Regulation of Corticotrophin-Releasing Factors in the Fetal Sheep Hypothalamus.

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Contents

Declaration v

Acknowledgements vi

Abstract vii

Abbreviations ix

Chapter 1. Introduction 1

Chapter 2. Review of the Literature 3

2.1. Overview of the Fetal Pituitary-Adrenal Axis in development. 4

2.1.1. Historical Perspective 4
2.1.2. The Preparturient Activation of the Fetal Pituitary-Adrenal Axis 5
2.1.3. Functions of Cortisol in the Fetal Sheep 6

2.2. Functional Development of the Fetal Hypothalamo-Pituitary-Adrenal Axis 8

2.2.1. ACTH in the Developing Fetal Pituitary 8
2.2.2. ACTH-Releasing Factors, ACTH, and Adrenal Steroids in Fetal Life 10
2.2.3. Integrated Function of the Fetal Hypothalamo-Pituitary Adrenal Axis 15

2.3. Hypothalamic Corticotrophin-Releasing Factors 17

2.3.1. Co-localisation of CRH and AVP in the Hypothalamus 18
2.3.2. Functional Divisions within the PVN 19
2.3.3. Hypothalamic Synthesis of CRH and AVP 20
2.3.4. Development of CRH and AVP Cells in the Fetal sheep PVN 21
2.3.5. CRH and AVP Immunoreactivity in the Fetal Sheep Hypothalamus 22

2.4. Neural Control of CRH and AVP Secretion 24

2.4.1. The Effects of Opioids on the Secretion of ACTH-Releasing Factors 24
2.4.2. Opioid Peptides and Pituitary-Adrenal Activity *in vivo* 26
2.4.3. Opioids and the Fetal Sheep Hypothalamo-Pituitary Adrenal Axis 28

2.4.4. Neuropeptide Y as a Regulator of Pituitary-Adrenal Activity 30
2.4.5. Neurotransmitter Regulation of ACTH-Releasing Factors 31

2.5. Negative Feedback Regulation of ACTH-Releasing Factors 33

2.5.1. Central Distribution of Glucocorticoid Receptors 34
2.5.2. Glucocorticoid Regulation of CRH and AVP Synthesis 35
2.5.3. Negative Feedback Regulation of CRH and AVP Secretion 36
2.5.4. Glucocorticoid Feedback Regulation in the Fetal Sheep Hypothalamus 37
2.5.5. Extrahypothalamic Glucocorticoid Negative Feedback 38

2.6. Aims of this Thesis 40

Chapter 3. General Materials and Methods 41

3.1. Animals and Husbandry 41

3.1.1. Sheep Management 41
3.1.2. Timed Mating 41

3.2. Materials for Cell Culture 42

3.2.1. Culture Media and Reagents 42
3.2.2. Glassware and Plastics 42
3.2.3. Stock solutions for Dispersion and Culture Media 43
3.2.4. Preparation of Media for Tissue Dispersion 45
3.2.5. Preparation of Serum-Free Medium For Cell Culture 45

3.3. Preparation of Fetal Sheep Hypothalamic Cell Cultures 46

3.3.1. Coating of Culture Dishes 46
3.3.2. Euthanasia 46
3.3.3. Dissection 46
3.3.4. Tissue Dispersion 47

3.4. Experimental Procedures 49

3.4.1. Preparation of Media and Supplements 49
3.4.2. General Experimental Procedure 49

3.5. Peptide Radioimmunoassays 50

3.5.1. Arginine Vasopressin 50
3.5.2. Corticotrophin-Releasing Hormone Immunoradiometric Assay 54
3.5.3. oCRH Radioimmunoassay 58

3.6. Sephadex Chromatography 61

3.7. Data Analysis 61

Chapter 4. Development of the Fetal Sheep Hypothalamic Cell Culture System 63

4.1. Introduction 63

4.2. Experimental Procedures 65

4.2.1. Optimisation of Culture Conditions 65
4.2.2. Assessment of Experimentally-Induced AVP Release 66

4.3. Results 67

4.3.1. Optimisation of Culture Conditions 67
4.3.2. Assessment of Experimentally-Induced AVP Release 70

4.4. Discussion

Chapter 5. Ontogeny of AVP and CRH Secretion from Fetal Sheep Hypothalamic Cells 75

5.1. Introduction 75

5.2. Experimental Procedures 76

5.2.1. AVP and CRH Release from the developing hypothalamus *in vitro* 77
5.2.2. Time and Calcium Dependency of AVP and CRH Release 78
5.2.3. AVP and CRH Content of Cell Cultures 79
5.2.4. Cell Numbers after 36 days *in vitro* 79
5.2.5. Immunocytochemistry; Antibody Characterisation 80
5.2.6. Validation of Immunocytochemical Procedures 82
5.2.7. Quantitative NSE Immunochemistry 84

5.3. Results 86

5.3.1. AVP and CRH release from the developing hypothalamus *in vitro* 86
5.3.2. Time and Calcium Dependency of AVP and CRH Release 90
5.3.3. Culture Contents of AVP and CRH 94
5.3.4. Cell Counting and Immunocytochemistry 94

5.4. Discussion 97

Chapter 6. Corticotrophin-Releasing Factors in the Hypothalamus of the Developing Fetal Sheep 102

6.1. Introduction 102

6.2. Experimental Procedures 104

6.2.1. Immunoreactive AVP and CRH in the Fetal Sheep Hypothalamus 104
6.2.2. Gel Chromatography of Fetal Corticotrophin Releasing Factors 105
6.2.3. Pituitary Cell Bioassay 106
6.2.4. ACTH Radioimmunoassay 107

6.3. Results 108

6.3.1. Immunoreactive AVP and CRH in the Fetal Hypothalamus 108
6.3.2. Gel Chromatography of Corticotrophin-Releasing Factors 111

6.4. Discussion 117

Chapter 7. Cortisol Negative Feedback at the level of the Fetal Hypothalamus *in vitro* 123

7.1. Introduction 123

7.2. Experimental Procedures 125

7.3. Results 127

7.4. Discussion 129

Chapter 8. Opioid Regulation of AVP Secretion in the Developing Fetal Sheep Hypothalamus 138

8.1. Introduction 138
8.2. Experimental Procedures 140

8.2.1. The Effects of the Selective Opioid Agonists on AVP Secretion from Fetal Rat Hypothalamic Cells 141
8.2.2. Opioid and Steroid Regulation of AVP Secretion by Fetal Sheep Hypothalamic Cells 144

8.3. Results 145

8.4. Discussion 150

Chapter 9. General Discussion 159

9.1. Introduction 159

9.2. Discussion 159

9.3. The Hypothalamic Cell Culture Model 163

Bibliography 165

Declaration

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Abstract

In many species a developmentally related increase in cortisol production from the fetal adrenal gland brings about the progressive maturation of fetal organ systems and triggers the onset of parturition. The fetal hypothalamus is thought to play a pivotal role in this process by secreting the neuropeptides, CRH and AVP which act upon the fetal pituitary gland to cause the secretion of ACTH. However, little is known about the ontogeny and neuroendocrine regulation of CRH and AVP secretion from the fetal hypothalamus. The experiments described in this thesis were therefore designed to investigate the maturation of CRH and AVP secretion from the fetal hypothalamus during fetal development.

In order to study the secretion of CRH and AVP from the fetal hypothalamus a method for the serum-free culture of fetal sheep hypothalamic neurones was developed. The system was optimised in terms of plating density and substrate requirements and cells were maintained in vitro for up to 35 days. The functional capacity of these cells was demonstrated by measuring enhanced AVP secretion after potassium-induced depolarization, a response which was time- and calcium-dependent.

To investigate the ontogeny of CRH and AVP secretion, cultured fetal sheep hypothalami removed at day 70, day 100 and day 130 of gestation (Term = day 145) were incubated with control and 56 mM potassium-containing medium. The results showed an overall reduction in basal and potassium-stimulated CRH and AVP release with advancing gestational age. These data suggested that hypothalamic secretion of CRH and AVP was reduced with advancing gestation.

To relate these results to the intact hypothalamus, the concentrations of immunoreactive and bioactive CRH and AVP were measured in age-matched fetal sheep hypothalami by RIA and pituitary cell bioassay respectively. In contrast to the reduction in CRH and AVP secretion observed in vitro, the concentrations of CRH and AVP in the fetal hypothalamus increased with advancing gestational age. These results suggest that extrahypothalamic inputs regulate the secretion of

CRH and AVP from the hypothalamus in vivo. Alternatively, changes in peptide secretion in vitro may reflect inherent maturational differences between hypothalamic cells removed at each gestational age.

The functional capacity of cultured fetal hypothalamic cells was assessed by studying the effects of cortisol and opioid peptides on AVP secretion at day 70 and day 100 of gestation. Cortisol inhibited basal and potassium-stimulated AVP secretion in day 70 but not in day 100 cells. Similarly, the kappa opioid agonist [D-Pro^{10}] Dynorphin (1-11) inhibited AVP secretion from day 70 cells via a naloxone-insensitive pathway, yet had no effect in day 100 cultures or in cells pre-treated with cortisol. These results suggest that both cortisol and opioid inhibition are diminished with advancing gestation and that there may be interactions between these inhibitory influences during development.

In conclusion, these studies have demonstrated the viability of an in vitro culture system for investigating developmentally related changes in neuropeptide secretion from the fetal sheep hypothalamus. As a result of these studies it is possible to conclude that CRH and AVP secretion from the fetal hypothalamus is likely to be under extrahypothalamic inhibitory control early in gestation. The gradual decline in this inhibition during fetal development would allow activation of the fetal pituitary-adrenal axis, the maturation of fetal organs and the onset of parturition.

[~ 45,000 words in main text]

Abbreviations

ACTH	adrenocorticotrophic hormone, corticotrophin
ANOVA	analysis of variance
AVP	arginine vasopressin
BSA	bovine serum albumin
ChAT	choline acetyltransferase
CRH	corticotrophin-releasing hormone
dH_2O	deionised water
DMEM	Dulbecco's modified Eagle's medium
DNAse	deoxyribonuclease
DPBS	Dulbecco's phosphate buffered saline
EBSS	Earle's balanced salt solution
EDC	N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide
EDTA	ethylenediaminetetraacetic acid
GAD	glutamic acid decarboxylase
HBSS	Hank's balanced salt solution
HPD	hypothalamo-pituitary disconnection
5-HT	Serotonin
ICC	immunocytochemistry
IRMA	immunoradiometric assay
KIU	Kallikrein inactivator units
MSH	melanocyte stimulating hormone
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y
NSE	neurone-specific enolase
NSS	normal swine serum
o	ovine (as in oCRH)
PBS	phosphate-buffered saline
Penstrep	penicillin/streptomycin
PMSG	pregnant mares serum gonadotrophin
POMC	pro-opiomelanocortin
RIA	radioimmunoassay
SARB	biotinylated swine anti-rabbit antibody
T3	triiodothyronine

TFA	trifluoroacetic acid

1. Introduction.

In the United Kingdom, 5-8% of all births occur preterm, and prematurity with immaturity accounts for the majority of perinatal mortality and morbidity in this country. However, the precise mechanisms which underlie the onset of prenatal maturation and the timing of parturition are unknown. The experimental evidence suggests that sequential maturation of fetal endocrine systems, specifically the adrenal axis, plays a significant role in prenatal development. Thus the developing fetal hypothalamus secretes corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP). These factors provoke the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland, which stimulates cortisol secretion from the fetal adrenal (Norman, Lye, Wlodek & Challis, 1985; Challis & Brooks, 1989). Cortisol thus secreted by the fetal adrenal cortex late in gestation enhances neonatal viability by stimulating the maturation of the fetal lung and gut. Further, high concentrations of fetal cortisol observed just before term induce specific biochemical changes in the placenta which cause a decline in progesterone and increase in oestrogen production. This ultimately leads to the enhanced myometrial contractility which culminates in birth. Therefore, increasing hypothalamic input to the fetal pituitary-adrenal axis late in gestation may play a pivotal role in the fetal preparations for extrauterine life.

In pioneering studies to examine the contribution of the fetal hypothalamo-pituitary-adrenal axis to the onset of parturition in the sheep, Liggins and colleagues determined the effects of various endocrine and surgical manipulations in the fetus on the timing of birth (see Liggins, Fairclough, Grieves, Kendall & Knox, 1973, for review). Separation of the fetal hypothalamus from the fetal pituitary by infundibular stalk section lead to prolonged pregnancy, and this was mimicked by electrocoagulation of the fetal pituitary gland (hypophysectomy). By contrast, intrafetal administration of ACTH or cortisol reliably brought about preterm delivery of the fetus within a few days. Recent studies have reported that disconnection of the hypothalamus from the pituitary gland in the fetal sheep prevents the

onset of labour at the normal time (Antolovich, Clarke, McMillen, Perry, Robinson, Silver & Young, 1990). Furthermore, specific ablation of the fetal hypothalamic paraventricular nucleus, which contains the cell bodies of the CRH and AVP neurones, invariably results in a failure of parturition at term (see Palca, 1991). However it is not known whether an increase in hypothalamic drive to ACTH secretion by the fetal pituitary accounts for these maturational processes.

The observations presented above lead to a general hypothesis regarding the function of the fetal sheep hypothalamus during development. It was considered that maturation of the fetal sheep hypothalamus gave rise to changes in hypothalamic secretion of CRH and AVP, and that these changes might underlie the preparurient increase in fetal plasma ACTH and cortisol. The purpose of this thesis was therefore to investigate the regulation of AVP and CRH secretion in the fetal sheep hypothalamus during development. However, it was not possible to measure AVP and CRH secretion directly. Therefore, the first experimental chapter describes the optimisation of the culture conditions required to maintain fetal sheep hypothalamic neurones *in vitro*. Subsequently, the development of fetal hypothalamic CRH and AVP was followed at several gestational ages by measuring CRH and AVP secretion *in vitro* and by assessing hypothalamic CRH and AVP content in fresh tissues. Later, the functional maturation of the fetal hypothalamus was investigated by assessing the responses to cortisol or an opioid peptide at different gestational ages.

The results of the above investigations are presented in chapters 4-8, each of which contains a discussion of the results relevant to that chapter. A synthesis of these results is presented in chapter 9, in which methodological limitations are discussed, along with those areas which warrant further investigation.

2. Review of the Literature.

The studies described in this thesis are centred around an investigation of corticotrophin-releasing factors in the developing fetal sheep hypothalamus. To illustrate the relationship of these studies to the general physiology of the hypothalamo-pituitary-adrenal axis, the following chapter describes the functional development of this axis in the fetal sheep. Moreover, detailed reviews are given of those aspects of hypothalamic biology most relevant to material presented in ensuing chapters of this thesis. Many of the reports concerning the hypothalamic secretion of corticotrophin-releasing factors have described data gathered in adult animals and in species other than the sheep. In order to present a more complete description of hypothalamic function in the fetal sheep, references have been made to these reports where the corresponding studies had not been carried out in the sheep fetus.

This chapter is divided into two general areas. The first part comprises sections 2.1 and 2.2, which contain information relating to the physiology of the hypothalamo-pituitary-adrenal axis in the fetal sheep. Therefore, an overview of the functional maturation of the pituitary-adrenal axis in the sheep fetus is presented in section 2.1, and in section 2.2, the interplay between hypothalamic releasing factors, pituitary ACTH and adrenal steroids during fetal development is discussed. The second part of this review comprises sections 2.4 to 2.5 and examines those areas of hypothalamic function most relevant to specific chapters in this thesis. Therefore, these sections examine factors such as the neuropeptides and adrenal steroids which regulate secretion of corticotrophin-releasing factors from the hypothalamus. These sections are not intended to be exhaustive documents of all research carried out in these fields. Instead, they serve to highlight areas of investigation which are most relevant to the subject matter of this thesis.

2.1 Overview of the Fetal Pituitary-Adrenal axis in development

2.1.1. Historical Perspective.

The significance of the fetal pituitary gland in fetal maturation and in the timing of parturition in the sheep came to be appreciated in the 1960's. Until this time, the question of whether the fetus or the mother originated the signal for parturition had remained a matter for speculation, though a paper by Binns and colleagues in 1960 had highlighted a fortuitous natural experiment implicating the fetus as the origin for this signal (Binns, Anderson & Sullivan, 1960). These researchers observed that certain sheep failed to deliver their fetuses at the normal gestational age. On examination, the unborn lambs were found to bear widespread lesions of the central nervous system and pituitary gland, which had been induced by maternal ingestion of teratogenic shrubs during early pregnancy. Reports appearing in the literature of the time had made parallel observations in guernsey cows, in which the fetuses had genetically-induced dysgenesis of the central nervous system and pituitary gland, and the mothers had greatly extended pregnancies (Kennedy, Kendrick and Stormont, 1957). Therefore, these data provided good evidence that the fetal central nervous system and pituitary were implicated in the initiation of parturition, in contrast to the role of the mother, which was essentially passive in this regard.

The observations described above lead Liggins and co-workers to investigate the role of the fetal hypothalamus and pituitary in parturition in the sheep. These authors removed hypothalamic input to the fetal pituitary gland by performing pituitary stalk section, and showed that fetuses were not delivered at term after this treatment (see Liggins *et al.* 1973, for review). In parallel, electrocoagulation of the fetal sheep pituitary gland significantly delayed parturition in fetuses in which 70% or more of the gland had been destroyed (Liggins, Kennedy & Holm, 1967). These studies did not, however, account for the many active principles contained in the fetal pituitary, and so could not determine which was responsible for normal parturition. Later experiments by

Liggins (1968) determined that administration of growth hormone, luteinising hormone, follicle-stimulating hormone and prolactin to the sheep fetus had no effect on the duration of pregnancy. However, administration of these hormones plus ACTH, or of ACTH alone, reliably gave rise to parturition within a few days. By contrast, concomitant administration of ACTH and metyrapone, the corticosteroid synthesis inhibitor, did not result in premature labour (Liggins, 1968).

The efficacy of intrafetal ACTH to induce premature delivery, and the dependence of this phenomenon on adrenal steroid synthesis, suggested that the fetal pituitary-adrenal axis formed an essential component in the initiation of parturition. In support of this, Drost & Holm (1968) showed that fetal adrenalectomy abolished parturition in the sheep, complementing the observation that intrafetal administration of cortisol, but not oestradiol, precipitated preterm delivery in this species (Liggins, 1968). The physiological significance of fetal adrenal activation was then confirmed in studies showing that fetal adrenal cortisol secretion was increased with advancing gestation in the sheep (Alexander, Britton, James, Nixon, Parker, Wintour & Wright, 1968), and that parturition was associated with dramatic elevations in fetal plasma cortisol levels, which preceded delivery by several days (Basset & Thorburn, 1969). These early studies laid the foundations for the current understanding, which holds that activation of the hypothalamo-pituitary-adrenal axis in the fetal sheep gives rise to parturition (see Challis & Brooks, 1989).

2.1.2. The preparturient activation of the fetal pituitary-adrenal axis.

The basic hypothesis outlined above suggests that pituitary drive to the fetal adrenal is increased with advancing gestation, and that it is this drive which increases the secretion of cortisol from the fetal adrenal. Until recently, however, experimental observations suggested that the progressive increase in fetal plasma cortisol levels which occurs in the last few weeks of gestation was not dependent on fetal plasma ACTH, which remained unchanged during this time. Thus, Rose and colleagues found that plasma ACTH concentrations in the fetal sheep between day 70 and day 140 of gestation showed no developmental trends, though they

clearly demonstrated increasing plasma cortisol levels over the last three weeks of gestation (Rose, MacDonald, Heymann & Rudolph, 1978). Such observations had lead other workers to consider that fetal ACTH was not implicated in the adrenal activation which occurs in the maturing sheep fetus (Rees, Jack, Thomas & Nathanielsz, 1975; Jones, Boddy & Robinson, 1977).

Recent improvements in assay techniques, coupled with more stable chronically instrumented preparations, have permitted a re-examination of fetal ACTH during the preparturient activation of the fetal adrenal axis. These studies have lead to the current appreciation that fetal plasma ACTH levels begin to increase around day 110-120 of gestation, well before fetal cortisol begins to rise at day 125, and that fetal ACTH continues to rise in parallel with cortisol as gestation proceeds towards term (Norman *et al.* 1985). Around day 140 of gestation, fetal plasma ACTH and cortisol undergo a further coincident elevation, as recorded by earlier workers (Rees *et al.* 1975; Jones *et al.* 1977; Rose *et al.* 1978). Ultimately, these endocrine developments stimulate fetal maturation and bring about the marked changes in placental steroid synthesis characteristic of the later stages of pregnancy and the onset of parturition in this species.

2.1.3. Functions of cortisol in the fetal sheep.

Early investigations were concerned with the function of the fetal hypothalamo-pituitary-adrenal axis in parturition. It was clear that fetal adrenal cortisol was important in the mechanisms of parturition, however, these studies did not elucidate how fetal cortisol might act in this regard. Serial plasma samples removed from the mother showed that spontaneous or glucocorticoid-induced labour in the sheep was preceded by a sharp rise in maternal plasma oestrogens and a concomitant fall in plasma progesterone (Bassett, Oxborrow, Smith & Thorburn, 1969; Challis, 1971; Flint, Anderson, Patten & Turnbull, 1974), suggesting that removal of the "progesterone block" on uterine contractility may have been involved (Csapo & Weist, 1969). Fetal placental membranes are the site of progesterone synthesis in the sheep placenta (Anderson, Flint & Turnbull, 1975), and fetal, but not maternal placental tissues collected

after intrafetal administration of glucocorticoids or from term fetuses during labour show much enhanced 17α-hydroxylase (Anderson *et al.* 1975) and C-17, 20 lyase activity (Steele, Flint & Turnbull, 1976). In concert with placental aromatase, these enzymes catalyse the synthesis of oestrone from progesterone (Steele *et al.* 1976). Therefore, glucocorticoids acting in the fetal compartment transform the placenta from a progesterone-synthesising organ to an oestrogen-synthesising organ near term. Previous experiments had shown that oestrogens and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) levels increase in parallel in uterine venous blood, and that oestrogen infusion to fetuses stimulates $PGF_{2\alpha}$ levels in uterine venous blood (Flint *et al.* 1974). Therefore, fetal glucocorticoids ultimately stimulated the release of a potent uterotonic prostaglandin, which is believed to play a major role in the onset of labour and parturition in many species (See Thorburn & Challis, 1979, for review).

In addition to the role of glucocorticoids in the placenta, it has become clear that fetal corticosteroids have equally important actions within the fetus, and are responsible for bringing about fetal organ maturation in late gestation (see Silver, 1990, for review). Liggins (1969) remarked on the precocious maturity of the lungs of fetal lambs infused with dexamethasone *in utero* and hypothesised that this may be due to increased production of pulmonary surfactant. It has since become clear that glucocorticoids act to induce functional differentiation of the type 2 pneumocytes which synthesise surfactant (Wang, Kotas, Avery, & Thurlbeck, 1971), and stimulate the activity of one of the key enzymes in surfactant production, choline phosphotransferase (Farrell & Zachman, 1973). Glucocorticoids may also have potent stimulatory effects on the maturation of other fetal organ systems. For example, cortisol infusion to the hypophysectomised fetal sheep restores hepatic glycogen levels (Barnes, Comline & Silver, 1978), induces the expression of gut disaccharidases, and enhances maturation of the fetal insulin response to intravenous glucose administration (see Liggins, 1976, for review).

Therefore, the current understanding suggests that increasing fetal plasma corticosteroids in late gestation induce fetal maturation, in preparation for extrauterine survival. Subsequently, the further elevated cortisol levels observed in the last few days of pregnancy have a different

function, which is to initiate the placental endocrine mechanisms which bring about parturition.

2.2 Functional development of the fetal hypothalamo-pituitary-adrenal axis.

The previous section has described the important functions of the fetal adrenal gland as a glucocorticoid-synthesising organ. However, the failure of parturition after fetal hypophysectomy clearly demonstrates the importance of pituitary drive in the normal preterm activation of the fetal adrenal gland. Therefore, increasing hypothalamic secretion of ACTH-releasing factors, and hence ACTH secretion from the fetal pituitary gland, are possibly more important factors in the timing of birth than the activity of the adrenal gland alone. The purpose of this section is therefore to examine pituitary ACTH secretion during fetal life, and to illustrate how hypothalamic ACTH-releasing factors might act at the fetal pituitary, to acutely stimulate ACTH release and to activate the pituitary-adrenal axis near term.

2.2.1. ACTH in the developing fetal pituitary.

ACTH is a 39 amino acid peptide which is processed from a large precursor in the corticotrophs of the anterior pituitary gland (Mains, Eipper & Ling, 1977; Phifer, Orth & Spicer, 1977;). Transcription of the pro-opiomelanocortin (POMC) gene gives rise to a high molecular weight peptide, which is cleaved at several processing sites to yield mature ACTH (1-39), in addition to several other bioactive peptides, such as β-lipotrophin and β-endorphin (Nakanishi, Inoue, Kita, Nakamura, Chang, Cohen, & Numa, 1979). Whilst the POMC gene product is synthesised in both the anterior and intermediate lobes of the pituitary gland, only the anterior pituitary contains substantial amounts of ACTH (see Eipper & Mains, 1980, for review).

Recent studies have investigated the maturation of corticotrophs in the fetal sheep pituitary gland. Using four antisera, each specific for a different POMC product, corticotrophs were observed to contain all of

ACTH, pro-γmelanocyte-stimulating hormone (pro-γMSH), γMSH and β-endorphin/β-lipotrophin by day 38 of gestation (Mulvogue, McMillen, Robinson & Perry, 1986). The pituitary glands from more mature fetuses showed the same staining pattern, however, the intensity of the immunostaining was increased, which was suggestive of increased pituitary content of the POMC peptides. In pituitary glands removed at day 38 of gestation, the immunostaining reaction products were located in large columnar cells (fetal corticotrophs). However, between day 87 and 127, small, immunoreactive stellate cells were found in increasing numbers (adult corticotrophs). After day 132 of pregnancy, only the stellate, adult-type corticotrophs were present.

Investigations carried out recently have suggested that maturation of the fetal sheep corticotrophs is under hypothalamic neuroendocrine control. Antolovich and colleagues reported that fetal ACTH-immuno-reactive cells adopt the adult, stellate appearance in normal fetuses between day 120 and day 135 of gestation (Antolovich, Perry, Trahair, Silver & Robinson, 1989). However, the majority of ACTH-immunoreactive cells in day 135 pituitaries show the columnar, fetal-type morphology if surgical disconnection of the hypothalamus and pituitary is carried out at day 108-112 of gestation (Antolovich, McMillen, Robinson, Silver, Young & Perry, 1991). Therefore, aside from ACTH-releasing activities, hypothalamic input to the fetal pituitary is likely to play a significant part in the maturation of the fetal pituitary corticotrophs during fetal life.

Development of adult-type corticotrophs may be associated with developmental changes in the biochemistry of ACTH synthesis. Extracts of anterior pitutiary glands removed from fetal and adult sheep contain several molecular weight species which are immunoreactive in ACTH radioimmunoassay (RIA) procedures (Silman, Holland, Chard, Lowry, Hope, Rees, Thomas & Nathanielsz, 1979). In fetal sheep pituitary glands removed around day 120 of gestation, most ACTH immuno-reactivity was accounted for by high molecular weight (> 20 kD) ACTH molecules, whereas the majority of ACTH-immunoreactivity in the adult pituitary gland co-eluted with synthetic ACTH(1-39) on Sephadex chromatography (Silman *et al.* 1979).

Jones & Roebuck (1980) carried out experiments to examine the bioactivity of the different ACTH peptides present in the fetal sheep pituitary gland. These authors purified the ACTH-immunoreactive substances in fetal pituitaries to yield 50-60 kD, 30 kD, 20 kD and 4.5 kD (ACTH 1-39) molecular weight species. They incubated fetal sheep adrenal cells removed at day 127 of gestation with the high molecular weight ACTH peptides, in the presence and absence of ACTH(1-39). The 30 kD peptide stimulated cortisol production to some degree, however, there was no additive effect with ACTH(1-39). By contrast, the 50-60 kD and 20 kD peptides had little effect alone but substantially inhibited ACTH(1-39)-induced cortisol secretion, suggesting that these substances may act as ACTH antagonists at the fetal adrenal gland *in vivo*.

These studies investigated the ACTH peptides present in pituitary extracts, and so did not reveal which ACTH-like molecules might exist in the circulation to act at the fetal adrenal gland *in vivo*. However, Jones & Roebuck (1980) pointed out that electrophoresis of fetal sheep plasma proteins reveals ACTH immunoreactivity to be present as equal amounts of "big" ACTH (50-60 kD, 30 kD and 20 kD) and small ACTH(1-39) molecules. Interestingly, these authors found that the proportion of big ACTH, relative to ACTH(1-39), was reduced in late pregnancy, and showed that ACTH(1-39) became the principal ACTH-immunoreactive species in fetal plasma in late gestation. These data are in agreement with more recent studies, which have shown that increased ACTH bioactivity, as secreted by fetal sheep pituitary cells, is associated with increasing proportions of low molecular weight ACTH relative to total ACTH immunoreactivity (Brieu & Durand, 1989). Therefore, the fetal sheep pituitary gland may secrete more bioactive ACTH, and less of the high molecular weight ACTH antagonists, and so provide increasing drive to fetal adrenal corticosteriod secretion near term.

2.2.2. ACTH-Releasing Factors, ACTH, and adrenal steroids in fetal life.

The identification of the corticotropin-releasing factor in the ovine hypothalamus by Vale's group in 1981 (Vale, Speiss, Rivier, & Rivier, 1981; Speiss, Rivier, Rivier & Vale, 1981) rapidly established the new corticotropin-releasing hormone (CRH) as a potent stimulator of pituitary

ACTH secretion in several species, including rat (Rivier, Brownstein, Speiss, Rivier, & Vale, 1982), sheep (Donald, Redekopp, Cameron, Nicolls, & Bolton, 1983), and man (Motomatsu, Takahashi, Ibayashi & Nobunaga, 1984). Ironically, the isolation of CRH permitted the identification of Arginine Vasopressin (AVP), long suspected to be an ACTH-releasing factor, as a physiologically significant peptide in the hypothalamic control of ACTH secretion (Linton, Tilders, Hodgkinson, Berkenbosch, Vermes & Lowry, 1985), which exhibited synergy in this regard with CRH (Gillies, Linton, & Lowry, 1982; Giguere & Labrie, 1982). Recent evidence suggests that AVP is a more potent ACTH-releasing factor in the sheep than CRH (Familiari, Smith, Smith & Funder, 1989), however, both factors are implicated in the physiological stimulation of ACTH release in this species (Engler, Pham, Fullerton, Ooi, Funder & Clarke, 1989). In the fetal sheep, it is not known whether hypophyseal portal plasma levels of AVP and CRH are increased during the preparturient activation of the pituitary-adrenal axis. However, administration of either of these peptides to the fetus *in vivo* rapidly stimulates fetal plasma ACTH levels (Norman & Challis, 1987a). Therefore, increasing hypothalamic secretion of CRH and AVP during development may play a significant role in stimulating ACTH secretion in the maturing sheep fetus.

To investigate releasing factor-induced ACTH secretion from the developing fetal sheep pituitary gland, Brieu & Durand (1987) prepared dispersed cell cultures from anterior pituitary glands, removed from fetal sheep at day 63, day 125-138 and day 144 of gestation. Cells were incubated with AVP, CRH, or AVP+CRH, and ACTH release was measured by RIA and adrenal cell bioassay. These studies showed that incubation with AVP or CRH elicited secretion of both immunoreactive and bioactive ACTH, and that the magnitudes of the secretory responses were increased with advancing gestational age. In cultures of rat anterior pituitary cells, AVP and CRH are synergistic in their action to stimulate ACTH secretion (Gillies *et al.* 1982). Similarly, AVP and CRH showed synergy in their actions to stimulate ACTH secretion by fetal sheep pituitary cell cultures, however, the magnitude of the synergistic effect appeared to be greatest in the cells removed at day 63 of gestation (Brieu & Durand, 1987).

Evidence has accumulated to suggest that the degree of synergistic interaction between AVP and CRH in the fetal sheep varies with

gestational age. Plasma ACTH concentrations have been measured in chronically catheterised fetal lambs treated with AVP, CRH, or AVP+CRH at day 110-115, day 125-130 and day 135-140 of gestation (Norman & Challis, 1987a). At day 110-115 of gestation, fetal plasma ACTH levels were elevated for 240 minutes after administration of CRH, whereas the fetal pituitary ACTH response to AVP lasted for 10 mintues, perhaps reflecting the much shorter half-life of AVP in the circulation (Wiriyathian, Porter, Naden & Rosenfeld, 1983; Brooks & Challis, 1988). By contrast, the ACTH response to AVP+CRH was prolonged, and was maintained at a level in excess of the response to CRH alone for the first 60 minutes. As gestation proceded, the ACTH responses to the various treatments were decreased, such that by day 125-130, there was no difference between the overall reponses to CRH and AVP+CRH. A little later in gestation, at day 135-140, the magnitudes of the ACTH responses were further diminished, though the patterns of response to the various treatments remained similar to those observed at day 125-130.

These data suggest that the effects of ACTH-releasing factors are diminished with advancing gestational age. Norman & Challis (1987b) provided evidence to corroborate the studies described above, and convincingly demonstrated the gestational reduction in pituitary ACTH response to ACTH-releasing factors. It is worthy of note that these data contrast with the *in vitro* studies discussed above (Brieu & Durand, 1987), wherein the secretion of bioactive and immunoreactive ACTH in response to CRH or AVP was increased with advancing gestation. In fact, the presence of endogenous corticosteroids, the plasma levels of which are elevated from day 125 onwards in the fetal sheep (Norman *et al.* 1985), is likely to account for the reduced ACTH response to CRH or AVP observed with advancing gestation *in vivo*. Previous studies have shown that cortisol or dexamethasone inhibits basal and AVP- or CRH-stimulated ACTH release from cultured fetal sheep pituitary cells *in vitro* (Durand, Cathiard, Dacheux, Naaman & Saez, 1986). *In vivo*, dexamethasone infusion abolishes the ACTH response to AVP, CRH, or AVP+CRH in the fetal sheep (Norman & Challis, 1987b). Re-examination of the data presented by Norman & Challis (1987a) shows that basal cortisol levels in the experimental animals were elevated at day 135-140, when compared to earlier gestational ages. Hence, the absence of a

pituitary response to ACTH-releasing factors in late gestation may be ascribed to the inhibitory effects of endogenous corticosteroids on ACTH release.

It is most interesting to consider that endogenous corticosteroids act to inhibit release of ACTH from the pituitary gland in response to ACTH-releasing factors. The normal preterm rise in fetal plasma ACTH in the sheep is dependent on hypothalamic input to the fetal pituitary gland (Antolovich *et al.* 1991), and yet this elevation in plasma ACTH occurs in the presence of increasing fetal plasma cortisol levels (Norman *et al.* 1985), which is at odds with an hypothesised inhibitory role for glucocorticoids. To investigate the effects of cortisol on endogenously-stimulated ACTH release from the fetal pituitary gland, Wood & Rudolph (1983) infused fetal sheep with either saline or cortisol at day 117-131 of gestation. Subsequently, they determined the changes in fetal plasma ACTH levels in these fetuses in response to nitroprusside-induced hypotension. The results showed that plasma ACTH levels in saline-pretreated fetuses were greatly elevated by hypotension. However, cortisol pretreatment completely abolished the fetal ACTH response to hypotensive stress. By contrast, a more recent study carried out by Wood (1988) used a similar protocol to reveal that cortisol did not inhibit the hypotension-induced release of ACTH in fetuses between day 132-142 of gestation.

These data are at odds with the findings of Norman and Challis (1987b), who showed that dexamethasone administration abolished fetal ACTH release stimulated by exogenous ACTH-releasing factors between day 110 and day 140 of gestation. However, Wood & Rudolph (1983) and Wood (1988) investigated ACTH release induced by an endogenous neural pathway, mediated by neurones in the fetal hypothalamic paraventricular nucleus (McDonald, Rose, Figueroa, Gluckman & Natanielsz, 1988). Furthermore, they used physiological levels of cortisol as the feedback stimulus, instead of the highly potent synthetic glucocorticoid favoured by Norman and Challis (1987b). Therefore, these data suggest that reductions in the negative feedback efficacy of cortisol may occur in the maturing fetal sheep. These observations may explain the paradoxical coincident rise in fetal plasma ACTH and cortisol which is observed in the fetal sheep in late gestation.

In contrast to the reduction in fetal pituitary responsiveness to ACTH-releasing factors with advancing gestational age, fetal adrenal sensitivity to ACTH increases in late pregnancy (Wintour, Brown, Denton, Hardy, McDougall, Oddie, & Whipp, 1975; Manchester & Challis, 1982). Thus Rose and associates found that fetal plasma levels of cortisol, for a given concentration of ACTH, were significantly greater after day 125 of gestation than before day 125 (Rose *et al.* 1978) and more directly, the increase in fetal plasma cortisol levels in response to a fixed bolus of ACTH becomes greater as fetuses mature (Rose, Meis, Urban & Greiss, 1982).

The mechanism which underlies the gestational increase in fetal adrenal sensitivity to ACTH has received considerable attention, as this pathway could serve to further elevate plasma cortisol levels in the fetal sheep in late gestation, in the absence of increased ACTH concentrations. Experimental evidence has indicated that adrenal cortisol itself may have a priming action on the fetal adrenal, to enhance cortisol secretion in response to ACTH. For example, fetal adrenal cells isolated from fetuses treated with ACTH and metyrapone, an 11β-hydroxylase inhibitor, accumulate less cyclic adenosine monophosphate (cAMP) in response to ACTH than cells from fetuses treated with ACTH alone (Lye & Challis, 1984). However, concomitant administration of glucocorticoids with the ACTH and metyrapone restores the cAMP response to ACTH of the adrenal cells (Challis, Huhtanen, Sprague, Mitchell & Lye, 1985). Similarly, fetal adrenal cells cultured for 48 hours in the presence of glucocorticoids show a greater cAMP response to ACTH than those maintained in control medium alone (Darbeida & Durand, 1987).

In conclusion, the fetal pituitary-adrenal axis undergoes progressive maturational changes during fetal life. ACTH released from the fetal pituitary becomes increasingly bioactive with advancing maturity, and the fetal adrenal gland shows greater sensitivity to pituitary ACTH in late gestation. It is not yet clear how plasma ACTH and corticosteroid concentrations increase in parallel in late gestation, though a reduction in the negative feedback effects of corticosteroids may permit an escape of the axis from feedback control. Most likely, several different mechanisms, including those described here, act in concert to bring about

the increasing activity of the fetal pituitary-adrenal axis during development.

2.2.3. Integrated function of the fetal hypothalamo-pituitary-adrenal axis.

The functional development of the hypothalamo-pituitary-adrenal endocrine system has been investigated in the sheep fetus, primarily by assessing the fetal plasma ACTH and steroid responses to various physiological stressors, which require mature afferent and efferent neural and endocrine pathways. Rose and associates (1978) demonstrated that plasma ACTH was not affected by a haemorrhagic stress in the fetal sheep before day 100 of gestation, but that fetal plasma ACTH was elevated after fetal blood withdrawal in fetuses older than 100 days, suggesting that the central mechanisms governing the pituitary ACTH response did not mature until day 100. By contrast, fetal pituitary ACTH secretion was shown to be powerfully stimulated by an hypovolaemic stress imposed on fetal sheep at day 64-90 of gestation (MacIsaac, Congiu, Levidiotis, McDougall & Wintour, 1989), providing good evidence that the fetal neuroendocrine reflexes were fully competent as early as day 64 of pregnancy.

The functional maturity of the fetal hypothalamo-pituitary-adrenal axis has also been assessed by infusing hypothalamic releasing factors into the sheep fetus, with a view to mimicking a hypothesised hypothalamic input to the fetal pituitary gland. For example, Wintour and colleagues have infused CRH into the the sheep fetus and reported that CRH increasingly stimulates ACTH secretion with advancing gestational age (Wintour, Bell, Fei, Southwell, Tregear & Wang, 1984). Furthermore, continuous infusions of high doses of CRH to fetuses at day 125 of gestation were reported by this group to induce parturition after 5-11 days (Wintour, Bell, Carson, MacIsaac, Tregear, Vale & Wang, 1986).

To study the phenomenon of releasing factor-induced parturition more closely, Brooks & White (1990) and Brooks, Challis & Norman (1987) infused ACTH-releasing factors every four hours for seven days to fetal sheep at day 117-120 of gestation. These authors found that AVP or CRH alone stimulated ACTH and cortisol release from the fetal pituitary and adrenal respectively. However, the ACTH response to CRH and AVP

combined was greater than the response to either peptide alone (Brooks & White, 1990). In fact, only CRH+AVP treatment resulted in elevated basal plasma ACTH and cortisol levels. As expected, the fetal ACTH response to CRH+AVP decreased as basal plasma cortisol levels increased, however, the cortisol response to CRH+AVP was not attenuated by the reduction in plasma ACTH, suggesting that an increase in adrenal sensitivity to ACTH had occurred. Despite the marked increase in pituitary-adrenal activity, as suggested by the increased adrenal weights in all peptide-treated fetuses, parturition was not induced by any of the experimental treatments (Brooks & White, 1990; Brooks *et al.* 1987).

Clearly, the results of Brooks & White (1990) and Brooks *et al.* (1987), as described above, are at odds with the observations of Wintour *et al.* (1984, 1986) who asserted that CRH increasingly stimulated plasma ACTH and that CRH could induce parturition in the fetal sheep. However, there are a number of differences evident between these studies. Re-examination of the data presented by Wintour and colleagues (1984) reveals that administration of CRH increasingly stimulated ACTH secretion in only 3 out of 6 fetuses. Further, in only one animal were plasma samples obtained for more than a few days before the animal was removed from the study, which presumably occurred secondary to fetal or maternal death, suggesting that elevated fetal plasma ACTH levels may not have been due to CRH administration. With regard to the induction of parturition by CRH, none of the experimental animals received a uniform treatment schedule, and so it would be difficult to conclude which particular dosage regime was most efficacious in the induction of parturition (Wintour *et al.* 1986). Perhaps of greater significance, maternal plasma progesterone levels did not fall prior to CRH-induced parturition in this study (Wintour *et al.* 1986). If parturition had occurred as a result of fetal pituitary-adrenal activation, then maternal plasma progesterone levels would undoubtedly have fallen, as explained in section 2.1.3. Therefore, these data suggest that parturition, as induced by CRH administration, was dissimilar to the natural mechanism of delivery in the sheep.

The data provided by Brooks and colleagues (Brooks *et al.* 1987; Brooks & White, 1990), as reviewed above, suggest that increased ACTH-releasing factor secretion by the fetal hypothalamus may not be

responsible for the initiation of parturition, as it was not possible to induce parturition by administering hypothalamic ACTH-releasing factors to fetal sheep. However, recent evidence obtained by Antolovich and associates serves to underline the significance of the fetal hypothalamus in parturition. These authors utilised the technique of fetal hypothalamo-pituitary disconnection (HPD), in which the neural tissue of the median eminence is removed, leaving intact the vascular supply of the anterior pituitary. After HPD had been performed on fetuses at day 110 of gestation, the ewes did not go into labour at the expected time (day 145), and showed no signs of labour when they were sacrificed at day 153 (Antolovich, Clarke, McMillen, Perry, Robinson, Silver & Young, 1990). More recent studies have repeated these findings (Antolovich *et al.* 1991), which provides very good evidence that the fetal hypothalamo-pituitary unit is intimately associated with the mechanisms of parturition.

2.3 Hypothalamic Corticotrophin-Releasing Factors.

The previous sections have illustrated how releasing factors, pituitary ACTH, and adrenal steroids may act as part of the fetal hypothalamo-pituitary-adrenal endocrine system to bring about fetal maturation and parturition in the sheep. ACTH-releasing factors were shown to have stimulatory effects on the fetal pituitary-adrenal system, and could substantially activate the pituitary-adrenal axis prior to term. The importance of hypothalamic drive to the fetal pitutary gland was indicated by the failure of normal endocrine changes prior to term, and the abolition of parturition, in response to fetal hypothalamo-pituitary disconnection. Available evidence, therefore, suggests that stimulatory hypothalamic input is of crucial importance in the preparturient activation of the fetal pituitary-adrenal axis. The purpose of the following sections is to examine ACTH-releasing factors in the hypothalamus, and to see how their secretion is controlled, with a view to understanding how ACTH release may be regulated during fetal life in the sheep.

2.3.1. Co-localisation of CRH and AVP in the hypothalamus.

The isolation of CRH (1-41) from ovine hypothalami (Vale *et al.* 1981) lead rapidly to the development of immunocytochemical methods capable of detecting CRH-containing cells (e.g. Pelletier, Desy, Cote, Lefevre, Vaudry & Labrie, 1982; Olscowka, O'Donohue, Mueller & Jacobowitz, 1982; Bloom, Battenberg, Rivier & Vale, 1982). Around 2000 CRH-immunopositive cells were detected in the hypothalamic paraventricular nucleus (PVN), principally in the medial parvocellular subdivisions which project massively to the median eminence (Wiegrand & Price, 1980; Swanson, Sawchenko, Rivier & Vale, 1983). CRH cells were also detected elsewhere in the CNS, however, in contrast to the cells of the PVN, the CRH-immunoreactivity in these cell groups was not increased by adrenalectomy (Swanson *et al.* 1983), indicating that these neurones were less directly involved in the regulation of the pituitary-adrenal axis.

Vandesande, Dierickx & De Mey (1977) had previously investigated the dense fibre projection from the PVN to the external lamina of the median eminence. In contrast to later work, their study showed that the putative hypophysiotropic fibres originating in the PVN were AVP-immunoreactive. Indeed, adrenalectomy caused a marked increase in AVP immunoreactivity in these fibres (Vandesande *et al.* 1977; Stillman, Recht, Rosario, Seif, Robinson & Zimmerman, 1977), however, these studies could not establish the origins of the AVP fibres, as the immunostaining methods were not sufficiently sensitive to detect cell bodies.

More recently, double-labelling studies have revealed that medial parvocellular CRH cell bodies in the PVN express AVP mRNA and become AVP-immunopositive after adrenalectomy (Kiss, Mezey & Skirboll, 1984; Tramu, Croix & Pillez, 1983; Sawchenko, Swanson & Vale, 1984; Wolfson, Manning, Davis, Arentzen & Baldino, 1985). Therefore, it would seem that there is no anatomical distinction between parvocellular CRH and AVP cells in the PVN. However, these investigations may be criticised on the grounds that they do not show whether AVP and CRH may exist in the same cell under normal conditions.

Electron micrcroscopy, coupled with double-label immunocytochemistry has recently been used to investigate the co-existence of AVP and CRH. Whitnall, Mezey & Gainer (1985) have determined that AVP is present in 50% of CRH neurosecretory vesicles in the external zone of the median eminence in normal rats, providing good evidence for co-release of these ACTH-releasing factors under physiological conditions. Further studies by these researchers have shown that CRH/AVP+ and CRH/AVP- cells have different distributions within the PVN (Whitnall and Gainer, 1988), and that stress selectively activates the AVP-containing subset of CRH-immunopositive cells (Whitnall, 1989). Therefore, the normal activation of the hypothalamo-pituitary-adrenal axis may involve the release of a potent, synergistic mixture of ACTH-releasing factors into the hypophysial portal vasculature.

2.3.2. Functional divisions within the PVN.

In addition to the fibres directed to the external zone of the median eminence, cells located in different areas of the PVN project widely throughout the neuraxis. Specific characteristics, such as cell size, afferent input and immunohistochemical staining have been used to divide the PVN into 3 magnocellular and 5 parvocellular subdivisions (Swanson & Kuypers, 1980; Swanson & Sawchenko, 1980). In conjunction with the supraoptic nucleus, the magnocellular areas contain the cell bodies of the vasopressinergic and oxytocinergic neurones which project to the posterior pituitary gland (Vandesande & Dierickx, 1975; Swanson & Sawchenko, 1983). Fibres originating in the dorsal aspect of medial parvocellular region project heavily to the external lamina of the median eminence (Wiegrand & Price, 1980), and these are the fibres containing CRH and AVP as discussed above. The PVN also projects to the spinal cord (Kuypers & Maisky, 1975), more sepcifically to the intermediolateral column of the cord (Saper, Loewy, Swanson & Cowan, 1976) and to the nucleus of the solitary tract and the dorsal motor nucleus of the vagal complex (Conrad & Pfaff, 1976; Saper *et al.* 1976). Therefore, efferent fibres of the PVN project to sites containing preganglionic neurones of sympathetic and parasympathetic nervous systems. The PVN is thus implicated in the orchestration of both endocrine and autonomic

responses to stress, and so is considered to function as an integrator of humoural and neural reponses to stress (see Swanson & Sawchenko, 1980, for review).

2.3.3. Hypothalamic synthesis of CRH and AVP.

The messenger RNA (mRNA) species which code for the CRH and AVP precursors have been sequenced in several species (Shibahara, Morimoto, Furutani, Notake, Takahashi, Shimizu, Horikawa & Numa, 1983; Furutani, Morimoto, Shibahara, Noda, Takahashi, Hoirose, Asai, Inayama, Hayashida, Miyata & Numa, 1983; Jingami, Mizuno, Takahashi, Shibahara, Furutani, Imura &Numa, 1985; Land, Schutz, Schmale & Richter, 1982; Schmale, Heinsohn & Richter, 1983). The CRH mRNA precursor in the sheep is approximately 1.2 kilobases in length and codes for a polypeptide of 190 amino acids, which contains CRH (1-41) at amino acid residues 148-188 (Furutani *et al.* 1983). Several proteolytic cleavage sites exist within the precursor structure, as indicated by the presence of paired basic amino acids (Arg-Arg or Arg-Lys) at positions 116-117, 144-145 and 146-147. Post translational processing of the nascent polypeptide involves transport to the Golgi apparatus, where it is packaged into secretory vesicles ready for transport to the axon terminal. En route to the median eminence, the peptide precursor is appropriately cleaved and undergoes C-terminal amidation, in order to yield fully bioactive CRH (1-41) (see Gainer, Russell, & Loh, 1985).

Data obtained from the fetal human hypothalamus indicate the presence of a high molecular weight CRH in early gestation (Ackland, Ratter, Bourne & Rees, 1986). This may reflect incomplete post-translational processing during development, as an 9kD peptide containing the CRH sequence would be liberated if cleavage were to occur at the Arg-Arg present at positions 123-124. A similar cleavage site is present in the sheep CRH precursor, at amino acids 116-117. In contrast to the human hypothalamus, available data indicate that CRH (1-41) is the sole immunoreactive CRH species present in the fetal sheep hypothalamus (Brooks, Power, Jones, Yang & Challis, 1989; Watabe, Levidiotis, Oldfield & Wintour, 1991).

Prepro-AVP mRNA is approximately 0.6 kilobases in length (Land *et al.* 1982). The 147 amino-acid polypeptide contains AVP at amino acids 1-9, and the AVP-binding protein, neurophysin-II, at positions 13-107. During transport to the median eminence, the C-terminal amino acids of AVP-Gly^{10}-Lys^{11} are cleaved, and the amino group from the glycine is sacrificed to form a carboxy-terminal amide, which is required for AVP bioactivity (Eipper & Mains, 1988). There have been no reports of purification of AVP immunoreactive species from fetal sheep or rat hypothalami, and so it is not clear whether AVP is present in the mature 1-9 form, or whether during development, AVP-Gly^{10}-Lys^{11} is present in measurable quantities. It is therefore not known whether bioactvity of fetal hypothalamic AVP varies at different fetal ages.

2.3.4. Development of CRH and AVP cells in the fetal sheep PVN.

The stereotaxic atlas of the fetal sheep brain shows the PVN as a paired midline structure, lying either side of the third cerebral ventricle (Gluckman & Parsons, 1983). At its most rostral extent, the PVN lies above and in close proximity to the optic chiasm. The nucleus passes dorsally and caudally over several millimetres, with the major part of the nucleus approaching the dorsal extent of the third ventricle.

To examine the development of hypothalamic neurones in the fetal sheep PVN, Levidiotis, Oldfield & Wintour (1987) used immunocytochemical techniques to visualise CRH and AVP cells in fetal sheep hypothalami removed at different gestational ages. AVP-immmunoreactive terminals were present in the external layer of the fetal median eminence at day 42 of gestation, whereas CRH-containing cells did not arise in the PVN until day 90, with fibres projecting to the median eminence by day 105. These data supported a more significant role for AVP in early gestation, in agreement with previous studies which had shown AVP to be a more potent ACTH-releasing factor than CRH in fetal sheep pituitary cells removed at midgestation (Brieu & Durand, 1987).

In contrast to the immunocytochemical data cited above, CRH was detected by radioimmunoassay (RIA) procedures in the extracts of fetal sheep hypothalami removed at day 63 of pregnancy (Brieu, Tonon, Lutz-

Bucher & Durand, 1989), suggesting the presence of CRH cells in the fetal hypothalamus by midgestation. In a re-appraisal of CRH-immunoreactive cells in the fetal sheep hypothalamus, Watabe *et al.* (1991) used more sensitive immunocytochemical techniques and found CRH-immunopositive cells in the fetal sheep hypothalamus at day 49 of pregnancy. These data do not reveal whether CRH or AVP is the more important ACTH-releasing factor in the fetal sheep hypothalamus. However, they do show that the anatomical and biochemical maturation of the hypothalamic ACTH-releasing cells is complete around day 50 of gestation in the sheep, which is very early in the development of this species. This is in contrast with observations made in the human fetus, in which immunoreactive CRH-immunoreactive cell bodies are detected in the fetal hypothalamus at week 19 (Term = 39 weeks; Bugnon, Fellman, Gouget, Bresson, Clavequin, Hadjiyiassemis & Cardot, 1984).

2.3.5. CRH and AVP immunoreactivity in the fetal sheep hypothalamus.

The fetal hypothalamic contents of CRH and AVP have been measured by RIA at different gestational ages in several species (Rundle & Funder, 1988; Ackland *et al.* 1986;). In the fetal sheep at midgestation, AVP and CRH levels in the hypothalamus are approximately 5% of the concentrations observed near term (Brieu *et al.* 1989). AVP and CRH concentrations rise slowly until day 100, and then increase more rapidly as gestation proceeds towards term (Brieu *et al.* 1989; Brooks *et al.* 1989), perhaps as a result of maturation of trophic neural input to the fetal peptidergic neurones (see Brooks & Challis, 1988). Further to the sharp increases in the hypothalamic contents of CRH and AVP in late gestation, CRH and AVP levels in the fetal sheep hypothalamus fall dramatically in the last few days of pregnancy. This has been attributed to increased glucocorticoid feedback late in gestation (Brooks *et al.* 1989), or to a massive increase in hypothalamic secretion of the two neuropeptides immediately before parturition (Brieu *et al.* 1989). Interestingly, extracts prepared from term fetal hypothalami do not show greatly reduced ACTH-releasing bioactivity (Brieu *et al.* 1989), despite the greatly reduced AVP and CRH content. However, the factors which are responsible for the ACTH-releasing activity present in the hypothalamic extracts have

not yet been determined, and so the significance of these observations is not clear.

The reduction in CRH and AVP immunoreactivity in the fetal sheep hypothalamus near term may reflect hypothalamic maturation, such that immunoreactive, but not bioactive, forms of AVP and CRH are selectively diminished in late development. In the human fetus prior to 20 weeks of gestation, hypothalamic CRH is present as two molecular species, one with a high molecular weight which is not bioactive, and another bioactive peptide which co-elutes with CRH (1-41) on Sephadex chromatography (Ackland *et al.* 1986). In contrast, the high molecular weight CRH species is absent from hypothalami collected after 20 weeks gestation. Immunoreactive CRH in the fetal sheep and rat hypothalamus has been fractionated using sephadex chromatography (Watabe *et al.* 1991; Chatelain, Boudouresque, Chautard, Dupouy & Oliver, 1988). In contrast to the human data, immunoreactive CRH in fetal sheep and rat hypothalami co-eluted with the synthetic CRH(1-41). However, neither of these studies assessed the bioactivity of the chromatography fractions, and so it remains to be determined whether the bioactivity of fetal hypothalamic CRH, or AVP, is enhanced with fetal maturation.

In conclusion, the data presented here have shown that AVP- and CRH-containing cells are present in the fetal sheep hypothalamus by day 50 of development. Whilst immunoreactive CRH and AVP have been detected by RIA in the fetal sheep hypothalamus at day 63 of gestation, it is not clear whether these peptides correspond to the genuine, mature forms of the ACTH-releasing factors. Evidence exists in other species that post-translational processing of ACTH-releasing factors may develop with increasing fetal maturity. This suggests that the biologically active forms may arise relatively late in development in the fetal sheep hypothalamus. Irrespective of the biological activity of hypothalamic CRH and AVP during fetal life, it is clear that fetal sheep hypothalamic contents of CRH and AVP increase dramatically through gestation, suggesting that the preparturient activation of the fetal pituitary-adrenal axis is accompanied by sharply increasing hypothalamic activity.

2.4. Neural control of CRH and AVP secretion.

The interface between the central nervous system and the anterior pituitary gland is formed by the neuroendocrine hypothalamus. Thus, neural signals converge on the hypothalamic peptidergic neurones and are transduced into a neuroendocrine signal, which then acts at the anterior pituitary to stimulate an endocrine response. Therefore, the central nervous system relays signals in the form of neurotransmitters and neuropeptides to the peptidergic cells. Recently, the endogenous opioid peptides have gained support as central regulators of the hypothalamo-pituitary-adrenal axis, in addition to the many other neuropeptides and neurotransmitters, such as neuropeptide Y, noradrenaline and serotonin, which act to stimulate or inhibit CRH and AVP release. The purpose of this section is to discuss the various regulatory substances which act at the AVP and CRH cells to control the activity of the pituitary-adrenal axis.

2.4.1. The effects of opioids on the secretion of ACTH-releasing factors.

The endogenous opioids are a family of peptides, each of which contains the sequence Tyr-Gly-Gly-Phe at the amino terminus (Hughes, Smith, Kosterlitz, Fothergill, Morgan & Morris, 1975; Li & Chung, 1976; Chavkin, James & Goldstein, 1982). The prototypical endogenous opioids are [Met^5]- and [Leu^5]-enkephalin, dynorphin (1-17) and β-endorphin, each of which is derived from a separate gene (Kakidani, Furutani, Takahashi, Noda, Morimoto, Hirose, Asai, Inayama, Nakanishi & Numa, 1982; Noda, Furutani, Takahashi, Toyosato, Hirose, Inayama, Nakanishi & Numa, 1982; Nakanishi *et al.* 1979), and interacts respectively with either delta (∂), kappa (k) or mu (μ) opioid receptors (Patterson, Robson & Kosterlitz, 1983). Compared to other brain regions, the hypothalamus shows remarkably high concentrations of the opioids (Bloom, Rossier, Battenberg, Bayon, French, Henriksen, Siggins, Segal, Browne, Ling & Guillemin, 1978; Miller, Chang & Cuatrecasas, 1978; Zamir, Palkovits & Brownstein, 1984). This is understood to reflect the presence of numerous opioid neurones in this region of the brain (Khachaturian, Lewis, Schafer & Watson, 1985).

