RADIOIMMUNOASSAY AND IMMUNOCYTOCHEMICAL STUDIES ON THE RECOVERY OF PINEAL INNERVATION AND FUNCTION FOLLOWING UNILATERAL DENERVATION

A thesis presented in partial fulfilment of the requirements for the
Degree of Master of Science in Physiology
at Massey University

JOHN RICHARD PRANGLEY

December, 1993

Thesis Abstract

The sympathetic noradrenergic neurons of the superior cervical ganglia provide the major source of innervation to the pineal gland. Studies described in this thesis were designed to further investigate the initial decline and subsequent recovery of pineal melatonin secretory capacity which has been reported in sheep after unilateral superior cervical ganglionectomy (Lapwood, 1993). Further to that, the compensatory mechanism proposed by Dornay, *et al* (1985), of re-innervation of denervated tissue by residual nerve fibres originating from the intact SCG, was investigated.

Melatonin secretory capacity is advocated as a superior index of pineal function with direct measurement of pineal output. Radioimmunoassay was used to measure dark period plasma levels of melatonin prior to and at 1, 3, 7, 14, 21 and 28 days after unilateral SCGX. Initial response to partial denervation was a reduction in secretory capacity by 80% of pre-operative levels, followed by a linear recovery to pre-operative levels at 21 days after surgery, which was sustained at 28 days.

Immunocytochemical localization of GAP-43 determined that nerve regeneration occurs in the pineal gland as a reponse to unilateral SCGX. GAP-43 in nerve fibres was most prominent at 3 days after surgery after which followed a linear decline to pre-operative levels in measurements taken at 28 days. An association between nerve terminals and the membranes of pinealocytes was observed at 28 days, suggesting those cells were the target of new nerve growth.

The presence of nerve growth maturity corresponded with the recovery in pineal function and for this reason the compensatory mechanism of re-innervation is reasoned to be responsible for that recovery.

Immunocytochemical localization of alpha tubulin established the presence of that component of microtubules in the cytoplasm of pinealocytes, where it is suggested to function in the process of hormone secretion. No variance in the presence of alpha tubulin was measured in any treatment group indicating that cell integrity was maintained and that atrophy did not occur, despite partial denervation.

The findings of this study have confirmed a role for re-innervation in the full recovery of pineal melatonin secretory capacity after unilateral SCGX and has demonstrated that the SCG-pineal complex is a very useful model for future studies correlating nerve growth and functional regeneration.

Acknowledgements

Special thanks to my supervisor, Dr K. R. Lapwood for enthused encouragement and support throughout this program.

Thanks to Dr D.H. Carr and Dr G.W. Reynolds who gave their time to oversee the induction and maintenance of anaesthesia during surgery.

I am also grateful to Mr E. Hunt for support in animal management, Mrs I. Hall for technical assistance with the surgical procedures and Ms. E. J. Candy for assistance with the radioimmunoassay for melatonin.

In the Histology Section it was a pleasure to be guided by Mr R.I. Sparksman who was of invaluable assistance with the photomicroscopy, Mrs A. Jolly for technical advice with microtomy and Mr M.J. Birtles for valued comment.

To Dr. K. Meiri (State University of New York Health Science Centre) thanks are due in appreciation for the kind donation of antisera for GAP-43.

This work was supported by grants from the Massey University Research Fund and the Department of Physiology and Anatomy and was approved by the Massey University Animal Ethics Committee.

Table of Contents

List of Abbreviations

5-HT	5-hydroxytrypyamine or serotonin
AT	Alpha tubulin
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
cAMP	cyclic Adenosine monophosphate
CGRP	Calcitonin gene related protein
ChAT	choline acetyl transferase
CNS	Central nervous system
CSF	Cerebrospinal fluid
CST	Cervical sympathetic trunk
CV	Coefficients of variation
DAB	Diaminobenzadine
d.f.	degrees of freedom
DIC	Differential contrast optics
DSIP	Delta sleep-inducing peptide
ECN	External carotid nerve
GAP-43	Growth associated protein (43 kD)
GFAP	Glial fibrillary acidic protein
GnRH	Gonadotrophin releasing hormone
HCl	Hydrochloric acid
HIOMT	Hydroxyindole O-methyltransferase
HP	High power magnification (313X)
hr	hour
ICC	Immunocytochemistry
ICN	Internal carotid nerve
IR	Immunoreactive or immunoreactivity
IU	International Units
LH	Luteinizing hormone
LHRH	Luteinising hormone releasing hormone
LP	Low powered magnification (125X)
M	Molar
min	minute
NaCl	Sodium chloride
NAT	N-acetyltransferase
NE	noradrenaline
NGF	Neural growth factor
NO	Normal optics
*n*m	nano meter
NPY	Neuropeptide-Y
NSE	Neuron specific enolase
NSE-LI	Neuron specific enolase-like immunoreactivity
OXT	Oxytocin
PKC	protein kinase C
PNMT	Phenylethanolamine N-methyltransferase
Post-op	Post-operative
Pre-op	Pre-operative
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
TRH	Thyroid releasing hormone
VIP	Vasoactive intestinal polypeptide

List of Figures

CHAPTER 1

Review of Literature

1. Introduction

The pineal gland is an unpaired organ, situated in the roof of the third ventricle of the brain, which controls a number of circadian and seasonal rhythms, through its secretion of melatonin at night (Reiter, *et al*, 1981). Its principle nerve supply is via post-ganglionic sympathetic fibres which originate in the superior cervical ganglia (Kappers, 1965). See Section 1.3.4.

As discussed in section 1.3.5, one technique which has been used to study the control of pineal gland function, is denervation by bilateral superior cervical ganglionectomy. Occasionally unilateral SCGX has also been utilized. In one such study Lapwood (1993) found that while melatonin secretory capacity was abolished after bilateral SCGX and was reduced to 92% of pre-operative levels on day 1 after unilateral SCGX, it recovered to 77% by day 14 after surgery for that group. It was suggested that recovery of function after unilateral surgery, may have been due to re-innervation of denervated pineal endocrine cells (pinealocytes) by collateral sprouting of nerve terminals originating from the remaining SCG.

The experiment described in this thesis investigated whether full restoration of pineal melatonin secretory capacity occurred if the post-surgery period was extended to 28 days. In addition, a study was undertaken to investigate whether evidence of re-innervation of the pineal could be demonstrated.

The aim of Chapter 1 is to provide an overview of the literature relating to both the pineal gland and the regeneration of nerves, as is pertinent to this thesis.

1.1 Early history of pineal research

Early anatomists held various views on the physiological function of the pineal in the human. This unique unpaired structure, that lies deeply recessed under the cerebral hemispheres of the brain, drew their attention and speculation. According to Kappers (1979) and Oksche (1984), Herophilas, an anatomist at the University of Alexandria in Egypt, was first to discover the pineal, around 300 BC. The philosopher Descartes considered it the "seat of the soul". The possible physiological significance of the pineal was first recognised by Heubner in 1898, who noted precocious sexual maturity in a young boy whose pineal was destroyed by a tumor. Holmgren (1917/1918) noted that the cells of the pineal gland of an elasmobranch were sensory-like in nature: the pinealocytes resembled the sensory cells of the retina. Because some reptiles possess a prominent "third eye" the pineal of mammals was considered a vestige of this primitive visual organ. The observation that the human pineal may become calcified at an early age further consolidated thought that the pineal was, indeed, a vestigial organ and therefore of little physiological consequence. However, in 1954, Kitay and Altschule reviewed the literature on human pineal tumors and described clinical correlations of pineal dysfunction with evidence clearly revealing that the pineal may in some way be related to reproductive functions in humans.

McCord and Allen (1917), interested in endocrine factors affecting morphogenesis, observed that bovine pineal extracts added to the water in which tadpoles swam caused the larvae to blanch. In 1958 dermatologist Aaron Lerner, in searching for a factor which might be responsible for vitiligo, was able to isolate and determine the structure of the bovine pineal extract as N-acetyl-5-methoxytryptamine, an indoleamine, which he named melatonin. This molecule can now be readily synthesized and made available for a variety of physiological studies.

1.2 Seasonal adaptive changes mediated by the pineal

1.2.1 Seasonal Reproduction

Many mammals in their natural habitat are seasonal breeders. Seasonal reproduction is one of the more conspicuous changes that natural populations of mammals rely on for their survival. It ensures the birth of the young during those seasons of the year in which their chances of survival are greatest (Bronson, 1988). Clearly, the most favourable seasons for supporting the survival of offspring are those in which food is accessible and environmental conditions are mild, in the spring and summer seasons (Karsch, *et al*, 1984). Diverse species mate during various seasons of the year so that birth occurs during those favourable seasons.

There are potent exogenous factors on which animals rely for the synchronization of their annual cycles. Most biometerological parameters change throughout the course of the year and animals could have selected any one of these to guide or determine their annual cycle of reproduction (Stonehouse, 1981). However, some factors change with greater regularity than others. One of the most dependably recurring phenomena in the environment is the photoperiod, consequently it has great predictive value in terms of anticipating the upcoming season. Hence, it is logical that many mammals have come to depend on the seasonal changes in photoperiod to synchronize their annual reproductive rhythms, as it is both essential and advantageous for these species to initiate reproduction at approximately the same time each year, before the optimal conditions for birth and rearing have arrived (Reiter, *et al*, 1981).

1.2.2 Photoperiod and the pineal gland

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 (Reiter, 1980). Central to seasonal reproductive adjustments in response to light is the pineal gland. Although the photic information is detected by the retinae of the lateral eyes (Moore, 1978), it is the pineal that tranduces (Wurtman, *et al*, 1968) the resultant neural information into a chemical signal that determines the level of reproductive activity.

The pineal is a small organ located near the centre of the brain, that functions as an endocrine organ which secretes melatonin. As an end organ of the visual system in mammals, the pineal gland's production and secretion of melatonin are affected by light which causes a drop in blood levels of the compound. As day length (and therefore night length) varies seasonally, the pineal gland, because of the secretion of melatonin, provides information concerning time of year to all other organs of the body. Thus in animals whose reproductive patterns fit into a specific seasonal scheme the pineal may play a pivotal role in the control of their gonadal function (Kauppila, *et al*, 1987). Hence the pineal gland is essential to the chronobiology that assists an animal in adapting to the external environment, both daily and seasonally (Reiter, 1991).

1.2.3 Reproductive seasonality in sheep

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 domesticated sheep, use the decreasing daily photoperiod of autumn to time the initiation of breeding activity and generally have long gestation periods eventuating in spring parturition (Nalbandov, 1976). These modern sheep breeds have developed as a result of controlled breeding programmes intended to improve meat and wool production, and to increase fecundity (Carter & Cox, 1982). Marshall in (1937) was the first to define the reproductive cycle of sheep, with Hammond (1944) later establishing the importance of photoperiod in regulating the onset and termination of reproductive activities. Yeates (1947, 1949) in early studies investigating seasonal reproduction in sheep, concluded that, seasonal variation in the length of photoperiod was the predominating factor determining the time of onset and the duration of the breeding season. A change from increasing to decreasing photoperiod induced in both rams and ewes to commence behavioural characteristics associated with the onset of reproductive activity. Ram behaviour associated with increasing reproductive activity occurs in conjunction with elevated testosterone secretion from the testes. Characteristic behaviour includes increased libido, inter-male aggression and the occurence of flehmen, the raising of the upper lip in order to facilitate the detection of olfactory stimuli originating from vaginal secretions (Lincoln & Short, 1980). Behavioural oestrus of ewes is characterised by sexual receptivity towards the ram, culminating in pro-active behaviour by some ewes. Conspicuous signs of behavioural oestrus are however, mostly absent, with rams detecting oestrous ewes by pheremonal signals from their vaginal secretions (Smith, 1982).

Seasonal reproductive capacity may also be measured by hormonal, physical and physiological changes in both rams and ewes. The initiation and cessation of reproductive activity is a reflection of the changing secretory profiles of pituitary gonadotrophins and gonadal steroids (Lincoln, *et al*, 1977). Ram testis size is greatest during the breeding season and least during sexual quiescence (Tulley & Burfening, 1983). Sperm output and quality (motility and percentage of live spermatazoa) (Dufour, *et al*, 1984; Boland, *et al*, 1985) and ejaculate volume (Sanford, *et al*, 1977; Barrell & Lapwood, 1978/1979a; Boland, *et al*, 1985) are highest during the breeding season and lowest during sexual quiescence. For ewes, the onset of breeding activity is initiated by cyclic changes in ovarian hormones 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).

In addition to light and pheremonal factors influencing reproductive seasonality in sheep, both nutritional and temperature variations may be observed. Through effects of inhibition of luteinizing hormone secretion, low levels of nutrition result in reduced levels of reproductive activity, delaying both the onset of puberty and of the breeding season. On the other hand high nutrition levels are associated with increased reproductive activity (Lindsay, *et al*, 1984; Bronson, 1988; Rhind, *et al*, 1989a). A study of the effects of temperature on the breeding cycle of Clun ewes has indicated that temperature, at least in this breed, may play a secondary, but important, role in timing the onset of breeding activity (Lees, 1971).

1.3 Pineal function

1.3.1 Pineal development and morphology

The vertebrate pineal, a part of the epithalamus, arises as a median evagination of the diencephalic roof of the embryonic brain (Oksche, 1965). In some mammals the pineal gland moves away from the roof of the third ventricle and loses connection with the brain except for a thin 'pineal' stalk. The gland is richly perfused with blood vessels derived from the posterior cerebral arteries. The venous drainage of the gland is directly into large venous sinuses which surround the organ (Reiter, 1991).

Pineal parenchymal cells, pinealocytes, are derived from the ependymal lining of the epithalamus; both light and dark parenchymal cells can be distinguished in the mammalian pineal gland (Oksche, 1965). The dark cells contain pigment granules of an unknown nature, as well as glycogen deposits of undefined physiological significance. Dark pinealocytes are interconnected by tight junctions, suggesting that electrical signals may be communicated between the cells (Reiter, 1977). The main body of the pinealocyte, the parikaryon, has either one or two processes emanating from it. These processes terminate in buds which lie in close proximity to pericapillary spaces or inter-cellular lacunae. The actual relationship of the terminals with the pericapillary space varies between species and is perhaps related to the mode of release of the secretory products (Reiter, 1977). The number of pinealocytes may decrease in advanced age, when calcium deposits, which can be visualised radiologically, also form in the gland (Reiter, 1991). Fibroblasts and glial cells make up the rest of the cellular components of the glandular mass which, in an adult sheep weighs about 60-80 mg and measures approximately 5-7mm in length, and 3-5 mm in width (Barrell & Lapwood, 1978/1979b; Vollrath, 1981).

1.3.2 Pineal indoleamine biosynthesis

The biochemistry of pineal indoleamine biosynthesis is well documented (Relkin, 1976; Sugden, 1989; Wurtman, *et al*, 1968). Indoleamine biosynthesis involves pinealocyte uptake of the amino acid, L-tryptophan, from the blood (King & Steinlechner, 1985). Conversion by hydroxylation to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase follows. The aromatic enzyme 5-hydroxytryptophan decarboxylase acts on the hydroxylated derivative to form 5-hydroxytryptamine. Serotonin concentrations are higher in the pineal than in any other organ or brain region (Quay, 1964). Serotonin is converted to N-acetylserotonin by the action of N-acetyltransferase (Klein & Weller, 1970). The N-acetylserotonin produced is O-methylated by hydroxyindole-O-methyltransferase to form N-acetyl-5-methoxytryptamine (melatonin) (Axelrod & Weissbach, 1960). The methyl group in this latter conversion is provided by S-adenosylmethionine.

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 & Steinlecher, 1985). It is considered that the increase in N-acetylserotonin concentration acts by a mass action effect to enhance the production of melatonin (Adrendt, 1985).

Although acetylation to N-acetylserotonin is a necessary step in the biosynthesis of melatonin, deamination of serotonin by monoamine oxidase can also occur in the pineal. The deaminated product may either be oxidized to 5-hydroxyindoleacetic acid or reduced to 5-hydroxytryptophol. The latter compounds can then become O-methylated by HIOMT to give 5-methoxyindole acetic acid and 5-methoxytryptaphol (Wilson, 1978).

The formation of melatonin may also occur from methoxytryptophan, although this is a minor synthetic pathway (Morton, 1987).

1.3.3 Effect of light on pineal indoleamine biosynthesis

Within the pineal conversion of serotonin to melatonin is a highly cyclic event which is closely related to the prevailing light : dark cycle to which animals are exposed. In all animals thus far studied, melatonin production is greatest within the pineal gland during the dark phase of the light : dark cycle (Quay, 1964; Lynch, 1971; Panke, *et al*, 1978). Pineal serotonin levels also reveal marked diurnal changes with highest levels noted during daylight hours and depressed levels during darkness.

Pineal enzyme activities are rapidly depressed by light (Reiter, *et al*, 1986). At night there is an increase in the activity of NAT in rat pineals which is 10- to nearly 100-fold greater than values in the light (Adrendt, 1985). The pineal concentration of N-acetylserotonin is subsequently increased to values ten to thirty times greater than observed under day conditions. HIOMT activity also increases, which results in nocturnally elevated levels of pineal melatonin (Adrendt, 1985).

In experimental conditions reversal of external lighting periods reverses the rhythm of pineal enzyme activity and indoleamine biosynthesis. Thus a diurnal rhythm of pineal melatonin synthesis is observed but with maximum levels measured during the true day when lights are off. Shaw, *et al* (1988) observed a cessation of melatonin production in sheep exposed to continuous light, with normal night time levels recurring within 10 mins of lights off.

Studies using monochromatic light have demonstrated that not all wavelengths are equally effective in suppressing pineal melatonin synthesis and secretion. 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 suppressors of pineal HIOMT activity. That review also reports between-species differences in effectiveness of various wavelengths in altering melatonin production.

In sheep, the intensity of light required to suppress nocturnal pineal melatonin levels in a dose-dependent manner has been shown to range between 1.02 to 88.60 lux, with 88.6 lux producing a >80% reduction (Arendt & Ravault, 1988). The duration of light exposure that can inhibit pineal melatonin synthesis during a period of darkness is very short, as little as 1 sec for the Syrian hamster. Return to night-time melatonin levels after a light pulse may take 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).

1.3.4 Neural control of pineal indoleamine biosynthesis

Melatonin is synthesised in response to norepinephrine released from postganglionic sympathetic neurons originating from the SCG's. Thus the pineal is considered to be a neuroendocrine transducer, as neural input to this organ is converted into an endocrine output (Wurtman, *et al*, 1968). Postganglionic stimulation of the pinealocyte cells depends on the absence of light activation of the retina of the lateral eyes. Light information perceived by the eyes is transduced into a neural signal by the retinal ganglion cells and then conveyed to the suprachiasmatic nuclei of the brain by way of the retinohypothalamic tract. This pathway is always bilateral and decussates at the optic chiasma innervating the contraleral SCN (Mess & Ruzsas, 1986).

Neuronal fibres from the SCN, which convey information to the pineal on the status of the environmental photoperiod, then course through the medial forebrain bundle down to the upper thoracic spinal cord. Axons from the preganglionic neurons, located in the intermediolateral cell columns of the spinal cord, synapse within the SCG. From these ganglia postganglionic fibres proceed to innervate the pinealocytes in the pineal gland (Mess & Ruzsas, 1986). Prior to their entrance into the gland many of the sympathetic fibres coalesce to form two bilaterally symmetrical nervi conarii which, in some mammals, fuse before entering the pineal. Within the pineal the fibres branch extensively and with the onset of darkness release noradrenaline from their terminals, followed by interaction of the catecholeamine with beta adrenergic receptors in the pinealocyte membrane (Pangerl, *et al*, 1990). Beta-adrenergic stimulation activates an adenylate cyclase enzyme via a stimulatory, guanine nucleotide-binding, regulatory protein (Spielgel, 1989). This results in a rapid and large (up to 60-fold in the rat pineal) increase in intracellular cyclic adenosine monophosphate. cAMP serves as a second messenger in the nocturnal elevation of melatonin biosynthesis by activating a cAMP

dependent protein kinase. Transcription of mRNA follows, initiating an eventual rise in serotonin NAT activity (Sugden, 1989).

In addition to sympathetic innervation controlling pineal biosynthesis there is also evidence for a possible central innervation of sheep pineals. Immunocytochemical studies have demonstrated immunoreactivity for various substances including somatostatin, GnRH, Substance P, CGRP, TRH, DSIP, NSE, AVP, OXT, GnRH NPY and VIP, as well as the enzymes ChAT and PNMT, within pineal nerve fibres, particularly in the stalk (Mockett, 1991). Also electrophysiological (Schapiro & Salas, 1971; Dafny, 1980; Reuss, *et al*, 1984; Reuss, 1987), retrograde neuron tracing (Guerillot, *et al*, 1982; Moller & Korf, 1983a, 1986) and lesion studies (Moller, *et al*, 1987b) indicate that various central structures have direct neural connections with the pineal in a range of species. For example, NPY-like immunoreactive nerve fibres projecting to the pineal from central nuclei have been demonstrated in the hypothalamus of cat, rat, monkey and golden hamster. Other peptidergic projections exhibited include rat amygdala, monkey limbic regions and rat hippocampal region (reviewed by Ebadi, *et al*, 1989). Catecholaminergic neurons with a central origin have been demonstrated in the habenular area (Bjorkland, *et al*, 1972; Wiklund, 1974), brainstem (Moore & Bloom, 1979) and hypothalamus (Culman, *et al*, 1987).

Although no function has yet been ascribed to these central innervations, it appears possible that they may influence pineal function indirectly as demonstated in 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 (Moller, *et al*, 1987b). Also, Morgan, *et al* (1988) demonstrated a VIP dose-dependent effect on cAMP in sheep pineal homogenates, suggesting that modification of enzyme activity by centrally derived nerves is possible in this species.

Similar conclusions may be drawn from the findings of Mockett (1991) who clearly demonstrated the presence of NPY, VIP and PNMT immunoreactive nerve fibres within the ovine pineal. While regulation of ovine pineal function is similar to that of most other mammalian species, in that it is primarily mediated by the sympathetic nervous system, it is unclear to what extent the two innervations interact to initiate or modify pineal secretory response. The various central structures having direct neural connections with the pineal are suggested to process or relay information about environmental or social conditions (e.g. 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.

1.3.5 Techniques used to evaluate the control of pineal melatonin synthesis.

That the pineal gland, through secretion of melatonin, is responsible for transducing photoperiodic cues into physiological adjustments to hormone profiles, is well established (Lincoln, 1980; Lincoln & Short, 1980; Karsch, et al, 1984). Four principal experimental methods have been used to investigate the relationships between control, function and biosynthesis associated with the gland. These are pinealectomy, superior cervical ganglionectomy, melatonin administration and photoperiod manipulation. Typical results from use of these methods, particularly with sheep, and the advantages and disadvantages of each, are given briefly in this section.

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 (Barrell & Lapwood, 1979b). Although pinealectomy is relatively difficult to perform in sheep (Roche & Dziuk, 1969), it does provide the only definitive means by which an animal's response to changes in environmental lighting, in the absence of pineal influences, can be measured. 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).

Denervation of the pineal gland by bilateral SCGX 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 (Barrell & Lapwood, 1978/1979b; Lincoln, 1979). The principal advantage of this surgical

method as compared to 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.

As mentioned in the Introduction (page 1), unilateral SCGX is a technique which occasionally has been used in studying the control of the pineal. More detail is given here on the results and conclusions from those studies, than for those using the other techniques mentioned in this section, because of the central place of unilateral SCGX in the studies described in this thesis.

Reiter, *et al* (1978) established that unilateral SCGX caused pineal NAT and melatonin levels to be intermediate between those in sham-operated control rats and those in rats from which both ganglia had been removed. From that experiment it was concluded that unilateral SCGX impaired the ability of a portion of the pinealocytes to respond to darkness. Zigmond, *et al* (1981) measured a 75% decrease in NAT activity immediately after unilateral SCGX, however, subsequent measurements determined a recovery in pineal NAT activity to levels similar to those measured prior to surgery. That group proposed that residual nerve fibres originating from the intact SCG had a "reserve capacity" to stimulate denervated pinealocytes, whereby the loss of approximately 50% of sympathetic nerve fibres caused a reduction in nerve "re-uptake" of NE. Subsequent increases in NE levels were suggested to cause an increase in stimulation of denervated pinealocytes and hence the recovery in NAT levels.

Later, Dornay, *et al* (1985) also measured a >50% decline, followed by a recovery, in both TH activity and NE uptake after unilateral SCGX. These results led to the proposal that the mechanism responsible for the recovery entailed "collateral sprouting" from residual nerve fibre terminals, culminating in the re-innervation of denervated pinealocytes. Anatomically, this process requires that nerve terminals of fibres from each

SCG are in close proximity to each other so that following unilateral SCGX and degeneration of the lesioned axon terminals, collateral sprouts developing from the intact nerve terminals were able to contact and re-innervate nearby denervated tissue. Evidence demonstrating that fibres from each SCG cross over to innervate the contralateral as well as the ipsilateral sides of the pineal, and that nerve terminals from each SCG are closely intermingled, has been demonstrated in rats (Dornay, *et al*, 1985; Zigmond, *et al*, 1981, 1985; Lingappa & Zigmond, 1985) and also in sheep (Mockett, 1991).

Mockett and Lapwood (unpublished) recently found that atrophy of NSE-IR presumptive pinealocytes, which was observed after bilateral SCGX, did not occur in animals killed 14 days after unilateral SCGX. In an attempt to explain why normal cell morphology was maintained after single SCGX, Lapwood (1993) took the physiological approach of measuring melatonin secretion profiles during exposure of sheep to darkness, before and periodically after unilateral SCGX. On the day after surgery there was a reduction of pineal melatonin secretory capacity to values 92% ($P< 0.001$) below those measured before surgery, however, succeeding measurements indicated a substantial recovery of that parameter of melatonin biosynthesis, to within 77% of pre-operative levels after 14 days. These results suggested that a full recovery in pineal secretory capacity was possible, despite a reduction of sympathetic nerve fibres in the pineal gland by approximately half.

Reviews by Wurtman, *et al* (1963) and Reiter (1980) have addressed the topic of potent antigonadal effects on the mammalian reproductive system in response to exogenous melatonin injection.

Several studies using administration of melatonin to sheep have focused on the effectiveness of that hormone in advancing the onset of ovarian cyclicity in seasonally anoestrus ewes. Collectively their results indicated that during the initial stages of

anestrus, 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 anestrus, 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).

As a separate technique or in conjunction with those described earlier, 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 described in this section, such as those by Hafez (1952), Barrell & Lapwood (1979a,b), Lincoln & Short, (1980) and Robinson & Karsh (1987), have involved combinations and/or variations of these techniques. Further studies investigating the role of light in sheep pineal physiology have experimented with a range of lighting regimes. Matthews, *et al* (1992) studying endogenous pacemaker effects on melatonin biosynthesis, housed sheep in conditions of acutely extended darkness with results suggesting a functional role for the suprachiasmatic nuclei in sensitizing the photoreceptive system to seasonal changes in photoperiod. Shaw, *et al* (1988) have demonstrated, by placing sheep in constant darkness following exposure to prolonged continuous light for 28 days, that the response in melatonin production is rapid, often commencing within 10 min, regardless of what time of the day dark conditions were imposed. This not only indicates that the synthetic mechanisms which generate melatonin are maintained in an inducible state under constant illumination, but that under these conditions the pattern of melatonin secretion is not dominated by the historical photoperiod.

Results derived from these experiments have all been instrumental in advancing our knowledge of pineal physiology, its control mechanisms and the gland's role in mediating the effects of photoperiod.

1.3.6 Post-ganglionic noradrenaline activity

With the onset of darkness, sympathetic neurons increase their firing rate (Taylor & Wilson, 1970) causing a rise in NE levels (Levitt, *et al*, 1965) and a significant increase in NE turnover (Brownstein & Axelrod, 1974). NE ultimately derives from tyrosine, which, in the pineal, is produced primarily from dietary phenylalanine by the rate limiting enzyme tryptophan hydroxylase (Bensinger, *et al*, 1974). Tyrosine is converted to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (Levitt, *et al*, 1974), the rate limiting step in NE synthesis. Tyrosine hydroxylase, which increases by more than 50% in the dark, is highly concentrated in pineal tissue (McGeer & McGeer, 1966). Conversion of DOPA to dopamine is followed by the conversion of that compound to NE by the enzyme dopamine beta-hydroxylase (Laduron & Belpaire, 1968).

On release into the axon terminal-pinealocyte cleft, NE can interact with the pineal's beta-adrenergic receptors. Deactivation occurs either by reuptake into the terminal neurons, diffusion away from the terminal-receptor environment, or through deactivating mechanisms related to enzymatic degradation to nonactive metabolites (reviewed by Backstrom & Wetterberg, 1972). A marked change in receptor numbers is apparent during each 24-hour period, with binding of labeled alprenolol (a beta-adrenergic drug) being twice as great at the end of the light period as it is at the beginning of the light period (Zatz, *et al*, 1976; Kebabian, *et al*, 1975).

1.3.7 Melatonin circulation and excretion

Plasma levels of melatonin in sheep and other animals (e.g. primates, pig, rat, cow, donkey, camel, chicken, salmon, lizard) are highest at middark and lowest at midlight periods (Vaughan, *et al*, 1978). In addition to the circadian rhythm of melatonin levels in blood there is also a diurnal melatonin rhythm in the cerebrospinal fluid of primates (Reppert, *et al*, 1979) and sheep (Shaw, *et al*, 1989). In primates night peak values of melatonin in CSF are 2 to 15 times higher than day values. This increase occurs soon after lights are turned off, and the decrease towards day values occurs rapidly after lights are turned on again. Changes in CSF melatonin concentrations appear to reflect daily changes in plasma melatonin concentrations (Vaughan, *et al*, 1978). A 24 hour rhythm in the concentration of CSF melatonin suggests the possibility that the CSF may be an important route of communication between the pineal gland and other parts of the brain (Li, *et al*, 1989).

HIOMT activity is also present in some blood cells, the harderian gland and the retina (Claustrat, *et al*, 1990), and melatonin may also be synthesized in other extrapineal sites such as the hypothalamus, retina and gastro-intestinal tract. These sites may assume some minor functional significance following pinealectomy.

Melatonin is metabolized in the liver to 6-hydroxymelatonin by melatonin hydroxylase and then conjugated to sulfate or to glucuronide (Kopin, *et al*, 1961). In the brain, melatonin is metabolized to a hallucinogenic agent N-acetyl-5-methoxykenurenamine (Hirata, *et al*, 1974). Both the hepatic and neural metabolites are discharged into the blood and are eventually excreted in the urine along with small quantities of unchanged melatonin (Kopin, *et al*, 1961).

1.4. Circadian rhythms, photoperiodism and the pineal gland

1.4.1 General

The majority of plants and animals have evolved, as part of their internal organisation, 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 the 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 (deVlamming & Olcese, 1981).

1.4.2 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).

1.4.3 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, of which the most important appears to be the suprachiasmatic nuclei (Moore, 1983), within the hypothalamus. 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 retinohypothalamic tract, is always bilateral and usually innervates the contralateral SCN (Mess & 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 & 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. The circadian rhythm in melatonin secretion is thought to be due to changes in synthesis generated by endogenous signals emanating from a neural centre located within the SCN (Rusak & Zucker, 1979). Light both suppresses melatonin synthesis and entrains the neural centre, so that the time of the increase and the decrease of melatonin secretion is related to the light/dark cycle (Tamarkin, *et el*, 1985).

1.5 Nerve Regeneration

The mechanisms underlying structural plasticity and regenerative growth in mature neurons, and the extraneural cues that regulate it remain largely undefined. As mentioned in Section 1.3.5., recovery of pineal gland function after unilateral SCGX, may occur as a result of pinealocyte innervation, by collateral sprouting of sympathetic nerve terminals, originating the intact SCG.

Section 1.5 provides a review of current knowledge of nerve regeneration processes and environmental interactions as they pertain to an interpretation of results of the experiments described in this thesis.

1.5.1 True regeneration

Under normal circumstances nerves continue to maintain connections with their targets throughout the life of an animal, suggesting that some cellular behaviours associated with developmental growth must persist in mature neurons. They may continually remodel their connections (Purves & Voyvodic, 1987), produce and transport to the nerve terminals many of the molecules needed for nerve growth (Lasek, *et al*, 1984), and they elongate considerably during body growth. Elongation of axons and active remodelling of their terminal arbors underlies the differentiation of neural circuits during development (Collinridge & Bliss, 1987), contributes to some forms of neural plasticity in adult brains (Merzenich, 1987; Singer, 1987) and may determine the success or failure of nerve regeneration (Fawcett & Keynes, 1990).

In the mature nervous system, with a few notable exceptions such as the olfactory system of most species (Graziadei, *et al*, 1980) and the vocal motor system of songbirds (Nottebohm, 1985), neurons do not develop either *de novo* nor migrate to new positions

(Mathew & Miller, 1990). However, new process outgrowth in the form of axonal regeneration or sprouting does occur in response to neural trauma or pathology (Brown, 1984; Seil, 1988), albeit a comparatively slow process Developing sympathetic neurons explanted from fetal or neonatal animals can reinitiate neurite outgrowth in a culture dish within a few hours (Argiro & Johnson, 1982; Collins & Lee, 1982). In contrast, adult neurons explanted to identical culture conditions do not extend neurites for several days (Agranoff, *et al*, 1976; Collins & Lee, 1982).

It is the cell soma that provides the essential metabolic machinery necessary for continued neuronal function at the onset of regeneration, through the expression of new genes and synthesis of proteins. Biochemical and structural changes, such as the swelling of nucleoli and proliferation of rough endoplasmic reticulum, indicating increased RNA activity and protein synthesis, are amongst the first signs that regeneration is starting (Stein, *et al*, 1974). Sequential gene expression is associated with commitment, migration, process outgrowth and synaptogenesis (Mathew & Miller, 1990). In general, the proteins produced during regeneration are the same as those associated with axonal growth in development. Substances that are abundant in developing axons, such as growth associated proteins (GAPs) and tubulin, have their synthesis enhanced during the migratory phase of nerve regeneration, whereas neurofilament proteins associated with later developments, when axons have connected with their targets and have expanded radially, are decreased (Fawcett & Keynes, 1990).

Primary terminology for nerve regeneration differentiates axonal growth which does not pass beyond the proximal surface of a lesion (Anderson, *et al*, 1971), and the actual reestablishment of point to point contacts (Guth, 1974). The former called collateral sprouting, the latter is true regeneration.

For true regeneration proximal axons of a cut nerve may regenerate through to an intact distal endoneural tube at distances of up to 1 cm, an attraction that is dependent on

the presence of live Schwann cells in the distal endoneural tube (Kuffler, 1986). Schwann cell multiplication is stimulated by machrophages and myelin debris, and is itself dependent on the successful degeneration of detached neural segments (Fawcett & Keynes, 1990).

In response to axotomy peripheral nerve fibres distal to the lesion and detached from cell body metabolic machinery, degenerate, by the process of anterograde ("wallerian") degeneration (Hallpike, 1976; Allt, 1976). Anterograde degeneration leads to the removal and recycling of axonal and myelin-derived material, and prepares the environment through which regenerating proximal axons will grow. Both the axon and the myelin degenerate, leaving behind dividing Schwann cells inside a basal lamina tube that had surrounded the original nerve fibre (Cragg, 1970; Grafstein & McQuarrie, 1978); these columns of Schwann cells, surrounded by the basal lamina, are known as endoneural tubes.

The proximal axon's initial response to axotomy is also degeneration, however, in contrast, the intact neuron then initiates a process of axonal regrowth along with its attendant metabolic changes (Cragg, 1970; McQuarrie, 1978), culminating in synaptogenesis with target tissue and, if successful, a return to operative functioning.

Regenerative growth occurs with greater success following crush rather than cut injuries (Sutherland, 1978). Endoneural tubes and Schwann cell basal lamina are left intact after crush injuries (Haftek & Thomas, 1968) and regenerating axons remaining in their parent tubes are guided directly back to their targets. If the tubes are disrupted, however, regenerating axons may enter inappropriate tubes in the distal stump, and so be guided to inappropriate targets (Muller, 1992) which may not be just ineffectual, but inhibitory.

1.5.2 Collateral regeneration

Both myelinated and unmyelinated regenerating axons exhibit repetitive sprouting, such that each axon proximal to a site of axotomy can give rise to several processes distal to it (Jenq, *et al*, 1987). However, not all regenerative growth survives; over the months following a nerve repair, some axons will enlarge and return to an approximately normal diameter, whereas others will disappear (Cragg & Thomas, 1964). Subsequent axon enlargement and maturation is dependent on connection with a target, and branches that disappear have presumably failed to do this (Aitken, 1949).

In culture, foetal and neonatal axons initiate regenerative sprouting within a few hours of axotomy (Argiro & Johnson, 1982; Collins & Lee, 1982). The first sprouts in myelinated axons are generally seen coming from the terminal nodes of Ranvier, through the gap left by partial retraction of Schwann cells (Meller, 1987); unmyelinated axons sprout equally as rapidly (Bray, *et al*, 1972). While collateral sprouts are forming, the cut tip of the axon swells, inflated with smooth endoplasmic reticulum, mitochondria, and eventually microtubules (Fawcett & Keynes, 1990).

A number of morphological variations of collateral regeneration are possible. Cotman & Nadler (1978) examining morphological variation in collateral sprouting have differentiated between 'paraterminal sprouting' and 'contact synaptogenesis'. In the former the terminal end-feet of an intact axon may simply enlarge and establish new synaptic contacts at sites left vacant as a result of damage to another neuron. Contrasted with 'contact synaptogenesis', in which an intact nerve axon in contact with a denervated cell would form new synapses at points of apposition where there were none previously. Alternatively, the formation of new synapses could follow a shift in position of an axon prior to creating new points of contact. These variations suggest that axon elongation

need not necessarily be a requirement for successful reinnervation if residual neurons were in close proximity to denervated cells.

Electron microscopy studies investigating collateral sprouting following partial denervation of the septal ganglia, indicated that "intact fibres from one system could replace the axons of another system". Termed 'heterotypic sprouting', that mechanism of regeneration occurs when axons passing close to a deafferented zone respond by developing and sending collaterals to occupy vacated synaptic sites, while retaining their own connections. Raisman (1969) suggested that the response was triggered by the absence or removal of the normal afferents to the area, and a role for both glial cells and 'neural growth factors' were also implicated.

1.5.3 Axonal dynamics

Although axons have some capacity for synthesis and processing of lipids and carbohydrates there is an absence of identifiable components for protein synthesis in neuronal processes (Koenig, 1967). Protein synthesis is generally thought to be restricted to cell bodies where protein synthesizing organelles are located. Sorting of proteins to specific membrane compartments occurs mostly at the level of the Golgi complex within the cell body (Stone & Hammerschlag, 1987). However, after assembly intracellular membranous organelles often must be moved considerable distances along an axonal or dendritic compartment before being delivered to terminal sites of action.

During neuronal growth axonal dynamics encompass the synthesis, packaging, sorting, targeting, and translocation of the proteins needed for neuronal function and migration. Consequently, intracellular transport is highly developed in neurons with a wide variety of polypeptides being delivered to the axonal and dendritic terminals. Brady

(1993) provides a review of the molecular mechanisms involved in nerve transport, including identification of potential mechanisms for regulating transport and characterization of the influence of different axonal environments on transport.

Three categories of cytoskeletal elements play distinct roles in both neuronal transport and growth: microtubules, neurofilaments and microfilaments. Each consists of a diverse set of polypeptides that are confined in various configurations to fill specific functions within the nervous system. There are overlapping functions between classes, while isoforms exist within each class of cytoskeletal protein; these may subtly change local properties of the cytoskeleton (reviewed by Brady, 1988).

