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A STUDY OF POSSIBLE INTERACTIONS BETWEEN THE PINEAL GLAND AND THE OPIOIDERGIC SYSTEM

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ABSTRACT

Recent observations suggest a link between the pineal gland and the opioid system. Possible areas of interaction between the pineal gland and the opioidergic system in Wistar rats were investigated.

The effect of opioids on the pineal gland in organ culture was monitored. Neither morphine, methadone nor the opioid antagonist naloxone was found to affect [^{14}C]-serotonin metabolism by the pineal gland *in vitro*.

Both the pineal gland and the opioid system are influenced by exposure to stressful stimuli. Morphine and melatonin had protective effects on stress-induced gastric lesions. The ability of melatonin to inhibit lesion formation was found not to be exerted via an opioidergic mechanism. Evidence has been obtained for a possible modulation of the stress response by the pineal gland.

The opioid drugs are the most potent analgesic agents available. A possible interaction between the opioid system and the pineal gland in the modulation of the response to noxious stimuli was investigated. An intact pineal gland was found to be necessary for the manifestation of the nocturnally increased response of rats to noxious stimuli.

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotopic Hormone
APUD	Amine Precursor Uptake and Decarboxylate
bw	Body Weight
BZP	Benzodiazepine
cAMP	Cyclic-Adenosine Monophosphate
cGMP	Cyclic-Guanosine Monophosphate
Ci	Curie
CNS	Central Nervous System
CSF	Cerebrospinal fluid
c.p.m.	Counts per minute
DA	Dopamine
d.p.m.	Disintegrations per minute
DZP	Diazepam
ESCR	External Standards Channel Ratio
FSH	Follicle Stimulating Hormone
GABA	Gamma-amino Butyric acid
GH	Growth Hormone
HCL	Hydrochloric Acid
HIOMT	Hydroxyindole-O-methyltransferase
HIAA	Hydroxyindole Acetic Acid
hr	Hour
5-HT	Serotonin
HTOH	Hydroxytryptophol
icv	intracerebroventricular
ip	Intraperitoneal
iv	Intravenous
MEL	Melatonin
MIAA	Methoxyindole acetic acid
MOR	Morphine
MTOH	Methoxytryptophol
NA	Noradrenaline
NAS	N-Acetyl Serotonin
NAL	Naloxone
NAT	N-Acetyl Transferase
nmol	nanomole
Pinx	Pinealectomized

pmol	picomole
PNS	Parasympathetic Nervous System
RNA	Ribonucleic acid
SNAT	Serotonin-N-Acetyl Transferase
SNS	Sympathetic Nervous System
TLC	Thin Layer Chromatography
μg	microgram
μl	microlitre

CHAPTER ONE

LITERATURE REVIEW

1 The Pineal Gland

History

The human pineal gland was first discovered in 325-280 BC by a famous anatomist, Herophilos. He described it as being a "tap" regulating the stream of the "pneuma" from the third to the fourth ventricle (Ariens Kappers,1979). Galen (130 - 300 A.D) termed the pineal gland "soma konoeides" because of its pine cone shape in man.

Rene Descartes' (1596 - 1650) concept of the pineal gland being the "seat of the soul" came at a time when philosophers and scientists had a keen interest in attempting to localize the soul.

In the second half of the 19th century, interest in pineal anatomy, histology and embryology grew as a result of the availability of more refined staining and sectioning techniques.

Studnicka (1905) described many of the cell types of the pineal gland which today are known to be present in the pineal organ of lower vertebrates.

Heubner (1898) first described a boy suffering from precocious puberty showing a pinealoma - and suggestions were made that the pineal gland could act to normally inhibit premature development of the reproductive system in infancy by an action on the hypothalamus.

An important study undertaken by Borgman described the anatomy, histology and cytology of the pineal organ in various animal species (Ariens Kappers, 1981).

In 1954, Kitay and Altschule (1954) edited a book on pineal physiology and suggested future lines of research in this field.

Considerable advances in pineal research have been made since then, and interest in this field is still gaining impetus.

Anatomy

Although the pineal gland was discovered and described before most other endocrine glands in the body, its function has remained unknown until recently.

The pineal gland originates in the brain of the developing embryo, but it loses direct nerve connection with the brain soon after birth (Axelrod, 1974). It is found outside the blood brain barrier between the two cerebral hemispheres of the brain and weighs approximately 100 mg in man and 1mg in rats. Postnatally, the pineal gland of the rat enlarges rapidly. Volumetric data indicates that depending on the strain, the pineal gland continues to enlarge until the the rat is 8.5 to 12 weeks old (Reiter, 1981). The superior saggital sinus and the transverse sinus cover the pineal gland, which is superficially located just below the skull.

The pineal gland of mammals displays much variation in size, location, and form.

Blood supply

The major blood supply to the pineal gland is provided by branches of the posterior choroidal arteries (Gladstone and Wakely, 1940). The arteries branch into the capsule of the pineal gland before they penetrate the parenchyma. In most mammals, the pineal glands are abundantly supplied with capillaries.

Hodde (1979) found that the pineal gland receives 2 - 6 arterial branches from posterior cerebral arteries. The capillary network forms vessels, which enter the septae, or merely run through the pineal tissue to the capsule.

The pineal gland has 12 - 16 superficial collecting veins (Hodde, 1979) which drain via the distal end of the great cerebral vein, into the superior saggital sinus in rodents.

A profuse blood supply reaches the pineal gland at a rate of flow of 4 ml/ min/ g - surpassed only by the kidney (Reiter, 1981). This blood supply is greater at night than during the day (Quay, 1972; Rollag, 1978).

Innervation

The pineal gland is innervated mainly by noradrenaline (NA) containing postganglionic sympathetic neurons, the cell bodies of which are found in the superior cervical ganglia (SCG) (Kappers, 1981). These nerve fibres reach the pineal gland along the internal carotid plexus. On nearing the pineal gland, the nerve fibres eventually form one or two discrete nerves called the nervii conarii (Kappers, 1981). They penetrate the pineal capsule at the apex of the gland, and ramify among the pinealocytes, ending between the parenchymal cells, especially in the pericapillary spaces (Romijn, 1973; Matsushima and Reiter, 1977).

The bulk of the NA within the pineal gland is confined to the sympathetic nerve terminals (Matsushima *et al*, 1981). The neurotransmitter is primarily released during darkness resulting in a stimulation of indole production within the pineal gland (Morgan *et al*, 1976).

