CONTROL OF ß-ENDORPHIN SECRETION INTO THE PERIPHERAL BLOOD OF THE SOAY RAM

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If then Socrates we find ourselves.....unable to make our discourse in every way wholly consistent and exact you must not be surprised. Nay, we must be well content if we can provide an account not less likely than another; we must remember that I who speak and you who are my audience are but men and should be satisfied to ask for no more than the likely story.

CONTENTS

DECLARATION

The experiments described in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has already been accepted for any other degree, nor is any part of it being concurrently submitted in candidature for another degree.

Edward Ssewannyana

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ABSTRACT

The major aim of the experiments described in this thesis was to investigate the putative mechanisms that are involved in the control of ß-endorphin (ß-END) secretion into the peripheral blood of the Soay ram. In addition, the effect of ß-END and adrenocorticotrophin (ACTH) on cortisol secretion was investigated.

A series of experiments was carried out to assess the influence of season, photoperiod, melatonin implantation, pinealectomy, castration and testosterone replacement on ß-END secretion. There were significant changes in the plasma concentrations of ß-END related to season and photoperiod in rams kept outside and inside, respectively. In outdoor rams ß-END levels were highest in autumn and lowest in winter; in indoor rams the levels were highest during short days and lowest during long days. Melatonin implantation in outdoor rams from May to August caused a significant increase in ß-END secretion, indicating a melatonin-induced short-day effect inspite of the prevailing long days. Pinealectomy disrupted the seasonal cycle in ß-END secretion. Castration and testostrone replacement in indoor rams did not influence ß-END secretion. These results indicate that ß-END secretion is strongly influenced by season and photoperiod (via melatonin from the pineal gland) and that testosterone plays no role in ß-END secretion.

In another series of experiments, the roles of arginine vasopressin (AVP), corticotrophin releasing factor (CRF), the synthetic glucocorticoid, dexamethasone (DEX) and the synthetic glucocorticoid antagonist, RU 486, in the control of the seasonal cycle in ß-END secretion were investigated in spring, summer, autumn and winter. AVP and CRF given alone or in

combination significantly stimulated ß-END secretion at all seasons and acted synergistically when given together. The responses were greater in summer and autumn than in winter and spring. DEX suppressed ß-END secretion at all seasons and the responses were also greater in summer and autumn. DEX also blocked the AVP-induced increase in ß-END secretion, indicating an action of DEX at the pituitary gland. RU 486 given in summer and winter significantly stimulated ß-END secretion only in winter, indicating a seasonal variation in the negative feedback action of endogenous glucocorticoids. In addition, ACTH, but not ß-END, significantly stimulated cortisol secretion at all seasons, with the greatest response in spring. These studies indicate that AVP, CRF and glucocorticoids are involved in the control of the seasonal cycle in ß-END secretion; and that ACTH rather than ß-END constitutes the " drive " to cortisol secretion.

The roles of dopamine (DA) and endogenous opioid peptides (EOP) in the control of ß-END secretion were also investigated. The mixed DA antagonist, pimozide, significantly increased ß-END secretion under long and short days; with a greater effect under long days.The D_2 agonist, bromocriptine, and the D_2 antagonist, sulpiride, significantly decreased and increased, respectively, ß-END secretion both under long and short days. The opioid antagonist, naloxone, had no effect on ß-END secretion. These studies indicate that DA exerts an inhibitory control over ß-END secretion while the EOP play no role in ß-END secretion.

Based on the current results and a survey of the relevant literature, a model is proposed in which AVP, CRF, glucocorticoids on one hand, and DA on the other hand, are involved in the control of the secretory activity of corticotrophs and melanotrophs in the pituitary gland. These central mechanisms are influenced by changes in photoperiod and other environmental factors to dictate the seasonal cycle in ß-END secretion in the Soay ram, which

is low in winter and high in autumn. To fully assess the importance of AVP, CRF and DA in the seasonal control of ß-END secretion, it will be necessary to directly measure the concentration of these hormones in the hypophysial portal circulation of Soay rams at different seasons.

LIST OF ABBREVIATIONS

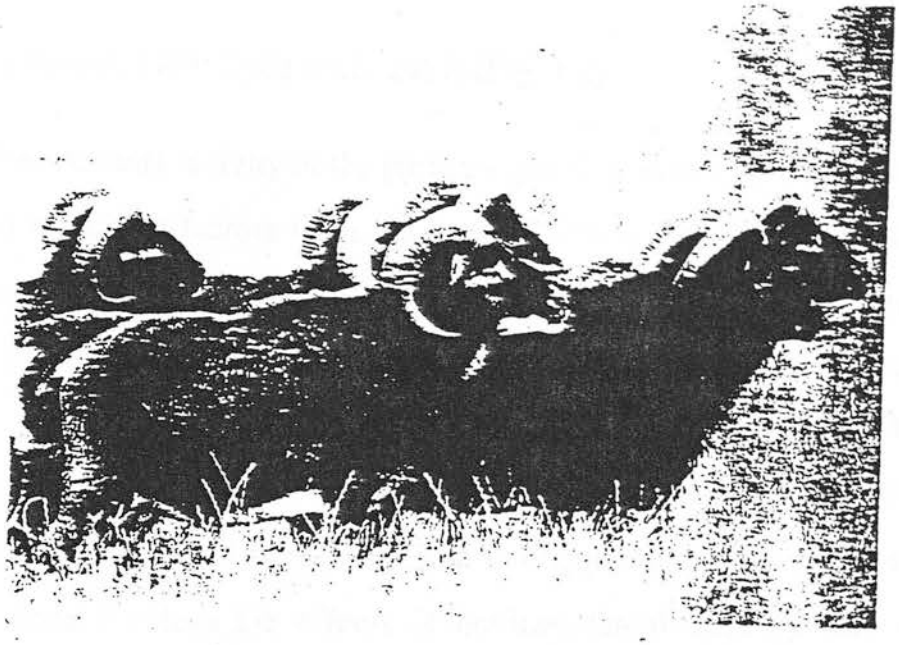
ACTH	adrenocorticotropin hormone
α-END	α-endorphin
α-MSH	α–melanocyte stimulating hormone
ANOVA	analysis of variance
AVP	arginine vasopressin
ß-END	ß-endorphin
ß-LPH	ß-lipotropin
cAMP	adenosine cyclic 3' 5' monophosphate
CCK	cholecystokinin
CLIP	corticotropin-like intermediate lobe peptide
CRF	corticotropin releasing factor
C. V.	coefficient of variation
DA	dopamine
FSH	follicle stimulating hormone
GABA	gamma aminobutyric acid
γ-END	γ- endorphin
γ-LPH	γ-lipotropin
GnRH	gonadotropin releasing hormone
i.c.v.	intracerebroventricularly
i.m.	intramuscularly
i.v.	intravenously
LH	luteinizing hormone
mg/kg	dose in milligrams per kilogram
µg/kg	dose in micrograms per kilogram

mRNA	messenger ribonucleic acid
Nac-ß-END	N-acetyl-ß-endorphin
NE	norepinephrine
PINX	pinealectomized
POMC	proopiomelanocortin
PRL	prolactin
RIA	radioimmunoassay
s.c.	subcutaneously
SCGX	superior cervical ganglionectomized
SCN	suprachiasmatic nucleus
TSH	thyroid stimulating hormone

CHAPTER 1

INTRODUCTION

The Soay breed of semi-domesticated sheep (*Ovis aries*) originates from the island of St. Kilda (58°N) in the north Atlantic Ocean (Fig. 1.1). Like other mammals living in cold and temperate climates, the Soay sheep show many adaptations to living in a seasonal environment in which there are marked changes in daylength, temperature and food supply from summer to winter (Lincoln, 1989a). These adaptations include the occurrence of a seasonal cycle in reproduction with a restricted mating period in autumn, which is timed to produce offspring at a time of abundant food supply and warm conditions in spring (Lincoln and Davidson, 1977; Lincoln and Short, 1980; Lincoln, 1989b). There are seasonal changes in pelage with a moult between summer and winter coats, allowing for a change in colour for camouflage and better insulation in winter (Ryder, 1971; Lincoln, 1989a). There are also conspicuous seasonal cycles in voluntary food intake, body growth and fattening to optimise the use of food which changes in both quantity and quality from summer to winter (Argo and Smith, 1983; Bronson, 1985). The physiological systems regulating these adaptative seasonal cycles are not well understood but clearly involve seasonal changes in the secretion of protein hormones from the pituitary gland. For example, there are seasonal changes in the circulating levels of FSH, LH, PRL and ß-END (Lincoln and Short, 1980; Ebling and Lincoln, 1987). The seasonal cycle in reproduction is regulated by FSH and LH (Lincoln, 1988; 1989b), while that in moulting is controlled by TSH and PRL (Sutherland and Irvine, 1974; Ryder, 1979; Lincoln, Klandorf and Anderson, 1980; Lincoln and Ebling, 1985; Lincoln, 1989a). The regulation of the seasonal cycles in voluntary food intake, body growth and fattening involves complex mechanisms; however, changes in TSH, PRL, ACTH and ß-END are thought to be involved in the regulation of these three seasonal variables (Ashmore and Morgan, 1967; Meier, 1977; Margules, 1979; Baile, Kein, Della-Fera and McLaughlin, 1981; Ryg and Jacobsen, 1982;



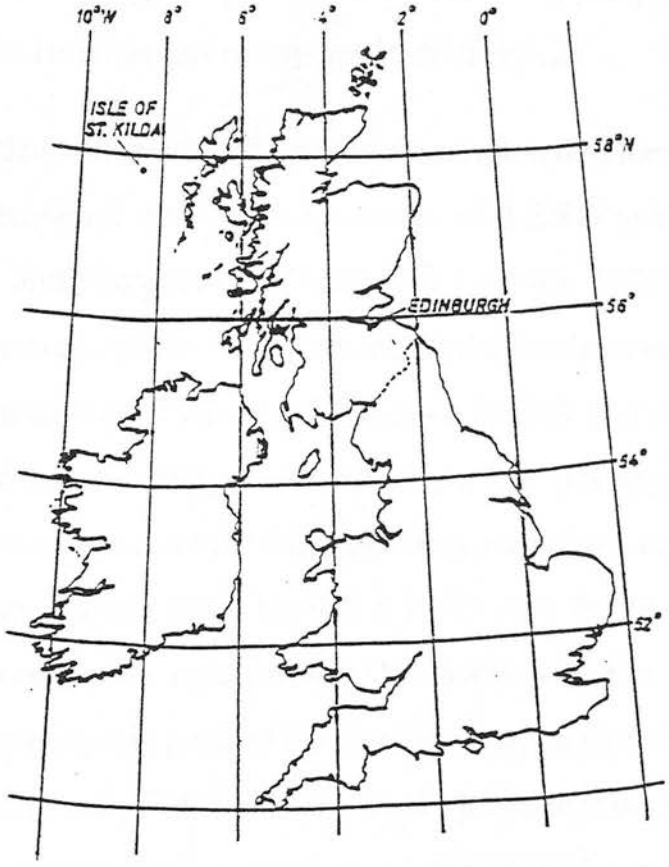


Fig. 1.1. A group of Soay rams which originate from the island of St. Kilda, 160 km from the mainland Scotland. They represent an early stage of domestication of sheep, having returned to the wild during the early settlement of the Western Isles, and are well adapted to living in a seasonal environment, showing seasonal cycles in reproduction, moulting, food intake, body growth and fattening. (With permission from Lincoln and Short, 1980).

Brinklow and Forbes, 1983; Baile *et al.*, 1987) (Fig. 1.2).

The secretory activity of the pituitary gland is, in turn, governed by releasing and inhibiting factors from the hypothalamus. However, the way hypothalamic factors control seasonal changes in the secretion of pituitary hormones is poorly understood. The most comprehensive studies have been made on the regulation of the secretion of FSH and LH, which is dictated by changes in the release of GnRH from neurones in the hypothalamus. The hypothalamus is thought to play a key role in regulating all the seasonal adaptations since it relays the effects of environmental cues by way of modulating the release of releasing and inhibiting factors, which control the secretion of a diverse range of protein hormones from the pituitary gland which have specific target tissues around the body (Fig.1.2).

Of particular interest is the recent observation that there are marked changes in the peripheral blood concentrations of ß-END in the Soay ram related to season and photoperiod (Ebling and Lincoln, 1987). In outdoor rams, there is a seasonal cycle in ß-END secretion; the levels increase about 20-fold from spring to autumn. This seasonal cycle in ß-END secretion is closely correlated with the seasonal changes in body weight and fattening; the increase in ß-END secretion occurs at a time when the rams are accumulating fat in the subcutaneous tissue, which suggests that ß-END may be involved in the deposition of fat reserves in anticipation of the harsh winter as proposed for other seasonal species by Margules (1979). In indoor rams, there is also a long-term cycle in ß-END secretion; the levels are high under short days and low under long days, showing that photoperiod is probably the most important environmental cue regulating the timing of the seasonal cycle in ß-END secretion under natural conditions (Ebling and Lincoln, 1987).

The ß-END in the peripheral circulation of the Soay ram is assumed to be secreted primarily from the pituitary gland since this tissue contains much

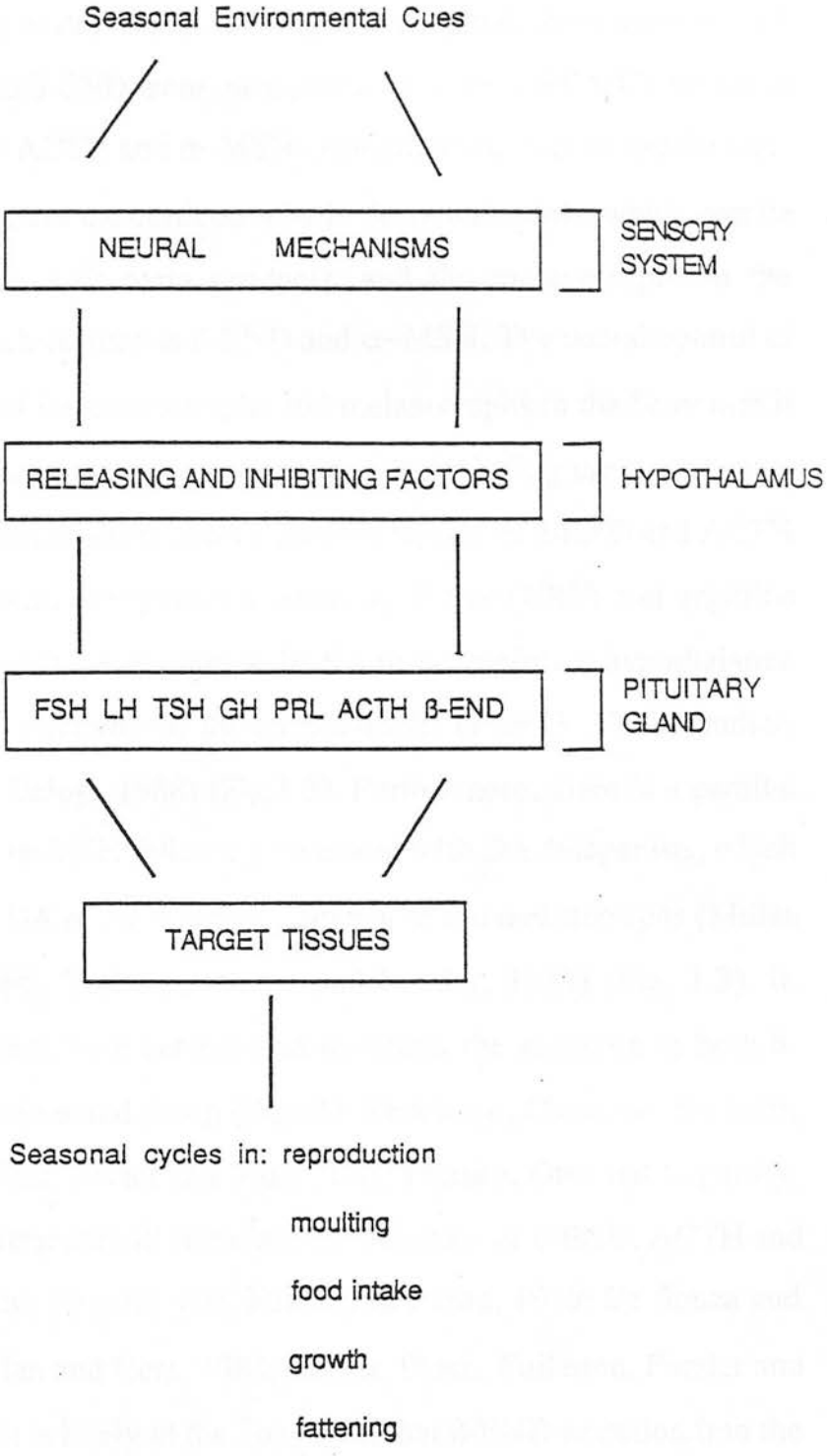


Fig. 1.2. Summary of the neuroendocrine relay by which environmental cues influence seasonal cycles in physiology and behaviour which represent adaptation to life in a seasonal environment.

larger amounts of ß-END compared with the hypothalamus (Ebling and Lincoln, 1987; Ebling *et al.*, 1987). In the pituitary gland, there are two cell types which synthesize ß-END from proopiomelanocortin (POMC), which is also the precursor for ACTH and α–MSH (Antoni, 1986; Smith and Funder, 1988) (Fig. 1.3). These are the corticotrophs in the anterior lobe which secrete ß-END and ACTH as their main products, and the melanotrophs in the intermediate lobe which co-secrete ß-END and α–MSH. The neural control of the secretory activity of the corticotrophs and melanotrophs in the Soay ram is unknown. However, studies in non-seasonal species, including the rat and man, and those in other breeds of sheep show a parallel release of ß-END and ACTH following treatment with corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) which are thought to be the most important hypothalamic peptides regulating the activity of the corticotrophs (Antoni, 1986; Pradier, Dalle, Tournaire and Delost, 1988) (Fig.1.3). Furthermore, there is a parallel release of ß-END and α–MSH following treatment with DA antagonists, which implicates the role of DA in the inhibitory control of the melanotrophs (Millan and Herz, 1985; Smith, Wallace, Clarke and Funder, 1989) (Fig. 1.3). In addition, glucocorticoids have been shown to inhibit the secretion of both ß-END and ACTH in the rat and sheep (Donald, Redekopp, Cameron, Nicholls, Bolton, Livesey, Espiner, Rivier and Vale, 1983; Plotsky, Otto and Sapolsky, 1986), and acute stressful stimuli stimulate the secretion of ß-END, ACTH and α-MSH (Nakao, Nakai, Jingami, Oki, Fukati and Imura, 1979; De Souza and Van Loon, 1985; Millan and Herz, 1985; Engler, Pham, Fullerton, Funder and Clarke, 1988). Thus, it is likely in the Soay ram, that ß-END secretion into the peripheral blood represents the combined secretion from the corticotrophs and melanotrophs, with the effects of photoperiod acting at the level of the hypothalamus to influence the timing of the cycle in ß-END secretion (Fig. 1.3).

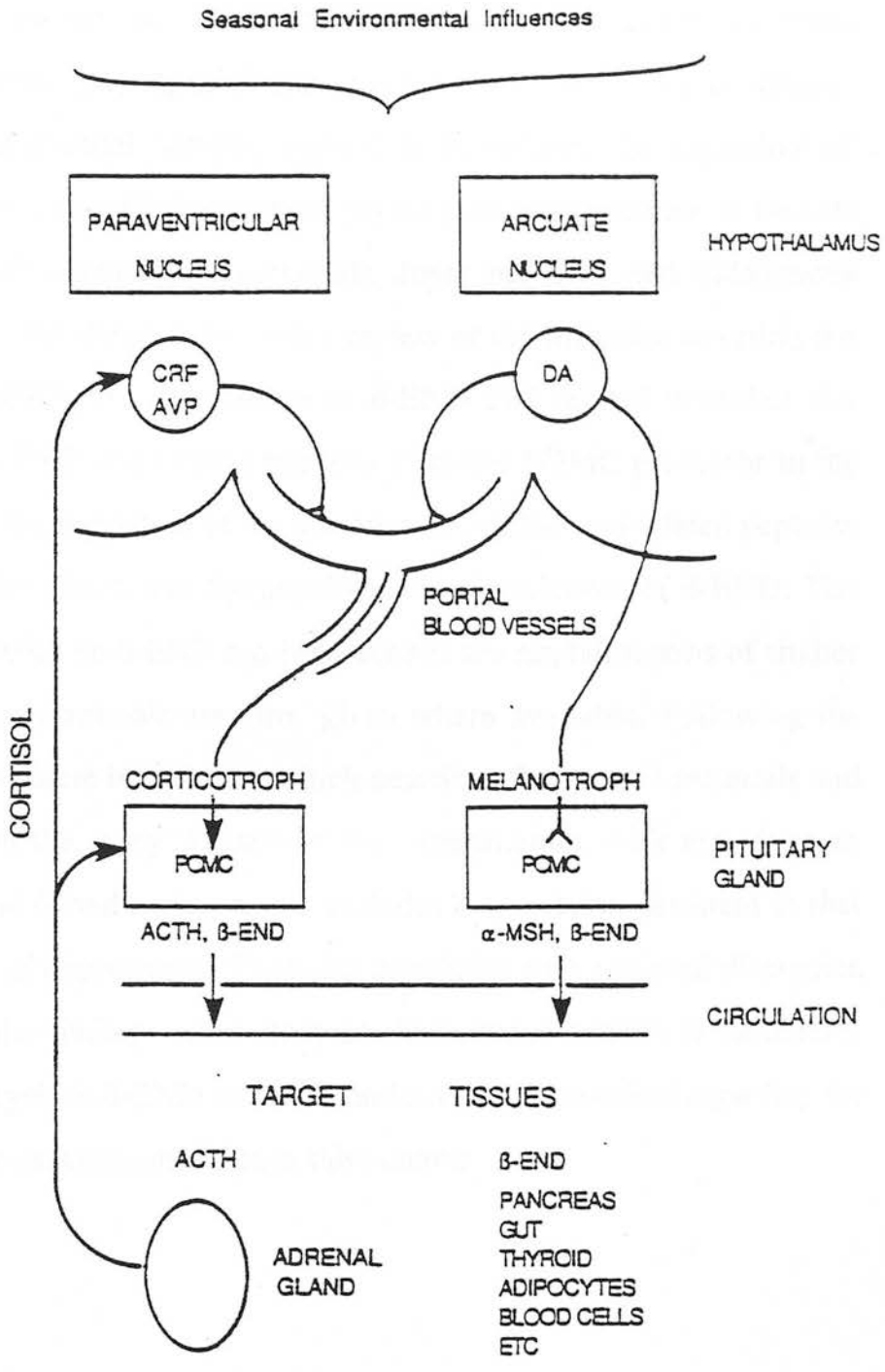


Fig. 1.3. General organisation of physiological mechanisms thought to control the secretion of ß-endorphin and related peptides from the pituitary gland and the possible target tissues for these peptides in the Soay ram.

This study was undertaken with two main objectives. First, to provide more information on the cycle in ß-END secretion under different physiological states, paying particular attention to the influence of season, photoperiod and gonadal steroids. Second, to investigate the physiological control of the cycle in ß-END secretion, paying particular attention to the role of CRF and AVP, adrenal glucocorticoids, dopamine (DA) and endogenous opioid peptides. The thesis starts with a review of the literature covering the isolation of ß-END; the distribution of ß-END and related peptides; the processing of ß-END and related peptides from the POMC precursor in the pituitary gland; the regulation of the secretion of ß-END and related peptides from the pituitary gland; and the physiological significance of ß-END. The majority of the work on ß-END has been done in the rat, but reports of studies in sheep and other animals are also given where available. Following the literature review, there is a chapter which describes the general materials and methods used in the study. Details of the experimental work are given in chapters 4, 5 and 6, and each chapter includes a discussion pertinent to that particular series of experiments. The thesis concludes with a general discussion in which the major findings of the study are discussed in relation to the control of the seasonal cycle in ß-END secretion and a model is proposed regarding the physiological mechanisms involved in this control.

CHAPTER 2

REVIEW OF THE LITERATURE

2.1 ISOLATION OF ß-END

ß-endorphin (ß-END) is a peptide which belongs to one of the three families of endogenous opioid peptides called the endorphins; the other two being the enkephalins and dynorphins. ß-END was first isolated as one of a series of fragments present in pig pituitary gland and was found to contain 31 amino acids (Bradbury, Smyth, Snell, Birdsall and Hulme,1976). One of the fragments isolated with ß-END was ß-LPH, a 91-residue peptide that had been identified previously (Li, Barnafi, Chretien and Chung, 1965) and whose C-terminal sequence was shown to be identical to the sequence of ß-END (residues 61-91) (Fig. 2.1); hence, ß-END was initially named the " C-fragment" of ß-LPH (Bradbury *et al.*, 1976). A second group of peptides isolated from porcine pituitary included ACTH $_{1-39}$ and its constituent fragments, α-MSH$_{1-13}$ and CLIP$_{18-39}$. Later, it was shown in both mouse pituitary tumour cells and in pituitary cell line At20/D$_{16v}$ cells that ß-END and the other pituitary peptides are derived from the same biosynthetic 31 kilodalton precursor called proopiomelanocortin (POMC) (Mains, Eipper and Ling, 1977; Roberts and Herbert, 1977) (Fig. 2.1).

Investigation of the biological activity of the 31-residue peptide using dihydromorphine and naloxone revealed that it possesses opiate properties to an exceptional degree, both *in vitro* (Bradbury et *al.*, 1976) and *in vivo* (Feldberg and Smyth, 1976, 1977; Loh, Tseng, Wei and Li, 1976); and remarkably this peptide, though isolated from the pituitary, was found to exhibit its morphine-like effects on the brain. In view of the similarity of its properties to those of morphine, the general term endorphin (endogenous morphine), which had earlier been proposed by E. J. Simon for peptides with opiate activity (Goldstein, 1976), seemed appropriate when applied to ß-LPH C-fragment and the name "ß-endorphin" has become generally accepted.

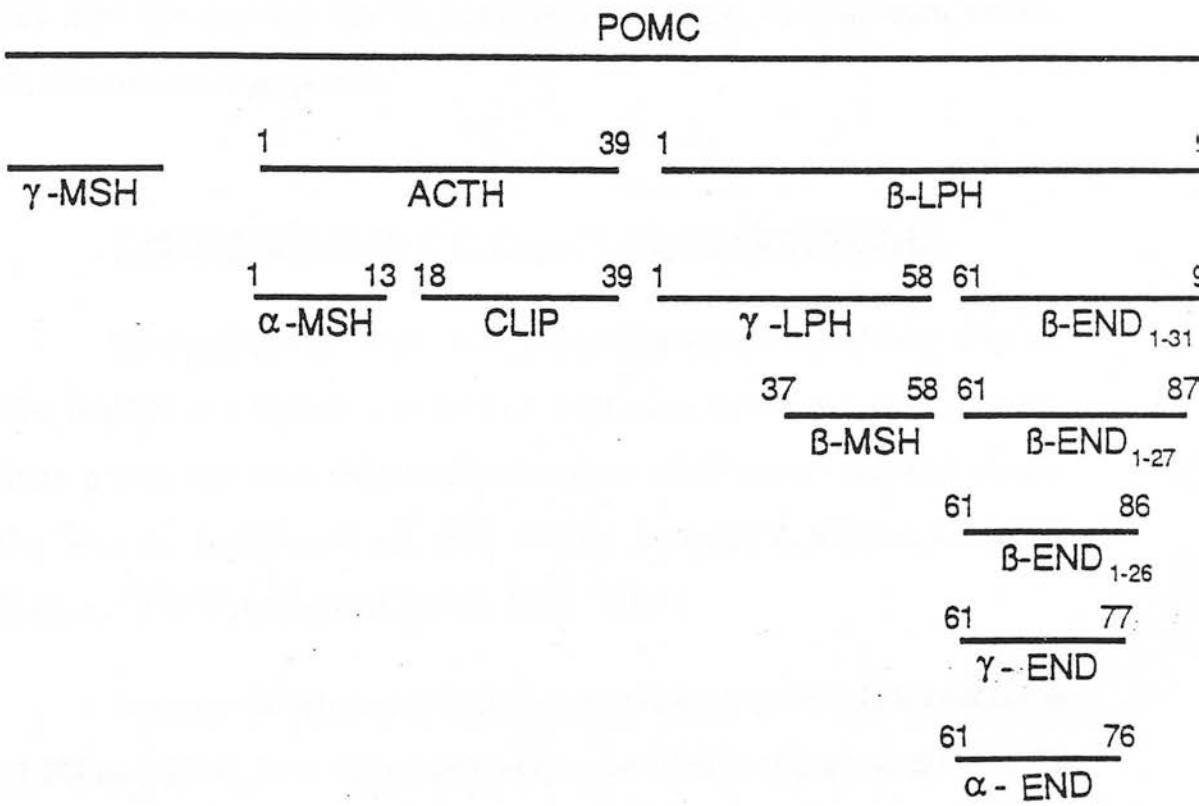


Fig. 2.1. Structural relationship between peptides derived from proopiomelano-cortin.

After the demonstration that ß-END was present in pig pituitary, it was isolated and sequenced in a variety of species, including the salmon (Kawauchi, Tubokawa and Muramoto, 1979; Kawauchi, 1983), frog (Martens, Civelli and Herbert, 1985), ostrich (Naude, Oelofsen and Maske, 1980), mouse (Uhler and Herbert, 1983), rat (Rubinstein, Stein and Udenfriend, 1978; Drouin and Goodman, 1980), sheep (Chretien, Benjannet, Dragon, Seidah and Lis, 1976; Ebling and Lincoln, 1987), camel (Li and Chung, 1976) and man (Chretien *et al.*, 1976; Dragon, Seidah, Lis, Routhier and Chretien , 1977; Cochet, Chang and Cohen, 1982). It is notable that the amino sequence is strongly conserved with about 95% homology between positions 1 and 25 (Fig. 2.2). Such a strong conservation of structure in diverse species indicates that the full sequence of ß-END may be important for its opioid and analgesic properties.

2.2 DISTRIBUTION OF ß-END AND RELATED PEPTIDES

Immunohistochemistry and radioimmunoassay have been used to localise ß-END and related peptides in a wide range of tissues, including the pituitary gland, the brain and peripheral organs (Guillemin, Ling and Vargo, 1977a; Watson, Barchas and Li, 1977; Bloom, Battenberg, Rossier, Ling and Guillemin, 1978; Zakarian and Smyth, 1979; 1982a).

Immunohistochemical studies of rat pituitary gland, with antibodies to ß-LPH or ß-END, have shown that only some of the cells are reactive in the anterior lobe whereas all the cells are reactive in the intermediate lobe (Watson *et al.*, 1977; Zakarian and Smyth, 1979; 1982a); a similar distribution is apparent in the sheep (Moon, Li and Jennings, 1973). Most recent evidence indicates that several POMC-derived peptides coexist in the same pituitary cell, which is consistent with processing from a common precursor; and that it is

	1																													31
Pig	Tyr-	Gly-	Gly-	Phe-	Met-	Thr-	Ser-	Glu-	Lys-	Ser-	Gln-	Thr-	Pro-	Leu-	Val-	Thr-	Leu-	Phe-	Lys-	Asn-	Ala-	Ile-	Val-	Lys-	Asn-	Ala-	His-	Lys-	--	Lys-	Gly-	Gln
	1																													31
Sheep (camel,cattle)	Tyr-	Gly-	Gly-	Phe-	Met-	Thr-	Ser-	Glu-	Lys-	Ser-	Gln-	Thr-	Pro-	Leu-	Val-	Thr-	Leu-	Phe-	Lys-	Asn-	Ala-	Ile-	Ile-	Lys-	Asn-	Ala-	His-	Lys-	Lys-	Gly-	Gln
	1																													31
Rat	Tyr-	Gly-	Gly-	Phe-	Met-	Thr-	Ser-	Glu-	Lys-	Ser-	Gln-	Thr-	Pro-	Leu-	Val-	Thr-	Leu-	Phe-	Lys-	Asn-	Ala-	Ile-	Ile-	Lys-	Asn-	Val-	His-	Lys-	Lys-	Gly-	Gln
	1																													31
Man	Tyr-	Gly-	Gly-	Phe-	Met-	Thr-	Ser-	Glu-	Lys-	Ser-	Gln-	Thr-	Pro-	Leu-	Val-	Thr-	Leu-	Phe-	Lys-	Asn-	Ala-	Ile-	Ile-	Lys-	Asn-	Ala-	Tyr-	Lys-	Lys-	Gly-	Glu

Fig. 2.2. Comparison of amino acid sequence of ß-endorphin in different mammalian species.

possible to divide pituitary cells into corticotrophs and melanotrophs according to the way they process the POMC precursor. The corticotrophs are mainly located in the anterior lobe of the pituitary gland and synthesize primarily ACTH and ß-END, whereas the melanotrophs are mainly located in the intermediate lobe and synthesize primarily α–MSH and ß-END (Antoni, 1986; Smith and Funder, 1988).

In the same studies that localised ß-END and related peptides in the pituitary gland, it was demonstrated that these peptides are also localised in specific regions of rat brain. The main concentrations of ß-END occur in the hypothalamus, and the cell bodies where biosynthesis takes place are located exclusively in this region, being concentrated in the arcuate nucleus. From the arcuate nucleus, nerve fibres extend to many regions of the brain, including the median eminence, amygdala, preoptic area, ventromedial nuclei, periaqueductal gray, reticular formation, stria terminalis, locus coeruleus, striatum and hippocampus (Watson *et al.*, 1977; Gramsch, Kleber, Hollt, Pasi, Mehraein and Herz, 1980). In addition, Ibata, Kawakami, Okamura, Obata-Tsuto, Morimoto and Zimmerman (1985) have shown in both light and electron immuno-histochemical studies that ß-END-positive staining neurons project to the portal capillaries of the external zone of the median eminence, suggesting the hypothalamus as a possible source of ß-END in hypophysial portal blood. Furthermore, these studies have shown fibres extending to the ependymal layer of the third ventricle, suggesting that ß-END and related peptides may be secreted into the cerebrospinal fluid.

Of particular relevancy to this thesis, is the distribution of ß-END in the hypothalamus of the Soay ram characterised by extraction followed by radio-immunoassay. High ß-END concentrations are detected in the mediobasal hypothalamus and median eminence; the immunoreactivity also extends to lateral and dorsal hypothalamus (Ebling, Lincoln, Martin and Taylor, 1987).

Although there are significant amounts of ß-END and related peptides in the hypothalamus, the total content is only a fraction (< 1%) of that found in the pituitary (Ebling and Lincoln, 1987; Ebling *et al.*, 1987; Smith and Funder, 1988), indicating that the pituitary gland is the major source of ß-END in the peripheral circulation.

ß-END and related peptides have also been localised in a variety of peripheral tissues, including the placenta (Genazzani, Hurlimann, Fioretti and Felber, 1974; Odagiri, Sherrell, Mount, Nicholson and Orth, 1979; Liotta, Loudes, McKelvy and Krieger, 1980), the thyroid gland (Clements, Funder, Tracy, Morgan, Campbell, Lewis and Hearn, 1982; Cheng, Smith and Funder, 1986), the adrenal gland (Vuolteenaho, Vakkuri and Leppaluoto, 1980; Evans, Erdelyi, Weber and Barchas, 1983), the antrum and pancreas (Fuerle, Weber and Helmstaedter, 1980; Tanaki, Yoshikatsu, Kazuwa, Oki, Masaki, Ohtsuki and Imura, 1982; Smyth, 1983), the spleen (Lolait, Lim, Toh and Funder, 1984a), the male and female reproductive tracts (Sharp, Pekary, Meyer and Hersham, 1980; Tsong, Phillips, Halmi, Liotta, Margioris, Bardin and Krieger, 1982; Lim, Lolait, Barlow, Zois, Toh and Funder, 1983; Lolait *et al.*, 1984b; Shaha, Margioris, Liotta, Krieger and Bardin, 1984; Cheng *et al.*, 1985; Li, Risbridger, Funder and Clements, 1989), the eye, pineal gland, kidney (Vuolteenaho *et al.*, 1980) and the lungs (Clements *et al.*, 1982).

2.3 PROCESSING OF ß-END AND RELATED PEPTIDES FROM THE POMC PRECURSOR IN THE PITUITARY GLAND

The pituitary gland is the major site of synthesis and secretion of circulating ß-END (Ebling and Lincoln, 1987; Ebling *et al.*, 1987; Smith and Funder, 1988); therefore, the way the POMC precursor is processed to produce ß-END and related peptides will be considered only for this tissue.

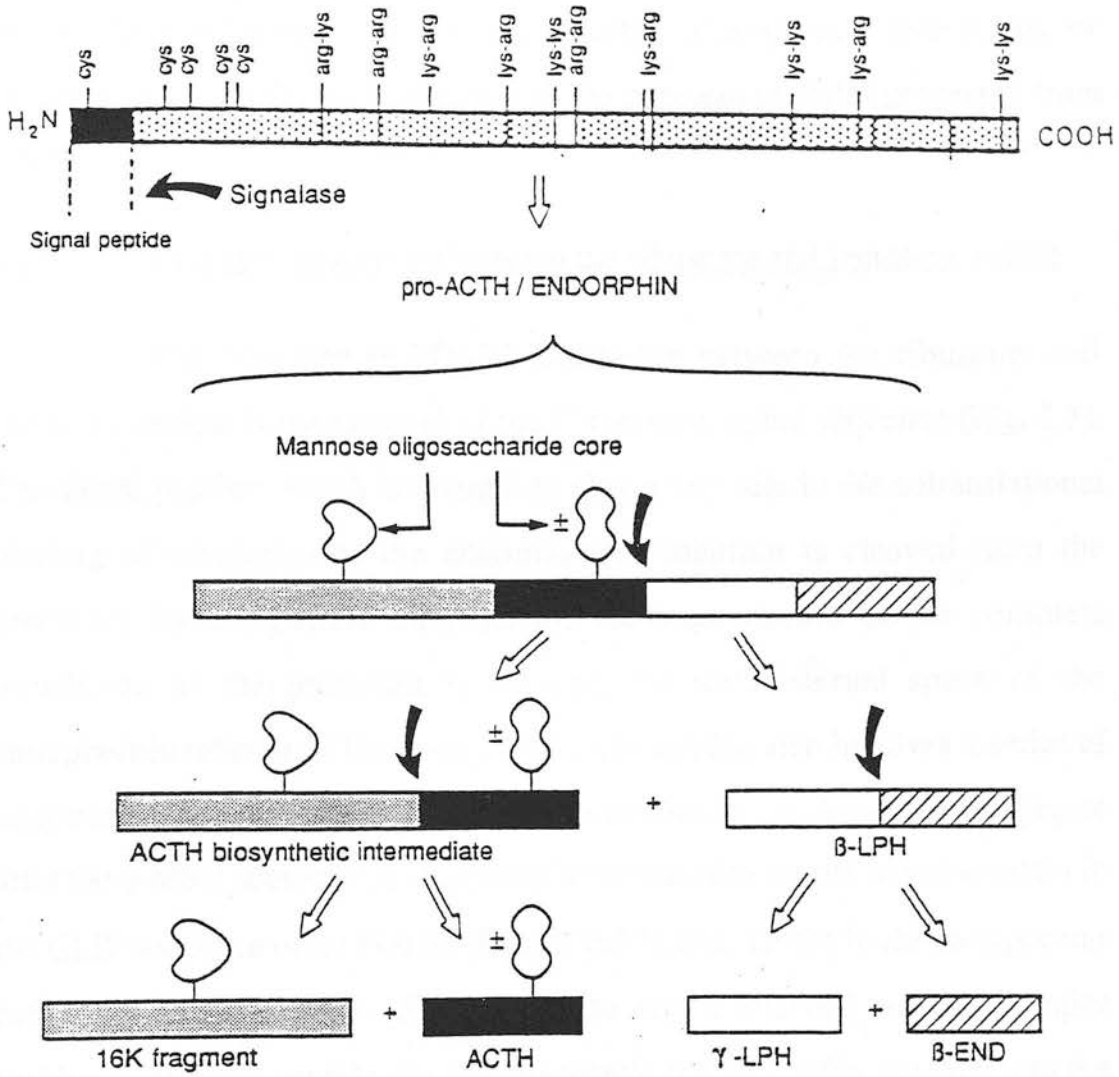


Fig. 2.3. Schematic diagram of pathways involved in the processing of the proopiomelanocortin precursor between the ribosome and the secretory vesicle.

The processing events occur in two parts. The first part involves processing the POMC precursor before its complete translation; this occurs between the ribosome and secretory vesicle and is sometimes referred to as "maturation". This part applies to all cells that synthesize POMC. The second part is posttranslational and is tissue-specific; it involves a multiplicity of enzymes necessary for the production of the different peptides processed from POMC.

2.3.1 First part: processing between the ribosome and secretory vesicle

The first step in POMC processing between the ribosome and secretory vesicle is the removal of the N-terminal signal sequence (Fig. 2.3). The signal peptide, which is thought to play a key role in the cotranslational binding of ribosomes to the endoplasmic reticulum is cleaved from the precursor by a signalase enzyme; the cleavage occurs before complete translation as the precursor is entering the intracisternal space of the endoplasmic reticulum (Harwood, 1980). The second step involves a series of enzymatic events in which the asparagine residue in the Asn-X-Ser sequence after the γ-MSH sequence is N-glycosylated; this also occurs to some extent in the CLIP sequence of rat POMC (Eipper and Mains, 1980). In the endoplasmic reticulum, a high mannose oligosaccharide core is attached to the asparagine residue of the polypeptide via a N-glycosidic linkage. After passage into the Golgi apparatus, glucose and mannose residues are "trimmed" from the core by glucosidases and mannosidases. In the third step, peripheral residues (N-acetyl-glucosamine, galactose and N-acetylneuraminic acid) are attached to the mannose residues of the core by glycosyl transferases. A less complex O-linked glycosylation (Lennarz, 1980) of Ser and Thr residues may also take place in the Golgi apparatus (Seidah and Chretien, 1981), where the attachment to the -OH group is probably through N-acetyl-galactosamine via O-glycosyl-transferases.

2.3.2 Second part: processing in secretory vesicle and after secretion

This part of the processing involves a number of posttranslational events, including endopeptidase and exopeptidase cleavages, α-amidation and acetylation and, to a lesser extent phosphorylation and sulphuration. However, the extent to which these various events occur and the nature and amount of the peptides processed from POMC differ markedly between the anterior and intermediate lobes; these being determined by the distribution and controlled actions of the specific enzymes involved in each of the two lobes.

2.3.2.1 Processing in anterior lobe

In the rat anterior lobe, the principal products processed from POMC are ß-LPH and ACTH; a significant portion of ß-LPH is further processed to produce ß-END (Mains *et al.*, 1977; Eipper and Mains, 1980) (Figs. 2.1 and 2.3). The principal form of ß-END is ß-END$_{1-31}$, while smaller amounts of ß-END$_{1-27}$ and ß-END$_{1-26}$ are produced. These are further metabolized to their respective N-acetylated derivatives, Nac-ß-END$_{1-31}$, Nac-ß-END$_{1-27}$ and Nac-ß-END$_{1-26}$ (Zakarian and Smyth, 1982b) (Fig. 2.4). Other studies in the rat and man have shown that two smaller forms of endorphin, ß-END$_{1-17}$ (γ-END) and ß-END$_{1-16}$ (α-END), are also present in the anterior lobe (Ling, Burgus and Guillemin, 1976; Burbach and Wiegant, 1984) (Fig. 2.1). A similar processing pattern observed in the rat has been found to occur in a range of species, including the mouse, guinea pig, armadillo, cat, pig, sheep, cattle, monkey, baboon and man (Weber, Evans, Chang and Barchas, 1982; Zakarian and Smyth, 1982b; Smith and Funder, 1988). In most species, there is a low level of acetylation in the anterior lobe (< 5%) (Smyth and Zakarian, 1980; Weber *et al.*, 1982;), while the sheep is the exception in producing up to 25% of ß-END in acetylated form (Smith and Funder, 1988).

