Impact of Environmental Factors on the Development of Corticotroph Subpopulations in the Fetal Sheep Pituitary.

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Declaration

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Abbreviations

11βHSD2	11βhydroxysteroid dehydrogenase type 2
ACTH	adrenocorticotropic hormone
AR	antigen retrieval
AVP	vasopressin
BP	bandpass
CRH	corticotropin releasing hormone
$CRHR_1$	corticotropin releasing hormone receptor 1
$CRHR_2$	corticotropin releasing hormone receptor 2
CLIP	corticotrophin-like intermediate lobe peptide
Cy	cyanine
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
GR	glucocorticoid receptor
HMW	high molecular weight
IgG	immunoglobulin G
irACTH	immunoreactive adrenocorticotropic hormone
JP	joining peptide
LMW	low molecular weight
LP	longpass
LPH	lipotrophin
MC_2R	melanocortin 2 receptor
MSH	melanocyte stimulating hormone
RHPA	reverse haemolytic plaque assay
RIA	radioimmunoassay

RIPA	radioimmunoprecipitation assay
PBS	phosphate buffered saline
PC1	prohormone convertase 1
PC2	prohormone convertase 2
PCUN	periconceptional undernutrition
PKA	protein kinase A
POMC	pro-opiomelanocortin
PR	placental restriction
ST-1	Nonapeptide of pro-opiomelanocortin spaning the cleavage point between adrenocorticotropic hormone and β-lipotrophin
V_{1b}	Vasopressin receptor 1b

Abstract

The prepartum surge in fetal plasma cortisol, essential for the maturation of organs in mammals and the normal timing of parturition in some species, including sheep, may result from an increase in the molar ratio of adrenocorticotropin (ACTH) to pro-opiomelanocortin (POMC) in the fetal circulation. Related to this, the cleavage of POMC to ACTH by the enzyme, prohormone convertase 1 (PC1), may be influenced by corticotrophin releasing hormone (CRH) stimulation. Accumulating evidence suggests that the capacity of individual corticotrophs to process POMC to ACTH may vary and individual corticotrophs are differentially responsive to CRH. It is not known, however, if there are separate corticotroph subpopulations in the fetal sheep pituitary which can be identified by differential colocalisation of POMC, ACTH and the CRH receptor 1, $CRHR_1$, nor if changes in the relative proportions of such subpopulations play a role in the molecular mechanisms underlying the overall changes in pituitary function described previously during gestation and in response to suboptimal uterine environments. To investigate these hypotheses, it was first necessary to develop novel methods for the simultaneous immunohistochemical labelling of POMC, ACTH and $CRHR_1$ in individual cells on sections of fetal sheep pituitary. In addition, I developed and validated an automated method to categorise and count individual cells to increase the quantitative power of this study.

Pituitary tissue was collected from control fetuses at 53-55 (n=6), 63-85 (n=6), 110 (n=4), 139-141 (n=4) and 144-145 (n=6) days gestation. Two

animal models, known to alter pituitary function in the fetal sheep, were used to investigate corticotrophic adaptations to suboptimal uterine environments. For the maternal periconceptional undernutrition (PCUN) model, maternal feed was reduced to 70% of maintenance requirements from at least 45 days before to 7 days after mating and fetal tissues were collected at 53-55 days gestation (n=7). For the placental restriction (PR) model, the majority of the placental attachment sites were removed in five ewes before mating and fetal tissues were collected at 140 (n=4) and 144 (n=4) days gestation. Pituitary sections were simultaneously labelled with antisera raised against full length POMC, ACTH and $CRHR_1$ and the proportions of pituitary cells with combinations of antisera were quantified.

Four subpopulations of corticotrophs were identified, which expressed either: POMC+ACTH+$CRHR_1$, ACTH+$CRHR_1$, POMC+ $CRHR_1$ or POMC-only. There was a significant decrease in the proportion of pituitary cells expressing POMC+ACTH+$CRHR_1$ between 53-55 and 65-85 days gestation, before an increase at 110 days gestation and a further marked decrease between 139-141 and 144-145 days gestation. In fetuses from the PCUN group, the proportion of pituitary cells expressing POMC+ACTH+$CRHR_1$ in early gestation was reduced. PR resulted in a significantly higher proportion of corticotrophs expressing POMC+ACTH+$CRHR_1$ during the prepartum period.

This work represents the discovery of the differential expression of POMC, ACTH and $CRHR_1$ in individual corticotrophs of the fetal sheep pituitary and the first insights into the pituitary adaptations to periconceptional

nutrient restriction and placental restriction at the level of individual corticotrophs.

Chapter 1: Introduction

Under the neuroendocrine regulation of the hypothalamus and pituitary, adrenal glucocorticoids contribute to maintaining homeostasis and are responsible for coordinating the physiological response to stress of the adult (Charmandari et al. 2005) and the fetus (Challis et al. 2001). Throughout gestation, glucocorticoids also play a crucial role in determining the rate of growth and differentiation of several fetal physiological systems (Ballard 1979). Therefore, untimely activation of the hypothalamic-pituitary adrenal (HPA) axis during critical windows of gestation has permanent effects on fetal organogenesis and the later health of the offspring (McMillen et al. 2001). Corticotroph cells of the pituitary occupy a pivotal position in the regulation of HPA axis activity. Several lines of evidence suggest that the corticotrophs are a heterogeneous population of cells which are differentially regulated. This chapter describes the current understanding of the physiological roles and regulation of the HPA axis, with a particular focus on corticotroph heterogeneity in the fetal sheep pituitary as a model of dynamic changes in the function of the HPA axis.

1.1 Physiological roles of the HPA axis

The HPA axis refers to the functional neuroendocrine signalling loop that links the hypothalamus, pituitary and adrenal glands. The hypothalamic corticotrophic neuropeptides, corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), are released into the hypothalamic-pituitary

portal circulation and stimulate pituitary cells via the membrane bound, G-protein coupled receptors, $CRHR_1$ (Aguilera et al. 2004) and V_{1b} (Jard et al. 1986), respectively. These signals regulate the synthesis and secretion of ACTH by cells of the anterior pituitary. In addition to its trophic activity, as predicted by Collip in 1934 (reprinted as Collip 1999) and subsequently established by others, ACTH stimulates adrenocortical cells via the melanocortin 2 receptor (MC_2R) to increase the production of glucocorticoids. Adrenal steroids with glucocorticoid activity are able to influence almost all cells in the body and alter the transcription of approximately 20% of the genes expressed on the human genome (Charmandari et al. 2005). Among the numerous systemic effects, glucocorticoids also inhibit further secretion of ACTH through negative feedback at the pituitary and hypothalamus.

Circulating glucocorticoid levels fluctuate in a pulsatile manner. However continuous sampling has demonstrated that the exact nature of the ultradian rhythm of ACTH and glucocorticoid secretion is an extremely complex and has a large degree of inter-individual variability (Gudmundsson and Carnes 1997). Unfortunately the physiological significance of such complex patterns are currently not well understood, although there is evidence that alterations in the pattern of HPA axis pulsatility are associated with neurobiological disorders such as depression (Lee et al. 2006) and addiction (Lovallo 2006). HPA axis activity is also under the influence of the suprachiasmatic nucleus and follows a diurnal pattern, where pulse amplitude increases just prior to the onset of the

active phase in mammals (Gudmundsson and Carnes 1997). This circadian rhythm is important in regulating the metabolic rates of numerous tissues including liver, kidney, and brain throughout the day.

In response to numerous challenges to homeostasis, there is an increased activation of the HPA axis and subsequent elevation in plasma glucocorticoid levels which act on a range of physiological systems including the immune, metabolic, gastrointestinal, reproductive and central nervous systems to restore homeostasis (Charmandari et al. 2005). Not surprisingly, different physical and psychological stressors have been shown to elicit distinct patterns of HPA activation that enable an appropriate physiological response to each stimulus (Romero and Sapolsky 1996). There is also evidence that HPA activation can cause long term adaptive changes in glucocorticoid-target organs which alter their responses to subsequent stressors (Charmandari et al. 2005).

1.1.1 Role of glucocorticoids in fetal development

In mammals, elevations in fetal plasma glucocorticoid levels promote the differentiation of the foregut and foregut-derived organs such as the liver, lungs, pancreas and small intestine (Liggins 1976) in addition to several other organs including the skin, retina, brain, and adrenal medulla and cortex (Ballard 1979). In hypophysectomised fetuses, tissues still differentiate, albeit at a slower rate, whereas a precocious rise in fetal plasma glucocorticoid levels accelerates the development of at least twelve different fetal tissues (Ballard 1979). There is a well characterised

exponential rise in fetal plasma cortisol levels during the prepartum period (Magyar et al. 1980; MacIsaac et al. 1985; Norman et al. 1985). In addition to preparing fetal tissues, such as the lungs, for the environment in all mammals, in fetal sheep, this surge in endogenous plasma cortisol levels during the prepartum period is important in determining the timing of parturition. Similarly, fetal stress (Ballard 1979) or administration of CRH (Wintour et al. 1984) or ACTH (Jones and Roebuck 1980) have been show to induce labour. In contrast, ligation of the fetal pituitary stalk and fetal hypophysectomy are known to delay parturition, and this delay can be prevented by continuous ACTH infusion (Antolovich et al. 1991; Jacobs et al. 1994).

The mechanism by which HPA activity controls the timing of parturition in the sheep is thought to involve multiple steps which begin with increased levels of cortisol stimulating changes in the placenta and end with increasing activity in the myometrium to initiate labour (Liggins et al. 1973). In sheep, cortisol activates placental enzymes responsible for the conversion of progesterone to estrogens. The increased ratio of estrogens to progesterone stimulates the release of prostaglandin $F_{2\alpha}$ in the maternal placenta and the myometrium. This potentiates the myometrial response to oxytocin which stimulates contractions and active labour (Liggins et al. 1977). Although HPA activity determines the timing of parturition in the sheep, in humans the initiation of parturition is not affected by fetal HPA dysfunction and is not induced by administration of glucocorticoids, except when pregnancy is extended beyond normal term (Liggins et al. 1977).

Interestingly, in humans the timing of the onset of parturition is thought to involve regulation by placental CRH, where an exponential increase in the bioavailability of placental CRH may trigger labour (McLean et al. 1995) through distinct $CRHR_1$ isoforms in the myometrium which may be capable of inducing myometrial contractions (Grammatopoulos et al. 1998).

1.2 Pituitary anatomy

The pituitary sits in the sella turcica, a depression on the upper surface of the sphenoid bone immediately below the base of the brain and is anatomically linked to the hypothalamus by the pituitary stalk in most mammals (Dubois et al. 1997). Part of the vasculature of the pituitary stalk is the portal blood supply which carries neurohormones that are released from axons in the median eminence to the anterior pituitary at undiluted concentrations (Daniel and Prichard 1957). The pituitary gland itself is comprised of the anterior lobe (also referred to as the adenohypophysis or *pars distalis*), the intermediate lobe (or *pars intermedia*) and the neuronal lobe (or *pars nervosa*) (Dubois et al. 1997).

1.2.1 Identifying corticotrophs

The first successful hypophysectomies provided clear evidence that the pituitary was responsible for controlling several physiological systems including the growth and function of the adrenal cortex, the genital organs, the mammary glands, the thyroid gland and overall somatic growth (Collip 1934 reprinted as Collip 1999). Different protein fractions from the pituitary were found to restore each physiological function, indicating that the

pituitary was responsible for secreting several trophic hormones. However, researchers had difficulty establishing which pituitary cells were responsible for producing each peptide hormone. Several approaches were taken to determine which cells produced the ACTH peptide. Adrenalectomy was found to result in marked changes in a small proportion of the pituitary cell population, however, basic histochemical techniques could not reliably characterise the responsive cells, noting changes in acidophils (Finerty and Briseno-Castrejon 1949), basophils (Leznoff et al. 1962) and chromophobes (Siperstein 1963). Electron microscopy allowed the differentiation of pituitary cells that underwent changes following adrenalectomy based on cell morphology and secretory granules size (Kurosumi 1968). Eventually, there was agreement amongst researchers that corticotrophs were characterised as stellate shaped cells with both electron dense and electron lucent secretory granules, varying in size between 100 and 300 μm (Kurosumi and Kobayashi 1966; Kurosumi 1968; Siperstein and Miller 1970; Moriarty and Halmi 1972).

As early as 1951 (Marshall), when only relatively crude pituitary extracts were available that contained ACTH along with many inert peptides, it was possible to develop antisera that were capable of immunochemically identifying corticotrophs in pituitary sections. As extracts became more purified (Leznoff et al. 1962), immunohistochemical detection of corticotrophs became more widely used (Baker et al. 1974). Immunohistochemical labelling with anti-ACTH was validated to be specific for ACTH based on changes at the level of individual cells in response to

adrenalectomy and cortisol treatment (Baker et al. 1970). In addition, cells labelled with anti-ACTH conformed to the stellate morphology with small secretory granules of varying electron densities described previously (Pelletier et al. 1978). Since the 1970s, immunohistochemistry has been the most routinely used, reliable method for identifying corticotrophs in the heterogeneous cell population of the pituitary.

1.3 ACTH biosynthesis

Although the trophic hormone for the adrenal cortex was purified from sheep pituitaries, validated chemically and biologically, and named ACTH in the 1940's (Li et al. 1943), successful synthesis of bioactive ACTH was not achieved for another twenty years (Schwyzer and Sieber 1963). Some of the difficulty in the purification and synthesis of ACTH was due to the large molecular weight range of the bioactive peptides. It was ultimately established that the 1-39 and 1-17 amino acid fragments of ACTH were the most potent adrenal and melanocyte stimulating peptides, respectively (Li 1963; Ramachandran et al. 1964). These peptides are now known to be derived from a much larger precursor, which was named 'pro-opiomelanocortin' (POMC). *In toto* ACTH, β-lipotrophin (βLPH) and β-melanocyte stimulating hormone (βMSH) were all determined to be derived from this common precursor (Chretien et al. 1979). A complete bovine cDNA sequence for POMC was published in 1979 (Nakanishi et al.) and ultimately each of the fragments shown in Figure 1.1 were demonstrated to be cleaved from POMC in *in vivo* and *in vitro* models (Eipper and Mains

1975; Roberts and Herbert 1977; Roberts and Herbert 1977; Crine et al. 1978; Roberts et al. 1979).

POMC is cleaved at pairs of basic residues to form pro-ACTH and then ACTH by prohormone convertase 1 (PC1) (Benjannet et al. 1991; Korner et al. 1991; Bertagna 1994) which belongs to the subtilisin family of serine proteinases (Seidah et al. 1992). Throughout this dissertation, POMC and pro-ACTH are referred to as ACTH precursors, while ACTH is used to indicate $ACTH_{1-39}$ and smaller bioactive fragments such as $ACTH_{1-24}$ and $ACTH_{1-17}$. The term, immunoreactive ACTH (irACTH), is used to signify all peptides containing all or part of the ACTH sequence that would be recognised by a promiscuous antibody.

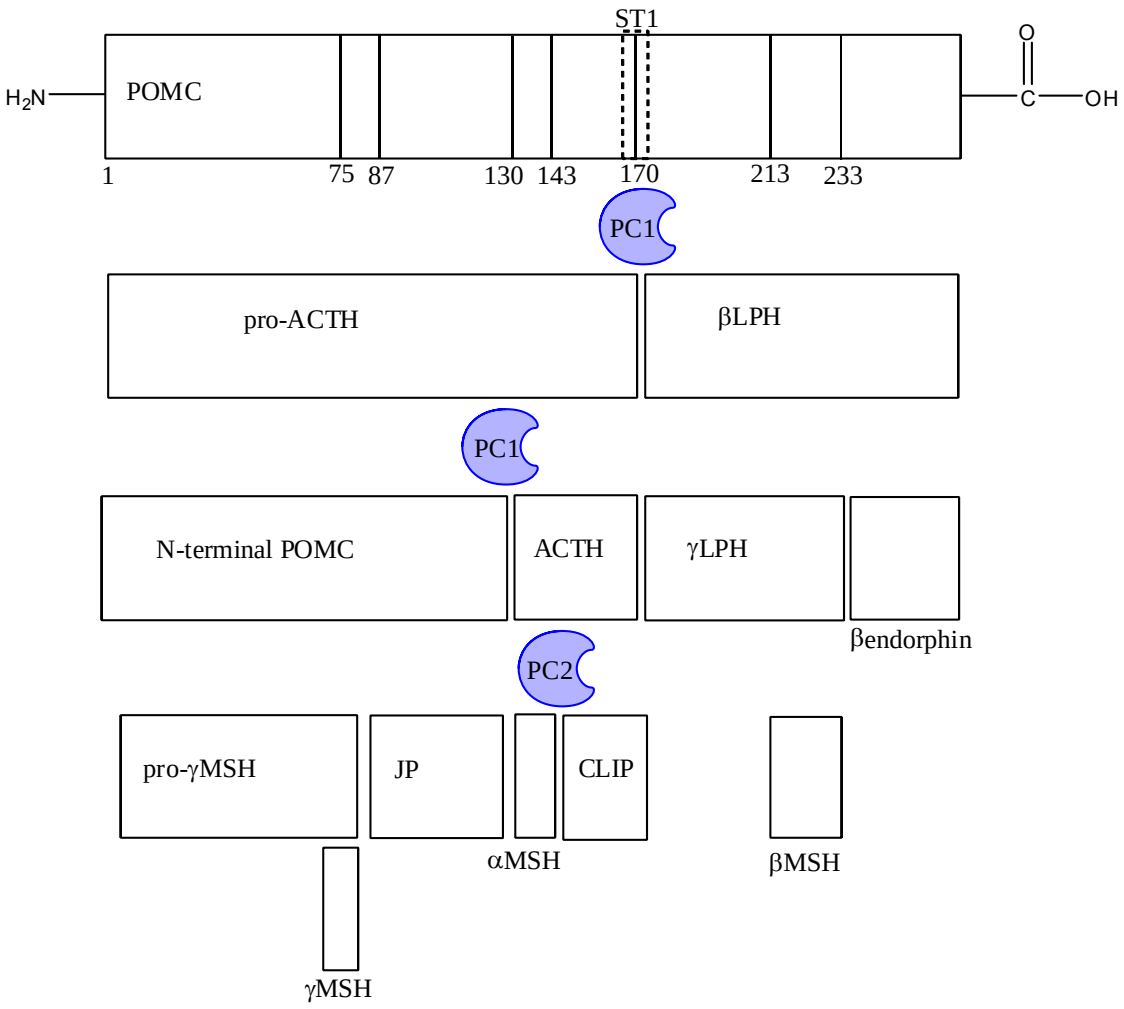


Figure 1.1 Schematic diagram of the POMC molecule denoting the POMC-derived peptides referred to in this dissertation. ST-1 refers to the nonapeptide recognised by the POMC antibody used in this dissertation. LPH, lipotrophin. JP, joining peptide. CLIP, corticotrophin-like intermediate lobe peptide. MSH, melanocyte stimulating hormone.

1.3.1 Post-translational processing of POMC

PC1 is only expressed in endocrine and neuroendocrine cells and has been identified in cells of the anterior pituitary (Seidah et al. 1990; Seidah et al. 1991). The closely related cleavage enzyme, prohormone convertase

2 (PC2), is known to cleave ACTH to αMSH and corticotrophin-like intermediate lobe peptide (CLIP), and this enzyme is expressed in the melanotrophs of the intermediate lobe of the pituitary (Seidah et al. 1991). Interestingly, several studies have reported cells in the anterior pituitary that express PC1 but not POMC or ACTH (Day et al. 1992; Marcinkiewicz et al. 1993). Evidence suggests that these cells may be gonadotrophs (Marcinkiewicz et al. 1993; Uehara et al. 2001) or lactotrophs (Muller et al. 1998). Using a very sensitive combination of *in situ* hybridisation and immunohistochemistry, PC1 mRNA was colocalised with prolactin, thyrotropin stimulating hormone β (the thyrotrophic specific subunit of the glycoprotein thyrotropin stimulating hormone) and β-luteinising hormone, but not growth hormone, suggesting that PC1 may cleave all granin peptides (Kato et al. 2004).

PC1 is synthesised as an 87kDa inactive precursor with an N-terminal pro-segment of 83 amino acids which is removed rapidly in acidic secretory vesicles to form the bioactive 66kDa form (Seidah et al. 1991; Vindrola and Lindberg 1992), possibly via an autocatalytic process (Zhou and Lindberg 1993). In an *in vitro* reconstituted system, inhibition of the vacuolar H+-ATPase in the Golgi apparatus causes POMC to be trapped within this organelle, but as soon as the pH is decreased below six, the trapped POMC is processed to ACTH (Moore et al. 2002). This evidence indicates that organelle acidification, and presumably subsequent PC1 activation, is required for the formation of the $ACTH_{1-39}$ fragment. Overall, there are

clearly several points at which the processing of POMC to ACTH may be regulated.

1.3.2 Regulation of POMC processing by CRH and glucocorticoids

CRH stimulation of cultures of the murine cell line, AtT-20, causes the secretion of the 66kDa active form of PC1, while unstimulated cultures only secreted the 87kDa PC1 precursor (Vindrola and Lindberg 1992). AtT-20 cells transfected with human PC1-promoter-luciferase reporter constructs have been used to demonstrate that CRH activation of a JAK-STAT-related pathway result in the increased PC1 gene expression (Li et al. 1999). Therefore, $CRHR_1$ activation is likely to play a role in PC1 synthesis, activation and subsequent processing of POMC to ACTH.

Adrenalectomy in adult rats does not alter the levels of anterior lobe PC1 mRNA. However, this is likely to be a measure of corticotrophic and non-corticotrophic PC1 gene expression (Day et al. 1992). In contrast, in AtT-20 cells, dexamethasone treatment causes a marked, although transient, decrease in PC1 mRNA levels (Day et al. 1992). This suggests that cortisol disruption of the POMC processing pathway by decreasing PC1 gene expression may be part of the slower pathway by which cortisol decreases the ACTH content of corticotrophs.

1.3.3 POMC processing during vesicle maturation

The time course of *de novo* POMC synthesis to the appearance of ACTH in secretory granule takes less than an hour (Gumbiner and Kelly 1981; Tooze et al. 1987). The specific vesicles in which certain processing steps

occur have been thoroughly characterised in the AtT-20 cell line. Specific antisera raised against POMC, pro-ACTH, joining peptide (JP)-amide, ACTH (Figure 1.1) and PC1 have been used to show that all of these antigens are present in the same granules. Although most of the POMC was localised to the Golgi cisternae, most of the labelling representing fully processed peptides was identified in mature secretory granules (Gumbiner and Kelly 1981; Schnabel et al. 1989). This suggests that processing of POMC to smaller fragments happens progressively throughout the vesicle maturation pathway. More detailed quantitative analysis has demonstrated that 25-30% of peripheral secretory granules contain unprocessed POMC (Tooze et al. 1987), indicating that the precursor may be secreted from cells of this particular murine cell line. These findings have been confirmed in tissue sections from the rat pituitary (Tanaka and Kurosumi 1992). Interestingly, POMC was identified in secretory granules of primary cultures of rat anterior pituitary cells, whereas, intermediate lobe cells only contained POMC in early stage vesicles, indicating differential processing of POMC between the pituitary lobes and that POMC secretion may occur from anterior but not intermediate pituitary cells (Tanaka et al. 1991).

1.3.4 Unstimulated corticotroph secretion

In vitro investigation of pituitary slices from late gestation fetal sheep indicate that the pituitary secretes ACTH precursors along with $ACTH_{1-39}$ under stimulated and unstimulated conditions (McMillen et al. 1995). In cultures of adult sheep pituitary cells, approximately half of the cells that express POMC also secrete the peptide *in vitro* (Young and Rose 2002).

Although this might be due to different levels of sensitivity of the immunohistochemical and immunoblotting procedures, it might also suggest in ovine pituitaries some corticotrophs can secrete POMC and others cannot.

1.3.5 Regulation of corticotroph activity by CRH, AVP and cortisol

CRH stimulates pituitary cells via the seven transmembrane G-protein coupled receptor, $CRHR_1$ (Aguilera et al. 2004). $CRHR_1$ is a 415 amino acid peptide with 98% homology among human, rat and mouse amino acid sequences (Chalmers et al. 1996) and has been identified in the anterior and intermediate lobes of the pituitary (Millan et al. 1987). Two families of CRH receptor have been identified, $CRHR_1$ and $CRHR_2$, which are transcribed from two different genes and have 70% similarity in their amino acid sequence (Grammatopoulos et al. 1998). Differential splicing of the mRNA for both $CRHR_1$ and $CRHR_2$ has also been demonstrated to give rise to multiple isoforms with varying functionality (Chalmers et al. 1996; Grammatopoulos et al. 1998). The $CRHR_1$ transcript is the predominant form found in the intermediate and anterior pituitary (Chalmers et al. 1996) and thought to be the form responsible for the single high affinity binding site found in membrane preparations from sheep pituitaries (Shen et al. 1990).

CRH binding to pituitary $CRHR_1$ activates the adenylyl cyclase/ cyclic AMP/protein kinase A (PKA) intracellular signalling system (Aguilera et al. 1983; Millan et al. 1987; Liu et al. 1990) to stimulate secretion of ACTH from primary cultures of adult (Liu et al. 1990; Kemppainen et al. 1993;

Levin et al. 1993) and fetal (Lu et al. 1994) sheep anterior pituitary cells. CRH also increases the transcription of POMC within 15 min of treatment of AtT-20 cells (Lutz-Bucher et al. 1987) and rat pituitary primary cultures (Gagner and Drouin 1985), and can maintain elevated POMC gene expression for in rat pituitary cultures for eighteen hours (Eberwine et al. 1987). In contrast, in cultures of adult (Levin et al. 1993) and prepartum fetal (Lu et al. 1994) sheep anterior pituitary cells, CRH does not alter POMC mRNA levels. CRH does induce increased ACTH protein levels in cultures of adult sheep anterior pituitary cells and this is abolished by the translation inhibitor, cycloheximide (Liu et al. 1990). This indicates that there may be some species specific differences in the effect of CRH on POMC gene expression and translation. In sheep at least, CRH is known to increase the production of POMC protein and secretion of ACTH.

AVP acts via V_{1b} receptors (Jard et al. 1986) and the protein kinase C (PKC) intracellular signalling pathway (Liu et al. 1990). Although extensive work was performed in attempts to identify the V_{1b} receptor on cells in tissue sections from fetal sheep pituitaries in this dissertation research, the library of antibodies currently available (through commercial sources and the generous donations of colleagues from the USA and UK) did not reliably detect the antigen. Therefore further reference will not be made to the regulation of corticotrophs by AVP unless it directly relates to regulatory action of CRH.

Glucocorticoids feed back primarily to the pituitary, but also to the hypothalamus, via glucocorticoid receptors (GR) (Charmandari et al. 2005).

Glucocorticoids decrease unstimulated ACTH secretion *in vivo* (Gemzell et al. 1951) and *in vitro* (Fleischer and Rawls 1970) and CRH-stimulated ACTH secretion *in vitro* (Gagner and Drouin 1985). In cultures of rat pituitary cells, glucocorticoid treatment decreases ACTH secretion at lower doses than those required to reduce the intracellular ACTH content (Fleischer and Rawls 1970). Glucocorticoids also directly inhibit the transcription of the ACTH precursor (Birnberg et al. 1983; Autelitano et al. 1987). In addition, there is evidence that pituitary cells may contain several different glucocorticoid binding sites to provide different pathways for the immediate inhibition of ACTH secretion and inhibition of ACTH synthesis which is delayed by 30 minutes (McEwen 1979).

Although it is possible that the corticotroph population may consist of a homogenous set of cells, all having the biosynthetic, secretory and response characteristics outlined above, there is now a substantial evidence base that corticotrophs are a heterogeneous population of cells with differential capacities to process POMC and respond to CRH, AVP and glucocorticoids. The following section reviews the current understanding of the heterogeneity of the corticotrophs, with particular reference to subpopulations of corticotrophs characterised in the fetal sheep pituitary.

1.4 Corticotroph heterogeneity

1.4.1 Heterogeneity of the response to CRH, AVP and cortisol amongst corticotrophs

In cultures of rat anterior pituitary cells, reverse haemolytic plaque assays (RHPA) provided the first evidence of functionally different corticotroph subpopulations at the level of individual cells. AVP treatment of RHPA cells resulted in small amount of ACTH secretion and therefore small plaques areas (Neill et al. 1987). In the presence of CRH, secretion by the cells that produced the small plaques continued but, in addition, another group of cells was recruited to secrete ACTH and did so in amounts that produced larger plaques (Neill et al. 1987). Thus, analyses of the distribution of plaque size indicated there were two populations of cells: one that secreted in the absence of secretagogue or in response to vasopressin, and a second that secreted in response to CRH (Figure 1.2). Only corticotrophs that secreted large amounts of ACTH in response to CRH were susceptible to inhibition by glucocorticoids (Neill et al. 1987). Subsequent investigation with this technique revealed four functionally distinct cell types: (1) cells that secreted ACTH without stimulation, (2) cells that increased ACTH secretion in response to CRH but not AVP, (3) cells that increased ACTH secretion in response to CRH or AVP, (4) cells that increased ACTH secretion in response to CRH and AVP in combination (Jia et al. 1991).

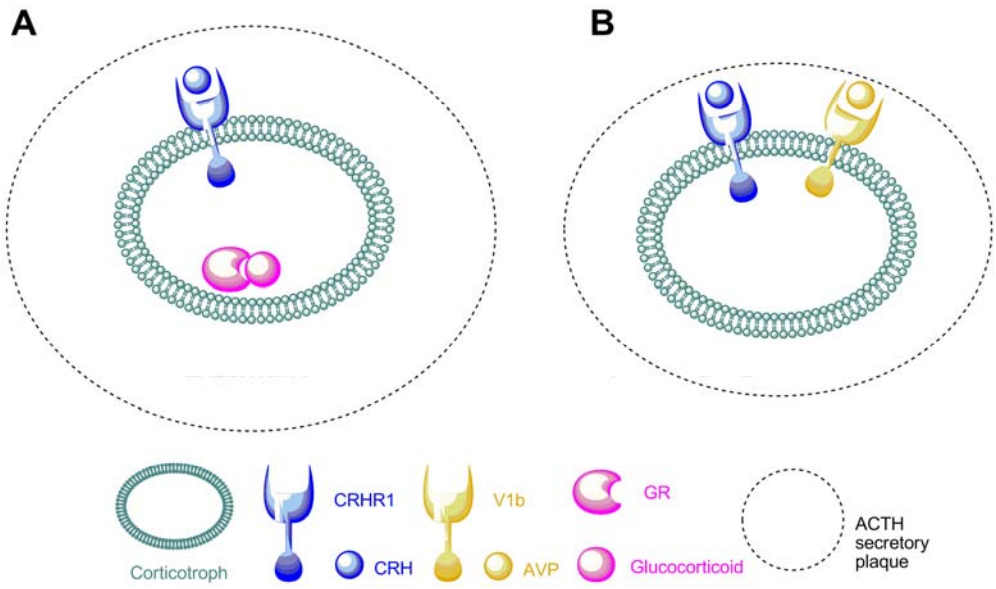


Figure 1.2 Schematic model of the mechanisms that might underlie the functional differences amongst corticotrophs suggested by observations described by Neill and colleagues (1987). (A) A subpopulation of corticotrophs that secrete a large amount of ACTH in response to CRH, are unresponsive to AVP. (B) A subpopulation of corticotrophs that secrete a small amount of ACTH in response to either CRH or AVP.

In cultures of anterior pituitary cells from several species including adult and fetal sheep, a cytotoxin conjugated to CRH has been used to eliminate all pituitary cells responsive to CRH (Schwartz et al. 1987; Schwartz and Vale 1988; Schwartz et al. 1991; van de Pavert et al. 1997; Butler et al. 1999; Butler et al. 2002). In cultures of fetal sheep anterior pituitary cells, this technique has demonstrated that ~70% of the ACTH is stored within corticotrophs that are sensitive to the CRH-cytotoxin (Butler et al. 1999). The CRH-cytotoxin completely abolishes the ACTH secretory response to CRH treatment as expected. However, removal of all the CRH-target cells attenuates, but does not ablate, the ACTH response to AVP (Schwartz et al. 1987; van de Pavert et al. 1997; Butler et al. 1999). This evidence confirms that in the fetal sheep pituitary there are at least two subpopulations of corticotrophs: (1) cells that respond to CRH and AVP and (2) cells that respond to AVP but not CRH (Figure 1.3). I therefore hypothesised that the corticotrophs in the fetal sheep pituitary which are not vulnerable to the CRH-cytotoxin in culture would be corticotrophs which do not express $CRHR_1$.

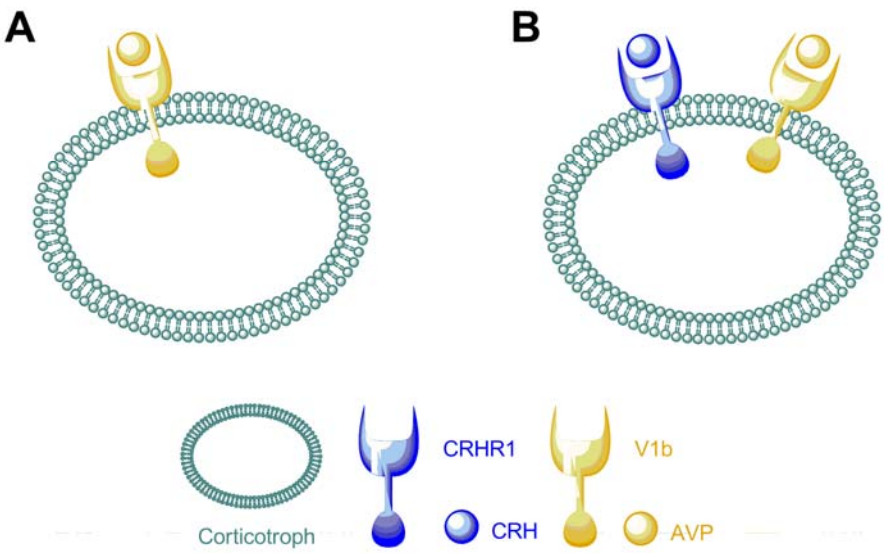


Figure 1.3 Schematic model of the mechanisms that might underlie the functional differences amongst corticotrophs described by Butler and colleagues (1999). (A) A subpopulation of corticotrophs that secrete ACTH in response to AVP, but are unresponsive to CRH. (B) A subpopulation of corticotrophs that secrete ACTH in response to either CRH or AVP.

Following CRH-cytotoxin, the remaining cells have elevated POMC mRNA levels and secrete a higher proportion of the ACTH in unstimulated conditions (van de Pavert et al. 1997). This has led the authors to suggest that the CRH-target population may secrete a factor which acts as an inhibitor at non CRH-targets (Figure 1.4). If this is the case, then the relative proportions of corticotroph subpopulations within the whole corticotroph population might also play a role in regulating ACTH secretion from individual cells.

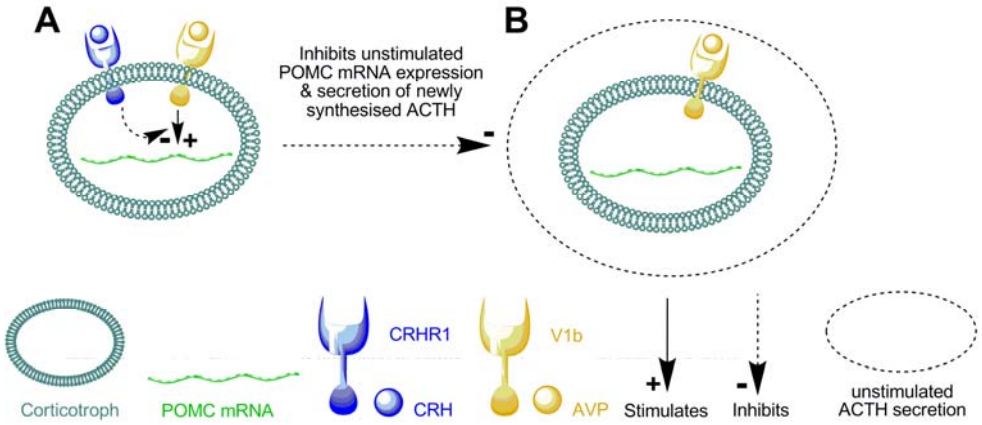


Figure 1.4 Schematic model of the mechanisms that might underlie the functional differences amongst corticotrophs described by van de Pavert and colleagues (1997). (A) A subpopulation of corticotrophs that respond to either CRH or AVP, where AVP-induced increases in POMC mRNA levels are inhibited by CRH stimulation. (B) A subpopulation of corticotrophs that respond to AVP, are unresponsive to CRH, and secrete ACTH in unstimulated conditions. Authors present evidence that subpopulation A may secrete a factor that inhibits the POMC mRNA expression and secretion of newly synthesised ACTH of subpopulation B.

In anterior pituitary cell cultures from the adult and fetal sheep, the cells remaining after CRH-cytotoxin treatment, that are responsive to AVP but not CRH, are also oblivious to the negative feedback effects of cortisol (Schwartz et al. 1994; Butler et al. 1999). This indicates that there are also subpopulations of corticotrophs that are differentially responsive to cortisol, and that CRH responsiveness is likely to be related to glucocorticoid responsiveness.

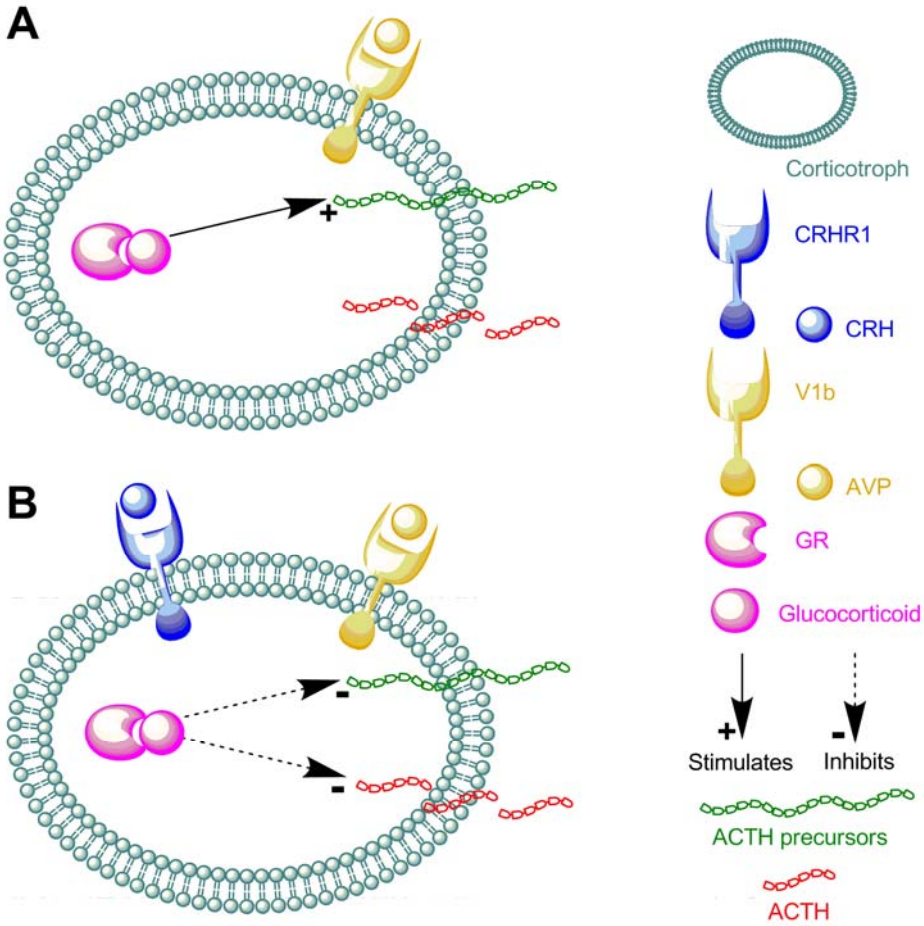


Figure 1.5 Schematic model of the mechanisms that might underlie the functional differences amongst corticotrophs described by Schwartz and colleagues (1994). (A) AVP responsive subpopulation in which glucocorticoids stimulate the secretion of ACTH precursors. (B) AVP and CRH responsive subpopulation in which glucocorticoids inhibit the secretion of ACTH precursors & ACTH.

1.4.2 Morphological heterogeneity amongst corticotrophs

As reviewed above, when corticotrophs were first identified using immunohistochemistry and electron microscopy, the typical corticotroph was found to have a stellate morphology with small electron dense and electron lucent secretory granules of varying sizes. However in several species, including the sheep, fetal and neonatal pituitaries have been

found to contain several morphologically distinct subpopulations of corticotrophs (Dacheux 1981; Kurosumi et al. 1984; Perry et al. 1985; Tanaka and Kurosumi 1986; Antolovich et al. 1989). These reports indicate that there are clear species-specific differences in the morphology of corticotrophs; in particular, fetal sheep pituitaries appear to have a unique combination of (1) columnar corticotrophs that are arranged in palisades, and (2) stellate corticotrophs that are sparsely distributed throughout the anterior pituitary (Perry et al. 1985). This group has later published data on the existence of an intermediate cell, but this third subpopulation accounted for less than 2% of the corticotroph population (Antolovich et al. 1989).

1.4.3 Heterogeneity of POMC processing amongst corticotrophs

Interestingly, cells have been identified in tissue sections of the late gestation fetal sheep pituitary that express the transcript for POMC but not for PC1 (Bell et al. 1998). This suggests that there may be corticotrophs that express POMC but do not process it to ACTH. I have been unsuccessful in my attempts to label fetal sheep pituitary sections with PC1 antisera. However, I have developed methods to differentially label the high and low molecular weight species of POMC, and have used this approach to investigate the differential processing of POMC in individual corticotrophs.

