THE IMPACT OF ACUTE MATERNAL NUTRIENT RESTRICTION ON THE DEVELOPMENT AND LONG-TERM FUNCTION OF THE HYPOTHALAMO-PITUITARY-ADRENAL AXIS

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Physiology
University of Toronto

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Rania I. Lingas

M.Sc. 2000, Department of Physiology, University of Toronto

ABSTRACT

We hypothesized that maternal nutrient restriction affects HPA development and function. Pregnant guinea pigs were deprived of food or fed normally on gestational days 50 and 51, and subsequently allowed to feed normally. One group of offspring was euthanized on postnatal day (pd) 18 (juveniles). Another group was implanted with carotid artery and jugular vein catheters on pd65 (adults), exposed to HPA challenges on alternate days, then euthanized. Prenatal nutrient restriction (NR) reduced ACTH and increased glucocorticoid receptor (GR) mRNA in the ventromedial hypothalamus of juvenile offspring. In adult offspring, female basal cortisol was increased, and male basal ACTH and cortisol was decreased by NR. Responses to HPA challenges were consistent with basal adrenocortical function. Limbic MR mRNA was elevated in NR males, and reduced in females. NR decreased pituitary GR mRNA in females. In conclusion, maternal nutrient restriction during pregnancy, permanently modifies offspring HPA function in a sex-specific manner.
SUMMARY

We hypothesized that acute maternal nutrient restriction affects HPA development and subsequent function. Pregnant guinea pigs were deprived of food or fed normally on days 50 and 51 of gestation, after which they were allowed to feed and deliver normally. One group of offspring was euthanized on postnatal day (pd) 18 (juveniles). Another group was surgically implanted with carotid artery and jugular vein catheters on pd65 (adults). In adult offspring, samples were collected to determine basal plasma ACTH and cortisol levels, and animals were treated on alternate days with human ACTH$_{1-24}$, CRH, insulin, or restraint stress. Following treatments, adults were euthanized. Prenatal nutrient restriction (NR) reduced birth weight and body weight at day 18, and increased the brain to body weight ratio. Plasma ACTH, but not cortisol was significantly reduced in NR juveniles. Glucocorticoid receptor (GR) mRNA levels were significantly higher in the ventromedial hypothalamus (VMH) of NR offspring, but not in the limbic system or the paraventricular nucleus (PVN) of the hypothalamus. Mineralocorticoid receptor (MR) mRNA was not different between groups. In adult offspring, there were opposite effects on HPA function between the sexes. Female basal cortisol, but not ACTH was significantly increased in NR female offspring, while in males both basal ACTH and basal cortisol levels were significantly decreased by NR. Cortisol responses to ACTH and CRH injection, as well as restraint stress were not different between groups in either sex. However, NR male offspring, but not female offspring, had a significantly reduced peak in cortisol in response to insulin injection. Limbic MR mRNA was significantly elevated in male NR offspring, suggesting an increase in glucocorticoid feedback, and hence a decrease in HPA function. Conversely, limbic MR mRNA was significantly reduced in
NR female offspring, as was pituitary GR mRNA, suggesting a decrease in glucocorticoid feedback, and hence an increase in HPA function. There were no differences observed between groups in GR mRNA in the PVN or VMH in either sex, and there was no effect of NR on male pituitary GR mRNA. In conclusion, 48h of maternal nutrient restriction during pregnancy, modifies the HPA function of juvenile and adult offspring, with juveniles exhibiting a reduction in plasma ACTH, and an increase in GR mRNA within the VMH, and adults exhibiting sex-specific differences in HPA function (hyperactive female HPA activity and reduced glucocorticoid feedback; hypoactive male HPA activity and increased glucocorticoid feedback).
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACTH</td>
<td>adrenocorticotrophin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine vasopressin</td>
</tr>
<tr>
<td>BNST</td>
<td>bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBG</td>
<td>corticosteroid-binding globulin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotrophin releasing hormone</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>dex</td>
<td>dexamethasone</td>
</tr>
<tr>
<td>DG</td>
<td>dentate gyrus</td>
</tr>
<tr>
<td>DTT</td>
<td>DL-dithiolthreitol</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
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<tr>
<td>G</td>
<td>gauge</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma aminobutyric acid</td>
</tr>
<tr>
<td>GAL</td>
<td>galanin</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>glucocorticoid response element</td>
</tr>
<tr>
<td>H</td>
<td>handled</td>
</tr>
<tr>
<td>H&lt;sup&gt;3&lt;/sup&gt;</td>
<td>tritium</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamo-pituitary-adrenal</td>
</tr>
<tr>
<td>11β-HSD</td>
<td>11β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>5-HT</td>
<td>serotonin</td>
</tr>
<tr>
<td>&lt;sup&gt;125&lt;/sup&gt;I</td>
<td>radiolabelled iodine</td>
</tr>
<tr>
<td>icv</td>
<td>intraventricular</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>in utero</td>
<td>intra-uterine</td>
</tr>
<tr>
<td>ir</td>
<td>immunoreactive</td>
</tr>
<tr>
<td>ISH</td>
<td><em>in situ</em> hybridization</td>
</tr>
<tr>
<td>IUGR</td>
<td>intra-uterine growth restriction</td>
</tr>
<tr>
<td>IVGTT</td>
<td>intra-venous glucose tolerance test</td>
</tr>
</tbody>
</table>
mdr  multi-drug resistance
mRNA  messenger ribonucleic acid
MR  mineralocorticoid receptor
MRI  magnetic resonance image
αMSH  α-melanocyte stimulating hormone

NH  non-handled
NIDDM  non-insulin dependent diabetes mellitus
NMDA  N-Methyl-D-aspartate
NPY  neuro-peptide Y
NSB  non-specific binding

P450scc  cytochrome P450
PBS  phosphate buffer saline
PBSG  phosphate buffered saline with gelatin
POMC  pro-opiomelanocortin
PTU  propylthiouracil
PVN  paraventricular nucleus
PVP  polyvinylpyrrolidine
P³²  radiolabelled phosphorus

RIA  radioimmunoassay
RNA  ribonucleic acid
ROD  relative optical density
rpm  revolutions per minute

³⁵S  radiolabelled sulfur
SEM  standard error of the mean
SDS  sodium dodecyl sulfate
SON  supra-optic nucleus
SSC  saline sodium citrate
StAR  steroidogenic acute regulatory protein

TC  total counts
TdT  terminal deoxynucleotidyl transferase
TLC  thin-layer chromatography
T³  tri-iodothyronine
T³  thyroxine

ZF  zona fasciculata
ZG  zona glomerulosa
ZR  zona reticularis

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CHAPTER 1: INTRODUCTION

1.1 Fetal Programming of Adult Disease

The concept of prenatal programming arose from epidemiological evidence linking low birth weight with adult pathologies such as coronary heart disease (CHD), hypertension, insulin resistance, and non-insulin dependent diabetes mellitus (NIDDM). It was suggested that fetal "programming" of physiological functions, such as hormone regulation, was responsible for this relationship (Seckl, 1997). Following studies showing associations between infant mortality and coronary heart disease (Barker & Osmond, 1986; Rose, 1964), several groups in the UK (records from Hertfordshire, Preston, and Sheffield) have examined how weight and body proportions relate to the onset of adult disease. A study in Hertfordshire traced 1,600 men and women, and found that those who had low birthweight were at twice the risk of developing CHD than those who were larger at birth (Osmond et al., 1993). The Sheffield cohort also had information on body proportions and showed that death rates from cardiovascular disease fell progressively with increasing birthweight, head circumference, and ponderal index (weight/length$^3$)(Barker et al., 1993). In this study, very large babies also demonstrated an increase in death rate, but this is now believed to have been due to infants from diabetic mothers (Dennison et al., 1997). It should be noted that these associations to CHD exist in those who were small for gestational age at birth or intra-uterine growth restricted (IUGR) from fetal undernutrition, rather than those who were born prematurely, and that significant correlations exist even within the range of normal birthweights (Barker, 1996). Within the normal birthweight range, differences in body
proportions such as thin (Barker et al., 1993b; Barker et al., 1993) or short (Barker et al., 1992) babies at birth are also strongly linked to CHD.

The link between birth weight and CHD has been supported by associations between low birthweight and the incidence of cardiovascular risk factor such as hypertension, NIDDM and insulin resistance, and high plasma concentrations of glucose, insulin, cholesterol, and fibrinogen (Barker, 1996; Barker et al., 1993a; Dennison et al., 1997). Several adult studies have shown a relationship between lower birthweight and impaired glucose tolerance (Barker, 1994; Hales et al., 1991; Phipps et al., 1993). One of the most recent studies from Southampton found a strong correlation between birth size and both insulin sensitivity and insulin secretion, with small infants having reduced insulin sensitivity and higher insulin secretion (Flanagan et al., 2000). Although, this study found no significant relationships in women, only in men, another study recently reported that women born with IUGR were hyperinsulinemic and less insulin sensitive than those with normal birth weight (Jaquet et al., 1999). Impaired β-cell development is also a feature of IUGR (Van Assche et al., 1977), and together with insulin resistance may be important in the pathogenesis of NIDDM.

Raised blood pressure is a common feature of small birthweight individuals, from infancy to adulthood, that has gained a great deal of attention. Several studies have reported a relationship between birthweight and adult hypertension (Barker, 1996; Barker et al., 1990; Law et al., 1993), especially in middle aged men (Phillips et al., 1998). Elevated blood pressure, which appears to begin in infancy (Law et al., 1993), is independent of the current size of the child, and is closely associated with maternal body weight and blood pressure (Barker, 1996). In general, children’s blood pressure is more
closely related to maternal blood pressure than paternal (Bengtsson et al., 1979; Law et al., 1991), suggesting that the intrauterine environment as well as possible X-linked genes have a strong impact on offspring blood pressure (Barker, 1996). The relationship between low birthweight and increased blood pressure appears to become stronger as individuals age. It has been hypothesized that this is due to amplification of initially raised in utero blood pressure (Barker, 1996). The mechanisms behind initial changes in blood pressure and the amplification process are not entirely understood, but may involve endocrine changes such as exposure to elevated glucocorticoids during fetal life.

The potential role of glucocorticoids in the programming of adult pathologies such as hypertension first arose from observations involving excess fetal glucocorticoid exposure in both humans and animals (animal models to be discussed in section 1.9.3). Fetuses showing IUGR have been shown to have increased cortisol levels (Goland et al., 1993), and exposure to excess glucocorticoids, including synthetic glucocorticoids such as prednisone, retards fetal growth (Seckl, 1994; Seckl et al., 1999). Recent studies in men have found birth weight to be negatively correlated with basal plasma cortisol, and cortisol levels to be positively correlated with systolic blood pressure (Phillips et al., 1998). Walker showed that increased dermal glucocorticoid sensitivity was associated with relative hypertension, insulin resistance, and hyperglycemia in both a cross-sectional study and in men predisposed to high blood pressure (Walker et al., 1998). Furthermore, in a population of 51-year-old Swedish men, those that lacked a normal hypothalamo-pituitary-adrenal (HPA) diurnal rhythm exhibited increased blood pressure, heart rate, and insulin levels, and a strong association existed between perturbed HPA activity and abdominal obesity (Rosmond & BJORNTORP, 1998). Thus, differences in circulating
cortisol levels may provide a link between low birth weight and hypertension. In this regard, resetting of the HPA axis and its feedback regulation, via changes in glucocorticoid receptor gene expression, may be a possible mechanism for the programming of adult disease.

The associations seen between low birth weight and elevated cortisol, as well as hypertension and NIDDM, appear to be more apparent in men versus women. This may be due partly to a lack of extensive studies in women compared to men. This is due to the complicating factor of the estrous cycle, which prevents easy assessment of factors such as plasma cortisol concentrations. However, the estrous cycle may also be responsible for the lack of relationships between birth size and adult disease in the studies that do exist. Since estrogens are known to interact with steroid regulation as well as growth and development, it is possible that they provide a protective affect, especially in women prior to menopause.

In addition to sex-specific effects, it has been argued that an adverse environment during pregnancy may be continued into the childhood and adult life of offspring, and that it is this latter environment that is responsible for the adult onset of disease. However, this notion has been examined directly in several studies, and found not to be a determining factor, since associations with cardiovascular risk factors are independent of known lifestyle. Most studies have been corrected for social class, cigarette smoking, alcohol consumption, and obesity, and still found associations between birth size and adult disease (Barker, 1996; Barker et al., 1993a). However, most do agree that adult lifestyle has an additive effect on the effects of fetal programming, since the highest risk
of NIDDM (Hales et al., 1991), and the highest blood pressures (Law et al., 1993) are found in individuals who had low birthweight and became obese as adults.

Racial differences may also alter the effects observed in IUGR. Since the majority of studies were performed in the UK, the observed trends may have been specific to the region examined. Also, different races have a genetic predisposition for different sized babies, an example being the long, thin babies common to India. In order to examine whether fetal programming is observed in different races a number of studies have been done in several distinct populations including Europe, Asia, the United States, and the Caribbean (Barker, 1994; Rich-Edwards et al., 1999; Thame et al., 2000; Yajnik et al., 1995). In the United States a cohort of 80,000 women showed a two-fold decrease in the risk of CHD as birthweight increased (Rich-Edwards et al., 1999). Four-year-old Indian children, had associated impaired glucose and insulin metabolism with decreased fetal growth (Yajnik et al., 1995). In the West Indies (Kingston, Jamaica), measures of maternal nutritional status were strongly related to birth weight and to placental volume, and in turn systolic blood pressure fell by 1.4 mmHg for every 1 kg increase in birth weight (Thame et al., 2000). Therefore, fetal programming of adult disease may be a common phenomenon throughout the world. However, future studies in additional populations would be required to accurately confirm the widespread applicability of fetal programming. Perhaps the examination of body weight relative to population averages and detailed investigations of body proportions will help reveal the importance of the intrauterine environment in different races. Even within the UK studies, different body proportions and abnormalities are observed depending on the timing of undernutrition. Undernutrition in early gestation appears to produce small but proportional babies, while
undernutrition in late gestation alters body proportions, thus producing thin or short babies (Barker et al., 1993a). Interestingly, thin babies tend to develop a disorder called syndrome X that includes symptoms such as insulin resistance, hypertension, NIDDM, and lipid disorders (Barker et al., 1993b), and short babies tend to develop hypertension and high plasma fibrinogen concentrations (Barker et al., 1992). Hence, phenotypic variations can occur even within the same population.

In summary, undernutrition during fetal life is strongly linked with adult diseases such as coronary heart disease, hypertension, and NIDDM. In this regard, as birth weight decreases, the incident and risk of adult disease progressively increases. This phenomenon appears to exist in a number of populations around the world, and adult lifestyle has an additive effect on fetal programming. At the moment more information is required regarding the extent and applicability of fetal programming globally, and most importantly the mechanisms behind these observed associations need to be elucidated.

1.2 Programming of HPA Function

With respect to the mechanisms involved in the fetal programming of adult disease, several animal studies have examined what the role of the HPA axis is in fetal programming, and how prenatal manipulations affect HPA function (Ader & Grota, 1973). Fetal glucocorticoid exposure has been examined in several species, both in terms of the relation of glucocorticoids to adult disease, as well as to examine the potential effects of synthetic glucocorticoid administration during pregnancy. Synthetic glucocorticoids such as dexamethasone and betamethasone are commonly given to women at risk of preterm delivery to induce lung maturation (NIH Consensus
development conference, 1995). We have shown that in the guinea pig, dexamethasone has immediate, sex-specific effects on fetal HPA function, and resulted in increased female plasma cortisol and decreased male plasma cortisol, as well as increased mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) mRNA in the limbic system (Dean & Matthews, 1999). In the rat, dexamethasone in the last week of pregnancy has been shown to have long-term effects on HPA function (Levitt et al., 1996). Adult rat offspring of dexamethasone treated mothers, had increased basal plasma cortisol, and reduced levels of hippocampal GR and MR mRNA (Levitt et al., 1996).

Prenatal stress has also been shown to program HPA function. In the rat, prenatal stress has been shown to increase the stress-induced ACTH (McCormick et al., 1995) and corticosterone (Maccari et al., 1995; McCormick et al., 1995) response of adult offspring. In addition, the handling of neonatal rats during the first three weeks of life has been shown to reduce basal and stress-induced levels of corticosterone and ACTH (Meaney et al., 1996). The mechanisms involved in the effects of neonatal handling have been examined, and found to be related to an increase in thyroid hormones that elevate serotonin turn-over, which in turn causes an increase in GR density (Meaney et al., 1994)(see section 1.9.4).

The majority of studies examining the role of prenatal nutrition in HPA programming involved protein restriction in the rat. Langley-Evans has shown that a low protein diet throughout pregnancy produces offspring with a blunted diurnal pattern of plasma adrenocorticotrophin (ACTH) concentrations, and an increase in the binding capacity and number of glucocorticoid receptors in the male hippocampus (Agmon et al.,
1996). Offspring of rats exposed to the low protein diet also had higher systolic blood pressures than control animals (Agmon et al., 1996; Langley-Evans et al., 1996).

In the guinea pig, a couple of studies have shown increases in fetal cortisol immediately following maternal nutrient restriction (Dwyer & Stickland, 1992) or uterine artery ligation (Jones et al., 1990). Although, one study has demonstrated an increase in blood pressure following uterine artery ligation, in adult guinea pig offspring, to date there is no information on the long-term effects of nutrient restriction on the guinea pig HPA axis.

1.3 Overview of the HPA axis

Glucocorticoids have many effects including altering metabolism, immune responses, secretion of several hormones and neuropeptides, as well as increasing the resistance to stress. In general, glucocorticoid levels will rise in response to any kind of threat to homeostasis or stress, in order to mount a first line of defence against such challenges (Munck et al., 1984). Eventually glucocorticoids control the body’s reactions to stress by enhancing the organism’s resistance to stress (Munck et al., 1984). The primary glucocorticoid for humans and most mammals, including guinea pigs is cortisol, while corticosterone serves the same function in most rodents, such as rats and mice. The hypothalamic-pituitary axis controls the release of glucocorticoids from the adrenal cortex, and in turn glucocorticoids regulate their own release through a negative feedback loop.

Stressors cause the release of stimulus-specific factors, such as corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) from specialized
neurosecretory cells in the paraventricular nucleus (PVN), into the hypophyseal portal system (Antoni, 1986; Plotsky et al., 1989; Whitnall, 1993). These factors stimulate the release of adrenocorticotropic hormone (ACTH) from the corticotroph cells within the anterior pituitary, as well as altering the synthesis of ACTH and its precursor proopiomelanocortin (POMC)(Dallman et al., 1987). ACTH in turn induces the synthesis and release of cortisol from the zona fasciculata of the adrenal cortex (Griffin & Ojeda, 1996).

There are diurnal fluctuations in cortisol, ACTH, and CRH levels, with episodic bursts of release throughout the day. Generally, for humans and most mammals there is a peak in HPA activity in the early morning, just before awakening, and a trough in the afternoon (Griffin & Ojeda, 1996). In guinea pigs, the peak in HPA activity, occurs between 4:00 and 8:00 am, with plasma cortisol levels being highest at this time (Garris, 1979). A wide range of basal plasma cortisol levels have been reported in the guinea pig (Dalle & Delost, 1974; Dalle et al., 1980; Fujieda et al., 1982; Kaiser & Sachser, 1998). Our laboratory has previously reported basal levels to range from 70 to 300ng/ml. Guinea pig cortisol is relatively high compared to other rodents such as the rat and mouse (Claman, 1972), due to a reduced sensitivity of the glucocorticoid receptor (GR) for cortisol (Keightley & Fuller, 1995).

It is generally agreed that ACTH is the main secretagogue that drives the zona fasciculata to produce and release glucocorticoids. Under basal conditions, ACTH is secreted episodically, and acts via a cyclic AMP pathway to increase steroidogenesis (Griffin J.E. & Ojeda, 1996). The actions of ACTH occur via ACTH receptors, and only a small percentage of receptors need be occupied to cause maximal steroidogenesis.
ACTH receptors can be downregulated with sustained ACTH exposure (Dallman et al., 1987), and in rats, chronic stimulation of the adrenals by ACTH increased the cAMP, pregnenolone, and corticosterone responses to ACTH as well as the expression and activity of steroidogenic enzymes such as p-450scc (Aguilera et al., 1996) and StAR (LeHoux et al., 1998).

Glucocorticoids can also regulate their own production in the adrenal gland (Mokuda et al., 1997; Peron et al., 1960; Salmenpera et al., 1976; Trung et al., 1984). In the guinea pig, basal cortisol production was decreased by the addition of cortisol to incubated guinea pig adrenal cells (Trung et al., 1984) and in isolated perfused adrenal glands (Mokuda et al., 1997). Cortisol pre-perfusion into isolated guinea pig adrenal glands also increased the cortisol response to ACTH, suggesting that glucocorticoids play a permissive role in the action of ACTH on adrenal steroidogenesis (Guo et al., 1997).

Conversely, in the rat, corticosterone reduced basal and ACTH-induced corticosterone production in the adrenal (Peron et al., 1960; Salmenpera et al., 1976), suggesting that the actions of glucocorticoids on the adrenal are profound, but likely species specific.

In addition, there is evidence that catecholamines (McDonald & Nathanielsz, 1998), as well as other hormones and cytokines (Chrousos, 1998), may participate in the regulation of cortisol synthesis and secretion. Neuronal mechanisms may also act directly on the adrenal, since there is extensive neuronal innervation of the adrenal gland by both afferent and efferent nerve terminals via the splachnic nerves, and it appears that adrenal innervation plays a significant role in adrenal responses to homeostatic challenges (McDonald & Nathanielsz, 1998).
CRH and AVP are the primary secretagogues controlling ACTH synthesis and secretion, and although CRH appears to be a more potent stimulator of ACTH release, it can act synergistically with AVP to produce a response greater than either alone (Dallman et al., 1987). CRH acts via an adenylate cyclase-linked membrane receptor (CRH receptor) that activates a cAMP/protein kinase A pathway, while AVP acts via the diacylglycerol/protein kinase C pathway (Liu, 1994). It appears that AVP acts to augment the ACTH response to CRH by potentiating the action of CRH on the cAMP pathway (Dallman et al., 1987). Studies in mouse anterior pituitary cells, showed that the CRH to AVP ratio may be an important regulator of ACTH release. In addition, it appears that CRH and AVP can regulate the reaction of the corticotrophs to subsequent stressors, depending of the nature of the challenge (Castro, 1993), and that the ACTH response to CRH and AVP varies during the day according to the circadian rhythm (Dallman et al., 1987). Other factors that can stimulate ACTH synthesis and release include certain endogenous opioids (Buckingham & Cooper, 1984), oxytocin, epinephrine, norepinephrine, and angiotensin II, albeit at a lesser extent than CRH or AVP (Vale et al., 1983). Serotonin has also been implicated in ACTH regulation, by altering POMC mRNA levels in the anterior and intermediate lobes (García-García et al., 1997). Inhibition of POMC gene expression occurs under dopaminergic control (via D₂ receptors) in the intermediate lobe (Loeffler et al., 1988) and by glucocorticoid feedback inhibition in the anterior lobe (Eberwine & Roberts, 1984).

Much of the stress response begins with the release of CRH and AVP from the hypothalamus. Both CRH and AVP are secreted into the portal system in a circadian and concordant pulsatile fashion, such that levels of both hormones are higher in the early
morning hours (Chrousos, 1998). The pulsatile release of CRH and AVP is controlled by pacemakers, which are not yet known (Chrousos, 1998), but may involve inputs from the suprachiasmatic nuclei (Dallman et al., 1987). Variations in hypothalamic activity occur with changes in lighting, feeding, and stress (Chrousos, 1998). In addition, during stress it has been shown that the ratio of CRH to AVP changes (Chrousos, 1998).

Several factors have been shown to regulate CRH secretion. Studies in vitro found that serotonin, acetylcholine, catecholamines, and neuropeptide Y stimulated CRH release, while the GABA/benzodiazepine system, β-endorphin, ACTH, and glucocorticoids all acted to inhibit CRH release (Chrousos, 1992). A few other factors that influenced CRH were α-MSH and corticotropin-like intermediate lobe peptide (CLIP) that had suppressive effects, and interleukin-1, tumour necrosis factor-α (TNF-α), platelet activating factor (PAF), and several eicosanoids that had stimulating effects. In vivo, the stimulating properties of serotonin, cholecystokinin, and neuropeptide Y have been demonstrated (Chrousos, 1992). In addition, estrogen response elements in the promoter of the CRH gene suggest that estrogen may stimulate CRH gene expression, thus resulting in increased HPA activity (Chrousos, 1998). The hippocampus also regulates PVN CRH and mostly acts to inhibit its activity (Chrousos, 1998)(see section 1.6).

Despite the many factors described above that regulate HPA activity, glucocorticoid feedback is by far the most influential regulator of HPA function. Glucocorticoid feedback occurs at many levels within the brain and pituitary via corticosteroid receptors (Keller-Wood & Dallman, 1984). The primary sites involved in glucocorticoid feedback are the hippocampus, hypothalamic PVN, and anterior pituitary (Dallman et al., 1987).
The central nervous system is involved in the regulation of the hypothalamo-pituitary-adrenal (HPA) axis by affecting the hypothalamic release of CRH (De Kloet et al., 1998). Glucocorticoid feedback is described in detail below.