It should be emphasized that, although the POMC precursor is processed into all these various forms of ß-END detectable in tissue extracts by immuno-precipitation, biosynthetic labelling experiments using intact cell suspensions have demonstrated that the major end product from POMC which is secreted is ß-END$_{1-31}$ as the other forms of ß-END are not detected in the medium after 6h to 48h of pulse-chase incubations; even after 48h of incubation 75% of the ß-END secreted is ß-END$_{1-31}$ (Mains and Eipper,

α-MSH and CLIP are the other products processed from POMC in the anterior lobe (Fig. 2.1). In the rat, α-MSH is present in low amounts and is largely in the desacetyl form (Smith and Funder, 1988). In the sheep, a significant amount (25%) of ACTH is further processed to α-MSH; this has been characterized as desacetyl-α-MSH (30%), α-MSH (40%) and diacetyl-α–MSH (30%) (Smith and Funder, 1988).

2.3.2.2 *Processing in intermediate lobe*

In the intermediate lobe, ß-LPH and ACTH derived from the POMC precursor are further processed to produce ß-END and γ-LPH (Crine, Gianoulakis, Seidah, Gossard, Pezalla, Lis and Chretien, 1978; Mains and Eipper, 1979) and α-MSH and CLIP (Crine *et al.*, 1979), respectively (Fig. 2.1). Both ß-END and α-MSH are extensively acetylated in the intermediate lobe. ß-END is rapidly α-N-acetylated at the N-terminal tyrosine residue to produce acetyl-ß-END$_{1-31}$; this is then slowly metabolized by proteolysis to produce acetyl ß-END$_{1-27}$ and acetyl-ß-END$_{1-26}$ (Eipper and Mains, 1981; Kizer, Bateman, Miller, Humm, Busby and Youngblood, 1986) (Fig. 2.4). α-MSH is acetylated at both the N and O positions of the amino-terminal serine residue (Rudman, Chawla and Hollins, 1979). These biochemical modifications have important effects on the biological activity of both ß-END and α-MSH. For example, α-N-acetylation renders ß-END inactive in terms of both opioid receptor binding and inducing analgesia after central administration (Smyth *et*

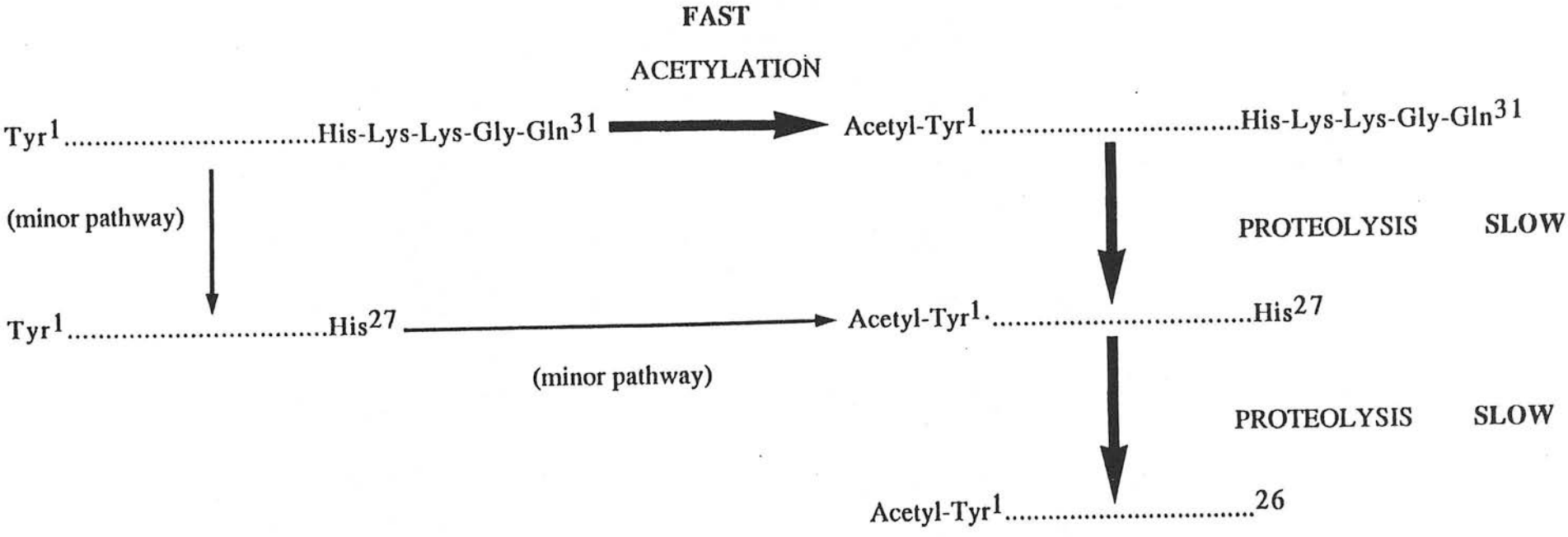


Fig. 2.4. Post-translational processing of ß-endorphin in rat intermediate lobe of the pituitary gland.

al., 1979). In contrast, acetylation of α-MSH markedly increases the potency of the peptide on melanocytes compared with desacetyl-α-MSH (Guttmann and Biossonnas, 1961). In the rat and sheep intermediate lobe, Nac-ß-END$_{1-27}$ is the principal form of acetylated endorphin as the there are two pathways for its production as illustrated in Fig. 2.4; Nac-ß-END$_{1-26}$ is also present in significant amounts (Smyth *et al.*, 1979; Eipper and Mains, 1981; Smith *et al.*, 1987). However, whereas the shorter Nac-α-END and the Nac-γ–END are relatively minor components in the rat, they constitute more than 60% of the total Nac-ß-END in the sheep intermediate lobe (Smith and Funder, 1988). In both the rat and sheep, Nac-ß-END$_{1-31}$ is a relatively minor component (Mains and Eipper, 1981; Smith and Funder, 1988).

In the rat, mouse and cattle intermediate lobe, α-MSH is diacetylated (Rudman *et al.*, 1979; Al-Noaemi, Biggins, Edwardson, McDermott and Smith, 1982; Goldman, Beaulieu, Kebabian and Eskay, 1983). In the sheep intermediate lobe, however, desacetyl-α-MSH and diacetyl-α–MSH are present in approximately equal amounts (Smith and Funder, 1988).

2.4 REGULATION OF THE SECRETION OF ß-END AND RELATED PEPTIDES FROM THE PITUITARY GLAND

There are important differences between the anterior and intermediate lobes of the pituitary gland in the way the hypothalamus regulates their secretory activity. The anterior lobe receives no direct neuronal inputs and is regulated by releasing and inhibiting factors which are released by the neurons in the hypothalamus into the portal blood vessels linking the median eminence with the pituitary gland. The intermediate lobe, in contrast, has a limited vascular supply and receives a neuronal input, in particular from the dopaminergic neurons in the arcuate nucleus, a population largely independent

of its counterpart running to the median eminence (Bjorklund, Moore, Nobin and Steveni, 1973; Millan and Herz, 1985; Moore, 1987). (Fig. 1.3)..

2.4.1 Regulation of the anterior lobe

2.4.1.1 Regulation by hypothalamic CRF and AVP

CRF is a 41-amino acid peptide isolated and characterized by Vale, Spiess, Rivier and Rivier (1981) from the hypothalamus of sheep, and is thought to play a key role in the regulation of the secretory activity of corticotrophs in the anterior lobe (Rivier, Brownstein, Spiess, Rivier and Vale, 1982; Antoni, 1986; Smith and Funder, 1988). It is present in high amounts in the external median eminence and portal plasma, its content in the paraventricular nucleus is increased by adrenolectomy, and it promotes the secretion of ß-END and related peptides from the anterior lobe (Rivier *et al.*, 1982; Burlet, Tonon, Tankosic, Coy and Vaudry, 1983; Swanson, Sawchenko, Rivier and Vale, 1983; Bruhn, Sutton, Rivier and Vale, 1984b; Antoni, 1986; Smith and Funder, 1988; Engler, Pham, Fullerton, Ooi, Funder and Clarke, 1989b). Furthermore, CRF antibodies block the release of ACTH *in vivo*, e.g. in response to stress (Antoni, 1986).

AVP is the other hypothalamic peptide implicated in the regulation of anterior lobe corticotrophic activity. AVP produced from the para-ventricular nucleus is similarly heavily represented in the extrenal median eminence (Levidiotis, Oldfield and Wintour, 1987; Whitnall, Smyth and Gainer, 1987) and its concentration in the paraventricular nucleus is modified by glucocorticoid manipulations (Antoni, 1986; Smith and Funder, 1988). It is also found in portal plasma in large quantities and is a potent stimulant of the release of ß-END and related peptides by a direct action on the anterior lobe (Buckingham,1987; Rivier *et al.*, 1984; Lutz-Bucher, Kovacs, Makara, Stark and Koch, 1986; Pradier, Dalle, Tournaire and Delost, 1988; Engler *et al.*,

1989b). Additional studies have found that AVP antisera block the ACTH releasing activity of median eminence extracts and introduction of antibodies against AVP in the brain partially blocks the release of ACTH induced by stimulation of the paraventricular nucleus (Carlson, Dornhorst, Seif, Robinson and Gann, 1982; Linton, Gillies and Lowry, 1983). Indeed, colocalization of CRF with AVP in a subpopulation of AVP neurons projecting to the external median eminence has been reported (Burlet *et al.*, 1983; Tramu, Croix and Pillez, 1983; Levidiotis *et al.*, 1987; Whitnall *et al.*, 1987).

Furthermore, both *in vitro* and *in vivo*, AVP potentiates the stimulatory action of CRF upon anterior lobe secretion (Rivier and Vale, 1983a; Redekopp, Livesey, Toth and Donald, 1985b; Pradier *et al.*, 1988). Thus, CRF and AVP coreleased in the median eminence may act synergistically on the anterior lobe to facilitate the secretion of ß-END and related peptides (Rivier and Vale, 1983a; Carlson and Gann, 1984). In addition, there is anatomical and physiological evidence that neural lobe stores of AVP may be involved in the modulation of anterior lobe corticotrophic activity (Millan and Herz, 1985; Antoni, 1986).

Mechanism of action : CRF stimulates the release of ACTH and ß-END from the corticotrophs and also induces an increase in the cellular levels of POMC mRNA and the synthesis of the POMC precursor (Bruhn *et al*, 1984b; Dallman, Makara, Roberts, Levin and Blum, 1985; Reisine, Rougon, Barbet and Affolter, 1985). The mechanism of action of CRF involves binding to specific, high affinity receptors in the anterior pituitary gland (Koch and Lutz-Bucher, 1983; Wynn, Aguilera, Morell and Catt, 1983; De Souza, Perrin, Rivier, Vale and Kuhar, 1984; Holmes, Antoni and Szentendrei, 1984; Perrin, Haas, Rivier and Vale, 1986). The binding of CRF to these receptors activates adenylate cyclase through a mechanism that requires magnesium ions and guanosine triphosphate (Aguilera, Harwood, Wilson, Morell, Brown and Catt,

1983; Holmes *et al.*, 1984; Perrin *et al.*, 1986), thereby increasing the intracellular levels of cAMP and enhancing the activity of cAMP-dependent protein kinase(s) (Labrie, Veilleux, Lafevre, Coy, Sueras-Diaz and Schally, 1982; Aguilera *et al.*, 1983; Erlichman, Litvin and Fleischer, 1984; Miyazaki, Reisine and Kebabian, 1984). Derivatives of cAMP, as well as agents that increase the intracellular levels of this second messenger, mimic the effects of CRF in all respects (Axelrod and Reisine, 1985). CRF also increases the levels of cytosolic free calcium and it has been suggested that influx of calcium from the extracellular medium is associated with the stimulation of ACTH release by CRF, and that this is caused by cAMP (Luini, Lewis, Guild, Corda and Axelrod, 1985). There is also a possibility that CRF acts via the arachidonic pathway. A mechanism alternative to cAMP, which may serve to increase intracellular calcium levels by causing influx of calcium ions from the extracellular medium, is the generation of arachidonic acid metabolites via the cyclooxygenase and/or lipoxygenase pathways of arachidonic acid metabolism (Naccache, Showell, Becker and Sha'afi, 1979).

There is evidence that the direct action of AVP on corticotrophs and the indirect action of AVP in potentiating the effect of CRF are mediated by two different types of receptor. That mediating the direct action of AVP on the anterior lobe corticotrophs has many properties in common with the V_1 pressor type AVP receptor (Manning and Sawyer, 1984), but can be clearly differentiated by its low affinity for potent V_1 receptor antagonists (Antoni, 1984). In fact, V_1 antagonists are partial agonists in stimulating ACTH release and they also act synergistically with CRF (Antoni *et al.*,1984; Knepel, Homolka, Vlaskovska and Nuto, 1984; Rivier *et al.*, 1984; Rivier and Vale, 1985; Buckingham, 1987). The AVP receptor involved in the potentiation of CRF activity is not fully characterized but appears to be neither V_1- nor V_2-receptor (Buckingham, 1987). Knepel *et al.* (1984) have suggested that the two

distinct actions of AVP on the anterior pituitary gland, which appear to be effected by different parts of the molecule, could be explained either by a single binding site mediating both responses by different mechanisms or, more feasibly, by the existence of two receptors with similar binding characteristics. Extracellular calcium is required for the stimulation of anterior lobe corticotrophic secretion by AVP (Zimmerman and Fleischer, 1970), but the direct action of AVP does not require cAMP (Axelrod and Reisine, 1985) instead, it seems to involve the activation of the calcium-calmodulin kinase C pathway. This is supported by a number of studies which explain the synergistic action between CRF and AVP in terms of an interaction between the cAMP-dependent and the calcium-calmodulin-kinase C-dependent pathways (for review see Rasmussen, Kojima, Kojima, Zawalich and Apfeldorf, 1984). Another site where the actions of CRF and AVP may converge is the arachidonic acid pathway. For example, the AVP-induced ACTH release has been shown to be inhibited by blockers of lipoxygenase and enhanced by indomethacin, a blocker of cyclooxygenase (Vlaskovska and Knepel, 1984; Vlaskovska *et al.*,1984).

2.4.1.2 *Regulation by adrenal glucocorticoids*

Glucocorticoids, secreted from the adrenal gland under the stimulus of ACTH have a negative feedback action on anterior lobe corticotrophic activity (Giguere, Labrie, Cote, Cory, Suerias-Diaz and Schally, 1982; Antoni, 1986; Plotsky *et al.*, 1986; Smith & Funder, 1988). Glucocorticoids not only act directly on the anterior lobe to suppress corticotrophic secretion, they also act centrally to suppress the release of CRF and AVP (Buckingham, 1979; Sakakura, Yoshioka, Kobayashi and Takebe, 1981; Carnes, Barksdale, Kalin, Brownfield and Lent, 1987). *In vitro* studies have clearly demonstrated the potent suppressive effects of glucocorticoids on both the CRF-induced release of ß-END and ACTH in cultured anterior lobe cells and the release and

generation of CRF (Guillemin, Vargo, Rossier, Minick, Ling, Rivier, Vale and Bloom, 1977b; Antoni, 1986). These studies have been corroborated by the observation that the synthetic glucocorticoid, dexamethasone, administered *in vivo* causes a rapid decrease in CRF content of rat median eminence (Suda, Tomori, Tozawa, Mouri, Damura and Shizume, 1984).

Mechanism of action : Glucocorticoids act at both the central and pituitary levels to regulate the secretion of ß-END and related peptides from the anterior lobe. Studies on the hypothalamic distribution of glucocorticoid receptors, either by immuno-histochemistry using monoclonal antibodies against the glucocorticoid receptor (Fuxe, Wikstrom, Okret, Agnati, Hafstrand, Yu, Granholm, Zdi, Vale and Gustafsson, 1985) or radioligand assays in micro-dissected areas (Reul and De Kloet, 1985), confirm that the paraventricular nucleus contains feedback type glucocorticoid receptors, which are apparently present in the parvicellular compartment (Reul and De Kloet, 1985). Systemic administration of glucocorticoids has been shown to prevent the adreno-lectomy-induced increase of CRF immunostaining (Sawchenko and Swanson, 1984) and to reduce the hybridizable AVP mRNA in the parvicellular neurons of the rat hypothalamus (Davis, Arentzen, Reid, Manning, Wolson, Lawrence and Baldino, 1986). In a most elegant experiment, Kovacs, Kiss and Makara (1986) showed that , in the rat, unilateral implantation of dexamethasone in the vicinity of the paraventricular nucleus blocked the increase in immunostaining of CRF and AVP after adrenalectomy. Implants in the central amygdala had no effect on the CRF cells locally or in the paraventricular nucleus; furthermore, implants in the dorsal hippocampus also failed to prevent the paraventricular nucleus response to adrenalectomy (Kovacs *et al.*, 1986). In addition, Kovacs and Mezey (1987) have demonstrated that unilateral implantation of dexamethasone around the paraventricular nucleus results also in a decrease of hybridizable CRF mRNA at the dexamethasone-implanted side. Dexamethasone implants into the cerebral cortex, dorsal hippocampus, ventral

subiculum, lateral septum or amygdala were without any effect on the CRF expression in the paraventricular nucleus. Interestingly, corticosterone did not result in any significant change in the CRF mRNA when implanted into the paraventricular region; however, when it was placed into the amygdala it slightly inhibited the CRF mRNA levels in the ipsilateral paraventricular nucleus in a few cases (Kovacs and Mezey, 1987). These observations strongly suggest that a glucocorticoid receptor system is present in the paraventricular nucleus and is involved in the regulation of the expression of CRF and AVP by hypophysiotrophic CRF neurons.

The precise mechanism of glucocorticoid feedback action at the pituitary level is not clear.There is evidence that the inhibitory feedback action of glucocorticoids on the pituitary gland involves the activation of genes through binding of the hormone-receptor complex to promoter element in cell nuclear chromatin (Lan, Karin, Nguyen, Weisz, Birnbaum, Eberhardt and Baxter, 1984). This is followed by the initiation of mRNA transcription from the activated gene(s) with subsequent synthesis of new proteins. One of the glucocorticoid-induced proteins is lipocortin, an inhibitor of phospholipase A_2 (Hirata, Schiffman, Venkatabramanian, Salomon and Axelrod, 1980). In principle, inhibition of this enzyme could reduce the ß-END/ACTH-releasing action of CRF and AVP (Vlaskovska and Knepel, 1984). On the other hand, since feedback inhibition is associated with a reduction of the POMC mRNA in corticotrophs (Eberwine and Roberts, 1984) while cAMP increases POMC mRNA, it has been suggested that the inhibition of corticotrophic secretion by glucocorticoids involves a reduction in the ability of the cAMP-dependent and C protein kinases to phosphorylate their specific substrates (Antoni, 1986). It should be noted, however, that glucocorticoids do not completely inhibit the corticotrophic-stimulating action of CRF (Bilezikian and Vale, 1983; Giguere *et al.*, 1982), which indicates that perhaps only one branch of the transducing pathway activated by CRF is affected by this feedback action of

glucocorticoids. A fast feedback inhibitory action of glucocorticoids on corticotrophic secretion has also been described (Keller-Wood and Dallman, 1984; Widmaier and Dallman, 1984), but the mechanism of action is yet to be identified.

2.4.2 Regulation of the intermediate lobe

2.4.2.1 Regulation by hypothalamic DA

The intermediate lobe of the pituitary gland is under tonic inhibition by DA. This is based on the observation that disconnection of the pituitary stalk results in hypertrophy of the cells of the intermediate lobe and increased secretion of ß-END and α–MSH (Clarke, Clements, Cummins, Dench, Smith, Robinson and Funder, 1986). These peculiarities are accounted for by the interruption of a tonic inhibitory influence acting upon the intermediate lobe. Indeed, a component of the tuberohypohysial dopaminergic system arises in the rostral arcuate nucleus and sends fibres to the intermediate lobe, where dopaminergic neurons make synapse-like contact with the pituitary cells (Moore, 1987). (Fig. 1.3). It is this component that inhibits intermediate lobe melanotrophic secretion of ß-END and α-MSH. For example, DA and congeners have been shown, *in vitro*, to dose-dependently block spontaneous action potentials and the release of ß-END and α-MSH via an action at DA receptors of the D_2 subtype (Vale *et al.*, 1979; Vermes, Mulder, Smelik and Tilders, 1980; Cote *et al.*, 1982a). *In vivo* studies have corroborated the picture of a dopaminergic brake upon melanotrophic secretion of ß-END and α-MSH. Levels of these peptides in the systemic circulation are increased by DA antagonists (Farah, Malcolm and Mueller, 1982; Chen, Dionne and Roberts, 1983; Kemppainen, 1986; Smith, Wallace, Clarke and Funder, 1989), and chronic exposure to these antagonists causes a rise in intermediate lobe content of ß-END and α–MSH and the amount of mRNA coding for POMC (Hollt and

Bergman, 1982; Chen *et al.*, 1983; Chronwall, Millington, Griffin, Unnerstall and O'Donohue, 1987). Jenks, Verburg van Kemenade, Tonon and Vaudry (1985) have also observed that in the frog (*Rana ridibunda*) intermediate lobe, DA inhibits the acetylation of α-MSH (and ß-END).

Mechanism of action : The action of DA on intermediate lobe melanotrophs has been shown to be mediated by D_2 receptors (Kebabian and Calne, 1979; Cote *et al.*, 1982a,b; Creese, Sibley, Hamblin and Leff, 1983; Farah and Mueller, 1989). The physiological consequence of stimulating these receptors is a reduction in both calcium entry and the synthesis of cAMP in the melanotrophs, which results in a decrease in secretion (Munemura, Cote, Tsuruta, Eskay and Kebabian, 1980a,b; Cote *et al.*, 1981; Cote *et al.*, 1982b).

Dopaminergic drugs have also been shown to alter the production of POMC mRNA and the rate of α-N-acetylation in the intermediate lobe. For example, Hollt, Haarmann, Seizinger and Herz (1982) and Chen *et al.* (1983) found that treatment of male rats with the DA antagonist, haloperidol, results in a time-dependent increase in POMC mRNA levels. More recently, Millington, O'Donohue, Chappell, Roberts and Mueller (1986) have also demonstrated that chronic treatment of rats with haloperidol not only increased levels of mRNA coding for POMC but also caused an essentially parallel rise in α-N-acetyl-transferase activity in the intermediate lobe.

2.5 MODULATION OF THE SECRETION OF ß-END AND RELATED PEPTIDES BY OPIOIDS AND GONADAL STEROIDS

2.5.1 Modulation by opioids

The administration of opioid agonists to rats induces the secretion of ACTH as reflected by a rise in glucocorticoid levels, an action which develops tolerance (Buckingham, 1979; Buckingham & Cooper, 1984; Jezova,

Jurcovicova, 1982; De Souza & Van Loon, 1982). This appears to be a central effect mediated via anterior lobe pools of ACTH since it is abolished by hypophysectomy, treatment with dexamethasone, or lesions of the median eminence. The effect is reproduced by administration of opioids into the brain, and is accompanied by a rise in serum ß-END and ACTH (Buckingham, 1979; Jezova *et al.*, 1982; De Souza & Van Loon, 1982; Antoni, 1986). These changes appear to involve the central action of opioids on the secretion of CRF and AVP since, *in vitro*, the anterior lobe secretion of ß-END and ACTH is not affected by opioids, whereas these compounds stimulate hypothalamic release of CRF (Buckingham, 1982; Buckingham and Cooper, 1984). Also, the rise in ACTH after opioids administration *in vivo* has been shown to be associated with changes in hypothalamic levels of CRF (Buckingham, 1982; Antoni, 1986). However, it is evident that the interaction of CRF and brain opioids is not a direct one, since CRF is predominantly produced in the paraventricular nucleus while high levels of opioid receptors occur in other areas including the preoptic area, the posterior hypothalamus and the locus coeruleus (Bardo, Bhatnagar and Gebhart, 1982; Tempel and Zukin, 1987). Furthermore, contradictory data has been obtained in man and sheep regarding the nature of opioid regulation of the anterior lobe. In man, opioid antagonists have been shown to elicit a release of glucocorticoids instead of inhibiting it, and the agonists have been shown to cause a suppression of ACTH and glucocorticoid release instead of causing a stimulation (Taylor, Dluhy and Williams, 1983). In sheep, Redekopp *et al.* (1985a) demonstrated an inhibition of ACTH using the enkephalin analogue, DAMME, while Wang, Coghlan, Congiu, Scoggins and Wintour (1986) showed that met-enkephalin stimulates the secretion of ACTH.

There is experimental evidence that endogenous opioids influence the secretory activity of the intermediate lobe of the pituitary gland by acting both directly on the melanotrophs and indirectly on hypothalamic DA neurons. For example, i.c.v. injection of ß-END causes a degranulation of melanotrophs

(Saland, Ortiz & Munger, 1982a), and an elevation of circulating levels of α-MSH (De Rotte, Van Wimersma Greidanus, Van Ree, Andringa-Bakker & De Weid, 1981; Van Wimersma Greidanus, Van Ree, Goedemans, Van Dam, Andringa-Bakker & De Weid, 1981); this is a central effect related to blockade of the inhibitory action of DA.(De Rotte *et al.*, 1981). In addition, Celis (1980) has shown that ß-END stimulates α-MSH both *in vitro* and *in vivo*. ß-END has also been shown to antagonize the DA-induced suppression of α-MSH release from the intermediate lobe in the frog *in vitro* (Saland *et al.*, 1982b). This effect could not, however, be observed in the rat (Voigt, Frank, Duker, Martin & Wuttke, 1983).

Mechanism of action: In the anterior lobe there are few, if any, opioid receptor binding sites in the rat (Lightman, Ninkovic, Hunt and Iversen, 1983), cow (Simantov and Snyder, 1977), sheep (Boublik and Clarke, 1984) and monkey (Wamsley, Zarbin, Young and Kuhar, 1982). The intermediate lobe has moderate levels of opioid binding sites (Simantov and Snyder, 1977; Wamsley *et al.*, 1982; Lightman *et al.*, 1983; Stojilkovic, Dufan and Catt, 1987). These observations point to the fact that the actions of opioids are, primarily, centrally mediated. Indeed, high levels of opioid receptors have been found in such areas as the medial and lateral preoptic areas, the posterior hypothalamus and the locus coeruleus (Bardo *et al.*, 1982; Tempel and Zukin, 1987). Opioid receptors are linked to the family of guanine nucleotide regulatory proteins (G-proteins) and these proteins have been shown to inhibit adenylate cyclase (Klee, Koski, Tocque and Simonds, 1984). Thus, the action of opioids on their receptors is not linked to the stimulation of cAMP (Rodbell, 1980; Jakobs, Aktories and Schultz, 1984). This action needs sodium ions (Blume, Lichtshtein and Boone, 1979), a feature characteristc of hormone systems coupled negatively to adenylate cyclase. While the G-mediated inhibition of adenylate cyclase remains the best characterized biochemical effect of opioid receptor activation, electrophysiological data suggest that alternative mechanisms

mechanisms account for opioid inhibition in many neuronal systems. Williams, Egan and North (1982) showed that opioid-induced hyperpolarization of rat locus coeruleus neurons resulted from an increased potassium conductance. This led to the suggestion that a G-protein mediates the opioid activation of potassium channels in these neurons through a pathway independent of cAMP-dependent protein kinase. Opioids action has also been shown to involve an inhibition of calcium channels (Werz and Macdonald, 1984; Macdonald and Werz, 1986).

All these actions are not in favour of a stimulatory role via CRF and would, therefore, indicate that opioids are primarily inhibitory in the modulation of ß-END secretion from the pituitary gland.

2.5.2 Modulation by gonadal steroids

Scant information is available on the possible influence of gonadal steroids on the regulation of ß-END secretion from the pituitary gland into the peripheral circulation. In male and female rats, a significant reduction in the plasma concentrations of ß-END has been observed 3-5 weeks after gonadectomy and replacement therapy with testosterone propionate or oestradiol benzoate partially restored the plasma levels of ß-END, respectively (Petraglia, Penalva, Locatelli, Cocchi, Panerai, Genazzani and Muller, 1982). In the same study, a decrease in ß-END content in the intermediate lobe of rats of both sexes was observed. In contrast, Lee, Panerai, Bellabarba and Friesen (1980) have reported a decrease in pituitary content of ß-END after gonadectomy in male but not in female rats. Furthermore, it has been reported in intact male rats that a 5-day treatment with testosterone propionate had no effect on ß-END concentrations in either pituitary lobe, whereas a 5-day treatment of intact female rats with oestradiol benzoate decreased intermediate lobe ß-END storage (Mueller, 1980). Thus, there appears to be sex differences

regarding the effect of gonadal steroids on ß-END secretion.. There are also age differences since old rats have higher ß-END contents in both the anterior and intermediate lobes in addition to elevated levels in the plasma compared with the levels found in young rats (Forman, Sonntag, Hylka and Meites, 1983; Missale, Govonis Croce, Bosio, Spano and Trabucchi, 1983). On the other hand, significant decreases in plasma concentrations of ß-END and ß-LPH have been described in women with physiological menopause (Genazzani, Facchinetti, Ricci-Danero, Parrini, Petraglia, La Rosa and D'Antona, 1981).

Mechanism of action : The mechanism of gonadal steroids action on the secretion of ß-END from the pituitary gland is poorly understood. One suggestion is that gonadal steroids act at the level of the pituitary gland to affect the processing of ß-END from the POMC precursor. This is based on the observation that the activity of trypsin-like proteinases which participate in the intracellular conversion of precursor hormones into the final product is considerably increased in the anterior lobe after gonadectomy (Kenessey, Paldi-Haris, Makara and Graf, 1979).

2.6 INFLUENCE OF ENVIRONMENTAL FACTORS

Many environmental factors influence the secretion of ß-END, apparently acting through stimulatory and inhibitory systems in the hypothalamus (Fig. 1.3). These environmental factors which may influence the cycle in ß-END secretion include photoperiod and stressful stimuli.

2.6.1 Influence of photoperiod

In seasonal animals such as white-footed mouse, hamster and sheep, the secretion of ß-END into the peripheral circulation is influenced by photoperiod. For example, the plasma levels of ß-END are markedly increased under short days, and decreased under long days (Kumar, Besch, Millard,

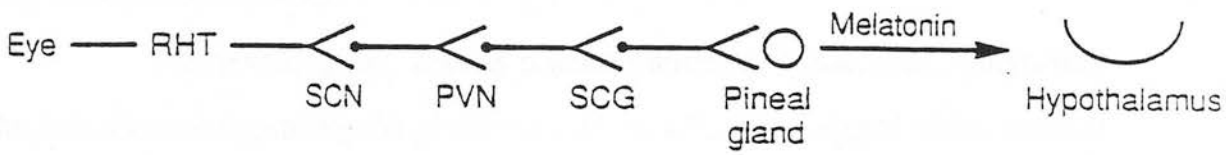


Fig. 2.5. Neural connections conducting light stimuli from the retina in the eye to the suprachiasmatic nucleus and then to the pineal gland via the superior cervical ganglia. The pineal gland acts as a neuroendocrine transducer, converting information regarding the light/dark cycle into a hormonal signal in the form of the 24h rhythm in melatonin secretion. The duration of period of melatonin secretion reflects night length and therefore daylength, and this acts on the hypothalamo-pituitary axis. RHT= retinohypothalamic tract; SCN= suprachiasmatic nucleus; PVN= paraventricular nucleus; SCG= superior cervical ganglia.

Sharp and Leadem, 1984; Ebling and Lincoln, 1987; Glass, Ferreira and Deaver, 1988). In the Soay ram, there are opposite changes in ß-END content in the pituitary gland (Ebling and Lincoln, 1987).

Mechanism of action : The pathway which transmits photic information to the hypothalamo-pituitary axis has been thoroughly investigated in the golden hamster (Reiter, 1980) as illustrated in Fig 2.5. Light stimulates photoreceptors in the retina of the eyes and the photic information is transmitted to the suprachiasmatic nucleus of the hypothalamus via a monosynaptic pathway known as the retino-hypothalamic tract. From the suprachiasmatic nucleus, the photic signal is relayed to the paraventricular nucleus and the pineal gland by way of the superior cervical ganglia. A similar photoneuronal pathway is evident in sheep (Legan and Winans, 1981; Pau, Kuehl and Jackson, 1982; Legan and Karsch, 1983).

The pineal gland acts as a neuroendocrine transducer, converting the information regarding the photoperiod into a humoral signal which acts on the hypothalamus to influence the secretion of the regulatory peptides and neuro-transmitters (Arendt, Symons and Land, 1981; Tamarkin, Baird and Almeida, 1985). Although several neuroactive peptides have been identified in the pineal gland, considerable evidence points to indolamine, melatonin (N-acetyl-5-methoxy-tryptamine), as the active neurohormone that mediates the effects of photoperiod.by influencing brain neurotransmitter systems For example, treatment with melatonin in the rat has been shown to elevate DA levels in the brain (Wendel, Waterburg and Pearce, 1974) as well as to inhibit DA release from the hypothalamus (Zisapel, Egozi and Laudon, 1983).The observation that melatonin treatment inhibits DA release from the hypoyhalamus raises the possibilty that short photoperiods increase ß-END secretion via the inhibitory action of melatonin on DA release.

The possibility that melatonin may also act directly on anterior lobe corticotrophs to influence ß-END secretion has been raised by *in vitro* studies

in the rat in which melatonin was shown to potentiate the effect of CRF on ß-END release (Samra, Dechand, Estour, Chalendar, Fevre-Montage, Pugeat and Tourniaire, 1985). More recently, the demonstration of melatonin-binding sites in the pars tuberalis of the rat and sheep pituitaries further suggests that melatonin acts directly on this part of the pituitary gland (Williams and Morgan, 1988; Morgan, Williams, Davidson, Lawson and Howell, 1989). However, the pars tuberalis contains cells which are morphologically different from those of the rest of the anterior pituitary and may not be involved in the secretion of ß-END.

2.6.2 Influence of stressful stimuli

The influence of acute stressful stimuli on ß-END secretion into the peripheral circulation has been demonstrated in a number of studies involving a variety of acute stressful stimuli. For example, in rats, elevated plasma levels of ß-END have been observed following footshock (Rossier, French, Rivier, Ling, Guillemin and Bloom, 1977a) and restraint stress (De Souza and Van Loon, 1985). In sheep plasma levels of ß-END have been elevated in response to acute haemorrhage (Smith, Owens, Lovelock, Chan and Falconer, 1986), electroimmobilization and shearing (Jephcott, McMillen and Rushen, 1987). There is also an increase in ß-END secretion in response to hypoglycaemia and audiovisual stimulus (barking dog) (Engler *et al.*, 1988) and during handling, transport and slaughter (Fordham, Lincoln, Ssewannyana and Rodway, 1989). Increases in plasma levels of ß-END and related peptides have also been demonstrated in other species, including man, following various acute stressful stimuli (Nakao *et al.*, 1979; for reviews see Antoni, 1986; Smith and Funder, 1988).

Chronic stress has also been shown to affect ß-END secretion. For example, in rats, daily footshock for 14 days increases the content of ACTH and ß-END in the anterior lobe of the pituitary gland (Young and Akil, 1985).

After14 days, the animals develop tolerance, indicating adaptation to the chronic stress. In a seasonal environment where extreme cold and food deprivation in winter may constitute chronic stress to the animal, such adaptation occurs. Of particular interest to this thesis are data obtained by exposing rats to an environment of a cold room, with marked hypothermia mimicking the effects of hibernation. In these circumstances, there was a decrease in the plasma levels of ACTH (Gibbs, 1985); the decrease was apparently due to a decline in AVP levels in pituitary portal blood as there was no change in the portal blood concentrations of CRF (Gibbs, 1985). With respect to the seasonal cycle in ß-END secretion, this might suggest that the low levels of ß-END seen in winter occur as a result of a decrease in the drive from the hypothalamus.

Mechanism of action : The stress-induced release of ß-END is associated with the activation of the paraventricular hypothalamus to release CRF, AVP, oxytocin and NE (Plotsky, Bruhn and Vale, 1985; Antoni, 1986). However, the type of factor(s) released from the hypothalamus depends on the type of stimulus. For example, ether stress, haemorrhage and insulin-induced hypoglycaemia involve the release of CRF, AVP, oxytocin and NE into the hypophysial portal blood (Rivier and Vale, 1983b; Bruhn, Plotsky and Vale, 1984a; Mezey, Reisine, Brownstein, Palkovits and Axelrod, 1984; Plotsky *et al.*, 1985); formalin and immobilization stress involve the release of CRF and AVP only (Linton, Tilders, Hodgkinson, Berkenbosch, Vermes and Lowry, 1985; Nakane, Andhya, Kaine and Hollander, 1985); and hypothermia seems to involve the release of AVP and oxytocin only (Gibbs, 1985). Whether this means that the hypophysiotrophic signal to a given stressful stimulus originates from several groups of anatomically distinct neurons (e.g. parvicellular CRF, magnocellular AVP and oxytocin neurons) remains to be investigated. The possible involvement of peripheral catecholamines in mediating the actions of

stress as CRF stimuli is still a matter of controversy (Cryer, Gerich, Dallman, Greer, Reisine, Mezey, Palkovits, Brownstein and Axelrod, 1986).

The effect of stressful stimuli on the hypothalamic systems controlling the release of CRF and AVP results in changes in the synthesis and release of ß-END from its precursor, POMC. There is evidence that both acute and chronic stress may modulate posttranslational processing of POMC and the release of ß-END and ACTH. Shiomi, Watson, Kelsey and Akil (1986) have demonstrated that, after an acute footshock stress in the rat, the rate of POMC translation is increased by 50%, and the rate of conversion to immunoreactive products is doubled. In the same study, when the animals were subjected to chronic stress, the ß-END/ACTH stores in the pituitary gland were increased, presumably due to an increase in transcription and POMC mRNA levels; however continued stress did not affect posttranslational processing. Furthermore, Hollt, Przewlocki, Haarmann, Almeida, Kley, Millan and Herz (1986), using an intermittent electrical footshock stress, saw an approximately 2-fold increase in POMC mRNA levels in the anterior lobe after 3 days of treatment and the levels remained elevated after 7 days of treatment; however, there were no significant differences in the intermediate lobe POMC mRNA levels. On the other hand, Dave, Eiden, Karanian and Eskay (1986) have demonstrated that POMC gene expression is inhibited in both the anterior and intermediate lobes by chronic exposure to ethanol stress. If the extreme cold and food deprivation that occur during winter are interpreted as chronic stress leading to adaptation in the animal's physiology, then the synthesis and processing of the POMC precursor may be inhibited with a resultant decrease in ß-END secretion, partially accounting for the low levels of ß-END that occur in winter.

2.7 PHYSIOLOGICAL SIGNIFICANCE OF ß-END

ß-END is widely distributed throughout the body (section 2.2); such a wide distribution is suggestive of a broad spectrum of target tissues whose function is regulated or modified by this peptide. It may have endocrine actions centrally on the brain or peripherally on target tissues or may have paracrine actions adjacent to its site of release.

2.7.1 Actions on the brain

The simplest evidence in support of an action of ß-END on brain function is the capacity of systemically administered ß-END, in both animal and clinical studies, to modify both endocrine and behavioural parameters integrated in higher centres of the brain (Millan and Herz, 1985); indeed, parenterally administered ß-END has been shown to cross the blood brain barrier (Merin, Hollt, Prezewlochi and Herz, 1980; Rapoport, Klee, Pettigrew and Ohno, 1980; Gerner, Sharp and Catlin, 1982; Ohlsson, Tsu-Ching, Jones, Martin and Dewley, 1982). ß-END, via the systemic circulation, may also interact with brain opioid receptors located externally of the blood brain barrier, for example, the subfornical organ and area postrema which lie in close proximity to autonomic regulatory centres in the hypothalamus and the brain stem, respectively (Millan and Herz, 1985). Furthermore, the median eminence and mediobasal-arcuate hypothalamus possess opioid receptors, whose activation by central or circulating ß-END may be involved in endocrine regulation by affecting the release of pituitary hormones. For example, the i.c.v. injection of ß-END suppresses the release of GnRH from hypothalamic neurons, resulting in a decrease in LH secretion (Schulz, Wilhelm, Pirke, Gramsch and Herz, 1981; Kalra and Kalra, 1983) and the introduction of antisera against ß-END into the mediobasal hypothalamus of immature female rats causes an increase in the plasma levels of LH (Schulz *et*

al., 1981). ß-END may also have antiovulatory effects via its suppressive action on hypothalamic GnRH. In female rats there is a proestrus surge of LH which underlies the initiation of ovulation. ß-END administered during the critical period of proestrus may prevent the occurrence of ovulation by interfering with the requisite LH surge based on the results obtained with morphine and naloxone (Pang, Zimmerman and Sawyer, 1977; Ieiri, Chen, Campbell and Meites, 1980). Furthermore, a clinical finding is the abnormally high incidence of relative infertility, for example, in heroin addicts, which may partially reflect a disruption of LH control (Meites, Bruni, Van Vugt and Smith, 1979).

Other pituitary hormones whose secretion is influenced by the action of ß-END on the brain are PRL, growth hormone and TSH. In the brain, ß-END interacts with DA to influence PRL secretion. Administration of ß-END i.c.v. or intracisternally increases the plasma levels of PRL and decreases hypophysial portal levels of DA (Grandison and Guidotti, 1977a; Rivier, Vale, Ling, Brown and Guillemin, 1977; Van Loon *et al.*, 1980a,b,c). In addition, the i.c.v. administration of antisera against ß-END suppresses basal levels of PRL in the circulation and attenuates the stress-elicited release of PRL, suggesting a role for ß-END in the control of PRL secretion (Ragavan and Frantz, 1981). ß-END injected i.v. or intracisternally has also been shown to be a potent stimulant of growth hormone (Rivier *et al.*, 1977). ß-END has also been implicated in the modulation of thyroid stimulating hormone in man but not in the rat (Grossman, Stubbs, Gaillard, Delitala, Rees and Besser, 1981; Grossman *et al.*, 1982), which suggests that ß-END is involved in metabolism.

2.7.2 Actions in the periphery

It is possible that ß-END may act locally in a paracrine way to influence the function of the tissues that produce it, or it may act in a conventional hormone-like fashion to activate receptors located in remote tissues. ß-END is locally produced in the gut and it may be involved in food

digestion, based on the evidence that naloxone reduces the secretion of gastric acid and gastric acid secretory response in man (Feldman, Walsh and Taylor, 1980). In addition, solutions of low pH have been shown to release ß-END *in vitro* together with secretin, motilin and vasoactive intestinal peptide from duodenal mucosa in man (Matsumura, Saito and Fujino, 1982a, Matsumura *et al.*, 1982b). ß-END is also involved in the regulation of pancreatic secretion. Infusion of ß-END in dogs elevates the plasma levels of glucagon and results in a transient hypoglycaemia (El-Tayeb, Gauthier, Brubaker, Lickley and Vranic, 1986), while the i.v. injection of ß-END in rabbits causes hypoinsulinaemia and hyperglycaemia (Knudtzon, 1986). ß-END has also been shown to stimulate the secretion of insulin and glucagon in humans (Reid and Yen, 1981). Of special note are data which show that ß-END can bind and/or modify the activity of adipocytes and also facilitate corticosterone formation in the adrenal cortex (Millan and Herz, 1985). These observations further highlight the view that ß-END is involved in carbohydrate and fat metabolism.

