STUDIES ON THE INNERVATION OF THE OVINE PINEAL AND THE REGULATION OF MELATONIN SECRETION

A thesis presented in partial fulfilment of the requirements for the

Degree of Doctor of Philosophy in Physiology

at Massey University

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1991

Thesis Abstract

The studies described in this thesis were designed to investigate the neural regulation of melatonin secretion from the ovine pineal gland.

Initial studies sought to establish the effects of a range of anaesthetics on the nocturnal rise in plasma melatonin levels in Romney rams and to identify the anaesthetic treatment most suitable for use in future acute studies; this was found to be halothane induction and maintenance.

To investigate the neural mediation of melatonin secretion from the ram pineal, a series of experiments was performed in which the pre-ganglionic sympathetic innervation was stimulated electrically. Acute stimulation during the night or day resulted in an immediate, sustained rise in plasma melatonin levels, with nighttime responses being significantly greater than daytime responses ($P<0.05$).

Similar studies in conscious rams were subsequently made possible by the development of a cuff electrode which could be implanted around the CST's and remain functional for at least six weeks. In these studies it was demonstrated that photoperiod (16L:8D or 8L:16D) did not influence melatonin output, while responsiveness to stimulation was highest during the middle, rather than at the beginning or end, of the photoperiod.

The second study employing chronically implanted electrodes was designed to evaluate the influence of various parameters of continuously applied CST stimulation on the pineal's melatonin secretory response. Unexpectedly, it was found that only small rises in plasma melatonin levels resulted from stimulation with any of the combinations of stimulus parameters tested and that no one combination was significantly more effective than any other in promoting increases in plasma melatonin levels. These findings appear to have resulted from inadequate performance of the electrodes and/or nerve damage.

The final study undertaken in this thesis was designed to identify the innervation of ovine pineal by immunocytochemically localizing the neuropeptides NPY & VIP, and the enzymes NSE & PNMT. All four antigens were observed in intrapineal nerve fibres, while NSE was also present in pinealocytes. SCGX reduced, but did not eliminate, this immunoreactivity suggesting that both central and peripheral regions innervate the pineal and that partial denervation initiates a neural compensatory mechanism.

Preface

Some material from Chapters 3 & 5 is contained in the following papers:

(1) **B.G. Mockett, K.R. Lapwood, R.J. Pack & D.H. Carr** (1991) Induction of pineal melatonin secretion in anaesthetized rams by bilateral electrical stimulation of the cervical sympathetic trunks. *Proceedings of the Physiological Society of New Zealand*, pp 32-33

(2) **B.G. Mockett, K.R. Lapwood, R.J. Pack & D.H. Carr** (1991) Innervation of the ovine pineal gland and the regulation of melatonin secretion: An electro-physiological and immunocytochemical study. *Advances in Pineal Research,* Vol 6, 13-16

(3) **B.G. Mockett, K.R. Lapwood, R.J. Pack & D.H. Carr** (1991) Regulation of ovine pineal gland function: An electrophysiological and immunocytochemical study. Proceedings of the *International Symposium on Pineal Hormones, Bowral, Australia.* pp 51.

Copies of these are contained in an envelope inside the back cover of this thesis.

Acknowledgements

I wish to gratefully acknowledge the help and guidance generously provided by my chief supervisor, Dr K.R. Lapwood, whose knowledge and understanding of pineal physiology and experimental design made the work presented in this thesis possible.

Further gratitude is due to Drs R.J. Pack and D.H. Carr for their roles as supervisors. I would particularly like to thank Dr Pack for his assistance in the application of the electrophysiological methods and Dr Carr for his skilful execution of the surgical procedures required for the nerve stimulation studies. Thanks are also due to Prof. R.E. Munford for his significant contribution to the statistical analyses of experimental data. Professor Munford initially, and later Professor D.J. Mellor, are acknowledged for provision of facilities within the Department of Physiology and Anatomy.

I would also like to thank Mr J. Elgar for his perseverance and interest whilst I was learning the intricacies of radioimmunoassay methodology. Mr M. Birtles is thanked for his valuable advise in the application of immunocytochemical procedures, as is Mr R. Sparksman for his assistance in the preparation of tissue and the performance of some immunocytochemistry. Thanks are also due to Mrs I. Hall for her technical assistance with the surgical procedures, and to Mr T.G. Law for the processing of photographic slides from which the prints in this thesis were taken. Appreciation is extended to Miss D. Anthony for producing much of the illustration work presented in the Chapter 2.

The kind gift of melatonin antiserum by Dr D.J. Kennaway (University of Adelaide, South Australia) is gratefully acknowledged.

Special thanks are due to Drs A.S. Davies and P.S. Davie who gave moral support in times of need and who freely gave of their time and energy to aid the completion of this thesis. I would also like to thank V. Davie for assistance with some of the illustration work.

The financial assistance of the Massey University Research Fund is gratefully acknowledged.

I would like to extend a special thanks to my good friend, Allan Nutman, who provided invaluable help and advice whilst engaged in his own demanding studies. Your help will also be remembered.

Finally, I would like to extend my deepest gratitude to my wife, Debbie, whose patience and perseverance has enabled me to complete these studies.

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List of Abbreviations

5-HT	Serotonin
ACh	Acetylcholine
APUD	Amine precursor uptake and decarboxylation
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
AVT	Arginine vasotocin
cAMP	cyclic Adenosine monophosphate
cGMP	cyclic Guanylate
CNS	Central nervous system
CSF	Cerebrospinal fluid
CST	Cervical sympathetic trunk
CV	Coefficient of Variation
DAB	Diaminobenzidine
DßH	Dopamine ß hydroxylase
d.f.	degrees of freedom
ECN	External carotid nerve
FSH	Follicle stimulating hormone
GABA	Gamma-aminobutyric acid
GnRH	Gonadotrophin releasing hormone
HCl	Hydrochloric acid
H/H	Halothane induction/halothane maintenance
HIOMT	Hydroxyindole O-methyltransferase
HNO_3	Nitric acid
hr	hour
ICC	Immunocytochemistry
ICN	Internal carotid nerve
ID	Internal diameter
IR	Immunoreactive
IU	International Units
LH	Luteinizing hormone
M	Molar
min	minute
NaCl	Sodium chloride
NAT	N-acetyltransferase
NPY	Neuropeptide-Y
NPY-LI	Neuropeptide-Y-like immunoreactivity
NSE	Neuron specific enolase
NSE-LI	Neuron specific enolase-like immunoreactivity
OD	Outside diameter
OXT	Oxytocin
PNMT	Phenylethanolamine N-methyltransferase
PNMT-LI	Phenylethanolamine N-methyltransferase-like immunoreactivity
PrL	Prolactin
PVN	Paraventricular nuclei
RIA	Radioimmunoassay
SCG	Superior cervical ganglia
SCGX	Superior cervical ganglionectomy
SCN	Suprachiasmatic nuclei
S.E.M	Standard error of the mean
S/H	Saffan induction/halothane maintenance
TH	Tyrosine hydroxylase
VIP	Vasoactive intestinal polypeptide
VIP-LI	Vasoactive intestinal polypeptide-like immunoreactivity

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CHAPTER 1

Introduction

1.1 Seasonality and survival

Temperate zone animals experience significant seasonal fluctuations in climatic and environmental factors, which, without adaptation, threatens their ability to survive and reproduce (Bronson, 1988). Seasonal reproduction is one of the more conspicuous adaptative changes that can be seen in most species at mid-latitudes. In order to successfully raise their offspring seasonal breeders typically give birth in the early spring at a time when environmental conditions favour the survival and growth of young and parent alike (Karsch et al, 1984). To this end, accurate timing of parturition requires that breeding activity occurs during a clearly defined time of the year determined, in evolutionary terms, by the length of gestation characteristic of each species (Rowlands & Weir, 1984). A rhythm of reproduction, intrinsic in nature, but modified by external factors, is therefore central to the timing of events associated with seasonal reproduction (Malpaux et al, 1988b). Cyclic changes in daily photoperiod, temperature, pheromones and nutrition are recognised as important environmental cues responsible for timing activities such as sexual behaviour, and physical development in a wide variety of mammals (Stonehouse, 1981; Rowlands & Weir, 1984). Both circadian and circannual rhythms in the duration of daily photoperiods have been shown to be the major factors influencing the timing of reproductive activity in almost all seasonally breeding mammalian species. Central to this function is the role of the pineal gland in transducing photoperiodic signals into endocrine responses, which act to modify the reproductive status of the individual (Karsch et al, 1984).

An initial interest in this aspect of neuroendocrinology thus led to the initiation of this thesis and the design of a series of experiments to investigate the neural control of pineal secretory activity in sheep. Since seasonal reproduction is mainly influenced by environmental photoperiod and hence the pineal, it is appropriate to review the endocrine control of reproductive seasonality and its mediation by melatonin, the pineal's principal secretory product.

1.1.1 Seasonal adaptative changes mediated by the pineal.

The timing of reproductive events is perhaps the most studied of seasonal physiological changes. Pineal-mediated seasonal reproductive changes occur through modification of the

pattern of gonadotrophin secretion (refer Section 1.3) and certainly include the onset of the breeding season (Kennaway, 1984; Robinson et al, 1985a), timing of puberty (Rodway et al, 1985; Yellon and Foster, 1985, 1986), and may also include pregnancy (Kennaway, 1984; Lew, 1987; Yellon and Longo, 1987) and the oestrous cycle (Kennaway, 1984). Other pineal-mediated changes are related to, although not totally dependent on, changes in levels of gonadal steroid hormones. These include changes in body weight, fat deposition and daily torpor, as studied in hamsters (Wade and Bartness, 1984; Vitale et al, 1985), induction of moult in Soay rams (Lincoln & Ebling, 1985), red deer (Kay & Ryder, 1978) and mink (Allain & Rougeot, 1980), antler growth and shedding in deer (Suttie et al, 1984) and hibernation in ground squirrels (Stanton et al, 1986, 1987).

1.1.2 Short and long day breeders.[1]

Seasonally breeding animals which use photoperiod to time their reproductive activity can generally be divided into two groups - short and long day breeders. Short day breeders, such as sheep, use the decreasing daily photoperiod of autumn to time the initiation of breeding activity. These animals generally have longer gestations and give birth the following spring. In contrast, long day breeders, such as rodents, initiate breeding during the longer days of summer and generally give birth and raise their young before the onset of winter (Nalbandov, 1976). Horses are unusual in this regard in that, although they have a well defined season of sexual activity during the long days of spring and summer, their 11 month gestation period means they do not give birth until the following spring/summer, after which they rapidly resume normal reproductive activity (Rowlands & Weir, 1984). These variations in response to changing photoperiods are adaptations related to the length of gestation of each species and ensure the spring/summer birth of offspring.

1.1.3 History of Pineal research

According to Kappers (1979) and Oksche (1984), in their reviews, the pineal gland in man was first discovered around 300 BC by Herophilas, a famous anatomist at the University of Alexandria in Egypt. Little true knowledge was gained about the pineal until the beginning of the twentieth century, although speculation, influenced by the prevailing philosophical systems and tradition, attributed many roles to it. One of the earliest scientific pineal studies,

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[1] The terms 'length of day' and 'daylength' as used in this field of research refer to the period of light occurring within each 24 hr day.

suggesting a secretory function, was carried out by McCord and Allen (1917), who demonstrated that bovine pineal extracts blanched the skin of frogs. Such a function was finally confirmed when Lerner and his colleagues (1958, 1960) discovered the principal hormone of the pineal and named it melatonin. Since then many functions, both speculative and established, have been attributed to the pineal gland of mammals. Included in these is the well established participation in the timing of seasonal reproduction. Other functions, as outlined below, involved a wide range of tissues and organs which were often essential for the animal's survival of seasonal environmental changes. These include facilitation of the onset or maintenance of sleep (Wurtman & Lieberman, 1985), reduction of glucose-stimulated insulin release from the pancreas (Nir, 1978), a possible role in parturition (Nir & Hirschmann, 1979; Kennaway, 1984), effects on locomotor activity and avoidance behaviour in rats, effects on the mental condition of humans, an inhibitory effect on the pituitary, a stimulatory effect on the thymus (Nir, 1978), a role in thermoregulation through adaptations such as pelage changes, nonshivering thermogenesis, brown adipose tissue adjustments, hibernation, daily torpor and coping with acute heat stress (see Heldmaier & Lynch, 1986 for review), and inhibitory effects on thyroid and adrenal gland functions (Nir, 1978; Lewinski, 1986). Other functions related to seasonal adaptive changes are described earlier in this section, while those functions expressed through the actions of pineal peptides are given in Section 1.5.2.

1.2 Reproductive seasonality in sheep.

Primitive breeds of sheep such as the Soay have breeding seasons which occur at precise times of the year and are of very restricted duration (2-3 months). Located on St Kilda, off the northwest coast of Scotland, Soay rams begin breeding activity with the rut which takes place in October-November; most ewes are sexually active and conceive during November and lambing begins in March, reaching a peak in April. Very little variation is seen in this pattern, which is likely to have evolved in response to harsh climatic conditions and limited food resources existing in this region (Lincoln & Short, 1980).

Modern (domesticated) sheep breeds have developed as the result of controlled breeding for meat, wool and fecundity (Carter & Cox, 1982). Despite continued efforts, however, no breed has been developed which will breed all year round (ie. which is aseasonal) or even twice yearly. Breeding activity (as described later in this section) is generally not as restricted as in the Soay, but is still confined to the autumn/winter period. Early research efforts by

workers such as Marshall (1937) and Hammond (1944) were the first to define the period of breeding activity as occurring during the autumn/winter months and to establish the importance of photoperiod in regulating the onset and termination of such activity.

The relationship between photoperiod and seasonal breeding in sheep is reviewed more fully in Sections 1.2.3 and 1.2.4.

1.2.1 Behavioural and physical characteristics of seasonality.

Both rams and ewes show a number of distinct behavioural and physical characteristics which are associated with breeding activity and sexual function. These and other aspects of reproductive seasonality in sheep are discussed in this section.

(I) **Behavioural**

(i) **Rams**

In rams, behavioural characteristics expressed during the breeding season are associated with elevated testosterone secretion from the testes. These include increased libido and inter-male aggression and the occurrence of flehmen - the raising of the upper lip in order to detect olfactory stimuli originating from vaginal secretions (Lincoln & Short, 1980).

Libido, as measured by the interest shown in sexual activity (Sanford et al, 1977; Dufour et al, 1984; Boland et al, 1985), decreases during the spring/summer period and increases during the short days of autumn and winter (Tulley & Burfening, 1983) to reach a maximum during the middle of the breeding season. Although this pattern is apparent in the majority of sheep breeds, significant differences have been found to exist between breeds in the magnitude of sexual activity at different times of the year, but not in the timing of changes in sexual activity relative to the breeding season (Dufour et al, 1984).

Inter-male aggression develops during the latter stages of summer and is particularly evident during the rut. For example, aggressive behaviour of Soay rams is first observed in July, shortly after the summer solstice and 3 months before rutting activity begins. Thereafter a linear increase in the aggressive behaviour index was observed up until the middle of the rutting period (November) when a gradual decline occurred, until minimal levels were reached during April. Inter-male aggression then remained at very low levels until the summer solstice had again passed (Lincoln & Davidson, 1977).

Flehmen is associated with short day stimulation of reproductive activity and is exhibited only when testosterone secretion is high. It is particularly evident when aggressive activity

peaks during the rut and continues to be expressed for 3-4 months, while plasma testosterone levels are declining (Lincoln & Davidson, 1977; Lincoln & Short, 1980).

(ii) **Ewes**

Females of most species generally do not engage in such active sexual behaviour, but do display more subtle signs of receptivity. Behavioural oestrus of ewes is the period of sexual receptivity during the oestrous cycle when she will accept service by rams and may actively seek their attention. Oestrus normally lasts for approximately 24 hrs and is associated with the pre-ovulatory rise in the level of oestrogen in the blood and changes in the reproductive tract (see later this section). Conspicuous signs of behavioural oestrus are absent and rams detect oestrous ewes by pheromonal signals from their vaginal secretions (Smith, 1982).

(II) **Physical and physiological changes associated with reproductive capacity**

a) **Rams**

Rams show a number of morphological characteristics, mostly related to sexual state, which fluctuate in a circannual rhythm. Testis size is greatest during the breeding season and least during sexual quiescence (Tulley & Burfening, 1983). Redevelopment of the testes begins well before the summer solstice and is completed during the rutting period, about 1 month before the winter solstice. Peak size is maintained for only a very short period of time (less than one week). Minimal size occurs approximately 3 months after the winter solstice and is maintained for a similarly short period of time (Lincoln & Short, 1980; Lincoln, 1989).

Change in testis size result from changes in the histological structure of the seminiferous tubules and coincide with changes in semen quality and quantity. Seasonal changes in the length of the seminiferous tubules occur in addition to the changes in diameter (Lincoln, 1989). During the period of full testicular development and maximal spermatogenic activity tubular diameter is at its greatest due to an increase in the number of epithelial germ cells undergoing mitosis and meiosis. When seasonal regression occurs germinal cells decline in number, spermatogenesis is inhibited and tubule length and diameter are reduced (Lincoln & Short, 1980; Lincoln, 1989). Sperm output and quality (motility and percentage of live spermatozoa) (Dufour et al, 1984; Boland et al, 1985), ejaculate volume (Sanford et al, 1977; Barrell & Lapwood, 1978/1979a; Boland et al, 1985) and seminal fructose levels (Barrell & Lapwood, 1978/1979a) are highest during the breeding season and lowest during sexual quiescence.

"Sexual flush" (cutaneous hyperaemia in the inguinal region) is most intense when blood testosterone concentrations are high in the breeding season and is not evident when concentrations are low (Lincoln, 1978b, 1989).

Other indications of sexual reproductive capacity such as epididymal size, penis sheath diameter and genital sensitivity to physical stimuli are greatest during the breeding season (Lincoln & Davidson, 1977).

b) **Ewes**

For ewes, the onset of breeding activity is not characterised by major physical changes as it is in rams. Physical changes which do occur do so in relationship to the oestrous cycle and are initiated by the cyclic changes in the ovarian hormone profiles. These include ovarian activation leading to follicle growth, ovulation and corpus luteum development, swelling of the uterus and vagina, an increase in the secretory activity of glandular tissue within these structures, and an increase in the secretion of mucus from the cervix (reviewed by Smith, 1982).

1.2.2 Ovine seasonal hormonal changes

(I) **Ram**

The initiation and cessation of reproductive activity in rams is a reflection of the changing secretory profiles of pituitary gonadotrophins and gonadal steroids. Temporal relationships between these hormones have been observed (in Soay rams) after an abrupt change from long photoperiods (under which levels of FSH, LH and testosterone are low and the testes have regressed to approximately 20% of their maximum size) to short photoperiods which stimulate sexual activity. Within 2-3 weeks gonadotrophin levels began to rise rapidly followed shortly thereafter by redevelopment of the testes and increases in plasma testosterone levels. FSH and LH levels peaked approximately three weeks later when the testes were still growing rapidly. Levels of both gonadotrophins exhibited a gradual decline following this peak while the size of the testes continued to increase and plasma testosterone levels became maximal after 14-16 weeks. Conversely, after an abrupt change to long photoperiods, testicular regression starts within 1 week followed by a rapid fall in testosterone levels. Within 11 weeks, plasma testosterone, LH and FSH content and testicular diameter are at their lowest levels (Lincoln et al, 1977; Lincoln & Peet, 1977). Similar patterns of hormonal and gonadal changes are observed in sheep experiencing natural photoperiodic cycles. Hence, Schanbacher & Ford (1976), and others (Lincoln & Short, 1980; Lincoln, 1989), recorded a

pattern of increasing gonadotrophin and gonadal steroid levels during the months of decreasing photoperiods immediately preceding the main breeding period, followed by decreasing levels when testicular redevelopment was complete and photoperiods were increasing. Also involved in the regulation of gonadotrophin and gonadal steroid levels is the endogenous rhythm of reproduction as discussed in Section 1.2.3.

Short term fluctuations in the mean plasma levels of testosterone are linked temporally to those of LH. Each peak in LH is invariably followed by a peak in testosterone within 20-60 minutes (Wilson & Lapwood, 1978). Longer term increases in plasma testosterone content appear to vary in parallel with reproductive capacity. For example, in rams exposed to an alternating sequence of long and short photoperiods, serum testosterone levels vary with scrotal circumference and peak 3-4 weeks after LH & FSH have reached their maximum levels (Langford et al, 1987).

Circannual changes in prolactin levels have often been assessed simultaneously with those hormonal patterns already described, although its role in mammalian reproduction remains uncertain. Such studies have consistently demonstrated a positive correlation between daylength and plasma prolactin content. During the short photoperiods of the autumn/winter breeding period, prolactin levels fall towards basal concentrations where they remain during the period of most intense sexual activity. As the winter solstice passes and daylength begins to increase so too does the plasma content of prolactin. This pattern of parallel change continues through to the summer solstice when daylength and prolactin levels again begin to decline (Wilson & Lapwood, 1978; Lincoln & Short, 1980).

(II) **Ewe**

Details of the hormonal changes occurring within the ovine oestrous cycle are beyond the scope of this review, but have been extensively studied and reviewed by a number of workers (Hauger et al, 1977; Goodman, 1988). The aim of this section is to detail the changes in hormonal patterns which occur during the onset and termination of the breeding season.

Studies by numerous workers in recent years have established that a characteristic of anoestrus in ewes is the occurrence in plasma of high amplitude LH and FSH pulses which are of insufficient frequency to cause ovulation (Goodman & Karsch, 1981). Comparison of

the patterns of tonic[2] LH secretion during seasonal anoestrus and the breeding season shows that the frequency of LH pulses during anoestrus is lower than that which occurs during both the luteal and follicular phases of the oestrous cycle, but the pulses of LH which do occur have greater amplitude than those of the breeding season (Scaramuzzi & Baird, 1977; Robinson et al, 1985a).

Onset of breeding activity in ewes is characterized by a transition period of 1-4 weeks, before the first full-length oestrous cycle, during which 1 or more increases in LH pulse frequency and in serum progesterone concentrations occur (I'Anson & Legan, 1988). In addition, Legan et al (1985b) observed that these brief increases in progesterone levels were associated with the appearance of ovarian structures which macroscopically resembled corpora lutea. Such ovarian changes during the initiation of breeding activity often occurred without the incidence of behavioural oestrus (ie. with no increase in oestradiol secretion (Fletcher & Lindsay,1971)), but resulted in a 'silent' oestrus (Kelly et al, 1976; Walton et al, 1977; Rowlands & Weir, 1984). It therefore appears likely that this progesterone secretion prior to the onset of normal oestrous cycling represents short luteal phases which result from ovulation of immature follicles, or luteinization of follicles, which have only been exposed to an increase in LH pulse frequency, without a subsequent pre-ovulatory surge (Walton et al, 1977; I'Anson & Legan, 1988).

Numerous studies have provided evidence which suggests that the transient rise in serum progesterone is essential for the subsequent development of normal luteal function and behaviour. This effect, known as progesterone priming, is likely to be achieved through either a direct action at the ovarian level (McLeod & Haresign, 1984) or by delaying the LH surge and thus lengthening the duration of gonadotrophin priming of follicles (Pearce et al, 1985). Regardless of which mechanism operates, prior exposure to progesterone ensures follicles mature in response to the pre-ovulatory LH surge, by increasing LH receptor numbers and the capacity for oestradiol secretion (Hunter et al, 1986). Patterns of FSH secretion during transitions in reproductive status have been more difficult to define with no clear pattern of change in pulse frequency or amplitude (Walton et al, 1977, 1980), but generally higher FSH levels are associated with increased levels of LH secretion.

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[2]The term 'tonic' is used to describe LH secretion that is controlled by the inhibitory effects of gonadal steroids. It includes all types of secretion except the pre-ovulatory surge of gonadotrophin (Goodman & Karsch, 1981a).

Prolactin secretory patterns also appear to be important in the return to oestrous cycling in ewes. Walton et al (1977) measured the concentration of prolactin during anoestrus and during the transition to oestrous cycling. They found that prolactin concentrations were raised throughout anoestrus, but always declined before the first occurrence of ovulation. Results such as these have been interpreted by some as indicating that prolactin may be involved in mediating the effects of long photoperiods on reproductive state. However, the precise mechanisms by which this occurs are yet to be determined (Munro et al, 1980).

By comparison with the onset of the breeding season, the transition to anoestrus appears to occur abruptly. Following the last occurrence of behavioural oestrus, plasma concentrations of LH and oestradiol remain at the low levels typical of the mid-luteal phase of the cyclic ewe. Progesterone levels do not rise as they normally would after ovulation, but remain at levels typical of the follicular phase (Rawlings et al, 1977).

1.2.3 Factors influencing reproductive seasonality.

(I) Photoperiod.

Early studies of seasonal reproduction in sheep, carried out by Yeates (1947,1949) and Hafez (1952), have become classics of their time. Yeates concluded that seasonal variations in the length of photoperiod was the chief factor determining the time of onset and the duration of the breeding season and that a change from increasing to decreasing photoperiod induced reproductive activity, while the reverse situation induced sexual quiescence. Hafez, through the use of artificial lighting, was able to accelerate the onset of the breeding season and demonstrated that even an abrupt change to a shorter daylength would induce the onset of the breeding season.

Further support for photoperiodic involvement in timing reproductive changes was gained from the work of Legan & Karsch (1980) who used 90 and 120 day alternating periods of short (8L:16D) and long (16:8D) days to accelerate the changes in reproductive capacity and thus produce two breeding seasons in one year. Manipulation of artificial photoperiods and measurement of various reproductive parameters (refer to Sections 1.2.1 & 1.2.2) have therefore clearly established that photoperiod is the primary environmental cue controlling both the timing and duration of the breeding season in sheep. The mechanisms whereby this occurs are discussed in Section 1.3.

Recent research into the effects of photoperiod on reproduction in sheep may be summarized as follows. Firstly, the way in which an animal responds to a change in

photoperiod depends on its photoperiodic history (Robinson & Karsch, 1987; Malpaux et al, 1988a). Therefore the effect of decreasing photoperiods from long to short days is to induce the onset of reproduction, while the reverse has an inhibitory effect. Secondly, photoperiod itself is not responsible for initiating breeding activity, but rather is the means by which reproductive events are timed to occur. This may be illustrated by interruption of the neuroendocrine pathway which mediates the effects of photoperiod, or by subjecting animals to long term, unchanging photoperiods. Thus, removal or denervation of the pineal gland prevents photoperiod-induced shifts in reproductive function in both ewes (Bittman et al, 1983) and rams (Barrell & Lapwood, 1979b), but does not completely abolish seasonal reproductive changes (Kennaway et al, 1981, 1984; Bittman et al, 1983). During prolonged exposure to unchanging inhibitory photoperiods, both ewes and rams become refractory to these photoperiods and enter the breeding season at a time not significantly different from those experiencing natural photoperiods (Robinson et al, 1985b; Jackson et al, 1988). An endogenous rhythm in reproduction therefore must exist in sheep and may be independent of photoperiodic cues and the pineal gland (Kennaway et al, 1984). The role of the pineal gland and its hormone melatonin in transducing photoperiodic signals is discussed in Section 1.4.

Further evidence for an endogenous rhythm of reproduction and a timing role for photoperiod is provided by the observation that sheep also become refractory to stimulatory photoperiods after prolonged periods of exposure. This was demonstrated by Lincoln (1978b) who observed that in rams living under natural field conditions, plasma levels of FSH and testosterone, and the size of the testes began to decrease while photoperiods were still stimulatory (decreasing) and increase while photoperiod were inhibitory (increasing).

Studies investigating the pattern of melatonin secretion during photorefractoriness indicated that secretion was maintained at levels appropriate to the photoperiods and did not change when refractoriness developed (Wayne et al, 1988; Malpaux et al, 1987, 1988). Photorefractoriness also developed in pinealectomized ewes in which infusions of melatonin simulating a short day pattern were maintained for a period similar to that required to induce photorefractoriness in pineal-intact sheep. These results indicate that refractoriness to a photoperiodic signal develops as a result of a change in the post-pineal processing of the melatonin signal and that the signal itself does not change.

(II) **Nutrition**

Plane of nutrition, through its effects on liveweight, can be very important in determining reproductive performance of domestic livestock. Whereas a high level of nutrition

may result in a high level of reproductive activity, poor nutrition may delay the onset of puberty or the onset of the breeding season, through inhibition of LH secretion (Lindsay et al, 1984; Bronson, 1988; Rhind et al, 1989a, b). Examples of nutritional effects on reproductive seasonality in sheep include delayed puberty in severely undernourished lambs (Foster & Olster, 1985), an increase in the ovulation rate in well-fed ewes (Smith, 1988) and a trend towards greater numbers of large (≥4 mm diameter) follicles in ewes of high body condition (Rhind & McNeilly, 1986). Montgomery et al (1988) observed that although differential nutritional status affected the ovulation rate of Coopworth ewes, there was no significant effect on the time ewes began cycling or when they entered anoestrus, although ewes on a low plane of nutrition did enter anoestrus up to 9 days earlier than those on a high plane of nutrition.

(III) Temperature.

Although the seasonal pattern of temperature variation is less consistent than that of photoperiod, it is still used by some seasonal breeders, often in conjunction with other environmental cues (eg circannual rhythm in photoperiod) to time seasonal reproductive activity. Animals which time seasonal reproduction in this manner are usually opportunistic breeders responding to favourable environmental conditions and include some mammals. For example, some species of mice, which are not sensitive to photoperiod, exhibited maximum breeding activity only within relatively narrow temperature ranges (e.g. -6°C - 3°C for Texas deer mice) (Bronson & Pryor, 1983).

In contrast to opportunistic breeders, photoperiod is considered to be the most important environmental cue for the majority of seasonal breeders, although temperature has been described, on a few occasions, to have a small, but measurable, influence on the time of reproductive transitions in these animals. In a study of the effects of temperature on the breeding cycle of sheep, Lees (1971) sought to explain why the average date of first oestrus for a group of mature Clun ewes varied from year to year by as much as 2 weeks. After examining local meteorological data Lees found that the mean ambient temperature was the only factor to have any significant effect: higher mean temperatures during the period in which breeding activity normally commenced resulted in a later onset of first oestrus. Over a five year period mean temperature varied by only 3.83 °F and this resulted in a 20 day variation in mean day of onset of breeding activity. This result indicated that temperature, at least in this breed, may play a secondary, but important, role in timing the onset of breeding activity.

(IV) **Pheromones.**

Pheromonal signals produced by rams may elicit a neuroendocrine response in ewes involving an increase in the frequency of pulses of GnRH secretion from the hypothalamus followed by high frequency, low amplitude, pulsatile LH release from the pituitary, which then results in stimulation of sexual function (Martin et al, 1980, 1986). This phenomenon, known as the 'ram effect', occurs in prepubertally, seasonally, and in lactationally anovulatory ewes preconditioned by a period of isolation from rams; in each situation ewes can be stimulated to ovulate by re-introduction of rams. This effect of rams is primarily androgen-dependent; the pheromone(s) originate from oily secretions produced by the sebaceous and apocrine tubular glands of the entire skin and which are stimulated by androgens. Urine and ante-orbital wax are not major sources of the pheromone(s) (Knight & Lynch, 1980; Martin et al, 1986). As yet the chemical identity of the pheromone(s) is unknown, but does not appear to be very specific for a given species since the odour from male goats can induce ovulation in anoestrous ewes (Martin et al, 1986). In addition to pheromonal communication a number of other factors are also involved in the ram effect. These include a behavioural component, possibly a degree of learning (ie. recognition of sexually active rams) and a diurnal variation in the responsiveness of the hypothalamic centres controlling LH secretion, such that introduction of rams in the morning results in more multiple ovulations than occurs if rams are introduced to ewes in the evening (Martin et al, 1985, 1986). Lindsay (1988) hypothesized that the advantage of a triggering mechanism such as the ram effect in sheep is to ensure that there is synchrony of ewes in oestrus and hence only a short period of time in spring when most lambs are born. This, according to Lindsay, might limit the period available for predation on new-born lambs and may enable ewes to collectively protect their young.

1.2.4 Seasonal reproduction in New Zealand sheep.

The period of peak reproductive activity in New Zealand rams begins in early autumn (March) and continues, on average, through to mid-winter (June) (Allison, 1982). Changes in the reproductive hormonal profiles, however, are evident well before this period of sexual activity. Barrell and Lapwood (1978/1979a) reported that under natural photoperiods mean plasma concentrations of LH exhibited a peak during December-February in Romney and Dorset, but not Merino rams. Levels began to rise toward the end of winter reaching a maximum around the middle of summer, then began to decrease. Mean FSH levels in

Romney rams began to rise during November, similar to the timing of the LH rise, and peaked during February. With the onset of the breeding season in March, FSH levels declined until reaching a minimum in May, where they remained until the following November (Barrell et al, 1987). Concomitant with the rise in LH was an elevation in mean plasma testosterone concentrations. Levels began to rise before the longest day, peaked about two months later (February) and remained relatively high for the next two months, after which they declined to minimum levels. Testosterone secretion remained low for the duration of winter and spring (May to November) and did not rise again until late spring (Barrell & Lapwood, 1987/1979a). As in the Canadian studies of Langford et al (1987), peak plasma testosterone concentrations occurred some 3-4 weeks after that of LH. In contrast to the hormonal profiles already described, plasma prolactin concentrations were approximately in phase with length of photoperiod. Mean plasma levels increased as daylength increased to reach a peak in November, slightly before the longest day and at about the time when LH, FSH and testosterone levels began to rise. Secretion of prolactin remained high for the following four months and then declined to basal levels at about the time the gonadotrophins and testosterone reached their minima in May (Barrell & Lapwood, 1978/1979a).

In addition to these seasonal hormonal profiles, Barrell & Lapwood (1978/1979a), also recorded parallel changes in some semen characteristics. All three breeds studied showed seasonal changes in ejaculate volumes, while only Romney and Merino rams exhibited seasonal changes in seminal fructose levels. Peaks in all parameters coincided with the onset of the breeding season.

Whereas rams of modern breeds have been shown to be capable of reproduction at all times of the year, with a period of intense activity during the autumn, ewes have a more restricted period lasting 5-6 months. Allison (1982) recorded peak ovulation rates during April and the first half of May, while Kelly et al (1976) recorded the breeding activity of three breeds (Romney, Coopworth and Perendale) as beginning in mid-March and extending through to mid July/early August. Significant between-breed differences in the timing of the onset and in the duration of the breeding season were recorded. First signs of oestrous cycling were observed earlier in Coopworth (19th of March) than in Romney (25th of March) or Perendale (26th of March) ewes. Coopworth ewes had the longest breeding season (137 $\pm$ 3 days), while Perendale and Romney ewes had successively shorter breeding seasons with 132 $\pm$ 3 and 123 $\pm$ 4 days, respectively.

1.3 Hypothalamo-Pituitary mechanisms mediating seasonal reproduction

Since the isolation of GnRH (Schally et al, 1966), it has been confirmed that this hypothalamic decapeptide passes by way of the hypothalamo-hypophyseal portal system to the anterior pituitary where it induces the release of both LH and FSH (Clarke, 1989).

The seasonal initiation and termination of reproduction in sheep is primarily controlled by the pulsatile secretion of LH from the pituitary which, in turn, is regulated by a hypothalamic GnRH pulse generator (Lincoln et al, 1985). During oestrous cycling in ewes, the follicular phase rise in LH secretion (leading to ovulation) results from increased bursts of neural activity from the GnRH pulse generator (Karsch et al, 1984). This leads to a higher frequency of pulsatile GnRH release from hypothalamic nerve terminals and hence of pulsatile LH release from the pituitary (Clarke et al, 1987; Clarke, 1989). During seasonal anoestrus, increasing photoperiods sensitize the hypothalamo-hypophyseal axis to the inhibitory effects of oestradiol on GnRH pulse frequency, resulting in a decreased number of LH and FSH pulses from the anterior pituitary and mean plasma LH levels insufficient to initiate follicular development and ovulation (Karsch et al, 1984; Robinson et al, 1985a).

Feedback mechanisms which control the activity of the pulse generator during the oestrous cycle have been reviewed by a number of authors (Karsch et al, 1984; Lincoln et al, 1985; Clarke, 1989) and will only be summarized here, while the seasonal changes in pulse generator activity and its influence on seasonal reproduction will be reviewed more fully.

1.3.1 Control of GnRH pulse generator activity by photoperiod.

Frequency of GnRH release is determined by the hypothalamic GnRH pulse generator which, in turn, is modulated by both steroid-dependent and steroid-independent effects on its neural activity. These effects are thought to be governed by photoperiod through its influence on the secretion of melatonin from the pineal gland (Karsch et al, 1984).

(I). **Direct action of photoperiod (Steroid independent effects)**

A direct action of photoperiod on GnRH pulse generator activity can be demonstrated in long term ovariectomized ewes subjected to natural or artificial changes in photoperiod. For example, Robinson et al (1985a) measured LH pulse frequency in ovariectomized ewes under long photoperiods (16L:8D) and after transfer to one of a graded series of shorter photoperiods (ie 8L:16D, 11.5L:12.5D, 13.5L:10.5D), observing that LH pulse frequency increased with decrease in photoperiod. Experiments with castrated rams subjected

to long or short photoperiods have produced similar results, thus LH pulse frequencies during the summer months are lower than those observed at other times of the year (Lincoln & Short, 1980; Olster & Foster, 1988).

(II) **Steroid negative feedback.**

(i) **Ewe.**

a) **Effects during the oestrous cycle.**

During the luteal phase of the oestrous cycle, when progesterone levels are high and oestradiol levels low, GnRH pulse generator activity is low and both GnRH and LH pulse frequencies are inadequate to raise oestradiol concentrations. This inhibitory effect is primarily achieved through the action of progesterone exerting a negative feedback effect on the GnRH pulse generator and does not appear to be a direct effect on the pituitary. As the transition into the follicular phase occurs, decreasing progesterone levels release the pulse generator from its negative feedback influence allowing an increase in GnRH and LH pulse frequencies. Follicular development then proceeds to a point at which raised oestradiol concentrations initiate an LH surge and ovulation (reviewed by Karsch et al, 1984). In addition, a recent study by Kaynard et al (1988) indicated that the increase in LH pulse frequency during this phase was due not only to the removal of progesterone's negative feedback influence, but also to a positive effect of oestradiol on GnRH pulse frequency.

b) **Control of seasonal breeding**

Legan et al (1977), who were among the first to present a hypothesis for the endocrine control of seasonal reproduction, treated ovariectomized ewes with oestradiol-17β implants over an extended period, a procedure which maintained oestradiol at constant mid-luteal levels for the duration of the experiment. Measurement of LH concentrations showed a high degree of seasonal variation, with low levels during anoestrus, rising more than 20-fold at the onset of the breading season. This change in sensitivity to the negative feedback effect of oestradiol on LH secretion has been demonstrated by many authors since that initial study (Goodman et al, 1981, 1982; Robinson et al, 1985a; Legan et al, 1985a). Further studies have revealed fundamental differences in the pattern of LH secretion during breeding and anoestrous periods. LH pulse frequency in anoestrus is significantly lower than in the breeding season, while the pulse amplitude is almost twice as great (Goodman et al, 1982; I'Anson & Legan, 1988).

These observations indicate that during anoestrus oestradiol acts on a GnRH pulse generator located in the brain to inhibit GnRH pulse frequency. More recent observations of

additional effects of oestradiol during the breeding season include effects on the pituitary to inhibit the amplitude of LH pulses (Clarke & Cummins, 1985) and on the hypothalamus to increase LH pulse frequency prior to the pre-ovulatory LH surge (Kaynard et al, 1988).

(ii) Ram

Changes in LH pulse frequency also occur during the period of seasonal reproductive activity in rams. When the testes are fully regressed LH pulse frequency is very low (1.1 ± 0.3/24 hrs), but as reproductive development occurs pulse frequency increases until it reaches a maximum (12.5 ± 0.4/24 hrs) at about the time the testes are fully developed (Lincoln & Short, 1980). As in the ewe, this change in LH pulse frequency is assumed to be related directly to an increase in the number of GnRH discharges reaching the pituitary (Lincoln & Short, 1980).

A model, similar to that used in ewes, has been employed to investigate the role and regulation of the GnRH pulse generator in rams. Results of experiments with castrated rams with steroid containing implants indicate that during the summer both testosterone and oestradiol inhibit LH pulse frequency, an effect which is reduced during autumn resulting in an increase in LH pulse frequency. The time of the decrease in response to steroid inhibition is similar to, but slightly delayed compared to that recorded from intact rams (Lincoln & Short, 1980; Olster & Foster, 1988). In addition, artificial photoperiods have also been found to affect the inhibitory steroid feedback on LH pulse frequency in a similar fashion (Pelletier & Ortavant, 1975).

Techniques such as immunoneutralization of endogenous oestradiol (Sanford, 1987a, b) and castration followed by steroid replacement therapy (D'Occhio et al, 1982; Schanbacher & D'Occhio, 1984) suggest that testosterone, through its androgenic (dihydrotestosterone) and oestrogenic (oestradiol) metabolites (D'Occhio et al, 1983), exerts a negative feedback effect on LH secretion by inhibiting GnRH discharge into the hypothalamo-hypophysial portal system, although it probably also has some direct effects on the pituitary (D'Occhio et al, 1982; Schanbacher et al, 1987).

1.3.2 **Neural mechanisms regulating seasonal reproduction.**

Studies of the neural mechanisms regulating seasonal patterns of GnRH release have focused on the neurotransmitters involved. GnRH neurons in sheep are found predominantly in the medial preoptic area and project, by at least two routes, to the median eminence

(Lehman et al, 1986) where interaction with other neural systems regulates the release of GnRH.

(I) **GnRH release during anoestrus**

Oestradiol-sensitive catecholaminergic neurons are able, during anoestrus, to inhibit GnRH release. Evidence supporting this concept has been obtained from studies using agonists and antagonists of dopaminergic and α-adrenergic receptors in intact ewes and indicates that dopamine may be the primary transmitter involved in the steroid-dependent inhibition of GnRH release during anoestrus (Meyer & Goodman, 1985, 1986). Alpha-adrenergic agonists were also effective in increasing LH levels, indicating a possible stimulatory role for adrenergic neurotransmitters (Meyer & Goodman, 1986).

The steroid independent effect on the GnRH pulse generator during anoestrus is likely to occur via serotoninergic neurons since only the serotoninergic antagonist Cyproheptadine was able to increase LH pulse frequency during anoestrus (Meyer & Goodman, 1986). Other antagonists tested in anoestrus were for receptors of muscarinic and nicotinic cholinergic, GABAergic, opioid and β-adrenergic neurotransmitters, all of which were ineffective in increasing LH pulse frequency.

The origin of the oestradiol sensitive catecholaminergic neurons, which inhibit LH pulse frequency during anoestrus, is likely to be within the lateral retrochiasmatic area. Application of oestradiol to this area during anoestrus results in a decrease in LH pulse frequency, while the presence of a neurotoxin (6-hydroxydopamine), specific for catecholaminergic cells, significantly diminishes this effect of oestradiol (Thiery et al, 1989).

(II) **GnRH release during the breeding season.**

Negative feedback control during the breeding season appears to be mediated by the central opioid peptide system (Horton et al, 1987) in both ewes (Yang et al, 1988) and rams (Schanbacher, 1985; Lincoln, 1988). In ewes, treatment with β-endorphin, a naturally occurring opioid peptide, results in inhibition of LH secretion during the breeding and non-breeding seasons. However, as shown in steroid treated ovariectomized ewes, gonadal steroids are an important component of this system and only appear to activate endogenous opioid peptide-mediated inhibition of LH secretion during the breeding season (Horton et al, 1989).

During the initiation of the breeding season of rams, naloxone, an opioid antagonist, is able to increase plasma LH concentrations by inducing low frequency, high amplitude LH pulses, an effect which is significantly diminished at other times of the year (Ebling & Lincoln, 1985; Lincoln, 1988) and which is likely to be involved in the steroid negative

feedback system (Schanbacher, 1985; Lincoln et al, 1987). The mechanism by which this inhibition is achieved is likely to involve modulation of GnRH secretion, as has been shown in sheep (Ebling et al, 1987; Horton et al, 1987) and rats (Malven, 1986).

1.4 Methods used to evaluate the role of the pineal gland and melatonin in mediating seasonal reproduction.

There is now compelling evidence showing that the pineal gland, through secretion of its hormone melatonin, is responsible for transducing photoperiodic cues into physiological events in seasonal breeders (Lincoln & Short, 1980; Karsch et al, 1984; Lincoln, 1989). Although early work on the pineal indicated that it was not required for ewes to 'read' photoperiodic cues (Roche et al, 1970), Karsch et al (1984) later explained that erroneous result as arising because the investigators did not appreciate the importance of pheromonal effects due to the close proximity of rams, nor the existence of an endogenous rhythm of reproduction.

In chronic animal experiments on the effects of the pineal and melatonin, four principal experimental methods have been used. These are pinealectomy, superior cervical ganglionectomy, melatonin administration and photoperiod manipulation. Typical results from the use of these methods, and the advantages and disadvantages of each, are given in this section. Other methods such as nerve stimulation and electrophysiology are appropriate to acute experiments and will be covered in section 1.6.2.

1.4.1 Pinealectomy

Removal of the pineal gland has provided conclusive evidence that this gland is necessary for induction of the normal pattern of reproductive seasonality in sheep. For example, Barrell & Lapwood (1979b) demonstrated that after pinealectomy of rams, testosterone, and to a lesser extent prolactin, did not display any changes in concentration when rams were exposed to a change in photoperiod, whereas sham operated rams responded in phase with the change in lighting. Also, FSH secretion continued in the absence of the pineal gland, but the pattern of secretion became unrelated to photoperiod and probably represented an endogenous rhythm (Barrell et al, 1985).

In addition to modifying seasonal hormonal secretory patterns, pinealectomy also alters the response of other reproductive parameters in rams. Seasonal cycles in diameter of the

testes and colouration of the sexual skin continue following pinealectomy, however peaks in these parameters occurred earlier and were of a lesser magnitude compared to those in control rams (Lincoln et al, 1989).

Similarly, changes in the normal circannual pattern of gonadal steroid and gonadotrophin secretion have been observed in pinealectomized ewes. For example, photoperiod-induced alterations in the mean plasma concentrations of LH and FSH in pinealectomized ewes are abolished, as are changes in hypothalamic sensitivity to the negative feedback effects of oestradiol. Also, while variations in the degree of sexual activity are still displayed, the timing of such activity became asynchronouswith environmental photoperiod and that recorded from intact ewes (Bittman et al, 1983; Kennaway et al, 1984).

Although pinealectomy is relatively difficult to perform in sheep, it provides the only definitive means by which an animal's response to changes in environmental lighting, in the absence of pineal influences, can be measured (Roche & Dziuk, 1969). Also, since this gland is the major source of melatonin, pinealectomy abolishes the circadian pattern of melatonin secretion and allows for the study of reproductive function in the absence of variations in levels of this hormone (Bittman et al, 1983).

1.4.2 **Superior Cervical Ganglionectomy.**

Denervation of the pineal gland by bilateral superior cervical ganglionectomy results in dysfunction of the pineal in sheep and as such has often been used as an alternative to pinealectomy (Lincoln et al, 1989). Studies employing this technique have produced results similar to those of experiments using pinealectomy, that is, sheep no longer respond to changes in photoperiod and display cycles in gonadotrophin secretion and sexual behaviour which are less pronounced and occur at random relative to those recorded from control animals (Barrel & Lapwood, 1978/1979; Lincoln, 1979).

The principal advantage of this method over pinealectomy is the relative ease with which the SCG can be located and removed. In addition, the health of ganglionectomized sheep does not appear to be compromised. They do, however, show characteristic signs of Horner's syndrome, including dryness of the nose and facial skin and slight ptosis (Lincoln, 1979). In addition, decreased blood flow through cerebral cortical blood vessels in rabbits (Aubineau et al, 1985) and pineal blood vessels in rats (Cardinali et al, 1982) has also been recorded following SCGX. These latter two effects, contrary to those which might be expected following loss of vasoconstrictor control, possibly resulted from "modification of

membrane mechanisms of the smooth muscle fibres" (Aubineau et al, 1985). While it may be suggested that the loss of vasoconstrictor control on blood vessels in the brain and the effects of ptosis on light reception by the eye are responsible for rendering the animals essentially non-photoperiodic, it is more likely this occurs as a result of pineal gland denervation (Lincoln, 1979).

Since this neural pathway is the sole sympathetic innervation of the pineal, SCGX will disrupt sympathetic, but not central input to the pineal and therefore is only appropriate for the study of how sympathetic denervation influences pineal physiology.

1.4.3 **Melatonin Administration.**

For experiments investigating the effects of melatonin in sheep, this hormone may be administered by feeding of melatonin impregnated pellets in the afternoon, by timed injections or by timed infusions, so that circulating levels are raised for long periods of the day, as occurs during short photoperiods. Alternatively melatonin may be administered from intravaginal (Nowak & Rodway, 1985) or subcutaneous (Lincoln & Ebling, 1985) implants, which result in continuously high plasma concentrations, at or above physiological nighttime levels.

Several studies using these methods have focused on the effectiveness of melatonin in advancing the onset of ovarian cyclicity in seasonally anoestrous ewes. Collectively their results indicated that during the initial stages of anoestrus ewes were insensitive to the effects of melatonin and therefore attempts to advance the onset of the breeding season were unsuccessful. During the latter stages of anoestrus, however, ewes again became sensitive to melatonin and implants or infusions at that time of year led to an advancement of the breeding season by 5-10 weeks (Kennaway et al, 1982; Nowak & Rodway, 1985; English et al, 1986). In other studies where pinealectomized ewes have received infusions of melatonin which mimic that secreted on short days or long days, it was observed that they produced seasonal cycles of LH secretion which were almost exactly the same as those which occurred in pineal intact controls experiencing the corresponding photoperiods. Contrasting with this, pinealectomized ewes without replacement melatonin treatment exhibited variable levels of plasma LH that were intermediate between those found in pineal intact ewes maintained in inhibitory and stimulatory photoperiods (Bittman & Karsch, 1984).

In summary, therefore, the pattern of administration of exogenous melatonin to pinealectomized animals can easily be designed to simulate endogenous secretory patterns, or

changed to simulate patterns characteristic of any particular daylength, regardless of existing lighting regimes.

1.4.4 Photoperiod Manipulation.

As a separate technique or in conjunction with those previously described, artificial manipulation of photoperiod has been an important feature of experiments designed to investigate the role of the pineal in mediating the effects of photoperiod on seasonal reproductive activity. Experiments such as those by Hafez (1952), Barrell & Lapwood (1979a, b), Lincoln & Short (1980) and Robinson & Karsch (1987) have involved combinations and/or variations of these techniques and have been instrumental in advancing our knowledge of pineal physiology to its present state. Sections 1.4.1 - 1.4.3 detail the techniques which have been used in conjunction with photoperiod manipulation and the results obtained from these studies.

1.5 Physiology and regulation of pineal function.

The physiology and regulation of pineal function involves many, if not most, organ systems of the body. As such it is not the purpose of this section to cover all aspects of this topic, but rather to review the neural regulation and the circadian and circannual rhythms of pineal activity.

1.5.1 Circadian rhythms, photoperiodism and the pineal gland

(I) General

The majority of plants and animals have evolved, as part of their internal organization, oscillating systems whose periods are closely matched to one or more of the major physical cycles in the environment. These oscillating systems, which include many behavioural and physiological processes, show significant daily (circadian) rhythms which are thought to confer a selective advantage such that events occur at the correct time of day. In mammals, light is the major entraining environmental cue for circadian and circannual rhythms, with temperature also being important to fishes, amphibians and reptiles (de Vlamming & Olcese, 1981).

(II) **Formal properties of circadian rhythms**

Several formal properties characterise circadian rhythms. Firstly, they are entrained by the light/dark cycle and show a regular period of 24 hours. Secondly, they do not vary greatly from the 24 hour period and resist entrainment to any other period. Finally, they are endogenously generated with a period close to 24 hours, even in the absence of entraining stimuli (Moore, 1978; Underwood, 1984). The light/dark cycle, therefore, imposes both period and phase on the endogenous rhythm.

The demonstration that the daily pineal rhythms in serotonin, NAT and melatonin content continue under conditions of constant darkness confirms that they are true circadian rhythms (Reiter, 1981).

(III) **Suprachiasmatic nuclei as circadian pacemakers.**

Generation of circadian rhythms and their entrainment by the light/dark cycle is a unique feature of the CNS. Endogenously generated circadian rhythms require an oscillating system which is made up of one or more circadian pacemakers, one of which appears to be the SCN (Moore, 1983).

Two observations provide evidence for the role of the SCN as a generator of photoperiod-entrained circadian rhythms. Firstly, a neural pathway from the photoreceptors (eyes) to the SCN has been identified in the majority of fish, amphibians and mammals studied. This pathway, the retinohypothalmic tract, is always bilateral and usually innervates the contralateral SCN (Mess and Ruzsas, 1986). Additionally, the ability of light to alter the neural activity of the SCN (Nishino et al, 1976) demonstrates that this pathway is functional and important. Secondly following ablation of the SCN there is a general loss of circadian rhythms including drinking behaviour, locomotor activity and adrenal corticosterone secretion in rats and hamsters (Moore, 1983). Circadian rhythms in both neural activity (Inouye and Kawamura, 1979) and metabolism (Schwartz et al, 1980) exist in the surgically isolated SCN, adding further support for the role of this structure as a circadian oscillator.

(IV) **Pineal-SCN interrelationship**

A strong functional relationship exists between the SCN and the pineal gland (Mess and Ruzsas, 1986). Numerous efferent neural fibres from the SCN innervate the paraventricular nuclei, which have direct and indirect (via the intermediolateral nuclei of the thoracic spinal cord and the cervical sympathetic nerves) connections with the pineal gland. Repetitive stimulation of both optic nerves, or light acting on the eyes, results in augmented activity in approximately half of the suprachiasmatic neurons, which in turn results in a strong

inhibition of electrical activity in cervical sympathetic nerves (Nishino et al, 1976). This inhibitory action causes a reduction in noradrenalin release by nerve fibres innervating the pineal and consequently a reduction in pineal enzyme production and activation. Transsection of these efferent fibres from the SCN abolishes the circadian rhythm of pineal NAT activity (Mess & Ruzsas, 1986). Collectively these results provide strong evidence for the role of the SCN as a central rhythm generator involved in regulating pineal circadian rhythms.

1.5.2 Indoles and Peptides - metabolism and function

(I) Indoles

Indole metabolism in the pineal gland of mammals, which displays a circadian rhythm with maximal synthetic activity occurring during the dark phase (Reiter, 1981), begins with the uptake of L-tryptophan from the blood (King and Steinlechner, 1985). Hydroxylation to 5-hydroxytryptophan followed by decarboxylation leads to the formation of serotonin, a common intermediary for several indole metabolic pathways (Balemans, 1979). Since the activity of the rate limiting, N-acetylating enzyme NAT is low during the light phase there is a build-up of serotonin and a decline in the N-acetylated product, N-acetylserotonin during this period (Steinlechner et al, 1983; Arendt, 1985). With the onset of darkness, the release of noradrenalin from sympathetic nerve endings increases and exerts a stimulatory effect on production of both tryptophan hydroxylase and NAT through an interaction with beta-adrenergic receptors located on the plasmalemma of pinealocytes. This association stimulates membrane bound adenylate cyclase to convert ATP to the second messenger cAMP, which in turn stimulates protein kinase activity. Increased activity of this enzyme results in new protein synthesis (increased tryptophan hydroxylase and N-acetyltransferase activity) and may activate existing NAT by phosphorylation (Balemans, 1979; Ebadi, 1984; King and Steinlecher, 1985). Conversion of serotonin to N-acetylserotonin by NAT occurs almost exclusively during the dark phase and is considered to be the rate limiting step in the production of melatonin, due to the lower Km value of this enzyme relative to those of other enzymes in the melatonin synthetic pathway (King and Steinlecher, 1985). The final step is the methylation of N-acetylserotonin by HIOMT to melatonin. The formation of melatonin may also occur from methoxytryptophan, although this is a minor synthetic pathway (Morton, 1987).

Little is known about the function of many of the other potential pineal hormones. Available evidence is often conflicting with many questions remaining unanswered. The methoxyindoles are all potential biologically active factors. For example, 5-methoxytryptamine

has been shown to be able to regulate sexual activity in those species so far studied and is able to duplicate the effects of photoperiod on the reproductive axis. These effects include inhibition of ovarian growth and suppression of GnRH-induced release of LH (Pevet, 1983). The hydroxy- and methoxy-tryptophols also appear to have potential antigonadotrophic effects, which include inhibition of compensatory ovarian hypertrophy and maturation of the ovaries, as well as inhibiting the growth of the sexual organs. These actions may or may not be effected through inhibition of LH and FSH synthesis and/or release (Reiter and Vaughan, 1977; Smith, 1983).

(II) **Peptides**

(i) **Neurohypophysial peptides**

Pineal AVT, AVP, and OXT have been identified in a number of mammalian species including hamsters (Prechel et al, 1984), sheep (Ebels & Noteborn, 1986; Liu & Burbach, 1987a, 1987b) and rats (Dogterom et al, 1979; McNulty et al, 1985; Liu & Burbach, 1987a, 1987b). Both AVT and AVP have been shown to exhibit seasonal rhythms in pineal levels with peaks occurring during the summer months. Liu & Burbach (1987a, 1987b) however, cast some doubt over the presence of AVT in mammalian pineal glands, suggesting that an antiserum of insufficient specificity had been used by previous workers, with both AVP and OXT being identified as AVT. Indeed the action of these peptides appears to be similar in many ways (ie antigonadotrophic) (Vaughan, 1984) with possible sites of action being the pineal and hypothalamus.

(ii) **Other peptides**

Numerous other peptides have been identified in the pineals of mammalian species. Roles for these peptides, which are often speculative or unknown, may include modulation of hormone secretion by somatostatin (Webb et al, 1984, 1985), modulation of neurotransmitter mediated pineal activation by VIP (Yuwiler, 1983a, 1983b), modification of FSH (Orts et al, 1980) and/or GnRH secretion (Root-Bernstein and Westall, 1986) by threonylseryllysine, regulation of NAT activity by NPY, possibly through presynaptic and postsynaptic mechanisms (Reuss & Schroder, 1987), regulation of pineal blood flow and the release of noradrenalin from nerve ending by angiotensin II (Vaughan, 1984). Other pineal peptides such as the "ovine luteinizing hormone release stimulating factor" (Noteborn et al, 1988b) and delta sleep-inducing peptide (Ebels and Noteborn, 1986) have not been characterized, chemically or functionally.

1.5.3 Environmental factors affecting pineal function.

(I) Light.

Biochemical and secretory activities of the pineal gland are primarily regulated by the light-dark cycle, with light being inhibitory to many of these processes (Reiter, 1985). Characteristics of the light perceived by the eyes which are important in determining its effect on the pineal include:

(i) Wavelength.

Studies using monochromatic light have demonstrated that not all wavelengths are equally effective in suppressing pineal melatonin synthesis and secretion. Between species there are also differences in effectiveness of various wavelengths in altering melatonin production. Reiter (1985), in a review of the effects of light characteristics on the pineal, identified green wavelengths (510-550 nm) as being the most potent suppressor of pineal HIOMT activity. Brainard et al (1986) reported a similar inhibitory relationship between reproductive capacity and exposure to wavelengths in the 555-350 nm range (green, blue, near ultraviolet) in Syrian hamsters. By way of contrast, in this and other species in which the effect of red light exposure has been investigated, no significant suppression from nighttime levels of pineal melatonin content (Reiter, 1985; Benshoff et al, 1987) or HIOMT activity (Cardinali et al, 1972; Reiter, 1985) has been detected.

(ii) Intensity.

The intensity of light required to suppress nocturnal pineal melatonin production varies greatly among species. For example, only 0.1 $\mu W/cm^2$ light intensity is required to cause a drop in the pineal melatonin content of Syrian hamsters, whereas an intensity of 1850 $\mu W/cm^2$ is required to suppress pineal melatonin content in the Richardson's ground squirrel (Reiter, 1985). In sheep, light intensities ranging from 0.30 to 26.32 $\mu W/cm^2$ (1.02 to 88.60 lux) have been demonstrated to suppress nocturnal plasma melatonin levels in a dose-response manner, with 88.6 lux producing a >80% reduction (Arendt & Ravault, 1988). A similar dose-response relationship has also been recorded in goats (Deveson et al, 1990). Under these circumstances there is a very rapid fall in pineal melatonin content with a half-life of less than 10 minutes (Lynch & Wurtman, 1981; Brainard et al, 1983; Reiter, 1985). Studies in humans indicate that exposure to light during the dark phase may result in a similar dose-response suppression of plasma melatonin levels. For example, McIntyre et al (1989) reported an intensity-dependant response to light in human subjects, in which a short exposure (1 hr) to

artificial light suppressed nocturnal melatonin secretion by 71%, 44%, 38%, and 16% with intensities of 3000, 1000, 500, 350 and 200 lux, respectively.