Early investigations of the effects of opioids indicated that morphine was stimulatory to the pituitary-adrenal axis (Briggs & Munson 1955), however, this effect was blocked in rats bearing hypothalamic lesions (George & Way, 1959), suggesting, with hindsight, that morphine acts to enhance the secretion of ACTH-releasing factors. More recently, this issue has been addressed directly by measuring the release of CRH or AVP from hypothalamic tissue pieces in the presence of opioid receptor agonists and antagonists.

Tsagarakis and colleagues investigated the effects of 202-250, U50, 488H or [D-Pen^{2,5}]-enkephalin, which are highly specific μ-, k- and ∂-receptor agonists respectively, on the secretion of CRH from rat hypothalamic tissue during short-term incubations (Tsagarakis, Rees, Besser & Grossman, 1990). These authors found no effects of the μ-, k- and ∂-receptor agonists on basal CRH release from the rat hypothalamus. However, CRH release evoked by 28 mM potassium stimulation was inhibited in a dose-dependent fashion by the μ- and κ- agonists, but remained unaffected by the ∂-agonist. These effects were shown to be opioid receptor-mediated when μ- and k-receptor antagonists selectively reversed the inhibitory effects. Previous authors had carried out similar investigations using a perifusion system to maintain rat hypothalami (Yajima, Suda, Tomori, Sumitomo, Nakagami, Ushiyama, Demura & Shizume, 1986). Interestingly, the results from the perifusion studies were in close agreement to those of Tsagarakis *et al.* (1990); the μ- and κ-agonists β-endorphin and dynorphin(1-17) inhibited CRH release, whereas [D-Ala^2, D-Ala^5]-enkephalin, a specific ∂-agonist, had no effect.

The effects of opioids on AVP secretion by the hypothalamus *in vitro* was recently assessed by Knepel & Reimann (1982). Superfused hypothalami secreted AVP under basal conditions, and responded to electrical stimulation with elevated AVP secretion, an effect which was calcium-dependent and tetrodotoxin-sensitive. The inclusion of morphine in the superfusate reduced AVP released in response to stimulation, and this effect was naloxone-reversible, suggesting that morphine inhibition was mediated via opioid receptors. The data also suggested that naloxone enhanced AVP release overall, however, the responses were too variable to reach statistical significance.

The data presented so far are at odds with the early findings of Briggs & Munson (1955), which suggested that hypothalamic drive to pituitary ACTH release was stimulated by opioid agonists. However, recent studies carried out *in vivo* have examined hypothalamic secretion of AVP and CRH into the portal vasculature, and the results from these investigations are in agreement with the inhibitory effects reported above. Thus, Plotsky (1986) showed that portal plasma CRH levels in anaesthetised rats were decreased in response to intravenous administration of β-endorphin or dynorphin(1-17). Similarly, Koenig and colleagues demonstrated that peripheral infusions of morphine to rats resulted in a naloxone-reversible decrease in portal plasma AVP concentrations (Koenig, Metlzer, Devane & Gudelsky, 1986).

The inhibitory actions of the opioids as described here conflict considerably with a number of reports from Buckingham, all of which support the hypothesis that opioids act to enhance the secretion of hypothalamic ACTH-releasing factors. Using an *in vitro* incubation system, Buckingham (1982; 1986) has shown that morphine, [Met^5]- or [Leu^5]-enkephalin and β-endorphin all stimulate the release of bioactive ACTH-releasing factors from the rat hypothalamus *in vitro*. The incubation system was very similar to that used by Tsagarakis *et al.* (1990), and careful scrutiny of the experimental procedures does not reveal a reason as to why the results should differ from Tsagarakis and colleagues. An explanation could be that Buckingham utilised a pituitary fragment bioassay to measure secretion of bioactive ACTH-releasing factors, rather than direct CRH and AVP RIA procedures as employed by other workers (Koenig *et al.* 1986; Yajima *et al.* 1986; Plotsky, 1986; Tsagarakis *et al.* 1990). Therefore, the effects of the opioids on the release of CRH or AVP may be obscured by the opioid-stimulated secretion of other hypothalamic ACTH-releasing factors.

2.4.2. Opioid peptides and pituitary-adrenal activity *in vivo*.

In contrast to the data obtained *in vitro*, a considerable body of evidence suggests that administration of opioids to rats *in vivo* is stimulatory to the hypothalamo-pituitary-adrenal axis. Intraperitoneal infusion of morphine rapidly results in elevation of plasma ACTH levels

(Buckingham, 1982). Similarly, in rats treated with either morphine, a μ-agonist, or U50, 488H, a κ-agonist, plasma corticosterone levels increase dramatically, and peak around 20 minutes after subcutaneous opioid administration (Ignar & Kuhn, 1990). These data are in agreement with a number of previous studies, which have shown that opioid receptor agonists stimulate ACTH and corticosterone release (von Voightlander, Lahti and Ludens, 1983; Hayes and Stewart, 1985; Pfeiffer, Hertz, Loriaux and Pfeiffer, 1985), and that these effects are probably mediated at a central site, as the anterior pituitary gland does not contain opioid receptors (Herkenham, Rice, Jacobson and Rothman, 1986).

Perhaps of greater physiological relevance are the effects of opioid receptor antagonists on the secretion of ACTH and corticosteroids. Administration of low doses of naloxone, the opioid antagonist, to rats has little effect on plasma ACTH levels (Eisenberg, 1980; Siegel, Chowers, Conforti, Feldman and Weidenfeld, 1982; Jezova, Vigas & Jurcovicova, 1982). However, when higher doses of naloxone are administered, plasma ACTH and corticosterone are greatly increased (Eisenberg, 1980; Siegel *et al.* 1982; Jezova *et al.* 1982), suggesting that endogenous, naloxone-resistant opioid pathways, such as ∂- or k- (Patterson *et al.* 1983), are involved in a tonic inhibition of the pituitary-adrenal axis.

In order to investigate whether endogenous opioids were active in regulating hypothalamo-pituitary-adrenal activity under basal and stressed conditions, Cover & Buckingham (1989) pretreated rats with μ-, ∂- or k- receptor selective antagonists and then subjected the animals to laparotomy stress. Whilst the ∂- antagonist had no effect on basal plasma levels of ACTH, the μ- and κ-antagonists stimulated basal ACTH release. The μ-antagonist, however, attenuated the pituitary and adrenal responses to stress, an effect opposite to the κ-antagonist, which enhanced the stress-induced ACTH and corticosterone secretion. By contrast, the ∂-antagonist had no effect on stress-induced activity of the pituitary-adrenal axis (Cover & Buckingham, 1989).

The data presented above suggest a very complex opioid regulation of the hypothalamo-pituitary-adrenal axis. However, taking these findings together, one can hypothesise that there are both stimulatory and inhibitory κ-receptor and μ-receptor pathways, whereas there is less evidence for the role of ∂-opioids. In an effort to resolve the paradoxical

similarities between the effects of opioid agonists and antagonists on the pituitary-adrenal axis, Nikolarakis and associates investigated the effects of opioid administration in rats pretreated with saline or CRH-antiserum (Nikolarakis, Pfeiffer, Stalla & Herz, 1987). These authors showed that the stimulatory effects of the opioid antagonist naloxone and the κ-agonist MR 2034 on ACTH secretion were abolished by CRH antiserum, whereas the stimulatory effects of morphine, the μ-agonist, were still present after antiserum pretreatment. These findings suggested that there was a tonic, inhibitory opioid pathway(s) regulating CRH release, whereas there may have been κ- and μ-mediated stimulatory pathways, in which the secretion of either CRH or another ACTH-releasing factor was stimulated by opioid administration. These data served to emphasise the complexity of opioid regulation of the hypothalamo-pituitary-adrenal axis, and suggest that futher studies should utilise specific RIA procedures, and highly specific opioid agonists and antagonists to clarify the physiological actions of these substances.

2.4.3. Opioids and the fetal sheep hypothalamo-pituitary-adrenal axis.

The pronounced activation of the fetal sheep pituitary-adrenal axis in late gestation may be driven by a stimulatory endogenous opioid input to the fetal hypothalamus. In order to determine whether endogenous opioids could act to elevate plasma ACTH and cortisol in the fetal sheep, [Leu^5]-enkephalin was administered to fetuses at day 120-125 of pregnancy (Bousquet, Lye & Challis, 1984). The results clearly showed a marked stimulation of plasma cortisol levels in the fetus in response to opioid administration. In view of the absence of opioid receptors in the fetal sheep pituitary gland (Yang & Challis, 1991), and the absence of an effect of [Leu^5]-enkephalin on fetal adrenal cortisol secretion (Bousquet *et al.* 1984), these data suggested that [Leu^5]-enkephalin stimulated the release of CRH and AVP into the fetal hypophysial portal blood. Furthermore, these results provided evidence that endogenous opioids might stimulate fetal ACTH and cortisol secretion during the preparturient phase, and so may participate in the initiation of parturition.

To investigate the development of opioid pathways in the fetal hypothalamus, Yang & Challis (1991) determined the binding of tritiated

naloxone to homogenates of fetal sheep hypothalami removed at day 110-115, day 125-130 and day 135-140 of gestation. Opioid receptors at all gestational ages investigated showed similar dissociation constants, suggesting that the same receptor type was present at each gestational age. However, opioid-binding capacity increased two-fold between day 110-115 and day 125-130, providing good evidence that opioid systems in the fetal sheep undergo maturational changes at this point in fetal life.

In order to examine the functional maturation of fetal sheep opioid systems, Brooks & Challis (1988) measured fetal plasma ACTH levels after opioid administration at day 110-115, day 125-130 or day 135-140 of gestation. These authors demonstrated that fetal plasma ACTH levels were stimulated by intrafetal administration of FK 33,824, a μ-selective enkephalin analogue (Roemer, Buescher, Hill, Pless, Cardinaux, Closse, Hauser & Huguenin, 1977), at day 125-130 and day 135-140 of gestation, and that this effect was abolished by naloxone pretreatment. However, there was no effect of the opioid on plasma ACTH concentrations at day 110-115. It is notable that the appearance of the fetal ACTH response to opioid administration coincides with the sharp increase in hypothalamic opioid receptors as described by Yang & Challis (1991).

The functional significance of endogenous opioid systems in the fetal sheep has been much clarified in a very recent study by Brooks & Challis (1991). These authors measured plasma ACTH levels in fetal sheep infused with saline or naloxone in the last few days of pregnancy. They clearly demonstrated that whilst the elevated fetal plasma ACTH levels present at this time were unaffected by saline treatment, chronic naloxone infusion caused a marked reduction in fetal ACTH levels, which provided good evidence that endogenous opioids in the fetal sheep are stimulatory to the fetal hypothalamo-pituitary-adrenal axis near term. Taking the above studies together, the data suggest that there is a maturation of fetal hypothalamic opioid systems during fetal life and that activation of the fetal opioid pathways in late gestation may drive the fetal hypothalamo-pituitary-adrenal axis towards term.

2.4.4. Neuropeptide Y as a regulator of pituitary-adrenal activity.

Neuropeptide Y (NPY), a 36 amino acid peptide, has recently been isolated from mammalian brain using a chemical assay for the the C-terminal amide group (Tatemoto, Carlquist, & Mutt, 1982), which it possesses, in common with a number of biologically active peptides (Eipper & Mains, 1988). Purification of the peptide, and the development of RIA procedures and immunolocalisation techniques, has revealed that NPY is widely distributed throughout the central nervous system (Allen, Adrian, Allen, Tatemoto, Crow, Bloom & Polak, 1983). More specifically, NPY-immunoreactivity is found in brainstem neurones in which NPY is co-localised with catecholamines (Everitt, Hokfelt, Terenius, Tatemoto, Mutt & Goldstein, 1984). There is also a population of NPY-immunoreactive cells in the arcuate nucleus of the hypothalamus which does not contain catecholamines (Bai, Yamano, Shiotani, Emson, Smith, Powell & Tohyama, 1985). Both groups of cells project heavily to the PVN (Bai *et al.* 1985; Sawchenko, Swanson, Grzanna, Howe, Bloom & Polak, 1985), in which NPY-immunoreactive structures innervate CRH-containing cell bodies (Liposits, Sievers & Paull, 1988), and may therefore be involved in the regulation of ACTH-releasing factors.

Recently, data have been obtained which support the function of NPY as a regulator of neuroendocrine input to the anterior pituitary corticotrophs. NPY infusion to the lateral cerebral ventricles of awake rats had a small stimulatory effect on circulating levels of corticosterone, however, direct administration of NPY to the PVN caused a massive increase in plasma ACTH and corticosteroid concentrations (Wahlstedt, Skagerberg, Ekman, Heilig, Sundler & Hakanson, 1987). Investigations of the effects of NPY on CRH release from hypothalami *in vitro* have shown that NPY is stimulatory to CRH release (Tsagarakis, Rees, Besser & Grossman, 1989). These data complement observations made in the sheep, which show that intracerebroventricular administration of NPY is a potent stimulator of plasma cortisol levels (Porter, 1988).

2.4.5. Neurotransmitter regulation of ACTH-releasing factors.

Presently, data are accumulating which describe the functions of the endogenous neuropeptides in the regulation of the hypothalamo-pituitary-axis. However, considerably more data are available regarding neurotransmitter control of this axis, presumably because central neurotransmitter pathways were described over 25 years ago (Dahlstrom & Fuxe, 1964; Anden, Dahlstrom, Fuxe, Larsson, Olson & Ungerstedt, 1966) and neurotransmitter systems have continued to receive attention since then. It has become clear that the parvocellular PVN receives catecholaminergic inputs which originate in the nucleus tractus solitarius (NTS), and in the C1 and C3 cell groups of the brainstem (Cunningham and Sawchenko, 1988; Cunningham, Bohn & Sawchenko, 1989). At the ultrastructural level, CRH- and AVP-immunoreactive neurones in the medial parvocellular subdivision of the PVN have been observed to form synapses with adrenergic and noradrenergic processes (Kitazawa, Shioda & Nakai, 1987; Ochia, Iwai & Nakai, 1988). Electrical stimulation of the brainstem catecholaminergic nuclei results in enhanced electrical activity of the PVN neurones (Day, Ferguson & Renaud, 1985), which suggests that the aminergic fibres form stimulatory junctions with CRH and AVP cells of the PVN.

In support of a stimulatory role for catecholamines, noradrenaline has been shown to elevate the release of CRH and AVP from serum-free cultures of fetal hypothalamic cells (Clarke & Gillies, 1988; Widmaier, Lim & Vale, 1989) and from hypothalamic tissue pieces incubated over the short term *in vitro* (Tsagarakis, Holly, Rees, Besser & Grossman, 1988; Hillhouse & Milton, 1989a). Furthermore, administration of noradrenaline or adrenaline into the third cerebral ventricle of the sheep stimulates ACTH release from the anterior pituitary gland (Liu, Clarke, Funder & Engler, 1991). This study did not reveal the mechanism of action of the catecholamines, however, the hypophysiotropic CRH and AVP neurones lie on either side of the third ventricle (Swanson and Sawchenko, 1983). In view of the small effect of catecholamines on the secretion of ACTH from anterior pituitary cells in culture (Liu *et al.* 1991), the stimulatory effects are probably mediated by the paraventricular CRH- and AVP-containing neurones.

In the fetal sheep, available evidence suggests that central catecholamine pathways are functional relatively early in gestation. Hypotensive stimuli cause massive increases in plasma ACTH in the fetal sheep from midgestation onwards (MacIsaac *et al.* 1989; Hargrave & Rose, 1985). The afferent fibres which relay arterial baroreceptor information pass to the NTS (Sumal, Blessing, Joh, Reis, & Pickel, 1983), which sends catecholaminergic fibres to the PVN (Cunningham &Sawchenko, 1988; Cunningham *et al.* 1989). Ablation of the NTS abolishes the ACTH response to hypotension (Darlington, Shinsako & Dallman, 1986). Therefore, these data suggest that in the fetal sheep, brainstem aminergic inputs to the fetal PVN are functionally mature by mid-gestation.

In addition to the catecholamines, there are proposed roles for other neurotransmitter substances in the central control of CRH and AVP secretion. *In vivo*, 5-HT agonists cause elevations of plasma ACTH and glucocorticoids in rat and man (Koenig, Gudelsky & Meltzer, 1987; Fuller & Snoddy, 1990; Petraglia, Faccinetti, Martignoni, Nappi, Volpe & Genazzani, 1984). Data gathered in the rat indicate that this effect is mediated at a supra-pituitary site, as administration of a CRH-antiserum blocks this response (Calogero, Bagdy, Szemeredi, Tartaglia, Gold & Chioursos, 1990). These data suggest the hypothesis that endogenous 5-HT is stimulatory to the secretion of ACTH-releasing factors. In support of this hypothesis, the 5-HT reuptake inhibitor Fluoxetine increases hypophyseal portal plasma concentrations of CRH and AVP (Gibbs & Vale, 1983). Further, the ACTH reponse to insulin-induced hypoglycaemia is attenuated by 5-HT receptor blockers or prior depletion of brain 5-HT (Yehuda & Meyer, 1984). Furthermore, 5-HT enhances the release of bioactive ACTH-releasing factors from hypothalamic tissue pieces (Jones, Hillhouse & Burden, 1976; Hillhouse & Milton, 1989a), and stimulates CRH release from fetal hypothalamic cell cultures *in vitro* (Clarke, 1988). Thus, considerable evidence favours the stimulatory role of 5-HT in the central regulation CRH and AVP secretion.

In contrast to the facilitatory effects of 5-HT, the inhibitory neurotransmitter γ-Amino Butyric Acid (GABA) is reported to have little effect on the basal release of CRH and AVP, either from cultured hypothalamic cells (Clarke, 1988), or from hypothalamic tissue pieces (Suda, Yajima, Tomori, Sumitomo, Nakagami, Ushiyama, Demura and

Shizume, 1987; Hillhouse & Milton, 1989b). On the other hand, GABA significantly inhibited the acetyl choline (ACh)-stimulated release of ACTH-releasing factors (Buckingham & Hodges, 1979) or CRH or AVP (Suda *et al.* 1987, Hillhouse & Milton, 1989b). Furthermore, the GABA antagonists bicuculline and picrotoxin stimulated the release of both AVP and CRH from cultured hypothalamic cells (Clarke, 1988). *In vivo*, administration of bicuculline and picrotoxin rapidly increases plasma ACTH levels (Ixart, Cryssogelou, Szafarczyk, Malaval & Assenmacher, 1983) and corticosterone concentrations (Makara & Stark, 1974). These data suggest that GABA can inhibit CRH and AVP secretion, and that a tonic inhibitory effect of GABA *in vivo* may act to antagonise stimulatory inputs as described above.

In conclusion, there are a large number of neuropeptide and neurotransmitter systems which act to stimulate and inhibit the function of the pituitary-adrenal axis by modulation of hypothalamic CRH and AVP. The central control of ACTH-releasing factors in the fetal sheep has received relatively litle attention, compared to other animal models, however, available data indicate the presence of a stimulatory opioid input to hypothalamic ACTH-releasing factors and that this input may be tonically active in late gestation.

2.5. Negative Feedback Regulation of ACTH-Releasing Factors.

Stimulatory drive to fetal ACTH secretion is provided by hypothalamic CRH and AVP. However, cortisol secreted by the fetal adrenal gland is able to act at both hypothalamic and pituitary sites to regulate the activity of the axis, in the manner of an endocrine negative feedback loop. In view of the parallel increase in fetal plasma ACTH and cortisol levels which are observed during the preparturient phase of gestation in sheep, it may be that mechanisms exist within the fetal hypothalamo-pituitary-adrenal system which override normal glucocorticoid negative feedback. Therefore, the purpose of this section is to assess the effects of glucocorticoids on hypothalamic CRH and AVP, and to discuss the effects of glucocorticoids on the fetal sheep hypothalamus in development.

2.5.1. Central distribution of glucocorticoid receptors.

It now accepted that glucocorticoids may only act to regulate gene expression, and hence secretion of CRH and AVP, after binding to the glucocorticoid receptor (see Gustaffson, Carlstedt-Duke, Poellinger, Okret, Wikstrom, Bronnegard, Gillner, Dong, Fuxe, Cintra, Harfstrand & Agnati, 1987, for review). To examine whether glucocorticoids could act directly at the level of hypothalamic neurones, Okret and colleagues raised a monoclonal antibody against rat liver glucocorticoid receptors (Okret, Wikstrom, Wrange, Andersson & Gustaffson, 1984). Immunocytochemical studies were then carried out to visualise glucocorticoid receptors in the telencephalon and diencephalon of the rat brain (Fuxe, Wikstrom, Okret, Agnati, Harfstrand, Yu, Granholm, Zoli, Vale & Gustaffson, 1985). The monoclonal antibody detected large numbers of glucocorticoid receptor-containing cells throughout the brain, with the most intensely staining cells located in the hypothalamic PVN, the mediobasal hypothalamus and in the CA1 and CA2 subregions of the hippocampal formation (Fuxe *et al.* 1985).

Glucocorticoid receptor immunoreactivity in the PVN was most concentrated in the medial parvocellular region, where the receptor-immunoreactivity overlapped the distribution of CRH-immunoreactive cells (Swanson *et al.* 1983; Fuxe *et al.* 1985). However, these studies did not show whether the peptidergic neurones themselves expressed glucocorticoid receptor. To address this issue, double-labelling immunocytochemistry was carried out using the monoclonal anti-glucocorticoid receptor antibodies and conventional anti-CRH antiserum. Again, intense glucocorticoid receptor immunostaining was detected in the hypothalmamic PVN. In addition, cells of the medial parvocellular PVN, which expressed glucocorticoid receptor immunoreactivity, were intensely immunostained with the anti-CRH antiserum (Gustaffson *et al.* 1987). These data clearly showed that circulating adrenal corticosteroids may act directly at the level of the paraventricular neurones to regulate stimulatory drive to anterior pituitary ACTH release.

2.5.2. Glucocorticoid regulation of CRH and AVP synthesis.

Primary evidence for the inhibitory effects of glucocorticoids on peptide synthesis has been provided in studies which sought to establish the co-localisation of CRH and AVP in hypothalamic cells. Several workers reported that hypothalamic AVP immunostaining was much enhanced in adrenalectomised animals (Vandesande *et al.* 1977; Stillman *et al.* 1977), and that the effects were specific to the parvocellular regions of the PVN (e.g. Tramu *et al.* 1983; Kiss *et al.* 1984). Furthermore, adrenalectomy permitted the co-localisation of AVP mRNA and CRH peptide within the same hypothalamic neurones (Wolfson *et al.* 1985). Therefore, the hypothalamic cells which control the pituitary-adrenal axis are disinhibited by diminished glucocorticoids, in keeping with the hypothesis of glucocorticoid negative feedback regulation.

More recent investigations have directly assessed the effects of glucocorticoid treatment on the expression of mRNA species coding for CRH or AVP. Using quantitative *in situ* hybridisation techniques, Davis and colleagues investigated the effects of adrenalectomy on the hypothalamic levels of AVP mRNA (Davis, Arentzen, Reid, Manning, Wolfson, Lawrence & Baldino, 1986). These workers clearly showed that removal of endogenous corticosteroids was associated with a doubling of AVP mRNA signal intensity in the hypothalamus. However, this effect was only observed in the parvocellular regions of the PVN, and was not present in adrenalectomised animals treated with dexamethasone.

Similar studies have been carried out to examine the responses of hypothalamic CRH mRNA to glucocorticoid removal. Jingami and co-workers showed that adrenalectomy induced an increase in hypothalamic CRH mRNA levels, which could be reversed by glucocorticoid administration (Jingami, Matsukura, Numa & Imura, 1985). Interestingly, these authors found that extrahypothalamic CRH mRNA levels were unaffected by either adrenalectomy or by glucocorticoid administration (Jingami *et al.* 1985). Further studies of CRH mRNA regulation in the rat central nervous system have confirmed the precise nature of the anatomical locus at which glucocorticoids act to inhibit CRH mRNA. Thus Beyer, Matta & Sharp (1988) showed that levels of CRH mRNA in the parvocellular PVN, but not in the bed nucleus of the stria

terminalis or the central nucleus of the amygdala, were decreased by glucocorticoid administration. Kovacs & Mezey (1987) utilised implants of dexamethasone to show that glucocorticoids act within the hypothalamus, but not in the hippocampus, cerebral cortex or amygdala, to reduce CRH mRNA levels.

Complementary investigations have been carried out to assess the effects of glucocorticoids on the peptide content of the hypothalamus under conditions of altered glucocorticoid negative feedback. Plotsky & Sawchenko (1987) found that after pharmacological adrenalectomy, in which adrenal corticosteroid synthesis was prevented by administration of metyrapone + aminoglutethimide, hypothalamic levels of CRH and AVP were initially decreased at 24 hours post treatment, and were then increased 72 hours later. These data were taken to mean that there was an initial outpouring of AVP and CRH, which accounted for the diminished peptide content in the first 24 hour period, followed by an adrenalectomy-induced elevation in hypothalamic peptide synthesis and storage. The reports of several authors indicate that the hypothalamic contents of CRH and AVP are greatly enhanced by adrenalectomy, as CRH and AVP immunostaining in the rat PVN are much more intense several days after removal of endogenous adrenal steroids (Swanson *et al.* 1983; Plotsky & Sawchenko, 1987). These data provide good evidence that physiological levels of glucocorticoids may exert a potent inhibitory influence on the synthesis of the hypothalamic factors which stimulate ACTH release.

2.5.3. Negative feedback regulation of CRH and AVP secretion.

In support of a direct inhibitory effect of glucocorticoids on the secretion of CRH and AVP, chronic glucocorticoid treatment has been shown to reduce CRH and AVP output from cultured rat hypothalamic cells *in vitro* (Clarke & Gillies, 1988). By contrast, the levels of AVP in hypophysial portal blood are rapidly elevated by adrenalectomy (Koenig *et al.* 1986; Eckland, Todd & Lightman, 1987), an effect which is paralleled by the increased portal levels of CRH observed under these conditions (Plotsky & Sawchenko, 1987). Furthermore, hypothalami removed from adrenalectomised animals secrete increased quantities of CRH and AVP

in vitro (Suda, Yajima, Tomori, Demura, & Shizume, 1985; Knepel, Nutto, Meyer & Vlaskovska, 1984). These changes in hypophysial portal ACTH-releasing factors may well underlie the massive elevation in peripheral plasma ACTH concentrations observed in adrenalectomised animals (Plotsky & Sawchenko, 1987), as surgical ablation of PVN projections to the median eminence abolishes the post-adrenalectomy increase in plasma ACTH (Levin, Shinsako & Dallman, 1988).

2.5.4. Glucocorticoid feedback regulation in the fetal sheep hypothalamus.

Glucocorticoid negative feedback in the hypothalamo-pituitary system of the fetal sheep is apparently diminished in late gestation, as plasma ACTH levels escalate in parallel with circulating cortisol concentrations towards term (Norman *et al.* 1985). However, it is not clear whether such a hypothetical reduction in glucocorticoid feedback occurs at a hypothalamic site, as data are not available which describe hypothalamic release of CRH and AVP in the fetal sheep *in vivo*. To determine how the efficacy of glucocorticoid negative feedback at the fetal hypothalamus might vary during development, Yang, Jones & Challis (1990) examined glucocorticoid receptor binding in the fetal sheep hypothalamus at different gestational ages. These authors found that between day 60 of gestation, the earliest gestational age studied, and term, there were no appreciable changes in the binding characterisitics of the fetal hypothalamic glucocorticoid receptor. In contrast, the glucocorticoid binding capacity of the fetal hypothalamus varied markedly with fetal development. Binding was dramatically elevated at day 100-110 of gestation when compared to other gestational ages. These observations suggested that there were maturational changes occurring in the expression of glucocoricoid receptors in the fetal sheep hypothalamus, and that this might be associated with an increase in the negative feedback effects of glucocorticoids around day 100-110.

To examine the developing negative feedback effects of glucocorticoids on CRH secretion by the fetal sheep hypothalamus, Brooks and co-workers (1989) measured CRH release from perifused fetal hypothalamic pieces removed at day 100 and day 140 of gestation. These workers found that the spontaneous release of CRH from day 140

hypothalami was greater than from day 100 tissues, suggestive of an increasing hypothalamic drive to ACTH release with advancing gestational age. In addition, they found that dexamethasone inhibited basal CRH release from hypothalami removed at day 140 of gestation, but had no effect on release from day 100 tissues. These data are at odds with the binding studies carried out by Yang and colleagues (1990), which showed very high levels of hypothalamic glucocorticoid receptors at day 100-110 of gestation. However, there are no studies which have determined the functionality of the hypothalamic glucocorticoid receptor in the developing fetal sheep. In a number of studies in which the effects of glucocorticoids on the fetal sheep hypothalamus have been assessed, the data are clearly supportive of a negative feedback effect of glucocorticoids. Thus, intrahypothalamic inplants of dexamethasone at day 108-111 of gestation cause marked reductions in AVP and CRH immunostaining in the fetal median eminence, and abolish the fetal ACTH response to stressors such as hypotension and hypoxaemia (McDonald, Hoffman, Myers & Nathanielsz, 1990). By contrast, adrenalectomy in the fetal sheep at day 118-121 of gestation gives rise to greatly elevated CRH mRNA levels in the fetal PVN (Myers, Ding & Nathanielsz, 1991). Therefore, the available evidence suggests that endogenous glucocorticoids are inhibitory to hypothalamic ACTH-releasing factors, though it is still not clear whether the efficacy of glucocorticoid feedback varies during fetal life.

2.5.5. Extrahypothalamic glucocorticoid negative feedback.

The original studies which described the distribution of glucocorticoid receptors in the CNS showed that receptors were present in several cell groups within the hypothalamus and in several brainstem nuclei (Fuxe *et al.* 1985). In addition to the direct inhibitory effects of glucocorticoids on the paraventricular CRH and AVP neurones, corticosteroid negative feedback on the hypothalamo-pituitary-adrenal axis may therefore include a component mediated by the cells which project to the PVN.

Noradrenergic, adrenergic and serotoninergic projections from the brainstem to the paraventricular cells of the PVN originate in the A2, C1-C3 and raphe nuclei respectively (Cunningham & Sawchenko, 1988;

Cunningham *et al.* 1989; Sawchenko, Swanson, Steinbusch & Verhofstad, 1983). These neurones may be differentiated in immunocytochemical procedures by their contents of specific enzymes or neurotransmitters. For example, noradrenergic fibres contain tyrosine hydroxylase (TH), but not phenylethanolamine-N-methyltransferase (PNMT), which is present in adrenergic cells, whereas serotonin (5-HT) may be visualised directly by conventional immunocytochemistry. A recent study by Harfstrand and colleagues investigated the presence of glucocorticoid receptor immunoreactivity in the brainstem nuclei, and further assessed whether TH, PNMT or 5-HT were co-localised in these cells. The vast majority of brainstem cells which contained TH, PNMT or 5-HT showed pronounced glucocorticoid receptor immunoreactivity (Harfstrand, Fuxe, Cintra, Agnati, Zini, Wikstrom, Okret, Yu, Goldstein, Steinbusch, Verhofstad & Gustaffson, 1986). Presently, it is not known whether the fetal sheep brainstem neurones which project to the PVN contain glucocorticoid receptors. However, glucocorticoid modulation of the input to the CRH and AVP-containing cells may be of significance in a global activation of the fetal hypothalamo-pituitary-adrenal system near term.

In addition to the brainstem neurones discussed above, the hippocampus may also be a major site for feedback regulation of the hypothalamo-pituitary-adrenal axis (see Jacobson & Sapolsky, 1991, for review). In the rat there is good evidence that the hippocampus mediates inhibitory actions of glucocorticoids, for example, there is a marked enhancement of portal CRH and AVP levels in animals bearing experimental fornix transection, which destroys the projections of the hippocampus to the hypothalamus (Sapolsky, Armanini, Sutton & Plotsky, 1989). Furthermore, portal plasma levels of AVP and CRH are inversley related to the occupancy of hippocampal glucocorticoid receptors (Sapolsky, Armanini, Packan, Sutton & Plotsky, 1990).

2.6 Aims of this thesis.

This review has discussed the regulation of the pituitary-adrenal axis during fetal life in the sheep, and has illustrated the various issues with information drawn from studies carried out in other animals. Based on the information obtained from these reports, it was decided to embark upon an investigation of the factors which might regulate the activity of the fetal sheep pituitary-adrenal axis during fetal life. Of specific interest were hypothalamic CRH and AVP which may control pituitary drive to the fetal adrenal gland. These factors are therefore implicated in the mechanisms which bring about fetal maturation in late gestation, and may have a pivotal role in the initiation of parturition. However, it is not possible to measure directly the hypothalamic secretion of AVP and CRH into the fetal portal vasculature. Therefore, the aims of this thesis were to develop a fetal sheep hypothalamic culture system (chapter 4), and to investigate the secretion of CRH and AVP at different gestational ages (chapter 5). Subsequently, the results of these studies were to be compared to the levels of immunoreactive and bioactive ACTH-releasing factors present in the intact hypothalamus (chapter 6). Latterly, the effects of glucocorticoids (chapter 7) and opioids (chapter 8) on the secretion of ACTH-releasing factors was to be assessed at varying gestational ages.

3. General Materials and Methods

This chapter describes the techniques common to a number of studies in this thesis. Other methodologies unique to specific experiments are elaborated upon in the relevant chapters.

3.1 Animals and Husbandry

3.1.1. Sheep Management.

The animals used for the studies presented in this thesis were mature ewes of mixed breed, obtained from the Macauley Land Use Research Institute at Sourhope Research Farm, Roxburghshire. Animals were kept indoors at the University of Edinburgh's Marshall Building, Roslin, Midlothian in groups of 10 in large pens (3m x 6m). Sheep had constant access to hay and water, and were fed a dietary supplement (500g Moredun Nuts, Dalgety Agriculture, Bristol,) each day.

3.1.2. Timed Mating.

Mature ewes were implanted intravaginally with sponges containing 60 mg medroxyprogesterone acetate ("Veramix Sheep Sponge", Upjohn Limited, Crawley, Sussex). After 13 days the sponges were withdrawn and 48 hours later the ewes were penned with proven rams. The ewes were removed after a further 24 hours, and these animals were considered to have a single date of insemination with day 1 of pregnancy taken as the date upon which the rams were introduced.

During seasonal anoestrus, ovulation was induced by treating animals with progesterone sponges as previously described, in conjunction with 300 I.U. pregnant mare serum gonadotrophin (PMSG, Intervet U.K. Ltd., Milton Road, Cambridge) administered at the time of

sponge withdrawal. Subsequently, the ewes were mated in the same way as before. Pregnant animals were returned to a separate pen where they were kept until required for tissue collection at the appropriate stage of gestation.

3.2 Materials for Cell Culture

3.2.1. Culture Media and Reagents.

Basic Culture Media. Dulbecco's Modified Eagle's Medium (with phenol red and without l-glutamine), Dulbecco's Phosphate Buffered Saline, Ham's F12 Medium (with phenol red and without l-glutamine), Earle's Balanced Salt solution (without phenol red) and Hank's Balanced Salt Solution (with calcium and magnesium and without calcium and magnesium) were purchased from ICN Flow Ltd. (Rickmansworth, Hertfordshire).

Culture Grade Biochemicals and Enzymes. Progesterone, oestradiol 17-β, sodium selenite, transferrin, putrescine, gentamycin, insulin, triiodothyronine (T3), glucose, bovine serum albumin (BSA; Fraction V), poly-l-lysine hydrobromide (MWt. 70,000 - 150,000), double processed tissue culture water, HEPES (1M stock) and deoxyribonuclease Type 1 (DNAse) were purchased from Sigma Chemical Co. Ltd., Poole, Dorset. penicillin/streptomycin mixture ("Penstrep", 5000 I.U./ml and 5000 µg/ml respectively) and glutamine were obtained from ICN Flow Ltd.. Dispase (cell culture grade Neutral Protease) was obtained from BCL Ltd, London U.K..

Miscellaneous Reagents. Glacial acetic acid and sodium hydroxide were obtained from BDH Ltd., Thornliebank, Glasgow.

3.2.2. Glassware and Plastics.

Supplies. Pasteur pipettes were obtained from John Poulten Ltd., Barking, Essex and glass storage bottles (Schott glass) came from BDH.

Gamma irradiated Nunc 6-well multidishes were obtained from Gibco Ltd., Paisley, Scotland. Aseptically prepared 7 ml bijou tubes and 30 ml universal tubes, sterile 60 ml and 150 ml plastic containers, Corning 500ml filter sterilisation units (0.2 micron) and sterile medium storage bottles were obtained from Mackay and Lynn Ltd., Edinburgh. Disposable, sterile plastic pipettes, syringes and needles were obtained from Becton Dickinson U.K. Ltd., Cowley, Oxford, and small 0.2 micron filters were obtained from Sartorius, Epsom, Surrey.

Glassware Treatment. In order to remove surface contamination from medium storage bottles or coverslips upon which cells were plated, such glassware was chemically cleaned before use. Articles were steeped overnight in 2% "Micro" detergent (International Products Ltd, Chislehurst, Kent). Next morning, they were rinsed and immersed in sterile water (Sigma) for several hours. Glassware was then washed in 1M hydrochloric acid (BDH), rinsed, and steeped in sterile water as before. Subsequently, several rinses of sterile water were applied, followed by a rinse in absolute ethanol (Hayman Ltd., Witham, Essex) and further rinses in sterile water. Finally, articles were autoclaved (2 atmospheres pressure for 30 minutes at 121°C) and stored until ready for use.

All glassware which was to come into contact with cell suspensions was siliconised before use, to prevent cell adhesion. Articles were briefly immersed in "Sigmacote" (Sigma) and then left to dry in a fume cupboard. Subsequently, these articles were rinsed once in ethanol and several times in sterile water before autoclave sterilisation.

Siliconised pasteur pipettes of varying diameters were required during the tissue dispersions. Pipettes were heated gently in a bunsen flame until the tips were red hot ("fire polished"). Varying the amount of heating produced pipettes of differing tip diameter.

3.2.3. Stock Solutions for Dispersion and Culture Media.

The media used for cell culture were made from stock solutions (stored at 4°C) and frozen concentrates (-20°C), prepared using a class 2 microbiological safety cabinet (Medical Air Technology, Denton, Manchester) to prevent microbial contamination. Whilst a few

concentrated reagents could be bought from commercial suppliers, most stock solutions were made up in the laboratory, as described in this section.

20% BSA. 10 g BSA were added to 50 ml pure water and the BSA was dissolved over several hours with gentle shaking. The solution was then filter-sterilised and aliquots of 1 ml were stored frozen in autoclaved 1.5 ml eppendorf tubes (BDH).

Penstrep. 100 ml penicillin/streptomycin mixture was thawed, 5 ml aliquots were placed in sterile bijou tubes and these were stored at -20°C.

Insulin. 20 mg of insulin were dissolved in sterile acetic acid solution (10% v/v) to yield an insulin concentration of 10 mg/ml. This was diluted to 5 mg/ml with sterile water and the solution was filter-sterilised and stored at 4°C for up to 1 month.

Triiodo-Thyronine (T_3). 5 mg T_3 were dissolved in 0.1M sterile sodium hydroxide solution resulting in a final T_3 concentration of 0.651 mg/ml ($1x10^{-3}$ M). This mixture was further diluted to a concentration of $1x10^{-6}$ M and was filter-sterilised and stored in a sterile universal at 4°C for up to 1 week.

Combined Supplement. 20 ml glutamine solution (29.2 mg/ml, 0.2 M) and 20 ml transferrin solution (10 mg/ml) were mixed together in a plastic container. 2 ml putrescine solution (16.11 mg/ml, 0.1M) and 20µl sodium selenite (0.52 mg/ml, $3x10^{-3}$M) were added to the above and the mixture was filter sterilised. Aliquots of 10.5ml were stored frozen for up to a month.

Oestradiol 17-ß. 3.67 ml sterile, absolute ethanol were added to 1 mg powdered hormone (supplied in a sterile 50 ml serum vial), giving a solution of $1x10^{-3}$M. This was diluted to a concentration of $1x10^{-6}$M, and 50µl of this solution was added to 50 ml Earle's Balanced Salt Solution (EBSS), resulting in a final concentration of $1x10^{-9}$M. This solution was stored in a tightly stoppered, autoclaved glass bottle at 4°C and was

replaced approximately every 3 months.

Progesterone. 1.59 ml sterile, absolute ethanol were added to 1 mg hormone powder in a sterile 50 ml serum vial, resulting in a concentration of $2x10^{-3}M$. 500µl of this solution were added to 50 ml EBSS, giving a stock concentration of $2x10^{-5}M$. The final solution was stored under the same conditions as oestradiol and replaced at the same time.

3.2.4. Preparation of Media for Tissue Dispersion.

Media were prepared one day in advance, filter-sterilised, then stored overnight at 4°C. The following morning, they were warmed to 37°C and the appropriate enzymes were added to the media as described below.

Collection Buffer. 2 ml of 1M HEPES, 2 ml Penstrep and 0.5 ml 20% BSA were added to 100 ml Hank's Balanced Salt Solution (HBSS; without calcium and magnesium).

Dispersion Medium. This buffer was made in the same way as collection buffer, however, 2 ml 20% BSA were added per 100 ml solution, instead of 0.5 ml, and this medium was supplemented with 20 mg Dispase and 25 mg DNAse per 100 ml before use.

DNAse Medium. Again, this solution was made up according to the formulation for collection buffer, however, the base was HBSS plus calcium and magnesium, and 50 mg DNAse were added per 100 ml solution before use.

3.2.5. Preparation of Serum-Free Medium for Cell Culture.

500 ml basic medium was made, containing 250 ml Ham's F12 medium (F12) and 250 ml Dulbecco's Modified Eagles Medium (DMEM). To this were added 500 µl triiodo-thyronine stock solution ($1x10^{-6}M$), 500 µl insulin solution (5 mg/ml), 500 µl oestradiol 17-ß ($1x10^{-9}M$), 500 µl progesterone stock ($2x10^{-5}M$), 500 µl gentamycin (50 mg/ml), 10 ml

penstrep concentrate and an aliquot of combined supplement (10.5 ml). The serum-free medium was then filter-sterilised and stored for up to a week at 4°C, and aliquots were withdrawn and heated to 37°C before use. Table 3.1 shows the composition of the serum-free culture medium.

3.3 Preparation of Fetal Sheep Hypothalamic Cell Cultures

3.3.1. Coating of Culture Dishes.

On the morning of the tissue dispersion, an aliquot of poly-l-lysine solution (Sigma, MWt. 70,000-150,000 kD; 10 mg polypeptide per ml sterile water, filter sterilised) was thawed and a 1000-fold dilution (10µg/ml) was prepared in sterile, culture grade water. 1 ml of this solution was placed in each 35 mm culture dish for 30 minutes, after which it was aspirated and the wells were washed twice with 1 ml sterile water. Subsequently, 1 ml of culture medium containing 20% fetal calf serum (ICN Flow Ltd.) was placed in each well. Finally, the plates were returned to a humidified incubator (Scotlab, Bellshill, Scotland) at 37°C, in an atmosphere of 95% air : 5% CO_2, until required approximately four hours later.

3.3.2. Euthanasia.

20 ml Euthesate (Pentobarbitone sodium, 200 mg/ml, Willows Francis Vetetrinary, A.H. Robbins Co. Ltd., Horsham, West Sussex) were administered as an intravenous bolus to pregnant ewes. After maternal death, the fetuses were rapidly delivered via abdominal and uterine incisions and were euthanised with intracardiac administration of 1 - 2ml Euthesate. The fetuses were weighed and transferred to a sterile surface for gross dissection of the brain.

3.3.3. Dissection.

The fetal brain was exposed by cutting around the skull at the level of the supraorbital ridges using either stout scissors or bone shears, depending on gestational age. The head was inverted and the brain was removed by

Table 3.1. Composition of the Serum-Free Culture Medium

Component	Final Concentration
Ham's F12/DMEM	50% : 50%
Penicillin/Streptomycin	100 I.U./ml, 100 µg/ml
Gentamicin	50 µg/ml
Glutamine	2 mmol/l
Insulin	5 µg/ml
Transferrin	100 µg/ml
Putrescine	100 µmol/l
Selenium	30 nmol/l
Triiodo-Thyronine	1 nmol/l
Progesterone	20 nmol/l
Oestradiol 17ß	1 pmol/l

blunt dissection.

The brain was placed ventral surface uppermost on an ice cold, sterile glass dish and irrigated with cold, sterile Hank's Balanced Salt Solution (HBSS, ICN Flow Ltd.). With reference to the stereotaxic atlas prepared for the fetal sheep (Gluckman & Parsons, 1983), the rostral edge of the mammillary bodies, the lateral hypothalamic sulci, the rostral edge of the optic chiasm and the most dorsal extent of the third ventricle were used as the landmarks defining the hypothalamus. Using sterile instruments, this block was dissected out from the rest of the brain tissue and placed in approximately 50ml sterile collection buffer on ice.* All tissue was collected within one hour of the start of dissection and was rapidly transported to the MRC Reproductive Biology Unit in chilled buffer for dispersion.

3.3.4. Tissue Dispersion.

The manipulations from this stage onwards were carried out in the class 2 microbiological safety cabinet to minimise the risks of airborne contamination.

Washing. The hypothalami were removed from the collection buffer and finely chopped in a sterile petri dish using a sterile scalpel blade. The tissue pieces were resuspended in 20 ml fresh, warmed (37°C) collection buffer in a universal container, which was rotated end over end to wash the tissue. The suspension was centrifuged (30 g, 1 minute) and the supernatant was aspirated. Fresh collection buffer (20 ml) was added and this process was repeated 3 times.

Stage 1. The tissue pieces were suspended in 40 ml warmed dispersion medium in a 50 ml Bellco stirrer flask (Arnold Horwell Ltd., West Hampstead, London) which was placed on a magnetic stirrer (Arnold Horwell Ltd.). This assembly was located in a conventional laboratory incubator (Gallenkamp Economy Incubator; Fisons Scientific Equipment, Loughborough, Leicestershire), and the suspension was stirred at 75 rpm for 30 minutes at 37° C. Solid pieces of tissue were allowed to settle and the supernatant was aspirated, placed in a sterile pot, and returned to the

* Average hypothalamic weights were 241 +/- 50 mg, 330 +/- 35 mg, and 462 +/- 58 mg at day 70, 100 and day 130 gestation respectively (mean +/- S.E., n = 5-6).

incubator for short term storage (Harvest 1).

Stage 2. A little fresh dispersion medium was added to the tissue pieces left over from Stage 1. These were triturated using two sterile, siliconised pasteur pipettes of decreasing diameter (fire polished- approximately 1 mm and 0.5 mm). The cell suspension was then made up to roughly 40 ml with dispersion medium, and the digestion process was repeated as described above.

The supernatant was then aspirated and stored in the incubator (Harvest 2). Remaining pieces of hypothalami were further triturated (pipettes of 0.5 mm and 0.2 mm) and the resultant suspension was added to Harvest 2.

Stage 3. Harvests 1 and 2 were centrifuged for 10 minutes at 200g. The pellets were resuspended and combined in 40 ml DNAse medium in a sterile plastic pot. The cell suspension was then returned to the incubator for 30 minutes with occasional end over end rotation. The cells were then centrifuged at 200g for 10 minutes.

The cell pellet was resuspended in 5 ml collection buffer. In order to remove debris, the suspension was then layered on top of 20 ml collection buffer containing 4 % BSA, then centrifuged at 200g for 10 minutes.

Plating Out. The pelleted cells were resuspended in a little culture medium containing 20% fetal calf serum. The total cell number and viability were assessed using a haemocytometer and trypan blue exclusion. The suspension was diluted to 2.5×10^6 cells/ml and 1 ml of suspension was added to each culture dish. Approximately 6 to 10 million cells were obtained from a single hypothalamus, according to gestational age, and the average viability was 96.75 +/- 0.53%, (Mean +/- S.E.M., n= 16).

After 2 days in culture, the medium was changed to serum-free culture medium alone and was thereafter replaced every 3 days, or more often as appropriate.

3.4 Experimental Procedures

Experiments carried out on cell cultures utilised incubation media based on Earle's Balanced Salt Solution (EBSS; ICN Flow Ltd.). EBSS was supplemented with antibiotics and protease inhibitors for use in peptide release experiments as described below.

3.4.1. Preparation of Media and Supplements.

Basal Medium. Supplemented EBSS was made by adding 1ml fresh, sterile, ascorbic acid solution (Sigma; 3 mg/ml in culture grade water, filter-sterilised), 1 ml sterile bacitracin solution (Sigma; 3 mg/ml, prepared as for ascorbic acid), 1 ml sterile HEPES (1 M stock), 800 µl Aprotinin solution ("Trasylol", Bayer U.K. Ltd., Newbury, 10,000 K.I.U./ml), a-D glucose (Sigma), up to a final concentration of 2 mg/ml, and 500µl sterile 20 % BSA solution to each 100ml of EBSS. The resultant solution was filter-sterilised and stored at 4°C for up to 1 week. The final concentrations of the various constituents of this medium are shown in Table 3.2 and Table 3.3.

56 mM Potassium-Containing Medium. EBSS, identical to the standard product except that it contained high potassium levels and isotonically reduced sodium levels, was made in the laboratory (see Table 3.2). The various components of the basic salt solution were accurately weighed, and all components, excepting calcium chloride, were added to 400 ml sterile, culture grade water. The calcium chloride was dissolved separately in 100 ml sterile water and this solution was added slowly, with vigorous mixing, to the 400 ml salt solution. Finally, the complete solution was filter sterilised and stored at room temperature. Supplemented, 56 mM potassium-containing medium was prepared in exactly the same way as basal medium and stored under the same conditions.

3.4.2. General Experimental Procedure.

Throughout this thesis, a common approach was used for peptide release

Table 3.2. Composition of EBSS and 56 mM potassium-containing medium.

Component	mg/litre		mmol/litre	
	EBSS	56 mM K+	EBSS	56 mM K+
NaCl	6800	3820	116	65
KCl	400	4170	5.4	56
$CaCl_2 \cdot 2H_2O$	265		1.8	
$MgSO_4$	97		0.8	
$NaH_2PO_4 \cdot 2H_2O$	158		1	
$NaHCO_3$	2200		24	
Glucose	2000		11	

Table 3.3. Supplements to the experimental media.

Supplement	Stock Concentration	Final Concentration
Ascorbic Acid	3 mg/ml	30 µg/ml
Bacitracin	3 mg/ml	30 µg/ml
Hepes	1 mol/l	1 mmol/l
Penstrep	5000 I.U. Penicillin	100 I.U./ml
	5000 µg/ml Streptomicin	100 µg/ml
BSA	20 % w/v	0.1%
Aprotinin	10,000 K.I.U.*/ml	80 K.I.U./ml

*K.I.U. = Kallikrein Inactivator Units.

experiments. Briefly, cultures were removed from the incubator and placed in the class 2 cabinet. The culture medium was aspirated and each dish was washed with 1 ml prewarmed basal medium. This medium was replaced and the cells were returned to the incubator for ten minutes. Subsequently, this medium was aspirated and replaced with 700µl fresh basal medium, and the cells were incubated for the determined experimental period.

After the basal release period, the medium was aspirated and collected in polystyrene sample tubes (Teklab, Sacriston, Durham) for storage at -20°C. 700µl 56 mM potassium-containing medium was then placed in each well and the cultures were returned to the incubator for a further incubation period. At the end of this time, the medium was collected and stored as before, and the cells were washed 3 times with 1 ml DMEM. Finally, this was replaced with 1 ml serum-free culture medium and the cells were returned to the incubator.

3.5 Peptide Radioimmunoassays

The following sections detail the procedures which were used to measure the peptide concentrations in culture media or the peptide content of tissue or culture extracts.

3.5.1. Arginine Vasopressin.

AVP was measured by a radioimmoassay procedure, originally described by Kasting, Carr, Martin, Blume & Bergland (1983). The assay has been somewhat modified for use in the studies presented in this thesis, and so is fully described below. The assay uses a rabbit anti-AVP antiserum (NK-2), which was kindly supplied by Dr. Norman J. Kasting (Department of Medical Physiology, University of British Columbia, Vancouver, Canada). This antiserum, raised in a New Zealand male rabbit, shows minimal cross reactivity with a variety of neuropeptides, including oxytocin, in RIA procedures (Oxytocin cross reactivity; <0.2%; Kasting *et al* ., 1983).

Assay Buffer. Assays were carried out in 0.05M phosphate buffer which contained NaCl (100 mmol/l), EDTA (1 mmol/l), NaN_3 (0.1%) and BSA (0.1%; Sigma, Fraction V, RIA Grade). Reagents were dissolved in deionised water which was made up to 1 litre with 100ml 0.5M phosphate buffer solution. The pH of the assay buffer was adjusted to 7.4.

Antiserum. The lyophilised antiserum was reconstituted in assay buffer, diluted to a concentration of 1:500 and stored in aliquots of 100µl at -20°C. Immediately prior to use, an aliquot was added to 25 ml assay buffer, giving a final dilution of 1:125,000.

AVP Standards. 1 mg of AVP peptide (Cambridge Research Biochemicals, Northwich, Cheshire) was dissolved in 2 ml 0.1 M acetic acid, giving a concentration of 500 µg/ml, and aliquots of 10 µl and 20 µl were stored at -20°C.

Iodination. AVP may be iodinated directly at the Tyr^2 position by the Chloramine-T method, originally described by Hunter & Greenwood (1962). Briefly, 15 µl 0.5 M phosphate buffer and 10 µl $Na^{125}I$ (1 mCi, Amersham International, Aylesbury, Buckinghamshire) were added to an aliquot of AVP (10µl containing 5 µg peptide). Fresh chloramine-T solution (BDH; 50 µg chloramine-T in 10µl 0.05M phosphate buffer) was added to start the reaction. The mixture was vortexed, and the reaction was terminated after 10 seconds by adding 600 µl 0.05M phosphate buffer containing 0.5 % BSA.

Trace Purification. A C-18 Sep-Pak cartridge (Millipore, Hertfordshire, U.K.) was attached to a 5 ml syringe and was wetted with 2 ml 1% Trifluoroacetic acid in water (1%TFA; Aldrich, Poole, Dorset), followed by 5 ml of a mixture of 4 parts methanol with 1 part 1% TFA. The column was washed with 3 ml 1% TFA and coated with polypep (Sigma; 1 ml of 1% TFA containing 0.1 mg polypep). The iodinated preparation was pipetted on to the column and allowed to wash in to the bed under gravity.

Unincorporated ^{125}I was washed off the column by adding 0.7 ml 1% TFA and gently pushing the solvent through the cartridge with the

syringe plunger. The iodinated peptide was eluted with an ascending, step gradient of methanol in 1% TFA, with between 10% and 80% methanol, in 700µl aliquots. A Mini-Assay gamma counter (Type 6-20, Mini Instruments, Burnham on Crouch, Essex) was used to count each fraction as it was eluted from the column. The fraction which showed the highest binding activity to the NK-2 antiserum was used as the trace for conventional assays and was stored at -20°C for up to 1 month (see Figure 3.1).

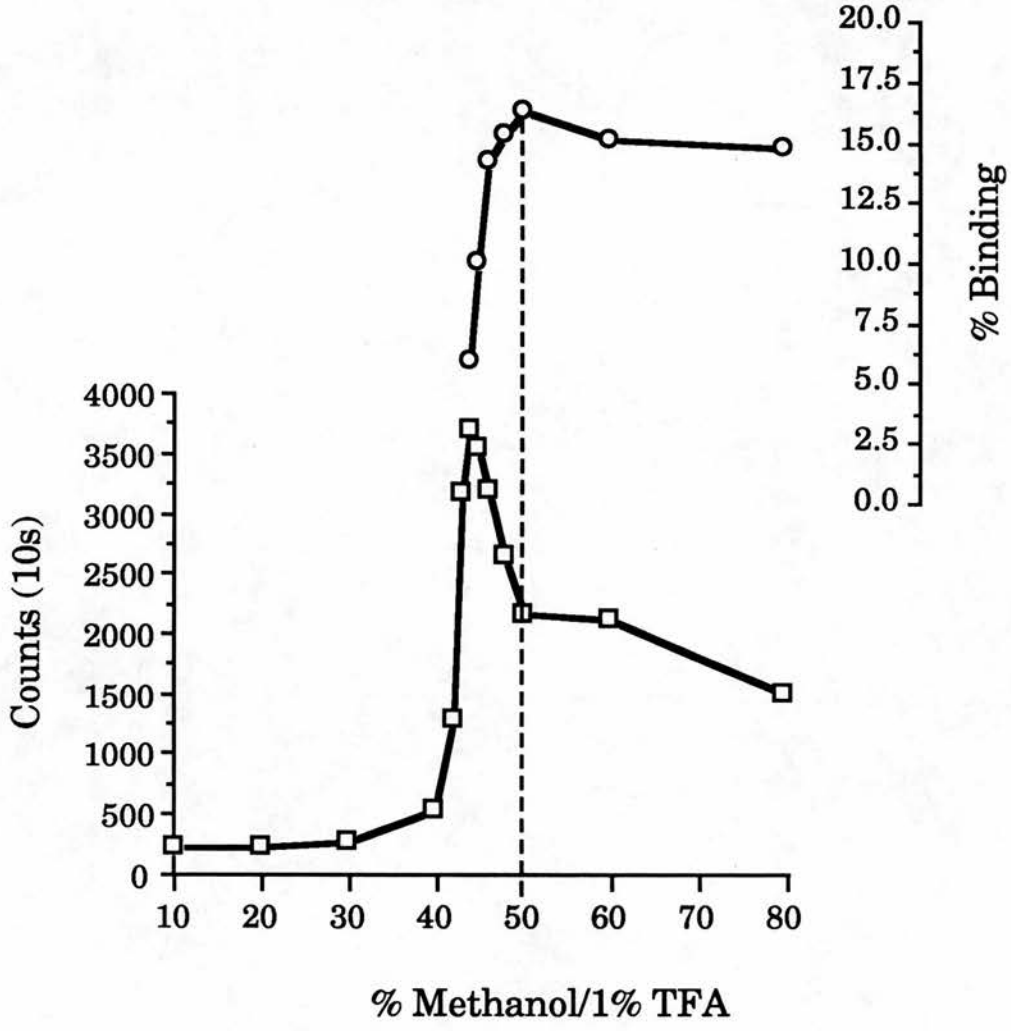
Assay Procedure. On day 1, 100µl aliquots of standards, samples and quality control media (Low and High QCs) were incubated with 200µl assay buffer and 100µl NK-2 antiserum (added at 1:125,000) in 3 ml polystyrene tubes (Sarstedt, Beaumont Leys, Leicester). There were always two or more standard curves, one at the beginning and one at the end, which were prepared from a freshly thawed standard aliquot of AVP (500µg/ml), giving a range of dilutions from 500pg/ml to 0.5 pg/ml. The samples were assayed in duplicate, whereas the standards were assayed in triplicate, as were the QCs. Included with the standard curves were tubes containing either 400µl buffer (NSB) or 300µl buffer plus 100µl antiserum (Bo). These were to be used to determine the non-specific binding of trace to the assay tubes (NSB) and the maximal binding of trace in the absence of AVP (Bo). Assays were vortexed and then incubated for 24 hours at 4°C.