ß-END is also involved in the regulation of respiratory and cardiovascular functions. For axample, i.v. injection of ß-END results in bradycardia and hypotension (Lemaire, Tseng and Lemaire, 1978). Blood vessels and blood cells are also targets of circulating ß-END. This peptide has been shown to cause vasodilatation of the microcirculatory system of hamster cheek pouch (Wong, Koo and Li, 1981) and to modify the activity of blood platelets, human complement, leucocytes, lymphocytes and monocytes, consonant with its role in the regulation of the immune system (Smith and Blalock, 1981; Gilman, Schwartz, Milner, Bloom and Feldman, 1982; Johnson, Smith, Torres and Blalock, 1982). It is interesting to note that the action of ß-END on human complement and lymphocytes is through the carboxy-terminus rather than the amino-terminus, which is involved in binding to opioid receptors (Gilman *et al.*, 1982; Mehrishi and Mills, 1983; Schweigerer, Bhakdi and Teschemacher, 1982). It is conceivable that the binding of ß-END to

complement is of special physiological relevance in the adaptation of the immune system to stress.

ß-END produced in the testis has been shown to act as a paracrine inhibitor of Sertoli cell function in cell cultures. Chronic ß-END treatment of Sertoli cells of immature and adult rat testes in culture significantly inhibited basal and FSH-stimulated androgen-binding protein production; this effect being prevented by the opioid antagonist, naloxone (Fabbri and Dufau, 1988). Furthermore, the production of ß-END in the follicular cells of the ovary may be related to the oestrous cycle, perhaps affecting maturation or transportation of the oocytes. This is based on the observation that significantly higher concentrations of ß-END are found in ovaries removed in the periovulatory period than in those removed from anoestrous ewes (Lim *et al.*, 1983), and also on the observation that high ß-END immunoreactivity is seen in corona radiata and cumulus oophorus cells of ovulated oocytes (Shaha *et al.*, 1984).

Functional significance of circulating ß-END in seasonal animals: The timing of the seasonal changes in ß-END secretion are associated with the changes in the reproductive axis, body growth and fattening. In the hamster, the plasma levels of ß-END are high under short days when the animals are sexually inactive and are low under long days when the animals are sexually active (Kumar *et al.*, 1984); on the other hand, in sheep, the plasma levels of ß-END are high under short days when the animals are sexually active and are low under long days when the animals are sexually inactive (Ebling and Lincoln, 1987). Thus, there is no consistent relationship between the cycle in peripheral secretion of ß-END and the cycle in reproduction for different species.

The timing of the seasonal changes in ß-END secretion observed in the Soay ram is positively correlated with the seasonal changes in body growth and fattening (Ebling and Lincoln, 1987). A positive correlation between cortisol secretion, body growth and fat deposition has also been observed in the Svalbard and Norwegian reindeer (Larsen, Nilsson and Blix, 1985; Nilssen,

Bye, Sundsfjord and Blix, 1985). These observations imply that ß-END is involved in body growth and fat deposition in autumn as part of the adaptations to survive during the period of food deprivation in winter.

Three lines of evidence support the view that ß-END plays an important role in the regulation of metabolism. Firstly, ß-END is released with ACTH from the anterior lobe of the pituitary gland. Since these peptides are generated together they may act together to coordinate functions in the periphery. ACTH is involved in metabolism by its stimulatory effect on the adrenal cortex to produce glucocorticoids which play an important role in the regulation of protein, carbohydrate and fat metabolism. ß-END also appears to play a role in the control of metabolism. For example, infusion of ß-END in the carotid artery and jugular vein of dogs has been shown to cause increases in glucagon and cortisol levels and a transient decrease in plasma glucose concentrations (El-Tayeb *et al.*, 1986). Secondly, it has been observed that animals with abnormalities in carbohydrate and fat metabolism, for example congenitally obese rats and mice, have high circulating levels of ß-END (Margules, Moisset, Lewis, Shibuya and Pert, 1978; Govoni and Yang, 1981). Thirdly, elevated plasma levels of ß-END are positively correlated with body weight in obese women (Givens, Wiedemann, Anderson and Kitabchi, 1980).

Thus, in seasonal animals like the Soay sheep, the most likely role of ß-END is to regulate body resources and energy in such a way that the animals eat more and accumulate fat in summer and autumn in anticipation of food restriction associated with winter.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1. ANIMALS

3.1.1 Soay rams

Rams of the Soay breed of sheep illustrated in Fig. 1.1 were used in the majority of the studies described in this thesis. The animals were obtained as yearlings from commercial farms (Mr. P. Mapson, Cambridgeshire and Dr. G. A. Lincoln, Fife). Some of the rams were kept outdoors while others were kept indoors.

Outdoor groups : Groups of eight to sixteen rams were kept outside in grass paddocks at Dryden Field Station of the Institute of Animal Physiology and Genetics near Edinburgh (56°N). These animals were kept as all-male groups and were provided with an indoor shelter and fed a supplement diet of hay and sheep nuts during winter (Table 3.1).

Indoor groups : Groups of five to eighteen rams were kept permanently indoors in light controlled rooms at the Marshall Building of the University of Edinburgh, Roslin. (Table 3.1). The rams were penned individually within visual and olfactory contact and were fed a diet of grass pellets and hay, and water was available *ad libitum*. Artificial daylight was provided by fluorescent strip lights (4 x 180 cm) with light intensity of 300-400 lux at the ram's eye level, and lights on and off were controlled by time clocks (Sangamo Western Ltd, Port, Glasgow, U. K.). The rams were normally exposed to an artificial lighting regimen of alternating 16-week periods of long days (16h light : 8h darkness, 16L : 8D) or short days (8L : 16D) which entrained the animals' physiological cycles to a period of 32 weeks (Ebling & Lincoln, 1987). In experiment 6 the rams were exposed to a lighting regimen of 30 weeks of long days followed by 10 weeks of short days; then 16 weeks of alternating long and short days, and finally to 14 weeks of long days. Changes from one

Table 3.1 Animal groups used in the studies described in this thesis

Outdoor rams	Experiments
12 Soay rams	1, 8, 9, 10, 11 and 13
7 Tasmanian Merino and 4 crossbred rams	2
8 Soay rams: 3 controls and 5 PINX	3
16 Soay rams: 8 controls and 8 melatonin-implanted	4

Indoor rams	Experiments
7 Soay rams	5
8 Soay rams: 4 controls and 4 melatonin-implanted	6
18 Soay rams: 6 controls, 6 castrates and 6 castrates + testosterone implants	7
5-8 Soay rams	12, 14, 15, 16, 17 and 18.

photoperiod to another were made by altering the time of lights off. The temperature in the rooms was maintained relatively constant by supplementary heating in winter months (daily mean ± SEM, 15.3 ± 0.3).

3.1.2 Other rams

A secondary study was also conducted on seven Tasmanian Merino rams obtained from the AFRC Institute of Animal Physiology and Genetics Research, Edinburgh, and four crossbred rams produced locally by mating one Tasmanian Merino ram with ten Soay ewes (Table 3.1). These rams were kept outside as all-male groups in grass paddocks at the Dryden field station.

3.2 BLOOD SAMPLING AND ROUTINE MEASUREMENTS

3.2.1 Outdoor rams

Routine 10 ml blood samples for hormone assays were collected every week by jugular venepuncture, using heparinized plastic tubes (Brunswick) and a plastic syringe (Plastipak) fitted with a 21G needle. For this, the rams were herded by a sheep dog into a pen in the field and allowed to rest for 1-2h before blood sampling; each ram was handled with minimum disturbance during blood sampling. Body weight and testis diameter were recorded every 2 weeks; whenever this was done, routine blood samples were collected before this procedure.

For the collection of serial blood samples at 10 or 15 min intervals, the rams were moved into pens and then transferred to crates on the day before the study. A polythene cannula (Braunula Luer, Armour Pharmaceuticals) was inserted into the jugular vein of each ram and was anchored to the skin of the neck with silk sutures (Ethicon). The cannula was connected to a three-way tap (Vygon-VG1) by a 60 cm polythene tube (Portex) and kept patent with 0.9% physiological saline (w/v; Travenol Labs.) containing 10,000 I. U. heparin /

litre (Wellcome Diagnostics). Blood samples were collected into 5ml plastic syringes (Plastipak) and transferred to heparinized plastic tubes.

3.2.2 Indoor rams

Routine 10 ml blood samples were collected every week as for outdoor rams. For serial blood samples at 10 or 15 min, the rams remained in their home pens and were cannulated a day before the study as for the outdoor rams.

In all cases, care was taken not to disturb the animals during blood sampling; the blood samples were collected on ice and centrifuged within 30-40 min for 30 min at 2500rpm. Plasma was removed and stored in 5 or 10 ml vials (Sardstedt) at 20°C until required for assay. In instances where ß-END and ACTH were to be measured in the same blood sample, the plasma was divided into two aliquots to avoid repeated thawing.

3.3 DRUGS AND SOLVENTS

AVP was purchased as a 10 I. U./ml solution from Sigma Chemical Company (Poole, Dorset, U. K.); CRF (Amunine, ovine 1-41), ß-END (human ß-LPH, 61-91) and ACTH (human, 1-24) were obtained from Bachem UK Ltd (Saffron, Walden, Essex, U. K.). CRF was dissolved in 0.003N HCL and both ß-END and ACTH were dissolved in 0.9% physiological saline (w/v) obtained from Travenol Labs. Ltd (Thatford, Norfolk, U. K.). DEX was obtained as a 4mg/ml solution of dexamethasone sodium sulphate from Merck Sharp & Dohme (MSD) Ltd (Hoddesdon, Herts, U. K.); RU 486 was kindly donated by Roussel-Uclaf (Paris, France) and was dissolved in 50% ethanol : propylene glycol (v/v); NAL was kindly donated by Sterling-Winthrop (Surbiton, Surrey, U. K.); DA was purchased from Sigma Chemical Company (Poole, Dorset, U. K.). Both NAL and DA were dissolved in physiological saline.

Bromocriptine and sulpiride were kindly donated by Drs. J. Curlewis and A. S. McNeilly (MRC Reproductive Biology Unit, Edinburgh) and were dissolved in 15% ethanol (v/v) and 0.01N NaOH, respectively. Bromocriptine was originally obtained from Sandoz Products Ltd (Feltham, Middlesex, U. K) and sulpiride was originally obtained from Delagrange (Paris, France). Heparin was purchased as a 25,000 I. U./ml solution from Leo Labs. Ltd (Risborough, Berkshire, U. K.). Lignocaine was obtained from Univet 2 Ltd (Bicester, Oxfordshire, U. K.).

3.4 ASSAYS

3.4.1 ß-END assay

Plasma concentrations of ß-END were measured in unextracted plasma by radioimmunoassay based on the assay described and validated for ovine plasma by Leshin and Malven (1984). The ß-END assay was always the first assay to be done on those samples collected for the measurement of several hormones.

Although the ß-END assay had been validated for unextracted ovine plasma, the effect of plasma was still checked as follows: (i) a portion of a pool of plasma from hypophysectomized sheep, provided by Dr. A. McNeilly (MRC Reproductive Biology Unit, Edinburgh) was assayed after extraction to confirm that there was no ß-END detectable in it. No ß-END was detected in the hypophysectomized plasma by this assay with a sensitivity of 24.40 pg/ml; (ii) standard ß-END (camel ß-END$_{1-31}$; 0.2 to 12.5 ng/ml) was added to two portions of the hypohysectomized plasma. One of these portions was extracted, reconstituted in buffer and assayed; the other portion was assayed directly. Recoveries of standard ß-END were 88.3 ± 4.9 % and 91.4 ± 7.8 % for direct and extraction assays respectively; (iii) two pools of whole plasma were used. One portion of each pool was extracted before assay and the other was assayed

directly. There was good agreement between estimates of ß-END concentration in whole plasma and in extracts of plasma (Table 3.2).

Taken together, these results indicate that the assay of whole ovine plasma or extracts of plasma will yield similar estimates of ß-END concentration. The assay of unextracted plasma is preferred because it does not introduce potential errors of differential extraction and elution from silica grass or silicic acid, and it is more adapted to the large numbers of samples which neuroendocrine studies involve.

Leshin and Malven (1984) observed that incubation of ^{125}I-ß-END with plasma under assay conditions resulted in the appearance of a labelled product of smaller molecular weight (1400 daltons) than ß-END (molecular weight = 3439). Approximately one-half of this small molecular weight product was bound and was displaced from the ß-END antiserum. They hypothesized that this condition may be a prerequisite for a valid ß-END RIA for whole plasma. However, they noted that the ratio of this product to intact ^{125}I-ß-END averaged only 0.29 ± 0.08 ($n = 10$) and that the generation of this small molecular weight product varied widely among different plasma pools. A test of the occurrence of this product was carried out on five pools of plasma following the method of Leshin and Malven (1984). No such small molecular weight product was observed.

Because ß-LPH exists in ovine plasma and since this RIA for ß-END is using an antiserum which cross-reacts about 30 % by weight with ß-LPH (Ebling & Lincoln, 1987), the estimates of ß-END should be assumed to contain a contribution from the endogenous ß-LPH. However, the ratio of ß-LPH to ß-END in the plasma of the Soay ram is low (ß-LPH : ß-END, 1 : 7.7 under short days; 1 : 3.4 under long days; Ebling and Lincoln, 1987).

Table 3.2. Comparison of radioimmunoassay of unextracted ovine plasma with that of extracted ovine plasma. Values are means ± SEM (pg/ml), n=12 determinations in duplicate per pool for each method.

	RIA of unextracted plasma	RIA of extracted plasma
Pool 1	560.02 ± 14.42	540.69 ± 31.90
Pool 2	580.76 ± 23.15	620.45 ± 26.32

3.4.1.1 Reagents

Buffer : The standard buffer contained 0.25 % (w/v) bovine serum albumin (RIA grade, Sigma) and 0.2 % (w/v) sodium azide (BDH, Poole) in 0.05M PO_4 buffered saline (pH 7.6). For assays, 0.1 % (v/v) α-2-mercaptoethanol (Sigma) was added to the standard buffer as a preservative and two drops of phenol red indicator (Flow Labs.) were also added to monitor the pH.

Standards : Camel ß-END_{1-31} (Sigma) was used for preparation of standards and for iodination. The structure of camel ß-END_{1-31} and ovine ß-END_{1-31} are identical (Li & Chung, 1976). Solid peptide was dissolved in 0.01 N HCl to give a 0.5 µg/µl stock solution. 10 µl (= 5 µg) aliquots were pipetted into plastic stoppered LP3 tubes (Sardstedt) and stored at -40^{0}C for use as iodination aliquots.

For assay standards, a 10 µl aliquot was further diluted with standard buffer to give a 50 ng/ml solution. 1 ml aliquots of this solution were pipetted into plastic stoppered LP3 tubes (Sardstedt) and stored at -40^{0}C for up to one year.

Antiserum : The antiserum 7.9.02 used in these studies was raised in Soay rams at the MRC Reproductive Biology Unit in Edinburgh against the synthetic ovine ß-END conjugated to porcine thyroglobulin with carbodiimide (Ebling & Lincoln, 1987). In the assays it was used at a final dilution of 1 : 99,600 which gave 50 % inhibition of specific binding of 100-120 pg/tube. This antiserum has negligible cross-reaction with α-END_{1-17} (< 0.19 %), γ-END_{1-16} (< 0.19 %), met-enkephalin (< 0.001 %), $dynorphin_{1-17}$ (< 0.05 %) and $ACTH_{1-39}$ (< 0.11 %). However, it crossreacts 100% with ß-LPH_{1-91} (Ebling and Lincoln, 1987). In the Soay ram ß-LPH_{1-91} has been previously shown to be present in low concentrations compared with ß-END

(Ebling and Lincoln, 1987). Thus, the hormone concentrations measured by this assay are assumed to be predominantly ß-END.

Quality controls : Three plasma quality controls, low (500-750 pg/ml), medium (800-1000 pg/ml) and high (1200-1800 pg/ml) were included in each assay. The low and high quality controls were prepared by taking a pool of blood plasma from outdoor Soay rams in winter (low plasma levels of ß-END) and in summer (high plasma levels of ß-END), respectively. A portion of the low quality control plasma and a portion of the high quality control plasma were mixed to produce the medium quality control plasma. The three quality controls were used to calculate the within and between assay C. Vs.; this was done by the Assay Zap computer program designed to calculate the assay results. The average intra- and inter-assay C. Vs. for the assay were 5.6% and 11.2%, respectively, based on 288 assays.

ß-END tracer : The chloramine T method of iodination was used to produce the tracer. A working solution of 10 mg in 10 µl 0.05M PO_4 buffer was prepared just prior to use. To the iodination aliquot of ß-END (5 µg) was added:

10 µl 0.25M PO_4 buffer (no bovine serum albumin or preservative)

10 µl Na ^{125}I (specific activity -15mCi/µg iodine, Amersham)

10 µl working solution of chloramine T (= 10 µg)

The mixture was allowed to react for 30 seconds then was diluted with 1 ml assay buffer, counted and immediately loaded onto a prepared Sep-Pak C18 column (Waters Assoc., MA, U. S. A). The column was prepared by washing it with 3 X1 ml 80% methanol : water containing 1% (v/v) trifluoroacetic acid (Sigma), then with 3 X1 ml 1% trifluoroacetic acid alone.

The reaction mixture was eluted with a stepped increasing gradient of methanol. The initial wash was with 1 ml 0.25M PO_4 buffer, then 1 ml methanol : water at concentrations of 30%, 40%, 50%, 60%, two X 65%, six X 70% and six X 80% (Fig. 3.3). All washes contained 1% trifluoroacetic acid and the eluate was collected in plastic LP3 tubes. The appropriate fractions were made up to 5 ml with 70% methanol : water and were stored liquid at -20°C for 4 weeks.

For assay and record purposes, the approximate specific activity of the tracer was calculated as follows, assuming that all the 5 µg of the iodination aliquot was in the column fractions counted:

volume of $Na^{125}I$ taken = 10 µl containing 1000 µCi

total counts = 14877

counts as ß-END = 12391

% iodination yield = counts as ß-END/ total counts X 100 = 12391/14877 X

= 83.6%

radioactivity incorporated into protein = % iodination yield/100 X original activity

= 83.6/100 X 1000= 835.71 µCi

specific activity = protein radioactivity/weight of protein = 835.71/5

= 167µCi/µg

Dextran-charcoal suspension: Because the ß-END antiserum 7.9.02 was raised in Soay rams and the measurements of plasma concentrations of ß-END were made in the same animals, the dextran-charcoal method of McLoughlin,

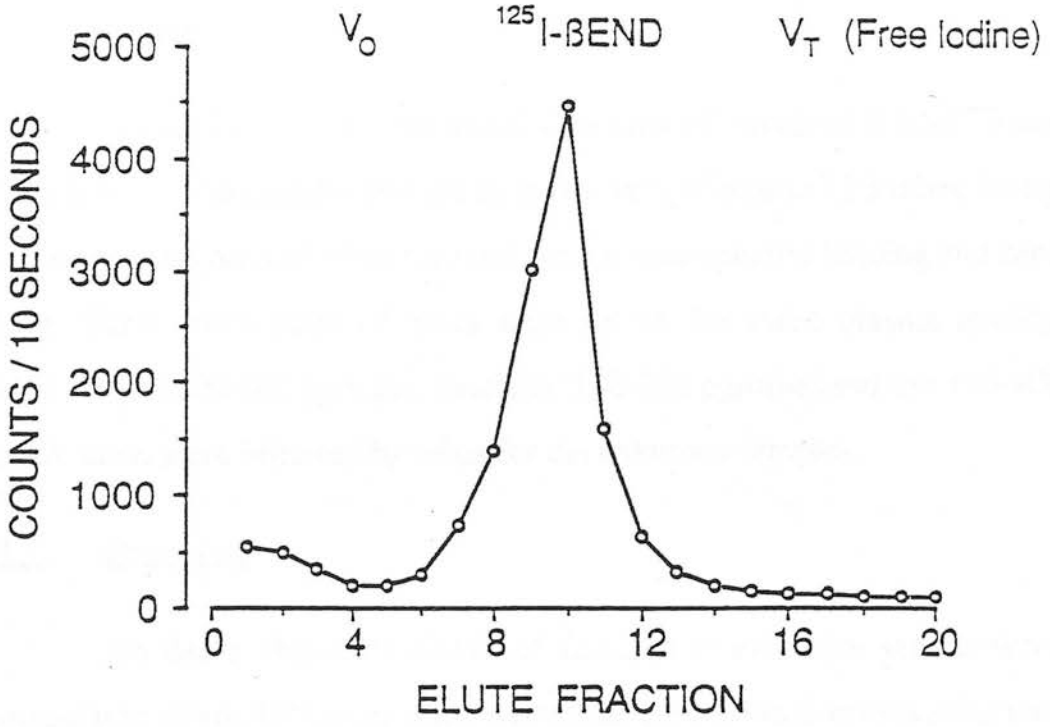


Fig. 3.3. Elution profile of ^{125}I-ß$_C$-endorphin tracer from a Sep-Pak C18 column. The column was first loaded with the iodination mixture, then washed with 0.25MPO4 buffer followed by washes with 30%, 40%, 50%, 60%, 2 X 65%, 6 X 70% and 6 X 80% methanol : water.

Lowry, Ratter, Besser & Rees (1980) was used to separate the bound from the free tracer. The dextran-charcoal suspension was prepared by first dissolving 0.75g Dextran T70 (Pharmacia) in 40 ml 0.05M PO4 buffer, then adding 10 ml normal sheep serum (Scottish Antibody Production Unit) and 3g charcoal (C-5260, Sigma). The suspension was stirred for 45-60 min in an ice bath before adding it to assay tubes.

3.4.1.2 Design

A standard curve with serial dilutions of standard ß-END from 2500 pg/tube to 4.88 pg/tube was set up in ten pairs of plastic LP3 tubes, being preceded by three pairs of tubes for total counts, non-specific binding and zero binding. Three more pairs of tubes were set up for three plasma quality controls : high (200-300 pg/tube), medium (140-180 pg/tube) and low (60-100 pg/tube); these were followed by tubes for the unknown samples.

3.4.1.3 Procedure

On day 1, duplicate 200 µl of standard or unknown sample were dispensed into plastic LP3 tubes using a microlab (Hamilton Bonaduz AG); then the ß-END antiserum was added at a dilution of 1 : 16,600 in 50 µl assay buffer. After thorough mixing, the tubes were covered and incubated at 4°C for 24h. Incubation of antiserum with standard (or unknown) for NOT less than 24h is a critical requirement to reliably detect the small quantities of standard ß-END (or unknown samples under long days). On day 2, 10-12,000 counts per min of ^{125}I-ß-END was added in 50 µl assay buffer. The tubes were vortexed, covered and incubated at 4°C for another 20-24h. On day 3, 250 µl of the cold dextran-charcoal suspension was added to separate the bound from the free hormone. The tubes were then vortexed, centrifuged at 2500 rpm at 4°C for 20 min. The supernatant (containing the bound hormone) was tipped into clean

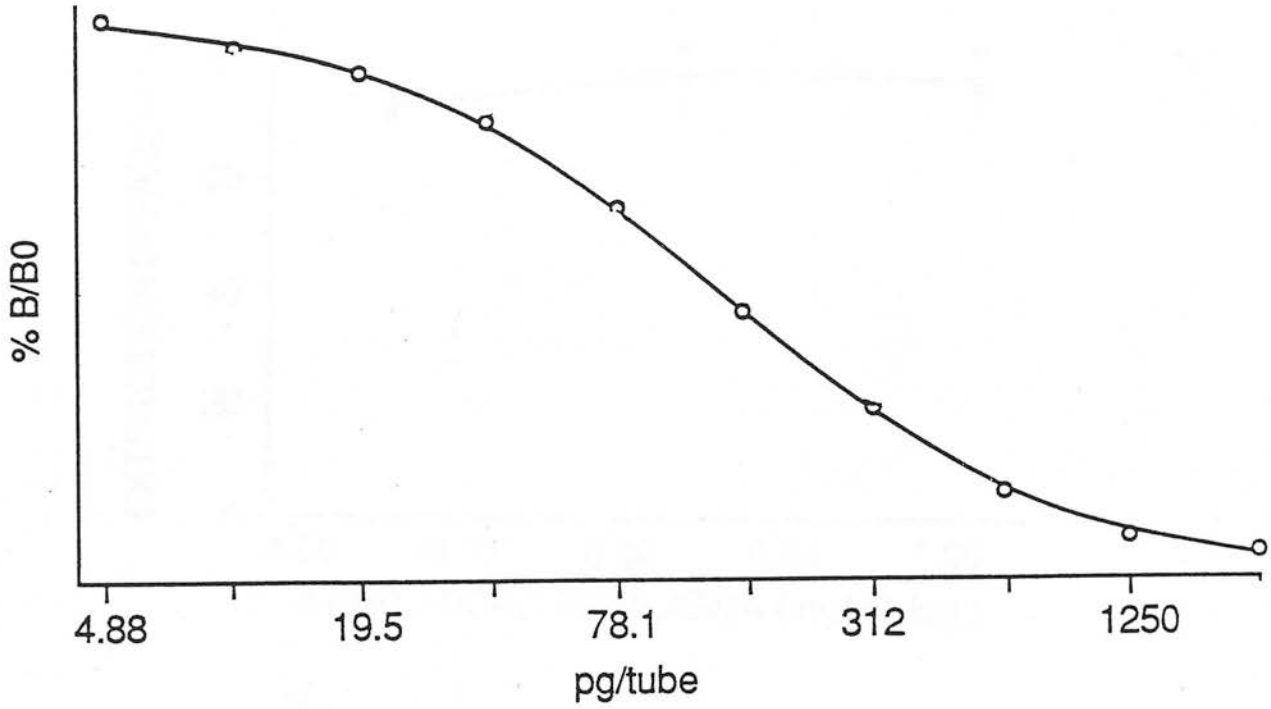


Fig. 3.4. Standard curve for ß$_C$-endorphin. Each point is the mean of duplicate determinations.

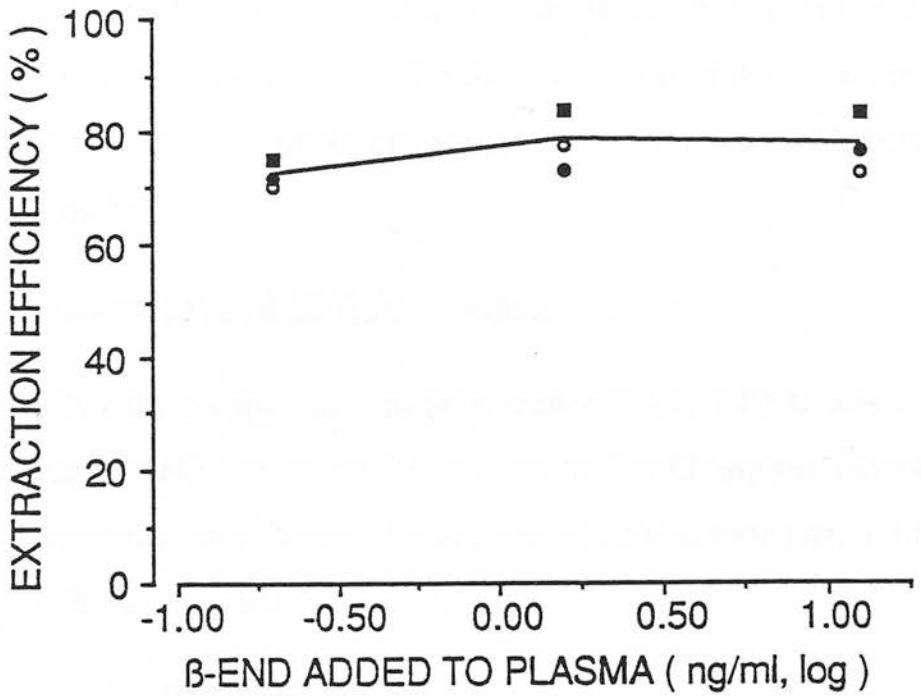


Fig. 3.5. Recovery of $ß_C$-endorphin standard added to 3ml of plasma as a proportion of the initial amount added.

LP3 tubes and counted using a NE 1600 gamma counter (Nuclear Enterprises, Edinburgh).

3.4.1.4 *Calculation of assay results*

Raw counts were processed by an Assay Zap program developed for Apple Macintosh microcomputer by Dr. P. L. Taylor (MRC Reproductive Biology Unit, Edinburgh). This program draws a standard curve, calculates the total binding for the assay, the quality controls along with the consequent within- and between-assay C. V. plus the calculation of the concentration of unknown samples. An example of a standard curve drawn by this program is shown in Fig. 3.4.

3.4.1.5 *Extraction of ß-END from plasma*

For the comparisons made in section 3.4.1, ß-END was extracted from plasma according to the procedure described by Ebling and Lincoln (1987). The extraction efficiency for this procedure was tested and found to be 75.8 ± 2.76%, mean ± SEM (Fig. 3.5).

3.4.2 Nac-ß-END assay

The conventional radioimmunoassay for measuring total ß-END was modified to measure Nac-ß-END by using human Nac-ß-END$_{1-31}$ instead of camel ß-END$_{1-31}$ for standards and iodination. The radioimmunoassay for measuring Nac-ß-END employed an antiserum (R1-5) specific for Nac-ß-END kindly donated by Dr. E. Weber, Stanford University School of Medicine, California, U. S. A. The cross-reactivity of this antiserum with camel ß-END was 0.17% based on the amount of unlabelled peptide needed to obtain a 50% displacement of ^{125}I-labelled Nac-ß-END$_{1-31}$ (Fig. 3.5).

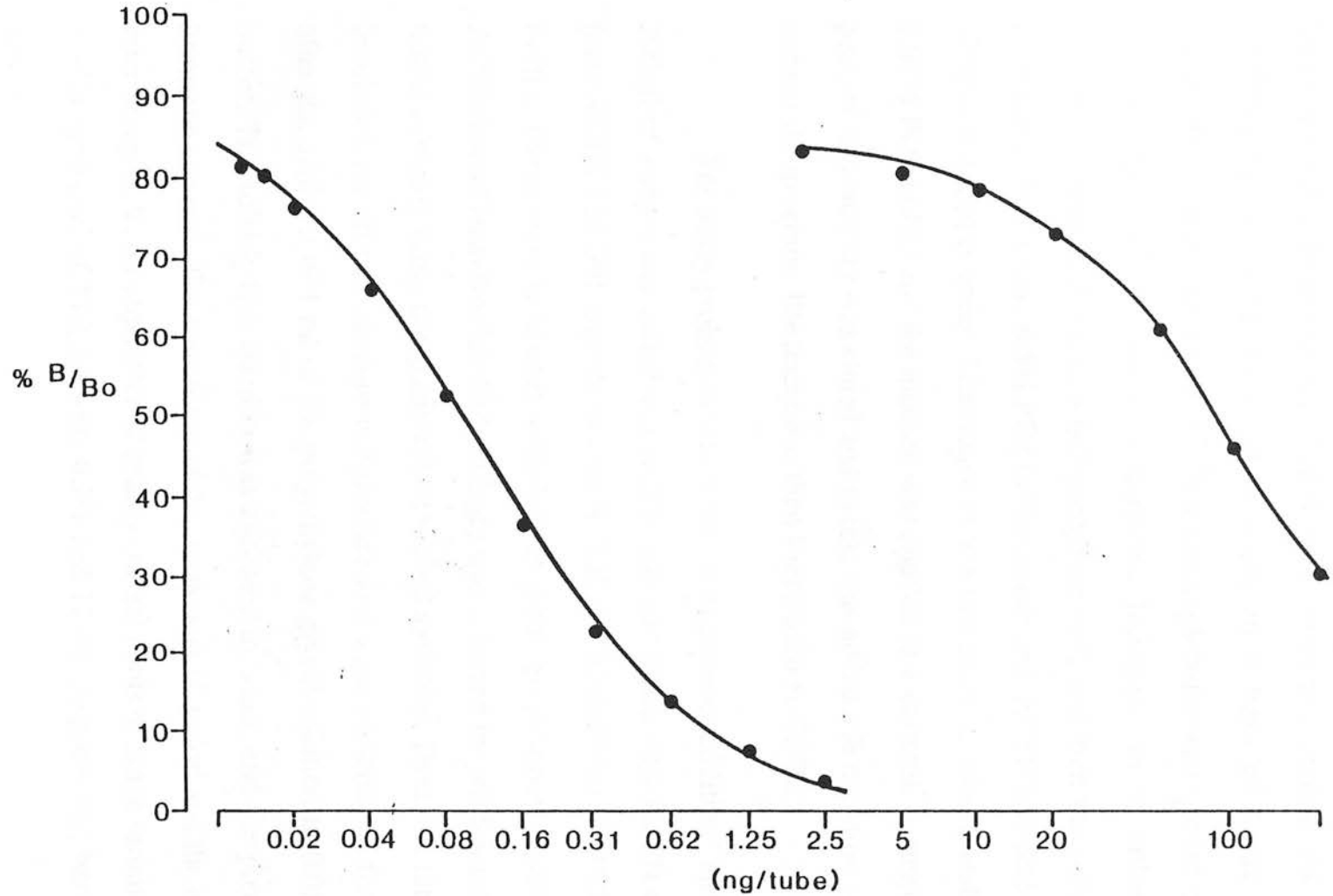


Fig. 3.6. Standard curve for N-acetyl-β_h-endorphin and inhibition curve for β_h-endorphin. Each point is the mean of duplicate determinations.

3.4.3 ACTH assay

The plasma concentrations of ACTH were measured in duplicate by the immunoradiometric assay described by Hodgkinson, Allolio, Landon and Lowry (1984). In this assay, 0.05M PO_4 buffer was used at pH 7.4 and $ACTH_{1-39}$ was used as standard made in a base of horse serum. Radioiodination of sheep anti-ACTH immunoglobulin was carried out by a modification of the Iodogen method. Iodogen, in a solution of dichloromethane, was put in a polypropylene vial, and then the solvent was evaporated. To the vial, 0.5M PO_4 buffer, sheep anti-ACTH IgG and sodium ^{125}I were added in order. After reaction, the vial contents were transferred to 0.05M PO_4 buffer and the mixture was applied to a column. A single major peak of radioactivity was eluted and to this was added 1% mannitol and 10% normal sheep serum. The tracer was then kept frozen at -20°C.

The assay procedure was of the "simultaneous addition" variety. To 200 µl of sample was added anti-ACTH IgG and rabbit anti-ACTH antibody (anti-ACTH {34-39} peptide or anti-ACTH {2-16} peptide) in 0.05M PO_4 buffer. These were incubated overnight at room temperature. Separation of ACTH-bound from free labelled antibody was achieved by precipitation of the bound complex using sheep anti-rabbit second antiboby. Then, the tubes were incubated for 30 min at room temperature and were centrifuged for 30 min after the addition of 1 ml of 2% polyethylene glycol solution in 0.05M PO_4 buffer. The soluble free fraction was aspirated to waste and the precipitated hormone counted. The sensitivity of the assay was 10 pg/ml and the intra-and inter-assay C. V. for duplicates of quality control pools of horse serum " spiked " with synthetic $ACTH_{1-39}$ were 6.1% and 12.4% respectively, based on 23 assays.

3.4.4 Cortisol assay

The plasma concentrations of cortisol were measured in duplicate by radioimmunoassay using the method of Abraham, Buster and Teller (1972), as modified by Hillier and Read (1975) and Hunter, Nars and Ruthersord (1975) for an iodinated tracer. In this assay, 0.1M PO4-gelatin buffered saline (PGBS) was used as buffer and synthetic cortisol (Sigma) was used for standard. Cortisol antiserum S004-201 (Scottish Antibody Production Unit) was used at a final dilution of 1 : 24,000. The tracer was prepared by Chloramine T method; this involved reacting ^{125}I, cortisol-histamine conjugate and chloramine T. The reaction was terminated by the addition of sodium metabisulphite and the labelled cortisol was extracted with ethyl acetate. After separation, the steroid fraction was run on a thin-layer-chromatographic plate. Then, the radioactive peak was transferred and stored in ethanol at 4°C.

The samples (50 µl) were extracted in 1 ml dichloromethane and dried down under nitrogen in a heating block at 40°C. The extract was reconstituted in 200 µl of 0.1M PGBS. To 100 µl of the reconstituted extract was added 100 µl of antiserum, and then 100 µl of tracer was added. The tubes were mixed and incubated overnight at 4°C before separating the bound from the free hormone by the dextran-charcoal method. The supernatant, containing the bound hormone, was then counted. The lower limit of detection of the assay was 2.3 ng/ml. The mean intra- and inter-assay C. V. were 5.5% and 7.8% respectively, based on 10 assays.

3.4.5 PRL assay

The plasma concentrations of PRL were measured in duplicate by RIA described by McNeilly and Andrews (1974). The buffer used for this

assay consisted of 1% bovine serum albumin/phosphate (BSA/PBS) (w/v) and the reference standard was NIH-P-S9 (NIAMDD, Bethesda, U. S. A.). The antiserum R50 was used at a final dilution of 1 : 422,400. The tracer was prepared by the Lactoperoxidase method; this involved reacting ^{125}I, ovine PRL, lactoperoxidase and hydrogen peroxide. The reaction was terminated by 1 ml of 0.1% BSA/PBS, then the reaction mixture was loaded onto a column. After separation, the reactive peak was kept frozen at -20°C.

The assay procedure involved taking 30 µl of sample to which was added 100 µl of buffer, 100 µl of first antibody and 100 µl of tracer. The mixture was then incubated overnight at 4°C. On the second day, 200 µl of second antibody was added and the tubes were again incubated overnight at 4°C. On the third day, the tubes were spun, the supernatant discarded and the precipitate counted. The lower limit of detection of the assay was 0.4 ng/ml. The mean intra- and inter-assay C. V. were 8.3% and 11.9% respectively, based on 78 assays.

3.4.6 Testosterone assay

The plasma concentrations of testosterone were measured in duplicate by radioimmunoassay using the method described by Corker and Davidson (1978), as modified by Sharpe and Bartlett (1985) for an iodinated tracer. This assay used 0.1M PGBS buffer and synthetic testosterone ((Sigma) was used for standards. The antiserum 505 was used at a final dilution of 1 : 2,800,000. The tracer was prepared by Chloramine T method. This involved reacting ^{125}I, testosterone-carboxymethyl-oxime histamine and chloramine T. The reaction was terminated by addition of sodium metabisulphate and the labelled testosterone extracted with ethyl acetate. After volume reduction, the steroid fraction run on a thin-layer-chromatographic plate. The radioactive peak was removed and kept in ethanol at 4°C.

The samples (50 µl) were extracted in hexane : ether and dried down under nitrogen. 200µl of 0.1M PGBS was added to the extract for reconstitution. To 100 µl of the reconstituted extract was added 100 µl of antiserum and then 100 µl of tracer. The tubes were then mixed and left on bench for 3h before adding 200 µl of the second antibody. The tubes were incubated overnight before centrifugation the following day. The supernatant was tipped off and the precipitate was counted. The lower limit of detection of the assay was 5 pg/ml and the mean intra- and inter- assay C. V. were 9.9% and 12.7%, respectively, based on 5 assays.

3.4.7 FSH assay

The plasma concentrations of FSH were measured in duplicate by RIA using the method described by McNeilly, McNeilly, Walton and Cunningham (1976). This assay used 0.1% BSA/PBS as buffer and the reference standard was NIAMDD FSH-14 (Bethesda, U. S.. A). The antiserum NIAMDD-Anti-$_o$FSH-1 (AFP-C5288113) (Bethesda, U. S. A.) was used at a final dilution of 1 : 48,000. The tracer was prepared by the Lactoperoxidase method as described for PRL assay.

The assay procedure involved taking 150 µl of sample to which was added 150 µl of buffer and 50 µl of first antibody. Then, the mixture was incubated at for a day at 4^{o}C. On the second day, 50 µl of tracer were added and the tubes were incubated overnight at 4^{o}C. On the third day, 200 µl of second antibody were added and the tubes were again incubated at 4^{o}C. On the fourth day, the tubes were spun and the supernatant was discarded while the precipitate was counted. The lower limit of detection of the assay was 4 ng/ml. All samples for FSH measurement were done in one assay.

3.5 STATISTICAL ANALYSIS

Results were tested for significance by analysis of variance (ANOVA) using the CLR ANOVA computer program (Clear Lake Research, Houston, USA), followed by Newman Keul's (NK) test or simple effects where appropriate. Further details of analyses are given under the respective experiments where necessary.

CHAPTER 4

CYCLES IN ß-END SECRETION: INFLUENCE OF SEASON, PHOTOPERIOD AND GONADAL STEROIDS

4.1 INTRODUCTION

A clearly defined cycle in the peripheral plasma concentrations of ß-END has recently been described in the Soay rams; the levels of ß-END are 10-to-20 times higher in late summer and autumn than in winter and spring. This cycle in ß-END secretion was also shown to be greatly influenced by changes in photoperiod; the levels of ß-END are high under short days and low under long days, showing a close correlation with the changes in the gonads and an inverse relationship with the levels of PRL (Ebling and Lincoln, 1987). The studies described in the first part of this chapter were undertaken: (i) to investigate in more detail the cycle in ß-END secretion in relation to body weight, reproduction and changes in the secretion of other hormones; (ii) to compare the amount of Nac-ß-END with that of total ß-END in the peripheral blood in order to determine the relative contribution by the anterior and intermediate lobes, based on the observation that Nac-ß-END is primarily secreted by the intermediate lobe (Antoni, 1986; Smith and Funder, 1988); and (iii) to establish whether the cycle in ß-END secretion seen in the Soay rams also applies to other breeds of sheep, for example the less seasonal Merino breed. The studies described in the second part of the chapter were carried out to complement the study of the cycle in ß-END secretion; for this, samples taken from other studies investigating the effect of photoperiod and melatonin on reproduction were used (Lincoln and Ebling, 1985; Lincoln, Libre and Merriam, 1989a).

4.2 EXPERIMENT 1- INFLUENCE OF SEASON IN SOAY RAMS LIVING OUTDOORS

4.2.1 Aims

The aims of this experiment were to confirm whether there is a clearly defined cycle in ß-END secretion related to season, and to determine the ratio of Nac-ß-END to total ß-END at three stages of the cycle. Also, to find out whether there is a cycle in ACTH and cortisol secretion paralleling the cycle in ß-END secretion.and to relate the cycle in ß-END secretion to other endocrine cycles, like those in PRL secretion and reproduction.

4.2.2 Materials and methods

A group of twelve adult Soay rams living outdoors in grass paddocks was used in this experiment. Blood samples were collected weekly and body weight and testis diameter were recorded for each ram fortnightly for a period of one year. Plasma was assayed for the measurement of ß-END, Nac-ß-END (measured in February, May and August), ACTH, cortisol, PRL, FSH and testosterone as previously described (chapter 3, section 3.4).

4.2.3 Results

The seasonal changes in the plasma concentrations of ß-END related to the seasonal changes in the plasma concentrations of ACTH, cortisol, PRL, and to changes in reproduction and body weight are illustrated in Fig. 4.1. There were highly significant ($P < 0.001$, ANOVA) changes with respect to time of the year in all the variables. The plasma concentrations of ß-END increased some 10-to-20-fold from spring to summer, with the seasonal peak occurring in August. At the peak of the seasonal cycle, the ratio of ß-END to Nac-ß-END was found to be 22 : 1 (Table 4.2). The plasma concentrations of

Fig. 4.1(a). Seasonal changes in the blood plasma concentrations of ß-endorphin, ACTH, corisol and prolactin in outdoor Soay rams. The values are means ± SEM, n=12. The broken line indicates the time of summer solstice.

