AN INVESTIGATION INTO CHOLINERGIC INTERACTIONS IN THE RAT PINEAL GLAND

THESIS

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by

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Oh, the depth of the riches

of the wisdom

and knowledge of God!

How unsearchable

his judgements,

and his paths

beyond tracing out!

"Who has known

the mind of the Lord?

Or who has been

his counsellor?"

"Who has ever given to God,

that God should repay him?"

For from him and through him

and to him are all things

To him be the glory for ever!

Amen

Romans 11: 33-36

ABSTRACT

The mammalian pineal gland is mainly innervated by the sympathetic nervous system which modulates the activity of indole pathway enzymes and the secretion of pineal hormones. Recently researchers have demonstrated and characterized the presence of muscarinic cholinergic receptors in the pineal gland. However the role of these receptors remains unclear. In an attempt to investigate the role of cholinergic receptors in the pineal gland, a number of studies were carried out on the various steps in the indole metabolic pathway, using various agents which act on the cholinergic system.

Investigations using pineal organ cultures showed that stimulation of these muscarinic cholinergic receptor sites with a parasympathomimetic agent, a rise in levels of aHT occurred without a concomitant increase in aMT levels. Further organ culture experiments using the cholinergic agonist acetylcholine and anticholinesterase agent physostigmine, produced a similar rise in aHT without altering aMT levels. This acetylcholine-induced rise in aHT levels were not altered by the ganglion blocking agent hexamethonium whilst the antimuscarinic agent atropine prevented the acetylcholine-induced rise in aHT levels. These findings suggest that cholinergic agents may play a role in regulating indoleamine synthesis in the pineal gland.

Cyclic-AMP assay studies showed that acetylcholine increases pineal cAMP levels significantly and does not influence the isoproterenol-induced cAMP rise in the pineal gland. The cAMP regulator cAMP-phosphodiesterase (cAMP-PDE) was found to increase significantly in the presence of the anticholinesterase agent physostigmine. NAT enzyme studies revealed that physostigmine does not affect NAT enzyme levels significantly and HIOMT studies showed that this agent does not inhibit HIOMT activity. The mechanism by which acetylcholine and physostigmine are able to cause a increase in aHT and not aMT levels needs to be researched further.

Acetylcholinesterase enzyme assay studies revealed that the AChE enzyme undergoes a diurnal rhythm in the pineal gland with activity being higher during the day and lower at night.

Investigations using the drug reserpine showed that this rhythm is not under the control of the sympathetic nervous system. Further research needs to be done however, in determining whether or not this enzyme is present in the pineal gland to regulate the levels of acetylcholine interacting with muscarinic receptors in the gland, or for some other reason.

Choline acetyltransferase studies demonstrate the presence of the enzyme in the rat brain cerebral cortex as well as showing that melatonin increases ChAT enzyme activity in this tissue. This suggests that melatonin plays a role in cholinergic transmission there. ChAT activity could not be measured in the pineal gland however. Muscarinic receptor binding studies also carried out on rat brain cerebral cortex show that melatonin enhances cholinergic receptor affinity and receptor number in this tissue.

In summary, data presented herein concur with proposals that:

i) the cholinergic system affects the indole metabolic pathway by causing a rise in aHT but not aMT levels.

ii) cholinergic agonist acetylcholine causes cAMP levels to rise with a concomitant increase in cAMP-PDE levels.

iii) the enzyme acetylcholinesterase undergoes a diurnal rhythm in the pineal gland which is not under the control of the sympathetic nervous system.

iv) the activity of the enzyme choline acetyltransferase is increased by melatonin in the rat brain cerebral cortex suggesting that melatonin facilitates cholinergic transmission in this tissue.

v) melatonin enhances cholinergic receptor affinity and receptor number in the cerebral cortex of rat brain.

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α	Alpha
Å	Angstrom
AChE	Acetylcholinesterase
ACh	Acetylcholine
aHT	N-acetylserotonin
ANOVA	Analysis of Variance
ANS	Autonomic Nervous System
5'-AMP	Adenosine monophosphate
aMT	Melatonin
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
Bq	Bequerel
b.wt	Body weight
°C	Celsius
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monosphate
[^{14}C]	Carbon-14 radiolabel
cGMP	Cyclic guanosine monophosphate
ChAT	Choline acetyltransferase
ChE	Cholinesterase
Ci	Curie
CNS	Central Nervous System
cpm	Counts per minute
CSF	Cerebrospinal fluid
DAG	Diacylglycerol
dpm	Disintegrations per minute
DSIP	Delta sleep inducing peptide
EGTA	Ethyleneglycol-bis-(ß-aminoethylether)-N,N,N',N'-tetraacetic acid
fmol	Femtomole
γ	Gamma
g	Gram
GABA	Gamma amino butyric acid
G-protein	Guanine nucleotide binding protein
GTP	Guanine triphosphate
h	Hour(s)
[^{3}H]	Tritium radiolabel
HA	5-Hydroxyindole acetic acid
HCl	Hydrochloric acid
HIOMT	Hydroxyindole O-methyltransferase
HL	5-Hydroxytryptophol
HT	Serotonin
K_d	Equilibrium constant
K_m	Michaelis-Menten constant
K^+	Potassium ion
kg	Kilogram
l	Litre
LL	Constant Light
LD 12:12	Light/dark cycle
m	Milli

M	Molar
MA	5-Methoxyindole acetic acid
MAO	Monoamine oxidase
mCi	Millicurie
mg	Milligram
min	Minute(s)
ML	5-Methoxytryptophol
ml	Millilitre
mm	Millimetre
mM	Millimolar
mol	Mole
MT	5-Methoxytryptamine
n	Nano
NA	Noradrenaline
NaOH	Sodium Hydroxide
NAT	N-Acetyltransferase
nm	Nanometre
nM	Nanomolar
nmol	Nanomole
NPY	Neuropeptide Y
NS	Not Significant
p	Pico
PBS	Phosphate Buffered Saline
PDE	Phosphodiesterase
PHI	Peptide histidine isoleucine
PI	Phosphoinositol
pmol	Picomole
RIA	Radioimmunoassay
SAD	Seasonal affective disorder
SCG	Superior cervical ganglion
SCGX	Superior cervical ganglionectomy
SCN	Suprachiasmatic nuclei
sec	Seconds
S.E.M.	Standard error of the mean
TCA	Tricholoroacetic acid
TLC	Thin layer chromatography
UV	Ultra-violet
w.r.t.	With respect to

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Pineal research has proceeded with ever increasing speed and investigators have had the advantage of being able to draw upon a substantial body of information. Researchers representing a variety of different disciplines have become involved in pineal studies, and this field has developed into an acknowledged area of the neurosciences. Although the existence of the mammalian pineal gland has been recognized for many centuries it was only thirty years ago that it was considered to be more than a "vestigial organ of little functional importance". The expansion of neuroscientific research has witnessed the pineal gland attain the status of one of the most important participants in the regulation of various physiological and behavioral processes.

The mammalian pineal gland enjoys a large measure of protection by virtue of its location and yet it is directly exposed to the vascular system, providing an ideal environment in which to monitor homeostasis. The pineal also exhibits other unique features such as a) receiving both peripheral and central innervations, b) being part of the brain, yet existing outside the blood brain barrier, and c) especially exhibiting an astonishing and often bewildering species-directed variation in their innervations and biochemical properties (See Korf and Moller, 1984; and Ebadi and Govitrapong, 1986a for reviews and references).

The pineal gland is also considered to be an active neuroendocrine transducer (Wurtman and Axelrod, 1965) converting neural input, namely a neurotransmitter released at a synapse, to a hormonal output ie. methoxyindoles of melatonin and polypeptides. In addition it appears that the pineal may itself be subjected to other hormonal signals suggesting endocrine-endocrine and endocrine-neural transduction mechanisms (Cardinali and Vacas, 1978). It was only subsequent to the isolation and characterization of melatonin - a secretion of the pineal gland, by Lerner and his

coworkers in 1958 (Lerner *et al.*, 1958) that a lot of the functional significance of this enigmatic gland has begun to be unravelled.

The cyclic nature of the gland's activity earned it the title of a 'biological clock', a switching mechanism regulating the activity of the sex glands in response to the changing seasons and exposure to light and dark. The pineal gland in all mammals produces melatonin in a rhythmic manner with peak levels occurring at night irrespective of whether the animal is nocturnal or diurnal in its behaviour pattern. The major functions of pineal melatonin appears to synchronize the seasonal and circadian rhythms of a variety of physiological events (Armstrong, 1989). These include effects on reproduction, coat growth, temperature regulation and locomotor activity in species where seasonal cycles depend on photoperiod (Reiter, 1980; Tamarkin *et al.*, 1985; Armstrong, 1989).

The pineal gland has inspired novel avenues of research due to its unique nature and its discrete association with the sympathetic nervous system. The idea that the pineal might respond when challenged by certain agents lent a new tool for biochemists and pharmacologists involved with this type of study and extensive investigation into the effects of administered substances on the pineal gland has been performed. Increased emphasis has been placed on the pineal gland in recent years, being chemically associated with the functions of other organs, and is perhaps appropriately labelled as the 'last great frontier' in the study of physiology.

The rat pineal gland was used in this study for a variety of reasons. It is situated outside the blood brain barrier and is thus accessible to drugs administered peripherally. It also has the ability to maintain its metabolic functions in organ culture, which enables investigations on the effects of various drugs on the gland. Pineal metabolites such as melatonin and its precursor N-acetylserotonin, as well as associated enzymes can be conveniently assayed using rat pineal gland. In this way important information on the effects of various cholinergic agents on the internal metabolism of the pinealocyte may be derived.

The pineal has a modulatory effect, changing the timing, amplitude or intensity of the response rather than a primary effect of initiating such responses. It is no longer a matter of what organs or organ systems the pineal and melatonin influences but rather it is a question of whether any escape its influence (Reiter, 1988).

Govitrapong *et al.*, (1989b) have recently identified and characterized the presence of muscarinic cholinergic receptors in the pineal glands of cow and swamp buffalo by using quinuclidinyl benzilate binding studies. The pineal gland is innervated almost exclusively by sympathetic nerves from the superior cervical ganglia and although recently muscarinic receptors have been found in the pineal glands, their function still remains a mystery. This study serves to explore the use of the rat pineal gland as a model for the investigation into cholinergic interactions in the pineal gland as well as to determine the role that these muscarinic cholinergic receptors play in this gland. A number of cholinergic agents will be used and their effect on pineal function and metabolic pathways examined.

1.2 History of Pineal Research

The discovery of the human pineal has generally been ascribed to Herophilos (325 to 280 BC), the "father of anatomy". During the medieval period around 400 BC when Galen and Hippocrates were the medical authorities, Galen coined the term "konareion" for the pineal gland because of its 'pine-cone'-like shape in man (refer to Ariens-Kappers, 1981 for references). The Renaissance heralded a more personal approach to the study of the pineal. Berangario de Carpi (1470-1530) was the first to examine the pineal gland of man more carefully from which Andreas Vesalius (1514-1564) in the famous paper *'De Humani Corporis Fabrica Libri Septem'* described the topography and consistency of the gland but little in terms of a suggested function was proposed.

René Descartes (1596-1650) is widely cited as stating that the pineal gland is the seat of the soul. At this time the problem of the localization of the soul was very much in the mind of both philosophers and scientists. Descartes reasoned that the soul, in principle, cannot be localized in any

precise region of the body because it is related with all of its parts, according to the doctrine of Aristotle. He however suggested that the soul would be more expressed in the pineal, the only unpaired part of the brain. According to Descartes, an optical image can pass from the retina to the pineal thereby inducing the pineal to transmit its spirits by moving in different directions to different motor nerves. The seat of the soul and ancient theories on the *spiritus* haunted neuroscience research for more than twenty centuries before new concepts cleaved a path for more useful research.

Interest in the pineal was revived in the latter nineteenth century after the discovery of endocrine glands by Claude Bernard (1818-1878) and Brown-Sequard (1817-1894). The medical profession continued to believe that the pineal was of no functional significance, and that its only medical value was to the neuro-radiologists who made use of the radio opaqueness of the acervuli as a welcome reference point (refer to Ariens-Kappers, 1981 for references). However the mammalian pineal gland was being considered as a serious candidate for hormone production as interest by physiologists and clinicians became more intense.

Heubner (1898) first described a boy suffering from a pinealoma and showing signs of precocious puberty. According to Marburg, the human pineal is an endocrine gland which in youth exerts an inhibitory effect on hypothalamic function. Berblinger (1920) and Engel (1936) were convinced that the pineal gland inhibits gonadal development being of the opinion that its extract was antigonadotropic. Bargman (1943) stressed the necessity of investigations on the influence of light on the pineal and Kitay and Altschule (1954) edited a book on pineal physiology and suggested future lines of research in this field.

1.3 Anatomy

1.3.1 Location and Characteristics

The pineal gland or the *epiphysis cerebri* is located between the cerebral cortex and the cerebellum. In the rat brain it is situated between the two cerebral hemispheres just forward of the cerebellum

(**Figure 1.1a** and **1.1b**). It is covered by the confluence of the superior sagittal and transverse sinuses. It is connected to the commissural region of the brain by a pineal stalk (Kappers, 1965). Topographically the stalk can be divided into three parts namely a proximal part, midpart and dorsal part. The proximal part lies between the habenulae and caudal commissures. The midpart is extremely thin and the distal part is the largest of the three structures, situated between the rostral and caudal collicles (Kappers, 1965). The gland consists of two parenchymal cell types, pinealocytes and intestinal cells (Wartenberg and Gusek, 1965) both of neuroectodermal origin. The pinealocytes are the neurosecretory cells of the gland.

The pineal gland is considered to lie outside the blood brain barrier, with its own arterial blood supply of up to four branches of the posterior cerebral artery (Hodde, 1979) and posterior choroidal artery (Gladstone and Wakely, 1940). Drainage consists of some sixteen veins into the great cerebral vein which leads to the systematic circulation via the *confluens sinuum* (Hodde, 1979). The pineal is thus sensitive to stimuli from the periphery, including hormones and those drugs not entering the brain (Axelrod, 1974; Tamarkin *et al.*, 1985). A profuse blood supply reaches the pineal gland at a rate of 4ml/min/g surpassed only by the kidney (Reiter, 1981). This blood supply is greater at night than during the day (Quay, 1972; Rollag *et al.*, 1977).

1.3.2 Pineal Innervation

From initial detailed studies of rat pineal gland innervation, which were carried out by Kappers (1960, 1965), it appears that innervation of the pineal gland is critical to its activity and function. Datta and King (1980) discovered sympathetic innervation destruction of the gland, destroying the ability of the gland to synthesize pineal hormones and the pineal is rendered non-functional by all currently measured parameters (Reiter, 1989). Pinealopetal orthosympathetic and pinealopetal parasympathetic fibres innervate the pineal gland. The function of the parasympathetic fibres is unclear, reaching the pineal gland by way of the facial and greater superficial petrosal nerves. Although the existence of central pinealopetal fibres which enter the gland via its stalk (Korf and Moller, 1984) has been well defined, their function also remains an enigma (Reiter, 1989). The

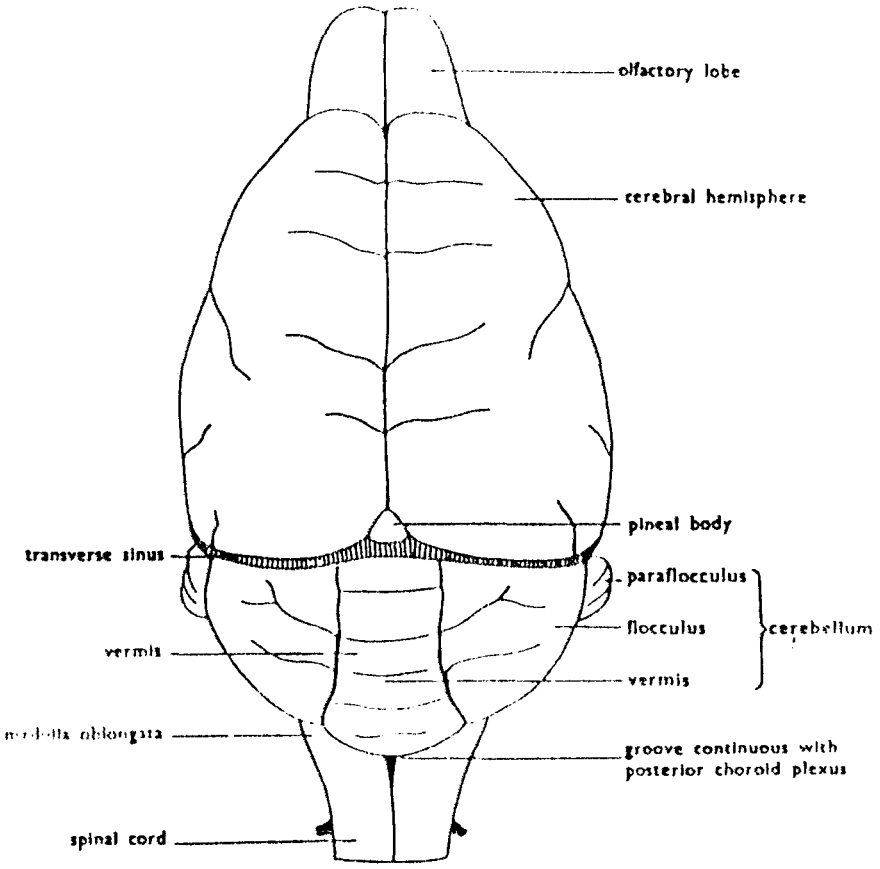


Figure 1.1a Dorsal view of the rat brain (Rowett, 1968).

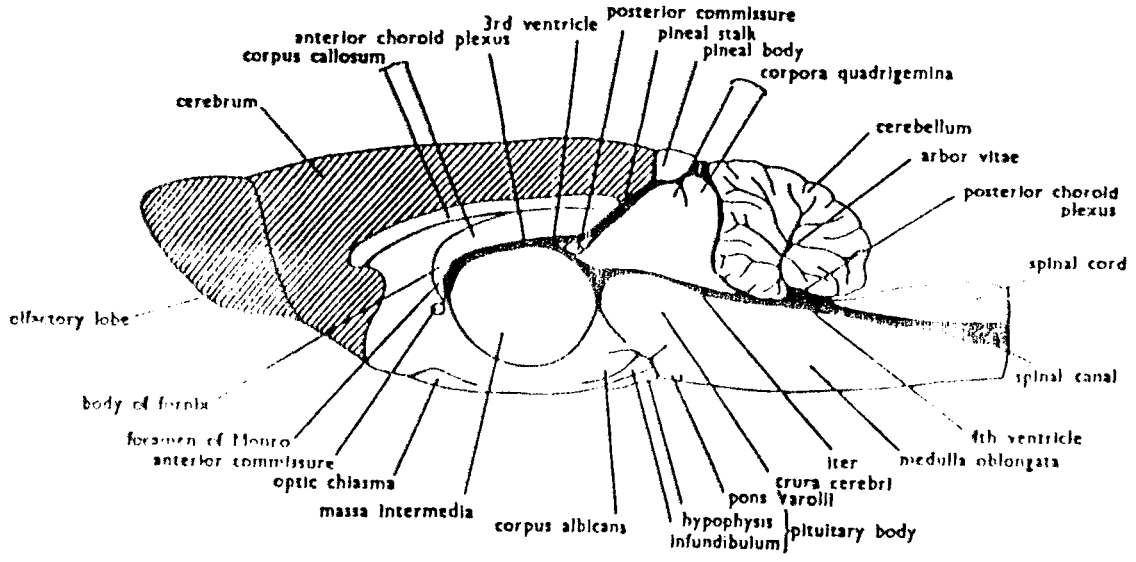


Figure 1.1b Median sagittal view of the rat brain (Rowett, 1968).

existence of a parasympathetic innervation was demonstrated in certain mammals (Kenny, 1961; Romijn, 1973; Romijn, 1975b). Both postganglionic noradrenergic sympathetic and postganglionic acetylcholinergic parasympathetic fibres do not form true synaptic contacts with the pinealocytes, ending either in the parenchyma or in the pericapillary spaces.

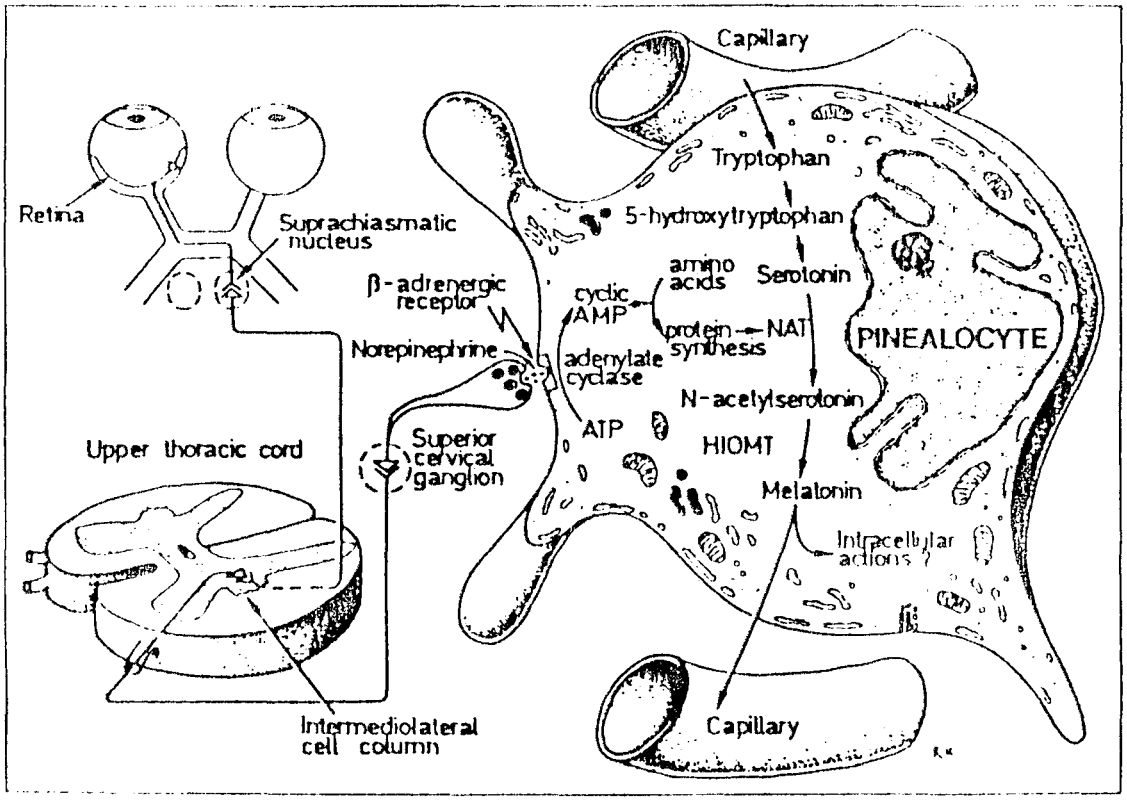
Wurtman *et al.*, (1963b) describes the pineal gland as a 'neuroendocrine transducer' whose hormonal output depends upon, or is mediated by, the light message reaching the pineal through its nerve supply. Although it appears that the major driving force of the pineal gland occurs due to the activity of the afferent sympathetic neurons, the gland can receive other hormonal effects through its blood supply. The hormonal effects have been found however to be subsidiary to, and modulated by, the sympathetic neuronal effects (Cardinali, 1981; Datta and King, 1980). Hence noradrenaline (NA) released from sympathetic nerves regulates the daily rhythms in enzyme activity and melatonin synthesis that occur in response to environmental lighting.

1.3.3 The Neural Pathway

In the rat pineal gland the neural pathways by which photosensory information reaches the pineal is well established. Unlike a number of other endocrine organs, the pineal relies heavily on its innervation for its endocrine activity (Reiter, 1989). In particular, sensory information perceived by the eyes is essential in determining the production of pineal melatonin (**Figure 1.2**). The first stage of the pathway is a monosynaptic projection from the retina to the hypothalamic suprachiasmatic nucleus (SCN) (Moore, 1983). Using radio-labelled amino acids, Moore (1973) was able to prove unequivocally the existence of this retina hypothalamic pathway. Moore also found that after transduction into a neural signal in the photoreceptors of the retina, the message is sent to the hypothalamus via ganglion cell axons which form part of the retinohypothalamic tract (Moore, 1978). At the level of the optic chiasma these fibres diverge from the classic optic system and terminate in the suprachiasmatic nuclei of the anterior hypothalamus (Sonofriew and Weindl, 1982). After synapsing in this location, fibres project possibly to the paraventricular nuclei of the hypothalamus (Bittman, 1984). Long descending axons then supposedly carry the neural message

Figure 1.2 Diagrammatic representation of the proposed neural connections between the eyes and a pinealocyte in the pineal gland.

The synthesis of MEL is shown in the pinealocyte. Interruption of the neural connections between the suprachiasmatic nucleus and the pineal eliminates cyclic MEL production. [Reiter (1988)].



into the intermediolateral cell column of the upper thoracic spinal cord (Swanson and Sawchenki, 1983). The axons of these preganglionic perikarya leave the spinal cord and pass up the sympathetic trunk to synapse on postganglionic fibres entering the skull and among other organs, terminate within the pineal gland (Kappers, 1965).

The SCN contains elements of the circadian timing system which generates pineal and other rhythms. Although the SCN can function in a cyclical manner autonomously, environmental lighting has strong effects on the clock.

There is a general agreement that the fibres do not form distinct synapses with the pinealocytes (Kappers, 1976) but end on the perivascular spaces and that the neurotransmitter simply diffuses to the surface of the pineal cells. A few processes might terminate between the perikarya of the pinealocytes (Matsushima *et al.*, 1981). Apart from the delivery of NA, the sympathetic nerves can also act as a sponge, taking up and concentrating catecholamines from the perivascular space (Kvetnansky *et al.*, 1979). This protective effect has been demonstrated when stressful treatments, known to elevate catecholamine levels have had little effect on the pineal or plasma melatonin levels (Vaughan *et al.*, 1982). This is true, provided that the uptake process is not blocked (Parfitt and Klein, 1976).

The sympathetic neural connections between the SCN and the pineal gland must remain intact in order for the pineal to function in its normal capacity. Interruption of the neural connection caudal to the SCN in either the central or peripheral nervous system interferes with the cyclic production of melatonin as well as with the endocrine capability of the pineal gland (Kneisley and Moskowitz, 1978).

1.3.4 Other Possible Pathways Modulating Neural Control of Pineal Function

Attention has been drawn recently to prostaglandins (PG's) as possible modulators of melatonin synthesis and release. Prostaglandins are produced and released by the pineal gland and by

stimulation of pineal α-adrenoreceptors, resulting in the activation of phospholipase A_2 (Ho and Klein, 1987) and enhancing the release of arachidonic acid and PGE_2. PGE_2 added *in vitro* increased rat pineal cAMP levels, NAT activity and melatonin release, while the addition of cyclo-oxygenase inhibitors decreased NA-induced melatonin release (Cardinali and Ritta, 1983).

Vasoactive intestinal polypeptide (VIP) is localized in nerve endings of the pineal gland and is one of several neuropeptides which have been reported to affect pineal melatonin biosynthesis (Moller and Mikkelsen, 1989). A rapid increase in pineal cAMP accumulation and NAT activity is induced when VIP interacts with specific receptor sites (Yuwiler, 1983; Kaku *et al.*, 1985,1986). VIP has an effect which has been shown to be potentiated by α-adrenoceptor activation by the same mechanism which potentiates β-adrenergic stimulation (Chik *et al.*, 1988).

Another neuropeptide, neuropeptide Y (NPY) has been found in pineal glands (Shiotani *et al.*, 1986) and may be located in nerve fibres together with noradrenaline. The use of immunohistochemical techniques have allowed the detection of NPY (Schon *et al.*, 1985; Schroder, 1986). The NPY have been shown to exert a dual effect in the pineal by enhancing the postsynaptic effects of NA at low concentrations whilst inhibiting sympathetic neurotransmission at high concentrations. It may even play a role in the transfer of light information as administration of NPY to the SCN area causes phase shifts in the circadian rhythm (Harrington and Eicberg, 1983). Vacas *et al.* (1987) showed that NPY enhanced the noradrenergic induced stimulation of melatonin production in the pineal and imparts the K^+ induced release of [^{3}H]-noradrenaline.

Substance *P* has been detected in the pineal glands of mammals. The characterization of receptors for substance *P* has occurred in bovine pineals but not in rat pineals (Govitrapong and Ebadi, 1986). The substance *P* plays a key role as a neurotransmitter or as a neuromodulator in the peripheral and central nervous system (for reviews see Euler and Pernow, 1977; Leeman *et al.*, 1977; Porter and O'Connor, 1982; Pernow, 1983; Jessell, 1983; Jordan and Oëhme, 1985). Euler and Gaddum (1931) identified in the brain a hypotensive and spasmogenic factor which to a certain extent resembled acetylcholine in its action, but whose effects could not be blocked by atropine, a muscarinic

cholinergic receptor blocking agent. The factor was called substance *P*.

There are many other candidate neurotransmitters and neuropeptides for which binding sites in the pineal have been reported and they include glutamate (Govitrapong *et al.*, 1986), dopamine (D_2) (Govitrapong *et al.*, 1984), acetylcholine (muscarinic) (Govitrapong *et al.*, 1986; Laitinen *et al.*, 1989a), substance *P* (Govitrapong and Ebadi, 1986) and peptide histidine isoleucine (PHI) (Tsuchiya *et al.*, 1987; Moller and Mikkelsen, 1989). However for many of these, an effect on pineal melatonin synthesis and release is yet to be demonstrated.

There is a peptide transmitter found in discrete populations in central synapses called delta sleep inducing peptide (DSIP) (Graf and Kastin, 1986). This peptide has been reported to have various effects on sleep as well as other physiological functions (Schoenenberger, 1984; Graf and Kastin, 1984). DSIP has been proposed as a "programming substance for circadian rhythms" (Graf *et al.*, 1982). It has been known to reduce the nocturnal rise in rat pineal NAT as well as reducing the stimulation of NAT in the presence of α- and β- adrenergic agonists both *in vivo* and *in vitro* (Graf and Schoenenberger, 1986). The enhancement of the effect of NA on NAT through a adrenoreceptor mechanism occurs through DSIP (Graf and Schoenenberger, 1987).

1.4 Circadian Rhythm

In mammals the circadian clock is a powerful device. Buried deep in the brain it tells us when to wake and when to sleep as well as regulating the temperature of our bodies. The body rhythms it sets is a puzzle that has kept a branch of physiology known as 'chronobiology' in business for more than two decades. Only now are researchers getting to grips with its most important environmental time keeper : daylight.

Left to its own devices the body clock completes its cycle over a period of about, but not exactly, 24 hours - hence the term circadian (circa-about; diem-day). In this free running state all that drives the clock is the activity of its constituent nerve cells. In everyday life, we never experience the

rhythms of this state because the clock is always entrained by time cues in the environment. These cues which chronobiologists call "zeitgebers" (the German word meaning 'time-givers') synchronize the clock with the daily solar cycle. Enviromental lighting has the effect of resetting and keeping the clock entrained to the light cycle (Reiter, 1991b).

The hormonal and biochemical rhythm that has been studied most intensely is that of melatonin production (Binkley, 1981; Klein, 1985). Melatonin may be speculated to hold the key to unlocking the location of the circadian clock since it is the best known hormone whose release is timed by it. Melatonin acts as a chemical time-keeper, being released by the pineal gland in darkness it sets both daily and annual hormonal rhythms.

The components of this circadian system in the rat and other mammals are anatomically distinct with a multisynaptic pathway connecting these components. The photoreceptors are the retina, the pacemaker is in the suprachiasmatic nucleus of the hypothalamus (Moore, 1983) and the chemical time-keeper melatonin is synthesized in the pineal gland. The rat pineal gland is neither rhythmic nor photosensitive *in vitro* but can be stimulated to make melatonin (Zatz, 1982; Klein, 1985).

1.4.1 Light - A Pineal Zeitgeber

The melatonin rhythm of the rat pineal gland is influenced largely by light. Light causes an acute reduction of amplitude (synthesis and output) of the melatonin rhythm and influences the carrying of the underlying pacemaker as revealed by changes in period or phase (Erlich and Apuzzo, 1985). A precipitous decline in pineal melatonin synthesis and release occurs due to acute exposure of animals or humans to light at night. The reduction to daytime levels requires only 20 to 30 minutes. Exposing the pineal gland to light at night suppresses melatonin production in the pineal of all animals tested, but the brightness (irradiance) of light required to achieve this result varies greatly among mammals (Reiter, 1985). An illustration as to how these species differ, the albino rat pineal gland can respond to as little as 0,0005$\mu W/cm^2$ of white light at night whereas the Richardson's ground squirrel requires light of about 1850$\mu W/cm^2$. The human pineal is intermediate between

these two extremes responding to about 150μW/cm^2 of white light at night. Normal room light averages 50-100μW/cm^2 while on a clear day the sunlight at noon can be as bright as 25 000 to 50 000 μW/cm^2 (Reiter, 1988).

Melatonin synthesis is not only suppressed by the brightness of the light but also due to its wavelength. Brainard (1985) found that light in the blue range 500-510Å seems to be most suppressive of pineal melatonin production. This includes light in the yellow-green range as reported by Wurtman and Moskowitz (1977).

Light controls the amplitude and length of melatonin production cycle and although it is the primary input in controlling the cycle, it appears that the levels of other hormones such as oestrogen, progesterone, glucocorticoids and prostaglandins are also important in the full expression of the final response (Cardinali and Vacas, 1987).

Melatonin synthesis may be activated by stress and hypoglycaemia (Lynch *et al.*, 1973; Sampson, 1975) demonstrating that environmental stimuli other than light may also be involved in the regulation of pineal melatonin synthesis. The effect of ambient temperature also has an effect on melatonin synthesis leading to a suggestion that the pineal gland may be involved in thermoregulation (Ralph *et al.*, 1979).

1.5 The Role of β- and α- Adrenergic Mechanisms in Melatonin Production

The most abundant neurotransmitter released from the nerve-endings is noradrenaline (Pellegrino de Iraldi and Zieher, 1966). Noradrenaline exhibits a circadian rhythm in the rat pineal gland (Wurtman and Axelrod, 1966) and when released, acts on adrenoreceptors present on the pineal gland. The cascade of events leading to the biosynthesis and release of melatonin together with other indoles and polypeptide compounds is then initiated.