1.5.4 Microtubules

Microtubules serve at least two main roles in the mature nervous system. First, they provide a structural framework for the axon (Freide & Samorajski, 1970), playing an important role in determining the size and morphology of neuronal processes. In small unmyelinated fibres and in many dendrites, both of which have few neurofilaments, microtubules act as the primary structural element. A second critical role for microtubules is connected with intracellular transport, mediated by kinesins and dyneins, the molecular 'motors' of the neuronal transport system (Brady, 1991). During axon elongation and regeneration the synthesis and transport of proteins associated with growth is increased.

The primary subunits of microtubules are alpha and beta tubulins, which are disassembled as part of the structural organisation of the cytoskeleton and then must be reassembled for neurite extension, such that the stability of assembled microtubules plays a critical role in effective regeneration (Brady & Black, 1986).

Tubulin is the major protein of vertebrate brain, representing 10-26% of total brain protein, whereas the protein content of other tissues is only 1-3% tubulin. Less than 5% of the protein derived from glia and other non-neural cells of the brain is tubulin, so most brain tubulin is derived from neurons (Hiller & Weber, 1978). The importance of the tubulins in regeneration can be inferred from changes in axonal transport of tubulin associated with regeneration (McQuarrie & Lasek, 1989) and the up-regulation of specific tubulin genes during axonal growth or regeneration (Wong & Oblinger, 1990; Mathew & Miller, 1993).

Relevant to this thesis, but not to this section on nerve regeneration, is the reported presence of microtubules and tubulin in pineal glands of some species. This matter is reviewed and discussed in Chapter 4.

1.5.5 Interactions between regenerating axons and their environment.

A complex interplay between extrinsic influences and mechanisms intrinsic to the neuron initiate and determine the pattern of regenerative processes.

Based upon a number of observations, the control of regeneration is considered to be independent of cell body mechanisms (Fawcett & Keynes, 1990). There is evidence that sprouting of mouse motor terminals may occur even when axons are disconnected from their cell bodies (Brown & Lunn, 1988), and that an axon completely disconnected from its cell body can elaborate a new growth cone in response to axotomy (Mason & Muller, 1982). Also, the earliest regenerative sprouting at the proximal axon tip can occur within a few hours of axotomy (Argiro & Johnson, 1982), too rapidly for the cell body to have been informed by a retrograde signal and to have sent its response, even by fast axonal transport (Collins & Lee, 1982). Similar considerations apply during development, during which it would be impossible for the frequent changes in the rate of axonal growth

to be regulated sufficiently rapidly from the cell body (Cowan, 1979a). These observations suggest that there may be controlling mechanisms for axon elongation, acting rapidly and locally at the axon terminal.

From transplantation studies, both *in vitro* and *in vivo* (for review see Hatton, 1985), the accepted scenario for initiation of axon growth involves extensive interactions between an axon and multiple environmental cues. Olsen and Malmfors (1970) showed that when transplanted into the anterior chamber of a host eye, a piece of iris, though deprived of its own nerve supply for 3 months, could evoke collateral sprouting from intact sympathetic nerves. Target tissue influence was implicated.

Patterns of neural activity are also dependent on the availability of glial cell surface molecules and extracellular matrix components (Muller, 1992). Glial cells function in the mainteance of ionic homeostasis (Kuffler, 1967; Walz & Hertz, 1983; Walz, 1989), the monitoring of presynaptic and postsynaptic neuronal activity (Kuffler, 1967; Orkand, *et al*, 1966; Wuttke, 1990), and in factors supporting and guiding growth and migration of regenerating nerves (Ard & Bunge, 1988; Hatten, *et al*, 1982; Levine & Card, 1987; Pixley, *et al*, 1987).

The intrinsic ability of a neuron to respond to environmental cues, and the nature of its response to any particular cue, depend on the neuron's expression of relevent receptors, signal tranducing proteins, and on the presence of structural materials to execute a response. Purves (1975) indicated that expression of some of the neural genes involved in axon growth and culminating in synaptogenesis, decreased sharply as neurons matured, so that the extracellular cues that stimulated axon elongation in developing neurons evoked different responses from mature neurons. Alternatively, repression of growth-related properties in many neurons during differentiation suggests that some of the genes involved in axon growth might be expressed transiently during development and be reinduced during successful nerve regeneration. Relevant studies have

concentrated on the synthesis of proteins destined for transport into axons and their terminals, the population of neural proteins most directly involved in axon functions. This has prompted a search for related genes using electrophoretic methods (Katz, *et al*, 1965) and phenotypic studies investigating expression of nerve growth factors by means of immunocytochemistry (Fantini and Johansson, 1992). In almost all neurons screened in this way, it has been possible to identify one or more axonal proteins whose synthesis is specifically increased an order of magnitude during developmental or regenerative growth.

1.5.6 Neural growth factors

Neuronal changes necessary for nerve regeneration generally lead to increases in the supply of substances necessary to rebuild the growing nerve fibre. Also there are extrinsic changes in the quantities of trophic (growth-promoting) factors that function in attracting axons to grow in the proper direction (Finger & Stein, 1982).

The first extrinsic factor to be isolated was nerve growth factor (Bueker, 1948; Levi-Montalcini and Hamburger, 1951), which has been observed to influence adrenergic neurons in the sympathetic nervous system and to promote an increase in the growth of axons from sympathetic ganglion towards a source of NGF (Campenot, 1977). Diamond, *et al* (1987) have demonstrated that intact sensory neurons sprout in response to denervation of adjacent sensory fields in the skin, an effect that is inhibited by systematically administered antibodies to NGF. Furthermore, increasing local concentrations of NGF at the terminals of developing sympathetic neurons promotes increased neural growth, both *in vivo* (Edwards, *et al*, 1989) and *in vitro* (Campenot, 1982). Also, administration of NGF to neonates causes both increased terminal sprouting

(Levi-Montalcini and Angeletti, 1968) and increased dendritic arborization of sympathetic neurons (Snider, 1988).

While very little NGF or the receptors for NGF are found in the normal mature nervous system, both are apparent in areas of nerve regeneration. When a nerve is cut or crushed, the level of both the NGF molecule, NGF receptors and their respective mRNAs in the region distal to the injury, increases enormously (Heumann, *et al*, 1987). The expression of NGF by Schwann cells is probably controlled by axonal contact; NGF receptor levels increase in the absence of axonal contact, and decrease when contact is restored (Taniushi, *et al*, 1988). Johnson and his colleagues suggest that the NGF receptor may act both in trophic support and as a cell adhesion molecule, the NGF having an association with NGF receptors on both axons and Schwann cells (Johnson, *et al*, 1988).

1.5.7 Growth cones

During target-directed outgrowth, when the nervous system is developing, or when a neuron is regenerating following injury, the growing axon elaborates a prominent, growth-specific structure at its leading edge. This 'growth cone' is able to translocate over a substrate and to make synaptic contacts with appropriate target neurons; it is highly specialised to perform a number of functions (for review, see Johnston & Wessels, 1980; Bunge, *et al*, 1983; Landis, 1983; Kater & Letourneau, 1985; Letourneau, 1985) including those of motility (Letourneau, 1975; Tosney & Wessels, 1983; Argiro, *et al*, 1984; Bray & Chapman, 1985), pathfinding (Bastiani & Goodman, 1986; Bently & Toroian-Raymond, 1986; Caudy & Bently, 1986; Kuwada, 1986), adhesion to the substrate (Letourneau, 1975; Hammerback & Letourneau, 1986), and membrane addition (Bray, 1970, 1973; Pfenninger & Maylie-Pfenninger, 1981a,b; Pfenninger & Johnson, 1983).

The molecular mechanisms that control growth cone formation and that regulate its function are unknown, however, a potential clue to the control of growth cone formation is that certain proteins, designated growth-associated proteins, or GAPs, are much more abundant in neurons with growing axons than in neurons where synaptic connections have already been established, suggesting that they may perform functions required at earlier stages during axonal growth. One of these, GAP-43, a phosphopeptide with a molecular weight of approximately 43 kDa (Basi, *et al*, 1987; Karns, *et al*, 1987), is synthesized and axonally transported at elevated levels during both developmental and regenerative axonal growth (Skene & Willard, 1981a,b).

Subcellular fractionation, electrophoresis and immunocytochemical experiments have demonstrated that GAP-43 is a principal component of the growth cone, and is highly enriched there (Meiri, *et al*, 1986; Skene, *et al*, 1986). Yet despite the extensive studies, both *in vitro* and *in vivo*, investigating GAP-43 associations with growth cones *per se* (Meiri, *et al*, 1986; Skene, *et al*, 1986; Meiri, *et al*, 1987; Goslin, *et al*, 1988; Goslin & Banker, 1990), to date only one investigation has focused on this protein's associations with axonal development during functional regeneration (Schreyer & Skene, 1993). Results from that experiment suggest that GAP-43 induction in the dorsal root ganglion of rats is caused by disconnection from target tissue and not by axon injury *per se*.

1.5.8 Growth associated protein - 43 (GAP-43)

GAP-43 is an axonally transported phosphoprotein found on the inside of the membrane of regenerating and growing axons, particularly near the growth cone (Meiri, *et al*, 1988), and comprises of the order of 1% of the total protein in growth cone membranes (Skene, *et al*, 1986). Biochemical determination indicates that GAP-43 is

approximately 10-fold more concentrated in both developing and regenerating nervous tissue than in adult nervous tissue (Meiri, *et al*, 1986). The protein is induced very rapidly following axotomy, with levels increasing up to 100 times and then decreasing again on reinnervation (Skene, *et al*, 1986). Newly synthesized GAP-43 is rapidly transported down axons as part of the fast axonal transport system (Skene & Willard, 1981c), which uses membrane-bound vesicles (20-60 nm) to deliver material from its site of synthesis in the cell body (Pfenninger & Johnson, 1983).

The time course of GAP-43 expression during axon regeneration is consistent with periods of axon elongation and the initial formation of an association between the terminal axon and target tissue. Meiri, *et al* (1988), working with a polyclonal antibody to GAP-43 in neonatal rat SCG tissue, reported a variation in the distribution of the protein as axons elongate. The cell body became progressively less immunoreactive, whereas the growth cone at the tip of the growing axon reacted more strongly. Finger & Stein (1982) suggested that the initial rise in GAP-43 synthesis coincided with, or slightly preceded, initiation of axon outgrowth in fish and amphibian optic nerves, but that the protein's synthesis did not peak or plateau until regeneration was well underway. In rat dorsal root ganglia, induction of GAP-43 mRNA is shown to begin between 1 and 2 days after sciatic nerve injury, corresponding to the end of a lag period preceding axon outgrowth (McQuarrie, *et al*, 1977). However, induction of GAP-43 is not considered to be a secondary consequence of outgrowth, as application of colchicine to rat dorsal root ganglia at the time of nerve injury, or at the end of a 2 day post-crush lag period, which should have prevented axon outgrowth, was found to have no effect on the time course or amplitude of GAP-43 induction (Skene, 1989).

Elevated GAP-43 expression continues throughout the period of axon elongation and also synaptogenesis in all developing and regenerating systems examined (Skene, 1989). GAP-43 induction in regenerating systems begins just early enough not to rule

out a role in the early phases of axon outgrowth, and persists just long enough not to rule out participation in later phases of synaptogenesis and maturation of the axon's terminal arbor. A slow decline in GAP-43 synthesis late in axon development or regeneration, could permit the protein to play some role in synaptogenesis, or in the active sorting out of the terminal arbor. Localization of the protein to growth cones and the distal portions of outgrowing neurites, however, does argue against direct GAP-43 participation in maturation of the axon structure behind the immature axon sprouts, or in the slow growth in axon diameter, myelination and maturation of the axon's electrical properties (Goslin & Banker, 1990).

1.5.9 Structure and Biochemical Characteristics of GAP-43

Structural and biochemical studies of GAP-43 reveal a novel protein with an amino acid sequence that is highly conserved among mammals (Goslin & Banker, 1990) and that appears to interact extensively with several intracellular messenger systems (Goslin, *et al*, 1988).

For a protein associated predominately with membranes, GAP-43 is surprisingly hydrophilic. GAP-43 is synthesized as a soluble protein, whose post-translational association with membranes is considered to be mediated by covalent attachment of fatty acid (Skene, 1989). A short hydrophobic region at the amino terminus of the protein is the most likely site of fatty acylation and membrane attachment (Basi, *et al*, 1987). The extreme hydrophilicity of GAP-43 and the nature of its membrane attachment are consistent with models that envision the protein extending away from the cytoplasmic surfaces of growth cones and synaptic membranes (Meiri, *et al*, 1988), in a position to interact with cytoplasmic or cytoskeletal proteins on one hand, and reversibly attached to the membrane on the other.

GAP-43 has been identified as a unique calmodulin-binding protein, binding calmodulin selectively in the absence of calcium, and releasing calmodulin at higher calcium concentrations. This 'reversed' calcium dependence for calmodulin binding contrasts to the usual mediation of calmodulin on the secondary messenger calcium in various physiological processes such as excitation-contraction coupling and excitation-secretion coupling (for review see Cheung, 1980). On the basis of its abundance, membrane binding properties, and reversed pattern of calmodulin binding; GAP-43 has been proposed to act under low calcium conditions to sequester calmodulin in certain regions of the neural membrane, then releasing the calmodulin upon influx or mobilization of free calcium (Cimler, *et al*, 1987). However, because the calcium-

dependent antagonism of GAP-43 binding with calmodulin is strongly affected by ionic strength (Alexander, *et al*, 1987), it is not clear whether calcium regulates calmodulin binding to GAP-43 under any or all physiological conditions.

An alternative regulator of calmodulin binding to GAP-43 is protein kinase C (PKC). Phosphorylation of GAP-43 by PKC strongly inhibits binding of that protein to calmodulin (Alexander, *et al*, 1987).

Signalling across growth cone membranes is an essential feature of axon elongation and synaptogenesis, and numerous studies have implicated PKC in these events (Spinelli, *et al*, 1982; Murphy, *et al*, 1983; Hsu, *et al*, 1984; Mattson, *et al*, 1988; Hall, *et al*, 1988; Girard & Kuo, 1990). As a prominent substrate for PKC, a functional role for GAP-43 could be mediation of some of the effects of PKC on growth cone function (Meiri, *et al*, 1988; Nelson, *et al*, 1989). The identification of GAP-43 as a major substrate of PKC and the induction of increased levels of GAP-43 in response to disconnection from target tissue (Schreyer & Skene, 1993), suggest that GAP-43 may be an element of, or may be regulated by, a transduction system that enables the growth cone to sample the environment and then to generate an internal response.

1.6 Aims of the present study

The principal aims of the studies described in this thesis were to:

(i) Confirm the use of melatonin secretory responses, to darkness exposure of sheep, as a parameter of pineal function.

(ii) Examine the effects of unilateral SCGX on the profiles of melatonin secretory capacity over a period of 28 days post-operatively, to explore whether or not full recovery of function occurred over that period.

(iii) Investigate the occurence of new neural growth in pineal tissue as a response to partial denervation, using immunocytochemical localization of GAP-43.

(iv) Use localization of alpha tubulin to determine whether pinealocyte cell integrity was maintained after unilateral SCGX.

CHAPTER 2

Materials and Methods

All of the aims of thesis were investigated in a single experiment incorporating radioimmunoassay techniques to measure plasma melatonin levels, for which specific methods have been described in Chapter 3. In Chapter 4 the technique of immunocytochemistry was used to investigate the occurence of both GAP-43 and alpha tubulin in pineal tissue. With this approach it was aimed to establish whether or not functional recovery was related to neural growth, which has not previously been reported in the literature.

While details of specific experimental techniques are described in Chapters 3 and 4, more general materials and methods (animal management, experimental design and surgical procedures) relevant to both those chapters, are given in Chapter 2.

2.1 Animal management and treatment groups

2.1.1 Animal management

Romney ewes, approximately 9 months of age and weighing 30 - 35 kg , were used in the experiment detailed in this thesis. The natural photoperiod at the time the animals were transferred to experimental conditions was 12 hr light : 12 hr dark (12L:12D), with dawn occuring at approximately 0600 hrs (New Zealand Nautical Almanac, 1992-1993).

All animals were constrained in pens and housed indoors in a well ventilated, light- proof room maintained at a constant 15^{O}C during autumn (May/June) 1993. Fluorescent lighting, which provided approximately 200 lux at the eye level of each animal, and was controlled by automatic time switches to provide an 8L:16D lighting regime, with lights on from 0500 to 1300 hr. Feed consisted of approximately 800 grams of lucerne chaff per day, with water available ad libitum. All animals were acclimatized to the 8L:16D photoperiod for 21 days prior to surgery.

2.1.2 Treatment groups

Twenty animals were randomly allocated to one of four groups (n = 5), with respect to a given time of sacrifice for harvesting of pineal glands.

Respective animal groups were subjected to the following experimental procedures:

Control Group: 15-20 min Saffan/Halothane anaesthesia, without sham SCGX.

3 Day Group: S/H anaesthesia and unilateral SCGX.

14 Day Group: S/H anaesthesia and unilateral SCGX.

28 Day Group: S/H anaesthesia and unilateral SCGX.

All animals were bled in the dark for 4 hr on the day before surgery or anaesthesia (see Section 3.2.2) to test their melatonin secretory capacity. Subsequent bleedings for this purpose were conducted as follows (i) on days 1, 3, 7, 14, 21 and 28 after surgery or anaesthesia for the 28 day and control groups; and (ii) on days 3 and 14, respectively, for the 3 and 14 day groups. At the end of those last bleedings, all animals were euthanased and pineal glands collected for immunocytochemical evaluation (Chapter 4).

2.2 Surgical techniques

2.2.1 General surgical techniques

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

b) Anaesthesia was induced with intravenous 'Saffan' (Glaxovet Ltd, Harefield, England) containing alphaxalone 0.9% w/v and alphadalone acetate 0.3% w/v, average dose 3mg/kg body weight. Anaethesia was maintained with 2-3% (v/v) halothane ('Fluorothane', ICI, Macclesfield, Cheshire, England) in oxygen, after intubation with a Magill endotracheal tube, and supplied at a rate of 2 l/min from a Fluotec 3 (Cyprane, Keighley, England) vapourizer.

c) After induction of anaesthesia, unilateral SCGX was performed according to the method of Appleton and Waites (1957) (Section 2.2.2). All surgical procedures were conducted under sterile conditions.

2.2.2 Superior cervical ganglionectomy

In this procedure a skin incision was started over the zygomatic process at a point mid-way between the canthus of the eye and the base of the ear, and was continued parallel with the mandible to the level of the thyroid cartilage. The thin subcutaneous platysma muscle was then incised along the same line, care being taken not to damage the external jugular vein, which lies just deep to the muscle at the ventral end of the incision.

The depressor auriculae muscle was incised with the platysma muscle. After reflecting the cut edges of this muscle the parotid salivary gland, lying in fat in the dorsal area of the operative field, and the external jugular vein became visible. The few vessels which pass caudally from the external jugular and internal maxillary veins were cut between ligatures, and the caudal and ventral borders of the parotid gland were then freed from underlying tissue to enable this gland to be retracted rostro-dorsally. The common carotid artery could then be identified under fat, deep to the external jugular vein, and its cranial course to where it is crossed by the hypoglossal nerve could be identified. Here the artery passes deep to the digastric muscle, dorsal to which is the fan-shaped jugulo-hyoid muscle. This latter muscle was partially cut through to enable retraction of the epihyoid bone. By retracting the bone and the cut ends of the muscle using self-retaining retractors, the superior cervical ganglion, the cervical sympathetic nerve and related structures could be readily freed from the fat which surrounds them.

Following removal of the superior cervical ganglia the operation was completed by suturing the cut ends of the jugulo-hyoid muscle, suturing the caudal border of the parotid gland into place and closing the platysma muscle and skin in layers. Each operation was completed in approximately 20 mins.

2.2.3 Post surgical care

Following recovery from anaesthesia, animals 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.

2.3 Blood collection, processing and melatonin radioimmunoassay

In Section 2 of Chapter 3 details are given for the periodic collection of blood, subsequent plasma extraction and radioimmunoassay protocol and data processing, used to determine pineal melatonin secretory capacity in response to unilateral SCGX.

2.4 Immunocytochemistry

Section 2 of Chapter 4 details the collection of pineal tissue, its processing and the subsequent localization of GAP-43 and alpha tubulin using immunocytochemical techniques.

2.5 Statistical analyses.

Analysis of variance (ANOVA) was used to assess data derived from both Chapters 3 and 4. In addition, paired *t*-tests were performed on data in Chapter 3. Details of statistical analyses are given in respective chapters.

Levels of significance in all statistical analyses are denoted thus:

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

Chapter 3.

Effects of unilateral superior cervical ganglionectomy on ovine melatonin secretory responses to darkness exposure

3.1 Introduction

As mentioned in Chapter 1, the pineal gland is innervated by noradrenergic sympathetic fibres originating from the right and left SCG's, the two nerves each providing approximately 50% of the pineal innervation, which is distributed equally over the two halves of the gland (Lingappa & Zigmond, 1987). Norepinephrine released at the nerve terminals regulates a number of aspects of pinealocyte biochemistry, with the most dramatic being the regulation of the synthesis of the hormone melatonin, during darkness (Reiter, 1976).

Studies investigating pineal gland innervation commonly have used the technique of bilateral SCGX, which eliminates rodent pineal rhythms of NAT activity and melatonin content (Reiter, *et al*, 1979), as well as sheep pineal melatonin secretory capacity (e.g. Lincoln, *et al*, 1982; Lapwood, 1993). It also causes a reduction in pineal weight (Barrell & Lapwood, 1978-9) and pinealocyte atrophy (Mockett & Lapwood, unpublished).

As discussed in section 1.3.5, the effects of unilateral SCGX also have been investigated, with most workers measuring pineal enzyme activity as their parameter of pineal function or secretory potential. Thus Reiter, *et al* (1979) reported that pineal NAT activity was reduced by half two days after surgery; Zigmond, *et al* (1981) on the other hand, found that while NAT levels were depressed 75% on the night after unilateral SCGX, effective recovery had occured the following night. Later Dornay, *et al* (1985) measured pineal tyrosine hydroxylase activity

and tritiated noradrenaline uptake after unilateral SCGX; both were depressed by >>50% 2 days after surgery, but increased to 80% of control levels by 10 days post-operatively.

Until recently only one report appears to have been published on effects of unilateral SCGX on melatonin production: Reiter, *et al* (1979) found that pineal melatonin content was reduced about 50% two days post-surgery.

Recently, Lapwood (1993) took the physiological approach of measuring melatonin secretion profiles during exposure of sheep to darkness, before and periodically after unilateral SCGX. On the day after surgery there was a reduction of pineal melatonin secretory capacity to values 92% ($P< 0.001$) below those measured before surgery, however, succeeding measurements indicated a substantial recovery of that parameter of melatonin biosynthesis, to within 77% of pre-operative levels after 14 days.

This current study also used measurement of melatonin secretion profiles during exposure of sheep to darkness, but was designed to examine the degree of recovery in pineal melatonin secretory capacity which occured when an extended post-surgery survival time of 28 days was utilized.

3.2 Materials and Methods

3.2.1 Group treatments

Animal management and treatment group allocation is detailed in Sections 2.1.1 and 2.1.2, respectively.

Surgical techniques are described in detail in Section 2.2

3.2.2 Blood collection and processing

Faint red illumination from a hand torch was used during the periods of darkness to aid in the blood sampling.

After the acclimatization period of 3 weeks, on the day before surgery blood was collected twice prior to lights off, then at 30 minute intervals for a four hour period in the dark, in order to measure pre-treatment melatonin secretory capacities. Blood samples were collected by jugular venipuncture into 7 ml vacutainer tubes containing l05 units of heparin, then immediately centrifuged at 3000 rpm for 15 mins at $4^{o}C$. Care was taken to prevent the haemolysis of blood samples as this has been reported to lead to a spurious increase in apparent melatonin levels (Fraser, *et al*, 1983). After separation from cells, plasma was stored at $-20^{o}C$ until thawing 24 hours prior to assay.

Blood sampling was again repeated at days 1, 3, 7, 14, 21 and 28 days post-surgery or post-anaesthesia, using the above bleeding schedule, to study melatonin secretory capacities during darkness exposure, so as to measure responses after partial denervation or anaesthesia. Animals to be killed 3 and 14 days after surgery were bled in such a manner only on the day before surgery and on the day they were killed.

Plasma samples were analysed for concentrations of melatonin as a direct measurement of the capacity of individual pineal glands to secrete that hormone. No account was taken of variable metabolic clearance rates for melatonin, consequently measurements are not claimed to be a direct quantification of pineal rate of production for the hormone.

3.2.3 Radioimmunoassay reagents

(i) **Tricine buffer.**

Contained 0.1M Tricine (Sigma Chemical Company, St Louis, MO, USA), 0.15 M sodium chloride (May & Baker New Zealand Ltd, Auckland, New Zealand), 1g/l gelatin (BOH Chemicals Ltd, Poole. U.K), and with 100 mg/l sodium azide (Riedel-De Haen AG, Seelze-Hannover, West Germany) included as an anti-bacterial preservative. Solution was adjusted to pH 7.7 with 4N NaOH and stored at $4^{O}C$.

(ii) **Activated charcoal.**

0.4% activated charcoal (Sigma Chemical Company, St Louis, MO, USA) with 0.004% Dextran T70 (Sigma Chemical Company, St Louis, MO, USA) in pH 7.7 tricine buffer, was prepared 1 day ahead of use and discarded after a week.

(iii) **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 added to make up 3 litres of scintillation fluid.

(iv) **Organic solvents.**

- Absolute ethanol (May & Baker Australia pty Ltd, Victoria, Australia)
- Toluene (Shell Chemicals New Zealand Ltd, Wellington, New Zealand)
- Triton X-100 (Rohm and Haas New Zealand Ltd, Auckland, New Zealand)

3.2.4 Preparation of standards, tracer & antibody.

Standard curves were made by diluting a melatonin stock standard solution (1 mg/ml in absolute ethanol) with melatonin free plasma to give a range of standards with melatonin concentrations from 20 to 500 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 [O-methyl-^{3}H] melatonin was supplied by Amersham International plc, (TRK.798, Little Chalfont, England, 85 Ci/m mol) and stored as a working solution by diluting 10ul in 1 ml of absolute ethanol. The working solution was dried down with oxygen free nitrogen (I.C.I Ltd, New Zealand) prior to further dilution for each assay to give 4 -5,000 cpm in 100ul of assay buffer, to be added to each tube.

Lyophilized melatonin antiserum (Guildhay Batch G/S/704 - 8483), raised in goats (for specificity see Table 3.1), was reconstituted in assay buffer to give a dilution of 1:25 which was stored frozen in 25 ul aliquots. Working dilution antibody was prepared by further dilution with assay buffer to 1 : 7500, to give a final dilution in the incubation mixture of 1 : 60,000.

3.2.5 Radioimmunoassay for melatonin.

All plasma samples were analyzed for melatonin concentrations using the radioimmunoassay method described by Fraser, *et al* (1983), as modified by Maxwell, *et al* (1989). All samples, standards, blanks and quality control samples within each assay

were assayed in duplicate, while all samples from an individual animal were assayed in the same assay.

After thawing samples were vortexed and 500 ul of each sample, quality control pool or standard was then added to individual assay tubes. Two hundred microlitres of antiserum was added to assay tubes, followed by a vortex mix and incubation at room temperature (dark) for 30 minutes. A further addition of 100 ul ^{3}H melatonin was made to each tube. Tubes were vortexed and incubated for 18 hours at $4^{o}C$ under foil, to avoid prolonged light exposure.

Separation of the antibody-bound melatonin (supernate) from the free fraction was induced by incubation for 20mins at $4^{o}C$ with 500ul dextran coated charcoal. Charcoal addition was done quickly with a multipipette from a constantly stirred slurry and the tubes were vortexed immediately, then stood still for the remainder of the incubation. Tubes were centrifuged at 3000 rpm for 15 min at $4^{o}C$ and the resultant supernatant fluid decanted, consistently, into scintillation vials. Eight millilitres of scintillation fluid was added, vials were shaken, capped and placed in a scintillation counter (Wallac 1409, Pharmacia, Finland) at a constant room temperature for estimation of radioactivity. Readings (cpm) for each sample were used to determine the concentration of melatonin (in pg/ml) for each sample 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 with the RIA 400 program.

3.2.6 Assay Performance.

Specificity of the melatonin antibody has been established by Guildhay Antisera Ltd. (Guildford, Surrey, U.K) as demonstrated in Table 3.1. In this present experiment, mean non-specific binding (NSB) was 5.6% +/- 2.7% (n=17). Mean assay sensitivity, defined as the minimal detectable concentration significantly different from zero (Burger, *et al*, 1972) was 7.4 +/- 3.1 pg/ml (n=17).

Table 3.1 Specificity of antibody (Guildhay Antisera No. G/S/704-8483) to melatonin as determined by comparative cross reaction.

Compound	Reactivity (%)
Melatonin	100
N-acetyl tryptamine	0.97
6 hydroxymelatonin	0.38
N-acetyl tryptophan	0.26
All other related comounds	< 0.07

Dilution with assay buffer did not affect the estimated melatonin content of quality control samples, as demonstrated in Table 3.2.

Table 3.2 Effects 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)
MS8	neat	106.4
"	1:1	52.9
MS9	neat	572.4
"	1:1	237.1
"	1:2	120.6

Reproducibility of assay results, estimated from two quality control plasma samples assayed twice in each of seventeen assays to assess assay performance, are shown in Table 3.3.

Table 3.3 **Between- and within- assay coeffiecients of variation (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 (%)
34	MS8	112.7	11.20	16.08
34	MS9	566.7	20.77	9.23

3.2.7 Computations and transformations

Computations using the Cricket graph program (Apple software Inc.) determined total melatonin secretory responses as areas under individual melatonin secretory response curves, by integrating calculated hormone concentrations in samples, with the time intervals between samplings, and were expressed as pg/ml.hr. Prior to statistical analyses all data for areas under curves were transformed to logarithms. This transformation was used to stabilize the variance, to ensure that the standard deviation did not vary directly with the mean (Snedecor & Cochran, 1967).

3.2.8 Statistical Analyses

To assess effects of anaesthesia and partial sympathetic denervation by unilateral SCGX on pineal gland responses to darkness, the significance of experimental main

effects, and their interaction, were examined by analyses of variance (Cochran & Cox, 1960). Some experimental hypotheses were tested using standard orthogonal coefficients, except when non-orthogonal matrices were constructed to test hypotheses which were not possible within the constraints of orthogonality (Fisher & Yates, 1963). Limitations are recognised in accepting statistical analyses based on non-orthogonal contrasts and only data represented by relatively high levels of significance ($P< 0.01$) will be referenced where non-orthogonal contrasts are discussed.

Paired *t*- tests (Mosteller & Rourke, 1973) were performed to measure any significant difference between pre-surgery and post-surgery mean integrated melatonin secretory responses measured from the two groups subjected to unilateral SCGX and sacrificed 3 and 14 days after surgery.

3.3 Results

See Tables 3.4 - 3.6 and Figures 3.1 - 3.4

At all sampling periods, both before and after surgery or anaesthesia, plasma samples collected during the period of light, immediately prior to darkness, had melatonin levels which were consistently below the limit of assay sensitivity (7.4 pg/ml) (Table 3.4).

Table 3.4 **Mean (± S.E.M) plasma melatonin levels (pg/ml) for sheep in light, immediately prior to darkness, during a 8L:16D lighting regime. Values are derived from both samples collected from each of five sheep per group at each sampling.**

	Day relative to surgery						
	-1	+1	+3	+7	+14	+21	+28
Experimental group							
Controls	7.18 (0.06)	6.68 (0.05)	6.62 (0.11)	4.41 (0.12)	2.18 (0.02)	6.17 (0.20)	4.88 (0.08)
Unilateral SCGX 28 Day Group	6.26 (0.06)	4.12 (0.03)	0.00 (0.00)	0.00 (0.00)	5.14 (0.05)	1.44 (0.44)	6.14 (0.01)
3 Day Group	1.52 (0.08)		0.00 (0.00)				
14 Day Group	0.00 (0.00)				1.91 (0.11)		

On the day before being subject to either anaesthesia or surgery, the mean integrated secretory responses from each group during the period of darkness were quite uniform, ranging from 572.9 ± 213.8 pg/ml.hr to 799.1 ± 272.7 pg/ml.hr. However, during that same period the melatonin secretory capacities for individual animals varied considerably ($P < 0.001$), for example ranging from 162.0 pg/ml.hr to 1588.8 pg/ml.hr in the control group of animals. Ranges for the other experimental groups were similarly extended, with respective standard errors of the means for the groups killed at 3 days, 14 days and 28 days after surgery being 213.8 pg/ml.hr, 147.5 pg/ml.hr and 163.2 pg/ml.hr. Regardless of any effects of the experimental treatments, such between-animal variability in secretory responsiveness was again seen in the post-surgery or post-anaesthesia periods, but individual animals tended to be consistent in their relative responsiveness at each sampling time. Extreme examples of this variability recorded for both control and unilateral SCGX animals killed at 28 days post-surgery, are illustrated in Fig 3.1.

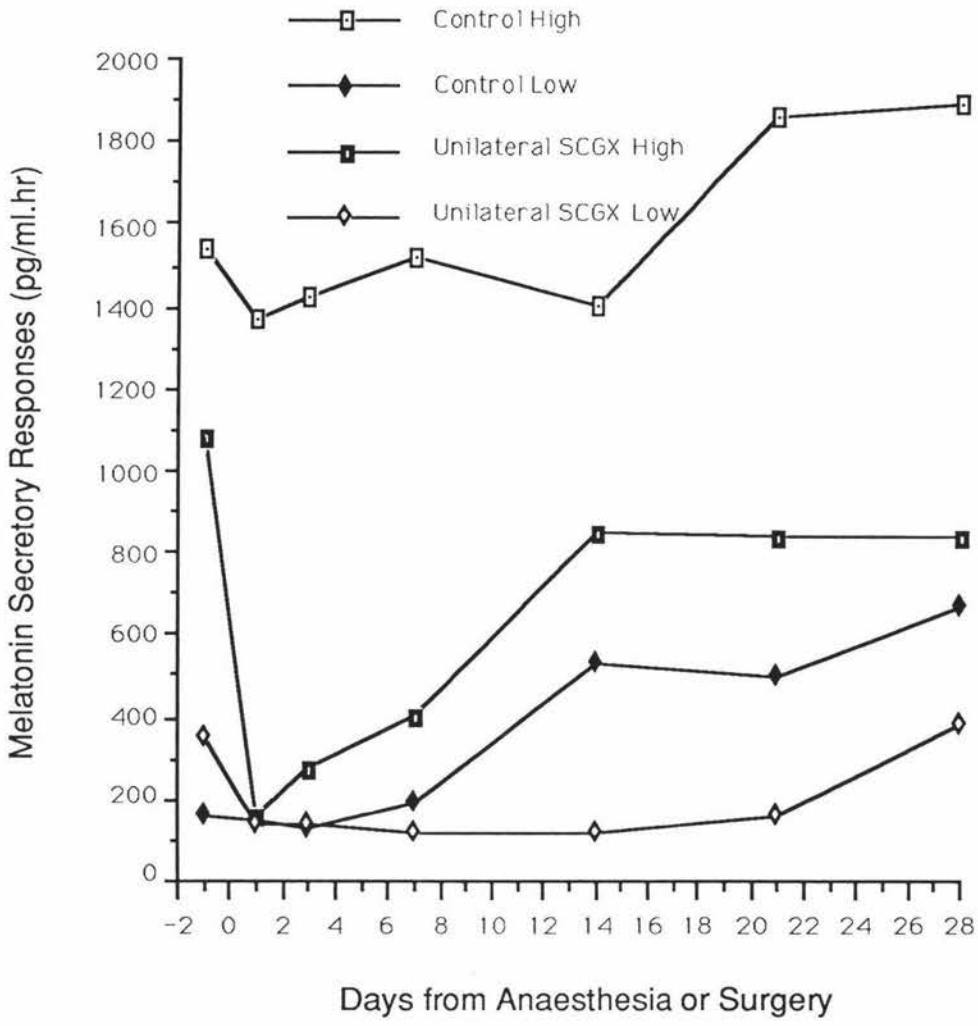


Fig 3.1 Range of melatonin secretory responses (pg/ml.hr) of sheep during 4 hrs of exposure to darkness, prior to and at 1, 3, 7, 14, 21 and 28 days after anaesthesia or surgery. Highest and lowest responses for both control and surgery groups are represented.

Table 3.5 **Mean (± S.E.M) integrated (n=5) melatonin secretory responses (pg/ml.hr) for sheep during the initial 4 hr period of dark within a 8L:16D lighting regime.**

	Day relative to surgery						
	-1	+1	+3	+7	+14	+21	+28
Experimental group							
Controls	799.1 (272.7)	780.5 (246.1)	933.9 (336.6)	1200.1 (305.0)	1176.7 (298.7)	1136.2 (293.3)	1325.8 (272.1)
Unilateral SCGX 28 day pineal harvest	645.2 (163.2)	132.0 (44.7)	323.4 (61.1)	370.3 (78.9)	608.5 (139.3)	652.6 (152.5)	701.8 (104.9)
3 Day pineal collection	572.9 (213.8)		278.9 (129.4)				
14 Day pineal collection	646.7 (147.5)				395.1 (107.7)		

Table 3.6 **Summary of analysis of variance of melatonin secretory response data from control and 28 day groups. Areas under individual melatonin response curves (both pre- and post-surgery) were log transformed prior to analysis.**

Source of Variation	D. F	Variance Ratios
A. Surgical Treatment (Controls vs Unilateral SCGX)	1	22.62***
B. Time	6	
Orthogonal contrasts		
Post-op Linear	1	11.20**
Remainder	5	0.96
Treatment x Time Interaction (Error mean square) +	6	0.24
Post-op L x Control vs Op	1	5.00*
Remainder	5	1.88
C. Animals within treatments	8	6.91***
Residual (Error mean square)	48	0.0934
Non orthogonal contrasts		
Time		
Unilateral SCGX : Post-Op Linear	1	11.85**
Unilateral SCGX: day -1 vs +1	1	24.46***
Comparisons at each day : Control vs SCGX		
Day +1	1	26.54***

+ Treatment x time interaction mean square was used to test the significance of "Time" components.

The mean integrated secretory capacity for the control group of animals was depressed by 2.3% on the day after anaesthesia ($P > 0.05$). After that initial depression, substantial increases in secretory capacity were measured from control animals, but when the variance of that non-orthogonal contrast was tested against the variance of the treatment x time interaction, the increase in melatonin secretory response in the post-anaesthetic period for control animals was shown to be non-significant ($P > 0.05$). Anaesthesia combined with unilateral SCGX dramatically reduced melatonin secretory capacity to 20% ($P < 0.001$) of pre-surgery levels, when measured the day after surgery. However, by post-surgery day +3 the secretory capacity of the operated animals had recovered to within 50% of pre-operative levels, an increase that in temporal terms realised the highest rate of functional recovery recorded throughout the experiment, with an increase from 132.0 pg/ml.hr to 323.4 pg/ml.hr within two days. That trend continued, melatonin secretion measuring 94% of pre-operative levels by 14 days after surgery, followed by a relative slowing in the rate of recovery for melatonin secretory capacity, which can be observed in Fig 3.2. Melatonin secretion had reached pre-operative levels by 21 days and by day 28 was actually slightly higher than prior to surgery. After surgery that recovery of function was reflected as a highly significant ($P < 0.01$) linear component in the ANOVA, with mean integrated secretory capacity rising from 132 pg/ml.hr to 701.8 pg/ml.hr. That recovery of secretory function after unilateral SCGX was more rapid than the comparable increase recorded at the same time from control animals (Treatment x Time Interaction : Post-op Linear x Control vs SCGX; $P < 0.05$). Also highly significant ($P < 0.01$) was the difference between the mean integrated secretory capacities for the two main groups on the day after surgery, with unilateral SCGX melatonin levels 83% being below those measured for controls. On succeeding experimental days secretory responses also were higher for the controls, rather that unilateral SCGX animals as indicated by the significant ($P < 0.001$) treatments main effect in the analysis of variance,

but non- orthogonal comparisons made on data from days 3 to 28 were non-significant ($P > 0.05$).