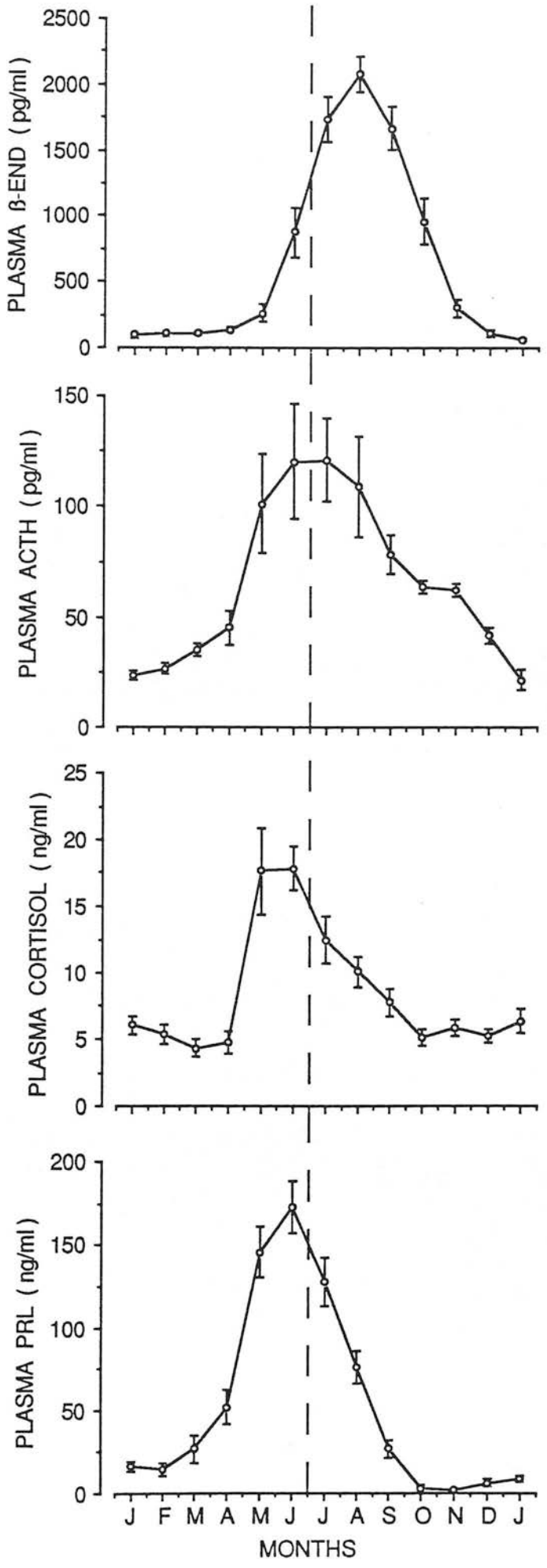

PLASMA β-END (pg/ml)
2500
2000
1500
1000
500
0
PLASMA ACTH (pg/ml)
150
100
50
0
PLASMA CORTISOL (ng/ml)
25
20
15
10
5
0
PLASMA PRL (ng/ml)
200
150
100
50
0
J F M A M J J A S O N D J
MONTHS

Fig. 4.1(b). Seasonal changes in the blood plasma concentrations of FSH and testosterone, diameter of the testes and body weight in outdoor Soay rams. The values are means ± SEM, n=12. The broken line indicates the time of summer solstice.

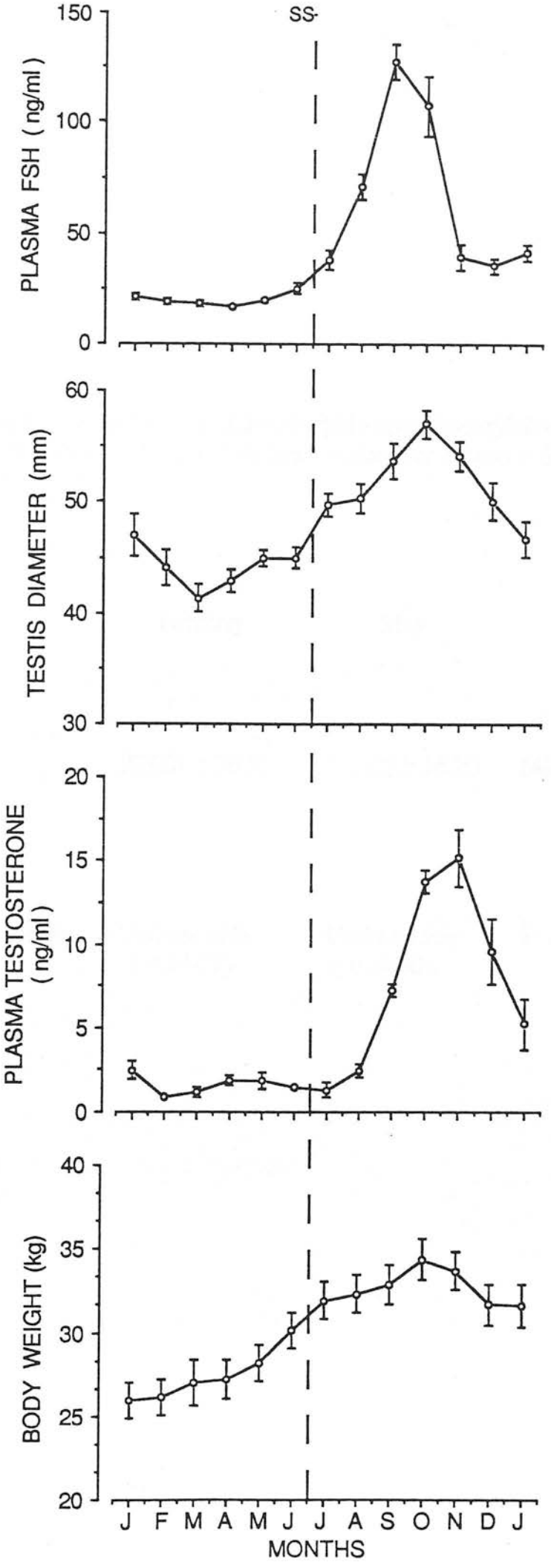

SS
PLASMA FSH (ng/ml)
150
100
50
0
TESTIS DIAMETER (mm)
60
50
40
30
PLASMA TESTOSTERONE (ng/ml)
20
15
10
5
0
BODY WEIGHT (kg)
40
35
30
25
20
J F M A M J J A S O N D J
MONTHS

Table 4.2. Plasma concentrations of ß-endorphin and N-acetyl-ß-endorphin in outdoor rams in February, May and August. Values are means ± SEM (pg/ml) n= 12 animals

	February	May	August
ß-endorphin	228.01 ± 26.85	313.05 ± 36.20	2499.90 ± 179.70
N-acetyl-ß-endorphin	Undetectable (< 24.40)	Undetectable (< 24.40)	111.65 ± 20.55

ACTH also increased markedly from spring to summer, reaching a seasonal peak in June and July some 1 to 2 months earlier than the peak in ß-END secretion. The plasma concentrations of cortisol increased abruptly in spring at a time coincident with the seasonal increase in the plasma concentrations of ACTH, and reached a peak in May and June. The plasma concentrations of PRL also increased from spring to summer, to reach a peak in June some 1 to 2 months earlier than the peak in ß-END secretion (Fig. 4.1(a)).

The plasma concentrations of FSH increased from summer to autumn and there were associated increases in testis diameter and the plasma concentrations of testosterone. The animals' overall body weight increased from spring to summer, with the highest weights occurring in autumn (Fig. 4.1(b)).

4.3 EXPERIMENT 2-INFLUENCE OF SEASON IN SOAY AND MERINO RAMS LIVING OUTDOORS

4.3.1 Aim

The aim of this experiment was to establish whether the cycle in ß-END secretion observed in the Soay breed also occurs in the Merino, which is a less seasonal breed in its reproduction, and to compare the two breeds with crossbred animals produced by mating a Merino ram with Soay ewes . The seasonal changes in PRL secretion were also monitored to provide a second example of a hormone influenced by season.

4.3.2 Materials and methods

This experiment involved a group of seven adult Merino rams and four adult Soay X Merino rams kept outdoors in grass paddocks. Blood samples were collected from each ram weekly for a period of one year, and

plasma was assayed for the measurement of ß-END and PRL as previously described (chapter 3, section 3.4). The results are compared with the data for purebred Soay rams described in experiment 1.

4.3.3 Results

The seasonal changes in the plasma concentrations of ß-END and PRL in the Soay, Merino and crossbred rams are illustrated in Fig. 4.3. Compared with the Soay rams, the Merino rams showed a poorly defined cycle in the plasma concentrations of ß-END (no significant changes in the plasma concentrations of ß-END with respect to time of the year), and the crossbred rams showed a pattern intermediate between that characteristic of the parents. In contrast, there was a clearly defined seasonal cycle in the plasma concentrations of PRL in the Merino rams, not significantly different in timing or amplitude from that in the Soay or crossbred rams(Fig. 4.3).

4.4 EXPERIMENT 3-INFLUENCE OF PINEALECTOMY IN SOAY RAMS LIVING OUTDOORS

4.4.1 Aim

The aim of the study was to establish whether the cycle in ß-END secretion in outdoor rams is modified by removal of the pineal gland.

4.4.2 Materials and methods

A group of eight adult Soay rams living outdoors in grass paddocks was studied for a period of one year. Five of these rams were PINX and the other three of similar age received a sham operation to act as controls. The surgical operation of pinealectomy was carried out by Dr. G. A. Lincoln following the method described by Roche, Karsch, Foster, Takagi and Dziuk

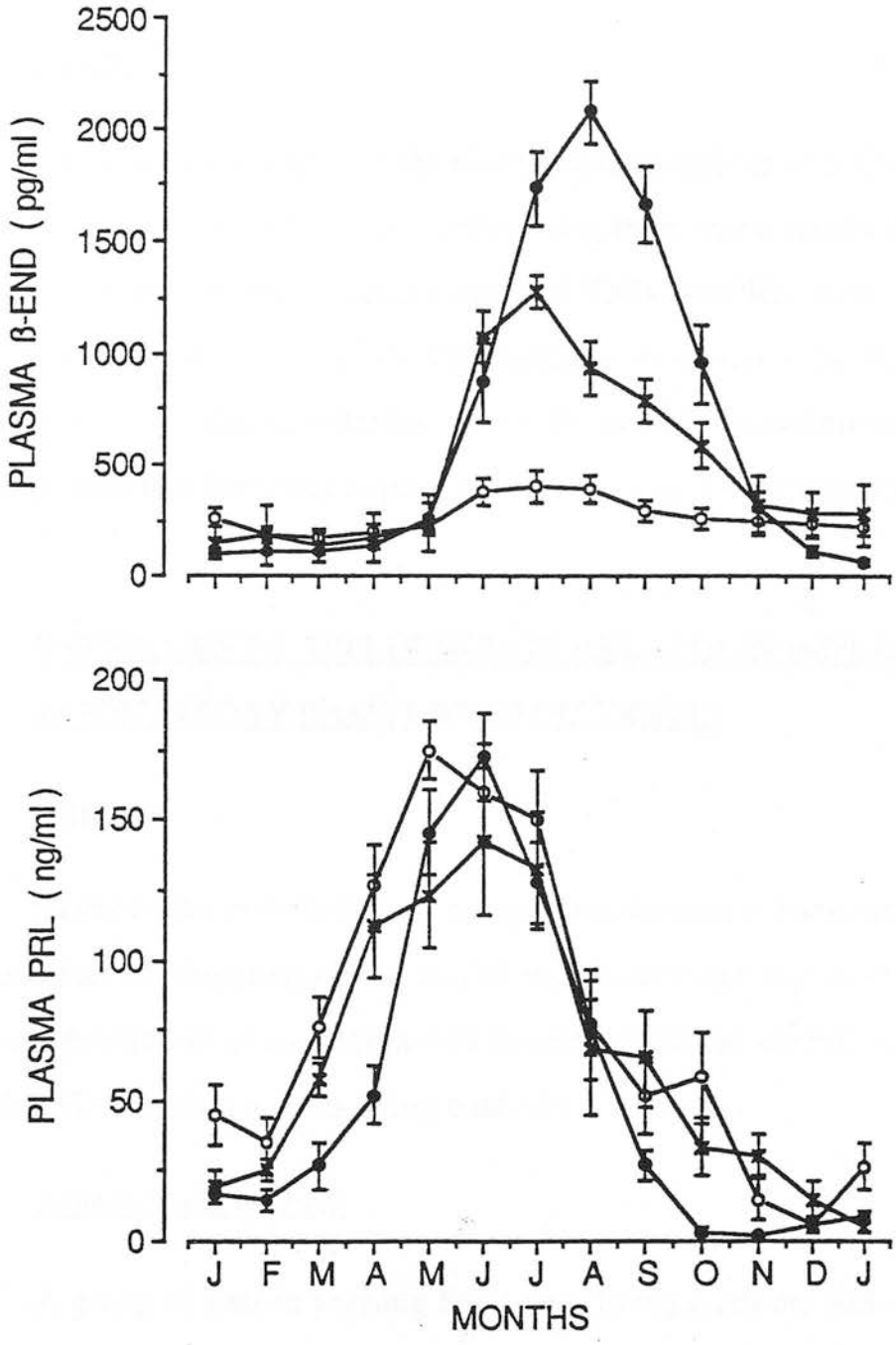


Fig. 4.3. Seasonal changes in the blood plasma concentrations of ß-endorphin and prolactin in outdoor Soay (●), Soay X Merino (X) and Merino (O) rams. The values are means ± SEM, n=12 Soay, 4 Soay X Merino and 7 Merino rams. The Soay X Merino rams were produced by mating a Merino ram with a group of Soay ewes.

(1970). Blood samples were collected from each ram fortnightly for a period of one year and plasma was assayed for the measurement of ß-END and PRL as previously described (chapter 3, section 3.4).

4.4.3 Results

The seasonal changes in the plasma concentrations of ß-END and PRL are illustrated in Fig. 4.4. In the control rams, there was a clearly defined seasonal cycle in the plasma concentrations of ß-END and PRL. In the PINX rams, the cycle in ß-END secretion was disrupted as revealed by ANOVA followed by simple effects, whereas the cycle in PRL secretion was not significantly different from that in the control rams (Fig. 4.4(a) and (b)).

4.5 EXPERIMENT 4- INFLUENCE OF MELATONIN IMPLANTATION IN SOAY RAMS LIVING OUTDOORS

4.5.1 Aim

Based on the knowledge that melatonin is the pineal hormone which relays the effect of photoperiod, the aim of this experiment was to establish whether administration of melatonin will interfere with the normal seasonal cycle in ß-END secretion in rams living outdoors

4.5.2 Materials and methods

A group of sixteen yearling Soay rams living outdoors was used in this experiment. Eight of these rams were treated for 10 weeks, starting in May (beginning of long days), with Silastic implants containing melatonin. The implants were prepared, according to the method of Lincoln & Ebling (1985), from Silastic sheeting (500-1 Dow Corning, Midland, MI, U. S. A) sealed into an envelope with a total surface area of 32-42 cm^2, containing 1-4 g melatonin

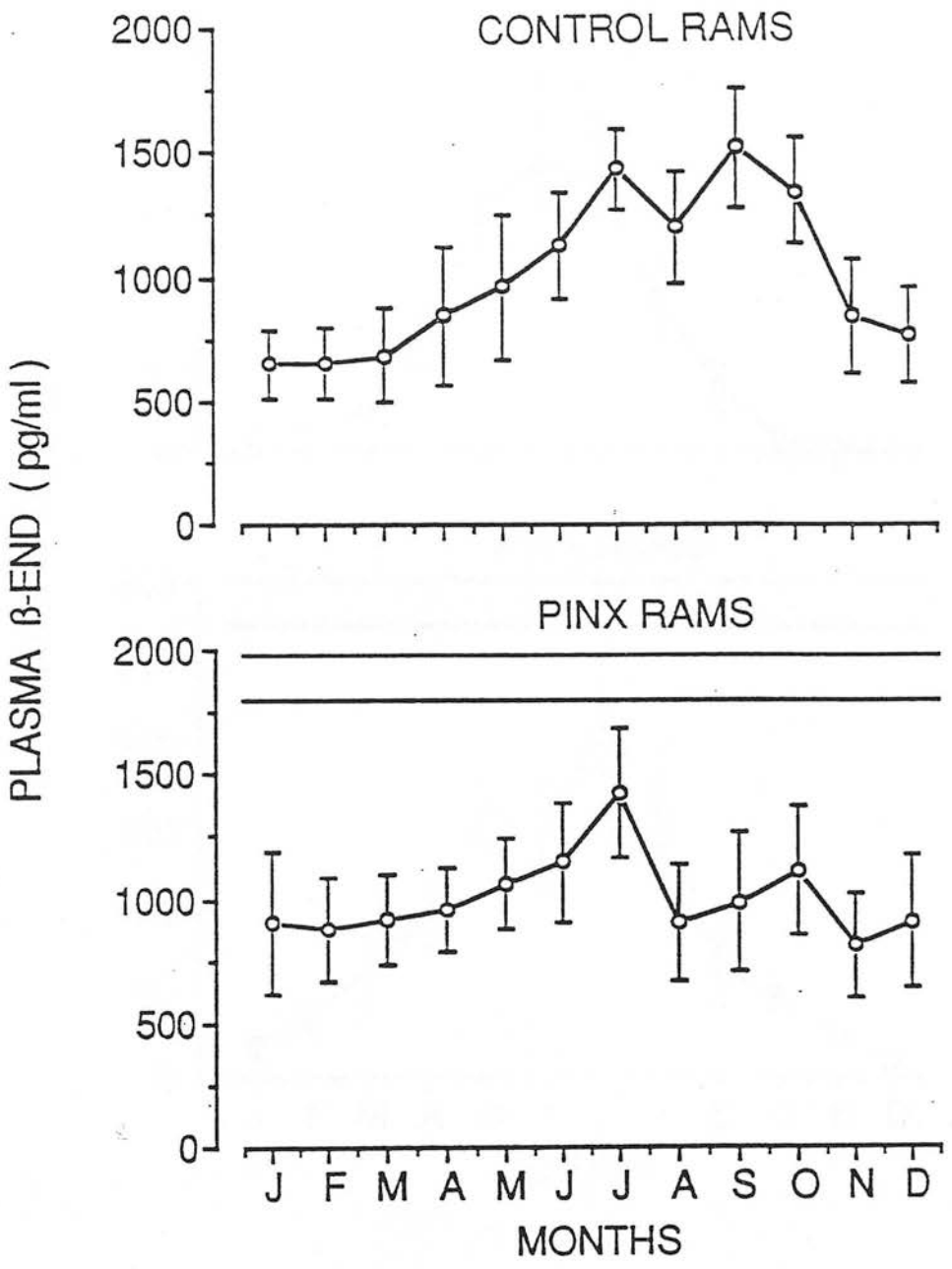


Fig. 4.4(a). Seasonal changes in the blood plasma concentrations of ß-endorphin in outdoor control and PINX rams. The values are means ± SEM, n=3 control and 5 PINX rams. The open bar indicates the period of pinealectomy operation.

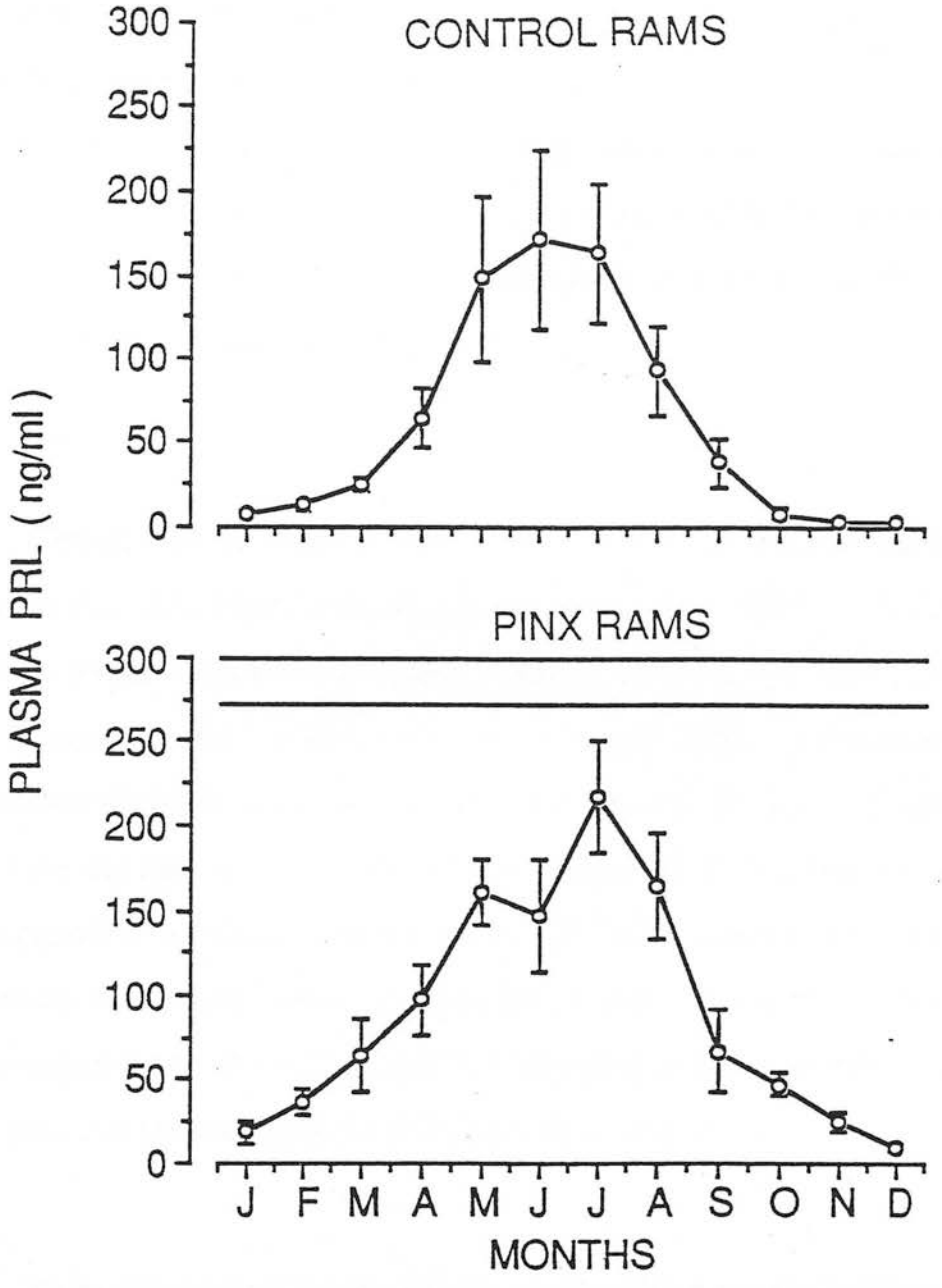


Fig. 4.4.(b). Seasonal changes in the blood plasma concentrations of prolactin in outdoor control and PINX rams. The values are means ± SEM, n=3 control and 5 PINX rams. The open bar indicates the period of pinealectomy.operation.

(Sigma Chemicals, Poole, Dorset, U. K), and were placed beneath the skin above the rib cage while the animals were locally anaesthetized with lignocaine. The implants maintained daytime melatonin concentrations of approximately 250 pg/ml compared with 34 pg/ml in untreated rams and night concentrations of 500 pg/ml compared with 200 pg/ml in control rams (Lincoln and Ebling, 1985). The other eight rams received empty implants to act as sham-operated controls. Blood samples were collected from each ram weekly for a period of 12 weeks and plasma assayed for the measurement of ß-END and PRL as previously described (chapter 3, section 3.4).

4.5.3 Results

Changes in the plasma concentrations of ß-END and PRL are illustrated in Fig. 4.5. There were highly significant ($P < 0.001$, ANOVA) changes with respect to time in the plasma concentrations of both hormones in the two groups. In the control rams, the seasonal peak in the plasma concentrations of ß-END was in August, while the peak in PRL was 1-2 months earlier in June and July, as seen in the previous experiments. Implantation with melatonin caused an acceleration in the increase of ß-END levels and a decrease in PRL levels. Within one week of implantation with melatonin the treated group was significantly ($P < 0.005$, ANOVA followed by Newman Keul's test) different from the control group for both hormones (Fig. 4.5).

4.6 EXPERIMENT 5- INFLUENCE OF PHOTOPERIOD IN SOAY RAMS LIVING INDOORS

4.6.1 Aim

To further investigate the role of photoperiod, a series of experiments were conducted on Soay rams living indoors. The aim of the first

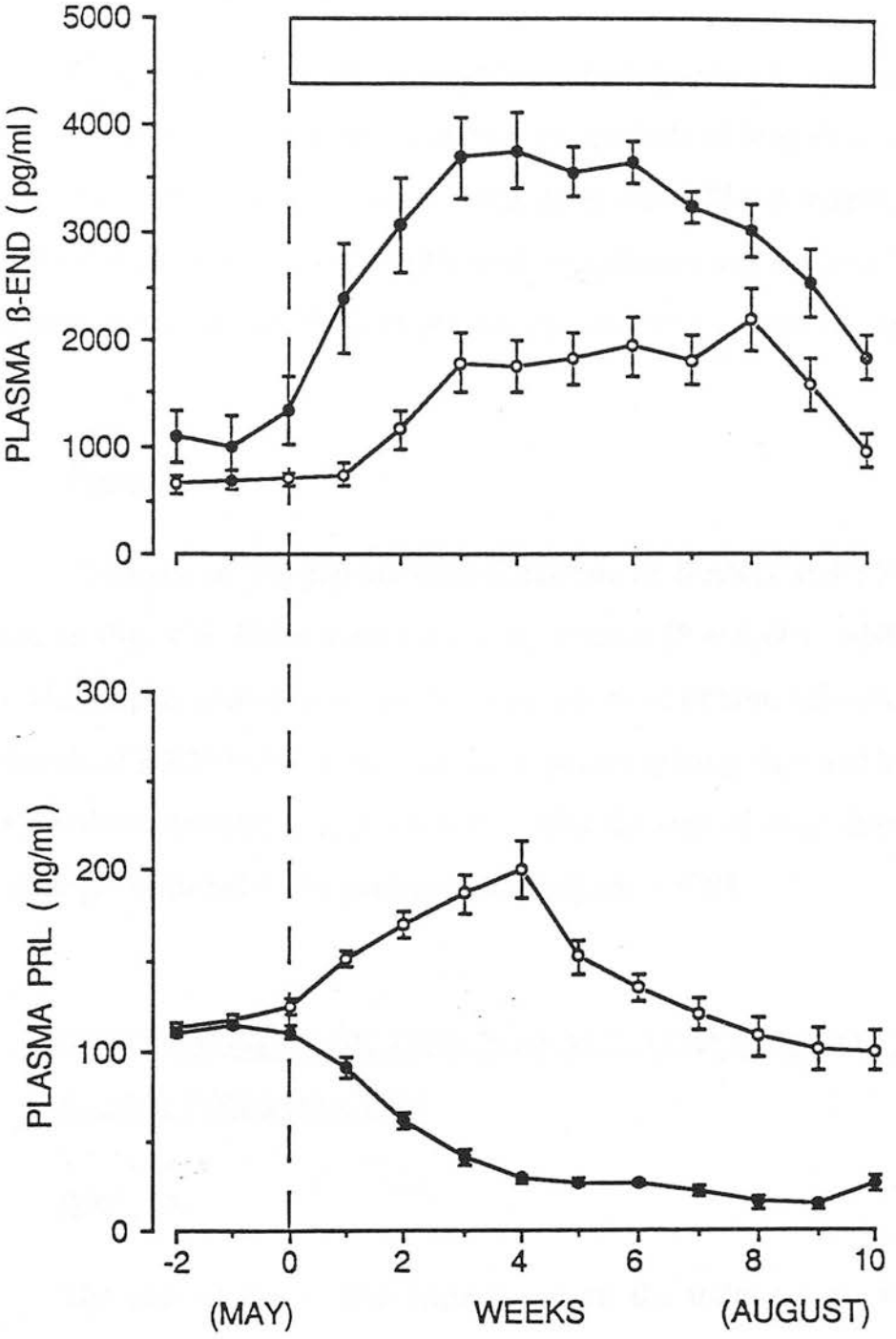


Fig. 4.5. Changes in the blood plasma concentrations of ß-endorphin and prolactin in outdoor control (O) and melatonin-implanted (●) Soay rams. The values are means ± SEM, n=8 control and 8 melatonin-implanted rams. The implants were introduced in May (week 0) and were left in place for 10 weeks. The rectangular box indicates the period of melatonin implantation.

experiment was to confirm the effect of exposing rams to an artificial lighting regimen of alternating 16-week periods of long days and short days on ß-END and PRL secretion.

4.6.2 Materials and methods

A group of seven adult Soay rams housed indoors and exposed to an artificial lighting regimen of alternating 16-week periods of long days (16L : 8D) and short days (8L : 16D) was used in this experiment. Blood samples were collected from each ram weekly for 39 weeks, and plasma was assayed for the measurement of ß-END and PRL as previously described (chapter 3, section 3.4).

4.6.3 Results

Changes in the plasma concentrations of ß-END and PRL are illustrated in Fig. 4.6. There were highly significant ($P < 0.001$, ANOVA) changes with respect to time in the plasma concentrations of both hormones; the plasma levels of ß-END were lowest during exposure to long days and highest during short days, reaching a peak 4-8 weeks after the start of short days. The reverse changes occurred in the plasma concentrations of PRL.

4.7 EXPERIMENT 6- INFLUENCE OF MELATONIN IN SOAY RAMS LIVING INDOORS

4.7.1 Aim

The aim of the second experiment on the indoor rams was to observe whether administration of melatonin will induce changes in the plasma concentrations of ß-END and PRL as observed in the outdoor rams, and to

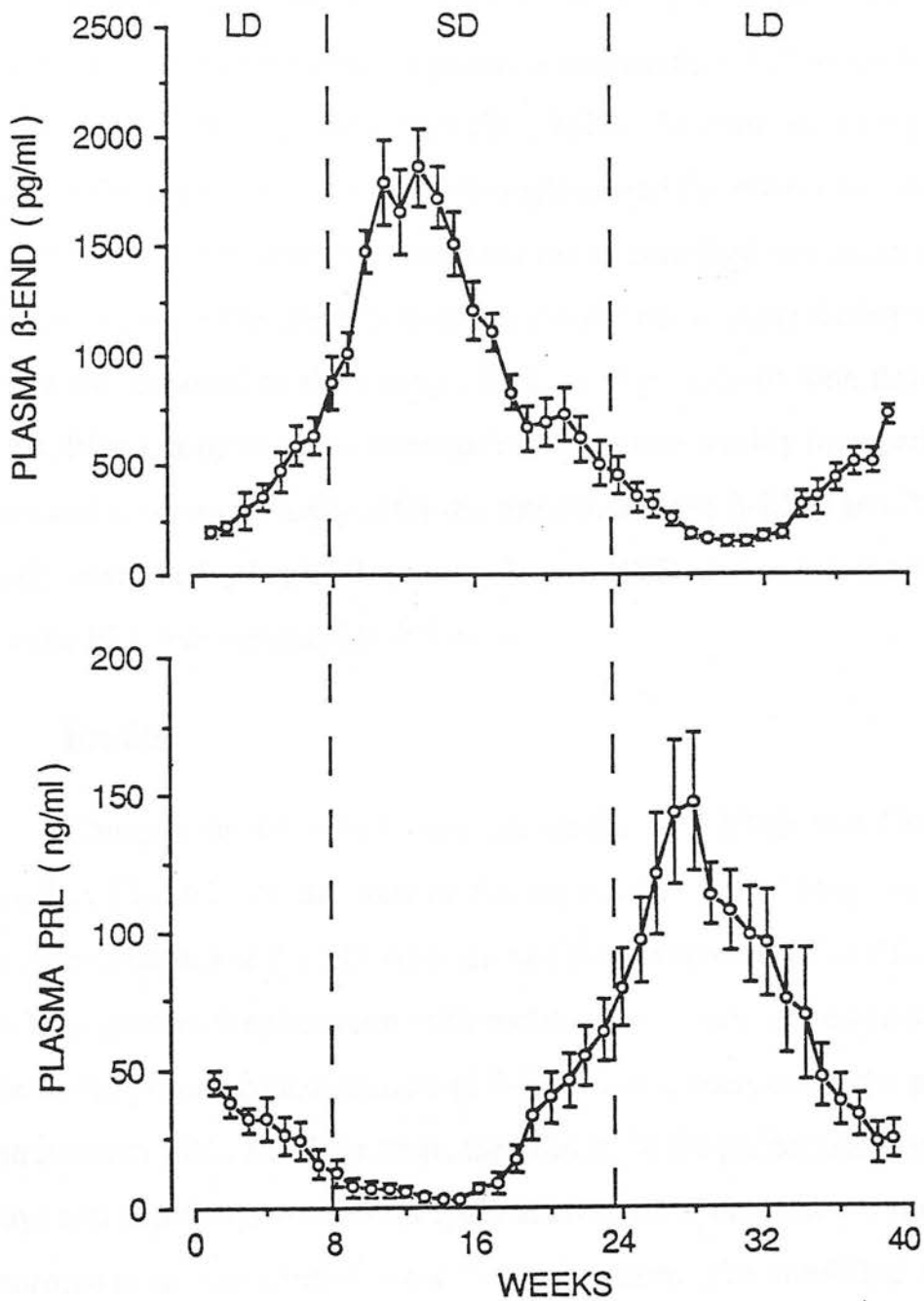


Fig. 4.6. Changes in the blood plasma concentrations of ß-endorphin and prolactin in indoor Soay rams exposed to alternating 16-week periods of long days (16h light : 8h darkness; 16L : 8D; LD) and short days (8L : 16D; SD) for 39 weeks. The values are means ± SEM, n=7 rams.

establish whether the long term treatment with melatonin blocks the photoperiodically induced cycles in ß-END and PRL secretion.

4.7.2 Materials and methods

A group of eight adult Soay rams housed indoors was pre-conditioned to an artificial lighting regimen of alternating 10-30-week periods of long days (16L : 8D) and short days (8L : 16D). At week 14 during long days, four of the rams received melatonin implants and the other four received empty implants to act as sham-operated controls as described in experiment 4. The implants remained in place throughout the period of study during which the rams were exposed to alternating 10-16 week periods of long days and short days. Blood samples were collected from each ram weekly for a period of 86 weeks and plasma was assayed for the measurement of ß-END and PRL as previously described (chapter 3, section 3.4). ß-END was measured for 86 weeks while PRL was measured for 67 weeks.

4.7.3 Results

Changes in the plasma concentrations of ß-END and PRL are illustrated in Fig. 4.7. At the start of the experiment under long days, the plasma concentrations of ß-END were low and the concentrations of PRL were high in both groups. Implantation with melatonin at week 14 resulted in an increase in the plasma concentrations of ß-END and a decrease in the plasma concentrations of PRL. At a later stage, the changes in the photoperiod between long days and short days resulted in cyclical changes in the concentrations of both hormones in the control rams but the pattern was modified in the melatonin-implanted rams. The plasma concentrations of ß-END in the melatonin-implanted rams remained relatively higher while the plasma concentrations of PRL remained relatively lower throughout the study and there was no correlation with alterations in photoperiod.

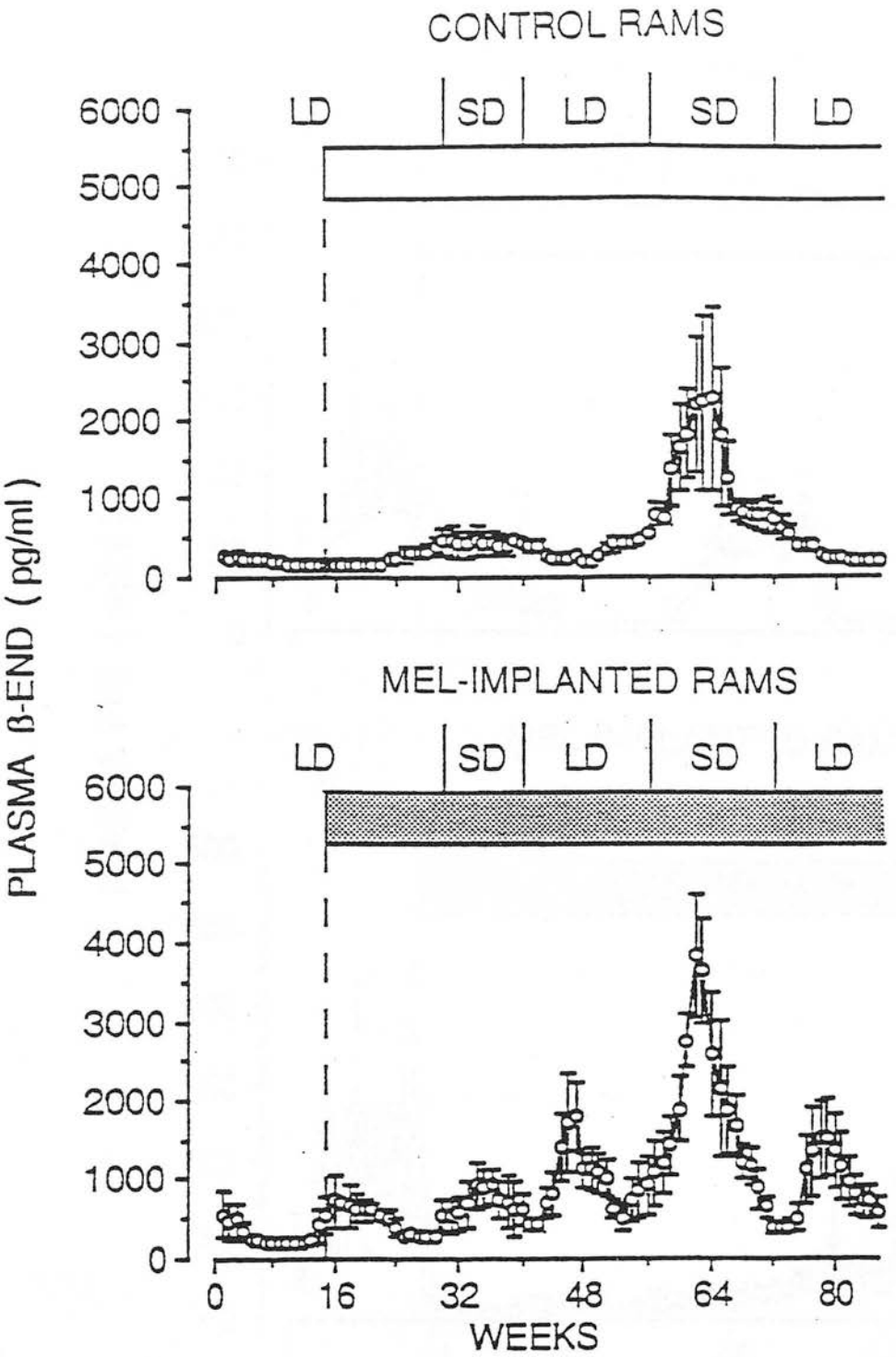


Fig. 4.7(a). Changes in the blood plasma concentrations of ß-endorphin in indoor control (upper panel) and melatonin-implanted (lower panel) Soay rams exposed to alternating 10-30 week periods of long days (16h light : 8h darkness; 16L : 8D; LD) and short days (8L : 16D; SD) for 86 weeks. The values are means ± SEM, n=4 control and 4 melatonin-implanted rams. The implants were introduced at week 14 during long days and left in place throughout the remainder of the study. The rectangular box indicates the period of implantation with empty implants (open) and with melatonin implants (dotted).

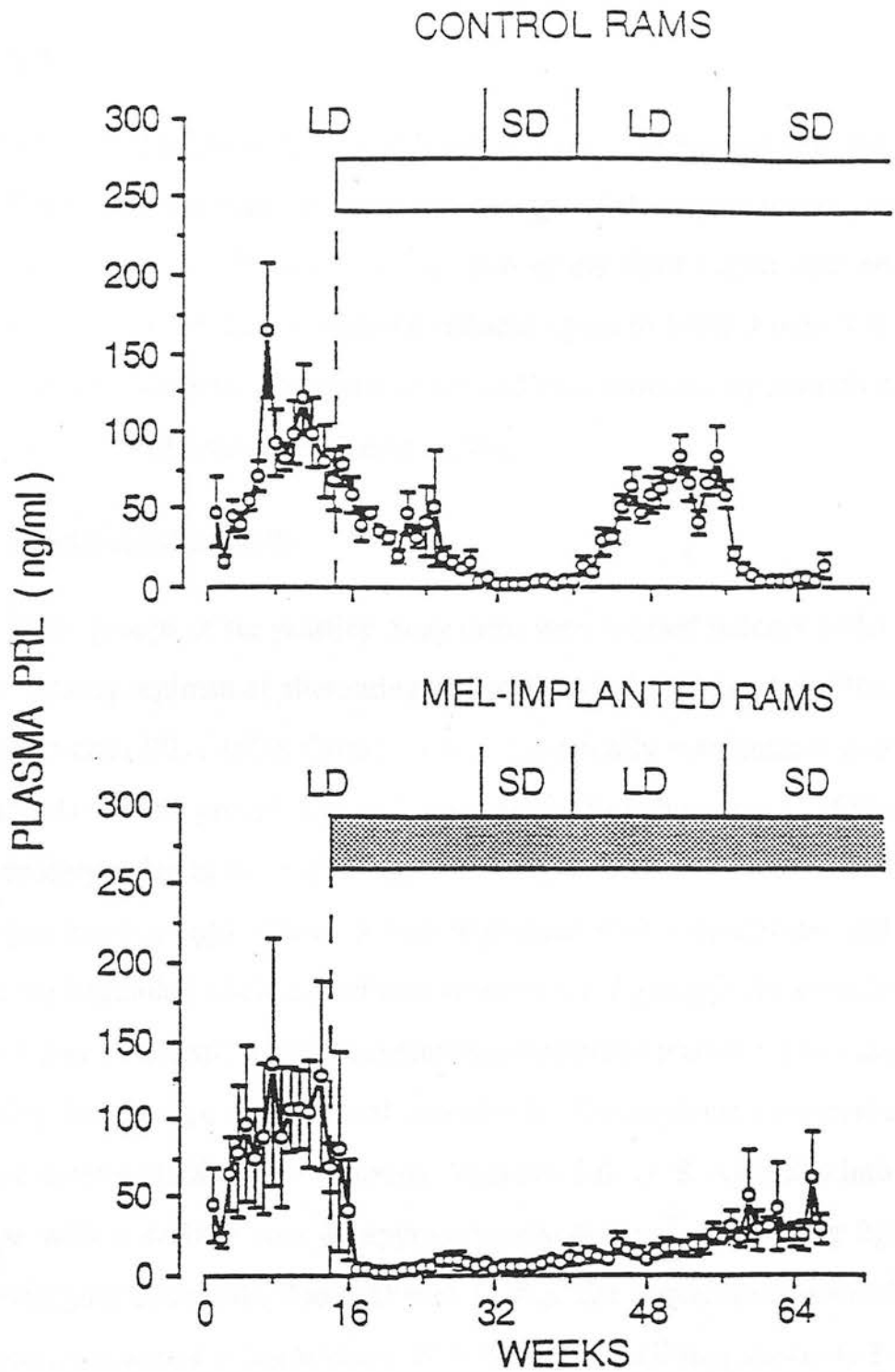


Fig. 4.7(b). Changes in the blood plasma concentrations of prolactin in indoor control (upper panel) and melatonin-implanted (lower panel) Soay rams exposed to alternating 10-30 week periods of long days (16h light : 8h darkness; 16L : 8D; LD) and short days (8L :16D; SD) for 67 weeks. The values are means ± SEM, n=4 control and 4 melatonin-implanted rams. The implants were introduced at week 14 during long days and left in place throughout the remainder of the study. The rectangular box indicates the period of implantation with empty implants (open) and with melatonin implants (dotted).