The α- and β- receptors exhibit 24 hour rhythms in their density (Pangerl *et al.*, 1990). Such

fluctuations are generally believed to be determined by NA itself which following its release, causes desensitization or the internalization of the receptors (Romero *et al.*, 1975; Gonzalez-Brito *et al.*, 1988; Pangerl *et al.*, 1989). The involvement of these two receptors in the regulation of intracellular cAMP is a feature common to many cells (Reiter, 1991a). Cyclic-AMP is the essential intracellular second messenger required for the rise in nocturnal melatonin production (Axelrod, 1974; Klein *et al.*, 1981a; Santana *et al.*, 1988).

Beta-adrenergic stimulation of the pinealocyte causes a rise in intracellular accumulation of cAMP due to its ability to increase the activity of adenylate cyclase (O'Dea and Zatz, 1976). Activation of β-adrenergic receptors alone induces up to 10-fold increase in cAMP in the pineal gland. Conversely stimulation of α-adrenergic receptors alone is without any effect on cyclic nucleotide accumulation (Vanacek *et al.*, 1985). However, when combined stimulation of both β- and α-adrenergic receptors by NA occurs, the rat pineal response in terms of cAMP is greatly potentiated with nucleotide content increasing up to 100-fold (Klein, 1985). These findings are consistent with the view that, in the pinealocyte, β-receptor activation is a requirement for cAMP accumulation with the α-receptor stimulation amplifying the response. The dual receptor regulation of cAMP in the pineal gland also translates into a similar stimulation (by β-adrenergic receptors) and augmentation (by α-adrenergic receptors) of the activity of the rate-limiting enzyme in melatonin production, N-acetyltransferase (Klein *et al.*, 1981a) as well as of melatonin itself (Santana *et al.*, 1988).

Noradrenaline released at night acts on the pinealocyte membrane to stimulate β-adrenoceptors. Beta-adrenoceptors have been identified in rat pinealocytes (Auerbach *et al.*, 1981) and in sheep and hamster pineal membranes (Foldes *et al.*, 1983; Craft *et al.*, 1985) using radioligand binding studies. Stimulation of these receptors results in the activation of the enzyme adenylate cyclase and in so doing causes cAMP synthesis. The increase in cAMP mediates the activation of a cAMP-dependent protein kinase, protein kinase A, which initiates the transcription of mRNA required for new synthesis of enzymes in the melatonin pathway (Axelrod, 1983; Ebadi, 1984). Thus an increase in cAMP mediates an increase in activity of serotonin N-acetyltransferase (NAT). This rate limiting

enzyme catalyses the conversion of serotonin to N-acetylserotonin which is a precursor for melatonin. Similarly the activity of the enzyme that converts N-acetylserotonin to melatonin, HIOMT, is also increased by cAMP.

By observing the fact that ribosylation of the G-protein by cholera toxin can substitute for β-adrenergic receptor activation (Sugden, 1989) it is believed that a G-protein is involved in β-receptor mediated stimulation of adenylate cyclase.

Activation of rat pineal α-adrenoceptor triggers the hydrolysis of phosphatidylinositol (PI) to yield diacylglycerol and inositol phosphates (mainly inositol monophosphates). In addition, α-adrenoreceptor stimulation produces an increase in intracellular Ca^{2+}, presumably by opening a ligand-dependent channel (Sugden *et al.*, 1987). Diacylglycerol is a potent activator of calcium and phospholipid dependent enzyme, protein kinase C (PKC). Activation of PKC mediates the amplification of the cAMP and NAT response.

1.6. Pineal Indole Biosynthesis and Metabolism

1.6.1 Synthesis of Melatonin

The biosynthesis of melatonin commences with the uptake of circulating tryptophan by the parenchymal cells of the pineal gland (**Figure 1.3**). A major portion of this is converted to 5-hydroxytryptophan. This conversion is catalysed by tryptophan hydroxylase (Lovenberg *et al.*, 1967; Jequier *et al.*, 1969) which may differ from the one in the brain (Nakamura *et al.*, 1965). It requires the presence of oxygen pervious irons and a reduced pteridine cofactor (Lovenberg *et al.*, 1962; Snyder and Axelrod, 1964b).

The 5-hydroxytryptophan is then decarboxylated to form serotonin (Lovenberg *et al.*, 1962; Snyder and Axelrod, 1964b), which is found in very high concentrations in the pineal (Giarman and Day, 1959). The enzyme involved here is the pyridoxal dependent aromatic L-amino acid decarboxylase (Snyder and Axelrod, 1964b). This enzyme has a concentration in the pineal greater than in any

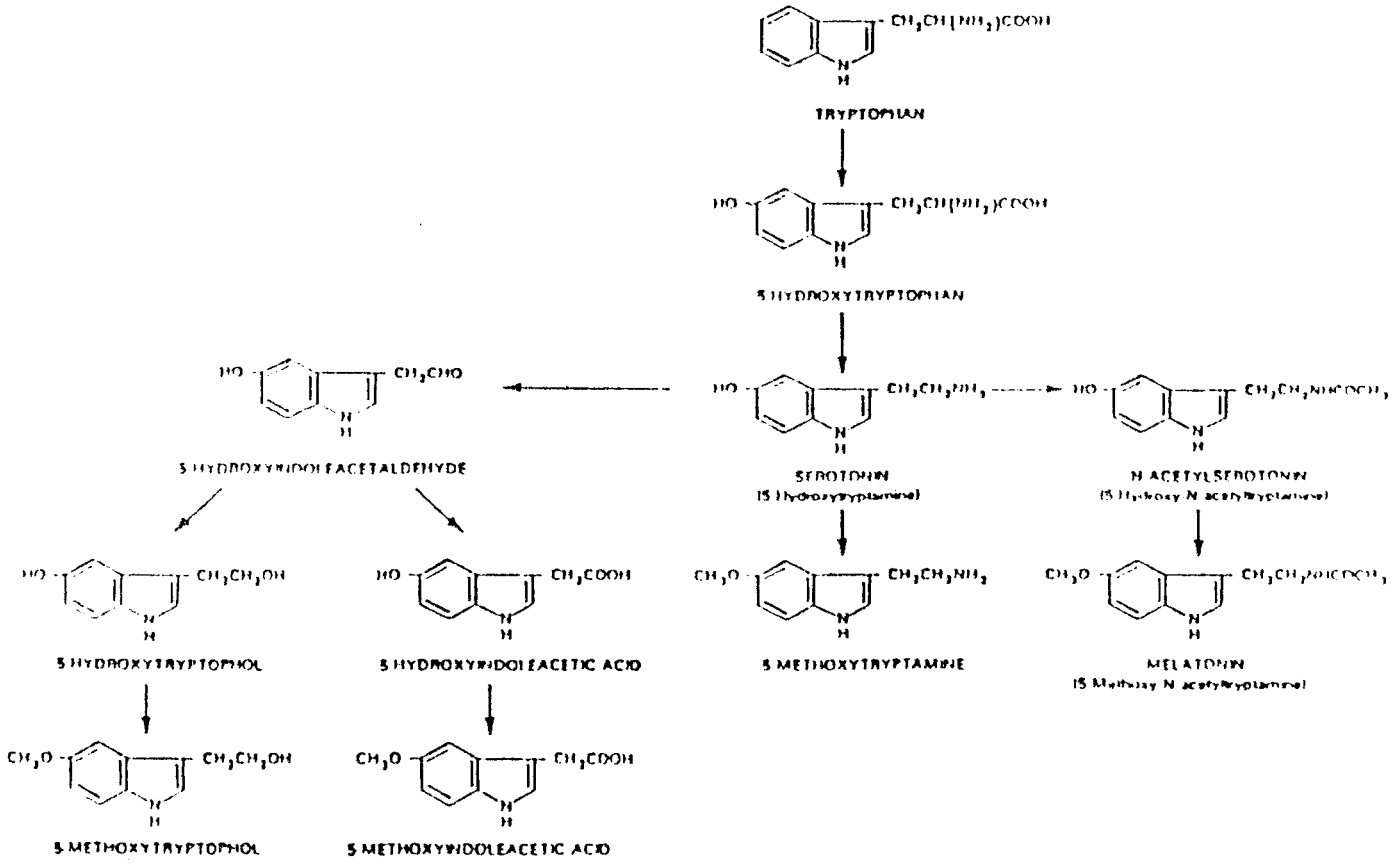


Figure 1.3 Pathway of pineal indole metabolism.

other tissues examined (Snyder and Axelrod, 1964b).

The next step involves the acetylation of serotonin to form N-acetylserotonin by the enzyme NAT, acetyl CoA being the donor of the acetyl group (Weissbach *et al.*, 1960). Finally, the N-acetylserotonin is converted to melatonin by O-methylation in the 5-position. This step is catalysed by the enzyme, HIOMT (Axelrod and Weissbach, 1960,1961; Cardinali and Wurtman, 1972).

1.6.2 Synthesis of Other Pineal Indoles

Serotonin is also oxidized to 5-hydroxyindole acetaldehyde by monoamine oxidase (Axelrod *et al.*, 1969). Some of this is then converted to 5-hydroxyindoleacetic acid by aldehyde dehydrogenase (Wurtman and Larin, 1968; Lerner and Case, 1960). This is then O-methylated in the 5-position by HIOMT to form 5-methoxyindole acetic acid (Wurtman and Axelrod, 1967). Some of the 5-hydroxyindole acetaldehyde is also converted to 5-hydroxytryptophol by alcohol dehydrogenase (McIsaac and Page, 1959) and is then methoxylated by HIOMT to form 5-methoxytryptophol (Wurtman and Axelrod, 1967). Serotonin can also be methoxylated by HIOMT to form 5-methoxytryptamine.

In addition to the pineal, several other organs have the capacity to produce melatonin. Most of the tissues that produce melatonin seem somehow to be related to the visual system, the pineal gland itself being an end organ of the visual system. Melatonin is produced in the retina (Pang and Allen, 1986), harderian gland (Bubenik *et al.*, 1976) and the extra-orbital lacrimal glands (Mhatre *et al.*, 1988). It appears that these organs lack the ability to secrete melatonin. Thus under normal circumstances the blood melatonin concentration almost exclusively reflects that of the pineal (Cardinali, 1981; Reiter, 1989).

Whereas melatonin's actions on mammals are no longer debated, the diversity and mechanisms of melatonin's effects are still being uncovered. Clearly, the consequences of the pineal and melatonin are extremely widespread, perhaps influencing every organ system in the body (Reiter, 1980;

Vriend, 1983; Heidmaier and Lynch, 1986; Watson-Whitmyre and Stetson, 1988; Maestroni *et al.*, 1989).

1.6.3 Pineal Enzymes Involved in Serotonin Metabolism

1.6.3.1 Monoamine Oxidase

Monoamine oxidase activity has been reported to be high in the pineal (Snyder *et al.*, 1965), the enzyme being mainly confined to the mitochondria. The principle function of this enzyme is to counterbalance amine production and to maintain a constant level of amine.

1.6.3.2 Alcohol dehydrogenase

This enzyme, alcohol dehydrogenase, transforms aldehydes into acids and requires an oxidized pyridine cofactor (Feldstein and Williamson, 1968) for its mechanism.

1.6.3.3 Serotonin N-acetyltransferase (NAT)

Serotonin N-acetyltransferase undergoes large daily changes (Axelrod, 1974). Rat NAT adversely is 30-70 fold higher during the night than during the day. Pineal NAT is found entirely in the cytoplasm, and acts to transfer an acetyl group from the cofactor acetyl CoA to acceptor amines (Weissbach *et al.*, 1961). The main acetyl group acceptor is considered to be serotonin, although 5-methoxytryptamine can also be N-acetylated. It has been speculated that a physiological disulphide in the pinealocyte is involved in NAT regulation. Pineal NAT activity is regulated by an adrenergic-cAMP mechanism (Klein *et al.*, 1978). Inhibition of cAMP destruction by drugs or cAMP analogs results in an increase in NAT activity (Klein *et al.*, 1970).

Cycloheximide and actinomycin D block these effects, indicating that the increase in NAT requires the synthesis of new RNA hyperpolarization is necessary for NAT stimulation. An additional

important piece of evidence concerning the role of cAMP is that propanolol treatment reduces the amount of cAMP in the cell as well as NAT (Klein *et al.*, 1978).

The amount of cAMP in the cell is a function of its rate of production, which is under adrenergic control. Pineal NAT activity is hence regulated by this second messenger cAMP, the production of which is controlled by a beta-adrenergic receptor associated with adenylate cyclase in the membrane of the pinealocyte.

Environmental factors known to regulate pineal NAT activity are drugs, light and stress. An analogy of noradrenaline, isoproterenol, is a drug commonly used *in vivo* to induce an increase in NAT activity. Environmental lighting exercises a marked effect on pineal NAT activity. During the day, when NAT activity is diminished, exposure to darkness does not induce an increase in NAT activity.

Stress has also been shown to induce small increases in NAT activity (Lynch *et al.*, 1973). It is important thus, for these factors to be strictly controlled during experimental procedures which directly or indirectly involve NAT. NAT activity is generally considered to be rate limiting in the production of melatonin (Klein, 1985). Thus the pineal melatonin content typically rises parallel to NAT levels. There are however notable exceptions where a large melatonin peak is present without a large increase in NAT activity (Menendez-Palaez *et al.*, 1990).

1.6.3.4 Hydroxindole-O-Methyltransferase (HIOMT)

In the pineal gland hydroxyindole-o-methyltransferase (HIOMT) catalyses the conversion of N-acetylserotonin to melatonin and to a lesser degree converts 5-hydroxyindole acetic acid to methoxyindole acetic acid, hydroxytryptamine and hydroxytryphophol to methoxytryptophol (Axelrod and Weissbach, 1961). The enzyme is located almost entirely in the cytosol of the pinealocyte.

HIOMT is regulated by a neural mechanism modulated by light and circulating steroids. Pineal HIOMT activity alters slowly over a period of days in response to changes in environmental lighting conditions (Axelrod and Wurtman, 1978). During exposure to constant darkness there is a gradual increase in HIOMT activity, and in constant light there is a gradual decrease in HIOMT activity. It is generally accepted that the signals generated endogenously on a circadian basis in the suprachiasmatic nucleus stimulates the release of transmitter in the pineal gland, resulting in an increase in HIOMT activity.

Light appears to block transmission of signals to the pineal gland although intracellular mechanisms involved in HIOMT regulation have not been entirely elucidated. No direct evidence implicating NA release in the stimulation of HIOMT exists. Attempts to stimulate HIOMT activity in organ culture with NA and cAMP have been unsuccessful (Berg and Klein, 1971). The increment in HIOMT activity observed in constant darkness is due to an increase in the number of HIOMT molecules (Yang and Neff, 1975).

N-acetylserotonin appears to occupy the role of a "fulcrum" in terms of melatonin production. At night, when NAT levels are high, and when saturating amounts of N-acetylserotonin are produced, it would appear that HIOMT would function as the rate limiting enzyme. During the day, however, when N-acetylserotonin levels are low, NAT would probably assume dominance in this role.

Several forms of HIOMT exist in pineal tissue (Jackson and Lovenberg, 1971) and unlike the enzyme NAT, it is restricted to only a few other organs. It is found in the retina (Cardinali and Rossier, 1971), the harderian gland (Vlahakes and Wurtman, 1972) and in the erythrocytes (Rosengarten *et al.*, 1972). Monoclonal antibodies to bovine HIOMT show a high degree of structural similarity with HIOMT from other species and have allowed the immunocytochemical cytosolic localization of this enzyme (Deguchi *et al.*, 1987).

1.7 Melatonin - a neuroendocrine modulator

Of all the pineal substances studied, melatonin is probably the most investigated substance. The importance of the pineal gland and melatonin as possible neuroendocrine modulators becomes apparent when studying their various physiological effects. In 1917 MCord and Allen observed a lightening of the skin of amphibians fed with bovine pineal extracts. Lerner *et al.*, (1958) first isolated and identified the pineal substance responsible for this effect, and called it melatonin (5-methoxy-N-acetyltryptamine). Melatonin is synthesized and secreted by the pineal gland of all the vertebrate species examined. As far as can be determined melatonin is not stored within the pineal gland in any appropriate quantity. Shortly after being synthesized it is released into circulation (Kennaway *et al.*, 1977). In some cases melatonin may even be released into the cerebral spinal fluid (Reppert *et al.*, 1988) and blood levels in the cerebrospinal fluid (CSF) follow those of the pineal gland.

Melatonin appears to be secreted by the pineal gland by simple diffusion. Although it is generally accepted that the blood of the pineal capillaries rather than the cerebro-spinal fluid (CSF) is the primary site of melatonin secretion (Cardinali *et al.*, 1983). Melatonin has a half life of about 20 minutes in the blood of rats (Gibbs and Vriend, 1981). A major portion of melatonin in blood is bound to plasma protein (Partridge and Mietus, 1980). The primary site for the metabolism of melatonin in experimental animals is the liver (Wurtman *et al.*, 1968). After being secreted into the blood, melatonin is loosely bound to plasma albumin and passes through the liver where it is rapidly conjugated with glucuronide (5%) and sulphate (70-80%) by hepatic microsomes and the metabolites are then excreted in the urine (see Kveder and McIsaac, 1961 and Reiter, 1989 for reviews and references).

N-acetylserotonin has been recently identified as a melatonin metabolite in the urine (Yang and Neff, 1975) suggesting that melatonin is demethylated back to its precursor and implying the existence of a complex feedback mechanism controlling melatonin synthesis. One of the metabolites in the urine, 6-hydroxy-melatonin-sulphate is sometimes measured to monitor pineal melatonin

production, and may serve to provide a simple, rapid, non-invasive technique of assessing melatonin levels in clinical situations (Aldous and Arendt, 1988). This technique would not however be able to pick up rapid changes of short duration.

1.7.1 Site and Mechanism of Action of Melatonin

There are many potential functions of the pineal gland and melatonin which have been suggested but possibly the most extensively documented effects of the pineal gland are those on the reproductive system. Reiter and colleagues (1988) were one of the first to observe an involution of the gonads and accessory sex organs of both male and female hamsters on exposure to constant light providing convincing data that the pineal gland is an organ of internal secretion.

Although the principle locus of melatonin activity is still a matter of controversy, most investigators agree that the central nervous system is probably the major site of melatonin action (Anton Tay, 1974; Cardinali, 1981; Sugden, 1983; Smith, 1985). The high density of melatonin binding sites in the suprachiasmatic nuclei (SCN) (Vanacek *et al.*, 1987; Reppert *et al.*, 1988) suggests that melatonin may affect circadian rhythms via this structure. It has recently been shown that lesions of the SCN disrupt locomotor activity and drinking rhythms and inhibit the effect of administered melatonin (Bartness and Goldman, 1989). Zisapel *et al.*, (1988) demonstrated a distinct diurnal variation in binding site density in the hypothalamus but not in the striatum. The diurnal variations in the density of melatonin binding sites at specific brain regions underlie the physiological response of the neuroendocrine system to melatonin.

It has been suggested that melatonin assists the entry into sleep and perhaps maintains undisturbed sleep throughout the night (Armstrong, 1989). Anton Tay *et al.*, (1971) noted an increase in REM sleep and a slowing down of the EEG rhythm in patients treated with melatonin. In humans a circadian secretory pattern is well established, with low daytime and high nighttime blood concentrations. An age related disease on nocturnal melatonin concentration has been observed (Touitou *et al.*, 1984; Carini *et al.*, 1987). The relation of secretory episodes to sleep stage cycles

remains controversial. Vaughan *et al.*, (1979) and Weinberg *et al.*, (1979) reported that nocturnal plasma melatonin levels exhibit an episodic pattern with no apparent relation to sleep stages. Spontaneous waking episodes are significantly correlated to the occurrence of melatonin peaks but not with other sleep stages.

Many reports suggest that the pineal gland may be involved in the modulation of epileptic seizures with melatonin being the principle anticonvulsant agent exerting its action possibly by reducing the ability of excited epileptic neurons to maintain activity (Pang *et al.*, 1976). An increase in the convulsive threshold in epileptic patients and an improvement in the clinical picture of Parkinsonian patients (Anton Tay *et al.*, 1971) was noted.

1.7.2 Influence of melatonin on man

Melatonin has been implicated in the immune reaction. Interleukin-2 cells inhibit pineal melatonin production *in vivo* (Eposti *et al.*, 1988) and melatonin may stimulate natural killer cell activity in humans and enhance humoral and cellular immune responses in mice (Fraschini *et al.*, 1988).

The pineal has been suggested to influence the spread and growth of malignant tumours (Tapp, 1980). Patients dying of breast cancer often have enlarged pineals (Tapp, 1982; Blask, 1984) while those dying of disseminated forms of cancer invariably have high HIOMT levels and less Ca^{2+} deposits in their pineals (Kerenyi *et al.*, 1977). In studies done on female rats, melatonin has emerged as a particularly important anti-neoplastic agent especially with respect to growth of breast cancer. Lissoni *et al.*, (1988) have suggested that an increase in plasma melatonin concentrations can be used as a prediction of the objective response of cancer patients' chemotherapy. These investigations have suggested that the anti-neoplastic effect of cytotoxic drugs may require the participation of the pineal gland. In rats pinealectomy generally promotes the growth of cancer tissue while melatonin administration does the opposite.

The therapeutic potential of melatonin in jet-lagged travellers who have passed through eight or more time zones is well established (Arendt *et al.*, 1987; Arendt and Aldous, 1988; Armstrong and Chesworth, 1987). The timed administration of melatonin to these individuals improves mood and synchronizes endogenous melatonin and cortisol rhythms. The most recent demonstration of melatonin receptors in the SCN of human brains (Reppert and Weaver, 1988) encourages the view that exogenous melatonin acts on this structure to synchronize disrupted circadian rhythms. By the administration of the drug triazolam, phase shifts or "jet-lag" caused by changes in environmental light input into the "clock" may be adjusted (Turek, 1987). The drug flumazenil, a specific benzodiazepine antagonist, may block this effect. Turek (1987) discovered this finding useful since it could shorten the time of readjustment to the circadian rhythm and thus altering the sleep wake cycle.

Due to exhaustive investigations done to determine the effect of blindness on the biological clock, studies done on blind patients suggest that melatonin may affect circadian systems. Sach *et al.*, (1987) found in a controlled study that the timed daily administration of melatonin to two blind subjects was found to phase advance the free running endogenous melatonin rhythm. In another study (Arendt *et al.*, 1988) involving a blind subject with a severely disrupted and possibly free running sleep wake rhythm, melatonin administration was found to synchronize the endogenous melatonin rhythm and improve synchronization of sleep onset.

Ageing has been related to a relative melatonin deficiency, a result of a gradual failure of the pineal gland. The hypothesis proposes that the persistence of serotonin in the midst of the melatonin decline results in the chain of neuro-endocrine and peripheral events which promote ageing (Rozencwaig *et al.*, 1987). Hasegawa *et al.*, (1987) described pineal decline and the presence of calcifications in the pineal. This study found however that although there existed a correlation between pineal weight and the degree of calcification, some pineals in patients over 90 years old showed no calcification and were indistinguishable from the ones of younger subjects. This suggested that the pineals of humans do not necessarily degenerate after involution.

Melatonin may be the most investigated substance in the pineal. It is unwise to assume that it is the only pineal factor with physiological consequences. Many other known or yet undiscovered pineal substances may prove to have roles equally as, or perhaps even more important than melatonin. For instance, melatonin free pineal extracts were observed to exert antigonadotropic effects (Damian *et al.*, 1981) which raises the possibility of other factors other than the methoxyindoles such as pteridines and pterins exerting an effect (Ebels, 1979). Pineal factors other than melatonin such as 5-methoxytryptophol and 5-methoxytryptamine (McIsaac *et al.*, 1964) also exert antigonadotropic effects even in the absence of an intact pineal gland (Pevet *et al.*, 1981).

To exhaustively review the vast literature available on the pineal gland would exceed the scope of this study. Newly discovered possible physiological functions for the pineal gland and its factors are constantly being suggested. Indeed pineal research is a dynamic field of study and certainly the wide range of data being collected from both animal and human sources indicates much interest in the pineal gland and its possible roles, very different from the lack of enthusiasm for the gland in the past.

1.8 The Autonomic Nervous System and the Pineal Gland

1.8.1 Introduction

A brief background into the autonomic nervous system, its characteristics as well as neurotransmission needs to be reviewed in order to put into perspective the role that the cholinergic system and its receptors play in the pineal gland.

The autonomic nervous system (ANS) supplies innervation to smooth muscles, glands and visceral organs. Termed the "involuntary nervous system" because of its control of physiological reaction not under conscious regulation, this communications network maintains homeostasis and facilitates appropriate bodily responses to environmental influences. The integrating action of the autonomic nervous system is of vital importance for the well-being of the organism. As Claude Bernard (1818-1879) and Cannon (1829-1932) have emphasized, that the constancy of the internal

environment of the organism is to a large extent controlled by the negative or autonomic nervous system.

1.8.2 Anatomy and Physiology of the Autonomic Nervous System (ANS)

The autonomic (or involuntary) nervous system is separated into two divisions based upon anatomic and physiological criteria: the parasympathetic and sympathetic nervous systems. Both ANS divisions innervate many organs. The dual autonomic innervation of the heart and intestines are important because of the opposing action of these two divisions. Effector cells in these organ systems may be activated to either elevate or suppress the characteristic inherent activity of the heart and intestines.

Changes in the organ system activity result from the interplay between the sympathetic and parasympathetic divisions. Drugs that affect the ANS may mimic the activity of one of the systems thereby eliciting a pharmacologic effect by an "active" mechanism. Conversely a "passive" mimetic effect on one of the divisions may result from blocking the influence of the opposing division.

1.8.3 Characteristics of the Autonomic Nervous System

The central nervous system (CNS) functions in concert with the ANS in both the maintenance of homeostasis and the body's reaction to environmental or internal stresses. Thus, centres in the medulla oblongata influence the integration of blood pressure control and respiration (Miller, 1978). Ganglia and plexuses are distinguishing factors in the ANS. Chemical mediators transmit information (nerve action potential) across gaps or spaces in the autonomic neuron pathways (Bullock and Hagiwara, 1957). These interruptions in physical communication are represented by autonomic ganglia in which a synaptic cleft separates the preganglionic fibres from postganglionic fibres and by the neuro-effector junction in which the autonomic postganglionic fibres do not physically attach to effector cells or receptor sites on smooth muscles and visceral organs. After release into the autonomic synapse or neuro-effector junction upon passage of a nerve action

potential, these chemicals (transmitters or neurohormones) attach to receptor sites on postganglionic membranes or effector cells on smooth muscles, glands and visceral organs. Combining a chemical transmitter with a receptor site initiates biochemical events that culminate in a physiological effect or response (Schmitt *et al.*, 1976).

Acetylcholine ACh is the chemical neurotransmitter at the following: all autonomic ganglia; the neuro-effector junction of postganglionic parasympathetic fibres and effector cells on smooth muscle, glands and visceral organs; the junction of autonomic fibres and the adrenal medulla; and the autonomic nerve-effector cell union on sweat glands and vasodilator vessels. Thus, these fibres are designated as cholinergic - as a consequence of a specific chemical (ACh) released from their endings upon passage of a nerve action potential. Various regions of the CNS differ markedly in their ability to synthesize ACh (Hebb, 1963).

Noradrenaline is the neurotransmitter released from postganglionic sympathetic nerves that innervate smooth muscles, glands and visceral organs. Thus, these fibres are termed "adrenergic fibres", indicating the chemical transmitter released from these sympathetic nerve endings.

1.9 Drug Action on ANS Neurotransmission

Upon the passage of a nerve impulse, neurotransmitters such as acetylcholine and noradrenaline are released from presynaptic sites and nerve terminals. Pharmacological agents alter neurotransmitter events at one or more sites on the neurotransmission sequence. Most drugs owe their activity to an effect on the synaptic or neuro-effector environment and act either directly or indirectly on the "breaks" or "gaps" in the neuron pathway by altering neurotransmitter influence at receptor sites on effector cells. A direct agonist or antagonist attaches to a receptor site to either elicit or block a neurotransmitter response, respectively. For example, isoproterenol is a direct acting beta-adrenergic receptor agonist that mimics the action of the neurotransmitter noradrenaline. A direct cholinergic receptor antagonist, eg. atropine, blocks cholinergic receptors to prevent the attachment of the neurotransmitter ACh.

1.10 Cholinergic Transmission

Acetylcholine is synthesized from acetyl coenzyme A (CoA) and choline by the action of choline acetyltransferase. The source of acetyl CoA is citrate, an intermediate in oxidative carbohydrate metabolism. Choline is either obtained from exogenous sources or "recycled" following synaptic hydrolysis of acetylcholine by active transport into the cholinergic nerve terminals (**Figure 1.4**). Choline acetyltransferase is found in the cytoplasm of nerve ending particles. The enzyme originates in the perikaryon and travels to the nerve terminal where it catalyses acetylcholine synthesis. Choline acetyltransferase (ChAT) has been partially purified from cell free extracts of the organ of the Amazonian electric eel by Nachmahnsohn and associates, playing a major role in elucidating its properties (Nachmahnsohn, 1959). Choline is the limiting factor in the synthesis of ACh and about 50% of the choline produced by cholinesterase activity is reutilized to synthesise new ACh. Choline acetyltransferase catalyses the final step in the synthesis of ACh i.e. the acetylation of choline with acetyl coenzyme A (Hebb, 1972; Collier *et al.*, 1976; Rossier, 1977).

1.10.1 The Mechanism of Cholinergic Transmission

After ACh synthesis, the neurotransmitter is stored in vesicles. The vesicles that house the newly synthesized ACh are one of the most consistent ultrastructural features of junctional tissues. In cholinergic nerve endings, the synaptic vesicles range in diameter from 200 - 400 **Å** units. These neurohumoral containers migrate toward the nerve terminal synaptic membrane during nerve stimulation and disgorge their ACh contents by exocytosis (**Figure 1.5**). Thus, the vesicle and synaptic membrane essentially fuse prior to the exocytotic emptying of ACh into the synaptic cleft.

Acetylcholine reversibly attaches to the postsynaptic membrane receptor and induces changes in the membrane character. The role of dissociation of the neurotransmitter-receptor union occurs rapidly to activate neuron activity with succeeding impulses. Upon release from cholinergic nerve endings, ACh is inactivated by several means. Enzymatic degradation of ACh is accomplished by cholinesterase, including true or specific cholinesterase. This enzyme, associated with and present

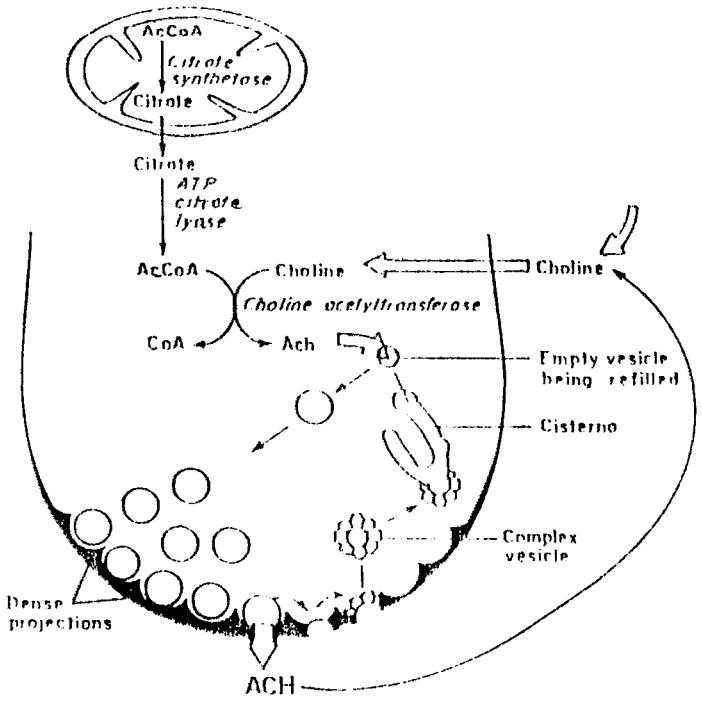


Figure 1.4 Postulated mechanisms of synthesis, storage and release of acetylcholine and the recycling of vesicles in cholinergic nerve endings (Bowman and Rand, 1980).

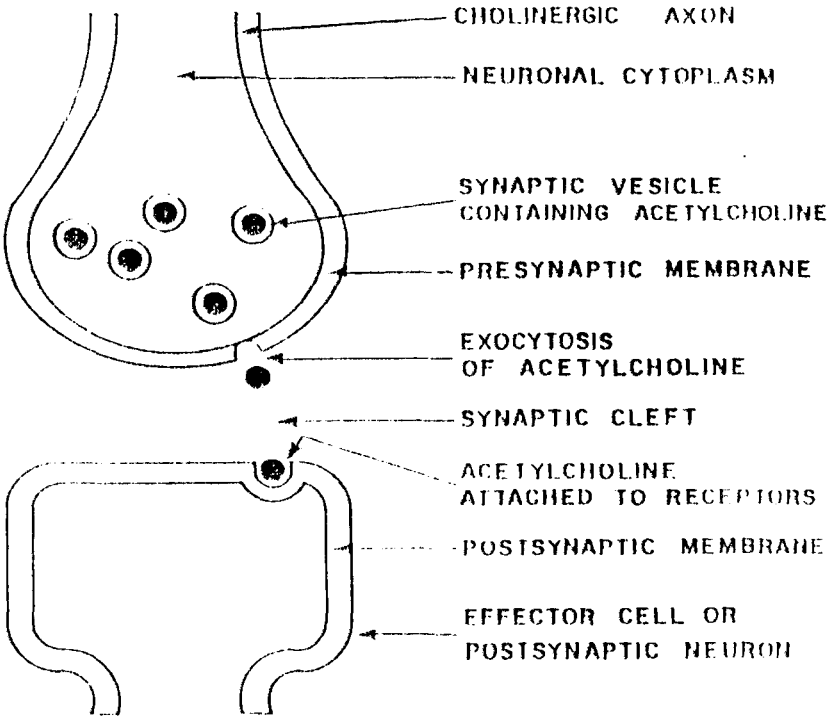


Figure 1.5 Cholinergic transmission.

at the surface of post-junctional membranes (Davis and Koelle, 1967; Couteaux, 1972), is preferential in its catabolism of ACh. Other sites containing true or specific cholinesterase (acetylcholinesterase) include the red blood cells and the placenta. Nonspecific cholinesterase catalyses the hydrolysis of other choline esters, especially butyrylcholine. For ACh to serve as the neurohumoral agent in peripheral junctional transmission, the ester must be removed or inactivated within the time limits imposed by the response characteristics of visceral neuro-effector junctions, motor end plate and various types of neurons (Dale, 1914).

