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FUNCTIONAL HETEROGENEITY OF THE CORTICOTROPH CELLS IN THE FETAL SHEEP PITUITARY

A thesis submitted for the degree of Doctor of Philosophy

to

The University of Adelaide

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Dedicated to Everyone's Patience,

My Own

&

Especially Cleo's

Character is like a tree and reputation like its shadow. The shadow is what we think of it; the tree is the real thing.

Abraham Lincoln

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DECLARATION

This body of scientific work contains no material that has been accepted for the award of any other degree or diploma in any other University or Tertiary Institution. To the best of my knowledge and understanding, this thesis contains no material previously published or written by any other person, except where due reference is made in the text.

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Timothy G. Butler

September, 2003

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The journey was well worth making.

PUBLICATIONS ARISING FROM THIS THESIS

BUTLER, T.G., SCHWARTZ, J. AND MCMILLEN, I.C. (1999) Functional heterogeneity of corticotrophs in the anterior pituitary of the sheep fetus. *The Journal of Physiology (London)*, **516**, 907-913.

BUTLER, T.G., SCHWARTZ, J. AND MCMILLEN, I.C. (2002) Differential effects of the early and late intrauterine environment on corticotrophic cell development. *The Journal of Clinical Investigation*, **110**, 783-791.

Submitted:

BUTLER, T.G., MCMILLEN, I.C., EDWARDS, L.J. AND SCHWARTZ, J. (2003) Chronic hypoglycaemia and the functional heterogeneity of corticotrophs in the fetal sheep pituitary. *Journal of Endocrinology*.

RELATED PUBLICATIONS

FORA, M.A., **BUTLER, T.G.**, ROSE, J.C. AND SCHWARTZ, J. (1996) Adrenocorticotropin secretion by fetal sheep anterior and intermediate lobe pituitary cells *in vitro*: Effects of gestation and adrenalectomy. *Endocrinology*, **137**, 3394-3400.

PHILLIPS, I.D., ANTHONY, R.V., **BUTLER, T.G.**, ROSS, J.T. AND MCMILLEN, I.C. (1997) Hepatic prolactin receptor gene expression increases in the fetus before birth and after cortisol infusion. *Endocrinology*, **138**, 1351-1354.

ADAMS, M.B., ROSS, J.T., **BUTLER, T.G.** AND MCMILLEN, I.C. (1999) Glucocorticoids decrease phenylethanolamine N-methyltransferase mRNA expression in the immature foetal sheep adrenal. *Journal of Neurochemistry*, **11**, 569-575.

EDWARDS, L.J., SYMONDS, M.E., WARNES, K.E., OWENS, J.A., **BUTLER, T.G.** JURISEVIC, A. AND MCMILLEN, I.C. (2001) Responses of the fetal pituitary-adrenal axis to acute and chronic hypoglycaemia during late gestation in the fetal sheep. *Endocrinology*, **142**, 1778-1785.

SUMMARY

It has been well established that the fetal corticotroph cells of the anterior pituitary synthesise and secrete adrenocorticotrophin (ACTH) during late gestation in response to stimulation by the hypothalamic secretagogues, corticotrophin releasing factor (CRH) and arginine vasopressin (AVP). There is evidence that the corticotroph cell population in the adult ovine anterior pituitary is comprised of subpopulations of corticotroph cells that have different functional characteristics. The aim of the series of experiments described in this thesis was to investigate the functional characteristics of the subpopulations of the corticotrophs in the fetal pituitary during normal development and after chronic intrauterine stress.

It was found that at 116 and at 140 – 145 days gestation, around 70% of the ACTH in the fetal anterior pituitary is stored within corticotrophs which are responsive to CRH alone (CRH-responsive cells). Intrafetal cortisol infusion resulted in a specific decrease in the amount of ACTH stored in the CRH-responsive corticotroph cells. The corticotroph cells responsive to AVP alone, were resistant to the negative actions of cortisol on ACTH synthesis. These findings presented in Chapter 2 suggest that two corticotrophic cell types exist in the sheep pituitary throughout late gestation, one which is responsive to CRH alone and one which is responsive to AVP alone. Given that circulating ACTH and cortisol concentrations increase in late gestation, the negative feedback effect of cortisol on ACTH synthesis in the CRH responsive cells must be counteracted by the stimulatory influence of the fetal hypothalamus, enabling the ACTH output to be maintained by the pituitary.

Surgical removal of the caruncles from the uterus of the ewe before conception, alters

the embryonic environment, resulting in the development of fetuses during late gestation that are either placentally restricted i.e. chronically hypoxaemic or fetuses that demonstrate compensatory placental growth i.e. normoxaemic. The results in Chapter 3 show that uterine caruncle ctomy resulted in the emergence of a population of non-corticotrophin-releasing hormone (non-CRH) target cells that secreted high amounts of adrenocorticotrophic hormone (ACTH) in the fetal pituitary. This change in corticotroph development was independent of late-gestation hypoxaemia. The presence of chronic hypoxaemia during late gestation in either the caruncle ctomised or non-caruncle ctomised uterine environments resulted in a reduction in the proportion of ACTH stored in CRH-target cells. These findings suggest that the early and late intrauterine environments program the development of specific corticotrophic cells types differentially in the fetal pituitary.

In Chapter 3 it was reported that, placental restriction in late gestation resulted in a decrease in the ACTH stored in CRH responsive corticotrophs. Placental restriction results in fetal hypoxaemia and hypoglycaemia and it is unclear whether the changes in the functional characteristics of the corticotrophs are related to either hypoxaemia or hypoglycaemia. The aim of Chapter 4 was therefore to determine whether fetal hypoglycaemia (defined as a mean plasma glucose concentration below 1.2 mmol l^{-1}), in the absence of fetal hypoxaemia, alters the ACTH synthetic or secretory capacity of the fetal corticotrophs. It was found that fetal hypoglycaemia had no effect on the ACTH synthetic and secretory characteristics of the fetal corticotrophs in late gestation. These observations suggest that the effects of placental restriction on the functional characteristics of the fetal corticotroph are probably due to the direct or indirect actions of fetal hypoxaemia and are not a result of the fetal hypoglycaemia.

In summary, this series of studies provide evidence that there are functionally distinct subpopulations of corticotroph cells within the fetal sheep pituitary and that these cells show plasticity in response to chronic intrauterine stress. These patterns of altered corticotroph development are important given the central role of the hypothalamo-pituitary-adrenal axis in the fetal adaptive response to intrauterine stress and in the early programming of adult disease.

COMMONLY USED ABBREVIATIONS

A B C

ACTH	adrenocorticotrophic hormone
$ACTH_{1-24}$	adrenocorticotrophic hormone *comprising only amino acids 1 to 24*
$ACTH_{1-39}$	adrenocorticotrophic hormone *comprising only amino acids 1 to 39*
ANOVA	analysis of variance
anti-ACTH antisera	anti-adrenocorticotrophic hormone antisera
AP	anterior pituitary
AVP	arginine vasopressin
cDNA	complementary deoxyribonucleic acid
Control	maintenance feeding regime
CR	carunclectomised fetal group
CRH	corticotrophin releasing hormone
C-TOX	[$Nle^{21,38}$, Arg^{36}] *r*CRH-gelonin conjugate
culture medium	DMEM/F12 (1:1) and serum

D E F G

d	day (s)
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
Euglycaemia	> 1.2 mmol l^{-1}
F12	Ham's F-12
GAR	goat anti rabbit serum
g	gravitational acceleration

H I J K L

h	hour(s)
h	human

hACTH$_{1\text{-}39}$	human adrenocorticotrophic hormone *comprising only amino acids 1 to 39*
$h\alpha$-LPH	human α-Lipotrophin
$h\alpha$-MSH	human α-Melanocyte stimulating hormone
Hb	arterial haemoglobin content
$h\beta$-EP	human β-Endorphin
$h\beta$-LPH	human β-Lipotrophin
$h\beta$-MSH	human β-Melanocyte stimulating hormone
HCl	hydrochloric acid *(hydrochloride)*
HDB	HEPES-dissociation buffer
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hg	mercury
HPA axis	hypothalamo-pituitary-adrenal axis
Hypoglycaemia	< 1.2 mmol l^{-1}
HX	hypoxaemic fetuses *(< 16 mmHg)*
^{125}I-Cortisol	iodinated-cortisol
^{125}I-hACTH$_{1\text{-}39}$	iodinated-human adrenocorticotrophic hormone *comprising only amino acids 1 to 39*
i.d.	internal diameter
I.M.	intra-muscularly
incubation medium	DMEM/F12 (1:1) and 0.2% polypep
irACTH	immuno reactive adrenocorticotrophic hormone
IU	international units
I.V.	intravenous
KCl	potassium chloride
KIU	kallikrein inhibitory units

M N O

mRNA	messenger ribonucleic acid
Milli Q H_2O	double distilled water
min	minute (s)
M	moles l^{-1}
n	number
NaCl	sodium chloride

NAD	nicotinamide adenine dinucleotide
NADH	reduced form of nicotinamide adenine dinucleotide
Na_2HPO_4	Sodium Phosphate dibasic anhydrous
NCR	non-carunclectomised fetal group
N-POMC	amino terminal of proopiomelanocortin
NRS	normal rabbit serum
NX	normoxaemic fetuses *(> 16 mmHg)*
o	ovine
*o*CRH	ovine corticotrophin releasing hormone
o.d.	outer diameter
140 - 145d	late gestation fetuses
116d + F	cortisol infused fetuses
116d + Sal	saline infused fetuses
O_2 content	arterial oxygen content

P Q R S

P_aCO_2	arterial partial pressure of carbon dioxide
P_aO_2	arterial partial pressure of oxygen
pH	acidity of arterial blood
POMC	proopiomelanocortin
r	rat
rRNA	ribosomal ribonucleic acid
S_aO_2	arterial oxygen saturation
S.E.M.	standard error of the mean
SPSSX	statistical package for social sciences on a vax mainframe computer

T U V W X Y Z

Tris-HCl	Tris[hydroxymethyl]-aminomethane hydrochloride
Undernutrition	restricted feeding regime

Mathematical Prefixes

k	kilo (10^3)
c	centi (10^{-1})
m	milli (10^{-3})
μ	micro (10^{-6})
n	nano (10^{-9})
p	pico (10^{-12})
f	fento (10^{-15})

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1 CORTICOTROPH CELLS IN THE FETUS

1.1 INTRODUCTION

It is well established in sheep that the normal timing of parturition and the maturation of key fetal organs including the lungs, kidneys and gut are dependent on both an increase in fetal plasma adrenocorticotrophin (ACTH) and cortisol concentrations occurring during the last 10 to 15 days of gestation (term = 147 ± 3 days gestation) (Challis *et al.*, 2000). Bilateral fetal adrenalectomy (Drost & Holm, 1968), fetal hypophysectomy (Liggins *et al.*, 1967; Liggins & Kennedy, 1968) or surgical disconnection of the hypothalamus from the pituitary at approximately 110 days gestation (Antolovich *et al.*, 1990) results in prolonged pregnancy. Whilst it is clear that the timing of parturition is dependent on a cascade of endocrine signals generated by an intact fetal hypothalamo-pituitary-adrenal (HPA) axis little is known about the relative roles of the hypothalamic secretagogues, arginine vasopressin (AVP) and corticotrophin releasing factor (CRH) in the stimulation of fetal ACTH synthesis and secretion during late gestation. There is evidence in the adult that pituitary corticotroph cells may be differentially responsive to CRH and AVP (Schwartz *et al.*, 1991b; Schwartz *et al.*, 1994). Furthermore the proportion of the corticotrophs in the adult pituitary which are responsive to CRH or AVP can vary depending on the presence or absence of acute or chronic stressors which stimulate the HPA axis (Schwartz *et al.*, 1994; van de Pavert *et al.*, 1997). There have been few studies, however, which have investigated whether there are changes in the functional characteristics of the pituitary corticotrophs during late gestation, or whether chronic intrauterine stress has an effect on the heterogeneity of the corticotroph population in the fetal pituitary. The studies outlined in this thesis provide evidence that there are functionally distinct subpopulations of corticotroph cells within the fetal sheep

pituitary and that there are changes in these subpopulations which may be of importance in the changes which occur in the fetal HPA axis, both before birth and in response to chronic intrauterine stress.

This literature review outlines the information available on the control of the synthesis and secretion of ACTH in the adult corticotroph and reviews the evidence that there are functionally distinct subpopulations of corticotrophs in the adult pituitary, which are differentially responsive to CRH and to AVP. The review then focuses on the development of the pituitary and the ACTH synthetic and secretory responses of the fetal pituitary during late gestation and during chronic intrauterine stress. The hypotheses and aims of the experimental studies described in this thesis are then discussed.

1.2 SYNTHESIS AND SECRETION OF ACTH IN THE ADULT

The pituitary gland or hypophysis (Hypophysis Cerebri), a small ovoid gland is located on the ventral surface of the brain, lying immediately below the base of the brain, to which it is linked by the pituitary stalk. The pituitary gland in most mammals occupies a depression on the upper surface on the sphenoid bone, the sella turcica (Dubois *et al.*, 1997). The gland is composed of three distinct parts or zones and a variety of anatomical designations have been applied to the pituitary gland (Table 1.1).

The ovine pituitary has a large anterior lobe, a small neural lobe, and a well developed intermediate lobe which is often separated from the anterior lobe by a cleft, considered to be the remnant of Rathke's pouch (Daniel & Prichard, 1957).

Table 1.1 Anatomical designations that have been applied to the pituitary gland

Anterior Lobe	Intermediate lobe	Neural Lobe
		(posterior lobe)
	Neurointermediate Lobe	
pars tuberalis	pars intermedia	pars nervosa
		(infundibular process)
pars distalis		infundibulum
		(neural stalk)
adenohypophysis		neurohypophysis

Each row indicates the primary anatomical names describing the three regions of the pituitary. Throughout the Thesis an effort has been made to use the same nomenclature consistently; with the anterior pituitary the primary focus of these studies.

1.2.1 Proopiomelanocortin (POMC)

In 1977 Mains (1977) and colleagues used a double-antibody immunoprecipitation technique and ^{3}H-labeled amino acids to demonstrate that the AtT-20 mouse pituitary tumor cell line contained a common 31 KDa glycoprotein precursor which was able to generate both endorphin and ACTH related peptides. Antisera raised against ACTH and β-lipotrophin recognized a 28.5 KDa protein synthesised in a cell free translation system from mRNA isolated from AtT-20 cells (Roberts & Herbert, 1977). Nakanishi and colleagues (1979) conclusively identified the nucleotide sequence of the bovine precursor protein with cloned complementary deoxyribonucleic acid (cDNA) and nucleotide sequence analysis. Initially termed corticotropin-β-lipotropin (Nakanishi *et al.*, 1979) the term, proopiomelanocortin (POMC), was quickly adopted to describe the three principal biological activities contained within the precursor protein sequence (Chrétien *et al.*, 1980).

The POMC gene is transcribed and POMC mRNA, associates with the membrane-bound polyribosomes on the rough endoplasmic reticulum (Roberts *et al.*, 1982). POMC mRNA is translated into the POMC precursor protein by the ribosomes, and the protein passes into the cisternae of the rough endoplasmic reticulum. Subsequent transfer into the Golgi apparatus allows the POMC precursor to be packaged into secretory granules. Post-translational processing results in cleavage of the precursor into the constituent active peptides (Roberts *et al.*, 1982). The post-translational processing has been shown to be different between the corticotrophs cells in the anterior lobe and the melanotroph cells of the intermediate lobe of the pituitary (Takahashi *et al.*, 1983).

The possible cleavage products from POMC in the anterior and intermediate lobe of the mammalian pituitary include, N-terminal proopiomelanocortin domain products (Pro-ACTH, Pro-γ-MSH, N-POMC), $ACTH_{1-39}$, β and γ-lipotrophin, β-endorphin, α, β and γ melanocyte-stimulating hormone, corticotropin-like intermediate lobe peptide and methionine-enkephalin (Figure 1.1: see reviews by Cohen *et al.*, 1980; Eipper & Mains, 1980; Whitfeld *et al.*, 1982; Seger & Bennet, 1986; Smith & Funder, 1988; Denef & Van Bael, 1998).

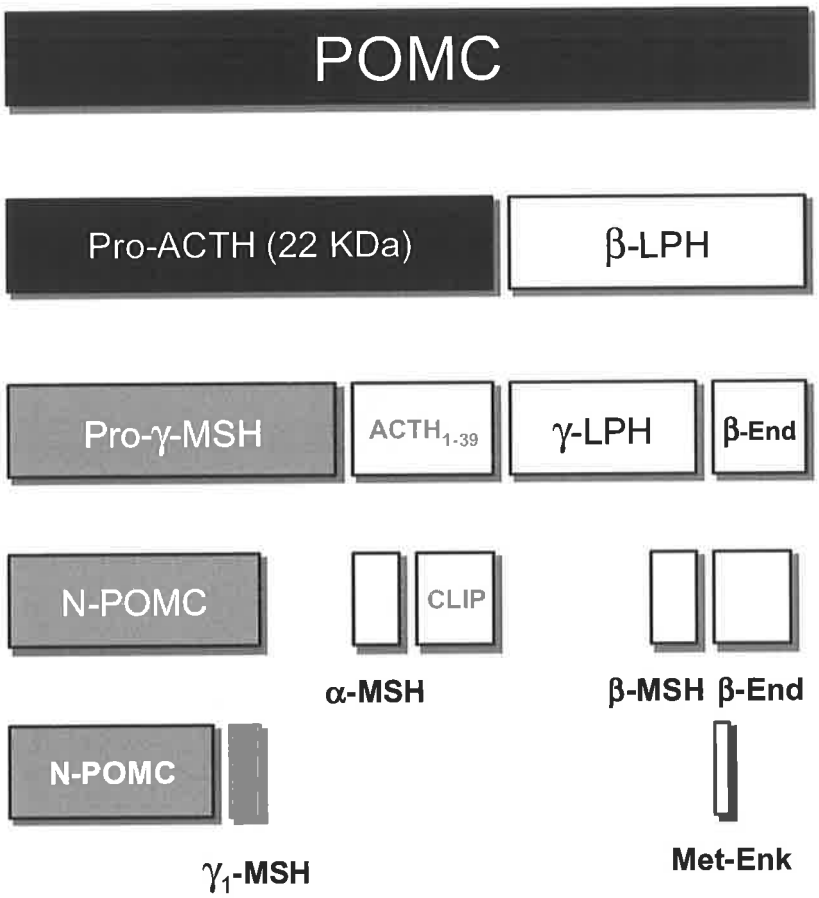


Figure 1.1 Precursor protein proopiomelanocortin.

Schematic representation of pro-opiomelanocortin. The products of post-translational processing include:

Pro-ACTH; Pro-Adrenocorticotropin
β-LPH; β-lipotrophin
Pro-γ-MSH; Pro-γ-melanocyte stimulating hormone
$ACTH_{1-39}$; Adrenocorticotropin
γ-LPH; γ-lipotrophin
β-End; β-endorphin
N-POMC; N-terminal proopiomelanocortin domain products
α-MSH; α-melanocyte stimulating hormone
CLIP; corticotropin-like intermediate lobe peptide
β-MSH; β-melanocyte-stimulating hormone
$\beta\text{-End}_{1-37}$; $\beta\text{-endorphin}_{1-37}$
γ-MSH; γ–melanocyte stimulating hormone
Met-Enk; methionine-enkephalin

1.2.2 Corticotropin Releasing Hormone (CRH)

Twenty five years after the demonstration that hypothalamic extracts stimulated ACTH secretion (Saffran & Schally, 1955, Guillemin, 1955 #206), Vale *et. al.* (1981) isolated a 41 amino acid peptide from ovine hypothalamic extracts, which specifically stimulated ACTH secretion in rat anterior pituitary cells in primary culture. The peptide has since been named corticotropin releasing hormone or CRH and is synthesised as a precursor peptide (prepro-CRH) of around 190 – 196 amino acids, depending on the species investigated (Furutani *et al.*, 1983; Shibahara *et al.*, 1983). CRH has been sequenced in a range of species, including the rat (Rivier *et al.*, 1983), pig (Patthy *et al.*, 1985) and human (Shibahara *et al.*, 1983). The CRH sequences of these species are highly conserved; the rat and human CRH sequences are identical and differ by only seven amino acids from the ovine (*o*)CRH sequence (Gillies & Grossman, 1985).

1.2.2.1 CRH Location

CRH has been localised using immunohistochemistry both in hypothalamic and extra-hypothalamic regions in the rat (Chappell *et al.*, 1986) and in the sheep brain (Kolodziejczyk *et al.*, 1983; Palkovits *et al.*, 1983). Using a combination of surgical lesioning, immunohistochemistry, and radioimmunoassays, Bruhn *et. al.* (1984) concluded that more than 90% of the CRH containing cells that project to the external lamina of the median eminence are derived from the parvicellular subdivisions of the hypothalamic paraventricular nucleus (PVN). These cells also express the mRNA that encodes the precursor for CRH (Young *et al.*, 1986). The hypothalamic PVN is a densely packed, wing-shaped nucleus that adjoins either side of the third ventricle and projects into the rostral region of the hypothalamus. The PVN is comprised of three functional regions; the medial parvicellular, intermediate mediocellular and the posterior magnocellular regions.

The magnocellular neurones predominantly project to the posterior pituitary, the mediocellular neurones project to the brainstem and spine whilst the parvicellular projections terminate in the external lamina of the median eminence (see Swanson *et al.*, 1986 for review). However, approximately 10% of the CRH projections to the external lamina of the median eminence are derived from the magnocellular neurons of the PVN, the supraoptic and the accessory magnocellular nuclei of the hypothalamus (see Makara *et al.*, 1984 for review). CRH synthesised in these neurons projecting to the external lamina, is transported through the axons and stored in the nerve terminals, once released into the primary capillary plexus, the CRH circulates in the sinuses of the anterior pituitary gland via the hypothalamic-hypophysial portal vessels. The levels of CRH detected in the rat and sheep hypophyseal portal blood is sufficient to stimulate ACTH secretion *in vivo* (Gibbs & Vale, 1982, Plotsky, 1984 #317; Engler *et al.*, 1989b).

1.2.2.2 CRH Receptors

CRH binding sites have been shown to be present in the rat (De Souza *et al.*, 1984b) and human pituitary (De Souza *et al.*, 1985), rat brain (De Souza *et al.*, 1984a; De Souza *et al.*, 1985), the primate adrenals (Udelsman *et al.*, 1986) and mouse spleen (Webster & De Souza, 1988). The CRH receptors belong to the family of Gs-protein-coupled receptors and are comprised of seven putative transmembrane helices (Perrin *et al.*, 1986; Chen *et al.*, 1993; Lovenberg *et al.*, 1995). Two distinct genes encode for CRH receptors (Chalmers *et al.*, 1996). The first receptor is designated as CRH_{R1} a 415 amino acid protein and was identified and cloned from several sources, including human ACTH-secreting pituitary adenoma (Chen *et al.*, 1993), AtT20 mouse pituitary tumour cells (Vita *et al.*, 1993), rat brain (Chang *et al.*, 1993; Perrin *et al.*, 1993) and human brain (Vita *et al.*, 1993). The second receptor designated CRH_{R2} has three distinct splice variants

($CRH_{2\alpha}$, $CRH_{2\beta}$ and $CRH_{2\gamma}$) encoding proteins of 411, 431 and 397 amino acids, respectively and was cloned from the rat brain (Lovenberg *et al.*, 1995), human (Liaw *et al.*, 1996; Sperle *et al.*, 1997) and mouse heart and skeletal muscle (Kishimoto *et al.*, 1995; Perrin *et al.*, 1995).

1.2.2.3 Cellular activity of CRH

Binding of CRH to the CRH_1 receptors (Engler *et al.*, 1999; Perrin & Vale, 1999 for reviews) activates the adenylate cyclase pathway (Labrie *et al.*, 1982; Aguilera *et al.*, 1983) through a mechanism requiring magnesium ions and ATP (Holmes *et al.*, 1984; Perrin *et al.*, 1986), which increases the intracellular level of cyclic 3',5'-adenosine monophosphate (cAMP), thereby enhancing the level of cAMP-dependent protein kinases (Labrie *et al.*, 1982; Aguilera *et al.*, 1983; Litvin *et al.*, 1984, Erlichman, 1984 #325, Miyazaki, 1984 #326). Activation of the intracellular pathway results in an increase in both POMC synthesis and ACTH secretion.

There is evidence to suggest that CRH stimulated cAMP activity results in an increased entry of extracellular calcium via voltage dependent calcium channels aiding in the release of ACTH (Luini *et al.*, 1985). The role of cAMP in the increase of intracellular calcium is controversial (Naccache *et al.*, 1979), and it has been proposed that arachidonic acid metabolites generated via the cyclooxygenase and/or lipoxygenase pathways are also involved in the calcium related ACTH secretion. Incubation of rat pituitary cells cultures with 100 μM arachidonic acid resulted in an approximate 16 fold increase in ACTH secretion (Abou-Samra *et al.*, 1986). This effect was partially blocked by inhibition of the lipoxygenase pathway, but enhanced by indomethacin, a cyclooxygenase inhibitor. The authors suggesting arachidonic acid is predominately metabolized to an ACTH stimulatory

metabolite through the lipoxygenase pathway, thus participating in the control of ACTH secretion.

Observations by Sobel (1986) using rat pituitary cells indicate that the calcium binding protein, calmodulin, is important in the secretion of ACTH, since calmodulin inhibitors, penfluridol and pimozide attenuated the CRH stimulated ACTH secretion. The importance of calmodulin in the response of the corticotroph cells to CRH was further indicated in a series of experiments by Won and Orth (1990) using perifused dispersed rat anterior pituitary cells in which extracellular and intracellular calcium was removed, calmodulin inhibited and blocking voltage sensitive calcium channels. Depletion of extracellular calcium and blocking the calcium channels resulted in a depressed ACTH response to both CRH and cAMP, supporting the notion that CRH acts principally through the adenylate cyclase pathway to synthesis POMC and secrete ACTH.

1.2.3 Arginine Vasopressin (AVP)

Du Vigneaud *et. al.* (1953) was the first to isolate and chemically characterise arginine vasopressin (AVP). AVP is a peptide that is comprised of 9 amino acids and is generated from a precursor form, preproAVP. This precursor is comprised of AVP at the N-terminal portion following the hydrophobic signal sequence, the carrier protein neurophysin, and the C-terminal glycopeptide (Land *et al.*, 1982; Schmale *et al.*, 1983; Ruppert *et al.*, 1984; Mohr *et al.*, 1985). PreproAVP and AVP are highly conserved between animal species, such as the rat, cow and human. In the rat and in man, however, AVP is a weak ACTH secretagogue, although it appears to be as potent as CRH in the bovine species and even more potent than CRH in the ovine species (Engler *et al.*, 1999).

1.2.3.1 AVP Location

The majority of vasopressinergic fibres in the hypothalamus originate in the magnocellular and supraoptic PVN, project through the pituitary stalk past the zona interna of the median eminence into the posterior pituitary (Silverman & Zimmerman, 1975; Zimmerman *et al.*, 1977). This pathway is mainly responsible for the neurosecretion of AVP into the circulation promoting its well-characterised renal and vascular actions. Vasopressinergic fibres also originate in the parvicellular region of the PVN and project to the zona externa of the median eminence and terminate at the portal capillary plexus (Szentágothai, 1964; Réthelyi & Halász, 1970; Zimmerman *et al.*, 1977). The concentrations of AVP released into the primary capillary plexus are sufficient to activate the secretion of ACTH from the pituitary corticotroph cells (Zimmerman *et al.*, 1973). Neurones in both the PVN and supraoptic nuclei have been shown to synthesise AVP (Rhodes *et al.*, 1981). Additional AVP neurons project to the areas of the central nervous system, including the brainstem and spinal cord (Sawchenko *et al.*, 1992; Raff, 1993).

1.2.3.2 AVP Binding Sites

Two main AVP receptor types have been described, the V_1, contained in the liver and on vascular smooth muscle cells and V_2, which mediates antidiuretic activity at the kidney (Altura & Altura, 1977; Michell *et al.*, 1979; Sawyer & Manning, 1982). The AVP receptors on pituitary corticotrophs show characteristics of both the V_1 and V_2 subtypes (Antoni, 1981; Gillies *et al.*, 1982; Antoni *et al.*, 1984; Baertschi & Friedli, 1985; Koch & Lutz-Bucher, 1985), and were later termed the V_{1b} receptor (Jard *et al.*, 1986).

1.2.3.3 Cellular Activity of AVP

AVP binds to the AVP receptors on the cell membrane of the corticotroph cells activating the phosphatidylinositol pathway (Berridge & Irvine, 1989; Nishizuka, 1992; Liu, 1996), stimulating phosphatidylinositol hydrolysis of the cell membrane, generating inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (Raymond *et al.*, 1985; Bilezikjian *et al.*, 1987a; Todd & Lightman, 1987). Diacylglycerol is required for the activation of protein kinase C a process enhanced by increases in intracellular calcium ions, which then causes the phosphorylation of a number of cellular substrates including the substrate protein, myristoylated alanine-rich C kinase (Bilezikjian *et al.*, 1987b; Carvallo & Aguilera, 1989; Oki *et al.*, 1990; Liu *et al.*, 1992, 1994b). Inositol 1,4,5-trisphosphate mobilizes internal calcium ions from both the mitochondria and the endoplasmic reticulum, and together with the influx of extracellular calcium ions, increases the intracellular calcium concentrations mediating the release of ACTH (see Leong, 1988 for review). The increase in internal calcium and protein kinase C each contribute to the stimulation of ACTH secretion by AVP.

1.2.4 Functional Heterogeneity of Corticotroph Cells

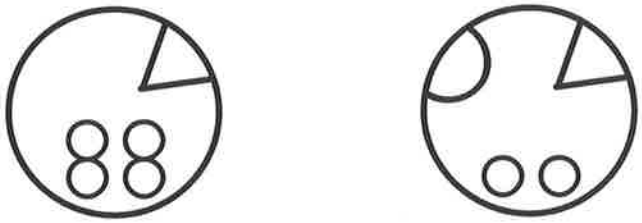
Several investigators have shown that there is functional heterogeneity of corticotrophic cells in the anterior pituitary in several species. Neill *et. al.* (1987) analysed the ACTH secretory responses of dissociated rat pituitary cells using the reverse haemolytic plaque assay and found that there were populations of pituitary cell which responded differentially to the actions of CRH and AVP. In these experiments, one population of corticotrophs secreted ACTH, in the absence of any secretagogue or in response to AVP, while a second population of more actively secreting cells was 'recruited' in response to CRH stimulation. These observations suggested the existence of two functional

subpopulations of corticotrophs, one of which was responsive to the stimulatory effects of CRH alone. Interestingly, ACTH secretion from this CRH responsive subpopulation of corticotrophs was preferentially inhibited by glucocorticoids (Neill *et al.*, 1987). Further studies using single and double reverse haemolytic plaque assays with antisera raised against ACTH resulted in the classification of corticotrophs on the basis of their secretory responses to various secretagogues. Using single haemolytic plaque assays, approximately 1% of dispersed rat pituitary cells formed plaques when exposed to medium alone, whereas 3% of the cells formed plaques after AVP stimulation and around 5% of the cells formed plaques in response to CRH stimulation. After administration of CRH and AVP in combination, 5 – 6% of the cells formed plaques. Studies with the double haemolytic plaque assay also showed that there were distinct groups of corticotrophs, those responding to CRH alone, those responding to either CRH or AVP and those responding only to CRH and AVP administered together (Jia *et al.*, 1991). The use of a cytotoxin (C-TOX) specific for CRH receptors has also been utilised in studies of corticotrophic heterogeneity. The toxic CRH-conjugate, is a synthetic hybrid molecule consisting of the CRH analogue, [$Nle^{21,38}$, Arg^{36}] rat (*r*)CRH conjugated with the cellular plant toxin gelonin. Anterior pituitary cells are treated with C-TOX prior to the treatment with hypothalamic secretagogues. Once the C-TOX is added to the culture medium, it binds to any corticotroph cells with CRH receptors and the toxic gelonin is internalised, disrupting the cells ribosomal activity. Normal ribosomal translation within the cell ceases and the cell then dies (Stirpe *et al.*, 1980; Blättler *et al.*, 1985; Schwartz *et al.*, 1987). Corticotroph cells that only respond to AVP are resistant to the effects of the C-TOX and are spared (Schwartz & Vale, 1988; Schwartz *et al.*, 1991b; Schwartz *et al.*, 1994). The efficiency of the cytotoxin treatment in eliminating CRH target cells is now well established and is usually demonstrated by the diminution of the ACTH secretory

response to CRH (Schwartz *et al.*, 1987; Schwartz *et al.*, 1991b). ACTH responses in adult ovine pituitary cells have been attributed to two different types of AVP responsive cells, those that respond to AVP (but not CRH) and those that respond to both CRH and AVP (Schwartz *et al.*, 1991b, see Figure 1.2).