4.8 EXPERIMENT 7- INFLUENCE OF GONADAL STEROIDS IN SOAY RAMS LIVING INDOORS

4.8.1 Aim

Seasonal changes in the reproductive axis occur in parallel with the changes in ß-END secretion and it is possible that gonadal steroids induce or modify the pattern of ß-END secretion. The aim of the third experiment on indoor rams was to record the photoperiod-induced cycle in ß-END secretion in groups of rams in which the gonadal steroids had been removed by castration or held constant by implantation with testosterone.

4.8.2 Materials and methods

Three groups of six yearling Soay rams were housed indoors under an artificial lighting regimen of alternating 16-week periods of long days (16L : 8D) and short days (8L : 16D). Group1 was not surgically manipulated and acted as controls (intacts group). Group 2 was castrated by the removal of the testes and epididymides at the beginning of the experiment at 12 weeks into long days (castrates group). Group 3 was implanted with testosterone and castrated at the beginning of the experiment (castrates + T group). The rams in group 3 were given a Silastic implant containing testosterone placed beneath the skin overlying the rib cage using a local anaesthetic. The implants were made from Silastic sheeting (500-1 Dow Corning, Midland, MI, U. S. A) sealed into an envelope with a surface area of approximately 40 cm^2, containing 2g testosterone (Sigma Chemicals, Poole, Dorset, U. K.). The implants maintained circulating concentrations of testosterone of 6-10 ng/ml, declining slowly to 3-6 ng/ml from 50-96 weeks. Blood samples were collected weekly from each ram in all the three groups for a period of 96 weeks starting 8 weeks into long days; and plasma was assayed for the measurement of ß-END and PRL as previously described (chapter 3, section 3.4).

Fig. 4.8(a). Long-term changes in the blood plasma concentrations of ß-endorphin in indoor Soay rams exposed to alternating 16-week periods of long days (16h light : 8h darkness; 16L : 8D; LD) and short days (8L : 16D; SD) for 96 weeks. The values are means ± SEM, n=6 rams per group. (a) One group of six rams was not surgically manipulated (intacts); (b) another group of six rams was castrated at week 12 during long days (castrates); (c) the third group was castrated and then implanted with testosterone at week 12 during long days (castrates + T). C indicates the time of castration and C + T indicates the time of castration plus testosterone implantation.

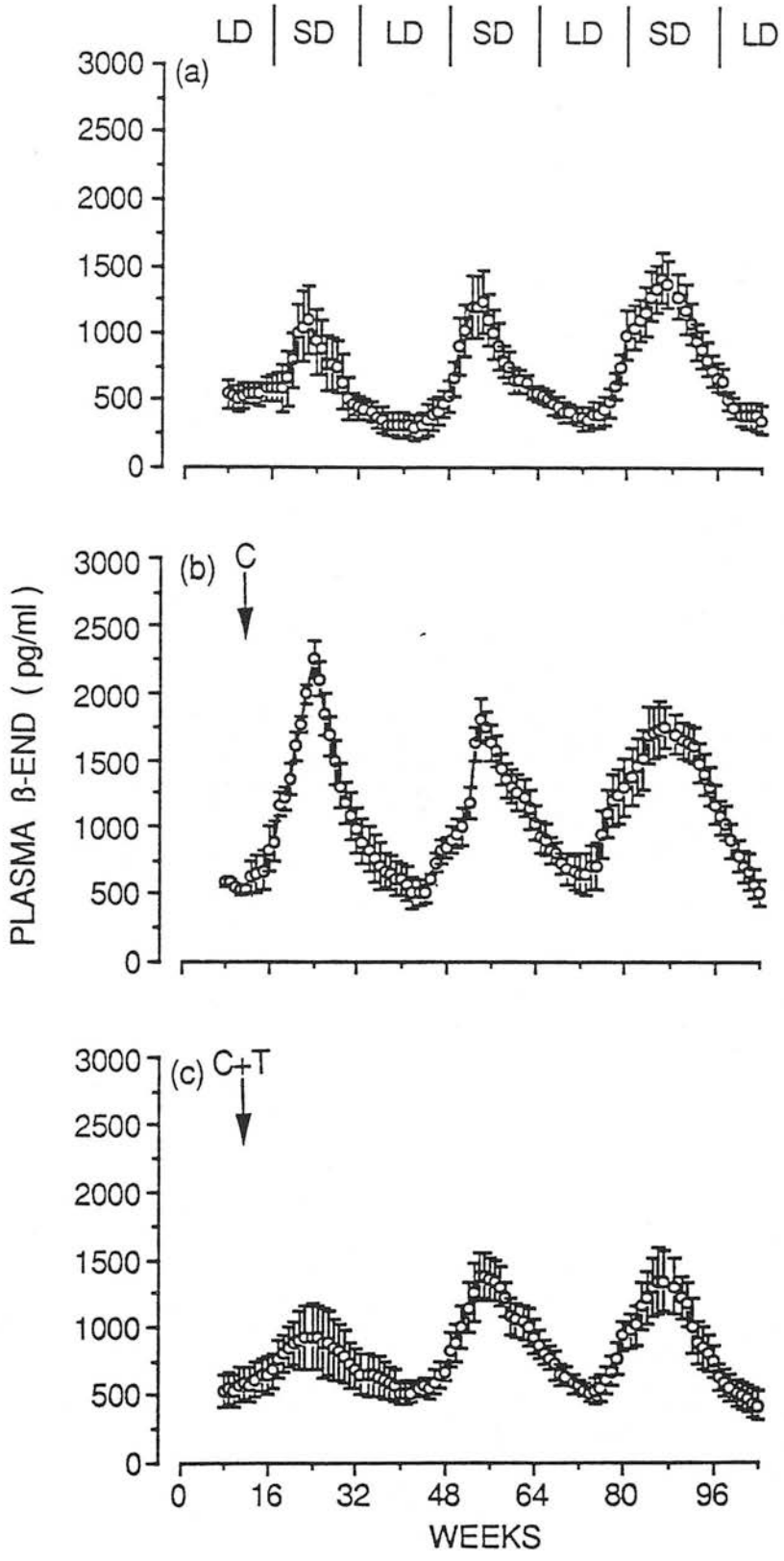
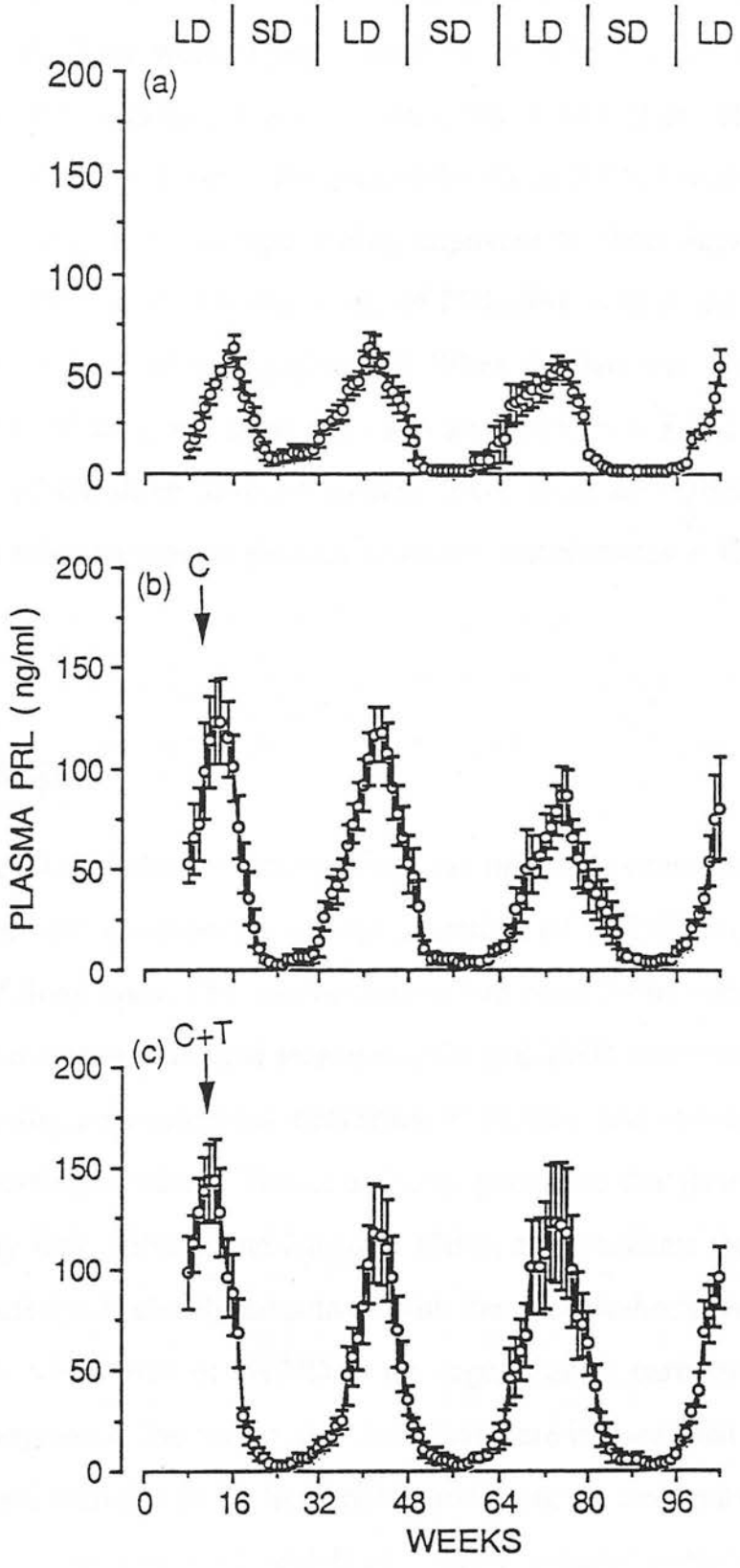

LD
SD
LD
SD
LD
SD
LD
(a)
(b)
C
(c)
C+T
PLASMA β-END (pg/ml)
3000
2500
2000
1500
1000
500
0
0
16
32
48
64
80
96
WEEKS

Fig. 4.8(b). Long-term changes in the blood plasma concentrations of prolactin in indoor Soay rams exposed to alternating 16-week periods of long days (16h light : 8h darkness; 16L : 8D; LD) and short days (8L : 16D; SD) for 96 weeks. The values are means ± SEM, n-6 per group. (a) One group of six rams was not surgically manipulated (intacts); (b) another group of six rams was castrated at week 12 during long days (castrates); (c) the third group was castrated and then implanted with testosterone at week 12 during long days (castrates + T). C indicates the time of castration and C + T indicates the time of castration plus testosterone implantation.


LD
SD
LD
SD
LD
SD
LD
(a)
200
150
100
50
0
(b)
C
(c)
C+T
PLASMA PRL (ng/ml)
0
16
32
48
64
80
96
WEEKS

4.8.3 Results

Changes in the plasma concentrations of ß-END and PRL are illustrated in Fig. 4.8. There were highly significant ($P < 0.001$, ANOVA) changes with respect to time in the plasma concentrations of ß-END and PRL in intact, castrate and castrate + T rams. The plasma levels of ß-END were low during exposure to long days and high during exposure to short days; the reverse changes occurred in the plasma levels of PRL (Fig. 4.8(a) and (b)), similar to the situation observed in experiment 5. When the data was divided into 16-week periods of long and short days and analysed by a 3 X 2 way ANOVA for each of the three 32-week cycles, there were no significant difference between the three groups (intacts, castrates and castrates + T; Fig. 4.8(a) and (b)).

4.9 DISCUSSION

The studies described in this chapter have primarily examined the influence of season and photoperiod on the secretion of ß-END into the peripheral blood of Soay rams. The results obtained on rams living outdoors illustrate that there is a clearly defined seasonal cycle in ß-END secretion with the highest circulating concentrations occurring in autumn and the lowest concentrations occurring in winter. This is a similar pattern to that described previously for Soay rams (Ebling and Lincoln, 1987), and confirms that the cycle in ß-END secretion is closely associated with the cycle in body weight, which is consistent with a role of ß-END in the regulation of carbohydrate metabolism and lipogenesis. The results also show that there is a seasonal cycle in ACTH and cortisol secretion in the Soay ram, paralleling the seasonal cycle in ß-END secretion (experiment 1), which indicates a parallel secretion of ACTH and ß-END from corticotrophs in the anterior lobe of the pituitary

gland. This close relationship between ACTH and ß-END secretion further confirms the role of ß-END in metabolism and fattening. In addition, a parallelism between the seasonal cycle in cortisol secretion, body weight and fat deposition has been observed in another seasonal species, the reindeer (Nilssen, Bye, Sundsford and Blix, 1985). If there is a close relationship between the seasonal cycle in ß-END, cortisol and body weight in the Soay ram, it is not unreasonable to further conclude that ß-END is involved in fat deposition in the ram.

The study comparing the seasonal cycle in ß-END secretion between the Soay and Merino breeds illustrates that Merino rams do not show a seasonal cycle in ß-END secretion, which reflects a lack of seasonality (experiment 2). This lack of seasonality is well known for the reproductive system and is likely to apply to other aspects of the animals' physiology. However, the observation that there was a seasonal cycle in PRL secretion indicates that the Merino breed is not entirely non-seasonal, which suggests that the genetic mechanisms responsible for the differences between the Soay and Merino breeds are specific to the reproductive axis and the corticotrophs/melanotrophs, but they do not seem to affect the lactotrophs. The crossbred rams, produced by mating Soay ewes with a Merino ram, had a seasonal cycle in ß-END secretion intermediate in amplitude between that characteristic of the parents, demonstrating the genetic basis for the differences between the Soay and Merino breeds (experiment 2).

The current results show clear evidence for the influence of photoperiod on ß-END secretion as previously described for the reproductive axis (Lincoln and Ebling, 1985). Also they show, for the first time, that treatment with melatonin affects the secretion of ß-END, which is consistent with the role of melatonin in relaying the effects of photoperiod on this endocrine system. Exposure to short days stimulates, and exposure to long days

inhibits, ß-END secretion, opposite to the effect on PRL secretion. Implantation with melatonin, initiated under long days, causes an increase in ß-END secretion both in outdoor (experiment 4) and in indoor (experiment 6) rams. This treatment, which results in continuous melatonin exposure, blocks the effect of long days and has the effect of mimicking the effect of short days in the long term. Melatonin implantation interferes with the photoperiodically-induced cycles in ß-END secretion, presumably because it obscures the endocrine signal produced by endogenous melatonin.

The timing of the seasonal cycle in outdoor rams relative to a change from long to short days is different from the timing of the long-term cycle seen in indoor rams. This is explained by the differences in the duration of exposure to long and short days in these two situations. In the outside environment, the rams are exposed to a natural light cycle of 52 weeks (26-week periods of alternating long and short days); in an indoor environment, the rams are exposed to an artificial light cycle of 32 weeks (16-week periods of alternating long and short days). As the animals remain exposed to the same photoperiod for a long time, they become refractory to the prevailing photoperiod. For example, prolonged exposure to long days causes a decrease in ß-END secretion but after 20 weeks the levels increase as the animals become refractory to the inhibitory long days (experiment 6). This also accounts for the rise in ß-END secretion seen in outdoor rams from May through the long days of June and July; a similar explanation can be invoked for the decline in ß-END secretion which occurs in September and November during short days (experiment 1).

The results also illustrate that, in the natural environment, photoperiod may not be the only environmental variable influencing the seasonal cycle in ß-END secretion. In the current study, pinealectomy disrupted the seasonal cycle in ß-END secretion but it did not render the

animals totally unresponsive to season (high plasma levels of ß-END in summer and low plasma levels in winter; experiment 3). The effect of pinealectomy has been studied in more detail in Soay rams for the control of reproduction and PRL secretion (Lincoln *et al.*,1989a). In these studies, the cycle in reproduction continued, although with a different timing, that in PRL secretion persisted after pinealectomy, indicating that these cycles were being driven by other non-photoperiodic environmental cues. For example, a change in temperature plays a major role in regulating the seasonal cycle in PRL secretion (Lincoln, 1989a), while a change to good quality nutrition reactivates testicular activity (Lincoln *et al.*, 1989a). Therefore, influence of changes in temperature and nutrition may account for the persistence of the seasonal changes in ß-END secretion after pinealectomy.

In the Soay ram, the seasonal changes in the reproductive axis occur in parallel with the seasonal changes in ß-END secretion (Ebling and Lincoln, 1987). However, there seems to be no consistent relationship between reproduction and ß-END secretion across seasonal species. For example, in the hamster, the reproductive axis is most active under long days when ß-END levels are low, and it is least active under short days when ß-END levels are high. In the current study, removal of testes or replacement with testosterone did not interfere with the photoperiod-driven cycle in ß-END secretion in indoor rams, which indicates that the control of ß-END secretion from the pituitary gland is independent of the reproductive axis (experiment 7).

4.10 SUMMARY

This first series of experiments has established the following:

1. There is a cycle in ß-END secretion in the Soay ram related to season and photoperiod.

2. There is also a seasonal cycle in the plasma concentrations of ACTH and cortisol secretion in the Soay ram.

3. There are genetic differences between breeds with regard to the seasonal cycle in ß-END secretion

4. Pinealectomy disrupts the seasonal cycle in ß-END secretion and treatment with melatonin modifies the photoperiodically-induced cycle in ß-END secretion, which is consistent with the role of melatonin from the pineal gland in mediating the effect of photoperiod on ß-END secretion.

5. Castration and testosterone replacement therapy does not influence the photoperiodically-induced cycle in ß-END secretion, indicating that the cycle in ß-END secretion is not dependent on the changes in the reproductive axis.

6. There is usually an inverse relationship between the cycle in ß-END and PRL secretion, which indicates that the two hormones may have independent control systems.

CHAPTER 5

CONTROL OF THE SEASONAL CYCLE IN ß-END SECRETION: ROLE OF AVP, CRF AND GLUCO-CORTICOIDS

5.1 INTRODUCTION

The ß-END in the peripheral blood is secreted primarily from the pituitary gland since this tissue contains much larger amounts of ß-END compared with other tissues in the body (Ebling and Lincoln, 1987; Ebling *et al.*, 1987; Smith and Funder, 1988). Both the corticotrophs and melanotrophs in the pituitary gland secrete ß-END (Antoni, 1986; Smith and Funder, 1988). The neural control of the secretory activity of the corticotrophs involves stimulation by hypothalamic AVP and CRF, and inhibition by adrenal glucocorticoids. In sheep, AVP and CRF have been measured in the pituitary portal blood system (Engler *et al.*, 1989b) and both these peptides have been shown to stimulate the secretion of ß-END, ACTH and cortisol in short-term studies (Donald *et al.*, 1983; Redekopp *et al.*, 1985b; Pradier *et al.*, 1986; Familari *et al.*, 1989).Also glucocorticoids have been shown to inhibit the secretion of both ß-END and ACTH (Engler *et al.*, 1989a). However, it is not clear whether these mechanisms, which involve a parallel change in adrenal axis, can account for the regulation of the seasonal cycle in ß-END secretion on a long-term basis.

The aims of the studies described in this chapter were to investigate the role of AVP, CRF and glucocorticoids in the control of the seasonal cycle in ß-END secretion in the ram and to examine more closely the relationship between ß-END, ACTH and cortisol secretion.

5.2 EXPERIMENT 8- DOSE RESPONSE TO AVP IN SUMMER AND WINTER

5.2.1 Aim

The aim of this experiment was to assess whether AVP would elicit a dose-dependent increase in the plasma concentrations of ß-END in the Soay ram and to compare the response at the peak and nadir of the seasonal cycle in ß-END secretion.

5.2.2 Materials and methods

The twelve outdoor rams used to study the seasonal cycle in ß-END secretion (experiment 1) were employed in this experiment. The rams were treated i.v with either saline or three doses of AVP (0.07, 0.33 and 1.67 µg/kg) in summer (July) and winter (December) of the first year of the study. The treatment design and bleeding protocol was as follows: each ram received saline or AVP at one of the doses on two consecutive days in a randomized design (six rams / treatment group). The low dose of AVP was selected on the basis of previous studies in sheep (Redekopp *et al.*, 1985). Blood samples were collected 20 min before and for 2h after the i.v. injection, starting at 13.40h (sampling times -20, -10, 0, 10, 20, 30, 40, 50, 60, 75, 90, 105 and 120 min relative to the injection). The treatments were carried out at the same time of the day in both summer and winter; this provided consistency although there is no clear evidence for a diurnal rhythm in the hypothalamic-pituitary-adrenal axis in the Soay ram (Lincoln, Almeida, Klandorf and Cunningham, 1982; Ebling and Lincoln, 1987). Plasma was assayed for the measurement of ß-END as previously described (chapter 3, section 3.4).

To assess the response to AVP the incremental changes were calculated for each animal using the mean pretreatment values (three samples

collected 0-20 min before the AVP injection) and subtracting this from the post-treatment values (ten samples collected from 10-120 min after the injection). A single mean value for the period 10-60 min was then calculated for each animal and the overall mean for the treatment group was determined to represent the mean incremental response. The statistical significance of the response was assessed by ANOVA (chapter 3, section 3.5).

5.2.3 Results

The incremental changes in the plasma concentrations of ß-END following treatment with saline, low, medium and high doses of AVP in summer (July, high endogenous ß-END concentrations) and winter (December, low endogenous ß-END concentration) are summarized in Table 5.1. In both seasons, AVP induced a dose-dependent increase in the plasma concentrations of ß-END. The slope of the response was greater in winter than in summer; in winter even the lowest dose of AVP (0.07 µg/kg) induced a significant increase in the plasma levels of ß-END.

There was a minor behavioural response following the injection of the medium and high doses of AVP. This involved the rams standing up and becoming slightly restless for 1 to 5 min after the injection. The behavioural response was more conspicuous in July than in December.

5.3 EXPERIMENT 9- EFFECT OF AVP AND CRF ON ß-END AND ACTH SECRETION AT THE FOUR SEASONS OF THE YEAR

5.3.1 Aim

The aim of this experiment was to investigate whether AVP and CRF given alone or in combination will induce an increase in ß-END and

Table 5.1. Changes in the plasma concentrations of ß-END in outdoor rams after receiving saline or three doses of arginine vasopressin in summer and winter. Values are means ± SEM (pg/ml), n=6 rams/treatment group.

	Plasma ß-endorphin (pg/ml)	
	Summer	Winter
Treatment		
Saline	186.20 ± 24.80	0.55 ± 9.60
AVP (0.07 µg/kg)	204.95 ± 47.70	38.40 ± 20.45*
AVP (0.33 µg/kg)	783.50 ± 250.20**	269.20 ± 140.65**
AVP (1.67 µg/kg)	2736.35 ± 758.00***	3816.60 ± 1471.45***

*P < 0.05, ** P < 0.01, *** P < 0.001 compared with the corresponding saline group, ANOVA followed by Newman Keul's test.

ACTH secretion and to measure the changes in response at different times of the year related to the seasonal cycle in ß-END secretion.

5.3.2 Materials and methods

The twelve outdoor rams used to study the seasonal cycle in ß-END and ACTH secretion (experiment 1) were employed in this experiment. The rams were treated with AVP and/or CRF in spring (April), summer (July), autumn (September) and winter (December) of the first year of the study. AVP was given i.v. at a dose of 0.33 µg/kg made up to 1 ml with physiological saline (dose selected from experiment 8) and CRF was given as an equimolar dose of 1.67 µg/kg. The treatment design and bleeding protocol was as in experiment 8. Plasma was assayed for the measurement of ß-END and ACTH as previously described (chapter 3, section 3.4). The incremental increases were calculated for the post-treatment period 10-60 min as described in experiment 8, and the significance of the change was assessed by ANOVA (chapter 3, section 3.5).

5.3.3 Results

The effects of AVP and CRF on the plasma concentrations of ß-END and ACTH in summer are illustrated in Fig. 5.2 and the incremental increases in hormone concentrations at the four seasons are summarized in Fig. 5.3. The i.v. injections of AVP and CRF given separately caused a significant ($P < 0.01$, ANOVA) increase in the plasma concentrations of ß-END; the levels were maximum 10 min after the injections and declined to pretreatment values within 60 min. The treatment with AVP plus CRF caused a significantly greater increase in the plasma concentrations of ß-END than when the drugs were given separately. There was also an increase in the plasma concentrations of ACTH after the i.v. injection of AVP alone or in combination with CRF but there was no significant response after the injection of CRF alone (Fig. 5.2). The ß-END and ACTH response to AVP and AVP plus CRF changed

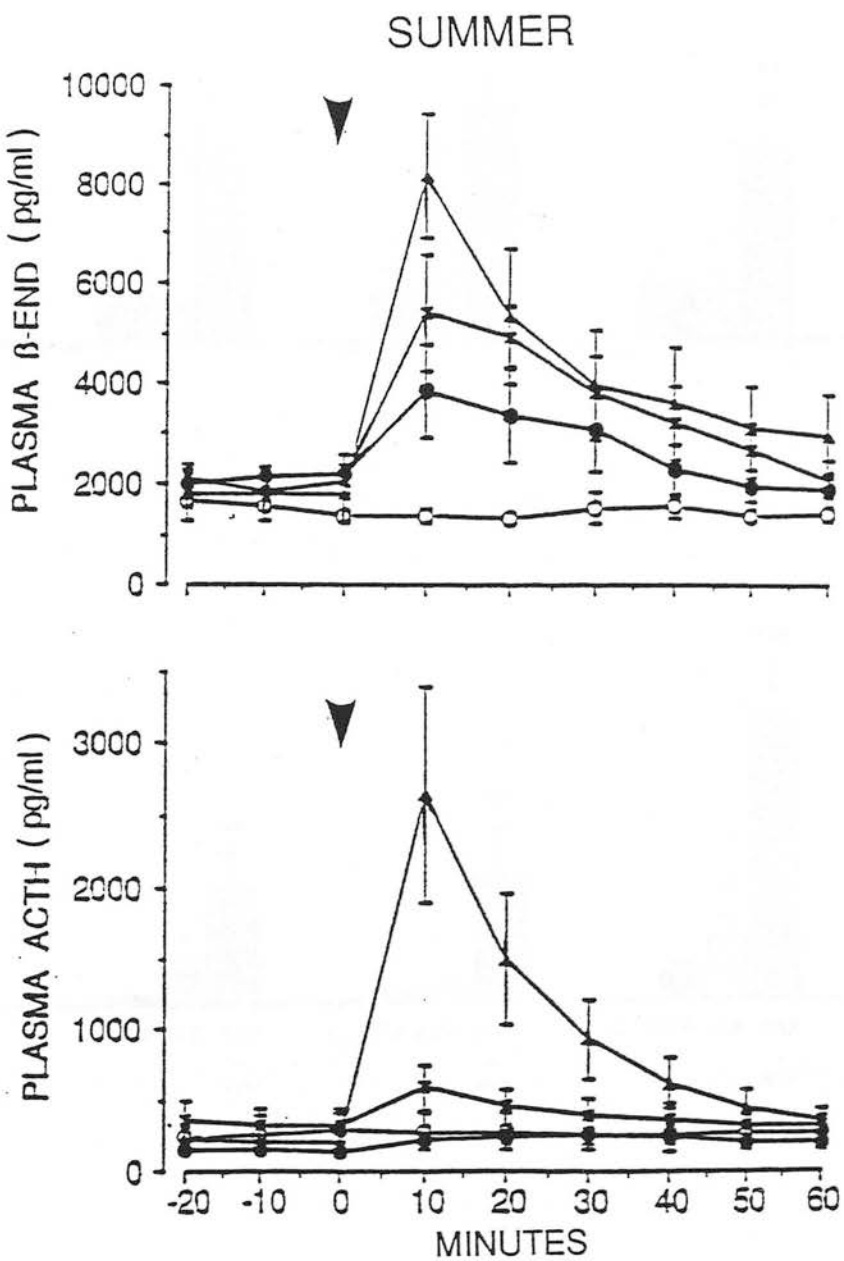


Fig. 5.2. Plasma concentrations of ß-endorphin and ACTH in outdoor Soay rams receiving a single bolus i.v. injection of either saline (○), 1.67 µg corticotropin-releasing factor (CRF)/kg (●), 0.33 µg arginine vasopressin (AVP)/kg (✕), or a combination of 1.67 µg CRF/kg and 0.33 µg AVP/kg (▲) in summer. Values are means ± SEM, n=6 rams per treatment group. The solid arrow indicates the point of drug administration at time 0.

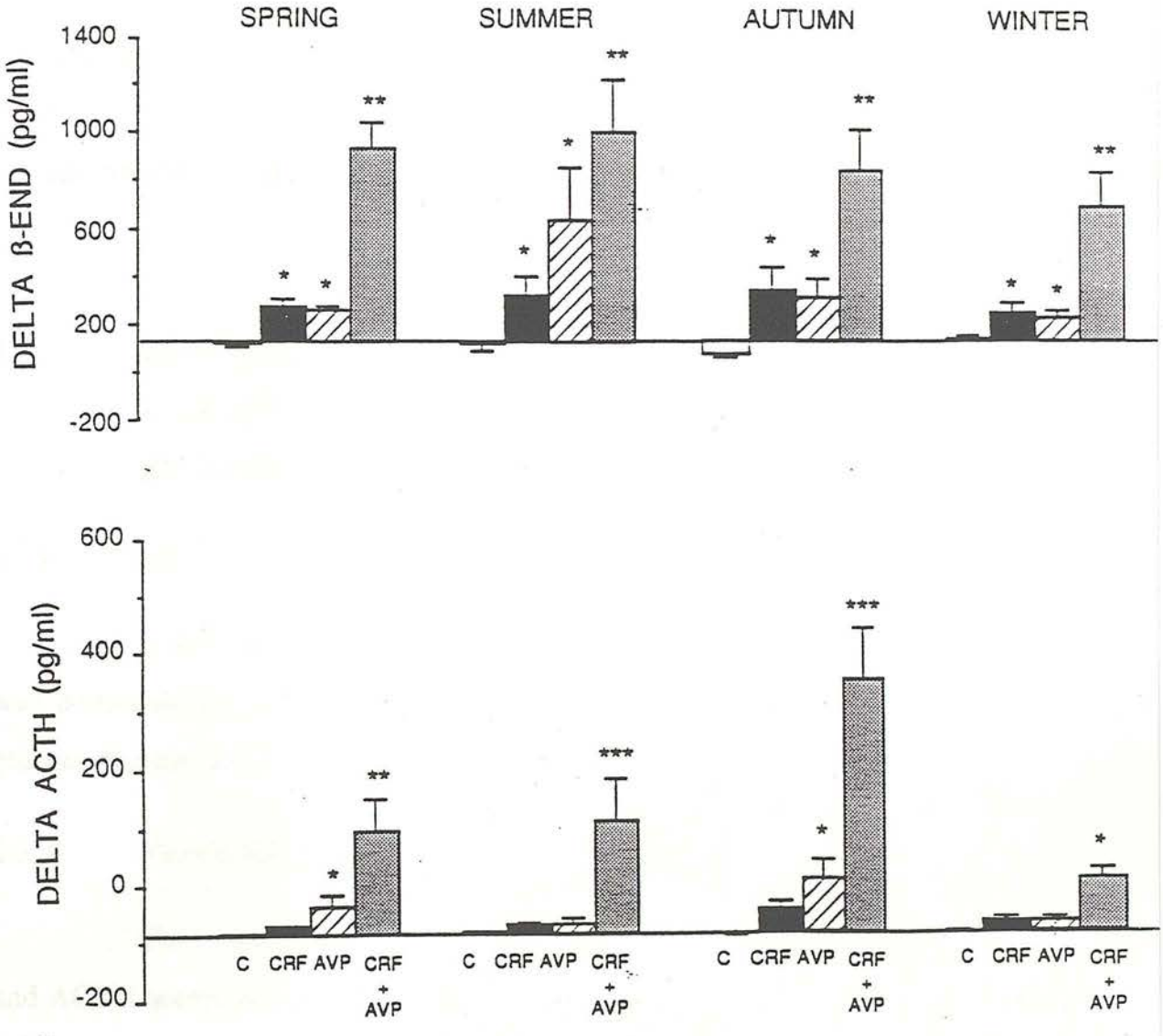


Fig. 5.3. Summary of the incremental changes in the plasma concentrations of ß-endorphin and ACTH in outdoor Soay rams after receiving a single bolus injection of either saline (C), 1.67 µg corticotrophin-releasing factor (CRF)/kg, 0.33 µg AVP/kg or a combination of 1.67 µg CRF/kg and 0.33 µg AVP/kg in spring, summer, autumn and winter. Values for each histogram are means ± SEM, n=6 rams per treatment group. Asterisks indicate significant effects of the treatments: * $P < 0.05$ compared with C; ** $P < 0.01$, *** $P < 0.005$ compared with CRF or AVP (Newman Keul's test following ANOVA).

significantly ($P < 0.01$, ANOVA) with season; the greatest response occurred in summer for ß-END and in autumn for ACTH. There was no significant seasonal change in the response to CRF given alone. At all seasons the synergistic effect of AVP plus CRF was clearly evident (Fig. 5.3).

The minor behavioural response of restlessness described in experiment 8 was observed following the injection of AVP and AVP plus CRF, but not following CRF alone. This response was more conspicuous in summer and autumn than in winter and spring.

5.4 EXPERIMENT 10- EFFECT OF DEXAMETHASONE ON ß-END AND ACTH SECRETION AT THE FOUR SEASONS OF THE YEAR

5.4.1 Aim

In this experiment, the synthetic glucocorticoid, dexamethasone, was administered at different times of the year to investigate the role of glucocorticoids in the inhibition of ß-END and ACTH secretion.

5.4.2 Materials and methods

The twelve outdoor rams used to study the seasonal cycle in ß-END and ACTH secretion (experiment 1) were employed in this experiment. Six of these rams were treated with dexamethasone in spring (April), summer (July), autumn (September) and winter (December) two weeks after each of the studies described in experiment 9. The other six rams received saline and acted as controls. The treatment design and bleeding protocol was as follows: dexamethasone was given i.v. at a dose of 5.67 µg/kg every 10 min for 2h (total dose 68.04 µg/kg). The dose of dexamethasone was selected on the basis of the

infusion rate of 1 mg/h used in man (Samra, Dechaud, Estour, Chalendar, Fevre-Montange, Pugeat & Tourniaire, 1985). Blood samples were collected at 10 min intervals for 2h before and 6h after the beginning of the dexamethasone treatments. Plasma was assayed for the measurement of ß-END and ACTH as previously described (chapter 3, section 3.4).

The incremental changes in the plasma concentrations of ß-END and ACTH were calculated for each animal using the mean pretreatment values (thirteen samples collected 0-2h before the injection of saline or dexamethasone) and subtracting this from the post-treatment values (thirty six samples collected 0-6h after the injection). A single mean value for the three periods, 0-2, 2-4 and 4-6h, was calculated for each animal and the overall mean for each 2h-period for the two treatments was determined. The statistical significance of the differences between the treatment groups was assessed by ANOVA (chapter3, section 3.5). In this experiment, the correlation between the short-term fluctuations in the plasma concentrations of ß-END and ACTH was also assessed using data for the 8h sampling period for the control rams in spring and summer during the seasonal period of increased secretion. For this, the peaks in plasma concentrations of ACTH were firstly defined as two consecutive high values, at least one of which exceeded the mean of the previous two baseline values by > 2 times the intra-assay C. V. The mean plasma concentrations of ACTH relative to all the defined peaks was then calculated for each animal and the corresponding ß-END values were also determined. A significant increase in the plasma concentrations of ß-END at a time coincident with the ACTH peak (ANOVA with Newman Keul's test) was used as evidence for a correlation between the fluctuations in the concentrations of the two hormones.

5.4.3 Results

The plasma ß-END and ACTH profiles for the 8h blood sampling period for representative rams treated with saline or dexamethasone in summer are illustrated in Fig. 5.4, and the overall results are summarized in Fig. 5.5. In the saline-treated rams the plasma concentrations of ß-END and ACTH fluctuated in a pulsatile manner but the mean levels for the group remained relatively constant throughout the 8h sampling period. The treatment with dexamethasone resulted in a slow decline in the concentrations of ß-END and ACTH, with the mean levels maximally suppressed at the end of the study, 6h after the initiation of drug treatment (Fig. 5.4). The suppressive effect of dexamethasone on the plasma concentrations of ß-END and ACTH changed significantly ($P < 0.001$, ANOVA; analysed for the last 2h of the study) with season, with the maximum responses for both hormones occurring in summer at the time of increased endogenous secretion (Fig. 5.5).

The temporal relationship in the short-term fluctuations in the plasma concentrations of ß-END and ACTH was analysed in the individual control rams in spring and summer, and the results for one ram in summer are illustrated in Fig. 5.6(a). In two of the six rams, in both spring and summer, the peaks in the plasma concentrations of ACTH were correlated with a significant ($P < 0.05$, ANOVA) simultaneous increase in the plasma concentrations of ß-END. This was also evident for the combined data for all animals (Fig. 5.6(b)). In no animal were all the ACTH peaks correlated with an increase in ß-END.

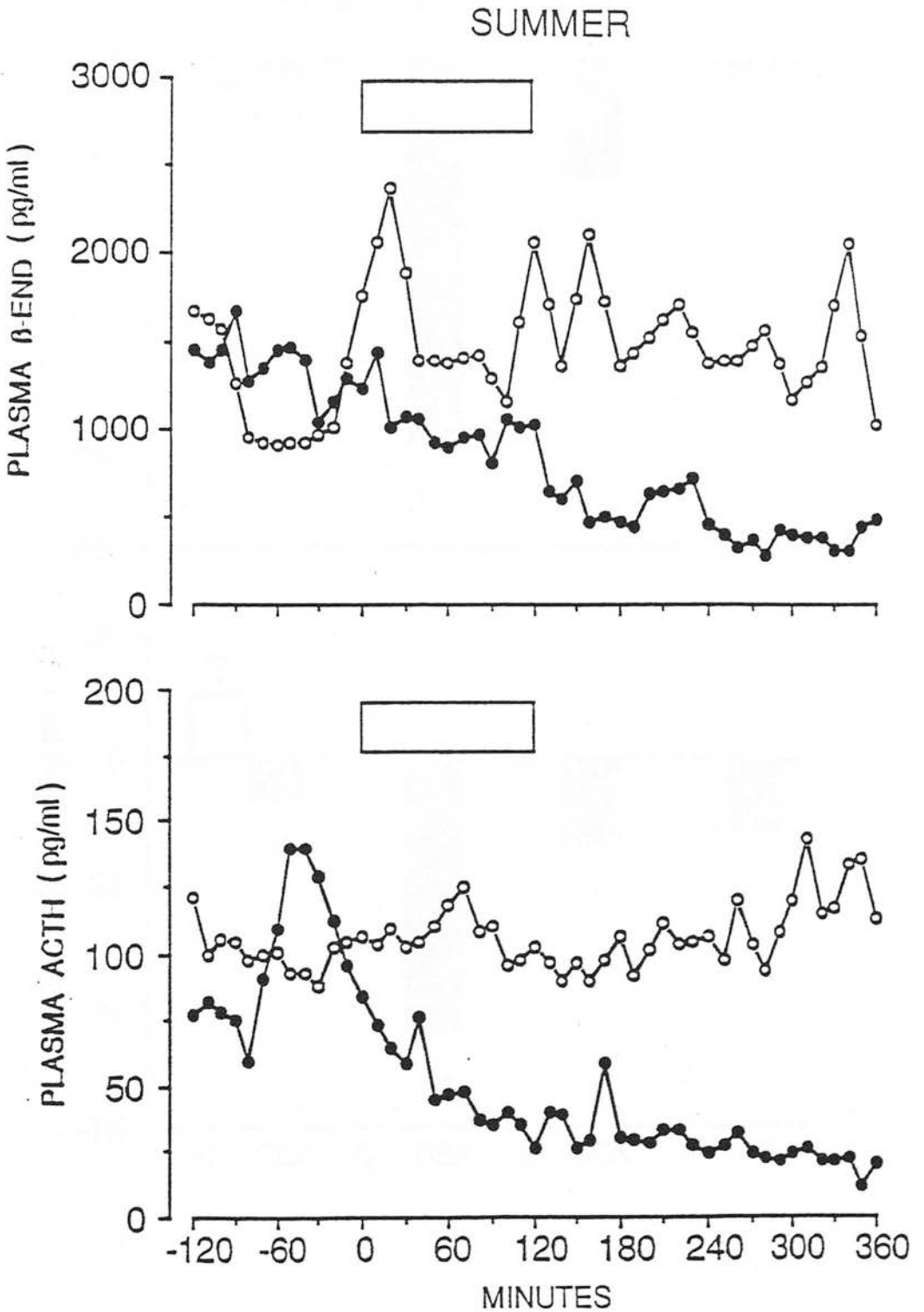


Fig. 5.4. Plasma (a) ß-endorphin and (b) ACTH profiles in a representative Soay ram treated with either saline (○) or dexamethasone (●) in summer. The open box indicates the period of dexamethasone administration given as i.v. injections of 5.67 µg/kg every 10 min for 2h (total dose of 68.0 µg/kg).

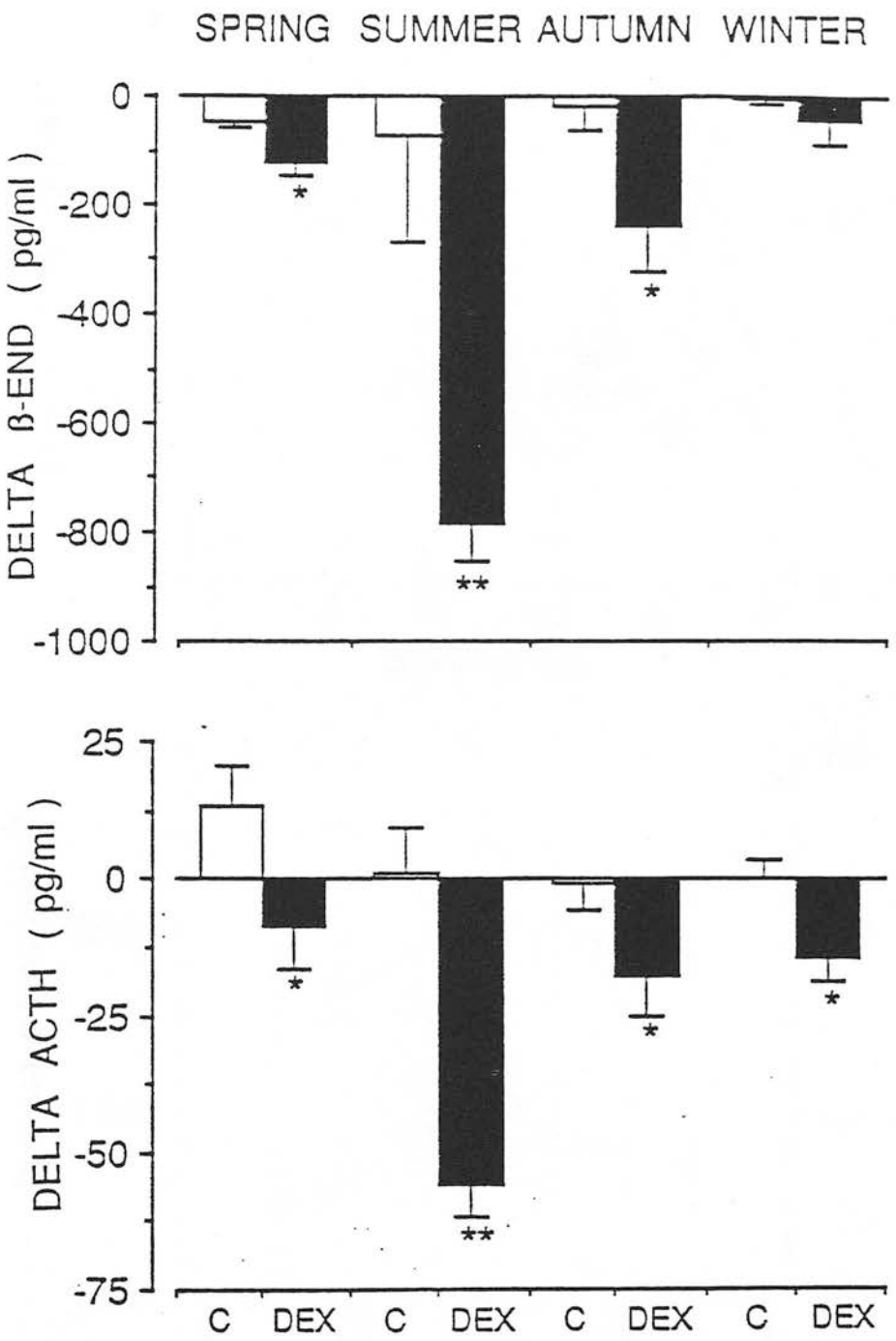


Fig. 5.5. Summary of the incremental changes in the plasma concentrations of ß-endorphin and ACTH in outdoor Soay rams after receiving an i.v. injection of saline (C) or 68.04 µg dexamethasone (DEX)/kg. Values are means ± SEM, n=6 rams per treatment group, calculated as the difference in hormone concentration between a 2h-period before treatment and the period between 4 and 6h after the start of administration of DEX. The asterisks indicate significant effects of the treatments: * $P < 0.05$, ** $P < 0.005$ compared with C (Newman Keul's test following ANOVA).