On day 2, 100µl of dilute trace (~15,000 cpm in 100µl) was added to all assay tubes. A further set of tubes, containing only trace, were included at this stage to determine the total amount of activity added to each assay tube (Total counts; TC). Assays were vortexed and then incubated at 4°C for a further 24 hours.

Separation of Bound and Free ^{125}I-AVP. 200µl DAR (Donkey Anti-Rabbit IGG Magnetic Particles; gift of Dr. Rodney Kelly, MRC Reproductive Biology Unit), diluted 1:16 in 0.05M phosphate buffer containing 0.015% Triton X-100 (BDH), was added to each assay tube (except TC). The tubes were placed in magnetic separation racks (Amerlex-M assay racks, Amersham), vortexed, and incubated at room temperature for 30 minutes. Racks were then placed on specially designed magnets

Figure 3.1. Purification of ^{125}I-AVP Trace; Elution Profile of Radioactivity and Antiserum Binding Activity.

The dashed line shows the fraction with the highest binding activity. A binding assay was carried out for each new trace, and only the fraction with the highest binding was used in RIA procedures.


20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
% Binding
4000
3500
3000
2500
2000
1500
1000
500
0
Counts (10s)
10
20
30
40
50
60
70
80
% Methanol/1% TFA

(Amerlex magnetic separator, Amersham) and incubated for a further 15 minutes to precipitate the immunocomplex. Subsequently, the rack and magnet assembly was inverted to pour off the supernatant.

300µl wash buffer (0.075 M phosphate-buffered saline, containing 0.075 M NaCl, 0.1% NaN_3 and 0.015% Triton X-100) was added to each tube (except TC). The tubes were vortexed, and the immunocomplex magnetically precipitated as previously described. Finally, the tubes were inverted, the supernatant was poured off and the tubes were allowed to stand upside down to drain for 30 minutes. The precipitate was then counted for 60 seconds on a gamma counter (1261 Multigamma, LKB, Wallac OY, Turku, Finland).

Data Processing. During counting, data were collected onto a floppy disk by an online recorder ("Datagrabber"; Mutek, Box, Wiltshire). Subsequently, these data formed the input for an assay calculation program (AssayZap; Biosoft, Cambridge) written for the Apple Macintosh computer. This program initially plots a simple standard curve to the data. Subsequently, it reiteratively adjusts the curve to fit best the experimental data and the theoretical shape of the assay curve. The assay results were generated directly by AssayZap, along with historical data about the assay, e.g. % tracer binding and QC values.

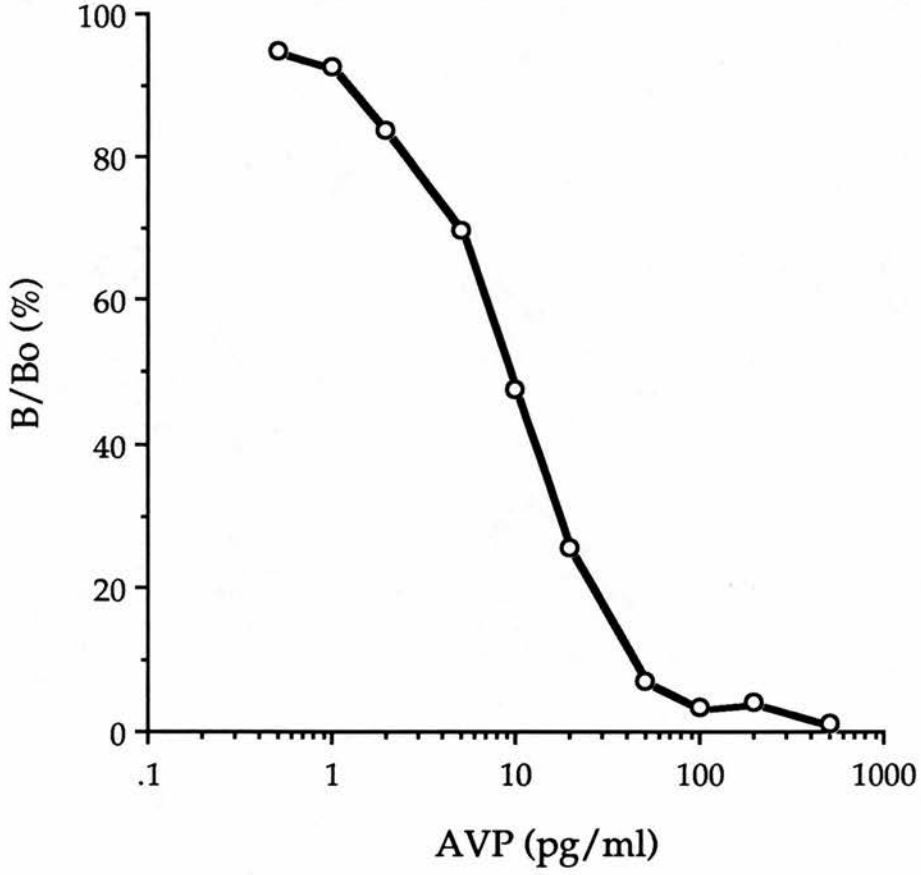
Assay Sensitivity and Variation. The limit of sensitivity of the assay, defined as 2 standard deviations from the zero point, was 1.75 pg/ml and the intraassay coefficient of variation was 16.0%. The interassay coefficients of variation for the low (8.46 pg/ml) and high (51.1 pg/ml) QCs were both 21.8%. It was noticed that different trace preparations gave somewhat different assay sensitivities, and this may account for what is felt to be a rather high limit of sensitivity overall. To minimise variation from such sources, all samples within a study were assayed using one batch of trace. A representative AVP standard curve is shown in Figure 3.2.

3.5.2. Corticotrophin-Releasing Hormone Immunoradiometric Assay.

Ovine Corticotrophin Releasing Hormone (oCRH) was measured by

Figure 3.2. A Representative AVP Standard Curve.

Non-specific binding was subtracted from the counts for each tube, and the results were expressed as a percentage of the values obtained in the absence of cold AVP (Bo). Binding values were then plotted against the quantity of cold AVP added.


100
80
60
40
20
0
B/Bo (%)
.1
1
10
100
1000
AVP (pg/ml)

immunoradiometric assay (IRMA) either alone or in conjunction with oCRH RIA (see 3.5.3). In contrast to RIA, an IRMA depends on the specific binding of two antibodies from different species to one molecule of the analyte (see Figure 3.3). One antibody (sheep anti-C terminal CRH) is radiolabelled; when it binds to the CRH, it effectively radiolabels the analyte. The second antibody (guinea pig anti-N terminal; "link") binds to another epitope on the same molecule, and the whole complex is precipitated with another antibody, directed against the link. In this way, precipitated radioactivity is proportional to the amount of intact hormone present, as it is the CRH molecule which joins the precipitating antibody to the radiolabel. The strength of the IRMA lies in its ability to detect only full size hormone, rather than fragments which may cross-react in an RIA.

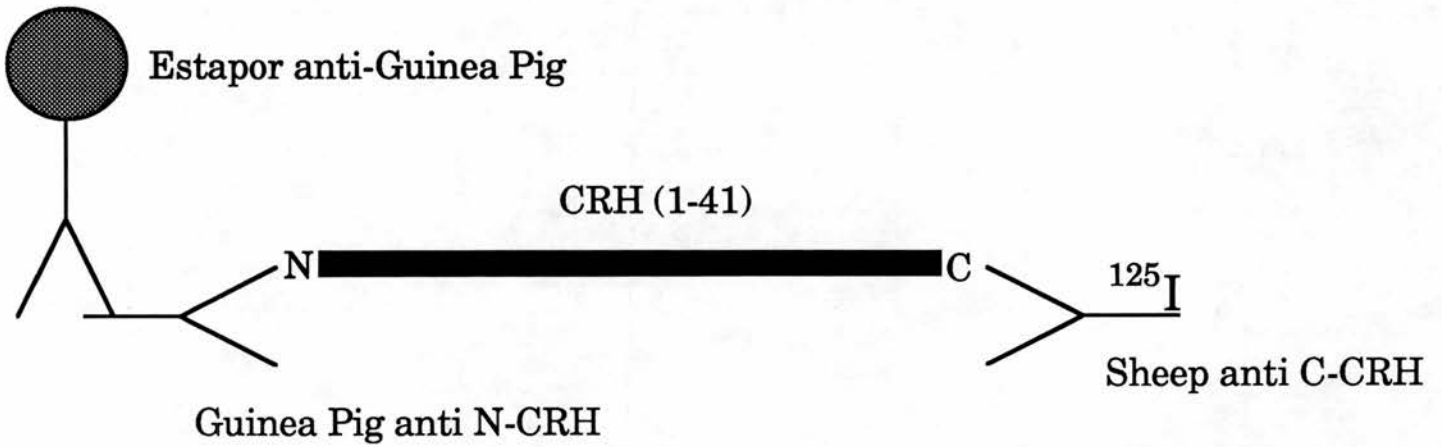
The methodology reported here is an adaptation of the assay first described by Linton & Lowry (1986). Dr. Peter Saphier (University of Reading, U.K.) very kindly prepared all the reagents used in these assays, which were carried out at the MRC Reproductive Biology Unit in Edinburgh.

200µl R1 reagent (0.05 M phosphate buffer containing 2% normal sheep serum, 0.5% BSA, guinea pig anti N-CRH at 1:5000 and ^{125}I-sheep anti C-CRH; ~100,000 cpm in 200µl) were added to 200µl of samples (duplicates), standards and QCs (triplicates). Standards were prepared in sample buffer in the same way as for the RIA, to give a range of 10,000pg/ml down to 10pg/ml. Included in the standard curve was a set of tubes containing 200µl sample buffer and 200µl R1; these were used to determine the background counts with no added hormone (3ml polystyrene tubes; as for RIA). The assay tubes were then vortexed and incubated at room temperature for 18 hours.

On day 2, 100µl of diluted, solid phase second antibody (Rabbit Anti-Guinea Pig, "Estapor"; 1:5 in 0.05M phosphate buffer) were added to each tube and the tubes were incubated for 30 minutes on the bench. The suspension was diluted with 2 ml wash buffer (0.05M phosphate buffer containing 150 mmol/l NaCl and 0.01% Triton X-100) per tube, and the tubes were centrifuged for 10 minutes at 3000 rpm (2110g). Subsequently, the assay tubes were placed in magnetic separation racks and the supernatant was decanted. Finally, the wash buffer, centrifugation and

Figure 3.3. Principles of the oCRH Immunoradiometric Assay.

The radiolabelled sheep antibody binds to the C-terminal end of the CRH molecule. A guinea pig antibody binds to the N-terminus, and the complex is immunoprecipitated using an anti-guinea pig antibody. Thus, precipitated counts are proportional to the number of molecules bearing both N- and C-epitopes.


Estapor anti-Guinea Pig
CRH (1-41)
N
C
125I
Sheep anti C-CRH
Guinea Pig anti N-CRH

decanting steps were repeated, and the tubes were left inverted to drain, before counting the precipitates as previously described.

The limit of sensitivity of the IRMA was 34 pg/tube, the intraassay coefficient of variation was 14.5%, and the interassay coefficients of variation for the low (123 pg/ml) and high (479 pg/ml) QCs were 18.5% and 16.9% respectively. Figure 3.4 shows a standard curve for the CRH IRMA.

3.5.3. oCRH Radioimmunoassay.

In some experiments presented in this thesis, oCRH was measured by conventional RIA with magnetic separation. The assay reported here is a modified version of that first described by Brooks *et al.* (1989) and is described below. Both CRH (1-41) standard peptide and [Tyr^0] CRH (1-41), for iodination, were purchased from Cambridge Research Biochemicals.

100µl rabbit anti-CRH antiserum (CRF-2G; 1:100,000) and 100µl assay buffer (0.05M phosphate buffer containing 0.1 mol/l NaCl, 25 mmol/l EDTA, 0.1% NaN_3, 0.1% Triton X-100 and 0.1% BSA,pH 7.4) were added to 200µl of samples, standards or QCs in 3 ml polystyrene tubes. Standard curves, containing NSB and Bo tubes, were prepared by diluting a freshly thawed aliquot of CRH (10µg/ml) in assay buffer to give a range from 10,000 pg/ml to 20 pg/ml (see Figure 3.5). Samples were assayed in duplicate, whereas standards and QCs were assayed in triplicate. All tubes were vortexed and incubated for 24 hours at 4^{o}C.

The next day, 100µl dilute ^{125}I-[Tyr^0] CRH trace (Brooks *et al.* 1989), containing ~15,000 cpm was added to each tube, including TC tubes. Subsequently, all tubes were vortexed and incubated overnight at 4^{o}C. On day 3, antibody-bound tracer was magnetically precipitated and the assay results were calculated as previously described.

The lower limit of sensitivity of the assay was 14pg/tube, the intraassay coefficient of variation was 16.2%, and the interassay coefficients of variation for the low (241 pg/ml) and high (948 pg/ml) QCs were 16.5% and 15.5% respectively. Figure 3.5 shows a CRH RIA standard curve.

Figure 3.4. A Representative oCRH IRMA Standard Curve. Counts measured in tubes containing no hormone were subtracted from all tubes, and the logs of the resultant figures were plotted against the quantities of added hormone.

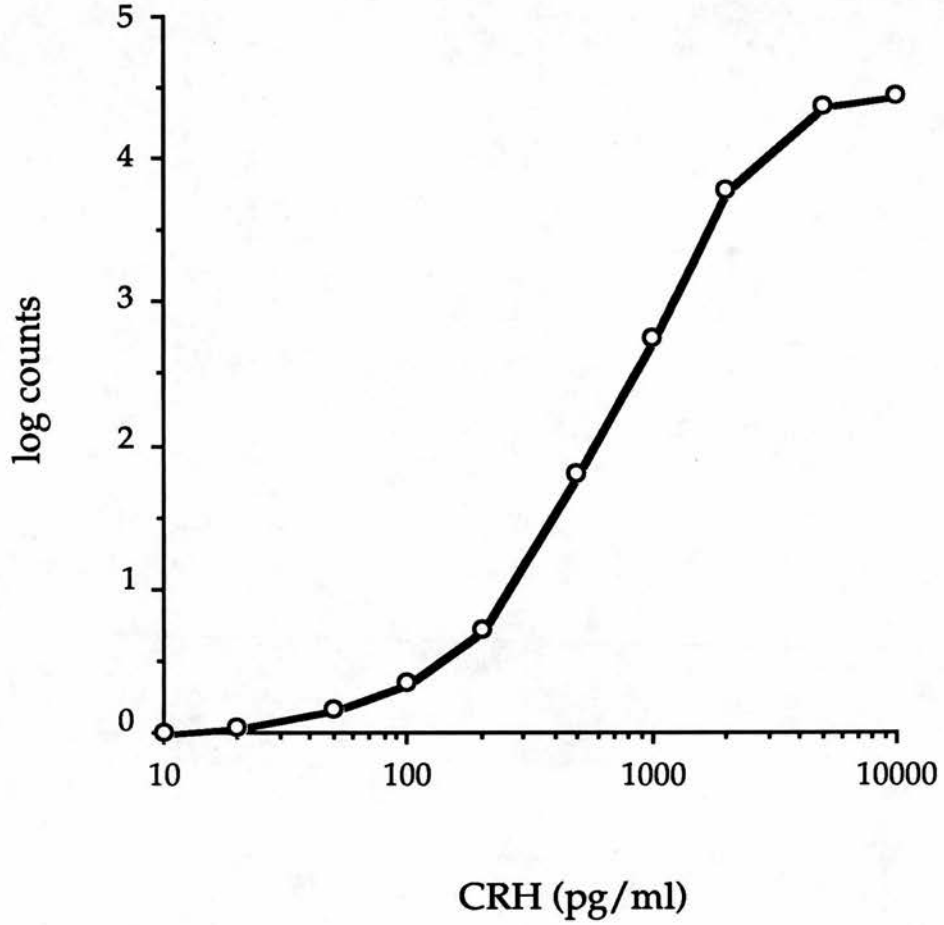
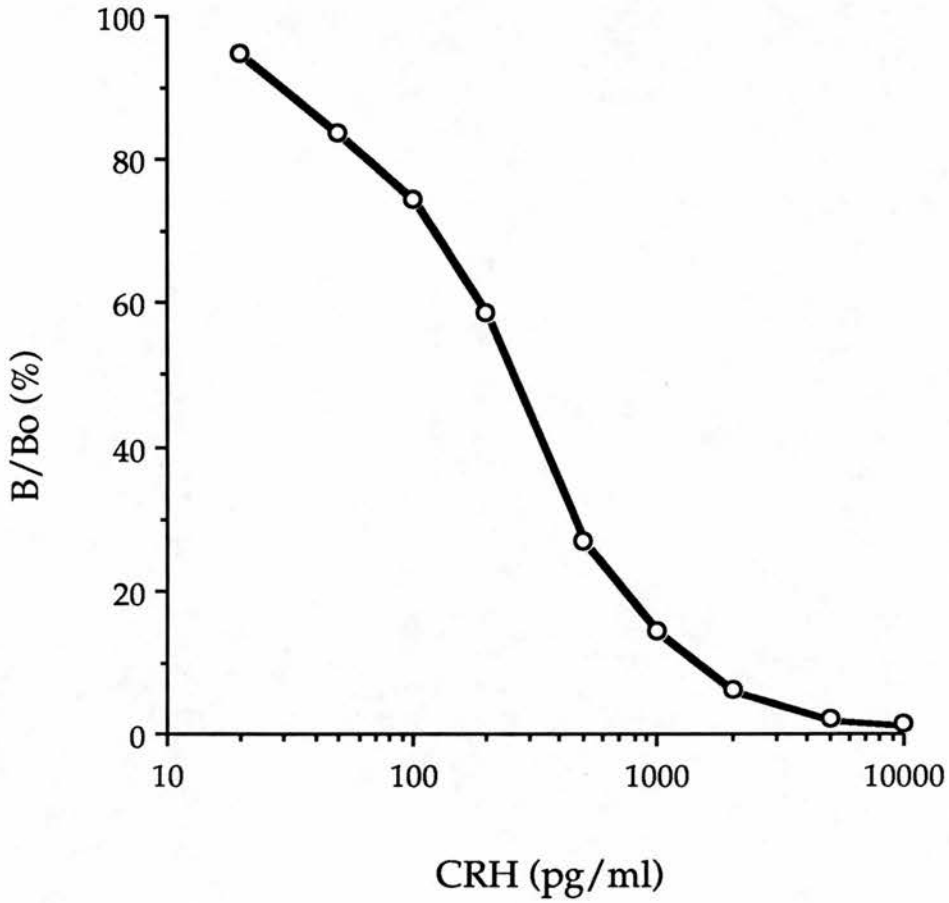

5
4
3
2
1
0
log counts
10
100
1000
10000
CRH (pg/ml)

Figure 3.5. A Representative oCRH RIA Standard Curve. Non-specific binding was subtracted from the the counts for each tube, and the results were expressed as a percentage of the values obtained in the absence of cold CRH (Bo). Binding values were plotted against the quantity of cold CRH added.


100
80
60
40
20
0
B/Bo (%)
10
100
1000
10000
CRH (pg/ml)

3.6. Sephadex Chromatography

Several experiments presented in this thesis required the characterisation of immunoreactive species present in tissue extracts or culture supernatant. In order to determine the approximate molecular weight of these molecules, Sepahdex chromatography was carried out followed by CRH or AVP RIA.

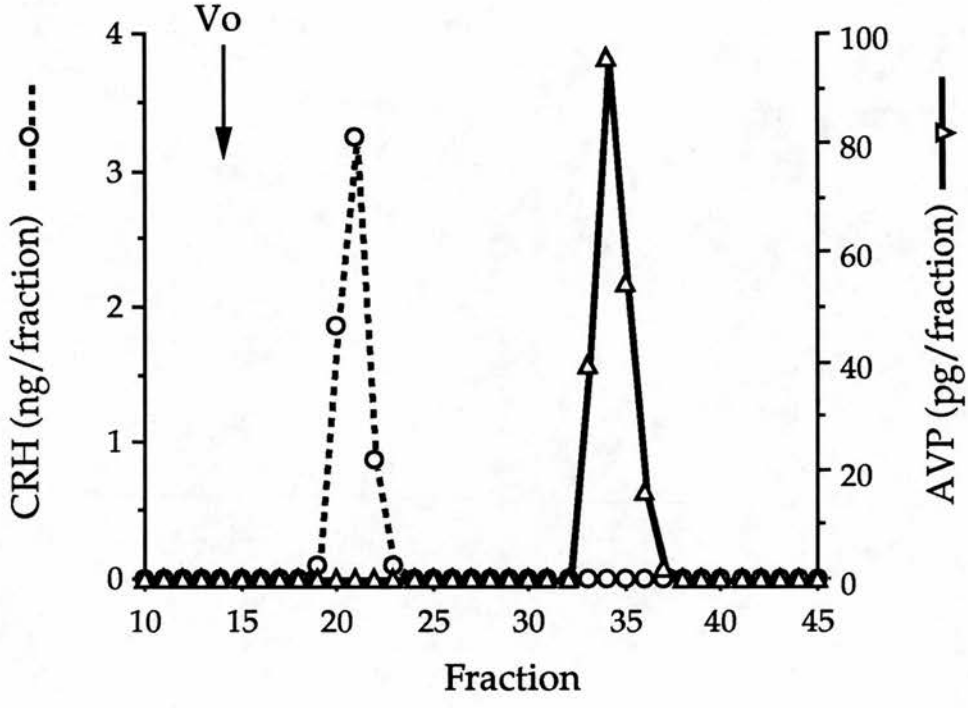
Sephadex G50 Fine (Pharmacia, Uppsala, Sweden) was swollen overnight in 0.1M HCl. The slurry was then packed into a glass column (50cm x 1cm internal dimensions; Amicon Ltd., Stonehouse, Glocestershire) under gravity at 4°C. The column was perfused for 24 hours (0.1M HCl, 3.5 ml/hour, Watson Marlowe Pump, Type 202U; Falmouth, Cornwall) and binding to non-specific sites in the gel matrix was prevented by priming the column with 1ml 20% BSA solution.

Standard 1ml aliquots containing 0.1% BSA and either AVP (500pg/ml), oCRH(1-41) (10,000pg/ml) were added to the column in duplicate. These were eluted with 0.1M HCL at a flow rate of 3.5ml/hour, and fractions were collected every 20 minutes in polystyrene tubes (10x75mm; Sarstedt) with a fraction collector (Gilson Microcol TDC 80; Scotlab, Bellshill, Scotland). Fractions were neutralised with 10M NaOH and the oCRH or AVP concentration was measured by RIA. Figure 3.6 shows the elution position of synthetic oCRH and AVP. Blue dextran (2mg/ml ; Pharmacia) exclusion was used to determine the void volume of the column.

3.7 Data Analysis

The effects of experimental manipulations were assessed in the first instance by Analysis of Variance (ANOVA), using a programme designed for the Macintosh computer called CLR ANOVA (Clear Lake Research, Houston, Texas, U.S.A.). The strategy of an analysis was dictated by the number of between- and within-subject variables, taking into account whether the data represented repeated measures on the same subjects or independent samples. For pairwise comparisons subsequent to ANOVA, data were compared using Duncan's multiple range test.

Figure 3.6. Sephadex G50 Chromatography.
0.1M HCl was used to elute AVP and CRH, which were then measured by RIA. Blue dextran was used to determine the void volume of the column (Vo).


Vo
CRH (ng/fraction)
AVP (pg/fraction)
Fraction
0
1
2
3
4
0
20
40
60
80
100
10
15
20
25
30
35
40
45

4. Development of the Fetal Sheep Hypothalamic Cell Culture System.

4.1 Introduction

The chronically instrumented fetal sheep model of Bassett & Thorburn (1969) has made possible the dynamic assessment of the fetal pituitary-adrenal axis *in utero*. However, this approach relies on the quantitation of systemic hormones such as ACTH and cortisol in the peripheral circulation, and so the developing hypothalamic control of the fetal pituitary-adrenal axis remains poorly understood.

There are several studies which have examined the maturation of the fetal sheep hypothalamus by indirect techniques. For example, the fetal hypothalamic contents of CRH and AVP have been investigated at different developmental ages (Brieu *et al.* 1989), and this research shows that the hypothalamic concentration of these ACTH-releasing factors is substantially increased with advancing gestation. In parallel with such changes in peptide content, immunocytochemistry has revealed the progressive maturation of CRH- and AVP-containing cells in the paraventricular nucleus of the fetal hypothalamus (Levidiotis *et al.* 1987). These studies give some insights to fetal hypothalamic activity during development, however, they are unable to show how control of the various releasing factors evolves with advancing gestational age.

Recently, Brooks *et al.* (1989) investigated developmental changes in the regulation of CRH release from fetal sheep hypothalamic tissue *in vitro* . These researchers were able to demonstrate that a glucocorticoid agonist, dexamethasone, inhibited CRH release from tissue removed at day 140 of gestation. However, they showed that there was no inhibitory effect of the agonist on CRH release from day 100 hypothalami. These results were in agreement with those of Clarke & Gillies (1988), who showed that CRH and AVP secretion from fetal rat hypothalamic neurones removed late in gestation were inhibited by corticosteroids. However, the latter authors utilised a cell culture system to maintain hypothalamic neurones over long periods *in vitro*. This technique made

possible more sophisticated investigations of hypothalamic neuronal function.

Hypothalamic cell culture has undergone a transformation with the introduction of serum-free culture media (Bottenstein & Sato, 1979, Faivre-Bauman, Rosenbaum, Puymirat, Grouselle & Tixier-Vidal, 1981, Puymirat, Barret, Picart, Vigny, Loudes, Faivre-Bauman & Tixier-Vidal, 1983). Release of AVP and CRH from fetal rat hypothalamic cultures is enhanced in serum-free medium when compared to serum-supplemented medium (Clarke & Gillies, 1988). Furthermore, peptide secretion in these cultures is stimulated by potassium-induced depolarisation in a calcium-dependent fashion. These recent developments have suggested that a cell culture system would be a most elegant means by which to investigate hypothalamic function in the fetal sheep.

In order to develop a system for the culture of fetal sheep hypothalamic cells, it was first necessary to assess the optimal culture conditions for maintaining these cells. Variations in plating density are known directly to modulate expression of neurotransmitters and proteins in cultured cells (Lucas, Edgar & Thoenen, 1979; Zurn & Mudry, 1986) and culture substrates which are reported to be optimal for certain cell types may be less so for the same cell type in different species. For example, Faivre-Bauman, Puymirat, Loudes, & Tixier-Vidal (1984) carried out a thorough investigation to determine the appropriate substrate for maintenance of mouse hypothalamic neurones *in vitro* . These authors reported that gelatin plus poly-l-lysine coating was far superior to gelatin or poly-l-lysine alone, whereas excellent results have been acheived in fetal rat hypothalamic cultures with a poly-l-lysine substrate (Clarke, Lowry & Gillies, 1987).

In order to assess the optimum conditions for maintaining fetal sheep hypothalamic cultures *in vitro,* cells were plated at densities between 1.5×10^5 and 3×10^5 cells/cm^2 on gelatin or poly-l-lysine substrates or on a combination of both. The accumulated AVP in the supernatant was measured as an indicator of cell function. A second culture was then maintained under the optimised conditions as suggested by the first experiment. These cells were then subject to experimental stimulations to assess functional responses after 2 weeks *in vitro.*

4.2 Experimental Procedures

4.2.1. Optimisation of Culture Conditions.

Cell Culture. Fetal sheep hypothalamic tissue was obtained from 7 fetuses at a gestational age of 50 days (Term = 145 days). The tissue was dispersed for cell culture as previously described.

Experimental Conditions. To establish the effect of cell plating density on subsequent AVP output, four dilutions of the final cell suspension were prepared, giving rise to cell densities of 1.5, 2, 2.5 and 3 x 10^5 cells/cm^2 when plated. The effect of culture substrate was assessed by plating cells on sterile glass coverslips (Chance Propper Size 3, 13 mm round; R.A. Lamb, London), previously coated with gelatin, poly-l-lysine, or a combination of both substrates, according to modifications of published methods (Faivre-Bauman*et al.* 1984), described in more detail below. Each combination of substrate and cell density was assessed in duplicate or triplicate wells.

Briefly, coverslips were immersed in a solution of gelatin (Sigma) in culture grade water (0.25 mg/ml) for 30 minutes. They were subsequently rinsed in sterile water, placed on edge in 4-well culture dishes (Nunc 4-well multidish, Gibco) and allowed to dry in the culture hood before use. Coverslips were coated with poly-l-lysine (Sigma) by incubating overnight in coating solution (100 µg/ml in sterile water), and then rinsing and drying as described previously. Where both treatments were applied, coverslips were first coated with gelatin and then with poly-l-lysine.

Coverslips were then incubated with 200 µl culture medium containing 20% fetal calf serum for 3 hours. Finally, this medium was aspirated, and the cells were plated out on the coverslips in a volume of 200 µl.

Culture Maintenance. Cells were maintained in serum-containing medium for 48 hours after plating. Subsequently, the cells were transferred to serum-free medium alone. This medium was changed every 3 or 4 days, at which times the culture supernatants were collected and stored at -20°C, for later measurement of AVP by RIA. Cultures were

frequently assessed under phase-contrast microscopy and a photographic record of the appearances of different cultures was maintained throughout this time.

Statistics. The AVP concentration in the culture supernatants was expressed as pg/dish. To compare AVP output from the various culture conditions, ANOVA was carried out with plating density and substrate as between-group variables and time *in vitro* as the within-group variable. This was followed by Duncan's multiple range test for pairwise comparisons, in order to assess which substrates or densities had enhanced AVP output in the culture medium. Data were log transformed prior to analysis to eliminate heterogeneity of variance.

4.2.2. Assessment of Experimentally-Induced AVP Release.

A hypothalamic tissue culture was prepared from fetal sheep at 100 days of gestation as previously described. Cells were plated in 35 mm wells on a substrate coated with poly-l-lysine, at a density of 2.5×10^5 cells/cm^2 (equal to 2.5×10^6 cells/dish), as detailed in Chapter 3.

On the 15th day *in vitro*, cells were incubated with control medium and with 56 mM potassium-containing medium for 3 hours each. On day 19, this protocol was repeated, except that cells received control medium containing 100 μM N-Methyl D-Aspartate (NMDA; Cambridge Research Biochemicals) instead of high potassium medium. Subsequently, the content of AVP in these samples was measured by RIA.
Statistical comparison of the response to potassium or NMDA with basal release was carried out in each case by one way ANOVA, where treatment was the within-group variable, followed by Duncan's multiple range test.

4.3 Results

4.3.1. Optimisation of Culture Conditions.

Observations. The cultures were first observed 7 hours after plating, at which time all cells were firmly adhered to the coverslips. Within 24 hours (day 2 *in vitro*), the hypothalamic cells had extended projections and formed a dense network throughout the culture dishes. On the 4th day *in vitro* cells grown on poly-l-lysine had spread over the substrate, showing some localised clumping. However, those grown on gelatin showed a greater degree of clumping, forming a net-like structure throughout the dish. These changes, which became more exaggerated with time, are shown in Figure 4.1.

Cell cultures were maintained up to 28 days *in vitro*. Cultures plated on poly-l-lysine remained attached to the coverslips throughout the culture period, however, cells plated on either gelatin or gelatin plus poly-l-lysine peeled away from their substrates between day 22 and 25. For this reason, AVP output data are shown only until day 22 of culture.

AVP Output. Figure 4.2 shows the AVP output from hypothalamic cells maintained under the various culture conditions. Analysis of variance revealed that there were highly significant effects of plating density ($p < 0.001$) and culture substrate ($p< 0.001$) on the quantity of AVP accumulated in culture supernatant. Individual comparisons on each day *in vitro* showed that medium removed from cells grown on gelatin had a significantly greater AVP content at all time points in culture, when compared to the combination of gelatin and poly-l-lysine ($p < 0.01$, except day 19, $p < 0.05$). Poly-l-lysine alone had enhancing effects similar to gelatin when compared to the combination substrate, although these were not as marked (significant enhancement on days 6 and 9, $p < 0.01$, and day 16, $p < 0.05$). Gelatin coating enhanced AVP accumulation in comparison to poly-l-lysine only on day 6 of culture ($p < 0.01$), thereafter, there were no differences between these groups.

AVP accumulation in culture media was very much elevated at high plating densities. At all time points, supernatant from high density cultures (2.5 and 3 x 10^5 cells/cm^2) contained significantly more AVP than

Figure 4.1. Appearance of cell cultures at different times after plating. **A.** Cells plated on poly-l-lysine. **B.** Cells plated on gelatin. **C.** Cells plated on gelatin plus poly-l-lysine. Representative photographs are shown of cultures on day 2, 4, 10 and 20 *in vitro*. Cells plated on gelatin show a marked clumping when compared to other substrates, which is exaggerated with time *in vitro* .

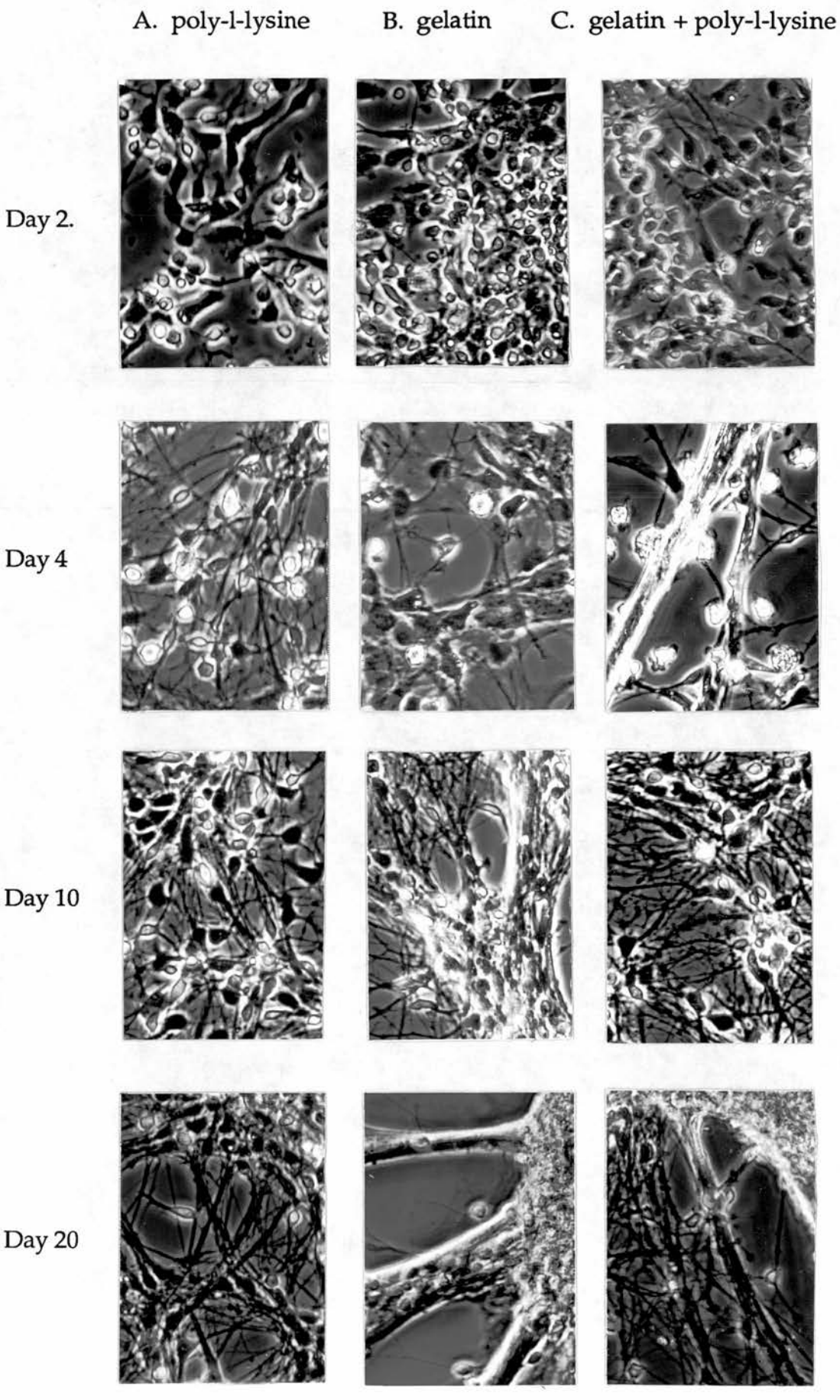
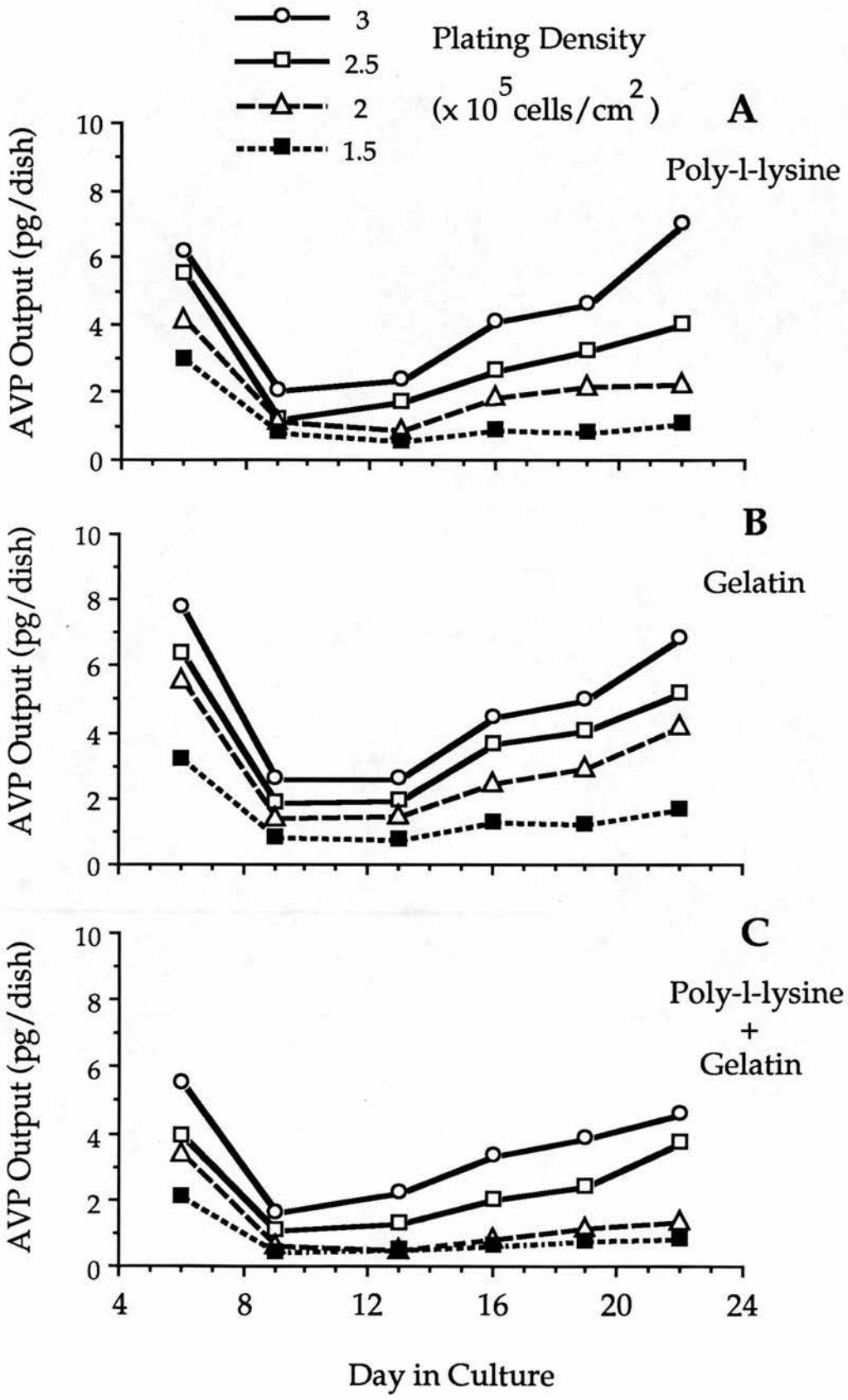

A. poly-l-lysine
B. gelatin
C. gelatin + poly-l-lysine
Day 2.
Day 4
Day 10
Day 20

Figure 4.2. The effects of plating density and culture substrate on AVP accumulation in hypothalamic culture supernatant. **A.** Cells plated on poly-l-lysine. **B.** Cells plated on gelatin. **C.** Cells plated on gelatin plus poly-l-lysine. Each graph shows the AVP contents of the media, collected from cultures of different densities. AVP accumulation is shown as pg/dish. Each data point represents the mean of 2 or 3 wells. Standard errors have been omitted for clarity.


3
2.5
2
1.5
Plating Density
(x 10^5 cells/cm^2)
A
Poly-l-lysine
B
Gelatin
C
Poly-l-lysine
+
Gelatin
AVP Output (pg/dish)
AVP Output (pg/dish)
AVP Output (pg/dish)
0
2
4
6
8
10
4
8
12
16
20
24
Day in Culture

did the lower density cultures ($p < 0.05$), whereas there were few differences between the two high density groups (output from 3×10^5 cells/cm^2 greater than 2.5×10^5 cells/cm^2 at days 6 and 9 only, $p < 0.01$).

4.3.2. Assessment of Experimentally-Induced AVP Release.

Figure 4.3 shows that AVP release from hypothalamic cultures was significantly elevated by both 56 mM potassium and 100 μM NMDA treatment ($p < 0.01$). This suggests that the AVP-secreting cells were capable of a secretory response to membrane depolarisation induced either by 56 mM potassium or receptor activation via the NMDA/glutamate receptor.

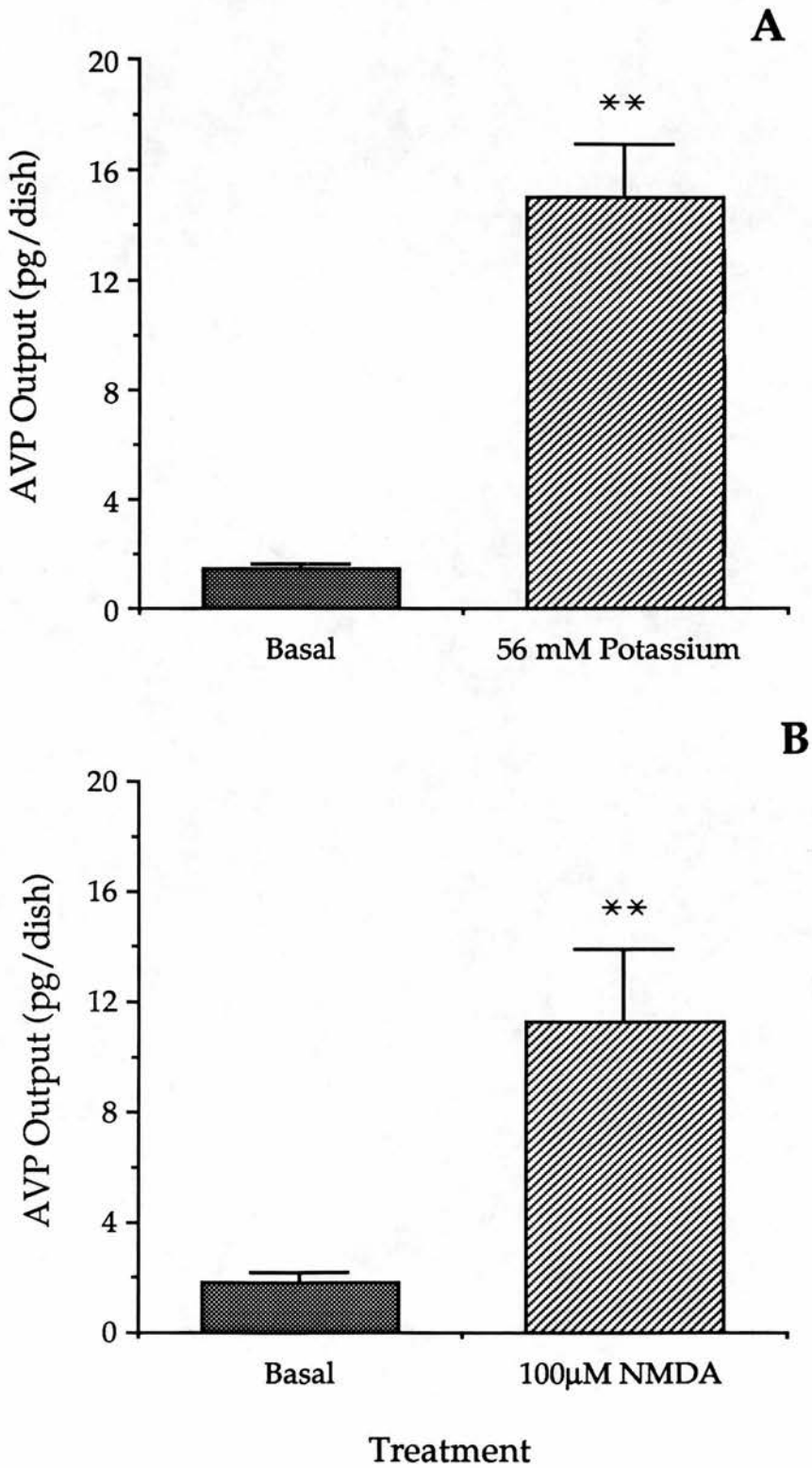
4.4 Discussion

The studies reported in this chapter examined the effects of different plating densities and growth substrates on the function of hypothalamic cell cultures, which was assessed by measuring accumulated AVP in the culture supernatant. Medium removed from cells plated at higher densities contained more AVP than supernatant aspirated from lower density cultures. Furthermore, AVP accumulation was accentuated in cultures plated on either gelatin or poly-l-lysine, in contrast to lower AVP levels observed in medium from cells grown on a combination of the two substrates. In fact, there was a tendency for cells plated on gelatin to show a greater AVP output than cells plated on poly-l-lysine, however, it was later found that gelatin coating was associated with failure of cell-substrate adhesion. In a second experiment to assess AVP secretion in response to different secretagogues, AVP output was significantly elevated in response to 56 mM potassium and to 100μM NMDA.

There was a dramatic difference in AVP levels between the cultures plated at 1.5×10^5 cells/cm^2 and 3×10^5 cells/cm^2 (see Figure 4.2), more so than could be explained by the two-fold difference in cell numbers. It may be that at the highest densities, AVP synthesis and secretion per neurone is enhanced. Adler & Black (1985, 1986) have demonstrated that a 30-fold increase in substance P content of rat

Figure 4.3. The effects of 56 mM potassium and 100 μM NMDA on AVP secretion by hypothalamic cell cultures. **A.** Secretory response to 56 mM potassium. **B.** NMDA response (100 μM). Each result is the mean of 6 wells, +/- S.E.M., $**p < 0.01$, significantly different from basal values.

Stimulated Release of AVP from cultured Fetal Hypothalamic Cells



sympathetic neuronal cultures occurs when density is increased 4-fold. Similar density-dependent effects have been reported in cultured chick sympathetic neurones (Zurn & Mudry, 1986), bovine adrenal chromaffin cells (Acheson & Thoenen, 1983) and the PC 12 phaeochromocytoma cell line (Lucas *et al.* 1979) for catecholamine synthesis, tyrosine hydroxylase activity and Choline Acetyl Transferase (ChAT) activity respectively. Alternatively, it may be that AVP accumulation is adversely affected in the lowest density cultures. Early work has shown that low plating density is associated with poor cell growth *in vitro* (Sanford, Earle & Likely, 1948) and it has become widely accepted that cells plated below a minimum density fail to thrive (Ham & McKeehan, 1979).

The effect of culture substrate on AVP levels could be explained in several ways. The most interesting may be that specific substrates induce greater expression of neuronal AVP synthesis, and hence secretion. Culture substrate has previously been examined as a factor regulating the expression of Glutamic Acid Decarboxylase (GAD) and ChAT activities in cultured chick neurones (Mangoura, Sakellaridis & Vernadakis, 1990). Whilst collagen selectively enhanced GAD activity, poly-l-lysine stimulated ChAT. Furthermore, increased GAD activity was associated with greater numbers of GAD-immunopositive cells, suggesting that substrate might exert a profound effect on cell function. Cell-substrate interactions have been found by other workers greatly to enhance or retard the differentiation of CNS cells in culture (Agresti, Aloisi, & Levi, 1991). These findings relate indirectly to the present study, however, in the absence of comparable data, the data suggest that peptidergic function of cultured AVP neurones may well be determined to some degree by substrate. Another explanation may be that the poor adhesive qualities of the gelatin plus poly-l-lysine-coated coverslips led to a progressive loss of cells, and so reduced AVP accumulation. However, cells plated on gelatin also detached from the substrate, and yet the data suggest that AVP levels in these cultures were greater than those observed in other cultures. The interesting possibility remains that the clumping of cells observed in cultures plated on gelatin effected a local increase in cell-cell contact, and so improved AVP secretion from these cells via a density-dependent mechanism.

Cells maintained on gelatin or gelatin plus poly-l-lysine but not

poly-l-lysine alone peeled away from the coverslips between day 22 and 25 of culture. It was observed that cells plated on a gelatin-based substrate tended to form tight clusters of cells connected by "cables" of neurites (Figure 4.2). As time *in vitro* progressed, these cables became detached from the substrate, and ultimately, all cultured tissues became detached from the substrate as the cells peeled away. Other authors have reported "stringiness" and peeling in serum-free cultures (Mangoura *et al.* 1990). On the same note, Faivre-Baumanet *al.* (1984) mention "cables" in association with gelatin-coated substrates. Substrate requirements may well vary between different species and this may account for the relative successes of different substrates with different preparations. In the work described here, poly-l-lysine was found to be the optimum substrate for fetal sheep cells.

56 mM potassium-induced neuropeptide release is widely used as a demonstration of culture viability (Clarke *et al* . 1987; Sarkar & Sakaguchi, 1990) and N-Methyl D-Aspartate (NMDA) has been shown to stimulate peptide release from cultured neurones via glutamate receptors (Tapia-Arancibia & Astier, 1988). In the present study, AVP release over a 3 hour period was elevated 6-fold and 10-fold in the presence of 100μM NMDA and 56mM potassium-containing medium respectively. Although each of the experiments reported in this chapter was carried out only once, these results provide good evidence that the cell culture system developed here is capable of maintaining functional neurosecretory cells over prolonged periods *in vitro.*

A major advantage of serum-free cell culture is the degree of control which is possible over the cellular environment. The medium used in the present study is based on that first described by Bottenstein & Sato (N2 medium; 1979), supplemented with Triiodo-thyronine (Puymirat *et al.* 1983) and Oestradiol-17β (Faivre-Bauman *et al.* 1981). No attempt was made to alter the reported composition, as the development of a specific medium for fetal sheep neurones would have been outside the scope of these studies. However, a description of some of the important components of the chosen medium is given here.

Transferrin, the major iron transport protein in the blood (Fletcher & Huehns, 1968), is present at high levels in this medium. Many cell types have a most stringent requirement for this additive, and fail to

proliferate or die in its absence (Bottenstein & Sato, 1979; Faivre-Bauman *et al.* 1981, Bottenstein, 1984). Similarly, triiodo-thyronine markedly enhances neurite extension and is obligatory for peptide secretion in hypothalamic cell cultures (Puymirat *et al.* 1983, Clarke & Gillies, 1988). Another important supplement is selenium, which was discovered to be the active factor in trace contaminants of early media (McKeehan & Ham, 1979). Again, previous workers have shown that selenium-deficient media do not support cell growth (Bottenstein & Sato, 1979, Bottenstein, 1984).

Insulin is present at high levels in this medium. It is localised in cultured neurones by immunocytochemistry (Schechter, Holtzclaw, Sadiq, Kahn & Devaskar, 1988), suggesting a paracrine role within the CNS. It may also be acting as a neurotrophic agent in culture, as the high concentrations present *in vitro* may result in cross-reactivity at IGF-1 receptors (Adamo, Raizada & LeRoith, 1989). Other additives include oestradiol-17β, which enhances the neurone: glial cell ratio (Faivre-Bauman *et al.* 1981); putrescine, found in greatly elevated quantities in fetal brain tissue and associated with rapid cellular growth (Seiler & Lamberty, 1975), and progesterone, which is trophic to neuroblastoma cells (Bottenstein & Sato, 1979) and is routinely added to neuronal culture media (Maurer, 1986).

In summary, a range of culture conditions have been assessed in order to develop a serum-free culture system for fetal sheep hypothalamic cells. Using available tissue, it was determined that AVP release was facilitated by plating cells at high densities, and that a poly-l-lysine substrate was superior in maintaining the integrity of cell cultures. Furthermore, cells plated under these conditions displayed a dramatic secretory response to membrane- or receptor-mediated depolarising stimuli after 2 weeks *in vitro,* suggesting that this culture system is perfectly suitable for subsequent investigations of fetal neuroendocrine function *in vitro.*

5. Ontogeny of AVP and CRH Secretion from Fetal Sheep Hypothalamic Cells.

The previous chapter decribed the development and optimisation of a serum-free culture system for the maintenance of fetal sheep hypothalamic cells. These cultured cells were shown to secrete the hypothalamic peptide AVP, and furthermore, could respond to depolarising stimuli with increased AVP secretion some 2 weeks after plating out. Previously, studies of the developing hypothalamic input to the pituitary corticotrophs in the fetal sheep were dependent on evaluation of ACTH levels, and were therefore based on indirect measurements. By contrast, the present chapter seeks to quantify directly the secretion of ACTH-releasing factors from the developing fetal sheep hypothalamus *in vitro* .

5.1 Introduction

Evidence has now accumulated to suggest that in fetal sheep, increasing hypothalamic drive to the pituitary is responsible for the preparturient activation of the fetal pituitary-adrenal axis. Around day 110-120 of gestation in the sheep fetus, plasma ACTH and cortisol concentrations begin to increase (Term= day 145; Norman *et al.* 1985) and undergo further sharp elevations in the last few days of pregnancy (Rose *et al.* 1978). Administration of CRH or AVP stimulates fetal plasma ACTH levels (Wintour *et al.* 1984; Norman & Challis, 1987a,b), and repeated infusion of boluses containing CRH+AVP to the fetus results in elevated basal plasma ACTH levels and significant adrenal corticosteroid secretion (Brooks & White, 1990).

In the intact fetal hypothalamus, CRH and AVP content is greatly increased with advancing gestational age (Brieu *et al.* 1989; Brooks *et al.* 1989). In addition, pituitary cell bioassays reveal that the ACTH-releasing activity of fetal hypothalamic extracts is augmented in parallel with CRH and AVP content (Brieu *et al.* 1989). Recently, Antolovich and colleagues

obtained good evidence to suggest that the fetal hypothalamus provides a crucial stimulatory input to the fetal pituitary-adrenal axis in late gestation. These workers showed that surgical disconnection of the fetal hypothalamus from the pituitary prevents the normal increase in fetal plasma ACTH and cortisol in late gestation, and hence abolishes parturition at the normal time (Antolovich *et al.* 1990; 1991).

The objective of the present chapter was therefore to investigate the gestational changes in secretion of ACTH-releasing factors from the fetal sheep hypothalamus. Hypothalamic cultures were prepared from tissues obtained at day 70, day 100 and day 130 of gestation. Basal and potassium-stimulated release of CRH and AVP were measured on several occasions from each culture, in order to assess more fully the secretory capacity at the different gestational ages. In addition, the facilitatory effects of repeated potassium stimulation on AVP and CRH output were determined in all cultures, as was the time and calcium dependency of AVP and CRH release. Finally, cultures were extracted for measurement of AVP and CRH content at each gestational age, and quantitative immunocytochemistry was carried out to examine numerical differences in cell types between different cultures.

5.2 Experimental Procedures

A total of 37 hypothalami were removed from fetal sheep at day 70 (5+4 hypothalami) day 100 (8+7 hypothalami) and day 130 (4+5 hypothalami) of gestation. Hypothalamic cell cultures were prepared as described previously, and the experiments described below were carried out in duplicate cultures at each gestational age, the results from which were combined for statistical analysis. In the experimental procedures described below, the statitistical analyses are described for AVP alone. CRH release data were assessed using the same procedures. To eliminate heterogeneity of variance, data were log-transformed where appropriate.

5.2.1. AVP and CRH release from the developing hypothalamus *in vitro* .

The objectives of this experiment were to assess whether potassium stimulation enhanced AVP and CRH output from the hypothalamic cultures, to assess the effects of gestational age on the basal and 56 mM potassium-stimulated secretion of AVP and CRH, and to examine the facilitatory effects of repeated potassium stimulation on AVP and CRH secretion.

Culture dishes were incubated with basal release medium for 3 hours and then with 56 mM potassium-containing medium for a further 3 hours, as detailed in section 3.5.2. These experimental treatments were repeated every 3 or 4 days, from day 7 to day 27 *in vitro* (Group 1 cells). To assess whether such incubations had a facilitatory effect on subsequent peptide release, AVP and CRH output from these cells was compared with output from a second group of wells, experimented upon from day 17 until day 27 *in vitro* (Group 2 cells). After each incubation, samples were stores at -20°C until measurement of AVP and CRH contents by RIA and IRMA respectively.

