CORT stress response
		E12-17	2x	60	Ward and Wainwright, 1988	neurobehavioural deficit
		E13-18	2x	30	Kinsley and Svare, 1986; 1988	↓ intermale aggression
		E8-19	1x	360	Chung et al., 2005	↑ CORT stress response ↓ hippocampal GR ↑ anxiety-like behaviour
					Son et al., 2006; 2007	DAergic hyperfunctionality ↓ NMDA-mediated synaptic plasticity
		E1-birth	1x	180	Alonso et al., 1994; 1997; 2000	↑ depressive-like behaviour DA abnormalities in nucleus accumbens
	CUMS	E1-birth	-	-	Pardon et al., 2000	↓ maternal behaviour
	Noise	E12+14	1x	24 hrs	Pincus-Knackstedt et al., 2006	↑ anxiety-like behaviour ↓ CRH in PVN
	Crowding	E12-17	-	-	Harvey and Chevins, 1984; 1987 Crump and Chevins, 1989	↓ sexual performance
	Footshock	E15-19	1x	-	Palermo-Neto et al., 2001; 2003	↑ anxiety-like behaviour ↑ CORT stress response

Table 1.1 (Cont.) Experimental models of prenatal stress reported in the literature
5-HT = serotonin; CORT = corticosterone; CRH = corticotropin-releasing hormone; CUMS = chronic ultramild stress; DA = dopamine; E = embryonic day; GR = glucocorticoid receptor; PVN = paraventricular nucleus

Species	GC	Timing	Daily Freq	Dose	Reference(s)	Primary Findings
Rat	DEX	Whole	1x	0.1 mg/kg sc	Celsi et al., 1998	hypertension (glomerular damage)
					Ahlbom et al., 2000	↑ neonatal brain sensitivity to oxidative stress
					Welberg et al., 2001	↑ anxiety-like behaviour
						↑ amygdala GR/MR mRNA
					Hadoke et al., 2006a	hypertension (↑ vascular tone)
					Chen et al., 2004	renal abnormalities
		E1-10	1x	0.1 mg/kg sc	Woods and Weeks, 2005	↓ maternal food intake
		E15-20				
		E1-7	1x	0.1 mg/kg sc	Nyirenda et al., 1998	hyperglycaemia, hyperinsulinemia
		E8-14				↓ glucose tolerance
		E15-21				↑ hepatic GR/PEPCK mRNA/activity
		E8-birth	1x	0.1 mg/kg ip	Emgard et al., 2007	↓ cognitive ability
		E9+11+13	1x	0.1 mg/kg ip	Swolin-Eide et al., 2002	abnormal skeletal growth
		E11+12	2x	0.2 mg/kg ip	Ortiz et al., 2001; 2003	hypertension (↓ nephron number)
		E13+14				
		E15+16				
		E17+18				
		E19+20				
		E14-19	1x	0.125 mg/kg sc	Shoener et al., 2006	↑ CORT stress response
						↓ hippocampal MR/↑ hippocampal 11β-HSD1 mRNA
		E14-21	1x	0.1 mg/kg sc	Holson et al., 1995	↓ sexual performance
					O'Regan et al., 2004	↑ hepatic GR/PEPCK mRNA/activity
						hypertension (↑ RAAS activity)
		E15-18	1x	0.2 mg/kg ip	Dagan et al., 2006	renal abnormalities
		E15-20	1x	0.1 mg/kg sc	Levitt et al., 1996	↑ CORT stress response
						hypertension
		E15-birth	-	2.5 ug/ml	Brabham et al., 2000	↓ cognitive ability
				drinking water		↑ CORT stress response
		E15-21	1x	0.1 mg/kg sc	Nyirenda et al., 2001; 2006	hyperglycaemia, hyperinsulinemia
						↓ glucose tolerance
						↑ hepatic GR/PEPCK mRNA/activity
					Welberg et al., 2001	↑ anxiety-like behaviour
						↓ hippocampal GR/MR mRNA
					Cleasby et al., 2003a; 2003b	↓ fat depot weight
					Drake et al., 2005	intergenerational metabolic programming
		E15-21	1x	0.1 mg/kg or 0.2 mg/kg	Langdown et al., 2001; 2003;	↓ cardiac function (altered cardiac protein expression)
				by osmotic pump	Sugden et al., 2001	
		F16-birth	1x	0.1 mg/kg im	Wan et al., 2005	↑ neonatal hippocampal 11β-HSD1 mRNA
		E17-19	1x	0.05 mg/kg	Navarro et al., 1989	impaired postnatal lung growth
				0.2 mg/kg	Carlos et al., 1991	↑ neonatal brain sensitivity to oxidative stress
				0.8 mg/kg	Slotkin et al., 1992; 1996; 2006	altered central monoaminergic content
					Kauffman et al., 1994	↓ neonatal cardiac tolerance to hypoxia
					Muneoka et al., 1997	monoaminergic disturbances during weaning period
					Kreider et al., 2005	↓ cognitive ability
		E18+19	1x	0.1 mg/kg sc	Oliveira et al., 2006	↓ DEX suppression
		E20+21	1x	0.4 mg/kg ip	Okajima et al., 2001	impaired postnatal lung growth
Rat	BET	E8-birth	1x	0.1 mg/kg ip	Emgard et al., 2007	↓ cognitive ability
Rat	CORT	E14+15	1x	0.8 mg/kg sc	Singh et al., 2007	hypertension (↓ nephron number)
		E14-21	3x	0.7 mg/kg sc	Holson et al., 1995	↓ sexual performance
		E16-birth	-	2.4 mg/day pellets	Diaz et al., 1995; 1997	↑ spontaneous activity
						altered response to amphetamine and apomorphine
		E18+19	1x	25 mg/kg sc	Oliveira et al., 2006	↓ DEX suppression

Table 1.2 Experimental models of prenatal GC administration reported in the literature
11β-HSD1 = 11β-hydroxysteroid dehydrogenase type 1; BET = betamethasone; CORT = corticosterone; DEX = dexamethasone; E = embryonic day; GR = glucocorticoid receptor; ip = intraperitoneal; MR = mineralocorticoid receptor; PEPCK = phosphoenolpyruvate carboxykinase; sc = subcutaneous

1.6.3 Effects of Prenatal programming

1.6.3.1 Effects on behaviour

PS and exogenous GC administration in rodent models are known to precipitate several behavioural abnormalities including anxious-, depressive- and schizophrenic-like behaviour in tests.

1.6.3.1.1 Anxiety

Several groups have investigated the anxiety-like behaviour in rodent offspring from different PS paradigms (see Table 1.1). The animal tests employed in the investigation of anxiety in these rodents include the elevated plus maze (EPM), open field (OF) and light/dark box (LD). The EPM is comprised of two open and two closed arms on an elevated platform. The test measures the conflict between the exploratory behaviour and the aversion to open spaces and has been validated for the measurement of anxiety levels in the rat (Pellow et al., 1985) and mouse (Lister, 1987). The open field test was originally described in 1934 (Hall) and since has become one of the most popular anxiety tests (reviewed in Prut and Belzung, 2003). Animals are assessed in an inescapable open arena, where the exposed centre presents a more anxiogenic environment for the rodent and the exploratory activity is measured. The LD test was initially described as an anxiety test in mice (Crawley and Goodwin, 1980) and has since been employed to measure emotional behaviour and evaluate anxiolytic drug efficacy (reviewed in Bourin and Hascoet, 2003). The LD test is based on the conflict of avoidance of brightly illuminated areas and the innate desire to explore a novel environment.

PS rat offspring have displayed heightened anxiety-like behaviour when assessed on the EPM (Fride and Weinstock, 1988; Zimmerberg and Blaskey, 1998; Poltyrev and Weinstock, 2004; Patin et al., 2005; Zagron and Weinstock, 2006; Barros et al., 2006b; Estanislau and Morato, 2006), OF (Peters, 1986; Lehmann et al., 2000; Fujioka et al., 2001; Bowman et al., 2004; Laviola et al., 2004; Van den Hove et al., 2005) or both (Poltyrev et al., 1996; Vallee et al., 1997; Poltyrev and Weinstock, 2004). Not all studies found a difference in spontaneous behaviour under basal conditions in these tests

(Rimondini et al., 2003; Poltyrev et al., 2005). However, PS offspring had an enhanced anxiety response to acute stress (Rimondini et al., 2003; Estanislau and Morato, 2006) and heightened sensitivity to an anxiolytic drug treatment (Poltyrev et al., 2005). The integral role of maternal GCs in the programming effects of PS have been illustrated by the normalisation of offspring by prior maternal adrenalectomy (Barbazanges et al., 1996; Zagron and Weinstock, 2006). Additional support for GCs mediating PS-induced behavioural programming comes from maternal administration studies. Prenatal DEX administration in rats increased anxiety-like behaviour on the EPM and OF when given throughout the final trimester (Welberg et al., 2001) or E18 and 19 only (Oliveira et al., 2006).

Increased anxiety-like behaviour in PS rat offspring has also been detected in fear conditioning (Griffin et al., 2003), defensive withdrawal (Dickerson et al., 2005) and the light/dark test, although only following aversive conditioning (Louvart et al., 2005). The acoustic startle response was unaltered by PS, although offspring displayed a reduced response to anxiogenic drugs acting via modulation of the serotonergic system (White and Birkle, 2001).

PS-mediated behavioural programming has been less studied in mice. Increased anxiety-like behaviour has been programmed by sound stress during E12 and 14 (Pincus-Knackstedt et al., 2006), and footshock stress daily between E15 and 19 (Palermo-Neto et al., 2001). Of particular note in the latter experiment was the detection of programmed effects despite unaltered birth weight (Palermo-Neto et al., 2001). Programmed consequences of prenatal GC treatment, in the absence of birth weight deficits have been also been observed in rats (Ortiz et al., 2001; 2003) and primates (de Vries et al., unpublished observations), indicating that this may not be absolute requirement following prenatal GC treatment. Following a severe PS regimen, mice were not found to display any alteration in behaviour, although exhibited enhanced anxiety following chronic postnatal stress (Chung et al., 2005). Single dose DEX or BET on E14 decreased juvenile anxiety, with the BET effects persisting through to adulthood (Rayburn et al., 1997). Multiple dose BET, however did not result in any

alteration in anxiety levels (Rayburn et al., 1998), or indeed cognitive ability (Christensen et al., 2001).

Studies conducted by the Matthews laboratory in Toronto have investigated prenatal GC programming in guinea-pigs and observed heightened anxiety-like behaviour in the OF following PS administration (Kapoor and Matthews, 2005), although not BET treatment (Owen and Matthews, 2007) despite the evidence of altered HPA axis regulation in response to both regimes (reviewed in Kapoor et al., 2006).

1.6.3.1.2 Depression

Investigators have also studied the depressive-like behaviour of prenatally manipulated offspring in the forced swim test originally described by Porsolt (1977; 1978). This test measures the behavioural despair of rodents placed in a inescapable cylinder of water and decreased immobility interpreted as antidepressant efficacy (reviewed in Cryan et al., 2005). Several studies revealed an increase in immobility in both rats (Alonso et al., 1997; Drago et al., 1999; Morley-Fletcher et al., 2003a; Frye and Wawrzycki, 2003; Smith et al., 2004; O'Mahony et al., 2006) and mice (Alonso et al., 2000), indicative of behavioural despair and depression-like behaviour. However, others studies have also found prenatally stressed offspring to display a reduced immobility (Stohr et al., 1998; Welberg et al., 2001). Whilst reduced immobility time can be interpreted as reduced depressive-like behaviour, this could alternatively represent an impaired coping strategy through cognitive deficits (Welberg et al., 2001).

1.6.3.1.3 Cognition

An elegant epidemiological study of the Lothian Birth Cohort reported a positive correlation between birth weight and cognitive ability at age 11 (Shenkin et al., 2001). Furthermore, this correlation was still apparent when cognitive ability was assessed at age 78 (Deary IJ, personal communication). Cognitive function has been assessed following PS or prenatal GC treatment in rodent species, most commonly in the Morris Water Maze (Morris, 1984). Programmed deficits in water maze performance, indicative of memory and/or learning impairment have been observed in rats (Hayashi et al., 1998; Brabham et al., 2000; Nishio et al., 2001; Meunier et al., 2004; Emgard et

al., 2007) and mice (Meek et al., 2000). Szuran et al. (1994; 2000) did not observed any change in cognitive performance when the water was at an ambient temperature (18-20°C), although when measured in cold water (10-12°C) male PS rat offspring showed a reduced capacity in the colder, more stress-provoking water compared to control offspring. However, CORT response was not seen to be significantly different depending on prenatal treatment or the temperature of the water (Szuran et al., 2000), indicating other alterations are likely to be responsible.

Several other rodent tests have detected a prenatal treatment-specific effect on cognitive ability. PS rat offspring had impaired radial arm maze performance (Vallee et al., 1999; Bowman et al., 2004; Kreider et al., 2005). Furthermore, decreased retention of passive (Lordi et al., 1997; 2000; Drago et al., 1999; Gue et al., 2004) and active (Lehmann et al., 2000) avoidance conditioning was observed following PS in rats. Behaviour in the Y- or T-maze has delivered further evidence of cognitive impairment (Vallee et al., 1999; Gue et al., 2004; Estanislau and Morato, 2005).

The tests mentioned above assess different aspects of cognitive function (Ehman and Moser, 2006) and divergent effects have been observed. One study detected water maze deficits although object recognition was undisturbed (Bowman et al., 2004). Another found Y- and radial arm maze impairment, despite unaltered water maze performance throughout the subject's lifetime (Vallee et al., 1997; 1999). This illustrates the importance of employing multiple tests in the analysis of cognitive phenotype. The PS effects on cognition and other features, may be dose-dependent and involve a postnatal component as mild PS improved memory performance on the radial arm maze and active avoidance paradigms, findings which were prevented by early adoption to a control dam (Fujioka et al., 2001).

1.6.3.1.4 Schizophrenia

An altered fetal GC environment has been proposed in the etiology of schizophrenia, supported by findings from animal models (Koenig, 2006). The psychological disorder is often associated with cognitive impairment (Friedman et al., 1999; Freedman, 2003), which was a common observation in the animal models previously described (see above). Other features seen in schizophrenic patients include deficits in prepulse inhibition (PPI) (Braff et al., 1992), reduced latent inhibition (LI) (Lubow and Gewirtz, 1995) and dopaminergic hyperactivity (Nieoullon, 2002). Studies have investigated these schizophrenia-associated phenomena in PS and GC administration models. Enhanced amphetamine-induced hyperactivity and PPI deficits were observed in offspring subjected to a variable PS paradigm in the final week (Koenig et al., 2005), seen without any alteration in anxiety-like behaviour in the OF. The nature of the prenatal stressor appears to be important in the programming of schizophrenic-like attentional deficits. Prenatal restraint stress did not yield similar observations on dopaminergic function and PPI (Koenig et al., 2005). Reduced LI was observed following final trimester CORT administration or repeated footshock, but not restraint stress (Shalev and Weiner, 2001). Another study observed no change in LI, whilst PPI was enhanced following final trimester restraint stress (Lehmann et al., 2000). Prenatal CORT administration has also been found to programme increased spontaneous activity and an altered response to amphetamine and apomorphine administration, with increased and decreased hyperactivity, respectively (Diaz et al., 1995; 1997). Most recently, prenatal DEX was not found to consistently impact offspring LI and PPI (Hauser et al., 2006). It thus appears that the specific nature of stress exposure is important in mediating effects upon schizophrenic-like behaviour.

1.6.3.1.5 Other behaviours

An effect of PS has been observed on the sleep-wake cycle in adult male rats, with a dramatic increase in paradoxical sleep and other abnormalities (Dugovic et al., 1999; Rao et al., 1999). A disruption in normal sexual activity has been observed following PS. Female rats displayed less socio-sexual contact with decreased lordosis and solicitation behaviour than controls (Frye and Orecki, 2002b), with differences further accentuated by acute restraint (Frye and Orecki, 2002a). PS also resulted in altered

estrous cycling within female mice (Harvey and Chevins, 1987). Male rats subject to PS or DEX exhibited a reduction in sexual performance (Holson et al., 1995) and abnormal behaviour including a reduced number of ejaculations (Gerardin et al., 2005). Male mice subjected to prenatal crowding stress displayed impaired intermale aggression (Kinsley and Svare, 1988), copulatory behaviour (Harvey and Chevins, 1984) and reduced fertility (Crump and Chevins, 1989), coupled with alterations in sexual partner preference (Meek et al., 2006), indicative of demasculinization of behaviour. Findings of alterations in sleep patterns and sexual performance have been observed in depressed patients (Ferguson, 2001; Tsuno et al., 2005) and are consistent with prenatal programming of depressive-like symptoms.

The findings discussed above provide significant evidence of the crucial role of the prenatal GC milieu in development and programming of the lifelong behavioural phenotype. Prenatal stress and GC administration has been found to be associated with elevated symptoms of anxiety, depression, cognitive deficits, schizophrenia, sleep disturbance and sexual dysfunction. CORT is well known to modulate behaviour and the observed behavioural abnormalities may be a related to programming effects upon the HPA axis.

1.6.3.2 Effects on the HPA axis

In humans, a negative correlation between adult HPA axis activity and birth weight has been observed (Phillips et al., 2000; Reynolds et al., 2001; 2005). The HPA axis has been well-studied in experimental models and evidence of similar prenatal HPA axis programming is available. Most often a hyperactivity of the axis has been observed although studies differ in specific abnormalities and gender dichotomy is common. Increased basal CORT has been observed in female rat offspring following prenatal restraint stress (Szuran et al., 2000), but the majority of studies have not reported any difference in either gender (McCormick et al., 1995; Vallee et al., 1996; 1997; 1999; Reznikov et al., 2001; Smith et al., 2004; Viltart et al., 2006) when examined at the diurnal nadir. However, a phase shift in the circadian rhymicity was detected with an earlier and enhanced diurnal peak in both genders following PS in rats (Koehl et al., 1997; 1999), which was prevented by a postnatal stressor during the first week of life (Koehl et al., 1997).

PS in rats has been found to programme an altered stress response. Following acute restraint, female PS offspring had an exaggerated and prolonged elevated of CORT (McCormick et al., 1995). Several studies have reported no exaggeration in the peak stress response, but rather a prolonged return to baseline (Vallee et al., 1996; 1997; 1999; Smith et al., 2004; Viltart et al., 2006) which was ameliorated by either environmental enrichment during adolescence (Morley-Fletcher et al., 2003b) or chronic tianeptine antidepressant treatment (Morley-Fletcher et al., 2003a). A decreased habituation to repeated stress was seen in male offspring (Bhatnagar et al., 2005). In a unique finding, female PS offspring displayed a prolonged stress-response, whilst males display a more rapid return to baseline than control offspring (Reznikov et al., 2001). Similarly, Fischer 344 PS male offspring had a reduced peak CORT response 20 and 60 mins post restraint (Van den Hove et al., 2005), indicating that similar to behaviour, diverging effects of PS on the HPA axis can occur in different strains (Stohr et al., 1998).

PS male mice had an exaggerated CORT stress response at weaning, which was prevented by chronic postnatal selective serotonin reuptake inhibitor (SSRI) treatment

(Ishiwata et al., 2005). In another study basal CORT was unaltered, although a prolonged acute stress response was observed in male PS offspring (Chung et al., 2005).

Similar programming effects on the HPA axis in rats have been seen following exposure to prenatal stressors, other than restraint. Noise and light PS resulted in elevated basal CORT and exaggerated and prolonged levels following acute stress in adulthood, together with increased plasma catecholamines and metabolites (Weinstock et al., 1992; 1998). Variable PS, which programmed schizophrenia-like behaviour also resulted in a prolonged stress response in male rat offspring (Kinnunen et al., 2003). Elevated basal CORT was observed in mice receiving footshock PS which was highest when the stressor was inescapable (Palermo-Neto et al., 2001; 2003).

Prenatal GC administration in rats has also exacerbated hyperactivity of the HPA axis. Some studies observed no difference in basal or stress-induced CORT in the morning in male prenatal DEX offspring (Nyirenda et al., 2001). However, similar studies did detect increased basal CORT at this time-point in male (Levitt et al., 1996), but not female prenatal DEX offspring (O'Regan et al., 2004) although stress levels were unaltered (Levitt et al., 1996). Prenatal DEX administration through drinking water led to unaltered basal or peak stress CORT, although the response was prolonged in both sexes (Brabham et al., 2000). This was abolished by postnatal adoption to a control mother. However, similar dysregulation was seen in control offspring fostered to a DEX drinking dam identifying the postnatal period as the critical locus of HPA axis programming in this study (Brabham et al., 2000). Another study found no difference of prenatal DEX or CORT administration on nadir and peak basal CORT levels, although both treatment offspring had reduced DEX suppression (Oliveira et al., 2006). Elevated basal and stress-induced ACTH and CORT was observed in male offspring during the rising phase following prenatal DEX injection on E14-19 (Shoener et al., 2006).

These rodent studies provide evidence of significant CORT hyperactivity in offspring following PS and prenatal GC administration indicating programming of the HPA axis.

1.6.3.3 Effects on the brain

1.6.3.3.1 Regulation of the HPA axis

PS and exogenous GC administration have been found to instigate alterations within the brain including mRNA transcript expression, neurochemistry and function. Corticosteroid receptors localised to the limbic system and PVN regulate behaviour and the HPA axis. Adult rats subjected to prenatal restraint stress had a reduction in hippocampal GR and MR mRNA expression in both sexes (Koehl et al., 1999) associated with a reduced ability to curtail the CORT response to acute stress (Henry et al., 1994). Adoption to a non-stressed mother ameliorated both the HPA axis hyperactivity and decreased central expression of the corticosteroid receptors, presumably through the increased maternal behaviour expressed by foster mothers (Maccari et al., 1995). Others have found alterations in GR binding in rat PS offspring, although only in females (Szuran et al., 2000). Another prenatal restraint paradigm (E15-19; 20 min/day) found no GR binding differences in the hippocampus, but an increase in the amygdala and septum with a decrease in the frontal cortex of PS rats (McCormick et al., 1995) although CRH content in the median eminence was unchanged (Smythe et al., 1996). Within the amygdala CRH receptor binding, content and depolarization-induced release were however elevated in offspring following final trimester saline injection stress (Cratty et al., 1995; Ward et al., 2000). Furthermore, both a mild stressor and intracerebroventricular (icv) CRH administration caused more marked acetylcholine (ACh) release in the hippocampus of PS male and female rat offspring, compared to controls (Day et al., 1998).

Prenatal DEX administration has also been observed to programme alterations in central HPA axis abnormalities. Male prenatal DEX rats had reduced mRNA expression of MR and GR within the hippocampus following DEX in the final trimester (Levitt et al., 1996), whilst administration throughout gestation did not elicit hippocampal changes. CRH mRNA was elevated following both treatments within the amygdala and PVN, although only treatment throughout gestation resulted in increased MR and GR mRNA in the amygdala (Welberg et al., 2001). Shoener et al. (2006) found reduced hippocampal MR, but not GR mRNA expression, whilst 11β-HSD1 mRNA was elevated during adulthood following prenatal DEX treatment. The expression of

hypothalamic CRH mRNA was upregulated, although GR and AVP were unaltered. An elevation in hippocampal 11β-HSD1 mRNA and protein content has also been found on P1 and P7 (Wan et al., 2005). In rats, final trimester DEX administration in drinking water reduced hippocampal GR and feedback sensitivity, although fostering to a control dam ameliorated this effect (Brabham et al., 2000).

In mice, restraint PS yielded reductions in GR and MR protein content within the hippocampus. CRH and GR content within the hypothalamus and amygdala was also elevated, although only when combined with a postnatal stressor (Chung et al., 2005).

Alterations in the expression of the corticosteroid receptors and CRH within the hippocampus, amygdala and PVN may underlie the observed CORT elevations following PS and prenatal GC treatment through increased forward drive and/or reduced negative feedback on the HPA axis. Additionally, the corticosteroid receptors and CRH are well-known to regulate behaviour and their modified expression may also be involved in the behavioural changes seen in prenatal programming models. However, modifications within other central neurochemical systems will also certainly play a mediating role too.

1.6.3.3.2 Neurochemistry

GCs and monoamine neurotransmitter systems possess reciprocal modulatory roles (reviewed in Chaouloff, 2000; Carrasco and Van de Kar, 2003; Van Craenenbroeck et al., 2005). Serotonergic and noradrenergic inputs to the HPA axis from the raphe nucleus and locus coeruleus, respectively are shown in Figure 1.1 (page 5). Therefore, it is expected that PS and prenatal exogenous GCs would have effects upon brain neurochemistry and neurotransmitter systems. GC effects may be mediated through activated corticosteroid receptor binding to GREs, such as located in the 5-HT_{1A} gene regulatory region which can inhibit the transcription of this neuronal receptor (Ou et al., 2001). Studies performed over 15 years ago by Peters in rats, identified PS effects on the serotonergic and noradrenergic systems. Alterations were observed at P16 (but not P9 or P23) in 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA) and noradrenaline (NA) levels were observed in the cortex and hypothalamus, but only the

latter had persistent alterations when measured at P60 (Peters, 1982). At P16, a transient reduction in α1, α2 and β-adrenoreceptor binding was observed in several brain regions. In adulthood, 5-HT binding was increased in the cortex, and reduced in the hippocampus, which also displayed reduced 5-HT synthesis from tryptophan (Peters, 1986). These PS offspring also showed altered behavioural responses to the 5-HT agonist 5-methoxy-N,N-dimethyltryptamine (5-MeODMT), dependent on a strong interaction with the postnatal rearing conditions (Peters, 1986; 1988a). Peters (1988b) identified the critical period for PS on programming of serotonergic dysfunction to be from P15 until birth, as PS from E3-14 had no observable impact. The central fetal and neonatal 5-HT environment was investigated in this experiment and found to be significantly increased in the PS group during this period and up to P10 (Peters, 1990). Prenatal DEX from E17-19 resulted in transient 5-HT/5-HIAA decrease in the hippocampus, neocortex, hypothalamus and medulla at P21 (Muneoka et al., 1997). Another group observed reduced 5-HT and elevated 5-HIAA levels in the hippocampus at P35 in PS rat offspring, concurrent with a reduction in synaptic density (Hayashi et al., 1998).

These studies indicate a significant influence of PS and DEX treatment on the serotonergic system. The underlying mechanism is unclear, although a recent study in adolescent rats (P23) reported alterations in the 5-HT system following 7-day CORT pellet implantation (Bush et al., 2003). This manipulation resulted in a flattening of the diurnal CORT rhythm such that circulating CORT levels throughout the cycle resembled those of the evening peak. 5-HT_{1A} binding and serotonin turnover (5-HIAA/5-HT) was reduced by CORT treatment in the cortex at 21 days following the start of treatment, but not 7 days after (Bush et al., 2003). This report indicates that exaggerated CORT can impact on the 5-HT system, although the effects may be delayed, similar to the observations of transient alterations following PS. No alterations were seen in hippocampal 5-HT_{1A} binding in the previous study (Bush et al., 2003), although another observed significantly reduced 5-HT_{1A} immunobinding in the ventral, but not dorsal hippocampus following PS, whilst 5-HT_{2A} immunobinding was unaffected (Van den Hove et al., 2006a). A decreased functional response to 5-HT_{1A} receptor blockade has also been reported (White and Birkle, 2001). A significant

increase in pre-synaptic 5-HT transporter was observed in the brainstem and cortex, where 5-HT_{1A} and 5-HT_2 (non-specific) expression was also increased in the absence of any change in 5-HT levels (Slotkin et al., 1996; 2006). The alterations of the serotonergic system, including neurotransmitter levels, turnover and receptor expression within the cortex and hippocampus may act to alter behaviour following prenatal programming. In addition, other neurotransmitter systems have displayed similar disturbance following PS or prenatal GC exposure, including NA and dopamine (DA).

Brainstem and midbrain noradrenergic and dopaminergic maturation was accelerated following prenatal DEX in rats from E17-19, although levelled off prematurely (Slotkin et al., 1992). Reduced DA turnover in the hypothalamus and lower NA content in the hippocampus was seen at P21 (Muneoka et al., 1997). In adulthood NA, 5-HT and DA content within the hypothalamus was unaltered, although 5-HT and DA levels were increased in the striatum of PS offspring (Gerardin et al., 2005). 5-HT and DA turnover and NA levels displayed alterations in the rat prefrontal cortex, hippocampus and amygdala following PS (Bowman et al., 2004). Gender-specific differences in catecholamines within the preoptic area and medial basal hypothalamus of 10 day-old rats were attenuated as a result of PS or exogenous GC administration, which was prevented by naltrexone, implicating endogenous opioids in the effect (Reznikov et al., 2003; 2004; 2005). The alterations in NA and DA content within the hypothalamus of PS rat offspring persisted through to adulthood, as was the icv NA-induced adrenal CORT release (Reznikov et al., 2001). DA abnormalities have also been observed in the nucleus accumbens (Alonso et al., 1994; 1997). Following final trimester prenatal restraint, D_2 and *N*-methyl-D-aspartic acid (NMDA) receptors were increased in the dorsal frontal cortex, medial prefrontal cortex, hippocampal CA1 and nucleus accumbens in adult rats. Similarly, metabotropic group III glutamate receptors were elevated in the dorsal frontal cortex and medial prefrontal cortex only (Berger et al., 2002). Several of these alterations could be prevented by cross-fostering (Barros et al., 2004). Benzodiazepine receptors were reduced in the central amygdala (CeA) and hippocampus, ameliorated by cross-fostering (Fride et al., 1985; Barros et al., 2006b). Microarray analysis identified significant changes in genes associated with the NMDA receptor/postsynaptic density complex and the vesicle exocytosis machinery in

offspring that received variable PS (Kinnunen et al., 2003). Chronic prenatal restraint in mice was also found to programme alterations suggestive of hyperfunctionality of the dopaminergic system (Son et al., 2007) and reduced NMDA receptor-mediated synaptic plasticity (Son et al., 2006).

The alterations observed in brain neurochemistry are likely to participate in the behavioural deficits that we have described following prenatal manipulation. For example, genetically modified mice lacking functional NMDA receptor expression in the dentate gyrus displayed spatial working memory deficits (Niewoehner et al., 2007). Furthermore, 5-HT_{1A} receptor deletion has been found to elicit heightened anxiety-like behaviour (Parks et al., 1998; Ramboz et al., 1998), whilst overexpression was found to modulate emotionality in the opposite direction (Kusserow et al., 2004).

1.6.3.3.3 Other effects on the brain

A deficit in neurogenesis within the dentate gyrus was detected in adult rats following PS and found to be associated with reduced hippocampal-dependent spatial learning (Lemaire et al., 2000), but could be entirely prevented with postnatal pup stimulation, mimicking maternal behaviour (Lemaire et al., 2006). This PS-induced impairment in neurogenesis was shown to be GR-mediated. In the same study a milder PS paradigm produced an opposite enhancement of neurogenesis, which was identified to be MR-mediated (Fujioka et al., 2006). Tolloid-like 1, a metalloprotease positively involved in neurogenesis and negatively regulated by GCs, was decreased in the hippocampus of female PS mice offspring (Tamura et al., 2005). Brain-derived neutrophic factor (BDNF) was found to be reduced in the cortex and striatum of PS offspring, and expression was dysregulated following chronic stress during adulthood (Fumagalli et al., 2004). The sensitivity to oxidative stress in neonates exposed to prenatal DEX treatment was enhanced throughout the brain (Carlos et al., 1991; Ahlbom et al., 2000). Hippocampal BDNF and the astroglial-specific neurotrophic factor, S100B were reduced following PS with a concomitant decrease in brain cell proliferation (Van den Hove et al., 2006b; 2006c). In adulthood PS induced a long-lasting astroglial reaction and reduced dendritic arborisation with global synaptic loss (Barros et al., 2006a; Murmu et al., 2006). PS exhibited a higher number of Fos-immunoreactive neurones in

the hippocampus and locus coeruleus indicative of increased regional activation. Following a mild stressor (15-min open arm exposure), control offspring displayed enhanced Fos expression, whilst PS offspring displayed no further rise in Fos, suggestive of dysregulated activity in HPA axis feedback sites of PS offspring (Viltart et al., 2006).

1.6.3.4 Effects on peripheral function

In humans, birth weight has been reported to be negatively correlated with metabolic abnormalities including hyperglycaemia/type II diabetes, hypertension, heart disease (Barker et al., 1990; 1991; 1993; Hales et al., 1991), features of the human metabolic syndrome. These features have been studied in rodent programming models and findings from these are presented below.

1.6.3.4.1 Body weight maintenance

The vast majority of studies have found PS or exogenous GC treatment to precipitate IUGR and a reduction in birth weight. Prenatal DEX administration was found to programme reduced body weight throughout life, associated with reduced fat deposition (Cleasby et al., 2003a), although most papers report a normalisation of body weight in adulthood (O'Regan et al., 2004; Drake et al., 2005). Evidence from human studies has indicated that catch-up growth may play a mechanistic role in low birth weight susceptibility to adult pathophysiology (Ong, 2006).

1.6.3.4.2 Liver and glucose homeostasis

Prenatal restraint stress induced hyperglycaemia, glucose intolerance and reduced basal leptin levels in aged rats. Body weight, fat deposition and basal food intake were unaltered, although food intake was higher in PS offspring following cessation of a 24-hr fast (Lesage et al., 2004). Another study found increased basal glucose, despite reduced food intake and body weight in adulthood (Vallee et al., 1996). Prenatal DEX treatment has been found to programme increased fasting glucose, reactive hyperglycaemia and hyperinsulinemia, although only when administered in the final week. This was associated with elevated hepatic GR mRNA and PEPCK mRNA/activity (Nyirenda et al., 1998; O'Regan et al., 2004). Interestingly, cross-

fostering after birth did not prevent the glycaemic dysregulation suggesting that programming occurs independently of the postnatal environment (Nyirenda et al., 2001). Recently, the hepatocyte nuclear factor 4 alpha (HNF4α) has been suggested to play a role in the GC-mediated induction of PEPCK, increased gluconeogenesis and altered glucose homeostasis (Nyirenda et al., 2006). The prenatal DEX-induced rise in PEPCK and glucose hyperactivity was also seen to be intergenerational (Drake et al., 2005).

1.6.3.4.3 Blood pressure and cardiac function

Human epidemiology has observed a robust link between low birthweight and subsequent hypertension in adulthood (Barker et al., 1990). Hypertension was observed in prenatal DEX offspring (Levitt et al., 1996) which was associated with hyperactivity of the RAAS in females (O'Regan et al., 2004; Hadoke et al., 2006a). The majority of studies have assessed blood pressure through direct manipulation causing unavoidable stress to the subject. A recent study has recorded mean arterial pressure via radiotelemetry and made intriguing observations. Opposite to that seen previously, prenatal DEX offspring of both genders displayed resting hypotension. In contrast, an exaggerated rise in mean arterial pressure during even brief disturbance or restraint stress was observed. This response was ameliorated by prior reserpine administration implicating a sympathetic component in the hypertensive mechanism (O'Regan et al., unpublished observations). Increased vascular tone to NA, AVP and potassium also reported (Hadoke et al., 2006a; O'Regan et al., unpublished observations). Basal blood pressure was unaltered in PS offspring, although markedly increased following restraint (Igosheva et al., 2004). Heart disease susceptibility has been negatively correlated to birth weight (Barker, 2002). PS offspring had altered cardiac protein expression, consistent with impaired function (Langdown et al., 2001; 2003; Sugden et al., 2001), whilst neonatal cardiac tolerance to hypoxia was lowered (Kauffman et al., 1994).

1.6.3.4.4 Kidney and renal function

Altered renal function has been reported in several models of prenatal programming and may underlie, at least in part, the hypertension observed. Maternal CORT programmed hypertension in both sexes which was found with a deficit in nephron number and renal

angiotensin receptors (Singh et al., 2007). Proximal tubular transport was increased, in part through stimulated Na^+/H^+ exchanger activity in prenatal DEX offspring (Dagan et al., 2007). This region was also found to be more susceptible to toxin-induced apoptosis (Chen et al., 2004). Hypertension and reduced nephron number was also observed following twice-daily prenatal DEX treatment on consecutive days, despite no alteration in birth weight (Ortiz et al., 2001; 2003). PS offspring from another study displayed glomerular damage and sodium retention along with hypertension (Celsi et al., 1998).

1.6.3.4.5 Others

A clear role for GCs in promoting lung development and function is known, although in excessive amounts they can be detrimental resulting in impaired normal postnatal lung growth (Navarro et al., 1989; Okajima et al., 2001). Immune function has been altered by prenatal GC manipulation including abnormalities in macrophage number and function (Palermo-Neto et al., 2001; 2003) and dysregulated cellular and humoural immune responses (Pincus-Knackstedt et al., 2006). Abnormal skeletal growth following prenatal DEX supports role for GCs in functional bone development (Swolin-Eide et al., 2002).

1.7 Perspectives in prenatal programming

1.7.1 Prenatal component

It is most likely that prenatal GCs elicit their offspring effects by acting at several different sites. A key component will be mediated through passage of GC across the 2placenta to directly impact on the developing fetus. Binding to corticosteroid receptors in different tissues will elicit downstream effects on fetal development and maturation, and GCs are known to regulate the expression of several key growth signals including insulin-like growth factors (IGFs) and growth hormone. PS was recently found to reduce fetal rat circulating GH at E21 (Mairesse et al., 2007). Another component of GC programming of the fetus may be indirect through GC effects on the placenta. Maternal DEX administration in drinking water was found to inhibit the normal rise in vascular endothelial growth factor in the placental labyrinthe zone towards the end of gestation and was associated with decreased placental vascularisation (Hewitt et al., 2006). Increased apoptosis has also been observed in the placenta following prenatal DEX or CBX in rats, although only in the basal, and not labyrinthine zone (Waddell et al., 2000). Maternal DEX also increased endothelin-1 and endothelin receptor expression in ovine placental arteries (Kutzler et al., 2003). These effects may serve to restrict the efficiency of the placenta and the transfer of nutrients to the fetus.

The 11β-HSD2 feto-placental 'barrier' has also been identified as a vital target of GC programming and will be discussed below. PS and prenatal GC adminstration may also induce alterations in maternal nutritional intake, and thereby fetal nutrient availability. Reduced food intake and body weight gain has been observed in prenatally manipulated dams (Kinsley and Svare, 1986; Woods and Weeks, 2005) and these were found to be highly correlated to neurobehavioural deficits (Ward and Wainwright, 1988). Indeed, lifelong programmed effects have been observed in response to maternal dietary manipulation, including nutrient restriction, low protein and high fat diets. Global maternal undernutrition has been found to downregulate fetal IGF1 leading to IUGR, reduced offspring birthweight and hypertension (Woodall et al., 1996a; 1996b; 1998). The programming effects of a low protein diet during gestation have been well studied and observed to include IUGR, low birth weight, HPA axis dysregulation, renal

damage, hypertension, glucose intolerance and obesity (Bertram and Hanson, 2001; Langley-Evans et al., 2005), but also alterations in hippocampal morphology (Cintra et al., 1997; Granados-Rojas et al., 2002).

1.7.2 Postnatal component

Although insults in the aforementioned studies occur during the prenatal period, evidence also indicates that a locus of effect may be in the susceptible postnatal period through altered maternal care. Dams stressed during gestation displayed a reduction in maternal behaviours including arched back nursing, nesting/grouping and pup retrieval behaviour (Fride et al., 1985; Pardon et al., 2000; Patin et al., 2002; Smith et al., 2004). The Meaney group in Montreal has investigated the lifelong effects of variations in maternal care by studying pups raised by dams with maternal behaviour at disparate ends of the normal distribution. This has revealed profound consequences of maternal care on lifelong welfare, potentially involving epigenetic mechanisms (Meaney, 2001; Meaney and Szyf, 2005). Neonatal programming has been found to elicit lifelong programmed effects and manipulations studied include neonatal handling and brief maternal separation, have been found to permanently programme positive consequences on the offspring, including reduced negative behaviour and improved cognitive ability (Levine, 2005). These consequences have generally been opposite to those observed in PS or GC programmed offspring and postulated to be mediated through improved maternal care during rest periods. Opposite, detrimental effects can be programmed by longer periods of maternal separation which decrease offspring nurture. Studies have found neonatal handling or brief maternal seperation to prevent the emergence of PS programmed effects (Wakshlak and Weinstock, 1990; Lemaire et al., 2006), possibly through normalisation of deficits in maternal behaviour.

Adoption has been seen to improve maternal care in rats (Maccari et al., 1995), although was not observed to prevent the anxiogenic behaviour induced in PS offspring (Barros et al., 2006b). Cross-fostering did however have effects upon anxiety-like behaviour (Barros et al., 2006b), central neurotransmitter regulation (Barros et al., 2004) and HPA axis activity (Maccari et al., 1995). In these studies the adult phenotype of the offspring was reliant on the mother's treatment during gestation, rather than the prenatal group of

the offspring *per se*. However, other parameters including hypertension, impaired glucose homeostasis and dopamine-dependent hyperactivity have not been prevented by cross-fostering (Diaz et al., 1995; Nyirenda et al., 2001; Igosheva et al., 2004). Other studies found no reversal of programmed deficits by cross-fostering, although abnormalities could be programmed in control offspring reared by a prenatally DEX-treated mother (Peters, 1988a; Brabham et al., 2000; Hauser et al., 2006). It is therefore apparent that in particular circumstances the impaired postnatal rearing environment can play a dominant or complementary role in 'prenatal' programming. In addition to altered physical mother-pup interactions, modified hormone levels delivered through the milk could be implicated in the mechanism of perinatal programming.

1.7.3 Reversibility and prevention

Prenatal GC programmed effects were found to be both preventable and reversible by pharmacological treatment during fetal (Meunier et al., 2004; Ishiwata et al., 2005) and in postnatal life (Zimmerberg and Blaskey, 1998; Reznikov et al., 2003; Poltyrev et al., 2005), respectively.

1.7.4 11β-HSD2: a common mechanistic link?

PS, GC administration and nutritional restriction appear to share similar features in the programming effects they elicit. A common link between these prenatal manipulations is the evasion of the protective enzymatic barrier, 11β-HSD2. Repeated PS results in maternal CORT levels that overwhelm the barrier, whilst DEX circumvents GC inactivation due to poor substrate specificity and has a relatively unrestricted journey into the fetal circulation (Brown et al., 1993). Studies in rats (Langley-Evans et al., 1996; Bertram et al., 2001) and sheep (Whorwood et al., 2001) have observed a downregulation of placental 11β-HSD2 following maternal nutrient restriction and recently a similar reduction was also observed following PS in rats (Mairesse et al., 2007). Furthermore, in humans the enzyme has been found to positively correlate with birth weight (Stewart et al., 1995; Shams et al., 1998; McTernan et al., 2001). The lowered barrier will therefore allow greater passage of GCs across the placenta.

The 11β-HSD inhibitor carbenoxolone (CBX) has been used to study the impact of enzyme blockade during gestation. Prenatal CBX treatment was found to programme similar alterations to those already described with PS or DEX administration, namely low birth weight, hypertension, glucose intolerance, HPA axis hyperactivity and altered behaviour (Lindsay et al., 1996; Saegusa et al., 1999; Welberg et al., 2000). Increased basal CORT and a prolonged stress response were observed despite unaltered hippocampal corticosteroid receptor mRNA expression. GR was upregulated in the amygdala, and reduced in the PVN where CRH was elevated. Behavioural alterations suggestive of heightened anxiety were also noted in the EPM and OF (Welberg et al., 2000). However, CBX is a non-specific inhibitor of the 11β-HSD type 1 and 2 enzymes (Stewart et al., 1990; Jellinck et al., 1993) and treatment with this pharmacological agent could have additional effects upon the pregnant dam. Therefore, we have chosen to investigate mice lacking the 11β-HSD2 enzyme, a potential model for prenatal GC programming.

1.8 Hypotheses and aims

The primary objective of this thesis was to further explore the phenomenon of prenatal GC programming and investigate the protective role of the 11β-HSD2 feto-placental barrier by addressing the following aims:

1.) Studies performed in our laboratory have detected elevated basal plasma CORT (O'Regan et al., 2004) and unearthed a heightened BP sensitivity to stress in prenatally DEX-treated rat offspring (O'Regan et al., unpublished observations). We hypothesise that similar enhanced stress sensitivity may be observed in anxiety-related behaviour, which could be related to altered patterns of brain activation. Therefore, we aim to extend previous findings in the prenatal DEX model through analysis of the diurnal CORT excretion, behavioural sensitivity to stress and brain activation following stressor exposure.

2.) The importance of the feto-placental 11β-HSD2 barrier has been demonstrated using the non-selective 11β-HSD inhibitor, CBX (Lindsay et al., 1996; Welberg et al., 2000). Similar to prenatally DEX-treated rat offspring (Welberg et al., 2001), prenatally CBX-treated rat offspring displayed heightened anxiety, altered plasma CORT and central HPA axis-associated gene expression (Welberg et al., 2000). Homozygous-bred 11β-HSD2 null mutant mice have been studied and shown to exhibit anxiety-like behaviour (Holmes et al., 2006). However, variations in maternal nurture and behaviour could account for these consequences of enzyme deletion. We hypothesise that a key locus of effect in 11β-HSD2 null mutant mice is the overexposure of GCs during the fetal period. In order to control for mothering influence on the offspring phenotype we aim to use the unique model of prenatal GC overexposure, the heterozygous-bred 11β-HSD2 knockout mouse, to study the consequence of global 11β-HSD2 enzyme deletion on the brain, behaviour and HPA axis.

3.) Prenatal DEX treatment has been found to programme metabolic disturbances including a lean body phenotype (Cleasby et al., 2003a) and impaired glucose homeostasis (Nyirenda et al., 1998; 2001; 2006; O'Regan et al., 2004; Drake et al., 2005). We hypothesise that the heterozygous-bred 11β-HSD2 knockout mouse will display similar alterations in metabolic profile. Therefore, we aim to perform initial characterisation of the metabolic phenotype.

CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

Unless otherwise stated, suppliers are located in the United Kingdom and all chemicals and reagents were purchased from ***Sigma-Aldrich Company Ltd., Poole, Dorset.***

2.1.1 General chemicals

BDH-Merck, Poole, Dorset:

Acetone, Formaldehyde, Glacial Acetic Acid, Hydrochloric Acid, Xylene

Bright Instruments Co Ltd., Huntingdon, Cambridgeshire:

Cryo-m-bed Embedding Compound

Cementone-Beaver Ltd., Buckingham:

Non-toxic Latex Liquid

Fisher Scientific, Loughborough, Leicester:

Duolite Mixed Resin

Fluka Chemicals Ltd., Gillingham, Dorset:

Acetic Anhydride, Triethanolamine

Hayman Ltd., Witham, Essex:

Ethanol

VWR International, Lutterworth, Leicester:

Paraformaldehyde, SuperFrost Plus Slides

Whatman® International Ltd., Maidstone, Kent:

Whatman 3mm Chromatography Paper, Whatman Filter Paper

2.1.2 Molecular Biology Reagents

Applied Biosystems, Warrington, Cheshire:

TaqMan® Gene Expression Assays

Amersham Biosciences, Chalfont, Buckinghamshire:

Anti Rabbit Scintillation Proximity Assay (SPA) reagent, Hybond-N Membrane, NICK Sephadex G-50 DNA column®, Rediprime® II Random Prime Labeling System

Anachem Ltd., Luton, Bedfordshire:

Rnaid Matrix, Rnaid Wash

Biowhittaker Molecular Applications, Wokingham, Surrey:

SeaKem® LE Agarose

H.A. West (X-Ray) Ltd., Edinburgh:

Amfix High Speed Fixer, Kodak Developer D-19

Invitrogen, Paisley, Renfrewshire:

1kb DNA Ladder, Low DNA Mass Ladder, Low Melting Point Agarose, Quant-iT™ RiboGreen® RNA Reagent and Kit, TRIzol®, Yeast tRNA

Macherey-Nagel, Düren, Germany:

NucleoSpin® Tissue

Merck Biosciences, Nottingham

Dithiothreitol (DTT)

Promega Ltd., Southampton, Hampshire:

Nuclease-free H_2O, Nucleotides, Restriction Enzymes (AvaI, EcoRI, HindIII and XbaI), Reverse Transcription System, RNA Polymerases (T3, T7 and SP6), RNase Inhibitor (RNasin), RQ1 DNase (RNase-free), Transcription Optimised Buffer (5x), Vectors (pGem-3, pGem-4)

Qiagen Ltd., Crawley, West Sussex:

HotStar*Taq* DNA Polymerase, QIAquick Gel Extraction Kit, QIAquick PCR Purification Kit

Roche Diagnostics Ltd., Lewes, East Sussex:

LightCycler® 480 Multiwell Plate 384, LightCycler® 480 Probes Master, Malate Dehydrogenase, RNase A

Sigma-Aldrich Company Ltd., Poole, Dorset:

Kodak Biomax MR Film, Kodak Biomax MS Film

Stratagene, Amsterdam, The Netherlands:

pBluescript KS Vector

Thermo Fischer Scientific, Salford, Manchester:

Infinity™ Glucose Hexokinase Liquid Stable Reagent

Viagen Biotech Ltd., Los Angeles, Calfornia, USA:

DirectPCR Lysis Reagent (Ear)

Zinsser Analytic, Maidenhead, Berkshire:

Aquasafe 300 Plus Scintillant

2.1.3 Radioisotopes

Amersham Biosciences, Chalfont, Buckinghamshire:

[1,2,6,7-^{3}H]-corticosterone (2.8-3.9 TBq/mmol), α^{32}P-dCTP (111 TBq/mmol), ^{35}S-UTP (30 TBq/mmol)

2.1.4 Animals

Charles River Laboratories, Margate, Kent:

Wistar Rats

Harlan, Bicester, Oxon:

C57BL/6J Mice

Special Diet Services (SDS), Witham, Essex:

Rat and Mouse Breeder and Grower (CRM(E)) Chow (62.0% carbohydrate, 18.6% protein, 3.4% oil)

Rat and Mouse No.1 Maintenance (RM1(E)) Chow (66.7% carbohydrate, 14.4% protein, 2.7% oil)

Rat and Mouse No.3 Breeding (RM3(E)) Chow (55.7% carbohydrate, 22.4% protein, 4.3% oil)

2.1.5 Materials for plasma corticosterone assay

Reagent of Dr. CJ Kenyon, Edinburgh:

Anti-Corticosterone

2.1.6 Equipment

Amersham Biosciences, Chalfont, Buckinghamshire:

GeneQuant RNA/DNA Calculator, Hypercassette™

Beckman Coulter Ltd., High Wycombe, Buckinghamshire:

Beckman Optima™ TLX Ultracentrifuge, TLA 100.3 rotor

Bio-Tek Instruments Inc., Winooski, Vermont, USA:
EL 312e Bio-Kinetics Microplate Reader

Carl Zeiss, Welwyn Garden City, Hertfordshire:
Zeiss Stemi 100 Dissection Microscope

Dage Inc., Michigan City, Indiana, USA:
Dage MTI CCD72S Imaging Camera

Eppendorf Ltd., Histon, Cambridge:
Centrifuge 5415R

Fuji Photo Film Company Ltd., Tokyo, Japan:
Phosphoimager FLA-2000, Phosphoimager screens

Gene Technologies Ltd., Braintree, Essex:
G-storm Thermal Cycler

Heraeus Sepatech GmBH, Osterode, Germany:
Megafuge 3.0R

Hook & Tucker Instruments, Croydon, Surrey:
Rotamixer

Hybaid Ltd., Basingstoke, Hampshire:
Hybaid Omni Slide Wash Module

Ika, Labortechnik, Staufen, Germany:
Ultra-Turrax T8 Auto-Homogeniser

Interfocus Imaging Ltd., Linton, Cambridgeshire:
Northern Light Illuminator

Jencons-PLS, East Grinstead, West Sussex:

Techne Dri-Block DB3, Techne Genius Thermal Cycler, Techne Hybridiser HBID, Techne Hybridisation Bottles

Konica Minolta, Milton Keynes, Buckinghamshire:

Konica SRX-101 X-ray Developer

Leica Microsystems Ltd., Milton Keynes, Buckinghamshire:

Leica Cryostat

Mettler-Toledo, Leicester:

Mettler HK60 Semi-Microbalance

Panlab, Barcelona, Spain:

Rat Elevated Plus Maze

PerkinElmer, LAS (UK) Ltd., Beaconsfield, Buckinghamshire:

Wallac 1450 Microbeta Plus Liquid Scintillation Counter, Wallac Victor2 1420 MultiLabel Counter

Roche Diagnostics Ltd., Lewes, East Sussex:

Roche Lightcycler® 480 Real-Time PCR System

Sarstedt, Numbrecht, Germany:

Microvette Tubes, EDTA-coated

Shimadzu UK Ltd., Milton Keynes, Buckinghamshire:

Shimadzu UV-160A Spectrophotometer

Spectronics Corporation, Westbury, New York, USA:

Spectrolinker XL-1500 UV Crosslinker

Techniplast UK Ltd, Kettering, Northamptonshire:

Mouse Wheel Running Cages, Rat Metabolic Cages

Ugo Basile, Comerio, Italy:

Passive Avoidance Apparatus

UVItec* Ltd., *Cambridge:

UVIpro Gel Documentation System

Weiss-Gallenkamp, Loughbourgh, Leicestershire:

Gallenkamp Fan Incubators

2.1.7 Software

Actimetrics Software, Wilmette, Illinois, USA:

ClockLab™, LimeLight™, WaterMaze™

InterFocus Imaging Ltd., Linton, Cambridgeshire:

MCID Analysis

Raytest Scientific Ltd., Sheffield:

Aida 2.0 Auto Image Data Analyser, Fujifilm Fluorescent Image Analyser FLA-200

Roche Diagnostics Ltd., Lewes, East Sussex:

Lightcyler® 480 Software

PerkinElmer, LAS (UK) Ltd., Beaconsfield, Buckinghamshire:

Multicalc Advanced v2.0f

The Mathworks Ltd., Cambridge:

MATLAB 6.5

UVItec Ltd., Cambridge:

UVIpro Acquisition Software

2.1.8 Buffers and Solutions

1.2% Agarose Formaldehyde Denaturing Gel (100ml): 17ml formaldehyde (BDH) added to 1.2g of agarose, pre-melted in DEPC-H_2O (73ml) and 10X MOPS buffer (10ml)

Box buffer (100ml): 20ml 20xSSC buffer, 50ml deionised formamide made up to 100ml in DEPC-H_2O

Deionised Formamide: 150ml formamide mixed with 15g mixed bed ion-exchange resin (Duolite) for at least 1 hr, filtered twice through Whatman filter paper and stored protected from light using sterile glassware

DEPC-H_2O: dH_2O mixed with diethylpyrocarbonate (DEPC; 1 drop/ 100ml), shaken and left for 1-24 hr prior to autoclaving

1M Dithiothreitol (DTT): 0.15g was added to 1ml DEPC-H_2O and filter sterilised, stored at -20°C

250mM EDTA (pH 8.0; 100ml): 80ml dH_2O was added to 9.3g $Na_2EDTA.2H_2O$. pH adjusted with NaOH and volume adjusted to 100ml. Autoclaved before use

Ethanol in Ammonium Acetate:

50% - 11.55g Ammonium Acetate dissolved in 250ml ethanol, made up to 500ml with dH_2O

70% - 11.55g Ammonium Acetate dissolved in 350ml ethanol, made up to 500ml with dH_2O

90% - 11.55g Ammonium Acetate dissolved in 450ml ethanol, made up to 500ml with dH_2O

HEPES/sucrose buffer (pH 7.4): 250mM sucrose, 5mM HEPES and stored at 4°C

2x Hybridisation Buffer: 1.2M NaCl, 20mM Tris-HCl (pH 7.5), 2x Denhardt's solution, 2mM EDTA (pH 8.0), 0.2mg salmon sperm DNA (11mg/ml), 0.2mg yeast tRNA (50 mg/ml) and 2g dextran sulphate made up to 10ml in DEPC-H_2O, stored at -20°C

Loading Buffer: 2g Ficoll® 400, 100mg SDS, 25mg bromophenol blue, 25mg xylene cyanol dissolved in 10ml 0.1M EDTA

10x MOPS Buffer (pH 7.0): 42g MOPS, 16.6ml 3M sodium acetate (pH 5.2) and 20ml 0.5M EDTA dissolved in 1L dH_2O; adjusted to pH 7.0 and autoclaved before use

4% Paraformaldehyde in 0.1M Phosphate Buffer (pH 7.5): 20mM NaH_2PO_4, 80mM Na_2HPO_4 in 1L DEPC-H_2O, heated to 80°C prior to addition of 40g paraformaldehyde using sterile glassware. Stirred for 1 hr to dissolve and stored at 4°C

Phosphate Buffer: 0.2M NaH_2PO_4, 0.6M Na_2HPO_4, 5mM EDTA. Autoclaved before use

PBS (Phosphate Buffered Saline; pH 7.4): 10mM phosphate buffer with 137mM NaCl, 2.7mM KCl in dH_2O. Autoclaved before use

2x Pre-hybridisation Buffer: 1.2M NaCl, 20mM Tris-HCl (pH 7.5), 2x Denhardt's solution, 2mM EDTA (pH 8.0), 10mg salmon sperm DNA (11mg/ml), 0.2mg yeast tRNA (50 mg/ml) made up to 10ml in DEPC-H_2O, stored at -20°C

RNA Sample Buffer: 50% deionised formamide, 18% formaldehyde, 1x MOPS buffer

RNase A Solution: 25mg RNase A (Roche) dissolved in 1mM Tris, 15mM NaCl made up to 2.5ml with dH_2O (10mg/ml). Heated to 100°C for 15 mins then cooled to room temperature and stored at -20°C. RNase A (10mg/ml) added to buffer (3µl/ml)

Rnase Buffer: 0.5M NaCl, 10mM Tris, 1mM EDTA in dH_2O

20x SSC (Saline Sodium Citrate Buffer; pH7.0): 3M NaCl, 0.3M Na citrate in 1L DEPC-H_2O, pH 7.0. Autoclaved before use

5M NaCl: 29.55g added to 100ml DEPC-water

10x TBE Buffer: 0.9M Tris, 0.9M Boric acid, 20mM EDTA (pH 8.0) in dH_2O. Autoclaved before use

10x TBS (Tris Buffered Saline; pH 7.6): 0.2M Tris, 1.4M NaCl in dH_2O

TE Buffer (pH8.0): 10mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0). Autoclaved before use

0.1M Triethanolamine (pH 8.0): 13.3ml Triethanolamine (Fluka) dissolved in 800ml DEPC-water, volume adjust to 1L, using sterile glassware

2.2 Methods

2.2.1 Animal maintenance

All studies were performed to the highest standards of humane animal care under the aegis of the United Kingdom Animals (Scientific Procedures) Act, 1986.

2.2.1.1 Rats

Studies employing rats were conducted in the Biological Research Facility (BRF) at the Western General Hospital (WGH). Female Wistar rats (200–250 g; Charles River) were maintained under conditions of controlled lighting (lights on 07.00–19.00 h) and temperature (21-22°C) and allowed *ad libitum* access to water and chow (CRM(E), Special Diets Services). After a week of acclimatization, rats were timed mated. A single virgin female was housed with a sexually-experienced male in breeding cages until an expelled vaginal plug was noted, designated embryonic day (E) 0. Females were then housed singly throughout pregnancy until delivery, which occurred on E21–22. Pregnant females were injected subcutaneously with a solution of 100μg/kg/day dexamethasone (DEX) in 0.9% NaCl containing 4% ethanol or with an equivalent volume of vehicle (VEH) between 09.00 and 11.00 h on E14-21, inclusive. On the day of birth, pups were weighed, sexed and culled to 8 pups per litter retaining equal numbers of each sex, where possible. Pups were then weighed weekly until weaning, which occurred on postnatal day (P) 21. Following weaning, pups were housed in single-sex, sibling groups of 2-4 and left undisturbed until testing, apart from routine cage maintenance, which involved a weekly change of cage.