The above evidence strongly supports the suggestion that the corticotrophs are a heterogeneous population of cells that are

phenotypically and functionally diverse. As indicated in the schematics used in this section, the differential expression of key molecules amongst corticotrophs might provide a mechanism to explain these findings. It is therefore the aim of this dissertation to characterise the differential expression of the key peptides by individual corticotrophs of the fetal sheep pituitary. However, to understand the roles and regulation of individual corticotrophs, it is also necessary to investigate well characterised models of dynamic changes in corticotroph appearance and function. Thus, the following sections review three models of physiological challenges in which the characteristics of individual corticotrophs are thought to be differentially affected.

1.5 Dynamic models known to alter corticotroph phenotype and function

1.5.1 Fetal development

1.5.1.1 Pituitary organogenesis

The pituitary gland develops from two primordia, a downgrowth from the floor of the diencephalon, which becomes the posterior and intermediate pituitary lobes, and an upgrowth of a single epithelial layer from roof of the oral cavity, which becomes Rathke's pouch, and subsequently, the anterior lobe of the pituitary (Dubois et al. 1997). The anterior and intermediate lobes are permanently separated by a remnant of Rathke's pouch (Daniel and Prichard 1957). In humans (term = 40 weeks), the corticotrophs are the first differentiated secretory cells to be identified (at 5 weeks gestation) (Kono et al. 2001) and cell cultures from the Rathke's pouch of the fetal rat

have been shown to contain differentiated corticotrophs (Dubois et al. 1997). In fetal sheep (term = 145-150 days), corticotrophs have been identified as early as 38 days gestation (Perry et al. 1985; Mulvogue et al. 1986).

1.5.1.2 Ontogenic changes in the corticotroph population

There is a lack of agreement among researchers concerning the ontogenic changes the corticotroph population undergoes in the fetal sheep pituitary. The study with the longest range of gestational ages shows that there is an increase in the proportion of pituitary cells labelled with anti-ACTH between 60 and 100 days gestation, and then no change to term (Matthews et al. 1994). In contrast, reports of the last third of gestation indicate that the proportion of pituitary cells that are labelled with ACTH antisera decreases between 115 and 135 days gestation (Antolovich et al. 1989) or increases between 126-130 and 134-136 days gestation (Braems et al. 1996). In primary cultures of pituitaries taken from fetal sheep at 100 and 135 days gestation, there appears to be an ontogenic decline in the proportion of cells that express ACTH (Perez et al. 1997). It is unlikely that these variations are due to differences in antibody binding to tissue, because each study demonstrates a change with gestational age for groups of tissues labelled with a single antibody. Alternatively, it is possible that the slight differences in gestational age windows used in each of these studies contribute to this variation between reports in ontogenic changes. This might suggest the corticotrophs are a very dynamic cell population that undergoes relatively rapid changes over short periods of gestation.

1.5.1.3 Ontogenic changes in the morphologically heterogeneous subpopulations of corticotrophs

In the fetal sheep, corticotrophs were described as being either 1) regularly shaped, columnar cells found in large palisades which are present exclusively from 38 days gestation, or, 2) stellate corticotrophs that appear at 90 days gestation (Perry et al. 1985). Both morphologies are present until 135 days gestation at which time there is a dramatic decrease in the columnar cells so that at term, almost all of the corticotrophs are the stellate type (Perry et al. 1985). Thus, as gestation proceeds, the relative distribution of columnar versus stellate cells within the corticotroph population decreases. The authors therefore referred to the columnar corticotrophs as the “fetal-type corticotrophs” and the stellate corticotrophs were named “adult-type corticotrophs”. Clearly, the maturation of fetal corticotrophs includes a change within the corticotroph population from one dominant of morphological subpopulation to another.

1.5.1.4 Ontogenic changes in the biological actions of precursors at adrenal cortex

In a number of studies, POMC and other peptides derived from POMC were shown to interact with ACTH receptors and/or the adrenocortical responses to ACTH. In cultures of rat adrenal cells, high molecular weight irACTH peptides (6.5-9kDa and 20-30kDa) compete with $ACTH_{1-39}$ standards for binding, and exhibit parallel dose-response curves to $ACTH_{1-39}$ (Eipper and Mains 1975). Interestingly, ACTH precursors also inhibit the

$ACTH_{1-24}$-induced increase in cortisol secretion by adrenal cell cultures taken from the fetal sheep, but not the adult sheep (Roebuck et al. 1980; Schwartz et al. 1995). The ACTH precursors, POMC and pro-ACTH, alone do not alter the secretion of cortisol from fetal adrenal cell cultures at low doses (Roebuck et al. 1980; Schwartz et al. 1995), but may have steroidogenic activity at higher doses (Roebuck et al. 1980). Therefore, although it is clear that the ACTH precursors interact with ACTH or MC_2R, the bioactivity of ACTH precursors at the adrenal depends on species, adrenal maturity and plasma concentrations of the precursor. In the late gestation fetal sheep, it is likely that ACTH precursors act as antagonists at the adrenal cortex, suggesting that the ratio of plasma ACTH to precursors may be more important than the concentration of ACTH alone in influencing the cortisol secretory activity of the fetal adrenal. Interestingly, the plasma precursors levels measured in fetal sheep are 10 fold higher than those of ACTH (Carr et al. 1995) and 1000 times higher than the precursor levels in adult humans (Crosby et al. 1988). To the best of my knowledge, there is no published data on the levels of ACTH precursors in adult sheep. It is possible that processing of POMC by the fetal and adult pituitaries is quite different.

1.5.1.5 Ontogenic changes in the ratio of $ACTH_{1-39}$ to its precursors in the fetus

Extensive sampling of fetal sheep plasma between 74 days gestation and 4 weeks after birth demonstrates a constant increase in the ratio of plasma ACTH to its precursors, POMC and pro-ACTH (Saoud and Wood 1996).

More specifically, there is no change in ACTH precursor concentrations in fetal sheep plasma between 110, 120, 130 and 140 days gestation although there is a significant increase in $ACTH_{1-39}$ across this window of gestation (Carr et al. 1995). Similarly, *in vitro* investigation of pituitary slices from late gestation fetal sheep indicates that the unstimulated secretion of $ACTH_{1-39}$ increases over the last third of gestation, whereas the unstimulated secretion of ACTH precursors does not change (McMillen et al. 1995). Together these findings suggest that the plasma concentrations of peptides derived from POMC are separately controlled during fetal maturation, possibly via different POMC processing pathways or via the regulation of individual corticotrophs that differently process POMC.

It has been shown that the maturational changes in the molecular weight profile of POMC-derived peptides in pituitary extracts occur primarily in the anterior lobe in sheep but in the intermediate lobe in primates (Silman et al. 1981). At the level of individual corticotrophs, cells containing the POMC fragments proγMSH, $ACTH_{1-24}$ and βendorphin/βLPH, have been reported at 38 days gestation (Mulvogue et al. 1986), indicating that these corticotrophs are not just synthesising POMC but also cleaving it into fragments.

Evidence suggests that in the mouse anterior pituitary, corticotroph maturation involves a change in the end product of POMC processing from αMSH to $ACTH_{1-39}$. (Chatelain et al. 1979; Marcinkiewicz et al. 1993). Similarly, the immature corticotrophs found in fetal and neonatal rat anterior pituitaries contain ACTH precursors and fragments of ACTH such

as CLIP and αMSH, while the mature corticotrophs of adult rats contain $ACTH_{1-39}$ almost exclusively (Noel and Mains 1991). To date, there are no published reports of differences in the extent of POMC processing in individual corticotrophs in the fetal sheep pituitary at the protein level at any gestational age.

At the level of POMC gene transcription in the fetal sheep pituitary, there is an overall increase in the pituitary PC1 mRNA content across the last third of gestation (Holloway et al. 2000), and more specifically, an increase in the proportion of POMC mRNA-expressing cells that also express the PC1 mRNA between 126-130 days gestation and term (144-147 days gestation in this breed) (Bell et al. 1998). Therefore, I hypothesised that in the time approaching term there would be an increase in the proportion of POMC-expressing cells that also express ACTH.

1.5.1.6 Ontogenic changes in the regulation of corticotrophs by CRH, AVP and glucocorticoids

There is a developmental increase in the $ACTH_{1-39}$ plasma levels following administration of CRH to fetal sheep between 110 and 120 days gestation and then no change in the response between 120 and 140 days gestation (Carr et al. 1995). In contrast, the ACTH precursor response to CRH administration increases from 110 days gestation to peak at 130 days gestation, before a partial decrease again at 140 days gestation (Carr et al. 1995). It is possible to explain these gestational changes as maturational developments that involve increased functional CRH receptors and increasingly efficient conversion of POMC to ACTH.

Within the fetal sheep pituitary, the total level of $CRHR_1$ mRNA and protein have been found to decrease between 102-105 and 137-139 days gestation and further in adulthood (Green et al. 2000). CRH binding sites determined by radioligand binding were barely detectable at 65-70 days gestation, peaked at 125-130 days gestation and then declined at term (Lu et al. 1991). This suggests that there may be an increase in the expression of $CRHR_1$ during the early gestation and a decrease during the prepartum period.

CRH-induced ACTH secretion in cultures of pituitary cells taken from fetuses at 110-115 days gestation is four times greater than unstimulated ACTH secretion, but this response decreases to a level not significantly different to unstimulated ACTH secretion when pituitaries are collected from fetuses at 138-145 days gestation (Fora et al. 1996). Interestingly, this loss of responsiveness to CRH does not occur if fetuses undergo adrenalectomy early in the last third of gestation (Fora et al. 1996), indicating the glucocorticoid feedback plays a role in the maturation of the pituitary response to CRH.

Current evidence from the CRH-cytotoxin experiments suggests that there is no change in the proportion of ACTH stored within CRH-target cells between 116 days gestation and 140-145 days gestation, while infusion of cortisol between 110-115 days gestation decreases the proportion of ACTH stored within CRH-target cells at 116 days gestation compared with saline treated controls (Butler et al. 1999). This suggests that the CRH-target corticotrophs are responsive to a precocious rise, but not the normal

ontogenic rise, in fetal plasma glucocorticoid levels. However, it is difficult to reconcile this evidence at the level of differentially responsive subpopulations of corticotrophs with previous reports of the ontogenic changes in pituitary cultures (Fora et al. 1996; Perez et al. 1997), because, even in cultures not treated with the cytotoxin, Butler and colleagues (1999) did not demonstrate an ontogenic decline in the ACTH-secretory response to CRH.

1.5.2 Perturbations

1.5.2.1 Fetal exposure to maternal glucocorticoids

Fetuses can be exposed to excess glucocorticoids at times of increased maternal HPA axis activity. Although injection of radiolabelled ACTH to pregnant ewes and fetal sheep has demonstrated that ACTH does not cross the placenta (Jones et al. 1975), cortisol can pass across the placenta (Wood and Rudolph 1984). It has also been shown that at 65 days gestation, administration of dexamethasone (a synthetic glucocorticoid) to the ewe reduces fetal plasma ACTH levels (Wintour et al. 1995). Therefore, the fetal HPA function is likely to be responsive to maternal glucocorticoid levels early in gestation. The degree of fetal exposure to maternal glucocorticoids is thought to be regulated by the expression and activity of placental 11βhydroxysteroid dehydrogenase type 2 (11βHSD2), which inactivates cortisol by converting it to cortisone. For example, experimental reduction of 11βHSD2 has been shown to increase fetal plasma cortisol levels and inhibit fetal growth (Seckl 1997).

1.5.2.2 Fetal stress response

Changes in fetal homeostatic variables are also known to increase activity of the fetal HPA axis. In sheep, acute fetal hypoxia for six hours at 135 days of gestation (term is approximately 150 days gestation in this breed) results in activation of the HPA axis at multiple levels, including increased hypothalamic CRH mRNA, pituitary POMC mRNA and plasma ACTH and glucocorticoids (Matthews and Challis 1995; Matthews and Challis 1995; Challis et al. 2001). Haemorrhage by withdrawal of 15% of blood volume in sheep fetuses between gestational days 117 and 144 has been shown to increase fetal ACTH and cortisol plasma levels significantly, but this response is not demonstrated at 70-100 days gestation (Rose et al. 1978). This might suggest that the fetal HPA is too immature to respond to stressors before 100 days gestation. Indeed, there is some evidence that that there is a maturational change between 121 days gestation, when the primary source of cortisol in the fetal circulation is of maternal origin, and 136 days gestation, when less than 12% of the fetal plasma cortisol is derived from the ewe (Hennessy et al. 1982).

1.5.2.3 Ontogenic changes in fetal HPA axis response to stress

The degree of stress-induced fetal HPA activation varies considerably with gestational age. Fetal hypotension results in peak increases in plasma AVP and cortisol at 130-144 days gestation, whereas peak ACTH response is at 120-130 days gestation. Interestingly, the ACTH response at 120-130 days does not significantly increase plasma cortisol levels (Rose et al. 1981). A similar pattern of response is seen in response to

administration of CRH to fetal sheep, with a peak ACTH plasma response between 95 and 113 days gestation, and a peak cortisol response between 122 and 136 days gestation (Hargrave and Rose 1986).

1.5.2.4 Long term effects of inappropriate exposure of the fetus to glucocorticoids

As reviewed above, the fetal or maternal response to stress during gestation causes the fetus to be exposed to excess glucocorticoids. Although this may increase the chances of survival of the fetus during the stressful event, it can cause significant alterations in the development of several fetal organs and lead to adverse outcomes after birth. Considering that the fetal plasma levels of glucocorticoids finely regulate the rates of growth and differentiation of a large number of fetal organs, it is not surprising that the timing of elevations in fetal plasma glucocorticoid level determines whether adaptations to intrauterine stressors will be reversible or irreversible (Ballard 1979). Irreversible changes in organogenesis suggests there is a permanent resetting of genetic expression (Ballard 1979). In addition, maximal adaptive responses are found when an inappropriate increase in fetal glucocorticoid exposure occurs just prior to a normal ontogenic increase (Ballard 1979). There is now a large body of experimental evidence which clearly indicates that there are specific windows in gestation when excess fetal glucocorticoid exposure will result in permanent changes in several physiological systems (Charmandari et al. 2005; McMillen and Robinson 2005; Nathanielsz 2006).

In sheep, 48 hour glucocorticoid infusion to the fetus early in gestation results in permanently elevated blood pressure in adulthood (Dodic et al. 1999). Maternal glucocorticoid administration in late gestation sheep results in fetal growth restriction, indicated by low birth weight, followed by the development of insulin resistance and an exaggerated cortisol response to stressors when the offspring are 6-12 months old (Challis et al. 2001). This indicates that fetal exposure to elevated glucocorticoid levels not only alters the development of the cardiovascular and metabolic systems, but also the HPA axis. Several different protocols of maternal and fetal stress and glucocorticoid administration have been reported to cause permanent changes at all levels of the HPA axis (Welberg and Seckl 2001; Theogaraj et al. 2005; Kapoor et al. 2006).

1.5.2.5 Maternal periconceptional undernutrition

In sheep, a 30% reduction in maternal nutrition for 2 months before mating until the end of the preimplantation period (day 7 of gestation) results in increased mean arterial blood pressure, systolic blood pressure and diastolic pressure in twin, but not singleton, fetal sheep at all ages investigated between 115 and 147 days gestation (Edwards and McMillen 2002). In these twin fetuses, basal plasma irACTH concentrations are also elevated between 115 and 147 days gestation, as is the cortisol, but not irACTH, response to CRH at 139-144 days gestation (Edwards and McMillen 2002). This indicates that the fetal pituitary is working at a higher basal level in PCUN fetuses but is not more responsive to CRH stimulation. A 15% reduction in material nutrient intake for the first seventy days of

gestation does not alter basal fetal plasma ACTH or cortisol levels in singleton fetuses (twins not investigated) (Hawkins et al. 1999). However, these PCUN singleton fetuses have attenuated ACTH and cortisol responses to CRH+AVP challenge at 113-116 days gestation (Hawkins et al. 1999) and, as three month old lambs, potentiated ACTH and cortisol responses to CRH+AVP administration (Hawkins et al. 2000). Taken together, these findings indicate that even small changes to the PCUN protocol have significant impacts on the adaptations of the HPA axis and that the phenotype of these adaptations depends on the age at which the offspring are investigated. In addition, these reports indicate that fetal number also has an impact on the translation of PCUN to altered HPA development, where twins seem to demonstrate changes in basal pituitary activity that singletons do not.

Although various PCUN protocols have been shown to alter HPA development and predispose the adult offspring to cardiovascular and metabolic disorders, the specific physiological adaptations of the fetal HPA axis are determined by the timing, duration, intensity and type of perturbation (Fowden and Forhead 2004; McMillen and Robinson 2005). To focus on developmental changes in corticotrophs, I selected as the best experimental model, a 30% reduction in maternal nutrient intake beginning 60 days before mating, returning to control levels of nutrition before blastocyst implantation. This perturbation is known to result in increased fetal plasma levels of ACTH in twins, but not singletons, at all times in late gestation from 115 days gestation (Edwards and McMillen 2002). I

examined the effects of this PCUN treatment regime on individual corticotrophs in twins for evidence of altered organogenesis early in gestation.

1.5.2.6 Placental restriction of nutrient supplies to the fetus

Placental insufficiency by maternal carunclectomy, which causes hypoxia and hypoglycaemia in fetal sheep from approximately 110 days gestation (Robinson et al. 1994), results in elevated fetal plasma cortisol levels from 127 days gestation without concomitant changes in fetal plasma ACTH concentrations and a decrease in POMC mRNA at 140 days gestation (Phillips et al. 1996). Conversely, severe maternal food restriction (50% of control diet) in late gestation results in elevated pituitary POMC mRNA levels but no change in circulating ACTH levels in lambs at 4 and 8 months old, with elevated cortisol levels at 8 months but not at 4 months (Sebaai et al. 2002). For these two models of IUGR, the fetal HPA axis makes very different specific adaptations which, like the PCUN models, depend on the timing, duration, intensity and type of perturbation, and, the resulting HPA phenotype may change with time after the perturbation has ceased.

There are several well characterised models of fetal growth restriction that result in markedly reduced birth weight, including severe maternal diet restriction during late gestation, and, placental insufficiency induced by surgical reduction of the placental attachment sites (McMillen et al. 2001). I investigated the effects of placental insufficiency on subpopulations of corticotrophs in the fetal sheep pituitary using a model of placental restriction known to induce severe growth restriction in late gestation,

evidenced by significantly reduced fetal body weight. This model of placental restriction involves the initial uterine carunclectomy and, in approximately 2/3 of the animals, subsequent hypoxia and hypoglycaemia in late gestation with the associated growth restriction evidenced by low fetal weights for gestational age.

The uterine carunclectomy and hypoxia have been found to have different effects on corticotroph development (Butler et al. 2002). Hypoxia, but not carunclectomy, specifically decreased the proportion of ACTH stored in those cells susceptible to the CRH-cytotoxin conjugate. In contrast, carunclectomy, independent of fetal oxygenation, caused an increase in the proportion of ACTH secreted *in vitro* under unstimulated conditions. In the studies described in this dissertation, carunclectomy, which resulted in hypoxia and significantly reduced fetal weight, was used to investigate the adaptations of the fetal pituitary. Therefore I hypothesised that this may alter the proportion of pituitary cells that express $CRHR_1$ in the pituitaries of PR fetal sheep.

1.6 Models of corticotroph subpopulations

This chapter has reviewed evidence for the existence of subpopulations of corticotrophs which differentially process POMC to ACTH and differentially express the CRH receptor, $CRHR_1$ in the fetal sheep anterior pituitary (Figure 1.6). Given the evidence that CRH increases the expression and action of the POMC cleavage enzyme, PC1, I expect that $CRHR_1$ might be primarily associated with corticotrophs that express ACTH.

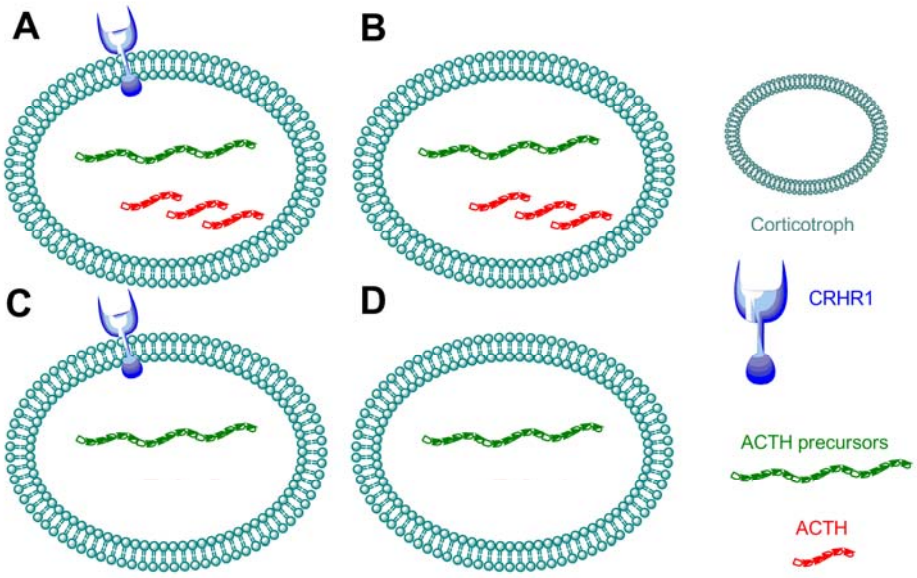


Figure 1.6 Schematic model of the four possible subpopulations of corticotrophs that differentially express POMC, ACTH and $CRHR_1$. (A) A subpopulation that expresses POMC, ACTH and $CRHR_1$. (B) A subpopulation that expresses POMC and ACTH. (C) A subpopulation that expresses POMC and $CRHR_1$. (D) A subpopulation that expresses POMC only. Development, optimisation and validation of the methods required to test whether these subpopulation exist in the fetal sheep pituitary are described in the next chapter.

It is likely that these corticotroph subpopulations undergo separate ontogenic changes throughout gestation and in the HPA axis reprogramming that occurs in response to poor intrauterine environments. Across the last third of gestation at least, it is expected that there will be a decrease in the subpopulations of corticotrophs that express $CRHR_1$ and an increase in the subpopulations of corticotrophs that express $ACTH_{1\text{-}39}$ (Figure 1.7).

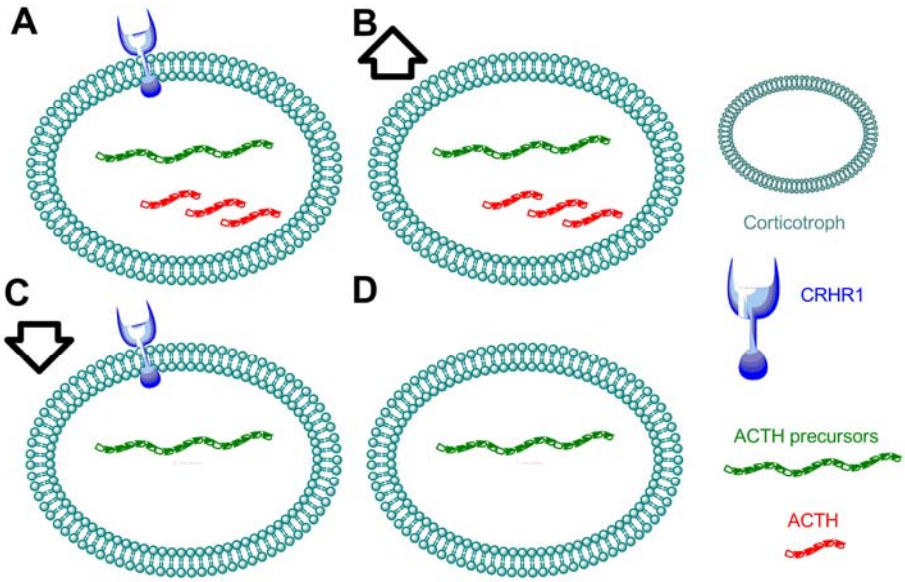


Figure 1.7 Schematic model of ontogenic changes in the hypothetical subpopulations of corticotrophs that differentially express POMC, ACTH and $CRHR_1$. Arrows indicate that there is expected to be no change in the proportion of pituitary cells that belong to (A) the subpopulation that expresses POMC, ACTH and $CRHR_1$, an increase in (B) the subpopulation that expresses POMC and ACTH, a decrease in (C) the subpopulation that expresses POMC and $CRHR_1$, and no change in (D) the subpopulation that expresses POMC only. Automated quantification methods are also developed, optimised and validated in the next chapter to test the changes postulated in this schematic.

The studies in this dissertation also employed two ovine models of the suboptimal intrauterine environment, PCUN which alters the uterine environment for a short period in early gestation before implantation of the blastocyst, and placental restriction (PR), which subjects the fetus to long term restriction of nutrient and oxygen supply. Although the fetal sheep model provides the opportunity for these interventions, and analysis of

pituitary development early in gestation (as early as 38 days), the expenses incurred with each fetus make large scale investigations difficult. Thus, while it would have been ideal to compare large groups of fetuses from the PCUN and PR models, in both early and late gestation, a more rationalised approach was necessary. Previous evidence indicated that PCUN caused changes in fetal HPA function before 110 days gestation (Edwards and McMillen 2002), possibly by 55 days gestation (MacLaughlin et al. 2005). Therefore PCUN fetuses were investigated at 55 days gestation. In contrast, evidence indicates that the greatest effects of PR on fetal sheep development occur late in gestation when fetal growth is restricted most by the limited supply of nutrients (Robinson et al. 1994). Therefore, PR fetuses were investigated in late gestation. Given the importance of the timing, duration, intensity and type of perturbation on the specific adaptations the HPA axis undergoes, it is expected that the adaptations of corticotroph subpopulations will be quite different in PCUN (Figure 1.8) and PR (Figure 1.9) animals.

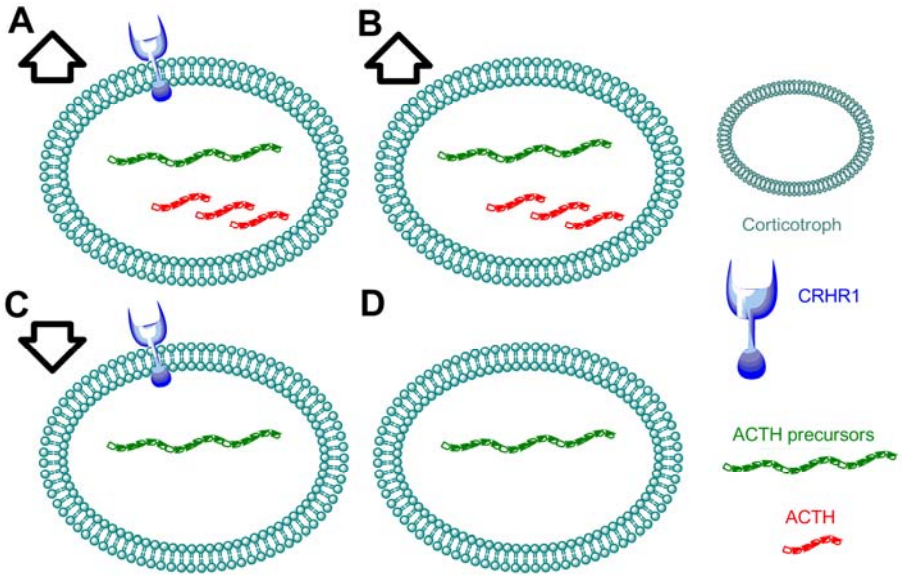


Figure 1.8 Schematic model of PCUN-induced changes in the hypothetical subpopulations of corticotrophs that differentially express POMC, ACTH and $CRHR_1$. Arrows indicate that there is expected to be an increase in the proportion of pituitary cells that belong to (A) the subpopulation that expresses POMC, ACTH and $CRHR_1$, and (B) the subpopulation that expresses POMC and ACTH, while there may be a decrease in (C) the subpopulation that expresses POMC and $CRHR_1$, and no change in (D) the subpopulation that expresses POMC only. The methods established in Chapter 2 are used to test this model in Chapter 4.

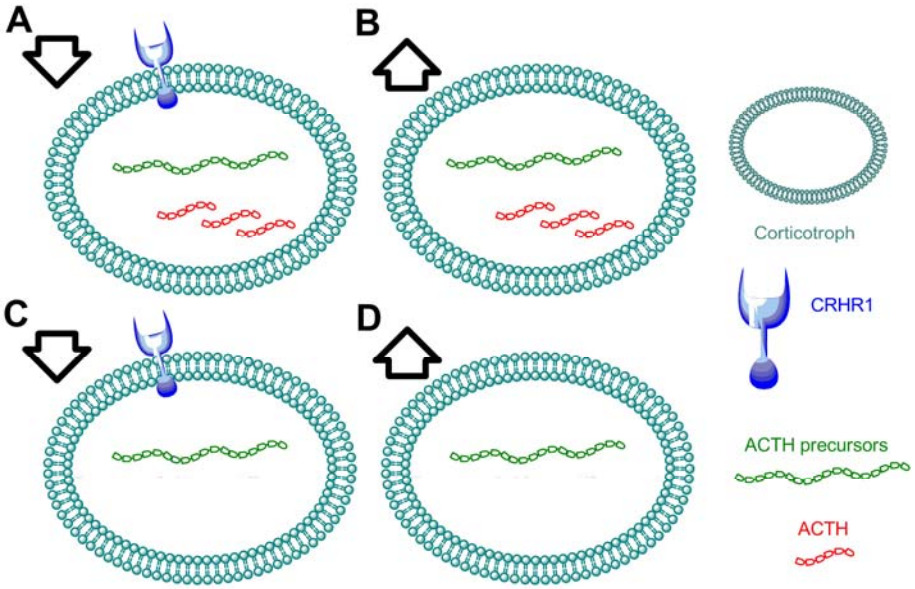


Figure 1.9 Schematic model of PR-induced changes in the hypothetical subpopulations of corticotrophs that differentially express POMC, ACTH and $CRHR_1$. Arrows indicate that there is expected to be a decrease in the proportion of pituitary cells that belong to (A) the subpopulation that expresses POMC, ACTH and $CRHR_1$, an increase in (B) the subpopulation that expresses POMC and ACTH, a decrease in (C) the subpopulation that expresses POMC and $CRHR_1$, and an increase in (D) the subpopulation that expresses POMC only. This model is tested in Chapter 5.

Chapter 2: Method development and validation

2.1 Introduction

As described in the previous chapter, there is evidence that not every individual fetal sheep corticotroph processes POMC to ACTH with identical cellular machinery (Bell et al. 1998), and that not all corticotrophs respond to CRH (Butler et al. 1999). In addition, studies using immunohistochemistry have established morphological characteristics that distinguish individual corticotrophs (Perry et al. 1985; Mulvogue et al. 1986). It was therefore expected that separate subpopulations of corticotrophs might differentially contain POMC, ACTH and $CRHR_1$. To investigate this hypothesis, multiple labelling immunohistochemistry and automated quantification methods were developed and validated.

To investigate the functional differences among corticotrophs in POMC processing and the capability to respond to CRH, I designed a series of studies to identify the expression of POMC, ACTH and $CRHR_1$ at the level of individual cells. The experimental approach involved multiple-label immunofluorescence to determine the degree of colocalisation of POMC, ACTH and $CRHR_1$ in individual corticotrophs.

There were two major conventional obstacles I had to overcome with this approach. Firstly, the formaldehyde-fixed tissue was intensely autofluorescent, and therefore before embarking on these studies I compared and optimised various bleaching techniques. Secondly, fixation masks some antigens (Shi et al. 1997; Shi et al. 2001), and therefore before commencing experimental work I systematically established an

optimised antigen retrieval system in the tissues I was using for all the proteins I was to identify by immunological methods.

Quantitative immunohistochemical investigation of the parenchymal cells of the anterior pituitary may be confounded by changes in cells size, morphology and vascularity, if a volumetric approach is taken (Levy 1999). Therefore, I decided to count the proportion of pituitary cells expressing each of the peptides instead of determining the proportion of pituitary volume positive for each peptide. To quantify the number of cells across seven possible subpopulations in pituitaries from replicate fetuses in several gestational age and treatment groups, manual quantification was unrealistic. Modern information technology and recent advances in image analysis software dramatically increased the consistency and speed of quantitative analysis of microscopy images. Most off-the-shelf software is designed for general use and requires some customisation to achieve maximum accuracy and speed for a specific application. Identifying and quantifying positive and negative particles corresponding to the number of cells within heterogeneous tissues such as the anterior pituitary provided some challenges for currently available image analysis systems. The problems included the accurate discrimination between positive and negative pixels, the correct identification of the overlap of three separate positive signals, representing POMC, ACTH and $CRHR_1$, and the accurate counting of the number of cells represented by a large positive particle representing contiguous corticotrophs. For these reasons I developed an

automated quantification method, which I validated against an extensive set of manual counts.

2.2 Methods

2.2.1 Animals

All procedures were approved by The University of Adelaide Animal Ethics Committee. South Australian Merino ewes were mated on designated dates. Those carrying twins were selected and cared for in the paddocks of the South Australian Research and Development Institute and the School of Agriculture and Wine of the University of Adelaide. At least one day prior to collection of the fetal pituitaries, the ewes were transferred to the Medical School Animal House.

2.2.1.1 Pituitary Collection and Processing

Ewes were killed by an intravenous overdose of sodium pentobarbitone (200mg/kg; Lethobarb; Virbac Pty Ltd., Peakhurst, New South Wales, Australia). The fetuses were delivered via laparotomy, weighed and killed by decapitation.

2.2.1.1.1 Tissue collected for western blotting

Pituitaries from two control fetal sheep at 140 days gestation were collected separately into liquid nitrogen and extracted independently. Samples of the tissues (50mg) were homogenized on ice in 250µl of radioimmunoprecipitation assay lysis buffer (RIPA; 50 mM Tris-HCl, 150

mM NaCl, pH 8.0, 0.1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1mM disodium EDTA and protease inhibitor cocktail). After homogenization, samples were centrifuged at 10,000 x g for 5 min at 4ºC and the clear supernatant collected. Protein concentrations were measured by Bio-Rad protein assay.

2.2.1.1.2 Tissue collected for immunohistochemistry

Pituitaries were excised intact and immediately fixed by immersion in 4% formaldehyde in 0.1M phosphate buffered saline (PBS; see Appendix A.1) at 4°C for 24h. Tissues were then washed twice in PBS and dehydrated in 70% ethanol. Whole pituitaries were bisected in the coronal plane, with the neurointermediate lobe kept intact so the neural lobe could be used as a negative control tissue and the intermediate lobe as a positive control tissue. Pituitaries were then processed into paraffin wax blocks, sections (5μm) were cut at 100μm intervals, and collected onto glass slides coated with poly-L-ornithine (Sigma, St Louis, MO).

2.2.2 Western Analysis

Protein samples (20μg/well) from the extracts of the two fetal pituitaries were resolved in separate lanes using 4-15% gradient-ready polyacrylamide gel (Bio-Rad, NSW, Australia) and then transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked with Tris-buffered saline (TBS) containing 5% (wt/vol) non-fat dried milk and incubated with rabbit anti-POMC immunoglobulin G (ST-1 IgG, 1:250,

kindly donated by Dr S. Tanaka, Japan) or anti-ACTH IgG (1:250, DAKO, Glostrup, Denmark). The POMC antibody is specific for the full length protein, being raised against the nine amino acid cleavage site between murine ACTH and β-LPH. The murine sequence is identical to the sheep sequence except for the ninth amino acid which is absent in ovine POMC (see Figure 1.1; Tanaka et al 1991). The ACTH antibody was raised against the 16 C-terminal amino acids of $ACTH_{1-39}$.

Membranes were washed and incubated with either horseradish peroxidase-coupled sheep anti-rabbit (1:3000, Silenus Laboratories, VIC, Australia) or goat anti-mouse antibody (1:2000, Sigma). Bound peroxidase activity was detected by the enhanced chemiluminescence system (ECL, Amersham Biosciences, England, UK) and visualized by exposure to radiographic film (Hyperfilm ECL, Amersham) for 1 minute for bands above 10 kDa molecular weight, and for 5 minutes to improve the visualization of the smallest peptide bound by anti-ACTH. Precision Plus dual colour protein standards (BioRad) were used as molecular weight markers.

The specificity of the labelling for the antisera raised against POMC and ACTH was confirmed by preabsorption with the ST-1 peptide (the POMC fragment as used to raise the POMC antibody) and $ACTH_{1-39}$ peptide (AUSPEP, VIC, Australia), respectively. Non-specific binding of the secondary antisera to membranes was determined by omission of the primary antisera.

2.2.3 Immunohistochemistry

2.2.3.1 Bleaching

In preliminary studies, the tissue sections were found to have autofluorescence of sufficient intensity to interfere with the accurate detection of the positive labelling with fluorophores. It was therefore necessary to reduce the autofluorescence before immunohistochemistry could be performed.

2.2.3.1.1 Chemical Reduction of Autofluorescence

Pituitary sections were rehydrated in histolene (Fronine, NSW, Australia) and equilibrated in a series of 100%, 90% and 70% ethanol rinses before washing in 0.1M phosphate buffered saline (PBS) three times for 5 min each. Sections were then treated with sodium borohydride (1% w/v, pH 7.5) for 30 min and washed in PBS.

2.2.3.1.2 Photo-bleaching

2.2.3.1.2.1 Globes

For 24hr, a slide was placed 5-8 cm below each of the following globes: 100W incandescent globe (Philips, Indonesia); 15W Reptistar (Sylvania, Germany); 15W energy efficient (Evamax Daylight, China); and 50W halogen (Gartner Superlux Ltd, Auckland, New Zealand). A second slide, which was not placed under any light source, served as a control. All slides were then rehydrated, washed in PBS and sealed with cover slips.

2.2.3.1.2.2 Duration

A slide was placed under the optimal globe (50W halogen) for each of the following durations: 0 (control), 6, 12, 24, 48 and 72h. All slides were then rehydrated, washed in PBS and cover slipped.

2.2.3.1.3 Autofluorescence measurements

Sections were visualized on a BioRad MRC1000uv laser scanning confocal microscope built around a Nikon DIAPHOT 300 inverted microscope. Green autofluorescence was detected using 488 nm laser for excitation and imaged using a 522/535 nm bandpass (BP) filter. Red autofluorescence was detected using the 514 nm laser for excitation and imaged through a 585 nm longpass (LP) filter. Far red autofluorescence, visualised using the 647 nm laser for excitation and imaged through a 680/732 BP filter, was found to have negligible autofluorescence and was therefore excluded from autofluorescence comparisons.

All images were collected using a 40x/1.15NA water immersion objective lens. Five fields were imaged with a 40x objective and 3x optical zoom spanning a straight line through the centre of the section from the superior to the inferior edges (Figure 2.1). A histogram was generated for each image that depicted the grey scales values of every pixel. The average pixel grey scale value was found to be a good estimate of the “brightness” of each field and was therefore used to compare the effects of the borohydride, different globes and different durations of photoirradiation in tissue autofluorescence intensity.

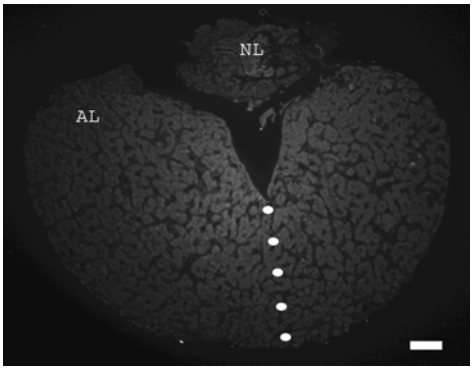


Figure 2.1 Image of pituitary section, AL = anterior lobe, NL = neuronal lobe. White circles indicate the positioning of fields for autofluorescence measurements. Scale bar = 100μm.

2.2.3.1.4 Optimal bleaching protocol

It was found that the optimal bleaching method was to place the slides 5-8cm below a 50W halogen light bulb for 24h to reduce tissue autofluorescence.

2.2.3.2 Antigen Retrieval

The antigen retrieval battery described by Shi et al (1997) was modified to use buffers (see Appendix A.2) with a pKa similar to the desired pH of the solution. Briefly, ten slides were bleached, rehydrated and washed. Nine slides were subjected to antigen retrieval under the conditions outlined in Table 2.1. The heating was performed in water baths because it provides more even and controlled heating than the microwave method prevalent in the histochemistry literature. The slides were then cooled for 20 min in a water bath before being washed in PBS. The tenth slide remained in PBS. Slides were then incubated with primary and secondary antisera (as

detailed below) and cover-slipped. Sections were examined under an epifluorescence microscope (AX70 Olympus, Tokyo, Japan) attached to a digital camera (Photometrics Cool Snap Fx Roper Scientific, Tucson, AZ, USA). The quality of staining was scored blindly from - (negative) to ++++ (positive with white saturation in images captured at 500 msec exposure).

Table 2.1 Battery of antigen retrieval conditions modified from Shi et al 1997.

The best results for all antibodies were achieved at the highest temperature. ACTH and CRH-R1 were most intense for the neutral to basic solutions, whereas POMC was most intense in the acidic solution. Therefore, a further trial was undertaken at 121°C in Tris-HCl at pH 6.6. This method of antigen retrieval was found to be suitable for detection of all three antigens, POMC, ACTH and CRHR1.

2.2.3.2.1 Optimal antigen retrieval

As a result of the screening trials, the AR process was refined as follows. Rehydration and washing were followed by antigen retrieval in Tris-HCl buffer (0.1M, pH 6.6) for 10 min at 121°C. Sections in a tanks containing PBS were then placed in a water bath at 22°C for 20 min to cool, and washed three more times in PBS.