(iii) **Duration**

Light exposure during the period of darkness can inhibit pineal melatonin synthesis, even if the duration of the exposure is very short. For example, as little as 5 sec of light exposure at night can suppress pineal melatonin production in the Richardson's ground squirrel, while 1 sec or less is sufficient in the Cotton rat and Syrian hamster. Return to night-time melatonin levels after the light pulse occurs over several hours in many rodent species, while in sheep there is a lag period of only 5-10 min (reviewed by Vollrath, 1981; Reiter, 1991).

(II) **Nutrition**

Level of nutrition available to an animal influences both the sensitivity of the pineal to sympathetic stimulation and the sensitivity of the neuroendocrine system to the effects of melatonin. Chik et al (1987), in a study of pineal NAT and melatonin, found that underfed Wistar rats responded in a delayed fashion to β-adrenergic (isoproterenol) stimulation such that levels of these parameters never did reach those recorded from controls. Other effects of a restricted food intake on pineal function have been reported to include a depression of pineal weight and an increase in the activities of HIOMT and NAT (reviewed by Vollrath, 1981). In addition, significant increases in sensitivity of the neuroendocrine-reproductive axis to melatonin are experienced by underfed Sprague-Dawley rats, as measured by gonadotrophin levels and reproductive development. Pituitary levels of PrL decreased, while those of FSH and LH were significantly increased in melatonin treated, underfed rats, resulting in a significant reduction in testicular and accessory sex organ weights (Blask et al, 1980; Nordio et al, 1989).

(III) **Other influences**

In addition to photic and nutritional influences on pineal function, a number of other environmental factors appear to affect the pineal in a variety of ways. These factors include ambient temperature, noise and vibration, olfactory stimuli, stressful stimuli such as isolation and immobilization, irradiation and electromagnetic fields (reviewed by Vollrath, 1981). Recent studies by Lerchl et al (1991) indicate that electromagnetic fields do not inhibit pineal melatonin production by their presence, but rather by eddy currents generated in the animals during rapid changes in electromagnetic direction or strength. Such studies are of

potential relevance in determining the effect of electromagnetic fields on human pineal function and on disease processes.

1.5.4 Melatonin's sites and mechanisms of action.

Although the effects of melatonin on reproduction have been well documented, the precise site(s) of its action is uncertain (Vanecek, 1988). Attempts to identify the sites of action have employed a number of techniques, which have indicated the following possibilities: pineal gland (Vacas & Cardinali, 1980; Holloway et al, 1985), SCN (Vanecek et al, 1987; Vanecek, 1988; Stehle et al, 1989, Laitinen et al, 1989), retrochiasmatic area (Glass & Lynch, 1981), median eminence (Vanecek & Jansky, 1989) anterior pituitary (Vanecek, 1988), retina (Vanecek, 1988), medial basal hypothalamus (Cardinali et al, 1979b; Morguenstern et al, 1984), anterior hypothalamic area and medial preoptic area (Glass & Knotts, 1987) and hippocampus (Zeise & Semm, 1985). Many of these areas have either been identified as potential sites which regulate pineal function (eg hippocampus and SCN for feedback regulation), or which are involved in regulating gonadotrophin release (eg retrochiasmatic area, medial preoptic area and median eminence - refer Sections 1.3 & 1.5.1).

Little is known about melatonin's mechanism(s) of action, although several studies have provided some insight. For example, Glass and Knotts (1987), in experiments using the White-footed mouse, demonstrated that melatonin's action on reproductive systems may involve an alteration of the release of GnRH. At the cellular level other studies have indicated an ability to decrease cAMP and increase cGMP levels (Vacas et al, 1981) and participate in the control of brain protein/peptide secretion (Haldar-Misra & Pevet, 1983). Modification of neural activity by melatonin has been the subject of several studies which have demonstrated both in vitro and in vivo effects on sympathetic neurons of the SCG (Papke et al, 1986) and heart (Viswanathan et al, 1986), and neurons of the eye and CNS (Quay, 1987). Of particular interest are several lines of evidence which suggest that the SCN, through its role as a circadian pacemaker, may be important in the expression of melatonin's effects on circadian and circannual rhythms (Cassone et al, 1987). The ultimate effect of melatonin on the reproductive system of seasonal breeders is thought to be through an action on the GnRH pulse generator, to alter its sensitivity to oestradiol negative feedback and hence the frequency of LH secretion, which then determines reproductive status (Bittman et al, 1985). Although melatonin receptors have been located in the anterior pituitary (Vanecek, 1988), indications are

that melatonin does not alter the response of the pituitary to GnRH pulses in sheep (Rodway & Swift, 1983; Bittman et al, 1985; Robinson et al, 1986) or hamsters (Bacon et al, 1981).

1.5.5 **Melatonin and the measurement of daylength.**

As yet the mechanism(s) by which melatonin codes for a particular photoperiod has not been resolved. Two hypotheses, however, have been put forward. Firstly, the duration hypothesis postulates that the duration of the elevated nocturnal melatonin release, which is proportional to the length of the night, determines the response to changing photoperiods (Karsch et al, 1988). Secondly, in the phase hypothesis, it is claimed that sheep measure daylength by using a circadian rhythm of photosensitivity. When the light sensitive phase is coincident with external light then a physiological response is initiated (Ortavant et al, 1988). Most available evidence supports the duration hypothesis.

Many studies which have investigated either the phase or duration hypotheses have used a common experimental model, involving removal of all ovarian/testicular and pineal tissue, followed by subcutaneous implantation of silastic capsules containing sufficient oestradiol to maintain physiological concentrations (2-5 pg/ml in serum) and melatonin infusions which can be adjusted to produce physiological patterns simulating either short or long days. Alternatively, gonadectomized photoperiodically manipulated sheep have also been used. Reproductive status has been assessed by measurement of hypothalamic responsiveness to the negative feedback effects of oestradiol on LH secretion.

The following evidence has been reported supporting the duration hypothesis.

1) Duration of elevated nocturnal melatonin secretion is proportional to the length of the night (Kennaway et al, 1983).

2) Reproductive status conforms to the length of time that melatonin is elevated each day, rather than to photoperiod (Bittman & Karsch, 1984; Lincoln & Ebling, 1985; Yellon et al, 1985).

3) Elevated melatonin levels during the day (ie., out of phase with the normal nighttime rise) are just as effective in initiating a reproductive response as those levels seen in intact animals at night (Wayne et al, 1988).

Evidence supporting the phase hypothesis has been obtained from resonance or night-interruption studies. Resonance experiments involve fixed short photoperiods with varying durations of darkness. Night-interruption experiments use short day treatments with the night being interrupted by a short period of light (Ortavant et al, 1988).

Results from these studies have been interpreted as indicating that a short day treatment may be 'read' as a long day if the dark phase is interrupted with a period of light (ie 7L:9D:1L:7D), or if the light-dark cycle is very short (4L:8D) or very long (4L:32D) (Ravault and Thimonier, 1988), suggesting that when light occurs during a 'light sensitive' phase a reproductive response is initiated.

Karsch et al (1988), in a review of evidence supporting the duration hypothesis, presented a slightly different phase hypothesis in which the circadian rhythm of sensitivity was for melatonin, rather than for light, as proposed by Ortavant et al (1988). Evidence presented by Karsch et al (1988), while supporting the duration hypothesis, does not totally preclude the phase theory.

1.5.6 Hormonal modulation of pineal function.

(I). Gonadal steroids.

(i) Oestradiol.

Feedback stimulation of pineal melatonin production by oestradiol has been demonstrated in ovariectomized rats (Cardinali et al, 1987). Studies attempting to define specific feedback mechanisms have shown that this effect may be mediated indirectly through the pituitary gland and in particular through oestradiol stimulating the release of prolactin, which in turn increases pineal serotonin synthesis (reviewed by King, 1986).

One mechanism by which oestradiol promotes melatonin production directly is thought to involve an increase in HIOMT activity (Daya & Potgieter, 1982) and protein (Mizobe & Kurokawa, 1978) synthesis. NAT activity appears to be largely unaffected by gonadal steroids, although Daya & Potgieter (1982) did report a stimulatory effect of testosterone on the nocturnal rise in activity of this enzyme. A further mechanism by which oestradiol may affect pineal function is through regulation of β-adrenergic receptors. Studies by Maxwell et al (1989a) revealed a seasonal variation in the sensitivity of ovine pineal β-adrenergic receptors to modification by gonadal steroids. These workers concluded, from this and other studies (Foldes et al, 1984, 1985), that pineal β-adrenergic receptors were relatively insensitive to the effect of oestradiol alone during the breeding season, but during anoestrus these receptors became highly sensitive.

(ii) Testosterone.

Early studies indicated a likely role for testosterone in the regulation of pineal physiology (Cardinali et al, 1975; Cardinali & Vacas, 1978). The presence of androgen

receptors in the bovine pineal provided indirect evidence for this as a potential site of action (Moeller et al, 1984). Further studies have obtained more direct evidence. Castration decreases the ability of the rat pineal gland to synthesize melatonin and 5-methoxytryptophol. Restoration to control levels is achieved by the administration of testosterone to castrated rats, an effect probably achieved through heightened HIOMT activity (Daya & Potgieter, 1985). Contrasting with these results, in vitro studies by Cardinali et al (1987) suggested that testosterone decreased pineal melatonin content in rats. Whether the effect is to increase or decrease pineal melatonin content, these results imply that testosterone may be involved in a feedback relationship with the pineal.

(II) **Corticosteroids.**

Corticosterone has been shown to cause a decrease in nighttime NAT activity and melatonin synthesis in adult rats (Troiani et al, 1988), the mechanism of which is unknown, but does not appear to involve cAMP or HIOMT (Yuwiler, 1985, 1989).

An American group of workers, led by T.H. Champney, contended that the pineal was outside the normal steroid feedback loops which modulate hypothalamic function (Champney, 1986). In support of this hypothesis they showed that manipulations of hamster gonadal and thyroidal endocrine status had no effect on the ability of the pineal to initiate gonadal regression in response to short photoperiod exposure (Champney, 1988). In addition, adrenalectomy of rats and hamsters had little or no effect on pineal NAT activity (Champney et al, 1985). The criticism applied by these workers to many previous studies, which have detected effects of steroids on pineal physiology, was that they have been performed in vitro and may have used inappropriate endpoints (alterations in pineal factors) which may not be involved in the regulation of pineal function (Champney, 1986).

1.6 Innervation and electrophysiology of the mammalian pineal gland.

(I) **Introduction.**

Since Kappers (1960) established that post-ganglionic, sympathetic neurons are the major source of innervation to the pineal of Albino rats, numerous studies have demonstrated the primary role of the sympathetic nervous system in maintaining the integrity of pineal function. More recently, researchers using electrophysiological techniques have provided evidence suggesting that the pineal also receives innervation from central loci, many of which

are involved in processing environmental information, including olfactory and visual stimuli. This section will provide a synopsis of the current knowledge of the central and peripheral innervation of the mammalian pineal gland and the methods by which this knowledge has been attained.

1.6.1 Neurological investigative techniques applicable to pineal research.

In addition to methods employing superior cervical ganglionectomy, as described in Section 1.4.2, a variety of other techniques have been employed to investigate the peripheral and central innervation of the pineal. Results from the application of these methods will be discussed in Sections 1.6.2 & 1.6.3, in terms of their contribution to our understanding of the innervation and electrophysiology of the mammalian pineal gland. This section will detail the techniques most commonly applied to the study of pineal function and discuss them in terms of their advantages and disadvantages.

(I) **Lesion studies.**

In attempts to investigate the role of various areas or nuclei of the brain in regulating pineal function, methods have been developed which interrupt the pineal's neural input through lesioning or destruction of specific brain sites in vivo by electrocoagulation or knife cuts. Such methods have provided valuable evidence implicating nuclei such as the SCN and PVN as areas involved in the control of seasonal reproductive cycles and pineal function. However, a number of inconsistencies have arisen in the literature which highlight the technical difficulties encountered in the application of these methods. As discussed by Klein et al (1983a), this is likely to be due to difficulties experienced in achieving accurate and/or total lesioning of the targeted site without damage to adjacent nuclei or pathways. It is clear from that and other studies (Rusak, 1980; Eskes et al, 1984) that the inability to achieve complete and accurate lesioning has been a major cause of inconsistent results between both animals and experiments, but this problem may be overcome through histological verification of the lesion site at post-mortemexamination.

(II) **Stimulation studies.**

1. Stimulation of the peripheral innervation involves placing electrodes around the pre-ganglionic CST, the SCG or the post-ganglionic ICN and applying stimuli (usually a monophasic square wave pulse) which elicit maximal compound action potentials in these nerves (Bowers & Zigmond, 1982).

2. Stimulation of the central innervation requires the use of stereotaxic techniques to accurately position stimulating electrodes in areas such as the habenular nuclei, SCN and PVN. For example, Reuss et al (1984) used bipolar (concentric), stainless steel electrodes to stimulate several brain loci and concurrently measured extracellular action potentials in the pineal. As in other stimulation studies (Pazo, 1981; Demaine & Semm 1984) the pulse applied was a monophasic, square-wave produced by a stimulator and delivered through a stimulus isolation unit. Such procedures, although technically difficult, can be used to mimic neural input to the pineal and hence reveal the role of those areas being stimulated in regulating pineal function.

While many parameters of pineal function have been measured following stimulation of central or peripheral innervation, the secretion of melatonin is by far the least studied. Most studies have investigated pineal enzyme levels, melatonin content or electrophysiological responses, as measures of pineal function and most of the few studies which have measured melatonin secretion have been pharmacological studies conducted in vitro (eg Morgan et al, 1988b). This is perhaps not surprising since the large majority of studies have been conducted in rats and other small mammals from which only a small amount of blood can be taken Refer to Section 1.6.3 (III) for details of a recent in vivo study measuring plasma melatonin content during CST stimulation.

(III) **Recording of neuronal/pinealocyte electrical activity.**

Various electrophysiological methods can be used to record electrical activity occurring in nerves and other electrically excitable tissues. These include single-unit recordings (both intra- and extracellular), multi-unit recordings, the detection of "brain waves", and evoked potentials (Reuss, 1987). Action potentials from single neurons can be detected by microelectrodes whose tip diameters are in the order of microns. Ionic currents occurring during the generation and conduction of an action potential produce voltages in the extracellular fluid which are detected by these electrodes and usually displayed on an oscilloscope after amplification. Electrodes are constructed of either metal (platinum or tungsten) or micropipettes containing an ionic solution and are often placed using stereotaxic methods. Application of extracellular recording techniques allow large numbers of individual neurons or pinealocytes to be isolated and studied for extended periods of time, without injury to the cells (reviewed by Frank & Becker, 1964; Delgado, 1964).

Although intracellular recordings provide more information on the nature of recorded action potentials (eg resting membrane potential and postsynaptic potential discharges) they are,

however, much more difficult to obtain than extracellular recordings. Thus, for technical reasons, all intracellular recordings from mammalian pinealocytes have been conducted in vitro and are very few in number (Reuss, 1987; Parkington et al, 1987).

Researchers using stimulation and recording techniques have typically stimulated a particular brain locus and recorded single cell extracellular action potentials in the pineal (eg Ronnekleiv et al, 1980; Reuss et al, 1984). In a few studies, to investigate possible efferent connections between the pineal and central areas, the pineal itself has been stimulated and recordings made in other areas of the brain (Semm et al, 1981).

(IV) Retrograde and Anterograde nerve fibre tracing

In vivo and in vitro retrograde tracer techniques enable visualization of nerve fibres and their perikarya projecting to the pineal from the brain via the pineal stalk. Following injection of a marker molecule, often horseradish peroxidase, into the area of interest, adjacent nerve terminals actively take up the marker by pinocytosis and transport it with the retrograde flow of axoplasm to the perikarya from which the fibres originate. Fibres containing horseradish peroxidase may then be visualized with DAB and hydrogen peroxide. This technique has proven to be extremely powerful and although limited by the amount of tracer nerve terminals can take up, especially in larger animals in which neurons travel for greater distances, it promises to greatly aid research into the location of perikarya in the brain whose fibres project to the pineal (reviewed by Møller & Korf, 1986).

Relatively few attempts have been made to use the anterograde transport of marker molecules to trace nerve fibres from their perikarya to the pineal. Those that have demonstrated that tracers such as horseradish peroxidase and the plant lectin *Phaseolus vulgaris leucoagglutinin* may be used successfully to identify various central nuclei which project fibres to the pineal and hence potentially influence its function (Reuss & Møller, 1986; Mikkelsen & Møller, 1990; Fink-Jensen & Møller, 1990; Larsen et al, 1991). One likely reason for the lack of studies employing this method is that it is relatively easier to locate and inject tracer into the pineal than it is to accurately and repeatedly position microelectrodes in central nuclei. Anterograde tracing studies investigating pineal innervation do however have the advantage of not requiring injection into the heavily vascularized pineal where some of the tracer could be lost into the blood (Larsen et al, 1991).

(V) Immunological techniques

For the measurement of indoles in biological fluids or for the location of a wide variety of proteins, indoles and other compounds (e.g. neurotransmitters) in pineal tissue,

immunological techniques have proven to be versatile, sensitive and easy to apply. These techniques, which include RIA, ICC and immunoneutralization (passive and active), all involve the application of highly specific antisera in order to detect the minute amounts of antigens often present in biological fluids and tissues. As with all measurement approaches, several difficulties are encountered with these techniques. Amongst these are the requirement for validation against a non-immunological method, determination of specificity in ICC methods when other methods lack a similar degree of sensitivity and retention, without chemical alteration, of antigens in tissues during fixation (reviewed by Brown et al, 1983).

(VI) **Pharmacological methods/studies.**

As applied to pineal research, pharmacological techniques have significantly advanced understanding of the receptor-mediated neural control of pinealocyte synthetic activity. An example of this was the discovery, in rats, that α_1-adrenoceptors directly potentiate β-adrenoceptor stimulation of cAMP production (Sugden et al, 1984). Interpretation of results from studies of this type are dependent, however, on the selection of receptor agonists/antagonists which are highly specific and have high affinity for the receptor under investigation.

1.6.2 Innervation.

(I) **Sympathetic (peripheral)**

A variety of techniques applied to many mammalian species have established that a multisynaptic neural pathway projecting through the CNS, which gives rise to the cervical sympathetic trunk and its post-ganglionic fibres, is the principal means by which the pineal gland receives neural signals and maintains its circadian rhythm of melatonin release. This neural pathway begins at the retina from which the retinohypothalamic tract passes to the SCN. Fibres then travel to the PVN, continue on to the tuberal hypothalamus, over the medial forebrain bundle and reticular formation, finally descending in the intermediolateral cell column of the spinal cord. Pre-ganglionic sympathetic fibres emerge from the thoracic section of the spinal cord, travel to the superior cervical ganglion in the cervical sympathetic trunk and synapse with post-ganglionic fibres which enter the pineal gland via the ICN (reviewed by Vollrath, 1981).

Various studies support the existence of this neuronal pathway in rats and other species. Numerous efferent projections from the SCN of rats have been shown to be intrahypothalamic and travel to such areas as the PVN, dorsomedial hypothalamic nucleus, retrochiasmatic area

and the ventral tuberal region (Berk & Finkelstein, 1981). Lesions of the SCN or PVN of hamsters prevent testicular regression in short photoperiods (Eskes et al, 1984), while in rats PVN lesions result in an almost total reduction of nighttime NAT and HIOMT activity (Klein et al, 1983a). Although this basic neural supply has been well established in rats and other small mammals there is a total lack of such studies in larger mammals such as sheep (Arendt, 1985).

(i) **Pineal Sympathetic Neurotransmitters.**

Sympathetic nerve endings in the pineal contain numerous granular and clear vesicles which are closely associated with the storage and release of the neurotransmitters noradrenalin and serotonin (Karasek, 1983). Nighttime induced stimulation of these nerves causes the release of noradrenalin from the nerve endings, which then stimulates melatonin production via β-adrenergic receptors and the adenyl cyclase-cyclic AMP system (Reiter, 1986, 1991). Comparatively little appears to be known about the significance of the serotonin also contained within these nerve terminals. This monoamine may either derive from pinealocytes and be stored in vesicles, or be synthesized in the fibres themselves. In the dog, at least, additional serotoninergic fibres appear to innervate the pineal from central areas. Evidence for this, however, is indirect since this contention is only based on the observation that some serotonin containing fibres remain after bilateral SCGX (Matsuura & Sano, 1983). Further evidence obtained from noradrenalin depletion studies indicates that these two transmitters, while existing in the same nerve terminals, are not localized in the same vesicles (Hess, 1981). Reports of serotonin containing fibres in the pineal of other species have been reviewed by Ebadi & Govitrapong (1986). Other compounds found within intrapineal nerve fibres (e.g. NPY & VIP) may function as neurotransmitters or serve to regulate the release and action of noradrenalin (see Section 1.5.2).

(ii) **Pineal adrenergic receptors.**

Pharmacological and photoperiod manipulation experiments have shown that the circadian rhythm in NAT synthesis and activity is controlled by β-adrenergic receptors (Klein & Weller, 1973) which act via the second messenger cAMP. The larger (30-50 fold) increase in nocturnal NAT activity contrasts with the relatively small (2-fold) increase in HIOMT which normally occurs at night and which is mediated by the sympathetic system (Sugden & Klein, 1983). Induction of cAMP synthesis by β-adrenergic receptor stimulation results in the activation of one or more protein kinases, which are likely to be involved in the induction of transcription and translation (Chan & Ebadi, 1980) and the activation of existing, but inactive,

NAT (Zatz et al, 1978). The normal integrity of β-adrenergic receptors and adenylate cyclase activity has been shown, in rats, to be dependent on an intact sympathetic innervation. Removal of this neural input results in a temporary sensitizing of adenylate cyclase to catecholamine stimulation, apparently due to an increased density of β-adrenergic receptors (Cantor et al, 1981).

Studies with adrenergic agonists and antagonists have indicated that noradrenalin acts through both α_1 and β_1 receptors (Klein et al, 1983b) and led to the identification of pineal α-adrenergic receptors. Alpha$_1$-agonist association with α_1-adrenergic receptors has been shown in rats and hamsters to potentiate the β-adrenergic stimulation of NAT approximately 3-fold (Klein et al, 1983b; Alphs & Lovenburg, 1984; Sugden et al, 1984). Whereas this potentiation in rat pineals is effected through increased cAMP production, Morgan et al (1989) reported that a different mechanism appears to operate in sheep pineals. Pharmacological studies by this group showed that α_1-adrenergic receptors can mediate an increase in melatonin release in the absence of changes in cAMP production, but only when there was some β-receptor stimulation. These observations suggest there must be some interaction between the two receptor types which occurs at a step other than cAMP. Earlier work by Sugden et al (1985a) indicated that ovine α_1-receptors may act at the level of methylation of N-acetylserotonin by HIOMT to form melatonin, although the mechanism by which this may occur is yet to be identified. A more recent study suggested that α_1-adrenergic receptors may have another role unrelated to β-adrenergic potentiation. Serotonin is known to be released and then taken-up by pinealocytes. Stimulation of α_1-receptors, but not α_2- or β-receptors, by agonists results in the release of 5-HT from cultured rat pineals. These results therefore indicate that serotonin release may be regulated by the activation of α_1-adrenergic receptors (Aloyo & Walker, 1988).

(II) Central.

Central innervation of the pineal gland has been demonstrated in a number of species including the Mongolian gerbil, hedgehog (Korf & Møller, 1984), rat (Dafny, 1980) and guinea-pig (Schneider et al, 1981), although the functional significance of this innervation has not been established. Tracing of these central fibres by various techniques has revealed a number of brain areas through which they either pass or in which they appear to originate. The habenular nuclei have been identified in many species as areas which project fibres to the pineal. Electrical stimulation of these nuclei in rats (Reuss et al, 1984) and guinea-pigs (Semm et al, 1981) modified electrical activity in the pineal gland, while the PVN has also

been identified as a source of innervation by these methods (Reuss et al, 1984). In addition to the habenular nuclei and PVN of rats and Mongolian gerbils, perikarya innervating the pineal are located in or project their axons through the stria medullaris, caudal commissure and dorsal nucleus of the lateral geniculate body (Møller & Korf, 1983a, b; 1986; Dafny, 1983). The presence of acetylcholinesterase in some of these central fibres in the pineal suggests there may be some cholinergic influence over pineal function (Møller & Korf, 1983b). As discussed in Section 1.5, fibres containing neuropeptides are also found in the pineal and appear to be of central origin.

The location of these perikarya in the brain may allow for a variety of external stimuli to influence the activity of the pineal gland. Light impulses may reach the pineal via the dorsal nucleus of the lateral geniculate body and the caudal commissure, while olfactory and limbic stimuli may be transmitted through connections with the lateral and medial habenular nuclei, respectively (Møller & Korf, 1986).

The presence of a variety of receptors for possible neurotransmitters in the pineal provides further evidence for neural input other than that from the sympathetic system. GABA, benzodiazepine, dopaminergic, cholinergic and serotoninergic receptors have all been identified in the pineals of a variety of species, including sheep. In most cases the role(s) of these potential transmitters and their interactions with their receptors are unknown (Ebadi & Govitrapong, 1986).

1.6.3 **Electrophysiology of the pineal gland.**

An understanding of the electrophysiological control of pineal gland physiology is central to an understanding of how this gland responds to environmental stimuli. Under influences of their central and sympathetic innervations, the cell membranes of pinealocytes show spontaneous and inducible depolarisation, which is likely to be coupled to altered secretory activity. The significance of changes in parameters of neural activity, such as frequency, duration, amplitude and timing, has not been an area of extensive research and is only partly understood (Reuss, 1987).

(I) **Patterns of electrical activity.**

(i) **Spontaneous.**

Spontaneous electrical activity occurring in pineal cells displays a variety of discharge patterns. Pinealocyte firing frequencies range from <1 to 100 Hz (Ronnekleiv et al, 1980; Reuss & Vollrath, 1984), while cells vary in firing patterns with regular, irregular or

bursting patterns having been recorded. Average frequencies usually range up to 10 Hz with levels of activity tending to increase during the night (Reuss & Vollrath, 1984). In rats, low amplitude, high frequency waves have been recorded from cells which periodically exhibited bursts of high amplitude activity lasting 1-3 seconds (Schapiro & Salas, 1971). A detailed study of these parameters was carried out by Stehle et al (1987) in Golden hamsters, which, in their opinion, appeared to give results similar to those recorded from other mammalian species. These workers recorded action potentials of 1-2 ms duration and frequencies of 0.2 to 25 Hz, with distinct time-dependent differences. During all of the light period (12 hrs) and the first 3 hours of the dark period frequencies were in the order of 2 Hz. This was followed by an increased level of activity up to 7 Hz for the next 6 hours. During the remaining 3 hours before lights on, a decrease to 4 Hz was observed. Most (95%) pinealocytes showed irregular patterns of firing. These results support the earlier findings of Reuss & Vollrath (1984), that there exists a circadian rhythm of electrical activity in pineal cells, with higher activity occurring during the night.

(ii) **Evoked activity.**

Spontaneous electrical activity of pinealocytes is under the influence of nerve fibres from both peripheral and central origins. As discussed below, stimuli, including photic and electrical, which alter electrical activity in these fibres also evoke a change in the recorded electrical activity of both pinealocytes and intrapineal nerve fibres.

(a) Photic stimulation modifies pinealocyte electrical activity on a time-dependent basis. Nighttime light stimulation of Golden hamsters caused a decrease in spontaneous electrical activity of those cells which responded (Stehle, 1987). Daytime photic stimulation has been reported to enhance spontaneous activity (Dafny & McClung, 1975), have no effect (Ronnekleiv et al, 1980), or to produce potentials which tended to oscillate (Schapiro & Salas, 1971). It would appear, therefore, that not all pinealocytes are affected by the same stimuli or to the same degree.

(b) Electrical stimulation of the sympathetic or central innervation of the pineal gland alters the spontaneous activity of some, but not all, pinealocytes (Reuss, 1987). Refer to part (III) of this section for details on the effects of sympathetic stimulation on electrophysiological activity. Stimulation of central loci thought to influence pineal function has been shown to modify the electrical activity of cells considered 'silent' in sympathetic stimulation experiments. For example stimulation of the habenular nuclei of rats induces pineal responses with alterations in the rate of spontaneous electrical activity and evoked

discharges of 'silent' cells (Ronnekleiv et al, 1980; Reuss et al, 1984). Also, the observation of PVN modification of electrical activity of pineal cells in the guinea-pig (Reuss et al, 1984) is in accordance with other studies showing inhibition of pineal synthetic activity when this nucleus is stimulated (Olcese et al, 1987). A number of other brain areas including the hippocampal formation (Reuss et al, 1984), superior and inferior colliculi (Reuss et al, 1984), septal area and optic tract (Pazo, 1981), also alter the spontaneous electrical activity of pineal cells during and after electrical stimulation, indicating possible functional links between these areas and the pineal gland.

(II) **Characteristics of stimulation affecting the pineal's response.**

(i) **Frequency, duration and amplitude.**

In the few studies which have investigated the effect of these parameters on pineal neuronal or synthetic activity, most investigators have chosen stimulus parameters which give a maximal response in terms of their end-point measurements. To date the effects of changes in frequency, duration or amplitude of the stimulus pulse have usually been measured using NAT levels as an end-point. Bowers & Zigmond (1982) reported that in rats the range of frequencies over which NAT is influenced by CST or ICN stimulation is very narrow. At 1 Hz there was little or no response, while at 5 Hz there was a maximal response. The strength of current required to produce a threshold compound action potential from the ICN appeared to be inversely proportional to the duration of the pulse. For example, Bowers & Zigmond (1982) found that the current required to produce a threshold response at 0.5 ms duration was sufficient to produce a near maximal response at 1 ms. In addition, they found that NAT levels were not significantly affected by increases in pulse duration at current strengths which produced maximal exophthalmus. As yet it is uncertain which of these stimulus parameters most influences the final response of the pineal, however results such as those described above suggest that frequency may be of considerable importance.

(ii) **Pattern of discharge.**

Electrophysiological studies investigating the importance of patterns of neural discharge in nerves innervating the pineal gland and other areas of the body have revealed a complex pattern of responses. For example, in the alimentary system, as reviewed by Edwards (1984), several organs gave greater responses if stimulated with intermittent rather than continuous stimulation. This effect usually was specific to a particular range of frequencies with lesser responses occurring outside of this range. In contrast, pre-ganglionic nerve stimulation of rat superior cervical ganglia caused long term changes in tyrosine hydroxylase

activity in the ganglia, which were dependent on the length of time for which the stimulus was applied and the number of pulses administered (Zigmond & Chalazonitis, 1979). Up to 60 minutes of continuous stimulation caused a progressive increase in tyrosine hydroxylase activity when measured 72 hours later. Longer periods of stimulation were no more effective. Interestingly, the number of pulses administered determined the response rather than the pattern of application (trains; 250 ms on/500 msec off or the same number of impulses administered continuously). Similar studies of pineal gland responses to neural stimulation have shown that changes in the pattern of sympathetic stimulation, as well as the total number of stimuli, play a significant role in determining the magnitude of the response. Bowers & Zigmond (1982) stimulated rat CST's continuously during both night and day and observed that NAT levels were comparable to those observed in control animals at night. When they applied patterns of stimuli which were not continuous, induced NAT levels were partially dependent on the pattern rather than the total number of stimuli. For example, NAT activity produced by patterns having the same number of stimuli (10 Hz for 2 sec every 20 sec, 5 Hz for 2 sec every 10 sec, 5 Hz for 4 sec every 20 sec) was greater than that measured after stimulation at 1 Hz continuously. It would therefore appear that within the SCG-pineal system some element is sensitive to the temporal relationship between stimuli which, at least in part, determines the pineal's response.

(III) **Effects of sympathetic nerve stimulation.**

(i) **Secretory activity**

Work recently reported by Chan et al (1989) is the only study to date investigating the effect of in vivo sympathetic nerve stimulation on plasma melatonin levels. This study, performed in rabbits, sought to investigate the effects of unilateral electrical stimulation of the pre-ganglionic sympathetic nerve trunk on cerebral venous plasma melatonin levels and hence pineal melatonin secretion. Performed in the light phase under pentobarbital anaesthesia and delivering trains of rectangular pulses of 0.5 ms duration at 300 Hz for 60 ms every 2 sec (low stimulation) or 7.5 sec every 20 sec (high stimulation), this treatment clearly demonstrated the ability of the pineal's sympathetic innervation to influence melatonin secretion in this species. Low stimulation did not have any significant effect on plasma melatonin, whereas high stimulation elicited small (1.2-, 1.5-, 1.6-, & 2.3-fold), but significant, increases in 4 of 6 animals, with the remaining 2 showing large (4.5- & 16-fold) and sustained rises in melatonin secretion.

(ii) **Morphological and biochemical changes.**

Stimulation of the CST or SCG, to investigate the role of this pineal innervation in mammals, has produced a variety of effects, in addition to those seen in NAT levels (eg., Bowers & Zigmond, 1982, see pages 38 & 39). For example, Reuss et al (1989a) found that bilateral stimulation of rat SCG had no effect on pineal synaptic ribbon numbers, size or location. In contrast, Gonzalez & Alvarez-Uria (1986) reported that similar treatment of cats caused an increase in synaptic ribbon numbers. Cyclic AMP concentrations are also under the influence of the sympathetic nervous system, as shown by Heydorn et al (1981): electrical stimulation of the SCG increased pineal cAMP concentrations, while β-antagonists blocked this effect. Noradrenalin release from presynaptic pineal nerve terminals, after bilateral electrical stimulation of pre-ganglionic fibres innervating the SCG, is presumed to mediate this effect (Jaim-Etcheverry & Zieher, 1980). Metabolism in the pineal is also increased by electrical stimulation of the sympathetic innervation. Thus, glucose utilization was increased 71% following unilateral stimulation of the CST, indicating a general metabolic activation (Ito et al, 1988).

(iii) **Electrophysiological changes.**

The influence of the sympathetic system on the electrophysiological activity of the pineal appears to be quite complex. Demaine & Patel (1989) recorded extracellular electrical activity from spontaneously active hamster pinealocytes during bilateral stimulation of the SCG. They observed responses which fell into three categories: non-responsive, excited or inhibited and went on to conclude that some, but not all, pinealocytes are influenced by neural inputs originating in the SCG. In a similar study, Reuss et al (1985) carried out unilateral and bilateral electrical stimulation of the rat SCG and recorded single cell electrical activity in the pineal. Of the cells investigated half of those with spontaneous activity were affected by unilateral stimulation, responding with either an increase or decrease in activity. Some cells without spontaneous activity were activated by this stimulation, although some cells failed to respond to either unilateral or bilateral stimulation and therefore did not appear to be under the control of the sympathetic nervous system. Bilateral stimulations showed that only a small number of cells received input from both ganglia suggesting, as other studies have (Lingappa & Zigmond, 1987), that one ganglion innervates only approximately half the pinealocytes of the gland and that only a few are innervated by both ganglia (Reuss et al, 1985). Further evidence for this pattern of sympathetic innervation comes from studies in which unilateral stimulation of the CST's produced NAT levels which were less than half those resulting from

bilateral stimulation and which also demonstrated that no significant differences existed between the capacities of the left and right CST's to activate NAT (Bowers & Zigmond, 1982).

While most cells appeared to be stimulated by sympathetic nerves, some were inhibited by noradrenalin in the circulation, suggesting dual excitatory and inhibitory control of the pineal by this catecholamine (Reyes-Vazquez & Dafny, 1985; Reyes-Vazquez et al, 1986).

(iv) **Circadian rhythm in NAT activation during sympathetic stimulation**

The magnitude of the increase in NAT activity during sympathetic stimulation, is dependent on the time of day as well as the frequency of stimuli. Responses during daytime occur at a much slower rate than those observed at night (Bowers & Zigmond, 1982). Nighttime electrical stimulation at 5 or 10 Hz produced significant linear increases in NAT activity during 2 hrs of stimulation, whereas daytime stimulation at 10 Hz produced a much slower non-linear increase in NAT activity. During the first hour this was equivalent to only 8% of that seen during the corresponding period of stimulation in animals at night, but reached levels comparable to those obtained during nighttime stimulations towards the end of the third hour (Bowers & Zigmond, 1980,1982). This daytime lag may be accounted for by the time needed for messenger RNA to be synthesized and transported into the cytoplasm of pineal cells prior to the initiation of protein synthesis (Zatz et al, 1976). A further possibility is that down-regulation of β-adrenergic receptors during the latter part of the night desensitizes the pineal to stimulation during the light phase (Gonzalez-Brito et al, 1988b).

(IV) **Inhibitory regulation of sympathetic nerve activity.**

Optic nerve stimulation, through electrical or photic means, is capable of inhibiting tonic CST activity. In particular an American group of workers has demonstrated that low frequency electrical stimulation (1 Hz) is more effective in producing this inhibition than are frequencies of around 10 Hz (Brooks et al, 1975). In addition electrophysiological, pharmacological and morphological studies have provided evidence for a sympathetic intraganglionic inhibitory system which is activated by high intensity stimulation of pre-ganglionic fibres and is mediated by the release of a catecholamine, probably dopamine (Greengard & Kebabian, 1974; Burnstock & Costa, 1975; Eranko, 1978). Cells of this inhibitory system in the SCG are likely to be interneurons (identified as small intensely fluorescent cells due to their formaldehyde-induced fluorescence), which are often associated with principal nerve cells originating in the SCG and innervating the pineal. The diverse pattern of association between these two cell types in the SCG may help to explain the range

of responses of single pinealocytes to electrical stimulation of the SCG (Reuss & Schroder, 1988).

1.7 Purpose of this study

The objectives of the experiments presented in this thesis were two-fold and were inspired by the opportunity to contribute towards an area of pineal physiology which has received little attention in the past, but which is of interest to all studying neuroendocrinology. That is, the relationship between neural activity and hormone secretion.

Initially, an investigation was carried out to determine the effects of various anaesthetics on the nocturnal secretion of melatonin from the pineal. The aim of this work was to find an anaesthetic treatment which, while providing surgical anaesthesia, did not interfere significantly with the nocturnal production and secretion of melatonin. Results of this work were applied to subsequent studies in which acutely prepared animals were used to determine the effect of electrical stimulation of the pineal's sympathetic innervation on melatonin secretion in anaesthetized sheep.

Subsequent experiments were designed to determine how the circadian timing of sympathetic neural stimulation and stimulus characteristics influenced pineal melatonin secretion. Previous studies of this nature have been conducted in vitro or in acutely prepared and anaesthetized animals. Development of electrodes which could be chronically implanted, however, allowed stimulation experiments to be performed in conscious sheep, without the potential inhibitory effects of anaesthetics interfering with the pineal secretory response. In addition, the use of melatonin secretion as an index of pineal secretory activity has, with the exception of one very recent study in rabbits, been restricted to a few in vitro studies using a variety of stimulation techniques. Virtually all other studies have used induced NAT activity as the parameter of pineal synthetic potential. Within the current series of studies, however, sequential changes in melatonin secretion were recorded through repeated blood sampling during the course of each experiment.

Finally, an immunocytochemical study was undertaken to characterize the innervation of the ovine pineal by identifying the presence and origin of various regulatory neuropeptides and enzymes and by determining the effects of unilateral or bilateral superior cervical ganglionectomy on these antigens. Unique characteristics of each antigen and their presence in pineal tissue enabled this study to detect morphological evidence for changes in sympathetic nervous system control of pineal function following partial or total sympathetic denervation.

CHAPTER 2

Materials and Methods

2.1 Animals and management.

Adult Romney rams, one to two years of age and weighing 45-55 kg., were used in all experiments detailed in this thesis.

All animals in Experiments 1, 2, 3, and 4 were maintained indoors during the course of each experiment. Rams used in Experiment 1 were held in a communal pen, while those in Experiments 2, 3 and 4 were held in individual animal crates. Animal accommodation was in well ventilated, light proof rooms maintained at a constant 15°C. Fluorescent lighting provided approximately 200 lux at the eye level of each ram, and was controlled by automatic time switches to provide the lighting regimes determined by each experimental design. Feed consisted of approximately 800 grams of lucerne chaff per day, with water available ad libitum.

Rams used in Experiment 5 were held on pasture after recovery from surgery and until euthanasia.

2.2 Surgical techniques.

2.2.1 General surgical procedures.

a) Food was withheld for at least 24 hours preceding surgery. Water continued to be available ad libitum during this period.

b) Except for Experiments 1 & 2, anaesthesia was induced with 'Saffan' (Glaxovet Ltd, Harefield, England) containing Alphaxalone 0.9% w/v and alphadalone acetate 0.3% w/v, administered as an intravenous bolus at the rate of 3 mg/kg body weight. Anaesthesia was maintained with 2-3% (v/v) halothane ('Fluothane', ICI, Macclesfield, Cheshire, England) in oxygen, delivered via an endotracheal tube at a rate of 2 l/min from a Fluotec 3 (Cyprane, Keighley, England) vaporizer.

Anaesthetics used in Experiment 1 varied according to the experimental design and are described in Chapter 3. Anaesthesia in Experiment 2 was induced and maintained with halothane.

c) Surgical procedures were conducted under sterile conditions, except in the acute studies (Experiment 2) in which sterile procedures were not required.

d) Following recovery from anaesthesia, rams were placed back in their crates and given prophylactic antibiotic treatment consisting of an initial 10 ml intramuscular injection of 'Streptopen' (Glaxo (NZ) Limited) followed by five consecutive daily treatments of 5 ml. This preparation contains procaine penicillin and dihydrostreptomycin, each at a concentration of 250 mg/ml.

2.2.2 Surgical preparation for acute stimulation and recording (Experiment 2)

- refer to Section 2.3 for details of the design of the relevant electrodes.

In order to stimulate the cervical sympathetic trunks at the level of the sixth cervical vertebra, anaesthetized rams were positioned in dorsal recumbency and a mid-ventral skin incision was made from a point 3-4 cm above the first costosternal joint, rostrally approximately 15-20 cm. Lateral retraction of the skin and subdermal connective tissue exposed the sternocephalic muscles which, when separated by blunt dissection and retracted, exposed the sternohyoid and sternothyroid muscles. These latter muscle groups were in turn separated in the midline and retracted to reveal the trachea.

Further dissection revealed the common carotid artery and associated vagus and cervical sympathetic trunks lying dorsolateral to the trachea. Connective tissue binding the common carotid to the two nerve trunks was then separated and a 10-15 cm length of cervical sympathetic nerve was dissected free from the connective tissue of the vagus and surrounding muscles. To verify the identity of this nerve trunk, electrodes were positioned under it and a short burst of electrical stimuli applied. Widening of the palpebral margin in response to this stimulation was taken to confirm that the dissected nerve was the sympathetic innervation to the head and, hence, the pineal.

Upon completion of this procedure, a metal ring, 110 mm in diameter, was positioned over the area and the skin edges sutured to it around its entire perimeter. When held in a position suspended above the surgical field with the skin drawn tight a well was formed in which pairs of stimulating electrodes were placed bilaterally in the caudal region of the surgical field. Blue glass hooks were then used to manoeuvre each CST over its respective electrodes. Care was taken to ensure the wires of the electrodes were orientated with the anode caudal to the cathode to prevent the development of an anodal block (Thompson, 1967)

rostral to the site of stimulation, which is capable of impeding the transmission of electrical impulses to the head and pineal. Approximately 10 cm cranial to the stimulating electrodes a further segment of sympathetic nerve was exposed and freshly flamed, platinum wire electrodes were placed under the nerves to enable recording of the generated compound action potential on an oscilloscope. In addition, an earth electrode, as described in Section 2.3.2, was situated between the stimulating and recording electrodes. Finally, the whole preparation was covered with warm (37°C) paraffin oil to a depth of 2-3 cm above the dissected nerves.

2.2.3 Chronic electrode implantation (Experiments 3 & 4)

- refer to Section 2.3 for details of electrode design.

A surgical technique, similar to that used in the acute stimulation experiments, but under sterile operating conditions, was used to isolate the cervical sympathetic trunks prior to chronic implantation of electrodes for stimulation of these nerves in conscious animals. After exposure of the CST's and confirmation of their identity as described above, electrodes were manoeuvred under the sympathetics, opened and passed around the nerves. In this way the electrode wires were held in contact with the nerve while still isolated from other tissue. Orientation of electrode wires was again with the anode caudal to the cathode. 3-0 suture material was used to secure the strip of silastic sheeting to local muscle tissue. A subcutaneous tunnel was formed (with the aid of a blunt ended trocar, a slightly curved, hollow, stainless steel tube with ID of 5 mm), from the operative site to the dorsum in the vicinity of the withers, where it was passed through a skin incision so that the electrode wires could be exteriorized at that point. Each anode and cathode wire was soldered to a multipin edge connecter secured to the ram's back with adhesive tape. Closure of the surgical site involved suturing of subcutaneous tissues with 3-0 suture thread and closure of the skin with Michel clips (Aesculap, Tuttlingen, Germany). A protective elastic netting (Systenet, International surgical netting S.P.A, Moncalieri, Italy), supplied as circular netting, was then passed over the animal's head and positioned so as to prevent the electrode wires and edge connector from snagging on animal crate fixtures.

2.2.4 Superior cervical ganglionectomy (Experiment 5)

Removal of one or both superior cervical ganglia was achieved using the lateral approach described by Appleton & Waites (1957). A skin incision was made beginning from

a point just rostral to and at the level of the auditory meatus and extended parallel to the mandible to the level of the thyroid cartilage. Superficial muscles lying under the skin were cut and reflected exposing the parotid gland and the external jugular vein. Those vessels which pass caudally from the external jugular and internal maxillary veins were ligated and cut. Retraction of the parotid gland enabled the common carotid artery to be identified and traced to the point where it is crossed by the hypoglossal nerve. Blunt dissection techniques were then used to locate and isolate the superior cervical ganglion from surrounding structures and adipose tissue, prior to excision of the ganglion with scissors. Completion of the operation entailed suturing muscles and the parotid gland into their original positions and layered closing of subcutaneous tissue and the skin. This procedure was repeated on the other side in those animals in which bilateral ganglionectomies were required.

2.3 Equipment for electrophysiological studies.

2.3.1 Stimulating electrodes.

(I) Acute stimulation.

The electrodes for the acute stimulation of CST's in anaesthetized rams in Experiment 2 were silver/silver chloride hook electrodes (diameter 0.5mm). Chloride coating was carried out, as described by Mayhew et al (1985) to prevent the formation of a charge gradient (otherwise called the electrode double layer) between the electrodes. Such a gradient results from the tendency, at any electrode/electrolyte interface, for the electrode to discharge ions into solution and for ions in the electrolyte to combine with the electrode thus forming polarized charging at the positive (attracting negatively charged ions) and negative (attracting positively charged ions) electrodes. The effect of a charge gradient is to impede the flow of electrons (charge) between the electrodes and hence diminish the current strength of the applied stimulus. The presence of the silver chloride coating prevents the formation of the electrode double layer by dissociating to silver and chloride ions which migrate between the electrode and electrolyte (Strong, 1971). Prior to chloriding, the silver wire was cleaned by repeated dipping in aqua regia (1 part concentrated HNO_3 : 3 parts concentrated HCl) until the surface was an even matt grey colour, followed by a wash in double distilled water. Coating with silver chloride was achieved by suspending the silver wire as the anode in 0.1 M NaCl and passing a current of 238 μA through the solution for 12 hours. A similar silver wire,

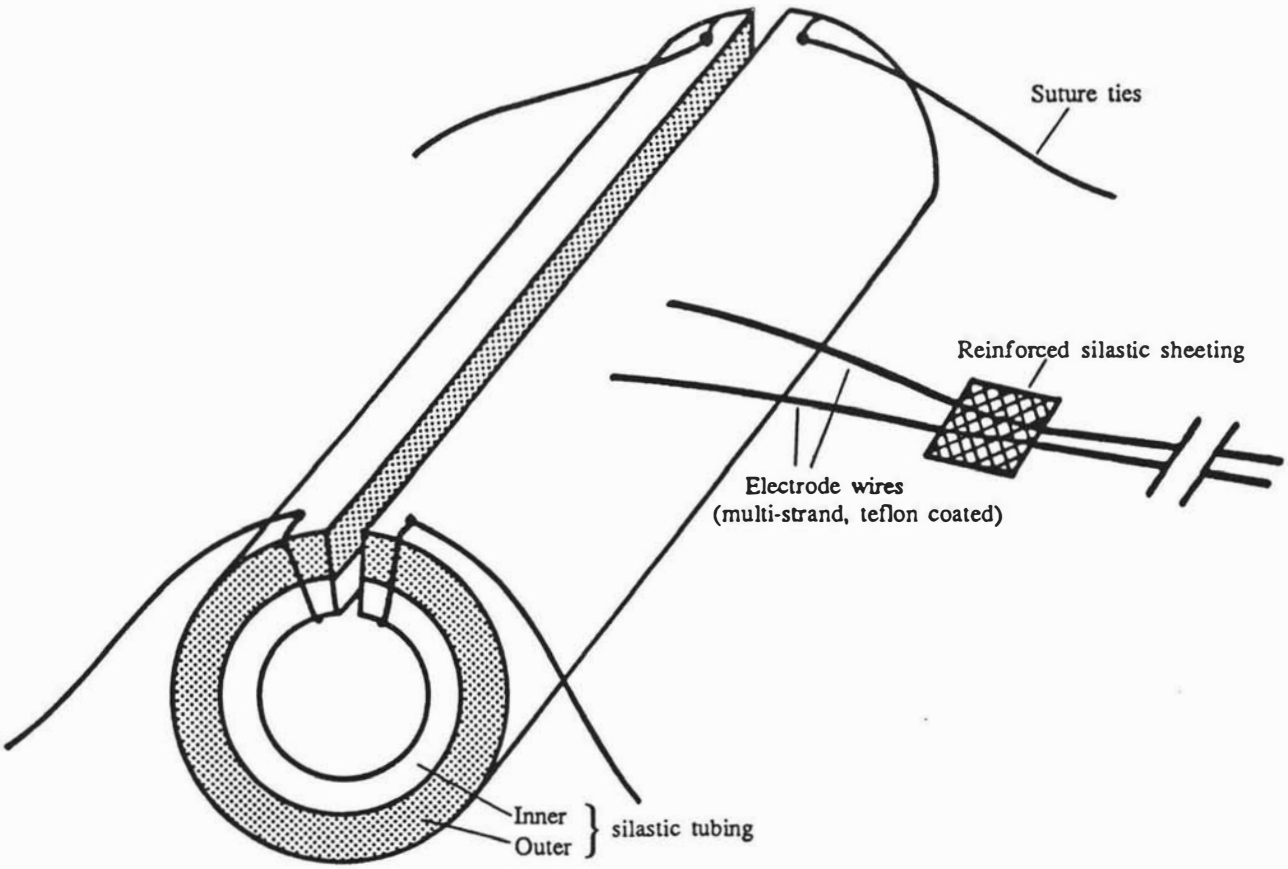
ring shaped and suspended in the NaCl solution so as to encircle the anode, acted as the cathode. This procedure was repeated prior to each experiment. Chlorided electrodes were then stored in the dark to prevent the reduction of silver chloride to metallic silver, by light.

(II) **Chronic stimulation (Experiments 3 & 4)**

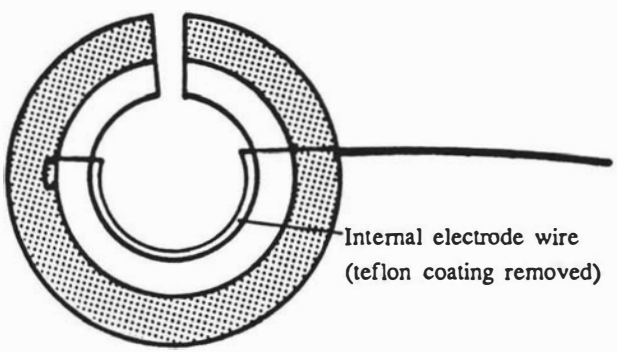
Electrodes for chronic implantation and stimulation of CST's in conscious rams in Experiments 3 and 4 were a modification of a design described by Loke et al (1986). Electrodes (illustrated in Fig 2.1), consisted of 2 lengths of Teflon coated, multistrand stainless steel wire (Biomed wire, Cooner wire company, Chatsworth, USA) positioned inside two layers of 1.5 cm long silastic tubing (Dow Corning Corporation, Midland, USA).

Five millimetres of the teflon coating was removed from the ends of each of the 50 cm long wires which represented the anode and cathode electrodes. The stripped section of each wire was passed, with the aid of a hypodermic needle, through the walls of the inner silastic tube (1D 1.95 mm, OD 3.125 mm) at a point approximately 0.5 - 0.75 mm above the midline of the tube. Wires were placed 5 mm apart and 5 mm from the nearest end of the tube. The bare wire passing through the inner space of the silastic tube was then moulded around the internal perimeter, so as to form a concave structure which was to partly encircle the nerve and ensure electrical contact was maintained. Exposed wire on the outside of the tube was glued to the external surface with a fast drying adhesive (Loctite, Loctite Corporation, Dublin, Ireland).

To protect the electrodes inside their silastic sheath from mechanical damage, a larger size silastic tube (OD 4.65 mm, ID 3.35 mm) was glued over the external surface of the smaller tube. This was achieved by cutting through one wall of the larger tube from end to end, filling it with a biologically inert silicone adhesive (Medical adhesive, Silicone type A, Dow Corning Corporation, Midland, USA) and inserting the smaller tube into the internal surface of the larger tube with the open face of the electrode wires orientated towards the cut. The electrode wires were then passed through the wall of the outer tube by the procedure described above for the inner tube. In this way all exposed surfaces were composed of biologically inert materials. Once the adhesive had cured the inner tube was cut along the same line as that of the outer tube, thus forming a potential opening through which the nerve



a) General view



b) Transverse section showing internal arrangement of electrode wires within silastic tubing

Figure 2.1 Schematic representation of the chronically implanted stimulating electrodes used in Experiments 3 and 4 for bilateral stimulation of the CST's of conscious rams.

could pass to lie in contact with the electrodes. At this stage one wire was designated as the anode and a knot tied at the free end for later identification. Suture threads, 2-3 cm long, were attached at both ends of the silastic tubing and on both sides of the cut to aid in the opening of the tubing for placement of the electrodes around the nerve. These threads were also tied together at each end after the electrode had been passed around the nerve to retain the nerve in place. Finally, a 1 x 0.5 cm strip of reinforced silastic sheeting (Dow Corning Corporation, Midland, USA) was secured to the electrode wires with silicon adhesive, approximately 5 cm from the silastic tubing, to allow suture fixation to muscle tissue in the neck so that tension on the electrodes and nerves was minimized.

2.3.2 **Recording electrodes.**

In a series of trial experiments (n=6), prior to Experiment 2, recordings were made of the generated compound action potential in the CST's, rostral to the site of stimulation. The recording electrodes consisted of hook-shaped, freshly flamed platinum wire (diameter 0.5 mm) positioned under the CST's approximately 8-10 cm rostral to the stimulating electrodes. An additional earthed silver/silver chloride electrode was placed under the nerve, between the stimulating and recording electrodes, and a pledget of saline-soaked cottonwool placed over the nerve and electrode to improve electrical contact. This electrode served to reduce the stimulus artifact in the recording so that the stimulus parameters which maximized the amplitude of the compound action potential could be established (see Section 3.2.1 for explanation).

2.4 **Stimulus generation and delivery.**

The 'Neurolog' system of modular instruments for electrophysiology (Digitimer, Welwyn Garden City, Hertfordshire, England) was used in all studies requiring nerve stimulation (Experiments 2-4). Four modules of this system (illustrated in Figure 2.2 in the arrangement for Experiment 3) were used to generate stimuli of the frequencies, durations and amplitudes determined by the experimental designs, as specified in the appropriate chapters. Each module was situated in a mother-board and received input or transferred output to/from neighbouring modules, either via internal connections at the back of each module, or via

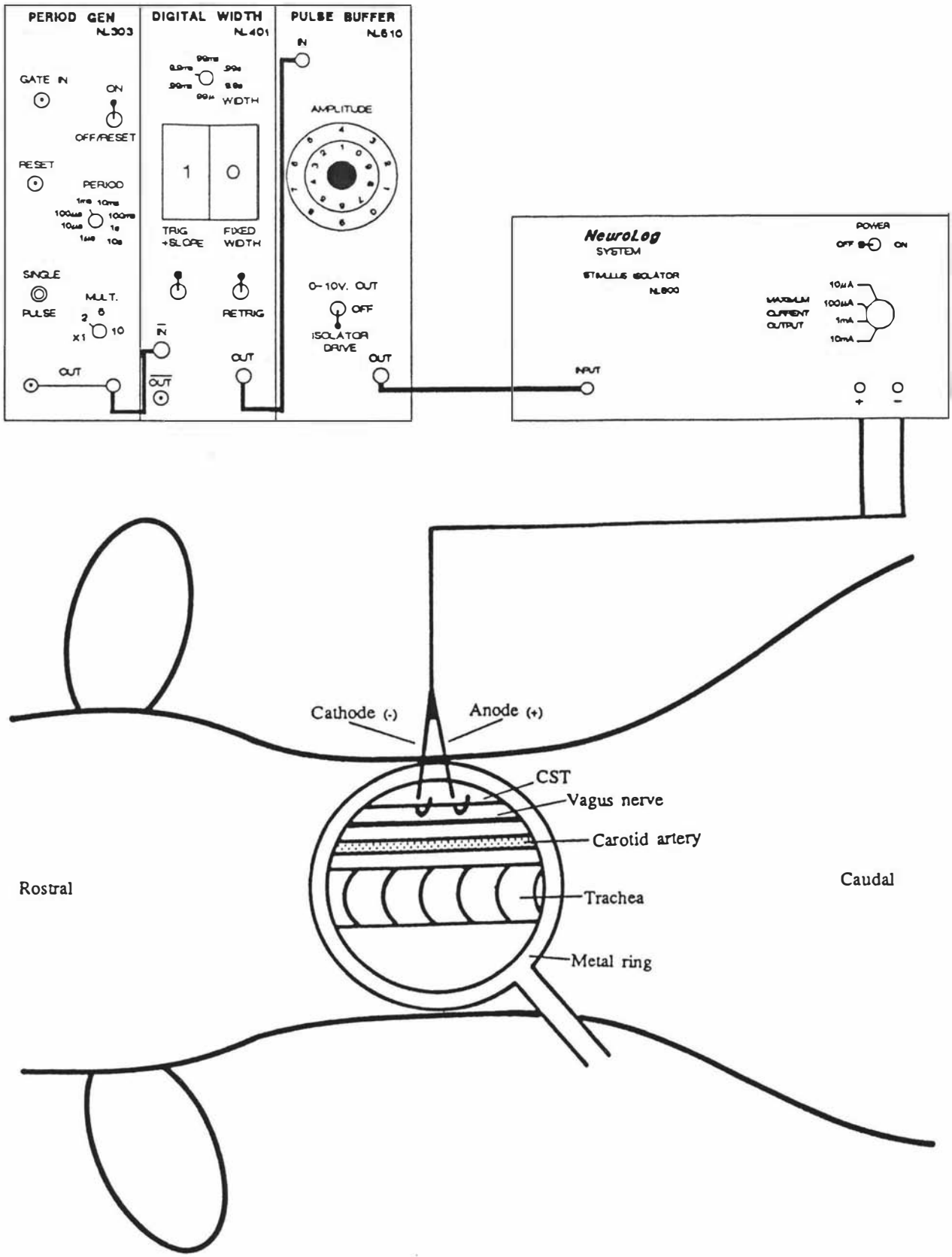


Figure 2.2 Schematic representation of the arrangement of Neurolog modules and electrodes for left side CST stimulation. For bilateral stimulation in Experiment 2, the right side was surgically prepared in a similar manner and electrodes placed under the right CST.

electrical leads attached to the face of each module. The method of generation of each stimulus parameter with the Neurolog system is outlined below.