Further investigations by Schwartz *et. al.* (1994) using ovine pituitary cells, and C-TOX in conjunction with immunoradiometric assays measuring either $ACTH_{1-39}$ or ACTH precursor peptides have supported the concept that these different types of pituitary corticotroph cells also exhibit functional heterogeneity, since they secrete different POMC cleavage products. Treatment of ovine pituitary cells with C-TOX had no significant effect on basal $ACTH_{1-39}$ secretion, but eliminated the $ACTH_{1-39}$ response to CRH and also significantly decreased the $ACTH_{1-39}$ response to AVP, in comparison to cells not treated with C-TOX (Schwartz *et al.*, 1994). Dexamethasone treatment (100 nmol l^{-1}) decreased the $ACTH_{1-39}$ secretory response to both CRH and AVP, in comparison to cells not treated with dexamethasone. Interestingly, even though the $ACTH_{1-39}$ secretion in response to AVP was significantly diminished by C-TOX treatment, the level of the $ACTH_{1-39}$ secretory response was the same in C-TOX and dexamethasone treated ovine pituitary cells. The secretion of ACTH precursors after dexamethasone treatment was significantly decreased in response to CRH but dexamethasone had no effect on basal ACTH precursor secretion or on the secretion of ACTH precursors in response to AVP, when compared to cells not treated with dexamethasone (Schwartz *et al.*, 1994). C-TOX treatment also had no effect on basal ACTH precursor secretion but it eliminated the ACTH precursor response to CRH and diminished the ACTH precursor release in response to AVP, in comparison to cells not treated with C-TOX. Interestingly dexamethasone significantly increased the secretion of ACTH precursors in response to AVP after C-TOX treatment.

These authors therefore concluded that the CRH responsive and CRH and AVP responsive corticotrophs primarily secrete $ACTH_{1-39}$ and are sensitive to glucocorticoid inhibition. In contrast the AVP responsive corticotrophs primarily secrete ACTH precursors and resist inhibition by glucocorticoids (Figure 1.2).



	AVP-only	**CRH/AVP dually responsive**
$ACTH_{1-39}$ secretion:	**Glucocorticoid-resistant**	**Glucocorticoid-sensitive (inhibition)**
ACTH precursor:	**Glucocorticoid-sensitive (stimulation)**	**Glucocorticoid-sensitive (inhibition)?**

Figure 1.2 Schematic summary of results concerning those subtypes of corticotrophs that respond to AVP.

The cell on the left responds to AVP, but not CRH; the cell on the right responds to CRH and AVP. Only the cell on the right would be eliminated by treatment with C-TOX (Schwartz *et al.*, 1994)

1.2.5 Cortisol

The cascade of hormonal events leading from the perception of stress to the metabolic responses of the body are moderated at several sites throughout the HPA axis by the final hormonal product, cortisol. Early studies showed increasing plasma concentrations of cortisol decreased the levels of ACTH in the blood thus inhibiting the stress response (Jones *et al.*, 1972; Sayers & Portanova, 1974). Investigators using various techniques including autoradiography with radiolabelled glucocorticoids, binding of radioactive

ligands to homogenised tissues, immunocytochemistry, the measurement of glucocorticoid receptor (GR) mRNA (for review see Jones & Gillham, 1988) and physiological experiments have demonstrated the presence of GR receptors in adenohypophyseal cells (Sayers & Portanova, 1974; Giguére *et al.*, 1982; Dupouy & Chatelain, 1984; Nicholson & Gillham, 1989), in areas of the hypothalamus (Dallman *et al.*, 1987; Jones & Gillham, 1988; Canny *et al.*, 1990) and in higher brain structures, such as the hippocampus, amygdala, lateral septum, and neuronal cell bodies in the motor nuclei of cranial and spinal nerves (Dallman *et al.*, 1987). These studies have provided the evidence at the cellular level that cortisol has the ability to act at several regions within the HPA axis. It has been demonstrated that cortisol acts in three specific time courses to inhibit ACTH release in the adult animal. These time courses are described as rapid (fast feedback), intermediate or delayed feedback or slow feedback.

Fast feedback of glucocorticoids, occurs within seconds or minutes after exposure to the steroid (Phillips & Tashjian, 1982; Jones & Gillham, 1988; Nicholson & Gillham, 1989). Fast negative feedback acts to inhibit stimulus-induced ACTH levels, but does not affect basal levels of ACTH (Roberts *et al.*, 1979; Phillips & Tashjian, 1982; Dallman *et al.*, 1987), suggesting that the fast negative – feedback only acts to decrease ACTH secretion but not synthesis. Nicholson and Gillham (1989) have shown that during fast feedback there is a reduced accumulation of cAMP and IP_3, concurrent with the reduced ACTH secretion, induced by CRH and AVP stimulation, respectively. Thus it appears that glucocorticoids acting in the fast time domain alter the second messenger responses to secretagogues by reducing the activity of these second messengers, thereby inhibiting ACTH release.

Intermediate or delayed feedback characteristically operates in a time frame 1 to 2 hours after glucocorticoid administration (Phillips & Tashjian, 1982; Dallman *et al.*, 1987; Jones & Gillham, 1988; Nicholson & Gillham, 1989; Canny *et al.*, 1990). The inhibition operates after glucocorticoids concentrations have returned to normal, but must decrease secretion of ACTH via inhibitors of the secretory rather than the synthesis process, since a reduction in cellular ACTH levels, due presumably to decreased POMC gene expression and translation, is only measured after a period of 6 hours or more (Roberts *et al.*, 1982). The inhibition in the intermediate or delayed feedback time frame is considered to be at a site distal to the second messenger, since the accumulation of cAMP and IP_3 was not attenuated by glucocorticoid treatment 90 minutes prior to stimulation by CRH and AVP, respectively, although ACTH release was inhibited (Nicholson & Gillham, 1989). The decrease in secretion of ACTH may be associated with an increase in lipocortins and annexins within the corticotrophs (Jones & Gillham, 1988). Lipocortin and annexin production increases within 30 to 60 minutes of glucocorticoid administration, and since lipocortin inhibits phospholipase A_2 activity, an enzyme involved in ACTH secretion, and annexins interfere with the process of ACTH exocytosis, the two peptides are prime candidates to disrupt ACTH secretion at a site distal to the second messengers.

Chronic treatment with glucocorticoids (6 or more hours) results in slow feedback, POMC mRNA transcription and translation are inhibited resulting in a subsequent fall in basal and stimulus induced ACTH concentrations (Roberts *et al.*, 1979; Roberts *et al.*, 1982). Glucocorticoids have also been shown to act at the hypothalamic level. The level of CRH in the parvicellular neurons of the hypothalamus is reduced after a 6 hour glucocorticoid treatment (Sakakura *et al.*, 1981) whilst dexamethasone implants near the PVN in rats also reduce the CRH content in the parvicellular neurons (Dallman *et al.*, 1987). This implies

that glucocorticoids may indirectly affect ACTH secretion and synthesis by decreasing the CRH stimulus to the corticotrophs (Sakakura *et al.*, 1981). Interestingly the hypothalamus may be integral in the inhibitory action of glucocorticoids on ACTH secretion since the levels of ACTH secretion remained depressed in hypothalamic-lesioned rats even after the removal of glucocorticoid feedback by adrenalectomy (Dallman *et al.*, 1985).

1.2.6 Acute and Chronic Stress

Engler and co-workers identified that the POMC peptides, ACTH, β-endorphin and α–melanocyte-stimulating hormone were secreted from the anterior pituitary in a pulsatile manner after measuring blood samples taken at 2 – and 10 – minute intervals from the external jugular vein, in conscious ovariectomised sheep (Engler *et al.*, 1989a). In a subsequent, elegant study the group increased the complexity of the protocol to determine if the pulsatile release of ACTH and the endorphin-related peptides coincided or mirrored the secretion of CRH and AVP by the hypothalamus. Paired blood samples were collected from the hypophysial portal and systemic circulations of five conscious ewes at 5 – 10 minute intervals over a 2 to 3 hour period. The ewes were then subjected to an audio–visual stress of a barking dog for 3 minutes and the effect monitored for 3 hours. A bolus dose of insulin (5 units kg^{-1}) was then administered intravenously and the induced hypoglycaemia monitored for an hour (Engler *et al.*, 1989b). The authors were able to conclude that the hypothalamus secretes CRH and AVP in a pulsatile manner and that audiovisual stimuli and insulin induced hypoglycaemia augment this secretion. They also noted that there was not a strict 1:1 relationship between the hypothalamic CRH/AVP release and the pituitary ACTH secretion during stress, which may be in part due to the hypothalamus secreting other ACTH releasing factors such as oxytocin. The basal hypophysial portal concentrations of AVP were always higher than that of CRH, however,

the secretion of CRH and AVP were each reliably increased by audiovisual stimuli and the insulin induced hypoglycaemia. Interestingly, the rise in AVP secretion was always greater than the rise in CRH resulting in an increased AVP/CRH ratio. Caraty *et. al.* (1990) also observed that insulin induced hypoglycaemia (0.2 IU kg^{-1}) in conscious, unrestrained, castrated rams resulted in an equal secretion of CRH and AVP into the hypophysial portal blood. At a higher concentration of insulin (2 IU kg^{-1}), CRH secretion was further stimulated, while AVP release was dramatically increased (see reviews by Engler, 1993; Engler *et al.*, 1999)

1.3 PITUITARY DEVELOPMENT

1.3.1 Embryological Origin

The classical view of the embryonic development of the pituitary gland has been that two tissue types form the gland. That the gland originates as two anlagens (Rathke, 1838) has not been disputed but there has been conjecture concerning the particular embryonic tissue forming the gland (Dubois *et al.*, 1997; Kawamura & Kikuyama, 1998). Historically, it has been assumed that the adenohypophysis (anterior and intermediate lobes) originate from one of the embryonic germ layers (extraneural part of the embryo); stomodeal ectoderm (Rathke, 1838), oral ectoderm (von Mihalkovics, 1874) or even the neural lobe (von Baer, 1828). Thus, the adenohypophysis develops as an upgrowth from the lining of the oral cavity (O´Rahilly, 1973; Svalander, 1974) with a funnel shaped extension developing from the roof of the upgrowth called the adenohypophyseal pouch or Rathke's pouch (Hamburger & Hamilton, 1951). Most of the mitotic activity is in the dorsal region of the pouch and accordingly the superior portion of the pouch undergoes considerable

modification. During this development, the connections with the oral cavity are lost and the pouch comes into close contact with the down growth of the neurohypophysis. The anterior wall of the pouch differentiates to form the pars distalis, while an upgrowth of this tissue surrounds the infundibular stem forming the pars tuberalis. The posterior wall of the pouch forms the intermediate lobe of the pituitary. In many species the hypophyseal cleft develops from the remnant of pouch's cavity (Wingstrand, 1966; see Dubois *et al.*, 1997 for a review).

The second anlage develops as a down growth from the floor of the diencephalon. This neural tissue forms not only the neurohypophysis but also forms the hypothalamus (Wingstrand, 1951, 1966). The down growth of neuroectodermal tissue forms a funnel like structure known as the infundibulum, the end of which eventually becomes the pars nervosa (Blake, 1984). As development continues the infundibulum and vascular tissue constitute the pituitary stalk, ultimately providing a neural connection between hypothalamus and the neural lobe (Wingstrand, 1966).

Whilst it is widely accepted that the neural lobe is derived from the neural ectoderm, the long held assertion that extraneural or stomatic cells form the anterior and intermediate lobe has recently been questioned, in part because it had never demonstrated in any species and the technical advances now allowed improved analysis (Dubois *et al.*, 1997; Kawamura & Kikuyama, 1998). Of the few investigators who had expressed their doubts about this view De Beer (1924) is the only investigator who insisted that the pituitary anlage arises before the appearance of the stomodeum in amphibia. Takor and Pearse (1975) demonstrated with avian embryos that the caudal portion of ventral neural ridge, the neuroectoderm gives rise to Rathke's pouch and hence to the adenohypophysis, leading

them to suggest that the hypothalmo-hypophysial complex should be regarded as a single rather than a composite entity. Several research groups using several species including quail-chick chimeras (Couly & Le Douarin, 1987), chick embryos (El Amraoui & Dubois, 1993) and even amphibia (Eagleson *et al.*, 1986; Kawamura & Kikuyama, 1992) have since indicated the adenohypophyseal tissue is precommitted from the anterior ridge of the neural plate. Researchers using gene-targeting experiments in mice, have also determined that the anterior pituitary in the mouse is precomitted to develop from the anterior midline region of the neural plate, the so called anterior neural ridge. The hypothalamus develops from the adjacent midline region of the neural plate. Interestingly, the future head ectoderm has the innate ability to develop into the anterior pituitary if experimentally brought into contact with the floor of the diencephalon (Treier & Rosenfeld, 1996). During normal development, the early phases of pituitary development do not occur if there is not direct contact between the overlying neural epithelium of the ventral diencephalon (the infundibulum) and Rathke's pouch (Treier & Rosenfeld, 1996).

Interestingly and perhaps not unexpectedly, Dasen and Rosenfeld (1999) have shown that the development of the anterior pituitary is also reliant on a repertoire of homeodomain transcription factors that interact in a signal dependent cascade, on the basis of their distinct expression patterns, the overlap of these expression patterns, and specific functional interactions. This signal dependent cascade or pathway ultimately leads to the development of five functionally distinct cell phenotypes of the anterior pituitary.

The five cell types distinguishable in the adenohypophysis include (Purves, 1966):

1. **Somatotrophs** – containing GH; growth hormone (somatotropin)
2. **Lactotrophs** – containing PRL; prolactin
3. **Gonadotrophs** – containing LH; luteinizing hormone & FSH; follicle stimulating hormone
4. **Thyrotrophs** – containing TSH; thyroid-stimulating hormone (thyrotropin)
5. **Corticotrophs** – containing $ACTH_{1\text{-}39}$; Adrenocorticotropin, LPH; lipotrophin & End; endorphin: MSH; melanocyte stimulating hormone

1.3.1.1 Morphological Heterogeneity of Pituitary Corticotrophs

Between 38 and 50 days gestation around 40% of the cells in fetal sheep pituitary contain immunoreactive ACTH (Mulvogue, 1984). The proportion of corticotroph cells decreases to 20-24% at 90-115 days gestation, 12-15% at 120 – 138 days gestation and to around 10% in the week preceding delivery (Mulvogue, 1984; Antolovich *et al.*, 1989; Antolovich, 1990; Antolovich *et al.*, 1991). The proportion of corticotrophs in the pituitary of the adult sheep (5%) is lower than the proportion present in fetal life (Mulvogue, 1984 #300). The observation that the proportion of corticotroph cells decreases with gestational age is supported by the immunocytochemical studies of Perez *et. al.* (1997). These authors found that the proportion of corticotrophs decreased from 22.0 ± 1.0% of all pituitary cells present in the fetal sheep pituitary at 100 days of gestation to 14.0 ± 1.0% at 135 days gestation. In this study the proportion of corticotrophs present in the adult sheep pituitary was around 9%.

There are two morphologically distinct types of corticotroph cells discernible by 87 days of gestation in the sheep pituitary (Perry *et al.*, 1985). The predominant corticotroph cell type between 105 and 125 days is large, lightly stained, and columnar (called the "fetal" type) whereas after 130 days, the predominant cell type is small, darkly stained and stellate (called the "adult" type). The "adult" type corticotroph cells in the fetal pituitary resemble those corticotroph cells observed in the adult sheep (Perry *et al.*, 1985; Mulvogue *et al.*, 1986; Antolovich *et al.*, 1989).

The impact of either fetal adrenalectomy or intrafetal infusion of cortisol on the development of corticotroph morphology in the fetal sheep pituitary has been investigated. After bilateral adrenalectomy at 120 days gestation the proportion of fetal (14.7 ± 0.3%) and adult (6.4 ± 0.3%) type corticotrophs in the anterior pituitary at 135 days gestation, was comparable with the proportion of fetal (14.9 ± 0.4%) and adult (5.7 ± 0.8%) type corticotrophs present in intact, control fetuses at 115 days gestation. After intrafetal cortisol infusion between 109 and 115 days gestation, the percentage of fetal (3. 8 ± 0.6%) and adult (12.4 ± 1.3%) type corticotrophs in the fetal pituitary at 115 days gestation was comparable to the percentage of fetal (2.3 ± 0.5%) and adult (11.9 ± 1.4%) type corticotrophs in non infused, control fetal sheep at 135 days gestation (Antolovich *et al.*, 1989). Furthermore in fetuses in which the fetal hypothalamus was surgically disconnected from the pituitary (HPD) at 108 – 112 days gestation, the proportion of fetal type corticotrophs (12.1 ± 1.0%) and adult type corticotrophs (3.7 ± 0.3%) in the pituitary at 135 days gestation (Antolovich *et al.*, 1991) was comparable to the proportions of these cell types in pituitaries of intact fetal sheep at 105 – 125 days gestation (Antolovich *et al.*, 1989; Antolovich *et al.*, 1992).

It is therefore clear that the morphology of the corticotrophic cell types within the fetal pituitary changes with increasing gestational age and that this ontogenetic profile is in part dependent on the fetal adrenal and fetal hypothalamus. Removal of the adrenals or fetal HPD appears to 'delay' the normal developmental pattern of the corticotroph cells. Exposure of the fetus to prepartum plasma cortisol concentrations during early gestation also causes an 'accelerated' development of the appearance of the adult type corticotroph as the predominant cell type. Thus cortisol and an intact hypothalamo-pituitary axis are each essential for the normal morphological development of the fetal corticotroph cells (Antolovich *et al.*, 1991, 1992). There has been considerable interest in whether these changes in the morphology of the corticotroph occur in parallel with changes in the functional characteristics of the corticotroph, such as their responsiveness to the hypothalamic secretagogues, which may in turn underlie the prepartum increase in ACTH secretion or the specific responses of the fetal corticotroph to chronic intrauterine stress. The ontogenic profile of the ACTH synthetic and secretory characteristics of the fetal pituitary are reviewed below, with a focus on the differential effects of CRH and AVP on fetal ACTH secretion during the periods in late gestation when there are changes in the morphology of the fetal corticotroph.

1.3.2 Hypothalamo-Pituitary axis in Fetal Sheep during Late Gestation

Whilst it has been well established that circulating immunoreactive ACTH (irACTH) levels increase during late gestation in the sheep fetus, controversy exists as to whether there is also a concomitant increase in POMC gene expression in the anterior pituitary in the sheep fetus. Some investigators have demonstrated that there is an increase in POMC mRNA levels in the fetal anterior pituitary during late gestation (Yang *et al.*, 1991; Myers *et al.*, 1993; Matthews *et al.*, 1994) while others have shown that there is a decrease in

POMC mRNA in late gestation (McMillen *et al.*, 1988; Brooks *et al.*, 1992; Mérei *et al.*, 1993). Yang *et. al.* (1991) observed that POMC mRNA could be detected as early as 60 days gestation and that POMC mRNA levels then increased progressively to term. Myers *et. al.* (1993) also found that POMC mRNA levels (normalised to the content of actin mRNA), increased between 105 – 107 days (14.1 ± 2.2) and 138 – 140 days (43.2 ± 6.0) gestation. Other researchers have used in situ hybridisation and radiolabelled POMC cDNA to determine the amount of POMC mRNA in the inferior portion of the pars distalis of the fetal sheep. They found that POMC mRNA levels in this zone were 2 fold greater between 145 – 147 than between 100 – 143 days gestation (Matthews *et al.*, 1994). Brooks *et. al.* (1992) however, found that POMC mRNA (expressed as a ratio of 18S RNA) increased to a maximum level at 130 days (ratio approximately 7.5) and then declined dramatically at 140 days gestation (ratio approximately 0.7). The prepartum decline observed by Brooks is further supported by Mérei *et. al.* (1993) who found that the ratio of POMC mRNA : 18S RNA was lower at 141 – 143 days (0.67 ± 0.07) than at 130 – 136 days (0.9 ± 0.08).

The disparity between these different studies may be due to differences in molecular techniques, variations in pituitary preparations, gestational age groupings and inclusion of animals in active labour in later gestational age groupings. There is however, a consensus that the steady state levels of pituitary POMC mRNA increase up until at least 135 days gestation in the fetal sheep. It may be, that as Levin suggested, steady state POMC mRNA is not the best indicator of ACTH synthesis in the sheep pituitary (Levin *et al.*, 1993) and that the synthesis of bioactive $ACTH_{1\text{-}39}$ in late gestation is more dependent on the translation of POMC mRNA and the subsequent cleavage of the POMC precursors to their active peptides and products.

1.3.2.1 Measurement of Immunoreactive and Bioactive ACTH in Fetal Plasma

Early studies found that there was no significant change in plasma immunoreactive (ir) ACTH concentrations, until after the start of the prepartum increase in plasma cortisol concentrations (Jones *et al.*, 1975; Rees *et al.*, 1975; Jones & Ritchie, 1977a). Later studies however showed that plasma irACTH concentrations increased earlier in gestation, from 110 to 121 days gestation, in the fetal sheep (Hennessy *et al.*, 1982b; Norman *et al.*, 1985). These variations in the gestational age profile of the plasma irACTH concentrations may be explained by investigators using different antisera which may recognise different molecular weight forms of ACTH in the fetal sheep plasma, which in turn may have different ontogenetic profiles.

Ozolins *et. al.* (1991) found that irACTH in fetal sheep plasma occurs in two main molecular weight ranges *i.e.* smaller or greater than 12K. The proportion of irACTH that eluted in the low molecular weight (LMW) range was significantly higher between 121 – 125 days (43.9 ± 4.2%) than between 126 – 139 days (26.8 ± 9.3%) and then increased to 29.9 ± 5.5% at 140 – 145 days gestation (Ozolins *et al.*, 1991). Jones and Roebuck (1980) reported that the ratio of 4.5K ACTH ($ACTH_{1-39}$): higher molecular weight forms of ACTH present in fetal sheep plasma also increased after 138 days gestation. These findings are further supported by Phillips *et. al.* (1996a) who measured the fetal plasma concentrations of $ACTH_{1-39}$ and of the ACTH precursors (POMC and pro-ACTH (the 22KDa N-terminal portion of POMC)) using specific immunoradiometric assays. The plasma concentrations of $ACTH_{1-39}$ increased between 120 – 125 days (1.5 ± 0.3 pmol l^{-1}) and 136 – 142 days (2.9 ± 0.3 pmol l^{-1}), whereas, the plasma concentrations of the ACTH precursors did not change between 120 – 125 days (20.8 ± 3.0 pmol l^{-1}) and 136 – 142 days (23.3 ± 5.0 pmol l^{-1}).

Interestingly, McMillen *et. al.* (1995) measured $ACTH_{1-39}$ and ACTH precursors secreted from slices of the anterior pituitary from fetal sheep during a similar gestational age range. Basal pituitary secretion of $ACTH_{1-39}$ increased between 120 - 136 days (1.04 ± 0.23 fmol $5min^{-1}$ mg^{-1}) and 140 – 143 days of gestation (3.08 ± 0.33 fmol $5min^{-1}$ mg^{-1}). In contrast the basal secretory rate of the ACTH precursors did not change significantly between 105 and 143 days and therefore the ratio of the output of ACTH precursors: $ACTH_{1-39}$ decreased from 19.10 ± 2.05 to 6.36 ± 0.58 between 120 and 143 days gestation. It therefore appears that the gestational profile of pituitary secretion of $ACTH_{1-39}$ and of the ACTH precursors is similar to the gestational profile of the ACTH containing peptides in the fetal sheep plasma.

Disconnection of the fetal hypothalamus and pituitary alters the gestational profile of $ACTH_{1-39}$ in fetal plasma. In contrast to intact, control fetal sheep, plasma concentrations of $ACTH_{1-39}$ did not change between 120 – 125 days (1.9 ± 0.3 pmol l^{-1}) and 136 – 142 days gestation (2.0 ± 0.2 pmol l^{-1}) after HPD (Phillips *et al.*, 1996a). There was also no change in the ACTH precursor concentrations between 120 – 125 days (20.5 ± 3.3 pmol l^{-1}) and 136 – 142 days (24.7 ± 4.0 pmol l^{-1}) after HPD. There was therefore no gestational increase in $ACTH_{1-39}$ concentrations or in the ratio of $ACTH_{1-39}$: ACTH precursor concentrations in the HPD fetus in late gestation. Thus the presence of an intact hypothalamo-pituitary axis is critical for the prepartum changes in the post-translational processing of POMC. Given that fetal sheep do not deliver at the normal time after HPD, this supports that these changes are also critical for the normal timing of delivery. It has also been demonstrated that bilateral ablation of the PVN within the fetal hypothalamus also results in the abolition of the prepartum increase in irACTH and the normal timing of parturition (Gluckman *et al.*, 1991; McDonald & Nathanielsz, 1991). It is not clear

whether there are similar changes in the post-translational processing of POMC within the pituitaries of fetal sheep after PVN lesions compared to HPD.

1.3.2.2 Effects of CRH/AVP on ACTH Secretion

Since the identification of CRH and AVP as neuropeptides that stimulate the release of ACTH from the corticotroph cells of the anterior pituitary, debate, speculation and conjecture has occurred around the role each hypothalamic secretagogue plays in the release of ACTH from the fetal pituitary. In part this complexity has arisen because there are marked species differences and gestational effects on corticotroph function. In the pituitary cells of most species studied, CRH stimulates ACTH secretion to a greater extent than does AVP (Vale *et al.*, 1983), however, the opposite effect has been observed in the adult sheep (Pradier *et al.*, 1986; Familari *et al.*, 1989 7; Liu *et al.*, 1990; Kemppainen *et al.*, 1993) although the responses of the fetal sheep pituitaries to CRH or AVP have not been consistent between studies.

In chronically catheterised fetal sheep it has been demonstrated that CRH is more potent than AVP in stimulating ACTH output from the pituitary tissue (Hargrave & Rose, 1986; Norman & Challis, 1987a). The response profiles, however, are quite different with AVP inducing a transient rise in plasma ACTH whilst CRH stimulates a more sustained increase (Pradier *et al.*, 1985; Norman & Challis, 1987a). Several investigators have observed that during late gestation in the ovine fetus, responses to CRH decrease (Wintour *et al.*, 1984; Norman *et al.*, 1985; Hargrave & Rose, 1986), whilst Pradier *et. al.* (1985) observed similar responses to CRH throughout late gestation. CRH infused for 15 minutes (50 or 500 ng kg^{-1} min^{-1}) produced a dose related increase in fetal plasma ACTH concentrations at day 95 of gestation, but by day 122 – 136 the ACTH response to the high

dose of CRH had diminished (Hargrave & Rose, 1986). CRH injections (1 μg – 10 μg) increased fetal plasma concentrations between 110 – 115 and 125 – 130 days gestation but the influence of CRH on the ACTH concentrations had diminished by 135 – 140 days gestation (Norman *et al.*, 1985). Pradier *et. al.* (1985) monitoring plasma ACTH concentrations for 3 hours after the injection of 10 μg oCRH in fetuses at 120, 130 and 137 days gestation observed that similar concentrations of ACTH were released, although the profiles differed over time. The majority of these studies suggest that the pituitary's responsiveness to CRH between days 95 and 130 is enhanced (Matthews *et al.*, 1995a) but that the negative feedback effect of elevated endogenous cortisol concentrations may be influencing the response to CRH in the late gestation fetal pituitary (Rose *et al.*, 1985). These increased cortisol concentrations during late gestation also occur with a decrease in CRH receptor population, which is maximal in the pituitary at 125 – 130 days (Lü *et al.*, 1991).

In vivo studies have described the effect of AVP on ACTH release in fetuses before 100 days. A significant increase in the ACTH plasma concentrations was detectable 10 minutes after injecting 200 ng of AVP (MacIsaac *et al.*, 1989) whilst at 98 and 124 day fetuses, infusing either low (1.6 ng kg^{-1} min^{-1}) or high (8 ng kg^{-1} min^{-1}) concentrations of AVP produced rate dependent increases in ACTH in both gestational ages (Harper & Rose, 1988). Norman and Challis (1987a) also observed that injecting 200 ng of AVP evoked significant rises in plasma ACTH on days 110 – 115 and 125 – 130, but not on days 135 – 140. After the bolus injection of AVP, the peak plasma concentration of ACTH was attained within 5 – 10 minutes, and basal (pre-injection) values were re-established by 30 to 60 minutes. The lack of response to AVP in late gestation is surprising considering the ACTH release over 3 hours in two year old ewes injected with

1 μg kg^{-1} of AVP were significantly greater than basal ACTH release (Pradier *et al.*, 1986). Significantly greater maximal ACTH responses to bolus injections of AVP were measured between 101 and 118 days gestation when compared with the ACTH responses to equimolar concentrations of CRH (MacIsaac *et al.*, 1989). The studies comparing CRH and AVP responses have opted for equimolar doses (Norman & Challis, 1987a; MacIsaac *et al.*, 1989) but perhaps administering comparable raw doses when observing the effects of CRH and AVP on ACTH release would be more appropriate since AVP concentrations are about five times that of CRH in the hypophyseal portal circulation of adult sheep (Liu *et al.*, 1994a), and it remains possible that the relative importance of AVP in fetal corticotroph activation in utero may be greater than that of CRH (Levidiotis *et al.*, 1989). However, the *in vivo* effects of exogenous AVP are always potentially more difficult to interpret than the responses to CRH, since AVP has the capacity to act on the pituitary via two distinct pathways, directly at the corticotroph cells or indirectly by stimulating CRH release via the hypothalamus.

Whichever secretagogue, CRH or AVP has the greatest ability to secrete ACTH is less relevant when the two secretagogues are administered together. Norman and Challis (1987a) have shown that between 110 and 115 days there is a synergistic ACTH response to 1 μg CRH and 200 ng AVP, especially 30 minutes after agonist injection. Using these concentrations of CRH and AVP in a different study, Norman and Challis (1987b) again found a synergism between the actions of CRH and AVP at 113 – 116 days on plasma ACTH concentrations, and a similar but not significant trend at 126 – 130 days gestation. MacIsaac *et. al.* (1989) also found that the ACTH response 10 minutes after the simultaneous administration of CRH and AVP was significantly greater than the sum of the responses to CRH and AVP, when administered separately. It appears *in vivo*, that the

administration of CRH and AVP results in an ACTH response that is greater than when either secretagogue is administered independently, and the interaction is synergistic in nature, at least, between 100 and 120 days gestation.

