Brain programming effects of glucocorticoids: implications for behaviour

Leonie Anna Maria Welberg

Doctor of Philosophy

University of Edinburgh

1999

And I gave my heart to know wisdom and to know madness and folly: I perceived that this also is vexation of spirit. For in much wisdom is much grief: and he that increaseth knowledge increaseth sorrow. (Eccl. 1:17-18)

En ik begaf mijn hart om wijsheid en wetenschap te weten, onzinnigheden en dwaasheid; ik ben gewaar geworden, dat ook dit een kwelling des geestes is. Want in veel wijsheid is veel verdriet; en die kennis vermeerdert, vermeerdert smart. (Pred. 1:17-18)

In memory of my father

Ter herinnering aan mijn vader

Declaration

I declare that this thesis has been written entirely by me 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 and is not currently submitted for any other degree.

Leonie Welberg

Acknowledgements

I am indebted to a great number of people without whom this work would not have seen the light of day. First of all I wish to thank my supervisors, Prof Jonathan Seckl and Dr Megan Holmes, for their continuing support, helpful suggestions and their capacity to show me the significance of and in my data. A special thanks to Jonathan for taking me to Elba – the best week of my PhD! I owe a lot to Prof Ron de Kloet and everybody in his lab in Leiden, for introducing me to the 'stress'-field. Their interest, advice and gossip over the past four years have been greatly appreciated. I would also like to thank the Wellcome Trust for their financial support.

Thanks to everyone in the Molecular Endocrinology lab for the great atmosphere they provide and for their helpfulness at all times. A special thanks to June Noble and Karen French for giving me the templates and cDNAs they subcloned and for their expert knowledge on in situ hybridisation. Keith Chalmers and Andrew Wilson deserve a special mention for their assistance with the animal experiments.

Thanks to my office mates Duncan, Amanda, Heather, and more recently Malcolm, Rebecca and David Mayo (bay 9) for putting up with my moods and providing the necessary distractions and cigarettes, and to Rhona, Maggie, Sue, Rebecca and Pauline for being such good friends in good times and in bad times.

Very important have been my friends and flatmates in Edinburgh who made the transition from Holland to Scotland very smooth indeed. And ofcourse, I owe thanks to my family and friends in Holland (especially my pseudo-paranymphs, Drs Lucres and Judith) for their many visits and for always making me feel like I haven't been away.

Finally, I would like to commemorate Dr Mags Carey, who died much too young. She stood at the basis of this thesis not only because she convinced me to take this step in the first place, but most of all because she inspired me with her dedication and her laugh.

Abstract

Increasing evidence suggests that an adverse *in utero* environment can result in disease later in life. Diseases such as hypertension and type 2 diabetes have recently, in epidemiological studies, been linked to low birth weight. This link has also been found in animal studies, and has led to the suggestion that factors acting very early in life may 'program' the setpoint of certain physiological systems. The brain is particularly sensitive to early environmental manipulations. Prenatal stress, maternal deprivation, and neonatal handling have long-term effects on brain functioning such as regulation of the hypothalamic-pituitary-adrenal (HPA) axis and stress-induced behaviour. The factor(s) underlying the link between low birth weight and later disease is unknown, but glucocorticoids have been implicated. Whereas low glucocorticoid levels are important for normal development, exposure to excessive levels can have deleterious effects. To maintain this critical balance 11β–hydroxysteroid dehydrogenase (11β-HSD) type 2 normally rapidly inactivates glucocorticoids in placenta and many fetal tissues, thus acting as a 'barrier' to maternal steroids.

We investigated the effect of bypassing (using dexamethasone, DEX) or inhibiting (using carbenoxolone, CBX) feto-placental 11β-HSD in rats, on subsequent offspring HPA axis regulation and stress-induced behaviour. Pregnant Wistar rats were injected with CBX, DEX or vehicle daily throughout pregnancy. A separate group received DEX injections in the last week of pregnancy only (DEX3), as this had previously been shown to contain the critical time window for programming of hypertension and hyperglycaemia. All treatments reduced birth weight (CBX 12%, DEX1-3 15%, DEX3 7%). This was reversed by 4 and 6 weeks of life in DEX3 and CBX offspring, respectively, while offspring of dams treated with DEX throughout pregnancy had reduced body weight throughout life (7% at 5 months of age). Prenatal exposure to glucocorticoids resulted in increased basal corticosterone (CORT) levels (CBX 0.70 μg/dl, DEX1-3 0.76 μg/dl, control 0.40 μg/dl) and a trend ($p=0.07$) towards increased corticotropin-releasing hormone (CRH) in the

hypothalamic paraventricular nucleus (PVN) in adult offspring. In addition, CBX offspring had reduced PVN glucocorticoid receptor (GR) mRNA and a prolonged stress response. Hippocampal GR and mineralocorticoid receptor (MR) mRNA expression was reduced only in DEX3 offspring, which also showed increased CRH mRNA levels in PVN. CBX and DEX3 rats displayed impaired non-spatial learning. In addition, exploratory behaviour in an open field was affected by prenatal glucocorticoid exposure, especially in DEX3 offspring, which showed reduced locomotion and rearing. The behavioural alterations are associated with increased expression of MR, GR and CRH mRNAs in the amygdala, a structure implicated in the expression of fear and anxiety. These data suggest that disturbance of the feto-placental enzymatic barrier to maternal glucocorticoids, or *in utero* exposure to DEX, reduces birth weight and produces permanent subtle alterations of the HPA axis combined with behavioural inhibition or impaired coping in aversive situations. Furthermore, the similar but not identical effects resulting from CBX, DEX1-3 and DEX3 administration suggest the existence of critical time windows for glucocorticoid programming, different programming mechanisms for CBX and DEX, or exposure to different concentrations of glucocorticoid *in utero*. The behavioural and HPA effects may reflect corticosteroid receptor and CRH gene programming in amygdala and hypothalamus, respectively. In conclusion, fetal overexposure to endogenous (reduced activity of feto-placental 11β-HSD) or exogenous (prenatal DEX therapy) glucocorticoids may represent a common link between the prenatal environment, fetal growth and adult neuroendocrine and affective disorders.

Contents

List of figures

List of tables

List of abbreviations

11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
11β-HSD2	11β-hydroxysteroid dehydrogenase type 2
ACh	acetyl choline
ACTH	adrenocorticotrophic hormone
ADX	adrenalectomy
ALDO	aldosterone
AME	apparent mineralocorticoid excess
ANOVA	analysis of variance
AVP	arginine vasopressin
BLA	basolateral nucleus of the amygdala
BNST	bed nucleus of the stria terminalis
CBX	carbenoxolone (3β-hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate)
cDNA	complementary deoxyribonucleic acid
CEA	central nucleus of the amygdala
CNS	central nervous system
CORT	corticosterone
CRH	corticotrophin releasing hormone
DA	dopamine
DEPC	diethyl pyrocarbonate
DEX	dexamethasone (9α-fluoro-16α-methylprednisolone)
dl	decilitre
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra acetic acid
g	gram
GD	gestational day
GR	glucocorticoid receptor
HPA	hypothalamus-pituitary-adrenal
5-HT	5-hydroxy tryptamine (serotonin)

I.c.v.	intracerebroventricular
kg	kilogram
l	litre
M	molar
MEA	medial nucleus of the amygdala
µg	microgram
ml	millilitre
mg	milligram
ME	median eminence
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NA	noradrenaline
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
nt	nucleotides
pnd	postnatal day
PCA	principal component analysis
POMC	proopiomelanocortin
PVN	paraventricular nucleus
RNA	ribonucleic acid
SHRP	stress-hyporesponsive period
SPA	scintillation proximity assay
SSC	saline sodium citrate
ST	stria terminalis
TRIS	tris (hydroxymethyl) methyl amine

Preface

Increasing evidence suggests that an adverse *in utero* environment can result in disease later in life. Diseases such as hypertension and type 2 diabetes, generally assumed to result from a combination of genetic predisposition and life style factors in adult life (smoking, lack of exercise, diet), have recently, in epidemiological studies, been linked to low birth weight. This link has also been found in animal studies, and has led to the suggestion that factors acting very early in life may 'program' the setpoint of certain physiological systems, in order to prepare the organism for life under sub-optimal conditions.

A system that has long been shown to be programmable is the central nervous system (CNS). Early environmental manipulations (e.g. prenatal stress, maternal deprivation, neonatal handling) alter brain development and have long-term effects on brain functioning. For example, prenatal stress induces permanent changes in offspring sexual behaviour (Holson et al., 1995), hormonal and behavioural responses to stress (Fride et al., 1986; Henry et al., 1994; Takahashi et al., 1988; Vallee et al., 1997; Weinstock et al., 1992) and brain monoamine systems (Day et al., 1998; Peters, 1984; Peters, 1990; Takahashi et al., 1992a). Disturbances in hypothalamic-pituitary-adrenal (HPA) regulation and brain monoamine levels are associated with affective and anxiety disorders in humans (Holsboer and Barden, 1996; Lopez et al., 1998). Thus, the majority of depressed patients display increased levels of corticotrophin releasing hormone (CRH) in CSF, elevated plasma levels of adrenocorticotrophic hormone (ACTH) and cortisol and an attenuated diurnal variation in HPA activity. This suggests that such conditions may be, in part, prenatally programmed. Indeed, several behavioural abnormalities have been reported in children exposed to 'prenatal stress' (Weinstock, 1997). Interestingly, prenatal stress also results in low birth weight offspring in rats (Kinley and Svare, 1986; Takahashi et al., 1988) and humans (Rothberg and Lits, 1991; Sandman et al., 1997; Wadhwa et al., 1993).

In the search for a mechanism underlying prenatal programming, fetal exposure to glucocorticoids has been implicated. Glucocorticoids are necessary for normal development [for review see (Meyer, 1985)]. They affect mitosis, myelination and differentiation in the CNS and have many other key regulatory functions during

development. However, excess exposure to glucocorticoids can have deleterious effects, inhibiting fetal body and brain as well as placental growth. For example, exposure to high levels of glucocorticoids inhibits neurogenesis, gliogenesis and dendrite and myelin formation in the CNS (Bohn et al., 1994; Cotterrell, 1972; Meyer, 1985). Thus, the inhibiting effects of high doses and the permissive effects of physiological low doses of glucocorticoids indicate the importance of keeping glucocorticoid concentrations within limits for normal development. It has recently become apparent that even moderate-dose glucocorticoid administration to pregnant rats has long-lasting effects and results in low birth weight offspring that develop hypertension and hyperglycaemia in adulthood (Lindsay et al., 1996a, 1996b; Nyirenda et al., 1998). The long-term effects of low levels of glucocorticoids administered prenatally on brain functioning have received little attention. It is important that these effects are established, since glucocorticoids are commonly administered in pregnancies where the fetus is potentially affected with congenital adrenal hyperplasia. Moreover, they are used in cases of threatened prematurity to prevent neonatal respiratory distress syndrome by promoting lung maturation.

Because glucocorticoids are lipophilic, they are assumed to readily pass the placenta from mother to fetus (Zarrow et al., 1972). However, a high-affinity glucocorticoid-inactivating enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) is present in both placenta and fetus (Brown et al., 1996; Stewart et al., 1994) and may function as a barrier protecting the fetus from high levels of maternal glucocorticoids. Synthetic glucocorticoids such as dexamethasone (DEX) are poor substrates for this enzyme. Inhibiting this barrier has been shown to program low birth weight and hypertension and hyperglycaemia later in life (Lindsay et al., 1996a; Lindsay et al., 1996b), similar to the effects of low-dose prenatal synthetic glucocorticoid administration.

This thesis aims to increase understanding of the effects of prenatal glucocorticoid exposure on HPA regulation and stress-induced behaviour. Furthermore, the role of feto-placental 11β-HSD was investigated in protecting the fetus from the effects of maternal glucocorticoids on these parameters. The findings may have important implications for prenatal glucocorticoid treatment in humans.

Chapter 1. Introduction

A. Glucocorticoids, their regulation and actions

A.1. Glucocorticoids

An organism responds to change in environmental or physiological circumstances in both a reactive and an anticipatory mode. Whereas a reactive response is set out in order to restore homeostasis, an anticipatory response is the result of a process called allostasis. Allostasis has been defined as 'the regulation of many variables over time in maintaining stability to meet changing circumstances' (Schulkin et al., 1994) or in short 'maintaining stability through change' (De Kloet, 1991). The physiological endpoint of both types of response is known as the 'stress response'. This reaction involves activation of the sympathetic nervous system as well as the release of glucocorticoid hormones.

Glucocorticoids have a wide range of actions in the body, and several theories have been put forward to explain the role of glucocorticoids in the stress response [for review see de Kloet, 1991]. Thus, it has been proposed that their main goal is to mobilise energy and suppress systems that are not essential in an emergency situation. Alternatively, they may have a permissive role in the actions of neurotransmitters released in reponse to stress. A third hypothesis postulates that glucocorticoids prevent damage to the organism caused by primary stress reactions by inhibiting these reactions (Munck et al., 1984). In metaphorical terms, they 'prevent water damage caused by the fire brigade'.

Among the effects of glucocorticoids are suppression of growth, bone production, immune- and reproductive functions, and increased glucose levels, blood pressure and cardiovascular tone. Obviously, these effects can all be considered adaptive in an acutely stressful situation. However, as all the above mentioned actions are potentially damaging, chronic elevation of glucocorticoid levels can have deleterious results. It is important that glucocorticoid levels are tightly regulated, so that they return to basal levels once the stressor has disappeared. Therefore, glucocorticoids are subject to homeostatic, negative-feedback control acting at several levels of the

system that mediates glucocorticoid release: the hypothalamic-pituitary-adrenal (HPA)-axis.

A.2. The hypothalamus-pituitary-adrenal axis

The HPA-axis regulates the release of glucocorticoids. The hypothalamus receives direct or indirect input from virtually all areas of the brain (Herman and Cullinan, 1997a) and thereby acts as the regulatory centre of the stress response. Thus, a variety of central inputs can trigger the hypothalamus to release CRH, arginine vasopressin (AVP) and several other peptides from its parvocellular paraventricular (PVN) neurones (Figure 1.1). Together these peptides are released into the hypophyseal portal circulation of the median eminence and trigger anterior pituitary corticotrophs to stimulate the release of proopiomelanocortin (POMC), the precursor molecule for ACTH and several other peptides. The effect of CRH is potentiated by AVP, which is colocalised with CRH in PVN neurones. Once released into the circulation, ACTH triggers the adrenal cortex to synthesise and secrete glucocorticoids (O'Riordan et al., 1988). In humans, the main glucocorticoid is cortisol, whereas in rats and mice it is corticosterone (CORT).

In addition to stress-induced release, CORT levels follow a diurnal variation, with peak levels occuring toward the start of the active period. This endogenous circadian rhythm is generated by the suprachiasmatic nucleus of the hypothalamus, which has projections to the hypothalamus, but variations in pituitary and adrenal responsiveness and splanchnic nerve activity also play a role (De Kloet, 1991; Dijkstra et al., 1996).

Glucocorticoids, via binding to their specific receptors, regulate their own secretion by negative feedback actions at the level of the hypothalamus and the pituitary, inhibiting the synthesis and release of CRH, AVP, POMC and ACTH, thus terminating the stress response (Figure 1.1). This is referred to as 'slow feedback' and its effects may last several days (de Kloet, 1991). There are also two non-genomic mechanisms of feedback. Fast feedback is thought to take place at the level of the cell membrane, while intermediate feedback may be underlain by steroid effects on neuronal excitability and intracellular signal transduction pathways (de Kloet, 1991).

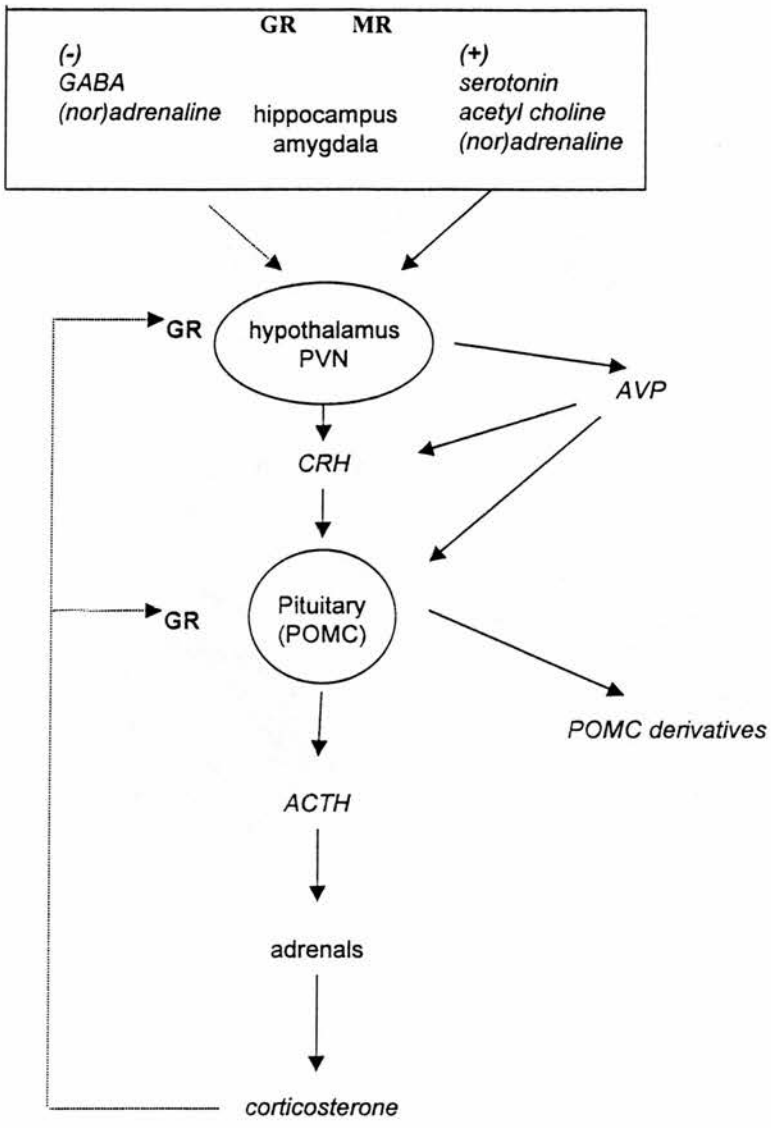

Figure 1.1 **Schematic representation of the HPA axis.**

→ stimulating effect; ---> inhibiting effect

A.3. Corticosteroid receptors

Corticosteroids exert most of their actions via binding to their intracellular receptors (De Kloet et al., 1998). Molecular and biochemical studies have revealed two receptor subtypes: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) (Reul and de Kloet, 1985). GRs are found in practically every tissue in

the body, whereas the distribution of MRs is restricted to specific mineralocorticoid target tissues such as kidney, skin and intestines. Within the brain, a widespread distribution of GR in neurones and glial cells is observed but MRs are found in relatively few areas, mainly in the limbic system. Thus, autoradiography, immunocytochemistry and *in situ* hybridisation studies have revealed that GRs are concentrated in hippocampus, septum, amygdala, PVN, supraoptic nucleus and arcuate nucleus of the hypothalamus, cerebral cortex, cerebellum and ascending monoaminergic neurones of the brainstem. High MR densities have been shown in neurones in hippocampus, septum, amygdala, olfactory nucleus, and brainstem sensory and motor neurones (De Kloet, 1991).

The physiological ligands for MR are aldosterone (ALDO) and CORT, for which MR has equal affinities ($K_d \approx 0.5$ nM). GR binds its natural ligand CORT with a ten times lesser affinity ($K_d \approx 5.0$ nM) (De Kloet et al., 1998), while it has a slightly higher affinity for its synthetic ligand dexamethasone (DEX, $K_d \approx 1.5$ nM) (Figure 1.2).

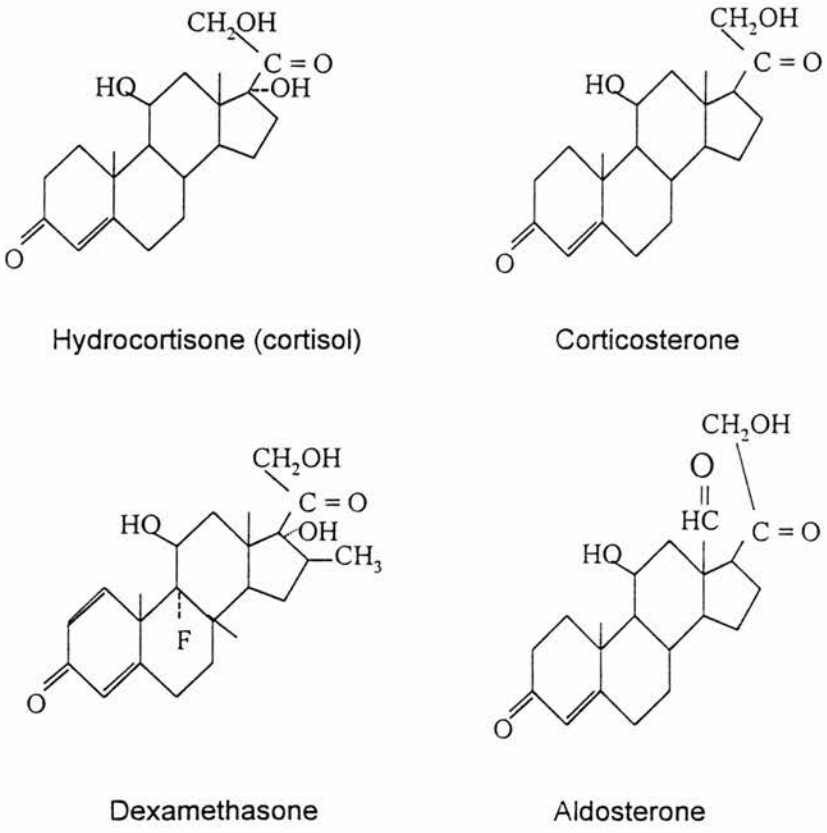

Figure 1.2 **Structures of MR and GR ligands.**

Corticosteroid receptors belong to a family of nuclear steroid hormone receptors, acting as transcription factors, which are characterised by three regions: a DNA-binding domain, a C-terminal ligand-binding domain and an N-terminal domain involved in transcriptional regulation or modulation (Evans and Arriza, 1989). The ligand-binding domain is associated with heatshock proteins. Upon binding of the receptor to its ligand, the hormone-receptor complex dissociates from the heatshock protein complex, the receptor becomes phosphorylated and translocates to the nucleus. There it forms homo- or heterodimers (Trapp et al., 1994) and binds to glucocorticoid response elements (GREs) on the DNA of target genes in order to regulate their transcription (Evans and Arriza, 1989). In addition to transcriptional regulation via steroid receptor-DNA interaction, activated steroid receptors may influence transcription by interaction with other transcription factors such as AP-1, NF-κB or CREBP (Pfahl, 1993).

Access of CORT to its receptors is regulated on three levels: 1) CBG and albumin in plasma bind > 95% of circulating CORT, rendering it biologically unavailable (De Kloet et al., 1998); 2) in- and reactivation of CORT by the enzyme 11β-HSD, types 1 and 2 (see section C of this chapter); 3) metabolism of CORT by 5α- and 5β-reductase ($t_{1/2}$ ~ 6 h).

Brain corticosteroid receptors are subject to autoregulation in a cell- and region-specific fashion (Herman, 1993): adrenalectomy (ADX) results in increased MR and GR mRNA (Herman, 1993; Herman and Spencer, 1998; Pfeiffer et al., 1991) and MR and GR binding (Reul et al., 1987) in the hippocampus, but not PVN (Peiffer et al., 1991). In contrast, high doses of dexamethasone or stress decrease hippocampal GR numbers (Herman, 1993; McEwen et al., 1986; Sapolsky et al., 1984), while high glucocorticoid levels decrease MR binding in some studies (Herman et al., 1995; Herman and Watson, 1995), but increase them (De Kloet, 1991) or leave them unaffected (Makino et al., 1995a; Reul et al., 1987) in others. The mechanisms underlying the specificity of corticosteroid receptor expression and regulation may involve transcriptional control via specific promoters as well as posttranscriptional and -translational control.

There is also evidence for neural control of brain corticosteroid receptors. Thus, alterations in noradrenergic (Kabbaj et al., 1995; Kabbaj et al., 1996; Maccari et al., 1992a; Maccari et al., 1992b; Yau and Seckl, 1992), serotonergic (Seckl et al., 1990; Weidenfeld and Feldman, 1991) or cholinergic (Yau et al., 1992) neurotransmission induce changes in brain MR and GR expression.

In addition to their genomic actions, steroids exert rapid non-genomic effects on neurotransmission and behaviour (Schumacher, 1990), perhaps via binding to membrane receptors (Orchinik et al., 1991). Proposed mechanisms include binding of steroid metabolites to neurotransmitter receptors on the neuronal membrane such as the $GABA_A$ receptor (Paul and Purdy, 1992; Puia et al., 1990).

A.4.1. Central corticosteroid action: hippocampus

A.4.1.1.Neuroendocrine regulation

There is considerable evidence that the hippocampus plays a role in regulating both stress-induced and circadian HPA-activity (Jacobson and Sapolsky, 1991), stemming from lesion studies, electrical stimulation of the hippocampus and administration of receptor agonists and antagonists.

Because of the abundant levels of MR and GR in this structure, hippocampal modulation of HPA-activity is likely to be influenced by occupation of these receptors. The different affinities of MR and GR for CORT imply that under basal resting conditions, when CORT levels are low, mainly MRs are occupied in the hippocampus, whereas at high CORT levels, such as during stress or at the circadian peak, MRs and GRs are simultaneously activated (Reul et al., 1987). In fact, the hippocampus is itself thought to play a role in the regulation of circadian HPA-activity, mainly through MR occupation (Bradbury et al., 1994; Levin et al., 1987).

Electrophysiological studies have shown that in the hippocampal CA1 area MR and GR activation interact and antagonise each other in the control of neuronal excitability (De Kloet et al., 1998; Joels and de Kloet, 1992). Similarly, MR and GR appear to have opposing effects on hippocampal cholinergic theta-activity (Murphy et al., 1998). The same might be true in complex spatial memory tasks associated with the hippocampus such as the Morris watermaze task (Oitzl et al., 1998). It has

recently been suggested that, similar to their effects on cell activity and behaviour, hippocampal MR and GR *co-ordinately* mediate hippocampal regulation of HPA-activity (De Kloet, 1991; De Kloet et al., 1998). Thus, activation of hippocampal MR mediates a tonic inhibitory control over basal HPA-activity. At higher CORT levels, hippocampal GRs are also activated and override the MR-mediated action, thereby *disinhibiting* HPA-activity. GR-mediated negative feedback on stress-induced HPA-activity would primarily take place at the levels of the PVN (Feldman et al., 1992; Feldman and Weidenfeld, 1995), and pituitary (De Kloet et al., 1988; Young et al., 1995), although hippocampal MRs may also play a role in glucocorticoid negative feedback (Dallman et al., 1992; Ratka et al., 1989; Spencer et al., 1998). Indeed, van Haarst *et al.* showed that intrahippocampal administration of the GR antagonist RU38486 *de*creased basal ACTH levels in the afternoon, whereas RU38486 given i.c.v. *in*creased basal HPA-activity in the afternoon and in response to stress (van Haarst et al., 1996; van Haarst et al., 1997), suggesting that the effect of the GR antagonist in the PVN overrides the effect of hippocampal GR blockade. In addition, Feldman *et al.* showed that intrahippocampal high concentration CORT implants failed to decrease the HPA-response following neural stimuli (Feldman et al., 1992). Taken together, it appears as though hippocampal GRs are not involved in glucocorticoid negative feedback of the HPA axis, and may even underlie stress-induced facilitation of HPA-activity.

However, early life manipulations that alter hippocampal, but not PVN or pituitary, GR levels affect glucocorticoid *negative* feedback (Henry et al., 1994; Meaney et al., 1989). Similarly, other correlative evidence as well as lesion studies suggest that hippocampal GRs mediate stress-induced corticosteroid negative feedback (Feldman and Weidenfeld, 1995; Herman et al., 1989; Jacobson and Sapolsky, 1991; Makino et al., 1995a; Sapolsky et al., 1984; Sapolsky et al., 1986). In these studies, elevated or decreased plasma CORT levels are consistently associated with respectively decreased or increased hippocampal GR levels, but whether altered CORT levels are cause or consequence of altered hippocampal GR levels is unclear. In a more direct study, Feldman *et al* showed that intrahippocampal high concentration CORT implants failed to decrease the HPA-response following neural stimuli (Feldman et al., 1992).

Thus, the role of hippocampal GR in mediating HPA-activity remains somewhat controversial. In any case, the hippocampus is certainly not the only site regulating HPA-activity. It has been suggested that although the hippocampus, via its GRs, may be involved in the *behavioural* response to stress, its role in corticosteroid feedback may only be modulatory (De Kloet et al., 1998).

A.4.1.2. Behaviour

The hippocampus has been shown to be a critical site in the regulation of learning and memory and in the behavioural response to novelty. The 'Porsolt' or 'forced swim' test (for details see Materials & Methods section) is an example of tests that involve activation of hippocampal MR and/or GR. The increased immobility rats acquire upon exposure to forced swimming has been interpreted as 'learned helplessness', but also as a successful means of passive 'behavioural adaptation' aimed to conserve energy in an inescapable situation. I.c.v. or intrahippocampal treatment with antiglucocorticoids or with antisense directed against GR mRNA as well as ADX reduces the time spent immobile (De Kloet et al., 1988; Korte et al., 1996; Mitchell and Meaney, 1991), and reduced immobility in ADX rats is restored by CORT-injections, suggesting a role for hippocampal corticosteroid receptors in this form of acquired behaviour.

The watermaze (for details see Materials & Methods section) is a spatial ability test that relies on hippocampal integrity (Morris et al., 1982) and activation of hippocampal MR and GR, although the exact roles of MR and GR in spatial memory are still a matter of debate. The implications of hippocampal corticosteroid receptor number for watermaze performance depend on the strain and the age of the rats used (Yau et al., 1994; Yau et al., 1995), but in general reduced corticosteroid receptor levels correlate with impaired spatial memory [e.g. (Issa et al., 1990)]. Accordingly, blockade of central GRs impairs spatial memory (Conrad et al., 1997; Oitzl and de Kloet, 1992).

However, not only receptor number but also levels of available ligand may be crucial in (especially) GR-mediated effects on watermaze performance. Thus, at low levels of plasma CORT, a lower percentage of GRs will be activated than at high plasma

CORT concentrations (Reul and Dekloet, 1985). Very low (ADX) as well as very high GR occupation impairs spatial memory measured in a Y-maze (Conrad et al., 1997), suggesting an inverted U-shape relationship between hippocampal GR occupation and spatial memory (Conrad et al., 1997). Accordingly, in a watermaze task acute posttraining intrahippocampal administration of antiglucocorticoids, imposing a reduction in GR occupation possibly to a level corresponding with the 'top' of the inverted U-shape, improves spatial memory (Oitzl et al., 1998), although these data were originally interpreted as GR occupation interfering with memory consolidation. Interestingly, a similar concentration-dependent biphasic influence of CORT levels has been found for the mechanisms thought to underlie learning and memory, namely hippocampal long-term potentiation (LTP) and primed-burst potentiation (PBP) (Diamond et al., 1992).

In contrast to hippocampal GRs, hippocampal MRs appear to play a less specific role in memory formation. Central injection of MR antagonists in Wistar rats only altered search strategy and not spatial memory (Oitzl and de Kloet, 1992), suggesting distinctly different functions for hippocampal MR and GR in this task, possibly through nonspecific effects such as increased arousal (Conrad et al., 1997; Oitzl and de Kloet, 1992; Sandi, 1998). Indeed, the function of hippocampal MR has been studied in a number of other behavioural tasks, such as an open field (De Kloet et al., 1993; Oitzl et al., 1994), forced swim test (Mitchell and Meaney, 1991), black-white box (Smythe et al., 1997), and elevated plusmaze (Bitran et al., 1998; Korte et al., 1996). They appear to facilitate memory formation by mediating adaptive behavioural responses to stressful challenges, through evaluation of the situation and selection of an appropriate strategy, a process called 'sensory integration' (Lupien and McEwen, 1997).

A.4.2. Central corticosteroid action: amygdala

A.4.2.1. Neuroendocrine regulation

Electrical stimulation and lesion studies have led to the hypothesis that the amygdala may have a facilitatory influence on HPA-activity. Stimulation of the central

amygdaloid nucleus (CEA) depletes the ME of CRH and increases plasma CORT and ACTH concentrations. Recent data suggest that hypothalamic 5-HT and NA mediate these effects via 5-HT_2 and α_1 receptors, respectively (Feldman and Weidenfeld, 1998) and that they are sensitive to inhibition by i.p. injections of dexamethasone (Weidenfeld et al., 1997). Furthermore, lesions of the CEA before a single learning trial of fear of inescapable footshock, abolish the CORT response to stress (Roozendaal et al., 1992). These amygdaloid influences on HPA-activity may be mediated by projections from the CEA to the PVN, both direct and indirect via the BNST and POA [for review see (Davis, 1992)]. It has been suggested that the inhibitory GABA-ergic tone in the PVN is inhibited by GABA-ergic neurones from the CEA, thus having a net stimulatory result on HPA-activity (de Kloet et al., 1998; Herman and Cullinan, 1997). Recently, evidence has emerged for a *reciprocal* influence between CEA and PVN, as direct innervations from PVN neurones were traced to CRH-producing CEA cells through the ST and through the lateral hypothalamus (Palkovits et al., 1998).

A.4.2.2. Fear & anxiety

The amygdala also plays a major role in the behavioural expression of fear and anxiety. Electrical stimulation of the amygdala results in fear-associated responses [see (Applegate et al., 1982; Davis et al., 1994)], whereas amygdaloid lesions attenuate fear-related behavioural responses (Kopchia et al., 1992). Furthermore, i.c.v. or intra-amygdaloid injections of CRH generally have anxiogenic effects (Dunne and Berridge, 1990), suggesting that CRH may be a mediator of anxiety. Since the amygdaloid complex contains both CRH and CRH receptors (Owens and Nemeroff, 1991; Potter et al., 1994), it may be the site of action for CRH-induced behavioural responses [for review see (Gray and Bingaman, 1996)]. CRH mRNA expression in CEA is facilitated by corticosteroids: chronic or acutely elevated CORT levels or chronic stress increased CRH mRNA in CEA (Albeck et al., 1997; Hatalski et al., 1998; Hsu et al., 1998; Makino et al., 1994a), whereas ADX caused a decrease of CRH mRNA in CEA (Makino et al., 1994b; Palkovits et al., 1998) (although CRH *binding* in whole amygdala did not alter after 4 days of CORT treatment (Hauger et al., 1987)). CRH *receptor* mRNA expression in the amygdala is

not affected by alterations in CORT levels (Makino et al., 1995b). Thus, CORT appears to increase the CRH signal in the CEA, thereby augmenting amygdala-modulated fear.

The presence of corticosteroid receptors in CRH-positive neurones in the CEA (Honkaniemi et al., 1992) suggests that CORT may directly regulate the level of CRH gene transcription through MR and/or GR in the amygdala. Additionally, there may be an indirect effect of CORT: the recent discovery of direct innervations from PVN to CEA (Palkovits et al., 1998), in combination with the opposite effect of plasma CORT manipulations on CRH mRNA expression observed in the PVN and CEA, respectively (Makino et al., 1994b; Palkovits et al., 1998), have led to speculations that this PVN input into the CEA has an inhibitory influence upon CEA CRH expression.

The CEA also receives input from the BLA. Whereas the BLA is involved in conditioned fear behaviour (Goldstein et al., 1996), this is probably not the case in innate fear, which in fact appears to be regulated by an activated CEA (Moller et al., 1997).

A.4.2.3. 'Emotional memory'

Increasing evidence suggests a role for the amygdala in the formation of 'emotional memory'. PET-scans of human subjects watching 'emotionally arousing' films reveal heightened activity in the amygdala. Moreover, three weeks later, a higher number of 'emotionally arousing' films was recollected than of neutral films, and these numbers correlated positively with PET-scan activity in the amygdala during the viewings (Cahill et al., 1996). A possible mechanism underlying this correlation is that glucocorticoids, secreted in response to a stressful, 'emotionally loaded' event, activate the amygdala, resulting in modulation of memory formation. Indeed, numerous animal studies by Roozendaal *et al* have shown that GR agonists administered systemically (Roozendaal and McGaugh, 1996), intrahippocampally (Roozendaal and McGaugh, 1997a) or into the BLA (Roozendaal and McGaugh, 1997b) immediately posttraining in an inhibitory-avoidance task enhance memory, and that this effect requires an intact BLA (Roozendaal and McGaugh, 1996). Similarly, BLA-lesions block learning deficits in a water maze task resulting from

pretraining i.c.v. administration of antiglucocorticoids or as a consequence of ADX (Roozendaal et al., 1996). BLA-lesions alone however, do not impair memory retention (Roozendaal and McGaugh, 1996), suggesting that the BLA modulates memory consolidation in other brain regions, such as the hippocampus (Quirarte et al., 1997). Indeed, although BLA-lesions block the memory-impairing effects of ADX in a spatial watermaze task, they do not affect ADX-induced dentate gyrus damage in the same animals (Roozendaal et al., 1998).

Summarising, increasing evidence suggests that the amygdala, via the CEA, modulates neuroendocrine as well as behavioural responses to stress, and, through the BLA, also plays a role in glucocorticoid-induced memory modulation. Taken together, it can be postulated that the amygdala is a critical site for integrating and interpreting incoming sensory signals, thus playing a role in the *perception* of fear and in modulation of memory storage of aversive events. This continuous evaluation of incoming information by the amygdala and the concomitant *anticipatory* behavioural and endocrine responses can be considered an allostatic mechanism (Schulkin et al., 1994).

B. Development and programming of HPA-axis, brain corticosteroid receptors and behaviour

B.1. Introduction

The components of the fetal HPA-axis are functional during late gestation, although developmental changes still take place in the postnatal period (Grino et al., 1995; Sapolsky and Meaney, 1986). HPA-activity during late gestation is represented by a gradual increase in fetal plasma ACTH levels, stimulated by signals from pituitary and placenta as well as from intrahypothalamic excitatory amino acids. This rise in plasma ACTH is followed by an increase in CORT concentrations (Brooks et al., 1996). The concomitant rise in ACTH and CORT during this period indicates the absence of negative feedback regulation at this time. The sensitivity to negative feedback regulation by circulating glucocorticoids develops postnatally, increasing with age (McEwen et al., 1986; Meaney et al., 1985). Relatively high HPA-activation during the late fetal period is followed by reduced activity after birth, resulting in a marked suppression from around postnatal day 4 until postnatal day 14. This period is known as the stress hyporesponsive period (SHRP) (Goldman et al., 1973; Sapolsky and Meaney, 1986; Walker et al., 1986). During the SHRP, most stressors evoke only a blunted HPA response, although the HPA axis is capable of eliciting an adult-like response to specific stressors (Walker et al., 1991). It was thought that this intriguing phenomenon might serve to protect the neonate from anti-proliferative effects of high levels of glucocorticoids during a critical time in development.

The physiological relevance of low HPA-activity during development has become clear from studies that investigated the effects of corticosteroids on neural development. Exposure to high levels of glucocorticoids in particular can have catabolic effects that interfere with brain growth and cell proliferation (Cotterrell, 1972; Weichsel, 1974)[for review see (Meyer, 1985)]. On the other hand, they are known to play an organisational role in neuronal development by acting as a differentiating signal as well as by stimulating synaptogenesis and regulating cell signalling (Slotkin et al., 1993; Slotkin et al., 1998; Takahashi and Goh, 1998)[for review see (Meyer, 1985)]. Thus, the inhibiting effects of high doses and the

permissive effects of moderate doses of glucocorticoids indicate the importance for normal neuronal development of keeping glucocorticoid concentrations within closely regulated limits.

Brain MR and GR levels, important in the regulation of HPA-activity, follow distinct developmental patterns. In early gestation both types of corticosteroid receptors are not expressed. Increases in receptor levels from midgestation onwards are the result of both an increase in the number of expressing cells and an increased expression per cell (Bohn et al., 1994; Rosenfeld et al., 1988a).

MR mRNA has not been detected until the last week of gestation (Brown et al., 1996; Diaz et al., 1998), with high levels at birth (Bohn et al., 1994). Immunocytochemistry studies showed that the high perinatal levels of MR immunoreactivity are followed by a sharp decline around pnd 10, after which immunoreactivity gradually increases until adult levels are reached around the time of weaning (Lawson et al., 1991). MR binding however is only detectable from pnd 8 (Rosenfeld et al., 1988b) at which time it has adult characteristics regarding affinity and capacity. The discrepancy between binding studies and mRNA expression can be explained by the fact that binding studies require prior ADX, which may influence receptor levels. Furthermore, binding studies measure functional receptors, whereas mRNA expression studies do not take into account possible posttranscriptional and posttranslational mechanisms that may determine the number of functional receptors.

GR mRNA expression has been demonstrated in rat brain from midgestation onwards (Diaz et al., 1998; Kitraki et al., 1996). GR immunoreactivity develops 1 or 2 days later (Cintra et al., 1993). The signal increases to high levels perinatally (Bohn et al., 1994), and a drop in GR mRNA expression and immunoreactivity is noted around pnd 10-12 (Lawson et al., 1991; Rosenfeld et al., 1988b; Rosenfeld et al., 1988c), although not in every study (Kalinyak et al., 1989). GR binding and immunoreactivity then gradually increase until adult levels have been reached around pnd 21 (Rosenfeld et al., 1988b; Rosenfeld et al., 1988a). Unlike in the adult animal, GR expression during development does not seem to be subject to autoregulation. For example, DEX-binding at pnd 10 is not altered by five days of either ADX or CORT-treatment (Meaney et al., 1985), and DEX failed to regulate GR mRNA expression in the early neonate (Kalinyak et al., 1989). Furthermore, after the SHRP

both GR number and circulating CORT levels increase in parallel. Thus, the potential for autoregulation seems to develop gradually with age.

In conclusion, the brain and the components of the HPA-axis are developing *in utero* until weeks after birth. The plasticity of these developing systems becomes evident when investigating the effect of environmental events that take place during these periods on their physiological or behavioural endpoints. How early life events, especially the intensively-studied prenatal stress paradigm, alter the development of the HPA- and behavioural responses to stress is described in the following paragraphs.

B.2. Prenatal programming of HPA axis and behaviour

B.2.1. Introduction

Developing physiological systems are sensitive to outside influences. The androgenic hormonal status *in utero* for example, can determine within a specific developmental stage or *'time window'* the development of brain and body in a 'male' or a 'female' direction (O'Riordan et al., 1988).

The HPA-axis is another well-known example of systems of which the 'setpoint' can be altered by early life events. One of the most intensively investigated early life manipulations of this system is prenatal stress. In this paradigm, a pregnant dam is exposed to stress, and effects of this manipulation are studied in the offspring. These effects are described in the next paragraphs. In short, prenatal stress has been shown to have profound effects on offspring sexual development and behaviour, hormonal and behavioural responses to stress and brain development, although results of different studies can be variable or even contradictory. This is probably due to high variability between studies in timing, duration and type of stressor applied during pregnancy (Fride and Weinstock, 1984). Many behavioural abnormalities associated with prenatal stress are also seen in humans [see (Weinstock et al., 1988; Weinstock, 1997)], although these studies are mostly retrospective.