2.2.3.3 Antibody binding

Sections were incubated for 30min in blocking solution (PBS, 0.01% azide, 10% normal donkey serum) at room temperature. Three-color immunofluorescence was performed by incubating the sections for 24hr at 4ºC with an antibody cocktail containing: mouse anti-ACTH IgG (1:50, DAKO, Glostrup, Denmark), goat anti-CRHR1 IgG (1:50 Santa Cruz Biotechnology, CA) and rabbit anti-POMC IgG (ST-1, 1:100) in antibody diluent (see Appendix A.3). After washing in PBS, slides were incubated for 2hr at room temperature with secondary antisera conjugated to distinct fluorophores: cyanine (Cy) 3 conjugated donkey anti-mouse IgG; Cy5 conjugated donkey anti-goat IgG; and Cy2 conjugated donkey anti-rabbit IgG (1:200 1:100, 1:100, respectively, Jackson ImmunoResearch, Luton, UK). The slides were washed in PBS and incubated with 3μM 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, Molecular Probes Inc, OR) before a final wash in PBS. Glass cover slips were attached with antifade fluorescent mounting medium (DAKO).

2.2.3.4 Controls

The following control experiments were conducted in conjunction with the experimental staining to determine the specificity of the immunohistochemical labelling for each peptide.

2.2.3.4.1 Preabsorption and replacement

The specificity of the staining for the antisera raised against POMC, ACTH and $CRHR_1$ was confirmed by preabsorption with the ST-1 peptide (the POMC fragment as used to raise the POMC antibody), $ACTH_{1-39}$ (AUSPEP, VIC, Australia) and the $CRHR_1$ peptide fragment used to raise the $CRHR_1$ antibody (Santa Cruz Biotechnology), respectively. Substitution of the monoclonal ACTH with another monoclonal antibody against a protein not expressed by the pituitary, namely, the A chain of human laminin (4C7, DAKO) was also performed to confirm the specificity of the antibody raised against ACTH.

2.2.3.4.2 Primary omission control

Each primary antibody was omitted and substituted with the diluent to test for nonspecific reactions between the tissue and the secondary antibodies.

2.2.3.4.3 Secondary antisera specificity

Sections were also incubated with a single primary antibody followed by incubation with the cocktail of all three secondary antibodies. This allowed us to confirm that each secondary antibody reacted only with the appropriate primary antibody, and that each dichroic mirror only transmitted light from the appropriate fluorophores. This was particularly important for the Cy3 and Cy5 mirrors as some versions of these mirrors are not specific enough to distinguish between Cy3 and Cy5 fluorophores.

Section	Primary Antibody	Secondary Antibody
1	mouse α ACTH	Cy2 donkey α rabbit IgG
2	mouse α ACTH	Cy5 donkey α goat IgG
3	rabbit α POMC	Cy3 donkey α mouse IgG
4	rabbit α POMC	Cy5 donkey α goat IgG
5	goat α CRHR1	Cy2 donkey α rabbit IgG
6	goat α CRHR1	Cy3 donkey α mouse IgG

Table 2.2 Sections 1-6 were incubated with the corresponding primary and secondary antisera to determine the inappropriate reactions between primary and secondary antibodies.

2.2.4 Imaging

2.2.4.1 Qualitative imaging

2.2.4.1.1 Intracellular localisation

To determine whether staining was localized to the cell membrane, cytoplasm or nucleus, confocal microscopy (for equipment specifications, see Section 2.2.3.1.3 was used to obtain a series of images of staining for nuclei, POMC, ACTH and $CRHR_1$ through the depth of the cells. DAPI signals were imaged using excitation of 351/363 nm laser and emission with 460 LP filter. Cy2 was detected using 488 nm laser for excitation and imaged using a 522/535 BP filter. Cy3 was detected using the 514 nm laser for excitation and imaged through a 585 LP filter. Cy5 was detected using the 647 nm laser for excitation and imaged through a 680/732 BP

filter. All images were collected using a 40x/1.15NA water immersion objective lens and a z-step of 0.5 µm through a depth of 10 µm.

2.2.4.2 Quantitative imaging

Four images per field, one for each of the fluorescent labels, were captured using an epifluorescence microscope and digital camera (see 2.2.3.2 Antigen Retrieval). Ten to fifteen non-overlapping fields were captured randomly from each pituitary section at 400x magnification using V++ (Total Turnkey Solutions, Mona Vale, NSW, Australia) and saved in Tagged Image File format (TIF). Each pixel in the images represented $30nm^2$ of tissue. Since images were captured as eight bit greyscale images, each pixel in the image was displayed as a shade of grey, ranging from 0 (black) to 255 (white) corresponding to the intensity of the light transmitted from the section to the camera.

2.2.5 AnalySIS module

This image analysis procedure was designed to use the greyscale values of pixels to determine the areas of each field corresponding to positive staining for each of the four labels, and to use the positive areas in each field to identify the proportion of pituitary cells that expressed all possible combinations of POMC, ACTH and $CRHR_1$. This two step procedure was repeated for approximately 10,000 images to collect the data reported in this thesis. In order to automate the repetitive steps I constructed mini-programs called modules. These modules function in the same fashion as

macros. A shortcut key is pressed to activate the module, which then performs a set series of commands such as opening up files, calibrating images, and determining the positive areas. The use of modules hastens image analysis because the computer can open menus, select options and enter values at machine speeds and accuracy. The computer will also perform repetitive steps reproducibly, eliminating the error inherent in human processing.

I constructed two modules in programming language C++ to run with the image analysis program, AnalySIS (Soft Imaging Systems, Münster, Germany). The first module (Appendix B.1) opens the four images for a field, calibrates the images, uses input from the user to select the positive greyscale values for each field, transforms the images into binary images and saves the binary images. This entire sequence is repeated for all fields from a section before moving onto the second module. The second module (Appendix B.2) opens the binary images for POMC, ACTH and $CRHR_1$ and produces a new set of binary images representing the pixels that are positive for all possible combinations of POMC, ACTH and $CRHR_1$ for each field. The binary image for the nuclei is then opened and all positive areas within all images are compared with a classification scheme to determine the number of pituitary cells and the number of cells expressing all possible combinations of POMC, ACTH and $CRHR_1$.

The following subsections describe the processes of calibration, selection of positive areas, colocalisation of POMC, ACTH and $CRHR_1$ and quantification in further detail.

2.2.5.1 Calibration

The calibration value was determined by photographing a graticule in the same manner as the tissue sections. The *calibrate* function within AnalySIS was used to draw a line between two scale lines and enter the distance this line represented. AnalySIS then calculated the calibration value in nm^2 per pixel, and this value was transferred into the module code.

2.2.5.2 Grey scale threshold

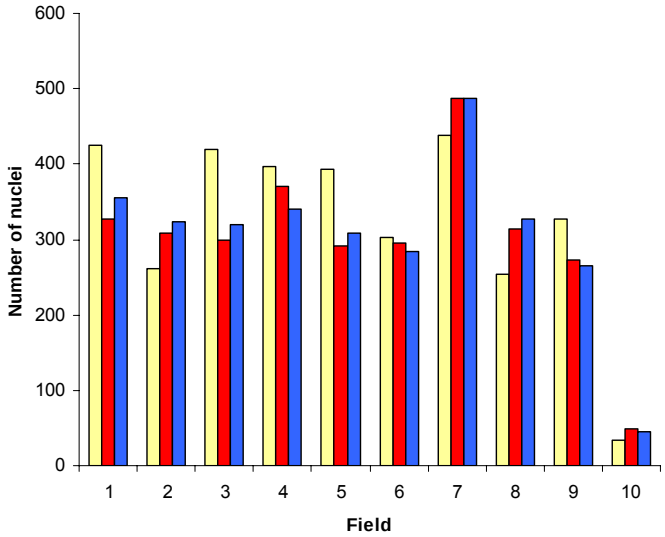
The *Threshold* function was used to view a histogram representing the number of pixels at each of the greyscale values between 0 and 255 (Figure 2.3). The operator selects the range of greyscale values that represents the positive stain. Since the positive stain is brighter than the background, and therefore represented by whiter greyscale values, an upper value of 255 remained constant. Within each image the operator selected the lower greyscale value to accurately highlight all of the positive stain whilst excluding the background. If a field was observed where the highest greyscale value of the background was greater than the lowest greyscale value of the positive stain, the field was discarded

The maximum greyscale values, or intensity, of the background for each of the fluorophores typically ranged between 45 and 60 (this is consistent with the background grey scale values reported for tissue after 24 hour of bleaching under a halogen lamp, Figure 2.10). However, even after bleaching, there was a variation in the background intensities across each tissue section. In general, the center of each section had a higher

background intensity than the edge of the tissue (this variation can be seen in Figure 2.1). It would have been convenient to use a standard threshold for each fluorophore, as this would avoid having the operator define the threshold for positive pixels for each image and would have completely removed user intervention from the quantification method. To determine if a standard threshold could be used, I determined the mean threshold used for ten fields for the nuclei and ACTH labels and used these grey scale values as the threshold for all ten fields. I then compared the number of nuclei and ACTH positive cells for each field for each method of quantification: manual counting; user defined threshold; and standard threshold (Figure 2.2). The coefficient of variation between the standard threshold method and the manual quantification was 14% for the nuclei and 33% for ACTH. In contrast, the coefficient of variation between the user defined method and manual quantification was 4% for the nuclei and 6% for ACTH. These results clearly indicate that using a user-defined threshold for each image is a more accurate approximation of manual quantification than using a standard threshold for each fluorophore.

After determining the grey scale values representing the positive stain for each image, AnalySIS produced a corresponding binary image. In the binary image, pixels from the original images that had greyscale values within the positive range were white (value = 1) while pixels that had greyscale values below the positive threshold (the background) were black (value = 0) (Figure 2.4).

A)



B)

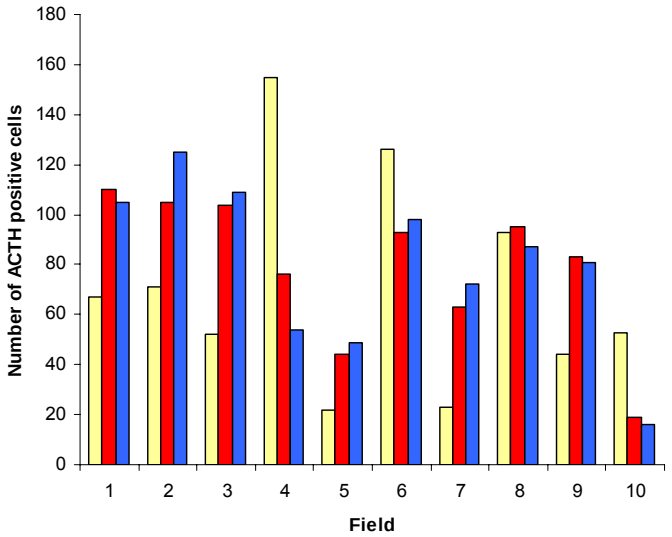


Figure 2.2 Number of nuclei (A) and ACTH positive cells (B) for each of ten fields examined by the automated method with standard thresholds (yellow), the manual counting method (red) and the automated method with user defined thresholds(blue).

A) B)

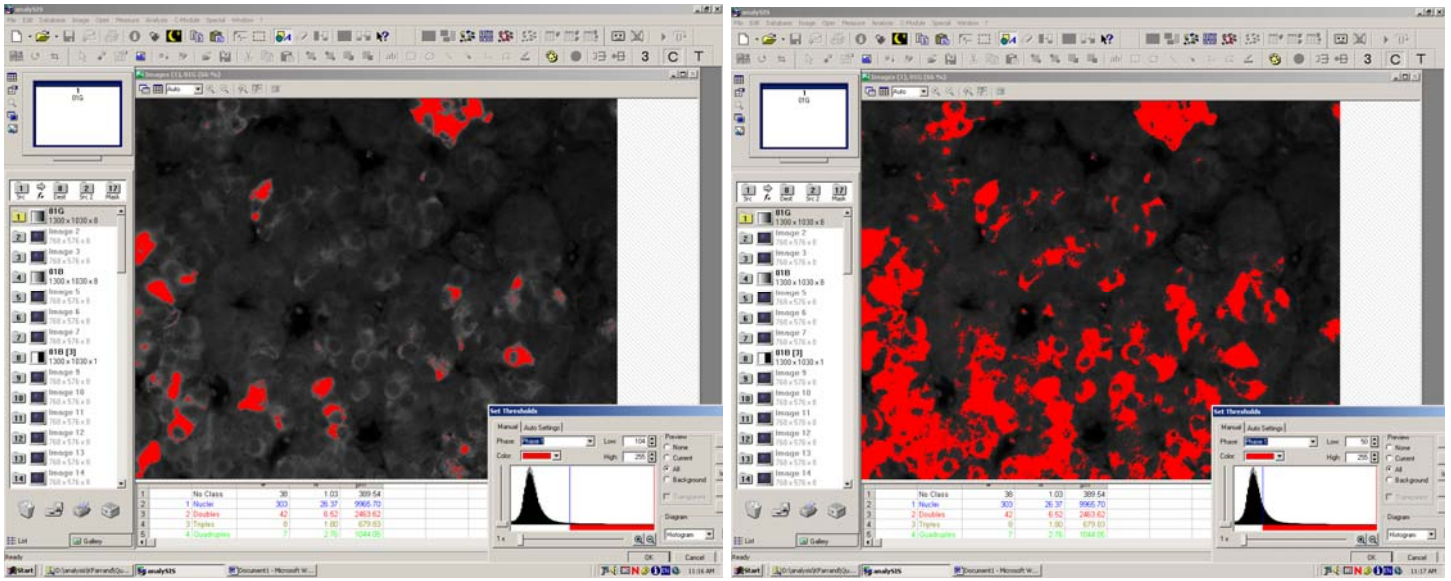


Figure 2.3 A) The *threshold* function was used to view a histogram (bottom left corner) representing the number of pixels at each of the greyscale values between 0 and 255. Moving the blue line along the x axis of the histogram selects the range of greyscale values that represents the positive stain. B) When the blue line is moved to the left, the operator has instructed the program to consider lower greyscale values as positive and more of the image is highlighted in red. The minimum greyscale value representing positive staining is determined when the operator is confident that all positive cells are covered in red and no background has been selected.

2.2.5.3 Colocalisation by subtraction

The *Subtraction* function within AnalySIS was used to identify pixels that were positive in more than one image. This function subtracts the value for each pixel in one image from the value for the corresponding pixel in a second image (Figure 2.4).

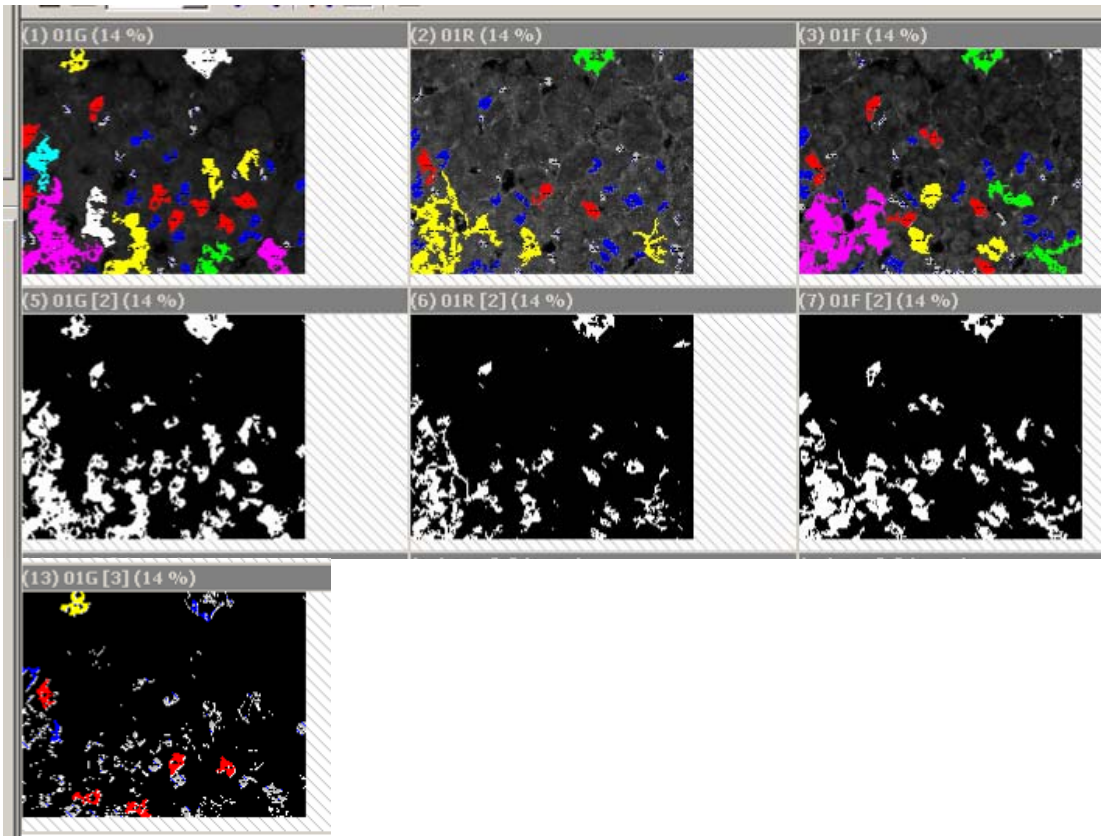


Figure 2.4 First row: coloured areas represent positive areas identified from the greyscale images for POMC, ACTH and $CRHR_1$ (left to right). Second row: binary images representing the positive staining for POMC, ACTH and $CRHR_1$ (left to right). Third row: image represents the areas positive for POMC but not ACTH or $CRHR_1$ i.e. POMC only cells.

Since the greyscale images had been reduced to binary format, each pixel was either 0 or 1, and displayed as black or white, representing negative or positive, respectively. When one binary image is subtracted from another binary image the resulting image only contains positive pixels (value = 1) where there was a positive pixel in the first image but not in the second image (i.e. 1 – 0 = 1). For example, if the ACTH image is subtracted from the POMC image, then the resulting image will contain positive pixels that were positive for POMC but not for ACTH. When the $CRHR_1$ image is subtracted from this image, then the resulting image has the pixels that were positive for POMC but not for ACTH or CRH-R_1 (i.e. POMC only, Figure 2.4 third row). The subtraction function was repeated for pairs of images to determine the positive areas for the seven possible combinations of POMC, ACTH and $CRHR_1$ (Table 2.3).

	Peptide combinations	Fluorophore Colours	Subtraction process
1	ACTH only	red only	ACTH minus POMC minus CRH-R_1
2	POMC only	green only	POMC minus ACTH minus CRH-R_1
3	CRH-R_1 only	far red only	CRH-R_1 minus POMC minus ACTH
4	ACTH+CRH-R_1	red and far red	ACTH minus POMC minus ACTH only
5	ACTH+POMC	red and green	ACTH minus CRH-R_1 minus ACTH only
6	POMC+CRH-R_1	far red and green	POMC minus ACTH minus POMC only
7	ACTH+POMC+CRH-R_1	red, green and far red	ACTH minus ACTH only minus ACTH+POMC minus ACTH+CRH-R_1

Table 2.3 Possible combinations of the positive labelling for POMC, ACTH and $CRHR_1$ with respective fluorophore emission colours and subtraction schedule for images.

2.2.5.4 Counting cells

Many corticotrophs were part of clusters of contiguous corticotrophs, which covered most of the image in some fields (Figure 2.4 first row: a cluster of twelve corticotrophs positive for POMC, ACTH and $CRHR_1$). Contiguous cells in fetal sheep pituitaries have been reported previously as palisades of columnar corticotrophs (Perry et al. 1985; Antolovich et al. 1989; Antolovich et al. 1991), which can be seen clearly in Figure 2.13 A. Contiguous positive pixels are grouped as a single *particle* by AnalySIS, and therefore scored as one *particle* by Analysis. To correct for the number of individual cells in clusters, I determined the size of individual cells within clusters of contiguous cells and demonstrated that individual cell size did not vary with cluster size (Figure 2.5). From the mean and range of sizes of

individual corticotrophs, I developed a size-based classification scheme (Figure 2.6) which coded *particles* detected by AnalySIS into class bins representing the number of cells present in the cluster, based on the size (area) of the *particle*. In this way, the automated method was able to determine consistently how many individual cells were represented by each positive *particle*. The classification scheme consisted of class bins ranging from single cells to the maximum number of corticotrophs that could fit into the field image if all cells were corticotrophs. In all chapters of this dissertation, the clusters identified in fetal pituitaries did not exceed 150 cells, which was smaller than the maximum class bin in the classification scheme. Accurate counting of individual cells within cluster was validated against manual quantification. To manually count the number of cells within each cluster, I printed the DAPI image onto a transparency and physically superimposed the nuclei image over the cytoplasmic image. Manual and automated counts of the number of individual cells within clusters were then compared. It would have been convenient to have AnalySIS superimpose the image of the nuclei over the cytoplasmic image and then determine the number of cells within clusters. Unfortunately, although the spherical morphology of nuclei made it easy to distinguish individual nuclei by eye, many nuclei also overlap within the images. As a result, AnalySIS also scored contiguous nuclei as a single particle and was therefore unable to determine the number of nuclei based on particle counts. Accordingly, I also determined the size of individual

nuclei using the DAPI images and developed a corresponding classification scheme to count the number of nuclei in each field.

The number of particles in each class of the scheme was the reported in a spreadsheet along with the total area for each class and the class labels (Figure 2.7, AnalySIS actually produces 11 separate spreadsheets per field, one for each antigen column in the Figure 2.7, I constructed a macro in Visual Basic to make Excel copy the results from each of the individual spreadsheets and compile them in the template as shown in the Figure 2.7. In addition to markedly accelerating the process of counting contiguous cells, this macro removed all human error. For each phenotypic subpopulation (Figure 2.7, Columns G to M), the number of particles in each class was multiplied by the number of cells each class represented (Figure 2.7, Column B) and the sum total number of cells in was determined (Figure 2.7, Row 22). The sum of all cells in each phenotypic subpopulation for all fields in each section was calculated at the bottom of the spreadsheet.

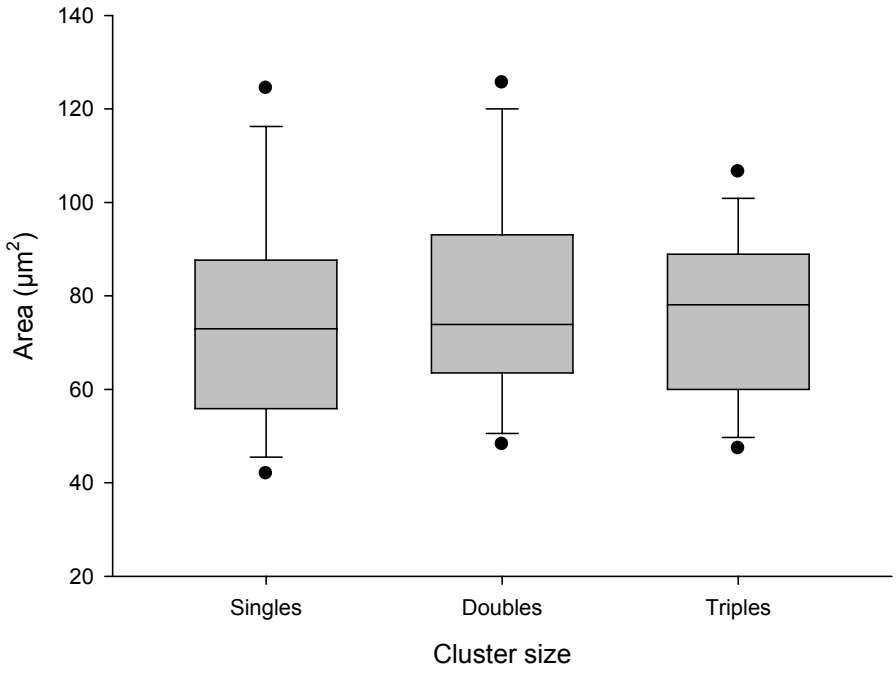


Figure 2.5 Cross-sectional area of individual cells identified as a single cell (Singles) or part of a cluster of two (Doubles) or three (Triples) cells. Box indicates range from 25th to 75th percentiles, line inside box indicates median, error bars represent the 10th and 90th percentiles and black circles represent 5th and 95th percentiles.

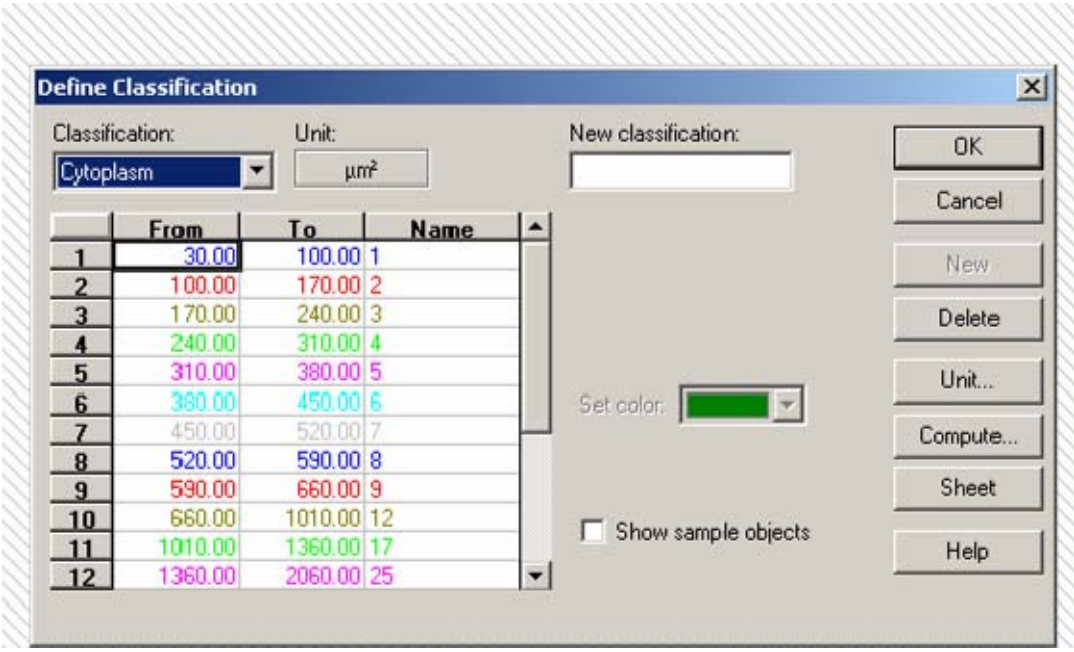


Figure 2.6 Classification scheme indicating the minimum (From) and maximum (To) area in µm^2 representing the first twelve class bins for single cells to clusters of approximately 25 labelled cells (Name).

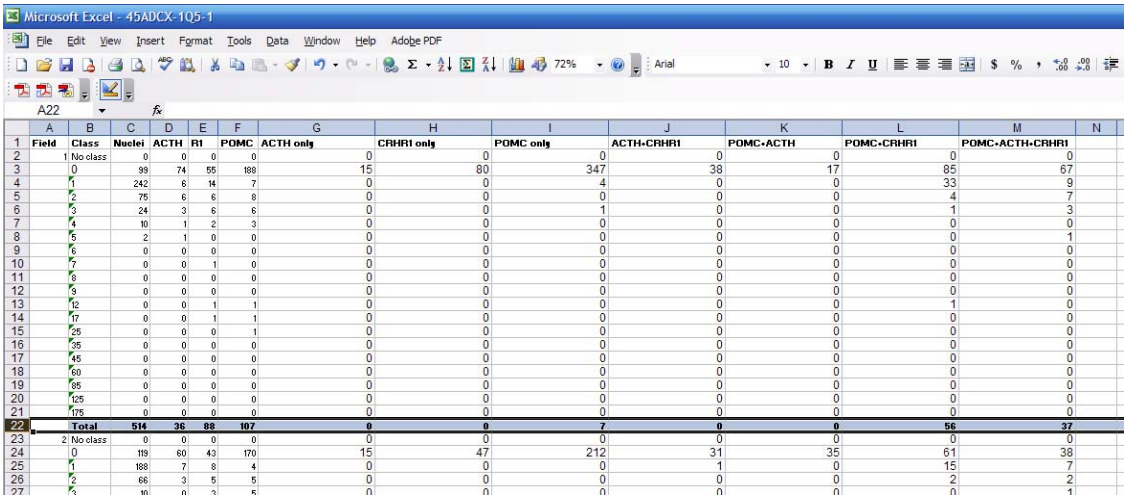


Figure 2.7 Spreadsheet summarising the number of particles in each class for each possible combination of POMC, ACTH and CRHR1. Row 22 (highlighted) shows the total number of cells expressing each peptide combination for Field 1.

2.2.5.4.1 Determining cell size for cytoplasmic stains

To determine the size of a single positive cell, the *Single Particle Result* function was used to measure the size of particles that clearly corresponded to a single cell. At least twenty cells from five different fields were used to find an average cell size as well as the maximum and minimum cell sizes. This was repeated for obvious double cells and triple cells to ensure that cells within a contiguous group were the same size as single cells. This determination of cell size was repeated for each gestational age and treatment group to check for cell hyperplasia.

There was no evidence of different cell sizes between animal groups, and individual cells within contiguous cluster were the same size of regardless of the number of cells within the cluster. Therefore the classification scheme was extrapolated to a maximum area covering most of the image.

2.2.5.5 Validation against manual counts

Automated counts using the procedure described above were compared with manual counts to confirm the accuracy of the automated method. Each image was printed out at A4 size to count manually the number of nuclei and the number of cells in each subpopulation in five fields. When there was a repeatable variation of less than 10% on either side between the quantification methods in determining the number of nuclei and number of cells in each subpopulation, the automated method was accepted as a reliable substitute for manual quantification.

2.2.6 Data analysis

Autofluorescence scores following chemical and photo bleaching were compared with unbleached controls using one-way ANOVA and, where indicated by ANOVA, with Tukey post hoc analysis. Significant differences were accepted at $P < 0.05$.

Adult type corticotrophs, have been described as being distributed "individually or in small clusters" while fetal corticotrophs are described as "usually arranged in palisades or clumps" (Antolovich et al. 1989). Inspection of the images published by Antolovich and colleagues (1989) reveals cells labelled by the authors as adult which would be part of a contiguous cluster of 3-5 cells by my automated quantification method. Therefore, I postulated that cluster of 1-4 cells in my analysis might be roughly representative of adult corticotrophs while clusters greater than 5 cells might be representative of fetal corticotrophs. I therefore divided the cells that stained positive for each peptide into small clusters (1 – 4 cells) and large clusters (more than 5 cells) to determine whether there are more cells positive for any of the three peptides in large or small clusters.

2.3 Results

2.3.1 Western Analysis

To determine the specificity of the POMC and ACTH antibodies, I performed Western blot analysis using extracts of pituitary tissues from control fetuses at 140 days gestation (Figure 1: lanes 1 and 3 represent the extract of one fetal pituitary; lanes 2 and 4 the extract of a second fetal

pituitary). The antibodies produced very discrete and unambiguous bands with no smearing. The results indicate that anti-POMC (lanes 1 and 2) strongly recognized an antigen of the expected molecular weights (~31 kDa). In addition, the POMC antibody produced a band at approximately twice the molecular weight of POMC and a much weaker staining of an unidentified band at ~19 kDa but no staining of any bands below 10 kDa even under extended exposure of the film to the Western membrane. In contrast, anti-ACTH (lanes 3 and 4) produced staining of bands at entirely different molecular weights, with no binding whatsoever to peptides above 25 kDa molecular weight, two distinct bands between 10 and 25 kDa, and, a single band below 10 kDa (which became obvious after extending the exposure time to 5 minutes). All bands visible in Figure 2.8 were abolished by the pre-absorption of primary antisera with the appropriate peptide and omission of the primary antisera (data not shown).

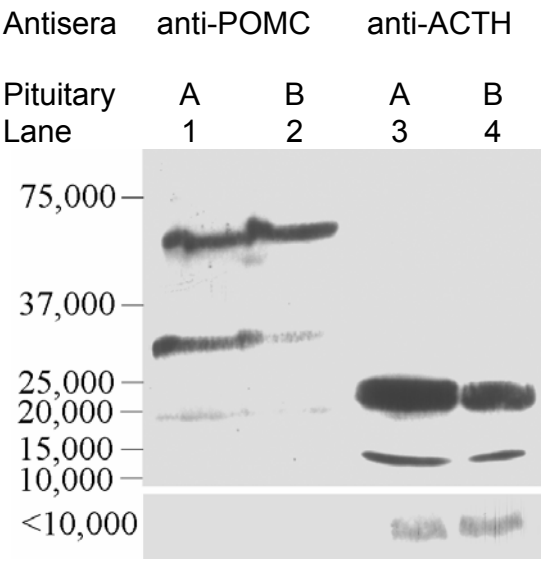


Figure 2.8 Western blot of binding of antibodies to extracts of two separate fetal sheep pituitaries. Lanes 1 and 3 represent one pituitary and lanes 2 and 4 represent the other. Lanes 1 and 2 show the binding of the POMC antibody. Lanes 3 and 4 show the binding of the ACTH antibody. Discontinuity of blot: upper panel represents 1 minute exposure, lower panel represents 5 minute exposure. Molecular weight standards indicated.

2.3.2 Bleaching

2.3.2.1 Sodium Borohydride

The green autofluorescence was not significantly reduced by pretreatment of sections with borohydride (control: 84±8 vs. borohydride: 79±12, $P = 0.991$). For red autofluorescence, the greyscale values for control (79±8) were significantly reduced by pretreatment with borohydride (29±3, $P < 0.05$).

2.3.2.2 Optimal Globe

All globes were found to reduce significantly the autofluorescence score of sections compared with no bleaching (Figure 2.9). While the halogen globe was no more effective in a statistical sense than the other globes, it was associated with the greatest overall reduction in tissue autofluorescence and was therefore used for all further experiments.

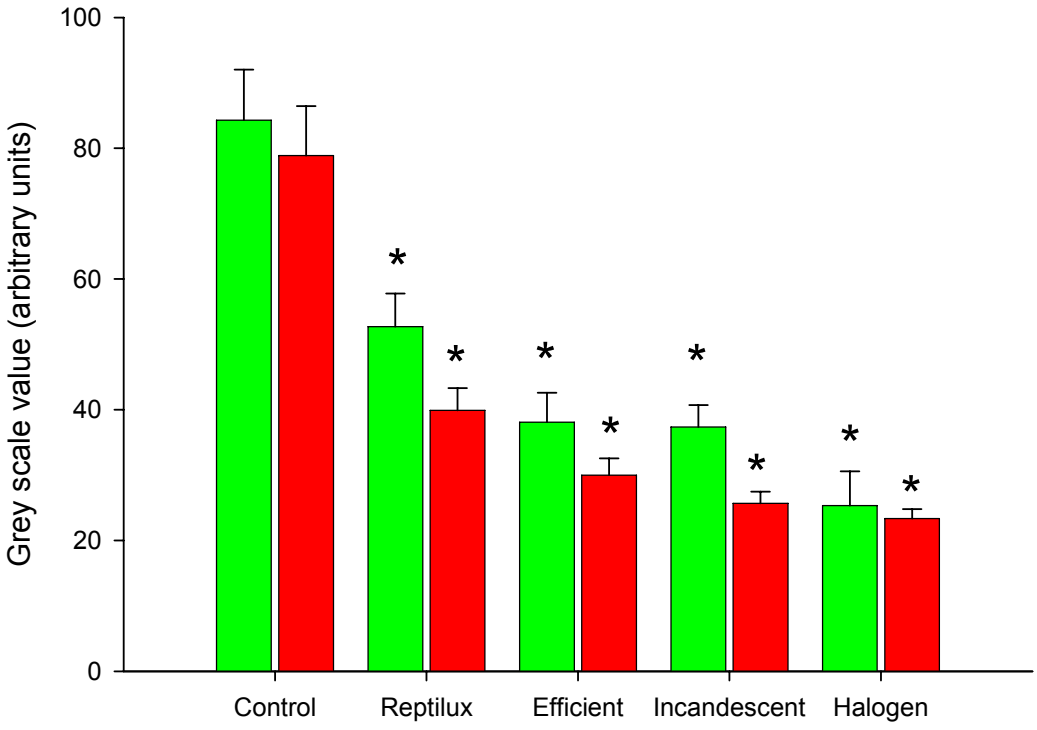


Figure 2.9 Average pixel grey scale value (mean ± SEM from five fields) for the green (green bars) and red (red bars) wavelengths from sections bleached under several lights and a non-bleached control. * indicates autofluorescence of bleached tissue is significantly less than control tissue ($P < 0.05$).

2.3.2.3 Optimal Duration

Within 24h of exposure to the halogen lamp, there was a significant reduction in intensity of autofluorescence compared with control sections not exposed to the halogen lamp (Figure 2.10). Further exposure had no additive effect, so all further experiments were performed on sections that had been photo-bleached under a 50W halogen light for 24hr. At the end of this period, the residual autofluorescence (noise) was negligible compared to the specific signal from the various fluorophores, and therefore it was possible to conduct image analyses.

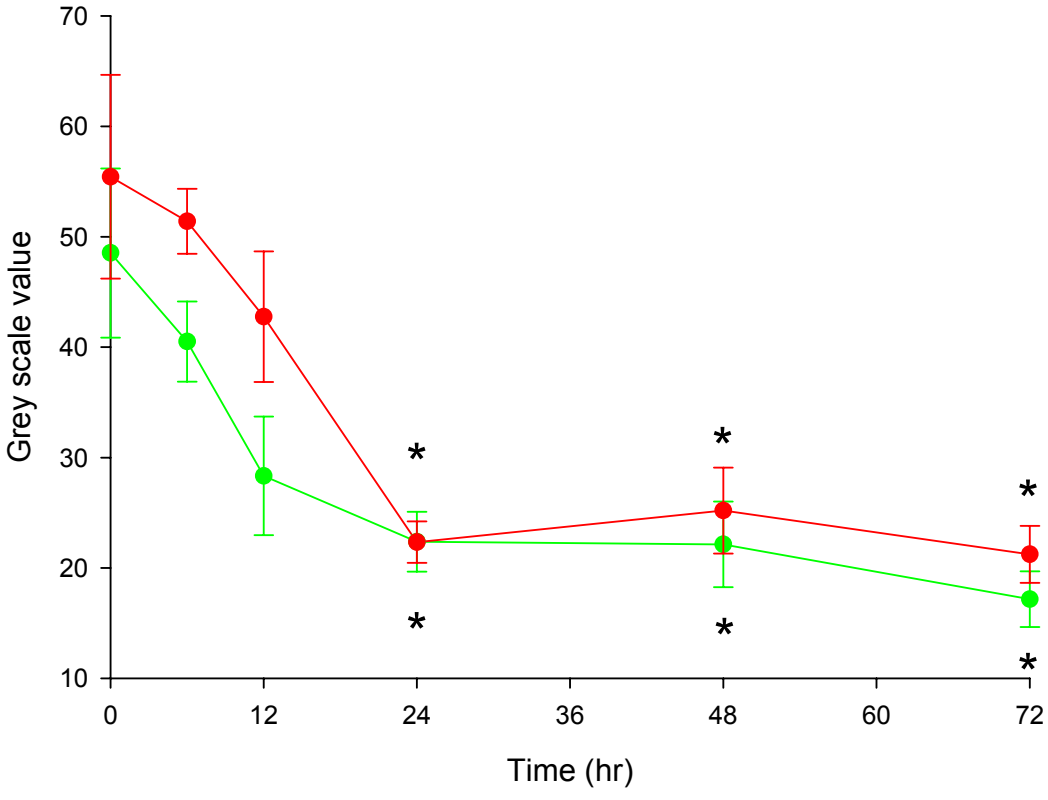


Figure 2.10 Mean ± SEM red (red line) and green (green line) autofluorescence scores after exposure to 50W halogen lamp for different durations. * indicates score is significantly different to control tissue not exposed to halogen lamp.

2.3.3 Antigen Retrieval (AR)

Table 2.4 summarises the results of screening numerous AR procedures on the immunostaining for the various antigens. In all cases, the most intense labelling for all antibodies was achieved after AR at the highest temperature (121ºC). ACTH and $CRHR_1$ antibodies produced the most intense staining after AR at 121ºC in the neutral to basic solutions, whereas POMC labelling was most intense after AR at 121ºC in the acidic solution. Therefore a further AR trial was performed at 121°C in Tris-HCl at pH 6.5 to determine whether a staining intensity of +++ or greater could be achieved for all three antibodies simultaneously (Table 2.4).

Section	Temp	AR solution	Primary Antibody	Score	Primary Antibody	Score	Primary Antibody	Score
1	80°C	pH 5: 0.1M Acetic acid	ACTH	+	POMC	++	$CRHR_1$	-
2		pH 7: 0.1M Tris-HCl	ACTH	++	POMC	+	$CRHR_1$	-
3		pH 9: 0.1M Tris-base	ACTH	+++	POMC	-	$CRHR_1$	+
4	100°C	pH 5: 0.1M Acetic acid	ACTH	++	POMC	+++	$CRHR_1$	+
5		pH 7: 0.1M Tris-HCl	ACTH	+++	POMC	+	$CRHR_1$	-
6		pH 9: 0.1M Tris-base	ACTH	+++	POMC	-	$CRHR_1$	++
7	121°C	pH 5: 0.1M Acetic acid	ACTH	+++	POMC	+++	$CRHR_1$	++
8		pH 7: 0.1M Tris-HCl	ACTH	++++	POMC	++	$CRHR_1$	+++
9		pH 9: 0.1M Tris-base	ACTH	++++	POMC	+	$CRHR_1$	++++
10	No AR	No AR	ACTH	+	POMC	-	$CRHR_1$	++
11	121°C	pH 6.5: 0.1M Tris-HCl	ACTH	++++	POMC	+++	$CRHR_1$	+++

Table 2.4 Qualitative scores for staining intensity for each antibody following a battery of antigen retrieval conditions.