(i) **Frequency.**

A single period generator (NL 303) was used to generate the frequency (Hz) of the stimuli. These units were responsible for 'driving' the others in the system and produced stimuli of precise and stable frequency.

(ii) **Duration.**

The digital width module (NL 401) received output from the period generator and produced pulses of adjustable duration. Output was fed into two pulse buffer modules.

(iii) **Amplitude.**

Amplitude was determined by two separate modules for each nerve being stimulated. Output from the digital width module was received by each of two pulse buffers (NL 510). The variable voltage output from each pulse buffer was adjusted with a 10-turn potentiometer to regulate the output of the stimulus isolator (NL 800). Output from the stimulus isolator was a constant current within the output range set by the switch on the NL 800 and the position of the potentiometer of the pulse buffer.

Prior to each experimental series the output from this system was tested by passing the output through an oscilloscope (type 564 storage oscilloscope, Tektronix Inc, USA), to ensure accurate delivery of the stimulus parameters.

2.5 Compound action potential recordings.

The compound action potentials recorded during the acute stimulation experiments were amplified by an AC preamplifier (Neurolog module NL 103), passed through a 50 Hz notch filter (Neurolog module NL 125) and displayed on the oscilloscope. See Section 3.2.1 for the purpose of this recording.

2.6 Blood collection.

Blood samples in Experiment 1 were collected into 10 ml vacutainer tubes containing 150 units of heparin following jugular venipuncture, while those in Experiment 2 were collected via 1.5 mm I.D polythene tubing (Dural Plastics & Engineering Pty Ltd, Dural, N.S.W, Australia) cannulae, inserted into a femoral vein following the induction of anaesthesia.

After collection, blood was heparinized at the rate of 15 I.U./ml to prevent clotting. Throughout Experiments 3 & 4 indwelling jugular cannulae were used to sample blood. These consisted of silicon tubing ("Silastic", Dow Corning Corporation, Midland, USA) with an internal diameter of 1.55 mm and were inserted at the time the electrodes were implanted or with the aid of a local anaesthetic ("No Pain", Ethical agents Ltd, Auckland, NZ) immediately prior to the experiment. Both jugular veins were cannulated to protect against cannula failure. Immediately following collection all blood samples were heparinized as described above.

In all experiments blood samples were centrifuged immediately after collection at 3000 rpm for 15 minutes and plasma was stored at -18°C until assayed for melatonin content. For Experiments 1 to 4, in which melatonin secretory profiles were measured, collection protocols are given in the materials and methods of each chapter.

2.7 Melatonin radioimmunoassay.

All plasma samples from Experiments 1 to 4 were analyzed for melatonin concentrations using the method described by Kennaway et al (1982). All samples, standards, blanks and quality control samples within each assay were assayed in duplicate.

2.7.1 Reagents.

(I) **Assay buffer,** pH 7.4, contained 0.08 M di-sodium hydrogen orthophosphate (Na_2HPO_4) (Univar,Ajax Chemicals, Sydney, Australia), 0.02 M sodium dihydrogen phosphate-dihydrate ($NaH_2PO_4.2H_2O$) (Riedel-De Haen AG, Seelze-Hannover, West Germany), 0.15 M sodium chloride (May & Baker New Zealand Ltd, Auckland, New Zealand), bovine serum albumin (Fraction V, Sigma Chemical Company, St Louis, MO, USA) 5 g/l, bovine gamma globulin (Cohn Fraction II, Sigma Chemical Company, St Louis, MO, USA) 500 mg/l and sodium azide (Riedel-De Haen AG, Seelze-Hannover, West Germany), 1 g/l, as a preservative. Between assays the buffer was stored at 4°C.

(II) **1% ethanol buffer.** Absolute ethanol was diluted to 1% by volume with assay buffer on the day of assay.

(III) **Borate Buffer.** 0.5 M borate buffer was made with 152 g/l potassium tetraborate ($K_2B_4O_7.4H_2O$) (Unilab, Ajax Chemicals, Sydney, Australia) in double distilled water and adjusted to pH 9.6.

(IV) **Saturated ammonium sulphate.** 520 g of ammonium sulphate (May & Baker Ltd, Dagenham, England) was dissolved in 1 l of double distilled water and stored at 4°C.

(V) **Scintillation fluid.** 9 grams of 2, 5-diphenyloxazole (PPO) (Sigma Chemical Company, St Louis, MO, USA) and 300 mg of (1,4-bis[2-(5 phenyloxazolyl)] benzene) (POPOP) (Sigma Chemical Company, St Louis, MO, USA) were dissolved in 2 litres of toluene. One litre of Triton X-100 was then mixed in to make up 3 l of scintillation fluid.

(VI) **Organic solvents**

- Absolute ethanol	(May & Baker Australia Pty Ltd, Victoria, Australia)
- Dichloromethane	(May & Baker Australia Pty Ltd, Victoria, Australia)
- Hexane	(Unilab, Ajax Chemicals, Auburn, N.S.W, Australia)
- Methanol	(May & Baker Australia Pty Ltd, Victoria, Australia)
- Petroleum spirit 60-80°C	(May & Baker Ltd, Dagenham, England)
- Toluene	(Shell Chemicals New Zealand Ltd, Wellington, New Zealand)
- Triton X-100	Rohm and Haas New Zealand Ltd, Auckland, New Zealand)

2.7.2 Preparation of standards, tracer & antibody

Standard curves were made by diluting a melatonin stock standard solution (1 mg/ml in absolute ethanol) with borate buffer to give a range of standards with melatonin concentrations from 20 to 1000 pg/ml, within which is the expected range of physiological values. The stock standard was made from crystalline melatonin (M5250, Sigma Chemical Company, St Louis, MO, USA) dissolved in absolute ethanol. Tracer was supplied by Amersham International plc, (TRK.798, Little Chalfont, England, 85 Ci/m mol) and stored as a working standard by diluting 20 µl in 1 ml of absolute ethanol. This was further diluted for each assay to give 10-12,000 cpm in 100 µl of assay buffer to be added to each tube. Lyophilized melatonin antiserum (GT280), raised in goats and donated by Dr D.J. Kennaway (University of Adelaide, Adelaide, South Australia), was reconstituted in assay buffer to give a dilution of 1:25 which was stored frozen in 50 µl aliquots. Working antibody dilution was prepared by further dilution with assay buffer to 1:7500, to give a final dilution in the incubation mixture of 1:60,000.

2.7.3 Assay protocol

Extraction of melatonin was necessary before RIA procedures were performed. This was accomplished by an initial gentle rocking of tubes (16 x 125 mm borosilicate culture tubes, Kimble, Vineland, Illinois, USA) containing 1 ml of plasma, quality control sample, assay blank or standard melatonin solution, with an added 1 ml borate buffer plus 6 mls of petroleum spirit (b.p. 60-80°C) for 30 mins to remove interfering lipids. After aspiration of the petroleum spirit, extraction of melatonin into 5 ml of a dichloromethane/hexane mixture (1:1) was achieved by further gentle rocking of tubes for 30 min. Following freezing of the aqueous phase, the organic phase was poured into 12 x 100 mm borosilicate glass assay tubes (Kimble, Vineland, Illinois, USA) and evaporated to dryness at 37°C in a thermostatically controlled heating block (Grant Instruments (Cambridge) Ltd, England) under a continuous stream of oxygen free, industrial grade, dry nitrogen. Crystalline melatonin was redissolved into 300 µl methanol, vortexed and evaporated to dryness in order to concentrate the melatonin at the bottom of the assay tube. Five hundred microliters of 1% ethanol in assay buffer, 100 µl antiserum, 100 µl ^{3}H melatonin and 100 µl of assay buffer was added, in the above order, to give a final incubation volume of 800 µl and an antibody dilution of 1:60,000. Incubation to equilibrium was achieved at 4°C overnight or at room temperature for 1 hour followed by a further hour at 4°C. Separation of bound and unbound melatonin was achieved using 800 µl of a saturated solution of ammonium sulphate (4°C) to precipitate the antibody-melatonin complex. After vortexing and incubation for 30 mins at 4°C, assay tubes were centrifuged at 3000 rpm for 30 mins. Assay tubes were kept on ice, the supernatant aspirated and the remaining pellet (precipitated antibody-antigen complex) dissolved by vortexing with 1 ml of distilled water. Nine hundred microliters were then transferred into 20 ml glass vials (Packard, Canberra, Australia), 5 ml of scintillation fluid was added, vials capped and placed in a scintillation counter (LS7500, Beckman, California, USA) for estimation of radioactivity. Readings (cpm) for each sample were used to determine the concentration of melatonin (in pg/ml) by the method of Burger et al (1972), as modified by Prof R.E Munford (Massey University, Palmerston North, New Zealand) for use on an IBM compatible computer.

2.7.4 Performance and validation.

Specificity of the melatonin antibody has been established by Dr D.J. Kennaway (University of Adelaide, Adelaide, South Australia) by demonstrating a low level of cross

reactivity with melatonin metabolites or synthetic intermediates (<1.2%) (Kennaway et al, 1982). Mean non-specific binding (blank as a percentage of the counts of the 0 pg/ml standard) for 57 assays was 10.5 ± 2.4%. Mean assay sensitivity, defined as the minimum detectable concentration significantly different from zero (Burger et al, 1972) was 6.05 ± 2.75 pg/ml (n=60).

Reproducibility of assay results, estimated from three quality control plasma samples assayed twice in each assay, is shown in the table below. In total, six quality control plasma pools were used to assess assay performance.

Table 2.1 **Between- and within-assay CV for melatonin radioimmunoassay based on repeated measurement of ovine plasma samples.**

Replication factor (n)	Control plasma	Mean melatonin level (pg/ml)	Within-assay C.V (%)	Between-assay C.V (%)
46	MP1	29.41	31.51	42.54
46	MP4	1192.73	9.63	8.47
46	MP5	333.07	13.02	17.38
36	MP6	30.48	13.69	36.58
60	MP7	84.98	10.47	24.71
58	MP8	172.92	8.09	19.02

Dilution with assay buffer did not affect the estimated melatonin content of two quality control plasma samples (Table 2.2).

Table 2.2 **Effect of dilution with assay buffer on estimates of melatonin concentration in two quality control plasma samples. All samples were assayed twice.**

Sample	Dilution factor	Melatonin concentration (pg/ml)
MP2	neat	663.9
"	1:1	331.4
MP4	neat	1335.4
"	1:1	640.7
"	1:3	316.0

2.8 Pineal tissue collection and processing.

2.8.1 Collection

In Experiment 5, pineal glands which were to be studied histologically, were collected at night between 2245 hours and 2400 hours, after the rams had been killed in the dark with a lethal dose (60 mg/kg) of sodium pentobarbitone ("Nembutal", Techvet Laboratories Ltd, Auckland, New Zealand). Each animal's head was immediately removed and the top of the cranium removed with the aid of a band-saw to expose the brain. The pineal gland, attached to the posterior roof of the third ventricle and lying in the sulcus between the rostral colliculi, in close association with the dorsally situated deep cerebral vein, was exposed by retraction of the occipital lobes of the cerebral hemispheres, dissected free from brain tissue surrounding its attachment and lifted out. Excess brain tissue was trimmed from the pineal leaving only a small amount of thalamic tissue on the anterior margin for purposes of orientation. The whole procedure from killing to immersion of the pineal in histological fixative was completed in approximately 6-8 min.

2.8.2 Processing prior to ICC

(I) Tissue fixation

Immediately after trimming of the aforementioned brain tissue, pineals were fixed for 12 hours in Bouin's fluid and then transferred to 70% ethyl alcohol. Prior to paraffin wax embedding pineals were cut in half down the midline, each half then being transected twice into 3 blocks, representing the stalk, middle and apex of the pineal. The first cut was made at the level of the stalk, while the second was made approximately through the middle of the body of the pineal.

(II) Paraffin wax embedding

All pineal tissue was paraffin wax embedded using an automatic tissue processor (Shandon Elliott, Liverpool, U.K). The tissues were dehydrated, cleared and impregnated with paraffin wax according to the schedule in Table 2.3 (Birtles, 1981).

Table 2.3 Paraffin wax processing schedule.

Process		Reagent	Time
1. Dehydration		70% ethyl alcohol	1hr
		95% " "	"
	Change 1.	100% " "	"
	" 2.	" " "	"
	" 3.	" " "	2hr
2. Clearing	Change 1.	Chloroform	1hr
	" 2.	Xylene	"
	" 3.	"	"
3. Impregnation	Temperature 58°C	Paraffin wax m.p 56°C	2hrs
	"	" " " "	"

Following impregnation with wax, tissue blocks were transferred to a tissue embedding centre (Tissue Tek II, Miles Laboratories Inc, Elkhart, Indiana, USA), embedded in paraffin wax and attached to plastic cassettes. Sections, 6 μm thick, were cut using a sliding microtome (Model OME, C.Reichert Optische Werke AG, Vienna, Austria) floated onto warm water, transferred to polyvinyl acetate (PVA) coated 75 × 25mm glass slides and air dried at 60°C.

2.8.3 Immunocytochemistry

Immunocytochemical methods were employed to investigate the presence of the following antigens in pineal tissue:

Table 2.4 Antibodies used in ovine pineal tissue immunocytochemistry.

Antigen	Antibody Source	Antibody #
1. VIP	Amersham International plc, Little Chalfont, UK	RPN1582
2. PNMT	Eugene Tech International Inc, Allendale, N.J, USA	TE104
3. NSE	Dako Corporation, Santa Barbara, USA	Dako A589
4. NPY	Amersham International plc, Little Chalfont, UK	RPN1702

Each antiserum has been shown to be specific for its antigen, with no detectable cross-reactivity with structurally related peptides or proteins (NPY & VIP data sheets; PNMT, Dr A.C. Towle, personal communication). NSE showed no cross-reactivity with alpha subunits (glial), but possible cross-reactivity with beta subunits (muscle) (NSE data sheet).

After initial application of the appropriate 1° antibody to the sections, tissue antigen-antibody binding sites were localised by the biotin-streptavidin detection system (Amersham International plc, Little Chalfont, U.K) and visualised under a light microscope. This detection system consisted of a two step process requiring initial binding of a biotinylated anti-rabbit immunoglobulin G (IgG) to the primary antibody, followed by binding of preformed biotinylated peroxidase-streptavidin complex to the immunoglobulin. The antigen-antibody reaction complex was visualised with the DAB-hydrogen peroxide reaction, which produced a brown, insoluble reaction product at binding sites. The following equation illustrates this latter reaction:

Peroxidase (HRP) + Peroxide (H_2O_2) ----> $HRP.H_2O_2$ $-^{D}-^{A}-^{B}->$ coloured reaction product + HRP + H_2O.

Table 2.5 outlines the immunocytochemical procedure in stepwise fashion:

Table 2.5 Immunocytochemical procedure for the identification of antigens in pineal tissue.

Step	Procedure
1.	Dewax sections: Two changes of xylene, 5 mins each.
2.	Rehydrate: Brief immersion in absolute and 70% alcohol to remove xylene followed by a wash in tap water.
3.	Wash in PBS[1] for 1 min.
4.	Blot slides dry with paper tissue.
5.	Cover sections with 1% bovine serum albumin (BSA) (Sigma Chemical Company, St Louis, MO, USA) in PBS to block non-specific binding sites. Place in humidity tray for 5 mins.
6.	Drain off BSA, cover sections with primary (1°) antibody[2] (except negative control which was covered with 1% BSA in PBS) and incubated in humidity tray for 1 hour at room temperature.
7.	Three 1 minute washes in PBS.
8.	Slides dried with paper tissue, covered with the secondary (2°) antibody (1:200 dilution of biotinylated anti-rabbit IgG with 1% BSA in PBS) and incubated in the humidity tray for 30 mins at room temperature.
9.	Three washes in PBS; 1 min each.
10.	Slides drained and wiped dry with paper tissue. Sections covered with the streptavidin peroxidase preformed complex (1:400 dilution with 1% BSA in PBS) and incubated in the humidity tray for 15 mins at room temperature.
11.	Three washes in PBS; 1 min each.
12.	Visualize reaction with DAB-hydrogen peroxide solution[3] (4 mg DAB dissolved in 7 ml PBS and 10 µl of hydrogen peroxide).
13.	Place in PBS to stop reaction.
14.	Rinse in tap water.
15.	Counterstain in haematoxylin (Mayer's haemalum[4]) for 30 sec.
16.	Wash off excess stain in tap water, blue in Scott's tap water for 2 mins.
17.	Dehydrate through 70% ethanol and two changes of absolute ethanol.

18. Clear in two changes of xylene.

19. Mount in DPX (synthetic mountant, BDH Chemicals, London, England).

Notes:

1. Phosphate buffered saline (PBS), pH 7.4, comprised 0.154 M sodium chloride, 0.002 M potassium dihydrogen orthophosphate and 0.008 M disodium hydrogen orthophosphate.

2. VIP antibody diluted 1:1000 with 1% BSA in PBS

   PNMT " " 1:200 " " " " "

   NSE " " 1:1000 " " " " "

   NPY " " 1:250 " " " " "

3. Reaction time varied from 4-6 mins. Some subjective assessment was involved.

4. Culling (1974)

The procedures described above resulted from initial studies to maximize the binding of each antibody to its antigen in pineal tissue. Previously, this laboratory had not undertaken studies using these antibodies specifically in pineal tissue, therefore it was deemed necessary to approach this study assuming established procedures were not necessarily optimized for individual antibodies in this tissue. In these initial studies each trial staining run included: (a) a positive control (a tissue other than pineal which was known to contain the antigen) to confirm the 1° antibody was binding to the antigen; (b) a negative control (pineal tissue without 1° antibody added) as a check on non-specific reaction product formation; and (c) non-experimental pineal tissue to which the 1° antibody was added to confirm antigen-antibody binding in this tissue. Positive control tissues were as follows: duodenum for NSE, VIP and NPY and adrenal for PNMT. Negative controls indicated that there were insignificant levels of non-specific binding. Non-experimental pineal tissue was used in trials aimed at optimizing antibody dilutions, the necessity for trypsin pretreatment, DAB reaction time and the need for enhancement techniques (eg heavy metal intensification utilising 50 µl of 1% cobalt chloride and 1% nickel chloride in distilled water added to the DAB solution or silver enhancement of the DAB-peroxidase complex) for detection of reaction product. It was concluded that trypsin pretreatment and enhancement procedures were not required for the NSE, PNMT or NPY reaction products because the peroxidase reactivity was strongly positive. Heavy metal

intensification was used to enhance the VIP reaction product. ICC procedures were considered optimized when the reaction gave strong specific staining without significant levels of background staining or non-specific binding.

2.8.4 Evaluation of antigenic immunoreactivity

Prior to examination, identity codes for each slide were covered with non-transparent, adhesive labels in order to facilitate unbiased evaluation of each antigen's immunoreactivity. Slides were then randomized prior to sequential numbering for purposes of record keeping.

Two sections, taken 60-90 μm apart, were examined from each of the three blocks that comprise each half pineal.

Methods used for the evaluation of each antibody's immunoreactivity in pineal tissue are described in Section 5.2.

2.9 Experimental design and analysis.

Detailed experimental designs are included in the relevant chapters, together with the statistical methods used to analyze the statistical significance of experimental results.

2.9.1 Missing data

Values for missing hormone data were calculated as the mean of the values derived from the preceding and succeeding plasma samples collected within an experiment.

2.9.2 Total melatonin secretion estimates

In Experiments 1, 2, 3 & 4, total melatonin secretory responses were calculated as areas under individual response curves. Areas were calculated by integrating hormone concentrations in samples with the time intervals between samplings and were expressed as pg/ml.hr.

2.9.3 Transformations.

Prior to statistical analyses all data in Experiments 1, 2, & 3, for areas under curves, were transformed to logarithms using the relationship: log area under curve = 10 $\log_{10}(x + 1.1)$, where x was the area under the curve in pg/ml.hr. This transformation was used to

stabilize the variance prior to statistical analyses to ensure that the standard deviation did not vary directly with the mean (Snedecor & Cochran, 1967).

2.9.4 Statistical analyses.

(I) Plasma melatonin profiles

The significance of experimental main effects, and their interactions, were examined by analyses of variance (Experiments 1 & 2) or by analyses of covariance (Experiments 3 & 4) in which orthogonal coefficients were used to partition sources of variation into single degree of freedom contrasts (Cochran & Cox, 1960). Standard orthogonal coefficients (Fisher & Yates, 1963) were used, except where orthogonal matrices were constructed a priori to test specific hypotheses. Non-orthogonal contrasts were constructed to test hypotheses which were not possible within the constraints of orthogonality.

(II) Immunoreactivity

(i) NSE

An analysis of variance, as described above, was used to examine the significance of experimental main effects and their interactions.

(ii) VIP, NPY & PNMT

Refer to Section 5.2.3

(III) Levels of significance

Levels of significance in all statistical analyses are denoted thus:

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

Chapter 3.

Induction of pineal melatonin secretion in anaesthetized rams by darkness or bilateral electrical stimulation of the cervical sympathetic trunks.

3.1 Introduction

As discussed in Section 1.6.3 (III) of Chapter 1, there has been only one report in the literature to date in which the authors have recorded in vivo changes in plasma melatonin concentrations during electrical stimulation of the CST's in a mammalian species (Chan et al, 1989). Those workers demonstrated that in rabbits, unilateral stimulation with intermittent pulses produced significant increases in pineal melatonin secretion into the cerebral venous blood. Results from earlier studies, mainly in rodents, which have investigated other parameters of pineal secretory potential (eg., NAT activity, Bowers & Zigmond, 1980, 1982) during bilateral CST stimulation, have indicated that increased melatonin secretion from the pineal may be expected to occur during such stimulation.

Although a number of studies have investigated the effects of peripheral nerve stimulation on the secretory function of various endocrine organs, including NPY release from calf adrenals (Allen et al, 1984), bombesin release in response to splanchnic nerve stimulation (Bloom & Edwards, 1984), and insulin and glucagon release following stimulation of the sympathetic innervation to the pancreas (Ahren et al, 1987b), little is known about the mechanisms mediating these effects. Given the paucity of such information the present study was designed to provide further insights into the neural mediation of endocrine gland function.

Studies described in this chapter were designed to investigate:-

a) whether it was possible to induce melatonin secretion through continuous, bilateral electrical stimulation of CST's of anaesthetized sheep, and

b) whether such stimulation caused a differential response depending on whether it was conducted during the light or dark phase of the day.

A preliminary study was conducted to determine which of a range of anaesthetics was least inhibitory to the normal nocturnal rise in plasma melatonin concentrations; such an anaesthetic presumably would allow maximal secretory responses from the pineal during electrical stimulation of its sympathetic innervation. Typically, anaesthetics have been shown to influence hormone secretion through inhibitory effects on neurons innervating neuroendocrine organs (Clarke & Doughton, 1983). These effects usually lead to a decrease

in plasma hormone levels, although in some instances where the activity of inhibitory neurons is reduced, increases in hormone secretion have been recorded (Goodman & Meyer, 1984).

3.2 Materials and methods.

3.2.1 Experimental procedures.

(1) Melatonin secretion in anaesthetized rams (Experiment 1).

The initial study, to determine the relative effects of various anaesthetics on the nocturnal rise in plasma melatonin levels, was conducted at two to six day intervals during April/May 1986 (late autumn/early winter). Six adult Romney rams, housed in a light controlled room (approximately 200 lux at eye level of rams) under a 16L:8D photoperiod (lights on at 0630 and off at 2230 hrs) were allocated, in turn, to each of the following five treatments, applied in randomised sequence:

1. Sodium Pentobarbitone induction and maintenance (30 mg/kg, 'Nembutal', South Island Chemicals Ltd, Christchurch, N.Z)
2. Halothane induction (8% in oxygen) and maintenance (2-3% in oxygen, 2 l/min)
3. Saffan induction (dose 3 mg/kg), Halothane maintenance (2-3% in oxygen, as above).
4. Chloral Hydrate induction and maintenance (230 mg/kg, National Dairy Association, Auckland, N.Z).
5. Control (no anaesthetic).

Rams were treated three at a time in a predetermined sequence which ensured that each ram had at least two days between successive treatments. Anaesthesia was induced, if required by the experimental design, immediately prior to lights off at 2230 hrs. Rams were then maintained anaesthetized in the dark for one hour during the initial phase of the expected nocturnal elevation of plasma melatonin levels. Animals were then allowed to recover.

Blood samples were collected by jugular venepuncture into 10 ml vacutainer tubes containing 150 units of heparin. To establish basal melatonin levels blood samples were collected at 15 min intervals for one hour before lights off. Anaesthesia (where required) was then induced, the lights were turned off, and then blood sampling was undertaken for a further hour, at 15 min intervals, with the aid of a dim red penlight torch, to establish nocturnal

levels of plasma melatonin. Anaesthetic depth was assessed at each sampling time during the second hour of the experiment with any additional drug requirement being administered at that time.

(2) Bilateral cervical sympathetic nerve stimulation (Experiment 2).

Experiment 2 utilized 16 adult Romney rams during late autumn/early winter of 1987. These animals were brought in from pasture, housed, fed and pre-operatively starved as detailed in Sections 2.1 and 2.2.1. Artificial lighting provided a 8L:16D photoperiod with lights on at 0830 and off at 1630. Rams were randomly allocated to the following treatments:

a) daytime bilateral cervical sympathetic nerve stimulation (n=6)

b) nighttime bilateral cervical sympathetic nerve stimulation (n=6)

c) daytime controls - surgically prepared for bilateral cervical sympathetic nerve stimulation, but not stimulated (n=2)

d) nighttime controls - surgically prepared for bilateral cervical sympathetic nerve stimulation, but not stimulated (n=2)

Anaesthesia was induced with 8% (v/v) halothane, and maintained with 2-3% (v/v) halothane, in oxygen (see Section 2.2.1 for details on the method and rate of administration). Rams were then surgically prepared for nerve stimulation as described in Section 2.2.2. In addition, 1.5 mm I.D polythene tubing cannulae were inserted into a femoral vein for blood sample collection. Daytime stimulation commenced between 1300 and 1330 hrs when natural light intensity in the laboratory was approximately 700 lux, while nighttime stimulation (in a well lit room with light intensity of approximately 200 lux) began between 2100 and 2130 hrs, 4.5-5 hours into the light and dark phases of the day, respectively, and continued for 3 hours.

Pre-stimulus blood sampling consisted of one sample taken immediately after the induction of anaesthesia and another immediately before the commencement of nerve stimulation, when both cervical sympathetic nerves were on the stimulating electrodes in the liquid paraffin pool. Blood samples were collected every five min for the first 30 min of stimulation, followed by 10 min sampling intervals for the remaining 2.5 hrs of stimulation. Post-stimulus samples were collected at 5 min intervals for the first 30 min and at 10 min intervals for the next 30 min, to monitor the expected decline in plasma melatonin levels. An identical blood sampling regime was applied to control, non-stimulated animals, except that they were not bled in the hour corresponding to the post-stimulus sampling period of the

stimulated rams (ie., samples were collected after anaesthesia, after completion of surgery and during the subsequent 3 hr "sham" stimulation period).

3.2.2 Stimulus Parameters.

Prior to the commencement of Experiment 2, a series of trial experiments, using the same techniques as outlined above in Section 3.2.1 (2) and in Sections 2.1 & 2.2, were conducted to determine the current strength which could elicit maximal compound action potentials in the CST's at a constant pulse duration and frequency. To achieve this the compound action potentials generated in the CST's during stimulation, with a range of current strengths, were recorded and displayed for measurement on an oscilloscope, as described in Section 2.4.

While observing the size and shape of the displayed compound action potential the current strength was progressively increased until a maximal compound action potential was achieved. This was taken to indicate that all nerve fibres of each type (predominantly B, but some C) were stimulated above their threshold levels, which are determined by fibre size (Jack, 1976). Then, to ensure that all nerve fibres in the CST's were stimulated, the current strength which maximized the compound action potential (2-2.3 mA), was doubled, a practice advocated by previous workers in this field (Bowers & Zigmond, 1982).

Pulse frequency and width were determined following a review of the relevant literature. According to Delgado (1964), maximal autonomic responses are obtained by stimulation of adrenergic nerves at 20-30 Hz, although lesser frequencies may still initiate significant effector responses. In pineal studies, stimulus frequencies in the 5-25 Hz range have frequently been shown to alter both neuronal (Jaim-Etcheverry & Zieher, 1983) and biochemical (Bowers & Zigmond, 1982; Bowers et al, 1984) parameters of pineal function in anaesthetized animals during continuous CST stimulation. In particular, studies by the latter group of authors have demonstrated that frequencies in the 5-10 Hz range are optimal for continuous CST stimulation in anaesthetized animals.

Studies by Bowers & Zigmond (1982), in anaesthetized rats, have shown that pulse widths in the range 0.5-1.0 ms were equally effective in stimulating maximum pineal NAT levels, irrespective of pulse frequency. Several subsequent studies found pulse durations in this range to be effective in modifying pineal physiology (eg., Jaim-Etcheverry & Zicher, 1983). Finally, as it has been shown that pulse widths greater than 1 ms provided no increase in

pineal response (Bowers & Zigmond, 1982) and may even result in tissue injury (Delgado, 1964), this value was taken as the upper limit of pulse width for Experiment 2.

For these reasons the following stimulus parameters were chosen for all stimulations in Experiment 2: 5 mA, 10 Hz and 1 ms, generated and delivered with the Neurolog system described in Section 2.3 and illustrated in Figure 2.2.

3.2.3 Statistical analysis

(1) Effects of anaesthetics on nocturnal melatonin secretion.

In order to evaluate the effect of anaesthesia, induced immediately prior to the onset of darkness, on the nocturnal rise in plasma melatonin content, Experiment 1 was divided into two time periods : pre-anaesthetic/lights on and anaesthetized/lights off. For each time period, areas under individual melatonin response curves were calculated and converted to logarithms using the formula described in Section 2.9.3; these data were then subjected to separate analyses of variance in which both orthogonal and non-orthogonal coefficients (Table 3.1) were used to partition the treatment effects (Cochran & Cox, 1957).

Table 3.1 Coefficients used in partitioning treatment effects in Experiment 1.

Contrast	Anaesthetic Treatment				
	Control	Pentobarbitone	Chloral hydrate	Halothane/ Halothane	Saffan/ Halothane
(i) Orthogonal					
Control vs Anaesthetic	4	-1	-1	-1	-1
Pentobarbitone vs Chloral hydrate	0	1	-1	0	0
Halothane/Halothane vs Saffan/Halothane	0	0	0	1	-1
Pentobarbitone + Chloral hydrate vs Halothane/Halothane + Saffan/Halothane	0	1	1	-1	-1
(ii) Non-orthogonal					
Control vs Pentobarbitone	1	-1	0	0	0
" vs Chloral hydrate	1	0	-1	0	0
" vs Halothane/Halothane	1	0	0	-1	0
" vs Saffan/Halothane	1	0	0	0	-1

(2) **Effects of acute CST stimulation on melatonin secretion.**

For analysis, data from each ram in Experiment 2 were partitioned into four groups, as follows:

1. Pre-surgery. The single sample taken after the induction of anaesthesia and during the initial stages of surgery.
2. Pre-stimulation. The single sample taken after surgery was completed, but before stimulation commenced.
3. Stimulation Period. The 3 hrs of sampling following surgery during which animals were stimulated or, in the case of the controls, not stimulated.
4. Post-stimulation. The 1 hour of sampling after completion of the stimulation period for those animals which were so treated (Non-stimulated animals were not sampled during that period).

Data in groups 1 and 2 served as measures of the effects of anaesthesia and surgery, respectively, on pineal melatonin secretion. Data in groups 3 and 4 were used to evaluate the effect of CST stimulation on plasma melatonin levels, and to monitor the expected decline following the termination of stimulation, respectively. Data analyzed in groups 1 and 2 were the logarithms of the plasma melatonin concentrations in the single samples, while that for data groups 3 and 4 were the logarithm transformed values of the areas under the melatonin response curves of individual animals. Data from each time period were then subject to analysis of variance (Cochran & Cox, 1957).

Analysis of pulsatile melatonin secretion was performed using the arbitrary method of Lincoln (1988). A melatonin pulse was thus defined as a single melatonin value which exceeded the previous value by a concentration greater than 2 times the intra-assay coefficient of variation or a series of melatonin values, each of which successively exceeded previous values by concentrations greater than than 2 times the intra-assay coefficient of variation. Melatonin values were reported as pulses only if they were above the limit of assay sensitivity. Student's t-tests were used to examine for significant differences in melatonin pulse frequencies during and after daytime and night-time CST stimulation.

3.3 Results.

3.3.1 Experiment 1 (The effects of anaesthetic agents on nocturnal melatonin secretion)

See Table 3.2 and Figures 3.1 and 3.2.

Mean (± S.E.M) pre-anaesthetic plasma melatonin levels exhibited little variability between control (0.9 ± 0.4 pg/ml) and anaesthetized groups (Pentobarbitone, 2.2 ± 0.9; H/H, 2.7 ± 0.6; S/H, 5.9 ± 4.2; Chloral hydrate, 0.2 ± 0.1 pg/ml). Individual sample values mostly were below the limit of assay sensitivity (6.2 ± 0.6 pg/ml), containing estimated melatonin concentrations ranging from 0.0 to 20.7 pg/ml. An exception was one animal prior to saffan/halothane anaesthesia, from which an abnormally high value was recorded for the 45 min sample (128.7 pg/ml). Secretion of melatonin from rams prior to the induction of anaesthesia was not significantly different from that of control rams during the same period.

During the one hour following induction of anaesthesia (or not for the control group) and lights off, plasma melatonin levels in the pentobarbitone treated group rose only slightly resulting in a mean melatonin secretory response which was significantly lower than that of the control group. In contrast, the halothane/halothane, saffan/halothane and chloral hydrate treated groups responded to lights off with higher mean plasma melatonin concentrations, so that areas under melatonin response curves for these groups were not significantly different from those of the control group (see Table 3.2 and Figures 3.1 & 3.2). Initial inspection of Figure 3.2 suggested that the Chloral hydrate group responded to lights out with a more pronounced rise in melatonin secretion than did the H/H group, but this was due to the response of one animal and not the group as a whole.

Table 3.2 Summary of analyses of variance of data from Experiment 1. Areas under individual melatonin response curves (both pre- and post-anaesthetic) were log transformed prior to analysis.

Source of Variation		D.F	Variance ratios	
			Pre-anaesthetic	Post-anaesthetic
A.	Treatment	4		
	Orthogonal coefficients			
	Control vs Anaesthetic	1	0.45	6.68*
	Remainder	3	1.91	0.65
	Non-orthogonal contrasts			
	Control vs Pentobarbitone	1		8.27**
	" vs Halothane/Halothane	1		2.51
	" vs Saffan/Halothane	1		3.60
	" vs Chloral hydrate	1		3.28
B.	Rams	5	0.74	0.33
	Error mean square	20	13.74	22.97

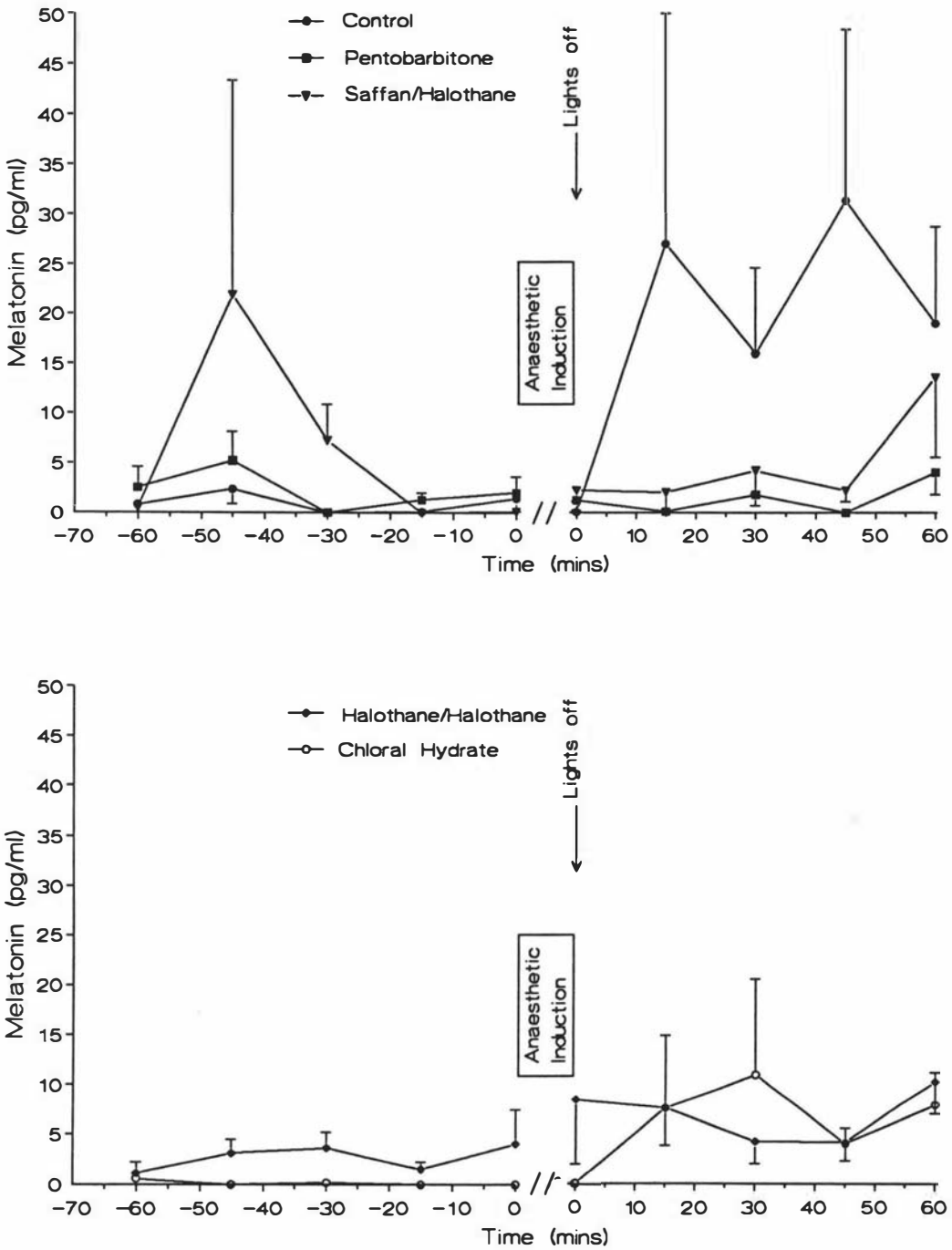


Figure 3.1 Effects of various anaesthetics on the nocturnal rise in plasma melatonin concentrations (mean +/− S.E.M) in adult Romney rams (n=6) following a 60 min pre-induction, lights on period.

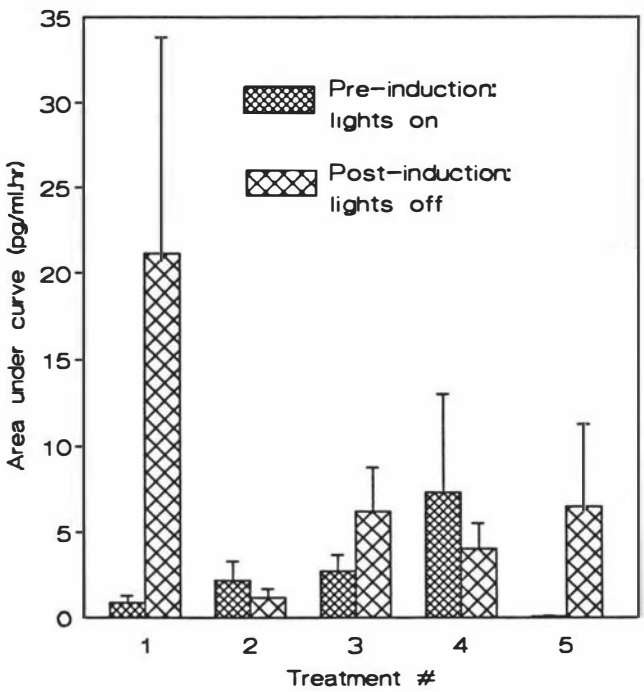


Figure 3.2 Effects of various anaesthetics on the average amount of melatonin secreted (indicated by each bar + S.E.M) in adult Romney rams (n=6) during a 60 min period beginning at lights off. Pre-induction levels were assessed during a similar time period immediately prior to lights off. Treatment numbers are as follows:

1. Control
2. Pentobarbitone induction and maintenance
3. Halothane induction and maintenance
4. Saffan induction/Halothane maintenance
5. Choral hydrate induction and maintenance

3.3.2 **Experiment 2.** (The effect of CST stimulation on melatonin secretion)

See Tables 3.3 & 3.4 and Figures 3.3-3.7.

Results of the analysis of variance of lights off data from Experiment 1 indicated that the halothane/halothane anaesthetic regimen was marginally less inhibitory to nocturnal melatonin secretion than the saffan/halothane and chloral hydrate treatments; consequently rams in Experiment 2 were anaesthetized and maintained with halothane.

(i) **Pre-stimulation.**

Figures 3.3 and 3.4 and Table 3.3 illustrate that mean pre-stimulation plasma melatonin levels (both pre-surgery and pre-stimulation) were low for all treatment groups, often being below the 7.6 ± 0.9 pg/ml limit of assay sensitivity. Mean (± S.E.M) concentrations ranged from 1.8 ± 1.8 pg/ml for daytime controls pre-surgery to 14.7 ± 3.5 pg/ml for nighttime controls at time 0. Between-group differences were non-significant at these times (Table 3.4).

Table 3.3 **Mean melatonin concentrations ± S.E.M (pg/ml) prior to stimulation and mean hourly areas under melatonin response curves ± S.E.M (pg/ml.hr) during and after acute or sham bilateral electrical stimulation of the CST's of anaesthetized rams in Experiment 2.**

1. Pre-stimulation (pg/ml)

	Pre-surgery	Pre-stimulation (time 0)
Stimulated - day	6.2 ± 4.7	7.6 ± 4.5
" - night	7.7 ± 2.5	13.6 ± 5.0
Non-stimulated - day	1.8 ± 1.8	3.0 ± 3.0
" - night	6.7 ± 6.7	14.7 ± 3.5

2. Post-surgery (pg/ml.hr)

	During stimulation	Post-stimulation
Stimulated - day	26.6 ± 7.0	15.8 ± 4.2
" - night	45.7 ± 6.3	44.6 ± 13.7
Non-stimulated - day	4.1 ± 2.2	
" - night	11.6 ± 2.2	

(ii) **During stimulation.**

Plasma melatonin content recorded in daytime groups (stimulated and control) during the 3 hr of stimulation was significantly less than values recorded during the same period in the corresponding nighttime groups ($P<0.05$). With the onset of stimulation, mean plasma melatonin concentrations began to increase rapidly in both daytime and nighttime

Table 3.4 **Summary of analyses of variance of data from Experiment 2. Pre-surgery and pre-stimulation levels were represented by single plasma samples (pg/ml), while responses during and after stimulation were represented by the areas under individual melatonin response curves (pg/ml.hr).**

Source of variation	D.F	Variance Ratios			
		Pre-surgery	Pre-stimulation	Stimulation	Post-stimulation
Treatment	3				
Day vs Night	1	1.20	2.64	6.13*	7.92*
Stimulated vs Non-stimulated	1	0.33	0.003	16.54**	
Interaction	1	0.01	0.42	0.27	
Error mean square	12	28.76	28.99	8.04	
	9				6.88

stimulated animals. During daytime stimulation plasma melatonin levels increased 4.7 fold during the first 10 mins of stimulation (ie. from 7.6 ± 4.5 pg/ml immediately prior to stimulation to 36.1 ± 9.2 pg/ml after 10 mins). Following this initial 10 min period, plasma melatonin levels plateaued and remained relatively constant throughout the remaining period of stimulation (Figure 3.3). Nighttime secretion of melatonin in response to CST stimulation was different, both quantitatively and qualitatively, from that of daytime secretion. Plasma levels began to rise immediately following the onset of stimulation, reaching mean concentrations after 10 mins similar to those achieved after the same period of daytime stimulation (29.4 ± 5.3 pg/ml recorded at night and 36.1 ± 9.21 pg/ml during the day). Thereafter mean plasma melatonin concentrations continued to increase, peaking at 64.2 ± 10.6 pg/ml after 80 mins of stimulation. Throughout the remaining 100 mins of stimulation, mean melatonin concentrations remained elevated at similar levels (Figure 3.4).

During the time corresponding to the stimulation period, melatonin levels recorded from both control groups were low and were highly significantly less ($P<0.01$) than those recorded from the corresponding stimulated groups. Mean stimulated daytime melatonin output was 26.6 ± 7.0 pg/ml.hr compared to 4.1 ± 2.2 pg/ml.hr in controls, while at nighttime comparable values were 45.7 ± 6.3 pg/ml.hr and 11.6 ± 2.2 pg/ml.hr, respectively. Individual melatonin secretory profiles of control animals (Figure 3.5) demonstrated that melatonin was secreted in pulses, but at frequencies lower than those of the corresponding stimulated groups (see below). Thus, pulse frequencies of 1 & 4 pulses/3 hr were recorded in the two daytime control rams while a higher frequency of 5 & 6 pulses/3 hr was recorded in the night-time control rams.

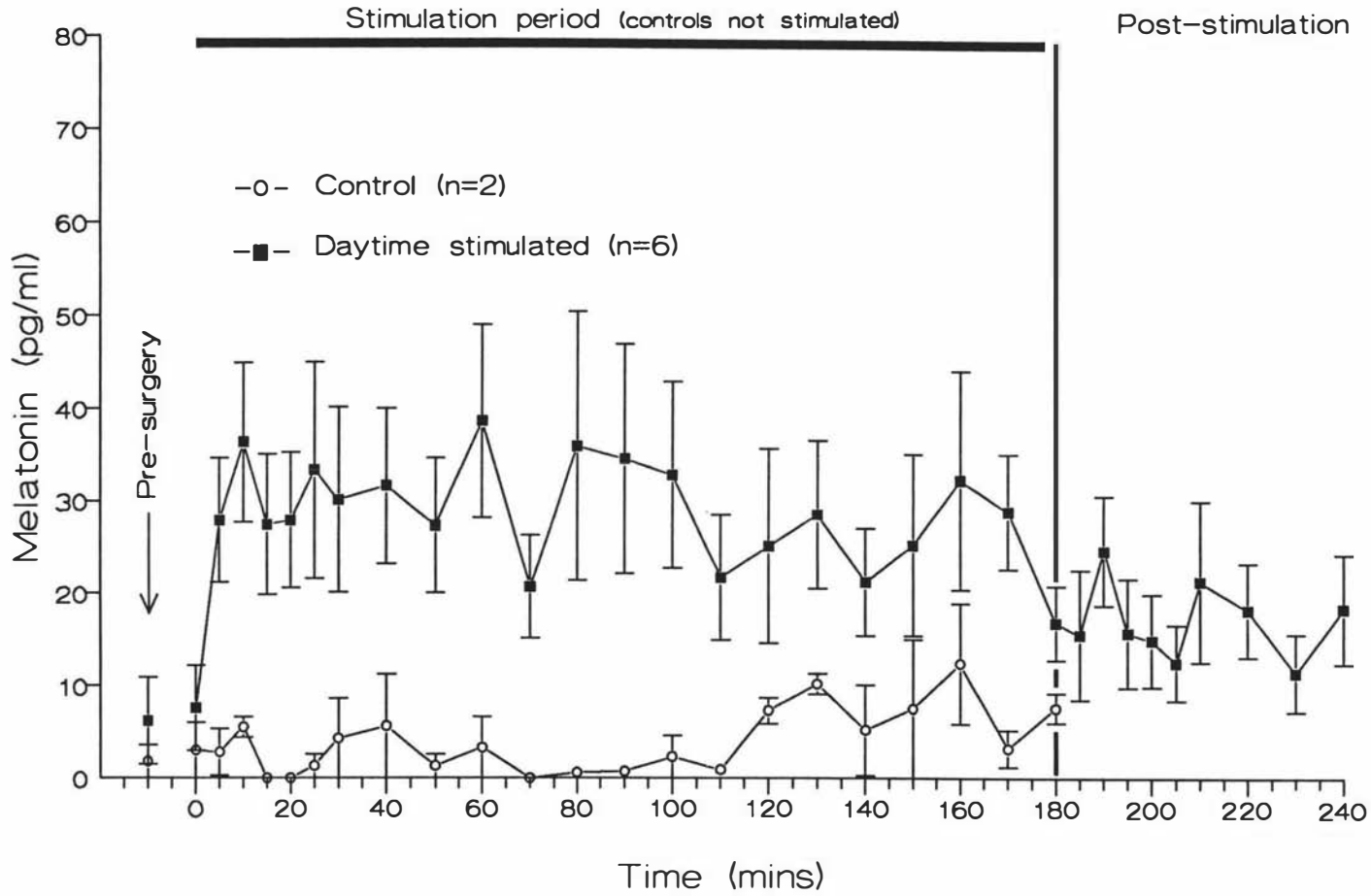


Figure 3.3 Daytime plasma melatonin levels (mean +/- S.E.M) before (pre-surgery & pre-stimulation (time 0) values), during and after acute bilaterial electrical stimulation of the CST's of anaesthetized rams in Experiment 2. Control rams were surgically prepared and electrodes placed under nerves, but not stimulated. All experimental procedures were conducted in a room illuminated by sunlight, with light intensity of approximately 700 lux.

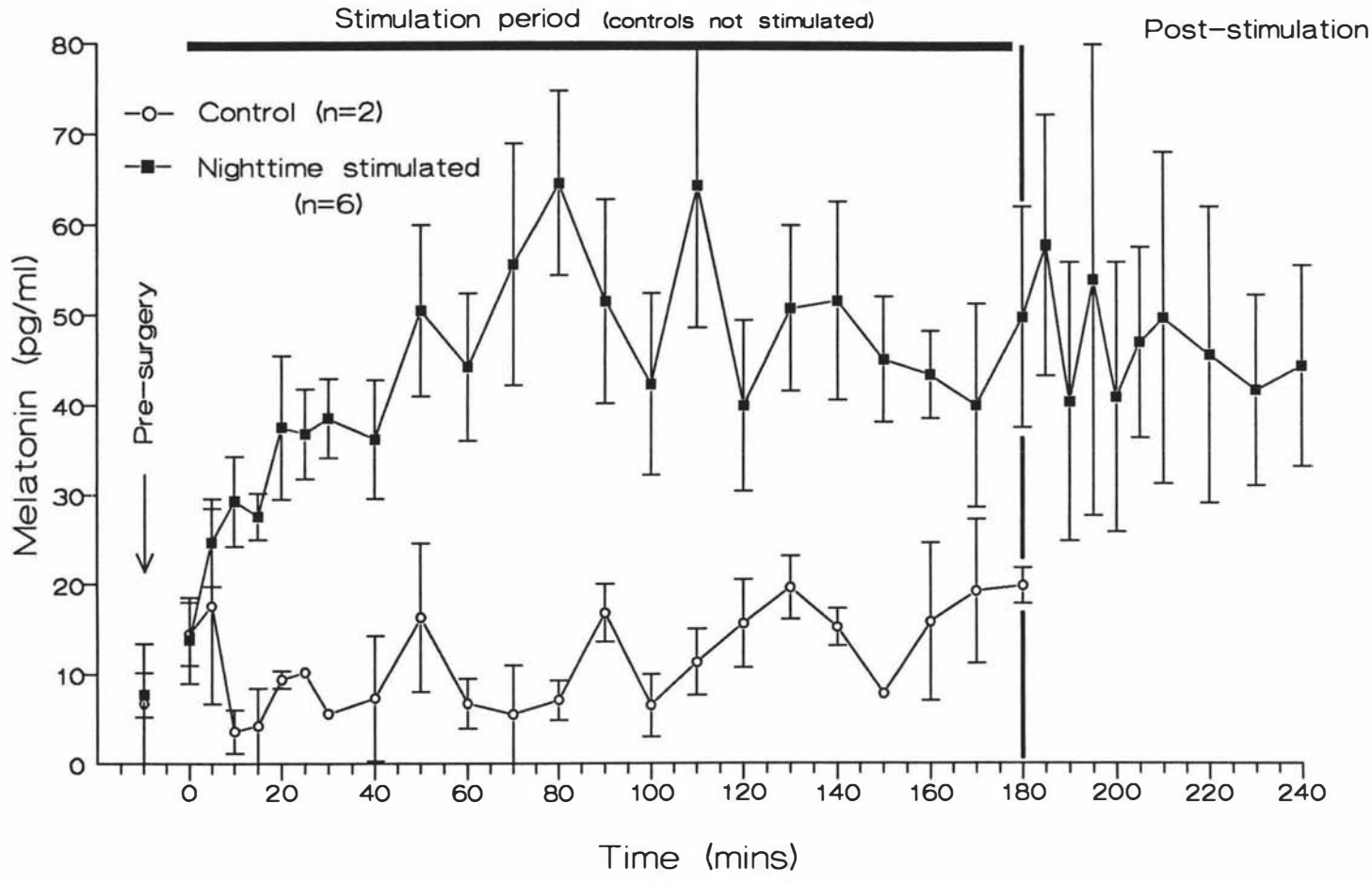


Figure 3.4 Nighttime plasma melatonin levels (mean +/- S.E.M) before (pre-surgery & pre-stimulation (time 0) values), during and after acute bilateral electrical stimulation of the CST's of anaesthetized rams in Experiment 2. Control animals were surgically prepared and electrodes placed under nerves, but not stimulated. All surgical, stimulation and sham-stimulation procedures were conducted in a well lit room with light intensity of approximately 200 lux.

Examination of plasma melatonin profiles of individual animals during stimulation revealed a highly variable pattern of responses (Figures 3.6 & 3.7). At one extreme daytime stimulated animal number 6 showed little evidence of any response, with values for only a few samples exceeding the limit of assay sensitivity. On the other hand nighttime stimulated ram number 4 showed a rapid increase to 77.4 pg/ml after 20 min stimulation, followed by substantial fluctuations in concentrations thereafter. In contrast, nighttime stimulated ram number 1 exhibited relatively constant melatonin levels during the period of stimulation, being in the range 31.6-51.3 pg/ml from 50-180 min of stimulation. One notable plasma melatonin profile was exhibited by nighttime stimulated ram number 5 which showed rapidly increasing plasma melatonin levels during the final 30 min of stimulation.

Analysis of pulsatile melatonin secretion during the period of stimulation revealed that all rams secreted melatonin in a pulsatile manner, but that there were irregular intervals between pulses (Figures 3.6 & 3.7). Also the magnitude of pulses varied considerably between animals: both day and night stimulated rams numbered 3 showed highly pulsatile fluctuations in melatonin levels, while pulses recorded from day stimulated ram number 6 and night stimulated ram number 1 showed much more subdued pulses. Despite these comments, there were no significant differences in the frequency of melatonin pulses during the stimulation periods ($t=1.664$ with 10 d.f, $P>0.1$), mean values being 5.5 pulses/3 hr (1.83 pulses/hr) during day stimulation (range 3-7 pulses) and 6.67 pulses/3 hr (2.22 pulses/hr) at night (range 6-8 pulses).

(iii) **Post-stimulation melatonin secretion.**

Mean plasma melatonin concentrations for both treatment groups exhibited little change during the 60 min of post-stimulation blood sampling (Figures 3.6 & 3.7; note that no post-stimulation data are shown for night-time stimulated ram# 6 which died at the beginning of this period). As with the period of stimulation, the amount of post-stimulation melatonin appearing in the plasma was significantly different according to time-of-day (Table 3.4) ($P<0.05$), being 15.8 ± 4.2 pg/ml.hr for daytime stimulated rams and 44.6 ± 13.7 pg/ml for nighttime stimulated rams (Table 3.3). Examination of individual ram melatonin secretory profiles showed that, as during the period of stimulation, melatonin was secreted into the plasma in an irregular, pulsatile manner. Melatonin pulse frequencies in the two treatment groups were not significantly different during the post-stimulation period ($t=0.717$ with 9 d.f, $P>0.3$) and ranged between 1-3 pulses/hr in daytime stimulated rams (mean 2.5 pulses/hr) and 2-3 pulses/hr in rams stimulated at night (mean 2.2 pulses/hr).