Plasma, liver and glial cells contain the nonspecific cholinesterase. Non-enzymatic means of ACh inactivation involve binding to nonreceptor sites, diffusion away from the neuron receptor site and dilution in extracellular fluids.

1.11 The Acetylcholine Receptor

The acetylcholine receptor translates acetylcholine binding into changes in ionic permeability of the post-synaptic membrane. Membrane-bound, the ACh receptor is concentrated mainly at synaptic sites. The isolation and characterization of the ACh receptor was accomplished by using materials with a high ACh receptor concentration, such as the intact electroplax of the electric eel (*Electrophorus electricus*) and frog skeletal muscle (Colquhoun, 1975; Rang, 1973).

Isolation by subcellular fractionation techniques purifies the receptor material five to ten fold. Certain snake neurotoxins, such as bungarotoxin, bind specifically to post-synaptic ACh receptors. ACh receptor affinity labels (such as "bungarotoxin") are introduced *in situ* to irreversibly bind to the membrane-bound ACh receptor. By using iontophoresis or by radioligand receptor-displacement assays it was found that ACh binds to a mixture of nicotinic and muscarinic cholinergic receptors on the synaptic membranes (Appelt and Appelt, 1988 for reviews).

1.11.1 Nicotinic Acetylcholine Receptor

Three points of attraction exist between acetylcholine and the nicotinic acetylcholine receptor. The affinity triad on the ACh molecule consists of the quaternary nitrogen (1), the atom bridge separating the quaternary nitrogen from the carbonyl oxygen (2) and the carbonyl function (3).

An anionic function on the receptor surface attracts the quaternary nitrogen of the ACh molecule. The anionic receptor function is probably the carboxylate anions of aspartic or glutamic acids. These dicarboxylic amino acids are negatively charged at physiological pH and are strong candidates as the anionic affinity source on the ACh receptor. Two of the methyl groups on the quaternary nitrogen apparently add stability by enhancing the receptor cavity fit and binding through van der Waals forces.

The distance between the quaternary nitrogen on the methonium compounds (R_3-N^+-$(CH_2)_n$-R_3), as determined by the number of the methylene groups (CH_2), differentiates between nicotinic receptors at autonomic ganglia and neuromuscular junctions. A four to eight group carbon separation in the quaternary nitrogen functions produces compounds that are primarily autonomic ganglia blockers. Dale (1914) suggests that the two nicotinic receptor anionic sites at the neuromuscular junctions are located further apart. Thus, a separation of $(CH_2)_{10}$ is the preferred distance for blocking nicotinic receptors at the neuromuscular junction.

The biological translation of the acetylcholine-receptor combination is initially evidenced by a rapid influx of sodium ions and efflux of potassium ions through channels in the synaptic membrane. Depolarization of the synaptic membrane results from the ion flux and a specific physiological event occurs.

1.11.2 Muscarinic Acetylcholine Receptor

Physiological responses to muscarinic receptor activation usually occurs more slowly and less dramatically than with nicotinic stimulation. Studies on the structure-activity relation of muscarinic receptor blocking drugs, such as atropine sulphate, have demonstrated certain features of the muscarinic receptor. The distance between the nitrogen atom and the carbonyl oxygen of the ester portion of atropine approaches the separation distance of these functional groups in the ACh molecule. Consequently the positively charged nitrogen on ACh agonists or antagonists forms an ionic bond with an anionic subsite on the muscarinic receptor. Hydrogen bonding occurs between the carbonyl function of ACh agonists and antagonists and a cavity located at the esteratic subsite on the receptor substance.

Muscarinic receptors are divided into M_1 and M_2 designations. M_1 receptors are present on interneurons in autonomic ganglia and in certain regions of the central nervous system, whereas M_2 receptors are primarily present in cardiac tissue and gastro-intestinal smooth muscle.

Biochemical changes at postsynaptic sites are noted upon muscarinic agonist attachment to the receptor substance. M_2 receptors may be linked to carbonyl cyclase, in which inhibition of this enzyme occurs when these receptors are activated. M_2 receptor interaction with a guanine nucleotide-binding regulatory protein that binds guanosine triphosphate (GTP) may explain the adenyl cyclase inhibition. Another related theory hypothesizes that the combination of ACh (or other cholinergic agonists) with the M_2 receptor activates guanylate cyclase, which forms guanosine 3',5'-monophosphate (cyclic GMP). Muscarinic receptor activation is also characterized by calcium ion influx across synaptic membranes. M_1 receptors probably regulate Ca^{++} fluxes and the synthesis of phosphorylated derivatives of inositol.

Understanding of the events associated with activation of the muscarinic receptor remains small. Accumulation of cyclic guanosine 3',5'-monophosphate (cyclic GMP) (George *et al.*, 1970), enhanced permeability of monovalent cations (Burgen and Spero, 1968) and increased turnover of

phosphoinositol (Jafferji and Michell, 1976) have each been implicated in the sequence of steps that follows occupation of the receptor.

1.12 Cholinergic Drugs

Cholinergic drugs elicit their pharmacological responses by activating cholinergic receptors. These ACh receptors are classified as either muscarinic or nicotinic and their agonist activity is classically defined in terms of pharmacological response. Cholinergic drugs act on the ANS by either a direct action on ACh receptors or by an indirect effect that increases endogenous ACh concentration at synaptic and neuro-effector junctions. A variety of cholinergic drugs such as the direct and indirect acting cholinergic drugs, the anticholinesterase agents as well as cholinergic receptor blocking agents have been used in this study. A brief overview of their characteristics, structural features and mode of action are carried out in the sections which follow.

1.12.1 Direct acting cholinergic drugs

These direct acting cholinergic drugs are agonists at muscarinic and nicotinic receptor sites. ACh is an example of this type of cholinergic drug and is also an endogenous neurotransmitter at cholinergic synapses. Rapid hydrolysis by plasma cholinesterase causes the transient and fleeting action of ACh. ACh directly stimulates both muscarinic and nicotinic receptors depending on accessibility to the receptor site and dosage. This drug produces rapid and complete miosis by intracellular administration.

Carbachol is another direct acting cholinergic drug and is an ester of carbamic acid (rather than acetic acid as in the case of ACh) and choline. Carbachol is a potent muscarinic agonist that is not readily hydrolysed by acetylcholinesterase or non-specific choline esterase (because of the resistance of the carbamoyl group to degradation). The release of endogenous ACh from cholinergic nerve terminals contributes to the cholinergic response.

1.12.2 Anticholinesterase Drugs (Indirect Acting Cholinergic Drugs)

Anticholinesterase drugs block the enzymatic degradation of acetylcholine and causes its accumulation at cholinergic receptor sites. Several of the anticholinesterase drugs are toxic agents employed as insecticides or promoted as potential "nerve gases" in chemical warfare. However, some of these agents (including physostigmine, neostigmine and pyridostigmine) are useful as therapeutic agents because of their indirect cholinergic action.

The active centre of acetylcholinesterase consists of anionic and esteratic subsites. The anionic subsite on AChE combines with the positive nitrogen charge of choline by means of electrostatic or ionic bonding, comprising one of the attachment sites of ACh to the enzyme. At the esteratic subsite, a covalent bond forms with the carbonyl group of the acetate portion of ACh. The attachment of ACh to AChE in this manner orients the ACh molecule so that nucleophilic attack on the acyl carbon occurs. Thus, acetylation of the enzyme is followed by rupture of the ester linkage of acetylcholine and the elimination of choline. The acetylated enzyme then reacts with water to form regenerated active enzyme that has the capacity to hydrolyse 3×10^5 ACh molecules per enzyme molecule per minute.

1.12.3 Acetylcholinesterase Inhibition

Reversible inhibitors of AChE combine with the active anionic subsite or with a peripheral anionic subsite that is spatially removed from the active centre of the enzyme. Quaternary reversible AChE inhibitors interact by means of the quaternary nitrogen with the anionic subsite on the active site and by hydrogen bonding to the nitrogen at the esteratic subsite. Reversible acetylcholinesterase inhibitors that contain a carbamyl ester grouping, such as physostigmine and neostigmine, attach to the anionic subsite because of their quaternary (neostigmine) or tertiary (physostigmine) nitrogen. A carbamylated enzyme forms by the bonding at the esteratic subsite of AChE. The carbamylated intermediate is stable and its formation precludes the rapid enzymatic hydrolysis of ACh for a period of time. The interaction of a reversible inhibitor (substrate) with AChE (enzyme) depends

upon the anionic site attraction and the nature of the esteratic site interaction. Subsequent rapid reversibility of binding (edrophonium) or the formation of a stable carbamylated intermediate (physostigmine and neostigmine) characterise the reversible inhibitor-AChE interaction.

Irreversible inhibitors of AChE, the organophosphates, do not routinely interact with the anionic subsite at the active centre of AChE. Rather, the organophosphates form a stable complex by phosphorylating the enzyme.

1.12.4 Reversible Anticholinesterase Agents

Reversible anticholinesterase drugs inhibit the degradation of acetylcholine and permit its accumulation at muscarinic and nicotinic receptor sites.

1.12.4.1 Physostigmine (Eserine): Physostigmine is a naturally occurring alkaloid obtained from the Calabar bean and is a reversible inhibitor of acetylcholinesterase that forms a carbamylated intermediate with the enzyme. Physostigmine reacts primarily with AChE. The carbamylated intermediate of the enzyme so formed prevents the ACh-AChE interaction and spares endogenous ACh from enzymatic hydrolysis. The accumulation of ACh at autonomic ganglia and cholinergic neuro-effector junctions causes the pharmacological responses to physostigmine. The predominant effects that occur in therapeutic doses of physostigmine are muscarinic in nature.

Physostigmine contains a tertiary amine and because it is lipid soluble it readily passes the blood brain barrier. Consequently it possesses both peripheral and central cholinergic activity.

1.12.4.2 Neostigmine: A reversible cholinesterase inhibitor, neostigmine contains a quaternary nitrogen. This structural feature limits its passage into the CNS and imparts a direct action on peripheral cholinergic receptor sites. Neostigmine inhibits the hydrolysis of ACh by competing with ACh for attachment sites on AChE. By forming a carbamylated intermediate with AChE, neostigmine elicits anticholinesterase activity. Additionally, neostigmine has a direct action at the

motor end plate on skeletal muscle. This direct cholinomimetic activity sets the drug apart from physostigmine and makes the neuromuscular actions of neostigmine more pronounced than those of physostigmine. As well as augmenting gastric secretions, neostigmine also increases the motor activity of the gastro-intestinal tract.

1.12.5 Muscarinic Receptor Blocking Agents

Antimuscarinic drugs exert their pharmacological effects by competitive blockade of ACh receptors at the neuro-effector junction of postganglionic parasympathetic fibres and effector cells on smooth muscle, glands and visceral organs. The classical antimuscarinic drug is atropine, an alkaloid obtained by extraction from the plant *Atropa belladonna* (Deadly Nightshade). Atropine inhibits the action of ACh on smooth muscles, glands and visceral organs by blocking muscarinic receptors. Due to its structural similarity to ACh, atropine binds to muscarinic receptors, but does not elicit the response noted when ACh-receptor interaction occurs. The atropine-receptor combination prevents access of ACh to receptor sites, which produces an effective muscarinic block. The degree of muscarinic block depends upon the relative concentrations of atropine and ACh present in the receptor vicinity and represents a classic example of competitive inhibition in drug mechanisms.

The atropine molecule does not produce the identical conformational changes in the muscarinic receptor substance that ACh elicits. Rather, atropine produces molecular perturbations in the receptor substance that are sufficiently different from ACh so that the typical pharmacological response is absent. In summary, atropine is structurally similar enough to ACh to bind to muscarinic receptors, but the receptor fit is not as "tight" and does not produce the response noted with the ACh-receptor interaction. The bulky substituent group connected to the carbonyl function in the atropine molecule apparently prevents a "proper" fit in the receptor substance cavity that accepts this molecular portion of muscarinic agonists and antagonists.

1.12.6 Drugs blocking Nicotinic Receptors

Drugs that block nicotinic receptors including nicotine, lobeline and succinylcholine, usually produce a transient stimulation at these sites. Nicotinic blocking drugs either prevent the depolarizing action of ACh or produce a persistent depolarization of the postsynaptic membrane receptor substance. Some drugs (such as nicotine) produce an initial stimulation of ganglionic nicotinic receptors before effecting a final blockade of these same receptors.

1.12.6.1 Hexamethonium: Hexamethonium is the prototype C_6 compound that led to the development of newer ganglionic blocking drugs. The drug acts as a competitive inhibitor of ACh at nicotinic receptor sites in autonomic ganglia. They produce a ganglionic blockage by occupying receptor sites and by stabilizing the postsynaptic membranes against the actions of ACh liberated from presynaptic nerve endings.

1.13 A Summary of Recent Studies Based on the Cholinergic System in the Pineal Gland

The innervation of the mammalian pineal gland is mainly sympathetic and modulates the activity of indole pathway enzymes and secretion of pineal hormones (Cardinali 1981). This fact arose from the classical studies of Ariens-Kappers (1965) who proposed the sympathetic nature of the innervation of the mammalian pineal gland, originating from the superior cervical ganglion and entering the pineal gland *via* the conarian nerve. Furthermore, the main neurotransmitter released from these sympathetic fibres was noradrenaline (Wurtman and Moskowitz, 1977), which interacted with pineal ß-adrenergic receptor sites.

In addition to these receptors, recent studies have shown that the pineal glands also contain GABAergic receptors (Ebadi *et al.*, 1989), benzodiazepine receptors (Lowenstein and Cardinali, 1983), glutamatergic receptors (Govitrapong *et al.*, 1986), D_2-dopaminergic receptors (Govitrapong

et al., 1984) and substance *P* receptors (Govipatrong and Ebadi, 1986). Furthermore, it has been shown that in addition to its sympathetic innervation, the mammalian pineal glands also receive a distinct central pinealopetal innervation (Korf and Moller, 1984). The possible existence of cholinergic pineal innervation in various species is controversial.

Cholinergic neurons are distributed widely in the autonomic nervous system. For examples, the preganglionic neurons of both sympathetic and parasympathetic nervous systems are cholinergic. Furthermore, the postganglionic parasympathetic neurons are mainly cholinergic. In addition while the majority of postganglionic sympathetic fibres are adrenergic, the fibres supplying the sweat glands, vessels of skeletal muscles in some species and vessels of tongue muscles are all cholinergic in nature (Dale, 1937; Gabella, 1976). The development and use of immunohistochemical methods for measuring both cholinesterase and choline acetyltransferase have provided excellent tools for the localization of cholinergic neurons in various areas of CNS (Butcher and Woolf, 1984; Nitecka and Frotsher, 1989).

The origin of cholinergic fibres innervating the bovine pineal gland, which may involve the habenula, needs to be studied and delineated carefully. Nevertheless, there is evidence that in addition to serotonin and noradrenaline, sympathetic fibres containing acetylcholinesterase do exist in the rat pineal gland (Eranko *et al.*, 1970; Eranko and Eranko, 1971). Moreover, neurohistological studies involving silver impregnation technique using mammalian pineal glands including those of humans (Scharenberg and Liss, 1965) have shown that nerve fibres passing *via* the habenula or posterior commissure may intermingle and associate functionally with pineal parenchyma. In addition, recent immunohistochemical studies have demonstrated that choline acetyltransferase immunoreactive cells are indeed present in the habenula (Vincent *et al.*, 1986).

The functions of ACh and cholinergic receptors in the pineal gland are not known. However fragmentary evidences gathered thus far denote that ACh does influence pineal functions. For examples:

(i) The injection of melatonin or ACh into the cisterna magna of rabbits increases the concentration of 3'5'cyclic guanosine monophosphate (cGMP) (Rudman, 1976).

(ii) The intraventricular injection of carbachol, a cholinergic receptor agonist, causes a rapid reduction in the activity of serotonin N-acetyltransferase. Furthermore, mimicking the effects of light exposure, carbachol also causes a shift to the right on the temporal patterns of NAT (Zatz and Brownstein, 1979).

(iii) Atropine methylbromide, a cholinergic receptor antagonist, inhibits the activity of HIOMT in constant darkness, whereas oxotremorine oxalate, a cholinergic receptor agonist, restores it (Wartman *et al.*, 1969).

(iv) Studies involving micro-electrophoretically applied melatonin, NA and ACh indicated that the responses of pinealocytes to these agents were non-uniform in nature (Semm and Vollrath, 1981) suggesting that pinealocytes may possess the biochemical mechanisms to respond uniquely and differently to various neurotransmitters.

(v) ACh is able to lighten some frogskins (Moller and Lerner, 1966; Goldman and Hadley, 1968), and atropine, the cholinergic receptor antagonist, causes melanosome dispersion (Tercafs, 1966).

(vi) In rat pineal glands in culture, ACh (10^{-6} M) stimulated the release of arginine vasotocin (Sartin *et al.*, 1979).

Schrier and Klein (1974) were unable to identify choline acetyltransferase (ChAT) activity in pineals of rat or rabbit, whereas Rodriguez de Lores Arnaiz and Pellegrino de Iraldi (1972) showed that the presence of ChAT activity in the rat pineal gland can be detected even after bilateral superior cervical ganglionectomy (SCGX). This suggests that the presence of cholinergic terminals do not originate in these ganglia. In addition, Moller and Korf (1983) and Romijn (1973) reported finding pineal nerve terminals containing small clear vesicles that stained intensely for AChE. These structures did not disappear after SCGX and were considered as cholinergic vesicles.

In fact, muscarinic binding sites found in the pineal gland were not distinguishable from those identified as muscarinic receptors in other tissues (Taylor *et al.*, 1980). The number of sites did

not decrease after SCGX, indicating that muscarinic receptors were not exclusively located in sympathetic terminals (Taylor *et al.*, 1980). The first positive evidence for the existence of functional muscarinic receptors in the pineal gland, however, was the demonstration of an acetylcholine-stimulated increase in the turnover rate of phosphatidylinositol (Basinka *et al.*, 1973). Laitinen *et al.*, (1989b) suggested that the activation of the muscarinic M_1-receptor by carbachol produced an accumulation of inositol monophosphates, a finding that confirms and extends the report by Basinka *et al.*, (1973).

Indirect evidences supporting the presence of cholinergic fibres (Labella and Shin, 1968; Eranko *et al.*, 1970; Eranko and Eranko, 1971; Trueman and Herbert, 1970; Rodriguez de Lores Arnaiz and Pellegrino de Iraldi, 1972; Romijn, 1973, 1975a; David and Herbert, 1973; David and Kumar, 1978; Matsushima and Reiter, 1978; Burt and Taylor, 1980) or the absence of cholinergic fibres (Schrier and Klein, 1974) in the pineal gland have appeared in the literature.

In addition to the large number of terminals containing NA and serotonin, other neurotransmitters such as gamma-aminobutyric acid, taurine, octopamine and dopamine have been found in the pineal gland (Ebadi, 1984). Only terminals containing small dense cored vesicles, catecholaminergic in nature, disappear with SCGX, while those containing mostly large dense-cored vesicles, probably peptidergic, and small clear vesicles, probably cholinergic, remained unaffected (Cardinali *et al.*, 1987). These observations imply a complex neuronal regulation of pineal activity. Furthermore, ChAT activity was detected in pineal homogenates, suggesting that the neurotransmitter could be synthesized *in situ.*

A necessary requirement for neurotransmitter-mediated effects on a target tissue is the presence of specific receptors. Accordingly, Finocchiaro *et al.*, (1989) found that [^{3}H]QNB-binding sites in the pineal membranes showed similar properties to muscarinic receptors in other tissues confirming a previous report by Taylor *et al.*, (1980). [^{3}H]QNB binding sites in the pineal described above are so similar to those identified as muscarinic receptors in other tissues eg. heart (Fields *et al.*, 1978), brain (Yamamura and Snyder, 1974b), ileum (Yamamura and Snyder, 1974a), parotid (Talamo *et*

al., 1979) and pituitary (Burt and Taylor, 1980), that there can be little doubt that they represent muscarinic receptors. There is, however, virtually an absence of corroborating functional data.

Indeed only the possible existence of ACh-stimulated increase in the turnover in phosphatidylinositol (Basinka *et al.*, 1973) regularly found to accompany muscarinic receptor activation in other tissues (Heilbronn and Bartfai, 1978) may be taken as positive functional evidence for muscarinic receptors in the pineal. Even here there is a counterbalancing negative report (Eichberg *et al.*, 1973) as well as a similar negative report for effects of ACh on pineal cyclic GMP content (Sharma and Banerjee, 1978).

Although pineal muscarinic receptors were found in relatively low concentrations, they have a definite functional activity. Their existence raises interesting questions about the possible location and functional role of the muscarinic receptors in the pineal. The likelihood remains that an appreciable portion of them are on sympathetic terminals where their activation may stimulate the release of noradrenaline, as demonstrated in the hypothalamus (Engberg and Stevensson, 1980) and septal area where ACh liberates serotonin through a mechanism mediated by NA (Pirola *et al.*, 1987). A postsynaptic action of the muscarinic agonist (Finocchiaro *et al.*, 1989) may be suggested since the number of muscarinic binding sites are not decreased after SCGX (Moller and Korf, 1983).

The study done by Finocchiaro *et al.*, (1989) rules out any action due to muscarinic receptors located in pineal vessels, as suggested in the case of muscarinic effects on blood flow associated with secretory stimuli in the posterior pituitary (Sooriyamoorthy and Livingston, 1972). Hence some of these receptors might therefore be located in pineal neurons or pinealocytes.

1.13.1 Muscarinic Acetylcholine Receptor Genes

Muscarinic receptors mediate many of the actions of ACh in the central and peripheral nervous systems (Nathanson, 1987). Two pharmacologically distinct classes of such receptors, M_1 and M_2,

have been defined on the basis of their affinities, high and low respectively, for the antagonist pirenzepine (Hammer *et al.*, 1980). However a variety of heterogeneous properties of muscarinic receptors have not always correlated well with this classification which suggests that there may be other classes. It has been unclear whether the apparently different receptors represent modifications of a single receptor or whether they are different proteins. Recently a porcine brain muscarinic receptor complementary DNA (cDNA) was cloned and expressed in *Xenopus* oocytes to produce functional receptors (Kubo *et al.*, 1986). The presence of related messenger RNA (mRNA) in tissues rich in M_1 receptors and its absence in tissues rich in M_2 receptors suggested that there are more than one muscarinic ACh receptor gene.

Muscarinic receptors are members of a large class of neurotransmitter hormone and light receptor which act through binding to and activation of guanosine 5'-triphosphate (G) binding proteins. The activated G proteins can stimulate or inhibit adenylate cyclase, stimulate phosphatidylinositol turnover and directly regulate ion channels thereby affecting a variety of cellular responses (Gilman, 1984; Stryer and Bourne, 1986; Spiegel, 1987).

1.13.2 Cholinergic Stimulation of Phosphoinositol Hydrolysis

Muscarinic receptors transduce agonist signals by activating G proteins to regulate ion channel activity and to generate second messengers. It has been reported that four different genes code for muscarinic receptors (Kerslavage *et al.*, 1987). Studies on second messenger coupling of muscarinic receptor subtypes (M_1 - M_4) suggest that the expression of rat muscarinic M_1 and human M_1 and M_4 receptor genes in cells lacking endogenous muscarinic receptors can lead to a complete appearance of the phosphoinositol (PI) signalling pathway and that the magnitude of the signal produced is proportional to the level of gene expression for these receptors (Dudley *et al.*, 1988; Peralta *et al.*, 1988). Expression of human M_1 and M_3 subtypes in embryonic kidney cells was coupled to PI hydrolysis to a lesser extent (Peralta *et al.*, 1988).

Laitinen *et al.*, (1989b) suggest the presence of a functional ACh receptor in the rat pineal which are coupled to the PI signalling pathway. Furthermore, the relative potency of muscarinic antagonists to block the PI accumulation suggests that these receptors are of M_1 subtype. These receptors, as judged by the PI response, were not affected by the removal of the sympathetic innervation to the gland.

In line with this, Taylor *et al.*, (1980) reported no changes in [^{3}H] quinuclidinyl benzylate ([^{3}H]QNB) binding in rat pineals after bilateral ganglionectomy. On the other hand, ganglionectomy resulted in enhanced adrenergic stimulation of pineal PI turnover. In other studies, both the PI response to NA (Zatz, 1985) and the density (but not the affinity) of α-adrenoreceptors (Sugden and Klein, 1985) have been shown to double after bilateral SCGX. All these results indicate supersensitivity of the rat pineal to adrenergic stimulus after removal of the sympathetic innervation.

Although the muscarinic receptor density in rat pineals (Taylor *et al.*, 1980) seems to be only a fraction of that reported for α-adreno receptors in the gland (Sugden and Klein, 1985), the PI response for submillimolar concentrations of both muscarinic acid and adrenergic agonists was very similar (Laitinen *et al.*, 1989b). The reason for this is unclear at the moment. Taken together, the results of the study done by Laitinen *et al.*, (1989b) suggest that ACh could modulate pineal function through the stimulation of PI turnover.

CHAPTER 2

ORGAN CULTURE STUDIES

2.1 Introduction

The pineal gland is one of the most highly perfused organs in the body and is highly vascularized second only to the kidney (Kappers, 1976). Various drugs are capable of affecting pineal indole synthesis and metabolism. Ideally, a technique is required that is sensitive enough to determine the normal functioning of the gland as well as to detect changes in activity upon drug manipulation *in vitro*.

Strangeways and Fell (1926) were amongst the first workers to employ the organ culture technique. At the time, the method was used chiefly for the culture of embryonic rudiments. More recently, refinements in biochemical techniques have led to an increase in the use of organ culture systems for the study of physiological and biochemical properties of living tissue. Trowell (1959) successfully employed a technique that allowed a number of fully differentiated organs, or parts thereof, to be kept viable *in vitro* without either growth or dedifferentiation, thus paving the way for many experimental studies.

Many researchers have successfully developed organ culture techniques for the pineal gland (Alphs *et al.*, 1980; Klein and Notides, 1969; Axelrod *et al.*, 1969; Shein and Wurtman, 1969). The technique of pineal gland organ culture is rendered suitable by virtue of its favourable size and accessibility (Trowell, 1959). It is also a useful technique since it eliminates the complexities of organ interaction and allows for direct pharmacological manipulation. Rat pineal glands cultured in our adequately aerated medium and incubated at 37°C have been shown to be viable for at least 6 days (Klein, 1969).

2.2 Theory of Assay

The pineal gland in culture is able to utilize an exogenous radioactive precursor (tryptophan, 5-hydroxytryptophan or serotonin) to synthesize various indole metabolites associated with pineal metabolism. It has been shown that approximately 95% of the synthesized radioactive indoles are secreted into the culture medium during the incubation period. It is usual to incubate rat pineal glands for 24 hours after which incubation is terminated by the removal of the gland from the medium (Klein and Rowe, 1970; Skene, 1985). The radioactive indoles are then separated, most commonly using bidimensional thin layer chromatography (TLC) (Klein and Notides, 1969; Balemans *et al.*, 1978; Daya and Potgieter, 1982) and quantitated with the aid of radiospectrometry.

In the following sections, the procedures for pineal organ culture, TLC separation of radioactive indole metabolites and quantification of labelled indoles using radiospectrometry are described. The pineal glands take up the radioactive precursor and incorporates it as part of its normal biosynthetic pathway, synthesizing radioactive metabolites and releasing them into the culture medium. The metabolites are then separated and quantitated using among other methods, bidimensional thin layer chromatography and liquid scintillometry. This method provides a more quantitative picture of pineal indole metabolism and has been shown to be sensitive to pharmacological manipulations (Klein and Rowe, 1970; Balemans *et al.*, 1983; Voisin *et al.*, 1983; Daya and Potgieter, 1985) as well as adequately detecting changes in pineal indole metabolism (Daya and Fata, 1986).

2.3 ORGAN CULTURE TECHNIQUE

2.3.1 Materials and Methods

2.3.1.1 Chemicals, drugs and reagents

5 Hydroxy (side-chain-2-^{14}C) tryptamine creatine sulphate (specific activity 56 mCi/mmol) was purchased from Amersham, England; aluminium TLC plates coated with silica gel 60 F_{254} (0.20mm) from Merck, Germany; and BGJ_b culture medium (Fitton-Jackson modification) from Gibco, Europe. Beckman Ready-Solv multipurpose premixed liquid scintillation solution was obtained from Beckman RIIC Ltd, Scotland. The antibiotics benzyl penicillin and streptomycin were purchased from Hoechst, SA; and amphotericin B from Squibb Laboratories, SA. All standard indoleamines, melatonin (aMT), N-acetyl serotonin (aHT) and hydroxy and methoxy indoles were purchased from Sigma Chemical Co, USA. All other chemicals and reagents were obtained from local commercial sources.

2.3.1.2 Animals

Inbred adult male albino rats of the Wistar strain were used throughout this study. Female rats were not included in the experiments because of the variation in the [^{14}C] serotonin metabolism noted in female rats in different stages of the estrus cycle (Daya, 1982). Variation was reported in the HIOMT sensitivity to oestradiol during the estrus cycle of female rats (Cardinali, 1981; Daya and Potgieter, 1982). All animals were maintained under artificial illumination with a daily photoperiod of 12 hours (lights on at 06h00). The light intensity during the light phase was approximately 300µWatts/cm^2 provided by cool white fluorescent tubes. As far as possible, the temperature of the living quarters was kept constant (20-24°C) and an extractor fan ensured the constant removal of stale air. All animals were housed in groups of 4 or 5 in opaque white plastic cages. A metal grid served as a floor in each cage. Animals were maintained on a diet of standard rat pellets *ad libitum* and tap water and weighed between 200-300g at the time of sacrifice.

Rats were killed swiftly by cervical dislocation and decapitation. Using a pair of scissors an incision was made through the bone from the foramen magnum to near the orbit. A clean, sterile dissecting forceps was inserted into this incision and the skull and adhering brain was lifted and folded back. The brain was gently peeled away from the dura and the skull, exposing the pineal gland. The small pearl white structure was often found adhering to the skull. Rarely, when this did not occur, the pineal gland could be found in the groove between the cerebral hemispheres, anterior and cephalic to the colliculi. Forceps were inserted into this groove and the pineal gland was easily removed, placed on a cold stainless steel spatula and dissected free of the pineal stalk and any adhering tissue. Once perfected this procedure could easily be accomplished within 30 seconds. Pineals were then placed in organ culture tubes as described in [**2.3.1.3**].

2.3.1.3 Organ Culture of Pineal Glands

Following decapitation, the rat pineal glands were exposed and removed as described in [**2.3.1.2**]. The glands were transferred to sterile (borosilicate 75x10mm) culture tubes containing 52µl culture medium. The culture medium consisted of BGJ_b medium (Filton-Jackson modification), supplemented with benzylpenicillin sodium, streptomycin sulphate and amphotericin B. The components of the culture medium and their respective concentrations are listed in **Table 2.1**. Each culture tube thus contained one pineal which floated just below the surface of the medium. To this, 8µl of the substrate [^{14}C] serotonin (0.4 µCi) was added. The air in the tube was displaced with carbogen (95%O_2/5%CO_2), and once the atmosphere was saturated the tubes were tightly sealed with parafilm. The culture tubes were placed in a Forma Scientific model 3028 incubator and the pineals were incubated in the dark at 37°C for 24 hours.

In experiments involving drug addition to the culture medium, 10µl of the drug was added to 52µl of the culture medium containing the pineal gland prior to the addition of the 8µl aliquot of [^{14}C] serotonin. Where basal pineal [^{14}C] serotonin metabolite levels had to be determined, 10µl of vehicle was substituted for the drug. After the 24 hour incubation period the reaction was stopped by the removal of the pineal glands from the culture medium.

TABLE 2.1 Constituents of BGJb culture medium (Fitton-Jackson modification)

CONTENTS	*CONCENTRATION (mg/1)*
Amino Acids	
L-Alanine	250.00
L-Arginine	175.00
L-Aspartic acid	150.00
L-Cysteine HCl	90.00
L-Glutamine	200.00
Glycine	800.00
L-Histidine	150.00
L-Isoleucine	30.00
L-Leucine	50.00
L-Lysine HCl	240.00
L-Methionine	50.00
L-Phenylalanine	50.00
L-Proline	400.00
L-Serine	200.00
L-Threonine	75.00
L-Tryptophane	40.00
L-Tyrosine	40.00
DL-Valine	65.00
Inorganic Salts	
Dihydrogen sodium ortho phosphate	90.00
Magnesium sulphate $7H_2O$	200.00
Potassium chloride	400.00
Potassium dihydrogen phosphate	160.00
Sodium bicarbonate	3 500.00
Sodium chloride	5 300.00

Continued on next page ...

CONTENTS	CONCENTRATION (mg/l)
Vitamins	
α-Tocopherol phosphate	1.00
Ascorbic acid	50.00
Biotin	0.20
Calcium pantothenate	0.20
Choline chloride	50.00
Folic acid	0.20
Inositol	0.20
Nicotinamide	20.00
Para aminobenzoic acid	2.00
Pyridoxal phosphate	0.20
Riboflavin	0.20
Thiamine HCl	4.00
Vitamin B_{12}	0.04
Other Components	
Calcium lactate	555.00
Glucose	10 000.00
Phenol red	20.00
Sodium acetate	50.00

2.3.1.4 Thin Layer Chromatography Separation of the Indoles

The technique employed was a minor modification of the method of Klein and Notides (1969). To measure the relative amounts of [^{14}C] indoles present, a 10µl aliquot of culture medium was spotted on a 10cm x 10cm TLC plate (Silica gel 60, Type F_{254} 0.25mm x 20cm x 20cm aluminium plates cut to 10cm x 10cm from Merck, Germany).

Following this, a solution containing synthetic unlabelled standards of all the pineal indoles to be measured was spotted on top of the culture medium spot. A total of 10µl of a solution containing 2µg of each standard was spotted. The indole standard solution was prepared as follows : 1mg of each standard indoleamine was dissolved together in a test tube containing 2.5ml of 95% ethanol. To this, 2.5ml of a solution of 1% ascorbic acid in 0.1N HCl was added. The ascorbic acid was included by Klein and Notides (1969) to protect indoles against oxidation. The solution was stored in darkness at -20°C. Morton (1987) reported no difference between plates spotted and run in normal light as opposed to those spotted and run in the dark (as suggested by the original method). In all instances the TLC plates were spotted in subdued light and a gentle stream of nitrogen was used to dry the spots which were no larger than 0.5cm to prevent atmospheric oxidation of the indole metabolites.