1.4 Glucocorticoid feedback

There are two known receptors for glucocorticoid feedback, the high affinity Type I receptor, also known as the mineralocorticoid receptor (MR), and the low affinity Type II receptor, also known as the glucocorticoid receptor (GR). These receptors are part of a cytoplasmic multiprotein complex, which includes one receptor molecule and several heat shock proteins (Smith & Toft, 1993). Once glucocorticoids bind to the receptor complex the heat shock proteins dissociate, several phosphorylation steps follow, and the receptor is translocated to the nucleus where it forms either MR or GR homodimers and possibly MR/GR heterodimers. Dimers then bind with high affinity to nuclear domains (Bamberger et al., 1996; Van Steensel et al., 1996). The actions of GRs and MRs on transcription involve the binding of the steroid receptors to glucocorticoid response elements (GRE) in the promoter region of glucocorticoid-responsive genes. Corticosteroid receptors can also interact with docking proteins and other transcription factors to alter gene expression (De Kloet et al., 1998). The access of glucocorticoids to brain glucocorticoid receptors is determined by several factors, including binding to proteins such as albumin and corticosteroid binding globulin (CBG), steroid-metabolizing enzymes such as 11β-hydroxysteroid-dehydrogenase (11β-HSD), and for synthetic glucocorticoids the mdr1A P-glycoprotein (De Kloet et al., 1998).
Generally, MRs bind glucocorticoids with a 10-fold higher affinity than GRs, and this is true for both the rat and the guinea pig (De Kloet et al., 1998; Funder, 1994). Interestingly, in the guinea pig, not only does the GR bind with a lower affinity to cortisol, but also comparatively to other species such as the rat and mouse it is "glucocorticoid resistant". Although the guinea pig GR binds has a 20-fold lower affinity for dexamethasone than mouse GR (Funder, 1994; Keightley & Fuller, 1995), it only has a 5-fold lower affinity than human GR (Keightley & Fuller, 1995). This is believed to be due to a difference in the ligand-binding domain of the guinea pig receptor compared to the human GR (Keightley & Fuller, 1995).

GRs are widely distributed in the brain, but are expressed at highest levels in the hypothalamic CRH neurons of the PVN, pituitary corticotrophs, and the limbic system. Conversely, MRs are found primarily in the hippocampus and lateral septum (Dallman et al., 1987; De Kloet et al., 1998; Matthews, 1998; van Eekelen et al., 1988). It is believed that MRs are primarily involved in the maintenance of basal HPA activity, while GRs are involved in activated HPA function and the recovery from stress (Dallman et al., 1987).

1.5 Feedback Regulation of the HPA Axis

At the level of the pituitary, glucocorticoids affect the synthesis and secretion of ACTH through different mechanisms and at different time domains. Multiple intermittent stressors produce fast, intermediate, and slow responses to glucocorticoid feedback that act to reduce the ACTH response to subsequent stress. Fast responses (seconds to minutes) to elevated glucocorticoid levels appear to be rate-sensitive and to affect ACTH secretion, without changing production. Hence, these rapid effects do not
involve a nuclear site of corticosteroid action, but rather likely involve an effect at the cell membrane. Intermediate and slow responses to glucocorticoid feedback involve inhibition of ACTH protein synthesis and POMC mRNA expression in addition to ACTH release. It appears as though only slow feedback can inhibit basal as well as stimulated ACTH secretion (Keller-Wood & Dallman, 1984). Glucocorticoids affect POMC mRNA expression by binding to GRs within the pituitary and form an active complex that interacts with a negative GRE to inhibit POMC transcription (Drouin et al., 1993). It should be noted that the circadian rhythm alters the sensitivity of the pituitary to corticosteroid feedback, just as it alters the response to CRH.

Glucocorticoids can also regulate ACTH responses by altering CRH receptor numbers in the corticotrophs. In intact rats, CRH binding sites were reduced by chronic administration of either corticosterone (Hauger et al., 1987) or the synthetic glucocorticoid dexamethasone (Wynn et al., 1985), in a dose-dependent manner. Glucocorticoid treatment in rats decreased CRH receptor mRNA levels in vitro (Iredale & Duman, 1997; Pozzoli et al., 1996; Rosendale et al., 1987; Sakai et al., 1996). Similarly to the glucocorticoid inhibition of POMC gene expression, glucocorticoids act via a GRE on the CRH receptor promoter to inhibit transcription (Iredale & Duman, 1997). Glucocorticoids can act to induce transcriptional repressors, such as the cAMP response element modulator and the Fas/Jun family of transcription factors (Angel & Karin, 1991; Iredale & Duman, 1997; Yang-Yen et al., 1990). Finally, glucocorticoid treatment has been shown to internalize CRH into corticotrophs via glucocorticoid mediated CRH receptor internalization (Childs et al., 1986; Leroux & Pelletier, 1984).
In the hypothalamus, glucocorticoids have been shown to down-regulate PVN CRH mRNA levels and CRH secretion (Chrousos, 1992). This inhibition occurs via GRs and occupation of these receptors serves to directly inhibit the CRH neuron from synthesizing and secreting CRH and AVP at the level of the PVN, and to indirectly inhibit via regulation of hippocampal activity (Dallman et al., 1987). The regulation of CRH by glucocorticoids may also be essential for maintaining CRH gene expression, especially in response to stress. Some abrupt transient stressors increase CRH mRNA (Darlington et al., 1992; Harbuz & Lightman, 1988; Imaki et al., 1991). In the rat, an absence of corticosterone caused by adrenalectomy, compromised the CRH gene response to a sustained stress. However, this was reversed with low doses of corticosterone, suggesting that low levels of corticosterone before stress, but not the stress-mediated elevation, are required to maintain CRH gene transcription during prolonged stress (Tanimura & Watts, 1998). Hence, glucocorticoids regulate CRH synthesis and release in many different ways, depending on the environmental circumstances present.

1.6 The Hippocampus and Glucocorticoid Feedback

The hippocampus is involved in many tasks including learning, memory, and of course HPA regulation. In regards to the hippocampal regulation of HPA function, the main factor controlling hippocampal output is glucocorticoid feedback. Since the hippocampus exhibits the highest levels of corticosteroid receptors of any brain region, and is one of the only brain regions to contain high levels of both GRs and MRs, it may represent a significant target for a wide range of corticosteroid concentrations (Jacobson
There is considerable evidence to suggest that the hippocampus influences HPA function in an inhibitory fashion, both during unstimulated and during stress-induced HPA activity (Jacobson & Sapolsky, 1991). In this regard, electrical stimulation of the rat hippocampus decreased basal plasma corticosteroids (Rubin et al., 1966; Slusher & Hyde, 1961), and inhibited the corticosterone response to several stressors (Dupont et al., 1972). Hippocampectomy or lesions of the hippocampus increased basal plasma corticosteroids and ACTH (Herman et al., 1989; Margarinos et al., 1987), and elevated the corticosteroid response to cannulation (Feldman & Conforti, 1980), while destruction of more than 50% of hippocampal neurons by stereotaxic kainate infusion enhanced restraint-induced increases in plasma corticosteroids (Sapolsky et al., 1984). Although there is conflicting data on this subject and several studies have found the hippocampus to be stimulatory rather than inhibitory (Conforti & Feldman, 1976; Feldman et al., 1987), it has been suggested that these differences are likely due to the effects of different cell fields of the hippocampus (Dunn & Orr, 1984), prior glucocorticoid exposure, or the result of stressed conditions during experimentation (Jacobson & Sapolsky, 1991).

It has been postulated that the hippocampus exerts control over the HPA axis by mediating glucocorticoid negative feedback sensitivity (Jacobson & Sapolsky, 1991), but it is unclear whether the hippocampus also exerts a tonic inhibitory influence (Jacobson & Sapolsky, 1991). Several studies suggest that glucocorticoid feedback is the primary regulator of hippocampal output (Feldman & Conforti, 1980; Kovacs & Makara, 1988), but more evidence is required to rule out tonic regulation entirely. The strong influence of corticosteroids on the hippocampus is supported by the fact that in all species studied,
including the guinea pig, human, non-human primate, and rat, the highest level of corticosteroid receptors in the brain are observed in the hippocampus (Matthews, 1998; McEwen, 1999; Reul & De Kloet, 1985; Sutanto & De Kloet, 1987).

Although the hippocampus does not have significant projections to the neurons of the hypothalamic PVN that are involved in HPA regulation (Cullinan et al., 1993; Sawchenko & Swanson, 1983), there are several regions that may serve as intermediaries to the PVN. Such regions include the lateral septum, the ventromedial hypothalamus (VMH), and the bed nucleus of the stria terminalis (BNST) (Herman et al., 1996). The BNST and its associated γ-aminobutyric acid (GABA) network (inhibitory input to PVN neurons) has gained acceptance as a major mediator of hippocampal effects (De Kloet et al., 1998). There is evidence that the hippocampus also influences CRH via the release of adrenaline from brainstem catecholaminergic nuclei, whose fibres innervate parvocellular fields of the PVN (Meibach & Seigel, 1977; Swanson & Cowan M.W., 1977; Swanson et al., 1981). In addition to hypothalamic effects, the hippocampus may also influence corticosteroid secretion independently from CRH, since the hippocampus can alter the activity of the splanchic nerve which innervates the adrenal medulla (Engeland & Gann, 1989). This is supported by the fact that hippocampal stimulation increased corticosterone secretion in hypophysectomized rats (Saito et al., 1989).

Therefore, the hippocampus influences the HPA axis in an inhibitory fashion, but may also under some circumstances facilitate adrenocortical function depending on the type of input and region of the hippocampus. It is believed that the hippocampus primarily mediates corticosteroid feedback via corticosteroid receptors, although the
possibility of additional non-glucocorticoid mediated tonic inhibition has not been ruled out.

With respect to glucocorticoid feedback, the expression of GRs and MRs is important since receptor levels likely influence HPA sensitivity to corticosteroids, by enhancing or diminishing the efficacy of corticosteroid feedback (De Kloet et al., 1998). Hippocampal mineralocorticoid receptors are believed to mediate feedback inhibition of basal HPA activity (Dallman et al., 1987). This conclusion first arose from the fact that De Kloet estimated these receptors to be 90% occupied under normal basal circulating cortisol levels (Reul & De Kloet, 1985). The role of the MR in the inhibition of basal HPA function is supported by studies involving receptor antagonists. In the rat, intraventricular (icv) administration of an MR antagonist increased basal (morning) trough levels of plasma corticosterone (Ratka et al., 1989), elevated afternoon basal ACTH and (Oitzl et al., 1995), and enhanced the adrenocortical response to a novel situation (Ratka et al., 1989). In man, similar results were obtained, with increased basal HPA activity resulting from systemic administration of spirinolactone (Born et al., 1991; Dodt et al., 1993). In addition, a corticosterone implant in the dorsal hippocampus of the rat abolished the adrenalectomy associated increase in ACTH (Van Haarst et al., 1997). Finally, rat strains with decreased MR expression (Oitzl et al., 1995) and aged rats that exhibit lower expression of hippocampal MRs (van Eekelen et al., 1991), have higher basal and stress-induced HPA activity. This evidence seems to suggest a role for hippocampal MRs in the maintenance of basal HPA activity.

During stress and the circadian rise in glucocorticoids, hippocampal GRs become progressively more occupied, and estimations of GR occupation range from 10% at the
circadian trough to 75% during stress (Reul & De Kloet, 1985). The involvement of GRs in the circadian peak or stress induced HPA activity is supported by the fact that intrahippocampal implants of the GR antagonist RU 486 decrease basal ACTH in the afternoon phase of the circadian cycle (Van Haarst et al., 1997). However, RU486 is also binds strongly to the progesterone receptor, thus acting as a progesterone antagonist (Rauch et al., 1985). Given that progesterone binds to the GR and enhances the dissociation of glucocorticoids from the GR, it is possible that progesterone is contributing to the RU486 induced decrease in ACTH during the circadian peak.

The GR in the hippocampus has traditionally been thought to be involved in negative feedback of glucocorticoids, thereby reducing HPA activity (Jacobson & Sapolsky, 1991). This is supported by several animal models, including aged, diabetic, and chronically stressed rats that have been shown to exhibit decreased hippocampal glucocorticoid receptor levels in conjunction with elevated plasma corticosterone, as well as studies that showed changes in receptor levels following synthetic glucocorticoid treatment or handling of rats (Jacobson & Sapolsky, 1991). Recently De Kloet suggested a different role for hippocampal GRs. He hypothesized that MR activation maintains hippocampal excitability, thus inhibiting the PVN through inhibitory projections, while GR activation suppresses hippocampal output, thus disinhibiting the PVN (De Kloet et al., 1998). Furthermore, De Kloet proposed that hippocampal GRs and MRs act in such a way to alter the conductances of ion channels, ionotropic receptors, and G-protein coupled receptors in neuronal membranes, thus inhibiting or exciting hippocampal neuronal output (De Kloet et al., 1998). Whether the GR is involved in activation of HPA activity, as De Kloet proposes, or whether the traditional view of HPA inhibition is true
remains to be determined. However, it is possible that the role of the GR changes in different circumstances, and that the roles of corticosteroid receptors overlap, since their ligands (glucocorticoid and mineralocorticoid) can alter the expression of each other's receptors in addition to their own (De Kloet et al., 1998).

Glucocorticoids may also alter hippocampal function by affecting neurogenesis within the pyramidal neurons and dentate gyrus (Cameron & Gould, 1994; McEwen, 1999). In addition, excitatory amino acids and NMDA receptors play an important role in the adaptive functional and structural changes produced in the hippocampus by glucocorticoids (McEwen, 1999). However, it is generally accepted that glucocorticoids are primarily involved in the control of hippocampal output to the PVN, and that this occurs via MRs and GRs.

1.7 Development of the HPA Axis

The guinea pig gives birth to precocious young, with the period of rapid brain growth occurring between 45 and 53 days of fetal life (term=70 days) (Dobbing & Sands, 1970). The guinea pig is similar to the human, whose brain growth spurt begins just prior to birth, and continues throughout the perinatal period (Dobbing & Sands, 1979). This is contrary to rodents such as the rat and mouse that undergo rapid brain growth during postnatal life (Dobbing & Sands, 1979). The brain growth spurt is a time of rapid neuroendocrine development, characterized by neuronal proliferation, synaptogenesis, dendritic arborization, and increased brain weight, and is a period of enhanced vulnerability to stressors such as nutrition and drugs (Dobbing & Sands, 1979). This is also a time when components of the HPA axis, such as the hippocampus, hypothalamus,
and pituitary are developing, and are sensitive to manipulation (Dobbing & Sands, 1971; Matthews, 1998).

There is not a great deal of information about the maturation of the guinea pig HPA axis, but a lot can be extrapolated from the large breadth of knowledge present for the sheep, another species where most rapid brain growth occurs in utero (Dobbing & Sands, 1979). In the fetal sheep, immunoreactive CRH has been detected as early as day 100 of gestation (term=140 days), and it has been demonstrated that ACTH is present in the pituitary and can stimulate cortisol output from day 60 of gestation (Matthews & Challis, 1995a). It has also been reported that the mechanisms for glucocorticoid feedback are present during fetal life in sheep as well as in the rat. Since the mechanisms for glucocorticoid feedback exist in the fetus, it is possible that glucocorticoid feedback is involved in the changes in HPA activity that occur during gestation. Specifically, the rise in cortisol, ACTH, and CRH levels observed just prior to term in many animals including the sheep, rat, and guinea pig, as well as the increase in adrenal and pituitary responsiveness during this time (Challis & Brooks, 1989). In the fetal guinea pig, the adrenal cortex responds to ACTH as early as day 40, and there is a large increase in the concentrations of ACTH and cortisol between day 55 and 60 of gestation. There is also a large increase in adrenal responsiveness to ACTH towards term (Jones & Roebuck, 1980a). After birth, cortisol levels in the guinea pig gradually decline to adult levels (Dalle & Delost, 1976; Malinowska et al., 1972).
1.7.1 The Developing Adrenal

The human adult adrenal cortex is derived from mesodermal tissue and is composed of three zones. The outer zona glomerulosa (ZG) is responsible for aldosterone production, the middle zona fasciculata (ZF) produces primarily cortisol, and the inner zona reticularis (ZR) produces primarily androgen. The adrenal medulla is derived from neuroectodermal cells and synthesizes and secretes catecholamines. During fetal life, the adrenal glands are larger relative to body size than in the adult, and in the human an outer subcapsular zone exists, called the fetal zone (Griffin & Ojeda). The zona fasciculata comprises most of the fetal guinea pig adrenal, and decreases in volume from 80% at day 60 of gestation to 71% at birth. Following birth the ZF grows until postnatal day 10 when it is 85% of adrenal volume, which is similar to that found in the adult (Dalle et al., 1985). As the adult guinea pig adrenal ages, the fractional mass of the ZR grows until it makes up nearly two-thirds of the gland. This change in fractional mass is accompanied by a reduction in cortisol secretion, which is believed to be attributed to a decrease in the cortisol secretory capacity of the ZR cells, as seen by decreases in several steroidogenic enzymes in the ZR (Colby et al., 1993).

In the fetal sheep, the adrenal gland is present by day 28 of gestation (term=140) (Wintour et al., 1975). Zonation of the sheep adrenal gland was apparent after day 60 of gestation (Webb, 1980), and while the fetal ZG and ZF can be clearly seen, the ZR does not appear in fetal life (Robinson et al., 1983). The cells of the ZF remain relatively immature until the last third of gestation, when they begin to resemble those of the adult (Robinson et al., 1983). In the sheep, the last third of gestation is associated with an increase fetal cortisol levels (Dalle et al., 1985). In the guinea pig, there was also a large
increase in fetal cortisol in the last part of gestation (Jones, 1974). There was also a rise in corticosteroid binding proteins in the guinea pig, such that the percentage of bound cortisol remained the same between days 44 and 66. Hence, not only did total plasma cortisol increase, but so did free cortisol concentrations (Jones, 1974). With respect to maternal cortisol, in the guinea pig maternal cortisol concentrations were much higher than fetal concentrations, increased progressively towards term, and decreased just prior to parturition (Jones, 1974). Conversely, in the sheep, maternal cortisol levels were much lower than fetal levels, and rose during the last third of gestation (Dalle et al., 1985).

It has been suggested that in species such as the sheep, which have a relatively quiescent adrenal gland during fetal life, there may be significant transfer of maternal glucocorticoids to the fetus (Dalle et al., 1985). Gurpide reported that 35% of sheep cortisol and 88% of guinea pig cortisol is of maternal origin according to \(^3\)H-cortisol infusion studies (Gurpide, 1972). Others also believe that a large fraction of fetal cortisol is of maternal origin (Dalle & Delost, 1979; Jones, 1974). Levels of glucocorticoids in the placenta and other tissues are regulated by 11\(\beta\)-hydroxysteroid-dehydrogenase. Two forms of this enzyme exist, 11\(\beta\)-HSD-1 acts to bidirectionally convert cortisol to cortisone inactive and vice versa, while 11\(\beta\)-HSD-2 only converts cortisol to cortisone (Yang, 1995). In the guinea pig placenta, only 11\(\beta\)-HSD-2 is expressed, and hence acts to protect the fetus from the high levels of maternal cortisol. However, the enzyme may not be sufficient to prevent the transfer of all cortisol, especially under conditions of maternal stress. In addition, the activity of 11\(\beta\)-HSD-2 has been reported to be high between days 40-45 and 50-60, and then to decrease significantly at term (Sampath-
Kumar et al., 1996). This decrease may facilitate cortisol transfer from the mother to the fetus prior to parturition.

In many rodent species, such as the rat, early neonatal life is associated with a ‘stress hyporesponsive’ period, during which time stimuli that are normally able to stimulate corticosterone secretion in the adult are unable to do so in the neonate (Levine S, 1994; Levine et al., 1967). A reduction in adrenal sensitivity is exhibited during the hyporesponsive period, and is characterized by a failure to exhibit an ACTH-induced corticosterone response (Levine et al., 1967). Conversely, precocial species, such as the guinea pig, do not demonstrate a stress hyporesponsive period during postnatal life, and in neonatal guinea pig, exogenous CRH or AVP increases plasma ACTH and cortisol levels (Pradier et al., 1990).

1.7.2 The Developing Pituitary

The pituitary gland is composed of two parts: the neurohypophysis of neural origin, and the adenohypophysis of ectodermal origin. The adenohypophysis can be divided into the pars distalis (anterior lobe), the pars intermedia (intermediate lobe), and the pars tuberalis (part of the stalk). The hypophyseal portal system of veins provides the main blood supply to the adenohypophysis (Griffin & Ojeda, 1996).

The anterior lobe contains corticotroph cells that are estimated to represent 3-10% of anterior pituitary cells. Within the corticotrophs of the anterior lobe, ACTH is synthesized from its large precursor protein, proopiomelanocortin (Dallman et al., 1987). ACTH is a single-chain of 39 amino acids with only the first 16 amino acids of the N-terminus required for minimal biological activity (Griffin & Ojeda, 1996). In the
intermediate lobe, POMC is processed primarily into α-melanocyte-stimulating hormone (α-MSH) and β-endorphin (Smith et al., 1989). In the fetal guinea pig, POMC mRNA was detected in the anterior and intermediate lobes by day 40 of gestation, with levels in the intermediate lobe being significantly higher than the anterior lobe (Matthews, 1998). As gestation progressed, there was an increase in POMC mRNA in the intermediate lobe, and a redistribution of POMC mRNA in anterior lobe near term, with levels being highest in the inferior region and lowest in the superior region of the gland. The high levels of POMC mRNA in the inferior region increased between day 50-55 and day 60-65, and these levels were maintained in the neonate (Matthews, 1998). The increase observed in both lobes suggests a possible role for the upregulation of POMC synthesis in the observed rise in plasma ACTH and cortisol concentrations near term (Jones & Roebuck, 1980b).

Considerably more is known about the development of the ovine pituitary. POMC mRNA has been found in high levels early in gestation (day 60) in both the pars distalis and pars intermedia, with levels in the pars intermedia some 10-fold higher than the pars distalis. Similar to the guinea pig, levels of POMC mRNA localized predominantly in the inferior part of the anterior lobe in late gestation. In addition, overall levels of POMC mRNA rose in the second half of gestation, with the greatest increase near term. After birth, POMC mRNA decreased in the pars distalis, but remains high in the pars intermedia (Matthews & Challis, 1996b). These changes in POMC mRNA reflect increases in ir-ACTH, and concentrations of ACTH in the fetal sheep (Kumar et al., 1991). It has also been shown that larger molecular weight forms of ACTH exist early in gestation, and that levels decrease towards term. It has been
suggested that these larger molecules compete with ACTH for ACTH receptors in the adrenal, acting as antagonists, and that the reduction in their levels would facilitate binding and action of ACTH, thus increasing adrenal glucocorticoid output (Kumar et al., 1991; McMillen et al., 1995). Ovine corticotrophs were most sensitive to the action of CRH, which stimulates ACTH secretion through an adenylate cyclase, cyclic AMP-mediated mechanism that also stimulates the synthesis of POMC. Administration of CRH induced a sustained increase in ACTH while AVP causes a transient rise. The responsiveness to CRH increased between days 110 and 125 and then decreased towards term, and this may be due to negative feedback by increased levels of glucocorticoid (Kumar et al., 1991). Perez et al showed that ovine corticotrophs in vitro have a diminished response to CRH and an increased AVP response during gestation and into adulthood, and suggest that maturational changes in fetal corticotrophs determine whether their response to CRH or AVP is caused by an increase in the proportion of cells secreting ACTH or an increase in the amount of hormone secreted by individual cells (Perez et al., 1997).

In the human, the fetal pituitary can secrete ACTH from 5 weeks of gestation (Siler-Khodr et al., 1974), and that pituitary corticotrophs were responsive to hypothalamic releasing factors, CRH and AVP from 10 weeks of gestation (Thliveris et al., 1980).

1.7.3 The Developing Hypothalamus

CRH and AVP are the primary factors controlling the synthesis and secretion of fetal ACTH (Kumar et al., 1991). Both hormones are of hypothalamic origin and the
hypothalamic neurons producing CRH are primarily found in the medial parvocellular portion of the paraventricular nucleus (Griffin & Ojeda, 1996). AVP is colocalized with CRH in the parvocellular region of the PVN, and in the fetal and adult sheep is also found in the magnocellular PVN and the magnocellular supraoptic nucleus (SON) (Matthews & Challis, 1995b). Paraventricular parvocellular neurons that contain CRH and AVP project to the median eminence and are released into the hypophyseal portal blood (Engler et al., 1989).

In the sheep, CRH and AVP mRNA have been detected in the fetal PVN by day 60 of gestation (term=147 days) (Matthews & Challis, 1995b), and ir-CRH and AVP have been found in fibres that terminate in the median eminence by day 100 (Brieu et al., 1989). CRH mRNA in the parvocellular PVN increased progressively towards term with a dramatic increase at term (Matthews & Challis, 1995b), then decreased after birth. Immunoreactivity of CRH follows a similar pattern to mRNA (Matthews & Challis, 1996a). Conversely, AVP mRNA in the magnocellular PVN and SON (AVP mRNA present by day 60) did not change during gestation, but increased after birth (Matthews & Challis, 1995b). Similarly to CRH, mRNA levels of AVP were closely associated to ir-AVP levels in the hypothalamus (Saoud & Wood, 1996).

In the human fetus, CRH immunoreactivity was detected as early as 12-13 weeks of gestation (term=38-42), but no regional analysis was performed to determine the presence of CRH in the PVN (Ackland et al., 1986). Circulating vasopressin was detected from 11 weeks of gestation (Burford & Robinson, 1982; Skowsky & Fisher, 1977). AVP mRNA has been identified as early as 21 weeks of gestation (Murayama et al., 1993), and ir-AVP was also identified during gestation in the human fetal
hypothalamus with levels increasing with gestational age (Ackland et al., 1986; Burford & Robinson, 1982; Skowsky & Fisher, 1977).

In the guinea pig, the hypothalamic PVN develops early in gestation. The PVN has been shown to be present by day 40 of gestation, and the size of the PVN did not change between gestational day 40 and postnatal day 7 (Matthews, 1998). Unfortunately, additional information on hypothalamic development in the guinea pig is limited. Since the guinea pig CRH gene has only recently been cloned, no information exists regarding CRH mRNA levels during development.