Reports have suggested that the pineal gland may also be innervated by fibres having their cell bodies in other areas of the CNS. Kappers (1960) showed that abberent commisural fibres of the habenular commisure often form hairpin loops in the pineal stalk or in the deep part of the pineal. Reiter (1982) has suggested that the axons of these cell bodies may pass into the vicinity of the habenular nucleus on their way up the pineal stalk.

Dafney (1980) has demonstrated short latency responses in the pineal body of the rat following amygdalic, ventromedial hypothalamic, olfactory, acoustic and photic stimulation - suggestive of neural connections between the pineal gland and neural structures in the brain of the rat. The transport of photic information via the habenular commisure to the pineal body is faster than that via the suprachiasmatic nucleus (SCN).

The pinealocyte

The pineal gland consists mainly of interstitial cells and pinealocytes which are the recepto-sensory components (Juillard and Collin, 1980). The biochemistry and function of the pinealocyte likens it to the APUD cell type series (Leong and Matthews, 1979) enabling the pineal to participate in the diffuse neuroendocrine system (Matthews and Leong, 1981). The pinealocyte has also been described as a paraneuron (Ueck and Wake, 1979).

Biochemistry

Klein and Moore (1979) described the neuronal pathway from the eye to the pineal gland. Photic stimuli sensed by the retina is sent as a neurally coded message by the optic nerve via the retino-hypothalamic tract to the SCN. From here, the information is sent via the spinal chord to the SCG, with the postsynaptic NA-ergic nerves synapsing in the pineal gland. The terminals of these fibres do not form true synapses with the pinealocytes, but end freely in the parenchyma.

The NA released from these fibres at night acts on highly specific receptors (Klein and Weller, 1970) of the β_1 subgroup (Backstrom, 1977).

The enzymatic capacity of pineal homogenates to convert adenosine triphospate (ATP) to cyclic AMP (cAMP) was an important finding for the understanding of pineal biochemistry. This reaction requires the enzyme adenylate cyclase. Unstimulated (basal) levels of the enzyme have been shown to undergo diurnal variations in the pineal gland (Weiss, 1971)

Stimulation of the β-receptor thus activates adenylate cyclase with a resultant increase in cAMP production (Klein *et al*, 1978).

The post-synaptic β-receptor is linked to adenyl cyclase - but it has been observed that not all of the effects of β-receptor stimulation are mediated exclusively by cAMP. Hyperpolarisation of the pinealocyte membrane due to the activation of the β-receptor changes the ion physiology in the pinealocyte, possibly independent

of cAMP (Parfitt *et al*, 1975). It has been suggested that both hyperpolarisation and increases in cAMP are necessary for the complete manifestation of the β-stimulatory effects in the pineal (Parfitt *et al*, 1975) - and Ca^{2+} ions have been shown to be essential for such activity (Zatz and Romero, 1978).

The amino acid taurine is found in high concentrations in the pineal gland when compared to other parts of the brain (Crabai *et al*, 1974) - and is located almost entirely in the pinealocyte. Stimulation of the β-receptor leads to a release of taurine - possibly via hyperpolarisation (Wheler and Klein, 1980) and cAMP (Wheler *et al*, 1979). Klein *et al* (1981) have suggested that taurine might act as an extracellular feedback messenger, modulating the amoumt of NA released in response to tonic neural stimulation.

The sensitivity of the β_1-adrenergic receptor in the pineal gland varies. Various manipulations including light exposure, treatment with reserpine, and superior cervical ganglionectomy have been reported to induce supersensitvity of the pineal β_1-adrenergic receptor (Reiter, 1981). Conversely, after 12 hrs of exposure to darkness (Romero *et al*, 1975) or either acute (Kebabian *et al*, 1975) or chronic (Moyer *et al*, 1980) exposure to β-adrenergic agonists reduces receptor sensitivity.

At the onset of the dark phase, these receptors exhibit supersensitivity, probably due to the decreased NA release during the light phase (Romero and Axelrod, 1974). Gonzales-Brito *et al* (1988) demonstrated an increase in β-receptor binding in the first half of the dark phase in hamsters, with peak binding around the mid-dark period and an abrupt decrease in binding one hour after lights on.

B-receptor stimulation causes a decrease in the number of receptors and in the concentration of hormone sensitive adenyl cyclase, suggesting that receptor sensitivity is dependent, at least to a certain extent, on the sensitivity of adenyl cyclase to stimulation (Kebabian *et al*, 1975)

Gonzales-Brito *et al* (1988) concluded that intrinsic mechanisms of up

and down regulation of pineal β-adrenergic receptors operate during a 24 hr LD cycle - resulting in a circadian variation in pinealocyte NA output (Browenstein and Axelrod, 1974).

SNAT

The adrenergic-cAMP mechanism described above regulates the activity of the pineal enzyme Serotonin-N-Acetyl-Transferase (SNAT) - the induction of which has been suggested to occur via two routes - transcription and enhancement of post-transcription events (translation).

Actinomycin D (inhibits transcription) and cycloheximide (inhibits translation) block the isoprenaline-stimulated or dark phase-induced increase in SNAT activity (Romero *et al*, 1975). A lag period prior to an increase in SNAT activity occurs after treatment with these drugs, possibly due to a lack of mRNA necessary for SNAT induction (Zatz *et al*, 1976). These findings thus suggest that both new RNA and protein are required for the synthesis of SNAT.

cAMP stimulates the synthesis of the specific new macromolecules - RNA and protein - required for the stimulation of SNAT and also stabilizes the enzyme and keeps it in an active state (Klein *et al*, 1970).

The activity of this enzyme undergoes large daily changes, with 30-100 fold increases having been noted in the dark phase (Axelrod, 1974). This enzyme is located mainly in the cytoplasm (Deguchi and Axelrod, 1972) and acts to transfer an acetyl group from the cofactor Acetyl Coenzyme A (CoA) to acceptor amines. It is responsible for the formation of N-Acetyl Serotonin (NAS) in the melatonin biosynthetic pathway.

The nocturnal induction of SNAT is dependent on increased pineal cAMP levels and the capacity of the intracellular mechanisms to respond to this increase (Romero and Axelrod, 1975).