Each spotted plate was then placed in a TLC tank and developed twice in the same direction in a solvent containing chloroform : methanol : acetic acid (93:7:1). The total front movement allowed during each development was 9cm. Once the solvent had reached this point (±25min) the plates were taken out of the TLC tanks and the position of the solvent front was marked. These were dried under nitrogen, then placed back into some solvent and run once again in the same direction up to the mark. Following this, the plate was developed once in ethyl acetate at right angles to the first direction and with a total front movement of 7cm. The plate was then dried under nitrogen evaporating off the ethyl acetate sprayed with Van Urk's reagent (lg of 4-dimethylamino benzaldehyde dissolved in 50ml of 25% HCl, followed by the addition of 50ml of 95% ethanol) and dried in an oven at 60°C for 20min to allow for development of the spots.

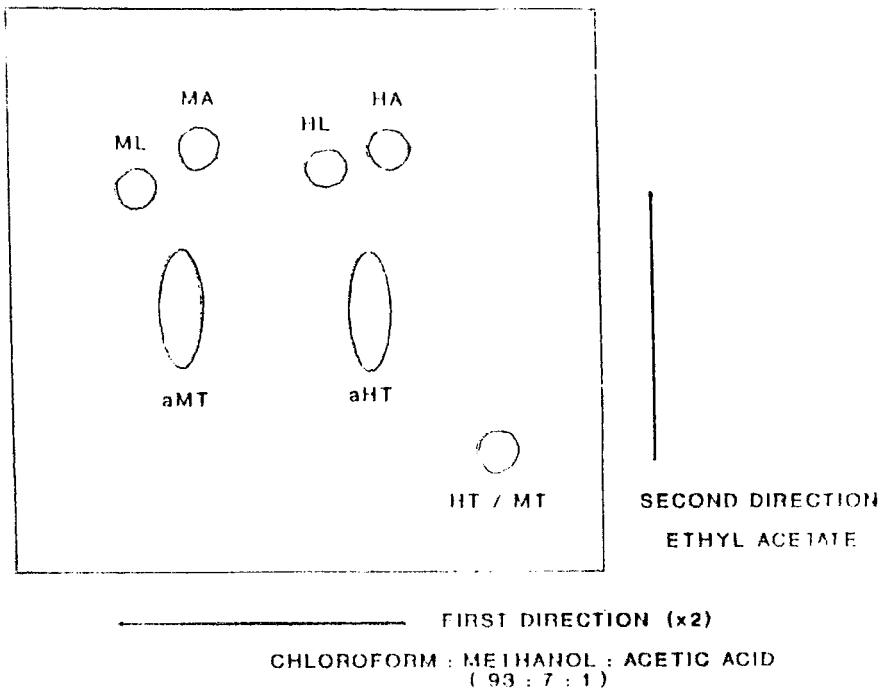
The spots were then cut out and placed into plastic scintillation vials. One millilitre of absolute ethanol was added to the vials together with 3ml scintillation cocktail fluid (Beckman Ready-Solv HP-B) and shaken on a Vortex Rotor-mixer Deluxe for 30 seconds. The radioactivity was quantitated at a counting efficiency of 79.58% in a Beckman LS 2800 scintillation counter and each vial was counted for a total of 5 minutes.

2.3.2 Results

A typical bidimensional thin layer chromatogram of the indole metabolites are presented in **Figure 2.1**. Adequate separation of all the indoles was achieved. Nomenclature for the pineal indoles follows the system suggested for international adoption by Smith (1982). A list of indoles together with abbreviations is presented in **Figure 2.1**.

2.3.3 Discussion

Using TLC the ability to separate N-acetylserotonin (aHT), melatonin (aMT), 5-hydroxyindole acetic acid (HA), 5-hydroxytryptophol (HL), 5-methoxyindole acetic acid (MA) and 5-methoxytryptophol (ML) was confirmed. This system has recently been validated by Skene (1985). This author was able to confirm that the removal of aliquots of medium present an accurate picture of the indole synthesis within the isolated pineal gland, as well as determining that the radioactivity on the TLC plates was present within the area of the standard spots. Skene was also able to demonstrate a recovery from the medium of expected metabolites in excess of 90%. Although Morton (1987) does not include acetic acid in solvent A, it was found that acetic acid was essential for good separation of the ML and MA indoles. Hence acetic acid was included in solvent A.



HT - Serotonin

MT - 5-Methoxytryptamine

aHT - N-Acetylserotonin

aMT - Melatonin

HA - 5-Hydroxyindole acetic acid

HL - 5-Hydroxytryptophol

MA - 5-Methoxyindole acetic acid

ML - 5-Methoxytryptophol

Figure 2.1 A trace of TLC plate showing the direction in which the solvents were run, and the subsequent location of the pineal indole metabolites. Nomenclature as to Smith (1982).

2.4 Experiment 1 : THE EFFECT OF CARBACHOL ON PINEAL INDOLE METABOLISM.

2.4.1 Introduction

Innervation of the mammalian pineal gland is mainly sympathetic and this modulates the activity of the indole pathway enzymes and secretion of pineal hormones (Cardinali, 1981). Noradrenaline is released from sympathetic nerves inducing the production of the pineal hormone melatonin through the rate limiting enzyme N-acetyltransferase (NAT). The diurnal rhythm in melatonin synthesis by the pineal is under direct control of this system (Cardinali, 1981). By using tritiated muscarinic receptor antagonist quinuclidinyl benzilate ([^{3}H] QNB) as a ligand, Govitrapong *et al.*, (1989b) identified and characterized muscarinic cholinergic receptors in the pineal glands of cattle. The aim of this experiment was to ascertain the effect of carbachol in the rat pineal gland using organ culture techniques, confirming the presence of these receptors in the pineal.

2.4.2 Materials and Methods

2.4.2.1 Materials

Carbachol was purchased from Sigma Chemical Co., USA; and noradrenaline was bought from Merck, Germany. All other chemicals and reagents were obtained from previously acknowledged sources [**2.3.1.1**].

2.4.2.2 Animals

Adult male Wistar rats (250-280 g.b.wt) were used for this series of experiments and were housed in groups of 4 to 5 animals per cage as described in [**2.3.1.2**]. The rats were killed by cervical dislocation and decapitation and their pineal glands removed.

2.4.2.3 Methods

The technique of pineal organ culture and the analysis of labelled indoles was performed as outlined in [**2.3.1.3**]. Fifteen pineal glands were rapidly collected and cultured individually in the BGJ_b culture medium containing 0.4 µCi of [^{14}C] serotonin. Five pineal glands were cultured in the presence of carbachol ($10^{-5}M$) alone, another five pineal glands in the presence of a combination of carbachol (10^{-5}) and noradrenaline ($10^{-5}M$) and a further five pineal glands were incubated in the absence of drug to act as a control group. The pineal glands were cultured at 37°C with a relative humidity of 95% and in an atmosphere of $5\%CO_2$:$95\%O_2$. Aliquots of culture medium was removed after 24 hours of incubation. The [^{14}C] indoles in the samples were isolated using bidimensional TLC and quantitated with the aid of liquid scintillometry as described in [**2.3.1.4**].

2.4.2.4 Data Analysis and Statistics

When using the pineal as an experimental tool it would seem logical to use the two indole metabolites aHT and aMT, as biological markers of pineal activity. These two metabolites represent the two products of two primary enzymes, NAT and HIOMT, in indole metabolic pathways. Skene (1985) suggested that an overall gauge of pineal activity could be the sum of the N-acetylated product (ie aHT and aMT). The results are therefore primarily analysed using these values. However it would not be fair to ignore the other indoles produced, especially the hydroxyindoles HA and HL which are produced in such great quantity. As these two hydroxyindoles are further methylated by HIOMT, to MA and ML, respectively, any correlations evident here could be used as a possible indicator of HIOMT activity.

Data are expressed as dpm/10µl medium/pineal gland calculated as the mean ± SEM(n=5). Owing to the multiple combinations of treatment within a single experiment a One-Way ANOVA followed by Neuman-Keuls Multiple Range test was applied for comparison of groups.

2.4.3 Results

The results are presented in **Table 2.2** showing all the metabolites separated by TLC, with the exception of the starting spot which contains both HT and MT. The muscarinic agonist carbachol increased aHT levels significantly ($p<0.05$) whilst aMT levels remained unaltered.

The presence of carbachol did not have any influence on the noradrenaline-induced rise in melatonin ($p<0.01$).

2.4.4 Discussion

Carbachol is a parasympathomimetic agent causing muscarinic cholinergic receptor stimulation. The drug caused a rise in the indole aHT and left aMT levels unaltered. The drug did not affect the adrenergic system with reference to melatonin synthesis. A daily rhythm in circulating melatonin occurs in all mammals (Klein, 1979). In the rat this is generated by a large rhythm in the activity of pineal arylamine N-acetyltransferase (Klein, 1979; Klein and Weller, 1970; Klein and Berg, 1970; Klein and Weller, 1973), the enzyme that converts serotonin to the intermediate precursor of melatonin, N-acetylserotonin. The activity of N-acetyltransferase is regulated by a neural circuit that stimulates the nocturnal release of noradrenaline from sympathetic nerves in the pineal gland (Klein *et al.*, 1971; Moore and Klein, 1974; Klein and Moore, 1979; Brownstein and Axelrod, 1974). Noradrenaline acts through an adrenergic mechanism to increase intracellular cAMP (Strada *et al.*, 1972; Deguchi and Axelrod, 1973) which induces and activates N-acetyl transferase (Klein and Berg, 1970; Klein and Weller, 1973) leading to an increase in melatonin production (Klein *et al.*, 1970; Shein and Wurtman 1969). The parasympathomimetic, carbachol was unable to affect this noradrenergic increase in melatonin production suggesting some other functional role for cholinergic receptors in the pineal gland. Laitinen *et al.*, (1989a) suggested that activation of the muscarinic M_1-receptor by carbachol produced an accumulation of inositol monophosphates, a finding that confirms and extends a previous report by Basinka *et al.*, (1973).

TABLE 2.2 The effect of carbachol (10^{-5}M) and a combination of carbachol and noradrenaline (CAR+NA) (10^{-5}M) on [^{14}C] serotonin metabolism in rat pineal glands.

Metabolites	Control	CAR	Significance	CAR + NA	Significance
ML	662 ± 325,76	477,25 ± 145,25	NS	179,20 ± 62,28	NS
MA	388,25 ± 130,61	224,50 ± 37,82	NS	174,20 ± 41,29	NS
aMT	1 619,25 ± 250,79	1 203,50 ± 365,21	NS	3 855,00 ± 777,20	(a) p<0.01
HL	8 594,50 ± 2 644,22	14 844,25 ± 2 534,51	NS	10 346,40 ± 3 368,85	NS
HA	27 663,75 ± 4 083,01	27 035,00 ± 4 437,87	NS	21 745,60 ± 3 408,38	NS
aHT	990,00 ± 207,67	4 477,75 ± 1 667,78	(a) p<0.05	3 504,00 ± 612,54	NS
5HT/5MT	67 020,00 ± 5 624,13	54 090,50 ± 4 287,82	NS	65 126,00 ± 8 951,35	NS

10µl aliquots of medium were chromatographed by TLC. Results are expressed as dpm/pineal gland (mean ± SEM; n=5 individually cultured pineal glands per group). Significance is computed using One-Way ANOVA followed by Neuman-Keuls Multiple Range test.

a) significantly increased w.r.t vehicle control.

2.5 Experiment 2: THE EFFECT OF ACETYLCHOLINE AND HEXAMETHONIUM ON PINEAL INDOLE METABOLISM.

2.5.1 Introduction

Identification of neurotransmitter receptor sites by chemical measurements of direct binding has been reported for nicotinic cholinergic receptors in the mammalian neuromuscular junction (Berg *et al.*, 1972; Fambrough and Hartzell, 1972; Hartzell and Fambrough, 1972; Miledi and Potter, 1971), and central nervous system (Bosman 1972; De Robertis, 1971; De Robertis *et al.*, 1969; Eldefrawi and O'Brien, 1970) and for the glycine receptor in the mammalian central nervous system (Young and Snyder, 1973).

The aim of this experiment was to determine whether the rise in the indole aHT induced by carbachol was indeed caused by muscarinic receptor stimulation and not through nicotinic receptors. Acetylcholine is a general agonist of the muscarinic and nicotinic receptors whilst the drug hexamethonium is a ganglion blocking agent inhibiting the transmission of the nicotinic receptor. By using these two drugs individually and in combination it is possible to investigate their effects on the pineal indole metabolic pathway.

2.5.2 Materials and Methods

2.5.2.1 Materials

Acetylcholine chloride as well as hexamethonium was purchased from Sigma Chemical Co., USA. All other chemicals and reagents were obtained from previously acknowledged sources [**2.3.1.1**].

2.5.2.2 Animals

Adult male Wistar rats (250-280g.b.wt) were used and maintained under an automatically regulated lighting cycle of LD12:12. Their living environment is described in detail in [**2.3.1.2**].

2.5.2.3 Methods

The technique of pineal organ culture and the analysis of labelled indoles was performed as outlined in [**2.3.1.3**]. Fifteen pineal glands were rapidly collected and cultured individually in BGJ_b culture medium containing 0.4µCi of [^{14}C] serotonin. Five pineal glands were cultured in the presence of acetylcholine (10^{-5}M), another five with a combination of hexamethonium (10^{-5}M) and acetylcholine (10^{-5}M) and a further five pineal glands were incubated in the absence of drug to act as a control group. The pineal glands were cultured at 37°C with a relative humidity of 95% and in an atmosphere of 5%CO_2:95%O_2. Aliquots of culture medium was removed after 24 hours of incubation. The [^{14}C] indoles in the samples were isolated using bidimensional TLC and quantitated with the aid of liquid scintillometry as described in [**2.3.1.4**]. The significantly different groups were identified using a One-Way ANOVA followed by Neuman-Keuls Multiple-Range test.

2.5.3 Results

The results are presented in **Table 2.3** showing all the metabolites separated by TLC with the exception of the starting spot which contains both HT and MT. Acetylcholine induced a rise in aHT production ($p<0.001$) whilst aMT levels remained unaltered. Hexamethonium does not alter the acetylcholine induced rise in aHT ($p<0.025$).

2.5.4 Discussion

The drug acetylcholine resulted in increased aHT levels whilst aMT levels remained unaltered. Hexamethonium did not alter this effect of acetylcholine and aHT levels still rose after applying the ganglion blocking agent. It may be suggested that the aHT increase is not induced at the nicotinic receptors. If the nicotinic receptors had played a role in the aHT increase, the hexamethonium would have suppressed any rise by acetylcholine. These results lead to a speculation that the rise in aHT occurred through muscarinic receptors. Acetylcholine may also be speculated to act on the enzyme NAT, inducing activation, and on the enzyme HIOMT to lower its activity thus reducing the conversion of aHT to aMT.

TABLE 2.3 The effect of acetylcholine (ACh)(10^{-5}M) and a combination of hexamethonium and acetylcholine (HEX + ACh) (10^{-5}M) on [^{14}C]-serotonin metabolism in rat pineal glands.

Metabolites	Control	ACh	Significance	HEX + ACh	Significance
ML	909,83 ± 139,28	426,33 ± 88,24	(a) p<0,005	323,5 ± 94,84	(a) p<0,005
MA	288,33 ± 29,38	202,67 ± 18,79	NS	179,4 ± 50,06	NS
aMT	1 615,00 ± 543,01	3 053,67 ± 615,36	NS	2 707,33 ± 578,79	NS
HL	15 482,50 ± 1 742,81	9 563,83 ± 1 316,93	(a) p<0,01	9 402,00 ± 1 424,40	(a) p<0,025
HA	24 372,83 ± 2 270,62	19 271,00 ± 2 520,89	NS	15 917,00 ± 2 161,84	(a) p<0,05
aHT	1 077,00 ± 187,51	3 278,00 ± 500,46	(b) p<0,001	1 932,50 ± 429,48	(b) p<0,025
5HT/5MT	57 062,67 ± 6 895,55	58 296,33 ± 4 723,89	(b) p<0,05	74 677,00 ± 5 816,60	(b) p<0,05

10µl aliquots of medium were chromatographed by TLC. Results are expressed as dpm/pineal gland (mean ± SEM; n=5 individually cultured glands per group). Significance is computed using a One-Way ANOVA followed by Neuman-Keuls Multiple Range test.

(a) Significantly decreased w.r.t vehicle control
(b) Significantly increased w.r.t vehicle control

2.6 Experiment 3: THE EFFECT OF ATROPINE ON PINEAL INDOLE METABOLISM.

2.6.1 Introduction

Antimuscarinic agents are competitive inhibitors of the action of acetylcholine at the muscarinic receptors of autonomic effector sites innervated by parasympathetic nerves as well as being inhibitors to some extent of the action of acetylcholine on smooth muscles lacking cholinergic innervation. Atropine is an antimuscarinic agent which competitively inhibits the action of acetylcholine at the muscarinic receptors, in so doing it may be possible to determine whether the rise in aHT occurred through the muscarinic receptor or not.

2.6.2 Materials and Methods

2.6.2.1 Materials

Atropine was purchased from BDH Chemicals Ltd, England. All other chemicals and reagents were obtained from previously acknowledged sources [**2.3.1.1**].

2.6.2.2 Animals

Adult male Wistar rats (250-280g.b.wt) were used and maintained under an automatically regulated lighting cycle of LD12:12. Their living environment is described in detail in [**2.3.1.2**]. All the animals were sacrificed at 11h00.

2.6.2.3 Methods

The technique of pineal organ culture and the analysis of labelled indoles was performed as outlined in [**2.3.1.3**]. Ten pineal glands were aseptically removed and individually cultured in the presence of 0.4µCi [^{14}C] serotonin. Five pineals were cultured in the presence of 10^{-5}M atropine and another

five with a combination of atropine and acetylcholine (10^{-5}M) and a further five in the absence of the drug to act as a control group, at 37°C with a relative humidity of 95% and in an atmosphere of 5%CO_2:95%O_2. Aliquots of culture medium was removed after 24 hours of incubation and the [^{14}C] indoles in the samples were isolated using bidimensional TLC and quantitated with the aid of liquid scintillometry as described in **[2.3.1.4]**. Statistical comparisons were made using a One-Way ANOVA followed by Neuman-Keuls Multiple Range test.

2.6.3 Results

The results are presented in **Table 2.4** showing all the metabolites separated by TLC with the exception of the starting spot which contains both HT and MT. Atropine was found to block the rise in aHT induced by acetylcholine.

2.6.4 Discussion

The antimuscarinic agent atropine antagonizes the muscarinic actions of acetylcholine, in so doing preventing the operation of the muscarinic cholinergic system. The results show that atropine blocked the aHT rise induced by acetylcholine, implying that acetylcholine acts on the pineal muscarinic receptor. [^{3}H] Quinuclidinyl benzilate ([^{3}H] QNB) binding sites in the rat pineal glands were found to show similar properties to cholinergic muscarinic receptors in other tissues (Sharma and Banerjee, 1978; Pirola *et al.*, 1986). Functionality of these receptors was demonstrated as *in vitro* muscarinic activation by pilocarpine increased the pineal metabolic production of the hydroxyindole derivatives 5-hydroxytryptophan and serotonin, with a slight effect on melatonin biosynthesis (Finocchiaro *et al.*, 1989). Electric field stimulation of pineal slices caused similar metabolic effects with the probable release of acetylcholine on excitation. These effects were inhibited by muscarinic blockade with atropine.

These results **[2.6.3]** suggest that acetylcholine plays a role in the pineal and that cholinergic activity may therefore regulate indole metabolism in the pineal gland. The mechanism by which this regulation occurs needs to be further examined.

TABLE 2.4 The effect of acetylcholine (ACh)(10^{-5}M) and a combination of atropine and acetylcholine (ATR + ACh) (10^{-5}M) on [^{14}C] serotonin metabolism in rat pineal glands.

Metabolites	Control	ACh	Significance	ATR + ACh	Significance
ML	324,09 ± 100,46	285,26 ± 209,20	NS	707,82 ± 195,48	NS
MA	31 009,53 ± 6 798,64	34 563,97 ± 5 323,86	NS	40 436,54 ± 3 777,13	NS
aMT	1 090,18 ± 327,75	1 666,67 ± 79,97	NS	612,74 ± 158,80	NS
HL	10 301,89 ± 51,85	4 383,22 ± 52,01	a) p<0.01	9 783,48 ± 50,44	NS
HA	2 309,08 ± 433,15	2 154,30 ± 587,47	NS	1 834,48 ± 280,34	NS
aHT	2 012,27 ± 1 630,69	4 288,11 ± 741,27	b) p<0.001	2 363,60 ± 870,21	NS
5HT/5MT	22 899,33 ± 5 383,02	29 752,53 ± 2 450,31	NS	29 285,36 ± 3 803,19	NS

10µl aliquots of medium were chromatographed by TLC. Results are expressed as dpm/pineal (mean ± SEM; n=5 individually cultured glands per group). Significance is computed using One-Way ANOVA followed by Neuman-Keuls Multiple Range test.

a) Significantly decreased w.r.t. vehicle control
b) Significantly increased w.r.t. vehicle control

2.7 Experiment 4 : THE EFFECT OF NEOSTIGMINE ON PINEAL INDOLE METABOLISM.

2.7.1 Introduction

Anticholinesterase drugs block the enzymatic degradation of acetylcholine and cause its accumulation at cholinergic receptor sites. Several anticholinesterase drugs are toxic agents employed as insecticides or used as 'nerve-gases' in chemical warfare. Reversibly acting agents such as physostigmine and neostigmine are useful in therapeutics because of their indirect cholinergic action. The aim of this experiment was to investigate the effect of an anticholinesterase agent neostigmine on indole metabolism in the pineal.

2.7.2 Materials and Methods

2.7.2.1 Materials

Neostigmine bromide was purchased from Sigma Chemical Co., USA. All other chemicals and reagents were obtained from previously acknowledged sources [**2.3.1.1**].

2.7.2.2 Methods

The technique of pineal organ culture and the analysis of labelled indoles was performed as outlined in [**2.3.1.3**]. Ten pineal glands were aseptically removed and individually cultured in the presence of 0.4μCi [^{14}C] serotonin. Five pineals were cultured in the presence of 10^{-5}M neostigmine and another five in the absence of drug to act as a control group, at 37°C with a relative humidity of 95% and in an atmosphere of 5%CO_2:95%O_2. Aliquots of culture medium was removed after 24 hours of incubation. The [^{14}C] indoles in the samples were isolated using bidimensional TLC and quantitated with the aid of liquid scintillometry as described in [**2.3.1.4**]. Statistical comparison made using Student's t-test.

2.7.3 Results

The results are presented in **Table 2.5** showing all the metabolites separated by TLC with the exception of the starting spot which contains both aHT and aMT. Neostigmine did not cause any significant changes in aMT and aHT levels.

2.7.4 Discussion

Neostigmine is a reversible cholinesterase inhibitor which contains a quaternary nitrogen. This structural feature limits its passage into the CNS and imparts a direct action on peripheral cholinergic receptor sites and at the motor end plate on skeletal muscle.

In general, compounds such as neostigmine which contain a quaternary ammonium group do not penetrate cell membranes readily. Hence anticholinesterase agents in this category are poorly absorbed from the gastrointestinal tract and are excluded by the blood brain barrier from exerting significant action on the CNS (Goodman and Gilmon, 1980).

The results show that neostigmine did not influence the pineal indoles in any significant way. Finocchiaro *et al.*, (1989) demonstrated the functionality of cholinergic muscarinic receptors in the rat pineal gland. These authors showed neostigmine to enhance the action of acetylcholine released by electric field stimulation resulting in a rise in 5-hydroxytryptophan and serotonin levels. However, 5-hydroxyindole 3-acetic acid (HA), N-acetylserotonin (aHT) and melatonin (aMT) showed no additional increase when compared with the electrically stimulated controls confirming our organ culture results above [**2.7.3**].

Although the effects of muscarinic activation may be due to increased tryptophan uptake or the activation of tryptophan hydroxylase, additional experiments are required to determine the mechanism involved and as to the reason why activation of indoles such as aHT and aMT do not occur.

TABLE 2.5 ***The effect of neostigmine (10^{-5}M) on [^{14}C]-serotonin metabolism in rat pineal glands.***

Metabolites	Control	Neostigmine	Significance
ML	1 241,71 ± 207,30	568,07 ± 79,77	a) p<0,01
MA	488,27 ± 54,05	404,26 ± 64,76	NS
aMT	843,69 ± 295,31	1 616,72 ± 309,03	NS
HL	8 740,34 ± 1 269,89	6 460,66 ± 718,45	NS
HA	23 436,72 ± 1 368,34	26 103,68 ± 4 322,48	NS
aHT	370,57 ± 135,99	638,04 ± 145,29	NS
5HT/5MT	20 834,75 ± 2 357,67	33 085,69 ± 5 351,78	NS

10µl aliquots of medium were chromatographed by TLC. Results are expressed as dpm/pineal gland (mean ± SEM; n=5 individually cultured glands per group). Significance is computed using the Student's t-test.

a) Significantly decreased w.r.t. vehicle control - Student's t-test.

Table 2.5 shows 5-methoxytryptophol levels to decline significantly when compared to control groups. Neostigmine could be speculated to influence methoxytryptophol levels by possibly inhibiting the HIOMT enzyme activity in some manner and in so doing limit the conversion of 5-hydroxytryptophol to 5-methoxytryptophol.

2.8 Experiment 5 : THE EFFECT OF PHYSOSTIGMINE ON PINEAL INDOLE METABOLISM

2.8.1 Introduction

The drug physostigmine is a reversible inhibitor of acetylcholinesterase that forms a carbamylated intermediate with the enzyme. Physostigmine reacts primarily with the true acetylcholinesterases. The carbamylated intermediate of the enzyme so formed prevents the acetylcholine-acetylcholinesterase interaction and spares endogenous acetylcholine from enzymatic hydrolysis. The accumulation of acetylcholine at autonomic ganglia and cholinergic neuroeffector junctions causes the pharmacologic responses to physostigmine. Physostigmine is a lipid-soluble agent possessing a tertiary amine and having a ubiquitous effect on central cholinergic receptor sites (Goodman and Gilman, 1980).

The aim of this experiment was to investigate the effects of a cholinesterase substrate inhibitor physostigmine on pineal indole metabolism.

2.8.2 Materials and Methods

2.8.2.1 Materials

Physostigmine was purchased from Merck, Germany. All other chemicals and reagents were obtained from previously acknowledged sources [**2.3.1.1**].

2.8.2.2 Animals

Adult male Wistar rats (250-280g.b.wt) were used for this series of experiments and were maintained under an automatically regulated lighting cycle of LD12:12. Their living environment is described in detail in [**2.3.1.2**]. All the animals were sacrificed at 11h00.

2.8.2.3 Methods

The technique of pineal organ culture and the analysis of labelled indoles was performed as outlined in [**2.3.1.3**]. Ten pineal glands were aseptically removed and individually cultured in the presence of 0,4µCi [^{14}C] serotonin. Five pineals were cultured in the presence of 10^{-5}M physostigmine and another five in the absence of drug to act as a control group, at 37°C with a relative humidity of 95% and in an atmosphere of 5%CO_2:95%O_2. Aliquots of culture medium was removed after 24 hours of incubation. The [^{14}C] indoles in the samples were isolated using bidimensional TLC and quantitated with the aid of liquid scintillometry as described in [**2.3.1.4**]. Statistical comparisons were made using Student's t-test.

2.8.3 Results

The results are presented in **Table 2.6** showing all the metabolites separated by TLC with the exception of the starting spot which contains serotonin (HT) and 5-methoxytryptamine (MT) respectively. Physostigmine was found to cause a significant rise in aHT ($p<0.005$) but not aMT.

2.8.4 Discussion

Our investigation found that the cholinesterase inhibitor physostigmine increased aHT levels significantly but not aMT. Physostigmine is a lipid-soluble drug containing a tertiary amine and readily passes the blood brain barrier. Consequently it has effects at both peripheral and central

TABLE 2.6 ***The effect of physostigmine [10^{-5}M] on [^{14}C]-serotonin metabolism in rat pineal glands.***

Metabolites	Control	Physostigmine	Significance
ML	1 275,62 ± 303,09	1 194,07 ± 71,74	NS
MA	883,94 ± 118,57	578,25 ± 96,14	a) p<0,05
aMT	450,24 ± 162,08	479,87 ± 165,77	NS
HL	9 961,65 ± 1 823,21	26 991,58 ± 4 758,53	b) p<0,005
HA	61 112,07 ± 1 307,56	58 285,04 ± 3 628,51	NS
aHT	460,02 ± 92,78	1 256,98 ± 177,04	b) p<0,005
5HT/5MT	18 030,61 ± 1 678,27	17 277,03 ± 2 280,59	NS

10µl aliquots of medium were chromatographed by TLC. Results are expressed as dpm/pineal gland (mean ± SEM; n=5 individually cultured glands per group). Significance is computed using the Student's t-test.

a) Significantly decreased w.r.t vehicle control
b) Significantly increased w.r.t vehicle control

cholinergic receptor sites. Although both neostigmine and physostigmine are anticholinesterases, neostigmine contains a quaternary nitrogen (Goodman and Gilman, 1980). This structural feature limits neostigmine's passage into the CNS and hence it imparts a direct action on peripheral cholinergic receptor sites only and not on central cholinergic receptor sites. This may be a reason why physostigmine causes aHT levels to rise significantly whilst neostigmine is unable to do so. By being lipid-soluble physostigmine is able to pass through the blood brain barrier and influence central cholinergic receptor sites in the pineal gland. These results further confirm the presence of cholinergic muscarinic receptors in the pineal gland. The physiological significance and mechanism behind the acetylcholine and physostigmine-induced rise in aHT without influencing aMT levels remains to be elucidated.

CHAPTER 3

CYCLIC AMP DETERMINATION TECHNIQUE

3.1 Introduction

Adenosine 3'5' cyclic monophosphate (cyclic AMP) has been identified in almost all forms of life, where it mediates intracellular responses to external stimuli (Fried, 1972). After receptor activation by an agonist, hormone receptor interaction occurs on the external cell surface. The membrane associated event usually triggers a secondary cascade of responses inside the cell to affect internal metabolic processes. In many cases the primary effect of the hormone receptor interaction is to cause a change in the level of some "second messenger" inside the cell (Fried, 1972; Yamamura and Enna, 1981). The second messenger can then directly or through a further cascade process affect the activity of appropriate metabolic enzymes or the expression of particular genes. Such a process appears to operate at all beta-adrenoreceptors and at some dopamine receptors. At these receptors, the enzyme adenylate cyclase is activated by the process of neurotransmitter receptor combination (Kruk and Pycock, 1979).

Various researchers have, at different times, employed a variety of techniques to measure cAMP levels. Early attempts, in this regard, were essentially indirect and involved determining the activity of the enzyme adenylate cyclase as an index of cAMP levels (Krishna *et al.*, 1968). This method involved measuring the rate of formation of cAMP from its radioactive precursor, [^{14}C] ATP. The cAMP thus formed was isolated by ion exchange chromatography followed by precipitation of all the nucleotides and inorganic phosphates. The radioactivity of the cAMP left in the supernatant was then measured.

A direct cAMP assay, using a purified protein from bovine muscle as the specific binding protein, was developed by Gilman (1970). Various workers have used this technique to measure rat pineal cAMP levels. Generally, the method involves prior trichloroacetic acid precipitation and ether

extraction before measuring cAMP levels. Deguchi (1973) found no difference in the amount of cAMP between purified and unpurified pineal tissue using the method of Gilman (1970).

Brown and co-workers (1971) have developed a saturation assay for cAMP using purified bovine adrenal protein as the specific binding protein. The assay was similar to the Gilman assay (1970), also involving trichloroacetic acid precipitation and ether extraction prior to measurement of cAMP levels. Although the two assays had similar sensitivity and specificity characteristics, the assay of Brown and co-workers (1971) was considerably simpler and less time-consuming.

More recently radioimmunoassays (RIAs) have been developed to measure rat pineal cAMP levels (Moyer *et al.*, 1979; Heydom *et al.*, 1980). The cAMP concentration is measured using [^{125}I] antigen and antiserum available commercially. The cAMP RIAs, although expensive, they have a high degree of specificity and sensitivity. In addition, these assays are rapid and simple to use.

The assay developed by Brown *et al.*, (1971) was chosen to measure pineal cAMP levels for the purposes of this study. The assay is sensitive, specific, easy to use and relatively inexpensive. The following section describes the preparation of the adrenal binding protein as well as the cAMP assay, adapted for the pineal gland.

3.1.1 Methods for the Determination of Cyclic AMP

Although several very sensitive radioimmunoassays for the determination of cAMP are available, their cost was prohibitive. Since the action of cAMP in all tissues involves its binding to some type of protein (Oye, 1973), several techniques have been devised which make use of a natural binding protein coupled to a radio isotope type of dilution assay, thereby giving greater sensitivity. The methods of Gilman (1970) and Brown *et al.*, (1971) are two well known examples of these so-called saturation binding assays.

Cyclic AMP assay kits are commercially available but the method by Brown *et al.*, (1971) was favoured by our laboratory. Modipane *et al.*, (1985) compared the efficiency, cost and working time between the commercial cAMP kit and the published laboratory method. The authors found the method by Brown *et al.*, (1971) had far greater advantages compared to the commercial kit, particularly with regard to cost since it was adaptable to tissues other than the pineal gland. Hence because of ever-increasing costs of biochemicals and the price of commercially available cyclic AMP kits, the method of Brown *et al.*, (1971) was used.

3.1.2 Theory of the Assay

The assay is based on the competition between unlabelled cAMP and a fixed quantity of the tritium labelled compound for binding to a protein which has a high specificity and affinity for cAMP. The amount of [^{3}H]-cAMP bound to this protein is inversely related to the amount of cAMP present in the assay sample. The amount of unlabelled cAMP in the sample is calculated by measurement of the protein-bound radioactivity. Separation of the protein-bound cAMP from the unbound nucleotide is achieved by centrifugation. The supernatant (which contains the protein-bound complex) is added to scintillation cocktail and its activity determined by liquid scintillometry. The concentration of unlabelled cAMP in the sample is then determined from a linear standard curve.