A paired *t*-test showed that for the animals sacrificed 3 days after unilateral SCGX, melatonin secretory capacity immediately before euthanasia was significantly ($P < 0.02$) less than that recorded pre-operatively. A comparable pre- versus 14 day post-operative comparison of secretory response data for the animals killed 14 days after surgery, was not significant. These results have been demonstrated in Figs. 3.3 and 3.4.

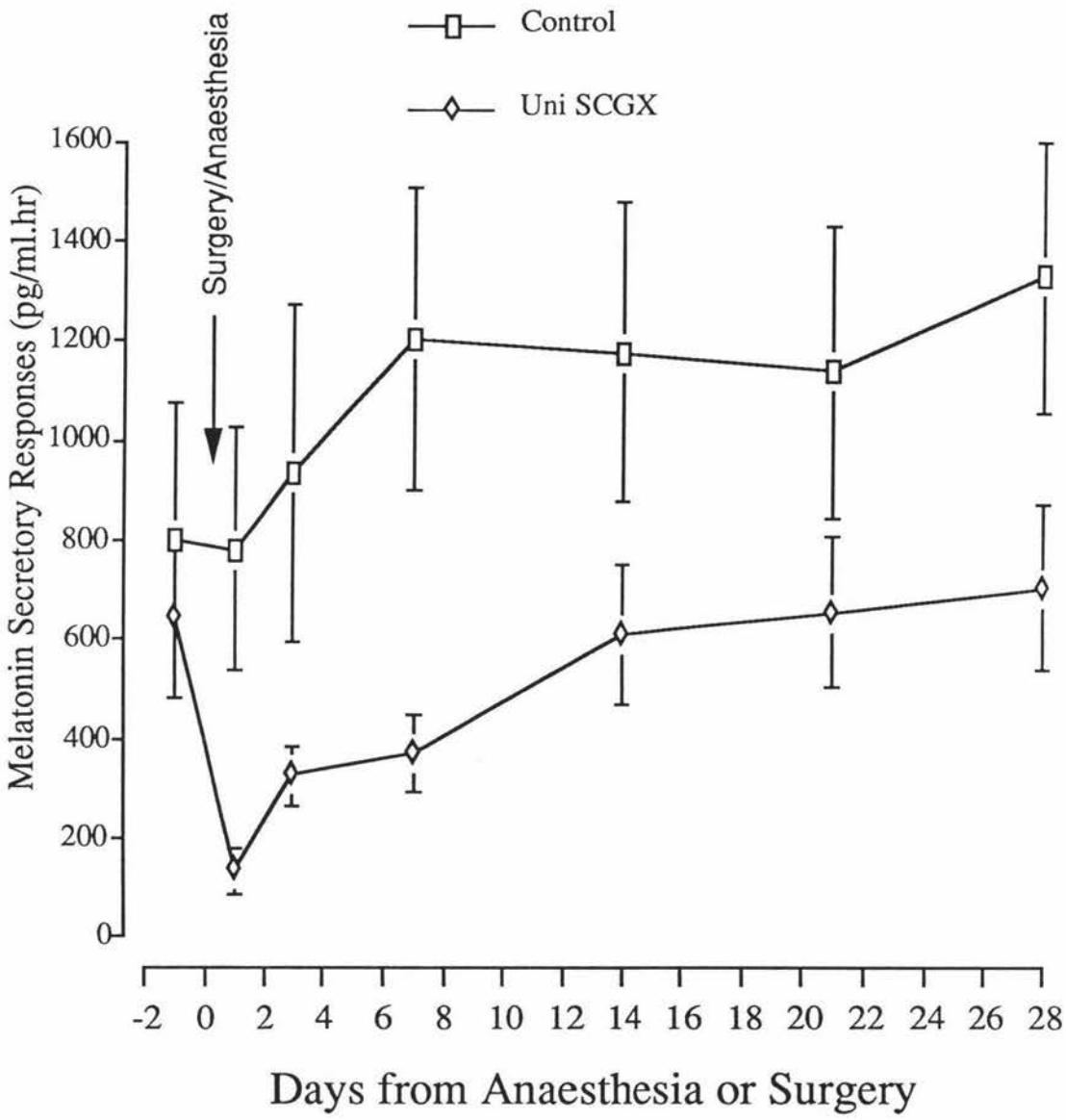
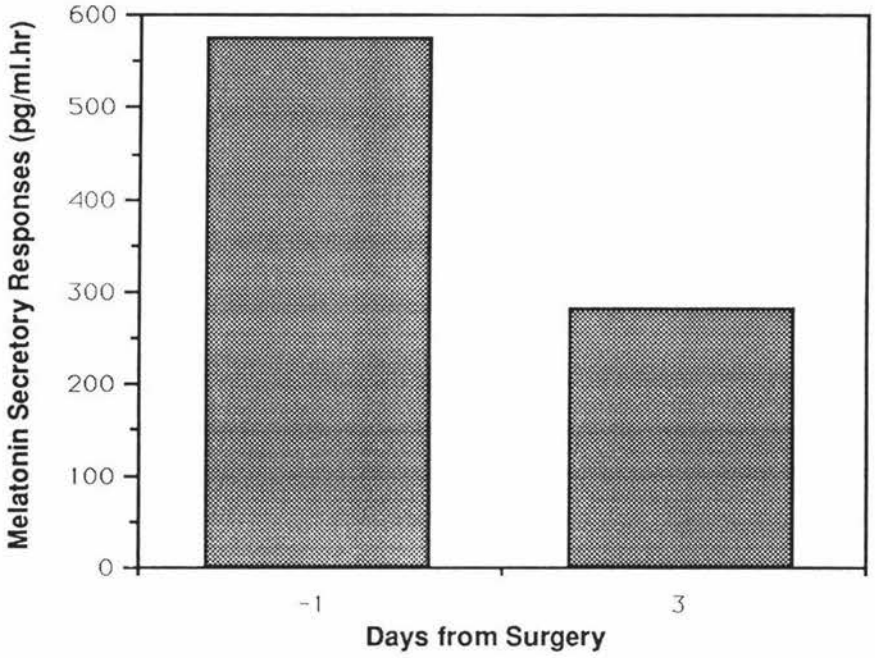
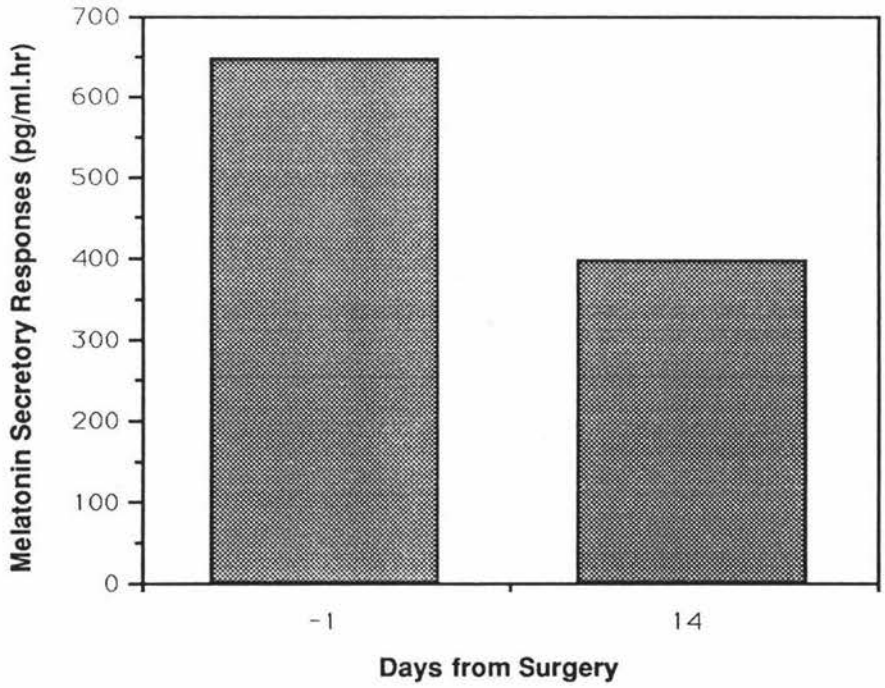


Fig 3.2 : Mean (±SEM) integrated melatonin secretory responses (pg/ml.hr) of sheep during 4 hrs of exposure to darkness, prior to and at 1, 3, 7, 14, 21 and 28 days after anaesthesia or surgery.





Figs. 3.3 & 3.4 Mean integrated melatonin secretory responses (pg/ml.hr) of sheep during 4 hrs of exposure to darkness, prior to and at 3 days (Fig 3.3) and 14 days (Fig 3.4) after unilateral SCGX. Data depicted is respectively, that from animals killed at 3 and 14 days after unilateral SCGX.

3.4 Discussion

The study described in this chapter was designed to further investigate the role of the sympathetic innervation to the sheep pineal gland in affecting biosynthesis of the indoleamine, melatonin. It was designed principally as an extension of the reported recovery (Lapwood, 1993) of pineal melatonin secretory capacity after partial denervation by unilateral SCGX, to investigate whether full recovery occurred if the post-operative period was extended to 28 days. It also re-examined the use of melatonin secretory profiles during 4 hr periods of darkness, as a parameter of pineal function.

3.4.1 Use of melatonin profiles as a parameter of pineal gland function

In view of variable metabolic clearance rates for melatonin, which undoubtedly exist between different animals, it is accepted that there are some limitations in the use of areas under melatoin secreteory responses (calculated by integrating hormone concentrations with time intervals between samplings), as a measure of pineal melatonin production rates. However, in this study the principal aim was to examine within-animals changes over time, relative to the time of treatments (unilateral SCGX or anaesthesia). Assuming that for a particular animal, the melatonin metabolism rate is relatively constant over time, it is considered these secretory responses are appropriate dynamic measures of changes in melatonin production rates, with time. Also, metabolism and clearance of melatonin is rapid (Kopin, *et al*, 1961), the major route of metabolism being 6-hydroxylation followed by conjugation with either sulphate (59-79%) or glucuronic acid (13-27%) within the liver, followed by excretion which is predominantly in the urine (Kennaway, *et al*, 1977).

Having acknowledged that limitation, it is appropriate to say that in terms of being an indication of pineal secretory capacity, the measurement of hormone concentrations in plasma and subsequent calculation of melatonin profiles are considered to be a superior indication of the pineal gland's secretory capacity, in contrast to other methods that were limited to indicating secretory potential, such as those that measured pineal NAT activity (e.g. Reiter, *et al*, 1979; Bowers, *et al*, 1984; Kuchel, *et al*, 1990). The reason for that assertion is that although Reiter, *et al* (1979) produced data that indicated that pineal NAT activity and melatonin content were highly correlated, there appears to be no data in the literature which demonstrates that either of those parameters translate into accurate measures of pineal melatonin output. To the contrary, it has been shown in rats that as little as 10% activation of NAT is sufficient to initiate full melatonin production (Whelar, *et al*, 1979), while in a later study by King, *et al* (1984) it was shown that maximum NAT activity may not necessarily initiate maximum melatonin production, if pineal serotonin production has been reduced.

It is through the use of larger non-rodent species, such as sheep, which can readily be subject to multiple blood sampling, that the dynamic estimate of melatonin secretion and its variation from time to time, can be measured for individual animals. The variability of melatonin secretory capacities between animals was highly significant ($P< 0.001$) and has been depicted in Fig. 3.1. For example, in the control group, on the day before anaesthesia, individual secretory capacities ranged from a low of 161.9 pg/ml.hr to a high of 1588.7 pg/ml.hr. Using this technique of measurement as a parameter of pineal function was, however, validated by the consistency of secretory levels for individual animals, where animals with low initial measurements remained comparatively low in comparison to those of animals with consistently high secretory levels.

3.4.2 Lack of effect of anaesthesia on dark period melatonin secretion

As clearly shown in Fig 3.2, no appreciable inhibitory anaesthetic effect on dark period melatonin secretion was measured in this experiment, despite indications to the contrary where in a corresponding study (Lapwood, 1993) had reported a relatively pronounced, though non-significant, post-anaesthetic effect. This result reiterates the importance of not over-emphasizing results that maybe substantial, but which are not significant, such as the apparent anaesthetic effect recorded by Lapwood (1993).

Anaesthetics have previously been reported to variably influence the secretory capacity of endocrine systems. For example, pentobarbitone reduces LH secretion in rats (Blake, 1975) and alters the secretion of gonadotrophins in ewes (Goodman & Meyer, 1984), whereas, saffan anaesthesia has no effect on gonadotrophin secretion in ewes (Clarke & Doughton, 1983). Those reports, in conjunction with the results of Lapwood (1993) in measuring a 36% post-anaesthesia depression in secretory capacity of melatonin for controls, suggested that possible anaesthetic effects should be acknowledged when designing experiments investigating pineal function. It is noteworthy that virtually all the past studies investigating pineal function in response to various surgical procedures have ignored the possibility of anaesthetic effects on the pineal gland and consequently had not anaesthetized control animals (e.g. Kuchel, *et al*, 1990).

Mockett (1991) measured the effects of a range of anaesthetics on the melatonin secretion response curves of sheep and, partially based upon those results, the anaesthetic combination of saffan induction and halothane maintenance was selected for use in this current experiment, as being the least inhibitory of the anaesthetic combinations tested to date.

3.4.3 Increase in plasma melatonin levels in controls

After day +1 after anaesthesia melatonin secretory capacity of control animals increased above pre-surgery levels and continued increasing through to 28 days post-surgery. In that time secretory output rose 70.0%, from 780.5 pg/ml.hr at day +1 to 1325.8 pg/ml.hr on day 28. Though substantial, that result was not, however, significant. That being so, this result does follow a similar trend to that of measurements for the control group of Lapwood (1993), in which melatonin secretion increased from 63.6% of the pre-operative levels on the day after unilateral SCGX to 125% on day +14 and was significant ($P< 0.01$). That author attributed at least part of the post-anaesthetic recovery in melatonin secretion to recovery from the depressant effects of anaesthesia. However, in the present experiment anaesthesia had little effect on melatonin production, so other factors which may have contributed to the post-anaesthetic increase must be considered. Possibilities include: (i) seasonal variations in melatonin secretion, (ii) the implementation of an insufficient acclimatization period, (iii) the disruption of endogenous oscillators and (iv) adaptation to the stress of handling .

Seasonal variations in melatonin secretion have been reported, particularly relevent are significantly elevated melatonin profiles during autumn, coinciding with the onset of endocrine, behavioural and social reproductive processes that occur through the breeding season, during which this experiment was performed. Reports on the effects of seasonal hormone changes on the melatonin secretory capacity for ewes are varied, however, it has been established that for Romney ewes the effect of changing seasons on mean melatonin levels during darkness are significant, with an increase of 153% for the autumn period over those measured during the preceeding season (Scott, *et al*, 1992).

As it is the photoperiodic signal which governs control of melatonin biosynthesis (Oksche, 1971) and also affects seasonal changes in melatonin secretion (Woodfill, *et al*,

1991), severe alteration of the lighting regime would presumably alter synthesis and secretory patterns for the hormone. In this experiment an acclimatization period of 3 weeks was implemented to stabilize melatonin secretion in synchrony with the new lighting regime. The consistency of the dark period secretory level means for the four treatment groups prior to anaesthesia /surgery (Table 3.5) would suggest that 3 weeks had been adequate in stabilizing secretory levels.

Endogenous oscillators emanating from the suprachiasmatic nuclei (Moore, 1983) have been implicated in maintenance of internal control of the rhythm of melatonin secretion and various models are proposed to explain the controlling mechanism. The theoretical two-oscillator model suggests that one oscillator coupled to dusk controls the evening rise in melatonin and another controls the morning fall (Illnerova & Vanecek, 1982). The imposed advance of 4 hours for the onset of dark in this current experiment may have caused asynchrony of any such model and adaptation to that change could cause some variation in melatonin levels.

As mentioned in Section 3.4.4, stress such as that due to animal handling, has been shown to inhibit pineal function. In the present experiment, it is possible that animal adaptation to handling stress over the 4 week sampling period, may have contributed to the increase in melatonin secretion recorded over that time.

3.4.4 Immediate (Day +1) effects of unilateral SCGX on pineal melatonin secretory capacity

On the day after surgery unilateral SCGX reduced pineal secretory capacity by 80%, despite only a halving of the sympathetic fibres innervating the pinealocytes. Corresponding to the reduction in melatonin secretory capacity in this current experiment, Dornay, *et al* (1985) have reported a > 50% reduction in pineal tyrosine

hydroxylase activity after unilateral SCGX and an approximate 50% reduction in noradrenaline uptake. Also, Zigmond, *et al* (1981) reported a 75% decrease in serotonin NAT activity and suggested that the effects of neurons in the two ganglia were not simply additive, but rather that an interaction of the two populations of neurons was necessary for the maintenance of normal rhythms in pineal NAT activity.

Mechanisms by which such a major effect (>> 50%) may have been produced could include secretory inhibition by anaesthesia as discussed in Section 3.4.2, in addition to probable 'stressors', for example handling (Cardinali & Romeo, 1991), which have been shown to decrease pineal NAT activity (Lynch, *et al*, 1973). The central role which the pineal gland plays in the neuro-hormonal axis does mean that any element of stress could disrupt pineal function, particularly in the immediate term. Also, Bjorklund, *et al* (1972) and Wiklund (1974) have demonstrated, using 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 catecholeamine-containing fibres, via the pineal stalk. Such a scenario, if present in sheep, could augment inhibitory effects on pineal function following unilateral SCGX, by the disruption of interactions between SCG's, through severance of a central relay to the contralateral, intact SCG.

3.4.5 Recovery in pineal function after unilateral SCGX.

Following unilateral SCGX, and the initial (Day +1) substantial decline ($P< 0.01$) in pineal melatonin secretory capacity, a linear recovery was measured through to 28 days after surgery. That linear recovery was a real experimental effect despite a corresponding increase in control pineals, as the rate of response was determined to be significantly greater ($P< 0.05$) for the SCGX group in contrast to that for the control group (Treatment x Time interaction; Post-op Linear x Control vs SCGX component). The capacity of the

unilateral SCGX animals to secrete melatonin had risen to within 94% of pre-operative levels by 14 days after surgery, which was a similar result to that measured by Lapwood (1993), who recorded a recovery to 77% over the same period. The continued measurement over an extended recovery period undertaken in this current experiment confirmed that full recovery occurs, reaching pre-operative levels by 21 days after partial denervation. A corresponding recovery has been repeatedly reported in rodent experiments too, however, in those experiments capacity to secrete melatonin was not measured, but rather parameters used were indications of potential to secrete melatonin, by measurement of pineal enzyme activities (Bowers, *et al*, 1984; Kuchel, *et al*, 1990; Reiter, *et al*, 1979; Dornay, *et al*, 1985 & Zigmond, *et al*, 1985), melatonin content (Reiter, *et al*, 1979) and measurement of urinary 6-hydroxymelatonin, conjugated with glucuronic acid (Kuchel, *et al*, 1990). Nevertheless, the recovery of different parameters of pineal function in response to partial denervation by unilateral SCGX does have universal verification. Very similar trends, in recovery of melatonin secretory capacities, also were recorded in the present experiment from the two groups of animals killed at 3 and 14 days after unilateral SCGX For convenience of calculation the melatonin secretory data from those two groups, which had been included for provision of pineal tissue, were not included in the ANOVA.

As mentioned earlier, anatomical, biochemical, and physiological evidence has determined that the two sympathetic tracts, originating from the SCGs, innervate the pineal gland to the same extent (Zigmond, *et al*, 1981; Bowers & Zigmond, 1982; King, *et al*, 1983, 1984b). Also, tracing of NE-containing fluorescent fibres (Dornay, *et al*, 1985) and anterograde tracing using horseradish peroxidase (Bowers, *et al*, 1984b), have established that nerve fibres from each SCG innervate both sides of the pineal. SCG stimulation studies have determined that only a small number of pinealocytes receive input from both ganglia (Lingappa & Zigmond, 1987), and that mostly one ganglia

innervates only approximately half the pinealocytes of the gland (Reuss, *et al*, 1985). With those concepts in mind, the initial effect of unilateral SCGX may be expected to render approximately 50% of the sympathetic neurons distributed throughout the gland 'electrophysiologically silent', and as a consequence approximately 50% of pinealocytes may be expected to cease to function.

The capacity to secrete melatonin in amounts above 50% of pre-operative levels, from 3 days after partial sympathetic denervation by unilateral SCGX, indicates that some compensatory mechanism(s) was/were functional. Several possible mechanisms have been cited to account for recovery of function after neural damage.

An increase in postjunctional sensitivity has been espoused with evidence of a rapid (24 hrs) increase in sensitivity of pinealocytes to *beta*-adrenergic stimulation after bilateral denervation of the pineal gland (Degushi & Axelrod, 1972c; Romero & Axelrod, 1974), with similar levels of hypersensitivity maintained at 14 days. However, Zigmond & Baldwin (unpublished, cited by Zigmond, *et al* (1985)) have determined that post-junctional sensitivity does not increase after unilateral SCGX. Increases in receptor density (Gonzalez-Brito, *et al*, 1988b; Pangerl, *et al*, 1990) also have been demonstrated to occur as a response to bilateral sympathetic denervation and possibly some degree of pinealocyte hypersensitivity does occur in sheep as a response to unilateral SCGX, functioning to alleviate the loss of innervation. Another hypothesized mechanism suggests an increase in impulse activity of nerves on the intact side (Zigmond, *et al*, 1981), compensating for the lack of transmitter release from the sectioned nerves. What retrograde signal would trigger such a mechanism, or how the signal would be sustained, and whether cell body organelles could be sufficiently responsive in producing necessary increases in NE levels, remain unanswered.

Alternatively, Zigmond, *et al* (1985) have proposed that an increase in the efficacy of residual nerves in stimulating denervated pinealocytes occurs. The reduction in the

number of sympathetic neurons effectively reduces available uptake sites for NE, resulting in increased junctional concentrations of the noradrenergic transmitter, culminating in an increased re-uptake by intact neurons and a subsequent increase in post-junctional effectiveness. In this current experiment that theory would suggest that NE uptake by sectioned neurons was initially maintained, and that the NE was ineffective, thus causing the preliminary decline in pineal function. However, as those nerves degenerate, and so too the ability to take up NE, junctional concentrations are increased, as is NE uptake and post-junctional stimulation by residual neurons, resulting in a recovery of pineal function. Topographical evidence to support this theory has included anterograde nerve tracing with horseradish peroxidase that has determined that nerve fibres from each SCG spread throughout the gland and merge with fibres originating from the contralateral SCG (Bowers, *et al*, 1984b).

The extent of the recovery, both quantitatively and temporally- (the recovery represents a long-lasting and probably permanent change), in melatonin secretory levels, suggests that the hormone producing machinery, within the pinealocyte cells, is not directly affected by the denervation, but remains intact and capable of re-establishing function. That the pinealocytes do not atrophy has recently been determined by morphological analysis of sheep pineals, where use of specific staining techniques has established the presence of a pinealocyte population, 14 days after unilateral SCGX, that was no different from that in unoperated controls (Mockett & Lapwood, unpublished). This present experiment makes a corresponding inference with functional regeneration establishing secretory capacity at levels above those which would be expected if pinealocyte degeneration had occurred.

To date, the most likely concept developed to explain recovery of pineal function after unilateral SCGX has been the re-innervation of denervated pinealocytes, by

regenerative growth from residual nerve terminals originating from the intact SCG. As evidence to support "compensatory collateral sprouting", Dornay, *et al* (1985) measured a gradual increase of choline acetyl transferase activity (a marker of cholinergic pre-ganglionic terminals) in the remaining 'intact' SCG, which occurred over the period corresponding to that during which an increase in pineal TH activity was measured. Further to that, corresponding with timing of the decline and recovery of both TH activity and ^{3}H noradrenaline uptake after partial denervation, those authors noted that the density of NE-containing fibres in the pineal glands (measured by NE histofluorescence intensity) was reduced at 2 days after unilateral SCGX, but partially recovered at 7 days.

Other studies have demonstrated that the sympathetic neurons of the SCG can undergo 'heterotypic collateral sprouting' in response to a selected partial denervation of a foreign target region in the brain (Loy & Moore, 1977; Stenevi & Bjorklund, 1978; Crutcher, *et al*, 1979). Presumably, cell bodies capable of heterotypic growth in response to some stimulatory signal would be equally capable of homotypic growth. NSE immunohistochemical studies have shown that 14 days after unilateral SCGX pinealocyte morphology remains intact (Lapwood & Mockett, unpublished), suggesting that collateral growth is at least anatomically possible as a viable compensatory mechanism.

Evidence in support of the concept that recovery of pineal function was the result of new nerve terminal growth, reinnervating denervated pinealocytes, is presented in Chapter 4, in the form of varying degrees of ICC detection of GAP-43 in nerve fibres in pineals of unoperated control animals and in the pineals collected at 3, 14 and 28 days post unilateral SCGX. That material in Chapter 4 provides the most convincing evidence to date that pineal recovery from semi denervation occurs as a result of pinealocyte re-innervation by collateral sprouting of residual nerve terminals, originating in the contralateral SCG.

3.5 Conclusions

Measurement, during the period of darkness, of melatonin secretory profiles in plasma has proved to be a practical method for monitoring pineal function. In this study, secretory rates have been measured periodically, following unilateral SCGX, as a parameter to determine the rate and extent of recovery in pineal function after partial denervation. It is considered that this approach is more appropriate to this kind of study than the previous use of estimates of pineal enzyme activity, because it provides a reasonably direct measure of hormone production, rather than of secretory potential.

Initial response to unilateral SCGX was a reduction in secretory capacity to 20% of pre-operative levels, which was followed by a full recovery, despite the loss of 50% of the regular sympathetic innervation to the gland. Measurements indicate that recovery begins by 3 days after unilateral SCGX, was clearly established at 14 days and that full secretory capacity was reached at approximately 21 days.

As demonstrated in previous experiments using bilateral SCGX, this experiment confirms that sympathetic innervation is essential to pinealocyte biosynthesis of the indoleamine, melatonin.

A number of mechanisms may contribute to establishing the recovery of pineal function after partial denervation. Post-junctional hypersensitivity and increasing levels of NE secretion from the residual neurons, combined with post-operative recovery, are possible initiating factors. However, measurements of a full recovery in secretory levels indicates that re-innervation of pinealocytes occurs as a result of collateral sprouting of sympathetic terminals from the contralateral SCG. Increasing enzyme activity in the

intact SCG indicates that residual nerve terminals may re-innervate pinealocytes from which ganglionectomized neurons had degenerated.

Chapter .4.

Neural growth in sheep pineal tissue following partial denervation by unilateral superior cervical ganglionectomy

4.1 Introduction

As discussed in Section 1.3 there have been numerous studies performed to investigate the mechanisms responsible for controlling pineal function and in particular melatonin biosynthesis. For all mammalian species studied, primary innervation is by post-ganglionic sympathetic neurons originating from the SCG's, as shown by the fact that bilateral SCGX disrupts rhythms of pineal metabolites and enzyme activity and also abolishes nocturnal melatonin secretion. Similarly, unilateral SCGX in sheep causes a >50% reduction in pineal secretory capacity for melatonin, however, that immediate reduction is succeeded by a substantial recovery to pre-operative levels, as reported by Lapwood (1993) and also exhibited in Fig 3.2.

Mechanisms which have been suggested to explain that recovery in pineal function after unilateral SCGX are discussed in Section 3.4 and include enhanced effectiveness of NE released by remaining nerve terminals (particularly due to an increased capacity for uptake of NE) (Zigmond, *et al*, 1981, 1984), increased impulse activity by residual neurons (Zigmond, *et al*, 1981), hypersensitivity of pineal beta-adrenergic receptors to NE (Romero & Axelrod, 1974), an increase in beta-adrenergic receptor density (Gonzalez-Brito, *et al*, 1988b; Pangerl, *et al*, 1990), and also collateral sprouting of the remaining nerve terminals to re-innervate denervated cells (Bowers, *et al*, 1984; Dornay, *et al*, 1985). It remains to be established which and to what extent each of these mechanisms is involved in the regeneration of pineal gland function after partial denervation.

Collateral sprouting of post-ganglionic neurons originating from the residual SCG is at present probably the most valid concept, as has been discussed in Section 3.4. However, to date, no definitive evidence has been presented that determines whether nerve regeneration does actually occur within the pineal gland. Re-innervation of pinealocytes by collateral growth originating from residual neurons could be implicated as a compensating mechanism if the presence of regenerating neurons were to be established during the period over which regeneration of pineal function has been demonstrated to occur.

Regenerative axon growth in mammalian peripheral nerves has been reported to be accompanied by increased or decreased synthesis and axonal transport/content of a number of proteins (for review see Skene, 1989). Prominent among these is the growth associated protein, GAP-43, against which antisera have recently been developed, enabling immunocytochemical visualization of that protein during both *in vitro* and *in vivo* studies of nerve growth, mostly investigating responses to traumatic injury of explanted neurons grown in culture (Meiri, *et al*, 1986; Skene, *et al*, 1986; Meiri, *et al*, 1987; Goslin, *et al*, 1988; Goslin & Banker, 1990). To date, the actual function of GAP-43 in growing axons is unknown, however, the high concentration of the protein in nerve growth cones (Widmer & Caroni, 1993) does suggest a functional role in either migration and/or environmental interaction and its presence in high concentration in neurons is interpreted as being indicative of growth (Gorio, 1993). Sections 1.5.7 to 1.5.9 review current literature on growth cones and GAP-43 which is pertinent to this experiment.

Microtubules, which are assembled from alpha and beta tubulins, are also integral components of growing neurites (Daniels, 1972) and, thus, essential building blocks during neuronal regeneration and sprouting. In addition to maintaining a structural role in association with the cytoskeleton, configurational modification of microtubules has been

reported in association with both dyneins and kinesins, an association involved in the function of axonal growth and in axonal transport (Brady, 1991). In the central nervous system the alpha tubulin (AT) component has mainly been associated with synaptic membranes and vesicles (Zisapel, *et al*, 1980), corresponding with a secretory role suggested for the presence of microtubules in cell lines from the pituitary gland (Kelly & Grote, 1993) and pancreas (Dentler & Suprenant, 1986). Two separate histochemical investigations have also established the presence of AT in pinealocytes of both baboons (Theron, *et al*, 1979) and guinea-pigs, but not in rats (Schroder, *et al*, 1990). No role for AT in pinealocytes has yet been demonstrated, but the association is not surprising considering the neuroendocrine characteristics which are a feature of pinealocytes (reviewed by Oksche, 1987) and a role for microtubules in hormonal secretion, similar to that exhibited in the pituitary (Shiino & Rennels, 1974; Labrie, *et al*, 1973) may be possible. Further to this, Mathew & Miller (1990) have reported an increase in expression of AT mRNA in the intact rat SCG following unilateral ganglionectomy, which has implications for a role of AT during the collateral growth of residual neurons in pineals as a response to partial denervation.

The aim of the study described in this chapter was to use antisera to both GAP-43 and alpha tubulin in an ICC study to investigate the presence of those proteins in sheep pineal tissue collected at 3, 14 and 28 days after unilateral SCGX, as well as in pineals from unoperated control sheep. GAP-43 ICC was undertaken to provide direct evidence of reinnervation as a possible mechanism responsible for the recovery of pineal secretory capacity after unilateral SCGX, while AT ICC was performed to determine whether any variations in nerve or parenchymal cell content/structure occurred in response to partial denervation. In addition, morphological associations with pineal tissue, specific to each protein, were investigated.

4.2 Materials and Methods

4.2.1 Animal treatment groups

The same animals and procedures as detailed in Section 2.2 were the subject of the following study.

4.2.2 Pineal tissue collection

At the end of the last post-operative bleeding period for each particular group, animals were killed in the dark, between 1730 and 1930 hours, with a lethal dose (60 mg/kg) of sodium pentobarbatone ("Pentobarb 500", Chemstock Animal Health, Ltd., Christchurch, New Zealand). Each animal's head was immediately removed and the top of the cranium opened 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, then dissected free from brain tissue surrounding its attachment and removed. Excess brain tissue was trimmed from the pineal leaving only a small amount of thalamic tissue on the anterior margin for the purpose of orientation. A saggital dissection ensued with each half of the gland placed in Bouin's fixative. The whole procedure from killing to immersion of the pineal in histological fixative was completed in approximately 6-8 min.

4.2.3 Tissue processing prior to immunocytochemistry.

(I) Tissue fixation

Both halves of each pineal were fixed for 12 hour's in Bouin's fluid and then transferred to 70% ethyl alcohol.

(II) Paraffin wax embedding

All pineal tissue was paraffin wax embedded using an automatic tissue processor (Shandon Elliot, Liverpool, U.K.). The tissues were dehydrated, cleared and impregnated with paraffin wax according to the schedule in Table 4.1 (Birtles, 1981).

Table 4.1 Paraffin wax processing schedule.

Process	Reagent	Time
1. Dehydration	Change 1. 70 % ethyl alcohol	1 hr
	" 2. 95 % " "	"
	" 3. 100 % " "	"
	" 4. " " "	"
	" 5. " " "	2 hr
2. Clearing	Change 1. Chloroform	1 hr
	" 2. Xylene	"
	" 3. "	"
3. Impregnation	Temperature 58^{o}C Paraffin wax m.p 56^{o}C	2 hr
	" " " " "	"

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.

(III) Sectioning

From each embedded pineal, 8 *um* thick sections were cut using a sliding microtome (Model OME, C. Reichert Optische Werke AG, Vienna, Austria), then floated onto warm water (45^{O}C). Sections taken from 60-90 *um* apart were transferred to polyvinyl acetate (PVA) coated 75 by 25mm glass slides and air dried at 60^{O}C.

4.2.4 Immunocytochemistry

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

Table 4.2 Antibodies used in ovine pineal tissue immunocytochemistry.

Antigen: GAP-43

Antibody source	Subclone No.
Meiri, K., State University of New York, Health Science Centre, Syracuse, N.Y., U.S.A	(i) 7B10/D4 (ii) 10E8/E7 Reacts against phosphorylated and dephosphorylated GAP-43

Form	Storage
Monoclonal supernatant	4^{O}C (not frozen)

Antigen: Alpha tubulin

Antibody source	Code No.
Amersham International, Little Chalfont, England.	N 356

Form	Storage
Monoclonal	-70^{O}C in Ascites fluid. 2-8 ^{O}C in dilution

The monoclonal GAP-43 antisera have been shown to be specific for their antigen, with no detectable cross-reactivity with structurally related peptides or proteins (Meiri, *et al*, 1991). The subclones used had not previously been tested against sheep neural tissue and this was first performed in this set of experiments in this laboratory, using foetal sheep cerebellum tissue.

Specificity of the monoclonal antibody to alpha tubulin has been confirmed using Western blot techniques by Blose, *et al* (1984).

Once optimal staining conditions were established for each antiserum, they were each used in 2 ICC runs to stain experimental tissue using the protocol detailed in Table 4.3; a complete set of slides from all animals was stained in each run.

Table 4.3 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.	Wipe slide around section with tissue paper.
5.	Cover sections with 1% bovine serum albumin (BSA) and 0.01% digitonin[2] (both 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^{o}) antibody[3,4] (except negative control which was covered with 1% BSA in PBS) and incubated in the humidity tray for 30 mins at room temperature.
7.	Three one min washes in PBS.

8. Slides dried with tissue paper, covered with the secondary (2^{O}) antibody[5] (1:200 dilution of biotinylated anti-mouse IgG with 1% BSA and 0.01% digitonin in PBS) and incubated in the humidity tray for 15 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[5] (1:200 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[6] (5 mg DAB dissolved in 10 ml PBS and 12 *ul* of hydrogen peroxide, plus one drop of heavy metal solution[7]).

13. Place in PBS to stop reaction.

14. Rinse in tap water.

15. Counterstain in eosin for 30 sec.

16. Wash off excess stain in tap water.

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. Digitonin at 0.01% to enhance cell permeability.

3. GAP-43 antibody diluted 1:35 with 1% BSA and 0.01% digitonin in PBS.

4. Alpha tubulin antibody diluted 1:500 with 1% BSA in PBS.

5. Source: Amersham International plc, Little Chalfont, U.K

6. Reaction time varied from 1-2 mins. Some subjective assessment was involved.

7. 50 *ul* of 1% cobalt chloride and 1% nickel chloride in distilled water.

4.2.5 Optimization of immunoreactivity

The procedures described above resulted from initial studies to maximize the binding of each antibody to its antigen in ovine pineal tissue. This laboratory had not previously undertaken studies using the GAP-43 or alpha tubulin antibodies, therefore it was deemed necessary to approach this study assuming established procedures were not necessarily optimized for individual antibodies in ovine pineal tissue. In these initial studies each trial staining run included: (a) a positive control (foetal sheep cerebellum [97 days] which was presumed to contain the antigen) to confirm the 1^{O} antibody was binding to the antigen; (b) a negative control (foetal sheep cerebellum without 1^{O} antibody added) as a check on non-specific reaction product formation; (c) non-experimental pineal tissue to which the 1^{O} antibody was added to confirm antigen-antibody binding in this tissue and (d) a negative control of non-experimental pineal tissue.

Negative controls indicated that there were no significant levels of non-specific binding. Non-experimental pineal tissue was used in trials aimed at optimizing antibody dilutions and DAB reaction time (2 min). Also the need for heavy metal intensification for detection of reaction product, was determined. ICC procedures were considered optimized when the reaction gave strong specific staining without significant levels of background or non-specific staining.

(I) GAP-43

(i) Subclone 7B10/D4

Starting from a stock antiserum solution dilutions ranging form 1:8 to 1:48 in 1% BSA in PBS, which covered the working concentrations (1:4 - 1:25) recommended

(K. Meiri, pers. comm), were tested. Having established a positive reaction at all those concentrations a further run was completed using concentrations at 1:30, 1:35, and 1:45 which defined the optimal working concentration to be 1:35 in 1% BSA in PBS and which was used on the experimental tissue.

(ii) Subclone 10E8/E7

No positive immunoreaction was observed using dilutions within the recommended working concentrations or with stock solution.

(II) Alpha tubulin

A similar series of immunohistochemical titration experiments, to those used above to optimize the GAP-43 reaction, were performed to establish the antibody for AT would form an immunoreaction with the corresponding antigen present in ovine tissue, in particular in both pineal and foetal cerebellum. The recommended working range for the AT antibody was 1:500 - 1:1000 in 1% BSA in PBS, this range was covered in a series of control experiments to determine the optimal concentration for use on the experimental tissue, which was established to be 1 : 500.

4.2.6 Microscopic evaluation of 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.

(I) GAP-43

To assess the presence of new neural growth in pineal tissue after partial denervation, an evaluation of GAP-43 IR for each of the four groups (n=5) was determined using an arbitrary scale of labelling intensity from 0 through to 10, where 0 was labelling intensity for -ve control cerebellum tissue and 10 was that in the dendritic terminals of Purkinje cells in +ve control cerebellum tissue. Subjective assessment was made using an Olympus CHS2 microscope (Olympus Optical Co. Ltd. Japan) at a magnification of 100X. Two sections from each pineal were assessed in this manner and the group data pooled for statistical analysis.