Maintenance of the elevated nocturnal activity of SNAT requires the continuous activation of the β-adrenergic receptor by NA - this is suggested by the rapid (Half-time = 3 minutes) decrease in SNAT

activity on exposure to light or β-receptor blockade (Deguchi and Axelrod, 1972).

The activity of SNAT is also very sensitive to drug manipulations affecting β-receptor adenylate cyclase-coupled regulating mechanisms. Agents such as isoprenaline, pargyline (MAO inhibitor), cocaine (NA reuptake blocker) increase SNAT activity (Deguchi and Axelrod, 1972).

Pre-synaptic α-adrenergic receptors have been characterized in the pineal gland . *In vivo* and *in vitro* experiments have shown that stimulation of these receptors leads to an accumulation of cGMP (Vanacek *et al*, 1986), as well as a potentiation of the stimulating effects of isoprenaline on SNAT activity (Klein *et al*, 1983) and cAMP production (Vanacek *et al*, 1985). Administration of the α_1-adrenergic antagonist prazosin inhibits the nighttime increase in melatonin release (Sugden *et al*, 1985a).

Mobilization of Calcium (Ca^{2+}) via the stimulation of the inositol lipid signalling pathway is reported to be necessary for α-adrenergic regulation of pineal activity (Exton, 1985).

It was suggested that NA released from the pineal sympathetic nerve endings may act on both β- and α-adrenergic receptors, with the effect on α-adrenergic receptors leading to a decrease in β-adrenergic receptor sensitivity (Klein *et al*, 1981). Gonzales-Brito *et al*, (1988), however, reported that a homologous (agonist-specific) mechanism of β-adrenergic receptor desensitization operates within the pineal gland. Thus, the NA acting on post-synaptic β-adrenergic receptors potentiates β-receptor stimulation induced increases in SNAT activity without modifying the sensitivity of the β-adrenergic receptor to the neurotransmitter (NA).

Recent studies have suggested that prostaglandin (PG) stimulation of SNAT may represent yet another mechanism for regulating melatonin production by the pineal gland (Ritta *et al*, 1981). It has been observed that α-adrenergic receptor stimulation of rat pineal explants results in PGE_2 release (Cardinali and Vacas, 1987). PG synthesis inhibitors block the nocturnal rise in pineal SNAT, and PGE_2 (*in vitro*) increases pineal SNAT activity, cAMP levels and

melatonin release (Cardinali and Vacas, 1987).

SNAT activity is also sensitive to stressful stimuli (Vaughan *et al*, 1978), possibly brought about due to the release of catecholamines from sympathetic nerve endings, and the adrenal medulla.

HIOMT

Hydroxy-indole-O-methyl transferase (HIOMT) was initially thought to be unique to the pineal gland (Axelrod *et al*, 1965) - however, it has since been localized in other areas, including the retina, Harderian gland, intestine and also enterochromaffin cells (Reiter, 1981).

Axelrod and Weissbach (1961) first purified HIOMT from bovine pineal glands. This enzyme transfers methyl groups from the cofactor S-adenosyl methionine to acceptor molecules (5-hydroxyindoles) - Reiter, 1981. In the pineal gland, HIOMT catalyzes the conversion of NAS to melatonin. The activity of the enzyme is possibly regulated *in vivo* by two mechanisms: a) Light - modulates neural stimulation (Klein and Moore, 1979); and b) circulating steroids (Preslock, 1977).

Although early reports suggested daily changes in enzyme activity, these findings were not reproducible. However, during exposure to constant darkness, a gradual increase in HIOMT activity occurs, and conversely, exposure to constant light results in a gradual reduction of HIOMT activity (Wurtman *et al*, 1963). It is now conceded that if HIOMT activity does vary throughout the 24 hr period, the rhythm is of very low magnitude (Klein, 1981).

Gonadal steroids (e.g. oestradiol) have been reported to increase pineal HIOMT activity by 80 - 90% using nM concentrations in an organ culture system (Reiter, 1981). Oestradiol, testosterone and progesterone show a biphasic dose-dependent stimulation of HIOMT activity (Daya, 1982).