3.2 CYCLIC-AMP DETERMINATION TECHNIQUE

3.2.1 Materials and Methods

3.2.1.1 Chemicals, Drugs and Reagents

[^{3}H] Adenosine 3'5'-cyclic phosphate, ammonium salt (specific activity 30 Ci/mmol) was purchased from Amersham, England; and the activated charcoal from Merck, Germany. Theophylline, bovine serum albumin, cyclic AMP and phosphodiesterase were obtained from Sigma Chemical Co., USA. The chemicals used in buffers were of analytical grade from commercial sources.

3.2.1.2 Animals

Male Wistar rats (200-250g.b.wt) were housed five per cage in a temperature controlled room under a fixed lighting cycle of LD12:12 (lights on 06h00) with food and water *ad libitum*. The rats were sacrificed between 10h30 and 11h30, maintaining uniformity with previous studies.

Male rats were chosen for the cAMP studies because they tended to be less anxious when handled, than the female rats. It was therefore felt that their cAMP levels would more accurately reflect the drug treatment, rather than exogenous stress influences. Skene (1985) has reported higher basal cAMP levels in female rats, which she ascribed to their more anxious nature.

3.2.1.3 Pineal Glands

The pineal glands were rapidly removed and transferred to prewarmed (37°C) BGJ_b culture medium (Fitton-Jackson modification).

3.2.1.4 Assay of cAMP in Rat Pineal Glands

3.2.1.4 (i) Collection and Preparation of Binding Protein

Bovine adrenals glands were collected from the local abattoir as soon after slaughter as possible and transported to the laboratory on ice. The cortices were separated from the medullas, chopped and homogenized in a glass homogenizer using 1.5 volumes of an ice-cold medium comprising of 0.25M sucrose; 50mM Tris HCl buffer, pH7.4; 25mM potassium chloride and 5mM magnesium chloride. The homogenate was centrifuged in a Hettich Universal 25 centrifuge at 2000 x g for 10 minutes at 4°C. The pellet was discarded and the supernatant respun at 5000g for 15 minutes at 4°C. The resulting supernatant was stored in 1ml eppendorf tubes at -20°C. A negligible loss of binding activity was found after at least 3 months of storage. The preparation, which contains a binding protein (protein kinase), was thawed and diluted as required with 50mM Tris-HCl buffer,

pH7.4 containing 8mM theophylline and 6mM 2-mercaptoethanol. This buffer was used for all subsequent procedures. The stored protein remained stable for the duration of this study.

3.2.1.4 (ii) Dilution of the Binding Protein

In order to determine the optimum dilution of the stock binding protein for the cAMP assay, aliquots of binding protein was thawed and diluted with an appropriate volume of cyclic AMP protein diluting buffer. Serial dilutions of binding protein in buffer were assayed for radioactivity by following the zero dose column in **Table 3.1** of the scheme for the cAMP assay of Brown *et al.*, (1971) and adding dilutions of binding protein. A protein dilution curve **Figure 3.1**, thus obtained shows that a 1:3 dilution provides optimum binding (85%) and radioactivity counts. This dilution factor was thus used in all subsequent cAMP assays with this particular batch of stock protein. Each time a new batch of binding protein was prepared a new dilution curve was determined. However a 1:3 dilution was found to be appropriate for all batches tested in this way. The method of Lowry *et al.*, (1951) for determination of protein in biological samples was used. A simple protocol for this method using bovine serum albumin as a standard is outlined in **Table 3.2**. **Figure 3.2** shows the protein standard curve obtained by this Folin Lowry method.

Briefly, a series of tubes were prepared for the Folin Lowry method which included: (1) a blank containing 1.0ml H_2O; (2) a set of standard tubes each containing appropriate aliquots of water and a protein standard solution (eg. 1mg BSA/ml) that will yield separate tubes containing 50, 100, 150, 200, 250 and 300 µg of protein, all in final volumes of 1.0ml, and (3) a set of assay tubes containing water and appropriate dilutions of the protein solution of unknown concentration (eg. 0.025 and 0.05ml of the unknown) plus water up to 1.2ml. Separately prepare 100ml of fresh alkaline copper reagent by mixing, in order, 1ml of 1% $CuSO_4 \cdot 5H_2O$, 1ml of 2% sodium tartrate and 98ml of 2% Na_2CO_3 in 0.1N NaOH. Add, and immediately mix in , 6ml of alkaline copper reagent to each tube. After 10 minutes at room temperature add, and immediately mix in, 0.3ml of Folin-Ciocalteu reagent to each tube. After 30 more minutes, read the absorbance of the standard and assay tubes at 500nm against the blank.

TABLE 3.1 ***Scheme of the cyclic AMP assay (Brown et al., 1971)***

cAMP standards (pmol)	Blank	0	0.25	0.5	1.0	2.0	4.0	8.0	Sample
Buffer* (µl)	250	150	150	150	150	150	150	150	150
µl Standard (1:10)	-	-	12,5	25	50	-	-	-	-
µl Standard (as is)	-	-	-	-	-	10	20	40	-
Sample (µl)	-	-	-	-	-	-	-	-	50
Distilled Water (µl)	50	50	37,5	25	-	40	30	10	-
[^{3}H]-cAMP (µl)	50	50	50	50	50	50	50	50	50
Vortex briefly									
µl diluted protein	-	100	100	100	100	100	100	100	100

Mix gently to avoid frothing

Incubate on ice for 100 minutes or in a cold room at 4°C									
Charcoal (µl)	100	100	100	100	100	100	100	100	100

Vortex briefly

Centrifuge at 1200 x g for 15 minutes at 4°C									
Supernatant (µl)	100	100	100	100	100	100	100	100	100

Carefully remove 100 µl of the supernatant.

Add supernatant to 3ml scintillation fluid and count radioactivity

* *Tris HCl (50 mM, pH 7.4) containing 8 mM theophylline and 6 mM 2-mercapto ethanol.*

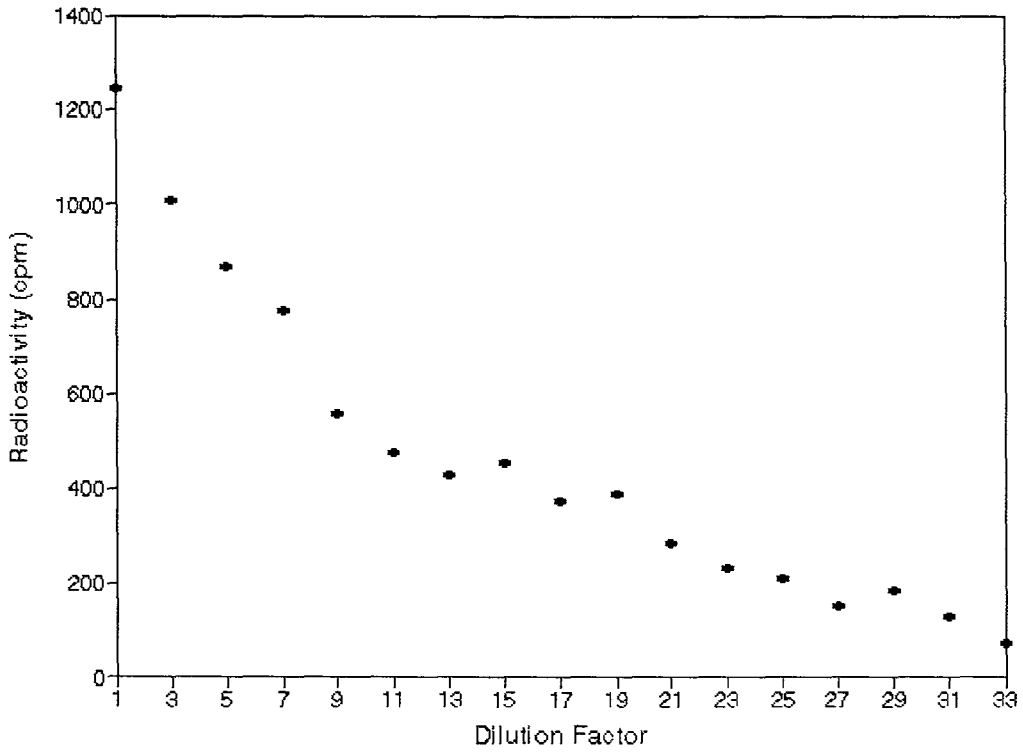


Figure 3.1: A typical protein dilution curve obtained in these experiments. Each point represents the mean of duplicate determinations.

TABLE 3.2 ***Scheme for the determination of protein (Lowry et al., 1951)***

Protein Concentration (µg/ml)	Blank	50	100	150	200	250	300	Sample 25	50
Protein Volume (ml)	0	0,05	0,1	0,15	0,2	0,25	0,3	0,025	0,05
Distilled Water (ml)	1	0,95	0,9	0,85	0,8	0,75	0,7	0,975	0,95
Alkaline* Copper reagent (ml)	6	6	6	6	6	6	6	6	6

Stand : 10 minutes at room temperature

Folin-Ciocalteu reagent (ml)	0,3	0,3	0,3	0,3	0,3	0,3	0,3	0,3	0,3
Total (ml)	7,3	7,3	7,3	7,3	7,3	7,3	7,3	7,3	7,3

Stand : 30 min at room temperature

Read absorbance of the standard and assay tubes at 500 nm against the blank.

* *1% $CuSO_4 \cdot 5H_2O$: 2% Sodium tartrate : 2% Na_2CO_3 in 0.1N NaOH*

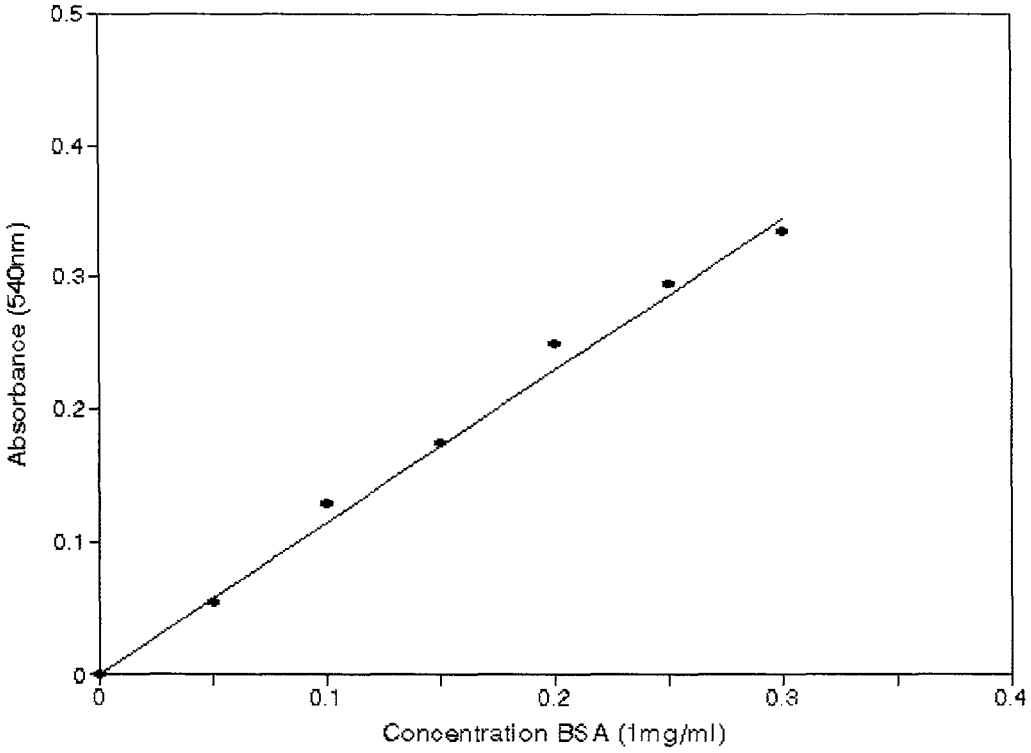


Figure 3.2: Protein standard curve (Folin Lowry method). Each point represents the mean of duplicate determinations.

3.2.1.5 Preparation of Pineal Tissue and Incubation Procedure

Pineal glands were rapidly removed and immediately placed in ice-cold physiological saline (0.9%). Pineals were then dissected free of adhering connective tissue. The pineals were then placed in 2ml of fresh prewarmed BGJ_b incubation culture medium (Filton-Jackson modification, Gibco (Europe) **[2.3.1.2]** and incubated at 37°C for 20 minutes. Following incubation the pineals were removed from the medium and homogenized in 100µl ice-cold cAMP protein diluting buffer. Duplicate aliquots (50µl) of pineal homogenate was transferred to reaction tubes and was used in the cAMP assay.

3.2.1.6 Cyclic AMP Assay

A simplified schematic representation of the saturation binding method of Brown *et al.*, (1971) is presented in **Table 3.1**. Each reaction tube contained 50µl of either a known amount (0-8pmol) of cAMP standard (Boehringer Mannheim) or of an unknown sample, 50µl of [^{3}H] cAMP (8nCi), 1000µl of diluted (1:3) binding protein and buffer (50mM Tris-HCl, pH7.4 containing 8mM theophylline and 6mM 2-mercaptoethanol) to a final volume of 350µl. The reaction tubes were placed on ice and incubated at 4°C for 100 minutes. Following incubation, 100µl of a 10% w/v suspension of charcoal in buffer containing 2% w/v of bovine serum albumin was added to each retention tube and the tubes were vortexed for 10 seconds, each on a Rotamixer Deluxe.

The charcoal was prepared by dissolving 400mg Bovine Serum Albumin (BSA) in 20ml of protein diluting buffer together with 2g of activated charcoal. After centrifugation at 1200 x g for 15 minutes at 4°C, a 100µl aliquot of the supernatant was added to plastic scintillation vials containing 3ml Ready-Solv HP (Beckman USA) scintillation fluid. The vials were briefly agitated on a Griffith flask shaker for 15 minutes and the radioactivity quantitated in a Beckman LS 2800 liquid scintillation counter at an efficiency of 61.12%.

3.2.1.7 Statistical Analysis

Cyclic AMP levels - the mean of duplicate determinations - were determined from the cAMP standard curve and expressed as pmol/pineal ± SEM. For each drug group the cAMP levels in those glands were compared to those of the control. The significance was evaluated using a One-Way ANOVA followed by Neuman-Keuls Multiple Range test.

3.2.2 Results

3.2.2.1 Calibration Curve

A calibration curve was set up for each assay and were plotted using the data obtained from the standard amounts of cAMP added to the tubes as outlined in **Table 3.1**. The amounts of unknown cAMP in samples were determined by reference to such curves. A typical cAMP calibration standard curve is given in **Figure 3.3**. The calibration curve was calculated in terms of the ratio Co/Cx against the concentration (pmoles/tube) of standard nucleotide. The Co/Cx ratio was obtained by dividing the radioactivity (cpm) of the zero standard (Co) by the radioactivity of the higher standards (Cx) after subtraction of blank values.

$$Co = \text{mean } cpm_{\text{zero standard}} - \text{mean } cpm_{\text{Blank}}$$

$$Cx = \text{mean } cpm_{\text{standard}} - \text{mean } cpm_{\text{Blank}}$$

or for samples

$$Cx = \text{mean } cpm_{\text{sample}} - \text{mean } cpm_{\text{Blank}}$$

3.2.2.2 Validation of Results

(i) After addition of tracer quantities of [^{3}H]-cAMP, BSA coated activated charcoal was added to the tubes. Assay of the supernatant showed all tracer had been removed.

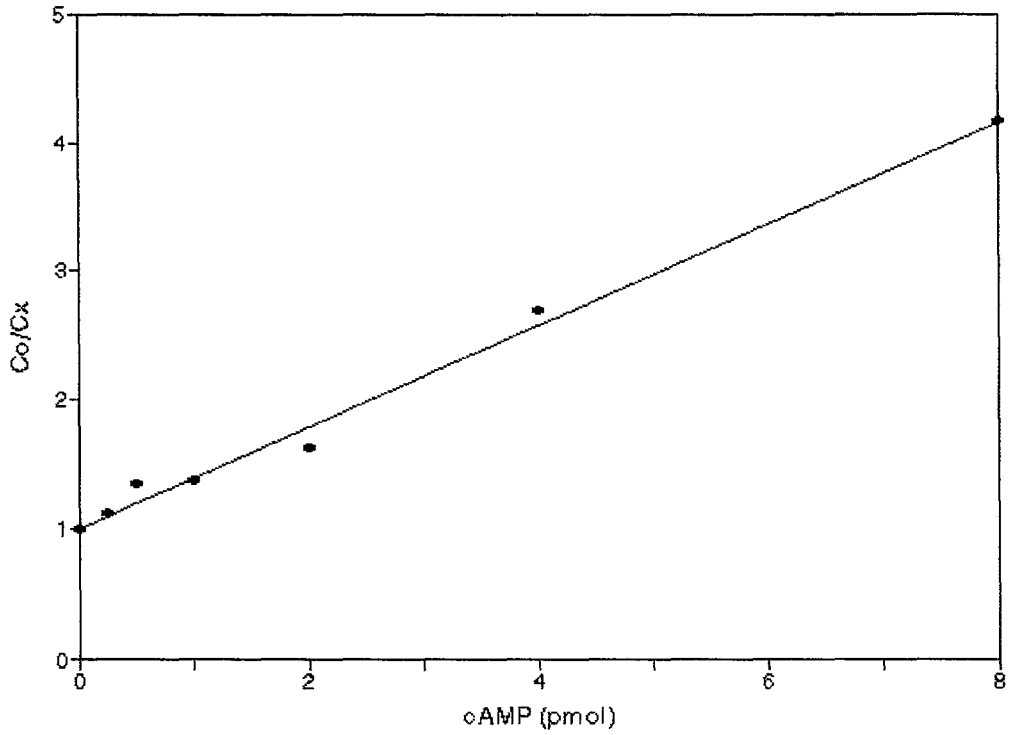


Figure 3.3: A typical cAMP calibration standard curve. Each point represents the mean of duplicate determinations.

(ii) Known quantities of standard cAMP were added to the tubes containing zero added cAMP. These were placed among the normal assay tubes, and the results from these were tested to determine whether they agreed with the calibration curve.

3.2.3 Discussion

The results presented indicate that the assay procedure described above is sufficiently sensitive and specific to measure cAMP levels in rat pineal tissue. In addition, the assay is considerably simpler than the method of Brown *et al.*, (1971) since it allows for the direct quantification of cAMP levels in pineal homogenates without prior trichloroacetic acid precipitation and ether extraction. The assay was therefore suitable for use in subsequent studies.

3.2.4 Problems Encountered with cAMP Assay

A series of problems were experienced when carrying out the cAMP assay by saturation binding method of Brown *et al.*, (1971). Adrenals were collected from the abattoir on two occasions, Batch 1 was collected three months earlier than Batch 2. It was disturbing to note the low counts per minute and the narrow range of the standard calibration curve for both batches. This reduces the sensitivity of the assay and diminishes the accuracy in reading the results off the curve.

To establish the cause of the low counts per minute the following avenues were investigated:

(i) The possibility that in separating out the cortices, some adrenal medulla remained, contaminating the cortices by releasing an unknown factor which disrupts and interferes with the binding protein. Careful removal of adrenal medulla took place on both occasions so the possibility of this being a factor was discarded.

(ii) Another possibility was that the binding protein was defective or damaged in some way resulting in inefficient binding of radiolabelled cAMP. This too would explain some of the

low counts per minute. Calibration curves done on Batch 1 and 2 indicate similar ranges and counts suggesting some other factor acting on the protein could possibly be the cause.

(iii) Another possibility is that the chemicals used in the assay were defective. The charcoal for example is required to strip extracellular proteins and unbound radiolabelled and non radiolabelled cAMP from the homogenate which leaves the binding protein with the cAMP attached to it in the supernatant. This process is essential in removing unbound radiolabelled cAMP which would otherwise interfere with scintillation readings. For this purpose activated charcoal from Merck, Germany is needed, not the neutral type which would be unable to bind the protein. The type of Bovine Serum Albumin (BSA) used is also important. BSA is used to saturate the assay homogenate medium in order to prevent charcoal from removing the binding protein. Whole BSA obtained from Sigma in crystallized form is used, not BSA Fraction V since it does not bind to the charcoal properly. The chemicals used were double-checked and found to be all correct and in so doing removed the possibility of it being the contributing factor to the low counts obtained.

(iv) The possibility of high endogenous cAMP in the protein homogenate was then suspected which would shift the equilibrium of the radiolabelled and non radiolabelled cAMP binding to the protein. More non-radiolabelled cAMP could be present due to circulating endogenous cAMP, displacing the radioactive cAMP from the binding protein resulting in low cpm's. In an attempt to remove endogenous cAMP a purification step was carried out in order to remove it. Protein homogenate and charcoal were mixed together in a ratio of 1:1 in Eppendorf tubes and vortexed. Centrifugation occurred at 1200g for 15 minutes. The 'purified' supernatant was then subsequently removed and used in the cAMP standard curve assay. The supernatant was diluted 1:3 and 100µl of the dilution was removed and used in the assay.

The purification step using charcoal produced a marked increase in binding. This provides strong evidence that endogenous cAMP was the possible factor contributing to the low binding. Batch 1

protein was compared with Batch 2 using the purification step and it was found that the counts were similar as well as being relatively high with a much wider range than before.

Thus in modifying the cAMP assay of Brown *et al.*, (1971), a unique relationship may be speculated to occur between endogenous cAMP and the physiological state of the animal. It is well known that during stress, catecholamines such as noradrenaline and adrenaline are released from the adrenal medulla. These possibly stimulate beta receptors in the adrenal cortex activating the enzyme adenylate cyclase which in turn causes an increase in the synthesis of cAMP from ATP. Hence high endogenous cAMP may be associated with the method of slaughter where it appears that the animal undergoes a great deal of stress resulting in increased levels of endogenous cAMP.

3.3 Experiment : TO DETERMINE THE EFFECT OF ACETYLCHOLINE ON PINEAL cAMP

3.3.1 Introduction

The possibility exists that the cholinergic system may influence cAMP levels without acting through the adrenergic system resulting in NAT enzyme activation and an increase in aHT levels. The aim of the experiment is to establish whether the cholinergic agonist acetylcholine is able to influence cAMP levels in any way.

3.3.2 Materials and Methods

3.3.2.1 Materials

All reagents used were of analytical grade from commercial sources. Isoproterenol and acetylcholine chloride were obtained from Sigma Chemical Co., USA. Sources of all other materials have been recorded in [**3.2.1.1**].

3.3.2.2 Method

A cAMP standard curve was constructed, and calibration curves were plotted Co/Cx against standard cAMP pmol/tube. Five rat pineals were exposed to isoproterenol (ISO)(10^{-4}M), another five to acetylcholine (ACh)(10^{-4}M), and a further five pineals to a combination of isoproterenol (10^{-4}M) and acetylcholine (10^{-4}M) (ISO+ACh) with a 20 minute incubation time period. The cAMP saturation binding assay of Brown *et al.*, (1971) with modifications, was used [**3.2.1.4**]. The significance was evaluated using a One-Way ANOVA followed by Neuman-Keuls Multiple Range test.

3.3.3 Results

The average cpm for each of the three treatment groups is shown in **Table 3.3** The pineals treated with acetylcholine (10^{-4}M) showed a significant increase when compared to the controls ($p<0.0001$). Acetylcholine was also found not to influence the isoproterenol induced rise in cAMP levels ($p<0.0001$) (**Figure 3.4**).

3.3.4 Discussion

The organ culture results [**2.5.3**] show that acetylcholine causes a significant rise in aHT levels without altering aMT levels significantly. By using hexamethonium, a ganglion blocking agent, the nicotinic receptor sites were blocked and this resulted in aHT levels still rising. When an antimuscarinic agent atropine was used, the rise in aHT was successfully blocked suggesting the presence of muscarinic cholinergic receptors in the pineal gland.

The results shown from **Table 3.3** of the cAMP assay reveal that the presence of acetylcholine caused a significant increase in cAMP levels in the pineal glands ($p<0.0001$). The rise in cAMP levels caused by isoproterenol was not affected in any way by the presence of acetylcholine. Hence the cholinomimetic agent, acetylcholine, influences the sympathetic pathway in a significant manner

Table 3.3 ***Cyclic AMP formed per rat pineal gland in the presence of vehicle or when stimulated with acetylcholine (ACh) (10^{-4}M), isoproterenol (ISO) (10^{-4}M), and a combination of the two drugs (ACh + ISO) (10^{-4}M) (mean ± SEM; n=5 glands per group).***

Treatment	cAMP formed (pmol/20min/pineal gland)
Control	1,815 ± 0,136
ISO	3,387 ± 0,138 *
ACh	2,667 ± 0,153 *
ISO + ACh	3,516 ± 0,388 *

In comparison with respect to vehicle control $p<^{*}0{,}0001$ (One-Way ANOVA followed by a Neuman-Keuls Multiple Range test)

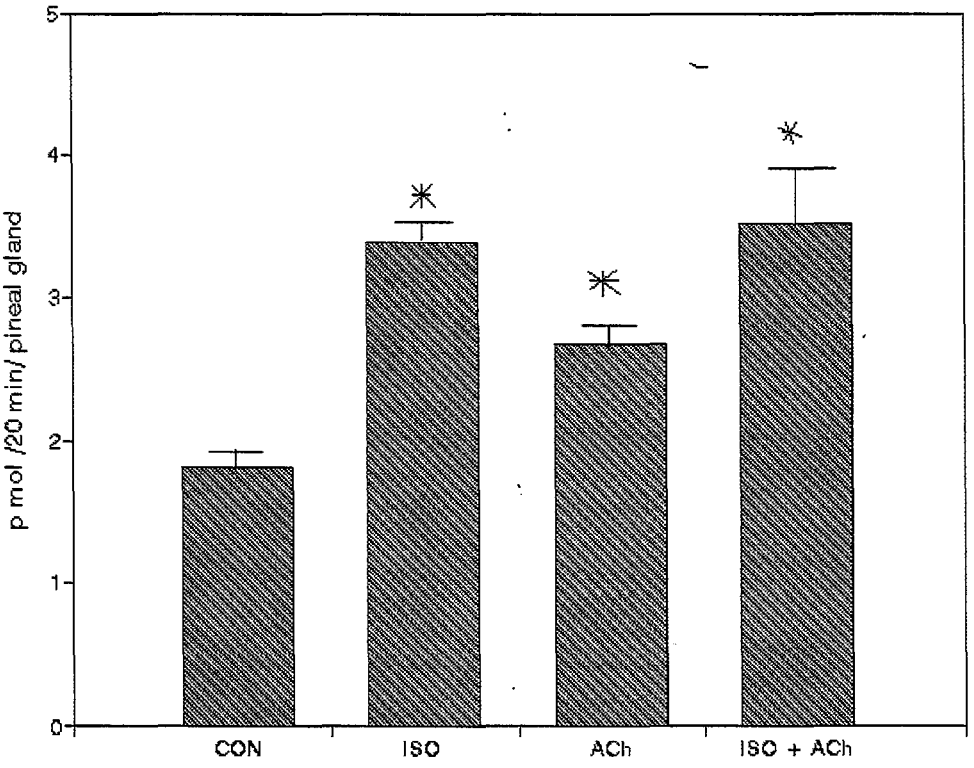


Figure 3.4 Cyclic AMP formed per rat pineal gland in the presence of vehicle or when stimulated with acetylcholine (ACh)(10^{-4}M), isoproterenol (ISO)(10^{-4}M) and a combination of the two drugs (ACh + ISO) (10^{-4}) (mean ± SEM; n=5 glands per group; $p<^{*}0.0001$ with respect to vehicle control: One-Way ANOVA followed by a Neuman-Keuls Multiple Range test).

with regard to cAMP. Noradrenaline is known to act by increasing intracellular cAMP in the pineal (Strada *et al.*, 1972; Deguchi and Axelrod, 1973) which in turn induces and activates the enzyme N-acetyltransferase (Klein and Berg, 1970; Klein and Weller, 1973). The rise in aHT (a precursor of aMT) due to the presence of acetylcholine in the organ culture studies [**2.5.3**] could be speculated to be caused by the stimulation of the adrenergic beta receptors resulting in the conversion of ATP to cAMP.

Another hypothesis that could be examined is the supply of acetate for acetylation of serotonin to melatonin. In the acetylation of serotonin to N-acetylserotonin, acetyl CoA is utilized. The adrenergic-cAMP mechanism regulates the activity of the pineal enzyme NAT. Cyclic-AMP stimulates the synthesis of specific new macromolecules, RNA and protein required for the stimulation of NAT (Klein and Weller, 1970). The NAT enzyme mainly acts in transferring an acetyl group from the co-factor acetyl CoA to acceptor amines and is responsible for the formation of N-acetylserotonin in the melatonin biosynthetic pathway (Axelrod, 1974). The possibility does exsist that acetylcholine could provide the acetate necessary for acetyl CoA formation, hence increasing acetyl group transfer and raising NAT activity. This could be another possible mechanism by which acetycholine increases aHT levels. The activity of the enzyme H10MT would hence not increase to produce a rise in melatonin levels since the enzyme is only able to transfer methyl groups from cofactor S-adensyl methionine to acceptor S-hydroxyindole molecules for conversion of aHT to aMT.

CHAPTER 4

cAMP-PHOSPHODIESTERASE STUDIES

4.1 Introduction

The regulation of many biological functions are under the control of cyclic nucleotides (Robison *et al.*, 1968) and their intracellular concentrations are delicately controlled (Teo *et al.*, 1973). The generation and quenching of the signal in any informational system must be considered equal. The biological actions of these cyclic nucleotides are terminated by the hydrolytic cleavage of the cyclic nucleotides to their corresponding 5'-nucleotides. This is the only mechanism by which termination of the biological actions of these nucleotides occur and they are catalysed by one or more types of phophodiesterase. Hence these cyclic nucleotides, which play a primary role in the control of intracellular events, are hydrolyzed and inactivated as second messengers by the cyclic nucleotide phosphodiesterases (PDE's).

The presence of phosphodiesterase was first demonstrated by Sutherland and Rall (1958). Butcher and Sutherland were able to partially purify the enzyme from bovine heart in 1967 and three years later in 1970, Kakiuchi and Yamazaki were able to show that rat brain phosphodiesterase could be inhibited by EGTA [ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N',-tetraacetic acid]. Cheung (1970) noted a percipitous drop in activity relative to his starting product after he passed the enzyme through an anion exchange column in order to purify it and enable better characterization. He found this to be due to dissociation of an activator protein from the enzyme. In independent studies Kakiuchi *et al.*, (1970) also demonstrated the presence of an activator. Teo and Wang (1973) were able to show that the activation of PDE was dependent on the presence of both the activator and Ca^{2+}. This activator protein has since been named calmodulin (Cheung, 1980).

These phosphodiesterase enzymes have been extensively studied in the brain and other tissue, and numerous assays have been devised to monitor their activity. The most widely used of these assays

are radiometric and involve the separation of the hydrolyzed 5'- nucleotide phosphate product from the substrate. The separation of the hydrolyzed radioactive product has been achieved in a number of ways, including batch or column anion exchange resin (Thompson and Appleman, 1971; Lynch and Cheung, 1975), paper chromatography (Nakai and Brooker, 1975) and precipitation (Le Donne and Coffee, 1979). These assays are all sensitive, relatively inexpensive and easy to use.

The batch method of Thompson and Appleman (1971) is popular because of its simplicity and economy of time. The method makes use of anionic exchange resin which presumably binds the unreacted cAMP (which carries a negative charge) but not the adenosine (which has no net charge). However Lynch and Cheung (1975) have shown that this method may underestimate the true cAMP-PDE activity, as the resin may sometimes not bind all the unreacted substrate. They feel that the column methods under certain circumstances may produce the same error.

The method of LeDonne and Coffee (1979) which employs a precipitation (ppt) technique, has been used in this laboratory and thin layer chomatography (TLC) method of Neuman (1983) was used for separation.

4.1.1 Summary of Assay

In this method cAMP and tritiated cAMP (tracer) are used as substrates. Pineal tissue homogenate is then added and the phosphodiesterase present in the homogenate hydrolyzes the cAMP substrate. The reaction is then terminated by the addition of trichloroacetic acid. An aliquot of the reaction mixture is spotted onto a TLC plate and the 5' AMP is then quantitated to be used as an indicator of PDE activity.

4.2 CYCLIC-AMP PDE DETERMINATION TECHNIQUE

4.2.1 Material and Methods

4.2.1.1 Chemicals, Drugs and Reagents

[8-^{3}H] Adenosine 3',5'-cyclic phosphate, ammonium salt (21,2 Ci/mmol) was purchased from Amersham, England. Adenosine 3',5'- cyclic phosphate and Adenosine 5'-monophosphate were obtained from Boehringer Mannheim, Germany. TLC plates (Alugram Sil G/ UV 254 0.025 x 20 x 20 cm) were obtained from Macherey-Nagel, West Germany. The solvents used for TLC and chemicals used in buffers were of analytical grade from commercial sources.

4.2.1.2 Animals

Male albino Wistar rats (200-280g.b.wt) were housed five per cage in a temperature controlled room under a fixed lighting cysle of LD12:12 (lights on 06h00) with food and water *ad libitum*. The rats were sacrificed by neck fracture between 10h30 and 11h30, to maintain uniformity with previous studies.

4.2.1.3 Pineal Glands

Pineal glands for cAMP-PDE determinations were swiftly removed as before **[2.3.1.2]** and placed in culture vessels containing 200 µl BGJ_b culture medium. Five pineal glands were transferred to each vessel.

4.2.1.4 Assay of cAMP-PDE in Rat Pineal Glands

4.2.1.4 (i) cAMP-PDE Assay

Five pineal glands were exposed to vehicle (control glands) while the remaining five glands were stimulated with the drug. These groups were incubated together with 200ml BGJ_b culture medium

at 37°C. The pineals were then removed from the vessels and each pineal gland was transferred to its own homogenization tube, for the measurement of cAMP-PDE activity as described below. The scheme for the cAMP-PDE assay is summarized in **Table 4.1.**

TABLE 4.1 Scheme for the cAMP - PDE assay (Neuman, 1983)

	Reaction vessel	Blank
Buffer	20µl	30µl
cAMP (1µM)	10µl	10µl
[^{3}H] - cAMP	10µl	10µl
Pineal homogenate	10µl	-
Incubate at 37°C for 30 minutes		
Add 30 µl of a 10% TCA		
Spot 10 µl onto TLC plates		

The pineal glands were individually homogenized in 100µl buffer (40µM Tris-HCl, pH8; 5µM Mg Cl_2). A 10µl aliquot of pineal homogenate was removed and transferred to a reaction tube. It was incubated at 37° with 1µM cAMP, 3 x10^5 cpm [^{3}H] cAMP and buffer (final volume 50µl). The reaction stopped after 30min by the addition of 30µl of 10% trichloroacetic acid (TCA) and the [^{3}H]-5'-AMP formed was separated from the [^{3}H] cAMP remaining on thin layer chromatography (TLC) plates, using a solvent system containing isopropyl alcohol, 25% water of NH_4OH and water (7:1:2).