(II) Alpha tubulin

One section from each pineal gland was evaluated. The immunoreaction product for alpha tubulin was principally associated with the cytoplasm of most parenchymal cells, assumed to be pinealocytes, as they could be distinguished from the other cells within the pineal tissue by their relatively large, oval shaped nucleus and nucleolus (Quay, 1965). Evaluation of IR for each tissue section was assessed through quantification of the cells which contained an IR product for alpha tubulin. This was acheived by using a 400 point grid, inserted into the eyepiece of an Olympus CHS2 microscope (Olympus Optical Co. Ltd., Japan), 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 location: parenchymal cell containing IR alpha tubulin (presumptive pinealocyte), parenchymal cell devoid of IR, or intercellular space. This approach was used as any changes in cell size or number following unilateral SCGX may be expected to be reflected as a change in frequency of cells observed lying beneath the

grid points (Mockett, 1991). For this purpose, two areas from each pineal section were evaluated at a magnification of 400-fold.

4.2.7 Photomicrography

High power (HP) microscopy was used to further investigate representative areas and cell associations of both GAP-43 IR and alpha tubulin IR present in pineals from each of the four groups. A Zeiss Axiophot microscope equipped with either normal or differential interference contrast optics, to enhance the visual perception of IR, was used to take photomicrographs (at both low and high powers) from tissue sections chosen for their high-quality tissue preservation and immunostaining, using Kodak Ektachrome Professional Film. Polarising lenses in the differential contrast optics (DIC) caused a bluish colour change to Figs 4.4 and 4.9, which was not a true representation of the eosin counterstain.

4.2.8 Statistical analysis

(I) GAP-43

In order to assess any variation in the presence of regenerating neurons within the pineal gland over the period corresponding to that in which a functional recovery had been measured, in response to partial denervation by unilateral SCGX, an analyses of variance as described in Section 2.5, were performed on results assessing GAP-43 IR, to examine the significance of experimental main effects. Contrasts between groups were determined from assessment of the intensity of IR in tissue from each group and as explained previously, in Section 4.2.6, were based solely on subjective assessment.

(II) Alpha tubulin

Analyses of variance were used on data derived from the alpha tubulin stained tissue, so as to establish whether partial denervation by unilateral SCGX caused any significant atrophy of pinealocytes, as indicated by treatment effects on the incidence of IR (presumptive) pinealocytes, non-IR parenchymal cells and intercellular space. Separate ANOVA's were performed on each of the three sets of data (i.e. IR cells, non IR cells and intercellular space).

4.3 Results

(I) GAP-43 immunoreactivity

See Tables 4.4 - 4.5 and Figures 4.1 - 4.9

Using anti GAP-43 serum subclone 7B10/D4, GAP-43 immunoreactivity was initially identified in cerebellum tissue collected from 97 day old foetal sheep, as shown in Figures 4.1 - 4.3. Three prominent layers of cells were seen in the cerebellar cortex and under low magnification, as shown in Fig. 4.1b, these were visibly differentiated by the presence or absence for GAP-43 IR, in distribution patterns that were specific to each cell layer. Compared to the consistency of magenta-eosin counter-staining across negative control tissue (Fig.4.1a), tissue treated with the primary antibody exhibited a diffuse and punctate pattern of dark IR in the granular layer (Fig.4.2b) and two definite bands of intense IR associated with the Purkinje cells, but the cells of the superficial layer were IR negative (Fig.4.3).

Deep to the molecular layer were the large flask-shaped Purkinje cells, from which a divergence of dendrites coursed into and terminated within the superficial molecular layer. The GAP-43 IR in those dendritic terminals appeared as one of the two distinct bands of IR seen in the cerebellum tissue. Molecular layer cells were not, however, IR for GAP-43. A second band of GAP-43 IR was observed at the base of the cell bodies of the Purkinje cells where neurons originating from the deeper granular layer terminated. The remainder of the gray matter of the cerebellar cortex consisted of the inner granular layer which exhibited a diffuse 'honeycomb' pattern of IR surrounding glial cells and contained intermittent punctate and intense IR which was contained within the cytoplasmic membrane of neuronal cells (Fig.4.2b). An interpretation of the patterns of GAP-43 IR in foetal cerebellum tissue is discussed in Section 4.4.1.

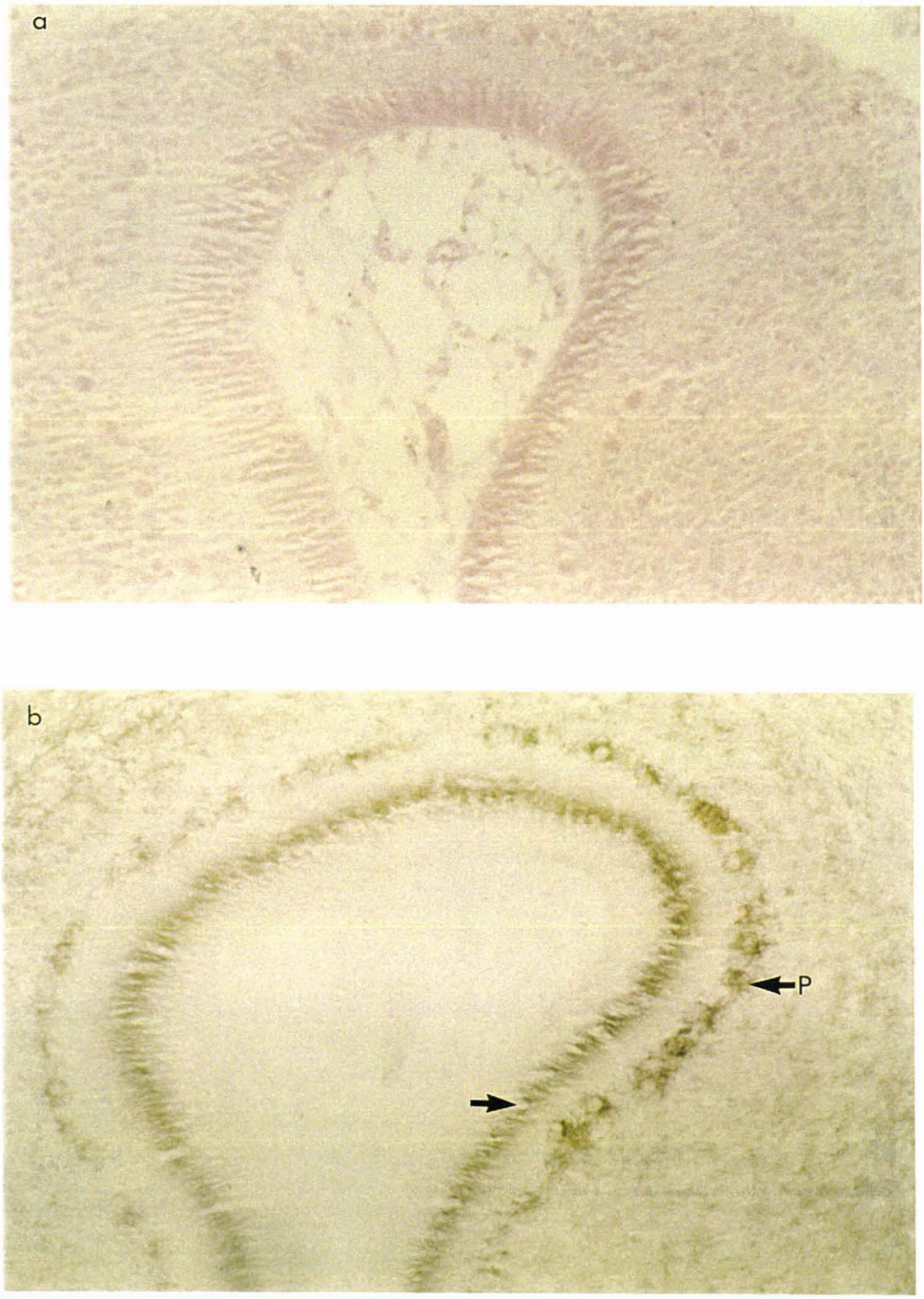


Figure 4.1 Serial sections of foetal (97 day) ovine cerebellum: negative (a) and positive (b) control tissue. Note the two main bands (arrows) of GAP-43 IR associated with both the Purkinje cell bodies (P) and dendrites. GAP-43 antiserum. Magnification 63 X.

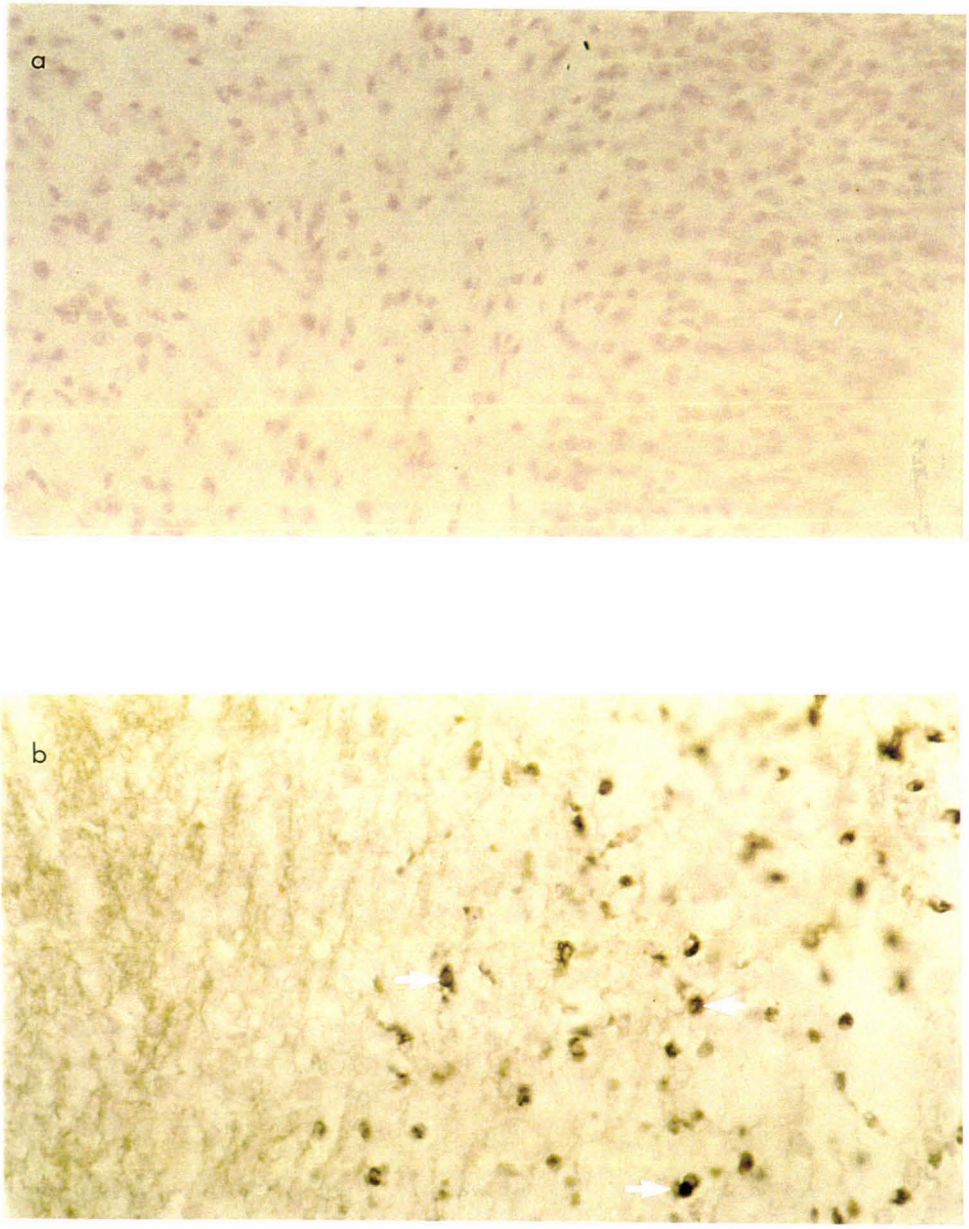


Figure 4.2 Negative (a) and positive (b) GAP-43 IR in foetal ovine cerebellum. Note the intense IR within some neuronal cell bodies within the granular layer (arrows). GAP-43 antiserum; Magnification 160 X.

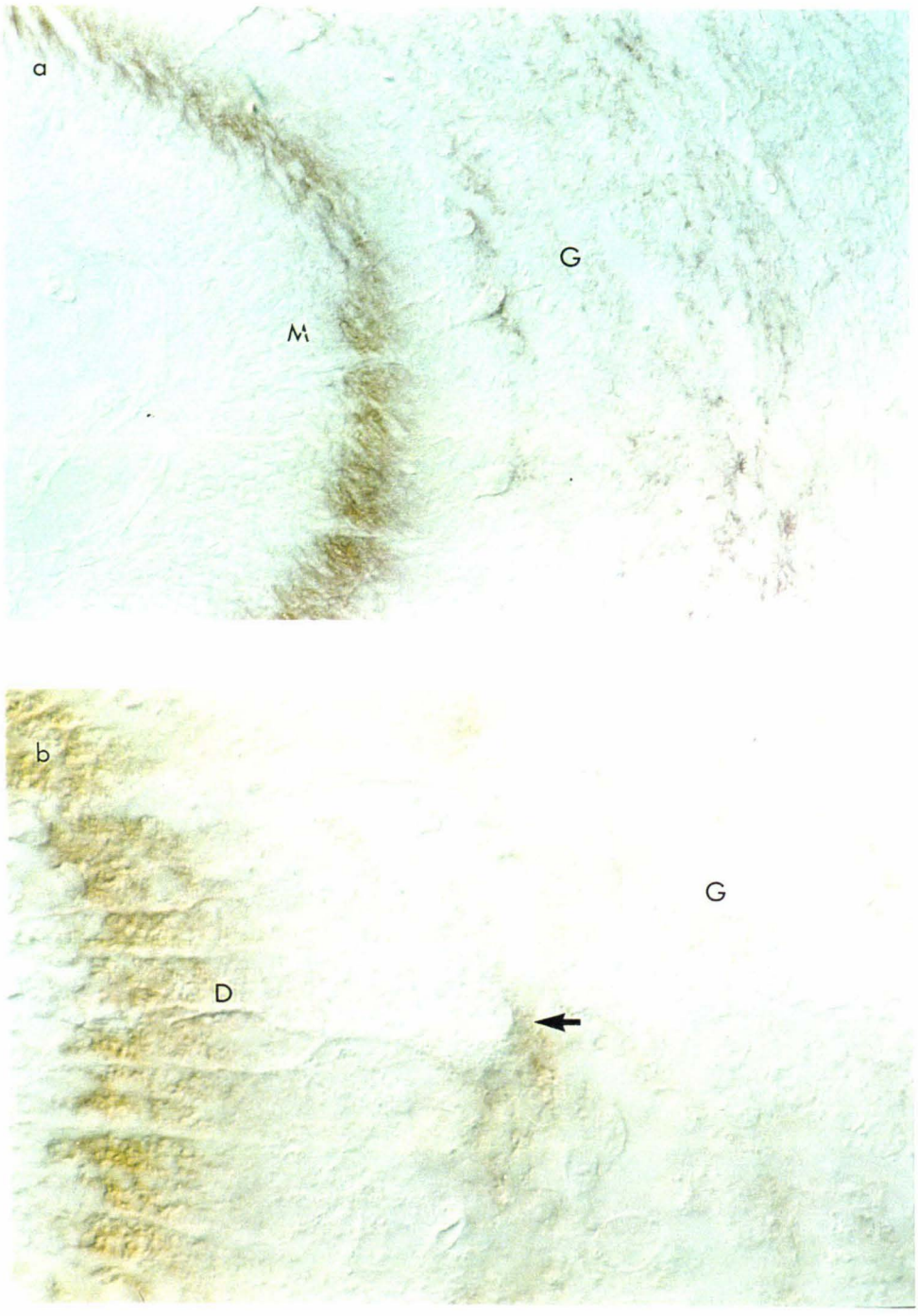


Figure 4.3 GAP-43 IR associated with the base of Purkinje cell bodies representing 'boutons' (arrow) of synapsing terminals from the deeper granular layer (G) and dendritic (D) nerve growth from the Purkinje axons apparent as a band of IR throughout the superficial molecular layer (M) of foetal cerebellum. Magnification 313 X (a) and 400 X (b). DIC.

Pineal tissue presumed to contain new neural growth did not exhibit IR when subjected to the ICC procedure described in Table 4.3, but with exclusion of the primary antibody 7B10/D4 for GAP-43 (Fig.4.4).

Application of subclone 10E8/E7 did not result in a positive immunoreaction in either foetal cerebellum tissue, nor in pineal tissue harvested from sheep that had been subjected to unilateral SCGX.

Use of subclone 7B10/D4 on pineal tissue from sheep which had previously been subjected to unilateral SCGX, resulted in a variable positive reaction which was dependent on the period of time elapsed between partial denervation and euthanasia (Fig.4.5 & 4.6) (see also Tables 4.3 & 4.4).

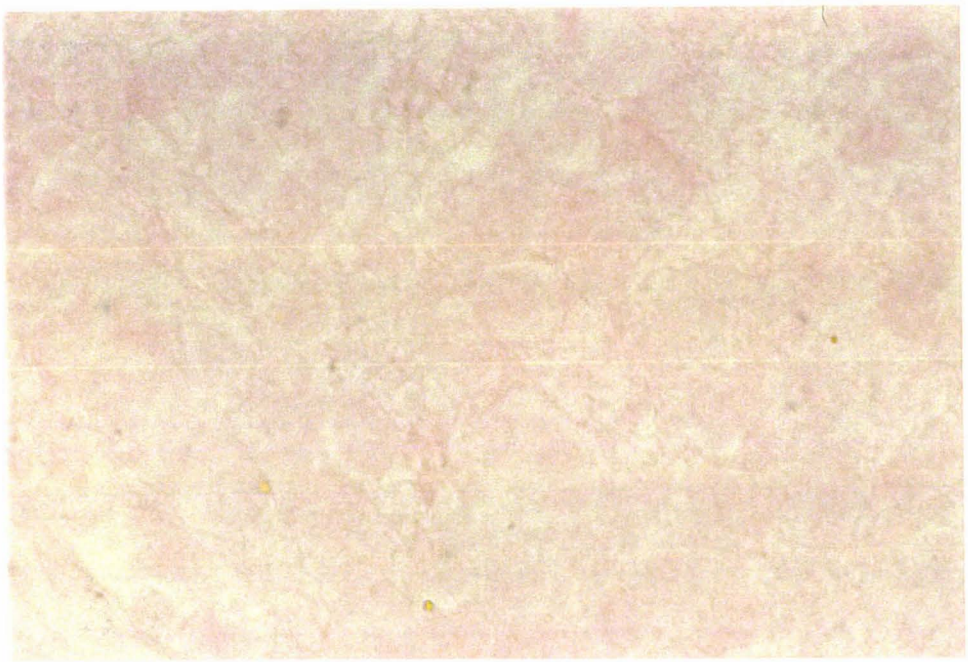


Figure 4.4 Pineal tissue collected 3 days after unilateral SCGX and used as negative control tissue. No GAP-43 antiserum had been applied. Yellow spots are melanin. Magnification 313 X.

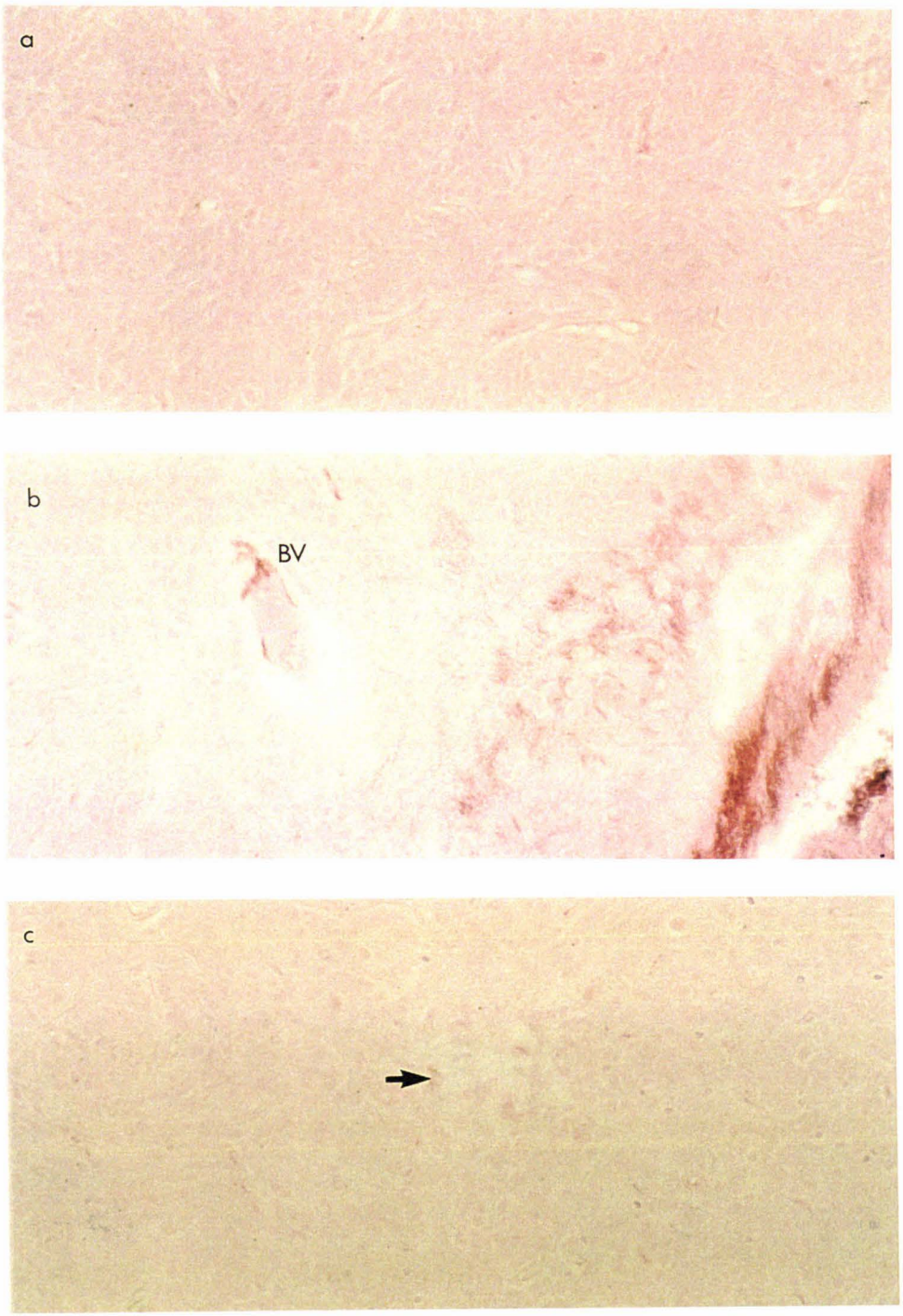


Figure 4.5 Representative examples of GAP-43 IR in pineal tissue collected from the control group (a) in addition to that from unilateral SCGX groups collected at 3 (b) and 28 days (c) after surgery. Note IR association with blood vessels (BV) in (b) and light IR (arrow) at 28 days. Note declining gradation of IR over the recovery period. GAP-43 antiserum. Magnification 63 X.

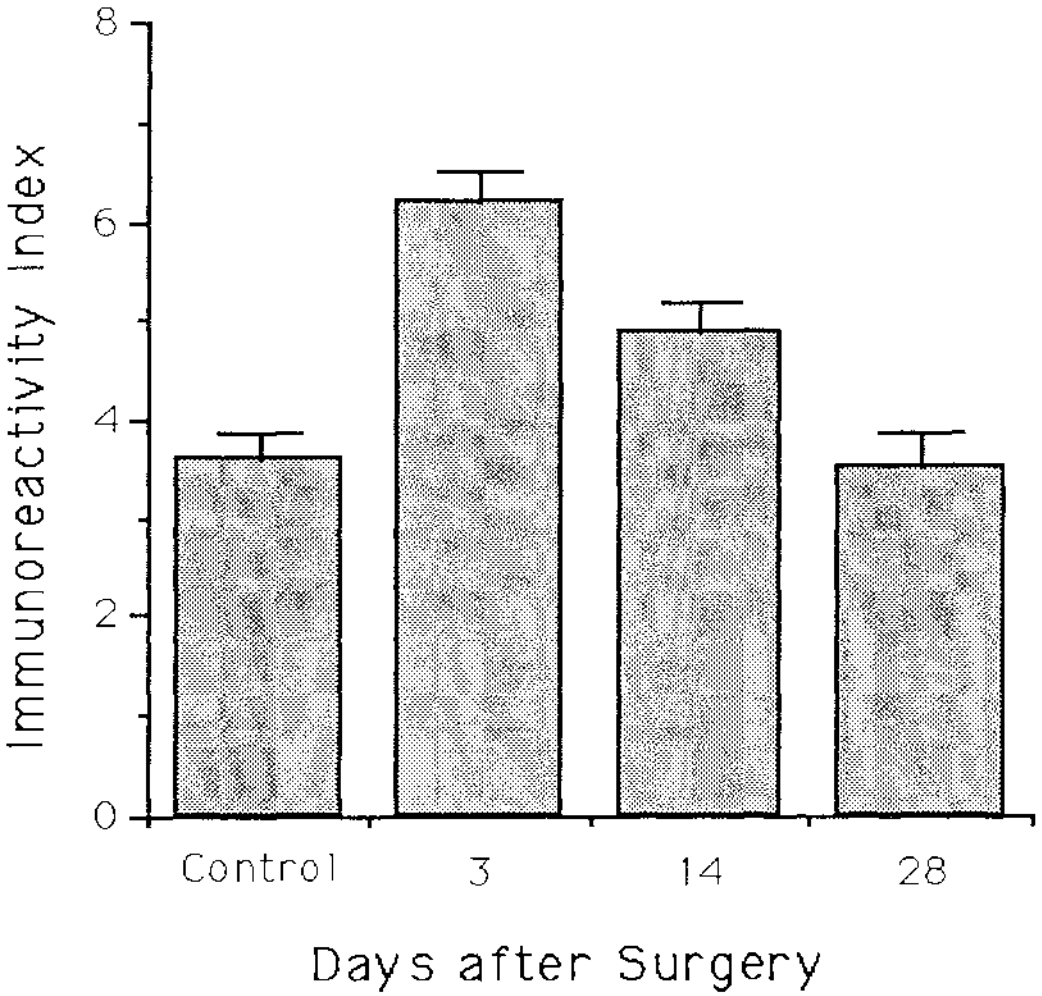


Fig. 4.6. Mean (± SEM) (n=5) Immunoreactivity of GAP-43 visualised in unilaterally denervated pineal glands collected 3, 14 and 28 days after surgery (SCGX),and in pineals of control animals euthanased 28 days after anaesthesia. Subjective assessment was made using an arbitrary scale from 0 - 10.

IR was significantly more intense ($p < 0.001$) in pineals harvested from animals at 3 days after unilateral SCGX (Fig.4.7b), than in pineals harvested from unoperated control animals (Fig.4.7a). The mean intensity of GAP-43 IR in pineal tissue collected from the control animals was less than the mean for the three operated groups, mainly due to the presence of intense IR in pineals harvested at three days post-surgery and a lesser, but strong, IR reaction in pineals collected at 14 days post-surgery.

Though sparse, under high power some IR was visible in some unoperated animal's pineals (Fig.4.8a), which had not been visible under lower magnification (125X). In contrast, GAP-43 IR present in some pineals harvested at 3 days after surgery was distributed throughout an area of >50% of the non-parenchymal tissue within the field of view (0.01mm.sq.) (Fig.4.8b). On the other hand, at 14 days GAP-43 IR was found to be dispersed among and in some instances associated with pinealocytes (Fig.4.8c). After a post-operative period of 28 days, GAP-43 IR was further reduced in intensity and had established definite associations with pinealocytes (Fig.4.8d), IR intensity being reduced to approximately the level seen in control pineals.

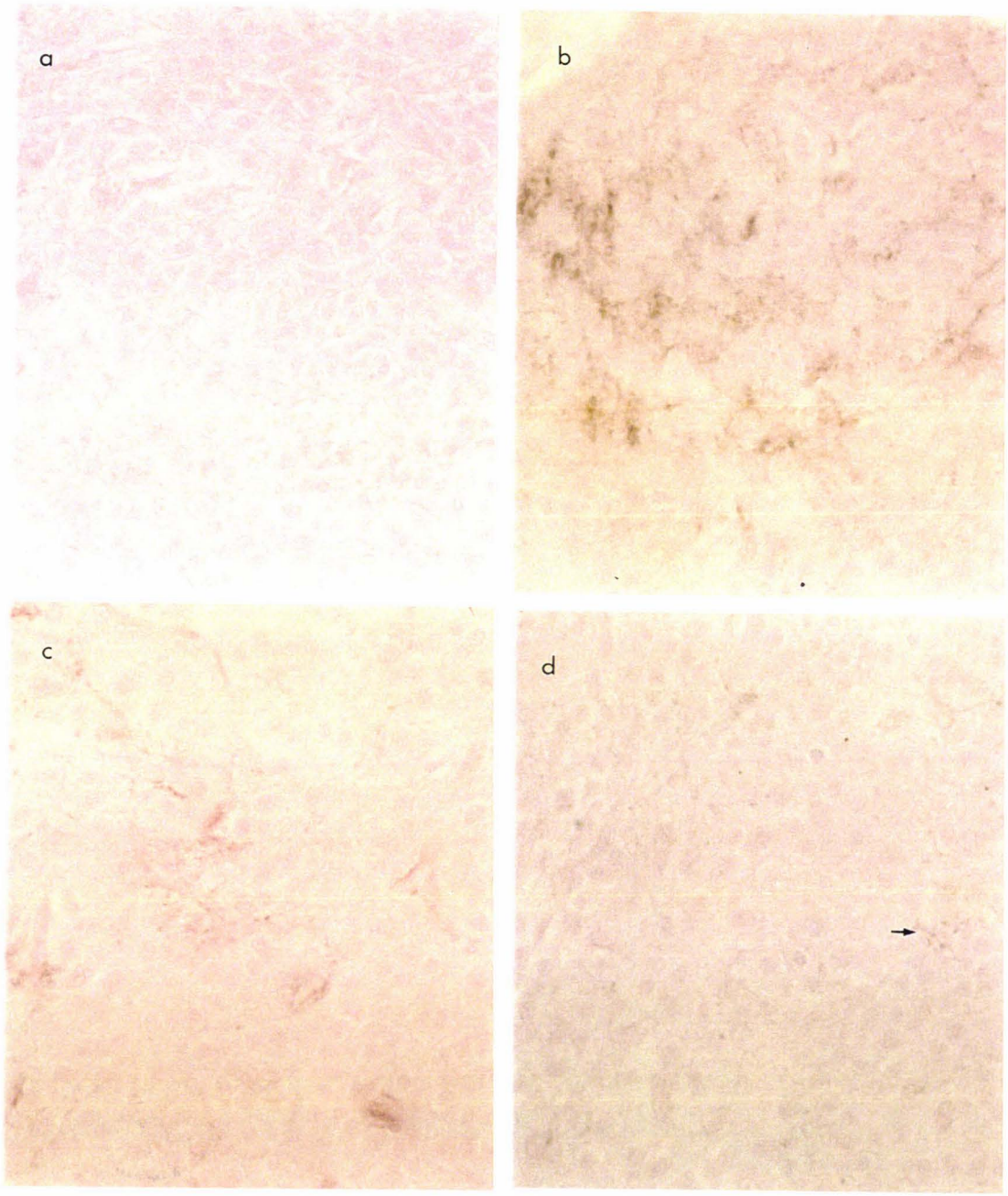


Figure 4.7 Representative examples of GAP-43 IR in pineal tissue collected from the control group (a) in addition to those from unilateral SCGX groups collected at 3 (b), 14 (c) and 28 days (d) after surgery. Note declining gradation of IR over the recovery period to slight IR at 28 days (arrow). GAP-43 antiserum. Magnification 125 X.

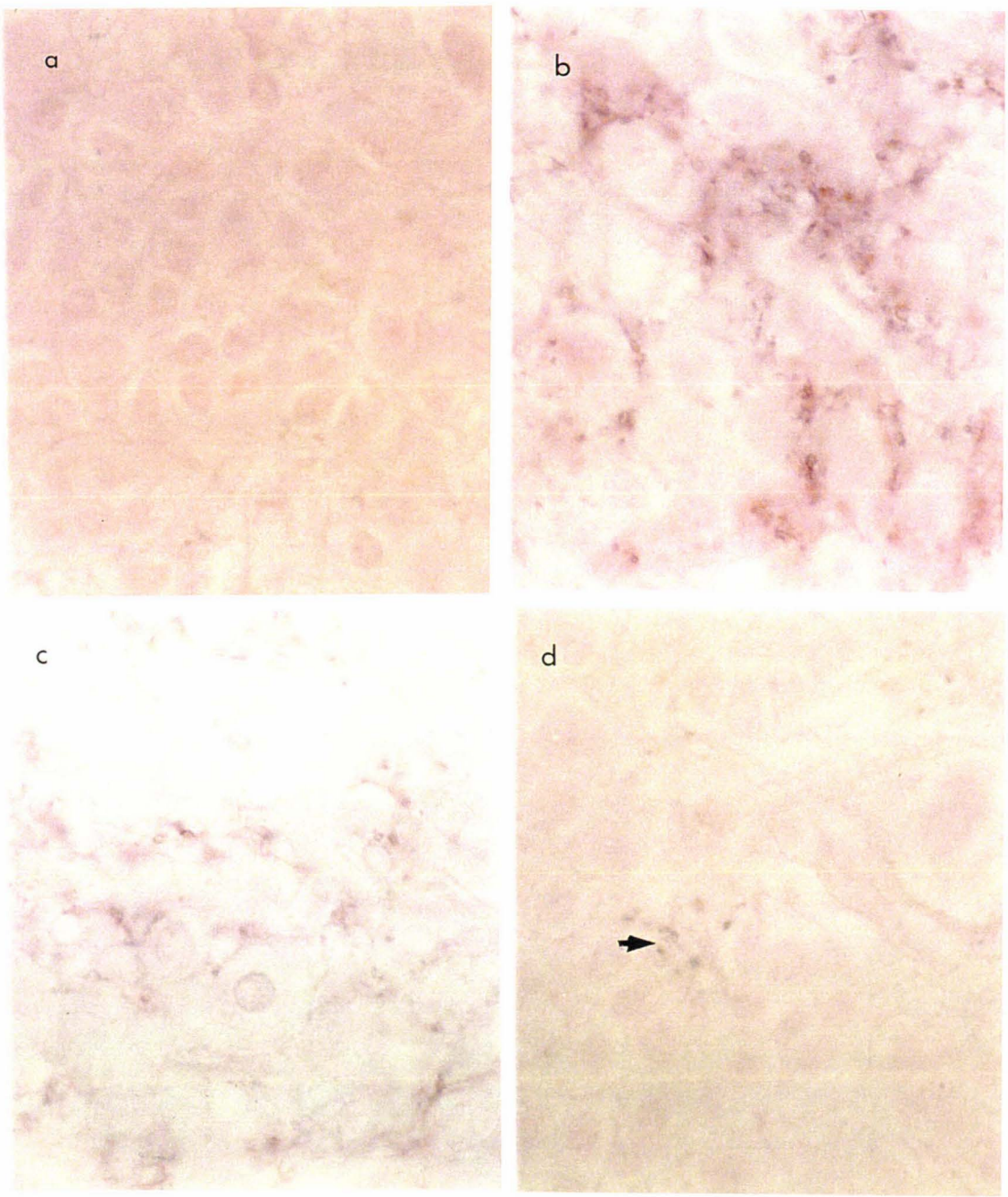


Figure 4.8 Representative examples of GAP-43 IR in pineal tissue collected from the control group (a) in addition to those from unilateral SCGX groups collected at 3 (b),14 (c) and 28 days (d) after surgery. Note declining gradation of IR over the recovery period to slight IR at 28 days (arrow). GAP-43 antiserum. Magnification 313 X.

ANOVA of IR scores for GAP-43 IR confirmed that staining was significantly more intense for the 3 day post-surgery group, than for the 28 day group ($p< 0.001$). In fact in tissue collected between 3 and 28 days post-surgery there was a linear decline in the presence of GAP-43 IR in pineal tissue, with no significant difference for measurements of IR in the means of pineal glands collected from the 3 day and 28 day groups, in comparison to the mean for the 14 day group.

Under low power, sites with reaction product were dispersed with any localization apparently confined to associations with blood vessels and were particularly visible around the area of the stalk (Fig. 4.5b). Under high power, the presence of IR was observed to be punctuate, particularly where reaction complexes were confined within transected neurons. Immunoreactivity which was diffuse most probably represented longitudinal and oblique perspectives of nerve fibres containing GAP-43 (Fig. 4.9).

For all pineal tissue collected from controls and all SCGX groups, no IR for GAP-43 was observed within any parenchymal cells.

Table 4.4 Mean levels of immunoreactivity (IR) for GAP-43 (± SEM) assessed in sheep pineal glands (n=5) collected 3, 14 and 28 days after partial denervation by unilateral SCGX, and in pineals from unoperated controls. Immunoreactivity was scored on a subjective scale of 0-10.

Experimental Group	IR level (+/-SEM)
Controls	3.6 (0.25)
3 Day post-surgery	6.2 (0.31)
14 Day post-surgery	4.9 (0.29)
28 Day post-surgery	3.5 (0.35)

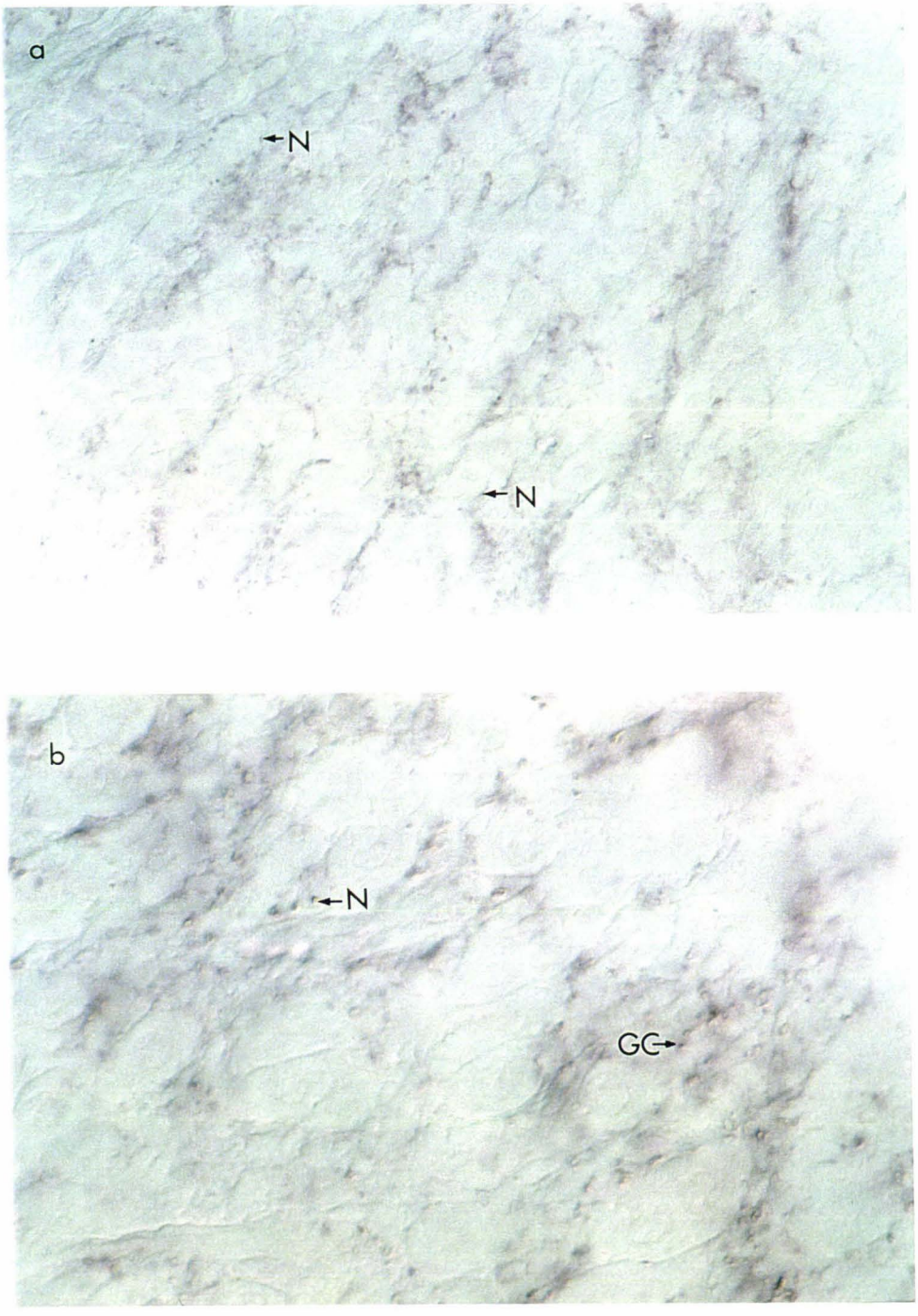


Figure 4.9 GAP-43 IR in pineal tissue collected 3 days after unilateral SCGX. Note the transection of neurons (N) and the transection of presumptive nerve growth cones (GC). GAP-43 antiserum; Magnification (a) 313 X and (b) 400 X. DIC.

