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.