2.3.4 Controls

POMC (Figure 2.11b), ACTH (Figure 2.11c) and $CRHR_1$ (Figure 2.11d) were found to be localized to the cytoplasm of anterior pituitary cells. There was no staining for any of these peptides in the neural lobe of the pituitary (Figure 2.11b-d). The cells of the intermediate lobe stained positively for POMC, ACTH and $CRHR_1$, (Figure 2.11b-d). No specific staining of anterior pituitary tissue was observed for the preabsorption controls for antisera against $CRHR_1$ (Figure 3), ACTH or POMC, nor for the replacement monoclonal antibody control (for ACTH, data not shown). None of the primary antisera were found to cause fluorescent signals that indicated inappropriate cross-reactions with secondary antisera or bleed-through of signals through the microscope filters (Table 2.5). Confocal microscopy revealed all three antigens were present throughout the entire cytoplasm. Localization of CRH-R_1 to the cell membrane could not be confirmed in any cells at any gestational age in controls or PR fetuses because the interface between the cytoplasm and cell membrane could not be identified. There was no alteration to the cellular localization of any antisera staining when the primary antisera were incubated with tissue sections separately instead of incubation with the cocktail of primary antisera.

During development of the automated quantification method, large rings were noted when the image from one antibody label was subtracted from another and cytoplasmic labelling for the two antisera did not exactly line up. After extensive investigation, I found that these rings did not provide consistent evidence of a differential intracellular localisation between POMC, ACTH and $CRHR_1$.

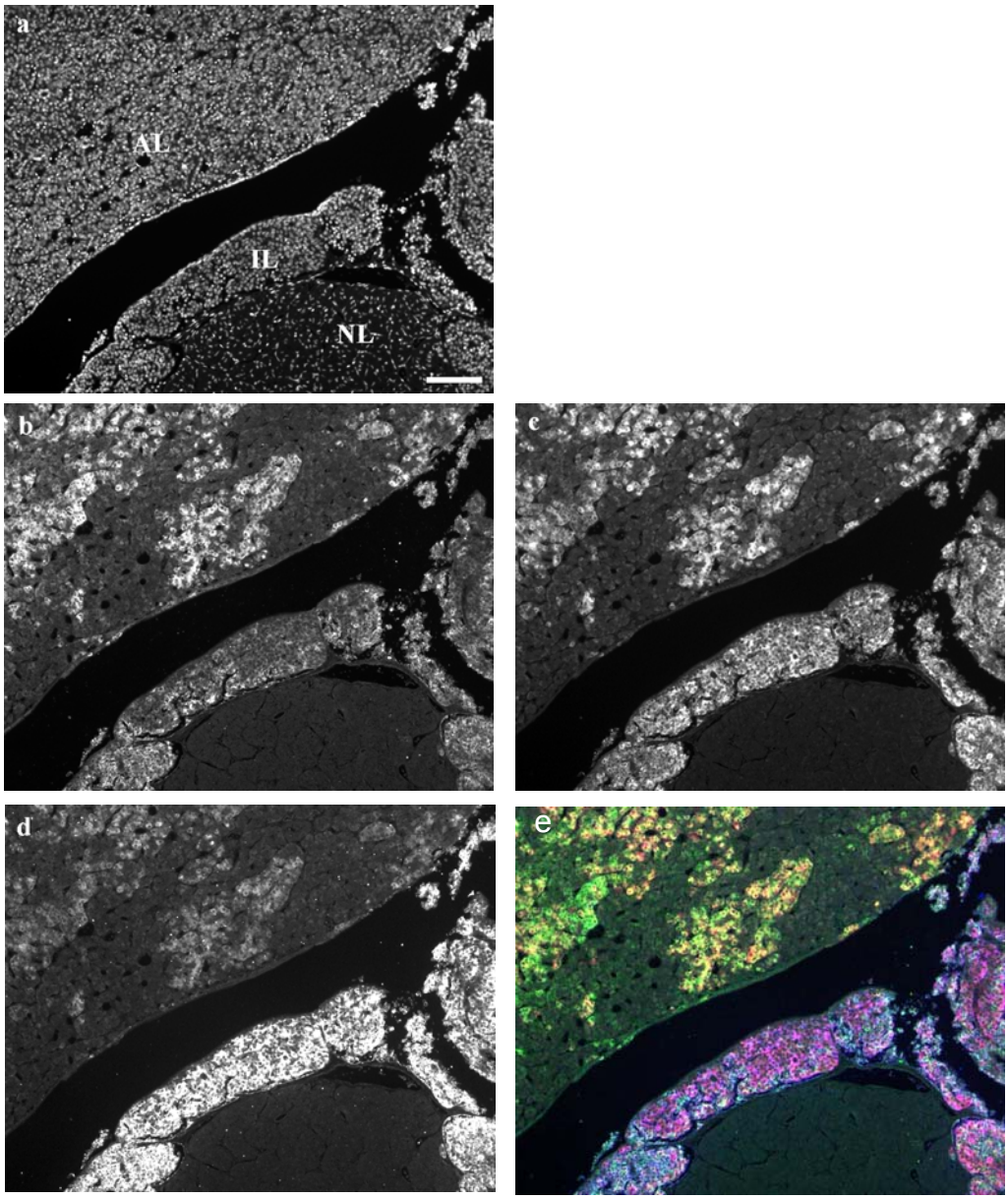


Figure 2.11 Labelling for nuclei (a), POMC (b, e: green), ACTH (c, e: red) and CRHR1 (d, e: blue) in 110d fetal sheep pituitary. AL = anterior lobe, IL = intermediate lobe, NL = neuronal lobe. Scale bar = 100μm. When viewing multi-fluorophore labelled sections through a series of dichroic mirrors, only one fluorophore can be viewed at a time (a, b, c, d). Colours visible in panel e are recreated from greyscale images of individual fluorophores in Adobe Photoshop, where a colour is assigned to each grey image and the images are merged to be viewed simultaneously.

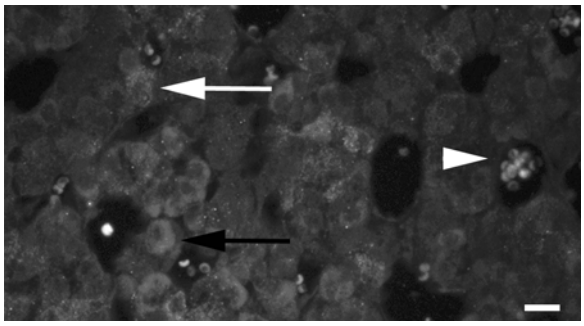


Figure 2.12 Pre-absorption control for $CRHR_1$: in the presence of blocking peptide (white arrow); autofluorescent cells (black arrow) and red blood cells (arrowhead). *Scale bar,* 10 µm.

Section	Primary Antibody	Secondary Antibody	Mirror	Score
1	Mouse anti-ACTH	Cy3 donkey anti-mouse	Cy3	++++
2	Mouse anti-ACTH	Cy2 donkey anti-rabbit	Cy2	-
3	Mouse anti-ACTH	Cy5 donkey anti-goat	Cy5	-
4	Rabbit anti-POMC	Cy3 donkey anti-mouse	Cy3	-
5	Rabbit anti-POMC	Cy2 donkey anti-rabbit	Cy2	+++
6	Rabbit anti-POMC	Cy5 donkey anti-goat	Cy5	-
7	Goat anti-CRH-R_1	Cy3 donkey anti-mouse	Cy3	-
8	Goat anti-CRH-R_1	Cy2 donkey anti-rabbit	Cy2	-
9	Goat anti-CRH-R_1	Cy5 donkey anti-goat	Cy5	+++

Table 2.5 Intensity of signal visualised through each dichroic mirror for each section incubated with a single primary antibody followed by all three secondary antisera.

2.3.5 Colocalisation

The majority of corticotrophs simultaneously stained positively for all three antisera and are referred to as POMC+ACTH+$CRHR_1$ cells (Figure 2.13 white arrow). There were also significant numbers of cells that stained positive: for ACTH and $CRHR_1$ but not POMC (ACTH+$CRHR_1$; Figure 2.13 fuchsia arrow); for POMC and $CRHR_1$ but not ACTH (POMC+$CRHR_1$; Figure 2.13 aqua arrow); and a further set of cells that were positive for POMC but not ACTH or CRH-R_1 (POMC only; Figure 2.13 green arrow). Thus, four major phenotypic subpopulations of corticotrophs were identified: POMC+ACTH+$CRHR_{1;}$ ACTH+$CRHR_{1;}$ POMC+$CRHR_{1;}$ and POMC only.

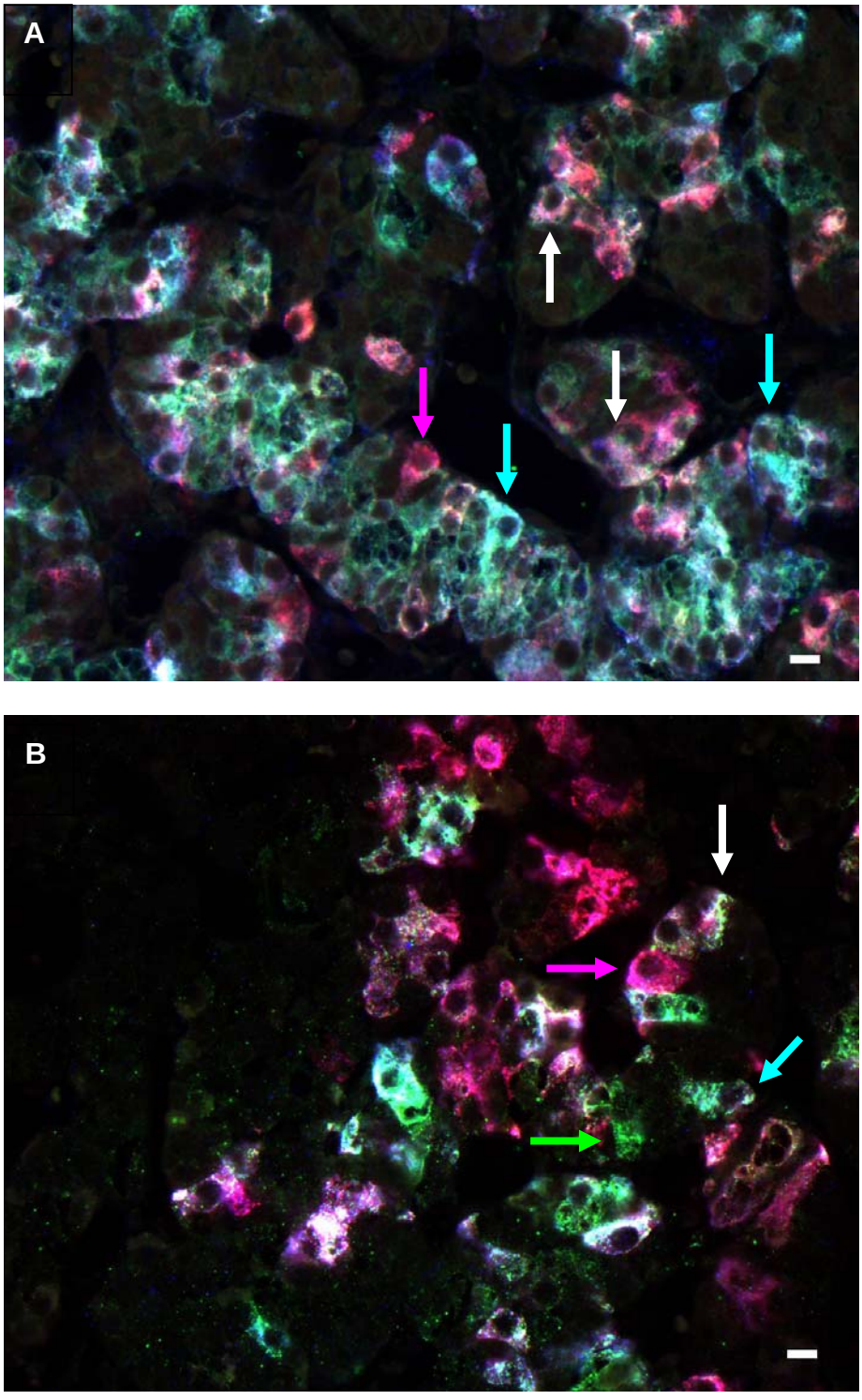


Figure 2.13 Cytoplasmic localization of POMC (green), ACTH (red) and $CRHR_1$ (blue) in the fetal sheep pituitaries at 63 (A) and 110 (B) days gestation. Green arrow indicates cells stained positive for POMC-only. Fuchsia arrow indicates cells stained positive for ACTH+$CRHR_1$. White arrow indicates cell stained positive for POMC+ACTH+$CRHR_1$. *Scale bars,* 10 µm.

2.3.6 Quantification of Corticotroph subpopulations

2.3.6.1 Corticotroph Cell Size and Cluster Size

There were no significant changes in the cross-sectional area of the cell bodies of individual corticotrophs as a function of either gestational age (see Chapter 3) or treatment (see Chapters 4 and 5). Therefore the same size-based classification scheme was used for the automated analysis of all fetal pituitaries. Positive *particles* ranged in size from single cells to clusters that covered most of the field image. Therefore the largest class used for the classification scheme reported clusters that covered the entire field image, approximately 350 cells. In all of the analyses reported in this dissertation, no field contained a cluster of positive cells greater than 150 cells.

Cells staining positive for each of the peptides, were evenly distributed amongst small clusters (1-4 cells) and large clusters (5-150 cells) (Figure 2.14). Conversely, examination of the subpopulations indicated that most cells in each subpopulation were not part of a large homogenous cluster, this was most striking for cells expressing POMC+$CRHR_1$ and ACTH+ $CRHR_1$ (Figure 2.15).

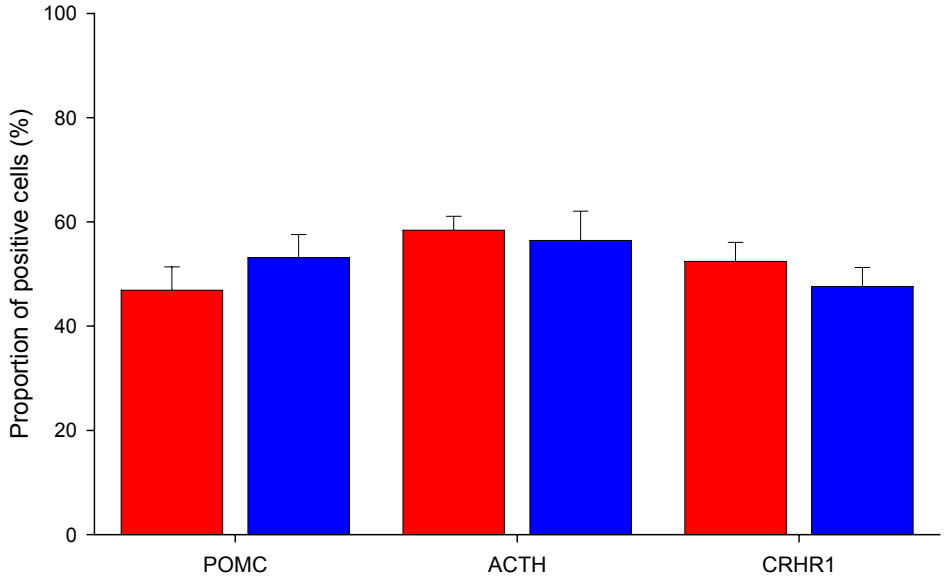


Figure 2.14 The proportion of cells positive for POMC, ACTH or $CRHR_1$ existing as part of small (red, 1-4 cells) or large (blue, 5-150 cells) clusters of cells. Data from 53-55 days gestation control fetuses is shown and is representative of all animals.

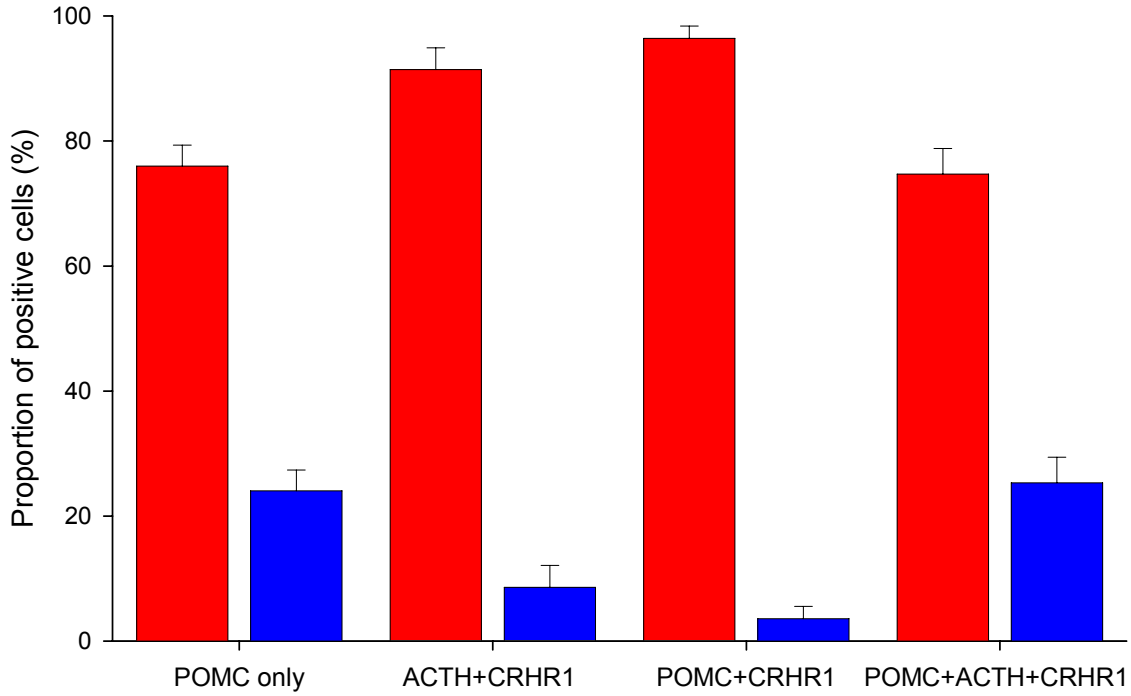


Figure 2.15 The proportion of cells in each subpopulation existing as part of small (red, 1-4 cells) or large (blue, 5-150 cells) clusters of cells. Data from 53-55 days gestation control fetuses is shown and is representative of all animals.

2.3.6.2 Validation of automated method against manual counts

As noted above, the first classification scheme, based solely on the extrapolation of measured cell sizes above, produced counts that were varied by greater than 10% from the manual counts. Therefore, I examined the differences between the cells counted manually from images and the images produced during the automated method of counting. In several fields, cells were being counted in the automated images for ACTH only, CRH-R_1 only and POMC only that were not noted as ACTH only, CRH-R_1 only and POMC only cells in the manual images.

Stepping through the images produced during the automated method it became clear that the subtraction step accounted for the deviation from the manual counting. Because the cytoplasmic locations of two peptides are not identical, electronic subtraction of one label from another left “rings” that represent part of a cell that was single labelled, where most of the cell’s cytoplasm was double labelled (Figure 2.16). This became a particular problem where contiguous cells had regions that were labelled for one peptide but not another. Areas of single label were therefore being counted as a whole cell even though the area represented only parts of two multi-labelled cells. The net effect of this was that the automated method was double counting some cells.

To correct for this double counting, I measured the average ring size using the Single Particle Result function and the lower class limit was increased from 40μm^2 to 50μm^2 to exclude the rings when counting the number of cells in images for ACTH only, CRH-R_1 only and POMC only.

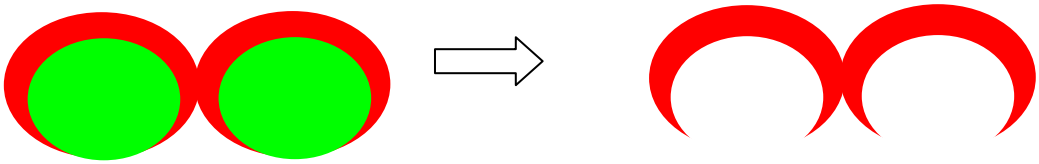


Figure 2.16 Subtraction of green pixels from red pixels leaves a ring of red pixels where part of the cytoplasm is only single labelled. When these cells are part of a contiguous cluster, some of the remaining rings are larger than some single cells.

Conversely, the area of pixels positive for two or three stains was found, in many instances, to be smaller than the whole cell. Therefore the class limit for a single cell was reduced to 15μm^2 rather than 40μm^2. These adjustments reduced the deviation between automated counts and manual counts to less than 10% for each subpopulation.

2.4 Discussion

I have successfully identified four major subpopulations of corticotrophs in the fetal sheep pituitary based on the differential coexpression of POMC, ACTH and $CRHR_1$ in individual pituitary cells. Importantly, subpopulations of corticotrophs differentially expressed POMC and ACTH, providing evidence that various subpopulations of cells may process POMC to ACTH at different rates, and therefore secrete the peptides at different rates. This finding is consistent with heterogeneity among corticotrophs with respect to the production and processing of POMC and the elaboration of CRH receptors. This may reflect heterogeneity among cells as they develop the functional molecular machinery associated with the activity of corticotrophs, as reported, for example, in the non-uniform distribution of PC1 among cells that express POMC.

2.4.1 Corticotroph subpopulations

I have identified cells expressing POMC, but too little ACTH to be detected by immunohistochemistry (i.e. the POMC only subpopulation). These cells obviously contain the full length POMC peptide but may not cleave the POMC into ACTH, consistent with the lack of PC1 transcript in some POMC mRNA expressing cells of the fetal sheep pituitary (Bell et al. 1998). I have also identified cells expressing ACTH, but too little POMC to be detected by immunohistochemistry (i.e. the ACTH+$CRHR_1$ subpopulation). These cells might process POMC to ACTH at a greater rate than those cells stained positively for both POMC and ACTH (i.e. the POMC+ACTH+$CRHR_1$ subpopulation) or those with POMC only. It must be noted that there may be POMC and ACTH present in both subpopulations of corticotrophs but that levels are below the detectable sensitivity of the method employed in this dissertation. With the use of antigen retrieval, which has been demonstrated to reveal very low levels of antigen (Shi et al. 1997), it is very likely that variations in the labelling for POMC and ACTH between individual cells represent differential rates of processing of POMC to ACTH amongst individual corticotrophs. This may be due to the differential expression and/or activation of PC1 which is produced as an 87kDa peptide and activated to a 66kDa form in acidic secretory vesicles (Vindrola and Lindberg 1992). Thus, the three major subpopulations of corticotrophs: POMC only, POMC+ACTH+$CRHR_1$, and ACTH+$CRHR_1$ expressing cells may differentially express and activate PC1, leading to the differential expression of POMC and ACTH.

Alternatively, it is also possible that individual cells observed to differentially express POMC and ACTH have different storage capacities for POMC and ACTH (Moore et al. 1983). In either case, this represents a capacity within the fetal pituitary to regulate separately the secretion of the two peptides by stimulating individual cells. The precursor forms of ACTH are secreted by fetal pituitaries *in vivo* and the present results suggest that plasma POMC levels may be maintained by secretion of POMC from cells that express POMC-only and/or POMC+ACTH+$CRHR_1$ while ACTH levels may be regulated by ACTH secretion from ACTH+$CRHR_1$ and/or POMC+ACTH+$CRHR_1$. Interestingly, a previous study using the same POMC antibody in pituitary cultures from adult sheep demonstrated that nearly half of the individual cells that stain positive for POMC also secreted POMC in unstimulated conditions and during treatment with CRH (Young and Rose 2002). Therefore, different POMC-expressing subpopulations might differentially secrete POMC.

The association of $CRHR_1$ with corticotrophs which apparently process POMC to ACTH at a faster rate, suggests there may be an association between $CRHR_1$ and the activity of PC1. CRH stimulation of cultures of the murine cell line, AtT-20, has been shown to cause secretion of the 66 kDa active form of PC1, whereas unstimulated cultures secrete only the 87 kDa precursor of PC1 (Vindrola and Lindberg 1992). Therefore CRH may stimulate POMC processing, via activation of PC1.

Interestingly, each peptide is expressed in cells that are equally distributed between small (one to four cells) and large (five to 150 cells) clusters while the majority of cells in each subpopulation did exist as part of a large homogenous cluster. Unfortunately, the fluorescent labelling used in this study often made it

difficult to define the borders of individual cells in clusters so I was unable to confirm the morphological identity of the positive cells. The development of the automated quantification system did, however, provide us with information about the proportion of positive cells in each class bin of the classification scheme. This allowed us to quantify the proportion of cells expressing POMC, ACTH or $CRHR_1$ that were part of small or large clusters. I reasoned that large clusters of corticotrophs identified in fetal pituitaries were likely to be the columnar palisades of fetal-type corticotrophs while small clusters and single cells represented adult-type stellate corticotrophs reported previously (Perry et al. 1985; Mulvogue et al. 1986; Antolovich et al. 1989; Antolovich et al. 1991). I found a relatively equal proportion of cells expressing POMC, ACTH or $CRHR_1$ in large and small clusters. This suggests that none of the peptides are more likely to be associated with either the fetal-type or adult-type corticotroph.

More interestingly, my findings suggest that each corticotroph within large palisades is likely to be neighboured by cells of different subpopulations. Such heterogeneity within clusters provides the spatial distribution necessary for paracrine communication between cells of different subpopulations. Paracrine inhibition of corticotroph secretory activity is known to exist *in vitro*, where decreased cell density increases the proportion of cells secreting ACTH in response to CRH (Jia et al. 1992). In addition, removal of CRH-responsive cells from pituitary cultures increases the basal secretion of ACTH by the remaining corticotrophs (Schwartz 1990; van de Pavert et al. 1997; Butler et al. 2002). Therefore, certain subpopulations of corticotrophs may release paracrine factors that regulate the basal and CRH-stimulated secretion of ACTH by neighbouring corticotrophs.

2.4.2 POMC and ACTH antisera specificity

The differential staining pattern in the western blots indicates that the POMC and ACTH antibodies react with epitopes on different molecules. In addition, the identification of individual cells that stain positively with either, but not both, antibodies is consistent with specific binding to appropriate antigens. The antibody used in this study to identify POMC recognises a sequence of POMC spanning the cleavage point between pro-ACTH and β-LPH. The specificity of this antisera to detect POMC and not pro-ACTH or ACTH has been previously validated in AtT-20 cells (Tanaka and Kurosumi 1992). The Western blot results clearly demonstrate that the POMC antisera reacts intensely with peptides at ~70 and ~31 kDa and reacts to a far lesser extent with a smaller peptide at ~19 kDa. This indicates that the POMC antibody reacted predominantly with full length POMC and possibly a POMC-homodimer, which has been previously reported to be present in pituitaries (Miller et al. 2003). Interestingly, the antibody produced a faint band at ~19 kDa, which was completely eliminated by pre-incubation with the antigenic peptide, suggesting the possible existence of a POMC fragment formed by cleavage at site(s) other than that between pro-ACTH and β-LPH. The reaction of the POMC antisera with a ~19 kDa peptide was unexpected given the results of studies in AtT-20 cells (Tanaka and Kurosumi 1992), but may indicate a different sequence of processing steps between the cell line versus fetal sheep pituitary cells. Elimination of this band in pre-absorption and primary omission controls suggests it was not a non-specific reaction; the identity of the 19 kDa antigen remains unknown and beyond the scope of this research.

The monoclonal ACTH antibody used in this dissertation was raised against residues 24 to 39 of $ACTH_{1-39}$ and is therefore specific for a single epitope at the carboxyl terminus of $ACTH_{1-39}$ (White et al. 1985). Previous investigation suggests this ACTH antibody is capable of binding to ACTH precursors such as POMC and pro-ACTH at high concentrations, when added to human serum (White et al. 1987). In the present western blots, the ACTH antibody reacted with peptides of molecular weights in the vicinity of that of pro-ACTH. It is possible that the free carboxyl terminus present in pro-ACTH may have reacted with the antibody. Notably, my western blot results indicate that the ACTH antibody does not recognise the full length precursor. Therefore anti-ACTH labelling in this study is a validated marker of POMC processing.

2.4.3 Intracellular localisation of antigens

As expected from previous reports (Tanaka et al. 1991; Imaki et al. 2001), the labelling for POMC, ACTH and $CRHR_1$ was distributed throughout the most of the cytoplasm with a punctate appearance which may represent the vesicular storage of the peptides. Thus, at the pixel level of images, the cytoplasmic localisation, for the three peptides did not always overlap perfectly and my analysis revealed that some parts of the cytoplasm of the multi-labelled cells were only labelled for one peptide. A significantly larger proportion of cells that expressed $CRHR_1$ or ACTH on the outer edge of the cell and POMC toward the center of the cell may have been consistent with membrane localisation of the receptor, or late vesicle docking of ACTH at the membrane. However, I did not find evidence of a consistent difference in the intracellular location of POMC,

ACTH or CRHR1 during close inspection of the subtraction images produced during the automated quantification method.

2.4.3.1 Cytoplasmic localisation of $CRHR_1$

Although I found that 12% of pituitary cells express the $CRHR_1$ peptide at 140 days gestation in control animals, it has been reported that fewer than 2.5% of pituitary cells actually bind CRH in dissociated fetal sheep cells at the same age (Young et al. 2003). In the current study, the $CRHR_1$ peptide was localised to the cytoplasm in all $CRHR_1$ positive cells, consistent with previous reports of the rat anterior pituitary (Imaki et al. 2001). Although confocal microscopy was used to confirm the cytoplasmic localisation of $CRHR_1$, this cytoplasmic localisation made it impossible to determine whether $CRHR_1$ was also present at the cell membrane in the $CRHR_1$ expressing cells. Differential intra-cellular localization of the $CRHR_1$ protein between individual cells may underlie the known differential regulation of $CRHR_1$ protein levels and CRH binding (Aguilera et al. 2004). It was unfortunate that I was unable to establish whether $CRHR_1$ was membrane bound, and therefore, whether all $CRHR_1$ expressing cells are functionally responsive to CRH.

There are other explanations for cytoplasmic localization of $CRHR_1$. Some of the cytoplasmic $CRHR_1$ identified in this study may represent receptors that were recently bound by CRH and are undergoing internalisation and degradation since binding of CRH to $CRHR_1$ has been shown to cause the complex to be internalized via endocytosis (Childs et al. 1986; Schwartz et al. 1987; Mason et al. 2002) and processed in lysosomes (Childs et al. 1986). Some of the cytoplasmic $CRHR_1$ identified in this study may be a mobilisable

source of $CRHR_1$ sequestered to the cell membrane when CRH stimulation is excessive. This may explain why 48h of CRH pre-treatment of pituitary cell cultures from fetal sheep, reduces subsequent CRH binding by 40-50% (Lu et al. 1994). In addition, the internalised $CRHR_1$ identified in this study may reflect ambient plasma cortisol at the time of tissue harvest, since glucocorticoid pretreatment of rat pituitary cell cultures reduces the proportion of cells that bind CRH by 70% (Childs et al. 1991).

2.4.4 Controls

The absence of staining for POMC, ACTH and $CRHR_1$ antisera in the neural lobe of the pituitary is consistent with previous reports (Millan et al. 1987; Matthews et al. 1994). In the intermediate lobe, intense POMC and lesser ACTH staining was present in most cells. Previous *in situ* hybridization has indicated intense POMC mRNA expression in nearly all of the cells of the intermediate lobe in the near term fetal sheep and intense immunohistochemical staining using a promiscuous ACTH antibody (Matthews et al. 1994) indicates that all cells of the intermediate lobe express ACTH-like peptides. Staining for anti-ACTH has also been reported in intermediate lobe cells of the 126-130 and 134-136 day fetal sheep pituitary (Braems et al. 1996). The intermediate lobe was stained intensely for $CRHR_1$, consistent with previous reports of immunohistochemical staining for $CRHR_1$ (Millan et al. 1987; Imaki et al. 2001) and *in vitro* evidence of secretion of immunoreactive ACTH in response to CRH (Fora et al. 1996).

Elimination of the labelling of tissue sections in the preabsorption and replacement controls also indicates that the staining seen in the anterior lobe

was specific for all three peptides. The lack of staining in the anterior lobe of fetal pituitaries when the primary antibodies were omitted indicates that there is little nonspecific binding between the secondary antibodies and the tissue.

2.4.5 Automated quantification method

The development and validation of an automated method for quantitative analysis of the cell populations made it achievable to accurately classify and count the ~2 million cells characterised in the next three chapters. Furthermore, the basic steps and code sequences developed are equally applicable to quantification of any homogeneous or heterogeneous cell populations from tissue sections and cultures with simple histological stains or multiple immunohistochemical labels.

2.5 Conclusions

Methods have been developed and validated to simultaneously identify POMC, ACTH and $CRHR_1$ in individual cells in fetal pituitary sections. This approach has identified four major subpopulations of corticotrophs. With the development and validation of an automated quantification method, it became possible to investigate the differential regulation and biological impact of these subpopulations by quantifying the changes associated with known alterations to HPA function. Specific gestational windows are known to be associated with different levels of HPA activation (Carr et al. 1995; Wintour et al. 1995), therefore I investigated the normal developmental changes in corticotroph subpopulations (Chapter 3). Since maternal periconceptional undernutrition (PCUN) is known to increase fetal plasma ACTH levels at the earliest

gestational age previously measured (Edwards and McMillen 2002), I investigated the effects of PCUN on fetal corticotroph subpopulations in early gestation (Chapter 4). Since PR is known to increase plasma ACTH levels beyond normal HPA axis activation in the fortnight before term (Phillips et al. 1996), and *in vitro* findings indicate that functionally distinct subpopulations of corticotrophs are differentially effected by PR during this period (Butler et al. 2002), I also investigated the effects of PR on fetal corticotroph subpopulations in late gestation fetal pituitaries (Chapter 5).

Chapter 3: Ontogeny

3.1 Introduction

Activation of the fetal hypothalamic-pituitary adrenal (HPA) axis during specific gestational windows and quiescence at others times is essential in mammals for proper rates of maturation and growth of several fetal organs (Ballard 1979). Adrenal steroidogenic enzyme activity is elevated between 40-90 days gestation, relatively low between 90-120 days gestation, then increases again between 120d-term (Wintour et al. 1995), and plasma cortisol levels increase over the last third of gestation with a surge at term required for correct timing of birth in the sheep (Norman et al. 1985). These major changes in adrenal activity are controlled by changes in plasma ACTH levels (Wintour et al. 1995) or, more specifically, by the relative plasma concentrations of ACTH and its biosynthetic precursors (Coulter et al. 2002). Plasma POMC and ACTH levels change independently over gestation (Carr et al. 1995; Saoud and Wood 1996) indicating there may be differential control of POMC and ACTH secretion. To support this claim, there is *in vitro* evidence that, at least over the last third of gestation, unstimulated pituitary secretion of $ACTH_{1-39}$ is increased while secretion of the precursor is not (McMillen et al. 1995). It is therefore reasonable to suggest that all corticotrophs differentially express POMC and ACTH at various stages of gestation or that the census of individual subpopulations of corticotrophs that express POMC or ACTH may fluctuate throughout gestation, in accordance with the major phases of HPA activity.

It has been noted that the morphology of fetal sheep corticotrophs changes over gestation from a predominance of large palisades of lightly stained columnar cells to single darkly stained stellate cells (Perry et al. 1985), in parallel with the

ontogenic changes ratio of low molecular weight ACTH to its high molecular weight precursors in the fetal sheep plasma (Saoud and Wood 1996). I tested the hypothesis that different morphologies were representative of differential POMC processing by quantifying and statistically comparing the proportion of pituitary cells expressing POMC or ACTH, in large and small clusters, early and late in gestation.

Overall, the binding of CRH to pituitary membrane preparations rises steadily from undetectable levels at 70 days gestation to a peak at 125 days gestation, then declining to lower levels at 145 days gestation (Lu et al. 1991). Accordingly, I hypothesised there would be a parallel increase in the proportion of pituitary cells expressing $CRHR_1$ between 60-80 and 110 days gestation, with a possible decrease at 145 days gestation. It is also known that the CRH-stimulated increases in plasma levels of $ACTH_{1-39}$ and precursors change as a function of age during the last third of gestation (Carr et al. 1995). For example, after 110 days gestation the ontogenic increase in the CRH-induced rise in plasma $ACTH_{1-39}$ levels occurs by 120 day gestation, and remains level to 140 days gestation. In contrast, the ontogenic increase in the CRH-induced rise in plasma POMC levels peaks slightly later, at 130 days gestation, and by 140 days gestation declines to a response indistinguishable from the response at 110 days gestation. This indicates that there are subtle, yet notable, ontogenic differences in the fetal sheep plasma levels of ACTH and its precursor following CRH administration. The research described in this chapter provided the opportunity to investigate whether the ontogenic change in pituitary function identified by Carr and colleagues (1995) is reflected in a greater increase in the

proportion of pituitary cells expressing ACTH+$CRHR_1$ compared with POMC+$CRHR_1$ between 110 and 145 days gestation.

CRH has been shown to control the biosynthesis and cleavage of POMC (Lutz-Bucher et al. 1987; Vindrola and Lindberg 1992; Lu et al. 1994; Li et al. 1999). Therefore, I expect that at gestational ages previously associated with high levels of hypothalamic CRH expression (Watabe et al. 1991) or high levels of CRH response (Carr et al. 1995) are likely there will be more corticotrophs that express POMC+ACTH+$CRHR_1$ or ACTH+$CRHR_1$ compared with other gestational ages.

Despite the extensive characterization of HPA activity in the fetal sheep throughout gestation, virtually nothing is known of changes to corticotrophs at the level of individual cells as a function of gestational age. Ontogenic changes that occur in the pituitary as a whole might be reflected in identical changes in whole cohorts of homogeneous cells. Alternatively, given the functional heterogeneity among corticotrophs, it is possible that the overall changes of the pituitary can be the result of changes in the relative numbers of specialised cells. A major thrust of the research described in this thesis challenges this view with the aim of determining functional changes in developing corticotrophs at the level of individual cells and relating the changes in size of the respective functionally distinct corticotrophs to the reported changes in pituitary function as a whole.

The experiments described in this chapter were designed to determine whether there are ontogenic changes in subpopulations of corticotrophs that differentially express POMC, ACTH and $CRHR_1$ coincident with the known developmental changes in pituitary secretion of POMC and ACTH and responsiveness to CRH.

I collected pituitaries at several gestational epochs representing each of the three phases of HPA activity. Considering that there are exponential changes in physiological parameters of HPA activity during the fortnight prepartum, I did not want to miss potentially rapid changes in pituitary architecture by pooling animals into groups with broad gestational age windows. Therefore fetuses were divided into groups at 140-141 and 144-145 days gestation (where term ~ 150 days gestation in this breed).

3.2 Methods

3.2.1 Animals

All procedures were approved by The University of Adelaide Animal Ethics Committee. Pregnant South Australian Merino ewes of known mating date carrying twins, were raised normally at the South Australian Research and Development Institute and then transferred, via the Waite Animal House facility, to the Medical School Animal House facility at least one day before tissue harvest. Ewes were killed by an intravenous overdose of sodium pentobarbitone (200mg/kg; Lethobarb; Virbac Pty Ltd., Peakhurst, New South Wales, Australia).

3.2.1.1 Pituitary Collection

Fetuses from twin pregnancies at 53-55 (n = 6, where n represents the number of fetuses), 63-84 (n = 6), 110-112 (n = 4), 140-141 (n = 4) and 144-145 (n = 6) days gestation were delivered via laparotomy, weighed and killed by decapitation. Fetal pituitary and adrenal weights were recorded before tissues were processed.

Pituitaries collected for immunohistochemistry were removed intact from the *sella turcica* and immediately fixed in 4% formaldehyde in 0.1M phosphate buffered saline (PBS) at 4°C for 24h. Tissues were then washed twice in PBS and dehydrated in 70% ethanol. Whole pituitaries were bisected in the coronal plane, and the neurointermediate lobe kept intact for the neural lobe to serve as a negative control tissue. Pituitaries were then processed into paraffin wax blocks. Sections (5μm) were cut at 100μm intervals, and collected onto glass slides coated with poly-L-ornithine (Sigma, St Louis, MO).

3.2.2 Immunohistochemistry

Slides were placed 5-8cm below a 50W halogen light bulb for 24h to reduce tissue autofluorescence (see Chapter 2). Sections were rehydrated in histolene (Fronine, NSW, Australia) and a series of 100%, 90% and 70% ethanol before washing in 0.1M phosphate buffered saline (PBS) three times for 5 min each. Antigen retrieval was performed in Tris-HCl buffer (0.1M, pH 6.6) for 10 min at 121°C. Sections were then placed in a water bath at room temperature for 20 min to cool and washed a further three times in PBS.

3.2.2.1 Antibody binding

Sections were incubated for 30min in blocking solution (PBS, 0.01% azide, 10% normal donkey serum) at room temperature. Three-color immunofluorescence was performed by incubating the sections for 24hr at 4ºC with an antibody cocktail containing: affinity-purified mouse anti-ACTH IgG (1:50, DAKO, Glostrup, Denmark), affinity-purified goat anti-$CRHR_1$ IgG (1:50 Santa Cruz Biotechnology, CA) and rabbit anti-POMC (ST-1, 1:100) in antibody diluent.

After washing in PBS, slides were incubated for 2hr at room temperature with secondary antisera conjugated to distinct fluorophores: Cy3 conjugated donkey anti-mouse IgG; Cy5 conjugated donkey anti-goat IgG; and Cy2 conjugated donkey anti-rabbit IgG (1:200 1:100, 1:100, respectively, Jackson ImmunoResearch, Luton, UK). The slides were washed in PBS and incubated with 3μM DAPI (Molecular Probes Inc, OR) before a final wash in PBS. Cover slips were attached with antifade fluorescent mounting medium (DAKO).