Table 4.5 Summary of analysis of variance of GAP-43 immunoreactivity scores in neurons within unilaterally denervated pineal glands (surgery), harvested at 3, 14 and 28 days post surgery (SCGX) and in control pineals harvested at 28 days after anaesthesia.

Source of Variation	D. F	Variance Ratios
A. Treatments	3	
Orthogonal contrasts		
(i) Control vs. SCGX	1	22.75^{***}
(ii) 3 Day vs. 28 Day (Linear)	1	68.90^{***}
(iii) 3Day & 28 Day vs. 14 Day (Quadratic)	1	0.03
B Between Animals	19	1.43
Within Animals (Error mean square)	17	0.53
Non orthogonal contrasts		
(i) Control vs 14 Day	1	8.45^{**}

(II) Alpha tubulin immunoreactivity

See Tables 4.6 - 4.7 and Figures 4.10 - 4.15

Alpha tubulin immunoreactivity was most prevalent and intense in the pre-migratory zone (Altman, 1972) of the molecular layer in foetal (97 day) ovine cerebellum (Fig.4.10b). The distribution of AT IR in that area of the molecular layer was only interrupted by dendrites originating from the deeper Purkinje fibres. Reactivity complexes representing the presence of AT also were dispersed through the granular layer, however, no differentiation between IR associations with different cell types could be made using light microscopy. AT IR in the granular layer was mostly present in proximity with the nuclear membrane of cells. No AT IR was present in negative control foetal cerebellum tissue (Fig 4.10a).

Figure 4.11 shows pineal tissue which had been subjected to the ICC procedure described in Section 4.2, without application of the primary antibody for alpha tubulin. Melanin can be seen in that micrograph as black and yellow specks, as is typical in ovine pineal tissue (Anderson, 1965).

Exhibited in Figure 4.12 are low power micrographs from each of the four experimental groups showing the distribution of AT IR positive presumptive pinealocytes throughout the pineal gland. One feature was the presence of these cells in groups, or lobules, however, there was not any distinct pattern of distribution with groups of IR cells varying in cell numbers and shape.

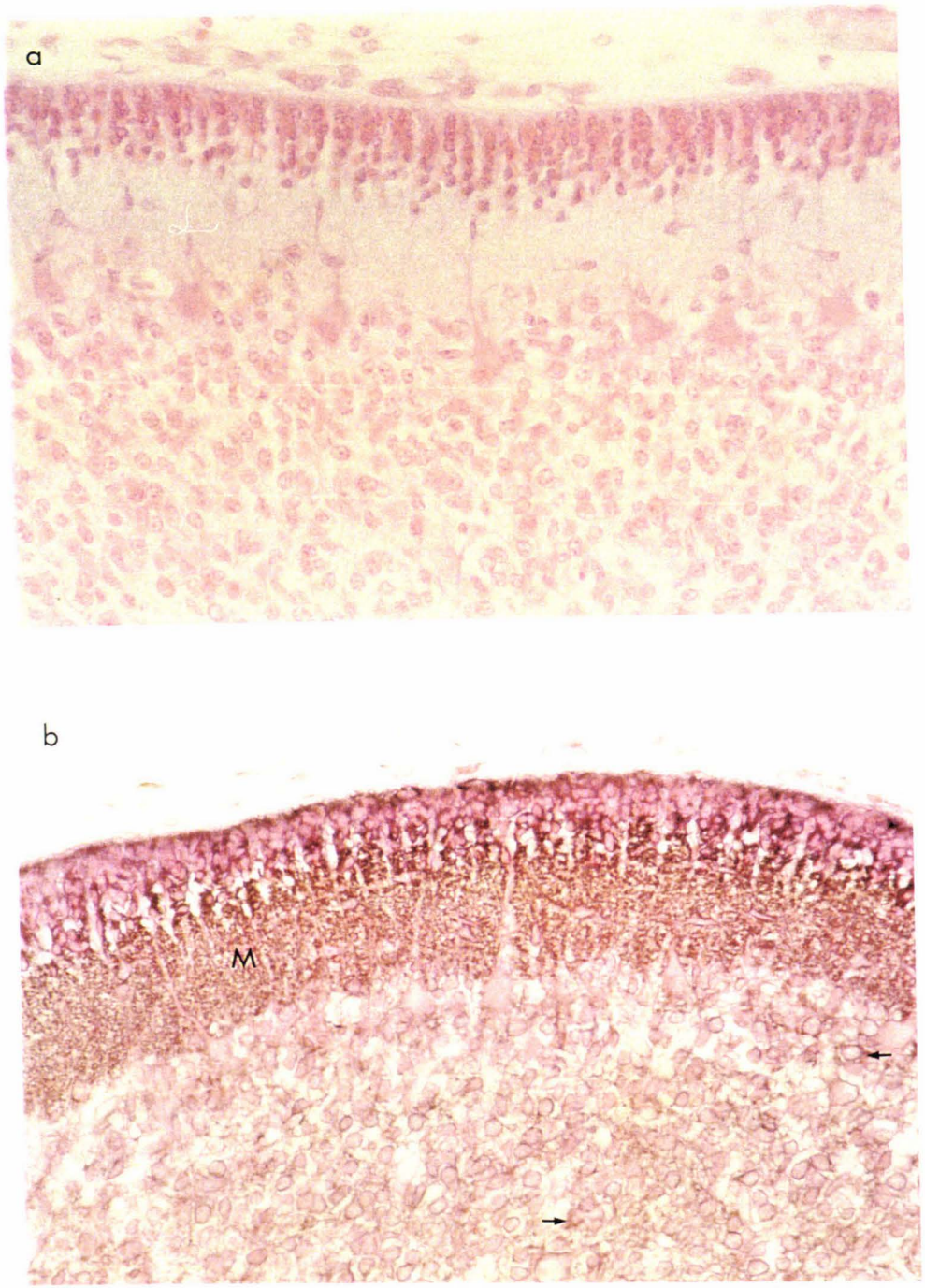


Figure 4.10 Negative (a) and positive (b) alpha tubulin immunoreactivity in foetal ovine cerebellum. Note the intense IR band in the migratory zone of the molecular layer (M) and slight IR surrounding some granular layer cells (arrows). Alpha tubulin antiserum; Magnification 125 X.

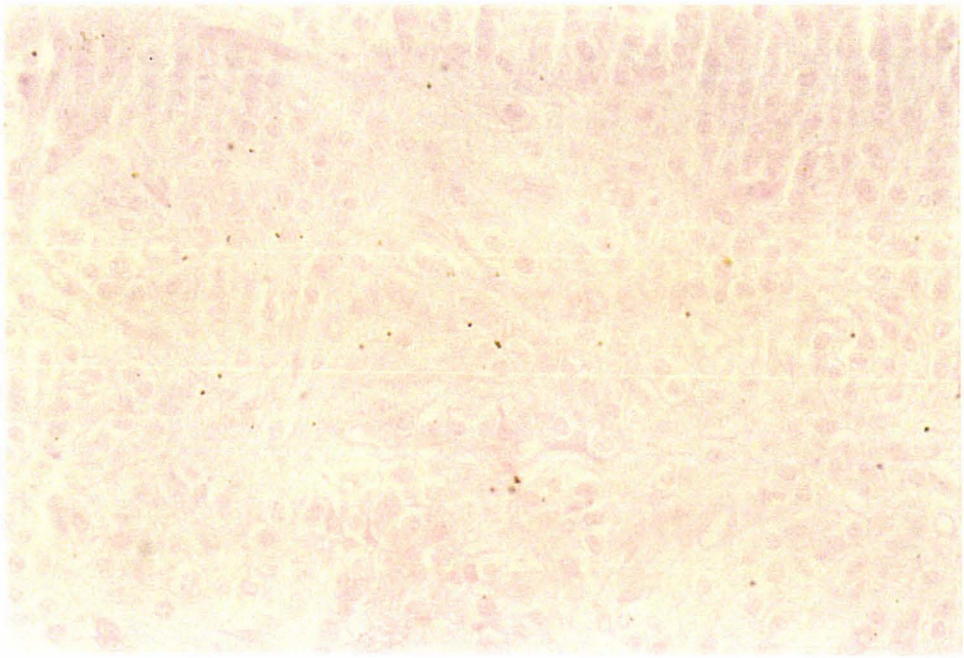


Figure 4.11 Pineal tissue collected 3 days after unilateral SCGX and used as negative control tissue. No alpha tubulin antiserum had been applied. Dark spots are melanin. Magnification 125 X.

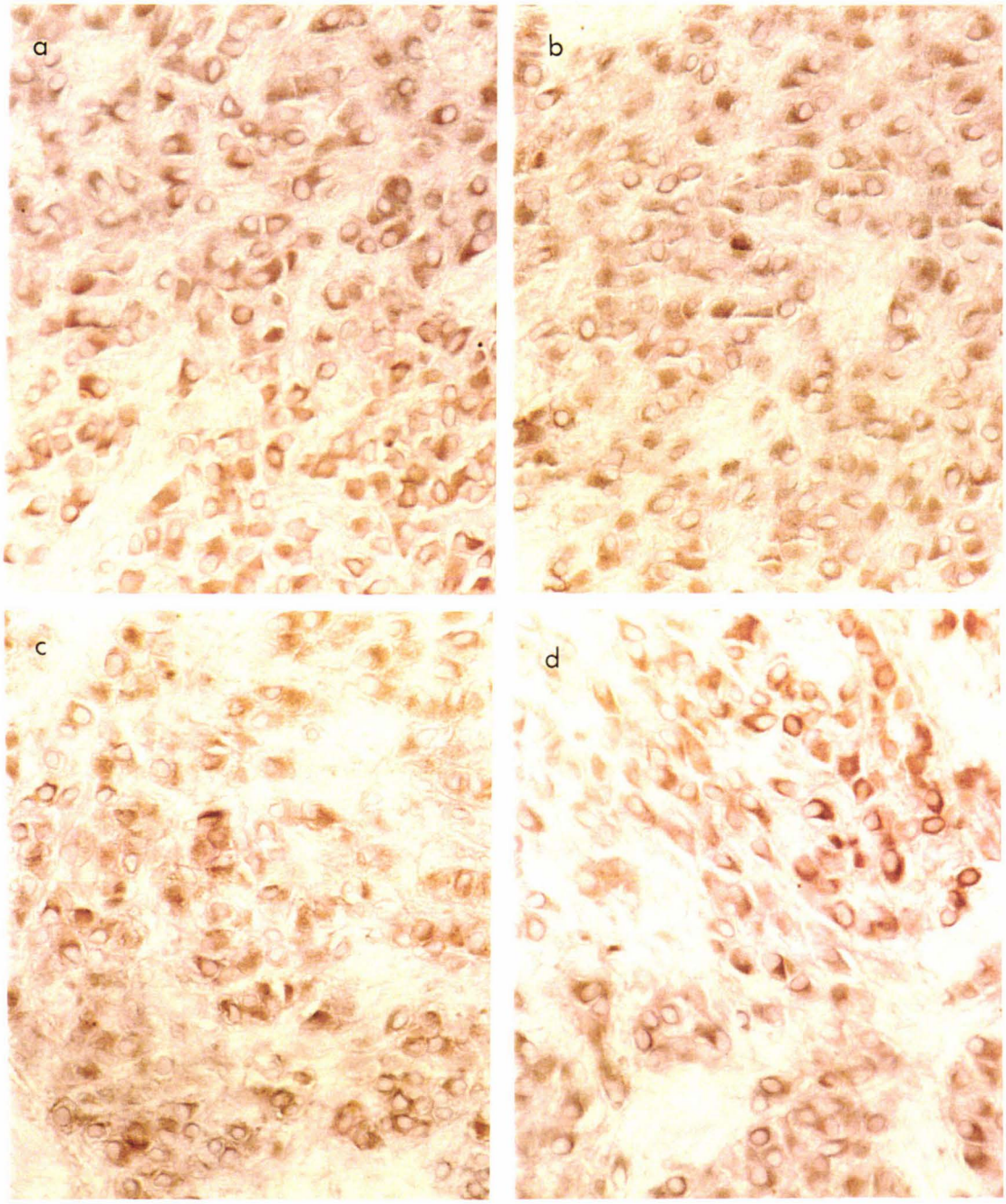


Figure 4.12 Representative examples of alpha tubulin IR in pineal tissue collected from the control group (a) in addition to those from unilateral SCGX groups collected at 3 (b), 14 (c) and 28 days (d) after surgery. Note the similarity between all experimental groups and the groups of IR cells (pinealocytes) divided by intercellular space. Alpha tubulin antiserum. Magnification 125 X.

The cytoplasm of the majority of parenchymal cells (presumptive pinealocytes) in pineals from all experimental groups in this experiment exhibited strong IR for AT, as shown under high power in Fig.4.13. Alpha tubulin was most often concentrated in one area of the cytoplasm in association with the nuclear membrane (Fig.4.13). Alpha tubulin IR in some presumptive pinealocytes was observed to extend into the cell processes (Fig.4.13) and in other cells reaction complexes were apparent throughout the cytoplasm, but with concentrations at both the cytoplasmic and the nuclear membranes (Fig.4.13).

Because of intense and widespread cellular staining throughout the tissue, it was not possible to distinguish immunoreactive nerve fibres from the stained pinealocytes and their processes.

Of the cells inspected in the body region of the pineal of control animals, 1316 (94%) exhibited IR for AT, while the remaining 91 (6%), which were intermittently dispersed among the IR cells, did not exhibit an immunoreaction. Unilateral SCGX did not significantly influence the incidence of AT-IR or non-IR pineal cells (Table 4.6), since neither the control group nor any of the three operated groups exhibited any significant variation in the frequency of either cell type (Table 4.7), indicating that partial denervation did not cause any immediate or delayed effect on the alpha tubulin content (Fig. 4.14), or by inference, the integrity of the pinealocyte population.

Measurements of interstitial space in this assessment also exhibited very little difference in incidence in pineals collected from the four experimental groups (Fig.4.15).

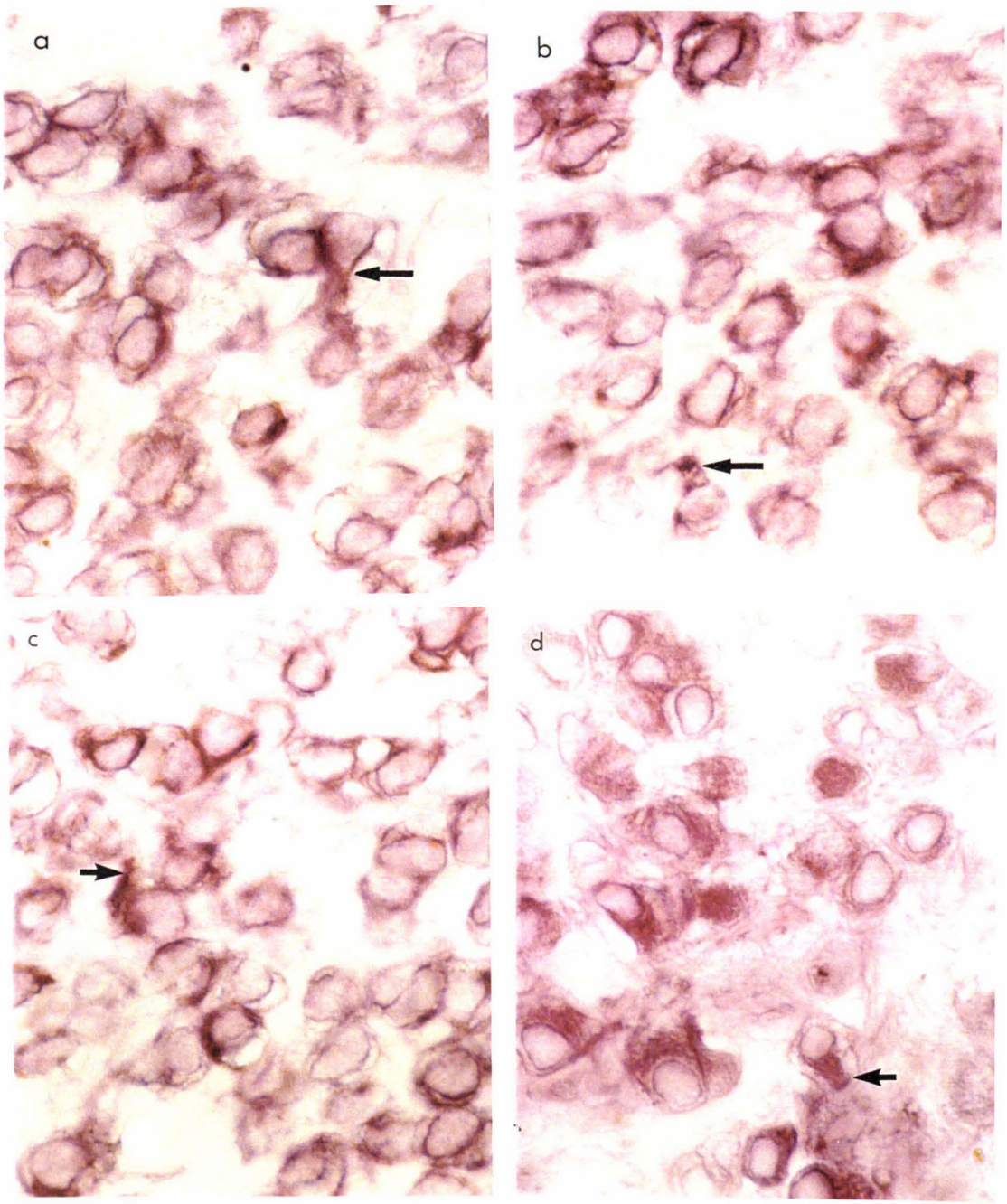


Figure 4.13 Representative examples of alpha tubulin IR in pineal tissue collected from the control group (a) in addition to those from unilateral SCGX groups collected at 3(b), 14 (c) and 28 days (d) after surgery. Note the localization of IR in the cytoplasm of pinealocytes in all experimental groups. Arrows indicate IR projecting into the processes of pinealocytes.
Alpha tubulin antiserum. Magnification 125 X.

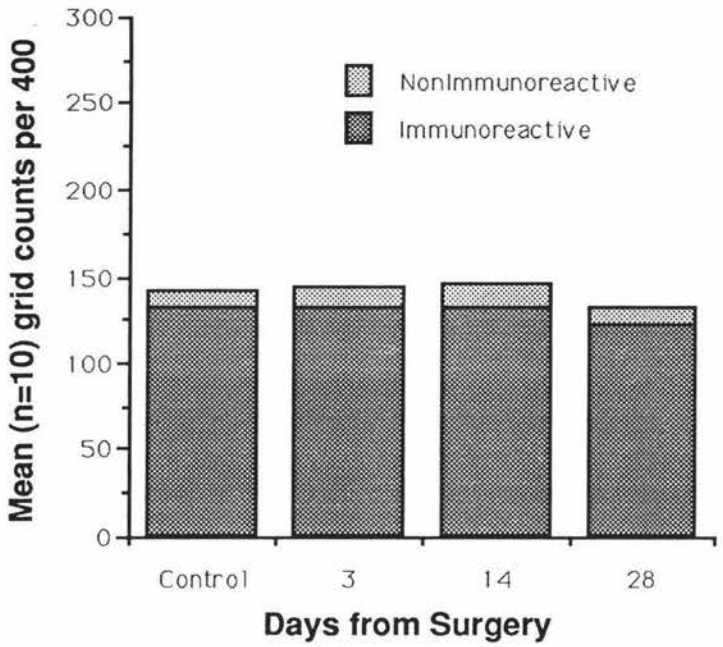


Fig.4.14 Mean counts (n=10) of immunoreactive- (IR) or non- IR pineal cells under 400 grid points superimposed on pineal tissue harvested from sheep 3, 14 & 28 days after unilateral SCGX, as well as from control animals.

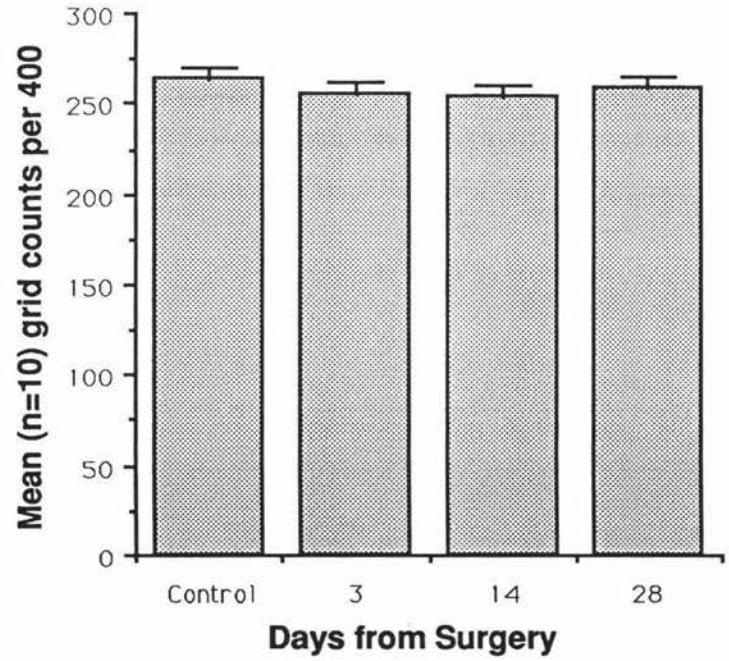


Fig.4.15 Mean counts (n=10) of intercellular space under 400 grid points superimposed on pineal tissue harvested from sheep 3, 14 & 28 days after unilateral SCGX, as well as from control sheep.

Table 4.6 Mean count (± SEM) of alpha tubulin immunoreactive cell-, non-reactive cell- and intercellular space- grid point intercepts per 400 grid points, in the pineal body region of sheep in Chapter 4. Each value is the average of 10 counts, being 1 each from two separate sections from the pineals of each of the five sheep in each treatment group.

	Control	Post-operative survival period		
		3 Day	14 Day	28 Day
+ve IR Cells	129.6 (6.35)	130.7 (6.24)	132.3 (7.44)	125.3 (3.60)
-ve IR Cells	9.1 (2.11)	14.3 (3.10)	13.4 (2.54)	12.2 (2.25)
Intercellular Space	261.3 (6.26)	255.0 (7.44)	254.3 (6.33)	262.5 (5.57)

Table 4.7 Summary of analyses of variance of data for alpha tubulin immunoreactive (IR)-, non-IR- pineal cells and intercellular space within unilaterally denervated pineal glands harvested at 3, 14 and 28 days post surgery (SCGX) and in control pineals harvested at 28 days after anaesthesia.

Source	D. F	Variance Ratios		
		+ve IR Cells	-ve IR Cells	Intercellular Space
Treatment	3	0.01	1.20	0.38
Residual (mean square)	36	367.7	56.6	424.8

4.4 Discussion

4.4.1 GAP-43 in foetal ovine cerebellum

This study has demonstrated that the GAP-43 antiserum, subclone 7B10/D4, which has previously been shown to be IR in rat, mouse, human, snake and cat neural tissue (K. Meiri, pers. comm), also forms complexes with this antigen in sheep neural tissue. Initial experiments performed in this laboratory demonstrated a strong positive reaction in two of the three main layers of cell types in sheep foetal (97 day) cerebellum tissue which was selected as positive control tissue, because it was presumed to contain developing neurons. To facilitate interpretation of experimental results an immunocytochemical procedure was developed which established the formation of an antibody-antigen complex, that was visible using LP light microscopy, with minimal interference from non-specific background counterstaining.

In the cerebellar cortex the association between GAP-43 IR and Purkinje cell bodies was predominant (Fig.4.3), particularly shown by the appearance of a band of IR situated between the outer molecular layer and the inner granular layer. The reaction product was most intense at the bases of the Purkinje cell bodies and was most probably representative of the 'boutons' of terminal axons originating from the deeper granular layer, synapsing with the cell bodies (Ham & Cormack, 1979). Dispersed, but intense, IR was also visible around Purkinje cell bodies, whereas, the intensity of reaction was reduced deeper to the granular layer, suggesting that growth from the neural cells of the granular layer was directed towards the Purkinje cell bodies. The distribution of new neurons in the granular layer was visible as a 'honey-comb' surrounding the glial cells, which were not immunoreactive. However, dispersed within the granular layer were some neuronal cells which contained an intense IR, apparently within the body of the cell,

indicating active protein synthesis (Zomzeley-Neurath & Walker, 1980). That staining pattern was similar to that observed in post-natal (8 days) rat cerebellum in which the distribution of GAP-43 IR in the granular layer has been suggested to represent inter-twining climbing fibres and parallel fibres between unstained cells in the granular layer (Oestreicher & Gispen, 1986).

Also in the cerebellum tissue one other prominent band of GAP-43 IR was observed, situated within and parallel with the surface of the superficial molecular layer, which was determined to be representative of a mass of terminals of dendritic growth originating from the deeper Purkinje cells. Though GAP-43 has been reported to be specific to axonal growth only, and not dendritic growth (Skene, 1989), the distinguishing factor between those two neural processes is primarily one of nomenclature describing variation in extent of migration, axons developing from dendritic growth and elongating to an extent that surpasses the rudimentary growth of dendrites (Finger & Stein, 1982). To explain how one neurite is selected to be the axon, Goslin & Banker (1989) suggested that a regulatory protein, such as GAP-43, may be present in limiting amounts that depend on axonal length. Because GAP-43 can enhance lamellipodial extension and accumulate selectively at axonal tips, it is suspected of playing a major role in dendrite to axon conversion. Use of differential interference contrast optics established that from each Purkinje cell body one process extended into the molecular layer where terminal growth was in a varicose form with multiple branching (Fig.4.3), typical of that conformation described in anatomical studies (Ham & Cormack, 1979). Oestreicher & Gispen (1986) similarly describe "dots of IR around the growing apical cones and on developing primary dendrites of the Purkinje cells", from observation of B-50 (GAP-43) IR in 8 day old rat cerebellum. The intrinsic cells of the molecular layer in sheep cerebellum were not IR for GAP-43 indicating that those nerve cells were probably not migrating, or alternatively a different protein could be associated with growth in some

neuronal cells. Altman (1972), in studies of the post-natal development of rat cerebellum, distinguished between the external germinal layer and the pre-migratory zone in the molecular layer. Only the pre-migratory zone stains intensely for GAP-43 in both 8 day post-natal rat (Oestreicher & Gispen, 1986) and foetal (97 day) ovine cerebellum.

These results of cerebellar GAP-43 staining, which were incidental to the main purpose of this study, have established the potential for using GAP-43 as a marker for tracing neural development by immunohistochemical localization for GAP-43 IR in ovine pineal tissue collected at periodic intervals after SCGX.

Attempts to form a visible reactive complex with the anti GAP-43 subclone 10E8/E7, in either developing sheep neural tissue or partially denervated pineals, were not successful. Cross reactivity of this monoclone has previously been demonstrated by Meiri, *et al* (1991) to be positive with both rat and mouse tissue, but has failed to cross react with human tissue, and as of April, 1993 had not been tested on tissue from other species (K. Meiri, pers. comm.).

4.4.2 Localization of GAP-43 in pineal tissue after unilateral SCGX

Results of this experiment have established ICC localization of GAP-43 in ovine pineals, particularly those collected after partial denervation, in which it was specifically confined within and often associated with the outer membrane of axons, as exhibited in Fig. 4.9. As described in Section 1.5.7 and reviewed by Skene (1989), GAP-43 is a product of protein synthesis in the cell body, which is transported along the axons in vesicles associated with the cytoskeleton, to be concentrated in the terminal growth cone. The association with both the terminal growth cone and the cytoskeleton of the neuron was observed using high power microscopy, under which transection through the growth

cones of neurons was envisaged by the presence of a dense circle of immunoreactive product, while upstream transection was visible as a peripheral ring of IR.

Neurons containing GAP-43 IR were observed in association with blood vessels (Fig.4.5b) in pineal tissue from all unilateral SCGX groups. That association was most apparent in the stalk region, most probably because the blood vessels are larger and more visible prior to divergence. Post-ganglionic sympathetic neurons have previously been reported to enter the pineal gland in close association with the blood supply around the area of the nervi conarii (Moore, 1978), an association that most probably is retained, as the blood vessels are, in part, controlled by sympathetic innervation (Ham & Cormack, 1979). Despite low oxygen consumption (Quay, 1962c) the blood flow to the pineal organ is high, exceeding that of most other endocrine organs at a rate of 4ml/min per gram of tissue (Vollrath, 1981). Partial denervation of blood vessels and consequential disruption of blood circulation may be an important factor in the reduction of pineal secretory capacity after unilateral SCGX. Goldman (1976) has shown that bilateral SCGX reduces pineal blood flow by one third. Re-innervation of denervated blood vessels by collateral growth of residual neurons originating from the intact SCG could re-establish pre-operative levels of blood circulation control and be a factor contributing to increasing secretory capacity. However, it is probable that re-innervation of pinealocytes is a much more important factor in this regard.

Another distinct association between neurons containing GAP-43 IR and tissue cytology was observed as the presence of intense punctate IR surrounding individual pinealocytes, which was considered to be representative of neuron terminals converging on targeted cells. Factors which influence and direct nerve regeneration are the topic of literature reviewed in Section 1.5.5, inclusive of research by Brown & Holland (1979), which suggests that denervated tissue attracts and directs neuronal growth. In this current experiment the presence of GAP-43 IR was observed to decline over the period from 3 to

28 days after unilateral SCGX and at 28 days neurons containing GAP-43 IR were mainly visible only at high power and in association with pinealocytes. This observation indicates that pinealocytes are most probably the target of the neuronal growth. Figure 4.8d distinctly shows pinealocyte cells, from 28 day post-operative pineal tissue, surrounded by neurons which either contain GAP-43 IR associated with the cytoskeleton, or which have been transected across the growth cone in which case the presence of GAP-43 IR was intense and punctate.

For most post-SCGX pineals no association between the presence of GAP-43 IR with any particular areas was observed, however, in some pineal glands a band of IR was observed to be situated parallel with the pineal body capsule (Fig.4.5b). In rodents, Ham & Cormack (1979) have described a band of trabeculae and incomplete septa that are situated parallel with the capsule, and that extend into the substance of the gland, conveying blood vessels and nerves along with them. It appears that a similar arrangement occurs in the sheep pineal, though that pattern of innervation has not been reported in this species (Vollrath, 1981), nor has the presence of a band of GAP-43 IR nerve fibres, a feature common to all post-SCGX pineals examined in this study.

For the most part the distribution of GAP-43 IR appeared to be intermittent throughout the gland which suggests that the nerve growth was associated with fibres distributed throughout the gland. Studies in rats have determined that post-ganglionic fibres originating from both SCG's are dispersed throughout the gland, with fibres arising from the cell bodies in each SCG innervating the contralateral as well as the ipsilateral half of the pineal (Dornay, *et al*, 1985; Lingappa & Zigmond, 1987; Zigmond, *et al*, 1981). Mockett (1991) suggested that the same pattern of pineal innervation probably was present in sheep, as inferred from measurements of neuron specific enolase IR associated with parenchymal cells in pineals collected at 14 days after unilateral SCGX. Tissue from

both sides of the pineals exhibited little variance in the presence of NSE-IR positive cells, which further indicates that sympathetic nerve fibres originating from each SCG cross-over and are dispersed throughout the ovine gland. Also Mockett, *et al* (1991) detected a reduction in number of nerve fibres containing IR for NPY, but no variation in distribution after unilateral SCGX.

4.4.3 Re-innervation of the pineal gland after unilateral SCGX

This study was designed to provide a temporal comparison of GAP-43 IR in pineal tissue which had been harvested from unoperated control animals at 28 days after anaesthesia, as well as in pineal tissue harvested at 3, 14, and 28 days after partial denervation by unilateral SCGX. Statistical analysis by ANOVA has determined a significant increase ($p< 0.001$) in the presence of GAP-43 in pineal tissue harvested from sheep 3 days after partial denervation, in contrast with that in the control group's pineals, indicating that at 3 days compensatory nerve growth had at least commenced as a response to unilateral SCGX. This is the most conclusive evidence to date in establishing neural plasticity as the most probable potential compensatory mechanism responsible for the measurements in Chapter 3 which determined that a recovery in pineal function occurred after unilateral SCGX, despite an initial decline in measurements of secretory capacity for melatonin by in excess of 50%. The novelty of testing for GAP-43 antigenicity in pineal tissue after unilateral SCGX confirms speculation, derived from previous experimental procedures (Zigmond, *et al*, 1981; Dornay, *et al*, 1985), which had suggested neural plasticity as a potential compensatory mechanism.

In unoperated control tissue the slight presence of GAP-43 IR probably indicated sites of nerve terminal remodelling, which is a characteristic of mature neural tissue (Collinridge & Bliss, 1987).

Dependency of post-ganglionic neurons on cell bodies that were removed by ganglionectomy means that subsequent nerve degeneration would lead to atrophy of approximately 50% of the sympathetic innervation in pineal glands that had been subjected to unilateral SCGX. Most probably the presence of new neural growth, which has been demonstrated in this experiment, had its origin from residual post-ganglionic neurons originating from the intact SCG. Such a concept for neuronal plasticity is similar to that proposed earlier by Dornay, *et al* (1985), on the basis of increasing enzyme activity (ChAT and TH) in the intact SCG over a recovery period of ten days, and resembles "homotypic collateral sprouting" described in pioneering experiments investigating nerve regeneration within muscle tissue (Edds, 1953). Assuming that the neural growth detected in this experiment was physiologically active and that synthesized neurotransmitters were compatible with membrane bound beta-adrenergic receptors of the pinealocyte cell, then a role for re-innervation in effecting a recovery in pineal melatonin secretory capacity seems highly likely. Future research, focused on the factors which instigate growth and the mechanisms by which growth occurs, will benefit from use of this excellent model on which to base further study of morphological and anatomical recovery of neural function.

The presence of high levels of GAP-43 IR in nerve fibres in pineals 3 days after unilateral SCGX confirms rapid synthesis of the protein and subsequent axonal transport as a response to denervation (Skene, 1989). In an immunofluorescence microscopy study investigating growth of cultured hippocampal cells, as a response to trauma, Goslin & Banker (1990) have established the presence of elevated concentrations of GAP-43 in new axonal growth cones by 12-30 min after axonal transection.

With a cross-over of nerve fibres from each SCG and their distribution throughout the pineal gland, presumably residual terminals only have short distances to grow to

reinnervate denervated cells by the process of collateral sprouting and hence accounts for full melatonin secretory recovery by 28 days. Growth rates of up to 0.8 mm per day have been reported for SCG post-ganglionic sympathetic fibres of neonatal rats, with Smith & Reddy (1990) suggesting that that rate was conservative. The actual rate could approach 1mm per day, similar to that measured for neurite elongation under optimal conditions *in vitro* (Campenot, 1982). That evidence, citing growth rates of 1mm per day in SCG post-ganglionic nerve fibres, combined with evidence from studies which have established the distribution of post ganglionic fibres throughout the pineal gland (Lapwood & Mockett, unpublished), suggests the possibility that homotypic collateral sprouting to re-innervate denervated pinealocytes may have been well advanced within 3 days of surgery. However, the presence of intense GAP-43 IR at 3 days suggests neuronal growth had not passed the migratory phase at that early stage of recovery. Whereas, the linear decline of GAP-43 IR intensity to 28days post-surgery, incorporating assessment at 14 days, does suggest that neural growth has entered a phase of maturing between 3 and 14 days post-surgery.

Together, these results suggest that the initial recovery in melatonin secretory capacity between 1 and 3 days was not an effect of re-innervation and most probably other compensatory mechanisms were involved in that initial recovery. Recovery over that period, however, was only to 50% of pre-operative levels and may only represent a recovery from the secondary inhibitory factors suggested in Section 3.4.4. Alternative mechanisms suggested to be responsible for measurements of a recovery in pineal enzyme activity after partial denervation include receptor hypersensitivity (Degushi & Axelrod, 1972c; Romero & Axelrod, 1974), an increase in receptor density (Gonzalez-Brito, *et al*, 1988b; Pangerl, *et al*, 1990), enhanced effectiveness of NE released by surviving neurons (Zigmond, *et al*, 1981, 1984), and increased activity by residual neurons

(Zigmond, *et al*, 1981), for which the relative merits of each hypothesis have been discussed in Section 3.4.5.

However, the linear decline in the presence of GAP-43 IR, which is indicative that neuron growth is maturing, corresponds with the same period for which a recovery in pineal melatonin secretory capacity was measured in excess of 50% of pre-operative levels. From 3 days after surgery to 28 days, the mean secretory capacity for the unilateral SCGX animals rose from 20.6% of pre-operative levels to 108.7%. That relationship between nerve growth maturation and increased melatonin secretory capacity is convincing evidence for establishing re-innervation as the primary mechanism responsible for the complete recovery in function after unilateral SCGX.

Numerous studies have established that the presence of GAP-43 is confined only to periods of migration and early synapse formation (Argiro & Johnson, 1982; Collins & Lee, 1982; Meiri, *et al*, 1988, 1991; Goslin, *et al*, 1988; Skene & Virag, 1989; Skene, 1989; Goslin & Banker, 1990). While the development of relationships between new nerve terminals and pinealocytes, after unilateral SCGX, is not strictly "synapse formation", the process of maturation of collateral growth most probably undertakes similar biochemical changes to those of synapse formation (Gorio, 1993) (e.g. phosphorylation by protein kinase C).

Baizer and Fishman (1987), experimenting with growing nerves in tissue cultures, have determined a decreasing expression of GAP-43 in neurons allowed to contact target tissue. Similarly in this experiment, the association of GAP-43 with pinealocytes was not apparent until a decreasing expression of GAP-43 IR had occurred after an extended recovery period of 28 days. However, the extent and intensity of IR present at 3 days may have obscured any similar relationships, between nerve terminal growth and pinealocytes, that may have occurred earlier.