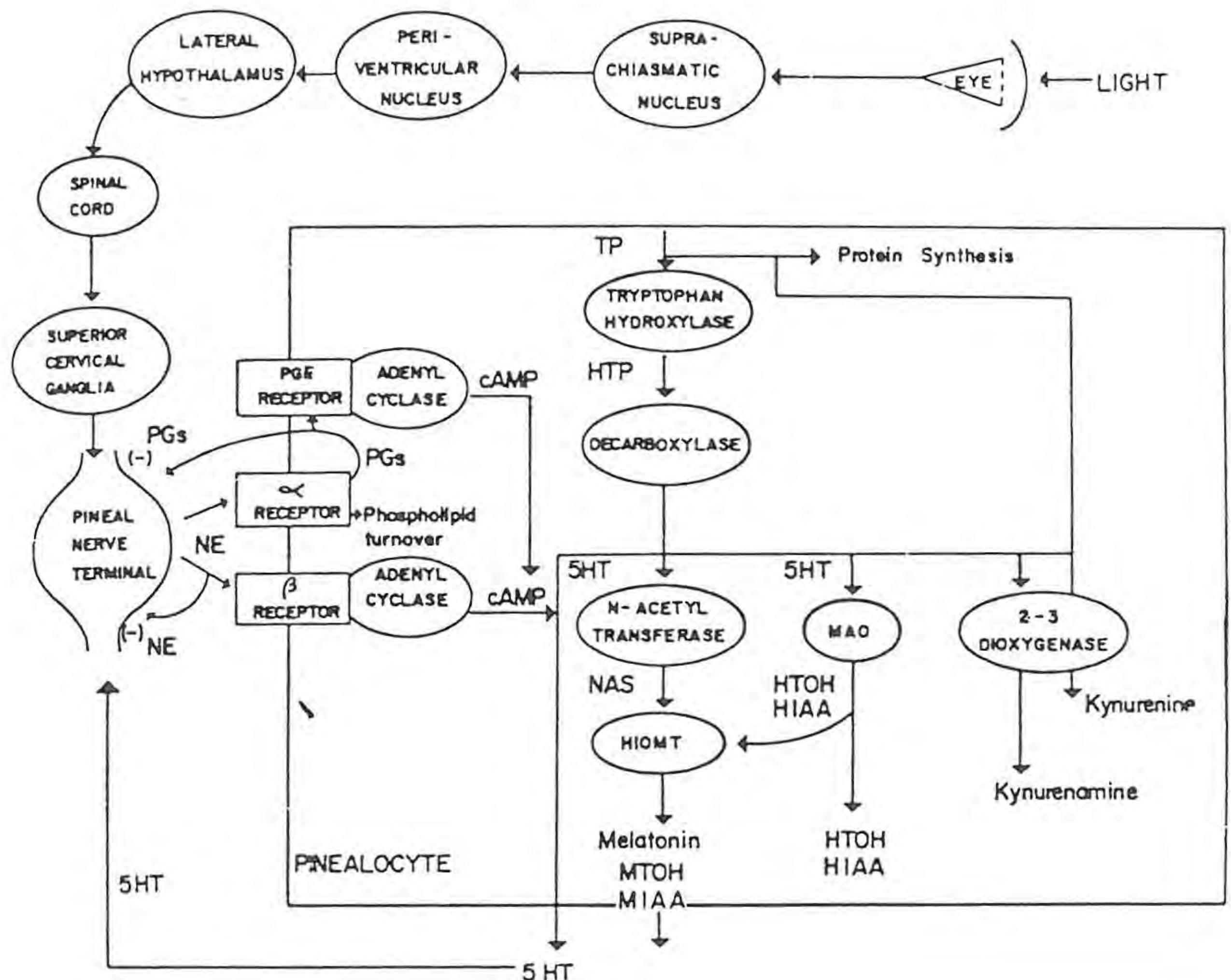

Fig 1.1: Diagram representing some of the subcellular mechanisms underlying neuroendocrine-transducing events in the mammalian pineal. TP: tryptophan; HTP: 5-hydroxytryptophan; 5HT: serotonin; NAS: N-acetylserotonin; HTOH: 5-hydroxytryptophol; HIAA: 5-hydroxyindoleacetic acid; MTOH: 5-methoxytryptophol; MIAA: 5-methoxyindloeacetic acid.

Adapted from Cardinali *et al*, 1982.

Melatonin

In 1917, McCord 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-acetyl tryptamine).

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 on studying their various physiological effects.

The generalized stimulation of the pineal gland at night results in a great increase in the concentration of melatonin within the gland. As far as can be determined, melatonin is not stored within the pineal gland in any appreciable quantity. Shortly after being synthesized, it is released into the circulation (Kennaway *et al*,1977; Rollag *et al*, 1977). In some cases, melatonin may even be released into the CSF (Reppert *et al*, 1978) - and CSF and blood levels of melatonin follow those of the pineal gland.

Wetterberg *et al* (1981) have observed that circulating levels of melatonin during the night are higher than those during the day in humans.

After being secreted into the blood, melatonin is loosely bound to plasma albumin, 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 (Reiter, 1981a). One of the metabolites in the urine - 6-hydroxymelatonin sulphate - is sometimes measured to monitor pineal melatonin production, and may serve to provide a simple, rapid and non-invasive technique of assessing melatonin levels in clinical situations (Aldhous and Arendt, 1988). This technique would not, however, be able to pick up rapid changes of short duration.

Many potential functions of the pineal gland and melatonin have been suggested. Possibly, the most extensively documented effects

of the pineal gland are those on the reproductive system. The first convincing data that the pineal gland was an organ of internal secretion came from the work of Reiter and colleagues (review, 1981a), who observed an involution of the gonads and accessory sex organs of both male and female hamsters on exposure to constant light. This effect was reversed by pinealectomy.

Other findings showed that melatonin is capable of suppressing reproduction (Cardinali *et al*, 1974) - and this effect requires an intact pineal gland (Reiter *et al*, 1976).

The growth of the ovarian interstitium is dependent on ample luteinizing hormone (LH) secretion from the anterior pituitary gland. LH and follicle stimulating hormone (FSH) exhibit a daily afternoon surge in light restricted female hamsters (Bridges and Goldman, 1975). It has been suggested that the pineal gland participates in linking this LH hormone surge to the photoperiod (Walker *et al*, 1982).

A melatonin free pineal extract was also observed to exert an anti-gonadotropic effect (Damian *et al*, 1981) which raises the possibility that factors other than the methoxyindoles e.g. pteridines and pterins might also exert this effect (Ebels, 1979). Other pineal factors, such as 5-methoxy tryptophol and 5-methoxytryptamine (McIsaac *et al*, 1964) also exert antigonadotropic effects, even in the absence of an intact pineal gland (Pevet *et al*, 1981).

The pineal gland has also been implicated as a target organ for androgens (Sem *et al*, 1981) suggesting a pineal gonadal feedback mechanism.

The anti-gonadal effects of melatonin have been well reported, and excellent reviews may be consulted for a more detailed perspective (Minneman and Wurtman, 1976; Reiter, 1981b).

Many other functions for melatonin and the pineal gland have been indicated in isolated reports. It has been suggested that melatonin affects melanin synthesis (Weatherhead and Logan, 1981), may serve a neuromodulatory role in the salivary glands (Bubenik, 1981), and may affect sperm motility in human semen (van Vuuren *et al*, 1988).

Abnormal rhythms of melatonin have been found in 47 disease states (Birau, 1981).

The CNS has also been implicated as a site of action of melatonin It has been suggested that melatonin assists the entry into sleep and perhaps maintains undisturbed sleep throughout the night (Armstrong *et al*, 1982).

Many reports suggest that the pineal gland may be involved in the modulation of epileptic seizures - with melatonin being the pineal anti-convulsant principle (Peterson *et al*, 1981), exerting its action possibly by reducing the ability of excited (epileptic) neurons to maintain activity, and/or via an indirect alteration of the EEG by an action on the hypothalamus (Pang *et al*, 1976).

Anton Tay *et al* (1971) noted an increase of REM sleep, and a slowing down of the EEG rhythm in patients treated with melatonin. An increase in the convulsive threshold was also noted in epileptic patients, and an improvement in the clinical picture of Parkinsonian patients was observed.

A feedback mechanism between the pineal gland and the hypothalamo-hypophyseal axis has been suggested (Karasek, 1979) with retinal melatonin also possibly affecting the hypothalamus (Grota *et al*, 1982). Evidence has been presented that melatonin may act on neurons in the preoptic and the anterior hypothalamic areas of the hypothalamus (Reiter, 1982a). Recently, English and Arendt (1988) characterized melatonin binding sites in the rat hypothalamus using [^{125}I]-Iodomelatonin. Melatonin may also influence GABA and 5HT concentrations of the hypothalamus and the midbrain (Reiter, 1982a).