Statistics. To assess whether potassium stimulation enhanced AVP release from the hypothalamic cultures, ANOVA was carried out on data obtained from group 1 cells at each gestational age individually with day *in vitro* (day 7, 10, 14, 17, 21, 24 and 27) and incubation (basal or stimulated) as the within-groups variables. Duncan's multiple range test was then employed for post-hoc comparisons, to determine whether potassium stimulated AVP release on each day *in vitro.* A similar analysis was applied to results from group 2 cells at each gestational age.

To examine the changes in basal peptide secretion with advancing gestational age, ANOVA was carried out on basal AVP release data from from group 1 cells. Gestational age (day 70, 100 or 130) was the between-groups variable, and day *in vitro* (day 7, 10, 14, 17, 21, 24 and 27) was the within-groups variable. The ANOVA was followed by Duncan's multiple range test to assess the overall differences in basal AVP secretion between hypothalamic cultures prepared at different gestational ages. An identically structured analysis was employed to compare potassium-stimulated AVP release at the different gestational ages.

In order to investigate the facilitatory effects of potassium stimulation on basal AVP secretion in the hypothalamic cultures, ANOVA was carried out to determine if there was an overall difference in basal AVP secretion between group 1 and group 2 cells at each gestational age. Cell group (1 or 2) was the between-groups variable and day *in vitro* (day 17, 21, 24 and 27) was the within-groups variable. Duncan's test was used to examine specific differences in basal AVP release between group 1 and 2 cells. Similar approaches were then used to investigate stimulated AVP release at each gestational age.

5.2.2. Time and Calcium dependency of AVP and CRH Release.

Time Dependency. In order to assess whether secretion of AVP or CRH was time-dependent, all wells were assigned to one of four groups at random on day 30 *in vitro.* These groups were incubated with basal release medium for either 30, 60, 120 or 240 minutes. Subsequently, wells were re-randomised, allocated to one of four new treatment groups, and incubated with 56 mM potassium-containing medium for a further 30, 60, 120 or 240 minutes. Media were collected and stored at -20°C until AVP and CRH were measured by RIA and IRMA respectively.

To examine the effects of increasing the duration of the experimental incubations, basal and stimulated AVP release data from the different time groups by were compared by ANOVA, with time (30, 60, 120 and 240 minutes) and incubation (basal or stimulated) as the between-groups variables, followed by Duncan's test to examine the differences between the group means.

Calcium Dependency. On day 33 *in vitro,* the calcium dependency of peptide release was examined. Dishes were incubated with basal release medium for 3 hours and then assigned to one of two groups at random. Group A was washed 3 times with 1 ml basal medium, then 700µl 56 mM potassium-containing medium was added to each dish for a further 3 hours. Group B was treated in the same way, except that washing and 56 mM potassium-containing media were calcium-free. Again, media were stored at -20°C for subsequent measurement of AVP and CRH by RIA and IRMA respectively.

An individual ANOVA, followed by Duncan's test, was then carried out at each gestational age to compare basal with potasssium-stimulated AVP release in group A and Group B cells.

5.2.3. AVP and CRH Content of Cell Cultures.

To assess whether cultures from different gestational ages contained similar quantities of AVP or CRH, cultured cells were resuspended by trypsinisation (section 5.2.4) on day 36 *in vitro,* and aliquots of the suspension were stored at -20°C for subsequent measurement of AVP and CRH content by RIA and IRMA respectively.

To determine the effects of gestational age on AVP content of the cultured cells, ANOVA was used with gestational age (day 70, 100 and 130) as the between-groups variable. Duncan's test was employed post-ANOVA to examine specific differences in content between the age groups.

5.2.4. Cell Numbers after 36 days *in vitro*.

The possibility existed that gestational differences in peptide secretion might reflect better survival of cells from a particular developmental age. To investigate cell survival after 36 days *in vitro,* cultures were trypsinised and the cells were counted by the following method.

All dishes were washed twice (2 x 1 ml Dulbecco's Phosphate-Buffered Saline without calcium and magnesium - DPBS; (ICN Flow Ltd.) and 500µl of the same medium, containing 0.02% EDTA (ICN Flow Ltd.) and 0.5 mg/ml trypsin (Sigma), were added to each well. 300µl excess medium was aspirated, and the dishes were incubated for 10 minutes at 37°C. 800µl DPBS, containing 150 µg/ml soybean trypsin inhibitor (Sigma) and supplemented with the same quantities of ascorbic acid, bacitracin, BSA and aprotinin as basal release medium (Section 3.4.1), were added to each well. The cells were triturated and 100µl of suspension was removed from each well for cell counting with a haemocytometer. The rest was used to determine the AVP and CRH contents of cultured cells (Section 5.2.3) and for immunocytochemistry (sections 5.2.5 - 5.2.7).

To examine differences between cell numbers at different gestational ages, ANOVA was carried out with gestational age as the between-group variable. Duncan's test was used to examine differences between individual means.

5.2.5. Immunocytochemistry; Antibody Characterisation.

Matutration of the fetal hypothalamus *in vitro* may be associated with an increasing number of the peptidergic neurones which synthesise CRH and AVP. To investigate the gestational differences in the numbers of hypothalamic neurones *in vitro,* and in the numbers of cells synthesising CRH or AVP, it was proposed to carry out immunocytochemical procedures to specifically stain cultured cells containing either Neurone-specific enolase (NSE), CRH or AVP.

Antisera. All antisera were raised in rabbits, either in the laboratory (oCRH antiserum; CRF-2G; see Brooks *et al.* 1989, AVP antiserum; AVP#26, gift of Miss Barbara Graham, MRC Reproductive Biology Unit) or by a commercial supplier (NSE; Cambridge Research Biochemicals). Immunostaining of NSE in fixed specimens was abolished when anti-NSE (Batch 01841) was preincubated with 10 nmol/ml NSE protein (Cambridge Research Biochemicals Data Sheet). The specificity of this antibody was therefore not investigated further, however, CRF-2G nor AVP#26 had previously been validated in immunocytochemical (ICC) procedures.

To show that immunostaining is due to the presence of a specific antigen, and not to non-specific interaction of the antibody with tissue proteins, it is neccessary to demonstrate that removal of antigen-specific antibodies abolishes the immunostaining. Therefore, specific antipeptide antibodies were removed by incubating CRF-2G and AVP#26 with magnetic particles coupled either to oCRH or AVP respectively, or with magnetic particles alone as a control. The supernatants were then used to carry out ICC procedures. The preparation of the magnetic particles and the preabsorbed antibodies were carried out as detailed below.

Preparation of Conjugates. 2 ml magnetic particles (bearing hexane-NH_2 groups; gift of Dr. Rodney Kelly, MRC Reproductive Biology Unit) were were washed in 50 ml 0.5M NaCl and then magnetically precipitated on an Amerlex-M separator (Amersham). The supernatant was decanted and the process was repeated 3 times. Finally the particles were washed in 50 ml deionised water (dH_20), pH 4.5, at 4°C, precipitated, and resuspended in 8 ml of the same medium. 200 µg AVP or CRH was added in 200µl dH_20, alternatively, control conjugate was prepared by adding 200µl water alone at this stage. N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; Sigma) was added dropwise (2 ml, 75 mg/ml in dH_20, pH 4.5, at 4°C) and the mixture was placed on a rotary mixer at 4°C for 18 hours.

The next day, the suspension was washed and pecipitated alternately in 50 ml acetate buffer (18% 0.1M Sodium Acetate, 82% ml 0.1M Acetic Acid, in 0.5M NaCl, pH 4) and 50ml bicarbonate buffer (0.1 M $NaHCO_3$ in 0.5M NaCl, pH 8.3) four times each. The conjugate was finally washed in dH_20 and resuspended in 5 ml 0.9% saline (containing 10mM phosphate buffer and 1% NaN_3) and stored at 4°C.

Preabsorbtion of Antisera. Control, AVP and CRH-conjugated magnetic particles were precipitated and resuspended in 1 ml of 10mM phosphate-buffered saline (PBS). To preabsorb the CRF-2G antiserum, CRF-2G (1:250) in 1 ml preabsorption buffer (40% Normal Swine Serum (NSS) and 4% BSA in PBS), was added to the CRH-conjugated magnetic particles. AVP#26 at 1:125 in 1 ml preabsorption buffer was added to the AVP-conjugate. Control preabsorptions were carried out in parallel by adding either CRF-2G or AVP#26 in preabsorption buffer to 1 ml of the control conjugate. The mixtures were then turned end over end for 18 hours at 4°C. The next day, the mixture was magnetically separated. The supernatants were divided into aliquots of 100µl and stored at -20°C.

Binding Characteristics of Antisera. Serial dilutions of the peptide or control-preabsorbed antisera were prepared in triplicate in either AVP or CRH RIA buffers. 100µl of diluted antiserum was added to 300µl assay buffer and 100µl ^{125}I CRH or AVP trace (Bo tubes). NSB and TC tubes, containing respectively 400µl buffer plus 100µl trace or 100µl trace alone

were prepared in parallel. All tubes were incubated overnight at 4°C. The next day, antisera were precipitated by conventional magnetic separation (Section 3.5.1.) and the precipitates were counted. The trace binding for each tube was calculated as (Bo-NSB)/TC.

Figure 5.1 overleaf shows the AVP tracer binding to AVP#26 antibody previously incubated with AVP- or control conjugate, and CRH trace binding to CRF-2G preabsorbed with CRH- or control magnetic particles. The results of this validation clearly show that substantial trace binding activity was present in antisera incubated with control conjugates, whereas no trace binding was observed in peptide-preabsorbed antisera. Therefore, the peptide-specific antibodies were absent from the peptide-preabsorbed antisera, but present in the control antisera.

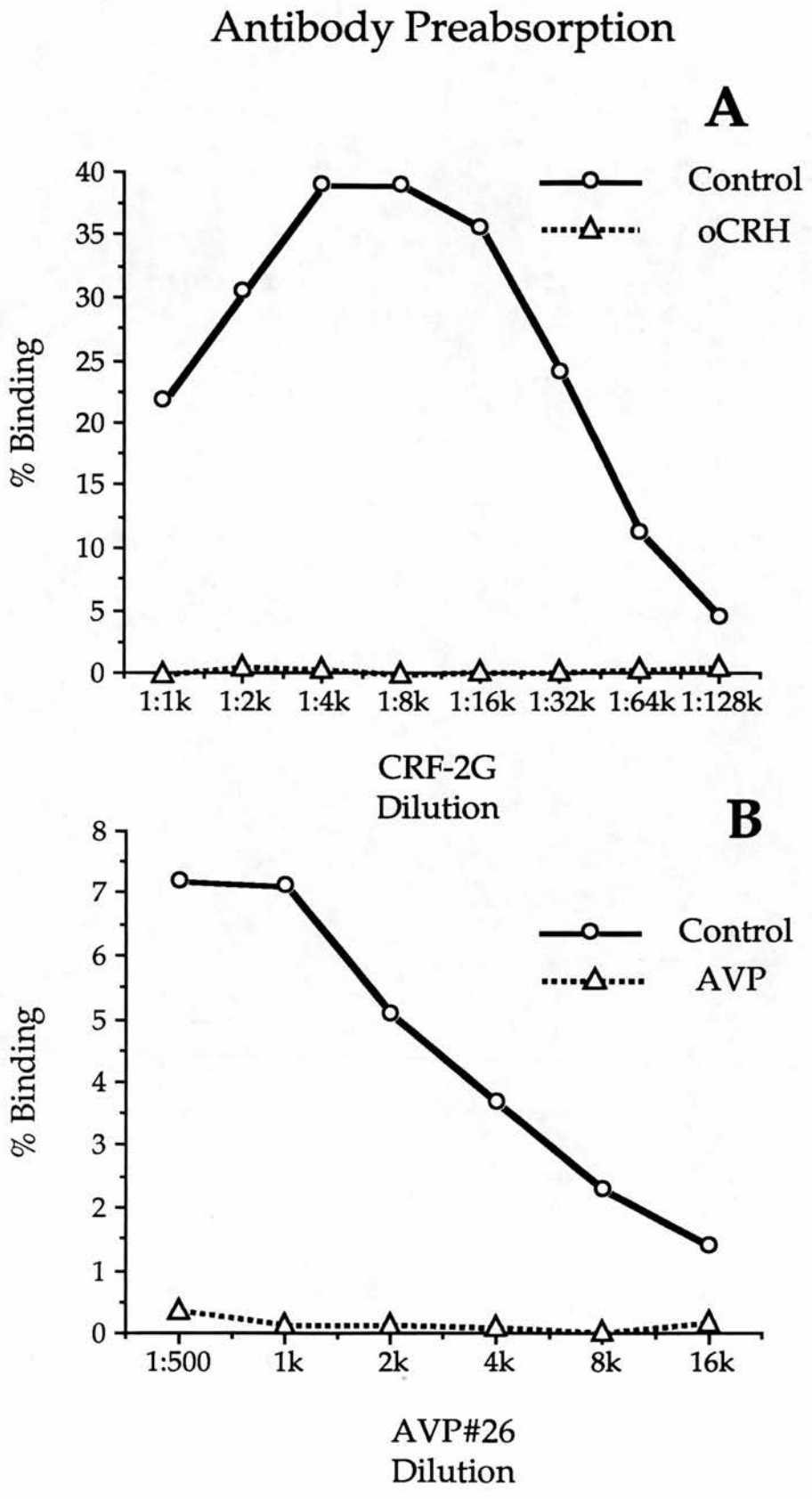
5.2.6. Validation of Immunocytochemical Procedures.

Fixation Procedure. Cells were resuspended by trypsinisation on day 36 *in vitro* (section 5.2.4). 4 x 5µl aliquots of suspension from each well were placed on glass microscope slides (C.A. Hendley, Essex). These smears were dried and were fixed by immersion for 10 minutes in Bouin's fluid. Slides were then washed once in water and 3 times in 70% ethanol. Finally, slides were dried and stored at -20°C.

Immunocytochemical Validation. To investigate the specificity of the antisera in immunocytochemical procedures, and to determine the optimum antibody dilution for strong specific staining, ICC was carried out in fetal sheep hypothalamic cells with peptide- and control-preabsobed CRF-2G and AVP#26 at different dilutions, according to the method described below. A similar validation was carried out for the NSE antibody, using non-immune rabbit serum as a parallel control.

Slides containing cell smears were incubated in a bath containing 0.1% Triton X-100 in PBS for 2 minutes. They were then rinsed and incubated in PBS alone for 10 minutes. Subsequently, a little blocking buffer (PBS containing 20% NSS and 2% BSA) was applied to each cell smear, and the slides were left to incubate in a humidified chamber at room temperature for 1 hour. The excess blocking buffer was removed, and primary antiserum diluted in blocking solution was added to the cell

Figure 5.1. Binding Characteristics of Preabsorbed Antisera. **A**. Various dilutions of CRF-2G, previously incubated with magnetic particles coupled to CRH or magnetic particles alone were incubated with CRH tracer and the trace binding was determined. **B**. Serial dilutions of AVP#26 preabsorbed with control- or AVP-conjugated magnetic particles were incubated with AVP tracer to determine the residual AVP-binding capacity of the preabsorbed antiserum. Neither antibody which had been preabsorbed with the appropriate peptide-conjugated particles showed any trace binding activity.


Antibody Preabsorption
A
Control
oCRH
% Binding
40
35
30
25
20
15
10
5
0
1:1k
1:2k
1:4k
1:8k
1:16k
1:32k
1:64k
1:128k
CRF-2G
Dilution
B
Control
AVP
% Binding
8
7
6
5
4
3
2
1
0
1:500
1k
2k
4k
8k
16k
AVP#26
Dilution

smears. Parallel controls were included which received blocking soluiton alone, or blocking solution containing non-immune rabbit serum. These slides were then incubated in the humidified chamber at 4°C for 21 hours.

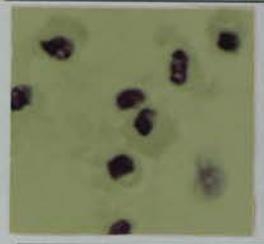
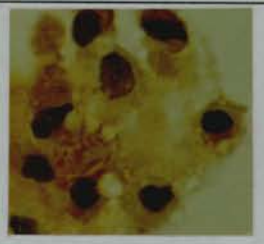
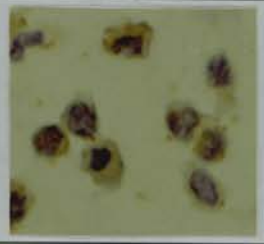
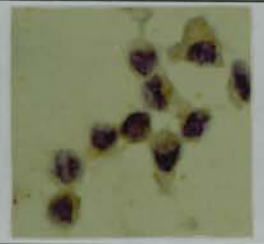
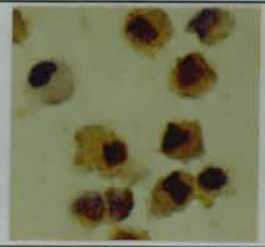
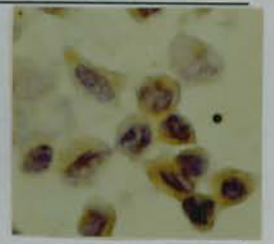
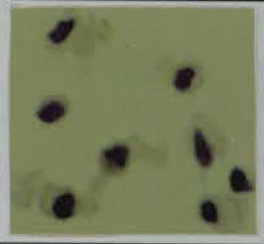
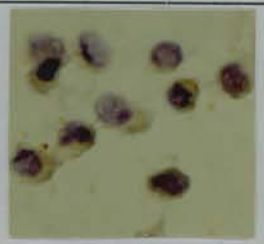
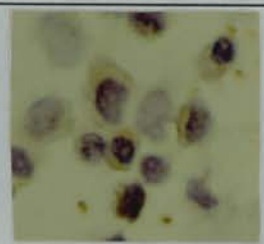
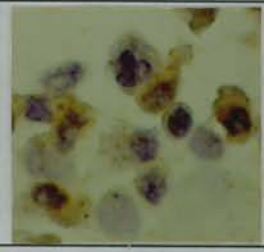
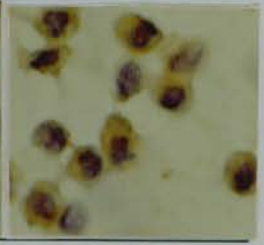
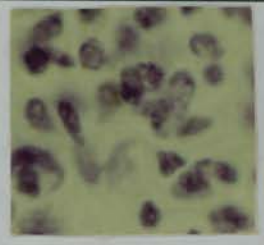
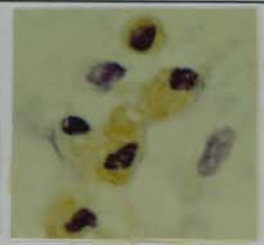
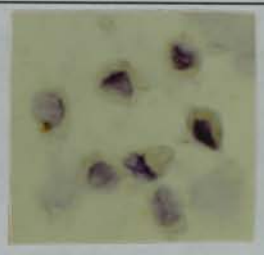
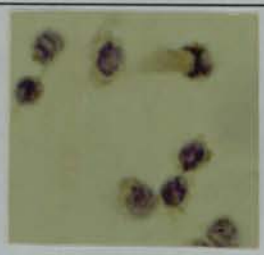
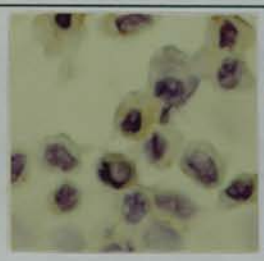
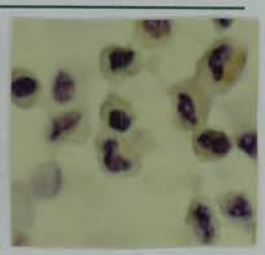
Next day, the slides were washed in PBS for 10 minutes and the second antibody, Biotinylated Swine Anti-Rabbit (SARB; DAKO, High Wycombe, Bucks), was applied to the smears at a dilution of 1:500 in blocking buffer. After 1 hour, unbound antibody was washed off in the same way as for the primary antiserum. Slides were then incubated for 30 minutes with Avidin-Biotin Complex (DAKO) inTris HCl buffer, pH 7.6. After a further 10 minute wash in PBS, chromagen solution (diaminobenzidine tetrahydrochloride; Sigma, 0.5 mg/ml in 0.05M Tris HCl buffer, pH 7.6, containing 0.02% H_2O_2) was added for 5 minutes. The reaction was than terminated with copious washing in tap water. Slides were than counterstained with Haematoxylin, dehydrated in ascending alcohols and mounted in Histomount (National Diagnostics, New Jersey, USA). The slides were then photographed, and the results from these studies are shown in Figure 5.2.

The results show that intense staining was observed with NSE antibody at a dilution of 1:500, whereas non-immune rabbit serum did not cause staining at this dilution, suggesting that the NSE staining was specific. Optimal specific staining with low background was observed with anti-NSE concentration of 1:1000, and so this concentration was used in further studies. In contrast, the staining with either CRF-2G or AVP#26 seemed to be weak and was not different between control- or peptide preabsorbed antisera. These observations suggested that the staining was not specific, and so it was decided not to utilise these antibodies further in immunostaining procedures.

5.2.7. Quantitative NSE Immunocytochemistry.

To determine if the numbers of neurones in culture, as defined by NSE immunocytochemistry, varied with gestational age, cells from 6 randomly chosen dishes at each gestational age were processed in quadruplicate for NSE ICC. Approximately 1000 stained and unstained cells were counted in total over several fields in the replicates from each

Figure 5.2. Immunocytochemical staining with anti-NSE, CRF-2G and AVP#26. Immunostaining was assessed with anti-NSE at various dilutions. Anti-NSE showed strong staining at 1:500, whereas non-immune rabbit serum (NRS) showed no staining at this concentration. Control slides which had received no primary antibody, or no primary anitbody and no secondary antibody did not stain. Optimal NSE immunostaining was observed at a dilution of 1:1000. CRF-2G ICC was carried out at several dilutions using control- and CRH-preabsorbed antibody. No difference in immunostaining was observed between control or CRH-preabsorbed antibody. AVP#26 ICC was carried out in the same fashion as for CRF-2G. Again, no difference in immunostaining was observed between control or AVP-preabsorbed antibody.

		CRF-2G		AVP#26	
Control	NSE	Control	Preabsorbed	Control	Preabsorbed
No 1°or 2°As	1:500	1:500	1:500	1:250	1:250
					
No 1°As	1:1000	1:750	1:750	1:500	1:500
					
NRS 1:500	1:2000	1:1000	1:1000	1:1000	1:1000
					

well and the number of stained cells as a percentage of the total was estimated. ANOVA followed by Duncan's test was used to compare the percentages of stained cells at each gestational age.

5.3 Results

5.3.1. AVP and CRH release from the developing hypothalamus *in vitro* .

Figures 5.3 to 5.5 show the basal and 56 mM potassium-stimulated release of AVP and CRH from cultured hypothalamic cells plated at days 70, 100 and 130 of gestation. Figures 5.3 and 5.4 show that potassium-induced depolarisation significantly ($p < 0.01$) enhanced AVP release from day 70 and day 100 hypothalamic cultures on each experimental occasion. However, Figure 5.5 shows that AVP release in day 130 cells was enhanced by 56 mM potassium only on day 7 ($p < 0.01$) and day 21 ($p < 0.05$)*in vitro* .

Figure 5.3 shows that CRH release in day 70 cultures was significantly stimulated by 56 mM potassium ($p < 0.01$) at days 7 and 21 *in vitro* . Overall analysis revealed that CRH release by day 100 cells was not significantly enhanced in response to this treatment (ANOVA; $p = 0.7$), as shown in Figure 5.4. Analysis of variance indicated that there was a significant effect of 56 mM potassium on CRH release from day 130 cultures (ANOVA; $p< 0.001$). Surprisingly, further analysis revealed that there was a significant inhibitory effect of elevated potassium on day 17 ($p < 0.01$) and day 27 ($p < 0.01$) *in vitro* , as shown in Figure 5.5.

Figures 5.3 to 5.5 demonstrate that advancing gestational age had a significant effect on AVP and CRH secretion. Overall analysis showed that basal and potassium-stimulated AVP secretion from day 70 cells was significantly greater than basal and stimulated release from day 100 cells ($p < 0.01$) , which in turn were greater than basal and potassium-induced release from day 130 cells ($p < 0.01$). A similar analysis showed that there was no difference in basal CRH release from day 70, day 100 and day 130 cells (ANOVA; $p = 0.3$). However, potassium-stimulated CRH release from day 70 cells was greater than from day 100 cultures ($p < 0.05$), which in turn exceeded that from day 130 cells ($p < 0.05$).

Figure 5.3. AVP and CRH secretion in fetal sheep hypothalamic cultures from 70 days of gestation. AVP and CRH secretion was measured in Group 1 cultures between day 7 and day 27 (A and C respectively) and in Group 2 cultures between day 17 and day 27 *in vitro* (B and D respectively). Data shown are means of 10 or more wells, +/- S.E.M. ** $p < 0.01$; significant difference between basal and 56 mM potassium incubations.

Day 70 Gestation

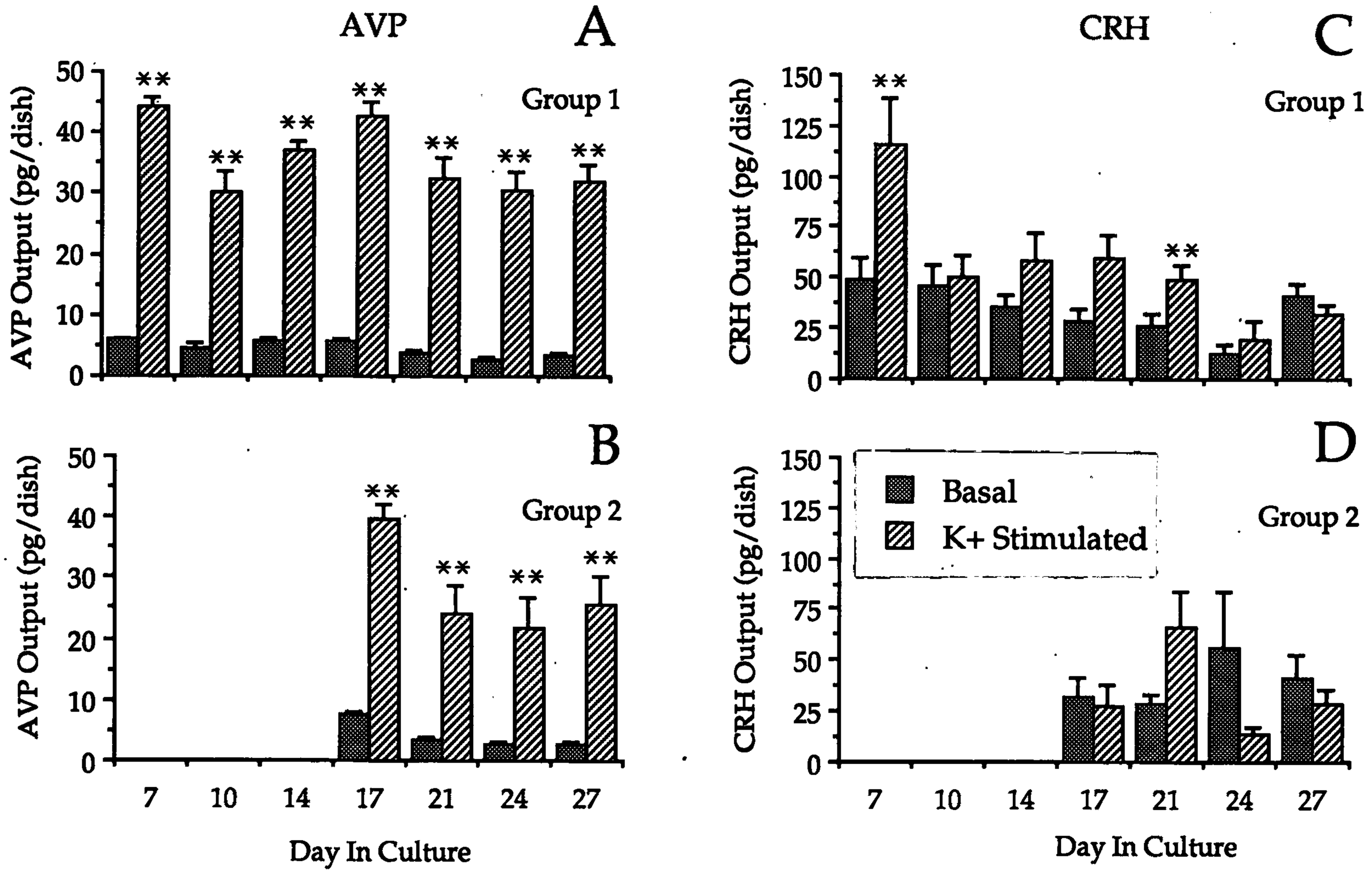
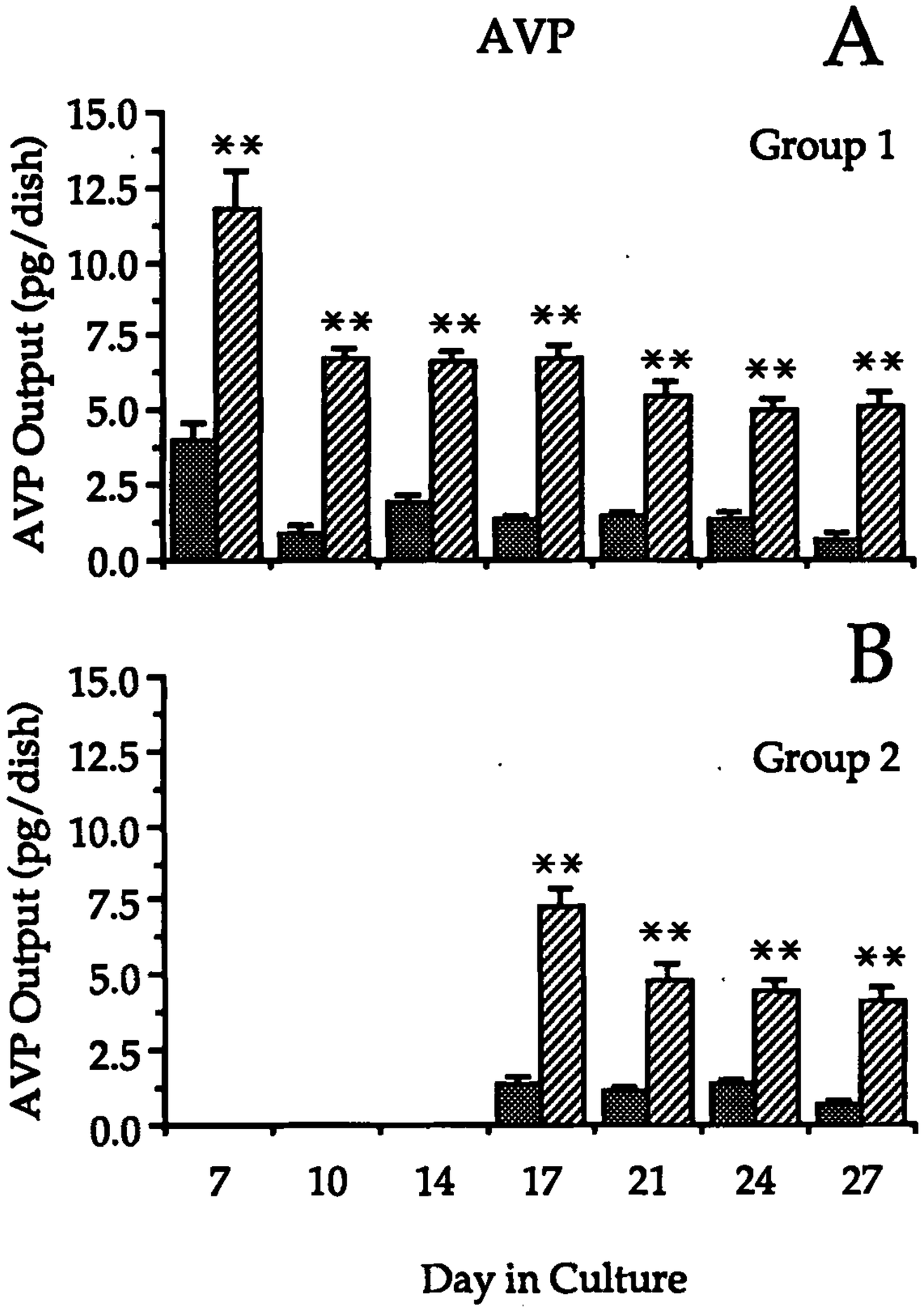


Figure 5.4. AVP and CRH secretion in fetal sheep hypothalamic cultures from 100 days of gestation. AVP and CRH secretion was measured in Group 1 cultures between day 7 and day 27 (**A** and **C** respectively) and in Group 2 cultures between day 17 and day 27 *in vitro* (**B** and **D** respectively). Data shown are means of 19 wells, +/- S.E.M. ** $p < 0.01$; significant difference between basal and 56 mM potassium incubations.

Day 100 Gestation



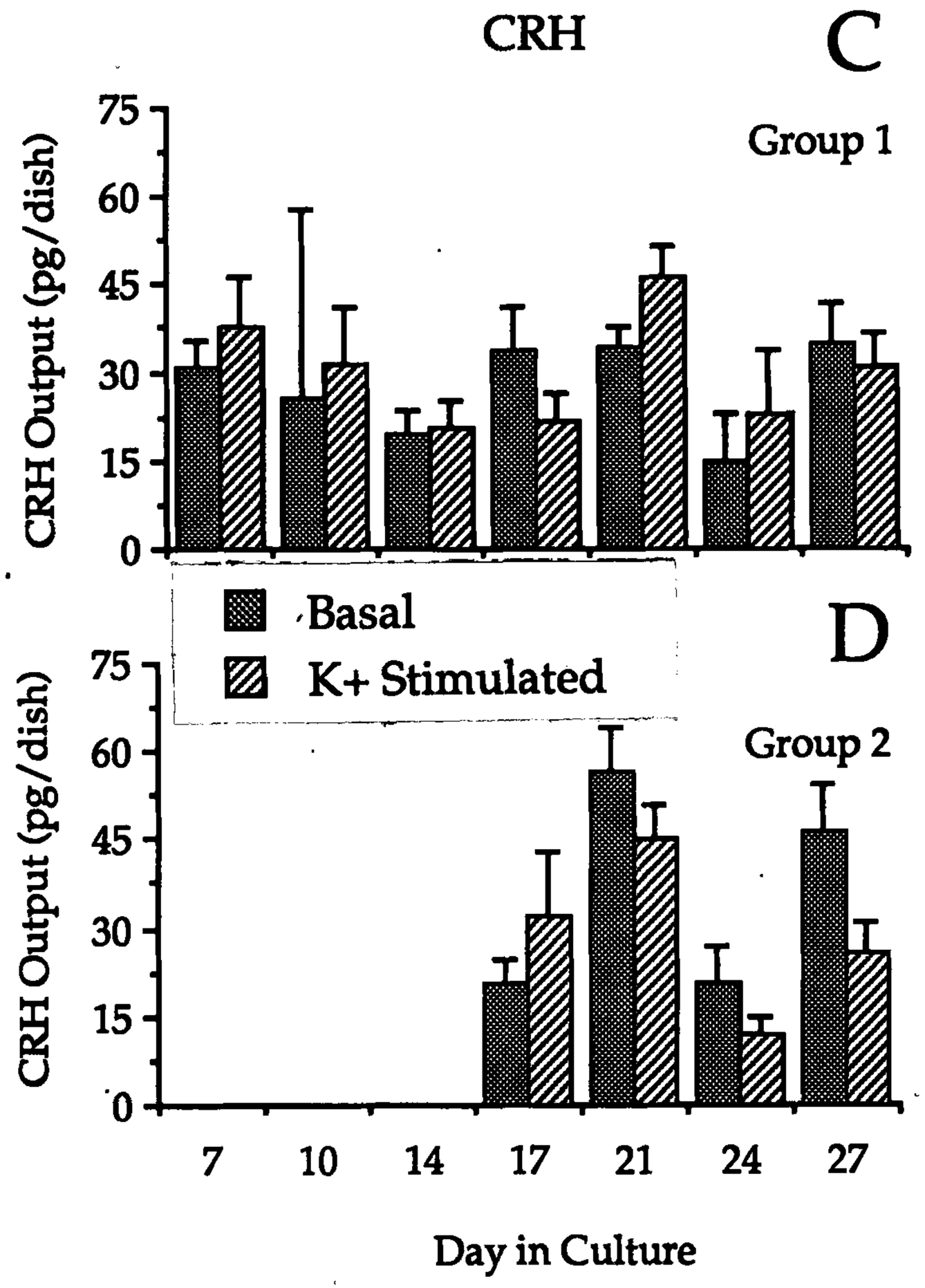
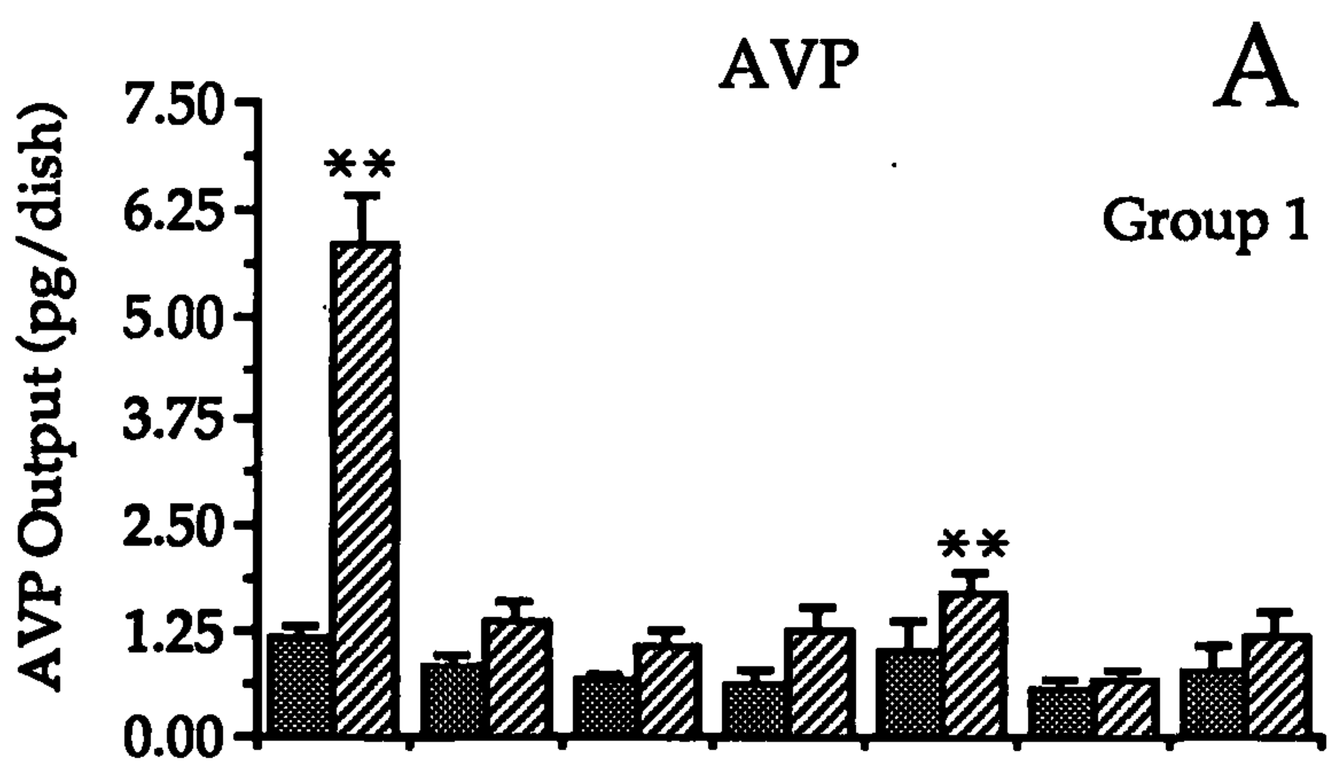
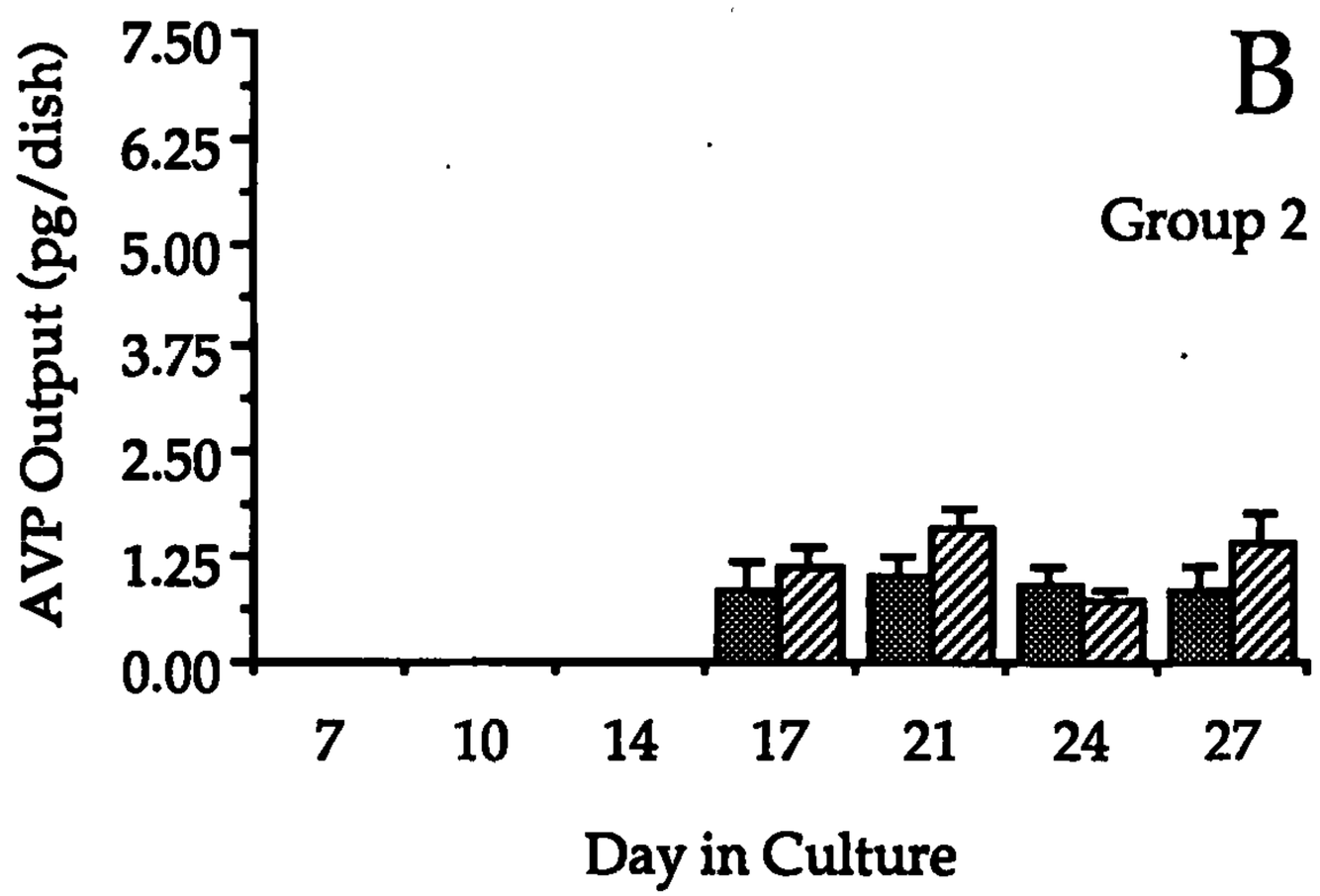
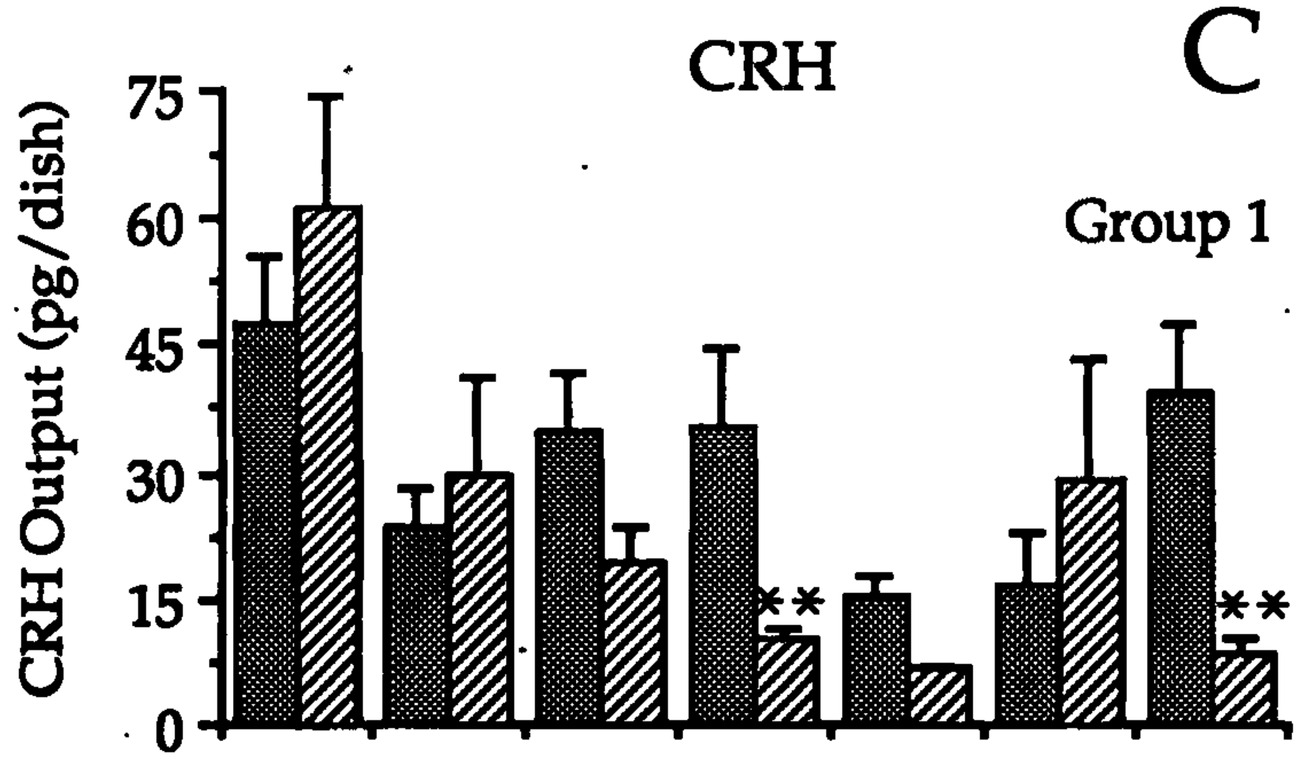


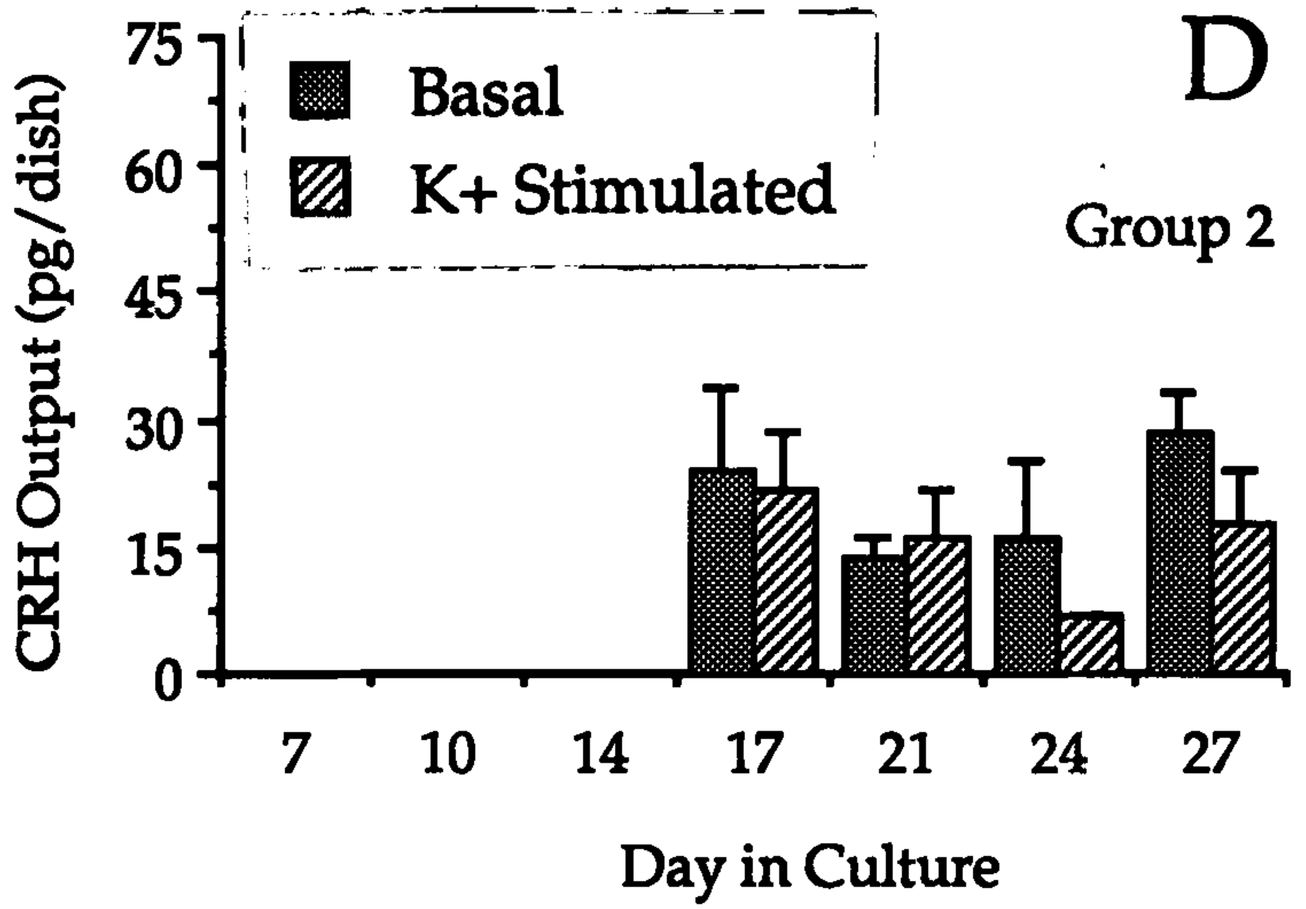
Figure 5.5. AVP and CRH secretion in fetal sheep hypothalamic cultures from 130 days of gestation. AVP and CRH secretion was measured in Group 1 cultures between day 7 and day 27 (A and C respectively) and in Group 2 cultures between day 17 and day 27 *in vitro* (B and D respectively). Data shown are means of 16 or more wells, +/- S.E.M. * $p < 0.05$, ** $p < 0.01$; significant difference between basal and 56 mM potassium incubations.

Day 130 Gestation









The design of the present study allowed a comparison between day 17 and 27 *in vitro* of AVP and CRH secretion by cells which either had (Group 1) or had not (Group 2) received previous experimental treatments, as shown also in figures 5.3 to 5.5. Overall analysis revealed that there were no significant differences in basal AVP secretion between the group 1 and group 2 cells in day 70, day 100 or day 130 cultures, neither was potassium-stimulated AVP secretion significantly different between the two groups at any gestational age. Similarly, there were no differences in basal or stimulated CRH secretion between the two groups at any gestational age.

5.3.2. Time and Calcium dependency of AVP and CRH Release.

The time course of AVP and CRH release was studied on day 30 *in vitro*, and on day 33, the calcium dependency of peptide scretion was assessed. Figures 5.6, 5.7 and 5.8 show the time dependency and calcium dependency of AVP and CRH release from cultures of day 70, 100 and 130 hypothalamic cells respectively.

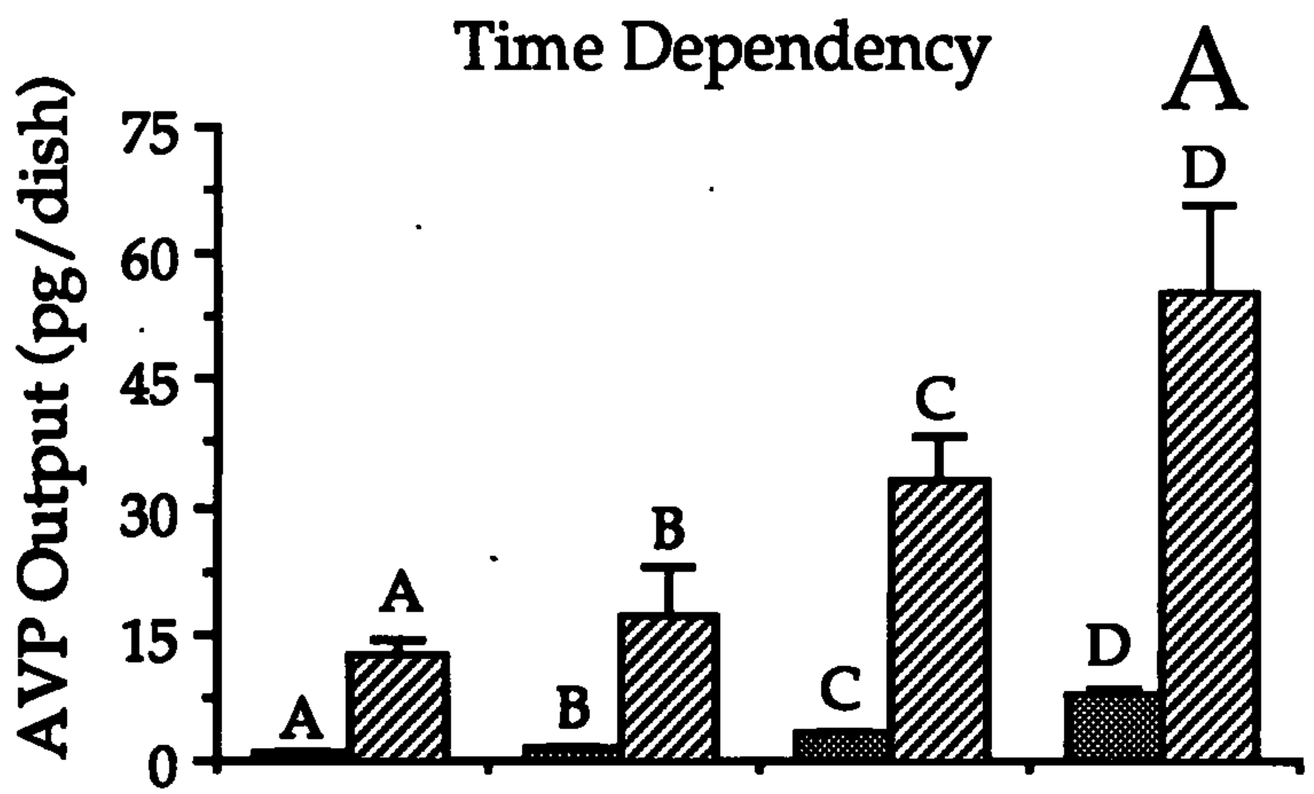
The quantity of AVP released from day 70 and day 100 cells was dependent on the duration of the incubation. With each increase in incubation length between 30 and 240 minutes in day 70 cultures, there was a significant increase in both basal ($p < 0.01$) and potassium-stimulated ($p < 0.01$) AVP release. In day 100 cells, basal and potassium-stimulated release of AVP was significantly increased between 60 and 120 minutes ($p < 0.01$), and between 120 and 240 minutes ($p < 0.01$). By contrast, there were no significant differences between the AVP contents of samples collected from day 130 cells after 30, 60, 120 or 240 minutes basal incubation. However, potassium stimulation for 240 minutes caused a significant elevation of AVP levels when compared to potassium stimulation for 30 minutes ($p < 0.05$).

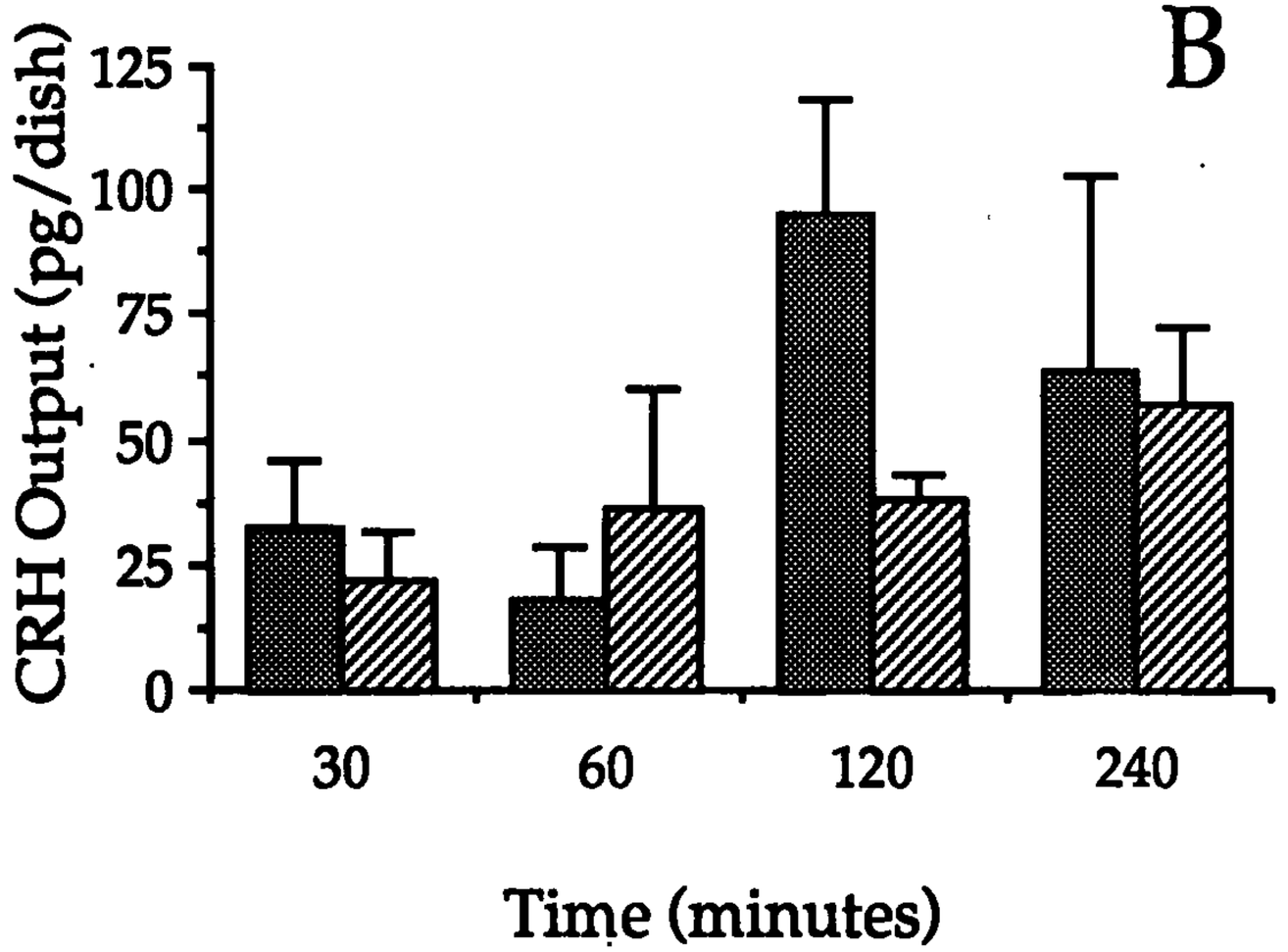
Overall analysis showed that there was no significant effect of incubation length on basal or potassium-stimulated CRH output from day 70, day 100 or day 130 hypothalamic cultures.