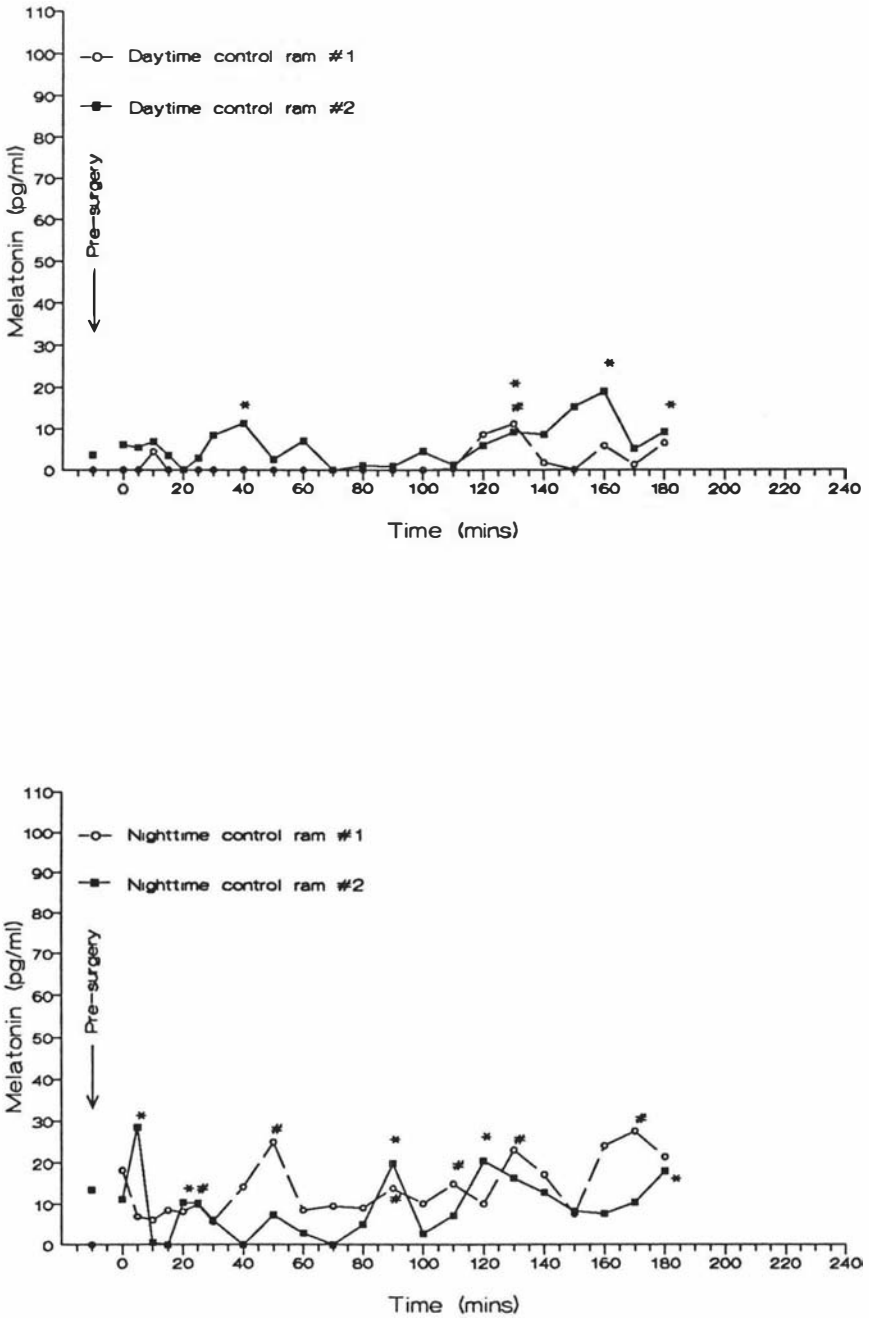


Figure 3.5 Plasma melatonin levels measured in individual control rams sampled during the period they would otherwise have been stimulated. Note that plasma levels in night-time control rams are slightly higher than in the corresponding daytime rams. (* or # = melatonin pulse)

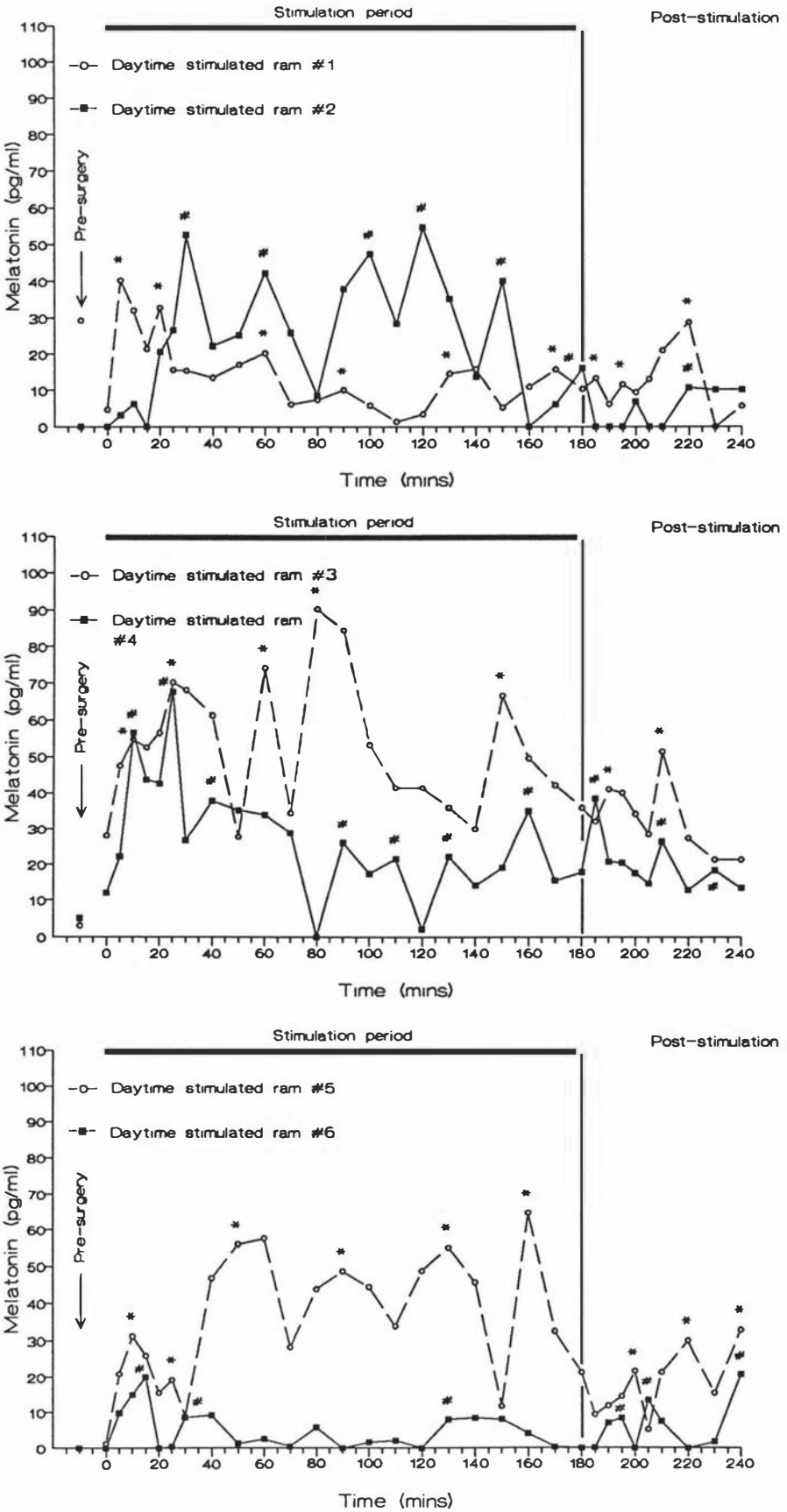


Figure 3.6 Plasma melatonin levels measured in individual rams stimulated during the day. Note that, with 2 exceptions (rams 1 & 6), melatonin levels remained elevated throughout the period of stimulation and that variations in these levels occurred at irregular intervals. (* or # = melatonin pulse)

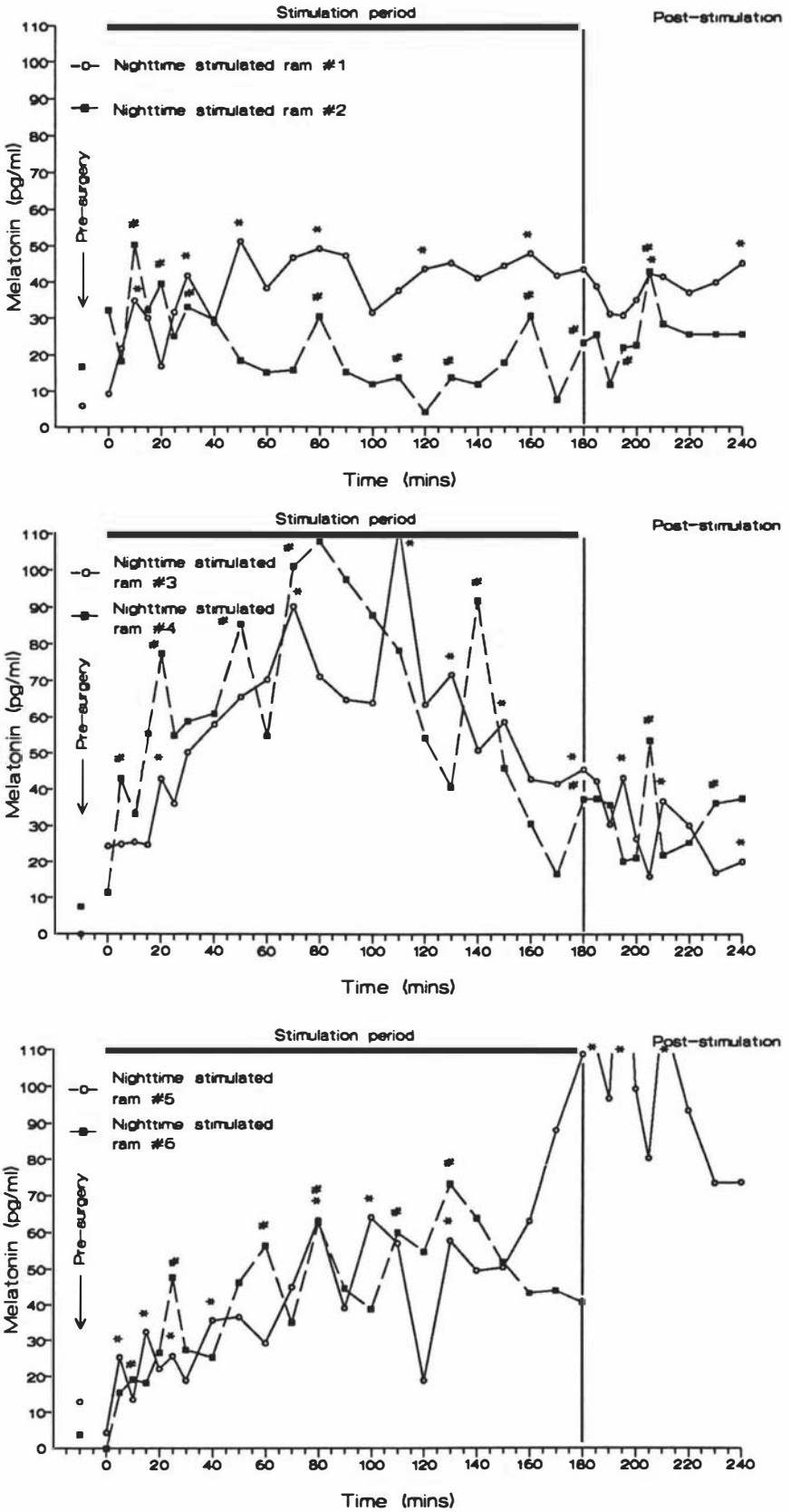


Figure 3.7 Plasma melatonin levels measured in individual rams stimulated during the night. Melatonin levels generally remained elevated throughout the period of stimulation although variations in these levels did occurr at irregular intervals. Note that ram 6 died at the beginning of the post-stimulation period and that post-stimulation values of ram 5 which exceeded the Y axis scaling at 185, 195, and 210 mins are 111.47, 153.40 and 120.33 pg/ml, respectively. (* or # = melatonin pulse)

3.4 Discussion

3.4.1 Anaesthetic action on nocturnal melatonin secretion.

The present work has shown that the anaesthetics investigated in Experiment 1 depressed the normal nighttime rise of plasma melatonin levels in rams. However, only pentobarbitone induction and maintenance resulted in a significant reduction in plasma melatonin levels during the lights-off period (as compared to values recorded from conscious rams). Since pineal melatonin secretion is initiated by excitation of its sympathetic innervation, those anaesthetics which selectively inhibit neural transmission in sympathetic nerves, or in central neurons which initiate the flow of action potentials in this division of the peripheral nervous system, are likely to also inhibit melatonin synthesis and secretion. All anaesthetics in this study fall into one or both of these two categories, as discussed below.

Anaesthetics have also been shown to influence the secretory activity of other endocrine systems. For example, pentobarbitone and thiopentone both alter the secretion of gonadotrophins and hypothalamic releasing hormones in ewes. This influence has been shown to be season-dependent, with increases in GnRH and gonadotrophin pulse frequencies being recorded during anaesthesia in anoestrus, when the effects of inhibitory neurons dominate the neural control of GnRH secretion, but decreases being recorded during anaesthesia in the breeding season, when stimulatory influences drive the secretion of these hormones (Clarke & Doughton, 1983; Goodman & Meyer, 1984). In rats, pentobarbitone has also been shown to rapidly reduce plasma LH concentrations, although this effect was temporary with LH pulses reappearing 50 min after anaesthetic induction and 90 min before the end of the treatment period (Blake, 1974). Conversely, some anaesthetics have no effect on the secretion of these hormones. Saffan anaesthesia, for example, had no effect on gonadotrophin secretion in ewes (Clarke & Doughton, 1983) or GnRH secretion in rats (Sherwood et al, 1980).

In the present study, the inhibitory effect of pentobarbitone is likely to have resulted from selective inhibition of synaptic transmission through the SCG's and blockade of conduction along nerve fibres (Larrabee & Posternak, 1952), while at the same time inhibiting synaptic transmission in the CNS (Barker & Ransom, 1978; MacDonald & Barker, 1978). Barbiturate anaesthetics, including pentobarbitone, have also been shown to modify the photic evoked responses of rat pineals (Dafny, 1980) and reduce nocturnal NAT activity (Zatz & Brownstein, 1979), although it was not determined whether this was a central or local effect or

an action at both sites. Pentobarbitone most likely achieves these effects by enhancing and mimicing the action of GABA, the principal inhibitory neurotransmitter in the mammalian CNS (MacDonald & Barker, 1978; Fragen & Avram, 1990). Bearing in mind the combined effects of pentobarbitone's actions on central and peripheral neurons and previous reports of its inhibition of hormone secretion and pineal NAT activity, the fact that it reduced the neurally mediated increase in nocturnal melatonin secretion to levels significantly less than those of controls, was not surprising.

Although nocturnal plasma melatonin levels in halothane anaesthetized rams were not significantly different from those in control rams, this anaesthetic does exert an inhibitory effect on sympathetic and central neuronal transmission (Wyke, 1965; Short, 1987; Koblin, 1990). However, the fact that this effect was not strong enough to significantly reduce melatonin secretion indicates that halothane did not disrupt neuronal function to the same extent as did pentobarbitone. Addition of saffan as an induction agent imposed further, albeit minor, inhibitory effects on melatonin secretion, so that this was slightly less than that recorded following halothane induction, but was not significantly less than that of control rams. Although relatively little is known about saffan's mechanism(s) of action, its effect on melatonin secretion may have arisen from non-selective depression of the central nervous system (Child et al, 1971).

Although chloral hydrate anaesthesia did not result in a significant reduction in nighttime plasma melatonin levels, there was a marked decrease in such levels in 4 of the 6 rams. The effect of chloral hydrate on melatonin secretion appears to have been achieved through inhibition of central and peripheral neuronal function. Like pentobarbitone, this anaesthetic has been demonstrated to inhibit synaptic transmission through the SCG (Brown, 1962), but in addition also exhibits a number of central effects, including depression of the cerebral cortex (Hall, 1971), enhancement of pre-synaptic inhibition (Miyahara et al, 1966), and reduction or complete block of responsiveness to environmental (auditory and visual) stimuli by serotoninergic neurons of the dorsal raphe nuclei (Trulson & Trulson, 1983; Heym et al, 1984). In contrast to the effects of pentobarbitone, chloral hydrate has been shown to have no significant effects on nocturnal NAT activity in rat pineals (Zatz & Brownstein, 1979).

Halothane induction and maintenance was marginally the least inhibitory anaesthetic tested and therefore was chosen as the anaesthetic regime for acute studies of the neural control of pineal melatonin secretion in sheep (Experiment 2).

3.4.2 Neural regulation of pineal secretory activity

Experiment 2 clearly demonstrated that continuous electrical stimulation of the pineal gland's pre-ganglionic sympathetic innervation in anaesthetized rams initiated and maintained elevated plasma levels of melatonin and that the pineal was differentially responsive to the effects of such stimulation, depending on whether it occurred at night or during the day.

Electrical stimulation of sympathetic nerve fibres innervating endocrine tissues, as applied in this study, does not appear to have been widely used in the study of other neurally regulated endocrine processes (see Section 4.4.1). Previous studies investigating the neural regulation of pineal function have measured parameters such as pineal enzyme activity (NAT (Volkman & Heller, 1971; Bowers & Zigmond, 1980, 1982; Reuss et al, 1989a), HIOMT (Brownstein & Heller, 1968; Reuss et al, 1989a)) and electrical activity of pinealocytes (Reyes-Vazquez & Dafny , 1985; Demaine & Patel, 1989), during sympathetic (CST, SCG or ICN) stimulation. However, at the time of conducting the present study, no reports had been published which directly investigated melatonin secretory responses during such stimulation in sheep, or in any other species.

Recording of plasma melatonin levels during CST stimulation has provided a direct measure of the pineal's secretory response to sympathetic stimulation. In rats, levels of NAT activity may not be an accurate predictor of the degree (magnitude) of pineal secretory activity, since the nocturnal rise in NAT activity is in the order of 50-fold (Rudeen et al, 1975; Bowers & Zigmond, 1982), whereas that of melatonin secretion is only 5-7 fold (Ho et al, 1984). Furthermore, it has been shown in rats that as little as 10% activation of NAT is sufficient to initiate full melatonin production (Wheler et al, 1979). In a later study by King et al, (1984) it was shown that maximal NAT activity may not necessarily initiate maximal melatonin production if pineal serotonin content has been reduced. Such results suggest that pineal NAT activity does not accurately reflect melatonin production and is only useful as an indicator of increased synthetic potential. Other indicators of the sympathetic nervous system's influence on pineal function, such as pineal cAMP concentrations (Heydorn, et al, 1981) and glucose metabolism (Ito et al, 1988), have also been shown to increase during CST or SCG

stimulation. Ultimately, however, the only absolute measure of pineal secretory activity is the level of melatonin appearing in the blood following a suitable stimulus, either normal nocturnally generated secretion, or during artificial nerve stimulation or pharmacological manipulation.

Recently, Chan et al (1989) reported a study in which unilateral electrical stimulation of rabbit CST's significantly increased output of melatonin from the pineal, as measured in samples taken from the confluens sinuum; that represents the only report in the literature to date which has demonstrated a direct relationship between sympathetic stimulation and pineal melatonin secretion. Those authors claimed their results indicated that the synthesis and secretion of pineal melatonin in rabbits was: (i) under sympathetic control and therefore (ii) was similar to that in rats. The first conclusion is indisputable, but their results showed one marked similarity with those presently recorded in sheep, that was that upon the commencement of stimulation plasma melatonin levels rose very rapidly for 20-25 mins without exhibiting the 1-2 hour lag phase characteristic of pineal NAT activity and melatonin content seen in a variety of rodent species, including rats, when exposed to photoperiodic or electrical stimulation of the pineal (eg, Goldman et al, 1981; Bowers & Zigmond, 1982). Thus the dynamics of the synthesis and secretion of pineal melatonin appear more similar in rabbits and sheep, than in rabbits and rats.

The initial rapid rise in plasma melatonin levels exhibited by both the stimulated groups in Experiment 2 was similar to that recorded previously from sheep during the initial 10-20 min of darkness (Sugden et al, 1985a) and appears to be due to a NAT-independent mechanism which results in elevated pineal melatonin synthesis in the absence of increased NAT activity (Namboodiri et al, 1985a, b). More recent studies, conducted in vitro with ovine pineal tissue, have shown that there is a 30-60 min delay in NAT synthesis following the addition of the sympathetic neurotransmitter, noradrenalin, to the perifusion medium, but a rapid rise (within 12-18 min) in the accumulation of melatonin (Morgan et al, 1988b). Thus, it is likely that in sheep, and possibly other species such as monkeys (Reppert et al, 1979) and rabbits (Chan et al, 1989), the initial rise in melatonin secretion is not dependent on a prior increase in NAT synthesis as it is in rats (Ho et al, 1984), but rather is mediated by NAT-independent mechanisms. NAT, however, is likely to be involved in maintaining elevated levels of melatonin production (Morgan et al, 1988b), such as recorded after the initial rapid rise following commencement of stimulation.

Melatonin levels recorded in control rams, during the period when they would otherwise have been stimulated, were significantly lower than those of stimulated rams ($P<0.01$), with many values around or below the limit of assay sensitivity. On a number of occasions, however, particularly for nighttime controls, melatonin secretion apparently increased to levels well above this limit, suggesting some form of pineal stimulation may have occurred. This nighttime secretion may have been due to mechanical activation of sympathetic nerve fibres in the CST's, at the time of day when pineal adrenoceptors of rats show maximal sensitivity to the effects of β-agonist stimulation (Romero & Axelrod, 1974). A similar degree of mechanical stimulation of sympathetic fibres probably occurred during daytime experiments, but at this time adrenoceptors are less sensitive to β-agonist stimulation; this may explain why little or no melatonin secretion occurred in daytime control rams. Regardless of whether there was mechanical stimulation or not, electrical stimulation of the CST's resulted in melatonin output which was far greater than in sham-stimulated rams.

Post-stimulation melatonin secretion.

Melatonin levels in most daytime and nighttime stimulated rams remained elevated during the 60 mins of post-stimulation sampling, at values similar to those recorded in the last sample taken immediately prior to the end of stimulation. Chan et al (1989) also found that the decline of plasma melatonin levels following electrical stimulation of the CST's was prolonged compared to that following the ending of exposure to darkness (Namboodiri et al, 1985a).

One possible explanation of this apparent continued secretion, that it was due to mechanical nerve stimulation by the stimulating electrodes, seems unlikely in light of the fact that plasma melatonin levels were only slightly elevated above pre-surgery levels at time 0, following 90 mins of surgery during which nerves were isolated and placed on the electrodes. A more likely cause involves halothane's effects in lowering blood pressure and inhibiting liver function (Maze, 1990), this organ being the principal site at which melatonin is removed from plasma (Kopin et al, 1961; Kveder & McIsaac, 1961). Such effects would have resulted in a reduced melatonin clearance rate and in a prolonged elevation of melatonin levels during the post-stimulation period. This hypothesis is further supported by the observation that chlorpromazine, a drug suggested to inhibit hepatic microsomal enzyme activity (Wurtman et al, 1968), also prolongs the clearance of melatonin from plasma (Wurtman & Axelrod, 1966b;

Ozaki et al, 1976). Further studies, investigating the clearance of plasma melatonin in conscious and anaesthetized rams, are needed to clarify this point.

Daytime vs nighttime secretory responses.

Elevation of melatonin secretion by CST stimulation at night differed markedly from that which occurred during daytime stimulation in two ways: (i) nighttime stimulated rams exhibited increasing mean plasma melatonin levels for the initial 80 minutes of stimulation compared to only the initial 10 minutes for the daytime stimulated group, and (ii) mean plasma melatonin levels attained during nighttime stimulation were greater than those attained during daytime stimulation.

Time-of-day differences in the concentrations of plasma melatonin recorded in the present study may have been due to a combination of factors which acted to limit daytime melatonin secretion. One such factor was suggested by Sugden et al (1985b) who observed that daytime intraperitoneal administration of L-5-hydroxytryptophan to sheep produced a marked rise in pineal serotonin and its metabolites, including melatonin. However, no change in NAT activity was observed. These observations led that group of authors to conclude that, in sheep, daytime serotonin availability may limit the production of melatonin. A second factor which may also have influenced the rate of melatonin production is the circadian rhythm of pineal adrenoceptor density (Gonzalez-Brito et al, 1988b) and sensitivity to pharmacological or electrical stimulation (Romero & Axelrod, 1974; Stehle et al, 1989). Both parameters of adrenoceptor function have been shown, in rodents, to exhibit down-regulation during the late dark phase and recovery to maximum levels during the late light and early dark phase. Although there appears to be no studies, reported in the literature, detailing the circadian pattern of pineal adrenoceptor density and sensitivity in short day breeders such as sheep, it is possible that a reduction in α- and/or β-adrenoceptor density and sensitivity during the first half of the light phase may have decreased the responsiveness of ram pineals to sympathetic stimulation and consequently reduced the rate of melatonin secretion. The circadian rhythms of adrenoceptor density and sensitivity in the ovine pineal will need to be studied in detail before they can be established as definitive causes of differential pineal responsiveness to sympathetic stimulation.

Episodic melatonin release

There can be little doubt from the results of the current study that melatonin is secreted from the ovine pineal in a pulsatile manner. Although all rams exhibited pulsatile release of melatonin, there was considerable between-animal variation in pulse frequency. This finding is in agreement with earlier studies investigating the pattern of melatonin secretion in a diverse range of species including rabbit (Chan et al, 1989, 1991), sheep (Arendt, 1985) and rats (Pang & Yip, 1988). For example, the melatonin content of pineal venous blood, sampled during both day and night from the cerebral sinuses and jugular veins of ewes was reported by Cozzi et al (1988) to peak every 15-20 min in some, but not all, animals tested. When pulsatile melatonin episodes were recorded they were more pronounced and more frequent in sinus plasma than in plasma collected from the peripheral circulation. These authors concluded that the sampling of blood that had just perfused the pineal was a key factor in establishing the pulsatile nature of melatonin secretion. In the current study, venous blood was sampled from the peripheral circulation which would have resulted in considerable dilution of melatonin and hence dampening or probably even obliteration of pulse peaks. Clearly, sampling of peripheral blood makes the detection of melatonin pulses more difficult, but against that there is a greater likelihood of inconsistent results when sampling central blood due to the difficulty of precise placement of cannulae in the cerebral sinuses (Cozzi et al, 1988).

The importance of the sampling frequency was demonstrated by Chan et al (1991) who found that high rates of venous sampling (every 2 or 4 min) of rabbits resulted in the detection of an apparently higher melatonin pulse frequency than that recorded in the present study. Other factors such as the alteration of melatonin metabolism and changes in pineal blood flow by anaesthetics also need to be considered when appraising the pulsatile nature of melatonin secretion.

Other authors have reported episodic melatonin secretion in sheep, but with time periods in the order of hours rather than minutes (reviewed by Arendt, 1985). Pulse rates comparable to those recorded by Cozzi et al (1988) have also been recorded in other species such as rabbits (Chan et al, 1989, 1991) and rats (Pang & Yip, 1988). In the present study the number of pulse peaks were 3-8 in each 3 hr stimulation period, which is a rate similar to that recorded by Cozzi et al in peripheral venous blood. Also, it is interesting to note that there were no significant differences in melatonin pulse frequencies in daytime or night-time

stimulated rams, suggesting that the mechanism of pulsatile secretion does not exhibit a circadian variation of sensitivity to pineal stimulation.

One problem encountered when attempting to compare rates of pulsatile release of hormones is that there is little consensus on methods for identifying each individual pulse of hormone secretion. Consequently, there have been significant differences in the approach adopted by various authors. For example, Cozzi et al (1988) assessed the pulsatile nature of melatonin secretion by visual appraisal of individual secretory profiles, Lincoln (1988) devised a method (used in this study) for the detection of LH pulses based on the intra-assay coefficient of variation, while Chan et al (1991) identified pulse peaks when they exceeded the average value of the preceding and succeeding troughs by 30%. These different approaches are likely to have contributed to the different pulse rates reported for melatonin secretion.

Neither the physiological significance nor the mechanism of pulsatile melatonin secretion is currently understood, and in fact it is generally agreed that it is the period of elevation of melatonin levels that is of greater physiological importance (Arendt, 1985). Also, as yet no definitive melatonin storage organelles have been identified in the pineal (Karasek, 1986). Furthermore, in vitro studies using sheep pineals have suggested that melatonin is released from the gland as it is synthesized (Morgan et al, 1988a, b). Clearly, a detailed, ultrastructural study is needed to establish if storage organelles are present in the ovine pineal and to correlate changes in their density with episodic changes in plasma melatonin levels.

3.5 Conclusions

The following conclusions have been drawn from these two experiments.

Experiment 1 (i) Of the 4 anaesthetic treatments administered, halothane induction and maintenance was marginally the least inhibitory to the normal nocturnal rise in plasma melatonin concentrations. For this reason all rams anaesthetized in Experiment 2 were induced and maintained with halothane.

Experiment 2 (i) Bilateral electrical stimulation of the CST's of anaesthetized rams resulted in an elevation of plasma melatonin content, presumably by increasing pineal melatonin synthesis and secretion.

(ii) The rapid and immediate increase in plasma melatonin concentrations observed in this study contrasts with the delayed rise in NAT activity observed in previous studies utilizing rodents and suggests that NAT-independent mechanisms may be involved in initiation of melatonin secretion from ovine pineals.

(iii) The ovine pineal is phase sensitive to neural stimulation with more pronounced increases in plasma melatonin levels occurring at night compared to those resulting from daytime stimulation.

(iv) Induced melatonin secretion was pulsatile in all rams, although considerable between-animal variation in pulse frequency was observed.

CHAPTER 4

Bilateral electrical stimulation of the CST's of conscious rams: Effects of photoperiod, time-of-day and stimulus parameters on pineal melatonin secretory responses.

4.1 Introduction.

As described in Chapter 3, bilateral electrical stimulation of the CST's in anaesthetized rams produced less pineal melatonin secretion when stimulation was carried out during the middle of the day rather than at night. This result suggested that the sensitivity of the ovine pineal varied according to the time of stimulation. However, that work was conducted in anaesthetized rams in which the sensitivity of the pineal to neural stimulation may have been reduced through the pharmacological action of the anaesthetic on neuronal activity (see Section 3.4.1). Experiment 3 was therefore designed, in part, to more accurately define the period(s) of greatest pineal sensitivity to neural stimulation, in the absence of anaesthetic modification of neuronal activity. This approach was made possible through the development of implantable stimulating electrodes (Loke et al, 1986) which allowed repeated stimulation of each animal over a 3-6 week period.

Numerous studies have demonstrated that the duration of pineal melatonin secretion in sheep is directly proportional to the length of the night (Kennaway, et al, 1983; Robinson & Karsch, 1987; Karsch et al, 1988). Others have shown that pineal sensitivity to sympathetic nerve stimulation varies according to the time-of-day, for example, Bowers & Zigmond (1982) demonstrated a greater rate of increase in pineal NAT activity during the initial 2 hrs of stimulation when it was applied during the early dark phase rather than during the early light phase. Possible interactions between length of photoperiod and time-of-day, in influencing pineal responsiveness, have received little attention. In order to examine any such interactions, rams in Experiment 3 were subjected to long (16L:8D) and short (8L:16D) photoperiods, and bilateral CST stimulations were undertaken at the beginning, middle and end of the light phase.

In addition to phase shifting of pineal sensitivity to neural stimulation, the pineal has also been shown to be sensitive to changes in the pattern and magnitude of stimuli applied to its sympathetic innervation (Bowers & Zigmond, 1982), although studies of this nature have only been conducted in anaesthetized rodents. Experiment 4 was thus designed to investigate the effect on melatonin secretion from the pineal, of changes in the current strength, frequency

and duration of stimuli delivered to the CST's. As in Experiment 3, chronically implanted electrodes enabled the study to be conducted in conscious rams over a 1-2 week period.

Prior to these two major experiments, several pilot studies were performed to examine whether it was possible to induce melatonin secretion by electrical stimulation of the CST's of conscious sheep, without causing them any apparent stress.

4.2 Materials and methods.

4.2.1 Preliminary studies to establish appropriate stimulus parameters.

Prior to the design of Experiment 3, a series of pilot studies was conducted to determine the stimulus parameters which optimized pineal melatonin secretion, without causing stress to the experimental animal. In these initial studies, each trial ram received either unilateral or bilateral electrode implants (as described in Section 2.2.3) and was allowed 4-7 days post-operative recovery time. Following this period the viability of each electrode was determined, under anaesthesia, by applying a short burst of stimuli via the stimulating system described in Section 2.4. Widening of the palpebral margins of the ipisilateral eye was taken to indicate that the applied stimulus was producing a cranial flow of neural impulses in the CST's and that the electrodes were functional. In the first pilot study positive responses were observed in three of the four rams treated in this way and these were then allowed 1-2 days recovery time before receiving a 2 hour period of stimulation with stimulus parameters set at 10 Hz, 4 ms and 1 mA. General behavioural responses of rams during these trial stimulations varied from an absence of signs of stress to immediate head shaking, foot stamping and body arching such that treatment was terminated immediately. Such reactions were likely to have been caused by stimulus flow to the muscles of the neck, via interstitial fluid. When these rams were stimulated again several days later they showed few signs of discomfort suggesting that any fluid had drained from the surgical site, thus removing electrical contact between the electrodes and surrounding soft tissues of the neck.

Plasma melatonin concentrations during the period of stimulation suggested that pineal activation occurred rapidly at the onset of stimulation and declined at an equal rate following the termination of stimulation (Figure 4.1). Similar responses were recorded in 2 of 3 such experiments. However these initial studies suggested that the stimulus parameters caused unwarranted stress to some animals and so a second pilot study was undertaken to determine a more suitable configuration of stimulation parameters.

Of the three stimulus parameters, pulse frequency and amplitude were held constant at levels previously shown to give effective stimulation in the sympathetic nervous system (Bowers and Zigmond, 1982), while only the pulse duration was varied. The following sets of stimulus parameters were applied during the day to one ram with bilateral CST electrode implants.

A. 1 mA, 10 Hz, 0.25 ms

B. 1 mA, 10 Hz, 0.50 ms

C. 1 mA, 10 Hz, 1 ms

These resulted in a graduated rise in melatonin secretory responses, which increased as stimulus duration increased (Figure 4.2). Areas under the melatonin response curve for each 30 min period of stimulation illustrated this point:

A. 4.7 pg/ml.hr

B. 12.1 pg/ml.hr

C. 19.3 pg/ml.hr

In addition, physical signs of discomfort did not occur during periods of stimulation. From these results it was concluded that the following stimulus parameters should be adopted for Experiment 3 proper, because they produced effective pineal stimulation without jeopardising animal welfare:

1 mA, 10 Hz, 1 ms.

4.2.2 Experiment 3: Animals, lighting regimes and treatment times.

Experiment 3 was conducted during late spring/early summer of 1987. Six Romney rams of approximately 15 months of age, were brought in from pasture and housed in a light-proof, temperature-regulated room as detailed in Section 2.1. Artificial lighting, at an intensity of 200 lux, initially provided a 16L:8D photoperiod, with lights on at 0630 and off at 2230. Following 3 weeks of adaptation to this lighting regime each animal was removed to an operating theatre for bilateral implantation of chronic stimulating electrodes around the CST's as described in Section 2.2.3. A further period of one week was allowed for post-operative recovery, before the commencement of stimulation treatments.

Under the 16L:8D initial lighting regime, rams were then subject to 2 hr periods of nerve stimulation, at the beginning, middle and end of the light phase, at the times shown in Table 4.1. Rams were divided into 3 groups of 2 for random allocation of treatment times.

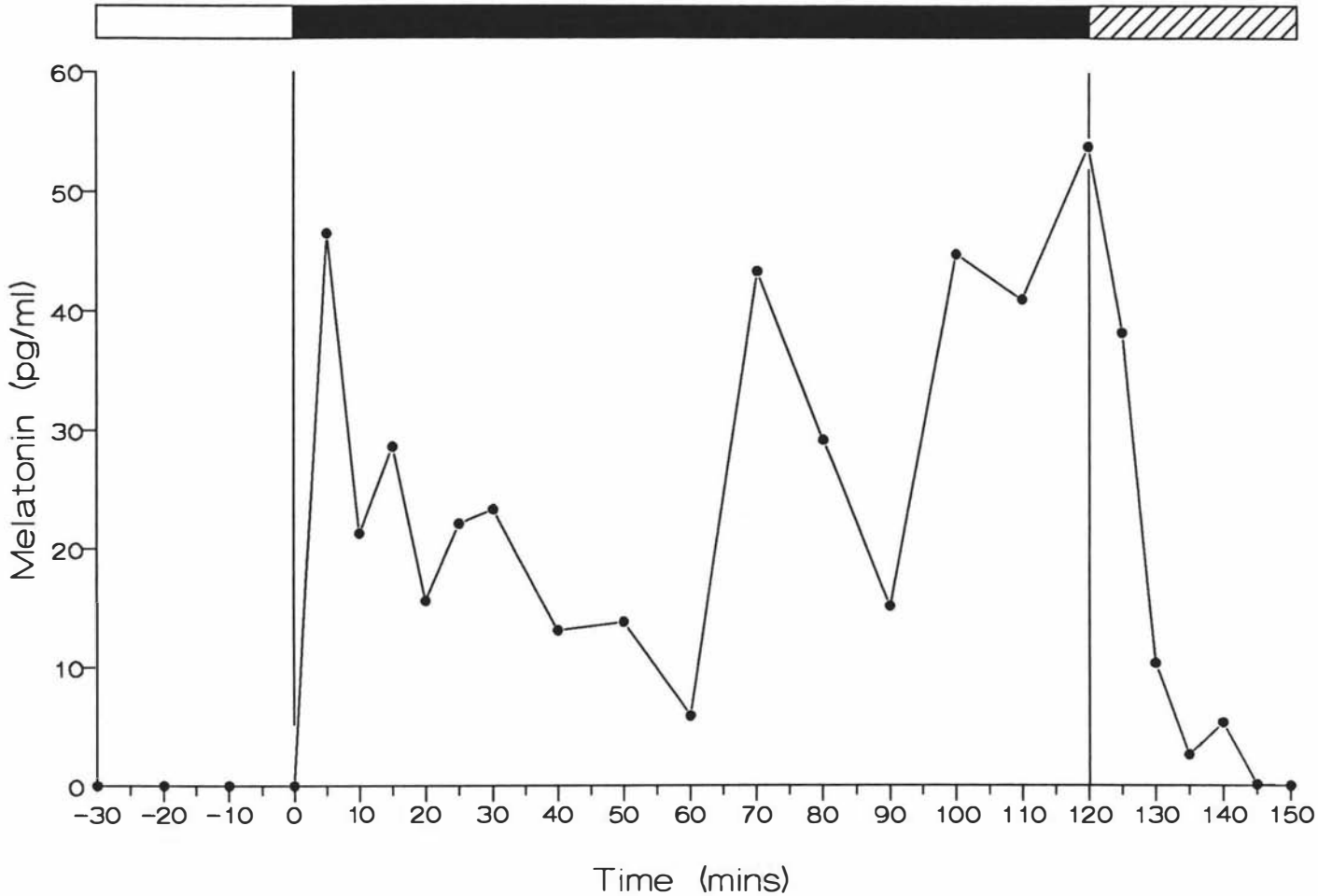


Figure 4.1 Effect of daytime, bilateral, electrical stimulation of the CST's of one conscious ram, with chronically implanted stimulating electrodes, at 10 Hz, with pulse duration and amplitude of 4 ms and 1 mA, respectively, on the plasma melatonin (pg/ml) profile.

[1]Sections of the bar at the top of each figure in this chapter indicate pre-stimulation (open), stimulation (shaded), and post-stimulation (cross-hatched) periods. Note also that axis scaling varies between figures.

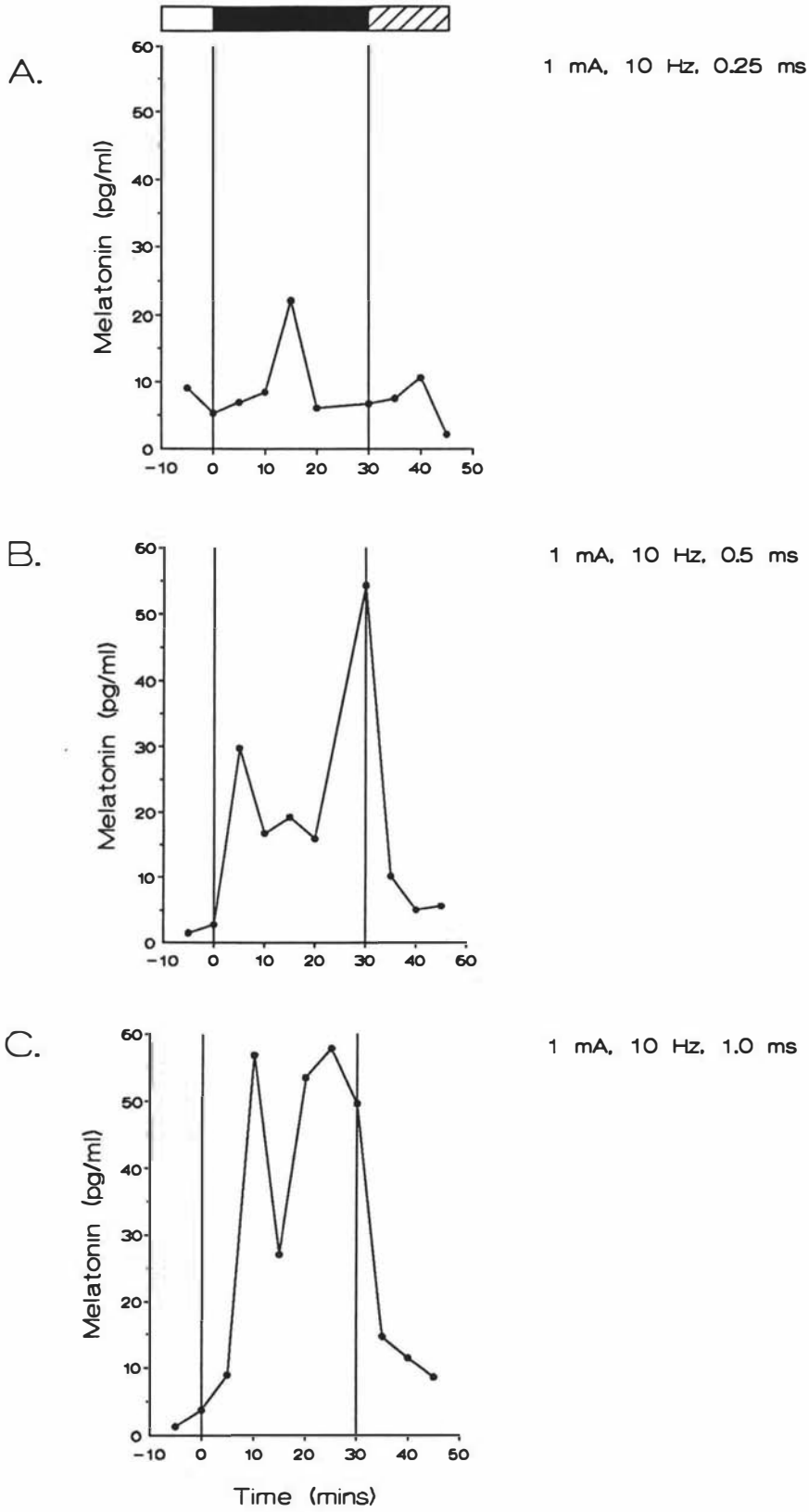


Figure 4.2 Effect of daytime, bilateral, electrical stimulation of the C.S.T's of one conscious ram, with chronically implanted stimulating electrodes, at a range of pulse durations (0.25, 0.5, and 1.0 ms – graphs A, B and C, respectively) on the plasma melatonin (pg/ml) profile. Current strength and frequency were held constant at 1 mA and 10 Hz.

Experiments were then performed on alternate days with 1 full day between treatments for each group, so that only 5 days were required to treat all animals. No adverse reactions of the rams were observed during the periods of stimulation.

Table 4.1 **Times for each photoperiod during which blood sampling occurred in Experiment 3.**

		Sampling Times		
Photoperiod	Time-of-day	Pre-stimulation	Stimulation	Post-stimulation
16L:8D	Beginning	0600 - 0630	0630 - 0830	0830 - 0900
	Middle	1400 - 1430	1430 - 1630	1630 - 1700
	End	2200 - 2230	2230 - 0030	0030 - 0100
8L:16D	Beginning	0730 - 0800	0800 - 1000	1000 - 1030
	Middle	1130 - 1200	1200 - 1400	1400 - 1430
	End	1530 - 1600	1600 - 1800	1800 - 1830

Following completion of stimulation treatments under the initial photoperiod, the light/dark schedule was adjusted to 8L:16D (lights on at 0800 hr and off at 1600 hr). After an exposure period of 2.5 weeks and immediately prior to the second round of stimulation treatments, each ram was anaesthetized and stimulated, as described earlier in this section. In each case nerve stimulation caused widening of the palpebral margins. Table 4.1 details the treatment times for this photoperiod. For each ram these treatment times were randomized as under the longer photoperiod.

Following the completion of all stimulation treatments the functional viability of both electrodes and nerves was again confirmed with each ram under anaesthesia, and following the procedure outlined in Section 2.2.2. The death of one ram a few days earlier from undetermined causes precluded it from this check. Of the remaining five rams, four responded with a pronounced widening of both palpebral margins, while one ram gave no response to stimulation of either CST. Melatonin secretory responses in the ram which died earlier and in the ram with no palpebral response were comparable to those of the remaining rams and therefore were included in the analyses of treatment effects.

Blood sampling regime.

Methods for the collection and processing of blood samples and storage of plasma are detailed in Section 2.6

The pattern of sample collection in Experiment 3 consisted of 10 min sampling for 30 min prior to stimulation, 5 min sampling for the first 30 min of stimulation and 10 min samples for the remaining 90 mins of stimulation. Post-stimulus samples were taken at 5 min intervals for 30 mins.

4.2.3 Experiment 4

(I) Animals and lighting regimes.

In general, melatonin secretory responses recorded in Experiment 3 were very limited, so Experiment 4 was designed to study whether greater responses could be produced by further attempts at optimizing the parameters of electrical stimulation. Experiment 4 was conducted during the early summer of 1988. Six Romney rams, similar in age and condition to those used in Experiment 3, were similarly maintained in a light-proof, temperature regulated room as detailed in Section 2.1. Artificial lighting provided a constant photoperiod of 16L:8D throughout this experiment, with lights on at 0800 hrs and off at 2400 hrs. This photoperiod was chosen as results from Experiment 3 indicated that pineal melatonin secretion was more readily evoked in response to CST stimulation under this photoperiod than during the shorter 8L:16D photoperiod. All rams were initially exposed to this photoperiod for 3 weeks before undergoing surgery for the bilateral implantation of stimulating electrodes around the CST's. As in Experiment 3, a further period of one week was allowed for post-operative recovery before the commencement of stimulation treatment.

(II) Stimulus parameters and treatment times.

In order to investigate the effects of current strength, frequency, and pulse duration on the secretion of melatonin from the pineal, each stimulus parameter was varied over the range reported to produce subthreshold to supramaximal stimulation of the CST's, or other sympathetic nerves, in terms of the compound action potential generated (Bowers & Zigmond, 1982) and effector responses (Bowers & Zigmond, 1980, 1982; McGeown et al, 1987). As each individual stimulus parameter was varied within its range, the two associated parameters were held constant. Table 4.2 lists the combinations of stimulus parameters applied in

Experiment 4. For each ram, each combination of stimulus parameters was applied in random order so as to eliminate any effect of treatment order on experimental results.

Table 4.2 **Combinations of electrical stimulus parameters applied to each ram in Experiment 4.**

Parameter set #	Stimulus parameter ms	mA	Hz
1	**0.5**	2	10
2	**2**	2	10
3	**4**	2	10
4	1	**0.5**	10
5	1	**1**	10
6	1	**4**	10
7	1	2	**5**
8	1	2	**10**
9	1	2	**20**

All stimulation treatments commenced at 0830 hrs, 30 mins after the beginning of the photophase, and continued for a period of 30 mins, concluding at 0900 hrs. This time of day was chosen as preliminary examination of results from Experiment 3 indicated that the pineal responded to CST stimulation at this time with a larger melatonin secretory response compared to that following CST stimulation during the middle or end of the photophase. No evidence of adverse or detrimental effects to the rams was recorded in this experiment.

Following completion of all stimulation treatments a visual inspection of each electrode was made and a histological examination of each CST performed to enable a morphological assessment of its condition. This approach enabled the integrity of the electrode/nerve complex to be evaluated both macroscopically and microscopically. Palpebral responses were not examined in this experiment as results from Experiment 3 indicated that the electrodes remained functional for at least three weeks and that in most cases electrodes remained functional for six weeks.

Each ram was killed by an overdose of sodium pentobarbitone, administered intravenously, the electrodes then being exposed in each ram and removed by severing the CST's rostrally and caudally to each electrode. Each nerve/electrode preparation was immediately immersed in Bouin's fluid for 12 hr and then stored in 70% ethyl alcohol. Prior to histological processing as described in Section 2.8.2 (II), the electrode wire and silastic sheathing was carefully removed from the CST's thus exposing a 2 cm length of nerve trunk.

This was then cut twice, once between the attachment points of the anode and cathode electrode wires and once rostral to the cathode electrode wire attachment point. Segments of nerve trunk were then processed, sectioned and mounted onto glass slides. Each section was stained with Masson's Green Tri-chrome (Culling, 1974) which specifically stains the following neural components: nuclei (blue/black) of Schwann cells, collagen (green) and myelin (red). Individual stained sections were examined under a microscope and compared with CST's obtained from sheep without electrode implants and prepared in a similar manner.

Blood sampling regime.

As in Experiment 3, all blood sampling was via indwelling cannulae, inserted bilaterally into the jugular veins at the time of electrode implantation, or under local anaesthetic immediately prior to the experiment. Details of cannulae size and type, anaesthetic used and blood processing following collection, can be found in Section 2.6. All blood sample volumes were 10 ml.

Samples in Experiment 4 were taken over a shorter time period as results from Experiment 3 indicated this was sufficient to assess any response. Pre-stimulus samples were collected over a 20 min period at 10 min intervals, while sampling during the 30 min period of stimulation was at 5 min intervals. Sampling at 10 min intervals continued during the 20 min post-stimulus period.

4.2.4 Statistical analysis

(I) Experiment 3

Following transformation to logarithms, as described in Section 2.9.3, the data for the pre-stimulation period were subjected to analyses of variance to examine the effects of photoperiod and time-of-day when stimulation was applied. The data for the stimulation and post-stimulation periods, transformed to logarithms, were examined for effects of photoperiod and time-of-day when stimulation was applied in analyses of covariance (Cochran & Cox, 1957). This form of analysis examined treatment effects on the responses in the two periods with any effects of differences in the pre-stimulation period removed. Elimination of this aspect of between-animal differences results in a reduction in the experimental error. In all analyses, orthogonal coefficients (Table 4.3) were used to partition the treatment effects into single degree of freedom contrasts.

Table 4.3 Orthogonal coefficients used in partitioning treatment effects in analysis of variance of data from Experiment 3.

	Photoperiod					
	16L:8D			8L:16D		
	Part of photophase when stimulation was applied					
Contrast	Beginning	Middle	End	Beginning	Middle	End
(a) Photoperiods	+1	+1	+1	-1	-1	-1
(b) Time						
(i) Beginning-of-day vs End-of-day (Linear)	+1		-1	+1		-1
(ii)Beginning & End-of-day vs Middle-of-day (Quadratic)	-1	+2	-1	-1	+2	-1
Interaction (i)	+1		-1	-1		+1
(ii)	-1	+2	-1	+1	-2	+1

(II) **Experiment 4**

As in Experiment 3, it was necessary to remove any relationship between pre-stimulation and stimulation period melatonin output before the significance of treatment effects could be assessed. This was achieved following the same procedure as that used in Experiment 3, except that transformation to logarithms was not necessary since the means and standard deviations were not linearly related. Post-stimulation data were also treated in this way, while pre-stimulation data were examined by analysis of variance. Again, orthogonal and non-orthogonal coefficients (Table 4.4) were used to partition the treatment effects into single degree of freedom contrasts.

Table 4.4 **Coefficients used in partitioning treatment effects in analyses of variance of data from Experiment 4 (Refer to Table 4.2 for characteristics of each stimulus parameter set).**

Contrast	Parameter set number								
	1	2	3	4	5	6	7	8	9
1. Orthogonal coefficients									
(i) Frequency:									
Linear - 5 vs 20 Hz							+1		-1
Quadratic - 5 + 20 vs 10 Hz							+1	-2	+1
(ii) Amplitude:									
Linear - 0.5 vs 4 mA				+1		-1			
Quadratic - 0.5 + 4 vs 1 mA				+1	-2	+1			
(iii) Duration:									
Linear - 0.5 vs 4 ms	+1		-1						
Quadratic - 0.5 + 4 vs 2 ms	+1	-2	+1						
(iv) Frequency vs Amplitude:									
5 + 10 + 20 Hz vs 0.5 + 1 + 4 mA				-1	-1	-1	+1	+1	+1
(v) Frequency + Amplitude vs Duration:									
5 + 10 + 20 Hz + 0.5 + 1 + 4 mA vs 0.5 + 2 + 4 ms	-2	-2	-2	+1	+1	+1	+1	+1	+1
2. Non-orthogonal coefficients[1]									
(i) Amplitude:									
Linear				-3	-1	+3		+1	
Quadratic				+1	-1	+1		-1	
Cubic				-1	+3	+1		-3	
(ii) Duration:									
Linear	-3	+1	+3					-1	
Quadratic	+1	-1	+1					-1	
Cubic	-1	-3	+1					+3	

[1] These non-orthogonal coefficients for amplitude and duration take account of the stimulus parameters in Parameter set #8 in which the levels of these factors were different from those tested by the orthogonal coefficients.

4.3 Results.

4.3.1 Experiment 3. (Effects of photoperiod and time-of-day)

See Tables 4.5-4.9 and Figures 4.3-4.15

(I) Pre-stimulation values

Pre-stimulation mean plasma melatonin levels for the three treatment times followed a similar pattern in both photoperiods. Sampling prior to stimulation, at the beginning of each photoperiod, was carried out prior to lights on and hence mean plasma melatonin content was

elevated throughout this period (73.0 ± 11.6 pg/ml under the 16L:8D photoperiod and 48.5 ± 12.9 pg/ml under the 8L:16D photoperiod). Mean pre-stimulation levels for the mid- and end-of-photoperiod treatment times were only slightly above the 5.1 ± 0.4 pg/ml limit of assay sensitivity (Table 4.5). The linear and quadratic effects of time-of-day were significant at the 0.1% and 1% levels, respectively (Table 4.6), because of the elevated values in samples collected at the end of the dark phase.

Table 4.5 **Mean (± S.E.M) pre- and post-stimulation plasma melatonin levels (pg/ml) and mean (± S.E.M) melatonin secretory responses to CST stimulation (pg/ml.hr) under 16L:8D and 8L:16D photoperiods, when nerve stimulation was performed at the beginning, middle or end of the photophase.**

	Mean pre-stimulation levels (pg/ml)	Mean melatonin secretory responses (pg/ml.hr)	& levels (pg/ml)	Mean post-stimulation levels (pg/ml)
16L:8D photoperiod				
Beginning of photophase	73.0 ± 11.6	117.1 ± 31.2	60.8 ± 26.3	54.2 ± 16.3
Middle " "	7.2 ± 2.0	59.0 ± 19.4	28.3 ± 13.7	31.5 ± 9.0
End " "	10.5 ± 1.5	47.2 ± 7.2	23.6 ± 7.9	14.0 ± 1.7
8L:16D photoperiod				
Beginning of photophase	48.5 ± 12.9	79.1 ± 34.0	40.5 ± 22.0	37.0 ± 13.3
Middle " "	8.5 ± 2.1	64.3 ± 23.1	31.2 ± 12.9	24.4 ± 7.8
End " "	5.9 ± 1.8	28.1 ± 10.8	15.8 ± 10.0	13.5 ± 3.9

Table 4.6 **Summary of analysis of variance of pre-stimulation data from Experiment 3. Prior to analysis mean melatonin levels (pg/ml) were transformed to logarithms.**

Source of variation	D.F	Variance Ratios
		Pre-stimulation
Between treatment groups	5	
Photoperiod: 16L:8D vs 8L:16D	1	3.02
Time-of-day		
- Linear (Beginning vs End)	1	26.29***
- Quadratic (Beginning & End vs Middle)	1	11.41**
Interaction	2	1.66
Within treatment groups		
Error mean square	27	0.130

(II) **Stimulation responses**

Bilateral electrical stimulation of CST's in conscious rams at the beginning of the photophase prevented the light-induced decline in mean plasma melatonin concentrations which normally occurs at this time. Similar stimulation during the middle and at the end of photophase increased mean plasma melatonin levels above pre-stimulation levels, and they

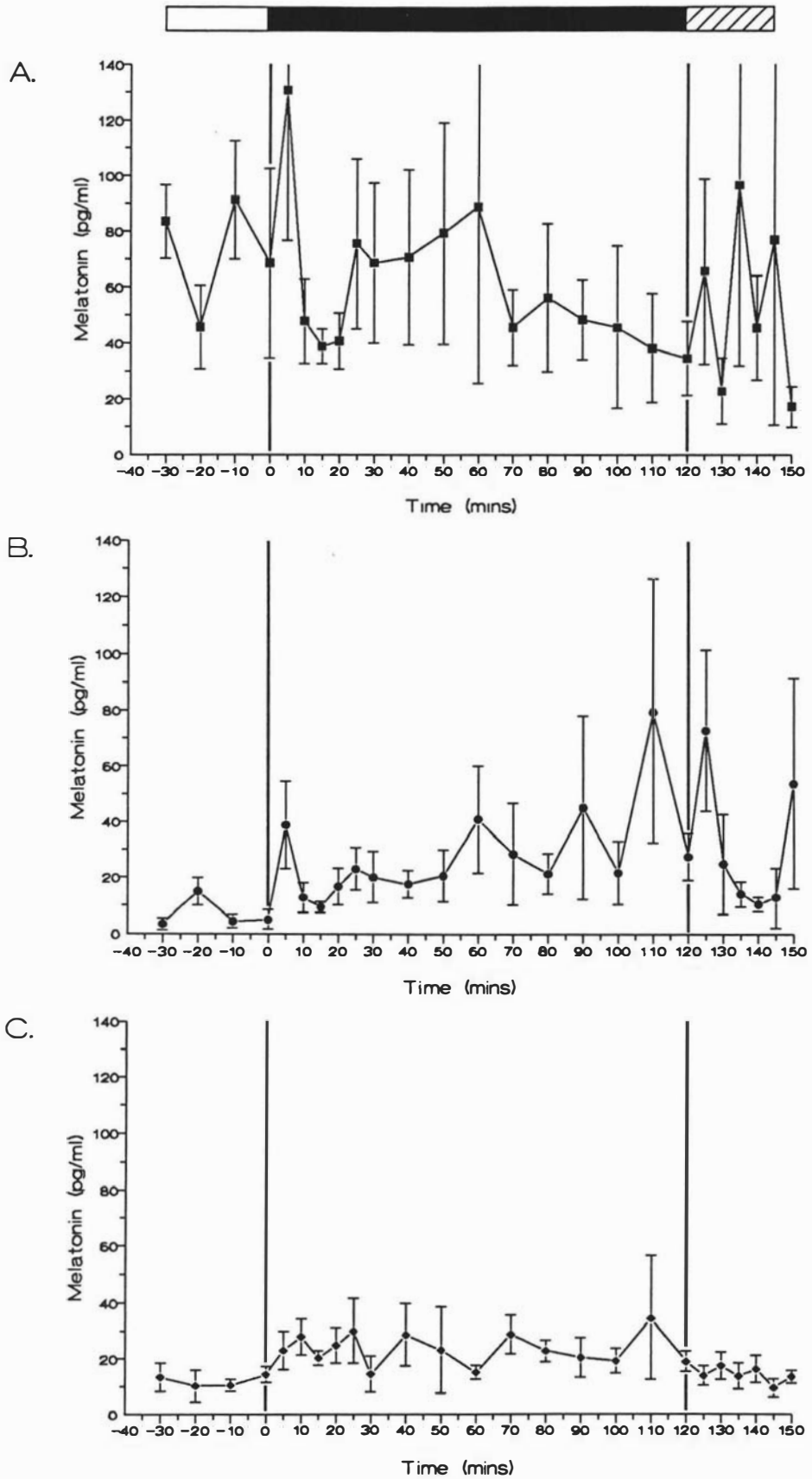


Figure 4.3 Mean plasma melatonin levels (+/− S.E.M) before, during and after bilateral, electrical stimulation of the CST's of conscious rams in a 16L:8D photoperiod. Stimulation commenced at the following times: A. beginning of the photophase (0630), B. during mid-photophase (1430), C. end of the photophase (2230), but with the lights remaining on until the end of the post-stimulation period.

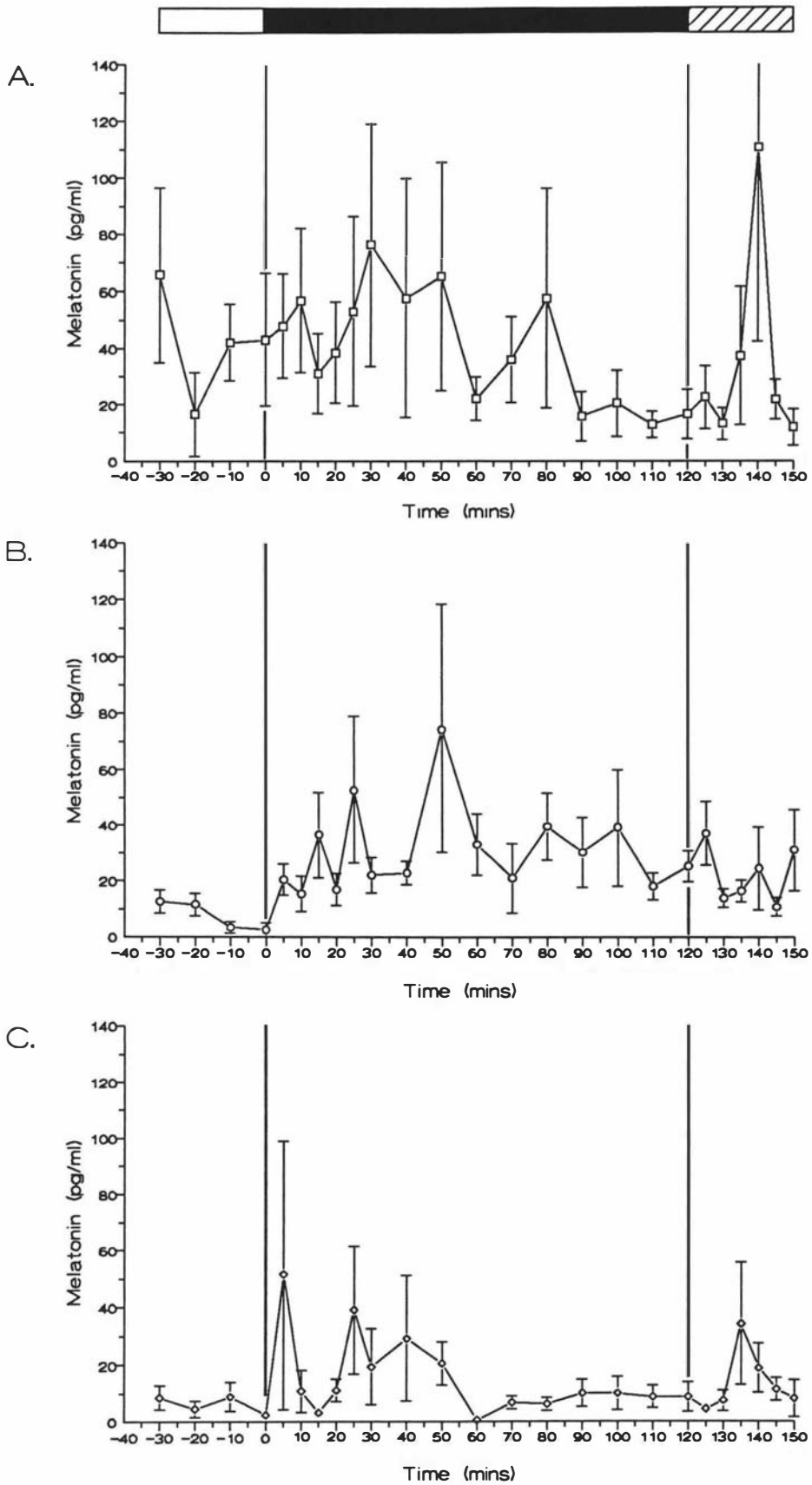


Figure 4.4 Mean plasma melatonin levels (+/− S.E.M) before, during and after bilateral, electrical stimulation of the CST's of conscious rams in a 8L:16D photoperiod. Stimulation commenced at the following times: A. beginning of the photophase (0800), B. during mid−photophase (1200), C. end of the photophase (1600), but with the lights remaining on until the end of the post−stimulation period.

generally remained elevated throughout the period of stimulation (Figures 4.3 & 4.4 & Table 4.5). Once adjusted for the regression of stimulated responses on pre-stimulation values (Table 4.7), it was found that overall responses were not significantly different between the two photoperiods. Within each photoperiod, however, there was a significant quadratic effect of time of stimulation. That is, mid-photophase melatonin secretory responses were significantly greater ($P<0.05$, Table 4.8) than either of the other responses, which did not differ significantly from each other. The highly significant regression ($P<0.001$, Table 4.8), determined by the between-animal variation, revealed a strong relationship between pre-stimulation and stimulation periods and resulted in a 43% reduction in experimental error.