Zisapel (1988) has observed specific melatonin binding sites in the rodent brain; with the presence of a diurnal variation in the hypothalamus, medulla, pons and the hippocampus. The ability to characterize melatonin binding sites in various brain areas will facilitate the understanding of the nature of the neuroendocrine effects of melatonin.

Vacas and Cardinali (1980) demonstrated specific, high affinity

binding of melatonin to crude membrane preparations from bovine pineal glands, suggesting that melatonin may have an action on the pineal gland.

It has been suggested that melatonin may exert some of its effects via a cAMP mediated mechanism (Brown and Niles, 1982) by indirectly influencing NA and DA sensitive adenylate cyclases in the brain, or by an action on its own receptor sites. Also, a direct action of melatonin at peripheral target organs has been proposed.

Convincing evidence for a role of the pineal gland in the control of thyroid function has been provided by Vriend and Reiter (1977). They have suggested that the pineal gland is capable of depressing both total plasma thyroxine concentrations as well as the free-active form of thyroxine. Pinealectomy was observed to increase Iodine accumulation in the thyroid gland (Csaba and Nagy, 1974).

Furthermore, the pineal gland has been suggested to influence the spread and growth of malignant tumours (Tapp, 1980). Patients with breast cancer have particularly large pineal glands (Tapp, 1980). In studies conducted on female rats, melatonin has emerged as a particularly important anti-neoplastic agent, especially with respect to the growth of breast cancer. Lissoni *et al* (1988) have suggested that an increase in plasma melatonin concentrations can be used as a predictor of the objective response of cancer patients to chemotherapy. These investigators have also suggested that the antineoplastic effect of cytotoxic drugs may require the participation of the pineal gland.

Melatonin has also been implicated in immune regulation. Interleukin-2 cells inhibit pineal melatonin production *in vitro* (Esposti *et al*, 1988a) and melatonin may stimulate Natural Killer cell activity in humans and enhance humoral and cellular immune responses in mice (Fraschini *et al*, 1988).

Extracts of the pineal gland are being used for the treatment of psychosis and schizophrenia (Young and Silman, 1982). Melatonin levels in the plasma correlate with mood - low in depression, high in mania, and scattered levels being observed in bipolar manic depression.

Fig 1.2: Illustration of Indole metabolism in the pineal gland. HIOMT: hydroxyindole-O-methyl transferase; SNAT: serotonin-N-acetyltransferase.

Adapted from Morton, (1982).

Although melatonin is the most investigated substance, 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.

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.

2 The Opioid System

Overview

The opium alkaloids are the structural prototypes of the modern potent analgesic agents. Opium is obtained from the milky exudate of the unripe seeds and capsule of the opium poppy - *Papaver somniferum* - now grown legally and illegally in many parts of the world.

Originally, opium was native to the Middle East and was used by the Assyrians to promote sleep. It was transported to the Far East (China) by the Arabs, and was used by the Chinese for dysentry. They later began to smoke the crude extract of the poppy. Chinese immigrants introduced opium to the West, and it was accepted by physicians as a useful remedy for pain. Addiction remained mild when smoking and eating opium became popular because the tarry extract decomposed thermally and was not well absorbed through the GIT. However, severe cases of addiction were noted, especially after the invention of the first hypodermic syringe (quill and bladder) enabling the intravenous (iv) administration of opium.

One of the opium alkaloids, morphine, was later extracted. It was observed not only to relieve pain, but also to produce a general feeling of well being, promote sleep and reduce anxiety (review, DeStevens, 1965). However, it had several disadvantages, producing respiratory depression, bradycardia, dependence, constipation and hypotension - and with repeated use, tolerance to certain of its

side effects.

The search then began for a potent analgesic agent without the side-effects of morphine - especially without the the ability to produce dependence. Extensive research introduced the phenylpiperidene and methadone congeners which were originally believed to be non-dependence producing drugs. These chemically synthesized drugs, however, were later found to have addictive potential.

The development of potent agonist-antagonist analgesics with low addiction potential resulted in part by accident and in part by design. In an attempt to develop a drug without the respiratory depressant actions of morphine, and possibly devoid of its addiction potential as well, Hart and McCawley (cited in Toro and Way, 1983) stumbled across a compound with antagonist properties to morphine. Replacing the piperidene-N-methyl group of the morphine compound with an allyl group gave rise to nalorphine. Lasagna and Beecher (1954) carried out a series of experiments to find a morphine-nalorphine combination that would maintain the analgesic properties of morphine and minimize its other side effects. A major breakthrough was made when, in the course of their work, they demonstrated that this opiate antagonist produces analgesic effects in man.

Although drug addiction studies confirmed that it would be unlikely to produce dependence in man (Wilker *et al*, 1953), nalorphine displayed psychotomimetic effects (including dysphoria) - (Isbell, 1956) that precluded its use as an analgesic.

This discovery, however, resulted in a search for other agonist antagonists that would cause pain relief without causing dysphoria. This led to the discovery of naloxone, a relatively pure antagonist compound, devoid of analgesic activity. Other agonist-antagonists having analgesic activity were later discovered. These include pentazocine, butorphanol, nalbuphine and buprenorphine (Toro and Way, 1983) all of which are now used clinically.

Beckett and Casy (1954) later proposed a model for an endogenous opioid receptor. The opioid receptor was later pharmacologically

(Kosterlitz and Waterfield, 1975) and chemically (Pert and Snyder, 1973) characterized using bioassays and ligand binding studies.

The characterization of the receptor led to the suggestion that an endogenous morphine-like substance should exist (Hughes, 1974) - and an exciting race to isolate such a ligand began.

Hughes(1975) and Terenius and Wahlstrom (1975) showed that a brain extract displayed opioid-like activity in bioassays using the mouse vas deferens. Met- and leu-enkephalin were purified (Hughes, 1975) later followed by the discovery of β-endorphin (Li and Chung, 1976).

These discoveries led to great advances being made in opioid pharmacology, some of which will be discussed below.

Characterization of the opioid receptor

The chemical characterization of the opioid receptor in 1973 led to a better understanding of the mechanisms of action of the opioid compounds (Snyder *et al*, 1973).