2.2.1.2 Mice

Studies employing mice were conducted in the BRF at the WGH or at Little France (LF). Where necessary for breeding, C57BL/6J mice were purchased from Harlan. Mice were maintained under conditions of controlled lighting (lights on 07.00–19.00 h) and temperature (21-22°C) and allowed *ad libitum* access to water and food (CRM(E) under all conditions in the BRF at the WGH; in the BRF at LF: RM3(E) during breeding and RM1(E) under normal maintenance conditions). The housing environment of the mice will be mentioned later in relation to individual experiments.

2.2.1.2.1 Generation of 11β-HSD2 null mutant mouse

A null mutation of the 11β-HSD2 locus was successfully generated by replacing the genomic fragment encompassing exons 2–5 with a neomycin resistance cassette through homologous recombination in mouse 129 embryonic stem cells (Kotelevtsev et al., 1999). Cells exhibiting homologous recombination were injected into blastocysts, which ultimately produced chimeras and 11β-HSD2−/− transgenic lines (intercrossed onto an outbred MF1 background). The targeted 11β-HSD2 allele has now been backcrossed for more than 10 generations onto the C57BL/6J strain. In homozygous-breeding experiments male and female 11β-HSD2 null or wild-type mice were pair-housed in breeding cages with bedding for nest building. In heterozygous-breeding experiments, male and female 11β-HSD2 heterozygous mice were pair-housed in breeding cages as above. Following weaning (P21) offspring pups were housed in single-sex, sibling groups of 2-6 and left undisturbed until testing, apart from routine cage maintenance.

2.2.1.2.2 Genotyping

Ear notches were taken at time of weaning and tail tips at time of cull for genotyping. Both were stored at -20°C until DNA extraction. Extraction was performed using two separate reagent kits: NucleoSpin® Tissue (Macherey-Nagel) or DirectPCR Lysis Reagent (Viagen Biotech) with proteinase K. Genotyping was performed by polymerase chain reaction (PCR) using a standard protocol and primers designed by Dr Janice Paterson. The following primers were used: B2Forw, AACGGGCTCCAAGTTGAGTC; B2Rev, GCTTCAGGCGAGGAGAACAGAGGTCACG; NEORev, CGCTTCCTCGTGCTTTACGGTATCGCCGCTCC (Eurogentec), to target exon 1, exon 2 and the neomycin resistance cassette, respectively. PCR was performed in a 100μl reaction mixture containing: 10μl 10xPCR buffer (Qiagen), 2μl dNTP mix (Invitrogen), 1μl primers (1:10), 0.5μl HotStarTaq DNA Polymerase (Qiagen), 83.5μl dH_2O and 1μl DNA (1:5). Thermal cycling conditions comprised an initial denaturation step at 95°C for 15 mins and 40 annealing and extension cycles of; 95°C for 30 secs,

55°C for 1 min and 72°C for 1 min followed by a final extension step at 72°C for 10 min in a Techne Genius thermal cycler (Jencons-PLS).

15µl PCR products were added to 2µl loading buffer and loaded onto a 1% agarose (Biowhittaker Molecular Applications) gel containing 0.5x TBE and ethidium bromide (1µl/100ml gel). A 1kb DNA ladder (Invitrogen) was run to assess size of the bands obtained. The gel was run at 100V until adequately separated and the results viewed on and photographed using the UVIpro gel documentation system (UVItec Ltd.). The presence of a 700 bp band indicates an 11β-HSD2 null mutant, a 600bp band represents a wild-type and the presence of both bands indicates an 11β-HSD2 heterozygous genotype. A representative gel picture taken under UV light is shown in Figure 2.1.

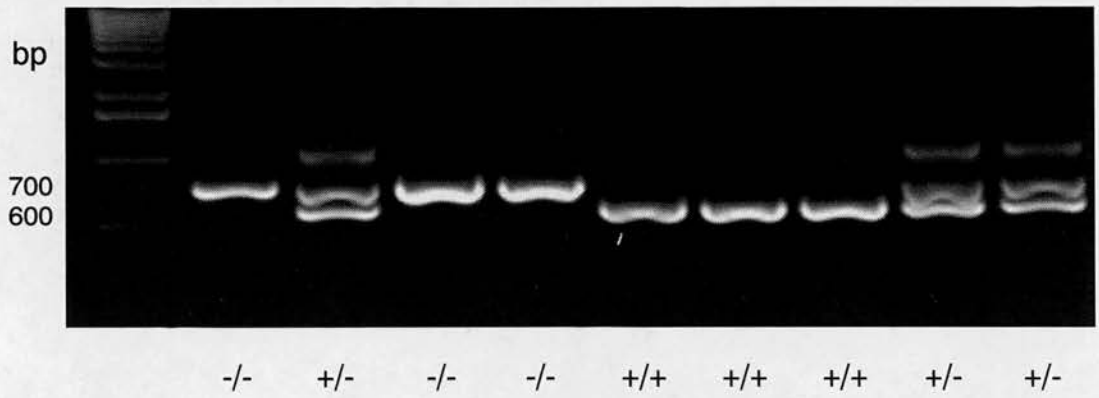


Figure 2.1 Picture of 11β-HSD2 genotyping PCR products separated by gel electrophoresis
+/+ = wild-type, +/- = 11β-HSD2 heterozygous and -/- = 11β-HSD2 null

2.2.2 Behavioural testing

All behavioural tests were conducted between 09.00 and 14.00 h, to coincide with the nadir of the corticosterone (CORT) circadian rhythm. Subjects were housed in groups of 2-3 with previous cagemates; no differences were noted in behavioural performance depending on the order run in the test. The apparatus was cleaned using 90% ethanol between subjects. Except for passive avoidance, behaviour was monitored by video camera and captured by a computer tracking programme (LimeLight™, Actimetrics Software for elevated plus maze and open field; WaterMaze™, Actimetrics Software for water maze) to allow for full spatiotemporal analysis.

2.2.2.1 Elevated plus maze

The elevated plus maze (EPM) is a common behavioural test that has been used extensively to investigate the psychological and neurochemical basis of anxiety and in the discovery of novel anxiolytic agents (Dawson & Tricklebank, 1995). The test has been validated in both rats (Pellow et al., 1985) and mice (Lister, 1987) and presents a conflict between the natural exploratory drive and the innate fear of open spaces in rodents. The EPM consists of two opposing open arms and two arms enclosed by high walls. The dimensions of the rat (Panlab) and mouse maze (made to order) used are shown in Table 2.1. The test animal was placed in the central area facing an open arm and allowed to freely explore the maze for 5 mins, whilst behaviour was recorded. The following spatiotemporal parameters were determined: the number of entries into the open and closed arms, time spent on the open arms and distance travelled on the open arms and in total.

Species	Length (cm)	Width (cm)	Height (cm)	Elevation (cm)	Material
Rat	100	10	50	64	Polymer Black arms Grey walls
Mouse	90	5	31	100	Polymer White arms White walls

Table 2.1 Dimensions and construction of the rat and mouse elevated plus maze

2.2.2.2 Open field test

The open field (OF) test measures the exploratory and anxiety-like behaviour in a large, square novel environment. It was originally described in 1934 (Hall) and has since been comprehensively used in psychopharmacology (reviewed in Prut and Belzung, 2003). The rat OF box was 100 x 100cm with 50cm high surrounding walls, and was made from grey-painted wood. The smaller mouse OF box was 60 x 60cm with 30cm surrounding walls, made from grey polymer. The OF was marked off into 25 equal squares using the tracking software. The outer rows, adjacent to the walls, are considered less anxiogenic than the inner squares. The animal was placed in the center

and allowed to explore freely for 5 mins whilst behaviour was recorded. The following parameters were determined: the total and inner zone crossings, time spent in the inner zone and distance travelled in the inner zone and in total.

2.2.2.3 Passive avoidance and modified light/dark test

The passive avoidance (PA) box (length – 39cm; width – 9.5cm; height – 16.5cm; Ugo Basile) features two chambers of equal sizes, both with gridded flooring. One chamber is made of white opaque polymer and is brightly lit, whilst the other is made of black polymer and is not illuminated. The two chambers are separated by a door which can be automatically opened and closed. On day one, subjects were placed in the light chamber facing away from the doorway, which was open, and allowed to freely explore both chambers for 5 mins, whilst recording the time spent in each, the latency to enter the dark chamber and crossings between the two. These parameters form the basis of our modified light/dark test of anxiety-like behaviour. On day two, subjects were placed in the light chamber facing away from the closed door. After 90 secs the door was opened and upon entering the dark chamber, the door closed and subjects received an inescapable 0.3mA footshock for 3 secs and were left for a further 5 secs before being removed. 6 hrs later this was repeated, although no footshock was administered. The latency to enter the dark chamber during both trials was noted and the increased latency to enter following footshock represents a measure of fear-related memory.

2.2.2.4 Water maze

Spatial learning and memory was assessed in a water maze test, pioneered by Professor Richard Morris (Morris, 1984). The water maze consistent of a circular tank (1.8m diameter, 60cm height) filled to a depth of 35cm with heated water (25 ± 1°C) made opaque with 50ml non-toxic latex liquid (Cementone-Beaver). The circular tank was arbitrarily divided into four quadrants (defined by the software) and a platform, 10cm in diameter, was submerged 2cm below the surface in the middle of one of these quadrants. The protocol employed is described in Figure 2.2. On the first two days mice were tested in the proximally-cued version of the maze where the circular platform position was indicated by a visible object with curtains drawn around the pool to exclude extramaze cues. This behavioural task requires the animal to associate the

object with escape from the water and provides a measure of associative learning. The training consisted of three trials per day for a total of five days, each trial lasting until the mouse reached the platform (escape latency) or 60 secs if the mouse had not previously escaped the water. After each trial the mice were allowed to remain on the platform for 30 secs. If the subject had not reached the platform during the 60 secs it was placed on it.

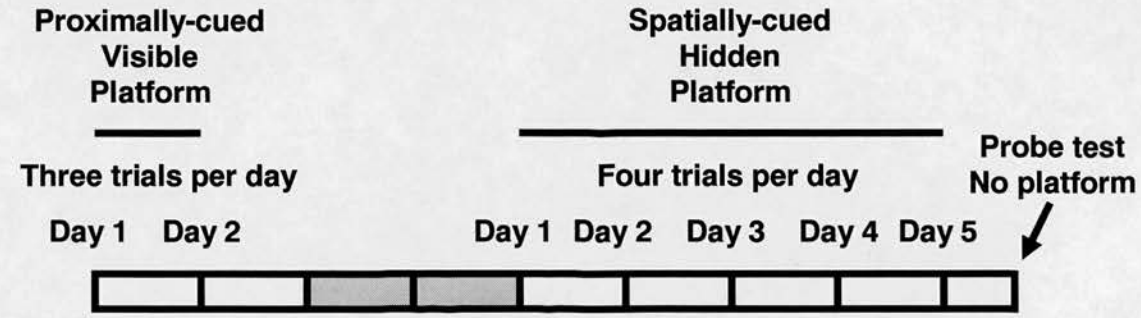


Figure 2.2 Schematic description of water maze protocol

Following two days of rest, the mice were then trained in the spatially-cued water maze in order to assess the spatial learning and memory. This consisted of four trials per day for five days, each trial lasting until the mouse reached the platform or 120 secs should the mouse not have previously escaped. After each trial the mice were allowed to remain on the platform for 30 secs. If the subject had not reached the platform it was placed on it. Mice were released in the pool from one of four equally spaced start positions along the perimeter of the pool in a predetermined, pseudorandom order. The location of the submerged platform was varied randomly from trial to trial. 90 mins following the fourth trial on the fifth day a probe test was carried out where the platform was removed. The time spent in the former platform quadrant and number of crossings of the former platform location was monitored in a 60 sec test. On all testing days, mice were returned to drying cages and warmed under a heat lamp between trials (intertrial interval 30 mins).

2.2.3 Metabolic cage study

Male rats were sibling pair housed in metabolic cages (Techniplast) at five to six weeks of age and allowed to habituate to the environment for two weeks prior to the start of collection. The animals were weighed, food and water intake, urine and faeces output

measured on four consecutive days and urine collected in 25µl 6M HCl and stored at -20°C until later analysis of CORT concentration.

2.2.4 Wheel running activity

Mice were single housed in wheel running cages (Techniplast) and allowed to habituate to the environment for one week. Activity was recorded for two weeks and data averaged over the last week with one wheel rotation equivalent to one count. The volume of water intake was also monitored to detect any polydipsia present in the 11β-HSD2 null mutant mice.

2.2.5 Blood collection, killing and harvesting of tissue

Basal blood samples were obtained in mice by tail nick at 09.00 h, at the diurnal CORT nadir and collected into EDTA-coated Microvette tubes (Sarstedt). Following 10-min restraint in a Perspex tube (30mm diameter) mice were culled by decapitation and trunk blood was collected to provide a peak stress sample. Tubes were later centrifuged (Eppendorf) at 1000*g* for 10 mins at 4°C, and then plasma supernatant was stored at –20°C. At time of killing, tissues (brain, liver, fat, right adrenal glands, tail tips) were collected, rapidly frozen on dry ice and stored at –80°C (except tail tips; stored at -20°C). Left adrenal glands were also collected in formamide, which were cleaned of fat under a dissecting microscope (Zeiss) and weighed on a semi-microbalance (Mettler). Rats were decapitated 30 mins following EPM exposure, brains were collected, rapidly frozen on dry ice and stored at -80°C.

2.2.6 Measurement of hepatic PECPK enzyme activity

2.2.6.1 Protein extraction

Approximately 100mg liver from freshly killed mice was placed in Eppendorf tubes and 1ml ice-cold 250mM sucrose/5mM HEPES buffer pH 7.4 was added. The samples were homogenised at 4°C and centrifuged at 13000*g* for 15 mins at 4°C. The cytosolic supernatant was removed into ultracentrifuge tubes and spun for 45 mins at 130000*g* at 4°C (Beckman Coulter). The supernatant was removed and aliquoted, either used for

quantification of protein concentration or stored at -20°C prior to measurement of enzyme activity.

2.2.6.2 Measurement of protein concentration

The protein concentration of tissue supernatants was determined colorimetrically using a protein assay kit (Bio-Rad) and bovine serum albumin (BSA) standards (0.05-0.5 mg protein/ml). Samples were diluted 1:50 and 1:100 in dH_2O to give concentrations in the standard range. 25µl BIO-RAD protein assay reagent A and 200µl Bio-Rad protein assay reagent B were added to duplicate 10µl aliquots of samples and standards in a 96 well plate. After 15 mins incubation at room temperature, the absorbance in each well was measured at 750nm (A_{750}) in a microplate reader. The concentration of protein in each sample was calculated from the standard curve (A_{750} vs. mg/ml protein concentration).

2.2.6.3 PEPCK enzyme activity assay

Within the liver, phosphoenolpyruvate carboxykinase (PEPCK) catalyses the decarboxylation and phosphorylation of oxaloacetate to form phosphenolpyruvate during gluconeogenesis. It also catalyses the reverse reaction (incorporation of carbon dioxide and dephosphorylation, with dGDP as the phosphate acceptor). This assay measures PEPCK activity by coupling oxaloacetate formation with its reduction by excess malate dehydrogenase to yield malate, with the equimolar oxidation of NADH. The rate of the decrease in NADH concentration can be measured spectrophotometrically and is directly proportional to the gluconeogenic activity of the PEPCK in a sample (Petrescu et al 1979). 1mg protein supernatant, diluted in HEPES/sucrose buffer (10mg/ml), was incubated at 30°C (Techne DB-3, Jencons-PLC) for 3 mins in a reaction mixture containing: 50µl NADH (3.6mg/ml), 40µl phosphoenolpyruvate (10.4mg/ml), 50µl malate dehydrogenase (1.5iu, Roche Diagnostics) and made up to 1ml with buffer (50mM HEPES pH 6.5, 50mM sodium bicarbonate and 1mM manganese (II) chloride). The reactions were initiated in a spectrophotometer (Shimadzu UK Ltd) using 0.15mM dGDP. The drop in absorbance at 340nm was followed for 3 mins. Reaction mixtures lacking bicarbonate were used as

negative controls. PEPCK activity was calculated from the rate of decrease in absorbance during the linear phase of the curve, representative of the breakdown of NADH.

2.2.7 RNA analysis of hepatic and adipose tissue

Total RNA was extracted from liver using TRIzol® Reagent (Invitrogen) and fat using RNeasy Lipid Tissue Mini Kit containing QIAzol® Lysis Reagent (Qiagen) - mono-phasic solutions containing phenol and guanidine isothiocyanate. The reagents maintain RNA integrity whilst disrupting cells and dissolving cell components.

2.2.7.1 Tissue homogenisation

Whilst still deep frozen, on dry ice, approximately 100mg tissue was added to 1ml TRIzol® (liver) or QIAzol® (fat) homogenised using a mechanical homogeniser (Ultra-Turrax T8, Ika); homogenising intermittently to avoid any sample heating.

2.2.7.2 Extraction of total RNA from liver

Total RNA was extracted with TRIzol® (Invitrogen), using a method derived from that of Chomczynski & Sacchi (1987). The mixture was allowed to stand for 5 mins at room temperature to permit the dissociation of nucleoprotein complexes. 300µl of chloroform per 1ml of TRIzol® was added and the tubes shaken for 15 secs. The mixture was allowed to stand at room temperature for another 2-3 mins and then left on ice for 15 mins. Samples were centrifuged at 13000*g* at 4°C for 15 mins and the upper aqueous phase was then transferred to another Eppendorf tube. 30µl Rnaid matrix (Anachem) was added and the mixture vortexed. Tubes were then agitated for 3-5 mins and spun at 13000*g* for 1 min to pellet. 500µl Rnaid wash (Anachem) was added and the pellet resuspended by mixing with a pipette tip and vortexing. The tubes were then centrifuged at 13000*g* for 1 min and the wash repeated twice. The resulting pellet was then resuspended in special DEPC-H_2O (10mM DTT; Rnasin), incubated at 55°C for 12 mins and then spun at 13000*g* for 1 min at 4°C. Samples were aliquoted, snap frozen and stored at -80°C.

2.2.7.3 Extraction of total RNA from fat

Total RNA was extracted from fat using the Lipid Tissue Mini Kit containing QIAzol® Lysis Reagent (Qiagen) following the protocol. The mixture was allowed to stand for 5 mins at room temperature to permit the dissociation of nucleoprotein complexes. 200µl of chloroform per 1ml of QIAzol® was added and the tubes shaken for 15 secs. The mixture was allowed to stand at room temperature for another 2-3 mins before being centrifuged at 13000*g* at 4°C for 15 mins and the upper aqueous phase transferred to another Eppendorf tube. 600µl 70% ethanol was added and tube vortexed vigorously. 700µl sample was pipetted into an RNeasy Mini Spin Column in a 2ml collecting tube and centrifuged at 9300*g* for 15 secs at room temperature with the flow-through discarded. The remainder of the sample was added and the spin repeated. 700µl Buffer RW1 was added to column and spun as before with the flow-through and collection tube discarded. The column was transferred to another 2ml collection tube. 500µl Buffer RPE (with ethanol) was added to the column and spun as before. The flow-through was discarded before another 500µl Buffer RPE (with ethanol) was added and spun at 9300*g* for 2 mins. The column was then placed in new collection tube and spun at 13000*g* for 1 min at room temperature to dry silica membrane. The column was transferred to a new 1.5ml collection tube and the RNA was eluted with 30µl nuclease-free H_2O (Promega) spun at 9300*g* for 1 min. The spin was repeated to elute the remaining RNA. Samples were then aliquoted, snap frozen and stored at -80°C.

2.2.7.4 Quantification and agarose gel electrophoresis of extracted RNA

2.2.7.4.1 RNA qualification

Agarose gel electrophoresis was used to ensure that the RNA was intact. A 1% agarose (Biowhittaker Molecular Applications) gel was used containing 0.5x TBE and ethidium bromide (1µl/100ml gel) using RNase-free materials. 1µl of RNA was loaded and electrophoresis performed in 0.5x TBE. The gel was run at 100V for approximately 1 hr and the results viewed on and picture taken using UVIpro gel documentation system. The presence of intact 28S, 18S and 5S ribosomal RNA bands indicated that the preparation was undegraded.

2.2.7.4.2 RNA quantification

2.2.7.4.2.1 GeneQuant

Prior to Northern blot analysis, RNA extracted from liver was quantified using a GeneQuant (Amersham Pharmacia) RNA/ DNA Calculator. 1µl of resuspended RNA from each sample was diluted with 99µl DEPC-H_2O and its UV absorbance at 260 and 280nm measured. The absorbance at 260nm (A_{260}) and 280nm (A_{280}) were determined to assess concentration and purity. Only RNA with an A_{260}/A_{280} of 1.8-2.0 was used.

2.2.7.4.2.2 Quant-iT™ RiboGreen® RNA assay

Prior to use in real-time PCR, RNA extracted from liver and fat was quantified using Quant-iT™ RiboGreen® RNA Reagent and Kit (Invitrogen). RNA standards were prepared in the linear range of 1-50ug/ml and samples were diluted 1:3000 (liver) and 1:500 (fat) in TE buffer to fall within the standard curve. 5µl standard or sample and 195µl reagent were added to a 96 well plate and incubated for 15 mins at room temperature. The concentration of each sample was estimated by comparison with the standard curve.

2.2.7.5 Northern analysis of RNA

2.2.7.5.1 RNA electrophoresis and capillary transfer

All electrophoresis equipment was soaked prior to use in NaOH for 30 mins and rinsed in DEPC-H_2O. Total RNA was separated by electrophoresis on a 1.2% agarose formaldehyde denaturing gel. A 100ml gel was prepared by melting 1.2g of agarose in 88ml DEPC-H_2O, adding 2ml formaldehyde and 10ml of 10x MOPS buffer, and pouring into a gel mould with appropriately sized combs in place. The gel was allowed to set and was then pre-run in 1x MOPS buffer in a gel tank for 10 mins at 60V. RNA was prepared for electrophoresis by aliquoting 10µg of RNA, with DEPC-H_2O to bring the total volume to 10µl, then adding deionised formamide (10µl), formaldehyde (2.5µl) and 10x MOPS buffer (2.5µl) to give a total volume of 25µl. The sample was mixed and the RNA denatured by incubating at 65°C for 15 mins. Ethidium bromide was added to the loading buffer (1µl/50µl loading buffer) and 2µl of this mix added to each sample of denatured RNA. The RNA was then loaded into the wells on the gel and

electrophoresed at 60V for 2-3 hrs until the front band of the loading buffer was ¾ of the way down the gel. The gel was soaked in 20x SSC buffer for 15min before blotting onto a nylon membrane (Hybond N, Amersham Biosciences). A wick of Whatman 3MM filter paper was placed over an upturned gel mould in a plastic tray containing 20x SSC buffer and the gel was placed on the top. A piece of nylon membrane cut to the same size as the gel and pre-wetted in 20x SSC was smoothed on top of the gel and this was covered with 3 layers of 3MM filter paper and approximately 5cm of paper towels. A plate of glass was placed on the top to secure the apparatus, and a weight balanced on the top. Capillary transfer was allowed to take place overnight at room temperature. The next day the membrane was washed in 20x SSC to remove any gel and the efficiency of transfer was checked by photographing the gel and the membrane under UV light (λ=254nm). The membrane was dried between two sheets of 3MM filter paper at 80°C for 30 mins, and the RNA cross-linked under UV light (Spectronics Corporation).

2.2.7.5.2 Hybridisation to ^{32}P-labelled cDNA

The nylon membrane was placed in Hybaid hybridisation bottle containing 10ml 20% SDS and 20ml phosphate buffer that had been warmed to 55°C (Techne Hybridiser HBID). To this was added denatured salmon sperm DNA (10mg/ml; 125µl). The membrane was prehybridised in a Hybaid hybridisation oven at 55°C for at least 2 hrs. 200µl ^{32}P-labelled cDNA probes (prepared as described below) were added to the prehybridisation buffer, and the membrane hybridised with the probe overnight at 55°C. The probe was disposed of and the membrane was washed as follows: three washes in 1x SSC: 0.1% SDS at 55°C for 30 mins, and two washes in 0.3x SSC: 0.1% SDS at 55°C for 30 mins. The membrane was wrapped in parafilm and exposed to a Fujifilm imaging screen for a variable duration depending on the probe used (see Table 2.2). The level of hybridized probe was quantified using a Fuji FLA2000 fluorescent image analyzer. The level of expression of the RNA of interest was then expressed as a ratio of the signal of the RNA of interest to the signal for eukaryotic ribosomal 18S RNA. Membranes were also exposed to Kodak Biomax MS film at -70°C for a variable

duration (see Table 2.2). Membranes were stripped after probing by washing in 0.1x SSC: 0.1% SDS at 67°C for 30 mins – 2 hrs and stored at -20°C before reprobing.

Probe	Specific Activity (cpm/μl)	Phosphoimager Screen Exposure Time	Autoradiographic Film Exposure Time
PEPCK	285 K	10 mins	2 hrs
11β-HSD1	210 K	15 mins	4 hrs
18S	54 K	15 secs	1 min

Table 2.2 ^{32}P-labelled probes, specific activity and exposure time
^{32}P-labelled probes used for Northern blot analysis (PEPCK – phosphoenolpyruvate carboxykinase; 11β-HSD1 - 11β-hydroxysteroid dehydrogenase type 1; 18S ribosomal RNA), their specific activity (200μl used of each) and exposure time on phosphoimager screen and Kodak Biomax MS film

2.2.7.5.3 ^{32}P labeling of cDNA

Approximately 25ng of DNA probe fragment of the target gene was aliquoted into an Eppendorf tube, diluted to 45μl with DEPC-H_2O and denatured at 100°C for 10 mins. The tube was cooled on ice and briefly centrifuged to bring the contents to the bottom. Rediprime™ II random prime labelling kit (5μl) and α^{32}P-dCTP (5μl) (both Amersham Biosciences) were added to give a total reaction volume of 55μl and the reaction incubated at 37°C for 1 hr. Unincorporated radioactivity was removed by passing the mixture over a NICK column (Amersham Biosciences). The NICK column was prepared by washing with 3ml TE before the reaction mixture was applied to the column. The column was washed with 400μl TE, the elutant discarded and the labelled DNA eluted from the column with a further 400μl TE. The specific activity of the probe was measured by mixing 2μl of probe with 1ml of scintillant (Zinsser Analytic) and counting in a beta-counter (PerkinElmer) for 2 mins. Probes were used if the specific activity was greater than 50,000cpm/μl and are shown in Table 2.2. The DNA probe was denatured before use by heating to 100°C for 5 mins followed by snap-cooling on ice for 5 mins.

2.2.7.6 Reverse transcriptase PCR

First strand cDNA synthesis was performed using a Reverse Transcription System (Promega). 500ng RNA (quantified as described above) was added to 0.2ml PCR tube. Nuclease-free H_2O was added to make volume up to 10µl. Samples were denatured at 70°C for 10 mins and kept on ice. 10µl PCR mastermix was prepared with: 4µl $MgCl_2$ (Promega), 2µl reverse transcription buffer, 2µl dNTP mix (Invitrogen), 1µl random primers (Promega), 0.5µl Rnasin (Promega), 0.5µl AMV reverse transcriptase (Promega) and samples were reverse transcribed under conditions of 42°C for 15 mins, 95°C for 5 mins and 4°C for 5 mins in a G-storm Thermal Cycler (Gene Technologies).

2.2.7.7 Real-time PCR

Real-time PCR exploits the 5'-3' exonuclease activity of *Taq* DNA Polymerase to cleave a probe during the PCR reaction. The *Taq*man® probes used in our studies incorporate 6-carboxyfluorescin (FAM) reporter dye covalently attached at the 5' end and nonfluorescent quencher (NFQ) at the 3´ end of the probe. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence. During the PCR reaction, if the target of interest is present the probe specifically anneals between the forward and reverse primer sites. The 5'-3' exonuclease activity of *Taq* DNA Polymerase then cleaves the probe separating the reporter dye and the quencher dye, which results in increased fluorescence of the reporter. Accumulation of PCR products is detected directly using the Roche Lightcycler® 480 Real-Time PCR System, which monitors the increase in fluorescence of the reporter dye. The cleaved probe fragments are displaced from the target, and polymerisation of the strand continues. This process occurs in every cycle and does not interfere with the exponential accumulation of product. The increase in fluorescence signal is detected only if the target sequence is complimentary to the probe and is amplified during PCR. Because of these requirements, any non-specific amplification is not detected.

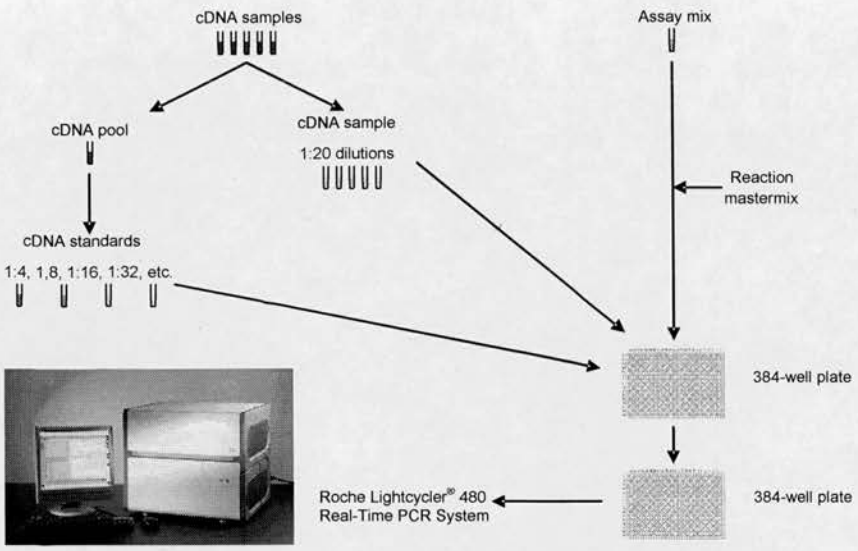


Figure 2.3 Schematic representation of real-time PCR set-up

Data analysis was carried out using Lightcycler® 480 Software. Absolute quantification analysis uses the sample crossing point (Cp) to determine the concentration of target gene. The Cp value is the cycle at which the fluorescence of the sample rises above the background fluorescence and corresponds to the maximum of the second derivative of the curve. The more target gene present in the sample the earlier it will be detected in the PCR cycles and the lower the Cp. A standard curve is constructed using serial dilutions of cDNA. Relative target gene mRNA abundance in samples is extrapolated from the standard curve (Cp value vs. target gene abundance) and expressed in arbitrary units. Target gene data are then normalised to an endogenous control, TATA-box binding protein (TBP) or eukaryotic 18S rRNA (18S), and calibrated to the control group in the study. The details of the real-time PCR assays used in this study are described in Table 2.3.

cDNA (standards and samples) and assay mastermix were added to 384-well plate and then centrifuged (Megafuge 3.0R, Heraeus Sepatech) at 1000*g* for 2 mins at 4°C to mix. The PCR was performed in a 10µl reaction mixture containing 5.0µl of LightCycler® 480 Probes Master (Roche Diagnostics), 2.5µl of LightCycler® 480 Probes Master H_2O, PCR-grade, 0.5µl *Taq*Man® Gene Expression Assay for the target gene (see Table 2.3 (Applied Biosystems) and 2µl of template (1:20 dilution of cDNA synthesised as

previously described) in a 384-well plate (LightCycler® 480 multiwell plate 384, Roche Diagnostics). In order to generate a standard curve; an aliquot of cDNA from each sample was collected and used to form stock cDNA. Serial dilutions (1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256) of the stock cDNA were included as standard samples on the plate in a similar manner to test samples. Non-template negative controls were also constructed by substitution of nuclease-free H_2O for cDNA. Each sample was assayed in triplicate. Thermal cycling conditions comprised an initial denaturation step at 95°C for 5 mins and 50 cycles of; 95°C for 10 secs and 60°C for 30 secs.

Target Gene	Assay	Accession No.	Amplicon Length
GR	Mm00433832_m1	NM_008173.2	68
PEPCK	Mm00440636_m1	NM_011044.1	88
11β-HSD1	Mm00476182_m1	NM_008288.1	67
LPL	Mm00434764_m1	NM_008509.1	61
18S	Hs99999901_s1	X03205.1	187
TBP	Mm00446973_m1	NM_013684.2	73

Table 2.3 TaqMan® Gene Expression Assays
Gene expression assays (GR – glucocorticoid receptor; PEPCK – phosphoenolpyruvate carboxykinase; 11β-HSD1 - 11β-hydroxysteroid dehydrogenase type 1; LPL – lipoprotein lipase; 18S ribosomal RNA; TBP – TATA binding protein) used in real-time PCR quantification of target gene mRNA expression

2.2.8 ^{35}S *In Situ* hybridisation histochemistry

In situ hybridisation histochemistry allows for the visualisation and quantification of specific gene transcripts by hybridisation of a ^{35}S-UTP labelled 'antisense' RNA probe to the mRNA of interest. ^{35}S-UTP labelled 'sense' RNA probes of similar length, nucleotide content and specific activity were included in experiments in order to assess the specificity of the hybridisation seen. Only RNase free, sterile solutions and equipment were used to prevent degradation of target mRNA by exogenous RNases.

2.2.8.1 Slide preparation

Slides were racked and washed in the following series of solutions; 0.2M HCl for 3 mins, DEPC-H_2O for 3 mins, 2% 3-aminopropyltriethoxysilane (dehydrated by filtering through $NaSO_4$) for 10 secs, acetone for 3 mins (twice), and finally DEPC-H_2O for 3 mins. Slides were air-dried for 30-60 mins before baking at 50°C (Gallenkamp Fan Incubator, Weiss-Gallenkamp) for 4-16 hr. Dried slides were wrapped in aluminium foil and stored for up to 3 months.

2.2.8.2 Tissue section preparation

Whole brains were routinely frozen on dry ice immediately after dissection from the animal and stored at -80°C until required. Brains were later placed in the cryostat (Leica) chamber at -20°C and allowed to equilibrate for approximately 30 mins. Following equilibration, the brains were embedded in Cryo-m-bed embedding compound (Bright Instruments) and positioned in the correct orientation for sectioning. 14μm thick coronal sections were collected and transferred to the prepared slides, and stored at -80°C until required.

2.2.8.3 Synthesis of ^{35}S-UTP labelled riboprobes

2.2.8.3.1 Preparation of cDNA templates by PCR

cDNA templates were prepared for various genes of interest, to be used subsequently for the synthesis of RNA probes for *in situ* hybridisation analysis, using one of three methods:

Method 1 - For MR, GR, CRH and AVP, plasmid vectors (pGem3/4; Bluescript KS) containing the cDNA fragment of interest were linearised using digestion by the appropriate restriction enzyme (see Table 2.4). The template was then purified using a QIAquick PCR Purification Kit (Qiagen).

Method 2 – For serum/glucocorticoid-regulated kinase 1 (Sgk1), FK506 binding protein 5 (Fkbp5) and brain-derived neurotrophic factor (BDNF), a standard PCR method was used to generate cDNA templates. Mouse brain cDNA was used as a template for a

'floppy' PCR where primers included 5' extensions containing phage polymerase consensus sites, with antisense and sense primer pairs incorporating T3 (ATTAACCCTCACTAAAGGGA) and T7 (TAATACGACTCACTATAGGG) sites, respectively (see Table 2.5). Thermal cycling conditions comprised an initial denaturation step at 95°C for 15 mins and 35 annealing and extension cycles of: 94°C for 45 secs, a variable annealing temperature (Sgk1 55°C; Fkbp5 50°C; BDNF 48°C) for 30 secs and 72°C for 45 secs followed by a final extension step at 72°C for 10 min in a G-storm thermal cycler. cDNA was pooled from several PCR reactions for each template and purified as above.

Probe	Antisense		Sense		Vector/Insert	Position (bp)	Length (bp)
	Restr Enzyme	RNA Polym	Restr Enzyme	RNA Polym			
MR	HindIII	SP6	EcoRI	T7	pGem4/ EcoRI	2769-3282	514
GR	AvaI	T7	EcoRI	SP6	pGem3/ Pst1-EcoRI	1743-2364	622
CRH	XbaI	T3	HindIII	T7	Bluescript KS/ PvuII-BamHI	1525-2043	519
AVP	EcoRI	SP6	HindIII	T7	pGem3/ HindIII-EcoRI	135-575	440

Table 2.4 *In situ* hybridisation riboprobe synthesis from plasmid vectors containing the cDNA of interest
Plasmid vectors were linearised with appropriate antisense or sense restriction enzyme and cDNA was then reverse transcribed with appropriate RNA polymerase to synthesise specific riboprobe. MR = mineralocorticoid receptor; GR = glucocorticoid receptor; CRH = corticotrophin-releasing hormone; AVP = arginine vasopressin

Method 3 – For c-fos, rat brain cDNA template was prepared by a nested PCR method. An initial PCR product was generated using the thermal cycling conditions detailed above, with an annealing temperature of 50°C, using nested primers. The product was used as the template for a second PCR reaction using 'floppy' primers. This PCR product was purified by QIAquick Gel Extraction Kit (Qiagen). Using this second purification method, the sample was first electrophoresed on a low melting point agarose gel. The DNA fragment was visualised under UV light, excised from the gel

using a scalpel, purified from the gel using the QIAquick Gel Extraction Kit and resuspended in 50µl DEPC-H_2O.

Recovery of the DNA fragment was assessed by electrophoresis of 1µl of the DNA solution through an agarose gel with a DNA mass ladder (Invitrogen) to obtain an approximate concentration of the purified cDNA fragment. Riboprobes cDNA fragments were checked by sequence analysis if they had not previously been used in our laboratory.

Probe	Accession nr	Position (bp)	Length (bp)	Primers (5' → 3')
c-fos	X06769	1203-1711	509	F – GCCCACCGAAAGGGCAGC R – GCTGCACTAGATACAATC
Sgk1	NM_011361	205-868	664	F – CCTATGCATGCAAACACGCTGAAGTTC R – TCAGGAGCCAGATACTCAGGCGTG
Fkbp5	NM_010220	672-1294	623	F – CTGGAAGGCTGCTGTGG R – GCCAACACCTTCTCGAAGTCG
BDNF	AY57908	843-1362	520	F – TGGCTGACACTTTTGAGC R - CTTATGAATCGCCTGCC

Table 2.5 cDNA template synthesis for the generation of *in situ* hybridisation riboprobes
Mouse (Sgk1, Fkbp5 and BDNF) or rat (c-fos) brain cDNA was used to generate cDNA templates of 509-664 bp in length using specific forward and reverse primers in a PCR. RNA polymerases were then used to synthesise riboprobes: T3 for antisense and T7 for sense. Sgk1 = serum/glucocorticoid-regulated kinase 1; Fkbp5 = FK506 binding protein 5; BDNF = brain derived neurotrophic factor; F = forward; R = reverse

2.2.8.4 ^{35}S-UTP labelling of cDNA

0.5-1µg of cDNA template was transcribed by incubation at 37°C (or 40°C for SP6 RNA Polymerase) for 60-90 mins in a reaction mixture containing 1mM ATP, CTP and GTP, 4µl ^{35}S-UTP (1.48 MBq/µl; Amersham Biosciences), 10mM DTT (Merck Biosciences), 0.4µl RNasin (40U/µl; Promega), and 1µl appropriate RNA polymerase (Promega) (see Table 2.4 and 2.5) in a total volume of 10µl 1x transcription optimised buffer (Promega). Following incubation, 1µl DNase 1 (RNase-free; Promega) was added and reactions incubated at 37°C for a further 10-15 mins to degrade the DNA

template, after which probes were placed on ice for 1-5 mins and purified using NICK columns, to separate unincorporated radioactivity. The column was prepared by washing through with 3ml TE buffer (pH 8.0). The probe mixture was then applied to the column. The column was washed with 400µl TE buffer and the initial elutant discarded. The radiolabelled probe was then eluted in an additional 400µl TE buffer.

For each probe, the total activity was estimated by counting 1µl of probe in 1ml scintillant fluid in a beta-counter (minimum activity required $2x10^5$ cpm/µl). Probes were stored at -20°C until required, for up to 3-4 weeks.

2.2.8.5 Tissue preparation and fixation

Slides were removed from the -80°C freezer and kept on dry ice until the start of the fixation procedure. Slides were fixed at 4°C in 4% paraformaldehyde in 0.1M phosphate buffer for 10 mins (to maintain tissue morphology and inhibit endogenous ribonucleases). Slides were rinsed twice in 1x PBS for 5 mins, acetylated in 0.1M triethanolamine with 0.25% acetic anhydride for 10 mins (to reduce non-specific binding of the probe to positively charged amino groups in tissues) and rinsed in 1x PBS for 3 mins. Following dehydration through a series of ethanol solutions (70, 80 and 95% ethanol in DEPC-H_2O) slides were air-dried for a minimum of 30 mins.

2.2.8.6 Prehybridisation and hybridisation

Following fixation, slides were pre-hybridised with 200µl/slide of 2x pre-hybridisation buffer diluted 1:1 with deionised formamide, at 50°C for 2 hrs. Dampening two layers of Whatman 3MM chromatography paper with box buffer humidified the slide boxes, preventing tissue sections from drying out. Probes were thawed and added to 2x hybridisation buffer diluted 1:1 in deionised formamide to give a final probe concentration of $10x10^6$ cpm/ml. Probes were denatured at 75°C for 10 mins and snap-cooled on ice before addition of 10mM DTT (10µl/ml probe solution). Pre-hybridisation buffer was drained from slides and 200µl appropriate probe in hybridisation buffer was applied to slides. Slides were hybridised in sealed, humidified boxes at 50°C for approximately 16 hrs.

2.2.8.7 RNase treatment and washes

Following hybridisation, slides were washed three times in 2x SSC for 5 mins and carefully wiped dry around the sections with lens tissue. 200µl of RNase A (30µg/ ml in Rnase buffer, Roche Diagnostics) were applied to each slide and slides were then incubated at 37°C for 1 hr in humidified boxes (1 layer of Whatman 3MM chromatography paper dampened with Rnase buffer) to remove unhybridised probe.

Following RNase treatment, slides were washed in 2x SSC at room temperature for 1 hr, then in 0.1x SSC at 60°C for 1 hr, and finally in 0.1x SSC heated to 60°C and allowed to cool to room temperature for the duration of the wash (1 hr). Following the washes, slides were dehydrated through a series of ethanol solutions containing 0.3M ammonium acetate (2 mins in each of 50, 70 and 90% ethanol) and air-dried.

2.2.8.8 Visualisation of hybridisation

Slides were exposed to Kodak Biomax-MR film for 1-10 days depending on the specific activity probe used and abundance of the gene transcript.

2.2.8.9 Image analysis

For image analysis autoradiographic films were scanned on a high resolution flatbed scanner and any damaged or inadequate sections were excluded. The background was low with sense section backgrounds having a greyscale level not significantly different to zero. Digital image analysis was performed using MCID image analysis system (InterFocus Imaging) by optical densitometry. The appropriate section was selected per animal, and ten measurements were taken per area (five from each hemisphere), with the background subtracted. The specific signal was measured in several different regions specified in the individual chapters.

2.2.9 Corticosterone radioimmunoassay

500µl urine was treated by ethyl acetate extraction and reconstituted in 200µl borate buffer and was run undiluted whilst plasma was diluted 1:10 in borate buffer containing 0.5% BSA. Samples were heated to 75-80°C for 30 mins to denature CORT-binding globulin and dissociate the steroid. ^{3}H-CORT was suspended in borate buffer to give between 10000 and 15000cpm per 50µl solution on the β-counter. Rabbit anti-CORT antibody was added to this mix in a 1:100 dilution. 20µl aliquots of diluted plasma sample or CORT standards, prepared in the linear range from 0.625-320 ng/ml, were incubated in duplicate with 50µl ^{3}H-CORT/primary antibody mix in 96 well plates. 50µl scintillation proximity assay beads (SPA; Amersham Biosciences) diluted with borate buffer was then added to each well, the plates were then sealed, shaken and left overnight at room temperature to equilibrate. Plates were counted on the beta-counter and the concentration of CORT in each sample was calculated from a graph of cpm versus [CORT] standards generated using the Multicalc programme (PerkinElmer). Intra- and inter-assay coefficients of variation were 9.4 and 9.2% respectively. In this assay, in comparison with CORT (100%), the cross-reactivities for progesterone, deoxyCORT and cortisol are 7.7, 6.5 and 5.3 %, respectively (Kenyon, 1993).

2.2.10 Glucose tolerance test

Adult male mice were fasted overnight (16 hrs) and weighed before and after the fasting period. Following the fast, subjects were given an intraperitoneal injection of D-glucose (2mg/g body weight in saline). Blood samples (15-20µl) were taken by tail venesection into EDTA-coated Microvette tubes at 0 min (just prior to glucose injection) and at 5, 15, 30, 60 and 120 mins following glucose load. Blood samples were centrifuged, plasma supernatant collected and stored at -20°C until glucose levels were assayed.

2.2.11 Glucose assay

Glucose was determined by an enzymatic method using the Infinity™ Glucose Hexokinase Liquid Stable Reagent (Thermo Fischer Scientific). Glucose standards (0-45 nM) were freshly prepared prior to the glucose assay. 1µl of plasma or glucose standard was added to 180µl reagent, in duplicate on a 96-well plate. The samples were

left to incubate at room temperature for 15 mins and then counted at 340nm in a plate reader. The sample glucose concentration was then calculated from the standard curve.

2.2.12 Statistical analysis

Data are expressed as mean±SEM. Comparisons between groups were analysed by the most appropriate method including either an unpaired t-test, one and two-way analysis of variance (ANOVA) or a repeated measures (RM) one-way ANOVA, with Student Newman-Keuls (SNK) *post-hoc* testing; $p<0.05$ was considered significant.

CHAPTER 3
EFFECT OF PRENATAL DEX ON THE HPA AXIS, BRAIN AND ANXIETY-RELATED BEHAVIOUR

3.1 Introduction

It has previously been reported that prenatal dexamethasone (DEX) treatment during the final trimester of fetal life resulted in hypothalamic pituitary adrenal (HPA) axis hyperactivity, hypertension and heightened anxiety-related behaviour in rat offspring (Levitt et al., 1996; Welberg et al., 2001; O'Regan et al., 2004). Plasma corticosterone (CORT) was found to be significantly elevated at the diurnal nadir, although in male DEX offspring only (O'Regan et al., 2004). However, following stress activation a similar response in DEX and vehicle (VEH) control offspring was seen, suggesting no stress-induced HPA axis hyperactivity was present (Nyirenda et al., 2001). More recently, blood pressure (BP) was measured in DEX and VEH offspring by radiotelemetry, which allows for remote quantification of BP without any necessary subject manipulation. Findings contradicted the hypertension previously detected by tail-cuff plethysmography, an undoubtedly stressful method (Levitt et al., 1996), and observed a resting decrease in BP in DEX offspring (O'Regan et al., unpublished observations). However, when disturbed and restrained an enhanced rise in BP was evident in DEX offspring indicating a heightened sensitivity to stress rather than underlying hypertension (O'Regan et al., unpublished observations). The stress-induced hypertension was blocked by reserpine, implicating increased sympathetic drive as the mechanism (O'Regan et al., unpublished observations). Prenatal DEX has also been found to increase anxiety-related behaviour when measured in the elevated plus maze (EPM) and open field (OF) and increased sympathetic drive may contribute.

In this chapter we aimed to investigate three aspects of the DEX-programmed rat as follows:

1.) We wished to explore the basal HPA axis activity in DEX offspring in order to confirm the previous indication of raised basal function. Therefore, male VEH and DEX offspring were housed in metabolic cages and following two weeks habituation we collected urine and subsequently measured CORT excretion.

2.) We wanted to extend the previous finding of enhanced stress sensitivity in DEX offspring and see whether this was also apparent in the behavioural response. The EPM presents a conflict between the natural exploratory drive and the innate fear of open spaces in rodents and has been extensively used to assess anxiety-related behaviour (Dawson and Tricklebank, 1995). In order to exacerbate differences in stress sensitivity VEH and DEX prenatally-treated offspring were subjected to either 30-min restraint stress or brief handling 24 hrs prior to testing on the EPM. This fear-potentiation has previously been elicited by several stressors including restraint, forced swim and inescapable footshock (Korte and De Boer, 2003). The timing and nature of stress exposure was selected based on the report that restraint stress exacerbated heightened anxiety-related behaviour when EPM was conducted 24 hrs, but not 1 or 2 hrs later (Padovan and Guimaraes, 2000).

3.) The transient expression of the immediate early gene, c-fos, or its encoded protein, Fos, have been extensively employed as neuroanatomical markers of activation in various situations including novelty, stressor exposure, hormone and drug treatment (Cullinan et al., 1995; Kovacs, 1998; Singewald et al., 2003; Miyata et al., 2005) and following EPM exploration (Duncan et al., 1996; Hinks et al., 1996). Furthermore, altered patterns of expression have been detected following acute restraint or EPM exposure in high and low anxiety behaviour-bred (Salome et al., 2004), neonatally-handled (Abraham and Kovacs, 2000; Park et al., 2003), prenatally-stressed (Viltart et al., 2006) and prenatal protein-restricted (Rosene et al., 2004) offspring. Therefore, we wished to investigate regional activation in prenatal VEH and DEX with the use of transient c-fos mRNA expression following EPM exposure. In particular, we were interested in the interaction of prenatal treatment with the prior acute stress.

3.2 Methods

Comprehensive details of methods can be found in chapter 2.

3.2.1 Prenatal treatment

In the biological research facility at the Western General Hospital, Wistar rats were time-mated and dams injected (sc) between 09.00 and 11.00 h on days 14-21 of pregnancy with either dexamethasone (DEX, 100µg/kg/day) or Vehicle (VEH, 0.9% NaCl-4% Ethanol). On the day of birth, pups were weighed, sexed and culled to 8 pups per litter keeping equal numbers of each sex, where possible. Pups were then weighed weekly until weaning, which occurred on postnatal day 21 (P21). After weaning, pups were housed in single-sex, sibling groups of 2-4 and left undisturbed until testing, apart from routine cage maintenance. Offspring were only subject to one of the following procedures.

3.2.2 Metabolic cage

Male rats, aged 5-6 weeks, were pair-housed in metabolic cages and, following two wks habituation, water/food ingestion and urine/faeces excretion was measured. Urine was collected and later analysed for CORT concentration.

3.2.3 Behaviour in EPM under basal and stress-potentiated conditions

At 8-9 months of age behavioural studies on the EPM were performed on male and female prenatally-treated offspring. No more than two offspring from each litter were used. All manipulations were commenced at 09.00 h. For four days, animals were brought into the experimental room and handled briefly to habituate them to the investigator and the testing environment. Following two days rest, the VEH and DEX subjects were randomly separated into two equal groups, control (CON) and STRESS. The CON group was handled in the experimental room as previously, whilst the STRESS group was subjected to restraint in plexiglass tubes for 30 mins on the day prior to behavioural testing. 24 hrs later, anxiety-related behaviour was assessed on the EPM during 5-min test, under normal lighting conditions (identical to housing rooms).

30 mins following the test, subjects were decapitated, brains collected and frozen on dry ice.

3.2.4 *In situ* hybridisation histochemistry

Coronal sections (14μm thickness) were taken from frozen male brains at the levels of the cortex, PVN, hippocampus, amygdala, raphe nucleus and locus coeruleus. *In situ* hybridisation was performed using a [^{35}S]-labeled antisense probe prepared from the c-fos cDNA template. Slides were exposed to Kodak Biomax MR film for four days and image analysis was performed by optical densitometry.

3.2.5 Statistical analysis

Data are expressed as mean±SEM, $p<0.05$ considered significant, and comparisons between groups analysed by most appropriate method: unpaired t-test, one or two-way analysis of variance (ANOVA), with Student Newman-Keuls (SNK) *post-hoc* testing.

3.3 Results

3.3.1 Prenatal treatment effects on maternal weight gain

VEH and DEX treatments were well tolerated and injections were not obviously uncomfortable for the pregnant dams. Weight gain during the final week of pregnancy (days 14-21 inclusive) was different between the two groups, with DEX-treated dams gaining significantly less weight (Table 3.1a; $p<0.05$).

	Maternal weight			Litter	Male:female	Birth Weight		
	before (g)	after (g)	gain (g)	size	ratio	group (g)	male (g)	female (g)
VEH	348.8±7.3 (n=8)	452.4±14.5	103.6±10.8	11.9±1.4	1.2±0.3	6.5±0.1 (n=95)	6.6±0.1 (n=47)	6.4±0.1 (n=48)
DEX	358.7±9.4 (n=7)	422.3±13.6*	63.6±7.7*	13.6±1.5	1.0±0.2	5.3±0.1** (n=95)	5.4±0.1** (n=46)	5.3±0.1** (n=49)

Table 3.1a Prenatal treatment effect on the dam and birthing parameters
Maternal weight before and after vehicle (VEH) or dexamethasone (DEX) treatment, maternal weight gain during the last week of pregnancy (days 14-21), litter size, male:female ratio, and group, male and female offspring birth weight. Results are presented as mean±SEM. *p<0.05; **p<0.0001 compared with VEH group.

3.3.2 Birth and growth parameters in VEH and DEX offspring

DEX treatment had no effect on gestation length (data not shown), litter size ($p=0.419$) and male:female ratio ($p=0.720$) compared to VEH (Table 3.1b). Birth weight was significantly reduced in both male ($p<0.0001$) and female ($p<0.0001$) DEX offspring compared to VEH, representing an 18.7% and 17.5% reduction, respectively. The reduction in body weight persisted through to 16-17 weeks. However, at 24-25 weeks no difference in body weight between prenatal treatment groups was observed in either male ($p=0.438$) or female offspring (Table 3.1b; $p=0.491$), suggesting catch-up growth had been achieved.

	1 wk		2 wks		3 wks	
	male (g)	female (g)	male (g)	female (g)	male (g)	female (g)
VEH	20.0±0.3 (n=32)	19.5±0.2 (n=30)	40.0±0.6	39.0±0.5	63.4±1.0	61.6±0.8
DEX	15.6±0.3** (n=23)	15.5±0.4** (n=23)	34.3±0.5** (n=27)	33.3±0.7** (n=27)	54.7±0.8**	52.8±1.0**

	4-5 wks	13-14 wks	16-17 wks	24-25 wks	
	male (g)	male (g)	male (g)	male (g)	female (g)
VEH	130.6±4.7 (n=8)	550.4±15.3 (n=8)	574.4±12.0 (n=11)	628.5±11.7 (n=15)	347.4±9.1 (n=10)
DEX	112.2±7.7* (n=8)	506.6±15.3* (n=8)	544.4±17.1* (n=11)	610.8±21.0 (n=11)	357.3±10.6 (n=10)

Table 3.1b Prenatal treatment effect on postnatal growth
Comparison of growth in prenatal vehicle (VEH) and dexamethasone (DEX) treated offspring, measured during first three weeks, and then periodically, in males and females. Results are presented as mean±SEM. *p<0.05; **p<0.0001 compared with VEH group, n=number of animals

3.3.3 Urinary CORT measurements in male VEH and DEX offspring

Following habituation to the metabolic cages, urine was collected daily for four days and CORT levels measured by RIA. The measured parameters and CORT concentration did not differ between collection days for either group so they were averaged. Urinary CORT excretion was significantly elevated in DEX offspring (Table 3.2; $p<0.05$). However, food/water intake ($p=0.235$; 0.428) and faeces/urine output ($p=0.213$; 0.596) were not significantly different between groups. DEX offspring had a reduced body weight prior to collection (Table 3.2; $p<0.05$), although the weight gain during collection was not different compared to VEH offspring ($p=0.775$).

	Initial Weight (g)	Weight Gain (g)	Intake Food (g/100g BW)	Intake Water (ml/100g BW)	Output Faeces (g/100g BW)	Output Urine (ml/100g BW)	CORT Excretion (ng/g BW)
VEH	198.5±5.2	9.38±0.36	12.9±0.6	17.2±0.8	5.8±0.2	7.2±0.4	36.6±2.2
DEX	172.9±7.2*	9.59±0.62	13.0±0.6	18.3±1.0	6.1±0.2	7.6±0.6	49.7±5.6*

Table 3.2 Daily ingestion and excretion in male VEH and DEX rat offspring
Initial weight, weight gain, daily food/water intake, urine/faeces output and CORT excretion measured daily during 4-day collection in prenatal vehicle (VEH) and dexamethasone (DEX) treated offspring. Results are presented as mean±SEM. *p<0.05 compared with VEH group. n=8 per group.

3.3.4 Anxiety-related behaviour in VEH and DEX offspring

3.3.4.1 Male offspring

Anxiety-related behaviour was measured during 5-min exploration of the EPM. Statistical analyses were performed by two-way ANOVA with SNK *post-hoc* testing, where appropriate.

There was no overall effect of prenatal treatment on the number of entries into the closed arms (Figure 3.1a; $F_{1,20}$=1.81, p=0.193), although there was a significant effect of acute treatment ($F_{1,20}$=6.55, p<0.05). *Post-hoc* testing revealed that closed arm entries were significantly reduced following prior stress in DEX offspring (DEX/STRESS) as compared to handled controls (DEX/CON) (p<0.05). There were no differences in open arm entries between VEH offspring that had been stressed (VEH/STRESS) and handled controls (VEH/CON) (p=0.442) or between VEH/CON and DEX/CON (p=0.852).

We found no overall effect of prenatal treatment (Figure 3.1a; $F_{1,20}$=0.07, p=0.784), whilst there was a tendency of acute restraint treatment ($F_{1,20}$=2.70, p=0.116) on the number of entries into the open arm. DEX/STRESS had a similar tendency for reduced open arm entries as compared to DEX/CON, but this was not significant (p=0.104). No differences were observed between any other groups.

An overall effect of prenatal treatment (Figure 3.1b; $F_{1,20}$=0.26, p=0.617) on the time spent on the open arms was absent, although a tendency of overall effect in acute treatment was observed ($F_{1,20}$=3.12, p=0.092).