In many studies birthweight of prenatally stressed rats was lower than that of control offspring (Kinsley and Svare, 1986; Peters, 1982; Takahashi et al., 1988; Williams et al., 1998), and in humans, maternal stress during the third trimester of pregnancy was

associated with a decrease in infant birthweight (Sandman et al., 1997). Low birthweight has been linked with an increased risk of hypertension, coronary heart disease, glucose intolerance and non-insulin-dependent diabetes mellitus, in epidemiological human studies (Barker, 1995; Fall et al., 1995; Hales et al., 1991; Stein et al., 1996) as well as in animal models (Langley and Jackson, 1994; Levitt et al., 1996; Lindsay et al., 1996a, 1996b; Nyirenda et al., 1998). It is unlikely that low birthweight *per se* causes this increased risk. Rather, there may be a common factor that influences birthweight as well as the setpoint of other physiological systems (see section B.3). The concept of a (non-genetic) factor early in life imprinting on physiological systems, such as in prenatal stress, is known as 'prenatal programming'.

The biological relevance of prenatal programming appears to be that plasticity of a physiological system allows environmental factors to 'hard-wire' that system in order to prepare the unborn animal for unfavourable environmental conditions in which it will grow up and spend its adult life. Thus, prenatal programming can be considered to be an allostatic mechanism. However, if the environmental circumstances in later life are not as anticipated, programming can have adverse effects and ultimately even lead to disease.

B.2.2. Prenatal stress: effects on HPA-activity

B.2.2.1. During pregnancy

Few studies have investigated the fetal and maternal HPA-responses to maternal stress, but most (Barbazanges et al., 1996; Holson et al., 1995; Ohkawa et al., 1991; Takahashi, 1998; Williams et al., 1999) found that maternal CORT levels remain elevated after repeated stress, suggesting that the HPA-axis of pregnant rats did not adapt to chronic stress in these experiments. Furthermore, fetal and maternal plasma CORT levels were positively correlated (Takahashi, 1998). Importantly, ACTH levels were also elevated in fetuses exposed to maternal stress. Because maternal ACTH cannot pass the placenta (Dupouy et al., 1980), ACTH measured in fetal plasma must be of fetal origin. Indeed, the same study found a decrease in fetal

pituitary ACTH-content (Ohkawa et al., 1991). This implies that the fetal HPA-axis is actually capable of responding to maternal stress, although different stress paradigms may result in different response patterns in the fetus (Weinstock et al., 1988). However, although the fetal HPA-axis is active by the end of gestation (Brooks et al., 1996) and capable of secreting CORT, a substantial part of CORT measured in fetal plasma is likely to be of maternal origin (Beitins et al., 1973; Zarrow et al., 1970).

Considering the elevated plasma CORT levels in maternally stressed fetuses, a possible mechanism for the decreased hippocampal corticosteroid receptor binding capacity often observed in adult prenatally stressed rats could be a permanent downregulation of those receptors (Sapolsky et al., 1984), initiated by elevated CORT levels during a critical period in development. Whether this is CORT of mainly maternal or fetal origin remains to be investigated, although an important study has revealed that the effects of prenatal stress on offspring HPA-activity and hippocampal MR binding are dependent upon the stress-induced increase in maternal CORT levels (Barbazanges et al., 1996).

B.2.2.2. During postnatal development

Studies into the effects of prenatal stress on HPA-activity during the first weeks of life have shown variable results, depending on the type and timing of the maternal stress. In some cases, male prenatally stressed rats have elevated basal CORT levels (Takahashi et al., 1988; Takahashi and Kalin, 1991) up until weaning, whereas in other cases, no effect of prenatal stress on basal CORT (Fride et al., 1986; Peters, 1982; Henry et al., 1994) or ACTH (Takahashi et al., 1990) was found. Stress-induced rises in CORT and ACTH are generally found to be higher in prenatally stressed animals throughout the preweaning period (Fride et al., 1986; Peters, 1982; Takahashi and Kalin, 1991), thus interfering with the SHRP normally seen in rats roughly between pnd 4 and pnd 14. Young prenatally stressed animals typically also have higher post-stress CORT levels compared to control animals (Henry et al., 1994; Takahashi and Kalin, 1991; Weinstock, 1997).

Hippocampal MR and GR binding is lower compared to controls in prenatally stressed rats at the time of weaning, but not on pnd 3 (Henry et al., 1994). Thus, the

increase in plasma CORT levels (already observed on pnd 3) in this study preceded the changes in hippocampal MR and GR binding. It is possible that the apparent absence of an SHRP, and thus elevated HPA activity in neonatal life, in these prenatally stressed rats in some way underlies the altered development of hippocampal MR and GR numbers, although (negative) autoregulation of GR does not appear to take place until after weaning (Kalinyak et al., 1989; Meaney et al., 1985).

B.2.2.3. In adult rats

Prenatal stress has long-lasting effects on offspring's HPA-axis. These effects are more marked in females than in males (McCormick et al., 1995; Weinstock et al., 1992). For example, basal CORT or ACTH levels in adult prenatally stressed male rats are unchanged compared to control rats in many studies (Barbazanges et al., 1996; McCormick et al., 1995; Stohr et al., 1998; Takahashi et al., 1992a; Vallee et al., 1996; Vallee et al., 1997; Weinstock et al., 1992), but increased in females (McCormick et al., 1995; Weinstock et al., 1992). Stress-induced CORT levels however, are elevated in both male and female prenatally stressed rats in most studies (Henry et al., 1994; Weinstock et al., 1992), especially in post-stress samples (Barbazanges et al., 1996; Maccari et al., 1995; Vallee et al., 1996; Vallee et al., 1997; Weinstock et al., 1998), indicating an impaired negative-feedback regulation. Although the effect of prenatal stress in rats is generally a hyperactive HPA-system, inconsistencies between studies investigating prenatally stressed effects on HPA-activity can be explained in terms of type, timing and predictability of the prenatal stress, as well as the stress paradigm and timing of the bloodsampling used when testing the offspring.

Several groups have shown that prenatal stress causes alterations in hippocampal corticosteroid binding sites: permanently decreased MR capacity in prenatally stressed males (Barbazanges et al., 1996; Henry et al., 1994; Maccari et al., 1995; Vallee et al., 1997), and decreased DEX-binding in females (Weinstock et al., 1992) have been observed. Hippocampal MR are involved in the control of basal HPA-activity and possibly also in the negative feedback regulation after stress (Dallman et

al., 1992; Ratka et al., 1989). Thus, a permanently reduced number of hippocampal MRs could be responsible for the increased poststress CORT levels observed in adult prenatally stressed animals. However, because corticosteroid receptors in adult rats are subject to autoregulation (Sapolsky et al., 1984), it is unclear whether increased CORT levels in prenatally stressed rats are actually the cause or the consequence of decreased hippocampal corticosteroid receptor levels. Moreover, the mechanism behind the decreased hippocampal receptor levels in prenatally stressed rats remains unclear. As mentioned earlier, increased CORT levels *in utero* could permanently alter the transcriptional level of corticosteroid receptors. Alternatively, they may influence developing monoaminergic systems which in turn regulate brain MR and GR expression [see (Kabbaj et al., 1995; Kabbaj et al., 1996; Maccari et al., 1992b; Seckl et al., 1990; Yau et al., 1992; Yau and Seckl, 1992)].

B.2.3. Prenatal stress: effects on behaviour

Prenatally stressed rats have often been described as having 'higher emotionality' (Fride et al., 1986). This conclusion stems from early open-field studies, where prenatally stressed rats typically show decreased locomotion and increased defecation (Fride et al., 1986; Pfister and Muir, 1992; Wakshlak and Weinstock, 1990)[see also (Weinstock et al., 1988)]. However, higher initial open-field activity has also been observed (Vallee et al., 1997), and this was explained as prenatally stressed rats having a higher level of escape behaviour, thus underlining the difficulty in interpreting animal behaviour. Furthermore, Vallee *et al.* showed avoidance of anxiogenic places in prenatally stressed animals, indicated by a low number of visits to the open arms on an elevated plusmaze (Vallee et al., 1997). Both types of behaviour were positively correlated with plasma CORT levels. These data, in combination with a reduced propensity to play and an increase in defensive freezing (Takahashi et al., 1992a; Takahashi et al., 1992b), reduced activity in an activity wheel (Lambert et al., 1995), and increased ultrasonic vocalisations in an open field (Williams et al., 1998b), led to the conclusion that prenatal stress causes *behavioural suppression* or *increased anxiety* in aversive situations. Interestingly, central CRH administration causes hyperanxiety-like behaviour similar to that seen in prenatally

stressed rats (Dunn and Berridge, 1990). Considering the role of the amygdala in this type of behaviour, it is tempting to speculate that the level of or sensitivity to CRH in the amygdala may be increased in prenatally stressed rats, resulting in a 'hyperemotional' state and reduced coping ability.

Cognitive abilities are also affected by prenatal stress. Acute and repeated prenatal stress reduced memory in an operant discrimination task (Grimm and Frieder, 1987). Furthermore, prenatal stress alters behaviour in a watermaze test: prenatally stressed rats spend more time looking for the platform in a reversal task in a watermaze-test in two studies (Hayashi et al., 1998; Szuran et al., 1994), indicating improved memory or altered search strategy. However, a third study failed to find a prenatal-stress effect on watermaze performance (Vallee et al., 1997). The amount of CORT secreted during the learning phase of this test may be of influence on retention expression, as only training in cold water (12°C) revealed differences between prenatally stressed and control rats (Szuran et al., 1994).

Because brain neurotransmitter systems and glucocorticoids interact to modulate both behaviour and HPA-activity (McEwen, 1987), it is possible that the effects of prenatal stress on these two parameters are mediated by prenatal stress-induced alterations in these systems. Indeed, prenatal stress alters brain 5-HT, NE and DA levels and turnover (Hayashi et al., 1998; Peters, 1982; Takahashi et al., 1992a), stress-induced hippocampal ACh levels (Day et al., 1998), and decreases synaptic density in the hippocampus, which is known to be regulated by 5-HT (Hayashi et al., 1998). Furthermore, in humans prenatal stress has been suggested to increase the risk of developing schizophrenia (van Os and Selten, 1998), a putative neurodevelopmental disorder that is associated with alterations in brain monoamine levels.

In conclusion, there is accumulating evidence to suggest that prenatal stress programs the HPA-axis as well as behaviour, and that plasticity of developing brain monoamine systems may underlie these changes. Ultimately, prenatal stress results in impaired coping and increased anxiety. Disturbances in HPA-regulation and brain monoamine levels have been associated with affective and anxiety disorders in humans (Holsbor and Barden, 1996; Lopez et al., 1998), suggesting that such conditions may be (partially) prenatally programmed. The mechanism underlying the

changes resulting from prenatal stress has not been convincingly established. In the search for a 'programming factor' imprinting on the development of fetal tissues, two major hypotheses have arisen which will be discussed in the next paragraphs.

B.3. Mechanisms of programming

B.3.1. Glucocorticoids

Because an important feature of the stress response is the secretion of high levels of CORT, this steroid has become an obvious candidate for the role of 'programming factor' in the prenatal stress paradigm. Moreover, prenatal glucocorticoid exposure has recently been implicated in the development of hyperglycaemia and hypertension, and may represent the epidemiological link observed between these disorders and low birth weight. Experiments describing the effects of injecting pregnant rats with dexamethasone (DEX), corticosterone (CORT) or ACTH have shed some light on the involvement of prenatal glucocorticoid exposure in fetal programming.

Daily administration of ACTH in the last week of pregnancy resulted in altered brain monoaminergic activity, reduced adrenal weight in combination with elevated basal CORT but reduced stress-induced CORT levels in plasma (Fameli et al., 1994). Similar to prenatal stress, prenatal ACTH injections had stronger effects in female than in male rats and caused an inability to adapt appropriately to stressful situations. Because ACTH does not pass the placental barrier (Dupouy et al., 1980), prenatal ACTH probably acts by increasing maternal CORT production. This has been tested by studying the effects of CORT administration to pregnant rats on their offspring. In two studies rats were injected with 21-28 mg CORT daily in the second (Lee and Rivier, 1992) or third (Holson et al., 1995) week of pregnancy, but in neither study was offspring HPA activity affected. In contrast, a third study administered a much lower dose of CORT (9 mg/day) to adrenalectomised dams following daily restraint procedures and observed effects identical to those of prenatal stress (Barbazanges et al., 1996).

Many more studies have been performed looking at the effects of prenatal exposure to the synthetic glucocorticoid DEX. A large number of animal studies have described the effects of DEX on normal growth and proliferation. Decreased brain weight is a well-established consequence of perinatal DEX administration (Benesova and Pavlik, 1989; DeKosky et al., 1982; Ferguson and Holson, 1999; Slotkin et al., 1993; but also Holson et al., 1995), and prenatal DEX administration on GD 17, 18 and 19 has been shown to influence brain development by altering the induction of nuclear transcription factors such as c-*fos* and AP-1 (Slotkin et al., 1998). Brain or hippocampal weights are rarely measured in prenatal stress studies, but Szuran *et al* showed decreased hippocampal wet weight in prenatally stressed males and females (Szuran et al., 1994). Furthermore, prenatal DEX-administration consistently reduces birth weight (Benediktsson et al., 1993; Celsi et al., 1998; Holson et al., 1995; Levitt et al., 1996; Muneoka et al., 1997; Slotkin et al., 1993), and perinatal DEX exposure is associated with (mostly transiently) reduced body weight (Dekosky et al., 1982; Ferguson and Holson, 1999, Muneoka et al., 1997; Slotkin et al., 1993). In addition, prenatal exposure to DEX increases blood pressure (Benediktsson et al., 1993; Celsi et al., 1998) and exposure only in the last week of pregnancy programs glucose intolerance (Nyirenda et al., 1998). This has led to the suggestion (Edwards et al., 1993) that prenatal glucocorticoid exposure may explain the link between low birthweight and later hypertension and diabetes found in epidemiologial studies [e.g. (Barker, 1995; Hales et al., 1991)].

A preliminary human study showed that prolonged prenatal low-dose DEX exposure did not affect cognition scores, but increased scores for shyness, internalising, avoidance, emotionality and decreased scores for sociability (Trautman et al., 1995). The similarities with prenatal stress-effects in rats are striking (Takahashi et al., 1992a; Takahashi et al., 1992b; Vallee et al., 1997; Weinstock, 1997). Alterations in brain monoamine systems may play a role in these effects on emotion. Indeed, late gestational DEX administration reduces brain 5-HT and DA turnover and elevates NA contents (Muneoka et al., 1997).

Behaviour in an open field was unaltered in offspring from mothers treated with DEX in the last week of pregnancy compared to offspring of untreated dams (Holson et al., 1995). In a different study however, DEX was only administered on GD

(gestational day) 17, 18 and 19 and offspring was compared to offspring of vehicle-treated dams. Here, an increased activity in an open field was observed in combination with reduced defecation, suggesting specific time windows for the effects of prenatal treatments as seen previously in prenatal stress procedures (Muneoka et al., 1997). However, this altered behaviour in an open field-test is actually opposite to what is usually observed in prenatally stressed rats (Fride et al., 1986; Wakshlak and Weinstock, 1990)[see also (Weinstock et al., 1988)]. In contrast, the effects of prenatal stress on male sexual behaviour could be imitated by injections with DEX in the last week of pregnancy, but not with CORT or ACTH (Holson et al., 1995). In the few studies investigating the effects of DEX-injections on HPA-activity, basal (Levitt et al., 1996; Muneoka et al., 1997), but not stress-induced (Holson et al., 1995) plasma CORT levels appear to be increased as a result of DEX-administration in the last week of pregnancy.

Summarising, prenatal DEX exposure clearly has programming capacities, acting centrally as well as peripherally. The effects of prenatal high *endogenous* glucocorticoid levels are less clear-cut and this raises the question whether maternal glucocorticoids reach the fetus to the same extent as DEX does. This question will be discussed in more detail in part C of this chapter.

B.3.2. Prenatal food restriction

The hypothesis of fetal (or maternal) undernutrition is based on the rationale that the fetus adapts to poor *in utero* energy availability by changing the set point of systems involved in metabolism. However, these permanent changes may be counterproductive if nutrition in the postnatal environment is normal and may ultimately even lead to disease. The relevance of this theory for HPA programming may lie in the capacity of glucocorticoids to regulate body weight and food intake (Spinedi and Gaillard, 1998; Tempel and Leibowitz, 1994), and effects of altered glucocorticoid levels due to nutritional programming on other parameters such as behaviour and learning may occur as a 'side effect'.

Food restriction or reduced protein intake during pregnancy programs offspring birth weight and blood pressure (Langley and Jackson, 1994; LangleyEvans, 1997;

Woodall et al., 1996). A correlation between maternal nutritional status during pregnancy and childhood blood pressure has also been observed in humans (Godfrey et al., 1994). Moreover, there is epidemiological evidence from persons conceived during the Dutch Hunger Winter of 1944-1945 that prenatal food restriction may be involved in obesity (Susser and Stein, 1994), glucose intolerance (Ravelli et al., 1998) and even in the origin of (some cases of) schizophrenia (Susser et al., 1996). Furthermore, early malnutrition reduces brain DNA content in children (Winick, 1971). Only one study has investigated stress-induced behaviour in prenatally protein-restricted rats, and found decreased anxiety or altered behavioural inhibition (Almeida et al., 1996). Interestingly, changes in behavioural suppression are also found in prenatally stressed rats (Weinstock, 1997), and stressed pregnant rats have been shown to eat and drink less than unstressed pregnant rats (Kinsley and Svare, 1986).

B.3.3. A common mechanism?

The similarities between the effects of prenatal DEX-injections and prenatal malnutrition or protein restriction, especially peripherally, suggest a common mechanism underlying these effects. One possibility is that DEX-injections reduce food intake in pregnant rats. Alternatively, food- or protein restriction could activate the dam's HPA-axis, thereby increasing CORT levels, as has been shown in primates (Recabarren et al., 1998), which in turn affect fetal development. In rats, HPA activation by undernutrition occurs at least in non-pregnant animals (Jacobson et al., 1997). A mutual interaction is not unlikely, since endogenous CORT is known to modulate food intake through its receptors, and increased energy requirement results in increased CORT levels (Tempel and Leibowitz, 1994). Accordingly, prenatally stressed rats, which have increased stress-induced HPA-activity (Henry et al., 1994), are lighter than control rats at 5 months of age and have reduced food intake (Vallee et al., 1996).

B.4. Interactions with the postnatal environment

It has been suggested that postnatal and later environmental events might modulate the effects of prenatal programming. Lifestyle factors such as smoking, exercise and diet clearly influence blood pressure and blood glucose levels. Where brain and HPA axis are concerned, early manipulations such as handling (Smythe et al., 1994; Wakshlak and Weinstock, 1990) and adoption (Maccari et al., 1995) reverse at least some of the effects of prenatal stress. This implies that postnatal manipulations and prenatal stress have at least some common neural targets on which they have opposite effects at different timepoints. Alternatively, it is possible that the effects of prenatal stress extend into the postnatal period and thus can be prevented or modulated by events within that same period. Interesting in this respect is the observation that an estimated 80% of hippocampal DG cells are formed in the first postnatal week (Takahashi and Goh, 1998).

Maternal behaviour is known to influence offspring HPA-activity and anxiety levels (Caldji et al., 1998; D'Amato et al., 1998). In fact, the effects on offspring of neonatal handling are due to increased licking/grooming of pups by the dam, initiated by the short daily separations that characterise the neonatal handling procedure (Lee and Williams, 1974), since the offspring of undisturbed mothers that have a naturally high licking/grooming behaviour are indistinguishable from handled rats (Liu et al., 1997). Moreover, stroking pups during a maternal deprivation procedure can abolish the effects of long-term maternal deprivation (van Oers et al., 1998), whereas maternal treatment with anxiolytics upon daily neonatal handling of the pups reverses the effects of the handling procedure on offspring emotionality (D'Amato et al., 1998). Thus, it may be possible that maternal stress during pregnancy has its effects through altered maternal behaviour in the early neonatal period, and that environmental manipulations during that period also influence maternal behaviour. At present it is not known whether stress during pregnancy does alter maternal behavioural patterns, although one paper suggested that at least the duration of anogenital licking of pups does not differ between previously stressed and unstressed mothers (Melniczek and Ward, 1994). Interestingly however, early adoption increases maternal licking behaviour and its effects on offspring HPA-activity are irrespective of the dams' experiences during pregnancy (Maccari et al., 1995),

indicating that possible stress effects on maternal licking behaviour are reversed by cross fostering of offspring at birth.

Additionally, stress during pregnancy and/or postpartum manipulations could alter the hormonal status of the dam's milk, and this in turn might influence the offspring's HPA-axis. Unpredictable stress to dams has been shown to increase plasma CORT levels up to 3 weeks after parturition [see (Weinstock, 1997)], which may be reflected in high levels of CORT in milk. However, when lactating dams are administered CORT through drinking water, their offspring's basal and stress-induced CORT levels are actually lower than those of control offspring from pnd 16 onwards, rendering it unlikely that prenatal stress effects on plasma CORT levels are indirectly caused by increased CORT levels in the mothers' milk (Catalani et al., 1993).

C. The feto-placental barrier

C.1. 11β-Hydroxysteroid dehydrogenase

As discussed earlier, maternal glucocorticoids are thought to, at least partly, underlie the effects of prenatal stress on the fetus (Barbazanges et al., 1996). The fact that glucocorticoids are highly lipophilic and therefore easily cross the placenta has contributed to this hypothesis. Indeed, many studies into the consequences of prenatal glucocorticoid exposure refer to a 1970 study by Zarrow (Zarrow et al., 1970), which claims to show the passage of ^{14}C-CORT from the rat mother to the fetus. However, this study, in which mothers were injected with ^{14}C-CORT, only measured the amount of radioactivity that had passed from mother to fetus, assuming it was still incorporated into CORT. The possibility that part of the radiolabel might have been incorporated into CORT-metabolites was not considered.

It is now well established that a CORT-metabolising enzyme is in fact found in the placenta (Brown et al., 1993, 1996; Lakshmi et al., 1993), as well as in many other organs (Monder, 1991; Walker et al., 1992). This 11β-hydroxysteroid dehydrogenase (11β-HSD) protein catalyses the interconversion of CORT and cortisol into 11-dehydroCORT and cortisone, respectively (Figure 1.2). These metabolites have very low affinity for corticosteroid receptors and can thus be considered to be 'inert'. In this way, 11β-HSD is thought to regulate corticosteroid access to its receptors. Two distinct isozymes of 11β-HSD have been characterised: the type 1 enzyme (11β-HSD1) is a widespread NADP(H)-dependent enzyme (Agarwal et al., 1989; Lakshmi and Monder, 1988) with relatively low affinity for CORT (K_m in low micromolar range), that catalyses in intact cells mainly the reductase reaction (Low et al., 1994a; Rajan et al., 1996) or is bi-directional (Agarwal et al., 1989). On the other hand, the type 2 enzyme (11β-HSD2) is an NAD-dependent exclusive dehydrogenase with a very high affinity for CORT (K_m in nanomolar range) (Albiston et al., 1994; Brown et al., 1993).

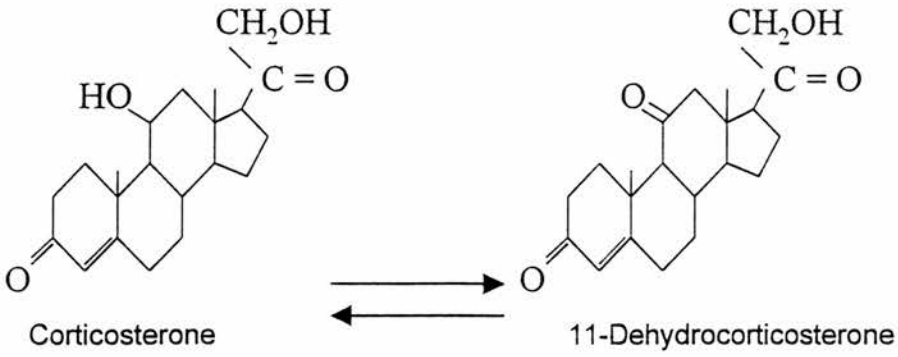

Figure 1.3 **Conversion of corticosterone and 11-dehydrocorticosterone by 11β-HSD.**

The crucial role of 11β-HSD in regulating corticosteroid access to MR and GR was discovered in ALDO-target tissues such as the distal nephron. Plasma levels of active CORT far exceed those of ALDO, even allowing for the majority of CORT being bound (and therefore presumed inactive) to CBG and other plasma proteins. In spite of these differences in concentration and their equal affinity for MR, ALDO and not CORT binds to MRs in certain tissues. It was revealed that 11β-HSD2 present in those tissues protects MRs from CORT by inactivating it, thus allowing ALDO access to the receptor (Edwards et al., 1988). ALDO is itself not a substrate for 11β-HSD due to its [11-18]hemiacetal bridge. When renal 11β-HSD2 is inhibited by liquorice or its derivatives (Stewart et al., 1987), or in case of a congenital absence of the enzyme (a syndrome called 'apparent mineralocorticoid excess') (DaveSharma et al., 1998), CORT is not inactivated and occupies renal MRs, causing overstimulation of these receptors which results in chronic hypertension (Edwards et al., 1988). Clearly, MR specificity *in vivo* is enzyme- and not receptor-mediated (Funder et al., 1988).

C.2. Placental 11β-HSD

The placenta is not an ALDO-target tissue, but placental 11β-HSD2 is thought to protect the fetus from excessive maternal CORT which, as described earlier, may have deleterious effects on normal growth and development. Consequently, in human

term fetuses. 75% of cortisol is of fetal origin, whereas cortisone is mainly of maternal origin (Beitins et al., 1973). The fetus itself also expresses 11β-HSD2 in a region- and time-specific manner (Brown et al., 1996). In addition to its protective role, feto-placental 11β-HSD2 is thought to locally regulate CORT access to GR in the developing fetus. A role for this enzyme in regulating CORT access to MR is unlikely as it is only expressed in high levels until midgestation, whereas MR do not appear in the fetus until the last few days of gestation (Brown et al., 1996).

Placental 11β-HSD activity has been shown to vary greatly between individuals (Benediktsson et al., 1993), possibly due to differences in the levels of local or circulating natural regulators of 11β-HSD activity (Buhler et al., 1994; Seckl, 1997; Souness et al., 1995). Interestingly, activity of placental 11β-HSD correlates positively with birth weight in rats (Benediktsson et al., 1993) and humans (Stewart et al., 1995) and humans with defective 11β-HSD2 often have low birth weight (Kitanaka et al., 1996; White et al., 1997). Indeed, inhibition of feto-placental 11β-HSD by administration of carbenoxolone (CBX) to pregnant rats decreases birthweight in the offspring, and programs hypertension and hyperglycaemia later in life (Lindsay et al., 1996a; Lindsay et al., 1996b).

The synthetic glucocorticoid DEX is relatively poorly metabolised by 11β-HSD2 (Kream et al., 1983; Siebe et al., 1993) and as mentioned before, when administered to pregnant rats it affects a range of developmental processes, resulting in growth retardation, hypertension, glucose intolerance and HPA-dysregulation (Benediktsson et al., 1993; Levitt et al., 1996; Nyirenda et al., 1998; Slotkin et al., 1993). Taken together, this suggests that feto-placental 11β-HSD2 may play an important role in modulating the programming effects of prenatal glucocorticoid exposure (Edwards et al, 1993; Seckl, 1997).

C.3. Ontogeny of 11β-HSD

High levels of 11β-HSD2 mRNA expression have been found in the fetal portion of mouse placenta until GD16.5 (Brown et al., 1996). Although residual 11β-HSD2 protein may continue to inactivate CORT in the last few days of gestation, it is likely that expression of the enzyme is reduced to allow glucocorticoids to act on

developing fetal tissues. Diaz *et al* (Diaz et al., 1998) described the expression of 11β-HSD types 1 and 2 in rat brain during fetal development. The type 1 enzyme mRNA is not expressed until the last week of gestation and activity is very low or undetectable, whereas high levels of type 2 enzyme mRNA and activity are expressed from midgestation onwards, reducing strongly in the last week of gestation, with exception of the thalamus and cerebellum. These patterns of expression suggest specific time-windows for glucocorticoid effects on the developing fetus. During midgestation 11β-HSD2 in fetus and placenta could be protecting the fetus from the growth-limiting effects of maternal CORT by inactivating it. Indeed, humans with defects in 11β-HSD2 have low birthweight (White et al., 1997), a feature often seen in animals that have been prenatally exposed to high glucocorticoid levels. By the end of gestation, when the fetal HPA-axis is active, glucocorticoids produced by the fetus are essential for differentiational and maturational processes. During this time window 11β-HSD1, the expression of which may be induced by rising CORT levels (Low et al., 1994b) may ensure the presence of active glucocorticoids, while discrete localisation of 11β-HSD2 postnatally (Robson et al., 1998) probably acts to restrict glucocorticoid access to specific brain areas.

The time- and tissue-specific expression of 11β-HSD during development may, in addition to different stages of maturity of the fetal target tissues, account for the variability of prenatal stress effects between studies in which maternal stress is often applied at different times during pregnancy.

D. Aim of the study

Numerous studies have described the epidemiological link that exists between birth weight and later hypertension (Leon et al., 1996), coronary heart disease (Barker, 1995) and glucose intolerance (Leon et al., 1996). This link has been confirmed in animal studies and can be induced by prenatally exposing animals to the synthetic glucocorticoid DEX (Levitt et al., 1996; Nyirenda et al., 1998), suggesting that glucocorticoids *in utero* may program birthweight and later cardiovascular disease and glucose intolerance. An alternative hypothesis proposes a role for prenatal undernutrition or protein restriction in fetal programming (Langley and Jackson, 1994).

The system that controls release of endogenous corticosteroids is the HPA axis and the activity of this axis is in turn (partly) controlled by the hippocampus. The HPA axis itself is perinatally programmable (Henry et al., 1994; Meaney et al, 1989; Weinstock et al., 1992; Rots et al., 1996), and the hippocampus is particularly sensitive to perinatal manipulations (Takahashi, 1998). Prenatal stress and maternal deprivation result in HPA hyperactivity in response to stress, whereas short-term daily neonatal handling has the opposite effect. These alterations in HPA responsiveness appear to be associated with alterations in hippocampal corticosteroid receptor levels. Therefore, the first aim of this thesis was to establish the long-term effects of prenatal exposure to the synthetic glucocorticoid DEX (figure 1.4) on HPA activity and regulation. Furthermore, because DEX has been shown to act in a specific time window on blood pressure and glucose metabolism (Levitt et al., 1996; Nyirenda et al., 1998), we also investigated whether a similar critical window was applicable here.

Glucocorticoids play an important role in CNS development: low levels promote mitosis, myelination and differentiation [for review see (Meyer, 1985)], but the catabolic actions of high glucocorticoid levels have deleterious effects on normal neural development. Therefore, it is important that corticosterone levels *in utero* are tightly regulated. The CORT-metabolising enzyme 11β-HSD2 present in both placenta and a range of fetal tissues may act to protect the fetus from adverse effects by high levels of maternal corticosterone. Inhibition of this barrier results in

hypertension and glucose intolerance in a similar way to DEX administration in pregnancy (Lindsay et al., 1996a; Lindsay et al., 1996b). The second aim of this thesis was therefore to investigate whether inhibition of the enzymatic barrier to maternal glucocorticoids using carbenoxolone (figure 1.4) has permanent effects on HPA activity and regulation similar to synthetic glucocorticoid exposure.

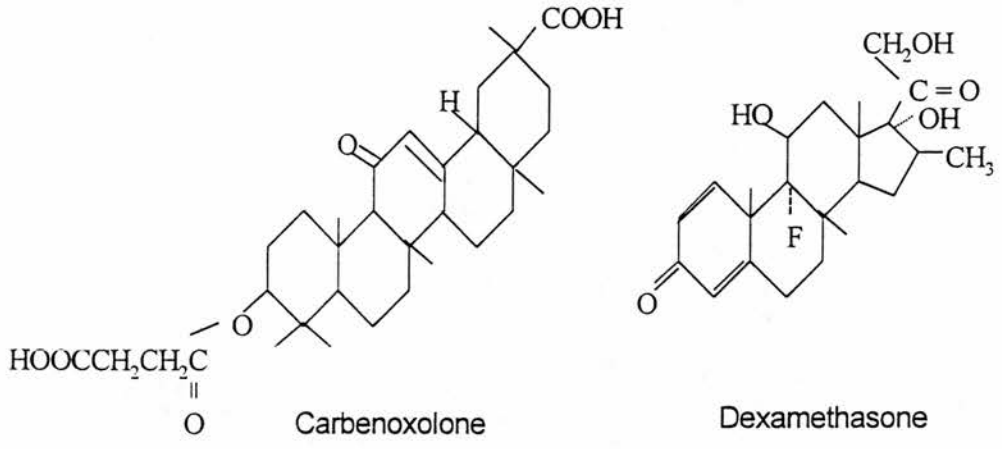

Figure 1.4. **Structures of carbenoxolone (CBX) and dexamethasone (DEX).**

Corticosteroids play an important role in cognition and behaviour by binding to their receptors in specific regions in the brain. The limbic system contains high levels of receptors and the hippocampus in particular is critically involved in learning and memory. Thus, corticosteroid modulation of hippocampal activity is thought to underlie some cognitive processes. The third aim of this thesis was to see if an altered HPA activity and hippocampal receptor expression resulting from prenatal glucocorticoid exposure had behavioural consequences. Firstly, the effect of the prenatal treatments on spatial (watermaze) and non-spatial (forced swim-test) learning and memory was investigated. Secondly, the animals' behaviour in adverse environments (open field, elevated plus-maze) was studied. Because the amygdala is involved in the expression of anxiety and also contains corticosteroid receptors, changes in this structure resulting from prenatal glucocorticoid exposure may be correlated with behavioural alterations.

This study has important clinical relevance. Long-term DEX therapy is not uncommon in pregnancies where congenital adrenal hyperplasia is suspected. Furthermore, short-term administration of DEX is often used perinatally in order to promote lung maturation in premature babies. Additionally, glucocorticoids are used as immunosuppressants for a number of maternal inflammatory diseases. The long-term consequences of maternal or fetal glucocorticoid therapy on the offspring have received little or no attention. Even subtle alterations in HPA activity and regulation resulting from prenatal exposure to glucocorticoids may have consequences for vulnerability to a number of affective disorders. For example, HPA abnormalities have been observed in depression (Holsboer and Barden, 1996b), schizophrenia (Jansen et al., 1998) and chronic fatigue syndrome (Demitrack et al., 1991).

E. Outline of the thesis

Chapter 3 describes the effects of prenatal exposure to glucocorticoids throughout pregnancy, either by administration of DEX or by inhibition of feto-placental 11β-HSD with CBX, on offspring HPA activity and regulation. In addition, maternal food intake and weight gain during pregnancy as well as offspring body weight gain were monitored. In order to establish whether cognitive function of these animals was affected as a result of the prenatal treatments they were tested for spatial ability in a Morris watermaze, as described in chapter 4. Because DEX has been shown to act in a specific time window on blood pressure and glucose metabolism, we investigated in chapter 5 whether a similar critical window was applicable here. Finally, in chapter 6, stress-induced behaviour was studied in a number of tests. The involvement of the amygdala in possible behavioural alterations resulting from prenatal glucocorticoid exposure was investigated by measuring the expression of mRNAs for corticosteroid receptors and CRH.

Chapter 2. Materials and methods

A. Materials

I. Chemicals

Acetic anhydride	Sigma-Aldrich Company Ltd., UK
Acetone	BDH [Merck Ltd.], UK
Amberlite resin	BDH [Merck Ltd.], UK
Amfix fixative	H.A. West Ltd., UK
Ammonium acetate	Sigma-Aldrich Company Ltd., UK
Ammonium persulphate	Sigma-Aldrich Company Ltd., UK
Boric acid	Sigma-Aldrich Company Ltd., UK
Bovine serum albumin	Sigma-Aldrich Company Ltd., UK
Calcium chloride (CaCl2)	Sigma-Aldrich Company Ltd., UK
Carbenoxolone	Sigma-Aldrich Company Ltd., UK
Cocktail T	Sigma-Aldrich Company Ltd., UK
Chloroform/isoamyl (24:1)	Sigma-Aldrich Company Ltd., UK
Citric acid	Sigma-Aldrich Company Ltd., UK
Corticosterone	Sigma-Aldrich Company Ltd., UK
D19 developer	Ilford Ltd., UK
Denhardt's 50×	Sigma-Aldrich Company Ltd., UK
DEPC	Sigma-Aldrich Company Ltd., UK
Dexamethasone	Sigma-Aldrich Company Ltd., UK
Dextran sulfate	Sigma-Aldrich Company Ltd., UK
Dipotassium hydrogen orthophosphate anhydrous (K_2HPO_4)	BDH [Merck Ltd.], UK
Disodium hydrogen orthophosphate anhydrous (Na_2HPO_4)	BDH [Merck Ltd.], UK
DNA (salmon sperm)	Sigma-Aldrich Company Ltd., UK
DTT	Sigma-Aldrich Company Ltd., UK
EDTA (disodium EDTA•$2H_2O$)	Sigma-Aldrich Company Ltd., UK
Enzymes (all)	Promega UK Ltd., UK

Ethanol	BDH, Merck Ltd., UK
Formamide	Sigma-Aldrich Company Ltd., UK
Gelatine	Sigma-Aldrich Company Ltd., UK
Hydrochloric acid (HCl)	BDH, Merck Ltd., UK
Hyperfilm β-max	Amersham Pharmacia Biotech UK
NICK columns	Amersham Pharmacia Biotech UK
NTB2	Kodak
Nucleotides	Life Sciences International, UK
Paraformaldehyde	Fisher Scientific, UK
Phenol/ChCl3	Sigma-Aldrich Company Ltd., UK
Poly-l-lysine	Sigma-Aldrich Company Ltd., UK
Potassium chloride (KCl)	BDH [Merck Ltd.], UK
Potassium dihydrogen orthophosphate (KH2PO4)	BDH [Merck Ltd.], UK
Potassium hydrogen carbonate (KHCO3)	BDH [Merck Ltd.], UK
Pyronin Y	Sigma-Aldrich Company Ltd., UK
Reaction buffers	Promega UK Ltd., UK
Sodium acetate	Sigma-Aldrich Company Ltd., UK
Sodium azide	Sigma-Aldrich Company Ltd., UK
Sodium bicarbonate (Na2CO3)	BDH [Merck Ltd.], UK
Sodium chloride (NaCl)	BDH [Merck Ltd.], UK
Sodium dihydrogen orthophosphate 1-hydrate ($NaH_2PO_4 \bullet H_2O$)	BDH [Merck Ltd.], UK
Sodium hydroxide (pellets)	Sigma-Aldrich Company Ltd., UK
SPA reagent	Amersham Pharmacia Biotech UK
SSC 20*	Sigma-Aldrich Company Ltd., UK
Transcription buffer	Promega UK Ltd., UK
Triethanolamine	Sigma-Aldrich Company Ltd., UK
TrisCL	BDH [Merck Ltd.], UK
Xylene	BDH [Merck Ltd.], UK
Yeast tRNA	Gibco BRL [Life Technologies Ltd.]

II. Radiochemicals

[^{3}H]-Corticosterone	Amersham Pharmacia Biotech UK
[^{35}S]-UTP	Amersham Pharmacia Biotech UK

III. Other

Coverslips	BDH [Merck Ltd.], UK
Cryostat	Bright Instruments Ltd., UK
Lens tissue	Whatman Labsales Ltd., UK
Microscope slides (frosted)	BDH [Merck Ltd.], UK
Microvette tubes	Sarstedt Ltd., UK
NICK columns, Sephadex G50	Amersham Pharmacia Biotech UK
Razorblades	BDH [Merck Ltd.], UK

IV. Behavioural equipment

Elevated plus-maze	Linton Instrumentation, UK
Open-field	Homebase
Video camera	

B. Methods

I. Subjects

Adult Wistar rats were obtained from Charles River UK Ltd. All experimental rats were housed in same-sex groups of 2-4. Animals had *ad libitum* access to food (standard rat chow: 56.3% carbohydrate, 18.3% protein, 0.7% NaCl; BS&S Scotland Ltd. Edinburgh). Rats were maintained under conditions of controlled temperature (22°C) and lighting (lights on 7:00 h, lights off 19:00 h). All experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986.

II. Prenatal treatments

Virgin female rats (225 g) were housed with adult male rats. Pregnancy was confirmed by the presence of a vaginal plug, checked every morning. Pregnant females were housed individually and randomly assigned to one of four treatment groups. In each experiment, pregnant rats received daily subcutaneous injections with 200 µl vehicle solution (4% ethanol in 0.9% saline), 12.5 mg/200 µl carbenoxolone (CBX), or 100 µg/kg dexamethasone (DEX1-3), respectively, throughout pregnancy. An additional treatment group received 200 µl vehicle during the first 2 weeks of pregnancy and 100 µg/kg dexamethasone during the last week of pregnancy (DEX3).

Body weight and food intake of pregnant rats were measured daily until the day before expected delivery. A single blood sample was taken from the tail vein (see below) on day 20 of pregnancy for determination of plasma CORT levels.

III. Litters

On the day of birth litters were weighed, sexed and culled to 8 pups per litter with a preference for male pups. Remaining pups were sacrificed by decapitation, their brains removed and quickly frozen on powdered dry ice and their blood collected for CORT measurement. Only litters containing 8 pups or more were selected for the studies. Unless stated otherwise, litters were then left undisturbed until weaning apart from routine maintenance. After weaning, pups from each litter were housed in same-sex groups of 2-4 animals and left undisturbed until the time of testing. In a separate experiment following the same prenatal- and birth protocol, one pup from each litter was sacrificed at 1 and 2 weeks of age and trunk blood was collected for determination of plasma CORT concentrations.

IV. Blood sampling

When repeated blood samples were required, rats were handled regularly during the week before the experiment by the experimenter and habituated to being held inside a folded tea towel. For blood sampling, rats were taken out of their home cage by the experimenter, quickly placed inside the folded tea towel and loosely restrained by a second experimenter. When only one blood sample per rat was required, a second experimenter held the rats while the first experimenter carried out the sampling procedure. A small cut was made approximately 1 cm from the tip of the tail, using a razorblade. Blood was gently pushed out of the tail vein by stroking the tail from the base to the tip and pressing it slightly just above the cut. Blood was collected in Microvette tubes, stored on ice until centrifuged at 4°C. Subsequently, plasma was stored at -20°C until assayed. The complete procedure took place within 2 minutes of removing the rat from its home cage. Using this method, low unstressed basal CORT levels could be obtained (0.5-1.5 μg/dl), and the effect of making the first cut on plasma CORT levels generally disappeared after 90 minutes (Figure 2.1). Unfortunately however, the volume of plasma obtained using this method was not enough to allow for ACTH measurements as well.

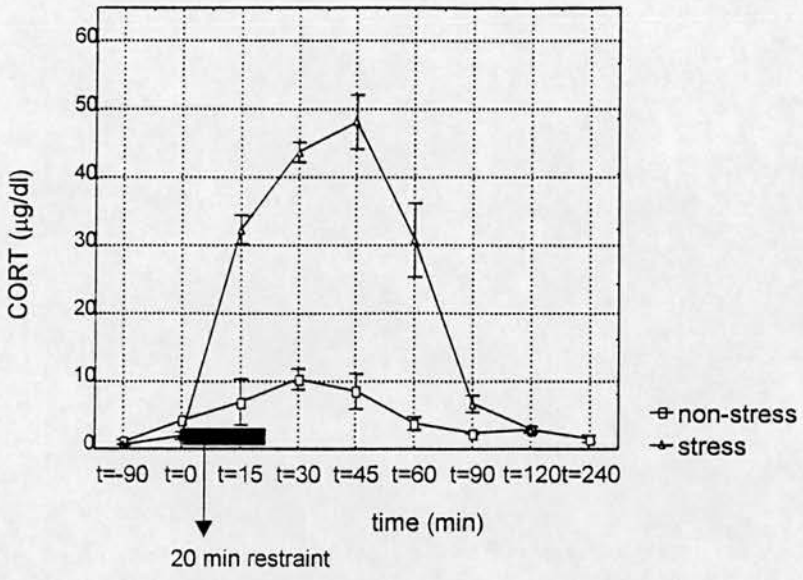

Figure 2.1 **Effect of single tail cut on plasma CORT levels.**
Plasma CORT levels are back to basal 90 minutes after the cut was made *(squares)*. The effect of a single tail cut plus 20 minutes restraint is also shown *(triangles)*. Blood samples were taken from a tail cut, made at time point t=-90.