Morphine is well suited for use as a receptor probe because of its rigid structure and the fact that its structure must describe at least a part of the morphology of the opioid receptor (Thorpe, 1984).

In the 1980's, pharmacologists began to recognize the structural characteristics shared by the potent narcotics (review, Thorpe, 1984). These included: 1) a tertiary nitrogen; 2) a quarternary carbon, separated from the basic nitrogen by an ethylene ($-CH_2-CH_2$) chain and attached to a phenyl group; and 3) the presence of a ketone (e.g. methadone) or a hydroxyl (e.g. morphine) group.

It has been suggested that the receptor site contains the amino acids tyrosine and phenylalanine. The following model for the receptor site has been proposed :

1) The receptor consists of two aromatic binding sites and one anionic site (for the binding of the basic nitrogen).

2) One of the receptor subsites - called the T site - binds hydroxylated rings (e.g. morphine) preferentially. The second aromatic subsite - the P site - binds non-hydroxylated rings (e.g. pethidine) but may also bind other groups (e.g. allyl, cyclopropyl).

3) A steric hump exists and serves to possibly elevate the nitrogen from its anionic site.

This is based on the operational model suggested by Beckett and Casy (1954), and although oversimplified, it facilitated the design of new compounds and explanations of opioid action.

Two major approaches are used to characterize the binding of opioids to the recognition (receptor) site. Competitive displacement assays are used to investigate the binding properties of the opioid, followed by saturation analysis to determine the maximum binding capacity of the different binding sites (Paterson *et al*, 1983).

Pharmacological characterization is also necessary to analyze the effects induced by interaction of the opioid receptor with the receptor site. A number of assays can be used to measure some of these effects, the most common being that using the mouse vas deferens, the guinea pig ileum, and the vas deferens of the rat and the rabbit (Paterson *etal*, 1983).

Ligand binding to the opioid receptor has been shown to be stereoselective (Goldstein *et al*, 1971) and sensitive to pH, ionic strength and temperature (Fisher and Medzihradsky, 1981).

Studies on the chemical nature of the opioid receptor have shown that it consists of both protein and lipids (Toro and Way, 1983). Cerebroside sulphate (Loh *et al*, 1978), phosphatidyl serine (Abood *et al*, 1975), and phosphatidyl insitol (Haregawa *et al*, 1981) are the lipids associated with the receptor. Not much is presently known about the nature of the protein moiety involved in opioid receptor activity.

Multiple Opioid Receptors

The multiplicity of the opioid receptors seem to match the complexity of the endogenous opioid ligands. Initially a single opioid receptor interacting with morphine was recognized. The most significant discovery that facilitated the conceptualization of multiple opioid receptors was the development of the narcotic antagonists. These antagonists became powerful tools for studying the specificity and mode of action of opioid action. The observation that nalorphine antagonized the analgesic effect of morphine as well as exerted an analgesic effect itself (Lasagna and Beecher, 1954), suggested the existence of another receptor for the mediation of nalorphine's effect. In order to explain the diverse actions of the opioids, it was necessary to postulate that either one receptor existed with more than one conformation, or that there were several types of receptors.

Martin *et al* (1976) did a series of experiments on dogs with chronic spinal transections and therefrom postulated the existence of multiple opioid receptors. The basic types defined by these investigators are:

The mu receptor - Mediates classic morphine-like effects such as analgesia, and is found abundantly in the guinea pig ileum and pain modulating brain regions.

The kappa receptor - so named for the effects of ketocyclazocine, and is thought to mediate opioid effects atypical of morphine e.g. hallucinations and dysphoria. Localized in the deep layers of the cerebral cortex and found abundantly in rabbit vas deferens

The sigma receptor - to which SKF-10047 binds, producing a general activation of the CNS (tachycardia, tachypnoea, mania). Naloxone insensitive - may not be a typical opioid receptor

Two other receptor types have also been postulated (Rodgers and Cooper, 1988):

The delta receptor - Found in the CNS, displays great affinity for the enkephalins. Also found abundantly in the mouse vas deferens

The epsilon receptor - for β-endorphin - has been characterized in the mouse vas deferens (Schulz *et al*, 1979). Although β-endorphin binds to the CNS (Akil *et al*, 1980), an epsilon receptor has not been described here.

Subtypes of these receptors may possibly exist (Pasternak *et al*, 1986).

Another argument favouring the existence of multiple opioid receptors is based on the experimental work done on cross-tolerance between various opioid compounds. Using the guinea pig ileum, Yoshimura *et al* (1982) were able to discriminate between various opioid agonists. They observed that ilea chronically treated with morphine exhibited complete cross-tolerance to β-endorphin, moderate cross-tolerance to enkephalins and no cross tolerance to dynorphin. Tolerance developing to a given opioid (in this case morphine) activating one class of receptors primarily will exhibit cross tolerance to other agonists acting on the same receptor type (in this case β-endorphin, and to a lesser extent enkephalin) but not to those acting at a different receptor type (dynorphin).

Behavioural studies (schedule controlled behaviour - Harris, 1980), drug discrimination (Teal and Holtzman, 1980) and locomotor activity studies (Iwamoto *et al*, 1981) have also provided evidence for the existence of receptor subtypes.

Evidence for a biochemical basis to support the existence of multiple opioid receptors has also been provided. Binding studies have shown that there may be more than one recognition site for opioid compounds (Smith and Loh, 1981; Lord *et al*, 1977), however, these studies do not indicate whether such sites are associated

with active or silent receptors. More research is necessary to correlate the activation of the binding site with "specific" pharmacological effects.

Mechanism of Action of Opioids

Many hypotheses have been advanced to explain the mode of action of the opioids. Several neurotransmitters have been implicated in opioid action. These include Ach, 5HT, DA, NA, GABA and Substance P (Chapman ann Way, 1980).

It could thus be proposed that a primary mechanism of action common to all these naturally occurring ligands may be involved in the opioid mechanism of action. Second messengers such as Ca^{2+}, cGMP, and cAMP have been suggested to be responsible for the transmission of opioid information to initiate or inhibit cellular processes.

Calcium

The neuronal Ca^{2+} concentration has been suggested to be critical for opioid action. Intracisternal administration of Ca^{2+} antagonizes morphine induced analgesia when tested on both the hot plate or in response to electrical stimulation (West and Miller, 1983). Lowering the Ca^{2+} level in the neuron (by intracisternal administration of EDTA, oxalate, citrate) enhances and may even mimic opioid action.