3.2.2.2 Microscopy and quantitative imaging

Four images per field, one for each of the fluorescent labels, were captured using an epifluorescence microscope and digital camera (see 2.2.3.2 Antigen Retrieval). Ten to fifteen non-overlapping fields were captured randomly from each pituitary section at 400x magnification using V++ (Total Turnkey Solutions, Mona Vale, NSW, Australia) and saved in Tagged Image File format (TIF). Each pixel in the images represented $30nm^2$ of tissue. Since images were captured as eight bit greyscale images, each pixel in the image was displayed as a shade of grey, ranging from 0 (black) to 255 (white) corresponding to the intensity of the light transmitted from the section to the camera.

The four grey scale images for each field were analysed for the proportion of pituitary cells labelled with POMC, ACTH and $CRHR_1$ and the proportion of pituitary cells labelled with all possible combinations of these peptides using the AnalySIS modules described in the last chapter (see section 2.2.5).

3.2.3 Data analysis

A corticotroph was defined as any pituitary cell that stained positively for either POMC or ACTH, and this total corticotroph population is presented as a proportion of the total number of pituitary cells. Each subpopulation of corticotrophs is presented as a proportion of pituitary cells (%) to identify the effects of gestational age and treatment on each subpopulation. Corticotrophs constitute only a small proportion of the total pituitary cells (~10% or ~100,000 cells) and each subpopulation is consequently smaller. Therefore, I also investigated the relative contribution each subpopulation made to the total corticotroph population by expressing each subpopulation as a proportion of corticotrophs (%). In all cases, results are expressed as the mean ± SEM.

A one-way ANOVA with a Tukey *post hoc* were used to analyse effects of gestational age on the proportion of pituitary cells expressing each subpopulation phenotype, and the proportion of corticotrophs expressing each subpopulation phenotype. Significant differences between gestational age groups are reported for $P < 0.05$.

3.3 Results

3.3.1 Fetal Growth

There were significant increases in fetal body, pituitary and adrenal weights between 63-84, 110-112 and 140-141 days gestation, although the rate of fetal somatic growth exceeded that of the pituitary and adrenal glands as gestation advanced (Table 3.1).

Gestational age (days gestation)	63-84	110-112	140-141	144-145
Body wt (kg)	0.22 ± 0.05	1.6 ± 0.2*	5.0 ± 0.4*	5.5 ± 0.2
Pituitary wt (mg)	16 ± 0.2	64 ± 3*	117 ± 12*	135 ± 10
Pituitary wt: body wt (mg/g)	83 ± 12	40 ± 2*	24 ± 2*	25 ± 2
Adrenal wt (mg)	38 ± 5	103 ± 8*	290 ± 13*	266 ± 10
Adrenal wt: body wt (mg/g)	198 ± 24	64 ± 9*	53 ± 16	48 ± 2

Table 3.1 Effects of gestational age on fetal growth. Pituitaries from 53-55 days gestation fetuses were too small to be weighed. * indicates value is significantly different to the previous gestational age group (P < 0.05)

3.3.2 Corticotroph Cell Size and Cluster Size

The cross-sectional area of the labelled cytoplasm of individual corticotrophs was 88±10, 81±4, 76±3 and 80±7μm^2 at 53-55, 110-112, 140-141 and 144-145 days gestation, respectively. There were no significant changes in cell size with gestational age (P > 0.05)

Throughout gestation, approximately half of the cells expressing either, POMC, ACTH or $CRHR_1$ were single cells or part of small clusters of corticotrophs (Figure 3.1), while the other half were part of larger clusters. There were no significant changes in the ratio of small : large clusters POMC or ACTH positive cells in throughout gestation. There were however significant increases in the proportion of $CRHR_1$ expressing cells that were in small clusters during the

gestational windows when HPA activity is greatest. The definition of small clusters at 1-4 cells and large clusters being greater than 5 cells was somewhat arbitrary and based on very vague descriptions from previous reports of corticotroph morphology in the fetal sheep pituitary (Perry et al. 1985; Antolovich et al. 1989). Therefore, I also checked several different limits for the boundary between small and large clusters. There was change to the above findings with alternative limits set for the small and large clusters (data not shown).

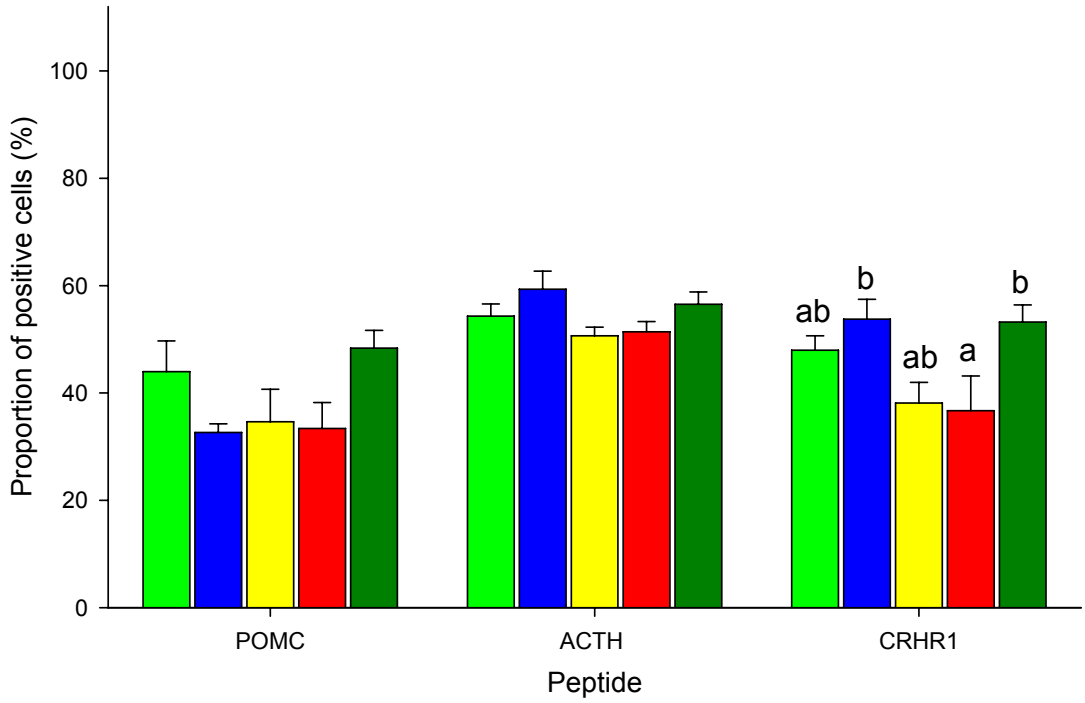


Figure 3.1 The proportion of cells positive for POMC ACTH or $CRHR_1$ existing as part of small clusters of cells (1-4 cells) at 53-5 (light green), 63-84 (blue), 110-2 (yellow), 140-1 (red) and 144-5 (dark green) days gestation. *Different superscript letters* indicate significant differences between animal groups (*P* > 0.05).

Another index, contact of two identical cell types, was applied to compare rates of cell division at various stages of gestation. The rationale for application of this index is that when one cell divides to from two daughter cells, these two cells will be contiguous cells of the same phenotype. Therefore I analysed the proportion of cells that did not neighbour any cells of the same subpopulation changes across gestation (Figure 3.2). This analysis demonstrated that the proportion of POMC+ACTH+CRHR$_1$ expressing cells that did not neighbour a like cell declined in mid gestation and rose in late gestation (Figure 3.2). Conversely, the proportion of cells expressing ACTH+CRHR$_1$ that did not neighbour a like cell peaked at 63-84 days gestation and then declined (Figure 3.2).

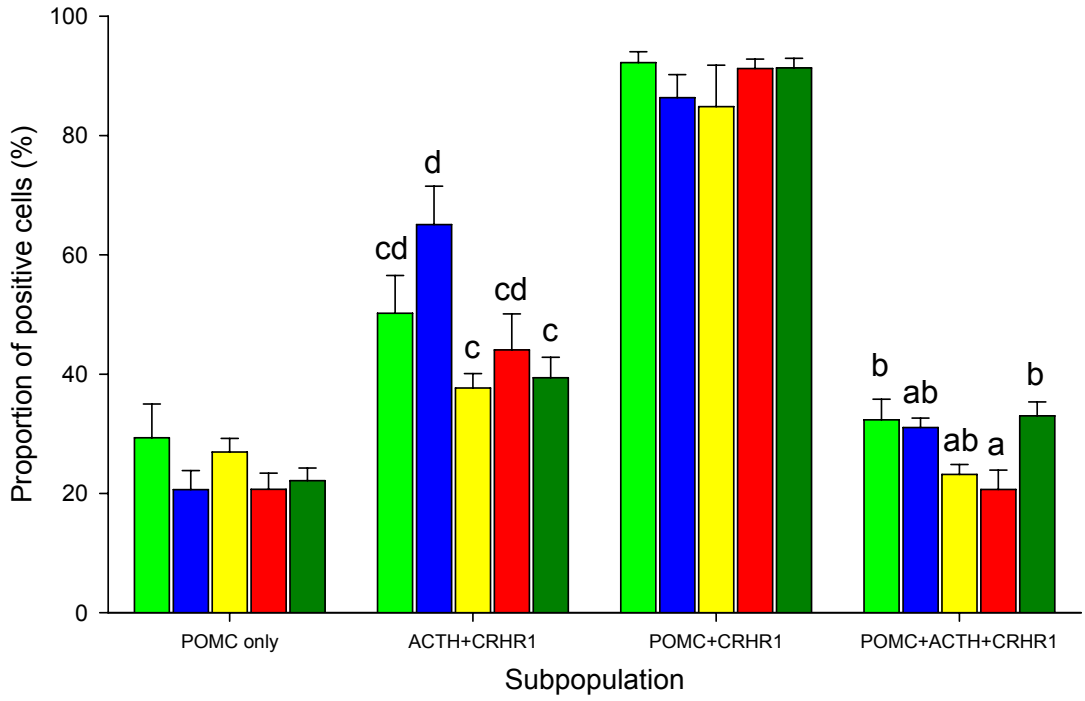


Figure 3.2 Mean ± SEM proportion of cells that do not contact another cell of the same subpopulation at 53-5 (light green), 63-84 (blue), 110-2 (yellow), 140-1 (red) and 144-5 (dark green) days gestation. *Different superscript letters* indicate significant differences between animal groups (*P* > 0.05).

3.3.3 Corticotroph Subpopulations

The following series of figures summarises the dynamics of the various subtypes of corticotrophs as a function of gestational age.

There was no significant effect of gestational age on the proportion of pituitary cells that expressed either POMC or ACTH (Figure 3.3).

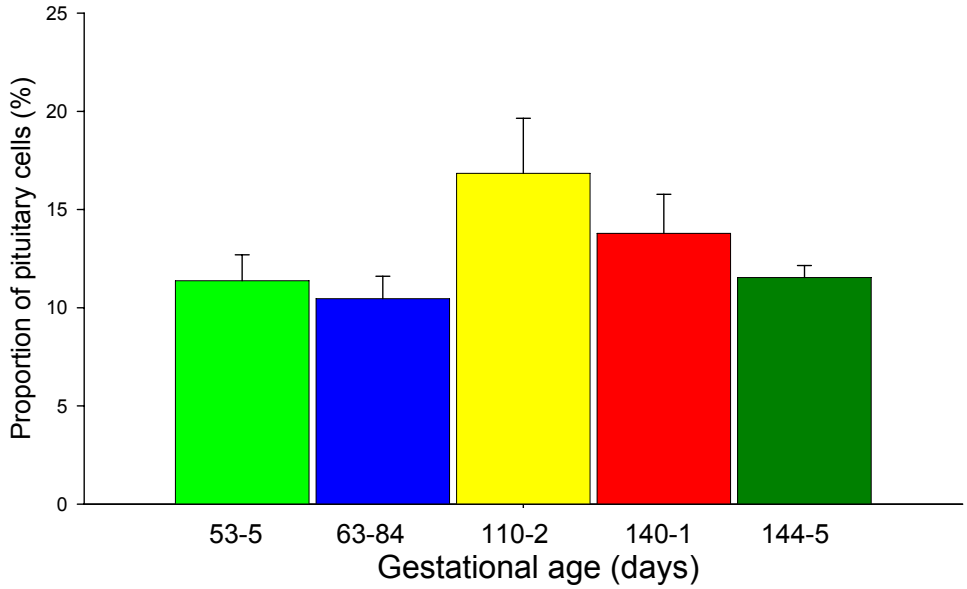


Figure 3.3 Mean ± SEM proportion of pituitary cells identified as corticotrophs at 53-5 (light green), 63-84 (blue), 110-2 (yellow), 140-1 (red) and 144-5 (dark green) days gestation fetuses.

The proportion of pituitary cells that expressed POMC, regardless of the presence of either ACTH or $CRHR_1$, did not change across gestation (Figure 3.4). In contrast, the proportion of pituitary cells that expressed either ACTH or $CRHR_1$ (and any other peptide) did undergo significant changes across (Figure 3.4). The proportion of corticotrophs expressing ACTH increased between 63-84 and 140-141 days gestation (Figure 3.5).

All four subpopulations were observed in varying numbers in every pituitary, regardless of gestational age (Figure 3.6, Figure 3.7).

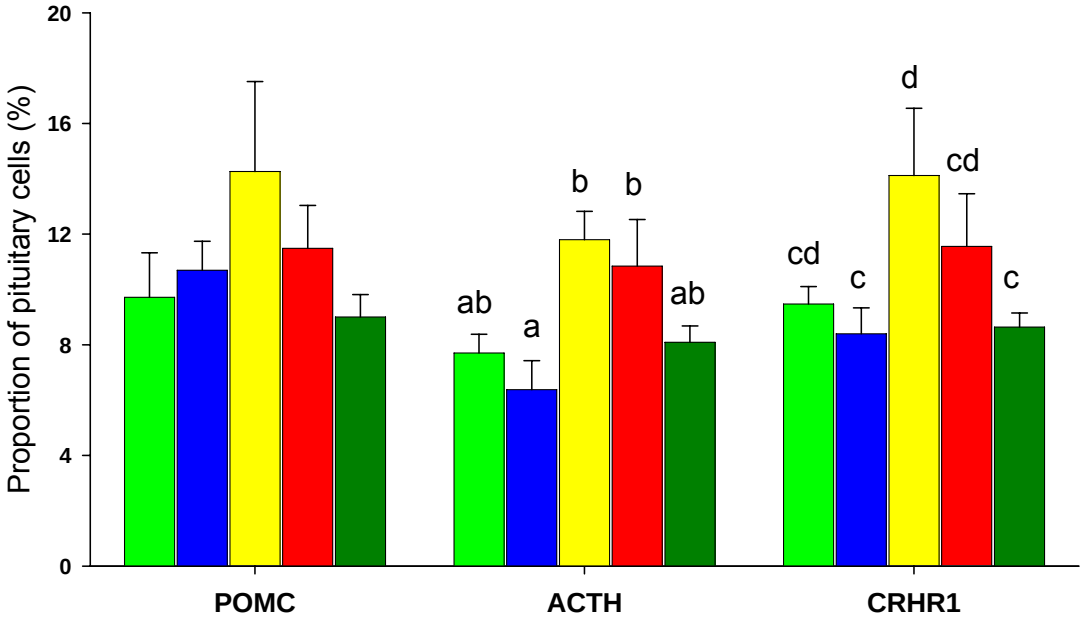


Figure 3.4 Mean ± SEM proportion of pituitary cells that express POMC, ACTH or $CRHR_1$, regardless of the coexpression of any other peptide, at 53-5 (light green), 63-84 (blue), 110-2 (yellow), 140-1 (red) and 144-5 (dark green) days gestation fetuses.

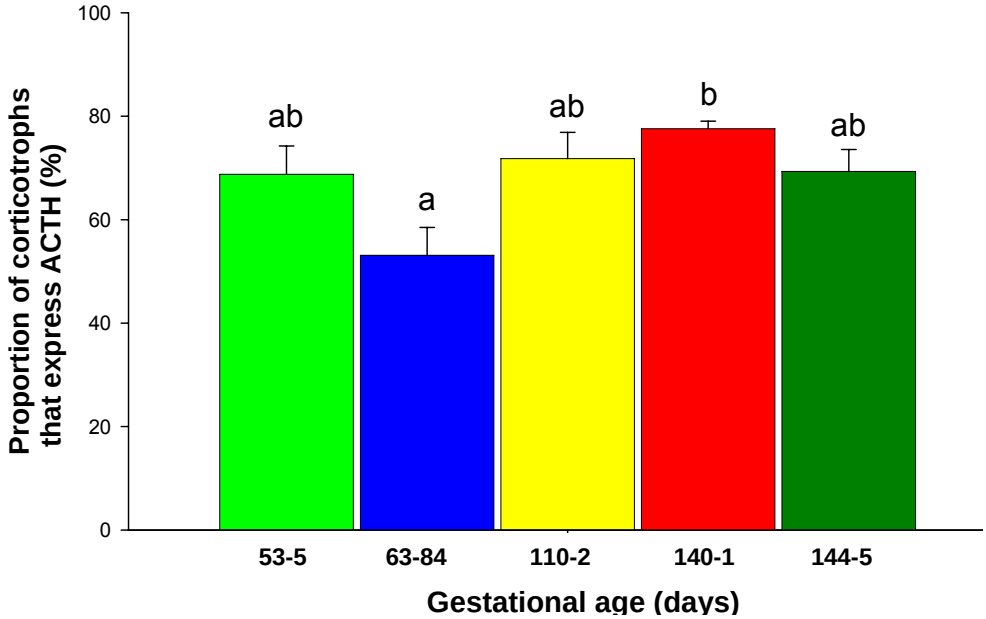
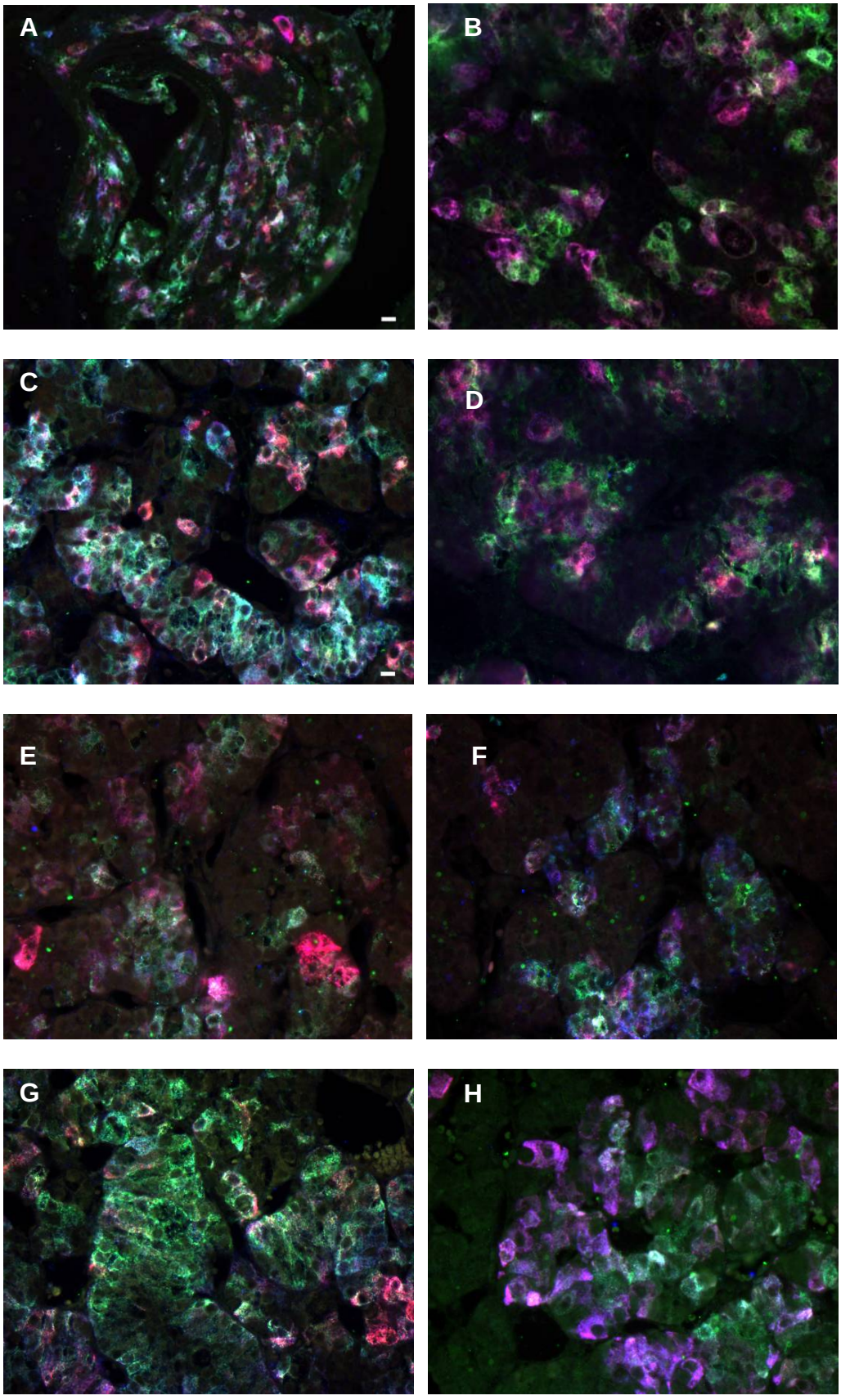


Figure 3.5 Mean ± SEM proportion of cells that express POMC (defined as all corticotrophs) that also express ACTH at 53-5 (light green), 63-84 (blue), 110-2 (yellow), 140-1 (red) and 144-5 (dark green) days gestation fetuses.


A
B
C
D
E
F
G
H

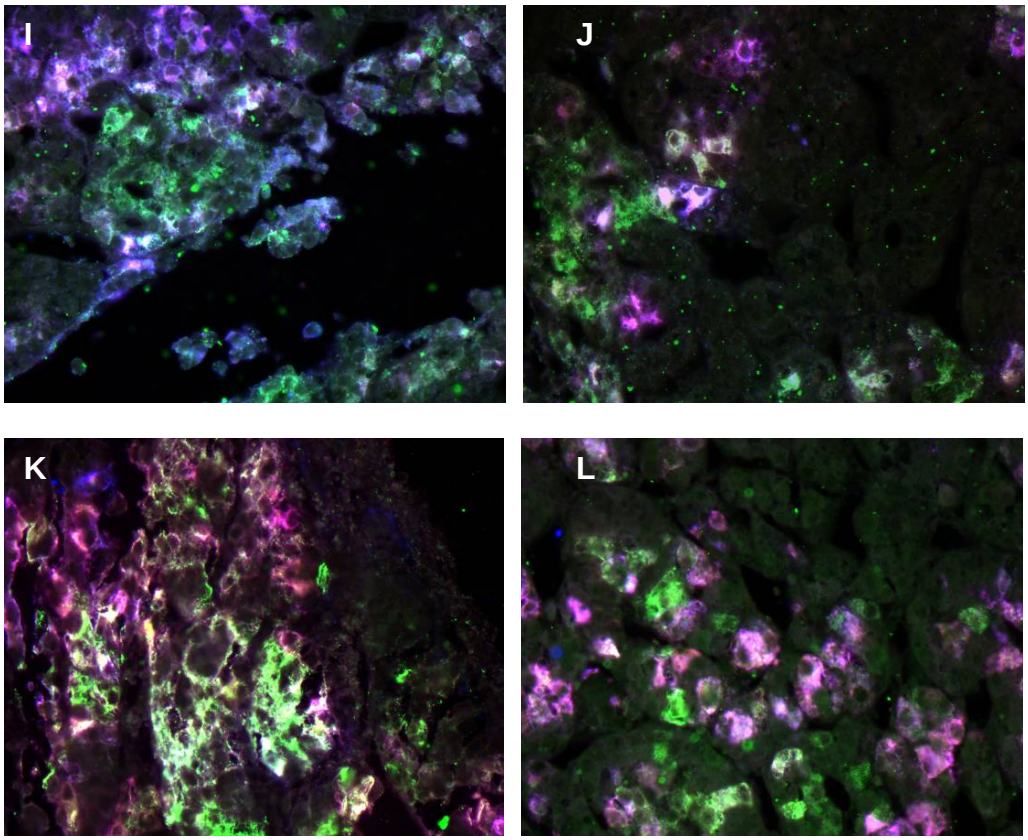


Figure 3.6 Pituitary cells stained for POMC (green), ACTH (red) and CRHR1 (blue) at 55 (A, B), 63 (C, D), 84 (E, F), 110 (G, H), 140 (I, J) and 145 (K, L) days gestation. A pair of field images for each gestational age group has been used to demonstrate the variation in cellular morphologies and peptide colocalisation amongst individual corticotrophs at each window of gestational. Scale bar = 10μm.

Throughout gestation the predominant subpopulation of corticotroph expressed all three antigens, POMC+ACTH+$CRHR_1$ (Figure 3.7). The proportion of pituitary cells in this subpopulation declined to a low at 63-84 days gestation, then peaked at 110-112 days gestation before falling again between 140-141 and 144-145 days gestation (Figure 3.7). The proportion of pituitary cells expressing ACTH+$CRHR_1$ was lowest at 63-84, but then peaked at 144-145 days gestation (Figure 3.7). In contrast there are no significant changes in the

proportion of pituitary cells that express POMC+CRHR$_1$ or POMC only throughout gestation (Figure 3.7).

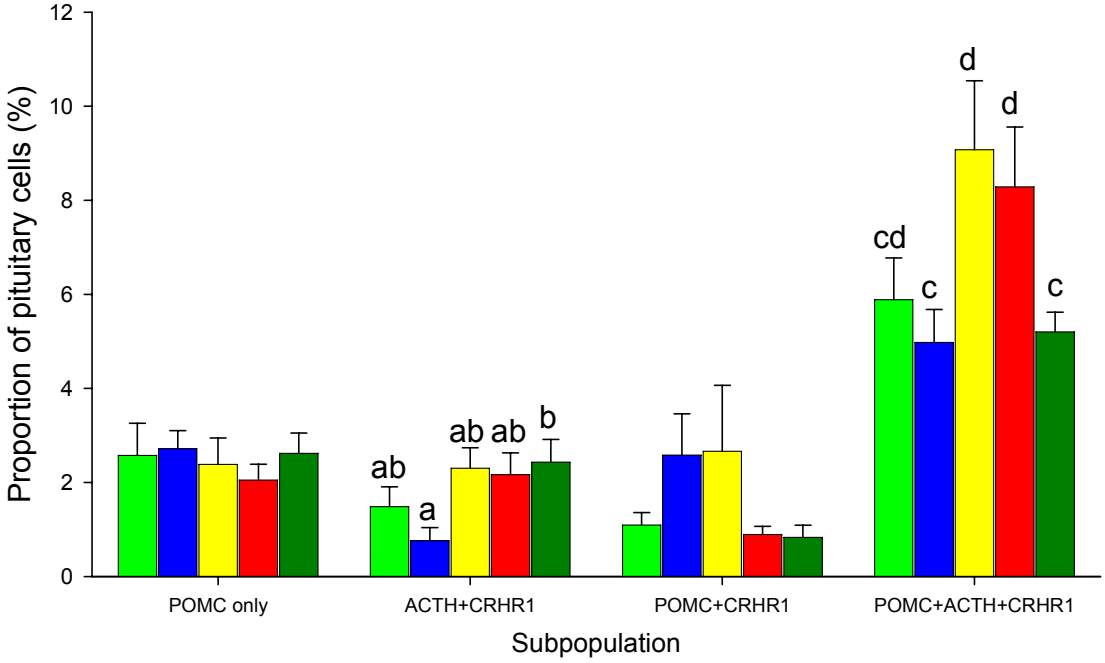


Figure 3.7 Mean ± SEM proportion of pituitary cells in each of the subpopulations at 53-5 (light green), 63-84 (blue), 110-112 (yellow), 140-141 (red) and 144-145 (dark green) days gestation fetuses. *Different superscript letters* indicate significant differences between animal groups (*P*_0.05).

3.4 Discussion

This chapter describes the characterisation of ontogenic changes in the subpopulations of corticotrophs in the fetal sheep pituitary between 53 and 145 days gestation. In the following section the findings of this chapter are discussed in relation to the three major hypotheses identified in the Introduction and relevant results in the scientific literature:

1) The proportion of corticotrophs that contain ACTH will increase across the last third of gestation.

2) The ontogenic changes in corticotroph morphology reported previously will be reflected in ontogenic changes in corticotroph cluster size.

3) $CRHR_1$ protein levels will increase in early gestation and decrease across the last third of gestation.

I then discuss the evidence for regulatory mechanisms that might play a role in the ontogenic changes in the corticotroph subpopulation, particularly the rapid loss of one specific subpopulation during the prepartum period.

3.4.1 Ontogenic changes in POMC processing

The proportion of corticotrophs that expressed ACTH increased from 63-84 to 140-141 days gestation. This suggests that the proportion of pituitary cells that synthesise POMC and cleave it to ACTH increases across this period of gestation. Interestingly, my findings present a slightly different time course for the ontogenic changes in ACTH expression by corticotrophs compared with the coexpression of transcript for POMC and the cleavage enzyme, PC1. The proportion of cells positive for mRNAs for both POMC and PC1 decreases between 107 and 126-130, and then rise by 144-147 days gestation to the levels observed at 107 days gestation (Bell et al. 1998). It must be noted that the gestational age windows used in my study and that of Bell and colleagues (1998) are not identical. The transcript data was collected from a flock of sheep where term is typically at 148 days gestation, therefore the proportion of POMC mRNA expressing cells that also express PC1 mRNA rises between 18 days before term and 1-4 days before term. In my sheep term is at ~150 days gestation and I investigated the differential expression of POMC and ACTH at 140-141 and 144-145 days gestation, which represents the period between 10

and 5 days before term. Unfortunately my window of gestation may have been too short to identify changes in the peptide expression by individual corticotrophs that parallel the ontogenic changes in the transcript levels of POMC and PC1. Most likely, the peptide expression might not be directly related transcript expression and activity. Unfortunately, Bell and colleagues (1998) provide only qualitative data on the coexpression of POMC and PC1 protein, no quantitative analyses were performed.

By examining fetuses at earlier gestational ages than the previous transcript investigation, I have identified a long term increase in the proportion of corticotrophs that contain the cleavage products of POMC processing. In general, this suggestion is broadly consistent with ontogenic data from fetal and neonatal rodents which indicate that the PC1 immunolabelling increases between postnatal days 3 and 5 and then again to adulthood (Marcinkiewicz et al. 1993).

There is an interesting body of evidence from the rodent models that indicates that POMC processing undergoes developmental changes. Fetal and neonatal rats have immature corticotrophs that contain precursors and fragments of ACTH such as CLIP and αMSH while adult rats have mature corticotrophs that contain $ACTH_{1\text{-}39}$ almost exclusively (Noel and Mains 1991). In the fetal mouse pituitary, αMSH appears earlier in gestation than $ACTH_{1\text{-}39}$ (Chatelain et al. 1979). This is supported by evidence from *in situ* hybridisation, which indicates that PC1 and PC2, the enzyme which catalyses formation of αMSH, detected by day 15 of gestation in the fetal mouse, and shows that PC1 increases with gestational age to adulthood while PC2 increases during the first two postnatal weeks and then decreases to adulthood (Marcinkiewicz et al. 1993). Given this

evidence, I might expect a developmental decrease in the subpopulations of corticotrophs expressing POMC, leaving only ACTH+$CRHR_1$ expressing corticotrophs in the adult. Compared with early gestational ages, I identified a reduction in POMC+ACTH+$CRHR_1$ expressing cells and an increase in ACTH+$CRHR_1$ expressing cells by 145 days gestation. It is possible that this pattern continues during postnatal development, and there are further losses of POMC only and POMC+$CRHR_1$ expressing cells, leaving only ACTH+$CRHR_1$ corticotrophs in the adult. Although this project did not extend to the analysis of corticotroph subpopulations in the adult, this is clearly an avenue for further investigation.

The emergence of the ACTH+$CRHR_1$ subpopulation is the greatest contributor to the increase in the proportion of corticotrophs that express ACTH across gestation. These changes parallel the ontogenic increase in the fetal plasma $ACTH_{1\text{-}39}$: high molecular weight ACTH ratio between 60 days gestation and term (Saoud and Wood 1996) may therefore represent a developmental increase in the net capacity of the pituitary to process POMC to ACTH.

In addition, the combined increase in ACTH+$CRHR_1$ cells and decrease in POMC+ACTH+$CRHR_1$ cells at 145d suggests there may be a marked increase in the net pituitary processing of POMC to ACTH in the immediate prepartum period. This ontogenic change is consistent with the increase in the ratio of low to high molecular weight ACTH being secreted from slices of fetal sheep pituitary *in vitro* (McMillen et al. 1995), and the change in fetal sheep plasma (Carr et al. 1995).

Compared with the ontogenic increase in the proportion of pituitary cells that express ACTH, there were no significant changes in the proportion of pituitary

cells that contained POMC across the same windows of gestation. The major contributor to this stability in overall pituitary POMC levels is the presence of a POMC-only subpopulation that represents a substantial proportion of corticotrophs and which does not vary considerably during gestation. Therefore, it is possible that *in vitro* POMC secretion (McMillen et al. 1995) and plasma levels (Carr et al. 1995) are maintained at a constant level over the last third of gestation primarily by the POMC-only corticotrophs.

In addition, I found a change in the proportion of pituitary cells expressing $CRHR_1$ across gestation. There is evidence from cultures of adult sheep cells that removal of all CRH-target cells does not alter the basal secretion of uncleaved ACTH precursors (Schwartz et al. 1994). The only subpopulation that does not express $CRHR_1$ is the POMC only subpopulation. Therefore, it is not unreasonable to speculate that the POMC only subpopulation may be chiefly responsible for maintaining pituitary POMC secretion.

3.4.2 Ontogenic changes in corticotroph morphology

In the previous chapter I suggested that the large clusters of corticotrophs observed in this study might correspond with the palisades of columnar corticotrophs described previously by Perry and colleagues as fetal-type corticotrophs, while the smaller clusters might represent the stellate corticotrophs known as adult-type corticotrophs (Perry et al. 1985; Antolovich et al. 1989). Similar morphological distinctions have also been reported in the rodent as large oval immature corticotrophs and small stellate mature corticotrophs (Kurosumi et al. 1989). However, I did not find evidence of changes in the size of clusters of cells expressing POMC or ACTH across

gestation. Although the specific staining of the ACTH and POMC antisera used in this study would not reflect precisely the staining produced by the single promiscuous antibody used previously, the ACTH label represents 53 - 77 % of corticotrophs and the POMC label represents between 77 - 92% of corticotrophs across the gestational age groups used in this chapter. It is unlikely that the large changes in morphology found previously would be missed by my investigation, if cluster size was a reliable differentiator of adult and fetal type corticotrophs.

The images of pituitary sections from each gestational age group used in this dissertation demonstrate some corticotrophs in palisades of columnar cells and other corticotrophs with a stellate morphology. Unfortunately immunofluorescent labelling lost some of the morphological detail demonstrated with protocols using peroxidase labelling. Thus most cells could not be reliably identified as fetal or adult type. In addition, the automated quantification method does not provide information on the shape of contiguous cells. My attempt to use cluster size to differentiate fetal from adult type cells is likely to have failed because of the stellate corticotrophs are also contiguous at the level of individual pixels. The contiguous nature of stellate corticotrophs is clearly demonstrated in the images presented in the results section of this chapter and is supported by numerous previous studies which report that stellate corticotrophs have long processes that wrap around other pituitary cells (Antolovich et al. 1989; Kurosumi et al. 1989).

There is a very interesting correlation incidence between the temporal changes in the major subpopulation of corticotrophs and the well established major phases of HPA activity (Wintour et al. 1995). The POMC+ACTH+CRHR$_1$

subpopulation represents ~5% of pituitary cells at 63-84 and 144-145 days gestation, and nearly doubles to 9% at 110 days gestation. Ovine fetal adrenal cells cocultured with fetal pituitary cells from the same gestational age, produce large cortisol secretory responses at 50 days gestation, reduced response at 100 days gestation, and an increased response between 130 days gestation and term (Glickman and Challis 1980). It is tempting to speculate that the POMC+ACTH+$CRHR_1$ expressing cells may modulate HPA activity by secreting an intrapituitary inhibitory factor which regulates the amount of ACTH secreted by neighbouring corticotrophs. Such paracrine regulation is certainly possible given the heterogeneous nature of the large clusters of corticotrophs described in the previous chapter and there is evidence for such interactions.

3.4.3 Ontogenic changes in $CRHR_1$ expressing cells

I have demonstrated a decline in the proportion of pituitary cells expressing the CRH receptor over the last third of gestation. This finding is consistent with previous reports at the level of individual cells (Perez et al. 1997; Young et al. 2003) and the whole pituitary (Fora et al. 1996; Green et al. 2000). Specifically, the proportion of cultured anterior pituitary cells that bind fluorescently labelled CRH increases between 100 and 120 days gestation, then falls again at 140 days gestation to levels identical to those at 100 days gestation (Young et al. 2003). At the level of the whole pituitary, there is a decline in the amount of $CRHR_1$ mRNA and protein, determined by Northern and Western blot analyses respectively, between 102-105 and 137-139 days gestation (Green et al. 2000). In culture, there is a decline in the ACTH secretory response to CRH stimulation between 110-115 and 138-145 days gestation (Fora et al. 1996). Therefore,

each level of evidence is consistent with my finding of a decrease in the proportion of pituitary cells expressing CRH receptors. In addition, I found a significant increase in the proportion of pituitary cells expressing $CRHR_1$ between 63-83 and 110-112 days gestation. This finding is consistent with data from radioligand binding of CRH to membrane preparations published previously (Lu et al. 1991).

As hypothesised, I found an increase in the proportion of pituitary cells that expressed ACTH+$CRHR_1$ alongside no change in the proportion of pituitary cells that expressed POMC+$CRHR_1$. This divergent change in POMC+$CRHR_1$ and ACTH+$CRHR_1$ expressing cells might underlie the divergent changes in CRH-induced increases in plasma POMC and ACTH across late gestation (Carr et al. 1995).

A decrease in the proportion of pituitary cells expressing POMC+ACTH+$CRHR_1$, with an increase ACTH+$CRHR_1$ expressing cells, and an increase in hypothalamic CRH content as gestation progresses (Saoud and Wood 1996), might result in a relative increase in CRH stimulation of the ACTH+$CRHR_1$ expressing cells. This would result in an increase in pituitary secretion of ACTH, if, as suggested in the previous chapter, these cells process POMC to ACTH at a faster rate than the other subpopulations of corticotrophs. This hypothesis is consistent with the increase in the average amount of ACTH secreted by individual cultured pituitary cells in response to an equal dose of CRH across the last third of gestation (Perez et al. 1997).

3.4.4 Mechanisms for the ontogenic changes in corticotroph subpopulations

The proportion of pituitary cells identified as corticotrophs in the present study is similar to that reported in previous studies in the fetal sheep (Antolovich et al. 1989; Matthews et al. 1994; Braems et al. 1996; Perez et al. 1997) and the neonatal rat (Childs et al. 1982). I did not find a statistically significant change in the proportion of pituitary cells identified as corticotrophs at any gestational age. This is not surprising considering the disparity between previous reports on the nature of the ontogenic changes in the proportion of fetal sheep pituitary cells identified as corticotrophs (Antolovich et al. 1989; Matthews et al. 1994; Braems et al. 1996; Perez et al. 1997).

Interestingly, I have found changes in a subpopulation of corticotrophs that parallels the maturational changes reported previously. I noted a statistical increase in the proportion of pituitary cells in the major subpopulation of corticotrophs, the POMC+ACTH+$CRHR_1$ expressing cells, between 5.0 ± 0.7 % of pituitary cells at 63-84 and 9.1 ± 1.5 % of pituitary cells at 110-112 days gestation and then no change to 9.9 ± 1.6 % of pituitary cells at 140-141 days gestation. Not only is the pattern similar to that reported by Matthews and colleagues (1994), it also represents similar proportions of pituitary cells, suggesting that overall, my method of corticotroph detection may have identified additional corticotrophs to those reported by Matthews and colleagues (1994). Variations in antibody binding between protocols have long been a limitation of quantitative immunohistochemistry. My method employed antigen retrieval, which has been reported to unmask antigens conjugated during the fixation process and increase the specific antibody binding capacity of most tissues (Shi

et al. 1997). Therefore I am confident that the immunohistochemical method has identified all of the pituitary cells expressing significant amounts of POMC, ACTH and $CRHR_1$.

The specific changes in the proportion of pituitary cells expressing ACTH+$CRHR_1$ or POMC+ACTH+$CRHR_1$ throughout gestation, and the particularly rapid loss of POMC+ACTH+$CRHR_1$ cells during the prepartum period, can be logically accounted for by 1) changes in the expression of one, two or all of the antigens or 2) apoptosis (Nolan et al. 1998) and proliferation (Taniguchi et al. 2000) of corticotroph subpopulations and other hormone producing cells of the pituitary.

I did not find evidence for the loss of only one or two antigens, since there were no reciprocal relationships between the ontogenic changes in different subpopulations across gestation. For example, the specific reduction in POMC+ACTH+$CRHR_1$ subpopulation between 140-141 and 144-145 days gestation did not coincide with an increase in any other corticotroph subpopulation.

Although the pituitary increases in weight, I have also demonstrated that corticotrophs do not undergo hypertrophy, and the proportion of pituitary cells that were identified as corticotrophs did not change over gestation. Taken together, these data suggest that the corticotroph population increases in proportion to total pituitary cellular increase. In the rat, the first corticotrophs have been found in Rathke's pouch (Kouki et al. 2001) and are derived from undifferentiated progenitors (Taniguchi et al. 2000). Therefore, even though the other cell types are known to appear after corticotrophs in all species studied (Dubois et al. 1997), there is no net outproliferation of corticotrophs by other

pituitary cell types during the gestation ages investigated in this study. It is therefore most likely that the ontogenic changes in corticotrophs expressing POMC+ACTH+$CRHR_1$ and ACTH+$CRHR_1$ result from separately regulated proliferation, differentiation and apoptosis of individual corticotrophs which occur in concert with changes in other pituitary cells.