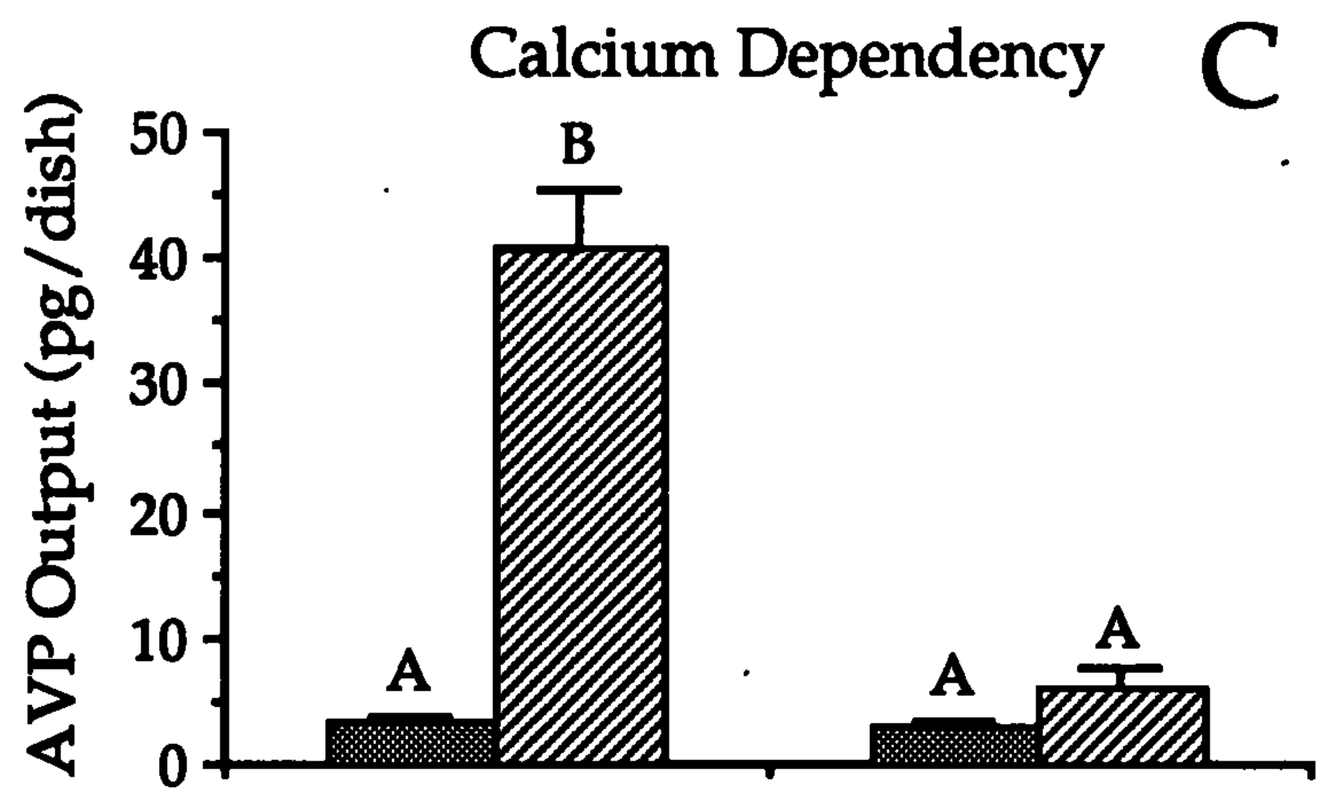
Figures 5.6-5.8 show that AVP secretion was calcium-dependent in cells from day 70 and day 100 of gestation. 56 mM potassium-containing medium significantly stimulated AVP output in these cells ($p < 0.01$)

Figure 5.6. Time and calcium dependency of AVP and CRH secretion by hypothalamic cultures from 70 days of gestation. A. Basal and potassium-stimulated AVP and B CRH secretion were measured in incubations of increasing duration, to show time dependency of peptide release. Data shown are means of 5-7 wells, +/- S.E.M. Data within either basal or potassium-stimulated groups were compared amongst their own groups. Data without common superscripts are significantly different ($p < 0.01$). C. AVP and D CRH secretion were determined under basal and potassium-stimulated conditions, and the effects of omitting calcium from the elevated potassium medium were examined. Data shown are means of 13 wells, +/- S.E.M. Data without common superscripts are significantly different (Upper case; $p < 0.01$; lower case; $p < 0.05$).

Day 70 Gestation







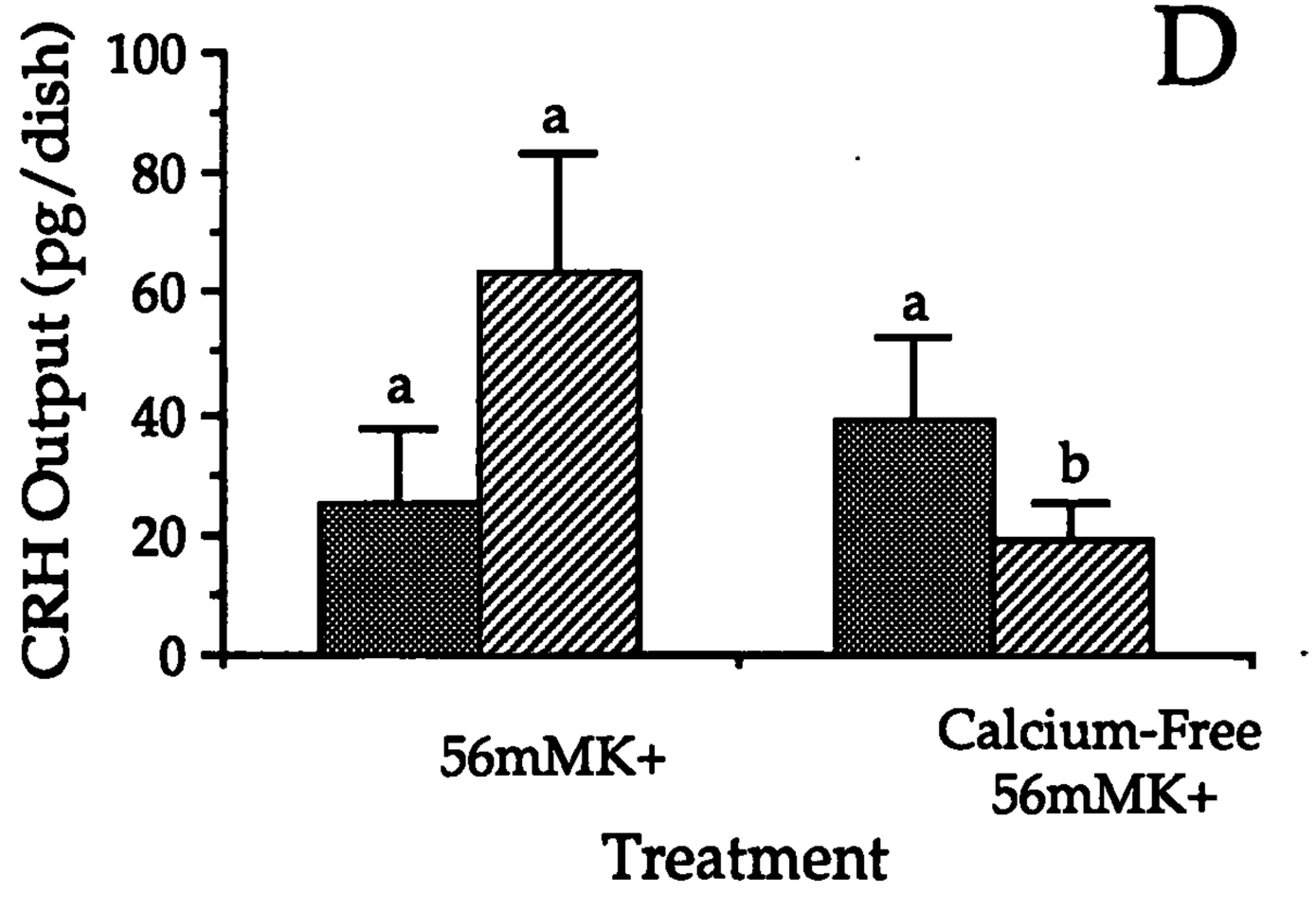
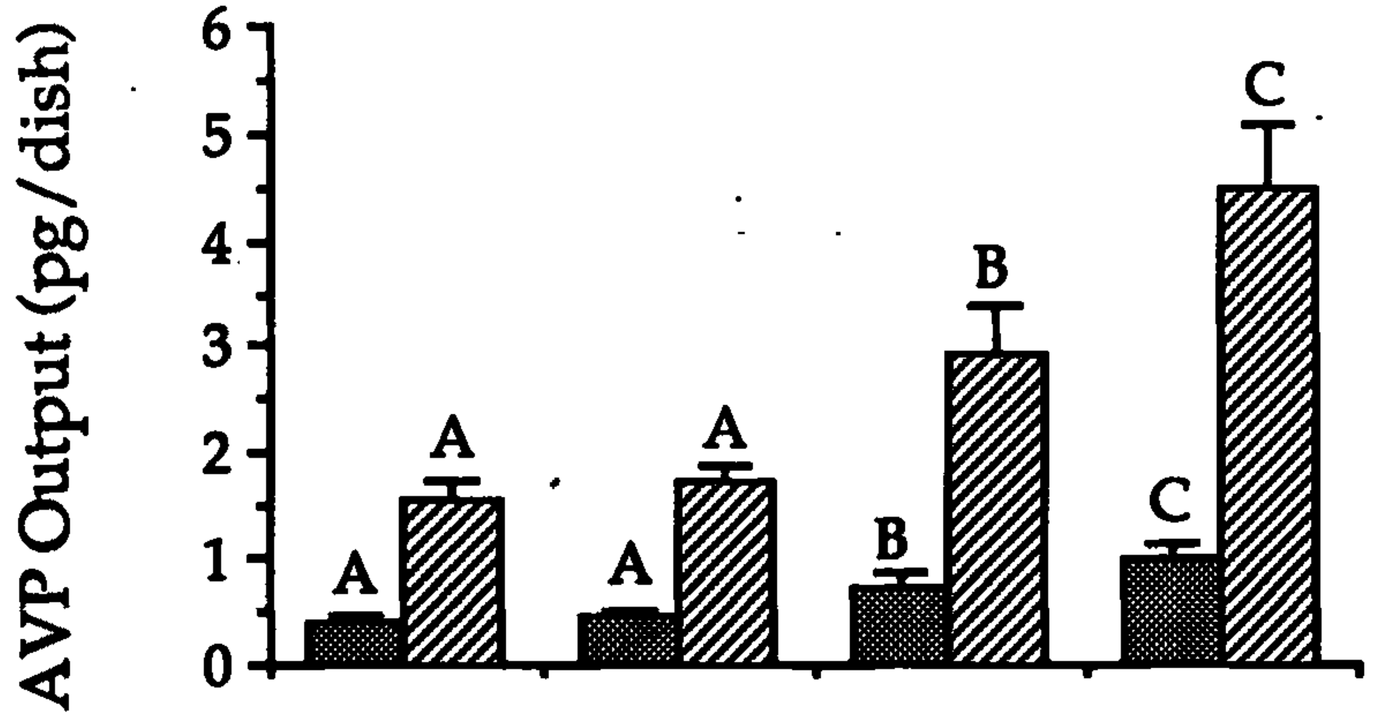


Figure 5.7. Time and calcium dependency of AVP and CRH secretion by hypothalamic cultures from 100 days of gestation. A. Basal and potassium-stimulated AVP and B CRH secretion were measured in incubations of increasing duration, to show time dependency of peptide release. Data shown are means of 9-10 wells, +/- S.E.M. Data within either basal or potassium-stimulated groups were compared amongst their own groups. Data without common superscripts are significantly different ($p < 0.01$). C. AVP and D CRH secretion were determined under basal and potassium-stimulated conditions, and the effects of omitting calcium from the elevated potassium medium were examined. Data shown are means of 18-19 wells, +/- S.E.M. Data without common superscripts are significantly different (Upper case; $p < 0.01$; lower case; $p < 0.05$).

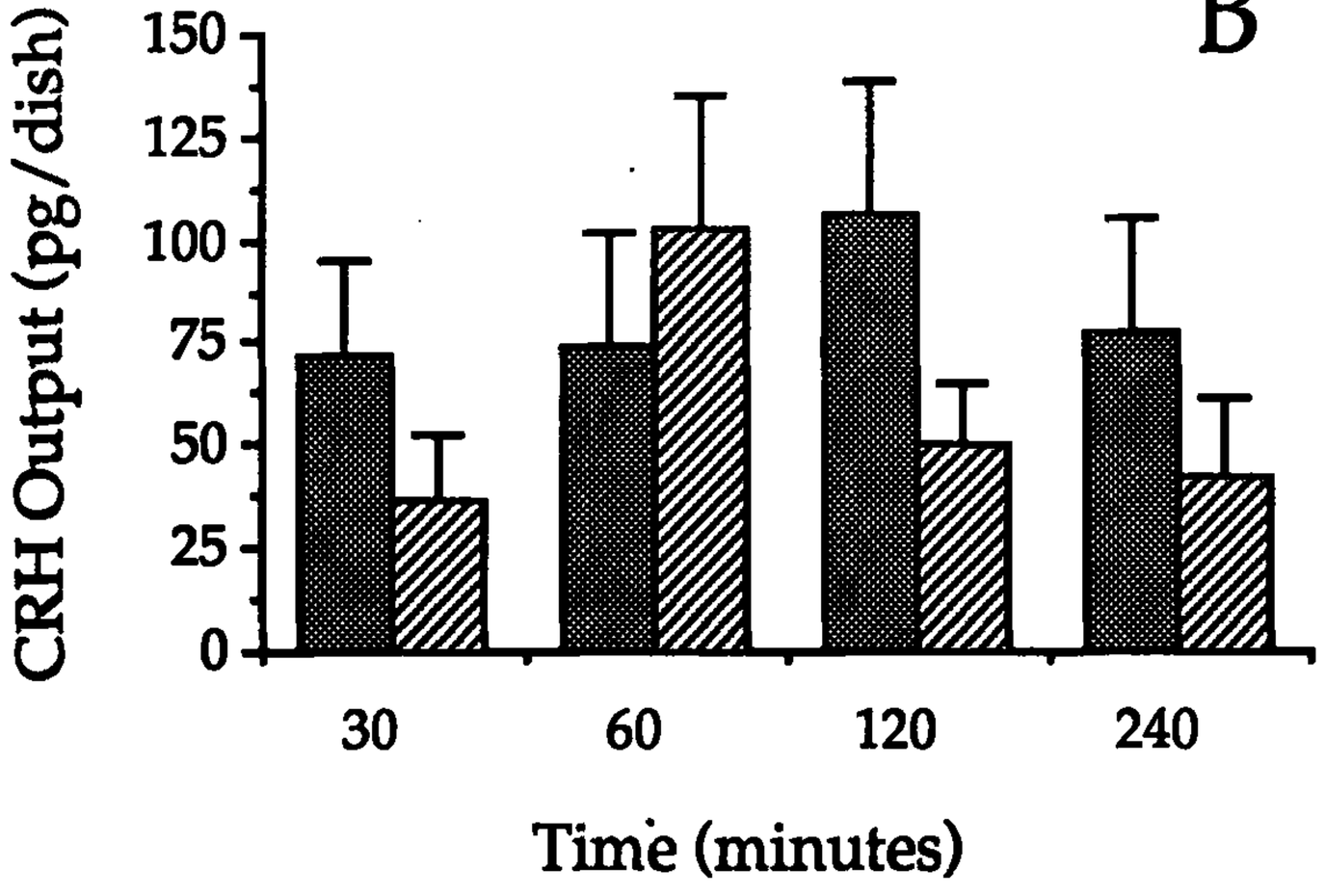
Day 100 Gestation

Time Dependency

A

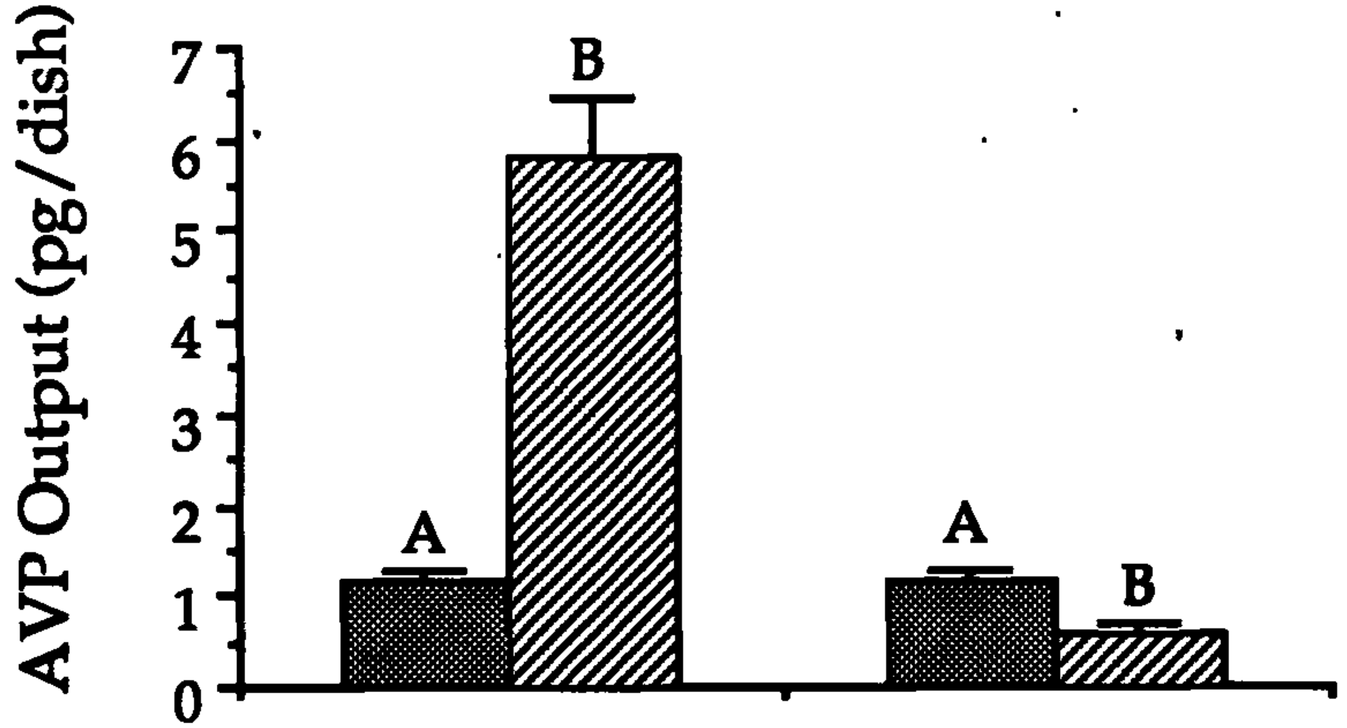


B



Calcium Dependency

C



D

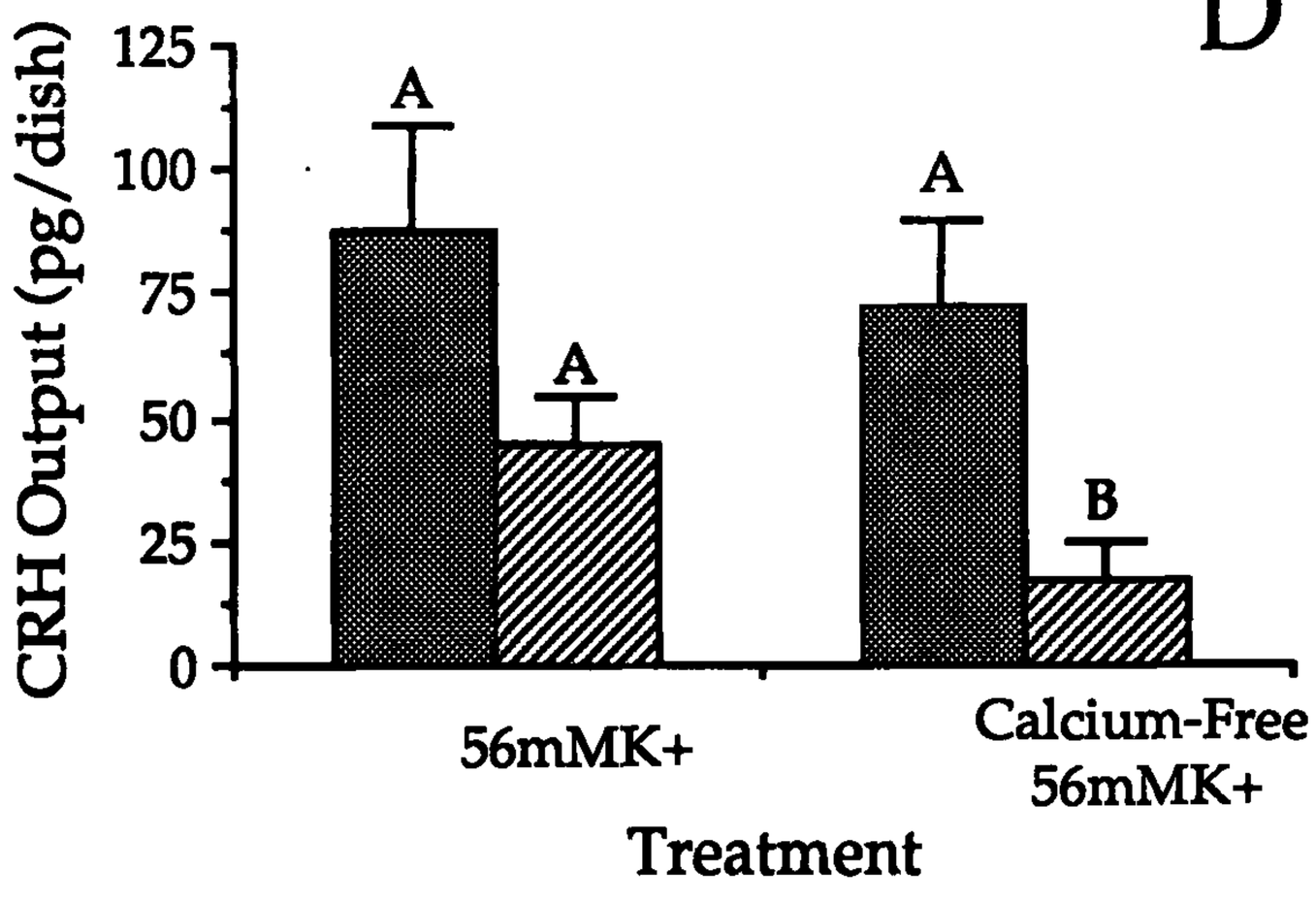
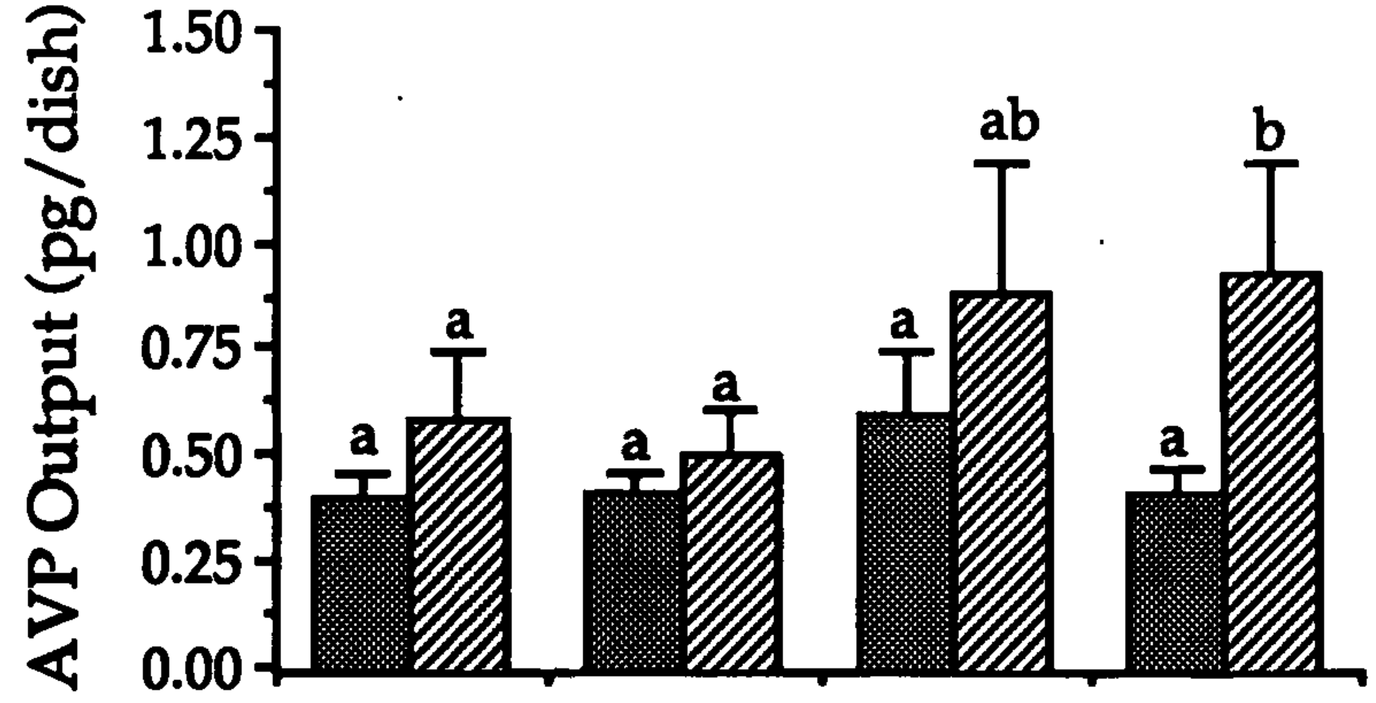
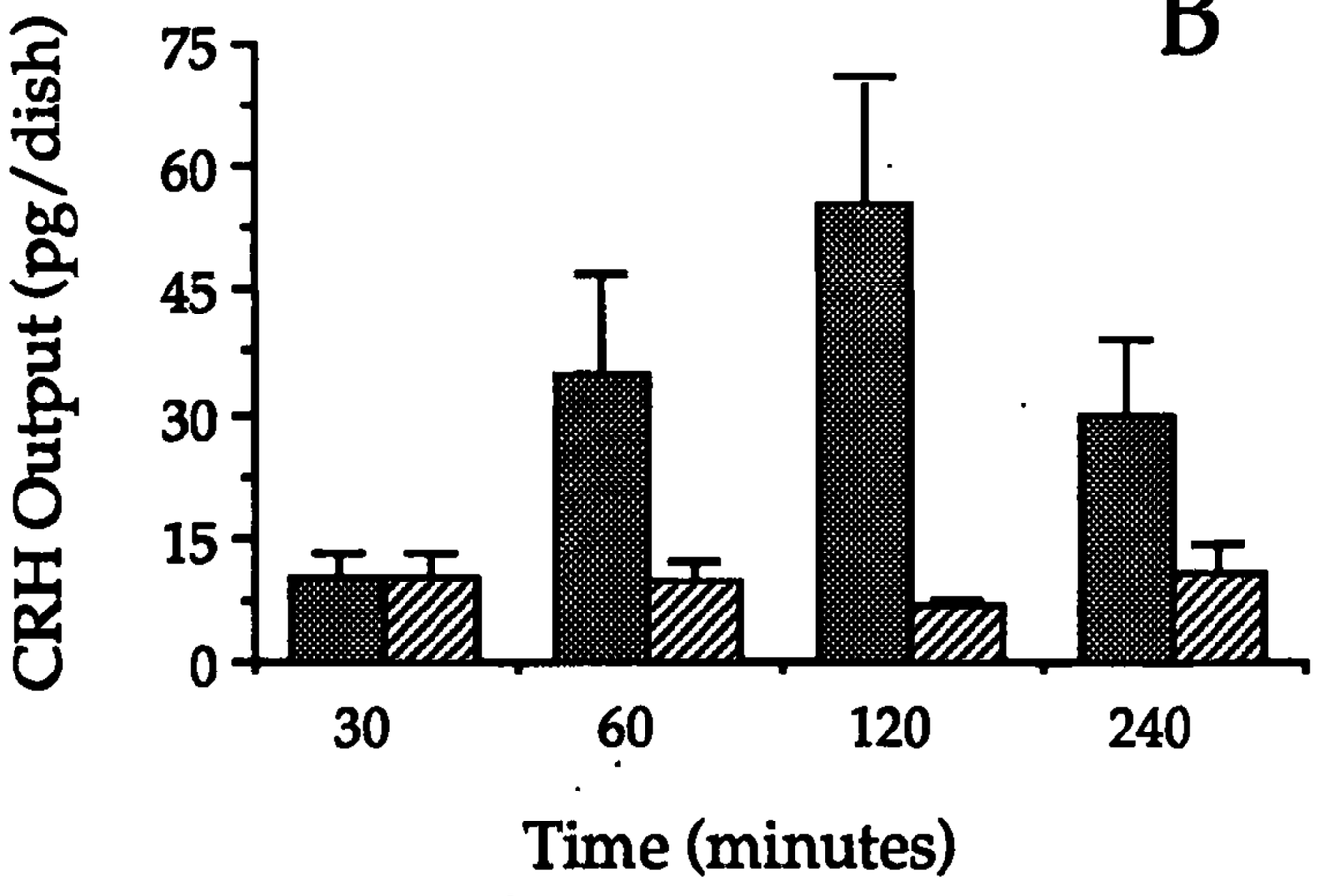


Figure 5.8. Time and calcium dependency of AVP and CRH secretion by hypothalamic cultures from 130 days of gestation. A. Basal and potassium-stimulated AVP and B CRH secretion were measured in incubations of increasing duration, to show time dependency of peptide release. Data shown are means of 8 wells, +/- S.E.M. Data within either basal or potassium-stimulated groups were compared amongst their own groups. Data without common superscripts are significantly different ($p < 0.01$). C. AVP and D CRH secretion were determined under basal and potassium-stimulated conditions, and the effects of omitting calcium from the elevated potassium medium were examined. Data shown are means of 14-15 wells, +/- S.E.M. Data without common superscripts are significantly different (Upper case; $p < 0.01$; lower case; $p < 0.05$).

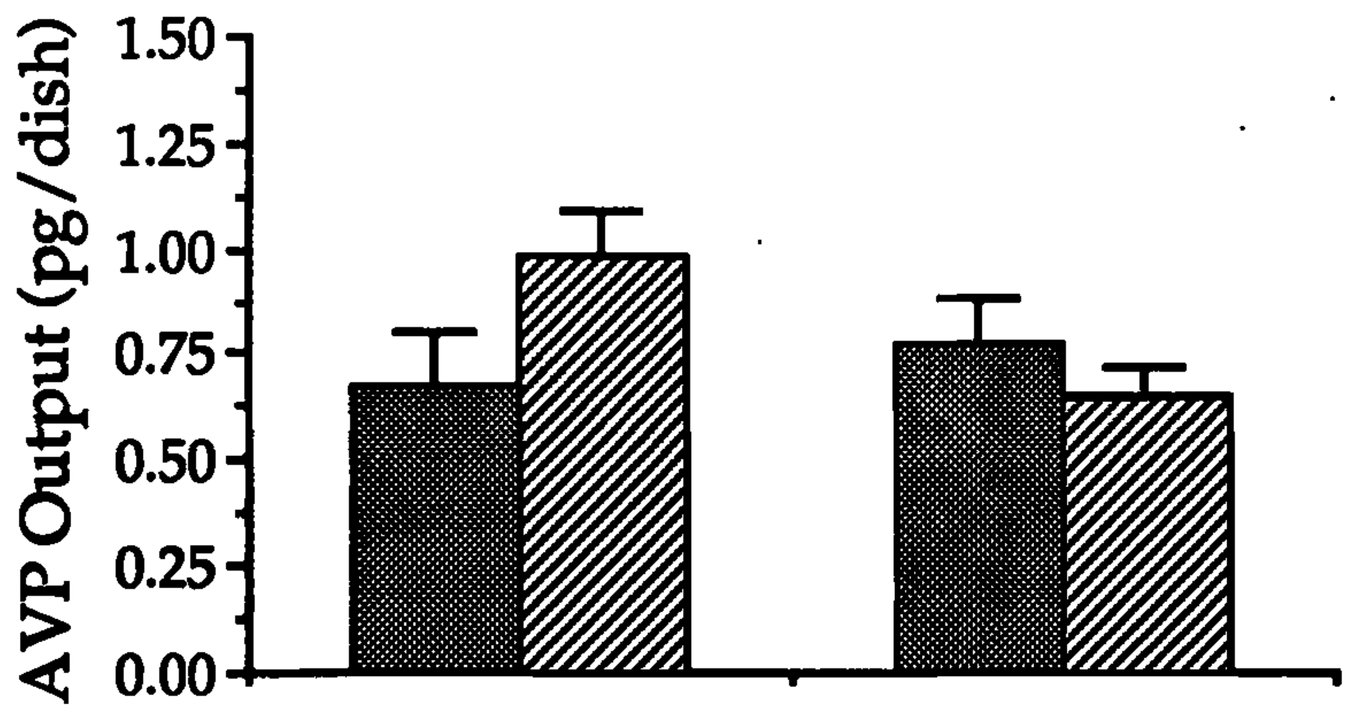
Day 130 Gestation

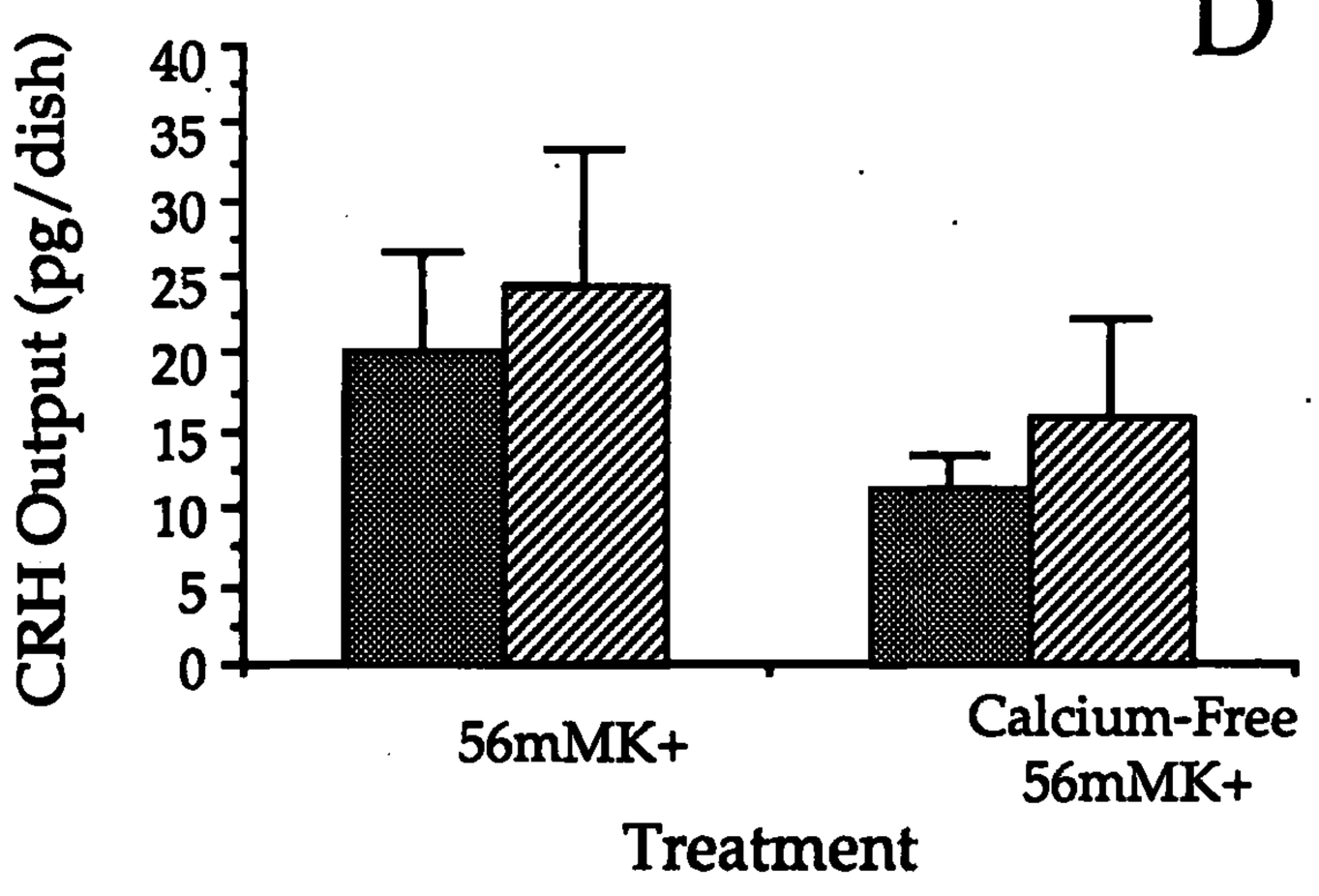
Time Dependency A





Calcium Dependency C





whereas calcium-free 56 mM potassium-containing medium either had no effect (day 70 cells) or was significantly inhibitory to AVP release (day 100 cells, $p < 0.01$) when compared to basal AVP secretion. Potassium-stimulated AVP secretion from day 130 cells was not significantly different from basal release, either in the presence or absence of calcium.

As depicted in figures 5.5-5.8, CRH release was not significantly stimulated in the presence of elevated potassium at any gestational age. However, in day 70 ($p < 0.05$) and day 100 ($p < 0.01$) cells, calcium-free 56 mM potassium-containing medium significantly inhibited CRH release .

5.3.3. Culture Contents of AVP and CRH.

At day 36 *in vitro* , the contents of CRH and AVP were measured in each well. Figure 5.9 shows that advancing gestational age was associated with significant reductions in the contents of AVP and CRH. AVP and CRH levels in day 100 cells were significantly less ($p < 0.01$) than the corresponding levels in day 70 cells. In day 130 cultures the AVP content had fallen significantly compared to day 100 cells ($p < 0.01$), however, there was no signficant difference between the CRH contents at these gestational ages.

5.3.4. Cell Counting and Immunocytochemistry.

On day 36*in vitro*,, resuspended cells were counted with a haemocytometer. Figure 5.10 shows that there was no significant difference in the number of cells between day 70 and day 100 cultures. However, a significant increase was observed between day 100 and day 130 cells ($p < 0.01$). Figure 5.10 also shows that there was a significant decline in the proportion of cells which were immunopositive for NSE with advancing gestational age. This technique demonstrated that whilst 65 +/- 0.5 % (mean +/- S.E.M.) of cells were labelled by this antibody in day 70 cultures, this proportion fell significantly ($p < 0.01$) to 51 +/- 1.4% in day 100 cells and again ($p < 0.01$) to 46 +/- 1.5% in day 130 cultures.

Figure 5.9. AVP and CRH content of hypothalamic cultures after 36 days *in vitro* .
Cultures were extracted on day 36 and A the AVP and B the CRH contents of the cell cultures were determined. Data shown are means of 20-28 wells, +/- S.E.M. Data without common superscripts are significantly different ($p < 0.01$).

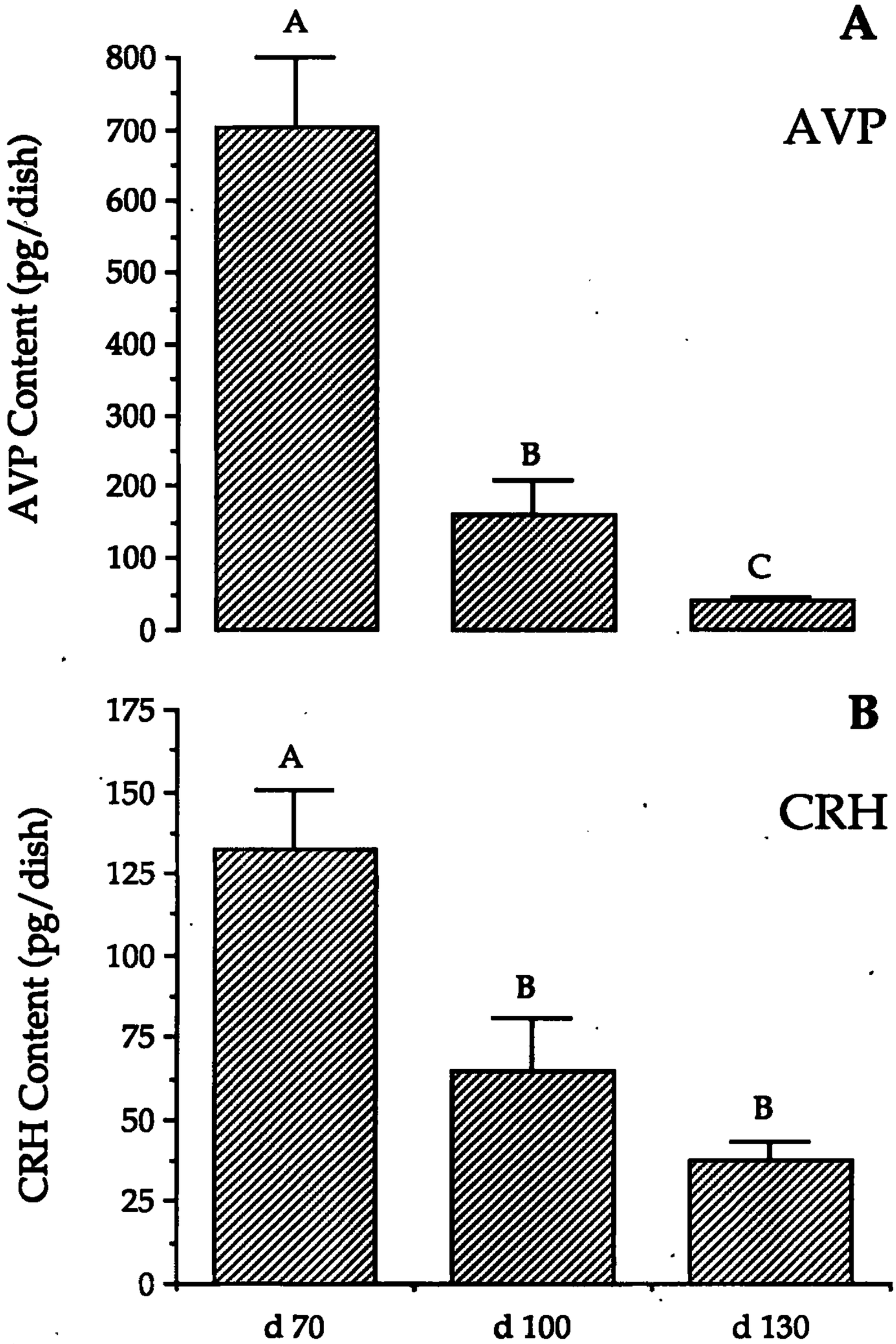
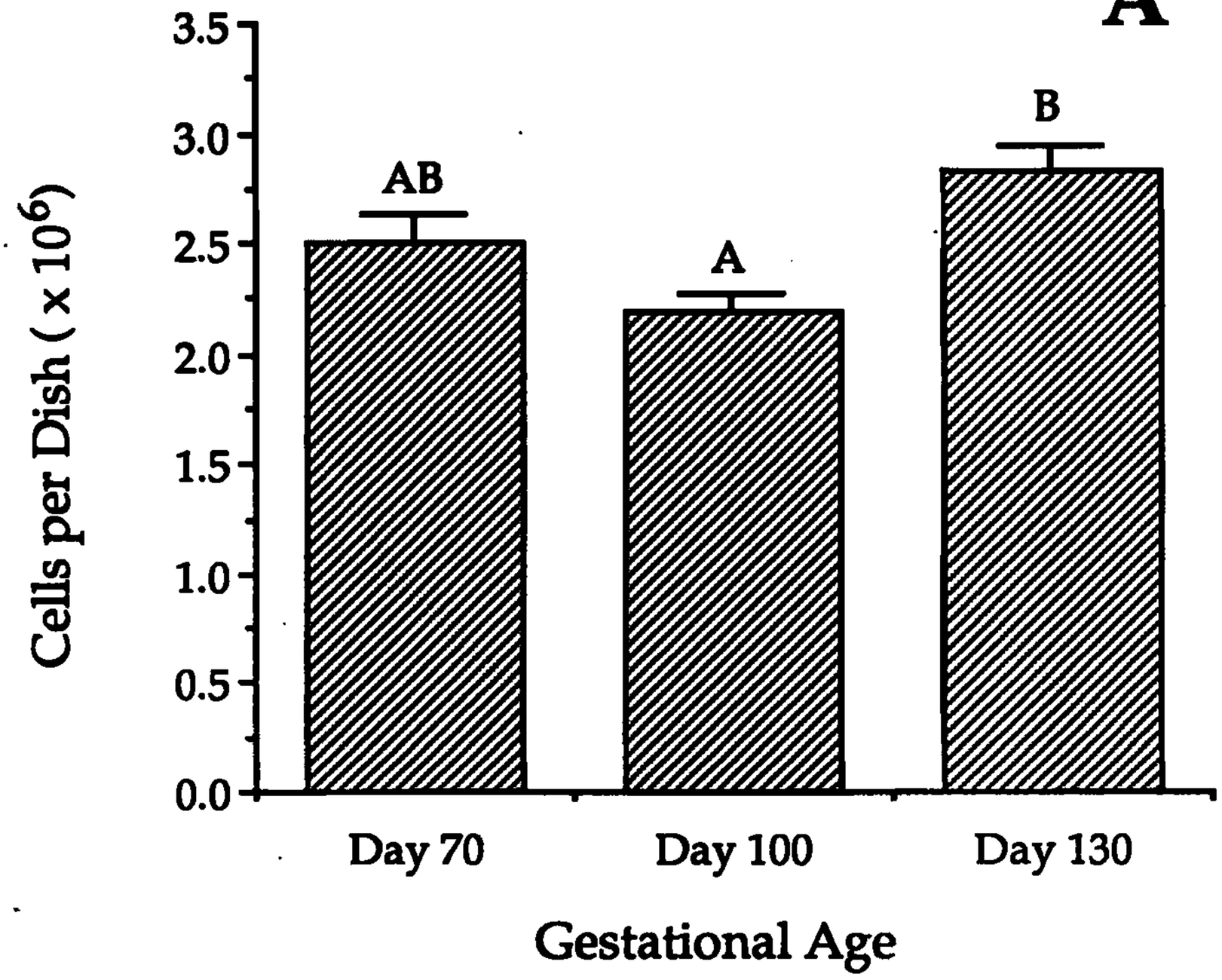
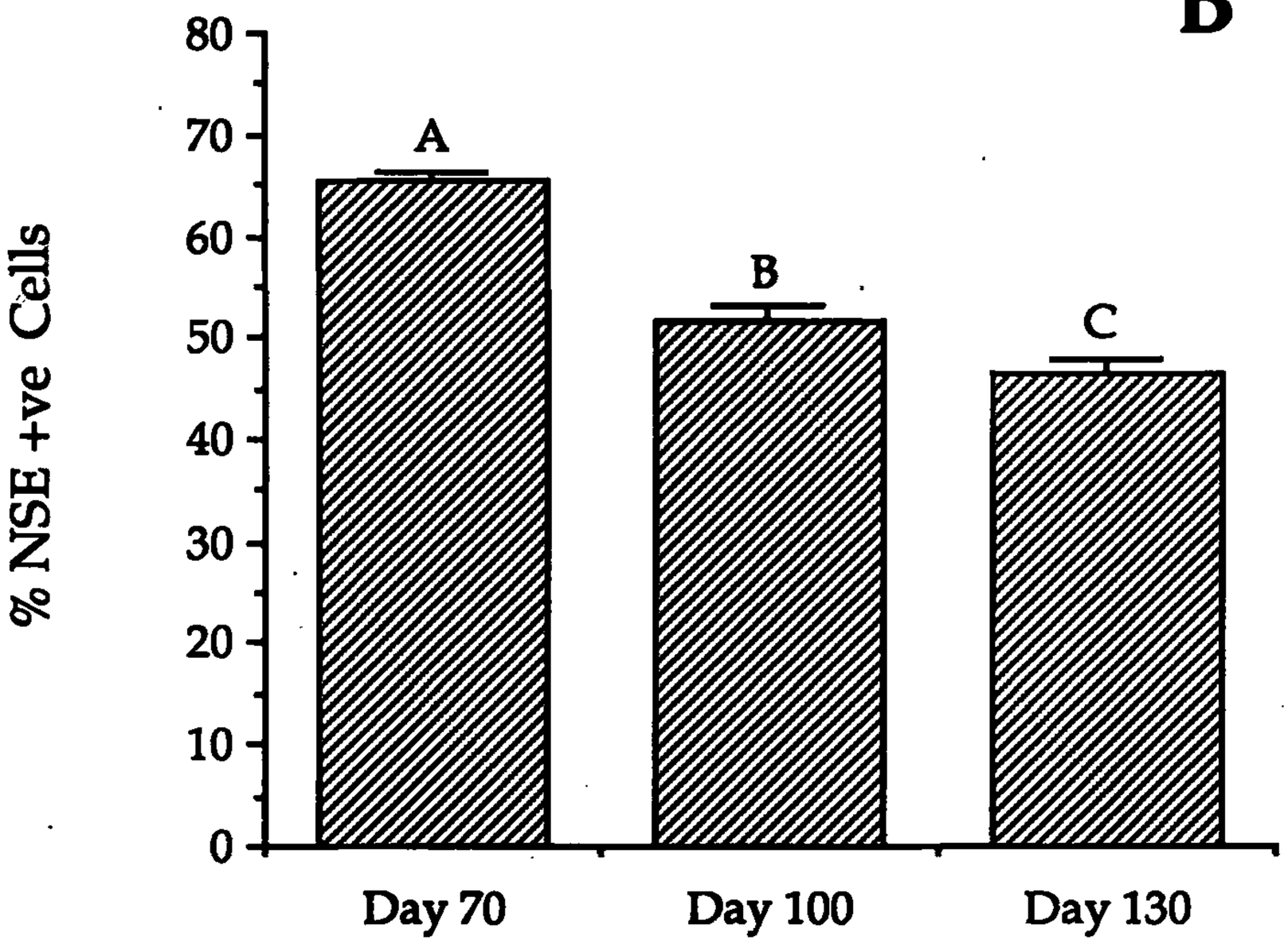

Peptide Content
A
AVP
AVP Content (pg/dish)
800
700
600
500
400
300
200
100
0
A
B
C
B
CRH
CRH Content (pg/dish)
175
150
125
100
75
50
25
0
A
B
B
d 70
d 100
d 130
Gestational Age

Figure 5.10. Total cell numbers present in cultures on day 36 *in vitro* and the proportion immunopositive for NSE. A. The number of cells per dish was determined in cultures at each gestational age. Data shown are means of 20-28 wells, +/- S.E.M. B. The propotion of cells which were immunopositive for NSE was assessed in representative wells in day 70, 100 and day 130 cultures. Data shown are means of 6 wells, +/- S.E.M. Data without common superscripts are significantly different ($p < 0.01$).

A


3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Cells per Dish (x 10^6)
AB
A
B
Day 70
Day 100
Day 130
Gestational Age

B


80
70
60
50
40
30
20
10
0
% NSE +ve Cells
A
B
C
Day 70
Day 100
Day 130
Gestational Age

5.4 Discussion

The objective of this study was to examine the secretion of AVP and CRH from cultured fetal sheep hypothalamic cells removed at day 70, day 100 and day 130 of gestation. AVP release from day 70 and day 100 cells was consistently stimulated by potassium-induced depolarisation, and these effects were shown to be time and calcium-dependent. In contrast, potassium rarely increased AVP secretion by day 130 cells, and AVP release from these cells was not convincingly shown to be time or calcium dependent. CRH release from day 70 cultures was sporadically enhanced by potassium stimulation, whereas no significant effect of potassium was seen in day 100 cells, although potassium treatment was inhibitory to CRH release from day 130 cultures on some occasions. However, none of the effects of potassium on CRH release were shown to be calcium and time dependent at any gestational age. An important finding made here was that in general, the secretion of AVP and CRH declined with advancing gestational age. Although this could be explained by a maturation-dependent reduction in the adaptability of the cells to the culture conditions, the large numbers of cells present *in vitro* after 36 days culture, and the considerable percentage of neurones found at this time in all cultures, suggested that reduction in peptide release was due to factors other than poor viability.

Considerable evidence has accumulated to suggest that hypothalamic drive to ACTH release is increased in fetal animals in late gestation. In the rat, fetal plasma ACTH levels are diminished by administration of CRH antibody to the fetus at day 19 of gestation (Term = day 22), but not in fetuses at day 17 (Boudouresque *et al.* 1988). ACTH-releasing bioactivities of fetal sheep hypothalamic extracts are much increased with advancing gestational age (Brieu *et al.* 1989), and the massive elevation in plasma ACTH levels which occurs in the last few days of gestation is abolished in fetuses in which the pituitary is disconnected from the hypothalamus (Antolovich *et al.* 1991). In contrast to these observations, the present study showed that overall, AVP and CRH secretion from day 100 cells was reduced when compared to day 70 cultures, and was further diminished in day 130 cells. In view of the observations made by previous authors, these data suggest that AVP

and CRH secretion from the cell cultures may not reflect the developmental pattern of hypothalamic secretion *in vivo*.

It was not possible to make a direct comparison of the results from the present study with data obtained in the intact animal, as there have been no studies to examine hypothalamic secretion of AVP and CRH into the portal blood of the sheep fetus. Nevertheless, Brooks and colleagues (1989) described CRH release from fetal sheep hypothalamic pieces maintained over the short term *in vitro*. These authors showed that CRH release from tissues removed at day 140 was significantly greater than CRH secretion from hypothalami collected at day 100 of gestation. These results suggested that that advancing gestation was associated with an increase in hypothalamic release of ACTH-releasing factors, in contrast to the present study, which showed that CRH and AVP release from day 130 cells was reduced when compared to output at day 100.

It was possible to explain the reduction in AVP and CRH output from the hypothalamic cultures in terms of the amounts of peptides contained by the cultured cells. The present study showed that the AVP and CRH contents of the cell cultures were decreased with advancing gestational age, in parallel with the reduction in secretion of these peptides. By contrast, a recent study has demonstrated that the contents of AVP and CRH in the intact fetal sheep hypothalamus were markedly increased with advancing gestational age (Brieu *et al*. 1989). Therefore, these results suggest that there may be factors present *in vivo* but not *in vitro*, which act to enhance the synthesis and storage of AVP and CRH with advancing gestational age. The present studies were not directed to reveal which endogenous substances might act to enhance peptide secretion from hypothalamic cells. However, cultured cells were removed from the normal presynaptic input which acts on hypothalamic neurones *in vivo*. Therefore, synthesis and storage of large quantities of AVP and CRH, as observed in intact hypothalami, may well be dependent on trophic stimulation from these presynaptic cells.

The possibility exists that decreasing output of AVP and CRH with advancing gestational age was due to a progressive reduction in culture viability. In order to test the hypothesis that gestational differences in peptide output arose through variations in culture viability, the total number of cells present in the culture dishes and the proportions of NSE-

immunopositive cells were quantified after 36 days *in vitro*. The results showed that regardless of gestational age, the number of cells remaining in culture on day 36 was remarkably similar to the number originally plated in each dish (2.5 x 10^6 cells). On the other hand, a smaller proportion of cells were NSE-immunopositive in cultures prepared from hypothalami collected later in gestation. These data suggested that the gestational reduction in peptide output was attributable to the presence of reduced numbers of neurones *in vitro*, although it was notable that the small decline in the percentage of NSE-positive cells did not parallel the dramatic reduction in peptide output. However, the possibility remains that the numbers of CRH and AVP cells were selectively attenuated in cultures prepared later in gestation.

Quantifying the numbers of AVP and CRH cells *in vitro* was one of the objectives of the present investigation, to which end immunocytochemical techniques were developed to detect AVP- and CRH-containing cells. Unfortunately, the final validation procedures for these techniques showed that the immunostaining was not specific to CRH or AVP cells, as the staining persisted when specific AVP and CRH antibodies were removed. It was therefore not possible to carry out the quantification of cultured AVP and CRH cells as planned. However, future studies, utilising techniques similar to those described here, could develop affinity-purified antibodies for use in immunostaining procedures. These antisera would be less likely to show the non-specific staining observed in the present studies.

Recent investigations have shown that potassium-induced depolarisation stimulates AVP and CRH secretion from fetal rat hypothalamic cells *in vitro* (Clarke, 1988). These studies showed that the response to potassium was augmented as the culture aged, and that aside from its direct stimulatory action on AVP and CRH release, potassium treatment acted in a trophic manner to effect an overall enhancement of AVP and CRH release. The present study was designed to investigate basal and potassium-stimulated release of AVP and CRH from fetal sheep hypothalamic cells over several weeks *in vitro*. In contrast to the results obtained in fetal rat hypothalamic cultures, there was no progressive increase in AVP or CRH secretion observed over these weeks *in vitro* at any gestational age. Rather, peptide secretion tended to be greatest on the

first experimental day, and then to plateau thereafter. To determine whether potassium stimulation could act in the trophic manner described in fetal rat hypothalamic cultures, the present studies also compared AVP and CRH secretion between cultured cells previously left untreated or potassium-stimulated 3 times. A rigourous statistical evaluation showed that there were no significant differences in basal or potassium-induced release of AVP or CRH between the 2 groups at any gestational age, which again contrasts with the enhancing effects of potassium reported in rat cells (Clarke, 1988).