Facility to use electron microscopic investigation of terminal neurons, particularly to study their association with the pinealocyte membranes, should establish the architectural characteristics of collateral growth and indicate the relationship at the interface of re-innervation. This concept will be developed in Chapter 5.

In Chapter 5 directions for future experimental studies, using this model, are recommended, with the aims of further establishing the role for re-innervation as a mechanism responsible for the full recovery of pineal function after unilateral SCGX and for elucidating the mechanisms of collateral growth.

4.4.4 Alpha tubulin in foetal sheep cerebellum

The presence of an intense band of alpha tubulin immunoreactivity in the migratory zone of the foetal cerebellar cortex indicates that structural changes, most probably due to proliferation, migration and differentiation, are under way in this area at 97 days (foetal). That band does not substantially differ in appearance from that in 8 day old rats (Oestreicher & Gispen, 1986), excepting that in that later developmental stage the band is concentrated at the tip of the growing folium and was otherwise reduced in intensity.

In the granular layer the association of AT IR with the nuclear membrane of many cells indicates alpha tubulin presence in a structural role.

4.4.5 Alpha tubulin in the sheep pineal

The current study is the first to demonstrate that the alpha tubulin (AT) component of microtubules is present in the sheep pineal, mainly in the cytoplasm of pinealocytes. The demonstration of AT antigenicity enabled presumptive pinealocytes to be distinguished from the non-endocrine cells, such as glial and endothelial cells, and intercellular space, all of which were not IR positive for AT. Complementary use of ICC detection for glial fibrillary acidic protein, to distinguish glial cells, could be implemented to confirm that AT IR stained cells were pinealocytes (Lapwood, K., pers. comm).

ICC localization of the AT component of microtubules in ovine pinealocytes corresponds with anatomical studies using electron microscopy which have previously determined the presence of microtubules in the cytoplasm of ovine pinealocytes (Anderson, 1965). Microtubules have also previously been observed in the cytoplasm of

baboon pinealocytes (Theron, *et al*, 1978), while alpha tubulin has been localized by ICC in the pinealocytes of guinea pigs, but not rats (Schroder, *et al*, 1990).

In this current study the presence of AT IR in proximity with the cell nucleus of pinealocytes was prominent and most often was concentrated to one side of the nucleus. The morphology of cells which synthesize proteins invariably incorporates an intracellular population of organelles involved in protein synthesis and located to one side of the nucleus, for example, endoplasmic reticulum and the golgi bodies (Hall & Cormack, 1979). AT IR present at both the nuclear and the cytoplasmic membranes may indicate an alternative conformity of AT, mainly involved in a structural role (Matus, 1988).

Electron microscopic observations of the microtubular population in the cytoplasm of ovine pinealocytes reported a different conformation of the structure, than reported for other cells, with the presence of a striated pattern. Anderson (1965) suggested the function of the microtubules in pinealocytes could be as structural elements which compartmentalize the cytoplasm and expedite intracellular transport. However, recent research has determined a more active role for microtubule components in intracellular transport in both nerves (Gorio, 1993) and in endocrine cells (Dentler & Suprenant, 1986).

The presence of microtubules in cells of endocrine organs has previously been reported for the pituitary gland (Labrie, *et al*, 1973; Warchol, 1974; Sherline, *et al*, 1977) and pancreas (Orci, *et al*, 1973; Dentler & Suprenant, 1986). Pharmacological and morphological studies have indicated that microtubules were associated with secretory granules in the islet cells of the pancreas and were necessary for normal secretory activities (Orci, *et al*, 1973). Likewise, Labrie, *et al* (1973) demonstrated that microtubules were contained in prolactin and growth hormone secreting cells in the pituitary and showed that agents which alter the process of secretion *in vitro* also produce changes in the microtubule system. Similarly, microtubules in the pineal, a neuro-

endocrine organ, could be involved in a secretory role by facilitating intracellular transport of secretory granules. In this current experiment, ICC localization of alpha tubulin in sheep pinealocytes has indicated a tendency for the protein to course into some processes, corresponding with observations, using electron microscopy, of microtubules in sheep pinealocyte processes (Anderson, 1965). Apparently, the movement of tubulin is from the nucleus associated site of synthesis, towards the process terminal. If the function of the tubulin is a secretory role, then presumably other proteins synthesized at the nucleus are transported in association with the alpha tubulin. The terminal structure of pinealocyte processes has been described as bulbous by Vollrath (1981) and shown to contain a large number of electron-lucent vesicles and dense-core vesicles, which may be a morphological correlate of secretory products. The bulbous endings of pinealocyte processes also contain many mitochondria in sheep pineals (Anderson, 1965).

While the assessment used in this experiment has not distinguished any variation in the alpha tubulin content of pineal gland parenchymal cells after unilateral SCGX, it has provided further evidence (Mockett, 1991) to confirm that cell atrophy does not occur in response to partial denervation. Cell integrity was maintained at 3 days after unilateral SCGX and also at both 14 and 28 days. Re-establishment of melatonin secretory levels to pre-operative levels after unilateral SCGX, also indicates that the pinealocyte population was not diminished. However, the lack of effect of unilateral SCGX on cell morphology measured in this experiment could be due to limitations in use of ICC for quantitating experimental effects. Alternatively, quantitative analysis of AT mRNA by electrophoretic fractionation (Mathew & Miller, 1990) may distinguish some variance in the expression of AT polypeptides over the recovery period. Alternatively, RIA techniques (Hiller & Weber, 1978) could be implemented to quantify any variation in the AT content of experimental group pineals as a result of SCGX.

Within pineal glands distinguishing nerves was not possible, with the majority of pinealocytes (94%) containing an intense reaction product which masked any variations in experimental effects on AT IR in nerve fibres. Hence it was not possible to use between-group evaluations of variations in AT IR in pineal nerve fibres, to confirm evidence provided by GAP-43 ICC, that recovery of secretory capacity was due to increased nerve terminal growth, although that would have been expected because of alpha tubulin's role in nerve growth and regeneration (Matus, 1988).

Since synthesis of proteins occurs at some distance from many functional domains of a neuron, transport to distal regions of the neuron is necessary for proper function. Materials moved in anterograde transport include membrane-associated enzyme activities, neurotransmitters, and neuropeptides. Many of the materials move down the axon by anterograde transport are returned by retrograde transport (Bisby, 1982), in some cases following modification in the terminal (Black & Lasek, 1977). Morphological studies of living cells and cell fractions have shown that microtubules are intimately associated with organelle movements (Weisenberg & Allen, 1984), and examination of fixed and sectioned cells has revealed examples of microtubules that were directly linked to organelle membranes by thin bridges (Raine, ***et al***, 1971). Gorio (1993) suggested that when movement of membrane bound organelles along microtubules occurs, that interrelationship means concurrent movement of microtubules will also occur. The proteins involved in axonal transport do not move as individual polypeptides, instead, polypeptides move as part of cytological structures or in association with a cytological structures. As a result, microtubules play critical roles in regeneration. They serve both for transporting required materials to the growth cone and as the primary structural element in growing neurites (Brady & Black, 1986). The importance of the tubulins in regeneration can be inferred from changes in tubulin during regeneration.

Specific tubulin genes are upregulated during axonal growth and regeneration (Mathew & Miller, 1990), during which processes there are characteristic changes in the axonal transport of tubulin (McQuarrie & Lasek, 1989).

Increases in alpha tubulin during the growth phase of nerve fibres (Tucker, 1990) may be observed using electron microscopy (Anderson, 1965, Brabander, *et al*, 1986), or quantified using gel electrophoresis techniques, providing the polypeptide population of alpha tubulin in growing nerves varied with that present in secretory cells (Ginzberg, *et al*, 1986). In Chapter 5 recommendations are made for incorporating these additional techniques in any future unilateral SCGX experiments.

4.5 Conclusions

The monoclonal antibody 7B10/D4 for GAP-43, a protein solely associated with neuron growth in both development and regeneration, reacts in both developing and regenerating sheep neural tissue.

Confirmation of neural regeneration in the pineal gland, following unilateral SCGX, has not previously been reported. The presence of the growth associated protein GAP-43 in neurons within pineal glands, previously subjected to partial denervation, establishes the presence of new neural growth.

Temporal variations in GAP-43 antigenicity indicate that regenerating neural growth in the ovine pineal gland declines from a peak at around 3 days after unilateral SCGX and has reached a state of maturity by 28 days.

GAP-43 ICC results from this experiment provide firm evidence for reinnervation in the regeneration of pineal function following partial denervation and confirms previous speculation regarding compensatory collateral sprouting of residual neurons as being an important mechanism in this process.

Re-innervation is unlikely to be the sole mechanism responsible for the recovery in pineal function after partial denervation. Recovery from secondary effects (e.g. reduced blood circulation) and the mechanism of increasing competency of residual neurons to take up NE, are other possible compensatory factors, though probably of lesser importance.

As will be expanded in Chapter 5, the model developed in this thesis, in which recovery of pineal function has been correlated with gland morphological changes in GAP-43 occurence, has the potential to become a very important means by which to elucidate mechanisms of nerve regeneration.

Alpha tubulin has not previously been demonstrated in sheep pineal tissue and reports for the presence of alpha tubulin in pineals of any species are sparse. The presence of alpha tubulin in the cytoplasm of ovine pinealocytes is most probably related to the function of secretion. Absence of variation in parenchymal cell content of alpha tubulin, after unilateral SCGX, suggests that cell atrophy does not occur in response to partial denervation.

The demonstration that pinealocytes contain a strong representation of the alpha tubulin component of microtubules in the cytoplasm is highly indicative of a secretory role within the cells. Recent research has established an active role for

microtubules in transport of cell products and a presence of microtubules has been shown in other endocrine organs.

Chapter 5

General Discussion and Conclusions

5.1 Introduction

As mentioned in the Introduction to Chapter 1, the main innervation to the pineal gland of mammals is via the sympathetic nervous system. Experiments presented in this thesis used the technique of unilateral superior cervical ganglionectomy, which removes the cell bodies from one of two post-ganglionic sympathetic nerve tracts innervating the pineal gland. The aim was to measure the effects of partial denervation on pineal function and to establish whether compensatory reinnervation of the gland occurred.

For the measurement of indoles in biological fluids, or for 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 relatively easy to apply. These techniques, which include both radioimmunoassay and immunocytochemistry, involve the application of highly specific antisera in order to detect the minute amounts of antigens often present in biological fluids and tissues. Both ICC and RIA techniques were utilized in this study of physiological and anatomical recovery from the effects of unilateral SCGX.

In the dark the pineal gland's main product of biosynthesis is the hormone melatonin. Recently, Lapwood (1993) reported that in sheep pineals the immediate effect of partial denervation is a dramatic reduction in melatonin secretory capacity, followed by a linear recovery to 77% of pre-operative levels at 14 days after surgery. Using RIA this study further investigated that recovery in function to determine whether there was full recovery

over an extended period, with the result that pre-operative melatonin secretory levels were shown to be reached by 21 days after partial denervation.

ICC localization of a protein that specifically concentrates in the terminating growth cones of both developing and regenerating axons, was used to investigate the pineal glands of unilaterally ganglionectomized animals for the presence of any compensatory regenerative neural growth. The presence of the immunoreaction complex formed between the GAP-43 antibody subclone 7B10/D4 and the endogenous antigen was established from 3 days after partial denervation. That evidence for the presence of compensatory nerve growth is the first to directly demonstrate re-innervation as a compensatory mechanism and confirms circumstantial evidence cited by Dornay, *et al* (1985). The decline in GAP-43, which occurred in pineal nerve fibres, between 3 and 28 days after unilateral SCGX occurred in the period during which melatonin secretion rates increased from 50% to over 100% of pre-operative levels. Both the decline in GAP-43 and increase in melatonin secretion almost certainly occurred in the period during which maturation of new nerve terminals, and their association with pinealocytes, occurred.

In addition, ICC procedures were used to detect the localization of alpha tubulin in ovine pineal tissue. AT is a constituent of microtubules, functioning both as a structural element and in the transport of proteins within cells, particularly neural cells. This study is the first to investigate the presence of AT in ovine pineals. An intense immunoreaction was found in the cytoplasm of most pineal parenchymal cells, but there was no significant effect of experimental treatments on the frequency of AT IR cells. That result indicates that cell atrophy had not occurred in response to unilateral SCGX, despite denervation to approximately 50% of the gland.

While the relationship between collateral sprouting and the recovery of function may be highly complex, the need to broaden and define knowledge in this field is elevated by the increasing inference of neural plasticity involvement in syndromes such

as Alzheimer's disease (Scheibel & Tomiyasu, 1978), as well as in recovery of function after neural injury (for review see Gorio, 1993).

5.2 Re-innervation and recovery of pineal melatonin secretory capacity after partial denervation by unilateral SCGX

In this study an 80% reduction of sheep pineal melatonin secretory capacity has been measured as an initial response to unilateral SCGX. In addition to confirming that the primary innervation to the pineal gland originates from the SCG's, this result also confirms the essential role of sympathetic nerve fibres in controlling pineal melatonin biosynthesis (Lincoln & Short, 1980; Lincoln, *et al*, 1982; Maxwell, *et al*, 1989b).

That initial effect of unilateral SCGX in causing a greater than 50% reduction in parameters of pineal function measured in this study and others (see Section 3.4.4), indicates that additional factors, other than the immediate effects of partial denervation, also are inhibitory. Lapwood (1993), in a corresponding study in sheep, had suggested the possibility of anaesthetic effects, particularly in consideration of a substantial, though non-significant, decline of secretory capacity measured in the control group of that experiment. Saffan induction and Halothane maintenance had been chosen as the favoured anaesthetic combination for least affecting pineal secretory capacities, as derived from comparative analysis of various anaesthetic combinations by Mockett (1991). In this current experiment S/H anaesthesia of controls had essentially no inhibitory effect, suggesting that other undefined inhibitory factors were involved in causing that initial post-operative reduction. Alternatively, Parfitt & Klein (1976) have shown that animals exposed to certain stressors (e.g. handling) have reduced pineal NAT activity. Also, in this current experiment, the presence of neural growth associated with blood vessels has indicated the possibility that blood circulation may have been reduced by the effects of

unilateral SCGX. Most likely the gross inhibitory effect on secretory capacity is an accumulation of the effects of partial denervation, anaesthesia, stress and a reduction in blood circulation, all of which have been shown to be elements affecting various parameters of pineal function (see Section 3.4.4). The main cause in reducing secretory potential, by at least 50%, is however, considered to be the immediate effect of removing the cell bodies of half the sympathetic fibres innervating the pineal gland.

Results of the present study and those conducted previously in both sheep (Lapwood, 1993) and rodents (Zigmond, *et al*, 1981, 1984; Bowers, *et al*, 1984; Dornay, *et al*, 1985; Kuchel, *et al*, 1990) have established that the initial inhibitory effects of unilateral SCGX on various parameters of pineal function are not permanent, but that substantial recovery occurs. This study, however, was the first to provide a direct measurement of pineal melatonin output and subsequently determined that a full recovery of pineal secretory capacity of melatonin occurred. Pre-operative levels of melatonin in plasma were re-established by 21 days after unilateral SCGX and were sustained at 28 days.

Several mechanisms have been proposed to be responsible for the recovery in pineal function after partial denervation, however, all proposals to date have been based upon inference. Most suggested mechanisms incorporate an increase in activities of the intact SCG post-ganglionic fibres. For example, Zigmond, *et al* (1985) suggested, that by some mechanism, unilateral denervation triggers an increase in nerve impulse activity in the contralateral intact sympathetic neurons and queried the possibility of any signal being effective in triggering such a selective response. Zigmond, *et al* (1981, 1984) and Kuchel, *et al* (1990), after measuring increased enzyme activity (NAT) in the pineal, have proposed that residual fibres increase in efficiency, by an ability to take up greater concentrations of NE, and consequently increase adrenergic stimulation of beta-adrenergic receptors. Dornay, *et al* (1985), however, proposed an alternative hypothesis,

citing the possibility that residual nerves may sprout collateral growth, termed "homotypic collateral sprouting", which terminates at and re-innervates denervated pinealocytes.

The introduction to this institution of antisera for GAP-43 has provided the opportunity to clarify the role of re-innervation as a possible compensatory mechanism responsible for recovery of pineal secretory capacity after unilateral SCGX. This study has determined the presence of nerve growth in partially denervated pineals during the same period for which measurements of a direct recovery in secretory capacity were also measured.

As mentioned in Section 1.5.8 the function of GAP-43, the growth-associated protein used as a marker for the presence of new neural growth, is closely linked to axon growth during both development and in regeneration of nerves. During those times it is present in growing axon tips at least at levels 10-fold greater than found in mature neurons, and it performs functions of motility during axogenesis and regeneration .

This experiment has made practical use of the specificity of GAP-43 by localization of antibody-antigen complexes as a marker for regenerating neurons. The IR complexes in pineal tissue often were visibly diffuse indicating that nerve fibres containing GAP-43 had been sectioned at various longitudinal and oblique angles. Because of the cytoskeletal association of GAP-43 during axonal transport, the presence of nerves with a peripheral ring of IR has been interpreted as cross-sectional transection of an elongating axon. That observation was able to be distinguished from transection through growth cones in which IR was condensed. Also by inference, the occurence of areas of intense reactivity may be considered to represent the convergence of a number of axons growing towards target tissue.

The occasional presence of sparse IR in control animal pineals suggested that nerve growth is not confined to development and that synapse re-modelling (Gorio, 1993) does occur in mature pineal tissue. Re-modelling of the nerve terminal-pinealocyte

relationship, observed in control tissue, infers that the suggested mechanism of re-innervation may involve up-regulation of an on-going process, albeit originating from homotypic nerve fibres.

Measurements of a maximum presence of GAP-43 in pineals at 3 days after unilateral SCGX, followed by a linear decline to 28 days, suggests that the majority of new growth seen at 3 days had eventually reached targeted cells by 28 days after surgery, that migration had ceased, and possibly that a maturing relationship between the nerve terminal and pinealocyte membranes had culminated in reinnervation. Observations of terminal growth cones present at the membranes of pinealocytes, particularly in pineal tissue collected after an extended recovery period, suggests those cells were the target of new nerve fibres. Nerve growth terminating at the membranes of pinealocytes exhibited intense points of GAP-43 IR resembling the presence of the IR product at areas where nerve synapses are known to occur in cerebellum tissue. Synapses in the 'basket' region of Purkinje cell bodies are known sites of stimulation (Oestreicher & Gispen, 1986) and in foetal tissue synaptogenesis was localized by the presence of condensed and intense areas of GAP-43 IR.

In this experiment, the presence of GAP-43 observed in the pineals subjected to unilateral SCGX has been found to correspond with measurements indicating that those same pineals had initiated a recovery in melatonin secretory capacity, albeit in an inverse relationship. As depicted in Figure 5.1 a recovery in function was initially apparent at 3 days after surgery, corresponding with observations citing an intense reactivity for the GAP-43 complex. Over the remaining experimental period, while melatonin secretory capacity increased GAP-43 IR declined. This relationship between the presence of GAP-43 in pineal tissue and recovery of pineal secretory capacity (output) is highly indicative evidence in support of the hypothesis that had suggested neural plasticity may be

involved as a compensatory mechanism in re-establishing pre-operative levels of pineal function, after unilateral SCGX.

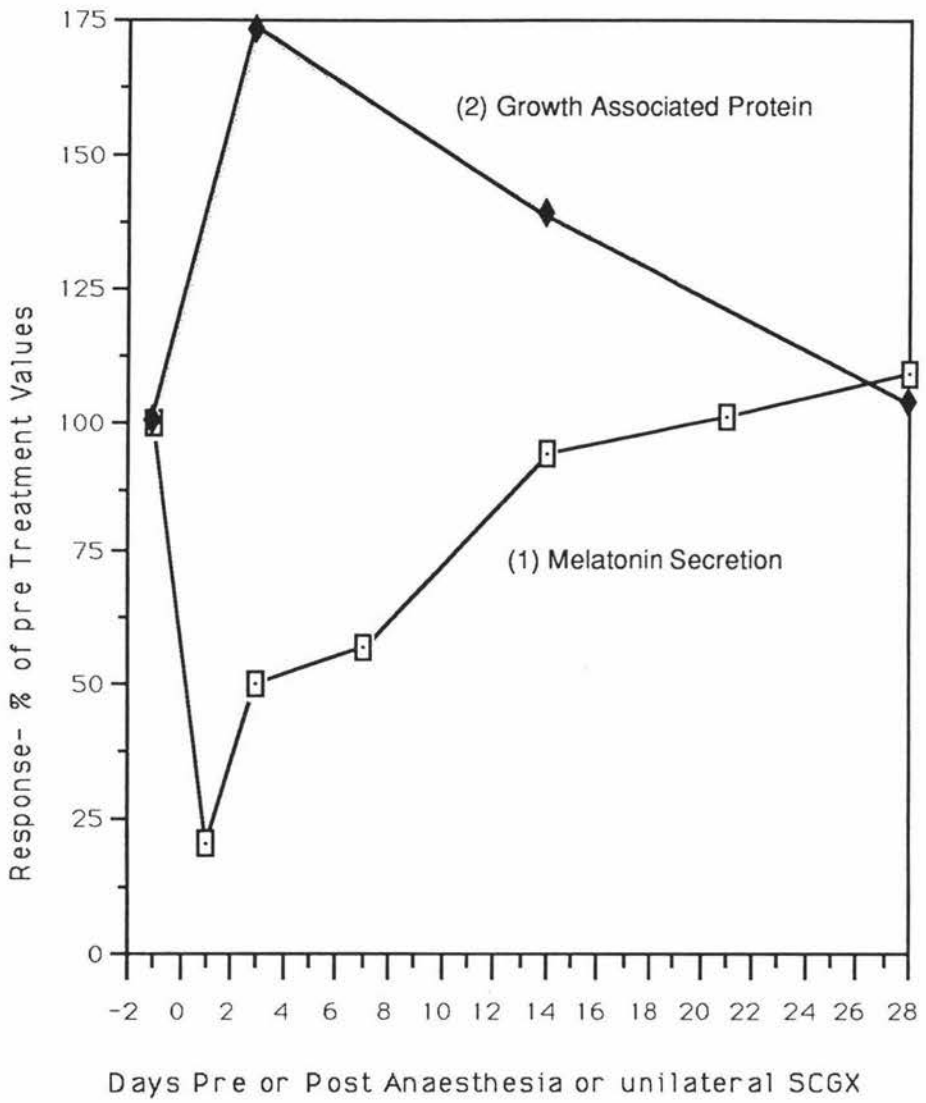


Fig. 5.1. Dual plot of: (1) Mean integrated melatonin secretory responses for 28 day group sheep calculated as percentages of pretreatment values. Measurements were recorded during 4 hrs of darkness, prior to and at 1, 3, 7, 14, 21 & 28 days after unilateral SCGX.
: (2) Mean integrated ICC intensity levels as percentages of control animal values of GAP-43 immunoreactivity in sheep pineal glands collected 3, 14 and 28 days after unilateral SCGX.

Further evidence for a role for re-innervation in the recovery of pineal function after unilateral SCGX can be derived from anatomical studies. It has been established in rodents that the sympathetic fibres arising from cell bodies in each SCG innervate the contralateral, as well as the ipsilateral half of the pineal gland (Dornay, *et al*, 1985; Zigmond, *et al*, 1981, 1985; Lingappa & Zigmond, 1987) and a similar pattern of innervation has been reported in sheep (Mockett & Lapwood, 1993). Within pineal tissue the intermingling of nerve terminals from each SCG has been established, with Reuss, *et al* (1985) suggesting that most pinealocytes are innervated by nerve fibres from only one SCG, not both. In sheep that pattern of innervation has been confirmed where the number of ICC localized NPY IR nerve fibres was reduced after unilateral SCGX, but the distribution of those fibres was still bilateral (Mockett, *et al*, 1991). NPY has commonly been shown to be localized in NE fibres (Ebadi, *et al*, 1989).

As bilateral SCGX has been shown to result in reductions in pinealocyte cell sizes, including diminishing profile areas of both cytoplasm (Peschke, *et al*, 1989) and the nucleus (Calvo, *et al*, 1990), it would appear that cell morphology, as well as function were dependent on an intact sympathetic nerve supply. That information and the pattern of sympathetic innervation suggests that 50% of pinealocytes in a pineal gland that had been unilaterally ganglionectomized may be expected to cease to function and ultimately atrophy. However, Mockett (1991), by inference from measurements of NSE-like IR, has established that cell atrophy does not occur in sheep pineals collected at 14 days after unilateral SCGX. Similarly, in this experiment, measurements of AT IR parenchymal cells has established that cell integrity is maintained up to 28 days after unilateral SCGX. These measurements indicate that the innervation was intact despite partial denervation, providing further evidence that re-innervation had occurred.

5.3 Alpha tubulin and secretion from pinealocytes of the sheep pineal gland

In Section 4.4.4 the presence of alpha tubulin in the cytoplasm of ovine pinealocytes was discussed, while it is exhibited in Figures 4.12-4.13. Other than a report of a similar localization in guinea pigs (but not in rats; Schroder, *et al*, 1990), the presence of AT in pinealocytes had not previously been reported. However, the presence of microtubules, of which alpha tubulin is a component, had been reported in the pinealocytes of rats (Friere & Cardinali, 1975), baboons (Theron, *et al*, 1979) and sheep (Anderson, 1965). In sheep the microtubules were reported to be striated (Anderson, 1965), an ultrastructural difference that may translate to a variation in function. In contrast, in a review on neuronal structures in a range of species, Brady (1993) has suggested that there were no obvious ultrastructural differences in the appearance of microtubules.

The function of microtubules in the cytoplasm of cells in other endocrine glands has previously been proposed to involve intracellular transport and exocytosis of secretory granules (Shiino & Rennels, 1974). Early pharmacological studies revealed that agents that depolymerize or superstabilize microtubules, also inhibited cell secretion (Boyd, *et al*, 1982). More recently, microtubules have been implicated to perform an active role in the secretory process of the endocrine pancreas, following observations of organelle movement along microtubules (Dentler & Suprenant, 1986).

The process of pinealocyte secretion has been reported by Pevet (1979), who described the presence of proteinaceous material and the formation of vacuoles in the cisternae of the granulated endoplasmic reticulum. At the Golgi bodies the synthesized substances are packaged for secretion and delivered to the pericapillary and intercellular spaces, prior to release by either exocytosis or diffusion. In an EM study on sheep pineals, Struwe & Vollrath (1990) reported the presence of 'synaptic' spherules at the membranes of pinealocytes. Presumably, the microtubule population observed in ovine

pinealocytes is involved in the "delivery" of secretory products, perhaps in 'synaptic' spherules. Facility to use E.M. to make observations, similar to those of Dentler & Suprenant (1986) in pancreatic beta cells, of microtubules in ovine pinealocytes would help to elucidate the mechanisms of melatonin secretion. The presence of AT in this current experiment and the observation of the protein coursing into pinealocyte processes is indicative of a secretory role, similar to that previously reported in both the pancreas and pituitary.

5.4 Future directions for research in nerve regeneration using sympathetic innervation to the sheep pineal gland as a model.

An understanding of the mechanisms of nerve regeneration in mature nerve tissue is potentially a valuable prerequisite to establishing effective means of treating both disease and trauma in the nervous system. Compared to developmental growth, mature nerve tissue is less inductive to regeneration, as exemplified by comparisons drawn from growth of nerves in culture, where in the same conditions neonatal neurites extend within hours (Argiro & Johnson, 1982; Colins & Lee, 1982), whereas mature neurites take days.

This current experiment has established that the sympathetic innervation to the sheep pineal gland is a relatively easy system to manipulate with the aim of investigating nerve regeneration in mature tissue. Continued research, using this model system, could no doubt be used to further elucidate the factors that are associated with promoting nerve growth and the mechanisms involved in both nerve migration and functional re-innervation.

An important element of this model is the choice of sheep as an experimental animal, a choice that was made for various practical and physiological reasons. Other species

such as goats and pigs, while also providing the benefits derived from using sheep are, for various behavioural and anatomical reasons, deemed less suitable candidates for such experiments (Rowan, 1981). Most experiments studying innervation and function of the pineal gland have been confined to measurements of indirect parameters of secretory capacity only, mostly pineal NAT and HIOMT activity (Bowers & Zigmond, 1980, 1982; Reuss, *et al*, 1989a), or gland melatonin content (Reiter, *et al*, 1979c), as imposed by constraints in use of rodents as the experimental species. The comparative size of sheep enables periodic blood collection for the measurement of sequential changes in concentrations of melatonin occuring in the blood of individual animals. Albeit not taking account of metabolic clearance, this method represented a significant advantage over those studies 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 capacities to secrete melatonin may have highlighted such changes. The relative merits of individual and average (mass) curves have been discussed elsewhere (Medawar, 1945; Sholl, 1954). Another important potential advantage of the use of sheep for such studies is the relatively large size of their pineal glands (approximately 70 mg; Barrell & Lapwood, 1978/9). Glands of this size can readily be sub-divided to provide tissue to be fixed for paraffin embedding, cryostat sectioning and electron microscopy, as well as for enzyme measurements. Choice of sheep as the experimental animal was also encouraged by the comparative wealth of anatomical and physiological information available for the species.

Experiments designed to elucidate the mechanisms of nerve regeneration in the sheep pineal gland will need to incorporate various methods of denervation in order to make comparative assessments of the effects of both unilateral and bilateral denervation. Not only should such studies involve SCGX, but in addition, sectioning of the cervical

sympathetic trunk (decentralization) could be implemented to establish the role of central fibres in recovery of function. ICN section in sheep would, however, not be recommended because of its inaccessability in this species.

RIA analysis of plasma levels of melatonin should be monitored in all experimental animals during periods of investigation, excepting after bilateral SCGX, following which secretion is abolished (Korf & Moller, 1984). Measurements of variation in capacity to secrete melatonin has been established as a suitable direct method of determining the effects of surgical manipulation on pineal function.

Other adaptations in the use of the sheep SCG-pineal model which would benefit this type of research would involve the development of further laboratory analytical techniques. For example, much more objective analysis of GAP-43 in histological studies would derive from use of image processing technology such as that from Jandell Scientific, which Schreyer and Skene (1993) used to assess labelling intensity in dorsal root ganglion neurones. Development of further RIA's, gel electrophoretic and ICC tests for relevant proteins, enzymes and transmitters, also may be beneficial, as would use of electron microscopy.

In addition to collecting pineal glands at the end of experiments, any remaining SCG's should also be collected for determining enzyme activities with respect to establishing the role of intact SCG's in the presence of nerve growth.

Maximum gain could be derived from experimental animals if experiments were aimed at an integrated investigation of: (i) the factors which induce neural growth, (ii) the source of new growth and (iii) the progression of nerve growth. Guidelines to investigating each aim are to follow:

(i) The nature and the source of the signal elicited by ganglionectomy, to initiate a recovery in pineal function, needs to be addressed.

Factors which stimulate nerve growth have previously been demonstrated to originate from both deafferented target tissue (Brown & Holland, 1979; Pockett & Slack, 1982) and the products of nerve degeneration, including various cellular elements, such as Schwann cells (Brown, 1984). Also, factors in the glial environment have been shown to influence growth (Muller, 1992), particularly, the presence of NGF, a neurotrophic protein which has been shown to affect many aspects of sympathetic neuron growth, including survival (Hendry, 1977), neuronal size (Hendry & Campbell, 1976), catecholamine biosynthetic enzyme and neuropeptide content (Hayashi, *et al*, 1985), axon outgrowth and guidance (Levi-Montalcini, 1966, 1976) and neurite maintenance (Campenot, 1977).

Detection systems are available for some neural growth factor receptors (e.g. NGF receptor antiserum; Amersham International plc, Little Chalfont, U.K). ICC application of antisera for localising NGF receptors (McNulty, *et al*, 1993) in ovine pineal tissue could establish the relationship between the presence of that factor and nerve growth. Presumably, the sequence of events leading to regenerative nerve growth in mature tissue means that after unilateral ganglionectomy the presence of NGF receptors might be expected to precede the presence of GAP-43. On the other hand, after bilateral ganglionectomy it might be expected that NGF receptor concentrations would be high, but would not be succeeded by the presence of GAP-43.

In addition to stimulating factors for nerve growth, inhibiting factors which originate from deafferented target tissue have been postulated (Diamond, *et al*, 1976). Myelin is reported to prevent cultured neurons from growing (Crutcher, 1989) and it has been suggested that continued characterization of such inhibitory factors, and the raising of antibodies to block their actions, could further unravel the question of whether regenerating connections are able to elaborate functional interactions.

(ii) Experiments must also be designed to confirm the origin of the neural growth that has been exhibited in unilaterally SCGX pineals. The demonstration of an increase in activity in the intact SCG by Dornay, *et al* (1985) has provided indirect evidence to implicate collateral growth of residual nerve fibres, originating from the intact SCG, as the source of nerve growth. Any combination of RIA, ICC or gel electrophoretic investigation into the presence of GAP-43 in pineals harvested at around 3 days post bilateral SCGX, could either confirm or refute that implication, for if nerve growth does originate from the intact SCG in unilateral SCGX animals, then presumably growth would be checked by bilateral SCGX and consequently levels of GAP-43 would not be seen to increase. Another approach would be to use injection of an anterograde neural tracer, such as Fluoro Ruby, into the SCG remaining after unilateral SCGX. This fluorescent tracer would then be expected to co-localize with ICC-detected GAP-43 in newly grown nerve terminals. A trial study would be required to determine the time for SCG to pineal transport of Fluoro Ruby, in order to determine when the tracer should be injected relative to the time of SCGX surgery.

Similarly, decentralization by CST transection could establish whether increased GAP-43 occurs in nerve fibres that retain cell body connections, but which receive no pre-ganglionic stimulation.

RIA analysis for ChAT in SCG tissue collected from animals at various periods after unilateral SCGX would establish the presence of any variance in SCG enzyme activity during the period over which nerve growth occurs. Alternatively, gel electrophoresis could be used to determine the presence of any polypeptide variations in the SCG during the recovery period. For example, in rat dorsal root ganglia, measurements of GAP-43 mRNA has demonstrated that protein induction begins within 1 and 2 days after sciatic nerve injury (Skene, 1989).

(iii) To further implicate neural regeneration, and so too the hypothesis for collateral sprouting, as a mechanism responsible for the functional recovery apparent in the pineal gland after unilateral SCGX, the period of onset of nerve growth needs to be better defined. Experimental procedures should be focused at measurements immediately subsequent to surgery. ICC investigation for GAP-43 in pineals harvested from day 1 post-surgery through to day 5, will establish when growth is initiated and perhaps indicate some anatomical contrasts for the presence of new neural growth over the period during which growth is apparently most prolific.

The monoclonal subclone 7B10/D4 antibody used in these experiments reacts against both phosphorylated and non-phosphorylated GAP-43 (Meiri, K. pers. comm), therefore localisation determines the presence of GAP-43 *per se*, however, this antibody does not differentiate the reactive state of the GAP-43 present. Phosphorylation of GAP-43 by PKC is considered to represent a further step in the maturing of migrating axons (Spinelli, *et al*, 1982; Murphy, *et al*, 1983; Hsu, *et al*, 1984) and has been implicated in the interaction between growth cones and target tissue (Mattson, *et al*, 1988; Hall, *et al*, 1988; Girard & Kuo, 1990; Schreyer & Skene, 1993). The possibility of using an alternative antibody (subclone 2G12/C7), which has been shown to be specific to just the phosphorylated form of GAP-43, could further establish any variance in the tertiary configuration of the protein over the period during which functional regeneration has been measured (Meiri, *et al*, 1991). The reactivity of 2G12/C7 has previously been demonstrated with rat pineal tissue (Meiri, *et al*, 1991) and if reactivity with sheep pineal tissue were possible, then application of that antibody to the pineal tissue harvested in this current experiment would further clarify the temporal sequence of later stages of maturity during nerve regenerative processes.

In addition, ICC techniques could be used to detect the presence of either dopamine beta-hydroxylase or tyrosine hydroxylase in pineal tissue at various periods after unilateral SCGX. Preferably co-localization of either of these two enzymes with the localization of GAP-43 could be investigated by use of an alternative immunoenzyme method. Peroxidase based labels could be retained for GAP-43 (black) and alkaline phosphatase with a fast blue counter-stain are recommended for best contrast (Larsson, 1989) and could be used for TH. ICC localization of TH for distinguishing active nerve fibres should prove to be superior to AT IR localization which was used in this experiment and proved to be of limited value, due to the presence of intense parenchymal cell AT IR, which obscured any nerve fibre localization. A similar approach using DBH ICC would confirm the noradrenergic nature of the new neural growth. Alternatively, AT ICC could be used in rats, as in that species, while pinealocytes have been demonstrated to be AT IR negative, positive AT staining of nerve fibres was recorded by Schroder, ***et al*** (1990)

Having confirmed that neural growth occurs in the pineal gland after unilateral SCGX, and having determined that functional regeneration occurs over the same period, it is concluded that these two processes are correlated as a cause and effect. Detailed examination of the relationship between terminal nerve growth and pinealocyte membranes would provide some indication of whether nerve growth does culminate in reinnervation. Collection, fixation and embedding of pineal tissue for the purpose of examination of growing nerve terminals at the *n*m range using electron microscopy, should provide further evidence for the presence of collateral growth, its architecture, and relationship with pinealocytes.

5.5 Conclusions

The viability of using melatonin secretory profiles (output) as a measure of pineal function, as an alternative to other indicators of potential to secrete melatonin, has been demonstrated. In response to unilateral SCGX measurements of dark period melatonin secretory capacity were depressed in excess of 50% indicating the presence of secondary inhibitory factors, in addition to the main effect of partial denervation. Recovery in secretory capacity has been confirmed and the potential to realise full recovery has been established.

ICC localization of the growth associated protein GAP-43 in ovine pineal tissue has confirmed the presence of neural growth which has served to consolidate the hypothesis that re-innervation of denervated pinealocytes, by collateral sprouting of residual neurons, is the most probable compensating mechanism responsible for the full recovery in pineal secretory capacity after partial denervation.

Assessment of a stable alpha tubulin component in the cytoplasm of pinealocytes after unilateral SCGX, has indicated that partial denervation does not have detrimental effects on cell morphology. The function of alpha tubulin in ovine pinealocytes is proposed to be an involvement in hormone secretion.

Conclusive evidence, the first to determine both the presence of new neural growth in pineal tissue, and a full recovery in pineal melatonin secretory capacity, has established the sympathetic innervation to the pineal gland as a useful model suitable for the continued investigation of nerve regeneration corresponding to functional regeneration.

Future avenues of research, using the SCG-pineal gland system as a model for study of anatomical and physiological recovery from denervation, have been discussed.

References:

Adrendt, J., Laud, C. A., Symons, A. M. & Pryde, S. J. (1983) Plasma melatonin in ewes after ovariectomy. *J. Reprod. Fert.* **68:** pp. 213-218.

Aitken, J. (1949) The effect of peripheral connections on the maturation of regenerating nerve fibres. *J. Anat* **83:** pp. 32-43.

Alexander, K. A., Cimler, B. M., Meier, K. E. & Storm, D. R. (1987) Regulation of calmodulin binding to P-57. *J. Biol. Chem* **262:** pp. 6108-6113.

Allt, G. (1976) Pathology of the peripheral nerve. In: *The Peripheral Nerve*. Ed: D. N. Lan

Altman, G. (1972) Post-natal development of the cerebellar cortex in the rat. *J. comp. Ne*

Anderson, E. (1965) The anatomy of ovine and bovine pineals. *J. of Ultrastructure Res.* **8:** pp. 18-26.

Anderson, P., Bliss, T. V. P. & Skrede, K. K. (1971) Lamellar organisation of hippocampal excitatory pathways. *Expt. Brain. Res.* **13:** pp. 222-238.