It has been suggested that throughout gestation and early postnatal life in rats, approximately 20% of the progenitors develop into corticotrophs while 25% of the ACTH-immunoreactive cells undergo proliferation (Taniguchi et al. 2000). This suggests that corticotrophs develop from at least two lineages. It would be possible to determine whether different corticotroph subpopulations develop from these different cell lineages if the fluorescent labelling protocol used in this study could be coupled with a marker of cellular proliferation. The markers for mitotic and apoptotic activity show very few, less than 1%, of the pituitary cells undergo these activities at any point in time (Levy 1999). This is likely to be due to the short durations of these activities, 80 min for mitotic figures and 44 min for apoptotic bodies (Levy 1999).

Given the methods currently available, I attempted a crude surrogate measure of corticotrophs that had recently undergone mitosis. Dividing cells share cytoplasm and would therefore be detected in my method as contiguous cells of the same subpopulation. Looking at the proportion of cells that did not touch another cell of the same subpopulation, I demonstrated that nearly none of the POMC+$CRHR_1$ expressing cells contact another POMC+$CRHR_1$ expressing cell, which was not surprising because POMC+$CRHR_1$ is the least common subpopulation throughout gestation. ACTH+$CRHR_1$ expressing cells are also relatively sparse with a high degree of separation. Interestingly, although

POMC+ACTH+$CRHR_1$ expressing cells represent twice the number of corticotrophs compared with POMC only cells, only 20-30% of both cell types do not touch another cell of the same subpopulation. This suggests that POMC only cells are more likely to be part of a homogeneous cluster than any other subpopulation. It is therefore possible that POMC only cells represent the 20% of corticotrophs undergoing proliferation documented in the developing rat pituitary (Taniguchi et al. 2000). If POMC only cells are those corticotrophs that have recently divided, it leads one to suspect that the next stage in development might be the expression of CRHR1, which through the activation by CRH causes cells to start processing POMC into ACTH. While this presents an interesting model of corticotroph maturation, I did not find any evidence of transdifferentiation between corticotroph subpopulations across gestation. Therefore it is more likely that corticotrophs develop from distinct cell lineages.

3.4.5 Regulation of the ontogenic changes in corticotroph subpopulations

The developmental changes in the proportion of pituitary cells expressing POMC+ACTH+$CRHR_1$ may be regulated by the changing cortisol levels over the gestation (Norman et al. 1985; Apostolakis et al. 1992) since adrenalectomy prevents the decline in the proportion of pituitary cells expressing ACTH-like peptides, over the last third of gestation (Antolovich et al. 1989). In the adult rat, dexamethasone treatment decreases the proportion of pituitary cells undergoing mitosis and can cause a burst of apoptotic activity if dexamethasone treatment is preceded by adrenalectomy (Nolan et al. 1998). It is unclear why a specific subpopulation of corticotrophs might be targeted by cortisol, although it has

been shown previously that cortisol specifically deceases the amount of ACTH in CRH-target cells in the fetal sheep pituitary at 116 days gestation but does not affect the ACTH content of non-CRH-target cells (Butler et al. 1999). Such differential regulation by cortisol may relate to differential expression of the glucocorticoid receptor in individual cells. Unfortunately, attempts to label the tissues used in this study with several antisera raised against the glucocorticoid receptor were unsuccessful, and this aspect remains to be investigated.

Previous investigation indicates that hypothalamic CRH content increases between its first appearance at 48 and 100 days gestation, and further at 140 days gestation and again before term (Watabe et al. 1991; Matthews and Challis 1995). In addition, hypothalamic input is required to decrease $CRHR_1$ protein levels in the fetal pituitary in late gestation (Young et al 2003). Therefore, increasing CRH stimulation may act on POMC+ACTH+$CRHR_1$ to cause the decrease in all the proteins over the last fortnight of gestation. Regulation by CRH alone, however, cannot totally explain the specific decrease in POMC+ACTH+$CRHR_1$ expressing cells and lack of change in ACTH+$CRHR_1$ expressing cells.

Interestingly, surgical disconnection of the hypothalamus from the pituitary (HPD) prevents the late gestational rise in fetal plasma cortisol levels and therefore represents a model in which both the hypothalamic and adrenal input into the pituitary are removed in late gestation. Indeed, a functional hypothalamic-pituitary axis is required during fetal development for an appropriate pituitary response to glucocorticoids (Ozolins et al. 1990). It is therefore possible that an interaction between hypothalamic and adrenal outputs controls the maturational changes in specific cells within a particular

subpopulation of corticotrophs as term approaches. Such an interaction has been observed in rat pituitary cell cultures where pre-treatment with glucocorticoids reduces the proportion of pituitary cells that bind CRH by approximately half (Childs et al. 1986; Schwartz et al. 1986), which suggests that only a subpopulation of CRH-target cells respond to negative feedback by cortisol.

3.4.6 Summary

This study has demonstrated that, at the level of individual corticotrophs, there is differential control of separate subpopulations throughout gestation. It is possible that changes in POMC+ACTH+$CRHR_1$ and ACTH+$CRHR_1$ are also influenced by intrapituitary factors, hypothalamic factors and cortisol. These factors may also play a role in the maintenance of POMC+$CRHR_1$ and POMC-only cell populations. The increase in plasma ratio of ACTH to precursors throughout gestation is likely to be a result of increasing CRH stimulation and combined changes in the POMC+ACTH+$CRHR_1$ and ACTH+$CRHR_1$ subpopulations.

Maternal periconceptional undernutrition has been shown to activate the HPA axis during the quiescent and activated windows of gestation. In the next chapter, I investigate the changes in corticotroph subpopulations following at an early stage of gestation during which HPA activity is already known to be high.

Chapter 4: Periconceptional Undernutrition

4.1 Introduction

In humans and experimental models of early programming, cardiovascular and metabolic disease in late life has been associated with maternal undernutrition during the periconceptional period (PCUN). In humans, this is typically defined as 1-2 months before conception to 1 week – 1 month after conception (Ravelli et al. 1999; Roseboom et al. 2000). In two experimental models, PCUN has been shown to result in increased arterial blood pressure in fetal sheep by 115 days gestation (Edwards and McMillen 2002) and altered fetal growth trajectory at 55 days gestation (MacLaughlin et al. 2005). As indicated in the previous chapter, timely activation of the HPA axis plays an important role in regulating the relative rates of growth and differentiation of several organ systems (Ballard 1979). Consequently, it is not surprising that researchers have found evidence that the changes in the timing of activation of the HPA axis are likely to play a role in permanent PCUN-induced changes to a variety of physiological regulatory systems (Fowden and Forhead 2004; McMillen and Robinson 2005).

Precocious activation of the HPA axis in late gestation is likely to facilitate the early differentiation of the fetal tissues required for the transition to extrauterine life (Fowden and Forhead 2004). PCUN has been shown to increase plasma ACTH levels in twin fetal sheep throughout late gestation, from at least 115 days gestation (Edwards and McMillen 2002). In another sheep study that involved more severe nutrient deprivation over a longer period, there was precocious activation of the HPA and premature delivery of half the lambs (Bloomfield et al 2003). Currently, the only information available on the impact of PCUN on the fetal pituitary suggests that PCUN does not alter the net

anterior pituitary expression of POMC mRNA in late gestation (Lingas and Matthews 2001; Bloomfield et al. 2004). Therefore, it is important to investigate PCUN-induced changes at the level of individual pituitary cells to determine if differential adaptations in subpopulations of corticotrophs are involved in PCUN-induced changes in fetal HPA activity.

The timing, intensity and duration of the PCUN treatment is known to influence the specific changes in HPA axis activity later in life (Nathanielsz 2006). I choose a PCUN treatment regime known to increase basal plasma ACTH levels at all points of gestation investigated thus far, the earliest point being 115 days gestation. To extend the findings of previous research, I investigated the fetal pituitary at an earlier stage of gestation, 53-55 days gestation.

In the last chapter I demonstrated that ontogenic changes in the activity of the fetal sheep HPA axis corresponded with changes in the relative proportions of pituitary corticotroph subpopulations that differentially express POMC, ACTH and $CRHR_1$ protein. Specifically, during periods of high HPA activity, corticotrophs that express POMC+ACTH+$CRHR_1$ were decreased, whereas ACTH+$CRHR_1$ increased throughout gestation. The PCUN model used in this chapter represents a model in which HPA activity is elevated throughout late gestation. Thus, the working hypothesis was that at 53-55 days gestation, PCUN fetal pituitaries would have fewer POMC+ACTH+$CRHR_1$ expressing cells or more ACTH+$CRHR_1$ expressing cells than controls.

4.2 Methods

4.2.1.1 PCUN treatment

All procedures were approved by The University of Adelaide Animal Ethics Committee. Pregnant South Australian Merino ewes of known mating date, carrying twins, were normally grown at the South Australian Research and Development Institute. PCUN ewes were fed 70% by volume of the diet required to meet maintenance energy requirements of the non pregnant ewe for at least 45 days prior to, and 7 days after, mating: control ewes were fed 100% of the diet, as described previously (MacLaughlin et al 2005). The diet consisted of lucerne chaff and pellets, containing straw, cereal, hay, clover, barley, oats, lupins, almond shells, oat husks and limestone. The lucerne chaff and pellet mixture provided 80% and 20% of the feeding requirements, respectively. All components shown in Table 4.1 were reduced proportionally for the restricted diet. PCUN ewes were then returned to the normal level of nutrition at 8 days gestation.

Feed	Energy (MJ/kg)	Crude protein (g/kg)	Dry matter (%)
Lucerne chaff	8.3	193	85
Pellet mixture	8.0	110	90

Table 4.1 Feed composition of the control diet for ewes. All components were reduced proportionally for the PCUN diet.

4.2.1.2 Pituitary Collection

At 53-55 days gestation all ewes were killed by an intravenous overdose of sodium pentobarbitone (200mg/kg; Lethobarb; Virbac Pty Ltd., Peakhurst, New South Wales, Australia). Both fetuses were delivered via laparotomy, weighed

and killed by decapitation. Pituitaries from control (n = 6) and PCUN (n = 7) fetuses (representing 4 and 5 pregnancies, respectively) were collected and processed for immunohistochemistry as described previously (see section 3.2.1.1).

4.2.2 Immunohistochemistry

Sections of fixed pituitary tissue were bleached and rehydrated as described previously (see section 3.2.2). Antigen retrieval was performed for all slides (see section 3.2.2) before serial incubation with primary and secondary antisera (see section 3.2.2.1). The sections were sealed under coverslips and quantitative microscopic analysis undertaken to determine the proportion of pituitary cells expressing POMC, ACTH and $CRHR_1$, alone and in all possible combinations, as well as the proportion of antibody-positive cells in small or large clusters, as detailed in Chapter 3, above (see section 3.2.2.2).

4.2.3 Data analysis

A corticotroph was defined as any pituitary cell that stained positively for POMC or ACTH, and this total corticotroph population is presented as a proportion of the total number of pituitary cells. Each subpopulation of corticotrophs is presented as a proportion of pituitary cells to identify the effects of treatment on each subpopulation. In all cases, results are expressed as the mean ± SEM. A Student's t-test was used to analyse the effects of maternal PCUN on the proportion of pituitary cells expressing each subpopulation phenotype. Significant differences are reported for $P < 0.05$.

4.3 Results

4.3.1 Maternal physiological parameters

There was no difference between the PCUN and control ewe in the changes in maternal weight across the PCUN feeding regime, nor at sacrifice at 53-55 days gestation (Table 4.2).

	45 days before – 7 days after mating (kg)	45 days before – 53-55 days after mating (kg)
Control	0.4 ± 1.1	1.4 ± 0.9
PCUN	-2.5 ± 1.7	1.0 ± 1.7

Table 4.2 Effect of PCUN on maternal weight changes from the beginning of the PCUN feeding regime at 45 days before mating to the end of the PCUN feeding regime, and 53-55 days gestation for control and PCUN ewes.

4.3.2 Fetal physiological parameters

The body weights of fetuses from the PCUN group (26.8 ± 2.5 g) were not significantly different to those from the control group (28.3 ± 1.1 g, $P>0.05$) and there was also no effect of PCUN on corticotroph cell size (control: 88±10, PCUN: 84±9 μm^2, $P>0.05$). PCUN did not alter the proportion of cells expressing POMC, ACTH or $CRHR_1$ in small or large clusters (data not shown) or the proportion of cells in each subpopulation that were not part of a homogenous cluster (data not shown).

4.3.3 Fetal corticotrophs

PCUN fetal pituitaries displayed the same distribution of POMC, ACTH and $CRHR_1$ as the controls with no staining of the neural lobe, intense staining of the intermediate lobe and clusters of positive cells distributed across the entire anterior lobe (data not shown). The same subpopulations of corticotrophs were

identified in PCUN pituitaries as the control, and all pituitaries described in the previous chapters (Figure 4.1). As in the other experiments, the vast majority of corticotrophs were of the phenotypes POMC+ACTH+$CRHR_1$, ACTH+$CRHR_1$, POMC+$CRHR_1$ or POMC only. Few, if any cells exhibited any of the remaining possible patterns of antigen expression, such as POMC+ACTH.

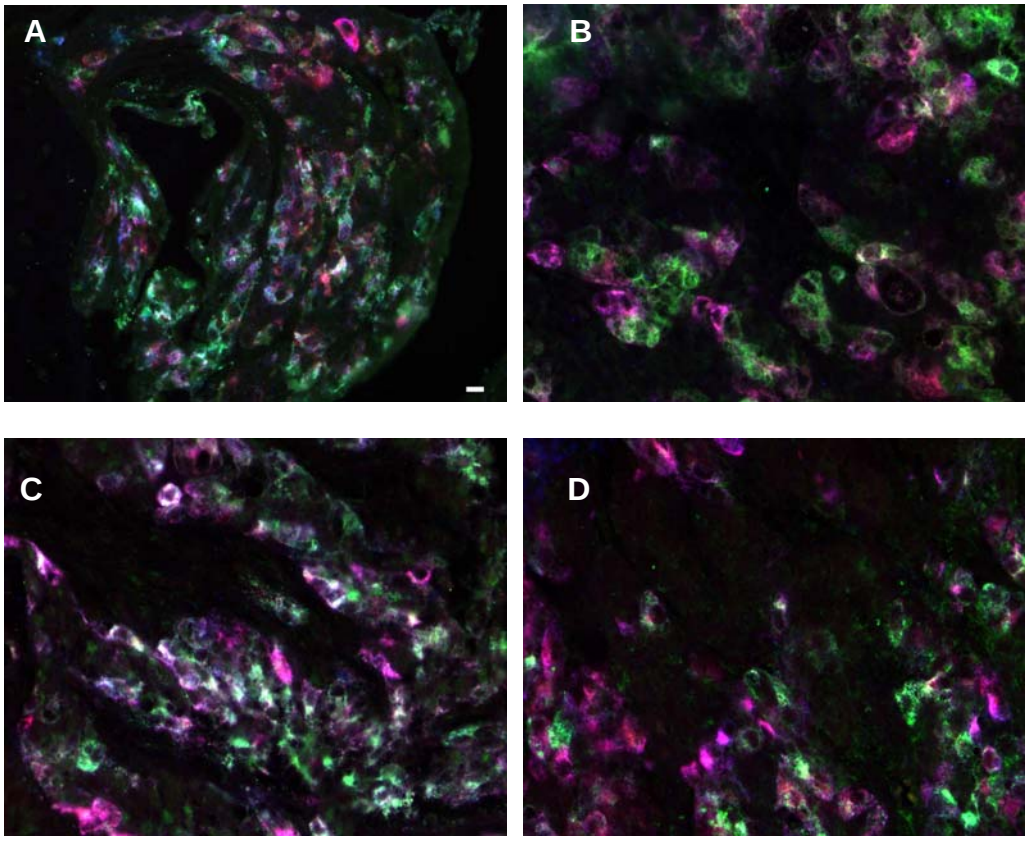


Figure 4.1 Pituitary cells stained for POMC (green), ACTH (red) and CRHR1 (blue) at 53-55 days gestation in control (A, B) and PCUN (C, D) animals. A pair of field images for each treatment group has been used to demonstrate the variation in cellular morphologies and peptide colocalisation amongst individual corticotrophs at each window of gestational. Scale bar = 10μm.

PCUN caused a significant decrease in the proportion of pituitary cells that were identifiable as corticotrophs (Figure 4.2). This was due to a specific decrease in the corticotrophs that expressed all three antigens, POMC+ACTH+$CRHR_1$, (Figure 4.3) without any concomitant changes in the levels of corticotrophs expressing POMC only, POMC+$CRHR_1$ or ACTH+$CRHR_1$.

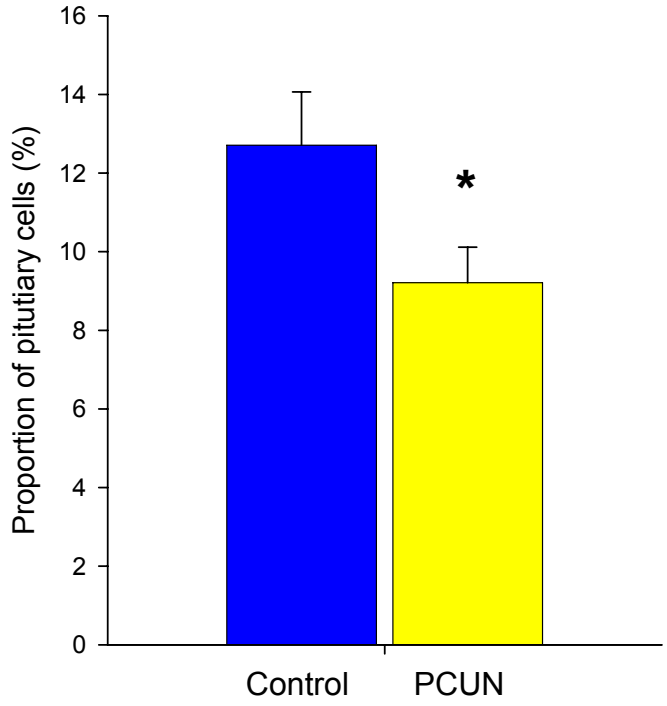


Figure 4.2 Mean ± SEM proportion of pituitary cells identified as corticotrophs in control (blue) and PCUN (yellow) fetuses at 53-5 days gestation. * indicates significant difference to control (P<0.05).

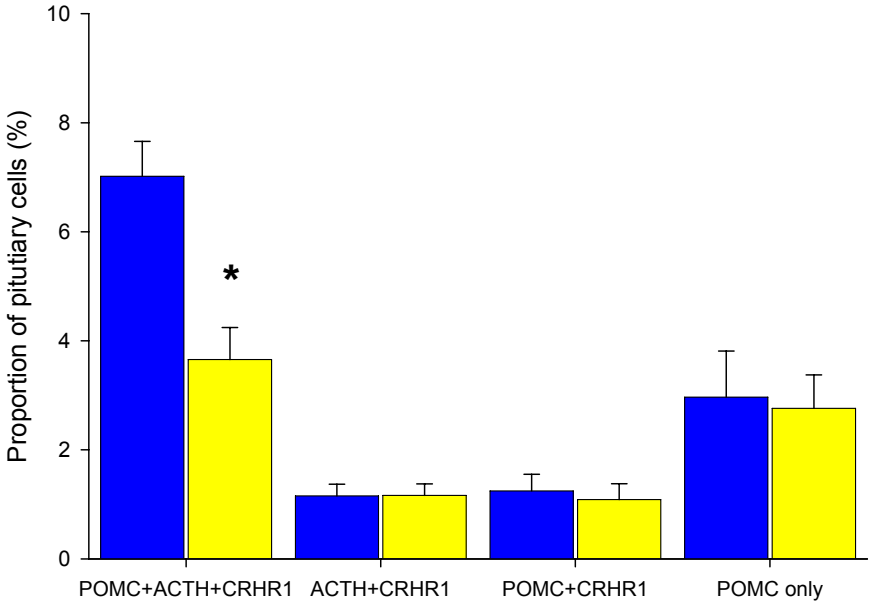


Figure 4.3 Mean ± SEM proportion of pituitary cells in each of the subpopulations in control (blue) and PCUN (yellow) fetuses at 53-5 days gestation. * indicates significant difference to control (P<0.05).

4.4 Discussion

The reduction in the proportion of pituitary cells identified as corticotrophs in PCUN fetal pituitaries was due to a specific reduction in the proportion of pituitary cells expressing POMC+ACTH+$CRHR_1$. This finding is strikingly similar to the ontogenic decline at term (144-145 days) in the same corticotroph subpopulation described in the previous chapter. Interestingly, PCUN is associated with precocious elevation of activity in the HPA axis. Taken together, these findings suggest that a relative decrease in the population of POMC+ACTH+$CRHR_1$ cells is associated with maturation of the HPA axis. A logical next step is to investigate how such a specific loss of one subpopulation of corticotrophs is regulated and the role played by such an adaptation in altering HPA activity.

I identified a change in the relative distribution of subtypes of corticotrophs at the earliest point of HPA axis development measured to date, when the tissue is likely to have been producing ACTH for possibly two weeks (Perry et al. 1985). PCUN occurs at a time when limitations of metabolic substrates cannot be considered an issue. It is possible that PCUN signals changes in fetal development through epigenetic regulation. That is, alterations to maternal and uterine hormone and nutrient levels alter the methylation of specific genes and promoters in the peri-implantation embryo (McMillen and Robinson 2005). PCUN-induced alterations in the number of cells in the blastocyst are also thought to play a role in the altered function of the cardiovascular system in late gestation (Kwong et al. 2000). This suggests that altered pituitary structure resulting in a relative decrease of POMC+ACTH+$CRHR_1$ expressing cells may be programmed by progenitor cells far before the tissue begins expressing hormones or reflects an interaction between the periconceptional environment and later conditions.

Although my findings indicate that there is reduction in the proportion of pituitary cells identified as corticotrophs in the PCUN fetal pituitary, the extremely early stage of development of the fetuses investigated in this chapter did not allow for accurate excision and weighing of the fetal pituitary. Therefore I am unable to compare the relative numbers of corticotrophs likely to be present in the PCUN and control pituitaries. Currently there is only one published report of the fetal pituitary weight following PCUN which indicates that there is no change in pituitary weight at 135 days gestation (Lutz et al. 2006). It is difficult to determine if this finding can be extrapolated to my study because of differences

in PCUN intensity, timing and duration between studies which are all known to alter the specific adaptations of the fetal HPA (Nathanielsz 2006).

In the previous chapter, I demonstrated that changes in the differential processing of POMC to ACTH suggested by the decrease in numbers of cells positive for POMC parallels changes in POMC processing by immature and mature corticotrophs as demonstrated in developing rodents (Chatelain et al. 1979; Noel and Mains 1991; Marcinkiewicz et al. 1993). This suggests that cells expressing POMC+ACTH+$CRHR_1$ represent an immature type of corticotroph while the ACTH+$CRHR_1$ phenotype might represent a more mature corticotroph subpopulation. It then follows, that a deficit in immature POMC+ACTH+$CRHR_1$ expressing corticotrophs would shift the corticotroph population to a more mature set point early in gestation. Such a proposition would be consistent with evidence that PCUN sets a trajectory for precocious maturation of the HPA axis (Bloomfield et al. 2003).

Early maturation of POMC processing to ACTH may play a significant role in the changes previously noted in HPA axis activity of PCUN fetus. In one study, PCUN itself has been associated with increases in basal fetal plasma cortisol levels without changes in ACTH plasma levels (Bloomfield et al. 2004). It is noteworthy that the ratio of ACTH to precursors was not investigated in that study, and this lack of resolution might underlie the apparent discrepancy between the reports of this group in 2003 and 2004 (Bloomfield et al. 2003; Bloomfield et al. 2004). This suggests that the decrease in POMC+ACTH+$CRHR_1$ expressing corticotrophs may cause a precious maturation of processing mechanisms in corticotrophs that produces ACTH from POMC and which may drive the adrenal to trigger early parturition.

Recent evidence indicates that at 55 days gestation, the fetal growth trajectory is altered (MacLaughlin et al. 2005) while adrenal growth and steroidogenic enzyme mRNA levels are not affected by PCUN (MacLaughlin et al. 2005). Given the information currently available on this PCUN model, it is reasonable to suggest that the loss of POMC+ACTH+$CRHR_1$ corticotrophs in PCUN fetuses may be involved in the production of elevated ACTH levels identified in late gestation (Edwards and McMillen 2002) but do not alter adrenal growth or steroidogenic capacity in early (MacLaughlin et al. 2005) or late (Edwards et al. 2002) gestation.

If a decrease in POMC+ACTH+$CRHR_1$ type corticotrophs is associated with elevated plasma ACTH levels, then high levels of POMC+ACTH+$CRHR_1$ corticotrophs inhibit HPA axis activity (Edwards and McMillen 2002; Bloomfield et al. 2004). The underlying mechanism for this inhibition might be that secretion of ACTH precursors by POMC+ACTH+$CRHR_1$ corticotrophs, which have been shown to inhibit the ACTH-induced increase in cortisol secretion by adrenal cell cultures taken from late gestation fetal sheep (Roebuck et al. 1980; Schwartz et al. 1995). There is currently no specific information available from this PCUN model on the fetal plasma levels of ACTH precursors, however, fetal plasma irACTH levels have been reported to be elevated (Edwards and McMillen 2002). This finding is not consistent with a decrease in pituitary secretion of ACTH precursors alone. Alternatively, it is possible that the POMC+ACTH+$CRHR_1$ subpopulation secretes a paracrine inhibitory factor that reduces the secretion of ACTH from neighbouring corticotrophs, and a reduction in this inhibitory subpopulation would increase net pituitary secretion of ACTH. Indeed, it has been previously demonstrated *in vitro* that the amount

of ACTH secreted in unstimulated conditions increases when CRH-target cells are removed (Schwartz et al. 1994).

There is no effect of PCUN on the CRH-induced increase in fetal plasma ACTH concentrations at 139-144 days gestation, however the cortisol response is exacerbated (Edwards and McMillen 2002). As this is currently the only information available concerning the effect of the PCUN model used in this dissertation on pituitary responsiveness to CRH, and my immunohistochemical method only identifies the presence $CRHR_1$, not its activity, it is impossible to speculate on the alterations to CRH-response of a decrement in POMC+ACTH+$CRHR_1$ corticotrophs at 55 days gestation.

Fetal weight was not altered by PCUN treatment, consistent with previous reports from this model (Edwards and McMillen 2002; MacLaughlin et al. 2005). Even with a diet restricted to 50% of control intake during the first month of gestation, researchers have shown that cardiovascular function is altered in the absence of changes in birth weight (Gardner et al. 2004). While there is a substantial literature base indicating that decreased birth weight is associated with cardiovascular and metabolic disorders in later life, the association between PCUN and these adult pathologies exists outside of the relationship with birth weight (Barker 1995; Kuzawa 2004). The relationship between corticotroph subpopulations and fetal growth restriction is the subject of the next chapter.

Evidence indicates that a suboptimal intrauterine environment even before blastocyst implantation signals the developing offspring and causes a reprogramming of fetal development which increases the offspring's chances of immediate survival (Simmons 2005; Nathanielsz 2006). Early fetal maturation

and birth increase the chances of survival for both the fetus and the mother, by limiting fetal growth, adequately preparing the fetal organs for extrauterine life and reducing the duration of fetal reliance on maternal substrate supply. Therefore it is not surprising that PCUN causes an early activation of the HPA axis in late gestation (Edwards and McMillen 2002; Bloomfield et al. 2003). I have identified a decrease in the proportion of pituitary cells that express POMC+ACTH+$CRHR_1$ in PCUN animals, which is likely to represent the early reprogramming of the fetal pituitary in response to signals of a suboptimal intrauterine environment.

Chapter 5: Placental Restriction

5.1 Introduction

A poor substrate supply during the last third of gestation that results in low birth weight has been shown to be associated with an increased risk of developing cardiovascular and metabolic disease later in life (Welberg and Seckl 2001). Activation of the HPA axis during critical windows in gestation is known to alter the development of several organs permanently (Fowden and Forhead 2004). There is now little doubt that fetal exposure to excess glucocorticoids during critical windows of development is involved in the cardiovascular and metabolic disorders that result from intrauterine growth restriction (IUGR) (McMillen et al. 2001; Nathanielsz 2006; Seckl and Meaney 2006).

Currently, research is focussed on using animal models of placental insufficiency to determine how the HPA adapts during intrauterine growth restriction to alter plasma glucocorticoids levels and programs adverse health outcomes in later life. As reviewed previously, the timing, duration, intensity and type of perturbation determines the specific fetal reprogramming mechanisms that are activated and the phenotype of the HPA axis adaptation. There is a well established experimental model of chronic fetal substrate restriction in sheep in which the majority of the placental attachment sites (caruncles) are surgically removed before mating. This procedure reduces the number of placentomes formed, and therefore, restricts the capacity of the placenta to deliver nutrients to the fetus during the subsequent pregnancy. Although the majority of fetuses are hypoxemic, hypoglycaemic and growth restricted in late gestation, in approximately one third of the fetuses, the remaining placentomes undergo sufficient growth to adequately supply nutrients to the fetuses and prevent

growth restriction (Robinson et al 1994). It is therefore important to select fetuses which are small for their gestational age to investigate the effects of chronic substrate deficiency on physiological systems.

Fetuses that undergo IUGR following carunclectomy show elevated basal cortisol levels after 127 days gestation without changes in plasma ACTH levels, measured as either total immunoreactive ACTH or $ACTH_{1-39}$ (Phillips et al. 1996). There is evidence of a decrease in the pituitary POMC mRNA in these fetuses (Phillips et al. 1996), however it is not known how the pituitary responds to the feedback from excess cortisol in late gestation without altering ACTH plasma levels at the level of individual corticotrophs. Therefore I have investigated the changes in subpopulations of corticotrophs in the fetal sheep pituitary in late gestation using the carunclectomy model growth restriction.

In another model of chronic fetal hypoxia, the basal plasma concentrations of both high molecular weight (HMW) and low molecular weight (LMW) ACTH were elevated compared with normoxic controls, with an overall increase in the ratio of LMW:HMW ACTH (Myers et al. 2005). At the cellular level, this suggests that the pituitary may adapt to chronic hypoxia by increasing the post-translational processing of POMC to ACTH, whilst overall increasing the secretion of both ACTH and its precursors. This suggests that there would be an increase in the subpopulations responsible for secreting POMC, but a greater increase in those subpopulations responsible for secreting ACTH. Interestingly, anterior pituitary protein levels of POMC, pro-ACTH, ACTH and $CRHR_1$ were found to be lower in chronically hypoxic sheep. Therefore the corticotroph subpopulation expressing POMC+ACTH+$CRHR_1$ might be reduced. Conversely, *in vitro* evidence indicates that hypoxia causes a specific

reduction in the proportion of ACTH stored in CRH-target cells (Butler et al. 2002) which might indicate that there will be a reduction in either ACTH+$CRHR_1$ and/or POMC+ACTH+$CRHR_1$ expressing corticotrophs in growth restricted fetuses.

5.2 Methods

5.2.1 Placental Restriction

In non pregnant ewes (Placental Restriction Group, PR), the majority of endometrial caruncles were surgically removed from the uterus as described previously (Robinson et al. 1979). This procedure restricts the number of placental cotyledons formed and subsequently limits placental, and hence fetal, growth. The carunclectomy procedure was performed under aseptic conditions with general anaesthesia induced by an intravenous injection of sodium thiopentone (1.25g/ml, Boehringer Ingelheim, NSW, Australia) and maintained with 3-4% halothane in oxygen. Ewes were kept under observation for 4-7 days post surgery. After a minimum of 10 weeks recovery the ewes entered a mating program and twin pregnancies were confirmed by ultrasound at approximately 50 days gestation.

5.2.1.1 Pituitary Collection

All ewes carried the fetuses through to sacrifice of the ewe by an intravenous overdose of sodium pentobarbitone (200mg/kg; Lethobarb; Virbac Pty Ltd., Peakhurst, New South Wales, Australia). Fetuses from twin pregnancies were delivered via laparotomy, weighed and killed by decapitation. Weights of the pituitaries and adrenal glands were also recorded. Pituitaries from control twin

fetuses at 139-141 (n = 4) and 144-145 (n = 6) days gestation, and PR twin fetuses at 139-141 (n = 4) and 144-145 (n = 4 ewes) days gestation, were collected and processed for immunohistochemistry as described in Chapter 3, above (see section 3.2.1.1).

5.2.2 Immunohistochemistry

Sections were bleached and rehydrated as described previously (see section 3.2.2). Antigen retrieval was performed for all slides (see section 3.2.2) before incubation with primary and then secondary antisera (see section 3.2.2.1). Finally sections were sealed under cover slips and quantitative microscopic analysis undertaken to determine the proportion of pituitary cells expressing POMC, ACTH and $CRHR_1$, alone and in all possible combinations, as well as the proportion of antibody-positive cells in small or large clusters (see section 3.2.2.2).

5.2.3 Data analysis

A corticotroph was defined as any pituitary cell that stained positively for POMC or ACTH, and this total corticotroph population is presented as a proportion of the total number of pituitary cells. Each subpopulation of corticotrophs is presented as a proportion of pituitary cells to identify the effects of treatment on each subpopulation. In addition, each subpopulation is expressed as a percentage of the whole corticotroph population to identify more subtle changes that alter the relative contribution of each subpopulation to the overall corticotroph population. In all cases, results are expressed as the mean ± SEM.

A two-way analysis of variance, with gestational age (140 vs. 144 days gestation) and treatment (PR vs. control) as the specified factors was used to determine whether there were differences between different groups in the average cross-sectional areas of the corticotrophs, the frequency of clusters of different sizes, and the proportion of cells in the different subpopulations of corticotrophs. Differences between gestational age groups or treatment groups were considered significant at $P < 0.05$. Results for gestational age are not reported here unless specifically related to changes resulting from PR treatment (ontogenic changes have been dealt with in Chapter 3).

5.3 Results

Pituitary and adrenal weights were significantly lower in the PR fetuses than the control fetuses at both gestational ages, although as a proportion of body weight, pituitary and adrenal weights were significantly higher in the PR fetuses (Table 5.1).

	140d		144d	
	control	PR	control	PR
Body wt (kg)	5.0 ± 0.4	2.1 ± 0.2*	5.5 ± 0.2	2.2 ± 0.3*
Pituitary wt (mg)	117 ± 12	76 ± 7*	135 ± 10	81 ± 8*
Pituitary wt: body wt (mg/kg)	24 ± 2	37 ± 6*	25 ± 2	37 ± 2*
Adrenal wt (mg)	290 ± 13	208 ± 9*	266 ± 10	202 ± 11*
Adrenal wt: body wt (mg/kg)	53 ± 16	86 ± 6*	48 ± 2	95 ± 14*

Table 5.1 Fetal body, pituitary and adrenal weights, and the ratios of pituitary and adrenal weights to body weight in groups of control and PR fetuses at 140 and 144 days gestation. * indicates significant differences between PR and control groups (P<0.05).

There was no difference in the cross-sectional area of the cell bodies of individual corticotrophs between PR (77.1±7.2 at 140 and 78.6±5.5μm^2 at 144 days gestation) and control animals (77.6±5.0 at 140 and 79.7±6.4μm^2 at 144 days gestation). PR did not alter the proportion of cells expressing POMC, ACTH or $CRHR_1$ in small or large clusters (data not shown). In PR pituitaries compared with controls, there was a specific increase in the proportion of cells expressing POMC only that were not part of a homogeneous cluster at 140 (control: 21±3 vs. PR: 29±4%, $P<0.05$) and 144 days gestation (control: 22±2% vs. PR: 28±2%, $P<0.05$), without changes in any other subpopulation.

PR fetal pituitaries displayed the same distribution of POMC, ACTH and $CRHR_1$ with no staining of the neuronal lobe, intense staining of the intermediate lobe and clusters of positive cells distributed across the entire anterior lobe (data not shown). The same subpopulations of corticotrophs were identified in PR pituitaries as the control, and in all tissues in the previous chapters (Figure 5.1)

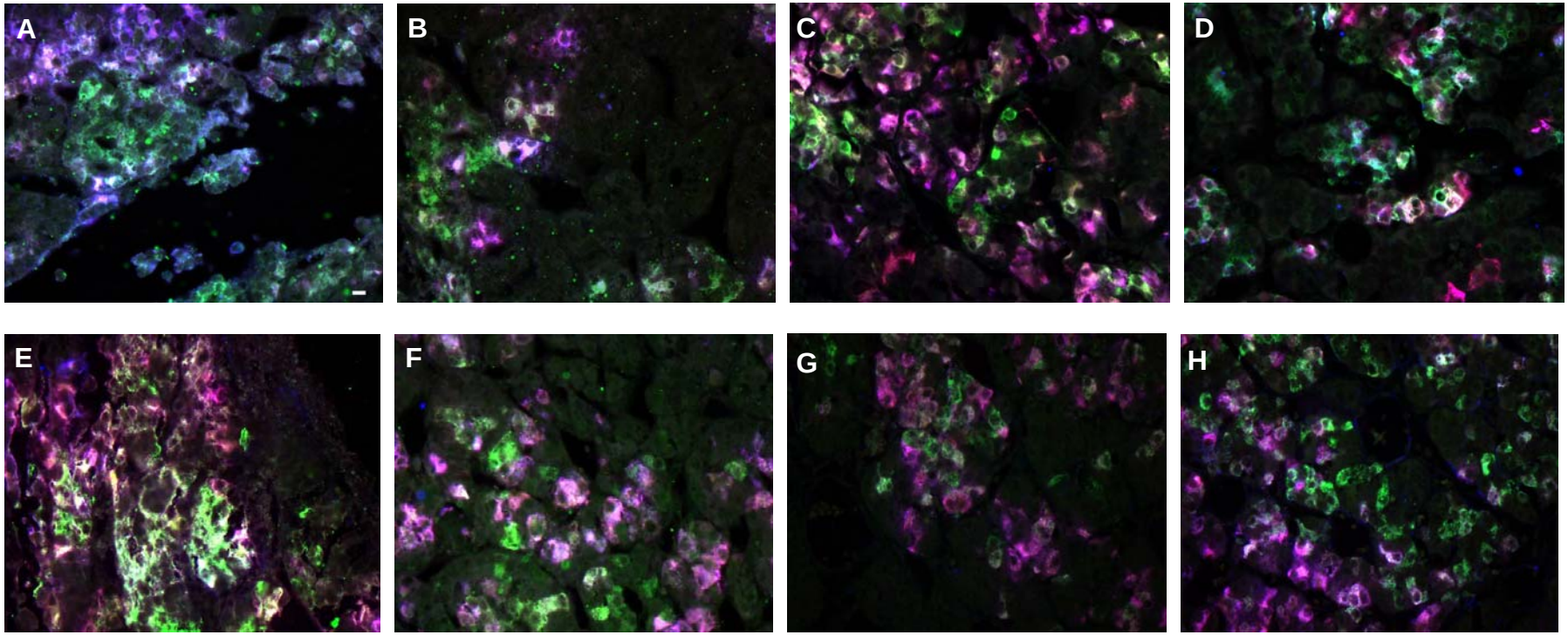


Figure 5.1 Pituitary cells stained for POMC (green), ACTH (red) and $CRHR_1$ (blue) at 140-141 days gestation in control (A, B), and PR (C, D), animals, and at 144-145 days gestation in control (E, F) and PR (G, H) animals. A pair of field images for each gestational age and treatment group has been used to demonstrate the variation in cellular morphologies and peptide colocalisation amongst individual corticotrophs at each window of gestational. Scale bar = 10μm.

Overall, there was no effect of PR on the proportion of corticotrophs present in the fetal pituitary at either 140 (control: 13.8±1.2 vs. PR: 14.9±1.6%) or 144 days gestation (control: 11.5±0.6% vs. PR: 9.8±0.7%,). PR fetuses underwent the same decline in proportion of pituitary cells of the POMC+ACTH+$CRHR_1$ phenotype (Table 5.2) as the control animals between 140 and 144 days gestation. Within the corticotroph population however, PR caused a significant shift in the distribution of subpopulations, resulting in a significant conservation of the corticotrophs expressing POMC+ACTH+$CRHR_1$, independent of the effects of gestation age (Figure 5.2).

	140		144	
	Control	PR	Control	PR
POMC+ACTH+$CRHR_1$	8.3±1.4[a]	9.9±1.6[a]	5.2±0.4[b]	5.6±0.9[b]
ACTH+$CRHR_1$	2.2±0.5	1.6±0.5	2.4±0.5	1.3±0.2
POMC+$CRHR_1$	0.9±0.2	0.4±0.1	0.8±0.3	0.9±0.2
POMC only	2.1±0.3	2.1±0.3	2.6±0.4	1.8±0.3

Table 5.2 The proportion of pituitary cells (%) expressing POMC+ACTH+$CRHR_1$, ACTH+$CRHR_1$, or POMC-only in control and PR fetuses at 140 and 144 days gestation. Different superscript letters indicate significant differences between animal groups (P<0.05).