V. HPA axis activity

To assess circadian variation in basal plasma CORT concentrations, 6 month-old rats were handled regularly during the week before the experiment. Using the method described above, blood samples were taken at 08:00 h, 14:00 h and 20:00 h in all rats. In order to avoid stress induced by making more than one tail cut on one day, sampling was spread out over several days for each rat. Evening samples were taken under red-light illumination.

Stress-induced activation of the HPA axis in adult rats was determined using a restraint procedure. Two versions of this procedure were used. In the first version, a blood sample was taken at 8:00 h for determination of basal CORT levels ('t=-120'). Rats were then left undisturbed in their home cages for 2 h. A second plasma sample was taken ('t=0'), immediately after which rats were placed in a Perspex cylinder for 20 minutes. Upon termination of the restraint procedure, rats were placed back in

their home cages. Further plasma samples were taken at the following time points: t=15, t=30, t=45, t=60, t=90, t=120, t=150.

In a second stress-experiment, a blood sample was taken at 9:00 h for determination of basal CORT levels. Rats were then left undisturbed for 3 hours, after which they were restrained in Perspex cylinders for 20 minutes. A second plasma sample was taken 10 minutes into the restraint procedure. Upon removal from the tube, rats were placed back in their home cages and were not disturbed until a third blood sample was taken 90 minutes after the restraint, to measure post-stress CORT levels.

In a separate experiment, we determined the sensitivity to the suppressive effects of DEX on stress-induced CORT secretion. Thus, rats were given a single bolus of DEX (40 μg/kg) s.c. before the first blood sample was drawn, using the procedure described above. This dose was chosen because it has been shown not to suppress the HPA axis completely and was thought to be suitable for detecting subtle changes in sensitivity to DEX (Meaney et al., 1989).

VI. Corticosterone assay

Plasma aliquots were freshly thawed for the analysis of corticosterone, using an in-house specific radioimmunoassay as described previously (MacPhee *et al*, 1989), and modified for microtiter plate scintillation proximity assay. Plasma samples were diluted in borate buffer (135 mM sodium borate (pH 7.4), 0.5% bovine serum albumin, 1% methanol, 0.1% ethylene glycol, pH 7.4) and heated to 70°C in order to inactivate plasma proteins. ^{3}H-CORT (s.a. 84 Ci/mmol) and rabbit antirat-CORT antiserum (kindly donated by Dr C. Kenyon; final antibody dilution 1:10000 in borate buffer containing 0.01% Na azide) were added to each sample. After a 1-h incubation at room temperature, a secondary antibody bound to fluomicrospheres (anti-rabbit SPA reagent) was added. [^{3}H-CORT]-labelled antibody bound to the secondary antibody is immobilised by the fluomicrospheres, which then emit light as detected by a β-scintillation counter 24 h later. Samples were assayed in duplicate and their CORT concentrations estimated by comparison to serial dilutions of CORT

in duplicate, ranging from 320 to 0.613 nM, using the 1224 Multicalc Software program. Results are presented as μg/dl plasma. The inter- and intra-assay coefficients of variation were <10% and <5 %, respectively, whilst the detection limit was 0.15 μg/dl.

VII. In situ hybridisation histochemistry

This technique allows visualisation of the exact cellular and/or structural location of specific mRNAs (indicating transcription of the corresponding genes) by hybridisation of a radiolabelled 'antisense' RNA probe to the mRNA of interest. Labelled RNA 'sense' probes of similar length, nucleotide content and specific activity were included in each experiment in order to asses the specificity of the probe and the hybridisation reaction.

Probes

RNA probes for mineralocorticoid receptor (MR), glucocorticoid receptor (GR) and corticotropin-releasing hormone (CRH) mRNAs were produced *in vitro* using T3, T7 or SP6 phage polymerase systems. The templates used in this study were a kind gift from Dr Arriza, San Diego (MR), Dr Miesfeld, San Francisco (GR), and Dr. Thompson, Portland (CRH), were contained in plasmids (listed in table 2.1) and where necessary subcloned by June Noble and Karen French. The cDNA fragments of interest, flanked by T3, T7 or SP6 promoter sequences (table 2.1), were linearised with the appropriate restriction enzymes (table 2.1). After phenol/chloroform extraction and ethanol precipitation, 0.5-1.0 μg of linear DNA template was transcribed by incubation with a ribonucleotide mix (ATP, CTP, GTP, UTP), ^{35}S-UTP (s.a. 800 Ci/mmol), 6.7 mM DTT, 0.5 μl RNase inhibitor and 1 μl of the appropriate polymerase (see table 2.1) in a total volume of 10 μl in transcription buffer (see table 2.1) for 1 h at 37°C (T3, T7) or 40°C (SP6). The proportions of labelled and cold UTP were adjusted to yield probes of the specific activity required. Adding 1 μl DNase I for 10 minutes at 37°C degraded the DNA templates, after which probes were purified using NICK columns (Sephadex G50). For each probe,

the total activity was estimated by counting 1 µl in a β-counter (minimum activity required 2.5×10^8 cpm/ml), and purity was determined by running 1 µl on a polyacrylamide gel and exposing the gel to Hyperfilm β-max, which should produce a single black band on the film. Finally, probes were stored at -20°C for a maximum of 4 days.

probe	*vector*	*insert*	*probe length (nt)*	*restriction enzyme*	*RNA polymerase*	*temp (°C)*
GR s	pGEM3	Pst1-EcoRI	673	Eco RI	SP6	40
GR as	pGEM3	Pst1-EcoRI	673	Ava I	T7	37
MR s	pGEM4	EcoRI	513	Eco RI	T7	37
MR as	pGEM4	EcoRI	513	Hind III	SP6	40
CRH s	Bluescript Ks	PvuII-BamHI	518	Hind III	T7	37
CRH as	Bluescript Ks	PvuII-BamHI	518	Xba I	T3	37

Table 2.1 **Characteristics of rat GR, MR and CRH riboprobe generation.**
s sense; *as* antisense.

Slide preparation

Before use, glass microscope slides were washed in 0.2 M HCl, DEPC-treated water (0.1% DEPC in ultrapure water) and acetone for 3 minutes each. Slides were then subbed in a solution containing 0.03% Na-azide and 15% gelatine and left to dry o/n at 50°C. The following day, slides were subbed in poly-L-lysine solution (100 mg in 500 ml DEPC-water) for 20 seconds, twice washed in DEPC-water for 10 minutes and left to dry at 50°C. Dried slides were wrapped in aluminium foil until further use.

Tissue preparation

Rats were decapitated between 09:00 h and 12:00 h. Brains were removed, rapidly frozen on powdered dry ice and stored at -80°C. Coronal cryosections (10 μm) were cut at a temperature of –18°C at the level of the PVN, hippocampus and amygdala (approx. bregma –2.00 mm, -2.80 mm, -2.30 mm, respectively (Paxinos and Watson, 1986)). Sections were immediately thaw-mounted onto gelatine-subbed, poly-L-lysine-coated slides and stored at -80°C until further use.

Post-fixation

Slides were kept on dry ice until the start of the post-fixation procedure. Sections were post-fixed for 10 minutes in pre-chilled 4% paraformaldehyde/0.1 M phosphate buffer (3.12 g NaH_2PO_4, 11.28 g Na_2HPO_4 in 1 l DEPC-water, with 40 g paraformaldehyde added at 80°C), rinsed twice in 1× phosphate buffered saline (PBS, 10× diluted from 10x PBS (80 g NaCl, 29 g Na_2HPO_4.2H2o, 2 g KH_2HPO_4, 2 g KCl in 1 l DEPC-water)), acetylated for 10 minutes in 0.1 M triethanolamine (13.3 ml triethanolamine in 1 l DECP-water, pH 8.0, 0.75 ml acetic anhydride added prior to transfer of slides), rinsed in 1× PBS and dehydrated in 70-95% ethanol solutions.

Pre-hybridisation

For CRH mRNA detection, sections were pre-hybridised with 200 μl/slide buffer (2× pre-hybridisation buffer consisting of 0.6 M NaCl, 10mM Tris-HCl pH 7.5, 1mM EDTA pH 7.5, 1× Denhardt's solution, 0.5 mg/ml denatured salmon sperm DNA and 0.1 mg/ml yeast tRNA diluted 1:1 with formamide (deionised using 10% amberlite resin) for 3 h at 50°C in humidified boxes (box buffer, 50% deionised formamide, 20% 20× SSC, 30% DEPC-water).

Hybridisation

Sense and antisense riboprobes were defrosted, added to hybridisation solution consisting of 50% deionised formamide and 50% 2× hybridisation buffer (10% dextran sulphate, 0.6 M NaCl, 10 mM Tris-Cl pH 7.5, 1mM EDTA pH 7.5, 1× Denhardt's solution, 0.1 mg/ml denatured salmon sperm DNA and 0.1 mg/ml yeast tRNA), and denatured at 75 °C. Pre-hybridisation buffer was drained from the slides and probes (10-15 x 10^6 cpm/ml, 200 µl/slide) were applied to sections and hybridised overnight at 50°C in humidified chambers (box buffer as above).

RNAse digestion of unbound probes

Slides were washed 3 times 5 minutes in 2×SSC and carefully wiped dry around the sections with lens tissue. RNase A (200 µl/slide, 30 mg/ml in RNase buffer, consisting of 10mM Tris-Cl pH 7.5, 1mM EDTA pH 7.5 and 0.5 M NaCl in ultrapure water) was applied to the sections and left to incubate for 1 h at 37°C in humidified (RNase buffer as above) boxes.

Washes and dehydration of sections

Slides were washed for 15-30 minutes in 2×SSC at room temperature, then twice in 0.1× SSC at 60°C and left to cool down to room temperature. Sections were then dehydrated through 50%, 70% and 90% ethanol in 0.3 M ammonium sulphate solutions for 2 minutes each and left to dry.

Visualisation of positive areas

Sections were exposed to autoradiographic film (Hyperfilm β-max) for 7-10 days. Afterwards, they were individually dipped in NTB-2 photographic emulsion (diluted 1:1 with ultrapure water at 40°C) and exposed in light-tight boxes at 4°C for 21-28 days. They were then developed in D19 solution (diluted 1:1 with water), fixed in Amfix solution (diluted 1:5 with water) and counterstained with 1% w/v pyronin Y.

Cellular MR, GR and CRH mRNA expression was quantified by blindly counting silver grains overlying identified neurones (~10 measurements per subfield in two replicate sections from each brain area) under bright-field illumination using an automatic image-analysis system (Seescan). Results were calculated as mean number of grains/cell area for each subregion after subtraction of background (counted over areas of white matter), which was always low, so that the number of grains in expressing cells was higher than 10× background expression. Alternatively, total mRNA expression per region was determined by measuring optical density (O.D.) of the relevant area on autoradiograms using a PC-based image analysis package (MCID, Research Imaging, Canada). Typical expression patterns for MR and GR mRNA in brain sections taken at the level of the hippocampus are shown in figure 2.2. The advantages and disadvantages of using *in situ* hybridisation analysis are discussed in the general discussion (chapter 7).

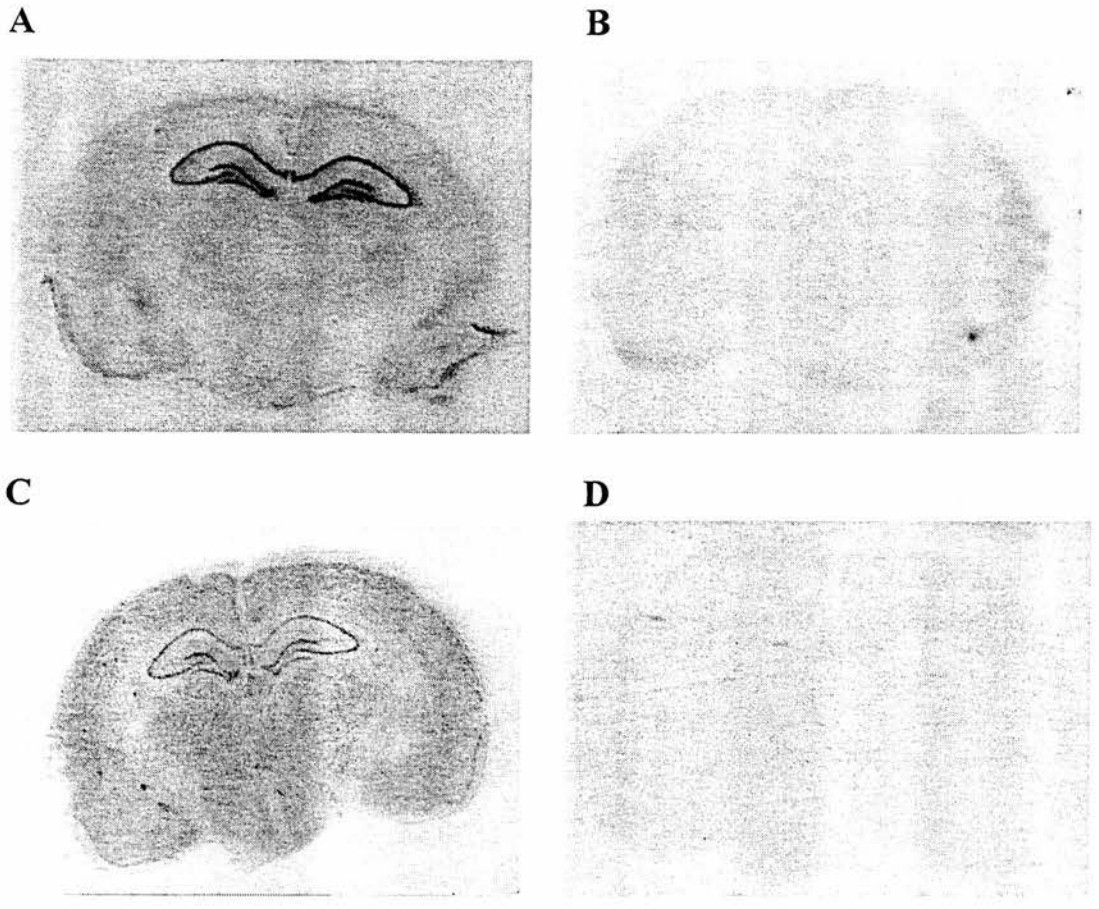

Figure 2.2 **Typical MR and GR mRNA expression patterns in rat brain at the level of the hippocampus.**
These autoradiographic images show the restricted distribution of MR mRNA when hybridised with antisense (*A*) and sense (*B*) probes. GR mRNA is more widely expressed, as shown by the strong signal obtained with an antisense (*C*) probe, whereas a sense probe (*D*) reveals no visible hybridisation.

VIII.1 Open-field test

Rats were tested for their exploratory and anxiety-like behaviour in a large novel environment. The apparatus consists of an octagonal field with a diameter of 1 m surrounded by 31-cm high walls. The field was divided into 16 parts: 4 central and 12 peripheral segments. Testing took place in an air-conditioned, reasonably soundproof, brightly lit room between 9:00 h and 15:00 h. Sixteen hours before testing, paired-housed animals were transferred in their home cages to a room adjacent to the testing room. All rats were weighed, as an indicator of their dominance or subordinance and immediately put in the centre of the open field. Animals were allowed to explore the field freely for 12 minutes, during which their behaviour was monitored by a video camera. Following each 12-minute session the field was thoroughly wiped with ethanol to remove any olfactory cues. The recorded behaviour was analysed by an experimenter blind to treatment conditions. The following parameters were considered: (1) total ambulation (expressed as the number of grids crossed), (2) number of centre entries, (3) time spent grooming, (4) number of rears and (5) defecation.

VIII.2 Elevated plus-maze test

This test is a common test often used to assess anxiety. It evokes an approach-avoidance response in rats, based on their natural exploratory drive and their innate fear of open spaces. Thus, a highly anxious animal will tend to avoid the open arms on an elevated plus-maze and spend the majority of time on the closed arms (Pellow and File, 1985, 1986). The elevated plus-maze is a plus-shaped platform made of black plastic. It consists of two opposing open arms (50 cm × 10 cm) and two arms of the same size but enclosed by 40-cm high walls. A central area of 10 cm^2 connects all 4 arms. The maze is elevated 64 cm from the floor.

At the start of the test, a rat was placed in the central area facing one of the open arms and allowed to explore the maze freely for 10 minutes. During this time, the following number of open- and closed-arm entries and the time spent on open and closed arms were determined. This was achieved by computer-controlled (MazeSoft software, Panlab, Spain) recording of the interruptions of infrared photo beams that were located at the beginning and halfway each arm. All rats were tested between 9:00 h and 12:00 h.

VIII.3 Forced-swim test

This test is used to obtain an indication of 'behavioural adaptation' and is regarded as a test for nonspatial learning. It is based on the fact that a rat will recognise the inescapability of a situation upon second exposure. A cylindrical bucket (height 40 cm, diameter 35 cm) was filled with 21°C water to a level of 25 cm. In two sessions, called 'acquisition' and 'retention test', respectively, 24 h apart, rats were placed in the water and remained there for 10 minutes. During this time, the duration of active escape behaviour (swimming, diving, climbing) and passive behaviour (floating or 'acquired immobility') was measured. Immobility typically shows after an initial period of escape behaviour (climbing, struggling, diving) and is thought to reflect the rats' ability to learn that escape is impossible. When the procedure is repeated 24 h later (retention test), rats will have retained the acquired immobility and show passive behaviour earlier than on the first exposure. After the second exposure, a blood sample was taken from the tail vein to measure corticosterone levels. After testing animals were thoroughly dried off with a towel and put back in their home cages. Between six and ten paired-housed rats from each treatment group were tested, but rats housed together were tested 4 days apart. All testing was carried out between 9:00 h and 12:00 h.

VIIII.4 Neophagia test

This test is a conflict-based test in which a food-deprived rat has to overcome its aversion of novel spaces in order to approach food (Britton and Britton, 1981).

Longer latencies to approach food and start eating reflect increased anxiety. Thus, rats were deprived of food for 18 hours. Subsequently they were placed in a novel environment, consisting of a large cage with Perspex walls and no bedding. Around 10 pellets of rat chow were placed in the centre of the cage and animals were allowed to freely explore the cage and eat the food for 5 minutes. This induced a conflict in rats between their appetite and novelty-induced fear, such that more fearful rats were expected to wait longer before moving to the centre of the cage to eat. In order to control for possible differences in basal appetite between the treatment groups, separate numbers of rats from each treatment group were provided with the same amount of food in their home cage. The experimenter observing the animals was unaware of their prenatal treatments. The following parameters were recorded: latency to approach the food, number of approaches, eating latency and time spent eating. All testing was carried out between 9:00 h and 12:00 h.

VIII.5 Watermaze test

Spatial learning and memory is often assessed in a watermaze test, introduced by Dr R. Morris (Morris, 1984). In this test, rats are trained in a circular pool to find a platform submerged under the water surface on 4 consecutive days (the *acquisition/consolidation* compound of the test). On the fifth day, the platform is removed and the amount of time spent around the former platform location is recorded (the *memory/strategy* compound of the test).

Apparatus

The watermaze consisted of a circular pool (∅ 2 m) filled with water of 26 ± 1°C, made opaque with latex, that was arbitrarily divided into four quadrants. An escape platform (∅ 10 cm), submerged 1.5 cm, was located in the middle of one of the quadrants. Visual cues (posters) were placed on the walls of the testing room and remained there for the duration of the test. The rats' behaviour was recorded using a video camera, connected to a computer analysis system, that was fixed above the

centre of the pool. Three days before the test started, rats were allowed to habituate to the water for a period of 2 minutes.

Acquisition procedure

During the acquisition phase of the test, rats were trained to locate the submerged platform, which was located at the 'north-east' (NE) position for 50% of rats, and located at 'south-west' (SW) for the other 50%, using visual cues. For each rat, the platform remained in the same position across trials. Each trial started with the rat placed into the pool facing the wall of the pool at one of four starting positions. It was then given 2 minutes to locate the platform. If a rat failed to find it within 120 seconds, the experimenter placed the rat on the platform manually. The rat was left there for 30 seconds before the next trial began. Each rat was subjected to four trials per day. The latency to find the platform ('escape latency') was monitored for each trial as an indicator of spatial learning. At the end of a daily session, the rats were dried off thoroughly and put back into their home cage. This procedure was repeated the following 3 days, during which the platform remained in the same position. The starting position of the rats however, was changed between trials so that each starting position was used once every day.

Retention test

On the fifth day of the test, the platform was removed and the animals' behaviour was recorded during a 1-minute free-swim trial (probe test). The following parameters were recorded: a) latency until the former platform location was reached; b) number of crossings of the former platform location; c) percentage of time spent swimming in the quadrant where the platform had been located; d) swim speed; e) total distance swum; f) percentage of time spent swimming along the walls of the pool (thigmotaxis).

IX. Data analysis

Image analysis: Seescan plc., Cambridge, UK

Image analysis: MCID, Research Imaging, Canada

Elevated Plus-maze: MAZE, Panlab, s.l., Barcelona, Spain

CORT-assay: 1224 Multicalc Software v2.0 (Wallac Software)

Watermaze: HVS image analyser, Acorn Archimedes computer

X. Statistics

Data are presented as mean value per group ± s.e.m. Normally distributed data from two treatment groups were compared using Student's t-test, data from three or more treatment groups were compared using ANOVA followed by an LSD test, where appropriate. Where the normality tests on data failed, an ANOVA on ranks was performed.

The combined behavioural data from chapter 6 were analysed by multivariate (factor) analysis using Statistica 5.1 package. This is a statistical approach that analyses the correlations between variables and tries to detect structure in them by identifying common underlying dimensions (factors). Thus, factor analysis aims to reduce the number of variables for a number of new indices that are linear combinations of the original variables. These indices are then independent from each other and represent the underlying dimensions of the data.

The principal factors were selected according to an eigenvalue > 1, which means that factors that accounted for only a small proportion of the total variability were ignored. The remaining factors were then subjected to a varimax rotation (so that the relative locations of the factors to each other remained unchanged), in order for their significance to be interpreted more easily.

Chapter 3. Effects of prenatal glucocorticoid exposure on offspring HPA activity (studies with CBX and DEX)

Introduction

Prenatal stress reduces birthweight in rats (Kinsley and Svare, 1986; Takahashi et al., 1988), and induces permanent effects on offspring sexual behaviour (Holson et al., 1995), hormonal and behavioural responses to stress (Fride et al., 1986; Henry et al., 1994; Takahashi et al., 1988; Vallee et al., 1997; Weinstock et al., 1992) and brain monoamine systems (Day et al., 1998; Peters, 1984; Peters, 1990; Takahashi et al., 1992a). In general, prenatal stress impairs coping and alters the HPA response in aversive situations.

The mechanisms underlying the effects of prenatal stress have not yet been convincingly established. Prenatal undernutrition clearly has programming capacities (Almeida et al., 1996; LangleyEvans et al., 1996; Woodall et al., 1996), and stressed pregnant rats eat and drink less than unstressed rats (Kinsley and Svare, 1986). According to an alternative theory, high levels of the glucocorticoid CORT secreted in response to stress render maternal CORT an obvious candidate 'programming factor' in this paradigm. In rats, prenatal exposure to the synthetic glucocorticoid dexamethasone (DEX) reduces birthweight, affects brain development (Slotkin et al., 1993) and programs hypertension and hyperglycaemia in adult offspring (Benediktsson et al., 1993; Nyirenda et al., 1998). Moreover, prenatal glucocorticoid exposure reduces birth weight in humans [see (Seckl, 1994)] and some preliminary studies have indicated that even small doses of DEX given prenatally alter neuropsychological parameters such as emotionality in humans (Lajic et al., 1998; Trautman et al., 1995).

The fetus is normally thought to be protected from the relatively high levels of maternal physiological glucocorticoids by 11β-HSD2, which catalyses the conversion of active CORT (cortisol in humans) into inert 11-dehydroCORT (cortisone), whereas DEX is a poor substrate for this enzyme (Siebe et al., 1993).

11β-HSD2 is highly expressed in the placenta, as well as in many fetal tissues (Brown et al., 1996; Stewart et al., 1994). Interestingly, placental 11β-HSD2 activity correlates positively with birthweight in rats (Benediktsson et al., 1993) and in some human studies (Stewart et al., 1995). Moreover, humans with mutations of the 11β-HSD2 gene have low birth weight (DaveSharma et al., 1998; Kitanaka et al., 1996; White et al., 1997). Inhibition of feto-placental 11β-HSD by administration of carbenoxolone (CBX) to pregnant rats decreases offspring birthweight and programs hypertension and hyperglycaemia later in life (Lindsay et al., 1996a; Lindsay et al., 1996b) in a similar way to DEX administration.

These data raise the hypothesis that when the feto-placental barrier to maternal CORT is defective, inhibited or bypassed, the resulting prenatal exposure to glucocorticoids may cause alterations in HPA activity similar to those seen in prenatally stressed rats. This hypothesis was tested by injecting pregnant females with the 11β-HSD inhibitor CBX and investigating the effects of this inhibition on offspring HPA activity and –regulation. Moreover, in order to establish whether the effects of CBX were due to overexposure to glucocorticoids, the effect of prenatal exposure to the synthetic glucocorticoid DEX on the same parameters was tested.

Prenatal treatments

As described in chapter 2. In short, pregnant females injected daily throughout pregnancy with carbenoxolone (CBX, 12.5 mg/200 µl) or dexamethasone (DEX, 100 µg/kg), dissolved in 4% ethanol-0.9% saline (n=6-8 per group in each of two separate experiments), or the vehicle solution (controls). Body weight and food intake of pregnant rats were measured daily. On day 20 of pregnancy a blood sample was taken from the tail vein (see below) for determination of plasma CORT levels.

Litters

As described in chapter 2. In short, on the day of birth, litters were weighed, sexed and culled to 8 pups per litter. Remaining pups were sacrificed by decapitation, their brains were removed and quickly frozen on powdered dry ice and their blood collected for CORT measurement. Offspring were weighed regularly, starting 4 weeks after birth. In a separate experiment with identical prenatal- and birth protocols, one pup from each litter was sacrificed at 1 and 2 weeks of age and blood was collected for determination of plasma CORT concentrations. In addition, brains from the 1 week-old sacrificed pups were removed and quickly frozen on dry ice.

Blood sampling

As described in Chapter 2. Pilot experiments (chapter 2) showed that using this method, very low (0.5-1.5 µg/dl) basal morning CORT levels can be obtained.

HPA axis activity

As described in chapter 2. In short, 6 month-old rats were handled regularly during the week before the experiment. Blood samples were taken at 08:00 h, 14:00 h and 20:00 h, but in order to avoid possible stress induced by making more than one tail

cut on one day, sampling was spread out over several days for each rat. Evening samples were taken under red-light illumination.

Stress-induced activation of the HPA axis was determined using a procedure in which adult rats were restrained in Perspex cylinders for 20 minutes. Plasma CORT levels were measured in blood samples taken from the tail vein at several times before, during and after termination of the restraint procedure

In a separate experiment, we determined the sensitivity to the suppressive effects of DEX on CORT secretion in the rats. Thus, rats were given a single bolus of DEX (40 μg/kg) s.c., after which the restraint procedure described above was performed.

Corticosterone assay

As described in Chapter 2.

In situ hybridisation histochemistry

In situ hybridisation was performed as described in Chapter 2 on sections taken at the level of the PVN (adult rats; GR and CRH riboprobes) and hippocampus (pups and adult rats; MR and GR riboprobes). Equivalent 'sense' probes were included as controls. MR, GR and CRH mRNA expression was quantified by counting silver grains overlying identified neurones under bright-field illumination using an automatic image-analysis system. Results were calculated as mean number of grains/cell area for each subregion after subtraction of background (counted over areas of white matter), which was always low.

Statistics

Data are presented as mean value per group ± s.e.m. Data from the three treatment groups were compared using a one-way or two-way ANOVA followed by an LSD test, where appropriate. Significance was set at $p < 0.05$.

Effects of daily injections with CBX or DEX on pregnant rats

Vehicle and DEX treatment were tolerated well. In contrast, multiple CBX injections in the same region caused sores at the site of injection, but not to a similar extent in all CBX-injected rats. Thus, in order to avoid distress and possible infection as much as possible, injection sites were varied daily for CBX rats.

Weight gain as well as food intake during pregnancy were different between the three groups (weight gain: $F_{2,19}=48.98$, $p<0.0001$; food intake: $F_{2,19}=17.02$, $p<0.0001$). Both parameters were lower in rats that received daily injections with CBX or with DEX (Table 3.1). The amount of food eaten *per g weight gain* during pregnancy also differed between groups ($F_{2,19}=22.97$, $p<0.0001$), with DEX- but not CBX- injected rats eating more food per g weight they gained, compared to control mums. Maternal plasma CORT levels on day 20 of pregnancy were influenced by the prenatal treatments ($H_{2,14}=12.36$, $p<0.002$) and varied highly in CBX-treated mums. Levels were higher in CBX-mums than in DEX-injected animals, but neither differed from concentrations in control dams (Table 3.1).

	Total food intake (g)	*Total weight gain (g)*	*Food intake (g) per g weight gain*	*Plasma [CORT] day 20 (μg/dl)*
control	483 ± 11	128 ± 5	3.8 ± 0.1	16.6 ± 2.5
CBX	421 ± 18**	103 ± 7**	4.2 ± 0.2	40.0 ± 10.4*
DEX1-3	377 ± 13***	57 ± 4***	6.8 ± 0.5***	3.7 ± 0.7

Table 3.1 **Effect of treatment on pregnant rats.**
Food intake and weight gain were measured daily from day 2 of pregnancy until the day before delivery. Note the high variation in CORT levels in CBX rats. *p<0.05, *versus* DEX1-3,** p<0.01, *** p<0.001, **** p<0.0001 *versus* control; n=7-8/group for weight gain and food intake; n=5-6/group for plasma [CORT].

Effects of prenatal injections with CBX or DEX on offspring

Prenatal injections with CBX or DEX had no effect on gestation length ($F_{2,32}=0.51$, $p=0.61$) or litter size ($F_{2,32}=0.72$, $p=0.49$). On the day of birth, plasma CORT concentrations were different between the three groups ($F_{2,16}=13.34$, $p<0.001$): CBX-offspring had higher plasma CORT concentrations than control pups. However,

plasma CORT levels taken during the SHRP (see Chapter 1) on pnd 7 and pnd 14 did not differ between groups (Table 3.2). Furthermore, prenatal treatment with CBX or with DEX affected birth weight ($F_{2,82}$=15.24, p<0.0001). Birth weights were reduced by 12% and 15%, respectively (Table 3.3). Reduced body weight persisted in DEX offspring until at least 5 months of age, whereas CBX rats showed catch-up growth and had similar body weight to controls from 6 weeks of age onwards (Table 3.3).

	Litter size (n)	*Gestation length (days)*	*[CORT] pnd1 (μg/dl)*	*[CORT] week1 (μg/dl)*	*[CORT] week2 (μg/dl)*
control	11.3 ± 0.5	21.9 ± 0.2	6.7 ± 1.5	1.4 ± 0.2	4.3 ±1.1
CBX	10.5 ± 0.5	21.9 ± 0.3	13.1 ± 1.1*	1.4 ± 0.3	3.1 ± 2.2
DEX1-3	10.8 ± 0.6	22.2 ± 0.1	5.2 ± 0.8	0.9 ± 0.1	3.1 ± 1.7

Table 3.2 **Effect of prenatal injections on gestation length, litter size and plasma CORT levels in pups.**
*p<0.01 *versus* control; n=11-12/group for gestation length and litter size, n=6-7/group for plasma CORT levels.

	Day 1	*Week 4*	*Week 6*	*Week 9*	*Week 12*	*Week 20*
control	5.79 ± 0.09	73.4 ± 0.9	153 ± 2	261 ± 2	319 ± 4	380 ± 5
CBX	5.46 ± 0.11*	67.6 ± 1.6*	150 ± 2	255 ± 3	314 ± 4	372 ± 6
DEX1-3	4.94 ± 0.12**	67.7 ± 2.0*	137 ± 2**	241 ± 4**	301± 5*	355 ± 8*

Table 3.3 **Effect of prenatal injections on body weight.**
Results are expressed as g bodyweight. *p<0.01; **p<0.005 *versus* control; n=18-47/group.

Brain MR, GR and CRH mRNA expression in 1 day and 1 week-old offspring

Slides containing sections of both ages were processed together in two *in situ hybridisation* procedures for MR mRNA and two for GR mRNA. Therefore, the measured expression per neurone is in figure 3.1 expressed as % expression in control rats.

Hippocampal GR mRNA levels increased by 2-3 fold during the first week of life. However, prenatal exposure to glucocorticoids had no effect on GR mRNA expression in male rats of either age (Figure 3.1, *top panel*).

Hippocampal MR mRNA expression was relatively high in newborn rats and was decreased slightly in 1 week-old pups. Prenatal treatments had no effect on neuronal MR mRNA levels in 1 day-old rats' hippocampi, but at 1 week of age a significant

treatment effect occurred in CA2 ($F_{2,14}$=7.58, $p<0.05$). *Post-hoc* analysis revealed that in this region, CBX offspring have higher MR mRNA expression than DEX offspring ($p<0.01$), but there are no significant differences compared with control offspring (Figure 3.1, *bottom panel*).

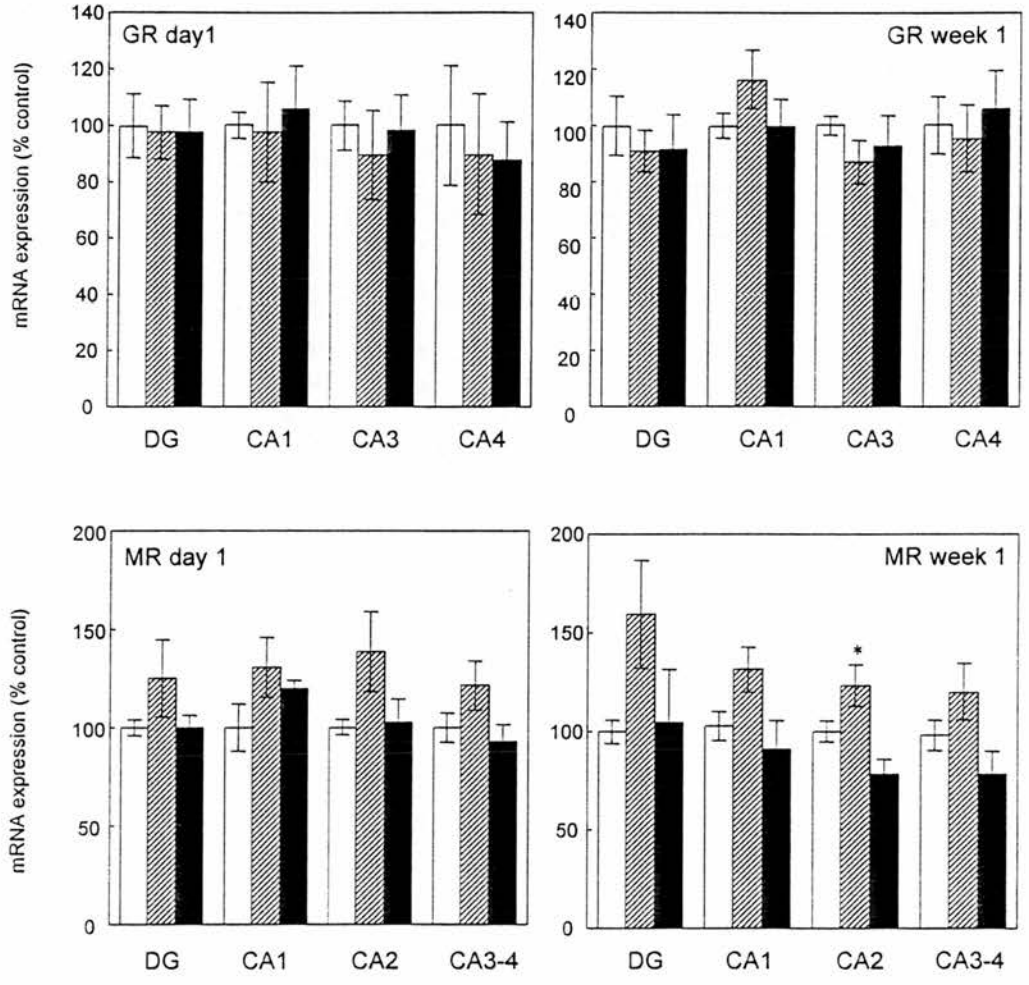

Figure 3.1 **Hippocampal GR and MR mRNA expression in 1 day-old and 1 week-old pups.** Data are expressed as % expression in control pups. *Open bars* control rats, *striped bars* CBX rats, *closed bars* DEX1-3 rats * $p<0.05$ *versus* DEX1-3 pups; n=5-7/group.

Prenatal exposure to glucocorticoids altered GR mRNA expression in PVN of 6 month-old rats ($F_{2,17}$=9.73, p<0.01), and *post-hoc* analysis revealed that prenatal CBX- but not DEX- injections were associated with a significant decrease in GR mRNA expression in the PVN (Figure 3.2, *left panel*; Figure 3.3). ANOVA for CRH mRNA expression in PVN approached significance ($F_{2,16}$=3.00, P=0.07), and figure 3.2 (Figure 3.2, *right panel*; Figure 3.4) shows a trend in both CBX and DEX offspring towards increased CRH mRNA expression in PVN that becomes significant when CBX- and DEX rats are compared to controls separately. Daily injections with either CBX or DEX had no effect on MR or GR mRNA expression in any of the hippocampal subfields in 6 month-old rats (Table 3.4).

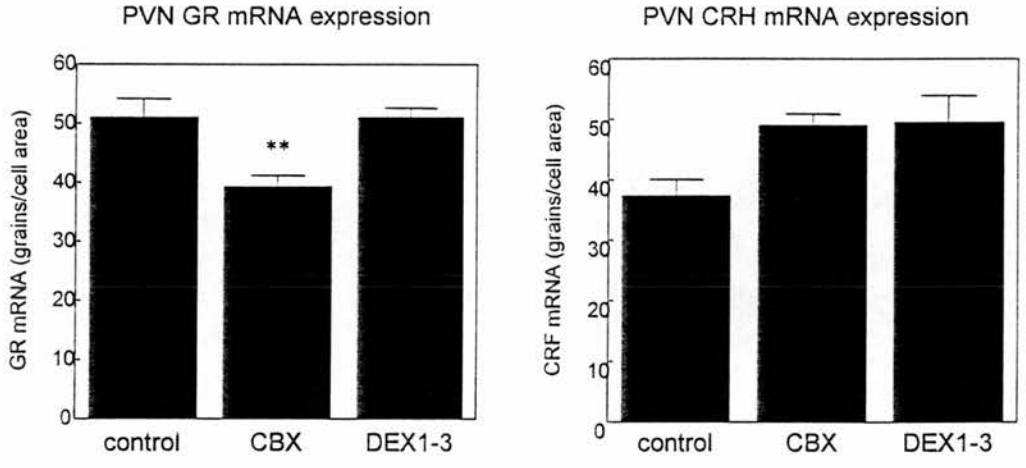

Figure 3.2 **GR and CRH mRNA expression in PVN of 6-month old rats.**
Left panel GR mRNA expression; *right panel* CRH mRNA expression. **p<0.01, n=6-7/group.

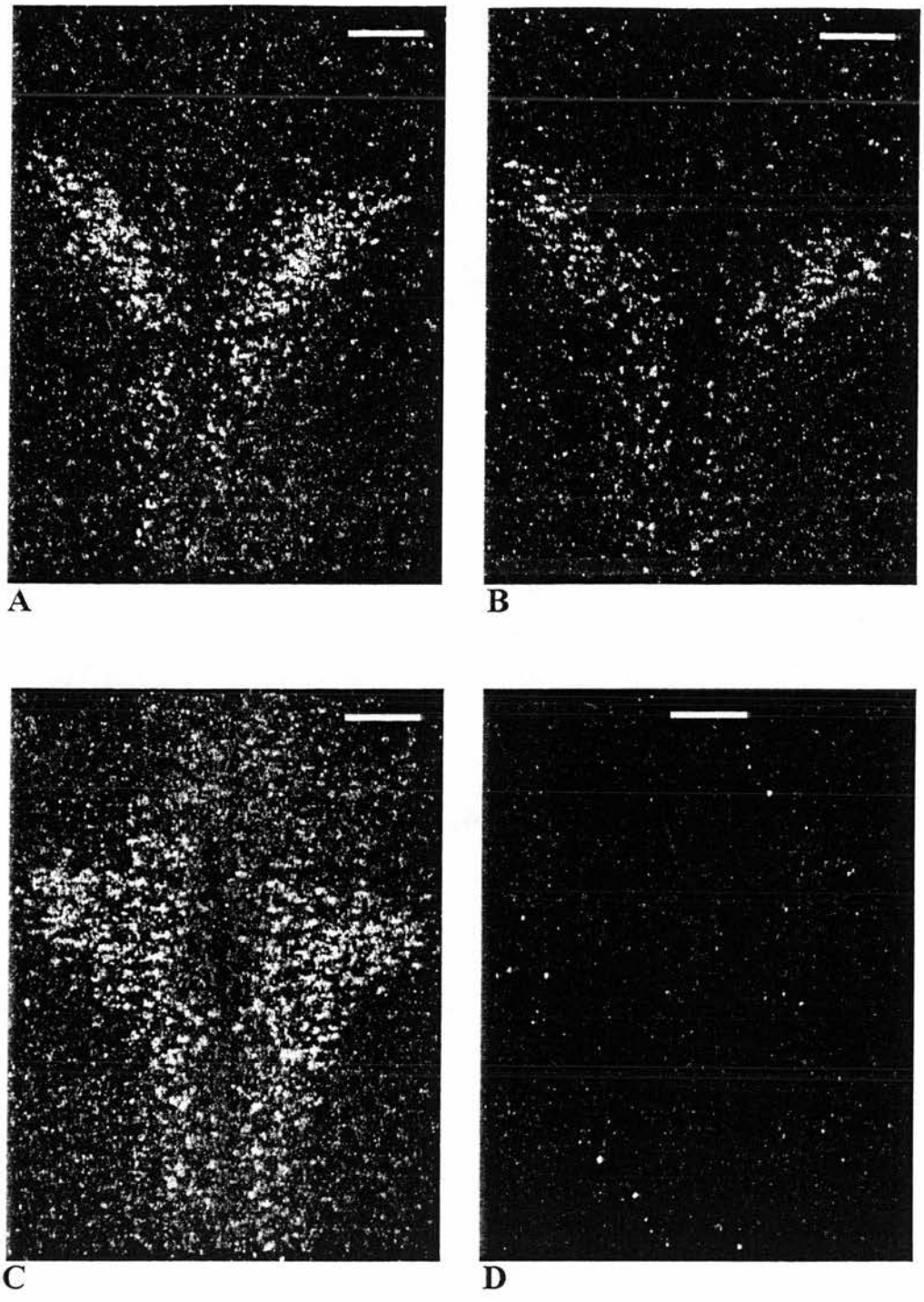

Figure 3.3 **Photomicrographs of GR mRNA expression in PVN of 6 month-old rats.**
Images show typical sections from animals in each treatment group. *A*-control, *B*-CBX, *C*-DEX1-3, *D*-sense. Scale bar represents 200μm.