Taken together, the data described above suggest that the responses of cultured fetal sheep and rat hypothalamic cells to repeated potassium stimulations are dissimilar in some respects. This may relate to differential effects of potassium stimulation on AVP and CRH gene transcription in the two species. Recently, it has become evident that potassium-induced depolarisation elevates intracellular calcium levels, resulting in phosphorylation of cAMP response element-binding protein (CREB; Sheng, McFadden & Greenberg, 1990). CREB may then activate the transcription of c-*fos* , one of a class of immediate early response genes which are thought to play a major role in the regulation of gene transcription in neuronal cells (Sheng & Greenberg, 1990). In addition, CREB may act directly to regulate neuropeptide gene transcription, as CREB binding sites have been located on the regulatory regions of the genes coding for somatostatin (Montminy & Bilezkijian, 1987) and preproenkephalin (Comb, Birnberg, Seasholtz, Herbert & Goodman, 1986). Therefore, the trophic effect of potassium stimulation observed in fetal rat hypothalamic cultures may be due to increases in AVP and CRH gene transcription. By contrast, the absence of this effect in the present studies suggests that potassium stimulation may not act in the same way in sheep cells. Future studies could investigate the regulation of transcription factors, including c-*fos* and CREB, in the fetal sheep hypothalamus. In this way, it may be possible to understand the mechanisms underlying the massive increases in hypothalamic AVP and CRH content observed in the fetal sheep hypothalamus before parturition (Brieu *et al.* 1989).

In conclusion, the studies described in this chapter investigated the secretion of AVP and CRH from fetal sheep hypothalamic cells cultured

at different gestational ages. The principal findings were that in cultures prepared at day 70 and day 100 of gestation, the secretion of AVP was stimulated by potassium-induced depolarisation in a time- and calcium-dependent fashion. By contrast, neither AVP release from day 130 cells nor CRH release from any of the cultures showed consistent responses to potassium stimulation. Overall, the results showed that secretion of AVP and CRH from fetal hypothalamic cells was reduced with advancing gestational age. In view of the apparent viability of the cultures in terms of the numbers of cells remaining after 36 days *in vitro* and the considerable percentage of neurones present, the data suggested that hypothalamic cells may be dependent on extrahypothalamic input to maintain the high levels of peptide content observed *in vivo*.

6. Corticotrophin-Releasing Factors in the hypothalamus of the developing fetal sheep.

In the previous chapter, the fetal sheep hypothalamic culture system optimised in chapter 4 was used to investigate the development of AVP and CRH secretion by the fetal sheep hypothalamus *in vitro*. The results of these studies clearly suggested that *in vitro* hypothalamic secretion of the ACTH-releasing factors AVP and CRH is much diminished with advancing gestational age in the sheep fetus. These data contrast with evidence obtained by other workers, which supports the hypothesis that hypothalamic drive to fetal pituitary ACTH secretion is increased with advancing gestational age. However, few reports have directly investigated ACTH-releasing factors in the fetal sheep hypothalamus, and none have examined the various molecular species which may be biologically active in this regard. Therefore, the current understanding of ACTH-releasing factors in the fetal sheep hypothalamus is not sufficient to relate the results of the previous chapter to hypothalamic function *in vivo*. Using a number of different approaches, such as radioimmunoassay, gel chromatography and pituitary cell bioassay, this chapter sought to catalogue more fully the ontogeny of ACTH-releasing factors in the fetal sheep hypothalamus.

6.1 Introduction

Prior to the advent of hypothalamic tissue culture, direct methods to study the developing hypothalamic drive to corticotropes in the fetus were not available, and so a number of authors assessed hypothalamic contents of ACTH-releasing factors as an index of the functional maturity of fetal hypophysiotropic neurones. In the fetal sheep, immunoreactive CRH and AVP have been detected by RIA in hypothalamic extracts as early as day 63-88 of gestation (Brieu *et al.* 1989). The hypothalamic concentration of CRH and AVP increased progressively from day 63-88 to day 138 of gestation, and these increases were correlated with increasing

ACTH-releasing bioactivity of the hypothalamic extracts. However, the AVP and CRH content fell by 80% and 50% respectively between day 138 and day 143, whereas ACTH-releasing activity fell only slightly during this time frame. To account for the discrepancy between reductions in hypothalamic CRH and AVP and bioactivity, these data suggest the presence of hypothalamic factors, other than CRH and AVP, which possess substantial ACTH-releasing activity in the late gestation hypothalamus. Alternatively, the hypothalamic contents of ACTH-releasing factors as measured by RIA may not accurately reflect bioactive CRH and AVP, as RIA does not differentiate between biologically inactive precursors or mature peptides bearing the same antigenic determinants.

Recently, data have been obtained in the fetal human hypothalamus to suggest that CRH immunoreactivity is accounted for by two forms of CRH. In addition to a small bioactive CRH which co-elutes with CRH(1-41) on Sephadex chromatography, there is present a large CRH of molecular weight 8-10kD which shows no ACTH-releasing activity (Ackland *et al.* 1986). Between 12-16 weeks of gestation, the high molecular weight species accounts for approximately 50% of CRH immunoreactivity. This proportion is reduced in more mature fetuses and is absent in fetal hypothalami removed after week 20 of gestation. Therefore, hypothalamic maturation in the fetal sheep may also be associated with substantial remodelling of CRH and AVP peptides. However, at the present time, no studies have been carried out to examine the developing immunoreactivity and bioactivity of individual hypothalamic releasing factors in the fetal sheep.

The aim of this chapter was therefore to examine the ontogeny of ACTH-releasing factors in the fetal sheep hypothalamus. AVP and CRH were extracted from hypothalamic tissues removed at day 70, 100 and 130 of gestation, and the hypothalamic contents of these peptides were determined by specific AVP RIA and CRH IRMA. In order to investigate the ontogeny of molecular weight species of AVP and CRH, the hypothalamic extracts were subjected to Sephadex chromatography. Fractions were collected and lyophilised, and the AVP and CRH immunoreactivity were determined in these fractions. In addition, the ontogeny of bioactive ACTH-releasing factors was examined by

reconstituting aliquots of these fractions in culture medium, and adding these to an adult sheep pituitary cell bioassay.

6.2 Experimental Procedures

Fetal sheep hypothalami were dissected as previously described for hypothalamic cultures. They were snap frozen on dry ice and stored at -70°C. A total of 16 hypothalami were collected from fetuses at day 70 (5), day 100 (6) and day 130 (5) of gestation to carry out the work described here.

6.2.1. Immunoreactive AVP and CRH in the Fetal Sheep Hypothalamus.

Extraction. Hypothalamic extracts were prepared according to published methods (Brooks *et al*. 1989). Tissue was weighed and added to 1 ml boiling acid mixture (0.1 M HCl, 1 M Acetic acid, 50:50). The hypothalami were boiled for 5 minutes, cooled on ice, and then homogenised in a glass homogeniser (Uniform Homogeniser; Jencons Scientific, Leighton Buzzard, Bedfordshire). The homogeniser was washed out with 1 ml acid mix, which was added to the tissue homogenate. The homogenate was then vortexed, and 50µl of the suspension was removed for measurement of protein content. The tissue extracts were centrifuged for 30 minutes at 10,000g (Beckman J2-21 Centrifuge; Beckman Instrument Inc., Palo Alto, California), and the supernatant was divided into a number of aliquots which were freeze-dried and stored at -70°C.

Protein Assay. Protein concentrations in the tissue homogenates were determined using a commercial kit (Biorad, California, US.A.). Tissue proteins were dissolved by adding 950µl 0.1M NaOH to 50µl of tissue homogenate and then by boiling for 30 minutes. A protein standard curve was prepared in distilled water using BSA (Sigma) at concentrations from 25µg/ml to 1.56µg/ml in doubling dilutions. 200µl chromagen was then added to 800µl sample or standard in duplicate, the tubes were vortexed and incubated for 30 minutes at room temperature, and the absorbance of the solutions was measured at 595 nm (Uvikon 880

Spectrophotometer, Zurich, Switzerland). Finally, the protein concentrations in the samples were calculated with reference to the absorbance readings for the standard curve.

Hypothalamic Peptide Content. Aliquots of tissue extracts were reconstituted in 1ml of the appropriate assay buffers for CRH IRMA and AVP RIA. To ensure that peptide concentrations were measured on the most sensitive parts of the assay curves, a range of dilutions were assayed for each peptide. Content was expressed as ng peptide/hypothalamus, ng peptide/g tissue and ng peptide/mg protein. CRH content of the hypothalamic extracts was also determined by RIA and the results from the two CRH assays were compared.

To assess whether immunoreactive AVP and CRH present in the fetal hypothalamus diluted in parallel with standard peptides in assay procedures, the CRH and AVP concentrations present in the diluted extracts, as prepared above, were plotted alongside the appropriate standard curves.

Data Analysis. AVP or CRH levels in hypothalami of different gestational ages were compared by ANOVA followed by Duncan's multiple range test. To determine whether the CRH IRMA and the CRH RIA measured similar quantities of hypothalamic CRH, CRH concentrations obtained by IRMA and RIA were compared by ANOVA and Duncan's test.

6.2.2. Gel Chromatography of Fetal Corticotrophin-Releasing Factors.

Tissue extracts were subject to Sephadex G50 chromatography in duplicate at each gestational age. AVP and CRH immunoreactivity and ACTH-releasing bioactivity in the resultant fractions were assayed by RIA and in a pituitary cell bioassay respectively.

Sephadex Chromatography. Tissue extracts from 2 or 3 hypothalami at each gestational age were reconstituted in a total volume of 1ml 0.1M HCl. The solution was centrifuged for 2 minutes at 10,000g and the supernatant was added to the Sephadex column. The column was

perfused with 0.1M HCl at 3.5 ml/hour and fractions were collected in polystyrene tubes every 20 minutes as previously described. Fractions were divided into aliquots of 200µl and 1ml for immunoreactivity and bioactivity measurements respectively, freeze-dried and then stored at -20°C.

Assay Procedures. The AVP and CRH contents of fractions were determined at several dilutions by RIA. Results were expressed as ng peptide/fraction. ACTH-releasing activity was determined using an adult sheep pituitary cell bioassay (Section 6.2.3).

To measure ACTH-releasing activity in the chromatography samples, lyophilised fractions were reconstituted in 250µl incubation medium and added to culture wells for 3 hours. Subsequently, ACTH output from the wells was measured by RIA (Section 6.2.4). Results were expressed as ng ACTH/dish. To eliminate interassay variation, all samples were assayed in one culture at the same time and all ACTH measurements were made in a single RIA. To show that the bioassay could respond to synthetic ACTH-releasing factors, dose-response curves of AVP and oCRH(1-41) were included in triplicate at ranges of 10^{-11} to 10^{-7} M, with parallel control wells receiving medium alone. ANOVA, with peptide (AVP or CRH) and dose (10^{-11} to 10^{-7} M) as the between groups-variables, followed by Duncan's test, was used to determine the minimum effective dose of AVP or CRH which stimulated ACTH release, and which of AVP or CRH was overall more potent in this regard.

6.2.3. Pituitary Cell Bioassay.

The ACTH-releasing activity in chromatographic fractions was determined using a sheep pituitary cell bioassay, prepared as described in the literature (Tsonis, McNeilly, Baird, 1986).

Culture Preparation. Heads from adult ewes were obtained from a local abbatoir within 1-2 hours of slaughter. Anterior pituitaries were washed in sterile Dulbecco's Phosphate Buffered Saline (DPBS) containing 7.5 mM glucose. Pituitaries were finely chopped and washed 6 times in

DPBS (supplemented with 7.5 mM glucose, 0.1% BSA). The tissue pieces were gently stirred in a spinner flask containing DPBS with 0.1% BSA and 0.5% Trypsin (Sigma) for 30 minutes at 37°C. The remaining pieces were washed four times with culture medium (DMEM containing 10% lamb serum, (ICN Flow Ltd.), 2.5% fetal calf serum, 2 mM glutamine, penicillin and streptomycin; 50 I.U./ml and 50 µg/ml respectively). The pieces were stirred in this medium for a further 30 minutes at 37°C then washed 4 times and stirred for 15 minutes in DPBS without calcium or magnesium ($DPBS^-$; ICN Flow), containing 0.1% BSA and 2 mM EDTA. Tissue was washed a further 4 times in $DPBS^-$ alone and the pieces were mechanically dispersed using siliconised glass pipettes of decreasing diameter (5mm to 2mm). The supernatant was aspirated and the cells were precipitated by centrifugation at 200g for 10 minutes. Cells were resuspended in culture medium and this procedure was repeated. Finally, viability was estimated using trypan blue exclusion. The suspension was diluted to 2×10^6 live cells/ml and 100µl suspension was added to each well (Nunc Multidish 24, Gibco), followed by 400 µl culture medium. Cultures were maintained in an incubator as described previously for neuronal cells.

Three days after plating, the culture medium was replaced with medium containing cortisol-free sera, to enhance the response to ACTH-releasing factors. Endogenous steroids were removed from sera by incubating the mixture of lamb serum and fetal calf serum (80:20) with Norit A charcoal (10mg/ml; Sigma) for 60 minutes at 4°C. Subsequently, the mixture was centrifuged at 3000g for 30 minutes and the supernatant was aspirated and filter sterilised.

Bioassay Procedure. On day 8 *in vitro,* cells were washed twice with 500µl DMEM supplemented with bacitracin (30µg/ml) and glutamine (2mM). Chromatography fractions were reconstituted in 250µl supplemented DMEM, and these were warmed and added to the cells for a further 3 hours. Afterwards, the culture supernatants were aspirated and stored at -20°C prior to the determination of ACTH concentrations by RIA.

6.2.4. ACTH Radioimmunoassay.

ACTH was measured by conventional RIA with magnetic separation. ACTH(1-39) peptide for standards and iodination was obtained from Cambridge Research Biochemicals, and the rabbit anti-ACTH antiserum were supplied by Dr. A. F. Parlow, National Hormone and Pituitary Program, National Institues of Health, Bethesda, Maryland, USA.

100µl rabbit anti-ACTH antibody (AFP 6328031; 1:19,000) in assay buffer (distilled water containing 8mM Na_2HPO_4, 12.5 mM EDTA, 30mM NaN_3, 0.1% Triton X-100 and 23µg/ml aprotinin (Sigma), pH 7.4) were added to 100µl samples in duplicate or standards in triplicate in 3 ml polystyrene tubes. Standard curves containing TC, NSB and Bo tubes and ranging between 5000pg/ml and 20pg/ml were prepared by serial dilutions of a freshly thawed aliquot of ACTH. All tubes were vortexed and incubated for 24 hours at 4°C.

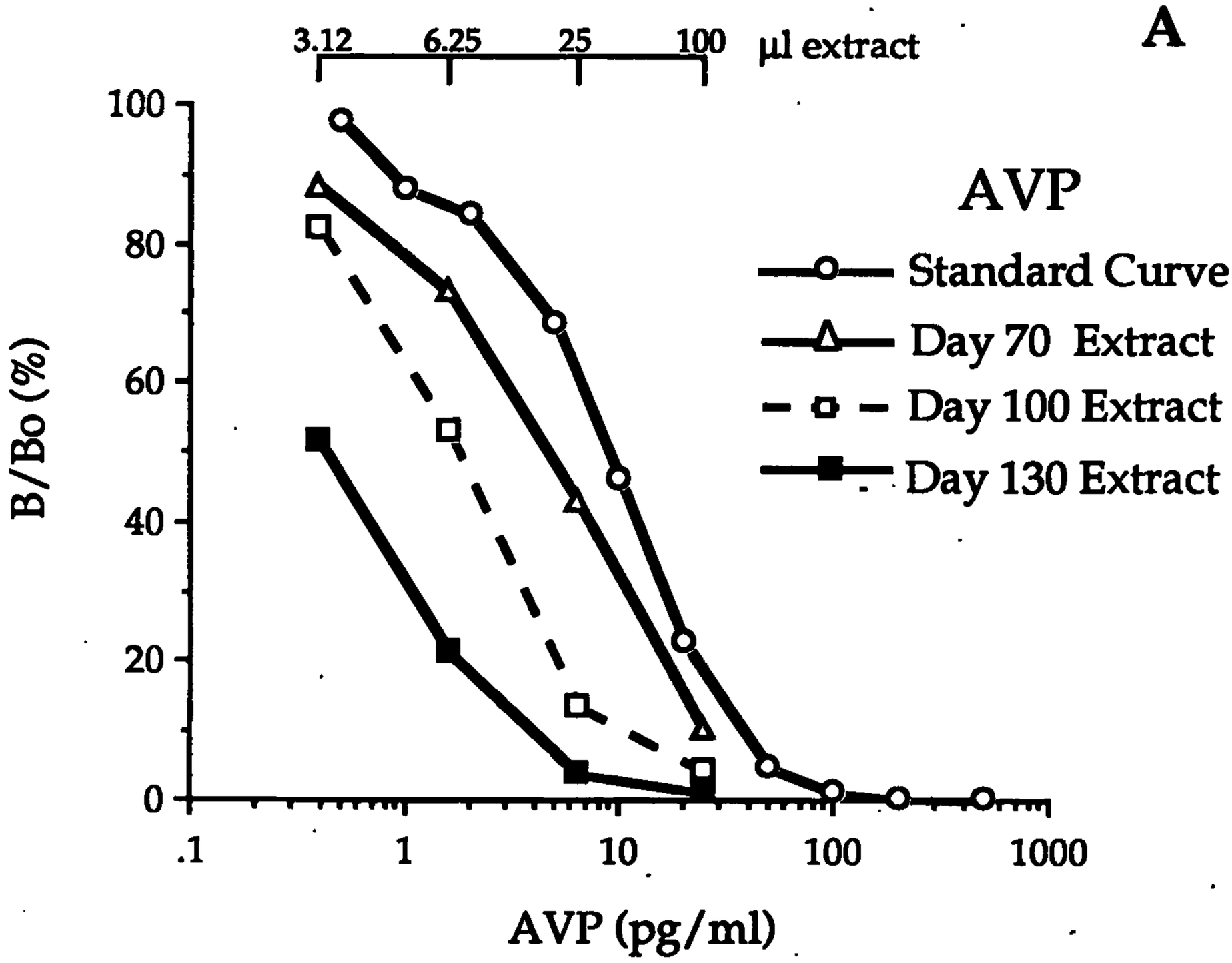
100µl ^{125}I-ACTH trace, iodinated by the chloramine-T method and purified by Sephadex G50 chromatography, was added to all tubes the next day. After a further 24 hours at 4°C, magnetic separation was carried out and the precipitate was counted as previously described (Section 3.6.1).

6.3 Results

6.3.1. Immunoreactive AVP and CRH in the Fetal Hypothalamus.

Immunoreactive AVP and CRH in fetal hypothalamic tissue diluted in parallel to the standard curves at all gestational ages studied, as shown in Figure 6.1. Figure 6.2 shows the hypothalamic contents of AVP and CRH, expressed as ng peptide/hypothalamus, ng peptide/g tissue and ng peptide/mg protein. These figures show that the hypothalamic contents of AVP and CRH increased significantly between day 70 and day 100 (p <0.01), and between day 100 and day 130 (p <0.01) of gestation. Similarly, hypothalamic concentrations of these peptides, expressed as ng/g tissue, were significantly increased between day 70 and day 100 (p <0.05), and day 100 and day 130 (p <0.01). However, when peptides were expressed as ng/mg protein, the rise from day 70 to day 100 was not

Figure 6.1. Parallel dilution of hypothalamic extracts in assay. Hypothalamic extracts were diluted and **A** immunoreactive AVP and **B** CRH were measured by RIA and IRMA respectively. Data shown are means of 5 or 6 extracts at each gestational age.


A
3.12
6.25
25
100
µl extract
100
80
60
40
20
0
B/Bo (%)
.1
1
10
100
1000
AVP (pg/ml)
AVP
Standard Curve
Day 70 Extract
Day 100 Extract
Day 130 Extract

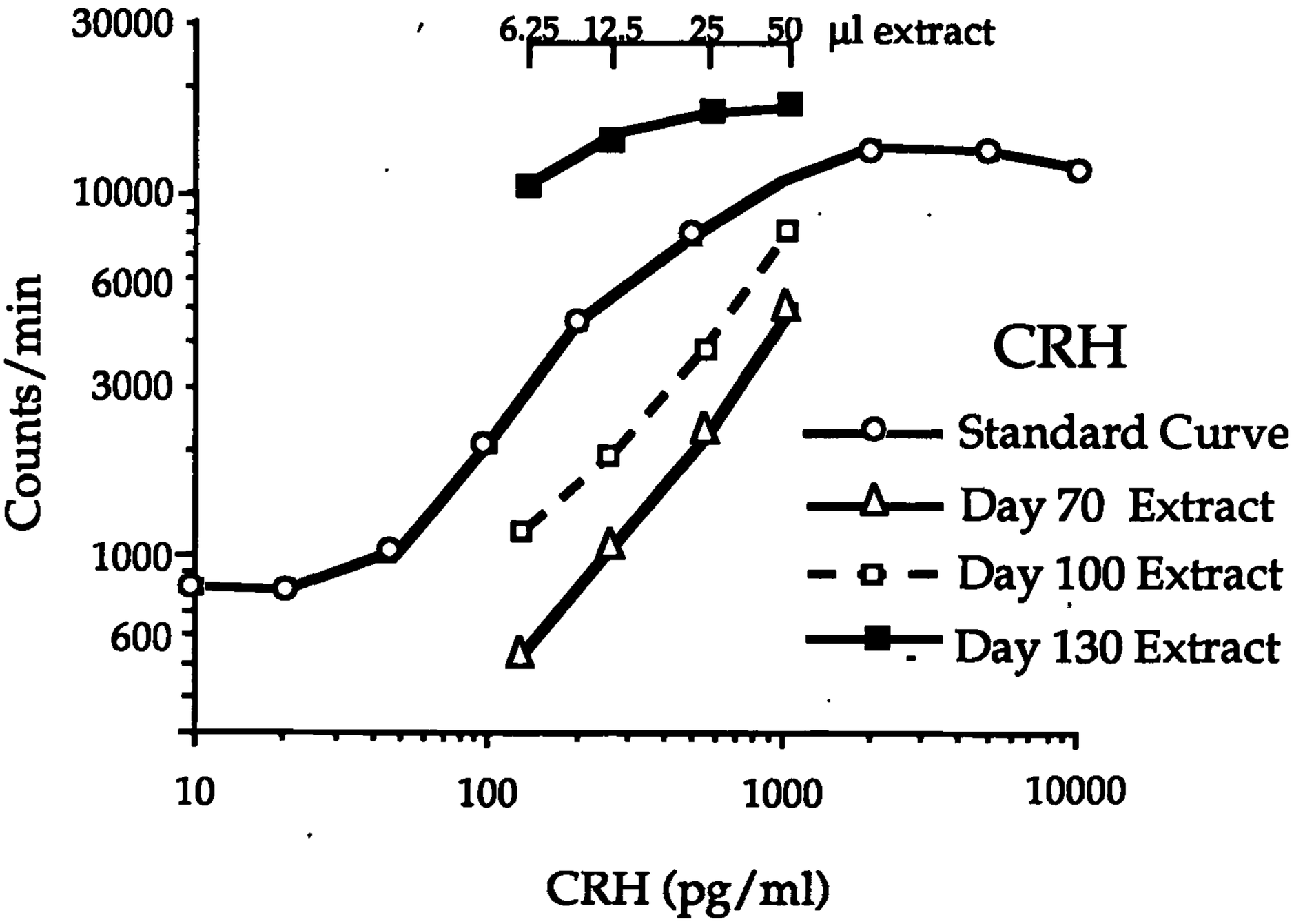
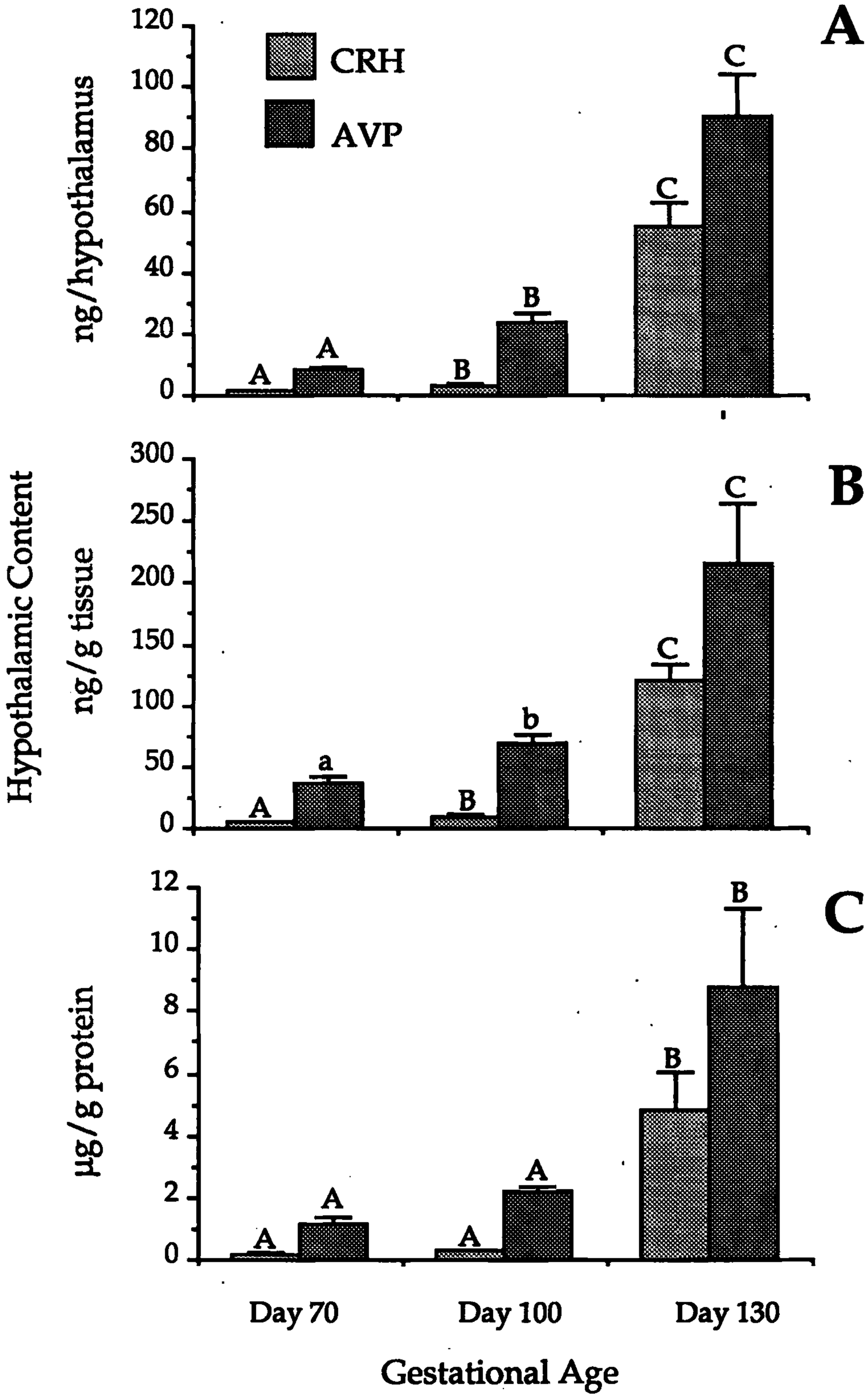

B
6.25
12.5
25
50
µl extract
30000
10000
6000
3000
1000
600
Counts/min
10
100
1000
10000
CRH (pg/ml)
CRH
Standard Curve
Day 70 Extract
Day 100 Extract
Day 130 Extract

Figure 6.2. AVP and CRH Content of the fetal hypothalamus with advancing gestational age. Peptide contents were expressed as **A**, ng/hypothalamus, **B**, ng/g tissue and **C**, ng/mg protein. Levels of AVP or CRH at different gestational ages were compared separately, and superscripts describe statistical differences within either AVP or CRH data groups. Data points without common superscripts are significantly different (Upper case; $p < 0.01$, lower case; $p < 0.05$). Means of 5 or 6 hypothalami are shown at each gestational age, +/- S.E.M.

Peptide Content of the Fetal Hypothalamus



significant for either CRH or AVP, whereas the increase from day 100 to day 130 remained highly significant in each case (p <0.01).

Measurement of CRH molecules other than CRH (1-41) after gel filtration required the use of an RIA rather than the IRMA, which could only detect intact CRH (1-41). To compare the RIA and the IRMA, hypothalamic contents of CRH were measured in both assays and the results were plotted against each other. Figure 6.3 shows the very close agreement between the assays. An overall ANOVA revealed that there was no effect of assay methodology on the results obtained for hypothalamic levels of CRH (ANOVA; p = 0.2608).

6.3.2. Gel Chromatography of Corticotrophin-Releasing Factors.

Figures 6.4 to 6.6 show that after gel chromatography, two immunoreactive peaks were detected in hypothalamic extracts at each gestational age. The first peak cross-reacted in the CRH RIA and eluted in fractions 20-24 (Void Volume = fraction 14), whereas the second peak eluted in fractions 33-38 and corresponded to immunoreactive AVP. At each gestational age, these immunoreactive peaks co-eluted with synthetic oCRH(1-41) and AVP respectively. As suggested by the increasing hypothalamic contents of CRH and AVP, successively greater quantities of these peptides were evident in the fractions with advancing gestational age.

A pituitary cell bioassay was employed to determine the bioactivity of fetal hypothalamic corticotrophin-releasing factors. The responses of the bioassay in terms of ACTH release to standard doses of AVP or CRH are shown in Figure 6.7. The minimum concentration at which AVP (p <0.01) or CRH (p <0.05) caused a significant increase in ACTH release when compared to control wells was 10^{-10}M. Overall analysis of variance followed by Duncan's test showed that AVP was significantly more potent than CRH in stimulating ACTH release from the pituitary cells (p <0.01).

At each gestational age, peaks of AVP and CRH immunoreactivity after Sephadex chromatography were associated with elevated ACTH output from the cells, as shown in Figures 6.4 to 6.6. Substantial bioactivity was also present in the void volume and in fractions 29-30 at

Figure 6.3. CRH Content of the Fetal Hypothalamus; comparison of CRH RIA and CRH IRMA. CRH in hypothalamic extracts was measured by CRH RIA and CRH IRMA. The results from the assays were compared, and an overall ANOVA showed that the results were not significantly different (ANOVA; $p = 0.2608$).

Comparison of CRH RIA and CRH IRMA

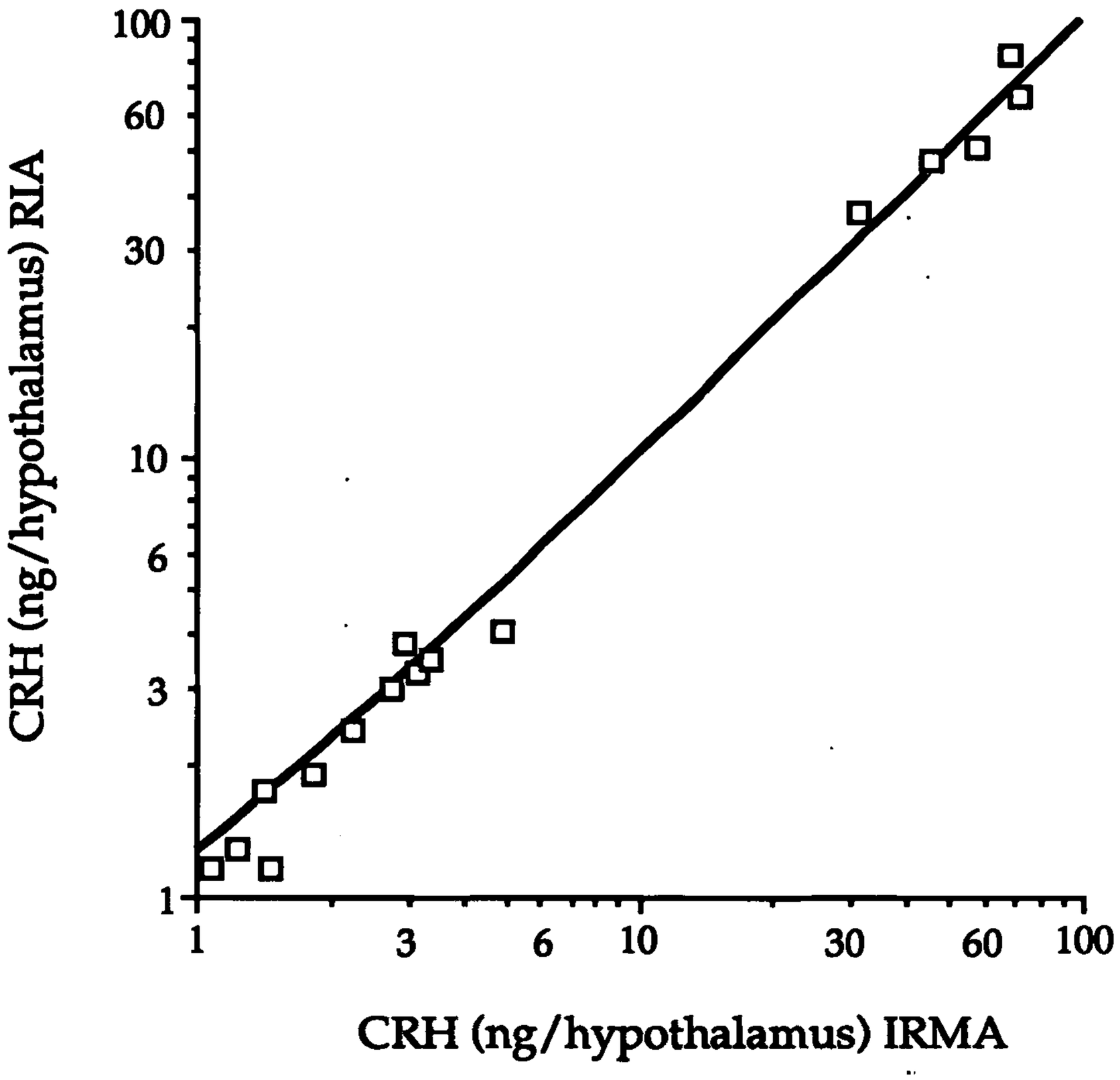


Figure 6.4. Chromatography of Corticotrophin-Releasing Factors in the Fetal Hypothalamus at day 70 of Gestation. AVP and CRH immunoreactivity were determined in fractions after chromatography of (A) 3 pooled and (C) 2 pooled hypothalami from 70 days of gestation. The elution positions of synthetic AVP and CRH are shown by the filled bars. Blue Dextran (M.Wt. =2 x 10^6) eluted at Vo.

ACTH-releasing activity was then determined in the fractions from pools A and C (B and D respectively). In the bioassay, the emtpy bars represent the bioactivity of the various fractions. Black bars correspond to fractions containing CRH immunoreactivity or AVP immunoreactivity. Where bioactivity exceeded the maximum shown on the graph, figures denote the peak levels detected. ND = Not determined.

Day 70

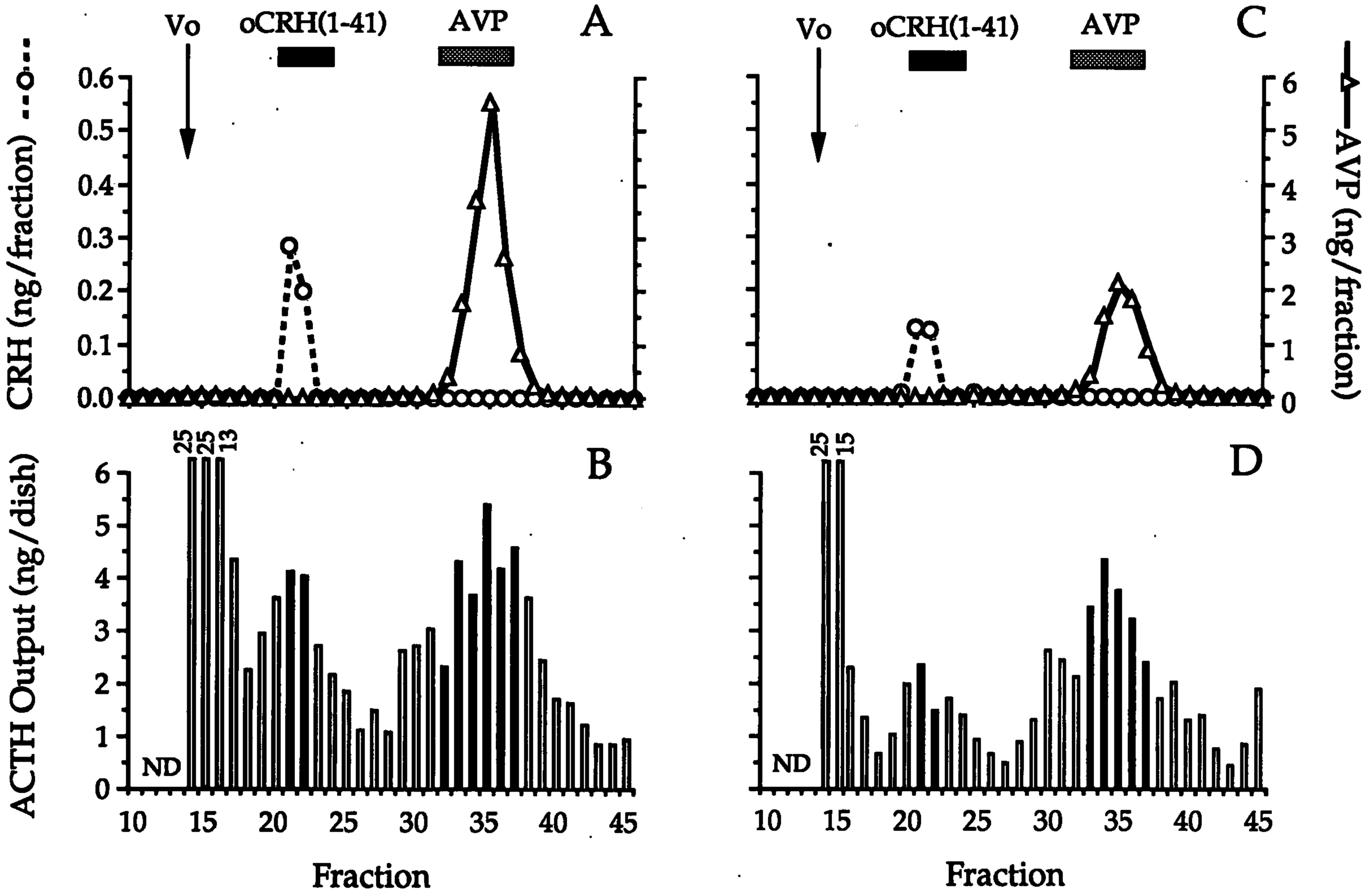

Vo
oCRH(1-41)
AVP
A
C
CRH (ng/fraction)
AVP (ng/fraction)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
6
5
4
3
2
1
0
ACTH Output (ng/dish)
25
25
13
25
15
ND
B
D
10
15
20
25
30
35
40
45
Fraction

Figure 6.5. Chromatography of Corticotrophin-Releasing Factors in the Fetal Hypothalamus at day 100 of Gestation. AVP and CRH immunoreactivity were determined in fractions after chromatography of (A) 3 pooled and (C) 3 pooled hypothalami from 100 days of gestation. The elution positions of synthetic AVP and CRH are shown by the filled bars. Blue Dextran (M.Wt. =2 x 10^6) eluted at Vo.

ACTH-releasing activity was then determined in the fractions from pools A and C (B and D respectively). In the bioassay, the emtpy bars represent the bioactivity of the various fractions. Black bars correspond to fractions containing CRH immunoreactivity or AVP immunoreactivity. Where bioactivity exceeded the maximum shown on the graph, figures denote the peak levels detected. ND = Not determined.

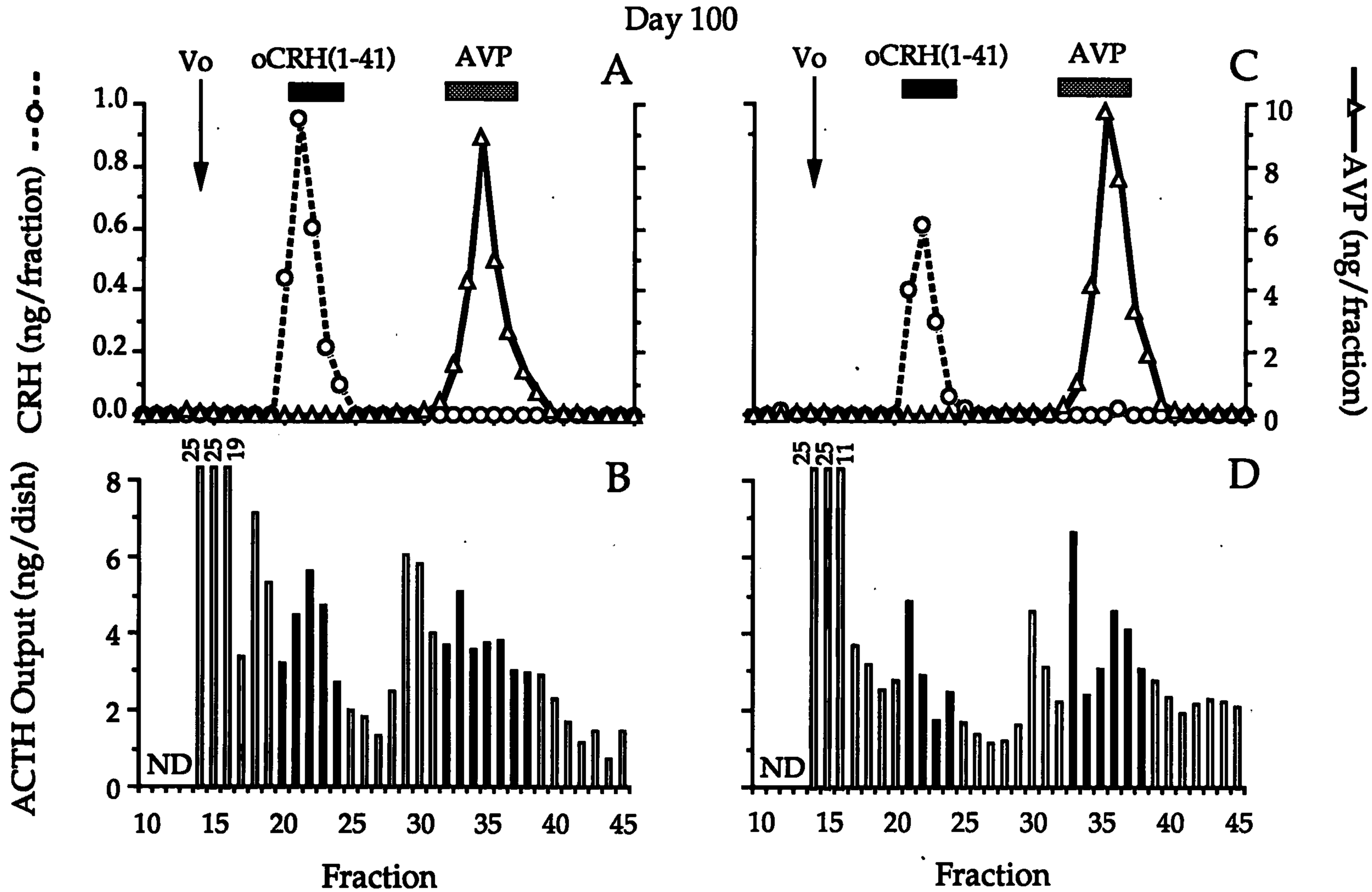

Day 100
Vo
oCRH(1-41)
AVP
A
C
oCRH (ng/fraction)
AVP (ng/fraction)
1.0
0.8
0.6
0.4
0.2
0.0
10
8
6
4
2
0
ACTH Output (ng/dish)
25
25
19
25
25
11
B
D
ND
8
6
4
2
0
10 15 20 25 30 35 40 45
Fraction

Figure 6.6. Chromatography of Corticotrophin-Releasing Factors in the Fetal Hypothalamus at day 130 of Gestation. AVP and CRH immunoreactivity were determined in fractions after chromatography of (A) 3 pooled and (C) 2 pooled hypothalami from 130 days of gestation. The elution positions of synthetic AVP and CRH are shown by the filled bars. Blue Dextran (M.Wt. =2 x 10^6) eluted at Vo.

ACTH-releasing activity was then determined in the fractions from pools A and C (B and D respectively). In the bioassay, the emtpy bars represent the bioactivity of the various fractions. Black bars correspond to fractions containing CRH immunoreactivity or AVP immunoreactivity. Where bioactivity exceeded the maximum shown on the graph, figures denote the peak levels detected. ND = Not determined.

Day 130

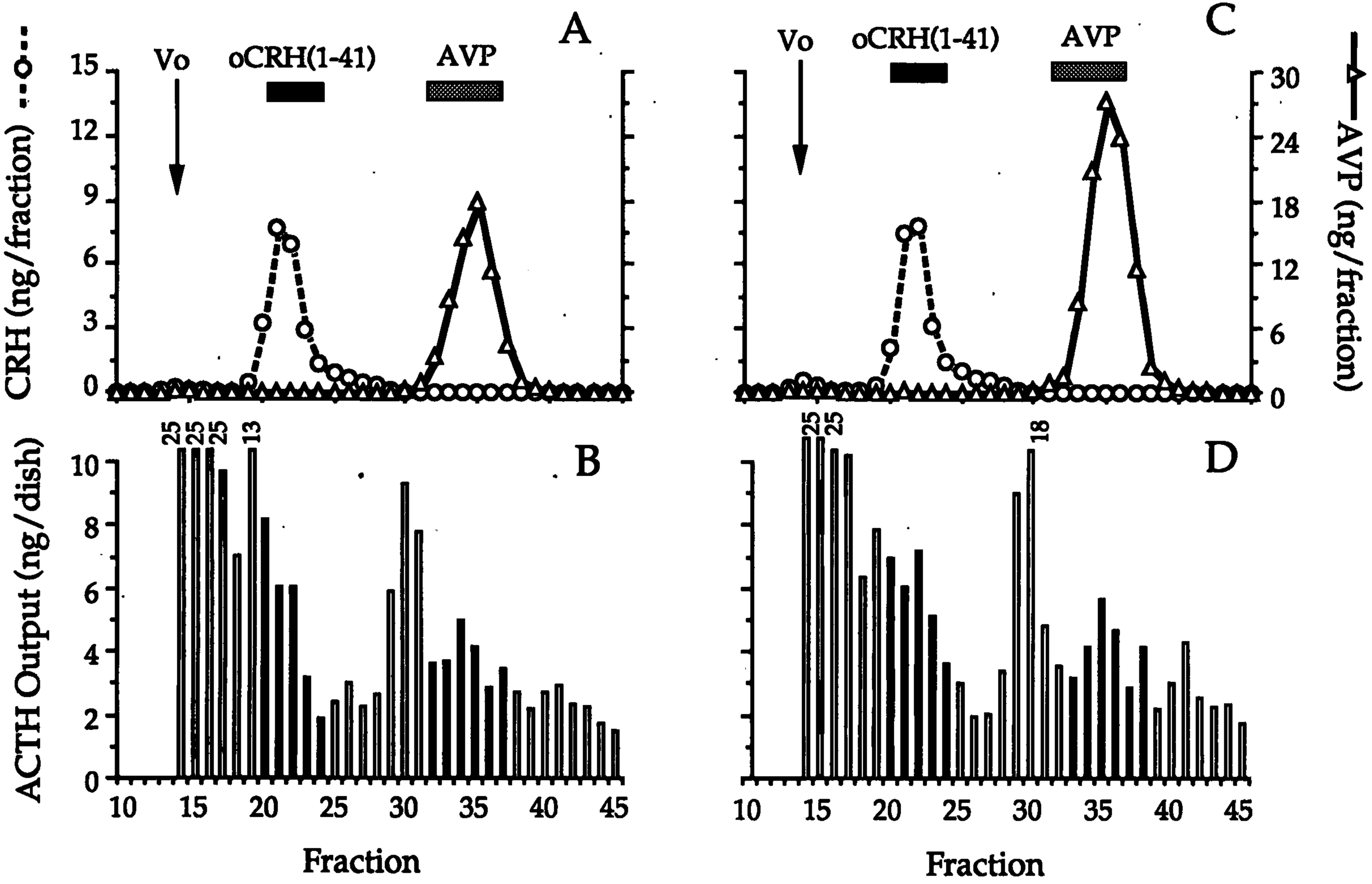
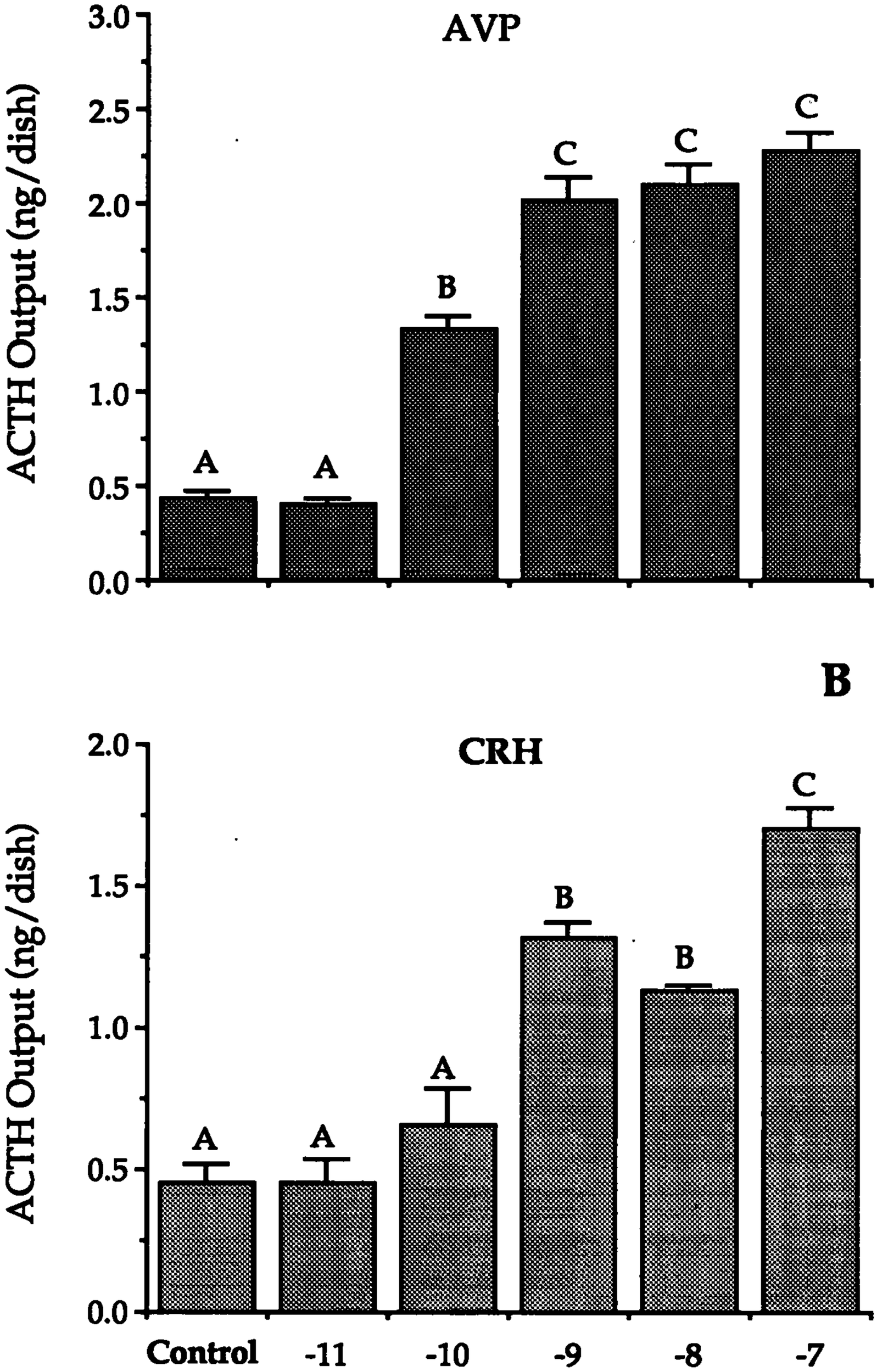

A
Vo
oCRH(1-41)
AVP
CRH (ng/fraction) --o--
15
12
9
6
3
0
B
ACTH Output (ng/dish)
25
25
25
13
10
8
6
4
2
0
10
15
20
25
30
35
40
45
Fraction
C
Vo
oCRH(1-41)
AVP
30
24
18
12
6
0
AVP (ng/fraction)
D
25
25
18
10
15
20
25
30
35
40
45
Fraction

Figure 6.7. Responses of Sheep Pituitary Cell Bioassay to AVP or CRH. Standard AVP (A) or CRH (B) were diluted in incubation medium immediately before use. Data represent means from triplicate wells, and are shown +/- S.E.M. Data without common superscripts are significantly different (p <0.01). Analysis of varicance showed that overall, AVP was significantly more potent than CRH in stimulating ACTH release (p <0.01).

Sheep Pituitary Cell Bioassay



each gestational age. Neither of these peaks was associated with significant CRH or AVP immunoreactivity.

6.4 Discussion.

The objective of the present study was to examine ACTH-releasing factors in intact fetal sheep hypothalami removed at different gestational ages. The major finding was that a progressive rise in the hypothalamic contents of immunoreactive AVP and CRH occurred with fetal maturation, and that this rise was especially striking between day 100 and day 130 of gestation. Gel permeation chromatography revealed that fetal hypothalamic CRH and AVP were present as single immunoreactive species, co-eluting with either synthetic oCRH or AVP, at all gestational ages studied. Bioactivity assessed in a pituitary cell culture system revealed marked ACTH-releasing activity in the fractions corresponding to immunoreactive CRH and AVP. In addition, significant bioactivity was present in other fractions which were devoid of CRH or AVP immunoreactivity.

The present work found a small increase in hypothalamic CRH and AVP concentrations between day 70 and day 100 of gestation, and a conspicuously large increase between day 100 and day 130. These data are in broad agreement with previous studies (Brieu *et al.* 1989), although the gestational increases in those studies were not as striking as those observed here. For example, the present work showed increases of approximately 6-fold and 20-fold respectively in the hypothalamic concentrations of immunoreactive AVP and CRH between day 70 and day 130 of gestation. The previous report suggested that hypothalamic CRH and AVP concentrations increased by a factor of 2 during this stage of development (Brieu *et al.* 1989). However, those authors collected hypothalami from the earliest gestational age over an interval of 25 days (day 63-88). The increases in hypothalamic peptide content between day 70 and day 100, as reported in the present study, suggest that marked changes in hypothalamic CRH and AVP may occur between day 63 and day 88. Therefore, the striking increases in CRH and AVP as described here may have been obscured in the previous investigation.

It is unlikely that the findings reported here arose through variation in tissue weight at different gestational ages. When corrected for the changes in tissue weight, the gestational increases in AVP and CRH concentration remained significant. Expressed as ng peptide/mg protein, only the rise in AVP and CRH between day 100 and day 130 was significant. These results indicate that early hypothalamic development is associated with a concomitant increase in peptide and protein expression, suggestive of hypothalamic growth. Whereas, the marked rise in AVP and CRH between day 100 and day 130 of gestation may be related to the increasing function of the hypothalamo-pituitary-adrenal axis over this time (see Norman *et al.* 1985).

CRH concentrations, expressed either as ng/g tissue or ng/mg protein, were consistently lower than AVP levels in the work reported here, although the CRH/AVP ratio rose from 0.17 at day 70 of gestation to 0.6 at day 130. This was likely due to the especially noticeable increase in hypothalamic CRH concentration between day 100 and day 130 of gestation. It is not clear why there was such an increase in hypothalamic CRH during this time. The present study utilised two independent assay methods to determine CRH levels in the fetal hypothalamic extracts. The IRMA, which detects only CRH molecules bearing both N and C-terminal epitopes, and the RIA, which could detect smaller immunoreactive fragments, produced results which were not significantly different. Therefore, it is unlikely that the present findings arose spuriously, as a result of methodological inadequacies.

Interestingly, a selective rise in CRH, compared to AVP, was reported at the slightly later gestational age of day 138 by Brieu and colleagues (Brieu *et al.* 1989). CRH is known to be less effective than AVP in stimulating ACTH release from pituitary cells isolated from young fetuses, whereas the converse is true in pituitary cells collected late in gestation (Brieu & Durand, 1987). The present study supports the hypothesis that AVP may be a more important ACTH-releasing factor in early gestation, and further suggests that either CRH alone or synergy between CRH and AVP may gain importance later in development. This is in contrast to findings in the adult sheep in which AVP is gaining acceptance as the major ACTH-releasing factor (see Shen, Clarke, Canny, Funder & Smith, 1990). In agreement with the results of other workers

(Familiari *et al.* 1989), the data presented here showed that AVP was more potent than CRH in releasing ACTH from the adult sheep pituitary cell bioassay.

Chapter 5 of this thesis quantified secretion of CRH and AVP from fetal sheep hypothalamic cells *in vitro*. Secretion of CRH and AVP by cultured cells removed at day 70, 100 or 130 of gestation was diminished with advancing gestational age, and this was associated with a reduction in the contents of AVP and CRH in the hypothalamic cultures. These data contrast with the results of the present study, which has shown that immunoreactive hypothalamic CRH and AVP are present in increasing quantities with fetal maturation. Taking these results together, the present study has shown that hypothalamic synthesis and/or storage of CRH and AVP is most probably dependent on factors which are present *in vivo* but not *in vitro*. These factors may take the form of neurotransmitter inputs to the PVN neurones, or they may be specific neurotrophic molecules or endocrine growth factors which act on the CRH and AVP cells. The previous chapter indicated that whilst the hypothalamic CRH and AVP cells *in vitro* show differences in function with advancing gestational age, suggestive of hypothalamic maturation, the cultured cells do not closely mimic neurones *in vivo* in terms of peptide content, as reported here. Therefore, it is perhaps more appropriate to study the qualitative responses of these cultured cells, rather than to ascribe significance to the quantities of peptides secreted.