Several investigators have measured the *in vitro* responses of fetal sheep pituitaries to CRH and AVP. Durand *et. al.* (1986) using pooled whole pituitary glands, including the neurointermediate lobe and plated at high density (3 x 10^5 cells 2 cm^{-2}) showed that the release of ACTH in response to a 3 hour exposure of CRH decreased two fold between 64 and 116 days gestation (7.89 ± 1.19 to 3.49 ± 0.88 ng ACTH 10^5 $corticotrophs^{-1}$ $3h^{-1}$), with the release of ACTH remaining approximately constant between 123 and 144 days gestation. The level of ACTH secreted in response to CRH in the neonatal group (30 – 120 days) was, however, similar to the 63 – 64 day fetal group. In the same study Durand and co-workers also observed that the corticotroph cells responded to 3 hour AVP treatment at 63 – 64 days. The pituitary cells were most responsive to AVP at 115 – 116 days gestation (7.02 ± 1.80 ng ACTH 10^5 $corticotrophs^{-1}$ $3h^{-1}$), but this response decreased dramatically in the older gestational age groups. The responses in the 144 day age group was not greater than the level of basal release although the pituitaries of young postnatal lambs responded significantly to AVP stimulation. The pooled pituitaries responded synergistically to CRH and AVP, with the ACTH release being more than the total of the ACTH responses to the maximal doses of CRH and AVP in all age groups, except the 133 and 144 day groups. Interestingly, the largest amount of ACTH secreted occurred in the two youngest gestational age groups (63 – 64 & 115 – 116 days) with the smallest amount of ACTH secreted in the later gestational groups (133 & 144 days). Durand's *in vitro* observations strongly support the *in vivo* observations already discussed in which CRH appears to be the primary modulator of ACTH release in late gestation.

Several other studies have observed that AVP does have a significant role in ACTH release during late gestation in the ovine fetus. CRH and AVP induced dose-dependent (0.1 – 1000 nmol l^{-1}) increases in ACTH secretion from fetal sheep pituitary cells (130 – 140 days) cultured for 6 days. ACTH secretion was also significantly increased after the addition of AVP compared with CRH at doses ranging between 1 and 100 nmol l^{-1} (Brooks & Gibson, 1992). Fora *et. al.* (1996) using dissociated anterior pituitary cells also observed that in early gestation (108 ± 5 days) ACTH was released predominantly in response to CRH rather than AVP and that in late gestation (139 ± 0 days), ACTH was released predominantly in response to AVP rather than CRH. In fetuses adrenalectomised at 120 days the ACTH secretion profile at 138 – 144 days gestation was similar to that of fetuses at an earlier gestational age. The net ACTH responses to AVP plus CRH in cells of both groups were significant, but the net ACTH responses to both secretagogues in combination were indistinguishable from the mathematical sums of the net responses to the individual secretagogues. Perez *et. al.* (1997) also treated fetal pituitary cells for 2 hours with vehicle, CRH (10^{-8} M) AVP (10^{-7} M) and both secretagogues and found a pattern of ACTH responses which was very consistent with the earlier findings of Fora and co-workers (1996). The fetal sheep corticotrophs again secreted ACTH predominantly in response CRH rather than AVP at 101 ± 1 days gestation but by 135 ± 4 days gestation and in adult sheep, ACTH was secreted predominately in response to AVP, rather than CRH.

In vivo and *in vitro* studies strongly suggest that the fetal pituitary is stimulated by both CRH and AVP during late gestation although there is a discrepancy between studies as to which of these secretagogues is more potent in late gestation. Firstly, CRH stimulates ACTH secretion between days 100 and 130 days, but this response decreases before term and the ACTH response to AVP similarly declines with increasing gestational age.

Secondly, several investigations have observed CRH is the prominent secretagogue in the release of ACTH in early gestation (~ 115 – 120 days) while AVP becomes the prominent secretagogue in late gestation (~ 140 – 145 days). These observations concur with findings in the adult sheep where AVP stimulates ACTH secretion to a greater extent than does CRH. Perhaps these different observations are as a consequence of using different plating techniques, cells from whole pituitaries (Durand *et al.*, 1986) versus anterior pituitaries (Fora *et al.*, 1996; Perez *et al.*, 1997), different cell plating densities (Durand *et al.*, 1986; Brooks & Gibson, 1992; Fora *et al.*, 1996; Perez *et al.*, 1997) or using different concentrations of CRH and AVP in the *in vivo* experiments (Norman & Challis, 1987a; MacIsaac *et al.*, 1989). Importantly, CRH and AVP are clearly the primary secretagogues which stimulate ACTH release from anterior pituitary cells of the ovine fetus.

1.3.2.3 Effects of Cortisol on ACTH Synthesis and Secretion

As discussed above, the normal changes in pituitary morphology are dependent on the presence of the fetal adrenals. Removing the fetal sheep adrenals at 120 days affects the normal morphological development. At 135 days the pituitaries of adrenalectomised fetuses display arrested development, with the morphology being typical of 115 day old fetal pituitaries (Antolovich *et al.*, 1989). Similarly exogenous cortisol infused between 109 and 115 days gestation, increases the circulating plasma concentrations of cortisol, an environment that leads to premature maturation of the pituitary morphology (Antolovich *et al.*, 1989), demonstrating a feedback relationship between the adrenal cortex, cortisol and the pars distalis of the fetal sheep. Fora *et. al.* (1996) has also measured the impact of cortisol removal on the *in vitro* corticotroph responses to CRH, AVP and CRH and AVP. These authors observed that after bilateral adrenalectomy at 120 days, the ACTH

responses at 138 – 146 days were similar to the ACTH responses at 108 days, with the corticotroph cells responding predominately to CRH, whereas cells from the intact fetuses responded predominately to AVP in late gestation.

McMillen *et. al.* (1990) has also observed that after fetal sheep were bilaterally adrenalectomised at 116 – 119 days gestation, the circulating ACTH concentrations in the adrenalectomised group (1838 ± 155 ng l^{-1}) were significantly higher than the intact control group (131 ± 25 ng l^{-1}) between 130 and 136 days gestation. The mean levels of POMC mRNA: 18s RNA measured in the adrenalectomised fetal sheep pituitaries were also significantly higher than the levels measured in the control fetal sheep pituitaries. Interestingly in this study, significantly reduced circulating cortisol concentrations were not measured in the adrenalectomised fetuses between 130 and 136 days gestation, perhaps reflecting placental transfer of cortisol from the maternal circulation (Hennessy *et al.*, 1982a), in both the adrenalectomised and control fetal groups. Interestingly, on the day of the post mortem (134, 135 or 136 days) the cortisol concentrations in the adrenalectomised fetuses were significantly lower than the control fetuses, a finding confirmed by Myers *et. al.* (1991) in 134 day adrenalectomised fetuses.

Myers *et. al.* (1991) also determined that adrenalectomy in the fetal sheep not only led to a significant increase in the expression of POMC mRNA in the anterior pituitary but also to an increase in the expression of CRH mRNA in the paraventricular nucleus. Importantly, the adrenalectomy procedure in the fetal sheep results in an increased concentration of circulating ACTH (Wintour *et al.*, 1980; Rose *et al.*, 1988; McMillen *et al.*, 1990), increased POMC mRNA expression (McMillen *et al.*, 1990; Myers *et al.*, 1991) and increased CRH mRNA expression (Myers *et al.*, 1991).

These studies in the adrenalectomised fetal sheep indicate the degree of negative feedback activity the glucocorticoids exert on the hypothalamic-pituitary-adrenal axis. Several investigators have pursued these observations further by infusing glucocorticoids into the fetus and monitoring basal and stimulated ACTH responses and the corresponding POMC mRNA levels. Between 115 – 116 days gestation, the synthetic glucocorticoid dexamethasone, administered as 2 hourly pulses during a 48 hour infusion period (694 ng 0.5 ml^{-1} 15 min^{-1}) had no effect on basal ACTH concentrations, however, basal ACTH concentrations after 125 days (128 – 130 and 138 – 140 days) were suppressed (Norman & Challis, 1987b). Hennessy *et. al.* (1982b) noted that infusing cortisol for 4 hours at 50 or 100 μg h^{-1} into fetuses (107 – 131 days) suppressed fetal plasma ACTH concentrations. Ozolins *et. al.* (1990) has observed that after a 4 hour intrafetal infusion of cortisol, basal irACTH concentrations were inhibited after 138 days but not before 128 days whilst the fetal ACTH concentration was significantly decreased in 140 day fetuses infused with hydrocortisone sodium succinate (10 μg min^{-1}) for 5 hours (Wood, 1991). Norman and Challis (1987b) also observed that after a minimum of 16 hours of dexamethasone infusion, the fetal responses to oCRH, AVP or oCRH and AVP were significantly suppressed in all three gestational age groups, 115 – 116, 128 – 130 and 138 – 140 days. Rose *et. al.* (1985) has also shown in the late gestation ovine fetus, cortisol infusion preceding bolus doses of CRH (10, 100 and 1000 ng kg^{-1}) suppresses the ACTH response. Infused glucocorticoids appear to suppress both basal and stimulated ACTH concentrations in late gestation fetuses although a 96 hour cortisol (5 μg min^{-1} or 3 ml h^{-1}) infusion starting at 126 ± 0.5 days significantly elevated the basal irACTH concentrations within 24 – 48 hours. The concentrations of irACTH remained significantly higher than the controls (10 fold) throughout the 96 hour experimental period whilst the level of pars distalis POMC mRNA decreased by 96% (Jeffray *et al.*, 1998).

1.3.2.4 Functional Heterogeneity of Fetal Corticotrophs

In their study on the responsiveness of the fetal pituitaries to CRH and AVP, Fora and colleagues noted that the increase in the ACTH responsiveness of the anterior pituitary cells to AVP in late gestation and early postnatal life coincided with the change in morphology of the corticotroph cells from predominately fetal cells to predominately adult cells (Perry *et al.*, 1985; Mulvogue *et al.*, 1986; Antolovich *et al.*, 1989, Antolovich, 1991 #2). Perez and co-workers (1997) also measured the same change in ACTH responsiveness to CRH and AVP stimulation in late gestation. Whether the morphology of the corticotrophs relates to this feature of functional heterogeneity is unknown although it has been well established in the adult sheep in which the pituitary is comprised of 'adult' type corticotrophs (Perry *et al.*, 1985; Mulvogue *et al.*, 1986; Antolovich *et al.*, 1989), the corticotroph cells respond to CRH only, AVP only or to either CRH or AVP (Schwartz, 1990; Schwartz *et al.*, 1994).

1.4 RESPONSES OF THE FETAL HPA AXIS TO ACUTE & CHRONIC STRESS

1.4.1 Acute Stress

During late gestation, the fetal pituitary-adrenal axis plays a major role in the response of the fetus to acute stress (Challis & Brooks, 1989). Acute episodes of fetal hypoxaemia are associated with increased CRH mRNA levels in the fetal hypothalamus, and of POMC, mRNA, in the fetal pituitary and in increased circulating ACTH and cortisol concentrations (Jones *et al.*, 1977b; Jones *et al.*, 1988; Akagi & Challis, 1990; Matthews & Challis, 1995b). Acute episodes of hypoglycaemia during late gestation have also been shown to increase the fetal plasma ACTH and cortisol concentrations (Ozolins *et al.*,

1992). The importance of a functional hypothalamo-pituitary connection in the fetus is demonstrated as there is no increase in the plasma concentrations of cortisol or ACTH, in HPD fetuses, in response to the acute episodes of either hypoxaemia or hypoglycaemia (Ozolins *et al.*, 1992). Furthermore, several investigators have shown that in association with the increased cortisol and ACTH concentrations in response to acute hypoxaemia, there is also an increase in the fetal concentrations of plasma catecholamines (Robinson *et al.*, 1977; Jones & Robinson, 1983; Widmark *et al.*, 1989; Cheung, 1990). During acute hypoxia the fetuses experience a catecholamine dependent re-distribution of blood flow, in favour of the brain, heart and adrenal glands, at the expense of the gut, liver and the kidneys (Jensen *et al.*, 1987; Yaffe *et al.*, 1987; Jansen *et al.*, 1989; Giussani *et al.*, 1993). The responses initiated by the fetal HPA axis to acute episodes of intrauterine stressors such as hypoxaemia or hypoglycaemia; presumably represents the primary endocrine responses necessary for the fetus to cope with chronic intrauterine stress.

1.4.2 Chronic Stress

A range of experimental approaches have been used to investigate the effects of chronic intra-uterine stress. These approaches include; maternal food restriction (Mellor, 1983; Edwards *et al.*, 2001a); reduction of uterine blood flow in pregnant ewes by uterine artery ligation (Boyle *et al.*, 1996); placental infarction (repetitive embolisation) (Creasy *et al.*, 1972; Clapp *et al.*, 1981; Clapp *et al.*, 1982; Block *et al.*, 1990; Murotsuki *et al.*, 1997) or limitation of placental growth and function from conception (Alexander, 1964; Robinson *et al.*, 1979; McMillen *et al.*, 2001).

One model of placental restriction involves the surgical excision of the majority of the potential placentome attachment sites from the uterus of the non-pregnant ewe (Alexander,

1964; Robinson *et al.*, 1979; McMillen *et al.*, 2001). During the subsequent pregnancy, a restricted number of placentomes form, and this subsequently limits placental and hence fetal growth. Several reviews have summarised the effects of this experimental method on oxygen and glucose delivery and subsequent consumption by the placenta and fetus (Owens *et al.*, 1989; Robinson *et al.*, 1994). Placentally restricted fetuses are generally hypoxaemic, hypoglycaemic and have increased blood lactate concentrations and, usually no change in fetal arterial pH (Owens *et al.*, 1989; Robinson *et al.*, 1994). Interestingly, the blood gas changes measured in the placentally restricted sheep fetuses are consistent with the measurements made in cordocentesis studies of human infants who are small for their gestational age (Economides *et al.*, 1991). Interestingly, Phillips *et. al.* (1996b) observed no differences in the concentrations of irACTH or $ACTH_{1\text{-}39}$ measured between the placentally restricted or control fetuses but found a significant decrease in the levels of POMC mRNA, and a significant increase in the cortisol plasma concentrations of the placentally restricted fetuses (140 days). The decrease in the level of POMC mRNA whilst maintaining the circulating irACTH or $ACTH_{1\text{-}39}$ plasma concentrations in the fetuses subjected to an extended period of chronic intrauterine stress, suggests the corticotroph cells have an innate plasticity. The increased concentration of circulating cortisol presumably inhibits the POMC mRNA synthesis, however, it would appear that the corticotrophs respond to the altered intrauterine environment by increasing the POMC turnover, thus maintaining the normal irACTH or $ACTH_{1\text{-}39}$ concentrations.

Not only is the impact of chronic hypoxia on the fetal corticotrophs unknown but the impact of chronic hypoglycaemia is also unknown. It has been established that maternal nutrient restrictions can have different consequences on the fetus dependent on the phase of gestation targeted; embryogenesis, placentation and period of rapid placental growth,

rapid phase of fetal growth or organ maturation up to the time of birth (Mellor & Murray, 1981; Faichney & White, 1987; McCrabb *et al.*, 1991; Roseboom *et al.*, 1999; Roseboom *et al.*, 2000; Symonds *et al.*, 2002). Reducing maternal food intake by 50% compared to the food intake of control pregnant ewes can result in a stimulation of the fetal HPA axis (Edwards *et al.*, 2001a; Edwards & McMillen, 2001b). In fetal sheep before 135 days gestation the counter regulatory cortisol response to either acute or chronic hypoglycaemia was only present when the fetal plasma glucose concentration fell below 1.0 $mmol^{-1}$. After 135 days gestation, the fetal HPA axis was stimulated when fetal glucose concentrations decreased below 1.2 $mmol^{-1}$ (Edwards *et al.*, 2001a).

1.5 AIMS AND HYPOTHESIS

The reviewed literature clearly establishes the importance of the corticotroph cells of the anterior pituitary in both the control of normal fetal development and in the generation of endocrine responses to physiological stress in utero. Little is known, however, about the relative proportions of the corticotroph cell subpopulations in the fetal anterior pituitary, although it is highly probable that the fetal, like the adult anterior pituitary, is comprised of different subpopulations of corticotroph cells which are differentially responsive to CRH or AVP. The effects of chronic intrauterine stress (hypoxia and hypoglycaemia) on the corticotroph cells in either fetal or adult life are also unknown. Thus the primary aim of this thesis was to investigate the functional characteristics of the subpopulations of corticotrophs in the fetal pituitary during normal development and during chronic intrauterine stress. The fetal sheep was utilised as the animal model since considerable experimental data on the development of the pituitary–adrenal axis has already been

compiled; it is a species with a long gestation period, often with only one fetus and is susceptible to both physical and environmental manipulations which result in chronic intrauterine stress.

1.5.1 Functional Heterogeneity of Corticotrophs in the Anterior Pituitary of the Sheep Fetus

It is well documented in the fetal sheep that a functional hypothalamo-pituitary-adrenal axis and a prepartum increase in the circulating cortisol concentrations are essential for parturition. It has also been demonstrated that the pituitary corticotroph cells display morphological heterogeneity during late gestation and that the predominant corticotroph cell type in late gestation, 'the adult corticotroph' can be induced earlier in gestation after infusing cortisol at concentrations that mimic the cortisol concentrations measured during the prepartum surge. Whether the morphological heterogeneity of the corticotroph cells reflects functional heterogeneity is unknown, however, it is clear that in several species the adult pituitary has distinct corticotroph subpopulations, which are distinguishable on the basis of responses to the hypothalamic secretagogues CRH and AVP.

Thus the hypotheses tested in Chapter 2 were:

That there are different corticotroph subpopulations in the anterior pituitary of the sheep fetus which are differentially responsive to CRH and AVP.

That the proportion of ACTH stored and secreted by corticotrophs which are responsive to CRH, AVP and/or CRH and AVP change during late gestation and after intrafetal cortisol infusion.

1.5.2 *Differential Effects of the Early and Late Intrauterine Environment on Corticotrophic Cell Development*

It is known that the surgical removal of the caruncles from the uterus of the ewe before conception, alters the embryonic environment, resulting in the development of fetuses during late gestation that are either placentally restricted and chronically hypoxaemic or fetuses that demonstrate compensatory placental growth and are normoxaemic. Whilst the developing embryo and fetus have the ability to respond to a range of intrauterine stressors, the effect of uterine carunclectomy and chronic intrauterine stress on the development of the corticotroph cells in the fetal pituitary are unknown.

Thus the hypothesis tested in Chapter 3 was:

That uterine carunclectomy and placental restriction will alter the proportion of ACTH stored in and secreted by corticotroph cells which are responsive to either CRH alone, AVP alone or to CRH and AVP in the fetal pituitary during late gestation.

1.5.3 *The Effect of Hypoglycaemia on the Functional Heterogeneity of Corticotrophs in the Fetal Sheep Pituitary*

Fetal hypoxaemia is not the only consequence of placental restriction. Placental restriction results in fetal hypoxaemia and hypoglycaemia and it is unclear which of these environmental stressors may possibly alter the functional characteristics of the corticotrophs. Fetal sheep were exposed to hypoglycaemia in the absence of hypoxia, allowing the specific effect of this stress on corticotroph cell development to be determined.

Thus the hypothesis tested in Chapter 4 was:

Fetal hypoglycaemia in late gestation will alter the proportion of ACTH stored and secreted by CRH, AVP and/or CRH and AVP responsive corticotroph cells.

2 FUNCTIONAL HETEROGENEITY OF CORTICOTROPHS IN THE ANTERIOR PITUITARY OF THE SHEEP FETUS

2.1 SUMMARY

i. Parturition in the sheep is dependent on a prepartum stimulation of the pituitary-adrenocortical axis and an increase in fetal plasma cortisol. However, it is unknown whether there are changes in the functional characteristics of the pituitary cells which secrete ACTH during this prepartum phase or in response to the increase in circulating cortisol.

ii. Pituitaries were collected from a late gestational group of animals (140 – 145 days; n = 10) for cell culture. Another group of fetal sheep were infused with either cortisol (2-3 mg·24 h^{-1}; n = 11) or saline (4.4 ml·24 h^{-1}; n = 10) between 109 and 116 days and their pituitaries were collected at the end of the infusion period. Cells in half the culture wells from each pituitary were treated with C-TOX (a cytotoxic analogue of corticotrophin releasing hormone (CRH)) to eliminate CRH-target cells before exposure to medium containing either vehicle, ovine (*o*)CRH (10^{-8} M), arginine vasopressin (AVP; 10^{-7} M) or *o*CRH + AVP.

iii. The results in this Chapter show that at 116 days gestation and at 140 – 145 days gestation around 70% of the adrenocorticotrophic hormone (ACTH) in the fetal anterior pituitary is stored within corticotrophs which are responsive to CRH alone (CRH-responsive cells). Infusion of cortisol resulted in a decrease in the amount of ACTH stored in the corticotroph cells which are CRH-responsive, whereas the corticotroph cells which are responsive to AVP alone were relatively resistant to

cortisol.

iv. The findings suggest that there are two corticotrophic cell types present in the fetal sheep pituitary throughout late gestation: those which are responsive to CRH alone and those which are responsive to AVP alone. Furthermore the stimulatory influence of the fetal hypothalamus must counteract the negative feedback effect of cortisol on ACTH synthesis in the CRH responsive cells to stimulate the increase in pituitary ACTH output, which occurs before delivery.

2.2 INTRODUCTION

In the sheep, an intact hypothalamo-pituitary-adrenal (HPA) axis and a prepartum increase in the fetal plasma concentrations of cortisol are required for parturition to occur at 147 ± 3 days gestation (Challis & Brooks, 1989). It has been demonstrated that there is morphological heterogeneity of corticotrophs in the anterior lobe of the fetal sheep pituitary in late gestation (Perry *et al.*, 1985; Mulvogue *et al.*, 1986). The main corticotrophic cell observed between 90 and 130 days gestation is a tall, columnar 'fetal' cell type, whereas after 135 days a small, stellate or 'adult' cell predominates. Intrafetal infusion of cortisol (2 mg day^{-1}) between 109 and 115 days gestation results in a precocious maturation of the corticotroph population in the fetal pituitary to a predominantly 'adult' type corticotroph (Antolovich *et al.*, 1989). It is unknown whether the morphological heterogeneity in the corticotrophic cell population in the fetal pituitary is associated with a functional heterogeneity which may be important in the prepartum stimulation of the fetal adrenal cortex. Studies using reverse haemolytic plaque assays or

a specific cytotoxic analogue of CRH have shown that there are separate sub populations of corticotrophic cells, distinguishable on the basis of response to hypothalamic secretagogues (Neill *et al.*, 1987; Schwartz & Vale, 1988; Jia *et al.*, 1991) in the adult pituitary. In the adult rat, cow and sheep pituitary there are corticotrophs which are responsive to either CRH or AVP alone or to both CRH and AVP (Neill *et al.*, 1987; Schwartz & Vale, 1988; Jia *et al.*, 1991). Interestingly, there appear to be major functional differences between these cell types in the synthetic and secretory pathways for ACTH and in their responsiveness to glucocorticoids (Schwartz *et al.*, 1994).

The present study was designed to investigate the different sub populations of corticotrophs in the fetal sheep pituitary and to determine whether there is any change in the functional corticotrophic cell types during late gestation. The effect of intra-fetal cortisol infusion on the ACTH synthetic and secretory capacity of the specific corticotrophic cell types has also been investigated. Pituitary cells collected from fetal sheep in early gestation after a 7 day infusion of either saline or cortisol between 109 and 116 days gestation, and in late gestation (140 - 145 days gestation) were cultured. During culture half of the cells from each pituitary were pretreated with a toxic CRH-conjugate, hereinafter referred to as C-TOX. The cytotoxin consists of an analogue of CRH coupled to a plant toxin, gelonin, that acts selectively to kill all CRH target cells (i.e. corticotrophs that respond only to CRH or those that respond to both CRH and AVP). Corticotrophs which only respond to AVP are resistant to the effects of C-TOX and are spared (Schwartz *et al.*, 1987). The proportion of ACTH stored and secreted by CRH responsive cells (i.e. C-TOX sensitive cells) was measured in late gestation and after cortisol infusion. The effects of gestational age and cortisol infusion on the ACTH responsiveness of

corticotrophs to the hypothalamic secretagogues, CRH and AVP in the presence and absence of the CRH-cytotoxin were also determined.

2.3 MATERIALS & METHODS

All experiments in the study were carried out according to the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Procedures and approved by the Standing Committee of Ethics and Animal Experimentation at the University of Adelaide.

2.3.1 Basic procedures

2.3.1.1 Animals and Surgery

Twenty seven pregnancy dated Merino × Border Leicester ewes were used in this study. Surgery was performed in theatre, using aseptic techniques, on twenty one ewes between 103 and 104 days gestation. General anaesthesia was induced by intravenous sodium thiopentone (1.25 g Pentothal; Rhone Merieux Australia, QLD, Australia). After intubation of the maternal trachea, anaesthesia was maintained with halothane (2.5-4.0% in oxygen; Fluothane, Zeneca, Macclesfield, United Kingdom). With the ewe in a supine position the abdominal wool was shorn and the skin disinfected. Before surgery, the ewes were administered ilium penstrep (2 ml: procaine penicillin, 250 mg ml^{-1}; dihydrostreptomycin sulphate, 250 mg ml^{-1}; procaine hydrochloride, 20 mg ml^{-1}: Troy Laboratories, Smithfield, NSW, Australia) intra-muscularly (I.M.). A midline laparotomy of approximately 15 cm was made to allow access to the uterus. The fetal head was then

palpated and brought to the surface. Once the uterus and fetal head had been positioned externally, a small incision was made into the uterine wall to expose the head and neck. Babcock clamps were used to minimize the loss of amniotic fluid by clamping the uterine wall together.

The fetal neck was positioned to allow a small 3-4 cm incision to be made below the larynx, approximately 1 cm from the midline. The carotid artery was exposed using blunt dissection and tied off rostrally. A single lumen polyvinyl catheter (i.d. 0.86 mm; o.d. 1.52 mm: Critchely Electrical Products, Silverwater, NSW, Australia) was inserted approximately 6.5 cm so the tip was in, or near the aorta. The procedure was repeated to position a catheter in the jugular vein with the tip in the superior vena cava. The neck incision was closed with a continuous silk stitch (3/0; Sherwood-Davis & Geck, St. Louis, MO, USA). An intra-ammniotic catheter (i.d. 1.50 mm; o.d. 2.70 mm: Critchely Electrical Products) was sutured to the fetal neck along with the carotid and jugular catheters. Ilium penstrep (2 ml; Troy Laboratories) was injected I.M. in the dorsal region of the neck and the head length was measured before returning the fetal head to the uterus. Once the fetal head was replaced in the uterus, the uterus and fetal membranes were closed in a single layer using catgut (2/0; Ethicon, Johnson and Johnson Medical, North Ryde, NSW, Australia) and then a layer of inverting sutures (2/0; Ethicon, Johnson and Johnson Medical) were subsequently placed in the myometrium. The uterus and fetus were then replaced in the abdominal cavity. The fetal catheters were filled with sterile heparinised saline (500 IU. ml^{-1}; Multiparin, Fisons Pharmaceuticals, Sydney, NSW, Australia), and exteriorised via a small incision (2 to 3 cm) in the ewes' flank. The peritoneum and rectus sheet were closed with discontinuous sutures followed by a precautionary continuous suture to the subcutaneous fat layer (2; Ethicon, Johnson and Johnson Medical). The skin

layer was closed with a continuous vetafil synthetic suture (Vetafil Bengen; 0.3 mm, Wirtschaftsgenossenschaft deutscher Tierärzte, Garbsen, Germany). Vinyl catheters (i.d. 1.50 mm; o.d. 2.70 mm: Critchely Electrical Products) were inserted surgically into the maternal carotid artery and jugular vein. After the operation, the ewes were returned to metabolic cages and maintained under a lighting regime of 12 h light, 12 h dark. Water was provided ad libitum and the ewes were fed once daily between 0900 and 1300 h.

2.3.1.2 Cortisol Infusion & Blood Sampling

After a postoperative recovery period of at least 5 days, cortisol (2-3 mg 24 h^{-1}: hydrocortisone succinate; Solucortef, Upjohn, Kalamazoo, MI, USA; 116d + F, n = 11) or saline (4.4 ml 24 h^{-1}: 116d + Sal, n = 10) was intravenously infused into the fetus between 109 and 116 days gestation using a MS 16A Syringe Driver (Graseby Medical, Gold Coast, QLD, Australia). Fetal (5 ml) and maternal (5 ml) arterial blood samples were collected twice daily (1000 h and 1700 h) starting between 105 and 107 days, into chilled tubes. Fetal blood (3ml) and all of the maternal blood were placed in separate tubes containing lithium heparin (125 I.U.; Disposable Products, Technology Park, SA, Australia) and aprotinin (1000 k.I.U. ml^{-1} blood; Sigma Chemical Company, St. Louis, MO, USA), whilst the remaining fetal blood was placed in a tube containing dipotassium ethylenediamine tetraacetic acid (EDTA: 18.6 g l^{-1} of whole blood; Sarstedt Australia, Technology Park, SA, Australia) and aprotinin (1000 k.I.U. ml^{-1} blood; Sigma Chemical Company). The blood samples were centrifuged at 1800 *g* for 10 min at 4°C and the plasma separated into aliquots and stored at -20°C. Arterial blood samples (0.3 ml) were also collected to assess the partial pressure of oxygen (P_aO_2) and carbon dioxide (P_aCO_2), pH, haemoglobin content (Hb) and oxygen saturation (S_aO_2), using an ABL 520 blood gas

analyser (Radiometer, Copenhagen, Denmark) with correction for the higher fetal body temperature.

2.3.1.3 Post Mortem & Tissue Collection

At 116 days, ewes were killed with an intravenous overdose of sodium pentobarbitone (200 mg kg^{-1}; Lethobarb, Syntex, Castle Hill, NSW, Australia). The fetal sheep, having been anaesthetized by the maternal overdose of sodium pentobarbitone, were delivered via laparotomy, weighed and then killed by decapitation. A separate group of 6 pregnant ewes and their 10 fetuses were also killed between 140 and 145 days gestation (140 - 145d). Pituitaries were quickly removed from the fetuses and immediately placed into HEPES-dissociation buffer (HDB: NaCl, 137 mM; KCl, 5 mM; Na_2HPO_4, 0.7 mM (Merk, Kilsyth, VIC, Australia); HEPES, 25 mM (Trace Biosciences, Castle Hill, NSW, Australia); pH 7.36).

2.3.1.4 Cell Preparation

Cultured anterior pituitary (AP) cells were prepared under sterile conditions as previously described by Fora *et. al.* (1996) in a laminar flow cabinet (Holten Lamin Air, Allerød, Denmark). The AP (pars distalis) and the neurointermediate lobes (pars nervosa and pars intermedia) of each pituitary were gently separated by blunt dissection. The AP tissue was minced into tissue fragments about 1 mm^3 using two scalpel blades. The fragments were washed in HDB solution, and then placed in HDB solution containing collagenase II (0.04%; Worthington Biochemical Corporation, Freehold, NJ, USA) and deoxyribonuclease I (Sigma Chemical Company). Polypropylene centrifuge tubes (15 ml; Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA), containing the digestion solution and tissue fragments, were gently rocked for 2.5 h at 37°C on an orbital shaker.