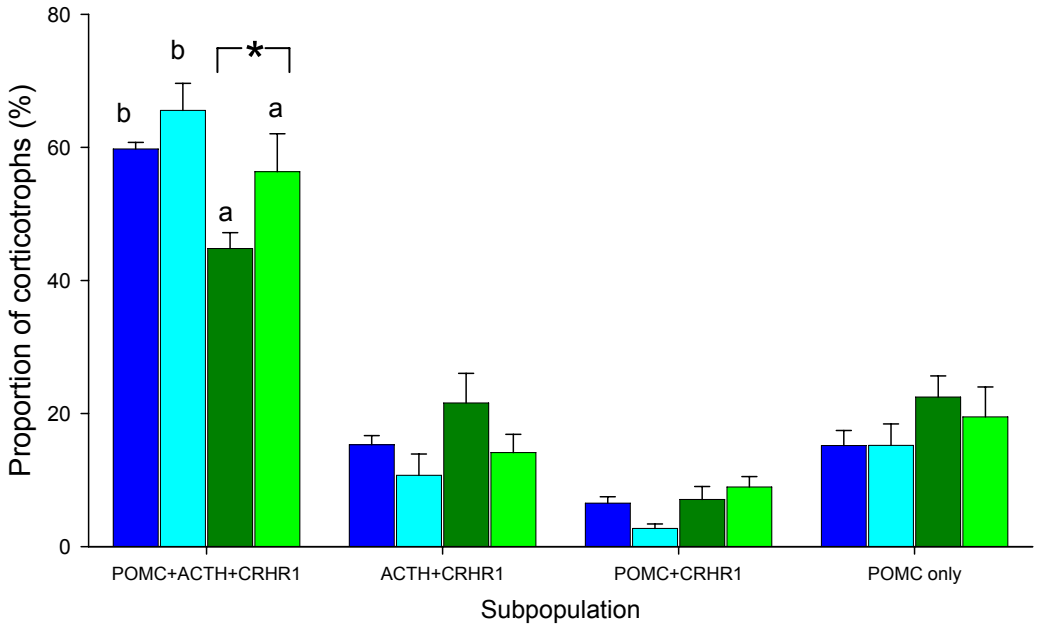


Figure 5.2 Mean ± SEM proportion of corticotroph cells in each of the subpopulations at 140 and 144 days gestation in groups of control (140d: dark blue; 144d: dark green) and PR (140d: light blue; 144d: light green) fetuses. Different superscript letters indicate significant differences between animal groups (P<0.05). * indicates significant differences between PR and control groups (P<0.05).

5.4 Discussion

The effects of gestational age and placental restriction on subpopulations of corticotrophs were found to be quite distinct. Fetuses subjected to restriction of placental nutrient supply underwent the same maturational reduction in the proportion of pituitary cells expressing POMC+ACTH+CRHR$_1$ as the controls. Independently, I found a higher proportion of corticotrophs expressed POMC+ACTH+CRHR$_1$ in pituitaries from PR fetuses compared with controls. Previous investigation of this model of growth restriction has shown that there is an earlier prepartum rise in the fetal plasma concentrations of cortisol (Phillips et al. 1996). Following the evidence from the preceding chapters that a decrease in the proportion of pituitary cells that express

POMC+ACTH+$CRHR_1$ is associated with elevated HPA activity and pituitary maturation, it is surprising that in PR fetuses, a model of precocious maturation, there is a conservation of the POMC+ACTH+$CRHR_1$ expressing corticotrophs. Therefore, it is unlikely that the growth restricted fetus is simply displaying a slower maturational decrease in POMC+ACTH+$CRHR_1$ expressing corticotrophs than the control fetus. It is much more likely that the pituitaries of PR fetuses have undergone a slightly different pattern of development, and may be operating at a different set-point from those of the control fetus (McMillen et al. 2001).

In growth restricted sheep fetuses, despite elevated levels of cortisol after 127 days gestation, the circulating levels of ACTH are not altered (Phillips et al. 1996). This dissociation between plasma levels of ACTH and cortisol is not uncommon in experimental cases of prolonged hypoxia, where ACTH levels have been shown to be transiently elevated before returning to baseline, while cortisol levels were elevated for the period of the hypoxia (Sug-Tang et al. 1992). In longer cases of hypoxia, from 30 to 141 days gestation, neither the basal cortisol nor ACTH plasma concentration were elevated compared with normoxic controls, however the hypoxic fetuses did show a potentiated cortisol response to a secondary period of acute hypoxia compared with normoxic controls, again without changes in the ACTH plasma concentration (Imamura et al. 2004). It is important to note the limitations of the plasma ACTH assays at this point. Several studies have shown a dissociation between plasma ACTH and cortisol levels in the fetal sheep. Specifically, the pulsatile changes in plasma ACTH

levels are not temporally related to the pulsatile changes in plasma cortisol levels (Apostolakis et al. 1992; Poore et al. 1998). In addition, infusion of $ACTH_{1-24}$ has been shown to cause changes in cortisol plasma levels without detectable changes in circulating ACTH levels (Poore et al. 1998). These reports highlight the limitations of current ACTH assays for fetal sheep plasma and suggest a high level of caution needs to be taken when interpreting the apparent dissociation between plasma ACTH and cortisol levels in fetal sheep reported by many investigators.

If indeed there is a dissociation between cortisol and ACTH levels in PR versus chronically hypoxic fetuses, this might indicate that the functions of the adrenal and pituitary glands may be undergoing differential adaptations to these two intrauterine perturbations. Specifically, the adrenal steroidogenic secretory capacity to elevate plasma cortisol levels is increased and ACTH plasma levels are maintained regardless of the elevations in cortisol feedback to the pituitary. In the PR model, non-ACTH mediated increases in adrenal growth and steroidogenic enzyme expression have been demonstrated, which have been proposed to involve angiotensin II stimulation or intra-adrenal insulin like growth factors (Coulter et al. 2002). Infustion of angiotensin II for 48 hours infusion of at doses capable of raising blood pressure in fetuses at 140 days gestation, does not alter the adrenal responsiveness to ACTH challenge (Poore et al. 1998). Therefore the involvement of angiotensin II in changes in steroidogenic capacity of the adrenal gland in PR animals remains uncertain.

It has been shown previously that PR fetuses have reduced levels of pituitary POMC mRNA during the prepartum period. Elevated fetal cortisol levels are known to decrease the rate of POMC transcription during some critical windows in gestation, such at 126 and 142 days gestation (Lu et al. 1994; Jeffray et al. 1998) but not others, such as 135 days gestation (Matthews and Challis 1995). As fetal plasma cortisol levels are elevated in PR animals from 127 days gestation to term (Phillips et al. 1996), it is likely that cortisol has an impact of pituitary development. The subtle changes in subpopulations of corticotrophs I have identified in the pituitaries in PR animals are likely to be part of the pituitary adaptation to chronic hypoxia that maintains fetal plasma ACTH levels amidst elevated fetal plasma cortisol levels.

Previous investigation of adrenalectomised fetuses has shown that elevated fetal plasma cortisol levels are required to increase the number of adult type (stellate shaped) corticotrophs in late gestation (Antolovich et al. 1989), suggesting that cortisol might influence the proliferation of this subset of corticotrophs. It is therefore tempting to speculate that cortisol might differentially regulate the rates of proliferation and differentiation of different corticotroph subpopulations.

I found no change in the proportion of pituitary cells that were identified as corticotrophs in PR fetuses, however long term hypoxia has been shown to decrease pituitary POMC, pro-ACTH, $ACTH_{1\text{-}39}$, and $CRHR_1$ protein content (Myers et al. 2005). It is possible that the apparent conservation of POMC+ACTH+$CRHR_1$ subpopulation represents both small increases in the proportion of pituitary cells that express POMC+ACTH+$CRHR_1$, and small decreases in the proportion of

pituitary cells in other corticotroph subpopulations. It might also suggest that POMC+ACTH+$CRHR_1$ expressing corticotrophs contain relatively less of each peptide than corticotrophs of the other subpopulation phenotypes. Unfortunately it was not possible to confirm this with the techniques used in this dissertation, as they did not allow for the accurate comparison of peptide content in individual corticotrophs between animals.

Alternatively, it might simply be that long term hypoxia represents a slightly different perturbation of the fetal environment to that of PR, where each causes unique adaptations at the level of individual corticotrophs in the fetal pituitary. Previous *in vitro* evidence indicates that chronic hypoxia, irrespective of whether carunclectomy was performed, decreases the proportion of ACTH stored in CRH-target cells, while carunclectomy, irrespective of fetal oxygen status, results in an increase in the proportion of ACTH secreted under unstimulated conditions (Butler et al. 2002). It is therefore expected that there might be a decrease in proportion of corticotrophs that are CRH targets and an increase in nonCRH target corticotrophs in my group of growth restricted fetuses from ewes that have undergone carunclectomy. I was unable to determine how much of the staining $CRHR_1$ I identified is membrane-associated, and therefore, which corticotrophs are actually functional CRH targets. As stated in Chapter 2, the fraction of fetal sheep pituitary cells that actually bind CRH (Young et al. 2003) is apparently less than the fraction that express $CRHR_1$ by immunohistochemistry. Therefore it is likely that only a subset of the corticotrophs I have identified to express the $CRHR_1$ protein might

actually respond to CRH. To determine whether CRH responsive corticotrophs are represented by a particular subpopulation or certain cells in all $CRHR_1$ expressing subpopulations, it would be necessary to pair the immunohistochemical and image analysis methods reported in this dissertation with a technique capable of identifying the secretion from individual cells, for example immunoblotting methods. In addition, such an approach may be able to determine factors that the non$CRHR_1$-expressing corticotrophs respond to.

PR fetuses used in this chapter were growth restricted, as shown by the low body weight of these fetuses compared with control animals. Interestingly, there was sparing of both the pituitary and adrenal glands in fetuses subjected to restriction of placental function, indicated by the higher weights of these organs as a proportion of fetal body weight. While the absolute weight of the pituitary was reduced in PR fetuses, this was not due to hypotrophy of the corticotrophs. In addition, the proportion of pituitary cells identified as corticotrophs was not altered by PR, suggesting that there is not a detectable degree of hypoplasia or hyperplasia of corticotrophs. Since an intact HPA axis is essential to produce the surge of fetal plasma cortisol levels required for parturition in the sheep (Challis and Brooks 1989) it is likely that the pituitary and adrenal sparing identified in this study may aid the production of the prepartum cortisol surge in the growth restricted fetus. An additional role of this organ sparing may be to produce the elevated fetal plasma cortisol concentrations identified in growth restricted fetuses compared with controls (Phillips et al. 1996).

There is a large amount of evidence to indicate that placental insufficiency during late gestation, which results in fetal hypoxia and growth restriction, is associated with a programming mechanism by which excess fetal glucocorticoid exposure during critical windows of development alters the development of key fetal physiological systems, such as the renin-angiotensin system, and predispose the offspring to develop cardiovascular disorders in later life (McMillen et al. 2001; Nathanielsz 2006). It is well established that during periods of intrauterine substrate restriction and chronic hypoxia there are changes in the distribution of blood flow which maintain perfusion of the fetal brain and heart at the expense of other organs such as the kidneys (Rurak et al. 1990). Therefore, the growth of certain organs may be altered more than others depending on their perfusion requirements at the time of perturbation. In addition, excess glucocorticoids are known to alter the rates of cellular differentiation and proliferation (Ballard 1979). Therefore the effects of greater cortisol levels on tissue development will depend on the stage of tissue development at the time of excess exposure.

It is unclear how the HPA axis adapts to IUGR to produce an increase in basal plasma cortisol during, and after, the chronic hypoxia associated with placental insufficiency. Evidence from the ovine carunclectomy model indicates that cortisol levels are elevated after 127 days gestation, without changes in plasma ACTH, and a deficit in pituitary POMC gene expression (Phillips et al. 1996). I have advanced this understanding to include a set of small changes in several corticotroph subpopulations that result in the conservation of the

POMC+ACTH+$CRHR_1$ expressing corticotrophs during the prepartum period. It is likely that such a subtle shift is regulated by greater cortisol feedback and is, at least in part, responsible for maintaining ACTH plasma levels.

Chapter 6: Discussion

6.1 Introduction

There are several lines of evidence that indicate corticotrophs are a heterogeneous cell population based on several characteristics. Morphologically diverse subpopulations of corticotrophs have been identified by immunohistochemistry (Perry et al. 1985; Antolovich et al. 1989; Antolovich et al. 1991) and electron microscopy (Dacheux 1984; Kurosumi et al. 1984; Kurosumi et al. 1989). Investigations using *in situ* hybridisation (Bell et al. 1998) and immunohistochemistry (Noel and Mains 1991; Marcinkiewicz et al. 1993) indicate that subpopulations of corticotrophs may differentially process POMC. Reverse haemolytic plaque assays (Neill et al. 1987; Jia et al. 1991) and a cytotoxin conjugated to CRH (Schwartz et al. 1986; Schwartz et al. 1987; Butler et al. 1999) have been used to provide *in vitro* evidence that there are subpopulations of corticotrophs that are differentially responsive to CRH, AVP and glucocorticoids.

Although I also found evidence of morphologically distinct corticotrophs, quantification of the changes in morphological subpopulations based on cluster size were not consistent with previous reports of reciprocal ontogenic changes in two corticotroph subpopulations (tall columnar versus stellate) across gestation (Perry et al. 1985; Antolovich et al. 1989; Antolovich et al. 1991). Therefore I was unable to extend the current understanding of morphological subpopulations of corticotrophs. My use of two specific antibodies, anti-POMC and anti-ACTH, which recognise full-length and processed peptides derived from the biosynthetic precursor POMC, respectively, have revealed differential

expression of the precursor and its fragments in individual corticotrophs. This finding suggests that subpopulations of corticotrophs may process POMC at different rates. The present research has also provided immunochemical evidence that not all corticotrophs express the CRH receptor, $CRHR_1$, suggesting at least one mechanism that might explain why some corticotrophs do not die when exposed to high levels of CRH conjugated cytotoxin (Schwartz et al. 1987; Butler et al. 1999). Extensive quantification of the differential labelling of individual corticotrophs with antisera for POMC, ACTH and $CRHR_1$, resulted in the identification of four subpopulations of corticotrophs that undergo very different changes across gestation and in response to suboptimal intrauterine environments induced by moderate restriction of the ewe's nutrient intake around the time of conception and carunclectomy-induced fetal growth restriction.

6.2 Differential expression of POMC, ACTH and $CRHR_1$

I have demonstrated the differential expression of POMC, ACTH and $CRHR_1$ in individual corticotrophs of the fetal sheep pituitary throughout gestation. Specifically I identified four distinct subpopulations of corticotrophs, cells that express (1) POMC+ACTH+$CRHR_1$, (2) ACTH+$CRHR_1$, (3) POMC+$CRHR_1$ and (4) POMC only (Figure 6.1). It is reasonable to link the differential expression of POMC and PC1 transcripts described previously in the fetal sheep pituitary (Bell et al. 1998) with corticotrophs that differentially process POMC to ACTH. I have also identified corticotrophs that do not express $CRHR_1$ which are likely to represent the non-CRH-target cells identified previously in

cultures of rat (Jia et al. 1991) and sheep pituitary cells (Schwartz et al. 1987; Butler et al. 1999).

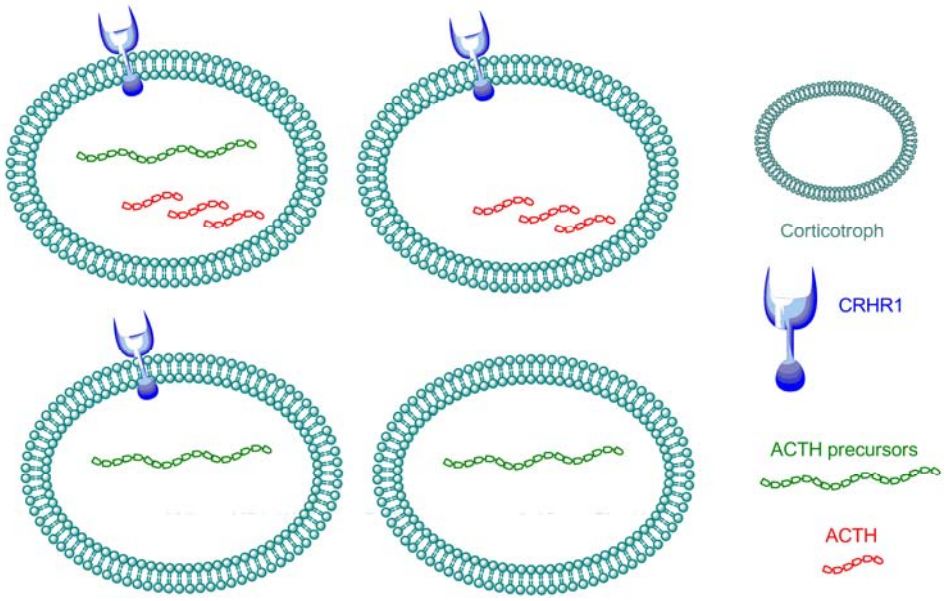


Figure 6.1 Schematic representation of the four subpopulations of corticotrophs identified in this dissertation, expressing (A) POMC+ACTH+CRHR$_1$, (B) ACTH+CRHR$_1$, (C) POMC+CRHR$_1$ and (D) POMC only.

In contrast to the model of corticotroph subpopulations proposed in Chapter 1, no corticotrophs were identified that express POMC and ACTH but not CRHR$_1$. In addition, a subpopulation that expresses ACTH and CRHR$_1$, but not detectable levels of POMC, was clearly evident in all pituitaries. Importantly, this revision of the model highlights the association of CRHR$_1$ with POMC processing to ACTH. This supports the suggestion that CRH activity may increase the processing of POMC to ACTH, which is consistent with previous reports that CRH is able to increase the rate of PC1 transcription (Li et al. 1999) and PC1 activation (Vindrola and Lindberg 1992). The present findings also suggest that there may be different corticotrophs that process POMC to ACTH at three distinct rates (1) no processing in POMC only and

POMC+CRHR$_1$ expressing cells, (2) moderate POMC processing in POMC+ACTH+CRHR$_1$ expressing cells and (3) immediate, complete processing in ACTH+CRHR$_1$ expressing cells.

Across all of the studies described in this dissertation, there is a strikingly consistent pattern of coexpression of POMC, ACTH and CRHR$_1$, in four subpopulations of a possible seven combinations of the peptides. This suggests that the selective colocalisation of these peptides plays an important biological role and is regulated. Consistent changes in the proportion of cells expressing all three peptides with gestational age and perturbation of the fetal environment, suggests that this subpopulation is most responsive to regulation. A coincidence of the changes in POMC+ACTH+CRHR$_1$ expressing cells with well established changes in adrenal activity and plasma cortisol levels across gestation, suggests that this subpopulation plays a role in controlling relatively rapid changes (at least over four days) in cortisol levels.

6.2.1 Corticotroph subpopulations

6.2.1.1 POMC processing

Previous investigations of corticotroph morphology and secretory granule characteristics have identified two distinct subpopulations of corticotrophs, and in most cases, a third, intermediate corticotroph phenotype (Dacheux 1981; Kurosumi et al. 1984; Tanaka and Kurosumi 1986; Antolovich et al. 1989). In the fetal sheep pituitary, the stellate corticotrophs increase with gestational age in step with fetal plasma levels of ACTH$_{1-39}$, and conversely, the levels of columnar corticotrophs

decline in late gestation (Perry et al. 1985). Thus there appears to be a temporal relationship between the change from columnar to stellate corticotrophs and the changes in proportions of POMC+ACTH+$CRHR_1$-expressing cells characterised in this dissertation. However, I did not find any evidence that correlates morphology to the corticotroph subpopulations differentially labelled with antisera against POMC, ACTH and $CRHR_1$.

In the rat, oval corticotrophs have been shown to contain electron lucent secretory granules while stellate corticotrophs contain electron dense secretory granules (Dacheux 1981; Kurosumi et al. 1984; Tanaka and Kurosumi 1986). Analysis of AtT-20 cells suggests that electron dense granules are mature secretory granules which contain processed peptides derived from POMC, whereas the electron lucent secretory granules are early stage vesicles and still contain full length POMC (Tanaka and Kurosumi 1992). Taken together these findings suggest that oval corticotrophs might process POMC to ACTH faster than stellate corticotrophs. Unfortunately the use of immunofluorescent labelling in this dissertation did not provide the resolution to determine corticotroph morphology or secretory granule characteristics, so I was unable to determine how morphology is related to POMC processing capacity. Thus, the relationships between corticotroph morphology and POMC processing remain unclear.

Previous evidence from the fetal sheep also suggested that columnar corticotrophs appeared in large clusters of cells while stellate corticotrophs tended to be found as single cells or in small clusters (Perry et al. 1985). In addition, the proportion of corticotrophs in large

clusters decreases between 40 and 145 days gestation, when the proportion of corticotrophs in small clusters increases (Perry et al. 1985). I investigated whether POMC and ACTH expressing cells were part of large or small clusters and found that approximately half of the cells expressing either peptide were part of each cluster category, (Perry et al. 1985; Antolovich et al. 1989). During the image analysis, it became obvious that individual cells from each of the four corticotroph subpopulations could be identified as part of large heterogeneous clusters, and as single cells. This indicates that there is no relationship between the subpopulations identified in this thesis and the morphologically distinct subpopulations described previously.

6.2.1.2 $CRHR_1$ expression

It is not surprising that I identified corticotrophs that expressed $CRHR_1$ and corticotrophs that did not, as there is substantial evidence for at least two populations of corticotrophs based on functional response to CRH (Schwartz et al. 1987; Jia et al. 1991; Butler et al. 1999). I have therefore provided evidence that at least one mechanism by which the responsiveness of individual corticotrophs to CRH might be controlled is through the differential expression of $CRHR_1$. In the present study, 6-14% of cells stained positively for $CRHR_1$. However, it was previously found that less than 2.5% of late gestation fetal sheep pituitary cells bind CRH *in vitro* (Young et al. 2003). It is likely that many of the cells I identified as $CRHR_1$-positive may not express functional receptors on the cell membrane and consequently not respond to CRH. Together, the findings provide indirect evidence that the CRH-responsiveness of

individual corticotrophs is also regulated by the distribution of $CRHR_1$ between the cytoplasm and the plasma membrane.

Another possibility is that only a particular subpopulation of $CRHR_1$-expressing cells might be CRH-responsive. The changes I found in the dominant corticotroph subpopulation, POMC+ACTH+$CRHR_1$, across gestation (Figure 6.2) are consistent with the broad changes in pituitary levels of $CRHR_1$ mRNA, protein (Green et al. 2000) and radioligand binding (Lu et al. 1991) described previously. Thus, the POMC+ACTH+$CRHR_1$ subpopulation may be primarily responsible for CRH receptor synthesis and CRH binding. It must be noted however, that the POMC+ACTH+$CRHR_1$ expressing cells do represent the major subpopulation of corticotrophs and I did find marked changes in the proportion of pituitary cells in this subpopulation. Therefore, regardless of whether the POMC+ACTH+$CRHR_1$ subpopulation are the only cells that bind CRH, or whether all $CRHR_1$ expressing cells bind CRH, the large changes in POMC+ACTH+$CRHR_1$ subpopulation would probably result in measurable changes in CRH binding.

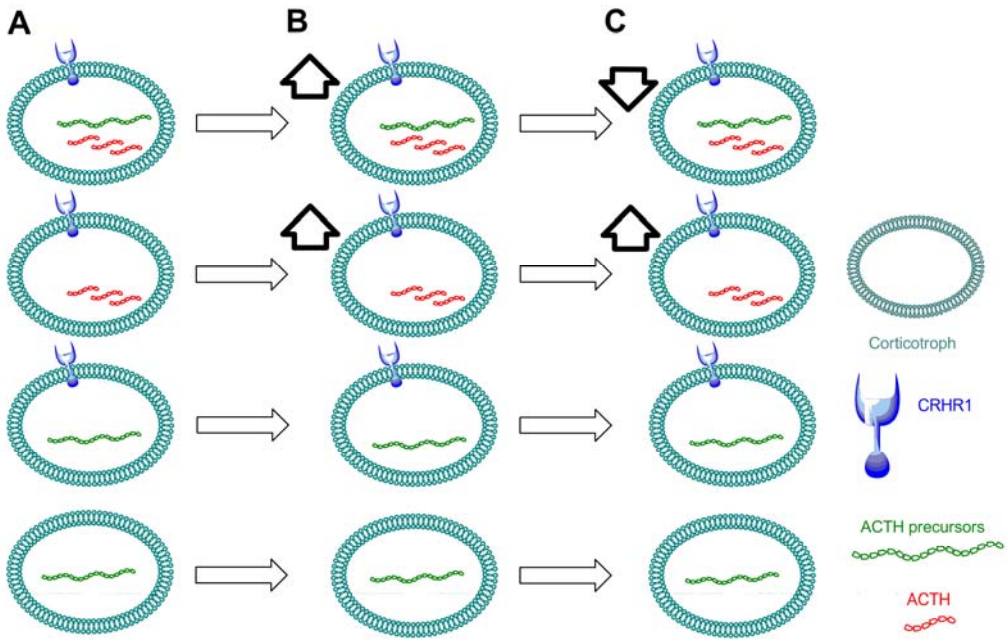


Figure 6.2 Schematic representation of the ontogenic changes in the subpopulations of corticotrophs that differentially express POMC, ACTH and $CRHR_1$. Arrows indicate that between (A) early gestation and (B) 110-140 days gestation, there are increases in the proportion of pituitary cells that belong to $POMC+ACTH+CRHR_1$ and $ACTH+CRHR_1$ subpopulations, and by (C) 144 days gestation, the $POMC+ACTH+CRHR_1$ subpopulation declines while the $ACTH+CRHR_1$ subpopulation continues to grow. Across these gestational windows there are no changes in the proportion of pituitary cells expressing $POMC+CRHR_1$ or POMC only.

Exogenous CRH increases the secretion of ACTH precursors in late gestation fetal sheep (Carr et al. 1995). It is possible that $POMC+CRHR_1$ or $POMC+ACTH+CRHR_1$ expressing cells, or both, secrete ACTH precursors in response to CRH. Interestingly, between 110 and 140 days gestation, previous investigations have found no change in the CRH-induced increase in fetal plasma precursor concentrations (Carr et al. 1995), and this is consistent with my findings

that there is no change in the proportion of pituitary cells expressing either POMC+$CRHR_1$ or POMC+ACTH+$CRHR_1$ across this gestational age window (Figure 6.2). Nevertheless, no direct correlation of the secretion of ACTH precursors and numbers of immunopositive cell has yet been performed.

The profile of the ontogenic changes in the proportion of pituitary cells that express ACTH+$CRHR_1$ and POMC+ACTH+$CRHR_1$ (Figure 6.2) suggests that ACTH+$CRHR_1$ might represent a more mature type of corticotroph while the POMC+ACTH+$CRHR_1$ subpopulation might represent an immature corticotroph. Similar developmental changes in the POMC processing within individual corticotrophs have been reported in rodent pituitaries (Chatelain et al. 1979; Noel and Mains 1991; Marcinkiewicz et al. 1993). Under this model, the shifts in the corticotroph population identified in the PCUN (Figure 6.3) and PR (Figure 6.4) fetuses might confer the HPA axis with a shift in maturity, which may represent part of the altered programming of the HPA axis that has been well established in these models of the suboptimal intrauterine environment (McMillen and Robinson 2005).

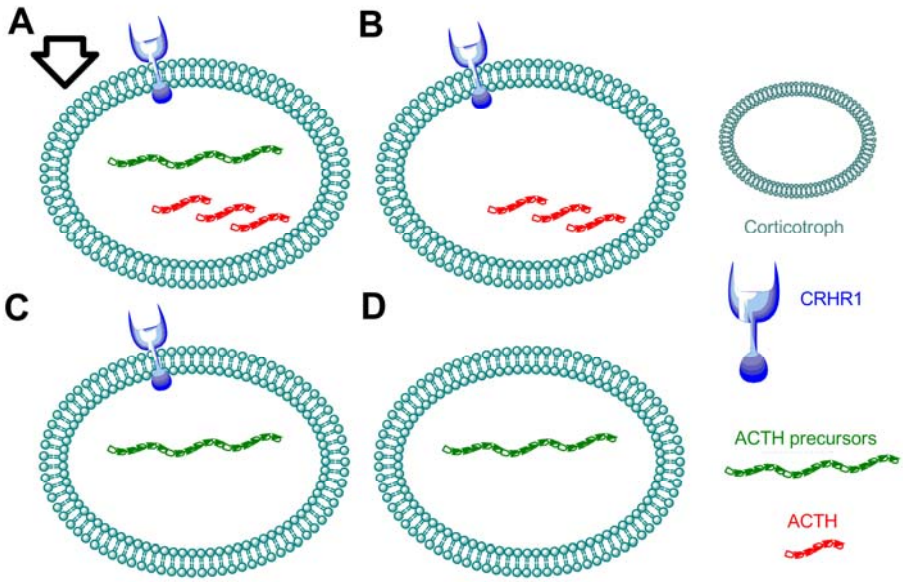


Figure 6.3 Schematic representation of PCUN-induced changes in the subpopulations of corticotrophs that differentially express POMC, ACTH and $CRHR_1$. Arrow indicates that there is a decrease in the proportion of pituitary cells that belong to (A) POMC+ACTH+$CRHR_1$, subpopulation but no changes in (B) the ACTH+$CRHR_1$ subpopulation (C) the POMC+$CRHR_1$ subpopulation or (D) the POMC only subpopulation in pituitaries of PCUN animals compared with normally grown controls.

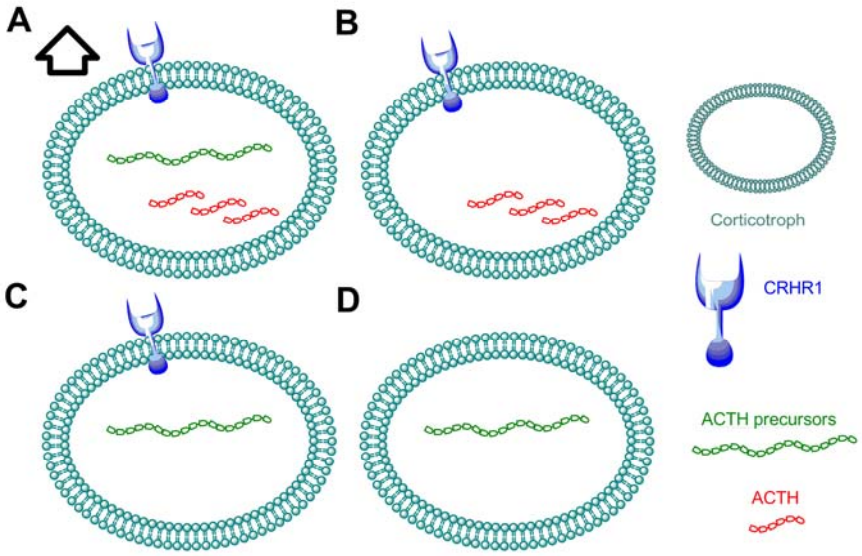


Figure 6.4 Schematic representation of PR-induced changes in the subpopulations of corticotrophs that differentially express POMC, ACTH and $CRHR_1$. Arrow indicates that there is an increase in the proportion of corticotrophs that belong to (A) the POMC+ACTH+$CRHR_1$ subpopulation relative to the (B) ACTH+$CRHR_1$ (C) POMC+$CRHR_1$ and (D) POMC only subpopulations in pituitaries of PR animals compared with normally grown controls.

I have provided the first evidence that PCUN decreases the proportion of pituitary cells expressing $CRHR_1$, which may reduce the responsiveness of the fetal pituitary to CRH. As investigations continue into the fetal adaptations to early perturbations of the fetal environment, it will be necessary to identify whether PCUN causes a permanent reduction in CRH responsiveness in later life. It is possible that reactions to stressors which principally rely on CRH-induced mechanisms might be blunted, while basal HPA activity might be elevated, as suggested by current reports (Edwards and McMillen 2002; Bloomfield et al. 2004; Gardner et al. 2006).

6.2.1.3 Transdifferentiation between corticotroph subpopulations

Beyond the static model that the ACTH+$CRHR_1$ subpopulation might represent a more mature type of corticotroph than the POMC+ACTH+$CRHR_1$ subpopulation, it is tempting to speculate that these corticotroph subpopulations represent sequential stages on a maturational continuum from the expression of POMC alone, to the additional expression of $CRHR_1$ in the POMC+$CRHR_1$ corticotrophs, through to the expression of POMC+ACTH+$CRHR_1$, and finally ACTH+$CRHR_1$. This model is supported by the *in vitro* evidence that CRH simulates PC1 synthesis and activation (Vindrola and Lindberg 1992; Li et al. 1999) and is likely to increase the POMC processing capacity of individual corticotrophs. At a certain level of CRH stimulation, the processing of POMC in POMC-only cells might increase to produce the POMC+ACTH+$CRHR_1$ corticotrophs. Finally, at maximal CRH stimulation, all of the POMC may be processed to ACTH forming ACTH+$CRHR_1$ corticotrophs. If this transdifferentiation mechanism exists, it might exist as a unidirectional maturation in which the POMC only cells are the first corticotroph cell type and the ACTH+$CRHR_1$ cells are the final cell type in the maturational process. Alternatively, the transdifferentiation mechanism might be bidirectional, where individual corticotrophs might transform back and forward throughout the continuum of distinct subpopulations in response to changes in the level of CRH stimulation. Ideally, live cell microscopy would be used to investigate transdifferentiation between corticotroph subpopulations. However, immunolabelling POMC, ACTH and $CRHR_1$ in live cells may inactivate the peptides and therefore alter the function of the

corticotrophs, which would make this approach difficult to develop. Overall, the evidence presented throughout this dissertation was devoid of any reciprocal changes in any pairs of corticotroph subpopulations, suggesting that corticotroph subpopulations might not undergo either form of transdifferentiation.

6.3 Roles of corticotroph subpopulations

In one model of differential corticotroph function, two corticotroph subpopulations show distinct characteristics: (1) a CRH-responsive corticotroph subpopulation that is responsible for the bulk of stored ACTH, contributes little to basal ACTH secretion and secretes an inhibitory paracrine factor, and, (2) an AVP-responsive corticotroph subpopulation that stores little ACTH, is responsible for most of the basal ACTH secretion, and secretes a stimulatory paracrine factor (Schwartz 1990). Such interactions between different corticotroph subpopulations via paracrine signalling would be facilitated by the heterogeneous distribution of different corticotroph subpopulations in large clusters identified in fetal sheep pituitaries.

6.3.1 Inhibitory corticotrophs

One observation in these studies is that across late gestation, the proportion of pituitary cells in the POMC+ACTH+$CRHR_1$ subpopulation is inversely correlated with previously reported changes in unstimulated HPA activity *in vivo*. This provides the first evidence that the POMC+ACTH+$CRHR_1$ subpopulation might secrete a paracrine factor that inhibits the secretion of ACTH from neighbouring corticotrophs,

resulting in a net lowering of basal ACTH secretion by the pituitary. This suggestion is consistent with previous *in vitro* evidence that CRH-target corticotrophs inhibit the basal secretion of ACTH from non-CRH target cells (Schwartz 1990) and that plating pituitary cells at decreasing density, increases unstimulated ACTH secretion (Schwartz et al. 1999). Thus, *in vitro* evidence indicates that intrapituitary paracrine signalling is only effective over relatively short distances.

Observations in my experimental models of suboptimal intrauterine environments are also consistent with the hypothesis that the POMC+ACTH+$CRHR_1$ subpopulation plays an inhibitory role in pituitary ACTH secretion. I found a decrease in the proportion of pituitary cells expressing POMC+ACTH+$CRHR_1$ in PCUN animals, which are known to have elevated fetal plasma ACTH levels at the earliest gestational age measured (Edwards and McMillen 2002). In addition, plasma ACTH levels in PR fetuses do not change over late gestation, and there is no change in the proportion of pituitary cells that express POMC+ACTH+$CRHR_1$. Therefore, the subtle shift I found to conserve the POMC+ACTH+$CRHR_1$ subpopulation may not be substantial enough to alter fetal plasma ACTH levels.

To investigate the role of paracrine signalling in the maintenance of particular corticotroph subpopulations, one possible experimental approach might be to adapt the immunohistochemical methods employed in this dissertation to pituitary cell cultures and then plate cultures at different cell densities. However, to examine the secretion by individual corticotrophs, this approach would need to be combined with

a protocol in which secretion by individual cells is measured, such as cellular immunoblotting or a reverse haemolytic plaque assay.

6.3.2 Stimulatory corticotrophs

Across gestation, I have demonstrated an increase in the proportion of pituitary cells that express ACTH+$CRHR_1$, which parallels the steady increase in the concentration of low molecular weight ACTH in the fetal sheep plasma described previously (Carr et al. 1995; Saoud and Wood 1996). I therefore hypothesise that these cells might be predominantly responsible for increasing the pituitary secretion of $ACTH_{1\text{-}39}$. Presumably, this might be a direct mechanism through which ACTH+$CRHR_1$-expressing cells synthesise and secrete large amounts of ACTH, and/or, an indirect mechanism through which ACTH+$CRHR_1$-expressing cells secrete a stimulatory paracrine factor which increases the secretion of ACTH from neighbouring cells. As there were no changes in this subpopulation with perturbations of the fetal environment, the ontogenic increase in ACTH+$CRHR_1$ subpopulation is likely to represent a developmental mechanism which is distinct from the adaptive mechanisms invoked in response to stress during gestation.

In a previously cited model of cell-cell interactions, the corticotroph subpopulation that secretes the stimulatory paracrine factor is responsive to AVP, but not CRH (Schwartz 1990). Therefore this subpopulation may be represented by the POMC only subpopulation, or alternatively, another subpopulation which expresses the peptide for $CRHR_1$ in the cytoplasm, but does not express the receptor on the cell

membrane and therefore does not respond to CRH. As mentioned previously, combining the current immunohistochemical approach with cellular immunoblotting might provide the evidence required to understand the paracrine signalling between the corticotroph subpopulations.

6.4 Response of corticotroph subpopulations to suboptimal intrauterine environments

Use of the PCUN and PR models to investigate individual corticotrophs has provided the unique opportunity for insight into the differential levels of control of pituitary function. In the PCUN model, changes in fetal ACTH plasma levels have been shown to precede changes in cortisol (Edwards and McMillen 2002; Bloomfield et al. 2003) indicating that the fetal pituitary may be driving the adaptation rather than the fetal adrenal. Conversely, PR results in an increase in fetal plasma cortisol levels without concomitant alterations in fetal plasma concentrations of ACTH or its precursors (Phillips et al. 1996). Therefore PR might represent a model in which changes at the adrenal, via non-ACTH controlled mechanisms, feedback and regulate the fetal pituitary. The following section primarily focuses on the differential adaptations identified in these two models of suboptimal uterine environment in an attempt to understand the regulation of corticotroph subpopulations in the fetal sheep pituitary.

In the PCUN model investigated in Chapter 4, the nutritional insult is completed before blastocyst implantation. It has been suggested that epigenetic adaptations in the embryo reprogram the progenitor cells of

several organ systems, including the fetal HPA axis, to develop in such a way that they maximise the fetus' chance for survival in nutrient restricted environments (McMillen and Robinson 2005; Seckl and Meaney 2006). I have provided evidence of structural changes in the fetal pituitary which may underlie the translation of a poor uterine environment during the periconceptional period into permanently altered HPA activity in later life. The data would suggest that this mechanism is likely to be responsible for the reduction in POMC+ACTH+$CRHR_1$ cells in PCUN fetuses rather than an increase in cortisol feedback, such as that seen between 140 and 144 days gestation in control animals. This is because the PCUN protocol used in this study has been previously demonstrated to cause an increase in fetal plasma ACTH levels without concomitant increases in fetal cortisol concentrations between 115 and 126 days gestation (Edwards and McMillen 2002).

In humans, twinning is associated with greater risk of IUGR, preterm birth and perinatal mortality (Imaizumi 2001; Lee et al. 2006). There is an early reprogramming of the fetal development in response to fetal number because a reduction of the embryo number during the first trimester does not disrupt the relationship between starting fetal number and risk of IUGR, miscarriage and preterm birth (Sebire et al. 1997). It is likely that signalling between fetuses and the uterine environment in multifetal pregnancies alters later fetal development via epigenetic mechanisms. In sheep, maturation of the fetal HPA axis is delayed in twins compared with singleton fetuses, as evidenced by lower plasma ACTH levels in late gestation, a later surge in fetal plasma cortisol levels and a decreased adrenal response to exogenous ACTH in late

gestation (Edwards and McMillen 2002; Gardner et al. 2004). In addition, recent evidence suggests that the fetal adrenal development is delayed by day 55 of gestation in twins compared with singletons (MacLaughlin et al. 2005). Due to the effect of fetal number on HPA axis development, I have only reported the results of twin fetuses in this dissertation. It would, however, be interesting to investigate the differences in maturational changes in subpopulations of corticotrophs between fetuses from twin and singleton pregnancies, particularly over short gestational age windows, to further our understanding of the regulation of the timing of HPA axis maturation. In addition, the effects of increased fetal number on the developmental trajectory of HPA axis is lost in the PCUN model (Edwards and McMillen 2002; MacLaughlin et al. 2005). Thus, the differential patterns of maturation of corticotroph subpopulations in singleton and twin fetuses following PCUN might provide further insights into the adaptations of the fetus to the intrauterine environment. Indeed the methods developed for this project could be applied to many other models of altered pituitary function to elucidate further the roles and regulation of the corticotroph subpopulations identified for the first time in this project.

The data presented in Chapter 5 demonstrates that PR results in a shift within the corticotroph population to conserve the corticotrophs that express POMC+ACTH+$CRHR_1$ during the prepartum period. This may represent a permanent resetting of corticotroph development because the elevated fetal plasma cortisol levels in growth restricted fetuses persist from late gestation (Phillips et al. 1996) to later life (Phillips et al. 1998; Sebaai et al. 2004). Growth restricted fetuses have elevated fetal

plasma cortisol concentrations during the prepartum period, without concomitant changes in either ACTH or its precursors (Phillips et al. 1996). Therefore, my finding that PR causes a shift in the corticotroph population to conserve the POMC+ACTH+$CRHR_1$ cells might represent the effect of increased cortisol feedback to the pituitary during late gestation. As it is well known that cortisol can affect corticotroph hyperplasia (Moriarty and Halmi 1972), it is likely that elevated cortisol levels hinder the hyperplasia of other subpopulations over the last twenty days of gestation and this results in subtle decreases in these subpopulations and an apparent conservation of the POMC+ACTH+$CRHR_1$ subpopulation. In support of this speculation, there is evidence that glucocorticoids are capable of producing rapid changes in the appearance of mitotic figures and apoptotic bodies in the rat pituitary (Nolan et al. 1998).