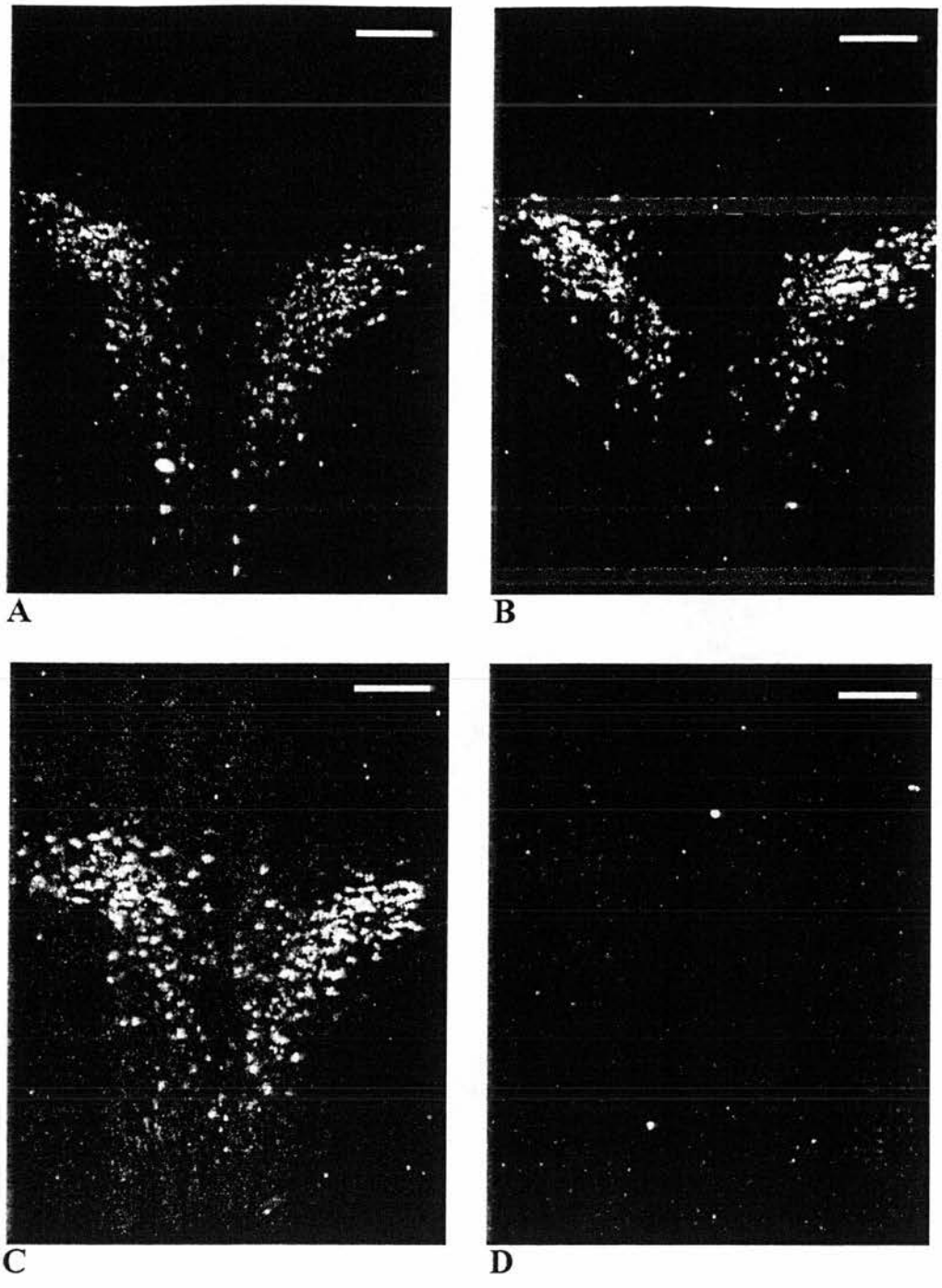

Figure 3.4 **Photomicrographs of CRH mRNA expression in PVN of 6 month-old rats.**
Pictures show typical sections from animals in each treatment group. *A*-control, *B*-CBX, *C*-DEX1-3, *D*-sense. Scale bar represents 200 μm.

MR mRNA	*DG*	*CA1*	*CA2*	*CA3-4*
control	127.9 ± 8.2	103.4 ± 3.9	143.7 ± 3.7	83.7 ± 3.0
CBX	124.9 ± 10.5	99.1 ± 4.7	139.1 ± 1.9	79.9 ± 2.2
DEX1-3	124.5 ± 9.5	100.8 ± 1.4	136.3 ± 2.5	81.3 ± 1.9

GR mRNA	*DG*	*CA1*	*CA3*	*CA4*
control	111.3 ± 2.9	111.0 ± 3.3	43.0 ± 2.2	33.8 ± 1.6
CBX	108.8 ± 1.7	103.7 ± 4.9	41.6 ± 1.4	34.3 ± 0.7
DEX1-3	109.2 ± 2.1	104.9 ± 3.8	43.1 ± 2.2	35.0 ± 2.2

Table 3.4 **Hippocampal MR and GR mRNA expression.**
Results are expressed as mean number of grains/cell area ± s.e.m. for each subregion after subtraction of background; n=7-8/group.

HPA axis activity in adult offspring

a. Circadian variation in plasma CORT levels

A number of samples taken at 14:00 h had CORT levels exceeding those of evening samples. Further investigation revealed that on some days rats had been fed and watered shortly before afternoon blood samples were taken. As values resulting from these particular samples did not reflect *basal* afternoon CORT levels, they were excluded from the analysis. Thus, samples sizes being different and multiple plasma

samples having been taken over several days, an ANOVA for repeated measures would not be the appropriate statistical method of analysis to use. One-way ANOVAs for each time point separately showed significant differences in CORT concentrations measured in morning (8:00 h) ($F_{2,49}$=3.33, $p<0.05$) and evening (20:00 h) ($F_{2,24}$=7.87, $p<0.01$) plasma samples, but not in afternoon (14:00 h) samples ($F_{2,12}$=0.07, p=0.93). Post-hoc analysis revealed that both CBX- and DEX-offspring have elevated basal morning CORT levels, at 0.70 ± 0.13 μg/dl and 0.76 ± 0.10 μg/dl, respectively, compared to controls (0.40 ± 0.07 μg/dl). In contrast, basal evening CORT levels are lower in DEX rats compared to controls, whereas CBX offspring did not differ from control rats (Figure 3.5).

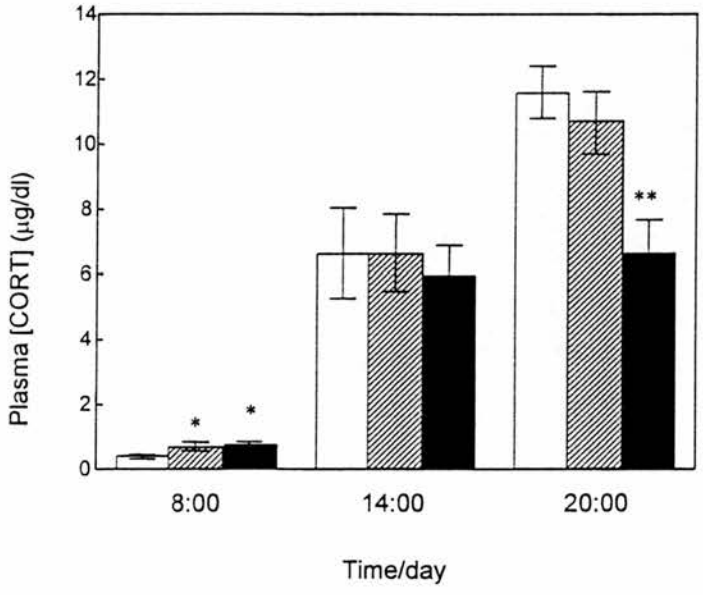

Figure 3.5 **Circadian variation in plasma CORT concentrations.**
Plasma CORT levels, expressed as μg/dl in adult rats prenatally exposed to CBX *(striped bars)*, DEX *(filled bars)*, or control rats *(open bars)*. *p<0.05, **p<0.01, n=16-18/group for 8:00 levels, n=5/group for 14:00 and 20:00 levels, n=9/group for 20:00 levels.

b. CORT response to restraint

Figure 3.6 shows the CORT concentrations in plasma samples taken before, during and after 20 minutes restraint. Rats prenatally treated with CBX or DEX did not respond to restraint differently from control rats. However, the maximum CORT levels measured were high in all groups (55-70 μg/dl), suggesting that ceiling levels of CORT output may have been reached. Furthermore, 90-150 minutes after initiation of the restraint procedure, none of the rats' plasma CORT concentrations had returned to basal levels, indicating that they may have been re-stressed. Therefore, a simplified version of this test was performed, and the results are shown in figure 3.7.

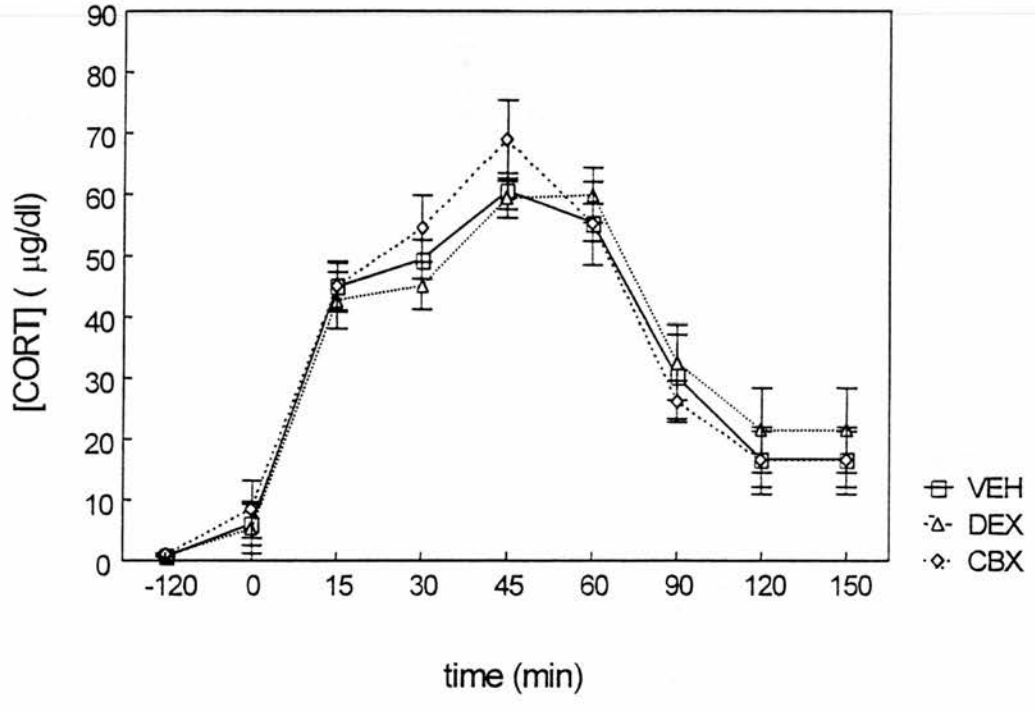

Figure 3.6 **Plasma CORT response to 20 minutes restraint.**
CORT concentrations measured in plasma samples taken before *(t=-120; t=0)*, 15 minutes into *(t=15)*, and at several time points after *(t=30-150)* 20 minutes restraint. n=9/group.

Figure 3.7 shows the CORT response to 20 minutes of restraint. An ANOVA for repeated measures revealed a time effect ($F_{2,30}$=33.02, $p< 0.001$), but no group effect, ($F_{2,15}$=1.09, p=0.36). There was a significant group×time interaction ($F_{4,30}$=3.2, $p<0.05$), and post-hoc analysis revealed that post-stress plasma CORT concentrations were elevated in CBX- but not DEX- offspring compared to control rats (Figure 3.7, *top panel*).

c. DEX-suppression of stress-induced CORT levels

A single bolus of DEX (40 μg/kg) given s.c. 3 hours before start of the restraint procedure, suppressed the CORT response to a similar extent in the three groups. ANOVA showed no group effect or group×time interaction (Figure 3.7, *bottom panel*), although there was a time effect ($F_{2,30}$=4.66, $p<0.05$), showing that the dose of DEX used had not suppressed the CORT response to restraint completely.

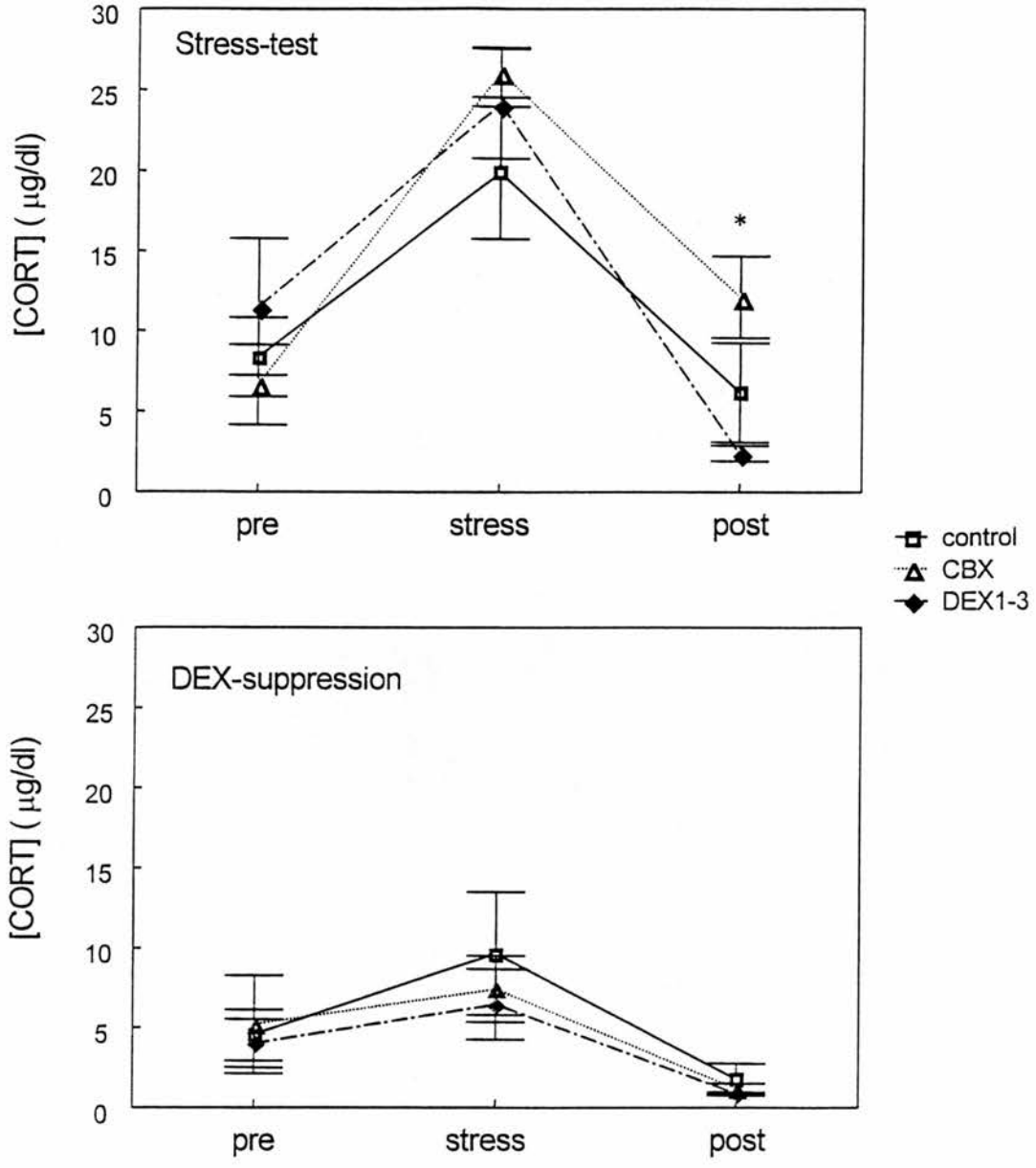

Figure 3.7 **Plasma CORT response to 20 minutes restraint.**
CORT concentrations measured in plasma samples taken before *(basal)*, 5 minutes into *(stress)*, and 90 minutes after *(post)* 20 minutes restraint *(top graph)*. The bottom graph shows the effect of a single DEX-injection (40 µg/kg) 3 h before start of the restraint procedure on the CORT response to restraint. *$p<0.05$, n=6/group.

Prenatal administration of CBX, an inhibitor of 11β-HSD, reduced birth weight and elevated basal and post-stress HPA activity. These findings are in accord with studies of prenatal stress, which causes persisting effects in adult life including reduced body weight and increased HPA activity (Fride et al., 1986; Takahashi and Kalin, 1991); (Vallee et al., 1996; Vallee et al., 1997), suggesting that a common mechanism may underlie these effects. Furthermore, prenatal exposure to DEX caused similar (although not identical) alterations. This indicates that glucocorticoid exposure *in utero* may be, at least partly, responsible for the changes observed in CBX offspring.

CBX is a potent inhibitor of 11β-HSD2, which rapidly converts CORT to inert 11-dehydroCORT (Brown et al., 1993) and is highly expressed in placenta and fetus (Brown et al., 1996). Previous experiments conducted in our laboratory showed that CBX treatment reduced placental 11β-HSD activity and increased ^{3}H-CORT levels in fetal tissues (Lindsay et al., 1996a). Thus, administration of CBX to pregnant animals is predicted to increase fetal exposure to CORT. Accordingly, in the present study plasma CORT levels were higher in newborn pups measured within 24 h of the last CBX-injection than in controls.

Previous studies showed that the effects of prenatal administration of CBX on blood pressure and blood glucose levels require intact maternal adrenal glands (Lindsay et al., 1996a, 1996b), and that they can be simulated by prenatal exposure to the synthetic glucocorticoid DEX (Benediktsson et al., 1993; Levitt et al., 1996; Nyirenda et al., 1998). This indicates that fetal exposure to maternal glucocorticoids underlies the changes observed in CBX offspring, as far as blood pressure and blood glucose levels are concerned. However, because adrenalectomy studies were not performed, it cannot be excluded that, in the present study, other factors than increased exposure to maternal glucocorticoids may have contributed to the effects of prenatal CBX described here. Indeed, prenatal administration of the synthetic glucocorticoid DEX did not result in identical changes in the parameters studied here. Several explanations are possible. Firstly, CBX is not an exclusive 11β-HSD

inhibitor and therefore may have affected other enzymes such as 3α-HSD (Baker, 1994). The 3α-HSD isozymes are involved in the production of neurosteroids, which influence brain functioning via interaction with the neurotransmitter $GABA_A$- and NMDA receptors (Paul and Purdy, 1992; Puia et al., 1990; Schumacher, 1990). Secondly, it is not unlikely that the CBX-paradigm may have acted not only to inhibit 11β-HSD activity in mother, placenta and fetus, but also as a maternal stress. CORT levels in CBX-injected rats varied greatly and may be related to the fact that most pregnant rats did not tolerate CBX injections well and that some developed sores at the site of injection. Consequently, factors released in response to infection or stress may underlie the alterations seen in CBX offspring. Stress-induced release of CORT in addition to inhibition of 11β-HSD may have exposed CBX-fetuses to much higher (endogenous) levels of glucocorticoids than the relatively low doses that DEX-fetuses were exposed to. Moreover, other factors associated with the possibly stressful CBX injections such as neurotransmitters or opioids may have played a role in programming CBX offspring. This possibility is supported by studies showing that the effects of prenatal stress on growth and behaviour in rats could be completely normalised by simultaneous benzodiazepine injections (Drago et al., 1999) and partly by blocking opiate receptors with naltrexone (Keshet and Weinstock, 1995).

Thirdly, although CBX and DEX were both administered daily throughout pregnancy, they may have acted at different times. Most fetal tissues show a rapid decline in 11β-HSD2 expression at the end of mid-gestation and even placental 11β-HSD2 mRNA begins to decline during the last week of pregnancy (Brown et al., 1996; Waddell et al., 1998). Thus, fetal tissue glucocorticoid exposure resulting from CBX-induced inhibition of fetal and placental 11β-HSD2 probably occurs mainly during the second week of pregnancy, whereas DEX may have acted for a longer period, GRs being expressed at least from midgestation onwards and increasing towards delivery (Bohn et al., 1994; Diaz et al., 1998; Kitraki et al., 1996).

Lastly, prenatal DEX only has a high affinity for GR, whereas increased CORT levels in the fetal circulation as a consequence of CBX administration may exert effects via both MR and GR, which have distinct and specific developmental expression [see chapter 1 and (Bohn et al., 1994; Diaz et al., 1998; Kitraki et al., 1996)] and gene targets. Thus, the patterns of 11β-HSD expression during

development, the stages of maturity of the fetal target tissues and the differential expression of corticosteroid receptors may underlie the time windows for specific effects of prenatal glucocorticoid exposure. This, in combination with the stressfulness of CBX injections may account for differential effects of prenatal DEX and CBX.

CBX and DEX treatment reduced body weight at birth and in DEX rats this effect persisted into adulthood. Insulin-like growth factors (IGFs) are important mediators of fetal growth, and the IGF system in turn is regulated by glucocorticoids (Li et al., 1993; Price et al., 1992). Indeed, IGF binding protein-1 (IGFBP-1), which inhibits the actions of IGF, was increased in fetuses that had been exposed to DEX (Price et al., 1992).
The mechanism underlying the persistence of the body weight reduction in DEX offspring may involve altered food intake and body weight regulation by glucocorticoids (Tempel and Leibowitz, 1994), possibly via modulation of leptin action (Inui, 1999; Spinedi and Gaillard, 1998). Further studies are needed to establish whether food intake is altered in DEX offspring, but interestingly, prenatally stressed rats, which in some studies also have permanently reduced body weight and increased HPA activity, show reduced food and water intake (Vallee et al., 1996).

An alternative hypothesis of prenatal programming has implicated undernutrition during pregnancy, in particular reduced protein intake (Almeida et al., 1996; Godfrey et al., 1994; Hales et al., 1991; Langley and Jackson, 1994; LangleyEvans et al., 1996a). The finding that daily CBX- and DEX injections throughout pregnancy reduced maternal food intake and body weight gain, similar to maternal stress during pregnancy (Kinsley and Svare, 1986), was therefore intriguing. It had already been shown that maternal protein restriction attenuates placental 11β-HSD2 activity in rats (LangleyEvans et al., 1996b) and increases maternal plasma cortisol levels in primates (Recabarren et al., 1998), suggesting that excessive exposure to maternal glucocorticoids may mediate, at least in part, the effects of prenatal undernutrition. The present study, however, shows that the opposite is also true: exposure to

glucocorticoids reduces maternal food intake. This indicates a reciprocal relationship between food intake and glucocorticoid levels during pregnancy, and the two hypotheses may not be mutually exclusive.

Prenatal CBX- and DEX administration increased basal CORT concentrations, indicating an increased central drive or reduced negative feedback of the HPA axis in the offspring. In addition, DEX rats had reduced evening CORT levels. Interestingly, subtle elevation of basal morning CORT concentrations has been shown to decrease evening CORT levels, thus ensuring that the mean daily output of CORT remained unaltered (Akana et al., 1992). The trend towards increased PVN CRH levels observed in both CBX- and DEX rats could indicate a slightly higher basal transcription rate of the CRH gene, resulting in greater release of CRH and concomitant elevation of basal CORT concentrations. However, ACTH levels in plasma and adrenal weights were not measured in this study. These would have provided important additional information regarding differences in HPA activity and adrenal sensitivity to ACTH in the three treatment groups.

Hippocampal corticosteroid receptors are involved in the regulation of basal HPA activity (De Kloet, 1991), but their mRNA levels were unaltered in CBX- and DEX rats compared to controls. This lack of effect was somewhat surprising, as DEX administration in the third week of pregnancy has been shown to reduce hippocampal MR and GR gene expression (Levitt et al., 1996). However, the hippocampus is not the only brain structure that mediates HPA activity. The prefrontal cortex (Herman and Cullinan, 1997; Sullivan and Gratton, 1999) and the amygdala (Weidenfeld et al., 1997) have also been implicated in the central control of HPA activity. Both structures contain moderate to high levels of corticosteroid receptors (De Kloet, 1991; McEwen et al., 1986). Thus, further studies are needed to establish whether inhibition of feto-placental 11β-HSD increases the central drive of the HPA axis by altering corticosteroid receptor expression in these areas.

In CBX offspring, levels of GR mRNA in the PVN were reduced. The PVN is considered to be one of the major sites for glucocorticoid negative feedback. Thus, it would not have been unlikely for CBX-rats to be less sensitive to DEX inhibition of stress-induced CORT secretion. However, sensitivity to the suppressive effects of a

single DEX-injection sufficiently low not to completely suppress the animals' HPA-axis, was similar in all groups. This discrepancy may be explained by the recent finding that DEX in low doses only poorly penetrates the brain (Meijer et al., 1998). If this was the case in our paradigm, then the DEX administered may have inhibited the HPA axis mainly at the pituitary level. Whether pituitary GR levels in CBX-DEX- and control offspring are similar has yet to be verified.

In contrast, post-stress plasma CORT levels were elevated in CBX offspring, indicating a reduced sensitivity to glucocorticoid negative-feedback that did not show up in the DEX-suppression test. This may be explained by the fact that in the stress test, *endogenous* CORT feeds back on GRs in pituitary *and* PVN, where a lower level of GR may be responsible for reduced negative-feedback sensitivity and thus elevated post-stress CORT levels.

The mechanisms whereby prenatal exposure to high levels of corticosterone alter corticosteroid receptor gene expression in the PVN have yet to be determined. Increased CORT levels *in utero* could directly modulate corticosteroid receptor transcription by acting on MR or GR gene promoters, as MR and GR gene transcription have been demonstrated in fetal brain (Diaz et al., 1998). Alternatively, they may influence developing monoaminergic systems which in turn regulate brain MR and GR expression (Kabbaj et al., 1995; Kabbaj et al., 1996; Maccari et al., 1992a; Seckl et al., 1990; Takahashi and Goh, 1998; Weidenfeld and Feldman, 1991; Yau et al., 1992; Yau and Seckl, 1992). It may be postulated that prenatal glucocorticoids program the *development* of the HPA axis rather than 'hardwire' it prenatally. This is supported by the finding that CBX rats showed a trend towards higher hippocampal MR gene expression at 1 week of age (which reached significance in CA2) which had disappeared completely in adult rats. At 1 week of age, CORT is maintained at very low levels and any actions of CORT are therefore likely to be mediated by MR. In this way, activation of increased numbers of MR may have affected neurogenesis. However, the increased MR mRNA expression in one week-old CBX offspring did not affect their plasma CORT levels at that stage and the SHRP was not affected by either of the prenatal treatments.

Taken together, data from this study suggest that prenatal CBX injections program subtle changes in adult HPA regulation and activity and that these are associated with reduced birthweight. Although CBX injections may have altered the *in utero* milieu in various ways, the similarity to the effects of prenatal DEX injections suggest that feto-placental 11β-HSD may normally function to protect the fetus from high levels of maternal CORT. However, the efficiency of this barrier may vary between individuals (Benediktsson et al., 1993), rendering some fetuses more susceptible to the programming effects of maternal CORT, such as in maternal stress.

Chapter 4. Effects of prenatal CBX and DEX on spatial memory

Introduction

In a population of humans or animals there are great individual variations in cognitive performance. Genetic background, early life experiences and ageing have all been shown to affect spatial memory as measured in a Morris watermaze (Issa et al., 1990; Lipp and Wolfer, 1998; Meaney et al., 1988; Zaharia et al., 1996). Performance in this task relies on hippocampal integrity (Morris et al., 1982) and functioning which in turn is for an important part regulated by numbers and occupation of MR and GR. Generally, reduced corticosteroid receptor numbers in hippocampus correlate with impaired spatial memory. However, from more direct studies using MR and GR agonists and antagonists to manipulate hippocampal receptor occupation, an inverted U-shaped relationship between GR occupancy (or plasma CORT levels) and spatial memory has been proposed (Lupien and McEwen, 1997). Interestingly, a similar correlation has been used to describe the relationship between CORT levels and LTP, the electrophysiological correlate of memory (Diamond et al., 1992). Hippocampal MR are thought to be involved in memory formation in a less specific way, through increased arousal and promotion of 'sensory integration'.

Prenatally stressed rats often have increased basal and stress-induced CORT levels and reduced hippocampal corticosteroid receptor numbers (Barbazanges et al., 1996; Henry et al., 1994; Maccari et al., 1995; Vallee et al., 1997). However, the implications of these findings on watermaze performance are less clear cut. Of the few investigations into cognitive performance in prenatally stressed rats, two reported improved memory (Fride et al., 1986; Szuran et al., 1994), whereas a third one failed to find an effect of prenatal stress on spatial memory (Vallee et al., 1997). Effects of prenatal glucocorticoid exposure *per se* on watermaze performance have thus far not been reported (although postnatal DEX administration impairs spatial learning (Dekosky et al., 1982; Vicedomini et al., 1986)) but provide an interesting

matter of study considering the elevated basal (in CBX and DEX rats) and post-stress (in CBX rats) CORT levels found in the previous chapter. At the time of this experiment, hippocampal MR and GR mRNA expression had not yet been measured in these animals, but a previous study (Levitt et al., 1996) had shown that exposure to DEX in the last week of pregnancy reduced both hippocampal MR and GR mRNA levels. With this in mind, this study aimed to establish whether prenatal exposure to glucocorticoids, by inhibiting feto-placental 11β-HSD or by injections with DEX throughout pregnancy, affects cognitive functioning measured in a watermaze task.

As described in Chapter 2. In short, rats (n=8/group) were trained in a circular pool to find a platform submerged under the water surface on 4 consecutive days using visual cues (acquisition phase - the *acquisition/consolidation* compound of the test). On the fifth day, the platform is removed and the amount of time spent around the former platform location was recorded (probe test - the *retention/strategy* compound of the test).

During the acquisition phase of the test, half the rats were trained to locate the submerged platform located at the 'north-east (NE) position, and the other half was trained to locate it at 'south-west' (SW). For each rat the platform remained in the same position across trials. Each trial started with the rat placed into the pool facing the wall of the pool at one of four starting positions. It was then given 2 minutes to locate the platform. If a rat failed to find it within 120 seconds, the experimenter placed the rat on the platform manually. The rat was left there for 30 seconds before the next trial began. Each rat was subjected to four trials per day, on three consecutive days. The latency to find the platform ('escape latency') was monitored for each trial as an indicator of spatial learning.

On the fifth day of the test, the platform was removed and the animals' behaviour was recorded during a 1-minute free-swim trial (probe test). The following parameters were recorded: a) latency until the former platform location was reached; b) number of crossings of the former platform location; c) percentage of time spent swimming in the quadrant where the platform had been located; d) swim speed; e) total distance swum; f) percentage of time spent swimming along the walls of the pool (thigmotaxis).

Training

All animals were able to swim and improved their performance over the course of 4 days of training (Figure 4.1). The results of 4 days of training (4 sessions/day) in the watermaze are depicted in figure 4.2. It shows the mean latency per day for control-, CBX- and DEX1-3 offspring to find the submerged platform. Ignoring the very first trial on the first day (trial 1, in which escape latency is determined by chance), a two-way ANOVA reveals a prenatal treatment effect ($F_{2,6}$=5.71, $p<0.05$), a day effect ($F_{3,18}$=39.32, $p<0.0001$) and a treatment × day interaction ($F_{6,18}$=5.27, $p<0.01$) on escape latency. *Post-hoc* analysis of the treatment × day interaction showed that CBX- and DEX1-3 rats needed more time to find the submerged platform than control rats on day 1, but not on any other day (Figure 4.2).

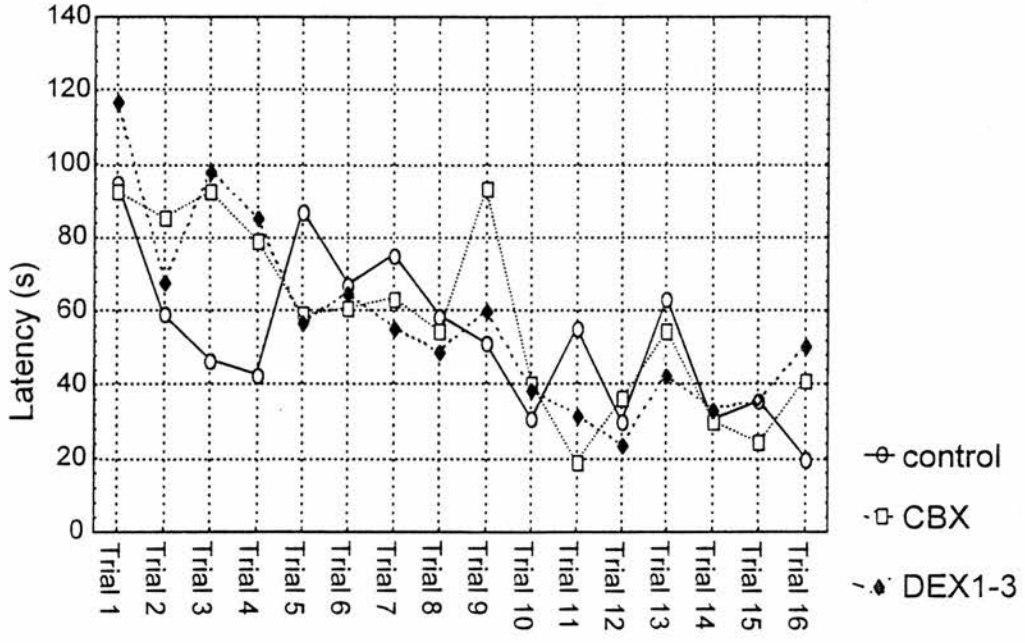

Figure 4.1 **Latency to find the submerged platform per group across 4 days of training.** An improvement over the course of 4 days can be seen in all three groups. Depicted are the mean latencies per group in each session. n=8/group.

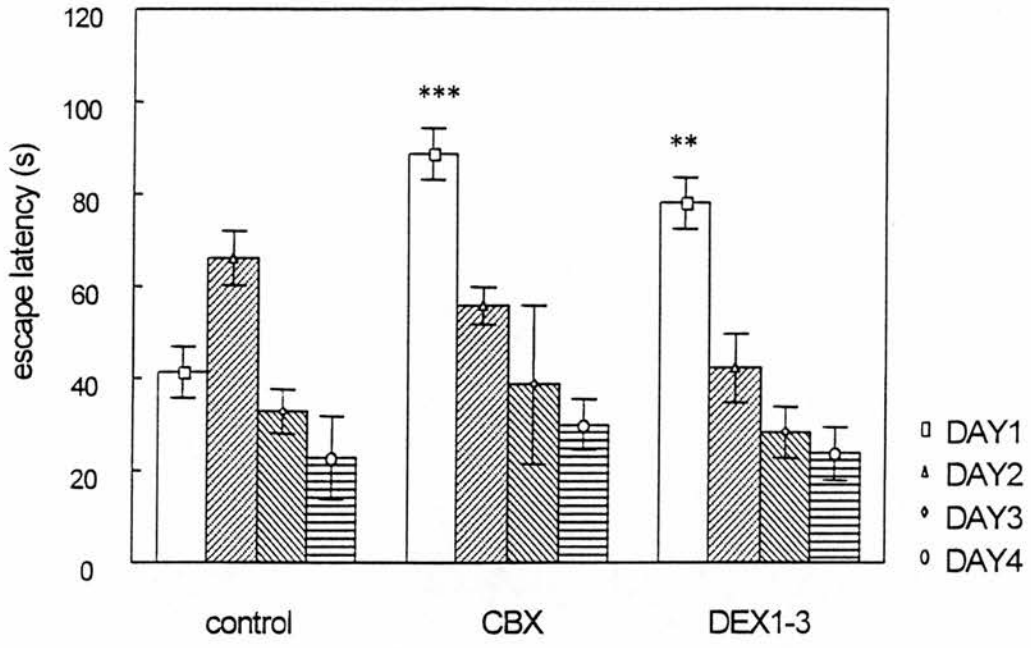

Figure 4.2 **Mean latency to find the submerged platform per group across 4 days of training.** Depicted are the mean latencies of 3 sessions (day 1) or 4 sessions (day 2-4) ± SEM.** $p<0.001$, *** $p<0.0001$ *versus* control; n=8/group.

Importantly however, the position of the platform (either at NE or SW) influenced escape latencies. In a three-way ANOVA with platform location and prenatal treatment as independent variables and trials as a repeated factor, no platform effect ($F_{1,18}=0.005$, $p=0.94$) or treatment effect ($F_{2,18}=0.16$, $p=0.85$) on escape latency was found, but there was a significant platform × treatment interaction ($F_{2,18}=3.67$, $p<0.05$) as well as a platform × treatment × trial interaction ($F_{28,252}=1.56$, $p<0.05$), indicating that the three treatment groups were differentially affected by the effect of platform location on escape latency. Therefore, normalisation of escape latencies to correct for the effect of platform location would not be appropriate. Furthermore, because of the low *n* per group per platform location (n=4), it is difficult to interpret data from this experiment, even when data from each platform position would be considered separately. In the probe test (next section), the possible effect of platform

position on retention was taken into account, and platform location during the training trials was therefore used as an independent factor in statistical analysis.

Probe test

Non-mnemonic parameters

To analyse the non-memory related parameters of the probe test on day 5, a two-way ANOVA was performed with prenatal treatment and platform location as independent variables, and swim speed, distance swum and thigmotaxis (time spent along the walls) as dependent factors. There were no significant treatment or platform effects on distance swum or swim speed, nor a treatment × platform interaction (Table 4.1a), although there was a trend for DEX1-3 rats to swim faster than CBX rats (Table 4.1a/b). With regard to the amount of time spent along the side walls of the pool, there were no main effects of treatment or platform location, but a significant interaction between the two existed (Table 4.1a), with control rats spending more time along the side walls when trained 'NE' and DEX1-3 rats showing more thigmotaxis when trained 'SW'.

effect	*Swim speed*	*Distance swum*	*%time at side walls*
Treatment (T)	$F_{2,18}$=3.20, p=0.06	$F_{2,18}$=2.34, p=0.12	$F_{2,18}$=0.00, p=1.00
Platform (PF)	$F_{1,18}$=0.54, p=0.47	$F_{1,18}$=0.08, p=0.78	$F_{1,18}$=0.06, p=0.81
T×PF interaction	$F_{2,18}$=1.38, p=0.28	$F_{2,18}$=1.92, p=0.18	$F_{2,18}$=4.65, p<0.05*

Table 4.1a **Statistical analysis of effects of treatment and platform location.**
n=8/group

	Mean speed (m/s)	*Total distance (m)*	*%time at side walls*
Control	0.22 ± 0.01	12.52 ± 1.00	34.5 ± 6.2
CBX	0.21 ± 0.02	12.08 ± 0.90	33.8 ± 4.9
DEX1-3	0.25 ± 0.08	14.36 ± 0.39	34.3 ± 7.9

Table 4.1b **Swimming parameters during 1-minute probe test.**
n=8/group

Mnemonic parameters

In the probe test, memory of rats was tested by observing (1) the number of times animals crossed the position where the platform was located during the training sessions, (2) time passed until the first crossing of that location ('latency'), and (3) percentage of time spent in the quadrant where the platform was previously located ('correct quadrant'). Two-way ANOVAs were performed to establish the effects of prenatal treatment and platform position during training on these parameters. Thus, prenatal treatment and platform position acted as independent factors.

There were significant main effects of both factors on the number of crossings, but no significant interaction (Table 4.2a). *Post-hoc* analysis revealed that DEX1-3 offspring have a higher number of crossings than control rats ($p<0.01$), and that rats trained at NE have more crossings than rats trained at SW ($p<0.01$), but the number of crossings was low in all groups (Table 4.2b; median 2, range 0-6). With respect to the latency until the first crossing of the former platform location, there were no effects of or interactions between prenatal treatment or platform position during training (Table 4.2a,b).

The percentage of time rats swam in the quadrant where the platform was located during the training sessions did not significantly differ between groups (Table 4.2a, no significant treatment effect). Platform location during training did influence the outcome of the probe test (Figure 4.3; Table 4.2a), with NE-trained rats performing better than SW-trained rats ($p<0.05$). However, there was no treatment × platform interaction (Table 4.2a), and raw data (Table 4.2b) were normalised to correct for the effect of platform position during training. A one-way ANOVA performed on normalised data (Table 4.2c) did not reveal significant differences between control and CBX- or DEX1-3 rats with respect to number of crossings ($F_{2,21}=2.81$, $p=0.08$), latency ($F_{2,21}=0.04$, $p=0.96$) or percentage of time swum in the 'correct' quadrant ($F_{2,21}=2.24$, $p=0.13$), although there was a trend for CBX and DEX1-3 offspring towards a higher number of crossings and greater amount of time spent in the 'correct' quadrant (Figure 4.4; Table 4.2c).

effect	*Number of crossings*	*Latency (s)*	*% time in correct quadrant*
Treatment (T)	$F_{2,18}$=4.45; p<0.05*	$F_{2,18}$=0.10; p=0.91	$F_{2,18}$=3.19; p=0.06
Platform (PF)	$F_{1,18}$=9.97; p<0.01**	$F_{1,18}$=0.18; p=0.68	$F_{1,18}$=8.3; p<0.05*
T×PF interaction	$F_{2,18}$=2.52; p=0.11	$F_{2,18}$=0.49; p=0.62	$F_{2,18}$=2.61; p=0.10

Table 4.2a **Effects of prenatal treatment and platform location on memory parameters in a 1-minute probe test.**
n=8/treatment group

	Number of crossings	*Latency (s)*	*% time in correct quadrant*
control	1.0 ± 0.3	31.7 ± 8.4	22.21 ± 3.67
CBX	2.0 ± 0.5	26.3 ± 8.5	32.04 ± 5.54
DEX1-3	2.6 ± 0.7	28.3 ± 7.3	34.50 ± 3.94

Table 4.2b **Memory parameters in a 1-minute probe test, not taking into account platform position during training sessions.**
n=8/treatment group

	Normalised number of crossings (% control)	*Normalised latency (% control)*	*Normalised % time in correct quadrant (% control)*
control	100 ± 27	100 ± 28	100 ± 16
CBX	200 ± 46	88 ± 30	143 ± 24
DEX1-3	260 ± 65	93 ± 27	155 ± 17

Table 4.2c **Memory parameters in a 1-minute probe test, normalised for platform position during training sessions.**
n=8/treatment group

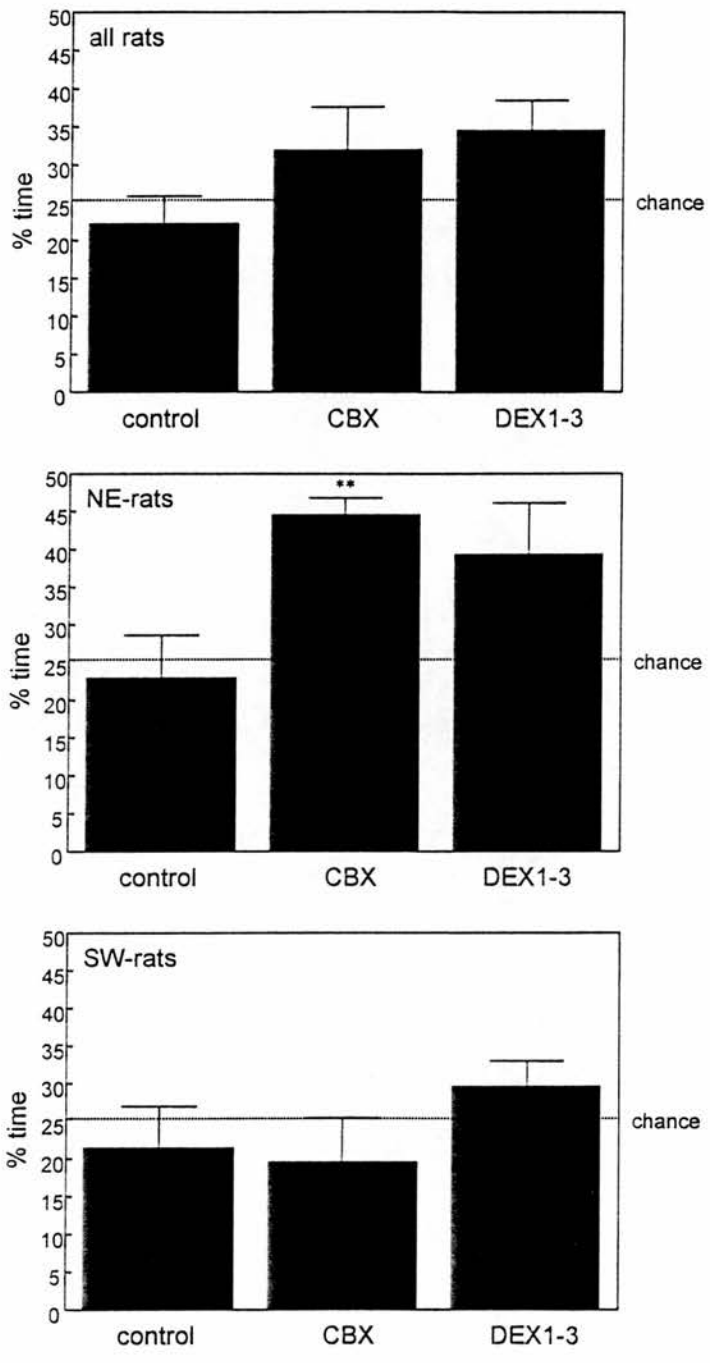

Figure 4.3 **Percentage of time spent in former platform quadrant.**
NE = trained with platform north-east, SW = trained with platform south-west; ** $p<0.01$; n=8/group.