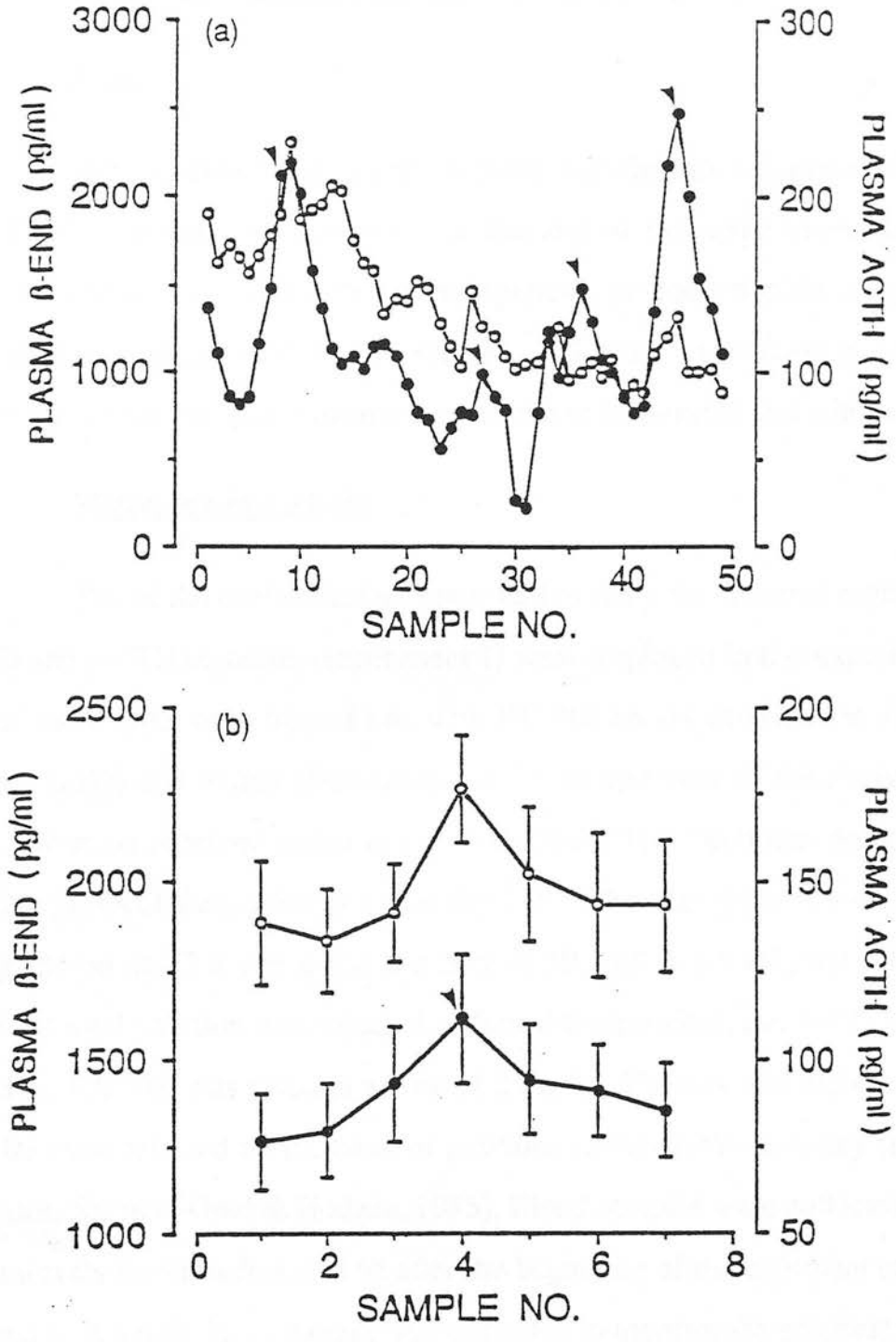


Fig. 5.6. (a) Temporal relationship between the peaks in plasma concentrations of ß-endorphin (○) and ACTH (●) in a representative Soay ram from the control group in summer. Each arrow depicts a significant ACTH peak as defined in the text. The values are based on blood samples collected at 10 min intervals for 8h. (b) Summary of the mean plasma concentration of ß-endorphin (○) and ACTH (●) related to the timing of all significant ACTH peaks for the data of all six control rams in summer.

5.5 EXPERIMENT 11- EFFECT OF RU 486 ON ß-END AND CORTISOL SECRETION IN SUMMER AND WINTER

5.5.1 Aim

Having shown that dexamethasone significantly inhibited ß-END and ACTH secretion in experiment 10, the aim of this experiment was to establish whether the blockade of endogenous glucocorticoids using the synthetic glucocorticoid antagonist, RU 486, will cause an increase in ß-END and cortisol secretion, and to measure the response in summer and winter.

5.5.2 Materials and methods

Ten of the twelve outdoor rams used to study the seasonal cycle in ß-END and ACTH secretion (experiment 1) were employed in this experiment. Five of these rams were treated i.m. with RU 486 on six consecutive days in summer (July) and winter (December) of the second year of the study. The other five rams received saline to act as controls. The treatment design and bleeding protocol was as follows: On day 1, RU 486 was given at a dose of 1 mg/kg and on day 2 it was given at a dose of 10 mg/kg in 8 ml total solution. The 8 ml total solution was injected at four different sites, i.e. 2 ml/site. On days 3-6, RU 486 was given at a dose of 2 mg/kg. The low and high doses of RU 486 were selected on the basis of previous studies in the monkey (Healy, Chrousos, Schulte, Gold & Hodgen, 1985). Blood samples were collected at 10 min intervals for 2h before and 6h after the beginning of the treatment on days 1, 2 and 6. A single blood sample was collected to monitor the progress of the treatment on days 3-5. Plasma was assayed for the measurement of ß-END and cortisol (indirect measure for ACTH) as previously described (chapter 3, section 3.4).

The mean plasma concentrations of ß-END and cortisol were calculated for each day based on the the 6h post-treament values (thirty six samples collected after the injection on days 1, 2 and 6), and the significance of the effect of RU 486 was assessed by ANOVA (chapter 3, section 3.5).

5.5.3 Results

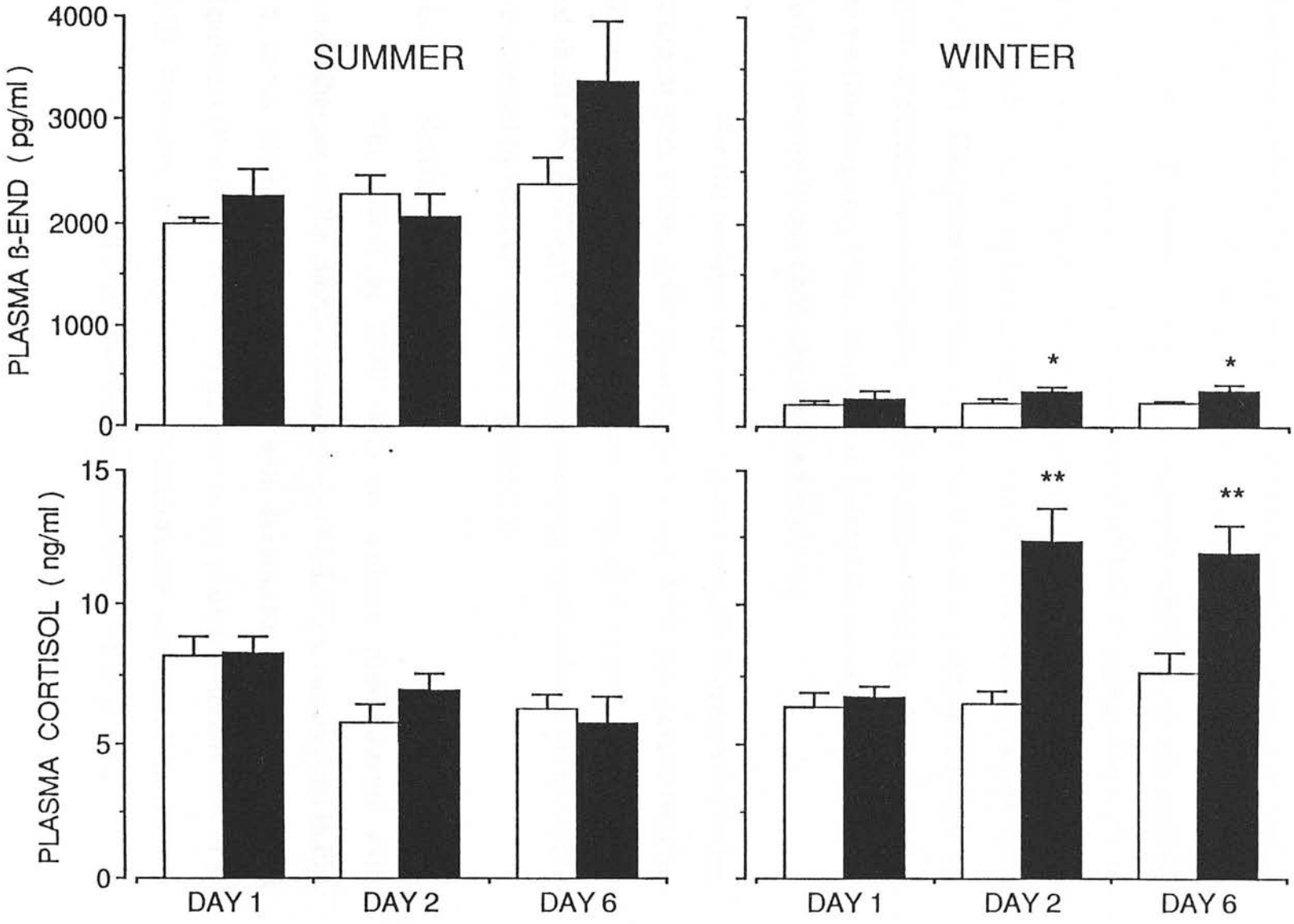
The effect of RU 486 on the mean plasma concentrations of ß-END and cortisol on days 1, 2 and 6 when serial blood samples were taken in summer amd winter is summarized in Fig. 5.7. In summer, the i.m. injection of RU 486 at all doses used did not affect the mean plasma concentrations of ß-END and cortisol. Although there was a tendency for the plasma levels of ß-END in the treated group to increase above the levels in the control group on day 6, this increase was not statistically significant. The single daily samples collected on days 3-5 also failed to show a significant effect of the treatment (results not shown). In winter, the i.m. injection of RU 486 did not affect the mean plasma concentrations of ß-END and cortisol on day 1. However, the treatment significantly ($P < 0.05$ for ß-END; $P < 0.001$ for cortisol, ANOVA) increased the plasma levels of both hormones on days 2 and 6. There was no difference in hormone response between the acute treatment (day 2) and the chronic treatment (day 6).

5.6 EXPERIMENT 12- STUDY OF THE SITE OF GLUCO-CORTICOIDS ACTION

5.6.1 Aim

The aim of this experiment was to assess whether glucocorticoids may act at the level of the pituitary gland to inhibit ß-END and ACTH secretion.

Fig. 5.7. Summary of the effects of saline (□) and RU 486 (■) on the plasma concentrations of ß-endorphin and cortisol in outdoor Soay rams in summer and winter. On day 1, the rams received i..m. injections of 2 mg RU 486/kg; on day 2, they received i.m. injections of 10 mg RU 486.kg. Then, they received daily i.m. injections of 2 mg RU 486/kg up to day 6. Values are means ± SEM, n=5 rams per group. The asterisks indicate the significant effects of RU 486 treratment: * $P < 0.05$, ** $P < 0.001$ compared with C (Newman Keul's test following ANOVA).


SUMMER
WINTER
PLASMA ß-END (pg/ml)
4000
3000
2000
1000
0
*
*
PLASMA CORTISOL (ng/ml)
15
10
5
0
**
**
DAY 1
DAY 2
DAY 6
DAY 1
DAY 2
DAY 6

5.6.2 Materials and methods

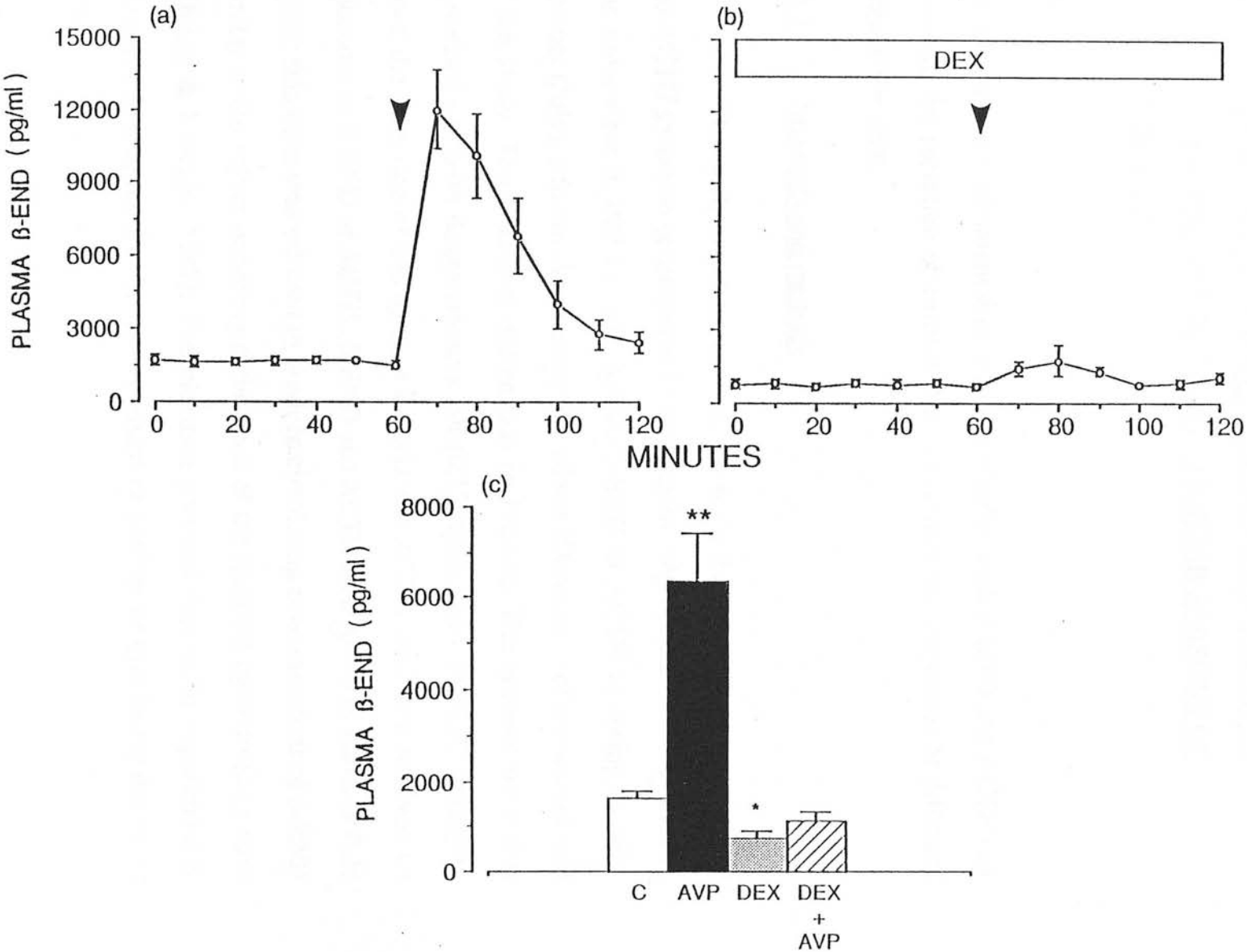
A group of five indoor rams was used in this experiment. The rams were treated with AVP with or without pretreatment with dexamethasone while maintained under short days (8 weeks after the change from long days to short days, equivalent to autumn in the outdoor animals). The treatment design and bleeding protocol was as follows: on day 1 the animals received no pretreatment with dexamethasone and were blood sampled at 10 min intervals for 1h before and 1h after the i.v. injection of AVP (1.07 μg/kg). On day 2 the animals were pretreated with dexamethasone 5h (0.13 μg/kg) and 1h (0.03 μg/kg) before receiving the i.v. injection of AVP and blood samples were taken as on day 1. The pretreatment dose of dexamethasone was selected based on the ability of dexamethasone to block ACTH secretion from the pituitary gland in the rat (Buckingham, 1987). Plasma was assayed for the measurement of ß-END as previously described (chapter 3, section 3.4).

For the analysis, the mean values for each 1h-period following treatment with saline, AVP, dexamethasone and AVP plus dexamethasone, were calculated based on 10-min samples collected for a period of 1h before and 1h after the AVP injections, and the statistical significance of the treatments was assessed by ANOVA (chapter 3, section 3.5).

5.6.3 Results

The effect of AVP with or without pretreatment with dexamethasone on the plasma concentrations of ß-END is summarized in Fig. 5.8. In the absence of pretreatment with dexamethasone, AVP caused a significant ($P < 0.001$, ANOVA) increase in the plasma concentrations of ß-END. However, pretreatment with dexamethasone suppressed the plasma concentrations of ß-END and blocked the response to AVP (Fig. 5.8).

Fig. 5.8. Plasma concentrations of ß-endorphin in indoor Soay rams (a) treated with a single bolus i.v. injection of 1 µg arginine vasopressin (AVP)/kg; (b) pretreated with i.v. injection of dexamethasone (DEX) (0.13 µg/kg 6h before and 0.03 µg/kg 1h before) and then treated with a single bolus i.v. injection of 1 µg AVP/kg ;(c) Summary of the effects of saline (C), AVP, DEX and DEX plus AVP. Values are means ± SEM, n=5 rams.The asterisks indicate significant effects of the treatments: * $P < 0.05$, ** $P < 0.005$ compared with C (Newman Keul's test following ANOVA). The arrow indicates the point of AVP administration.


(a)
(b)
DEX
PLASMA ß-END (pg/ml)
15000
12000
9000
6000
3000
0
0
20
40
60
80
100
120
MINUTES
(c)
8000
6000
4000
2000
0
**
*
C
AVP
DEX
DEX + AVP

The minor behavioural response of restlessness described in experiment 8 was observed following the injection of AVP, both with and without pretreatment with dexamethasone.

5.7 EXPERIMENT 13- EFFECT OF ß-END AND ACTH ON CORTISOL SECRETION AT THE FOUR SEASONS OF THE YEAR

5.7.1 Aim

The aim of this experiment was to assess whether both ß-END and ACTH can stimulate the secretion of cortisol and to compare the responses at different times of the year.

5.7.2 Materials and methods

The twelve outdoor rams used to study the seasonal cycle in ß-END and ACTH secretion (experiment 1) were again employed in this experiment. The rams were treated i.v. with saline, ß-END or ACTH in spring (April), summer (July), autumn (September) and winter (December) of the second year of the study. The treatment design was as follows: The animals were first pretreated i.v. with dexamethasone 5h (0.13 µg/kg) and 1h (0.03 µg/kg) to block the secretion of endogenous ß-END and ACTH, and then received i.v injections of ß-END or ACTH . ß-END and ACTH were given at a dose of 0.33 µg/kg; this dose was selected to produce circulating concentrations of ß-END similar to the values occurring at the peak of the seasonal cycle in Soay rams (Ebling & Lincoln, 1987). The bleeding protocol was as in experiment 8. Plasma was assayed for the measurement of cortisol as previously described (chapter 3, section 3.4).

Mean incremental responses were calculated as before (experiment 8) and the statistical significance of the treatments was assessed for the period 10-60 min post-treatment by ANOVA (chapter 3, section 3.5).

5.7.3 Results

The incremental changes in the plasma concentrations of cortisol following treatment with saline, ß-END or ACTH in spring, summer, autumn and winter are illustrated in Fig. 5.9. While ACTH significantly ($P < 0.001$, ANOVA) increased the plasma concentrations of cortisol at all the seasons, ß-END had no effect. The cortisol response to ACTH varied significantly ($P < 0.01$, ANOVA) with season; this was greatest in spring at the peak of the seasonal cycle in cortisol secretion (Fig. 5.9).

5.8 DISCUSSION

The peptides AVP and CRF are located in the neurosecretory cells of the hypothalamus and are thought to be the principal regulators of ß-END and ACTH secretion from the pituitary gland (Rivier & Vale, 1983a; Antoni, 1986; Smith & Funder, 1988). The current studies show that both AVP and CRF stimulate the release of ß-END and ACTH in the Soay ram, and the pituitary response is increased in summer and autumn at the time of increased endogenous secretion. This is consistent with the view that seasonal changes in AVP and/or CRF may dictate the seasonal cycle in ß-END and ACTH secretion. AVP was relatively more potent than CRF, on a molar basis, in stimulating the secretion of ß-END and ACTH, which is consistent with previous studies in other breeds of sheep (Pradier *et al.*, 1986; Familari *et al.*, 1989; experiments 8 and 9). Measurements of the concentrations of AVP and CRF in the pituitary portal blood system of sheep have revealed relatively higher concentrations of AVP than CRF, which could be taken to indicate that AVP is the more

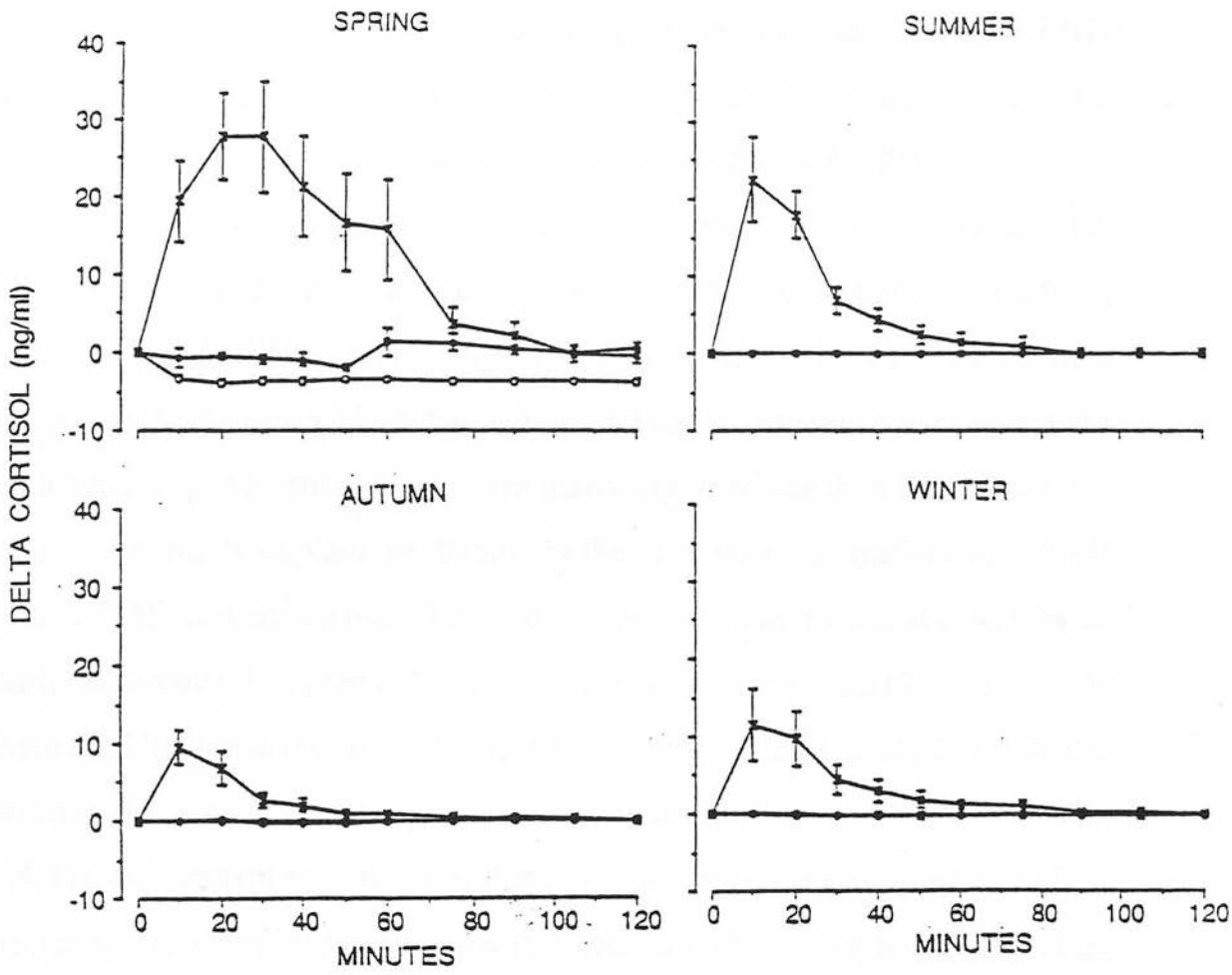


Fig. 5.9. Changes in the plasma concentrations of cortisol in outdoor Soay rams following a single bolus injection of either saline (○), 0.33 µg ß-endorphin/kg (●) or 0.33 µg ACTH/kg (X) in spring, summer, autumn and winter. Values are means ± SEM, n=6 rams per treatment group. The injection was given at time 0.

important physiological regulator of this endocrine system (Engler *et al.*, 1989b). However, the observation of a synergistic response to AVP and CRF in the Soay ram at all seasons of the year is consistent with a role for both hypothalamic peptides in the control of ß-END and ACTH secretion.

The studies also illustrate the involvement of the adrenal glucocorticoids in the negative feedback regulation of the secretion of ß-END and ACTH in the Soay ram. The administration of dexamethasone caused a decrease in the plasma concentrations of both ß-END and ACTH at all seasons of the year. Furthermore, the administration of the glucocorticoid antagonist, RU 486, increased the plasma concentrations of ß-END and cortisol (indirect measure for ACTH) in winter but not in summer. This observation of a significant response to RU 486 in winter and not in summer indicates that the endogenous glucocorticoids are more inhibitory in winter than in summer, and this may partially explain the decline in the plasma concentrations of ß-END and ACTH seen in winter. This study is the first one to use RU 486 as an antiglucocorticoid in sheep. The doses used in this experiment had previously been used in female monkeys (*Macaca fascicularis)* (Healy *et al.*, 1985). In this species, doses of 1 and 10 mg/kg were shown to cause a significant increase in ACTH and cortisol secretion but there was no significant increase in ß-END secretion. The current results show that the dose of 1 mg/kg had no effect on the plasma concentrations of both ß-END and cortisol while that of 10 mg/kg significantly increased the plasma concentrations of ß-END and cortisol in the ram. It is not clear whether these discrepancies are due to differences in methodology or sex.

The site of action of the negative feedback effect of glucocorticoids appears to be the pituitary gland based on the observation that dexamethasone pretreatment blocked the ability of AVP to stimulate the secretion of ß-END (experiment 12). This, however, does not exclude an additional negative

feedback action at the level of the hypothalamus affecting the release of AVP and/or CRF as has been demonstrated in the rat (Antoni, 1986; Plotsky *et al.*, 1986; Carnes *et al.*, 1987). The studies in the ram also show that ACTH and not ß-END stimulates the secretion of cortisol. If cortisol is the glucocorticoid which normally acts to provide homoeostasis, it follows that the control by ß-END is mediated indirectly via the secretion of ACTH (experiment 13). The lack of effect of ß-END on cortisol secretion does not appear to be a question of the dose used because this was selected to result in circulating levels in the physiological range seen at the peak of the seasonal cycle (Ebling and Lincoln, 1987).

The parallel changes in the plasma concentrations of ß-END and ACTH following the acute treatments with AVP, CRF and dexamethasone indicate a close link in the regulation of these two hormones, which are possibly cosecreted from the corticotrophs in the anterior pituitary gland. This is consistent with the observation that the ß-END in the peripheral circulation in the rams is largely in the non-acetylated form as released from the anterior pituitary gland (Smith & Funder,1988) (chapter 4, experiment 1). Furthermore, there was a temporal correlation between the spontaneous episodic peaks in the plasma profiles of ß-END and ACTH. This indicates a common control possibly involving the pulsatile release of peptides such as AVP and CRF from the hypothalamus.

However, the observation that not all episodic peaks of ß-END were correlated with those of ACTH indicates a more complex control of these two pituitary hormones. Detailed studies of the short-term fluctuations in the plasma concentrations of ß-END, ACTH and α-MSH in Merino sheep have indicated a complex differential regulation of these hormones. In particular, only a proportion (about 30%) of the episodic peaks in the plasma concentration of

ß-END was correlated with peaks in the plasma concentration of ACTH and some peaks in ACTH occurred with no corresponding increase in either ß-END or α-MSH (Engler *et al.*,1989a). These temporal associations indicate that the concentration in the peripheral blood is the result of the combined secretion from the different cell types in the pituitary gland which process the POMC precursor. Since the peaks in the peripheral blood most probably constitute secretory episodes induced by the hypothalamus, there must be a hypothalamic mechanism which separately regulates the secretion of ß-END and ACTH. One possibility is that there is a seasonal change in the pattern of secretion of AVP and CRF, or the ratio of these two peptides in the pituitary portal blood, which produces the differential control. To establish this it will be necessary to measure the levels of AVP and CRF in the portal blood in the Soay ram at different times of the year and correlate this with the seasonal cycle in the secretion of ß-END and ACTH.

5.9 SUMMARY

This second series of experiments has established the following:

1. AVP and CRF stimulate the secretion of ß-END and ACTH in the Soay ram and the responses are greatest in summer at the time of maximum endogenous secretion.

2. Dexamethasone (synthetic glucocorticoid) suppresses the secretion of ß-END and ACTH and the responses are also greatest in summer. RU 486 (synthetic glucocorticoid antagonist) stimulates ß-END and cortisol (indirect measure for ACTH) secretion and the responses are greater in winter than in summer.

3. Glucocorticoids act at the level of the pituitary gland to inhibit the secretion of ß-END and ACTH in the Soay ram.

4. ACTH, but not ß-END, stimulates the secretion of cortisol and the response is greater in spring, at the time of high endogenous secretion, than at other seasons of the year.

CHAPTER 6

CONTROL OF THE CYCLE IN ß-END SECRETION: ROLE OF DA AND ENDOGENOUS OPIOIDS

6.1 INTRODUCTION

Both the corticotrophs and melanotrophs of the pituitary gland contribute to the total ß-END in the peripheral blood (Antoni, 1986; Smith and Funder, 1988). The neural control of the secretory activity of the corticotrophs has been investigated in chapter 5; this involves stimulation by AVP and CRF and inhibition by glucocorticoids. In contrast, the control of the secretory activity of the melanotrophs appears to involve an inhibitory influence from the hypothalamus. This is based on the observation that experimental disconnection of the pituitary gland from the hypothalamus in sheep leads to hypertrophy of the intermediate lobe of the pituitary gland and an increase in the circulating concentrations of ß-END (Clarke *et al.*, 1986). In this situation, bromocriptine (DA agonist) suppresses ß-END secretion, indicating the possible involvement of DA as the inhibitory factor released from the hypothalamus. Indeed, short-term studies in intact sheep have established a dopaminergic inhibition upon melanotrophic secretion of ß-END and α-MSH (Newman, Wardlaw, Stark, Daniel and Frantz, 1987; Smith *et al.*, 1989). However, it is not known whether the results of these short-term studies would apply to the long-term control of the cycle in ß-END secretion in the Soay ram.

The functional activity of the hypothalamo-pituitary-adreno-cortical axis is also influenced by endogenous opioid peptides and opiate drugs in many species (e.g. frog: Saland *et al.*, 1982b: rat: Buckingham, 1979; Buckingham & Cooper, 1984; 1986; sheep: Redekopp *et al.*, 1985a; Wang *et al.*, 1986). However, the results from the various studies are often contradictory. For example, Redekopp *et al.* (1985a) demonstrated an inhibition of ACTH in sheep using the enkephalin analogue, DAMME, while Wang *et al.* (1986) showed that met-enkephalin stimulates the secretion of

ACTH. Stress is another factor which profoundly influences the the hypothalamo-pituitary-adrenocortical axis. For example, in rats, elevated plasma levels of ß-END and ACTH are observed following footshock and ether stress, respectively (Rossier et al., 1977; Rivier and Vale, 1983b). In sheep, a number of stressful stimuli, including acute haemorrhage, electroimmobilization and shearing, hypoglycaemia and audiovisual stimulus (barking dog), handling, transport and slaughter, have also been shown to elevate the plasma levels of ß-END (chapter 2, section 2.6.2).

The aims of the studies described in this chapter were to investigate the inhibitory role of DA in the control of the cycle in ß-END secretion in the Soay ram, and to assess whether endogenous opioids modulate this control. A small study was also conducted involving the stress response to exposure to a sheep dog. PRL was also monitored since it is well established that DA, opioids and stress influence PRL secretion.

6.2 EXPERIMENT 14- EFFECT OF DA, PIMOZIDE AND NALOXONE ON ß-END AND PRL SECRETION

6.2.1 Aim

The aim of this experiment was to assess the effect of DA, pimozide (DA antagonist) and naloxone (opioid antagonist) on ß-END and PRL secretion in rams with low or high endogenous hormone secretion induced by exposure to long and short days.

6.2.2 Materials and methods

A group of six adult rams housed indoors was used in this experiment

carried out both under long days (10-12 weeks after the change from short to long days; low plasma levels of ß-END and high plasma levels of PRL) and under short days (10-12 weeks after the change from long to short days; high plasma levels of ß-END and low plasma levels of PRL). On both occasions, the animals were treated i.v. with DA and naloxone in the first part of the experiment (10 weeks), and with naloxone and pimozide in the second part of the experiment (12 weeks). The treatment design and bleeding protocol was as follows:

Part one: on day 1, the animals were blood sampled at 10 min intervals for 4h before and 4h after the i.v. injection of naloxone given at a dose of 1.6 mg/kg; the dose of naloxone was selected on the basis of previous studies in sheep (Lincoln & Ebling, 1985; Lincoln *et al.*, 1987). On day 2, this was repeated but the animals also received i.v. injections of DA at a dose of 6.6 µg/kg every10 min throughout the 8h period (total dose 316.8 µg/kg); the dose of DA was also selected on the basis of previous studies in sheep (Deaver & Dailey, 1982).

Part two: on day 1, the animals were blood sampled at 10 min intervals for 4h before and 4h after the i.v. injection of naloxone given at a dose of 1.6 mg/kg. On day 2, this was repeated but the animals also received i.m. injections of pimozide at a dose of 0.08 mg/kg every 2h throughout the 8h period (total dose 0.32 mg/kg); the dose of pimozide was selected on the basis of previous studies in sheep (Meyer & Goodman, 1985; 1986).

Plasma concentrations of ß-END and PRL were measured by RIA as previously described (chapter 3, section 3.4). The results were analysed by calculating the mean plasma concentrations of ß-END and PRL for each treatment, based on 10 min samples collected for 4h-periods after the initiation of each treatment, and the statistical significance of the treatments was assessed by ANOVA (chapter 3, section 3.5).

6.2.3 Results

The effects of DA, pimozide and naloxone on the plasma concentrations of ß-END and PRL under long days and short days are illustrated in Fig. 6.1 and in Fig. 6.2, respectively. Under long days, the i.v. injections of DA did not significantly affect the mean plasma concentrations of ß-END and PRL assessed over the 4h period after the initiation of the treatment; however, there was a small increase in the plasma levels of ß-END within 1.5h of its administration. The i.v. injection of naloxone did not affect the plasma concentrations of ß-END and PRL; nor did naloxone given at the same time as DA have an effect on the plasma levels of the two hormones (Fig. 6.1(a)). The i.m. injections of pimozide caused a significant ($P< 0.001$, ANOVA) increase in the plasma concentrations of ß-END and PRL compared with the corresponding saline control period; the levels became significantly increased 1h after the first pimozide injection and remained markedly increased throughout the rest of the study. The injection of naloxone at the same time as pimozide did not affect the plasma concentrations of ß-END and PRL (Fig. 6.1(b)).

Under short days, the i.v. injections of DA and naloxone did not influence the plasma concentrations of ß-END and PRL (Fig. 6.2(a)). The i.m. injections of pimozide had no effect on the plasma concentrations of ß-END and PRL. However, the injection of naloxone at the same time as pimozide caused a significant ($P < 0.05$, ANOVA) increase in the plasma concentrations of ß-END and PRL (Fig. 6.2(b)).

There was a minor behavioural effect after the second and subsequent injections of pimozide; this involved the animals lowering their heads and shivering for about 20 min after each injection. This effect was more evident under long days than under short days.

Fig. 6.1(a). Plasma concentrations of ß-endorphin and prolactin in indoor Soay rams treated with saline plus naloxone and dopamine plus naloxone, at 10 weeks during long days. On day 1, the rams received saline and a single i.v. injetion of 1.6 mg naloxone/kg; on day 2, they received 6.6 µg dopamine/kg every 10 min for 8h and a single i.v. injection of naloxone given as on day 1. The values are means ± SEM, n=6 rams. NAL=naloxone and the arrow indicates the point of naloxone administration.

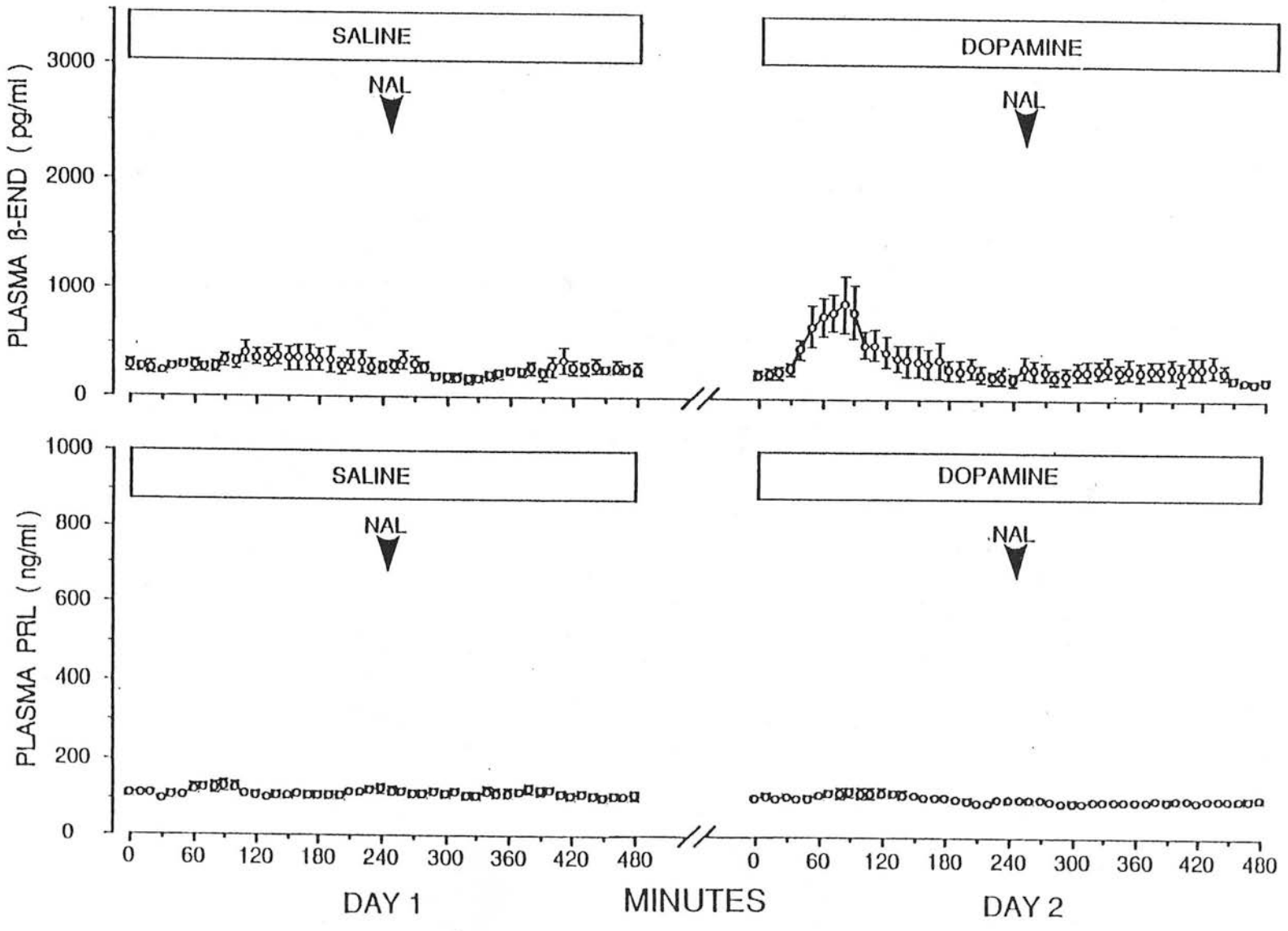

SALINE
NAL
DOPAMINE
NAL
PLASMA ß-END (pg/ml)
3000
2000
1000
0
SALINE
NAL
DOPAMINE
NAL
PLASMA PRL (ng/ml)
1000
800
600
400
200
0
0 60 120 180 240 300 360 420 480
0 60 120 180 240 300 360 420 480
DAY 1
MINUTES
DAY 2

Fig. 6.1(b). Plasma concentrations of ß-endorphin and prolactin in indoor Soay rams treated with saline plus naloxone and pimozide plus naloxone, at 12 weeks during long days. On day 1, the rams received saline and a single i.v. injection of 1.6 mg naloxone/kg; on day 2, they received 0.08 mg pimozide/kg every 2h for 8h and an i.v. injection of naloxone given as on day 1. The values are means ± SEM, n=6 rams. NAL=naloxone and the arrow indicates the point of naloxone administration.