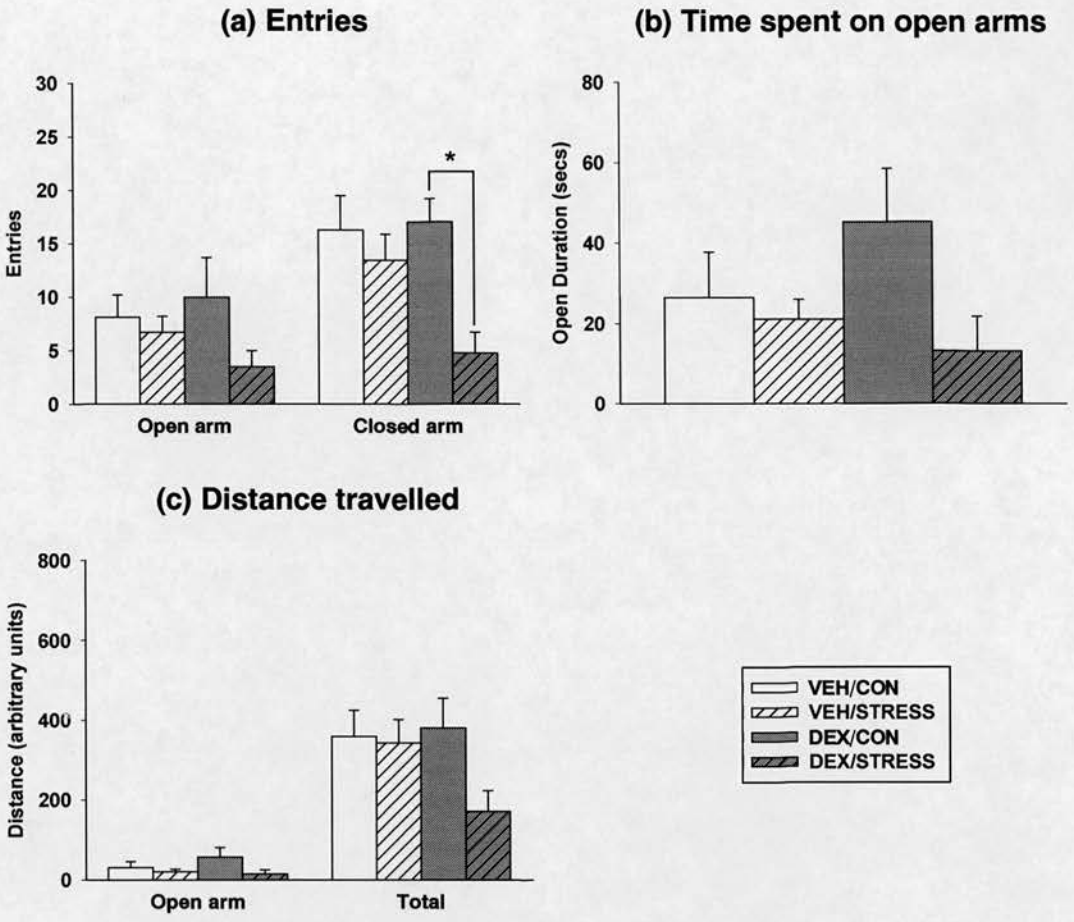


Figure 3.1 Behaviour of male VEH and DEX rat offspring in the elevated plus maze under normal and stress-potentiated conditions
Effects of prenatal dexamethasone (DEX) or vehicle (VEH) with (STRESS) or without (CON) 30-min restraint stress 24 hrs prior on (a) number of entries into the open and closed arms, (b) time spent on open arms and (c) distance travelled on the open arms and in total during a 5-min EPM test. *p<0.05 vs DEX/CON; n=4-8 per group.

No overall effect was observed in the total distance and that travelled on the open arms by prenatal treatment (Figure 3.1c; Total: $F_{1,20}$=1.18, p=0.291; Open arm: $F_{1,20}$=0.42, p=0.523). There was an overall effect tendency for acute treatment in the total and open arm distance travelled (Total: $F_{1,20}$=2.68, p=0.117, Open: $F_{1,20}$=2.81, p=0.109). *Post-hoc* testing revealed a tendency for reduced total and open arm distance travelled in DEX/STRESS compared to DEX/CON (Figure 3.1c; p=0.069; 0.102). No differences were observed between any other groups.

3.3.4.2 Female offspring

No significant differences were observed in female behaviour in the EPM. Open arm and closed arm entries (Figure 3.2a), open duration (Figure 3.2b), total and open arm distance (Figure 3.2c) were all unchanged by prenatal or acute treatment.

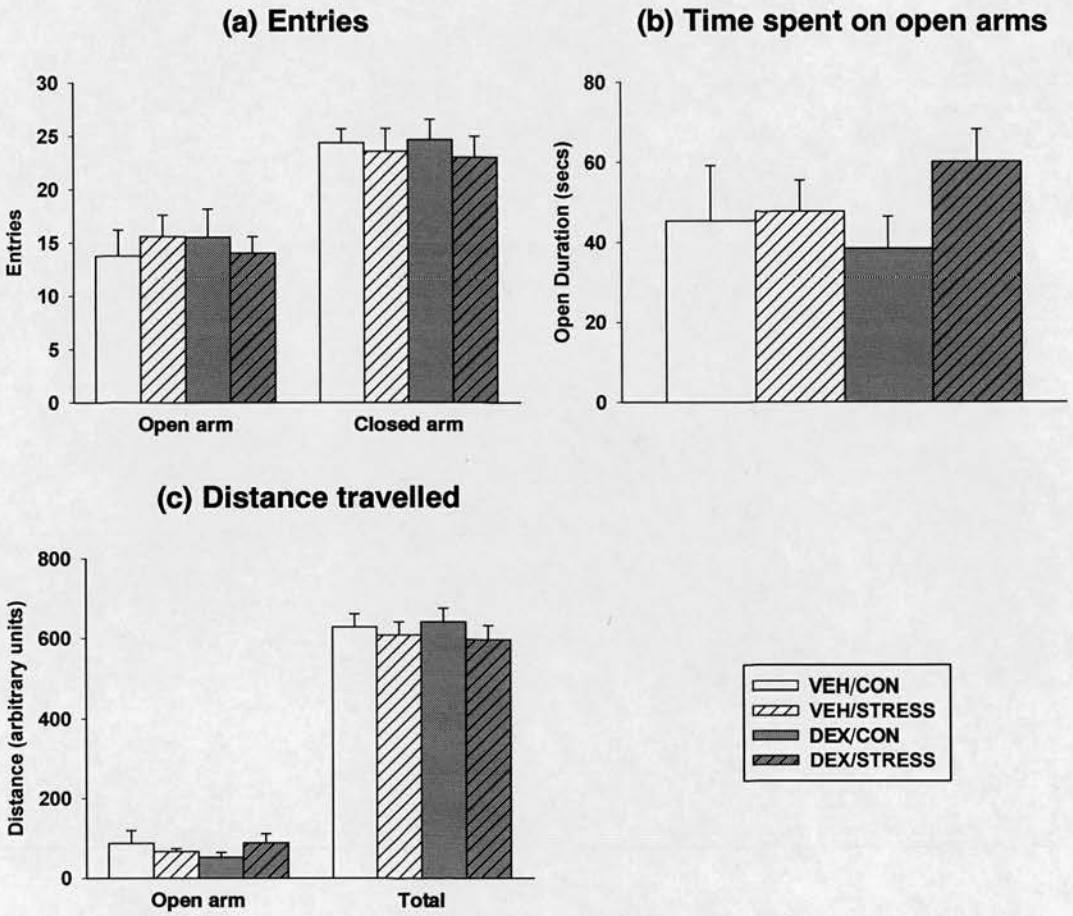


Figure 3.2 Behaviour of female VEH and DEX rat offspring in the elevated plus maze under normal and stress-potentiated conditions
Effects of prenatal dexamethasone (DEX) or vehicle (VEH) with (STRESS) or without (CON) 30-min restraint stress 24 hrs prior on (a) number of entries into the open and closed arms, (b) time spent on open arms and (c) distance travelled on the open arms and in total during a 5-min EPM test. n=5-8 per group.

3.3.5 Expression of c-fos mRNA following EPM exploration

The transient expression of the c-fos IEG is believed to be proportional to the level of cellular activation (Cullinan et al., 1995). We therefore measured c-fos mRNA expression in brains taken 30 mins following EPM exploration in male offspring in order to assess the regional brain activation elicited by this mild stressor in VEH and

DEX offspring subjected to prior stress or handled controls. Following [^{35}S] *in situ* hybridisation and exposure of slides to film, c-fos mRNA expression was analysed from a single section for each subject by optical densitometry and quantified in several regions. These regions include the cingulate and frontal cortex, PVN, hippocampus, raphe nucleus and locus coeruleus which are all implicated in the stress response. Statistical analyses were performed by two-way ANOVA with SNK *post-hoc* testing, where appropriate. Expression of c-fos mRNA in the cingulate and frontal cortex is shown in Figure 3.3.

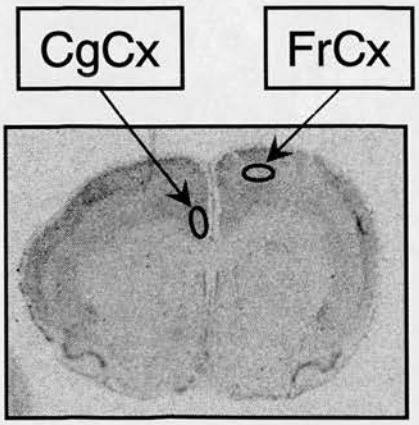


Figure 3.3 Autoradiograph of c-fos mRNA expression in male rat brain at the level of the cingulate (CgCx) and frontal cortex (FrCx)

There was a weak tendency of an overall effect of prenatal treatment on c-fos mRNA expression in the cingulate (Figure 3.4; $F_{1,21}$=2.55, p=0.125) and frontal (Figure 3.4; $F_{1,21}$=2.03, p=0.169) cortex. However, no overall effect of acute treatment was observed in either the cingulate ($F_{1,21}$=0.01, p=0.905) or frontal ($F_{1,12}$=0.14, p=0.712) cortex.

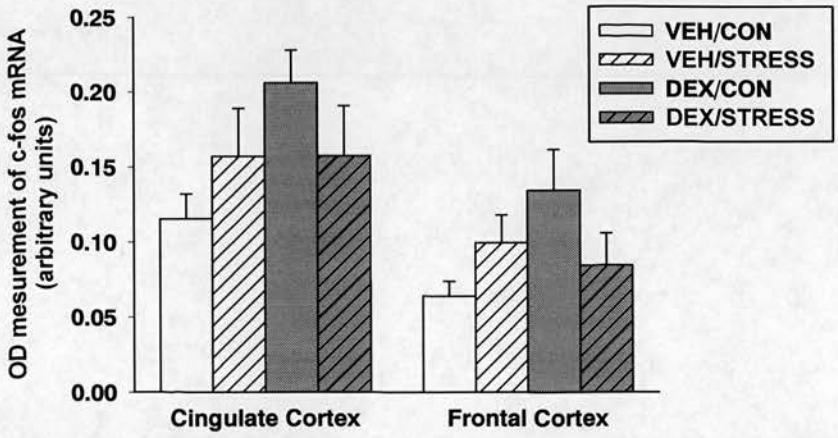


Figure 3.4 c-fos mRNA expression in cingulate and frontal cortex of male VEH and DEX rat offspring following EPM exposure
In situ hybridisation using a [^{35}S]-labeled riboprobe for c-fos mRNA. Coronal sections were taken from brains obtained 30 mins following 5-min EPM exposure in prenatal vehicle (VEH) and dexamethasone (DEX) treated offspring with (STRESS) or without (CON) 30-min restraint stress 24 hrs prior. Expression measured by optical densitometry of autoradiography film. n=4-8 per group.

In other regions quantified: hippocampus, raphe nucleus and locus coeruleus c-fos mRNA expression was evident, although no differences were detected between treatment groups (Table 3.3). In all other regions of the brain the level of expression was below our limit of detection, including PVN and amygdala.

	Hippocampus				Raphe Nucleus		Locus
	CA1	**CA2**	**CA3**	**DG**	**Median**	**Dorsal**	**Coeruleus**
VEH/CON	0.13±0.02 (n=7)	0.13±0.02	0.13±0.02	0.17±0.03	0.09±0.01 (n=8)	0.10±0.01	0.15±0.02 (n=8)
VEH/STRESS	0.13±0.03 (n=7)	0.13±0.04	0.13±0.03	0.17±0.04	0.11±0.02 (n=6)	0.12±0.02	0.12±0.02 (n=6)
DEX/CON	0.13±0.05 (n=4)	0.13±0.04	0.11±0.03	0.17±0.06	0.08±0.01 (n=4)	0.09±0.02	0.15±0.02 (n=5)
DEX/STRESS	0.14±0.02 (n=5)	0.13±0.03	0.12±0.02	0.14±0.02	0.09±0.03 (n=4)	0.09±0.01	0.16±0.02 (n=5)

Table 3.3 c-fos mRNA expression in hippocampus, raphe nucleus and locus coeruleus of male VEH and DEX rat offspring following EPM exposure
Expression measured by optical densitometry (arbitrary units). Sections were cut from brains obtained 30 mins following 5-min EPM exposure in VEH and DEX prenatally-treated offspring with (STRESS) or without (CON) 30-min restraint stress 24 hrs prior. Results are presented as mean±SEM.

3.4 Discussion

Similar to previous studies, we report a reduced birth weight in offspring following prenatal DEX treatment in the final trimester (Levitt et al., 1996; Nyirenda et al., 1998; 2001; Welberg et al., 2001; O'Regan et al., 2004; Drake et al., 2005). We observed increased urinary CORT excretion, further supporting the previously described finding of elevated HPA axis activity as a consequence of prenatal DEX exposure (Levitt et al., 1996; O'Regan et al., 2004). Exploration of the EPM following prior acute stress revealed anxiety-like behaviour in male DEX offspring compared to handled controls. In contrast, no effect of prior restraint was noted in VEH offspring. No effect of either prenatal (VEH/DEX) or postnatal (CON/STRESS) treatment was observed in female offspring. The expression of c-fos mRNA was quantified throughout the brain, which revealed enhanced induction in the frontal and cingulate cortex following EPM exposure.

Prenatal DEX administration lead to intrauterine growth restriction (IUGR) and a reduction of birth weight in both male and female offspring. We observed an 18.7% and 17.5% reduction in birth weight compared to VEH offspring in males and females, respectively. Interestingly, a large variation in the proportion of growth restriction induced by final trimester prenatal DEX treatment has been reported in studies performed in Edinburgh (8.8-25.1%) (Nyirenda et al., 1998; 2001; Cleasby et al., 2003a; 2003b; O'Regan et al., 2004; Drake et al., 2005). The difference in birth weight between VEH and DEX offspring in our study therefore reflects growth restriction in the middle of that range, indicating a robust effect of prenatal GC treatment. The reduced weight in DEX, compared to VEH, offspring persisted until approximately 6 months of age. Catch-up growth was therefore achieved in DEX offspring between 4-6 months. Previous studies have also reported differences in postnatal growth with full catch-up growth being observed by either 3 weeks (Nyirenda et al., 1998; O'Regan et al., 2004; Drake et al., 2005), 6 months (Nyirenda et al., 2001) or not at all (Cleasby et al., 2003a). Evidence from human observations indicates that catch-up growth may play a mechanistic role in prenatally programmed effects (Ong, 2006), although the involvement in experimental models is less clear. The discrepant findings

from almost identical treatment paradigms on both birth weight and postnatal growth are an indication of the sensitivity of this model to outside environmental influence. This may include the investigator performing the procedure or the general activity in the housing conditions.

Previous indications of HPA axis hyperactivity in DEX offspring have been obtained from the reports of elevated basal CORT at the diurnal nadir (Levitt et al., 1996; O'Regan et al., 2004). In this study, we detected elevated CORT excretion in DEX compared to VEH offspring, when averaged over the daily cycle, providing further evidence of enhanced basal HPA axis activity. It should be noted that Heidbreder et al. (2000) report elevated circulating CORT in rats reared in grid-floored compared to sawdust-filled cages, suggesting that housing in the metabolic cages may be a stressful experience *per se*. However, Erikkson et al. (2004) reported no increase in CORT excretion when 8 week old rats were placed in metabolic cages for three days, indicating a stress response is not always elicited. We observed no difference in CORT excretion during our four collection days in either prenatal treatment group, indicating that the offspring had habituated to the cages. Together with the previous report of no difference in the CORT response to acute restraint between DEX and VEH offspring (Nyirenda et al., 2001) and our finding of increased urinary CORT excretion over the whole daily cycle in young, immature male DEX offspring, it suggests that basal HPA axis hyperactivity is present following prenatal DEX treatment from early adulthood.

Previous studies have detected heightened anxiety-related behaviour on the EPM in male DEX offspring (Welberg et al., 2001). However, we report a lack of effect of prenatal DEX treatment on spontaneous anxiety-related behaviour on the EPM. Differences in prenatal treatment protocols could account for the apparently conflicting results. In the present study, prenatal treatment only commenced on embryonic day (E) 14 and DEX or VEH was administered daily until E21. However, Welberg et al. (2001) administered VEH from E0 onwards, and then split treatment groups on E15: continuing to administer VEH to one

group whilst dispensing DEX to the other, until E21. Indeed when DEX was administered throughout gestation offspring displayed no difference in EPM behaviour compared to VEH controls (Welberg, 1999). Saline injection *per se* acts as a mild prenatal stressor when administered in the final trimester and has been found to induce alterations in behaviour including enhanced conditioned fear (Griffin et al., 2003). Whilst the precise impact of early VEH (Saline-4% Ethanol) treatment has not been investigated, it is not impossible that it could elicit wider behavioural differences between VEH and DEX offspring. However, it is also likely that other uncontrollable, external factors such as variations in animal technicians, investigators and the general environment in the biological research facility (BRF) could contribute.

Fear-potentiation induced by a 60-min restraint immediately prior to EPM testing revealed enhanced anxiety-like behaviour in response to acute stress in PS offspring (Estanislau and Morato, 2005). The behavioural reaction to acute stress has not previously been studied following prenatal DEX treatment. Following our acute stress procedure (30-min restraint, 24 hrs prior) no change was seen in EPM exploration in VEH offspring. However, in DEX offspring, behavioural inhibition was observed in the EPM, including a significant reduction in closed arm entries and a tendency for reduced open arm entries and distance travelled in the whole maze and the open arms. Together these findings are suggestive of heightened stress-sensitivity and state anxiety in DEX offspring. These data support the previous discovery of an increased hypertensive response to stress in DEX offspring (O'Regan et al., unpublished observations).

It should be mentioned that the measure of closed arm entries is traditionally thought to reflect purely motor activity (Fernandes et al., 1999). However, several papers have reported a similar reduction in closed arm entries on the EPM following restraint stress in naïve adult rats (McBlane and Handley, 1994; Netto and Guimaraes, 1996; Padovan and Guimaraes, 2000), suggesting there is a stress-induced component. FG-7142, an anxiogenic benzodiazepine inverse agonist, was also found to induce hypoactivity in the

EPM, independent of sedation. Additionally, a recent factor analysis in male rats found closed arm entries to reflect a measure of anxiety (Doremus et al., 2006). Therefore, it appears that measures of general activity may also reflect the anxiety-like behaviour.

The absence of stress-induced behavioural inhibition in VEH offspring appears to contradict previous literature (reviewed in Korte and De Boer, 2003) reporting an anxiogenic response to restraint performed 24 hr prior to EPM exposure in treatment naïve, control animals (Guimaraes et al., 1993; McBlane and Handley, 1994; Martijena et al., 1997; Mendonca and Guimaraes, 1998; Padovan and Guimaraes, 2000). The duration of restraint does not appear to be responsible for this discrepancy as studies observed enhanced anxiety-related behaviour 24 hrs after a 15-min (Martijena et al., 1997), 1-hr (McBlane and Handley, 1994) or 2-hr restraint session (Guimaraes et al., 1993; Mendonca and Guimaraes, 1998; Padovan and Guimaraes, 2000). However, as stated above it should be remembered that final trimester prenatal VEH treatment *per se* is a mild prenatal stressor (Griffin et al., 2003) and our VEH subjects hence cannot be considered untreated. The anxiogenic response to 15-min acute restraint stress (24 hrs prior to testing on the EPM) was prevented by inhibition of CORT synthesis with metyrapone (Calvo et al., 1998), adrenalectomy and GR or MR antagonism (Calvo and Volosin, 2001), confirming the involvement of CORT in the restraint-induced fear-potentiation. It is possible therefore, that the heightened CORT levels in male DEX offspring compared to VEH offspring contributes to the stress sensitisation observed.

Similar to male offspring, no difference in spontaneous anxiety-like behaviour on the EPM was recorded in female DEX compared to VEH offspring. However, in contrast to males, female DEX offspring did not display sensitivity to the prior acute stress exposure. Female DEX offspring from our laboratory have not previously been assessed on the EPM, although they were not found to exhibit the impaired glucose homeostasis and elevated basal CORT measured in male DEX offspring (O'Regan et al., 2004). The unaltered CORT and normal stress-potentiated anxiety-like behaviour in female offspring further points

towards a possible role of heightened CORT in the programmed anxiety. However, BP alterations have been observed in both genders (O'Regan et al., unpublished observations), indicating that whilst perhaps less susceptible to prenatal GC programming, some effects are still apparent in female offspring. Gender differences in behavioural tests are widely reported (reviewed by Palanza, 2001) and PS has been shown to programme sex-specific effects on anxiety-related behaviour (Bowman et al., 2004; Richardson et al., 2006) and response to anxiolytic drug treatment (Poltyrev and Weinstock, 2004).

A caveat should be mentioned in that we did not correct for the phase of the oestrus cycle, which has previously been shown to affect behaviour in the EPM (Mora et al., 1996; Frye et al., 2000; Marcondes et al., 2001). However, the variability was relatively small in the parameters measured during the test suggesting this factor may not explain the lack of measured anxiety.

The quantification of c-fos mRNA was assessed 30 mins following the start of the 5-min EPM test, shown to represent the peak of c-fos mRNA induction (Cullinan et al., 1995). The induction of c-fos mRNA was enhanced in male DEX compared to VEH offspring in the cingulate and frontal cortex following EPM exposure. Abnormalities in these regions have been reported in human depression, anxiety and HPA axis regulation. Functional magnetic resonance imaging (fMRI) studies in patients suffering from anxiety disorders have revealed increased baseline activity in the cingulate cortex (Osuch et al., 2000) and increased brain activity in response to anxiety-provoking stimuli in the frontal cortex (Davidson et al., 1999). Additionally, a negative correlation between anxiety severity and metabolism has been identified in both regions (Kennedy et al., 2003). Furthermore, the activity within the anterior cingulate was also seen to be a predictor of treatment response in major depression (Pizzagalli et al., 2001). A reduced left anterior cingulate volume was found to be associated with dysregulated HPA axis function in elderly male patients (Maclullich et al., 2006). In experimental studies, anxiety-modulating and antidepressant drugs have been found to modulate activity in the rat cingulate cortex when measured by

both c-fos mRNA or Fos protein expression (Miyata et al., 2005; Salchner et al., 2006) and oxidative metabolism (Singewald et al., 2003; Gonzalez-Pardo et al., 2006). Furthermore, the expression of c-fos mRNA differed markedly in high and low anxiety-behaviour bred (HAB and LAB) Wistar rats following exploration of the light/dark anxiety test (Kabbaj and Akil, 2001). When compared LAB, HAB rats showed low expression of c-fos mRNA in the hippocampus, but high c-fos mRNA levels in the olfactory area, the orbital cortex, the cingulate cortex, the dorsal striatum and the PVN. Perinatal manipulation has been found to alter c-fos mRNA induction. Following acute restraint stress prenatal protein malnourished offspring displayed an enhanced Fos protein induction within the anterior cingulate (Rosene et al., 2004). Additionally, neonatally-handled offspring displayed a reduced induction of Fos protein in the cingulate cortex following restraint, but also in the hippocampus, PVN and amygdala (Abraham and Kovacs, 2000).

The expression of c-fos mRNA following EPM exposure was also detected in the hippocampus, raphe nucleus and locus coeruleus (key regions in central 5-HT and NA function), although the induction following EPM exposure was similar in DEX and VEH offspring. Previous findings indicate that prenatal DEX administration reduced the hippocampal GR and MR expression in adult offspring (Welberg et al., 2001). We therefore anticipated altered activation within this structure that is fundamentally involved in the modulation of HPA axis activity, memory and emotion (de Kloet et al., 2005), however none was observed. GR and c-fos mRNA have been found to co-localise within the medial prefrontal cortical neurons (Ostrander et al., 2003). It would be interesting to observe whether the prenatal DEX-induced alterations in corticosteroid receptor expression are also apparent in the cortex. We intended to quantify this, although unfortunately were unable to do so as a result of a freezer failure making stored tissue unusable.

c-fos mRNA expression 30-min following EPM exposure was below the level of detection in the PVN and amygdala. Previous studies have reported undetectable levels of c-fos mRNA following EPM exposure (Hinks et al., 1996), although a low level of Fos

immunoreactivity was observed (Duncan et al., 1996). In contrast, both swim and restraint stress elicited profound induction within these regions when measured 30 mins following the onset of the stressor (Cullinan et al., 1995). The release of ACTH and CORT appeared to correlate with the level of c-fos mRNA expression within the PVN, reflecting the perceived stressful quality of an experience (Pace et al., 2005). Thus, our employed stressor of EPM appears to be too mild in nature to elicit induction of c-fos mRNA in the PVN. It would therefore be interesting to study the c-fos mRNA expression in VEH and DEX offspring following more severe restraint stress to assess the impact of prenatal treatment on c-fos mRNA induction within these two regions previously found to express elevated CRH mRNA in DEX offspring (Welberg et al., 2001).

The enhanced induction of c-fos mRNA expression the cingulate and frontal cortex of DEX compared to VEH offspring following EPM exposure was no longer evident when restraint had been administered 24-hrs prior. Thus the pattern of c-fos mRNA expression appears disconnected to the anxiety-related behaviour recorded on the EPM *per se*, although may signal disparate stress reponsiveness between VEH and DEX offspring. Lesions within the cingulate cortex in rats have not been found to alter EPM behaviour (Bissiere et al., 2006). Together with our findings, this suggests that c-fos mRNA expression detected may be a reflection of the stressful experience of EPM exposure, rather than an indication of regions involved in generating altered exploration of the maze.

It should be noted that our assessment of regional activation measured c-fos mRNA expression at a 30-min timepoint, in order to measure the peak induction (Cullinan et al., 1995). It would be interesting to assess the timecourse of c-fos mRNA induction to observe whether spatiotemporal differences are apparent following prenatal GC administration. MRI technology, previously only employed in clinical studies (Sizonenko et al., 2006), has developed rapidly and can now be applied to rodents. The advantages of such a technique are several: measurements can be performed throughout the whole brain, rats can be assessed at different ages during life and the timecourse of regional activation

can be determined. The application of this technology to prenatal GC-treated offspring has the potential to rapidly develop and expand our knowledge regarding functional neuroanatomy as a consequence of altered fetal GC exposure.

In this chapter we have reported a reduction in birth weight, increased CORT excretion, enhanced sensitivity to stress-induced anxiety behaviour and increased cellular activation within the cingulate and frontal cortex following EPM exposure in animals exposed prenatally to increased GCs.

CHAPTER 4

COGNITIVE AND ANXIETY-RELATED BEHAVIOUR IN HETEROZYGOUS-BRED 11β-HSD2 KNOCKOUT MICE

4.1 Introduction

In the previous chapter we described behavioural studies in rat offspring following prenatal dexamethasone (DEX) treatment where we observed heightened stress-potentiated anxiety. Other studies have also reported elevated anxiety-like behaviour in offspring following prenatal stress (PS) (Vallee et al., 1997; Bowman et al., 2004; Patin et al., 2005; Van den Hove et al., 2005; Barros et al., 2006b) or DEX administration (Welberg et al., 2001; Oliveira et al., 2006). In addition, cognitive impairment has also been found in PS and/or DEX treated offspring using the water maze (Brabham et al., 2000; Meunier et al., 2004) and passive avoidance (Lordi et al., 1997; 2000; Gue et al., 2004) tests, although no difference in water maze performance was seen in prenatal DEX offspring in our laboratory (Welberg, 1999).

The developing fetus is normally protected from GC overexposure *in utero* through tight regulation of feto-placental GC transport by the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) enzyme (Seckl, 1997). This enzyme acts as a high affinity and high capacity dehydrogenase which inactivates CORT through conversion to its 11-keto metabolite, 11-dehydrocorticosterone (11-DHC), at least in rodents (cortisol to cortisone in humans) (Draper and Stewart, 2005). During early gestation the enzyme is highly expressed in the rodent placenta, but also many fetal tissues including the brain (Brown et al., 1996b; Burton et al., 1996; Waddell et al., 1998). However, from mid-gestation expression rapidly decreases to minimal levels in the placenta and most tissues, in accordance with a greater requirement for GC-mediated maturation and development (Diaz et al., 1998). The enzyme is thought to function as a protective barrier from the maternal GCs, which are much higher than those in the fetal circulation, thereby opposing the concentration gradient. Placental 11β-HSD2 activity is known to correlate with birthweight in both rats (Benediktsson et al., 1993) and humans (Stewart et al., 1995; Murphy et al., 2002), illustrating the importance of the enzyme in regulating the fetal endocrine and growth environment.

Evasion of the 11β-HSD2 feto-placental barrier appears to be a common feature in different models of prenatal programming including PS, prenatal GC administration and maternal dietary manipulation (Seckl, 1997). Stress exposure has been found to elevate CORT within the pregnant dam, which will 'overpower' the enzymatic barrier to cause GC overexpose to the developing fetus. DEX is a poor substrate for 11β-HSD2 and does not undergo the rapid inactivation that occurs with CORT (Brown et al., 1996a). The synthetic GC therefore passes readily into the fetal circulation where it can mediate effects during development. The importance of the 11β-HSD2 barrier and consequences of its inhibition have previously been addressed in our laboratory. Welberg et al. (2000) treated dams with the 11β-HSD inhibitor carbenoxolone (CBX) throughout pregnancy. Prenatal CBX offspring displayed similar programmed effects to those previously observed in DEX offspring (Welberg et al., 2001), including reduced birth weight, heightened anxiety-like behaviour and HPA axis dysregulation. However, previous investigations into GC programming and the role of 11β-HSD2 have been subject to certain limitations:

1.) Studies involve unavoidable manipulations of the pregnant dam in order to exert prenatal effects upon the offspring. These manipulations will impact on maternal physiology including reduced food intake (Kinsley and Svare, 1986; Woods and Weeks, 2005) which will almost certainly affect fetal nutrient supply.
2.) Several groups have reported a negative influence on the level of maternal behaviour following gestational stress (Patin et al., 2002; Smith et al., 2004). Indeed, impaired maternal care *per se* has been found to programme negative lifelong outcomes (Liu et al., 1997; Meaney, 2001). Additionally, control offspring could be programmed in a similar manner to prenatal DEX offspring when cross-fostered to a DEX-treated mother, indicating the early postnatal period to be a critical window of sensitivity (Brabham et al., 2000; Hauser et al., 2006). Additionally, the nutritional and hormonal content of maternal milk could be different in prenatally-manipulated dams. Thus, the early postnatal period may be an important period of effect in the mechanism of 'prenatal' programming.

3.) With regard to prenatal CBX treatment, this inhibitor is relatively non-specific for the 11β-HSDs (Stewart et al., 1990; Jellinck et al., 1993), so therefore the maternal blockade of the type 1 enzyme may also involved in the observed effects. Additionally, the injection stress may also have an impact on maternal physiology.

Genetic deletion of 11β-HSD2 is an ideal tool to further study the role of this enzyme in protecting the fetus from GC overexposure during development and as a model of prenatal GC programming in absence of maternal manipulation. Homozygous-bred 11β-HSD2 null mutant mice have significantly reduced birth weights and males displayed heightened anxiety-like behaviour on the elevated plus maze (EPM) and open field (OF) (Holmes et al., 2006a). However, as a consequence of 'illicit' mineralocorticoid receptor (MR) activation these mice displayed a phenotype of apparent mineralocorticoid excess (AME). This adult phenotype is comprised of electrolyte imbalance, hypertonic polyuria and hypertension (Kotelevtsev et al., 1999), factors that may affect nutrient provision both *in utero* and during the early postnatal period. In contrast, the 11β-HSD2 heterozygous mice appear physiologically normal. Therefore, to control for a variation in pre- and postnatal nutrient supply, offspring were obtained from heterozygous breeding which delivers 11β-HSD2 null mutant, heterozygous and wild-type mice within the same litter. Thus, we are able to study the direct effect on the fetus of a total and approximately 50% loss of enzyme activity independent of any maternal manipulation or postulated altered pup rearing.

In this chapter we aimed to investigate the anxiety-related and cognitive behaviour in the heterozgyous-bred 11β-HSD2 null mutant mouse, a potential model of prenatal GC programming. We describe behaviour in three separate validated tests of anxiety: EPM, OF and light/dark (LD) test; and two different tests of memory: the water maze and passive avoidance tests. Due to unavoidable circumstances, these studies were performed in two different biological research facilities (BRFs), the implications of which shall be discussed.

4.2 Methods

Comprehensive details of methods can be found in chapter 2.

4.2.1 Animal maintenance and behavioural testing

11β-HSD2 heterozygous breeding and behavioural analyses of offspring were conducted in both the Biological Research Facility (BRF) at the Western General (WGH) and later in the BRF at Little France (LF). This was necessary due to relocation of the Endocrinology Unit. Three heterozygous breeding cohorts were generated and studied, the first in the BRF at the WGH and remainder in the BRF at LF.

4.2.1.1 Cohort one

Breeding and subsequent behavioural analyses were performed in the BRF at the WGH. Male and female 11β-HSD2 heterozygous mice were pair-housed in breeding cages and left undisturbed until parturition. Cages were checked daily between 09.00 and 11.00 h for delivery of litters. On delivery, pups were counted and sexed. Litters were left undisturbed with the mother (apart from routine cage maintenance) until weaning at postnatal day (P) 21, when they were ear-notched (for identification and genotyping), housed in single-sex groups of 2-6 and left undisturbed until testing.

One week (wk) prior to behavioural testing male and female offspring were re-housed in groups of 2-3 with previous cagemates. Commencing at the age of 8-9 wks in males and 11-12 wks in females, subjects were tested weekly on behavioural tasks measuring anxiety-like behaviour, in the order of the elevated plus maze (EPM), open field (OF) and light/dark (LD) tests followed by the passive avoidance (PA) test. Subjects were brought into the experimental room immediately prior to testing.

A further set of male offspring at 29-31 wks of age were rehoused in pairs with previous cagemates a wk prior to testing in the water maze. All subjects were brought into experimental room daily, immediately prior to testing of the first subject.

4.2.1.2 Cohort two

Breeding and subsequent behavioural analyses were performed in the BRF at LF. Male and female 11β-HSD2 heterozygous mice were pair-housed in breeding cages. Females were subsequently checked daily for a vaginal plug which was used as evidence of copulation. Upon identification of a plug the day was recorded and designated embryonic day (E) 0. Males were removed and females singly housed throughout pregnancy, until delivery which occurred on days 18-19. Pregnant females were randomly divided into two groups: Control and Restraint. Dams in the Control group (n=14) were left undisturbed until parturition, whilst those in the Restraint group (n=8) were subjected to restraint stress during pregnancy. Restraint was performed in a plexiglass tube daily for 1 hr between 09.00 and 11.00 h from E7 until parturition, in a separate procedure room. Cages were checked each morning for delivery of litters. Following birth, pups were counted, sexed and weighed. Pups were tattooed on paws using a coded system to allow for continued recording of weights in individual offspring, which were weighed at P7, P14 and P21. At P21 offspring were weaned in single-sex groups of 2-6 and left undisturbed until testing, apart from routine cage maintenance.

One wk prior to behavioural testing, male and female offspring were divided into groups of 2-3 with previous cagemates and moved to a holding room adjacent to the experimental room. At 13 wks of age male and female subjects were tested weekly in behavioural tests commencing with the EPM, followed by the OF and LD test.

4.2.1.3 Cohort three

Breeding and subsequent behavioural analyses were performed in the BRF at LF except there was no further manipulation of the mice during pregnancy or the pre-weaning period, thus the protocol was similar to that described for cohort one. One wk prior to behavioural testing male and female offspring were divided into groups of 2-3 with previous cagemates and moved to a holding room adjacent to the experimental room. Male and female subjects at 10-11 wks of age were tested on the EPM.

4.2.1.4 Genotyping

On weaning ears were notched for identification. DNA was extracted from ear notches and used for genotyping of 11β-HSD2 null, heterozygous and wild-type mice by PCR and gel electrophoresis. The presence of an approximately 700 bp band indicates an 11β-HSD2 null mutant, an approximately 600bp band represents a wild-type and the presence of both bands indicates an 11β-HSD2 heterozygous genotype. A representative gel picture taken under UV light is shown on page 63.

4.2.1.5 Diet

Mice in both biological research facilities were allowed food and water *ad libitum*. However, the food differed between the two locations. Mice housed in the BRF at WGH were fed CRM(E) chow whilst breeding and under normal maintenance conditions. In contrast mice at the BRF at LF were fed RM3(E) during breeding and lactation, and the RM1(E) under normal maintenance conditions.

4.2.2 Statistical analysis

Data are expressed as mean±SEM. Comparisons between groups and prenatal manipulations were analysed by the most appropriate method including one- and two-way analysis of variance (ANOVA), with Student Newman-Keuls (SNK) *post-hoc* testing. Repeated measures ANOVAs were used to analyse data obtained in the passive avoidance and water maze paradigms.

4.3 Results

4.3.1 Cohort one

Male and female 11β-HSD2+/+, +/- and -/- mice were tested in several behavioural tests including EPM, OF and LD in order to measure anxiety-related behaviour. Data were statistically analysed using a one-way ANOVA with SNK *post-hoc* testing. Additionally, fear-related memory was determined in the PA test following initial exploration of the LD box. Male mice were also tested in the water maze in order to assess spatial learning and memory. Behaviour in the PA and water maze tests was analysed using repeated measures ANOVA with SNK *post-hoc* testing.

4.3.1.1 Behaviour of male 11β-HSD2 transgenic mice

4.3.1.1.1 Anxiety-related behaviour in the elevated plus maze

Genotype was found to have an effect on behaviour in the EPM. The entries into the more anxiogenic open arms were found to differ between genotype (Figure 4.1a; $F_{2,30}=9.73$, $p<0.001$), with 11β-HSD2-/- offspring making significantly fewer entries compared to both +/+ and +/- ($p<0.001$). There was no significant difference of entries into the closed arms of the maze between genotypes ($F_{2,30}=1.88$, $p=0.170$). The time spent on the open arms was significantly different between genotypes (Figure 4.1b; $F_{2,30}=3.61$; $p<0.01$), with 11β-HSD2-/- spending significantly less time on the open arms than +/- ($p<0.05$), whilst 11β-HSD2-/- displayed a tendancy compared to +/+ ($p=0.065$). The distance travelled on both the open arms (Figure 4.1c; $F_{2,30}=5.12$; $p<0.05$) and on the whole maze ($F_{2,30}=6.56$, $p<0.05$) were dependent on genotype. The 11β-HSD2-/- group travelled less on both the open arms ($p<0.05$ vs +/+ and +/-) and the entire maze ($p<0.01$ vs +/+ and +/-). These findings indicate heightened anxiety-like behaviour in the EPM test in male 11β-HSD2-/- mice.

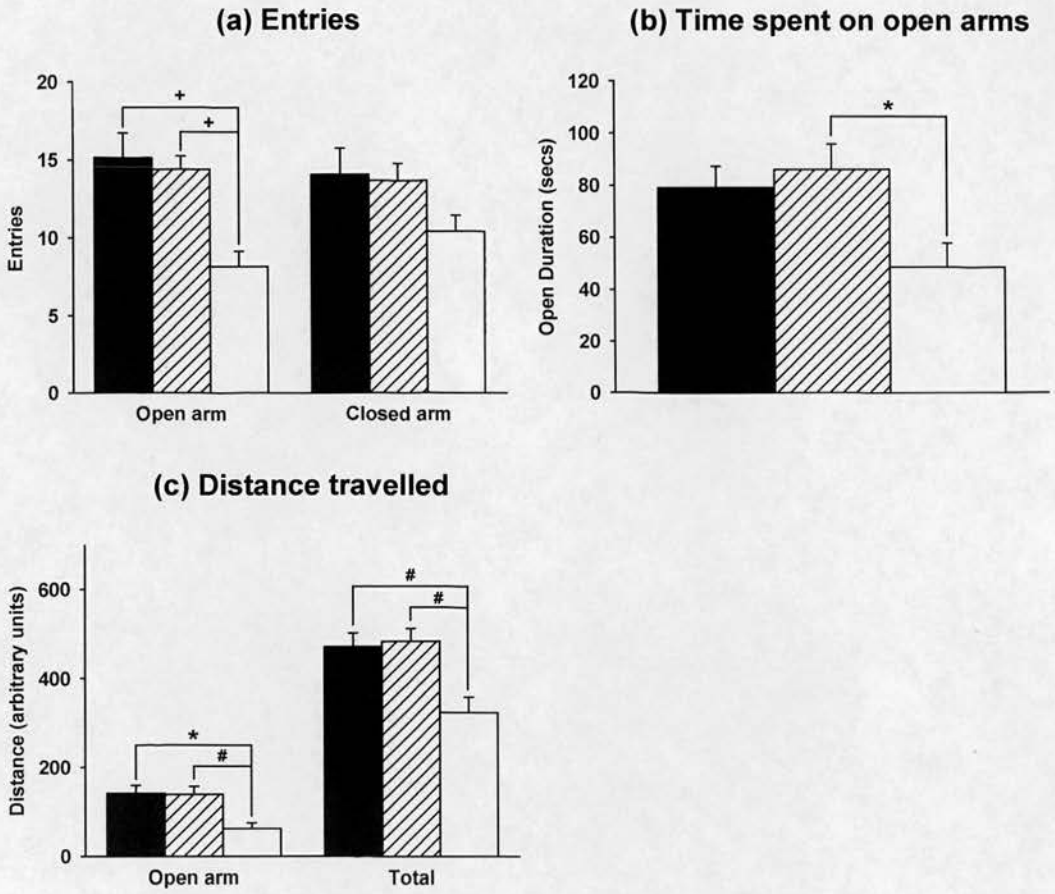


Figure 4.1 Behaviour of male 11β-HSD2 transgenic mice in the elevated plus maze
Effects of 11β-HSD2 genotype (+/+ ■, +/- ▨ and -/- □) on the (a) number of entries made onto the open and closed arms, (b) time spent on the open arms and (c) distance travelled on the open arms and the whole maze during a 5-min EPM test. Data are expressed as mean±SEM and analysed by one-way ANOVA with SNK *post-hoc* testing; n=8-16 per group. *p<0.05, #p<0.01, +p<0.001.

4.3.1.1.2 Anxiety-related behaviour in the open field test

During 5-min exploration of the OF, we did not detect any statistical differences in assessed behaviour between genotypes in male mice. Crossings within the more anxiogenic inner zone of the OF box (Figure 4.2a; $F_{2,27}$=0.96, p=0.397) and crossings in the whole box ($F_{2,27}$=2.28; p=0.122), time spent in the inner zone (Figure 4.2b; $F_{2,28}$=0.68, p=0.514), distance travelled in the inner zone (Figure 4.2c; $F_{2,27}$=0.74, p=0.486) and during the entire test ($F_{2,27}$=2.92; p=0.071) were not statistically different between genotypes. The 11β-HSD2-/- mice had a tendency to show increased total crossings and distance travelled as

compared to +/+ and +/- mice. The 11β-HSD2-/- mice do not show elevated anxiety-related behaviour in this test.

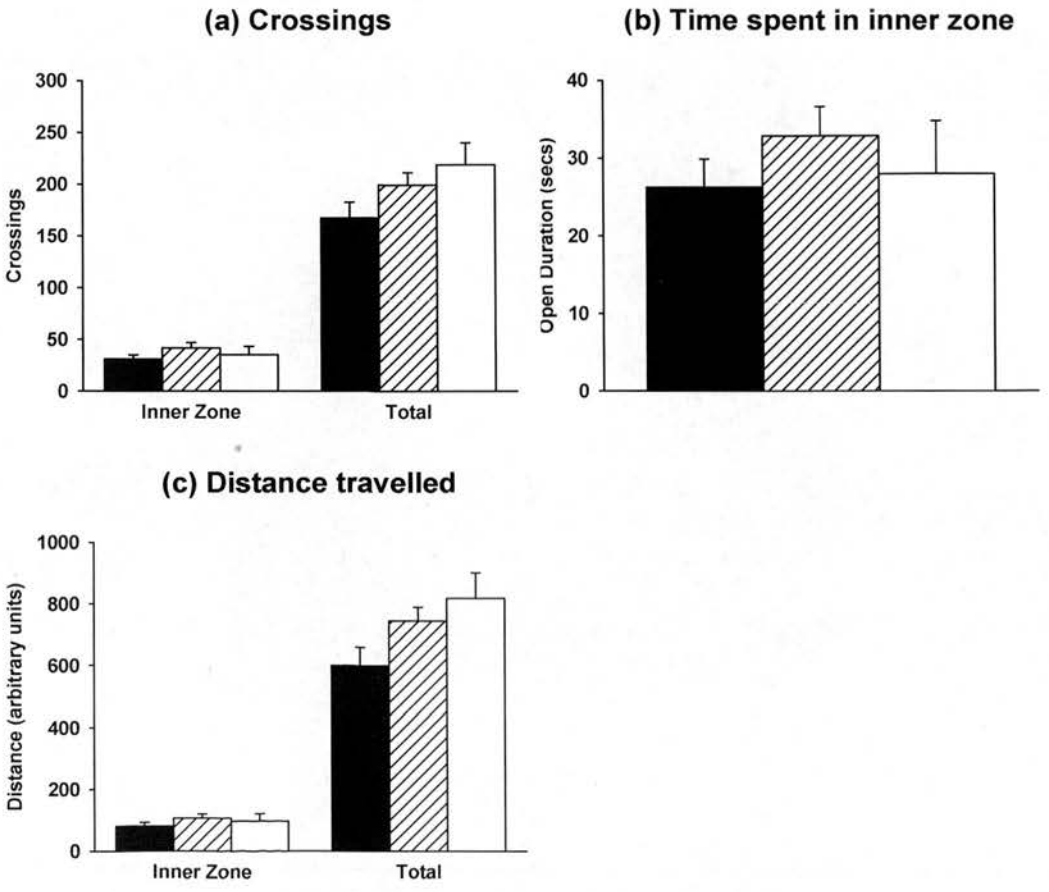


Figure 4.2 Behaviour of male 11β-HSD2 transgenic mice in the open field test
Effects of 11β-HSD2 genotype (+/+ ■, +/- ▨ and -/- □) on the (a) number of crossings in the inner zone and in total, (b) time spent in the inner zone and (c) distance travelled in the inner zone and in total during a 5-min OF test. Data are expressed as mean±SEM and analysed by one-way ANOVA; n=7-15 per group.

4.3.1.1.3 Anxiety-related behaviour in the light/dark test and fear-related memory in the passive avoidance test

Significant behavioural differences between genotypes were detected in the LD anxiety test in male mice. The number of crossings between the two compartments of the apparatus were different between groups (Figure 4.3a; $F_{2,23}=4.53$; $p<0.05$), with 11β-HSD2-/- mice

making significantly fewer crossings compared to +/+ and +/- ($p<0.05$). The time spent in the more anxiety-provoking light compartment displayed a tendency to be altered by genotype (Figure 4.3b; $F_{2,24}=3.19$; $p=0.059$). The latency to enter the dark compartment was unaltered between genotypes ($F_{2,24}=0.036$, $p<0.965$; +/+: 11.4±1.5; +/-: 11.8±2.1; -/-: 10.7±5.3 secs).

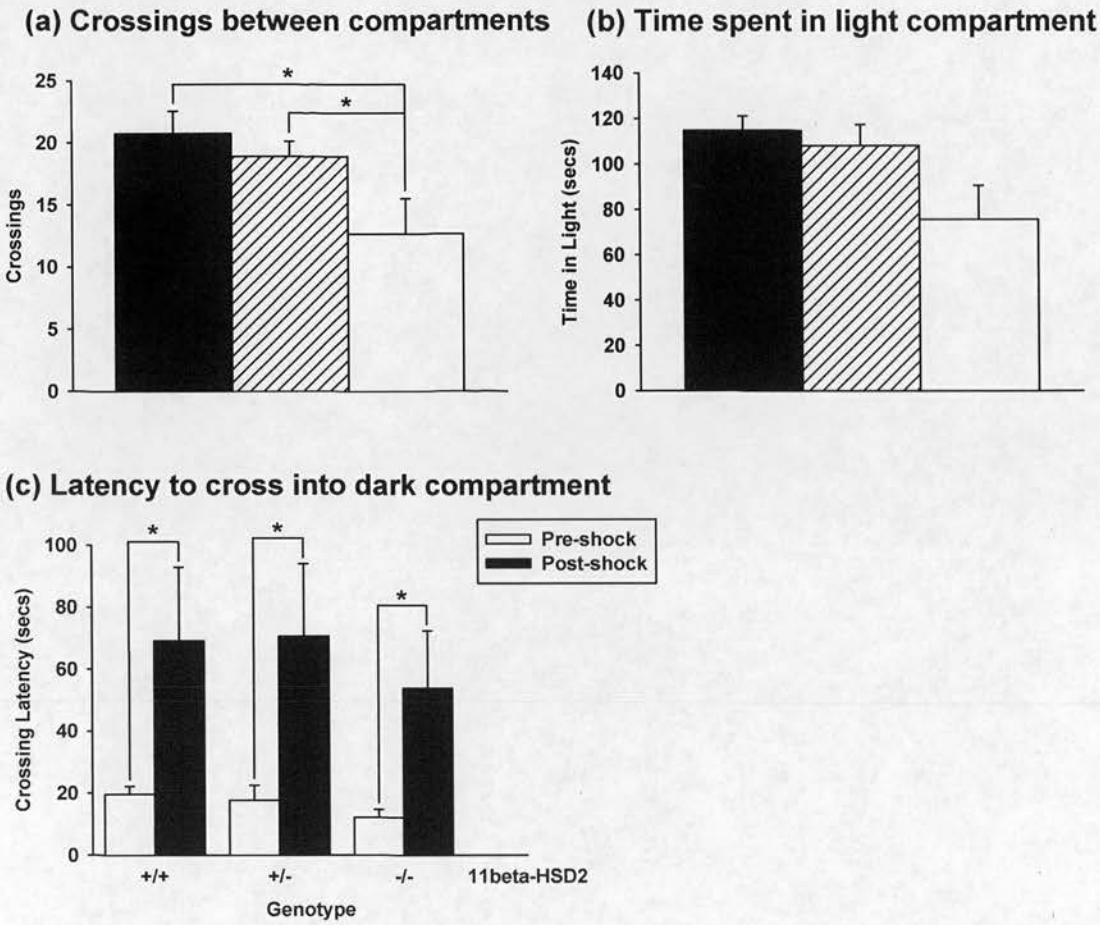


Figure 4.3 Behaviour of male 11β-HSD2 transgenic mice in the light/dark anxiety test and conditioned passive avoidance paradigm
Effects of 11β-HSD2 genotype (+/+ ■, +/- ▨ and -/- □) on the (a) number of crossings between the two compartments, (b) time spent in the light compartment during a 5-min free exploration on Day 1 and (c) latency to cross into the dark compartment before (□) and six hrs after (■) footshock on Day 2. Data are expressed as mean±SEM and analysed by one-way ANOVA with SNK *post-hoc* testing (a,b) and two-way repeated measures ANOVA with SNK *post-hoc* testing (c); n=9-15 per group for (a,b); n=5-9 per group for (c). *p<0.05

The response of mice to passive avoidance conditioning was analysed by two-way repeated measures ANOVA. Foot shock increased the latency to enter the dark compartment (Figure 4.3c; $F_{1,17}=14.72$, $p<0.001$) although this was not affected by genotype (Figure 4.3c; $F_{2,17}=0.24$; $p=0.789$). Data from the LD test implies heightened anxiety-related behaviour in male 11β-HSD2-/- mice. In contrast, no change in fear-related memory is suggested from the PA test.

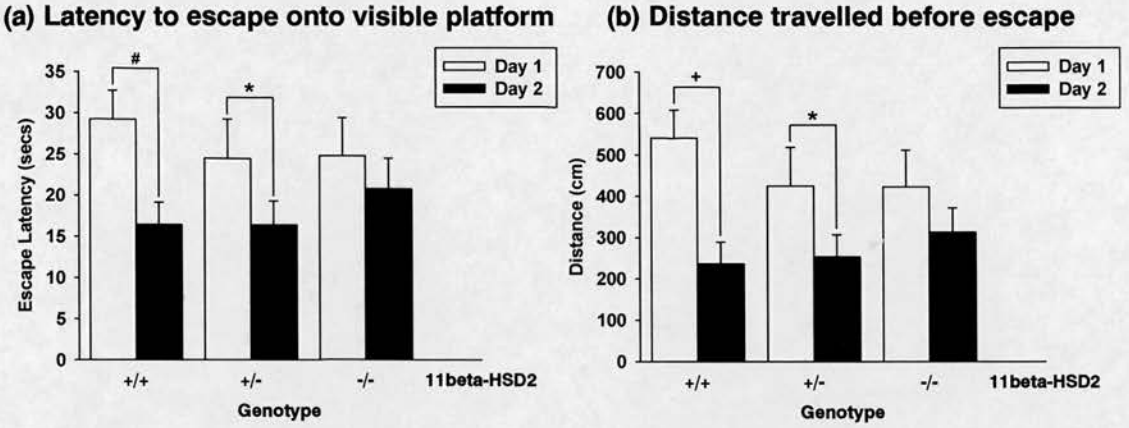


Figure 4.4 Behaviour of male 11β-HSD2 transgenic mice in the two-day water maze test with the visibly-cued platform

Effects of 11β-HSD2 genotype (+/+, +/- and -/-) on the (a) latency to escape the water maze onto the submerged platform and (b) distance travelled until escape measured in the visible water maze performed over two days. Data are expressed as mean±SEM and analysed by two-way repeated measures ANOVA with SNK *post-hoc* testing; n=9-10 per group. *p<0.05, #p<0.01, +p<0.001.

4.3.1.1.4 Spatial learning and memory behaviour in the water maze test

Behaviour in the water maze test was studied to assess spatial learning and memory ability in 11β-HSD2+/+, +/- and -/- male mice. In the visibly-cued water maze there was a significant decrease in the time taken to escape onto the platform (Figure 4.4a; $F_{1,24}=13.48$; $p<0.001$) and in the distances swum until escape between day one and two (Figure 4.4b; $F_{1,24}=19.24$; $p<0.001$). There was no overall effect of genotype in either escape latency ($F_{2,24}=0.34$, $p=0.719$) or distance travelled ($F_{2,24}=0.39$, $p=0.682$). However, 11β-HSD2+/+ and +/- displayed a significant reduction in escape latency between days one and two (Figure 4.4a; +/+: $P<0.01$; +/- $P<0.05$), although no reduction was noted in -/- ($P=0.283$).

The distance travelled showed a similar pattern, with significant reduction in +/+ (Figure 4.4b; $P<0.001$) and +/- ($P<0.05$), whilst the distance travelled on day two was not significantly less than from day one for -/- ($P=0.172$).

In the spatially-cued water maze (with a hidden platform), as expected, a significant reduction in escape latency (Figure 4.5a; $F_{4,96}=28.28$; $p<0.001$) and distance travelled (Figure 4.5b; $F_{4,94}=42.38$; $p<0.01$) were observed during the test. There was no overall statistical difference between genotypes in escape latency ($F_{2,96}=1.83$, $p=0.182$) or distance travelled ($F_{2,94}=0.34$, $p=0.715$). However, whilst 11β-HSD2+/+ and +/- displayed a significant reduction between day one and two (Figure 4.5a; +/+: $p<0.001$; +/-: $p<0.01$) no decrease was observed in escape latency between days one and two in -/- mice ($p=0.142$). Similarly, +/+ and +/- ($p<0.001$) swam a shorter distance on day two compared to one, whilst the reduction was not statistically significant in the -/- group ($p=0.077$). The average overall swim speed was not statistically different between genotypes (Table 4.1a; $F_{2,96}=2.16$, $p=0.138$) and slowed during the test in all groups ($p<0.001$). On days two, three and five 11β-HSD2-/- swam significantly slower than +/+ and +/- ($p<0.05$). On sequential days in all groups, the time spent near the walls of the circular tank decreased ($F_{4,96}=34.01$, $p<0.001$), whilst the time in quadrant containing the platform increased during the testing days ($F_{4,96}=5.05$, $p<0.001$) but did not differ significantly between genotypes ($F_{2,96}=0.74$, $p=0.486$; $F_{2,96}=0.24$, $p=0.792$). These findings suggest a mild impairment of spatial learning and/or memory in male 11β-HSD2-/- mice.

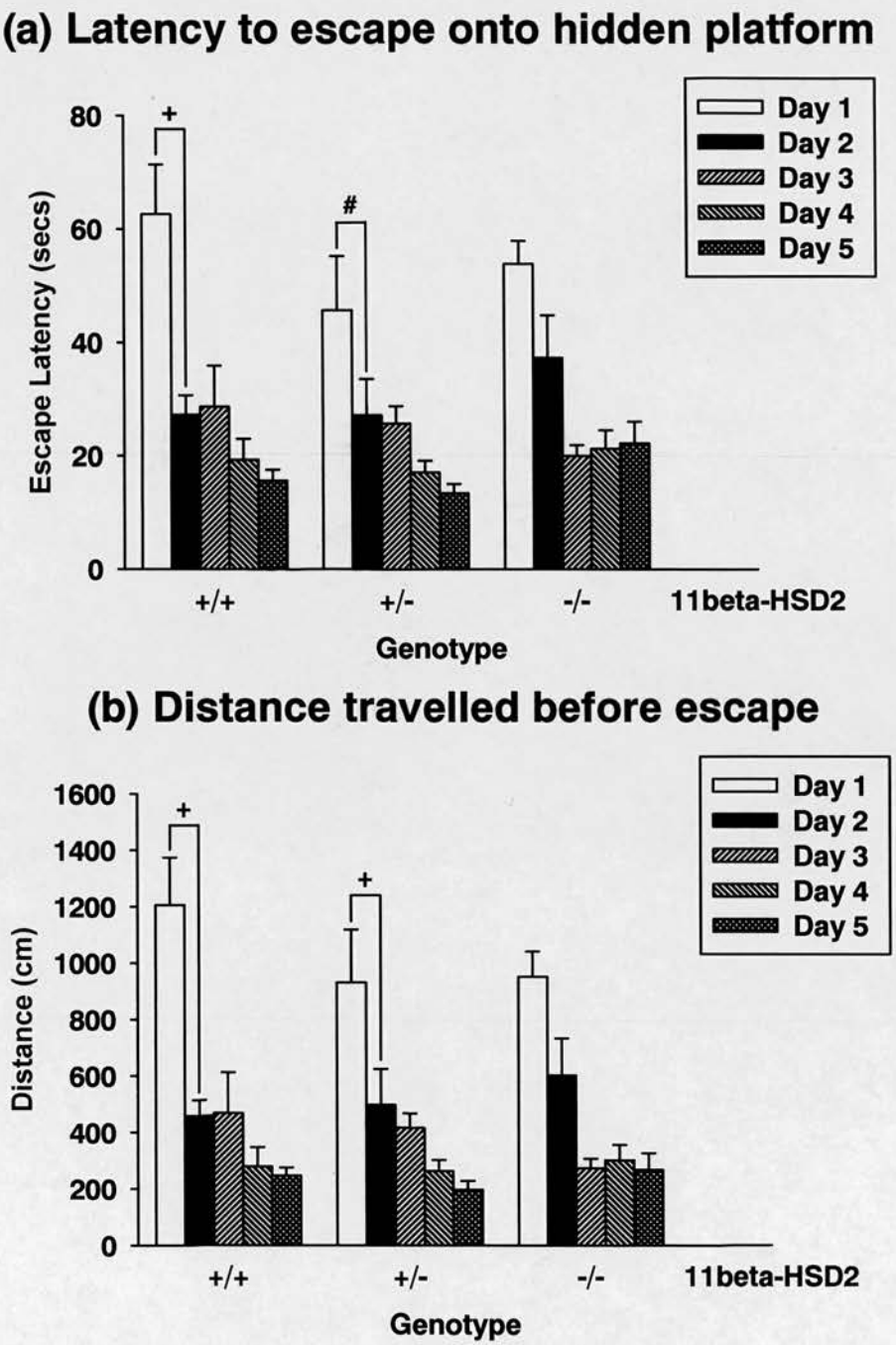


Figure 4.5 Behaviour of male 11β-HSD2 transgenic mice in the five-day spatially-cued water maze test with a hidden platform

Effects of 11β-HSD2 genotype (+/+, +/- and -/-) on the (a) latency to escape the water maze onto the submerged platform and (b) distance travelled until escape measured in the spatial water maze repeated over five days. Data are expressed as mean±SEM and analysed by two-way repeated measures ANOVA with SNK *post-hoc* test; n=8-10 per group. $^{\#}p<0.01$, $^{+}p<0.001$.