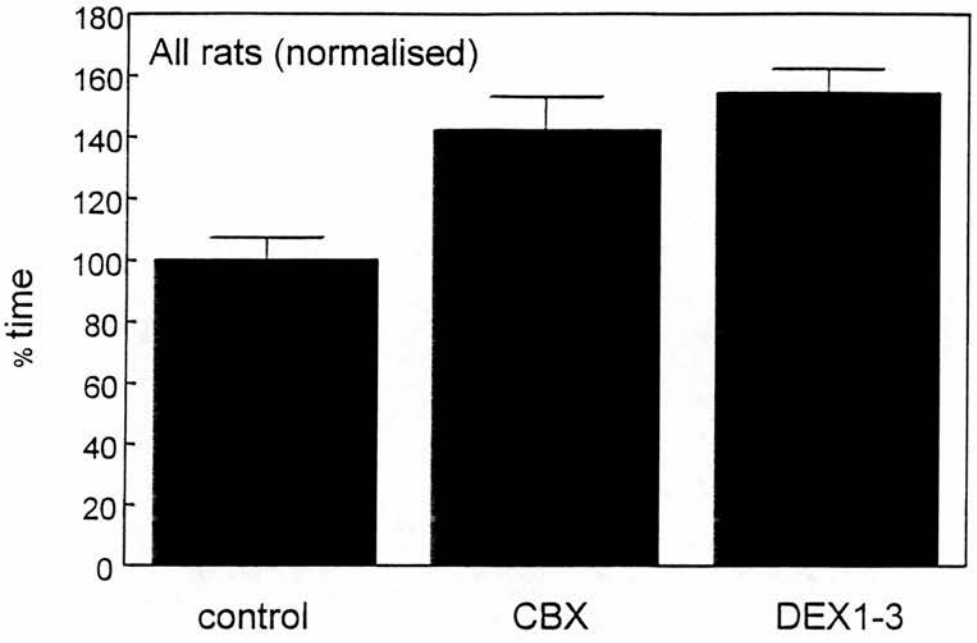

Figure 4.4 **Percentage of time spent in the 'correct' quadrant of the water maze in a 1-minute probe test, normalised for platform position during training sessions.**
n=8/group

Although all rats showed improved performance over the four days of training, they generally performed poorly. Wistar rats are known to be worse in this task compared to other rat strains e.g. Lister hooded rats (Yau et al., 1994). The prenatal treatments with CBX and DEX appeared to have an impairing effect on the first day of training, as rats from these groups had higher mean latencies to find the platform than control rats. This difference had however disappeared by day 2, suggesting that the negative effect of the treatments on watermaze performance is related to short-term rather than long-term memory.

In the 1-minute probe test, both CBX and DEX1-3 rats tended to spend more time in the quadrant where the platform had been located, although this trend did not reach significance. Some researchers interpret this as 'good memory', because the rats appear to remember the position of the platform during the training trials (Morris, 1984; Yau et al., 1994). However, a possibly better measure of memory would be the latency until rats cross the former platform location, which was similar in the three groups. Latency is determined by swim speed and navigation, and as swim speed was not affected by prenatal treatments, it appears that prenatal CBX or DEX treatment did not affect the amount of time it took rats to find the former platform position in the probe test.

The number of crossings of the former platform location in the probe test was very low in all groups. This does not necessarily imply poor memory. A high number of crossings could also be interpreted as proof of a rigid search strategy, whereas a low number of crossings might mean that the animal has discovered the platform is not where it expected it to be, and then decides to look for it in other parts of the watermaze. The same is true for the amount of time spent in the quadrant where the platform was located during the training trials (perhaps confusingly called 'correct quadrant'). The trend towards spending more time in this quadrant in CBX and DEX rats implies an altered (more rigid) search strategy compared to control rats. Interestingly, two studies examining the effects of prenatal stress on watermaze

performance also failed to find an effect in the acquisition phase of the test, but in a reversal task (in which the platform is moved to a different position) or probe test, prenatally stressed rats spent more time in the quadrant where the platform was previously located (Hayashi et al., 1998; Szuran et al., 1994). Exploratory behaviour in this task has been associated with hippocampal MR occupation (Oitzl and de Kloet, 1992), and decreased MR binding has been reported in prenatally stressed adult male rats (Barbazanges et al., 1996; Henry et al., 1994; Maccari et al., 1995; Vallee et al., 1997). Although MR (or GR) mRNA expression in this study were not affected by the prenatal treatments, this does not rule out alterations in posttranscriptional or –translational mechanisms resulting in a reduced number of MR binding sites in CBX or DEX1-3 rats. However, this is only speculation and none of the trends in the probe tests reached significance, so this interpretation should be taken with caution.

In fact, the lack of significant effects of prenatal glucocorticoid exposure on watermaze performance may be explained by considering the inverted U-shaped relationship between spatial memory and hippocampal GR occupation (Conrad et al., 1997). This relationship implies that both plasma CORT concentration and hippocampal corticosteroid receptor levels are determinants of watermaze performance. There were no differences in stress levels of plasma CORT between CBX-, DEX1-3 and control rats as measured using a restraint procedure (see previous chapter), but CORT levels were not determined in the study described here. Water temperature in the maze has been shown to be an important factor in acquisition and retention, probably by increasing (lower temperature) or decreasing (higher temperature) the stressfulness of the task. Rats appear to learn better at lower temperatures (Sandi et al., 1997), indicating that (moderately) low temperatures are beneficial for acquisition and retention. Interestingly, in one study differences between prenatally stressed and unstressed rats only appeared when the watermaze task was performed at a very low temperature. Further experiments are needed to establish whether this could be the case for the treatments used in this study.

However, assuming that CORT levels of DEX-, CBX- and control rats were similar during the watermaze procedure, only increased or decreased hippocampal MR or

GR levels could account for differences in performance. However, as shown in the previous chapter, stress-levels of plasma CORT were similar in the three groups, and hippocampal MR and GR mRNA expression were not affected by prenatal treatment with CBX or DEX (see chapter 3). Thus, the lack of differences in spatial memory in this study was not completely unexpected. In fact, in one study a reduction of approximately 25% in hippocampal MR or GR binding by i.c.v. administration of specific antisense mRNAs did not result in impaired watermaze performance in Brown Norway rats (Engelmann et al., 1998), indicating that only very large alterations in receptor number may have any functional impact in this test.

The most surprising finding from this experiment was the difference in performance between rats trained to find a platform located at 'NE' and at 'SW'. It is unclear what may have caused this difference. Interestingly, the three treatment groups were differentially influenced by platform positions during the training period. It appears as though CBX and DEX1-3 rats were more attracted to the NE-side of the pool, possibly by an unpleasant cue nearer the SW side, causing the rats to avoid this part of the pool, even if the platform was located there. This would suggest an extremely aversive cue, preventing them from escaping the water by staying on the platform. One possibility is that the sound produced by the air conditioning in the testing room, although not very loud or unpleasant to human ears, may have been perceived (perhaps ultrasonically) as aversive by rats. Unfortunately, because of the low *n* per group per platform location (n=4), it is difficult to interpret data from this experiment when data from each platform position are considered separately.

In conclusion, this study showed that prenatal glucocorticoid exposure via CBX of DEX administration throughout pregnancy does not significantly affect spatial memory in a watermaze task, but may have increased the rats' sensitivity to an (as yet unidentified) unpleasant cue in the testing room. It is possible that an increased level of anxiety in CBX and DEX rats underlies this heightened sensitivity. Chapter 6 of this thesis will explore anxiety-like behaviour in these animals in greater detail.

Chapter 5. Prenatal dex: a specific time window?

Introduction

The finding that exposure to DEX throughout gestation did not result in major alterations in HPA activity or hippocampal MR or GR gene expression was somewhat surprising considering the robust effects, peripherally as well as centrally, of perinatal DEX administration that have been described in the literature on the one hand (see chapter 1), and the sensitivity of hippocampal corticosteroid receptors to early manipulations on the other (Henry et al., 1994; Levine et al., 1994; Maccari et al., 1995; Meaney et al., 1989). However, earlier studies with DEX varied greatly with respect to timing and dosage of DEX administration, and timing of the exposure may be critical for effects to occur. Indeed, the existence of a critical 'time window' in the third week of gestation for DEX effects on blood pressure (Levitt et al., 1996) and glucose homeostasis (Nyirenda et al., 1998) has been established.

Similarly, most studies describing the effects of prenatal DEX on brain functioning have used late gestational or neonatal exposure. Thus, low-dose DEX administered on gestational days 17, 18 and 19 altered 5-hydroxytryptamine turnover and noradrenaline content in specific brain regions measured at 14 weeks of life. Moreover, these animals had altered behaviour and elevated plasma CORT levels in a novel environment (Muneoka et al., 1997). Another study reported the disruption of brain nuclear transcription factors due to late gestational DEX exposure (Slotkin et al., 1998), but beneficial effects of prenatal DEX administration have also been reported (Slotkin et al., 1993). A greater number of studies exist with regard to *neonatal* DEX injections. Depending on timing (between postnatal days 1 and 7, varying from 1 to 3 injections) and dose (0.04-100 mg/kg bodyweight), neonatal DEX exposure resulted in behavioural suppression (Felszeghy et al., 1993), hyperactivity (Ferguson and Holson, 1999), spatial learning deficits (Dekosky et al., 1982; Vicedomini et al., 1986), decreased brain GR levels (Felszeghy et al., 1996), but unaltered plasma CORT concentrations (Felszeghy et al., 1996).

In humans, perinatal glucocorticoid therapy is usually given to accelerate lung maturation in fetuses to prevent neonatal respiratory distress syndrome and chronic lung disease in cases of (threatened) prematurity. The long-term effects of perinatal glucocorticoid treatment on cognition and behaviour have received little or no attention, but preliminary data regarding *early* dexamethasone exposure in cases of suspected congenital adrenal hyperplasia show that there may be unfavourable effects on the behavioural development (emotional and social) of children (Lajic et al., 1998; Trautman et al., 1995). Animal models have shown that these aspects of behaviour are modulated by corticosteroids (see chapter 1). Therefore, this chapter aims to establish the long-term effects of late gestational DEX administration on HPA activity and regulation in rats.

Methods

Prenatal treatments

Pregnant females (225 g) were injected s.c. with dexamethasone (DEX, 100 μg/kg; dissolved in 4% ethanol-0.9% saline) daily in the third week of pregnancy, and with vehicle injections in the first two weeks. A control group received vehicle injections throughout pregnancy. On day 20 of pregnancy a blood sample was taken from the tail vein (see below) for determination of plasma CORT levels.

Litters

Only litters containing 8 pups or more were selected for the studies. On the day of birth, litters were weighed, sexed and culled to 8 pups per litter. Offspring were weighed regularly, starting 4 weeks after birth.

Blood sampling

As described in Chapter 2.

HPA axis activity

Stress-induced activation of the HPA axis was determined using a restraint procedure, in which adult rats were restrained in Perspex cylinders for 20 minutes. Plasma CORT levels were measured in blood samples taken from the tail vein 3 h before (at 9:00 h), 10 minutes into and 90 minutes after the restraint.

In a separate experiment, we determined the sensitivity to the suppressive effects of DEX on CORT secretion in the rats. Thus, rats were given a single bolus of DEX (40 μg/kg) s.c., after which the restraint procedure described above was performed.

Corticosterone assay

As described in Chapter 2.

In situ hybridisation histochemistry

In situ hybridisation was performed as described in Chapter 2 on sections taken at the level of the PVN (GR and CRH riboprobes) and hippocampus (MR and GR riboprobes). Equivalent 'sense' probes were included as controls. MR, GR and CRH mRNA expression was quantified by counting silver grains overlying identified neurones under bright-field illumination using an automatic image-analysis system. Results were calculated as mean number of grains/cell area for each subregion after subtraction of background (counted over areas of white matter), which was always low.

Statistics

Data are presented as mean value per group ± s.e.m. Data from the two treatment groups were compared using Student's t-test or an ANOVA for repeated measures followed by a LSD test, where appropriate. Significance was set at $p < 0.05$.

Effects of DEX injections in week 3 of pregnancy on pregnant rats

Vehicle and DEX treatment were tolerated well. Maternal plasma CORT levels on day 20 of pregnancy were significantly lower in animals receiving DEX injections (control 16.6 ± 2.5 μg/dl, DEX3 6.5 ± 1.6 μg/dl, $p<0.01$).

Effects of DEX injections in week 3 of gestation on offspring

Prenatal injections with DEX in the third week of gestation had no effect on gestation length (control 22.4 ± 0.24 days, DEX3 21.7 ± 0.29 days; $p=0.12$) or litter size (control 10.8 ± 0.54, DEX3 9.8 ± 0.58; $p=0.23$). However, treatment with DEX in the third week of gestation significantly reduced birth weight (Table 5.1). From week 4 of life onwards, DEX3 and control animals had similar body weights (Table 5.1).

	Day 1	*Week 4*	*Week 6*	*Week 9*	*Week 12*	*Week 20*
control	5.79 ± 0.09	73.4 ± 0.9	153 ± 2	261 ± 2	319 ± 4	380 ± 5
DEX3	5.37 ± 0.12**	71.3 ± 0.8	151 ± 3	253 ± 5	309± 6	364 ± 9

Table 5.1 **Effect of prenatal injections on body weight.**
Results are expressed as g bodyweight. **p<0.01 *versus* control; n=13-47/group.

Brain MR, GR and CRH mRNA expression in adult offspring

Exposure to DEX during the last week of pregnancy altered corticosteroid receptor gene expression in specific hippocampal subfields at 6 months of age (Figure 5.1, Figure 5.3). In DEX3 offspring MR mRNA levels were significantly reduced in CA1 and CA2 (Figure 5.1, *top panel*), whereas GR mRNA expression was lower in DG and CA1 compared to controls (Figure 5.1, *bottom panel*). Furthermore, DEX exposure did not alter GR mRNA expression in PVN of 6 month-old rats (Figure 5.2 *left panel*; Figure 5.4), but increased CRH mRNA levels (Figure 5.2 *right panel*, Figure 5.5).

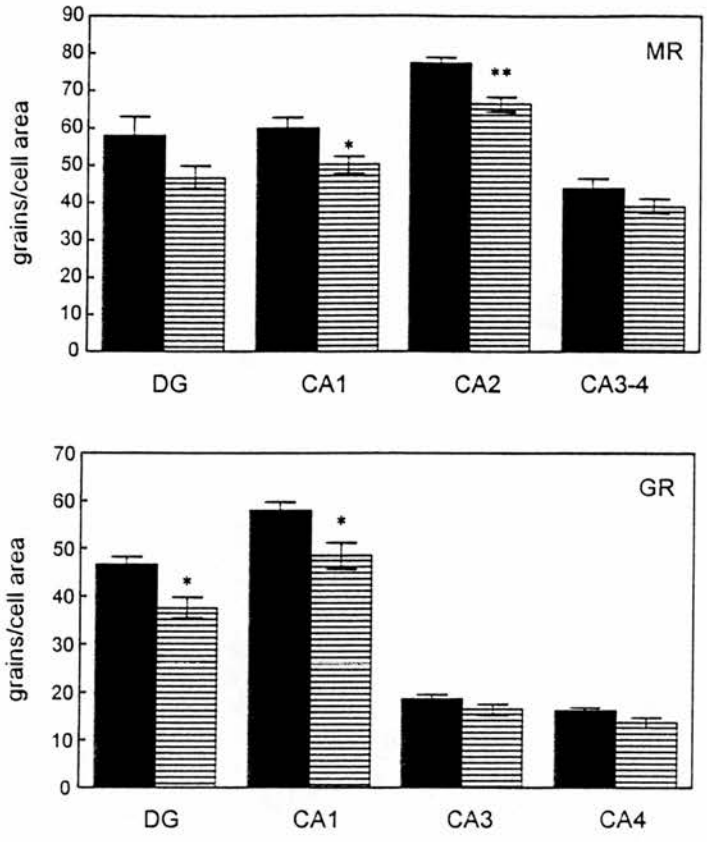

Figure 5.1 **Hippocampal MR and GR mRNA expression.**
Effect of prenatal treatments on hippocampal MR (n=8-13/group) and GR (n=8-11/group) mRNA expression in 6 month-old rats. Results are expressed as mean number of grains/cell area ± s.e.m. for each subregion after subtraction of background. *p<0.05, **p<0.01

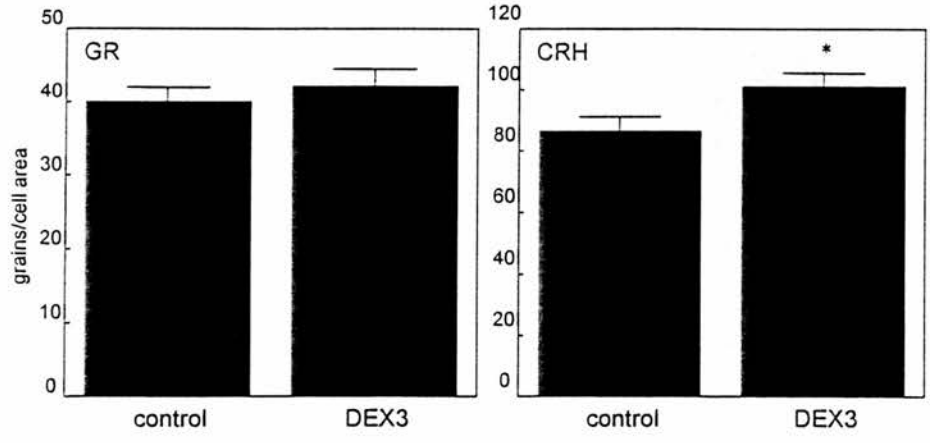

Figure 5.2 **GR and CRH mRNA expression in PVN of 6-month old rats.**
Left panel GR mRNA expression, *right panel* CRH mRNA expression; *p<0.05; n=7/treatment group

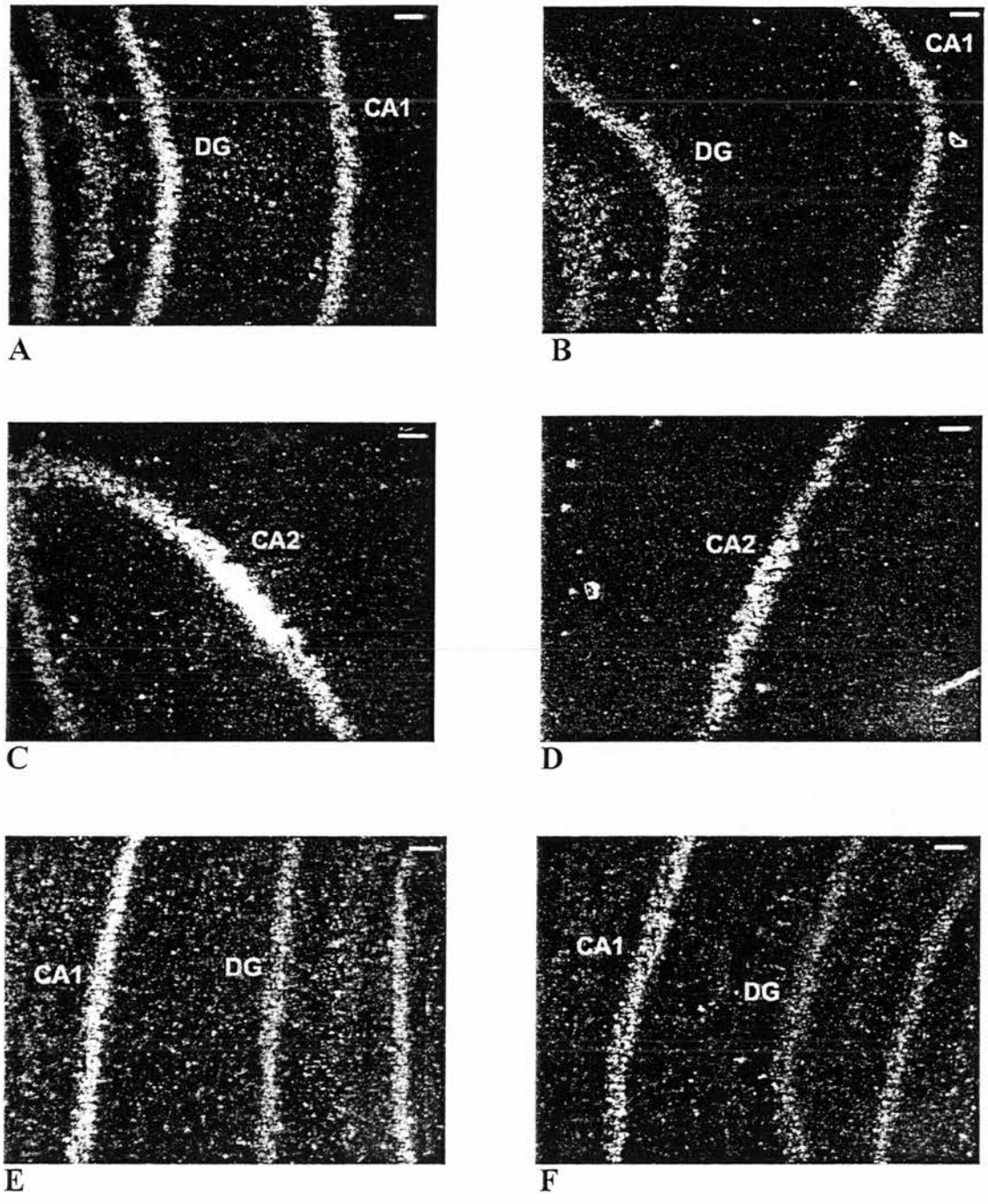

Figure 5.3 **Photomicrographs of hippocampal MR and GR mRNA expression.**
Left panels show typical sections from control rats, right panels show typical sections from DEX3 rats. *A* and *B*: MR mRNA in DG and CA1; *C* and *D*: MR mRNA in CA2; *E* and *F*: GR mRNA in DG and CA1. Scale bar represents 100 μm.

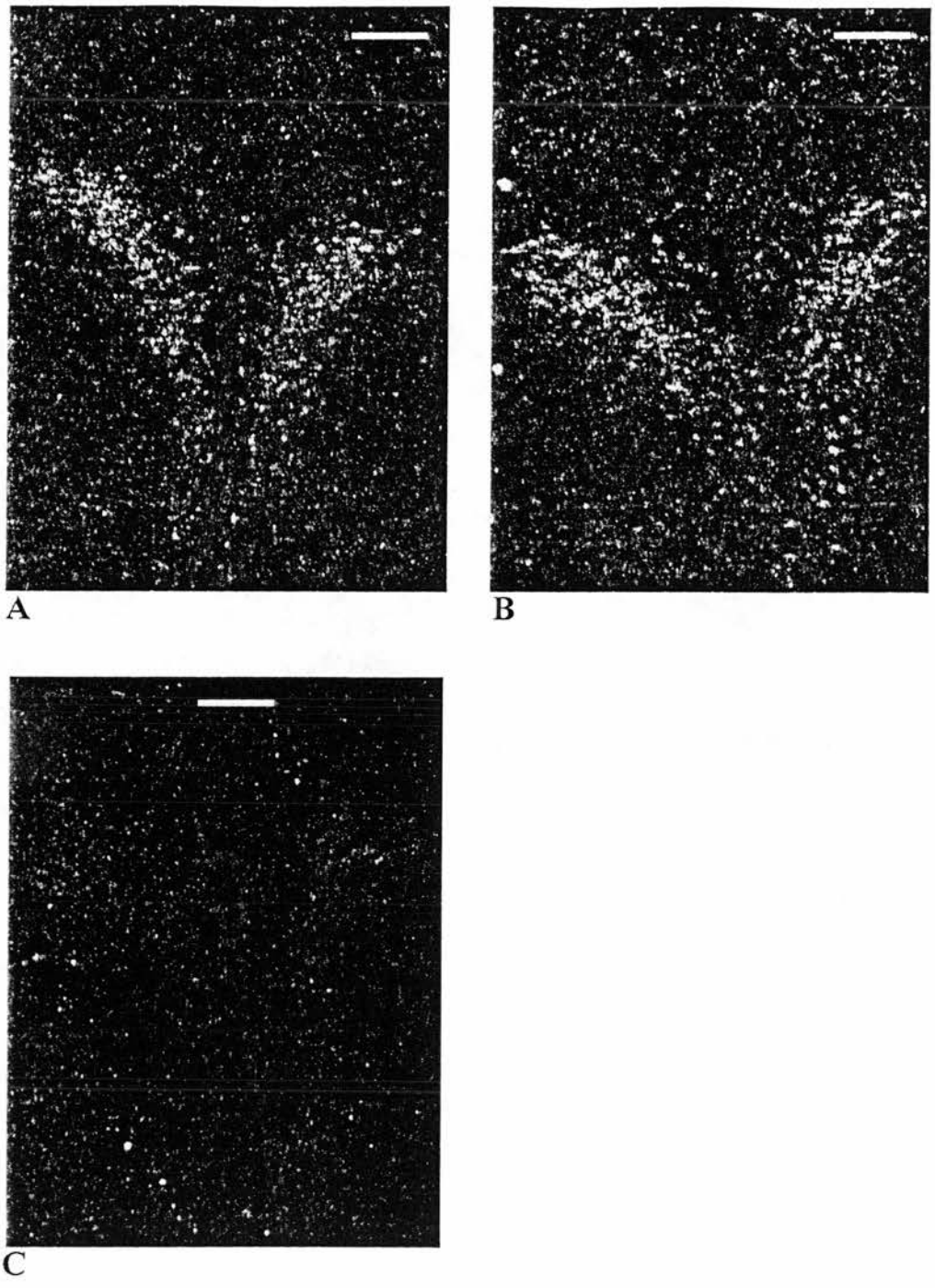

Figure 5.4 **Photomicrographs of GR mRNA expression in PVN.**
Images show typical sections from control and DEX3 rats. *A*-control, *B*-DEX3, *C*-sense. Scale bar represents 200 μm.

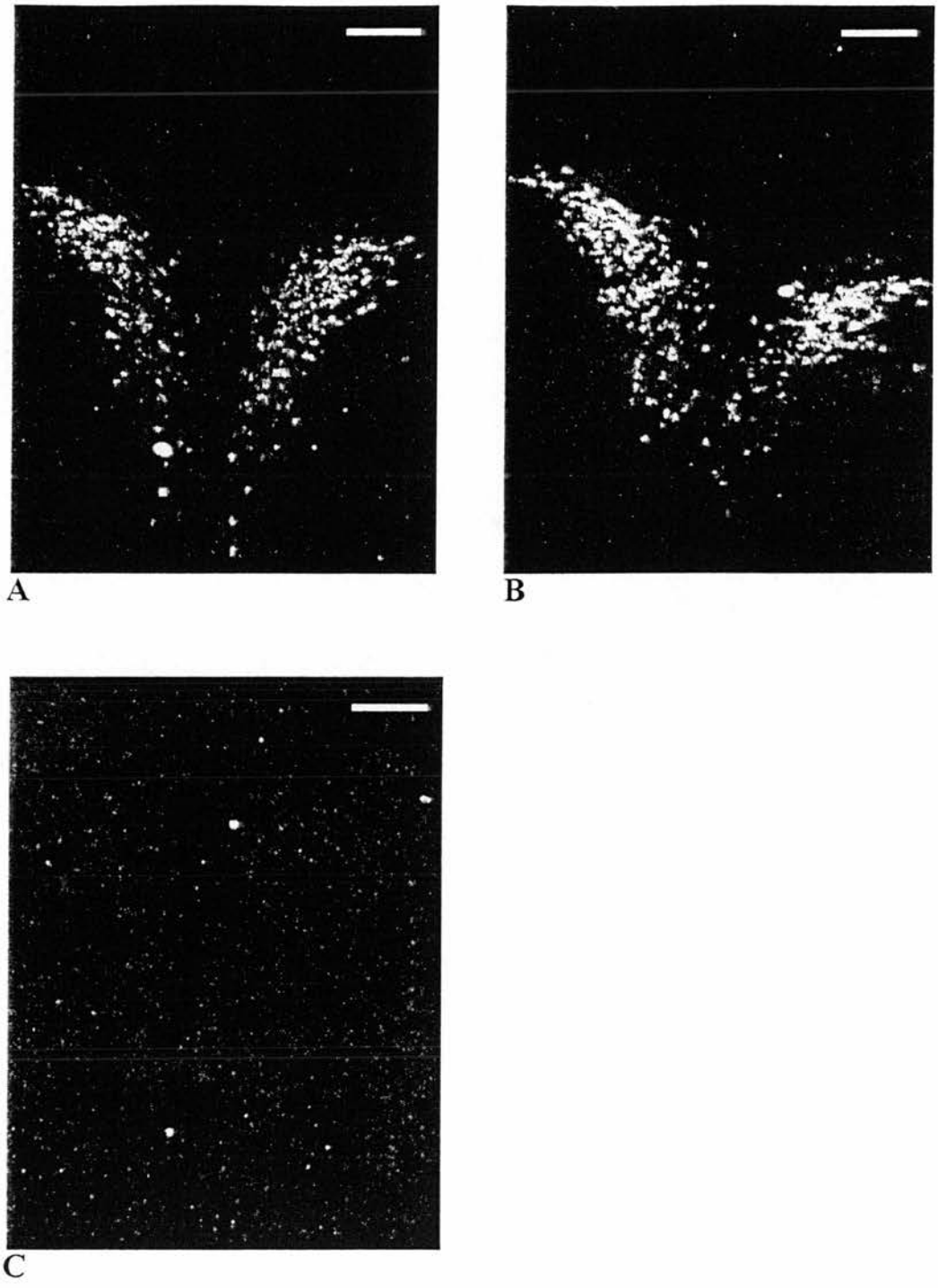

Figure 5.5 **Photomicrographs of CRH mRNA expression in PVN.**
Pictures show typical sections from control and DEX3 rats. *A*-control, *B*-DEX3, *C*-sense. Scale bar represents 200 μm.

a. CORT response to restraint

Figure 5.6 shows the CORT response to 20 minutes of restraint. A three-way ANOVA for repeated measures revealed a time effect ($F_{2,20}$=17.08, p< 0.001), but no treatment effect, ($F_{1,10}$=0.007, p=0.94) or treatment×time interaction ($F_{2,20}$=0.10, p=0.91) (Figure 5.6, *top panel*). Thus, DEX-administration in the third week of gestation did not affect the CORT response to restraint.

b. DEX-suppression of stress-induced CORT levels

A single bolus of DEX (40 μg/kg) given s.c. 3 hours before start of the restraint procedure, suppressed the CORT response to a similar extent in the two treatment groups. ANOVA showed no treatment effect ($F_{1,10}$=0.008, p=0.93) or treatment×time interaction ($F_{2,20}$=0.64, p=0.53) (Figure 5.6, *bottom panel*), although there was a time effect ($F_{2,20}$=3.60, p<0.05), showing that the dose of DEX used had not suppressed the CORT response to restraint completely. Clearly, exposure to DEX in the last week of gestation did not affect the sensitivity to the suppressive effects of DEX on stress-induced CORT secretion.

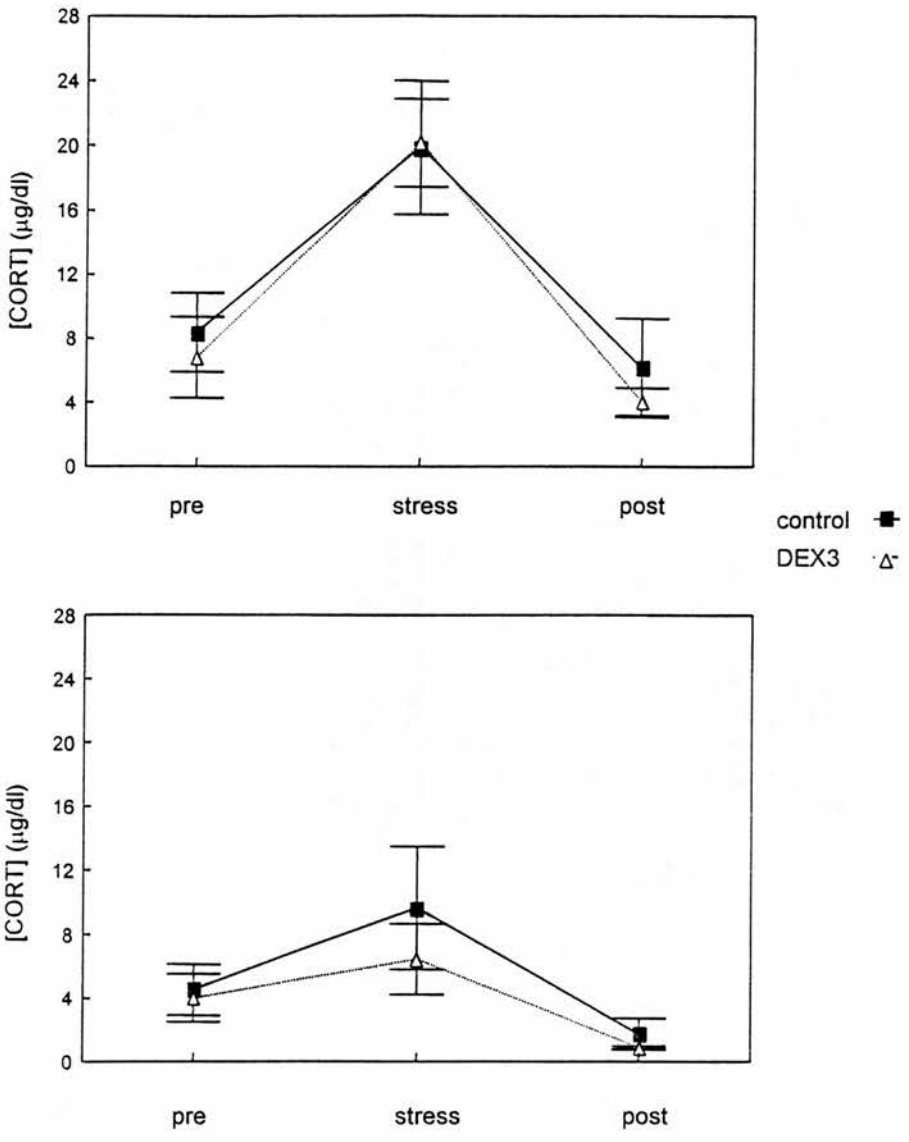

Figure 5.6 **Plasma CORT response to 20 minutes restraint.**
CORT concentrations measured in plasma samples taken before *(pre)*, 5 minutes into *(stress)*, and 90 minutes after *(post)* 20 minutes restraint *(top graph)*. The bottom graph depicts the effect of a single DEX-injection (40 µg/kg) 3 h before start of the restraint procedure on the CORT response to restraint. n=6/group.

This study showed that DEX exposure in the last week of pregnancy reduces hippocampal MR and GR gene expression in specific subregions and increases CRH mRNA in the PVN. However, stress-induced activity of the HPA axis and suppression of HPA activity by an acute DEX injection did not differ between DEX offspring and control animals.

Earlier work from our laboratory showed that basal CORT levels in DEX3 rats are elevated when compared to controls (Levitt et al., 1996). This was not verified in the present study. However, CORT levels in plasma samples taken 3 h before the start of the restraint procedure at 9:00 h were 8.4 μg/dl for controls and 6.8 μg/dl for DEX3 rats, respectively. Unfortunately, these levels cannot be considered basal, especially compared with basal levels shown in chapter 3, which were around 1 μg/dl (figure 3.5). The latter samples were taken in a different animal unit under slightly different conditions, and it may be suspected that the rats used in the present experiment were not completely unstressed under basal circumstances. Therefore, these data do not give conclusive evidence about the effect of DEX administration in the last week of gestation on basal CORT concentrations.

Although DEX3 rats had increased CRH mRNA expression in the PVN, their GR mRNA levels in this region were unchanged, indicating that a reduced glucocorticoid negative feedback action at PVN level is unlikely to account for the higher CRH transcription rate. This suggests an increased central drive to the HPA axis that may explain the elevated basal CORT levels found in DEX3 rats in an earlier study (Levitt et al., 1996). DEX3 rats had reduced MR and GR gene expression in specific regions of the hippocampus. Hippocampal MRs in particular are thought to play an important role in the regulation of basal HPA activity. This conclusion was based on the observation that basal ACTH levels are elevated after administration of mineralocorticoid antagonists (Bradbury et al., 1994; Ratka et al., 1989). However, *numbers* of MRs have never actually been shown to be associated with basal HPA

activity. This is surprising considering the fact that the MR is a 'high affinity-low capacity' receptor for which the number of receptors rather than the amount of ligand (CORT) determines the receptor-mediated actions of CORT. In the majority of studies, hippocampal corticosteroid receptor *numbers* are associated with stress-induced rather than basal HPA activity [e.g. Henry et al., 1994; Maccari et al., 1995; Meaney et al., 1989], whereas MR *occupation* appears to determine basal ACTH and CORT concentrations (Bradbury et al., 1994; Ratka et al., 1989). In this study however, the reduced levels of MR mRNA in the CA1 and CA2 regions of the hippocampus may have resulted in an increased basal transcription rate of CRH in the PVN. Although ACTH levels were not measured in this experiment, and the CORT levels obtained could not be considered truly basal, we may speculate that exposure to DEX in the last week of gestation permanently alters the 'set point' of the HPA axis at the level of the hippocampus, resulting in an increased basal HPA activity. HPA disturbances are associated with psychiatric disorders (Barden, 1999) and increased circulating glucocorticoid levels may impair cognition, as has been observed in patients with Cushing's disease. Certainly in many animal studies CORT levels and hippocampal MR and GR activity have been shown to influence learning and memory in a watermaze. Further experiments are needed to show the consequences of DEX-programmed alterations in hippocampus and HPA axis on these parameters.

GR gene transcription in the hippocampus was also reduced as a consequence of prenatal DEX exposure in the last week of gestation. As mentioned before, hippocampal GR occupation and numbers have been correlated to stress-induced HPA activity in many studies. In the present study however, no differences between control and DEX3 offspring were found in stress-induced plasma CORT levels as concentrations during as well as 90 minutes after stress were similar in both groups. Several explanations for this lack of difference are possible. Firstly, it has been suggested that glucocorticoid negative feedback takes place at the pituitary and hypothalamic level rather than in the hippocampus (De Kloet et al., 1998). GR expression in the PVN was unaltered by prenatal DEX treatment, and the same may be the case in the pituitary. This explanation is supported by the finding that DEX-induced suppression of HPA activity, the sensitivity to which would be determined

primarily at the level of the pituitary because DEX poorly penetrates the brain (De Kloet, 1997), also did not differ between DEX3 and control offspring. Thus, the impact of reduced hippocampal GR mRNA levels on HPA activity may have been marginal in this study. Secondly, the HPA response may be stressor-specific and differences in peak- or post-stress CORT levels may have become apparent in other experimental paradigms. In addition, a more detailed profile of the stress response may have been informative, as a prolonged CORT release to stress may last up to 120 minutes after exposure to the stressor. Thirdly, an increased GR gene expression may not necessarily result in higher number of receptors. Posttranscriptional and/or - translational mechanisms may have regulated the amount of receptor protein to a similar level in control and DEX3 rats.

Taken together, results from this study and from chapter 3 show that the effects of prenatal DEX administration are dependent on the timing of the exposure. A striking effect upon adult hippocampal MR and GR gene expression is only observed in rats exposed to DEX in the last week of gestation and may be associated with an increased central drive to the HPA axis. It may seem surprising that DEX administration throughout pregnancy does not have the same effect, considering that exposure in this paradigm also contains the critical time window (week 3). It is possible that in the three weeks of gestation several time windows for hippocampal corticosteroid receptor development occur during which glucocorticoid exposure has opposing effects. This is not an uncommon phenomenon: for example, prenatal stress and neonatal stress (short daily maternal deprivations) have opposite effects on hippocampal corticosteroid receptor binding and stress-induced HPA activity (Henry et al., 1994; Meaney et al., 1989). MR and GR have distinct developmental patterns of expression (see chapter 1), and hippocampal subregions do not develop at equal rates. Thus, the impact of high glucocorticoid levels is likely to depend on the stages of maturity of the fetal brain areas and this may underlie the time windows for specific effects of prenatal glucocorticoid exposure. A similar critical time window in the last week of gestation has been observed for the programming properties of prenatal DEX on blood pressure (Levitt et al., 1996) and glucose metabolism (Nyirenda et al., 1998). Alternatively, prolonged exposure to DEX may continuously affect growth and maturation throughout gestation without altering the relative rates

of development. In contrast, DEX administration in the last week of gestation only may result in disproportionate development that is persistent throughout the animal's lifespan. Whether the subtle alterations hippocampus and pituitary resulting from DEX exposure in the last week of pregnancy affect stress-associated behaviour will be investigated in the next chapter.

Chapter 6. Prenatal glucocorticoid exposure and stress-induced behaviour.

Introduction

In humans low birth weight is a risk factor for mental disorders later in life, such as schizophrenia (Hultman et al., 1997; Jones et al., 1998) and poor premorbid social adjustment and cognitive ability (Cannon et al., 1997; Foerster et al., 1991; Rifkin et al., 1994). In one study, 22% of a cohort of low birth weight children had a psychiatric disorder at the age of 6 (Whitaker et al., 1997).

Animal models have suggested that birthweight as well as the susceptibility to behavioural and cognitive abnormalities may be (partially) determined by early life experiences. Indeed, prenatal stress reduces birth weight in many studies (Kinsley and Svare, 1986; Peters, 1982; Takahashi et al., 1988; Williams et al, 1998b) and generally induces 'behavioural inhibition' (Lambert et al., 1995), such as an increased latency to play (Takahashi et al., 1992b), a high level of defensive freezing (Takahashi et al., 1992a), and reduced activity in an open field (Fride et al., 1986). In many cases prenatal stress is associated with increased anxiety later in life (Vallee et al., 1997; Wakshlak and Weinstock, 1990; Weinstock, 1997).

Because corticosteroids modulate behaviour in many of the tests used to measure anxiety levels, it is not unlikely that alterations in corticosteroid concentration or action may underlie the behavioural changes observed in prenatally stressed rats. Indeed, a hyperactive HPA axis and alterations in the hippocampus have been shown in a number of studies (Henry et al., 1994; McCormick et al., 1995; Takahashi, 1998; Weinstock et al., 1992). Similarly, as described in chapters 3 and 5, glucocorticoid exposure *in utero* had subtle effects on HPA activity, and administration of DEX in the last week of pregnancy reduced hippocampal corticosteroid receptor expression. Thus, these alterations may affect stress-induced behaviour, or 'emotionality', in these animals. Because emotionality is a complex concept, data from a single behavioural test may not give a correct interpretation concerning the animal's

emotionality. We therefore decided to perform multiple behavioural tests that may measure different aspects of emotionality (Ramos and Mormede, 1998).