1.7.4 The Developing Hippocampus

The hippocampal formation includes the hippocampus proper, the dentate gyrus, and the subiculum (Meibach & Seigel, 1977). The hippocampus resembles two interlocking “C”s, with one being the granule cell layer of the dentate gyrus and the second the pyramidal cell fields of the hippocampus. These pyramidal cell fields have been defined as CA1-4 from the subiculum to the dentate gyrus. Although it was long believed that the connections in the hippocampus were primarily unidirectional from the dentate gyrus to the subiculum (Amaral & Witter, 1989; Andersen et al., 1971), new evidence suggests that there is considerable cross-talk within and between cell fields ((Amaral & Witter, 1989). The main efferents from the hippocampus and subiculum are to the septum, cerebral cortex, anterior, and lateral thalamic nuclei, VMH, mamillary complex, BNST, accumbens nucleus, and anterior olfactory nucleus (Swanson & Cowan, 1977). In the fetal guinea pig, morphological studies have shown an increase in brain and
hippocampal size between day 40 and 50 of gestation (Dobbing & Sands, 1979; Matthews, 1998).

1.8 Development of Glucocorticoid Feedback

In the guinea pig pituitary, GR mRNA has been shown to be present in the anterior lobe, but not the intermediate lobe by day 40 of gestation, and to decrease significantly near term. In the PVN, the levels of GR mRNA significantly decreased as gestation progressed, with levels at days 50-55 being significantly lower than days 40-45, and levels at days 60-65 being significantly lower than days 50-55. Following birth, GR mRNA increased slightly by postnatal day 7 (Matthews, 1998). The high levels of GR mRNA early in gestation may be involved in neuronal differentiation, since glucocorticoids have been linked to this process. The observed decrease in GR mRNA towards term in both the anterior pituitary and PVN suggests that glucocorticoid feedback is reduced at this time. This may account for the rises in POMC mRNA synthesis, and ACTH release observed at this time, thus providing a possible mechanism for ACTH concentrations to remain high in late gestation (Matthews, 1998).

Both glucocorticoid receptor and mineralocorticoid receptor mRNA in the guinea pig limbic system were present by day 40 of gestation. GR mRNA levels were similar in the CA1 and CA3 pyramidal layers, and mRNA levels increased in the hippocampus and dentate gyrus between gestational day (gd) 40-45 and gd50-55. Hippocampal GR mRNA was at its peak near term, and then decreased slightly in day 7 neonates. Conversely, MR mRNA expression was significantly higher in the CA3 region compared to CA1, and mRNA decreased in the hippocampus and dentate gyrus between gd40-45 and gd50-55.
There was a slight recovery of MR mRNA levels in the neonate in the CA1 region and the dentate gyrus, but not in the CA3 region (Matthews, 1998).

The development of hippocampal corticosteroid receptors in fetal life in the guinea pig differs from the rat, where development of GR and MR occurs in postnatal life (Bohn et al., 1994; Meaney et al., 1985; Rosenfeld et al., 1988). In the rat, GR mRNA was expressed in the hippocampus by embryonic day 18 (Bohn et al., 1994), and binding sites increased gradually after birth until 4 weeks of postnatal life when they reached adult levels (Bohn et al., 1994). Hippocampal MR mRNA was three-fold higher than GR mRNA and remained high until postnatal day 60. Both hippocampal GR and MR mRNA and protein increase in parallel until they reach adult levels (Bohn et al., 1994).

In the fetal sheep, GR mRNA was present at high levels by day 120 of gestation, and dramatically increased by day 130 (Matthews, 1997). Following the increase, mRNA levels decreased in the last 15 days of gestation, and at term levels were similar to those seen in adult sheep (Matthews, 1997). There is no information regarding MR mRNA or protein expression in the fetal sheep.

The dynamic changes that occur in development of hippocampal corticosteroid receptors in the guinea pig, rat and sheep, may be involved in the activation and regulation of fetal HPA function. Furthermore, these changes may effect the HPA axis in such a way to sustain the observed increase in circulating glucocorticoids near term. Conversely, the changes observed in corticosteroid receptor mRNA may reflect regulation of receptor levels by glucocorticoids.
1.9 Animal Models of Fetal Programming:

1.9.1 Prenatal Nutritional Restriction and the HPA Axis

One of the most well studied forms of nutrient restriction involves protein restriction. In the rat, protein restriction during pregnancy significantly blunted the diurnal pattern of ACTH concentrations of offspring, but did not to alter corticosterone concentrations. In addition, glucocorticoid receptor, but not mineralocorticoid receptor, binding capacity and receptor numbers were elevated in male offspring of protein restricted mothers (Langley-Evans et al., 1996). The authors suggest that the adrenal of rat offspring may be hyper-responsive to ACTH, thus allowing for normal corticosterone levels in the presence of blunted ACTH, and conclude that programming of the HPA axis is caused by low protein diet. They also speculate that changes in the binding of GR sites in vascular tissue may provide a direct mechanism for the modulation of blood pressure by glucocorticoids, since blood pressure has been shown to be increased in offspring of protein restricted mothers (Langley-Evans et al., 1996). The role of the HPA axis was investigated in another rat study that used pair fed adult rats, thus eliminating the complicating factor of reduced food intake that is displayed by many species with low protein diets. Dietary protein deficiency, but not pair-feeding increased basal plasma corticosterone secretion, but did not alter restraint-induced ACTH secretion of adult adrenalectomized rats (Jacobson et al., 1997). The same study found that adrenalectomy or adrenalectomy with low level corticosterone replacement increased basal plasma ACTH and anterior pituitary POMC mRNA in protein deprived rats (Jacobson et al., 1997). Therefore, the brain and pituitary may play a role in increasing glucocorticoid production during malnutrition.
The production of hypertensive offspring has also been shown to occur in response to nutritional manipulation, and in a number of different species. In the rat, Langley-Evans reported that protein restriction produced hypertensive offspring (Langley-Evans et al., 1996; Langley-Evans et al., 1996). A high saturated fat, or low linoleic acid intake during pregnancy in the rat has also been shown to decrease weight, and increase the blood pressure of 7 week old offspring (Langley-Evans, 1996). This suggests that this type of diet may also be involved in the programming of hypertension in the rat. However, the authors believed that the mechanisms involved are different than those initiated by protein restriction (Langley-Evans, 1996). Recent criticism of these studies has arisen, since a tail cuff was used to measure blood pressure. In order to measure blood pressure with a tail cuff, it is necessary to expose the rat to heat, a procedure that is quite stressful. Therefore, the blood pressure measurements produced by Langley-Evans likely reflecting stress-induced blood pressure measurements.

In the guinea pig, unilateral uterine artery ligation resulted in a 20% reduction in birth weight, and produced an increase in blood pressure in chronically catheterized adult offspring. Adult offspring were 3-4 months of age and at this time those that displayed IUGR still had reduced (17%) body weights. There was also a negative correlation between heart rate and birth weight (Persson & Jansson, 1992). Both these studies support the hypothesis that the maternal environment, and specifically maternal nutrition, can program the fetus, resulting in differences in the onset of adult disease.

As for the mechanisms involved in nutritional fetal programming, some insight has been gained in the examination of the immediate effects of nutrient restriction. A recent study in the fetal sheep demonstrated that a 15% reduction in nutritional intake
over the first 70 days of gestation reduced the fetal ACTH response to CRH and AVP at gd 113-116, and reduced the cortisol response to CRH and AVP at both gd 113-116 and gd 125-127. Nutrient restriction also decreased the cortisol response to ACTH, but did not alter basal ACTH and cortisol concentrations (Hawkins et al., 1999). The effects of a 15% nutrient restriction were further investigated by examining the HPA response to acute isocapnic hypoxaemia in fetal sheep at gd 114-129, 120-123, and 126-129. Once again, basal plasma cortisol concentrations were not altered by restriction. Nor were HPA responses to hypoxaemia at gd114-115 or gd 120-123. However at gd 126-129, both plasma ACTH and cortisol responses to hypoxaemia were reduced in fetuses that had been nutrient restricted in early gestation (Hawkins et al., 2000). Hence, nutrient restriction not only alters adrenocortical responses to exogenous administration of CRH+AVP and ACTH, but it reduces the pituitary-adrenal response to endogenous stimuli.

Several studies in the guinea pig have examined the immediate effects of nutrient restriction. Jones et al. demonstrated that uterine artery ligation depressed fetal growth rate by >50% and was associated with reductions in plasma insulin, IGF-1, cortisol, thyroid hormone, glucose, acetate and free fatty acid concentrations, and elevations in IGF-2, glucagon and amino acids. The same study showed that 2 days of food withdrawal at gd 43-44 resulted in mild fetal growth retardation and similar changes in plasma constituents to artery ligation, except that plasma IGF-2 was now decreased (Jones et al., 1990). The emphasis of this report was on IGF-1 and its positive correlation with fetal growth rate (IGF-2 was not correlated with growth), but did not closely examine glucocorticoids or their regulation following nutritional deprivation. Similarly,
another study showed that a 40% reduction in maternal feed intake decreased fetal serum IGF-1 throughout gestation and diminished peak IGF-2 at day 55 of gestation. This study also showed decreases in thyroid hormones, and increases in plasma cortisol, as well as reductions in fetal and placental weight (35%) (Dwyer & Stickland, 1992). Once again, emphasis was placed on the role of IGF-1 in mediating the effects of undernutrition on the feto-placental unit. However, the authors also suggested that cortisol may play a role via its effect on IGFs, since IGF-2 is known to be depressed by glucocorticoids (Beck et al., 1988), and in this study plasma IGF-1 levels were inversely correlated with plasma cortisol levels.

Our laboratory has also reported IUGR associated increased cortisol as a result of acute nutrient deprivation (48 hours) (Lingas et al., 1999). Nutrient deprivation significantly decreased maternal and fetal glucose, decreased fetal thyroxine, and increased both maternal and fetal cortisol levels (Figure 1.1). However, plasma ACTH levels were only elevated in maternal blood. We propose that the discrepancy between fetal and maternal plasma ACTH indicates that the increase in fetal cortisol resulted from the placental transfer of stress-induced cortisol levels to the mother. We also demonstrated a significant decrease in hypothalamic (PVN) and hippocampal (CA1-2) GR mRNA in females, and decreased hippocampal (CA1-2) GR mRNA in male fetuses (Figure 1.2). We concluded that acute nutrient deprivation alters fetal HPA function and brain glucocorticoid receptor levels associated with glucocorticoid feedback, suggesting that programming of the HPA axis may occur at this time.
1.9.2 11β-HSD and Prenatal Programming

In addition to regulating glucocorticoid access to mineralocorticoid receptors, 11β-HSD plays an important role in the placenta in excluding maternal cortisol from the fetus (Lopez-Bernal et al., 1980). However, 11β-HSD is not able to effectively metabolize synthetic glucocorticoids such as dex (Brown et al., 1995). Two isoforms of the enzyme exist: the low affinity NADP(H)-dependent 11β-HSD-1 that acts bidirectionally to interconvert cortisol to cortisone and vice versa in the human, guinea pig, and sheep (corticosterone to 11-dehydrocorticosterone in rats) (Moore et al., 1993; Stewart et al., 1994), and the high affinity NAD-dependent 11β-HSD-2 that primarily converts cortisol to its inactive metabolite cortisone (corticosterone to 11-dehydrocorticosterone in rats) (Brown et al., 1993; Walker et al., 1992). The guinea pig placenta only expresses 11β-HSD-2, and the activity of the enzyme was found to be high between days 40-45 and 50-60 of gestation, but subsequently decreased at term (Sampath-Kumar et al., 1996).

It has been proposed that a deficiency in placental 11β-HSD-2 would allow an increased amount of maternal glucocorticoids to cross the placenta to the fetus (Seckl, 1997), thus representing a mechanism by which fetal plasma cortisol levels could be increased (Dalle & Delost, 1979; Jones & Roebuck, 1980b). Increased cortisol would in turn decrease growth and program the onset of adult disease (see section 1.1) (Seckl, 1997). This is supported by the observation that the lowest placental 11β-HSD activity (and likely the highest fetal glucocorticoid exposure) in the rat is seen in the smallest fetuses with the largest placentae (Seckl, 1997).
Studies involving the 11β-HSD (type 1&2) inhibitor carbenoxolone, have shown reduced birth weight and higher blood pressures in adult offspring whose mothers were treated with carbenoxolone (Langley-Evans, 1997b; Lindsay et al., 1996). Since, the effect of carbenoxolone is abolished by maternal adrenalectomy, this suggests that maternal glucocorticoids are necessary to observe results, and that effects are mediated via inhibition by 11β-HSD (Seckl, 1997). In addition, carbenoxolone treatment increased the insulin response to an oral glucose load in adult offspring (Lindsay et al., 1996). This suggests that excess maternal glucocorticoid exposure may be a common mechanism through which maternal environment factors can program the fetus. 11β-HSD deficiency may mediate the effect of maternal factors, since low protein diets reduce 11β-HSD activity (Langley-Evans et al., 1996) as well as lowering birthweight and producing hypertensive offspring (Langley-Evans, 1997a; Langley-Evans et al., 1996; Langley-Evans & Jackson, 1994; Langley-Evans et al., 1996).

Therefore, there is strong evidence to suggest that 11β-HSD, and specifically its deficiency, plays an important role the programming of adult disease, via an increase in the transfer of maternal glucocorticoids to the fetus.

1.9.3 Prenatal Stress and Glucocorticoid Exposure

Several different forms of prenatal stress have been studied in the rat. Takahashi showed higher levels of resting and stress induced (10 min separation or foot shock) ACTH and corticosterone in 14 day old rat pups born to pre-natally stressed (social isolation and chronic tail shock) compared to controls (Takahashi et al., 1988). Prenatal restraint stress during the last week of pregnancy (term=21 days) caused prolonged stress-
induced corticosterone secretion in adult offspring (Maccari et al., 1995). Another study involving prenatal restraint stress observed increased basal plasma ACTH concentrations with no change in corticosterone, but only in female offspring (McCormick et al., 1995). The MR has been suggested as a mediator for increased adrenocortical responses in the rat, since it is also decreased in adult offspring of mothers exposed to prenatal stress (Barbazanges et al., 1996; McCormick et al., 1995).

In the guinea pig, psychosomatic stress (exposure to a flashing light, novel environment/mild restraint stress) in pregnant guinea pigs (day 60 or 67 of gestation) increased maternal and fetal plasma cortisol levels (Dauprat et al., 1984). The offspring (12 and 60 days of age) of mothers exposed to this psychosomatic stress had reduced basal plasma ACTH as well as stress-induced ACTH and cortisol concentrations compared to controls (Cadet et al., 1986). In contrast to the decrease in HPA activity in the guinea pig, the juvenile offspring of pregnant primates exposed to unpredictable noise during mid-to late gestation exhibited increased basal ACTH and cortisol, and elevated stress-induced ACTH levels, but not cortisol (Clarke et al., 1994).

These studies suggest that prenatal stress can permanently alter the HPA function of offspring in several species including the rat, guinea pig, and primate. However, effects on the HPA axis differ depending on the type and extent of stress, as well as the species. In addition, the difference between fetal and juvenile/adult cortisol in the guinea pig suggests that fetal responses to prenatal stress do not necessarily reflect juvenile or adult HPA activity. This may reflect the fact that the HPA axis dynamically changes during development. Another possibility may be that during maternal stress, excess glucocorticoids cross the placenta from the mother to the fetus. Although the fetus is
normally protected from maternal glucocorticoids by 11β-HSD, it may not be able to deal with the very high levels seen in response to certain stressors (see section 1.7.2).

With respect to the transfer of maternal glucocorticoids to the fetus, studies involving excess prenatal glucocorticoid exposure have reported similar results to those of prenatal stress. 11β-HSD does not metabolize synthetic glucocorticoids, and therefore cannot protect the fetus from their effects (Siebe et al., 1993). This is important since pregnant women at risk of preterm delivery are often administered synthetic glucocorticoids to mature the fetal respiratory system (Liggins & Howie, 1972). Therefore, several animal studies have been performed to determine how fetal glucocorticoid administration affects development and whether it programs adult HPA function.

Our laboratory has shown that dexamethasone (dex) administration on day 50 and 51 of gestation in pregnant guinea pigs, produces an immediate change in basal adrenocortical activity. Although ACTH was unchanged following dex treatment, female plasma cortisol was significantly increased, and male plasma cortisol was significantly decreased in dex exposed animals compared to control. Dex treatment also increased GR and MR mRNA in the CA1 region of the hippocampus in female fetuses. Since plasma T4 was elevated in both sexes, changes in GRs may involve the proposed mechanism by Meaney that implicated increased 5-HT turnover caused by the increase in T4 (Dean & Matthews, 1999). Gestation length was increased by dex treatment and in juvenile (pd18) female offspring resulted in decreased GR mRNA in the hippocampus (CA3 & CA4), while in male offspring it increased GR mRNA in the cingulate cortex and hippocampal CA3. Dex-treated male offspring also demonstrated elevated basal plasma
cortisol concentrations compared to control males (Dean et al., 2000). The discrepancy between fetal and juvenile corticosteroid receptors in dex-treated animals, suggests that the regulation of GRs and MRs is highly dynamic, and that corticosteroid receptor levels directly following prenatal treatment do not necessarily reflect juvenile or adult receptor compliment in the hippocampus. This is further supported by recent preliminary data in adult offspring of dex-treated mothers. Male offspring of dex-treated mothers had reduced basal cortisol levels, and appeared to have an increase in hypothalamic (PVN) and hippocampal MR mRNA. Conversely, female offspring had increased basal cortisol levels, and decreased hippocampal MR mRNA (Liu and Matthews, unpublished observation). Therefore, dex treatment had a significant long-term effect on HPA function, which changes over the course of postnatal development, and is sex-specific.

In the rat, fetal exposure to synthetic glucocorticoids (0.1mg/kg) during the last week of gestation (gd15-20) increased basal plasma corticosterone in adult male offspring. Dex treatment also increased adult blood pressure, and reduced birth weight, without altering gestation length (Levitt et al., 1996). Altered glucocorticoid feedback was proposed to be the cause for elevated corticosterone levels, since dex treated males had significantly reduced levels of hippocampal GR mRNA and MR mRNA (Levitt et al., 1996). Another study that administered the same dexamethasone dose, found both male and female dex offspring to have increased blood pressure compared to controls (Benediktsson et al., 1993). A smaller dose of dex on gd 17, 18, and 19, resulted in adult male offspring with an increased corticosterone response to stress (Muneoka et al., 1997). Finally, a larger dex dose (0.4mg/kg) on gd 17 and 19 did not alter basal HPA function in prepubertal offspring, but did change the ratio of AVP to CRH in the median eminence.
(Bakker et al., 1995), indicating subtle long-term effects on HPA regulation (Matthews, 2000). Therefore, in utero programming of HPA regulation can also occur in the rat, and similarly to the guinea pig, effects appear to be sex-specific, since changes occurred primarily in males. In addition, the timing and dose of dexamethasone treatment is critical, and differences in time and dose can differentially program the HPA axis.

Cortisol infusion in pregnant sheep (0.48mg/h for 48 hrs) on gd27 (term=146 days), but not on gd 64, increased mean arterial blood pressure as well as glomerular filtration rate, urine flow rate, and free water clearance in the fetus (Dodic & Wintour, 1994). Daily intramuscular dexamethasone treatments in the rhesus minkey (4x1.25mg/kg) commencing on gd 132, caused an immediate dose-dependent degeneration and depletion of pyramidal neurons in the CA2 and CA3 regions of the hippocampus and in the granular neurons of the dentate gyrus (Uno et al., 1990). At 10 months of age, offspring born to dex treated mothers had elevated basal and stress-induced cortisol levels (Uno et al., 1990). These studies in the sheep and primate further support the hypothesis that short term exposure to elevated endogenous glucocorticoids or synthetic glucocorticoids, during critical times, can program the HPA axis, and thus alter its regulation in later life.

In addition to changes in HPA regulation, which can have profound effects on the onset of adult disease (see section 1.5), prenatal glucocorticoids in excess can alter brain structure and adult behaviour (for review see (Matthews, 2000)). Therefore, synthetic glucocorticoid administration to women at risk of preterm delivery, or excess glucocorticoids from maternal stress could have profound clinical implications.
1.9.4 Neonatal Handling in the Rat

Another procedure that examined the long-term effects of stress during development on HPA function involved the handling of neonatal rats during the first three weeks of life (Meaney et al., 1996). Although this is not a fetal manipulation, the rat gives birth to immature young, and the period of rapid brain growth associated with HPA development occurs after birth in the neonatal period (Dobbing & Sands, 1979). In these studies, rat pups were removed from their cage, placed together in small containers, and following a set amount of time, returned to their cage and their mothers. The animals were then allowed to mature to adulthood and tested for basal corticosterone and ACTH levels, as well as responses to stress (Meaney et al., 1996). Although handled (H) animals did not differ from non-handled (NH) in either basal ACTH or corticosterone at any time during the diurnal cycle (Meaney et al., 1989a), they did exhibit a significant attenuation of their adrenocortical responses to a number of stressors, and returned to basal levels faster than the termination of stress (Bhatnagar et al., 1995; Meaney & Aitken, 1985; Meaney et al., 1992; Meaney et al., 1989b; Viau et al., 1993). Decreased secretion of ACTH and corticosterone appears to be responsible for the attenuated response in H animals, since there were no differences in adrenal sensitivity, pituitary sensitivity, CBG, or metabolic clearance of ACTH and corticosterone (Meaney et al., 1992). These effects appeared to be long-term, since they were apparent as late as 24-26 months of age, hence differences in adrenocortical responses to stress persisted over the entire life of the animal (Meaney et al., 1992). Interestingly, the effect of handling during the first week of life was as effective at altering adrenocortical responses (Levine
S, 1975), with handling in the second or third week alone being less effective than the first. Hence, the HPA axis, is particularly sensitive during the first week of life.

Although basal ACTH and corticosterone do not differ in H and NH rats, resting-state CRH and AVP as significantly higher in NH rats (Bhatnagar et al., 1995), as are CRH mRNA levels (Plotsky & Meaney, 1993). In addition, CRH release in response to stress was higher in NH rats compared to H rats. From these observation, Meaney concluded that changes in CRH and AVP represent differences in their readily releasable storage pools in the PVN, and that this results in greater CRH and AVP release in NH animals, and that the regulator of CRH synthesis that leads to changes in the storage pool, is believed to be the hippocampus, and specifically GRs within the hippocampus (Meaney et al., 1996).

The observed differences in the adrenocortical response to stress between H and NH rats were suggested to be due to a difference in glucocorticoid negative-feedback sensitivity, and that this in turn must be due to changes in glucocorticoid receptors (Meaney et al., 1996). Corticosterone or dexamethasone administration 3 hours prior to a 20 min restraint stress suppressed the ACTH response in H rats nore than NH suggesting a role for negative-feedback (Meaney et al., 1989a). Furthermore, several studies have shown H animals to have increased glucocorticoid receptor binding capacity in the hippocampus and frontal cortex (Bhatnagar et al., 1995; Meaney & Aitken, 1985; Meaney et al., 1992; Meaney et al., 1989a; Sarrieau et al., 1988), as well as increased GR mRNA expression throughout the hippocampus (O'Donnell et al., 1994). The role of hippocampal GRs in mediating the effects of handling is further supported by the fact that the pattern of normal glucocorticoid receptor density development in the rat (for review
see (Meaney et al., 1994) corresponds to the ‘critical’ period of handling (first weeks of life).

The mechanism behind the observed increases in glucocorticoid receptors is believed to involve an increase in thyroid activity during handling that increases GR gene expression indirectly via changes in serotonin (Meaney et al., 1994). It has been shown that handling increases thyroxine (T₄) and triiodothyronine (T₃) (Meaney et al., 1996). Furthermore, T₄ or T₃ administration during neonatal life increased glucocorticoid receptor binding in later life (Meaney et al., 1987), indicating a link between the thyroid hormones and GR levels. In the same study, treatment of neonates for two weeks with the thyroid hormone synthesis inhibitor propylthiouracil (PTU) blocked the effects of handling on hippocampal GRs (Meaney et al., 1987). When cultured hippocampal cells were treated with thyroid hormones, no changes occurred in glucocorticoid receptors in this in vitro system (Meaney et al., 1994). However, since thyroid hormones increase serotonin turnover in the hippocampus of the neonatal rat (Mitchell et al., 1990) it was proposed that this was the mechanism increasing GR density. Lesions of the raphe 5-HT neurons decreased serotonergic input to the hippocampus, and administration of 5-HT to rat pups on day 2 decreased GR receptor density when examined in later life (Mitchell et al., 1990). This supports the role of 5-HT in regulating GR density. The treatment of hippocampal cell cultures resulted in a substantial increase in glucocorticoid receptor binding and mRNA, and this was blocked by the 5-HT₂ receptor antagonist ketanserin. Similarly, in vivo handling increased 5-HT turnover in the hippocampus (Smythe et al., 1994), and administration of ketanserin blocked handling-induced (Mitchell et al., 1990) and thyroid hormone induced (Meaney et al., 1996) increases in hippocampal GRs.
Therefore, handling increases 5-HT turnover via increased thyroid hormone levels, which in turn increases hippocampal GR levels via a 5-HT₂ receptor.