The present study utilised Sephadex chromatography to examine the multiplicity of molecular species in the fetal sheep hypothalamus which were immunoreactive in CRH and AVP RIA procedures. Importantly, only one immunoreactive peak was detected in the CRH assay and one in the AVP assay at all gestational ages studied, each of which co-eluted with the appropriate synthetic peptide. The CRH and AVP precursors contain several paired basic amino acids at which cleavage occurs during post-translational modification (Furutani *et al.* 1983; Land *et al.* 1982), therefore, several extended molecular forms of CRH and AVP can exist, depending on the degree to which post-translational cleavage occurs. The present study provides good evidence that as early as midgestation (day 70), fetal sheep hypothalamic neurones

correctly process the CRH and AVP precursors, giving rise to CRH(1-41) and AVP(1-9).

By contrast to the above, immunoreactive CRH in the human fetal hypothalamus at an equivalent developmental age (prior to week 20; Term = 39 weeks) elutes as two peaks on Sephadex chromatography, one corresponding to a molecular weight of 8-10kD and another which co-elutes with oCRH(1-41) (Ackland *et al.* 1986). The large molecular form may represent an intermediate between prepro-CRH and CRH(1-41). Cleavage of the precursor at amino acids 123-124 (CRH(1-41) present at amino acids 154 to 194; Shibahara *et al.* 1983) would give rise to a CRH(1-41)-containing polypeptide of 71 amino acids, with a molecular weight of around 9kD. A similar cleavage site exists at amino acids 116-117 in the ovine CRH precursor (Furutani *et al.* 1983). However, the absence of large CRH molecules as reported here suggests that immunoreactive prepro-CRH molecules are present in very low abundance in the fetal sheep hypothalamus. Alternatively, the antibody used in the present work may not cross react with this form of CRH. To examine prepro-CRH in the hypothalamus of the fetal sheep, a recent study used Sephadex chromatography followed by RIA with multiple anti-CRH antibodies to screen CRH molecules in hypothalami removed as early as day 48 of gestation. These workers convincingly demonstrated that CRH-like immunoreactivity co-eluted with CRH(1-41) at all gestational ages investigated (Watabe *et al.* 1991). Therefore, in contrast to the human, these data suggest that the fetal sheep hypothalamus may not store appreciable amounts of prepro-CRH.

The work reported here showed that the peaks of CRH and AVP imunoreactivity after Sephadex chromatography were associated with peaks of ACTH-releasing activity, as determined by pituitary cell bioassay. This was most obvious in extracts prepared from hypothalami removed at day 70 of gestation. Prior to these studies, it was not clear whether CRH and AVP synthesised by the developing hypothalamus contained biological activity. Both CRH and AVP depend on C-terminal amidation for biological activity (Sossin, Fisher & Scheller, 1989), and there are no studies which have assessed the activity of peptidyl-glycine α-amidating monooxygenase, the enzyme which catalyses α-amidation (Eipper & Mains, 1988), in the fetal sheep hypothalamus during development.

Therefore, the present work strongly suggests that as early as day 70 of gestation, the fetal sheep hypothalamus correctly cleaves and amidates the CRH and AVP precursors to give rise to the bioactive peptides.

It is also possible that the ACTH-releasing activity observed in hypothalamic extracts may be attributable to molecular species other than CRH or AVP. Oxytocin (Antoni, Holmes & Jones, 1983), angiotensin II (Ganong & Marakami, 1987) and galanin (Hoo, Maiter, Martin & Koenig, 1990) have been implicated as ACTH-releasing factors, and these substances may co-elute with CRH or AVP on Sephadex chromatography. The present work does not distinguish between these various ACTH-releasing factors, and confirmatory studies, such as HPLC purification followed by antibody quenching experiments (Gillies & Lowry, 1982), will have to be carried out to identify further the contributions of these substances.

The work reported here shows that substantial ACTH-releasing activity elutes in fractions containing little or no detectable AVP or CRH at each gestational age. For example, bioactivity was present in the column void volume and in fractions 29 and 30 in each case, and these activities tended to increase with advancing gestation. Brieu and colleagues (1989) observed a gestational age-dependent increase in the bioactivity of hypothalamic extracts, which they suggested was due to the rising levels of AVP and CRH. However, their study showed similar ACTH-releasing activity in hypothalami from day 138 and day 143, despite a large reduction in hypothalamic AVP and CRH content over this time. These authors were unable to explain this observation, however, the present study clearly shows that both high molecular weight (void volume) and low molecular weight (fractions 29 and 30) species may contribute significantly to ACTH-releasing bioactivity of hypothalamic extracts.

A high molecular weight ACTH-releasing factor has previously been reported in extracts from adult bovine hypothalami (Yasuda, McClung & Greer, 1978). Interestingly, low molecular weight ACTH-releasing factor (corresponding to CRH) was generated from the high molecular weight molecule on storage (Yasuda *et al.* 1978). The void volume species in the present report could be a CRH precursor, which is cleaved by proteolytic enzymes present in the bioassay to yield CRH(1-41).

Whilst a protease inhibitor was included, it is not clear whether all such enzymes would have been inactivated in the bioassay. It would be interesting to re-examine CRH immunoreactivity in the fractions after incubation with pituitary cells.

The identity of the low molecular weight ACTH-releasing factor in the present study is unknown. Fractions 29 and 30 were relatively acidic, but were neutralised before incubation with pituitary cells. It is therefore likely that this represents an intrinsic ACTH-releasing activity in this fraction rather than a non-specific stimulus such as hydrogen ions. It remains an exciting prospect that a fetus-specific CRF exists, neither CRH nor AVP, which could override cortisol negative feedback during the preparturient phase.

In conclusion, this chapter has examined the ontogeny of ACTH-releasing factors in the fetal sheep hypothalamus. Hypothalamic AVP and CRH corresponded to mature, biologically active peptides, as demonstrated by Sephadex chromatography, RIA and pituitary cell bioassay, as early as day 70 of gestation. It is concluded that in terms of peptide synthesis and storage, the fetal hypothalamus displays functional competence by midgestation. Moreover, the present chapter found that fetal maturation was associated with progressive increases in AVP and CRH, which is consistent with the functional maturation and activation of the hyopthalamo-pituitary-adrenal axis in fetal life.

7. Cortisol Negative Feedback at the level of the fetal Hypothalamus *in vitro*.

To examine the ontogeny of AVP and CRH in the fetal sheep hypothalamus, the previous 2 chapters have investigated AVP and CRH release from cultured hypothalamic cells *in vitro* and the levels of these peptides in the intact hypothalamus at several gestational ages. These studies have revealed a progressive reduction in AVP and CRH secretion *in vitro* with advancing gestational age, and furthermore, that AVP secretion was calcium- and time-dependent in day 70 and day 100 cultures, whereas CRH secretion did not conform to these criteria at any gestational age. In contrast to these observations, the fetal hypothalamus *in vivo* was found to contain very large amounts of immunoreactive and bioactive AVP and CRH peptides as early as day 70 of gestation, the levels of which increased with advancing gestational age.

Whilst these chapters have revealed aspects of the functionality of fetal hypothalamic cells at different stages in development, they have not assessed physiological aspects of AVP and CRH regulation, such as the responses to adrenal steroids or neuropeptides. The remit of the remaining experimental chapters in this thesis is to examine these issues. Furthermore, as reliable measurement of CRH *in vitro* has been shown to be problematic, and certainly not possible at different gestational ages, it was decided to pursue these studies quantifying AVP alone.

7.1 Introduction

The fetal sheep hypothalamus contains high levels of immunoreactive and bioactive AVP and CRH from day 70 of gestation onwards (Brieu *et al.* 1989; see Chapter 6, this thesis) and either of these peptides alone or in combination stimulates ACTH release by fetal sheep pituitary cells from day 63 of gestation onwards (Durand *et al.* 1986; Brieu & Durand, 1987). However, fetal ACTH levels remain low until rather later in gestation (Rose, Meis & Morris, 1981; Norman *et al.*, 1985), suggesting that, early in

development, the fetal hypothalamo-corticotrope system is under inhibitory control, possibly through the negative feedback actions of adrenal glucocorticoids. These data also suggest the hypothesis that a reduction in the effectiveness of glucocorticoid negative feedback may underlie increases in ACTH levels in the fetal sheep in late gestation.

The effects of glucocorticoids on fetal sheep pituitary ACTH release at different times in gestation have recently been investigated, and available data indicate that cortisol or dexamethasone inhibits the release of ACTH from the fetal pituitary over a wide range of gestational ages, both *in vivo* (Norman & Challis, 1987b) and *in vitro* (Brieu *et al.* 1986). However, the development of negative feedback regulation of ACTH-releasing factors in the fetal sheep hypothalamus is less well studied. The available evidence suggests that feedback may well regulate synthesis and secretion of ACTH-releasimg factors at certain times in development. For example, implants of dexamethasone to the fetal PVN at day 108-111 of gestation abolish the elevation in fetal ACTH after fetal hypotension or hypoxaemia at day 120 (McDonald *et al.* 1990), whereas fetal adrenal-ectomy at day 118-121 increases PVN CRH mRNA levels within a few days (Myers *et al.* 1991). In contrast to investigations of fetal pituitary ACTH secretion, these studies have not attempted a quantitative description of glucocorticoid feedback at the fetal hypothalamus. Therefore, the question of whether negative feedback becomes more or less effective with advancing gestation remains unanswered.

Recently, evidence has been provided that the secretion of ACTH-releasing factors from the fetal sheep hypothalamus is more sensitive to glucocorticoid inhibition in late gestation (Brooks *et al.* 1989). These authors showed that basal CRH secretion from perifused fetal sheep hypothalami removed at day 140 of gestation was inhibited by glucocorticoids, whereas this treatment had no effect on basal CRH release from tissues obtained at day 100. These data may contrast with receptor binding studies which show that glucocorticoid receptors in the sheep hypothalamus are present at lower concentrations in late gestation fetuses (Yang *et al.* 1990). However, both studies provide very good evidence that the efficacy of glucocorticoid negative feedback at the fetal hypothalamus may vary during fetal development.

To investigate the hypothesis that the efficacy of glucocorticoid negative feedback at the fetal hypothalamus undergoes change during development, the present study assessed the secretion of AVP from cultured fetal sheep hypothalamic cells removed at day 70 and day 100 of gestation, in the presence and absence of a glucocorticoid. Cultures were divided into two groups and maintained in serum-free medium, or in serum-free medium containing cortisol and AVP release was measured under basal conditions and in response to incubation with 56 mM potassium-containing medium. At the end of the experiment, cultures were extracted to determine the effects of cortisol on AVP content. Finally, the secreted AVP was characterised by gel chromatography and RIA to assess identity with synthetic AVP.

7.2 Experimental Procedures

Hypothalami were removed from fetal sheep at day 70 and day 100 of gestation, and 2 cultures were prepared at each gestational age as previously described. 2 groups of 5 fetuses and 2 groups of 3 fetuses were used for day 70 and day 100 hypothalamic cultures respectively, and the results from duplicate cultures were combined for analysis. Where appropriate, data were logarithmically transformed to eliminate heterogeneity of variance.

Experimental Design. The wells in each culture were divided into 2 groups at the time of plating. 48 hours later, serum-free medium was added to all cells, however 1 group received medium containing 150nM cortisol (Sigma; 50 μM stock in 0.1% ethanol). Subsequently, cortisol was routinely added to all culture medium and experimental media which were prepared for this group of cells, whilst the other group remained cortisol-free.

Between day 7 and day 18 after plating, all cultures were incubated every 3 or 4 days with basal medium for 3 hours and then with 56 mM potassium-containing medium for a further 3 hours. Incubations were carried out as previously described, in the presence or absence of 150nM cortisol, as appropriate to the experimental group. All media were stored

at -20°C prior to measurement of AVP by RIA. To assist detection of low levels of AVP, basal samples from each well were frozen as 2 aliquots of 300µl. These were freeze-dried and reconstituted in a smaller volume at the time of assay. On day 21, cultures were photographed, and wells in each group were washed 3 times with basal release medium and extracted in 1ml of this solution.

The samples from this study were assayed for AVP by RIA as described previously, except that the lyophylised basal release samples were reconstituted in 100 µl deionised water immediately prior to assay. All results were expressed as pg/dish. The AVP contents in cell extracts from different cultures within the same gestational age group showed marked variation. To reduce this variation, the AVP contents of cells from each well in a culture were expressed as a percentage of the average AVP content of all the wells in that particular culture. The percentage values for the two cultures from each gestational age were then combined for statistical analysis.

Statistics. The multiple between- and within-groups variables in the present study precluded a simple statistical analysis to determine the effects of cortisol on basal and potassium-stimulated release of AVP. Therefore, a preliminary ANOVA was carried out to determine the overall effects of and interactions between cortisol and potassium stimulation, gestational age and age in culture, on AVP release.

To determine whether potassium stimulation enhanced AVP secretion from the cortisol-free wells in day 70 cultures, basal AVP release was compared to stimulated release by ANOVA, followed by Duncan's multiple range test, with day in culture (day 8, 11, 15 and 18) and incubation (basal or stimulated) as the within-groups variables. Identical analyses were then carried out for the cortisol-supplemented day 70 cultures, and for both groups of day 100 cells.

Similarly, ANOVA, followed by Duncan's multiple range test, was carried out to determine the effects of cortisol on basal AVP release, with gestational age (day 70 and day 100) and cortisol (present or absent) as the between-groups variables an day in culture (day 8, 11, 15 and 18) as the within-groups variable. Similar analyses were utilised to determine the effects of cortisol on potassium-stimulated AVP release. Finally, the

effects of cortisol on the AVP contents of the cultured cells was assessed by one-way ANOVA, followed by Duncan's multiple range test, with gestational age (day 70 and day 100) and cortisol (present or absent) as the between-groups variables.

Characterisation of Secreted AVP. In order to assess parallelism of secreted AVP with the AVP standard curve in RIA procedures, doubling dilutions of culture supernatant (excess, cortisol-free 56 mM potassium-containing medium) were prepared in duplicate in AVP assay buffer. After conventional AVP RIA procedures were carried out, the degree of tracer binding in these tubes was expressed as a percentage of the Bo levels, and these results were plotted alongside the corresponding values for a standard curve.

Size fractionation of AVP immunoreactivity was carried out by Sephadex chromatography, essentially as previously described. However, some modifications were required in view of the much reduced peptide levels. A pool of excess, cortisol-free 56 mM potassium-containing media (~3ml) was collected from each culture. This was freeze-dried, and reconstituted in 1ml assay buffer. Each was added to a Sephadex G50 column, 30 x 1cm, and eluted with 0.01M HCl, containing 0.9% NaCl, at a rate of 3.5 ml/hour. Fractions were collected every 12 minutes (0.7 ml) and were immediately assayed for AVP. Results were expressed as pg/fraction.

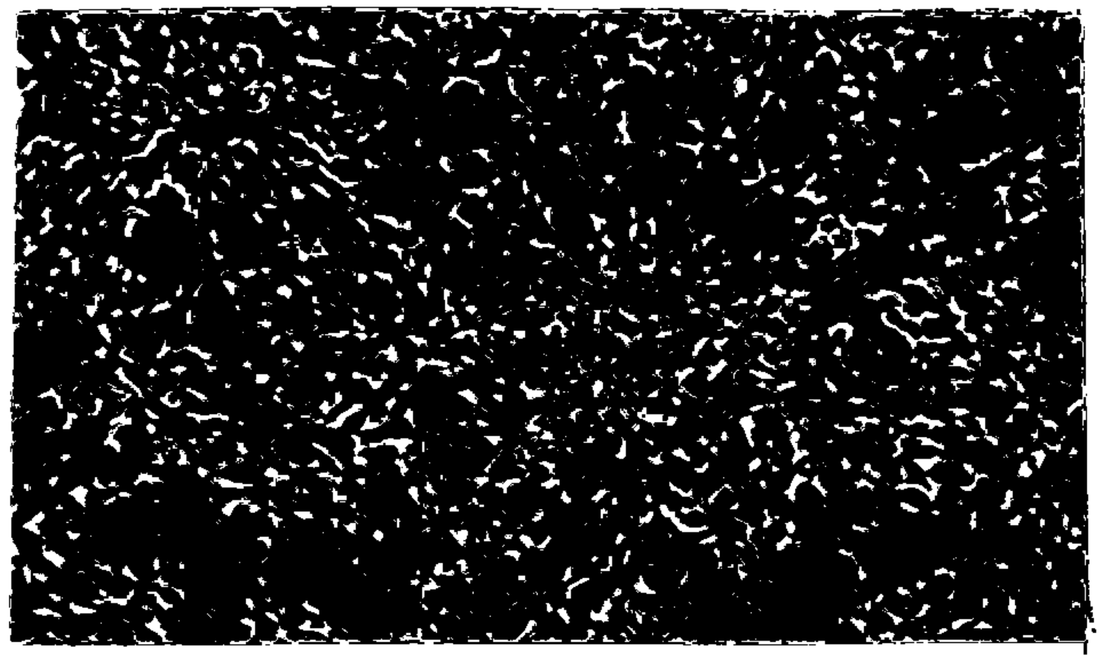
7.3 Results

Figure 7.1 shows phase contrast micrographs of cultured cells at day 21 *in vitro,* immediately before culture extraction. The viability of the cultures was subjectively assessed in terms of the cell density and the phase characteristics (bright or dark cells) of the hypothalamic cells. This procedure suggested that neither gestational age nor the presence of glucocorticoids adversely affected the viability of these cultures.

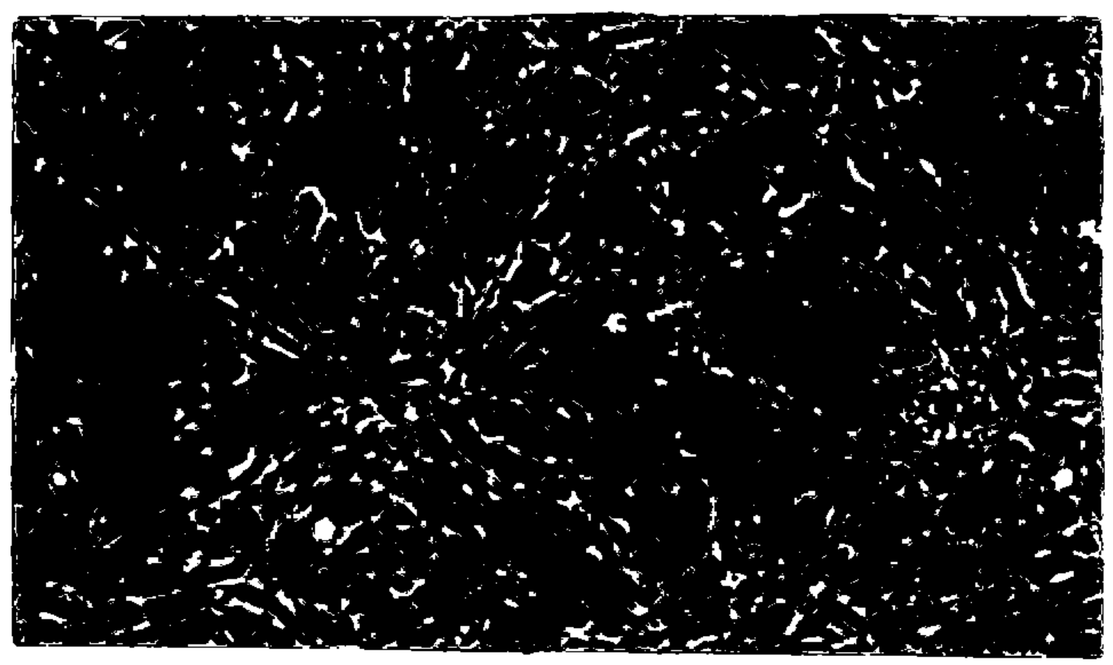
Figure 7.1. Day 70 and day 100 fetal sheep hypothalamic cells cultured in the presence or absence of 150 nM cortisol. The phase-contrast photomicrographs show day 70 cells in **A** the absence or **B** the presence of 150 nM cortisol. Similarly, **C** and **D** show day 100 cells in the absence and presence of cortisol (150nM) respectively. Subjective assessment, in terms of cell density and phase contrast appearance of the cells, suggested there was no detrimental effect of corticosteroid treatment on the hypothalamic cultures.

Day 70 Cells

A Without Cortisol

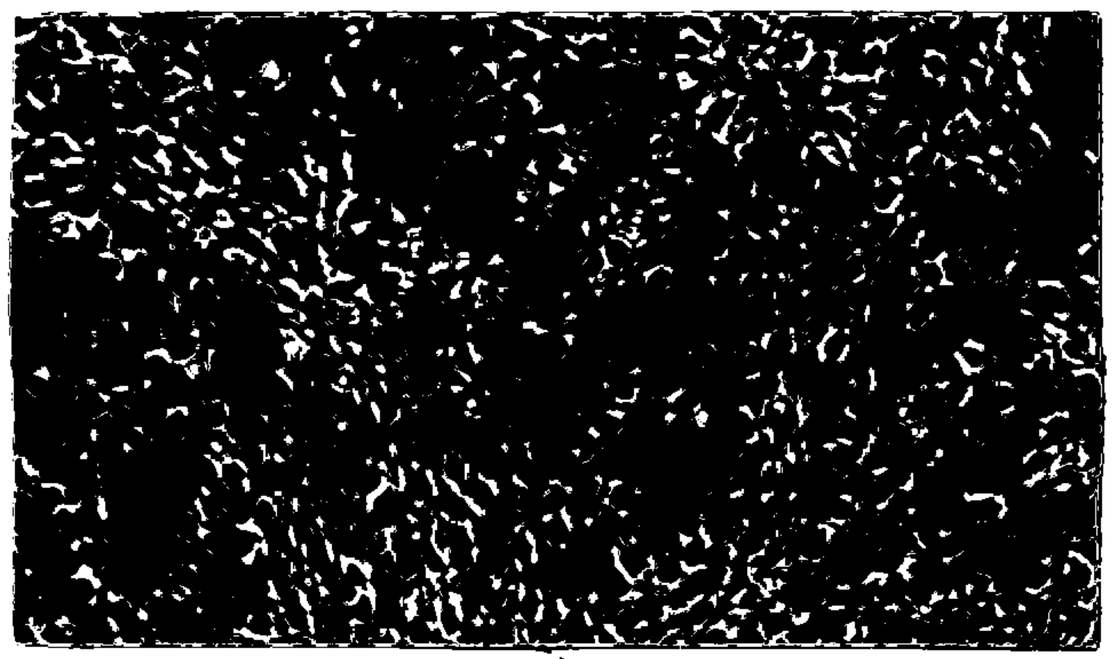


B With 150 nM Cortisol

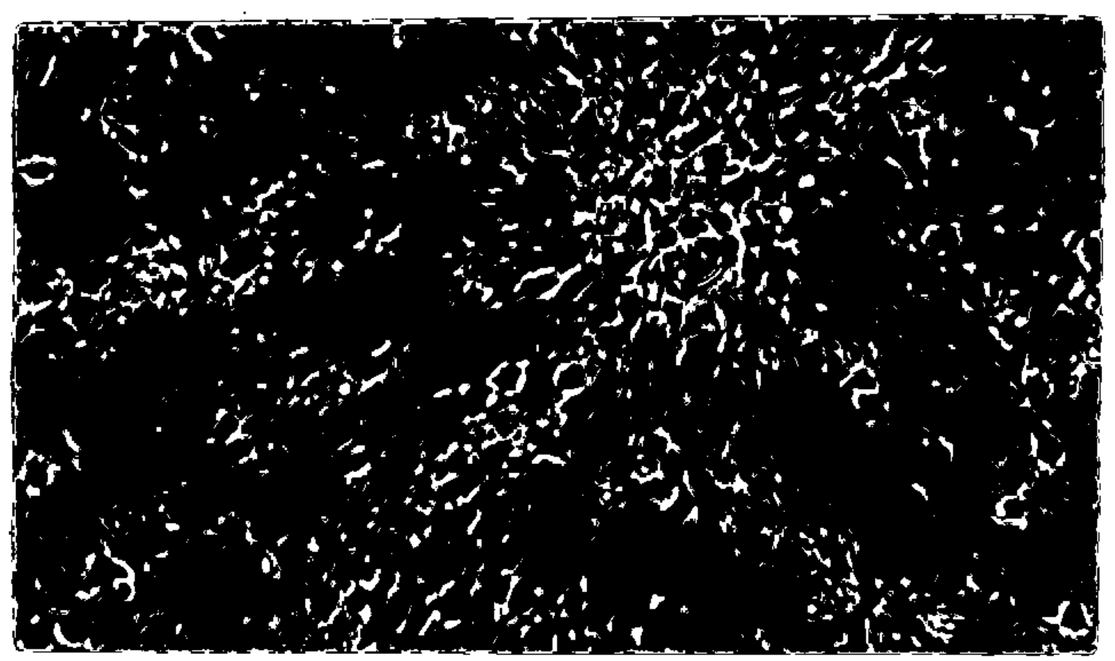


Day 100 Cells

C



D



A preliminary analysis of variance suggested that both elevated potassium stimulation (ANOVA; p <0.001) and cortisol (ANOVA; *p* <0.001) had profound effects on the secretion of AVP from fetal sheep hypothalamic cultures. Further statistical analyses established that in day 70 and day 100 hypothalamic cells cultures, either in the presence or absence of cortisol, incubation with elevated potassium-containing medium significantly stimulated AVP output on each experimental day *in vitro* (*p* <0.01), as shown in Figure 7.2. However, cortisol significantly and profoundly inhibited basal (*p* <0.01) and potassium-stimulated (*p* <0.01) AVP output from cells removed at day 70 gestation. In contrast cortisol had no significant effect on either basal or potassium-stimulated AVP secretion from day 100 cultures.

A marked variation characterised the AVP contents of wells in different cultures, even within the same gestational age grouping. To eliminate such variation, the AVP contents of wells in each culture were expressed as a percentage of the average content for that particular culture. These normalised data revealed that 150nM cortisol significantly reduced the AVP content of fetal sheep hypothalamic cells cultured at day 70 of gestation (*p* <0.05), whereas it had no effect on the AVP content of cells removed at day 100 of gestation, as shown in Figure 7.3.

The immunoreactive AVP present in the supernatant of fetal sheep hypothalamic cultures, prepared either at day 70 or at day 100 of gestation, reproducibly showed parallelism with the synthetic peptide in RIA procedures, as shown in Figure 7.4. This figure also shows that AVP in the supernatant from cultures of day 70 or day 100 hypothalamic cells consistently co-eluted with standard AVP on Sephadex chromatography.

7.4. Discussion.

The aims of the present study were to investigate the ability of cortisol to inhibit AVP secretion from cultures of fetal sheep hypothalamic cells, and to assess the gestational age-dependent changes in hypothalamic sensitivity to this steroid by investigating the effects of cortisol in cells from day 70 and day 100 of gestation.

The results showed that basal and potassium-stimulated AVP

Figure 7.2. The effects of 150 nM Cortisol on AVP secretion from cultured day 70 and day 100 fetal sheep hypothalamic cells. **A** and **B** show AVP secretion from day 70 cells cultured in the absence or presence of 150 nM cortisol repectively, and **C** and **D** show the corresponding AVP released from day 100 cells. Data shown are means of 12 or 13 wells (+/- S.E.M.) combined from 2 separate cultures at each gestational age. Cortisol significantly inhibited basal ($p < 0.01$) and potassium-stimulated ($p < 0.01$) AVP secretion from day 70 cells, but was without effect on either basal or stimulated AVP secretion in day 100 cultures. At each time point *in vitro*, 56 mM potassium treatment significantly elevated AVP release from all cultures (** $p < 0.01$).

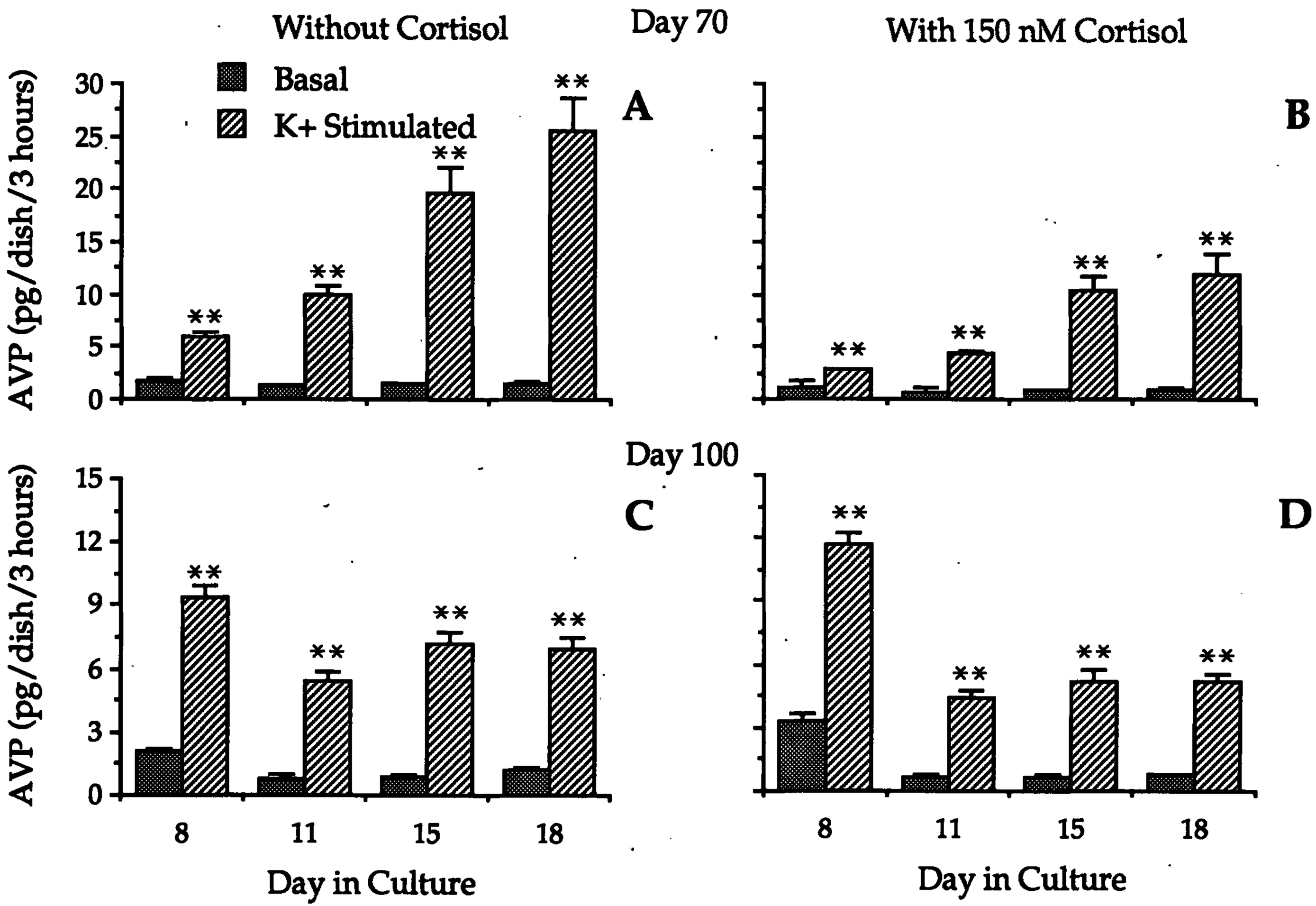

Without Cortisol
Day 70
With 150 nM Cortisol
Basal
K+ Stimulated
A
B
AVP (pg/dish/3 hours)
30
25
20
15
10
5
0
**
Day 100
C
D
12
9
6
3
8
11
15
18
Day in Culture

Figure 7.3. The effects of 150 nM Cortisol on AVP content of cultured day 70 and day 100 fetal sheep hypothalamic cells. Data shown are means (+/- S.E.M.) of 6 or 7 wells combined from 2 different cultures at each gestational age. AVP content in each well of a culture was expressed as a percentage of the average AVP content for that culture, and the data from 2 cultures at the same gestational age were combined for analysis. Cortisol significantly reduced AVP content in day 70 cells (*$p < 0.05$), but had no significant effect in day 100 cultures.

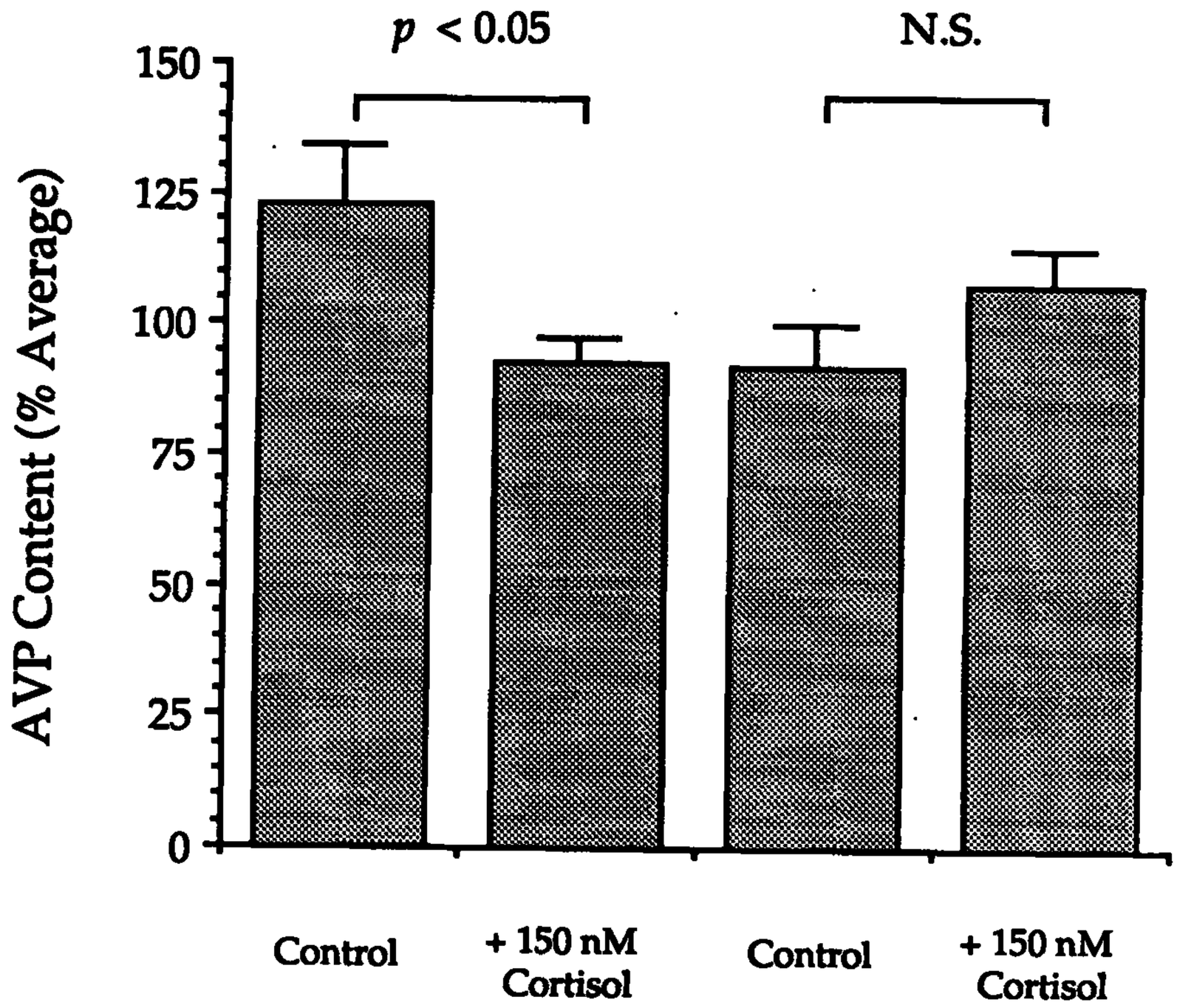
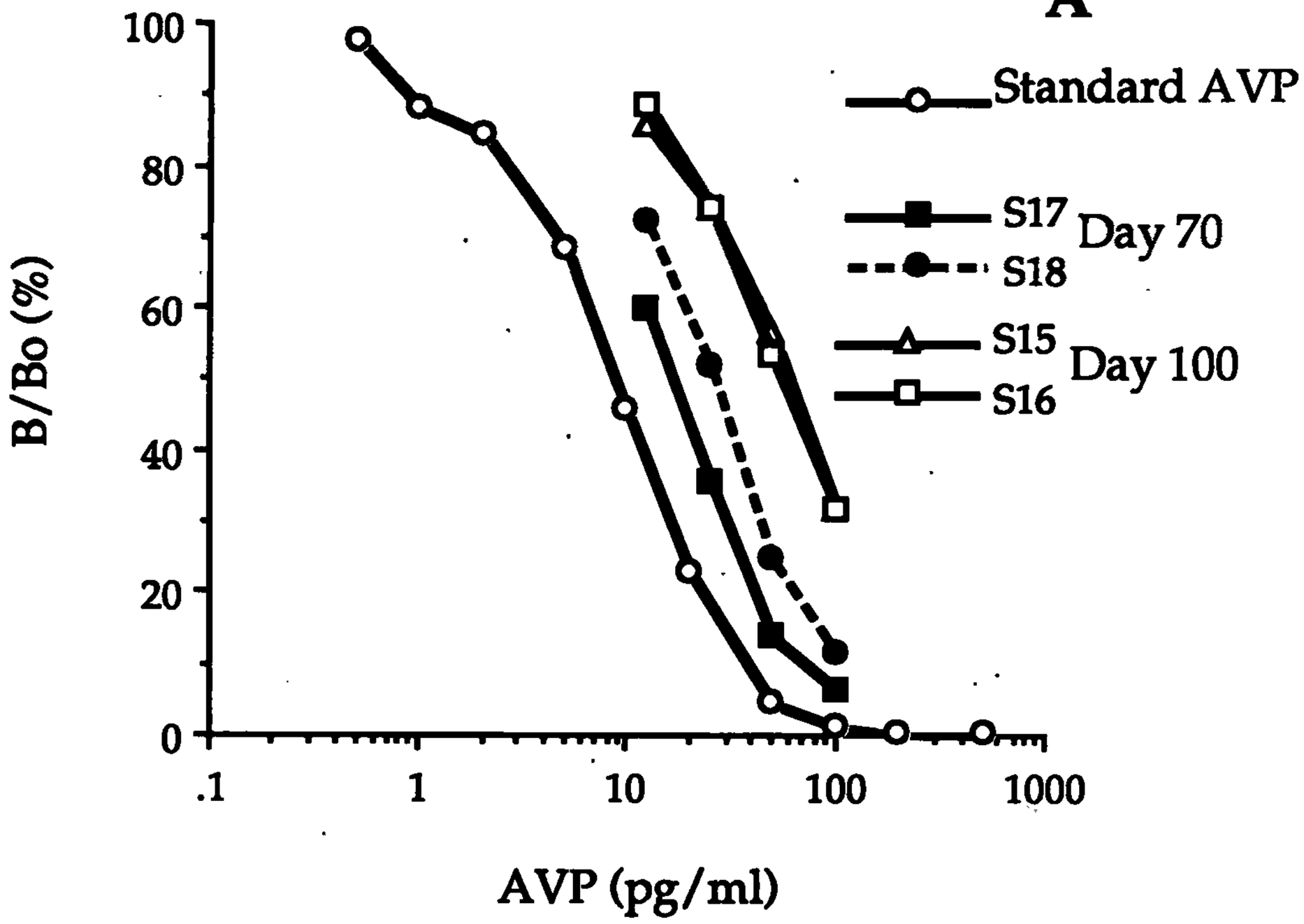
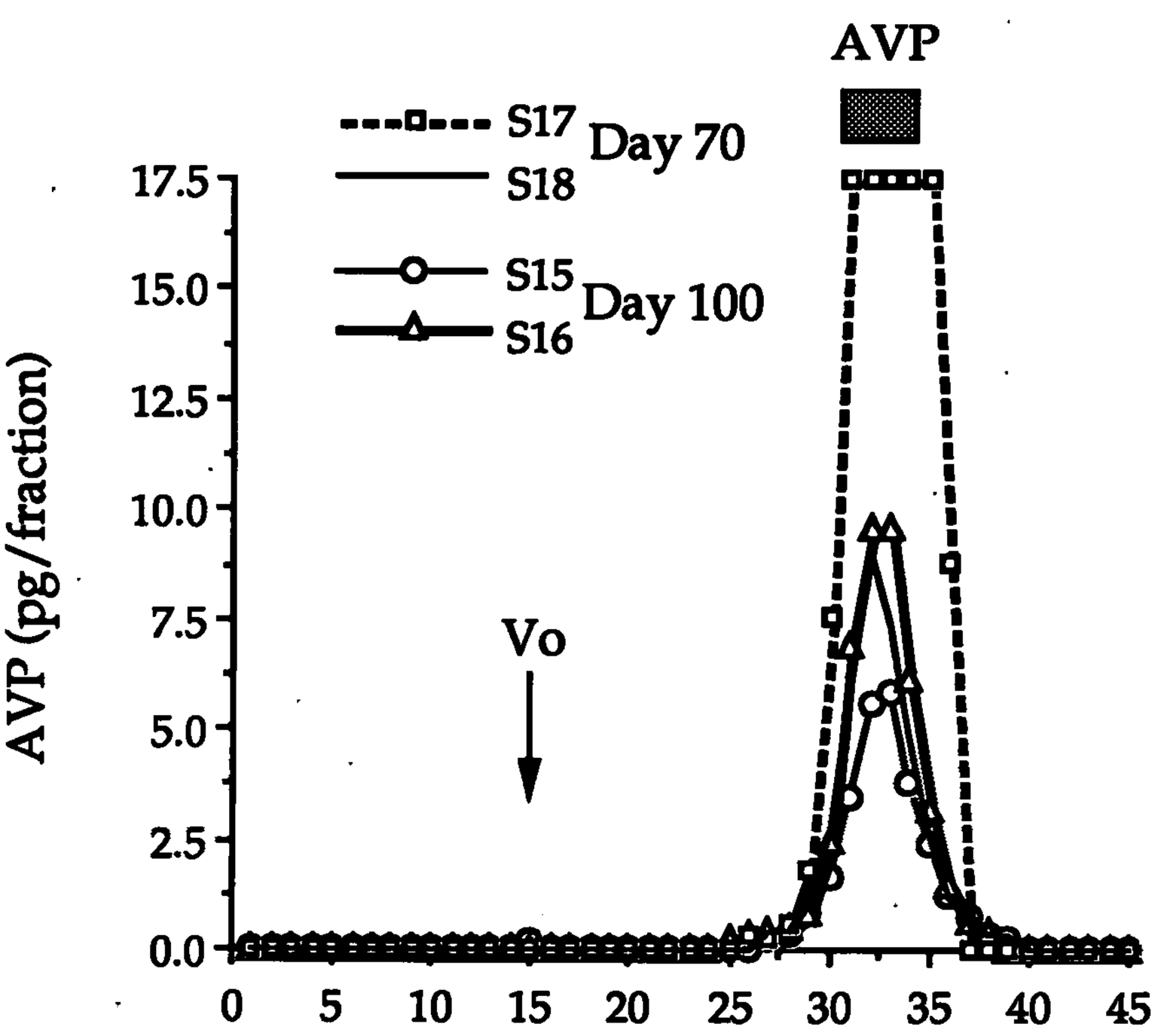

p < 0.05
N.S.
AVP Content (% Average)
150
125
100
75
50
25
0
Control
+ 150 nM Cortisol
Control
+ 150 nM Cortisol
Day 70
Day 100

Figure 7.4. Characterisation of AVP secreted by day 70 and day 100 fetal sheep hypothalamic cultures. A. Parallelism of the secreted peptide with the standard curve in an AVP RIA. In all cases, hypothalamic culture supernatant diluted in parallel with the standard peptide.
B. Immunoreactive profile of culture supernatant subjected to Sephadex G50 chromatography and AVP RIA. Supernatant from cultures showed one peak of immunoreactive AVP which co-eluted with the standard AVP in each case.

Characterisation of AVP





secretion by hypothalamic cultures were inhibited by cortisol in cultures from day 70 of gestation, but not in cultures from day 100. This effect in day 70 cells was characterised by a 25% fall in AVP content of the cells and a 50% reduction in AVP output. Whilst the effect of cortisol differed at the two gestational ages, the nature of AVP secreted from the cells was identical, both in terms of its parallelism with synthetic AVP in the RIA and its molecular size as assessed by Sephadex chromatography.

AVP is present as a 20-fold molar excess when compared to CRH in the hypothalamus of the ovine fetus at midgestation (Term = 145 days; see Chapter 6). Well before this developmental age, neuronal tracts containing AVP project from the hypothalamus to the external lamina of the median eminence (Levidiotis *et al.* 1987). In the present study, AVP secretion from cultured fetal sheep hypothalamic cells was significantly inhibited in the presence of cortisol, which is consistent with the hypothesis that hypothalamic AVP is a site of negative feedback regulation of the pituitary-adrenal axis in the ovine fetus.

To investigate this hypothesis, the effects of glucocorticoid administration to the hypothalamic PVN have recently been examined in the chronically catheterised fetal sheep (McDonald *et al.* 1990). Fetuses responded to hypotension or hypoxaemia with a significant elevations in circulating ACTH levels. However, dexamethasone crystals placed bilaterally beside the fetal PVN completely abolished these responses, without affecting basal ACTH release. Furthermore, AVP immuno-staining in the fetal median eminence was dramatically reduced after dexamethasone treatment, consistent with a reduction in synthesis and secretion of this peptide. The data presented here confirm and extend these observations, and firmly support the hypothesis that glucocorticoids during development negatively regulate the fetal hypothalamo-pituitary-adrenal axis at a hypothalamic level.

Currently, it is believed that expression of glucocorticoid receptors is a prerequisite for the action of glucocorticoids in a particular cell (see Gustaffson *et al.* 1987, for review). The present study revealed a profound inhibitory effect of cortisol on AVP release from day 70 cells whereas there was no inhibition of AVP secretion from day 100 cultures. Paradoxically, Yang and colleagues (1990) have shown that the concentration of glucocorticoid receptors in the fetal sheep hypothalamus

is much lower at days 60-70, when compared to day 100. However, these workers did not determine the distribution of glucocorticoid receptors within this tissue, and it may be that glucocorticoid receptor expression by cells outwith the PVN contributed significantly to hypothalamic corticosteroid binding in this study. On the other hand, glucocorticoid receptor expression in the hypothalamus of the rat is most dense within the medial parvocellular PVN (Gustaffson *et al.* 1987), the area containing the AVP and CRH cells which regulate ACTH release (Antoni, 1986).

The observation in the present study that glucocorticoids had minimal effects in tissues removed at day 100 of gestation is difficult to reconcile with the glucocorticoid receptor binding studies, as described above. However, substantial remodelling of hypothalamic glucocorticoid receptors may occur during development, such that the PVN at day 100 of gestation may not contain glucocorticoid receptors. Further histological studies co-localising the glucocorticoid receptor and AVP mRNA throughout develoment would be required to clarify these issues.

In order to determine whether glucococoticoid treatment had effects on both basal and stimulated AVP release, the present report measured AVP secretion under basal and 56 mM potassium-stimulated conditions in cells receiving chronic exposure to either cortisol-supplemented culture medium or to control medium. The results clearly showed that cortisol-mediated inhibition affected both basal and potassium-stimulated release of AVP from the hypothalamic cultures, and was associated with a substantial reduction in the AVP content of the cells. However, acute administration of the potent glucocorticoid agonist dexamethasone is known to inhibit basal but not potassium stimulated CRH release. (Brooks *et al.* 1989). These data suggest that the acute and chronic inhibitory effects of glucocorticoids on the secretion of ACTH-releasing factors may operate through different mechanisms.

The rapid onset of dexamethasone-induced inhibition of CRH release (within 30 minutes) suggested that the mechanism does not involve a reduction in CRH synthesis at the cell body and reduced CRH transport to nerve terminals. Rather, the reversal of the effects of glucocorticoids by potassium-induced depolarisation suggests that acute dexamethasone treatment directly inhibited CRH release from the axon terminals. By contrast, the reduction in basal and potassium-stimulated

release of AVP in concert with a reduction in AVP content, as reported in the present work, is consistent with a cortisol-induced reduction in AVP synthesis.

In a recent study, Schilling, Schmale, Oeding & Pilgrim (1991) examined glucocorticoid effects on mRNA transcription. Using cultured fetal rat hypothalamic cells, these workers showed that AVP mRNA but not oxytocin mRNA expression was much increased when RU 486, a glucocorticoid antagonist, was added to the serum-containing culture medium. In parallel, Clarke & Gillies (1988) have shown that corticosterone inhibits AVP and CRH secretion but not somatostatin in serum-free cultures of fetal rat hypothalamic cells. These data are very supportive of a highly specific, inhibitory effect of glucocorticoids on AVP synthesis which subsequently leads to reduced AVP secretion. Further *in vitro* studies, utilising northern blotting techniques to visualise AVP mRNA (see Schilling *et al.*, 1991), and [^{35}S] methionine-labelling of nascent proteins (see Gainer, Sarne, & Brownstein, 1977), may reveal more precisely the nature of glucocorticoid effects on the synthesis and secretion of hypothalamic AVP.

The AVP content of the fetal sheep hypothalamus increases between days 70, 100 and day 130 of gestation, moreover, the peptide elutes as a single immunoreactive and bioactive peak on Sephadex chromatography, corresponding to synthetic AVP, at each gestational age (Chapter 6, this thesis). In the present study, it was not possible to assess the bioactivity of AVP collected from cultured hypothalamic cells. However, gel filtration of supernatant from day 70 and day 100 cultures revealed that secreted AVP co-eluted with the synthetic peptide at each gestational age. It is therefore likely that the complex biochemistry required to fully process the high molecular weight AVP precursor to the mature peptide is present in the fetal hypothalamus *in vitro*. Therefore, the reduction in AVP secretion observed with advancing gestational age, as seen in this chapter and in Chapter 5, is unlikely to be associated with a loss of differentiated function in these cultured cells.

The present report does not reveal whether the fetal hypothalamus is under the inhibitory influence of glucocorticoids in vivo. Plasma cortisol levels early in fetal sheep development are very low compared to late gestation (Rose *et al.* 1978), which possibly reflects the finding that

prior to day 120 of pregnancy, fetal cortisol is largely of maternal origin (Hennessy, Coghlan, Hardy, Scoggins & Wintour, 1982). Until the onset of labour, a further 90% of this cortisol is bound to corticosteroid binding globulin (Ballard, Kitterman, Bland, Clyman, Gluckman, Platzker, Kaplan & Grumbach, 1982). Therefore, free bioactive cortisol levels in the fetal sheep are likely to be extremely low until late in fetal development.

On the other hand, lung, liver, and gut maturation occurs well before parturition in this species, and is dependent on cortisol from the fetal adrenal (Liggins, 1976; Silver, 1990). Moreover, the ratio of adult to fetal corticotropes in the fetal pituitary gland is increased by cortisol (Antolovich *et al.* 1989), and the increase *in vivo* is apparent from day 87 of gestation (Mulvogue *et al.* 1986). These data suggest that biologically active cortisol is present in the fetal circulation from mid-gestation at least, and this could be implicated in the negative feedback regulation of the fetal pituitary-adrenal axis. These data also present the intriguing hypothesis that maternal cortisol inhibits the activity of the fetal hypothalamo-pituitary-adrenal axis in early development. This may have some physiological importance, as the fetal adrenal before day 50 of gestation is extremely sensitive to ACTH (Wintour *et al.* 1975), and so premature hypothalamic drive to pituitary ACTH release could have disasterous consequences for the very immature fetus.

The data presented in this study are subject to a number of alternative interpretations to those suggested in the body of this discussion. It is shown here that cortisol inhibited AVP output from cells removed at 70 days of gestation, and yet the steroid had no effect on cells from later in development. These differential effects of cortisol cannot be explained by a reduction in the potency of the stock solution over time in storage, as cultures from day 100 of gestation were experimented upon first, and these cultures showed no effect of cortisol on AVP secretion. These observations would tend to rule out suggestions that variability in the cortisol stock solution lead to the discrepancies between day 70 and day 100 cells.

It is also possible to explain the effect of cortisol on AVP output from day 70 cells in terms of a toxic effect on the cultures. If this were so, the same responses would be expected in day 100 cells. Clearly, this was not the case. Furthermore, the viability of the cultures was demonstrated

by treatment with 56mM potassium-containing medium, and throughout the culture period, depolarisation continued to stimulate AVP release from cells in the presence or absence of cortisol. On the other hand, these studies do not rule out a cortisol-mediated selective toxicity on neuronal cells. However, the concentration of cortisol used in these studies is less than the plasma level observed at term in the fetal sheep (Basset & Thorburn, 1969), suggesting that the dose was physiological, rather than toxic.

In conlcusion, this chapter has demonstrated that cortisol may act at a hypothalamic level as a negative feedback regulator of the developing fetal pituitary-adrenal axis. Interestingly, the inhibitory effects of cortisol on hypothalamic cells were highly dependent on the gestational age of the tissue from which the hypothalamic cultures were prepared. Both basal and potassium-stimulated AVP secretion by day 70 cells was inhibited by cortisol, which suggested that AVP synthesis was inhibited in these cells, whereas this treatment was without effect in cultures prepared from day 100 hypothalami. The present study also determined by RIA and gel chromatography that mature AVP peptide is secreted by cultured cells removed either at day 70 or day 100 of gestation, which suggest the cells retain differentiated function and biochemical maturity *in vitro*. The data presented in this chapter support the hypothesis that changes in hypothalamic glucocortcoid sensitivity may underlie activation of the pituitary-adrenal axis which is observed in the developing fetal sheep.

8. Opioid regulation of AVP secretion in the developing fetal sheep hypothalamus *in vitro*.

In the previous chapter, the effects of cortisol on basal and 56 mM potassium-stimulated AVP secretion from cultured fetal sheep hypothalamic neurones were investigated. The principal findings were that cortisol treatment *in vitro* significantly inhibited AVP release and reduced AVP content of cultured hypothalami removed at day 70 of gestation, whereas these effects were absent in hypothalamic cells cultured at day 100 of gestation. These data suggest that changes in the cortisol sensitivity of the fetal hypothalamus may underlie the pre-parturient activation of the pituitary-adrenal axis. On the other hand, a reduction of inhibitory neural input may account for the increase in fetal hypothalamo-pituitary-adrenal activity. What is more likely is that several factors interact to regulate the pituitary-adrenal axis at central and peripheral sites. The objective of this chapter was therefore to investigate the effects of one of these putative factors, the opioids, on AVP secretion from the fetal sheep hypothalamus. To examine the interactions between neuropeptides, steroids and development, the hypothalamic responses to opioids were to be determined in the presence and absence of cortisol at different fetal ages.

8.1 Introduction

The fetal sheep hypothalamus releases CRH and AVP (Brooks *et al.* 1989; Chapter 5), which act to stimulate ACTH release from the fetal sheep pituitary gland (see Brooks & White, 1990). ACTH release from the fetal pituitary may also be stimulated by administration of opioids (Brooks & Challis, 1988), an effect which is probably mediated via hypothalamic CRH and AVP, as the fetal pituitary gland does not contain opioid receptors (Yang & Challis, 1991). A hypothalamic site of action for the ACTH-releasing activities of the opioids is supported by studies in adult animals, which show that opioids greatly elevate secretion of bioactive

ACTH-releasing factors from adult hypothalami *in vitro* (Buckingham, 1982).

The studies reviewed above suggest that the fetal sheep pituitary-adrenal axis is under stimulatory opioid control at the level of the hypothalamus. However, they do not demonstrate whether endogenous opioid peptides are involved in the physiological regulation of the fetal pituitary-adrenal axis. Brooks & Challis (1988) found that intravenous administration of a bolus of naloxone to the fetal sheep had no effect on fetal plasma ACTH concentration, suggesting that endogenous opioids were not tonically active. However, these authors demonstrated in a recent study that naloxone infusion to the fetal sheep near term profoundly inhibits fetal plasma ACTH levels (Brooks & Challis, 1991), strongly suggesting that endogenous opioids are stimulatory to the fetal pituitary-adrenal axis.

In the fetal sheep hypothalamus, opioid receptor binding increases with advancing gestational age (Yang & Challis, 1991), in parallel with maturation of the fetal ACTH response to exogenous opioid treatment (Brooks & Challis, 1988). It is notable that these maturational changes occur at the time when fetal plasma cortisol levels begin to rise towards term (Norman *et al.* 1985). These data suggest that there is a progressive maturation of the hypothalamic opioid system in the fetal sheep. Further, fetal plasma cortisol concentration may increase as a result of hypothalamic maturation, or act to enhance hypothalamic development. Indeed, cortisol may be stimulatory to the endogenous opioids, and so form a positive feedback loop which maintains the rise of fetal plasma ACTH and cortisol towards term.

In order to investigate the actions of opioids on the fetal hypothalamus and the effects of cortisol on the developing opioid responses, fetal sheep hypothalamic cells were removed at day 70 and day 100 of gestation and cultured in the presence and absence of cortisol. Cells were subjected to incubations with the highly specific κ-agonist [D-Pro^{10}] Dynorphin (1-11), the opioid antagonist naloxone, or both treatments together. It was not possible to characterise the dose-dependent effects of selective mu- (μ), delta- (∂) and kappa- (κ) receptor agonists in fetal sheep cultures, as there were insufficient numbers of fetal sheep available. Therefore, the effects of different doses of these drugs were fully

investigated in preliminary experiments, carried out with fetal rat hypothalamic cell cultures.

8.2 Experimental Procedures

Cultures of fetal rat hypothalamic cells were prepared using adaptations of the methods described in Chapter 3. For the experiments reported here, cultures were prepared on 6 different occasions, using a cumulative total of 412 fetal rat hypothalami removed at day 18 of gestation (Term = 22 days). The experiments on fetal sheep hypothalamic cells reported here were carried out on the cultures described in the previous chapter.

Preparation of Fetal Rat Hypothalamic Cell Cultures. Vaginal cytology was used to detect oestrus in Female Wistar rats. These animals were placed with proven males the next day (Dioestrus) and removed at the time of next oestrus (72 hours later). Females were then kept isolated until required on day 18 of pregnancy. Animals were then asphyxiated with 100% CO_2 and the cervical vertebrae were dislocated. The fetuses were dissected out, decapitated and the brains were removed. Hypothalami were dissected away from the base of the brain and placed in ice cold, sterile collection buffer.

Fetal rat hypothalamic cell cultures were prepared in the same fashion as fetal sheep hypothalamic cell cultures (Chapter 3), except that only half the volumes of enzyme-containing media were used and the cells were plated out in culture medium containing 10% fetal calf serum. For the studies reported in this chapter, the average viability at plating was 96.2 +/- 0.35 % (mean +/- S.E.M., n = 6)

As with the fetal sheep cell preparations, cultures were placed in serum-containing medium for the first 48 hours of culture and serum-free medium thereafter.