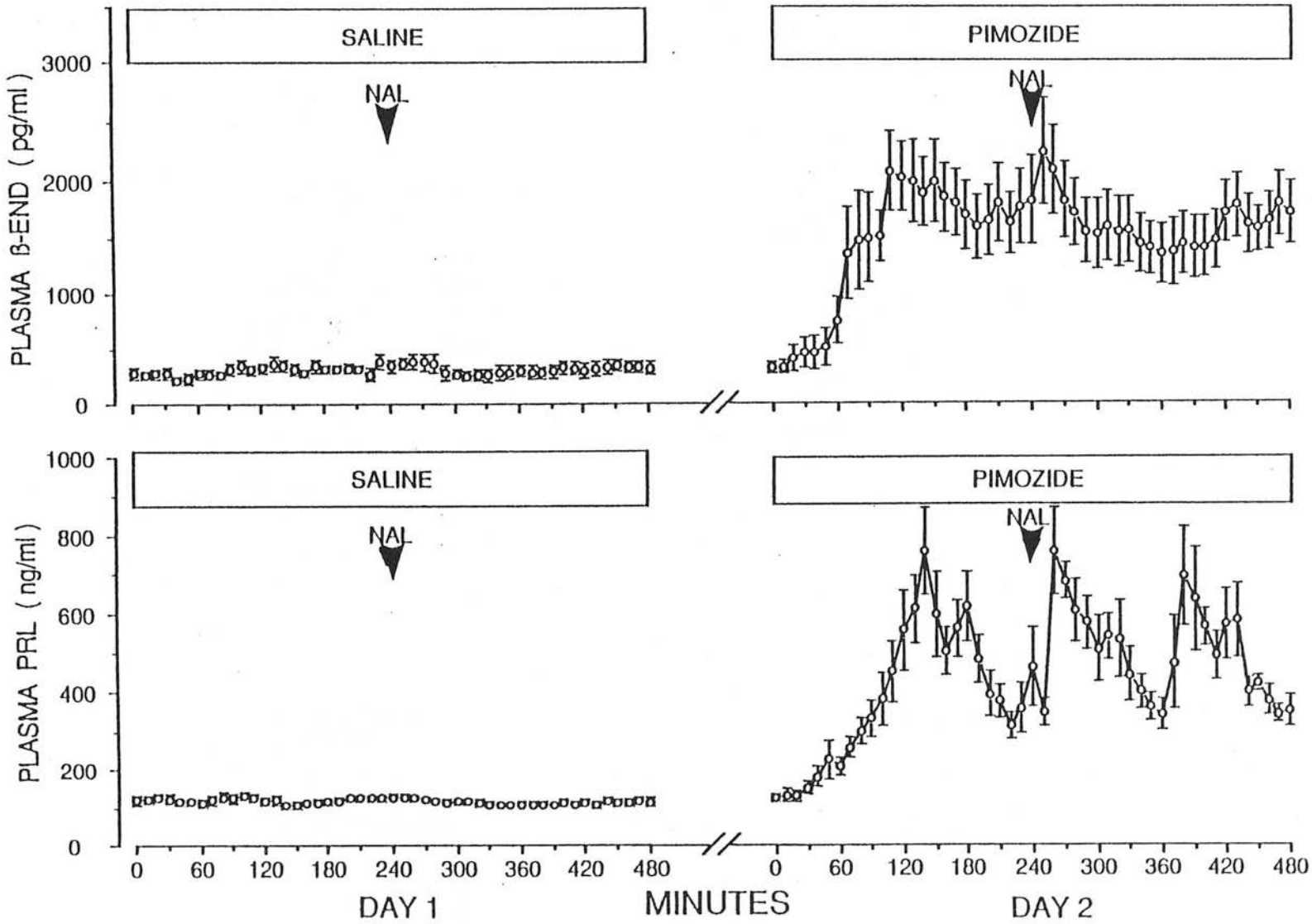

SALINE
PIMOZIDE
NAL
NAL
PLASMA β-END (pg/ml)
3000
2000
1000
0
SALINE
PIMOZIDE
NAL
NAL
PLASMA PRL (ng/ml)
1000
800
600
400
200
0
0 60 120 180 240 300 360 420 480
0 60 120 180 240 300 360 420 480
DAY 1
MINUTES
DAY 2

Fig. 6.2(a). Plasma concentrations of ß-endorphin and prolactin in indoor Soay rams treated with saline plus naloxone and dopamine plus naloxone, at 10 weeks during short days. On day 1, the rams received saline and a single i.v. injection of 1.6 mg naloxone/kg; on day 2, they received 6.6 µg dopamine/kg every 10 min for 8h plus an i.v. injection of naloxone given as on day 1. The values are means ± SEM, n=6 rams. NAL=naloxone and the arrow indicates the point of naloxone administration.

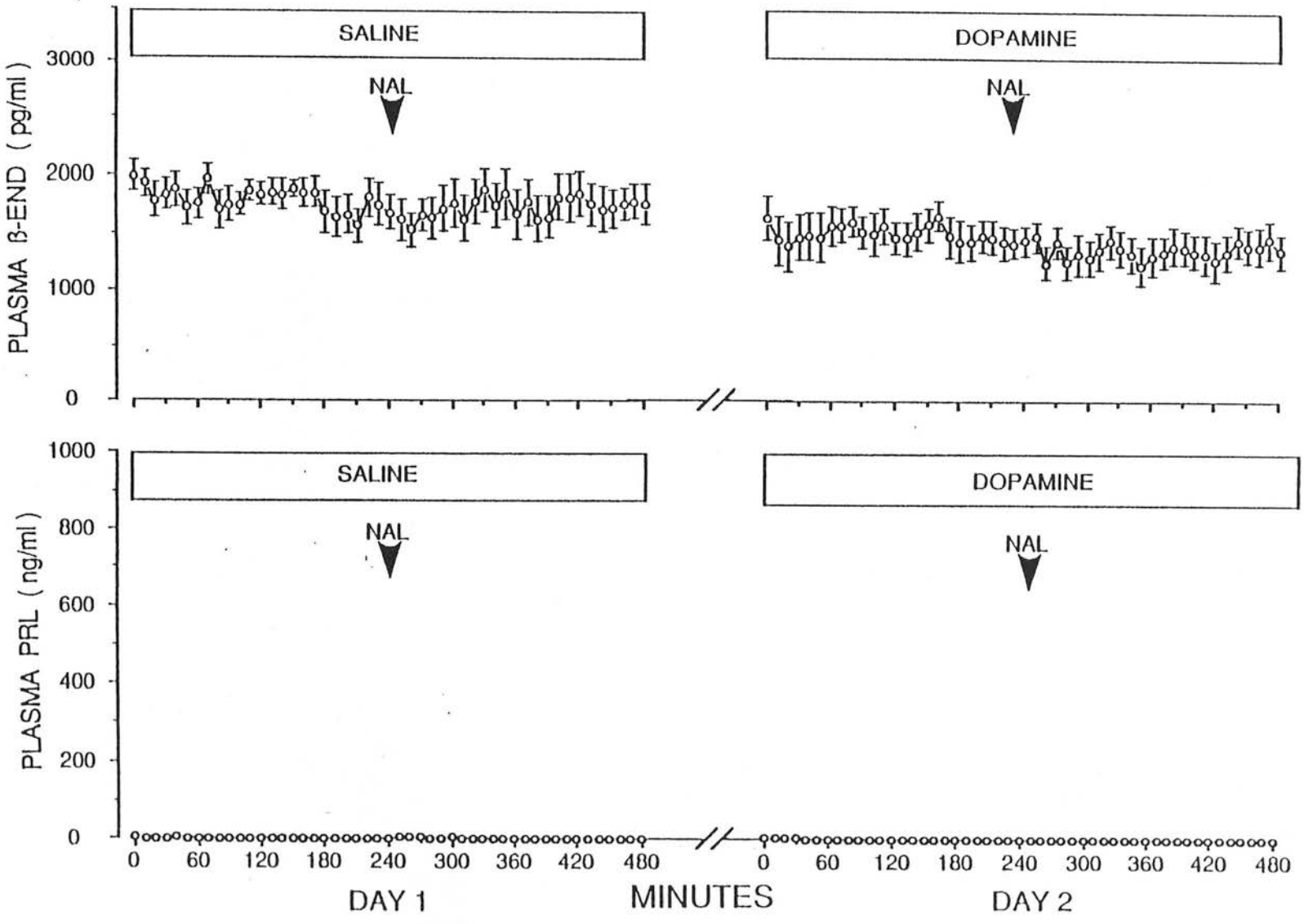
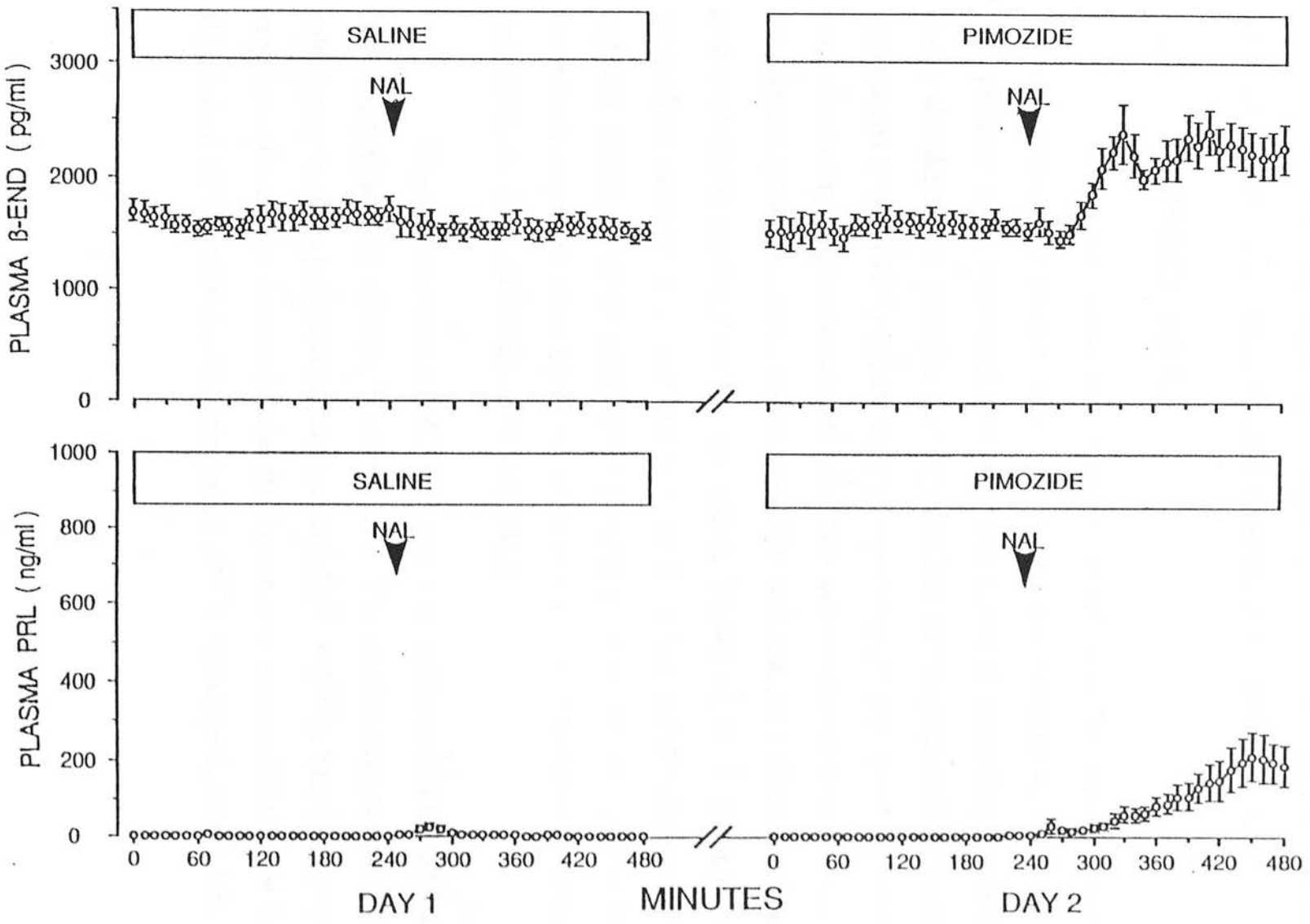

SALINE
DOPAMINE
NAL
NAL
PLASMA ß-END (pg/ml)
3000
2000
1000
0
SALINE
DOPAMINE
NAL
NAL
PLASMA PRL (ng/ml)
1000
800
600
400
200
0
0 60 120 180 240 300 360 420 480
0 60 120 180 240 300 360 420 480
DAY 1
MINUTES
DAY 2

Fig. 6.2(b). Plasma concentrations of ß-endorphin and prolactin in indoor Soay rams treated with saline plus naloxone and pimozide plus naloxone, at 12 weeks during short days. On day 1, the rams received saline and a single i.v. injection of 1.6 mg naloxone/kg; on day 2, they received 0.08 mg pimozide/kg every 2h for 8h plus an i.v. injection of naloxone given as on day 1. The values are means ± SEM, n-6 rams. NAL=naloxone and the arrow indicates the point of naloxone administration.


SALINE
PIMOZIDE
NAL
NAL
PLASMA β-END (pg/ml)
3000
2000
1000
0
SALINE
PIMOZIDE
NAL
NAL
PLASMA PRL (ng/ml)
1000
800
600
400
200
0
0 60 120 180 240 300 360 420 480
0 60 120 180 240 300 360 420 480
DAY 1
MINUTES
DAY 2

6.3 EXPERIMENT 15- EFFECT OF DA AND NALOXONE ON ß-END AND PRL SECRETION

6.3.1 Aim

In this experiment, a 10-fold higher dose of DA was used than in the previous study to assess whether this will inhibit ß-END and PRL secretion.

6.3.2 Materials and methods

The six adult rams used to study the effect of DA, pimozide and naloxone (experiment 14) were employed in this experiment during a different photoperiodic cycle. The experiment was carried out both under long days (10 weeks after the change from short to long days; low plasma levels of ß-END and high plasma levels of PRL) and under short days (10 weeks after the change from long to short days; high plasma levels of ß-END and low plasma levels of PRL). On both occasions, the animals were treated i.v. with DA and naloxone and the treatment design and bleeding protocol was as follows: on day 1, the animals were blood sampled at 10 min intervals for 4h before and 4h after the i.v. injection of naloxone given at a dose of 1.6 mg/kg. On day 2, this was repeated but the animals also received i.v. injections of DA at a dose of 66 µg/kg every 10 min throughout the 8h period (total dose 3168 µg/kg).

Plasma concentrations of ß-END and PRL were measured by RIA as previously described (chapter 3, section 3.4). The results were analysed by calculating the mean plasma concentrations of ß-END and PRL, based on 10 min samples collected for 4h-periods after the initiation of each treatment, and the significance of the treatments was assessed by ANOVA (chapter 3, section 3.5).

6.3.3 Results

The effects of DA and naloxone on the plasma concentrations of ß-END and PRL under long days and short days are illustrated in Fig. 6.3 and Fig. 6.4, respectively. The i.v. injections of DA at the high dose decreased the plasma concentrations of ß-END and PRL both under long and short days. However, this decrease was not statistically significant (Figs. 6.3 and 6.4). The i.v. injection of naloxone did not affect the plasma concentrations of ß-END and PRL as previously demonstrated (Figs. 6.3 and 6.4).

6.4 EXPERIMENT 16- EFFECT OF ACUTE BROMOCRIPTINE TREATMENT ON ß-END AND PRL SECRETION

6.4.1 Aim

In this experiment, the DA agonist, bromocriptine, which has high affinity for D_2 DA receptors was used to further assess the possible role of DA in the inhibitory control of ß-END and PRL secretion.

6.4.2 Materials and methods

A group of eight adult rams housed indoors was used in this experiment. The rams were treated s.c. with bromocriptine while maintained under long days (12 weeks after the change from short to long days; low plasma levels of ß-END and high plasma levels of PRL) or short days (12 weeks after the change from long to short days; high plasma levels of ß-END and low plasma levels of PRL). The treatment design and bleeding protocol was as follows: on day 1, the animals were blood sampled at 10 min intervals for 8h. On day 2, the animals were sampled in the same way but were treated with a s.c. injection of

Fig. 6.3. Plasma concentrations of ß-endorphin and prolactin in indoor Soay rams treated with saline plus naloxone and dopamine plus naloxone, at 10 weeks during long days. On day 1, the rams received saline and a single i.v. injection of 1.6 mg naloxone/kg; on day 2, they received 66 µg dopamine/kg every 10 min for 8h plus a single i.v. injection of naloxone given as on day 1. The values are means ± SEM, n=6 rams. NAL=naloxone and the arrow indicates the point of naloxone administration.

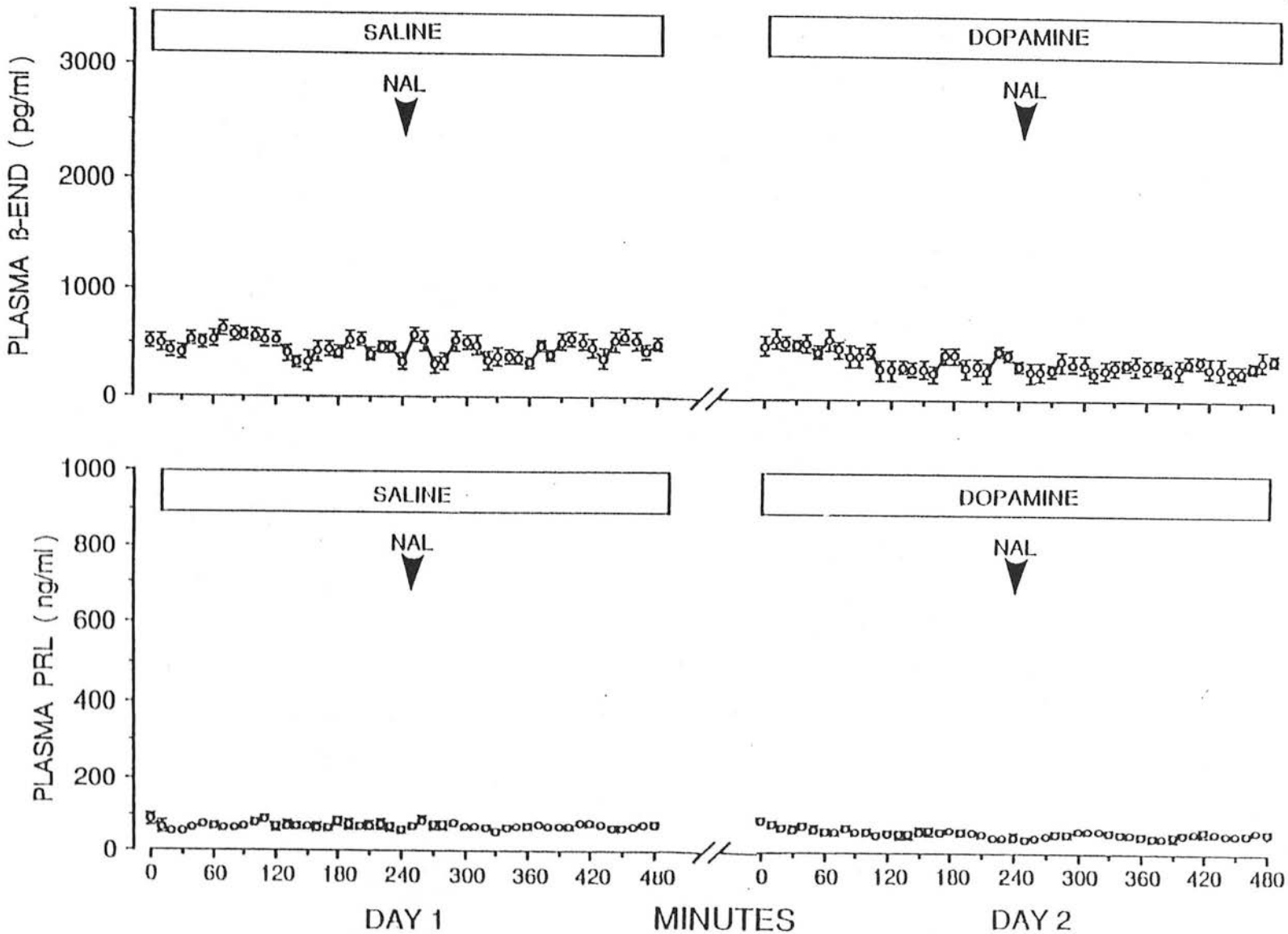
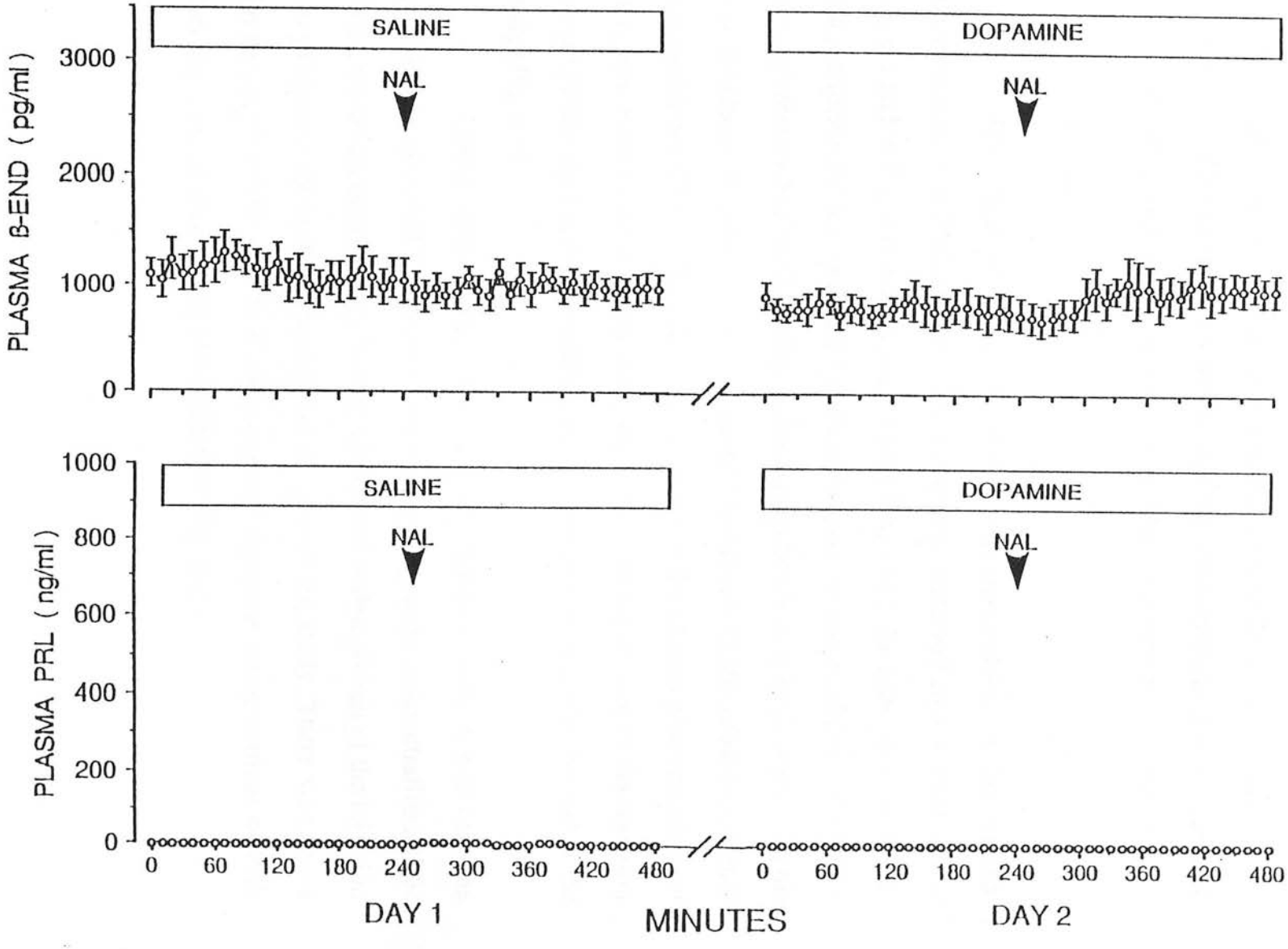

SALINE
NAL
DOPAMINE
NAL
PLASMA β-END (pg/ml)
3000
2000
1000
0
SALINE
NAL
DOPAMINE
NAL
PLASMA PRL (ng/ml)
1000
800
600
400
200
0
0 60 120 180 240 300 360 420 480
0 60 120 180 240 300 360 420 480
DAY 1
MINUTES
DAY 2

Fig. 6.4. Plasma concentrations of ß-endorphin and prolactin in indoor Soay rams treated with saline plus naloxone and dopamine plus naloxone, at 10 weeks during short days. On day 1, the rams received saline and a single i.v. injection of 1.6 mg naloxone.kg; on day 2, they received 66 µg dopamine/kg every 10 min for 8h plus a single i.v. injection of naloxone given as on day 1. The values are means ± SEM, n=6 rams. NAL=naloxone and the arrow indicates the point of naloxone administration.


SALINE
DOPAMINE
NAL
NAL
PLASMA β-END (pg/ml)
3000
2000
1000
0
SALINE
DOPAMINE
NAL
NAL
PLASMA PRL (ng/ml)
1000
800
600
400
200
0
0 60 120 180 240 300 360 420 480
0 60 120 180 240 300 360 420 480
DAY 1
MINUTES
DAY 2

bromocriptine was given at a dose of 0.06 mg/kg. The dose of bromocriptine was selected based on previous studies in sheep (McNeilly & Land, 1979).

The plasma concentrations of ß-END and PRL were measured by RIA as previously described (chapter 3, section 3.4). The results were analysed by calculating the mean plasma concentrations of ß-END and PRL based on 10 min samples collected for the 8h period after the bromocriptine injection, and the significance of the treatment was assessed by ANOVA (chapter 3, section 3.5).

6.4.3 Results

The effect of the s.c. injection of bromocriptine on the plasma concentrations of ß-END and PRL under long days and short days is illustrated in Fig. 6.5 and in Fig. 6.6, respectively. Under long days, the acute treatment with bromocriptine did not significantly affect the plasma concentrations of ß-END although there was a small decline in the plasma levels of ß-END from 4h to 8h post-treatment. In contrast, the s.c. injection of bromocriptine caused an abrupt and significant ($P < 0.001$, ANOVA) decrease in the plasma concentrations of PRL; the levels became significantly suppressed within 60 min of the injection and progressively declined to undetectable levels (< 0.4 ng/ml) by the end of the study (Fig. 6.5).

Under short days, the acute treatment with bromocriptine significantly ($P < 0.001$, ANOVA) decreased the plasma concentrations of ß-END ; the levels became significantly suppressed within 60 min of the injection and progressively declined throughout the rest of the study. There was also a significant ($P < 0.05$, ANOVA) decrease in the plasma concentrations of PRL after the acute treatment with bromocriptine (Fig. 6.6).

Fig. 6.5. Plasma concentrations of ß-endorphin and prolactin in indoor Soay rams treated with saline and the D_2 dopamine agonist, bromocriptine, at 12 weeks during long days. On day 1, the rams received saline and, on day 2, they received a single s.c. injection of 0.06 mg bromocriptine/kg. The values are means ± SEM, n=8 rams. The arrow indicates the point of bromocriptine administration.

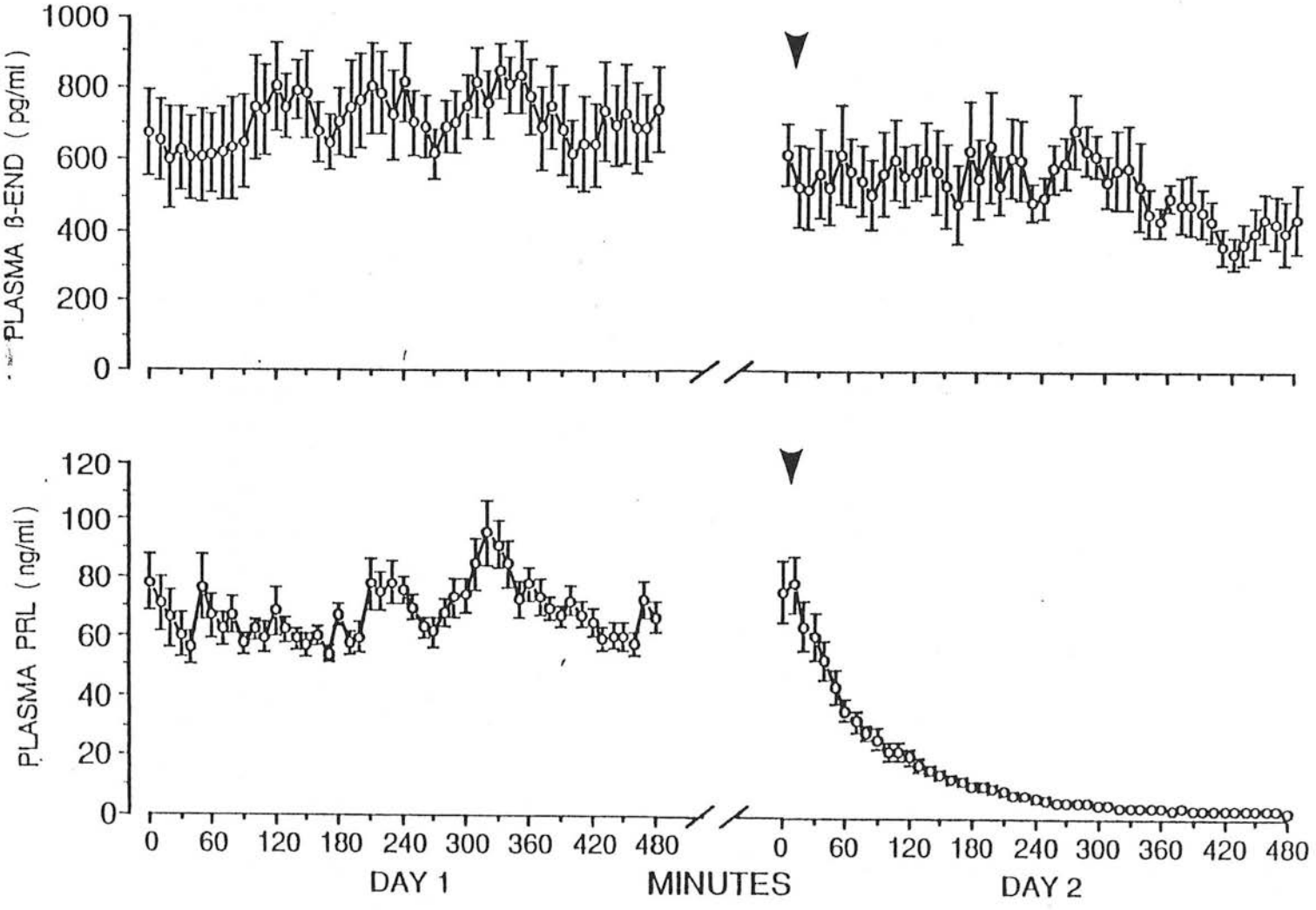
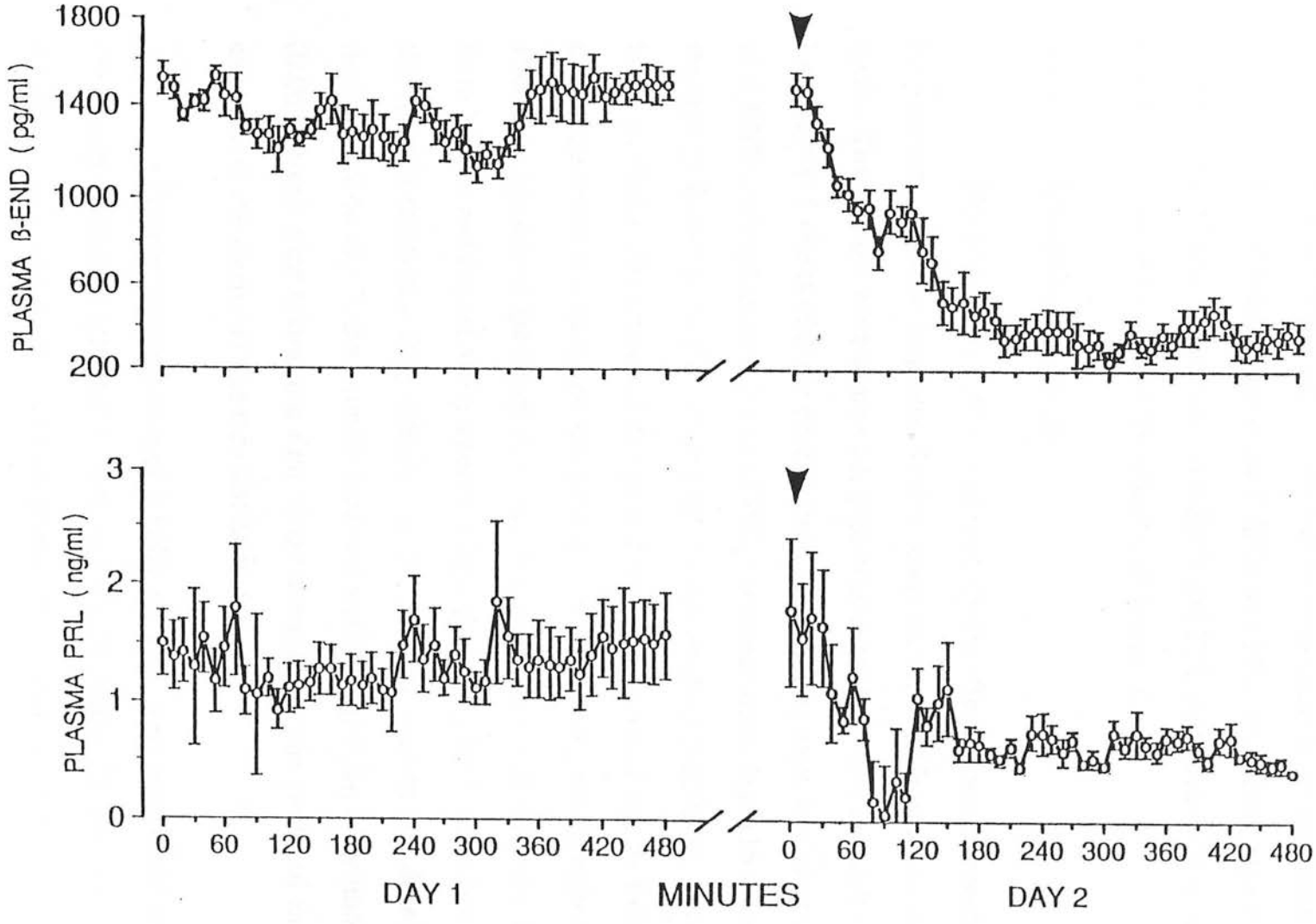

PLASMA ß-END (pg/ml)
1000
800
600
400
200
0
PLASMA PRL (ng/ml)
120
100
80
60
40
20
0
0 60 120 180 240 300 360 420 480
DAY 1
MINUTES
0 60 120 180 240 300 360 420 480
DAY 2

Fig. 6.6. Plasma concentrations of ß-endorphin and prolactin in indoor Soay rams treated with saline and the D_2 dopamine agonist, bromocriptine, at 12 weeks during short days. On day 1, the rams received saline and, on day 2, they received a single s.c. injection of 0.06 mg bromocriptine/kg. The values are means ± SEM, n=8. The arrow indicates the point of bromocriptine administration.


PLASMA ß-END (pg/ml)
1800
1400
1000
600
200
PLASMA PRL (ng/ml)
3
2
1
0
0 60 120 180 240 300 360 420 480
0 60 120 180 240 300 360 420 480
DAY 1
MINUTES
DAY 2

6.5 EXPERIMENT 17- EFFECT OF CHRONIC BROMOCRIPTINE TREATMENT AND STRESS ON ß-END AND PRL SECRETION

6.5.1 Aim

The aim of this experiment was to investigate the effect of the chronic administration of bromocriptine on ß-END and PRL secretion. Also to assess whether stress-induced increases in ß-END and PRL secretion after exposure to a sheep dog can be blocked by treatment with bromocriptine.

6.5.2 Materials and methods

The eight indoor rams used to study the effect of acute bromocriptine (experiment 15) were employed in this study during a different photoperiodic cycle.. The animals were treated s.c. with bromocriptine while maintained under long days (15 weeks after the change from short to long days; low plasma levels of ß-END and high plasma levels of PRL) or under short days (15 weeks after the change from long to short days; high plasma levels of ß-END and low plasma levels of PRL). The treatment design and bleeding protocol was as follows: on day 1, the animals were blood sampled at 10 min intervals for 4h before and 2h after the exposure to the sheep dog. For this, each individual ram was removed from his pen and herded along a corridor by a sheep dog for 5 min. On days 2-6, the animals received a daily injection of bromocriptine given at a dose of 0.06 mg/kg, and on day 7, the animals received a single injection of bromocriptine (0.06 mg/kg) after taking the first blood sample and the protocol involving exposure to the sheep dog was repeated as on day 1.

Plasma concentrations of ß-END and PRL were measured by RIA as previously described (chapter 3, section 3.4). To assess the effect of chronic treatment with bromocriptine, mean plasma concentrations of ß-END and PRL were calculated based on 10 min samples collected for 4h-periods before

exposure to the sheep dog; and to assess the effect of exposure to the sheep dog, mean plasma concentrations were calculated based on 10 min samples collected for 1h-periods before and after the exposure to the dog. The statistical significances of the treatment with bromocriptine and that of the effect of exposure to the sheep dog were assessed by ANOVA (chapter 3, section 3.5).

6.5.3 Results

The effects of chronic treatment with bromocriptine and the effect of stress induced by the sheep dog on the plasma concentrations of ß-END and PRL under long days and short days are illustrated in fig. Fig. 6.7 and in Fig. 6.8, respectively. Under long days, chronic treatment with bromocriptine significantly ($P < 0.01$, ANOVA) decreased the plasma concentrations of ß-END. There was also a significant ($P < 0.001$, ANOVA) decrease in the plasma concentrations of PRL and the levels became undetectable (< 0.4 ng/ml) after 6 days of treatment (Fig. 6.7). The stimulus of the sheep dog significantly ($P < 0.001$, ANOVA) increased the plasma concentrations of ß-END both before and after the chronic treatment with bromocriptine, with no difference between the ß-END responses on the two occasions (Fig. 6.7). The stimulus of the sheep dog also significantly ($P < 0.001$, ANOVA) increased the plasma concentrations of PRL before the chronic treatment with bromocriptine; but there was no response after the chronic treatment with bromocriptine (Fig. 6.7).

Under short days, the chronic treatment with bromocriptine significantly ($P < 0.005$, ANOVA) decreased the plasma concentrations of ß-END. The plasma concentrations of PRL were already very low on this occasion and chronic treatment with bromocriptine produced no effect. The stimulus of the sheep dog significantly ($P< 0.05$, ANOVA) increased the plasma concentrations of ß-END both before and after the chronic treatment with bromocriptine, with no difference in the responses on the two occasions.

Fig. 6.7. Plasma concentrations of ß-endorphin and prolactin in indoor Soay rams treated with saline plus exposure to a sheep dog, and then chronically treated with bromocriptine plus exposure to a sheep dog, at 15 weeks during long days. On day 1, the rams received saline for 4h before and 2h after the exposure to the sheep dog; on days 2-6, they received a daily s.c. injection of 0.06 mg bromocriptine/kg; on day 7, the rams received a single s.c. injection of 0.06 mg bromocriptine/kg after taking the first blood sample, and then the protocol involving exposure to the sheep dog was repeated as on day 1. The values are means ± SEM, n=8. The arrow indicates the point of exposure to the sheep dog and the open box indicates the period of chronic treatment with bromocriptine, with the injection given on day 7 inclusive.

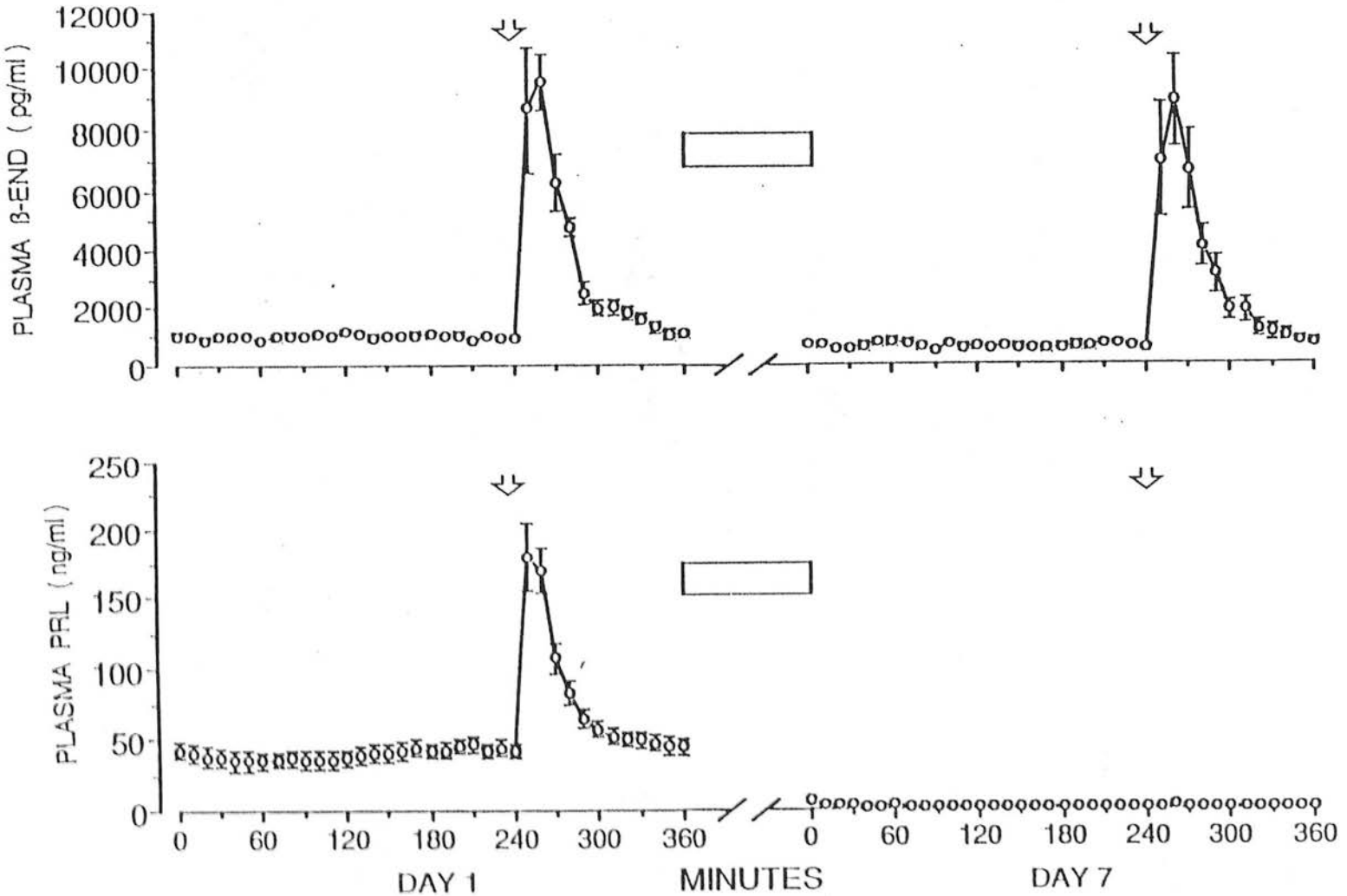
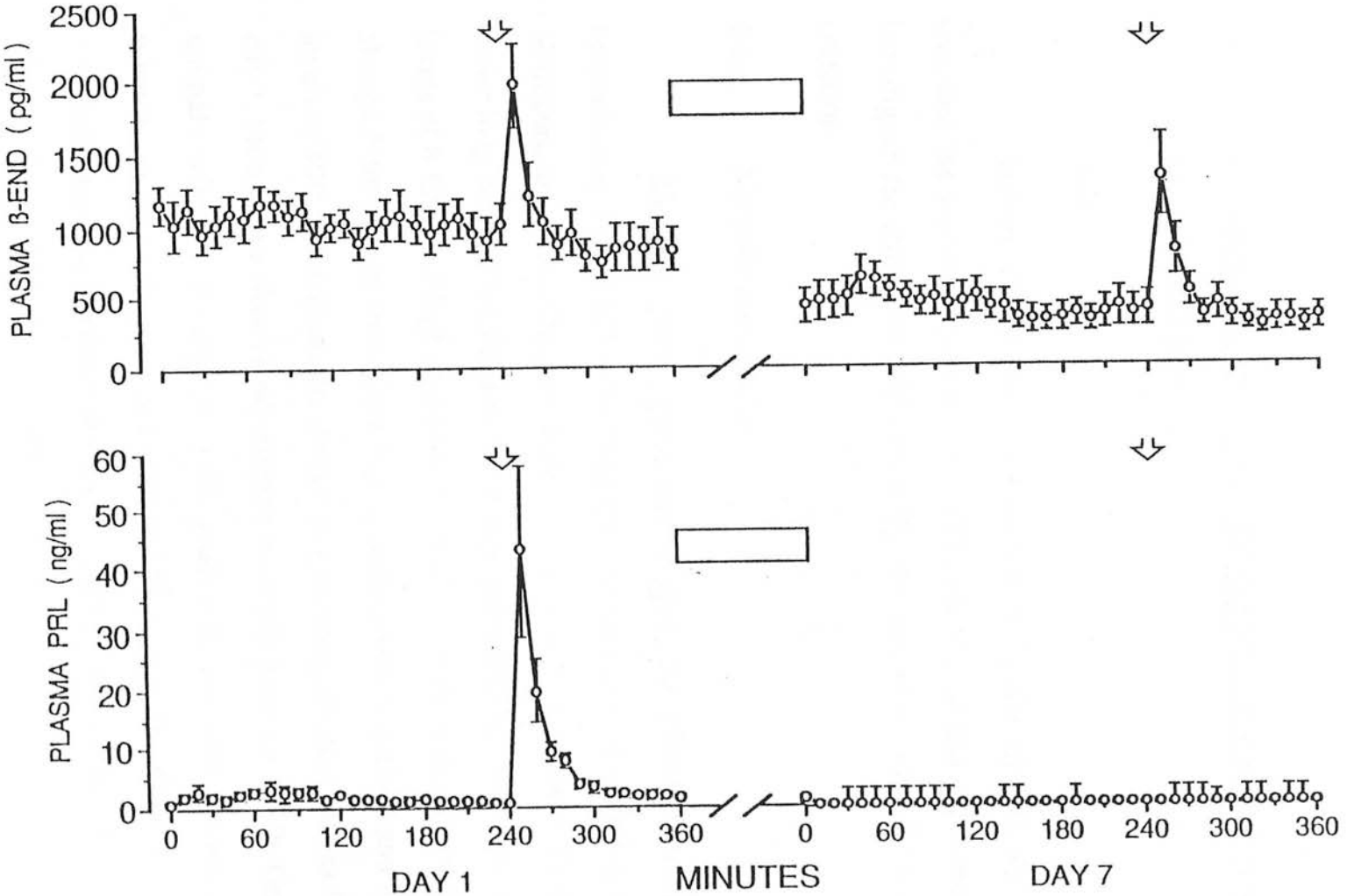

PLASMA β-END (pg/ml)
12000
10000
8000
6000
4000
2000
0
PLASMA PRL (ng/ml)
250
200
150
100
50
0
0 60 120 180 240 300 360
0 60 120 180 240 300 360
DAY 1
MINUTES
DAY 7

Fig. 6.8. Plasma concentrations of ß-endorphin and prolactin in indoor Soay rams treated with saline plus exposure to a sheep dog, and then chronically treated with bromocriptine plus exposure to a sheep dog, at 15 weeks during short days. On day 1, the rams received saline for 4h before and 2h after the exposure to the sheep dog; on days 2-6, they received a daily s.c. injection of 0.06 mg bromocriptine/kg; on day 7, the rams received a single s.c. injection of 0.06 mg bromocriptine/kg after taking the first blood sample, and then the protocol involving exposure to the sheep dog was repeated as on day 1. The values are means ± SEM, n=8. The arrow indicates the point of exposure to the sheep dog and the open box indicates the period of chronic treatment with bromocriptine, with the injection given on day 7 inclusive.