4.2.1.4 (ii) Thin Layer Chromatography

10µl aliquots of each sample was spotted in duplicate 15mm from the bottom of the plates, and in the centre of 15mm wide channels inscribed on the silica gel TLC plates. The mobility of cAMP and 5'AMP was monitored routinely by spotting 10µl aliquots of standard solutions containing 0.2mg/ml authentic cAMP or 5'-AMP, dissolved in distilled water, in adjacent channels near one end of the plate. Following development of the plates (± 2 hours), the plates were dried under a gentle stream of nitrogen, then placed in a oven at 60°C (10min) to dry the plates.

4.2.1.5 Quantitation

Visualization of these markers was accomplished under short wave ultraviolet light. The radioactivity in the identified spots was determined by scraping off the spots into a scintillation vial containing 400µl 0.01M KOH (Beckman Information Brochure).

After the KOH had solubilized the scrapings, 3ml of scintillation cocktail (Beckman Ready-Solv HP/b) was added to the vials. These were then shaken on a Griffith Flask shaker for 20 min. after which the vials were placed in a Beckman LS 2800 scintillation counter and each vial was counted for a total of 5 minutes.

4.2.1.6 Data Analysis and Statistics

The mean dpm's were determined for each sample. Blanks were run in exactly the same way except that TCA was added to the tubes before tissue was included. Thompson *et al,*. (1974) described several factors which could influence blank values. These authors suggest that the method of terminating the reaction by boiling for 2 minutes could possibly increase blanks in tritium based assays, apparently due to formation of tritiated water. Wells and Hardman (1977) suggest that the construction of the appropriate blanks is best achieved by the addition of the stopping mixture prior to the addition of tissue and they reported that this should equal blank values.

Blank values, which were very low, were first subtracted from the results before these were finally expressed as nmol cAMP hydrolyzed/30 min/pineal gland.

4.2.1.7 Statistical Analysis

Cyclic-AMP PDE levels (the mean of triplicate determinations) for the assays are expressed as nmol cAMP hydrolyzed/30 min/pineal gland. The significance between those glands stimulated with drug and control glands was evaluated using the two-tailed Student's t-test.

4.2.2 Results and Discussion

The radioactivity being applied to the TLC plates was checked to determine if it was being recovered in order to validate the assay. In order to assess the recovery of cAMP from silica gel TLC plates in the assay system, sample tubes were made up containing the equivalent amounts of reaction mixture that they would have contained under assay conditions. The TCA was added first to inactivate any PDE in the pineal tissue homogenate used. Aliquots (10µl) were then removed from these sample tubes and applied to TLC plates, either before or after development, or directly to scintillation vials. The quantitation and counting procedures were then followed as described above [**4.2.1.5**].

The radioactivity remaining at the origin or at the 5' AMP region was negligible in the TCA blanks with the major radioactivity being recovered at the [^{3}H]-cAMP region, thus validating this assay.

4.3 Experiment: TO DETERMINE THE EFFECT OF PHYSOSTIGMINE ON PDE ACTIVITY

4.3.1 Introduction

The relative rate of formation and degradation of the cyclic nucleotides determine the steady state levels of these compounds. Robison *et al.*, (1971) proposed that inhibition of cAMP-PDE should potentiate the effect of hormones acting via cAMP and has often been used in attempts to demonstrate cAMP involvement in a hormone response (Wells and Kramer, 1981). Alternatively the opposite is true, that by demonstrating cAMP potentiation in the presence of the hormone is due to cAMP-PDE inhibition. The cAMP studies [**3.3.3**] showed that a cholinomimetic agent acetylcholine increased cAMP levels significantly. In this study the anticholinesterase physostigmine was used to investigate whether the drug has any effect on the cyclic AMP-PDE activity.

4.3.2 Materials and Methods

4.3.2.1 Materials

Physostigmine was purchased from Sigma Chemical Co., USA. Sources of all other materials were recorded in [**4.2.1.1**].

4.3.2.2 Animals

Male Wistar rates (200-280g.b.wt) were used in this study. Their living environment has been described in [**2.3.1.2**]. The rats were sacrificed by neck fracture between 10h30 and 11h30. This was at the same time as in other studies thus maintaining uniformity.

4.3.2.3 Method

After decapitation, pineals were swiftly removed aseptically as before in [**2.3.1.2**] and placed in culture vessels containing BGJ_b culture medium at 37°C in an atmosphere of $5\%CO_2$:$95\%O_2$ (carbogen). Five pineal glands were transferred to vessels containing vehicle in the case of the control, and a further five to vessels containing physostigmine ($IO^{-5}M$) drug in the other. The vessels contained a final incubation volume of 200µl. After a 3 hour incubation period at 37°C the pineals were removed from the vessels and each transferred to its own homogenization tube containing "cAMP-PDE buffer". The cAMP-PDE activity was then assayed as described above [**4.2.1.4**]. The significance was evaluated using Student's t-test.

4.3.3 Results

Physostigmine caused a significant increase in PDE activity ($p<0.05$) when compared to the controls (**Table 4.2** and **Figure 4.1**).

TABLE 4.2 Cyclic AMP-phosphodiesterase activity per rat pineal gland in the presence of vehicle or physostigmine (10^{-5}M) (mean ± SEM; n=5 glands per group.

Treatment	CAMP hydrolyzed (nmol/30min/pineal gland)
Control	83,65 ± 23,27
Physostigmine	129,85 ± 37,28*

* *In comparison with respect to the vehicle control, p < *0.05 (Student's t-test).*

4.3.4 Discussion

The anticholinesterase physostigmine caused an increase in phosphodiesterase activity ($p<0.05$). In parallel experiments, organ culture studies [**2.7.3**] showed an increase in aHT and not aMT. In cAMP studies [**3.3.3**] cholinomimetic agent acetylcholine caused a significant rise in cAMP levels. It is a well known finding that the rise in intracellular concentration of cAMP is coupled to a corresponding increase in its regular cAMP-phosphodiesterase (PDE) (Oleshansky and Neff, 1975). It may be thus speculated that an increase in cAMP occurs due to cholinergic agents with a concomitant rise in PDE activity levels. In so doing these drugs may possibly induce a rise in NAT activity and aHT levels.

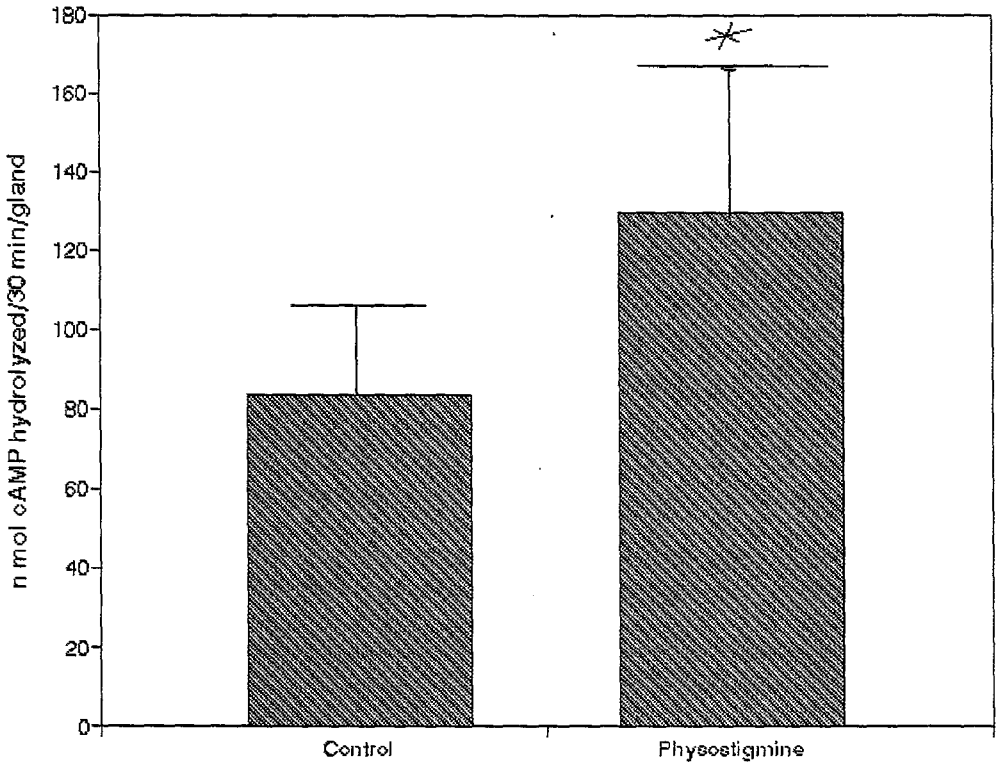


Figure 4.1 Cyclic AMP - Phosphodiesterase activity per rat pineal gland in the presence of vehicle or physostigmine (10^{-5}M) (mean ± SEM; n=5 glands per group; $p<^{*}0.05$ Student's t-test)

CHAPTER 5

N-ACETYLTRANSFERASE STUDIES

5.1 Introduction

The hormone melatonin, isolated by Lerner *et al.*, (1958) is produced in the pineal gland by O-methylation of N-acetylserotonin (Axelrod and Weissbach, 1960). The enzyme responsible for O-methylation in the pineal, hydroxyindole-o-methyltransferase, can only utilize serotonin one tenth as efficiently as it can N-acetylserotonin implying that N-acetylation preceeds O-methylation (Axelrod and Weissbach, 1960). Serotonin has shown to be N-acetylated to N-acetylserotonin *in vivo* (McIsaac and Page, 1959) and this reaction is catalyzed by an N-acetyltransferase enzyme (Weissbach *et al.*, 1960).

Investigations carried out in rat pineal glands have shown that the release of noradrenaline from the nerve terminals innervates the gland and regulates N-acetyltransferase activity (Klein *et al.*, 1981a; Moore and Klein, 1974; Klein and Moore, 1979; Brownstein and Axelrod, 1974). The release causes ß receptor stimulation leading to an elevation of intracellualar cAMP (Strada *et al.*, 1972; Deguchi and Axelrod, 1973). The elevated cAMP levels stimulates a series of protein phosphorylations (Morgan *et al.*, 1988) which induces the activity of the arylalkylamine N-acetyltransferase (NAT), the rate limiting enzyme in the conversion of serotonin to melatonin (Klein *et al.*, 1981a). The ∝- and β-receptors play a crucial role in the mechanism by which NA controls cAMP. Sugden and Klein (1984) confirmed the presence of ∝-receptors in the pineal gland. Noradrenaline has been found to regulate cAMP and cGMP through an unusual synergistic mechanism. Both cAMP and cGMP are increased through β-adrenoceptor stimulation whilst ∝-adrenoceptor stimulation of the pineal gland alone produces no detectable change in the levels of these two nucleotides (Vanacek *et al.*, 1986).

The α-adrenoceptor stimulation does however amplify the β-adrenoceptor response by potentiating the accumulation of cAMP and cGMP (Klein *et al.*, 1983; Vanacek *et al.*, 1985; Klein, 1985). Beta-adrenergic activity sufficient for the induction of NAT (Klein, 1978; Zatz, 1982) has been shown to be potentiated by α-adrenoreceptor stimulation (Klein *et al.*, 1983) although the effect is not as pronounced as that seen with cAMP levels (Illnerova *et al.*, 1983; Klein, 1985; Illnerova, 1985).

Before the development of the very sensitive radioimmunoassays for melatonin the most common indication of pineal function and especially of melatonin production were the activities of NAT (Klein and Weller, 1970) and HIOMT (Wurtman *et al.*, 1963b). NAT was thought to be the rate limiting enzyme in melatonin formation (Klein and Weller, 1970; 1973) since it had been shown to best correlate with melatonin production (Panke *et al.*, 1978; Wilkinson *et al.*, 1977; Morton, 1987).

The accumulation of some evidence shows that NAT activity does not always mirror the production of melatonin. By subjecting rats to stress produces varied NAT results in comparison to melatonin (Seggie *et al.*, 1985; Vollrath and Welker, 1988). The pineal appears to modulate the body's response to stress via a mechanism involving the release of melatonin.

Tannenbaum *et al.*, (1988) supported this by demonstrating decreased NAT levels in the presence of raised aMT. Kahn *et al.*, (1990) have demonstrated that gastric ulcers induced by cold stress are reduced in the presence of melatonin.

5.1.1 Theory of the Assay

Deguchi and Axelrod (1972a) have developed a radiochemical method of assaying serotonin N-acetyltransferase and is based on the acetylation of tryptamine with radioactive acetyl coenzyme A by NAT to form radioactive N-acetyltryptamine. Tryptamine is used in place of the natural substrate 5-hydroxytryptamine as the enzyme can utilize it far more readily than 5-hydroxytryptamine. The radioactive N-acetyltryptamine thus formed can be extracted into an

organic solvent and measured by liquid scintillometry. The use of this assay permits a rapid and sensitive evaluation of NAT activity and also enables a more accurate interpretation of any pharmacological effects on the enzyme (Deguchi and Axelrod, 1972a).

In the sections that follow the method of Deguchi and Axelrod (1972a) with minor modifications, is described.

5.2 NAT ASSAY TECHNIQUE

5.2.1 Materials and Methods

5.2.1.1 Animals

Adult male Wistar rats (200-250g) were used. The animals were maintained under normal laboratory conditions as detailed in [**2.3.1.2**]. Ilnerova (1975) showed NAT in rats to be insensitive to oestradiol treatment. Hence female albino Wistar rats may be used throughout the NAT studies as NAT, unlike HIOMT, has been shown not to be influenced by the various stages of the estrus cycle (Cardinali and Vacas, 1978). Male rats were however used to maintain uniformity with previous studies.

The rats were sacrificed between 10h30 and 11h30 by neck fracture. This was done to avoid possible interference with β-receptor sensitivity changes. It is well known that the increase on NAT activity following adrenergic stimulation is dependent on the time of day (Romero and Axelrod, 1974). The β-receptors show down regulation after the full stimulation of the night, a rhythm in receptor density which is well documented (Reiter *et al.*, 1985; Gonzalez Brito *et al.*, 1988; Santana *et al.*, 1988).

5.2.1.2 Chemicals and Reagents

Batches of tritiated acetyl coenzyme A (specific activity 3.9 Ci/mmol) were purchased, as required, from Amersham Laboratories (England); Tryptamine-HCl and l-isoprenaline-HCl from Sigma Chemical Co. (USA); and unlabelled acetyl coenzyme A from Boehringer Mannheim (Germany). All other chemicals and reagents were obtained from local commercial sources and were of analytical grade.

5.2.1.3 NAT Assay Procedure

The assay used is a modification of that originally described by Deguchi and Axelrod (1972a). Pineal glands were rapidly excised from rats as described previously [**2.3.1.2**]. Each pineal was individually placed in a small glass homogenizer containing 100µl of ice-cold 50mM phosphate buffer (pH 6.8) and homogenized for 30 seconds (about 15 strokes). The reaction mixture contained 20µl homogenate which was removed and transferred to the bottom of corresponding separate cold microfuge tubes with 10µl of the working solution. The working solution was made up of 5.6mM tryptamine, 800µM acetyl CoA and 40nCi [^{3}H] acetyl coenzyme A in a total volume of 10µl per sample tube.

All manipulations and additions up to this stage were performed on ice. Samples were then incubated for 45 minutes at 37°C on a waterbath and the reaction terminated by the addition of 100µl chilled 0.2M borate buffer (pH10.0). A blank containing no tissue, was also included in each series of incubations. Using a Pasteur pipette the reaction mixture was then transferred to a tube containing 1ml of the toluene : isoamyl alcohol (97:3). The tubes were sealed and shaken for 5 minutes using a Griffith flask shaker. The emulsion formed as a result was dispersed by centrifugation in a microfuge for 30 seconds. Chilled borate buffer (100µl) was then added to each tube to wash out any contamination. Once again tubes were shaken for 5 minutes as before and centrifuged for 30 seconds. Aliquots of 500µl were transferred from the organic phase to a plastic scintillation vial containing 3ml scintillation fluid (Ready-Solv HP/B Beckman, USA).

The radioactivity in each sample was quantitated using liquid scintillometry spectroscopy in a Beckman LS 2500 scintillation counter at an efficiency of 61.15%. Counting efficiency, using computer-assisted analysis was determined by the external channel ratio method of quench correction. A correction factor was built into the quench correction program to allow for tritium decay. This enabled an accurate assessment of the label being quantitated. Radioactivity counts did not appear to fluctuate significantly after storing vials for up to two weeks. However, since scintillation fluid does evaporate from plastic vials with time, all samples were quantitated within 24 hours of preparation. Blank values were subtracted from the assay values before expressing the results as picomoles [^{3}H]-N-acetyltryptamine formed/gland/hour (Deguchi and Axelrod, 1972a).

5.2.1.4 *In Vitro* Studies

For studies aimed at evaluating the effect of pharmacological manipulation, *in vitro*, on NAT activity, groups of 5 pineals were incubated in sterile 12mm diameter glass culture vessels containing 490µl of the culture medium (BGJ_b Filton-Jackson modification), Gibco (Europe) [**2.3.1.3**]. The culture medium was placed in vessels of sterile 20ml glass vials during incubation. Drugs were added to the culture medium as 10µl aliquots. The tubes were sealed and incubated at 37°C for a specified time period. Following incubation, the glands were removed, homogenized and the enzyme activity determined as described above.

5.2.2 Results

The linear relationship between the amount of [^{3}H]-N-acetyltryptamine formed and the incubation period in each assay is depicted in **Figure 5.1** - the time course experiment. In all subsequent assays a 45 minute incubation period was used as it gave the greatest dpm counts. Blank values were obtained by incubation without enzyme.

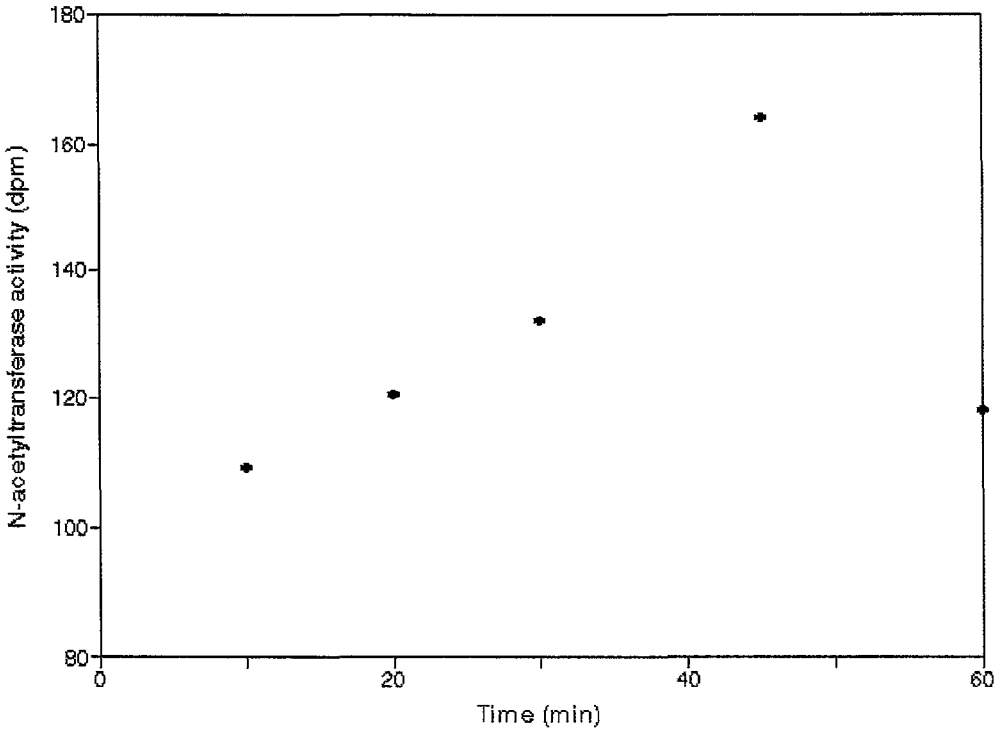


Figure 5.1 Effect of incubation time on N-acetyltransferase (NAT) activity.
Five pineal glands were collected and homogenized in 50mM phosphate buffer (pH 6.8). A 20µl aliquot of homogenate was used in the assay in a total volume of 30µl. Samples were incubated at 37°C for varying time periods. Each point represents the mean of duplicate determinations.

5.2.3 Discussion

In the assay, the use of tryptamine and radioactive acetyl coenzyme A as substrates makes it possible to extract the enzymically formed N-acetyltryptamine into a nonpolar solvent which does not extract acetyl coenzyme A. Within the conditions described above, the assay proved to be linear with time and homogenate concentration. In addition, the assay affords the measurement of enzyme activity in a single rat pineal gland.

5.3 Experiment: TO DETERMINE THE EFFECT OF PHYSOSTIGMINE ON NAT ACTIVITY IN RAT PINEAL GLANDS.

5.3.1 Introduction

The anticholinesterase agent physostigmine produced a significant rise in indole levels of N-acetylserotonin in organ culture studies [**2.7.4**]. N-acetyltransferase (NAT) is the enzyme responsible for the conversion of serotonin to N-acetylserotonin. The aim of this investigation is to determine whether the cholinergic system acts through the enzyme NAT in order to cause a rise in N-acetylserotonin levels without influencing melatonin levels. The drug physostigmine, a anticholinesterase agent was used.

5.3.2 Materials and Methods

5.3.2.1 Materials

l-Isoproterenol-HCl and physostigmine were purchased from the Sigma Chemical Co., USA. All other items are described in [**5.2.1.2**].

5.3.2.2 Animals

Adult male albino Wistar rats (200-250g.b.wt) were used in this study. Their environment is described in [**2.3.1.2**] and were housed five per cage in a temperature controlled room under a fixed lighting cycle of LD12:12 (lights on at 06h00) with food and water *ad libitum*. The rats were sacrificed between 10h30 and 11h30. This maintained uniformity with previous studies.

5.3.2.3 Methods

After the rats (n=15) were sacrificed their pineals were removed and transferred to sterile culture vessels containing BGJ_b culture medium. (Fitton-Jackson modification) at the required volume [**5.2.1.4**]. Five pineals were transferred to each of three incubation vessels containing BGJ_b culture medium. To one group of pineals isoproterenol ($IO^{-5}M$) was added to the medium, to another physostigmine ($IO^{-5}M$) and in the final group of pineals vehicle was added to give a final incubation volume of 500µl in each of the three vessels. The atmosphere in the vessels was saturated with carbogen ($5\%CO_2$:$95\%O_2$) before the taps were firmly secured. The vials were placed in a Forma Scientifica incubator and incubated in the dark at 37°C for 6 hours. After 6 hours the incubation was terminated by removing the glands from the vessels and placing them in individual 1ml glass homogenization tubes. They were assayed by the technique described in [**5.2.1.3**].

5.3.2.4 Data Analysis and Statistics

Data are expressed as pmol N-acetyltransferase activity/pineal gland/hour, with each point representing the mean ± SEM for 5 glands. Statistical comparisons are made using the One-Way ANOVA followed by Neuman-Keuls Multiple Range test.

5.3.3 Results

The results are represented in **Table 5.1**. Isoproterenol (10^{-5}M) caused the expected rise in NAT activity ($p<0.02$) whilst physostigmine did not cause NAT activity to rise significantly above basal levels.

TABLE 5.1 N-acetyltransferase activity per rat pineal gland in the presence of vehicle or when stimulated with either isoproterenol (10^{-5}M) or physostigmine (10^{-5}M) (mean ± SEM; n=5 glands per group).

Treatment	N-acetyltryptamine formed (pmol/pineal/hour)
Control	0,234 ± 0,070
Isoproterenol	0,344 ± 0,046*
physostigmine	0,266 ± 0,035

*In comparison with respect to vehicle control, p<*0,02 (One-Way ANOVA followed by Neuman-Keuls Multiple Range test)*

5.3.4 Discussion

Isoproterenol produced an increase in NAT as a result of adrenergic stimulation. NAT activation is dependent on the activation of adenylyl cyclase enzyme (Klein *et al.*, 1970; Klein *et al.*, 1978) leading to increased cAMP levels. These are controlled by β receptor stimulation which may be activated by the β receptor agonist isoproterenol, resulting in increased NAT activity. Physostigmine did not alter basal NAT activity suggesting that the cholinergic activated increase in the aHT did not occur due to the stimulation of the enzyme NAT.

The cAMP assay studies [**3.3.3**] revealed that the rise in aHT and not aMT due to the presence of acetylcholine in the organ culture studies [**2.7.3**] was possibly caused by the activation of the adrenergic β receptors resulting in the conversion of ATP to cAMP. Also, the adrenergic system is known to act by increasing intracellular cAMP in the pineal which in turn activates NAT enzyme

activity. The above NAT results [**5.3.3**] however show that NAT activity was not increased in the presence of an anticholinesterase agent. The cAMP-PDE studies revealed a significant rise in cAMP-PDE enzyme activity in the presence of an anticholinesterase agent physostigmine. The rise in cAMP levels could possibly be speculated to cause a transient increase in NAT enzyme activity thus explaining the aHT rise and not aMT.

The reasons for the acetylcholine inducing a rise in pineal cAMP without inducing a rise in pineal NAT activity remains unclear, but could however be related to a recent finding by Olivieri and Daya (1992) that raised intracellular calcium could reduce or inhibit pineal NAT activity. The synthesis of the pineal hormone melatonin, is regulated primarily by a classical ß-adrenoceptor system, calcium playing a important role in this regulatory mechanism (Brown *et al.*, 1989). During the synthesis of melatonin in the pineal gland, the enzyme NAT catalyzes the conversion of serotonin to N-acetylserotonin, the precursor of melatonin (Weissbach *et al.*, 1960; Axelrod and Weissbach, 1961). The activity of the enzyme is presently used as an indicator of pineal activity (Rodriguez-Cabello *et al.*, 1990).

Exposure of rats to the light during the dark phase of diurnal light cycle induces a rapid decline in pineal NAT activity (Klein and Weller, 1972). Such a decline in NAT activity has also been observed during homogenization of stimulated pineal glands (Binkley *et al.*, 1976). Studies have shown that calcium channel blockers reduce the nocturnal rise in pineal activity (Zawilska and Nowak, 1991). Chelation of extracellular calcium results in a reduction of NAT activity due to a reduced ß-adrenoceptor sensitivity as well as to reduce NAT protein synthesis (Zatz and Romero, 1978). A recent study using the chelating agent ethyleneglycol-bis-N,N,N',N',-tetraacetic acid (EGTA) in homogenates of pineal from isoproterenol treated rats have shown that EGTA reduces the rate of deactivation of NAT (Rodriguez-Cabello *et al.*, 1990).

Olivieri and Daya (1992) support the view that EGTA, a chelating agent, enhances NAT activity. In addition, the authors suggest that the addition of calcium at high concentrations to pineal homogenates reduces pineal NAT activity. It appears therefore that the concentration of calcium in

pinealocytes is important for the regulation of pineal NAT. Acetylcholine is known to trigger the inositol phosphate signalling system in the pineal gland. This signalling system is known to result in a elevation of diacylglycerol (DAG) as well as intracellular calcium providing a possible way by which acetylcholine induces a rise in pineal cAMP without inducing a rise in pineal NAT activity.

CHAPTER 6

HYDROXYINDOLE-O-METHYLTRANSFERASE (HIOMT) STUDIES

6.1 Introduction

6.1.1 Properties of HIOMT

Hydroxyindole-O-methyltransferase was first detected in bovine pineal glands by Axelrod and Weissbach (1960) and has since been found in the pineal glands of other mammals (Quay, 1966), birds, reptiles and amphibians (Quay, 1965). Until recently it was thought to be unique to the pineal gland but has since been found in the retina and harderian gland (Cardinali and Wurtman, 1972) and may occur in enterochromaffin cells (Raikhlin *et al.*, 1975). Retinal hydroxyindole-O-methyltransferase exhibits similar properties to the pineal enzymes while that from the harderian gland is notably different, showing a different spectrum of substrate specificities (Cardinali and Wurtman, 1972).

HIOMT is capable of transferring the methyl group of S-adenosyl methionine to the hydroxy group of N-acetylserotonin. Axelrod and Weissbach (1961) showed that unlike catechol-O-methyltransferase, this enzyme had no requirement for a metal. The optimum enzyme activity occurs between pH 7.5 and 8.3 and N-acetylserotonin proved to be the best substrate. Other 5-hydroxyindoleamines, serotonin and its N-methylated derivatives, as well as 5-hydroxyindole acetic acid are also O-methylated, but at a much reduced rate.

Deguchi and Barchas (1971) showed that S-adenosyl-homocysteine, the product which results when S-adenosylmethionine donates its methyl group, is a potent inhibitor of HIOMT activity and binds to HIOMT with a higher affinity than S-adenosyl methionine.

Cardinali and Wurtman (1972) demonstrated the presence of HIOMT in the rat pineal gland, retina and harderian gland. They found that Mg^{++} enhances HIOMT activity in the harderian gland but

does not affect HIOMT activity in the pineal and retina. These authors concluded that the pineal and retinal HIOMT enzymes were very similar but differed from that in the harderian gland in requirements for storage, pH optima and metal ions.

Hydroxyindole-O-methyltransferase catalyses the formation of a variety of methoxyindoles including 5-methoxytryptophan and 5-methoxytryptamine (Balemans *et al.*, 1980), melatonin (Axelrod and Weissbach, 1960), 5-methoxytryptophol (McIsaac *et al.*, 1965) and 5-methoxyindole-3-acetic acid (Axelrod and Weissbach, 1961). It has been suggested that several such enzymes exist in the pineal (Cremer-Bartels, 1979), activity being regulated by pterins and pteridines (Cremer-Bartels, 1979; Balemans *et al.*, 1980).

6.1.2 Effect of Light on HIOMT Rhythms

There is a diurnal fluctuation in pineal HIOMT activity. HIOMT activity increases during the night and drops during the day (Axelrod *et al.*, 1965). The presence of light suppresses the activity of this enzyme (Wurtman *et al.*, 1963a). The nocturnal rise of the enzyme is abolished by bilateral superior cervical ganglionectomy (Axelrod *et al.*, 1965).

Yochim and Wallen (1974) showed that although HIOMT activity rises and remains high in rats placed in continuous darkness (Wurtman and Axelrod, 1965), an estrus cycle pattern of HIOMT activity remains. The presence of such a rhythm in the dark suggests an endogenous aspect of the rhythm. They described the pattern of enzyme activity as the interaction of two component rhythms with slightly different periodicities whose characters are endogenous, synchronized with the estrus cycle, yet modified by light.

Pevet *et al.*, (1980) found that the rat pineal HIOMT activity involved in the synthesis of melatonin is high at the end of the dark period and at the middle of the light period. The pineal HIOMT activity concerned with the production of 5-methoxytryptophol is only observed during the light period. Comparing the peaks, these authors conclude that the pineal produces more 5-

methoxytryptophol than melatonin. They suggest that different HIOMT enzymes are involved in the synthesis of 5-methoxytryptophol and melatonin. Balemans *et al.*, (1980) found that in rat pineals and harderian glands a larger amount of 5-methoxytryptophol than melatonin is synthesized, confirming the proposal by Pevet *et al.*, (1980).

Jackson and Lovenberg (1971) speculate that the diurnal variation observed in the activity of HIOMT might be due to an association-dissociation phenomenon while Yang and Neff (1976) showed that the diurnal variation is due to an alteration of enzyme molecule numbers rather than a change in enzyme kinetics.

Although studies have evaluated some kinetic parameters (Cardinali and Wurtman, 1972) and several groups have obtained pure enzyme (Jackson and Lovenberg, 1971; Kuwano *et al.*, 1978) only one report has suggested a catalytic mechanism (Satake and Morton, 1979). This report proposed an ordered BiBi mechanism for the enzyme.

6.1.3 Theory of the Assay

The assay technique used for the determination of HIOMT activity was the one by Champney *et al.*, (1984). The assay is based on the transfer of a radioactive [^{14}C]-methyl group from the methyl donor, S-adenosyl-L-[methyl-^{14}C] methionine to the substrate, N-acetylserotonin to form radioactive melatonin (N-acetyl-5-[methoxy-^{14}C] tryptamine). In the pineal gland, HIOMT O-methylates N-acetylserotonin to form melatonin. By adding radioactive S-adenosyl methionine to pineal homogenates incubated with N-acetylserotonin, radioactive melatonin is formed which can be extracted and measured by liquid scintillometry. The amount of radioactive melatonin formed can be used as an indication of HIOMT activity.

In the sections that follow the method described by Champney *et al.*, (1984) with minor modifications, is used.

6.2 HIOMT ASSAY TECHNIQUE

6.2.1 Materials and Methods

6.2.1.1 Animals

Adult male Wistar rats (200-250g) were used. The animals were maintained under normal laboratory conditions as detailed in [**2.3.1.2**]. Wurtman *et al.*, (1963a) showed that rat pineal HIOMT activity varies during the estrus cycle and in light of this finding only male rats were used to maintain a uniformity with previous studies. The rats were sacrificed between 10h30 and 11h30 by neck fracture, at the same time as the rats used in the organ culture studies. This was done to avoid possible interference with β receptor sensitivity changes.

6.2.1.2 Chemicals and Reagents

Radioactive S-adenosyl-L-[methyl-^{14}C] methionine (Specific Activity 56mCi/mmol) was purchased as required from Amersham Laboratories (England). N-acetylserotonin and cold S-adenosyl methionine were purchased from Sigma Chemical Co. (USA). All other chemicals and reagents were obtained from local commercial sources and were of analytical grade.