The AP fragments were then gently triturated and centrifuged for 5 min at 400 *g*. The HDB solution was removed and the enzymatic reaction stopped by suspending the pellet in culture medium (7.5 ml) and centrifuged. Culture medium consists of (Dulbecco's Modified Eagle's Medium (DMEM) plus Ham's F12 medium (F12, 1:1; Gibco BRL, Life Technologies, Grand Island, NY, USA) and charcoal-stripped fetal calf serum and heat inactivated horse serum both added to a final concentration of 10%). The cell suspension was again washed in culture medium and centrifuged. Cells were plated in 1 ml of culture medium, at 2.0-3.0 $\times 10^5$ cells $well^{-1}$ in 48-well tissue culture plates (Falcon). Cells were cultured at 37°C in a water-saturated 5% CO_2 atmosphere (BB16 Gas Incubator, Haraeus Instruments, Hanau, Germany).

2.3.2 Experimental Protocol

Twenty four hours after plating the cells, the cells in half the wells from each pituitary were treated with either vehicle or the C-TOX that specifically eliminates CRH target cells at a final concentration of 25 nmol l^{-1}. C-TOX was synthesized as previously described (conjugate of $Nle^{21,38}$, Arg^{36} rat CRH and the gelonin toxin) (Schwartz *et al.*, 1987; Schwartz & Vale, 1989). After overnight exposure (approximately 18 h) to either C-TOX or vehicle, cells were washed with culture medium and returned to culture. After a further 48 h, the cells were washed 3 times with incubation medium [DMEM/F12 (1:1; Gibco BRL, Life Technologies) and 0.2% Polypep (Trace Biosciences, Castle Hill, NSW, Australia)] and allowed to equilibrate to serum-free conditions for 1 h. Cells were then washed in incubation medium and treated for 3 h with either medium containing vehicle (also termed basal; n = 31), oCRH (10^{-8} M; n = 16), AVP (10^{-7} M; n = 20) or oCRH + AVP (n = 18) (Peninsula Laboratories, Belmont, CA, USA). The concentrations of CRH and AVP used in these experiments were selected on the basis of previous *in vitro* studies

with adult sheep pituitary cells and *in vivo* measurements of CRH and AVP concentrations in portal blood (Engler *et al.*, 1989b; Kemppainen *et al.*, 1993). After 3 h the culture media was collected and stored at -20°C, and the cells were stored in 0.1 M HCl (1 ml) at -80°C for ACTH radioimmunoassay. Cellular extracts of ACTH were obtained by thawing and refreezing the cells to -80°C three times and using mechanical trituration on either the second or third cycle. The extracts were stored at -20°C.

2.3.3 Analyses

2.3.3.1 ACTH Radioimmunoassay

The concentrations of immunoreactive (ir)ACTH in the cell culture media and in the cellular extracts, referred to as ACTH throughout this thesis, were measured using a modified version of the double antibody radioimmunoassay previously described by McMillen *et. al.* (1990).

2.3.3.1.1 BUFFERS

The first step in the preparation of 0.05 M phosphate buffer was made by titrating 0.5 M anhydrous di-sodium hydrogen orthophosphate (35.49 g·500 ml^{-1}; Merck) and 0.5 M sodium dihydrogen orthophosphate (7.8 g·100 ml^{-1}; Merck) in an approximate ratio of 5:1, to a final pH of 7.4. The complete buffer was made by adding bovine albumin (0.5 g; CSL, Parkville, VIC, Australia), 2-mercaptoethanol (2.5 ml; BDH, Poole, England) and 50 ml of the 0.5 M phosphate solution to 447.5 ml of Milli-Q water, and then adjusting the pH to 7.5.

The 0.1 M Tris[hydroxymethyl]-aminomethane hydrochloride buffer (Tris-HCl) consisted of Tris-HCl (7.9 g; TRIZMA Hydrochloride, Sigma Chemical Company) and sodium azide (0.5 g; Sigma Chemical Company) dissolved in 500 ml of Milli-Q water, and adjusted to pH to 7.4.

2.3.3.1.2 STANDARDS

Standards were made using synthetic human (*h*)$ACTH_{1-39}$ (Peninsula Laboratories). One hundred µg of the *h*$ACTH_{1-39}$ was weighed and dissolved in 175 mM acetic acid solution, containing 0.1% gelatin. The *h*$ACTH_{1-39}$ stock solution aliquots (10 µg·100 μl^{-1}) were then stored at -80°C. During an assay, a stock solution aliquot was serially doubly diluted with 0.05 M phosphate buffer to generate a standard curve, with a range of *h*$ACTH_{1-39}$ between 1.95 and 500 pg $tube^{-1}$.

2.3.3.1.3 ANTISERA

Anti-ACTH antisera (Anti-ACTH 1000T; ICN Biomedicals, Costa Mesa, CA, USA) was raised in rabbits against a purified porcine ACTH-conjugate. The antisera was reconstituted from the supplied powder with 100 µl of Milli-Q water (1:1) before being stored in 25 to 30 µl aliquots at -20°C. To use the antisera in the assay, aliquots were diluted with 0.1 M Tris-HCl buffer, and used at a final dilution 1:850. In the absence of unlabelled ACTH, the antisera bound between 10 and 28% of the iodinated-synthetic human (^{125}I-*h*$ACTH_{1-39}$). ICN Biomedicals stated that the anti-ACTH antisera cross-reacted with $ACTH_{1-39}$ and $ACTH_{1-24}$ 100% and the human peptides β-Lipotrophin (*h*β-LPH) 0.8%, *h*α-LPH 0.1%, α-Melanocyte stimulating hormone (*h*α-MSH), *h*β-MSH and β-Endorphin (*h*β-EP) < 0.1%.

2.3.3.1.4 TRACER

The *h*ACTH$_{1-39}$ (Peninsula Laboratories) was iodinated by Dr David Casely (Department of Medicine, The University of Melbourne, Heidelberg, VIC, Australia). The ^{125}I-*h*ACTH$_{1-39}$ was purified via low pressure liquid chromatography and stored in methanol (volume ranging between 20.0 to 80.0%) at -20°C. Each supplied vial of ^{125}I-*h*ACTH$_{1-39}$ had an activity of approximately 400 kBq and was diluted for the assay in 0.05 M phosphate buffer (dilution ranging between 1:30 and 1:80) to contain approximately 11000 to 11500 total counts per min tube^{-1}.

2.3.3.1.5 SECONDARY ANTIBODIES

The goat anti rabbit serum (GAR; generously donated by Dr Ross Young, Department of Physiology, Monash University, Clayton, VIC, Australia) and normal rabbit serum (NRS; CSL, Edwardstown, SA, Australia) were used to precipitate the irACTH/anti-ACTH sera/^{125}I-*h*ACTH$_{1-39}$ complex. The GAR and NRS were both diluted in 0.05 M phosphate buffer 1:10 and 1:60 respectively, before addition to the assay.

2.3.3.1.6 ASSAY

Duplicate samples of cell culture media, cellular extracts (50 or 100 µl; diluted prior to addition 1:5 to 1:20) or ACTH standards (100 µl) were added to tubes containing anti-ACTH sera (100 µl), along with the appropriate volume of 0.05 M phosphate buffer to produce a final tube volume of 200 µl. Tubes were vortexed and incubated overnight (~ 20 h) at 4°C. After the addition of ^{125}I-*h*ACTH$_{1-39}$ (100 µl) the tubes were vortexed again and incubated overnight (~ 20 h) at 4°C. The vortexing and overnight incubation (~ 20 h) at 4°C were repeated after the addition of GAR and NRS (100 µl respectively). On the fourth day, the tubes were centrifuged for 30 min at 2500 *g* and 4°C, the supernatant

aspirated and the precipitate counted with a multidetector gamma system (RIASTAR; Packard Instrument Company, Meriden, CT, USA).

Human $ACTH_{1-39}$ (7.8 - 31.2 pg $tube^{-1}$) added to incubation medium was quantitatively recovered (109 ± 5%; n = 9). The sensitivity of the assay was 1.95 pg $tube^{-1}$. The interassay coefficient of variation was 20.9%, and intraassay coefficient was less than 10%.

2.3.3.2 Cortisol Radioimmunoassay

Cortisol was measured after extraction from fetal plasma with dichloromethane (Merck, Kilsyth, VIC, Australia) as previously described by Bocking *et. al.* (1986) and using the Orion Diagnostica cortisol radioimmunoassay kit (Orion Diagnostica, Espoo, Finland: (Phillips *et al.*, 1996b)).

2.3.3.2.1 ASSAY PREPARATION

The 0.1 M Tris-HCl buffer for the cortisol assay was made with Tris-HCl (7.9 g; Sigma Chemical Company), bovine albumin (1.0 g; CSL, Parkville) and sodium azide (0.5 g; Sigma Chemical Company) dissolved in 500 ml of Milli-Q water, and pH adjusted to 7.4. The cortisol standards were made from lyophilised cortisol-free human serum (either 2000 or 1000 nmol l^{-1}) reconstituted in Mill-Q water (1 ml). After being left to stand for 1 h the stock standard was then diluted in 0.1 M Tris-HCl buffer to provide a working solution of 100 pmol ml^{-1}. For the assay the working solution was serially diluted 1:2 to generate standards, ranging between 10.0 and 0.078 pg $tube^{-1}$. The cortisol-antisera raised in rabbits against a bovine albumin conjugate of cortisol-3-carboxy-methoxylamine was also supplied in lyophilised form and reconstituted with 11 ml buffer (1:1). It was diluted

with buffer before being added to the assay at a final concentration of 1:10. Iodinated cortisol (^{125}I-Cortisol) containing serum blocking agents and 0.1% sodium azide was supplied ready to use, with a vial containing < 400 kBq. The ^{125}I-Cortisol was diluted with 0.1 M Tris-HCl buffer (between 1:6 and 1:3) to emit approximately 10000 to 11000 counts per min tube^{-1}. The secondary precipitant agent, 20% polyethylene glycol 6000 (PEG; BDH Laboratory Supplies, Poole, England) was made by adding 20 g of PEG per 100 ml of Milli-Q water.

2.3.3.2.2 Sample Preparation

Duplicate fetal samples were extracted as follows: Plasma (100 µl), Milli-Q water (100 µl) and dichloromethane (2 ml; Merck) were added to glass tubes and vortexed for 1 min. The tubes were left to stand for 5 min until two layers formed. The vortexing was repeated, and the aqueous layer removed by aspiration. One ml of the remaining organic layer was pippetted into a new glass tube and evaporated with forced air and heat (37°C) using a heating module (Reacti-Therm III; Pierce, Rockford, IL, USA). The efficiency of the extraction of cortisol from these fetal sheep plasma samples was 84 ± 1%.

2.3.3.2.3 Assay

Duplicate tubes contained sample extracts, standards (100 µl), anti-cortisol sera (100 µl), ^{125}I-Cortisol (100 µl) and the appropriate volume of Tris-HCl buffer (final tube volume of 400 µl). Tubes were vortexed and incubated for 1 h at 37°C. After allowing 10 min equilibration at room temperature 1 ml of PEG (BDH Laboratory Supplies) was added to all tubes except the tubes containing the total counts and spun for 30 min at 3300 *g* and 6°C. The supernatant was aspirated and the precipitate counted with a multidetector gamma system (RIASTAR; Packard Instrument Company). The sensitivity of the assay

was determined, as 0.078 pmol $tube^{-1}$ and the inter– and intraassay coefficients of variation were both less than 10%.

2.3.3.3 Statistics

2.3.3.3.1 *IN VIVO EXPERIMENTS*

Fetal blood gas values are expressed as mean ± standard error of the mean (S.E.M.). The mean P_aO_2, P_aCO_2, pH, Hb, S_aO_2 and cortisol concentrations were calculated for each fetus as the average of all P_aO_2, P_aCO_2, pH, Hb, S_aO_2 and cortisol values obtained between 104 and 109 days gestation (Basal period) and 110 and 117 days gestation (Infusion period). Arterial oxygen content (O_2 content) per 100 ml blood (ml dl^{-1}) was also calculated for the basal and infusion periods for each fetus using the following equation (Block *et al.*, 1989, Edwards, 1999 #177):

$$O_2\,\text{content} = (P_aO_2 \times 0.003) + \left[[Hb] \times \left(\frac{S_aO_2}{100} \right) \times 1.39 \right]$$

where;

P_aO_2 x 0.003 equals the dissolved fraction of oxygen in plasma and the constant (1.39) is the volume of oxygen able to bind to each g of haemoglobin (ml g^{-1}).

The blood gas and cortisol levels during the basal and infusion periods in the 116d + Sal and 116d + F groups were compared, using multifactorial analysis of variance (ANOVA) using a Statistical Package for Social Sciences on a Vax mainframe computer (SPSSX).

2.3.3.3.2 IN VITRO EXPERIMENTS

In the *in vitro* experiments, each treatment was completed in duplicate and the duplicate values of ACTH from either the culture media (secreted) or cellular extracts (stored) were

averaged to yield an n = 1 for each pituitary, in each treatment group. The total ACTH represents the arithmetic sum of the secreted ACTH and stored intracellular ACTH. All values are expressed as mean ± S.E.M.

The effects of C-TOX on the total ACTH (per well) and the amount of total ACTH secreted by the corticotrophic cells (per 10^4 cells) during basal conditions in the three treatment groups (116d + Sal; 116d + F and 140 – 145d) were compared using ANOVA with group and C-TOX pretreatment as the statistical parameters. The percentage of total ACTH secreted under basal conditions was also analysed using a multifactorial ANOVA with C-TOX and treatment group as the statistical parameters.

The proportion (%) of total ACTH present in the CRH-responsive cells (i.e. C-TOX sensitive cells) was calculated as the ratio of:

$$\frac{V - Cyto}{V} \times 100$$

where;
V = total ACTH in vehicle-treated wells under basal conditions
Cyto = total ACTH in C-TOX-treated wells under basal conditions

The effect of the treatments (116d + Sal; 116d + F and 140 – 145d) on the proportion of ACTH stored in CRH target cells was analysed using a one way ANOVA.

The ACTH secretory responses to vehicle, CRH (10^{-8} M), AVP (10^{-7} M) and CRH + AVP (expressed as a percentage of their respective total ACTH) were compared in the 3 treatment groups, in the presence or absence of C-TOX pretreatment, using multifactorial ANOVA. The ACTH secretory responses to CRH (10^{-8} M), AVP (10^{-7} M) and CRH +

AVP were also expressed as fold changes above basal ACTH secretion. The differences among pituitary cells from the 3 *in vivo* treatment groups on the ACTH fold changes, in the presence and absence of C-TOX were also analysed using multifactorial ANOVA

For all analyses, where the Cochran's and Bartlett-Box tests identified significant heterogeneity of variance, the data were logarithmically transformed prior to ANOVA. For significant interactions between major variables identified by the multifactorial ANOVA, the data were split on the basis of the interactions and reanalysed. When the ANOVA indicated differences between the groups or treatments, a Duncan's *post hoc* test was used to identify the significant differences between the mean values. For all differences $P < 0.05$ was taken to be significant.

2.4 RESULTS

2.4.1 Fetal Outcome

There were no changes in the mean fetal P_aO_2 or Hb during the saline or cortisol infusion periods (Table 2.1). There was an increase in the mean fetal P_aCO_2, and decrease in the mean fetal arterial pH during the infusion period in both the cortisol and saline infused fetuses (Table 2.1). The S_aO_2 and O_2 content were significantly lower during the infusion period when compared with basal values in the 116d + Sal fetuses. These changes did not occur, however in the cortisol infused group (Table 2.1). Fetal plasma concentrations of cortisol increased significantly during infusion of cortisol in the 116d + F fetuses (Table 2.1). There was no difference between the weight of the saline and cortisol infused fetuses (116d + Sal 2.06 ± 0.10 kg; 116d + F 2.20 ± 0.09 kg).

2.4.2 *Impact of age, cortisol and C-TOX on total ACTH and secretion*

There was no difference in the total ACTH (secreted + stored) between the pituitary cells of the 3 treatment groups when expressed as either total ACTH $(10^4 \text{ cells})^{-1}$ (116d + Sal; 0.62 ± 0.20 ng $(10^4 \text{ cells})^{-1}$: 116d + F; 0.46 ± 0.08 ng $(10^4 \text{ cells})^{-1}$: 140 - 145d, 0.78 ± 0.24 ng $(10^4 \text{ cells})^{-1}$) or as the percentage of total ACTH secreted during the 3 h control period (116d + Sal, 1.16 ± 0.23%; 116d + F, 2.45 ± 0.95%; 140 – 145d, 1.73 ± 0.25% respectively) (Figure 2.1).

Treatment with C-TOX resulted in a significant decrease ($F = 142.8$, $p < 0.001$) in the amount of total ACTH present in pituitaries of 116d + Sal (no C-TOX, 16.9 ± 5.5 ng ACTH $well^{-1}$; C-TOX, 6.5 ± 3.1 ng ACTH $well^{-1}$) and of 140 – 145d fetuses (no C-TOX, 22.9 ± 7.7 ng ACTH $well^{-1}$; C-TOX, 7.1 ± 2.9 ng ACTH $well^{-1}$). The effect of C-TOX on total ACTH was significantly greater ($P < 0.05$) in these groups, however, than in the 116d group which had been infused with cortisol *in vivo* (116d + F; no C-TOX, 12.7 ± 4.0 ng ACTH $well^{-1}$; C-TOX, 8.2 ± 3.3 ng ACTH $well^{-1}$).

There was no significant difference in the proportion of total ACTH present in C-TOX sensitive cells between the 116d + Sal group (71.0 ± 4.5%) and the 140 – 145d gestation group (71.0 ± 4.5%). After cortisol infusion *in vivo*, however, there was a significant reduction ($P < 0.01$) in the proportion of total ACTH present in the C-TOX sensitive cells (46.0 ± 7.5%) during basal conditions, compared with either the 116d + Sal or the 140 - 145d groups (Figure 2.2).

After C-TOX treatment, there was a similar and significant increase ($F = 65.4$, $P < 0.001$) in the percentage of total ACTH which was secreted under basal conditions in the 3 h

TREATMENT		P_aO_2 (mmHg)	P_aCO_2 (mmHg)	pH	Hb (g dl^{-1})	S_aO_2 (%)	O_2 Content (ml dl^{-1})	Cortisol (mmol l^{-1})
SALINE	Basal	22.4 ± 0.6	44.7 ± 1.2	7.357 ± 0.009	9.0 ± 0.3	71.2 ± 2.9	8.9 ± 0.2	1.75 ± 0.31
	Infusion	21.4 ± 0.8	48.3 ± 1.3$^{\varphi}$	7.343 ± 0.011$^{\varphi}$	8.7 ± 0.4	66.4 ± 3.4$^{\varphi}$	8.0 ± 0.3$^{\varphi}$	1.65 ± 0.17
CORTISOL	Basal	22.5 ± 0.8	45.4 ± 0.7	7.360 ± 0.006	8.9 ± 0.2	70.7 ± 2.6	8.8 ± 0.3	1.50 ± 0.12
	Infusion	23.5 ± 1.0	47.7 ± 0.7$^{\varphi}$	7.354 ± 0.006$^{\varphi}$	8.8 ± 0.4	71.2 ± 2.2	8.8 ± 0.4	36.50 ± 4.44$^{\varphi}$

Table 2.1 Effect of saline and cortisol infusion on fetal blood gas status and cortisol concentrations

Mean values for arterial blood gas variables and cortisol in Saline (116d + F) and Cortisol (116d + Sal) infused fetal sheep. All values are expressed as mean ± S.E.M. Superscript $^{(\varphi)}$ indicates significant differences between mean values during the Basal and Infusion periods within each treatment group, ($P< 0.05$).

period in all three treatment groups (116d + Sal, 6.30 ± 1.49%; 116d + F, 4.05 ± 1.33%, 140 – 145d, 5.80 ± 1.65%) (Figure 1).

2.4.3 Effect of C-TOX on ACTH secretory responses to CRH and AVP

Gestational age or cortisol infusion *in vivo* had no effect on the ACTH secretory responses to the hypothalamic secretagogues.

There was a significant interaction ($P < 0.001$) between the effects of C-TOX and the effects of the secretagogues on the ACTH secretory response. In the absence of C-TOX treatment, the percentage of total ACTH secreted was significantly higher ($P < 0.05$) in response to CRH + AVP in all three groups than in response to either CRH or AVP alone or to vehicle (Table 2.2). The percentage of ACTH secreted in response to AVP was also significantly higher ($P < 0.05$) than in response to CRH (10^{-8} M) or to vehicle (Table 2.2).

After treatment with C-TOX, the percentage of total ACTH secreted in response to CRH was not significantly greater than the ACTH response to vehicle. Furthermore, the ACTH secretory responses to CRH + AVP and to AVP were similar, and significantly greater than the responses to either CRH or to vehicle (Table 2.2, Figure 2.3).

There was also no significant difference in the ACTH responses to AVP (expressed as fold change from baseline) between cells not exposed or exposed to C-TOX treatment (116d + Sal; 5.5 ± 1.7 vs 5.8 ± 1.3 respectively: 116d + F; 4.6 ± 1.9 vs 7.7 ± 3.1 respectively: 140 - 145d; 3.6 ± 1.0 vs 4.9 ± 1.5 respectively).

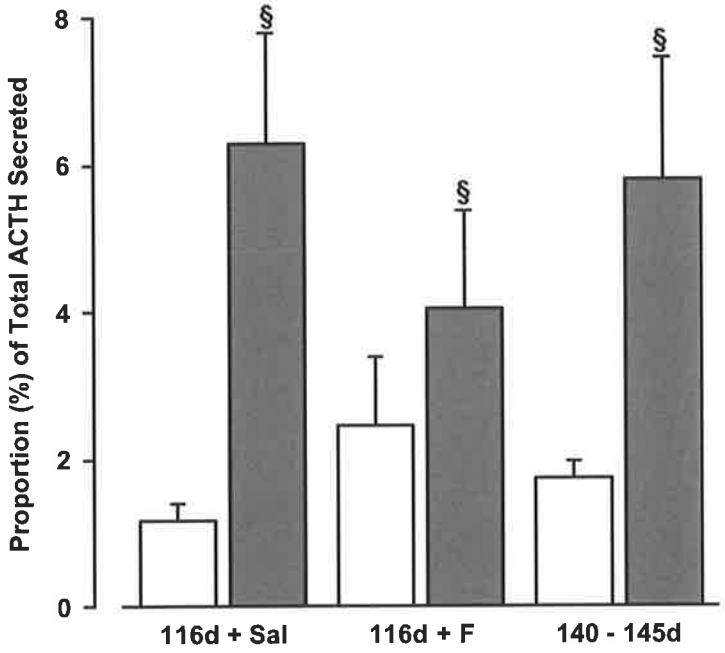


Figure 2.1 Effect of C-TOX on basal ACTH secretion

The percentage (means ± S.E.M.) of total ACTH secreted during basal conditions in the three treatment groups (116d + Sal, n= 10; 116d + F, n= 11; 140 - 145d, n = 10) following treatment with vehicle (open bars) or C-TOX (dark bars). The superscript (§) indicates the significant effect of C-TOX on the percentage of total ACTH secreted, ($P < 0.05$).

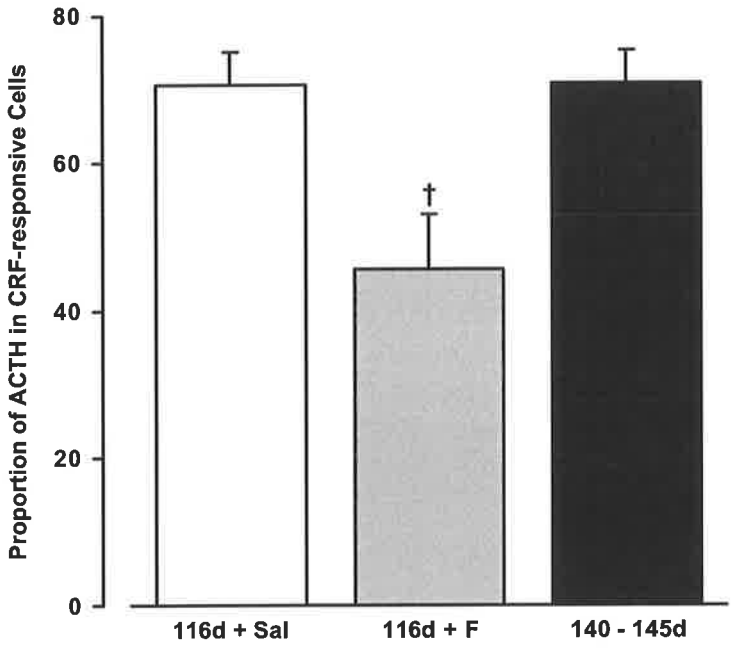


Figure 2.2 Proportion of total ACTH contained in CRH-responsive Cells

The proportion (means ± S.E.M.) of total ACTH present in CRH-responsive cells in each of the three treatment groups. The superscript (†) indicates that the proportion of ACTH present in the CRH-responsive cells was significantly lower in the 116d + F group (n = 11) than in either the 116d + Sal (n = 10) or 140 - 145d (n = 10) groups, ($P < 0.05$).

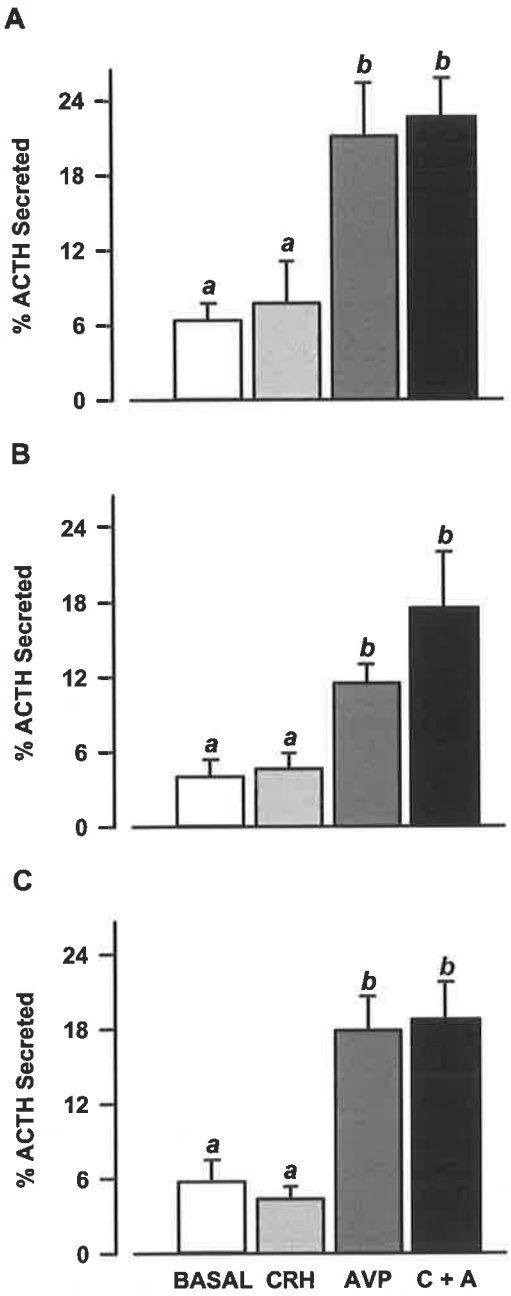


Figure 2.3 The percentage of total ACTH secreted from pituitary cells in response to vehicle, CRH, AVP or CRH + AVP with C-TOX treatment

Values represent the percentage (means ± S.E.M.) of ACTH secreted in response to the hypothalamic secretagogues or vehicle in (**A**) 116d + Sal, (**B**) 116d + F and (**C**) 140 – 145d treatment groups after C-TOX treatment. Different superscript letters denote significant differences between the ACTH secretory responses within each treatment group, ($P < 0.05$).

	Vehicle Treatment			C-TOX Treatment		
	116d + Sal	**116d + F**	**140 - 145d**	**116d + Sal**	**116d + F**	**140 - 145d**
Basal	1.2 ± 0.2^{a}	2.5 ± 0.9^{a}	1.7 ± 0.2^{a}	6.3 ± 1.5^{a}	4.0 ± 1.3^{a}	5.8 ± 1.6^{a}
CRH	3.5 ± 1.2^{b}	2.8 ± 1.1^{b}	2.2 ± 0.8^{b}	7.7 ± 3.4^{a}	4.6 ± 1.3^{a}	4.4 ± 1.0^{a}
AVP	5.0 ± 1.6^{c}	3.4 ± 1.1^{c}	5.5 ± 1.5^{c}	21.1 ± 4.3^{b}	11.5 ± 1.5^{b}	17.8 ± 2.7^{b}
CRH + AVP	9.8 ± 2.7^{d}	11.5 ± 4.7^{d}	14.7 ± 3.9^{d}	22.5 ± 3.2^{b}	17.4 ± 4.5^{b}	18.8 ± 2.9^{b}

Table 2.2 The percentage of total ACTH secreted from pituitary cells in response to CRH, AVP or CRH + AVP after vehicle or C-TOX treatment

Values represent the percentage (means ± S.E.M.) of ACTH secreted in response to the hypothalamic secretagogues and basal treatments in each of the three fetal pituitary groups after C-TOX or vehicle treatment. Different superscript letters denote significant differences between the ACTH secretory responses within each fetal treatment (vehicle or C-TOX) group, ($P < 0.05$).

2.5 DISCUSSION

2.5.1 Total ACTH

There was no significant change in the total amount of ACTH present in the fetal pituitary cells or in the percentage of ACTH that was secreted under basal conditions between 116 and 145 days gestation. Several studies have shown, however, that basal plasma ACTH concentrations increase significantly between 116 and 145 days gestation in the fetal sheep (Challis & Brooks, 1989; Ozolins *et al.*, 1991). It is known that there is a relative decrease in the proportion of ACTH secreting cells in the fetal sheep pituitary between 115 and 140 days gestation (Perry *et al.*, 1985; Perez *et al.*, 1997). Thus in the present study whilst there is no change in the total amount of ACTH stored or secreted from a fixed number of pituitary cells between 116 and 145 days gestation, there may be an increase in amount of ACTH stored and secreted per cell when the data are expressed in relation to the number of fetal corticotrophs present within the culture well. There are conflicting reports of whether there is or is not an increase in the ACTH synthetic capacity of the corticotrophic cells in the fetal pituitary before birth. There are reports of an increase (Yang *et al.*, 1991; Myers *et al.*, 1993; Matthews *et al.*, 1994) and a decrease (McMillen *et al.*, 1988; Brooks *et al.*, 1992; Mérei *et al.*, 1993) in the POMC mRNA levels in the anterior pituitary of the fetal sheep in the week before delivery. It is also possible that the similar basal secretion rates in the cells from the 116d and 140 – 145d pituitaries may be a result of the absence of stimulatory effects of the hypothalamic drive after the fetal pituitary cells have been in culture for 4 days. It has been shown that the increase in plasma ACTH concentrations in late gestation is dependent on a functional fetal hypothalamus (Phillips *et al.*, 1996a).

Treatment with C-TOX resulted in an increase in the percentage of ACTH secreted *in vitro*, under basal conditions. This was a consequence of maintained ACTH secretion in the face of a decrease in cellular ACTH content, meaning, the remaining AVP responsive cells secrete the same or more ACTH as in the intact population, although fewer corticotrophs are present (van de Pavert *et al.*, 1997). It has been reported that treatment of adult sheep pituitary cells with C-TOX also increased the basal levels of POMC mRNA and ACTH secretion (van de Pavert *et al.*, 1997). Thus, in the present study, the data also suggests that under basal or unstimulated conditions, ACTH may be secreted primarily from a sub population of corticotrophs in the fetal pituitary which are not CRH responsive (i.e. the AVP responsive corticotrophs).