The behavioural expression of fear is thought to be regulated by the actions of CRH since central administration of CRH induces anxiety-like responses [for review see Dunn and Berridge, 1996]. The amygdala is a likely site of action for CRH: it contains CRH receptors and also produces CRH (Gray and Bingaman, 1996). Indeed, electrical stimulation of the amygdala results in fear-associated responses [see (Applegate et al., 1982; Davis et al., 1994)], while amygdaloid lesions attenuate fear-related behavioural responses (Kopchia et al., 1992). Brain CRH mRNA expression is regulated by corticosteroids (Makino et al., 1994a; Makino et al., 1995b), possibly via stimulation of transcription by activated corticosteroid receptors, the mRNAs of which have been localised in several amygdaloid nuclei (Honkaniemi et al., 1992).

The aim of this experiment was firstly to establish whether prenatal glucocorticoid exposure affects the expression of corticosteroid receptor and CRH mRNA in amygdaloid nuclei, and secondly to test whether these possible changes affect the animals' behaviour in aversive environments.

Prenatal treatments

Pregnant females (n=7-8 per group in each of two separate experiments) received daily subcutaneous injections with either 200 µl vehicle solution (control, 4% ethanol in 0.9% saline), 12.5 mg/200 µl carbenoxolone (CBX), or 100 µg/kg dexamethasone (DEX1-3), respectively, throughout pregnancy. An additional treatment group received 200 µl vehicle solution during the first 2 weeks of pregnancy and 100 µg/kg dexamethasone during the last week of pregnancy (DEX3).

Litters

On the day of birth, litters were weighed, sexed and culled to 8 pups per litter. Only litters containing 8 pups or more were selected for the studies. Unless stated otherwise, litters were then left undisturbed until weaning, apart from routine maintenance. After weaning, male pups from each litter were housed in same-sex groups of 2-4 and left undisturbed until the time of testing. Experiments were performed on male rats selected randomly from each litter.

Blood sampling

Blood samples were taken from the tail vein, collected in Microvette tubes and stored on ice until centrifuged at 4°C. Subsequently, plasma was stored at -20°C until assayed. Plasma aliquots were freshly thawed for the analysis of corticosterone, using an in-house specific radioimmunoassay as described in Chapter 2. Results are presented as µg/dl plasma.

In situ hybridisation histochemistry

In situ hybridisation was performed as described in Chapter 2 on amygdala sections from 6 month-old rats, using MR, GR and CRH riboprobes. Equivalent 'sense' probes were included as controls.

MR and GR mRNA expression was quantified by counting silver grains overlying identified neurones under bright-field illumination using an automatic image-analysis system. Results were calculated as mean number of grains/cell area for each

subregion and mean optical density per area, respectively, after subtraction of background (counted over areas of white matter), which was always low. CRH mRNA expression was determined by measuring optical density (O.D.) of the relevant area on autoradiograms.

Open field

As described in Chapter 2.

Elevated plus-maze

As described in Chapter 2.

Forced swim test

As described in Chapter 2.

Neophagia

As described in Chapter 2.

Statistics

Data are presented as mean value per group ± s.e.m. In most cases, data were compared using ANOVA followed by an LSD test, where appropriate. However, a non-parametrical statistical analysis was used on data from the forced-swim test because of great between-group variability. Thus, *between*-group differences in immobility were analysed using Kruskal-Wallis rank-sum tests separately for the acquisition and the retention phase, followed by Dunn's multiple-comparison test for ranked sums. Furthermore, to compare measurements from the acquisition and the retention phase of the forced-swim test *within* a treatment group, data from this test were submitted to a Mann-Whitney rank sum test. In all cases, significance was set at $p< 0.05$.

Because 'emotionality' or 'anxiety' is unlikely to be a unidimensional characteristic and the behavioural tests described in this chapter may thus have measured different aspects of this characteristic, the combined behavioural data were analysed by multivariate (factor) analysis using a Statistica 5.1 package. This is a statistical

approach that analyses the correlations between variables and tries to detect structure in them by identifying common underlying dimensions (factors). Thus, factor analysis aims to reduce the number of variables to a number of new indices that are linear combinations of the original variables. These indices are then independent from each other and represent the underlying dimensions of the data.

The principal factors were selected according to an eigenvalue > 1, which meant that factors that accounted for only a small proportion of the total variability were ignored. The remaining factors were then subjected to a varimax rotation (so that the relative locations of the factors to each other remained unchanged), in order for their significance to be interpreted more easily.

Amygdala MR, GR and CRH mRNA expression in adult offspring

A low level of MR mRNA expression was found in BLA and CEA, whereas expression in MEA was practically absent (Figure 6.1). An ANOVA revealed no significant effects of prenatal treatment on MR mRNA in either BLA ($F_{3,28}$=1.42, p=0.26) or CEA ($F_{3,26}$=1.34, p=0.28), although there was a clear trend towards increased expression in both nuclei in DEX1-3 offspring (Figure 6.3a) which became significant in CEA when DEX1-3 rats were compared with control rats separately.

GR mRNA was moderately expressed in BLA, CEA and MEA (Figure 6.2). Prenatal treatments significantly altered GR mRNA expression per cell in BLA ($F_{3,24}$=7.23, p<0.005) and CEA ($F_{2,23}$=6.00, p<0.01), but not MEA ($F_{3,24}$=6.01, p=0.07). *Post-hoc* analysis revealed a significant increase in GR mRNA expression in amygdala of CBX offspring compared to control animals (BLA and CEA: p<0.001 and p<0.01 *versus* control, respectively) and DEX-treated animals (BLA p<0.01 *versus* DEX3; CEA p<0.01 *versus* DEX1-3 and DEX3). DEX1-3 animals also had significantly higher GR mRNA expression compared to control and DEX3 animals, but only in BLA (p<0.05) (Fig 6.3b).

CRH mRNA expression was restricted to CEA only (Figure 6.4). Analysis of total CRH RNA expression in CEA revealed significant differences between groups ($F_{3,34}$=6.58; p<0.005). *Post-hoc* analysis showed that prenatal exposure to DEX, either throughout pregnancy or in the last week only, increased CRH mRNA expression in CEA, with a similar trend in CBX offspring (Figure 6.5).

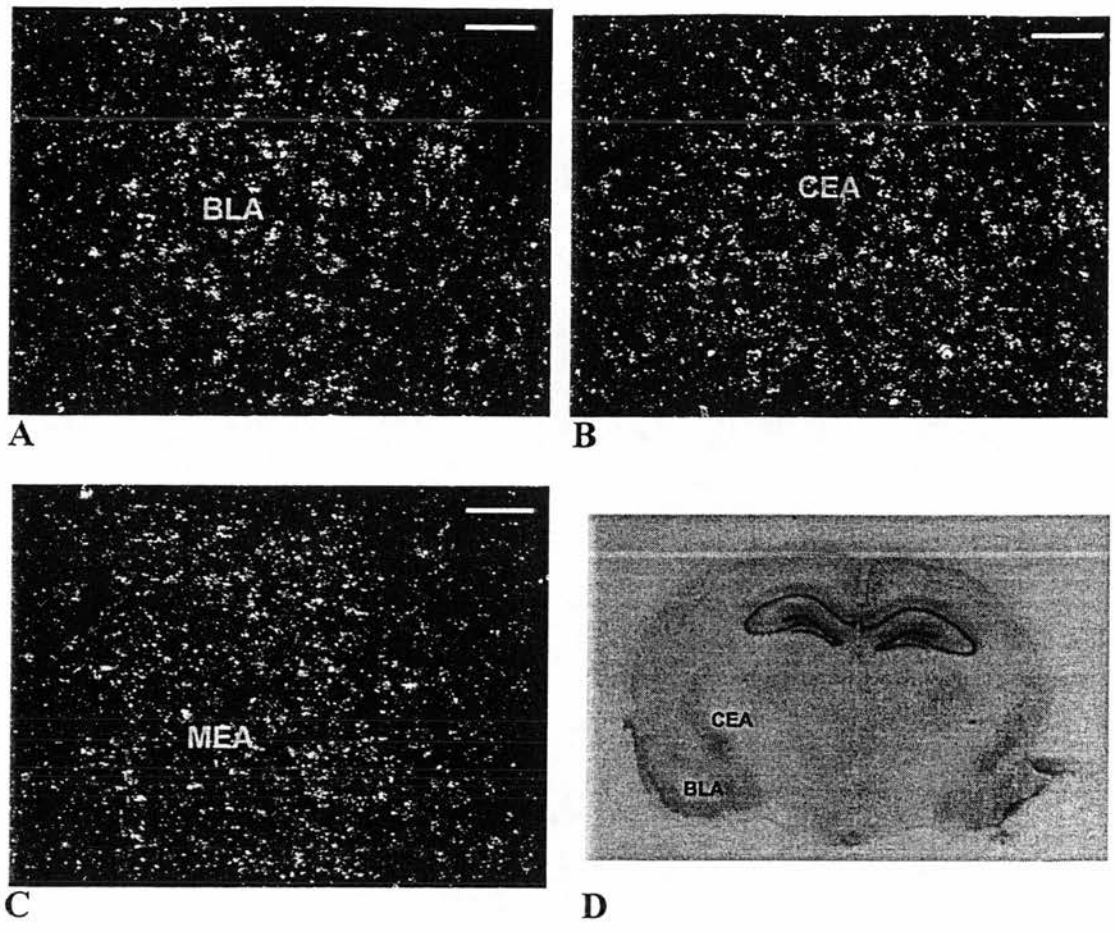

Figure 6.1 **Typical MR mRNA expression pattern in amygdaloid nuclei.**
Photomicroscopic (*A*, *B* and *C*) and autoradiographic (*D*) images show MR mRNA expression in BLA (*A*) and CEA (*B*), but not MEA (*C*). Scale bar represents 50 μm.

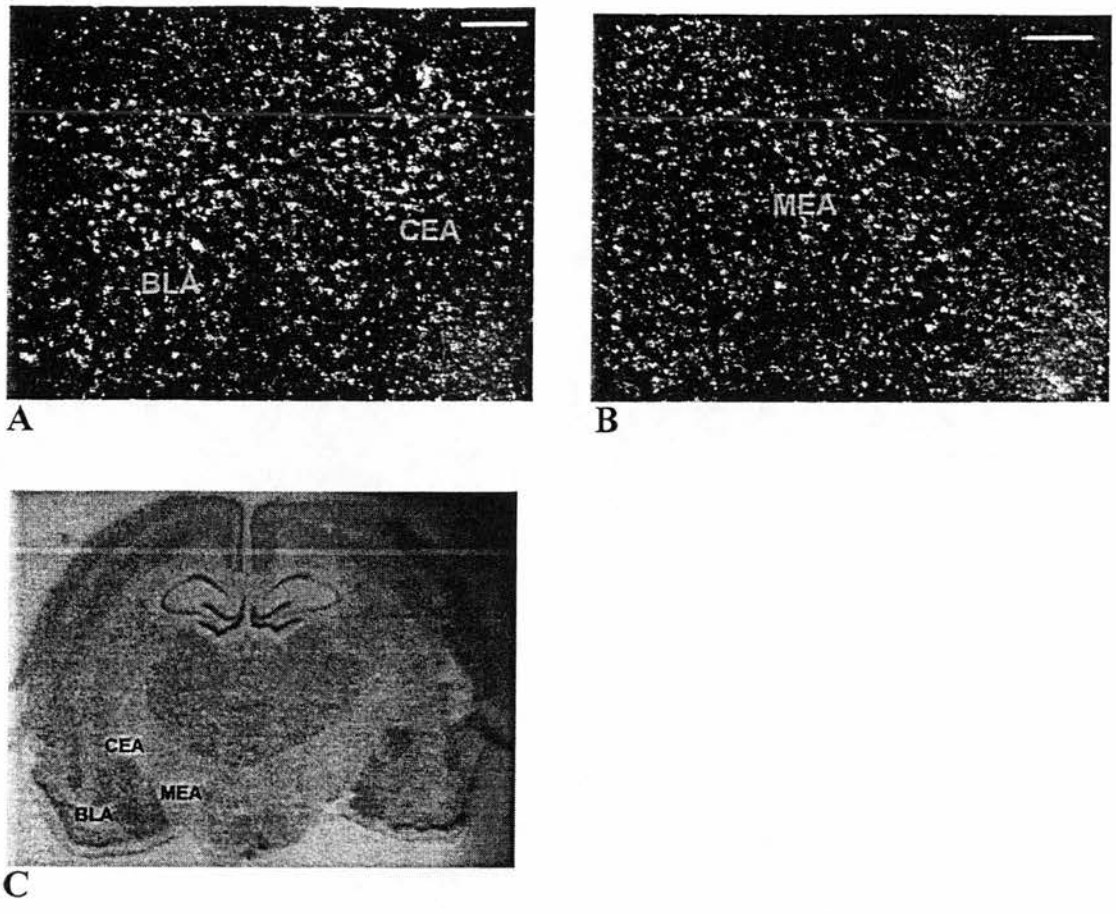

Figure 6.2 **Typical GR mRNA expression pattern in amygdaloid nuclei.**
Photomicroscopic (A and B) and autoradiographic (C) images show GR mRNA expression in BLA and CEA (A) and in MEA (B). Scale bar represents 50 μm.

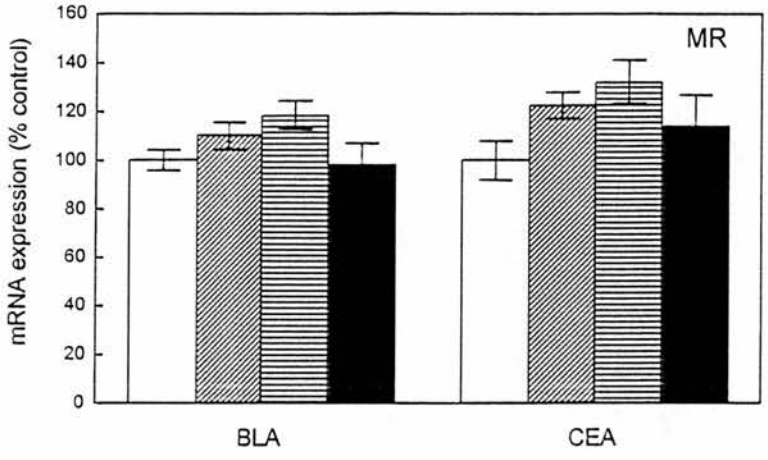

Figure 6.3a **MR mRNA expression in BLA and CEA.**
Effects of prenatal injections on amygdaloid GR mRNA expression. Data are expressed at % control. *Open bars* control, *diagonally striped bars* CBX, *horizontally striped bars* DEX1-3, *closed bars* DEX3, n=6-10/group.

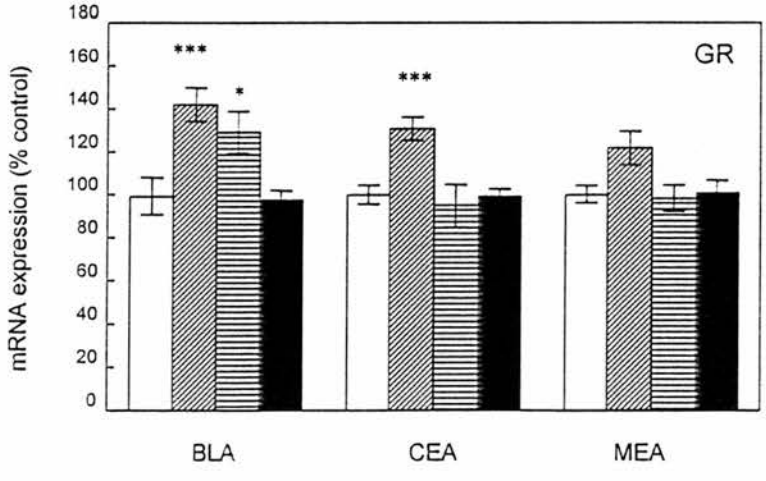

Figure 6.3b **GR mRNA expression in amygdala.**
Effects of prenatal injections on amygdaloid GR mRNA expression. Data are expressed at % control. *Open bars* control, *diagonally striped bars* CBX, *horizontally striped bars* DEX1-3, *closed bars* DEX3. *p<0.05, ***p<0.005; n=6-9/group

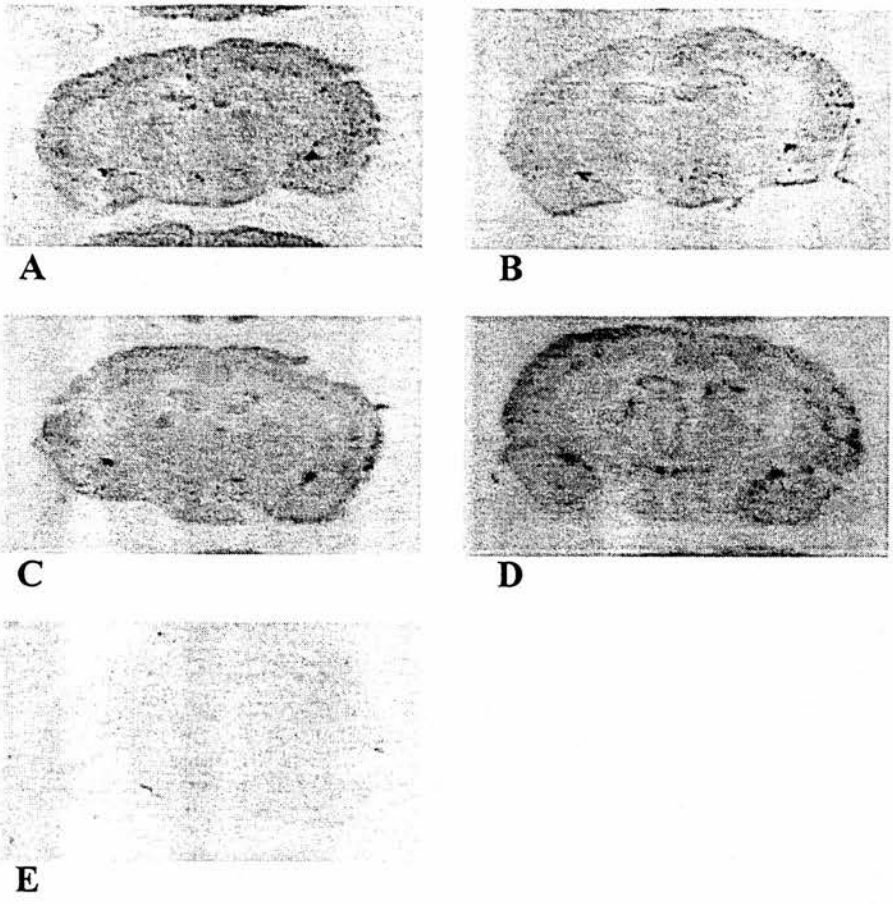

Figure 6.4 **Autoradiographic images of CRH mRNA expression in CEA.**
Images show typical sections from animals in each treatment group.
A-control, *B*-CBX, *C*-DEX1-3, *D*-DEX3, *E*-sense.

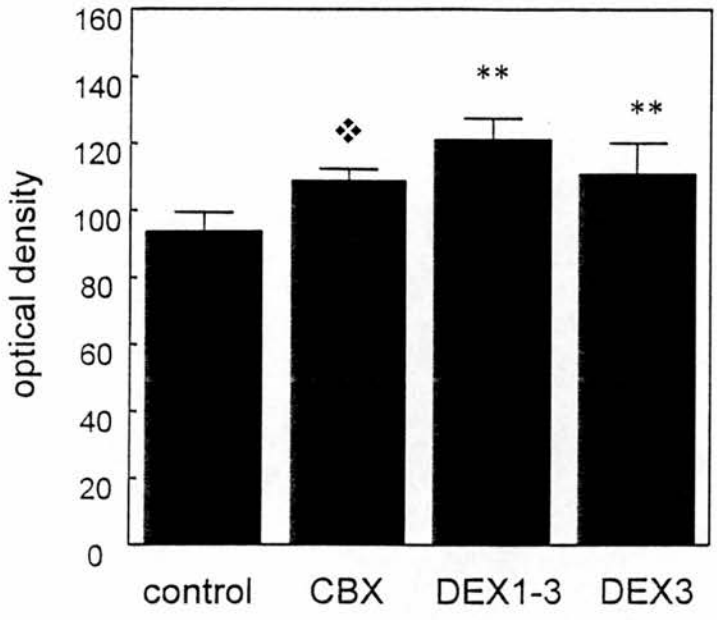

Figure 6.5 **CRH mRNA expression in CEA.**
This graph shows the effect of prenatal treatments on total CRH mRNA expression in CEA. ❖P=0.07; **p<0.01 *versus* control.

Behavioural reactivity in adult offspring

Open field

In a 12-minute open field test, locomotion ($F_{3,32}$=3.17, p<0.05) and rearing ($F_{3,32}$=4.94, p<0.01) were reduced in DEX-exposed animals, and this effect was most pronounced in DEX3 offspring. There was also a tendency for DEX-treated rats to avoid the centre of the open field (Figure 6.6a). Prenatal CBX treatment did not significantly affect behaviour in this test, although the slight trend towards reduced rearing and grooming (Figure 6.6a) was also observed in an earlier 10-minute open field test with a different population of CBX-offspring (Figure 6.6b).

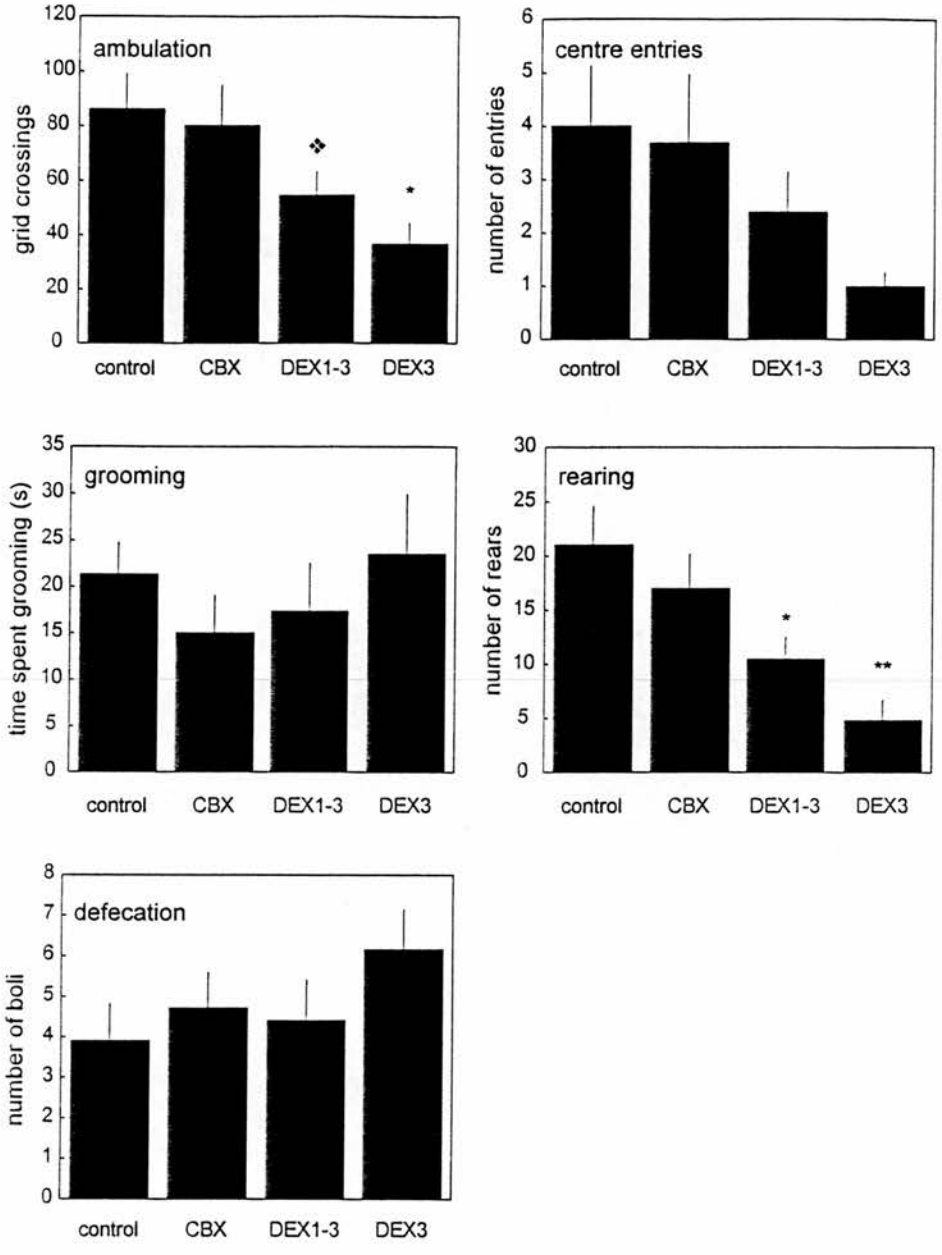

Figure 6.6a **Behaviour in a 12-minute open field test.**
Shown are the effects of prenatal treatments on ambulation, approaches into the centre area, grooming, rearing, and defecation in an open field later in life.* $p<0.05$, ** $p<0.01$, ❖$p=0.06$ *versus* control; n=8-9/group.

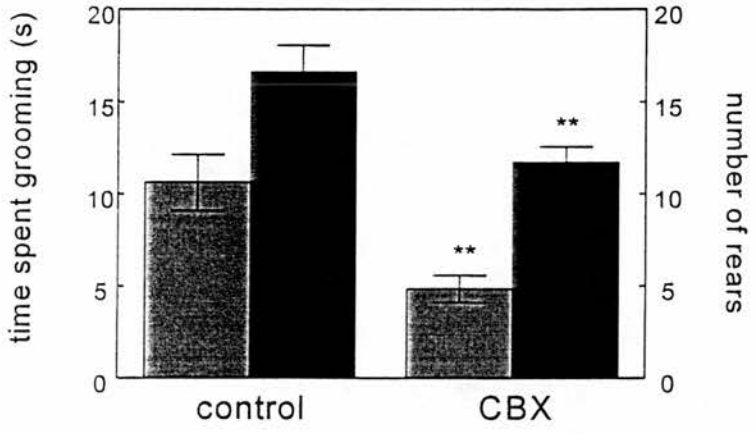

Figure 6.6b **Effect of prenatal CBX on grooming and rearing in a 10-min open field test.**
** p<0.01 *versus* control; *grey bars* grooming; *black bars* rearing; n=19/group.

Elevated plus-maze

Analysis of variance did not reveal any significant differences between prenatally glucocorticoid-exposed- and control offspring on the elevated plus-maze. However, the overall picture reveals a tendency towards reduced locomotion in CBX- and DEX3 rats (Table 6.1) and avoidance of open arms in DEX3 rats (Figure 6.4), whereas DEX1-3 animals tended to spend more time 'decision-making' in the central area of the plus-maze (Figure 6.7).

	open arm entries	*closed arm entries*	*base entries*	*total entries*
control	5.3 ± 1.4	25.8 ± 4.2	8.0 ± 2.0	39.1 ± 5.6
CBX	2.9 ± 1.2	16.3 ± 4.9	6.0 ± 1.8	25.1 ± 6.4
DEX1-3	3.3 ± 1.0	21.6 ± 2.7	8.3 ± 2.2	33.1 ± 5.1
DEX3	1.1 ± 0.6	14.3 ± 2.8	4.0 ± 0.8	19.4 ± 1.0
	p=0.10	p=0.14	p=0.33	p=0.05

Table 6.1 **Behaviour in a 5-minute elevated plus-maze.**
n=8-10/group

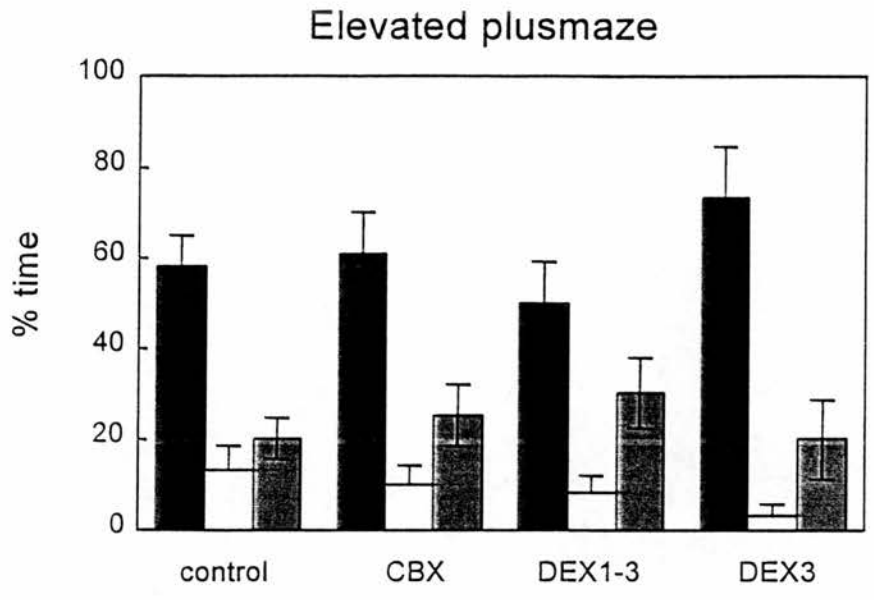

Figure 6.7 **Percentage of time spent on different compartments of an elevated plus-maze.** *Black bars* closed arms; *white bars* open arms; *grey bars* base; n=8-10/group.

Forced swim test

Both the acquisition phase (day 1) and the retention phase (day 2) of the forced-swim test showed significant differences in immobility between treatment groups ($p=0.012$ and $p=0.017$, respectively). Further analysis revealed that in both phases of the test DEX3 rats, but not CBX or DEX1-3 rats, were significantly less immobile than control rats (Table 6.2). In order to test whether all four groups were able to 'acquire' immobility, data from the acquisition phase were compared with those from the retention phase. Control rats and DEX1-3 rats spent significantly more time floating on the second day of the test (Table 6.2). In contrast, CBX- and DEX3 rats were not significantly more immobile in the retention test than in the acquisition test ($p=0.08$ and $p=0.24$, respectively), although there was a trend for CBX rats towards greater immobility on the second day (Figure 6.8). Plasma CORT levels measured in samples taken immediately after the retention test did not differ between groups (Table 6.2).

	Floating time day 1 (s)	*Floating time day 2 (s)*	*Difference (s)*	*Plasma [CORT] day 2 (μg/dl)*
Control	215.6 ± 2.3	250.7 ± 7.9	35.1 ± 8.8**	28.9 ± 8.6
CBX	178.7 ± 16.3	208.3 ± 12.4	29.6 ± 9.0	20.8 ± 3.2
DEX1-3	203.0 ± 7.4	234.6 ± 8.0	31.6 ± 7.7**	25.6 ± 8.5
DEX3	127.8 ± 28.6❀	169.0± 29.7❀	41.3 ± 10.7	25.8 ± 4.2

Table 6.2 **Immobility in a forced swim test.**
Effect of prenatal glucocorticoid exposure on immobility in acquisition and retention phase of a forced swim test and post-swimming plasma CORT levels (n=6-10/group). ❀ $p<0.05$ *versus* control same day, ** $p<0.01$ day 1 *versus* day 2.

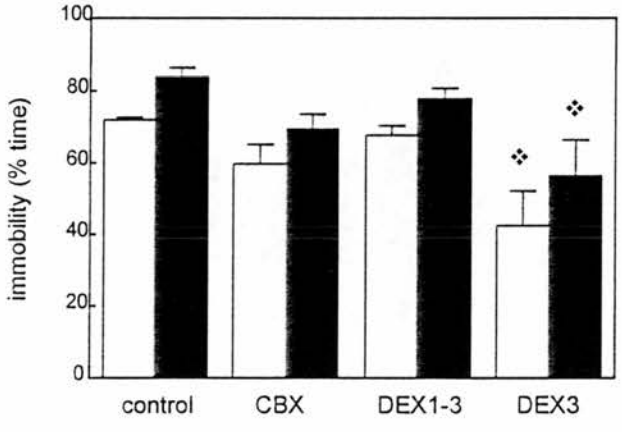

Figure 6.8 **Percentage of time spent floating in a 5-minute forced-swim test.**
Open bars day 1; *closed bars* day 2; ❖ $p<0.05$ compared to control same day; n=6-10/group.

Neophagia

Table 6.3 displays the results from the neophagia conflict test. Overall, variation was large in all parameters measured. There were no effects of treatment ($F_{1,64}$=0.18, p=0.67), novelty (new cage *versus* home cage) ($F_{3,62}$=0.95, p=0.42) or treatment × novelty interaction ($F_{3,64}$=0.84, p=0.48) with regard to the latency to approach the food pellets. The same was true for the number of approaches (treatment $F_{3,64}$=0.65, p=0.59; novelty $F_{1,64}$=0.90, p=0.35; interaction $F_{3,64}$=0.13, p=0.94). In contrast, the time it took to start eating was increased in a novel cage (novelty $F_{1,64}$=85.0, p<0.00001), but prenatal treatment did not affect this (treatment $F_{3,64}$=0.17, p=0.92; treatment × novelty interaction $F_{3,64}$=0.17, p=0.92). Similarly, the time spent eating was less for rats in a novel cage compared to rats in their home cage (novelty $F_{1,62}$=74.85, p<0.00001), but there was no significant treatment effect ($F_{3,64}$=2.23, p=0.09), or novelty × treatment interaction ($F_{3,64}$=1.61, p=0.20). Summarising, there were no significant effects of prenatal treatment on feeding parameters either in the home cage or in a novel cage, although DEX3 rats appear to spend less time eating than rats from the other groups. In general, animals in the novel cage showed a longer latency to start eating and spent less time eating.

Group/cage	*Approach latency (s)*	*Number of approaches*	*Eating latency (s)*	*Time spent eating (s)*
Control/home	69.6 ± 35.2	4.0 ± 0.9	84.8 ± 31.9	175.2 ± 20.4
Control/new	57.4 ± 17.0	5.0 ± 0.8	268.0 ± 21.1	19.5 ± 13.8
CBX/home	33.4 ± 12.1	3.5 ± 0.5	87.7 ± 31.1	140.8 ± 25.9
CBX/new	39.0 ± 8.5	3.7 ± 0.5	251.4 ± 21.2	36.8 ± 17.5
DEX1-3/home	54.5 ± 25.7	3.7 ± 0.7	83.6 ± 31.4	168.0 ± 4.1
DEX1-3/new	87.5 ± 35.2	4.0 ± 0.8	267.3 ± 18.9	7.8 ± 6.9
DEX3/home	111.8 ± 58.8	4.0 ± 1.1	87.8 ± 43.9	85.2 ± 33.6
DEX3/new	51.2 ± 14.1	4.5 ± 0.6	294.0 ± 6.0	1.0 ± 1.0

Table 6.3 **Food deprivation- and novelty induced feeding behaviour.**

Correlations

No correlations were found between data from the forced swim or neophagia test and the other behavioural tests. However, when data from the open field- and elevated plus-maze test were compared, significant correlations were found between ambulation in the open field and the number of entries (open arms, closed arms, base and total number of entries) on the elevated plus-maze. Borderline significance was found for the correlation between defecation in the open field and closed-arm and base entries on the plus-maze (Table 6.4a).

		ELEVATED PLUS-MAZE			
	variables	*OA entries*	*CA entries*	*Base entries*	*Total no. entries*
OPEN FIELD	*ambulation*	r=0.43 p=0.02*	r=0.45 p=0.02*	r=0.42 p=0.03*	r=0.47 p=0.01*
	centre entries	r=0.17 p=0.39	r=0.32 p=0.10	r=0.20 p=0.31	r=0.29 p=0.13
	grooming	r=0.19 p=0.34	r=0.14 p=0.50	r=0.16 p=0.43	r=0.09 p=0.64
	rearing	r=0.20 p=0.31	r=0.08 p=0.68	r=-0.01 p=0.96	r=0.60 p=0.76
	defecation	r=-1.5 p=0.45	r=-0.37 p=0.05•	r=-0.36 p=0.06•	r=-0.31 p=0.11

Table 6.4a **Correlations between behavioural variables in open field and elevated plus-maze.** *OA* open arms; *CA* closed arms; • borderline significance.

When the percentage of time spent on the arms of the plus-maze was compared with open field behaviour, a strong correlation was found between rearing in the open

field and the time spent on the open arms of the elevated plus-maze. Furthermore, there was a trend for rats that displayed a high level of ambulation in the open field to spend more time on the open arms and base compartment of the plus-maze (Table 6.4b).

		ELEVATED PLUS-MAZE		
	variables	*% time OA*	*% time CA*	*% time base*
OPEN FIELD	*ambulation*	r=0.35	r=-0.17	r=0.12
		p=0.07•	p=0.40	p=0.05•
	centre	r=0.26	r=-0.08	r=0.06
	entries	p=0.18	p=0.69	p=0.77
	grooming	r=0.07	r=0.21	r=-0.20
		p=0.72	p=0.29	p=0.32
	rearing	r=0.56	r=-0.19	r=-0.01
		p=0.00***	p=0.32	p=0.98
	defecation	r=0.01	r=0.13	r=-0.13
		p=0.94	p=0.50	p=0.51

Table 6.4b **Correlations between behavioural variables in open field and elevated plus-maze.** *OA* open arms; *CA* closed arms; • borderline significance.

Lastly, the level of CRH mRNA expressed in the central nucleus of the amygdala was negatively correlated with the total number of entries made on the elevated plus-maze and with the number of closed arm entries. Moreover, borderline significance was found for correlations between CRH mRNA expression and the number of base entries on the plus-maze and ambulation in the open field (Table 6.5).

	% time OA	*% time CA*	*% time base*
CEA CRH mRNA	r=-0.20	r=0.21	r=-0.32
	p=0.45	p=0.44	p=0.23

	OA entries	*CA entries*	*base entries*	*total no. entries*
CEA CRH mRNA	r=-0.36	r=-0.56	r=-0.49	r=-0.61
	p=0.17	p=0.02*	p=0.06•	p=0.01*

	ambulation	*centre entries*	*grooming*	*rearing*	*defecation*
CEA CRH mRNA	r=-0.37	r=0.36	r=0.03	r=-0.21	r=0.25
	p=0.09•	p=0.80	p=0.89	p=0.33	p=0.26

Table 6.5 **Correlations between CEA CRH mRNA expression and behaviour in elevated plus-maze and open field.**
OA open arms; *CA* closed arms; • borderline significance.

Characterisation of behavioural reactivity

Although some of the variables in the behavioural tests correlated to each other, this does not necessarily mean that they represent the same aspect of 'emotionality' or 'anxiety'. Furthermore, a number of borderline correlations suggest that some, *but not all* animals that show for example high defecation in the open field have a high number of closed arm entries in the elevated plus-maze. In other words, there may be a variation between rats in the 'sensitivity' to the challenges in different behavioural tests. In order to detect structure in data from the behavioural tests by identifying common underlying dimensions, a principal component analysis (PCA) was performed. However, because the results from the neophagia test had large variations and did not correlate with data from any other experiment, it was decided to exclude this test from the factor analysis. Therefore, a PCA was performed on data from the open field- and elevated plus-maze test. Thus, 5 factors with an eigenvalue > 1 were retained which together accounted for 90% of the total variance (Table 6.6). These

were then subjected to a varimax rotation (so that the relative locations of the factors to each other remained unchanged), in order for their significance to be interpreted more easily.

factor	*eigenvalue*	*% total variance*	*cumulative % variance*
1	4.53	41.21	41.21
2	1.77	16.08	57.28
3	1.40	12.77	70.05
4	1.11	10.12	80.18
5	1.09	9.87	90.04

Table 6.6 **Characteristics of each factor for PCA analysis.**
% variance percentage of variation explained by each factor.

It appears that factor 1 represents variables from the elevated plus-maze test, whereas factor 2 represents some of the variables measured in the open field. Factor 3 is associated with avoidance of the open arms on the elevated plus-maze. Factors 4 and 5 both represent a single variable, defecation and grooming in the open field, respectively (Table 6.7).

variables	*Factor 1*	*Factor 2*	*Factor 3*	*Factor 4*	*Factor 5*
Ambulation	.27	.88*	.16	.19	-.01
Centre entries	.18	.88*	.02	-.24	-.05
Defecation	-.28	.07	.05	-.89*	.02
Rearing	-.13	.64	.19	.62	.11
Grooming	.09	-.02	.02	.02	.97*
CA entries	.95*	.08	.03	.11	.04
OA entries	.50	.12	.75*	.04	.17
Base entries	.95*	.06	.25	.10	.05
Total entries	.95*	.15	.24	.06	.01
% time CA	-.37	.08	-.71*	-.06	.36
% time OA	.08	.21	.91*	-.03	.07

Table 6.7 **Factor loadings of each variable.**
This table shows the correlation between each variable and the factors in the factor analysis. *CA* closed arms; *OA* open arms; * loadings > 0.70.

From table 6.7 it is clear that the elevated plus-maze and open field test paradigms test for different aspects of 'emotionality', as variables from the two tests do not load on the same factors. Interpretation of extracted factors is arbitrary, but in the present analysis, factor 1 appears to be involved with the number of arm visits on the plus-maze (with the exception of the open arms), and can be interpreted as representing 'general activity' in this particular test. Factor 2 is associated with ambulation, entering the centre of the open field and to a lesser extent rearing. This factor represents 'exploration', although rearing also loads on factor 4 and may have an additional interpretation. Factor 3 comprises the number and time spent on the open arms of the plus-maze and time spent on the closed arms (negatively related) and is termed ' – anxiety', because a *negative* score on this factor suggests anxiety. Defecation in the open field loads highly (but negatively) on factor 4, which can be interpreted as 'fearlessness'. This factor also has moderate (positive) loading from rearing in the open field. Finally, factor 5 is only associated with grooming, and may represent 'relaxed' domestic activity. The location of all variables on factors 1, 2 and 3 is shown in figure 6.9.