Morphine administration also reduces Ca^{2+} content in synaptosomes of brain homogenate by approximately 30% after acute treatment , possibly by inhibiting Ca^{2+} uptake and binding. The converse is found after chronic opioid treatment, where an increase in synaptosomal Ca^{2+} is observed, localized mainly in the vesicular fractions of the synaptosome.

Lanthanum blocks Ca^{2+} effects in many systems (Weiss, 1974), and displays naloxone sensitive analgesia in mice (West and Miller, 1983). This cation (La^{3+}) also reduces naloxone precipitated jumping in morphine dependent mice (Harris, 1975), and a possible cross tolerance between morphine and lanthanum has been described.

The opioids are suggested to reduce Ca^{2+} binding to synaptic membranes possibly by inhibiting the interaction of Ca^{2+} with certain phospholipids (thus for e.g., altering serine incorporation into phosphatidylserine) - (West and Miller, 1983).

High Ca^{2+} concentrations in the neuron possibly inhibit morphine's effect by hyperpolarisation of the synaptic plasma membrane with a resultant reduction in the effect of morphine on transmitter release (Szerb, 1980)

Cyclic AMP

In 1974, Collier and Roy reported that morphine and morphine-like drugs inhibit the stimulation by PGE of cAMP formation in rats brain homogenate, without affecting basal (unstimulated) levels. These investigators suggested that the inhibition of cAMP may represent a mechanism whereby morphine-like drugs exert their analgesic and other effects.

Ho *et al* (1973) observed that intravenous (iv) or intraventricular administration of cAMP antagonizes morphine analgesia in tolerant and non-tolerant animals. Administration of cAMP or PDE inhibitors increases naloxone precipated opioid withdrawal jumping (Collier and Francis, 1975).

Law *et al* (1981) observed that a number of opioids inhibit basal adenylate cyclase activity in the striatum.

These findings suggested a role for cAMP in opioid neurotransmission.

Cyclic GMP

Reports have indicated that opioid agents may increase cGMP levels in the striatum and in cultured cells (Racagni *et al*, 1976; Minneman and Iversen, 1977) and in the plasma after icv administration (of morphine to mice) - (Muraki *et al*, 1982). Dibutyryl cGMP has been reported to have analgesic effects in mice (Vocci *et al*, 1978).

Tolerance and Dependence

Criteria for tolerance and dependence are set out in table 1. Tolerance is generally agreed to be a lessened response to a drug after its continued application. Dependence is more difficult to define, but it includes behavioural disturbances induced by withdrawal of certain drugs after their continued application.

TABLE 1 : Criteria for tolerance and dependence

Tolerance	1. Decreased response to the same dose of drug occurs. 2. Increase in the dose of drug to yield the same response is required. 3. Lessened response to the drug occurs after continued exposure.
Dependence	1. "Spontaneous" behavioural disturbance occurs when the drug is removed. This effect is suppressed by replacing the drug. 2. The specific antagonist of the drug displays an increased potency. 3. Administration of the specific antagonist precipitates the behavioural disturbance (1)

Adapted and modified from Collier, 1981a and 1981b.

The opioids are drugs *par excellence* for illustrating the phenomenon of tolerance and dependence. Both *in vitro* (guinea pig ileum) and *in vivo* (self administration by animals) can be used to display these effects (Iversen and Iversen, 1975), and *in vivo* and *in vitro* techniques of dependence induction have many similarities (table 2).

TABLE 2: Similar characteristics of dependence displayed in *in vivo* and *in vitro* studies

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1) Dependence is only manifested after a period of continued exposure to the opioid.
2) Dependence is accompanied by tolerance to the acute effects of the opioid.
3) Opioid withdrawal results in disturbances that can be suppressed by retreatment with the opioid.
4) A similar disturbance can be elicited by treatment with an opioid antagonist.
5) A specific as well as stereospecific opioid receptor is activated to produce opioid dependence

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Adapted from Collier *et al*, 1981a

The exact mechanisms of opioid tolerance and dependence have not been discovered. Two hypotheses have enjoyed much research, and these will be discussed below.

The Ca^{2+} Hypothesis

It has been suggested that the nociceptive state is regulated by the Ca^{2+} level within the neuron (West and Miller, 1983) with opioids having a primary action of lowering these levels.

The lowering of these neuronal Ca^{2+} levels after acute administration is counterbalanced by a homeostatic mechanism which tends to reverse this effect. This process is cumulative, so that with chronic opioid administration there is a gradual accumulation of intraneuronal Ca^{2+}, establishing a new elevated steady state for Ca^{2+}. Lowering of this steady state becomes more difficult but maintenance of this steady state requires the opioid (dependence).

An abstinence syndrome would then reflect sequestered Ca^{2+} release (normally inhibited by the opioid) or a supersensitive state due to the absence of the opioid.

This hypothesis has been forwarded by Chapman and Way, (1980); Way and Rezvani, (1980); West and Miller, (1983).

The cAMP Hypothesis

Most of the data supporting a role for cAMP in opioid tolerance and dependence mechanisms have been undertaken *in vitro* using cultured hybrid neuroblastoma x glioma cells. The opioid receptors characterized on these cell lines are of the σ-type.

Sharma *et al* (1975) reported that the addition of morphine to cultured cells results in a rapid inhibition of adenylate cyclase activity with a resultant decrease in cAMP. As the cells are incubated for longer periods with the opioid, a gradual return to the normal cAMP level occurs. Changes in enzyme (adenylate cyclase) activity parallel those of the cAMP levels, and this stage is likened to tolerance. A late compensatory increase of adenylate

cyclase activity occurs. Challenge with naltrexone or abrupt withdrawal of the opioid at this point generates high levels of cAMP - possibly reflecting dependence. With sufficient deprivation of the opioid, the cAMP levels and adenylate cyclase activity return to normal.

The actual physiological significance of alterations in cAMP levels in opioid tolerance and dependence is not clear.

A Unitary Hypothesis?

It has been suggested that the Ca^{2+} and cAMP hypotheses may be related in some causal way.

Reports have suggested that calmodulin (Ca^{2+}-dependent regulatory protein) stimulates brain adenylate cyclase activity (Lynch *et al*, 1977) - suggesting that the reduced Ca^{2+} uptake induced by opioids could directly inhibit adenylate cyclase.