In the probe test, neither time taken to reach the former platform position (Table 4.1b; $F_{2,24}$=1.25; p=0.304), the number of times the former platform position was crossed ($F_{2,24}$=0.17, p=0.849) or time spent in the former platform quadrant ($F_{2,24}$, p=0.25, p=0.783) were altered by genotype, although there was a significant difference between genotypes for swim speed ($F_{2,24}$=7.00, p<0.01), where 11β-HSD2-/- mice swam more slowly than +/+ (P<0.05) and +/- (P<0.01).

11β-HSD2 Genotype	Day 1	Day 2	Day 3	Day 4	Day 5
(a) Average speed (cm/sec)					
+/+	18.3±0.6	16.0±0.5	14.7±0.8	13.5±0.8	14.2±0.6
+/-	16.9±0.7	17.5±0.7	15.5±0.7	15.0±0.8	14.2±1.1
-/-	17.1±1.0	15.0±0.8*	12.9±0.8*	14.0±0.8	12.6±0.9*
(b) Twalls (%)					
+/+	56.2±3.3	37.3±2.6	28.5±3.6	24.1±2.3	25.2±3.7
+/-	49.0±4.2	39.9±3.6	32.8±4.0	31.9±3.4	27.1±4.7
-/-	47.4±3.2	31.4±2.4	27.9±3.0	25.1±3.0	20.1±2.6
(c) Tquad (%)					
+/+	33.8±4.9	39.5±2.9	45.9±4.6	41.3±4.0	53.3±1.9
+/-	43.9±5.1	41.1±4.7	41.1±2.9	46.9±4.6	49.7±3.0
-/-	34.1±6.5	39.1±5.7	51.4±4.3	47.5±3.3	44.3±4.8

Table 4.1a Average swim speed, time spent near walls of maze and time spent in platform quadrant of male 11β-HSD2 transgenic mice, measured in the five-day spatially-cued water maze test with a hidden platform

Effects of 11β-HSD2 genotype (+/+, +/- and -/-) on the average swim speed, time spent near walls of maze (Twalls) and time spent in platform quadrant (Tquad) measured in the spatial water maze repeated over five days. Data are expressed as mean±SEM and analysed by two-way repeated measures ANOVA with SNK *post-hoc* testing; n=8-10 per group. *p<0.05 vs +/+ and +/-.

11β-HSD2 Genotype	Tplatform (secs)	Platform Crossings	Tquad (%)	Speed (cm/sec)
+/+	14.5±3.3	3.5±0.5	41.7±3.5	17.3±0.7
+/-	12.1±2.4	3.8±0.6	40.9±3.7	19.2±0.9
-/-	21.3±5.8	3.3±0.4	44.9±5.3	15.5±0.9*,#

Table 4.1b Behaviour of male 11β-HSD2 transgenic mice in the spatially-cued water maze probe test
Effects of 11β-HSD2 genotype (+/+, +/- and -/-) on the latency to reach former platform position (Tplatform), number of crossings of former platform position, time spent in former platform quadrant (Tquad) and average swim speed during 60-sec spatial water maze probe test performed 90 mins following last water maze exposure. Data are expressed as mean±SEM and analysed by one-way ANOVA with SNK post-hoc testing; n=9-10 per group. *p<0.05 vs +/-, #p<0.01

4.3.1.2 Behaviour of female 11β-HSD2 transgenic mice

4.3.1.2.1 Anxiety-related behaviour in the elevated plus maze

Alterations in behaviour between genotypes in female offspring were detected on the EPM. The number of open arm entries were found to be different depending on genotype (Figure 4.6a; $F_{2,21}$=4.05, p<0.05). A reduction in open arm entries was observed in 11β-HSD2-/- which was significant when compared to +/- (P<0.05), but not +/+ (p=0.097). However, no difference was found between the groups on entries into the closed arms ($F_{2,21}$=0.26, p=0.772). A tendency for altered open arm duration between genotypes was observed (Figure 4.6b; $F_{2,21}$=3.17, p=0.062). Whilst 11β-HSD2-/- were not different from +/+ mice (p=0.613), +/- had a tendency to spend longer in the open arms compared to -/- (p=0.062) and +/+ (p=0.139) mice. No statistically significant difference between genotypes was seen in open arm (Figure 4.6c; $F_{2,21}$=1.45, p=0.256) and total distance ($F_{2,21}$=1.95, p=0.167) travelled. The findings point towards increased anxiety behaviour in female 11β-HSD2-/- mice.

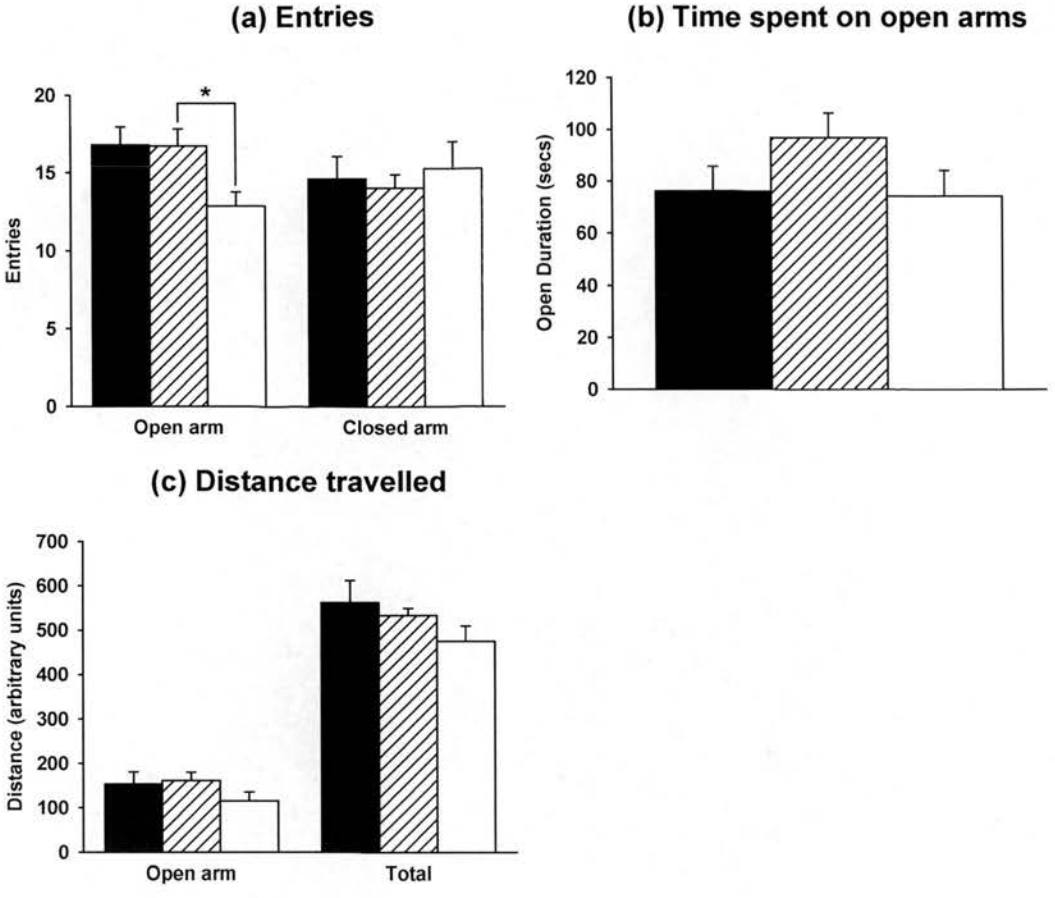


Figure 4.6 Behaviour of female 11β-HSD2 transgenic mice in the elevated plus maze
Effects of 11β-HSD2 genotype (+/+ ■, +/- ▨ and -/- □) on the (a) number of entries made onto the open and closed arms, (b) time spent on the open arms and (c) distance travelled on the open arms and the whole maze during a 5-min EPM test. Data are expressed as mean±SEM and analysed by one-way ANOVA with SNK *post-hoc* testing; n=5-11 per group. *p<0.05.

4.3.1.2.2 Anxiety-related behaviour in the open field test

In the OF anxiety test in female mice, groups displayed no statistical differences in inner zone (Figure 4.7a; $F_{2,21}$=1.73, p=0.201) or total crossings ($F_{2,21}$=0.35, p=0.701), inner duration (Figure 4.7b; $F_{2,21}$=1.75, p=0.198), inner zone (Figure 4.7c; $F_{2,21}$=1.43, p=0.261) or total crossings ($F_{2,21}$=0.26, p=0.772) during the 5-min exploration of the apparatus. Therefore, no indication of altered anxiety was seen in the OF behavioural test.

Figure 4.7 Behaviour of female 11β-HSD2 transgenic mice in the open field test
Effects of 11β-HSD2 genotype (+/+ ■, +/- ▨ and -/- □) on the (a) number of crossings in the inner zone and in total, (b) time spent in the inner zone and (c) distance travelled in the inner zone and in total during a 5-min OF test. Data are expressed as mean±SEM and analysed by one-way ANOVA; n=5-11 per group.

4.3.1.2.3 Anxiety-related behaviour in the light/dark test and fear-related memory in the passive avoidance test

Female mice of each 11β-HSD2 genotype did not differ in crossings between compartments (Figure 4.8a; $F_{2,18}$=0.62, p=0.538) or time spent in the light compartment (Figure 4.8b; $F_{2,18}$=0.53, p=0.600) during the LD anxiety test. The latency to enter the dark compartment was also unaltered by genotype ($F_{2,18}$=0.16, p=0.850; +/+: 11.9±1.6; +/-: 14.2±2.7; -/-: 12.8±3.0 secs). As analysed by two-way repeated measures ANOVA with SNK *post-hoc* testing there was an increased latency to enter the dark compartment post-shock compared to pre-shock (Figure 4.8c; $F_{1,15}$=21.90, p<0.001), although this was

significant in the +/- and -/- groups only ($p<0.01$). The latency to cross into the dark compartment pre-shock did not differ between genotypes, although testing following shock administration revealed a reduced latency in +/+ mice compared to both +/- and -/- ($p<0.05$). These results do not support an alteration of anxiety or fear-related memory in 11β-HSD2-/- female mice.

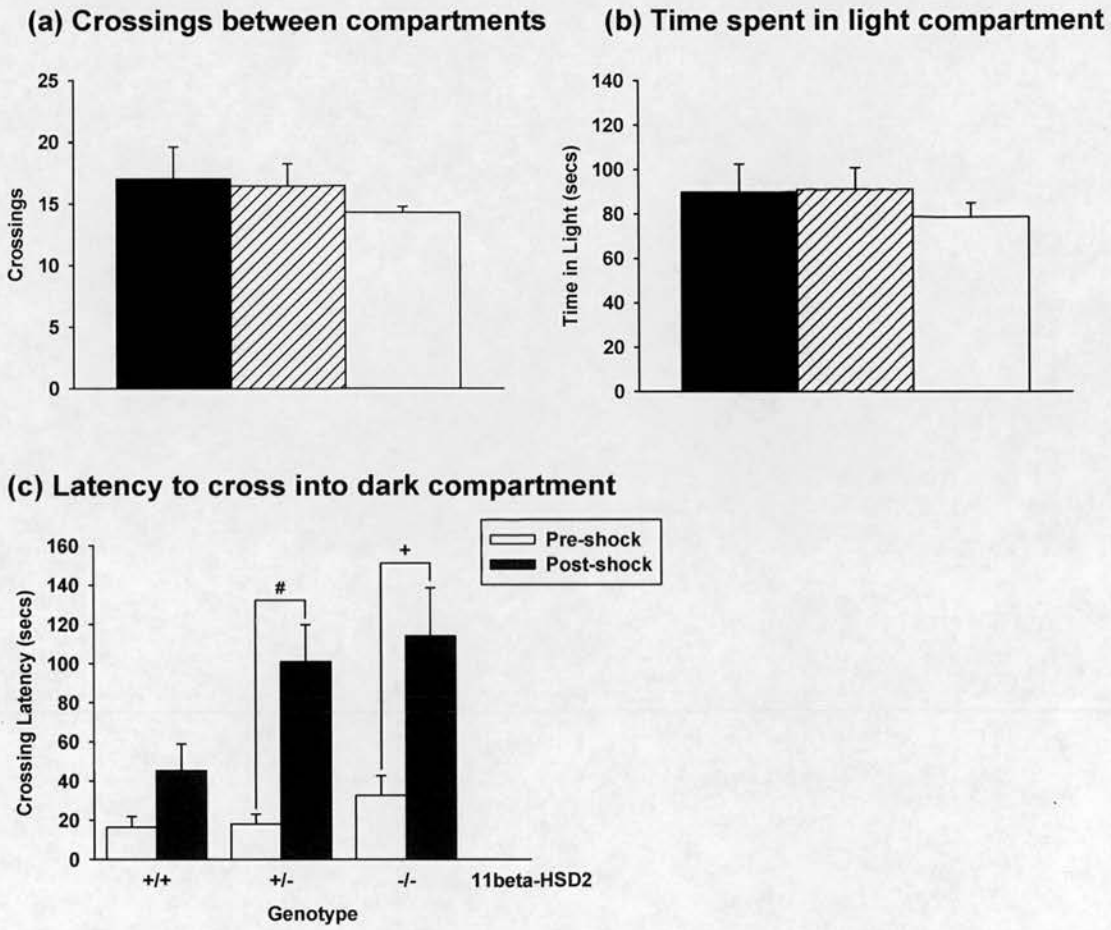


Figure 4.8 Behaviour of female 11β-HSD2 transgenic mice in the light/dark anxiety test and conditioned passive avoidance paradigm
Effects of 11β-HSD2 genotype (+/+ ■, +/- ▨ and -/- □) on the (a) number of crossings between the two compartments, (b) time spent in the light compartment during a 5-min free exploration on Day 1 and (c) latency to cross into the dark compartment before (□) and six hrs after (■) footshock on Day 2. Data are expressed as mean±SEM and analysed by one-way ANOVA (a,b) and two-way repeated measures ANOVA with SNK *post-hoc* testing (c); n=5-11 per group for (a,b); n=4-8 per group for (c) with n=4 for +/+. $^{+}p<0.01$, #$p<0.001$.

4.3.2 Cohort two

4.3.2.1 Birth weight and early postnatal growth in 11β-HSD2 transgenic mice

The length of gestation, litter size and male:female ratio were unaltered by prenatal manipulation (data not shown). Birth and early postnatal weights were analysed by two-way ANOVA. There was no effect of gender so therefore males and females were grouped for statistical analysis. Birth weight was significantly different depending on genotype (Table 4.2; $F_{2,111}=9.78$, $p<0.001$) and prenatal manipulation ($F_{1,111}=4.02$, $p<0.05$).

11β-HSD2 Genotype	Prenatal Manipulation	Birth Weight group (g)	Birth Weight male (g)	Birth Weight female (g)	P7 group (g)	P14 group (g)	P21 group (g)
+/+	CONTROL	1.27±0.02 (n=22)	1.29±0.03 (n=9)	1.25±0.03 (n=13)	3.83±0.05	7.00±0.09	8.20±0.12
+/-		1.28±0.02 (n=34)	1.31±0.02 (n=19)	1.25±0.03 (n=15)	**4.00±0.05$^{\#}$**	7.26±0.10	8.68±0.16
-/-		**1.16±0.02^{+}** (n=20)	1.18±0.03 (n=10)	1.14±0.03 (n=10)	3.81±0.10	7.18±0.13	8.38±0.17
+/+	RESTRAINT	1.26±0.02 (n=12)	1.26±0.02 (n=8)	1.26±0.06 (n=4)	3.74±0.07	**6.45±0.14^{a}**	**7.28±0.33^{a}**
+/-		**1.20±0.02^{x,a}** (n=24)	1.19±0.02 (n=14)	1.21±0.02 (n=10)	**3.68±0.06^{a}**	**6.47±0.14^{a}**	**7.55±0.23^{a}**
-/-		**1.14±0.04$^{\#}$** (n=7)	1.15±0.08 (n=3)	1.13±0.06 (n=4)	**3.35±0.10^{*,a}**	**6.43±0.20^{a}**	**7.48±0.30^{a}**

Table 4.2 Birth and pre-weaning weights in 11β-HSD2 transgenic offspring subjected to prenatal restraint or unmanipulated controls
Birth and early postnatal weights in 11β-HSD2 offspring (+/+, +/- and -/-) subjected to prenatal restraint or unmanipulated controls. Data are expressed as mean±SEM and analysed by two-way ANOVA with SNK *post-hoc* testing; n=20-34 for control offspring; n=7-24 for restraint offspring. $^{*}p<0.05$ vs +/+ and +/-, $^{+}p<0.001$ vs +/+ and +/-, $^{x}p<0.05$ vs +/+, $^{a}p<0.05$ vs Control

11β-HSD2-/- offspring born to control dams were significantly lighter than both +/+ and +/- offspring ($p<0.001$, SNK *post-hoc* test). Following prenatal restraint stress 11β-HSD2-/- mice remained significantly lighter than +/+ ($p<0.05$), although not +/- offspring ($p=0.138$). Additionally, the 11β-HSD2+/- mice tended to be lighter than +/+ offspring

(p=0.062). Prenatal restraint significantly reduced 11β-HSD2+/- offspring birth weight compared with offspring of the same genotype born to unstressed control dams (p<0.001).

4.3.2.2 Body weight at postnatal day 7

There was an overall effect of genotype (Table 4.2; $F_{2,112}$=5.02, p<0.01) and treatment ($F_{1,112}$=19.09, p<0.001) on body weight at P7. Offspring from control dams, 11β-HSD2-/- mice tended to be lighter than +/- (p=0.068) although no different from +/+ (p=0.836). +/- were significantly heavier than +/+ mice (p<0.05). 11β-HSD2-/- offspring from restraint dams were significantly lighter than +/+ and +/- offspring (p<0.05). Comparing across prenatal manipulation, 11β-HSD2-/- and +/-, although not +/+ offspring (p=0.420) from dams receiving restraint were significantly lighter than offspring from control, unmanipulated dams (p<0.001)

4.3.2.3 Body weight at postnatal day 14

There was no significant overall effect of genotype on body weight at P14 (Table 4.2; $F_{2,112}$=0.71, p=0.496), although there was a significant treatment effect ($F_{1,112}$=34.75, p<0.001). Offspring of all genotypes subjected to prenatal restraint were significantly lighter than those from unstressed control dams (+/-: p<0.001; +/+ and -/-: p<0.01).

4.3.2.4 Body weight at postnatal day 21

No significant overall effect of genotype was observed on body weight at P21 (Table 4.2; $F_{2,111}$=2.01, p=0.139), although there was a significant effect of restraint ($F_{1,111}$=29.20, p<0.001). Offspring of all genotypes born to mothers receiving restraint were significantly lighter than offspring from control dams (+/-: p<0.001; +/+: p<0.01; -/-: p<0.05).

4.3.2.5 Behaviour of male 11β-HSD2 transgenic mice

In contrast to results obtained in cohort one, no change in behaviour was observed in male 11β-HSD2-/- mice in the EPM, OF or LD anxiety tests. Results obtained in male mice on the EPM are shown in Table 4.3a,b.

11β-HSD2 Genotype	Prenatal Manipulation	Entries		Distance (au)		Open Duration (secs)
		Open	Closed	Open	Total	
+/+	CONTROL	17.5±2.5	21.4±2.7	169±32	1028±104	57.2±8.1
+/-		12.2±2.2	20.1±2.3	162±56	930±109	53.6±17.4
-/-		16.4±3.2	18.5±1.1	203±42	958±83	74.8±14.1
+/+	RESTRAINT	15.3±3.1	20.6±3.1	187±43	1076±75	58.6±11.2
+/-		14.9±2.3	18.5±1.6	208±33	964±87	88.2±17.8

Table 4.3a Behaviour in the elevated plus maze of male 11β-HSD2 transgenic mice subjected to prenatal restraint or unmanipulated controls
Effects of 11β-HSD2 genotype (+/+, +/- and -/-) and prenatal manipulation on the number of entries made onto the open and closed arms, distance travelled on the open arms and the whole maze, and time spent on the open arms during a 5-min EPM test. Data are expressed as mean±SEM and analysed by two-way ANOVA; n=7-8 per group. au = arbitrary units

EPM Measure	11β-HSD2 Genotype	Prenatal Manipulation
Open arm entries	$F_{2,35}$=0.56, p=0.575	$F_{1,35}$=0.00, p=0.975
Closed arm entries	$F_{2,35}$=0.85, p=0.437	$F_{1,35}$=0.37, p=0.548
Open duration	$F_{2,35}$=1.08, p=0.351	$F_{1,35}$=1.51, p=0.228
Open distance	$F_{2,35}$=0.31, p=0.739	$F_{1,35}$=0.61, p=0.439
Total distance	$F_{2,35}$=0.70, p=0.505	$F_{1,35}$=0.20, p=0.655

Table 4.3b Statistical analysis of elevated plus maze behaviour shown in Table 4.3a as analysed by two-way ANOVA with SNK *post-hoc* testing

Behaviour in the OF was unaltered by either 11β-HSD2 genotype or prenatal manipulation and is shown in Table 4.4a,b.

11β-HSD2 Genotype	Prenatal Manipulation	Crossings		Inner Zone Duration (secs)
		Inner Zone	Total	
+/+	CONTROL	51.0±11.2	468±36	21.6±4.9
+/-		57.9±15.2	523±30	23.5±5.0
-/-		66.9±10.5	496±19	33.2±4.3
+/+	RESTRAINT	45.7±15.7	557±37	17.7±6.5
+/-		75.4±13.0	518±18	32.7±4.2

Table 4.4a Behaviour in the open field test of male 11β-HSD2 transgenic mice subjected to prenatal restraint or unmanipulated controls
Effects of 11β-HSD2 genotype (+/+, +/- and -/-) and prenatal manipulation on the number of crossings in the inner zone and in total, and time spent in the inner zone during a 5-min OF test. Data are expressed as mean±SEM and analysed by two-way ANOVA; n=7-8 per group.

OF Measure	11β-HSD2 Genotype	Prenatal Manipulation
Inner zone crossings	$F_{2,35}$=0.27, p=0.767	$F_{1,35}$=0.05, p=0.822
Total crossings	$F_{2,35}$=0.06, p=0.943	$F_{1,35}$=1.86, p=0.181
Inner zone duration	$F_{2,35}$=2.87, p=0.070	$F_{1,35}$=0.32, p=0.576

Table 4.4b Statistical analysis of open field behaviour shown in Table 4.4a as analysed by two-way ANOVA with SNK *post-hoc* testing

Furthermore, no change in LD behaviour was observed, shown in Table 4.5a,b.

11β-HSD2 Genotype	Prenatal Manipulation	Latency (secs)	Crossings	Time in Light (secs)
+/+	CONTROL	11.8±3.1	14.9±1.7	86.0±1.6
+/-		9.7±1.5	17.0±2.3	95.3±10.3
-/-		10.2±1.4	19.0±1.4	102.0±11.9
+/+	RESTRAINT	14.2±2.8	14.8±2.3	102.8±19.7
+/-		10.0±3.0	16.0±1.6	101.4±16.2

Table 4.5a Behaviour in the light/dark anxiety test of male 11β-HSD2 transgenic mice subjected to prenatal restraint or unmanipulated controls
Effects of 11β-HSD2 genotype (+/+, +/- and -/-) and prenatal manipulation on the latency to cross into the dark compartment, number of crossings between the two compartments and time spent in the light compartment. Data are expressed as mean±SEM and analysed by two-way ANOVA; n=7-8 per group.

LD Measure	11β-HSD2 Genotype	Prenatal Manipulation
Latency to cross	$F_{2,29}$=0.73, p=0.489	$F_{1,29}$=0.29, p=0.592
Crossings	$F_{2,29}$=1.37, p=0.271	$F_{1,29}$=0.08, p=0.785
Time in light	$F_{2,29}$=0.28, p=0.761	$F_{1,29}$=0.69, p=0.413

Table 4.5b Statistical analysis of light/dark behaviour shown in Table 4.5a as analysed by two-way ANOVA with SNK *post-hoc* testing

4.3.2.6 Behaviour of female 11β-HSD2 transgenic mice

Similar to males no behavioural differences were seen between genotypes and prenatal treatment groups in the EPM (Table 4.6a,b), OF (Table 4.7a,b) or LD (Table 4.8a,b) anxiety tests.

11β-HSD2 Genotype	Prenatal Manipulation	Entries		Distance (au)		Open Duration (secs)
		Open	Closed	Open	Total	
+/+	CONTROL	14.8±1.4	19.9±1.1	122±34	921±77	42.4±8.1
+/-		12.3±1.3	18.9±1.4	103±21	878±83	36.7±6.5
-/-		14.3±1.5	20.8±1.5	168±27	994±61	60.9±9.1
+/-	RESTRAINT	13.8±2.6	18.5±2.4	127±29	858±77	43.2±8.8

Table 4.6a Behaviour in the elevated plus maze of female 11β-HSD2 transgenic mice subjected to prenatal restraint or unmanipulated controls
Effects of 11β-HSD2 genotype (+/+, +/- and -/-) and prenatal manipulation on the number of entries made onto the open and closed arms, distance travelled on the open arms and the whole maze, and time spent on the open arms during a 5-min EPM test. Data are expressed as mean±SEM and analysed by two-way ANOVA; n=8 per group.

EPM Measure	11β-HSD2 Genotype	Prenatal Manipulation
Open arm entries	$F_{2,28}$=0.55, p=0.584	$F_{1,28}$=0.35, p=0.558
Closed arm entries	$F_{2,28}$=0.32, p=0.728	$F_{1,28}$=0.03, p=0.874
Open duration	$F_{2,28}$=2.40, p=0.109	$F_{1,28}$=0.32, p=0.557
Open distance	$F_{2,28}$=1.45, p=0.252	$F_{1,28}$=0.37, p=0.551
Total distance	$F_{2,28}$=0.62, p=0.547	$F_{1,28}$=0.03, p=0.855

Table 4.6b Statistical analysis of elevated plus maze behaviour shown in Table 4.6a as analysed by two-way ANOVA with SNK *post-hoc* testing

11β-HSD2 Genotype	Prenatal Manipulation	Crossings		Inner Zone Duration (secs)
		Inner Zone	Total	
+/+		40.3±9.0	519±34	17.8±4.6
+/-	CONTROL	42.4±11.3	490±37	19.7±4.3
-/-		50.4±14.3	418±32	22.9±6.3
+/-	RESTRAINT	46.9±8.2	413±23	21.4±4.4

Table 4.7a Behaviour in the open field test of female 11β-HSD2 transgenic mice subjected to prenatal restraint or unmanipulated controls
Effects of 11β-HSD2 genotype (+/+, +/- and -/-) and prenatal manipulation on the number of crossings in the inner zone and in total, and time spent in the inner zone during a 5-min OF test. Data are expressed as mean±SEM and analysed by two-way ANOVA; n=8 per group

OF Measure	11β-HSD2 Genotype	Prenatal Manipulation
Inner zone crossings	$F_{2,28}$=0.24, p=0.791	$F_{1,28}$=0.08, p=0.774
Total crossings	$F_{2,28}$=2.69, p=0.085	$F_{1,28}$=2.93, p=0.098
Inner zone duration	$F_{2,28}$=0.27, p=0.767	$F_{1,28}$=0.05, p=0.822

Table 4.7b Statistical analysis of open field behaviour shown in Table 4.7a as analysed by two-way ANOVA with SNK *post-hoc* testing

11β-HSD2 Genotype	Prenatal Manipulation	Latency (secs)	Crossings	Time in Light (secs)
+/+		15.8±2.1	11.4±1.4	58.4±7.2
+/-	CONTROL	19.2±3.7	10.5±2.5	61.3±7.5
-/-		14.5±2.9	14.1±1.4	68.5±6.4
+/-	RESTRAINT	16.0±2.1	12.1±2.5	54.9±9.5

Table 4.8a Behaviour in the light/dark anxiety test of female 11β-HSD2 transgenic mice subjected to prenatal restraint or unmanipulated controls
Effects of 11β-HSD2 genotype (+/+, +/- and -/-) and prenatal manipulation on the latency to cross into the dark compartment, number of crossings between the two compartments and time spent in the light compartment. Data are expressed as mean±SEM and analysed by two-way ANOVA; n=8 per group.

LD Measure	11β-HSD2 Genotype	Prenatal Manipulation
Latency to cross	$F_{2,26}$=0.73, p=0.489	$F_{1,26}$=0.65, p=0.651
Crossings	$F_{2,26}$=0.90, p=0.419	$F_{1,26}$=0.31, p=0.582
Time in light	$F_{2,26}$=0.47, p=0.632	$F_{1,26}$=0.31, p=0.581

Table 4.8b Statistical analysis of open field behaviour shown in Table 4.8a as analysed by two-way ANOVA with SNK *post-hoc* testing

4.3.3 Cohort three

To test whether increased handling of mice postnatally (cohort two) reversed the phenotype observed in cohort one, the anxiety-related behaviour of unmanipulated 11β-HSD2 transgenic mice was analysed.

4.3.3.1 Behaviour of male 11β-HSD2 transgenic mice

In the EPM anxiety test, there was no statistical difference of genotype in the measured parameters. Open entries (Table 4.9; $F_{2,29}$=0.13; p=0.880), closed entries ($F_{2,29}$=1.20, p=0.315), open duration ($F_{2,29}$=0.91, p=0.414), distance travelled on open arms ($F_{2,29}$=0.68, p=0.515) and entire maze ($F_{2,29}$=0.05, p=0.955) were not different between genotypes.

11β-HSD2 Genotype	Entries		Distance (au)		Open Duration (secs)
	Open	Closed	Open	Total	
+/+	12.8±1.9	18.7±1.2	120±34	901±87	40.7±10.5
+/-	13.7±1.7	23.2±1.8	124±33	882±50	45.9±10.9
-/-	14.2±2.3	20.2±3.4	175±40	868±85	62.9±13.2

Table 4.9 Behaviour of male 11β-HSD2 transgenic mice in the elevated plus maze
Effects of 11β-HSD2 genotype (+/+, +/- and -/-) on the number of entries made onto the open and closed arms, distance travelled on the open arms and the whole maze, and time spent on the open arms during a 5-min EPM test. Data are expressed as mean±SEM and analysed by one-way ANOVA; n=9-13 per group. au = arbitrary units

4.3.3.2 Behaviour of female 11β-HSD2 transgenic mice

In the EPM anxiety test performed on female offspring, closed arm entries displayed an overall effect of genotype (4.10; $F_{2,22}$=4.39; p<0.05) with 11β-HSD2-/- group making significantly fewer closed entries than +/+ and +/- groups (P<0.05). No overall effect of genotype was observed on open entries ($F_{2,22}$=0.07, p=0.931), open duration ($F_{2,22}$=0.52, p=0.603), distance travelled on the open arms ($F_{2,22}$=0.49, 0.618) or entire maze ($F_{2,22}$=0.33, p=0.719).

11β-HSD2 Genotype	Entries		Distance (au)		Open Duration (secs)
	Open	Closed	Open	Total	
+/+	16.6±4.4	22.4±1.7	99±37	957±82	35.0±10.6
+/-	15.1±2.3	21.9±1.1	149±29	944±58	47.8±7.2
-/-	15.0±3.0	16.0±2.1*	147±58	870±86	49.9±16.7

Table 4.10 Behaviour of female 11β-HSD2 transgenic mice in the elevated plus maze
Effects of 11β-HSD2 genotype (+/+, +/- and -/-) on the number of entries made onto the open and closed arms, distance travelled on the open arms and the whole maze, and time spent on the open arms during a 5-min EPM test. Data are expressed as mean±SEM and analysed by one-way ANOVA with SNK *post-hoc* testing; n=6-12 per group. *p<0.05 vs +/+ and +/-. AU = arbitrary units

These results indicate that the early postnatal handling of pups is not responsible for the loss of heightened anxiety-like phenotype previous observed in 11β-HSD2-/- mice.

4.4 Discussion

In this chapter we describe work carried out in the behavioural assessment of mice bred through 11β-HSD2 heterozygous parents, thus generating 11β-HSD2 null, heterozygous and wild-type offspring whilst controlling for any influence of altered pre- or post-natal maternal nurture. We have shown that 11β-HSD2 null mutant mice from heterozygous mothers display behavioural alterations in anxiety and spatial learning and memory tests. In brief, male 11β-HSD2 knockout mice displayed heightened anxiety-like behaviour in the EPM and LD test, although not the OF test. Female 11β-HSD2 knockout mice displayed anxiety-like behaviour in the EPM only. The results also suggest that there may be a slight deficit in the spatial learning and memory ability of male 11β-HSD2 null mice in the water maze test. However, 11β-HSD2 null mice of both genders did not display alterations in fear-related memory when assessed in the passive avoidance test.

It has been previously reported that PS or GC administration programs lifelong alterations in offspring behaviour including heightened anxiety (Vallee et al., 1997; Welberg et al., 2001; Van den Hove et al., 2005; Oliveira et al., 2006) and cognitive impairment (Lordi et al., 1997; 2000; Brabham et al., 2000). The presence of the 11β-HSD2 enzyme in the placenta is thought to be a critical regulator of GC maternal-fetal transfer, acting to restrict the free passage of CORT into the fetal circulation (Seckl, 1997). 11β-HSD2 null deletion results in similar effects to prenatal programming including low birth weight and anxiety-related behaviour in the EPM and OF in male mice (Holmes et al., 2006a). However, these mice also display symptoms of AME, including hypertension, electrolyte imbalance and hypotonic polyuria (Kotelevtsev et al., 1999). This may severely impair both the prenatal nutrient supply and postnatal pup rearing behaviour of the 11β-HSD2 null mother, effects that may be involved in the observed programmed phenotype. In contrast, 11β-HSD2 heterozygous mice appear 'normal' and do not display signs of AME. Therefore, the heterozygous mothers are not thought to have deficits in maternal nurture.

4.4.1 Anxiety-related behaviour

11β-HSD2 null male mice from our studies displayed a similar increase in anxiety-like behaviour in the EPM reported in 11β-HSD2 null male mice bred through homozygous matings (Holmes et al., 2006a). However, in contrast to that reported in homozygous-bred 11β-HSD2 knockouts (Holmes et al., 2006a), male mice in our studies did not exhibit anxiety-like behaviour on the OF. This may indicate a reduced severity of programmed anxiety in the heterozygous-bred 11β-HSD2 null mice compared to their homozygous-bred equals. Thus, it is possible that the altered maternal physiology, as a consequence of AME (Kotelevtsev et al., 1999) and also enhanced anxiety (Holmes et al., 2006a), may contribute to the severity of the anxious phenotype of the offspring. This altered physiology may compromise nutrient provision during fetal and early postnatal life and/or maternal nurturing behaviour. Alternatively, the lack of any measurable difference in the OF may reflect the insensitivity of the test for assessing affective function in mice.

Female 11β-HSD2 null mice had not previously been examined in anxiety tests. In contrast to males, our studies do not present concrete evidence for programmed anxiety behaviour in female offspring, however it should be highlighted that group sizes were small for female behavioural studies. Due to the relatively high biological variability traditionally noted in anxiety testing, any subtle alteration in anxiety-related behaviours may be masked. More studies should be performed obtaining evidence to confirm or deny an anxiety-like phenotype in female 11β-HSD2 null mice. Nevertheless, we can confirm that anxiety-like behaviour in heterozygous-bred 11β-HSD2 null mice is more apparent in males than females. As discussed in the previous chapter, gender differences on both anxiety tests and sensitivity to prenatal programmed alterations in behaviour are not uncommon.

4.4.2 Learning and memory behaviour

In this study we evaluated the behaviour of heterozygous-bred male 11β-HSD2 null, heterozygous and wild-type mice in the water maze and passive avoidance tests, to assess cognitive learning and memory. Performance in the water maze has previously indicated

impairment of spatial memory in PS offspring (Szuran et al., 1994; 2000; Aleksandrov et al., 2001; Zagron and Weinstock, 2006; Yang et al., 2007) and following prenatal DEX (Brabham et al., 2000). However no significant difference in water maze exploration was reported in offspring prenatally-treated with either CBX or DEX throughout gestation (Welberg, 1999). The water maze test involves placing the subject into a circular pool of water containing a submerged platform. During the first two days of the test, the platform was cued with the addition of a visual object allowing mice to find the submerged platform and associate the platform with escape from the maze. The following five days the platform is hidden, although extramaze cues are present to allow acquisition and memory of platform location.

In both the cued and hidden platform parts of the test 11β-HSD2 null mice displayed reduced improvement in escape latency and distance travelled between the first two days, compared to other groups. This finding suggests these offspring possess a mild impairment in spatial associative learning and memory, at least in this paradigm. 11β-HSD2 heterozygous and wild-type mice displayed a significant reduction in escape latency and distance travelled between the first two days in both the cued and hidden platform sections of the test, a finding indicative of significant associative and spatial learning in these mice (Morris, 1984). However, this reduction in escape latency and distance swum was not found to be significant in 11β-HSD2 null mice, which suggests this group may possess associative and spatial learning impairments.

The average swim speed was found to be reduced in the 11β-HSD2 null mice. This finding could be indicative of an altered search and escape strategy of these mice in the maze or alternatively, be explained by a motor impairment in mutant offspring. 11β-HSD2 mutant mice have been reported to display reduced cerebellar size during the early postnatal period which was associated with delayed developmental landmarks (Holmes et al., 2006b). Although we have not examined the cerebellum in adult 11β-HSD2 mutant mice, developmental abnormalities in this critical structure mediating motor coordination

(Swinny et al., 2005) could be reflected in deficits in adulthood. Data from wheel running activity indicated that male 11β-HSD2 null mice are spontaneously hypoactive over a 24-hr period (Chapter 6). However, we did not measure acute motor function in our 11β-HSD2 null mice, although will be important to do so in the future. Several tests have been validated for this purpose including the rotarod test (Fujimoto et al., 2004). We observed a reduction in measures that reflect general locomotor activity in the EPM (number of closed arm entries and total distance travelled) and LD (crossings between compartments) anxiety tests. However, these factors are also dependent upon by anxiety levels in the mice, which we believe to be heightened. Indeed, in the OF test, where locomotor activity is thought to have a large influence on behaviour (Prut and Belzung, 2003), no difference was detected between genotypes. The behaviour described above in our anxiety tests does not support a role for motor impairment in the observed effects. The water maze procedure has been shown to be extremely stressful (Sandi et al., 1997; Engelmann et al., 2006) and it is possible that the reduced swim speed may suggest a heightened anxiety response. The time spent near the walls of the maze (thigmotaxis) has been proposed to be a measure of anxiety in the water maze (Simon et al., 1994). However, despite evidence of heightened anxiety-like behaviour on the EPM and LD test we saw no alteration of thigmotaxis in 11β-HSD2 null mice. Together, the strongest evidence suggests that the reduced swim speed is due to a less proactive approach in escaping the water.

Fear-related memory was assessed in the passive avoidance test in both male and female 11β-HSD2 null mice. This test involves the conditioned pairing of entry into the less anxiogenic dark compartment of the LD box with a footshock. The association is subsequently tested and normally expressed as an increased post-shock latency compared to pre-shock latency to cross into the dark compartment. We observed no significant difference between male 11β-HSD2 genotypes in the magnitude of avoidance conditioning, implying that memory consolidation of the aversive experience was unimpaired in 11β-HSD2 null mutants. A peculiar observation in female mice subjected to the passive avoidance apparatus was noted. Whilst, 11β-HSD2 heterozygous and null mice displayed

significant avoidance conditioning, this was not evident in wild-type offspring. The reason for this is unclear, although as previously mentioned the sample size in our female studies was small and in particular the wild-type group (n=4). We were unable to repeat this test due to restricted numbers of mice available.

Additionally, care should be taken in attributing the altered behaviour in the 11β-HSD2 null mutant to cognitive impairments. These mice also display heightened anxiety which may confound the interpretation of behaviour in the water maze.

4.4.3 Behaviour of 11β-HSD2 heterozygous mice

In our different murine tests we saw no differences in anxiety-like and cognitive behaviour in 11β-HSD2 heterozygous mice of either gender. Several possible explanations exist:

1.) The approximate 50% activity of the enzyme within placenta (Kotelevtsev et al., 1999), and presumably the developing brain regions, provides sufficient regulation of feto-placental CORT transfer to prevent fetal overexposure to maternal CORT and subsequent programmed effects observed in the null offspring.

2.) 11β-HSD2 heterozygous mice lack the symptoms of AME (hypotonic polyuria, hypertension, electrolyte imbalance) observed in null mice (Kotelevtsev et al., 1999) suggesting that this may underlie the heightened anxiety-like behaviour and cognitive impairment observed.

3.) The tests employed were not sensitive enough to detect small differences in anxiety-related behaviour.

The placental expression of 11β-HSD2 in the mouse declines rapidly around E15-16 (Brown et al., 1996a). Additionally, there is a marked surge in maternal CORT during the last week and dams become incapable of mounting a further increase following 10-min

stress exposure (Douglas et al., 2003; Holmes et al., 2003). We therefore decided to commence daily restraint during the more susceptible mid-gestational period, during which stress can elicit a response (Holmes et al., 2003) and when 11β-HSD2 is normally highly expressed (Brown et al., 1996b). We hypothesised that increased levels of CORT generated by maternal restraint stress would breach the more fragile feto-placental barrier in 11β-HSD2 heterozygous offspring. To test this, we subjected pregnant 11β-HSD2 heterozygous dams to restraint stress (E7-19, 1 hr daily) and compared offspring to those born to unmanipulated dams. 11β-HSD2 null mice were lighter at birth, irrespective of prenatal treatment. No difference in birthweight was observed in wild-type or 11β-HSD2 null mice when compared to prenatally-unmanipulated offspring of the same genotype. However, an effect of prenatal restraint was seen in 11β-HSD2 heterozygous offspring who were lighter than those born to unmanipulated control dams. This susceptibility to increased maternal CORT may reflect a breach of the weakened defensive barrier in this genotype, whilst a lack of effect on birth weight following prenatal restraint in wild-type indicates a more robust protection in these mice. Additionally, the absence of a further reduction in birth weight in 11β-HSD2 null mice following prenatal restraint points towards a ceiling effect of maternal CORT action on growth restriction *in utero*.

The prenatal restraint procedure performed in our studies (1 hr/day; E7-19) was less severe than has been employed in mice by other groups (Sternberg and Ridgway, 2003; Chung et al., 2005; Son et al., 2006) and did not cause growth restriction in the wild-type fetus. However, when combined with the susceptibility of heterozygous offspring to CORT overexposure, the prenatal stressor yielded a reduction in birth weight. Despite similar birth weights of wild-type and 11β-HSD2 null mice following prenatal stress compared to unstressed controls, offspring of all genotypes born and reared by a restraint-stressed dam were found to be lighter at weaning. This suggests the presence of a deficient postnatal environment in these offspring. PS has been reported to alter maternal behavioural in rats (Patin et al., 2002; Smith et al., 2004) and mice (Meek et al., 2001), including the impairment of nursing behaviour. In our studies we did not monitor maternal behaviour,

although PS may have altered both the physical care and/or the nutrient provision to offspring. The effect on body weight was not permanent as a reduction was no longer evident at the testing age of 13 wks.

In our studies heterozygous-bred 11β-HSD2 null mutant mice displayed a normalisation of the reduced body weight at birth by P14, indicating rapid catch-up growth had occurred. Homozygous-bred 11β-HSD2 knockout mice had a similar magnitude of birth weight reduction to that observed in heterozygous-bred mutants. However, homozygous-bred 11β-HSD2 knockout mice still displayed a marked, approximately 20%, reduction in body weight into midlife (Holmes et al., 2006a). Whilst a similar impairment of 11β-HSD2 null fetal growth was observed independent of maternal genotype (11β-HSD2 null or heterozygous), the key difference in the effects on offspring growth seems to lie in the postnatal period. The putative deficit in postnatal rearing of offspring thus appears additive to the prenatal GC environment and may contribute to the more visible anxious phenotype observed in homozygous- compared to heterozygous-bred 11β-HSD2 null male offspring. Catch-up growth has previously been thought to have a mechanistic involvement in programmed phenotype, through the metabolic tax placed on offspring during the rapid growth phase (Ong, 2006). However, this does not appear to be involved in the programming of anxiety-related behaviour which was apparent with or without catch-up growth in offspring.

It was highly disappointing when we were unable to detect any difference in behaviour in the anxiety tests between any 11β-HSD2 genotype or prenatal treatment when studies were conducted in the BRF at LF. Genotypes were confirmed twice and 11β-HSD2 null mutant mice displayed symptoms of AME, including polyuria and polydipsia. The absence of heightened anxiety-like behaviour in 11β-HSD2 null mice, a result confirmed by several investigators, contradicts the findings from studies conducted in cohort one, bred and studied in the BRF at the WGH. The reasons for this are uncertain, although a number of possible explanations exist:

1.) The procedures at LF required handling of pups and dam during early postnatal life during paw-marking and weighing;
2.) The nutritional content of the *ad libitum* fed diet was altered;
3.) The experimental and adjacent holding rooms were different;
4.) The general environment of the BRF and animal technicians were unavoidably distinct.

Handling of neonatal offspring daily is known to positively influence adult stress reactivity, emotional behaviour and cognitive performance in both rats (Fernandez-Teruel et al., 1997) and mice (Zaharia et al., 1996; Anisman et al., 1998; Parfitt et al., 2004). Additionally, handling has been observed to attenuate negative effects programmed through prenatal stress exposure in both species (Wakshlak and Weinstock, 1990; Meaney et al., 1991; Vallee et al., 1997; Sternberg and Ridgway, 2003), presumably mediated through an increase in mother-infant interactions (Liu et al., 1997; Francis et al., 1999). In view of this, we conducted behavioural assessment on offspring born and reared in the BRF at the LF, but under similar, undisturbed conditions previously employed in the BRF at the WGH. However, the results showed no differences between genotype groups in their behavioural phenotype which excludes postnatal handling as the sole candidate for an absence of measurable anxiety-related behaviour in 11β-HSD2 null mice bred and assessed in the BRF at LF.

The content of the diet fed to mice differed between the two biological research facilities. Dams and offspring were fed the CRM(E) diet at the WGH, which is comprised of: 62.0% carbohydrate, 18.6% protein and 3.4% oil. However, at LF dams were fed the RM3(E) diet during breeding and lactation, which is comprised of: 55.7% carbohydrate, 22.4% protein and 4.3% oil. In the BRF at LF, offspring were maintained on the RM1(E) diet which contained 66.7% carbohydrate, 14.4% protein and 2.7% oil. There is ample evidence that maternal diet during the perinatal period is capable of programming offspring adult physiology and behaviour (Buckley et al., 2005). Both high and low protein maternal diets

during pregnancy and lactation have been reported to programme negative consequences on the metabolic phenotype of offspring including: reduced birth weight, HPA axis dysregulation, renal damage, hypertension, glucose intolerance and obesity (Langley-Evans et al., 1996; Bertram and Hanson, 2001; Daenzer et al., 2002; Thone-Reineke et al., 2006). In addition, changes in hippocampal morphology have been observed (Cintra et al., 1997; Granados-Rojas et al., 2002). Daenzer et al. (2002) compared a 40% protein (high) diet with a 20% protein (normal) diet and observed decreased energy expenditure and increased adiposity in the high prenatal protein diet-fed group. Langley-Evans et al. (1996) compared a 9% (low) protein diet to a 18% (normal) protein diet, and detected hypertension in offspring that had received the prenatal low protein diet. These studies have compared offspring receiving markedly different protein concentrations in their diet. However, they illustrate that variation in prenatal protein can have a significant impact upon offspring and an effect of the different maternal protein diets (18.6% at the WGH; 22.4% at LF) on the offspring phenotype is not impossible. Additionally, the influence of weaning onto a different diet at LF (protein 14.4%) may play a role in an altered anxiety phenotype in adulthood. However, protein concentration is not the only difference in composition between these diets, but also the levels of carbohydrate and fat. It was recently shown that the intake of a diet high in omega-3 fatty acids pre- and post-weaning reversed the hypertension observed following prenatal DEX treatment (Wyrwoll et al., 2006). The potential impact of the altered nutrition during both breeding and later adult life in our studies is unknown, and whilst this may be a factor in the prevention of our previously observed adult phenotype of anxiety-like behaviour this is merely speculation. It would be of interest to explore a possible dietary involvement in heightened anxiety, although in future studies experimental animals will be given the CRM(E) diet, previously used in the BRF at the WGH, where both DEX-programmed rats and 11β-HSD2 null mice had showed changes in anxiety-related behaviour.

The experimental rooms were obviously different between research facilities, but the testing apparatus was identical. We took care to setup conditions as similar as possible,

including two uplift lamps providing a dim lighting. Lighting levels have been shown to significantly affect the exploratory behaviour anxiety tests (Griebel et al., 1993; Rodgers and Dalvi, 1997; Garcia et al., 2005) and the lighting was kept dim to provide a low anxiety baseline in our animals, thereby providing for a large window in which to measure any heightened anxiety.

An elaborate study was performed involving a series of behavioural tests, including the EPM and OF, on six mouse strains in three separate animal facilities (Crabbe et al., 1999). Care was taken to standardise testing apparatus, protocols and other environmental variables. The findings showed that not only did the strains differ in behavioural performance, but measurable anxiety-like behaviour in 5-HT_{1B} knockout mice was present only in one of the three test sites. Emotionality appears to be highly sensitive to variations between different research facilities and laboratories (Wahlsten et al., 2003). Therefore, differences in the individual animal technicians involved and the general environment, including noise levels and the size of the holding rooms are amongst variables that could have an influence on the measured anxiety-like behaviour in 11β-HSD2 null mice.

As discussed above, several factors may influence the presentation of an anxious phenotype in the 11β-HSD2 null mice offspring. Whilst postnatal handling has been excluded as the lone variable, mice in the future will be returned to the single diet during pregnancy, lactation and in adulthood used in the BRF at the WGH. Further investigation is underway to identify the underlying variables responsible for the absence of measurable anxiety we report here. However, such studies are beyond the scope of this thesis due to time constraints. Due to the problems showing anxiety in 11β-HSD2 null mutant mice the experiment conducted to determine whether prenatal stress could tip the heterozygous mice into a null phenotype was not able to produce any useable behavioural data. However, the weight differences did suggest that the 11β-HSD2 heterozygous mice displayed increased susceptibility to PS-induced growth restriction.

We have shown that heterozygous-bred 11β-HSD2 null mice display low birth weight, heightened anxiety and mild cognitive impairment compared to wild-type and 11β-HSD2 heterozygous mice. This phenotype was more apparent in males, although appears to be sensitive to housing conditions and was not found to be robust across biological research facilities. 11β-HSD2 null mice therefore display characteristics of prenatal GC programming similar to those observed with prenatal DEX treatment (Welberg et al., 2001) and prenatal 11β-HSD2 enzyme inhibition with CBX (Welberg et al., 2000). We observed these deficits in behaviour due to the direct effect of 11β-HSD2 enzyme loss independent of any alteration in maternal physiology or nurture, indicating that altered fetal and pup nutrition and maternal care is not a necessity for the programming of adult behavioural deficits. However, there is still the slight posibility that an 11β-HSD2 heterozygous dam could treat lighter (ie 11β-HSD2 null) offspring differently; the 11β-HSD2 null pup may not be able to get the same level of care and nipple access for feeding compared to heavier, more developed littermates. Additionally, 11β-HSD2 null mice also display symptoms of AME (hypotonic polyuria, electrolyte imbalance and hypertension) and a contribution of these factors to altered behaviour cannot be ruled out. Furthermore, 11β-HSD2 knockout mice will have dramatically altered GC metabolism and breakdown throughout life, potentially altering kinetics of GC/receptor interactions. This model of prenatal GC programming has significant advantages over other models which involve maternal manipulation, although limitations exist which preclude the attribution of GC programming as the sole explanation of observed phenotype. As will be further discussed in chapter 7, tissue-specific 11β-HSD2 mutations will be crucial in the separation of individual components capable of mediating the behavioural phenotype in adulthood.

CHAPTER 5
HPA AXIS IN 11β-HSD2 KNOCKOUT MICE IN ADULTHOOD AND THE PRE-WEANING PERIOD

5.1 Introduction

In the previous chapter we observed heightened anxiety-like behaviour and cognitive impairments in 11β-hydroxsteroid dehydrogenase type 2 (11β-HSD2) null mice, thought to be a result of prenatal glucocorticoid (GC) overexposure. Programmed effects have also been observed on the hypothalamic-pituitary-adrenal (HPA) axis function throughout life in previous models. Prenatal overexposure to GCs due to maternal stress, exogenous GC administration or 11β-HSD2 inhibition lead to hypothalamic-pituitary adrenal (HPA) axis alterations characterised by heightened basal, exaggerated and/or prolonged response to stress (Levitt et al., 1996; Weinstock et al., 1998; Vallee et al., 1999; O'Regan et al., 2004). Altered circulating corticosterone (CORT) levels have been found in combination with the altered expression of corticosteroid receptors and corticotrophin-releasing hormone (CRH) within the hippocampus, amygdala and paraventricular nucleus (PVN) of the hypothalamus (Welberg et al., 2000; 2001). Studies have shown that circulating CORT levels modulate a wide variety of behaviours in rodents, including those related to anxiety (Korte, 2001) and cognition (McEwen and Sapolsky, 1995). Additionally, genetic modulation of receptor expression within the brain has been found to modulate GC-mediated effects on behaviour (Steckler et al., 1999; Tronche et al., 1999; Gass et al., 2001; Wei et al., 2004; Berger et al., 2006). The observed HPA axis hyperactivity in prenatal dexamethasone (DEX)-treated offspring was postulated to play a role in the anxious behaviour (Welberg et al., 2001). We therefore wished to investigate the HPA axis and central expression of corticosteroid receptors and CRH in 11β-HSD2 null mutant mice to observe whether similar mechanisms may underlie our observed phenotype of heightened anxiety-like behaviour and memory impairments.

In addition we looked at three candidate genes involved in GC action and regulation of anxiety: Serum/glucocorticoid-regulated kinase 1 (Sgk1), FK506 binding protein 51 (Fkbp5) and Brain-derived neurotrophic factor (BDNF). Sgk1 mRNA expression was found to be responsive to GCs and stress exposure in various cell lines and in the brain (Naray-Fejes-Toth et al., 2000; Leong et al., 2003; Koya et al., 2005; Murata et al., 2005).

This kinase has also been implicated in the regulation of ion channels through modulation of cell surface expression (reviewed in Lang et al., 2003). Sgk1 mRNA expression has been detected in the rat (van Gemert et al., 2006) and murine hippocampus (Murata et al., 2005), from the late embryonic stage (Lee et al., 2001). Sgk1 mRNA expression could therefore mediate GC-induced effects on cell survival and neuronal excitability within the hippocampus.

Fkbp5 is an immunophilin, which acts as a co-chaperone of heat shock protein 90 (hsp90), forming part of the machinery governing nuclear translocation of GR. Fkbp5 is induced by GR activation and can act to negatively modulate steroid hormone signalling (Cheung and Smith, 2000). The relative expression of Fkbp5 is thought to be a cause of GC resistance observed in new world primates (Scammell et al., 2001). Single nucleotide polymorphisms (SNPs) in the human Fkbp5 gene have been detected and were found to result in altered recurrence of depressive episodes and response to antidepressant treatment (Binder et al., 2004). These studies suggest that this downstream target of GR activation could mediate GC effects upon behaviour and the HPA axis. Whilst hippocampal expression of other FK506 binding proteins have been reported in the mouse (Kato et al., 2000; Nielsen et al., 2004), no previous literature regarding hippocampal Fkbp5 mRNA expression in any species is available.

BDNF has a well-established role in the CNS regulating survival, growth and differentiation of neurones during development, being expressed in mice from embryonic day (E) 15 and peaking during second postnatal week (Ivanova and Beyer, 2001). The neutrophic factor is also expressed in adulthood, where it may function to modulate neuroprotection and synaptic plasticity (Castren, 2004; Marini et al., 2004). Expression within the murine brain is relatively ubiquitous, with the highest level observed within the hippocampus and cortex (Hofer et al., 1990). In the hippocampus, GCs have been shown to modulate the BDNF mRNA expression although not in all subfields. Activation of MR and GR may have opposing effects upon mRNA expression of BDNF (Chao and McEwen,

1994; Chao et al., 1998). BDNF is thought to play a key role in learning and memory (Yamada et al., 2002) as well as anxiety; in mice, anxiety-like behaviour on the EPM were correlated to hippocampal BDNF levels (Yee et al., 2006) and a genetic variant polymorphism in mice has yielded heightened anxiety (Chen et al., 2006). Reduced mRNA expression levels of BDNF were detected within the prefrontal cortex and striatum of adult rats subject to prenatal restraint stress (Fumagalli et al., 2004), although no alterations were seen in the hippocampus in either mRNA (Fumagalli et al., 2004) or protein (Koo et al., 2003) expression. In contrast, transiently reduced BDNF mRNA was observed at postnatal day (P) 5 in the neonatal rat hippocampus following prenatal restraint (Van den Hove et al., 2006c). From studies mentioned above it appears that Sgk1, Fkbp5 and BDNF are downstream targets of GC action and are therefore candidate genes for mediating the consequence of heightened HPA axis function. We therefore decided to explore the expression of these gene transcripts in addition to those of GR, MR, CRH and AVP in 11β-HSD2 null mutants to observe whether they may be dysregulated as a consequence of prenatal GC programming.

In addition to the state of HPA axis-associated genes during adulthood, we wished to study the postnatal ontogeny in 11β-HSD2 null mutant mice. The pre-weaning period is a time of significant growth and maturation of the limbic system and exhibits heightened sensitivity to programmed effects. Rat offspring prenatally treated with carbenoxolone (CBX) displayed increased hippocampal mRNA expression of MR at one week of age, significant only in the CA2 subfield. A trend towards increased GR was also evident, although only in the CA1 (Welberg, 1999). The altered corticosteroid receptor expression was resolved by adulthood when no changes within the hippocampus were evident (Welberg et al., 2000). We therefore studied the postnatal ontogeny of corticosteroid receptor mRNA expression weekly until weaning. In addition we explored the expression of the GC-induced transcripts (Sgk1, Fkbp5, and BDNF) during this period. Due to experimental limitations this was performed in mice bred through homozygous matings, the significance of which will be discussed later.

5.2 Methods

Comprehensive details of methods can be found in chapter 2. Except where specified, breeding and studies were performed in the BRF at the WGH.

5.2.1 Study of HPA axis in 11β-HSD2 transgenic mice

5.2.1.1 Heterozygous-bred adult offspring

Male and female 11β-HSD2 heterozygous mice were pair-housed in breeding cages and left undisturbed until parturition. Cages were checked daily between 09.00 and 11.00 h for delivery of litters. Following delivery, pups were counted and sexed. Litters were then left undisturbed with the dam until weaning at postnatal day (P) 21, when they were ear-notched (for identification and genotyping), housed in single-sex groups of 2-6 and left undisturbed until testing, apart from routine cage maintenance.