2.5.2 CRH-responsive corticotrophs

Whilst the basal level of ACTH secretion is maintained after C-TOX treatment, there is a significant decrease in total ACTH, consistent with the selective removal of a population of cells which are CRH-responsive. It is particularly interesting that the same proportion (approximately 70%) of total ACTH was present in CRH-responsive corticotrophs at 116 days and after 140 days gestation. Whilst the fetal sheep in the early gestational age group were exposed to fetal surgery and catheterisation at around 103 days gestation, it is unlikely that there were any persistent effects of this surgery on the fetal corticotrophs, given the low circulating levels of fetal cortisol which were present in this group at 116 days gestation. During late gestation in the fetal sheep pituitary, there are morphological changes in the corticotrophic cells (Perry *et al.*, 1985; Mulvogue *et al.*, 1986; Antolovich *et al.*, 1989). The predominant corticotroph observed between 90 and 130 days gestation is a tall, columnar cell or "fetal" corticotroph, which is arranged in large clumps or palisades. After 135 days gestation, the predominant corticotroph in the fetal pituitary is

the small angular and intensely ACTH immunoreactive "adult" cell type (Perry *et al.*, 1985; Mulvogue *et al.*, 1986). The present data indicate that, despite the change in the predominance of the fetal cell type in mid gestation to the adult cell type in late gestation, there is no substantial change in the proportion of ACTH stored in corticotrophs which are CRH-responsive. This suggests that there is no direct relationship between the predominance of the morphologically distinct corticotrophs and the proportion of ACTH present in CRH-responsive corticotrophs in the fetal sheep pituitary.

There is evidence from studies in the adult sheep, that in CRH-responsive cells, the classically regulated protein secretory pathway operates, which has a time-course allowing for the greater processing of the parent precursor protein, POMC to $ACTH_{1-39}$ (Schwartz *et al.*, 1991a). Further, there is evidence that AVP responsive corticotrophs appear to synthesize and secrete ACTH via an alternately regulated pathway which results in less opportunity for processing of the ACTH precursors (Schwartz *et al.*, 1991a; Schwartz *et al.*, 1994). There is evidence from *in vitro* and *in vivo* studies, using separate immunoradiometric assays specific for $ACTH_{1-39}$ and the ACTH precursors, that there is an increase in the pituitary secretion of $ACTH_{1-39}$ relative to the ACTH precursors in late gestation (Carr *et al.*, 1995; McMillen *et al.*, 1995; Phillips *et al.*, 1996a). Given that the bulk of ACTH appears to be within the CRH responsive corticotrophs throughout late gestation, the change in the output of $ACTH_{1-39}$ after 140 days gestation, may be due either to maturation of post translational processing events within these corticotrophs or to increased hypothalamic stimulation of these cell types in late gestation.

Intrafetal infusion of cortisol for 7 days significantly decreased the proportion of ACTH present in CRH-responsive cells in the fetal pituitary. Matthews and Challis (1997) have

reported that, whilst cortisol decreased both CRH and AVP stimulated ACTH secretion in fetal pituitary cells in culture, this effect was more profound on CRH-stimulated secretion. It has been previously shown in intact populations of pituitary cells from adult sheep that pretreatment with dexamethasone for 16 to 18 hours decreased the ACTH secretory responses to AVP or CRH, but did not decrease the residual ACTH response to AVP in populations previously treated with CRH-cytotoxin (Schwartz *et al.*, 1994). Neill and co-workers (1987) also concluded from studies with the reverse haemolytic plaque assay that there are two functional sub populations of corticotrophs, one of which is differentially responsive to CRH and preferentially inhibited by glucocorticoids. The results of the present study are also consistent with cortisol acting preferentially to inhibit ACTH synthesis in fetal pituitary corticotrophic cells which are CRH-responsive. Previously, perifusion studies have found that cortisol acts *in vitro* in a short time domain (within 10 minutes) to suppress the secretion of $ACTH_{1\text{-}39}$ but not the ACTH precursors in late gestation (McMillen *et al.*, 1995). This provides further evidence that cortisol acts primarily on corticotrophic cell types which secrete relatively more $ACTH_{1\text{-}39}$. Whilst cortisol decreased the proportion of ACTH present in the CRH responsive cells, it had no effect on the amount of ACTH present in the C-TOX-resistant cells. Therefore, it can be concluded that the AVP responsive cells in the fetal pituitary are relatively resistant to the negative actions of cortisol on ACTH synthesis and secretion.

In the present study, whilst fetal cortisol concentrations achieved at 116 days gestation were similar to those present after 140 days gestation, the proportion of ACTH in the CRH-responsive corticotrophs in the cortisol treated group was significantly lower than that after 140 days gestation. It may be that after 140 days gestation, the stimulatory influence of the fetal hypothalamus overrides the negative feedback effect of cortisol on

ACTH synthesis in the CRH responsive cells. It is also noteworthy that electrolytic lesions of the paraventricular nuclei or surgical disconnection of the fetal hypothalamus and pituitary each prevent the normal prepartum increase in ACTH and delay parturition (McDonald & Nathanielsz, 1991; Phillips *et al.*, 1996a).

2.5.3 ACTH responses to CRH and AVP

In the present study, the ACTH secretory responses to AVP and to CRH + AVP were significantly greater than the responses to CRH throughout late gestation and after intrafetal cortisol infusion. Previous studies have also reported that the ACTH secretory responses to CRH + AVP and to AVP were greater than to CRH after 138 days gestation (Fora *et al.*, 1996). The ACTH secretory response to CRH, in these studies, however was reported to be greater than to AVP at around 108 days gestation (Fora *et al.*, 1996). In the present study, whilst the ACTH response to AVP was higher than the response to CRH independent of gestational age, there was a trend towards a greater ACTH response to CRH in early gestation; and the inclusion of all variables (age, G-TOX pretreatment, secretagogues) in the multifactorial ANOVA may have limited the capacity to define gestational trends in the ACTH secretory responses to CRH. Perez and co-workers have used immunoblotting techniques to study the effects of development on the number of cells secreting ACTH in response to CRH and AVP in fetal sheep pituitary cells (Perez *et al.*, 1997). At both 120 and 135 days gestation, AVP, alone or in combination with CRH, increased the proportion of secreting corticotrophs by about 30%. At 135 days gestation, AVP or AVP + CRH treatment also increased the amount of ACTH secreted by each cell. In contrast, at 120 days, CRH stimulated a subpopulation of corticotrophs to release ACTH, but at around 135 days, the ACTH response to CRH had changed to one that involved an increased output from cells which were already secreting ACTH. The authors

postulated that a number of changes, including an increase in AVP receptor expression (Shen *et al.*, 1990) and a decrease in CRH receptor expression (Lü *et al.*, 1991) may explain the changes in pituitary responsiveness to the hypothalamic secretagogues.

In the present study, it is interesting to note that the ACTH response to AVP remained constant at around 4 – 8 fold above basal output, even after C-TOX treatment i.e. after the elimination of CRH responsive cells. One explanation for this result, is that, in the fetal sheep pituitary, the majority of corticotrophs which are AVP responsive are responsive to AVP only, rather than to CRH + AVP.

2.5.4 Summary

In summary, therefore, separate subpopulations of corticotrophs in the anterior pituitary of the fetal sheep have been clearly defined, which are CRH and AVP responsive. The data demonstrated that approximately 70% of the ACTH in the fetal anterior pituitary is stored within corticotrophs which are CRH responsive. Whilst there was no change in this pattern with increasing gestation, cortisol acts preferentially to inhibit ACTH synthesis in fetal pituitary corticotroph cells which are CRH-responsive and that the AVP-responsive cells are relatively resistant to the negative effects of cortisol. The data also suggest that the stimulatory influence of the fetal hypothalamus must counteract the negative feedback effect of cortisol on ACTH synthesis in the CRH responsive cells to stimulate the increase in pituitary $ACTH_{1\text{-}39}$ output, which occurs before delivery.

3 DIFFERENTIAL EFFECTS OF THE EARLY AND LATE INTRAUTERINE ENVIRONMENT ON CORTICOTROPHIC CELL DEVELOPMENT

3.1 SUMMARY

i. The effect of chronic intrauterine stress on the programmed development of the pituitary corticotroph cells has not been established. Surgical removal of the caruncles from the uterus of the ewe before conception, alters the embryonic environment, resulting in the development of fetuses during late gestation that are either placentally restricted and chronically hypoxaemic or fetuses that demonstrate compensatory placental growth and are able to maintain a normoxeamic environment.

ii. In fifteen ewes the majority of endometrial caruncles (carunclectomised; CR) were removed before mating while eleven ewes were not carunclectomised (NCR). Nine fetuses in the CR, and four in the NCR group had a mean arterial partial pressure of oxygen (P_aO_2) less than 16 mmHg throughout late gestation and were classified as hypoxaemic (HX), while the remainder of fetuses were classified as normoxaemic (NX). Pituitaries were collected from all fetuses between 138 and 145 days gestation for cell culture. Cells in half the culture wells from each pituitary were treated with a selective cytotoxin (C-TOX) to eliminate corticotrophin releasing hormone (CRH)-target cells before treatment with either medium containing vehicle, ovine (*o*)CRH (10^{-7} M), arginine vasopressin (AVP; 10^{-7} M) or *o*CRH + AVP.

iii. The results in this Chapter show that uterine carunclectomy resulted in the emergence of a population of non-corticotrophin-releasing hormone (non-CRH) target cells that

secreted high amounts of adrenocorticotrophic hormone (ACTH) in the fetal pituitary. This change in corticotroph development was independent of late-gestation hypoxaemia. The presence of chronic hypoxaemia during late gestation in both the caruncleclectomised or non-caruncleclectomised uterine environments resulted in a reduction in the proportion of ACTH stored in CRH-target cells.

iv. The findings suggest that the early and late intrauterine environments program the development of specific corticotrophic cells types in separate ways. These patterns of altered corticotroph development are important given the central roles of the hypothalamo-pituitary-adrenal axis in the fetal adaptive response to intrauterine stress and in the early programming of adult disease.

3.2 INTRODUCTION

In the preceding Chapter it was reported that fetal sheep pituitary cells are either CRH or AVP responsive and that about 70% of the total ACTH in the fetal anterior pituitary is stored in CRH responsive corticotrophs during the last 4-5 weeks of gestation. Although, it is well established that the fetal HPA axis is activated in response to a range of acute intrauterine stressors including hypoxaemia, hemorrhage, and insulin-induced hypoglycaemia (Rose *et al.*, 1978; Wood *et al.*, 1988; Gardner *et al.*, 2001; Edwards *et al.*, 2001a), the effects of chronic intrauterine stress on the fetal HPA axis are less well understood. A model of chronic intrauterine stress has been developed in which the majority of the placental attachment sites, the uterine caruncles, are surgically excised from the uterus of the non-pregnant ewe (Alexander, 1964; Robinson *et al.*, 1979;

McMillen *et al.*, 2001). During the subsequent pregnancy, a restricted number of placentomes form, and this results in a chronic placental restriction of fetal substrate supply, hypoxaemia, and fetal growth restriction throughout late gestation (Robinson *et al.*, 1979; McMillen *et al.*, 2001). Circulating cortisol concentrations are higher in growth restricted fetuses of carunclectomised ewes, but surprisingly, there is no associated increase in the fetal plasma concentrations of either immunoreactive adrenocorticotrophic hormone (ACTH) or $ACTH_{(1-39)}$, and the expression of proopiomelanocortin messenger ribonucleic acid (mRNA) is decreased in the anterior pituitary of the growth-restricted fetuses (Phillips *et al.*, 1996b). As discussed in Chapter 2, different subpopulations of corticotrophs, which are responsive to either corticotrophin-releasing hormone (CRH) or to arginine vasopressin (AVP) and which are differentially sensitive to negative feedback to cortisol, have been described in the adult and fetal sheep pituitary (Schwartz *et al.*, 1994; Perez *et al.*, 1997; van de Pavert *et al.*, 1997; Butler *et al.*, 1999). One possibility is that the uterine carunclectomy programs a change in the developmental characteristics of the pool of corticotroph cells within the pituitary to maintain ACTH secretion in the face of elevated cortisol concentrations during late gestation.

One important feature of uterine carunclectomy is that it does not inevitably lead to chronic fetal hypoxaemia in late gestation, as there is a degree of compensatory growth of the remaining placentomes which often results in the maintenance of a relatively normoxaemic, well grown fetus (Robinson *et al.*, 1979; Owens & Robinson, 1988; Owens *et al.*, 1994; McMillen *et al.*, 2001). Therefore, among the effects of uterine carunclectomy on the fetal HPA axis, it is possible to separate out those that may be due to a perturbation of the intrauterine environment of the early embryo from those associated with the development of chronic substrate restriction and subsequent fetal growth

restriction. In this chapter, we have determined whether uterine carunclectomy alters the functional heterogeneity of corticotrophic cell types within the fetal pituitary, and whether these changes are related to the perturbation of the early intrauterine environment associated with uterine carunclectomy or are solely due to the impact of chronic restriction of fetal substrate supply in late gestation. The impact of uterine carunclectomy associated with either fetal hypoxaemia or fetal normoxeamia on the ACTH-secretory characteristics of pituitary corticotrophic cells and on the proportion of corticotrophic cells in the fetal anterior pituitary that are CRH-responsive has also been determined. These results provide insight into the mechanisms by which perturbations of the early and late intrauterine environment may result in changes in functional characteristics of corticotrophs in the developing pituitary.

3.3 MATERIALS & METHODS

All experiments in the study were carried out according to the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Procedures and approved by the Standing Committee on Ethics and Animal Experimentation at the University of Adelaide.

3.3.1 Basic procedures

3.3.1.1 Animals & Surgery

Twenty six pregnancy dated Merino × Border Leicester ewes were used in this study. In fifteen non-pregnant ewes (carunclectomised group; CR), the majority of visible

endometrial caruncles were surgically removed as previously described by Alexander (1964), Robinson et al. (1979) and Edwards et al. (1999). This procedure restricts the number of placental cotyledons that would normally form after implantation, subsequently limiting placental, and hence fetal, growth (Robinson *et al.*, 1979). Caruncle ctomy was performed under aseptic conditions with general anaesthesia induced by intravenous sodium thiopentone (1.25 g; Pentothal, Rhone Merieux Australia, Pinkenba, QLD, Australia) and maintained with halothane (2.0-2.5% in oxygen; Fluothane, Zeneca, Macclesfield, United Kingdom). With the ewe in a supine position, 2 ml ilium penstrep was administered intra-muscularly (I.M.; procaine penicillin, 250 mg ml^{-1}; dihydrostreptomycin sulphate, 250 mg ml^{-1}; procaine hydrochloride, 20 mg ml^{-1}: Troy Laboratories, Smithfield, NSW, Australia), the abdominal wool shorn and the skin disinfected. A low midline abdominal incision (approximately 8 cm) allowed access to the uterus. After locating the uterus and positioning it externally, the first horn was opened along the antimesometrial border from the level of the cervix to the uterotubule junction. The majority of visible caruncles (86 to 95%) were then excised. The uterus was closed with continuous inverting sutures of 2/0 catgut (Ethicon; Johnson and Johnson Medical, North Ryde, NSW, Australia) through the myometrium and serosa. To reduce the formation of adhesions, the internal uterine wall was swabbed clean while the inverting sutures were completed and the procedure was repeated for the other horn. On completion of the suturing of the second horn, the uterus was replaced in the abdominal cavity. The abdomen was closed in two layers, first the peritoneum and rectus sheet and secondly the skin and subcutaneous tissue with continuous sutures using 4/0 coated Vicryl (Ethicon; Johnson and Johnson Medical). The ewes were then kept under close observation for 4 – 7 days after surgery before returning to the farm prior to mating. After a minimum of 9 weeks` recovery from surgery the ewes entered a mating program and singleton

pregnancies were confirmed by ultrasound at approximately 50 days of gestation. The 11 remaining ewes underwent no uterine surgery (non-carunclectomised; NCR), but were mated under the same conditions as the CR ewes.

The pregnant ewes were brought back to the University Animal House between days 98 and 117 of pregnancy. The CR fetuses (n = 15) and NCR (n = 11) were catheterised between 110 and 120 days gestation as described in Chapter 2. Vascular catheters were inserted into a fetal and maternal jugular vein and/or carotid artery and into the amniotic cavity. All catheters were filled with sterile heparinised saline (500 I.U. ml^{-1}; Multiparin, Fisons Pharmaceuticals, Sydney, NSW, Australia), and exteriorised via an incision in the ewe's flank.

The ewes were housed under a 12 h light-dark cycle and fed once daily between 0900 and 1300h with water available *ad libitum*.

3.3.1.2 Blood sampling

Fetal arterial blood samples (0.3 ml) were collected for the first four days following surgery and then three times per week until and including the day of the fetal post mortem. The arterial blood samples were used to measure the partial pressure of oxygen (P_aO_2) and carbon dioxide (P_aCO_2), pH, haemoglobin content (Hb) and oxygen saturation (S_aO_2) using an ABL 520 blood gas analyser (Radiometer, Copenhagen, Denmark) with correction for the higher value of fetal body temperature. Fetal arterial blood samples (2 – 3.5 ml) were also collected for plasma cortisol determination. One to two ml of fetal blood was placed in a tube containing dipotassium ethylenediamine tetraacetic acid (EDTA: 18.6 g l^{-1} of whole blood; Disposable Products, Technology Park, SA, Australia) and the remaining

fetal blood was placed in a tube containing lithium heparin (125 I.U.; Disposable Products) with both tubes also containing aprotinin (1000 k.I.U. ml^{-1} blood; Sigma Chemical Company, St Louis, MO, USA). The blood samples were centrifuged at 1800 *g* for 10 min at 4°C and the plasma separated into aliquots and stored at -20°C.

3.3.1.3 Post Mortem & Pituitary Collection

Between 138 and 145 days of gestation, ewes were killed with an intravenous overdose of sodium pentobarbitone (200 mg kg^{-1}; Lethobarb, Syntex, Castle Hill, NSW, Australia). The fetal sheep were delivered via laparotomy, weighed and then killed by decapitation. Each pituitary was quickly removed and immediately placed into cold HEPES-dissociation buffer (HDB: NaCl, 137 mM; KCl, 5 mM; Na_2HPO_4, 0.7 mM (Merk, Kilsyth, VIC, Australia); HEPES, 25 mM (Trace Biosciences, Castle Hill, NSW, Australia); pH 7.36).

3.3.1.4 Cell Preparation

Cultured anterior pituitary (AP) cells were prepared as previously described by Fora et al. (1996) with minor alterations of the methods described in Chapter 2. The AP (pars distalis) and the neurointermediate lobes (pars nervosa and pars intermedia) of each pituitary were gently separated by blunt dissection and the AP tissue was subsequently minced into small fragments. The fragments were washed in HDB and then placed in HDB solution containing collagenase II (0.04%; Worthington Biochemical Corporation, Freehold, NJ, USA) and deoxyribonuclease I (Sigma Chemical Company) and gently rocked for 2.5 h at 37°C on an orbital shaker. The AP fragments were then dispersed into individual cells by gentle trituration, which were then centrifuged for 5 min at 400 *g*. The

cell pellet was washed by suspension in 5.0 ml culture medium [Dulbecco's Modified Eagle's Medium (DMEM) plus Ham's F12 medium (F12, 1:1; Gibco BRL, Life Technologies, Grand Island, NY, USA) and charcoal-stripped fetal calf serum (10% of culture medium)] and centrifuged. The cell washing cycle was repeated a further two times. The cells were then plated in culture medium (1 ml), at approximately 2.0×10^5 cells $well^{-1}$ in 48-well tissue culture plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) and incubated at 37°C in a water saturated 5% CO_2 atmosphere (BB 16 Gas Incubator, Haraeus Instruments, Hanau, Germany).

3.3.2 Experimental Protocol

Twenty four hours after plating the cells, the cells in half the wells from each pituitary were treated with either vehicle or C-TOX (final concentration of 25 nmol l^{-1}), the toxin that specifically eliminates CRH-target cells described in Chapter 2. All cells were washed extensively in culture medium 18 to 24 h later (following day) to remove the C-TOX or vehicle treatments. Two days after that, the cells were again washed extensively with incubation medium (DMEM/F12 mixed 1:1; Gibco BRL, Life Technologies) containing 0.2% Polypep (Trace Biosciences, Castle Hill, NSW, Australia) and allowed to equilibrate to serum-free conditions for 1 h. For the experimental incubations, the cells were again washed in incubation medium and treated for 5 h with either medium containing vehicle (also termed basal; n = 26), oCRH (10^{-7} M; n = 26), AVP (10^{-7} M; n = 23) or oCRH + AVP (n = 19; Auspep, Richmond, VIC, Australia). After 5 h the culture media were collected and stored at -20°C and the cells in the tissue culture plates were stored at -80°C both in preparation for assaying. Later 0.1 M HCl (1 ml) was added to each well of frozen cells. Cellular extracts of ACTH were subsequently obtained by

thawing and refreezing the cells three times (-80°C) and using mechanical trituration on the third cycle. These extracts were stored at -20°C.

3.3.3 Analyses

3.3.3.1 ACTH Radioimmunoassay

The concentration of irACTH in the cell culture media and cellular extracts, referred to as ACTH throughout the Chapter, were measured using a double antibody radioimmunoassay as previously described and validated in Chapter 2 and Butler *et al.* (1999). The standards (1.95 - 500 pg tube^{-1}) were made from synthetic human (*h*)ACTH$_{1\text{-}39}$ (Peninsula Laboratories, Belmont, CA, USA) and the ACTH antisera (Anti-ACTH 1000T) was supplied by ICN Biomedicals (Costa Mesa, CA, USA). The sensitivity of the assay was 1.95 pg tube^{-1}. The interassay coefficient of variation was 18.3%, and the intraassay coefficient of variation was less than 10%.

3.3.3.2 Cortisol Radioimmunoassay

Cortisol was extracted from fetal plasma with dichloromethane (Merck, Kilsyth, VIC, Australia) as previously described by Bocking *et. al.* (1986) and measured using the Orion Diagnostica cortisol radioimmunoassay kit (Orion Diagnostica, Espoo, Finland; (Phillips *et al.*, 1996b)) as described in Chapter 2. The cortisol standards ranged between 0.078 and 10.0 pg tube^{-1} and the sensitivity of the assay was determined as 0.078 pg tube^{-1}. The efficiency of the recovery of the iodinated cortisol (^{125}I-Cortisol) was always greater than 90% whilst the interassay coefficient of variation was less than 10%, and the intraassay coefficient of variation was less than 10%.

3.3.3.3 Statistics

3.3.3.3.1 *IN VIVO* EXPERIMENTS

Fetal blood gas values are expressed as mean ± standard error of the mean (S.E.M.). The mean P_aO_2, P_aCO_2, pH, Hb and S_aO_2 were calculated for each fetus as the average of all P_aO_2, P_aCO_2, pH, Hb and S_aO_2 values obtained between 111 and 145 days gestation. Arterial oxygen content (O_2 content) per 100 ml blood (ml dl^{-1}) was calculated for each fetus as described in Chapter 2.

Nine of the fifteen fetuses (60%) in the CR group, and four of the eleven fetuses (40.0%) in the NCR group had a mean gestational P_aO_2 of less than 16 mmHg and these fetuses were categorised as being hypoxaemic (HX). The remaining fetuses in the CR and NCR groups were categorised as normoxaemic (NX).

The separate and combined effects of caruncleclectomy and hypoxaemia on the mean gestational values for fetal arterial blood gas and pH were analysed using multifactorial analysis of variance (ANOVA), with a Statistical Package for Social Sciences on a Vax mainframe computer (SPSSX; SPSS Inc., Chicago, Illinois, USA).

Similarly, the effects of caruncleclectomy and hypoxaemia on the gestational age profile of the fetal arterial blood gas and pH values were determined using a multifactorial ANOVA using repeated measures, with caruncleclectomy, hypoxaemia and gestational age (grouped into 5-day windows) as the specified factors and repeated measures.

3.3.3.3.2 IN VITRO EXPERIMENTS

For the cell culture experiments, each treatment was completed in replicate, and the replicate values for the measured ACTH in either the culture media (secreted) or ACTH in the cellular extracts (stored) were averaged to yield an n = 1 for each pituitary in each treatment group. In this Chapter (and following Chapters), total ACTH refers to the arithmetic sum of secreted ACTH and stored intracellular ACTH under basal conditions. All values are expressed as mean ± S.E.M.

The effects of caruncleclectomy, hypoxaemia or C-TOX on the total ACTH (per well), the amount of total ACTH secreted by the corticotrophic cells (per 10^{-4}) and the absolute amount of ACTH (secreted) during the basal conditions were compared using multifactorial ANOVA (with CR vs NCR, hypoxaemia vs normoxaemia and with vs without C-TOX pretreatment as the specified variables). The percentage of total ACTH secreted under basal conditions was also analysed using a multifactorial ANOVA with caruncleclectomy, hypoxaemia and C-TOX as the statistical parameters.

The proportion (%) of total ACTH present in the CRH-responsive cells (i.e. C-TOX-sensitive cells) was calculated using the following equation:

$$\frac{V - Cyto}{V} \times 100$$

where:

V = total ACTH in vehicle-treated wells under basal conditions

Cyto = total ACTH in C-TOX treated wells under basal conditions

The effects of caruncleclectomy and hypoxaemia on the proportion of ACTH stored in CRH-target cells were analysed using a multifactorial ANOVA.

The ACTH secretory responses to CRH (10^{-7} M), AVP (10^{-7} M) and CRH + AVP (expressed as a percentage of total ACTH) were expressed as fold changes above basal ACTH secretion. The effects of carunclectomy, hypoxaemia and G-TOX on the fold changes in ACTH secretion after CRH, AVP and CRH +AVP stimulation were analysed using multifactorial ANOVA.

A fetal blood sample was available from 17 fetal sheep during the week before death (137 – 145 days; NCR or 138 – 144 days; CR) and the separate and combined effects of carunclectomy and hypoxaemia on the plasma cortisol concentrations in these fetuses were determined using multifactorial ANOVA. Simple linear regression analysis was also used to determine relationships between plasma cortisol concentrations and the percentage of ACTH secreted under basal conditions, the fold changes in ACTH secretion after secretagogues, and the proportion of ACTH stored in the CRH-responsive corticotrophs.

For all analyses, where the Cochrans and Bartlett-Box tests identified significant heterogeneity of variance, the data were logarithmically transformed prior to ANOVA. Where the multifactorial ANOVA's identified significant interactions between major variables, the data was split on the basis of the interactions and reanalysed. When the ANOVA's indicated there was differences between the groups, the Duncan's post hoc test was used to identify the significant differences between the mean values. For all differences $P < 0.05$ was considered to be significant.

3.4 RESULTS

3.4.1 Fetal outcomes

Carunclectomy and hypoxaemia each independently resulted in a significant reduction in fetal body weight (Table 3.1).

The S_aO_2, arterial O_2 content and pH were significantly lower in animals in the CR group (i.e. CR-normoxaemic and CR-hypoxaemic fetuses) when compared with the NCR group (i.e. NCR-normoxaemic and NCR-hypoxaemic fetuses) (Table 3.1; Figure 3.1). The mean gestational P_aO_2, S_aO_2, and arterial O_2 content were also significantly lower, and Hb content was significantly higher, in the hypoxaemic fetuses in each group when compared with the normoxaemic fetuses in the CR and NCR groups (Table 3.1; Figure 3.1).

3.4.2 Plasma cortisol concentrations

There were no significant separate or combined effects of either hypoxaemia or carunclectomy on plasma cortisol concentrations, which were 90.6 ± 31.5 nmol l^{-1} (n=7) in the hypoxaemic fetuses and 45.2 ± 21.0 $nmol^{-1}$ (n=10) in the normoxaemic group.

3.4.3 Effect of carunclectomy and hypoxaemia on total ACTH content

There was no significant effect of carunclectomy on the total ACTH content when expressed as either total ACTH per 10^4 cells or ng ACTH per well. The ACTH content measured in ng per 10^4 cells was: NCR–normoxaemic, 2.26 ± 0.86; NCR–hypoxaemic, 1.34 ± 0.36: CR–normoxaemic, 1.26 ± 0.29; CR–hypoxaemic, 0.89 ± 0.20 (Figure 3.2).

TREATMENT		P_aO_2 (mmHg)	P_aCO_2 (mmHg)	pH	Hb (g dl^{-1})	S_aO_2 (%)	O_2 Content (ml dl^{-1})	Weight (kg)
NCR	NX *(n=7)*	19.3 ± 0.9	45.8 ± 1.0	7.409 ± 0.006	9.9 ± 0.5	66.3 ± 3.4	9.1 ± 0.4	4.49 ± 0.33
	HX *(n=4)*	13.8 ± 1.1$^{\#}$	44.6 ± 0.9	7.402 ± 0.005	11.4 ± 0.7$^{\#}$	54.0 ± 3.1$^{\#}$	8.5 ± 0.5$^{\#}$	3.45 ± 0.52$^{\#}$
CR	NX *(n=6)*	19.1 ± 0.3	46.2 ± 1.7	7.395 ± 0.007†	10.0 ± 0.5	63.0 ± 1.2†	8.8 ± 0.4†	4.04 ± 0.11†
	HX *(n=9)*	13.5 ± 0.7$^{\#}$	46.5 ± 1.6	7.382 ± 0.007†	12.2 ± 0.5$^{\#}$	43.9 ± 3.2$^{\dagger\#}$	7.4 ± 0.4$^{\dagger\#}$	2.75 ± 0.38$^{\dagger\#}$

Table 3.1 Effect of caruncleclectomy and hypoxaemia on fetal blood gas status and fetal body weight

Mean values for arterial blood gas variables between 111 and 145 days gestation and body weight in NCR and CR fetal sheep. All values are expressed as mean ± S.E.M. Superscripts indicate significant differences between the mean values in the NCR and CR groups (†) or between the mean values in the normoxaemic (NX) and hypoxaemic (HX) groups (#), ($P < 0.05$).

There was also no effect of hypoxaemia on the total ACTH present in the pituitary cells in either the CR or NCR groups (Figure 3.2).

3.4.4 Effect of caruncleclectomy and hypoxaemia on ACTH secretion

The percentage of total ACTH that was secreted during the 5 h basal period was significantly greater ($F = 5.0$, $P < 0.05$) in pituitary cells from CR fetal sheep than in those from NCR fetal sheep (Table 3.2). Hypoxaemia itself, however, had no separate effect on basal ACTH secretion in the CR and NCR groups (Table 3.2). There was also no significant correlation between plasma cortisol concentrations and basal ACTH secretion.

The ACTH-secretory responses by intact populations of control cells (NCR, normoxemic, no C-TOX) were robust and indicative of normal, healthy *in vitro* responsiveness. In cells from NCR fetuses (Figure 3.3), CRH stimulated ACTH secretion 9.4 ± 1.5 fold and AVP stimulated ACTH secretion 10.6 ± 2.0 fold. The response to CRH and AVP in combination, 16.1 ± 4.3 fold, was significantly greater than the responses to either peptide alone. Caruncleclectomy attenuated the ACTH-secretory responses to the hypothalamic peptides, such that the respective increases in ACTH secretion in the presence of CRH, AVP or CRH + AVP were significantly lower in the CR than NCR group (Figure 3.3). Despite this attenuation, the responses represent a significant increase over basal ACTH secretion, and the response to CRH + AVP in combination remained significantly greater than the response to either peptide alone.