In summary, handling of neonatal rats during a critical time (first three weeks) in development alters adrenocortical function via increased hippocampal glucocorticoid receptor densities. The effects of handling on HPA feedback and function are sustained, such that they last through an animal’s lifetime. The increase in hippocampal GR is mediated by elevated thyroid hormone levels that increase 5-HT turnover. This mechanism may have mediated changes in HPA function in our model of nutrient restriction, since we showed an increase in T₄ immediately following nutrient restriction in the fetal guinea pig, in association with significant increases in limbic GR mRNA.
Figure 1.1: Maternal and fetal plasma a) glucose, b) adrenocorticotrophin (ACTH), c) cortisol (note: maternal x 10^{-1}) and d) thyroxine concentrations in control animals (open bars) and after 48h of nutrient deprivation (solid bars). Results are expressed as mean±SEM. * (p<0.05), ** (p<0.001) indicates statistical significance from control.
Figure 1.2: Densitometric analysis of glucocorticoid receptor (GR) mRNA in the hypothalamic PVN and specific limbic structures in control animals (open bars) and after 48h of nutrient deprivation (solid bars) in female (a, control n=5; deprived n=7) and male (b, control n=3; deprived n=6) fetuses. Results are expressed as mean±SEM relative optical density (ROD). * (p<0.05), ** (p<0.001) indicates statistical significance from control.
Figure 1.2

(a) Female

(b) Male

![Graph showing GR mRNA (ROD) levels in different brain regions for females and males.](image-url)
CHAPTER 2: RATIONALE AND HYPOTHESIS

2.1 RATIONALE

In humans, low birth weight has been strongly linked with an increased incidence of adult diseases such as coronary heart disease, hypertension, hyperlipidemia, and non-insulin dependent diabetes mellitus (Barker, 1995). IUGR can result from placental insufficiency, as in preeclampsia, and from reduced maternal nutrient intake. In the rat, a reduced protein diet during pregnancy results in hypertensive adult offspring (Langley-Evans & Jackson, 1994). Unilateral uterine artery ligation in the guinea pig also produces adult offspring with elevated blood pressure (Persson & Jansson, 1992). In addition, human studies indicate that elevated maternal glucocorticoids during pregnancy are associated with many of the same traits as IUGR, such as low birth weight, developmental delays, and neurological disorders (Field et al, 1985) (Ward, 1991). Therefore, it has been suggested that the resetting of major hormonal axes controlling growth and development may be responsible for the programming of adult disease (Barker et al., 1993).

The hypothalamo-pituitary-adrenal axis has emerged as a possible major player in fetal programming, since elevated plasma cortisol concentrations in adult males is associated with both low birth weight, as well as hypertension and insulin resistance (Phillips et al., 1998). Although several animal studies have examined changes in HPA function following nutrient restriction (Dwyer & Stickland, 1992; Hawkins P. et al., 1999; Langley-Evans et al., 1996) there is very little information regarding the central mechanisms mediating the effects of glucocorticoids during fetal programming. It has been proposed that altered development of corticosteroid receptor systems leads to the
development of adult pathologies, such as those observed with IUGR (Matthews, 1998). Furthermore, the *in utero* programming of adult disease is believed to occur during critical phases of development that are more sensitive to manipulation (Barker, 1994). Therefore, we wanted to investigate the effects of an acute nutrient restriction during the critical phase of the development of HPA regulatory systems. We also set out to establish a small mammalian model for IUGR that closely resembles the human. The guinea pig is similar to the human in that it is a prenatal brain developer that undergoes rapid neuroendocrine development in late gestation (Dobbing & Sands, 1970; Jones & Roebuck, 1980; Matthews, 1998). Conversely, species such as the rat and mouse undergo rapid neuroendocrine development in early postnatal life (Dobbing & Sands, 1979). Our laboratory has recently demonstrated that dynamic changes occur in development of GR and MR throughout gestation (Matthews, 1998). Therefore, we performed an acute nutrient restriction of 48h at this time (days 50-52 of gestation) in order to examine the involvement of the HPA axis in the programming of adult disease, and to investigate the possible mechanisms for observed changes in HPA activity.

2.2 HYPOTHESIS

In the present study we hypothesized that acute maternal nutrient restriction during rapid brain growth, permanently alters the HPA function guinea pig offspring, and that these changes are mediated by alterations in central corticosteroid receptor systems within the brain.

In experiments described in chapter 4 (juvenile study), we tested the hypothesis that maternal nutrient restriction has lasting effects, beyond fetal life, in juvenile offspring.
that are pre-pubertal and have not undergone the stress associated with weaning. Specifically, we propose that the modification of central corticosteroid receptor levels that we have previously reported in the fetus (Lingas et al., 1999) are maintained in the juvenile, and result in altered levels of basal plasma ACTH and cortisol.

In experiments described in chapter 5 (adult study), we wished to demonstrate that maternal nutrient restriction has long-lasting effects on HPA function and regulation, into young adulthood. Specifically, we examined HPA activity in detail, by measuring basal levels of ACTH and cortisol, as well as the pituitary-adrenal response to a number of HPA stimulants. We also propose that any differences in HPA activity result from differences in corticosteroid receptor levels between control and treated groups.
CHAPTER 3: GENERAL METHODS

3.1 Animal Studies

Female adult guinea pigs (450-500g) (Charles River Canada, St. Constant, Quebec, Canada) for breeding were housed separately in stainless steel cages measuring 24” width, 33.75” length, and 17.5” high, consisting of a tray containing Pro-chip/Bed-o Cobs (The Andersons, Industrial Products Group, Frisco, Ent., Bolton, Ontario, Canada) as bedding. Weanling and catheterized adult guinea pigs were housed separately in small plastic cages containing Pro-chip/Bed-o Cobs, with a stainless steel cover that had two prongs removed, to allow movement of the swivel mechanism (Lomir, Notre-Dame-de-l’Ile-Perrot, Quebec, Canada). Guinea pigs were maintained under conditions of controlled lighting (lights on 07.00 to 19.00h) and temperature (22°C), and allowed free access to food (Guinea Pig Chows 5052, Ralston Purina International, Leis Pet Distributing Inc., Wellesley, Ontario, Canada) and tap water. Pregnant guinea pigs remained undisturbed except for weekly cage maintenance. Weanling and catheterized adult guinea pigs remained undisturbed except for biweekly cage maintenance.

3.1.1 Breeding

The breeding of guinea pigs involved the observation of the vaginal membrane, which opens and closes in relation to the oestrus cycle. The oestrus cycle of a guinea pig is approximately 14-16 days, and the vaginal membrane starts to rupture at the beginning of the cycle. The initial tear is described as quarter open, and lasts a few hours to a day. The membrane then opens further to the half open stage and then finally to the fully open stage, where a very moist, enlarged and vascular vagina is observed. It is during the fully
open stage that the female guinea pig allows mating. This stage lasts 1-3 days until the animal has ovulated and the vagina begins to close to the half closed and quarter closed stages (Elvidge, 1972).

Guinea pigs were observed daily for changes in the vaginal membrane, and the status of the membrane recorded. When the membrane was observed to be half open, females were marked for identification, and caged with one of three male guinea pigs. This day was marked as day 0 of gestation. The guinea pigs were left undisturbed for three days, and then the status of the vaginal opening observed. When the membrane closed, female guinea pigs were taken from the male guinea pig and housed in their individual cages. The cycle of each guinea pig was monitored following mating until gestational day 30. If the membrane does not rupture within these thirty days the guinea pig was considered pregnant. Pregnant guinea pigs were left undisturbed, except to be palpated at approximately day 45, until they underwent treatment. The mean gestation length was 70 days.

3.1.2 Experimental Paradigms

For juvenile studies (chapter 4) pregnant guinea pigs were either deprived of all food (water ad libitum), or allowed to feed normally on days 50 and 51 of gestation. Pregnant guinea pigs were allowed to deliver normally and nurse undisturbed until
postnatal day 18, when juveniles were euthanized and tissues collected (see 3.1.5). In the adult studies (chapter 5) an identical nutrient deprivation protocol was undertaken and once again pregnant guinea pigs delivered normally. However, this time juveniles remained with their mother until postnatal day 30. At this time, juveniles were weaned and placed in individual plastic cages (see 3.1). On postnatal day 65, catheters were surgically implanted in the jugular vein and carotid artery. Animals were placed in a cage and the swivel system secured above. This allowed full rotation of the catheter and unrestricted movement of the guinea pig. Catheters were filled with heparinized saline and were flushed on a daily basis. Subsequent blood sampling ensued following a five day recovery. Surgery and sampling was performed during the same stage of the estrous cycle for female guinea pigs. Following completion of catheterized testing, guinea pigs were euthanized and tissues removed.

3.1.3 Catheterization

Before beginning the surgical cannulation, a catheter was prepared. 2.5 ft of polyethylene tubing (PE-50; OD 0.97mm; ID 0.58mm) was cut, and connected to a 22G needle. This was then fitted into a disposable tuberculin syringe, and filled with heparinized saline (10 units/cc). The end of the tubing was cut on a 45° angle to create a bevel. For carotid artery catheters a 0.5cm cuff of silastic tubing was placed 3.25cm from the end, and for jugular vein catheters a 3.25cm piece of silastic tubing with one end cut on a 45° angle was placed on the end of the catheter.

The guinea pig was anaesthetized with an intramuscular injection of ketamine (40 mg/kg I.M., MTC Pharmaceuticals) and xylazine (4 mg/kg I.M., Bayer Inc.). Once
anaesthetized the animal was shaved all around the neck area. The animal was then placed in a supine position, and secured so that its arms and legs were outstretched. The surgical site was cleaned with betadine solution and 70% propanol for surgery. A scalpel was used to cut a small horizontal incision on the skin of the neck, just above the sternum. The left jugular vein and right carotid artery was then catheterized.

The jugular vein lies close to the surface of the skin, and the carotid artery lies just lateral to the trachea. The carotid was distinguished by a pulsing that could be felt and seen. The vagus nerve lies alongside the carotid, and was separated from the artery before the artery was cut. This was done carefully to avoid stimulating the nerve and affecting the heart rate.

Each vessel was dissected separately using blunt dissection to move away fat and smooth muscle. Small forceps were used to bring the vessel to the surface where it could be worked with. The vessel was cleaned first to create a clean working area. The adventitia was gently pulled off the vessel with forceps. Two ligatures were placed around the vessel using 5-0 silk (braided, nonabsorbable surgical suture, Ethicon, Inc.); one at the cranial end, and one at the caudal end. The cranial ligature was tied off, and the caudal ligature was clamped with hemostats using gentle tension to temporarily occlude the vessel. Before cutting into the vessel 2% lidocaine (Abbott Laboratories Limited, Saint Laurent, Quebec) was placed on the artery. Lidocaine is a vasodilator and a local anaesthetic that served to dilate the vessel for easier insertion, and to prevent severe vasospasm. Small scissors (spring controlled vascular scissors) were used to make a small incision on the vessel wall close to the cranial ligature. The chamfered end of the catheter was immediately inserted towards the heart, through the small incision into the
lumen of the vessel. Small forceps were used to open the hole, while another pair of forceps were used to put the catheter in. The caudal suture was slowly loosened while the catheter was passed to allow for easier insertion. The catheter was passed all the way to the heart.

Once the catheter was inserted, and in the desired position, the caudal ligature was tied off and a third ligature was placed and tied around the vessel between the cranial and caudal ligatures, securing the catheter in place. For carotid cannulation, the ligatures were tied to the cuff. The line was then flushed with 1 ml of heparinized saline (100 units/ml) to prevent the formation of blood clots. Stoppers were placed on the end of each catheter.

The catheters were then burrowed subcutaneously around the neck using blunt dissection, and exteriorized in the interscapulary region, by inserting an angiocatheter, removing the needle, and passing the tubing through the flexible catheter. Local anaesthetic (lidocaine hydrochloride, Abbot Laboratorie) was administered as necessary. A small jacket and attached spring was then fitted to the guinea pig and the catheters passed up through the spring and attached to a Teflon swivel (Lomir Biolmedical Inc.). Following surgery the animals were treated with yohimbine (0.1 mg/kg I.V., Lloyd Laboratories) to reverse the actions of xylazine, along with 5 mls of heparinized saline (100 units/ml). Animals were placed in a cage and the swivel system secured above. This allowed full rotation of the catheter and unrestricted movement of the guinea pig. Catheters were filled with heparinized saline (200u/ml) and were flushed on a daily basis.
3.1.4 Catheterized Testing

In the first 2 days following surgery, guinea pigs were given 5mls of normal saline once a day to replace any lost fluids lost as a result of surgery. Following 5 days recovery blood samples were collected at 8am, 1pm, and 6pm to determine basal plasma ACTH and cortisol levels.

For all sampling, 150μl of blood was removed using a 1ml syringe and then placed in an eppendorf tube on ice. 400μl of heparinized saline (0.05u/ml) was flushed back into the catheter to return blood volume to normal and fill the catheter with fresh saline. Blood samples were spun at 3000rpm for 5min, at 4°C. Plasma was then separated and placed in titer-tubes (BIORAD, Mississauga, Ontario, Canada) that were subsequently stored at -20°C.

Animals were exposed to five treatments, which were carried out in the same order for each animal: (1) insulin (5 units/kg) followed by 5g food at 240 min, (2) ACTH₁₋₂₄ (0.5 μg/kg), (3) intra-venous glucose tolerance test (IVGTT)(500 mg/kg glucose), (4) 30 min restraint stress, (5) CRH (0.5 μg/kg). ACTH₁₋₂₄ and hCRH were purchased from Peninsula Laboratories (Belmont, CA, USA), insulin from and dextrose 50% (25g/50ml) injection from Abbott Laboratories (Montreal, Canada). Treatments 1 and 3 were commenced between 9.00 and 14.00 h following an overnight fast and blood samples (200μl) were taken at -30, 0, 5, 15, 30, 60, 90, 120, 240 , and 300 min for the insulin treatment and at -30, 0, 2, 5, 10, 15, 20, 30, and 60 for the IVGTT. Treatments 2, 4, and 5 were commenced between 13.00 and 15.00 h and blood samples (150 μl) were taken at -30, 0, 5, 15, 30, 60, 90, and 120 min for all treatments. At least 2 days recovery was allowed between treatments. All treatments were administered via the jugular vein and
samples were removed via the carotid artery. Treatments were administered after the base sample was drawn.

Blood pressure was measured on rest days via the carotid artery cannula. A small displacement pressure transducer was connected to a MacLab/4e data acquisition system (AD Instruments, Castle Hill, Australia) and a PowerMac Macintosh computer driven by MacLab Chart 3.5.6 software. Blood pressure was measured for five minutes between 12:00 and 1:00 pm.

Animals were given 2 days recovery following completion of treatments and were subsequently euthanized.

3.1.5 Plasma and Tissue Collection

Juvenile guinea pigs were quickly transferred from their cages to the necropsy room within 60 seconds, and decapitated with a guillotine. Trunk blood was collected immediately following decapitation into syringes containing heparin and placed on ice until they were centrifuged (Sorvall RT 6000 D) at 3500 rpm for 10 min at 4°C. Plasma was separated and stored at -20°C until analysis.

Each juvenile was weighed following decapitation. A midline incision was made on the ventral side of guinea pig, and the following organs were rapidly removed in sequential order: brain, pituitary, adrenal, kidney, liver, gonads, lung, heart, thymus gland, thyroid gland. All the organs were weighed, and the brain, pituitary, left adrenal, left kidney, two lobes of the liver, left gonad, left lung, left thymus, and left thyroid were slow frozen on dry ice, while the right adrenal, right kidney, one lobe of the liver, right gonad, right lung, heart, right thymus, and right thyroid were fixed in 4% paraformaldehyde solution. The pituitary
was frozen on a drop of mounting compound (Tissue Tek, Miles Canada, Etobicoke, Ontario, Canada) frozen on dry ice. The frozen tissues were wrapped in parafilm (American National Can™, CT, U.S.A.) or placed into sterile eppendorf tubes. Frozen tissues were stored at -80°C. Fixed tissues were washed once a day, for three days, twice in PBS, and once in 70% ethanol. Fixed tissues were stored in 70% ethanol, at 4°C for use in experiments related to, but not included in this thesis.

Adult guinea pigs were taken to the necropsy room in their cages, with their catheters disconnected, and euthanized with T61 (Hoechst Canada Inc., Regina Saskatchewan, Canada) via the jugular vein catheter. The jacket was then removed and the animal was decapitated using a guillotine. Guinea pigs were weighed following decapitation. A midline incision was made similarly to the juvenile necropsy and the following organs were removed and slow frozen on dry ice: brain, pituitary, left adrenal, left kidney, pancreas, heart. Organs were wrapped and stored in exactly the same way as juvenile organs.

3.2 Endocrine Analysis

Juvenile samples were analyzed in the same radioimmunoassay (RIA), as were all adult samples from each challenge (e.g. ACTH challenge, hypoglycemia etc.), in order to avoid inter-assay variation. Blood glucose was measured in adult samples from the hypoglycemia test and IVGTT using a One Touch blood glucose meter (Lifescan, Milpitas, California, USA). All procedures were performed at room temperature (21°C) unless otherwise indicated.
3.2.1 Adrenocorticotropic (ACTH)

A double-antibody RIA (Diasorin, Stillwater, MN, USA) was used to determine levels of immunoreactive (ir) plasma ACTH. This assay has been previously used in the guinea pig (Matthews 1998). The primary antiserum was raised in rabbits against human ACTH₁₋₃₉, and the secondary antiserum was an anti-rabbit IgG raised in goats. The cross-reactivity of this assay, measuring human ACTH₁₋₃₉, with α-melanocortin stimulating hormone, β-endorphin, β-lipotrophin, leucine enkephaline, methionin enkephalin, bombesin, calcitonin, parathyroid hormone, follicular stimulating hormone, oxytocin, and substance P is <0.01%. The procedure was as follows:

1. Ice-thawed plasma samples (20µl) were placed in test tubes along with phosphate buffer saline (PBS; see appendix)(80µl).

2. ACTH antiserum (200µl) was added to all samples and standards (16.4, 45.3, 99.6, 207, 463 pg/ml), followed by the addition of ¹²⁵I-ACTH (200µl; 10,000cpm) to all tubes including non-specific binding (NSB) and total counts (TC), and an overnight incubation at 4°C.

3. The secondary antiserum (500µl) was added to all the tubes excluding TC, and the tubes were gently vortexed.

4. Following a 15-20min incubation at room temperature, the tubes were centrifuged (Sorvall RC 3C Plus, Dupont, Canada) for 20min (15-20°C) at 3000rpm.

5. The supernatant was aspirated from each tube except TC, and the remaining precipitate was counted on a γ-counter (Packard Instruments Company Inc., Illinois, USA).

6. All samples and standards were assayed in duplicate, hence the duplicates were averaged for analysis. The average counts of the NSB tubes was subtracted from all
counts, and then the corrected counts were divided by the 0 standard.

\[
\%B/B_0 = \frac{\text{cpm of standard or unknown sample} - \text{cpm of NSB}}{\text{cpm of 0 standard} - \text{cpm of NSB}} \times 100
\]

A standard curve was plotted using the percentage bound (%B/B₀) of the standards versus the corresponding ACTH levels (pg/ml). Sample ACTH (pg/ml) were automatically calculated by a computer linked to the γ-counter.

### 3.2.2 Cortisol

Total plasma cortisol levels were measured using a radioimmunoassay protocol (Challis et al., 1981). The antibody was made kindly obtained from the laboratory of Dr. J Challis at the University of Toronto. Cross-reactivity of the antibody has been previously characterized as follows: cortisol sulphate 76%; 11-deoxy cortisol 35%; 21-deoxy cortisol 33%; cortisone 0.6%; corticosterone 0.84%; progesterone 0.31%; pregnelone, 17α-hydroxypregelone, and 11α-hydroxyprogesterone <0.1% (Norman et al, 1985). Prior to use, tritiated [³H] cortisol (NEN, Boston, MA, USA) was purified using thin-layer chromatography (TLC). The purification procedure was performed as follows:

1. Whatman filter paper was placed around the inside of a clean TLC tank (cleaned with methanol), and 100mls of solvent (95% chloroform, 5% ethanol) was poured into the tank. The TLC plate (Fisher Scientific) was placed in the tank.
2. Once the solvent reached the top of the plate, the plate was removed and allowed to dry. Then lines were marked down the middle of the plate, approximately one inch from each side, and across the top just underneath the front of impurities.

3. Approximately 50μl of cold steroid (1mg/ml stock) was spotted on the bottom of the left lane (10μl at a time), and the procedure was repeated in the right lane using \[^{3}H\] cortisol.

4. The plate was once again placed in the tank until the solvent almost reached the top, then removed and left to dry.

5. Using a U.V. light, the position of the cold steroid was found which corresponded to the position of the \[^{3}H\] cortisol. The powder containing the \[^{3}H\] cortisol was removed, placed in two test tubes, and centrifuged (Sorvall RC 3C Plus, Dupont, Canada) twice at 2500 rpm, 4°C, for 5 min., using 2 ml of ethyl acetate per test tube.

6. Purified \[^{3}H\] cortisol was counted on a β-scintillation counter (Liquid Scintillation Analyzer, Tri-CARB2100-TR, Packard, Mississauga, Ont, Canada) and stored at -20°C.

Purified \[^{3}H\] cortisol was used for the radioimmunoassay, and the assay was performed in duplicate for samples and in triplicate for standards. The radioimmunoassay procedure was performed as follows:

1. Ice-thawed plasma samples (10μl) were placed in extraction tubes (16×125mm) along with PBSG (100μl; see appendix). Diethyl ether (2ml) was then added to each tube, and the tubes were shaken at low speed for 1 hour at room temperature.
2. Extraction of the aqueous phase from the organic phase was done by placing each tube into a freezing acetone bath (dry ice pellets in acetone to cool) and then pouring the extracted liquid into new test tubes.

3. The samples were then air dried on a heating block, and reconstituted in 1ml of PBSG by vortexing every 5min during a 15min incubation on ice.

4. The reconstituted samples were aliquoted into test tubes in duplicate (100μl per tube).

5. Rabbit anti-cortisol antiserum (100μl) was added to all samples and standards (standard concentrations: 0, 10, 20, 40, 80, 160, 320, 640, and 1280μl).

6. Tritiated [3H] cortisol (10,000 counts/100μl in PBSG) was then added to all samples and standards, including non-specific binding (NSB) and total counts (TC) tubes.

7. The tubes were gently vortexed and incubated overnight at 4°C.

8. The following day, dextran-coated charcoal solution (200μl; see appendix) was added to all the tubes excluding TC, vortexed and incubated for 10min at 4°C.

9. The tubes were then centrifuged for 10min (4°C) at 2500 rpm (Sorvall RC 3C Plus, Dupont, Canada).

10. The supernatant was decanted into scintillation vials containing scintillation fluid, capped and shaken.

11. A β-scintillation counter (Liquid Scintillation Analyzer, Tri-CARB2100-TR, Packard, Mississauga, Ont, Canada) was used to count the radioactivity in each tube.
12. Standard triplicates and sample duplicates were averaged for analysis. The dose calculation was carried out using an ‘RIA data reduction system’ analysis program.

13. To determine the efficiency of the extraction a percentage of recovery was calculated and data was corrected for the recovery estimate.

\[
\%\text{Recovery} = \frac{\text{Recovery estimate in 1ml of plasma}}{\text{Recovery Total}}
\]

3.2.3 Progesterone

A double-antibody RIA (ICN, Montreal, Quebec, Canada) was used to determine levels of immunoreactive (ir) plasma progesterone. The primary antiserum was raised in rabbits against 11α-Hydroxyprogesterone-11α-hemisuccinate-HAS, and the secondary antiserum was an combination of goat ant-rabbit gamma globulin and PEG. Cross-reactivity of this assay, measuring human progesterone was as follows: 20α-dihydroprogesterone 5.41%; desoxycorticosterone 3.80%; corticosterone 0.70%; 17α-hydroxyprogesterone 0.67%; pregnenolone 0.41%; androstenedione 0.23%; testosterone 0.16%; 11-desoxycortisol, pregnenolone sulfate, cholesterol, dihydroepiandrosterone, ethiocholanolone, estradiol-17β, estradiol-17α, estrone, estriol, androsterone, aldosterone, cortisol, and DHEA-SDHEA-S <0.01%. The procedure was as follows:

1. Ice-thawed plasma samples (100µl) were placed in extraction tubes (16x125mm) along with PBS(ACTH)(100µl; see appendix). Diethyl ether (2ml) was then added to each tube, and the tubes were shaken at low speed for 1 hour at room temperature.
2. Extraction of the aqueous phase from the organic phase was done by placing each tube into a freezing acetone bath (dry ice pellets in acetone to cool) and then pouring the solution into a new test tubes.

3. The samples were then air dried on a heating block, and reconstituted in 100μl of PBSG by vortexing every 5min during a 15min incubation on ice.

4. The primary anti-progesterone antibody (500μl) was added to all samples and standards (standard concentrations: 0, 0.2, 0.5, 2.0, 5.0, 10.0, 25.0, and 50.0ng/ml).

5. Progesterone $^{125}$I was then added to all samples and standards, including non-specific binding (NSB) tubes.

6. The tubes were gently vortexed and incubated for 60min at 37°C.

7. Following the incubation, the secondary antibody (also known as precipitant solution; 500μl) was added to all the tubes.

8. The tubes were vortexed and then centrifuged for 20min (4°C) at 3000rpm (Sorvall RC 3C Plus, Dupont, Canada).

9. The supernatant was aspirated from each tube except TC, and the remaining precipitate was counted on a γ-counter (Packard Instruments Company Inc., Illinois, USA).

11. All samples and standards were assayed in duplicate, hence the duplicates were averaged for analysis. The standard curve was made using a data reduction system attached to the γ-counter, and employed the same formula used in the ACTH RIA.

\[
\%B/B_0 = \frac{\text{cpm of standard or unknown sample} - \text{cpm of NSB}}{\text{cpm of 0 standard} - \text{cpm of NSB}} \times 100
\]
A standard curve was plotted using the percentage bound (%B/ B₀) of the standards versus the corresponding progesterone levels (ng/ml) and sample progesterone (ng/ml) were automatically calculated.