Table 4.7 **Plasma melatonin secretory responses (log pg/ml.hr & pg/ml.hr) to bilateral CST stimulation adjusted for the regression of stimulated responses (log pg/ml.hr) on pre-stimulated responses (log pg/ml).**

	Mean plasma melatonin content or response			
	Pre-stimulation Log pg/ml	Stimulation Log pg/ml.hr	Adjusted stimulation Log pg/ml.hr	Adjusted stimulation pg/ml.hr
16L:8D photoperiod				
Beginning of photophase	1.832	1.959	1.498	31.5
Middle " "	0.817	1.627	1.857	71.9
End " "	1.115	1.660	1.687	48.9
8L:16D photoperiod				
Beginning of photophase	1.552	1.735	1.465	29.2
Middle " "	0.871	1.730	1.924	83.9
End " "	0.684	1.345	1.665	46.2

Table 4.8 **Summary of analyses of residual variance of stimulation and post-stimulation data from Experiment 3. Prior to analysis mean melatonin responses (pg/ml.hr) were transformed to logarithms and then adjusted for the regression of responses on pre-stimulation values in an analysis of covariance.**

Source of variation	D.F	Variance Ratios	
		Stimulation	Post-stimulation
Between treatment groups	5		
Photoperiod: 16L:8D vs 8L:16D	1	<1	<1
Time-of-day			
- Linear (Beginning vs End)	1	1.41	2.56
- Quadratic (Beginning & End vs Middle)	1	6.79*	6.20*
Interaction	2	<1	<1
Within treatment groups	27		
Regression	1	22.4***	12.21*
Error mean square	26	0.728	0.233

Comparison of the plasma melatonin concentrations during stimulation revealed a number of trends within each treatment time. Firstly, stimulation at the beginning of each

photoperiod, at a time when melatonin levels already were high, initially caused a transient increase in melatonin secretion lasting 5-15 min, followed by a fall to levels below those recorded prior to the commencement of stimulation. Therefore, although some rises in mean levels were recorded during the remaining period of stimulation, the overall trend was for a gradual decrease in the plasma melatonin content even while stimulation continued (see Figures 4.3A & 4.4A). As measured by the change in mean melatonin levels relative to the pre-stimulation period, this amounted to a net decrease of 14.5 ± 4.8 pg/ml during stimulation at the beginning of the short photoperiod and a 8.5 ± 13.5 pg/ml decrease during stimulation at the beginning of the long photoperiod.

Stimulation during the mid-photoperiod of either lighting regime initiated a rise in melatonin secretion, which was maintained above daytime, pre-stimulation levels throughout the stimulation period. The mean initial response to stimulation was a rise above mean pre-stimulation levels during the first 10 mins followed by a fall to near pre-stimulation levels. Subsequent to this the overall pattern was for progressively increasing plasma melatonin levels during all of the stimulation period under a 16L:8D photoperiod and for the initial 1 hr of stimulation under a 8L:16D photoperiod. The overall effect of this pattern of response during stimulation was for a net increase to occur in the mean plasma melatonin level compared to that recorded during the pre-stimulation period (ie., a mean increase of 21.1 ± 8.4 pg/ml under the long photoperiod and 22.7 ± 11.4 pg/ml under the short photoperiod). These patterns represented the greatest increases in melatonin secretion initiated by electrical stimulation during any of the three treatment times (see Figures 4.3B & 4.4B).

As with beginning- and mid-photoperiod stimulation, stimulation at the end of the photoperiod, conducted in the light, but continuing into what was normally the dark phase, produced a small increase in the mean plasma melatonin content during the initial 5-10 mins of stimulation followed by a sustained level of secretion which was only slightly above that of the pre-stimulation period. This relatively modest response to electrical stimulation is illustrated by the net increases in mean plasma melatonin concentrations of 13.1 ± 2.5 pg/ml under the long photoperiod and 8.3 ± 4.0 pg/ml under the short photoperiod (Figures 4.3C & 4.4C).

(III) Individual animal stimulation responses

Examination of individual melatonin secretory profiles revealed that all rams responded to CST stimulation, but that these responses were highly variable both within and between

animals. In those rams most sensitive to CST stimulation, large increases in plasma melatonin content were recorded during most, but not all, periods of stimulation (eg., Figure 4.5). These elevations in melatonin secretion usually were apparent within 15-20 minutes. A few rams exhibited relatively small increases in plasma melatonin content during CST stimulation, but again this was not consistent in each period of stimulation (eg., Figure 4.10). Although brief periods of rapidly changing melatonin levels (increases followed by decreases) were recorded during the stimulation period, these occurred at irregular intervals and appeared to be of a similar episodic nature to those which occurred in Experiment 2. Figures 4.5-4.15 show the melatonin secretory profiles of individual rams in Experiment 3 and illustrate the episodic pattern of melatonin secretion in these rams. Identification of pulse peaks was achieved by the method described previously in Chapter 3. No data are shown for Ram #11 under the 8L:16D photoperiod as catheter failure prevented it from being used in this part of the experiment.

(IV) **Post-stimulation values**

Mean plasma melatonin levels during the post-stimulation sampling period followed a similar pattern in both photoperiods. During the 30 min following stimulation at the beginning of the photophase mean plasma content exhibited a gradual fall to near daytime pre-stimulation levels. A few animals (eg., #32, Figure 4.15A) exhibited a pulse of melatonin secretion during this period, which contributed to a substantial between-animal variation and also to apparent continued secretion of melatonin. Mean plasma melatonin levels following stimulation during the middle or at the end of the photophase were generally similar to or

Table 4.9 **Plasma melatonin secretory responses (log pg/ml & pg/ml) following bilateral CST stimulation adjusted for the regression of post-stimulation responses (log pg/ml) on pre-stimulated responses (log pg/ml).**

	Mean plasma melatonin content or response			
	Pre-stimulation Log pg/ml	Post-stimulation Log pg/ml	Adjusted post-stimulation Log pg/ml	pg/ml
16L:8D photoperiod				
Beginning of photophase	1.832	1.559	1.047	11.2
Middle " "	0.817	1.287	1.543	34.9
End " "	1.115	1.061	1.091	12.3
8L:16D photoperiod				
Beginning of photophase	1.552	1.260	0.960	9.1
Middle " "	0.871	1.316	1.531	34.0
End " "	0.684	0.948	1.304	20.2

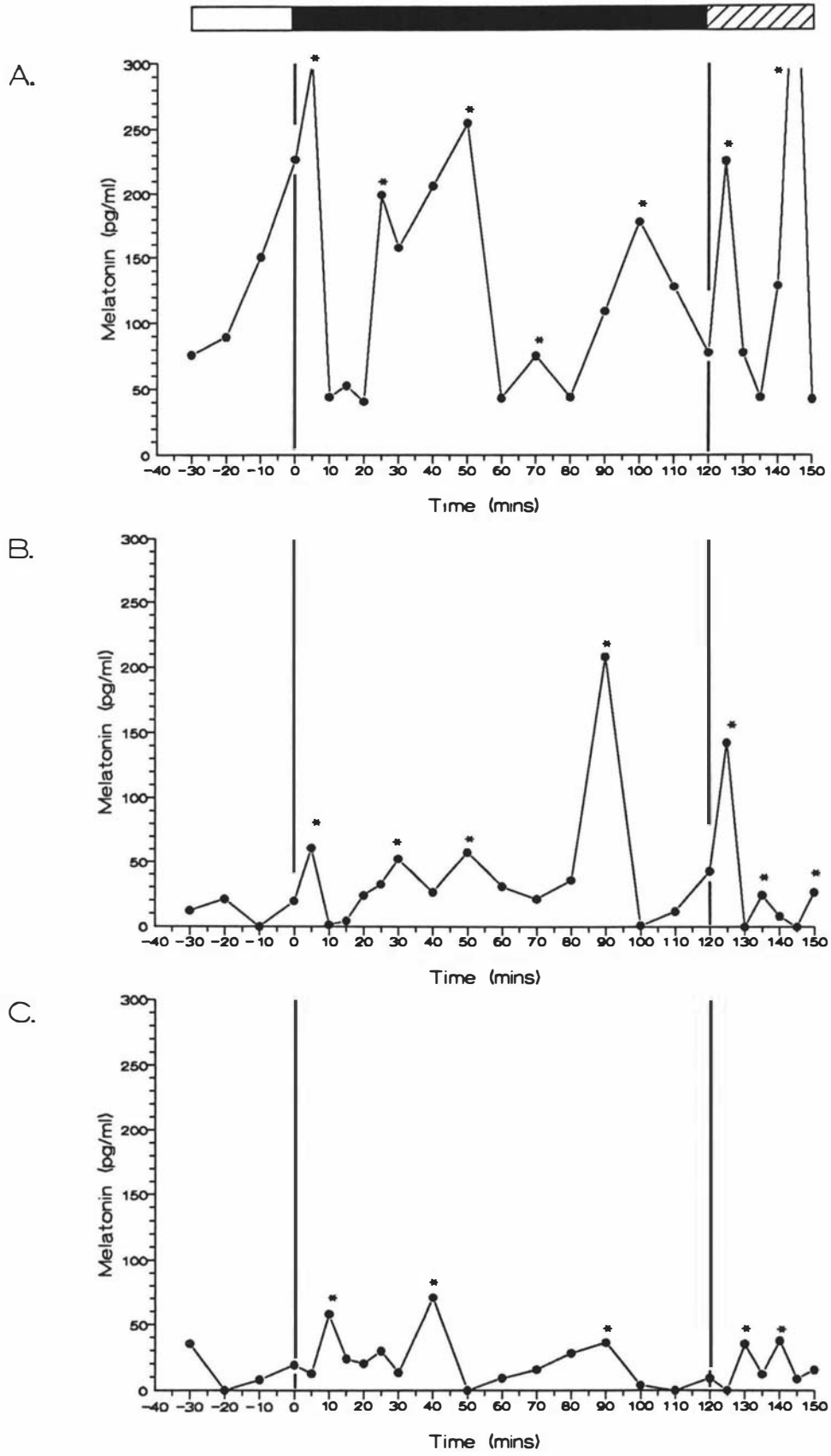


Figure 4.5 Plasma melatonin levels (pg/ml) before, during and after bilateral, electrical stimulation of the CST's of ram #10 in a 16L:8D photoperiod. A. stimulation commenced at lights on (0630). B. stimulation commenced during the mid-photophase (1400) C. stimulation commenced at lights off (2200) in a room with light intensity of 200 lux. Note that for all graphs in Expt 3 an asterix (*) indicates a melatonin pulse peak.

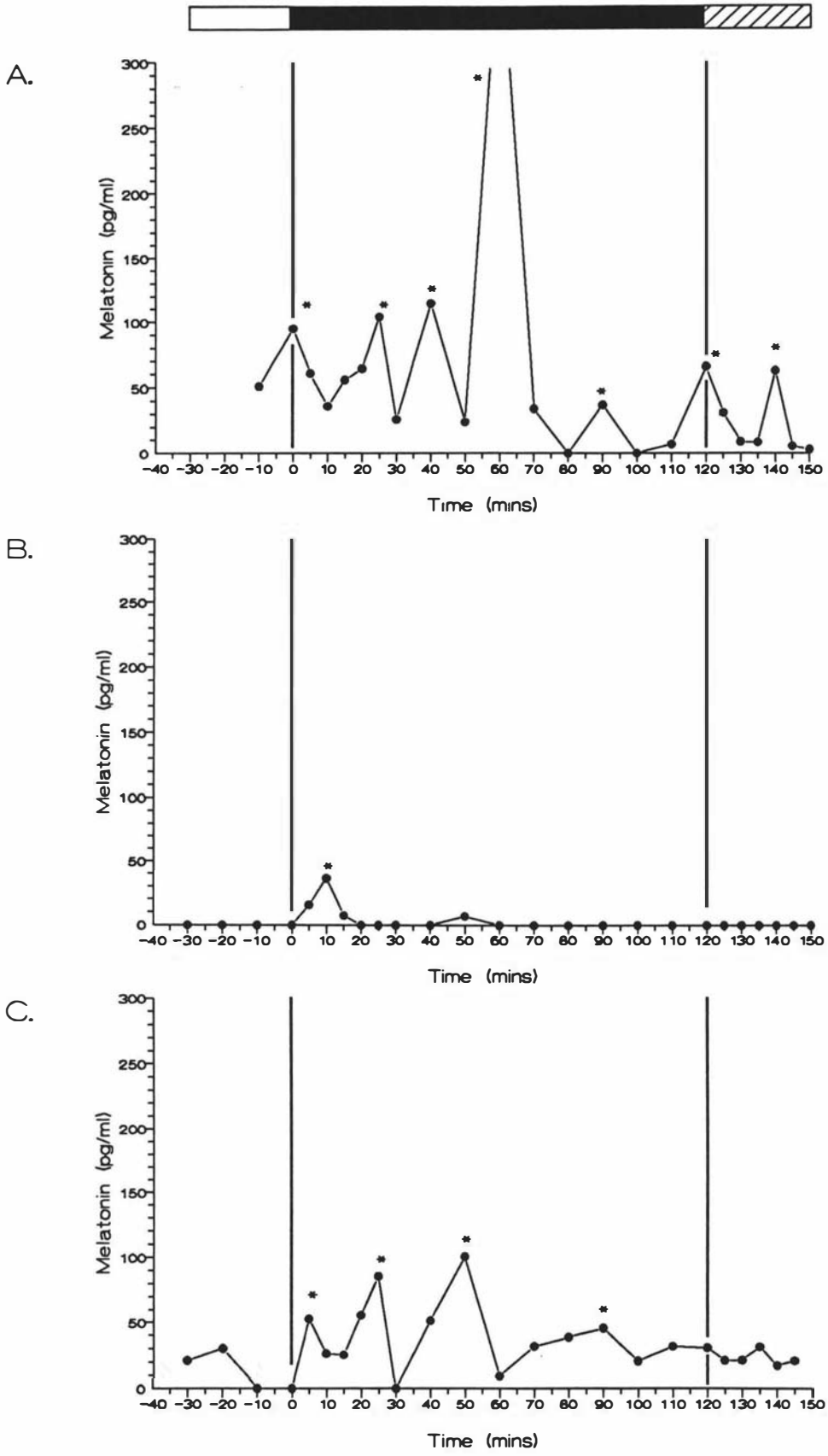


Figure 4.6 Plasma melatonin levels (pg/ml) before, during and after bilateral, electrical stimulation of the CST's of ram #11 in a 16L:8D photoperiod. A. stimulation commenced at lights on (0630). B. stimulation commenced during the mid-photophase (1400) C. stimulation commenced at lights off (2200) in a room with light intensity of 200 lux.

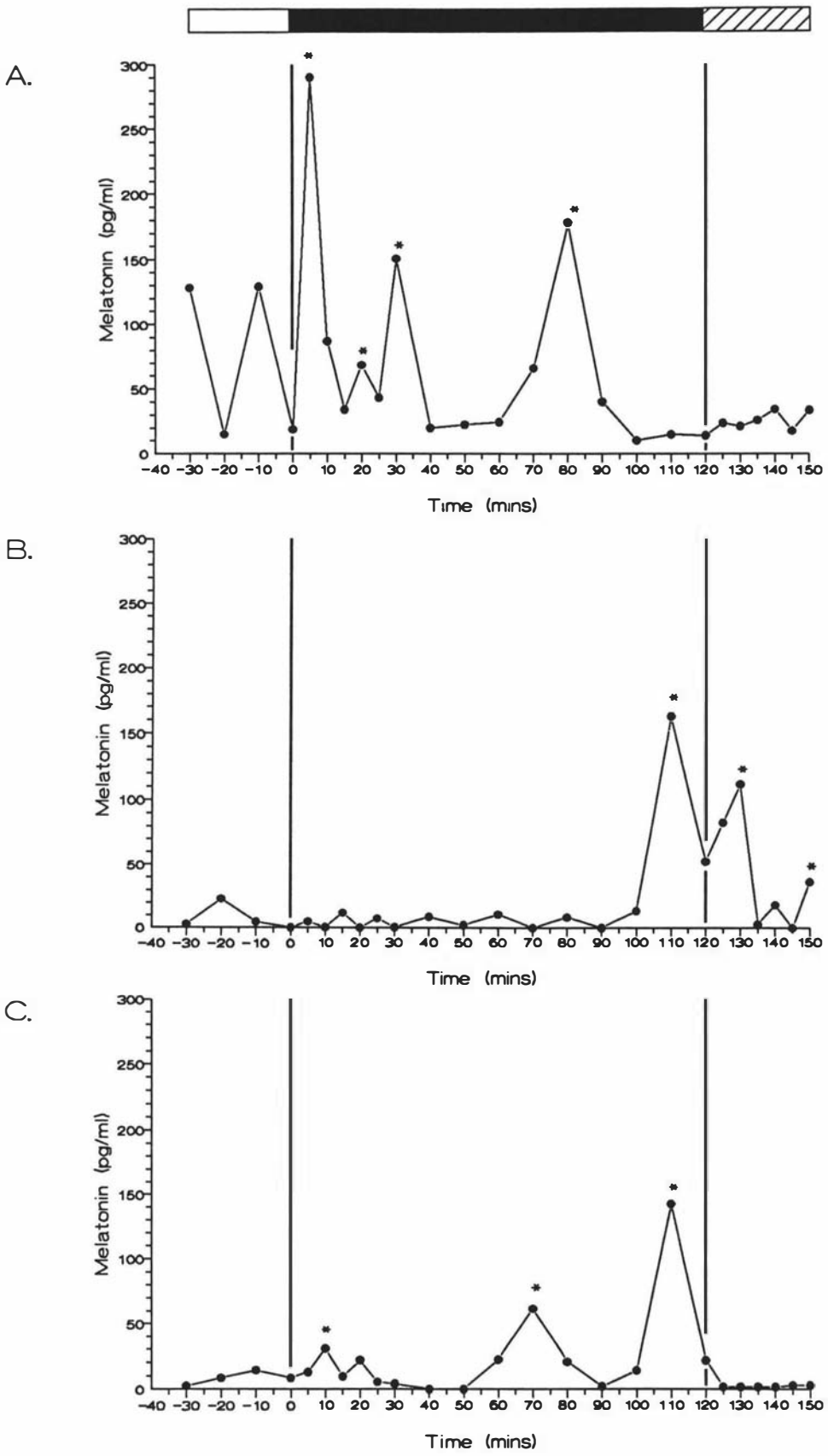


Figure 4.7 Plasma melatonin levels (pg/ml) before, during and after bilateral, electrical stimulation of the CST's of ram #18 in a 16L:8D photoperiod. A. stimulation commenced at lights on (0630). B. stimulation commenced during the mid-photophase (1400) C. stimulation commenced at lights off (2200) in a room with light intensity of 200 lux.

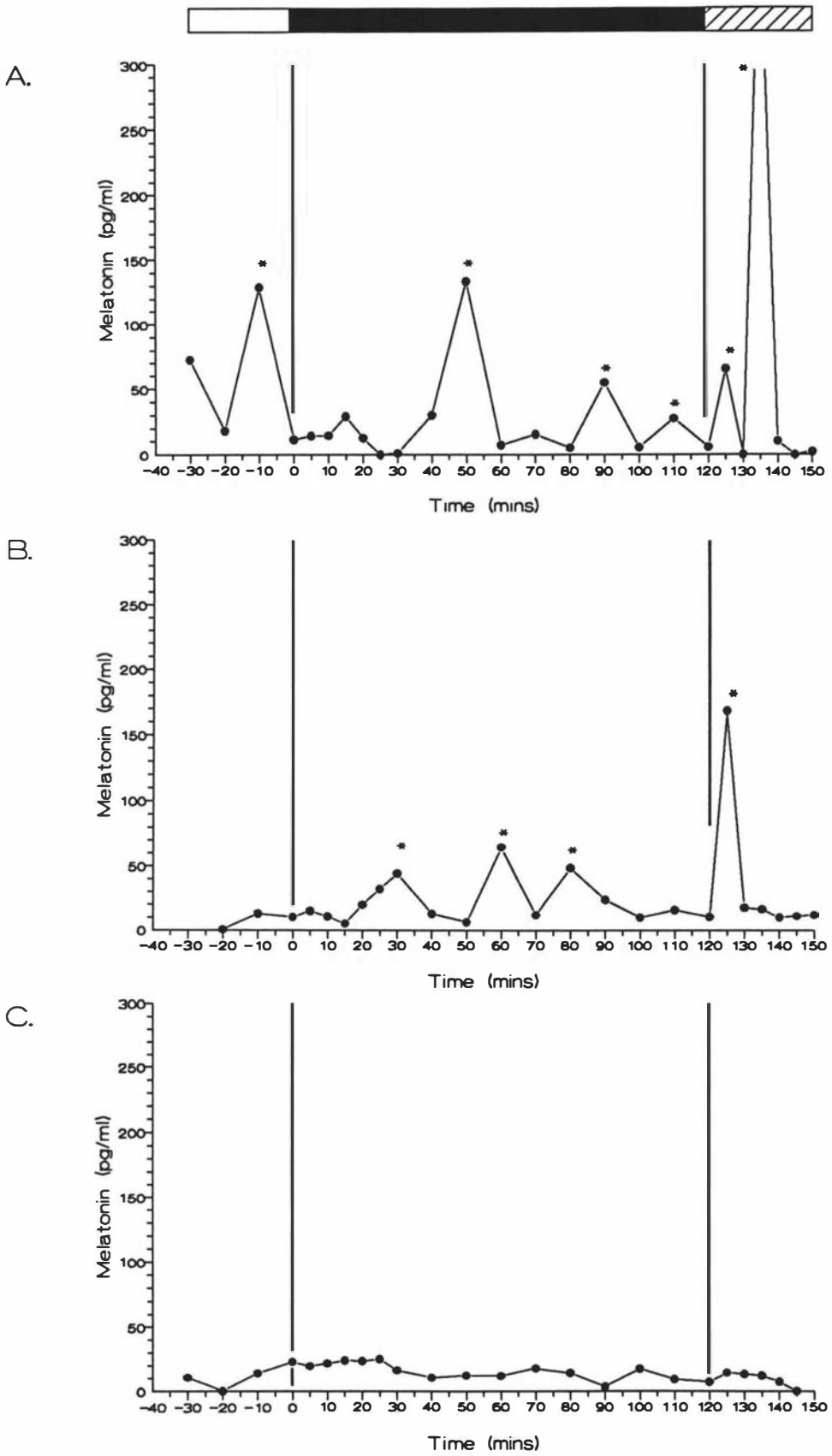


Figure 4.8 Plasma melatonin levels (pg/ml) before, during and after bilateral, electrical stimulation of the CST's of ram #22 in a 16L:8D photoperiod. A. stimulation commenced at lights on (0630). B. stimulation commenced during the mid-photophase (1400) C. stimulation commenced at lights off (2200) in a room with light intensity of 200 lux.

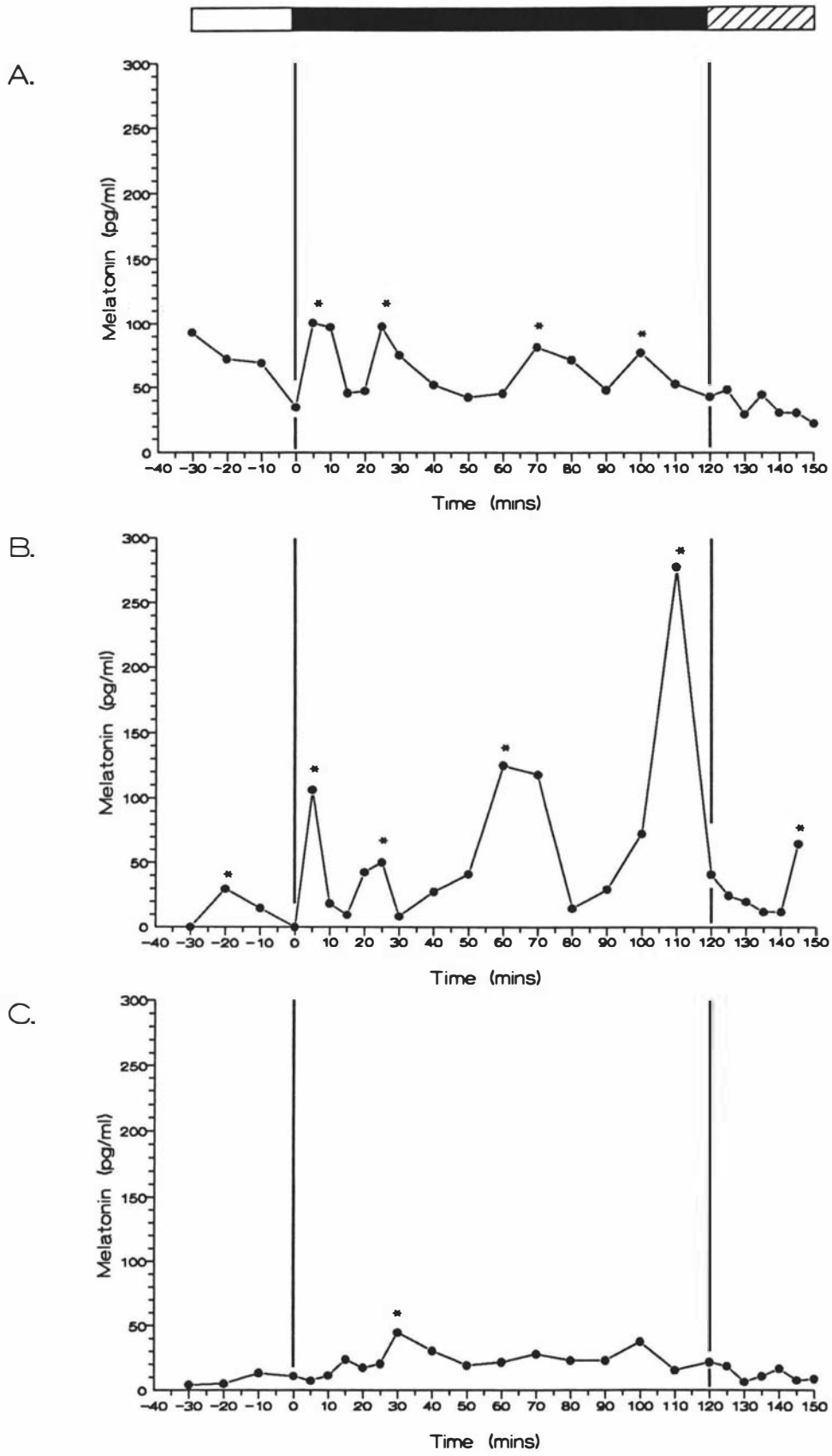


Figure 4.9 Plasma melatonin levels (pg/ml) before, during and after bilateral, electrical stimulation of the CST's of ram #28 in a 16L:8D photoperiod. A. stimulation commenced at lights on (06 30). B. stimulation commenced during the mid-photophase (1400) C. stimulation commenced at lights off (2200) in a room with light intensity of 200 lux.

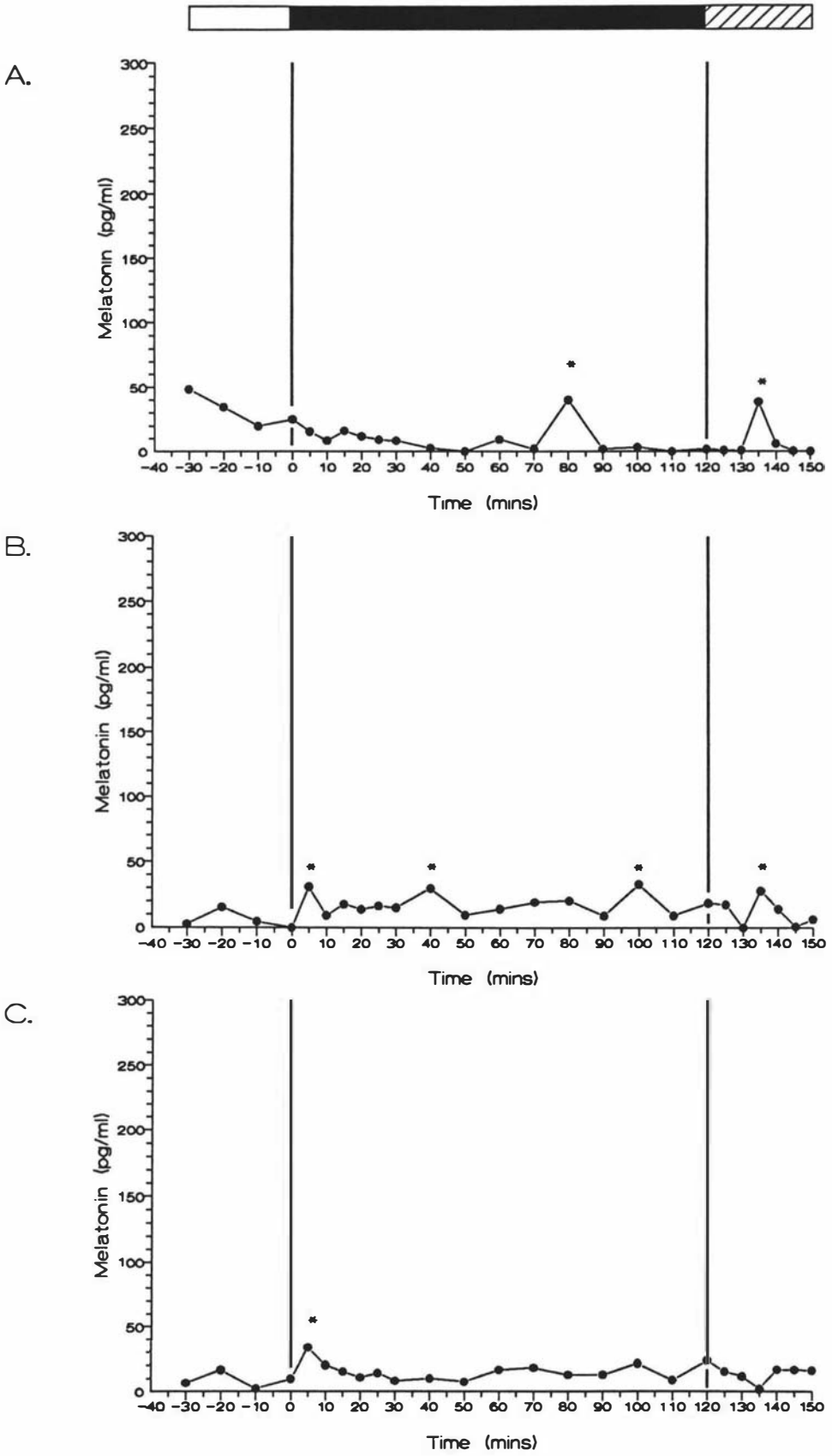


Figure 4.10 Plasma melatonin levels (pg/ml) before, during and after bilateral, electrical stimulation of the CST's of ram #32 in a 16L:8D photoperiod. A. stimulation commenced at lights on (0630). B. stimulation commenced during the mid-photophase (1400) C. stimulation commenced at lights off (2200) in a room with light intensity of 200 lux.

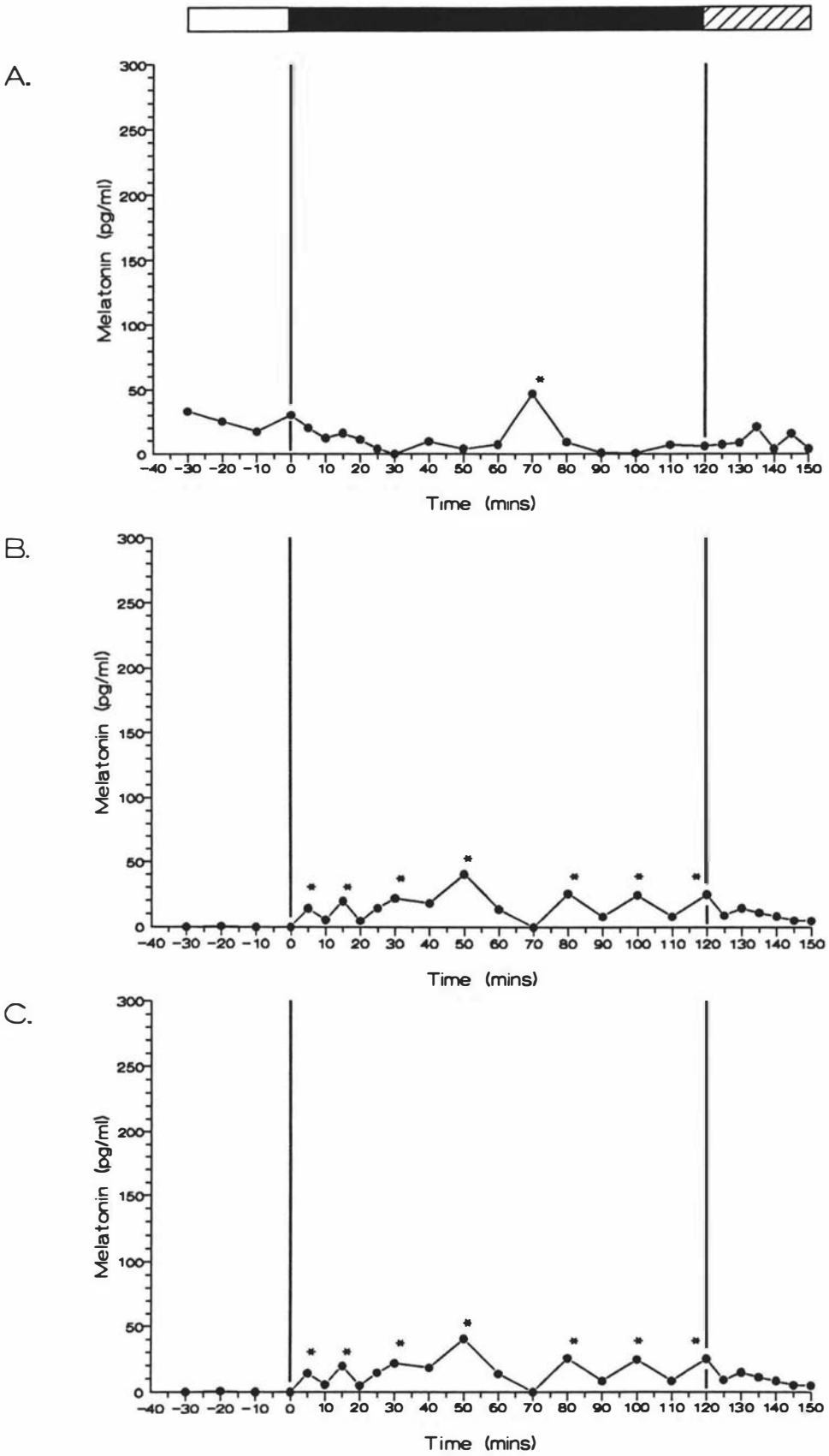


Figure 4.11 Plasma melatonin levels (pg/ml) before, during and after bilateral, electrical stimulation of the CST's of ram #10 in a 8L:16D photoperiod. A. stimulation commenced at lights on (0730). B. stimulation commenced during the mid-photophase (1130) C. stimulation commenced at lights off (1530) in a room with light intensity of 200 lux.

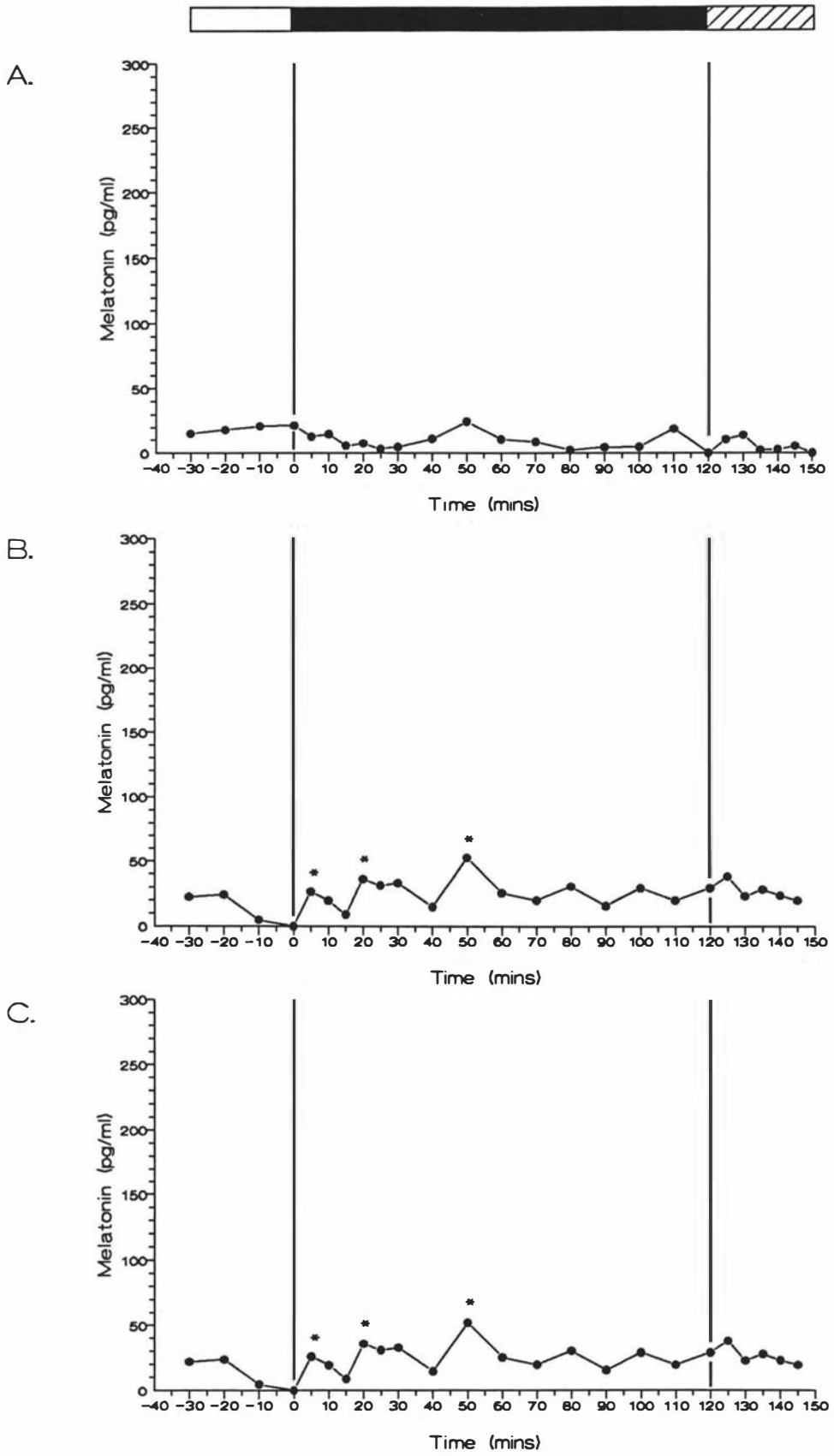


Figure 4.12 Plasma melatonin levels (pg/ml) before, during and after bilateral, electrical stimulation of the CST's of ram #18 in a 8L:16D photoperiod. A. stimulation commenced at lights on (0730). B. stimulation commenced during the mid-photophase (1130) C. stimulation commenced at lights off (1530) in a room with light intensity of 200 lux.

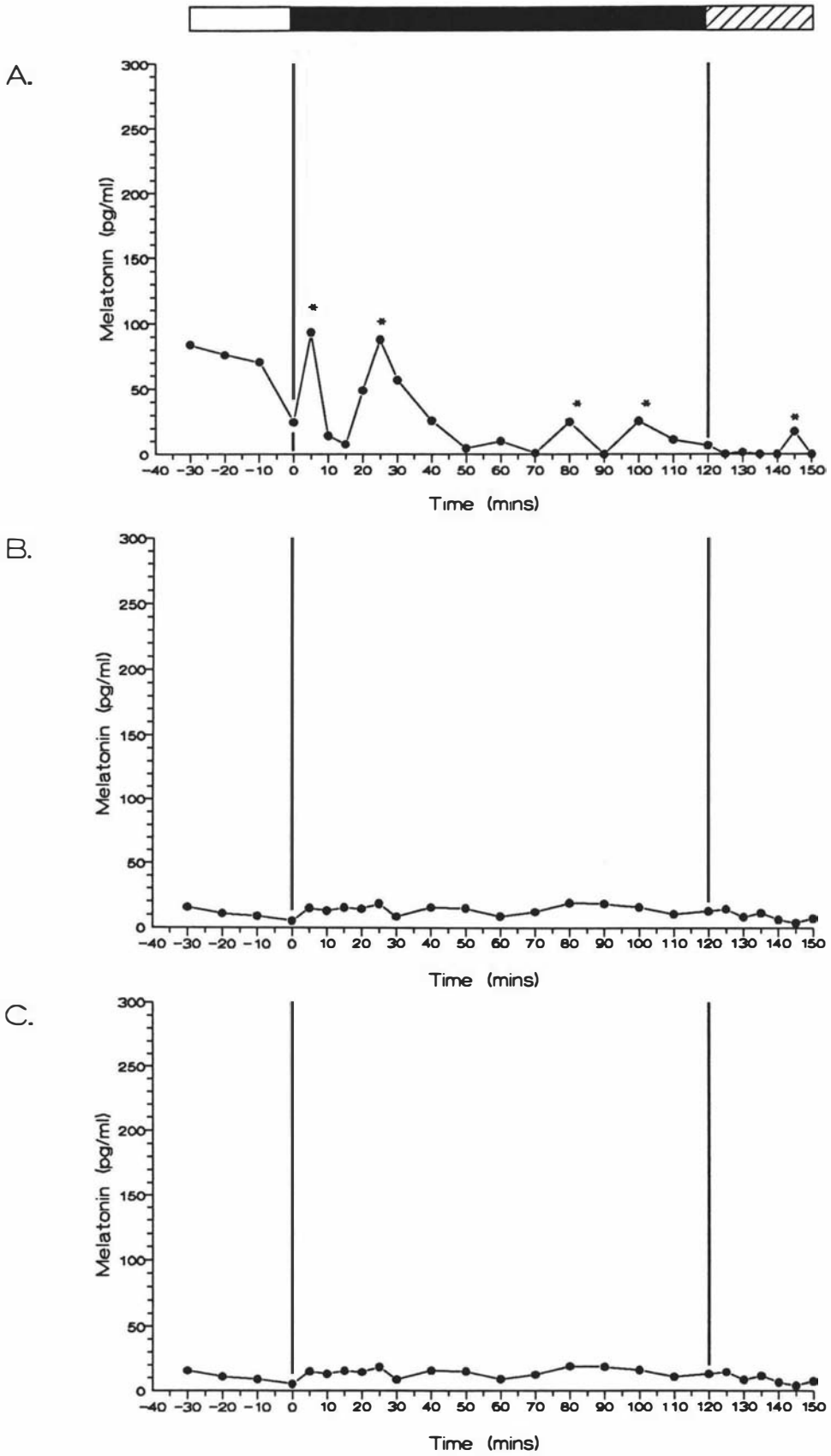


Figure 4.13 Plasma melatonin levels (pg/ml) before, during and after bilateral, electrical stimulation of the CST's of ram #22 in a 8L:16D photoperiod. A. stimulation commenced at lights on (0730). B. stimulation commenced during the mid-photophase (1130) C. stimulation commenced at lights off (1530) in a room with light intensity of 200 lux.

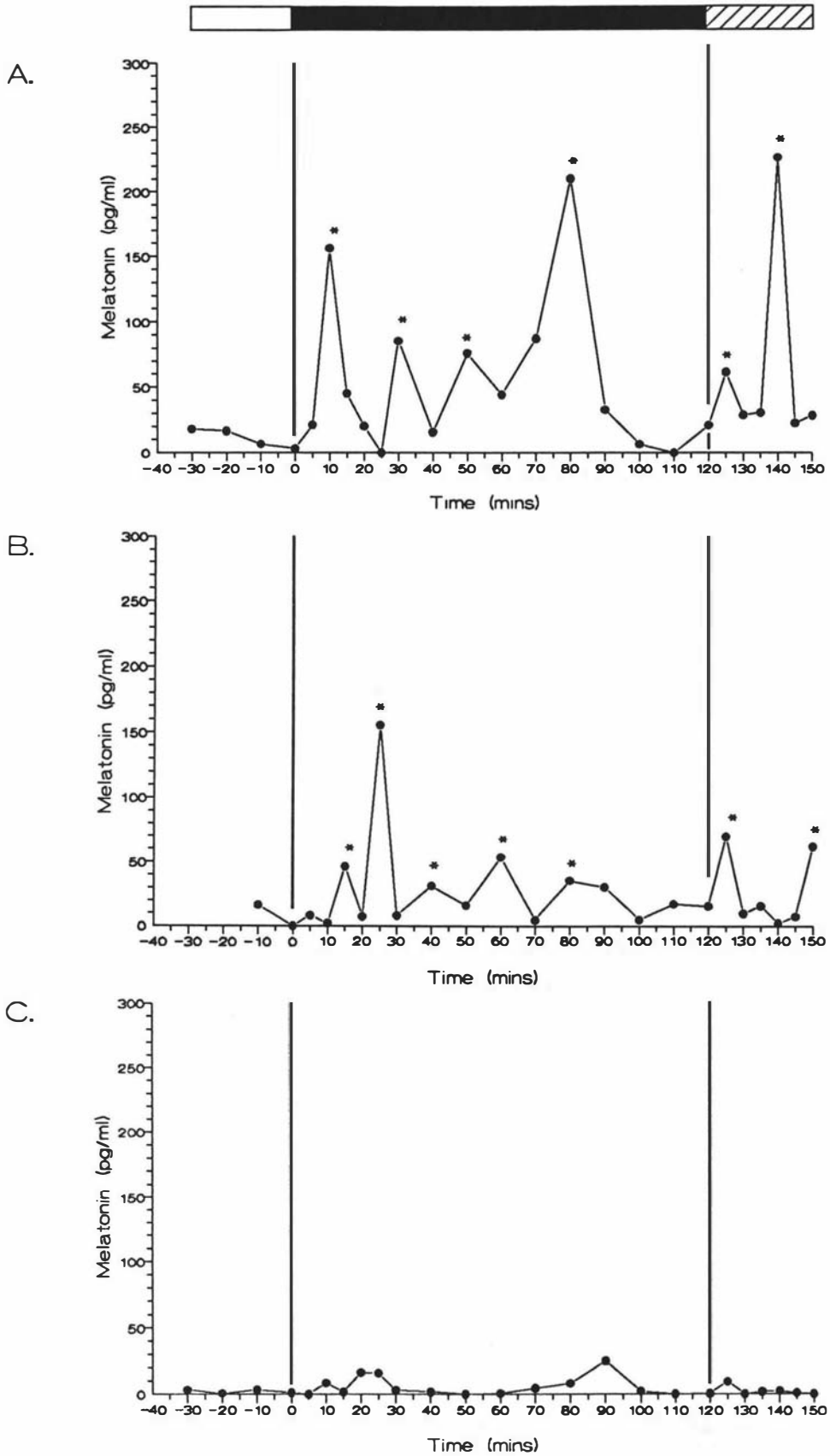


Figure 4.14 Plasma melatonin levels (pg/ml) before, during and after bilateral, electrical stimulation of the CST's of ram #28 in a 8L:16D photoperiod. A. stimulation commenced at lights on (0730). B. stimulation commenced during the mid-photophase (1130) C. stimulation commenced at lights off (1530) in a room with light intensity of 200 lux.

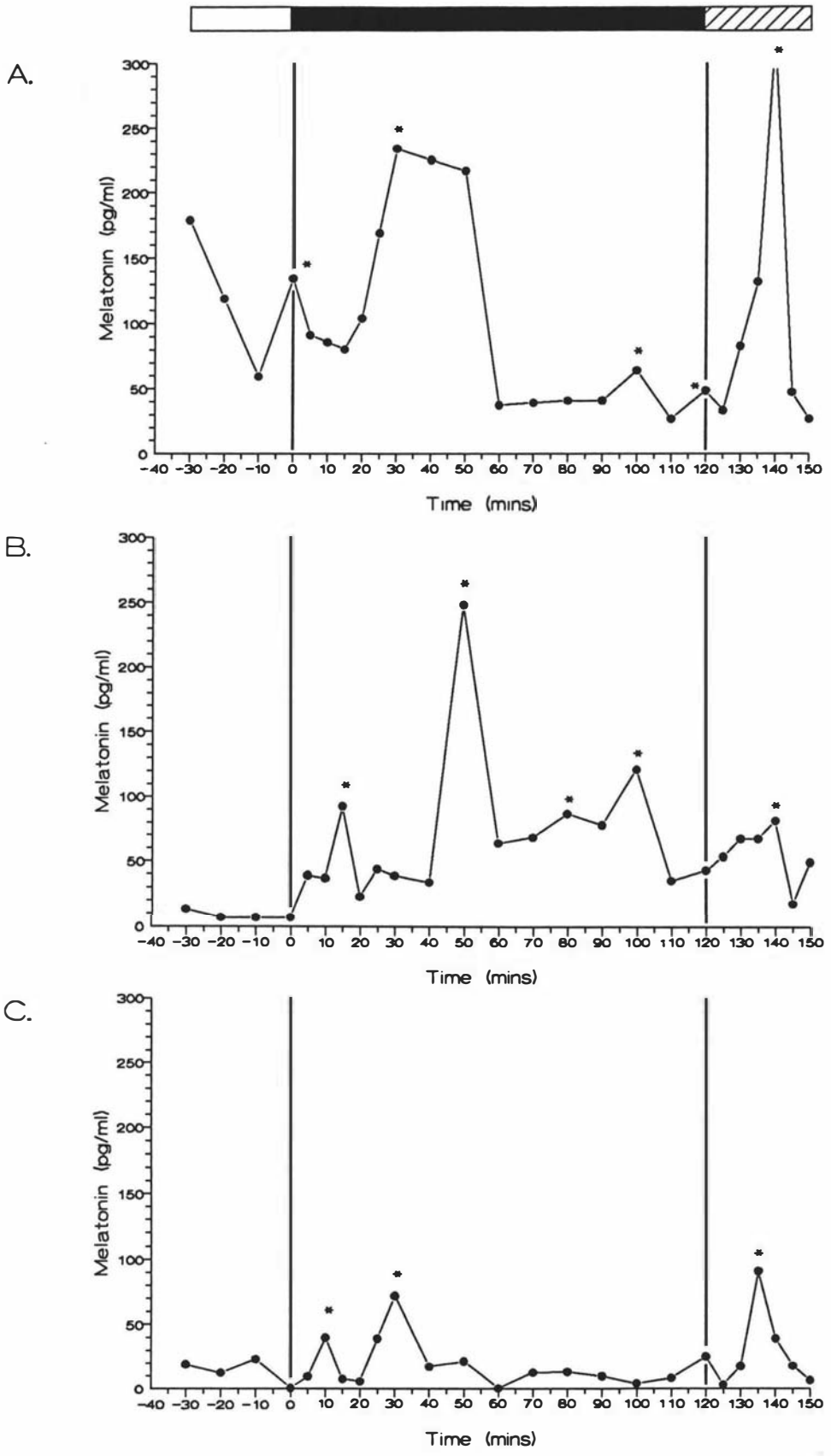


Figure 4.15 Plasma melatonin levels (pg/ml) before, during and after bilateral, electrical stimulation of the CST's of ram #32 in a 8L:16D photoperiod. A. stimulation commenced at lights on (0730). B. stimulation commenced during the mid–photophase (1130) C. stimulation commenced at lights off (1530) in a room with light intensity of 200 lux.

only slightly elevated above pre-stimulation levels, due to low levels occurring at the end of the respective stimulation periods (Figures 4.3 & 4.4). Once adjusted for the regression of post-stimulation responses on pre-stimulation responses (Table 4.9) it was found that there were no significant differences between photoperiods, but that there was a significant quadratic effect for time-of-day, with higher post-stimulation values being recorded following mid-photoperiod stimulation, than after stimulation at the other two times of the photoperiod ($P<0.05$, Table 4.8). The regression of post-stimulation responses on pre-stimulation responses was significant ($P<0.05$, Table 4.8) and resulted in a 29.4% reduction in experimental error.

4.3.2 **Experiment 4.** (The effect of stimulus parameters)

See Tables 4.10-4.13 and Figures 4.16-4.22.

Note: Since very little variation was seen in plasma melatonin levels before, during or after stimulation with any of the parameter sets, only a representative selection of individual melatonin secretory profiles have been included in this chapter. For similar reasons these profiles were not examined for pulsatile melatonin release.

(I) **Pre-stimulation values**

During the period of pre-stimulation sampling mean plasma melatonin levels (Table 4.10) were elevated above the limit of assay sensitivity (5.2 ± 0.7 pg/ml), presumably because melatonin secreted into the blood during the latter part of the dark phase had not yet been cleared entirely by the liver. Analysis of variance (Table 4.11) revealed that mean pre-stimulation levels were similar prior to the application of each set of stimulus parameters. Examination of each set of individual ram melatonin secretory profiles (eg., Figures 4.17, 4.19 & 4.21) revealed that most levels were less than 40 pg/ml at the beginning of the pre-stimulation period and less than 20 pg/ml immediately prior to the start of stimulation.

Table 4.10 **Mean (± S.E.M) pre- and post-stimulation plasma melatonin levels (pg/ml) and mean (± S.E.M) melatonin secretory responses (pg/ml.hr) and levels (pg/ml) to 30 min of CST stimulation in Experiment 4.**

Parameter set #	Stimulus parameters (ms, mA, Hz)	Mean pre-stimulation levels (pg/ml)	Mean melatonin secretory responses (pg/ml.hr)	Mean melatonin levels (pg/ml)	Mean post-stimulation level (pg/ml)
1	**0.5**, 2, 10	14.5 ± 1.9	9.7 ± 2.8	18.0 ± 1.1	10.9 ± 1.8
2	**2**, 2, 10	14.4 ± 5.7	6.5 ± 1.7	13.2 ± 0.9	14.0 ± 4.0
3	**4**, 2, 10	15.5 ± 4.1	10.3 ± 3.6	15.9 ± 1.1	12.1 ± 3.7
4	1, **0.5**, 10	14.0 ± 2.9	6.5 ± 1.4	15.3 ± 0.7	10.1 ± 2.7
5	1, **1**, 10	17.8 ± 3.1	7.6 ± 1.8	18.7 ± 1.9	11.9 ± 2.2
6	1, **4**, 10	15.5 ± 3.2	7.6 ± 1.7	14.9 ± 0.7	11.1 ± 2.1
7	1, 2, **5**	16.8 ± 3.4	8.8 ± 2.6	18.6 ± 1.8	13.8 ± 2.7
8	1, 2, **10**	18.7 ± 3.4	9.2 ± 2.2	20.1 ± 1.3	20.3 ± 4.0
9	1, 2, **20**	14.9 ± 2.4	8.1 ± 2.7	13.4 ± 0.8	14.6 ± 3.7

Table 4.11 Summary of analysis of variance of pre-stimulation data from Experiment 4.

Source of variation	D.F	Variance Ratios
Between Parameter sets	8	0.22
Within Parameter sets Error mean square	45	73.2260

(II) **Stimulated responses**

In general, electrically stimulated melatonin secretory responses in Experiment 4 were few and of very limited magnitude (Table 4.10 & Figures 4.16, 4.18 & 4.20). Following adjustment for the regression of stimulated responses on pre-stimulation melatonin levels (Table 4.12), it was revealed that there was no significant effect on mean melatonin output resulting from varying stimulation frequencies (5-20 Hz), pulse durations (0.5-4 ms) or current strengths (0.5-4 mA). Examples of the effects of varying stimulus duration, strength and frequency, on melatonin secretory profiles, are illustrated in Figures 4.17, 4.19 & 4.21, respectively. Comparison of the mean effects of stimulus frequency, amplitude and duration (contrasts (iv) and (v) in Table 4.4), demonstrated that there were no significant differences in the effectiveness of these parameters in increasing melatonin output (Table 4.13). Results of all non-orthogonal contrasts listed in Table 4.4 were also non-significant. The highly significant regression revealed a strong relationship between melatonin output in the pre-stimulation and stimulation periods and resulted in a 48% reduction in experimental error.

Table 4.12 Mean plasma melatonin secretory responses (pg/ml.hr) to bilateral CST stimulation and mean post-stimulation responses (pg/ml) adjusted for the regression of stimulated responses (pg/ml.hr) on pre-stimulation responses.

Para-meter set #	Stimulus parameters (ms, mA, Hz)	Mean melatonin response Stimulation (pg/ml.hr)	Adjusted stimulation	Post-stimulation (pg/ml)	Adjusted Post-stimulation
1	**0.5**, 2, 10	9.7	10.3	10.9	11.7
2	**2**, 2, 10	6.5	7.2	14.0	14.9
3	**4**, 2, 10	10.3	10.5	12.1	12.3
4	1, **0.5**, 10	6.5	7.4	10.1	11.1
5	1, **1**, 10	7.6	6.7	11.9	10.7
6	1, **4**, 10	7.6	7.8	11.1	11.2
7	1, 2, **5**	8.8	8.4	13.7	13.2
8	1, 2, **10**	9.2	7.8	20.3	18.5
9	1, 2, **20**	8.1	8.5	14.6	15.1

Table 4.13 **Summary of analyses of residual variance of stimulation (pg/ml.hr) and post-stimulation (pg/ml) data from Experiment 4. Prior to analysis, mean melatonin responses were adjusted for the regression of stimulation and post-stimulation responses on pre-stimulation levels (pg/ml) in an analysis of covariance.**

Source of variation	D.F	Variance Ratios	
		Stimulation	Post-stimulation
Between Parameter sets	8	<0.1	1.19
Within Parameter sets	45		
Regression	1	43.13***	34.25***
Error mean square	44	17.4682	32.9573

(III) **Post-stimulation values**

During the 20 min of post-stimulation sampling mean plasma melatonin levels were similar in all groups (Table 4.10) and exhibited little change, although in most groups there was a tendency for levels to decline slightly (Figures 4.16, 4.18 & 4.20). After the post-stimulation responses were adjusted for the regression on pre-stimulation levels (Table 4.12), it was found that there were no significant differences between treatment groups during this period (Table 4.13). The highly significant regression ($P<0.001$, Table 4.13) resulted in 42.5% reduction in experimental error.

(IV) **CST Morphology**

Histological examination of CST's obtained from rams in Experiment 4 revealed that substantial degenerative changes had occurred. Compared to CST's obtained from rams without electrode implants (Figure 4.22A), the CST's of experimental rams exhibited a cellular proliferation of neurolemmal cells, macrophages and fibrocytes (Guth, 1956; Fawcett & Keynes, 1990) in nerve segments both between the two electrode wires (Figure 4.22B) and rostral to the cathode electrode wire (Figure 4.22C). Furthermore, a marked increase in endoneurial connective tissue and a resultant reduction in the diameter of the axonal space was observed in these regions, particularly in the more rostral segments (Figure 4.22C). The feature most indicative of axonal degeneration, however, was the loss of the myelin sheath surrounding individual axons. This loss was most apparent in Figure 4.22C in which myelin is almost completely absent, but was less obvious between the anode and cathode (Figure 4.22B). All four features of axonal degeneration described here occurred in all CST's examined with only small variations in the extent of these changes.