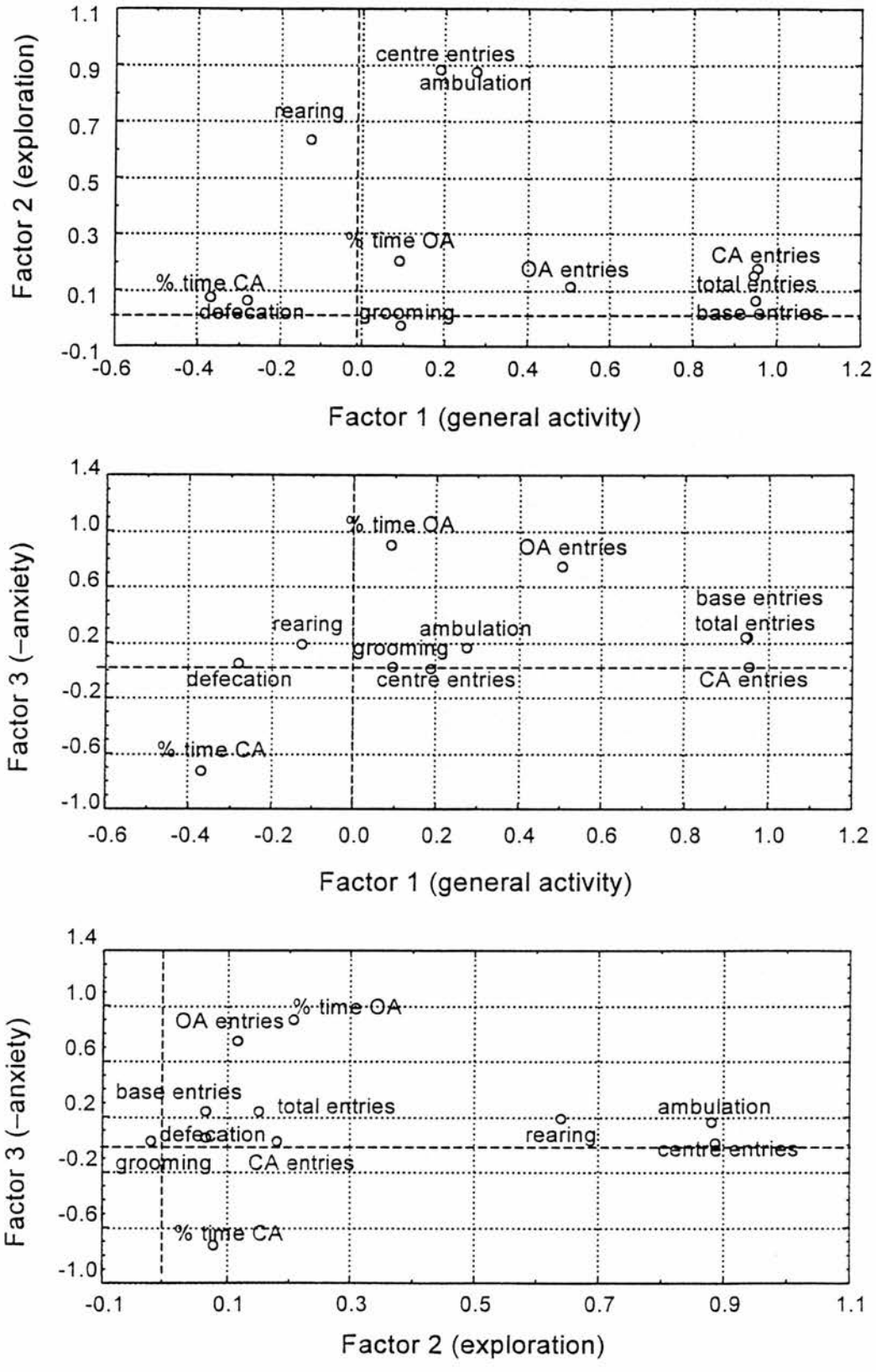

Figure 6.9 **PCA of behavioural variables.**
Three of the five extracted factors are plotted here. Factors 4 and 5 represent defecation and grooming, respectively. *CA* closed arms; *OA* open arms.

These experiments showed that fetal exposure to DEX programmed decreased exploration in an open field, and when administered in the last week of gestation only, programmed decreased acquired immobility in a forced swim test and a trend towards reduced locomotion and increased anxiety on the elevated plus-maze. CBX-exposed offspring displayed reduced grooming and rearing in an open field, tended to be less active on the elevated plus-maze and showed impaired (non-spatial) learning in a forced swim test. Taken together, these data suggest that fetal glucocorticoid exposure programmes 'behavioural inhibition' later in life. Furthermore, neuronal GR gene transcription was increased in BLA and CEA of CBX offspring, whereas DEX1-3, but not DEX3 offspring had higher GR mRNA expression in BLA and a trend towards higher MR mRNA levels in BLA and CEA. Total CRH mRNA levels were increased in CEA of DEX-exposed rats.

Several studies have shown that corticosteroids facilitate CRH mRNA expression in CEA (Albeck et al., 1997; Hatalski et al., 1998; Hsu et al., 1998; Makino et al., 1994a). The CEA contains corticosteroid receptors, which are expressed in CRH-positive neurones (Honkaniemi et al., 1992), suggesting that CORT may directly regulate the level of CRH gene transcription in CEA through activated MR and/or GR. Thus, increased corticosterone levels and/or increased expression of its receptors may have contributed to the elevated levels of CRH gene transcription observed in prenatally-glucocorticoid exposed rats in the present study: GR mRNA expression was increased in the CEA of CBX rats and in the BLA (which projects to the CEA) of CBX and DEX1-3 rats. DEX1-3 also tended to have higher MR mRNA levels in both nuclei. A direct relationship between brain corticosteroid receptor levels and anxiety-like behaviour was also shown in a recent paper by Tronche *et al.*, who observed reduced anxiety in mice with disrupted GR expression in the nervous system (Tronche et al., 1999). Furthermore, both CBX and DEX1-3 offspring (and possibly DEX3 rats, see Levitt et al., 1996) had increased basal circulating CORT. Thus, because basal, but not stress levels of CORT were elevated by the prenatal treatments (see chapter 3), these animals may have a higher basal CRH transcription,

resulting in an increased amygdaloid CRH content. Considering that central or intra-amygdaloid administration of CRH is anxiogenic [see Dunn and Berridge, 1990], it may be postulated that high CEA CRH levels in rats exposed to glucocorticoids *in utero* cause 'allostatic overload' (Schulkin et al., 1994), creating a permanent 'hyperemotional' state that is expressed behaviourally in adverse environments. Indeed, negative correlations were found between CRH mRNA levels in CEA and some measures of general activity and exploration in the behavioural tests. Interestingly, upregulation of CRH (Cratty et al., 1995) and GR mRNA (McCormick et al., 1995) was also found in prenatally stressed rats, which show hyperanxiety and impaired coping behaviour (Weinstock, 1997).

CRH-containing CEA neurones project to a number of brain regions including the BNST, hypothalamus, locus coeruleus and the nucleus of the *tractus solitarius* (Gray and Bingaman, 1996). They also express CRH receptors themselves (Grigoriadis et al., 1993), suggesting CRH may act in a paracrine fashion to modulate behaviour. The amygdala also modulates HPA activity (see chapter 1) and is thought to have a tonic stimulatory influence, possibly mediated by direct and indirect projections from the CEA to the PVN [for review see (Davis, 1992)]. Thus, activation of increased numbers of MR and/or GR in the CEA of CBX and DEX offspring by circulating CORT may have played a role in elevating basal CORT levels in these animals.

In the forced swim test, rats exposed to DEX throughout gestation did not behave differently from control animals. In contrast, DEX3 and CBX rats were affected by their respective prenatal treatments. The immobility rats acquire upon exposure to forced swimming is regarded as a successful means of passive 'behavioural adaptation' aimed to conserve energy in an inescapable situation. Activation of hippocampal GR has been implicated in this coping mechanism. Interestingly, DEX3 rats had reduced GR mRNA expression in the hippocampus and spent less time floating both in the initial and the retention test. This is in accord with a study from Korte *et al.*, who showed that treatment with antisense directed against GR mRNA in the dentate gyrus of the hippocampus reduces the time spent immobile both in the initial and the retest 24 h later (Korte et al., 1996). The same is true for mice with disrupted GR gene expression in the nervous system (Tronche et al., 1999).

Furthermore, DEX3- as well as CBX rats, did not improve their coping strategy upon a second exposure to forced swimming, indicating impaired consolidation and/or retrieval of information. Similar to initial acquisition, both consolidation and retrieval are modulated by glucocorticoid levels (De Kloet et al., 1988; Mitchell and Meaney, 1991). It may be postulated that in DEX3 rats, reduced hippocampal GR levels are responsible for this impairment, whereas in CBX rats, which had normal hippocampal corticosteroid receptor expression, increased circulating CORT levels 90 minutes post-stress (see chapter 3) interfered with the consolidation process. This would imply an inverted U-shaped relationship between hippocampal corticosteroid receptor occupation and memory in a forced swim test, where low GR occupation (such as in DEX3 rats or after ADX with low-dose CORT replacement, see (De Kloet et al., 1988; Mitchell and Meaney, 1991)) and very high GR occupation (such as when post-testing CORT levels remained elevated) interfere with the memory process. Importantly, the hippocampus is not the only brain region involved in the behavioural response to forced swimming. Increased activity in the locus coeruleus for example, also induces immobility in this test [see (West, 1990)]. The locus coeruleus receives input from the amygdala, which had increased GR mRNA expression in CBX rats. Thus, altered activity of locus coeruleus neurones may also have played a role in reduced performance of CBX rats in the forced swim test.

Adult rats exposed to DEX *in utero* display reduced ambulation and rearing and appear to make less entries into the centre of an open field. In addition, DEX3 have a tendency to defecate more. These parameters are generally assumed to represent 'anxiety' or 'emotionality'. Many studies measure only two variables in this test, and some only use defecation as an indication of 'emotionality'. Similarly, in the elevated plus-maze test, the amount of time spent on and the number of visits to the open arms are used as indicators of anxiety. Indeed, administration of anxiolytics increases the score for these parameters (Pellow and File, 1986). However, factor analysis clearly showed that it is not correct to draw conclusions about a rat's emotionality on the basis of one parameter or even one behavioural test. In the two tests used in this study, no less than five factors could be extracted that underlay the rats' behaviour.

Although the results of the two tests were only significant for 2 of the variables measured, DEX3 rats showed trends towards altered behaviour compared to control rats on almost every parameter. Interpreted in the factor analysis, this implies that DEX rats show reduced activity (factor 1), reduced exploration (factor 2) and increased anxiety (factors 3 and 4). Taken together, this points to 'behavioural inhibition' in aversive environments like an open field or an elevated maze. Similarly, the reduced immobility in the forced swim test can be regarded as behavioural inhibition, as the normal coping response would be to float and DEX3 rats are 'inhibited' from doing so. Behavioural inhibition can be regarded as an example of reduced coping ability, as it is not necessarily beneficial for an animal to remain passive in a challenging situation.

Rats treated with DEX throughout gestation have learning capacities similar to control rats in a forced swim test. On the elevated plus-maze there is a tendency to spend more time in the central area, but this was not significant. The only significant behavioural effect of DEX exposure throughout gestation was reduced rearing in an open field. In the factor analysis, rearing loaded moderately high on 'exploration' (factor 1) and negatively on 'fearlessness' (factor 4), indicating that DEX1-3 rats have a somewhat higher level of emotionality in some, but not all aspects. The programming properties of prenatal DEX on behaviour appear to be active mainly in the third week of gestation, similar to the critical windows observed for programming of hippocampal corticosteroid receptor expression, hypertension and glucose metabolism (Levitt et al., 1996; Nyirenda et al., 1998). Interestingly, this time window also applies for the effects of gestational stress on the serotonergic system in rats (Peters, 1988), which may be one of the systems underlying the behavioural alterations seen in prenatally stressed, and perhaps also in prenatal glucocorticoid-exposed rats.

Summarising, prenatal exposure to DEX, especially in the last week of gestation, results in behaviourally inhibited offspring that display reduced coping in aversive situations. Administration of CBX throughout pregnancy mainly impairs learning and/or memory in a non-spatial task like the forced swim test. As discussed in chapter 3, there are several factors that may explain why DEX and CBX offspring,

although both prenatally exposed to glucocorticoids, are not affected by this exposure in the same way. The patterns of 11β-HSD expression during development, the stages of maturity of the fetal target tissues, the differential expression of corticosteroid receptors and the stressfulness of CBX injections may underlie the time windows for specific effects of DEX and CBX administration *in utero*.

The long-term effects of fetal glucocorticoid exposure in humans have not been investigated. A preliminary study in children showed that prenatal DEX-exposure did not affect cognition scores, but increased scores for shyness, internalising, avoidance, emotionality and decreased scores for sociability (Trautman et al., 1995). Another study reported adverse events such as failure to thrive and delayed psychomotor development (Lajic et al., 1998). Although it is difficult to find the rodent equivalent of 'shyness' or 'internalising', the similarities with results from this study point to behavioural inhibition or reduced coping possibly related to increased anxiety. In conclusion, fetal overexposure to endogenous glucocorticoids may underlie the link between the prenatal environment and adult behavioural disorders.

Chapter 7. General discussion

The aim of this thesis was to establish the effects of prenatal glucocorticoid exposure on HPA axis regulation and stress-induced behaviour, and to study the role of feto-placental 11β-HSD2 in protecting the fetus from the effects of maternal glucocorticoids on these parameters. Chapter 3 described how inhibition of the feto-placental barrier to glucocorticoids reduced birth weight and increased basal morning and post-stress HPA activity, which may be associated with reduced GR mRNA expression in the PVN. Furthermore, fetal exposure to the synthetic glucocorticoid DEX also elevated basal morning but not stress-induced CORT concentrations. In chapter 4 it was shown that spatial learning and memory in a watermaze were not affected by these prenatal treatments. Chapter 5 revealed that DEX exposure in the last week of gestation reduces hippocampal MR and GR gene expression, an effect not seen in animals exposed to DEX throughout pregnancy. In contrast, GR mRNA levels in the PVN remained unchanged, but CRH mRNA levels were increased. Stress-induced CORT levels were not altered in these rats. As described in chapter 6, prenatal DEX administration reduced exploratory behaviour and general activity in adult offspring, especially after late-gestational exposure. CBX and DEX3 rats also showed an impaired behavioural response in a forced swim test. Taken together, it may be suggested that prenatal glucocorticoid exposure during specific 'critical' time windows, programs behavioural inhibition and impaired coping in aversive situations.

	CBX	*DEX1-3*	*DEX3*
Birth weight	↓	↓	↓
Basal CORT	↑	↑	n.m.
Hippocampus MR	=	=	↓
Hippocampus GR	=	=	↓
Amygdala MR	=	↑	=
Amygdala GR	↑	↑	=
Amygdala CRH	↑	↑	↑
PVN GR	↓	=	=
PVN CRH	↑	↑	↑
Coping (forced swim)	↓	=	↓
Exploration (open field)	=	↓	↓
General activity (plusmaze)	↓	=	↓
Anxiety (plusmaze)	↓	=	↓
Fearfulness (open field)	=	=	↑

Table 7.1 **Summary of results.**
Bold arrows represent significant effects; small arrows represent trends; n.m. = not measured.

An association between low birthweight and later disease has been shown in many epidemiological as well as animal studies and has led to the concept of 'prenatal programming'. The quest for the factors involved in prenatal programming has resulted in two hypotheses. One hypothesis proposes that maternal undernutrition interferes with fetal development, ultimately resulting in increased blood pressure (Godfrey et al., 1994; Langley and Jackson, 1994; Woodall et al., 1996), obesity (Susser and Stein, 1994), glucose intolerance (Ravelli et al., 1998) and even in the origin of (some cases of) schizophrenia (Susser et al., 1996). The second hypothesis is based on the importance of glucocorticoids in maintaining homeostasis and the regulatory role they play in development. It implicates excessive glucocorticoid

exposure *in utero* in fetal programming. Thus, earlier work from our lab showed that administration of DEX in the third week of pregnancy results in hypertension and hyperglycaemia (Levitt et al., 1996; Nyirenda et al., 1998).

Rats exposed to DEX or to CBX during pregnancy in this study had lower food intake than control rats. Undernutrition activates the HPA axis, as has been shown in non-pregnant rats (Jacobson et al., 1997), and reduces placental 11β-HSD activity (LangleyEvans et al., 1996b), suggesting that CBX injections increase *in utero* exposure to CORT levels in an indirect way as well as directly via inhibiting feto-placental 11β-HSD activity. Unfortunately, a lack of time prevented us from testing this by adrenalectomising CBX-injected pregnant rats. This should have been able to reveal whether the effects of CBX injections on offspring are dependent on maternal CORT, as is the case for hypertension and hyperglycaemia in CBX offspring (Lindsay et al., 1996a; Lindsay et al., 1996b). Moreover, adrenalectomy of vehicle-injected 'pair-fed' dams receiving a restricted amount of food similar to that eaten by adrenalectomised CBX-injected dams should demonstrate whether possible effects on offspring of undernutrition *per se* are dependent on maternal glucocorticoids as well. Similarly, in order to establish whether undernutrition *per se* could have caused the observed effects of DEX injections, this study would require an additional 'pair-fed' vehicle group with a constant low amount of circulating glucocorticoids.

Thus, the effects of the daily injections on pregnant females described in this thesis do not exclude the possibility that prenatal undernutrition may have (at least partly) underlain the effects seen in CBX- and DEX offspring. Both undernutrition and excessive glucocorticoid exposure may have induced fetal growth retardation by modulation of the IGF system (Oliver et al., 1993; Price et al., 1992), which is an important regulator of fetal and neonatal growth and development. In addition, glucocorticoid-induced reduction in placental glucose-transporter expression (Hahn et al., 1999) may reduce placental glucose transfer and thus contribute to fetal growth restriction.

One of the most important findings from this study is that prenatal CBX injections result in subtle changes in HPA axis regulation, whereas fetal DEX administration

mainly alters behaviour in aversive environments. This suggests that prenatal CBX and DEX are not two different versions of the same model. Other studies investigating the effects of DEX-injections on HPA-activity found that prenatal DEX treatment increased basal (Levitt et al., 1996; Muneoka et al., 1997), but not stress-induced (Holson et al., 1995) plasma CORT levels, whereas the opposite pattern is usually found in prenatally stressed animals (Henry et al., 1994; Weinstock et al., 1992). Apparently, prenatal glucocorticoid exposure is not the only component of maternal stress that programs offspring HPA-activity. CBX offspring had elevated basal *and* post-stress circulating CORT and decreased GR gene expression in PVN. There are several possible explanations for the differences in HPA regulation observed in CBX and DEX offspring. Firstly, glucocorticoid exposure through inhibition of CBX and through administration of DEX may have acted during different time windows. Placental 11β-HSD2 is highly expressed from midgestation onwards, but levels and activity drop in the last week of gestation (Brown et al., 1996; Waddell et al., 1998), suggesting that inhibition of this enzyme would be effective mainly during the second trimester. In contrast, DEX is poorly metabolised by 11β-HSD and readily reaches the fetal circulation. Thus, DEX1-3 animals were exposed to high glucocorticoid levels from early in gestation and DEX3 animals in the last week only. Brain GR mRNA and protein have been demonstrated from midgestation onwards (Bohn et al., 1994; Diaz et al., 1998; Kitraki et al., 1996), and although it is not known whether GRs are physiologically active at that time, it is possible that in DEX1-3 rats DEX effects through activation of GR have taken place in the second and third week of gestation. Thus, the three paradigms used in this experiment are likely to have provided three different time windows for glucocorticoid action: week 2 in CBX rats, week 2+3 in DEX1-3 rats, and week 3 in DEX3 rats. The components of the HPA axis and different brain areas do not all mature at a similar rate, and distinct development patterns in the expression of corticosteroid receptors have been demonstrated in brain (Bohn et al., 1994; Rosenfeld et al., 1988a; Rosenfeld et al., 1988b). Clearly, the timing of glucocorticoid exposure is likely to be of great importance for its effects on development. A specific prenatal time window has also been shown for

glucocorticoid programming of hyperglycaemia (Nyirenda et al., 1998) and hypertension in rats (Levitt et al., 1996) and sheep (Dodic et al., 1998).

A second explanation for the differences observed between the treatments might be that the dose of glucocorticoids that the fetuses were exposed to was not equal in all groups. The amount of CORT reaching the fetal circulation after inhibition of placental 11β-HSD depends on maternal CORT levels, which were highly variable in CBX-treated dams as shown in chapter 3. Thus, CBX-fetuses may have been exposed to much higher (endogenous) levels of glucocorticoids than the relatively low doses that DEX-fetuses were exposed to.

Thirdly, CBX and DEX may have acted through different mechanisms of programming: CBX is not an exclusive 11β-HSD2 inhibitor and may have affected other enzymes apart from 11β-HSD2, such as 11β-HSD1, 15-hydroxyprostaglandin dehydrogenase and 3α-HSDs (Baker, 1994). The latter are involved in the production of neurosteroids, which influence brain functioning via interaction with the neurotransmitter $GABA_A$- and NMDA receptors (Paul and Purdy, 1992; Puia et al., 1990; Schumacher, 1990). In addition, CBX may affect differentiation by blocking intercellular communication through gap junctions (BaniYaghoub et al., 1999). Furthermore, CBX-induced inhibition of 11β-HSD allows access of maternal CORT to the fetus, which may activate not only GR but also MR. MR mRNA and immunoreactivity are present from gestational day 13 onwards (Brown et al., 1996). This contrasts with administration of DEX, which only has high affinity for GR. The literature does not report any investigations into programming by specific MR agonists (i.e. other than CORT), but a preliminary study shows alterations in hippocampal 5-HT1a receptor mRNA levels by prenatal aldosterone injections (Diaz, Welberg and Holmes, unpublished observations), suggesting that MR activation may be responsible for at least some of the programming effects observed in CBX offspring.

In addition, CBX may have inhibited 11β-HSD activity in tissues other than placenta. 11β-HSD is expressed in many different tissues in the adult rat, such as kidney, liver, testis, heart, aorta, colon and specific brain areas (Monder, 1991;

Walker et al., 1992). In the mother, inhibiting 11β-HSD may have altered the level of circulating or local 'active' glucocorticoids, which could have effects on the mother's physiology and may have indirectly affected fetal growth and development. For example, CBX could have influenced glucocorticoid negative feedback on the maternal HPA axis. Indeed, administration of glycyrrhetinic acid, a different 11β-HSD inhibitor, decreased CRF-41 and increased AVP and oxytocin release into hypophysial portal blood at least in male rats (Seckl et al., 1993). Furthermore, if CBX is capable of crossing the placenta, inhibition of fetal 11β-HSD2, which is expressed from midgestation onwards (Brown et al., 1996), would increase the fetal level of glucocorticoids. Thus, CBX injections may have not only increased fetal exposure to maternal CORT, but also amplified the glucocorticoid signal locally in the fetal brain. Since 11β-HSD1 acitivity appears to be absent from the fetal brain (Diaz et al., 1998), reactivation of CORT (or inhibition thereof by CBX) is unlikely to play a role in fetal glucocorticoid programming.

Lastly, it is not unlikely that the CBX-paradigm has acted not only to inhibit 11β-HSD activity in mother, placenta and fetus, but also as a maternal stressor. CORT levels in CBX-injected rats varied greatly and this may be related to the fact that most pregnant rats did not tolerate CBX injections well and that some developed sores at the site of injection. This may have been due to poor solubility of CBX in the concentration used in this study. Other researchers (Robson, PhD thesis, Edinburgh University) have noted similar problems in neonates. A lower concentration of CBX (2.5 mg/day) has been tested, but did not reduce birthweight (Lindsay et al., unpublished observations), which is why the higher dose of 12.5 mg/day was continued in the present study. Consequently, factors released in response to infection or stress may have (partly) underlain the alterations seen in CBX offspring. Indeed, exposure to a prenatal immune challenge has been shown to program HPA axis activity (Reul et al., 1994). Furthermore, as mentioned earlier, stress-induced release of CORT in addition to inhibition of 11β-HSD may have exposed CBX-fetuses to much higher (endogenous) levels of glucocorticoids than the relatively low doses that DEX-fetuses were exposed to. Moreover, other factors associated with the possibly stressful CBX injections such as neurotransmitters or opioids may have

played a role in programming CBX offspring. Indeed, the opioid receptor inhibitor naltrexone prevents prenatal stress-induced alterations in rats (Keshet and Weinstock, 1995).

Interestingly, dissociation between prenatal exposure to 'stress' and 'glucocorticoids' has also been demonstrated in other studies. In two studies adrenalectomised rats were injected with 21-28 mg CORT daily in the second (Lee and Rivier, 1992) or third (Holson et al., 1995) week of pregnancy, but in neither case was offspring HPA activity affected. In contrast, a third study administered a much lower dose of CORT (9 mg/day) following daily restraint procedures and observed effects identical to those of prenatal stress (Barbazanges et al., 1996). Prenatal stress had no effects in offspring of adrenalectomised rats with constant low-dose CORT replacement or with no CORT administration at all (Barbazanges et al., 1996). Although the authors concluded that stress-induced increases in maternal glucocorticoids may be the mechanism by which prenatal stress impairs the development of the adult offspring's glucocorticoid response, it appears as though the *combination* of stress and high CORT levels was critical for the observed effects. Thus, an interaction between CORT and other stress-induced factors (e.g. neurotransmitters, opioids, neurosteroids) may be critical for inducing changes in prenatally stressed animals, as well as in CBX offspring in the present study.

Summarising, the differential effects of prenatal DEX and CBX may be attributed to the pattern of 11β-HSD expression during development and the stages of maturity of the fetal target tissues, while the stress and possible infection caused by CBX injections and the nonspecificity of this inhibitor may also have played a role.

The behavioural studies of this thesis were somewhat disappointing in the sense that they revealed many trends, but few of them reached significance. Although the tests were carefully designed and conditions kept as stable as possible, variability in the behavioural parameters measured was high in all tests. In future, a higher number of animals in each experimental group would be recommended to reduce the 'noise' caused by individual variability such that differences between experimental groups can be observed more clearly. Moreover, it might allow a clearer interpretation of the

data, as loading on the different factors in the principal component analysis would be greater. Furthermore, it may be interesting to test prenatally treated offspring at different ages. It has been demonstrated that cognitive function is influenced by perinatal manipulations, but this influence does not always become apparent until old age. For example, the effect of neonatal handling, which increases hippocampal GR levels, on watermaze performance can be demonstrated in 24 month-old, but not 6 or 12 month-old rats (Meaney et al., 1988), suggesting that handling interferes with the ageing process rather than directly influencing cognitive function. Similarly, prenatally stressed rats, which have elevated HPA axis activity in adulthood, only show cognitive impairments at old age (Vallee et al., 1999). Interestingly, Rowe *et al.* (Rowe et al., 1998) noted that cognitively impaired aged rats also display reduced exploratory behaviour in a novel environment, while another group demonstrated that reactivity to novelty in young animals predicts cognitive impairment later in life (Dellu et al., 1996). Thus, although the effects of prenatal injections with CBX or DEX on behaviour, particularly cognitive function, may only be subtle (or non-significant) at the age the rats were tested in the present study, they may become more apparent in old age.

An alternative mechanism that may explain the effects observed in CBX and DEX offspring which has not been addressed in this study, is the possibility that daily injections during pregnancy altered maternal behaviour after birth. This question may be resolved by cross-fostering studies, whereby prenatally CBX- or DEX exposed pups are adopted by control dams and vice versa. Adoption has been shown to reverse the effects of prenatal stress on HPA axis activity (Maccari et al., 1995) as does neonatal handling (Wakshlak and Weinstock, 1990), which probably acts through increased maternal behaviour (Liu et al., 1997). Furthermore, it would be interesting to investigate whether the effects of prenatal glucocorticoid exposure are transmissible across generations. Females were not tested in this study, but they appear to be more susceptible to effects of early manipulations such as prenatal stress (McCormick et al., 1995; Weinstock et al., 1992). Interestingly, Montano *et al.* described greater transport of CORT across the placenta in female than in male mouse fetuses, suggesting that placental 11β-HSD may be less active in female fetuses (Montano et al., 1993). If increased prenatal glucocorticoid exposure

permanently elevates circulating CORT levels in female offspring, then this may affect the *in utero* environment of the next generation.

Both CBX and DEX3 offspring showed some alterations in HPA axis regulation. CBX rats had elevated basal and post-stress plasma CORT levels in combination with reduced GR gene expression and a trend towards increased CRH expression in PVN. DEX3 rats had reduced hippocampal MR and GR and increased PVN CRH mRNA levels, but stress-induced CORT concentrations were unaltered. These results show that prenatal glucocorticoid exposure subtly increases HPA activity. However, the mechanisms underlying this increase could have been more thoroughly investigated.

For example, to state that a trend towards increased CRH mRNA expression in PVN leads to elevated plasma CORT levels is speculative, as there are many 'steps' in between these two parameters which have not been addressed. CRH stimulates the release of ACTH from the pituitary into the circulation. ACTH levels can be measured in plasma by radioimmunoassay but they rise quickly after an animal has been disturbed or stressed. Thus, they are only informative if the plasma sample is taken within 60 seconds from removing the rat from its cage, and unfortunately using the sampling method described in chapter 2, we were not able to obtain enough plasma in this short time span for measuring both CORT and ACTH.

Moreover, CRH is not the only secretagogue for ACTH but acts in concert with AVP. The latter potentiates the stimulatory effects of CRH (for review see Aguilera, 1993), and several physiological and especially psychological stimuli increase the median eminence AVP content (Romero and Sapolsky, 1996). Measurements of AVP expression in the parvocellular PVN would have revealed a more complete picture of possible alterations in the central drive of the HPA axis resulting from prenatal glucocorticoid exposure.

Furthermore, increased gene expression does not necessarily result in higher amounts of biologically active protein. The CRH content in the median eminence of the experimental rats was not measured, but it is possible that posttranscriptional or – translational mechanisms may have modified CRH protein levels. Similarly,

decreased mRNA levels of corticosteroid receptors in PVN, hippocampus or amygdala may not be of any influence on protein levels. The capacity and affinity of receptors can be measured using specific binding assays, but certain disadvantages are attached to these assays. Firstly, they require dissection of the brain region of interest immediately after decapitation and do not allow for distinction between separate hippocampal areas or amygdaloid nuclei as it is not possible to convincingly dissect these subregions. Secondly, some protocols for steroid binding assays require prior removal of endogenous ligands by adrenalectomy. Because circulating CORT regulates MR and GR, adrenalectomy is likely to affect the result of the binding assay. Indeed, adrenalectomy induces increased MR and GR mRNA (Herman, 1993; Herman and Spencer, 1998; Pfeiffer et al., 1991) and MR and GR binding (Reul et al., 1987) in the hippocampus. Thirdly, in order to obtain enough material to perform a binding assay, tissues from separate rats need to be pooled. As a consequence, the data obtained cannot be correlated to behavioural measures of individual rats. Because *in situ* hybridisation provides mRNA levels for each rat separately, amygdala CRH levels could be related to behaviour in open field and elevated plus-maze (see chapter 6). Unfortunately, MR and GR mRNA levels in amygdala and hippocampus were measured in tissues taken from a different population of rats, rendering correlational analysis impossible.

Results obtained from the *in situ* hybridisation experiments should be interpreted with caution. *In situ* hybridisation is only a semi-quantitative technique, as the number of grains expressed per neurone or the optical density on an autoradiogram cannot be directly related to an absolute amount of mRNA. It may therefore not be the best method available for quantifying mRNA levels. In future experiments, competitive quantitative PCR may be a useful alternative to measure alterations in gene expression, although this technique would not detect changes in specific subfields of the hippocampus or amygdala.

Summarising, it would be worth-while to repeat the behavioural tests with more animals at different ages, followed by measuring both gene transcription and protein levels of GR, MR, CRH, and AVP in the relevant brain regions so that these can be correlated to the behavioural measures. This would provide more direct information

regarding the role of these gene products in the rats' behavioural response in aversive situations.

In conclusion, this study provides another link between low birth weight and adult disorders, showing that prenatal glucocorticoid exposure not only increases blood pressure and plasma glucose levels (Levitt et al., 1996; Nyirenda et al., 1998), but also increases HPA axis activity and impairs coping in aversive situations. This reflects the crucial role of glucocorticoids in development and the long-term consequences of exposure to low dose DEX administration *in utero*. Chronic steroid treatment is not uncommon in cases of suspected congenital adrenal hyperplasia, whereas short-term DEX treatment is often given to postpone delivery of- and to help surfactant production in premature babies. From the rat model used in the present study it may be followed that such steroid therapy may predispose to later disorders by causing subtle alterations in brain and behaviour. A single (preliminary) follow-up study reports increased shyness, emotionality and avoidance with less sociability in children after early gestational exposure to DEX because of suspected congenital adrenal hyperplasia (Trautman et al., 1995).

Furthermore, this thesis shows the importance of feto-placental 11β-HSD in protecting the fetus from high endogenous glucocorticoids. Considering the high variation in placental 11β-HSD activity observed in rats (Benediktsson et al., 1993), low activity of this enzyme may render an individual more vulnerable to negative effects of maternal glucocorticoids on fetal development. Indeed, 11β-HSD activity correlates with birthweight in rats (Benediktsson et al., 1993) and humans (Stewart et al., 1995), and humans with defective 11β-HSD2 often have low birth weight (Kitanaka et al., 1996; White et al., 1997). However, the reduced food intake of CBX- and DEX treated dams and the stressfulness of CBX injections may also have played a role in programming of offspring.

In conclusion, this study has shown that fetal overexposure to endogenous (reduced activity of feto-placental 11β-HSD) or exogenous (prenatal DEX therapy) glucocorticoids may represent a common link between the prenatal environment, fetal growth and adult neuroendocrine and affective disorders.

References

Agarwal AK, Monder C, Eckstein B, White PC (1989) Cloning and expression of rat cDNA-encoding corticosteroid 11-beta- dehydrogenase. Journal of Biological Chemistry 264: 18939-18943.

Akana SF, Scribner KA, Bradbury MJ, Strack AM, Walker CD, Dallman MF (1992) Feedback sensitivity of the rat hypothalamo-pituitary-adrenal axis and its capacity to adjust to exogenous corticosterone. Endocrinology 131: 585-594.

Albeck DS, McKittrick CR, Blanchard DC, Blanchard RJ, Nikulina J, McEwen BS, Sakai RR (1997) Chronic social stress alters levels of corticotropin-releasing factor and arginine vasopressin mRNA in rat brain. Journal of Neuroscience 17: 4895-4903.

Albiston AL, Obeyesekere VR, Smith RE, Krozowski ZS (1994) Cloning and tissue distribution of the human 11-beta-hydroxysteroid dehydrogenase type-2 enzyme. Molecular and Cellular Endocrinology 105: R11-R17.

Almeida SS, Tonkiss J, Galler JR (1996) Prenatal protein malnutrition affects exploratory behavior of female rats in the elevated plus-maze test. Physiology & Behavior 60: 675-680.

Applegate CD, Frysinger RC, Kapp BS, Gallagher M (1982) Multiple unit-activity recorded from amygdala central nucleus during Pavlovian heart-rate conditioning in rabbit. Brain Research 238: 457-462.

Baker ME (1994) Licorice and enzymes other than 11-beta-hydroxysteroid dehydrogenase - an evolutionary perspective. Steroids 59: 136-141.

BaniYaghoub M, Underhill TM, Naus CCG (1999) Gap junction blockage interferes with neuronal and astroglial differentiation of mouse P19 embryonal carcinoma cells. Developmental Genetics 24: 69-81.

Barbazanges A, Piazza PV, LeMoal M, Maccari S (1996) Maternal glucocorticoid secretion mediates long-term effects of prenatal stress. Journal of Neuroscience 16: 3943-3949.

Barden N (1999) Regulation of corticosteroid receptor gene expression in depression and antidepressant action. Journal of Psychiatry & Neuroscience 24: 25-39.

Barker DJP (1995) Fetal origins of coronary heart disease. British Medical Journal 311: 171-174.

Beitins IZ, Bayard F, Ances IG, Kowarski A, Migeon CJ (1973) The metabolic clearance rats, blood production, interconversion and transplacental passage of cortisol and cortisone in pregnancy near term. Pediatric Research 7: 509-519.

Benediktsson R, Lindsay RS, Noble J, Seckl JR, Edwards CRW (1993) Glucocorticoid exposure inutero - new model for adult hypertension. Lancet 341: 339-341.

Benesova O, Pavlik A (1989) Perinatal treatment with glucocorticoids and the risk of maldevelopment of the brain. Neuropharmacology 28: 89-97.

Bitran D, Shiekh M, Dowd JA, Dugan MM, Renda P (1998) Corticosterone is permissive to the anxiolytic effect that results from the blockade of hippocampal mineralocorticoid receptors. Pharmacology, Biochemistry ond Behavior 60: 879-887.

Bohn MC, Dean D, Hussain S, Giuliano R (1994) Development of messenger-RNAs for glucocorticoid and mineralocorticoid receptors in rat hippocampus. Developmental Brain Research 77: 157-162.

Bradbury MJ, Akana SF, Dallman MF (1994) Roles of type-i and type-ii corticosteroid receptors in regulation of basal activity in the hypothalamo-pituitary-adrenal axis during the diurnal trough and the peak - evidence for a nonadditive effect of combined receptor occupation. Endocrinology 134: 1286-1296.

Britton DR, Britton KT (1981) A sensitive open-field measure of anxiolytic drug activity. Pharmacology, Biochemistry and Behavior 15: 577-582.

Brooks AN, Hagan DM, Howe DC (1996) Neuroendocrine regulation of pituitary-adrenal function during fetal life. European Journal of Endocrinology 135: 153-165.

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

Buhler H, Perschel FH, Fitzner R, Hierholzer K (1994) Endogenous inhibitors of 11-beta-OHSD - existence and possible significance. Steroids 59: 131-135.

Cahill L, Haier RJ, Fallon J, Alkire MT, Tang C, Keator D, Wu J, McGaugh JL (1996) Amygdala activity at encoding correlated with long-term, free recall of emotional information. Proceedings of the National Academy of Sciences of the United States of America 93: 8016-8021.

Caldji C, Tannenbaum B, Sharma S, Francis D, Plotsky PM, Meaney MJ (1998) Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. Proceedings of the National Academy of Sciences of the United States of America 95: 5335-5340.

Cannon M, Jones P, Gilvarry C, Rifkin L, McKenzie K, Foerster A, Murray RM (1997) Premorbid social functioning in schizophrenia and bipolar disorder: Similarity and differences. American Journal of Psychiatry 154: 1544-1550.

Catalani A, Marinelli M, Scaccianoce S, Nicolai R, Muscolo LAA, Porcu A, Koranyi L, Piazza PV, Angelucci L (1993) Progeny of mothers drinking corticosterone during lactation has lower stress-induced corticosterone secretion and better cognitive performance. Brain Research 624: 209-215.

Celsi G, Kistner A, Aizman R, Eklof AC, Ceccatelli S, DeSantiago A, Jacobson SH (1998) Prenatal dexamethasone causes oligonephronia, sodium retention, and higher blood pressure in the offspring. Pediatric Research 44: 317-322.

Cintra A, Solfrini V, Bunnemann B, Okret S, Bortolotti F, Gustafsson JA, Fuxe K (1993) Prenatal development of glucocorticoid receptor gene-expression and immunoreactivity in the rat-brain and pituitary-gland - a combined in-situ hybridization and immunocytochemical analysis. Neuroendocrinology 57: 1133-1147.

Conrad CD, Lupien SJ, Thanasoulis LC, McEwen BS (1997) The effects of Type I and Type II corticosteroid receptor agonists on exploratory behavior and spatial memory in the Y-maze. Brain Research 759: 76-83.

Cotterrell M, Balaz R, Johnson AL (1972) Effects of corticosteroids on the biochemical maturation of rat brain: postnatal cell formation. Journal of Neurochemistry 19: 2151-2167.

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 Research 675: 297-302.

Dallman MF, Akana SF, Scribner KA, Bradbury MJ, Walker CD, Strack AM, Cascio CS (1992) Stress, feedback and facilitation in the hypothalamopituitary-adrenal axis. Journal of Neuroendocrinology 4: 517-526.

D'Amato FR, Cabib S, Ventura R, Orsini C (1998) Long-term effects of postnatal manipulation on emotionality are prevented by maternal anxiolytic treatment in mice. Developmental Psychobiology 32: 225-234.

DaveSharma S, Wilson RC, Harbison MD, Newfield R, Azar MR, Krozowski ZS, Funder JW, Shackleton CHL, Bradlow HL, Wei JQ, Hertecant J, Moran A, Neiberger RE, Balfe JW, Fattah A, Daneman D, Akkurt HI, DeSantis C, New MI (1998) Extensive personal experience - Examination of genotype and phenotype

relationships in 14 patients with apparent mineralocorticoid excess. Journal Of Clinical Endocrinology and Metabolism 83: 2244-2254.

Davis M (1992) The role of the amygdala in fear and anxiety. Annual Review of Neuroscience 15: 353-375.

Davis M, Rainnie D, Cassell M (1994) Neurotransmission in the rat amygdala related to fear and anxiety. Trends in Neurosciences 17: 208-214.

Day JC, Koehl M, Deroche V, LeMoal M, Maccari S (1998) Prenatal stress enhances stress- and corticotropin-releasing factor- induced stimulation of hippocampal acetylcholine release in adult rats. Journal of Neuroscience 18: 1886-1892.

De Kloet ER (1991) Brain corticosteroid receptor balance and homeostatic control. Frontiers in Neuroendocrinology 12: 95-164.

De Kloet, ER (1997) Why dexamethasone poorly penetrates in brain. Stress 2: 13-19.

De Kloet ER, Dekock S, Schild V, Veldhuis HD (1988) Antiglucocorticoid RU-38486 attenuates retention of a behavior and disinhibits the hypothalamic-pituitary adrenal axis at different brain sites. Neuroendocrinology 47: 109-115.

De Kloet ER, Sutanto W, Vandenberg DTWM, Carey MP, van Haarst AD, Hornsby CD, Meijer OC, Rots NY, Oitzl MS (1993) Brain mineralocorticoid receptor diversity - functional implications. Journal of Steroid Biochemistry and Molecular Biology 47: 183-190.

De Kloet ER, Vreugdenhil E, Oitzl MS, Joels M (1998) Brain corticosteroid receptor balance in health and disease. Endocrine Reviews 19: 269-301.

Dekosky ST, Nonneman AJ, Scheff SW (1982) Morphologic and behavioral-effects of perinatal glucocorticoid administration. Physiology & Behavior 29: 895-900.

Dellu F, Mayo W, Vallee M, Maccari S, Piazza PV, LeMoal M, Simon H (1996) Behavioral reactivity to novelty during youth as a predictive factor of stress-induced

corticosterone secretion in the elderly - A life- span study in rats. Psychoneuroendocrinology 21: 441-453.

Demitrack MA, Dale JK, Straus SE, Laue L, Listwak SJ, Kruesi MJP, Chrousos GP, Gold PW (1991) Evidence for impaired activation of the hypothalamic-pituitary-adrenal axis in patients with chronic fatigue syndrome. Journal of Clinical Endocrinology and Metabolism 73: 1224-1234.

Diamond DM, Bennett MC, Fleshner M, Rose GM (1992) Inverted-u relationship between the level of peripheral corticosterone and the magnitude of hippocampal primed burst potentiation. Hippocampus 2: 421-430.

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

Dijkstra I, Binnekade R, Tilders FJH (1996) Diurnal variation in resting levels of corticosterone is not mediated by variation in adrenal responsiveness to adrenocorticotropin but involves splanchnic nerve integrity. Endocrinology 137: 540-547.

Dodic M, May CN, Wintour EM, Coghlan JP (1998) An early prenatal exposure to excess glucocorticoid leads to hypertensive offspring in sheep. Clinical Science 94: 149-155.

Drago F, Dileo F, Giardina L (1999) Prenatal stress induces body weight deficit and behavioural alterations in rats: the effect of diazepam. European Neuropsychopharmacology 9: 239-245.

Dunn AJ, Berridge CW (1990) Physiological and behavioral-responses to corticotropin-releasing factor administration - is CRF a mediator of anxiety or stress responses? Brain Research Reviews 15: 71-100.

Dupouy J-P, Chatelain A, Allaume P (1980) Absence of transplacental passage of ACTH in the rat: direct experimental proof. Biology of the Neonate 37, 96-102.

Edwards CRW, Benediktsson R, Lindsay RS, Seckl JR (1993) Dysfunction of placental glucocorticoid barrier - link between fetal environment and adult hypertension. Lancet 341: 355-357.

Edwards CRW, Burt D, Mcintyre MA, Dekloet ER, Stewart PM, Brett L, Sutanto WS, Monder C (1988) Localization of 11-beta-hydroxysteroid dehydrogenase- tissue specific protector of the mineralocorticoid receptor. Lancet 2: 986-989.

Engelmann M, Landgraf R, Lorscher P, Conzelmann C, Probst JC, Holsboer F, Reul JMHM (1998) Downregulation of brain mineralocorticoid and glucocorticoid receptor by antisense oligodeoxynucleotide treatment fails to alter spatial navigation in rats. European Journal of Pharmacology 361: 17-26.

Evans RM, Arriza JL (1989) A molecular framework for the actions of glucocorticoid hormones in the nervous-system. Neuron 2: 1105-1112.

Fall CHD, Osmond C, Barker DJP, Clark PMS, Hales CN, Stirling Y, Meade TW (1995) Fetal and infant growth and cardiovascular risk-factors in women. British Medical Journal 310: 428-432.