3.2.4 Testosterone

A double-antibody RIA (ICN, Montreal, Quebec, Canada) was used to determine levels of immunoreactive (ir) plasma testosterone. The primary antiserum was raised in rabbits against testosterone-19-carboxymethylether-BSA. Cross-reactivity of this assay, measuring human testosterone was as follows: 5α-dihydrotestosterone 3.40%; 5α-androstane-3β, 17β-diol 2.20%; 11-oxotestosterone 2.00%; 6β-hydroxytestosterone 0.95%; 5β-androstane-3β, 17β-diol 0.71%; 5β-dihydrotestosterone 0.63%; androstenedione 0.56%; epiandrosterone 0.20%; 11β-hydroxyandrostenedione, 11β-hydroxytestosterone, androsterone, aldosterone, 5α-androstane-3, 17-dione, 5β- androstane-3, 17-dione, 5α-androstane-3α, 17β-dione, dihydroepiandrosterone, estrone, estradiol-17β, estriol, progesterone, corticosterone, deoxycorticosterone <0.01%. The procedure was as follows:

1. Ice-thawed plasma samples (50μl) were placed in test tubes along with 500μl of diluent buffer.
2. SBGI solution (antiserum)(100μl) was added to all samples and standards (0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0 ng/ml), followed by the addition of 125I-testosterone (500μl; 40,000cpm) to all tubes including non-specific binding (NSB), and the tubes were incubated at 37°C for 120 min.
3. The secondary antiserum (100µl) was added to all the tubes, and the tubes were gently vortexed and incubated at 37°C for 60 min.

4. The tubes were then centrifuged (Sorvall RC 3C Plus, Dupont, Canada) for 15 min (15-20°C) at 2500 rpm.

5. The supernatant was aspirated from each tube, and the remaining precipitate was counted on a γ-counter (Packard Instruments Company Inc., Illinois, USA).

6. All samples and standards were assayed in duplicate, hence the duplicates were averaged for analysis. The average counts of the NSB tubes was subtracted from all counts, and then the corrected counts were divided by the 0 standard.

\[
\%B/B_0 = \frac{\text{cpm of standard or unknown sample} - \text{cpm of NSB}}{\text{cpm of 0 standard} - \text{cpm of NSB}} \times 100
\]

A standard curve was plotted using the percentage bound (%B/ B₀) of the standards versus the corresponding testosterone levels (ng/ml). Sample testosterone (pg/ml) was automatically calculated by a computer linked to the γ-counter.

3.3 Molecular Analysis: In Situ Hybridization

3.3.1 Tissue Preparation and Sectioning

The in situ hybridization procedure (ISH) has been used extensively in our laboratory (Matthews et al, 1993; 1995; 1998). Frozen guinea pig brains were mounted using OCT mounting compound, and placed in a cryostat (Leica, Germany) at approximately -20°C. Brains were coronally sectioned (10µm) on the cryostat, and then thaw-mounted onto poly-L-lysine coated glass microscopic slides. The slides were prepared by first baking them at 200°C for three hours and then coating with a poly-L-
lysine and DEPC water solution. Once the sections were mounted, they were allowed to dry at room temperature and then fixed in a 4% paraformaldehyde solution (4°C) for 5 min. The slides were then washed twice in phosphate buffer saline (PBS) for 1 min., and dehydrated in 70% and 95% ethanol (1 min). All procedures were undertaken using sterile, autoclaved glassware and equipment, and fresh DEPC-treated solutions.

3.3.2 Oligonucleotide Probes

The antisense GR, MR, POMC, and CRH oligonucleotide probes were synthesized using an Applied Biosystems DNA synthesizer (model 392). The antisense probes were complementary to bases 1-45 of the coding sequence of guinea pig GR mRNA (Keightley & Fuller, 1994), bases 2942-2986 of the coding sequence of human MR mRNA (Arriza et al., 1987), and bases 1-45 of the coding sequence of POMC mRNA (Keightley et al., 1991). A fragment of the guinea pig CRH gene, corresponding to bases 679-719 of the coding sequence of human CRH mRNA (Go and Matthews, unpublished observation) was used to assess CRH mRNA within the guinea pig brain. I would like to acknowledge the work of Kathee Go in sequencing of the CRH probe. The probes were labelled with [\(^{35}\)S]-deoxyadenosine-5'-\(\alpha\)-thiotriphosphate (1300 Ci/mmol, NEN, Du Pont Canada Inc., Mississauga, Ontario, Canada) using terminal deoxynucleotidyl transferase (TdT; 26 units/\(\mu\)l, Gibco, Burlington, Ontario, Canada).

3.3.3 Probe Labelling

The labelling procedure was carried out on a cold block, behind a plastic shield. Tailing buffer (2\(\mu\)l) (Gibco, Burlington, Ontario, Canada) was added to the reaction tube,
followed by 1μl of the oligonucleotide (10ng/μl). Then 35S deoxyadenosine-5'-α-thiotriphosphate (1μl) was added to the reaction tube, followed by TdT (1.5μl) and 14μl of DEPC-treated water. The contents of the reaction tube were gently mixed by passing the mixture up and down the pipette a few times, and the mixture was incubated in a water bath (32.5°C) for 1 hour. The reaction was terminated by adding 2μl of 0.5M EDTA and 28.5μl of STE buffer. The labelled probe was separated from unlabelled probe using micro-columns that had been centrifuged (Micromax, International Equipment Company (IEC), Needham Heights, MA, USA) for 2min at 750G (3000rpm). The reaction mixture was placed into the micro-columns (ProbeQuant G-50 Micro Columns, AmershamPharmacia, Biotech Inc., Piscataway, NJ, USA) and the columns were centrifuged again at 750G for 2min. DTT (2μl) was added to the collected probe immediately following centrifugation, to prevent disulphide bond formation. The specific activity of the final labelled probe was measured using a β-scintillation counter (Liquid Scintillation Analyzer, Tri-CARB2100-TR, Packard, Mississauga, Ont, Canada), and not used for hybridization if the counts did not exceed 100,000cpm/μl. Successfully labelled probes were stored at -20°C.

3.3.4 Hybridization

Slides containing desired sections for each probe were placed in hybridization chambers. Each slide was covered with 180μl of hybridization buffer (see appendix), containing approximately 100,000 cpm of labelled probe. A piece of parafilm was used as a coverslip to cover each slide. Kimwipes dipped in a soaking solution (see appendix) were used to maintain moisture levels within each incubation chamber. The slides were
incubated overnight at 42°C to allow for hybridization of the radiolabelled probe to specific complementary RNA sequences contained in the brain section.

3.3.5 Wash

The next day, slides were washed for 20 min at room temperature in a 1XSSC solution (see appendix) containing β-mercaptoethanol (0.05%) (SIGMA Chemical Co., St. Louis MO, USA). The slides were then washed in an identical solution at 55°C for 35 min. Throughout both washes, the slides were agitated in a shaking water bath. The slides were further washed and dehydrated by passing them (10 seconds in each) through 1XSSC (X2), and 0.1XSSC washes followed by 70% and 95% ethanol.

3.3.6 Image Analysis

Dry slides were exposed to autoradiographic film (Biomax, Eastman Kodak Co., Rochester, N.Y., USA) in film cassettes along with 14C standards (American Radiochemical Inc., St. Louis, M.O., USA). Cassettes were stored at room temperature, undisturbed for the appropriate incubation time (Juveniles: GR=21 days, MR=10 days, GR(pituitary)=7 days, POMC (intermediate lobe)=24 hours, POMC(anterior lobe)=3 days; Adults: GR=4 weeks, MR=14 days, GR (pituitary)=7 days, POMC(anterior lobe)=4 hours, POMC(intermediate lobe)=3 days, CRH=8 weeks). Films were developed using an automatic processor (Kodak M35A – OMAT processor, Interscience, Markham, Ont, Canada). Image analysis of autoradiographic film was performed on a computerized image analysis system (Imaging Research Inc., St. Catherines, Ontario, Canada). All sections to be compared were processed in the same ISH, and exposed simultaneously to
allow direct comparisons to be made. The $^{14}$C standard was used to determine the linear range of the autoradiographic film for the exposure period. GR mRNA levels were measured in the hippocampus (CA1-2, CA3, and CA4 subfields), dentate gyrus, paraventricular nucleus, ventromedial hypothalamus, thalamus, and anterior pituitary. MR mRNA levels were measured in the hippocampus (as for GR) and the dentate gyrus. POMC mRNA was measured in the intermediate lobe and anterior lobe (superior and inferior regions) of the pituitary. CRH mRNA was measured in the hypothalamus. Relative optical density was measured in at least 6 sections per region, and averaged for further statistical analysis. See figure below for analytical criteria in the hippocampus.

3.4 Statistical Analysis

Analysis of radioimmunoassay results was performed in consultation with the Statistical Consulting Service, University of Toronto. Analysis was performed using SAS, and several statistical tests, including t-tests and regression analyses were used, to address several questions. 1) To determine differences in baseline cortisol levels between test and control groups, a t-test was performed on baseline ACTH and cortisol measurements. 2) To determine differences in the rate of hormonal increase, a t-test was performed on the slope of cortisol levels between time –30 and the peak. 3) To determine differences in the absolute difference from baseline to peak, a t-test was
performed on the difference between the peak and time \(-30\). 4) To determine differences in the rate of hormonal decline, a t-test was performed on the slope of cortisol levels between the peak and the end of the experiment. 5) To determine differences in the overall level of cortisol exposure, a t-test was performed on the area under each of the curves of hormone levels.

Statistical analysis for in situ hybridization results was performed using Statistica (Release 5, 97 Edition, Oklahoma, USA). The effects of treatment (nutrient restricted vs control), sex (female vs male) and subfield (CA1-2, CA3, CA4, and DG) on juvenile and adult brain GR mRNA and MR mRNA were determined using a three-way analysis of variance (three-way ANOVA) with Student-Newman-Keuls test post hoc. A three-way ANOVA was also used to determine the effects of treatment, sex, and subfield (anterior lobe) on juvenile and adult POMC mRNA. Differences in juvenile body weight, organ weights, organ/body weight ratios, PVN GR mRNA, VMH GR mRNA, thalamic GR mRNA, pituitary GR mRNA, and hypothalamic CRH mRNA were assessed using a two-way ANOVA, with treatment (nutrient restricted vs control) and sex (female vs male) being the independent variables, and the Duncan’s Multiple Range test post hoc. All results for RIA, ISH, and physical parameters are expressed as Mean ± standard error of the mean (SEM). Statistical significance was set at \(P<0.05\).
3.5 APPENDIX

3.5.1 Radioimmunoassay

PBS for ACTH assay (500ml)

2.99g \( \text{Na}_2\text{HPO}_4 \)

0.56g \( \text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O} \)

2.7g \( \text{NaCl} \)

0.5g BSA (Bovine Serum Albumin) (SIGMA Chemical Co., St Louis, MO)

The buffer was made in 500ml of millipore water, and stored at 4°C.

PBSG cortisol assay (2L)

10.76g \( \text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O} \) (monobasic)

32.70g \( \text{Na}_2\text{HPO}_4\cdot7\text{H}_2\text{O} \) (dibasic)

18.00g \( \text{NaCl} \)

2.00g Sodium azide (SIGMA Chemical Co., St Louis, MO, USA)

2.00g gelatin (Fisher Scientific, Fair Lawn, NJ, USA)

Gelatin was pre-heated in 500ml millipore water. The remaining reagents were made-up in 1.5L of millipore water and the gelatin was added once it was completely dissolved. The pH of the solution was adjusted to 7.0 and stored at 4°C.

Charcoal (100ml)

0.0625g dextran T70 (Pharmacia Biotech, Uppsala, Sweden)

0.625g carbon, decolorizing, Norit neutral (Fisher Scientific, Fair Lawn, NJ)
100ml PBSG (see above)

The dextran was added to the PBSG and stirred for several hours until completely dissolved, before the charcoal was added. The solution was stored at 4°C, and stirred for at least 1 hour prior to use.

3.5.2 *In Situ* Hybridization

Molecular biology grade reagents (from BDH Laboratory Supplies, Poole, England, or from SIGMA Chemical Co., St Louis, MO, USA) were used for preparation of all the following solutions, and DEPC-treated water was used in all solutions including the HCL and NaOH used for adjusting pH. All containers and equipment were autoclaved prior to use.

**Diethyl pyrocarbonate (DEPC) treatment**

DEPC was added to millipore water or buffers, at a concentration of 500μL/L. The addition of DEPC was performed under the fumehood, and solutions were allowed to stand for 3 hours before being autoclaved.

**Paraformaldehyde**

Paraformaldehyde (40g) was dissolved in either millipore water for fixation of necropsy tissues, or DEPC water for cryosectioned tissues, by heating to 60°C under the fumehood. Several drops of NaOH were added to the heated solution to further dissolve the paraformaldehyde. The pH was adjusted to 7.5, then the solution was filtered and stored at 4°C.
Phosphate Buffered Saline (PBS)

8.00g  NaCl
0.20g  KCl
1.44g  Na$_2$HPO$_4$
0.24g  KH$_2$PO$_4$

The reagents were dissolved in 1L of millipore water, the pH was adjusted to 7.4, and treated with DEPC. The solution was stored at room temperature.

DL-Dithiothreitol (DTT)

3.09g  DTT
20ml  sodium acetate (0.01M)

The mixture was filtered and stored in 1ml aliquots at -20°C.

EDTA (0.5M)

93.05g  EDTA (Gibco BRL, Grand Island, NY, USA)
500ml  millipore water

The solution was stirred until completely dissolved and the pH adjusted to 8.0. The solution was DEPC-treated and stored at room temperature.

Deionized Formamide

100ml  formamide (Gibco BRL, Grand Island, NY, USA)
5g  mixed bed resin
The mixed bed resin was added to the formamide in an Erlenmeyer flask and stirred under the fume hood for 2 hours. The mixture was filtered through sterile filter paper and stored at -20°C in 25ml aliquots.

**Saline Sodium Citrate (20X SSC)**

175.3g NaCl
88.20g sodium citrate

The reagents were dissolved in 1L of millipore water, DEPC-treated, and stored at room temperature.

**Sodium Phosphate (0.5M)**

Na$_2$HPO$_4$(0.5M)
NaH$_2$PO$_4$(0.5M)

The solutions were mixed and the pH adjusted to 7.0. The solution was then DEPC-treated and stored at room temperature.

**Sodium Pyrophosphate (0.1M)**

**Denhardts Solution (100X)**

0.5g polyvinylpyrrolidine (PVP)
0.5g bovine serum albumin
0.5g Ficoll
50ml DEPC water
Reagents were dissolved in water and stored in 6 ml aliquots at -20°C.

Salmon Sperm DNA

Salmon sperm was purchased from Sigma in a ready to use form (10 mg/ml).

Polyadenylic Acid

100 mg Polyadenylic acid (potassium salt)

The salt was dissolved in 20 ml DEPC water and stored in 1 ml aliquots at -20°C.

Sodium Heparin

Freeze dried sodium heparin was dissolved in DEPC-treated water (120 mg/ml) and stored in 50 μl aliquots at -20°C.

Hybridization Buffer

25 ml deionized formamide
10 ml 10X SSC
2.5 ml 0.5M sodium phosphate
0.5 ml 0.1M sodium pyrophosphate
2.5 ml 100X Denhardts solution
1.0 ml salmon sperm DNA (10 mg/ml)
1.0 ml polyadenylic acid (5 mg/ml)
50 μl sodium heparin (120 mg/ml)
5.0 g Dextran sulphate (Amersham Pharmacia Biotech, Uppsala, Sweden)
All the reagents were mixed together and the volume adjusted to 50ml with DEPC water. The solution was made the day prior to *in situ* hybridization and stored overnight at 4°C.

**Soaking Solution**

- 25ml formamide
- 12.5ml 20X SSC
- 12.5ml DEPC water
CHAPTER 4: JUVENILE STUDY

4.1 INTRODUCTION

Epidemiological studies have demonstrated strong associations between low birth weight or intra-uterine growth restriction (IUGR) and adult diseases such as hypertension, coronary heart disease, and diabetes (Barker, 1995; Barker, 1996; Barker et al., 1993; Phillips et al., 1998). It has been suggested that "programming" of adult disease occurs during critical periods of pre-natal development associated with rapid cell proliferation (Dobbing & Sands, 1970; Dobbing & Sands, 1971). Furthermore, studies in men showing significant correlations between low birth weight and basal plasma cortisol, and between cortisol levels and systolic blood pressure, have implicated the resetting of the hypothalamo-pituitary-adrenal (HPA) axis as a possible mechanism for programming of adult disease (Phillips et al., 1998; Walker et al., 1998). Altered development of the HPA axis could result in chronically elevated cortisol, which in turn leads to growth restriction, hypertension, immunosuppression, neurological damage, and diabetes (Munck et al., 1984; Sapolsky, 1994; Walker & Williams, 1992).

Studies in rats have further supported the possibility that the HPA axis forms a link between reduced birth weight and adult disease. Maternal nutrient restriction or increases in glucocorticoids have been shown to decrease birth weight and significantly elevate blood pressure in the adult rat (Langley-Evans, 1997; Langley-Evans et al., 1996; Levitt et al., 1996). It has been suggested that these changes occur via alterations in glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) expression in the limbic system, which effect the feedback regulation of adrenocorticotropic (ACTH) and cortisol (Jacobson & Sapolsky, 1991; Liu et al., 1997; Meaney et al., 1988). However,
the rat gives birth to immature young and extensive neuroendocrine development occurs after birth (Dobbing & Sands, 1979). In contrast, the guinea pig gives birth to precocious young. Rapid brain growth occurs during the prenatal period (Dobbing & Sands, 1979), and GR and MR systems develop rapidly in late gestation (Matthews, 1998). In the human, rapid brain growth also occurs in late gestation (Dobbing & Sands, 1979).

We have previously shown that short periods of nutrient restriction (48h) during the brain growth spurt (gd50) cause IUGR. Furthermore, this perturbation led to a significant reduction in hippocampal and hypothalamic GR expression in the fetal guinea pig (Lingas et al., 1999). Although, one study has demonstrated that IUGR is associated with elevated adult blood pressure in the guinea pig (Persson & Jansson, 1992), there is no information concerning the long-term effect of short periods of nutrient restriction on HPA function in any species. In the present study, we hypothesize that a short period of acute nutrient restriction during fetal life has a long-term effect on growth, endocrine function and brain corticosteroid receptor systems in the pre-pubertal, juvenile guinea pig.

4.2 MATERIALS AND METHODS

4.2.1 Animals and treatments

Female guinea pigs were mated with males in our animal facility using a technique developed at the Nuffield Institute for Medical Research, Oxford, UK. (Elvidge, 1972). This method produces accurately time-dated pregnant guinea pigs. These studies were performed according to protocols approved by the Animal Care Committee at the University of Toronto, in accordance with the Canadian Council for Animal Care. Pregnant guinea pigs
were deprived of all food for 48 hours on gd50 and 51 (n=12), or allowed to feed normally (control, n=8). Water was available ad libitum. On gd52, pregnant guinea pigs were all fed normally. Neonates were weighed at birth and remained with their mother until day 18 of postnatal life, when juvenile guinea pigs were euthanized by decapitation. Trunk blood samples were taken and the plasma was separated and stored at -20°C. Juvenile body weights were recorded and brains and pituitaries were removed and weighed. Brains and pituitaries were frozen and stored at -80°C until processing.

4.2.2 Plasma adrenocorticotrophin and cortisol

Basal plasma adrenocorticotrophin (ACTH) and cortisol were measured by radioimmunoassay (RIA) as described previously (Li & Matthews, 1999).

4.2.3 In situ hybridization

The method for in situ hybridization has been described in detail, previously (Matthews & Challis, 1995; Matthews et al., 1995). Briefly, coronal brain and pituitary cryosections (12μm) were mounted onto (poly)-L-lysine (Sigma Chemical Company, St. Louis, MO) coated slides, dried and post-fixed with paraformaldehyde (4%). The antisense GR, MR, POMC, and CRH oligonucleotide probes were synthesized using an Applied Biosystems DNA synthesizer (model 392). The antisense probes were complementary to bases 1-45 of the coding sequence of guinea pig GR mRNA (Keightley & Fuller, 1994), bases 2942-2986 of the coding sequence of human MR mRNA (Arriza et al., 1987), bases 1-45 of the coding sequence of POMC mRNA (Keightley et al., 1991), and bases 679-719 of the coding sequence of CRH mRNA (Go and Matthews, unpublished observation). The probes were
labeled using terminal deoxynucleotidyl transferase (Gibco, Burlington, Ontario, Canada) and [\(^{35}\)S]-deoxyadenosine 5'- (a-thio)triphosphate (1300 Ci/mmol, NEN, Du Pont Canada Inc., Mississauga, Ontario, Canada) to a specific activity of 1.0 \( \times \) \( 10^9 \) cpm/\( \mu \)g. Labeled probe in hybridization buffer (200\( \mu \)l) was applied to slides at a concentration of 1.0 \( \times \) \( 10^3 \) cpm/\( \mu \)l. Slides were incubated overnight in a moist chamber at 42°C. After washing in 1 x SSC (30 min at room temperature), 1 x SSC (30 min at 55°C), the slides were rinsed once with 1 x SSC and with 0.1 x SSC at room temperature, then dehydrated in ethanol, dried and exposed to autoradiographic film (Biomax, Kodak). The films were developed using standard procedures (exposure: GR 21 days, MR 10 days). The oligonucleotide probes for GR and MR have been comprehensively characterized previously (Matthews, 1998). A control, 45 base sense oligonucleotide probe demonstrated no hybridization when incubated with sections known to contain POMC mRNA (Figures 4.1a and 4.1b).

4.2.4 Northern blot analysis (performed in collaboration with Marcus Andrews)

Northern blot analysis was carried out to further confirm the specificity of the probe for POMC mRNA. Total RNA (30\( \mu \)g) was extracted from a single term fetal guinea pig pituitary and subjected to electrophoretic separation. Analysis was performed using \(^{32}\)P-labelled POMC oligonucleotides, and overnight incubation, as described previously (Matthews et al., 1995). The same probe as that described for in situ hybridization was used. Examination of autoradiographic film (X-Omat AR, Kodak) after 4 hours exposure revealed distinct bands of hybridization at 1.3kb (Fig. 1c). This is consistent with the known size of the POMC mRNA transcript (Keightley et al., 1991).
4.2.5 Data Analysis

For *in situ* hybridization analysis, brain sections were processed simultaneously to allow direct comparison between groups. The sections were exposed together with $^{14}$C-standards (American Radiochemical Company, MO, USA) to ensure analysis in the linear region of the autoradiographic film. The relative optical density of the signal on autoradiographic film was quantified, after subtraction of background values, using a computerized image analysis system (Imaging Research Inc., St. Catharines, Ontario, Canada) (Matthews, 1998; Matthews & Challis, 1995; Matthews & Challis, 1995). GR mRNA levels were measured in the hippocampus (CA1/2, CA3, and CA4 regions), dentate gyrus, thalamus, the hypothalamic paraventricular nucleus (PVN), and the ventromedial hypothalamus (VMH), as well as the pituitary pars distalis. MR mRNA levels were determined in the hippocampus (as for GR), and dentate gyrus. POMC mRNA levels were measured in the pars distalis (superior and inferior regions measured separately) and pars intermedia of the pituitary (see Figure 4.1a) (Matthews et al., 1996). CRH mRNA levels were measured in the hypothalamic PVN. There were no differences between male and female values for any of the parameters measured, and so male and female data have been grouped for all subsequent analysis. Group data are presented as means±SEM and were statistically analyzed using a one-(treatment), two-(treatment × region) or three-(treatment × sex × region) way ANOVA followed by the Duncan’s method of post-hoc comparison. Statistical significance was set at $p<0.05$.

4.3 RESULTS

Nutritional deprivation for 48 hours during rapid neuroendocrine development resulted
in a significant reduction in birth weight (20%; <0.0001) (Figure 4.2a) but did not alter gestation length (Figure 4.2b). By postnatal day 18, juveniles that had been nutrient deprived during fetal life had significantly reduced body weight (10%; p<0.05) compared with controls (Table 4.1). The brain to body weight ratio of nutrient-deprived guinea pigs was significantly higher than that of control (p<0.006), indicating a sparing effect on the brain. There were no differences in weight or organ to body weight ratios of any other organs (Table 4.1). Sex-effects were not observed in either plasma ACTH or cortisol, hence males and females have been grouped for the presentation of endocrine and molecular data. Guinea pigs that were nutrient-deprived had significantly (p<0.05) lower basal plasma ACTH (Figure 4.3a), though there was no significant effect on basal plasma cortisol levels (Figure 4.3b).

GR mRNA was expressed widely throughout the brain, and at highest levels in the limbic system (CA1/2 regions of the hippocampus, dentate gyrus), the ventromedial hypothalamus, the thalamus, and the paraventricular nucleus (PVN) of the hypothalamus. MR mRNA was expressed almost exclusively in the limbic system, with highest levels in the CA1-2 region of the hippocampus and the dentate gyrus. Two-way ANOVA (treatment × sex × region) revealed no effect of prenatal treatment on MR mRNA (Table 4.2) in any areas of the brain, or on limbic GR mRNA (Table 4.3). However, there was a significant increase in levels of GR mRNA in the VMH (p<0.05) (Figures 4.4 and 4.5), but no treatment effect in the PVN or the thalamus (Figure 4.5).

GR mRNA was expressed in the pars distalis, while POMC was expressed in both the pars distalis and pars intermedia of the pituitary. Table 4.4 displays pituitary levels of GR mRNA and POMC mRNA levels in the different groups. There was no significant
difference in pituitary GR mRNA or POMC mRNA levels between nutrient-deprived and control guinea pigs in any region measured. CRH mRNA was highly expressed within the paraventricular nucleus of the hypothalamus, and to a lesser degree in surrounding cortical areas (Figure 4.6). CRH mRNA levels are shown in Table 4.5. Levels of CRH mRNA were not different between control animals and offspring of nutrient-deprived mothers, or between.