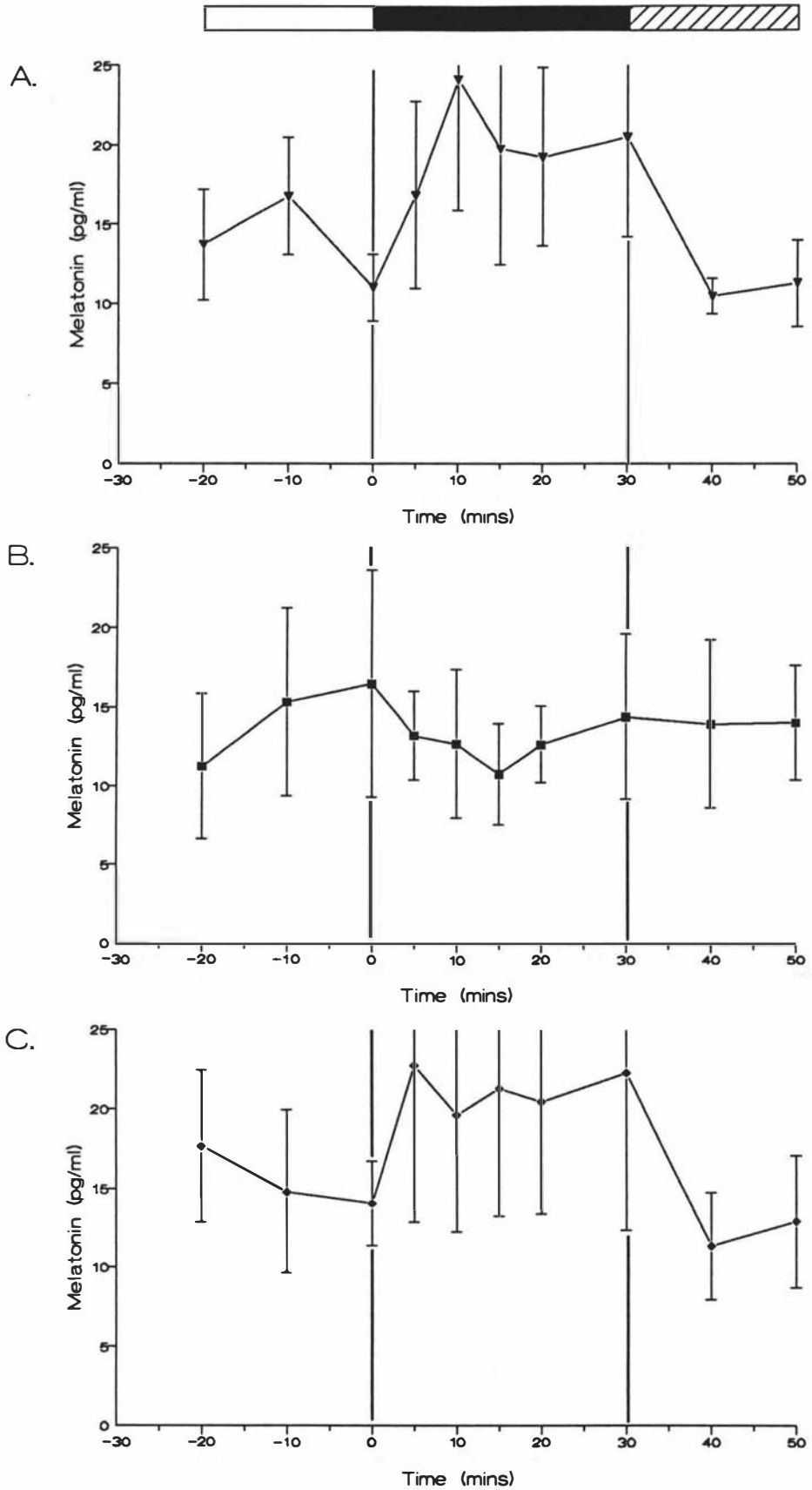


Figure 4.16 Effect of 30 min continuous electrical stimulation of CST's of conscious rams (Expt 4), with stimuli of varying pulse duration (A, 0.5 ms; B, 2.0 ms; C, 4.0 ms) on mean (+/- S.E.M) plasma melatonin levels. Stimulation started 30 min after the onset of light in a 16L:8D photoperiod. Stimulus strength (2 mA) and frequency (10 Hz) were held constant.

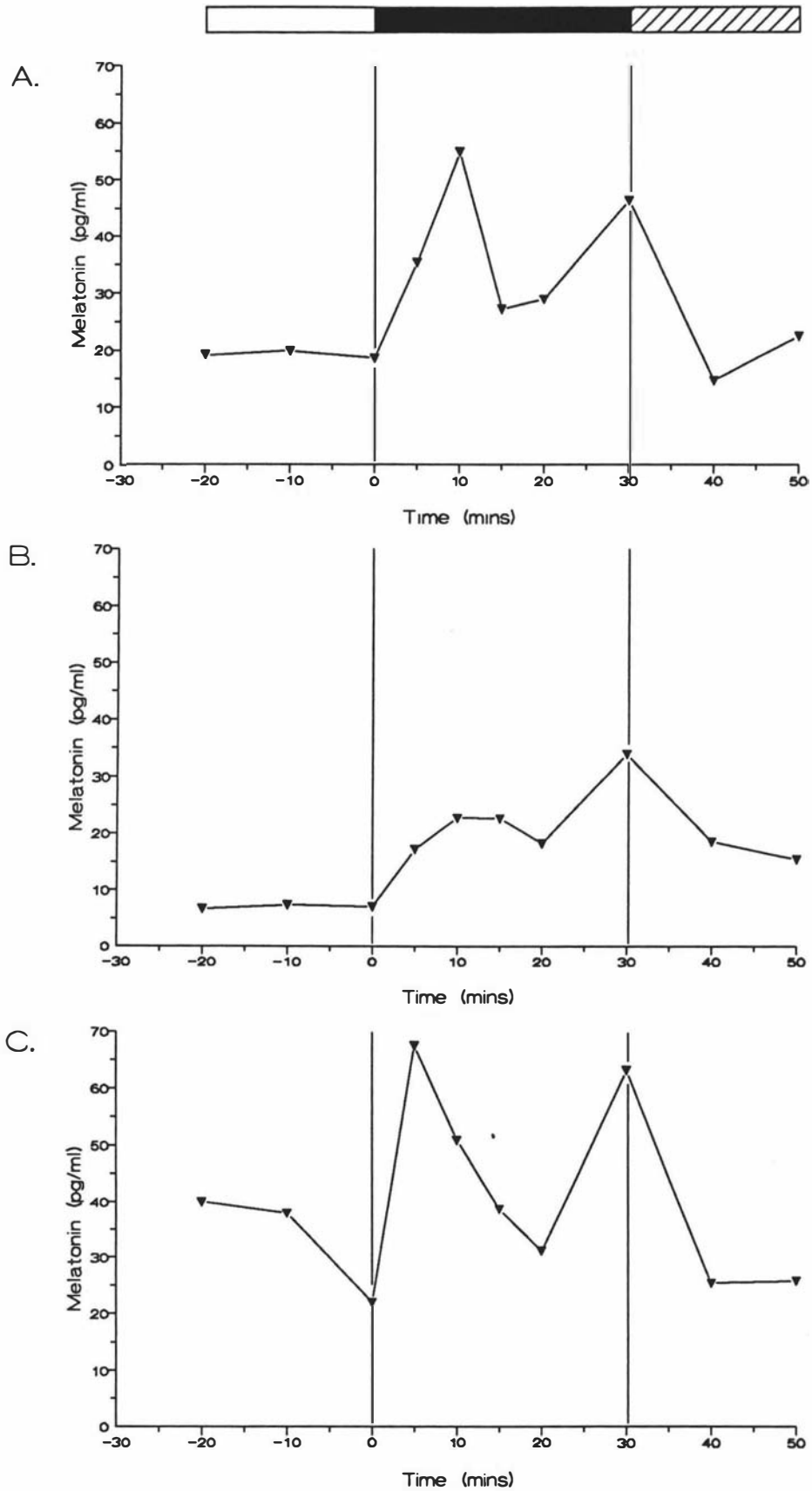


Figure 4.17 Effect of 30 min continuous electrical stimulation of CST's of sheep D (Expt 4), with stimuli of varying pulse duration (A, 0.5 ms; B, 2.0 ms; C, 4.0 ms), on plasma melatonin levels. Stimulation started 30 min after the onset of light in a 16L:8D photoperiod. Stimulus strength (2 mA) and frequency (10 Hz) were held constant.

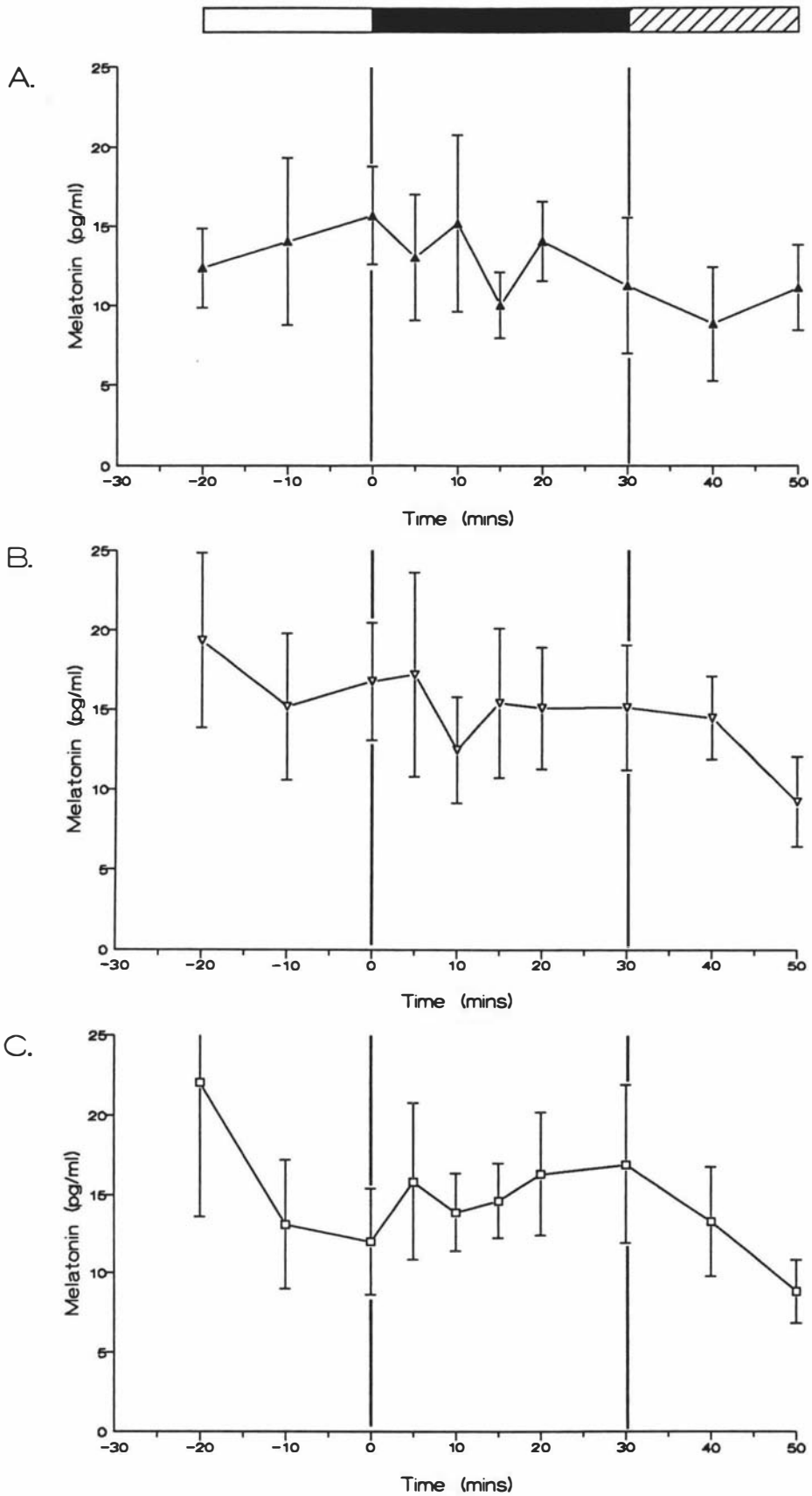


Figure 4.18 Effect of 30 min continuous electrical stimulation of CST's of conscious rams (Expt 4), with stimuli of varying current strength (A, 0.5 mA; B, 1.0 mA; C, 4.0 mA) on mean (+/− S.E.M) plasma melatonin levels. Stimulation started 30 min after the onset of light in a 16L:8D photoperiod. Pulse duration (1 ms) and frequency (10 Hz) were held constant.

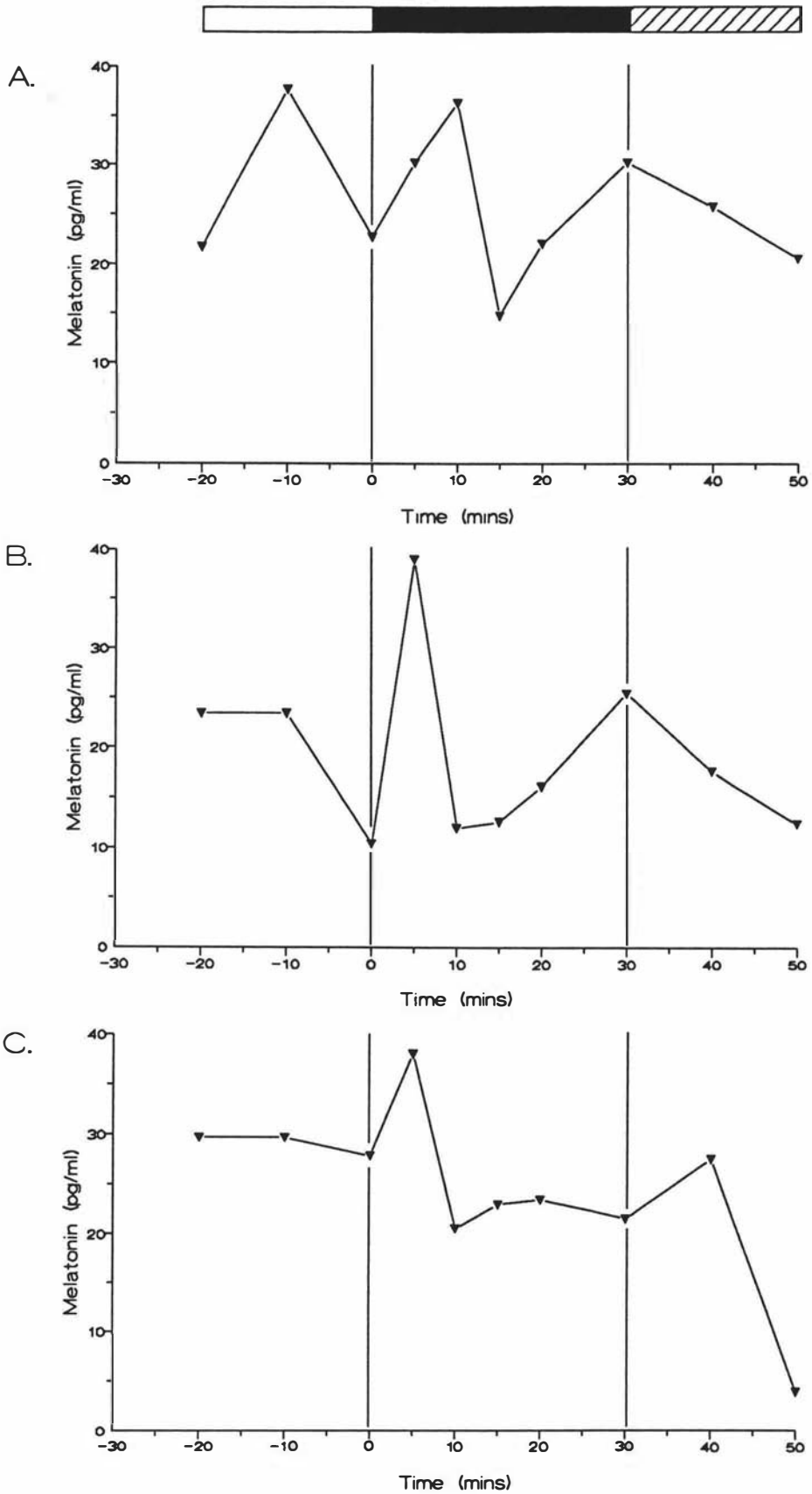


Figure 4.19 Effect of 30 min of continuous electrical stimulation of CST's of sheep F (Expt 4), with stimuli of varying current strength (A, 0.5 mA; B, 1.0 mA; C, 4.0 mA), on plasma melatonin levels. Stimulation started 30 min after the onset of light in a 16L:8D photoperiod. Pulse duration (1 ms) and frequency (10 Hz) were held constant.

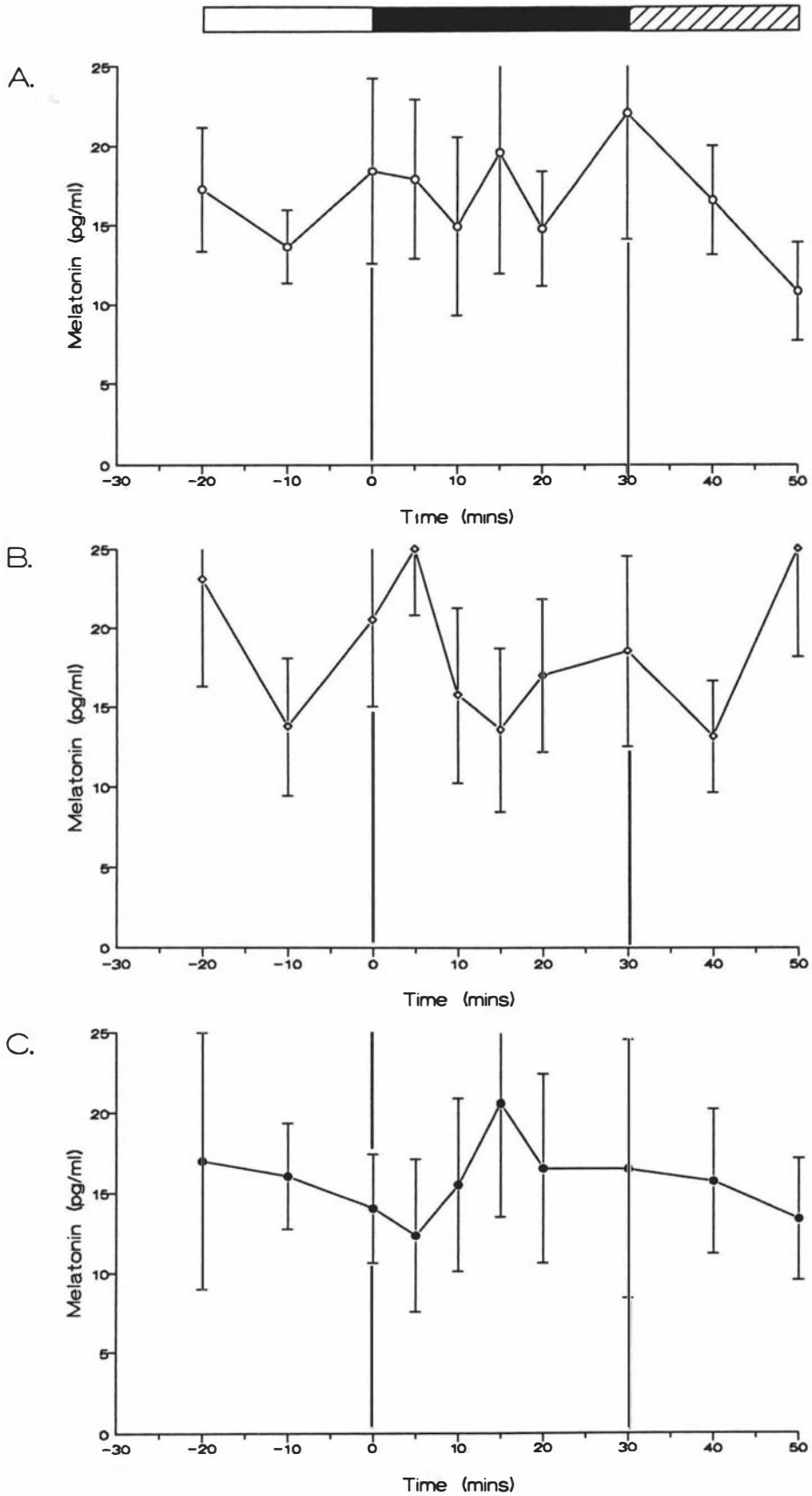


Figure 4.20 Effect of 30 min of continuous electrical stimulation of CST's of conscious rams (Expt 4), with stimuli of varying pulse frequency (A, 5 Hz; B, 10 Hz; C, 20 Hz), on mean (+/− S.E.M) plasma melatonin levels. Stimulation started 30 min after the onset of light in a 16L:8D photoperiod. Pulse duration (1 ms) and current strength (2 mA) were held constant.

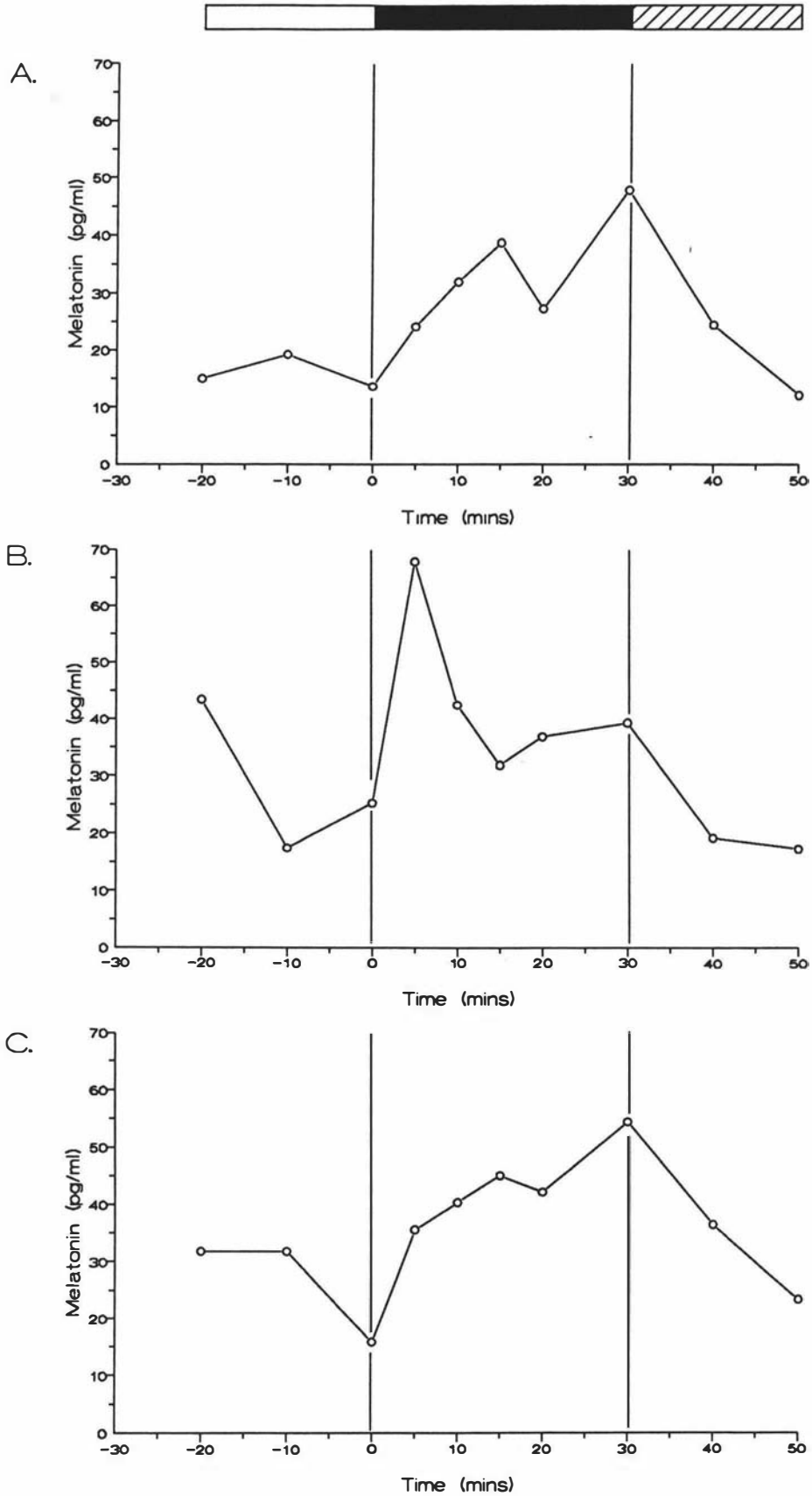
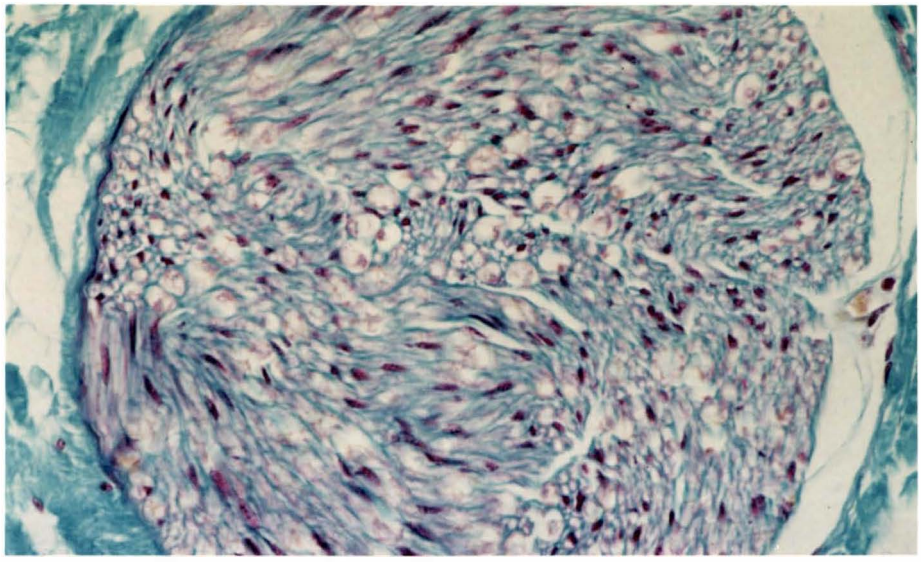
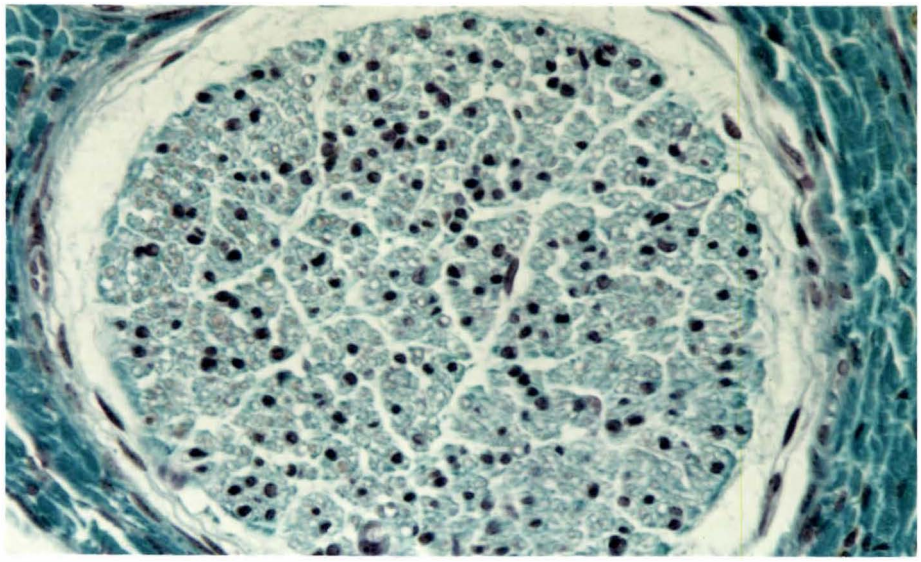
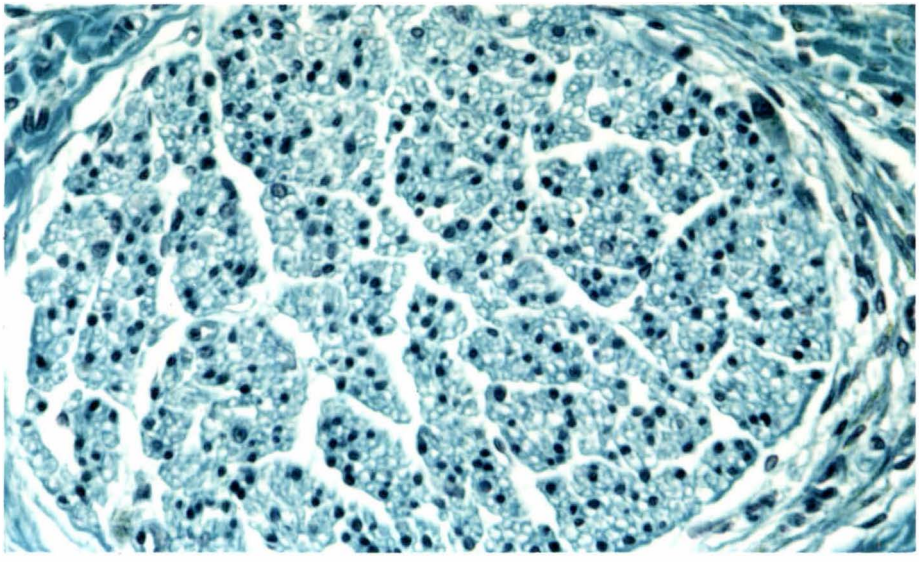


Figure 4.21 Effect of 30 min of continuous electrical stimulation of CST's of sheep D (Expt 4), with stimuli of varying pulse frequency (A, 5 Hz; B, 10 Hz; C, 20 Hz), on plasma melatonin levels. Stimulation started 30 min after the onset of light in a 16L:8D photoperiod. Pulse duration (1 ms) and current strength (2 mA) were held constant.

Figure 4.22 Transversely sectioned CST's stained with Masson's Green Trichrome to show cell nuclei (blue/black), collagen (green) and myelin (red). A. CST from a ram which did not receive electrode implants. Note that myelin is present throughout the nerve and that cell nuclei are relatively sparsely distributed. B. CST taken from between the electrodes of ram B in Experiment 4 showing intermediate nerve degeneration. Compared to A above there is an appreciable reduction in the presence of myelin while the number of cell nuclei and the amount of collagen has increased. C. CST from ram B distal to the stimulating electrodes. Nerve degeneration is extensive with almost no myelin present and an increase in the epineurial and endoneurial connective tissue. Magnification 560X.







Gross examination of the electrode/nerve complexes indicated that all electrodes were correctly positioned around the CST's and had not been dislodged during the experiment. Fibrotic sheaths which were found to encapsulate the electrodes and nerves appeared to contribute towards maintaining the electrodes in place.

4.4 Discussion.

4.4.1 Photoperiod and time-of-day

The experiments described in this chapter were designed to further investigate the influence of the sympathetic nervous system on pineal melatonin secretion in conscious rams. Such in vivo studies have previously only been attempted by pharmacological manipulation of pineal function through, for example, the administration of adrenoceptor agonists and/or antagonists (Sugden et al, 1985a) or steroid implants (Maxwell et al 1989a, b), and by sympathetic denervation by transection of the CST's or ICN's, or by removal of the SCG's (Reiter, 1979; Lincoln & Almeida, 1982; Maxwell et al 1989a, b; Kuchel et al, 1990). These earlier studies have provided direct evidence that the sympathetic innervation of the pineal regulates the circadian rhythm of melatonin synthesis and secretion, but have not revealed the relationship between neural activity in the sympathetic innervation, or the timing of such activity, and the subsequent pattern of melatonin secretion.

One of the major aims of the experiments described in this chapter was to evoke melatonin secretion in conscious rams by electrical stimulation of the pre-ganglionic sympathetic innervation of the pineal to achieve plasma levels similar to those reported to occur in intact sheep at night (58-255 pg/ml; Shaw et al, 1989). In Experiment 2, in which anaesthetized rams were acutely stimulated, mean nighttime plasma melatonin levels were at the lower end of that range (between 60-70 pg/ml) possibly because of anaesthetic modification of neural transmission and/or pineal sensitivity to CST stimulation (see Section 3.4.1). It was envisaged that without such inhibitory effects plasma melatonin levels during CST stimulation would be similar to those reported by Shaw & colleagues. Hence, it was decided to conduct further experiments in conscious rams in an attempt to produce melatonin secretory responses of magnitudes near to those recorded on exposure of sheep to darkness.

Investigations of this nature were made possible by the development of stimulating electrodes (similar to those used by Loke et al, (1986) to stimulate fetal, newborn and adult sheep sciatic nerves), which could be chronically implanted around the CST's of each ram and

remain functional for the duration of the experiment. This represented a significant refinement of the approach used in Experiment 2 and also enabled each ram to be utilized in successive stimulation treatments in each experiment. This aimed to remove some variation in experimental results due to between-animal differences and hence also reduce the experimental error. Although the long-term use of chronically implanted electrodes presented a number of potential technical difficulties, such as mechanical failure and electrolytically induced degenerative changes in the nerve (Delgado, 1964), preliminary experiments to establish stimulus parameters for Experiment 3 indicated that these problems were apparently of little importance and that stimulation during the three weeks the electrodes were implanted induced an immediate and sustained increase in pineal melatonin secretion (Pilot studies 1 & 2). Furthermore, these pilot studies established that the pineal was sensitive to changes in pulse duration, at least in the range of 0.25 to 1 ms. These findings provided sufficient confidence in this experimental approach to warrant further investigations through Experiments 3 and 4, in which the aims were to maximize the melatonin secretory responses.

Experiment 3 was designed to examine the influence of photoperiod and time-of-day on melatonin secretory responses to CST stimulation via chronically implanted stimulating electrodes. In contrast to expectations, however, melatonin secretory responses in this experiment were, at best, only similar to those induced in Experiment 2 and were less than reported nocturnal levels (Maxwell et al, 1989b; Shaw et al, 1989).

Several possibilities may provide an explanation for these limited responses. Firstly, the reduction in pulse amplitude from 5 mA in Experiment 2 to 1 mA in the present study may not have provided threshold stimulation of all nerve fibres in the CST's and consequently diminished the excitatory influence of the sympathetic nervous system on pineal melatonin secretion. This reduction, however, was necessary to ensure that no stress was experienced by the rams during periods of stimulation. Secondly, the delivery of stimuli continuously, as in the present experiments, or in intermittent bursts may affect the effectiveness of the stimuli in evoking a secretory response. Intermittent pulses have been employed in many studies of endocrine organs, including pineal (Bowers & Zigmond, 1982), adrenal (Edwards, 1982; Bloom et al, 1988), neurohypophysis (Dutton & Dyball, 1979; Bicknell et al, 1982) and pancreas (Bloom et al, 1984; Bloom & Edwards, 1985) and in comparison to stimuli delivered continuously have elicited greater secretory responses. The two points discussed here are, of course, interrelated. The magnitude of the various stimulus parameters used to elicit a

maximum response depends on the pattern in which they are delivered in addition to other considerations such as nerve size (Dutton & Dyball, 1979).

A third possibility is that inhibitory interneurons in the SCG blocked neural transmission through those ganglia (Greengard & Kebabian, 1974; Williams et al, 1975) and hence reduced the flow of action potentials to the pineal. This, however, appears to be unlikely since melatonin secretion resulted from CST stimulation in Experiment 2 and palpebral margin widening could be evoked at the end of this experiment. That test, however, does not preclude the possibility that the presence of inhibitory central influences, or the absence of excitatory central influences, affected the synthesis and secretion of pineal melatonin during CST stimulation. Lesion studies in the rat, for example, have shown that the CNS is likely to exert a direct influence on the pineal's secretory activity (Møller et al, 1987b). Similar studies in sheep are needed to determine if the CNS in this species is able to influence pineal melatonin secretion.

Finally, the possibility that the electrode/nerve complexes did not remain intact for the duration of the experiment, and hence failed to stimulate the nerves on all occasions, must also be considered. This, however, appears unlikely since it was confirmed that the electrodes were still functioning at the end of this experiment by observing the palpebral response during CST stimulation in each anaesthetized ram. All, but one responded with a widening of the palpebral margin. While this is not conclusive evidence that sympathetic fibres innervating the pineal were being stimulated, it does demonstrate that the electrodes were producing a cranial flow of action potentials in the CST's of most rams up until the end of the experiment. It is unlikely that post-ganglionic fibres innervating the pineal would not be activated if those to the eyelids were activated.

Despite limited responses recorded in Experiment 3, two findings from that study were of particular interest. Firstly, it was found that the duration of the photoperiod did not influence melatonin output in response to CST stimulation, with similar patterns and levels of secretion being observed in both long (16L:8D) and short (8L:16D) photoperiods. This observation is in agreement with previous studies which have shown that in sheep held under artificial (Kennaway et al, 1983; Malpaux et al, 1988) or natural (Maxwell et al, 1989b) photoperiods the onset of nocturnal melatonin secretion and the plasma melatonin levels attained did not vary significantly with changes in the lighting regime or seasonal changes in daylength. Thus, it is likely that the sensitivity of the pineal to stimulation via its sympathetic

innervation, either by electrical stimulation of the CST's or nocturnally induced activation, does not alter with respect to daylength.

The second finding, that of greatest pineal sensitivity to CST stimulation during the middle of the photophase (quadratic component of time-of-day effect, Table 4.8), is more difficult to account for. As discussed more fully in Chapter 3, evidence to date indicates that rat pineal sensitivity to sympathetic stimulation (electrical or pharmacological) increases during the latter part of the day and peaks at night before declining to minimum levels (Bowers & Zigmond, 1982; Gonzalez-Brito, 1988b). Although similar studies have not been conducted in sheep, the results of Experiment 2 indicated that the ovine pineal follows a similar pattern. In the present study stimulation at the beginning of the photophase may have coincided with a period of decreased sensitivity to stimulation thus contributing to the decline in plasma melatonin levels. A second factor which may also have contributed to this response is a reduced level of pineal serotonin at this time (Namboodiri et al, 1985a), which has been suggested to limit the production of melatonin in sheep pineals (Sugden et al, 1985b). With continued exposure to light during the photophase, pineal serotonin (Namboodiri et al, 1985a) and possibly adrenoceptor sensitivity (Gonzalez-Brito, 1988b) increase and hence this may provide an explanation for the increased mean plasma melatonin levels evoked during mid-photophase stimulation.

The low nighttime response to CST stimulation, however, was not expected in view of results from Experiment 2 and given that pineal NAT content and plasma melatonin levels in sheep exhibit a rapid increase at the onset of darkness (Namboodiri et al, 1985a, b). In Experiment 2 it was found that similar stimulation of the CST's of anaesthetized rams during the mid-photophase evoked less melatonin secretion than when stimulation was conducted after approximately 5 hours of darkness. In consideration of this finding it appeared likely that pineal sensitivity was lower during the day than at night and that CST stimulation in conscious rams at the beginning of the dark phase would evoke greater output of melatonin than during daytime stimulation. That the pattern of response observed in Experiment 3 was a physiological one and not an artifact of the experiment is suggested by the fact that the mean pattern of response during each of the three stimulation periods was consistent in both photoperiods. Characteristic of stimulation during the early light phase of both photoperiods was a gradual decline in the mean plasma melatonin content, while mid- and end-of-photoperiod stimulation elicited increases of similar magnitude for each photoperiod.

While it is difficult to explain this finding in view of our current understanding of ovine pineal regulatory mechanisms, it is worthwhile considering where shortfalls in our knowledge exist. For example, very little is known about the influence of the central nervous system on pineal function in any species. It is now well established that there are neural connections between various brain regions and the pineal (Korf & Møller, 1984). Further studies may reveal, for example, that in the absence of darkness it is necessary to stimulate both CST's as well as particular brain regions in order to achieve full melatonin synthesis and secretion. Furthermore, little is understood about the circadian rhythms of ovine pineal sensitivity and responsiveness to nocturnal or electrical stimulation. Pharmacological (Gonzalez-Brito et al, 1988b) and electrophysiological (Stehle, et al, 1989) studies in rodents have demonstrated that various parameters of pinealocyte activity did not increase with the dark phase as might be expected, suggesting that we are far from understanding all the factors that influence pineal function. Finally, it is possible, even likely, that the stimulus parameters used in this experiment were not optimal for pineal stimulation and resulted in low levels of melatonin secretion. Studies aimed at characterizing the optimal stimulus parameters will enable a stimulation protocol to be developed which will minimize electrode polarization, electrode deterioration due to current flow, and histological degenerative changes in the nerve and at the same time provide stimuli which initiate full pineal melatonin production. Once this goal is achieved, it will then be possible to initiate studies investigating the interaction between the sympathetic innervation and other influences on pineal secretory function. One example is the influence of the central nervous system which might be investigated in conscious sheep by concurrent CST stimulation and stimulation of specific brain sites such as the SCN, PVN or habenular nuclei, to examine the possibility that they have the potential to modify pineal melatonin production (Møller et al, 1987b).

4.4.2 **Stimulus parameters**

As mentioned earlier in this discussion, preliminary experiments established that the pineal was sensitive at least to changes in pulse duration in the range 0.25 to 1 ms. The finding of Experiment 4 that variation of the stimulus parameters (pulse width, amplitude and frequency) had no significant effects on plasma melatonin levels, was therefore contrary to expectations. When the individual melatonin secretory responses of each ram were examined, only 2 of the 6 rams responded to electrical stimulation with an increase in plasma melatonin levels. In those circumstances no significant results from varying individual stimulus

parameters could be expected. Furthermore, when melatonin profiles from individual rams, which did respond to stimulation, were studied no consistent pattern of results was revealed (eg., ram F exhibited no variation in melatonin output to changes in pulse amplitude) although plasma melatonin levels were elevated during periods of stimulation. Results from this experiment were therefore inconsistent with those of encouraging preliminary experiments, and no significant conclusions could be drawn from it.

One possible explanation for this lack of response is that the time-of-day chosen for the stimulation period was not the most appropriate. Selection of the early light phase for the stimulation period was made after preliminary examination of results from Experiment 3, although it later became clear that a mid-light phase stimulation period would have been more appropriate. In spite of this it is unlikely that the almost total lack of pineal response could be attributed to this choice. During early light phase stimulation in Experiment 3 plasma melatonin levels exhibited only a slight drop compared to pre-stimulation nocturnal levels thus indicating that elevated levels of melatonin secretion could be achieved at this time and that precursor depletion (Sugden et al, 1985b) probably was not a factor limiting the secretory response.

As discussed below, probably the major reason for the common lack of secretory responses in Experiment 4 was neural damage resulting either from implantation of the stimulating electrodes or from the application of stimuli. This neural damage and/or failure of the electrodes was suspected before the neural histological results became available and led to a time-series evaluation of results from individual animals to examine for evidence of progressive damage to the CST's or electrodes. However, no clear pattern of response was present which could be related to the chronological order of stimulation. This suggests that any damage suffered by the CST's occurred in the early stages of the experiment, either at the time of electrode implantation or within the first week after surgery.

The histological assessment of the CST's from rams in Experiment 4 demonstrated that at the end of the experiment degenerative changes had occurred both between and rostral to the stimulating electrodes. The most significant changes were the loss of myelin normally surrounding each pre-ganglionic axon and the apparent degeneration of many axons. This most likely led to impaired CST function in some rams and perhaps total loss of function in others, thus limiting the capacity of the CST's to transmit action potentials cranially and consequently reducing the stimulation of pineal melatonin secretion. What is not clear,

however, is why the CST's of most rams in Experiment 3 were still functioning at the end of that experiment, when those of rams in Experiment 4 were in various stages of degeneration by the end of all stimulation treatments. Given the longer duration of Experiment 3 and that the CST's were still functioning after six weeks it was considered probable that after the three weeks needed to complete Experiment 4, the CST's would still be functional and that the palpebral widening tests were not necessary. Therefore, only a histological assessment of the electrode\nerve complex was performed. In future experiments of this nature, any deterioration of the CST's induced by implanted electrodes could be examined by measuring plasma melatonin levels in experimental animals following exposure to darkness. Those that do not respond with an increase in plasma melatonin levels are likely to have suffered CST damage and should not be included in the analysis of experimental results.

Previous reports have shown that chronically implanted electrodes of this nature do not normally induce such effects. According to Delgado (1964) and others (Babb & Kupfer, 1984; Bowman & Erickson, 1985) stainless steel electrodes, as used in Experiment 4, are well suited for nerve stimulation because they are biologically well tolerated and may remain in tissue for long periods of time without modification to their electrical properties. Despite the problems of electrode polarization (Delgado, 1964) chronically implanted stainless steel electrodes have been employed successfully to repeatedly stimulate the brain (Delgado, 1959), heart (Delgado, 1952) and peripheral nerves (Loke et al, 1986), for periods ranging from a few days to several months.

Histologically, neural tissue has been shown to exhibit little evidence of degeneration following long term electrode (most commonly stainless steel or platinum) implantation. In peripheral nerves, for example, chronically implanted stainless steel intraneural electrodes (Bowman & Erickson, 1985) and platinum cuff electrodes (Jonzon et al, 1988) were shown to cause a minimum of damage to nerve fibres while inducing some connective tissue proliferation. Furthermore, threshold current and conduction velocity did not change significantly in nerves receiving long-term stainless steel electrode implants, suggesting that no loss of function had occurred (Bowman & Erickson, 1985). Similar histological studies of rat brains have shown that stainless steel electrodes are also well tolerated, causing few degenerative changes after 63 days of implantation (Babb & Kupfer, 1984).

It thus appears that the nerve fibre degeneration observed in Experiment 4 was not caused by a local reaction to the electrode material or to the effects of surgery to implant the

electrodes, but resulted from as yet unidentified influences. The difficulty in identifying these influences is further compounded when the effects of nerve stimulation in Experiments 3 and 4 are compared. The only significant differences between these experiments, apart from the stimulation parameters, was the total period of stimulation, being 12 hours per ram in Experiment 3 and 4.5 hours per ram in Experiment 4, and the duration of each experiment as discussed earlier. If factors such as electrode deterioration by current flow (Delgado, 1964) were significant then it would be expected that the cumulative effect would have been greater in Experiment 3 and that lesser melatonin secretory responses would be recorded in that Experiment rather than Experiment 4.

The cause(s) of the apparent failure to stimulate pineal melatonin secretion and the histological changes observed in the CST's cannot be identified from the current study, but require further investigations using electrophysiological and histological methods. For example, to assess whether or not the nerve damage occurred simply as a result of contact between the electrode and the nerve, electrodes could be implanted in two groups of rams, only one of which would receive electrical stimulation. After a period of 3-6 weeks histological examination of the CST's of all rams may reveal whether electrical stimulation or simply the electrodes themselves induced the degenerative neural changes.

One final consideration for future experiments is whether or not the melatonin secretory profile is a suitable parameter for assessment of the effects of CST stimulation on pineal secretory activity. While this parameter provides a direct measure of pineal melatonin secretion, the large between-animal variations in the melatonin responses may have contributed to the difficulties experienced in detecting significant treatment effects, especially in Experiments 3 & 4. In particular, the experimental design of Experiment 4 relied on the detection of potentially small changes in plasma melatonin levels following a change in stimulus parameters. Consideration should be given, therefore, to the use of other indicators of secretory function, such as NAT, which show less between-animal variability (Namboodiri et al, 1985a, b) and may more accurately reflect treatment effects. However, the significant advantage of having a direct measure of secretory activity, such as plasma melatonin levels, will make it difficult to justify changing to a parameter, which, at best, is only an indicator of secretory potential.

4.5 Conclusions

In conclusion, these studies have demonstrated that it is possible to increase plasma melatonin levels in conscious rams during stimulation of the pineal's pre-ganglionic sympathetic innervation via chronically implanted electrodes. The sensitivity of the pineal to this stimulation was not altered by changes in photoperiod, but did exhibit changes related to the time-of-day. Results from Experiment 4, however, were quite unsatisfactory, with many stimulations resulting in little or no induced melatonin secretion and others revealing no clear pattern of effects of varying stimulus parameters. Undoubtedly nerve damage was the major reason for the lack of secretory responses in that experiment. If the causes of that damage can be revealed and eliminated, this method of pineal study will be able to be utilized to further explore the neural regulation of the pineal gland.

CHAPTER 5

Immunocytochemical evaluation of the peripheral and central innervation of the ovine pineal gland and the effects of unilateral or bilateral superior cervical ganglionectomy.

5.1 Introduction

The major innervation of the mammalian pineal gland is now well established to be sympathetic in nature and to originate in the superior cervical ganglia (see Section 1.6.2). In species such as rat (Marangos et al, 1979a, b; McClure et al, 1986), hamster (Schroder, 1986; Cozzi & Møller, 1988), gerbil (Møller et al, 1979; Shiotani et al, 1986), and dog (Matsuura & Sano, 1983, 1986), immunocytochemical investigations of the pattern of the pineal's innervation and its morphology have demonstrated that components of the peripheral and central innervation can be visualized and that cell types may be distinguished by their staining characteristics. In addition, numerous studies, particularly in rats, have demonstrated that denervation through SCGX causes morphological degradation of some neuronal components within the pineal (Pellegrino de Iraldi et al, 1965; Arstila, 1967) and disruption of pineal circadian rhythms (Bittman, 1984). As far as sheep are concerned, relatively few immunocytochemical studies of the pineal have been reported. These have included the demonstration of NPY (Williams et al, 1989), thyrotropin (Lew, 1989), somatostatin (Lew & Lawson-Willey, 1987), serotonin (Tillet et al, 1986; Tillet, 1987), melatonin (Tillet et al, 1986) & VIP (Cozzi et al, 1990). To date only one study has been reported in which the effects of sympathetic denervation on the ovine pineal gland were assessed using immuncytochemical methods (Ravault et al, 1990).

The present study was designed to investigate the morphological pattern of ovine pineal innervation and also to characterize, and where possible quantify, the effects of unilateral or bilateral SCGX on intrapineal nerve fibres and pinealocyte structure, in order to better define the role of the post-ganglionic sympathetic fibres in maintaining normal pineal function in sheep. This was achieved through the application of specific immunocytochemical staining methods which indicate the presence of neuroendocrine cells, specific neurotransmitters and nerve fibres of the pineal (see Sections 1.5.2 & 1.6.2 for discussion on the function of these pineal constituents). To this end antibodies to NSE, PNMT, NPY and VIP were used to localize each of these antigens in pineal tissue from rams in which both, one or neither SCG had been removed.

Relevant characteristics of each antigen are as follows:

(i) NSE is an isoenzyme of the glycolytic enzyme, enolase and is restricted to and is a marker for neurons and neuron-like endocrine cells (Marangos et al, 1979a, b) and therefore any changes in its distribution within the pineal following SCGX will indicate that the sympathetic nervous system plays a role in maintaining the morphology of this gland.

(ii) PNMT is the enzyme which methylates noradrenalin to form adrenalin (Fuller, 1973) and therefore staining of this antigen will provide an indication of the density of adrenergic nerve fibres, which presumably contain both neurotransmitters. The presence of this enzyme in ovine pineal tissue would suggest that adrenalin is involved in the regulation of melatonin synthesis as it may do in rat pineals (Axelrod et al, 1969).

(iii) NPY is a regulatory neuropeptide found to occur in some nerve fibres in the pineal which originate from the SCG; this has been interpreted as indicating that NPY may have a role in regulation of pineal function (Ebadi et al, 1989). In other tissues NPY has been shown to coexist with (Hakanson et al, 1986) and be released simultaneously with noradrenalin (Pernow, 1988).

(iv) VIP is a neuropeptide found in the pineals of all species studied to date (Møller et al, 1987a), including that of sheep (Cozzi et al, 1990), in which it has been shown to increase cAMP concentrations (Morgan et al, 1988a). A stimulatory effect on pineal secretory activity appears likely.

5.2 Materials and Methods.

5.2.1 Experimental procedure

Experiment 5 was conducted during late winter (August) of 1987. Sixteen adult Romney rams were randomly divided into four groups of four, each group then being allocated to one of the following 4 treatments: (1) excision of the left SCG, (2) excision of the right SCG, (3) excision of both SCG's or (4) unoperated controls. See Section 2.2.4 for details of the surgical procedure employed to perform these ganglionectomies.

Surgical operations were performed on 4 days over a 3 week period and included one animal from each surgical treatment group on each day. Following recovery from surgery and return to pasture, a survival period of 14 days elapsed before each ram was returned to the laboratory where it was held in a light-proof room prior to sacrificing in the dark by the

method and at the times detailed in Section 2.8.1. One control ram was included in each group of rams sacrificed on each of the 4 nights required to complete this phase of the experiment. In this way each ram was killed in the dark between 5 and 6 hrs after lights off, at a time when pineal neural and secretory activity is maximal (Taylor & Wilson, 1970; Shaw et al, 1989).

Procedures for pineal tissue collection and immunocytochemical processing are detailed in Section 2.8.

All photomicrographs, except Figure 5.1, were taken with Kodak Ektachrome Professional Film through a Zeiss Axiophot photomicroscope and using differential interference contrast optics to enhance the visual perception of immunoreactivity. Figure 5.1 was taken under bright field illumination through an Olympus camera (model OM-2N) fitted with a 50 mm macro lens and neutral density filters.

5.2.2 Evaluation of antigenic immunoreactivity

Prior to examination, identity codes for each slide were covered with non-transparent, adhesive labels in order to facilitate unbiased evaluation of immunoreactivity. Slides were then randomized prior to sequential numbering for purposes of record keeping.

For each antibody, two sections, taken 60-90 μm apart, were examined from each of the three tissue blocks that comprised each half pineal (see Section 2.8.2).

(I) NSE

Immunoreactive NSE was principally contained in two elements of pineal tissue, nerve fibres and parenchymal cells, presumably pinealocytes[1]. Evaluation of immunoreactivity in each section from the middle and apex of the pineal was achieved through quantification of pinealocytes containing immunoreactive NSE. This was achieved by using a 400 point grid, inserted into the eyepiece of the microscope, so as to superimpose it on the section. In representative areas of pineal tissue, each grid point was inspected for its position over the following three types of locations: parenchymal cell containing immunoreactive NSE, parenchymal cell containing no immunoreactive material (principally glial cells, but not red blood cells), or intercellular space. For this purpose, one area from each pineal section was evaluated at a magnification of 400 X. Because of intense and widespread cellular staining in

[1]The principal feature by which pinealocytes may be distinguished histologically from other cells of the pineal is their relatively large, oval shaped nucleus and nucleolus (Quay, 1965).

these two regions it was not possible to distinguish immunoreactive nerve fibres from stained cells and their processes. The pineal stalk however, was characterized by a sparse population of immunoreactive pinealocytes and one or more tracts of immunoreactive nerve fibres. For this reason only an assessment of nerve fibre density was performed for this region. This was achieved by viewing the entire stalk region and subjectively assessing the density and pattern of immunoreactive nerve fibres.

(II) PNMT

PNMT-like immunoreactivity was restricted to nerve fibres, which appeared in distinct, but widely scattered, bundles. Evaluation, therefore was restricted to low magnification (160 X) examination of sections and assessment of the pattern and location of each fibre bundle and subjective assessment of their density. Analysis by a point scoring system was considered inappropriate because the uneven distribution of immunoreactivity introduced the possibility that a randomly selected area of pineal tissue may not have accurately represented the immunoreactivity occurring in the whole pineal (Snedecor & Cochran, 1967).

(III) NPY

The distribution of NPY-like immunoreactivity was similar to that of PNMT, in that it was mainly confined to nerve fibres and was highly regionalized, but in this case occurring principally in fibres associated with arterial blood vessels and only occasionally in the parenchyma of the pineal. This regional distribution, together with the small diameter and high density of fibres precluded accurate analytical evaluation of their number. Thus, evaluation was again restricted to low magnification (160 X) observations similar to those already described for PNMT.

(IV) VIP

VIP-like immunoreactivity was found only in perivascular nerve fibres. For reasons similar to those given above for NPY and PNMT, evaluation was restricted to low magnification (160 X) observations and assessment of the distribution and density of immunoreactivity.

5.2.3 Statistical analyses

(I) NSE

Analyses of variance, as described in Section 2.9, were performed to examine the significance of experimental main effects and their interactions. Data from each pineal half (apex and middle regions only) were examined separately to determine whether the innervation originating from one SCG was ipsilaterally distributed within the pineal or crossed over to supply both sides. As stated previously (Section 5.2.2 (I)) evaluation of pineal stalk immunoreactivity was by subjective assessment only. Coefficients used in the partitioning of treatment effects are given in Table 5.1.

Table 5.1 **Coefficients used in partitioning treatment effects on NSE-LI for pineal apex and body data from Experiment 5.**

	Contrast	Treatment			
		Control	Left SCGX	Right SCGX	Bilateral SCGX
(i)	Left pineal half				
	Orthogonal coefficients				
	(a) Control vs Right SCGX	+1		-1	
	(b) Left SCGX vs Bilateral SCGX		+1		-1
	(c) Control + Right SCGX vs Left SCGX + Bilateral SCGX	+1	-1	+1	-1
	Non-orthogonal coefficients				
	(a) Control vs Left SCGX	+1	-1		
	(b) Control + Bilateral SCGX vs Left SCGX	+1	-2		+1
(ii)	Right pineal half				
	Orthogonal coefficients				
	(a) Control vs left SCGX	+1	-1		
	(b) Right SCGX vs Bilateral SCGX			+1	-1
	(c) Control + Left SCGX vs Right SCGX + Bilateral SCGX	+1	+1	-1	-1
	Non-orthogonal coefficients				
	(a) Control vs Right SCGX	+1		-1	
	(b) Control + Bilateral SCGX vs Right SCGX	+1		-2	+1

Note: Explanation of orthogonal contrasts:

(i) Contrasts labelled (a) examine for differences in IR in tissue from pineal sides which were not 'denervated', but from animals with both or only one SCG intact.

(ii) Contrasts labelled (b) examine for differences in IR in tissue from pineal sides which were 'denervated', but from animals with only one or neither SCG intact.

(iii) Contrasts labelled (c) examine for differences in IR in tissue from pineal sides which were not denervated, or which were denervated.
Contrasts (a) & (b) were designed to evaluate significant cross-over of innervation from side to side within the pineal, while contrasts (c) evaluate the effect of denervation.

(iv) Non-orthogonal contrasts examine the effects of unilateral ganglionectomy.

(II) PNMT, NPY & VIP

No statistical analyses were performed on results for these antigens as evaluation of immunoreactivity was solely by subjective observation.

5.3 Results.

To orient the reader as to the position of the ovine pineal and the levels at which it was transected, as well as to provide a general picture of its structure, a paramedian section of the epithalamic region of sheep brain and associated structures was prepared, stained with haematoxylin and eosin, and mounted on a glass slide as described in Section 2.8. No immunocytochemical procedures were performed on this tissue. As can be seen in the photomicrograph of this section (Figure 5.1) and as is illustrated in Figure 5.2, the pineal gland is located on the posterodorsal roof of the third ventricle and is situated between and attached to the habenular and caudal commissures. In adult sheep, the pineal is ovoid in shape, measures approximately 5-7 mm long by 3-5 mm wide and on average weighs 60-80 mg (Barrell & Lapwood, 1978\1979b; Vollrath, 1981). Its histological structure is similar to that of many other mammals in that there is a uniform distribution of cell types (pinealocytes and supporting (mainly glial) cells) throughout the body of the pineal, with no evidence of regional congregations of particular cell types (Quay, 1965).

5.3.1 NSE

See Tables 5.2-5.5 & Figures 5.3-5.7

Pineal glands of all sheep in Experiment 5 exhibited strongly staining, NSE-LI cells as shown for the pineal apex in Figure 5.3. Of the cells inspected in the body and apex regions of the pineals of control rams, 3950 (81%) exhibited NSE-LI, with the remaining 912 (19%) being non-immunoreactive. Unstained cells were evident in all areas of the pineal body, usually closely associated with NSE-LI cells.