Appleton, A. B. & Waites, G. M. H. (1957) A surgical approach to the superior cervical ganglion and related structures in the sheep. *J. Physiol.* **135:** pp. 52-57.

Ard, M. D. & Bunge, R. P. (1988) *J. Neurosci.* **8:** pp. 2844-2858.

Arendt, J. (1985) Mammalian pineal rhythms. *Pineal res. rev.* **3:** pp. 161-213.

Arendt, J. & Ravault, J. P. (1988) Suppression of melatonin secretion in Ile-de-France rams by different light intensities. *J. of Pineal Res.* **5:** pp. 245-250.

Argiro, V., Bunge, M. B. & Johnson, M. I. (1984) Correlation between growth cone form and movement and their dependence on neuronal age. *J. Neurosci.* **4:** pp. 3051-3062.

Argiro, V. & Johnson, M. I. (1982) Patterns and kinetics of neurite extension from sympathetic neurons in culture are age dependent. *J. Neurosci.* **2:** pp. 503-512.

Armstrong, S., Ng, K. T. & Coleman, G. J. (1982) Influence of the pineal gland on brain-behaviour relationships. In: *The Pineal Gland*. Vol. III. Extra-Reproductive Effects. Ed: R, J, Reiter. CRC Press. Florida. pp. 87-94.

Arstila, A. V. (1967) Electron microscopic studies on the structure and histochemistry of the pineal gland of the rat. *Neuroendocr.* **2:** pp. 1-101.

Aubineau, P., Reynier-Rebuffel, A. M., Bouchard, C., Jousseaume, O. & Seylaz J. (1985) Long-term effects of superior cervical ganglionectomy on cortical blood flow of non-anesthetized rabbits in resting and hypertensive conditions. *Brain Res.* **338:** pp. 13-23.

Axelrod, J. & Weissbach, H. (1960) Enzymatic O-methylation of N-acetylserotonin to melatonin. *Science.* **131:** pp. 1312- 1316.

Backstrom, M. & Wetterberg, L. (1972) Catechol-O-methyltransferase, histamine-N-methyltransferase and methanol forming enzyme in the rat pineal gland. *Life Sci.* **11:** pp. 293-299.

Baehr, M. (1991) *Exp. Neurol.* **111:** pp. 65-73.

Baehr, M. & Bunge, R. P. (1990) *Glia.* **3:** pp. 293-300.

Barrell, G. K. & Lapwood, K. R. (1979a) Effects of various lighting regimes and pinealectomy on semen production in Romney rams. *J. of Repr. and Fert.* **57:** pp. 273-279.

Barrell, G. K. & Lapwood, K. R. (1979b) Effects of pinealectomy on the secretion of luteinizing hormone, testosterone and prolactin in rams exposed to various lighting regimes. *J. of Endocr.* **80:** pp. 397-405.

Barrell, G. K. & Lapwood, K. R. (1978/1979a) Seasonality of semen production and plasma luteinizing hormone, testosterone and prolactin levels in Romney, Merino and Polled Dorset rams. *Anim. Repr. Sci.* **1:** pp. 213-248.

Barrell, G. K. & Lapwood, K. R. (1978/1979b) Effects of modifying olfactory and pineal gland function on the seasonality of semen production and plasma luteinizing hormone, testosterone and prolactin levels in rams. *Anim. Repr. Sci.* **1:** pp. 229-243.

Barrell, G. K., Lapwood, K. R. & Elgar, H. J. (1985) FSH secretion in pinealectomized rams subjected to artificial photoperiods. *J. of Endocr.* **106:** pp. 167-171.

Basi, G. S., Jacobson, R. D., Virag, I., Schilling, J. & Skene, J. H. P. (1987) Primary structure and transcriptional regulation of GAP-43, a protein associated with nerve growth. *Cell* **49:** pp. 785-791.

Bastiani, M. J. & Goodman, C. S. (1986) Guidance of neuronal growth cones in the grasshopper embryo. III. Recognition of specific glial pathways. *J. Neurosci.* **6:** pp. 3532-3541.

Benfey, M. & Aguayo, A. J. (1982) *Nature* (London) **296:** pp. 150-152.

Benfey, M., Bunger, U. R., Vidal-Sanz, M., Bray, G. M. & Aguayo, A. J. (1985) *J. Neurocytol.* **14:** pp. 279-296.

Benowitz, L. I. & Routtenbourg, A. (1987) A membrane phosphoprotein associated with neural development, axonal regeneration, phospholipid metabolism and synaptic plasticity. *Trends Neurosci.* **10:** pp. 527-532.

Bensinger, R. E., Klein, D. C., Weller, J. L. & Lovenberg, W. (1974) Radiometric assay of total tryptophan hydroxylation by intact cultured pineal glands. *J. Neurochem.* **23:** pp. 111-117.

Bently, D. & Toroian-Raymond, A. (1986) Disorientated pathfinding by pioneer neurone growth cones deprived of filipodia by cytochalasin treatment. *Nature.* **323:** pp. 712-715.

Birtles, M. J. (1981) Histological studies of the secretory processes in bovine salivary glands. Massey University, Palmerston North : Masterate thesis. 33.

Bisby, M. A. (1982) Retrograde axonal transport of endogenous proteins. In: *Axoplasmic transport.* Ed: D. Weiss. Springer-Verlag. NY. pp. 193-205.

Bittman, E. L., Karsch, F. J. & Hopkins, J. W. (1983) Role of the pineal gland in ovine photoperiodism: Regulation of seasonal breeding and negative feedback effects of estradiol upon luteinizing hormone secretion. *Endocrinology.* **113:** pp. 329-336.

Bittman, E. L. & Karsch, F. J. (1984) Nightly duration of pineal melatonin secretion determines the reproductive response to inhibitory day length in the ewe. *Biol. of Repr.* **30:** pp. 585-593.

Bittman, E. L. (1984) Melatonin and photoperiodic time measurement: Evidence from rodents and ruminants. In: *The Pineal Gland.* Eds: R. J. Reiter. New York : Raven Press. pp. 155-192.

Bjorklund, A., Owman, C. & West, K. A. (1972) Peripheral sympathetic innervation and serotonin cells in the habenular region of the rat brain. *Zeitschrift fur Zelforschung and Mikroskopische Anatomie.* **127:** pp. 570-579.

Black, M. M. & Lasek, R. (1977) The presence of transfer RNA in the axoplasm of the squid giant axon. *J. Neurobiol.* **8:** 229-237.

Blake, C. A. (1975) Localization of the inhibitory actions of ovulation-blocking drugs on release of luteinizing hormone in ovariectomized rats. *Endocrinology.* **95:** pp. 999-1004.

Blask, D. E. (1981) Potential sites of action of pineal hormones within the neuroendocrine-reproductive axis. In: *The Pineal Gland.* Vol II. Reproductive Effects. Ed: R. J. Reiter. CRC Press. Inc. Florida. pp. 189-216.

Blose, S. H., Meltzer, D. I & Feramisco, J. R. (1984) 10-nm filaments are induced to collapse in living cells microinjected with monoclonal and polyclonal antibodies against tubulin. *J. Cell Biol.* **98 (3) :** pp. 847-858.

Boland, M. P., Al-Kamali, A., A., Crosby, T. F., Haynes, N. B., Howles, C. M., Kellher, D. L. & Gordon, I. (1985) The influence of breed, season and photoperiod on semen characteristics, testicular size, libido and plasma hormone concentrations in rams. *Anim. Repr. Sc.* **9:** pp. 241-252.

Bowers, C. W. & Zigmond, R. E. (1982) The influence of the frequency and pattern of sympathetic nerve activity on serotonin N-acetyltransferase in the rat pineal gland. *J. Physiol.* (Lond) **330:** pp. 279-296.
Bowers, C. W., Dham, L. & Zigmond, R. E. (1984b) The number and distribution of sympathetic neurons that innervate the rat pineal gland. *Neuroscience* **13:** pp. 87-96.
Boyd, A. E., Bolton, W. E., & Brinkley, B. R. (1982) Microtubules and beta cell function: effect of colchicine on microtubules and insulin secretion *in vitro* by mouse beta cells. *J. Cell Biol.* **92:** pp. 425-434.
Brabander, D. E., Geuens, G., Nuydens, M., Moeremans, M. & De Mey, J. (1986) Microtubule-dependent intracellular moyility investigated with nanometer particle video ultramicroscopy. In: *Dynamic aspects of microtubule biology.* Ed: D. Soifer. *Annals of N.Y. acad. of Sci.* **466:** pp. 666-668.
Brady, S. T. (1988) Cytotypic specializations of the neuronal cytoskeleton and cytomatrix: implications for neuronal growth and regeneration. In : *Cellular and molecular aspects of neural development and regeneration.* Eds: B. Haber & A. Gorio. Springer-Verlag. N. Y. pp. 311-322.
Brady, S. T. (1991) Molecular motors in the nervous system. *Neuron.* **7:** pp. 521-533.
Brady, S. T & Black, M. M. (1986) Axonal transport of microtubule proteins: cytotypic variation of tubulin and MAPs in neurons. *Ann. NY. Acad. Sci.* **466:** pp. 199-217.
Brady, S.T. (1993) Axonal dynamics and regeneration. In: *NeuroRegeneration.* Ed: A. Gorio. Raven Press. Ltd. NY. pp. 7-36.
Braizer, L. & Fishman, M. C. (1987) Recognition of specific targets by cultured dorsal root ganglion neurons. *J. Neurosci.* **7:** pp. 2305-2311.
Bray, D. & Hollenbeck, P. J. (1988) Growth cone motility and guidance. *Ann. Rev. Cell Biol.* **4:** pp. 43-61.
Bray, D. & Chapman, K. (1985) Analysis of microspike movements in the neuronal growth cone. *J. Neurosci* **5:** pp. 3204-3213.
Bray, G. M., Peyronnard, J. M. & Aguayo, A. J. (1972) Reactions of unmyelinated nerve fibres to injury- an ultrastructural study. *Brain Res.* **42:** pp. 297-309.
Bray, D. (1970) Surface movements during the growth of single explanted neurons. *Proc. Natl. Acad. Sci.* USA. **65:** pp. 4516-4519.
Bronson, F. H. (1988) Seasonal regulation of reproduction in mammals. In: *The Physiology of Reproduction*, Vol. 2, Eds: E. Knobil & J. Neill. New York: Raven Press Ltd. pp. 1831-1871.
Brown, M. C. & Holland, R. L. (1979) A central role for denervated tissues in causing nerve sprouting. *Nature* **282:** pp. 724-726.
Brown, M. C., Holland, R. L. & Hopkins, W. G. (1981) Motor nerve sprouting. *Ann. Rev. Neurosci.* **4:** pp. 17-42.
Brownstein, M. J. & Axelrod, J. (1974) Pineal gland: a 24-hour rhythm in norepinephrine turnover. *Science* **184:** pp. 163-165.
Bueker, E. D. (1948) *Anat. Rec.* **102:** pp. 368-390.
Bunge, M. B. (1973) Fine structure of nerve fibres and growth cones of isolated sympathetic neurons in culture. *J. Cell Biol.* **56:** pp. 713-735.
Bunge, M. B., Johnson, M. I. & Argiro, V. J. (1983) Studies of regenerating nerve fibres and growth cones. In: *Spinal cord regeneration.* Eds: C. C. Kao., R. P. Bunge & P. J. Reier. Raven, New York. pp. 99-120.
Burger, H. G., Lee V. W. K. & Rennie, G. C. (1972) A generalised computer program for the treatment of data from competitive protein-binding assays including radioimmunoassays. *J. of Lab. and Clin. Med.*. **80:** pp. 302-312.
Burnstock, G. & Coster, M. (1975) Structure of adrenergic neurons and related cells. In: *Adrenergic neurons.* Ed: G. Burnstock. pp. 19-37.

Calvo, J. L., Boya, J. & Garcia-Maurino, J. E. (1990) Ultrastructural changes in the rat pineal gland after sympathetic denervation. Quantitative study. *Histol.Histopathol.* **5:** pp. 155-160.
Campenot, R. (1982) Development of sympathetic neurons in compartmentalized cultures.1. Local control of neurite growth by nerve growth factor. *Dev. Biol.* **93:** pp. 1-12.
Campenot, R. B. (1977) Local control of neurite development by nerve growth factor. *Proc. Natl. Acad. Sci. USA.* **74:** pp. 4516-4519.
Cardinali, D. P., Pisarev, M. A., Barontini, M., Juvenal, G. J., Boado, R. J. & Vacas, M. I. (1982) Efferent neuroendocrine pathways of sympathetic superior cervical ganglia. *Neuroendocr.* **35:** pp. 248-254.
Cardinali, D. P. (1974) Melatonin and the endocrine role of the pineal organ. In: Current Topics in Experimental Endocrinology. Vol 2. Eds: V. H. T. James & L. Martini. Academic Press. New York. pp. 107-128.
Cardinali, D. P. & Romeo, H. E. (1991) The autonomic nervous system of the cervical region as a channel of neuroendocrine communication. *Frontiers in Neuroendocr.* **12 (3):** pp. 287-297.
Carter, A. H. & Cox, E. H. (1982) Sheep breeds in New Zealand. In: *Sheep Production: Breeding and Reproduction.* Vol 1. Eds: G. A. Wickham & M. F. McDonald. Auckland, Bay Richards. pp. 11-38.
Caudy, M. & Bently, D. (1986) Pioneer growth cone steering along a series of neuronal and non-neuronal cues of different affinities. *J. Neurosci.* **6:** pp. 1781-1795.
Cheung, N. Y. (1980) Calmodulin-Calcium interactions. *Science.* **207:** pp. 19-27.
Cimler, B. M., Giebelhaus, D. H., Wakim, B. T., Storm, D. R. & Moon, R. T. (1987) Characterization of murine cDNAs encoding P-57 , a neural-specific calmodulin-binding protein. *J. Biol. Chem.* **262:** pp. 12158-12163.
Clarke, K. A. (1989) In: *Neurophysiology, applications in the behavioural and biomedical sciences.* Ed: A. J. Turner. John Wiley, New York. pp. 126-128.
Clarke, I. J. & Doughton, B. W. (1983) Effect of various anaesthetics on resting plasma concentrations of luteinizing hormone, follicle-stimulating hormone and prolactin in ovariectomized ewes. *J. of Endocr.* **98:** pp. 79-89.
Claustrat, B., Brun, J. & Chazot, G. (1990) Melatonin in humans, neuroendocrinological and pharmacological aspects. *Nucl. Med. Biol.* **27:** pp. 625-632.
Cochran, W. G. & Cox, G. M. (1957) In: *Experimental Designs.* John Wiley & Sons, Inc. New York. pp. 45-90.
Collingridge, G. L. & Bliss, T. V. P. (1987) *TINS* **10:** pp. 288-293.
Collins, F. & Lee, M. R. (1982) A reversible developmental change in the ability of ciliary ganglion neurons to extend neurites in culture. *J. Neurosci.* **2:** pp. 424-430.
Cotman, C. W. & Nadler, J. V. (1978) Reactive synaptogenesis in the hippocampus. In: *Neural plasticity* Ed: C. W. Cotman . Raven press. New York. pp. 227-271.
Cowan, W. M. (1979a) The development of the brain. *Sci. Amer.* **241:** pp. 112-133.
Cozzi, B. & Moller, M. (1988) Indications for the presence of two populations of serotonin-containing pinealocytes in the pineal complex of the Golden hamster (*Mesocricetus auratus*). *Cell and Tiss. Res.* **252:** pp. 115-122.
Cozzi, B., Mikkelsen, J. D., Merati, D., Capsoni, S. & Moller, M. (1990) Vasoactive intestinal peptide-like immunoreactive nerve fibres in the pineal gland of the sheep. *J. of Pineal. Res.* **8:** pp. 41-47.
Crag, B. G. & Thomas, P. K. (1964) The conduction velocity of regenerated peripheral nerve fibres. *J. Physiol.* **171:** pp. 164-75.
Cragg, B. G. (1970) What is the signal for chromatolysis? *Brain Res.* **23:** pp. 1-21.

Culman, J., Torda, T. & Weise, V. K. (1987) A radiometric assay for phenylethanolamine N-methyltransferase and catechol O-methyltransferase in a single tissue sample: Application to rat hypothalamic nuclei, pineal gland and heart. *Analyt. Biochem.* **164:** pp. 345-354.

Czyba, J. C., Girod, C. & Durand, N. (1964) Sur l'antagonisme epiphysohypophysaire et les variations saisonnieres de la spermatogenese chez le hamster dore. (*Mesocricetus auratus*). *C. R. Seanc. Soc. Biol.* **158:** pp. 742-745.

Dahlstrom, A. & Heiwall, P. O. (1975) Intra-axonal transport of transmittors in mammalian neurons. *J. Neural transm.* **12:** 97-114.

Dafny, N. (1980) Two photo pathways contribute to pineal evoked responses. *Life Sci.* **26:** pp. 737-742.

Daniels, M. P. (1972) Colchicine inhibition of nerve fibre formation *in vitro*. *J. Cell Biol.* **53:** pp. 164-176.

David, S. & Aguayo, A. J. (1981) *Science.* **214:** pp. 931-933.

Degushi, T. & Axelro,d J. (1972c) Induction and superinduction of serotonin N-acetyltransferase by adrenergic drugs and denervation in rat pineal gland. *Proc. Natl. Acad. Sci.* USA. **69:** pp. 2208-2211.

Dentler, W. L. & Suprenant, K. A. (1986) Isolation of microtubule-secretory granule complexes from the anglerfish endocrine pancreas. In: *Dynamic aspects of microtubule biology.* Ed: D. Soifer. *Annals N. Y Acad. of Sci.* **446:** pp. 813-831.

Diamond, J., Cooper, E., Turner, C. & MacIntyre, L. (1976) Trophic regulation of nerve sprouting. Neuron-target interactions and spatial relations control sensory nerve fields in salamander skin. *Science.* **193:** pp. 371-377.

Diamond, J., Coughlin, M., MacIntyre, L., Holmes, M. & Visheau, B. (1987) Evidence that endogenous *B* nerve growth factor is responsible for the collateral sprouting, but not the regeneration, of nociceptive axons in adult rats. *Proc. Natl. Acad. Sci.* USA. **84:** pp. 6596-6600.

Dornay, M., Gilad, V. H. & Gilad, G. M. (1985) Compensatory changes in contralateral sympathetic neurons, superior cervical ganglia and in their terminals in the pineal gland following unilateral ganglionectomy. *J. Neurosci.* **5:** pp. 1522-1526.

Dufour, J. J., Fahmy, M. H. & Minvielle, F. (1984) Seasonal changes in breeding activity, testicular size, testosterone concentration and seminal characteristics in rams with long or short breeding season. *J. of Anim. Sci.* **58:** pp. 416-422.

Ebadi, M., Hexum, T. D., Pfieffer, R. F. & Govitrapong, P. (1989) Pineal and retinal peptides and their receptors. *Pineal Res. Revs.* **7:** pp. 1-156.

Edds, M. V. (1953) Collateral nerve regeneration. *Q. Rev. Biol.* **28:** pp. 260-275.

Edwards, R. M., Rutter, W. J. & Hanahan, D. (1989) Directed expression of NGF to pancreatic *B* cells in transgenic mice leads to selective hyperinnervation of the islets. *Cells* **58:** pp. 161-170.

English, J., Poultan, A. L., Arendt, J. & Symons, A. M. (1986) A comparison of the efficiency of melatonin treatments in advancing oestrous in ewes. *J. of Repr. and Fert.* **77:** pp. 321-327.

English, J., Bojkowski, C. J., Poulton, A. L., Symons, A. M., & Arendt, J. (1987) Metabolism and pharmokinetics of melatonin in the ewe. *J. of Pin. Res.* **4:** pp. 351-358.

Fawcett, J. W. & Keynes, R. J. (1990) Peripheral nerve regeneration. *Ann. Rev. Neurosci.* **13:** pp. 43-60.

Fields, R. D. & Nelson, P. G. (1991) Activity-dependent development of the vertebrate nervous system. *Int. Rev. of Neurobiol.* **34:** pp. 133-215.

Finger, S. & Stein, D. G. (1982) In: *Brain Damage and Recovery.* Academic Press, London Ltd. U. K. pp. 10-165.

Fisher, R. A. & Yates, F. (1963) In: *Statistical tables for biological, agricultural and medical research.* London: Oliver & Boyd.

Fox, J. E. B. (1987) The platelet cytoskeleton. *Thromb. Haemostasis.* pp. 175-225.

Fraser, S., Cowan, P., Franklin, M., Franey, C. & Arendt, J. (1983) Direct radioimmunoassay for melatonin plasma. *Clinical Chemistry.* **29(2):** pp. 396-397.

Freeman, J. A. (1986) A protein induced during nerve growth (GAP-43) is a major component of growth-cone membranes. *Science* **233:** pp. 783-786.

Freire, F. & Cardinali, D. P. (1975) Effects of melatonin treatment and environmental lighting on the ultrastructural appearance, melatonin synthesis, norepinephrine turnover and microtubule protein content of the rat pineal gland. *J. Neural. Transm.* **37:** pp. 237-257.

Friede, R. L. & Samorajski, T. (1970) Axon caliber related to neurofilaments and microtubules in sciatic nerve fibres of rats and mice. *Anat. Rec.* **167:** pp. 379-388.

Gilad, G. M. & Reis, D. J. (1979) Collateral sprouting in central mesolimbic dopamine neurons: Biochemical and immunocytochemical evidence of changes in the activity and distribution of tyrosine hydroxylase in terminal fields and in cell bodies of A10 neurons. *Brain Res.* **160:** pp. 17-36.

Ginzburg, I. Teichman, A. & Littauer, U. Z. (1986) Isolation and characterization of two rat alpha tubulin isotypes. In: *Dynamic aspects of microtubule biology.* Ed: D. Soifer. *Annals of N.Y Acad. of Sci.* **466:** pp. 31-40.

Girard, P. & Kuo, J. F. (1990) *J. Neurochem.* **54:** pp. 300-306.

Glass, J. D. & Lynch, R. (1981) Melatonin : identification of sites of antigonadal action in mouse brain. *Science* **214:** pp. 821-823.

Goldman, H. (1967) The nervous control of blood flow to the pineal gland. *Life Sci.* **6:** pp. 2017-2077.

Gonzalez-Brito, A., Reiter, R. J., Menendez-Palaez, A., Guerrero, J. M., Santana, C. & Jones, D. J. (1988b) Darkness induced changes in noradrenergic input determine the 24 hour variation in beta-adrenergic receptor density in the rat pineal gland: *In vivo* pharmacological and physiological evidence. *Life Sci.* **43:** pp. 707-714.

Goodman, R. L. & Meyer, S. L. (1984) Effects of pentobarbital anaesthesia on tonic luteinizing hormone secretion in the ewe: evidence of active inhibition of luteinizing hormone in anestrus. *Biology of Reproduction.* **30:** pp. 374-381.

Goslin, K. & Banker, G. (1990) Rapid changes in the distribution of GAP-43 correlate with the expression of neural polarity during normal development and under experimental conditions. *J. of Cell Biol.* **110:** pp. 1319-1331.

Goslin, K., Schreyer, D. J. Skene, J. P. H. & Banker, G. (1988) Development of neuronal polarity: GAP-43 distinguishes axonal from dendritic growth cones. *Nature.* **336:** pp. 672-674.

Grafstein, B. & McQuarrie, I. G. (1978) In: *Neuronal Plasticity.* Ed: C. W. Cotman. Raven, New York. pp. 155-195.

Guerillot, C., Pfister, A., Muller, J. & Da Lage, C. (1982) Recherche de l'origine des fibres nerveuses extra-orthosympathiques innervant l'epiphyse du rat (etude du transport retrograde de la peroxydase de raifort). *Repr. Nutr. Dev.* **22:** pp. 371-378.

Guth, L. (1974) Axonal regeneration and functional plasticity in the central nervous system. *Exp. Neurol.* **4:** pp. 606-654.

Guth, L. & Bernstein, J. J. (1961) Selectivity in the re-establishment of synapses in the superior cervical sympathetic ganglia of the cat. *Exp. Neurol.* **4:** pp. 59-69.

Hadley, M. E. (1992) In: *Endocrinology.* 3rd edn. Prentice Hall. New Jersey. USA.

Hafez, E. S. E. (1952) Studies on the breeding season and reproduction of the ewe. *J. of Agr. Sci.* **42:** pp. 189-265.

Haftek, J. & Thomas, P. K. (1968) Electron microscope observations on the effects of localised crush injuries on the connective tissues of peripheral nerves. *J. Anat.* **103:** pp. 233-43.

Halberg, F., Bakken, E., Cornelissen, G., Halberg, J., Halberg, E & Delmore, P. (1988) Blood pressure assessment in a broad chronobiologic perspective. In: *Heart Brain & Brain Heart*. Eds: Refsum, H., Sulg, J. A. & Rasmussen, K. Berlin : Springer-Verlag. pp. 142-162.

Hall, F. L., Fernyhough, P., Ishii, D. N. & Vulliet, P. R. (1988) *J. Biol. Chem.* **263:** pp. 4460-4466.

Hallpike, J. F. (1976) Histochemistry of peripheral nerves and nerve terminals. In: *The Peripheral Nerve*. Ed: D. N. Landon. Chapman & Hall. London. pp. 605- 665.

Ham, A. W. & Cormack, D. H. (1979) The endocrine system. In: *Histology*. Ed: A. W. Ham. Lippincott Co. Ltd. pp. 828-830.

Hammerback, J. A. & Letourneau, P. C. (1986) Neurite extensions across regions of low cell-substratum adhesivity: Implications for the guidepost hypothesis of axonal pathfinding. *Dev. Biol.* **117:** pp. 655-662.

Hammond, J. (1944) On the breeding season in the sheep *J. ofAgr. Sc. (Cambridge).* **34:** pp. 97-105.

Handa, Y., Caner, H., Hayashi, M., Tamamaki, N. & Nojyo, Y. (1990) The distribution pattern of the sympathetic nerve fibres to the cerebral arterial system in rat as revealed by anterograde labelling with WGA-HRP. *Exp. Brain. Res.* **82:** pp. 493-498.

Harrison, R. G. (1907) Observations on the living developing nerve fibre. *Anat. Rec.* **1:** pp. 116-118.

Hatten, M. E., Furie, M. B. & Rifkin, D. B. (1982) *J. Neurosci.* **2:** pp. 1195-1206.

Hatton, G. I (1985) In: *Synaptic Plasticity*. Ed: C. W. Cotman. Guilford, New York. pp. 373-404.

Hayashi, M., Edgar, D. & Thoenen, H. (1985) Nerve growth factor changes the relative levels of neuropeptides in developing sensory and sympathetic ganglia of the chick embryo. *Dev. Biol.* **108:** pp. 49-55.

Hedlund, L., Lischko, M. M., Rollag, M. D., Niswender, M. D. (1977) Melatonin: Daily cycles in plasma and cerebrospinal fluid of calves. *Science* **195:** pp. 686-687.

Hendry, I. A. (1977) Cell division in the developing sympathetic nervous system. *J. Neurocytol.* **6:** pp. 299-309.

Hendry, I. A. & Campbell, J. (1976) Morphometric analysis of rat superior cervical ganglion after axotomy and nerve growth factor treatment. *J. Neurocytol.* **5:** pp. 351-360.

Henrot, A. (1883) Notes de clinique medicale. *Gaz.d'Hop. de Paris.*

Herbert, J. (1989) Neural systems underlying photoperiodic time assessment: a blueprint. *Experientia.* **45:** pp. 965-972.

Heubner, O. (1898) Tumor der glandula pinealis. *Dtsch. Med. Wochenschr.* **24(2):** pp. 211-214.

Heumann, R., Lindholm, D., Bandtlow, C., Meyer, M., Radeke, M. J., Misko, T. P. Shooter, E. & Thoenen, H. (1987) Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerve during development, degeneration, and regeneration: Role of machrophages. *Proc. Natl. Acad. Sci. USA* **84:** pp. 8735-8739.

Heydorn, W. E., Fraser, A. & Weiss, B. (1981) Electrical stimulation of sympathetic nerves increases the concentration of cyclic AMP in rat pineal gland. *Proc. Natl. Sci.* USA **78:** pp. 7176 -7179.

Hiller, G. & Weber, K. (1978) Radioimunoassay for tubulin: A quantitative comparison of the protein content of different established tissue culture cells and tissues. *Cell.* **14:** pp. 795-804.

Hirata, F., Hayaishi, O., Tokoyama, T. & Senoh, S. (1974) *In vitro* and *in vivo* information of two new metabolites of melatonin. *J. Bio. Chem.* **249:** pp. 1311-1313.

Holmgren, N. (1917/1918) Zur Kenntnis der Parietalorgane von *(Rana temporaria). Arkiv Zool.* **11:** pp. 1-13.

Hoyle, G. W., Mercer, E. H., Palmiter, H. D. & Brinster, R. L. (1993) Expression of NGF in sympathetic neurons leads to excessive axon outgrowth from ganglia but decreased terminal innervation within tissues. *Neuron.* **10:** pp. 1019-1034.

Hsu, L., Natyzak, D. & Layskin, J. D. (1984) *Cancer Res.* **44:** pp. 4607-4614.

Jenq, C. B., Jenq, L. L. & Coggeshall, R. E. (1987) Numerical patterns of axon regeneration that follow sciatic nerve crush in the neonatal rat. *Exp. Neurol.* **95:** pp. 492-499.

Illnerova, H. & Vanecek, J. (1982) Complex control of the circadian rhythm in N-acetyltransferase activity in the rat pineal gland. In: *Vertebrate circadian systems.* Eds: J. Aschoff., S. Daan. & G, Groos. Springer-Verlag, Berlin. pp. 285.

Johnson, E. M., Taniuchi, M. & DiStefano, P. S. (1988) Expression and possible function of nerve growth factor receptors on Schwann cells. *Trends Neurosci.* **11:** pp. 299-304.

Johnston, R. N. & Wessells, N. K. (1980) Regulation of the elongating nerve fibre. In *Current topics in Dev. Biol.* Vol 16. Ed: R. K. Hunt. Academic, New York. pp. 165-206.

Kappers, J. A. (1979) Short history of pineal discovery and research. *Prog. in Brain Res.* **52:** pp. 3-22.

Karasek, M., Petterborg, L. J., King, T. S., Hansen, J. T., Reiter, R. J. (1983) Effect of superior cervical ganglionectomy on the ultrastructure of the pinealocyte in the cotton rat (*Sigmodon hispidus*). *Gen. Comp. Endocrinol.* **51:** pp. 131-137.

Karns, L. R., Ng, S. C. Freeman, J. A. & Fishman, M. C. (1987) Cloning of complimentary DNA for GAP-43, a neuronal growth-related protein. *Science* **236:** pp. 597-600.

Karsch, F. J., Bittman, E. L., Foster, D. L., Goodman, R. L., Legan, S. J. & Robinson, J. E. (1984) Neuroendocrine basis of seasonal reproduction. *Prog. in Horm. Res.* **40:** pp. 185-232.

Kater, S. B., Mattson, M. P., Cohan, C. & Connor, J. (1988) Calcium regulation of the neuronal growth cone. *Trends Neurosci* **11:** pp. 315-321.

Kater, S. B. & Letourneau, P. (1985) Biology of the nerve growth cone. *J. Neurosci. Res.***13:** pp. 124-127.

Katz, F., Ellis, L. & Pfenninger, K. (1985) Nerve growth cones isolated from fetal rat brain : Calcium-dependent protein phosphorylation. *J. Neurosci.* **5:** pp. 1402-1411.

Kauppila, A., Kivela, A., Pakarinen, A. & Vakkuri, O. (1987) Inverse seasonal relationship between melatonin and ovarian activity in humans in a region with a strong seasonal contrast in luminosity. *J. Clin. Endicronol. Metabol.* **65:** pp. 823-828.

Kebabian, J. W., Zatz, M., Romero, J. A. & Axelrod, J. (1975) Rapid changes in rat pineal beta-adrenergic receptor. Alterations in 1-(^{3}H) alprenolol binding and adenylate cyclase. *Proc. Natl. Acad. Sci.* USA **72:** pp. 3735-3739.

Kelly, R. & Grote, E. (1993) Protein targeting in the neuron. *Ann. Rev. of Neurosci.* **16:** pp. 95-127.

Kennaway, D. J., Firth, R. G., Phillipou, G., Matthews, C. D. & Seamark, E. F. (1977) A specific radioimmunoassay for melatonin in biological tissue and fluid and its validation by gas chromatography-mass spectrometry. *Endocrinology* **101:** pp. 119-124.

Kennaway, D. J., Gilmore, T. A. & Seamark, R. F. (1982) Effects of melatonin on serum prolactin and gonadotropin levels and the onset of seasonal estrous cyclicity in sheep. *Endocrinology.* **108:** pp. 1766-1772.

Kennaway, D. J., Dunstan, E. A., Gilmore, T. A., Seamark, R. F. (1984) Effects of pinealectomy, oestradiol and melatonin on plasma prolactin and LH secretion in ovariectomized sheep. *J. of Endocr.* **102:** pp. 199-207.

King, T. S. & Steinlechner, S. (1985) Pineal indolalkylamine synthesis and metabolism: Kinetic considerations. *Pineal Res. Revs.* **3:** pp. 69-113.

King, T. S., Steinlechner, S. & Reiter, R. J. (1984) Does maximal serotonin N-acetyltransferase activity necessarily reflect maximal melatonin production in the rat pineal gland? *Neuroscience Letters.* **48:** pp. 343-347.

King, T. S., Karasek, M., Steger, R. W., Hansen, J. T. & Reiter, R. J. (1983) Effect of acute or chronic unilateral superior cervical ganglionectomy on the adrenergic innervation of rat pineal gland. In: *Developing and Regenerating Vertebrate Nervous Systems.* Eds: P. W. Coates., R. R. Markwald. & A. E. Kenny., New York., Alan. R. Liss. Inc. pp. 223-230.

Kitay, J. I. & Altschule, D. (1954) *The Pineal Gland.* Cambridge. Mass: Harvard University Press.

Klein, D. C. (1985) Photoperiod regulation of the mammalian pineal gland. In: *Photoperiodism, melatonin and the pineal.* Pitman. London. pp. 38-51.

Klein, D. C. (1979) Circadian rhythms in the pineal gland. In: *Endocrine rhythms.* Ed: D. T. Kreiger. Raven, New York.

Klein, D. C. & Weller, J. L. (1970) Indole metabolism in the pineal gland: A circadian rhythm in N-acetyltransferase. *Science* **169:** pp. 1093-1099

Koenig, E. (1967) Synthetic innervations in the axon: IV. *In vitro* incorporation of ^{3}H precursors into axonal protein and RNA. *J. Neurochem.* **14:** pp. 437-446.

Kopin, I. J., Pare, C. M. B., Axelrod, J. & Weissbach, H. (1961) The fate of melatonin in animals. *J. Biol. Chem.* **236:** pp. 3072-3075.

Korf, H. W. & Moller, M. (1984) The innervation of the mammalian pineal gland with special reference to central pinealopetal projections. *Pin. Res. Rev.* **2:** pp. 41-86.

Kuchel, G. A , Sherman, R. L. & Zigmond, R. E. (1990) Recovery of function following unilateral denervation, but not unilateral decentralization, of the pineal gland as indicated by measurements of pineal melatonin content and urinary melatonin metabolites. *Neurosci.* **37:** pp. 413-420.

Kuchel, G. A. & Zigmond, R. E. (1991) Functional recovery and collateral neuronal sprouting examined in young and aged rats following a partial neural lesion. *Brain Res.* **540:** pp. 195-203

Kuffler, D. P. (1986) Isolated sattelite cells of a peripheral nerve direct the growth of regenerating frog axons. *J. Comp. Neurol.* **249:** pp. 57-64.

Kuffler, S. W. (1967) *Proc. Repr. Sci. London. Ser.* **168:** pp. 1-21.

Kuwada, J. Y. (1986) Cell recognition by neuronal growth cones in a simple vertebrate embryo. *Science.* **223:** pp. 740-746.

Labrie, F., Gauthier, M., Pelletier, G., Borgeat, P., Lemay, A. & Gouge, J. (1973) Role of microtubules in basal and stimulated release of growth hormone and prolactin in rat adenohypophysis. *Endocrinol.* **93 (4):** pp. 903-914.

Laduron, P. & Belpaire, F. (1968) Transport of noradrenaline and dopa-beta-hydroxylase in sympathetic nerves. *Life Sci.* **7:** pp. 1-7.

Landis, S. C. (1986) Neuronal growth cones. *Ann. Rev. Physiol.* **45:** pp. 567-580.

Lapwood, K. R. (1993) Effects of unilateral or bilateral superior cervical ganglionectomy (SCGX) on endogenous melatonin secretory capacity of sheep. *Proc. of Aust. & N.Z. Soc. Endocr.* Vol. 36. Abs. 19.

Larrson, L. I. (1989) Immunocytochemistry: Theory and Practise. CRC Press Inc. Florida, U.S.A.

Lasek, R. J., Garner, J. A. & Brady, S. T. (1984) Axonal transport of the cytoplasmic matrix. *J. Cell Biol.* **99:** pp. 212-221.

Lees, J. L. (1971) Some aspects of reproductive efficiency in sheep. *The Veterinary Record.* **88:** pp. 86-95.

Lerner, A. B., Case, J. D. & Takahashi, Y. (1960) Isolation of melatonin and 5-methoxyindole-3-acetic acid from bovine pineal glands. *J. Biol. Chem.* **235:** 1992-1996.

Letourneau, P. C. (1975) Cell-to-substratum adhesion and guidance of axonal elongation. *Dev. Biol.* **44:** pp. 92-101.