4.4 DISCUSSION

In the present study, we have demonstrated that 48h of nutrient deprivation during fetal development has a long-term effect on growth and function of the juvenile guinea pig HPA axis. Juvenile guinea pigs that were nutrient deprived on gd50 had reduced body weights and increased brain to body weight ratios compared with control animals. Nutrient deprivation significantly reduced basal plasma ACTH levels in pnd18 juveniles, but did not alter pituitary POMC mRNA or plasma cortisol levels. We have also shown a significant region-specific increase in GR mRNA in the VMH of juvenile guinea pigs that were nutrient deprived. However, the reductions in GR mRNA following nutrient deprivation previously observed in the fetal brain (hippocampus, PVN) (Lingas et al., 1999), are no longer present by post-natal day 18.

The regulation of the HPA axis is altered in juvenile offspring born to nutrient-deprived mothers. The reduction in plasma ACTH with little change in plasma cortisol may indicate a change in adrenal sensitivity. In this connection, previous studies have shown that glucocorticoid infusion may alter adrenal sensitivity to ACTH in this species (Dallman et al., 1987; Mokuda et al., 1997). It is possible that the adrenal cortex has
compensated for a reduction in ACTH, with an increase in sensitivity to ACTH. Alternatively, evidence exists to suggest that other hormones and/or cytokines from the adrenal medulla or systemic circulation, as well as neural inputs to the adrenal play an important role in cortisol secretion (Chrousos, 1998). It is possible that these systems may have been affected by nutrient deprivation. There may also be a discrepancy between ACTH and cortisol levels at this time, because the HPA axis is still developing on d18, and hence may not be as tightly regulated as in the adult.

The decrease in plasma ACTH appears to be a consequence of reduced release, rather than a decrease in synthesis, since POMC mRNA levels were unaltered in juvenile guinea pigs that had been nutrient deprived. In the mouse, CRH and AVP have been shown to increase ACTH secretion without changing POMC mRNA levels (Castro, 1993). This is not unusual since CRH and AVP can affect both ACTH release and POMC gene expression through a number of different pathways, and therefore can affect ACTH release without altering gene expression (Castro, 1993). Also, both POMC and ACTH are stored in large quantities in the corticotrophs (Antoni, 1986). Therefore, modest decreases or increases in ACTH release and POMC processing can occur without significant changes in POMC mRNA.

In order to determine whether the reduction in plasma ACTH is a consequence of effects on the pituitary or higher centres such as the hypothalamus, the expression of CRH mRNA in the PVN was determined. CRH mRNA measured in the PVN as a whole, or specifically in the parvocellular region where it is most highly expressed, was not different between groups. Therefore, it is unlikely that ACTH is reduced as a result of altered CRH levels. However, AVP also strongly influences ACTH secretion, and acts to
augment the ACTH response to CRH (Dallman et al., 1987). Studies in mice also indicate that the CRH to AVP ratio may be an important regulator of ACTH release (Castro, 1993). Therefore, in order to rule out a decrease in ACTH release as the cause for reduced plasma ACTH levels, AVP mRNA in the PVN and SON would also have to be determined. Specifically, AVP expression within the parvocellular region of the PVN (AVP is expressed in both the magnocellular and parvocellular regions of the PVN) that is coexpressed with CRH would be of particular relevance. These studies have not been performed since the sequence of guinea pig AVP has yet to be elucidated.

Glucocorticoid negative feedback is a key regulator of the HPA axis, and occurs via the high affinity type I corticosteroid receptor (MR), and the low affinity type II corticosteroid receptor (GR) (Dallman et al., 1987; Jacobson & Sapolsky, 1991). The day 50 guinea pig fetus is undergoing rapid brain growth, associated with neurological and neuroendocrine maturation (Dobbing & Sands, 1970; Dobbing & Sands, 1979; Jones & Roebuck, 1980). At this time, transitions occur in MR and GR levels, that may be sensitive to manipulations, resulting in the programming of glucocorticoid feedback (Matthews, 1998). We have recently demonstrated that GR mRNA levels in the hippocampus (CA1/2) and the hypothalamus (PVN) are reduced on gd52, immediately following 48h of nutrient deprivation (Lingas et al., 1999), indicating that glucocorticoid feedback can be manipulated at this time. In the present study, we have shown that there were no significant differences in either MR mRNA levels or GR mRNA levels in any of the key areas associated with glucocorticoid regulation. Though a quite different model, studies in the rat have also shown reductions in hippocampal and hypothalamic GR mRNA immediately following maternal deprivation during neonatal development.
(Avishai-Eliner et al., 1999; Van Oers et al., 1997). There is currently no information on the long-term effect of nutrient deprivation on corticosteroid receptor mRNA in any species. We have shown for the first time, that GR mRNA levels in the hippocampal CA1/2 and the hypothalamic PVN return to normal levels by d18, despite earlier reductions (Lingas et al., 1999).

The mechanism by which corticosteroid receptors are influenced by nutrient deprivation is not clear. We have previously associated increased fetal cortisol with reduced hippocampal GR mRNA (Lingas et al., 1999). It is possible that post-natal nutrition may partially compensate any central effects of prenatal nutrient availability. Also, there may be implications of fetal nutrient deprivation on postnatal nutrition, since although GR mRNA levels were unchanged in the hypothalamic PVN, nutrient deprivation resulted in significant increases in GR mRNA in the hypothalamic VMH. It has been shown that the guinea pig VMH expresses high levels of GR mRNA (Matthews, 1998), and others have shown extensive connection between the VMH and the PVN. Changes in GR mRNA in the VMH may be functionally important, since this is an area associated with glucose sensing and satiety (Canteras et al., 1994; Suemaru et al., 1995).

Evidence suggesting the VMH as a satiety centre come from experiments showing that VMH lesions cause hyperphagia, obesity, and hyperactivity of the HPA system (Choi et al., 1996; Suemaru et al., 1995). Although there is no evidence suggesting that the VMH produces either orexigenic or anorexigenic signals, the fact that destruction of cell bodies in the VMH produces hyperphagia and obesity does suggest that neuronal elements within the VMH regulate appetite. The VMH contains receptors for appetite-regulating signal molecules such as neuro-peptide Y (NPY), and galanin
(GAL), and it is believed that hyperphagia may be due to increased sensitivity to NPY, via increases in Y₁ receptors, as well as increasing leptin resistance, by disrupting leptin feedback. The VMH is also neurally linked with several hypothalamic sites involved in feeding, including the arcuate nucleus, the dorsomedial nucleus, and the PVN. Such connections indicate that disruption of the VMH may disturb the flow of information to and from other hypothalamic sites, consequently resulting in unregulated feeding (Kalra et al., 1999).

In addition to altering feeding, manipulations of the VMH have been shown to affect the release of hormones associated with glucose homeostasis. VMH lesions prevent the release of counterregulatory hormones such as glucagon, epinephrine and norepinephrine during hypoglycemia (Borg et al., 1994), an effect that is also seen with local glucose perfusion to the VMH (Borg et al., 1997). Also, electrical stimulation in the VMH significantly elevates serum glucose concentrations (Frohman & Bernardis, 1971; Gisel & Innes, 1999). Hence, the VMH plays a vital role in the maintenance of blood glucose levels.

The connection between the VMH and the HPA axis is not entirely understood, but Dallman has shown that VMH lesions increase basal corticosterone concentrations but decrease the sensitivity of ACTH to corticosterone feedback in adrenalectomized rats (Suemaru et al., 1995). They suggest that the access of information from a feedback site to CRH/AVP neurons in the PVN is blocked by the lesion (Suemaru et al., 1995). Another study by Dallman involving colchicine-injected rats also showed increased basal corticosterone, but reduced corticosterone responses to restraint (Choi et al., 1996). It has been suggested that the increase in basal corticosterone is either due to additional ACTH
release, or a block in corticosteroid feedback (King et al., 1993). Further associations between the VMH and the HPA axis involve the presence of corticosteroid receptors in the VMH. It has been suggested that corticosteroid receptors present in the VMH play a critical role in the regulation of the PVN by the VMH (Suemaru et al., 1995). The role of corticosteroid receptors in the VMH is not understood, but Langley and York have reported that RU 486, a GR antagonist, prevents the development of obesity in fa/fa Zucker rats, thus strongly connecting GRs with the VMH and glucose homeostasis (Langley & York, 1999). Since the levels of GR mRNA in the VMH are elevated in juvenile guinea pigs that had been nutrient deprived as fetuses, there are possible implications for glucose homeostasis both in the juvenile and later on in the adult. Unfortunately, due to the fact that plasma samples were taken from non-fasted juveniles, it was not possible to accurately assess the levels of glucose and counterregulatory hormones in the juvenile. However, future studies in the catheterized adult, will allow for fasted plasma samples to be obtained.

The mechanisms behind this increase in GR mRNA in the VMH is not known; however, nutrient restriction has been shown to alter gene expression and responsiveness in the VMH, as well as other hypothalamic nuclei (Nishimura et al., 1996; Xu et al., 1999). Restricting food intake of rats to 4 h daily for four weeks abolishes the daily patterns of gene expression and availability of orexigenic peptides, such as NPY, GAL, leptin, and POMC, that are normally observed in freely feeding rats (Xu et al., 1999). Food deprivation for 48 h in adult male rats decreases the facilitatory response of VMH neurons to serotonin, further indicating the nutrient deprivation can have profound effects on hypothalamic function (Nishimura et al., 1996). Whether the effects observed in these
studies are maintained, or whether nutrient deprivation has long-term effects on hypothalamic function remains to be determined. However, these observations, as well as our own observed increases in VMH GR mRNA, do support the fact that nutrient deprivation can significantly affect the VMH.

It should be noted that in the present study our analysis is restricted to the level of corticosteroid receptor mRNA. Although, we have shown that immunoreactive (ir)-GR is present in the guinea pig (Liu and Matthews, unpublished observation), rat (Cintra & Bortolotti, 1992), and sheep brain (Matthews, 1997), we have not carried out the extensive studies required to correlate mRNA with ir-receptor. GR protein levels could be measured via immunohistochemistry or ligand binding studies. However, due to difficulties in quantifying immunohistochemical ligand, we were not able to perform this analysis. In addition, ligand binding studies were precluded, since they require the adrenalectomy of animals to remove endogenous ligand, a procedure that would prevent the analysis of normal HPA function in this model. Given these limitations, it is important to acknowledge that there may be some discrepancies between mRNA, ir-receptor, and active binding sites. Future studies are required to establish these important associations in the juvenile guinea pig.

We have previously shown that 48h of nutrient deprivation on gd50 causes IUGR and brain sparing (Lingas et al., 1999). The present study shows that this growth restriction is maintained until postnatal day 18. Juveniles that had been nutrient-deprived as fetuses had a decrease in body weight and an increase in brain to body weight ratio. Hence, offspring from nutrient-deprived mothers are unable to catch-up to controls. There are strong associations between low birth weight and adult hypertension in the
guinea pig (Persson & Jansson, 1992), rat (Gardner et al., 1998; Langley-Evans, 1997; Langley-Evans et al., 1996), and the human (Barker, 1996). The ability of nutrient-deprived animals to catch-up has also been suggested as a determining factor for hypertension and heart disease (Barker, 1994; Persson & Jansson, 1992). Therefore, it is highly likely that guinea pigs that were growth restricted due to nutrient deprivation will have elevated blood pressure as adults. Unfortunately, it was not possible to catheterize the young guinea pigs to enable measurement of blood pressure.

In conclusion, we have shown that short periods of acute nutrient deprivation during rapid brain growth significantly alters HPA function and increases GR mRNA levels in the VMH of the juvenile guinea pig. We have also shown that the decreases in GR mRNA that were previously reported in the fetus following nutrient deprivation (Lingas et al., 1999), no longer exist in the juvenile, but that growth restriction is still apparent by d18.
Table 4.1 The effect of nutrient deprivation (48h) at day 50 of gestation on juvenile (postnatal day 18) weights and organ to body weight ratios. Results are expressed as mean±SEM. * (p<0.05) indicates statistical difference from control.

<table>
<thead>
<tr>
<th></th>
<th>Weight (g)</th>
<th>Organ/bodyweight x100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Deprived</td>
</tr>
<tr>
<td>Body</td>
<td>247.2 +/- 22.0</td>
<td>192.9 +/- 7.9*</td>
</tr>
<tr>
<td>Brain</td>
<td>3.31 +/- 0.05</td>
<td>3.12 +/- 0.06</td>
</tr>
<tr>
<td>Adrenal (x10)</td>
<td>0.49 +/- 0.02</td>
<td>0.41 +/- 0.02</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.22 +/- 0.11</td>
<td>1.02 +/- 0.04</td>
</tr>
<tr>
<td>Thyroid (x10)</td>
<td>0.29 +/- 0.04</td>
<td>0.25 +/- 0.02</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.77 +/- 0.09</td>
<td>0.74 +/- 0.04</td>
</tr>
<tr>
<td>Liver (/10)</td>
<td>1.02 +/- 0.11</td>
<td>0.75 +/- 0.00</td>
</tr>
<tr>
<td>Lung</td>
<td>1.86 +/- 0.11</td>
<td>1.50 +/- 0.06</td>
</tr>
<tr>
<td>Heart</td>
<td>0.97 +/- 0.09</td>
<td>0.76 +/- 0.03</td>
</tr>
<tr>
<td>Gonad</td>
<td>0.03 +/- 0.01</td>
<td>0.02 +/- 0.00</td>
</tr>
</tbody>
</table>
Table 4.2 The effect of nutrient deprivation (48h) at day 50 of gestation on mineralocorticoid receptor (MR) mRNA in the limbic system of juvenile guinea pigs. Results are expressed as mean±SEM relative optical density (ROD).

<table>
<thead>
<tr>
<th>MR mRNA</th>
<th>(ROD)</th>
<th>Region</th>
<th>Control (n=12)</th>
<th>Deprived (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hippocampus:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA1/2</td>
<td>13.3 +/- 1.4</td>
<td>12.4 +/- 1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA3</td>
<td>9.7 +/- 0.8</td>
<td>8.2 +/- 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA4</td>
<td>3.1 +/- 0.2</td>
<td>2.8 +/- 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D. Gyrus</td>
<td>12.8 +/- 1.1</td>
<td>12.1 +/- 1.8</td>
</tr>
</tbody>
</table>
Table 4.3. The effect of nutrient deprivation (48h) at day 50 of gestation on glucocorticoid receptor (GR) mRNA in the limbic system of juvenile guinea pigs. Results are expressed as mean±SEM relative optical density (ROD).

<table>
<thead>
<tr>
<th>Region</th>
<th>Control (n=12)</th>
<th>Deprived (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocampus:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA1/2</td>
<td>5.4 +/- 0.4</td>
<td>5.4 +/- 0.6</td>
</tr>
<tr>
<td>CA3</td>
<td>1.5 +/- 0.1</td>
<td>1.7 +/- 0.2</td>
</tr>
<tr>
<td>CA4</td>
<td>1.1 +/- 0.1</td>
<td>1.2 +/- 0.1</td>
</tr>
<tr>
<td>D. Gyrus</td>
<td>2.4 +/- 0.2</td>
<td>2.3 +/- 0.3</td>
</tr>
</tbody>
</table>
Table 4.4 The effect of nutrient deprivation (48h) at day 50 of gestation on juvenile (pnd18) pituitary proopiomelanocortin (POMC) mRNA and glucocorticoid receptor (GR) mRNA levels. Results are expressed as mean±SEM relative optical density (ROD).

<table>
<thead>
<tr>
<th></th>
<th>Control (n=12)</th>
<th>Deprived (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>POMC mRNA (ROD):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pars intermedia</td>
<td>92.1 +/- 13.0</td>
<td>91.2 +/- 12.6</td>
</tr>
<tr>
<td>Pars distalis:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4.2 +/- 0.3</td>
<td>4.8 +/- 0.3</td>
</tr>
<tr>
<td>Superior</td>
<td>2.9 +/- 0.2</td>
<td>3.5 +/- 0.4</td>
</tr>
<tr>
<td>Inferior</td>
<td>5.2 +/- 0.4</td>
<td>5.6 +/- 0.3</td>
</tr>
<tr>
<td><strong>GR mRNA (ROD):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pars distalis</td>
<td>11.2 +/- 0.5</td>
<td>11.3 +/- 0.4</td>
</tr>
</tbody>
</table>
Table 4.5 The effect of nutrient deprivation (48h) at day 50 of gestation on juvenile (pnd18) corticotrophin releasing hormone (CRH) mRNA levels. Results are expressed as mean±SEM relative optical density (ROD).

<table>
<thead>
<tr>
<th>CRH mRNA (ROD)</th>
<th>Control (n=11)</th>
<th>Deprived (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>8.1 +/- 0.4</td>
<td>8.8 +/- 0.8</td>
</tr>
<tr>
<td>Paraventricular nucleus:</td>
<td>8.9 +/- 0.7</td>
<td>8.5 +/- 0.7</td>
</tr>
<tr>
<td>Total</td>
<td>19.0 +/- 1.5</td>
<td>17.0 +/- 1.3</td>
</tr>
<tr>
<td>Parvocellular</td>
<td>19.0 +/- 1.5</td>
<td>17.0 +/- 1.3</td>
</tr>
</tbody>
</table>
**Figure 4.1:** A) Localization of pro-opiomelanocortin (POMC) mRNA in a coronal pituitary section after *in situ* hybridization, using an $^{35}$S-labelled antisense probe specific for POMC mRNA. Digitized image of autoradiographic film showing expression of POMC mRNA in the pars intermedia (PI), and superior (PDs) and inferior (PDi) regions of the pars distalis. B) Digitized image of an adjacent pituitary section that had been incubated with a sense oligonucleotide probe. Scale bar: 0.5mm. C) Image of an autoradiographic film demonstrating a single 1.3Kb transcript following Northern blot analysis using $^{32}$P-labelled antisense POMC probe and 30µg pf pituitary total RNA. Exposure time: A&B) 48h, C) 4h.
**Figure 4.2:** Guinea pig A) birth weight in grams and B) gestation length in days following either control (open bars) or 48h of nutrient deprivation (solid bars) on day 50 of gestation. Results are expressed as mean±SEM. ** (p<0.0001) indicates statistical difference from control.
Figure 4.2

(a) Birth weight (grams)

(b) Gestation length (days)

control deprived

control deprived
Figure 4.3: A) Plasma adrenocorticotrophin (ACTH) and B) cortisol concentrations in control juvenile offspring (n=13; open bars) and in young guinea pigs whose mothers were nutrient deprived for 48h (n=17; solid bars) on day 50 of gestation. Results are expressed as mean±SEM. * (p<0.05) indicates statistical difference from control.
Figure 4.4: Digitized image showing the localization and relative levels of glucocorticoid receptor (GR) mRNA in the VMH after in situ hybridization in A) control juveniles and B) juveniles whose mothers were nutrient deprived during gestation. Arrows indicate location of the VMH. Exposure time: 21 days. Scale bar: 0.5mm.
Figure 4.5: Densitometric analysis of glucocorticoid receptor (GR) mRNA in the hypothalamic paraventricular nucleus (PVN), ventromedial hypothalamus (VMH) and thalamus in control juveniles (n=12; open bars) and after 48h of nutrient deprivation (n=12; solid bars) on day 50 of gestation. Results are expressed as mean±SEM relative optical density (ROD). * (p<0.05) indicates statistical difference from control.
Figure 4.5
Figure 4.6: Localization of corticotrophin-releasing hormone (CRH) mRNA in coronal brain sections after in situ hybridization. Digitized images of autoradiographic film showing expression of CRH mRNA in the paraventricular nucleus (PVN) of the hypothalamus. Exposure time: 8 weeks. Scale bar:
CHAPTER 5: ADULT STUDY

5.1 INTRODUCTION

Studies in both the human and animals have suggested that changes in the \textit{in utero} environment can program physiological functions, such as hormonal regulation, and alter their activity in adult life (Barker, 1995; Seckl, 1997). Animal studies have shown that the hypothalamo-pituitary-adrenal (HPA) axis is vulnerable to manipulation during fetal life, and that its regulation can be permanently altered. In the rat, stress, glucocorticoid treatment, or a protein restricted diet during the prenatal period all result in adult offspring with hyperactive HPA function (Langley-Evans \textit{et al.}, 1996; Levitt \textit{et al.}, 1996; Takahashi \textit{et al.}, 1988). Studies in our lab have also shown that prenatal glucocorticoid treatment or nutrient restriction in the guinea pig alters HPA function in pre-pubertal juvenile offspring (Dean \textit{et al.}, 2000)(previous chapter)(Lingas \textit{et al.}, 2000). Altered HPA function may be implicated in the development of adult diseases such as hypertension and non-insulin dependent diabetes mellitus (NIDDM), since in the rat, prenatal glucocorticoids (Benediktsson \textit{et al.}, 1993) and protein restriction (Langley-Evans, 1997; Langley-Evans \& Jackson, 1994) also result in offspring with elevated blood pressure and insulin resistance.

We have selected the guinea pig as our animal model, since it has many similarities to the human in terms of development. Unlike the rat, the human and guinea pig give birth to mature young, and most neurological and neuroendocrine development occurs prenatally (Dobbing \& Sands, 1970; Dobbing \& Sands, 1979; Jones \& Roebuck, 1980; Matthews, 1998). Rapid brain growth, which occurs at approximately day 50 in the guinea pig (Matthews, 1998) is associated with rapid neuroendocrine development.
and is a period of vulnerability to prenatal insults (Dobbing & Sands, 1979). This is also a time when GR and MR systems develop rapidly (Matthews, 1998). We have previously shown that nutrient restriction (48h) during the brain growth spurt causes IUGR in the fetal guinea pig, and reduces hippocampal and hypothalamic GR expression (Lingas et al., 1999). Cortisol levels are also increased in the fetus, and coincide with elevated maternal cortisol levels (Lingas et al., 1999). In the previous chapter, we showed that nutrient restriction has a long-term effect on HPA function, since juvenile offspring have reduced basal ACTH levels, and elevated GR expression in the ventromedial hypothalamus (VMH)(previous chapter). We now wish to examine whether the changes in HPA function observed in nutrient-restricted juvenile offspring are maintained after puberty in adult guinea pigs.

In this study we examined the long-term effects of acute nutrient restriction (48h) on HPA function in adult guinea pig offspring. Persson and Jansson have demonstrated that intrauterine artery ligation, a severe form of nutrient restriction, in the guinea pig results in adult offspring with elevated blood pressure (Persson & Jansson, 1992). However, to date there are no studies examining the long-term effect of nutrient restriction on adult HPA function. Specifically we wished to measure basal HPA function, and to examine whether activated HPA function was altered by nutrient restriction, by exposing adult offspring to a number of challenges, such as hypoglycemia (a potent stimulator of the HPA axis) and restraint (a mild HPA stimulant). An examination of the responsiveness of the adrenal gland and pituitary gland to ACTH and CRH challenge respectively was also performed. In addition, given the fact that cortisol is naturally higher in female guinea pigs compared to males (El Hani et al., 1980a; El
Hani et al., 1980b), and observed sex-differences in adult guinea pig offspring exposed to dexamethasone treatment during gestation (Liu & Matthews, 2000), we also examined whether sex-specific changes occur in the HPA axis. We hypothesize that acute nutrient restriction alters the basal and activated HPA function of adult offspring, as well as HPA regulation via changes in corticosteroid levels, in a sex-specific manner.

5.2 MATERIALS AND METHODS

5.2.1 Animals and treatments

Female guinea pigs were mated with males in our animal facility using a technique developed at the Nuffield Institute for Medical Research, Oxford, UK. (Elvidge, 1972). This method produces accurately time-dated pregnant guinea pigs. These studies were performed according to protocols approved by the Animal Care Committee at the University of Toronto, in accordance with the Canadian Council for Animal Care. Pregnant guinea pigs were deprived of all food for 48 hours on gd50 and 51 (n=12), or allowed to feed normally (control, n=8). Water was available ad libitum. On gd52, pregnant guinea pigs were all fed normally. Neonates were weighed at birth and remained with their mother until day 30 of postnatal life, when juvenile guinea pigs were weaned. At approximately postnatal day 65 (females at same stage of estrous cycle) adult guinea pig offspring were catheterized under general anaesthesia using ketamine (40mg/kg) and xylazine (4mg/kg). Surgery was carried out under aseptic conditions, and polyvinyl catheters were implanted into the jugular vein and the carotid artery. The catheters were exteriorized in the intrascapulary region. Local anaesthetic (lidocaine hydrochloride was administered as necessary). Following surgery, animals were treated with yohimbine (0.1mg/kg) to reverse the actions of xylazine, and a
jacket and attached spring were fitted to the guinea pig. The catheters were passed through the spring and attached to a swivel. Following 48h recovery, guinea pigs were exposed to five tests, in sequential: (1) insulin (5 units/kg) followed by 5g food at 240 min, (2) ACTH$_{1-24}$ (0.5 μg/kg), (3) intra-venous glucose tolerance test (500 mg/kg glucose), (4) 30 min restraint stress, (5) CRH (0.5 μg/kg). ACTH$_{1-24}$ and hCRH were purchased from peninsula Laboratories. Glucose during the hypoglycemia test and IVGTT was measured using a One Touch blood glucose meter (Lifescan, Milpitas, California, USA). Tests 1 and 3 were commenced between 9.00 and 14.00 h following an overnight fast and blood samples (200μl) were taken at -30, 0, 5, 15, 30, 60, 90, 120, 240 , and 300 min for the insulin treatment and at -30, 0, 2, 5, 10, 15, 20, 30, and 60 for the IVGTT. Tests 2, 4, and 5 were commenced between 13.00 and 15.00 h and blood samples (150 μl) were taken at -30, 0, 5, 15, 30, 60, 90, and 120 min for all treatments. At least 2 days recovery was allowed between treatments. Following the completion of all tests, guinea pigs were euthanized. Adult body weight was recorded and the brain, pituitary, left adrenal, left kidney, pancreas, and heart were removed and weighed. Organs were frozen and stored at -80°C until processing. For female guinea pig offspring, it was ensured that surgery, catheterized tests, and tissue harvesting was performed at similar stages of the estrous cycle.