At the age of 15-16 weeks blood was collected between 09.00 and 10.00 h by tail nick to obtain an unstressed basal sample at the diurnal nadir. Two weeks later mice were restrained in plexiglass tubes for 10 min before decapitation and collection of trunk blood to obtain a peak stress sample. Tubes were centrifuged and plasma supernatant was stored at –20°C, until the measurement of CORT by radioimmunoassay. Brains were removed from males, rapidly frozen on dry ice and kept at –80°C until sectioned on the cryostat. Adrenals were removed from males, stored in formamide, later cleaned of fat by microdissection and weighed on a semi-microbalance.

At the age of 11-12 weeks, the left adrenals from male and female mice from cohort two bred in the BRF at LF (see Chapter 4, page 109) were removed, cleaned and weighed as above.

5.2.1.2 Homozygous-bred pre-weaning offspring

Male and female 11β-HSD2 homozygous or wild-type mice were pair-housed in breeding cages and left undisturbed until parturition. Cages were checked daily between 09.00 and 11.00 h for delivery of litters. Following delivery, pups were counted and sexed. No more than three pups per litter were removed at one week (P7), two weeks (P14) or three weeks (P21) of age and decapitated. Brains were removed, rapidly frozen on dry ice and stored at –80°C.

5.2.2 *In Situ* Hybridisation histochemistry

Coronal cryosections (14μm thickness) from adult male and mixed-gender pre-weaning brains were cut at the level of the paraventricular nucleus (PVN) and hippocampus, which both included amygdala regions (approx. bregma -0.70 to -1.90 mm). Sections were thaw-mounted onto prepared slides and stored at -80°C until further use. *In situ* hybridisation was performed on these sections using the antisense riboprobe for the transcript of interest shown in Table 5.1 (see also Chapter 2). Antisense riboprobes for GR, MR, CRH and AVP have previously been validated and used in our laboratory (Holmes et al., 2006; Welberg et al., 2000; 2001). Antisense riboprobes for Sgk1, Fkbp5 and BDNF had not previously been validated or used in our laboratory. The cDNA templates were sequenced and found to match the desired sequence.

Equivalent sense probes were included as controls. Previous studies and initial experiments indicated that Sgk1 and MR mRNA expression in the PVN is below our threshold for quantification and hippocampal CRH mRNA expression is very low. Therefore, *in situ* hybridisation was not performed for these gene transcripts in those regions.

The level of mRNA expression was quantified by optical densitometry of autoradiographs from sections containing the specific regions of interest. Individual expression was calculated from a mean of 5-10 measurements for each region, taken from both sides of the brain.

	PVN	Hippocampus
Adult	GR CRH AVP Fkbp5 BDNF	GR MR Sgk1 Fkbp5 BDNF
Pre-weaning	GR CRH Fkbp5 BDNF	GR MR Sgk1 Fkbp5 BDNF

Table 5.1 *In situ* hybridisation with riboprobes performed in adult and pre-weaning brain sections
Experiments performed in paraventricular nucleus (PVN) and hippocampus with riboprobes for glucocorticoid receptor (GR), mineralocorticoid receptor (MR), corticotrophin-releasing hormone (CRH), arginine vasopressin (AVP), serum/glucocorticoid-regulated kinase (Sgk1), FK-506 binding protein 5 (Fkbp5) and brain-derived neurotrophic factor (BDNF) mRNA.

5.2.3 Corticosterone radioimmunoassay

Plasma was diluted 1:10 in borate buffer containing 0.5% BSA and denatured at 75-80°C for 30 mins. 20µl of 1:10 plasma or CORT standards were incubated with 50µl ^{3}H-CORT/primary antibody mix. SPA beads (50µl) were then added and left overnight to equilibrate, following which plates were counted on a beta-counter. Sample CORT concentration was calculated from the Multicalc-prepared graph of cpm versus [CORT].

5.2.4 Statistical analysis

Data are expressed as mean±SEM. In studies on heterozygous-bred adult offspring, comparisons between genotype were analysed by one-way analysis of variance (ANOVA), with Student Newman-Keuls (SNK) *post-hoc* testing. In studies on homozygous-bred pre-weaning offspring, comparisons in each region, or hippocampal subfield, between genotype and age were analysed by two-way ANOVA, with SNK *post-hoc* testing. Where expression was only detected at a single age an unpaired t-test was performed.

5.3 Results

Offspring from litters of less than five were excluded from further analysis. Due to the semi-quantitative nature of *in situ* hybridisation, the direct comparison of expression levels between individual gene transcripts is not possible.

5.3.1 Heterozygous-bred adult male 11β-HSD2 transgenic mice

5.3.1.1 Brain mRNA expression of genes involved in the regulation of GC action and HPA axis activity

Representative *in situ* hybridisation autoradiograph pictures are shown in Figure 5.1.

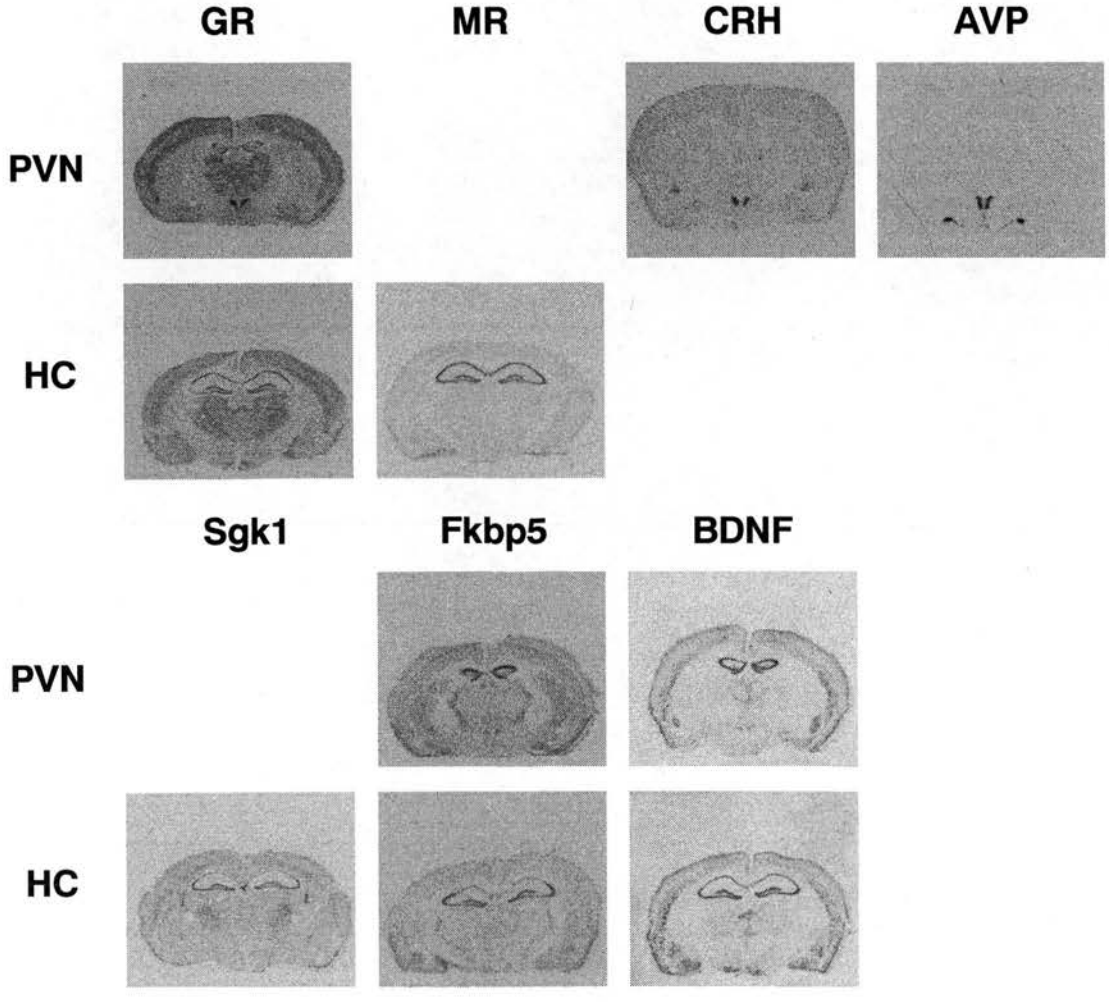


Figure 5.1 Autoradiograph pictures following GR, MR, CRH, AVP, Sgk1, Fkbp5 and BDNF *in situ* hybridisation at the level of the PVN and hippocampus (HC) in adult male mouse brain

Hippocampal mRNA expression of GR (Figure 5.2a), MR (Figure 5.2b), Sgk1, Fkbp5 and BDNF (Table 5.2) did not display any significant differences within any subfield between genotypes in adult male mice.

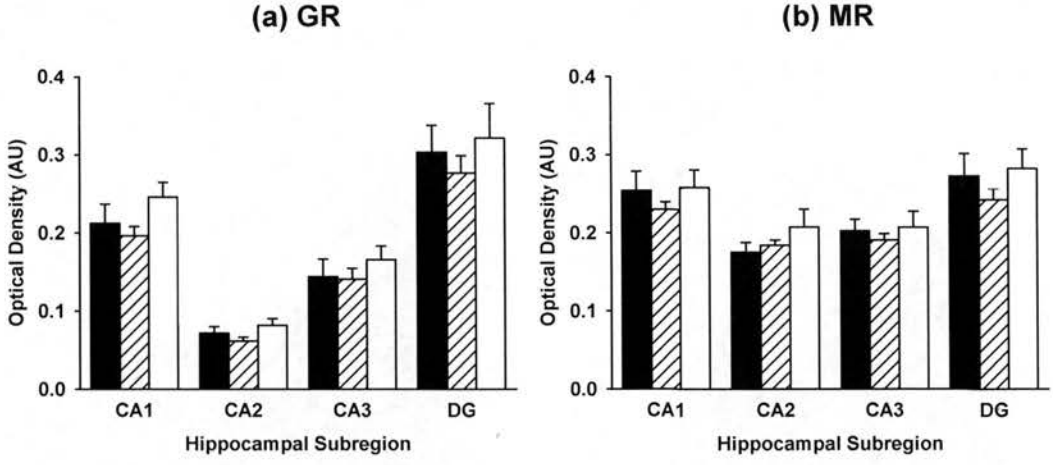


Figure 5.2 Hippocampal GR and MR mRNA expression in adult male 11β-HSD2 transgenic mice

Effect of 11β-HSD2 genotype (+/+ ■, +/- ▨, -/- □) on the hippocampal mRNA expression of the (a) glucocorticoid receptor (GR) and (b) mineralocorticoid receptor (MR). Expression levels were measured by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM in arbitrary units (AU) and analysed by one-way ANOVA for each subfield; n=7-15 per group.

11β-HSD2 Genotype	Optical Density (AU) CA1	CA2	CA3	DG
(a) Sgk1 mRNA				
+/+	0.23±0.02	0.44±0.01	0.54±0.02	0.45±0.03
+/-	0.22±0.01	0.43±0.02	0.51±0.02	0.43±0.02
-/-	0.25±0.03	0.47±0.02	0.53±0.03	0.44±0.03
(b) Fkbp5 mRNA				
+/+	0.31±0.04	0.72±0.03	0.31±0.04	0.62±0.04
+/-	0.28±0.02	0.74±0.02	0.31±0.02	0.58±0.03
-/-	0.34±0.03	0.74±0.03	0.32±0.02	0.62±0.02
(c) BDNF mRNA				
+/+	0.26±0.02	0.25±0.03	0.41±0.02	0.48±0.03
+/-	0.24±0.03	0.26±0.02	0.41±0.03	0.42±0.02
-/-	0.23±0.02	0.25±0.02	0.40±0.03	0.40±0.02

Table 5.2 Hippocampal mRNA expression of Sgk1, Fkbp5 and BDNF in adult male 11β-HSD2 transgenic mice
Effect of 11β-HSD2 genotype (+/+, +/-, -/-) on the hippocampal mRNA expression of the (a) serum/glucocorticoid-regulated kinase (Sgk1), (b) FK506 binding protein 5 (Fkbp5) and (c) brain derived neurotrophic factor (BDNF). Expression levels were measured by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM in arbitrary units (AU) and analysed by one-way ANOVA for each subfield; (a) n=6-13, (b) n=7-16 and (c) n=7-15 per group.

Furthermore, PVN mRNA expression of GR, CRH, Fkbp5, BDNF and AVP (Table 5.3) was unaltered by genotype. CRH, Sgk1, Fkbp5 and BDNF mRNA expression within the amygdala regions was also unchanged by genotype (Table 5.4). Sgk1 mRNA expression was only observed in the central amygdala (CeA), whilst Fkbp5 and BDNF mRNAs were only expressed in the medial amygdala (MeA) and basolateral amygdala (BlA). The expression of MR and GR mRNA was below our threshold for quantification and therefore was not measured.

11β-HSD2 Genotype	mRNA Optical Density (AU) (a) GR	(b) CRH	(c) Fkbp5	(d) BDNF	(e) AVP
+/+	0.60±0.03	0.47±0.04	0.21±0.02	0.21±0.03	0.61±0.06
+/-	0.56±0.03	0.43±0.03	0.20±0.02	0.25±0.01	0.56±0.03
-/-	0.62±0.03	0.39±0.04	0.19±0.02	0.21±0.02	0.62±0.03

Table 5.3 PVN mRNA expression of CRH, AVP, GR, Fkbp5 and BDNF in adult male 11β-HSD2 transgenic mice
Effect of 11β-HSD2 genotype (+/+, +/-, -/-) on the mRNA expression in the paraventricular nucleus (PVN) of the hypothalamus of (a) glucocorticoid receptor (GR), (b) corticotropin-releasing hormone (CRH), (c) FK506 binding protein 5 (Fkbp5), (d) brain derived neurotrophic factor (BDNF) and (e) arginine vasopressin. Expression levels were measured by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM in arbitrary units (AU) and analysed by one-way ANOVA; (a) n=4-10, (b,d) n=7-16, (c) n=6-16 and (e) n=6-13 per group.

11β-HSD2 Genotype	Optical Density (AU) CeA	MeA	BIA
(a) CRH mRNA			
+/+	0.18±0.01	below	below
+/-	0.17±0.01	quantification	quantification
-/-	0.18±0.02	threshold	threshold
(b) Sgk1 mRNA			
+/+	0.27±0.03	below	below
+/-	0.22±0.01	quantification	quantification
-/-	0.26±0.01	threshold	threshold
(c) Fkbp5 mRNA			
+/+	below	0.26±0.03	0.25±0.03
+/-	quantification	0.28±0.02	0.23±0.01
-/-	threshold	0.23±0.03	0.25±0.01
(d) BDNF mRNA			
+/+	below	0.29±0.05	0.37±0.02
+/-	quantification	0.27±0.02	0.36±0.02
-/-	threshold	0.28±0.02	0.37±0.04

Table 5.4 Amygdala mRNA expression of Sgk1, Fkbp5 and BDNF in adult male 11β-HSD2 transgenic mice
Effect of 11β-HSD2 genotype (+/+, +/-, -/-) on the mRNA expression in the central (CeA), medial (MeA) and basolateral (BIA) amygdala of (a) corticotrophin-releasing hormone (CRH), (b) serum/glucocorticoid-regulated kinase (Sgk1), (c) FK506 binding protein 5 (Fkbp5) and (d) brain derived neurotrophic factor (BDNF). Expression levels were measured by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM in arbitrary units (AU) and analysed by one-way ANOVA for each subfield; (a) n=7-16, (b) n=5-7 (MeA)/n=7-13 (BIA) and (c) n=4-11 (MeA)/n=6-13 (BIA) per group.

5.3.1.2 HPA axis

Plasma CORT was not altered by genotype under either basal or stress conditions in adult male (Figure 5.3a) or female (Figure 5.3b) mice. Relative left adrenal weight was significantly different between genotype (Figure 5.4; $F_{2,30}$=4.40, $p<0.05$), being reduced in male 11β-HSD2-/- compared to +/+ and +/- offspring ($p<0.05$). However, no difference was found between genotype in right adrenal weight ($F_{2,27}$=0.24, p=0.788).

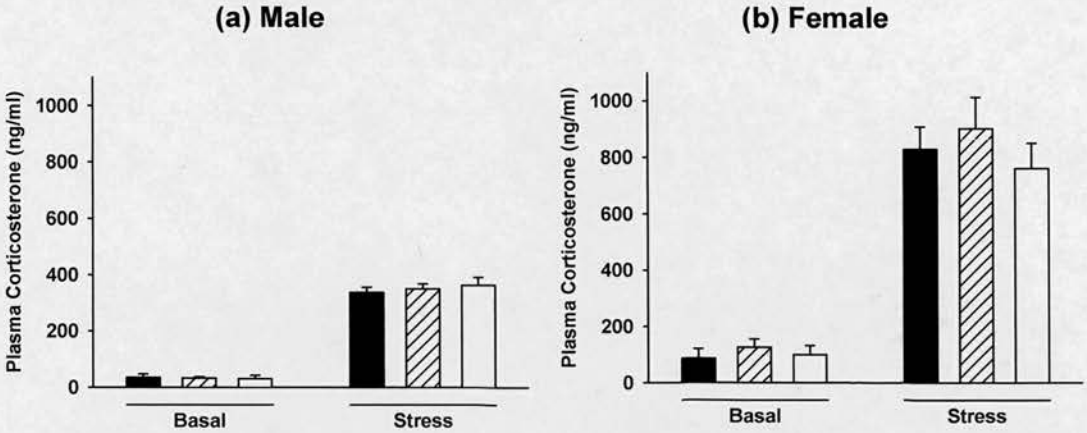


Figure 5.3 Plasma corticosterone under basal and stress conditions in adult male and female 11β-HSD2 transgenic mice
Effect of 11β-HSD2 genotype (+/+ ■, +/- ▨, -/- □) on basal plasma corticosterone and following 10-minute restraint (stress) in (a) male and (b) female adult mice. Data are expressed as mean±SEM in mg/ml and analysed by one-way ANOVA; (a) n=7-15 (basal)/n=8-14 (stress) and (b) n=5-8 (basal)/n=6-7 (stress) per group.

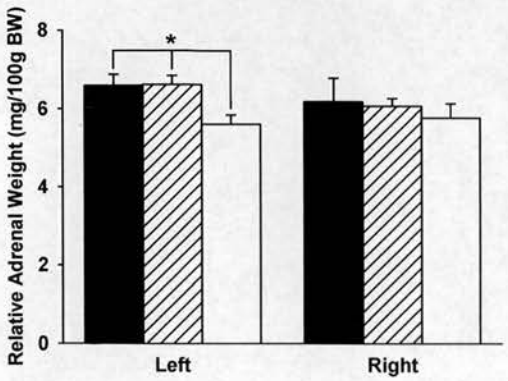


Figure 5.4 Adrenal gland weight in adult male 11β-HSD2 transgenic mice
Effect of 11β-HSD2 genotype (+/+ ■, +/- ▨, -/- □) on left and right adrenal gland weight relative to body weight (BW). Data are expressed as mean±SEM in mg/100g BW and analysed by one-way ANOVA with SNK *post-hoc* testing; n=8-16 (left)/n=7-14 (right) per group. *p<0.05

Due to the unexpected behavioural results in male and female 11β-HSD2 transgenic mice bred and maintained in the BRF at LF from cohort two (see chapter 4), we analysed adrenal glands from this cohort to check whether alterations in this aspect of their phenotype were also apparent. Male left adrenals were significantly different between genotype (Table 5.5; $F_{2,24}$=5.89, p<0.01), being significantly lighter in 11β-HSD2-/- compared to +/+ (p<0.01) and +/- offspring (p<0.05) consistent with the observations made in the 15-16 wk old mice (Figure 5.4). In contrast to males, there was no significant difference in adrenal weight between genotype in female mice ($F_{2,22}$=0.89, p=0.427).

11β-HSD2 Genotype	Relative Adrenal Weight (mg/100 g BW) Male	Female
+/+	6.31±0.19	11.84±0.44
+/-	6.14±0.25	11.65±0.29
-/-	**5.17±0.28*,#**	11.08±0.43

Table 5.5 Left adrenal gland weight in adult male and female 11β-HSD2 transgenic mice
Effect of 11β-HSD2 genotype (+/+, +/-, -/-) on left adrenal gland weight relative to body weight (BW). Data are expressed as mean±SEM in mg/100g BW and analysed by one-way ANOVA with SNK *post-hoc* testing; n=7-10 per group. *p<0.05 vs +/-, #p<0.01 vs +/+

5.3.2 Homozygous-bred 11β-HSD2 null pre-weaning offspring

5.3.2.1 Hippocampal mRNA expression

Representative autoradiograph pictures following GR and MR *in situ* hybridisation in pre-weaning mice brains at the level of the hippocampus are shown in Figure 5.5.

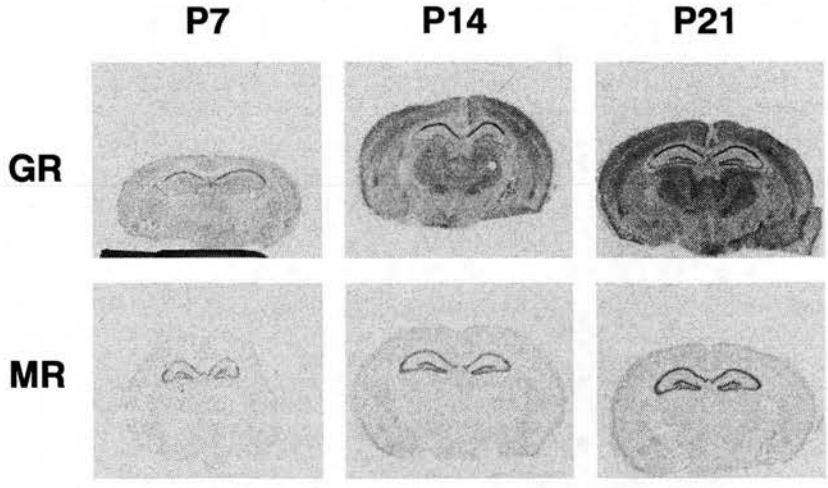


Figure 5.5 Autoradiograph pictures following GR and MR *in situ* hybridisation at the level of the hippocampus in pre-weaning mice

There was an age-dependent increase in GR mRNA expression throughout the pre-weaning period irrespective of genotype. 11β-HSD2-/- displayed significant alterations in hippocampal GR mRNA expression during early postnatal period (Figure 5.6). At P14 significant differences between -/- and +/+ mice in GR mRNA expression were seen in all subfields, with -/- mice displaying elevated expression (CA1: $p<0.01$; CA2/CA3/DG: $p<0.05$). Expression at P7 was only detected in CA1 region and was not different between genotype. No significant difference in GR mRNA expression was observed in any subfield at P21.

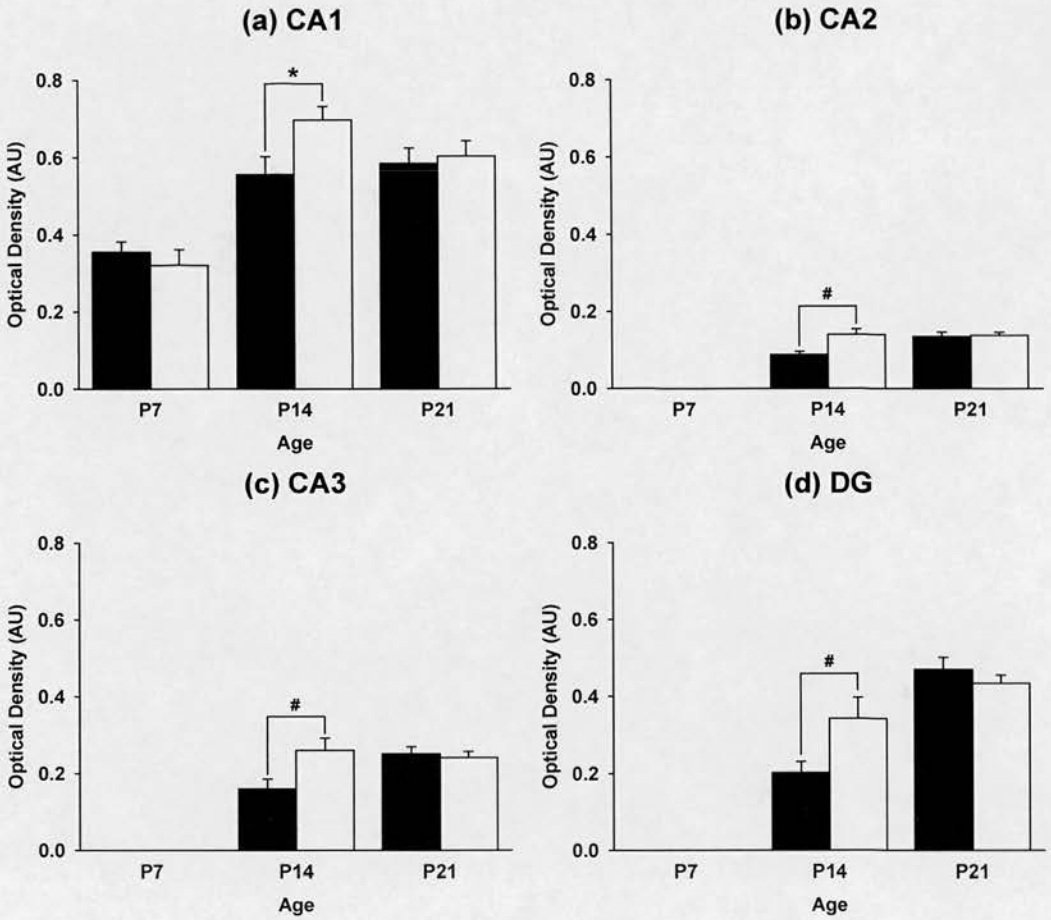


Figure 5.6 Hippocampal GR mRNA expression during pre-weaning period in 11β-HSD2 null mice

Effect of 11β-HSD2 genotype (+/+ ■, -/- □) on glucocorticoid receptor (GR) mRNA expression in the (a) CA1, (b) CA2, (c) CA3 and (d) dentate gyrus (DG) hippocampal subfields. Expression levels were measured in one-week (P7), two-week (P14) and three-week (P21) old mice by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM in arbitrary units (AU) and analysed by two-way ANOVA with SNK *post-hoc* testing for each subfield; n=5-10 per group. *$p<0.05$, #$p<0.01$ vs +/+

Hippocampal MR mRNA expression was different in 11β-HSD2-/- during early postnatal period (Figure 5.7). MR mRNA expression was elevated at P7 in the CA2 subfield only (Figure 5.7b; $p<0.05$), whilst at P14 it was significantly elevated in both the CA2 and DG ($p<0.05$), whilst displaying a tendency in the CA1 subfield ($p=0.080$). No differences were seen in any hippocampal subfield at P21.

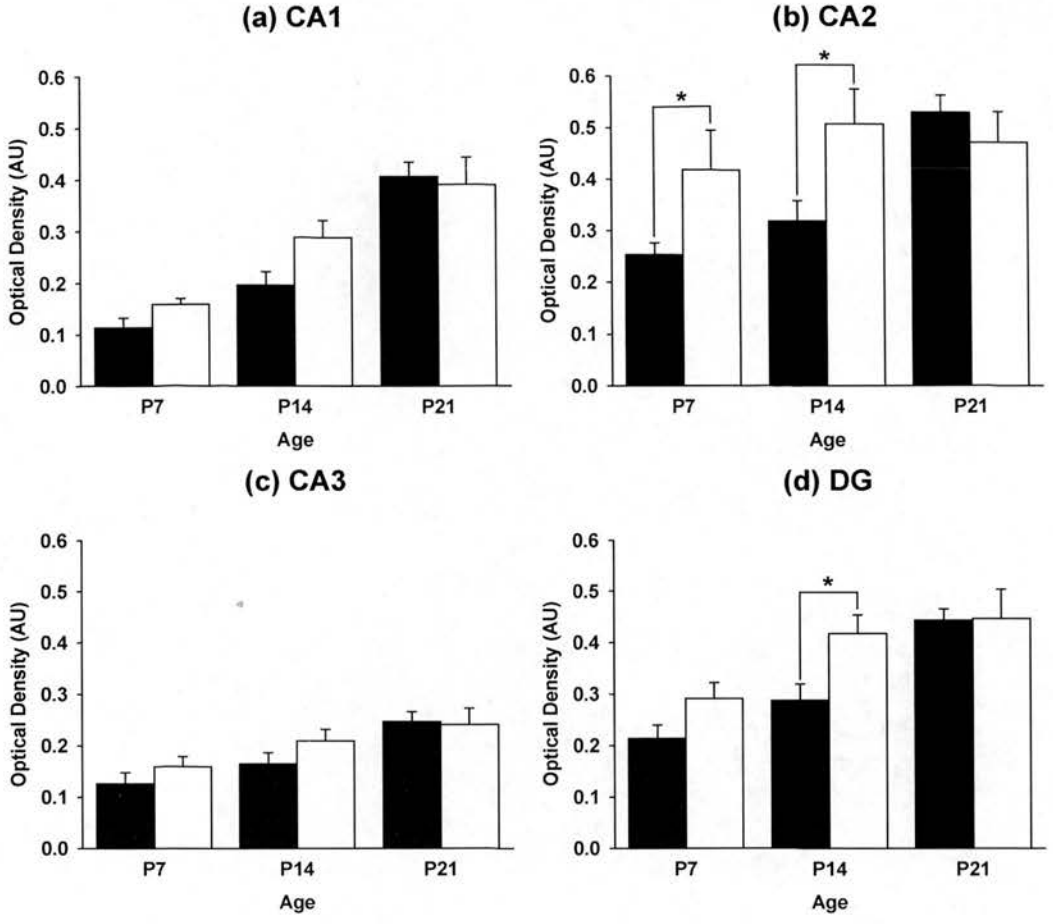


Figure 5.7 Hippocampal MR mRNA expression during pre-weaning period in 11β-HSD2 null mice

Effect of 11β-HSD2 genotype (+/+ ■, -/- □) on mineralocorticoid receptor (GR) mRNA expression in the (a) CA1, (b) CA2, (c) CA3 and (d) dentate gyrus (DG) hippocampal subfields. Expression levels were measured in one-week (P7), two-week (P14) and three-week (P21) old mice by optical densitometry of in situ hybridisation autoradiographs. Data are expressed as mean±SEM in arbitrary units (AU) and analysed by two-way ANOVA with SNK post-hoc testing for each subfield; n=5-10 per group. *p<0.05

Representative autoradiograph pictures following *in situ* hybridisation for Sgk1, Fkbp5 and BDNF mRNAs at the level of the murine hippocampus in the pre-weaning period are shown in Figure 5.8. Sgk1 mRNA expression was unaltered at P7 compared to 11β-HSD2+/+ offspring. However, a strong trend toward increased Sgk1 mRNA in 11β-HSD2-/- vs +/+ offspring at P14 was observed in the CA2 (Table 5.6; p=0.084), CA3 (p=0.083) and DG (p=0.068) subfields. At P21 Sgk1 mRNA expression was significantly elevated in

11β-HSD2-/- offspring within the CA1 ($p<0.05$), whilst being unaltered in the other subfields.

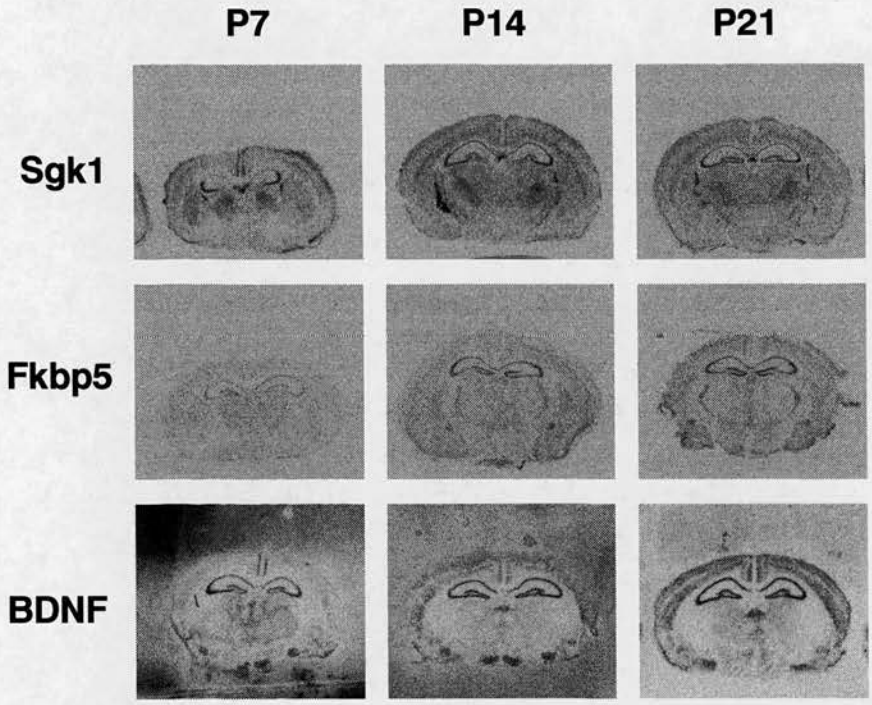


Figure 5.8 Autoradiograph pictures following Sgk1, Fkbp5 and BDNF *in situ* hybridisation at the level of the hippocampus in pre-weaning mice

11β-HSD2 Genotype	mRNA Optical Density (AU) P7	P14	P21
(a) CA1			
+/+	0.06±0.02	0.31±0.03	**0.19±0.03**
-/-	0.07±0.03	0.30±0.02	**0.27±0.02***
(b) CA2			
+/+	0.59±0.05	0.49±0.03	0.42±0.04
-/-	0.63±0.04	0.60±0.03	0.46±0.02
(c) CA3			
+/+	0.48±0.02	0.54±0.04	0.48±0.05
-/-	0.49±0.04	0.63±0.04	0.53±0.02
(d) DG			
+/+	0.06±0.02	0.33±0.04	0.39±0.05
-/-	0.06±0.02	0.41±0.02	0.43±0.01

Table 5.6 Hippocampal Sgk1 mRNA expression during pre-weaning period in 11β-HSD2 null mice
Effect of 11β-HSD2 genotype (+/+ and -/-) on Serum/Glucocorticoid-regulated kinase (Sgk1) mRNA expression in the (a) CA1, (b) CA2, (c) CA3 and (d) dentate gyrus (DG) hippocampal subfields. Expression levels were measured in one-week (P7), two-week (P14) and three-week (P21) old mice by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM in arbitrary units and analysed by two-way ANOVA with SNK *post-hoc* testing for each subfield; n=6-10 per group. *$p<0.05$

Fkbp5 mRNA was elevated in 11β-HSD2-/- compared to +/+ at P14 significantly in the CA2 (Table 5.7; $p<0.05$) and a tendency noted in the CA1 ($p=0.072$). At P21 a significant increase in Fkbp5 mRNA was observed in the CA1 of 11β-HSD2-/-, whilst within the CA2 ($p=0.058$) and DG ($p=0.056$) expression approached significance. No change in expression was found at P7.

11β-HSD2 Genotype	mRNA Optical Density (AU) P7	P14	P21
(a) CA1			
+/+	0.19±0.02	0.24±0.03	**0.23±0.02**
-/-	0.23±0.01	0.31±0.04	**0.32±0.02#**
(b) CA2			
+/+	0.33±0.03	**0.54±0.04**	0.50±0.04
-/-	0.36±0.04	**0.68±0.06***	0.60±0.05
(c) CA3			
+/+	0.15±0.02	0.21±0.02	0.21±0.02
-/-	0.19±0.02	0.25±0.03	0.23±0.02
(d) DG			
+/+	0.23±0.02	0.41±0.03	0.48±0.04
-/-	0.25±0.02	0.50±0.05	0.56±0.04

Table 5.7 Hippocampal Fkbp5 mRNA expression during pre-weaning period in 11β-HSD2 null mice

Effect of 11β-HSD2 genotype (+/+ and -/-) on FK506 binding protein 5 (Fkbp5) mRNA expression in the (a) CA1, (b) CA2, (c) CA3 and (d) dentate gyrus (DG) hippocampal subfields. Expression levels were measured in one-week (P7), two-week (P14) and three-week (P21) old mice by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM in arbitrary units and analysed by two-way ANOVA with SNK *post-hoc* testing for each subfield; n=3-9 per group. *$p<0.05$, #$p<0.01$ vs +/+

BDNF mRNA expression was also altered by genotype, being significantly elevated within the CA2 and DG subfields in 11β-HSD2-/- mice at P14 (Table 5.8; $p<0.05$). Expression was unchanged at either P7 or P21 in any hippocampal subfield.

11β-HSD2 Genotype	mRNA Optical Density (AU) P7	P14	P21
(a) CA1			
+/+	0.33±0.02	0.56±0.03	0.47±0.02
-/-	0.33±0.03	0.60±0.03	0.51±0.03
(b) CA2			
+/+	0.36±0.03	**0.49±0.03**	0.41±0.02
-/-	0.36±0.03	**0.57±0.02***	0.43±0.02
(c) CA3			
+/+	0.63±0.04	0.81±0.03	0.68±0.04
-/-	0.68±0.03	0.87±0.03	0.69±0.02
(d) DG			
+/+	0.47±0.05	**0.63±0.02**	0.67±0.03
-/-	0.50±0.01	**0.73±0.03***	0.69±0.02

Table 5.8 Hippocampal BDNF mRNA expression during pre-weaning period in 11β-HSD2 null mice

Effect of 11β-HSD2 genotype (+/+ and -/-) on brain derived neurotrophic factor (BDNF) mRNA expression in the (a) CA1, (b) CA2, (c) CA3 and (d) dentate gyrus (DG) hippocampal subfields. Expression levels were measured in one-week (P7), two-week (P14) and three-week (P21) old mice by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM in arbitrary units and analysed by two-way ANOVA with SNK *post-hoc* testing for each subfield; n=4-7 per group. *p<0.05

5.3.2.2 PVN and amygdala mRNA expression

Representative *in situ* hybridisation autoradiograph pictures at the level of the PVN are shown in Figure 5.9.

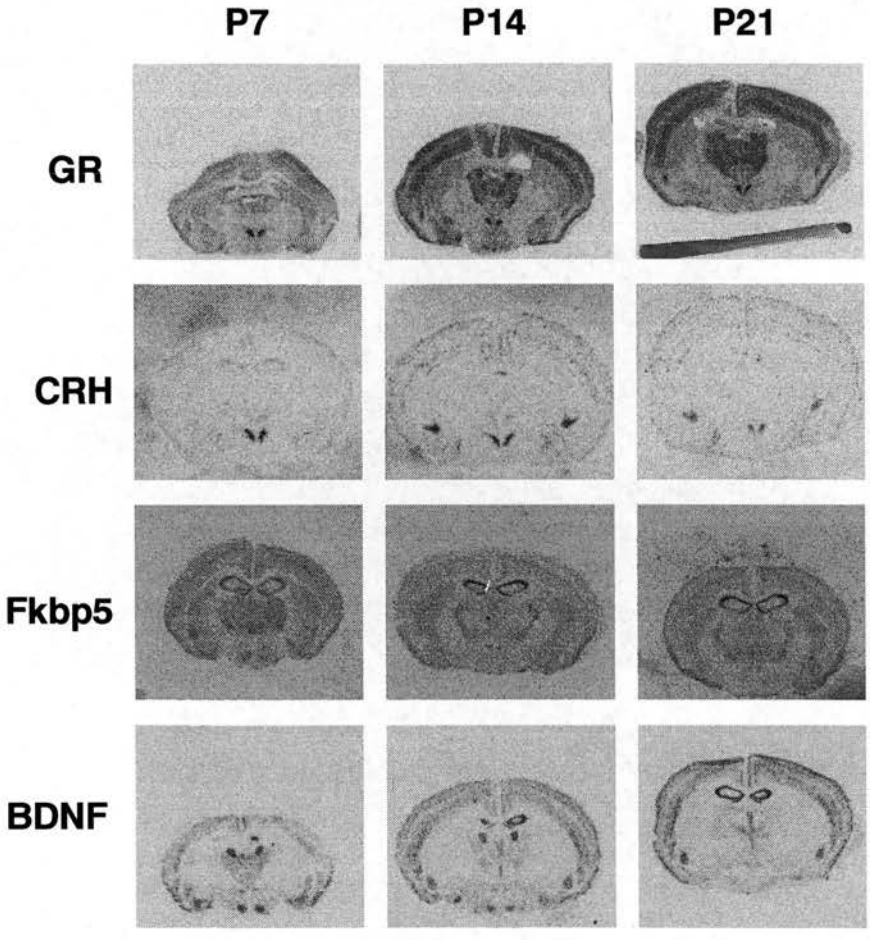


Figure 5.9 Autoradiograph pictures following GR, CRH, Fkbp5 and BDNF *in situ* hybridisation at the level of the PVN in pre-weaning mice

GR mRNA expression was significantly increased in 11β-HSD2-/- compared to +/+ mice at all measured ages: P7, P14 and P21 (Table 5.9; $p<0.05$). No significant difference at any age was observed in the expression of CRH, Fkbp5 or BDNF mRNA within the PVN between genotypes.

11β-HSD2 Genotype	Optical Density (AU) P7	P14	P21
(a) GR mRNA			
+/+	**0.62±0.03**	**0.53±0.04**	**0.49±0.03**
-/-	**0.76±0.05***	**0.64±0.04***	**0.61±0.02***
(b) CRH mRNA			
+/+	1.32±0.13	0.76±0.05	0.60±0.06
-/-	1.27±0.15	0.67±0.06	0.45±0.03
(c) Fkbp5 mRNA	below		
+/+	quantification	0.31±0.02	0.34±0.05
-/-	threshold	0.36±0.05	0.28±0.03
(d) BDNF mRNA			
+/+	0.29±0.04	0.27±0.02	0.30±0.02
-/-	0.32±0.07	0.29±0.04	0.27±0.02

Table 5.9 PVN mRNA expression of GR, CRH, Fkbp5 and BDNF during pre-weaning period in 11β-HSD2 null mice

Effect of 11β-HSD2 genotype (+/+ and -/-) on the mRNA expression in the paraventricular nucleus (PVN) of the hypothalamus of (a) glucocorticoid receptor (GR), (b) corticotropin-releasing hormone (CRH), (c) FK506 binding protein 5 (Fkbp5) and (d) brain derived neurotrophic factor (BDNF). Expression levels were measured in one-week (P7), two-week (P14) and three-week (P21) old mice by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM in arbitrary units and analysed by two-way ANOVA with SNK *post-hoc* testing; (a) n=5-10, (b) n=4-10, (c) n=3-10 and (d) n=4-8 per group. *p<0.05

Expression of CRH, Sgk1, Fkbp5 and BDNF mRNA within the amygdala was quantified and no differences were observed between 11β-HSD2 genotypes in any of the regions (Table 5.10 CeA; Table 5.11 MeA and Table 5.12 BlA) at any age (P7, P14 and P21). As observed in adult mice, the expression of MR and GR mRNA was below our threshold for quantification and was therefore not measured.

11β-HSD2 Genotype	Optical Density (AU) P7	P14	P21
(a) CRH mRNA	below	0.45±0.03	0.34±0.02
+/+	quantification	0.53±0.08	0.36±0.02
-/-	threshold		
(b) Sgk1 mRNA	below		
+/+	quantification	0.20±0.02	0.25±0.02
-/-	threshold	0.24±0.02	0.30±0.03
(c) Fkbp5 mRNA	below		
+/+	quantification	0.28±0.04	0.27±0.02
-/-	threshold	0.27±0.03	0.29±0.02

Table 5.10 CeA mRNA expression of Sgk1, Fkbp5 and BDNF during pre-weaning period in 11β-HSD2 null mice

Effect of 11β-HSD2 genotype (+/+ and -/-) on the mRNA expression in the central amygdala (CeA) of (a) corticotropin-releasing hormone (CRH), serum/glucocorticoid-regulated kinase (Sgk1) and (c) FK506 binding protein 5 (Fkbp5). Expression levels were measured in one-week (P7), two-week (P14) and three-week (P21) old mice by optical densitometry of in situ hybridisation autoradiographs. Data are expressed as mean±SEM in arbitrary units and analysed by two-way ANOVA; (a) n=5-10, (b) n=3-7 and (c) n=4-7 per group.

11β-HSD2 Genotype	Optical Density (AU) P7	P14	P21
BDNF mRNA			
+/+	0.45±0.05	0.48±0.04	0.53±0.03
-/-	0.52±0.01	0.55±0.05	0.47±0.05

Table 5.11 MeA mRNA expression of Fkbp5 and BDNF during pre-weaning period in 11β-HSD2 null mice

Effect of 11β-HSD2 genotype (+/+ and -/-) on the mRNA expression in the medial amygdala (MeA) of brain derived neurotrophic factor (BDNF). Expression levels were measured in one-week (P7), two-week (P14) and three-week (P21) old mice by optical densitometry of in situ hybridisation autoradiographs. Data are expressed as mean±SEM in arbitrary units and analysed by two-way ANOVA; n=4-10 per group.

11β-HSD2 Genotype	Optical Density (AU) P7	P14	P21
(a) Fkbp5 mRNA		below	below
+/+	0.37±0.02	quantification	quantification
-/-	0.39±0.03	threshold	threshold
(b) BDNF mRNA			
+/+	0.32±0.01	0.32±0.03	0.39±0.01
-/-	0.27±0.04	0.38±0.02	0.33±0.03

Table 5.12 BIA mRNA expression of Fkbp5 and BDNF during pre-weaning period in 11β-HSD2 null mice

Effect of 11β-HSD2 genotype (+/+ and -/-) on the mRNA expression in the basolateral amygdala (BIA) of (a) FK506 binding protein 5 (Fkbp5) and (b) brain derived neurotrophic factor (BDNF). Expression levels were measured in one-week (P7), two-week (P14) and three-week (P21) old mice by optical densitometry of *in situ* hybridisation autoradiographs. Data are expressed as mean±SEM in arbitrary units and analysed by (a) unpaired t-test or (b) two-way ANOVA; (a) n=5 and (b) n=4-9 per group.

5.4 Discussion

Results described in the previous chapter point to heightened anxiety-related behaviour and learning impairments in adult 11β-HSD2 null mice, indicative of lifelong programming through overexposure to maternal GCs during fetal life. In addition to behavioural alterations, studies in rat offspring subjected to prenatal stress (PS), DEX or CBX show evidence of HPA axis hyperactivity characterised by enhanced CORT levels under basal conditions and an exaggerated or a prolonged response to aversive situations (Levitt et al., 1996; Weinstock et al., 1998; Vallee et al., 1999; Welberg et al., 2000; O'Regan et al., 2004). Altered central expression of HPA axis-associated transcripts in key GC feedback sites, including the hippocampus, is thought to underlie this dysregulation, including reduced expression of the corticosteroid receptors within the hippocampus (Henry et al., 1994; Levitt et al., 1996; Koehl et al., 1999; Welberg et al., 2001). This reduction in receptors will impair negative feedback regulation of the HPA axis and influence emotionality and cognitive ability in the offspring. Enhanced forward drive on the HPA axis through elevated CRH in the PVN and amygdala has also been reported (Welberg et al., 2000; 2001) and may further contribute to the hyperactivity. We therefore investigated the HPA axis and expression of HPA axis-associated genes, candidates for the altered behaviour observed in the 11β-HSD2 null mutants.

In contrast to the HPA axis hyperactivity described above, plasma CORT levels were not altered under basal or peak stress conditions in heterozygous-bred 11β-HSD2 null mice. Furthermore, in adulthood there were no differences in the limbic expression of the corticosteroid receptors or our other measured transcripts: CRH, AVP, Sgk1, Fkbp5 and BDNF. It should be noted that a similar absence of altered HPA axis parameters has been made in our laboratory in adult homozygous-bred 11β-HSD2 null mutants (Holmes et al., 2006a). Thus, the putative altered maternal nurture provided by 11β-HSD2 null mothers is discounted from a role in programming the HPA axis. It is worth mentioning that we did not assess the extended stress response and recovery to baseline. However, the unaltered limbic expression of the corticosteroid receptors in adult 11β-HSD2 null mice makes

alterations unlikely. Although GC programming of the HPA axis has been well documented in rats, there have been fewer studies conducted in mice. Following a severe PS paradigm (6-hr daily restraint; E8 until delivery), adult male ICR mice displayed normal plasma CORT and adrenal weights (Chung et al., 2005). However, GR receptor density within the limbic system was reduced and application of a postnatal chronic stressor exacerbated abnormalities in HPA axis regulation and heightened anxiety (Chung et al., 2005). A less severe prenatal stressor in Balb/c mice resulted in heightened anxiety and decreased CRH mRNA within PVN of female offspring (Pincus-Knackstedt et al., 2006). Unfortunately circulating CORT levels or corticosteroid receptor expression were not measured and/or reported. Significant differences between rats and mice exist in the regulation of CORT in the pregnant dam and the pre- and postnatal ontogeny of corticosteroid receptors. Whilst the maternal CORT levels remain low throughout pregnancy in the rat (Johnstone et al., 2000), pregnant mice experience a profound surge of CORT towards term (Douglas et al., 2003; Holmes et al., 2003). In both species, hippocampal MR mRNA expression was identified from around E15 onwards (Diaz et al., 1998). However, in the murine hippocampus mRNA expression of GR was not detected until the first few days of postnatal life (Schmidt et al., 2003; Speirs et al., 2004; Noorlander et al., 2006), whilst GR is detected from midgestation onwards in the fetal rat (Diaz et al., 1998). These differences may decrease murine susceptibility to prenatal GC programming of the HPA axis and this should be kept in mind when comparing effects between species.

In male 11β-HSD2 null mutants, we observed significant hypotrophy of the adrenal gland, suggestive of HPA axis dysregulation. Reduced adrenal weight is indicative of decreased activity of the HPA axis, although no change in circulating CORT was detected. Several explanations could account for this observed HPA axis profile. Firstly, loss of 11β-HSD2 results in apparent mineralocorticoid excess (AME), characterised by hypertension, electrolyte imbalance and hypotonic polyuria, which could function to reset the HPA axis in these mice. However, how this could occur is unknown. Secondly, mice may be less

susceptible to programming of the HPA axis in general, as discussed above, or our background strain (C57BL/6) may be more resistant to HPA axis dysregulation, as has been observed in 11β-HSD1 null mice (Carter et al., unpublished observations). Thirdly, loss of the enzyme removes a key clearance pathway of CORT. Therefore a lower rate of synthesis is required to maintain normal circulating levels. One would expect a smaller adrenal gland to be reflected in reduced CORT response to acute stress, although we observed similar stress levels to wild-type mice. Perhaps prenatally programmed HPA axis hyperactivity is actually present in 11β-HSD2 null mice, as normal circulating CORT levels are seen when a hypoactivity would be anticipated. The observation of programmed behavioural alterations in the 11β-HSD2 null mice, independently of HPA axis hyperactivity, does indicate that elevated adult GCs are not the primary determinant of the anxiety phenotype. Interestingly, hypotrophy was only observed in the left adrenal gland. Although not well studied, adrenal asymmetry has been noted by other investigators (Droste et al., 2003; Perel'muter and Paderov, 2004). However, the reason for a selective weight reduction on the left hand side is unknown. Left adrenal glands from 11β-HSD2 mutant females did not display significant hypotrophy, although were approximately twice the size of males, a finding that correlates well with the roughly two-fold higher levels of basal circulating CORT in female compared to male mice that we observed and has been reported in the literature (Grad and Khalid, 1968). The apparent reduced sensitivity to 11β-HSD2 deletion in female mice is consistent with observations made on anxiety-like behaviour.

We examined the HPA axis and associated gene expression profile during the first three weeks of postnatal life. This represents a highly sensitive period in development, during which events can determine lifelong programmed effects (Meaney, 2001; Levine, 2005). Similar to adulthood, no alterations in circulating CORT under basal or stress conditions were observed at two weeks of age (Holmes et al., 2006a), although we uncovered significant changes in HPA axis-associated gene expression within the limbic system. The

postnatal ontogeny of the corticosteroid receptors and CRH was found to be in general agreement with previous studies in mice (Schmidt et al., 2003; Noorlander et al., 2006). We detected MR mRNA expression in all hippocampal subfields at P7, the abundance of which increased towards P21. However, 11β-HSD2 null mice displayed a significantly altered developmental trajectory of MR mRNA expression compared to wild-type mice. The MR transcript was significantly elevated at P7 although only in the CA2 subfield. At P14, increased expression of MR mRNA was seen in all but the CA3 subfield. We observed GR mRNA expression at P7 although only in the CA1 subfield. Similar to MR, 11β-HSD2 null mice had an altered trajectory of GR ontogeny during the pre-weaning period. Transient elevations in all hippocampal subfields were detected at P14 in 11β-HSD2 null mutants, whilst no changes were seen at P7 or P21 compared to wild-types.

Hippocampal elevations in GR mRNA have been observed previously following neonatal handling of rat offspring. This increase was detected as early as P7 (Meaney and Aitken, 1985) and has been found to persist throughout adulthood (Meaney et al., 1992). Enhanced GR within the hippocampus has been postulated to play a mechanistic role in the 'positive' consequences of neonatal handling, which include a less reactive HPA axis, reduced anxiety-like behaviour and prevention of age-related spatial memory impairment (Meaney et al., 1989; Vallee et al., 1997). In contrast to the lifelong hippocampal modification of GR mRNA expression following neonatal handling (which were opposite to those seen following prenatal DEX), the alterations observed in the 11β-HSD2 mutant mice were transient and not apparent in adulthood. Prenatal CBX was also found to transiently elevate hippocampal MR and GR in rat offspring at P7, although other pre-weaning ages were not studied (Welberg, 1999). The explanation for, or indeed the downstream effects of, the altered developmental trajectory are unclear.

The transient elevation in GR and MR is indicative of enhanced GC action in the hippocampus during this period. The neonatal cerebellum has previously been studied in 11β-HSD2 null mice. An altered cerebellar growth trajectory was observed during the pre-

weaning period in combination with a delay in developmental landmarks. Additionally, 11β-HSD2 null mutants were found to exhibit enhanced growth restriction following neonatal CORT treatment (Holmes et al., 2006b). In contrast to the cerebellum which displayed appreciable postnatal 11β-HSD2 mRNA expression (Roland et al., 1995b; Robson et al., 1998), hippocampal expression was found to be restricted to fetal life (Brown et al., 1996b; Diaz et al., 1998). Therefore, the hippocampal elevation of MR and GR mRNA is unlikely represent a direct intracellular effect of enzyme loss. Several possible explanations exist; it may be a result of prenatal GC overexposure and/or deficient pre- and postnatal nurture by the 11β-HSD2 null mother. Alternatively, the removal of CORT breakdown and distorted GC metabolism may be responsible. The hippocampus is a region that also exhibits considerable postnatal maturation (Leinekugel, 2003). Intricately involved in this maturation is CORT, identified as an important regulator of neurogenesis and neural development both in adulthood (Cameron and Gould, 1994), but also early postnatal life (Gould et al., 1991).

Corticosteroid receptors have been shown to have contrasting effects on neurogenesis in the hippocampus: whilst MR activation enhanced, GR activation inhibited neurogenesis *in vivo* (Fischer et al., 2002; Avital et al., 2006) and *in vitro* (Yu et al., 2004). This makes the relative balance of GR and MR an important functional determinant of CORT signalling, particularly within the hippocampus (de Kloet et al., 1998; Meijer and de Kloet, 1998). Fujioka et al. (2006) recently reported opposite effects of a mild or severe PS on neurogenesis. Severe PS caused a GR-mediated inhibition, whilst mild PS resulted in a MR-mediated enhancement of neurogenesis in the dentate gyrus of two week old rat offspring indicating a profound impact of prenatal manipulation on the postnatal hippocampus.

The net effect of the transient upregulation of both corticosteroid receptors in 11β-HSD2 null mice is uncertain. We did not assess the hippocampal morphometry or proliferation during the pre-weaning period in 11β-HSD2 null mice although this will be important in

identifying the functional consequences. The elevation of GR mRNA expression during the second and third week of postnatal life suggests that neurogenesis and the development of neuronal processes may have been inhibited. However, during this period MR was also increased in 11β-HSD2 null offspring which could cancel out the GR-mediated effect on neuronal maturation within the hippocampus. Elevations in the hippocampal transcripts of Sgk1, Fkbp5 and BDNF were also detected in 11β-HSD2 knockouts at P14. Sgk1 and Fkbp5 mRNA expression remained elevated at P21. The upregulation of these GC-inducible genes at these time-points provides support for elevated hippocampal GC signalling in 11β-HSD2 null mutants during the pre-weaning period. Whether these genes are involved in the downstream effects of the transient elevations in GC signalling is unknown. Whole brain Fkbp5 and Sgk1 mRNA were found to be elevated in methyl-CpG binding protein (Mecp2) null mice (Nuber et al., 2005). These mice displayed heightened anxiety despite unaltered circulating CORT (a similar scenario to that observed in the 11β-HSD2 null mutant). The potential contribution of these GC-inducible genes to the development of altered behaviour was postulated (Nuber et al., 2005).

Sgk1 is involved in regulating cell surface expression of ion channels (Lang et al., 2003) and is a key target of both MR and GR (Bhargava and Pearce, 2004). Therefore, the transient upregulation may be a consequence of activation of either corticosteroid receptor and could act to modulate neuronal excitability. Fkbp5 is induced by GR activation and may feed back to suppress steroid hormone signalling (Cheung and Smith, 2000). Fkbp5 may therefore be involved in dampening the induction of GR, although we can only speculate that it could be responsible for quenching the transient elevation we observed in 11β-HSD2 null mice. The question arises whether the transcriptional elevation we observed in the pre-weaning period occurs as a direct result of the prenatal GC effects (and possible downstream mediating effects on adult anxiety and cognitive behaviour) or as compensation for them. BDNF is known to positively influence growth and differentiation of neurones during development, being expressed in mice from E15 and peaking during second postnatal week (Ivanova and Beyer, 2001). Transiently reduced hippocampal

BDNF mRNA was observed at postnatal day (P) 5 in the neonatal rat hippocampus following prenatal restraint (Van den Hove et al., 2006c). BDNF mRNA expression has been found to be upregulated following aldosterone-mediated MR activation, and downregulated following GR activation (Chao et al., 1998). Therefore, our observation of elevated BDNF mRNA suggests a possible MR-mediated compensatory effect.

GCs and monoamine transmitter systems are known to exhibit reciprocal modulation (reviewed in Chaouloff, 2000). It was beyond the scope of this thesis to study monoamine systems in the 11β-HSD2 null mutants, although alterations have been documented following PS and DEX exposure and which could play a role in observed behavioural phenotype (Takahashi, 1998). PS in mice elicited a transient decrease in intrahippocampal 5-HT levels, observed at P21 and 42 but not at P7 or 63 (Ishiwata et al., 2005). Intriguingly, SSRI administration in the first three weeks normalised both the reduced 5-HT levels and decreased spine densities within the CA3 subfield observed at postnatal day 21, and prevented learning disability in the water maze seen during adulthood (Ishiwata et al., 2005). Administration between week 4 and 6 did not correct the aforementioned prenatally programmed deficits. These studies complement findings we have made in 11β-HSD2 null mutants and highlight the importance of the early postnatal development and alterations in predetermining later behavioural effects.