PLASMA ß-END (pg/ml)
2500
2000
1500
1000
500
0
PLASMA PRL (ng/ml)
60
50
40
30
20
10
0
0
60
120
180
240
300
360
DAY 1
MINUTES
DAY 7

The ß-END responses were reduced compared with those seen under long days. The stimulus of the sheep dog also significantly ($P < 0.001$, ANOVA) increased the plasma concentrations of PRL before the chronic treatment with bromocriptine, but there was no response after the treatment (Fig. 6.8).

6.6 EXPERIMENT 18- EFFECT OF SULPIRIDE ON ß-END AND PRL SECRETION

6.6.1 Aim

Having shown that bromocriptine, a D_2 DA agonist, significantly inhibited the secretion of ß-END and PRL, the aim of this experiment was to investigate the effects of sulpiride, a D_2 DA antagonist on ß-END and PRL secretion.

6.6.2 Materials and methods

The eight indoor rams used to study the effect of bromocriptine (experiments 16 and 17) were employed in this experiment during a different photoperiodic cycle.. The rams were treated s.c. with sulpiride while maintained under long days (9 weeks after the change from short to long days; low plasma levels of ß-END and high plasma levels of PRL) or short days (9 weeks after the change from long to short days; high plasma levels of ß-END and low plasma levels of PRL). The treatment design and bleeding protocol was as follows: on day 1, the animals were blood sampled at 10 min intervals for 8h. On day 2, the animals were blood sampled in the same way but were treated with a s.c. injection of sulpiride given at a dose of 0.59 mg/kg. The dose of sulpiride was selected based on previous studies in women (McMurdo, McEwen, Lewis, Marnie, Howie & McNeilly, 1985).

Fig. 6.9. Plasma concentrations of ß-endorphin and prolactin in indoor Soay rams treated with saline and the D_2 dopamine antagonist, sulpiride , at 9 weeks during long days. On day 1, the rams received saline and, on day 2, they received a single s.c. injection of 0.59 mg sulpiride/kg. The values are means ± SEM, n=8. The arrow indicates the point of sulpiride administration.

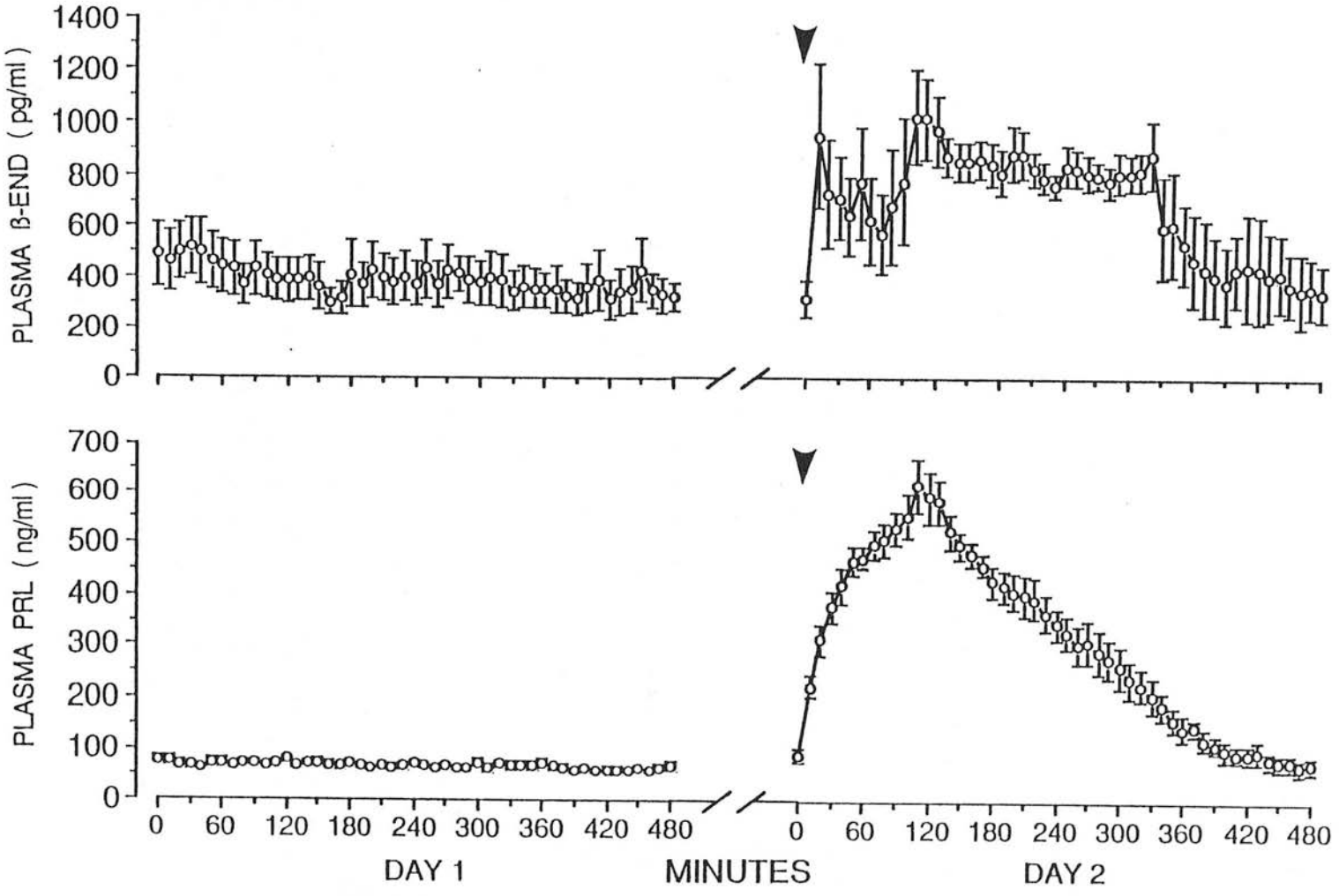
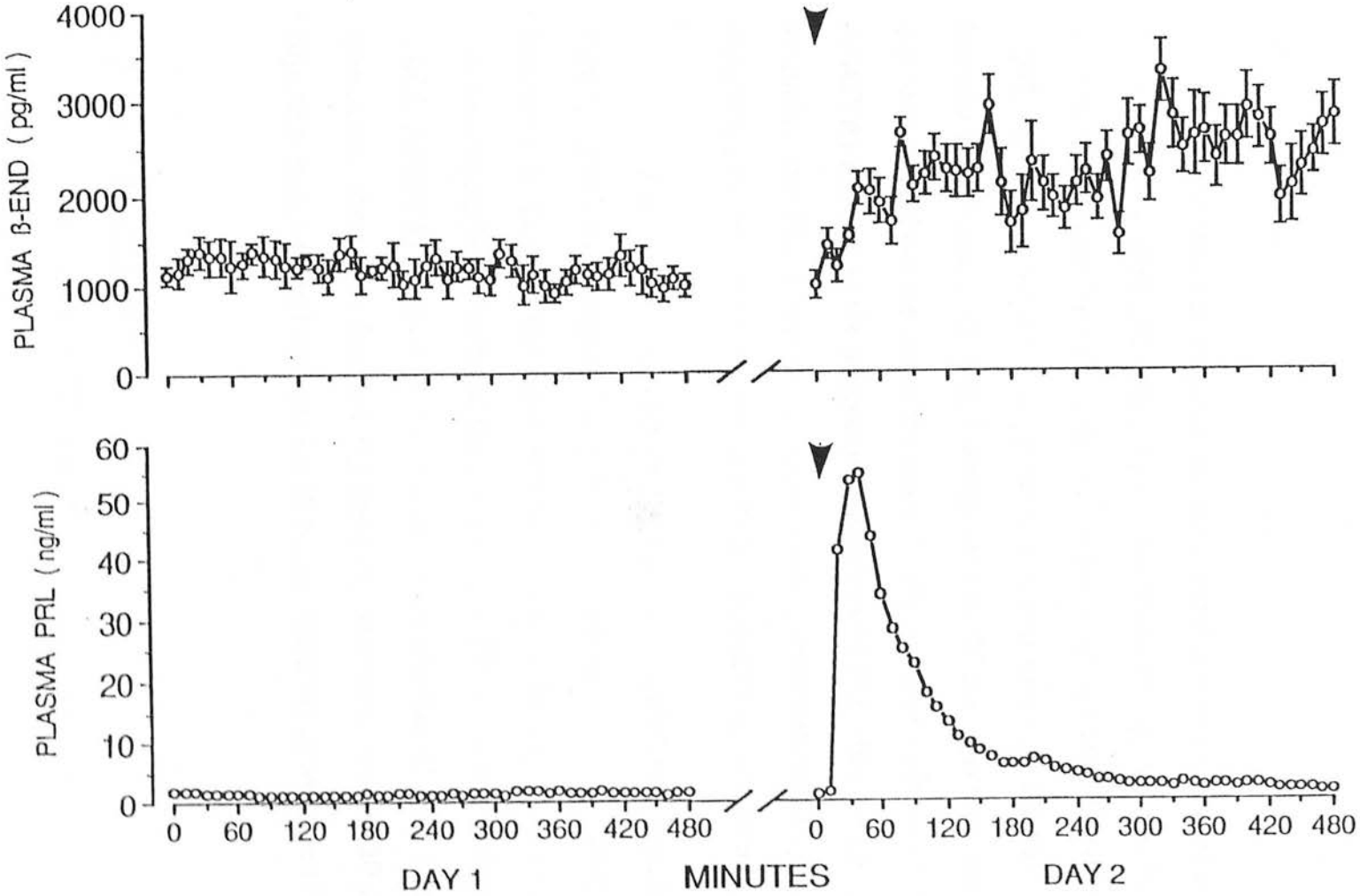

PLASMA β-END (pg/ml)
1400
1200
1000
800
600
400
200
0
PLASMA PRL (ng/ml)
700
600
500
400
300
200
100
0
0 60 120 180 240 300 360 420 480
0 60 120 180 240 300 360 420 480
DAY 1
MINUTES
DAY 2

Fig. 6.10. Plasma concentrations of ß-endorphin and prolactin in indoor Soay rams treated with saline and the D_2 dopamine antagonist, sulpiride , at 9 weeks during short days. On day 1, the rams received saline and, on day 2, they received a single s.c. injection of 0.59 mg sulpiride/kg. The values are means ± SEM, n=8. The arrow indicates the point of sulpiride administration.


PLASMA ß-END (pg/ml)
4000
3000
2000
1000
0
PLASMA PRL (ng/ml)
60
50
40
30
20
10
0
0 60 120 180 240 300 360 420 480
0 60 120 180 240 300 360 420 480
DAY 1
MINUTES
DAY 2

Plasma concentrations of ß-END and PRL were measured by RIA as previously described (chapter 3, section 3.4). The results were analysed by calculating the mean plasma concentrations of ß-END and PRL based on 10 min samples collected for the 8h period after the sulpiride injection, and the statistical significance of the treatment was assessed by ANOVA (chapter 3, section 3.5).

6.6.3 Results

The effect of sulpiride on the plasma concentrations of ß-END and PRL under long days and short days is illustrated in Fig. 6.9 and in Fig. 6.10, respectively. Under long days, the s.c. injection of sulpiride significantly ($P < 0.005$, ANOVA) increased the plasma concentrations of ß-END; the levels became significantly increased within 10 min of the injection and remained elevated throughout the rest of the study. There was also a significant ($P < 0.001$, ANOVA) increase in the plasma concentrations of PRL after the s.c. injection of sulpiride; the PRL levels became significantly increased within 10 min of the injection and remained elevated for 6.5h before declining to baseline (Fig. 6.9).

Under short days, the s.c. injection of sulpiride also significantly ($P < 0.005$, ANOVA) increased the plasma concentrations of ß-END; the levels became significantly increased within 40 min of the injection and remained elevated throughout the rest of the study (Fig. 6.10). There was a significant ($P < 0.005$, ANOVA) increase in the plasma concentrations of PRL after sulpiride treatment; the levels became significantly increased within 20 min of the injection and remained elevated for 7h before declining to baseline (Fig. 6.10 .

6.7 DISCUSSION

The studies described in this chapter illustrate that acute and

chronic treatment with bromocriptine inhibited ß-END and PRL secretion; the effect on ß-END was not so dramatic as for PRL but was evident especially under short days when endogenous secretion is increased (experiment 16 and 17). These results provide evidence for the involvement of DA in the control of ß-END and PRL secretion in the Soay ram, and are consistent with the studies in Merino sheep (Engler *et al.*, 1989a; Smith *et al.*, 1989). The observation that both ß-END and PRL were inhibited by bromocriptine treatment suggests that the action of DA on these two pituitary hormones is mediated by D_2 receptors. Support for this view is derived from studies in which both cAMP production and α–MSH secretion from the intermediate lobe cells were measured in response to both D_1- and D_2-receptor-specific dopaminergic analogues (Munemura *et al.*, 1980b; Beaulieu, Felder & Kebabian, 1986). These studies clearly demonstrated that the inhibitory effect of bromocriptine on α-MSH secretion is a D_2-receptor-mediated event. Since α–MSH and ß-END are co-secreted from the intermediate lobe cells, it is reasonable to conclude that D_2 receptors are involved in ß-END secretion. With regard to PRL, recent evidence has shown that the capacity of bromocriptine to reduce phosphoinositide phosphorylation is a unique property of the D_2-receptor, representing an aspect of the chronic mechanism by which DA inhibits PRL release (Jarvis, Judd & MacLeod, 1988).

The wide spectrum DA antagonist, pimozide, increased ß-END and PRL secretion both under long and short days (experiment 14). The concomitant increase in the secretion of ß-END and PRL after pimozide treatment indicates that endogenous DA inhibits the secretion of these two pituitary hormones; which is consistent with previous studies in other breeds of sheep (Meyer & Goodman, 1985). The more specific D_2 receptor antagonist, sulpiride, also increased ß-END and PRL secretion both under

long and short days, which provides more evidence for the role of DA in the control of ß-END and PRL secretion (experiment 18). These results also render further proof that the D_2 receptor is involved in the regulation of ß-END and PRL secretion in the Soay ram.

The i.v. injections of DA had no effect on ß-END and PRL secretion both under long and short days (experiments 14 and 15). This lack of effect by exogenous DA could have been due to degradation of the amine before reaching its receptors. Previous studies have shown that it may be necessary to use degradation enzyme blockers or antioxidants such as pargyline and ascorbic acid (as done in *in vitro* studies) in order to observe an effect (Lewis, Dieguez, Lewis and Scanlon, 1987; Jarvis *et al.*, 1988). Alternatively, the lack of effect by DA on ß-END and PRL secretion could have been due to the mode of DA administration. DA infused every min, at a dose similar to that used in experiment 14 and without the addition of enzyme blockers or antioxidants, has been shown to significantly reduce the plasma concentrations of PRL in crossbred sheep (Deaver & Dailey, 1982). Furthermore, Thomas, Cummins, Smythe, Gleeson & Clarke (1986) have shown a 70% reduction in plasma levels of PRL in hypothalamo-pituitary disconnected Merino sheep infused with DA every min. Thus, the possibility that DA administered every min instead of every 10 min may have been effective cannot be discounted.

The ß-END in the peripheral circulation is derived from both the anterior and intermediate lobes of the pituitary gland, and it is likely that DA affects the secretion in both lobes. Hypothalamic DA neurons are known to regulate circulating levels of ß-END by directly inhibiting hormone secretion from the intermediate lobe (Chen *et al.*, 1983; Farah & Mueller, 1989). In contrast, the anterior lobe is not directly innervated by dopaminergic neurons (Vermes *et al.*, 1980; Farah & Mueller, 1989); thus, the action of DA on the

anterior lobe is mediated either humorally via the hypohysial portal system or centrally within the central nervous system. So far no DA receptors have been identified on anterior lobe corticotrophs that secrete ß-END, and a very recent study has reported an absence of DA in the hypophysial portal blood in sheep (Thomas *et al.*, 1989). These two observations rule out the humoral action of DA in sheep. Therefore, the influence of DA on anterior lobe corticotrophs must be centrally mediated by its action on some other hypothalamic substances, most likely AVP and CRF, the two principal physiological regulators of ß-END secretion from the anterior lobe. In support of this view is the report that DA inhibits the secretion of CRF *in vitro* in the rat (Hillhouse, Burden & Jones, 1975). Moreover, it has also been demonstrated in the rat that the ability of the D_2 DA agonist, LY 141865, to influence circulating levels of ß-END is blocked by pretreatment with dexamethasone, which predominantly inhibits anterior lobe but not intermediate lobe secretion of POMC-derived peptides (Farah & Mueller, 1989). This is a central action mediated by the CRF pathway.

The studies also illustrate that there was no evidence for opioids involvement in the control of ß-END and PRL secretion as judged by the administration of naloxone alone. However, they provide evidence that an interaction between naloxone and pimozide occurred, indicating that opioids play an inhibitory role in ß-END and PRL secretion in the Soay ram.. An interaction between opioids and DA on PRL secretion has previously been reported in the rat, involving both a stimulatory and inhibitory action. For example, administration of opioids systemically, i.c.v. or into discrete brain regions elevates hypophysial portal blood levels of DA and suppresses the plasma levels of PRL (Haskins *et al.*, 1981; Johnson, 1982; Reymond *et al.*, 1983). On the other hand, opioids have been shown to inhibit the turnover, synthesis and release of DA in the hypothalamus (Deyo *et al.*, 1979; Van Loon

et al., 1980a; Wilkes and Yen, 1980), with a resultant increase in PRL secretion (Van Loon *et al*, 1980b). Thus, opioids appear to modulate the effect of DA on ß-END and PRL secretion.

Stress increased ß-END and PRL secretion before the chronic treatment with bromocriptine (experiment 17). After the treatment, however, the stress-induced increase in PRL secretion was inhibited while that in ß-END secretion was not blocked. This indicates differential control of ß-END and PRL systems and may partially explain the lack of correlation between the cycle in ß-END secretion and that in PRL secretion. The anterior lobe corticotrophs are stimulated by a number of hypothalamic factors such as AVP, CRF, oxytocin and adrenaline which are secreted into the hypophysial portal circulation in response to stress (Plotsky *et al.*, 1985; Antoni, 1986). In contrast, the intermediate lobe melanotrophs are stimulated by increases in circulating adrenaline that occur during stress, and this effect is mediated via the ß-adrenergic receptors (Antoni, 1986). The stress-induced increase in ß-END secretion could have involved any of these factors. The view that AVP and CRF were involved is supported by the recent study of Engler *et al.* (1989b) who demonstrated that a 3-min audiovisual stress (barking dog) rapidly increased the concentrations of AVP and CRF in the hypohysial portal circulation of conscious sheep. However, the observation by Donald *et al.* (1983) that CRF does not affect PRL secretion suggests the involvement of other hypothalamic factors in the PRL response to stress. This conclusion is partially supported, again, by the studies of Engler *et al.* (1989b) who noted an absence of a strict 1:1 concordance between hypothalamic AVP/CRF release and that of ACTH secretion during stress. These workers concluded from this observation that the release of additional hypothalamic ACTH secretagogues were partly responsible for this lack of concordance. Therefore, there is a strong possibility that heterogeneous hypothalamic responses were elicited by the sheep dog to increase the secretion of both

ß-END and PRL in the current study.

The series of experiments described in this chapter was carried out both under long and short days to establish whether the inhibitory action of DA and the modulatory influence of endogenous opioids change relative to a change in photoperiod. The acute administration of pimozide caused a greater increase in the secretion of ß-END and PRL under long days than under short days, which may suggest a greater inhibitory influence of endogenous DA over ß-END and PRL secretion under long days. While this may be true for ß-END, accounting for the low plasma levels of ß-END observed under long days, it is not true for PRL since there are higher plasma levels of PRL under long days than under short days. The administration of sulpiride caused a similar response in ß-END secretion both under long and short days; however, the response in PRL secretion was greater and more sustained under long days than under short days. Furthermore, the injection of naloxone at the same time as pimozide significantly increased the plasma concentrations of ß-END and PRL under short days but not under long days. This effect suggests a greater inhibitory influence of endogenous opioids on ß-END and PRL secretion under short days. However, as for the effect of pimozide, this seems to be true only for PRL secretion, probably accounting, in part, for the low plasma levels of PRL observed under short days, but it does not appear to be true for ß-END since there are higher plasma levels of ß-END under short days than under long days. These observations indicate that the action of endogenous DA and its interaction with endogenous opioids on ß-END and PRL secretion as related to photoperiod appears to involve more complex mechanisms on a long-term basis, which may further highlight the lack of correlation between the cycle in ß-END secretion and that in PRL secretion.

6.8 SUMMARY

This third series of experiments has established the following:

1. Bromocriptine (specific D_2 agonist) inhibits the secretion of ß-END and PRL in the Soay ram, and the response in ß-END secretion is greater under short days while that in PRL secretion is greater under long days.

2. Pimozide (non-specific DA antagonist) increases the secretion of ß-END and PRL and the responses are greater under long days than under short days.

3. Sulpiride (specific D2 antagonist) increases the secretion of ß-END and PRL, and the response in ß-END secretion is similar under long and short days for ß-END while that in PRL secretion is greater under long days than under short days.

4. Endogenous opioids interact with DA under short days to increase the secretion of ß-END and PRL.

5. Acute stress increases the secretion of ß-END and PRL, and the responses are greater under long days than under short days.

CHAPTER 7

GENERAL DISCUSSION

The current study demonstrates that Soay rams show a conspicuous seasonal cycle in the peripheral plasma concentrations of ß-END; the levels of ß-END are 10-to-20 times higher in summer and autumn than in winter and spring, which is consistent with a pattern previously described for Soay rams (Ebling and Lincoln, 1987). Merino rams do not show a seasonal cycle in ß-END secretion and crossbred rams, produced by mating Soay ewes with a Merino ram, show a seasonal cycle in ß-END secretion intermediate in amplitude between that characterisctic of the parents, demonstrating the genetic basis for the differences between the Soay and Merino breeds. The breed variation reflects a different degree of seasonality between the Soay and Merino sheep. The Soay sheep are semi-domesticated and show marked seasonality in physiology and behaviour like their ancestors, the wild Mouflons. They are very seasonal in appetite, body growth, moulting, fattening and reproduction (Ryder, 1971; Argo and Smith, 1983; Lincoln and Ebling, 1985; Ebling and Lincoln, 1987; Lincoln, 1989b,c). On the other hand, Merino sheep are domesticated animals originating from the Mediterranian areas, and are selected for production of fine wool, which grows continuously throughout the year. They do not show a seasonal cycle in moulting and also have a less conspicuous seasonality in reproduction (Amir and Volcani, 1965; Barrell and Lapwood, 1978/1979; Ryder, 1983; Lincoln, 1989c). The putative role of ß-END in the control of aspects of metabolism fits in with the breed differences. In the Soay sheep, the plasma levels of ß-END peak in autumn, coinciding with a phase of maximum body weight and fattening. This is an adaptation in which ß-END participates to regulate body resources and energy in order for the animals to survive through winter when food supply is limited.

The studies on outdoor rams revealed that there is a seasonal cycle in the peripheral plasma concentrations of ACTH and cortisol; the levels were minimum in winter and maximum in summer, about 2 months in advance of the peak in ß-END secretion. Furthermore, the studies involving the treatments

with AVP, CRF, dexamethasone and RU 486 indicated a parallel control of ß-END and ACTH. AVP and CRF stimulated the secretion of both ß-END and ACTH while dexamethasone suppressed and RU 486 enhanced their secretion. The seasonal changes in the responses to all these treatments occurred in parallel for both ß-END and ACTH, and a close analysis of endogenous secretion revealed a temporal correlation between some of the endogenous pulses in the plasma profiles of ß-END and those of ACTH secretion. All these observations provide evidence that ß-END and ACTH are cosecreted from the corticotrophs of the anterior pituitary gland. This is consistent with the observation that most circulating ß-END is non-acetylated as released from the anterior pituitary gland (Antoni, 1986; Smith and Funder, 1988). Since the pituitary gland contains higher amounts of ß-END than the brain and peripheral tissues (Ebling and Lincoln, 1987; Ebling *et al.*, 1987; Smith and Funder, 1988), the major source of circulating ß-END in the Soay ram seems to be the anterior pituitary gland.

The control of the secretory activity of the anterior lobe corticotrophs involves stimulation by AVP and CRF from the hypothalamus and inhibition by glucocorticoids from the adrenal gland. The seasonal cycle in ß-END secretion may, therefore, be dictated by a seasonal change in the 'drive' from the hypothalamus which stimulates an increase in the secretory activity of corticotrophs in summer and autumn, or may be due to a seasonal change in the negative feedback control from the adrenal gland which inhibits the secretory activity of corticotrophs in winter and spring. However, the control of the seasonal cycle in ß-END secretion is apparently more complex as this simple interpretation does not account, for example, the notable difference in the timing of the seasonal cycle in ß-END and ACTH secretion. The seasonal increase in the plasma concentrations of ACTH occurred in advance of that for ß-END, and the seasonal maximum was 2 months earlier in the year. This

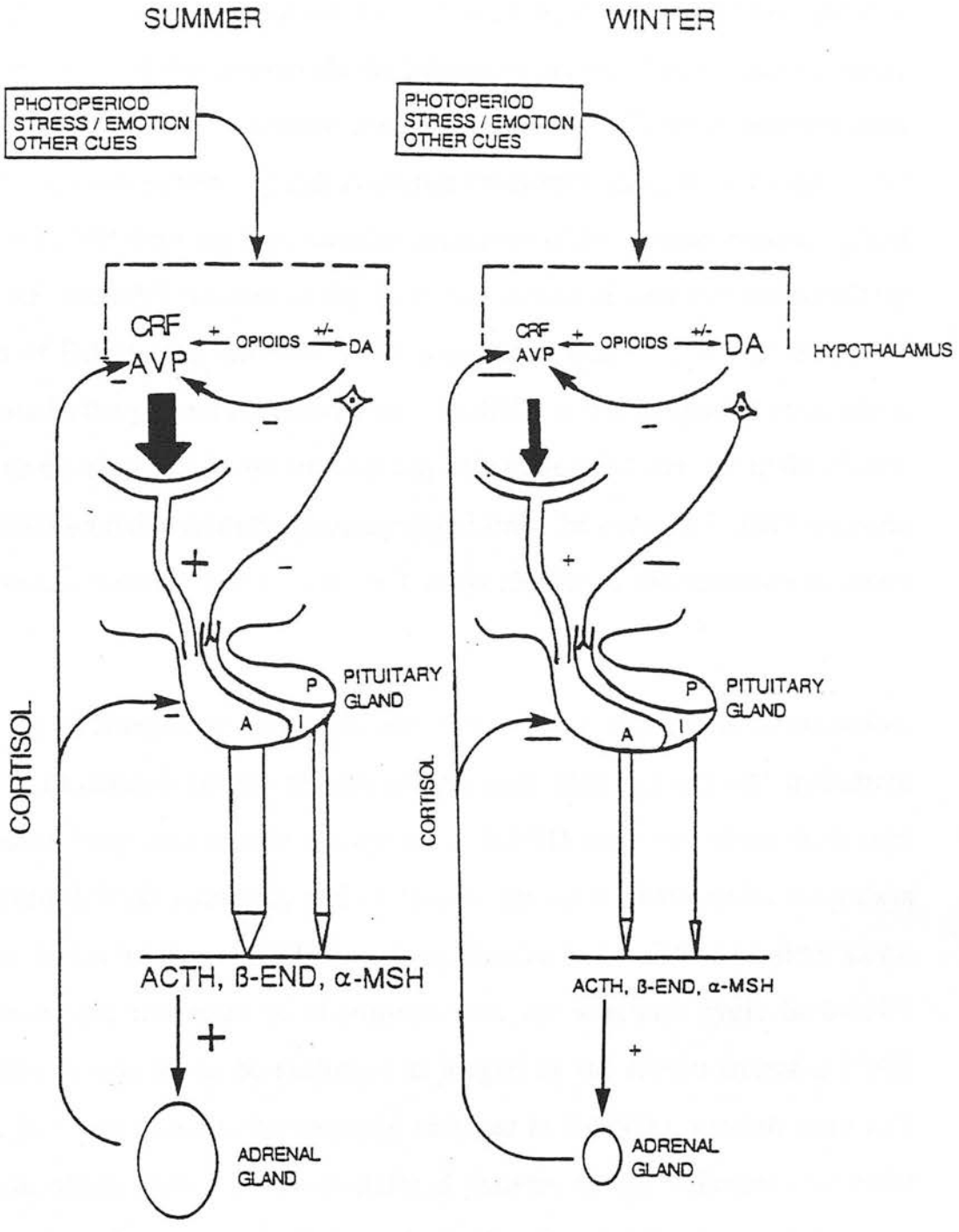
difference in the release of the two anterior pituitary hormones may involve changes in the activity of the corticotrophs which preferentially secrete ACTH in summer and ß-END in autumn. This may result from a seasonal change in the processing of the POMC precursor in the corticotrophs related to the level of hypothalamic stimulation and glucocorticoid feedback, or a seasonal change in the subpopulations of corticotrophs which may respond to a change in the ratio of AVP:CRF to secrete more ß-END or ACTH. An alternative explanation for the differential regulation, is that there is a separate control of the corticotrophs in the anterior pituitary gland which secrete ACTH in summer, and the melanotrophs in the intermediate lobe of the pituitary gland which secrete ß-END in the autumn.

The melanotrophs in the intermediate lobe of the pituitary gland form an additional contribution to the circulating pool of ß-END. They are under the inhibitory control of DA (Farah *et al.*, 1982; Chen *et al.*, 1983; Newman *et al.*, 1987; Smith *et al.*, 1989). The current studies, using the DA agonist, bromocriptine, and the DA antagonists, pimozide and sulpiride, illustrate the role of DA in the inhibition of ß-END secretion. Bromocriptine decreased the plasma concentrations of ß-END while both pimozide and sulpiride increased ß-END secretion. The results obtained with the specific D_2 receptor DA antagonist, sulpiride, provide further evidence as to which DA receptor type is involved in the action of DA. Thus, it is likely that the DA system is involved in the control of the secretory activity of melanotrophs in the Soay ram, and a seasonal change in the activity of hypothalamic dopaminergic neurons may influence the seasonal cycle in ß-END secretion by causing differential secretion of ß-END from melanotrophs as opposed to the secretion from the corticotrophs. Detailed studies of the short-term fluctuations in the plasma concentrations of ß-END, ACTH and α-MSH in Merino sheep have also indicated a complex differential regulation of these

hormones. In particular, only a proportion (about 30%) of the episodic peaks in the plasma concentrations of ß-END was correlated with peaks in the plasma concentrations of ACTH and some peaks in ACTH occurred with no corresponding increase in either ß-END or α–MSH (Engler *et al.*,1989a). These temporal associations indicate that the hormone concentrations in the peripheral blood is the result of the combined secretion from the corticotrophs and melanotrophs which process the POMC precursor in the two lobes of the pituitary gland.

Based on the current results and a survey of the relevant literature, a model is proposed to explain the control of the seasonal cycle in ß-END secretion in the Soay ram, and a simplistic illustrtation is given in Fig. 7.1. In this model, hypothalamic AVP, CRF and DA, and adrenal cortisol are involved. On one hand, AVP and CRF stimulate the release of ß-END and ACTH from the anterior pituitary gland. ACTH, but not ß-END, stimulates the adrenal gland to release cortisol, which, in turn, negatively regulates the secretion of ß-END and ACTH from the anterior pituitary gland as well as inhibiting the release of AVP and CRF from the hypothalamus. On the other hand, DA inhibits the release of ß-END and α-MSH from the intermediate pituitary gland. Within the hypothalamus, there is a possibility of DA influencing the secretion of ß-END by inhibiting the release of AVP and CRF (Farah *et al.*, 1982; Murburg *et al.*, 1986), and endogenous opioids may control the secretion of ß-END by modulating the release of AVP, CRF and DA from the hypothalamus (Buckingham, 1982; Buckingham and Cooper, 1984; Murburg *et al.*, 1986). For example, opiates have been shown to stimulate CRF (Buckingham and Cooper, 1986), and to either stimulate (Haskins *et al.*, 1981; Johnson, 1982) or inhibit (Deyo *et al.*, 1979; Wilkes and Yen, 1980; Reymond *et al.*, 1983) the release of DA. The extent to which these different mechanisms participate in the control of ß-END secretion apparently vary relative to a

Fig. 7.1. A model proposed to explain the control of the seasonal cycle in ß-endorphin secretion in the Soay ram, based on the current study and the relevant literature. i) Hypothalamic AVP and CRF stimulate the secretion of ß-endorphin and ACTH from the anterior pituitary gland., ACTH stimulates the adrenal gland to secrete cortisol, which, in turn, directly inhibits the secretion of ß-enorphin and ACTH from the pituitary gland and indirectly inhibits he release of AVP and CRF from the hypothalamus. ii) Dopamine inhibits the secretion of ß-endorphin from the intermediate pituitary gland. and within the hypothalamus, there is a possibility of dopamine influencing the secretion of ß-endorphin from the anterior pituitary gland by inhibiting the release of AVP and CRF. Endogenous opioid peptides may also influence the secretion of ß-endorphin by modulating the release of AVP, CRF and dopamine from the hypothalamus. The extent to which these different mechanisms participate in the control of ß-endorphin secretion varies between summer and winter relative to the change from summer and winter. In summer, the hypothalamus releases a lot of CRF and AVP which cause greater secretion of ß-endorphin and ACTH from the anterior pituitary gland. The ACTH stimulates greater quantities of cortisol which inhibit ß-endorphin and ACTH secretion at the pituitary and hypothalamic levels. The stimulatory hypothalamic drive seems to override the inhibitory action of cortisol in summer. Dopamine is less inhibitory in summer, leading to some ß-endorphin being secreted from the intermediate pituitary gland. A change from summer to winter reduces the CRF and AVP drive but increases the sensitivity of the anterior pituitary gland and CRF and AVP neurons to the inhibitory action of cortisol; the inhibitory action of dopamine is also increased from summer to winter. The end result is a reduction in the plasma concentrations of ß-endorphin in the peripheral circulation. A= anterior; I= intermediate; P= posterior.


SUMMER
WINTER
PHOTOPERIOD
STRESS / EMOTION
OTHER CUES
PHOTOPERIOD
STRESS / EMOTION
OTHER CUES
CRF
AVP
OPIOIDS
DA
CRF
AVP
OPIOIDS
DA
HYPOTHALAMUS
PITUITARY
GLAND
PITUITARY
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I
CORTISOL
CORTISOL
ACTH, ß-END, α-MSH
ACTH, ß-END, α-MSH
ADRENAL
GLAND
ADRENAL
GLAND

change from summer to winter as illustrated in Fig. 7.1. In summer, the hypothalamus releases a lot of CRF and AVP which cause greater secretion of ß-END and ACTH from the anterior pituitary gland. The ACTH stimulates greater quantities of cortisol which inhibits the secretion of ß-END and ACTH at the pituitary and hypothalamic levels. The stimulatory hypothalamic drive of CRF and AVP seems to override the inhibitory action of cortisol in summer. DA is less inhibitory in summer, leading to some ß-END being secreted from the intermediate pituitary gland. A change from summer to winter reduces the CRF and AVP drive but increases the sensitivity of the anterior pituitary gland and CRF and AVP neurons to the inhibitory action of cortisol; the inhibitory action of DA is also increased from summer to winter. The end result is a decrease in the plasma concentrations of ß-END in the peripheral circulation. The current study could not attribute any role to gonadal steroids in the control of ß-END secretion from the pituitary gland since the cycle in ß-END secretion occurred in castrate and castrate + T rams similar to the situation in intact rams.

Photoperiod apparently acts to time the cycle in ß-END secretion. This is illustrated by the effects of artificial photoperiod and melatonin treatment; long days caused a decrease in ß-END secretion while short days increased ß-END secretion, and melatonin implants given under long days caused an increase in ß-END secretion, similar to the effect of short days. However, the influence of photoperiod on the seasonal cycle in ß-END secretion seems not to be exclusive as judged by the results from the PINX rams. In these animals, the seasonal chnages in ß-END secretion were still evident, albeit with a less well defined pattern, which indicates that other environmental cues were operative. A similar situation has been observed in PINX and SCGX rams with respect to long-term cycles in pituitary and testicular activity (Lincoln *et al.*, 1989a). In this situation, the animals are

apparently unable to respond to changes in photoperiod in a normal way due to the loss of a functional pineal gland but they remain competent to respond to other environmental cues such as changes in nutrition and temperature.

Another environmental factor that influences the secretion of ß-END is that of stress. The exposure of rams to the sheep dog for 5 min significantly increased the plasma concentrations of ß-END, indicating an immediate activation of the hypothalamo-pituitary axis. Stressful farm procedures such as handling, transport, electroimmobilization and shearing are routinely experienced by sheep. These procedures cause acute stress which is associated with increased levels of ß-END both in cerebrospinal fluid and in the peripheral circulation (Nakao *et al.*, 1979; Jephcott *et al.*, 1987; Fordham *et al.*, 1989); and it has been proposed that secretion of ß-END during stress may be part of the mechanism of stress-induced pain suppression (Akil *et al.*, 1978; Shiomi and Akil, 1982; Terenius, 1982). The stress-induced release of ß-END is associated with the activation of the paraventricular hypothalamus, which releases a number of neuromodulatory factors, most importantly AVP and CRF, that regulate the secretory activity of the pituitary gland (Plotsky *et al.*, 1985; Antoni, 1986; Engler *et al.*, 1989b). In a seasonal environment, there are other environmental factors such as coldness, wettness and lack of food, which may have a chronic influence on the hypothalamo-pituitary axis. These various factors may also constitute a long-term environmental control over the seasonal cycle in ß-END secretion by acting via the hypothalamus.

In the Soay ram, the plasma concentrations of ß-END are high in summer and autumn and are low in winter and spring. The current evidence is consistent with the control of the seasonal cycle in ß-END secretion by the hypothalamus; AVP and CRF stimulate ß-END secretion from the anterior lobe corticotrophs while DA inhibits ß-END release from the intermediate lobe melanotrophs. However, the extent to which these two systems are

involved in the seasonality of ß-END secretion is not clear. For example, are the marked seasonal changes in ß-END secretion largely due to seasonal changes in the concentrations of AVP and CRF in the hypophysial portal system, or are they due to differential processing of the POMC precursor by the corticotrophs and melanotrophs at different times of the year?. To dissociate these issues, it will be necessary to obtain more detailed evidence, for example, on the following. First, on the peptides present in the hypophysial portal blood system linking the hypothalamus to the pituitary gland. The prediction is that there is an increase in AVP and CRF from spring to autumn which constitutes the drive to ß-END increase at this time of the year. Second, on the pattern of secretion of ß-END, ACTH, α-MSH and Nac-ß-END in the peripheral circulation at different times of the year, and to correlate the peripheral pattern of secretion with the seasonal changes in the concentrations of AVP and CRF in the hypophysial portal system. Third, on the effects of differentially manipulating the content or secretion of corticotrophs and melanotrophs, for example, by using specific cytotoxic techniques which selectively eliminate one cell type and leave the other type (Schwartz, Penke, Rivier and Vale, 1987; Schwartz and Vale, 1988), or by carrying out selective adeno- and neurointermedio-hypophysectomy (Przewlocki, Millan, Gramsch, Millan and Herz, 1982).

In conclusion, the studies described in this thesis have demonstrated that there is a clearly defined cycle in ß-END secretion in the peripheral blood of the Soay ram. The control of this cycle involves complex mechanisms, including hypothalamic AVP, CRF, DA and endogenous opioid peptides, and adrenal glucocorticoids. Photoperiod and other environmental factors act to influence the hypothalamic mechanisms and, thus, modulate ß-END secretion.

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