5.2.2 Plasma adrenocorticotrophin and cortisol: determination and data analysis.

Basal plasma adrenocorticotrophin (ACTH), cortisol, progesterone, and testosterone were measured by radioimmunoassay (RIA). Plasma samples from each catheterized test were measured in the same assay to prevent inter-assay variability. Analysis of basal ACTH and cortisol was performed using a two-way ANOVA (treatment x sex), and
analysis of progesterone and testosterone was performed using a one-way ANOVA (statistical significance was set at p<0.05). Analysis of cortisol responses to the various challenges was performed in consultation with the Statistical Consulting Service, University of Toronto. Statistical analysis was performed on SAS, and several statistical tests, including t-tests and regression analyses were used, to address several questions. 1) To determine differences in baseline cortisol levels between test and control groups, a t-test was performed on baseline ACTH and cortisol measurements. 2) To determine differences in the rate of hormonal increase, a t-test was performed on the slope of cortisol levels between time −30 and time 30 (peak). 3) To determine differences in the absolute difference from baseline to peak, a t-test was performed on the difference between time 30 and time −30. 4) To determine differences in the rate of hormonal decline, a t-test was performed on the slope of cortisol levels between time 30 and the end of the experiment. 5) To determine differences in the overall level of cortisol exposure, a t-test was performed on the area under each of the curves of hormone levels. Statistical significance was set at p<0.05.

Plasma progesterone levels were measured in female guinea pigs from samples taken throughout catheterized testing, to examine possible differences in the female estrous cycle. Low, medium, and high progesterone values were compared by one-way ANOVA. Overall plasma testosterone was measured in male offspring, and statistical analysis was performed using a one-way ANOVA.

5.2.3 In situ hybridization

The method for in situ hybridization has been described in previously in chapter 4.
5.2.4 *In situ* data analysis

For *in situ* hybridization analysis, brain sections were processed simultaneously to allow direct comparison between groups. The sections were exposed together with \(^{14}\)C-standards (American Radiochemical Company, MO, USA) to ensure analysis in the linear region of the autoradiographic film. The relative optical density of the signal on autoradiographic film was quantified, after subtraction of background values, using a computerized image analysis system (Imaging Research Inc., St. Catharines, Ontario, Canada) (Matthews, 1998; Matthews & Challis, 1995; Matthews, 1998). GR mRNA levels were measured in the hippocampus (CA1/2, CA3, and CA4 regions), dentate gyrus, thalamus, the hypothalamic paraventricular nucleus (PVN), and the ventromedial hypothalamus (VMH), as well as the pituitary pars distalis. MR mRNA levels were determined in the hippocampus (as for GR), and dentate gyrus. POMC mRNA levels were measured in the pars distalis (superior and inferior regions measured separately) and pars intermedia of the pituitary (Matthews et al., 1996). CRH mRNA levels were measured in the hypothalamic PVN. Data are presented as means±SEM and were statistically analyzed using a one-(treatment), two-(treatment × region) or three-(treatment × sex × region) way ANOVA followed by the Duncan's method of post-hoc comparison. Statistical significance was set at *p*<0.05.

5.3 RESULTS

Two-way ANOVA revealed no effect of treatment on body weight or organ weights, but did reveal a significant sex effect on body weight (*p*<0.001), brain weight
(p<0.05), and pituitary weight (p<0.05), with males having significantly higher body and organ weights compared with females (Table 5.1). There was also a significant sex effect in both systolic (p<0.005) and diastolic (p<0.05) blood pressure, but no effect of treatment in either sex (Table 5.2).

As there was a significant sex effect found by two-way ANOVA in basal cortisol (p<0.01) concentrations, the endocrine and molecular data were analysed separately for each sex. There was no effect of treatment on female basal plasma ACTH (post-hoc also did not reveal differences at 8:00 am, 1:00 pm, or 6:00 pm ) (Figure 5.1a), or cortisol, but post-hoc analysis did reveal a significant elevation in basal plasma cortisol levels of nutrient-restricted female offspring at 1:00 pm (p<0.05) (Figure 5.1b). Conversely, there was a significant treatment effect on male basal plasma ACTH (p<0.05) and post hoc analysis revealed a significantly reduction at both 1:00 pm (p<0.05) and 6:00 pm (p<0.005) (Figure 5.2a). Although, there was no effect of treatment on basal plasma cortisol, post-hoc analysis did reveal a significant reduction at 1:00pm (p<0.05) (Figure 5.2b) in offspring whose mothers had been nutrient restricted. During the hypoglycemia test, female cortisol levels were not different between control and nutrient-restricted animals in either basal levels, rate of increase or decrease, peak levels, or overall exposure to cortisol, though there was a very strong trend for an increase in female nutrient-restricted offspring (Figure 5.3a). Male basal cortisol was also not different between groups prior to the hypoglycemia test, but was fairly close to significance (p=0.06). Although the rate of increase or decrease, or the overall cortisol exposure was not different from control in males whose mothers had been nutrient-restricted, both peak cortisol (p<0.05), and the absolute peak (p<0.01), was significantly lower in nutrient-
restricted males (Figure 5.3b). The cortisol response to the ACTH challenge was not different between control females and females whose mothers had been nutrient restricted, in either basal levels, rates of increase or decrease, peak levels, or overall cortisol exposure, though there was a very strong trend for an increase in female nutrient-restricted offspring (Figure 5.4a). Male basal cortisol was significantly lower in nutrient-restricted offspring (p<0.05). There was no difference in rates of increase or decrease, peak levels, or overall cortisol exposure during the ACTH challenge, though there was a strong trend for a decrease in nutrient-restricted males (Figure 5.4b). During the restraint test there were no significant differences in either females (Figure 5.5a) or males (Figure 5.5b) in any of the parameters analyzed. Female basal plasma cortisol was increased just prior to the CRH challenge (p<0.05), but none of the other parameters examined were significant (Figure 5.6a). Male cortisol levels were not different between groups in any regard during the CRH challenge (Figure 5.6b). However, there was a strong trend for a decrease in nutrient-restricted males, and the p-values for male basal cortisol (p=0.07), as well as the rate of cortisol increase in females (p=0.09) and males (p=0.06), were fairly small, which may indicate a difference between groups if a larger sample were to be used.

ACTH concentrations in response to the CRH challenge and hypoglycemia test were performed and found to be highly variable, and showed no response to either challenge. During the CRH challenge plasma ACTH levels in control female offspring, 30 minutes before injection and 30 min after injection, were 2193±328ng/ml and 1563±21.5ng/ml respectively, and were 3069±140ng/ml and 2271±254ng/ml respectively in control male offspring.
Finally, fasting glucose levels in females whose mothers had been nutrient restricted were significantly lower than control, in the fasted state, just prior to the intravenous glucose tolerance test (p<0.05), but did not differ in the response to the IVGTT (Figure 5.7a). Male glucose levels were not different between groups at any point during the IVGTT (Figure 5.7b). Glucose levels were also not different between groups at any point during the hypoglycemia test in either sex (Figure 5.8).

Low, middle, and high female progesterone levels were compared and found to be not significantly different between control and nutrient-restricted offspring. In addition when progesterone levels were correlated with corresponding cortisol levels, once again no significant difference was found (Figure 5.9a). Male testosterone levels were also measured and were not significantly different between control and nutrient-restricted animals (Figure 5.9b).

*In situ* hybridization revealed that MR mRNA was expressed almost exclusively in the limbic system, with high levels in the CA1-2, CA3 subfields of the hippocampus, and the dentate gyrus (DG)(Figure 5.10a). Two-way ANOVA (treatment x region) revealed a significant effect of regional distribution in females (p<0.001) and males (p<0.00001). Post hoc analysis indicated that MR mRNA levels in the CA4 region were significantly lower than the CA1-2, CA3, or DG. There was also a significant treatment effect in both females and males, with female offspring of nutrient-restricted mothers having significantly lower MR mRNA levels than control (p<0.005)(Figure 5.11a), and male offspring having significantly higher MR mRNA levels (p<0.001)(Figure 5.11b). Post hoc analysis did not show any significant differences in individual regions.
GR mRNA was expressed widely throughout the brain, and at highest levels in the CA1-2, CA3, and CA4 subfields of the hippocampus, the dentate gyrus, and the hypothalamic paraventricular nucleus (PVN) (Figure 5.10b). Other brain regions that also expressed high levels of GR mRNA included the ventromedial hypothalamus (VMH), and thalamus. In addition, GR mRNA was also measured in the pituitary, and found to be expressed exclusively in the pars distalis. In the limbic system, two-way ANOVA (treatment x region) revealed a significant effect of regional distribution in females (p<0.00001) and males (p<0.00001). Post hoc analysis indicated that GR mRNA was highest in the CA1-2 region of the hippocampus and the dentate gyrus. There was no effect of treatment on limbic GR mRNA levels between offspring of nutrient-restricted mothers and control, in either females or males (Figure 5.12). One-way ANOVA (treatment) on GR mRNA in the PVN, VMH, and thalamus did not indicate any significant difference between groups in either females or males (Figure 5.13). However, one-way ANOVA of anterior pituitary GR mRNA levels did reveal a significant treatment effect (p<0.05) in females, with nutrient-restricted offspring having significantly reduced GR mRNA compared to control (Figure 5.13a). There was no treatment effect in male offspring (Figure 5.13b).

POMC mRNA was highly expressed in both the pars distalis and pars intermedia of the pituitary. In the pars distalis, POMC mRNA was higher in the inferior region compared with the superior region. There was no significant difference in pituitary POMC mRNA in females or males, in either the pars intermedia or the superior and inferior regions of the pars distalis (Table 5.3). CRH mRNA was highly expressed within the paraventricular nucleus of the hypothalamus, and to a lesser degree in surrounding
cortical areas. Despite a trend towards a decrease in female parvocellular CRH mRNA (p=0.07), there was no significant difference in either hypothalamic or cortical CRH mRNA in either sex (Table 5.4).

5.4 DISCUSSION

The present study demonstrates for the first time that 48h of maternal nutrient restriction during gestation has a long-term effect on the HPA function of adult guinea pig offspring, and that this effect is sex-specific. Adult offspring of nutrient-restricted mothers had caught-up to controls in terms of body and organ weights, and had similar blood pressures. Nutrient-restricted female offspring that had been chronically catheterized, had hyperactive basal pituitary-adrenal function, with significantly elevated cortisol levels, but were similar to control animals in their responses to stress. Conversely, male offspring had hypoactive basal pituitary-adrenal function, with significantly reduced ACTH and cortisol levels, and also had a decreased response to hypoglycemia. We have also shown a significant reduction in limbic MR mRNA and pituitary GR mRNA in female offspring of nutrient-restricted mothers, as well as a significant increase in limbic MR mRNA in nutrient-restricted male offspring. These results differ from those previously found in juvenile offspring of nutrient-restricted mothers (chapter 4).

We report that the previous reduction in body weight seen immediately following nutrient restriction in the fetus (Lingas et al., 1999) and in pre-pubertal juvenile offspring (pd18) (Lingas et al., 2000) is no longer present in adult offspring. Hence, nutrient-restricted offspring were able to catch-up in growth to control animals. A previous study
in the guinea pig showed that uterine artery ligation caused a continued reduction (17% decrease) in body weight well into adulthood (Persson & Jansson, 1992). However, uterine artery ligation is a very severe form of nutrient deprivation, and is chronic compared to an acute nutrient restriction of 48h. Hence, the degree of nutrient restriction, which differs between these two studies, may determine the extent of IUGR, and whether the reduced body weight is maintained. Persson and Jansson also reported a significant increase in blood pressure in adult offspring that had been nutrient deprived (Persson & Jansson, 1992). Several studies in the rat have shown that protein restriction during pregnancy produces hypertensive adult offspring (Langley-Evans, 1997; Langley-Evans & Jackson, 1994). In addition, dexamethasone treatment during the last week of pregnancy significantly increases blood pressure in adult rat offspring (Levitt et al., 1996). We do not report any significant differences in blood pressure in either sex. Once again, we performed an acute nutrient restriction (48h), compared to the chronic nutrient or protein restriction of the previous studies, and the five-day dexamethasone treatment. Therefore the fact that we did not observe differences in blood pressure may be due to the type of prenatal manipulation, as well as the degree of nutrient restriction. In addition, epidemiological studies suggest that hypertension resulting from IUGR is amplified as subjects age (Bradbury et al., 1997). Hence, since our experiments were performed on young adult guinea pigs, it is possible that differences in blood pressure may arise as the guinea pigs become older.

This is the first study to examine the long-term effects of in utero nutrient restriction on adult HPA regulation in any species. In the human, birth-weight has been shown to be negatively correlated with basal plasma cortisol levels in adult males.
(Phillips et al., 1998). In addition, our lab has performed a similar study to that described here, where guinea pigs were treated with dexamethasone in late gestation (Liu & Matthews, 2000), as has another study in the rat (Levitt et al., 1996). However, we are the first to show an effect of maternal nutrient restriction on adult HPA function in an animal model, and to examine the mechanisms behind these observed changes. Female offspring of nutrient-restricted mothers had significantly higher basal plasma cortisol levels compared with control, but had normal levels of basal plasma ACTH. The observed increase in cortisol, without a change in ACTH may be due to an increase in adrenal sensitivity. This would be consistent with our previous observations in juvenile offspring (Lingas et al., 1999), showing a decrease in ACTH with no change in cortisol, thus also suggesting an increase in the sensitivity of the adrenal gland. Alternatively, it is possible that differences in ACTH were not observed due to a lack of assay sensitivity. Levels of ACTH were found to be quite variable, and given that the assay was designed for human, not guinea pig, ACTH, it may be that only relatively large differences in ACTH would be detectable.

Conversely, male offspring of nutrient-restricted mothers had significantly reduced levels of both ACTH and cortisol. Therefore, in male offspring changes in adrenal and pituitary activity correlate closely. However, plasma cortisol concentrations in nutrient restricted offspring was only significantly decreased at 1:00pm, while ACTH was reduced at both 1:00pm and 6:00pm. Therefore, it is possible that a difference in adrenal sensitivity also exists in male offspring, and that this varies with the time of day. An examination of adrenocorticotropin receptor expression in the adrenal cortex may
help determine whether adrenal sensitivity is a factor in both male and female cortisol production.

Cortisol responses to the ACTH challenge, CRH challenge, and hypoglycemia in nutrient-restricted female offspring appear to be proportional to basal levels of cortisol, since the rate of increase and decrease, and the peak response is not significantly different between control and treatment groups. There also was no significant difference in cortisol levels between control and nutrient-restricted offspring during the restraint test. However, it should be noted that there was no apparent cortisol response to restraint stress in either control or nutrient-restricted offspring. We have previously observed that 30 minutes of restraint stress causes a mild activation of the HPA axis (Liu & Matthews, 2000). However, in this study very little response to restraint was observed. This may be due to differences in the handling of animals, since the animals in this study underwent several tests, and thus were handled more often than the animals in the previous study where only one or two tests were performed on each animal. In addition, it is possible that the restraint stress itself does not produce a significant response, but that handling, while placing each guinea pig in the restraint apparatus, elicits a stress response.

The similar responses to challenges observed between nutrient-restricted female offspring and controls is consistent with the Levitt study, which examined the adult offspring of dexamethasone treated mothers, and found the cortisol response to restraint stress to be identical between groups, despite differences in basal plasma cortisol (Levitt et al., 1996). Cortisol responses to the ACTH and CRH challenges in nutrient-restricted male offspring were also proportional to basal levels of cortisol. However, the peak response to hypoglycemia was significantly reduced in nutrient-restricted males. Of all
the tests performed, the hypoglycemia test is one of the more potent stimulators of HPA function. The difference in peak cortisol during this test suggests a reduced male response to hypoglycemia, which may have implications on glucose homeostasis, since glucocorticoids are known to influence counterregulatory hormones such as glucagon and insulin (Munck et al., 1984). Although, male glucose levels are not different between groups at any point during the hypoglycemia test, it is possible that levels of insulin and glucagon have been altered by maternal nutrient restriction or by the existing reduced circulating cortisol levels, despite normal glucose levels. Further investigation into the exact levels of counterregulatory hormones would be required to accurately determine potential alterations in glucose homeostasis. Interestingly, female basal glucose is significantly decreased in nutrient-restricted offspring, prior to the IVGTT. However, female glucose levels did not differ during the hypoglycemia test. The discrepancy between female fasted levels of glucose during the IVGTT and hypoglycemia, may result from differences caused by the estrous cycle, since the tests were performed at least four days apart. In the rat, glucose utilization in the brain and as a whole was measured by quantitative 2-[14C]deoxyglucose autoradiography, and was found to vary with the estrous cycle, with higher levels during proestrous and metestrous and lower levels during estrous and diestrous (Nehlig et al., 1985). Progesterone has also been shown to increase islet-cell proliferation in cyclic and pregnant rats (Nieuwenhuizen et al., 1998). Although progesterone did not differ between groups, changes in progesterone and estrogen levels during the estrous cycle may alter blood glucose levels, resulting in differences between control and nutrient deprived offspring becoming more pronounced.
at certain times during the estrous cycle. Unfortunately, there is no information regarding estrous cycle variations of glucose in the guinea pig.

Liu and Matthews have previously reported that the ACTH response to CRH injection and hypoglycemia is considerably smaller than the response observed in cortisol concentrations (Liu & Matthews, 1999). However, in the present study, ACTH responses to the CRH challenge and hypoglycemia test were highly variable, and showed no response to either challenge. It is possible that the nature of ACTH release in response to stress is different from other rodents, such that it is highly pulsatile and therefore difficult to quantitate. The nature of the response would need to be deciphered with repeated, rapid blood sampling to determine if the guinea pig ACTH response actually differs from other rodents. The lack of response to the CRH injection may also be due to the sensitivity of the assay, which although has been shown to measure guinea pig ACTH (Liu & Matthews, 1999), is nevertheless, an assay made for human plasma and not specifically for the guinea pig.

The observed differences in basal plasma cortisol levels could have profound effects on many aspects of physiological function, including altering metabolism, immune responses, and cardiovascular function. Elevated glucocorticoids have been linked with increased blood pressure both in humans and in animal models. In adult males, that also had reduced birth weight, cortisol levels were positively correlated with systolic blood pressure (Phillips et al., 1998). Also, in both a cross-sectional study and in men predisposed to high blood pressure, increased dermal glucocorticoid sensitivity was linked with hypertension, insulin resistance, and hyperglycemia (Walker et al., 1998). In pregnant sheep, cortisol infusion increased fetal mean arterial blood pressure (Dodic &
Wintour, 1994), and in the rat, fetal exposure to dexamethasone during the last week of gestation increased the blood pressure of adult offspring (Levitt et al., 1996). Although blood pressure was not different between groups during this study, it is possible that continued exposure to elevated cortisol in female offspring that had been nutrient-restricted may result in hypertension in later life. Furthermore, the differences in pituitary-adrenal function may lead to an increased incidence of coronary heart disease and diabetes. The fetal programming of hypertension, may occur via changes in HPA sensitivity (Dodic et al., 1998; Rosmond & Bjorntorp, 1998), altered activity and expression of glucocorticoid inducible enzymes and genes (Gardner et al., 1998), changes in vascular wall constituents and structural properties of vascular smooth muscle (Bendeck et al., 1994; Berry, 1978; Keeley & Johnson, 1987) and changes in densities of glucocorticoid receptors in the periphery (Gardner et al., 1998). In the adult, elevated glucocorticoid levels increase blood pressure by increasing sodium retention, cardiac output, peripheral resistance, and vascular reactivity (Kelly et al., 1998). Therefore, continued exposure to increased cortisol in female offspring and decreased cortisol in male offspring of nutrient-restricted mothers may lead to altered blood pressure in later life. Other physiological consequences of hyperactive or hypoactive HPA function have not been examined in detail in this study. However, an examination of the phenotype of nutrient-restricted offspring, such as body composition (i.e. fat accumulation), immune function, and cardiovascular function could provide greater insight into the physiological consequences of high or low cortisol in these animals.

Despite the observed differences in basal plasma ACTH and cortisol, there were no significant differences in POMC mRNA or CRH mRNA. In nutrient-restricted male
offspring, the reduction in circulating ACTH, with no change in POMC, likely results from a decrease in ACTH release, rather than a reduction in synthesis. As discussed in Chapter 4, CRH and AVP have been shown to increase ACTH secretion, without altering POMC mRNA (Castro, 1993), and ACTH is known to be stored in large quantities in the corticotrophs (Antoni, 1986). Therefore, changes in plasma ACTH need not reflect changes in ACTH synthesis or POMC mRNA. The lack of change in CRH mRNA, despite differences in pituitary function in nutrient-restricted males, may also not reflect changes in CRH release. CRH and AVP are stored in hypothalamic neurons until they are released into the hypophyseal portal system in a pulsatile fashion (Chrousos, 1998). Hence, changes in CRH release may occur without significant changes in CRH synthesis. Also, CRH mRNA may not reflect levels of immunoreactive protein. Although CRH mRNA has been shown to be closely related to ir-CRH in the sheep (Saoud & Wood, 1996), the guinea pig CRH gene has only recently been cloned in our lab (Go and Matthews, unpublished observation), hence we cannot conclude with certainty that CRH mRNA coincides with ir-CRH in this study. In addition, AVP plays a significant role in the regulation of ACTH release, and is thought to augment the ACTH response to CRH (Dallman et al., 1987). Therefore, AVP may be the hormone responsible for the observed decrease in male ACTH. At the present time our lab is in the process of cloning the AVP gene in order to investigate this further. In addition to the above mechanisms, changes in ACTH release may result from an increase in the pituitary response to CRH, independent of AVP. This may result from an increase in CRH receptor numbers at the level of the pituitary, or the involvement of other factors such as endogenous opioids (Buckingham, 1986), oxytocin, epinephrine, norepinephrine, and angiotension II, which
are known to be partially involved in ACTH release (Vale et al., 1983). Finally, considering that there is a trend for a decrease in female parvocellular CRH mRNA, it is possible that differences in CRH mRNA may become significant if a larger sample size is analyzed.

As previously described, glucocorticoid negative feedback occurs via GR and MR. We have previously shown that 48h of maternal nutrient restriction did not alter MR mRNA or GR mRNA levels in the key areas associated with glucocorticoid control in juvenile offspring, but did increase GR mRNA in the VMH (chapter 4). Although there were no longer elevations in brain GR mRNA in adult offspring, there was a significant decrease in pituitary GR mRNA in female offspring whose mothers had been nutrient-restricted during pregnancy. This would suggest a decrease in glucocorticoid feedback, and thus result in increased ACTH secretion. However, female basal and activated ACTH was not altered by nutrient restriction in this study. As previously mentioned it may be that the differences in ACTH were simply not detectable by the assay. Alternatively, other regulators of ACTH release such as CRH and AVP, as well as other hormones and cytokines (Chrousos, 1998) may be involved in normalizing female ACTH secretion.

There were also significant changes in limbic MR mRNA in both females and males, albeit in opposing directions. Several other studies in the rat, involving prenatal dexamethasone treatment (Levitt et al., 1996) or stress (Henry et al., 1994; Maccari et al., 1995) examined the long-term effects of prenatal manipulation on corticosteroid receptors. These studies also report changes in MR (decreased gene expression), but show concurrent attenuated GR gene expression. Therefore our results differ, in that only
limbic MR is affected, and MR is decreased in female offspring, and increased in male offspring. The MR in the limbic system is believed to be involved in basal HPA activity, while the GR is involved in activated HPA activity (Dallman et al., 1987). Reul and De Kloet reported that the MR is 90% occupied under normal basal circulating cortisol levels (Reul & De Kloet, 1985). Several receptor antagonist studies have demonstrated an inhibitory role for the MR in basal HPA function (Born et al., 1991; Dodt et al., 1993; Oitzl et al., 1995; Ratka et al., 1989). This coincides with our endocrine data, where basal pituitary-adrenal function was altered by nutrient restriction during gestation, but activated pituitary-adrenal function was not greatly different. Specifically, a reduction in MR mRNA in nutrient-restricted female offspring would decrease the inhibitory actions of the limbic system on the HPA axis, thus resulting in increased HPA activity, while an elevation of MR mRNA in male offspring would increase the inhibitory tone of the hippocampus on the HPA axis, and increase HPA function. The MR within the limbic system can alter HPA function by influencing the release of hypothalamic CRH (De Kloet et al., 1998) or it can change adrenocortical production directly via the splanchnic nerve, which innervates the adrenal medulla (Engeland W.C. & Gann, 1989).

It should be noted that in the present study our analysis is restricted to the level of corticosteroid receptor mRNA. Although, we have shown that immunoreactive (ir)-GR is present in the guinea pig (Liu and Matthews, unpublished observation), rat (Cintra & Bortolotti, 1992), and sheep brain (Matthews, 1997), we have not carried out the extensive studies required to correlate mRNA with ir-receptor. GR protein levels could be measured via immunohistochemistry or ligand binding studies. However, due to difficulties in quantifying immunohistochemical ligand, we were not able to perform this
analysis. In addition, ligand binding studies were precluded, since they require the adrenalectomy of animals to remove endogenous ligand, a procedure that would prevent the analysis of normal HPA function in this model. Given these limitations, it is important to acknowledge that there may be some discrepancies between mRNA, ir-receptor, and active binding sites. Future studies are required to establish these important associations in the adult guinea pig.