8.2.1. The Effects of Selective Opioid Agonists on AVP Secretion from Fetal Rat Hypothalamic Cells.

Overall Experimental Design. Four independent fetal rat hypothalamic cell cultures were used to carry out the two opioid experiments described in the present section. Each experiment was performed in all cultures, and the mean results from each culture were combined for analysis (n = 4 cultures). Cultures were routinely incubated with basal and 56 mM potassium-containing medium every 3 or 4 days, starting at 8 days *in vitro*, to enhance AVP output from the cells (Clarke & Gillies, 1988). The experiments were then carried out on either day 17, day 21, day 24, day 28 or day 31 *in vitro*, and to randomise the effects of advancing time *in vitro*, the three replicate experiments in the other cultures were performed on different days across this time span. To avoid any carry-over effects of previous treatments, wells were randomised into new treatment groups at the start of each experiment. Finally, each sample from a particular experiment was assayed at the same time to avoid interassay variation.

General Protocol. Cultures were incubated with basal medium for 2 hours (B1) and then immediately afterwards with 56 mM potassium-containing medium for a further 2 hours (S1). After a recovery period, this procedure was repeated (B2 and S2) in the presence or absence of the various opioids. AVP output from each well during B2 and S2 was expressed relative to output during B1 and S1 (B2/B1 and S2/S1), and the values of these ratios were compared for wells receiving control or opioid-containing media during B2 and S2. Therefore, opioid-mediated stimulation or inhibition would reveal itself as an increase or decrease in these ratios respectively.

In order to validate the experimental approach described here, it was necessary to establish the optimum interval between the control incubations such that the B2/B1 and S2/S1 ratios were approximately equal to 1. Using fetal rat hypothalamic cultures which were entirely different from those used for the opioid experiments, 6 groups of 3-4 wells were incubated with basal and 56 mM potassium medium for 2 hours each as described above (B1 and S1) on day 24 *in vitro*. After

intervals of either 1, 2, 4, 8, 12 or 20 hours, cells were incubated again with basal and 56 mM potassium-containing medium (B2 and S2). Media were stored at -20°C until they were assayed for AVP by RIA, and the B2/B1 and S2/S1 ratios were then calculated for each groups of wells. The results from these wells were combined with those from a duplicate experiment carried out in a separate culture. Data were expressed as the means of the ratios (B2/B1 and S2/S1) calculated for each time group, +/- S.E.M. (n = 6-8 wells).

To determine the intervals after which the B2/B1 and S2/S1 ratios equalled 1, one-way ANOVA, followed by Duncan's Multiple Range Test, was used to compare the means of B2 and B1 at each time point (also S2 and S1). The ratio was taken to be unity when the two means were not significantly different.

Differential Regulation of AVP Secretion by Selective Opioid Agonists. This experiment was designed to examine the AVP secretory responses of cultured fetal rat hypothalamic cells to various selective opioid receptor agonists. On day 17 *in vitro,* cells were incubated with basal and 56 mM potassium-containing medium for 2 hours each as previously described. 20 hours later, cultures were randomised into 10 treatment groups and incubated with basal and elevated potassium-containing media, in the presence of medium alone (control) or either the μ/∂-agonist β-Endorphin (1-31) (β-End; Cambridge Research Biochemicals Ltd, Northwich, Cheshire), the ∂-agonist [D-Ala^2, D-Leu^5] Enkephalin (DADLE; Cambridge Research Biochemicals) or the κ-agonist [D-Pro^{10}]-Dynorphin A (1-11) (Dyn 1-11; Bachem, Saffron Walden, Essex) at concentrations of 10^{-12}M, 10^{-9}M, or 10^{-6} M. All drug solutions were prepared immediately before use from concentrated stock solutions (1mg/ml in sterile water) stored at -20°C. The modified dynorphin analogue [D-Pro^{10}]-Dynorphin A (1-11) was chosen as a κ-agonist in the present studies as dynorphin(1-17), the endogenous κ-ligand, (Chavkin *et al.* 1982) showed a surprising cross-reactivity in the AVP RIA, whereas Dyn (1-11) did not show this cross-reactivity.

The AVP contents of the media were determined by RIA, and the B2/B1 and S2/S1 ratios were calculated for each well as previously described. This experiment was carried out in duplicate or triplicate wells

and the mean B2/B1 and S2/S1 ratios for each treatment group were calculated. This experiment was repeated in a further 3 cultures at 3 different times between day 17 and day 31 *in vitro,* as described above, and the means from each culture were averaged and expressed +/- S.E.M. (n = 4 cultures).

To assess whether there were significant responses to the opioids when compared to controls, B2/B1 and S2/S1 data were analysed separately by one-way ANOVA. The between-groups variable was treatment (control, β-End, DADLE and Dyn (1-11) at 10^{-12}M, 10^{-9}M and 10^{-6} M). Individual means were then compared to each other using Duncan's multiple range test.

Naloxone Antagonism of the Effects of the Opioid Agonists. This experiment was designed to investigate whether the opioid agonists were acting through naloxone-sensitive opioid receptors. Cultures were incubated with basal and 56 mM potassium-containing media on day 21 *in vitro* as previously described. Wells were allocated to 8 treatment groups and 20 hours later, the cells were incubated sequentially with basal and elevated potassium-containing media in the absence of opioid (control), or in the presence of β-End, DADLE or Dyn 1-11 at 10^{-6} M. Four other groups of cells received the same treatments in the presence of naloxone (Sigma) at a concentration of 10^{-5} M. As before, this experiment was carried out in duplicate or triplicate wells and repeated in 3 other cultures of different ages *in vitro.* The B2/B1 and S2/S1 ratios were calculated for each treatment group within each culture, and the overall means were averaged and expressed +/- S.E.M. (n = 4 cultures).

ANOVA was carried out on the B2/B1 and S2/S1 data separately, with Drug (Control, β-End, DADLE or Dyn 1-11) and naloxone (present or absent) as the between-groups variables. Duncan's multiple range test was used to reveal whether concomitant naloxone administration had abolished the opioid responses, and whether naloxone alone had significant effects when compared to controls.

8.2.2. Opioid and Steroid Regulation of AVP Secretion by Fetal Sheep Hypothalamic Cells.

The three experiments described below were carried out in duplicate cultures prepared from fetal sheep hypothalami removed at 70 days and 100 days of gestation. Each culture was divided two days after plating, and half the wells were maintained in serum-free medium and the other half were cultured in serum-free medium plus cortisol (150 nM), as described in Chapter 7. Cells were incubated with basal and 56 mM potassium-containing medium for 3 hours each on day 7 *in vitro*, and the experiments were carried out on days 10, 14 or 17 *in vitro*. As described in the previous chapter, basal incubation samples were collected as 2 aliquots of 300µl. These were freeze-dried and reconstituted in 100µl distilled water for measurement of AVP by RIA.

Within a particular culture, each experiment was carried out in triplicate or quadruplicate wells in the cortisol-free and cortisol-supplemented groups in parallel. As for the fetal rat hypothalamic cultures, duplicate experiments performed in separate cultures were carried out on different experimental days to randomise the effects of age *in vitro*. All samples from an experiment were assayed at the same time, and the results from the two cultures were combined for analysis.

The Effects of Dynorphin A (1-11) on AVP Secretion. Experiments in the fetal rat hypothalamic cultures indicated that Dyn (1-11) was the most potent inhibitor of AVP secretion. Therefore, this drug was chosen as suitable for investigations of opioid action in fetal sheep hypothalamic cells. In order to assess the developmental effects of Dyn (1-11) on AVP secretion, in the presence and absence of cortisol, cultures were incubated for 3 hours in basal medium and then for a further 3 hours in 56 mM potassium-containing medium on day 10 *in vitro*. 20 hours later, the cells were incubated sequentially with basal and elevated potassium medium each containing Dyn (1-11) at 10^{-6}M for 3 hours each. This experiment was carried out in parallel in the wells cultured in cortisol-containing medium, in which case all media contained cortisol (150 nM) in addition to the experimental treatments. This experiment was repeated in duplicate day 70 and day 100 fetal hypothalamic cultures, and

samples were stored at -20°C for later assay. The AVP contents of the samples were determined by RIA, the B2/B1 and S2/S1 ratios were calculated for individual wells and the means of these ratios were expressed +/- S.E.M., (n = 6-7 wells).

One-way ANOVA and Duncan's test were then employed to examine B2/B1 and S2/S1 ratios individually and to assess significant differences between the various groups and their respective controls. Cortisol (present or absent), gestational age (day 70 or day 100) and dynorphin (present or absent) were the between-groups variables.

Naloxone Antagonism of the Effects of Dynorphin A (1-11). To determine whether Dyn (1-11) affected AVP secretion via naloxone-sensitive opioid receptors, the experiment described above was repeated on day 14 *in vitro,* except that naloxone (10^{-5}M) was added to the media containing Dyn (1-11). The results preparation and statistical analysis were carried out as described for the experiment with Dyn (1-11) alone, and the pairwise comparisons were made to assess whether naloxone had abolished the effect of Dyn (1-11) under the various conditions.

The Effects of Naloxone on AVP Secretion. In order to interpret the data obtained during the experiments described above, it was necessary to assess the effects of naloxone treatment alone on AVP secretion from the cell cultures. Therefore, the protocol described above (The Effects of Dynorphin (1-11) on AVP Secretion) was carried out on day 17 *in vitro,* except that Naloxone (10^{-5}M) took the place of Dyn (1-11). The statistical analysis was as described for the Dyn (1-11) experiment, and the data were examined to reveal whether there was a significant effect of naloxone alone in the different cultures.

8.3 Results

Figure 8.1 shows the recovery of basal and potassium-stimulated AVP secretion from fetal rat hypothalamic cells after previous incubations with basal and 56 mM potassium-containing media. Basal AVP secretion during B2 was significantly less than secretion measured in B1 at all time

Figure 8.1. Recovery of basal and stimulated AVP output after previous basal and potassium-stimulated Incubations in fetal rat hypothalamic cells. Cells were incubated with basal (B) and 56 mM potassium-containing media (S) on 2 ocassions (B1, S1 and B2, S2), separated by varying intervals. **A.** Basal AVP output during B2 was expressed as a ratio of B1 (B2/B1) and **B.** potassium-stimulated AVP output during S2 was expressed as a ratio of S1 (S2/S1). AVP secretion in B2 was compared with output in B1 at each time point, and a similar analysis was carried out for S2 and S1. Data shown are means of 6-8 wells, +/- S.E.M. ** p <0.01, Output in second incubation significantly different from corresponding output in first (i.e. Ratio $\neq 1$).

Basal

56 mM Potassium

A

B2/B1 Ratio

1.2
1.0
0.8
0.6
0.4
0.2
0.0

**

B

S2/S1 Ratio

1.2
1.0
0.8
0.6
0.4
0.2
0.0

**

1 2 4 8 12 20

Interval (hours)

points except 20 hours (B2/B1 not equal to 1; $p < 0.01$), whereas potassium-stimulated AVP release had recovered to S1 levels by 12 hours (S2/S1 not equal to 1 at 1, 2, 4 and 8 hour intervals only; $p < 0.01$).

Figures 8.2 A and B show the effects of β-End, DADLE or Dyn (1-11) at doses from 10^{-12}M to 10^{-6} M on AVP secretion from cultured fetal rat hypothalamic cells, under basal and potassium stimulated conditions respectively. None of the drugs had any significant effects on AVP release at 10^{-12}M or 10^{-9}M. However, compared with the control ratio, β-End ($p < 0.05$), DADLE ($p < 0.05$) and Dyn (1-11) ($p < 0.01$) significantly inhibited basal AVP release at a dose of 10^{-6} M. Overall analysis of variance revealed that there was no significant effect of any opioid treatment in the presence of elevated potassium (ANOVA; $p = 0.8910$).

To determine if the effects of the opioids were naloxone-reversible, fetal rat hypothalamic cells were incubated either with basal medium, β-End, DADLE or Dyn (1-11) at 10^{-6} M in the presence or absence of naloxone at 10^{-5} M. Figure 8.2 C and D show that β-End ($p < 0.05$), DADLE ($p < 0.05$) and Dyn (1-11) ($p < 0.01$) significantly inhibited basal AVP release at a dose of 10^{-6} M, whereas naloxone alone at 10^{-5} M had no significant effect. However, in each case, concurrent naloxone administration abolished the opioid-mediated inhibition of basal AVP To determine if the effects of the opioids were naloxone-reversible, fetal rat hypothalamic cells were incubated either with basal medium, β-End, DADLE or Dyn (1-11) at 10^{-6} M in the presence or absence of naloxone, had no significant effects when added in the presence of 56 mM potassium-containing medium (ANOVA; $p = 0.7464$).

Figure 8.3 shows the effects of Dyn (1-11) on AVP release from fetal sheep hypothalamic cell cultures from day 70 and day 100 of gestation, in the presence or absence of cortisol. Dyn (1-11) significantly inhibited basal AVP secretion ($p < 0.05$) in day 70 hypothalamic cultures, however, these cultures did not show an inhibitory response to the opioid when maintained in cortisol-containing media. In day 100 cultures, basal AVP release was not significantly inhibited by Dyn (1-11), either in cells cultured in cortisol-supplemented or cortisol-free medium. In both day 70 and day 100 cells, overall analysis of variance showed that Dyn (1-11) had no effect on 56 mM potassium-stimulated AVP release, either in the presence or absence of glucocorticoid (ANOVA; $p = 0.4142$).

Figure 8.2. The effects of Selective Opioid Agonists on AVP secretion from fetal rat hypothalamic cell cultures. Cells were treated with various doses of the opioids as shown overleaf under basal (A) and potassium-stimulated (B) conditions. Cells were treated with control medium or opioids at 10^{-6}M, in the presence and absence of naloxone (10^{-5}M), in basal (C) and potassium-stimulated (D) incubations. Basal and stimulated ratios (B2/B1 and S2/S1) were compared to corresponding control values. Data shown are means of 4 cultures, +/- S.E.M. * $p < 0.05$, ** $p < 0.01$, Significantly different from controls.

Dose Response to Opioids

Basal

56 mM Potassium

Naloxone Reversibility

A

Ratio (B2/B1)

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0

*
**
*

B

Ratio (S2/S1)

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0

-12 -9 -6 -12 -9 -6 -12 -9 -6

log Dose (M) log Dose (M) log Dose (M)

Control β-End Dyn (1-11) DADLE

C

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0

*
**
*

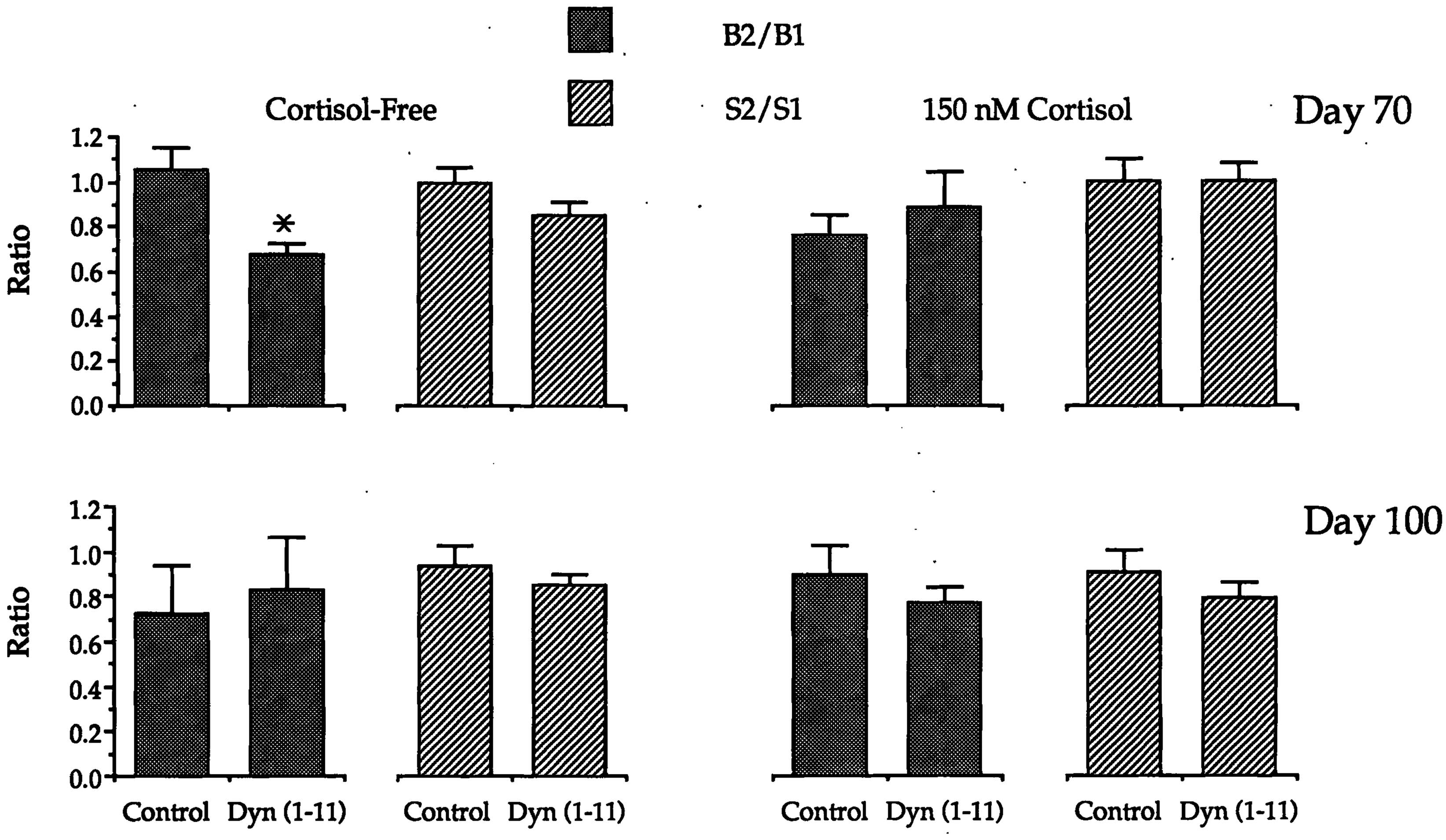
D

1.2
1.0
0.8
0.6
0.4
0.2
0.0

Control β-End Dyn (1-11) DADLE

Nal β-End+Nal Dyn (1-11) +Nal DADLE + Nal

Figure 8.3. The Effects of [D-Pro^{10}]Dynorphin (1-11) on AVP secretion from fetal sheep hypothalamic cells, in the presence and absence of cortisol. Cells maintained in the presence or absence of cortisol from day 70 or day 100 of gestation were incubated with Dyn (1-11) at 10^{-6}M or control media under basal (B2/B1) and potassium-stimulated (S2/S1) conditions. Data shown are means of 6-7 wells from 2 cultures, +/- S.E.M. Data were compared with the corresponding control groups in each case. * p <0.05, significantly different from corresponding control.


B2/B1
S2/S1
Cortisol-Free
150 nM Cortisol
Day 70
Day 100
Ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Dyn (1-11)
Control
Dyn (1-11)
Control
Dyn (1-11)
Control
Dyn (1-11)

In contrast to the effects of Dyn (1-11), naloxone had no inhibitory or stimulatory effects on AVP secretion from fetal sheep hypothalamic cultures, as shown in Figure 8.4. Either in the presence or absence of cortisol, overall analysis of variance revealed that basal AVP release was not affected by naloxone in day 70 or day 100 cells (ANOVA; $p = 0.6100$). A similar analysis showed that AVP secretion during potassium stimulation remained unchanged in the presence of naloxone, irrespective of glucocorticoid status, at either gestational age (ANOVA; $p = 0.2793$).

Figure 8.5 shows the effects of Dyn (1-11) plus naloxone on the secretion of AVP from fetal sheep hypothalamic cultures. There were no significant effects of this combined treatment on either basal or potassium-stimulated AVP release from day 100 cells, in the presence or absence of cortisol. However, this treatment schedule resulted in a significant inhibition ($p < 0.05$) of basal AVP release from day 70 cultures in the absence of cortisol. Overall analysis of variance revealed that there were no significant effects of Dyn (1-11) plus naloxone on AVP release in any of the experimantal groups during the 56 mM potassium incubations (ANOVA;$p = 0.2591$).

8.4 Discussion.

The study reported in this chapter investigated the opioid regulation of AVP secretion from fetal sheep hypothalamic cells *in vitro*, and was further structured to assess the effects of gestational age and cortisol on the opioid responses. During preliminary investigations with fetal rat hypothalamic cells, experiments showed that basal AVP secretion was significantly inhibited by the μ/∂-agonist β-End, the ∂-agonist DADLE and the κ-agonist Dyn (1-11), and that these effects were naloxone-reversible. In fetal sheep hypothalamic cultures, Dyn (1-11) inhibited basal AVP release from day 70 cells, but not in day 70 cells maintained in cortisol-containing medium. In day 100 fetal sheep cells, Dyn (1-11) had no effect on AVP secretion, either in cortisol-free or cortisol-treated cells. Further experiments showed that the Dyn (1-11)-mediated inhibition of AVP release in day 70 cells was not naloxone-reversible. In contrast to the

Figure 8.4. The Effects of Naloxone, plus or minus cortisol, on AVP Secretion from fetal sheep hypothalamic cell cultures. Cells maintained in the presence or absence of cortisol from day 70 or day 100 of gestation were incubated with Dyn (1-11) at 10^{-6}M or control media under basal (B2/B1) and potassium-stimulated (S2/S1) conditions. Data shown are means of 6-7 wells from 2 cultures, +/- S.E.M. Data were compared with the corresponding control groups in each case. There was no significant difference between the naloxone-treated and control groups (ANOVA, B2/B1 p = 0.8478, S2/S1 p = 0.6197).

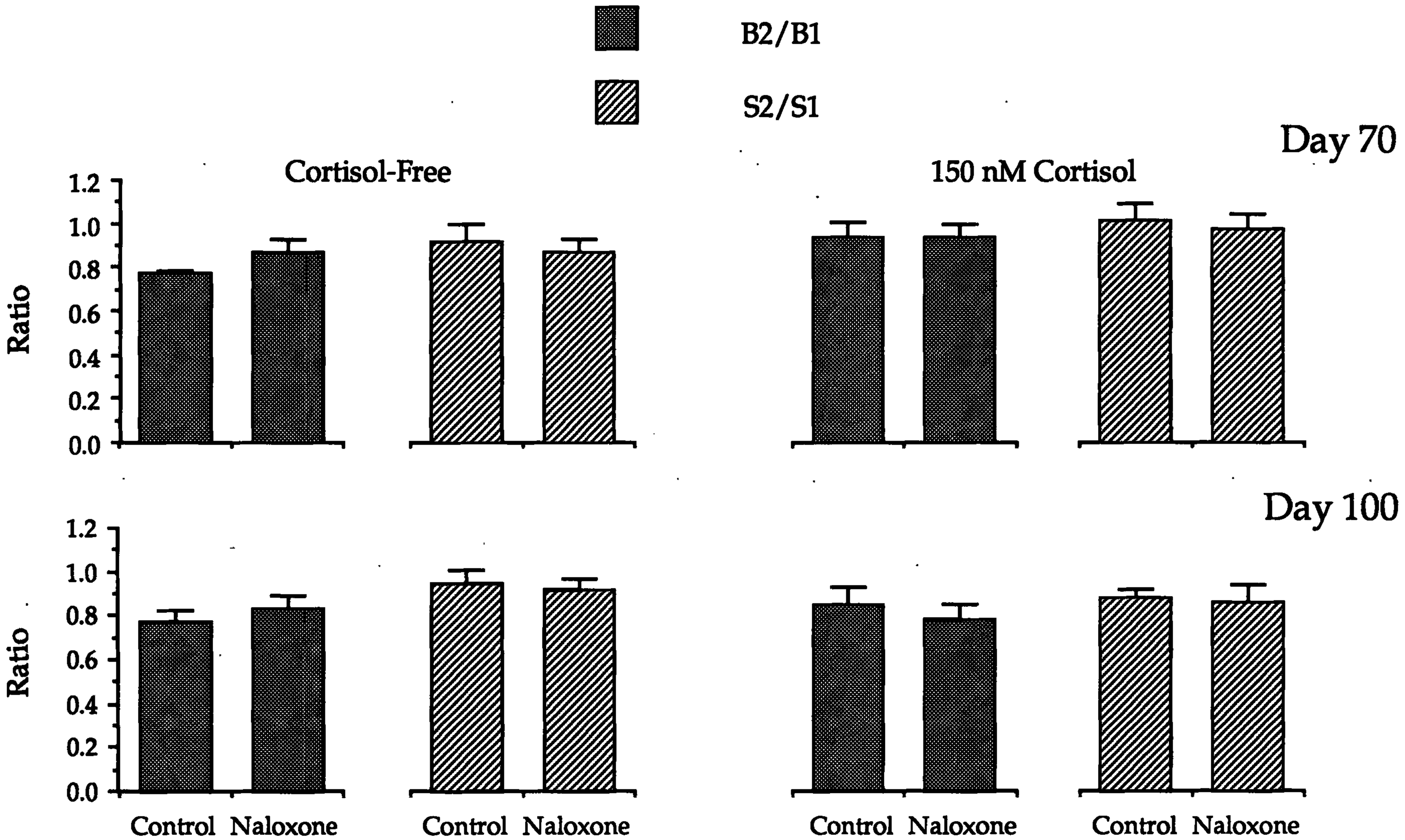
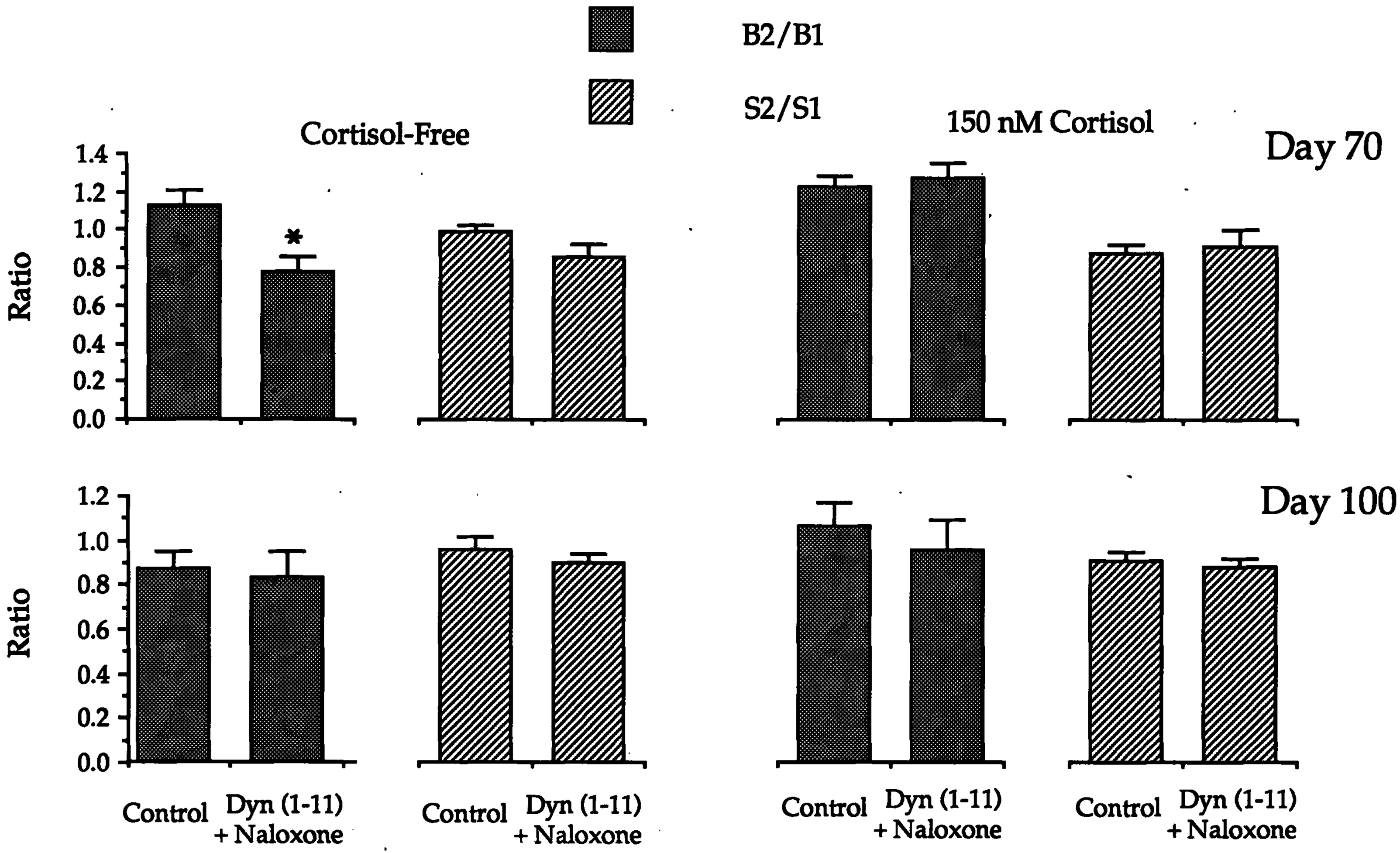

B2/B1
S2/S1
Day 70
Cortisol-Free
150 nM Cortisol
Ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Day 100
Control
Naloxone

Figure 8.5. The Effects of [D-Pro^{10}]Dynorphin (1-11) and Naloxone on AVP Secretion from fetal sheep hypothalamic cell cultures in the presence and absence of cortisol. Cells maintained in the presence or absence of cortisol from day 70 or day 100 of gestation were incubated with Dyn (1-11) at 10^{-6}M or control media under basal (B2/B1) and potassium-stimulated (S2/S1) conditions. Data shown are means of 6-7 wells from 2 cultures, +/- S.E.M. Data were compared with the corresponding control groups in each case. * p <0.05, significantly different from corresponding control.


B2/B1
S2/S1
Cortisol-Free
150 nM Cortisol
Day 70
Day 100
Ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Dyn (1-11)
+ Naloxone

opioid-induced suppression of basal AVP release observed in fetal sheep and rat hypothalamic cultures, opioid effects on AVP secretion were not observed in the presence of 56 mM potassium-containing medium in cell cultures from either species.

Intravenous opioid administration to fetal sheep stimulates ACTH secretion from the fetal sheep pituitary gland (Bousquet *et al.* 1984; Brooks & Challis, 1988). This opioid effect is most likely mediated indirectly, by stimulation of hypothalamic CRH and AVP secretion, as the fetal sheep pituitary contains no opioid receptors (Yang & Challis, 1991). By contrast, the work reported here showed that a specific κ-receptor agonist, Dyn (1-11) (Gairin, Gouarderes, Mazarguil, Alvinerie & Cros, 1984), was inhibitory to basal AVP secretion from fetal sheep hypothalamic cells. This was mirrorred by the effects of Dyn (1-11), β-End and DADLE on basal AVP secretion from fetal rat hypothalamic cells. The present study therefore contrasts with recent reports suggesting a stimulatory effect of opioids on the hypothalamic drive to fetal sheep pituitary ACTH secretion. However, previous authors employed either the μ-selective Met-enkephalin analogue FK33,824 (Brooks & Challis, 1988) or the ∂-agonist Leu-enkephalin (Bousquet *et al.* 1984), rather than the κ-agonist Dyn (1-11) used the present report. Therefore, separate opioid pathways may exist to mediate opioid-induced stimulation and inhibition of the hypothalamic input to the fetal sheep corticotropes.

Studies carried out in other species have examined more closely the various opioid mechanisms which regulate secretion of hypothalamic ACTH-releasing factors (see chapter 2). In a recent report, Cover & Buckingham (1989) determined that administration of naltrexone, a μ-opioid antagonist, attenuated the ACTH response to surgical stress in adult rats, whereas MR2266, a k-opioid antagonist, elevated basal ACTH secretion and enhanced the ACTH response to surgical stress. These data suggest that endogenous μ-opioids acutely stimulate and endogenous k-opioids tonically inhibit the hypothalamo-pituitary-adrenal axis, which is in agreement with the findings of Brooks & Challis (1988) and the present report.

The literature also contains reports which contrast with the present results, showing for example that administration of κ-opioids stimulates the hypothalamo-pituitary-adrenal axis (Pfeiffer *et al.* 1985). Such

contradictions may arise in part from the use of different animal preparations and assay methodologies, amd may also stem from differential opioid regulation of CRH and AVP. However, in studies which have specifically addressed hypothalamic AVP secretion, opioids have been shown to inhibit AVP release from medial basal hypothalamic fragments *in vitro* (Knepel & Reimann, 1982) and to reduce hypophyseal portal plasma concentrations of AVP *in vivo* (Koenig *et al* . 1986). Therefore, the results of the present study are consistent with κ-opioid inhibition of the fetal hypothalamo-pituitary-adrenal axis in early development.

It is worthy of consideration that the present results reflect the inhibitory effects of opioids on the secretion of AVP by magnocelluar hypothalamic neurones at least in day 70 cultures. κ-opioids *in vivo* have a characteristic diuretic action (see Von Voightlander *et al*. 1983), which is strongly suggestive of an inhibitory opioid effect on posterior pituitary AVP secretion. Studies which have investigated systemic levels of AVP have clearly shown that κ-opioid administration inhibits plasma concentrations of AVP (Leander, Zerbe & Hart, 1985; Oiso, Iwasaki, Kondo, Takatsuki & Tomita, 1988). Interestingly, there is a considerable projection of AVP-containing fibres in the developing fetal sheep hypothalamus which arise in the supraoptic nucleus and pass to the external layer of the median eminence (Levidiotis *et al*. 1987). Therefore, the normal segregation between AVP cells projecting to the posterior pituitary or to the median eminence may be less well defined in the fetal sheep hypothalamus.

In contrast to the inhibitory effects of Dyn (1-11) on basal AVP release in day 70 cells, the opioid had no effect on basal AVP release in day 100 cultures. These data suggest a gestational-age dependent decrease in hypothalamic sensitivity to opioid peptides, which could be mediated by reduced expression, or functional uncoupling, of opioid receptors. Dunlap, Christ & Rose (1986) carried out quantitative opioid receptor binding studies in the fetal sheep brain between day 77 and day 127 of gestation to determine how opioid receptors were regulated during development. These authors showed a marked increase in opioid binding with advancing gestational age, in agreement with the results of a more recent study of the fetal sheep hypothalamus (Yang & Challis,

1991). It is therefore not clear why cell cultures from day 70 of gestation showed a greater opioid response than day 100 cells in the present investigation. However, it may be that the ligands chosen in the binding studies did not detect changes in the κ-receptor to which Dyn (1-11) preferentially binds, as neither dihydromorphine, DADLE (see Dunlap *et al* ., 1986) nor naloxone (see Yang & Challis, 1991) are κ-selective opioid ligands (Patterson *et al*. 1983). Data from other species indicates that κ-receptors are present in the brain in early development and that specific κ-ligand binding in the CNS increases towards term (Ruis, Barg, Bem, Coscia & Loh, 1991; Magnan & Tiberi, 1989), suggesting that the κ-opioid system assumes greater significance in late gestation. By inference, the present report suggests that the number of inhibitory, fetal sheep hypothalamic κ-receptors is decreased with advancing gestational age. Such a reduction in hypothalamic κ-receptors would permit increased activity of the AVP neurones in the fetal hypothalamus, which could increasingly stimulate ACTH release from the fetal pituitary gland. However, the present results do not permit the conclusion that such a mechanism is operative in the fetal sheep *in vivo*. It would be necessary to carry out quantitative binding studies in fetal hypothalamic tissue, utilising specific κ-selective ligands, such as [^{3}H]U-69593 (see Kitchen, Kelly & Viveros, 1990), to examine this hypothesis in a subsequent investigation.

A surprising finding in the present report was that the inhibitory effects of Dyn (1-11) on basal AVP secretion in fetal sheep hypothalamic cultures were not naloxone-reversible. This result may be explained if the concentration of naloxone employed here (10 μM) was too low, as 10 μM naloxone only partially reversed the inhibitory effects of a specific κ-agonist in a previous study (Tsagarakis *et al* . 1990). However, the concentration of naloxone used here abolished Dyn (1-11)-mediated suppression of AVP release in fetal rat hypothalamic cultures, as described in this chapter. Therefore, the experiments on fetal sheep hypothalamic cells were carried out using this concentration of naloxone.

If it is presumed that Dyn (1-11) acts through κ-receptors in the sheep hypothalamic cultures, the naloxone-insensitivity of the effects of Dyn (1-11) may be explained by differing κ-receptor affinities in the two species, such that Dyn (1-11) is less easily displaced by naloxone from the

sheep κ-site. Competitive binding studies could be performed in the rat and sheep hypothalamus using the tritiated κ-ligand [^{3}H]U-69593, Dyn (1-11) and naloxone in order to reveal such a species difference. Alternatively, Dyn (1-11) may be toxic, or may not be a specific opioid ligand. However, the data obtained in the fetal rat hypothalamic cultures, which showed naloxone-reversibility of Dyn (1-11), would tend to refute these interpretations. In order to confirm these suggestions, day 70 fetal sheep hypothalamic cultures could be treated with Dyn (1-11) and increasing concentrations of naloxone or more κ-selective opioid antagonists, such as MR 2266 (Patterson *et al.* 1983). If neither naloxone nor a selective κ-antagonist abolished the effects of Dyn (1-11), then the interpretation would have to be that Dyn (1-11) acted via a non-opioid pathway in day 70 fetal sheep hypothalamic cells.

As gestation proceeds in the fetal sheep, plasma glucocorticoid levels increase concurrently with plasma ACTH (Norman *et al.* 1985). Such data suggest that negative feedback regulation of the pituitary-adrenal axis may be overcome near term (see Wood, 1988). This process may be effected by rising fetal plasma cortisol levels, which could act to increase stimulatory input or decrease inhibitory input to the fetal hypothalamus, so increasing the drive to pituitary ACTH secretion. In the present study, the inhibitory effects of Dyn (1-11) on AVP secretion were absent in cells treated with cortisol, which supports the hypothesis that cortisol reduces the inhibitory input to the fetal hypothalamus.

The data do not reveal how the cortisol effect was brought about. It may be that cortisol and Dyn (1-11) act through the same mechanism to inhibit AVP release, and hence no further effect is observed when both are added together. This is unlikely, as the principal action of glucocorticoids is to decrease AVP mRNA levels (Davis *et al.* 1986; Schilling *et al.* 1991), whereas opioids act at membrane-bound receptors to regulate ion fluxes (see North, 1986). Nevertheless, glucocorticoids acutely inhibit the secretion of ACTH-releasing factors from fetal sheep hypothalami (Brooks *et al.* 1989). This effect has been attributed to stabilisation of the cell membranes and inhibition of calcium fluxes (Keller-Wood & Dallman, 1984). These are the same mechanisms by which opioids reduce neuronal activity (North, 1986).

Glucocorticoids may also act to attenuate neuronal responses to opioids. Thus, the decrease in hypothalamic dopamine turnover caused by morphine is diminished by pretreatment with glucocorticoids (Fekete, Kanyicska, Szentendrai, Simoyi & Stark, 1984). Therefore, these data, in concert with the present finding that Dyn (1-11)-mediated inhibition of AVP release is absent in cortisol-treated cells, suggest that increasing plasma glucocortioids in the fetal sheep may be associated with a reduction in the inhibitory effects of opioids on the fetal hypothalmao-pituitary-adrenal axis.

In contrast to the modulatory effects of cortisol, the present report demonstrated that naloxone had no effect on AVP secretion from day 70 or day 100 fetal sheep hypothalamic cultures, under any of the experimental conditions applied. In a recent study, Brooks & Challis (1988) examined the effects of fetal naloxone administration on plasma concentrations of ACTH between day 115-140 of gestation. An intravenous bolus of the opioid antagonist did not alter fetal plasma ACTH levels, whereas this treatment completely abolished the stimulatory effects of the μ-agonist FK 33,824. According to these data, the hypothalamo-pituitary-adrenal axis is not under tonic endogenous opioid control in the fetal sheep. The present findings are in agreement with the data of Brooks & Challis (1988), suggesting that there is no endogenous opioid activity in fetal sheep hypothalamic cultures. What is more likely is that opioids are secreted by hypothalamic cultures, but that the amounts of opioids secreted are in the low picomolar range (Sarkar & Sakaguchi, 1990). The present report clearly shows that these concentrations are ineffective in suppressing AVP secretion from fetal rat hypothalamic cultures. Therefore, the fetal sheep hypothalamic cells may well secrete opioid peptides, but not in sufficient quantity to exert effects on AVP release.

Interestingly, the inhibitory effects of Dyn (1-11) on AVP secretion from day 70 hypothalamic cells were not present in cells incubated with 56mM potassium-containing solution. In fetal rat hypothalamic cultures, none of the opioid peptides shown to inhibit basal AVP release had any efffect in the presence of 56 mM potassium. Therefore, the abolition of the effects of opioids in 56 mM potassium solution was observed in several hypothalamic cultures from two species, confirming that this was

not a random occurrence. This finding contrasts with reports that opioids continue to inhibit hypothalamic ACTH-releasing factor secretion in the presence of elevated potassium solutions (Tsagarakis *et al.* 1989; Tsagarakis *et al* . 1990). Perhaps the difference between the present study and those cited above is that the present experiments were carried out with hypothalamic cell cultures, which lack the organotypic structure of the hypothalamic fragments utilised by Tsagarakis and colleagues (1989; 1990). This suggests that the responses to opioids in elevated potassium solutions are dependent on a specific presynaptic input. It would certainly be interesting to repeat these studies using short term incubations of hypothalamic pieces, to assess whether the responses to opioids were different in organotypic explants.

It is possible that the presence of elevated potassium *per se* interfered with the inhibitory actions of the opioids. Opioid peptides are known to hyperpolarise cells by increasing potassium channel conductivity (North, Williams, Surprenant & Christie, 1987). Williams, Egan & North (1983) have shown that opioid-induced hyperpolarisations are diminished or even reversed in elevated potassium solutions. Therefore, opioid inhibition may disappear in the presence of 56 mM potassium. Such data suggest that other forms of stimulation, such as acetyl choline treatment (Clarke & Gillies, 1988), may be more appropriate for investigations of opioid inhibition of AVP release.

In conclusion, the present report examined several variables which may act at the fetal sheep hypothalamus during development to regulate the fetal pituitary-adrenal axis. The studies indicated that the inhibitory effects of opioids on the fetal sheep hypothalamus are diminished with advancing gestational age, and that elevated cortisol levels may act to reduce any inhibitory effects of opioids. These results suggest a progressive reduction in inhibitory influences acting on fetal hypothalamic ACTH-releasing factors, and hence are consistent with an activation of the fetal sheep hypothalamo-pituitary-adrenal axis with advancing gestational age.

9. General Discussion.

9.1 Introduction

There is a gradual increase in the activity of the pituitary-adrenal axis in the fetal sheep in late gestation. Plasma ACTH and cortisol levels rise towards term, beginning around day 110-120 (Norman *et al.* 1985), and increasing further at day 140 (Rose *et al.* 1978). Whilst direct measurements have not been made of the concentrations of CRH and AVP in the fetal sheep hypophyseal portal blood, available evidence indicates that the increased pituitary-adrenal activity in the fetus in late gestation is due to elevated hypothalamic stimulation of the anterior pituitary (see Antolovich *et al.* 1991). These data are consistent with the progressive increase in bioactive CRH and AVP present in the fetal hypothalamus with advancing gestation (see chapter 6), which may be secreted to stimulate ACTH release from the fetal pituitary.

9.2 Discussion

A general observation in the hypothalamic culture experiments presented in this thesis was that secretion of ACTH-releasing factors was decreased with advancing gestational age (see Chapters 5 and 7). To incorporate these observations into a unifying hypothesis, whereby the net hypothalamic input to the anterior pituitary corticotropes is stimulatory late in gestation, it is neccessary to suppose the existence of a hypothalamic inhibitory factor, the secretion of which is also decreased in late pregnancy. Recently, Antolovich and associates have provided evidence for the existence of such a factor during fetal life (Antolovich *et al.* 1991). These authors surgically disconnected the fetal sheep hypothalamus from the pituitary gland (hypothalamo-pituitary disconnection; HPD) at day 110 of gestation and noticed a marked and sustained increase in circulating ACTH levels when compared to sham-operated fetuses. These data suggest that ACTH release from the fetal

anterior pituitary gland is under tonic inhibitory control, originating in the fetal hypothalamus. Recently, evidence has accumulated to suggest that atrial natriuretic peptide (ANP) may be such a physiological inhibitor of ACTH release.

ANP is a 28 amino acid peptide originally isolated from the cardiac atria (de Bold, Borenstein, Veress & Sonnenberg, 1981) which is detected in the hypothalamus by immunocytochemistry and RIA (Kawata, Nakao, Morii, Kiso, Yamashita, Imura & Sano, 1985). ANP neurones originate in the PVN (Palkovits, Eskay, & Antoni, 1987), and project to the median eminence where they release ANP into portal blood (Lim, Sheward, Copolov, Windmill, & Fink, 1990). ANP is found to have little effect on basal ACTH secretion, but to have potent inhibitory effects on stimulated release of ACTH (Dayanithi & Antoni, 1990). If this factor is incorporated into hypothalamic control of ACTH secretion in the fetus, then decreasing hypothalamic ANP release in late gestation would permit the full activity of CRH and AVP to stimulate ACTH secretion, resulting in the preparturient activation of the fetal adrenal axis.

The chromatographic fractionation of fetal hypothalamic extracts (chapter 6) provided good evidence for the presence of ACTH-releasing factors in the fetal sheep hypothalamus, however, the findings did not suggest the existence of a fetal corticotrophin release-inhibiting factor (CRIF). ANP (1-28) has a molecular weight of 3062, therefore it would have been expected to elute from the Sephadex column in between CRH (M.Wt. 4671) and AVP (M.Wt. 1084). Re-examination of the data presented in chapter 6 argues against the presence of such a CRIF in the fetal sheep hypothalamus. However, the tissue extract which was applied to the column was not purified, and so there was a good deal of background ACTH-releasing activity in the chromatography fractions which may have obscured an inhibitory effect of a putative CRIF. Future studies to investigate a fetal hypothalamic CRIF should therefore utilise an extract, purified initially, for example, by solvent extraction. The peptide extract could then be fractionated by Sephadex chromatography and the bioactivity of the various fractions measured in a pituitary cell bioassay in which ACTH output is stimulated with CRH and AVP, in order to detect inhibition of ACTH release (see Dayanithi and Antoni, 1990).

Whilst the chromatographic purification of fetal hypothalamic ACTH-releasing factors in chapter 6 did not reveal an ACTH release-inhibiting factor, these studies provided evidence for an ACTH-releasing factor, neither CRH nor AVP, which was present in apparently greater quantities in late gestation. There are no previous reports of the chromatographic purification of novel ACTH-releasing bioactivities in the fetal sheep hypothalamus. Therefore, it would be neccessary to carry out very thorough validation experiments in order to establish this substance as a physiological ACTH-releasing factor. For example, the chromatographic purification could be repeated with adult hypothalamic extracts, to see if the factor was found only in the fetal hypothalamus. The ACTH-releasing activity, which has an apparent molecular weight between those of AVP and CRH (1-5kD), could then be dialysed against water to remove residual chromatography buffer (0.1M HCl), which may have intrinsic ACTH-releasing activity. If the ACTH-releasing bioactivity is present after dialysis and is specific to the fetus, it is more likely to be a physiological, fetal ACTH-releasing factor. The attractiveness of a specific, novel, fetal ACTH-releasing factor is that its synthesis and secretion may not be negatively regulated by cortisol, in contrast to CRH and AVP, and so this factor could act to stimulate further ACTH release in the late gestation fetus, when endogenous cortisol levels are already elevated.

The reduction in the secretion of ACTH-releasing factors from hypothalamic cultures *in vitro* with advancing gestational age was in contrast to the increased hypothalamic contents of bioactive AVP and CRH in intact hypothalami (chapter 6). The simplest explanation for this is that the hypothalamic cells from later in gestation were increasingly unable to adapt to the culture system, which may be reflective of their increased maturity and differentiation. However, the quantitative immunocytochemical staining for Neurone-Specific Enolase (see chapter 5) suggested that there were relatively small differences between the numbers of neurones in cultures from different fetal ages, relative to the differences in peptide output. The possibility remains that a selective reduction in CRH- and AVP-containing cells occurs in culture.

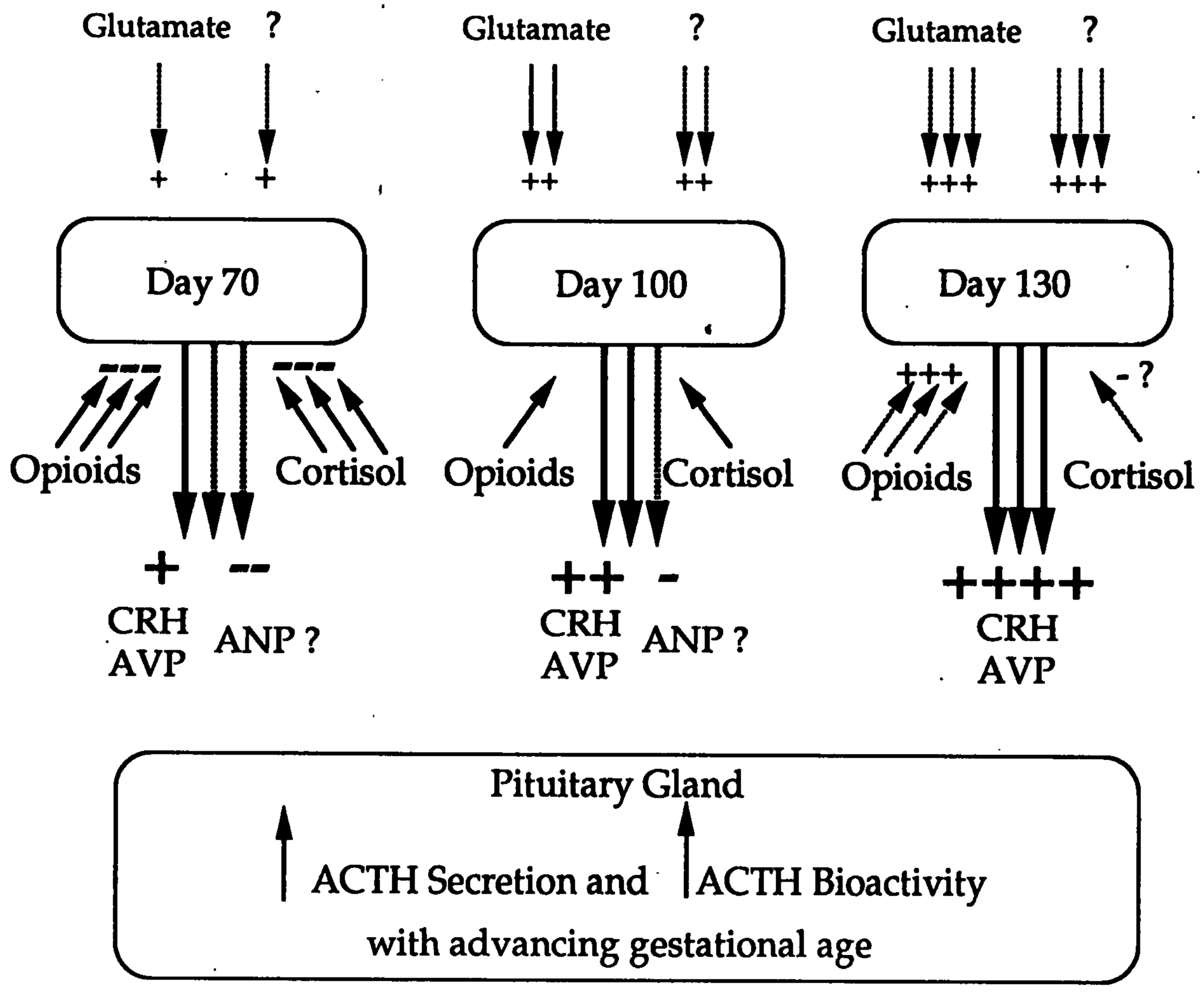
What is more likely is that the synthesis and secretion of AVP and CRH in the fetal hypothalamus becomes dependent on trophic presynaptic inputs, the contribution of which grows increasingly

significant as the fetal nervous system matures. In adult rats for example, destruction of the catecholamine input to the PVN with the 6-hydroxy dopamine results in a marked reduction in the numbers of CRH immunopositive neurones in the PVN (Alonso, Szafarczyk, Balmefrezol & Assenmacher, 1986), and a reduction in portal plasma CRH levels (Assenmacher, Szafarczyk, Alonso, Ixart & Barbanel, 1987), suggesting that these brainstem inputs maintain CRH synthesis and release. By contrast, these inputs are not implicated in the development of hypothalamic CRH in the rat fetus, as the catecholaminergic projections do not reach the PVN until after birth (Plotsky *et al.* 1989).

The hypothetical trophic stimulus to the CRH and AVP neurones may take the form of a excitatory amino acid input. Excitatory amino acids are implicated in the increase in hypothalamic drive to the pituitary at puberty (see Urbanski & Ojeda, 1990), a time when the negative feedback actions of gonadal steroids are reduced. In chapter 4, cell cultures from hypothalami removed at day 100 of gestation were treated with N-methyl D-Aspartate (NMDA), a specific glutamate receptor agonist (Cotman & Iversen, 1987). NMDA stimulation evoked AVP secretion which was several fold greater than basal release. Interestingly, fetal sheep hypothalamic cultures initiated at day 100 of gestation were later shown to be insensitive to adrenal steroids. It is not clear whether the NMDA response and the absence of steroid inhibition are linked. Unfortunately, the experiments which would have permitted a comparison of the NMDA responses in day 70 and day 100 hypothalamic cultures were not carried out. In order to assess whether endogenous glutamate is implicated in parturition, it would be very interesting to examine the sensitivities of fetal hypothalami to NMDA stimulation at different gestational ages. Possibly the best approach would involve incubation of hypothalamic pieces over the short term *in vitro,* as secretion of peptides from cultured hypothalami removed late in gestation is too low to measure reliably.

As previously described, the studies in this thesis showed that AVP secretion from day 70 hypothalamic cultures was inhibited by cortisol, whereas AVP release from day 100 hypothalamic cells was unaffected by this adrenal steroid. The mechanism whereby day 100 cells do not respond to cortisol is unclear, as hypothalamic cells express greater

Figure 9.1. Summary Diagram, indicating some of the stimulatory and inhibitory influences on hypothalamic drive to fetal pituitary ACTH secretion at different stages of fetal development. Solid arrows indicate results obtained in this thesis, and dashed arrows represent the work of others, or as yet hypothetical factors regulating ACTH release from the fetal pituitary gland. Ultimately, various trophic influences acting at central and hypophysial-sites cause increased release of ACTH, which drives the fetal adrenal gland to bring about fetal maturation and initiate parturition.


Glutamate ?
+ +
Day 70
Opioids
Cortisol
+ --
CRH
AVP
ANP ?
Glutamate ?
++ ++
Day 100
Opioids
Cortisol
++ -
CRH
AVP
ANP ?
Glutamate ?
+++ +++
Day 130
+++
- ?
Opioids
Cortisol
++++
CRH
AVP
Pituitary Gland
ACTH Secretion and
ACTH Bioactivity
with advancing gestational age

quantities of glucocorticoid receptors at day 100 than at day 70 of gestation (Yang *et al.* 1990). It may be that day 100 hypothalamic cells in culture no longer express glucocorticoid receptors, in which case, it would be interesting to immunostain hypothalamic cultures with a monoclonal anti-glucocorticoid receptor antibody (see Gustaffson *et al.* 1987) to confirm the presence of the glucocorticoid receptor.

The studies reported in chapter 8 showed that cortisol could abolish the inhibitory effects of an opioid peptide on AVP secretion. There are several reports in the literature which describe the interactions of glucocorticoids and opioids, confirming the validity of these observations (see Gustaffson *et al.* 1987; Fekete *et al.* 1984). However, in the fetal sheep near term, Brooks & Challis (1991) have demonstrated a physiological stimulatory opioid input to the fetal hypothalamus-pituitary unit, in spite of the much elevated fetal plasma levels of cortisol. This may suggest that cortisol is not biologically active within the fetal hypothalamus immediately before term.

Recently, evidence has accumulated to suggest that glucocorticoid receptors interact with other factors within the cell to bring about their biological effects (Akerblom, Slater, Beato, Baxter & Mellon, 1988). Schüle and colleagues have suggested that an intracellular transcription factor c-jun antagonises the effects of glucocorticoids by binding to the glucocorticoid receptor and preventing an action at the genome (see Schüle, Rangarajan, Kliewer, Ransone, Bolado, Yang, Verma & Evans, 1990). Presumably, any stimulus which enhanced the synthesis of c-jun could lead to a reduction in the inhibitory effects of glucocorticoids. These data suggest that an investigation of c-jun at the molecular level in the fetal sheep hypothalamus may clarify the mechanisms of glucocorticoid negative feedback during development.

9.3 The Hypothalamic Cell Culture Model

This thesis has developed and exploited a fetal sheep hypothalamic cell culture system in order to investigate fetal hypothalamic activity during fetal development. A serum-free system was chosen to permit complete

control over the chemical environment of the hypothalamic cells. In this way, it was possible to minimise the confounding effects of batch-to-batch variations in reagent quality on the characteristics of the cultured cells.

The cell culture system has permitted the detailed investigation of fetal sheep hypothalamic cells over 5 weeks *in vitro*, an investigation which would not have been possible using other techniques. During this time, the secretion of neuropeptides from the hypothalamic cells was measured in response to various experimental treatments. A limitation of this technique became apparent when the secretion and culture content of CRH and AVP was reduced with advancing gestational age, which was at odds with the implications of data gathered using other methodologies. Therefore, some properties of the cultured cells may not accurately reflect the functions of the cells *in vivo*.

Cell culture is most effective as a component of the investigation of fetal hypothalamic function. Other approaches could include short-term hypothalamic tissue incubations *in vitro* and the use of the chronically catheterised fetal sheep model. The main strengths of the cell culture system are the degree of control over the cellular environment and the ready accessibility of the hypothalamic neurones. This control has permitted investigations of the neuropeptide secretion from the fetal sheep hypothalmamus which have hitherto not been possible. Furthermore, the culture system will permit future studies of the molecular mechanisms of hypothalamic function in the fetal sheep.

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