Unilateral SCGX did not significantly influence the incidence of NSE-LI exhibited by pinealocytes in these ram pineals, since neither the NSE-LI recorded in the ipsilateral nor in the contralateral half of the pineal from each group of unilaterally ganglionectomized rams was significantly different from controls (Tables 5.2 & 5.3 (orthogonal contrasts (a) and non-orthogonal contrasts (a))). Furthermore, non-orthogonal contrasts (b) (Table 5.3) revealed that the mean number of immunoreactive cells of control plus bilaterally SCGX rams was significantly different from that in the 'denervated' pineal half of each group of unilaterally SCGX rams, indicating that the latter treatment did not produce an effect intermediate between those observed in control and bilaterally SCGX rams; the significance of these contrasts was

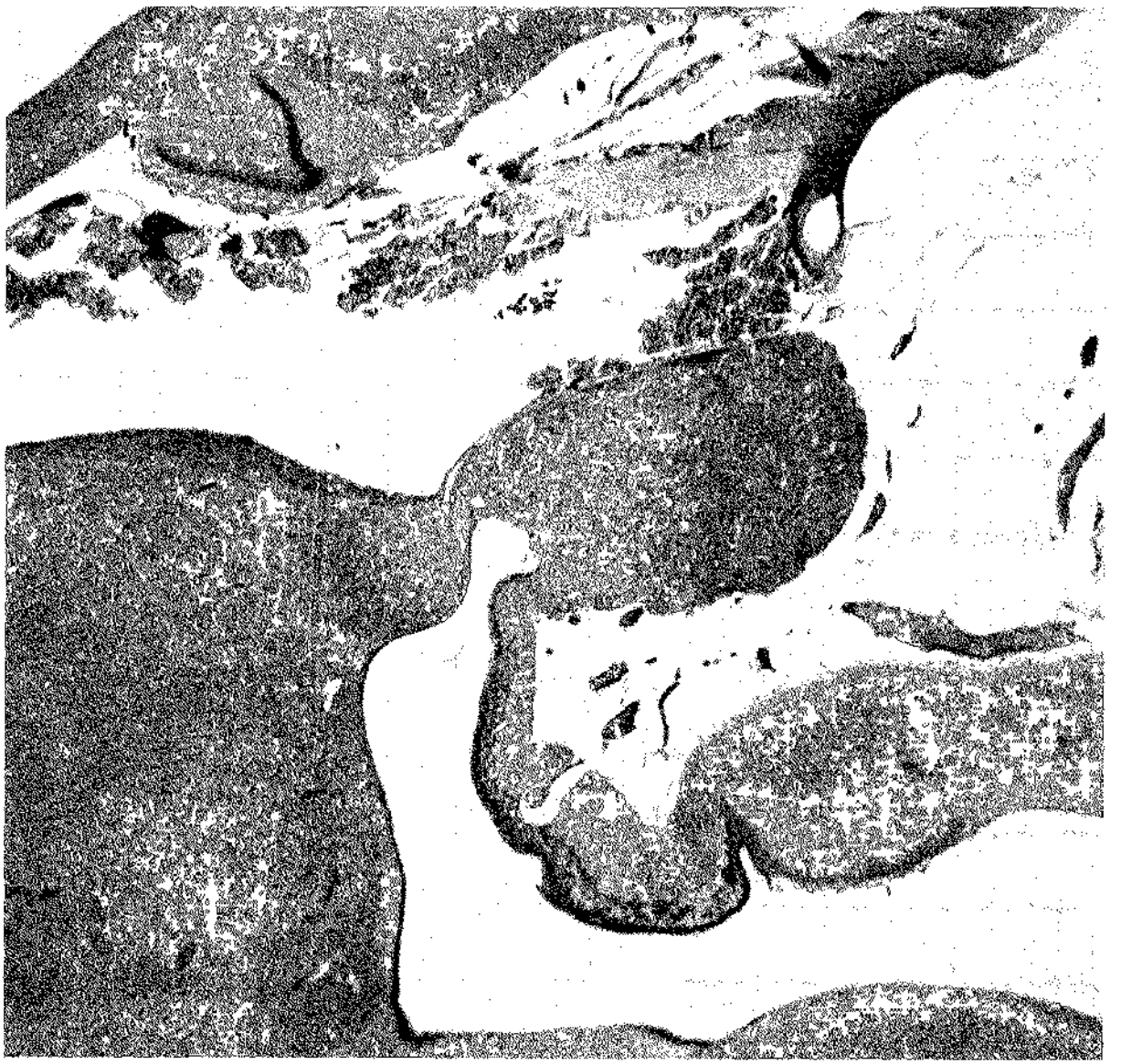


Figure 5.1 Paramedian section through the epithalamic region of the sheep brain showing the position of the pineal gland and associated structures. Stained with haematoxylin and eosin and magnified approximately 10 X.

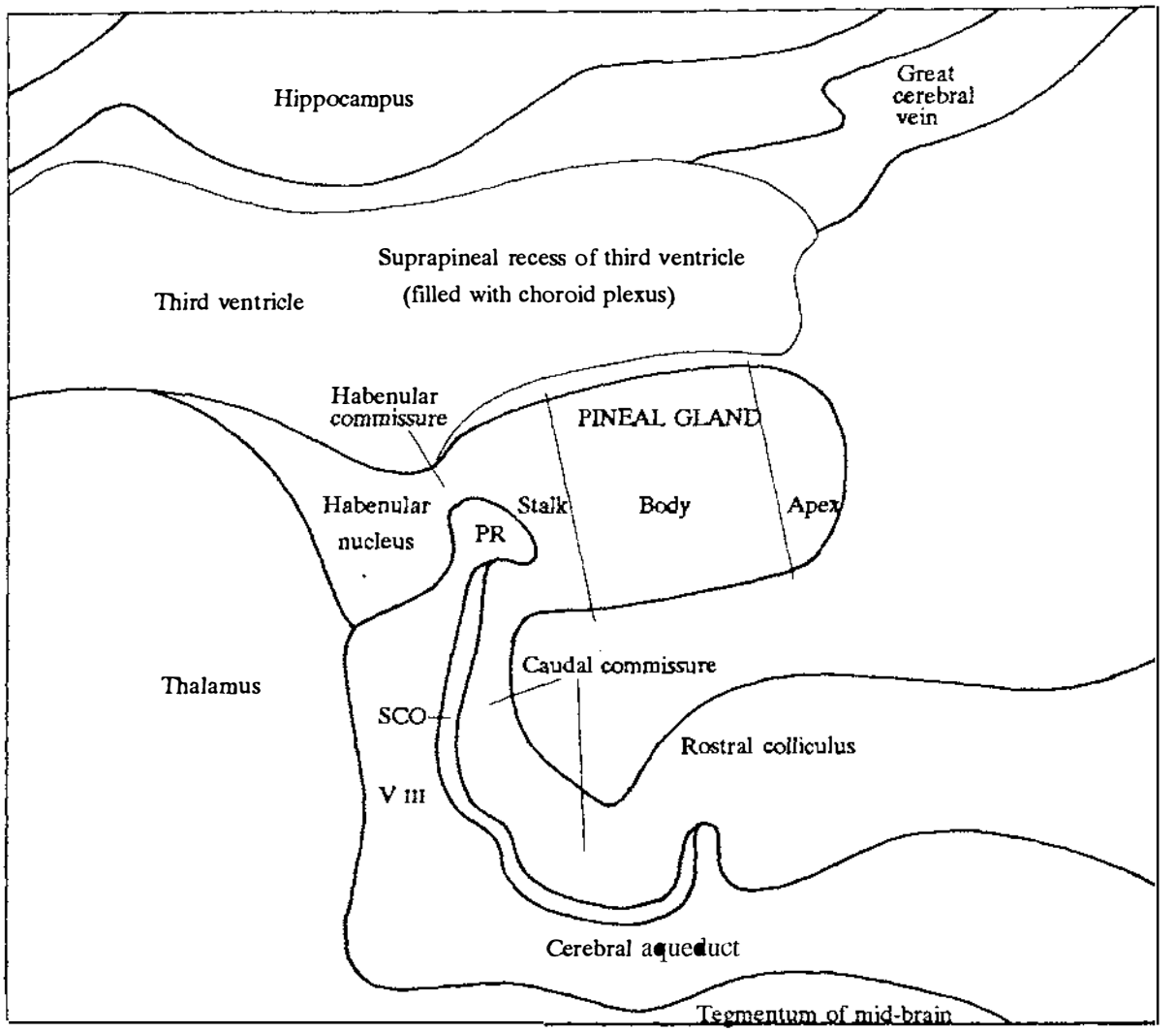
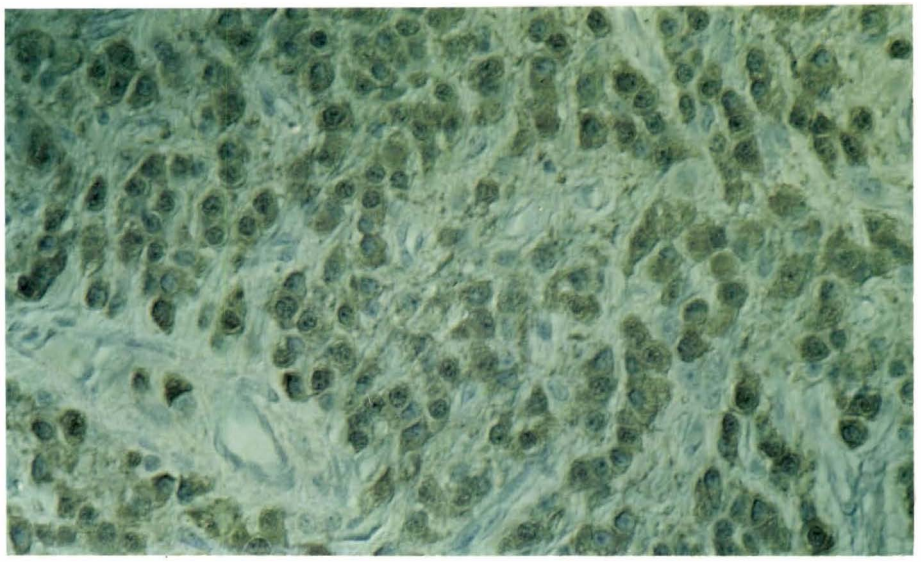
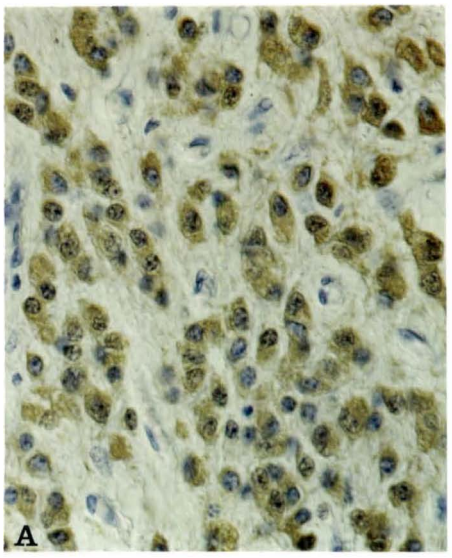


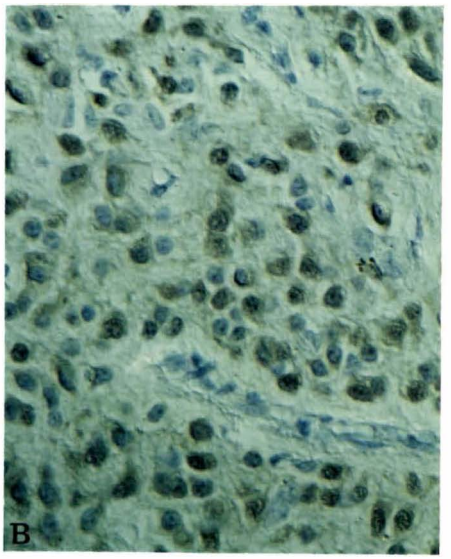
Figure 5.2 Outline diagram to show the anatomical position of the pineal gland and associated structures in the epithalamic region of the sheep brain. This diagram was traced from the photomicrograph shown in Figure 5.1 and is approximately 10 times actual size. SCO = Subcommissural organ; V III = Third ventricle; PR = Pineal recess

Figure 5.3 NSE-like immunoreactivity in the apex of a pineal gland with intact sympathetic innervation. Note that most cells are immunoreactive. Control; Left pineal half; NSE antiserum; Magnification 560 X.

Figure 5.4 NSE-like immunoreactivity in the apex of sympathetically denervated pineal glands. Fig 5.4a Right ganglionectomy, left pineal half. Fig 5.4b Bilateral ganglionectomy, Left pineal half. Note that in comparison to Figure 5.3, the density of NSE-LI cells has decreased in Figure 5.4b, but not Figure 5.4a. NSE antiserum; Magnification 560 X.




A


B

due solely to a reduction in the number of NSE-LI cells in bilaterally SCGX rams. That reduction in cellular immunoreactivity following double SCGX also was responsible for the significance of orthogonal contrasts (b) and of orthogonal contrast (c) for right half pineal data, in Table 5.3. It is illustrated by comparison of Figures 5.3 and 5.4a, with Figure 5.4b.

In order to further elucidate the histological basis for the observed differences, the changes in numbers of non-immunoreactive cell- and interstitial space-grid point intercepts following SCGX, were investigated (Table 5.2). It was found that, compared to the values recorded from control rams, the number of non-immunoreactive cells was not significantly changed in the 'non-denervated' pineal half following either left or right (unilateral) SCGX, nor in the 'denervated' pineal half following right SCGX, but was significantly reduced in the corresponding half of left SCGX rams (Table 5.4, non-orthogonal contrast (a) for left pineal data). Bilateral SCGX also produced significant effects (reductions in incidence of non-IR cells), although these were not consistent in both pineal halves. Whereas in the right half there was a significant difference between the effects of right SCGX and bilateral SCGX ($P<0.05$), this was not repeated in the comparison of left SCGX and bilateral SCGX in the left pineal half (orthogonal contrasts (b), Table 5.4). Furthermore, the significant difference between the combined values from the 'innervated' left pineal halves vs those of 'denervated' left pineal halves (contrasts (c)) was not significant in the right pineal half. Inspection of data in Table 5.2 reveals that these inconsistencies were due to bilateral SCGX reducing the incidence of NSE-LI cells in both pineal halves, as did left SCGX in the left pineal, but right SCGX had no effect on this parameter in the right pineal half. The variability of these results indicates that this parameter is not a reliable index of the effects of SCGX on pineal morphology. Contrasting with this result, analysis of the interstitial space data showed a more consistent pattern in response to SCGX. In the left pineal half, left SCGX did not significantly influence the number of interstitial space points counted, whereas bilateral SCGX resulted in a very significant increase ($P<0.01$, contrast (b), Table 5.5). A similar pattern was also recorded in the right pineal half, although the effect of bilateral SCGX attained an even higher degree of significance ($P<0.001$, contrast (b), Table 5.5). This effect of bilateral SCGX also was responsible for the significance of both contrasts (c).

For each of the above three parameters, similar results were obtained from the apex and body of the pineal (Tables 5.3, 5.4 & 5.5).

NSE-like immunoreactivity in the stalk of the pineal was present in most of the few pinealocytes present and in nerve fibres, the latter usually being present as well defined tracts originating from both the habenular and caudal commissures. Immunoreactivity in the cells and nerve fibres of the stalk of the pineal following unilateral or bilateral SCGX appeared not to vary appreciably from that observed in control rams (Figures 5.5, 5.6 & 5.7).

Table 5.2 **Mean count ± s.e.m of NSE-like immunoreactive cell-, non-immunoreactive cell- and interstitial space-grid point intercepts per 400 grid points in the apex and pineal body regions of rams in Experiment 5. Each value is the average of eight counts, being 1 each from two separate sections from the pineals of each of the four rams in each surgical treatment group. Combined (Apex + Body) means ± s.e.m are printed in bold at the bottom of each column.**

(i) Right pineal half

	Control	Left SCGX	Right SCGX	Bilateral SCGX
NSE-LI cells				
Apex	122.8 ± 5.6	122.5 ± 8.8	127.3 ± 8.2	106.8 ± 4.1
Body	121.4 ± 7.0	145.9 ± 8.8	127.0 ± 7.4	108.1 ± 5.0
Apex + Body	**122.1 ± 4.4**	**134.2 ± 6.7**	**127.1 ± 5.3**	**107.4 ± 3.1**
Non-IR cells				
Apex	29.0 ± 3.7	29.5 ± 3.3	31.1 ± 2.1	23.8 ± 2.2
Body	29.6 ± 3.6	27.9 ± 2.0	29.6 ± 3.6	23.6 ± 3.0
Apex + Body	**29.3 ± 2.5**	**28.7 ± 1.9**	**30.4 ± 2.0**	**23.7 ± 1.8**
Interstitial space				
Apex	248.3 ± 4.7	248.0 ± 8.2	241.6 ± 8.8	269.5 ± 3.6
Body	249.0 ± 5.3	226.3 ± 9.1	243.4 ± 10.4	268.3 ± 6.2
Apex + Body	**248.6 ± 3.5**	**237.1 ± 6.6**	**242.5 ± 6.6**	**268.9 ± 3.5**

(ii) Left pineal half

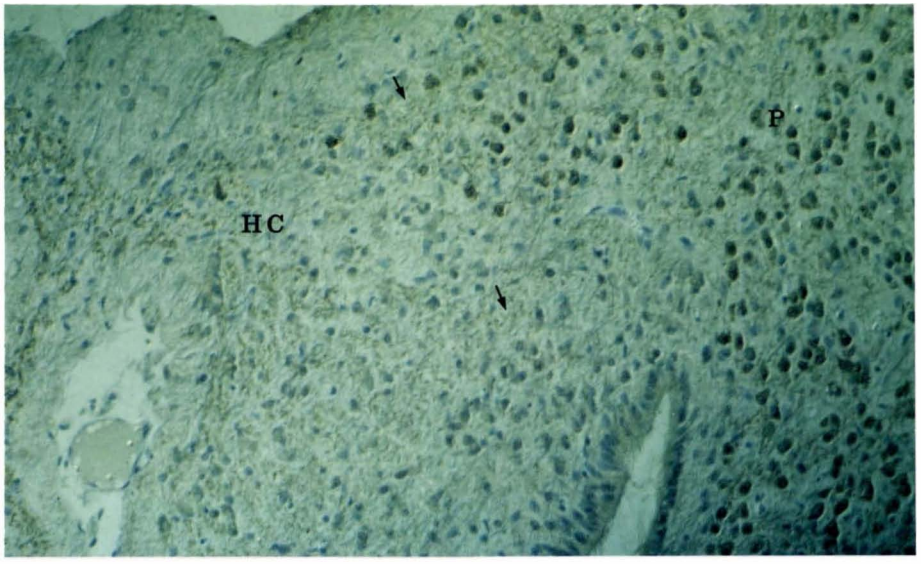
	Control	Left SCGX	Right SCGX	Bilateral SCGX
NSE-LI cells				
Apex	122.4 ± 8.6	129.3 ± 8.5	128.0 ± 9.4	99.5 ± 10.2
Body	127.3 ± 14.3	141.3 ± 11.6	136.5 ± 7.0	105.9 ± 10.7
Apex + Body	**124.8 ± 8.1**	**135.3 ± 7.1**	**132.3 ± 5.8**	**102.7 ± 7.2**
Non-IR cells				
Apex	26.9 ± 2.1	23.3 ± 3.1	31.0 ± 3.5	27.1 ± 2.6
Body	28.5 ± 3.1	20.1 ± 2.1	29.4 ± 2.8	20.8 ± 2.0
Apex + Body	**27.7 ± 1.8**	**21.7 ± 1.9**	**30.2 ± 2.2**	**23.9 ± 1.8**
Interstitial space				
Apex	250.8 ± 7.6	247.5 ± 9.7	241.0 ± 11.3	273.4 ± 9.9
Body	244.3 ± 12.7	238.6 ± 11.4	234.1 ± 8.0	273.4 ± 10.8
Apex + Body	**247.5 ± 7.2**	**243.1 ± 7.3**	**237.6 ± 6.8**	**273.4 ± 7.1**

Note:
NSE-LI = NSE-like immunoreactive
Non-IR = Non-immunoreactive

Figure 5.5 NSE-LI cells and nerve fibres in the habenular region of the pineal stalk. Note that immunoreactive nerve fibres (arrows) of the pineal gland (P) and the habenular commissure (HC) appear to be continuous, with no clear demarcation between them. Bilateral ganglionectomy; Right pineal half; NSE antiserum; Magnification 280 X.

Figure 5.6 NSE-LI cells and nerve fibres in the caudal commissure (CC) and pineal stalk (PS). Note the broad band of immunoreactive nerve fibres (arrow) in the commissure and their close association with the cells of the pineal stalk. Bilateral ganglionectomy; Left pineal half; NSE antiserum; Magnification 280 X.

Figure 5.7 NSE-LI cells and nerve fibres (arrows) in the habenular region of the stalk of a pineal (PS) with intact sympathetic innervation. Note that other than a greater staining intensity in some cells there is little discernible difference between this region and that of the bilaterally denervated pineal shown in Figure 5.5. Control; Right pineal half; NSE antiserum; Magnification 280 X. P = Pineal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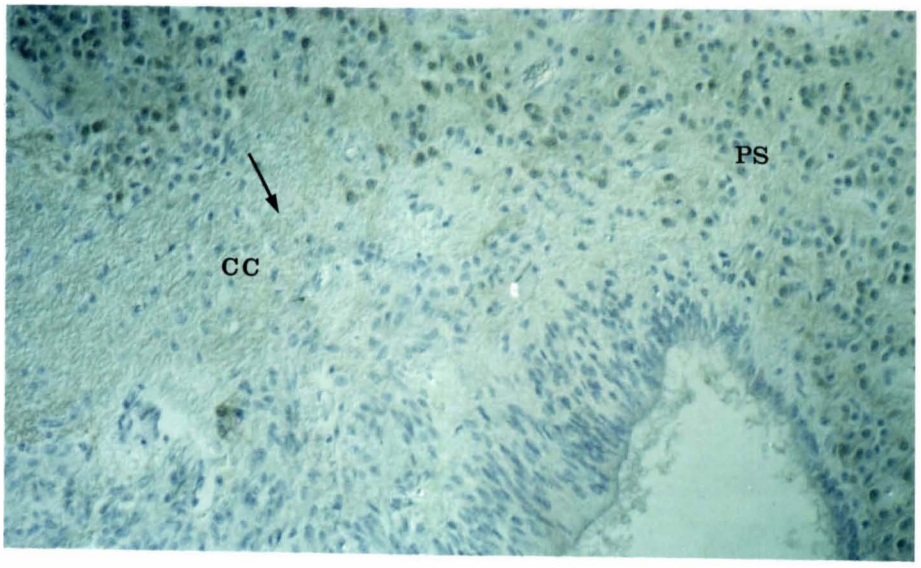
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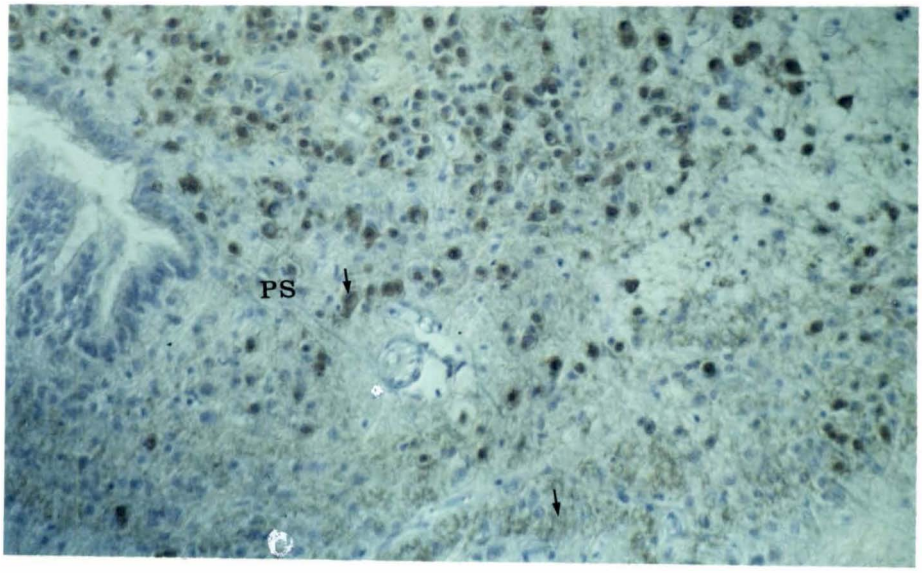
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Table 5.3 **Summary of analyses of variance of NSE-like immunoreactive cell data from the apex and body of the pineal.**

Source of Variation	D.F	Variance Ratios Pineal	
		Right half	Left half
A. Level (Apex vs Body)	1	1.34	1.20
B. Surgical Treatment	3		
Orthogonal contrasts			
(i) (a) Control vs Left SCGX	1	2.94**	
(b) Right SCGX vs Bilateral SCGX	1	7.75**	
(c) Control + Left SCGX vs Right SCGX + Bilateral SCGX	1	4.70*	
(ii)(a) Control vs Right SCGX	1		0.53**
(b) Left SCGX vs Bilateral SCGX	1		10.10**
(c) Control + Right SCGX vs Left SCGX + Bilateral SCGX	1		1.74
Non-orthogonal contrasts			
(i) (a) Control vs Right SCGX	1	0.51*	
(b) Control + Bilateral vs Right SCGX	1	4.08*	
(ii)(a) Control vs Left SCGX	1		1.04*
(b) Control + Bilateral vs Left SCGX	1		5.87*
Interaction (AxB)	3	1.39	0.05
Error mean square	56	400.18	839.45

Table 5.4 **Summary of analyses of variance of Non-NSE immunoreactive cell data from the apex and body of the pineal.**

Source of Variation	D.F	Variance Ratios Pineal	
		Right half	Left half
A. Level (Apex vs Body)	1	0.10	1.52
B. Surgical Treatment	3		
Orthogonal contrasts			
(i) (a) Control vs Left SCGX	1	0.04*	
(b) Right SCGX vs Bilateral SCGX	1	4.96*	
(c) Control + Left SCGX vs Right SCGX + Bilateral SCGX	1	0.86	
(ii)(a) Control vs Right SCGX	1		0.84
(b) Left SCGX vs Bilateral SCGX	1		0.68
(c) Control + Right SCGX vs Left SCGX + Bilateral SCGX	1		10.13**
Non-orthogonal contrasts			
(i) (a) Control vs Right SCGX	1	0.13	
(b) Control + Bilateral vs Right SCGX	1	2.22	
(ii)(a) Control vs Left SCGX	1		4.86*
(b) Control + Bilateral vs Left SCGX	1		3.06
Interaction (AxB)	3	0.07	0.75
Error mean square	56	72.09	59.27

Table 5.5 **Summary of analyses of variance of interstitial space data from the apex and body of the pineal.**

Source of Variation	D.F	Variance Ratios Pineal	
		Right half	Left half
A. Level (Apex vs Body)	1	0.96	0.53
B. Surgical Treatment	3		
Orthogonal contrasts			
(i) (a) Control vs Left SCGX	1	2.42	
(b) Right SCGX vs Bilateral SCGX	1	12.74***	
(c) Control + Left SCGX vs Right SCGX + Bilateral SCGX	1	6.01*	
(ii)(a) Control vs Right SCGX	1		0.93
(b) Left SCGX vs Bilateral SCGX	1		8.64**
(c) Control + Right SCGX vs Left SCGX + Bilateral SCGX	1		4.63*
Non-orthogonal contrasts			
(i) (a) Control vs Right SCGX	1	0.69	
(b) Control + Bilateral vs Right SCGX	1	6.45*	
(ii)(a) Control vs Left SCGX	1		0.19
(b) Control + Bilateral vs Left SCGX	1		5.87*
Interaction (AxB)	3	1.14	0.07
Error mean square	56	436.83	850.46

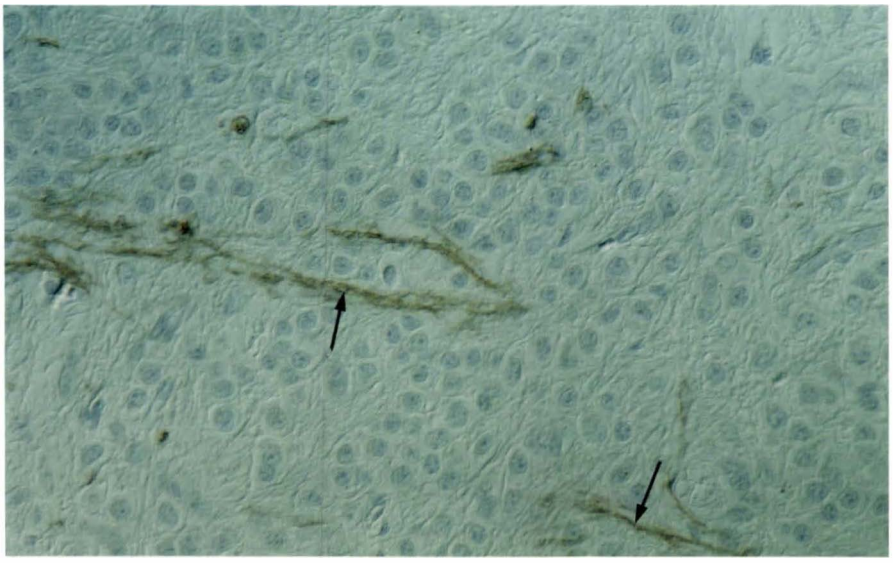
5.3.2 PNMT

See Figures 5.8-5.11

PNMT-LI was found to occur in small nerve fibres which generally travelled as distinct bundles throughout the parenchyma of the pineal (Figure 5.8). Occasionally small bundles of fibres were observed closely associated with blood vessels, but these were relatively rare and less intensely stained than those described above. Generally, PNMT-LI fibres were scattered in small groups of longitudinally or transversely sectioned bundles which, due to their close association and similar orientation, appeared to be part of a nerve tract travelling through the plane of section. Within the control group the majority of nerve fibre bundles were observed in the body of the pineal, while relatively few bundles were observed in the apex. Generally, fibre bundle thickness did not vary between regions, but rather the number of bundles appeared greater and fibre tracts were more frequent in the body of the pineal.

Figure 5.8 Bundles of PNMT-LI fibres (arrows) travelling through the interstitial tissue of the body of the pineal. Control; Right pineal half; PNMT antiserum; Magnification 560 X.

Figure 5.9 Transversely sectioned PNMT-LI fibres (arrows) emerging from the habenular commissure (HC) and travelling into the stalk of the pineal gland (P). Double ganglionectomy; Right pineal half; PNMT antiserum; Magnification 280 X.



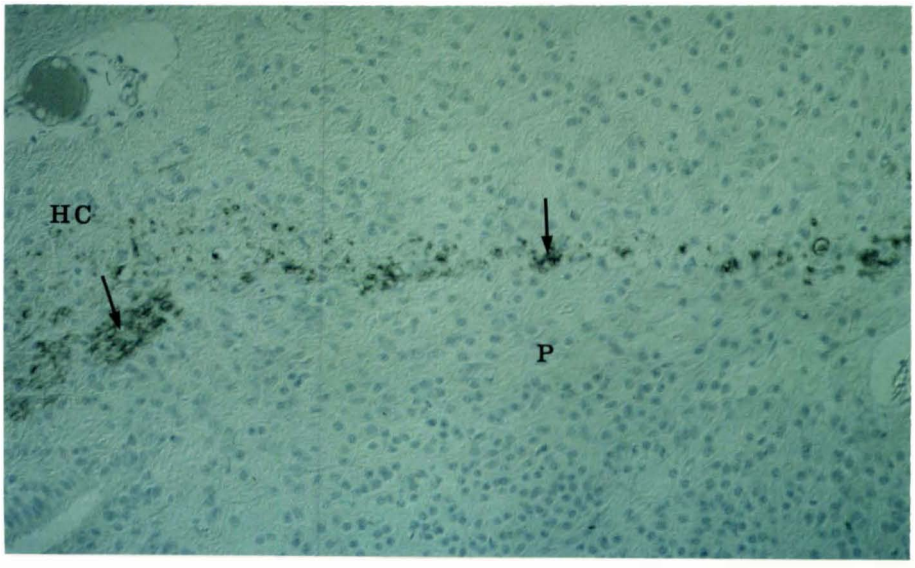
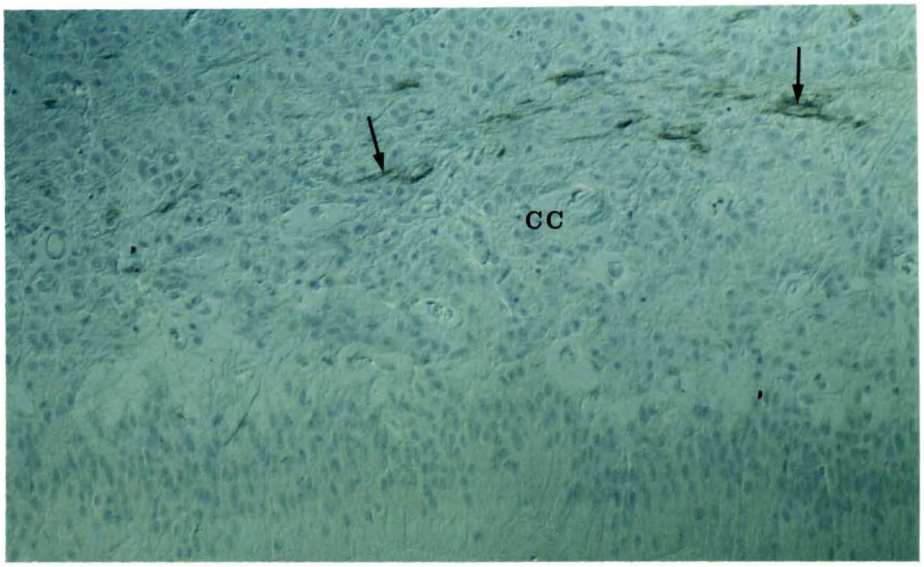

HC
P

Figure 5.10 PNMT-LI fibres (arrows) in the caudal commissure (CC). In some instances these fibres could be observed entering the pineal gland. Right ganglionectomy; Left pineal half; PNMT antiserum; Magnification 280 X.

Figure 5.11 Parallel tracts of PNMT-LI fibres (sub-capsular (large arrows) and parenchymal (small arrows)) in the apex of the pineal gland. Tracts similar to these were observed in one animal from each of the treatment groups. Right ganglionectomy; Left pineal half; PNMT antiserum; Magnification 280 X.


CC

Approximately one third of sections from the apex and pineal body regions exhibited little or no PNMT-LI fibres.

Unilateral and bilateral SCGX had no discernible effect on the presence of PNMT-LI fibres in pineal tissue, relative to that in control animal tissue.

All treatment groups exhibited PNMT-LI fibres in both the habenular and caudal commissures which, on occasion, could be observed projecting into the pineal for a short distance (Figures 5.9 & 5.10). The pattern of PNMT-LI fibres in both parts of the pineal stalk varied from well defined tracts of densely packed fibres to a diffuse network of lightly stained fibres only loosely aggregated together. No vascular association was observed in the two commissures.

One further pattern of PNMT-LI fibres occurred in one section in all four treatment groups. This consisted of a tract travelling under the pineal capsule with a second tract in the pineal parenchyma travelling parallel to the first tract (Figure 5.11).

5.3.3 NPY

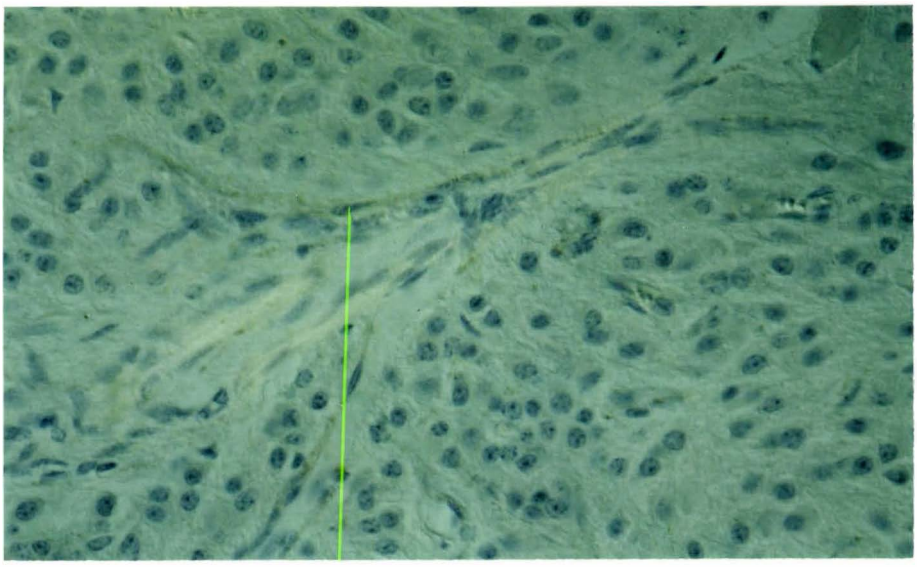
See Figures 5.12-5.18

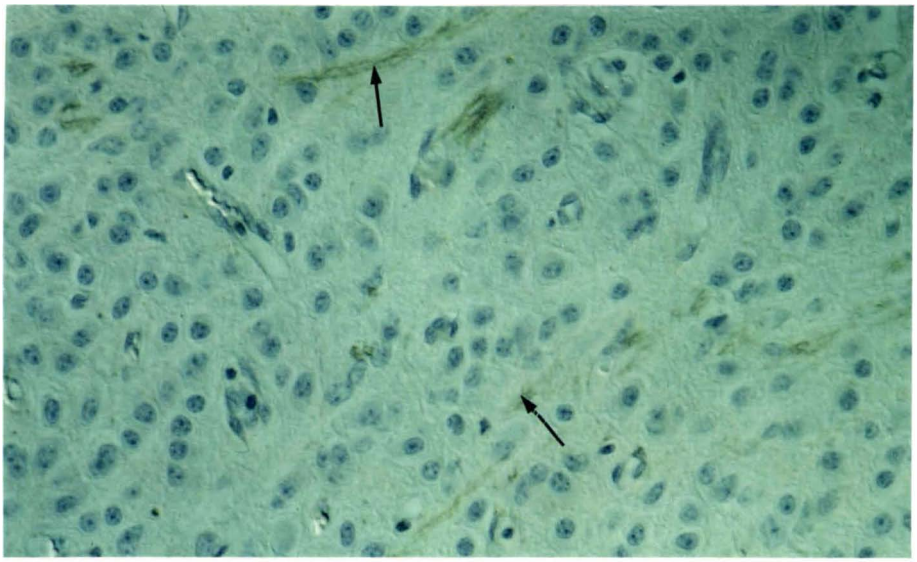
Pineal tissue obtained from control rams exhibited a dense network of NPY-LI fibres, most often in close association with arterial blood vessels, including arteries which enter the pineal over its surface, pre-capillaries and occasionally capillaries (Figure 5.12). While it could not be determined whether any of these fibres were within the perivascular space, it was clear that many travelled outside this space and were associated with the perivascular sleeve of connective tissue which surrounded each blood vessel. This pattern was observed in all but one control ram, which had only a few sites of immunoreactivity in one section and no reactivity in any other section. All other rams from this group showed approximately equal densities and distributions of immunoreactivity within both left and right halves of the apex and middle regions of the pineal. However, not all blood vessels of the pineal had associated NPY-LI fibres. In one section, an area of NPY-LI fibres was observed, which was not associated with any blood vessels (Figure 5.13), but which appeared as a nerve tract travelling in an apex-stalk direction. This tract extended for only a short distance in the mid-pineal region before diverging from the plane of section. In addition, tracts of immunoreactive nerve fibres often were observed in both the habenular (Figure 5.14) and caudal (Figure 5.15) commissures, but only occasionally were observed to enter the pineal.

Figure 5.12 NPY-LI fibres shown in close association with blood vessels in the apex of the pineal gland. Control; Right pineal half; NPY antiserum; Magnification 560 X

Figure 5.13 NPY-LI fibres (arrows) in the interstitial tissue of the mid-pineal region. Control; Left pineal half; NPY antiserum; Magnification 560 X.

Figure 5.14 NPY-LI fibres (arrows), without vascular association, travelling through the habenular commissure (HC) and projecting into the stalk of the pineal gland (PS). Control; Left pineal half; NPY antiserum, Magnification 140 X.





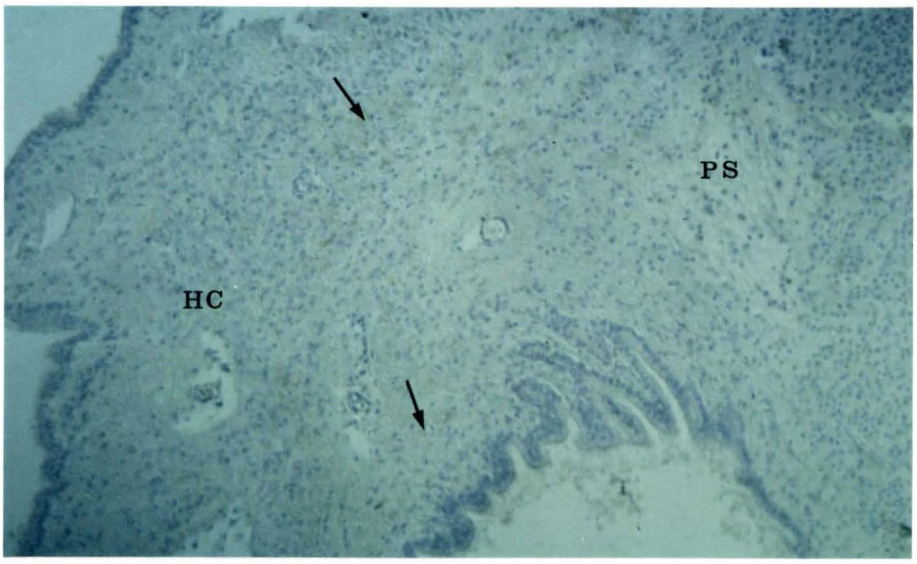
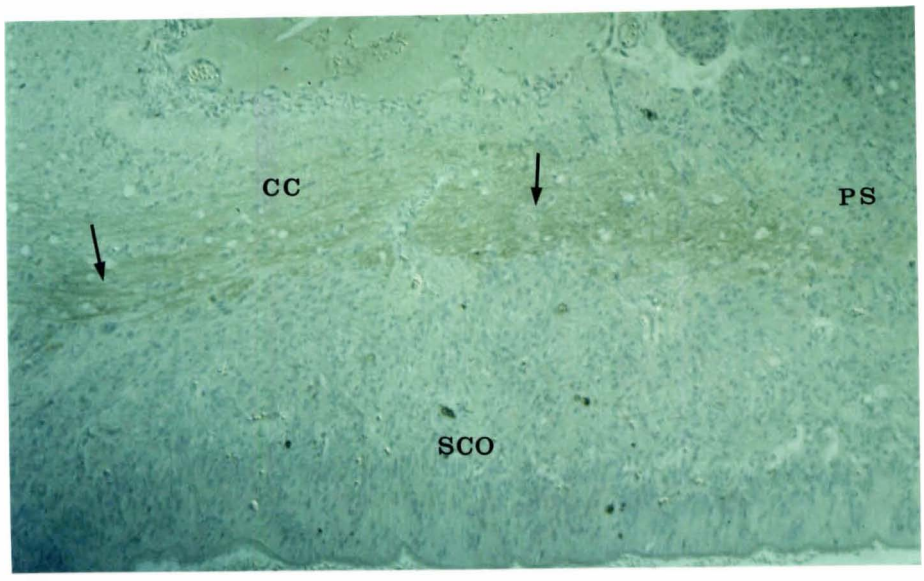

PS
HC

Figure 5.15 NPY-LI fibres (arrows), without vascular association, travelling through the caudal commissure (CC) and projecting into the stalk of the pineal gland (PS). SCO = subcommissural organ; Control; Left pineal half; NPY antiserum; Magnification 140 X.

Figure 5.16 NPY-LI associated with blood vessels in the pineals of unilaterally SCGX rams. Note that in comparison to Figure 5.12, there is a reduction in the density of immunoreactive nerve fibres around the blood vessels. a) Left ganglionectomy; Left pineal half; b) Right ganglionectomy; Left pineal half; NPY antiserum; Magnification 560 X.

Figure 5.17 NPY-LI in the pineal gland of a bilaterally ganglionectomized ram. Note that there is a tract of immunoreactive nerve fibres projecting through the interstitial tissue and that few perivascular immunoreactive nerve fibres are present. Right pineal half; NPY antiserum; Magnification 560 X.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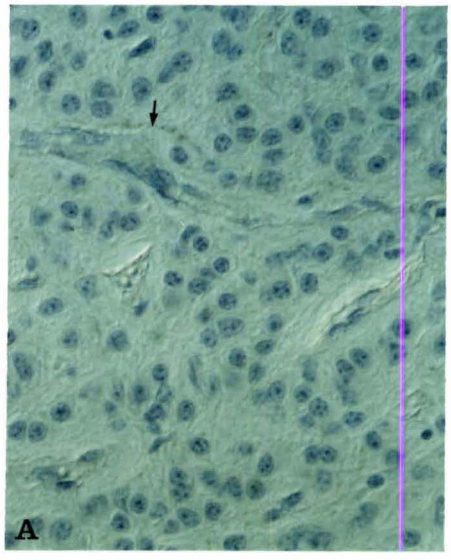
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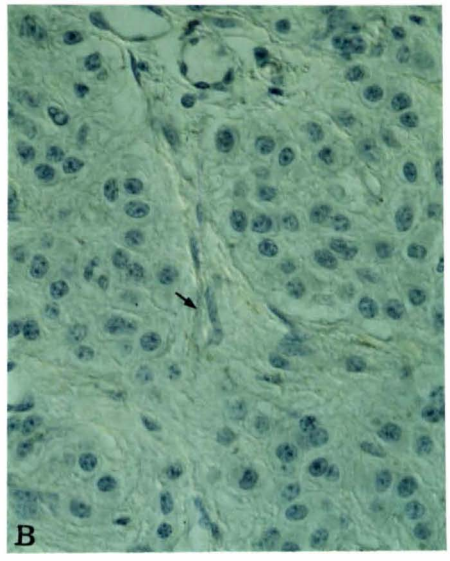
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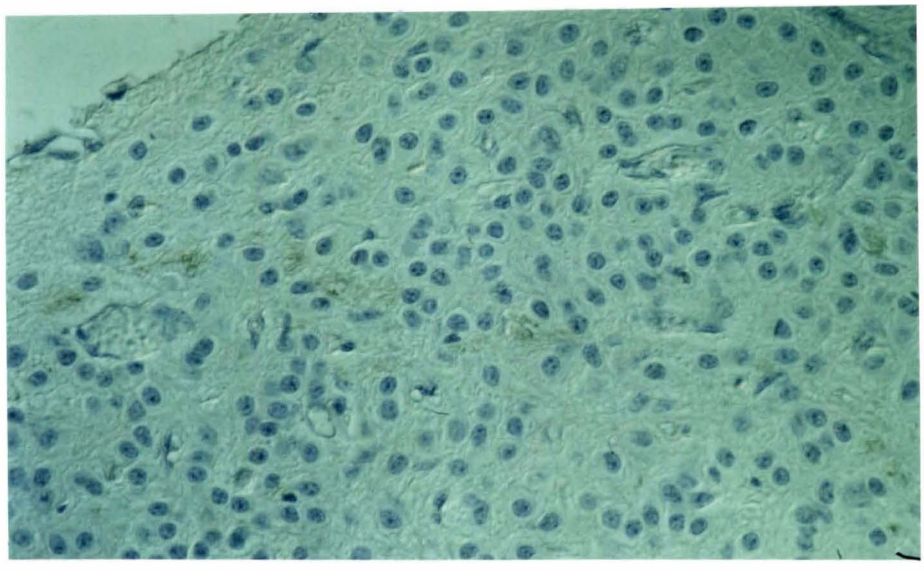


In contrast to pineal tissue taken from control rams, that taken from left or right SCGX rams showed considerably less NPY-LI, although the pattern of immunoreactivity present was much the same (Figure 5.16). In general, comparison of left and right pineal halves, or apex and middle regions of the pineal showed no differences in the pattern or distribution of NPY-LI fibres. Differences which did occur relative to controls were classed as being of three types. Firstly, approximately 40% of sections obtained from the 8 animals which comprised these two groups, exhibited no immunoreactivity at all or exhibited only a few sites which could be identified as exhibiting NPY-LI. Secondly, immunoreactivity associated with blood vessels was less intense, suggesting fewer NPY nerve fibres were present. Thirdly, in most sections, a greater proportion of blood vessels were completely devoid of NPY-LI. Whereas sections from control rams typically showed NPY-LI in association with approximately three-quarters of blood vessels, those from unilaterally ganglionectomized rams exhibited immunoreactivity in association with only about one-quarter of arterial blood vessels. These changes were evident in sections taken from both halves of the pineals obtained from these two treatment groups, regardless of whether the ganglionectomy had been ipsilateral or contralateral.

Bilateral SCGX almost entirely eliminated the occurrence of fibres exhibiting NPY-LI from the pineal. In sections from the apex and mid-pineal regions there was either no reactivity or only a few immunostained fibres could be identified. This immunoreactivity was exclusively found in association with blood vessels in all but one section, in which a tract of fibres in the parenchyma of the pineal was observed (Figure 5.17).

NPY-LI in the habenular and caudal commissures of unilaterally and bilaterally ganglionectomized rams was very similar to that observed in control rams. In each group of ganglionectomized rams, NPY-LI nerve fibres were observed in both the caudal and habenular commissures, and on occassion were observed to project into the pineal (Figure 5.18).

5.3.4 VIP

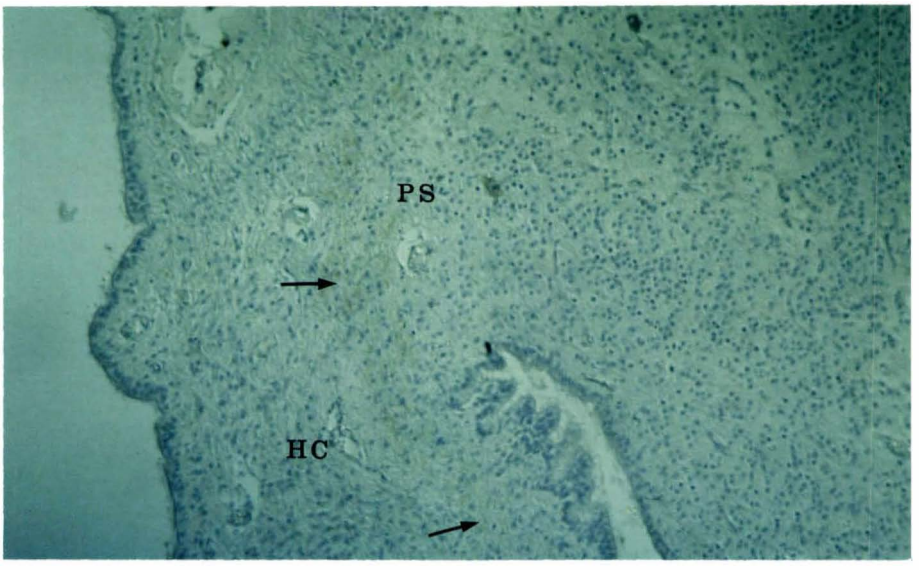
See Figures 5.19-5.25

Pineal tissue obtained from control rams exhibited a dense network of VIP-LI nerve fibres within the apex and body of the pineal (Figure 5.19). No cellular elements of the pineal exhibited immunostaining. Immunoreactive nerve fibres were always associated

Figure 5.18 NPY-LI fibres (arrows), without vascular association, travelling through the habenular commissure (HC) and projecting into the stalk of the pineal gland (PS). Left ganglionectomy; Left pineal half; NPY antiserum; Magnification 140 X.

Figure 5.19 VIP-LI nerve fibres (arrows) associated with blood vessels in the pineal of a control ram. Note that no cells are immunoreactive and that no intra-parenchymal nerve fibres are immunoreactive. Control; Right pineal half; VIP antiserum; Magnification 140 X.

Figure 5.20 VIP-LI nerve fibres (arrows) in close association with a blood vessel in the pineal of a control ram. Control; Right pineal half; VIP antiserum; Magnification 560 X.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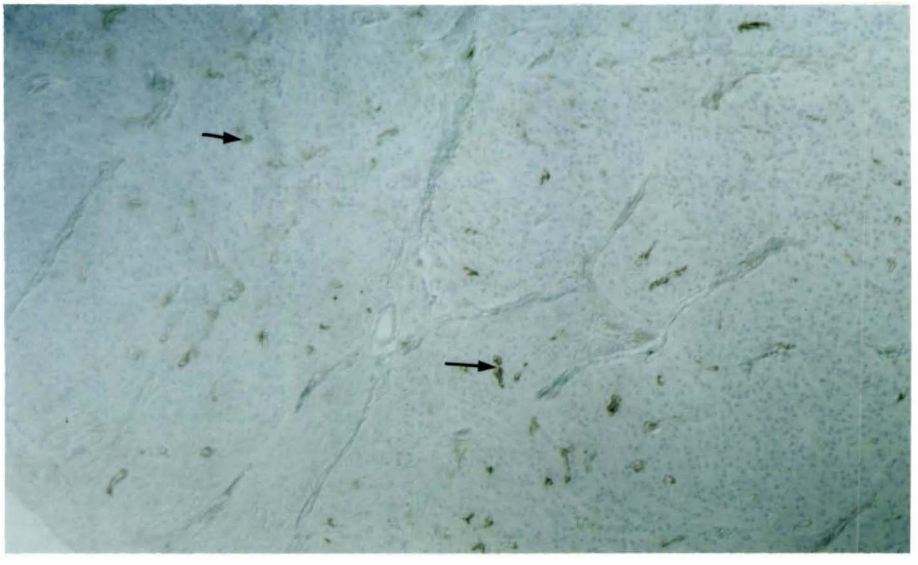
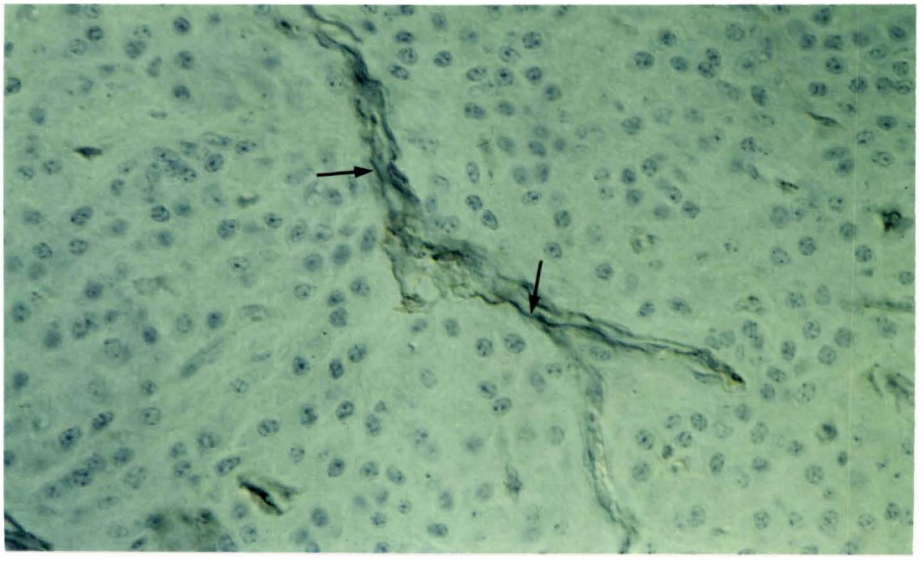




with the vasculature of the pineal and, like NPY, appeared to travel in the perivascular sleeve of connective tissue which surrounded each blood vessel (Figure 5.20). No immunoreactive nerve fibres were observed to travel intraparenchymally between pinealocytes.

The distribution of VIP-LI fibres varied; in approximately 50% of sections they were concentrated in the periphery (Figure 5.21), while in the remainder they were evenly distributed throughout the sections. This pattern applied in both the apex and body, and in the left and right pineal halves.

In contrast to the effects of partial or total sympathectomy on pineal NPY immunoreactivity, bilateral or unilateral (left or right) SCGX resulted in only a minor reduction in VIP-LI in the apex and body of the pineal. That reduction in immunoreactivity was greater in bilaterally SCGX rams as compared to rams with only one SCG removed (Figures 5.22 & 5.23). Although these effects of ganglionectomy were appreciable, pineal sections from treated rams exhibited extensive immunoreactivity similar in pattern and distribution to that observed in pineal tissue from control rams.

In all pineals taken from control rams, and from unilaterally and bilaterally ganglionectomized rams, the pineal stalk and the habenular and caudal commissures were totally devoid of VIP-LI nerve fibres or cells (Figures 5.24 & 5.25).

5.4 Discussion.

5.4.1 NSE

The current study demonstrated that NSE-LI is found in the majority of ovine pineal cells, presumably pinealocytes, and in intrapineal nerve fibres. This is in agreement with previous studies investigating the occurrence of this enzyme in APUD cells. Marangos et al (1979a, b), employing both RIA and ICC, demonstrated regional variations in NSE concentrations in rat, monkey and human neural and endocrine tissue. Highest concentrations were found in the CNS and some APUD cells, including those of the pineal gland (ie., pinealocytes) and pars intermedia of the pituitary. Other glands in these three species shown to contain NSE were the thyroid, pancreas, adrenal medulla and testes. More recently Kuwano et al (1983) demonstrated the presence of immunoreactive NSE in the majority of bovine pinealocytes, while McClure et al (1986) reported that some pinealocytes in the rat pineal

Figure 5.21 Low magnification photomicrograph to show the regionalized distribution of VIP-LI nerve fibres (arrows) which was observed in some pineals. Immunoreactive nerve fibres are concentrated in the periphery of one third of the section. Control; Right pineal half; VIP antiserum; Magnification 35 X.

Figure 5.22 VIP-LI nerve fibres (arrows) in the pineal of a unilaterally ganglionectomized ram. Note that there appears to be little difference in the amount of immunoreactivity shown in this photomicrograph compared to that shown in Figure 5.19. Left ganglionectomy; Left pineal half; VIP antiserum; Magnification 140 X.

Figure 5.23 VIP-LI nerve fibres (arrows) in the pineal of bilaterally ganglionectomized ram. As in Figure 5.22 there appears to be little reduction in immunoreactivity compared to that in control rams. Bilateral ganglionectomy; Left pineal half; VIP antiserum; Magnification 140 X.

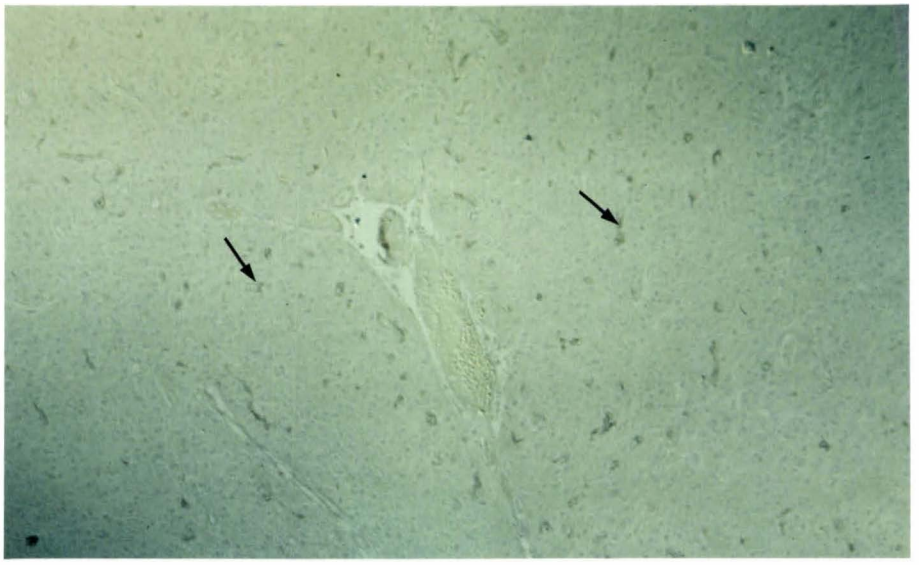
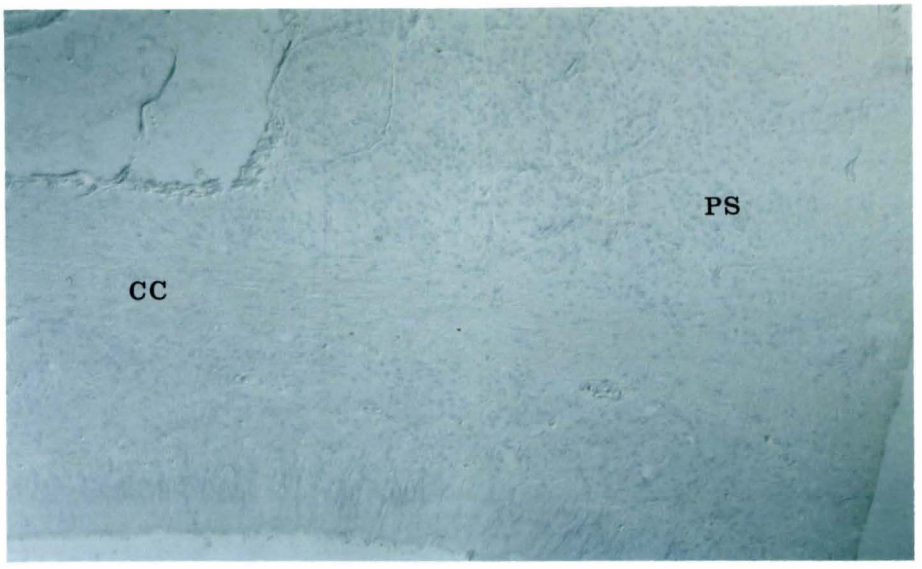


Figure 5.24 Caudal commissure (CC) of the pineal stalk of a control ram. Note that this region is totally devoid of VIP-LI. Control; Right pineal half; VIP antiserum; Magnification 140 X.