Interestingly, in these intact (no C-TOX) populations there was no separate effect of hypoxaemia on the ACTH responses to the peptides in either the CR or the NCR group. There was also no significant correlation between plasma cortisol concentrations and the

ACTH secretory responses to CRH, AVP or to CRH + AVP when the fetuses from all groups were combined.

Table 3.2 The percentage of total ACTH secreted under basal conditions following prior treatment with vehicle or C-TOX

TREATMENT		No C-TOX	C-TOX
NCR	NX	1.52 ± 0.31	5.02 ± 0.96§
	HX	1.85 ± 0.24	4.21 ± 0.65§
CR	NX	2.55 ± 0.61†	5.78 ± 1.35†§
	HX	5.30 ± 1.52†	8.38 ± 2.31†§

All values are expressed as means ± S.E.M. Superscripts indicate significant differences between the mean values in the NCR and CR groups (†) or between the mean values in the vehicle and C-TOX groups (§), ($P < 0.05$).

3.4.5 Effect of C-TOX and hypoxaemia on Total ACTH content

After treatment with G-TOX, the elimination of CRH-target cells was associated with a marked decrease in the total ACTH in the pituitary cells in both the CR and NCR groups, and there was no effect of carunclectomy on this decrease (Figure 3.2). In contrast, there was a significant interaction between the effects of G-TOX treatment and the effects of hypoxaemia on the total ACTH. The relative decrease in total ACTH after G-TOX treatment was attenuated in the hypoxaemic groups (CR-hypoxaemic and NCR-hypoxaemic) than in the normoxemic groups (Figure 3.2). The proportion of ACTH contained in CRH-target cells (i.e. cells susceptible to C-TOX treatment) was therefore less in the hypoxaemic than in the normoxemic fetuses in both the CR and NCR groups

(Figure 3.4). There was no significant correlation, however, between plasma cortisol concentrations and the proportion of ACTH contained in the CRH target cells when the CR and NCR groups were combined.

3.4.6 Effects of C-TOX on Basal and Stimulated ACTH Secretion

After C-TOX treatment, there was a similar and significant increase in the fraction of the total ACTH secreted during basal conditions in the CR and the NCR groups, and this increase was not influenced by hypoxaemia (Table 3.2). After elimination of the CRH-target cells, basal ACTH secretion was significantly higher in the CR group than in the NCR group (Table 3.2). Importantly, there was no separate effect of hypoxaemia on basal ACTH secretion after C-TOX treatment (Table 3.2). The increased proportion of ACTH secreted after C-TOX treatment was primarily a result of the decrease in total ACTH, as C-TOX treatment did not alter the absolute amount of ACTH secreted.

Elimination of CRH-target cells significantly decreased the ACTH responses to all secretagogues. Significantly, there were differences in the effect of C-TOX treatment among the various experimental groups (Figure 3.5A & B). In the NCR groups, there was a small residual response to the hypothalamic peptides (Figure 3.5A). Although these responses were much attenuated when compared with the corresponding ACTH responses in the intact (no C-TOX) populations, there were significant responses to CRH and AVP alone and a greater response to CRH + AVP in combination. Interestingly, the presence or absence of hypoxaemia made no significant difference to the fold changes in ACTH secretion. Pituitary cells from the CR fetuses, however, behaved more like cells from the adult sheep pituitary in that there was no response to CRH following elimination of the CRH-target cells (Figure 3.5B). In addition, there was no response to AVP alone and no

effect of CRH + AVP in combination. As in the NCR group, there was no significant difference between the CR hypoxaemic and CR normoxemic fetal pituitary cells.

The addition of CRH, AVP or CRH + AVP to the incubation medium significantly increased ($F = 135.0$, $P < 0.001$) ACTH secretion above the basal levels in the CR and NCR groups (Figure 3.5A & B). The fold change in ACTH secretion (above baseline) after CRH + AVP was significantly greater ($P < 0.05$) than the ACTH response to either CRH or AVP alone in both the CR and the NCR groups (Figure 3.5A). The fold changes in ACTH secretion after CRH, AVP and CRH + AVP were significantly lower ($F = 4.46$, $P < 0.05$), however, in the CR (Figure 3.5B) than in the NCR group. There was no separate effect of hypoxaemia on the fold changes in ACTH in response to these secretagogues in either the CR or NCR group.

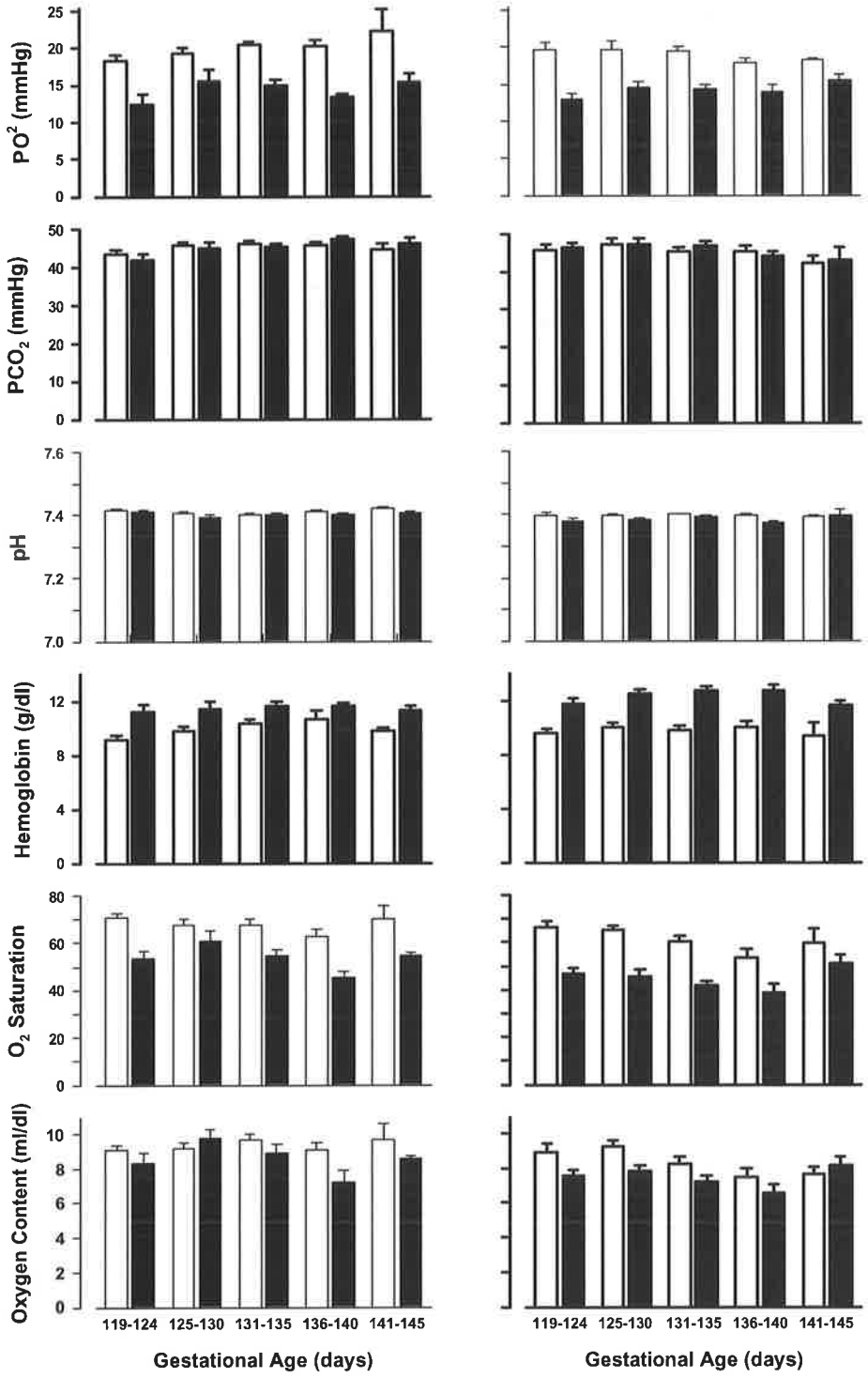


Figure 3.1 Effect of caruncleclectomy and hypoxemia on the gestational fetal blood gas profile
Mean arterial blood gases as a function of gestational age in NCR (left panels) and CR (right panels) groups. Open bars illustrate the values obtained in the normoxaemic (NX) fetuses and solid bars illustrate the values in the hypoxaemic (HX) fetuses. All values are expressed as mean ± S.E.M. The statistical analyses are summarised in Table 3.1.

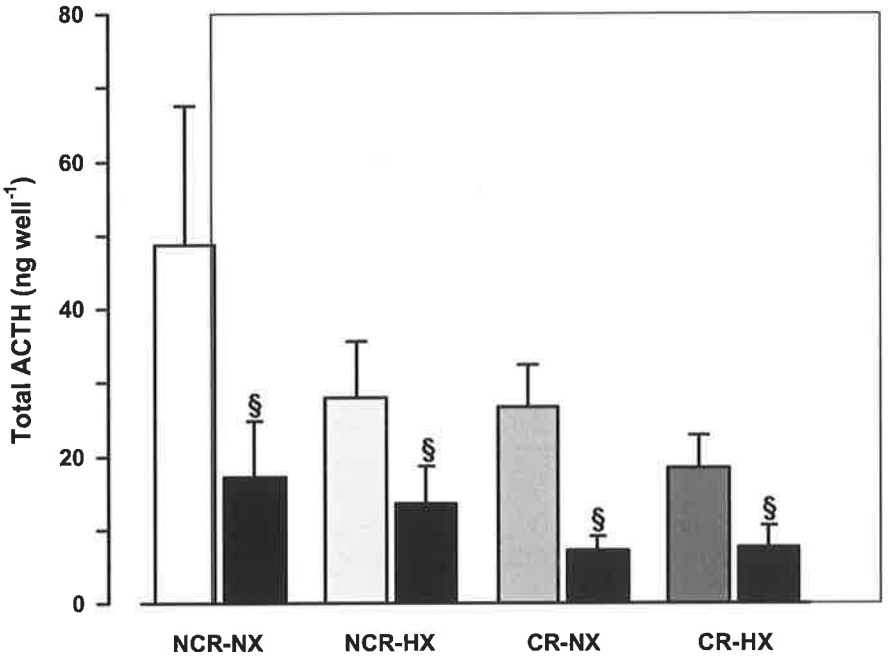


Figure 3.2 Effect of C-TOX on total ACTH content

The total ACTH (ng well $^{-1}$; means ± S.E.M.) measured during basal conditions in the NCR-normoxaemic (NCR-NX) and CR-NX groups and in the NCR-hypoxaemic (NCR-HX) and CR-HX groups after vehicle (open bars) or C-TOX treatment (dark bars). Open, normoxaemia; light grey, hypoxaemia; medium grey, normoxaemia and maternal carunclectomy; dark grey, hypoxaemia and maternal carunclectomy. The superscripts indicate the significant effect of C-TOX on total ACTH content, ($P < 0.05$).

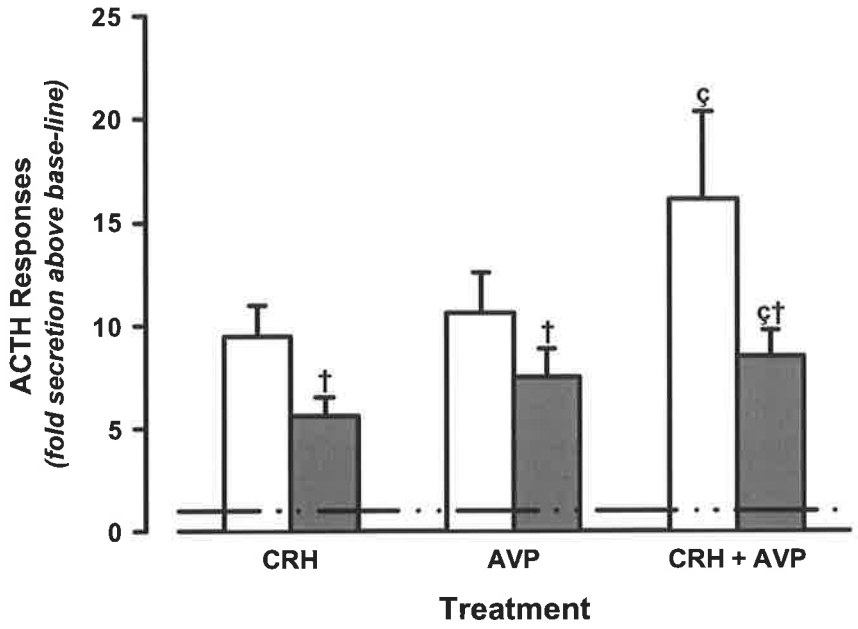


Figure 3.3 The ACTH response to the hypothalamic secretagogues in the absence of C-TOX pretreatment

The change in ACTH (means ± S.E.M.) secretion in response to CRH, AVP, or CRH + AVP administration in the NCR (open bars) and CR (shaded bars) groups in the absence of C-TOX treatment. The horizontal dashed line denotes basal secretion. The superscript (†) indicates that the response to the hypothalamic secretagogues was significantly higher in the NCR group than in the CR group, whilst (ç) indicates that the response to CRH + AVP was significantly higher than the response to CRH or AVP in both the NCR and CR groups, ($P < 0.05$).

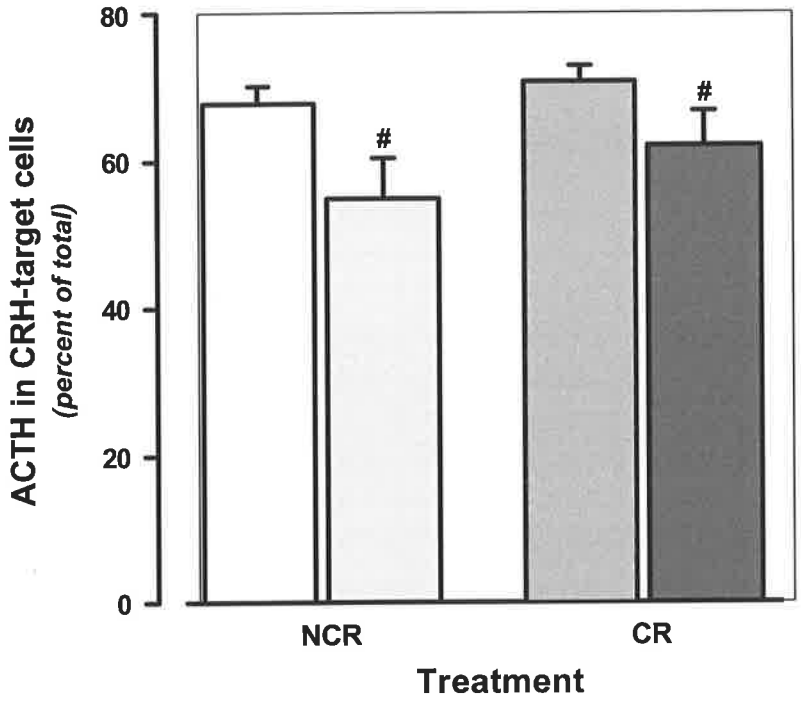


Figure 3.4 Proportion of total ACTH contained in CRH-responsive Cells

The proportion of total ACTH (means ± S.E.M.) present in CRH-responsive cells in the NCR and CR treatment groups. Open, normoxaemia; light grey, hypoxaemia; medium grey, normoxaemia and maternal carunclectomy; dark grey, hypoxaemia and maternal carunclectomy. The superscript indicates the proportion of ACTH present in the CRH-responsive cells was significantly lower in the hypoxaemic than in the normoxaemic pituitaries in both the NCR and CR groups, ($P < 0.05$).

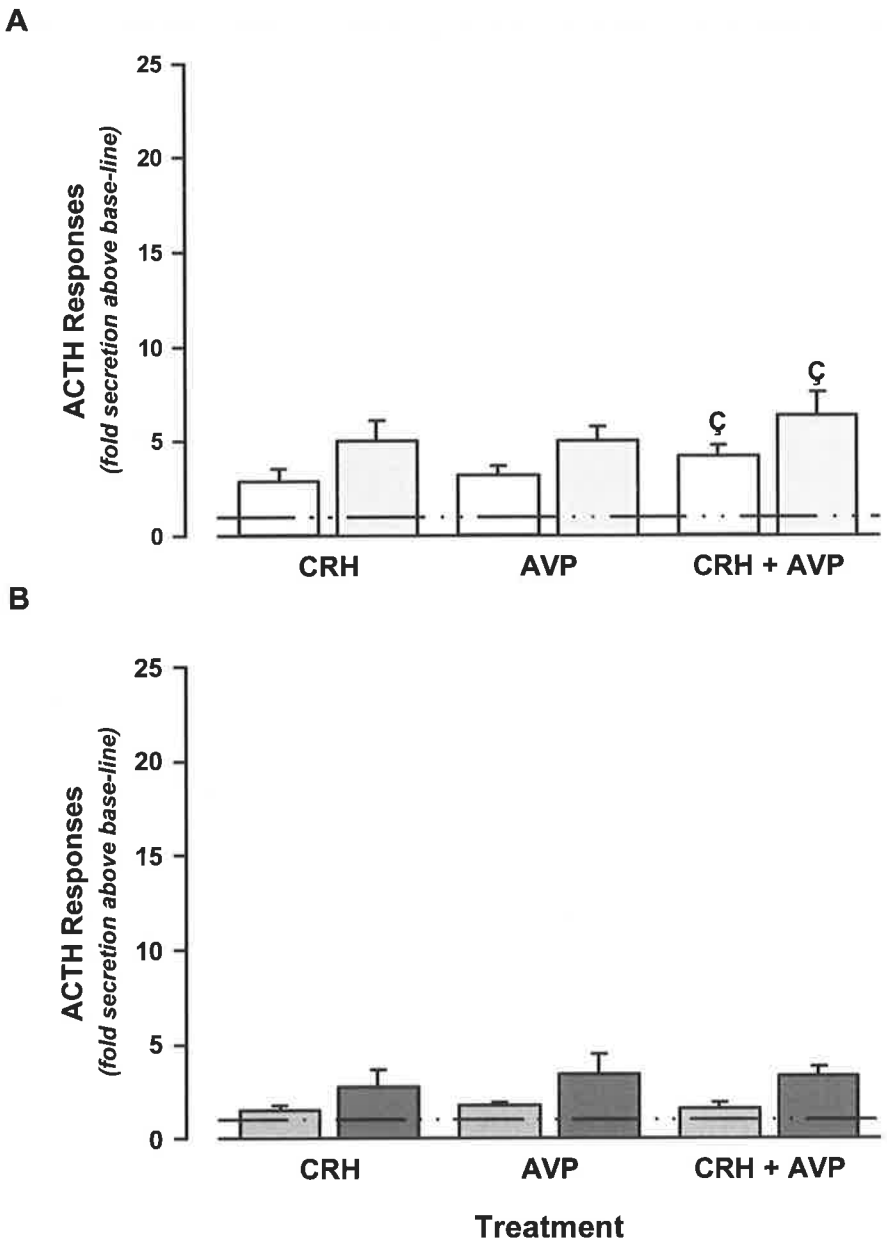


Figure 3.5 The ACTH fold change above baseline in response to the hypothalamic secretagogues in the presence of C-TOX pretreatment

(**A**) Fold change in ACTH secretion (means ± S.E.M.) after CRH, AVP, or CRH + AVP administration in the NCR group after C-TOX treatment. Open, normoxaemia; light grey, hypoxaemia. The superscript (ç) indicates that the fold change in response to CRH + AVP was significantly higher than the fold changes in response to CRH or AVP alone, ($P < 0.05$). (**B**) Fold change in ACTH secretion (means ± S.E.M.) after CRH, AVP, or CRH + AVP administration in the CR group after C-TOX treatment. Medium grey, normoxaemia and maternal carunclectomy; dark grey, hypoxaemia and maternal carunclectomy. The horizontal dashed lines denote basal secretion.

3.5 DISCUSSION

This study has found that there are differential effects of altering the early and late intrauterine environment on the functional characteristics of the corticotrophic cells in late gestation. The results are consistent with the emergence of a population of non-CRH-target cells that secrete high amounts of ACTH in the pituitaries of fetuses that were exposed to uterine carunclectomy in early gestation. The emergence of this subpopulation of corticotrophs in the CR group during late gestation occurred whether or not these fetuses were hypoxaemic throughout late gestation. Exposure to chronic hypoxaemia during late gestation, however, independently resulted in a specific reduction in the proportion of ACTH stored in CRH-target cells. Thus, the ACTH-synthetic capacity of the CRH-target cells, which appear to contribute relatively less to basal ACTH secretion, but which secrete ACTH in response to a superimposed acute intrauterine stress, may be diminished in the chronically hypoxaemic fetus during late gestation.

3.5.1 Carunclectomy and fetal substrate restriction

In the current study, 9 (of 15) fetuses in the CR and 4 (of 11) fetuses within the NCR group were chronically hypoxaemic with mean gestational P_aO_2 values of $\leq$ 16 mmHg (Char & Creasy, 1977; Gardner *et al.*, 2002). In the remaining fetal sheep the mean P_aO_2 was over 19 mmHg i.e. well within the normoxaemic range reported for healthy fetal sheep (Char & Creasy, 1977; Robinson *et al.*, 1979; Phillips *et al.*, 1996b; Edwards *et al.*, 1999; Gardner *et al.*, 2002). While the mean values for fetal arterial pH were marginally less in the CR, than NCR group overall, it should be noted that the arterial pH values in both the CR-NX and CR-HX groups were well within the normal range for healthy fetal sheep in late gestation, i.e., this group was not acidaemic. Fetal acidaemia is therefore not a

contributing factor to the changes that occurred in the ACTH-synthetic and -secretory characteristics of the corticotrophs in the CR group. It can be proposed that the changes in the ACTH-secretory characteristics of the corticotrophs in the CR groups are a consequence of the early intrauterine intervention, as there is no evidence for restriction of a fetal substrate present in both the CR-normoxaemic and CR-hypoxaemic groups.

3.5.2 Impact of carunclectomy on basal ACTH secretion

The percentage of ACTH secreted under basal conditions was greater in the CR than NCR fetuses and this effect was independent of the prevailing fetal P_aO_2. As argued above, it is therefore unlikely that this increase in basal ACTH secretion is related to the effects of placental restriction experienced by fetuses *in vivo* in late gestation in the CR group. Interestingly, the higher basal output of ACTH was maintained in the CR group after C-TOX treatment, reflecting maintained ACTH secretion despite a decrease in total ACTH content.

It has been concluded that in adult rat and sheep pituitaries there are at least two populations of corticotrophs, one responsive to CRH and AVP (and susceptible to C-TOX), and one that secretes the majority of ACTH under basal conditions, responding to AVP but not CRH (Jia *et al.*, 1991; Jia *et al.*, 1992; Schwartz *et al.*, 1994; Perez *et al.*, 1997; van de Pavert *et al.*, 1997; Butler *et al.*, 1999). The maintenance of basal ACTH secretion after treatment with C-TOX is consistent with findings reported in Chapter 2 and supports the presence of at least one type of corticotroph which accounts for basal ACTH secretion and is resistant to C-TOX pre-treatment (Butler *et al.*, 1999).

In the current study, there was no difference in the ACTH secretory responses to AVP alone or in combination with CRH in the CR group after C-TOX treatment. This is in contrast to the NCR group, where the ACTH responses to AVP in combination with CRH were greater than with CRH alone after C-TOX treatment. This implies that the ACTH response to AVP in the pituitaries of CR fetuses in the absence of C-TOX treatment is derived from cells that are responsive to both AVP and CRH. While previous studies with C-TOX in pituitary cells from the fetal and adult sheep have not provided evidence for a population of corticotrophs that secretes ACTH under basal conditions but does not respond to AVP, such cells have been described in adult rat anterior pituitary cells using the reverse hemolytic plaque assay (Jia *et al.*, 1991).

In the present study pituitary cells from normoxaemic NCR fetuses had similar levels of basal ACTH secretion to those measured in Chapter 2 pituitary cells collected from healthy, term fetal sheep (Butler *et al.*, 1999). Consistent with the previously observed effects of C-TOX in Chapter 2, pituitaries from normoxaemic NCR and CR fetuses in the present studies showed an increase in basal ACTH secretion in the normoxaemic NCR group after C-TOX treatment.

Interestingly, the ACTH responses to CRH and to CRH + AVP in the NCR group were higher after C-TOX than in the CR group. This contrasts with previous studies with C-TOX in adult ovine pituitary cells (Schwartz *et al.*, 1991b; Schwartz *et al.*, 1994) and in the study in fetal ovine pituitary cells reported in Chapter 2, in which there was no response to CRH, either alone or in combination with AVP, in the CRH-target-depleted populations. One explanation is that the C-TOX was ineffective in eliminating CRH-target cells. This is apparently not the case, since pituitaries removed from NCR and CR fetuses

were treated identically – with different effects in the two groups. In the current study, pituitary cells were exposed to the actions of the hypothalamic secretagogues for 5 h in culture, whereas in Chapter 2 the pituitary cells were treated for 3 h with the secretagogues. A possible explanation may be increased functional plasticity in the healthy developing pituitary, such that cells which do not have CRH receptors at the time of treatment with C-TOX and are therefore not susceptible to the toxin survive the treatment and subsequently become CRH-target corticotrophs. These surviving cells might be characterised as normally secreting ACTH under basal conditions, but only develop CRH receptors and CRH sensitivity after exposure to the actions of CRH in culture for periods exceeding 3 h. Alternatively, there might be some type of multipotential or stem cell that becomes a corticotroph after the treatment with C-TOX (Jia *et al.*, 1992). Schwartz and Vale (1988) have shown in rat corticotrophs, that a limited response to CRH reemerges at around 3 days after C-TOX treatment. In addition, Jia and coworkers (1992) have described the emergence of a new population of CRH-responsive corticotrophs following the elimination of the existing CRH-target cells by photoablation. Although the reemergence of an ACTH response to CRH has not been observed previously in ovine cells, it should be noted that the developmental plasticity of fetal ovine pituitary cells is likely to be higher than that of adult cells. During development the turnover rate for corticotrophs is extremely high, and probably involves mitosis and apoptosis as well as differentiation (Levy, 1999; Taniguchi *et al.*, 2000). Numerous other examples exist of plasticity in the anterior pituitary involving melanotrophs, somatotrophs, lactotrophs and gonadotrophs, in which there is interchange or transdifferentiation in the course of normal physiological adjustments (Goth *et al.*, 1996; Childs, 2000; Schwartz, 2000; Vazquez-Martinez *et al.*, 2001).

It is particularly noteworthy that the greater response to CRH after C-TOX treatment is a characteristic of pituitary cells from NCR, rather than CR fetuses. This suggests that the plasticity of the fetal pituitary, apparently characteristic of the control fetuses, is lacking in CR fetuses. Whether or not CR specifically has an effect on multipotential pituitary cells or transdifferentiation of other cells remains an interesting question.

3.5.3 *Impact of carunclectomy on corticotrophic cell types in the fetal pituitary in late gestation*

Although the basal output of ACTH was greater in the CR group, there was no difference in the proportion of ACTH present in CRH-target cells between the CR and NCR groups. The proportion of ACTH stored in CRH-responsive cells (i.e. C-TOX-sensitive cells) was around 70% of the total ACTH content in pituitaries from normoxaemic fetal sheep in both the CR and NCR groups, consistent with CRH-responsive cells of the pituitary of the healthy, term sheep fetus as reported in Chapter 2. The likeliest explanation of these results is that perturbation of the early intrauterine environment by carunclectomy results in the development of a population of non-CRH-target corticotrophs that secrete high amounts of ACTH during basal conditions but does not alter the ACTH content of the population of the CRH-target cells. Recent studies have found that nutritional or hormonal manipulation of the intrauterine environment or of the embryo during preimplantation period results in alterations in fetal growth patterns, fetal HPA function, and gestation length (Kleemann *et al.*, 1994; Kwong *et al.*, 2000; Edwards & McMillen, 2002). Thus the reprogramming of early pituitary development may be a key response to perturbations of the interaction between the uterine endometrium and the developing embryo.

3.5.4 *Impact of hypoxaemia on corticotrophic cell types*

Chronic hypoxaemia was associated with a decrease in the amount of ACTH stored in CRH-responsive cells and with a trend towards the reemergence of CRH sensitivity after C-TOX treatment. This effect was present to a greater extent in the NCR group. It has been demonstrated that fetal plasma cortisol concentrations are higher in the chronically hypoxaemic fetus in late gestation (Phillips *et al.*, 1996b; Gardner *et al.*, 2002). As reported in Chapter 2, cortisol can suppress ACTH synthesis in CRH-target corticotrophs in the fetal pituitary. In the present study, however, while there was a trend toward higher circulating cortisol concentrations in the hypoxaemic fetuses, the evidence did not support an inverse correlation between circulating cortisol and the proportion of ACTH stored in the CRH-responsive cells in the pituitaries from the fetal sheep in the NCR and CR groups. Alternatively the effect of chronic hypoxaemia on the amount of ACTH stored in the CRH-target cells may reflect a prior history of hypothalamic stimulation *in vivo* as a consequence of the low P_aO_2.

3.5.5 *Summary*

In summary, the results are consistent with the emergence of a population of non-CRH-target cells that secrete high amounts of ACTH in the pituitaries of fetuses that were exposed to uterine caruncleclectomy in early gestation. This may represent a reprogramming of the pattern of corticotroph development to ensure that fetal ACTH secretion can be maintained throughout late gestation independently of whether placental restriction of fetal substrate supply subsequently ensues. Given that altered corticotroph development persists in otherwise metabolically healthy fetuses in the CR group for up to five months after the early intrauterine intervention, it may also persist after birth to result in a maintained ACTH response to postnatal stressors.

While exposure to chronic hypoxaemia during late gestation did not appear to alter the subpopulations of corticotroph cell types present within the fetal pituitary, it did result in a specific reduction in the proportion of ACTH stored in CRH-target cells. Thus, the ACTH-synthetic capacity of the CRH-target cells, which do not appear to contribute to basal ACTH secretion, but which secrete ACTH in response to a superimposed acute intrauterine stress, may be relatively diminished in the chronically hypoxaemic fetus during late gestation. It has previously been reported that there is a decrease in proopiomelanocortin (POMC) mRNA levels, while basal circulating immunoreactive (ir) ACTH concentrations are maintained in the hypoxaemic CR fetus in late gestation (Phillips *et al.*, 1996b; Gardner *et al.*, 2002). Thus it is possible that circulating ACTH levels are maintained as a consequence of secretion from non-CRH-target corticotrophs whilst the expression of POMC is suppressed in the CRH-target cells. These patterns of altered corticotroph development are important given the central roles of the HPA axis in the fetal adaptive response to intrauterine stress and in the early programming of adult cardiovascular and metabolic disease (Barker, 1992 #312; Barker, 1998 #313].

4 THE EFFECT OF HYPOGLYCAEMIA ON THE FUNCTIONAL HETEROGENEITY OF CORTICOTROPHS IN THE FETAL SHEEP PITUITARY

4.1 SUMMARY

i. In Chapter 3 it was reported that, placental restriction in late gestation resulted in a decrease in the ACTH stored in CRH responsive corticotrophs. Placental restriction results in fetal hypoxaemia and hypoglycaemia and it is unclear whether the changes in the functional characteristics of the corticotrophs are related to either fetal hypoxaemia or hypoglycaemia. The aim of the present study was therefore to determine whether fetal hypoglycaemia, in the absence of fetal hypoxaemia, alters the ACTH synthetic or secretory capacity of the fetal corticotrophs.

ii. Ewes were maintained on either of two feeding regimes (Control, n = 7; Undernutrition; n =8). The mean plasma glucose concentrations were less than 1.2 mmol l^{-1} in six of the fifteen fetuses throughout late gestation and these fetuses were therefore classified as hypoglycaemic. Pituitaries were collected from all fetuses between 139 and 146 days gestation for cell culture. Cells in half the culture wells from each pituitary were treated with a selective cytotoxin (C-TOX) to eliminate corticotrophin releasing hormone (CRH)-target cells before treatment with either medium containing vehicle, ovine (*o*)CRH (10^{-7} M), arginine vasopressin (AVP; 10^{-7} M) or *o*CRH + AVP.

iii. There was no effect of either maternal undernutrition or fetal hypoglycaemia on the ACTH synthetic and secretory characteristics of the fetal corticotrophs in late

gestation. It therefore appears that the effects of placental restriction on the functional characteristics of the fetal corticotroph may be due to the direct or indirect actions of fetal hypoxaemia.