6.2.1.3 HIOMT Assay Procedure

Pineal glands were rapidly excised from rats aseptically as described previously [**2.3.1.2**]. Each pineal was placed in a small glass homogenizer containing 100µl of ice-cold 50mM sodium phosphate buffer (pH7.9) and homogenized for 30 seconds (about 15 strokes). The reaction mixture contained 10µl homogenate which was removed and transferred to the bottom of corresponding separate cold microfuge tubes together with 10µl of the working solution. The working solution contained 3mM N-acetylserotonin, 200mM sodium phosphate (pH7.9) buffer and [^{14}C] SAM in a total volume of 10µl per sample tube.

All manipulations and additions up to this stage were performed on ice. Samples were then incubated for 30 minutes at 30°C on a waterbath and the reaction terminated by the addition of 100µl chilled 0.3M borate buffer (pH10.0). A blank containing no tissue was also included in each series of incubations.

Using a Pasteur pipette the reaction mixture was then transferred to a tube containing 1ml of the toluene : isoamyl alcohol (97:3). The tubes were sealed and shaken for 5 minutes using a Griffith flask shaker. The emulsion formed as a result was dispersed by centrifugation in a microfuge for 30 seconds. Chilled borate buffer (100µl) was then added to each tube to wash out any contamination. Once again tubes were shaken for 5 minutes as before and centrifuged for 30 seconds. Aliquots of 500µl of the organic phase were transferred to plastic scintillation vials containing 3ml scintillation fluid (Ready-Solv HP/B Beckman, USA).

The radioactivity in each sample was quantitated using liquid scintillometry in a Beckman LS 2500 scintillation counter at an efficiency of 61,15%. Counting efficiency, using computer-assisted analysis was determined by the external channel ratio method of quench correction. Blank values were subtracted from the assay values before expressing the results as picomoles [^{14}C] melatonin formed/gland/hour (Champney *et al.*, 1984).

6.2.1.4 *In Vitro* Studies

For studies aimed at evaluating the effect of pharmacological manipulation, *in vitro*, on HIOMT activity, groups of 5 pineals were incubated in sterile 12mm diameter glass culture vessels containing 490µl of the culture medium (BGJ_b Fitton-Jackson modification, Gibco (Europe) **[2.3.1.3]**. The culture medium was placed in vessels of sterile 20ml glass vials during incubation and prewarmed. Drugs were then added to the culture medium as 10µl aliquots. The tubes were sealed and incubated at 37°C for a specified time period. Following incubation, the glands were removed, homogenized and the enzyme activity determined as described above.

6.2.2 Results

The HIOMT assay was carried out using a 45 minute incubation period. Blank values were obtained by incubating the tubes without the enzyme present.

6.2.3 Discussion

In the assay the use of radioactive S-adenosyl-methionine (SAM) and N-acetylserotonin as substrates makes it possible to extract the enzymically formed melatonin (N-acetyl-5-[methoxy ^{14}C] tryptamine) into a nonpolar solvent. The amounts of radioactive melatonin formed gives an indication of the amount of HIOMT activity. This assay affords the measurement of enzyme activity in a single rat pineal gland.

6.3 Experiment: TO DETERMINE THE EFFECT OF PHYSOSTIGMINE ON HIOMT ACTIVITY IN THE RAT PINEAL GLAND

6.3.1 Introduction

The anticholinesterase enzyme physostigmine produced a significant increase in N-acetylserotonin levels but not melatonin [**2.8.3**]. The N-acetyltransferase enzyme studies [**5.3.3**] showed that the rise in aHT was not caused by the stimulation of the enzyme NAT. The aim of this investigation is to determine whether physostigmine inhibits enzyme HIOMT to reduce conversion of the high levels of aHT to aMT.

6.3.2 Materials and Methods

6.3.2.1 Materials

Physostigmine was purchased from the Sigma Chemical Co., USA. All other chemicals and reagents are described in [**6.2.1.2**].

6.3.2.2 Animals

Adult male albino Wistar rats (200-250g.b.wt) were used in this study. The rats were housed five per cage in a temperature controlled room under a fixed lighting cycle of LD12:12 (lights on at 06h00) with food and water *ad libitum*. The rats were sacrificed between 10h30 and 11h30. This maintained uniformity with previous studies.

6.3.2.3 Methods

After the rats (n=10) were sacrificed their pineals were removed and transferred to sterile culture vessels containing BGJ_b culture medium (Fitton-Jackson modification) at the required volume **[6.2.1.4]**. Five pineals were added to both incubation vessels which contained BGJ_b culture medium. To one group of pineals physostigmine ($IO^{-5}M$) was added and to the other vessel vehicle was added and acted as the control group. The final incubation volume in each vessel was 500µl. The atmosphere in the vessels was saturated with carbogen ($5\%CO_2$:$95\%O_2$) before the taps were firmly secured. The vials were placed in a Forma Scientifica incubator and incubated in the dark at 37°C for 6 hours. After 6 hours the incubation was terminated by removing the glands from the vessels and placing them in individual 1ml glass homogenization tubes. They were assayed by the usual technique **[6.2.1.3]**.

6.3.2.4 Data Analysis and Statistics

Data are expressed as pmol hydroxyindole-O-methyltransferase activity/pineal gland/hour with each point representing the mean ±SEM for five pineal glands. Statistical comparisons were made using the Students *t*-test.

6.3.3 Results

The HIOMT activity for the physostigmine stimulated group was not significantly different to the controls. The results are tabulated in **Table 6.1**.

TABLE 6.1 Hydroxyindole-o-methyltransferase activity per rat pineal gland in the presence of vehicle, or when stimulated with physostigmine (10^{-5}M) (mean ± SEM; n=5 glands per group).

Treatment	melatonin formed pmol/pineal gland/hour
Control	8,165 ± 0,553
Physostigmine	9,695 ± 1,483*

* *Not significant in comparison with respect to the vehicle control.*

6.3.4 Discussion

The results show that physostigmine did not cause any significant changes in basal H10MT activity. This indicates that physostigmine did not inhibit the enzyme H10MT to reduce the conversion of the high levels of N-acetylserotonin induced by this drug, to melatonin.

Hence the mechanism by which the cholinergic agonist acetylcholine and the anticholinesterase agent physostigmine is able to increase aHT levels and not aMT levels still remains to be elucidated. A number of possible mechanisms causing this phenomenon may be examined :

(i) The occupation of the cell surface receptors by cholinergic molecules may be speculated to occur leading to a generation of intracellular second messengers eg. cyclic nucleotide, Ca^{2+} and diacylglycerol (DAG) which act as allosteric effectors to activate a variety of protein kinases (Shenolikar, 1987). These secondary messengers may hold the answer as to how aHT levels are able to rise without an increase in aMT levels.

(ii) The neutral lipid diacylglycerol is normally absent from membranes. On receptor stimulation, it appears transiently and disappears within seconds due to its conversion back to inositol phospholipids and its degradation to arachidonic acid (AA) which in turn can generate other messengers eg. the prostaglandins. The diacylglycerol formation resultant from phosphoinositol breakdown, following ∝-adrenergic stimulation, and the subsequent effects on cyclic nucleotide (Sugden *et al.*, 1985) and NAT activation (Zatz, 1985) have been adequately demonstrated in rats. The transient action of diacylglycerol may be a mechanism by which a transient increase in NAT activity occurred and by which levels of aHT rose without causing a concomitant rise in aMT.

(iii) Another mechanism which needs to be considered is the phosphoinositides. A compelling amount of evidence indicates that the ubiquitous inositol lipid signalling pathway plays a key role in neurotransmission and neuro-modulation (Exton, 1985). Laitinen *et al.*, (1989b) suggest the presence of a functional acetylcholine receptor in the rat pineal which are coupled to the phosphoinositide signalling pathway. Although the muscarinic receptor density in rat pineals (Taylor *et al.*, 1980) seems to be only a fraction of that reported for ∝-adrenoreceptors in the gland (Sugden and Klein, 1985), the phosphoinositide response for submillimolar concentrations of both muscarinic and adrenergic agonists were very similar (Laitinen *et al.*, 1989b). The results of the study done by Laitinen *et al.*, (1989b) suggest that acetylcholine could modulate pineal function through the stimulation of phosphoinositide turnover. This signalling pathway may in some way influence aHT levels to rise without aMT being affected.

(iv) It has been reported that four different genes code for muscarinic receptors (Kerslavage *et al.*, 1987). Studies on second messenger coupling of muscarinic receptors subtypes (M_1-M_4) suggest that the expression of rat muscarinic M_1 and human M_1 and M_4 receptor genes in cells lacking endogenous muscarinic receptors can lead to a complete appearance of the phosphoinositide (PI) signalling pathway and that the magnitude of the signal produced is proportional to the level of gene expression for these receptors (Dudley *et al.*, 1988; Peralta

et al., 1988). Hence muscarinic gene activation may also be in some way linked to the N-acetylserotonin levels rising without a rise in melatonin levels.

(v) Cyclic-GMP is a secondary messenger whose concentration is regulated in a manner somewhat similar to cAMP (Vanacek *et al.*, 1985). A fascinating feature of the pineal gland is that there are reciprocal changes in the magnitudes of cAMP and cGMP responses produced by changes in chronic neural stimulation (Deguchi and Axelrod, 1973; Klein *et al.*, 1981b). Following long periods of stimulus deprivation, produced by keeping animals in constant lighting or denervating the pineal gland, the cAMP response increases two-fold, an example of denervation supersensitivity. This is similar to what is generally seen in neural and hormonal regulation. In contrast the pineal cGMP response exhibits the opposite, a denervation subsensitivity . These effects of a supersensitive cAMP response and a subsensitive cGMP response are termed "see-saw" signal processing. The importance of this has not been established, but it may have important implications because it shifts the pineal gland between a mono- and a bi-cyclic nucleotide second messenger system. Assuming that each regulates different processes, one can imagine that the two cyclic nucleotides might be responsible for turning "off" and "on" different hormone systems eg while cAMP controls aMT production, cGMP might regulate another hormone. Hence cGMP may be speculated to have some influence on aHT and a closer examination of this secondary messenger needs to be carried out.

(vi) Prostaglandin stimulation of NAT may represent another mechanism by which the pineal is able to regulate its aHT and aMT production. Cardinali and Vacas (1987) have shown that prostaglandin E (*in vitro*) affects pineal NAT activity. The prostaglandins could hence also provide a possible mechanism by which increased aHT levels could be obtained without a rise in aMT levels.

(vii) Stress and the release of catecholamines from synaptic nerve endings and adrenal medulla could influence the rise in NAT activity and cause a increase in aHT levels. Vaughan *et*

al., (1978) found NAT activity to be sensitive to stressful stimuli and provides a possible way by which N-acetylserotonin levels are increased.

(viii) The rise may also be speculated to be caused by non-cholinergic agents. NAT activity is very sensitive to drug manipulations which affect beta-receptor adenylate cyclase coupled regulating mechanisms. Agents such as isoproterenol, pargyline (monoamine oxidase enzyme inhibitor), cocaine (noradrenaline reuptake blocker) have all been found to affect NAT activity (Deguchi and Axelrod, 1972b). This provides another mechanism by which aHT levels could be influenced.

(ix) The reasons for actylcholine inducing a rise in pineal cAMP without inducing a rise in pineal NAT activity remains unclear, but could however be related to a recent finding by Olivieri and Daya (1992) that raised intracellular calcium levels could reduce or inhibit pineal NAT activity. Acetylcholine is known to trigger the inositol phosphate signalling system in the pineal. The signalling system is known to result in a elevation of diacylgycerol (DAG) as well as intracellular calcium, and hence could play a role in influencing the rise in aHT.

The physiological significance of the rise in aHT by acetylcholine without influencing aMT levels remains to be elucidated and the mechanism by which it occurs needs to be researched further.

CHAPTER 7

ACETYLCHOLINESTERASE STUDIES

7.1 Introduction

Acetylcholinesterase is the enzyme which breaks down and inactivates the neurotransmitter acetylcholine. Hence, the presence of acetylcholinesterase (AChE) is one indicator of the neural nature of a tissue (Cooper *et al.*, 1982). This however, cannot be the sole indicator and additional evidence is necessary for exceptions to this generality are common. The study of Manocha (1970) demonstrated the complexity of the distribution of AchE (also called true or specific cholinesterase) and nonspecific cholinesterase (also called pseudo- or butyrylcholinesterase) in neural tissue.

When considering the pineal gland, AChE has been shown in a number of non mammalian animals, where pineal neurons are present and stain for AchE. Examples of these are anurans (Wake *et al.*, 1974), goldfish (Wake, 1973), rainbow trout (Korf, 1974), eel (Matsuura and Herwig, 1981) and birds (Ueck and Kobayashi, 1972). In mammals, AchE is also present in the pineal gland in some species. The enzyme in the bovine pineal was detectable biochemically (23μM/g/hr, LaBella and Shin, 1968). Rodriguez De Lores Arnaiz and Pellegrino De Iraldi (1972) estimated that the enzyme was equally distributed between pinealocytes and nerve fibres, since superior cervical ganglionectomy was followed by a 50% decrease of enzyme activity (Pellegrino De Iraldi and Rodriguez De Lores Arnaiz, 1972).

Histochemically the enzyme was regularly demonstrated in intrapineal sympathetic nerve fibres (Eränkö *et al.*, 1970; Trueman and Herbert, 1970a; Machado and Lemos, 1971). A mild to moderate reaction showed in the pinealocytes of the squirrel monkey (Manocha, 1970).

A few researchers have found however that the pinealocytes of rats (Arstila, 1967) and ferrets (Trueman and Herbert, 1970a) are negative for AChE. Also, Vollrath (1981) citing unpublished

work by Köhl, reported that guinea pig pinealocytes lacked AChE activity, even though the nerve fibres tested positive. These studies were performed using light microscopy. Luo *et al.*, (1990) recently discovered through the ultrastructural localization of AchE in the guinea pig pineal gland that the AChE-positive pinealocytes synthesize AChE. The AChE reaction product was also seen in the intracellular space between pinealocyte processes.

Acetylcholinesterase has been reported to possess peptidase activity and can hydrolyze both substance *P* (Chubb *et al.*, 1980) and leu- and metenkephalin (Chubb *et al.*, 1982). Substance *P* was revealed in the pineal gland with radioimmunoassay studies (Powel *et al.*, 1977), and substance *P* fibres were also found in the pineal organ, pineal stalk and deep pineal glands (Ronnekleiv and Kelly, 1984). However it is not known if the pineal AchE hydrolyzes the pineal substance *P*.

Luo *et al.*, (1990) recently provided evidence through the ultrastructural localization of AChE in the guinea pig pineal gland that the AChE-positive pinealocytes synthesize AChE. The AChE reaction product was also seen in the intracellular space between pinealocyte processes, confirming the enzyme's presence in the pineal gland.

7.1.1 Theory of the Assay

The principle role of AChE is believed to be the termination of nerve impulse transmission by hydrolysis of the neurotransmitter acetylcholine. The basis of the assay is the observation, by spectrophotometry, of a change in colour and hence absorption when the substrate acetylthiocholine is enzymatically broken down by acetylcholinesterase to acetate and thiocholine.

7.2 ACETYLCHOLINESTERASE DETERMINATION TECHNIQUE

7.2.1 Materials and Methods

7.2.1.1 Chemicals, Drugs and Reagents

Acetylthiocholine chloride, acetylcholine chloride, reserpine and 5,5 dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from the Sigma Chemical Co., USA. All other inorganic and buffer chemicals were of reagent grade.

7.2.1.2 Animals

Male rats of the Wistar strain weighing 200-250g were purchased from the South African Institute for Medical Research and housed five per cage under a diurnal lighting cycle of LD12:12 with food and water *ad libitum* for at least two weeks prior to the experiment. The animals were maintained and sacrificed as previously described in [**2.3.1.2**].

7.2.1.3 Preparation of Rat Brain and Pineal Glands

After removal of the rat brain cerebral cortex and pineal gland they were each individually homogenized. The brains were homogenized in 3ml and the pineals in 150µl of a 0.1M phosphate buffer, pH7. To this Triton X-100 was added, 3ml for brain tissue and 150µl for pineal tissue. The mixture was then centrifuged at 3000 x g for 15 minutes and 50µl aliquots were assayed for AChE.

7.2.1.4 The Acetylcholinesterase Assay

Hydrolysis of acetylthiocholine by acetylcholinesterase was recorded at 410nm at room temperature in accordance with the method described by Ellman *et al.*, (1961). The assay mixture (3.14ml) contained phosphate buffer (0.1M, pH7), buffered Ellman's reagent (DTNB 10 mM), $NaHCO_3$

(17.85mM, pH7) and acetylthiocholine chloride (0.2 mM). Aliquots were made up to 3.12ml with phosphate buffer (pH8) and the reaction was initiated by the addition of 50µl pineal or brain supernatant as described above [**7.2.1.3**]. The enzyme concentration was determined using an absorption coefficient (A_1cm) of 16.1 at 280nm and a Mr 260 000 (Silman, 1976). The protein concentration of the rat brain was determined according to a procedure developed by Lowry *et al.*, (1951) using bovine serum albumin as a standard. A typical protein calibration curve as well as the scheme of the protein assay may be found in [**3.2.1.4**].

7.2.2 Results

Acetylcholinesterase activity was detected in both the rat brain cerebral cortex and pineal glands confirming the sensitivity of the assay method. The results are shown in **Table 7.1**.

TABLE 7.1 Acetylcholinesterase activity measured in the rat brain and pineal gland.

	Acetylcholinesterase activity
Brain	0,978 ± 0,416 µmol min^{-1}/mg protein
Pineal Gland	0,079 ± 0,050 µmol min^{-1}/gland

7.2.3 Discussion

The results show that AChE activity is detectable in both the rat brain and pineal glands. This supports the findings of Luo *et al.*, (1990). These authors carried out ultrastructural localization studies in the pineal glands of guinea pigs and located AChE-positive pinealocytes near blood vessels which were located and distributed in small groups. The method by Ellman *et al.*, (1961) was thus sensitive enough to detect acetylcholinesterase activity in the pineal glands of rats.

7.3 Experiment: TO DETERMINE THE EFFECT OF RESERPINE ON THE AChE DIURNAL RHYTHM IN RAT PINEAL GLANDS

7.3.1 Introduction

The purpose of this experiment was to determine whether the acetylcholinesterase enzyme activity changes over a 24 hour period and to investigate the effect of reserpine on enzyme activity.

7.3.2 Materials and Methods

7.3.2.1 Materials

Reserpine was purchased from the Sigma Chemical Co., USA. Sources of other materials are described in [**7.2.1.1**].

7.3.2.2 Animals

Twenty adult male Wistar rats (200-250g.b.wt) were used for this study. They were housed as described in [**2.3.1.2**]. Ten animals were injected with reserpine ($5mg.kg^{-1}$) one day prior to being killed. The remaining ten animals received saline at the same time and served as the controls. For the day time study five reserpine-treated and five saline-treated rats were killed by neck fracture at between 10h30 and 11h00 and the brain and pineals were rapidly removed. For the night time study, five reserpine-treated and five saline-treated animals were killed at 01h00 by neck fracture and pineals were rapidly removed [**2.3.1.2**]. The procedure for the acetylcholinesterase assay was carried out in accordance with the method described by Ellman *et al.*, (1961) [**7.2.1.4**]. Significance was evaluated using Student's t-test.

7.3.3 Results

As shown in **Table 7.2** acetylcholinesterase was detected in the rat pineal glands. There was a diurnal variation in acetylcholinesterase activity with lower activity at night and higher activity during the day. Reserpine treatment did not alter this diurnal rhythm, nor did it change the activity of the enzyme significantly.

TABLE 7.2 Acetylcholinesterase activity (μmol.min^{-1}/gland) during the day and night and the effect of reserpine.

Treatment	Day	Night
Controls	0,079 ± 0,05 *	0,035 ± 0,02
Reserpine	0,081 ± 0,02 *	0,059 ± 0,02

** As compared with night-time, p <*0.05 (Student's t-test)*

Acetylcholinesterase activity was measured in the brain cerebral cortex and no diurnal variation was detected in the activity of this enzyme in brain tissue.

7.3.4 Discussion

Although a cholinergic innervation of the pineal gland has not been clearly demonstrated, a number of studies have demonstrated the presence of cholinergic markers such as ChAT (Rodriquez De Lores Arnaiz and Pellegrino De Iraldi, 1972), muscarinic receptors (Taylor *et al.*, 1980) and AChE (Moller and Korf, 1983; Romijn, 1973) in the pineal. Moller and Korf (1983) were the first to demonstrate pineal nerve terminals containing vesicles which stained for acetylcholinesterase. Although the presence of the enzyme in the pineal does not necessarily imply that the pineal gland has a cholinergic innervation, it does suggest that acetylcholine plays a role in pineal function.

The results of this study show that using the method described by Ellman *et al.*, (1961) it was possible to detect AChE activity in the rat pineal gland. In addition, the activity follows a diurnal

rhythm, with higher activity during the day and lower activity at night. The possibility of this being under the control of the sympathetic nervous system was refuted by using catecholamine depletor, reserpine. Reserpine treatment did not alter the diurnal rhythm in the activity of the enzyme, neither did it change the activity significantly. These results support the view that disruption of the sympathetic innervation of the pineal gland does not interfere with cholinergic components in the pineal gland (Romijn, 1973; Taylor, 1980). Basinka *et al.*, (1973) have established that functional muscarinic receptors associated with acetylcholine-induced increases in the turnover of phosphatidylinositol occur in the pineal gland. Whether acetylcholinesterase is present in the pineal to regulate the level of acetylcholine interacting with the muscarinic receptors in the gland remains to be established.

CHAPTER 8

CHOLINE ACETYLTRANSFERASE STUDIES

8.1 Introduction

The enzyme acetyl-CoA : choline O-acetyltransferase (ChAT) catalyses the synthesis of the neurotransmitter acetylcholine from acetyl CoA and choline. According to its important role in the function of the nervous system, considerable effort has been invested to develop sensitive, quick and accurate procedures for assaying this enzyme.

Studies with this enzyme choline acetyltransferase were limited by the availability of the bioassays described by Chang and Gaddum (1933) and the much less sensitive method of Hestrin (1949). Shuberth (1963, 1966) developed a radiochemical assay for the enzyme, but the method required an acetyl CoA generating system since it did not distinguish acetyl CoA from acetylcholine. Selective precipitation methods were developed by McCaman and Hunt (1965) and Alpert *et al.*, (1966) in which ACh is precipitated as the reineckate, and by Fonnum (1966) in which tetraphenylborate salt is formed. These precipitation methods are specific and their sensitivity is limited only by the available specific activity of the substrate, but the tedium of washing and centrifuging the precipitated product remains. A gas chromatographic method by Stavinoha and Ryan (1965) is cumbersome for use in enzyme purification studies.

Schrier and Shuster (1967) developed an assay method for choline acetyltransferase which is rapid and precise taking advantage of the fact that the enzyme transfers an acetyl moiety from an anionic substrate acetyl CoA, to a cationic substrate choline, resulting in a cationic product acetylcholine. The two classes of ions can be separated by use of an anion exchange resin. This method was found however, unable to separate acetylcholine from acetylcarnitine and produces an artificially high value for the activity of choline acetyltransferase (White and Wu, 1973; Hamprecht and Amano, 1974).

Fonnum (1969) developed a radiochemical assay for choline acetyltransferase based on reproducible, rapid and specific procedures for isolating acetylcholine from the incubation mixture. This was achieved by isolating labelled ACh by liquid cation exchange using sodium tetraphenylboron (Kalignost) in ethyl butyl ketone. Labelled ACh can be determined by liquid scintillation counting at high efficiency in the biphasic aqueous : toluene scintillation solution mixture.

8.1.1 Theory of the Assay

The method is based on the observation that choline esters and sodium tetraphenylboron (Kalignost) form complexes that are insoluble in water but soluble in organic solvents such as nitrates, higher ketones and benzyl alcohol. The extraction procedure is an example of liquid cation exchange where tetraphenylboron is the cation exchange group. The proportion of choline esters extracted depends on the type and total amount of cation in the aqueous phase and the amount of sodium tetraphenylboron in the organic solvent. The proportion of choline esters extracted is independent of the choline ester concentration, the pH between 3 and 8, and the relative volumes of the two phases. The affinity of sodium tetraphenylboron for choline esters increases with an increase in the size of the acyl group. The choline esters extracted can be released into an aqueous solution by treatment with strong acid, silver salts and anion exchange resins. This procedure by Fonnum extracts acetylcholine selectively (Rossier, 1977).

8.2 ChAT ASSAY TECHNIQUE

8.2.1 Materials and Methods

8.2.1.1 Chemicals, Drugs and Reagents

Tritium-labelled acetyl coenzyme A (specific activity 3,9Ci/mmol) was obtained from Amersham Laboratories, England. Acetyl coenzyme A (acetyl CoA), physostigmine (eserine) and sodium

tetraphenylboron (Kalignost) were purchased from Sigma Chemical Co., USA. Acetonitrile was obtained from Saarchem Pty Ltd (South Africa) and butyronitrile from Merck (Germany). All other chemicals were of the purest analytical grade available.

8.2.1.2 Animals

Adult male Wistar rats (200-250g) were used. The animals were maintained under normal laboratory conditions as detailed in [**2.3.1.2**]. The rats were sacrificed between 10h30 and 11h30 maintaining the uniformity with previous studies.

8.2.1.3 Preparation of Tissue Extraction

Rat pineal glands were dissected from freshly obtained brains by the procedure described in [**2.3.1.2**]. The tissues were homogenized with a glass homogenizer in 5-10 volumes of ice-cold sodium phosphate buffer (50mM, pH7.4) containing 1mM EDTA (50µl/pineal). The homogenates were then centrifuged at 900 x g for 10 minutes at 4°C in a refridgerated centrifuge. To obtain full enzymatic activity 1% (v/v) Triton X-100 was added to each supernatant (5µl) (Fonnum, 1966). Solutions were kept on ice for 15 minutes with occasional shaking. The control blank groups consisted of heat inactivated enzymes.

8.2.1.4 The Choline Acetyltransferase Assay

The activity of choline acetyltransferase was assayed by a technique which measures the transfer of [^{3}H] acetyl moiety from [^{3}H] acetyl CoA to choline to yield [^{3}H] acetylcholine (Fonnum 1969, 1975). The resulting [^{3}H] acetylcholine was then isolated from aqueous solution by applying liquid cation exchange extraction procedure and by using Kalignost as an organic solvent.

The incubation mixtures contained (final concentrations) 10mM choline chloride, 300 mM sodium chloride, 10mM EDTA, 50mM sodium phosphate buffer (pH7.4), 0.1mM eserine, [^{3}H] acetyl CoA

and 0.5mM unlabelled acetyl CoA to give a final concentration of 0.5mM. Aliquots of 10µl incubation mixtures were transferred to test tubes which already contained 10µl of the enzyme solution. In all studies protein concentrations ranging between 2.5-3.0mg/ml were used. Reaction mixtures were incubated at 37°C for 15 minutes and terminated by adding sequentially and immediately 2.5ml of a 10mM sodium phosphate buffer (pH7.4) and 1ml butyronitrile containing 15mg Kalignost. After shaking (vortexing) for 10 seconds and centrifuging the reaction mixtures, aliquots of 0.5ml of organic layers were transferred to scintillation vials containing 3ml of toluene fluor and 1ml acetonitrile. The radioactivity was then counted by liquid scintillation spectrometry at 61,12% efficiency. The activity of choline acetyltransferase was expressed as nmol [^{3}H] acetylcholine formed /mg protein/hour.

8.2.1.5 Kinetic Studies

In order to carry out kinetic studies, the final concentrations of choline were varied from 20, 10, 5, 2.5, 1.25, to 1.0mM while keeping the concentration of acetyl CoA at a constant level of 0.5mM. Kinetic studies were also carrried out on melatonin stimulated brain tissue and the results are summarised in **Figure 8.1**.

The present recovery of the newly synthesized [^{3}H] acetylcholine using the Kalignost butyronitrile extraction procedure was 98% as judged by assessing the radioactivity of known quantities of [^{3}H] acetylcholine determined directly and following extraction procedures.

8.2.1.6 Protein Determination

The protein concentration of solutions were determined according to a technique developed by Lowry *et al.*, (1951) using bovine serum albumin as a standard. A typical protein calibration curve in these experiments as well as the scheme for the determination of protein by the method of Lowry *et al.*, (1951) may be found in [**3.2.1.4**].

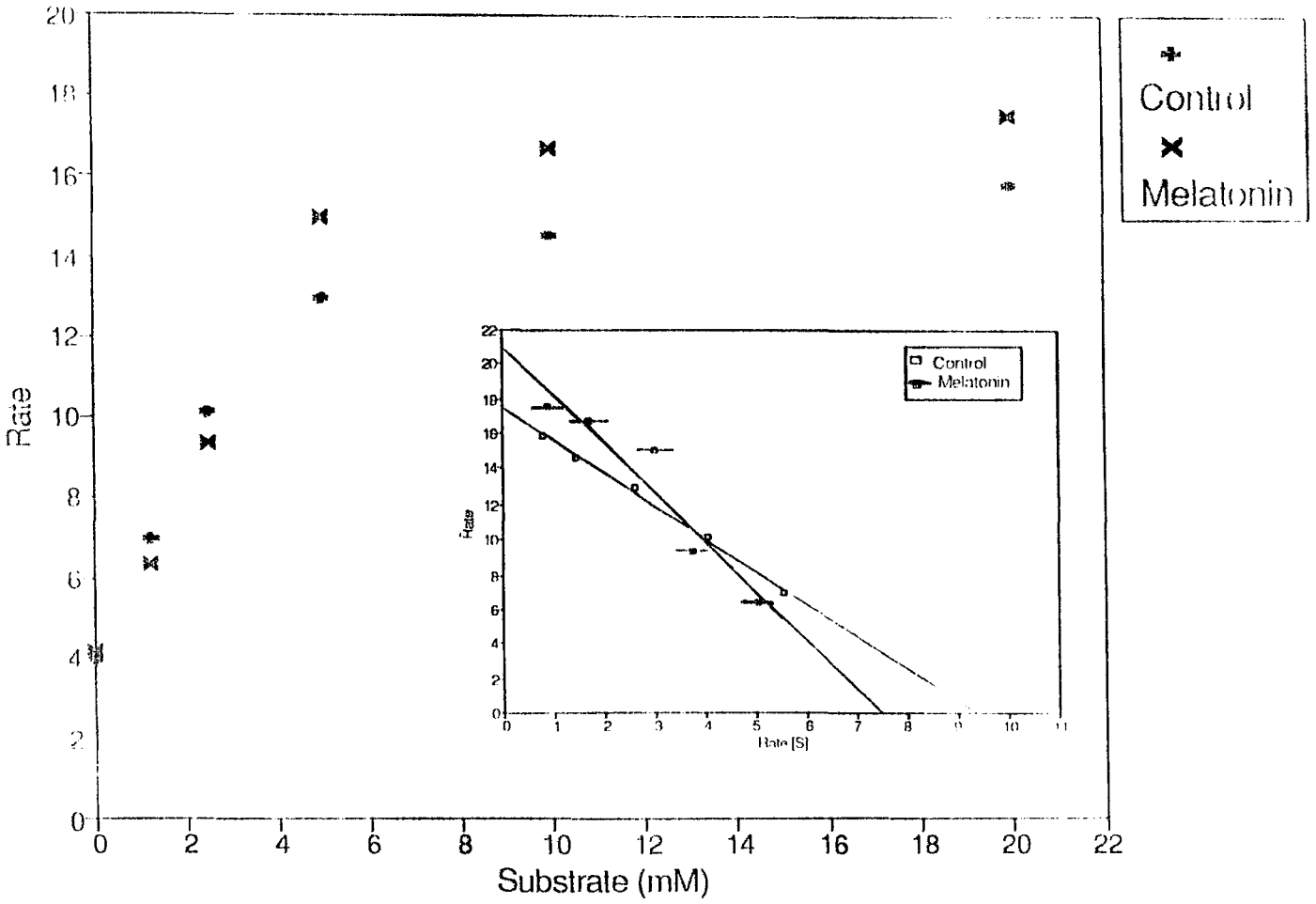


Figure 8.1 Eadie Hofstee plot of Choline acetyl transferase of the rat brain in the presence or absence of melatonin. These studies were carried out by varying the concentration of choline chloride from 20,10,5, 2.5 and 1.25 mM and by using fixed concentrations of acetyl CoA at 0.5mM. The velocity (rate) was expressed as nmol acetylcholine synthesised/mg protein/hour according to the procedure described in **[8.2.1.4]**. Each value represents the mean calculated from four experiments.

8.2.2 Results

The ChAT assay by Fonnum (1976) was sensitive enough to detect ChAT enzyme activity in the rat brain cerebral cortex. The kinetic studies revealed K_m value of 312.5 ± 44.05 µM and a Vmax value of 0.209 ± 0.014 nmol/mg protein/hour for melatonin treated brains and for the control, K_m was 196 ± 23.91 µM and Vmax was 0.172 ± 0.0034 nmol/mg protein/hour.

8.2.3 Discussion

Cholinergic neurons are distributed widely in the autonomic nervous system. For example, the preganglionic neurons of both sympathetic and parasympathetic nervous systems are cholinergic. Furthermore, the postganglionic parasympathetic neurons are mainly cholinergic. In addition, while the majority of postganglionic sympathetic fibers are adrenergic, the fibers supplying the sweat glands, vessels of the skeletal muscles in some species, and vessels of tongue muscles are all cholinergic in nature (Dale, 1937; Gabella, 1976). The development and use of immunohistochemical methods for measuring both cholinesterase and choline acetyltransferase have provided excellent tools for the localization of cholinergic neurons in various areas of CNS (Butcher and Woolf, 1984; Nitecka and Frotscher, 1989).

The ChAT assay by Fonnum (1975) is a rapid, specific and sensitive procedure based on the direct extraction of synthesized ACh into the scintillation mixture. There is no interference by AChE resistant acetylation products formed in liver and heart tissues, such as acetylcarnitine. This is apparently a serious problem for several other published ChAT assay methods.

8.3 Experiment: TO DETERMINE THE EFFECT OF PHYSOSTIGMINE AND MELATONIN ON ChAT ACTIVITY IN THE RAT BRAIN CEREBRAL CORTEX

8.3.1 Introduction

In recent studies Phansuwan-Pujito *et al.*, (1989) and Govitrapong *et al.*, (1989b) have identified muscarinic cholinergic receptors in the bovine pineal gland. They proceeded to measure the activity of choline acetyltransferase, determining its kinetic properties and thus substantiating the existence of cholinergic fibers in the bovine pineal gland.