The mechanisms behind the observed changes in MR mRNA are not entirely understood, particularly since they vary between the sexes, and have changed from our previous reports in the fetus and juvenile. Considerably more information is known about GR regulation than MR. The mechanism behind the observed elevation of GR density following post-natal handling involves increased serotonin turnover (Meaney et al., 1994), and possibly the induction of specific transcription factors (Olsson et al., 1994; Olsson et al., 1995). Also levels of GR may be altered by glucocorticoids themselves (Burnstein et al., 1991). It is possible that the increase in plasma cortisol seen immediately following nutrient restriction in the fetal guinea pig (Lingas et al., 1999) had a direct effect on MR mRNA levels, since dexamethasone has been shown to increase MR expression in adrenalectomized rats (Reul et al., 1987). The GR may also mediate changes in MR expression, since MR and GR have been shown to colocalize in neurons of the limbic system (Matthews, 1998) and MR/GR heterodimers are believed to exist (De Kloet et al., 1998). However, both these mechanisms are unlikely given the changes in GRs and MRs from fetal life to adulthood, and the fact that cortisol would exert an immediate alteration of MR. Also, a direct effect of glucocorticoids on the MR without
any changes in GR seems unlikely. A great deal is yet to be learned about the regulation of the MR within the limbic system, and how the MR influences HPA function.

One observation regarding MR regulation, which helps to explain the profound sex effects in this study, is that estrogens lower hippocampal MR mRNA levels and binding capacity, and progesterone causes a decrease in MR binding affinity (Carey et al., 1995). Conversely, gonadectomy in female rats decreases GR mRNA levels, suggesting that estrogen increases limbic GR gene expression (Almeida et al., 1997). Therefore, estrogen appears to affect MR and GR gene expression in opposing directions.

The strong interaction between gonadal steroid hormones and HPA function is further supported by the fact that sex differences exist in basal cortisol levels and the HPA response to stress in a number of species, with females generally exhibiting higher cortisol levels than males. Human plasma cortisol concentrations are similar in healthy men and women, but males have an increased production and clearance of cortisol (Vierhapper et al., 1998). In the guinea pig, sexual dimorphism begins during puberty and results in an increased level of plasma cortisol and cortisol binding in females compared to males (El Hani et al., 1980a; El Hani et al., 1980b). In the rat, the female HPA response to physical and psychological stresses is greater than in males, with increased production of both corticosterone and ACTH (Handa et al., 1994). Basal plasma corticosterone and ACTH are also higher in females compared to males, but only at certain points during the estrous cycle (Atkinson & Waddell, 1997). Several studies have shown that basal and activated ACTH and cortisol increase progressively as estrogen levels rise from estrous to proestrus in the rat (Atkinson & Waddell, 1997; Raps et al., 1971; Viau & Meaney, 1991). This observation, as well as studies in both
humans and rats of both sexes, showing that estrogen administration increases HPA function, indicates that estrogen increases HPA function (Handa et al., 1994; Magiakou et al., 1997). Conversely, testosterone is believed to inhibit HPA activity, since castration of male guinea pigs, rats, and hamsters has been shown to increase ACTH and cortisol production (El Hani et al., 1980a; Handa et al., 1994; McCormick et al., 1998), as well as CRH, an effect that can be reversed by androgen administration (Handa et al., 1994; McCormick et al., 1998). Interestingly, castration of male rats also decreases GR and increases MR binding within the pituitary (McCormick et al., 1998), and estrogen alters the ability of glucocorticoids to feedback regulate and to autoregulate GR mRNA levels (Handa et al., 1994). Therefore, sex steroids potentially alter glucocorticoid feedback, thus providing a possible mechanism for their effects on the HPA axis.

The actions of sex steroids occur via androgen and estrogen receptors. In their connection to the HPA axis, androgen receptors have been found within the hippocampal CA1 pyramidal cells (Handa et al., 1994), and estrogen receptors have been seen in the parvocellular PVN (Simerly et al., 1990) and to a small extent in the hippocampus (O'Keefe & Handa, 1990). The recent cloning of a second estrogen receptor (ERβ) has led to more extensive studies in the rat brain. Of the areas relevant to HPA regulation in the rat brain, ERα was found in the CA1-3 pyramidal layer of the hippocampus, numerous amygdaloid nuclei, the lateral nucleus, the bed nucleus of the stria terminalis (BnST), and the paraventricular nucleus (Laflamme et al., 1998). The ERβ was generally found to be less abundant than the α form, but was highly localized within the limbic system, and also found in several amygdaloid nuclei, the lateral nucleus, the BNST, and the PVN and SON nuclei of the hypothalamus (Laflamme et al., 1998). Within the caudal
PVN, the large majority of the ERβ-positive cells were colocalized with CRH-ir perikarya. This observation, combined with the positive correlation between estrogen and CRH mRNA during the estrous cycle (Bohler et al., 1990), and the estrogen response elements demonstrated on the CRH gene (Vamvakopoulos & Chrousos, 1993), indicate that estrogen may be able to directly affect the genetic expression of CRH, and hence influence HPA function. Although ERα and ERβ are also expressed in the rat pituitary gland (intermediate and anterior lobes), and 35% of corticotrophs express ERα and 25% of corticotrophs express ERβ, as of yet there is little evidence for a direct action of estrogen on corticotroph function (Mitchner et al., 1998). In terms of sex differences in ER density, Laflamme observed very few differences in ERα distribution and intensity, and no differences in ERβ distribution and intensity between the sexes (Laflamme et al., 1998). However, several other studies in rats, mice, and guinea pigs have reported sex differences in the number of estrogen receptors in various brain areas, that are species-specific in terms of distribution (Brown et al., 1990; Brown et al., 1996). In the guinea pig, higher levels of ER were found in the female hypothalamus compared with males (Brown et al., 1996). Therefore, differences in ER density and distribution between the sexes may provide a possible mechanism for the sex-specific effects observed following several in utero manipulations such as prenatal stress (McCormick et al., 1995), dexamethasone treatment (Liu & Matthews, 2000), and nutrient restriction.

To examine whether sex steroids could be involved in the sex differences observed in pituitary-adrenal function and corticosteroid receptor levels, we measured male testosterone and female progesterone levels. We did not find significant differences in either male testosterone levels, or female progesterone levels throughout the cycle, of
offspring whose mothers had been nutrient restricted during gestation. This observation, in addition to the fact that female progesterone levels were not correlated with basal plasma cortisol levels suggests that testosterone and progesterone are not responsible for the observed sex differences in the HPA function of adult offspring. Female testosterone and estrogen levels in both sexes were not measured due to a limited quantity of plasma available. Given the strong associations between estrogen and testosterone and HPA function, it is possible that estrogen or female androgen levels in adult offspring could be facilitating sex-specific effects. In addition, the determination of sex steroid receptor mRNA levels and binding affinity within the guinea pig brains of treated and untreated offspring could provide information on whether sex steroids, even at normal levels could alter HPA activity via changes in receptors. Furthermore, it is possible that differences in sexual differentiation between the sexes during development would result in a greater sensitivity of one sex over the other to nutrient restriction. Sexual differentiation within the central nervous system (CNS) contains a critical period of sensitivity to manipulations, and the distribution and concentrations of estrogen receptors is not static, but rather change during development (MacLusky & Naftolin, 1981). Sex differences also exist in CNS morphology, such as differences in cellular or synaptic organelles, synaptic or dendritic organization, and gross volume of defined cell groups, which likely depend on gonadal hormone secretions (MacLusky & Naftolin, 1981). Therefore, depending on the state of sexual maturity within the CNS, differences in sex steroid levels, and receptor numbers and distribution, females and males may react differently to in utero manipulations. In the fetal guinea pig, plasma estrogen levels are similar between the sexes, but plasma testosterone is significantly higher in males compared to
females on day 50 of gestation (Buhl et al., 1979; Toyooka et al., 1991). It is possible that testosterone interacts with the developing limbic system and HPA axis in fetuses exposed to maternal nutrient restriction. Given the belief that testosterone inhibits and estrogen stimulates HPA function, as well as the presence of androgen and estrogen receptors within the hippocampus and PVN, it is also possible that sex steroids alter the response of the hypothalamus, pituitary, and adrenal to nutrient restriction, by altering gene expression of HPA hormones and receptors such as GR, MR, and ACTH receptors. In order to examine the exact mechanisms involved in our observed sex-differences in HPA function, an examination of fetal, juvenile, and adult sex differences would have to be performed.

The changes we have observed in the hippocampus may have significant implications on behaviour, since the hippocampus is associated with learning and memory, and corticosteroids affect cognition (for review see (Schmidt & Meyer, 1994)). Changes in hippocampal development have been shown to alter adult behaviour in both humans and animal models. In the human, male combat veterans with post-traumatic stress disorder had a reduction in MRI-derived right side hippocampal volume (Bremner et al., 1995), and women who had experienced childhood sexual abuse also had a reduction in hippocampal volume compared to control subjects (Stein et al., 1997). Also, in the rhesus monkey, fetal glucocorticoid exposure causes considerable hippocampal damage (Uno et al., 1994).

The direct effects of elevated glucocorticoid exposure during gestation (as seen immediately following nutrient restriction (Lingas et al., 1999) on behaviour, have been examined in both humans and animals. There are several human studies, synthetic
glucocorticoids are often given to pregnant women at risk of preterm labour to induce fetal lung maturation (NIH Consensus development conference, 1995). However, the long-term effects of glucocorticoids on human behaviour are somewhat difficult to interpret, since most studies were undertaken in children born preterm (MacArthur et al., 1981; MacArthur et al., 1982; Salokorpi et al., 1997; Smolders-de Hass et al., 1990). However, one study involving children at risk of congenital adrenal hyperplasia, who were not born preterm, showed significant increases in emotionality, unsociability, avoidance, and behaviour problems in children exposed to dex in early pregnancy (Trautman et al., 1995).

Glucocorticoid-exposure during pregnancy also alters behaviour in rodent models. Pregnant rats treated with corticosterone produced adult offspring with sex-specific alterations in spontaneous and apomorphine-induced motor activity (Diaz et al., 1997). Also in the rat, dexamethasone treatment during the last week of gestation altered the sexual behaviour of offspring, such that males became demasculinized (Holson et al., 1995). Similarly, in mice dex-treatment over the last week of gestation caused specific differences in the anxiety, memory, and socialization of offspring compared with control (Rayburn et al., 1997). Presently, there are no studies in the guinea pig examining the effects of prenatal glucocorticoid exposure, or prenatal manipulation in general, on the behaviour of offspring. We have not examined whether changes in behaviour and cognition occur in our model as a result of in utero nutrient restriction. However, we speculate that behavioral differences between control and nutrient-restricted animals may exist, and may become more pronounced in older guinea pigs, who have been exposed to lifelong changes in HPA function. In addition, given that alterations in behaviour, such
as increased anxiety, or changes in feeding behaviour may effect the development of the HPA axis in the neonate, it would be very useful to examine the behaviour of offspring in our model of nutrient restriction.

In conclusion, we have demonstrated that maternal nutrient restriction (48h) during fetal neuroendocrine development produces lasting effects on the HPA activity of offspring that last into adulthood and are sex-specific. Nutrient restriction essentially produces female offspring with hyperactive HPA function, and male offspring with hypoactive HPA function. We have also shown significant changes in the regulation of the HPA axis via differences in MR mRNA in the limbic system, and GR mRNA in the female pituitary, which correlate with the observed differences in HPA activity.
Table 5.1 The effect of nutrient deprivation (48h) at day 50 of gestation on adult weights in females and males. Results are expressed as mean±SEM. *(p<0.05)***(p<0.001) indicates statistical significance from opposite sex.

<table>
<thead>
<tr>
<th></th>
<th>Female Weight (g)</th>
<th>Male Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Deprived</td>
</tr>
<tr>
<td>Body</td>
<td>501.1 +/- 25.3***</td>
<td>502.0 +/- 15.1</td>
</tr>
<tr>
<td>Brain</td>
<td>3.57 +/- 0.10*</td>
<td>3.68 +/- 0.07</td>
</tr>
<tr>
<td>Pituitaryx100</td>
<td>1.35 +/- 0.10*</td>
<td>1.06 +/- 0.08</td>
</tr>
<tr>
<td>Adrenalx10</td>
<td>1.54 +/- 0.10</td>
<td>1.65 +/- 0.08</td>
</tr>
<tr>
<td>Heart</td>
<td>1.72 +/- 0.11</td>
<td>1.69 +/- 0.08</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1.44 +/- 0.25</td>
<td>1.16 +/- 0.08</td>
</tr>
<tr>
<td>Gonad</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5.2 The effect of nutrient deprivation (48h) at day 50 of gestation on adult systolic and diastolic blood pressure, and heart rate.*(p<0.05)**(p<0.01) indicates statistical significance from opposite sex.

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Deprived</td>
</tr>
<tr>
<td><strong>Systole</strong></td>
<td>52.7 +/- 2.6*</td>
<td>52.7 +/- 3.2</td>
</tr>
<tr>
<td><strong>Diastole</strong></td>
<td>30.6 +/- 1.12</td>
<td>30.3 +/- 2.7</td>
</tr>
<tr>
<td><strong>Heart Rate</strong></td>
<td>27.9 +/- 15.</td>
<td>35.5 +/- 6.7</td>
</tr>
</tbody>
</table>
Table 5.3 The effect of nutrient deprivation (48h) at day 50 of gestation on adult pituitary proopiomelanocortin (POMC) mRNA levels. Results are expressed as mean±SEM relative optical density (ROD).

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th></th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=7)</td>
<td>Deprived (n=8)</td>
<td>Control (n=8)</td>
</tr>
<tr>
<td>POMC mRNA (ROD):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pars intermedia</td>
<td>64.8 +/- 8.4</td>
<td>78.7 +/- 19.2</td>
<td>78.4 +/- 9.0</td>
</tr>
<tr>
<td>Pars distalis:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8.2 +/- 1.2</td>
<td>10.2 +/- 1.7</td>
<td>7.6 +/- 1.7</td>
</tr>
<tr>
<td>Superior</td>
<td>4.2 +/- 0.6</td>
<td>5.7 +/- 0.7</td>
<td>4.2 +/- 1.1</td>
</tr>
<tr>
<td>Inferior</td>
<td>11.3 +/- 1.6</td>
<td>13.5 +/- 2.1</td>
<td>10.9 +/- 2.6</td>
</tr>
</tbody>
</table>
Table 5.4 The effect of nutrient deprivation (48h) at day 50 of gestation on adult corticotrophin releasing hormone (CRH) mRNA levels. Results are expressed as mean±SEM relative optical density (ROD).

<table>
<thead>
<tr>
<th>CRH mRNA (ROD):</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=7)</td>
<td>Deprived (n=6)</td>
</tr>
<tr>
<td>Cortex</td>
<td>5.6 +/- 0.5</td>
<td>5.4 +/- 0.5</td>
</tr>
<tr>
<td>Paraventricular nucleus:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>10.5 +/- 1.5</td>
<td>8.4 +/- 1.0</td>
</tr>
<tr>
<td>Parvocellular</td>
<td>27.0 +/- 3.5</td>
<td>19.1 +/- 2.1</td>
</tr>
</tbody>
</table>
Figure 5.1: A) Basal plasma adrenocorticotropic (ACTH) and B) cortisol concentrations at 8:00 am, 1:00 pm, and 6:00 pm in control adult female offspring (n=7; open bars) and female offspring whose mothers were nutrient restricted for 48h (n=9; solid bars) on day 50 of gestation. Results are expressed as mean±SEM. * (p<0.05) indicates statistical difference from control.
Figure 5.1

(a) ACTH (pg/ml) at 8AM, 1PM, and 6PM.

(b) Cortisol (ng/ml) at 8AM, 1PM, and 6PM, with an asterisk indicating a significant difference.
**Figure 5.2:** A) Basal plasma adrenocorticotrophin (ACTH) and B) cortisol concentrations at 8:00 am, 1:00 pm, and 6:00 pm in control adult male offspring (n=10; open bars) and male offspring whose mothers were nutrient restricted for 48h (n=9; solid bars) on day 50 of gestation. Results are expressed as mean±SEM. * (p<0.05) **(p<0.005) indicates statistical difference from control.
Figure 5.3: A) Female and B) male plasma cortisol concentrations in control offspring (open bars) (female: n=6; male: n=9) and offspring whose mothers were nutrient restricted for 48h during gestation (solid bars)(female: n=11; male: n=8) in the fasted state, prior to and following intravenous injection of 5units/kg insulin. Results are expressed as mean±SEM. Solid arrow indicates time of treatment, open arrow indicates time of food (5g) administration. * (p<0.05) indicates statistical difference in peak cortisol from control. ** (p<0.01) indicates statistical difference in absolute peak (peak minus basal) cortisol from control.
Figure 5.4: A) Female and B) male plasma cortisol concentrations in control offspring (open bars) (female: n=6; male: n=9) and offspring whose mothers were nutrient restricted for 48h during gestation (solid bars)(female: n=5; male: n=6) prior to and following intravenous injection of 1μg/kg ACTH. Results are expressed as mean±SEM. Arrow indicates time of treatment. * (p<0.05) indicates statistical difference from control.

A) Female and B) male plasma cortisol concentrations in control offspring (open bars) (female: n=6; male: n=9) and offspring whose mothers were nutrient restricted for 48h during gestation (solid bars)(female: n=5; male: n=6) prior to and following intravenous injection of 1μg/kg CRH. Results are expressed as mean±SEM. Arrow indicates time of treatment. * (p<0.05) indicates statistical difference from control.
Figure 5.5: A) Female and B) male plasma cortisol concentrations in control offspring (open bars) (female: n=6; male: n=8) and offspring whose mothers were nutrient restricted for 48h during gestation (solid bars) (female: n=9; male: n=7) prior to and following 30 minutes of restraint. Results are expressed as mean±SEM. Arrow indicates time of beginning of 30 minute restraint.
Figure 5.6: A) Female and B) male plasma cortisol concentrations in control offspring (open bars) (female: n=6; male: n=9) and offspring whose mothers were nutrient restricted for 48h during gestation (solid bars) (female: n=5; male: n=6) prior to and following intravenous injection of 1μg/kg CRH. Results are expressed as mean±SEM. Arrow indicates time of treatment. * (p<0.05) indicates statistical difference from control.
**Figure 5.7:** A) Female and B) male blood glucose concentrations in control offspring (open bars) (female: n=6; male: n=10) and offspring whose mothers were nutrient restricted for 48h during gestation (solid bars) (female: n=9; male: n=7) in the fasted state, prior to and following intravenous injection of 500 mg/kg glucose. Results are expressed as mean±SEM. Arrow indicates time of treatment. * (p<0.05) indicates statistical difference from control.
Figure 5.7

(a) Glucose (mMOL/L)

(b) Glucose (mMOL/L)

T-30  T0  T2  T5  T10  T15  T20  T30  T60
Figure 5.8: A) Female and B) male blood glucose concentrations in control offspring (open bars) (female: n=6; male: n=9) and offspring whose mothers were nutrient restricted for 48h during gestation (solid bars)(female: n=10; male: n=8) in the fasted state, prior to and following intravenous injection of 5 units/kg insulin. Results are expressed as mean±SEM. Solid arrow indicates time of treatment, open arrow indicates time of food (5g) administration.
Figure 5.8

(a) Glucose (mMol/L) over time for different conditions labeled as T-30, T15, T30, T60, T120, T240, T300.

(b) Glucose (mMol/L) over time for different conditions labeled as T-30, T15, T30, T60, T120, T240, T300.
Figure 5.9: A) Female progesterone concentrations in control offspring (pink line; n=9) and offspring whose mothers were nutrient restricted during gestation (blue line; n=5) at three points during the estrous cycle. Low, middle, and high levels of plasma progesterone have been plotted on the x-axis, against corresponding basal plasma cortisol levels on the y-axis, to show any correlation (or lack of) between plasma progesterone and basal plasma cortisol.

B) Male testosterone concentrations in control offspring (open bars; n=8) and offspring whose mothers were nutrient restricted during gestation (solid bars; n=8).

Results are expressed as mean±SEM.
Figure 5.9

(a) Cortisol (ng/ml) vs. Progesterone (ng/ml)

(b) Testosterone (ng/ml) for CONTROL and DEPRIVED groups
Figure 5.10: Localization of A) mineralocorticoid receptor (MR) mRNA and B) glucocorticoid receptor (GR) mRNA in coronal brain sections after in situ hybridization. Digitized images of autoradiographic film showing expression of MR in the CA1 and CA3 regions of the hippocampus, and the dentate gyrus (DG), and expression of GR in the CA1 and CA3 regions of the hippocampus, the dentate gyrus (DG), and the hypothalamic paraventricular nucleus (PVN). Exposure time: MR 14 days, GR=4 weeks. Scale bar: 1.5mm
Figure 5.11: Densitometric analysis of A) female mineralocorticoid receptor (MR) mRNA and B) male MR mRNA in the limbic system (CA1-2, CA3, CA4 regions of the hippocampus and dentate gyrus (DG)) in control offspring (open bars)(female: n=7; male: n=8) and offspring whose mothers were nutrient restricted for 48h during gestation (solid bars)(female: n=8; male: n=8). Results are expressed as mean±SEM relative optical density (ROD). Two-way ANOVA (treatment x region) revealed a significant effect of regional distribution in females (p<0.001) and males (p<0.00001), and a significant treatment effect in both females (p<0.005) and males (p<0.001).
Figure 5.12: Densitometric analysis of A) female glucocorticoid receptor (GR) mRNA and B) male GR mRNA in the limbic system (CA1-2, CA3, CA4 regions of the hippocampus and dentate gyrus (DG)) in control offspring (open bars)(female: n=7; male: n=8) and offspring whose mothers were nutrient restricted for 48h during gestation (solid bars)(female: n=8; male: n=8). Results are expressed as mean±SEM relative optical density (ROD). Two-way ANOVA (treatment x region) revealed a significant effect of regional distribution in females (p<0.00001) and males (p<0.00001).
Figure 5.12

(a) ROD values for CA1-2, CA3, CA4, and DG regions.

(b) ROD values for CA1-2, CA3, CA4, and DG regions.
Figure 5.13: Densitometric analysis of glucocorticoid receptor (GR) mRNA in the hypothalamic paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), thalamus, and anterior pituitary in control offspring (open bars)(female: n=7; male: n=8) and offspring whose mothers were nutrient restricted for 48h during gestation (solid bars)(female: n=8; male: n=8). Results are expressed as mean±SEM relative optical density (Rod). * (p<0.05) indicates statistical difference from control.
Figure 5.13

(a) and (b) show the ROD (Relative Optical Density) for PVN, VMH, Thalamus, and Pituitary. The chart indicates a significant difference (*) in the Pituitary compared to the other regions.
CHAPTER 6: SUMMARY

Acute maternal nutrient restriction (48h) had a major impact on the HPA function of both juvenile and adult offspring, an effect that was associated with changes in glucocorticoid feedback via corticosteroid receptors. Juvenile offspring of nutrient-restricted mothers had significantly reduced plasma ACTH levels compared to control animals, but did not differ in plasma cortisol levels, suggesting a possible change in adrenal sensitivity. MR mRNA and GR mRNA levels in hippocampi, paraventricular nucleus, thalamus, and pituitary, as well as POMC mRNA in the pituitary and CRH mRNA in the PVN, were not different between control and nutrient-restricted offspring. However, GR mRNA levels in the ventromedial hypothalamus (VMH) were significantly higher in offspring from nutrient-restricted mothers, and therefore may have implications on glucose sensing and satiety.

Adult offspring of nutrient-restricted mothers had sex-specific alterations in pituitary adrenal activity. Adult female offspring of nutrient-restricted mothers had significantly increased basal plasma cortisol without significant changes in ACTH, while adult male offspring had significantly reduced levels of both basal plasma ACTH and cortisol. Cortisol responses to ACTH and CRH injection, as well as restraint stress were not different between groups in either sex. However, nutrient-restricted male offspring, but not female offspring, had a significantly reduced peak in cortisol in response to the hypoglycemia test. The increase in female HPA activity and decrease in male HPA activity observed in adult nutrient-restricted offspring results from changes in corticosteroid receptor levels within the brain and pituitary. Sex-differences were apparent in levels of corticosteroid receptor mRNA, with nutrient restriction resulting in
decreased MR mRNA in females and increased MR mRNA in males. GR mRNA was decreased in the pituitary of females, but not males, whose mothers had been nutrient restricted. In nutrient-restricted female offspring, a decrease in limbic MR mRNA and pituitary GR mRNA would result in reduced glucocorticoid feedback, and consequently an increase in adrenocortical activity. Conversely, in male offspring, an increase in limbic MR mRNA would result in increased glucocorticoid feedback, and consequently a decrease in adrenocortical activity. Therefore, changes in corticosteroid receptors correspond to changes in pituitary-adrenal function in both sexes. There were no significant differences found limbic or hypothalamic GR mRNA, in pituitary POMC mRNA, or hypothalamic CRH mRNA.

Differences in basal pituitary-adrenal activity between control and nutrient-restricted offspring change from juvenile to adult life. Specifically, changes in ACTH and cortisol become more pronounced in nutrient restricted adults, and sex differences emerge following puberty. This may reflect alterations in pituitary-adrenal function as the HPA axis matures, or may involve sex steroids, such as estrogen and testosterone, which become more influential following puberty. Plasma progesterone levels in adult female offspring, and plasma testosterone levels in adult male offspring were measured and found to be not different between groups. However, plasma estrogen levels, as well as the role of sex steroid receptors within the brain, has not been assessed, and may provide insight into the observed sex-differences arising from in utero nutrient restriction.

We conclude that the period of rapid brain growth spurt in the guinea pig is a time when the developing HPA axis can be permanently modified by an acute nutrient restriction. Maternal nutrient restriction results in changes in the adrenocortical function.
of offspring, which are apparent into adult life. Given the strong links that exist between IUGR, HPA function, and adult disease, we speculate that changes in HPA activity are strongly involved in the development of adult diseases, such as coronary heart disease, NIDDM, and hypertension.
MANUSCRIPTS

Manuscripts arising from this thesis include:

**Lingas, R.I., Matthews, S.G.** Nutrient deprivation during fetal life alters hypothalamo-pituitary-adrenal function in the juvenile guinea pig. (submitted: Experimental Physiology)

**Lingas, R.I., Matthews, S.G.** Maternal nutrient restriction during late gestation alters HPA function, and corticosteroid receptor numbers in adult guinea pig offspring. (to be submitted)
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