4.2 INTRODUCTION

In the preceding Chapter it was shown that in fetuses that experienced a chronic substrate restriction in late gestation, the proportion of ACTH stored in CRH responsive cells in the fetal pituitaries was reduced and that this effect occurred irrespective of whether the pregnant ewe had, or had not, been previously carunclectomised.

It has been previously shown that spontaneous or experimental restriction of placental substrate supply is associated with both fetal hypoxaemia and hypoglycaemia (Simonetta *et al.*, 1997). Simonetta and colleagues reported that fetal plasma glucose concentrations were less than 1.2 mmol l^{-1} in hypoxaemic, placentally restricted fetal sheep and greater than 1.2 mmol l^{-1} in normoxaemic, normally grown fetuses during late gestation. It has also been shown that there is a fetal ACTH response when plasma glucose concentrations fall below a threshold value of approximately 1.2 – 1.3 mmol l^{-1} after 135 days gestation (Edwards *et al.*, 2001a). The fetal ACTH response occurs independently of whether the fetal hypoglycaemia is induced acutely by fetal insulin infusion or chronically as a consequence of maternal undernutrition (Edwards *et al.*, 2001a). One possibility for the decrease in the amount of ACTH stored in the CRH responsive corticotrophs in the pituitaries of the placentally restricted fetal sheep may be the low circulating glucose concentrations present in these fetuses, rather than the associated chronic hypoxaemia.

The aim of the present study therefore was to determine whether chronic fetal hypoglycaemia (defined as less than 1.2 mmol l^{-1}) in late gestation, in the absence of fetal hypoxaemia, altered the ACTH synthetic and secretory capacity of the fetal corticotrophs.

In a large cohort of pregnant ewes it was determined that fetal glucose concentrations are dependent on both maternal nutrient intake and fetal number (Edwards *et al.*, 2001a). Edwards *et. al.* (2001a) found that fetal glucose concentrations in ewes maintained on a maintenance diet, were 1.5 mmol l^{-1} in singleton and 1.1 mmol l^{-1} in twin fetuses respectively. When ewes were fed approximately 50% of their normal diet, fetal plasma glucose concentrations fell to 1.2 mmol l^{1} in singleton and 0.7 mmol l^{1} in twin fetuses. Therefore, pregnant ewes were placed on a diet, which provided 100% of the energy requirements (Control group), or 50% of the energy requirements (Undernutrition group) and the effects of fetal hypoglycaemia on the ACTH synthetic and secretory capacity of the corticotrophs cells were determined. Based on the study by Edwards *et. al.* (2001a) fetuses with a plasma glucose concentration below a threshold value of 1.2 mmol l^{-1} were defined as being 'hypoglycaemic' and similarly fetuses with plasma glucose concentrations above this threshold were defined as being 'euglycaemic'.

4.3 MATERIALS & METHODS

All experiments in the study were carried out according to the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Procedures and approved by the Standing Committee on Ethics and Animal Experimentation at the University of Adelaide.

4.3.1 Basic Procedures

4.3.1.1 Animals & Surgery

Fifteen dated pregnant Merino × Border Leicester ewes were used in this study. The fetuses in the maintenance feeding regime (Control; n = 7) and restricted feeding regime (Undernutrition; n = 8) underwent surgery between 110 and 119 days gestation as described in Chapter 2. Vascular catheters were inserted into the carotid artery and jugular vein of the fetus and carotid artery and/or jugular vein of the ewe. A catheter was also placed into the amniotic cavity of each ewe. All catheters were filled with sterile heparinised saline (500 I.U. ml^{-1}; Multiparin, Fisons Pharmaceuticals, Sydney, NSW, Australia), and exteriorised via an incision in the ewe's flank.

The ewes were housed under a 12 h light-dark cycle and fed once daily after 1100 h with water available *ad libitum*. Fetal blood gas status was monitored from the day following surgery, and animals were allowed 1 to 4 days recovery from surgery, before collecting fetal arterial blood samples.

4.3.1.2 Feeding Regime & Blood Sampling

Ewes were weighed between 110 and 116 days gestation to determine their feed requirements. The ewes were either placed on a maintenance diet (Control group: 17 - 27 g lucerne kg^{-1} and 3-5 g oats kg^{-1} per day) or a reduced energy diet (Undernutrition group: 10 g lucerne kg^{-1} and 1.5 g oats kg^{-1} per day) from 115 days gestation. The feed allowance was increased by 15%, every 9 – 10 days for all the ewes in the cohort. Fetal arterial blood samples (3.5 ml) were collected three times per week before the daily feeding of the ewes, for the measurement of glucose throughout late gestation. Blood (2.5 ml) was placed into a tube containing lithium heparin (Sarstedt Australia, Technology Park, SA,

Australia) and into a tube (1 ml) containing dipotassium ethylenediamine tetraacetic acid (EDTA; Sarstedt Australia) and aprotinin (1000 k.I.U. ml^{-1} blood; Sigma Chemical Company, St. Louis, MO, USA). The blood samples were centrifuged at 1800 *g* for 10 min at 4°C and the plasma separated into aliquots and stored at -20°C. Fetal arterial blood samples (0.5 ml) were also collected for the measurement of partial pressure of oxygen (P_aO_2) and carbon dioxide (P_aCO_2), pH, haemoglobin content (Hb), oxygen saturation (S_aO_2) and glucose using an ABL 520 blood gas analyser (Radiometer, Copenhagen, Denmark).

4.3.1.3 Post Mortem & Tissue Collection

Between 139 and 146 days, ewes were killed by an intravenous overdose of sodium pentobarbitone (200 mg kg^{-1}; Lethobarb, Syntex, Castle Hill, NSW, Australia). The anaesthetised fetal sheep were delivered via laparotomy, weighed and then killed by decapitation. Each pituitary was quickly removed and immediately placed into cold HEPES-dissociation buffer (HDB: NaCl, 137 mM; KCl, 5 mM; Na_2HPO_4, 0.7 mM (Merk, Kilsyth, VIC, Australia); HEPES, 25 mM (Trace Biosciences, Castle Hill, NSW, Australia); pH 7.36).

4.3.1.4 Cell Preparation

Cultured anterior pituitary (AP) cells were prepared as previously described in Chapter 3 and by Fora *et. al.* (1996). The AP cells were plated in culture medium [1ml; Dulbecco's Modified Eagle's Medium (DMEM) plus Ham's F12 medium (F12, 1:1; Gibco BRL, Life Technologies, Grand Island, NY, USA) and charcoal-stripped fetal calf serum (10% of culture medium)], at approximately 2.0×10^5 cells $well^{-1}$ in 48-well tissue culture plates

(Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA). The plates were cultured at 37°C in a water saturated 5% CO_2 atmosphere (BB16 Gas Incubator, Haraeus Instruments, Hanau, Germany).

4.3.2 Experimental Protocols

Twenty four hours after plating the cells, the cells in half the wells from each pituitary were treated as described in Chapter 2 with either vehicle or C-TOX (final concentration of 25 nmol l^{-1}) that specifically eliminates CRH target cells. All cells were washed extensively in culture medium 18 to 24 h later (following day) removing the C-TOX or vehicle treatments. Two days after removing the C-TOX or vehicle treatment, the cells were washed extensively with incubation medium [DMEM/F12 (1:1) (Gibco BRL, Life Technologies) and 0.2% Polypep (Trace Biosciences, Castle Hill, NSW, Australia)] and allowed to equilibrate to serum-free conditions for 1 h. For the experimental incubations, cells were again washed in incubation medium and treated for 5 h with either medium containing vehicle (also termed basal; n = 15), *o*CRH (10^{-7} M; n = 15), AVP (10^{-7} M; n = 15) or *o*CRH + AVP (n = 12; Auspep, Parkville, VIC, Australia). After 5 h the culture media were collected and stored at -20°C, and the cells in the wells of the tissue culture plates stored at -80°C in preparation for assaying. Later 0.1 M HCl (1 ml) was added to each well of frozen cells. Cellular extracts of ACTH were subsequently obtained by thawing and refreezing (-80°C) the cells three times and using mechanical trituration on the third cycle. The extracts were stored at -20°C.

4.3.3 Analyses

4.3.3.1 Photometric Glucose Assay

Fetal plasma glucose concentrations were determined by an *in vitro* automated diagnostic reagent assay (COBAS MIRA; F.Hoffmann-La Roche, Diagnostica, Basle, Switzerland). The photometric assay system uses hexokinase to convert glucose to glucose-6-phosphate, which in the presence of glucose-6-phosphate dehydrogenase forms the reduced form of nicotinamide adenine dinucleotide (NADH). The level of NADH produced is thus directly related to the concentrations of glucose in the plasma samples.

The COBAS MIRA analyser injects 200 µl of reagent (Tris 85 mM; adenosine triphosphate 3.6 mM; nicotinamide adenine dinucleotide (NAD) 1.8 mM; hexokinase ≥ 20 µkat l^{-1}; glucose-6-phosphate dehydrogenase ≥ 10 µkat l^{-1}: F.Hoffmann-La Roche) and 4 µl of sample [diluted in 30 µl Milli-Q water (1:8.50)], into a cuvette contained within the analyser turntable. Once mixed, the reagent and sample are incubated at 25°C, over 10 cycles (each cycle = 25 seconds, ~ 4.5 min). The formation of NADH is photometrically quantified at 340 nm, and compared against a standard curve with glucose concentrations between 0.5 mmol l^{-1} to 5.0 mmol l^{-1}. Each sample was assayed in duplicate. The intra- and interassay coefficients of variation were less than 5%.

4.3.3.2 ACTH Radioimmunoassy

The concentrations of immunoreactive ACTH in the cell culture media and cellular extracts, referred to as ACTH throughout the Chapter, were measured using a double antibody radioimmunoassay as previously described and validated in Chapter 2 and Butler *et al.* (1999). The standards (1.95 - 500 pg $tube^{-1}$) were made from synthetic human

$ACTH_{1-39}$ (Peninsula Laboratories, Belmont, CA, USA) and the ACTH antisera (Anti-ACTH 1000T) was supplied by ICN Biomedicals (Costa Mesa, CA, USA). The sensitivity of the assay was 1.95 pg $tube^{-1}$. The interassay coefficient of variation was 21%, and the intraassay coefficient of variation was less than 10%.

4.3.3.3 Statistics

4.3.3.3.1 IN VIVO EXPERIMENTS

Fetal blood gas values are expressed as mean ± standard error of the mean (S.E.M.). The mean P_aO_2, P_aCO_2, pH, Hb and S_aO_2 were calculated for each fetus as the average of all P_aO_2, P_aCO_2, pH, Hb and S_aO_2 values obtained between 111 and 146 days gestation. Arterial oxygen content (O_2 content) per 100 ml blood (ml dl^{-1}) was calculated for each fetus as described in Chapter 2.

The mean plasma concentration of glucose for each ewe and fetus was also calculated as the average of all glucose values obtained between 116 and 146 days gestation.

Fetal sheep within the Control (n = 7) and Undernutrition (n = 8) groups were further subdivided into 2 groups, based on their mean plasma glucose concentration (> 1.2 mmol l^{-1}; euglycaemic range; (n = 9): or < 1.2 mmol l^{-1}; hypoglycaemic range; (n = 6), (Table 4.1)).

	EUGLYCAEMIA			HYPOGLYCAEMIA		
	Fetus	Plasma Glucose ($mmol\ l^{-1}$)	Weight (kg)	Fetus	Plasma Glucose ($mmol\ l^{-1}$)	Weight (kg)
CONTROL	0011	1.34	5.28	*0198:A	0.98	3.23
	0172	1.42	5.68	*0407:A	1.17	5.38
	0211	1.81	5.22	*0951:A	1.17	4.30
	*0846:A	1.44	4.12			
UNDERNUTRITION	0015	1.35	4.44	0146	1.02	3.52
	0135	1.27	4.77	0148	0.92	4.24
	0353	1.37	5.28	0714	1.19	4.99
	0922	1.43	5.21			
	0954	1.49	4.40			

Table 4.1 Fetal plasma glucose concentrations (mean values between 116 and 146 days gestation) and body weights at postmortem in the control and undernutrition groups

An asterisk denotes fetuses, which are twins or triplets.

The effects of feeding group and of hypoglycaemia on fetal blood gas status were determined using multifactorial analysis of variance (ANOVA), with a Statistical Package for Social Sciences on a Vax mainframe computer (SPSSX). Factors specified in this analysis included feeding group (Control vs Undernutrition) and fetal glucose concentration (euglycaemia vs hypoglycaemia).

4.3.3.3.2 *In Vitro Experiments*

In the *in vitro* experiments, each treatment was completed in replicate and the replicate values for the measured ACTH in either the culture media (secreted) or ACTH in the cellular extracts (stored) were averaged to yield an n = 1 for each pituitary in each treatment group. The total ACTH represents the arithmetic sum of secreted ACTH and stored intracellular ACTH under basal conditions. All values are expressed as mean ± S.E.M.

The effects of feeding group, hypoglycaemia and C-TOX on the total ACTH (per well), the amount of total ACTH secreted by the corticotrophic cells (per 10^4), the absolute amount and percentage of total ACTH secreted during the basal conditions and the proportion of ACTH stored in CRH target cells were compared using multifactorial ANOVA.

The proportion (%) of total ACTH present in the CRH-responsive cells (i.e. C-TOX sensitive cells) was calculated as the ratio of:

$$\frac{V - Cyto}{V} \times 100$$

where:

V = total ACTH in vehicle-treated wells under basal conditions

Cyto = total ACTH in C-TOX pretreated wells under basal conditions

The effects of feeding group, hypoglycaemia and C-TOX treatment on the ACTH secretory responses to CRH (10^{-7} M), AVP (10^{-7} M) and CRH + AVP (expressed as a percentage of total ACTH) were analysed using multifactorial ANOVA. The ACTH secretory responses to CRH (10^{-7} M), AVP (10^{-7} M) and CRH + AVP were also expressed as fold changes above basal ACTH secretion and analysed using multifactorial ANOVA with feeding group, hypoglycaemia and C-TOX as the specified parameters.

For all analyses, where the Cochran's and Bartlett-Box tests identified heterogeneity of variance, the data were logarithmically transformed prior to ANOVA. If significant interactions between major variables were identified by the multifactorial ANOVA, the data were split on the basis of the interactions and reanalysed. When the ANOVA indicated there was differences between the groups, the Duncan's *post hoc* test was used to identify the significant differences between the mean values. For all differences $P < 0.05$ was considered to be significant.

4.4 RESULTS

4.4.1 Fetal Outcome

There was no effect of either maternal undernutrition or of fetal hypoglycaemia on fetal body weight (Table 4.2).

There were no significant differences between the mean fetal plasma concentrations of glucose in the Control and Undernutrition groups (Table 4.2). As expected, fetuses

classified as hypoglycaemic had mean glucose concentrations significantly lower in both feeding groups (Table 4.2).

The mean fetal P_aO_2 was significantly higher in the Undernutrition group and the mean fetal P_aO_2 and S_aO_2 were lower whilst Hb content was higher in the fetuses which were hypoglycaemic in both feeding groups (Table 4.2).

4.4.2 Total ACTH and ACTH secretion

There was no effect of either maternal undernutrition or fetal hypoglycaemia on the total ACTH contained in pituitary cells expressed as either total ACTH $(10^4 \text{ cells})^{-1}$ (Euglycaemia; 1.92 ± 0.52 ng $(10^4 \text{ cells})^{-1}$: Hypoglycaemia; 1.00 ± 0.17 ng $(10^4 \text{ cells})^{-1}$) or as total ACTH $well^{-1}$ (ng) (Figure 4.1) or on the proportion of total ACTH secreted during the 5 h basal period (Table 4.3).

Similarly, feeding group and the level of fetal glucose had no effect on the ACTH responses (expressed as percentage ACTH secreted or fold changes above baseline) to CRH, AVP or CRH + AVP stimulation. The percentage of total ACTH secreted increased significantly after CRH, AVP and CRH + AVP stimulation (Figure 4.3). The ACTH responses to CRH + AVP and AVP were not different whilst the ACTH response to CRH + AVP was significantly greater than the ACTH response to CRH alone. (Figure 4.3). The fold changes in ACTH secretion were significantly greater ($P = 0.05$) after CRH + AVP than after either AVP or CRH.

TREATMENT		Maternal Glucose (mmol l^{-1})	Fetal Glucose (mmol l^{-1})	P_aO_2 (mmHg)	P_aCO_2 (mmHg)	PH	Hb (g dl^{-1})	S_aO_2 (%)	O_2 Content (ml dl^{-1})	Weight (kg)
CONTROL	Euglycaemia *(n=4)*	2.56 ± 0.20	1.50 ± 0.10	21.8 ± 0.7	45.9 ± 1.1	7.403 ± 0.007	9.4 ± 0.6	71.8 ± 3.2	9.4 ± 0.6	5.08 ± 0.33
	Hypoglycaemia *(n=3)*	2.39 ± 0.04	1.11 ± 0.06$^{\#}$	19.9 ± 0.1$^{\#}$	46.5 ± 0.8	7.376 ± 0.012	10.7 ± 0.5$^{\#}$	62.0 ± 3.2$^{\#}$	9.2 ± 0.1	4.30 ± 0.62
UNDERNUTRITION	Euglycaemia *(n=5)*	2.34 ± 0.11	1.38 ± 0.04	25.1 ± 0.8†	43.8 ± 0.9	7.394 ± 0.009	8.9 ± 0.2	76.0 ± 1.3	9.5 ± 0.1	4.82 ± 0.19
	Hypoglycaemia *(n=3)*	2.53 ± 0.17	1.04 ± 0.08$^{\#}$	21.0 ± 2.1$^{\dagger\#}$	46.6 ± 2.6	7.420 ± 0.011	10.8 ± 0.5$^{\#}$	66.4 ± 6.5$^{\#}$	10.0 ± 0.5	4.25 ± 0.42

Table 4.2 Effect of undernutrition and hypoglycaemia on fetal blood gas status and fetal body weight

Mean values for arterial blood gas variables between 111 and 146 days gestation and body weight in control and undernutrition fetal sheep. All values are expressed as ± S.E.M. and maternal glucose concentrations are included. Superscripts indicate significant differences between the Control and Undernutrition groups (†) or between the Euglycaemic and Hypoglycaemic groups (#), ($P < 0.05$).

Table 4.3 The percentage of total ACTH secreted over 5 hours under basal conditions after initial treatment with vehicle or C-TOX

TREATMENT	Vehicle	C-TOX
Euglycaemia *(n=9)*	1.99 ± 0.26	5.89 ± 0.72§
Hypoglycaemia *(n=6)*	3.19 ± 0.77	5.54 ± 0.90§

There was no difference between the two feeding groups in the percentage of total ACTH secreted and the data are therefore grouped on the basis of fetal glucose concentrations. Superscript (§) indicates significant differences between the vehicle and C-TOX groups, (P< 0.05).

4.4.3 Effect of C-TOX on total ACTH

After treatment with C-TOX, there was a significant decrease ($P < 0.001$) in total ACTH, which occurred independently of feeding group or fetal glucose concentrations (Figure 4.1).

There was no significant effect of either maternal undernutrition or of fetal hypoglycaemia on the proportion of ACTH stored in the CRH responsive cells (Figure 4.2).

4.4.4 Effect of C-TOX on ACTH Secretion

After C-TOX treatment, there was a significant increase ($P < 0.001$) in the proportion of total ACTH that was secreted during basal conditions, and this increase was similar in both feeding groups and was not affected by the fetal glucose concentrations (Table 4.3). This increase in the proportion of ACTH secreted was a consequence of the decrease in total ACTH content as there was no change in the absolute amount of ACTH secreted after

C-TOX (vehicle treatment; Euglycaemia; 0.68 ± 0.13 ng well^{-1}: Hypoglycaemia; 0.64 ± 0.17 ng well^{-1}: C-TOX treatment; Euglycaemia; 0.86 ± 0.23 ng well^{-1}: Hypoglycaemia 0.39 ± 0.04 ng well^{-1}) (Figure 4.1).

The fold changes in ACTH secretion after CRH, AVP and CRH + AVP stimulation were each significantly decreased ($P < 0.001$) after C-TOX treatment. After C-TOX, there was no difference in the fold changes of ACTH secretion between CRH + AVP and AVP stimulation, although both of these responses were significantly higher ($P = 0.001$) than the fold change in ACTH after CRH stimulation (Figure 4.4 a & b).

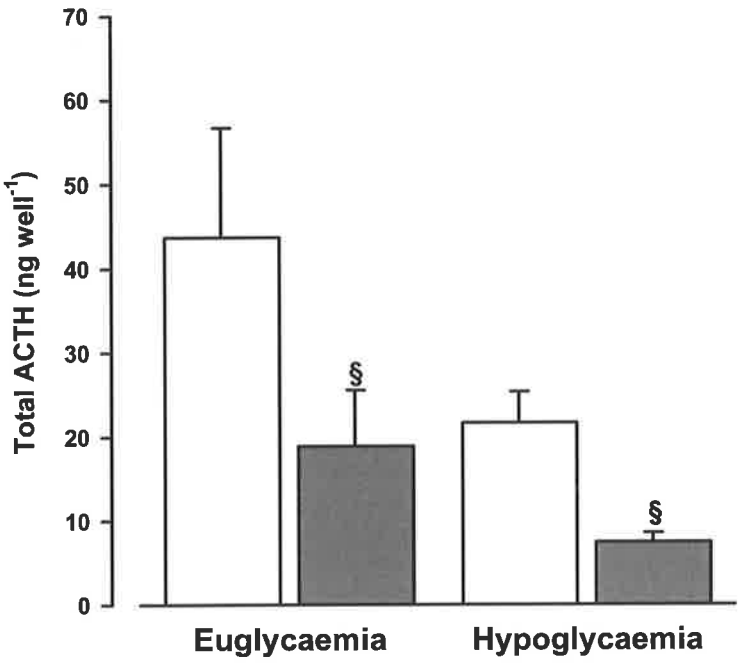


Figure 4.1 Effect of C-TOX on total ACTH

There was no effect of feeding group on the total ACTH content (ng well $^{-1}$; means ± S.E.M.) and the data are therefore grouped on the basis of fetal glucose concentrations. The superscript (§) indicates the significant effect of C-TOX treatment (shaded bars) on total ACTH content compared to vehicle treatment (open bars), ($P < 0.05$).

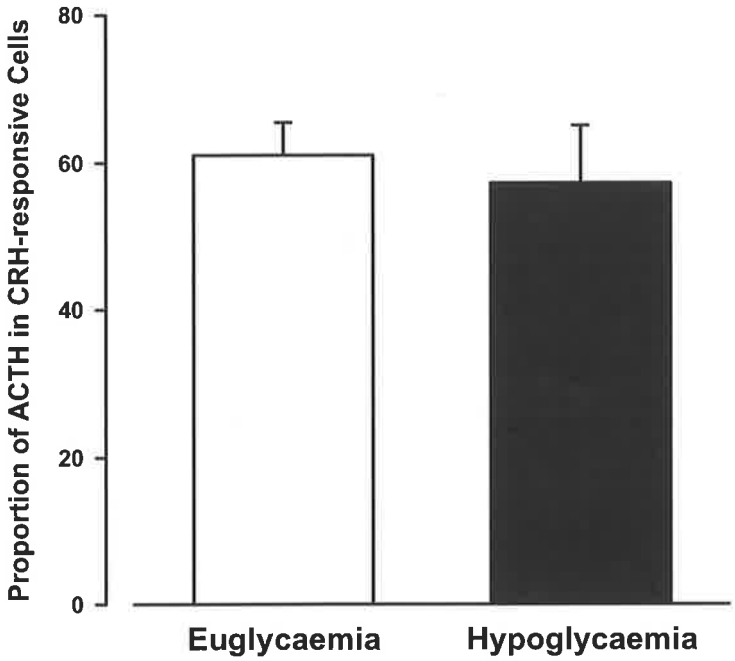


Figure 4.2 Proportion of ACTH contained in CRH-responsive Cells

The proportion (means ± S.E.M.) of ACTH present in CRH-responsive cells in Euglycaemic (open bar) and Hypoglycaemic (dark bar) fetal groups.

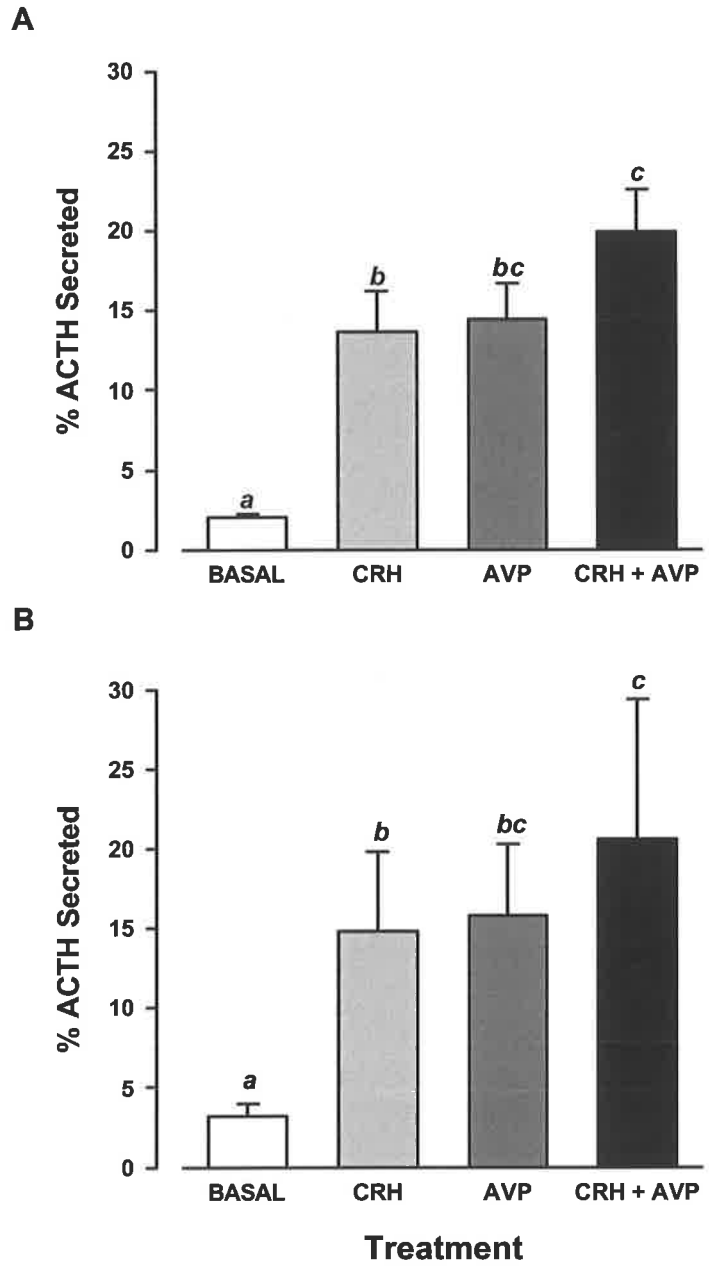


Figure 4.3 The percentage of total ACTH secreted from pituitary cells in response to hypothalamic secretagogues in the absence of C-TOX pretreatment

The percentage of total ACTH (means ± S.E.M.) secreted in response to vehicle, CRH, AVP, CRH + AVP grouped on the basis of (**A**) Euglycaemia and (**B**) Hypoglycaemia, in the absence of C-TOX treatment. Open, vehicle; grey, CRH; dark grey, AVP; black, CRH + AVP. Different superscript letters denote mean values that are statistically different within each treatment group, ($P < 0.05$).

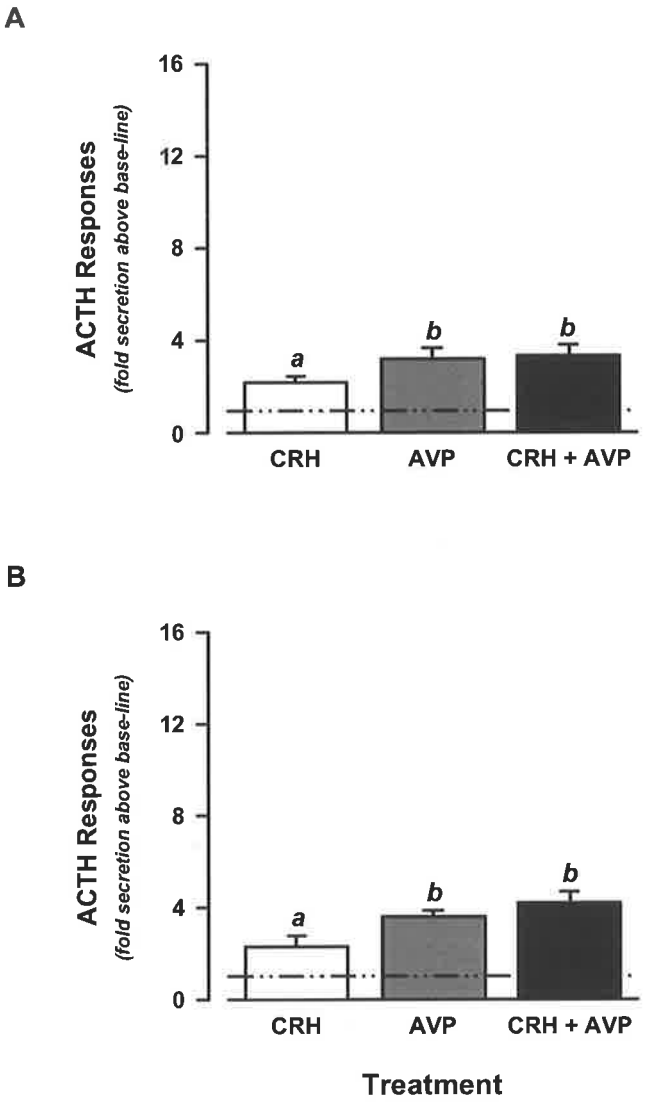


Figure 4.4 The fold change in ACTH secretion above baseline in response to the hypothalamic secretagogues in the presence of C-TOX treatment

The proportion of total ACTH (means ± S.E.M.) secreted in response to CRH, AVP, CRH + AVP expressed as a fold change in the Euglycaemic (**A**) and Hypoglycaemic (**B**) groups. Different superscript letters denote mean values that are statistically different within each treatment group, ($P< 0.05$; the horizontal dashed lines denote basal secretion).

4.5 DISCUSSION

In this chapter it has been demonstrated that maternal undernutrition and fetal hypoglycaemia did not alter the ACTH synthetic and secretory responses of the pituitary corticotrophic cells in the late gestation sheep fetus.