Prenatal DEX and CBX treatment has previously been found to elicit alterations in expression of HPA axis-associated genes within the PVN and amygdala (Welberg et al., 2000; 2001). Throughout the first three weeks of life we observed a significant rise in GR mRNA coupled with a slight, although non-significant, decrease in CRH mRNA expression in the PVN at P21 in 11β-HSD2 null mutants. However, the levels of Fkbp5 and BDNF mRNA expression were not affected, whilst Sgk1 expression, similar to in adulthood, was below our threshold of quantification. The amygdala is a key mediator of anxiety which is positively influenced by stress and corticosteroids (Schulkin et al., 1994), possibly through enhanced expression of CRH (Makino et al., 1994; Hsu et al., 1998). Prenatal DEX

treatment lead to increased CRH mRNA in the amygdala (Welberg et al., 2001). Furthermore, elevated CRH content of the amygdala has been observed in the rat (Cratty et al., 1995) and mouse (Chung et al., 2005) following PS. Expression within the amygdala regions in both the adult and early postnatal period was quantified, where possible. However, we did not measure any alteration in transcript levels within these regions in the 11β-HSD2 null mutant mice. Our results do not indicate any positive drive to the HPA axis as a consequence of 11β-HSD2 deletion.

In summary we observed transient elevations of corticosteroid receptors and GC-inducible transcripts in 11β-HSD2 null mutants during the early postnatal period. We postulate that elevated GC signalling during this critical developmental stage may inhibit neurogenesis and maturation of neuronal processes, later reflected in altered anxiety-related and cognitive behaviour in adulthood. Fetal GC overexposure may have programmed the altered developmental trajectory of the limbic system, particularly the hippocampus rather than hardwiring it prenatally. The offspring used to study the postnatal maturation of the limbic system were bred through homozygous matings. Therefore, maternal care and nutrition provided by the 11β-HSD2 mutant mother could play a significant part in the observed deficits, in addition to the excess GC milieu during fetal life. It would be interesting to follow-up our studies in offspring bred through heterozygous matings to identify the relative contribution of altered maternal nurture to our observations. A significant limitation of using 11β-HSD2 null mice with global enzyme deletion is that the effects of enzyme deficiency during fetal life cannot be dissociated from the obvious AME in these mice. This is especially pertinent in the study of the HPA axis where removal of the CORT inactivation pathway may mask any prenatally determined effects. This removal will prevent the accumulation of 11β-HSD1 substrate, 11-dehydrocorticosterone (11-DHC) and prevent the normal intracellular CORT regeneration and amplification of signalling, thus generating a state of 11β-HSD1 pseudo-inhibition and local CORT hypoactivity. The consequence of this will be discussed further in Chapter 7. Tissue-specific mutations will allow for the investigation of perinatally programmed effects, independent of additional deletion of the enzyme during adulthood. This will be a crucial step in confirming the locus of GC actions responsible for our observed findings.

CHAPTER 6
METABOLIC PHENOTYPE IN HETEROZYGOUS-BRED 11β-HSD2 KNOCKOUT MICE

6.1 Introduction

Although the main emphasis of this thesis is on the HPA axis, brain and behaviour we made observations during the course of our studies on a metabolic phenotype of 11β-HSD2 null mice, which may be relevant to the programmed phenotype. However, the results presented in this chapter really express a 'snapshot', demonstrating the importance of this model for future work. Prenatal dexamethasone (DEX) administration has previously been reported to programme hypertension, hyperglycaemia and impaired glucose tolerance in adult offspring (Nyirenda et al., 1998; 2001; 2006; O'Regan et al., 2004; Drake et al., 2005). The impaired glycaemic control is thought to be mediated through elevated GC signalling within the liver by heightened basal circulating plasma corticosterone (CORT) levels and/or enhanced hepatic glucocorticoid receptor (GR) mRNA expression (Nyirenda et al., 1998; 2001). Induction of the GC-regulated rate-limiting enzyme of gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK) is thought to underlie this (Nyirenda et al., 1998; 2001; O'Regan et al., 2004; Drake et al., 2005) as glucose uptake into the muscle or adipose tissue was found to be unaffected by prenatal DEX treatment (Cleasby et al., 2003a). Altered body composition was identified in DEX-treated offspring which displayed a 'lean' phenotype of reduced epididymal fat depot weight at 7-8 months of age (Cleasby et al., 2003a). Elevated GR mRNA expression was observed in this depot, although not in subcutaneous fat. Lipoprotein lipase (LPL) mRNA is known to be positively regulated by GCs and insulin (Fried et al., 1993; Oliver and Rogers, 1993). However, LPL mRNA expression was reduced in prenatal DEX epididymal fat (Cleasby et al., 2003a), suggesting this effect may be a secondary to elevated CORT and adipose GR, perhaps as a consequence of insulin resistance (Nyirenda et al., 1998). LPL overexpression has been seen to drive lipid accumulation in adipose depots (reviewed in Montague and O'Rahilly, 2000). Therefore, the reduction in LPL mRNA could contribute to the decreased adiposity through reduced fatty acid uptake from plasma.

11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) mRNA expression within the body is widespread. The highest transcript and activity levels have been observed in the liver,

although abundant expression has also been seen in the brain and adipose tissue (Monder and Lakshmi, 1990; Rajan et al., 1995). Through the local regeneration of CORT, 11β-HSD1 plays a key role in the amplification of GC signalling within these tissues. Obese humans and several rodent models of monogenic obesity have displayed elevated 11β-HSD1 mRNA expression and enzyme activity within adipose tissue (Livingstone et al., 2000a; 2000b; Rask et al., 2001; 2002; Lindsay et al., 2003). The generation and study of transgenic mice has been invaluable in exploring the role of the enzyme in metabolic balance. 11β-HSD1 null mutant mice displayed a 'cardioprotective' metabolic profile, characterised by increased hepatic and adipose insulin sensitivity, glucose tolerance, a favourable lipoprotein profile and resistance to increased weight gain following a high fat diet, even despite hyperphagia (Morton et al., 2001; 2004a). Although 11β-HSD1 mutant mice had adrenal hyperplasia and elevated circulating levels of CORT, they resisted hyperglycaemia upon high-fat diet (HFD) or stress challenge (Kotelevtsev et al., 1997).

Adipocyte-specific overexpression of 11β-HSD1 in transgenic mice resulted in a modest increase in GC regeneration but with marked metabolic changes. These mice displayed hypertension (Masuzaki et al., 2003), central obesity, glucose intolerance and insulin-resistant diabetes (Masuzaki et al., 2001). These findings recapitulate those observed in the clinical 'metabolic syndrome' supporting the involvement of excess GC signalling in the disorder. Whilst circulating CORT levels were normal, those in the portal blood were elevated approximately three-fold (Masuzaki et al., 2001) suggesting the locus of effect may include the liver in addition to adipose tissue. The involvement of increased intra-hepatic GC levels was explored through the generation and subsequent investigation of liver-specific 11β-HSD1 overexpressing transgenic mice. These mice were not found to exhibit obesity, but displayed more subtle alterations of metabolism including hypertension, insulin resistance, fatty liver and dyslipidemia (Paterson et al., 2004). However, no elevation in hepatic PEPCK mRNA was observed indicating that there was no major activation of the gluconeogenic pathway, despite elevated intrahepatic GC regeneration (Paterson et al., 2004). More recently, a transgenic mouse with adipocyte-

specific expression of the human 11β-HSD2 gene was created, resulting in appreciable CORT inactivation in fat depots where normal endogenous levels of the enzyme are minimal (Kershaw et al., 2005). These mice displayed a similar phenotype to the 11β-HSD1 deficient mouse (Morton et al., 2004a), resisting weight gain on a HFD and displaying reduced fat mass, improved glucose tolerance and insulin sensitivity although food intake was decreased (Kershaw et al., 2005), in contrast to the hyperphagia observed as a result of 11β-HSD1 deficiency (Morton et al., 2001). This discrepancy was later explained by alterations in the hypothalamic appetite and energy expenditure pathways in 11β-HSD1 null mutant mice (Densmore et al., 2006). Findings from studies investigating 11β-HSD transgenic mice have been consistent with a positive correlation between intracellular CORT and detrimental metabolic consequence, including obesity and the dysregulation of glycaemic control.

11β-HSD2 null mutant mice could display alterations in their metabolic phenotype, similar to either prenatal DEX-treated offspring or in 11β-HSD1 null mutant mice, as a consequence of prenatal GC overexposure and adult 11β-HSD1 pseudo-inhibition (11β-HSD2 null mice lack 11-dehydrocorticosterone (11-DHC), the substrate for 11β-HSD1). Also, they have altered GC kinetics which may affect GC regulation of metabolism. Therefore, we aimed to study the metabolic profile resulting from global 11β-HSD2 deletion. Although we were not able to obtain a comprehensive picture of this metabolic profile within 11β-HSD2 null mutants we have analysed the regional fat distribution, spontaneous voluntary activity, glucose tolerance and the expression of several GC-regulated genes within the liver and epididymal adipose tissue.

6.2 Methods

Comprehensive details of methods can be found in chapter 2.

6.2.1 Heterozygous-bred 11β-HSD2 null animal maintenance

Mice were bred and maintained in the biological research facility (BRF) at the Western General Hospital (WGH). Male and female 11β-HSD2 heterozygous mice were pair-housed in breeding cages and left undisturbed until parturition. Cages were checked daily between 09.00 and 11.00 h for delivery of litters. Following delivery, pups were counted and sexed. Litters were then left undisturbed until weaning at postnatal day (P) 21, ear-notched (for identification and genotyping), housed in single-sex groups of 2-6 and left undisturbed until testing in the water maze (see Chapter 4), apart from routine cage maintenance. Following testing at an approximate age of 8 months, mice were culled and tissue (fat: epididymal, subcutaneous, mesenteric; liver) was dissected and rapidly frozen on dry ice. A small section of liver was collected in HEPES/sucrose buffer and kept on ice, until the sample was homogenised and protein extracted (see below). Prior to storage at -80°C fat samples were weighed.

Male and female mice from which fat samples were obtained at 3 months of age were 11β-HSD2 heterozygous-bred and maintained in the BRF at Little France (LF). These mice had previously been subjected to a battery of behavioural testing (see Chapter 4; cohort three). Male mice studied for their spontaneous activity at 4 months and dynamic glucose response at 6 months of age had also been subject to a variety of behavioural tests (see Chapter 4; cohort two).

6.2.2 Wheel running

Adult male mice were single housed in wheel running cages and allowed to habituate to the novel environment for a week. Activity was recorded for one week and data averaged for this period.

6.2.3 Glucose tolerance test

Adult male mice were fasted overnight (16 hrs) and weighed before and after the fasting period. Following the fast, subjects were given an intraperitoneal injection of D-glucose (2mg/g body weight in saline). Blood samples (15-20μl) were taken by tail venesection into EDTA-coated Microvette tubes at 0 min (just prior to glucose injection) and at 5, 15, 30, 60 and 120 mins following glucose load. Blood samples were centrifuged (1000*g* for 10 mins at 4°C), plasma supernatant collected and stored at -20°C until glucose levels were assayed.

6.2.4 Hepatic PEPCK enzyme activity

Hepatic tissue was homogenised in HEPES/sucrose buffer and protein was extracted following centrifugation. Protein concentration was assayed using a Bio-Rad protein assay kit and 1mg protein was used in each reaction. The reaction mixture was incubated for 3 min at 30°C prior to measurement of absorbance at 340 nm in the spectrophotometer. The absorbance drop reflects the breakdown of NADH and is proportional to PEPCK enzyme activity.

6.2.5 Real-time PCR and northern analysis

RNA was extracted from liver and adipose samples by two different methods, appropriate to the properties of the individual tissues. RNA was quantified by GeneQuant prior to Northern analysis and Quant-iT for real-time PCR. Liver and adipose RNA samples were run by real-time PCR using probes for the following mRNA: PEPCK, GR, 11β-HSD1 and lipoprotein lipase (LPL). Gene expression was corrected for RNA loading using a probe for TATA binding protein (TBP) as an internal control.

Probes for PEPCK and 11beta-HSD1 were radiolabelled with ^{32}P and used to quantify the hepatic expression by Northern analysis. Gene expression was corrected for RNA loading using a probe for ribosomal 18S RNA as an internal control. Expression data are presented in arbitrary units relative to control (11β-HSD2+/+ = 1.0).

6.2.6 Glucose assay

Glucose was determined by an enzymatic method using the Infinity Glucose Hexokinase Liquid Stable Reagent.

6.2.7 Statistical analysis

Data are expressed as mean±SEM. Comparisons between genotype were analysed by unpaired t-test, one-way analysis of variance (ANOVA) or two-way repeated measures ANOVA, with Student Newman-Keuls (SNK) *post-hoc* testing, where appropriate.

6.3 Results

6.3.1 Adipose depot weight

The weight of each individual fat depot was compared between 11β-HSD2 genotypes by one-way ANOVA with SNK *post-hoc* testing.

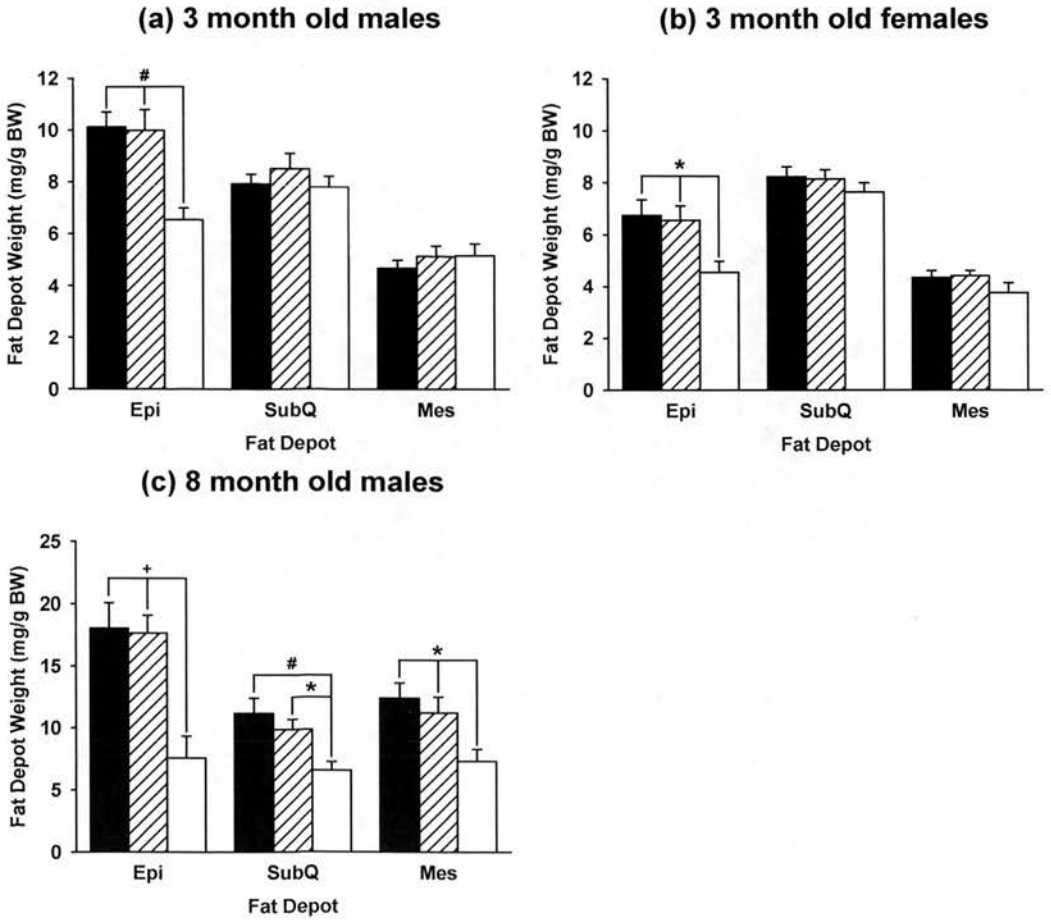


Figure 6.1 Adipose depot weights in adult 11β-HSD2 transgenic mice
Effect of 11β-HSD2 genotype (+/+ ■, +/- ▨, -/- □) on the epididymal (Epi) (or perigonadal in females), subcutaneous (SubQ) and mesenteric (Mes) adipose depot weights in (a) 3 month old males, (b) 3 month old females and (c) 8 month old males. Data are expressed as mean±SEM in mg relative to body weight (g) and analysed by one-way ANOVA with Student Newman-Keuls (SNK) *post-hoc* testing; (a) n=9-12, (b) n=6-11 and (c) n=8-10 per group. $^{+}p<0.001$, $^{\#}p<0.01$, $^{*}p<0.05$

The weight of the epididymal (or perigonadal in females) fat depot was found to be significantly altered by genotype in both male (Figure 6.1a; $F_{2,28}$=8.40, p<0.001) and female (Figure 6.1b; $F_{2,23}$=3.66, p<0.05) three month old mice. 11β-HSD2-/- mice of both genders had a significantly smaller epididymal or perigonadal fat depot weight compared to +/+ and +/- (male: p<0.01; female: p<0.05). However, no difference was observed between genotypes in the weight of the subcutaneous or mesenteric adipose depots at three months in either males or females. In contrast, in male mice at eight months of age, all three depots were found to be different according to genotype (Figure 6.1c; epididymal: $F_{2,24}$=10.24, p<0.001; subcutaneous: $F_{2,24}$=5.61, p<0.01; mesenteric: $F_{2,24}$=4.69, p<0.05). Thus, the fat depot weight, relative to body weight, was reduced in 11β-HSD2-/- mice compared to both +/+ and +/- in the epididymal (p<0.001), subcutaneous (+/+: p<0.01; +/-: p<0.05) and mesenteric depots (p<0.05). 11β-HSD2-/- mice appear to display a 'lean' phenotype, although body weight was not significantly different in adulthood as illustrate by the unaltered pre-fast weight measured (Table 6.1).

11β-HSD2 Genotype	Pre-fast weight (g)	Post-fast weight (g)	Weight loss (g)
+/+	31.6±0.6	27.9±0.7	3.7±0.2
-/-	30.6±0.5	25.8±0.5*	4.7±0.2*

Table 6.1 Body weights of 6 month old male 11β-HSD2 transgenic mice prior to and following a 16-hr fast
Effect of 11β-HSD2 genotype (+/+ and -/-) on the body weight of adult male mice before and after a 16-hr fast. Data are expressed as mean±SEM and analysed by unpaired t-test; n=8 per group. *p<0.05

6.3.2 Wheel running activity

Comparative analysis of wheel running activity between 11β-HSD2-/- and +/+ were made by unpaired t-test. The average daily wheel running activity was significantly reduced in 11β-HSD2-/- compared to +/+ mice (Figure 6.2, p<0.05). Both genotypes were significantly more active in the dark period, but during both the light and dark periods -/- mice travelled significantly less on the wheels compared to +/+ (p<0.05). Therefore, our results indicate 11β-HSD2-/- mice display a hypoactivity in voluntary spontaneous exercise.

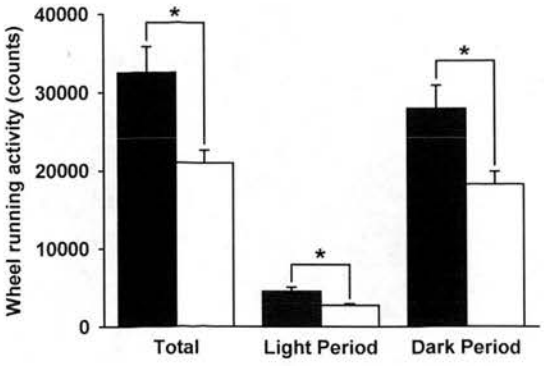


Figure 6.2 Wheel running activity in 4 month old male 11β-HSD2 transgenic mice
Effect of 11β-HSD2 genotype (+/+ ■, -/- □) on wheel running activity during the daily cycle, divided into the light and dark periods. Data are expressed as mean±SEM and analysed by unpaired t-test; n=6 per group. *p<0.05

6.3.3 Adipose gene expression measured by real-time PCR

Relative gene expression of PEPCK in epididymal adipose tissue was altered by genotype (Figure 6.3; $F_{2,17}$=3.94, p<0.05). Both 11β-HSD2+/- and -/- groups had significantly elevated levels of PEPCK mRNA expression compared to +/+ (p<0.05), whilst +/- and -/- were not statistically different (p=0.977). There was no significant difference between genotypes for expression of GR, 11β-HSD1 or LPL.

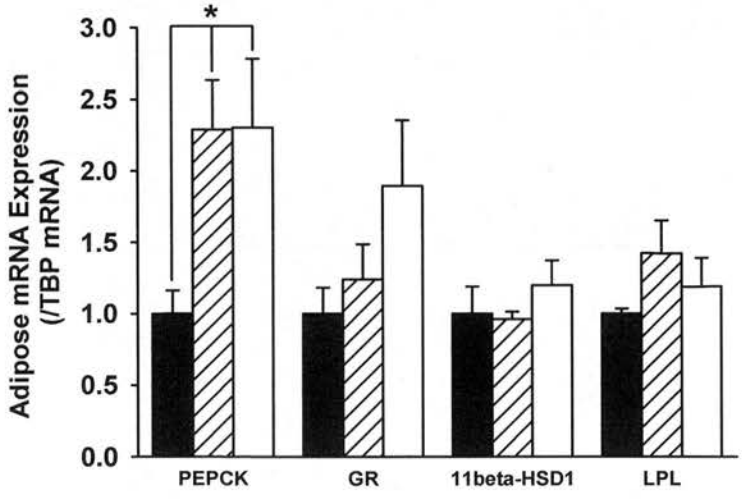
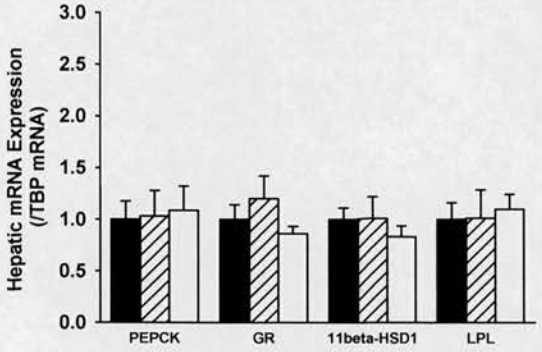


Figure 6.3 Epididymal adipose tissue mRNA expression measured by real-time PCR in 8 month old male 11β-HSD2 transgenic mice
Effect of 11β-HSD2 genotype (+/+ ■, +/- ▨, -/- □) on the epididymal adipose tissue expression of phosphoenolpyruvate carboxykinase (PEPCK), glucocorticoid receptor (GR), 11beta-hydroxysteroid dehydrogenase type 1 (11beta-HSD1) and lipoprotein lipase (LPL) mRNA. Data are expressed as mean±SEM corrected for expression of the housekeeping gene TATA binding protein (TBP) and relative to +/+ = 1.0. Data are analysed by one-way ANOVA with SNK *post-hoc* testing; n=5-8 per group. *p<0.05

6.3.4 Hepatic gene expression and PEPCK enzyme activity

There was no significant difference in the hepatic mRNA expression of PEPCK, GR, 11β-HSD1 and LPL as measured by real-time PCR (Figure 6.4a). In addition, neither PEPCK nor 11β-HSD1 mRNA was found to be altered by genotype when expression was measured by Northern analysis (Figure 6.4b). PEPCK enzyme activity was also not found to be different between genotypes (Table 6.2).

(a) Gene expression measured by real-time PCR



(b) Gene expression measured by Northern Analysis

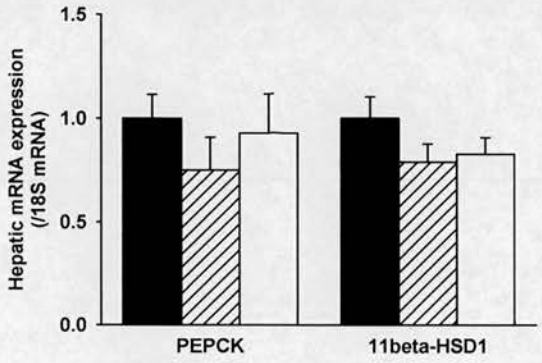


Figure 6.4 Hepatic mRNA expression measured by real-time PCR and Northern analysis in 8 month old male 11β-HSD2 transgenic mice
Effect of 11β-HSD2 genotype (+/+ ■, +/- ▨, -/- □) on the hepatic expression of phosphoenolpyruvate carboxykinase (PEPCK), glucocorticoid receptor (GR), 11beta-hydroxysteroid dehydrogenase type 1 (11beta-HSD1) and lipoprotein lipase (LPL) mRNA as measured by (a) real-time PCR and PEPCK and 11beta-HSD1 as measured by (b) Northern analysis. Data are expressed as mean±SEM corrected for expression of the housekeeping gene (a) TATA binding protein (TBP) or (b) ribosomal 18S (18S) relative to +/+ = 1.0. Data are analysed by one-way ANOVA with SNK post-hoc testing; (a) n=5-9 and (b) n=7-10 per group.

Table 6.2 Hepatic PEPCK enzyme activity in 8 month old male 11β-HSD2 transgenic mice
Effect of 11β-HSD2 genotype (+/+, +/- and -/-) on the activity of the phosphoenolpyruvate carboxykinase (PEPCK) enzyme in hepatic tissue homogenates from adult male mice. Data are expressed as mean±SEM in nmol/min/mg protein and analysed by one-way ANOVA with SNK post-hoc testing; n=6-8 per group.

11β-HSD2 Genotype	PEPCK Enzyme Activity (nmol/min/mg protein)
+/+	2.87±0.19
+/-	2.93±0.31
-/-	3.39±0.14

6.3.5 Dynamic glucose response to intraperitoneal glucose load

When plasma glucose levels were assessed immediately prior to and 5, 15, 30, 60 and 120 mins following an intraperitoneal glucose injection there was no difference between 11β-HSD2+/+ and -/- mice either at any timepoint or over the entire period (Figure 6.5; $F_{1,47}$=1.22, p=0.285) as analysed by two-way repeated measures ANOVA. There was no difference in body weights immediately prior to the fast (p=0.215). However, intriguingly 11β-HSD2-/- mice lost significantly more weight during the 16-hr fast (Table 6.1; p<0.05) such that they were smaller (p<0.05) prior to the glucose injection and tolerance test. 11β-HSD2-/- mice appear to have heightened sensitivity to starvation induced weight loss, albeit with normal glycaemic control.

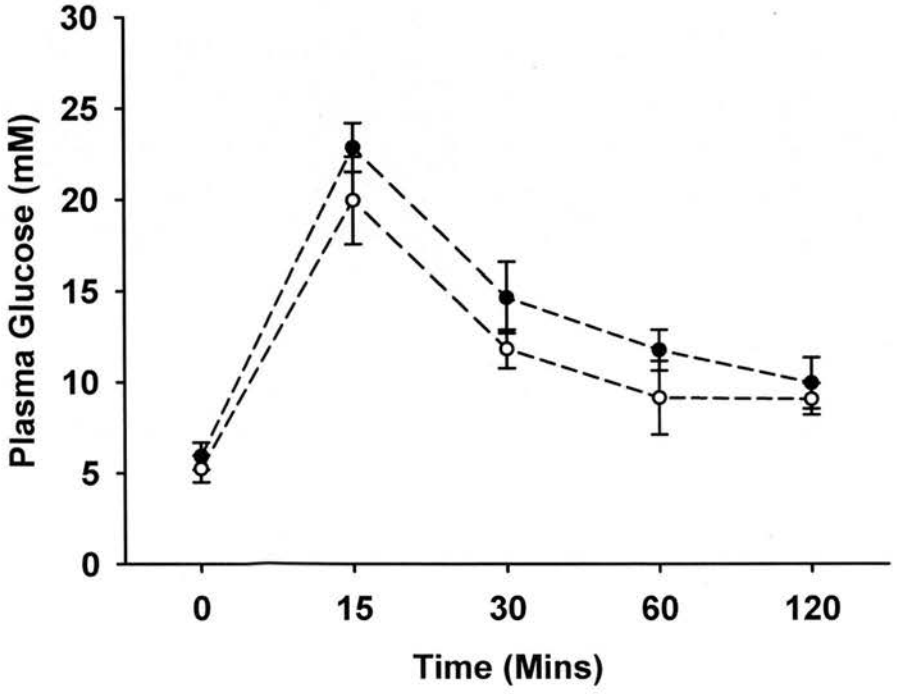


Figure 6.5 Dynamics of glucose disposal in a glucose tolerance test in 6 month old male 11β-HSD2 transgenic mice

Dynamics of glucose disposal upon intraperitoneal glucose injection (2mg/kg body weight) following a 16-hr fast in adult male wild-type (●) and 11β-HSD2 null (○) male mice. Data are expressed as mean±SEM and analysed by two-way repeated measures ANOVA with SNK *post-hoc* testing; n=5-8 per group.

6.4 Discussion

Metabolic dysfunction has previously been observed in rats following prenatal DEX exposure (Nyirenda et al., 1998; 2001; 2006; Cleasby et al., 2003a; O'Regan et al., 2004; Drake et al., 2005) and 11β-HSD1 transgenic mice (Kotelevtsev et al., 1997; Masuzaki et al., 2001; 2003; Morton et al., 2001; 2004a; Paterson et al., 2004). In this Chapter we present findings from initial investigations into the metabolic consequence of 11β-HSD2 null deletion.

We measured the accumulation of body fat in male and female mice at 3 months and from males at 8 months of age. At the younger age 11β-HSD2 knockout mice of both genders had a significantly reduction in epididymal (or perigonadal in females) depot weight, although the subcutaneous and mesenteric pads appeared normal in size. However, by 8 months of age all three measured depots were significantly lighter in 11β-HSD2 null males (females were not assessed). The specific explanation for this reduction in fat depot mass is unclear, although could be due to one or more of the following:

1.) Local alterations in GC action due to the loss of 11β-HSD2
2.) Local alterations in GC action due to loss of the 11-DHC substrate for 11β-HSD1
3.) Prenatal GC programmed effect
4.) Altered appetite and feeding behaviour or energy expenditure

6.4.1 Local alterations in GC action due to the loss of 11β-HSD2

Until recently, the 11β-HSD2 enzyme was not thought to be expressed in adipose tissue (Walker, 2004). However, several groups have detected 11β-HSD2 mRNA within adipose tissue from rats (Milagro et al., 2007), mice (Kershaw et al., 2005) and humans (Engeli et al., 2004) using real-time PCR, albeit at a much lower expression level than 11β-HSD1. Milagro et al. (2007) observed a significant increase in both GR and 11β-HSD2 mRNA expression following a HFD, although only in subcutaneous and not retroperitoneal fat. The investigators postulated that the increased 11β-HSD2 may play a compensatory or

protective role against adiposity. Transgenic overexpression of 11β-HSD2 in adipocytes has been observed to mediate improved glucose tolerance, insulin sensitivity and a resistance to HFD-induced adiposity (Kershaw et al., 2005). The presence of 11β-HSD2 in adipocytes is still controversial and as a result of a low level of expression 11β-HSD2 probably does not play a significant role in intra-adipose CORT metabolism. A direct effect of enzyme loss from these cells is therefore unlikely to explain the reduced adiposity we observed in the 11β-HSD2 null mutant mice.

6.4.2 Local alterations in GC action due to loss of the 11-DHC substrate for 11β-HSD1

In the 11β-HSD2 null mutants the functional loss of the enzyme from the kidney removes a key pathway of CORT clearance, via breakdown of the active steroid to its inert metabolite 11-DHC. This inactive metabolite is thought to be the sole substrate for 11β-HSD1-mediated CORT regeneration and the subsequent intracellular amplification of GC action (Paterson et al., 2005). As evidenced by mutation and tissue-specific overexpression of 11β-HSD1, this amplification of CORT is a key component of GC action and integral to its regulatory role in adipose and liver metabolism. Thus 11β-HSD2 null mutants, that lack the 11-DHC substrate pool, display 11β-HSD1 pseudo-inhibition. The result of this will be a downregulation of local GC signalling despite the 'normal' circulating levels of CORT we observed in 11β-HSD2 null mutants (see Chapter 5). Therefore, the 11β-HSD2 null mutant may be expected to bear similarities to 11β-HSD1 knockout mice and mimic an 11β-HSD double knockout, despite the opposite functional *in vivo* directionality of the enzymes.

11β-HSD1 null mutants were found to be resistant to HFD-induced weight gain despite hyperphagia and displayed a favourable lipoprotein profile, increased hepatic and adipose insulin sensitivity and improved glucose tolerance (Kotelevtsev et al., 1997; Morton et al., 2001; 2004a). These findings were observed in the face of elevated circulating levels of CORT, thought to be caused by impaired negative feedback of the HPA axis in the

hippocampus through lower intraneuronal CORT levels due to lack of 11-DHC reactivation (Yau et al., 2001).

6.4.3 Prenatal GC programmed effect

It is possible that the 'lean' phenotype of the 11β-HSD2 knockout mutants can be attributed to prenatal overexposure to GCs, a hypothesis we wish to investigate further. Final trimester DEX administration to pregnant rats led to offspring which displayed a reduced epididymal fat weight at 7-8 months of age. This was found in combination with elevated retroperitoneal GR and reduced LPL mRNA expression (Cleasby et al., 2003a). Combined with HPA axis hyperactivity that has been reported in prenatally DEX programmed offspring these findings suggest that the elevated GC signalling is central to the 'lean' phenotype observed. However, LPL is known to be positively regulated by GCs (Oliver and Rogers, 1993) suggesting that the reduced LPL mRNA expression may be mediated through the insulin resistance observed (Nyirenda et al., 1998). In contrast to findings in prenatal DEX rats, 11β-HSD2 null mice displayed unaltered circulating CORT levels and no change in GR mRNA expression levels in epididymal adipose tissue. Furthermore, the expression of 11β-HSD1 and LPL mRNA was not different in the 11β-HSD2 null mutants. However, the expression of PEPCK mRNA was significantly elevated which we shall discuss later.

6.4.4 Altered appetite and feeding behaviour or energy expenditure

In order to assess the spontaneous activity of our 11β-HSD2 null male mice we exposed them to wheel running cages. We found the 11β-HSD2 knockout males to be significantly hypoactive. Thus, increased energy expenditure does not appear to underlie the reduced adiposity in our mice. However, the spontaneous hypoactivity could be an outcome of reduced energy availability due to decreased adiposity. Alternatively, the hypoactivity may reflect an, as yet unidentified, dysregulation in energy homeostasis, even though glucose levels appear to be maintained during starvation and is normally tolerated following glucose loading. It is possible that the hypoactivity may be indicative of and mediated

through GC programming in the brain. However, prenatally CORT-treated rats display long-term spontaneous hyperactivity, associated with altered dopamine-mediated motor responses (Diaz et al., 1997). More recently, mice subject to prenatal stress were found to be hyperactive in spontaneous wheel running, an effect associated with alterations in the midbrain dopaminergic system suggestive of a hyperfunctional state (Son et al., 2007). We did not investigate the central dopaminergic system in our studies, although in adults we observed no change in corticosteroid receptors or CRH mRNA expression in limbic regions (see Chapter 5). We did not monitor the feeding behaviour in the 11β-HSD2 null mice. 11β-HSD1 knockout mice were found to resist HFD-induced weight gain despite hyperphagia (Morton et al., 2004a). Alterations in the hypothalamic appetite and energy expenditure pathways in 11β-HSD1 null mutant mice were later found to underlie these observations (Densmore et al., 2006). As described above, due to the 11β-HSD1 pseudo-inhibition it is possible that the 11β-HSD2 null mice may have similar differences in the central regulation of appetite and energy expenditure, which could be responsible, at least partially, for the lean body composition we observed.

As previously mentioned, fat depot weight was reduced in all the measured adipose pads in 11β-HSD2 null males at 8 months, although only the epididymal pad was significantly lighter at 3 months of age. The epididymal (or perigonadal in females) depot-specific decrease in fat accumulation at 3 months of age may reflect a heightened sensitivity of this pad to the consequence of global 11β-HSD2 deletion. In mice, both the mRNA expression and activity of 11β-HSD1 was found to be highest in subcutaneous, followed by epididymal and then lowest in mesenteric adipose tissue (Morton et al., 2004b). In spite of this, intra-adipose CORT levels were not found to vary between depots (Masuzaki et al., 2001), although were significantly decreased in 11β-HSD1 null mice to a similar degree in both the visceral and epididymal depots (Morton et al., 2004a). However, on normal chow 11β-HSD1 null mice had a decreased epididymal, but not mesenteric depot size (Morton et al., 2004a). Thus, it appears possible that the epididymal depot may be more sensitive than the mesenteric and subcutaneous depots to the postulated reduced intra-adipose CORT

action in 11β-HSD2 null mutants. Nevertheless, at 8 months all the depots were significantly lighter in 11β-HSD2 null mutants suggesting that the mesenteric and subcutaneous depots are also sensitive. It should be noted though that the interpretation of these findings is confounded by the fact that the two separate cohorts of mice were bred and studied in different biological research facilities where the normal chow differed in composition (see Chapter 4).

Whilst the relative size of the adipose depots were found to be smaller in the 11β-HSD2 knockout mice these animals were not found to be smaller in adulthood with catch-up growth having been achieved at an early age (see Chapter 4). However, these mice did lose a greater proportion of body weight (15.5% vs 11.8%) during a 16-hr fast. This impaired ability to maintain body weight under fasting conditions is intriguing. As has been mentioned previously in this thesis 11β-HSD2 null mice have hypotonic polyuria, electrolyte imbalance and hypertension, as a consequence of apparent mineralocorticoid excess (AME) through illicit activation of MR within the kidney (Kotelevtsev et al., 1999). It is possible that the hypotonic polyuria removes nutrients from these mice. One could postulate that 11β-HSD2 knockout mice maintain body weight under normal conditions through increased food intake. In such a situation, the removal of food would exacerbate a proportionately greater loss of weight in the 11β-HSD2 null mutants compared to wild-type mice. This is however speculation and food intake in these animals should be monitored and recorded to support or reject this possibility.

6.4.5 Role of PEPCK in tissues

PEPCK is a GC-regulated enzyme that is expressed in several tissues including the liver and fat, although there are distinct tissue-specific differences in function and regulation (Hanson and Reshef, 1997). Within hepatocytes PEPCK is the rate limiting enzyme in gluconeogenesis and catalyses the *de novo* synthesis of glucose from precursors (Barthel and Schmoll, 2003). During periods of starvation this is a central mechanism in maintaining circulating glucose levels. Within adipocytes, which are unable to synthesise

glucose, PEPCK functions as the rate-limiting enzyme in the process of glyceroneogenesis (Hanson and Reshef, 2003). In both tissues, PEPCK is induced by starvation signals and inhibited by insulin, although GCs have tissue-specific regulation of the enzyme in opposite directions. Whilst GCs induce PEPCK expression within the liver (Sasaki et al., 1984), they inhibit it within adipose tissue (Nechushtan et al., 1987). Thus, dysregulated intracellular GC action could have effects upon metabolism through the concert of opposite effects upon PEPCK in the adipose and hepatic tissues.

6.4.5.1 PEPCK mRNA expression in adipose tissue

Despite unaltered expression of GR and 11β-HSD1 mRNA, we observed an upregulation of PEPCK mRNA expression within epididymal fat in the 11β-HSD2 null mice. This elevation in PEPCK mRNA is consistent with a reduction in GC-mediated suppression within adipocytes. Thus, the reduced intra-adipose GC levels through the postulated 11β-HSD1 pseudo-inhibition may underlie this observation. The PEPCK enzyme determines the rate of free fatty acid (FFA) re-esterification in adipose tissue by regulating the availability of the glycerol 3-P substrate (reviewed in Hanson and Reshef, 2003). PEPCK overexpression in adipose tissue in transgenic mice led to the increased FFA esterification, higher adipocyte size, increased fat mass and body weight (Franckhauser et al., 2002). In contrast, the transgenic ablation of PEPCK in adipose tissue resulted in reduced adipose tissue size and fat content in mice (Olswang et al., 2002). Therefore, the induction of PEPCK is not likely to explain the reduced adipose mass in the epididymal depot, but may be involved in a compensatory mechanism due to the reduced adipose depot size. We also detected an increase in PEPCK mRNA expression in 11β-HSD2 heterozygous mice, despite no change in the epididymal fat depot mass in this genotype. This finding is therefore difficult to reconcile and may have represented an anomaly.

6.4.5.2 Glucose homeostasis and hepatic PEPCK mRNA expression and activity

When we investigated the fasting glycaemia and glucose tolerance in our mice, we observed no differences in either parameter. Furthermore, despite postulated lower intracellular CORT levels in 11β-HSD2 knockout mice there were no differences in hepatic PEPCK, GR or 11β-HSD1 mRNA expression. In addition, the enzyme activity of PEPCK was also unchanged. The hepatic PEPCK mRNA expression and activity were enhanced in prenatal DEX-treated offspring (Nyirenda et al., 1998; 2001; O'Regan et al., 2004), an effect which has even displayed intergenerational inheritance (Drake et al., 2005). However, these offspring also displayed elevated basal circulating CORT, opposite to the postulated intracellular CORT hypoactivity in 11β-HSD2 knockout mice. We might have anticipated a down-regulation of PEPCK as the enzyme is known to be positively regulated by GC in liver (Sasaki et al., 1984). However, 11β-HSD1 knockout mice had no differences in basal expression or activity, although in a fasted state they displayed reduced induction of PEPCK , associated with improved glucose tolerance (Kotelevtsev et al., 1997; Morton et al., 2001). Unaltered glucose tolerance in our studies in 11β-HSD2 knockout mice would argue against dysregulation of PEPCK following fasting, although this remains to be confirmed. The importance of gluconeogenesis, and its key rate-limiting enzyme PEPCK, in maintaining normal glycaemic control has been undermined by findings obtained in hepatocyte-specific PEPCK null mutants. These mutants displayed markedly impaired gluconeogenesis, although they were able to maintain near-normal glucose kinetics (She et al., 2003; Burgess et al., 2004). However, when hepatic PEPCK is approximately 2-fold overexpressed, mice display glucose intolerance and hyperinsulinaemia (Sun et al., 2002). These findings suggest that whilst of minimal importance in maintaining basal glucose levels, PEPCK and the gluconeogenic pathway play a more integral role in upregulating, rather than limiting, glucose output. Thus, PEPCK may be more susceptible to induction in situations of GC excess and insulin resistance rather than suppression in opposite circumstances.

6.4.6 Summary

We have observed that 11β-HSD2 knockout mice display a 'lean' phenotype of decreased fat storage in several distinct depots, which was seen in combination with increased fast-induced weight loss and spontaneous hypoactivity. Adipose PEPCK, a key regulator of glyceroneogenesis and fatty acid re-esterification was elevated suggestive of intra-adipose GC hypoactivity, although this probably not a mediator of the reduced fat deposition. The metabolic phenotype of the 11β-HSD2 null mutant is likely to be an interplay between three features, which may be complimentary or contradictory depending on the tissue studied. The first aspect is the prenatal overexposure to GCs through removal of the feto-placental 11β-HSD2 'barrier'. The second factor will be the 11β-HSD1 pseudo-inhibition due to the removal of 11-DHC and subsequent loss of intracellular CORT regeneration. Lastly, the AME adds an additional factor into the 11β-HSD2 mutant mouse equation, in which the contribution of the hypotonic polyuria, electrolyte imbalance and hypertension is difficult to quantify. The 'lean' adipose tissue distribution may thus reflect the complimentary effect of prenatal GC programming and 11β-HSD1 inhibition as both prenatal DEX and 11β-HSD1 null mutation were seen to reduce fat deposition (Cleasby et al., 2003a; Morton et al., 2004a). In hepatic glucose regulation prenatal DEX was observed to yield glucose intolerance, hyperglycaemia and insulin resistance associated with enhanced PEPCK and gluconeogenesis (Nyirenda et al., 1998; 2001), whilst 11β-HSD1 null mutation improved glucose tolerance, insulin sensitivity and reduced stress or HFD induction of PEPCK (Kotelevtsev et al., 1997; Morton et al., 2001). Therefore, we propose that the normal glycaemic control following 11β-HSD2 null mutation may reflect the net result of these two contradictory mechanisms. Generation of tissue-specific enzyme knockouts of 11β-HSD2 within placenta, brain and kidney will be crucial in separating these factors and allow for investigation of GC programmed phenotype independent of other confounding variables.

CHAPTER 7
DISCUSSION

7.1 Thesis summary

The aim of this thesis was to further explore the phenomenon of prenatal glucocorticoid (GC) programming and to study the involvement of the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) feto-placental barrier in regulating fetal exposure to GCs. Chapter 3 described how prenatal dexamethasone (DEX) treatment resulted in exaggerated urinary corticosterone (CORT) excretion, heightened stress-potentiation of anxiety and increased cortical activation following elevated plus maze (EPM) exposure. In chapter 4 we progressed to study prenatal GC programming in a unique model independent of maternal manipulation and potential altered maternal behaviour, the heterozygous-bred 11β-HSD2 null mutant mouse. We identified heightened anxiety-like behaviour and mild cognitive impairment in male 11β-HSD2 knockout mice. Chapter 5 reported findings on the hypothalamic-pituitary adrenal (HPA) axis and central transcript expression of associated genes. Plasma CORT was unaltered in adulthood as was the mRNA expression of the corticosteroid receptors within the hippocampus. Corticotrophin-releasing hormone (CRH) and glucocorticoid receptor (GR) transcript levels within the paraventricular nucleus (PVN) were similarly unchanged, although the left adrenal weight was significantly reduced. However, transient elevations of GR and mineralocorticoid receptor (MR) mRNA were seen in homozygous-bred 11β-HSD2 null mice during the pre-weaning period along with those of the GC-inducible transcripts: serum/glucocorticoid-regulated kinase 1 (Sgk1), FK506 binding protein 51 (Fkbp5) and brain-derived neutrophic factor (BDNF) suggesting a transient enhancement of GC action. In chapter 6 we presented initial studies into the metabolic phenotype of heterozygous-bred 11β-HSD2 null mice. We observed spontaneous hypoactivity and reduced adipose deposition, associated with an elevation in phosphoenolpyruvate carboxykinase (PEPCK) mRNA expression in epididymal fat. However, fasting plasma glucose and tolerance was unaltered despite a greater fast-induced weight loss. The hepatic expression of PEPCK, GR and 11β-HSD1 was also unchanged.

Taken together, our results indicate altered behaviour and a mild dysregulation in HPA axis function along with metabolic abnormalities in 11β-HSD2 null mice. This phenotype is reminiscent of those previously observed following prenatal GC programming, although additional factors resulting from the loss of 11β-HSD2 from postnatal tissues are likely to contribute to our results and shall be discussed in detail.

7.2 Discussion

Prenatal DEX exposure in rats has previously been found to elicit low birth weight, heightened anxiety, HPA axis hyperactivity, altered blood pressure (BP) regulation, impaired glucose homeostasis and a lean body composition in studies conducted in our laboratory (Levitt et al., 1996; Nyirenda et al., 1998; 2001; Welberg et al., 2001; Cleasby et al., 2003a; O'Regan et al., 2004; Drake et al., 2005; Hadoke et al., 2006a) (see Table 7.1). We have extended these findings of HPA axis hyperactivity with the report of enhanced 24-hr urinary CORT excretion in DEX offspring. O'Regan et al. (unpublished observations) recently explored BP regulation in DEX offspring by radiotelemetry and made intriguing observations of hypotension at rest, but hypertension following restraint suggesting an exaggerated BP sensitivity to stress rather than an underlying elevation. Our findings complement these and indicate that the DEX offspring also exhibit an exaggerated anxiety response to stress. Additionally, enhanced c-fos induction akin to cellular activation was observed in the cortex following EPM exposure, which was ameliorated by prior stress exposure, indicative of altered central reactivity to stress.

Our studies in heterozygous-bred 11β-HSD2 recapitulated some of the consequences of prenatal DEX and carbenoxlone (CBX) treatment (see Table 7.1), such as reduced birth weight and anxiety-like behaviour and reduced fat deposition, indicating aspects of prenatal GC programming are observed following deletion of 11β-HSD2. However, several discrepancies exist: firstly, no evidence of HPA axis hyperactivity was obtained in 11β-HSD2 null mice (although adrenal weight was found to be reduced). Secondly, the central transcript expression of GR, MR and CRH was found to be unaltered in 11β-HSD2 null

mice, whereas reductions in hippocampal GR and MR were previously observed in prenatal DEX-treated rats along with increased CRH within the PVN and amygdala (Welberg et al., 2000; Welberg et al., 2001), findings consistent with enhanced positive drive and reduced negative feedback on the HPA axis. Thirdly, no difference in glucose homeostasis was seen in contrast to the fasting hyperglycaemia, impaired glucose tolerance associated with increased PEPCK mRNA and activity reported in prenatal DEX rats (Nyirenda et al., 1998). It is possible that species differences may contribute to the less evident programming in the 11β-HSD2 null mouse. Indeed, the GC milieu during pregnancy has been found to vary markedly between rats and mice. The rat maintains low CORT levels throughout pregnancy and becomes hyporesponsive to stress towards term (Neumann et al., 1998). In contrast the pregnant mouse experiences a surge of CORT towards the end of gestation, becoming resistant to further increase following stress exposure (Douglas et al., 2003; Holmes et al., 2003). Therefore it appears that the fetal mouse has a much elevated requirement for CORT to enable key maturational events and we speculate, therefore that the mouse may be less susceptible to the programming effects of GC overexposure than the rat.

The loss of 11β-HSD2 from adult tissues is likely to have a significant impact on the phenotype with interacting effects mediated through prenatal GC overexposure. Apparent mineralocorticoid excess (AME) was observed in the 11β-HSD2 null mutant characterised by electrolyte imbalance, hypotonic polyuria, polydipsia and hypertension (Kotelevtsev et al., 1999; Bailey et al., unpublished observations). The impact of AME, and its mechanistic involvement in the phenotype we reported is unclear, albeit not unlikely. In addition to the AME, loss of 11β-HSD2 also removed a key clearance pathway of CORT, through inactivation to the inert metabolite 11-dehydrocorticosterone (11-DHC) in the kidney (Figure 7.1).

Outcome	11β-HSD2-/- mice	11β-HSD1-/- mice	DEX rat	CBX rat
Birth weight	↓	↔	↓	↓
Anxiety	↑	↑	↑	↑
Cognition	↓	↑ attenuated age-related cognitive decline	↔	nd
HPA axis	↔ basal CORT ↔ stress CORT ↓ adrenal weight	↔ basal CORT[#] ↑ stress CORT (mild) ↑ adrenal weight	↑ basal CORT ↔ stress CORT	↑ basal CORT ↑ stress CORT
Central HPA transcript expression	↔ hippocampal GR/MR ↔ PVN GR/MR/CRH ↔ amygdala CRH	↑ hippocampal GR/MR[#] ↑ PVN GR/CRH[#]	↓ hippcampal GR/MR[##] ↑ PVN CRH ↑ amygdala CRH	↔ hippcampal GR/MR ↑ PVN CRH / ↓ GR ↑ amygdala MR/GR
Blood pressure	↑	↔	↑ (↓)	↑
Glucose homeostasis	↔ fasting glycaemia ↔ glucose tolerance ↔ PECPK mRNA/activity	↓ fasting glycaemia ↑ glucose tolerance ↑ insulin sensitivity ↓ HFD or stress-induced PEPCK mRNA induction	↑ fasting glycaemia[##] ↓ glucose tolerance[##] reactive hyperinsulinaemia[##] ↑ PEPCK mRNA/activity[##]	↑ fasting glycaemia ↓ glucose tolerance reactive hyperinsulinemia
Body composition	↔ adult body weight* ↓ fat mass ↑ starvation-induced weight loss	↔ adult body weight ↓ fat mass ↓ HFD-induced weight gain	↔ adult body weight**,[##] ↓ fat mass	↔ adult body weight nd

Table 7.1 Comparison table of 11β-HSD2 null mutant mice with 11β-HSD1 null mice, prenatal DEX and CBX-treated rats
CORT = corticosterone; GR = glucocorticoid receptor; MR = mineralocorticoid receptor; CRH = corticotrophin-releasing hormone; PVN = paraventricular nucleus; PEPCK = phosphoenolpyruvate carboxykinase; HFD = high-fat diet; nd = not determined; *homozygous-bred offspring had ↓; [#]found on the C57BL/6J background strain; **some cohorts have displayed ↓; [##]following final trimester DEX treatment (Levitt et al., 1996; Lindsay et al., 1996; Kotelevtsev et al., 1997; 1999; Nyirenda et al., 1998; Welberg et al., 2000; 2001; Morton et al., 2001; 2004a; Yau et al., 2001; Cleasby et al. 2003a; Holmes et al., 2006; unpublished observations; Carter et al., unpublished observations; O'Regan et al., unpublished observations)

As a consequence of 11-DHC loss, lower CORT synthesis and release from the adrenal cortex was required to maintain normal plasma CORT levels. Consistent with this we observed adrenal hypotrophy in the 11β-HSD2 knockout mice. However, enlarged adrenal weights have been observed previously as a result of prenatal DEX treatment, in association with HPA axis hyperactivity. It is possible that the adult loss of 11β-HSD2, and consequent AME, may have masked any programmed HPA axis hyperactivity. The deficiency of 11-DHC will also remove the substrate for 11β-HSD1 and prevent the intracellular regeneration of active CORT. Thus, the 11β-HSD2 null mutant will actually be in a state of 11β-HSD1 pseudo-inhibition and reduced intracellular CORT action in tissues which only express the 11β-HSD1 isozyme (Figure 7.2).

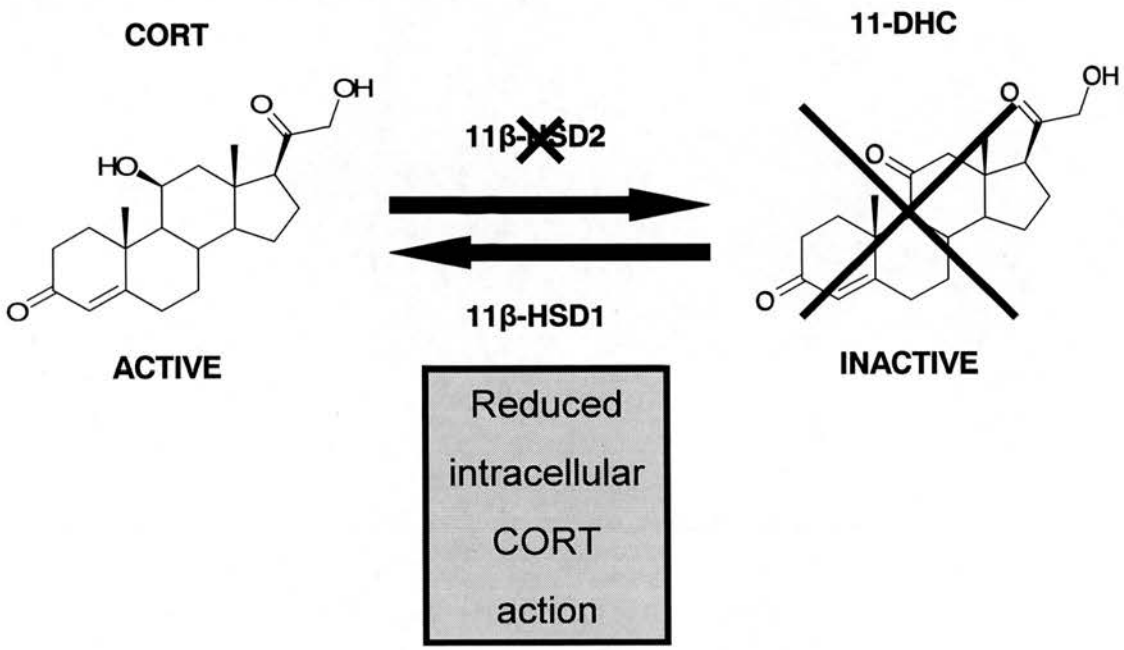


Figure 7.1 11β-HSD1 pseudo-inhibition as a consequence of 11β-HSD2 null mutation
Absence of the renal 11β-HSD2 corticosterone (CORT) clearance pathway results in a loss of circulating 11-dehydrocorticosterone (11-DHC) substrate pool for 11β-HSD1. This prevents the regeneration of CORT and leads to reduction in intracellular CORT action.

11β-HSD1 null mutant mice have been studied in detail (Table 7.1) and were found to exhibit heightened anxiety (Holmes et al., unpublished observations) but were resistant to normal age-related cognitive decline (Yau et al., 2001). On the C57BL/6J

background they were seen to have normal basal circulating CORT, although mild stress-induced CORT elevation was observed (Carter et al., unpublished observations). Additionally, hippocampal GR and MR mRNA was found to be elevated within the hippocampus in addition to GR and CRH within the PVN (Carter et al., unpublished observations). Metabolically, the 11β-HSD1 knockout mice were found to have unaltered adult body weight, despite reduced epipdidymal fat distribution and they displayed a resistance to high-fat diet (HFD)-induced weight gain (Morton et al., 2004a). These mice also displayed reduced fasting glycaemia, improved glucose tolerance and insulin sensitivity (Morton et al., 2001), and reduced PEPCK induction following stress or HFD (Kotelevtsev et al., 1997). It becomes evident that when comparing the phenotype that we and others (Kotelevtsev et al., 1999; Bailey et al., unpublished observations) have observed in 11β-HSD2 null mice, that the pseudo-inhibition of 11β-HSD1 has a significant influence on the phenotype. It appears in certain areas such as the anxiety-like behaviour and body composition that this influence may combine with prenatal GC overexposure to yield an anxious and lean mouse (Table 7.1). However, in other parameters such as central corticosteroid receptor expression and glucose homeostasis, the absence of a difference between 11β-HSD2 null and wild-type mice may reflect contrasting influences, each acting to cancel out the other (Table 7.1).

It therefore appears that the 11β-HSD2 null mouse is subject to combination of three influences which have complimentary or contrasting phenotypic influences, dependent on the specific targets considered (Figure 7.2). We therefore conclude that, despite the obvious existence of prenatal GC overexposure in 11β-HSD2 knockout mice, they represent a rather imperfect model of prenatal GC programming subject to significant limitations.