Letourneau, P. C. (1985) Axonal growth and guidance. In: *Molecular Bases of Neuronal Development.* Ed: G. M. Edelman. Wiley, New York. pp. 269-293.
Levi-Montalcini, R. (1987) The Nerve Growth Factor thirty-five years later. *Science.* **237:** pp. 1154-1162.
Levi-Montalcini, R. (1966) The nerve growth factor: its mode of action on sensory and sympathetic nerve cells. *Harvey Lect.* **60:** pp. 217-259.
Levi-Montalcini, R. (1976) The nerve growth factor: its role in growth, differentiation and function of the sympathetic adrenergic neuron. *Prog. Brain Res.* **45:** pp.235-258.
Levi-Montalcini, R. & Hamburger, V. (1951) *J. Exp. Biol.* **116:** pp. 321-362.
Levi-Montalcini, R. & Angeletti, P. U. (1968) Nerve growth factor. *Physiol. Rev.* **48:** pp. 534-569.
Levi-Montalcini, R. & Angeletti, P. U. (1963) Essential role of the nerve growth factor in the survival and maintenance of dissociated sensory and sympathetic embryonic nerve cells *in vitro. Dev. Biol.* **7:** pp. 653-659.
Levitt, M., Spector, S., Sjoerdsma, A. & Udenfriend, S. (1965) Elucidation of the rate-limiting step in norepinephrine biosynthesis in the perfused guinea pig heart. *J. Pharmacol. Exp. Ther.* **148:** pp. 1-8.
Lew, G. M. (1989) An immunocytochemical study of thyrotropin releasing hormone in the porcine, ovine and rodent pineal gland. *Histochemistry* **91:** pp. 43-46.
Lew, G. M., Lawson-Willey, A. (1987) An immunocytochemical study of somatostatin in the ovine, porcine and rodent pineal gland. *Histochemistry* **86:** pp. 591-593.
Li, Y., Jiang, D. H., Wang, M. L., Jiao, D. R. & Pang, S. F. (1989) Rhythms of serum melatonin in patients with spinal lesions at the cervical, thoracic or lumbar region. *Clin. Endocrinol.* **30:** pp. 47-56.
Lincoln, G. A. (1979) Photoperiodic control of seasonal breeding in rams: Participation in the cranial sympathetic nervous system. *Jour. of Endocr.* **82:** pp. 135-147.
Lincoln, G. A., Almeida, O. X. F., Klandorf, H. & Cunningham, R. A. (1982) *J. Endocr.* **92:** pp. 237-250.
Lincoln, G. A., Libre, E. A. & Merriam, G. R. (1989) Long-term reproductive cycles in rams after pinealectomy or superior cervical ganglionectomy. *J. of Repr. and Fert.* **85:** pp. 687-704.
Lincoln, G. A. & Almeida, O. F. X. (1982) Inhibition of reproduction in rams by long daylengths and the acute effect of superior cervical ganglionectomy. *Jour. of Repr. and Fert.* **66:** pp. 417-423.
Lincoln, G. A. & Ebling, F. J. P. (1985) Effect of constant release explants of melatonin on seasonal cycles in reproduction, prolactin secretion and moulting in rams. *J. of Repr. and Fert.* **73:** 241-253.
Lincoln, G. A. & Short, R. V. (1980) Seasonal breeding: Nature's contraceptive. *Rec. Prog. in Horm. Res.* **36:** pp. 1-52.
Lincoln, G. A., Peet, M. J. & Cunningham, R. A. (1977) Seasonal and circadian changes in the episodic release of follicle-stimulating hormone, luteinizing hormone and testosterone in rams exposed to artificial photoperiods. *J. of Endocr.* **72:** pp. 337-349.
Lindsay, D. R., Pelletier, J., Pisselet, C. & Courot, M. (1984) Changes in photoperiod and nutrition and their effects on testicular growth of rams. *J. of Repr. and Fert.* **71:** pp. 351-356.
Lingappa, J. R. & Zigmond, R. E. (1987) A histochemical study of the adrenergic innervation of the rat pineal gland: evidence for the overlap of the innervation from the two superior cervical ganglia and for sprouting following unilateral denervation. *Neuroscience.* **21:** pp. 893-901.
Loy, R. & Moore, R. J. (1977) Anomalous innervation of the hippocampal formation by peripheral sympathetic axons following mechanical injury. *Exp. Neurol.* **57:** pp. 645-650.
Lynch, H. J. (1971) Diurnal oscillations in pineal melatonin content. *Life Sci* **10:** 791-797.

Lynch, H. J., Ho, M. & Wurtman, R. J. (1973) Control of pineal indole biosynthesis by changes in sympathetic tone caused by factors other than environmental lighting. *Proc. Natl. Acad. Sci.* USA. **70:** pp. 1704-1707.
Maehlen, J. & Nja, A. (1982) The effects of electrical stimulation on sprouting after partial denervation of Guinea pig sympathetic ganglion cells. *J. Physiol.* **322:** pp. 151-166.
Marangos, P., J., Schmechel, D., Parma, A. M., Clark, A. L. & Goodwin, F. K. (1979a) Measurement of neuron-specific (NSE) isoenzymes of enolase in rat, monkey and human nervous tissue. *J. of Neuroch.* **33:** pp. 319-329.
Marangos, P. J., Schmechel, D., Zis, A. P. & Goodwin, F. K. (1979b) The existence and neurobiological significance of neuronal and glial forms of the glycolytic enzyme enolase. *J. of Neurochem* **14:** 563-579.
Marshall, F. H. A. (1937) On the change over in the oestrous cycle in animals after transference across the equator, with further observations on the incidence of the breeding seasons and the factors controlling breeding periodicity. *Proc. of the Royal Soc. of Lond. Series B. Bio. Sci.* **122:** pp. 413-428.
Mason, A. & Muller, K. J. (1982) Axon segments sprout at both ends: Tracking growth with fluorescent D-peptides. *Nature* **296:** pp. 655-657.
Mathew, T. C. & Miller, F. D. (1990) Increased expression of T alpha 1 alpha-tubulin mRNA during collateral and NGF-induced sprouting of sympathetic neurons. *Dev. Biol.* **141:** pp. 84-92.
Matsuura, T. & Sano, Y. (1983) Distribution of monoamine-containing nerve fibres in the pineal organ of untreated and sympathectomized dogs. *Cell & Tiss. Res.* **234:** pp. 519-531.
Matsuura, T. & Sano, Y. (1986) Characteristic patterns of monoaminergic nerve fibres in the pineal organ of the monkey *(Macaca fuscata). Cell & Tiss. Res.* **245:** pp. 453-456.
Mattson, M. P. Guthrie, P. B. & Kater, S. B. (1988) *J. Neurosci. Res.* **21:** pp. 447-464.
Matthews, C. D., Seamark, R. F. & Guerin, M. V. (1992) Plasma melatonin profiles of Romney Marsh sheep in natural photoperiod and in acutely extended darkness. *J. Repr. Fert.* **95:** pp. 869-875.
Matus, A. (1988) Microtubule-associated proteins: Their potential role in determining neuronal morphology. *Ann. Rev. Neurosci.* **11:** pp. 29-44.
Maxwell, C. A., Foldes, A., Downing, J. A., Scaramuzzi, R. J. & Carter, N. B. (1989a) Seasonal modification of ovine pineal function. I. Interaction of gonadal steroids and sympathetic innervation on *B*-adrenoceptors. *Neuroendo.* **49:** pp. 65-70.
Maxwell, C. A., Rintoul, A. J., Foldes, A., Downing, J. A., Scaramuzzi, R. J. & Carter, N, B. (1989b) Seasonal modification of ovine pineal function. II. Steroidal effects on melatonin and prolactin profiles. *Neuroendo.* **50:** pp. 274-279
McCord, C. P. & Allen, F. P. (1917) Evidence associating pineal function with alterations in pigmentation. *J. Exp. Zool.* **23:** pp. 207-224.
McGeer, E. G. & McGeer, P. L. (1966) Circadian rhythm in pineal tyrosine hydroxylase. *Science* **153:** pp. 73-74.
McNulty, J. A., Fox, L. A. & Silberman, S. (1993) Immunocytochemical demonstration of nerve growth factor (NGF) receptor in the pineal gland: effect of NGF on pinealocyte neurite formation. *Brain Research.* **610:** pp. 108-114.
McQuarrie, I. G., Grafstein, B. & Gershon, M. D. (1977) Axon regeneration in the rat sciatic nerve: Effect of a conditioning lesion and of dbcAMP. *Brain Res.* **132:** pp. 443-453.
McQuarrie, I. & Lasek, R. J. (1989) Transport of cytoskeletal elements from parent axons into regenerating daughter axons. *J. Neurosci.* **9:** pp. 436-446.
Medawar, P. B. (1945) Size, shape and age. In: *Essays on growth and form.* Eds: W. E. Le Gros., F. Clark & P. B. Medawar. Clarendon Press, Oxford. pp. 157-187.

Meiri, K. F., Willard, M. & Johnson, M. I. (1988) Distribution and phosphorylation of the growth associated protein GAP-43 in regenerating sympathetic neurons in culture. *J. Neurosci* **8:** pp. 2571-2581.
Meiri, K. F., Pfenninger, K. H. & Willard, M. (1986) Growth associated protein, GAP-43, a polypeptide that is induced when neurons extend axons, is a component of growth cones and corresponds to pp46 a major polypeptide of a subcellular fraction that is enriched in growth cones. *Proc. Natl. Acad. Sci.* USA. **83:** pp. 3537-3541.
Meiri, K. F. & Gordon-Weeks, P. R. (1990) The growth associated protein, GAP-43 is a component of the growth cone membrane skeleton. *J. Neurosci.* **10:** pp. 256-265.
Meller, K. (1987) Early structural changes in the axoplasmic cytoskeleton after axotomy studied by cryofixation. *Cell Tiss. Res.* **250:** pp. 663-672.
Merzenich, M. M. (1987) In: *The Neural and Molecular Bases of Learning.* Eds: J. P. Changeux & M. Konishi. Wiley, Chichester, England. pp. 337-358.
Mess, B. & Ruzsas, C. (1986) Relationship between suprachiasmatic nuclei and rhythmic activity of the pineal gland. *Adv. in Pineal Res.* **1:** pp. 149-158.
Milcu, I. & Nanu, L. (1979) Glanda pineala ca organ metabolic. *Editura Academiei* Republicu Socialiste Romania, Bucharest.
Mockett, B. G., Lapwood, K. R., Pack, R., J. & Carr, D. H. (1991) Innervation of the ovine pineal gland and the regulation of melatonin secretion: an electrophysiological and immunocytochemical study. *Adv. in Pineal Res.* Eds: A. Foldes & R. J. Reiter **6:** pp. 13-16.
Mockett, B. G. (1991) Studies on the innervation of the ovine pineal and the regulation of melatonin secretion. Massey University, Palmerston North, New Zealand: Thesis.
Moller, M., Nielsen, J. T. & van Veen, T. (1979) Effect of superior cervical ganglionectomy on monoamine content in the epithalamic area of the Mongolian gerbil (*Meriones unguiculatus*): a fluorescence histochemical study. *Cell and Tiss. Res.* **201**: pp. 1-9.
Moller, M., Reuss, S., Olcese, J., Stehle, J. & Vollrath, L. (1987b) Central neural control of pineal melatonin synthesis in the rat. *Experientia.* **43:** pp. 186-188.
Moller, M. & Korf, H. W. (1983a) The origin of central pinealopetal nerve fibres in the Mongolian gerbril as demonstrated by the retrograde transport of horseradish peroxidase. *Cell and Tiss. Res.* **230:** pp. 273-287.
Moller, M. & Korf, H. W. (1986) Tracing of pinealopetal neuronal projections by use of *in vivo* and *in vitro* retrograde horseradish peroxidse techniques. *Adv. in Pineal Res.* **1:** pp. 51-59.
Moore, R. J. (1974) Central regeneration and recovery of function: The problem of collateral reinnervation. In: *Plasticity and Recovery of Function in the Central Nervous System.* Eds: D. G. Stein., J. J. Rosen. & N. Butters. Academic Press, New York. pp. 111-128.
Moore, R. J. (1978) The innervation of the mammalian pineal gland. *Prog. Repr. Biol.* **4:** pp. 1-29.
Moore, R. J. & Bloom, F. E. (1979) Central catecholeamine neuron systems: Anatomy and physiology of the norepinephrine and epinephrine systems. *Ann. Rev. of Neurosci.* **2:** pp. 113-168.
Morgan, P. J., Williams, L. M., Lawson, W. & Riddoch, G. (1988a) Adrenergic and VIP stimulation of cyclic AMP accumulation in ovine pineals. *Brain Res.* **447:** pp. 279-286.
Morgan, P. J., Williams, L. M., Lawson, W. & Riddoch, G. (1988b) Stimulation of melatonin synthesis in ovine pineals *in vitro*. *J. of Neurochem.* **50:** pp. 75-81.
Morgan, P. J. & Williams, L. M. (1989) Central melatonin receptors: implications for a mode of action. *Experentia* **45:** pp. 955-965.
Morton, D. J. (1987) Production of methoxyindoles *in vitro* from methoxytryptophan by rat pineal gland. *J. of Pineal Res.* **4:** pp. 7-11.

Mosteller, F. & Rourke, R. E. K. (1973) *Sturdy statistics.* Addison-Wesley. Mass. U.S.A.
Muller, C. M. (1992) A role for glia in activity-dependent plasticity. *Int. Rev. of Neurobio.* Vol 34. pp. 215-281.
Murphy, K. M. M., Gould, R, G., Oster-Granite, M. L., Gearhart, J. D. & Snyder, S. H. (1983) *Science* **222:** pp. 1036-1038.
Nalbandov, A. V. (1976) The endocrinology of reproduction. In: *Reproductive Physiology of Mammals and Birds.* Ed: G. W. Salisbury. San Francisco. W. H. Freeman & Co Ltd. pp. 51-120.
Nelson, R. B., Linden, D. L., Hyman, C., Pfenninger, K. H. & Routtenberg, A. (1989) The two major phosphoproteins in growth cones are probably identical to two protein kinase C substrates correlated with persistence of long-term potentiation. *J. Neurosci.* **9:** pp. 381-389.
New Zealand Nautical Almanac (1992-1993) Marine Transport Division. Ministry of Transport, Wellington.
Nottebohn, F. (1985) *Ann. N. Y. Acad. Sci.* **457:** pp. 1046-1048.
Nowak, R. & Rodway, R. G. (1985) Effect of intravaginal implants of melatonin on the onset of ovarian activity in adult and prepubertal ewes. *J. of Repr. and Fert.* **74:** pp. 287-293.
Oestreicher, A. B. & Gispen, W. H. (1986) Comparison of the immunocytochemical distribution of the phosphoprotein B-50 in the cerebellum and hippocampus of immature and adult rat brain. *Brain Res.* **375:** pp. 267-279.
Oksche, A. (1965) Survey of the development and comparative morphology of the pineal organ. *Prog.in Brain Res.* **10:** pp. 3-29.
Oksche, A. (1984) Evolution of the pineal complex: Correlation of structure and function. *Opthalm. Res.* **16:** pp. 88-95.
Olsen, L. & Malmfors, T. (1970) *Acta. Physiol. Scanda. Suppl.* **348:** pp. 1-45.
Orkand, R. K., Nicholls, J. G. & Kuffler, S. W. (1966) *J. Neurophysiol.* **29:** pp. 788-806.
Orci, L., Like, A. A, Amherdt, B.,Blondel, Y., Kanazawa, E. B., Marliss, A. E., Lambert, C. B., Wollheim, B. & Renold, A. E. (1973) Monolayer cell culture of neonatal rat pancreas: an ultrastructural and biochemical study of functioning endocrine cells. *J. Ultrastrc. Res.* **43:** pp. 270-297.
Owen, D. J., Logan, A. & Robinson, P. P. (1989) A role for nerve growth factor in collateral re-innervation from sensory nerves in the guinea pig. *Brain Res.***476:** pp. 248-255.
Ozaki, Y., Lynch, H. J. (1976) Presence of melatonin in plasma and urine of pinealectomized rats. *Endocrin.* **99:** pp. 641-646.
Pangerl, B., Pangerl, A. & Reiter, R. J. (1990) Circadian variations of adrenergic receptors in the mammalian pineal gland: a review. *J. Neural. Trans.* **81:** pp. 17-27.
Panke, E. S., Rollag, M. D., Reiter, R. J., Panke, T. W. (1978) Pineal serotonin N-acetyltransferase activity and melatonin concentrations in prepubertal and adult Syrian hamsters exposed to short daily photoperiods. *Endocr. Res. Commun.* **5:** pp. 311-319.
Parfitt, A. G. & Klein, D. C. (1976) Sympathetic nerve endings in the pineal gland protect against acute stress-induced increase in N-acetyltransferase (EC 2.1.3.5) activity. *Endocrinology.* **99:** pp. 840-851.
Pellegrino de Iraldi, A., Zieher, L. M. & de Robertis, E. (1965) Ultrastructure and pharmacological studies of nerve endings in the pineal organ. In: *Structure and Function of the Epiphysis cerebri.* Vol 10. Eds: J. A. Kappers & J. P. Schade. Elsevier Publishing Company, Amsterdam. pp. 389-421.
Penny, R. (1982) Melatonin excretion in normal males and females: increase during puberty. *Metabolism* **31:** pp. 816-23.

Peschke, E., Schon, M., Tertsch, S., Perschke, D., & Peil, J. (1989) Morphometric investigations of the pineal gland after ganglionectomy and thyroidectomy under the aspect of circadian and seasonal variations. *J. Hirnforsch.* **30:** pp. 399-407.
Pevet, P. (1979) Secretory processes in the mammalian pinealocyte under natural and experimental conditions. *Prog. Brain Res.* **52:** pp. 149-159.
Pfenninger, K. H. & Johnson, M. P. (1983) Membrane biogenesis in the sprouting neuron. I. Selective transfer of newly synthesized phospholipid into the growing neurite. *J. Cell Biol.* **87:** pp. 1038-1042.
Pfenninger, K. H. & Maylie-Pfenninger, M. F. (1981a) Lectin labelling of sprouting neurons. I. Regional distribution of surface glycoconjugates. *J. Cell Biol.* **89:** pp. 536-546.
Pfeninnger, K. H. & Maylie-Pfeninnger, M. F. (1981b) Lectin labelling of sprouting neurons. II. Relative movement and appearance of glycoconjugates during plasmalemmal expansion. *J. Cell Biol.* **89:** pp. 547-559.
Pittendrigh, C. S. & Daan, S. (1976) A functional analysis of circadian pacemakers in nocturnal rodents. *J. Comp. Physiol. Psychol.* **106:** pp. 333-338.
Pixley, S. K. R., Nieto-Sampedro, M. & Cotman, C. (1987) *J. Neurosci. Res.* **18:** pp. 402-406.
Pomerat, C. M., Hendleman, W. J., Raiborn, C. W. & Massey, J. F. (1967) Dynamic activities of nervous tissue *in vitro*. In: *The Neuron*. Ed: H. Hyde. Elsevier, New York. pp. 119-178.
Poulton, A. L. (1988) The proposed use of melatonin in controlled sheep breeding. *Aust. J. Biol. Sci.* **41:** pp. 87-96.
Purves, D. (1975) Functional and structural changes in mammalian sympathetic neurones following interruption of their axons. *J. Physiol.* **252:** pp. 429-463.
Purves, D. & Voyvodic, J. T. (1987) Imaging mammalian nerve cells and their connections over time in living animals. *Trends Neuroscience* **10:** pp. 398-404.
Purves, D., Snider, W. D. & Voyvodic, J. T. (1988) Trophic regulation of nerve cell morphology and innervation in the autonomic nervous system. *Nature* **336:** pp. 123-128.
Quay, W. B. (1962c) The respiration of ependyma and pineal cells *in vitro* with diverse substrates and concentrations of inorganic ions. *Amer. Zooligist.* **2:** pp. 439.
Quay, W. B. (1964) Circadian and estrous rhythms in pineal melatonin and 5-hydroxyindole-3-acetic acid. *Proc. Soc. Exp. Biol. Med.* **115:** pp. 710-715.
Raine, C. S., Ghetti, B. & Shelanski, M.L. (1971) On the association between microtubules and mitichondria within axons. *Brain Res.***84:** pp. 389-393.
Raisman, G. (1969) Neural plasticity in the septal nuclei of the adult brain. *Brain Res.* **14:** pp. 25-48.
Ralph, C. F., Firth, B., Gern, W. A. & Owens, D. W. (1979) The pineal complex and thermoregulation. *Biol. Rev.* **54:** pp. 47-72.
Ramon y Cajal, S. (1928) *Degeneration and Regeneration of the Nervous System.* English translation and reprint: Hafner, New York. 1959.
Ramon y Cajal, S. (1890) A quelle epoque apparaissent les expensions des cellules nerveuses de la moelle epinere du poulet ? *Anat. Anz.* **5:** pp. 609-613.
Ravault, J. P., Cozzi, B., Fahrenkrug, J., Mikkelsen, D. & Moller, M. (1990) Presence of VIP- and PHI- containing nerve fibres in the pineal gland of ganglionectomized sheep. *Vth Colloquium of the European pineal Study Group.* Abstract #81.
Reiter, R. J. (1991) Encyclopedia of Human Biology. Vol. 6. Academic Press. USA.
Reiter, R. J (1988) Comparative aspects of pineal melatonin rhythms in mammals. *ISI Atlas of Science: Animal and Plant Sciences.* **1:** pp. 111-116.
Reiter, R. J., Joshi, B. N., Heinzeller, T. & Nurnberger, F. (1986) A simple 1- or 5-second light pulse at night inhibits hamster pineal melatonin. *Endocrinology* **118:** pp. 1906-1909.

Reiter, R. J., Vaughan, M. K., Vaughan, G. M., Sorrentimo, S. J. & Donofrio, R. J. (1975) The pineal gland as an organ of internal secretion. In: *Frontiers of Pineal Physiology*. Ed: M. D. Altschule. MIT Press, Cambridge, Mass.

Reiter, R. J. (1985) Action spectra, dose-response relationships, and temporal aspects of light's effects on the pineal gland. *Anns. N. Y. Acad. of Sci.* **453:** pp. 215-230.

Reiter, R. J., Rudeen, P. K., Banks, A. F. & Rollag, M. D. (1978) Acute effects of unilateral or bilateral superior cervical ganglionectomy on rat pineal N-acetyltransferase activity and melatonin content. *Experentia* **35/5:** pp. 691-692.

Reiter, R. J. (1980) The pineal gland and its hormones in the control of reproduction in mammals. *Endocr. Rev.* **1:** pp. 109-131.

Reiter, R. J., Rudeen, P. K., Banks, A. F. & Rollag, M. D. (1979c) Acute effects of unilateral or bilateral superior cervical ganglionectomy on rat pineal N-acetyltransferase activity and melatonin content. *Experentia.* **35:** pp. 691-692.

Reiter, R. J., Petterborg, L. J., Brainard, G. C., de los Santos, R.,. Guerra, J. C., Dinh, D. T. (1981) The photoperiod and melatonin in the control of the annual cycle of reproduction in hamsters. In: *Pineal Function.* Eds: C. D. Matthews & R. F. Seamark., Elsevier, North-Holland Biomedical Press. pp. 95-102

Reiter, R. J. (1977) In: *The Pineal.* Ed: D. F. Horrobin. Eden Press, Canada.

Relkin, R. (1976) *The Pineal.* Ed: T. W. Perret. Eden Press, Canada.

Reppert, S. M., Perlow, J., Tamarkin, L. & Klein, K. C. (1979) A diurnal rhythm in primate cerebrospinal fluid. *Endocr.* **104:** pp. 295-301.

Reuss, S. (1987) Electrical activity of the mammalian pineal gland. *Pineal Res. Revs.* **5:** pp. 153-189.

Reuss, S., Semm, P. & Vollrath, L. (1985) Changes in the electrical activity of the rat pineal gland following stimulation of the cervical sympathetic ganglia. *J. of the Auton. Nerv. Syst.* **12:** pp. 281-288.

Reuss, S., Semm, P. & Vollrath, L. (1984) Electrophysiological investigations on the central innervation of the rat and guinea-pig pineal gland. *J. of Neur. Trans.* **60:** pp. 31-43.

Rhind, S. M., Martin, G. B., McMillen, S. Tsonis, T. G. & McNeilly, A. S. (1989a) Effect of level of food intake of ewes on the secretion of LH and FSH and the pituitary response to gonadotrophin-releasing hormone. *J. of Endocr.* **120:** pp. 325-330.

Richardson, P. M., McGuinness, U. M. & Aguayo, A. J. (1980) Axons from CNS neurons regenerate into PNS grafts. *Nature* **296:** pp. 264-265.

Robinson, J. E. & Karsch, F. J. (1987) Photoperiodic history and a changing melatonin pattern can determine the endocrine response of the ewe to daylength. *J. of Repr. and Fert.* **80:** pp. 159-165.

Roche, J. F. & Dziuk, P. J. (1969) A technique for pinealectomy of the ewe. *Amer. J. of Vet. Res.* **30:** pp. 2031-2035.

Rollag, M. D. & Niswender, G. D. (1976) Radioimmunoassay of serum concentrations of melatonin in sheep exposed to different lighting regimes. *Endocrinology* **98:** pp. 482-490.

Rollag, M. D., Panke, E. S., Trakulrungsi, W. K., Trankulrungsi, C., Reiter, R. J. (1980) Quantification of daily melatonin synthesis in the hamster pineal gland. *Endocrinology* **106:** 231-235.

Romero, J. A. & Axelrod, J. (1974) Pineal *B*-adrenergic receptor: Diurnal variation in sensitivity. *Science.* **184:** pp. 1091-1092.

Romijn, H. J. (1975) The ultrastructure of rabbit pineal gland after sympathectomy, parasympathectomy, continuous illumination, and continuous darkness. *J. Neural Transm.* **36:** pp. 183-194.

Rowan, A. (1981) In : *Animals in research.* Ed : D. Sperlinger. John Wiley & Sons. N.Y. pp. 257-284.

Rudeen, P. K., Reiter, R. J. & Vaughan, M. K. (1975) Pineal serotonin-N-acetyltransferase in four mammalian species. *Neurosci Letters* **1:** pp. 225-228.

Rusak, B. & Zucker, I. (1979) Neural regulation of circadian rhythms. *Physiol. Rev.* **59:** pp. 449-526.
Sadlier, R. M. F. S. (1969) In: *The Ecology of Reproduction in Wild and Domestic Mammals.* Methuen, London.
Sanford, L. M., Palmer, W. M. & Howland, B. E. (1977) Changes in the profiles of serum LH, FSH and testosterone, and in mating performance and ejaculate volume in the ram during the ovine breeding season. *J. of Anim. Sci.* **45:** pp. 1382-1391.
Schapiro, S. & Salas, M. (1971) Effects of age, light and sympathetic innervation on electrical activity of the rat pineal gland. *Brain Res.* **28:** pp. 47-55.
Scheibel, A.B. & Tomiyasu, U. (1978) Dendritic sprouting in Alzheimer's pre-senile dementia. *Exp. Neurology.* **60:** pp. 1-8.
Scheving, L. E., Halberg, F. & Ehret, C. F. (1987) In: *Chronobiotechnology and Chronobiological Engineering.* Martinus Nijhoff. The Netherlands.
Schreyer, D. J. & Skene, F. H. P. (1993) Injury-association induction of GAP-43 expression displays axon branch specificity in rat dorsal root ganglion neurons. *J. of Neurobio.* **24 (7):** pp. 959-970.
Schroder, H. (1986) Neuropeptide Y (NPY)-like immunoreactivity in peripheral and central nerve fibres of the Golden hamster (*Mesocricetus auratus*) with special aspect to pineal gland innervation. *Histochemistry.* **94:** pp. 309-314.
Schroder, H., Bendig, A., Dahl, D., Groeschel-Stewart, U., & Vollrath, L. (1990) Neuronal markers in the rodent pineal gland - an immunohistochemical investigation. *Histochem.* **94:** pp. 309-314.
Scott, I. C., Heath, D. & Hawker, H. (1992) Photoperiod and melatonin secretion in ewes. *Proc. N.Z. Soc. Anim. Prod.*
Shaw, P. F., Kennaway, D. J. & Seamark, R. F. (1989) Evidence of high concentrations of melatonin in lateral ventricular cerebrospinal fluid of sheep. *J. of Pineal. Res.* **6:** pp. 201-208.
Shaw, P. F., Kennaway, D. J. & Seamark, R. F. (1988) Effects of prior exposure to prolonged continuous light on the pattern of melatonin secretion in sheep held under continuous darkness. *J. of Pineal. Res.* **5:** pp. 469-477.
Sheikheldin, M. A., Howland, B. A & Palmer, W. M. (1992) Seasonal profiles of melatonin in adult rams. *J. Pineal Res.* **12:** pp. 58-63.
Sherline, P., Lee, Y. C. & Jacobs, L. C. (1977) Binding of microtubules to pituitary secretory granules and secretory granule membranes. *J. Cell Biol.* **72:** pp. 380-389.
Shiino, M. & Rennels, E. G. (1974) Paracrystalline aggregates of microtubules in the anterior pituitary cells of the chinchilla. *Amer. J. of Anat 139:* pp. 135-140.
Shiotani, Y., Yamano, M., Shiosaka, S., Emson, P. C., Hillyard, C. J., Girgis, S. & MacIntyre, I. (1986) Distribution and origins of substance P (SP)-, calcitonin gene-related peptide (CGRP)-, vasoactive intestinal peptide (VIP)- and neuropeptide Y (NPY)-containing nerve fibres in the pineal gland of gerbrils. *Neuroscience Letters.* **70:** pp. 187-192.
Sholl, D. A. (1954) Regularities in growth curves, including rhythms and allometry. In: *Dynamics of growth processes.* Eds: E. J. Boell. Princeton University Press. pp. 224-241.
Singer, W. (1987) In: *The Neural and Molecular Base of Learning.* Eds: J. P. Changeux & M. Konishi. Wiley, Chichester, England. pp. 301-335.
Skene, J. H. P., Jacobson, R. D., Snipes, G. J., McGuire, C. B., Norden, J. J. & Freeman, J. A. (1986) A protein induced during nerve growth (GAP-43) is a major component of growth-cone membranes. *Science* **233:** pp. 783-786.
Skene, J. H. P. & Willard, M. (1981a) Axonally transported proteins associated with growth in rabbit central and peripheral nervous system. *J. Cell Biol.* **89:** pp. 96-103.
Skene, J. H. P. & Willard, M. (1981b) Electrophoretic analysis of axonally transported proteins in toad retinal ganglion cells. *J. Neurochem.* **37:** pp. 79-87.

Skene, J. H. P. (1989) Axonal growth-associated proteins. *Ann. Rev. Neurosci* **12:** pp. 127-156.
Smith, J. F. (1982) Principles of reproduction. In: *Sheep Production: Breeding and Reproduction.* Vol 1. Eds: G. A. Wickham & M. F. McDonald. Auckland: Ray Richards. pp. 211-237.
Smith, P. G. & Reddy, H. (1990) Reorganisation of cranial sympathetic pathways following neonatal ganglionectomy in the rat. *J.of Comp. Neurology.* **301:** pp. 490-500.
Snedecor, G. W. & Cochran, W. G. (1967) In: *Statistical Methods.* Ames, The Iowa State University Press.
Snider, W. D. (1988) Nerve growth factor enhances dendritic arborization of sympathetic ganglia cells in developing mammals. *Neurosci.* **8(7):** pp. 2628-2634.
So, K. F. & Aguayo, A. J. (1985) *Brain Res.* **328:** pp. 349-354.
Sperry, R. W. (1965) Mechanisms of neuronal maturation. In: *Handbook of experimental neurology.* Ed: S. S. Stevens. Wiley. New York.
Spiegel, A. M. (1989) Receptor-effector coupling by G-proteins: implications for endocrinology. *Tr.Endocr. Met.* **1:** pp. 72-79.
Spinelli, W., Sonnenfeld, K. H. & Ishii, D. N. (1982) *Cancer Res.* **42:** pp. 5067-5073.
Stein, D. G., Rosen, J. J. & Butters, N. (1974) *Plasticity and recovery of function in the central nervous system.* Academic press. New York.
Stenevi, U. & Bjorklund, A. (1978) Growth of vascular sympathetic axons into the hippocampus after lesions of the septohippocampal pathway: A pitfall in brain lesion studies. *Neurosci. Letters.* **7:** pp. 219-224.
Stone, G. C. & Hammerschlag, R. (1987) Molecular mechanisms involved in sorting of fast-transported proteins. In : *Axonal transport.* Eds : R. S. Smith & N. Busby. Alan. R. Liss. Inc. N.Y. pp. 15-36.
Stonehouse, B. (1981) Introduction: Mammals and their reproduction. In: *Environmental Factors in Mammal Reproduction.* Eds: D. Gilmore & B. Cook, London: Macmillan Publishers Ltd. pp. 1-10
Sugden, D. (1989) Melatonin biosynthesis in the mammalian pineal gland. *Experientia* **45:** pp. 922-932.
Sutherland, S. (1978) In: *Nerves and Nerve Injury.* 2nd ed. Edinburgh. Chuchill Livingstone.
Tamarkin, L., Baird, C. J. & Almeida, O. F. X. (1985) Melatonin; a co-ordinating signal for mammalian reproduction. *Science* **227:** pp. 714-720.
Tamarkin, L., Westrom, W. K., Hammil, A. I., Goldman, B. D. (1976) Effect of melatonin on the reproductive system of male and female Syrian hamsters: A diurnal rhythm in sensitivity to melatonin. *Endocrinology* **99:** pp. 1534-1539.
Taniuchi, M., Clark, H. B. & Johnson, E. M. Jr. (1988) Expression of nerve growth factor receptors by Schwann cells of axotomized peripheral nerves: Ultrastructural location, suppression by axonal contact, and binding properties. *J. Neurosci.* **8:** pp. 664-681.
Tello, F. (1911) *Trab. Lab. Invest. Biol.* **9:** pp. 123-159.
Theron, J. J., Biagio, R., Meyer, A. C. & Boekkooi, S. (1979) Microfilaments, the smooth endoplasmic reticulum and synaptic ribbon fields in the pinealocytes of the baboon *Papio ursinus. Amer. J. of Anat.* **154:** pp. 151-162.
Tillet, Y. (1987) Immunocytochemical localization of serotonin-containing neurons in the myelencephalon, brainstem and diencephalon of the sheep. *Neuroscience.* **23:** pp. 501-527.
Tosney, K. W. & Wessels, N. K. (1983) Neuronal motility: The ultrastructure of veils and microspikes correlates with their motile activities. *J. Cell Sci.* **61:** pp. 389-411.
Tucker, R. P. (1990) The roles of microtubule associated proteins in brain morphogenesis: a review. *Brain Res. Rev.* **15:** pp. 101-120.

Tulley, D. & Burfening, P. J. (1983) Libido and scrotal circumference of rams as effected by season of the year and altered photoperiod. *Theriogenology.* **20:** pp. 435-448.
Van Hoof, C. O. M., DeGraan, P. N. E. Oestreicher, A. B. & Gispen, W. H. (1989) Muscarinic receptor activation stimulates B50/GAP-43 phosphorylation in isolated nerve growth cones. *J. Neurosci.* **9:** pp. 3753-3760.
Vaughan, G. M., Meyer, G. G. & Reiter, R. J. (1978) Evidence for a pineal gonad relationship in the human. *Prog. Repr. Biol.* **4:** pp. 191-223.
Vidal-Sanz, M., Bray, G. M., Villegas-Perez, M. P., Thanos, S. & Aguayo, A. J. (1987) *J. Neurosci.***7:** pp. 2894-2909.
Vollrath, L. (1981) In: *The Pineal Organ.* Berlin: Springer-Verlag.
Warchol, J. B., Herbert, D. C. & Rennels, E. G. (1974) An improved fixation procedure for microtubules and microfilaments in cells of the anterior pituitary gland. *Am. J. Anat.* **141:** pp. 427-432.
Webley, G. E., Mehl, H. & Willey, K. P. (1985) Validation of a sensitive direct assay for melatonin for investigation of circadian rhythms in different species. (1985) *J. Endocrinol* **106:** pp. 387-394.
Weisenberg, R. C. & Allen, R. D (1984) ATP dependent motility *in vitro* by isolated calf brain microtubule proteins. *J. Cell. Biol.* **99:** pp. 239
Weskamp, G. & Otten, U. (1987) An enzyme-linked immunoassay for nerve growth factor (NFG): a tool for studying regulatory mechanisms involved in NGF production in brain and peripheral tissues. *J. Neurochem.* **48:** pp. 1779-1786.
Whelar, G. H. T., Weller, J. L. & Klein, D. C. (1979) Taurine: Stimulation of pineal N-acetyltransferase activity and melatonin production via a beta-adrenergic mechanism. *Brain Res.* **166:** pp. 65-74.
Widmer, F. & Caroni, P. (1993) Phosphorylation-site mutagenesis of the growth-associated protein GAP-43 modulates its effects on cell spreading and morphology. *J. of Cell Biol* **120 (2) :** pp. 503-512.
Wiklund, L. (1974) Development of serotonin-containing cells and the sympathetic innervation of the habenular region in the rat brain. *Cell and Tiss. Res.* **155:** 231-243.
Wilkinson, M., Arendt, J., Bradtke, J., deZiegler, D. (1977) Determination of a dark-induced increase of pineal N-acetyltransferase and simultaneous radioimmunoassay of melatonin in pineal, serum and pituitary tissue of the male rat. *J. Endocrinol.* **72:** pp. 243-249.
Willard, M., Meiri, K. F. & Johnson, M. I. (1987) The role of GAP-43 in axon growth. In: *Axonal transport, Neurology and Neurobiology.* Vol 25. Eds: R. S. Smith & M. S. Bisby. Alan. R. Liss Inc. pp. 407-421.
Williams, L. M., Morgan, P. J., Pelletier, G., Riddoch, G. I., Lawson, W. & Davidson, G. R. (1989) Neuropeptide Y (NPY) innervation of the ovine pineal gland. *J. of Pineal. Res.* **7:** pp. 345-353.
Wilson, B. W. (1978) The application of mass spectrometry to the study of the pineal gland. *J. Neural. Transm.* **13:** pp. 279-284.
Withyachumnarnkul, B., Reiter, R. J., Lerchl, A., Nonaka, K. O. & Stokkan, K. A. (1991) Evidence that interferon-gamma alters pineal metabolism both indirectly via sympathetic nerves and directly on the pinealocytes. *Int. J. Biochem.* **33:** pp. 1397-1401.
Wong, J. & Oblinger, M. (1990) A comparison of peripheral and central axotomy effects on neurofilament and tubulin gene expression in rat dorsal root ganglion neurons. *J. Neurosci.* **10:** pp. 2215-2223.
Woodfill, C. J. I., Robinson, J. E, Malpaux, B. & Karsch, F. J. (1991) Synchronization of the circannual reproductive rhythm of the ewe by discrete photoperiodic signals. *Biol. of Reprod.* **45:** pp. 110-121.

Wu, W., Mathew, T. C. & Miller, F. D. (1993) Evidence that the loss of homeostatic signals induces regeneration-associated alterations in neuronal gene expression. *Dev. Biol.* **158:** pp. 456-466.
Wurtman, R. J., Axelrod, J. & Kelly, D. E. (1968) In: *The Pineal.* New York: Academic Press. Inc.
Wurtman, R. J., Axelrod, J. & Chu, E. W. (1963) Melatonin a pineal substance: effect on rat ovary. *Sci. Wash. DC.* **141:** pp. 277-278.
Wuttke, W. A. (1990) *J. Neurophysiol.* **63:** pp. 1089-1097.
Yamada, K. M., Spooner, B. S. & Wessels, N. K. (1971) Ultrastructure and function of growth cones and axons of cultured nerve cells. *J. Cell Biol.* **49:** pp. 614-635.
Yan, Q., Snider, W. D., Pinzone, J. J. & Johnson, E. M. (1988) Retrograde transport of nerve growth factor in motorneurons of developing rats: Assessment of potential neurotrophic effects. *Neuron* **1:** pp. 335-343.
Yanker, B., Benowitz, L. Villa-Komaroff, L. & Neve, R. (1990) Transfection of PC12 cells with the human GAP-43 gene: effects on neurite outgrowth and regeneration. *Mol. Brain Res.* 7: pp. 39-44.
Yeates, N. T. M. (1947) Influence of variation in length of day upon the breeding season in sheep. *Nature.* **160:** pp. 429-430.
Yeates, N. T. M. (1949) The breeding season of the sheep with particular reference to its modification by artificial means using light. *J. of Agr. Sc.* **39:** pp. 1-43.
Zatz, M., Kebabian, J. W., Romero, J. A., Lefkowitz, R. J. & Axelrod, J. (1976) Pineal adrenergic receptor: correlation of binding of ^{3}H-alprenolol with stimulation of adenylate cyclase. *J. Pharmac. Exp. Ther.* **196:** pp. 714-722.
Zigmond, R. E., Baldwin, C. & Bowers, C. W. (1981) Rapid recovery of function after partial denervation of the rat pineal gland suggests a novel mechanism for neural plasticity. *Proc. Natl. Acad. Sci.* USA. **78:** pp. 3959-3963.
Zisapel, N., Levi, M. & Gozes, I. (1980) Tubulin : An integral protein of mammalian synaptic vesicle membranes. *J. of Neurochem.* **34 (1):** pp. 26-32.
Zomzeley-Neurath, C. & Walker, W. (1980) Nervous system specific proteins. In: *Proteins of the nervous system.* Eds: R. A. Bradshaw & D. M. Schneider. Raven Press N.Y. pp. 1-48.