Figure 5.25 Habenular region (HC) of ganglionectomized rams. Note that, as for controls, this region is totally devoid of VIP-LI. VIP antiserum; Magnification 140 X

a) Right ganglionectomy; Left pineal half

b) Bilaterally ganglionectomy; Left pineal half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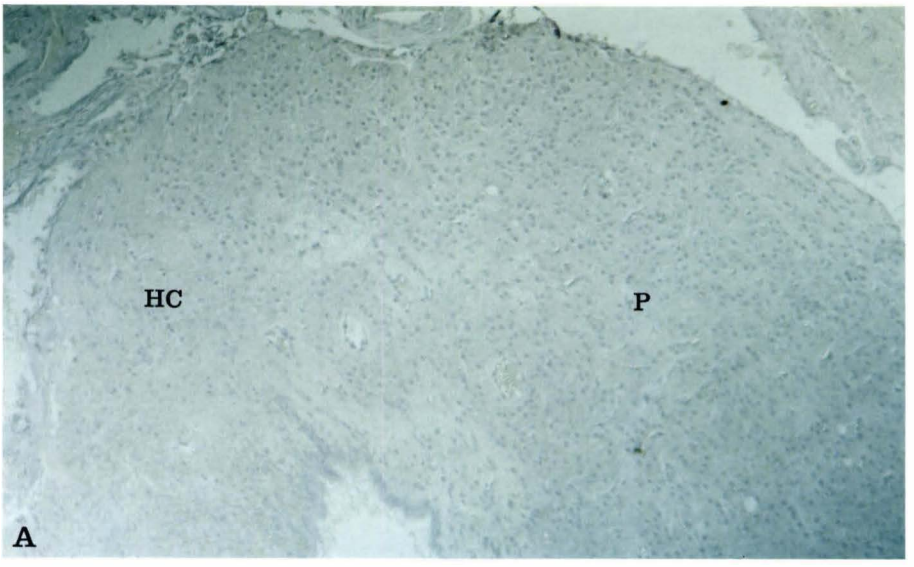
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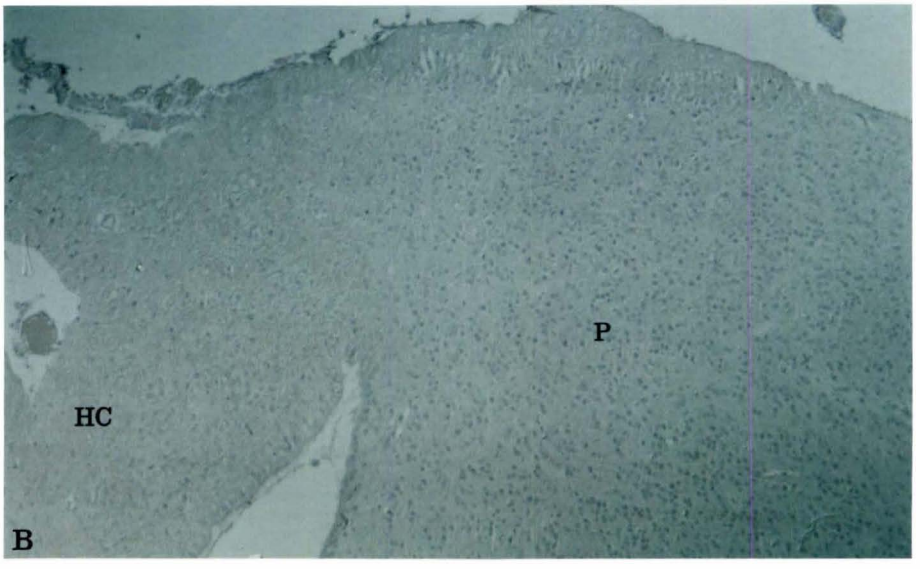
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exhibited more intense NSE staining than others and that there was a gradient of decreasing staining intensity from apex to stalk. Given that NSE is ubiquitous in its distribution in nervous tissue and given its role as a glycolytic enzyme resistant to inactivation by high neuronal chloride concentrations (Marangos et al, 1979a, b), these results lend further support to the hypothesized neuronal origin of pinealocytes. As discussed in Section 6.5.3, however, there is an increasing volume of evidence indicating the NSE may not be a definitive marker of neuronal cells since both APUD cells and neurons stain for this enzyme.

These findings had two implications in the present study. Firstly, the demonstration of NSE antigenicity enabled presumptive pinealocytes to be identified and allowed them to be differentiated from the non-neuroendocrine cells such as glial and endothelial cells (Marangos et al, 1979a, b). Secondly, the effect of sympathetic denervation on the occurrence of NSE-LI and non-immunoreactive cells could be evaluated to provide an indirect assessment of neural plasticity[2] in the ovine pineal.

Unilateral SCGX had no statistically significant effect on the number of NSE-LI cells counted. However, following bilateral removal of all known peripheral adrenergic input to the pineal, there was a significant reduction in the number of NSE-LI cells counted. Examination of the non-immunoreactive cell data suggests that that parameter is influenced by a variety of factors and may not be an accurate index of the effects of sympathetic denervation. Hence, whereas right SCGX was found to have no effect on the occurrence of non-immunoreactive cells, left SCGX reduced the frequency of such cells, but then only in the left pineal half and only at the 5% level of significance. Furthermore, the ipsilateral pineal half of left, but not right, SCGX rams exhibited similar numbers of non-immunoreactive cells to those of bilaterally SCGX rams. This variation in treatment effect on the number of non-immunoreactive cells may have been due to the fact that in addition to the resident supporting cells (eg., glial cells) the pineal contains a variable number of migratory cells such as lymphocytes, macrophages, mast cells, fibroblasts and fibrocytes (Quay, 1965), which are not uniformly distributed throughout the organ. In contrast, the frequency of interstitial space counts was found to be significantly increased throughout the pineal by bilateral SCGX, but remained unchanged following unilateral SCGX. Thus, it was concluded that bilateral, but not unilateral, SCGX resulted in a decrease in the number of NSE-LI cells counted with a

[2]The term "neural plasticity" refers to anatomical changes in the nervous system that occur in response either to neural damage or to changes in an animal's environment (Zigmond & Bowers, 1981).

corresponding increase in the volume of interstitial space. Furthermore, these results indicate that unilateral sympathetic innervation of the pineal is sufficient for maintenance of normal pinealocyte morphology in this species.

The observation that nerve fibres in the pineal originating from one SCG are able to compensate for the loss of innervation from the contralateral SCG and prevent degenerative changes from occurring indicates that these fibres expressed the property of neural plasticity (Zigmond & Bowers, 1981). Dornay et al (1985) were among the first to investigate the neural mechanism behind this compensation in the pineal and provided evidence to indicate that nerve endings of the intact sympathetic neurons branched to re-innervate cells where lesioned axons had degenerated; this process was essentially complete in 10 days and was termed "compensatory collateral sprouting". Functional recovery following unilateral denervation was evident by the return to control levels of nocturnal pineal NAT activity (Zigmond et al, 1985; Kuchel et al, 1990) and serotonin (Vassilieff et al, 1982), N-acetylserotonin and melatonin (Kuchel et al, 1990) content within a similar time-frame to that of the anatomical changes.

In the current study, the first to examine pineal morphology of sheep following unilateral SCGX, such neural compensatory mechanisms would explain why unilaterally denervated ram pineals were morphologically similar to those of control rams. Thus at the time of collection (14 days post-ganglionectomy) those pinealocytes of unilaterally denervated pineals, which had initially lost their neural contacts, had been reinnervated by collateral-sprouts from the contralaterally derived residual terminals, thus preventing a loss of pinealocyte function and subsequent degeneration. Furthermore, it appears that sympathetic fibres arising from cell bodies in each SCG innervate the contralateral, as well as the ipsilateral half of the pineal, since left and right SCGX produced very similar results in both halves. Similarly, in the rat pineal, peripheral sympathetic nerve fibres from each SCG are thought to innervate both sides of the pineal (Dornay et al, 1985; Lingappa & Zigmond, 1987).

In contrast to the effects of unilateral denervation, bilateral sympathectomy, which removed all peripheral noradrenergic input to the pineal and prevents the initiation of compensatory neural sprouting (Lingappa & Zigmond, 1987), resulted in a reduction in cell size of presumptive pinealocytes. Such an effect has been observed with the electron microscope in other species, such as rat (Karasek et al, 1983; Peschke et al, 1989; Calvo et al, 1990), hamster (Lin et al, 1975), gerbil (Welsh et al, 1979) and rabbit (Romijn, 1975) and

appears to be due to the degradation or loss of cellular components (Welsh et al, 1979) such as dense-core vesicles of the Golgi apparatus & rough endoplasmic reticulum (Romijn, 1975), nucleoli & smooth endoplasmic reticulum (Lin et al, 1975; Calvo et al, 1990), nuclei (Peschke et al, 1989), lipid droplets (both size and number, Calvo et al, 1990) and mitochondria (Karasek et al, 1983). Organelles associated with autolytic processes, such as lysosomes and autophagosomes, show a corresponding increase in number (Lin et al, 1975). Ultrastructurally, bilateral decentralization (CST section) or denervation (SCGX) of the rat pineal gland (Calvo et al, 1990) and denervation of the gerbil pineal (Welsh et al, 1979), also results in an enlargement of the interstitial space and a decrease in pinealocyte cytoplasmic volume. In sheep, bilateral SCGX results in a reduction in pineal weights (Barrell & Lapwood, 1978/79b) as has been shown to occur in rats (Peschke et al, 1989). These previous findings and those of the present study indicate that bilateral SCGX resulted in a reduction of the size of pinealocytes (NSE-LI cells of the ovine pineal) and increased the interstitial space, secondary to the degeneration of sympathetic nerve terminals.

In conclusion, the pattern of NSE-LI exhibited in the ovine pineal gland suggests that parenchymal cells have some neuron-like characteristics and establishes that the sympathetic innervation is necessary for the maintenance of normal pineal morphology. These results also provide evidence that the sympathetic nervous system has a built in compensatory mechanism, which enables residual neurons to establish neural connections with denervated cells and restore function after partial denervation. In the case of the pineal, this mechanism ensures that unilateral denervation results in no detectable long term morphological or functional alterations.

5.4.2 PNMT

PNMT-LI nerve fibres in this study were shown to occur in the parenchyma of most, although not all, ram pineals, indicating that some capacity for adrenalin production exists in this organ. However, given the relatively sparse distribution of fibre bundles it would appear that the formation of adrenalin by methylation of noradrenalin via PNMT (Fuller, 1973), is not a major function of the sympathetic fibres that innervate the ovine pineal.

Comparison with similar studies in other species supports this conclusion. For example, Jin et al (1988), in a study of hamster, rat and gerbil pineals by immunostaining with antisera to TH, DβH and PNMT, found a dense network of fibres reacting to TH and DβH, but observed that no fibres reacted to PNMT. Furthermore, levels of adrenalin

appearing in the rat pineal are also low (Saavedra et al, 1982; Saavedra & Alexander, 1983), whereas high noradrenalin content and turnover have consistently been reported to occur in these pineals, especially during the night (Wurtman & Axelrod, 1966a; Brownstein & Axelrod, 1974; Craft et al, 1984). While, Culman et al (1987) successfully measured PNMT levels in rat pineals by radiometric assay, these were between 10 & 34 times less than measured in other areas of the brain.

Interpretation of the present results in terms of adrenalin and PNMT content in the ovine pineal requires some caution as several factors may influence the apparent accuracy of these findings. For example, as suggested by Schroder & Vollrath (1985), many sympathetic nerve fibres may contain enzyme levels below the sensitivity limit of the immunocytochemical detection system and hence result in underestimation of their number and distribution. This may explain why Jin et al (1988) were unable to demonstrate PNMT-LI nerve fibres in pineals of a range of rodent species, even though a previous study had shown it to be present (Culman et al, 1987). Furthermore, the pineal content of PNMT may not reflect the adrenalin levels in sympathetic nerve fibres due to the neuronal uptake of adrenalin from the circulation and its storage in nerve terminals (Wurtman, 1966; Opacka-Juffry et al, 1991). In addition, the adrenalin content of intrapineal nerve fibres may also be enhanced by its synthesis in the neuron cell bodies located in the SCG and transport along their axons to the pineal (Livett et al, 1968).

Given the sparseness of PNMT-LI nerve fibres, results of the current study indicate that, as in other species, only minor amounts of adrenalin are synthesized in the ovine pineal. Early studies by Axelrod et al (1969), which established noradrenalin as the principal neurotransmitter of the pineal, also demonstrated that adrenalin had the potential to influence pineal biosynthetic processes by significantly increasing the concentration of serotonin, apparently through an inhibitory effect on its deamination by monoamine oxidase. Thus, adrenalin may be involved in the regulation of melatonin synthesis by indirectly promoting the metabolism of serotonin via N-acetylation and O-methylation. By demonstrating PNMT immunoreactivity in ovine pineals, this study has shown that this species may also possess a limited capacity to regulate melatonin synthesis via pineal derived adrenalin.

The finding that PNMT-LI fibres entered the pineal through the habenular and caudal commissures, and did not appear to be significantly reduced after bilateral SCGX, indicates that catecholamine-containing nerve fibres innervate the pineal principally from central regions,

although some input from peripheral sources cannot be excluded. This finding is in agreement with those of earlier studies. Bjorklund et al (1972) and Wiklund (1974) demonstrated by fluorescent microscopy that in addition to sending axons to the pineal, neurons of the rat SCG also innervated the habenular nuclei, which in turn appeared to innervate the pineal with catecholamine-containing fibres (adrenalin and noradrenalin were not differentiated) via the pineal stalk. Since only some of these fibres disappeared after bilateral SCGX it was concluded that the remainder were of central origin. More recently, Schroder & Vollrath (1985) reported that SCGX did not eliminate all DβH-containing nerve fibres from the rat pineal, leading them conclude that the remainder were derived from central loci. In the present study some of the immunoreactive fibres may have originated from the medulla oblongata, which both possesses PNMT positive neurons and projects such fibres to a variety of central nuclei (Moore & Bloom, 1979). Collectively these results add to the growing weight of evidence which indicates that there is a significant central innervation of the pineal, in addition to the now well established peripheral innervation, and furthermore, demonstrates that some intrapineal nerve fibres of central origin have the capacity to synthesize adrenalin by methylation of noradrenalin.

In conclusion, PNMT-LI fibres have been shown to be sparsely distributed throughout the ram pineal. Their origin appears to be from central loci via the habenular and caudal commissures. Given the low levels of PNMT activity and adrenalin concentrations and high levels of noradrenalin found in the pineals of other species, it is likely that the pineal has a limited capacity to produce adrenalin and that it probably does not play a critical role in regulating pineal function. It is unfortunate that in the present work the only DβH antiserum available did not perform satisfactorily, as an evaluation of its intrapineal distribution may have been valuable in determining what proportion of nerves capable of producing noradrenalin also exhibited the potential to produce adrenalin. A comparative analysis of DβH and PNMT immunoreactivity (through use of double-staining or staining of serially cut sections) would thus have provided an assessment of the relative importance of locally synthesized sympathetic neurotransmitters in the neural regulation of pineal function and, in conjuction with SCGX, would have shown if there was a differential origin of the two types of fibres.

5.4.3 NPY

The results of this study clearly demonstrated a dense, mainly perivascular, network of NPY-LI nerve fibres in the ovine pineal gland, similar to that reported to occur in gerbil pineals (Shiotani, 1986). This pattern contrasts with that observed in human (Moore & Sibony, 1988), rat (Schon et al, 1985; Reuss & Moore, 1989), mink (Møller et al, 1990), guinea pig (Schroder & Vollrath, 1986) and hamster (Schroder, 1986) pineals, all of which exhibited a relatively sparse, non-uniform distribution of fibres.

A recent study by Williams et al (1989) also established that the ovine pineal contains a dense, perivascular network of NPY-LI fibres. However, whereas in the present study both a relatively uniform distribution of immunoreactive fibres throughout all regions of the pineal, and the occurrence of non-vascular NPY-LI nerve fibres in the pineal stalk were recorded, Williams et al (1989) reported a non-uniform distribution with dorsal and peripheral pineal regions exhibiting a more extensive network of immunoreactive fibres than did the central region. Also, those authors found the stalk to be almost totally devoid of NPY-LI fibres, although some beaded fibres were present. These differences in results between the present experiment and that of Williams et al, (1989) may be explained by the differences in the time of day of pineal gland collection. Whereas pineal glands in the present study were obtained 5-6 hrs after the onset of darkness, those obtained by Williams et al (1989) were collected during the middle of the day. Hence, although a circadian rhythm of pineal NPY content or synthesis has not yet been demonstrated in any species, the production and pineal concentration of this neuropeptide may exhibit a circadian rhythm similar to that of rat pineal noradrenalin (Wurtman and Axelrod 1966a; Craft et al, 1984; Racke et al, 1989) since their storage and release from sympathetic nerve terminals has been shown to be closely related (Hakanson et al, 1986; Ebadi et al, 1989; Linton-Dahlof, 1989).

The results of the sympathetic denervation study indicated that most, but not all, ovine pineal NPY-LI nerve fibres have their neuron cell bodies located in the SCG, since removal of one or both ganglia resulted in a very substantial decline in, but not total loss of, pineal NPY-LI nerve fibres. Previously Schon et al (1985) demonstrated a similar effect in rat pineals following bilateral SCGX, although in that study both NPY-LI fibres and radio-immunoassable NPY completely disappeared. Thus it would appear that neuron cell bodies of NPY-LI fibres innervating the rat pineal are located solely in the SCG, but in sheep, while these fibres originate predominantly in the SCG, others have their perikarya elsewhere. Further evidence

supporting the SCG origin of most pineal NPY-LI nerve fibres has been obtained from retrograde axonal transport studies in which the injection of a fluorescent or wheat germ tracer into rat (Reuss & Moore, 1989) or gerbil (Shiotani et al, 1986) pineals resulted in the detection of labelled neuron cell bodies in the SCG, up to three-quarters of which exhibited NPY-LI. Upon leaving the SCG these fibres appear to travel in association with the internal carotid artery and supratentorial vessels, before entering the pineal with the vascular supply (Schon et al, 1985; Schroder, 1986; Reuss & Moore, 1989).

The origin of pineal NPY-LI fibres resistant to the effects of uni- or bilateral SCGX is less certain. In the present studies these fibres appeared to enter the pineal predominantly through the habenular region of the stalk, but were also observed to enter via the caudal commissure and with some arterial vessels. Reuss and Moore (1989), who observed NPY-LI fibres in the rat pineal stalk, hypothesized that these represented a group of aberrant sympathetic fibres as first suggested by Kappers (1960). However, several central loci, previously demonstrated to influence pineal electrical activity (Korf & Møller, 1984), have been shown to also contain NPY-LI fibres and cell bodies; hence it is possible that these loci influence pineal function via the projection of fibres containing NPY. In particular, Chromwall et al (1985) have shown by indirect immunofluorescence and radioimmunoassay techniques, the existence of relatively high concentrations of immunoreactive NPY and high densities of NPY-LI fibres and/or cell bodies in rat paraventricular, suprachiasmatic and habenular nuclei. More recently, Møller et al, (1990) has demonstrated that the NPY innervation of mink pineals originates largely from the central nervous system and hypothesized that their neuron cell bodies were located in the intergeniculate leaflet of the lateral geniculate body. Thus, there appears to be a multitude of central loci which may influence pineal function via the direct projection of NPY containing nerve fibres; the location of such loci in sheep apparently has not been studied. A further possible source of NPY fibres are the sphenopalatine and otic ganglia. These cranial parasympathetic ganglia have been demonstrated in the rat to contain NPY immunoreactive cell bodies (Leblanc et al, 1987), although it has not been established if these ganglia send fibres to the ovine pineal as they do in gerbils (Shiotani et al, 1986). Such a parasympathetic innervation of the ovine pineal would support the study of Taylor et al (1980) in which the presence of muscarinic receptors was demonstrated.

The function of NPY in ovine pineals is uncertain. In vitro studies have shown NPY to have no effect on ovine pineal melatonin release (Morgan et al, 1988b; Williams et al, 1989) or cAMP synthesis (Morgan et al, 1988a; Williams et al, 1989). In contrast, in vitro (Vacas et al, 1987) and in vivo (Reuss & Schroder, 1987) effects of NPY on rat pineals include an increase in NAT and HIOMT activity and in melatonin production, while a reduction in blood flow in rabbit pineals has been recorded during NPY infusion (Nilsson, 1991). In other tissues, NPY has been shown to be released during sympathetic nerve stimulation (Pernow, 1988; Linton-Dahlof, 1989) and to have a variety of effects (Lundberg et al, 1982; Agnati et al, 1983; Dahlof et al, 1985; Linton-Dahlof, 1989), many of which may be exerted in the pineal during periods of heightened sympathetic activity.

In view of its many actions and the wide distribution of NPY in the peripheral and central nervous system (Ebadi et al, 1989), as well as its presence in the pineal, it is likely this neuropeptide plays an important, but as yet undetermined, physiological role, possibly as a neurotransmitter.

5.4.4 VIP

The present study has demonstrated that the sheep pineal gland is innervated by VIP-LI nerve fibres which are associated solely with the vasculature of the pineal. A previous study investigating VIP immunoreactivity in sheep pineal glands reported a similar distribution of immunoreactive nerve fibres (Cozzi et al, 1990). The presence of a VIP innervation of the pineal is also in accordance with that observed in other species such as the gerbil (Møller et al, 1985; Shiotani et al, 1986), cat (Uddman et al, 1980; Møller et al, 1981), rat (Mikkelsen, 1989), rabbit and pig (Uddman et al, 1980), although some differences in the distribution and density of immunoreactivity have been reported. For example, whereas in the present study no VIP-LI nerve fibres were reported in the pineal stalk or in the habenular or caudal commissures, Møller et al (1981) has described VIP fibres penetrating deeply into the cat pineal from the anterior and caudal commissural areas. Similarly, Uddman et al (1980)have also reported VIP nerve fibres in the cat pineal stalk. In gerbils the pineal stalk appears to be a significant region of immunoreactivity (Shiotani et al, 1986). Furthermore, the only previous study examining VIP-LI in sheep pineals reported that a few fibres were observed in the pineal stalk, although these were not confirmed as definitely entering the pineal (Cozzi et al, 1990). Species differences in the density of VIP-LI nerve fibres have also been reported

(Uddman et al, 1980) suggesting that there is some variability in the number of VIP-containing nerve fibres innervating the pineal of various species.

A feature of pineal VIP-LI reported in other studies, but not observed in the current study, is the occurrence of VIP-LI nerve fibres in the parenchyma of the pineal, between the pinealocytes and without association with blood vessels. This pattern has been observed in the pineal of several species (Uddman et al, 1980; Mikkelsen, 1989), including sheep (Cozzi et al, 1990). In the latter study, the use of cryoprotected thick (12-14 μm) sections may have aided the detection of the small number of intraparenchymal VIP-LI nerve fibres not visualized with the immunocytochemical methods employed in the current study.

Bilateral and unilateral SCGX, performed to determine the origin of VIP-LI nerve fibres innervating the sheep pineal, revealed that the principal origin appears to be located within the central nervous system, although SCGX did appear to cause some decrease in VIP-LI fibres. This is in general agreement with the previous study of Shiotani et al (1986) and that of Ravault et al (1990). In gerbils, for example, VIP-containing neuron cell bodies have been demonstrated in the pterygopalatine ganglion (Shiotani et al, 1986), which projects axons to the pineal, while the apparent lack of effect of bilateral SCGX on pineal VIP-LI nerve fibres lead Ravault et al (1990) to conclude that in sheep this peptidergic innervation is independent of the SCG.

Numerous studies have identified a variety of other cranial structures and central loci which both contain VIP-LI neuron cell bodies and are known to, or thought to, project axons to the pineal. These include hypothalamic nuclei such as the SCN (Card & Moore, 1984; Abrams et al, 1985; Antonopoulos et al, 1987; Reuss et al, 1989b; Morin et al, 1991; Okamoto et al, 1991) and PVN (Antonopoulos et al, 1987; Morin et al, 1991), the dorsal hippocampus (Abrams et al (1985) and cranial parasympathetic ganglia such as the pterygopalatine and otic ganglia (Shiotani et al, 1986; Leblanc et al, 1987; Møller et al, 1987a). Techniques such as retrograde neuronal tracing in combination with immunocytochemical detection of VIP in labelled neuron cell bodies are required to determine which of these loci project VIP-containing nerve fibres to the pineal of sheep.

In contrast to the previously mentioned study of Ravault et al (1990), the present study found that there apparently was a small reduction in VIP-LI in the sheep pineal gland following bilateral or unilateral SCGX. This suggests that a small number of VIP-containing fibres which innervate the pineal originate from the SCG. Several studies lend support to this

hypothesis. Immunocytochemical evaluation of VIP-LI in the rat SCG by Sasek & Zigmond (1989) demonstrated that not only were there a small number of VIP-LI neuron cell bodies in these ganglia, but also that its three major trunks (CST, ICN & ECN) all exhibited immunoreactive nerve fibres. In addition, Uemura et al (1987) reported that many neuron cell bodies of the dog SCG exhibited VIP-LI and that following bilateral SCGX there was a substantial reduction in the number of VIP-LI nerve fibres found in association with the major cerebral blood vessels. Given such species differences in the incidence of SCG VIP immunoreactivity, there is a clear need to examine its presence in sheep SCG and to determine whether or not VIP-LI nerve fibres project to the pineal from the SCG.

The presence of VIP in the pineal indicates that this neuropeptide is of some functional importance. To date VIP has been shown to be a potent stimulator of pineal NAT activity under both in vivo (Yuwiler, 1983b; Schroder et al, 1989) and in vitro (Yuwiler, 1983a, b; Kaku et al, 1985) conditions and to increase melatonin output from perifused rat pineal glands in a dose dependent manner (Simonneaux, 1990). Furthermore, VIP increased cAMP content of sheep pineals and augmented the cAMP response to isoprenaline infusion (Morgan et al, 1988a). In addition, cGMP has also been demonstrated to accumulate in isolated rat pinealocytes in response to VIP infusion (Ho et al, 1987). Evidence that VIP may also act directly on sympathetic nerve terminals came from studies by Schwarchild & Zigmond (1989, 1991) in which VIP was found to increase tyrosine hydroxylase activity in the pineal, iris, submaxillary gland and heart. These effects are likely to have been achieved through interaction with VIP-specific receptors (Yuwiler, 1983b; Møller et al, 1985; Morgan et al, 1988a; Simonneaux et al, 1990) and augment those achieved with α and ß adrenergic agonists (Yuwiler, 1983b, 1987; Ho et al, 1987; Morgan et al, 1988a). In addition, there also appears to be a diurnal rhythm of sensitivity of the pineal to the effects of VIP with prior light exposure increasing the in vitro NAT and cAMP responses to VIP infusion (Kaku et al, 1985). These findings prompted Kaku et al (1985) to suggest that environmental lighting may alter the function of VIP as a neuromodulator.

Finally, the presence of VIP and its coexistence with ACh in neuron cell bodies of cranial parasympathetic ganglia (Leblanc et al, 1987) known to project nerve fibres to the pineal (Shiotani et al, 1986), suggests that the pineal receives a parasympathetic innervation. This supports earlier studies which demonstrated that there are muscarinic receptors in the sheep pineal at approximately 5% of the density of ß-adrenergic receptors (Taylor et al, 1980).

This finding further demonstrates that regulation of pineal function is achieved through the complex interaction of all three divisions of the nervous system (central, sympathetic & parasympathetic) and not solely through the activity of sympathetic nerves, as once thought.

5.5 Conclusions

In the present study, immunocytochemical evaluation of intact and sympathetically denervated ovine pineal glands has demonstrated that its innervation originates from at least two sources (peripheral sympathetic and central loci) and has provided morphological evidence of pineal dysfunction following total sympathectomy. In contrast, partial denervation by unilateral SCGX did not result in appreciable morphological changes (as evidenced by NSE-LI) indicating that remaining nerve terminals exhibited a degree of neural plasticity through the process of compensatory collateral sprouting. Furthermore, the present study has shown that in sheep the methylated derivative of noradrenalin (adrenalin) and the neuropeptides NPY and VIP may influence pineal function, in addition to the effects of the principal neurotransmitter of the pineal, noradrenalin. These results support the growing weight of evidence suggesting that the regulation of pineal function is not simply achieved through the release of noradrenalin from sympathetic nerve terminals, but is the result of both central and peripheral influences acting through the release of a multitude of neurochemicals. The contribution of the central nervous system to the range of such neural agents in the pineal is poorly understood, as is its influence on pineal secretory function. Further studies employing immunological and electrophysiological methods are needed to fully understand how peripheral and central influences are integrated to regulate pineal secretory activity.

CHAPTER 6

General Discussion and Conclusions

6.1 Introduction

The experiments presented in this thesis used both electrical nerve stimulation and selective denervation techniques to investigate the involvement of the sympathetic nervous system in regulation of ovine pineal gland function. At the time these studies were initiated electrical stimulation of the pineal's sympathetic innervation in acutely and chronically prepared animals, with concurrent sampling of peripheral venous blood for melatonin assay, was an entirely novel approach to the study of the neural regulation of pineal secretory function. Since then one report has been published in which the authors measured plasma melatonin levels during CST stimulation in acutely prepared animals, arguably employing a more direct measure of pineal secretion by sampling cranial venous blood (Chan et al, 1989). The chronic implantation of stimulating electrodes and subsequent induction of melatonin secretion in conscious rams remains a unique achievement in the study of pineal physiology, albeit with only limited success. Sympathetic denervation of the pineal by SCGX was used in a complementary study to investigate the relationship between pineal morphology (both parenchymal and neural) and its sympathetic innervation.

From these stimulation and denervation experiments it was concluded that: (i) normal pineal function in rams is dependent on an intact sympathetic innervation, which both regulates the level of melatonin secretion and maintains the structural integrity of pinealocytes, (ii) the ram pineal receives a degree of central innervation and (iii) induction of melatonin secretion through activation of sympathetic fibres innervating the ovine pineal produces a melatonin secretory response markedly different from that of many other species, especially rodents.

The choice of sheep, and in particular rams, as the experimental animals for these studies was made for two main reasons:

(1) The effect of CST stimulation could be evaluated through assessment of concurrent changes in levels of melatonin occurring in the blood. As a consequence of the constraints of animal size and RIA methodology, previous rodent studies of this nature have only been able to measure parameters of secretory potential (ie., pineal NAT & HIOMT activity, eg., Bowers & Zigmond, 1980, 1982; Reuss et al, 1989a) rather than sequential

changes in concentrations of melatonin occurring in the blood as in this thesis. This represented a significant advantage over previous studies, such as those cited above, in which each data-point was generated by averaging the values obtained from 6-10 animals. In those studies, it was possible that the between-animal variation concealed or dampened changes in the parameter of interest where otherwise individual curves may have highlighted such changes. The relative merits of individual and average (mass) curves have been discussed elsewhere (Medawar, 1945; Sholl, 1954).

Other species, while also providing the benefits derived from using sheep, may, for various practical and physiological reasons, be deemed less suitable for such experiments. For example, goats are generally more difficult to maintain in a laboratory setting, being more agile, socially interactive and aggressive than sheep, and thus more likely to dislodge any chronically attached experimental devices such as cannulae, electrical connectors and electrodes. Anatomically, pigs are also less well suited for these experiments since their thick layers of subcutaneous adipose tissue, deeply situated vagosympathetic trunks and smooth body contours make the placement and maintenance of electrodes more difficult and provide little purchase for the external attachment of electrical connectors. Moreover, much less is known about the physiology of the pineal of these and other candidate species, thus making interpretation of experimental results more difficult.

(2) Rams were chosen for these experiments, rather than ewes, because current evidence indicated that in ewes gonadal steroids exert some feedback control on pineal function through changes in pineal β-adrenoceptor characteristics (density and binding affinity) (Foldes et al, 1984, 1985). Concerns that this may lead to variable levels of pineal sensitivity to CST stimulation, especially during the 16-17 day oestrous cycle, when plasma ovarian steroid levels exhibit substantial fluctuations (Goodman, 1988), have recently been justified (Maxwell et al, 1989a, b). Similar cyclic fluctuations do not occur in rams (Lincoln & Short, 1980) and hence reproductive steroids were unlikely to have had such substantial effects on pineal function during the course of an experiment.

6.2 Sympathetic regulation of ovine pineal gland function

The acute and chronic stimulation experiments together demonstrated that increased sympathetic nerve activity increased plasma melatonin levels. While it might be argued that this melatonin may have originated from sites other than the pineal (eg., retina and areas of

the gastrointestinal tract), which are known to have the potential to synthesize melatonin (Quay, 1965; Gern & Karn, 1983), it is generally accepted that these extra-pineal sites secrete insignificant amounts of melatonin compared to that from the pineal (Pang, 1985). Indeed, sympathetic denervation of the pineal gland eliminates the nighttime rise of melatonin levels in the blood of sheep (Lincoln & Short, 1980; Lincoln et al, 1982; Maxwell et al, 1989b), indicating that most nocturnal melatonin in peripheral plasma originates from the pineal gland. In order to more dramatically demonstrate the effects of CST stimulation on pineal melatonin secretion, it may (in theory) have been appropriate to employ the method of Colombo et al (1987), in order to sample cerebral venous blood which had just perfused the sheep pineal; this should more accurately reflect the level of melatonin secretion by the pineal, since it would overcome dilution effects in the peripheral circulation. However, results of the study by Colombo et al (1987) highlighted the need to ensure that the cannula tip is correctly positioned in the sagittal sinus in order to collect pineal venous blood.

Results of the present stimulation studies (Experiments 2, 3 & 4) provided direct evidence of a link between increased sympathetic nerve activity and increased pineal melatonin secretion, whereas previously this has only been surmised from indirect observations. See Sections 1.6.3 & 3.4.2 for examples of such observations.

The presence of a diurnal rhythm of pineal sensitivity to CST stimulation (Experiment 2) extends the results of earlier studies which have demonstrated similar rhythms in various parameters of pineal function or secretory potential (eg., rat pineal NAT activity increases more rapidly in response to CST stimulation at night rather than during the day (Bowers & Zigmond, 1982)), while pineal β-adrenoceptor sensitivity (Romero & Axelrod, 1974) and density (Gonzalez-Brito et al, 1988b; Pangerl et al, 1989) increase during periods of low sympathetic nerve activity.

The finding in the first chronic stimulation experiment (Experiment 3) that length of photoperiod had no significant influence on plasma melatonin content is in agreement with previous studies which demonstrated that there were no significant differences in nocturnal plasma melatonin concentrations between sheep exposed to different artificial photoperiods (Kennaway et al, 1983), or between sheep held outdoors and sampled throughout the year (Maxwell et al, 1989b). Thus, the length of photoperiod does not appear to influence the level of nocturnally or electrically induced melatonin secretion.

The immunocytochemical study (Experiment 5) was designed to complement the preceding stimulation studies by identifying specific antigens (NSE, PNMT, NPY & VIP) in intact and sympathetically denervated ovine pineals. This study demonstrated that: (i) the sympathetic innervation of the ovine pineal includes a peptidergic (NPY) element, which originates principally from the SCG, (ii) a second peptidergic innervation (VIP) originates principally from cranial (CNS and/or parasympathetic ganglia) loci; both NPY and VIP fibres appear to innervate the whole of the pineal, (iii) nerve fibres originating in the SCG play an essential role in maintaining the morphology of the pineal (NSE results), and (iv) the pineal is innervated by adrenalin-containing nerve fibres which appear to originate exclusively from central regions (PNMT results).

It is apparent from these experiments (2, 3, 4 & 5) that the pineal's sympathetic innervation plays an essential role in the initiation and maintenance of melatonin secretion and that this is likely to be achieved by the release of noradrenalin and NPY (and possibly VIP) from sympathetic nerve terminals. Adrenalin and VIP release from nerve fibres originating from the CNS and/or cranial ganglia may also play a role, albeit minor, in regulation of melatonin secretion.

Comparative influence and distribution of pineal sympathetic nerve fibres

Results of the present studies and those conducted previously in sheep indicate that the regulation of melatonin secretion by the pineal's sympathetic innervation and the distribution of sympathetic nerve fibres within the pineal exhibits some marked differences compared to those of some commonly studied rodent species. These differences may be summarized as follows:

(1) Induction of melatonin production and/or secretion by electrical or pharmacological stimulation of the sympathetic innervation occurs within minutes of the onset of stimulation (Namboodiri et al, 1985a, b; Morgan et al, 1988b; Experiments 2, 3 & 4) and does not exhibit the time lag observed in rodents for NAT synthesis (Bowers & Zigmond, 1982; Reiter, 1988; Reuss et al, 1989a), or the re-activation of NAT activity following a pulse of light at night (Namboodiri et al, 1985a). Thus, an NAT-independent mechanism, not previously described in other species, appears to regulate the initial period of melatonin synthesis and release in sheep (Namboodiri et al, 1985a, b).

(2) In addition to the established β-adrenoceptor mediated increase in cAMP concentrations (Morgan et al, 1988a; Howell & Morgan, 1991), there is evidence suggesting

that control of melatonin secretion in ovine pineals may occur primarily through α-adrenoceptors at a step beyond serotonin N-acetylation (Sugden et al, 1985a). However this has been disputed in subsequent papers (Morgan et al, 1988a, b) and a role of lesser importance has been ascribed to that receptor subtype (Morgan et al, 1989).

(3) The pattern of aminergic innervation observed in the sheep pineal consists of a few fibres associated with the vasculature, but with the vast majority coursing through the parenchyma of the gland (Owman, 1965; PNMT results, Chapter 5). A review of the literature by Vollrath (1981) has highlighted that this is only one of a variety of patterns present in the pineals of a diverse range of species.

(4) The peptidergic innervation of the sheep pineal also exhibits some differences compared to that of other species. For example, NPY-LI nerve fibres were shown in Chapter 5 to project intraparenchymally as well as perivascularly, which contrasts with the non-uniform distribution of NPY fibres in other species (see Section 5.4.3). Similarly, VIP-LI nerve fibres, while confined to a perivascular location in sheep pineals, have been observed in the pineal stalk of other species without any vascular association (see Section 5.4.4).

Characteristics of neural stimulation influencing melatonin secretion

One aim of experiments in this thesis in investigating the role of the pineal's sympathetic innervation was to induce melatonin secretion by electrical stimulation of the CST's in anaesthetized and conscious rams and thus produce plasma melatonin levels similar to those recorded at night. Comparison of plasma melatonin concentrations recorded in control rams in Experiment 1, with those recorded from stimulated rams in Experiment 2, revealed that they were similar both in magnitude and pattern of change and that there was a similar degree of between-animal variation. To a lesser extent this was also true in Experiments 3 & 4, although, on average, plasma melatonin concentrations were decidedly less than those recorded in Experiment 2.

Given the latter results, it is appropriate to consider what components of the neural signal may contribute to a more effective stimulus in terms of pineal melatonin secretion.

The component of neural stimulation most often shown to influence endocrine responses is the pattern of neural stimuli delivered to the gland under study (ie., intermittent or continuous stimulation). Bowers & Zigmond (1982), in what appears to be the only study comparing the effects of intermittent and continuous stimulation on indicators of pineal secretory potential, demonstrated that stimulation of the CST's with intermittent stimuli of

frequencies averaging 2.5 Hz could be up to twice as effective in increasing NAT activity when compared to continuous stimulation at 2.5 Hz. Similarly, the pattern of neural stimulation has been shown to be important in control of secretion by other endocrine organs (see Section 4.4). Also, the finding of Iggo & Vogt (1960), that impulses in individual cat CST fibres occur in bursts and are not generally continuous, indicates that the pineal probably receives a similar pattern of stimuli.

An additional feature of impulse transmission in this section of the sympathetic nervous system is signal processing by the SCG. As demonstrated in electrophysiological (Birks & Isacoff, 1988) and biochemical (Birks, 1979, 1982) studies, transmission through the SCG is significantly enhanced when using intermittent bursts of stimuli rather than during continuously applied stimuli. Studies by Bowers & Zigmond (1982) have demonstrated that such SCG processing may influence the onset of NAT activity in the rat pineal. Thus it appears that both the SCG and the pineal are sensitive to the temporal pattern between stimuli and exhibit enhanced responses to intermittent stimuli compared to continuous stimuli of the same mean frequency. Further studies are needed to determine whether or not this increased responsiveness to intermittently applied stimuli also enhances pineal melatonin synthesis and secretion.

6.3 Central influences on pineal function.

Results from Experiment 5 provided evidence for a possible central innervation of sheep pineals (ie, following bilateral SCGX pineal glands continued to exhibit immunoreactive NSE, NPY, VIP and PNMT nerve fibres, particularly in the stalk). As discussed in Chapters 1 & 5, there is also an increasing volume of evidence from electrophysiological (Schapiro & Salas, 1971; Dafny, 1980; Reuss et al, 1984; Reuss, 1987), retrograde neuron tracing (Guerillot et al, 1982; Møller & Korf, 1983a, 1986) and lesion studies (Møller et al, 1987b) indicating that various central structures have direct neural connections with the pineal. Many of these sites are suggested to process or relay information about environmental or social conditions (eg visual processing by the dorsal nucleus of the lateral geniculate body) and hence may act as secondary routes for information of this nature to influence pineal function. To date, studies which have identified these possible sources of central innervation have been performed almost exclusively in rats and other rodents. In the current study however, the origin of immunoreactive nerve fibres was not determined, although in other species a number of central

nuclei known to project fibres to the pineal have also been demonstrated to contain catecholaminergic and peptidergic immunoreactive neuron cell bodies. For example, NPY-like immunoreactive nerve fibres have been demonstrated in cat, rat, monkey and g olden hamster hypothalamus, rat amygdala, monkey limbic regions and rat retrohippocampal region (reviewed by Ebadi et al, 1989), while nerve fibres containing catecholamines or enzymes involved in their metabolism have been demonstrated in the habenular area (Bjorklund et al, 1972; Wiklund, 1974), brainstem (Moore & Bloom, 1979) and hypothalamus (Culman et al, 1987). Further studies using neuronal tracing techniques and double immunostaining are needed to determine which neurochemicals are present in nerve fibres projecting to the pineal from these regions.

Although no function has yet been ascribed to these central innervations it appears possible that they may influence pineal function indirectly through effects such as the regulation of blood flow (Pernow, 1988), modification of the density of pineal α-adrenoceptors, as has been suggested for NPY in the medulla oblongata of the rat (Agnati et al, 1983), increasing sheep pineal cAMP content by VIP (Morgan et al, 1988a) or by enhancing the adrenoceptor-mediated response to sympathetic stimulation, as shown to occur in the heart in the presence of NPY (Dahlof et al, 1985). The observations that bilateral SCGX of sheep completely abolished the circadian rhythm of melatonin release and that melatonin is not detectable in the blood after this procedure (Maxwell et al, 1989b) indicates that, at least in sheep, fibres innervating the pineal from other loci do not exert significant stimulatory influences on melatonin secretion.

An indirect influence of the central input resulting in an enhancement of melatonin synthesis has been demonstrated in at least one lesion study conducted in rats in which disruption of central fibres from the PVN and hippocampus resulted in a significant reduction in nocturnal levels of NAT and HIOMT activity (Møller et al, 1987b). Although similar studies have not been conducted in sheep, a similar modification of enzyme activity cannot be discounted in this species since the findings of Experiment 5 clearly demonstrated that the ovine pineal is innervated by centrally derived nerve fibres. One means by which to investigate the influence of these fibres would be to electrically stimulate the cranial end of sectioned CST's at night, both in the dark and under lights. Comparison of plasma melatonin levels and pineal enzyme activity under these conditions may demonstrate that central fibres

entering through the stalk influence the night-time stimulated (via the CST's) secretion of melatonin and that this effect is reduced by light.

6.4 Integrated neural control of ovine pineal function

Although it is clear from this and other studies that the sympathetic innervation plays the principal role in the regulation of melatonin secretion, it is unclear how the two innervations interact to initiate or modify pineal secretory responses to environmental or autonomic stimuli. Regulation of ovine pineal function is similar to that of other mammalian species in that it is primarily mediated by the sympathetic nervous system. To date, only one immunocytochemical study has been published which demonstrated a second source of innervation (VIP) originating from the brain of this species (Ravault et al, 1990). As stated previously, there is currently no definitive data available on the role of this central innervation.

Other influences on ovine pineal physiology are not well understood. Of the neuropeptides which have been identified in ovine pineals only vasoactive intestinal peptide has been shown to influence pineal function by augmenting the isoproterenol stimulated increase in cAMP levels in vitro (Morgan et al, 1988a). Arginine vasopressin, luteinizing hormone-releasing hormone, NPY, thyrotropin releasing hormone, melanocyte stimulating hormone, melanocyte inhibitory factor and somatostatin were without effect (Morgan et al, 1988a). NPY has also been identified in ovine pineals (Williams et al, 1989; Experiment 5), but thus far its role in the pineal physiology of this species has not been determined (Morgan et al, 1988a, b; Williams et al, 1989). The present denervation study has also demonstrated the existence of a central innervation of the ovine pineal but its function is currently unknown.

6.5 Future directions for ovine pineal research

The current state of our understanding of ovine pineal physiology and particularly its control by the sympathetic and central innervation is limited. To date, the vast majority of pineal studies have utilized small mammals even though such studies are generally limited in the type of sample which can be obtained from each animal (ie., the pineal gland and a single blood sample) (eg., Ho et al, 1984; Tang et al, 1985; Chick et al, 1987). In contrast to the extensive use of sheep in experiments designed to determine the influence of the pineal on such events as seasonal breeding, relatively few research groups have used sheep to study

fundamental pineal physiology and consequently basic research in these animals is not as advanced as it is in some rodent species. A significant advantage of using sheep is their ability to tolerate serial blood sampling. This allows each animal to be used throughout an experiment and hence improve the precision of the results. Furthermore, it has been shown (Experiments 2 & 3) that the effect of a treatment can be measured not only in terms of an indicator of secretory potential, which may not accurately reflect final output (Wheler et al, 1979), but also in terms of melatonin secreted into the blood and hence a more accurate measure of the secretory response. Additional sampling of CSF may serve to quantify a second, potentially important, route of melatonin secretion (Rollag et al, 1978; Shaw et al, 1989). Future areas of ovine pineal research which could utilize these advantages and advance our overall knowledge of pineal physiology are discussed below.

6.5.1 Melatonin secretion during CST stimulation

Initial studies need to be directed towards optimizing the pineal secretory response during acute or chronic CST stimulation. At least two areas should be investigated as follows:

(i) enhancement of the performance of the stimulating electrodes by the use of different wires (eg., platinum) or adoption of electrode designs similar to those reported by other researchers (Bowman & Erickson, 1985). This aspect has been more fully discussed in Chapter 4.

(ii) Optimization of the pattern and parameters of stimulation. As discussed earlier in this chapter the pattern of stimulation may be particularly important in determining the level of response from the pineal as well as other neuroendocrine organs, with intermittent generally being more effective than continuously applied stimuli. Even if intermittent bursts of stimuli increase secretory responses, the effects of several other stimulus parameters need to examined. For example, Dutton & Dyball (1979), in their study of the rat neurohypophysis, concluded that the increased efficiency of intermittent bursts of stimuli in inducing in vitro vasopressin release was correlated with the firing rate within a burst and the interpulse interval, and was not related to the mean pulse frequency or the total number of bursts. Similarly, Birks (1978) demonstrated that it was not the number of stimuli or continuous high frequency stimuli that increased SCG ACh stores (a measure of ganglionic transmission), but rather the presence of a high frequency (10-40 Hz) component within the intermittent firing pattern of fibres in the

CST's. These components of stimulation need to be thoroughly investigated in order to fully evaluate their influences on pineal secretory responses.

6.5.2 Electrical activity, central innervation and secretory activity of the ovine pineal gland

To date, very little is known about the electrical activity and central innervation of the pineal of mammals, other than that of a few rodent species. While the present thesis has established that the ovine pineal receives both a peripheral and central innervation, no studies have been reported which have investigated the influence of neural activity on pineal electrical activity or the relationship between electrical activity and secretory responses. The latter point has been an area of much speculation and debate in recent years, especially in the field of rodent pineal physiology, although no resolution of this question has been reached (Reuss, 1987).

The following areas of study may help to elucidate the relationship between these aspects of pineal physiology as well as establishing the sources of the central innervation.

(i) Electrical activity

Although defined under a variety of conditions in some species of rodents (Taylor & Wilson, 1970; Ronnekleiv et al, 1980; Stehle et al, 1987), no attempt has been made to establish the ovine pineal's electrical properties, although this species offers some significant advantages for such studies (eg., correlation of electrical and secretory activity in response to nocturnal, electrical or pharmacological stimuli). Initial studies in this species might thus attempt to define any circadian rhythm of spontaneous electrical activity as shown to be exhibited by pinealocytes of other species (eg., Ronnekleiv et al, 1980; Stehle et al, 1987) and for the first time correlate such activity with plasma and/or CSF melatonin levels in each animal.

In addition to recording spontaneous electrical activity, many studies investigating pineal electrophysiology have attempted to evoke electrical responses from pinealocytes by stimulation of the pineal's sympathetic (SCG) (Reyes-Vazquez & Dafny, 1985; Reuss, 1987; Demaine & Patel, 1989) or central (Pazo, 1981; Semm et al, 1981; Reuss et al, 1984; Stehle et al, 1987) innervation, or by the iontophoretic application of drugs, usually neurotransmitters (Demaine & Patel, 1989; Stehle et al, 1989), and have shown that while many pinealocytes are under the influence of the sympathetic innervation, other 'silent' pinealocytes may be influenced by

central inputs. Such studies also need to be conducted in sheep to determine if the pineal is sensitive to a similar range of influences. An additional study which could be conducted in sheep would involve concurrent stimulation of the sympathetic and central innervation of the pineal to examine the interaction of these neural sources and their effects on pinealocyte electrical activity, enzyme levels (eg., NAT, HIOMT), second messenger concentrations (eg., cAMP) and plasma melatonin levels, as well as the identification, by RIA, of changes in biochemical indicators of altered secretory function. Morphological studies should be conducted using both light and electron microscopy in order to precisely identify the location and type of cellular elements involved in any changes evoked by the experimental treatment. In this way a significant contribution may be made to our understanding of the influences affecting pineal electrical and secretory activity and, perhaps more importantly, the way in which the central innervation acts to modify the secretory potential of the pineal.

(ii) Identification of central inputs

As a first step in defining the role of the ovine pineal's central innervation it is first necessary to identify the location of cell bodies projecting fibres to the pineal. Techniques such as retrograde and anterograde neuronal tracing (LaVail & LaVail, 1972; (Guerillot et al, 1982; Møller & Korf, 1983a, 1987; Reuss & Møller, 1986; Tamamaki & Nojyo, 1987), lesioning of selected brain areas (Møller & Korf, 1983b; Chafetz & Gage, 1983; Korf & Møller, 1984; Møller et al, 1987b), stimulation of central nuclei (Pazo, 1981; Semm et al, 1981; Reuss et al, 1984; Stehle et al, 1987) immunocytochemistry (Bjorklund et al, 1972; Wiklund, 1974; Korf & Møller, 1984), and various combinations of these methods, have been used extensively to determine the type and location of central and peripheral fibres innervating rodent pineals. Their application to the study of ovine pineal innervation is currently confined to immunocytochemical studies and requires significantly more attention to better define the central inputs in particular. Several technical problems will need to be addressed before they can be used successfully in sheep (eg., retrograde neuron tracer dilution in long nerve fibres and relative lack of stereotaxic atlases for accurate placement of brain lesions and stimulating electrodes in available breeds, sex and weights of sheep), but with perseverance and determination these can be overcome to extend our knowledge of pineal central innervation in a species whose pineal physiology appears to quite different from that of rodents.

6.5.3 Immunocytochemistry and its role in ovine pineal studies

In addition to the findings of Chapter 5, the application of immunocytochemical methods to the study of ovine pineal physiology and morphology has demonstrated that a wide variety of peptides and proteins are localized within this gland. Substances not studied in this thesis, but which have been detected in ovine pineals, include photoreceptor-like proteins (Foster et al, 1986), neurohypophyseal hormone-like factors (Noteborn et al, 1988b), neurophysins (Reinharz et al, 1985), peptide histidine isoleucine (Ravault et al, 1990), somatostatin (Lew & Lawson-Willey, 1987), glycoproteins (Meiniel et al, 1986) and delta-sleep-inducing peptide (Noteborn et al, 1988a). Serotonin and melatonin have also been identified in the ovine pineal by immunocytochemistry (Tillet et al, 1986).

Further studies in which immunocytochemistry would prove useful include:

(1) Determination of the neuron-like characteristics of pinealocytes. Recent studies attempting to determine if mammalian pinealocytes possess neuron-like characteristics have shown that while there are similarities in the morphology of neurons and pinealocytes (Vollrath & Schroder, 1987; Oksche, 1987), differences in electrophysiological properties speak against the neuronal nature of these cells (Vollrath & Schroder, 1987). This issue remains unresolved, although immunocytochemical studies using antibodies raised against neuron-specific compounds (e.g., NSE, neurofilaments, neural adhesion molecule, synaptophysin and tubulin) have provided further, but not conclusive, evidence supporting a neuron-like nature of pinealocytes (McClure et al, 1986; Vollrath & Schroder, 1987; Schroder et al, 1990; Chapter 5, NSE results). Further immunocytochemical studies using highly specific antibodies (e.g., protein gene product 9.5 (Wilson et al, 1988; Ramieri et al, 1990)) raised against neuron-specific antigens, in association with morphological and electrophysiological studies, are needed to determine what similarities exist between pinealocytes and neurons.

(2) Studies on neural plasticity and neural development in the pineal. As discussed in Chapter 5 (NSE results), intrapineal nerve fibres of the ovine pineal appeared to exhibit the characteristic known as neural plasticity. Because of its dense innervation, the ovine pineal appears to be a good model for further studies of this property of nerve fibres and of the development of the pineal's sympathetic innervation in newborn lambs. These studies might include:

(i) The effects of unilateral SCGX on pineal immunoreactivity (e.g., DßH, PNMT, acetylcholine transferase), NAT activity and plasma melatonin profiles in adult rams 1, 5, 14

and 21 days after the operation. In rats, axonal injury such as this has been shown to result in a temporary reduction in neural function within the pineal (Kuchel & Zigmond, 1991). Bilateral denervation, on the other hand, results in a permanent reduction in immunoreactivity exhibited by intrapineal nerves (Zhang et al, 1991). A detailed study of the reinnervation and recovery of function of the pineal following unilateral SCGX has not yet been done in any species.

(ii) The effects of pre-ganglionic nerve lesions (CST section) compared to those following SCGX (resulting in post-ganglionic nerve lesion) on pineal immunoreactivity, NAT activity and melatonin secretion. Previous studies in rats have shown that these treatments do not result in a similar degree of pineal dysfunction (Kuchel et al, 1990).

(iii) Postnatal development of ovine pineal immunoreactivity relative to the maturation of the nocturnal plasma melatonin profile and development of increased night-time NAT activity. Recent work by Li & Welsh (1991) has shown that in newborn hamsters and gerbils there is a gradual increase in numbers of pineal TH and NPY immunoreactive nerve fibres during the first 7 days of life. However, this has not been correlated with the maturation of the secretory function of the pineal. Furthermore, it would be of interest to examine the effects of unilateral SCGX in these animals on the development of pineal immunoreactivity and on plasma melatonin profiles as there appear to be differences in the ability of newborn and adult animals to respond to this type of lesion (Kuchel & Zigmond, 1991).

(3) As yet there appear to be no reports of studies attempting to characterize the presence or content of neurotransmitters (e.g., adrenalin, noradrenalin, ACh) or neuropeptides (e.g., VIP, NPY) in the sheep SCG using immunocytochemistry or radioimmunoassay. Given that the major sympathetic innervation and at least one peptidergic innervation (NPY) arise from the SCG, this type of study would provide valuable supporting evidence for the SCG as a source of other neurochemicals found in the pineal (e.g., VIP).

6.5.4 *In vivo* pharmacological manipulation of neural activity.

Although pineal α- and β-adrenoceptor functions have been studied extensively in rodents (Sugden et al, 1984; Gonzalez-Brito et al, 1988a, b), a similar state of knowledge has not yet been attained in sheep in which precise functions of these receptors are uncertain (Sugden et al, 1985a; Morgan et al, 1989). The methods developed in this thesis may be

useful in defining the role(s) of each receptor type. For example, the administration of β-antagonists during CST stimulation (or dark exposure) may reveal whether or not β-adrenoceptors are important in the initiation of melatonin secretion, as might the administration of α-antagonists with respect to α-adrenoceptor function. A further area of interest would be to investigate the effects of chronic CST stimulation on adrenoceptor (α & β) density and sensitivity, to establish whether these receptors in sheep are influenced by sympathetic activity as they are in rats (Romero & Axelrod, 1974; Gonzalez-Brito et al, 1988b).

Comparatively little is known about possible routes, other than neuronal, by which neuroregulatory peptides might influence pineal function. Previous studies have shown that intra-arterial injection of NPY increased daytime NAT activity in rat pineals (Reuss & Schroder (1987) and that intracerebroventricular administration of VIP results in a similar response in Mongolian gerbil pineals (Schroder et al, 1989). Studies of this nature in sheep may help to elucidate the role of these neuropeptides in regulating pineal function.

6.5.5 Anaesthesia and its role in pineal studies

While in any acute experiment anaesthesia is an absolute requirement for invasive surgery, the present studies have shown that various anaesthetics inhibit melatonin secretion to varying degrees depending on their ability to inhibit neural transmission. It may also be of interest to investigate the effect of other anaesthetics on nocturnal melatonin secretion (eg., chloralose, which is often used as the anaesthetic of choice in acute physiological experiments, G.W. Reynolds, personal communication) as their selective action to inhibit or not inhibit pineal function may be of value in subsequent studies. For example, urethane has been shown to have little affect on the induction of pineal NAT activity suggesting that the neural pathway mediating this event is not inhibited by this anaesthetic agent (Shivers, 1978), xylocaine as a local anaesthetic can be used to selectively block activity in the pineal's sympathetic innervation (Dafny , 1980) and pentobarbitone may be useful in determining how reduced blood flow affects pineal function (Goldman & Sapirstein, 1973). Thus, selective use of various anaesthetics may prove to be a valuable tool in the study of pineal function.

6.6 Conclusion

In conclusion, it is now apparent that while the physiology of the ovine pineal gland shares some similarities with the more commonly studied rodent species, many of its features

are markedly different. This thesis has gone some way in providing a clearer understanding of these differences, but, like most studies, has raised as many if not more questions than it has attempted to answer. There now appears to be an array of new opportunities to study the mammalian pineal, limited only by the imagination and skills of those who accept the challenge.

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