Fameli M, Kitraki E, Stylianopoulou F (1994) Effects of hyperactivity of the maternal hypothalamic-pituitary-adrenal (HPA) axis during pregnancy on the development of the HPA axis and brain monoamines of the offspring. International Journal of Developmental Neuroscience 12: 651-659.

Feldman S, Saphier D, Weidenfeld J (1992) Corticosterone implants in the paraventricular nucleus inhibit ACTH and corticosterone responses and the release of corticotropin- releasing factor following neural stimuli. Brain Research 578: 251-255.

Feldman S, Weidenfeld J (1995) Neural mechanisms involved in the corticosteroid feedback effects on the hypothalamo-pituitary-adrenocortical axis. Progress in Neurobiology 45: 129.

Feldman S, Weidenfeld J (1998) The excitatory effects of the amygdala on hypothalamo-pituitary- adrenocortical responses are mediated by hypothalamic norepinephrine, serotonin, and CRF-41. Brain Research Bulletin 45: 389-393.

Felszeghy K, Gaspar E, Nyakas C (1996) Long-term selective down-regulation of brain glucocorticoid receptors after neonatal dexamethasone treatment in rats. Journal of Neuroendocrinology 8: 493-499.

Felszeghy K, Sasvari M, Nyakas C (1993) Behavioral depression - opposite effects of neonatal dexamethasone and ACTH-(4-9) analog (Org 2766) treatments in the rat. Hormones and Behavior 27: 380-396.

Ferguson SA, Holson RR (1999) Neonatal dexamethasone on day 7 causes mild hyperactivity and cerebellar stunting. Neurotoxicology and Teratology 21: 71-76.

Foerster A, Lewis SW, Owen MJ, Murray RM (1991) Low-birth-weight and a family history of schizophrenia predict poor premorbid functioning in psychosis. Schizophrenia Research 5: 13-20.

Fride E, Dan Y, Feldon J, Halevy G, Weinstock M (1986) Effects of prenatal stress on vulnerability to stress in prepubertal and adult-rats. Physiology & Behavior 37: 681-687.

Fride E, Weinstock M (1984) The effects of prenatal exposure to predictable or unpredictable stress on early development in the rat. Developmental Psychobiology 17: 651-660.

Funder JW, Pearce PT, Smith R, Smith AI (1988) Mineralocorticoid action - target tissue-specificity is enzyme, not receptor, mediated. Science 242: 583-585.

Godfrey KM, Forrester T, Barker DJP, Jackson AA, Landman JP, Hall JS, Cox V, Osmond C (1994) Maternal nutritional-status in pregnancy and blood-pressure in childhood. British Journal of Obstetrics and Gynaecology 101: 398-403.

Goldman L, Winget C, Holingshead GW, Levine S (1973) Postweaning development of negative feedback in the pituitary-adrenal system of the rat. Neuroendocrinology 12: 199-211.

Goldstein LE, Rasmusson AM, Bunney BS, Roth RH (1996) Role of the amygdala in the coordination of behavioral, neuroendocrine, and prefrontal cortical monoamine responses to psychological stress in the rat. Journal of Neuroscience 16: 4787-4798.

Gray TS, Bingaman EW (1996) The amygdala: Corticotropin-releasing factor, steroids, and stress. Critical Reviews in Neurobiology 10: 155-168.

Grigoriadis DE, Heroux JA, Desouza EB (1993) Characterization and regulation of corticotropin-releasing factor receptors in the central nervous, endocrine and immune-systems. Ciba Foundation Symposia 172: 85-107.

Grimm VE, Frieder B (1987) The effects of mild maternal stress during pregnancy on the behavior of rat pups. International Journal of Neuroscience 35: 65-72.

Grino M, PaulmyerLacroix O, Anglade G, Oliver C (1995) Molecular aspects of the regulation of the hypothalamo-pituitary- adrenal axis during development in the rat. Annals of the New York Academy of Sciences 771: 339-351.

Hahn T, Barth S, Graf R, Engelmann M, Beslagic D, Reul JMHM, Holsboer F, Dohr G, Desoye G (1999) Placental glucose transporter expression is regulated by glucocorticoids. Journal of Clinical Endocrinology and Metabolism 84: 1445-1452.

Hales CN, Barker DJP, Clark PMS, Cox LJ, Fall C, Osmond C, Winter PD (1991) Fetal and infant growth and impaired glucose-tolerance at age 64. British Medical Journal 303: 1019-1022.

Hatalski CG, Guirguis C, Baram TZ (1998) Corticotropin releasing factor mRNA expression in the hypothalamic paraventricular nucleus and the central nucleus of the amygdala is modulated by repeated acute stress in the immature rat. Journal of Neuroendocrinology 10: 663-669.

Hauger RL, Millan MA, Catt KJ, Aguilera G (1987) Differential regulation of brain and pituitary corticotropin- releasing factor receptors by corticosterone. Endocrinology 120: 1527-1533.

Hayashi A, Nagaoka M, Yamada K, Ichitani Y, Miake Y, Okado N (1998) Maternal stress induces synaptic loss and developmental disabilities of offspring. International Journal of Developmental Neuroscience 16: 209-216.

Henry C, Kabbaj M, Simon H, LeMoal M, Maccari S (1994) Prenatal stress increases the hypothalamo-pituitary-adrenal axis response in young and adult-rats. Journal of Neuroendocrinology 6: 341-345.

Herman JP (1993) Regulation of adrenocorticosteroid receptor messenger-RNA expression in the central-nervous-system. Cellular and Molecular Neurobiology 13: 349-372.

Herman JP, Adams D, Prewitt C (1995) Regulatory changes in neuroendocrine stress integrative circuitry produced by a variable stress paradigm. Neuroendocrinology 61: 180-190.

Herman JP, Cullinan WE (1997) Neurocircuitry of stress: Central control of the hypothalamo- pituitary-adrenocortical axis. Trends in Neurosciences 20: 78-84.

Herman JP, Schafer MKH, Young EA, Thompson R, Douglass J, Akil H, Watson SJ (1989) Evidence for hippocampal regulation of neuro-endocrine neurons of the hypothalamo pituitary adrenocortical axis. Journal of Neuroscience 9: 3072-3082.

Herman JP, Spencer R (1998) Regulation of hippocampal glucocorticoid receptor gene transcription and protein expression in vivo. Journal of Neuroscience 18: 7462-7473.

Herman JP, Watson SJ (1995) Stress regulation of mineralocorticoid receptor heteronuclear RNA in rat hippocampus. Brain Research 677: 243-249.

Holsboer F, Barden N (1996) Antidepressants and hypothalamic pituitary adrenocortical regulation. Endocrine Reviews 17: 187-205.

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. Neurotoxicology and Teratology 17: 393-401.

Honkaniemi J, Peltohuikko M, Rechardt L, Isola J, Lammi A, Fuxe K, Gustafsson JA, Wikstrom AC, Hokfelt T (1992) Colocalization of peptide and glucocorticoid receptor immunoreactivities in rat central amygdaloid nucleus. Neuroendocrinology 55: 451-459.

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 Research 788: 305-310.

Hultman CM, Ohman A, Cnattingius S, Wieselgren IM, Lindstrom LH (1997) Prenatal and neonatal risk factors for schizophrenia. British Journal of Psychiatry 170: 128-133.

Inui A (1999) Feeding and body-weight regulation by hypothalamic neuropeptides - mediation of the actions of leptin. Trends in Neurosciences 22: 62-67.

Issa AM, Rowe W, Gauthier S, Meaney MJ (1990) Hypothalamic-pituitary-adrenal activity in aged, cognitively impaired and cognitively unimpaired rats. Journal of Neuroscience 10: 3247-3254.

Jacobson L, Sapolsky R (1991) The role of the hippocampus in feedback-regulation of the hypothalamic-pituitary-adrenocortical axis. Endocrine Reviews 12: 118-134.

Jacobson L, Zurakowski D, Majzoub JA (1997) Protein malnutrition increases plasma adrenocorticotropin and anterior pituitary proopiomelanocortin messenger ribonucleic acid in the rat. Endocrinology 138: 1048-1057.

Jansen LMC, Gispen-de Wied CC, Gademan PJ, de Jonge RCJ, van der Linden JA, Kahn RS (1998) Blunted cortisol response to a psychosocial stressor in schizophrenia. Schizophrenia Research 33: 87-94.

Joels M, de Kloet ER (1992) Control of neuronal excitability by corticosteroid hormones. Trends in Neurosciences 15: 25-30.

Jones PB, Rantakallio P, Hartikainen AL, Isohanni M, Sipila P (1998) Schizophrenia as a long-term outcome of pregnancy, delivery, and perinatal complications: A 28-year follow-up of the 1966 North Finland general population birth cohort. American Journal of Psychiatry 155: 355-364.

Kabbaj M, LeMoal M, Maccari S (1996) Hippocampal type I and type II corticosteroid receptors are differentially regulated by chronic prazosin treatment. Neuroscience 73: 963-970.

Kabbaj M, Piazza PV, Simon H, LeMoal M, Maccari S (1995) Opposite effects on hippocampal corticosteroid receptors induced by stimulation of beta-noradrenergic and alpha-1-noradrenergic receptors. Neuroscience 66: 539-545.

Kalinyak JE, Griffin CA, Hamilton RW, Bradshaw JG, Perlman AJ, Hoffman AR (1989) Developmental and hormonal-regulation of glucocorticoid receptor messenger-RNA in the rat. Journal of Clinical Investigation 84: 1843-1848.

Keshet GI, Weinstock M (1995) Maternal naltrexone prevents morphological and behavioral alterations induced in rats by prenatal stress. Pharmacology, Biochemistry and Behavior 50: 413-419.

Kinsley C, Svare B (1986) Prenatal stress effects - are they mediated by reductions in maternal food and water-intake and body-weight gain. Physiology & Behavior 37: 191-193.

Kitanaka S, Tanae A, Hibi I (1996) Apparent mineralocorticoid excess due to 11 beta-hydroxysteroid dehydrogenase deficiency: A possible cause of intrauterine growth retardation. Clinical Endocrinology 44: 353-359.

Kitraki E, Alexis MN, Papalopoulou M, Stylianopoulou F (1996) Glucocorticoid receptor gene expression in the embryonic rat brain. Neuroendocrinology 63: 305-317.

Kopchia KL, Altman HJ, Commissaris RL (1992) Effects of lesions of the central nucleus of the amygdala on anxiety- like behaviors in the rat. Pharmacology, Biochemistry and Behavior 43: 453-461.

Korte SM, de Kloet ER, Buwalda B, Bouman SD, Bohus B (1996) Antisense to the glucocorticoid receptor in hippocampal dentate gyrus reduces immobility in forced swim test. European Journal of Pharmacology 301: 19-25.

Kream J, Mulay S, Fukushima DK, Solomon S (1983) Determination of plasma dexamethasone in the mother and the newborn after administration of the hormone in a clinical-trial. Journal of Clinical Endocrinology and Metabolism 56: 127-133.

Lajic S, Wedell A, Bui TH, Ritzen EM, Holst M (1998) Long-term somatic follow-up of prenatally treated children with congenital adrenal hyperplasia. Journal of Clinical Endocrinology and Metabolism 83: 3872-3880.

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, Nath N, Muneyyircidelale O (1993) Characterization of 11-beta-hydroxysteroid dehydrogenase of human placenta - evidence for the existence of 2 species of 11-beta- hydroxysteroid dehydrogenase. Journal of Steroid Biochemistry and Molecular Biology 45: 391-397.

Lambert KG, Kinsley CH, Jones HE, Klein SL, Peretti SN, Stewart KM (1995) Prenatal stress attenuates ulceration in the activity stress paradigm. Physiology & Behavior 57: 989-994.

Langley SC, Jackson AA (1994) Increased systolic blood-pressure in adult-rats induced by fetal exposure to maternal low-protein diets. Clinical Science 86: 217-222.

LangleyEvans SC (1997) Intrauterine programming of hypertension by glucocorticoids. Life Sciences 60: 1213-1221.

LangleyEvans SC, Gardner DS, Jackson AA (1996a) Maternal protein restriction influences the programming of the rat hypothalamic-pituitary-adrenal axis. Journal of Nutrition 126: 1578-1585.

LangleyEvans SC, Phillips GJ, Benediktsson R, Gardner DS, Edwards CRW, Jackson AA, Seckl JR (1996b) Protein intake in pregnancy, placental glucocorticoid metabolism and the programming of hypertension in the rat. Placenta 17: 169-172.

Lawson A, Ahima R, Krozowski Z, Harlan R (1991) Postnatal-development of corticosteroid receptor immunoreactivity in the rat hippocampus. Developmental Brain Research 62: 69-79.

Lee MHS, Williams DI (1974) Chances in licking behaviour of rat mother following handling of young. Animal Behaviour 22: 679-681.

Lee S, Rivier C (1992) Administration of corticosterone to pregnant adrenalectomized dams does not alter the hypothalamic-pituitary-adrenal axis activity of the offspring. Molecular and Cellular Neuroscience 3: 118-123.

Leon DA, Koupilova I, Lithell HO, Berglund L, Mohsen R, Vagero D, Lithell UB, McKeigue PM (1996) Failure to realise growth potential in utero and adult obesity in relation to blood pressure in 50 year old Swedish men. British Medical Journal 312: 401-406.

Levin N, Akana SF, Jacobson L, Kuhn RW, Siiteri PK, Dallman MF (1987) Plasma adrenocorticotropin is more sensitive than transcortin production or thymus weight to inhibition by corticosterone in rats. Endocrinology 121: 1104-1110.

Levine S (1994) The ontogeny of the hypothalamic-pituitary-adrenal axis - the influence of maternal factors. Annals of the New York Academy of Sciences 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.

Li J, Saunders JC, Gilmour RS, Silver M, Fowden AL (1993) Insulin-like growth factor-ii messenger-ribonucleic-acid expression in fetal tissues of the sheep during late gestation - effects of cortisol. Endocrinology 132: 2083-2089.

Lindsay RS, Lindsay RM, Edwards CRW, Seckl JR (1996a) Inhibition of 11 beta-hydroxysteroid dehydrogenase in pregnant rats and the programming of blood pressure in the offspring. Hypertension 27: 1200-1204.

Lindsay RS, Lindsay RM, Waddell BJ, Seckl JR (1996b) Prenatal glucocorticoid exposure leads to offspring hyperglycaemia in the rat: Studies with the 11 beta-hydroxysteroid dehydrogenase inhibitor carbenoxolone. Diabetologia 39: 1299-1305.

Lipp HP, Wolfer DP (1998) Genetically modified mice and cognition. Current Opinion in Neurobiology 8: 272-280.

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.

Lopez JF, Chalmers DT, Little KY, Watson SJ (1998) Regulation of serotonin(1A), glucocorticoid, and mineralocorticoid receptor in rat and human hippocampus: Implications for the neurobiology of depression. Biological Psychiatry 43: 547-573.

Low SC, Chapman KE, Edwards CRW, Seckl JR (1994a) Liver-type 11-beta-hydroxysteroid dehydrogenase cDNA encodes reductase but not dehydrogenase-activity in intact mammalian cos-7 cells. Journal of Molecular Endocrinology 13: 167-174.

Low SC, Moisan MP, Noble JM, Edwards CRW, Seckl JR (1994b) Glucocorticoids regulate hippocampal 11-beta-hydroxysteroid dehydrogenase-activity and gene-expression in-vivo in the rat. Journal of Neuroendocrinology 6: 285-290.

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

Maccari S, Mormede P, Piazza PV, Simon H, Angelucci L, LeMoal M (1992a) Hippocampal type-i and type-ii corticosteroid receptors are modulated by central noradrenergic systems. Psychoneuroendocrinology 17: 103-112.

Maccari S, Piazza PV, Kabbaj M, Barbazanges A, Simon H, LeMoal M (1995) Adoption reverses the long-term impairment in glucocorticoid feedback induced by prenatal stress. Journal of Neuroscience 15: 110-116.

Maccari S, Piazza PV, Rougepont F, Angelucci L, Simon H, LeMoal M (1992b) Noradrenergic regulation of type-i and type-ii corticosteroid receptors in amygdala and hypothalamus. Brain Research 587: 313-318.

Makino S, Gold PW, Schulkin J (1994a) Corticosterone effects on corticotropin-releasing hormone messenger-RNA in the central nucleus of the amygdala and the parvocellular region of the paraventricular nucleus of the hypothalamus. Brain Research 640: 105-112.

Makino S, Gold PW, Schulkin J (1994b) Effects of corticosterone on CRH messenger-RNA and content in the bed nucleus of the stria terminalis - comparison with the effects in the central nucleus of the amygdala and the paraventricular nucleus of the hypothalamus. Brain Research 657: 141-149.

Makino S, Schulkin J, Smith MA, Pacak K, Palkovits M, Gold PW (1995b) Regulation of corticotropin-releasing hormone-receptor messenger-ribonucleic-acid in the rat-brain and pituitary by glucocorticoids and stress. Endocrinology 136: 4517-4525.

Makino S, Smith MA, Gold PW (1995a) Increased expression of corticotropin-releasing hormone and vasopressin messenger-ribonucleic-acid (messenger-RNA) In the hypothalamic paraventricular nucleus during repeated stress - association with reduction in glucocorticoid receptor messenger-rna levels. Endocrinology 136: 3299-3309.

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 Research Developmental Brain Research. 84: 55-61.

McEwen BS (1987) Glucocorticoid-biogenic amine interactions in relation to mood and behavior. Biochemical Pharmacology 36: 1755-1763.

McEwen BS, de Kloet ER, Rostene W (1986) Adrenal-steroid receptors and actions in the nervous-system. Physiological Reviews 66: 1121-1188.

Meaney MJ, Aitken DH, Vanberkel C, Bhatnagar S, Sapolsky RM (1988) Effect of neonatal handling on age-related impairments associated with the hippocampus. Science 239: 766-768.

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, Sapolsky RM, McEwen BS (1985) The development of the glucocorticoid receptor system in the rat limbic brain. 1. Ontogeny and auto-regulation. Developmental Brain Research 18: 159-164.

Meijer OC, de Lange ECM, Breimer DD, de Boer AG, Workel JO, de Kloet ER (1998) Penetration of dexamethasone into brain glucocorticoid targets is enhanced in mdr1A P-glycoprotein knockout mice. Endocrinology 139: 1789-1793.

Melniczek JR, Ward IL (1994) Patterns of anogenital licking mother rats exhibit toward prenatally stressed neonates. Physiology & Behavior 56: 457-461.

Meyer JS (1985) Biochemical effects of corticosteroids on neural tissues. Physiological Reviews 65: 946-1020.

Mitchell JB, Meaney MJ (1991) Effects of corticosterone on response consolidation and retrieval in the forced swim test. Behavioral Neuroscience 105: 798-803.

Moller C, Wiklund L, Sommer W, Thorsell A, Heilig M (1997) Decreased experimental anxiety and voluntary ethanol consumption in rats following central but not basolateral amygdala lesions. Brain Research 760: 94-101.

Monder C (1991) Heterogeneity of 11-beta-hydroxysteroid dehydrogenase in rat-tissues. Journal of Steroid Biochemistry and Molecular Biology 40: 533-536.

Montano MM, Wang MH, Vomsaal FS (1993) Sex-differences in plasma-corticosterone in mouse fetuses are mediated by differential placental transport from the mother and eliminated by maternal adrenalectomy or stress. Journal of Reproduction and Fertility 99: 283-290.

Morris R (1984) Development of a water-maze procedure for studying spatial-learning in the rat. Journal of Neuroscience Methods 11: 47-60.

Morris RMG, Garrud P, Rawlins JNP, O'Keefe J (1982) Place navigation impaired in rats with hippocampal lesions. Nature 297: 681-683.

Munck A, Guyre PM, Holbrook NJ (1984) Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocrine Reviews 5: 25-44.

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. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 42: R1669-R1675.

Murphy D, Costall B, Smythe JW (1998) Regulation of hippocampal theta activity by corticosterone: Opposing functions of mineralocorticoid and glucocorticoid receptors. Brain Research Bulletin 45: 631-635.

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. Journal of Clinical Investigation 101: 2174-2181.

O'Riordan JLH, Malan PG, Gould RP (Eds.) (1988) Essentials of endocrinology; Blackwell Scientific Publications; second edition.

Ohkawa T, Rohde W, Takeshita S, Dorner G, Arai K, Okinaga S (1991) Effect of an acute maternal stress on the fetal hypothalamo- pituitary-adrenal system in late gestational life of the rat. Experimental and Clinical Endocrinology 98: 123-129.

Oitzl MS, de Kloet ER (1992) Selective corticosteroid antagonists modulate specific aspects of spatial orientation learning. Behavioral Neuroscience 106: 62-71.

Oitzl MS, Fluttert M, de Kloet ER (1994) The effect of corticosterone on reactivity to spatial novelty is mediated by central mineralocorticosteroid receptors. European Journal of Neuroscience 6: 1072-1079.

Oitzl MS, Fluttert M, de Kloet ER (1998) Acute blockade of hippocampal glucocorticoid receptors facilitates spatial learning in rats. Brain Research 797: 159-162.

Oliver MH, Harding JE, Breier BH, Evans PC, Gluckman PD (1993) Glucose but not a mixed amino-acid infusion regulates plasma insulin- like growth factor-i concentrations in fetal sheep. Pediatric Research 34: 62-65.

Orchinik M, Murray TF, Moore FL (1991) A corticosteroid receptor in neuronal membranes. Science 252: 1848-1851.

Owens MJ, Nemeroff CB (1991) Physiology and pharmacology of corticotropin-releasing factor. Pharmacological Reviews 43: 425-473.

Palkovits M, Young WS, Kovacs K, Toth ZS, Makara GB (1998) Alterations in corticotropin-releasing hormone gene expression of central amygdaloid neurons following long-term paraventricular lesions and adrenalectomy. Neuroscience 85: 135-147.

Paul SM, Purdy RH (1992) Neuroactive steroids. FASEB Journal 6: 2311-2322.

Paxinos, G and Watson, C (1986) The rat brain in stereotaxic coordinates; second edition. Academic Press, Inc.

Peiffer A, Lapointe B, Barden N (1991) Hormonal-regulation of type-ii glucocorticoid receptor messenger- ribonucleic-acid in rat-brain. Endocrinology 129: 2166-2174.

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. Journal of Neuroscience Methods 14: 149-167.

Pellow S, File SE (1986) Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze - a novel test of anxiety in the rat. Pharmacology, Biochemistry and Behavior 24: 525-529.

Peters DAV (1982) Prenatal stress - effects on brain biogenic-amine and plasma-corticosterone levels. Pharmacology Biochemistry And Behavior 17: 721-725.

Peters DAV (1984) Prenatal stress: Effect on development of rat brain adrenergic receptors. Pharmacology, Biochemistry & Behavior 21.

Peters DAV (1988) Effects of maternal stress during different gestational periods on the serotonergic system in adult-rat offspring. Pharmacology, Biochemistry and Behavior 31: 839-843.

Peters DAV (1990) Maternal stress increases fetal brain and neonatal cerebral cortex 5-hydroxytryptamine synthesis in rats: A possible mechanism by which stress influences brain development. Pharmacology, Biochemistry and Behavior 35: 943-947.

Pfahl M (1993) Nuclear receptor/ap-1 interaction. Endocrine Reviews 14: 651-658.

Pfister HP, Muir JL (1992) Prenatal exposure to predictable and unpredictable novelty stress and oxytocin treatment affects offspring development and behavior in rats. International Journal of Neuroscience 62: 227-241.

Potter E, Sutton S, Donaldson C, Chen R, Perrin M, Lewis K, Sawchenko PE, Vale W (1994) Distribution of corticotropin-releasing factor-receptor messenger-RNA expression in the rat-brain and pituitary. Proceedings of the National Academy of Sciences of the United States of America 91: 8777-8781.

Price WA, Stiles AD, Moats-Staats BM, D'Ercole AJ (1992) Gene expression of insulin-like growth factors (IGFs), the type 1 IGF receptor, and IGF-binding proteins in dexamethasone-induced fetal growth retardation. Endocrinology 130: 1424-1432.

Puia G, Santi MR, Vicini S, Pritchett DB, Purdy RH, Paul SM, Seeburg PH, Costa E (1990) Neurosteroids act on recombinant human GABA-a receptors. Neuron 4: 759-765.

Quirarte GL, Roozendaal B, McGaugh JL (1997) Glucocorticoid enhancement of memory storage involves noradrenergic activation in the basolateral amygdala. Proceedings of the National Academy of Sciences of the United States of America 94: 14048-14053.

Rajan V, Edwards CRW, Seckl JR (1996) 11 beta-hydroxysteroid dehydrogenase in cultured hippocampal cells reactivates inert 11-dehydrocorticosterone, potentiating neurotoxicity. Journal of Neuroscience 16: 65-70.

Ramos A, Mormede P (1998) Stress and emotionality: a multidimensional and genetic approach. Neuroscience and Biobehavioral Reviews 22: 33-57.

Ratka A, Sutanto W, Bloemers M, Dekloet ER (1989) On the role of brain mineralocorticoid (Type-i) and glucocorticoid (Type-ii) receptors in neuro-endocrine regulation. Neuroendocrinology 50: 117-123.

Ravelli ACJ, vanderMeulen JHP, Michels RPJ, Osmond C, Barker DJP, Hales CN, Bleker OP (1998) Glucose tolerance in adults after prenatal exposure to famine. Lancet 351: 173-177.

Recabarren MP, Valenzuela GJ, SeronFerre M (1998) Proteic-caloric restriction during pregnancy affects the adrenal- placental axis and decreases birth weight in a

primate. Cebus apella, without affecting gestational length. Prenatal and Neonatal Medicine 3: 309-313.

Reul JMHM, de Kloet ER (1985) 2 Receptor systems for corticosterone in rat-brain - microdistribution and differential occupation. Endocrinology 117: 2505-2511.

Reul JMHM, Stec I, Wiegers GJ, Labeur MS, Linthorst ACE, Arzt E, Holsboer F (1994) Prenatal immune challenge alters the hypothalamic-pituitary- adrenocortical axis in adult-rats. Journal of Clinical Investigation 93: 2600-2607.

Reul JMHM, Vandenbosch FR, de Kloet ER (1987) Relative occupation of type-I and type-II corticosteroid receptors in rat-brain following stress and dexamethasone treatment - functional implications. Journal of Endocrinology 115: 459-467.

Rifkin L, Lewis S, Jones P, Toone B, Murray R (1994) Low birth weight and schizophrenia. British Journal of Psychiatry 165: 357-362.

Robson AC, Leckie CM, Seckl JR, Holmes MC (1998) 11beta-Hydroxysteroid dehydrogenase type 2 in the postnatal and adult rat brain. Molecular Brain Research 61: 1-10.

Romero LM and Sapolsky RM (1996) Patterns of ACTH secretagog secretion in response to psychological stimuli. Journal of Neuroendocrinology 8: 243-258.

Roozendaal B, Koolhaas JM, Bohus B (1992) Central amygdaloid involvement in neuroendocrine correlates of conditioned stress responses. Journal Of Neuroendocrinology 4: 483-489.

Roozendaal B, McGaugh JL (1996) Amygdaloid nuclei lesions differentially affect glucocorticoid- induced memory enhancement in an inhibitory avoidance task. Neurobiology of Learning and Memory 65: 1-8.

Roozendaal B, McGaugh JL (1997a) Basolateral amygdala lesions block the memory-enhancing effect of glucocorticoid administration in the dorsal hippocampus of rats. European Journal of Neuroscience 9: 76-83.

Roozendaal B, McGaugh JL (1997b) Glucocorticoid receptor agonist and antagonist administration into the basolateral but not central amygdala modulates memory storage. Neurobiology of Learning and Memory 67: 176-179.

Roozendaal B, PortilloMarquez G, McGaugh JL (1996) Basolateral amygdala lesions block glucocorticoid-induced modulation of memory for spatial learning. Behavioral Neuroscience 110: 1074-1083.

Roozendaal B, Sapolsky RM, McGaugh JL (1998) Basolateral amygdala lesions block the disruptive effects of long- term adrenalectomy on spatial memory. Neuroscience 84: 453-465.

Rosenfeld P, de Kloet ER, van Eekelen JAM, Sutanto W, Levine S (1988c) Ontogeny of corticosteroid receptors in the rat-brain. Neuroendocrinology Letters 10: 271.

Rosenfeld P, Sutanto W, Levine S, de Kloet ER (1988b) Ontogeny of type-i and type-ii corticosteroid receptors in the rat hippocampus. Developmental Brain Research 42: 113-118.

Rosenfeld P, van Eekelen JAM, Levine S, de Kloet ER (1988a) Ontogeny of the type-2 glucocorticoid receptor in discrete rat-brain regions - an immunocytochemical study. Developmental Brain Research 42: 119-127.

Rothberg AD, Lits B (1991) Psychosocial support for maternal stress during pregnancy - effect on birth-weight. American Journal of Obstetrics and Gynecology 165: 403-407.

Rots NY, de Jong J, Workel JO, Levine S, Cools AR, de Kloet ER (1996) Neonatal maternally deprived rats have as adults elevated basal pituitary-adrenal activity and enhanced susceptibility to apomorphine. Journal of Neuroendocrinology 8: 501-506.

Rowe WB, Spreekmeester E, Meaney MJ, Quirion R, Rochford J (1998) Reactivity to novelty in cognitively-impaired and cognitively- unimpaired aged rats and young rats. Neuroscience 83: 669-680.

Sandi C (1998) The role and mechanisms of action of glucocorticoid involvement in memory storage. Neural Plasticity 6: 41-52.

Sandi C, Loscertales M, Guaza C (1997) Experience-dependent facilitating effect of corticosterone on spatial memory formation in the water maze. European Journal of Neuroscience 9: 637-642.

Sandman CA, Wadhwa PD, Chicz-deMet A, Dunkel-Schetter C, Porto M (1997) Maternal stress, HPA activity, and fetal/infant outcome. Annals of the New York Academy of Sciences 814.

Sapolsky RM, Krey LC, McEwen BS (1984) Stress down-regulates corticosterone receptors in a site-specific manner in the brain. Endocrinology 114: 287-292.

Sapolsky RM, Krey LC, McEwen BS (1986) The neuroendocrinology of stress and aging - the glucocorticoid cascade hypothesis. Endocrine Reviews 7: 284-301.

Sapolsky RM, Meaney MJ (1986) Maturation of the adrenocortical stress response - neuroendocrine control mechanisms and the stress hyporesponsive period. Brain Research Reviews 11: 65-76.

Schulkin J, McEwen BS, Gold PW (1994) Allostasis, amygdala, and anticipatory angst. Neuroscience & Biobehavioral Reviews 18: 385-396.

Schumacher M (1990) Rapid membrane effects of steroid-hormones - an emerging concept in neuroendocrinology. Trends in Neurosciences 13: 359-362.

Seckl JR (1994) Glucocorticoids and small babies. Quarterly Journal of Medicine 87: 259-262.

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, Dow RC, Low SC, Edwards CRW, Fink G (1993) The 11-beta-hydroxysteroid dehydrogenase inhibitor glycyrrhetinic acid affects corticosteroid

feedback-regulation of hypothalamic corticotropin-releasing peptides in rats. Journal of Endocrinology 136: 471-477.

Seckl JR, Dickson KL, Fink G (1990) Central 5,7-dihydroxytryptamine lesions decrease hippocampal glucocorticoid and mineralocorticoid receptor messenger ribonucleic acid expression. Journal of Neuroendocrinology 2: 911-916.

Siebe H, Baude G, Lichtenstein I, Wang D, Buhler H, Hoyer G-A, Hierholzer K (1993) Metabolism of dexamethasone: Sites and activity in mammalian tissues. Renal Physiology & Biochemistry 16: 79-88.

Slotkin TA, McCook EC, Seidler FJ (1993) Glucocorticoids regulate the development of intracellular signaling: Enhanced forebrain adenylate cyclase catalytic subunit activity after fetal dexamethasone exposure. Brain Research Bulletin 32: 359-364.

Slotkin TA, Zhang J, McCook EC, Seidler FJ (1998) Glucocorticoid administration alters nuclear transcription factors in fetal rat brain: implications for the use of antenatal steroids. Developmental Brain Research 111: 11-24.

Smythe JW, McCormick CM, Rochford J, Meaney MJ (1994) The interaction between prenatal stress and neonatal handling on nociceptive response latencies in male and female rats. Physiology & Behavior 55: 971-974.

Smythe JW, Murphy D, Timothy C, Costall B (1997) Hippocampal mineralocorticoid, but not glucocorticoid, receptors modulate anxiety-like behavior in rats. Pharmacology, Biochemistry and Behavior 56: 507-513.

Souness GW, Latif SA, Laurenzo JL, Morris DJ (1995) 11-Alpha-hydroxyprogesterone and 11-beta-hydroxyprogesterone, potent inhibitors of 11-beta-hydroxysteroid dehydrogenase (isoform-1 and isoform-2), confer marked mineralocorticoid activity on corticosterone in the adx rat. Endocrinology 136: 1809-1812.

Spencer RL, Kim PJ, Kalman BA, Cole MA (1998) Evidence for mineralocorticoid receptor facilitation of glucocorticoid receptor-dependent regulation of hypothalamic- pituitary-adrenal axis activity. Endocrinology 139: 2718-2726.

Spinedi E, Gaillard RC (1998) A regulatory loop between the hypothalamo-pituitary-adrenal (HPA) axis and circulating leptin: A physiological role of ACTH. Endocrinology 139: 4016-4020.

Stein CE, Fall CHD, Kumaran K, Osmond C, Cox V, Barker DJP (1996) Fetal growth and coronary heart disease in South India. Lancet 348: 1269-1273.

Stewart PM, Murry BA, Mason JI (1994) Type 2 11beta-hydroxysteroid dehydrogenase in human fetal tissue. Journal of Clinical Endocrinology & Metabolism 78: 1529-1532.

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. Journal of Clinical Endocrinology and Metabolism 80: 885-890.

Stewart PM, Wallace AM, Valentino R, Burt D, Shackleton CHL, Edwards CRW (1987) Mineralocorticoid activity of licorice - 11-beta-hydroxysteroid dehydrogenase-deficiency comes of age. Lancet 2: 821-824.

Stohr T, Wermeling DS, Szuran T, Pliska V, Domeney A, Welzl H, Weiner I, Feldon J (1998) Differential effects of prenatal stress in two inbred strains of rats. Pharmacology, Biochemistry and Behavior 59: 799-805.

Sullivan RM, Gratton A (1999) Lateralized effects of medial prefrontal cortex lesions on neuroendocrine and autonomic stress responses in rats. Journal of Neuroscience 19: 2834-2840.

Susser E, Neugebauer R, Hoek HW, Brown AS, Lin S, Labovitz D, Gorman, JM (1996) Schizophrenia after prenatal famine. Further evidence. Archives of General Psychiatry 53: 25-31.

Susser M, Stein Z (1994) Timing in prenatal nutrition - a reprise of the Dutch famine study. Nutrition Reviews 52: 84-94.

Szuran T, Zimmermann E, Welzl H (1994) Water maze performance and hippocampal weight of prenatally stressed rats. Behavioural Brain Research 65: 153-155.

Takahashi LK (1998) Prenatal stress: Consequences of glucocorticoids on hippocampal development and function. International Journal of Developmental Neuroscience 16: 199-207.

Takahashi LK, Baker EW, Kalin NH (1990) Ontogeny of behavioral and hormonal responses to stress in prenatally stressed male-rat pups. Physiology & Behavior 47: 357-364.

Takahashi LK, Goh C-S (1998) Glucocorticoid facilitation of cholinergic development in the rat hippocampus. Neuroscience 83: 1145-1153.

Takahashi LK, Haglin C, Kalin NH (1992b) Prenatal stress potentiates stress-induced behavior and reduces the propensity to play in juvenile rats. Physiology & Behavior 51: 319-323.

Takahashi LK, Kalin NH (1991) Early developmental and temporal characteristics of stress-induced secretion of pituitary-adrenal hormones in prenatally stressed rat pups. Brain Research 558: 75-78.

Takahashi LK, Kalin NH, Barksdale CM, Vanden Burgt JA, Brownfield MS (1988) Stressor controllability during pregnancy influences pituitary-adrenal hormone concentrations and analgesic responsiveness in offspring. Physiology & Behavior 42: 323-329.

Takahashi LK, Turner JG, Kalin NH (1992a) Prenatal stress alters brain catecholaminergic activity and potentiates stress-induced behavior in adult rats. Brain Research 574: 131-137.

Tempel DL, Leibowitz SF (1994) Adrenal-steroid receptors - interactions with brain neuropeptide systems in relation to nutrient intake and metabolism. Journal of Neuroendocrinology 6: 479-501.

Trapp T, Rupprecht R, Castren M, Reul JMHM, Holsboer F (1994) Heterodimerization between mineralocorticoid and glucocorticoid receptor - a new principle of glucocorticoid action in the CNS. Neuron 13: 1457-1462.

Trautman PD, Meyer-Bahlburg HFL, Postelnek J, New MI (1995) Effects of early prenatal dexamethasone on the cognitive and behavioral development of young children: Results of a pilot study. Psychoneuroendocrinology 20: 439-449.

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. Nature Genetics 23: 99-103.

Vallee M, Maccari S, Dellu F, Simon H, LeMoal M, 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. European Journal of Neuroscience 11: 2906-2916.

Vallee M, Mayo W, Dellu F, Le Moal M, Simon H, Maccari (1997) Prenatal stress induces high anxiety and postnatal handling induces low anxiety in adult offspring: Correlation with stress-induced corticosterone secretion. Journal of Neuroscience 17: 2626-2636.

Vallee M, Mayo W, Maccari S, Le Moal M, Simon H (1996) Long-term effects of prenatal stress and handling on metabolic parameters: Relationship to corticosterone secretion response. Brain Research 712: 287-292.

van Os J, Selten JP (1998) Prenatal exposure to maternal stress and subsequent schizophrenia - The May 1940 invasion of The Netherlands. British Journal of Psychiatry 172: 324-326.

Van Haarst AD, Oitzl MS, de Kloet ER (1997) Facilitation of feedback inhibition through blockade of glucocorticoid receptors in the hippocampus. Neurochemical Research 22: 1323-1328.

Van Haarst AD, Oitzl MS, Workel JO, de Kloet ER (1996) Chronic brain glucocorticoid receptor blockade enhances the rise in circadian and stress-induced pituitary-adrenal activity. Endocrinology 137: 4935-4943.

Van Oers HJJ, de Kloet ER, Whelan T, Levine S (1998) Maternal deprivation effect on the infant's neural stress markers is reversed by tactile stimulation and feeding but not by suppressing corticosterone. Journal of Neuroscience 18: 10171-10179.

Vicedomini JP, Nonneman AJ, Dekosky ST, Scheff SW (1986) Perinatal glucocorticoids disrupt learning - a sexually dimorphic response. Physiology & Behavior 36: 145-149.

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.

Wadhwa PD, Sandman CA, Porto M, Dunkel-Schetter C, Garite TJ (1993) The association between prenatal stress and infant birth weight and gestational age at birth: A prospective investigation. American Journal of Obstetrics & Gynecology 169: 858-865.

Wakshlak A, Weinstock M (1990) Neonatal handling reverses behavioral abnormalities induced in rats by prenatal stress. Physiology & Behavior 48: 289-292.

Walker BR, Campbell JC, Williams BC, Edwards CRW (1992) Tissue-specific distribution of the NAD+-dependent isoform of 11- beta-hydroxysteroid dehydrogenase. Endocrinology 131: 970-972.

Walker CD, Perrin M, Vale W, Rivier C (1986) Ontogeny of the stress response in the rat - role of the pituitary and the hypothalamus. Endocrinology 118: 1445-1451.

Walker CD, Scribner KA, Cascio CS, Dallman MF (1991) The pituitary-adrenocortical system of neonatal rats is responsive to stress throughout development in a time-dependent and stressor- specific fashion. Endocrinology 128: 1385-1395.

Weichsel MEjr (1974) Glucocorticoid effect upon thymidine kinase in the developing cerebellum. Pediatric Research 8: 843-847.

Weidenfeld J, Feldman S (1991) Effect of 6-hydroxydopamine and 5,7-dihydroxytryptamine on tissue uptake and cell nuclear retention of corticosterone in the rat hypothalamus. Brain Research 566: 140-145.

Weidenfeld J, Itzik A, Feldman S (1997) Effect of glucocorticoids on the adrenocortical axis responses to electrical stimulation of the amygdala and the ventral noradrenergic bundle. Brain Research 754: 187-194.

Weinstock M (1997) Does prenatal stress impair coping and regulation of hypothalamic- pituitary-adrenal axis? Neuroscience & Biobehavioral Reviews 21: 1-10.

Weinstock M, Fride E, Hertzberg R (1988) Prenatal stress effects on functional-development of the offspring. Progress in Brain Research 73: 319-331.

Weinstock M, Matlina E, Maor GI, Rosen H, McEwen BS (1992) Prenatal stress selectivity alters the reactivity of the hypothalamic-pituitary adrenal system in the female rat. Brain Research 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. Physiology & Behavior 64: 439-444.

West AP (1990) Neurobehavioral studies of forced swimming: The role of learning and memory in the forced swim test. Progress in Neuro-Psychopharmacology & Biological Psychiatry 14: 863-877.

Whitaker AH, Van Rossem R, Feldman JF, Sam S, I, Pinto-Martin JA, Torre C, Shaffer D, Paneth N (1997) Psychiatric outcomes in low-birth-weight children at age

6 years: Relation to neonatal cranial ultrasound abnormalities. Archives of General Psychiatry 54: 847-856.

White PC, Mune T, Rogerson FM, Kayes KM, Agarwal AK (1997) Molecular analysis of 11beta-hydroxysteroid dehydrogenase and its role in the syndrome of apparent mineralocorticoid excess. Steroids 62: 83-88.

Williams MT, Davis HN, Mccrea AE, Hennessy MB (1998a) The distribution of radiolabeled corticotropin-releasing factor in pregnant rats: An investigation of placental transfer to the fetuses. International Journal of Developmental Neuroscience 16: 229-234.

Williams MT, Davis HN, Mccrea AE, Long SJ, Hennessy MB (1999) Changes in the hormonal concentrations of pregnant rats and their fetuses following multiple exposures to a stressor during the third trimester. Neurotoxicology and Teratology 21: 403-414.

Williams MT, Hennessy MB, Davis HN (1998b) Stress during pregnancy alters rat offspring morphology and ultrasonic vocalizations. Physiology & Behavior 63: 337-343.

Winick M (1971) Cellular growth during early malnutrition. Pediatrics 47: 969-978.

Woodall SM, Johnston BM, Breier BH, Gluckman PD (1996) Chronic maternal undernutrition in the rat leads to delayed postnatal growth and elevated blood pressure of offspring. Pediatric Research 40: 438-443.

Yau JLW, Dow RC, Fink G, Seckl JR (1992) Medial septal cholinergic lesions increase hippocampal mineralocorticoid and glucocorticoid receptor messenger RNA expression. Brain Research 577: 155-160.

Yau JLW, Morris RGM, Seckl JR (1994) Hippocampal corticosteroid receptor messenger-RNA expression and spatial-learning in the aged wistar rat. Brain Research 657: 59-64.

Yau JLW, Olsson T, Morris RGM, Meaney MJ, Seckl JR (1995) Glucocorticoids, hippocampal corticosteroid receptor gene-expression and antidepressant treatment - relationship with spatial-learning in young and aged rats. Neuroscience 66: 571-581.

Yau JLW, Seckl JR (1992) Central 6-hydroxydopamine lesions decrease mineralocorticoid, but not glucocorticoid receptor gene expression in the rat hippocampus. Neuroscience Letters 142: 159-162.

Young EA, Kwak SP, Kottak J (1995) Negative feedback-regulation following administration of chronic exogenous corticosterone. Journal of Neuroendocrinology 7: 37-45.

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.

Zarrow MX, Philpott JE, Denenberg VH (1970) Passage of ^{14}C-4-Corticosterone from the rat mother to the foetus and neonate. Nature 226: 1058-1059.