4.5.1 Maternal Nutrition and Fetal Glucose concentrations

In the present study, fetal plasma glucose concentrations varied and were, in part, dependent on maternal nutrition and on fetal number. There were 3 fetuses in the Control nutrition group which had circulating glucose concentrations <1.2 mmol l^{-1} and these fetuses were all from multiple pregnancies. Similarly 4 fetuses in the Undernutrition group had circulating glucose concentrations >1.2 mmol l^{-1} and these fetuses were all singletons. These data are consistent with the study of Edwards and co-workers (2001a), in which there were lower plasma glucose concentrations in twin fetuses in ewes fed a Control diet. In the present study, the only specific effect of the level of maternal nutrition on the fetal arterial blood gas or metabolic status appeared to be the marginal increase in fetal PaO_2 which occurred in both euglycaemic and hypoglycaemic fetuses in the Undernutrition group. One possible explanation is that the decrease in maternal nutrient intake is associated with a compensatory increase in placental transfer capacity. Whilst fetal PaO_2 and S_aO_2 were marginally decreased in the hypoglycaemic fetuses in Control and Undernourished ewes, importantly these values were still well within the normal physiological range reported for normoxaemic, normally grown fetal sheep in late gestation (Chapter 3 Simonetta *et al.*, 1997).

4.5.2 *Hypoglycaemia and the ACTH synthetic and secretory capacity of the fetal corticotrophs*

In the present study, there was no significant effect of fetal hypoglycaemia on the total ACTH stored in fetal pituitary cells, the proportion of the stored ACTH which was secreted under basal conditions or in the proportion of ACTH which was stored in CRH responsive cells. It has previously been shown that there is a fetal ACTH response when fetal glucose concentrations fall below a threshold value of around 1.2 – 1.3 mmol l^{-1} after 135 days gestation, and that this response occurs independently of whether the fetal hypoglycaemia is induced acutely or chronically (Edwards *et al.*, 2001a). Interestingly, these authors found that the fetal ACTH responsiveness to acute or chronically induced fetal hypoglycaemia increased after 135 days gestation and they suggested that there was an increased capacity of the fetal hypothalamo-pituitary axis to sense low fetal glucose concentrations after 135 days gestation. It has been shown in the adult sheep that insulin induced acute hypoglycaemia stimulates secretion of both CRH and AVP from the hypothalamus, resulting in an increased ACTH release from the pituitary gland (Engler *et al.*, 1989b). In the fetal as in the adult sheep, an intact hypothalamus and a functional hypothalamo-pituitary connection are required to generate an ACTH response to insulin induced hypoglycaemia in the late gestation sheep fetus (Ozolins *et al.*, 1992). Based on the results from the present study, it appears that chronic fetal hypoglycaemia does not alter the amount of ACTH stored and secreted under basal conditions from fetal pituitary cells. In the present study there was also no effect of either maternal undernutrition or fetal hypoglycaemia on the amount of ACTH stored in the CRH responsive corticotrophs or on the pituitary ACTH responses to CRH, AVP or CRH + AVP before or after removal of the CRH responsive corticotroph population. The profile of the ACTH secretory responses to the hypothalamic secretagogues were similar in this Chapter with those reported in Chapter 2 and were consistent with the presence of a subpopulations of cells

which were responsive to either CRH alone or AVP alone. The lack of an effect of maternal undernutrition or fetal hypoglycaemia on the ACTH synthetic or secretory characteristics of the fetal corticotrophs suggests that the actions of hypoglycaemia on the fetal hypothalamo–pituitary axis are predominantly at the fetal hypothalamus to stimulate the increase in fetal plasma ACTH concentrations in the sheep fetus *in vivo* during late gestation. Furthermore it appears that the changes in the functional characteristics of the corticotrophs in the placentally restricted sheep fetus are unlikely to be due to the presence of fetal hypoglycaemia in these animals.

5 INFLUENCES ON CORTICOTROPHIC CELL DEVELOPMENT

Fetal corticotroph cells of the anterior pituitary synthesise and secrete adrenocorticotrophin (ACTH) during late gestation in response to stimulation by the hypothalamic secretagogues, corticotrophin releasing factor (CRH) and arginine vasopressin (AVP) (for a review see Challis *et al.*, 2000). The fetal pituitary and ACTH synthesis and secretion are not only crucial for the control of the process of parturition but also play an important role in the fetal response to chronic intrauterine stress. Interestingly, no previous studies have investigated the impact of intrauterine stress such as chronic hypoxaemia or hypoglycaemia on the characteristics of the fetal corticotrophs, *in vitro*. Given that the fetus is required to respond to changes in the uterine environment, the response of the fetal pituitary to such intrauterine stressors is an important area of investigation. The studies presented in this thesis have examined the functional heterogeneity of the corticotroph cells during late gestation and the impact of intrafetal cortisol infusion and chronic intrauterine stress (placental restriction and maternal undernutrition) on the functional characteristics of the corticotroph cells in the pituitary of the fetal sheep. Thus, this final Chapter will summarise the main findings and implications of this series of experiments and briefly discuss several *in vivo* and *in vitro* experiments that could be undertaken in the future.

5.1 SUBPOPULATIONS OF CORTICOTROPH CELLS

5.1.1 Subpopulations of Corticotroph Cells Identified in the Adult Sheep

Schwartz *et. al.* (1994) in a series of studies using adult rat, bovine and ovine corticotroph cells proposed that the ovine corticotrophs were probably comprised of three

subpopulations of corticotroph cells. These were corticotrophs that were responsive to CRH alone, responsive to AVP alone or responsive to both CRH and AVP (Figure 5.1).

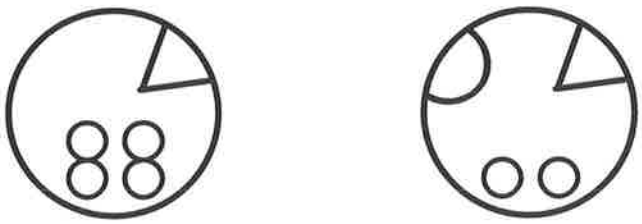
After measuring the pattern of secretion of POMC products including ACTH in response to CRH, AVP, dexamethasone and C-TOX, it was concluded, however, that the ovine corticotroph cells were comprised of either AVP responsive, or CRH and AVP responsive subpopulations (Figure 5.2). In the sheep fetus, several research groups have reported that there are a range of changes in the late gestation anterior pituitary including the morphology of the fetal corticotroph cells and ACTH secretory patterns. Based on these observations, this thesis has investigated whether the fetal sheep pituitary also has distinct subpopulations of corticotroph cells, the relative distribution of which is dependent on the intrauterine environment.

Figure 5.1 Schematic representation of three corticotroph cells types possible in the adult sheep.

The three types of corticotroph cells as proposed by Schwartz et al. (1994). The cell on the left responds to CRH, but not AVP; the cell in the middle responds to AVP, but not CRH; the cell on the right responds to CRH and AVP. Only the cells that respond to CRH are susceptible to elimination by C-TOX (CRH-target cells).

5.1.2 *Subpopulations of Corticotroph Cells Observed in the Sheep Fetus During Late Gestation*

In Chapter 2 it was observed that at 116 and at 140 – 145 days gestation, around 70% of the ACTH in the fetal anterior pituitary is stored within corticotrophs which are responsive to CRH alone. Interestingly the pituitary cells displayed the same ACTH secretory patterns at the two age groups, responding similarly to CRH, AVP and CRH and AVP, with and without C-TOX treatment. The data reported in Chapter 2 supported the conclusion that the fetal corticotrophs in late gestation are comprised of two subpopulations, cells responsive to CRH alone and cells responsive to AVP alone (Figure 5.3). Whilst both the adult and fetal pituitary contained a subpopulation of corticotrophs responsive to AVP alone, the remaining subpopulation of fetal corticotrophs were CRH responsive, whilst in the adult, the corticotrophs were responsive to both CRH and AVP.

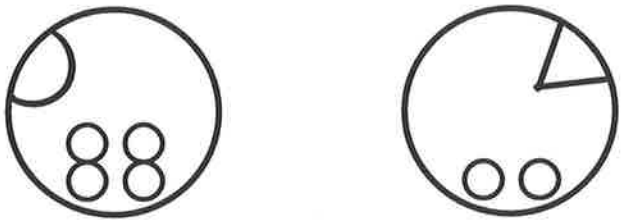


	AVP-only	CRH/AVP dually responsive
$ACTH_{1-39}$ secretion:	**Glucocorticoid-resistant**	**Glucocorticoid-sensitive (inhibition)**
ACTH precursor:	**Glucocorticoid-sensitive (stimulation)**	**Glucocorticoid-sensitive (inhibition)?**

Figure 5.2 Schematic summary of results concerning those subtypes of corticotrophs that respond to AVP in the adult sheep.

The cell on the left responds to AVP, but not CRH; the cell on the right responds to CRH and AVP. Only the cell on the right would be eliminated by treatment with C-TOX (Schwartz *et al.*, 1994)

Interestingly, intrafetal infusion of cortisol at 116 days resulted in a specific decrease in the amount of ACTH stored in the CRH-responsive corticotroph cells. The corticotroph cells which were responsive to AVP alone, however, were resistant to the negative feedback actions of cortisol on ACTH synthesis. Importantly, the influence of the precocious increase in cortisol concentrations indicated that during late gestation when the circulating ACTH and cortisol concentrations increase, the negative feedback effect of cortisol on ACTH synthesis in the CRH responsive cells must be counteracted by the stimulatory influence of the fetal hypothalamus. Thus, the pituitary maintains ACTH output throughout late gestation.



	CRH-only	AVP-only
C-TOX:	**sensitive**	**resistant**
Glucocorticoid:	**sensitive**	**resistant**
Total ACTH:	**70%**	**30%**
Basal ACTH secretion:	**–**	**+**

Figure 5.3 Schematic illustration of the types of corticotroph cell present in the fetal sheep during late gestation.

The cell on the left responds to CRH, but not AVP; and 70 % of the total ACTH is stored in this cell type while the cell on the right responds to AVP, but not CRH; the remaining proportion of total ACTH is stored in these cells. After cortisol infusion the total ACTH stored in the CRH responsive cells had diminished (60%) but the data indicated the two corticotrophs subtypes were unchanged.

5.1.3 *Subpopulations of Corticotroph Cell Observed in the Sheep Fetus after Chronic Intrauterine Stress*

Throughout Chapter 3 and 4, the intention of the experiments was to determine whether fetuses exposed to chronic intrauterine stress resulted in a 'reprogramming' of the corticotroph subpopulations as a consequence of the impact of the intrauterine environment. In Chapter 3 the modification of the fetal internal environment was achieved by surgically removing the caruncles from the uterus of the ewe before conception. The procedure alters the embryonic environment, resulting in the development of fetuses during late gestation that are either placentally restricted i.e. chronically hypoxaemic or fetuses that demonstrate compensatory placental growth i.e. are normoxaemic. Interestingly, uterine carunclectomy and the presence of hypoxaemia in late gestation each influenced the corticotroph cells. Uterine carunclectomy resulted in the emergence of a population of non-CRH responsive cells that secreted high amounts of ACTH in the fetal pituitary. This change in corticotroph development was independent of the presence of hypoxaemia in late gestation. The presence of chronic hypoxaemia during late gestation, in either the carunclectomised or non-carunclectomised uterine environments, resulted in a reduction in the proportion of ACTH stored in CRH-target cells.

5.1.3.1 *Subpopulations of corticotroph cells in normoxaemic, carunclectomised fetal sheep*

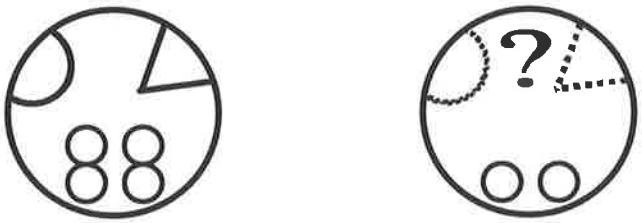
The proportion of ACTH stored in CRH-responsive cells was around 70% in pituitaries from normoxaemic fetal sheep in both the CR and NCR groups. This was the same percentage of ACTH reported to be present in the CRH responsive cells of the late gestation fetal pituitaries in Chapter 2 (Table 5.1). These cells from the normoxaemic CR group also secreted ACTH in response to CRH and AVP alone or in combination, and the

ACTH response was eliminated after C-TOX treatment. It appears therefore that this subpopulation of corticotrophs must express both CRH and AVP receptors (Figure 5.4).

As in Chapter 2, the remaining cells in the normoxaemic CR and NCR groups contained around 30% of the stored ACTH. In Chapter 2, it was possible to conclude that there was a population of corticotrophic cells in the fetal pituitary in late gestation, which was responsive to AVP alone. The responses derived from the normoxaemic CR pituitaries meant that the populations of corticotrophs in these fetuses were different from those reported in Chapter 2. Firstly, the percentage of ACTH secreted under basal conditions was greater, in the CR fetuses when compared to the NCR group and this effect was independent of the prevailing fetal P_aO_2. Interestingly, the higher basal output of ACTH was maintained in the CR group after C-TOX treatment (Table 5.1). Secondly, there was surprisingly no difference in the ACTH secretory responses after CRH, AVP or CRH + AVP administration in the CR group following C-TOX treatment. These responses were different to Chapter 2, where the ACTH responses to AVP or to CRH + AVP were maintained after C-TOX treatment in the fetal pituitaries. Thus, there is no evidence for a pool of corticotrophs that are responsive to AVP alone in the fetal pituitaries from the normoxaemic CR group.

One possible explanation for these findings is that the early intrauterine environment results in the presence of a population of corticotroph cell types in the fetal pituitary in late gestation, which constitutively secrete increased amounts of ACTH under basal conditions, and which are either not responsive, or relatively resistant to, the actions of CRH and AVP (Figure 5.4). It may even be possible that the impact of the intrauterine environment resulted in a predominant corticotroph subpopulation responsive to CRH and

AVP alone or in combination and the remaining corticotroph cells could be considered a transient population. These transient corticotroph cells appear to contain ACTH and secrete basal ACTH at a greater rate than the NCR and late gestation fetal pituitaries but not express CRF and AVP receptors and thus are unaffected by the C-TOX treatment.



	CRH/AVP dually responsive	Not responsive to/resistant to actions of CRH or AVP
C-TOX:	**sensitive**	**resistant**
Total ACTH:	**70%**	**30%**
Basal ACTH secretion:	**—**	**++**

Figure 5.4 Schematic illustration of the corticotroph cells types present in the normoxaemic CR fetal sheep

The cell on the left responds to CRH or AVP, alone or in combination; and 70 % of the total ACTH is stored in this cell type while the cell on the right is not responsive or resistant to the actions of CRH and AVP; the remaining proportion of total ACTH is stored in these cells.

5.1.3.2 Subpopulations of corticotroph cells in normoxaemic, non-carunclectomised fetal sheep

As stated earlier in the discussion, the normoxaemic NCR and CR groups appear to have a subpopulation of corticotrophs that express both CRH and AVP receptors (Figure 5.4 or 5.5). However, as with the normoxaemic CR group, the normoxaemic NCR group did not contain a subpopulation of corticotrophs easily identifiable as AVP responsive. Interestingly, like each group of pituitary cells throughout the thesis, the basal ACTH secretion in the NCR normoxaemic group increased after C-TOX treatment, however the ACTH responses to CRH, AVP and to CRH + AVP were also higher after C-TOX in this

group of fetuses compared to the CR fetuses. Two possible explanations have been proposed to explain these observations. The first explanation is that in the NCR group, AVP responsive cells normally secrete ACTH under basal conditions, survive pretreatment with the C-TOX but then develop CRH receptors and CRH sensitivity subsequently in culture (Figure 5.5A). The second explanation originates from a study by Perez *et. al.* (1997) where immunocytochemical analysis of individual corticotroph cells in the fetal sheep at various ages during late gestation suggested that not all of the corticotroph population secrete ACTH in response to CRH, AVP or CRH + AVP. Approximately 10% of the corticotrophs did not respond to those secretagogues and it may be possible that these corticotrophs could be considered a transient population. These transient corticotroph cells described earlier, appear to contain ACTH but do not express CRH and AVP receptors and thus are unaffected by C-TOX treatment. With the removal of CRH corticotroph cells it is possible that these transient cells in the normoxaemic NCR fetal sheep begin to express either CRH or AVP receptors, reestablishing the generic distribution of corticotroph cell subpopulations and the ability to respond to hypothalamic secretagogues (Figure 5.5B).

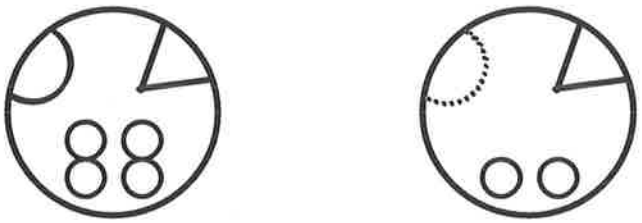
5.1.3.3 Subpopulations of corticotroph cells in hypoxaemic fetal sheep

Exposure to chronic hypoxaemia *in vivo* affected the ACTH synthetic and secretory characteristics of the fetal corticotrophs *in vitro* in both the NCR and CR fetuses. Chronic hypoxaemia was associated with a decrease in the amount of ACTH stored in CRH responsive cells, and yet there was a trend towards the reemergence of CRH sensitivity after C-TOX treatment. This effect was more pronounced in the NCR group and mirrored the response after C-TOX in the normoxaemic NCR pituitaries. It would appear that the subpopulation of corticotrophs proposed for the normoxaemic NCR cells also applies to

the hypoxaemic NCR and CR groups (see Figures 5.5A & B). The main difference between the normoxaemic NCR cells and the hypoxaemic NCR and CR groups is the amount of ACTH stored in the CRH and AVP responsive cells. The percentage of ACTH stored in the CRH responsive cells was similar to the percentage stored in the 116 day pituitaries after infusion of cortisol (Chapter 2; Table 5.1). It has also been established that the circulating concentrations of cortisol in growth restricted, chronically hypoxaemic fetus are significantly increased, and the expression of POMC mRNA measured in the anterior pituitary is significantly decreased (Phillips *et al.*, 1996b), thus a hypoxaemic induced increase in cortisol may be responsible for the decrease in ACTH stored in CRH responsive cells. The ACTH secretory responses to the hypothalamic secretagogues actually increased after C-TOX treatment, a response which was not observed at 116 days after treatment with cortisol. The ACTH secretory responses, however, were interesting in the absence of C-TOX treatment since the responses in the normoxaemic and hypoxaemic groups were similar in both the NCR and CR fetuses. Perhaps this infers that corticotroph cells that have experienced hypoxic conditions have a reprogrammed membrane potential (i.e. a relative depolarisation) thus maintaining the same ACTH output, but removal of the CRH responsive cells with C-TOX allows the corticotroph cells to reestablish CRH sensitivity with an increased capacity to respond to the hypothalamic secretagogues.

In summary, the data suggests that the early intrauterine environment alters the development of the subpopulations of corticotroph cells whereas the presence of chronic hypoxaemia in late gestation results in a specific decrease in the amount of ACTH stored in CRH responsive corticotrophs. These observations from the NCR and CR pituitaries

A

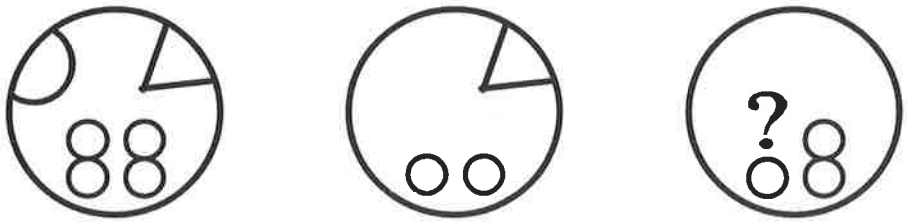


	CRH/AVP dually responsive	AVP-only
C-TOX:	**sensitive**	**resistant**
Total ACTH:	**70%**	**30%**
Basal ACTH secretion:	**—**	**+**

Figure 5.5A Schematic illustration of the possible corticotroph cell types present in the normoxaemic NCR fetal sheep

The cell on the left responds to CRH or AVP, alone or in combination and 70 % of the total ACTH is stored in this cell type. The cell on the right is only responsive to AVP initially; the remaining proportion of total ACTH is stored in these cells and these cells have the plasticity to reestablish CRH sensitivity.

B



	CRH/AVP dually responsive	AVP-only	Transient
C-TOX:	**sensitive**	**resistant**	**resistant**
Total ACTH: ?%	**70%**	**30%?**	
Basal ACTH:	**—**	**+**	**?+**

Figure 5.5B Schematic illustration of the possible corticotroph cell types present in the normoxaemic NCR fetal sheep

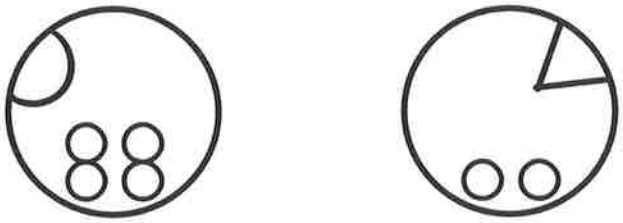
The cell on the left responds to CRH or AVP, alone or in combination; and 70 % of the total ACTH is stored in this cell type. The cell in the middle is not responsive or resistant to the actions of C-TOX; and possibly the remaining proportion of total ACTH is stored in these cells. The cell on the right does not respond to CRH or AVP initially and stores a potential proportion of total ACTH, these cells have the plasticity to develop CRH and AVP receptors after C-TOX pretreatment.

suggest that intrauterine stress in early pregnancy can sufficiently reprogram the fetal pituitary such that these characteristics of the corticotroph cells persist into late gestation. These findings suggest that the early and late intrauterine environments differentially program the development of specific corticotrophic cells types in the fetal pituitary.

5.1.3.4 Subpopulations of corticotroph cells in hypoglycaemic fetal sheep

Fetal hypoxaemia is not the only consequence of placental restriction. Placental restriction results in fetal hypoxaemia and hypoglycaemia and it was unclear in Chapter 3 whether the changes in the functional characteristics of the corticotrophs were related to either hypoxaemia or hypoglycaemia. Interestingly, fetal hypoglycaemia had no effect on the ACTH synthetic and secretory characteristics of the fetal corticotrophs in late gestation. Basal ACTH release and ACTH release in response to the hypothalamic secretagogues, with and without C-TOX treatment in the euglycaemic and hypoglycaemic fetuses, however, was extremely similar to the fetal ACTH response patterns described in Chapter 2. Before C-TOX treatment the cells responded to CRH and AVP and after the C-TOX treatment the basal secretion increased significantly and the cells only responded to AVP. Thus there appeared to be a subpopulation of CRH responsive corticotrophs and a subpopulation of AVP responsive corticotrophs (Figure 5.6). The only major difference between Chapter 2 and 4 was that the proportion of ACTH stored in the CRH responsive cells was approximately 60% in both the euglycaemic and hypoglycaemic fetuses in Chapter 4 instead of the 70% measured in the late gestation fetuses in Chapter 2. The change in the proportion of ACTH stored in the CRH responsive cells may be as a consequence of these pituitaries having been exposed to cortisol for an additional 3 days (144) in late gestation in comparison to the pituitaries of Chapter 2 (141 days). The observations in Chapter 4 indicate that the effects of placental restriction on the fetal

corticotroph subpopulations are most likely due to the direct or indirect actions of fetal hypoxaemia and are not a result of the fetal hypoglycaemia.



	CRH-only	AVP-only
C-TOX:	**sensitive**	**resistant**
Total ACTH:	**60%**	**40%**
Basal ACTH secretion:	**—**	**+**

Figure 5.6 Schematic illustration of the types of corticotroph cells present in the hypoglycaemic fetal sheep.

The cell on the left responds to CRH, but not AVP; and 60 % of the total ACTH is stored in this cell type while the cell on the right responds to AVP, but not CRH; the remaining proportion of total ACTH is stored in these cells.

In summary, this series of studies provide evidence that there are functionally distinct subpopulations of corticotroph cells within the fetal sheep pituitary and that these cells show plasticity in response to chronic intrauterine stress. These patterns of altered corticotroph development are important given the central role of the hypothalamo-pituitary-adrenal axis in the fetal adaptive response to intrauterine stress and in the early programming of adult disease (Seckl, 2001).

5.2 *Potential Subpopulations of Corticotroph Cells in the Ovine Fetuses*

This series of studies has provided evidence that there are functionally distinct subpopulations of corticotroph cells within the fetal sheep pituitary and that the

proportions of corticotroph cell types are influenced by different intrauterine conditions. Throughout this series of experiments, however, the corticotroph subpopulations have been deduced by measuring the ACTH responses to vehicle, CRH, AVP, CRH + AVP and C-TOX treatments and inferring which of the corticotroph subpopulations are responsible for a particular irACTH secretory profile. To move the interpretation of these observations beyond experimental inference requires a further series of studies, including experiments which would measure the irACTH, $ACTH_{1-39}$ and POMC precursors in the culture media, immunostaining of corticotroph cells and localisation of the CRH and AVP receptors. Compiling these new observations would precisely elucidate the distribution of corticotroph cells in the fetal pituitary during late gestation and in response to different intrauterine stressors. Considering the literature relating to the corticotroph cells in the adult and fetal sheep, in other species and in this series of experiments I consider that it is highly probable that four subpopulations of corticotroph cells are present in the fetal sheep pituitary. These include a population that responds to CRH, a population that responds to AVP, a population that responds to CRH or AVP alone or in combination and a population of transient corticotrophs. The transient corticotroph population is probably unable to respond to CRH or AVP but has the capacity to express either CRH or AVP receptors alone or in combination. Thus the transient corticotrophs are potentially the primary source of the other corticotroph subpopulations. Each of these corticotroph cell subpopulations has been observed under different circumstances in the preceding three chapters and it appears that the corticotroph cells in the fetal pituitary display plasticity and that the final composition of the population of these cells is influenced by the intrauterine environment.

5.3 FUTURE EXPERIMENTAL DIRECTIONS

This thesis has raised a number of important questions. In order to develop a further understanding about the functional heterogeneity of the fetal corticotrophs and the ability of these cells to adapt to stress associated with the intrauterine environment a carefully chosen research plan could now be undertaken using a range of both *in vivo* and *in vitro* experimental techniques.

5.3.1.1 Potential In vivo experiments

The experiments throughout the thesis had an *in vitro* focus so one possibility for a new series of experiments would be to use a whole animal model, which would help to identify, how the fetus responds to its internal environment. Using normal, placentally restricted and undernourished fetuses, CRH, AVP, and CRH + AVP could be infused into the fetuses before and after the prepartum cortisol surge. The ACTH and POMC peptide secretory responses could then be measured after the hypothalamic secretagogue treatment. One new approach might be to restrict the feed intake of ewes before conception and/or during early gestation since a recent study demonstrated that periconceptional undernutrition results in changes in the fetal pituitary–adrenal axis in late gestation (Edwards & McMillen, 2002). A study or series of studies measuring the basal concentrations of CRH and AVP in the hypophyseal portal circulation of the fetus in these different animal models would provide new information. Finally measurement of CRH and AVP in the portal circulation would also allow the definition of the fetal hypothalamic responses to specific stressors such as insulin-induced hypoglycaemia or hypoxia. In any of these fetal experiments, activation of the HPA axis will also result in a cortisol

response and the subsequent negative feedback actions of cortisol, would clearly increase the complexity of the responses and their interpretation.

5.3.1.2 Potential In vitro Experiments

The aim of a new series of *in vitro* experiments would be to extend the experiments already undertaken in this thesis. As was suggested for the *in vivo* experiments, the animal models of preference would again include normal, placentally restricted and undernourished fetuses. Each fetus would be catheterised and every effort made to monitor the fetal blood gas status and collect blood samples to measure irACTH, $ACTH_{1-39}$ and the POMC precursors and cortisol concentrations. The pituitaries would be removed from the fetuses during late gestation (140-145 days), although using pituitaries before the prepartum cortisol surge would also have strong merit. In preparing these pituitaries from these fetuses the researcher is always confronted by the problems of the small size of the fetal pituitary, the possible number of cells that can be prepared for plating, and the potential loss of cells through infection in the cell culture media. It would be anticipated in this series of experiments that the C-TOX treatment be incorporated whenever possible.

The initial experiment would be a repeat of the experiments in this thesis but instead of giving a fixed dose of CRH and AVP, the doses of the hypothalamic secretagogues would be varied and irACTH, $ACTH_{1-39}$ and POMC precursors in the culture media and the cellular protein content would be measured. A second series of experiments could be undertaken using the techniques of Perez *et. al.* (1997) in which pituitary cells are prepared on glass cover slips, cell plates or protein capturing membranes. The preparation of the pituitary cells on the different surfaces generates three pieces of distinct

experimental data from each pituitary. The data generated from each pituitary includes calculating the proportion of corticotroph cells in the pituitary, the measurement of ACTH release in response to CRH, AVP or CRH and AVP stimulation and the observation of the particular corticotrophs responding to the different hypothalamic secretagogues. Using this experimental approach and C-TOX the concept of plasticity and a transient population of corticotrophs could be explored in the different animal models alluded to in the previous section. Another series of experiments using C-TOX could measure the expression of POMC mRNA, whilst measuring the irACTH, $ACTH_{1-39}$ and POMC peptide secretory responses in the various models outlined.

5.3.1.3 Novel Experiments

The potential experiments discussed above would provide significantly more information about the plasticity of the corticotroph cells present in the fetal sheep pituitary under different environmental circumstances. It would be useful, however, to explore the development of a number of new techniques to elucidate the characteristics of these cell types to a greater extent.

Throughout the past 17 years, C-TOX treatment has been used to remove subpopulations of corticotroph cells that are CRH responsive and as an experimental tool this has been highly successful (Schwartz *et al.*, 1987; Schwartz & Vale, 1988, 1989; Schwartz *et al.*, 1991b; Schwartz *et al.*, 1994). It would clearly be worthwhile to design an AVP 'cytotoxin' that would act in a similar manner to the CRH-cytotoxin, i.e. to selectively remove AVP responsive corticotrophs. A different new approach would be to localise the CRH and AVP receptors on the corticotroph cells in fixed sections of fetal pituitaries

using immunostaining techniques. Immunostaining of corticotrophs for irACTH would also allow the localisation of $ACTH_{1-39}$ and POMC precursors in these specific cell types.

A further approach might be to obtain pituitaries from sheep in earlier gestation (eg. less than 50 days gestation) to determine how these subpopulations of corticotrophs develop in the pituitary and investigate their subsequent development after the fetuses have been exposed to either a nutrient deficiency or hypoxia. This would help in understanding how the unique population of corticotroph cells present in the carunclectomy fetuses developed early in gestation.

To complete any of the experiments however the research will require patience, time and resources. Whoever has the inclination to undertake any of these proposed experiments, I wish them luck since I am sure the rewards are worth the effort. Perhaps in the future I will have the opportunity to be involved in some of these experiments, time will tell.

	CHAPTER 2	CHAPTER 3			
	140 – 145d	NCR		CR	
		Normoxaemia	Hypoxaemia	Normoxaemia	Hypoxaemia
% Basal Secretion	1.73 ± 0.25	1.52 ± 0.31	1.85 ± 0.24	2.55 ± 0.61	5.30 ± 1.52
% Basal Secretion after C-TOX	5.80 ± 1.65	5.02 ± 0.96	4.21 ± 0.65	5.78 ± 1.35	8.38 ± 2.31
% in CRH Cells	71.0 ± 4.50	68.0 ± 2.30	55.0 ± 5.40	71.0 ± 2.20	62.0 ± 4.80

Table 5.1 Comparison of the ACTH release from late gestation pituitary cells after 3 h (Chapter 2) or 5 h (Chapter 3) Basal treatment

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What we got here.....is a failure to communicate.

Lucas Jackson; Cool Hand Luke, 1967