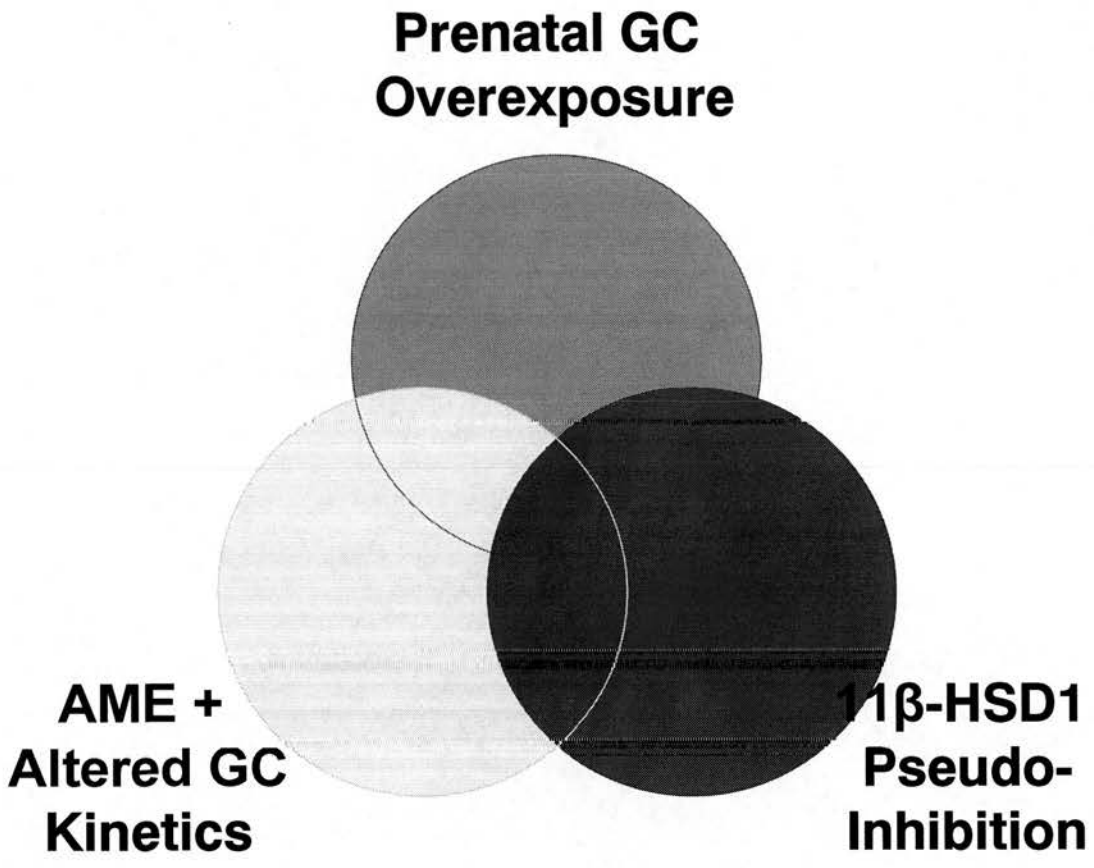


Figure 7.2 Determining influences on the phenotype of the 11β-HSD2 null mutant mouse

7.3 Future perspectives

The global deletion of 11β-HSD2 in mice provides a rather difficult and limiting model with which to study the programming effects of prenatal GC overexposure and importance of the feto-placental barrier due to the confounding influences on adult physiology discussed above. Therefore, our laboratory and other groups within Edinburgh are involved in generating transgenic mice with tissue-specific mutations of 11β-HSD2 within the:

1.) Placenta
2.) Brain
3.) Kidney

Placental-specific 11β-HSD2 null mutation will generate an excellent model of prenatal GC programming which will have the benefits of heterozygous-bred global mutant (including non-manipulation of the dam and unaltered maternal pre- or postnatal nurture), without the confounding influence of AME and 11β-HSD2 inhibition. 11β-HSD2 expression within the brain has a limited distribution in the adult mouse, being only found in the nucleus tractus solitarius (Holmes and Seckl, 2006). However, the enzyme plays a role in restricting CORT exposure in developing brain regions (Brown et al., 1996b). Therefore, brain-specific 11β-HSD2 null mutation will allow us to investigate the importance of this additional barrier and study the effects on the brain, behaviour and HPA axis regulation in the absence of GC overexposure to the periphery. The kidney represents the primary site of 11β-HSD2 in the adult mouse (Condon et al., 1997), where it functions to protect MR from illicit activation by CORT. The kidney-specific 11β-HSD2 knockout will allow investigators to study the role of the resulting AME and 11β-HSD1 pseudo-inhibition, independent of effects mediated through prenatal GC exposure and also identify any role that the AME could play on behavioural alterations noted in the global 11β-HSD2 knockout mouse.

These tissue-specific mutations will be instrumental in developing our understanding of 11β-HSD2 during both fetal and adult life.

It will be important to continue the work which we have performed in the thesis by utilising these tissue-specific mutants. In particular we will want to identify whether the results we have demonstrated are consequences of fetal GC programming. We predict that the placental-specific 11β-HSD2 null mutants will exhibit heightened anxiety, cognitive impairment and HPA axis dysregulation including elevated plasma CORT, impaired feedback and decreased hippocampal corticosteroid receptor expression.

CHAPTER 8
BIBLIOGRAPHY

Abraham IM, Kovacs KJ, 2000. Postnatal handling alters the activation of stress-related neuronal circuitries. Eur J Neurosci 12: 3003-3014.

Agarwal AK, Monder C, Eckstein B, White PC, 1989. Cloning and expression of rat cDNA encoding corticosteroid 11 beta-dehydrogenase. J Biol Chem 264: 18939-18943.

Agarwal AK, Mune T, Monder C, White PC, 1994. NAD(+)-dependent isoform of 11 beta-hydroxysteroid dehydrogenase. Cloning and characterization of cDNA from sheep kidney. J Biol Chem 269: 25959-25962.

Aguilera G, 1994. Regulation of pituitary ACTH secretion during chronic stress. Front Neuroendocrinol 15: 321-350.

Ahima R, Krozowski Z, Harlan R, 1991. Type I corticosteroid receptor-like immunoreactivity in the rat CNS: distribution and regulation by corticosteroids. J Comp Neurol 313: 522-538.

Ahlbom E, Gogvadze V, Chen M, Celsi G, Ceccatelli S, 2000. Prenatal exposure to high levels of glucocorticoids increases the susceptibility of cerebellar granule cells to oxidative stress-induced cell death. Proc Natl Acad Sci USA 97: 14726-14730.

Albiston AL, Obeyesekere VR, Smith RE, Krozowski ZS, 1994. Cloning and tissue distribution of the human 11 beta-hydroxysteroid dehydrogenase type 2 enzyme. Mol. Cell Endocrinol 105: R11-R17.

Aleksandrov AA, Polyakova ON, Batuev AS, 2001. The effects of prenatal stress on learning in rats in a Morris maze. Neurosci Behav Physiol 31: 71-74.

Alonso SJ, Damas C, Navarro E, 2000. Behavioral despair in mice after prenatal stress. J Physiol Biochem 56: 77-82.

Alonso SJ, Navarro E, Rodriguez M, 1994. Permanent dopaminergic alterations in the n. accumbens after prenatal stress. Pharmacol Biochem Behav 49: 353-358.

Alonso SJ, Navarro E, Santana C, Rodriguez M, 1997. Motor lateralization, behavioral despair and dopaminergic brain asymmetry after prenatal stress. Pharmacol Biochem Behav 58: 443-448.

Amelung D, Hubener HJ, Roka L, Meyerheim G, 1953. Conversion of cortisone to compound F. J Clin Endocrinol Metab 13: 1125-1126.

Anisman H, Zaharia MD, Meaney MJ, Merali Z, 1998. Do early-life events permanently alter behavioral and hormonal responses to stressors? Int J Dev Neurosci 16: 149-164.

Armanini D, Strasser T, Weber PC, 1985. Characterization of aldosterone binding sites in circulating human mononuclear leukocytes. Am J Physiol 248: E388-E390.

Avital A, Segal M, Richter-Levin G, 2006. Contrasting roles of corticosteroid receptors in hippocampal plasticity. J Neurosci 26: 9130-9134.

Barbazanges A, Piazza PV, Le MM, Maccari S, 1996. Maternal glucocorticoid secretion mediates long-term effects of prenatal stress. J Neurosci 16: 3943-3949.

Barden N, 2004. Implication of the hypothalamic-pituitary-adrenal axis in the physiopathology of depression. J Psychiatry Neurosci 29: 185-193.

Barden N, Stec IS, Montkowski A, Holsboer F, Reul JM, 1997. Endocrine profile and neuroendocrine challenge tests in transgenic mice expressing antisense RNA against the glucocorticoid receptor. Neuroendocrinology 66: 212-220.

Barker DJ, 2002. Fetal programming of coronary heart disease. Trends Endocrinol Metab 13: 364-368.

Barker DJ, 1991. Deprivation in infancy and risk of ischaemic heart disease. Lancet 337: 981.

Barker DJ, Bull AR, Osmond C, Simmonds SJ, 1990. Fetal and placental size and risk of hypertension in adult life. Br Med J 301: 259-262.

Barker DJ, Hales CN, Fall CH, Osmond C, Phipps K, Clark PM, 1993. Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. Diabetologia 36: 62-67.

Barker DJ Ed., 1992. Fetal and infant origins of adult disease. London: Br Med J books.

Barros VG, Berger MA, Martijena ID, Sarchi MI, Perez AA, Molina VA, Tarazi FI, Antonelli MC, 2004. Early adoption modifies the effects of prenatal stress on dopamine and glutamate receptors in adult rat brain. J Neurosci Res 76: 488-496.

Barros VG, Duhalde-Vega M, Caltana L, Brusco A, Antonelli MC, 2006a. Astrocyte-neuron vulnerability to prenatal stress in the adult rat brain. J Neurosci Res 83: 787-800.

Barros VG, Rodriguez P, Martijena ID, Perez A, Molina VA, Antonelli MC, 2006b. Prenatal stress and early adoption effects on benzodiazepine receptors and anxiogenic behavior in the adult rat brain. Synapse 60: 609-618.

Barthel A, Schmoll D, 2003. Novel concepts in insulin regulation of hepatic gluconeogenesis Am J Physiol Endocrinol. Metab 285: E685-E692.

Beggah AT, Escoubet B, Puttini S, Cailmail S, Delage V, Ouvrard-Pascaud A, Bocchi B, Peuchmaur M, Delcayre C, Farman N, Jaisser F, 2002. Reversible cardiac fibrosis and heart failure induced by conditional expression of an antisense mRNA of the mineralocorticoid receptor in cardiomyocytes. Proc Natl Acad Sci USA 99: 7160-7165.

Benediktsson R, Lindsay RS, Noble J, Seckl JR, Edwards CR, 1993. Glucocorticoid exposure in utero: new model for adult hypertension. Lancet 341: 339-341.

Berger MA, Barros VG, Sarchi MI, Tarazi FI, Antonelli MC, 2002. Long-term effects of prenatal stress on dopamine and glutamate receptors in adult rat brain. Neurochem Res 27: 1525-1533.

Berger S, Bleich M, Schmid W, Cole TJ, Peters J, Watanabe H, Kriz W, Warth R, Greger R, Schutz G, 1998. Mineralocorticoid receptor knockout mice: pathophysiology of Na+ metabolism. Proc Natl Acad Sci USA 95: 9424-9429.

Berger S, Bleich M, Schmid W, Greger R, Schutz G, 2000. Mineralocorticoid receptor knockout mice: lessons on Na+ metabolism. Kidney Int 57: 1295-1298.

Berger S, Wolfer DP, Selbach O, Alter H, Erdmann G, Reichardt HM, Chepkova AN, Welzl H, Haas HL, Lipp HP, Schutz G, 2006. Loss of the limbic mineralocorticoid receptor impairs behavioral plasticity. Proc Natl Acad Sci USA 103: 195-200.

Bertram C, Trowern AR, Copin N, Jackson AA, Whorwood CB, 2001. The Maternal Diet during Pregnancy Programs Altered Expression of the Glucocorticoid Receptor and Type 2 11beta-hydroxysteroid dehydrogenase: potential molecular mechanisms underlying the programming of hypertension in utero. Endocrinology 142: 2841-2853.

Bertram CE, Hanson MA, 2001. Animal models and programming of the metabolic syndrome: Type 2 diabetes. Br Med Bull 60: 103-121.

Bhargava A, Pearce D, 2004. Mechanisms of mineralocorticoid action: determinants of receptor specificity and actions of regulated gene products. Trends Endocrinol Metab 15: 147-153.

Bhatnagar S, Lee TM, Vining C, 2005. Prenatal stress differentially affects habituation of corticosterone responses to repeated stress in adult male and female rats. Horm Behav 47: 430-438.

Binder EB, Salyakina D, Lichtner P, Wochnik GM, Ising M, Putz B, Papiol S, Seaman S, Lucae S, Kohli MA, Nickel T, Kunzel HE, Fuchs B, Majer M, Pfennig A, Kern N, Brunner J, Modell S, Baghai T, Deiml T, Zill P, Bondy B, Rupprecht R, Messer T, Kohnlein O, Dabitz H, Bruckl T, Muller N, Pfister H, Lieb R, Mueller JC, Lohmussaar E, Strom TM, Bettecken T, Meitinger T, Uhr M, Rein T, Holsboer F, Muller-Myhsok B, 2004. Polymorphisms in FKBP5 are associated with increased recurrence of depressive episodes and rapid response to antidepressant treatment. Nat Genet 36: 1319-1325.

Bissiere S, McAllister KH, Olpe HR, Cryan JF, 2006. The rostral anterior cingulate cortex modulates depression but not anxiety-related behaviour in the rat. Behav Brain Res 175: 195-199.

Bleich M, Warth R, Schmidt-Hieber M, Schulz-Baldes A, Hasselblatt P, Fisch D, Berger S, Kunzelmann K, Kriz W, Schutz G, Greger R, 1999. Rescue of the mineralocorticoid receptor knock-out mouse. Pflugers Arch 438: 245-254.

Bloom SL, Sheffield JS, McIntire DD, Leveno KJ, 2001. Antenatal dexamethasone and decreased birth weight. Obstet Gynecol 97: 485-490.

Bourin M, Hascoet M, 2003. The mouse light/dark box test. Eur J Pharmacol 463: 55-65.

Bowman RE, MacLusky NJ, Sarmiento Y, Frankfurt M, Gordon M, Luine VN, 2004. Sexually dimorphic effects of prenatal stress on cognition, hormonal responses, and central neurotransmitters. Endocrinology 145: 3778-3787.

Boyle MP, Brewer JA, Funatsu M, Wozniak DF, Tsien JZ, Izumi Y, Muglia LJ, 2005. Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behavior. Proc Natl Acad Sci USA 102: 473-478.

Boyle MP, Kolber BJ, Vogt SK, Wozniak DF, Muglia LJ, 2006. Forebrain glucocorticoid receptors modulate anxiety-associated locomotor activation and adrenal responsiveness. J Neurosci 26: 1971-1978.

Brabham T, Phelka A, Zimmer C, Nash A, Lopez JF, Vazquez DM, 2000. Effects of prenatal dexamethasone on spatial learning and response to stress is influenced by maternal factors. Am J Physiol Regul Integr Comp Physiol 279: R1899-R1909.

Bray PJ, Cotton RG, 2003. Variations of the human glucocorticoid receptor gene (NR3C1): pathological and in vitro mutations and polymorphisms. Hum Mutat 21: 557-568.

Breuner CW, Orchinik M, 2002. Plasma binding proteins as mediators of corticosteroid action in vertebrates. J Endocrinol 175: 99-112.

Brown RW, Chapman KE, Edwards CR, Seckl JR, 1993. Human placental 11 beta-hydroxysteroid dehydrogenase: evidence for and partial purification of a distinct NAD-dependent isoform. Endocrinology 132: 2614-2621.

Brown RW, Chapman KE, Kotelevtsev Y, Yau JL, Lindsay RS, Brett L, Leckie C, Murad P, Lyons V, Mullins JJ, Edwards CR, Seckl JR, 1996a. Cloning and production of antisera to human placental 11 beta-hydroxysteroid dehydrogenase type 2. J Biochem 313 (Pt 3): 1007-1017.

Brown RW, Diaz R, Robson AC, Kotelevtsev YV, Mullins JJ, Kaufman MH, Seckl JR, 1996b. The ontogeny of 11 beta-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene expression reveal intricate control of glucocorticoid action in development. Endocrinology 137: 794-797.

Buckley AJ, Jaquiery AL, Harding JE, 2005. Nutritional programming of adult disease. Cell Tissue Res 322: 73-79.

Burgess SC, Hausler N, Merritt M, Jeffrey FM, Storey C, Milde A, Koshy S, Lindner J, Magnuson MA, Malloy CR, Sherry AD, 2004. Impaired tricarboxylic acid cycle activity in mouse livers lacking cytosolic phosphoenolpyruvate carboxykinase. J Biol Chem 279: 48941-48949.

Burton PJ, Smith RE, Krozowski ZS, Waddell BJ, 1996. Zonal distribution of 11 beta-hydroxysteroid dehydrogenase types 1 and 2 messenger ribonucleic acid expression in the rat placenta and decidua during late pregnancy. Biol Reprod 55: 1023-1028.

Bush VL, Middlemiss DN, Marsden CA, Fone KCF, 2003. Implantation of a slow release corticosterone pellet induces long-term alterations in serotonergic neurochemistry in the rat brain. J Neuroendocrinol 15: 607-613.

Calvo N, Martijena ID, Molina VA, Volosin M, 1998. Metyrapone pretreatment prevents the behavioral and neurochemical sequelae induced by stress. Brain Res 800: 227-235.

Calvo N, Volosin M, 2001. Glucocorticoid and mineralocorticoid receptors are involved in the facilitation of anxiety-like response induced by restraint. Neuroendocrinology 73: 261-271.

Cameron HA, Gould E, 1994. Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. Neuroscience 61: 203-209.

Carlos RQ, Seidler FJ, Slotkin TA, 1991. Fetal dexamethasone exposure sensitizes neonatal rat brain to hypoxia: effects on protein and DNA synthesis. Brain Res Dev Brain Res 64: 161-166.

Carrasco GA, Van de Kar LD, 2003. Neuroendocrine pharmacology of stress. Eur J Pharmacol 463: 235-272.

Castren E, 2004. Neurotrophins as mediators of drug effects on mood, addiction, and neuroprotection. Mol Neurobiol 29: 289-302.

Celsi G, Kistner A, Aizman R, Eklof AC, Ceccatelli S, de SA, Jacobson SH, 1998. Prenatal dexamethasone causes oligonephronia, sodium retention, and higher blood pressure in the offspring. Pediatr Res 44: 317-322.

Chao HM, McEwen BS, 1994. Glucocorticoids and the expression of mRNAs for neurotrophins, their receptors and GAP-43 in the rat hippocampus. Mol Brain Res 26: 271-276.

Chao HM, Sakai RR, Ma LY, McEwen BS, 1998. Adrenal steroid regulation of neurotrophic factor expression in the rat hippocampus. Endocrinology 139: 3112-3118.

Chaouloff F, 2000. Serotonin, stress and corticoids. J Psychopharmacol 14: 139-151.

Chen M, Bao W, Ceccatelli S, Celsi G, 2004. Prenatal exposure to high level of glucocorticoids increases the susceptibility of renal proximal tubular cells to apoptosis induced by uropathogenic Escherichia coli toxins. Am J Nephrol 24: 497-502.

Chen ZY, Jing D, Bath KG, Ieraci A, Khan T, Siao CJ, Herrera DG, Toth M, Yang C, McEwen BS, Hempstead BL, Lee FS, 2006. Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. Science 314: 140-143.

Cheung J, Smith DF, 2000. Molecular chaperone interactions with steroid receptors: an update. Mol Endocrinol 14: 939-946.

Chomczynski P, Sacchi N, 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-159.

Christensen HD, Gonzalez CL, Stewart JD, Rayburn WF, 2001. Multiple courses of antenatal betamethasone and cognitive development of mice offspring. J Matern Fetal Med 10: 269-276.

Christy C, Hadoke PWF, Paterson JM, Mullins JJ, Seckl JR, Walker BR, 2003. 11beta-hydroxysteroid dehydrogenase type 2 in mouse aorta: localization and influence on response to glucocorticoids. Hypertension 42: 580-587.

Chung S, Son GH, Park SH, Park E, Lee KH, Geum D, Kim K, 2005. Differential adaptive responses to chronic stress of maternally stressed male mice offspring. Endocrinology 146: 3202-3210.

Cintra L, Aguilar A, Granados L, Galvan A, Kemper T, DeBassio W, Galler J, Morgane P, Duran P, az-Cintra S, 1997. Effects of prenatal protein malnutrition on hippocampal CA1 pyramidal cells in rats of four age groups. Hippocampus 7: 192-203.

Cleasby ME, Kelly PA, Walker BR, Seckl JR, 2003a. Programming of rat muscle and fat metabolism by in utero overexposure to glucocorticoids. Endocrinology 144: 999-1007.

Cleasby ME, Livingstone DE, Nyirenda MJ, Seckl JR, Walker BR, 2003b. Is programming of glucocorticoid receptor expression by prenatal dexamethasone in the rat secondary to metabolic derangement in adulthood? Eur J Endocrinol 148: 129-138.

Cole TJ, Blendy JA, Monaghan AP, Krieglstein K, Schmid W, Aguzzi A, Fantuzzi G, Hummler E, Unsicker K, Schutz G, 1995. Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes Dev 9: 1608-1621.

Condon J, Gosden C, Gardener D, Nickson P, Hewison M, Howie AJ, Stewart PM, 1998. Expression of type 2 11beta-hydroxysteroid dehydrogenase and corticosteroid hormone receptors in early human fetal life. J Clin Endocrinol Metab 83: 4490-4497.

Condon J, Ricketts ML, Whorwood CB, Stewart PM, 1997. Ontogeny and sexual dimorphic expression of mouse type 2 11beta-hydroxysteroid dehydrogenase. Mol. Cell Endocrinol 127: 121-128.

Crabbe JC, Wahlsten D, Dudek BC, 1999. Genetics of mouse behavior: interactions with laboratory environment. Science 284: 1670-1672.

Cratty MS, Ward HE, Johnson EA, Azzaro AJ, Birkle DL, 1995. Prenatal stress increases corticotropin-releasing factor (CRF) content and release in rat amygdala minces. Brain Res 675: 297-302.

Crawley J, Goodwin FK, 1980. Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. Pharmacol Biochem Behav 13: 167-170.

Cross JC, 2000. Genetic insights into trophoblast differentiation and placental morphogenesis. Semin Cell Dev Biol. 11: 105-113.

Crump CJ, Chevins PF, 1989. Prenatal stress reduces fertility of male offspring in mice, without affecting their adult testosterone levels. Horm Behav 23: 333-343.

Cryan JF, Valentino RJ, Lucki I, 2005. Assessing substrates underlying the behavioral effects of antidepressants using the modified rat forced swimming test. Neurosci Biobehav Rev 29: 547-569.

Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ, 1995. Pattern and time course of immediate early gene expression in rat brain following acute stress. Neuroscience 64: 477-505.

Daenzer M, Ortmann S, Klaus S, Metges CC, 2002. Prenatal high protein exposure decreases energy expenditure and increases adiposity in young rats. J Nutr 132: 142-144.

Dagan A, Gattineni J, Cook V, Baum M, 2007. Prenatal programming of rat proximal tubule Na+/H+ exchanger by dexamethasone. Am J Physiol Regul Integr Comp Physiol 292: R1230-R1235.

Dallman MF, 2005. Fast glucocorticoid actions on brain: back to the future. Front Neuroendocrinol 26: 103-108.

Davidson RJ, Abercrombie H, Nitschke JB, Putnam K, 1999. Regional brain function, emotion and disorders of emotion. Curr Opin Neurobiol 9: 228-234.

Dawson GR, Tricklebank MD, 1995. Use of the elevated plus maze in the search for novel anxiolytic agents. Trends Pharmacol Sci 16: 33-36.

Day JC, Koehl M, Deroche V, Le MM, Maccari S, 1998. Prenatal stress enhances stress- and corticotropin-releasing factor-induced stimulation of hippocampal acetylcholine release in adult rats. J Neurosci 18: 1886-1892.

de Bosscher K, Vanden BW, 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: 488-522.

de Kloet ER, Joels M, Holsboer F, 2005. Stress and the brain: from adaptation to disease. Nat Rev Neurosci 6: 463-475.

de Kloet ER, Vreugdenhil E, Oitzl MS, Joels M, 1998. Brain corticosteroid receptor balance in health and disease. Endocr Rev 19: 269-301.

de Quervain DJ, Poirier R, Wollmer MA, Grimaldi LM, Tsolaki M, Streffer JR, Hock C, Nitsch RM, Mohajeri MH, Papassotiropoulos A, 2004. Glucocorticoid-related genetic susceptibility for Alzheimer's disease. Hum Mol Genet 13: 47-52.

Densmore VS, Morton NM, Mullins JJ, Seckl JR, 2006. 11 beta-hydroxysteroid dehydrogenase type 1 induction in the arcuate nucleus by high-fat feeding: A novel constraint to hyperphagia? Endocrinology 147: 4486-4495.

DeRijk RH, Schaaf M, de Kloet ER, 2002. Glucocorticoid receptor variants: clinical implications. J Steroid Biochem Mol Biol 81: 103-122.

DeRijk RH, Wust S, Meijer OC, Zennaro MC, Federenko IS, Hellhammer DH, Giacchetti G, Vreugdenhil E, Zitman FG, de Kloet ER, 2006. A common polymorphism in the mineralocorticoid receptor modulates stress responsiveness. J Clin Endocrinol Metab 91: 5083-5089.

Diaz R, Brown RW, Seckl JR, 1998. Distinct ontogeny of glucocorticoid and mineralocorticoid receptor and 11beta-hydroxysteroid dehydrogenase types I and II mRNAs in the fetal rat brain suggest a complex control of glucocorticoid actions. J Neurosci 18: 2570-2580.

Diaz R, Fuxe K, Ogren SO, 1997. Prenatal corticosterone treatment induces long-term changes in spontaneous and apomorphine-mediated motor activity in male and female rats. Neuroscience 81: 129-140.

Diaz R, Ogren SO, Blum M, Fuxe K, 1995. Prenatal corticosterone increases spontaneous and d-amphetamine induced locomotor activity and brain dopamine metabolism in prepubertal male and female rats. Neuroscience 66: 467-473.

Dickerson PA, Lally BE, Gunnel E, Birkle DL, Salm AK, 2005. Early emergence of increased fearful behavior in prenatally stressed rats. Physiol Behav 86: 586-593.

Doremus TL, Varlinskaya EI, Spear LP, 2006. Factor analysis of elevated plus-maze behavior in adolescent and adult rats. Pharmacol Biochem Behav 83: 570-577.

Douglas AJ, Brunton PJ, Bosch OJ, Russell JA, Neumann ID, 2003. Neuroendocrine responses to stress in mice: hyporesponsiveness in pregnancy and parturition. Endocrinology 144: 5268-5276.

Drago F, Di LF, Giardina L, 1999. Prenatal stress induces body weight deficit and behavioural alterations in rats: the effect of diazepam. Eur Neuropsychopharmacol 9: 239-245.

Drake AJ, Walker BR, Seckl JR, 2005. Intergenerational consequences of fetal programming by in utero exposure to glucocorticoids in rats. Am J Physiol Regul Integr Comp Physiol 288: R34-R38.

Draper N, Echwald SM, Lavery GG, Walker EA, Fraser R, Davies E, Sorensen TI, Astrup A, Adamski J, Hewison M, Connell JM, Pedersen O, Stewart PM, 2002. Association studies between microsatellite markers within the gene encoding human 11beta-hydroxysteroid dehydrogenase type 1 and body mass index, waist to hip ratio, and glucocorticoid metabolism. J Clin Endocrinol Metab 87: 4984-4990.

Draper N, Stewart PM, 2005. 11beta-hydroxysteroid dehydrogenase and the pre-receptor regulation of corticosteroid hormone action. J Endocrinol 186: 251-271.

Droste SK, Gesing A, Ulbricht S, Muller MB, Linthorst ACE, Reul JMHM, 2003. Effects of long-term voluntary exercise on the mouse hypothalamic-pituitary-adrenocortical axis. Endocrinology 144: 3012-3023.

Dugovic C, Maccari S, Weibel L, Turek FW, Van RO, 1999. High corticosterone levels in prenatally stressed rats predict persistent paradoxical sleep alterations. J Neurosci 19: 8656-8664.

Duncan GE, Knapp DJ, Breese GR, 1996. Neuroanatomical characterization of Fos induction in rat behavioral models of anxiety. Brain Res 713: 79-91.

Edwards CH, Cole OJ, Oyemade UJ, Knight EM, Johnson AA, Westney OE, Laryea H, West W, Jones S, Westney LS, 1994. Maternal stress and pregnancy outcomes in a prenatal clinic population. J Nutr 124: 1006S-1021S.

Ehman KD, Moser VC, 2006. Evaluation of cognitive function in weanling rats: a review of methods suitable for chemical screening. Neurotoxicol Teratol 28: 144-161.

Emgard M, Paradisi M, Pirondi S, Fernandez M, Giardino L, Calza L, 2007. Prenatal glucocorticoid exposure affects learning and vulnerability of cholinergic neurons. Neurobiol Aging 28: 112-121.

Engeli S, Bohnke J, Feldpausch M, Gorzelniak K, Heintze U, Janke J, Luft FC, Sharma AM, 2004. Regulation of 11beta-HSD genes in human adipose tissue: influence of central obesity and weight loss. Obes Res 12: 9-17.

Engelmann M, Ebner K, Landgraf R, Wotjak CT, 2006. Effects of Morris water maze testing on the neuroendocrine stress response and intrahypothalamic release of vasopressin and oxytocin in the rat. Horm Behav 50: 496-501.

Eriksson E, Royo F, Lyberg K, Carlsson HE, Hau J, 2004. Effect of metabolic cage housing on immunoglobulin A and corticosterone excretion in faeces and urine of young male rats. Exp Physiol 89: 427-433.

Estanislau C, Morato S, 2005. Prenatal stress produces more behavioral alterations than maternal separation in the elevated plus-maze and in the elevated T-maze. Behav Brain Res 163: 70-77.

Estanislau C, Morato S, 2006. Behavior ontogeny in the elevated plus-maze: prenatal stress effects. Int J Dev Neurosci. 24: 255-262.

Farman N, Oblin ME, Lombes M, Delahaye F, Westphal HM, Bonvalet JP, Gasc JM, 1991. Immunolocalization of gluco- and mineralocorticoid receptors in rabbit kidney. Am J Physiol 260: C226-C233.

Ferguson JM, 2001. The effects of antidepressants on sexual functioning in depressed patients: a review. J Clin Psychiatry 62 Suppl 3: 22-34.

Fernandes C, Gonzalez MI, Wilson CA, File SE, 1999. Factor analysis shows that female rat behaviour is characterized primarily by activity, male rats are driven by sex and anxiety. Pharmacol Biochem Behav 64: 731-736.

Fernandez-Teruel A, Escorihuela RM, Castellano B, Gonzalez B, Tobena A, 1997. Neonatal handling and environmental enrichment effects on emotionality, novelty/reward seeking, and age-related cognitive and hippocampal impairments: focus on the Roman rat lines. Behav Genet 27: 513-526.

Finotto S, Krieglstein K, Schober A, Deimling F, Lindner K, Bruhl B, Beier K, Metz J, Garcia-Arraras JE, Roig-Lopez JL, Monaghan P, Schmid W, Cole TJ, Kellendonk C, Tronche F, Schutz G, Unsicker K, 1999. Analysis of mice carrying targeted mutations of

the glucocorticoid receptor gene argues against an essential role of glucocorticoid signalling for generating adrenal chromaffin cells. Development 126: 2935-2944.

Fischer AK, von Rosenstiel P, Fuchs E, Goula D, Almeida OFX, Czeh B, 2002. The prototypic mineralocorticoid receptor agonist aldosterone influences neurogenesis in the dentate gyrus of the adrenalectomized rat. Brain Res 947: 290-293.

Francis D, Diorio J, Liu D, Meaney MJ, 1999. Nongenomic transmission across generations of maternal behavior and stress responses in the rat. Science 286: 1155-1158.

Franckhauser S, Munoz S, Pujol A, Casellas A, Riu E, Otaegui P, Su B, Bosch F, 2002. Increased fatty acid re-esterification by PEPCK overexpression in adipose tissue leads to obesity without insulin resistance. Diabetes 51: 624-630.

Freedman R, 2003. Schizophrenia. N Engl J Med 349: 1738-1749.

French NP, Hagan R, Evans SF, Godfrey M, Newnham JP, 1999. Repeated antenatal corticosteroids: size at birth and subsequent development. Am J Obstet Gynecol. 180: 114-121.

Fride E, Dan Y, Gavish M, Weinstock M, 1985. Prenatal stress impairs maternal behavior in a conflict situation and reduces hippocampal benzodiazepine receptors. Life Sci 36: 2103-2109.

Fride E, Weinstock M, 1988. Prenatal stress increases anxiety related behavior and alters cerebral lateralization of dopamine activity. Life Sci 42: 1059-1065.

Fried SK, Russell CD, Grauso NL, Brolin RE, 1993. Lipoprotein lipase regulation by insulin and glucocorticoid in subcutaneous and omental adipose tissues of obese women and men. J Clin Invest 92: 2191-2198.

Friedman JI, Temporini H, Davis KL, 1999. Pharmacologic strategies for augmenting cognitive performance in schizophrenia. Biol Psychiatry 45: 1-16.

Frye CA, Orecki ZA, 2002a. Prenatal stress alters reproductive responses of rats in behavioral estrus and paced mating of hormone-primed rats. Horm Behav 42: 472-483.

Frye CA, Orecki ZA, 2002b. Prenatal stress produces deficits in socio-sexual behavior of cycling, but not hormone-primed, Long-Evans rats. Pharmacol Biochem Behav 73: 53-60.

Frye CA, Wawrzycki J, 2003. Effect of prenatal stress and gonadal hormone condition on depressive behaviors of female and male rats. Horm Behav 44: 319-326.

Frye CA, Petralia SM, Rhodes ME, 2000. Estrous cycle and sex differences in performance on anxiety tasks coincide with increases in hippocampal progesterone and 3alpha,5alpha-THP. Pharmacol Biochem Behav 67: 587-596.

Fujimoto ST, Longhi L, Saatman KE, McIntosh TK, 2004. Motor and cognitive function evaluation following experimental traumatic brain injury. Neurosci Biobehav Rev 28: 365-378.

Fujioka A, Fujioka T, Ishida Y, Maekawa T, Nakamura S, 2006. Differential effects of prenatal stress on the morphological maturation of hippocampal neurons. Neuroscience 141: 907-915.

Fujioka T, Fujioka A, Tan N, Chowdhury GM, Mouri H, Sakata Y, Nakamura S, 2001. Mild prenatal stress enhances learning performance in the non-adopted rat offspring. Neuroscience 103: 301-307.

Fumagalli F, Bedogni F, Perez J, Racagni G, Riva MA, 2004. Corticostriatal brain-derived neurotrophic factor dysregulation in adult rats following prenatal stress. Eur J Neurosci 20: 1348-1354.

Funder JW, 2005. Mineralocorticoid receptors: distribution and activation. Heart Fail Rev 10: 15-22.

Funder JW, Feldman D, Edelman IS, 1972. Specific aldosterone binding in rat kidney and parotid. J Steroid Biochem 3: 209-218.

Galeeva A, Ordyan N, Pivina S, Pelto-Huikko M, 2006. Expression of glucocorticoid receptors in the hippocampal region of the rat brain during postnatal development. J Chem Neuroanat 31: 216-225.

Gambineri A, Vicennati V, Genghini S, Tomassoni F, Pagotto U, Pasquali R, Walker BR, 2006. Genetic variation in 11beta-hydroxysteroid dehydrogenase type 1 predicts adrenal hyperandrogenism among lean women with polycystic ovary syndrome. J Clin Endocrinol Metab 91: 2295-2302.

Garcia AMB, Cardenas FP, Morato S, 2005. Effect of different illumination levels on rat behavior in the elevated plus-maze. Physiol Behav 85: 265-270.

Gass P, Reichardt HM, Strekalova T, Henn F, Tronche F, 2001. Mice with targeted mutations of glucocorticoid and mineralocorticoid receptors: models for depression and anxiety? Physiol Behav 73: 811-825.

Gerardin DC, Pereira OC, Kempinas WG, Florio JC, Moreira EG, Bernardi MM, 2005. Sexual behavior, neuroendocrine, and neurochemical aspects in male rats exposed prenatally to stress. Physiol Behav 84: 97-104.

Gomez-Sanchez EP, Gomez-Sanchez CE, 2003. Is aldosterone synthesized in the CNS regulated and functional? Trends Endocrinol Metab 14: 444-446.

Gonzalez-Pardo H, Conejo NM, Arias JL, 2006. Oxidative metabolism of limbic structures after acute administration of diazepam, alprazolam and zolpidem. Prog. Neuropsychopharmacol Biol Psychiatry 30: 1020-1026.

Gould E, Woolley CS, Cameron HA, Daniels DC, McEwen BS, 1991. Adrenal steroids regulate postnatal development of the rat dentate gyrus: II. Effects of glucocorticoids and mineralocorticoids on cell birth. J Comp Neurol 313: 486-493.

Grad B, Khalid R, 1968. Circulating corticosterone levels of young and old, male and female C57B1/6J mice. J Gerontol 23: 522-528.

Granados-Rojas L, Larriva-Sahd J, Cintra L, Gutierrez-Ospina G, Rondan A, Cintra S, 2002. Prenatal protein malnutrition decreases mossy fibers-CA3 thorny excrescences asymmetrical synapses in adult rats. Brain Res 933: 164-171.

Griebel G, Moreau JL, Jenck F, Martin JR, Misslin R, 1993. Some critical determinants of the behaviour of rats in the elevated plus-maze. Behav Processes 29: 37-47.

Griffin WC3, Skinner HD, Salm AK, Birkle DL, 2003. Mild prenatal stress in rats is associated with enhanced conditioned fear. Physiol Behav 79: 209-215.

Gue M, Bravard A, Meunier J, Veyrier R, Gaillet S, Recasens M, Maurice T, 2004. Sex differences in learning deficits induced by prenatal stress in juvenile rats. Behav Brain Res 150: 149-157.

Guimaraes FS, Del Bel EA, Padovan CM, Netto SM, de Almeida RT, 1993. Hippocampal 5-HT receptors and consolidation of stressful memories. Behav Brain Res 58: 133-139.

Hadoke PW, Lindsay RS, Seckl JR, Walker BR, Kenyon CJ, 2006a. Altered vascular contractility in adult female rats with hypertension programmed by prenatal glucocorticoid exposure. J Endocrinol 188: 435-442.

Hadoke PW, Macdonald L, Logie JJ, Small GR, Dover AR, Walker BR, 2006b. Intravascular glucocorticoid metabolism as a modulator of vascular structure and function. Cell Mol Life Sci 63: 565-578.

Hadoke PWF, Christy C, Kotelevtsev YV, Williams BC, Kenyon CJ, Seckl JR, Mullins JJ, Walker BR, 2001. Endothelial cell dysfunction in mice after transgenic knockout of type 2, but not type 1, 11beta-hydroxysteroid dehydrogenase. Circulation 104: 2832-2837.

Hales CN, Barker DJ, Clark PM, Cox LJ, Fall C, Osmond C, Winter PD, 1991. Fetal and infant growth and impaired glucose tolerance at age 64. Br Med J 303: 1019-1022.

Hall CS, 1934. Emotional behavior in the rat. I. Defecation and urination as measures of individual differences in emotionality. J Comp Psychol 18: 385-403.

Hanson RW, Reshef L, 2003. Glyceroneogenesis revisited. Biochimie 85: 1199-1205.

Hanson RW, Reshef L, 1997. Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. Annu Rev Biochem 66: 581-611.

Harris HJ, Kotelevtsev Y, Mullins JJ, Seckl JR, Holmes MC, 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-IISD-1-deficient mice. Endocrinology 142: 114-120.

Harvey PW, Chevins PF, 1984. Crowding or ACTH treatment of pregnant mice affects adult copulatory behavior of male offspring. Horm Behav 18: 101-110.

Harvey PW, Chevins PF, 1987. Crowding during pregnancy delays puberty and alters estrous cycles of female offspring in mice. Experientia 43: 306-308.

Hauser J, Feldon J, Pryce CR, 2006. Prenatal dexamethasone exposure, postnatal development, and adulthood prepulse inhibition and latent inhibition in Wistar rats. Behav Brain Res 175: 51-61.

Hayashi A, Nagaoka M, Yamada K, Ichitani Y, Miake Y, Okado N, 1998. Maternal stress induces synaptic loss and developmental disabilities of offspring. Int J Dev Neurosci 16: 209-216.

Heidbreder CA, Weiss IC, Domeney AM, Pryce C, Homberg J, Hedou G, Feldon J, Moran MC, Nelson P, 2000. Behavioral, neurochemical and endocrinological characterization of the early social isolation syndrome. Neuroscience 100: 749-768.

Henning SJ, 1978. Plasma concentrations of total and free corticosterone during development in the rat. Am J Physiol 235: E451-E456.

Henry C, Kabbaj M, Simon H, Le MM, Maccari S, 1994. Prenatal stress increases the hypothalamo-pituitary-adrenal axis response in young and adult rats. J Neuroendocrinol 6: 341-345.

Herman JP, 1993. Regulation of adrenocorticosteroid receptor mRNA expression in the central nervous system. Cell Mol Neurobiol 13: 349-372.

Herman JP, Patel PD, Akil H, Watson SJ, 1989. Localization and regulation of glucocorticoid and mineralocorticoid receptor messenger RNAs in the hippocampal formation of the rat. Mol Endocrinol 3: 1886-1894.

Hewitt DP, Mark PJ, Waddell BJ, 2006. Glucocorticoids prevent the normal increase in placental vascular endothelial growth factor expression and placental vascularity during late pregnancy in the rat. Endocrinology 147: 5568-5574.

Hinks GL, Brown P, Field M, Poat JA, Hughes J, 1996. The anxiolytics CI-988 and chlordiazepoxide fail to reduce immediate early gene mRNA stimulation following exposure to the rat elevated X-maze. Eur J Pharmacol 312: 153-161.

Hirasawa G, Sasano H, Takahashi K, Fukushima K, Suzuki T, Hiwatashi N, Toyota T, Krozowski ZS, Nagura H, 1997. Colocalization of 11 beta-hydroxysteroid dehydrogenase type II and mineralocorticoid receptor in human epithelia. J Clin. Endocrinol Metab 82: 3859-3863.

Hofer M, Pagliusi SR, Hohn A, Leibrock J, Barde YA, 1990. Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. EMBO J 9: 2459-2464.

Holmes MC, Abrahamsen CT, French KL, Paterson JM, Mullins JJ, Seckl JR, 2006a. 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: 3840-3844.

Holmes MC, Sangra M, French KL, Whittle IR, Paterson J, Mullins JJ, Seckl JR, 2006b. 11beta-Hydroxysteroid dehydrogenase type 2 protects the neonatal cerebellum from deleterious effects of glucocorticoids. Neuroscience 137: 865-873.

Holmes MC, Seckl JR, 2006. The role of 11beta-hydroxysteroid dehydrogenases in the brain. Mol Cell Endocrinol 248: 9-14.

Holmes MC, Yau JL, Kotelevtsev Y, Mullins JJ, Seckl JR, 2003. 11 Beta-hydroxysteroid dehydrogenases in the brain: two enzymes two roles. Ann N Y Acad Sci 1007: 357-366.

Holson RR, Gough B, Sullivan P, Badger T, Sheehan DM, 1995. Prenatal dexamethasone or stress but not ACTH or corticosterone alter sexual behavior in male rats. Neurotoxicol Teratol 17: 393-401.

Hsu DT, Chen FL, Takahashi LK, Kalin NH, 1998. Rapid stress-induced elevations in corticotropin-releasing hormone mRNA in rat central amygdala nucleus and hypothalamic paraventricular nucleus: an in situ hybridization analysis. Brain Res 788: 305-310.

Hundertmark S, Dill A, Ebert A, Zimmermann B, Kotelevtsev YV, Mullins JJ, Seckl JR, 2002. Foetal lung maturation in 11beta-hydroxysteroid dehydrogenase type 1 knockout mice. Horm Metab Res 34: 545-549.

Igosheva N, Klimova O, Anishchenko T, Glover V, 2004. Prenatal stress alters cardiovascular responses in adult rats. J Physiol 557: 273-285.

Ishiwata H, Shiga T, Okado N, 2005. Selective serotonin reuptake inhibitor treatment of early postnatal mice reverses their prenatal stress-induced brain dysfunction. Neuroscience 133: 893-901.

Ivanova T, Beyer C, 2001. Pre- and postnatal expression of brain-derived neurotrophic factor mRNA/protein and tyrosine protein kinase receptor B mRNA in the mouse hippocampus. Neurosci Lett 307: 21-24.

Jamieson PM, Chapman KE, Edwards CR, Seckl JR, 1995. 11 beta-hydroxysteroid dehydrogenase is an exclusive 11 beta- reductase in primary cultures of rat hepatocytes: effect of physicochemical and hormonal manipulations. Endocrinology 136: 4754-4761.

Jamieson PM, Walker BR, Chapman KE, Andrew R, Rossiter S, Seckl JR, 2000. 11 beta-hydroxysteroid dehydrogenase type 1 is a predominant 11 beta-reductase in the intact perfused rat liver. J Endocrinol 165: 685-692.

Jellinck PH, Monder C, McEwen BS, Sakai RR, 1993. Differential inhibition of 11 beta-hydroxysteroid dehydrogenase by carbenoxolone in rat brain regions and peripheral tissues. J Steroid Biochem. Mol Biol 46: 209-213.

Jia D, O'Brien CA, Stewart SA, Manolagas SC, Weinstein RS, 2006. Glucocorticoids act directly on osteoclasts to increase their life span and reduce bone density. Endocrinology 147: 5592-5599.

Johnson EO, Brady L, Gold PW, Chrousos GP, 1996. Distribution of hippocampal mineralocorticoid and glucocorticoid receptor mRNA in a glucocorticoid resistant nonhuman primate. Steroids 61: 69-73.

Johnstone HA, Wigger A, Douglas AJ, Neumann ID, Landgraf R, Seckl JR, Russell JA, 2000. Attenuation of hypothalamic-pituitary-adrenal axis stress responses in late pregnancy: changes in feedforward and feedback mechanisms. J Neuroendocrinol 12: 811-822.

Jones MT, Hillhouse EW, Burden JL, 1977. Dynamics and mechanics of corticosteroid feedback at the hypothalamus and anterior pituitary gland. J Endocrinol 73: 405-417.

Justesen J, Mosekilde L, Holmes M, Stenderup K, Gasser J, Mullins JJ, Seckl JR, Kassem M, 2004. Mice deficient in 11beta-hydroxysteroid dehydrogenase type 1 lack

bone marrow adipocytes, but maintain normal bone formation. Endocrinology 145: 1916-1925.

Kabbaj M, Akil H, 2001. Individual differences in novelty-seeking behavior in rats: a c-fos study. Neuroscience 106: 535-545.

Kapoor A, Matthews SG, 2005. Short periods of prenatal stress affect growth, behaviour and hypothalamo-pituitary-adrenal axis activity in male guinea pig offspring. J Physiol 566: 967-977.

Kapoor A, Dunn E, Kostaki A, Andrews MH, Matthews SG, 2006. Fetal programming of hypothalamo-pituitary-adrenal function: prenatal stress and glucocorticoids. J Physiol 572: 31-44.

Karanth S, Linthorst AC, Stalla GK, Barden N, Holsboer F, Reul JM, 1997. Hypothalamic-pituitary-adrenocortical axis changes in a transgenic mouse with impaired glucocorticoid receptor function. Endocrinology 138: 3476-3485.

Kato H, Oikawa T, Otsuka K, Takahashi A, Itoyama Y, 2000. Postischemic changes in the immunophilin FKBP12 in the rat brain. Mol Brain Res 84: 58-66.

Kauffman KS, Seidler FJ, Slotkin TA, 1994. Prenatal dexamethasone exposure causes loss of neonatal hypoxia tolerance: cellular mechanisms. Pediatr Res. 35: 515-522.

Kennedy SH, Rafi-Tari S, Meyer JH, Mayberg HS, Evans K, Houle S, Vaccarino FJ, 2003. Regional cerebral metabolism is influenced by age, anxiety, and melancholia in major depressive disorder. Brain Cogn 51: 211-213.

Kenouch S, Lombes M, Delahaye F, Eugene E, Bonvalet JP, Farman N, 1994. Human skin as target for aldosterone: coexpression of mineralocorticoid receptors and 11 beta-hydroxysteroid dehydrogenase. J Clin Endocrinol Metab 79: 1334-1341.

Kershaw EE, Morton NM, Dhillon H, Ramage L, Seckl JR, Flier JS, 2005. Adipocyte-Specific Glucocorticoid Inactivation Protects Against Diet-Induced Obesity. Diabetes 54: 1023-1031.

Kinnunen AK, Koenig JI, Bilbe G, 2003. Repeated variable prenatal stress alters pre- and postsynaptic gene expression in the rat frontal pole. J Neurochem 86: 736-748.

Kinsley C, Svare B, 1988. Prenatal stress alters maternal aggression in mice. Physiol Behav 42: 7-13.

Kinsley C, Svare B, 1986. Prenatal stress effects: are they mediated by reductions in maternal food and water intake and body weight gain? Physiol Behav 37: 191-193.

Koehl M, Barbazanges A, Le MM, Maccari S, 1997. Prenatal stress induces a phase advance of circadian corticosterone rhythm in adult rats which is prevented by postnatal stress. Brain Res 759: 317-320.

Koehl M, Darnaudery M, Dulluc J, Van RO, Le MM, Maccari S, 1999. Prenatal stress alters circadian activity of hypothalamo-pituitary-adrenal axis and hippocampal corticosteroid receptors in adult rats of both gender. J Neurobiol 40: 302-315.

Koenig JI, 2006. Schizophrenia: a unique translational opportunity in behavioral neuroendocrinology. Horm Behav 50: 602-611.

Koenig JI, Elmer GI, Shepard PD, Lee PR, Mayo C, Joy B, Hercher E, Brady DL, 2005. Prenatal exposure to a repeated variable stress paradigm elicits behavioral and neuroendocrinological changes in the adult offspring: potential relevance to schizophrenia. Behav Brain Res 156: 251-261.

Koo JW, Park CH, Choi SH, Kim NJ, Kim HS, Choe JC, Suh YH, 2003. The postnatal environment can counteract prenatal effects on cognitive ability, cell proliferation, and synaptic protein expression. FASEB J 17: 1556-1558.

Korte SM, 2001. Corticosteroids in relation to fear, anxiety and psychopathology. Neurosci Biobehav Rev 25: 117-142.

Korte SM, De Boer SF, 2003. A robust animal model of state anxiety: fear-potentiated behaviour in the elevated plus-maze. Eur J Pharmacol 463: 163-175.

Kotelevtsev Y, Brown RW, Fleming S, Kenyon C, Edwards CR, Seckl JR, Mullins JJ, 1999. Hypertension in mice lacking 11beta-hydroxysteroid dehydrogenase type 2. J Clin Invest 103: 683-689.

Kotelevtsev Y, Holmes MC, Burchell A, Houston PM, Schmoll D, Jamieson P, Best R, Brown R, Edwards CR, Seckl JR, Mullins JJ, 1997. 11beta-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. Proc Natl Acad Sci USA 94: 14924-14929.

Kovacs KJ, 1998. c-Fos as a transcription factor: a stressful (re)view from a functional map. Neurochem Int 33: 287-297.

Koya E, Spijker S, Homberg JR, Voorn P, Schoffelmeer AN, De Vries TJ, Smit AB, 2005. Molecular reactivity of mesocorticolimbic brain areas of high and low grooming rats after elevated plus maze exposure. Brain Res Mol Brain Res 137: 184-192.

Kreider ML, Levin ED, Seidler FJ, Slotkin TA, 2005. Gestational dexamethasone treatment elicits sex-dependent alterations in locomotor activity, reward-based memory

and hippocampal cholinergic function in adolescent and adult rats. Neuropsychopharmacology 30: 1617-1623.

Kretz O, Schmid W, Berger S, Gass P, 2001. The mineralocorticoid receptor expression in the mouse CNS is conserved during development. Neuroreport 12: 1133-1137.

Krozowski Z, MaGuire JA, Stein-Oakley AN, Dowling J, Smith RE, Andrews RK, 1995. Immunohistochemical localization of the 11 beta-hydroxysteroid dehydrogenase type II enzyme in human kidney and placenta. J Clin. Endocrinol Metab 80: 2203-2209.

Kusserow H, Davies B, Hortnagl H, Voigt I, Stroh T, Bert B, Deng DR, Fink H, Veh RW, Theuring F, 2004. Reduced anxiety-related behaviour in transgenic mice overexpressing serotonin1A receptors. Mol Brain Res 129: 104-116.

Kutzler MA, Molnar J, Schlafer DH, Kuc RE, Davenport AP, Nathanielsz PW, 2003. Maternal dexamethasone increases endothelin-1 sensitivity and endothelin a receptor expression in ovine foetal placental arteries. Placenta 24: 392-402.

Lakshmi V, Monder C, 1985. Extraction of 11 beta-hydroxysteroid dehydrogenase from rat liver microsomes by detergents. J Steroid Biochem 22: 331-340.

Lakshmi V, Monder C, 1988. Purification and characterization of the corticosteroid 11 beta-dehydrogenase component of the rat liver 11 beta-hydroxysteroid dehydrogenase complex. Endocrinology 123: 2390-2398.

Lakshmi V, Sakai RR, McEwen BS, Monder C, 1991. Regional distribution of 11 beta-hydroxysteroid dehydrogenase in rat brain. Endocrinology 128: 1741-1748.

Lang F, Henke G, Embark HM, Waldegger S, Palmada M, Bohmer C, Vallon V, 2003. Regulation of channels by the serum and glucocorticoid-inducible kinase - implications for transport, excitability and cell proliferation. Cell Physiol Biochem 13: 41-50.

Langdown ML, Holness MJ, Sugden MC, 2003. Effects of prenatal glucocorticoid exposure on cardiac calreticulin and calsequestrin protein expression during early development and in adulthood. Biochem J 371: 61-69.

Langdown ML, Smith ND, Sugden MC, Holness MJ, 2001. Excessive glucocorticoid exposure during late intrauterine development modulates the expression of cardiac uncoupling proteins in adult hypertensive male offspring. Pflugers Arch 442: 248-255.

Langley-Evans SC, Phillips GJ, Benediktsson R, Gardner DS, Edwards CR, Jackson AA, Seckl JR, 1996. Protein intake in pregnancy, placental glucocorticoid metabolism and the programming of hypertension in the rat. Placenta 17: 169-172.

Langley-Evans SC, Bellinger L, McMullen S, 2005. Animal models of programming: early life influences on appetite and feeding behaviour. Matern Child Nutr 1: 142-148.

Laviola G, Rea M, Morley-Fletcher S, Di CS, Bacosi A, De SR, Bertini M, Pacifici R, 2004. Beneficial effects of enriched environment on adolescent rats from stressed pregnancies. Eur J Neurosci 20: 1655-1664.

Le Menuet D, Isnard R, Bichara M, Viengchareun S, Muffat-Joly M, Walker F, Zennaro MC, Lombes M, 2001. Alteration of cardiac and renal functions in transgenic mice overexpressing human mineralocorticoid receptor. J Biol Chem 276: 38911-38920.

Le Menuet D, Viengchareun S, Muffat-Joly M, Zennaro MC, Lombes M, 2004. Expression and function of the human mineralocorticoid receptor: lessons from transgenic mouse models. Mol Cell Endocrinol 217: 127-136.

Lee E, Lein ES, Firestone GL, 2001. Tissue-specific expression of the transcriptionally regulated serum and glucocorticoid-inducible protein kinase (Sgk) during mouse embryogenesis. Mech Dev 103: 177-181.

Lehmann J, Stohr T, Feldon J, 2000. Long-term effects of prenatal stress experiences and postnatal maternal separation on emotionality and attentional processes. Behav Brain Res 107: 133-144.

Leinekugel X, 2003. Developmental patterns and plasticities: the hippocampal model. J Physiol 97: 27-37.

Lemaire V, Koehl M, Le MM, Abrous DN, 2000. Prenatal stress produces learning deficits associated with an inhibition of neurogenesis in the hippocampus. Proc Natl Acad Sci USA 97: 11032-11037.

Lemaire V, Lamarque S, Le MM, Piazza PV, Abrous DN, 2006. Postnatal stimulation of the pups counteracts prenatal stress-induced deficits in hippocampal neurogenesis. Biol Psychiatry 59: 786-792.

Leong ML, Maiyar AC, Kim B, O'Keeffe BA, Firestone GL, 2003. Expression of the serum- and glucocorticoid-inducible protein kinase, Sgk, is a cell survival response to multiple types of environmental stress stimuli in mammary epithelial cells. J Biol Chem 278: 5871-5882.

Lesage J, Del-Favero F, Leonhardt M, Louvart H, Maccari S, Vieau D, Darnaudery M, 2004. Prenatal stress induces intrauterine growth restriction and programmes glucose intolerance and feeding behaviour disturbances in the aged rat. J Endocrinol 181: 291-296.

Levine S, 2005. Developmental determinants of sensitivity and resistance to stress. Psychoneuroendocrinology 30: 939-946.

Levine S, 1994. The ontogeny of the hypothalamic-pituitary-adrenal axis. The influence of maternal factors. Ann N Y Acad Sci 746: 275-288.

Levitt NS, Lindsay RS, Holmes MC, Seckl JR, 1996. Dexamethasone in the last week of pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates blood pressure in the adult offspring in the rat. Neuroendocrinology 64: 412-418.

Lindsay RS, Lindsay RM, Waddell BJ, Seckl JR, 1996. Prenatal glucocorticoid exposure leads to offspring hyperglycaemia in the rat: studies with the 11 beta-hydroxysteroid dehydrogenase inhibitor carbenoxolone. Diabetologia 39: 1299-1305.

Lindsay RS, Wake DJ, Nair S, Bunt J, Livingstone DE, Permana PA, Tataranni PA, Walker BR, 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: 2738-2744.

Lister RG, 1987. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology 92: 180-185.

Liu D, Diorio J, Tannenbaum B, Caldji C, Francis D, Freedman A, Sharma S, Pearson D, Plotsky PM, Meaney MJ, 1997. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science 277: 1659-1662.

Livingstone DE, Jones GC, Smith K, Jamieson PM, Andrew R, Kenyon CJ, Walker BR, 2000a. Understanding the role of glucocorticoids in obesity: tissue-specific alterations of corticosterone metabolism in obese Zucker rats. Endocrinology 141: 560-563.

Livingstone DE, Kenyon CJ, Walker BR, 2000b. Mechanisms of dysregulation of 11 beta-hydroxysteroid dehydrogenase type 1 in obese Zucker rats. J Endocrinol 167: 533-539.

Lombes M, Alfaidy N, Eugene E, Lessana A, Farman N, Bonvalet JP, 1995. Prerequisite for cardiac aldosterone action. Mineralocorticoid receptor and 11 beta-hydroxysteroid dehydrogenase in the human heart. Circulation 92: 175-182.

Lombes M, Oblin ME, Gasc JM, Baulieu EE, Farman N, Bonvalet JP, 1992. Immunohistochemical and biochemical evidence for a cardiovascular mineralocorticoid receptor. Circ Res 71: 503-510.

Lordi B, Patin V, Protais P, Mellier D, Caston J, 2000. Chronic stress in pregnant rats: effects on growth rate, anxiety and memory capabilities of the offspring. Int J Psychophysiol 37: 195-205.

Lordi B, Protais P, Mellier D, Caston J, 1997. Acute stress in pregnant rats: effects on growth rate, learning, and memory capabilities of the offspring. Physiol Behav 62: 1087-1092.

Louvart H, Maccari S, Darnaudery M, 2005. Prenatal stress affects behavioral reactivity to an intense stress in adult female rats. Brain Res 1031: 67-73.

Low SC, Chapman KE, Edwards CR, Seckl JR, 1994. 'Liver-type' 11 beta-hydroxysteroid dehydrogenase cDNA encodes reductase but not dehydrogenase activity in intact mammalian COS-7 cells. J Mol Endocrinol 13: 167-174.

Lubow RE, Gewirtz JC, 1995. Latent inhibition in humans: data, theory, and implications for schizophrenia. Psychol Bull 117: 87-103.

Maccari S, Piazza PV, Kabbaj M, Barbazanges A, Simon H, Le MM, 1995. Adoption reverses the long-term impairment in glucocorticoid feedback induced by prenatal stress. J Neurosci 15: 110-116.

MacLullich AM, Ferguson KJ, Wardlaw JM, Starr JM, Deary IJ, Seckl JR, 2006. Smaller left anterior cingulate cortex volumes are associated with impaired hypothalamic-pituitary-adrenal axis regulation in healthy elderly men. J Clin Endocrinol Metab 91: 1591-1594.

Mairesse J, Lesage J, Breton C, Breant B, Hahn T, Darnaudery M, Dickson SL, Seckl J, Blondeau B, Vieau D, Maccari S, Viltart O, 2007. Maternal stress alters endocrine function of the feto-placental unit in rats. Am J Physiol Endocrinol Metab Epub.

Makino S, Gold PW, Schulkin J, 1994. Corticosterone effects on corticotropin-releasing hormone mRNA in the central nucleus of the amygdala and the parvocellular region of the paraventricular nucleus of the hypothalamus. Brain Res 640: 105-112.