In the present study investigations were carried out into whether it was possible to detect choline acetyltransferase activity in the rat brain cerebral cortex and pineal gland as well as determining whether melatonin influences the production of acetylcholine in a significant manner, in these organs.

8.3.2 Materials and Methods

8.3.2.1 Materials

Melatonin was obtained from Sigma Chemical Co., USA. All other chemicals and reagents used are as described in [**8.2.1.1**].

8.3.2.2 Methods

In order to determine the effect of melatonin on acetylcholine transferase enzyme activity a 10^{-5}M solution of melatonin was prepared and added to the incubation mixture. The procedure for the choline acetyltransferase assay was as described in [**8.2.1.4**]. The significance was evaluated using Student's t-test.

8.3.3 Results

ChAT activity in the rat brain cerebral cortex was found to increase significantly in the presence of melatonin (**Table 8.1**). ChAT activity was unable to be detected in the pineal gland.

TABLE 8.1 ***The effect of melatonin on ChAT activity in the rat brain cerebral cortex.***

Treatment	ChAT activity
Control	4.202 ± 0.664
Melatonin	9.516 ± 1.817 *

*In comparison with respect to vehicle control p<*0,0001 (Student's t-test). ChAT activity was expressed as nmol [^{3}H] acetylcholine formed/mg protein/hour.*

8.3.4 Discussion

Acetylcholine is generally accepted as an important neurotransmitter in the peripheral and central cholinergic synapses (Tucek, 1979). It is synthesized by the reversible transfer of an acetyl group from acetyl CoA to choline, catalyzed by the enzyme choline acetyltransferase which was discovered initially in 1943 by Nachmansohn and Machado.

Studies by Tucek (1985) have verified that there exists a direct relationship between the relative specific activity of the enzyme and the density of cholinergic fibers in different regions of the CNS. Studies by Govitrapong *et al.*, (1989a) have identified in the bovine pineal gland, muscarinic cholinergic receptors with Bmax values of 69.75 ± 20.91 fmol/mg protein. The low density of cholinergic fibers in the bovine gland is indeed consistent with the low activity of choline acetyltransferase as being reported in this study of rat brain and pineal gland. Furthermore it is also in keeping with the low density of other characterized receptors for amino acids, biogenic amines and peptides in the pineal gland (Ebadi and Govitrapong, 1986 a and b; Ebadi *et al.*, 1989).

Melatonin was found to cause an increase in brain ChAT activity ($p<0.0001$) suggesting that it could in some way enhance cholinergic transmission in the brain. Pineal ChAT activity was unable to be detected suggesting that the enzyme was either not present or that its concentration was too low to be detected by the assay technique used.

CHAPTER 9

MUSCARINIC RECEPTOR [^{3}H]QNB BINDING STUDIES

9.1 Introduction

Receptor binding studies have become commonplace among many researchers, particularly neuroscientists. Indeed its advent has led to a subsequent exponential growth in the volume of publications and our knowledge of neurotransmission and receptor interaction.

Identification of neurotransmitter receptor sites by chemical measurements of direct binding has been reported for nicotinic cholinergic receptors of invertebrate electric organs (Changeux *et al.*, 1970; Eldefrawi *et al.*, 1971; Klett *et al.*, 1973; Miledi *et al.*, 1971; O'Brien and Gilmour, 1969; Raftery *et al.*, 1971), mammalian neuromuscular junction (Berg *et al.*, 1972; Fambrough and Hartzell, 1972; Miledi and Potter, 1971) and central nervous system (Bosman, 1972; De Robertis, 1971; De Robertis *et al.*, 1969; Eldefrawi and O'Brien, 1970) and for the glycerine receptor in the mammalian central nervous system (Young and Snyder, 1973). Attempts to study the muscarinic cholinergic receptor biochemically have involved measuring the binding of atropine to the guinea pig intestine (Paton and Rang, 1965; Rang, 1967) and subcellular fractions of the rat brain (Farrow and O'Brien, 1973).

A number of studies have demonstrated the presence of high affinity binding sites for radiolabelled muscarinic antagonists in subcellular fractions from brain and smooth muscle and in intact tissues. A major part of such binding satisfies criteria which suggest that it occurs specifically to muscarinic receptors (Hulme *et al.*, 1978). The specific binding of [^{3}H] QNB to homogenates of rat brain has many characteristics which might be expected of interactions with muscarinic cholinergic receptors in the brain. Displacement of [^{3}H] QNB binding is greatest with muscarinic antagonists and the relative affinity of muscarinic cholinergic agonists tend to parallel their pharmacological potency (Yamamura and Snyder, 1972). By contrast nicotinic and non-cholinergic drugs have much less

affinity for these binding sites. The extremely high affinity in the nanomolar range of the muscarinic antagonists for QNB binding sites is quite similar to their molar affinity for muscarinic receptors as demonstrated in smooth muscle. Atropine can interact with the specific choline uptake system of cholinergic neurons in the brain but only at concentrations several orders of magnitude greater than its affinity for QNB binding sites (Yamamura and Snyder, 1972).

Muscarinic antagonists have negligible affinity for choline acetyltransferase, cholinesterase or acetylcholine storage sites. Thus the selectively high affinity of muscarinic anticholinergic drugs for muscarinic receptors and for QNB binding sites strongly favours the identity of the binding sites with muscarinic cholinergic receptors in the brain.

9.1.1 Methods for Receptor Binding

For many years neuroscientists from many disciplines have been investigating the properties of neurotransmitter receptors. In order to do so indirect methods such as observing electrical, behavioural and biochemical changes after systematic administration of drugs have been used.

This type of methodology, while substantiating the existence of receptors for drugs and endogenous compounds, cannot reliably reveal the direct interaction between the ligand in question and the receptor nor determine the absolute amount of receptors in a tissue.

The radioreceptor assay is a method that avoids the major problems associated with the indirect methods. With the use of a radioactive ligand one can monitor the direct interaction of this substance with a receptor. The subsequent biological events are not monitored and cannot interfere with the observed receptor-ligand interaction. Thus direct observations of the ligand-receptor interaction are possible. Because only the binding of the ligand to the receptor is being monitored, the stability of the receptor and the ligand is all that is essential for a successful assay. Intact cellular structure and composition are not necessary as long as the receptor retains its native ability to interact potently with drugs and neurotransmitters. Tissue preparations, such as homogenization

that destroy the integrity of complex biological tissues do not necessarily destroy the binding properties of receptors.

9.1.2 Theory of the Assay

The equilibrium between the drug and the receptor is based on the dissociation and association rates. Experimentally it is extremely difficult to measure association rates directly as high affinity drugs have very rapid rates of association. Dissociation rates can be measured by allowing the tritiated drug to equilibrate with the receptor and then instituting a procedure that prevents reassociation of the radioactive drug onto the receptor after it has dissociated. The easiest and most satisfactory method is the addition of a large excess of nonradioactive competing drug to the equilibrated tritiated drug-membrane solution. As radioactive drug dissociates from the receptor, nonradioactive drug occupies the receptor. The rate of reduction of specific binding over time after addition of nonradioactive drugs is directly related to the dissociation rate of the radioactive drug from the receptor.

9.2 MUSCARINIC RECEPTOR [^{3}H] QNB BINDING ASSAY

9.2.1 Materials and Methods

9.2.1.1 Chemicals, Drugs and Reagents

Tritium labelled L-quinuclidinyl [phenyl 4-^{3}H]-benzilate ([^{3}H] QNB)(specific activity 50 Ci/mmol) was purchased from Amersham Corporation, England. Atropine was obtained from Sigma Chemical Co. (St Louis, USA). All other drugs and chemicals used were of the purest grade available and all solutions were prepared fresh.

9.2.1.2 Animals

Male Wistar rats (220-250g.b.wt) were housed five per cage in a temperature controlled room under a fixed lighting cycle of LD12:12 (lights on 06h00) with food and water *ad libitum* [**2.3.1.2**]. The rats were sacrificed between 10h30 and 11h30. This maintained uniformity with previous studies.

9.2.1.3 Preparation of Tissues

Brain cerebral cortex tissues were obtained fresh from the male Wistar rats. 1-2g of tissue were homogenized in 20 vol of ice-cold Tris Buffer, pH7.4. The homogenates were centrifuged at 20 000 rpm for 60 minutes at 4°C in a refridgerated centrifuge (HITACHI SCR 20 B). The supernatant was discarded and the pellets were resuspended in 20 volumes of ice-cold Tris buffer pH7.4 and then rehomogenized to get a smooth suspension. The crude pineal membrane preparations were stored at -20°C and used within one week.

9.2.1.4 Radiogland Binding Assay

A scheme for the muscarinic receptor [^{3}H]QNB binding assay is presented in **Table 9.1**. All binding assays were carried out using sufficient membrane preparations to provide a protein concentration of 1mg/ml. The protein concentration of solutions was determined according to the method of Lowry *et al.*, (1951) using bovine serum albumin as a standard. A typical protein standard curve as well as the scheme of the protein assay may be found in [**3.2.1.4**]. Radioligand receptor binding assays were carried out by the addition of 400µl aliquots of membrane suspension (1mg protein/ml) to the glass tubes containing 80µl Tris buffer or appropriate drug atropine (10^{-5}M) in buffer. The reactions were started by adding 20µl of the [^{3}H]QNB to give a final concentration of 5.0nM. Relevant dilutions were made to give eight concentrations of ^{3}H(-) QNB ranging from 0.25nM - 5.0nM. The assays were performed at 25°C for 100 minutes. The binding reactions were terminated by filtering the incubation media through Whatman GF-C filter paper under vacuum.

Table 9.1 Scheme for the [^{3}H]QNB binding assay (Yamamura and Snyder, 1972)

A. TOTAL BINDING

[^{3}H]QNB Concentration (nM)	0.25	0.5	1.0	1.5	2.0	3.0	4.0	5.0
Protein (µl)	400	400	400	400	400	400	400	400
Tris buffer (µl)	80	80	80	80	80	80	80	80
[^{3}H]QNB (µl)	1	2	4	6	8	12	16	20
Tris buffer (µl)	19	18	16	14	12	8	4	-
Total Vol (µl)	500	500	500	500	500	500	500	500

B. NON SPECIFIC BINDING

[^{3}H]QNB concentration (nM)	0.25	0.5	1.0	1.5	2.0	3.0	4.0	5.0
Protein (µl)	400	400	400	400	400	400	400	400
Tris buffer (µl)	60	60	60	60	60	60	60	60
[^{3}H]QNB (µl)	1	2	4	6	8	12	16	20
Tris buffer (µl)	19	18	16	14	12	8	4	-
Atropine (µl)	20	20	20	20	20	20	20	20
Total vol (µl)	500	500	500	500	500	500	500	500

The nonspecific ligand binding was assessed in the presence of the drug. Subsequently each filter was washed three times with 3ml ice-cold Tris buffer (pH7.4). Receptor-bound radioactivity which was trapped on the filters was counted by liquid scintillation spectrometry in 3ml scintillation cocktail Beckman Ready-Solv^{TM} MP multipurpose premixed liquid scintillation solution. The protein concentration of the rat brain cerebral cortex was determined according to a procedure developed by Lowry *et al.*, (1951) using bovine serum albumin as a standard [**3.2.1.4**].

9.2.1.5 Analysis of Data

Saturation curves were initially analysed by the method of Scatchard (1949). All experiments were replicated a minimum of four times in each individual condition (**Figure 9.1**). A time course profile was also carried out and the results are summarised in **Figure 9.2**. An example of a Scatchard analysis plot of [^{3}H]QNB binding to rat brain membranes may be found in **Figure 9.3** and from which Kd and Bmax values were determined.

9.2.2 Results

The kinetics of ^{3}H(-) QNB binding to rat brain membranes showed that [^{3}H]QNB associated with its binding sites slowly at 25°C reaching equilibrium by 60 minutes and remaining constant for more than one and a half hours (**Figure 9.1**). The time course profile of specific ^{3}H(-)QNB binding to rat brain membranes is shown in **Figure 9.2**. The membrane suspension containing 1mg protein/ml was incubated at 25°C in the presence or absence of 10μM atropine. The reactions were started by the addition of 0.85nM ^{3}H(-) QNB and incubated from 0 to 90 minutes at 25°C. The reactions were terminated by filtration as described in [**9.2.1.4**].

9.2.2.1 Ligand Saturation Studies

The binding of ^{3}H(-) QNB ranging from 0.25nM-5.0nM along with 10μM atropine to account for the nonspecific equilibrium dissociation constant (Kd) and the receptor density (Bmax) was

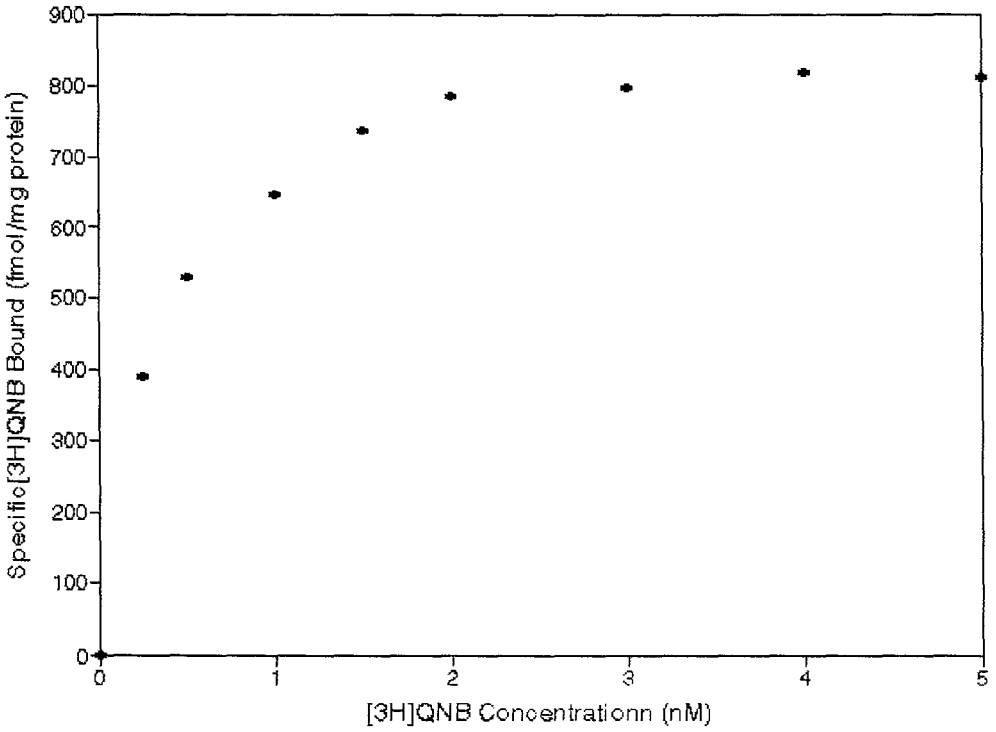


Figure 9.1 Saturation curve of [^{3}H]QNB binding to rat brain membranes, which was carried out using eight concentrations of [^{3}H]QNB, ranging from 0.25-5.0 nM. The plots that have been obtained from four determinations represent the specific binding of [^{3}H]QNB, ie the difference between total and non specific binding.

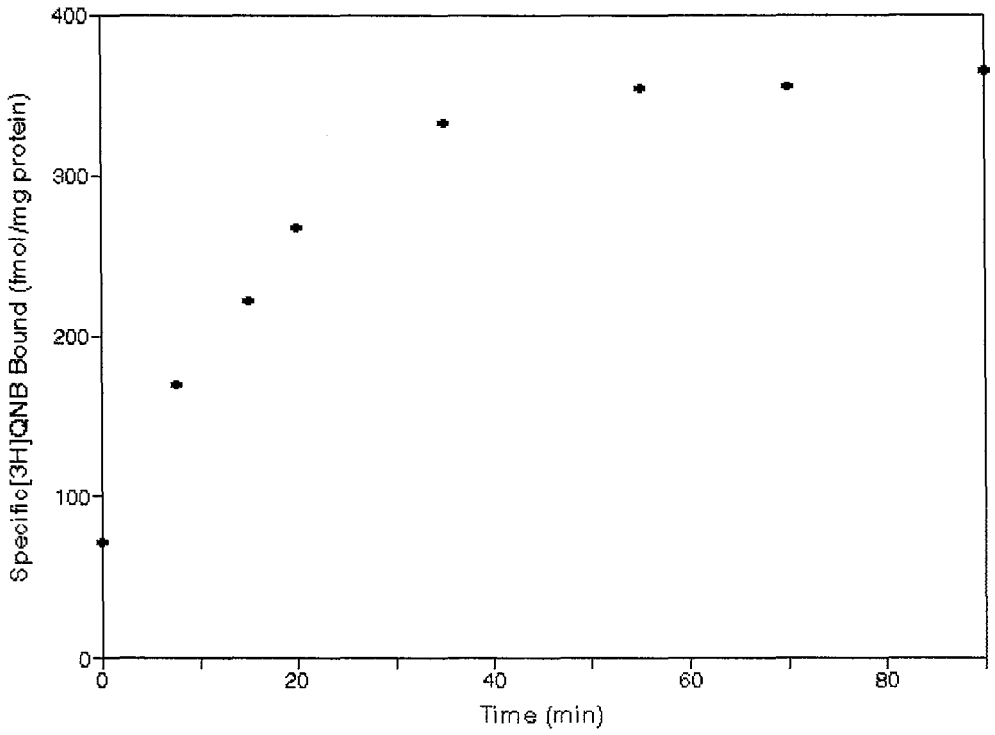


Figure 9.2 Time course of specific [^{3}H]QNB binding to the rat brain membranes. Membrane suspension containing 1mg protein/ml were incubated at 25°C in the presence or absence of 10^{-5}M atropine. The reactions were started by the addition of 0.85nM [^{3}H]QNB and incubated from 0 to 90 min at 25°C. The reactions were terminated by filtration as described in [9.2.1]. Each point represents the mean of duplicate determinations.

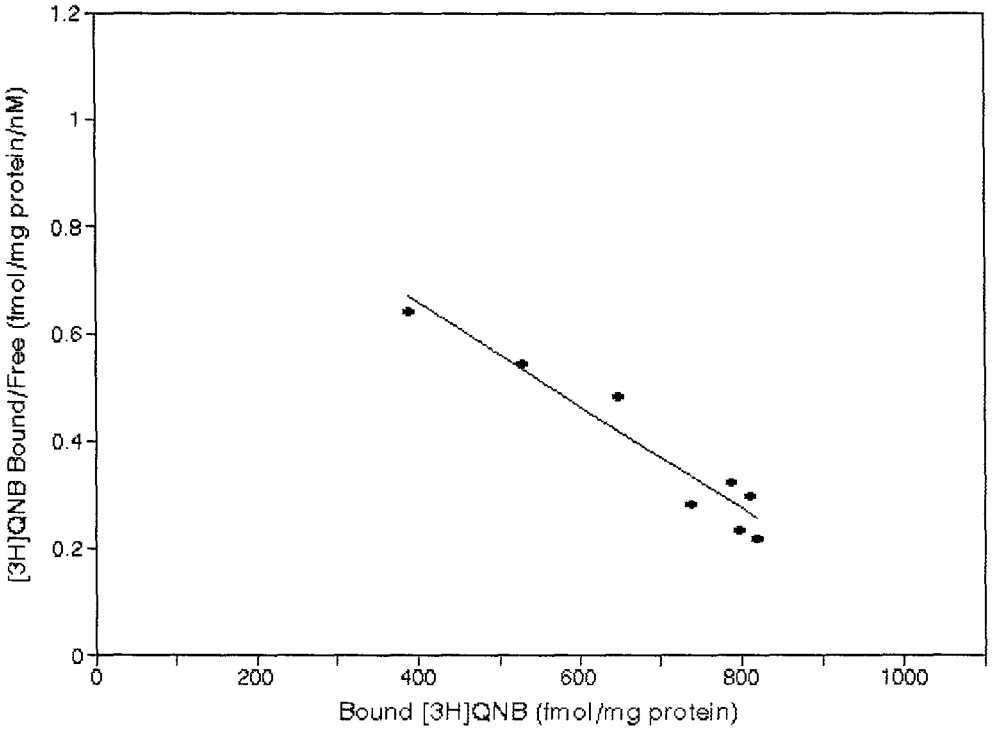


Figure 9.3 An example of a Scatchard analysis plot of [^{3}H]QNB binding to rat brain membranes, which was carried out using eight concentrations of [^{3}H]QNB, ranging from 0.25-5.0 nM. Each point represents the mean triplicate determinations.

determined. The Kd values for ^{3}H(-) QNB in the saturation studies from four individual experiments were 2.33 x 10^{-9}M whilst Bmax values were 1091.85 fmol/mg protein. The saturation curve **(Figure 9.1)** gives an indication of the concentration range we will be working in. The plateau indicates the saturation zone where receptor sites are being saturated.

9.2.3 Discussion

The [^{3}H]-3-quinuclidinyl benzilate ([^{3}H] QNB) has been used successfully to isolate muscarinic receptor sites in the brain since its initial introduction by Yamamura and Snyder (1974b) as well as in the heart (Fields *et al.*, 1978; Ehlert *et al.*, 1983; Watson *et al.*, 1986). Pirenzepine ([^{3}H] PZ) was also used as a selective muscarinic antagonist and it was with this ligand that the concept of muscarinic receptor subtypes was first postulated (Hammer *et al.*, 1980; Luthin and Wolfe, 1984). These and other studies (Giraldo *et al.*, 1988) revealed the presence of three distinct receptor subtypes in human tissues designated M1, M2 and M3 which have been found in neurons, heart and glands respectively. [^{3}H] QNB was employed as a simple, sensitive and specific label to quantify specific muscarinic cholinergic receptor binding in the rat brain (Yamamura and Snyder, 1972).

9.3 Experiment: TO DETERMINE THE EFFECT OF MELATONIN ON RAT BRAIN CEREBRAL CORTEX CHOLINERGIC RECEPTOR NUMBER AND AFFINITY.

9.3.1 Introduction

Quinuclidinyl benzilate is a potent muscarinic antagonist in the central nervous system (Albanus, 1970; Meyerhoffer, 1972) and peripheral nervous system (Becker, 1964). Govitrapong *et al.*, (1989b) used quinuclidinyl benzilate as a ligand in binding studies when identifying and characterizing the muscarinic cholinergic receptors in bovine pineal glands. The purpose of this experiment was to investigate the effects of melatonin on rat brain cerebral cortex cholinergic receptor number and affinity using [^{3}H] QNB as a ligand in receptor binding studies.

9.3.2 Materials and Methods

9.3.2.1 Materials

Acetylcholine chloride was purchased from Sigma Chemical Co., USA. All other drugs and reagents used were as described in [**9.2.1.1**] and all solutions were prepared fresh prior to use.

9.3.2.2 Animals

Male Wistar rats weighing between 200-250g were treated with melatonin by injecting them subcutaneously with melatonin (5mg/kg) daily at 11h00 for 2 weeks. Rats were then sacrificed 14 days after first treating them and the brains were removed as described in [**9.2.1.2**].

9.3.2.3 [^{3}H] QNB Radioligand Binding Assay

All binding assays were carried out using sufficient brain cerebral cortex membrane preparations to provide a protein concentration of 1mg/ml. The assay procedure was carried out as in [**9.2.1.3**]. Significance was evaluated using Student's t-test.

9.3.3 Results

The results of the investigation were obtained from Scatchard plots. The Kd values for [^{3}H] QNB obtained from four individual experiments were 2.057 ± 0.076nM for the melatonin-treated brains and 1.10 ± 0.120nM for the controls. The Bmax values for the [^{3}H] QNB were 2614 ± 145.60nM for the melatonin-treated brains and 1720 ± 91.65nM for the control as shown in **Table 9.2**.

TABLE 9.2 The effect of melatonin on [^{3}H]QNB muscarinic cholinergic receptor site affinity as well as receptor site availability for binding (mean ± SEM; Kd and Bmax values obtained from Scatchard plots of four individual experiments)

Treatment	Mean Kd (nM)	Mean Bmax (fmol/mg protein)
Control	1,10 ± 0,120	1720 ± 91,65
Melatonin	2,06 ± 0,076 *	2614 ± 145,60 **

*In comparison with respect to vehicle control p<*0,0005; ** 0,005 (Student's t-test)*

9.3.4 Discussion

In this study Scatchard plots indicate a higher mean Kd value for the brain cerebral cortex from melatonin-treated rats in comparison to the controls ($p<0.0005$) suggesting that melatonin increases the affinity of acetylcholine for its receptors. The Bmax values for the melatonin-treated brains also rose significantly in comparison to control brains ($p<0.005$) indicating an increase in receptor availability. Hence it may be suggested that melatonin increases acetylcholine receptor availability. These results show that melatonin may enhance cholinergic transmission in the brain cerebral cortex. There is a possibility that melatonin increases Bmax of available receptors by increasing gene expression for receptor production. Bonner *et al.*, (1987) discovered complementary DNA for three different muscarinic cholinergic receptors isolated from a rat cerebral cortex library. Analysis of human and rat genomic clones indicate that there are at least four functional muscarinic receptor genes and this gene family provides a new basis for evaluating the diversity of muscarinic mechanisms in the nervous system (Bonner, 1987).

CHAPTER 10

SUMMARY OF RESULTS

CHAPTER 1

Armstrong *et al.*, (1982) suggests that the functioning of the pineal gland must be at a higher level of integration since "considering the multitude of biochemical, physiological, endocrinological and behaviourial changes attributable to the pineal, it is unlikely to have so many specific functions". This suggestion coupled with others, such as the proposals that the pineal gland acts as a 'regulator of regulators' (Reiter and Hester, 1966) and that melatonin acts as a 'central inhibitory modulator', 'neuro-endocrine transducer' or 'biological clock' (Datta and King, 1980) supports this hypothesis proposed by Armstrong *et al.*, (1982), that the pineal is a 'synchronizer of regulators' with environmental lighting acting as the main zeitgeber (entraining agent or synchronizer).

The pineal is ideally placed anatomically to collect, integrate and compare information from both extracranial sources and intracranial sites. The regulatory function of the pineal gland on the body as a whole (including the brain) appears to be by melatonin release (although other pineal secretions and peptidase may also be involved). Studies which have examined the pineal indole metabolism on adrenergic regulation of melatonin production, N-acetyl transferase activity and cAMP showed that noradrenaline affected this biosynthetic pathway and is mainly mediated by ß-adrenergic receptors. Govitrapong and coworkers (1989b) have recently determined the presence of cholinergic receptor sites in the pineal gland although the physiological role of these receptors are not well characterized. Literature published on the pineal gland in recent years, as well as a background survey of the autonomic nervous system and the role and functioning of the cholinergic system in the pineal gland has been reviewed in this chapter. This has been done in order to put into perspective the full implications of this investigation into cholinergic interactions as well as in determining the role of cholinergic muscarinic receptors in the pineal gland.

CHAPTER 2

The organ culture studies show that carbachol induces a rise in the indole, N-acetylserotonin (aHT) without altering melatonin (aMT) levels. Carbachol did not affect the adrenergic system with reference to aMT. Acetylcholine, a general agonist of both muscarinic and nicotinic receptors also induced a rise in aHT levels without altering aMT levels. The ganglion blocking agent hexamethonium did not alter the acetylcholine induced rise in aHT levels suggesting that the aHT increase is not induced via the nicotinic receptors. The rise in aHT and not aMT may be speculated to occur through the muscarinic receptors. This was further confirmed by using the anti muscarinic agent atropine in organ culture studies. Atropine blocked the aHT rise induced by acetylcholine implying that the cholinergic system acts on the pineal muscarinic receptor. The anticholinesterase drug neostigmine was found not to influence the pineal indoles in any significant manner although a related anticholinesterase agent physostigmine caused aHT levels to rise, again without altering aMT. Although both drugs are anticholinesterase agents neostigmine possesses a quaternary nitrogen which limits its passage into the CNS. Physostigmine on the other hand is lipid soluble, possessing a tertiary amine and is hence able to pass through the blood brain barrier and influence central cholinergic receptor sites. These results from organ culture studies further confirms the presence of muscarinic cholinergic receptors in the pineal gland, suggesting that cholinergic agents may play a role in regulating indoleamine synthesis in the pineal gland.

CHAPTER 3

Cyclic-AMP studies revealed that the presence of acetylcholine caused a significant increase in cAMP levels ($p<0.0001$) in the pineal gland and did not alter the isoproterenol-induced rise in cAMP levels in the gland ($p<0.0001$). Hence, the cholinomimetic agent acetylcholine influences the sympathetic pathway in a significant manner with regard to cAMP.

CHAPTER 4

The cAMP-phosphodiesterase studies showed that physostigmine, an anticholinesterase agent, causes a significant increase in cAMP-phosphodiesterase enzyme activity ($p<0.05$). The cAMP-phosphodiesterase activity increased in the presence of an anticholinesterase agent with a simultaneous rise in cAMP levels.

CHAPTER 5

The NAT enzyme assay studies showed that physostigmine does not alter basal NAT enzyme activity significantly suggesting that the cholinergic activated increase in aHT did not occur due to the stimulation of the enzyme NAT.

CHAPTER 6

The HIOMT enzyme assay studies revealed that physostigmine did not cause a significant change in basal HIOMT activity. Physostigmine thus does not inhibit the enzyme HIOMT to reduce the conversion of N-acetylserotonin to melatonin. The mechanism by which the cholinergic agonist acetylcholine and anticholinesterase agent physostigmine are able to increase aHT levels and not aMT levels still remains to be elucidated. The reasons for acetylcholine inducing a rise in pineal cAMP without inducing a rise in pineal NAT activity remains unclear but could however be related to a recent finding that raised intracellular calcium levels reduce or inhibit pineal NAT activity. Acetylcholine is known to trigger the inositol phosphate signalling pathway system in the pineal resulting in a elevation of diacylglycerol (DAG) as well as intracellular calcium.

CHAPTER 7

Acetylcholinesterase enzyme studies reveal that the enzyme acetylcholinesterase undergoes a diurnal rhythm with a higher activity during the day and lower at night. The possiblity of this rhythm being under the control of sympathetic nervous system was refuted using the catecholamine depleting agent reserpine. Reserpine did not alter the diurnal rhythm of the acetylcholinesterase enzyme activity. Whether the acetylcholinesterase enzyme is present in the pineal to regulate the levels of acetylcholine interacting with the muscarinic receptors in the gland also remains to be elucidated.

CHAPTER 8

Choline acetyltransferase enzyme activity was detected in rat brain tissue but not in the rat pineal gland. Melatonin was found to increase ChAT enzyme activity in the brain cerebral cortex suggesting that it somehow enhances cholinergic activity there. A series of kinetic studies were carried out in brain tissue using melatonin.

CHAPTER 9

The muscarinic binding studies using [^{3}H]QNB were carried out on rat brain cerebral cortex and a saturation curve as well as a time trial were carried out. Scatchard plots produced the required Kd and Bmax values. The Kd results indicated that melatonin causes an increase in affinity of acetylcholine for its receptors whilst Bmax results suggest melatonin causes an increase in cholinergic receptor number in the brain. Melatonin may thus be speculated to enhance cholinergic receptor affinity and receptor number in the brain cerebral cortex of rats.

CHAPTER 11

CONCLUSION

The existence of muscarinic cholinergic receptors sites in the pineal gland have been identified and characterized. Stimulation of these muscarinic cholinergic receptor sites, using a parasympathomimetic agent carbachol, caused a rise in aHT levels leaving aMT levels unaltered. Further, organ culture studies using cholinergic agonists, ganglion blocking drugs, anti-muscarinic and anticholinesterase agents confirmed the presence of a cholinergic innervation in the pineal. The rise in aHT induced by acetylcholine and physostigmine was investigated and the results from studies using these two cholinergic agents show that pineal cAMP as well as cAMP-PDE levels rose without a concommitant increase in NAT. The reason for this is unclear, but it could be related to intracellular calcium levels causing a reduction or inhibition of pineal NAT activity. The rise in aHT was found however, not to be due to a reduction in the conversion of aHT to aMT by inhibiting the enzyme HIOMT. The mechanism by which aHT increases and not aMT still remains to be elucidated. Possible avenues which need to be investigated in order to shed more light on this mechanism are:

(i) The intracellular secondary messenger diacylglycerol, which appears transiently and then disappears within seconds due to its conversion back to the inositol phospholipid and its degradation to arachidonic acid which in turn can generate other messengers.

(ii) the phosphoinositides which participate in the inositol lipid signalling pathway.

(iii) the secondary messenger cGMP which is regulated in a similar manner to cAMP.

(iv) the possibility of muscarinic receptor gene activation which could cause the appearance of the phosphoinositide signalling pathway and result in levels of aHT rising.

(v) the role of prostaglandins in the stimulation of NAT.

(vi) stress and the release of catecholamines from synaptic nerve endings and adrenal medulla which could influence the rise of NAT and cause aHT levels to rise.

(vii) the possibility of non cholinergic agents influencing NAT activity.

(viii) the role that intracellular calcium levels play in reducing or inhibiting NAT activity. Acetylcholine is known to trigger the inositol phosphate signalling pathway system in the pineal which results in a elevation of diacylglycerol as well as intracellular calcium and could play a role in causing a rise in aHT levels.

Acetylcholinesterase, the enzyme which inactivates the neurotransmitter acetylcholine by breaking it down to choline and acetate, undergoes a diurnal rhythm in the pineal gland. The activity of this enzyme is higher during the day and lower at night. This diurnal rhythm is not under the control of the sympathetic nervous system. Further work needs to be done in determining whether the acetylcholinesterase enzyme is present in the pineal to regulate levels of acetylcholine interacting with muscarinic receptors or not.

The enzyme choline acetyltransferase catalyses the synthesis of the neurotransmitter acetylcholine from acetyl CoA and choline. This enzyme was not measured in the pineal gland although activity was detected in brain cerebral cortex tissue. Melatonin was found to increase ChAT activity in brain cerebral cortex suggesting that it somehow enhances cholinergic activity in this tissue. Muscarinic receptor binding studies were carried out on brain cerebral cortex tissue of rats and the results show that melatonin enhances receptor affinity and numbers there.

Pineal research is a dynamic and exciting field of study in the neurosciences as new discoveries shed more and more light on this unique gland. In investigating the role of cholinergic muscarinic receptors as well as cholinergic interactions in the pineal gland more facts have been added to the pool of existing information on this gland. This is just the tip of the iceberg however, and more avenues of research need to be pursued in order to fully understand the full functioning and mechanism of the cholinergic system as well as the role that these muscarinic receptors play in the pineal gland.

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