6.5 Conclusions

Overall, based on the differential expression of POMC, ACTH and $CRHR_1$, I have clearly demonstrated that there are four subpopulations of corticotrophs in the fetal sheep pituitary. I have provided evidence that individual corticotrophs can be immunopositive for the precursor, POMC and or the product ACTH, suggesting that corticotrophs process POMC to ACTH at different rates, and that this may be regulated by the expression of $CRHR_1$. In addition, the differential responsiveness of subpopulations of corticotrophs to CRH appears to be regulated, at least in part, by the expression of $CRHR_1$ and the distribution of $CRHR_1$ between the cytoplasm and the cell membrane.

The proportion of pituitary cells expressing POMC+ACTH+$CRHR_1$ varied across gestation inversely with known changes in HPA activity. Combined with findings from the PCUN fetuses, this suggests that this subpopulation may be responsible for inhibition of HPA axis activity. It remains to be determined whether this subpopulation secretes an inhibitory paracrine factor, which reduces the secretion of ACTH from neighbouring corticotroph levels, or secretes high levels of ACTH precursors, which inhibit the action of $ACTH_{1-39}$ at the fetal adrenal gland. There is an increase in the proportion of fetal sheep pituitary cells that express ACTH+$CRHR_1$ throughout the last third of gestation, which may be responsible for increasing fetal plasma $ACTH_{1-39}$ levels, and may even stimulate the secretion of this peptide from neighbouring cells. It is likely that the corticotrophs labelled with the POMC antibody are capable of secreting POMC and maintaining the fetal plasma levels of ACTH precursors, just as it is likely that POMC+$CRHR_1$ and/or POMC+ACTH+$CRHR_1$ expressing cells are capable of secreting POMC in response to CRH stimulation. Clearly, an important next step is to investigate the secretory profiles of ACTH precursors and ACTH of each subpopulation identified in this dissertation.

I have demonstrated that both PCUN and PR result in changes in specific corticotroph subpopulations, which are likely to play a role in the changes to basal and stimulated corticotroph function described previously in these models of suboptimal uterine environments. My findings suggest that adaptation to PCUN, possibly through epigenetic programming of the blastocyst, may translate into a deficiency in the proportion of pituitary cells expressing POMC+ACTH+$CRHR_1$ in early

gestation which results in increased basal pituitary secretion of irACTH by 115 days gestation. In contrast, I have suggested that elevated fetal plasma cortisol levels during the prepartum period in PR animals decreases the hyperplasia of several corticotroph subpopulations, resulting in a conservation of the POMC+ACTH+$CRHR_1$ expressing corticotrophs.

The novel methods developed during this project have provided further evidence for the heterogeneity of the corticotroph population and the differential development of corticotroph subpopulations. Furthermore, extensive quantification of these phenotypic subpopulations has provided further evidence of HPA axis reprogramming in suboptimal intrauterine environments.

Appendix A: Solutions

A.1 5x Phosphate Buffered Solution (PBS)

Chemical	Symbol	To make 2L	To make 5L
Sodium Chloride	NaCl	160 g	400 g
Sodium Phosphate Dibasic	Na_2HPO_4	11.5 g	28.75 g
Potassium Chloride	KCl	2 g	5 g
Potassium Phosphate Monobasic	KH_2PO_4	2 g	5 g

Make up to 2 or 5 L with MQ H_2O.

Store at room temperature.

To use, make up **1x PBS** (200ml 5x PBS per 1L MQ H_2O).

A.2 Antigen Retrieval Buffers

A.2.1 Citric Acid Buffer (100mM)

Chemical	Symbol	To make 1 L
Trisodium Citrate Dihydrate	$Na_3C_6H_5O_7.2H_2O$	25.7 g
Citric Acid Monohydrate	$C_6H_8O_7.H_2O$	2.65 g

Make up to 1 L with MQ H_2O.

Store at room temperature.

To use, make up **10mM CAB** (100ml 10x CAB per 1L MQ H_2O).

A.2.2 Acetic Acid Buffer (10mM)

pKa: 4.76

Chemical	Symbol	To make 200ml	To make 500ml
Acetic Acid, glacial	CH_3COOH	114µl	285µl

Make up to 200 or 500 mL with MQ H_2O.

Adjust pH to 4.76 with concentrated HCl or NaOH.

Store at room temperature.

A.2.3 HEPES Buffer (10mM)

pKa: 7.66

Chemical	Symbol	To make 200ml	To make 500ml
HEPES	$C_8H_{18}N_2O_4S$	0.476g	1.19g

Make up to 200 or 500 mL with MQ H_2O.

Adjust pH to 7.66 with concentrated HCl or NaOH.

Store at room temperature.

A.2.4 Trisma-Base Buffer (10mM)

pKa: 8.06

Chemical	Symbol	To make 200ml	To make 500ml
Tris Base	$C_4H_{11}NO_3$	0.242g	6.05g

Make up to 200 or 500 mL with MQ H_2O.

Adjust pH to 9.6 with concentrated HCl or NaOH.

Store at room temperature.

A.3 Antibody Diluent

Chemical	Symbol	To make 250 mL
Sodium Chloride	NaCl	4.25 g
Sodium Phosphate Dibasic	Na_2HPO_4	0.2675 g
Sodium Phosphate Monobasic	$NaH_2PO_4.2H_2O$	0.0975 g
Sodium Azide - 10%	NaN_3	250 µl

Make up to 250 mL with distilled H_2O and check pH is 7.1

Store at 2-8°C.

Appendix B: AnalySIS module codes

B.1 Detection of positive areas in grey scale images

```
//* The following group of variables is used to open, process and save files sequentially
//* i is a counter and counts from nstart (first file number, e.g. 1) to nfinish (last file number, e.g. 20)
//* stip is a string of characters equal to the folder path of all files to be opened and processed
//* stpp is a string of characters equal to the folder path where the processed files are to be saved
//* st is a string that contains the file number (e.g. 01, 02, 03, ....)
//* sifn is a string that contains the full file name of the file to be opened and processed.  That is, the path, file number, file letter (B, F, G, R) and the extension (.tif)
//* sofn is a string that contains the full file name of the file to be saved.

int i;
int nfinish, nstart;
char stip[255];
char stpp[255];
char st[4];
char sifn[255];
char sofn[255];

//*  The following group of variables is used to define the scale of the images
//*  iun is the units of the scale (um)
//*  scv is the value of 1 pixel in scale units
STUNIT iun;
double scv;

//* Define start and finish file numbers
nstart=1;
nfinish=20;

//* Define input and output paths
strcpy(stip,"D:\\analysis\\KFarrand\\TBD\\");
strcpy(stpp,"D:\\analysis\\KFarrand\\TBD\\Detected\\");

//* Define scale as micron (um) and um per pixel as 0.16801
untSet(&iun,UNIT_METER,US_MICRO,UP_LINEAR);
scv=0.16801;

//* Start processing
cls();
DeleteAllImages();
//* Loop over all file numbers in steps of 1
for (i=nstart;i<=nfinish;++i)
{
```

```
DeleteAllImages();

//**********************************Nuclei**************************************
****
Op.Display=4;
//* place the full name of file to be opened into sifn by concatenating all
the parts into 1
strcpy(sifn,stip);
sprintf(st,"%02d",i);
strcat(sifn,st);
strcat(sifn,"B.tif");

//* place the full name of file to be saved into sofn by concatenating all
the parts into 1
strcpy(sofn,stpp);
strcat(sofn,st);
strcat(sofn,"B.tif");

//*  Check if file to be opened exists. If exists process, if not skip
if (sysExistsFile(sifn))
{
printf("Processing %s\n",sifn);
//*  The following hides the command window
Op.Display=Op.Display;
Open(FileName:=sifn);
//* Open(FileName:="D:\\analysis\\KFarrand\\TBD\\01B.tif");
//*  Set scale for current display (4). X and Y are the same

imgSetUnit (Op.Display, IMGP_XUNIT, &iun);
imgSetUnit (Op.Display, IMGP_YUNIT, &iun);
imgSetScaleX(Op.Display, scv);
imgSetScaleY(Op.Display, scv);
//* SetMagnification(5952, US_MICRO);

//*  Could have used the following to set units
//*  imgSetUnitX (Op.Display, UNIT_METER,US_MICRO,UP_LINEAR);
//*  imgSetUnitY (Op.Display, UNIT_METER,US_MICRO,UP_LINEAR);
//*  This would have removed the need to define the variable iun
//*  but the use of iun is an example of passing a long pointer

Op.Dest=8;
Op.MaskEnable=FALSE;
{
          HTHRESHOLD hThr = thrCreate (70, 255 );
         thrSave(hThr, NULL);
         thrDestroy(&hThr);
}
SetGrayThresholds$(Thresholds:=NULL, AutoName:=NULL);
BinarizeGrayScaleImage(Thresholds:=NULL, Phase:=-1);

Op.Display=8;
Op.Dest=8;
```

```
MorphClose();

Op.Display=8;
Op.Dest=8;
SeparateParticles();

Op.Display=8;
claDeleteClassification ("Nuclei detect");
{
  CLASSINFO wItems [] = {
    {0, 575, "0", OVL_WHITE},
  };
  DefineClassification(Name:="Nuclei detect", Unit:="µm²",
    ClassArray:=wItems, Bins:=sizeof (wItems) / sizeof (CLASSINFO),
    Min:=0, Max:=1000, Linear:=1);
}
DefineDetection(ROIs:=0, Border:=ANA_BORDER_INCLUDE,
  Inclusions:=TRUE, MinPixel:=50, Min:=0, Max:=DBL_MAX,
  Unit:="µm", Connectivity:=ANA_CON_ADJBORDERS,
  UseRanges:=FALSE);
ClassifyParticles(Criterium:=M_AREA, Classification:="Nuclei detect",
  LabelMinPixel:=1, OutlineStyle:=0, LabelType:=0,
  LabelColor:=2, ShowLabelName:=FALSE, ShowLabelUnit:=FALSE);
Detect();

Op.Display=8;
Op.Dest=12;
DistanceBright();
Op.Display=12;
BurnOverlay();

Op.Display=12;
Op.Dest=12;
BinarizeGrayScaleImage(Thresholds:=NULL, Phase:=0);
Op.Display=12;

SaveAs(FileName:=sofn);
//* SaveAs(FileName:="D:\\analysis\\KFarrand\\TBD\\Detected\\01B.tif");
}
else
printf("Not processing %s. File doesn't exist\n",sifn);
//**********************************R**************************************
Op.Display=1;
Op.MaskEnable=FALSE;

strcpy(sifn,stip);
sprintf(st,"%02d",i);
strcat(sifn,st);
strcat(sifn,"R.tif");

strcpy(sofn,stpp);
strcat(sofn,st);
```

```
strcat(sofn,"R.tif");
if (sysExistsFile(sifn))
{
printf("Processing %s\n",sifn);
Open(FileName:=sifn);
//* Open(FileName:="D:\\analysis\\KFarrand\\TBD\\01R.tif");

imgSetUnit (Op.Display, IMGP_XUNIT, &iun);
imgSetUnit (Op.Display, IMGP_YUNIT, &iun);

imgSetScaleX(Op.Display, scv);
imgSetScaleY(Op.Display, scv);
//*  SetMagnification(5952, US_MICRO);
{
          HTHRESHOLD hThr = thrCreate (120, 255 );
         thrSave(hThr, NULL);
         thrDestroy(&hThr);
}
SetGrayThresholds$(Thresholds:=NULL, AutoName:=NULL);
claDeleteClassification ("Cyto detect");
{
  CLASSINFO wItems [] = {
    {0, 16030, "0", OVL_WHITE},
  };
  DefineClassification(Name:="Cyto detect", Unit:="µm²",
    ClassArray:=wItems, Bins:=sizeof (wItems) / sizeof (CLASSINFO),
    Min:=0, Max:=2000, Linear:=1);
}
DefineDetection(ROIs:=0, Border:=ANA_BORDER_INCLUDE,
  Inclusions:=TRUE, MinPixel:=50, Min:=0, Max:=DBL_MAX,
  Unit:="µm", Connectivity:=ANA_CON_ADJBORDERS,
  UseRanges:=FALSE);
ClassifyParticles(Criterium:=M_AREA, Classification:="Cyto detect",
  LabelMinPixel:=0, OutlineStyle:=0, LabelType:=0,
  LabelColor:=1, ShowLabelName:=FALSE, ShowLabelUnit:=FALSE);
Detect();

Op.Dest=5;
DistanceBright();
Op.Display=5;
BurnOverlay();

Op.Display=5;
Op.Dest=5;
BinarizeGrayScaleImage(Thresholds:=NULL, Phase:=0);
Op.Display=5;

SaveAs(FileName:=sofn);
//*  SaveAs(FileName:="D:\\analysis\\KFarrand\\TBD\\Detected\\01R.tif");
}
else
printf("Not processing %s. File doesn't exist\n",sifn);
```

```
//*********************************F*************************************
Op.Display=2;
Op.MaskEnable=FALSE;

strcpy(sifn,stip);
sprintf(st,"%02d",i);
strcat(sifn,st);
strcat(sifn,"F.tif");

strcpy(sofn,stpp);
strcat(sofn,st);
strcat(sofn,"F.tif");

if (sysExistsFile(sifn))
{
printf("Processing %s\n",sifn);
Open(FileName:=sifn);
//* Open(FileName:="D:\\analysis\\KFarrand\\TBD\\01F.tif");

imgSetUnit (Op.Display, IMGP_XUNIT, &iun);
imgSetUnit (Op.Display, IMGP_YUNIT, &iun);

imgSetScaleX(Op.Display, scv);
imgSetScaleY(Op.Display, scv);
//*  SetMagnification(5952, US_MICRO);

{
          HTHRESHOLD hThr = thrCreate (120, 255 );
         thrSave(hThr, NULL);
         thrDestroy(&hThr);
}
SetGrayThresholds$(Thresholds:=NULL, AutoName:=NULL);
claDeleteClassification ("Cyto detect");
{
 CLASSINFO wItems [] = {
  {0, 16030, "0", OVL_WHITE},
 };
 DefineClassification(Name:="Cyto detect", Unit:="µm²",
  ClassArray:=wItems, Bins:=sizeof (wItems) / sizeof (CLASSINFO),
  Min:=0, Max:=2000, Linear:=1);
}
DefineDetection(ROIs:=0, Border:=ANA_BORDER_INCLUDE,
 Inclusions:=TRUE, MinPixel:=50, Min:=0, Max:=DBL_MAX,
 Unit:="µm", Connectivity:=ANA_CON_ADJBORDERS,
 UseRanges:=FALSE);
ClassifyParticles(Criterium:=M_AREA, Classification:="Cyto detect",
 LabelMinPixel:=0, OutlineStyle:=0, LabelType:=0,
 LabelColor:=1, ShowLabelName:=FALSE, ShowLabelUnit:=FALSE);
Detect();
```

```
Op.Dest=6;
DistanceBright();
Op.Display=6;
BurnOverlay();

Op.Display=6;
Op.Dest=6;
BinarizeGrayScaleImage(Thresholds:=NULL, Phase:=0);
Op.Display=6;

SaveAs(FileName:=sofn);
//* SaveAs(FileName:="D:\\analysis\\KFarrand\\TBD\\Detected\\01F.tif");
}
else

printf("Not processing %s. File doesn't exist\n",sifn);
//**********************************G*****************************************
Op.Display=3;
Op.MaskEnable=FALSE;

strcpy(sifn,stip);
sprintf(st,"%02d",i);
strcat(sifn,st);
strcat(sifn,"G.tif");

strcpy(sofn,stpp);
strcat(sofn,st);
strcat(sofn,"G.tif");

if (sysExistsFile(sifn))
{
printf("Processing %s\n",sifn);
Open(FileName:=sifn);

//* Open(FileName:="D:\\analysis\\KFarrand\\TBD\\01G.tif");

imgSetUnit (Op.Display, IMGP_XUNIT, &iun);
imgSetUnit (Op.Display, IMGP_YUNIT, &iun);

imgSetScaleX(Op.Display, scv);
imgSetScaleY(Op.Display, scv);

//*  SetMagnification(5952, US_MICRO);
{
          HTHRESHOLD hThr = thrCreate (120, 255 );
         thrSave(hThr, NULL);
         thrDestroy(&hThr);
}
SetGrayThresholds$(Thresholds:=NULL, AutoName:=NULL);
claDeleteClassification ("Cyto detect");
{
  CLASSINFO wItems [] = {
```

```
    {0, 16030, "0", OVL_WHITE},
  };
  DefineClassification(Name:="Cyto detect", Unit:="µm²",
    ClassArray:=wItems, Bins:=sizeof (wItems) / sizeof (CLASSINFO),
    Min:=0, Max:=2000, Linear:=1);
}
DefineDetection(ROIs:=0, Border:=ANA_BORDER_INCLUDE,
  Inclusions:=TRUE, MinPixel:=50, Min:=0, Max:=DBL_MAX,
  Unit:="µm", Connectivity:=ANA_CON_ADJBORDERS,
  UseRanges:=FALSE);
ClassifyParticles(Criterium:=M_AREA, Classification:="Cyto detect",
  LabelMinPixel:=0, OutlineStyle:=0, LabelType:=0,
  LabelColor:=1, ShowLabelName:=FALSE, ShowLabelUnit:=FALSE);
Detect();

Op.Dest=7;
DistanceBright();
Op.Display=7;
BurnOverlay();

Op.Display=7;
Op.Dest=7;
BinarizeGrayScaleImage(Thresholds:=NULL, Phase:=0);
Op.Display=7;

SaveAs(FileName:=sofn);
//*  SaveAs(FileName:="D:\\analysis\\KFarrand\\TBD\\Detected\\01G.tif");
}
else
printf("Not processing %s. File doesn't exist\n",sifn);
}
```

B.2 Quantification of individual cells with multiple labels

```
int i;
int ngroups;
char stip[255];
char stpp[255];
char st[4];
char sifn[255];
char sofn[255];

ngroups=20;

strcpy(stip,"D:\\analysis\\KFarrand\\TBD\\Detected\\");
strcpy(stpp,"D:\\analysis\\KFarrand\\TBD\\Analysed\\");

for (i=1;i<=ngroups;++i)
{
printf("Processing %s\n",sifn);
DeleteAllImages();
```

```
//*********************************Open Images************************
//*********************************Nuclei*****************************************
Op.Display=8;
Op.MaskEnable=FALSE;

strcpy(sifn,stip);
sprintf(st,"%02d",i);
strcat(sifn,st);
strcat(sifn,"B.tif");
if (sysExistsFile(sifn))
{
Open(FileName:=sifn);
//* Open(FileName:="D:\\analysis\\KFarrand\\TBD\\Detected\\14B.tif");

claDeleteClassification ("Nuclei");
{
  CLASSINFO wItems [] = {
    {0, 15, "0", OVL_WHITE},
    {15, 50, "1", OVL_BLUE},
    {50, 85, "2", OVL_RED},
    {85, 120, "3", OVL_YELLOW},
    {120, 155, "4", OVL_GREEN},
    {155, 190, "5", OVL_MAGENTA},
    {190, 225, "6", OVL_CYAN},
    {225, 260, "7", OVL_WHITE},
    {260, 295, "8", OVL_BLUE},
    {295, 330, "9", OVL_RED},
    {330, 435, "12", OVL_YELLOW},
    {435, 575, "17", OVL_GREEN},
  };
  DefineClassification(Name:="Nuclei", Unit:="µm²",
    ClassArray:=wItems, Bins:=sizeof (wItems) / sizeof (CLASSINFO),
    Min:=0, Max:=1000, Linear:=1);
}
DefineDetection(ROIs:=0, Border:=ANA_BORDER_INCLUDE,
  Inclusions:=TRUE, MinPixel:=50, Min:=0, Max:=DBL_MAX,
  Unit:="µm", Connectivity:=ANA_CON_ADJBORDERS,
  UseRanges:=FALSE);
ClassifyParticles(Criterium:=M_AREA, Classification:="Nuclei",
  LabelMinPixel:=1, OutlineStyle:=0, LabelType:=0,
  LabelColor:=2, ShowLabelName:=FALSE, ShowLabelUnit:=FALSE);

Detect();
ClassResults$();

strcpy(sofn,stpp);
strcat(sofn,st);
strcat(sofn,"B.xls");

SaveAs(FileName:=sofn);
//*
SaveAs(FileName:="D:\\analysis\\KFarrand\\TBD\\Analysed\\14B.xls");
```

```
//**********************************ACTH**************************************
Op.Display=5;
Op.MaskEnable=FALSE;

strcpy(sifn,stip);
//* sprintf(st,"%02d",i);
strcat(sifn,st);
strcat(sifn,"R.tif");

Open(FileName:=sifn);
//* Open(FileName:="D:\\analysis\\KFarrand\\TBD\\Detected\\14R.tif");

//**********************************CRH
R1**************************************
Op.Display=6;
Op.MaskEnable=FALSE;

strcpy(sifn,stip);
//* sprintf(st,"%02d",i);
strcat(sifn,st);
strcat(sifn,"F.tif");

Open(FileName:=sifn);
//* Open(FileName:="D:\\analysis\\KFarrand\\TBD\\Detected\\14F.tif");

//**********************************POMC**************************************
***
Op.Display=7;
Op.MaskEnable=FALSE;

strcpy(sifn,stip);
//* sprintf(st,"%02d",i);
strcat(sifn,st);
strcat(sifn,"G.tif");

Open(FileName:=sifn);
//* Open(FileName:="D:\\analysis\\KFarrand\\TBD\\Detected\\14G.tif");

//**********************************R, F, G************************

//**********************************R************************

Op.Display=5;
claDeleteClassification ("Cytoplasm 1");
{
  CLASSINFO wItems [] = {
    {0, 50, "0", OVL_WHITE},
    {50, 110, "1", OVL_BLUE},
    {110, 190, "2", OVL_RED},
    {190, 270, "3", OVL_YELLOW},
    {270, 350, "4", OVL_GREEN},
```

```
    {350, 430, "5", OVL_MAGENTA},
    {430, 510, "6", OVL_CYAN},
    {510, 590, "7", OVL_WHITE},
    {590, 670, "8", OVL_BLUE},
    {670, 750, "9", OVL_RED},
    {750, 1150, "12", OVL_YELLOW},
    {1150, 1550, "17", OVL_GREEN},
    {1550, 2350, "25", OVL_MAGENTA},
    {2350, 3150, "35", OVL_CYAN},
    {3150, 3950, "45", OVL_WHITE},
    {3950, 5550, "60", OVL_BLUE},
    {5550, 7950, "85", OVL_RED},
    {7950, 11950, "125", OVL_YELLOW},
    {11950, 16030, "175", OVL_GREEN},
  };
  DefineClassification(Name:="Cytoplasm 1", Unit:="µm²",
    ClassArray:=wItems, Bins:=sizeof (wItems) / sizeof (CLASSINFO),
    Min:=0, Max:=2000, Linear:=1);
}
DefineDetection(ROIs:=0, Border:=ANA_BORDER_INCLUDE,
  Inclusions:=TRUE, MinPixel:=50, Min:=0, Max:=DBL_MAX,
  Unit:="µm", Connectivity:=ANA_CON_ADJBORDERS,
  UseRanges:=FALSE);
ClassifyParticles(Criterium:=M_AREA, Classification:="Cytoplasm 1",
  LabelMinPixel:=0, OutlineStyle:=0, LabelType:=0,
  LabelColor:=1, ShowLabelName:=FALSE, ShowLabelUnit:=FALSE);

Detect();
ClassResults$();

strcpy(sofn,stpp);
strcat(sofn,st);
strcat(sofn,"R.xls");

SaveAs(FileName:=sofn);
//*
SaveAs(FileName:="D:\\analysis\\KFarrand\\TBD\\Analysed\\14R.xls");

//*********************************F************************
Op.Display=6;
claDeleteClassification ("Cytoplasm 1");
{
  CLASSINFO wItems [] = {
    {0, 50, "0", OVL_WHITE},
    {50, 110, "1", OVL_BLUE},
    {110, 190, "2", OVL_RED},
    {190, 270, "3", OVL_YELLOW},
    {270, 350, "4", OVL_GREEN},
    {350, 430, "5", OVL_MAGENTA},
    {430, 510, "6", OVL_CYAN},
    {510, 590, "7", OVL_WHITE},
    {590, 670, "8", OVL_BLUE},
```

```
    {670, 750, "9", OVL_RED},
    {750, 1150, "12", OVL_YELLOW},
    {1150, 1550, "17", OVL_GREEN},
    {1550, 2350, "25", OVL_MAGENTA},
    {2350, 3150, "35", OVL_CYAN},
    {3150, 3950, "45", OVL_WHITE},
    {3950, 5550, "60", OVL_BLUE},
    {5550, 7950, "85", OVL_RED},
    {7950, 11950, "125", OVL_YELLOW},
    {11950, 16030, "175", OVL_GREEN},
   };
   DefineClassification(Name:="Cytoplasm 1", Unit:="µm²",
    ClassArray:=wItems, Bins:=sizeof (wItems) / sizeof (CLASSINFO),
    Min:=0, Max:=2000, Linear:=1);
}
DefineDetection(ROIs:=0, Border:=ANA_BORDER_INCLUDE,
  Inclusions:=TRUE, MinPixel:=50, Min:=0, Max:=DBL_MAX,
  Unit:="µm", Connectivity:=ANA_CON_ADJBORDERS,
  UseRanges:=FALSE);
ClassifyParticles(Criterium:=M_AREA, Classification:="Cytoplasm 1",
  LabelMinPixel:=0, OutlineStyle:=0, LabelType:=0,
  LabelColor:=1, ShowLabelName:=FALSE, ShowLabelUnit:=FALSE);

Detect();
ClassResults$();

strcpy(sofn,stpp);
strcat(sofn,st);
strcat(sofn,"F.xls");

SaveAs(FileName:=sofn);
//*
SaveAs(FileName:="D:\\analysis\\KFarrand\\TBD\\Analysed\\14F.xls");

//*********************************G************************
Op.Display=7;
claDeleteClassification ("Cytoplasm 1");
{
  CLASSINFO wItems [] = {
    {0, 50, "0", OVL_WHITE},
    {50, 110, "1", OVL_BLUE},
    {110, 190, "2", OVL_RED},
    {190, 270, "3", OVL_YELLOW},
    {270, 350, "4", OVL_GREEN},
    {350, 430, "5", OVL_MAGENTA},
    {430, 510, "6", OVL_CYAN},
    {510, 590, "7", OVL_WHITE},
    {590, 670, "8", OVL_BLUE},
    {670, 750, "9", OVL_RED},
    {750, 1150, "12", OVL_YELLOW},
    {1150, 1550, "17", OVL_GREEN},
    {1550, 2350, "25", OVL_MAGENTA},
```

```
    {2350, 3150, "35", OVL_CYAN},
    {3150, 3950, "45", OVL_WHITE},
    {3950, 5550, "60", OVL_BLUE},
    {5550, 7950, "85", OVL_RED},
    {7950, 11950, "125", OVL_YELLOW},
    {11950, 16030, "175", OVL_GREEN},
  };
  DefineClassification(Name:="Cytoplasm 1", Unit:="µm²",
    ClassArray:=wItems, Bins:=sizeof (wItems) / sizeof (CLASSINFO),
    Min:=0, Max:=2000, Linear:=1);
}
DefineDetection(ROIs:=0, Border:=ANA_BORDER_INCLUDE,
  Inclusions:=TRUE, MinPixel:=50, Min:=0, Max:=DBL_MAX,
  Unit:="µm", Connectivity:=ANA_CON_ADJBORDERS,
  UseRanges:=FALSE);
ClassifyParticles(Criterium:=M_AREA, Classification:="Cytoplasm 1",
  LabelMinPixel:=0, OutlineStyle:=0, LabelType:=0,
  LabelColor:=1, ShowLabelName:=FALSE, ShowLabelUnit:=FALSE);

Detect();
ClassResults$();

strcpy(sofn,stpp);
strcat(sofn,st);
strcat(sofn,"G.xls");

SaveAs(FileName:=sofn);
//*
SaveAs(FileName:="D:\\analysis\\KFarrand\\TBD\\Analysed\\14G.xls");

//**********************************Calculations************************
//**********************************R-F************************
Op.Source1=5;
Op.Source2=6;
Op.Dest=9;
AbsoluteSubtraction();

//**********************************R-G************************
Op.Source1=5;
Op.Source2=7;
Op.Dest=13;
AbsoluteSubtraction();

//**********************************R-F-G************************
Op.Source1=9;
Op.Source2=7;
Op.Dest=17;
AbsoluteSubtraction();

//**********************************F-R************************
Op.Source1=6;
Op.Source2=5;
```

```
Op.Dest=10;
AbsoluteSubtraction();

//*********************************F-R-G************************
Op.Source1=10;
Op.Source2=7;
Op.Dest=18;
AbsoluteSubtraction();

//*********************************G-R************************
Op.Source1=7;
Op.Source2=5;
Op.Dest=11;
AbsoluteSubtraction();

//*********************************G-R-F************************
Op.Source1=11;
Op.Source2=6;
Op.Dest=19;
AbsoluteSubtraction();

//*********************************RF=(R-G)-(R-F-G)************************
Op.Source1=13;
Op.Source2=17;
Op.Dest=21;
AbsoluteSubtraction();

//*********************************RG=(R-F)-(R-F-G)************************
Op.Source1=9;
Op.Source2=17;
Op.Dest=25;
AbsoluteSubtraction();

//*********************************FG=(F-R)-(F-R-G)************************
Op.Source1=10;
Op.Source2=18;
Op.Dest=26;
AbsoluteSubtraction();

//*********************************RFG:(Ro+RF)************************
Op.Source1=17;
Op.Source2=21;
Op.Dest=12;
AbsoluteAddition();

//*********************************RFG:((Ro+RF)+RG)************************
**
Op.Source1=12;
Op.Source2=25;
Op.Dest=16;
AbsoluteAddition();
```

```
//*********************************RFG=R-
(Ro+RF+RG)************************
Op.Source1=5;
Op.Source2=16;
Op.Dest=20;
AbsoluteSubtraction();

//*********************************Detections************************

//*********************************Ro=(R-F-G)************************
Op.Display=17;
claDeleteClassification ("Cytoplasm 2");
{
  CLASSINFO wItems [] = {
    {0, 60, "0", OVL_WHITE},
    {60, 100, "1", OVL_BLUE},
    {100, 170, "2", OVL_RED},
    {170, 240, "3", OVL_YELLOW},
    {240, 310, "4", OVL_GREEN},
    {310, 380, "5", OVL_MAGENTA},
    {380, 450, "6", OVL_CYAN},
    {450, 520, "7", OVL_WHITE},
    {520, 590, "8", OVL_BLUE},
    {590, 660, "9", OVL_RED},
    {660, 1010, "12", OVL_YELLOW},
    {1010, 1360, "17", OVL_GREEN},
    {1360, 2060, "25", OVL_MAGENTA},
    {2060, 2760, "35", OVL_CYAN},
    {2760, 3460, "45", OVL_WHITE},
    {3460, 4860, "60", OVL_BLUE},
    {4860, 6960, "85", OVL_RED},
    {6960, 10460, "125", OVL_YELLOW},
    {10460, 14030, "175", OVL_GREEN},
  };
  DefineClassification(Name:="Cytoplasm 2", Unit:="µm²",
    ClassArray:=wItems, Bins:=sizeof (wItems) / sizeof (CLASSINFO),
    Min:=0, Max:=2000, Linear:=1);
}
DefineDetection(ROIs:=0, Border:=ANA_BORDER_INCLUDE,
  Inclusions:=TRUE, MinPixel:=50, Min:=0, Max:=DBL_MAX,
  Unit:="µm", Connectivity:=ANA_CON_ADJBORDERS,
  UseRanges:=FALSE);
ClassifyParticles(Criterium:=M_AREA, Classification:="Cytoplasm 2",
  LabelMinPixel:=0, OutlineStyle:=0, LabelType:=0,
  LabelColor:=1, ShowLabelName:=FALSE, ShowLabelUnit:=FALSE);

Detect();
ClassResults$();

strcpy(sofn,stpp);
strcat(sofn,st);
strcat(sofn,"RO.xls");
```

```
SaveAs(FileName:=sofn);
//*
SaveAs(FileName:="D:\\analysis\\KFarrand\\TBD\\Analysed\\14RO.xls");

//*********************************Fo=(F-R-G)************************
Op.Display=18;

claDeleteClassification ("Cytoplasm 2");
{
  CLASSINFO wItems [] = {
    {0, 60, "0", OVL_WHITE},
    {60, 100, "1", OVL_BLUE},
    {100, 170, "2", OVL_RED},
    {170, 240, "3", OVL_YELLOW},
    {240, 310, "4", OVL_GREEN},
    {310, 380, "5", OVL_MAGENTA},
    {380, 450, "6", OVL_CYAN},
    {450, 520, "7", OVL_WHITE},
    {520, 590, "8", OVL_BLUE},
    {590, 660, "9", OVL_RED},
    {660, 1010, "12", OVL_YELLOW},
    {1010, 1360, "17", OVL_GREEN},
    {1360, 2060, "25", OVL_MAGENTA},
    {2060, 2760, "35", OVL_CYAN},
    {2760, 3460, "45", OVL_WHITE},
    {3460, 4860, "60", OVL_BLUE},
    {4860, 6960, "85", OVL_RED},
    {6960, 10460, "125", OVL_YELLOW},
    {10460, 14030, "175", OVL_GREEN},



  };
  DefineClassification(Name:="Cytoplasm 2", Unit:="µm²",
    ClassArray:=wItems, Bins:=sizeof (wItems) / sizeof (CLASSINFO),
    Min:=0, Max:=2000, Linear:=1);
}
DefineDetection(ROIs:=0, Border:=ANA_BORDER_INCLUDE,
  Inclusions:=TRUE, MinPixel:=50, Min:=0, Max:=DBL_MAX,
  Unit:="µm", Connectivity:=ANA_CON_ADJBORDERS,
  UseRanges:=FALSE);
ClassifyParticles(Criterium:=M_AREA, Classification:="Cytoplasm 2",
  LabelMinPixel:=0, OutlineStyle:=0, LabelType:=0,
  LabelColor:=1, ShowLabelName:=FALSE, ShowLabelUnit:=FALSE);

Detect();
ClassResults$();

strcpy(sofn,stpp);
strcat(sofn,st);
strcat(sofn,"FO.xls");
```

```
SaveAs(FileName:=sofn);
//*
SaveAs(FileName:="D:\\analysis\\KFarrand\\TBD\\Analysed\\14FO.xls");

//*********************************Go=(G-R-F)************************
Op.Display=19;
claDeleteClassification ("Cytoplasm 2");
{
  CLASSINFO wItems [] = {
    {0, 60, "0", OVL_WHITE},
    {60, 100, "1", OVL_BLUE},
    {100, 170, "2", OVL_RED},
    {170, 240, "3", OVL_YELLOW},
    {240, 310, "4", OVL_GREEN},
    {310, 380, "5", OVL_MAGENTA},
    {380, 450, "6", OVL_CYAN},
    {450, 520, "7", OVL_WHITE},
    {520, 590, "8", OVL_BLUE},
    {590, 660, "9", OVL_RED},
    {660, 1010, "12", OVL_YELLOW},
    {1010, 1360, "17", OVL_GREEN},
    {1360, 2060, "25", OVL_MAGENTA},
    {2060, 2760, "35", OVL_CYAN},
    {2760, 3460, "45", OVL_WHITE},
    {3460, 4860, "60", OVL_BLUE},
    {4860, 6960, "85", OVL_RED},
    {6960, 10460, "125", OVL_YELLOW},
    {10460, 14030, "175", OVL_GREEN},
  };
  DefineClassification(Name:="Cytoplasm 2", Unit:="µm²",
    ClassArray:=wItems, Bins:=sizeof (wItems) / sizeof (CLASSINFO),
    Min:=0, Max:=2000, Linear:=1);
}
DefineDetection(ROIs:=0, Border:=ANA_BORDER_INCLUDE,
  Inclusions:=TRUE, MinPixel:=50, Min:=0, Max:=DBL_MAX,
  Unit:="µm", Connectivity:=ANA_CON_ADJBORDERS,
  UseRanges:=FALSE);
ClassifyParticles(Criterium:=M_AREA, Classification:="Cytoplasm 2",
  LabelMinPixel:=0, OutlineStyle:=0, LabelType:=0,
  LabelColor:=1, ShowLabelName:=FALSE, ShowLabelUnit:=FALSE);

Detect();
ClassResults$();

strcpy(sofn,stpp);
strcat(sofn,st);
strcat(sofn,"GO.xls");

SaveAs(FileName:=sofn);
//*
SaveAs(FileName:="D:\\analysis\\KFarrand\\TBD\\Analysed\\14GO.xls");
```

```
//*********************************RF************************
Op.Display=21;
claDeleteClassification ("Cytoplasm 2");
{
  CLASSINFO wItems [] = {
    {0, 40, "0", OVL_WHITE},
    {40, 100, "1", OVL_BLUE},
    {100, 170, "2", OVL_RED},
    {170, 240, "3", OVL_YELLOW},
    {240, 310, "4", OVL_GREEN},
    {310, 380, "5", OVL_MAGENTA},
    {380, 450, "6", OVL_CYAN},
    {450, 520, "7", OVL_WHITE},
    {520, 590, "8", OVL_BLUE},
    {590, 660, "9", OVL_RED},
    {660, 1010, "12", OVL_YELLOW},
    {1010, 1360, "17", OVL_GREEN},
    {1360, 2060, "25", OVL_MAGENTA},
    {2060, 2760, "35", OVL_CYAN},
    {2760, 3460, "45", OVL_WHITE},
    {3460, 4860, "60", OVL_BLUE},
    {4860, 6960, "85", OVL_RED},
    {6960, 10460, "125", OVL_YELLOW},
    {10460, 14030, "175", OVL_GREEN},
  };
  DefineClassification(Name:="Cytoplasm 2", Unit:="µm²",
    ClassArray:=wItems, Bins:=sizeof (wItems) / sizeof (CLASSINFO),
    Min:=0, Max:=2000, Linear:=1);
}
DefineDetection(ROIs:=0, Border:=ANA_BORDER_INCLUDE,
  Inclusions:=TRUE, MinPixel:=50, Min:=0, Max:=DBL_MAX,
  Unit:="µm", Connectivity:=ANA_CON_ADJBORDERS,
  UseRanges:=FALSE);
ClassifyParticles(Criterium:=M_AREA, Classification:="Cytoplasm 2",
  LabelMinPixel:=0, OutlineStyle:=0, LabelType:=0,
  LabelColor:=1, ShowLabelName:=FALSE, ShowLabelUnit:=FALSE);

Detect();
ClassResults$();

strcpy(sofn,stpp);
strcat(sofn,st);
strcat(sofn,"RF.xls");

SaveAs(FileName:=sofn);
//*
SaveAs(FileName:="D:\\analysis\\KFarrand\\TBD\\Analysed\\14RF.xls");

//*********************************RG************************
Op.Display=25;
claDeleteClassification ("Cytoplasm 2");
```

```
{
  CLASSINFO wItems [] = {
    {0, 40, "0", OVL_WHITE},
    {40, 100, "1", OVL_BLUE},
    {100, 170, "2", OVL_RED},
    {170, 240, "3", OVL_YELLOW},
    {240, 310, "4", OVL_GREEN},
    {310, 380, "5", OVL_MAGENTA},
    {380, 450, "6", OVL_CYAN},
    {450, 520, "7", OVL_WHITE},
    {520, 590, "8", OVL_BLUE},
    {590, 660, "9", OVL_RED},
    {660, 1010, "12", OVL_YELLOW},
    {1010, 1360, "17", OVL_GREEN},
    {1360, 2060, "25", OVL_MAGENTA},
    {2060, 2760, "35", OVL_CYAN},
    {2760, 3460, "45", OVL_WHITE},
    {3460, 4860, "60", OVL_BLUE},
    {4860, 6960, "85", OVL_RED},
    {6960, 10460, "125", OVL_YELLOW},
    {10460, 14030, "175", OVL_GREEN},
  };
  DefineClassification(Name:="Cytoplasm 2", Unit:="µm²",
    ClassArray:=wItems, Bins:=sizeof (wItems) / sizeof (CLASSINFO),
    Min:=0, Max:=2000, Linear:=1);
}
DefineDetection(ROIs:=0, Border:=ANA_BORDER_INCLUDE,
  Inclusions:=TRUE, MinPixel:=50, Min:=0, Max:=DBL_MAX,
  Unit:="µm", Connectivity:=ANA_CON_ADJBORDERS,
  UseRanges:=FALSE);
ClassifyParticles(Criterium:=M_AREA, Classification:="Cytoplasm 2",
  LabelMinPixel:=0, OutlineStyle:=0, LabelType:=0,
  LabelColor:=1, ShowLabelName:=FALSE, ShowLabelUnit:=FALSE);

Detect();
ClassResults$();

strcpy(sofn,stpp);
strcat(sofn,st);
strcat(sofn,"RG.xls");

SaveAs(FileName:=sofn);
//*
SaveAs(FileName:="D:\\analysis\\KFarrand\\TBD\\Analysed\\14RG.xls");

//**********************************FG************************
Op.Display=26;

claDeleteClassification ("Cytoplasm 2b");
{
  CLASSINFO wItems [] = {
    {0, 15, "0", OVL_WHITE},
```

```
    {15, 100, "1", OVL_BLUE},
    {100, 170, "2", OVL_RED},
    {170, 240, "3", OVL_YELLOW},
    {240, 310, "4", OVL_GREEN},
    {310, 380, "5", OVL_MAGENTA},
    {380, 450, "6", OVL_CYAN},
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