Marcondes FK, Miguel KJ, Melo LL, Spadari-Bratfisch RC, 2001. Estrous cycle influences the response of female rats in the elevated plus-maze test. Physiol Behav 74: 435-440.

Marini AM, Jiang X, Wu X, Tian F, Zhu D, Okagaki P, Lipsky RH, 2004. Role of brain-derived neurotrophic factor and NF-kappaB in neuronal plasticity and survival: From genes to phenotype. Restor Neurol Neurosci 22: 121-130.

Martijena ID, Calvo N, Volosin M, Molina VA, 1997. Prior exposure to a brief restraint session facilitates the occurrence of fear in response to a conflict situation: behavioral and neurochemical correlates. Brain Res 752: 136-142.

Masuzaki H, Paterson J, Shinyama H, Morton NM, Mullins JJ, Seckl JR, Flier JS, 2001. A transgenic model of visceral obesity and the metabolic syndrome. Science 294: 2166-2170.

Masuzaki H, Yamamoto H, Kenyon CJ, Elmquist JK, Morton NM, Paterson JM, Shinyama H, Sharp MG, Fleming S, Mullins JJ, Seckl JR, Flier JS, 2003. Transgenic amplification of glucocorticoid action in adipose tissue causes high blood pressure in mice. J Clin Invest 112: 83-90.

McBlane JW, Handley SL, 1994. Effects of two stressors on behaviour in the elevated X-maze: preliminary investigation of their interaction with 8-OH-DPAT. Psychopharmacology 116: 173-182.

McCormick CM, Smythe JW, Sharma S, Meaney MJ, 1995. Sex-specific effects of prenatal stress on hypothalamic-pituitary-adrenal responses to stress and brain glucocorticoid receptor density in adult rats. Brain Res Dev Brain Res 84: 55-61.

McEwen BS, 2004. Protection and damage from acute and chronic stress: allostasis and allostatic overload and relevance to the pathophysiology of psychiatric disorders. Ann N Y Acad. Sci 1032: 1-7.

McEwen BS, 1991. Non-genomic and genomic effects of steroids on neural activity. Trends Pharmacol Sci 12: 141-147.

McEwen BS, 1998. Stress, adaptation, and disease. Allostasis and allostatic load. Ann N Y Acad Sci 840: 33-44.

McEwen BS, Sapolsky RM, 1995. Stress and cognitive function. Curr Opin Neurobiol 5: 205-216.

McEwen BS, Wingfield JC, 2003. The concept of allostasis in biology and biomedicine. Horm Behav 43: 2-15.

McTernan CL, Draper N, Nicholson H, Chalder SM, Driver P, Hewison M, Kilby MD, Stewart PM, 2001. Reduced placental 11beta-hydroxysteroid dehydrogenase type 2 mRNA levels in human pregnancies complicated by intrauterine growth restriction: an analysis of possible mechanisms. J Clin Endocrinol Metab 86: 4979-4983.

Meaney MJ, 2001. Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annu Rev Neurosci 24: 1161-1192.

Meaney MJ, Aitken DH, 1985. The effects of early postnatal handling on hippocampal glucocorticoid receptor concentrations: temporal parameters. Brain Res. 354: 301-304.

Meaney MJ, Aitken DH, Bhatnagar S, Sapolsky RM, 1991. Postnatal handling attenuates certain neuroendocrine, anatomical, and cognitive dysfunctions associated with aging in female rats. Neurobiol Aging 12: 31-38.

Meaney MJ, Aitken DH, Sharma S, 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: 204-213.

Meaney MJ, Aitken DH, Viau V, Sharma S, Sarrieau A, 1989. Neonatal handling alters adrenocortical negative feedback sensitivity and hippocampal type II glucocorticoid receptor binding in the rat. Neuroendocrinology 50: 597-604.

Meaney MJ, Szyf M, 2005. Environmental programming of stress responses through DNA methylation: life at the interface between a dynamic environment and a fixed genome. Dialogues Clin Neurosci 7: 103-123.

Meek LR, Burda KM, Paster E, 2000. Effects of prenatal stress on development in mice: maturation and learning. Physiol Behav 71: 543-549.

Meek LR, Dittel PL, Sheehan MC, Chan JY, Kjolhaug SR, 2001. Effects of stress during pregnancy on maternal behavior in mice. Physiol Behav 72: 473-479.

Meek LR, Schulz KM, Keith CA, 2006. Effects of prenatal stress on sexual partner preference in mice. Physiol Behav 89: 133-138.

Meijer OC, de Kloet ER, 1998. Corticosterone and serotonergic neurotransmission in the hippocampus: functional implications of central corticosteroid receptor diversity. Crit Rev Neurobiol 12: 1-20.

Mendonca FH, Guimaraes FS, 1998. Intra-hippocampal administration of cycloheximide attenuates the restraint-induced exploratory deficit of an elevated plus maze. Behav Brain Res 91: 207-211.

Meunier J, Gue M, Recasens M, Maurice T, 2004. Attenuation by a sigma1 receptor agonist of the learning and memory deficits induced by a prenatal restraint stress in juvenile rats. Br J Pharmacol 142: 689-700.

Meyer U, Kruhoffer M, Flugge G, Fuchs E, 1998. Cloning of glucocorticoid receptor and mineralocorticoid receptor cDNA and gene expression in the central nervous system of the tree shrew (Tupaia belangeri). Brain Res Mol Brain Res 55: 243-253.

Milagro FI, Campion J, Martinez JA, 2007. 11-beta Hydroxysteroid dehydrogenase type 2 expression in white adipose tissue is strongly correlated with adiposity. J Steroid Biochem Mol Biol. Epub

Miyata S, Hamamura T, Lee Y, Miki M, Habara T, Oka T, Endo S, Taoka H, Kuroda S, 2005. Contrasting Fos expression induced by acute reboxetine and fluoxetine in the rat forebrain: neuroanatomical substrates for the antidepressant effect. Psychopharmacology 177: 289-295.

Moisan MP, Seckl JR, Edwards CR, 1990. 11 beta-hydroxysteroid dehydrogenase bioactivity and messenger RNA expression in rat forebrain: localization in hypothalamus, hippocampus, and cortex. Endocrinology 127: 1450-1455.

Monder C, Lakshmi V, 1990. Corticosteroid 11 beta-dehydrogenase of rat tissues: immunological studies. Endocrinology 126: 2435-2443.

Monder C, Shackleton CH, 1984. 11 beta-hydroxysteroid dehydrogenase: fact or fancy? Steroids 44: 383-417.

Montague CT, O'Rahilly S, 2000. The perils of portliness: causes and consequences of visceral adiposity. Diabetes 49: 883-888.

Montkowski A, Barden N, Wotjak C, Stec I, Ganster J, Meaney M, Engelmann M, Reul JM, Landgraf R, Holsboer F, 1995. Long-term antidepressant treatment reduces behavioural deficits in transgenic mice with impaired glucocorticoid receptor function. J Neuroendocrinol 7: 841-845.

Moore XL, Hoong I, Cole TJ, 2000. Expression of the 11beta-hydroxysteroid dehydrogenase 2 gene in the mouse. Kidney Int 57: 1307-1312.

Mora S, Dussaubat N, az-Veliz G, 1996. Effects of the estrous cycle and ovarian hormones on behavioral indices of anxiety in female rats. Psychoneuroendocrinology 21: 609-620.

Morley-Fletcher S, Darnaudery M, Koehl M, Casolini P, Van RO, Maccari S, 2003a. Prenatal stress in rats predicts immobility behavior in the forced swim test. Effects of a chronic treatment with tianeptine. Brain Res 989: 246-251.

Morley-Fletcher S, Rea M, Maccari S, Laviola G, 2003b. Environmental enrichment during adolescence reverses the effects of prenatal stress on play behaviour and HPA axis reactivity in rats. Eur J Neurosci 18: 3367-3374.

Morris R, 1984. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11: 47-60.

Morton NM, Holmes MC, Fievet C, Staels B, Tailleux A, Mullins JJ, Seckl JR, 2001. Improved lipid and lipoprotein profile, hepatic insulin sensitivity, and glucose tolerance in 11beta-hydroxysteroid dehydrogenase type 1 null mice. J Biol Chem 276: 41293-41300.

Morton NM, Paterson JM, Masuzaki H, Holmes MC, Staels B, Fievet C, Walker BR, Flier JS, Mullins JJ, Seckl JR, 2004a. Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11 beta-hydroxysteroid dehydrogenase type 1-deficient mice. Diabetes 53: 931-938.

Morton NM, Ramage L, Seckl JR, 2004b. Down-regulation of adipose 11beta-hydroxysteroid dehydrogenase type 1 by high-fat feeding in mice: a potential adaptive mechanism counteracting metabolic disease. Endocrinology 145: 2707-2712.

Muller M, Holsboer F, Keck ME, 2002. Genetic modification of corticosteroid receptor signalling: novel insights into pathophysiology and treatment strategies of human affective disorders. Neuropeptides 36: 117-131.

Muneoka K, Mikuni M, Ogawa T, Kitera K, Kamei K, Takigawa M, Takahashi K, 1997. Prenatal dexamethasone exposure alters brain monoamine metabolism and adrenocortical response in rat offspring. Am J Physiol 273: R1669-R1675.

Murata S, Yoshiara T, Lim CR, Sugino M, Kogure M, Ohnuki T, Komurasaki T, Matsubara K, 2005. Psychophysiological stress-regulated gene expression in mice. FEBS Lett 579: 2137-2142.

Murmu MS, Salomon S, Biala Y, Weinstock M, Braun K, Bock J, 2006. Changes of spine density and dendritic complexity in the prefrontal cortex in offspring of mothers exposed to stress during pregnancy. Eur J Neurosci 24: 1477-1487.

Murphy VE, Zakar T, Smith R, Giles WB, Gibson PG, Clifton VL, 2002. Reduced 11beta-hydroxysteroid dehydrogenase type 2 activity is associated with decreased birth weight centile in pregnancies complicated by asthma. J Clin Endocrinol Metab 87: 1660-1668.

Mutale TH, Creed FR, Maresh MI, Hunt LI, 1991. Life events and low birthweight-analysis by infants preterm and small for gestational age. Br J Obstet Gynaecol 98: 166-172.

Nair S, Lee YH, Lindsay RS, Walker BR, Tataranni PA, Bogardus C, Baier LJ, Permana PA, 2004. 11beta-Hydroxysteroid dehydrogenase Type 1: genetic polymorphisms are associated with Type 2 diabetes in Pima Indians independently of obesity and expression in adipocyte and muscle. Diabetologia 47: 1088-1095.

Naray-Fejes-Toth A, Fejes-Toth G, Volk KA, Stokes JB, 2000. SGK is a primary glucocorticoid-induced gene in the human. J Steroid Biochem Mol Biol 75: 51-56.

Nathanielsz PW, 2006. Animal models that elucidate basic principles of the developmental origins of adult diseases. ILAR J 47: 73-82.

Navarro HA, Kudlacz EM, Eylers JP, Slotkin TA, 1989. Prenatal dexamethasone administration disrupts the pattern of cellular development in rat lung. Teratology 40: 433-438.

Nechushtan H, Benvenisty N, Brandeis R, Reshef L, 1987. Glucocorticoids control phosphoenolpyruvate carboxykinase gene expression in a tissue specific manner. Nucleic Acids Res. 15: 6405-6417.

Netto SM, Guimaraes FS, 1996. Role of hippocampal 5-HT1A receptors on elevated plus maze exploration after a single restraint experience. Behav Brain Res 77: 215-218.

Neumann ID, Johnstone HA, Hatzinger M, Liebsch G, Shipston M, Russell JA, Landgraf R, Douglas AJ, 1998. Attenuated neuroendocrine responses to emotional and physical stressors in pregnant rats involve adenohypophysial changes. J Physiol 508: 289-300.

Nielsen JV, Mitchelmore C, Pedersen KM, Kjaerulff KM, Finsen B, Jensen NA, 2004. Fkbp8: novel isoforms, genomic organization, and characterization of a forebrain promoter in transgenic mice. Genomics 83: 181-192.

Nieoullon A, 2002. Dopamine and the regulation of cognition and attention. Prog Neurobiol 67: 53-83.

Niewoehner B, Single FN, Hvalby O, Jensen V, Meyer zum Alten Borgloh S, Seeburg PH, Rawlins JNP, Sprengel R, Bannerman DM, 2007. Impaired spatial working memory but spared spatial reference memory following functional loss of NMDA receptors in the dentate gyrus. Eur J Neurosci 25: 837-846.

Nishio H, Kasuga S, Ushijima M, Harada Y, 2001. Prenatal stress and postnatal development of neonatal rats--sex-dependent effects on emotional behavior and learning ability of neonatal rats. Int J Dev Neurosci 19: 37-45.

Noorlander CW, De Graan PN, Middeldorp J, Van Beers JJ, Visser GH, 2006. Ontogeny of hippocampal corticosteroid receptors: effects of antenatal glucocorticoids in human and mouse. J Comp Neurol 499: 924-932.

Nuber UA, Kriaucionis S, Roloff TC, Guy J, Selfridge J, Steinhoff C, Schulz R, Lipkowitz B, Ropers HH, Holmes MC, Bird A, 2005. Up-regulation of glucocorticoid-regulated genes in a mouse model of Rett syndrome. Hum Mol Genet 14: 2247-2256.

Nyirenda MJ, Dean S, Lyons V, Chapman KE, Seckl JR, 2006. Prenatal programming of hepatocyte nuclear factor 4a in the rat: a key mechanism in the 'foetal origins of hyperglycaemia'? Diabetologia 49: 1412-1420.

Nyirenda MJ, Lindsay RS, Kenyon CJ, Burchell A, Seckl JR, 1998. Glucocorticoid exposure in late gestation permanently programs rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. J Clin Invest 101: 2174-2181.

Nyirenda MJ, Welberg LA, Seckl JR, 2001. Programming hyperglycaemia in the rat through prenatal exposure to glucocorticoids-fetal effect or maternal influence? J Endocrinol 170: 653-660.

O'Brien CA, Jia D, Plotkin LI, Bellido T, Powers CC, Stewart SA, Manolagas SC, Weinstein RS, 2004. Glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength. Endocrinology 145: 1835-1841.

O'Mahony SM, Myint AM, van den HD, Desbonnet L, Steinbusch H, Leonard BE, 2006. Gestational stress leads to depressive-like behavioural and immunological changes in the rat. Neuroimmunomodulation 13: 82-88.

O'Regan D, Kenyon CJ, Seckl JR, Holmes MC, 2004. Glucocorticoid exposure in late gestation in the rat permanently programs gender-specific differences in adult cardiovascular and metabolic physiology. Am J Physiol Endocrinol Metab 287: E863-E870.

Okajima S, Matsuda T, Cho K, Matsumoto Y, Kobayashi Y, Fujimoto S, 2001. Antenatal dexamethasone administration impairs normal postnatal lung growth in rats. Pediatr Res. 49: 777-781.

Oliveira M, Bessa JM, Mesquita A, Tavares H, Carvalho A, Silva R, Pego JM, Cerqueira JJ, Palha JA, Almeida OF, Sousa N, 2006. Induction of a hyperanxious state by antenatal dexamethasone: a case for less detrimental natural corticosteroids. Biol Psychiatry 59: 844-852.

Oliver JD, Rogers MP, 1993. Stimulation of lipoprotein lipase synthesis by refeeding, insulin and dexamethasone. Biochem J 292 (Pt 2): 525-530.

Olswang Y, Cohen H, Papo O, Cassuto H, Croniger CM, Hakimi P, Tilghman SM, Hanson RW, Reshef L, 2002. A mutation in the peroxisome proliferator-activated receptor gamma -binding site in the gene for the cytosolic form of phosphoenolpyruvate carboxykinase reduces adipose tissue size and fat content in mice. Proc Nat Acad Sci 99: 625-630.

Ong KK, 2006. Size at birth, postnatal growth and risk of obesity. Horm Res. 65 Suppl 3: 65-69.

Opherk C, Tronche F, Kellendonk C, Kohlmuller D, Schulze A, Schmid W, Schutz G, 2004. Inactivation of the glucocorticoid receptor in hepatocytes leads to fasting hypoglycemia and ameliorates hyperglycemia in streptozotocin-induced diabetes mellitus. Mol Endocrinol 18: 1346-1353.

Ortiz LA, Quan A, Weinberg A, Baum M, 2001. Effect of prenatal dexamethasone on rat renal development. Kidney Int 59: 1663-1669.

Ortiz LA, Quan A, Zarzar F, Weinberg A, Baum M, 2003. Prenatal dexamethasone programs hypertension and renal injury in the rat. Hypertension 41: 328-334.

Ostrander MM, Richtand NM, Herman JP, 2003. Stress and amphetamine induce Fos expression in medial prefrontal cortex neurons containing glucocorticoid receptors. Brain Res 990: 209-214.

Osuch EA, Ketter TA, Kimbrell TA, George MS, Benson BE, Willis MW, Herscovitch P, Post RM, 2000. Regional cerebral metabolism associated with anxiety symptoms in affective disorder patients. Biol Psychiatry 48: 1020-1023.

Ou XM, Storring JM, Kushwaha N, Albert PR, 2001. Heterodimerization of mineralocorticoid and glucocorticoid receptors at a novel negative response element of the 5-HT1A receptor gene. J Biol Chem 276: 14299-14307.

Ouvrard-Pascaud A, Sainte-Marie Y, Benitah JP, Perrier R, Soukaseum C, Cat AN, Royer A, Le QK, Charpentier F, Demolombe S, Mechta-Grigoriou F, Beggah AT, Maison-Blanche P, Oblin ME, Delcayre C, Fishman GI, Farman N, Escoubet B, Jaisser F, 2005. Conditional mineralocorticoid receptor expression in the heart leads to life-threatening arrhythmias. Circulation 111: 3025-3033.

Owen D, Matthews SG, 2007. Repeated maternal glucocorticoid treatment affects activity and hippocampal NMDA receptor expression in juvenile guinea pigs. J Physiol 578: 249-257.

Oyemade UJ, Cole OJ, Johnson AA, Knight EM, Westney OE, Laryea H, Hill G, Cannon E, Fomufod A, Westney LS, Jones S, Edwards CH, 1994. Prenatal predictors of performance on the Brazelton neonatal behavioral assessment scale. J Nutr 124: 994S-999S.

Pace TW, Gaylord R, Topczewski F, Girotti M, Rubin B, Spencer RL, 2005. Immediate-early gene induction in hippocampus and cortex as a result of novel experience is not directly related to the stressfulness of that experience. Eur J Neurosci 22: 1679-1690.

Padovan CM, Guimaraes FS, 2000. Restraint-induced hypoactivity in an elevated plus-maze. Braz J Med Biol Res 33: 79-83.

Palanza P, 2001. Animal models of anxiety and depression: how are females different? Neurosci Biobehav Rev. 25: 219-233.

Palermo-Neto J, de Oliveira MC, Robespierre de SW, 2003. Effects of physical and psychological stressors on behavior, macrophage activity, and Ehrlich tumor growth. Brain Behav Immun 17: 43-54.

Palermo-Neto J, Massoco CO, Favare RC, 2001. Effects of maternal stress on anxiety levels, macrophage activity, and Ehrlich tumor growth. Neurotoxicol Teratol 23: 497-507.

Pardon M, Gerardin P, Joubert C, Perez-Diaz F, Cohen-Salmon C, 2000. Influence of prepartum chronic ultramild stress on maternal pup care behavior in mice. Biol Psychiatry 47: 858-863.

Parfitt DB, Levin JK, Saltstein KP, Klayman AS, Greer LM, Helmreich DL, 2004. Differential early rearing environments can accentuate or attenuate the responses to stress in male C57BL/6 mice. Brain Res 1016: 111-118.

Park MK, Hoang TA, Belluzzi JD, Leslie FM, 2003. Gender Specific Effect of Neonatal Handling on Stress Reactivity of Adolescent Rats. J Neuroendocrinol 15: 289-295.

Parks CL, Robinson PS, Sibille E, Shenk T, Toth M, 1998. Increased anxiety of mice lacking the serotonin1A receptor. Proc Natl Acad Sci USA 95: 10734-10739.

Patacchioli FR, Puglisi-Allegra S, Maccari S, Casolini P, Angelucci L, Cabib S, Oliverio A, 1990. Strain-dependent differences in hippocampal glucocorticoid binding capacity and active avoidance in the mouse. Behav Brain Res 37: 185-188.

Patel PD, Lopez JF, Lyons DM, Burke S, Wallace M, Schatzberg AF, 2000. Glucocorticoid and mineralocorticoid receptor mRNA expression in squirrel monkey brain. J Psychiatr Res 34: 383-392.

Paterson JM, Holmes MC, Kenyon CJ, Carter R, Mullins JJ, Seckl JR, 2007. Liver-selective transgene rescue of hypothalamic-pituitary-adrenal axis dysfunction in 11beta-hydroxysteroid dehydrogenase type 1 deficient mice. Endocrinology 148: 961-966.

Paterson JM, Morton NM, Fievet C, Kenyon CJ, Holmes MC, Staels B, Seckl JR, Mullins JJ, 2004. Metabolic syndrome without obesity: Hepatic overexpression of 11beta-hydroxysteroid dehydrogenase type 1 in transgenic mice. Proc Natl Acad Sci USA 101: 7088-7093.

Paterson JM, Seckl JR, Mullins JJ, 2005. Genetic manipulation of 11beta-hydroxysteroid dehydrogenases in mice. Am J Physiol Regul Integr Comp Physiol 289: R642-R652.

Patin V, Lordi B, Vincent A, Caston J, 2005. Effects of prenatal stress on anxiety and social interactions in adult rats. Brain Res Dev Brain Res 160: 265-274.

Patin V, Lordi B, Vincent A, Thoumas JL, Vaudry H, Caston J, 2002. Effects of prenatal stress on maternal behavior in the rat. Brain Res Dev Brain Res 139: 1-8.

Pearce P, Funder JW, 1987. High affinity aldosterone binding sites (type I receptors) in rat heart. Clin. Exp Pharmacol Physiol 14: 859-866.

Pellow S, Chopin P, File SE, Briley M, 1985. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14: 149-167.

Pepe GJ, Burch MG, Albrecht ED, 2001. Localization and developmental regulation of 11beta-hydroxysteroid dehydrogenase-1 and -2 in the baboon syncytiotrophoblast. Endocrinology 142: 68-80.

Pepe GJ, Burch MG, Albrecht ED, 1999. Expression of the 11beta-hydroxysteroid dehydrogenase types 1 and 2 proteins in human and baboon placental syncytiotrophoblast. Placenta 20: 575-582.

Pepin MC, Pothier F, Barden N, 1992. Impaired type II glucocorticoid-receptor function in mice bearing antisense RNA transgene. Nature 355: 725-728.

Perel'muter VM, Paderov YM, 2004. Initial morphofunctional asymmetry of the adrenal glands in CBA/Lacy mice. Bull Exp Biol Med 137: 392-394.

Peters DA, 1986. Prenatal stress increases the behavioral response to serotonin agonists and alters open field behavior in the rat. Pharmacol Biochem Behav 25: 873-877.

Peters DA, 1988a. Both prenatal and postnatal factors contribute to the effects of maternal stress on offspring behavior and central 5-hydroxytryptamine receptors in the rat. Pharmacol Biochem Behav 30: 669-673.

Peters DA, 1988b. Effects of maternal stress during different gestational periods on the serotonergic system in adult rat offspring. Pharmacol Biochem Behav 31: 839-843.

Peters DA, 1990. Maternal stress increases fetal brain and neonatal cerebral cortex 5-hydroxytryptamine synthesis in rats: a possible mechanism by which stress influences brain development. Pharmacol Biochem Behav 35: 943-947.

Peters DA, 1982. Prenatal stress: effects on brain biogenic amine and plasma corticosterone levels. Pharmacol Biochem Behav 17: 721-725.

Phillips DI, Walker BR, Reynolds RM, Flanagan DE, Wood PJ, Osmond C, Barker DJ, Whorwood CB, 2000. Low birth weight predicts elevated plasma cortisol concentrations in adults from 3 populations. Hypertension 35: 1301-1306.

Pietranera L, Saravia FE, McEwen BS, Lucas LL, Johnson AK, De Nicola AF, 2001. Changes in Fos expression in various brain regions during deoxycorticosterone acetate treatment: relation to salt appetite, vasopressin mRNA and the mineralocorticoid receptor. Neuroendocrinology 74: 396-406.

Pincus-Knackstedt MK, Joachim RA, Blois SM, Douglas AJ, Orsal AS, Klapp BF, Wahn U, Hamelmann E, Arck PC, 2006. Prenatal stress enhances susceptibility of murine adult offspring toward airway inflammation. J Immunol 177: 8484-8492.

Pizzagalli D, Pascual-Marqui RD, Nitschke JB, Oakes TR, Larson CL, Abercrombie HC, Schaefer SM, Koger JV, Benca RM, Davidson RJ, 2001. Anterior cingulate activity

as a predictor of degree of treatment response in major depression: evidence from brain electrical tomography analysis. Am J Psychiatry 158: 405-415.

Poltyrev T, Gorodetsky E, Bejar C, Schorer-Apelbaum D, Weinstock M, 2005. Effect of chronic treatment with ladostigil (TV-3326) on anxiogenic and depressive-like behaviour and on activity of the hypothalamic-pituitary-adrenal axis in male and female prenatally stressed rats. Psychopharmacology 181: 118-125.

Poltyrev T, Keshet GI, Kay G, Weinstock M, 1996. Role of experimental conditions in determining differences in exploratory behavior of prenatally stressed rats. Dev Psychobiol 29: 453-462.

Poltyrev T, Weinstock M, 2004. Gender difference in the prevention of hyperanxiety in adult prenatally stressed rats by chronic treatment with amitriptyline. Psychopharmacology 171: 270-276.

Porsolt RD, Anton G, Blavet N, Jalfre M, 1978. Behavioural despair in rats: a new model sensitive to antidepressant treatments. Eur J Pharmacol 47: 379-391.

Porsolt RD, Le PM, Jalfre M, 1977. Depression: a new animal model sensitive to antidepressant treatments. Nature 266: 730-732.

Pratt WB, Toft DO, 1997. Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev 18: 306-360.

Pressley L, Funder JW, 1975. Glucocorticoid and mineralocorticoid receptors in gut mucosa. Endocrinology 97: 588-596.

Prut L, Belzung C, 2003. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol 463: 3-33.

Pryce CR, Feldon J, Fuchs E, Knuesel I, Oertle T, Sengstag C, Spengler M, Weber E, Weston A, Jongen-Relo A, 2005. Postnatal ontogeny of hippocampal expression of the mineralocorticoid and glucocorticoid receptors in the common marmoset monkey. Eur J Neurosci 21: 1521-1535.

Pryce CR, Ruedi-Bettschen D, Dettling AC, Feldon J, 2002. Early Life Stress: Long-Term Physiological Impact in Rodents and Primates. News Physiol Sci 17: 150-155.

Qin W, Rudolph AE, Bond BR, Rocha R, Blomme EA, Goellner JJ, Funder JW, McMahon EG, 2003. Transgenic model of aldosterone-driven cardiac hypertrophy and heart failure. Circ Res 93: 69-76.

Rafestin-Oblin ME, Lombes M, Michiel JB, Michaud A, Claire M, 1984. Mineralocorticoid receptors in the epithelial cells of human colon and ileum. J Steroid Biochem 20: 311-315.

Rajan V, Chapman KE, Lyons V, Jamieson P, Mullins JJ, Edwards CR, Seckl JR, 1995. Cloning, sequencing and tissue-distribution of mouse 11 beta-hydroxysteroid dehydrogenase-1 cDNA. J Steroid Biochem Mol Biol 52: 141-147.

Rajan V, Edwards CR, Seckl JR, 1996. 11 beta-Hydroxysteroid dehydrogenase in cultured hippocampal cells reactivates inert 11-dehydrocorticosterone, potentiating neurotoxicity. J Neurosci 16: 65-70.

Ramboz S, Oosting R, Amara DA, Kung HF, Blier P, Mendelsohn M, Mann JJ, Brunner D, Hen R, 1998. Serotonin receptor 1A knockout: an animal model of anxiety-related disorder. Proc Natl Acad Sci USA 95: 14476-14481.

Rao U, McGinty DJ, Shinde A, McCracken JT, Poland RE, 1999. Prenatal stress is associated with depression-related electroencephalographic sleep changes in adult male rats: a preliminary report. Prog Neuropsychopharmacol Biol Psychiatry 23: 929-939.

Rask E, Olsson T, Soderberg S, Andrew R, Livingstone DE, Johnson O, Walker BR, 2001. Tissue-specific dysregulation of cortisol metabolism in human obesity. J Clin Endocrinol Metab 86: 1418-1421.

Rask E, Walker BR, Soderberg S, Livingstone DE, Eliasson M, Johnson O, Andrew R, 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: 3330-3336.

Rayburn WF, Christensen HD, Gonzalez CL, 1997. A placebo-controlled comparison between betamethasone and dexamethasone for fetal maturation: differences in neurobehavioral development of mice offspring. Am J Obstet Gynecol 176: 842-850.

Rayburn WF, Christensen HD, Gonzalez CL, Rayburn LA, Stewart JD, 1998. Effect of in utero exposure to betamethasone on motivation/anxiety testing in mice offspring. Neurotoxicol Teratol 20: 475-481.

Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, Gass P, Schmid W, Herrlich P, Angel P, Schutz G, 1998a. DNA binding of the glucocorticoid receptor is not essential for survival. Cell 93: 531-541.

Reichardt HM, Kaestner KH, Wessely O, Gass P, Schmid W, Schutz G, 1998b. Analysis of glucocorticoid signalling by gene targeting. J Steroid Biochem Mol Biol 65: 111-115.

Reichardt HM, Umland T, Bauer A, Kretz O, Schutz G, 2000. Mice with an increased glucocorticoid receptor gene dosage show enhanced resistance to stress and endotoxic shock. Mol Cell Biol 20: 9009-9017.

Reynolds RM, Walker BR, Syddall HE, Andrew R, Wood PJ, Phillips DI, 2005. Is there a gender difference in the associations of birthweight and adult hypothalamic-pituitary-adrenal axis activity? Eur J Endocrinol 152: 249-253.

Reynolds RM, Walker BR, Syddall HE, Andrew R, Wood PJ, Whorwood CB, Phillips DI, 2001. Altered control of cortisol secretion in adult men with low birth weight and cardiovascular risk factors. J Clin Endocrinol Metab 86: 245-250.

Reznikov AG, Nosenko ND, Tarasenko LV, 2005. Opioids are responsible for neurochemical feminization of the brain in prenatally stressed male rats. Neuro Endocrinol Lett 26: 35-38.

Reznikov AG, Nosenko ND, Tarasenko LV, 2004. Early postnatal effects of prenatal exposure to glucocorticoids on testosterone metabolism and biogenic monoamines in discrete neuroendocrine regions of the rat brain. Comp Biochem Physiol C Toxicol Pharmacol 138: 169-175.

Reznikov AG, Nosenko ND, Tarasenko LV, 2003. Participation of endogenous opioids in pathogenesis of early neuroendocrine manifestations of prenatal stress syndrome. Bull Exp Biol Med 135: 421-423.

Reznikov AG, Nosenko ND, Tarasenko LV, Sinitsyn PV, Polyakova LI, 2001. Early and long-term neuroendocrine effects of prenatal stress in male and female rats. Neurosci Behav Physiol 31: 1-5.

Richardson HN, Zorrilla EP, Mandyam CD, Rivier CL, 2006. Exposure to repetitive versus varied stress during prenatal development generates two distinct anxiogenic and neuroendocrine profiles in adulthood. Endocrinology 147: 2506-2517.

Rimondini R, Agren G, Borjesson S, Sommer W, Heilig M, 2003. Persistent behavioral and autonomic supersensitivity to stress following prenatal stress exposure in rats. Behav Brain Res 140: 75-80.

Robson AC, Leckie CM, Seckl JR, Holmes MC, 1998. 11 Beta-hydroxysteroid dehydrogenase type 2 in the postnatal and adult rat brain. Brain Res Mol Brain Res 61: 1-10.

Rodgers RJ, Dalvi A, 1997. Anxiety, defence and the elevated plus-maze. Neurosci Biobehav Rev 21: 801-810.

Roland BL, Funder JW, 1996. Localization of 11beta-hydroxysteroid dehydrogenase type 2 in rat tissues: in situ studies. Endocrinology 137: 1123-1128.

Roland BL, Krozowski ZS, Funder JW, 1995a. Glucocorticoid receptor, mineralocorticoid receptors, 11 beta-hydroxysteroid dehydrogenase-1 and -2 expression in rat brain and kidney: in situ studies. Mol Cell Endocrinol 111: R1-R7.

Roland BL, Li KX, Funder JW, 1995b. Hybridization histochemical localization of 11 beta-hydroxysteroid dehydrogenase type 2 in rat brain. Endocrinology 136: 4697-4700.

Rosene DL, Lister JP, Schwagerl AL, Tonkiss J, McCormick CM, Galler JR, 2004. Prenatal protein malnutrition in rats alters the c-Fos response of neurons in the anterior cingulate and medial prefrontal region to behavioral stress. Nutr Neurosci 7: 281-289.

Rowland NE, Fregly MJ, 1988. Sodium appetite: species and strain differences and role of renin-angiotensin-aldosterone system. Appetite 11: 143-178.

Saegusa H, Nakagawa Y, Liu YJ, Ohzeki T, 1999. Influence of placental 11beta-hydroxysteroid dehydrogenase (11beta-HSD) inhibition on glucose metabolism and 11beta-HSD regulation in adult offspring of rats. Metabolism 48: 1584-1588.

Salchner P, Sartori SB, Sinner C, Wigger A, Frank E, Landgraf R, Singewald N, 2006. Airjet and FG-7142-induced Fos expression differs in rats selectively bred for high and low anxiety-related behavior. Neuropharmacology 50: 1048-1058.

Salome N, Salchner P, Viltart O, Sequeira H, Wigger A, Landgraf R, Singewald N, 2004. Neurobiological correlates of high (HAB) versus low anxiety-related behavior (LAB): differential Fos expression in HAB and LAB rats. Biol Psychiatry 55: 715-723.

Sandi C, Loscertales M, Guaza C, 1997. Experience-dependent facilitating effect of corticosterone on spatial memory formation in the water maze. Eur J Neurosci 9: 637-642.

Sapolsky RM, Romero LM, Munck AU, 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 21: 55-89.

Sasaki K, Cripe TP, Koch SR, Andreone TL, Petersen DD, Beale EG, Granner DK, 1984. Multihormonal regulation of phosphoenolpyruvate carboxykinase gene transcription. The dominant role of insulin. J Biol Chem 259: 15242-15251.

Scammell JG, Denny WB, Valentine DL, Smith DF, 2001. Overexpression of the FK506-binding immunophilin FKBP51 is the common cause of glucocorticoid resistance in three New World primates. Gen Comp Endocrinol 124: 152-165.

Schmidt MV, Enthoven L, van der MM, Levine S, de Kloet ER, Oitzl MS, 2003. The postnatal development of the hypothalamic-pituitary-adrenal axis in the mouse. Int J Dev Neurosci 21: 125-132.

Schmidt MV, Levine S, Oitzl MS, van der MM, Muller MB, Holsboer F, de Kloet ER, 2005. Glucocorticoid receptor blockade disinhibits pituitary-adrenal activity during the stress hyporesponsive period of the mouse. Endocrinology 146: 1458-1464.

Schoneveld OJLM, Gaemers IC, Lamers WH, 2004. Mechanisms of glucocorticoid signalling. Biochim Biophys Acta 1680: 114-128.

Schulkin J, McEwen BS, Gold PW, 1994. Allostasis, amygdala, and anticipatory angst. Neurosci Biobehav Rev 18: 385-396.

Schulz-Baldes A, Berger S, Grahammer F, Warth R, Goldschmidt I, Peters J, Schutz G, Greger R, Bleich M, 2001. Induction of the epithelial Na+ channel via glucocorticoids in mineralocorticoid receptor knockout mice. Pflugers Arch 443: 297-305.

Seckl JR, 1997. Glucocorticoids, feto-placental 11 beta-hydroxysteroid dehydrogenase type 2, and the early life origins of adult disease. Steroids 62: 89-94.

Seckl JR, Dickson KL, Yates C, Fink G, 1991. Distribution of glucocorticoid and mineralocorticoid receptor messenger RNA expression in human postmortem hippocampus. Brain Res 561: 332-337.

Seckl JR, Meaney MJ, 2004. Glucocorticoid programming. Ann NY Acad Sci 1032: 63-84.

Shalev U, Weiner I, 2001. Gender-dependent differences in latent inhibition following prenatal stress and corticosterone administration. Behav Brain Res 126: 57-63.

Shams M, Kilby MD, Somerset DA, Howie AJ, Gupta A, Wood PJ, Afnan M, Stewart PM, 1998. 11Beta-hydroxysteroid dehydrogenase type 2 in human pregnancy and reduced expression in intrauterine growth restriction. Hum Reprod 13: 799-804.

She P, Burgess SC, Shiota M, Flakoll P, Donahue EP, Malloy CR, Sherry AD, Magnuson MA, 2003. Mechanisms by which liver-specific PEPCK knockout mice preserve euglycemia during starvation. Diabetes 52: 1649-1654.

Shenkin SD, Starr JM, Pattie A, Rush MA, Whalley LJ, Deary IJ, 2001. Birth weight and cognitive function at age 11 years: the Scottish Mental Survey 1932. Arch Dis Child 85: 189-196.

Sher LB, Woitge HW, Adams DJ, Gronowicz GA, Krozowski Z, Harrison JR, Kream BE, 2004. Transgenic expression of 11beta-hydroxysteroid dehydrogenase type 2 in osteoblasts reveals an anabolic role for endogenous glucocorticoids in bone. Endocrinology 145: 922-929.

Shimojo M, Whorwood CB, Stewart PM, 1996. 11 beta-Hydroxysteroid dehydrogenase in the rat adrenal. J Mol Endocrinol 17: 121-130.

Shoener JA, Baig R, Page KC, 2006. Prenatal exposure to dexamethasone alters hippocampal drive on hypothalamic-pituitary-adrenal axis activity in adult male rats. Am J Physiol Regul Integr Comp Physiol 290: R1366-R1373.

Simon P, Dupuis R, Costentin J, 1994. Thigmotaxis as an index of anxiety in mice. Influence of dopaminergic transmissions. Behav Brain Res 61: 59-64.

Singewald N, Salchner P, Sharp T, 2003. Induction of c-Fos expression in specific areas of the fear circuitry in rat forebrain by anxiogenic drugs. Biol Psychiatry 53: 275-283.

Singh RR, Cullen-McEwen LA, Kett MM, Boon WM, Dowling J, Bertram JF, Moritz KM, 2007. Prenatal corticosterone exposure results in altered AT1/AT2, nephron deficit And hypertension in the rat offspring. J Physiol 579(Pt 2): 503-13.

Sizonenko SV, Borradori-Tolsa C, Bauthay DM, Lodygensky G, Lazeyras F, Huppi P, 2006. Impact of intrauterine growth restriction and glucocorticoids on brain development: insights using advanced magnetic resonance imaging. Mol Cell Endocrinol 254-255: 163-171.

Slotkin TA, Barnes GA, McCook EC, Seidler FJ, 1996. Programming of brainstem serotonin transporter development by prenatal glucocorticoids. Brain Res Dev Brain Res 93: 155-161.

Slotkin TA, Kreider ML, Tate CA, Seidler FJ, 2006. Critical prenatal and postnatal periods for persistent effects of dexamethasone on serotonergic and dopaminergic systems. Neuropsychopharmacology 31: 904-911.

Slotkin TA, Lappi SE, McCook EC, Tayyeb MI, Eylers JP, Seidler FJ, 1992. Glucocorticoids and the development of neuronal function: effects of prenatal dexamethasone exposure on central noradrenergic activity. Biol Neonate 61: 326-336.

Smith JW, Seckl JR, Evans AT, Costall B, Smythe JW, 2004. Gestational stress induces post-partum depression-like behaviour and alters maternal care in rats. Psychoneuroendocrinology 29: 227-244.

Smythe JW, McCormick CM, Meaney MJ, 1996. Median eminence corticotrophin-releasing hormone content following prenatal stress and neonatal handling. Brain Res Bull 40: 195-199.

Son GH, Chung S, Geum D, Kang SS, Choi WS, Kim K, Choi S, 2007. Hyperactivity and alteration of the midbrain dopaminergic system in maternally stressed male mice offspring. Biochem Biophys Res Commun 352: 823-829.

Son GH, Geum D, Chung S, Kim EJ, Jo JH, Kim CM, Lee KH, Kim H, Choi S, Kim HT, Lee CJ, Kim K, 2006. Maternal stress produces learning deficits associated with impairment of NMDA receptor-mediated synaptic plasticity. J Neurosci 26: 3309-3318.

Speirs HJ, Seckl JR, Brown RW, 2004. Ontogeny of glucocorticoid receptor and 11beta-hydroxysteroid dehydrogenase type-1 gene expression identifies potential critical periods of glucocorticoid susceptibility during development. J Endocrinol 181: 105-116.

Stec I, Barden N, Reul JM, Holsboer F, 1994. Dexamethasone nonsuppression in transgenic mice expressing antisense RNA to the glucocorticoid receptor. J Psychiatr Res 28: 1-5.

Steckler T, Rammes G, Sauvage M, van Gaalen MM, Weis C, Zieglgansberger W, Holsboer F, 2001. Effects of the monoamine oxidase A inhibitor moclobemide on hippocampal plasticity in GR-impaired transgenic mice. J Psychiatr Res 35: 29-42.

Steckler T, Weis C, Sauvage M, Mederer A, Holsboer F, 1999. Disrupted allocentric but preserved egocentric spatial learning in transgenic mice with impaired glucocorticoid receptor function. Behav Brain Res 100: 77-89.

Sternberg WF, Ridgway CG, 2003. Effects of gestational stress and neonatal handling on pain, analgesia, and stress behavior of adult mice. Physiol Behav 78: 375-383.

Stewart PM, Corrie JE, Shackleton CH, Edwards CR, 1988. Syndrome of apparent mineralocorticoid excess. A defect in the cortisol-cortisone shuttle. J Clin Invest 82: 340-349.

Stewart PM, Rogerson FM, Mason JI, 1995. Type 2 11 beta-hydroxysteroid dehydrogenase messenger ribonucleic acid and activity in human placenta and fetal membranes: its relationship to birth weight and putative role in fetal adrenal steroidogenesis. J Clin Endocrinol Metab 80: 885-890.

Stewart PM, Wallace AM, Atherden SM, Shearing CH, Edwards CR, 1990. Mineralocorticoid activity of carbenoxolone: contrasting effects of carbenoxolone and liquorice on 11 beta-hydroxysteroid dehydrogenase activity in man. Clin Sci 78: 49-54.

Stocklin E, Wissler M, Gouilleux F, Groner B, 1996. Functional interactions between Stat5 and the glucocorticoid receptor. Nature 383: 726-728.

Stohr T, Schulte WD, Szuran T, Pliska V, Domeney A, Welzl H, Weiner I, Feldon J, 1998. Differential effects of prenatal stress in two inbred strains of rats. Pharmacol Biochem Behav 59: 799-805.

Strohle A, Poettig M, Barden N, Holsboer F, Montkowski A, 1998. Age- and stimulus-dependent changes in anxiety-related behaviour of transgenic mice with GR dysfunction. Neuroreport 9: 2099-2102.

Sugden MC, Langdown ML, Munns MJ, Holness MJ, 2001. Maternal glucocorticoid treatment modulates placental leptin and leptin receptor expression and materno-fetal leptin physiology during late pregnancy, and elicits hypertension associated with hyperleptinaemia in the early-growth-retarded adult offspring. Eur J Endocrinol 145: 529-539.

Sun K, Yang K, Challis JR, 1997. Differential expression of 11 beta-hydroxysteroid dehydrogenase types 1 and 2 in human placenta and fetal membranes 20. J Clin Endocrinol Metab 82: 300-305.

Sun Y, Liu S, Ferguson S, Wang L, Klepcyk P, Yun JS, Friedman JE, 2002. Phosphoenolpyruvate carboxykinase overexpression selectively attenuates insulin signaling and hepatic insulin sensitivity in transgenic mice. J Biol Chem 277: 23301-23307.

Sutanto W, van Eekelen JA, Reul JM, de Kloet ER, 1988. Species-specific topography of corticosteroid receptor types in rat and hamster brain. Neuroendocrinology 47: 398-404.

Swinny JD, van der Want JJ, Gramsbergen A, 2005. Cerebellar development and plasticity: perspectives for motor coordination strategies, for motor skills, and for therapy. Neural Plast 12: 153-160.

Swolin-Eide D, Dahlgren J, Nilsson C, Albertsson WK, Holmang A, Ohlsson C, 2002. Affected skeletal growth but normal bone mineralization in rat offspring after prenatal dexamethasone exposure. J Endocrinol 174: 411-418.

Szuran T, Zimmermann E, Welzl H, 1994. Water maze performance and hippocampal weight of prenatally stressed rats. Behav Brain Res 65: 153-155.

Szuran TF, Pliska V, Pokorny J, Welzl H, 2000. Prenatal stress in rats: effects on plasma corticosterone, hippocampal glucocorticoid receptors, and maze performance. Physiol Behav 71: 353-362.

Takahashi LK, 1998. Prenatal stress: consequences of glucocorticoids on hippocampal development and function. Int J Dev Neurosci 16: 199-207.

Tamura G, Olson D, Miron J, Clark TG, 2005. Tolloid-like 1 is negatively regulated by stress and glucocorticoids. Brain Res Mol Brain Res 142: 81-90.

Thompson A, Han VK, Yang K, 2002. Spatial and temporal patterns of expression of 11beta-hydroxysteroid dehydrogenase types 1 and 2 messenger RNA and glucocorticoid receptor protein in the murine placenta and uterus during late pregnancy. Biol Reprod 67: 1708-1718.

Thone-Reineke C, Kalk P, Dorn M, Klaus S, Simon K, Pfab T, Godes M, Persson P, Unger T, Hocher B, 2006. High-protein nutrition during pregnancy and lactation programs blood pressure, food efficiency, and body weight of the offspring in a sex-dependent manner. Am J Physiol Regul Integr Comp Physiol 291: R1025-R1030.

Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, Bock R, Klein R, Schutz G, 1999. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat Genet 23: 99-103.

Tronche F, Opherk C, Moriggl R, Kellendonk C, Reimann A, Schwake L, Reichardt HM, Stangl K, Gau D, Hoeflich A, Beug H, Schmid W, Schutz G, 2004. Glucocorticoid receptor function in hepatocytes is essential to promote postnatal body growth. Genes Dev 18: 492-497.

Tsuno N, Besset A, Ritchie K, 2005. Sleep and depression. J Clin Psychiatry 66: 1254-1269.

Ulick S, Levine LS, Gunczler P, Zanconato G, Ramirez LC, Rauh W, Rosler A, Bradlow HL, New MI, 1979. A syndrome of apparent mineralocorticoid excess associated with defects in the peripheral metabolism of cortisol. J Clin Endocrinol Metab 49: 757-764.

Vallee M, Maccari S, Dellu F, Simon H, Le MM, Mayo W, 1999. Long-term effects of prenatal stress and postnatal handling on age-related glucocorticoid secretion and cognitive performance: a longitudinal study in the rat. Eur J Neurosci 11: 2906-2916.

Vallee M, Mayo W, Dellu F, Le MM, Simon H, Maccari S, 1997. Prenatal stress induces high anxiety and postnatal handling induces low anxiety in adult offspring: correlation with stress-induced corticosterone secretion. J Neurosci 17: 2626-2636.

Vallee M, Mayo W, Maccari S, Le MM, Simon H, 1996. Long-term effects of prenatal stress and handling on metabolic parameters: relationship to corticosterone secretion response. Brain Res 712: 287-292.

Van Craenenbroeck K, de Bosscher K, Van den Berghe W, Van Hoenacker P, Haegeman G, 2005. Role of glucocorticoids in dopamine-related neuropsychiatric disorders. Mol Cell Endocrinol 245: 10-22.

Van den Bergh BRH, Mulder EJH, Mennes M, Glover V, 2005. Antenatal maternal anxiety and stress and the neurobehavioural development of the fetus and child: links and possible mechanisms. A review. Neurosci Biobehav Rev 29: 237-258.

Van den Hove DL, Blanco CE, Aendekerk B, Desbonnet L, Bruschettini M, Steinbusch HP, Prickaerts J, Steinbusch HW, 2005. Prenatal restraint stress and long-term affective consequences. Dev Neurosci 27: 313-320.

Van den Hove DL, Lauder JM, Scheepens A, Prickaerts J, Blanco CE, Steinbusch HW, 2006a. Prenatal stress in the rat alters 5-HT1A receptor binding in the ventral hippocampus. Brain Res 1090: 29-34.

Van den Hove DL, Steinbusch HW, Bruschettini M, Gazzolo D, Frulio R, Scheepens A, Prickaerts J, Blanco CE, 2006b. Prenatal stress reduces S100B in the neonatal rat hippocampus. Neuroreport 17: 1077-1080.

Van den Hove DL, Steinbusch HW, Scheepens A, Van de Berg WD, Kooiman LA, Boosten BJ, Prickaerts J, Blanco CE, 2006c. Prenatal stress and neonatal rat brain development. Neuroscience 137: 145-155.

van Eekelen JA, Bohn MC, de Kloet ER, 1991. Postnatal ontogeny of mineralocorticoid and glucocorticoid receptor gene expression in regions of the rat tel- and diencephalon. Brain Res Dev Brain Res 61: 33-43.

van Eekelen JA, Jiang W, de Kloet ER, Bohn MC, 1988. Distribution of the mineralocorticoid and the glucocorticoid receptor mRNAs in the rat hippocampus. J Neurosci Res 21: 88-94.

van Gemert NG, van RE, Meijer OC, Fehr S, Schachner M, Joels M, 2006. No effect of prolonged corticosterone over-exposure on NCAM, SGK1, and RGS4 mRNA expression in rat hippocampus. Brain Res 1093: 161-166.

van Rossum EFC, Binder EB, Majer M, Koper JW, Ising M, Modell S, Salyakina D, Lamberts SWJ, Holsboer F, 2006. Polymorphisms of the glucocorticoid receptor gene and major depression. Biol Psychiatry 59: 681-688.

van Rossum EFC, Lamberts SWJ, 2004. Polymorphisms in the glucocorticoid receptor gene and their associations with metabolic parameters and body composition. Recent Prog Horm Res 59: 333-357.

Viltart O, Mairesse J, Darnaudery M, Louvart H, Vanbesien-Mailliot C, Catalani A, Maccari S, 2006. Prenatal stress alters Fos protein expression in hippocampus and locus coeruleus stress-related brain structures. Psychoneuroendocrinology 31: 769-780.

Waddell BJ, Benediktsson R, Brown RW, Seckl JR, 1998. Tissue-specific messenger ribonucleic acid expression of 11beta-hydroxysteroid dehydrogenase types 1 and 2 and the glucocorticoid receptor within rat placenta suggests exquisite local control of glucocorticoid action. Endocrinology 139: 1517-1523.

Waddell BJ, Hisheh S, Dharmarajan AM, Burton PJ, 2000. Apoptosis in rat placenta is zone-dependent and stimulated by glucocorticoids. Biol Reprod 63: 1913-1917.

Wahlsten D, Metten P, Phillips TJ, Boehm SL, Burkhart-Kasch S, Dorow J, Doerksen S, Downing C, Fogarty J, Rodd-Henricks K, Hen R, McKinnon CS, Merrill CM, Nolte C, Schalomon M, Schlumbohm JP, Sibert JR, Wenger CD, Dudek BC, Crabbe JC, 2003. Different data from different labs: lessons from studies of gene-environment interaction. J Neurobiol 54: 283-311.

Wakshlak A, Weinstock M, 1990. Neonatal handling reverses behavioral abnormalities induced in rats by prenatal stress. Physiol Behav 48: 289-292.

Walker BR, Stewart PM, Shackleton CH, Padfield PL, Edwards CR, 1993. Deficient inactivation of cortisol by 11 beta-hydroxysteroid dehydrogenase in essential hypertension. Clin Endocrinol 39: 221-227.

Walker BR, 2004. 11beta-Hydroxysteroid Dehydrogenase Type 1 in Obesity. Obesity Res 12: 1-3.

Wan S, Hao R, Sun K, 2005. Repeated maternal dexamethasone treatments in late gestation increases 11beta-hydroxysteroid dehydrogenase type 1 expression in the hippocampus of the newborn rat. Neurosci Lett 382: 96-101.

Ward GR, Wainwright PE, 1988. Reductions in maternal food and water intake account for prenatal stress effects on neurobehavioral development in B6D2F2 mice. Physiol Behav 44: 781-786.

Ward HE, Johnson EA, Salm AK, Birkle DL, 2000. Effects of prenatal stress on defensive withdrawal behavior and corticotropin releasing factor systems in rat brain. Physiol Behav 70: 359-366.

Wei Q, Lu XY, Liu L, Schafer G, Shieh KR, Burke S, Robinson TE, Watson SJ, Seasholtz AF, Akil H, 2004. Glucocorticoid receptor overexpression in forebrain: a mouse model of increased emotional lability. Proc Natl Acad Sci USA 101: 11851-11856.

Weinstock M, 2001. Alterations induced by gestational stress in brain morphology and behaviour of the offspring. Prog Neurobiol 65: 427-451.

Weinstock M, Matlina E, Maor GI, Rosen H, McEwen BS, 1992. Prenatal stress selectively alters the reactivity of the hypothalamic-pituitary adrenal system in the female rat. Brain Res 595: 195-200.

Weinstock M, Poltyrev T, Schorer-Apelbaum D, Men D, McCarty R, 1998. Effect of prenatal stress on plasma corticosterone and catecholamines in response to footshock in rats. Physiol Behav 64: 439-444.

Welberg LA. Brain programming effects of glucocorticoids: implications for behaviour. 1999. PhD Thesis University of Edinburgh

Welberg LA, Seckl JR, Holmes MC, 2001. Prenatal glucocorticoid programming of brain corticosteroid receptors and corticotrophin-releasing hormone: possible implications for behaviour. Neuroscience 104: 71-79.

Welberg LA, Seckl JR, Holmes MC, 2000. Inhibition of 11beta-hydroxysteroid dehydrogenase, the foeto-placental barrier to maternal glucocorticoids, permanently programs amygdala GR mRNA expression and anxiety-like behaviour in the offspring. Eur J Neurosci 12: 1047-1054.

White DA, Birkle DL, 2001. The differential effects of prenatal stress in rats on the acoustic startle reflex under baseline conditions and in response to anxiogenic drugs. Psychopharmacology 154: 169-176.

White PC, Mune T, Agarwal AK, 1997. 11 beta-hydroxysteroid dehydrogenase and the syndrome of apparent mineralocorticoid excess. Endocr Rev 18: 135-156.

Whorwood CB, Firth KM, Budge H, Symonds ME, 2001. Maternal undernutrition during early to midgestation programs tissue-specific alterations in the expression of the glucocorticoid receptor, 11beta-hydroxysteroid dehydrogenase isoforms, and type 1 angiotensin ii receptor in neonatal sheep. Endocrinology 142: 2854-2864.

Whorwood CB, Franklyn JA, Sheppard MC, Stewart PM, 1992. Tissue localization of 11 beta-hydroxysteroid dehydrogenase and its relationship to the glucocorticoid receptor. J Steroid Biochem Mol Biol 41: 21-28.

Whorwood CB, Mason JI, Ricketts ML, Howie AJ, Stewart PM, 1995. Detection of human 11 beta-hydroxysteroid dehydrogenase isoforms using reverse-transcriptase-polymerase chain reaction and localization of the type 2 isoform to renal collecting ducts. Mol Cell Endocrinol 110: R7-12.

Whorwood CB, Ricketts ML, Stewart PM, 1994. Epithelial cell localization of type 2 11 beta-hydroxysteroid dehydrogenase in rat and human colon. Endocrinology 135: 2533-2541.

Wintermantel TM, Berger S, Greiner EF, Schutz G, 2005. Evaluation of steroid receptor function by gene targeting in mice. J Steroid Biochem Mol Biol 93: 107-112.

Woodall SM, Bassett NS, Gluckman PD, Breier BH, 1998. Consequences of maternal undernutrition for fetal and postnatal hepatic insulin-like growth factor-I, growth hormone receptor and growth hormone binding protein gene regulation in the rat. J Mol Endocrinol 20: 313-326.

Woodall SM, Breier BH, Johnston BM, Gluckman PD, 1996a. A model of intrauterine growth retardation caused by chronic maternal undernutrition in the rat: effects on the somatotrophic axis and postnatal growth. J Endocrinol 150: 231-242.

Woodall SM, Johnston BM, Breier BH, Gluckman PD, 1996b. Chronic maternal undernutrition in the rat leads to delayed postnatal growth and elevated blood pressure of offspring. Pediatr Res 40: 438-443.

Woods LL, Weeks DA, 2005. Prenatal programming of adult blood pressure: role of maternal corticosteroids. Am J Physiol Regul Integr Comp Physiol 289: R955-R962.

Wyrwoll CS, Mark PJ, Mori TA, Puddey IB, Waddell BJ, 2006. Prevention of Programmed Hyperleptinemia and Hypertension by Postnatal Dietary omega-3 Fatty Acids. Endocrinology 147: 599-606.

Yamada K, Mizuno M, Nabeshima T, 2002. Role for brain-derived neurotrophic factor in learning and memory. Life Sci 70: 735-744.

Yang J, Hou C, Ma N, Liu J, Zhang Y, Zhou J, Xu L, Li L, 2007. Enriched environment treatment restores impaired hippocampal synaptic plasticity and cognitive deficits induced by prenatal chronic stress. Neurobiol Learn Mem 87: 257-63.

Yau JL, Noble J, Kenyon CJ, Hibberd C, Kotelevtsev Y, Mullins JJ, Seckl JR, 2001. Lack of tissue glucocorticoid reactivation in 11beta -hydroxysteroid dehydrogenase type 1 knockout mice ameliorates age-related learning impairments. Proc Natl Acad Sci USA 98: 4716-4721.

Yee BK, Zhu SW, Mohammed AH, Feldon J, 2006. Levels of neurotrophic factors in the hippocampus and amygdala correlate with anxiety- and fear-related behaviour in C57BL6 mice. J Neural Transm Epub

Yu IT, Lee SH, Lee YS, Son H, 2004. Differential effects of corticosterone and dexamethasone on hippocampal neurogenesis in vitro. Biochem Biophys Res Commun 317: 484-490.

Zagron G, Weinstock M, 2006. Maternal adrenal hormone secretion mediates behavioural alterations induced by prenatal stress in male and female rats. Behav Brain Res 175: 323-328.

Zaharia MD, Kulczycki J, Shanks N, Meaney MJ, Anisman H, 1996. The effects of early postnatal stimulation on Morris water-maze acquisition in adult mice: genetic and maternal factors. Psychopharmacology 128: 227-239.

Zhou MY, Gomez-Sanchez EP, Cox DL, Cosby D, Gomez-Sanchez CE, 1995. Cloning, expression, and tissue distribution of the rat nicotinamide adenine dinucleotide-dependent 11 beta-hydroxysteroid dehydrogenase. Endocrinology 136: 3729-3734.

Zimmerberg B, Blaskey LG, 1998. Prenatal stress effects are partially ameliorated by prenatal administration of the neurosteroid allopregnanolone. Pharmacol Biochem Behav 59: 819-827.