The Endogenous Opioid System

Opioid peptides are those "naturally occurring peptides with opioid-like biological properties" (Morley, 1983) - the effects of which can be reversed by opioid antagonists.

Five main groups of endogenous opioid peptides are characterized (adapted from Morley, 1983):

1) The two pentapetides derived from pro-enkephalin A :- methionine- and leucine-enkephalin.

2) Peptides arising from enkephalin precursors - these include [Met]-enkephalinyl-Arg-Phe and peptide E from adrenal pro-enkephalin; and dynorphin, α-neo-endorphin and rimorphin arising from other pro-enkephalins.

3) β-endorphin - as well as related α-, γ, and σ- endorphins arising from pro-opiomelanocortin.

4) Pronase resistant peptides e.g. β-casomorphin 5 and 7 (found in bovine milk) and anodynin (found in blood).

5) Other peptides - Opioid-like properties of these peptides do not arise from direct interaction with opioid receptors e.g. kyotorphin.

The Enkephalins

The first opioipeptins to be discovered were the pentapeptides met- and leu- enkephalin (Hughes, 1975). The first four amino acids of these peptides are similar in sequence (Try-Gly-Gly-Phe) and they differ only in the terminal carboxy amino acid residue, one being methionine and the other leucine.

These enkephalins are derived from pro-enkephalins in the brain (Beaumont *et al*, 1982) and in the adrenal glands (Schultzberg *et al*, 1978). Although met-enkephalin is contained in the terminal sequence of β-endorphin, it cannot be derived from this endorphin.

Enkephalins have a short half life in the body and can be degraded by a variety of peptidases (Hughes, 1975). In the guinea pig ileum, these enkephalins are about 3 times less potent than morphine, but 60 to 80 times more potent than morphine in the mouse vas deferens.

Enkephalinase inhibitors such as thiorphan have been synthesized - and protect the enkephalins from degradation *in vitro*, as well as produce analgesia *in vivo* probably due to the inhibition of enkephalin degradation (Roques *et al*, 1980). Synthetic enkephalin analogues have also been developed, some of which are relatively resistant to enzymatic degradation and exhibit strong analgesic properties (Morley, 1980a; Pert *et al*, 1976).

A few long enkephalinergic fibres do exist, however, these peptides are generally found primarily in interneurons with short axons (Frederickson, 1977), and immunoflourescence techniques have been used to localize them in the brain stem nuclei, periaqueductal grey, substantia nigra, medial lemniscus, hypothalamic nuclei (globus pallidus, nucleus accumbens) and the substantia gelatinosa

of the spinal chord (Atweh and Kuhar, 1983).

Peripherally, they have been localized in the colon, myenteric plexus, adrenal glands, various autonomic nerves, the retina, the cochlea, and the peripheral ganglia (Toro and Way, 1983). The physiological significance of this peripheral location is unknown. Evidence has been provided to suggest that catecholamines and the enkephalins may be stored in the same chromaffin vesicles in the adrenal glands and may be co-released on stimulation (Wilson *et al*, 1980).

Binding assays have localized the enkephalins to synaptosomal fractions - characterisic of neurotransmitter substances (Frederickson, 1977). The enkephalins probably act at postsynaptic sites making axo-dendritic or axo-somatic synapses on single neurons (Duggan, 1983) and their effects can be antagonized by naloxone (Frederickson and Morris, 1976).

The enkephalins elicit analgesic responses in mice (tail flick and hot-plate) - and a good correlation exists between the ability of analogues of enkephalin to produce physical dependence and their analgesic activity (Morley, 1983).

Administration of enkephalins near the bodies of neurons in the dorsal horn of the spinal chord results in a naloxone reversible depression of cell firing (Duggan, 1983). This inhibitory action of the enkephalins could reduce the response to both noxious and non-noxious inputs.

A simple hypothesis has been advanced to describe the postsynaptic mode of action of the enkephalins. It suggests that the enkephalins are inhibitory transmitters, and the main inhibitory effect of the enkephalins is not on the excited neurons, but instead, on tonically active adjacent inhibitory interneurons (Duggan, 1983).

Considerable processing of sensory information occurs within the retina and the olfactory bulb. The enkephalins act to increase the firing of the input neurons in these areas through a block of inhibition (Nicoll, 1980).

Enkephalins have also been suggested to cause an excitatory effect on pyramidal cells when administered iontophoretically near hippocampal cells (Nicoll, 1980). Most of the evidence on this effect favours disinhibition as being responsible for the excitation of the hippocampal cells (Duggan, 1983). Although these excitatory effects are said to resemble epileptiform spiking of neuronal activity, no evidence has been forwarded to link it to human epilepsy. Recent evidence has been forwarded suggesting that these endogenous peptides may actually function to spontaneously arrest seizures (Tortella, 1988).

The functional and physiological significance of enkephalinergic action remains to be determined. It would appear that these peptides have a wider role in the body than just the control of nociception.

The Endorphins

In most cases, endogenous transmitters or hormones were discovered before their receptors were characterized (review; Offermeier and Van Rooyen, 1984), but this was not so for the endogenous opioid ligands. The opioid receptor was characterised in 1954 (Beckett and Casy) followed many years later by the discovery of the endogenous opioid ligands (Hughes, 1975; Li and Chung, 1975).

β-endorphin is a 31 amino acid peptide fragment of β-lipotropin (Li and Chung, 1975). It is derived via selective degradation of β-lipotropin, which also contains the sequence of ACTH and part of MSH (Toro and Way, 1983). Immunohistochemical and biochemical techniques have localized β-endorphin in the arcuate nucleus of the hypothalamus and certain limbic structures (Watkins and Akil, 1981) and also in the anterior pituitary lobe.

β-endorphin exists in conjunction with ACTH and both may be concommitantly released (Guilleman *et al*, 1977). This endorphin is also more resistant to enzymatic degradation than the enkephalins, and in most cases is more potent than these peptides (Delitala *et al*, 1984).

Possible roles for the endorphins

An explosion of research into the possible physiological functions of the endogenous opioids followed their discovery, and investigation in this field is still gaining impetus. Literature on possible roles for the endorphins increases at a rapid rate, and various excellent reviews are available on this subject (Morley *et al*, 1983; Rodgers and Cooper, 1988; Clement-Jones and Rees, 1982).

Food intake

Until comparatively recently, the pharmacology of food intake was largely concerned with "anorectic drugs" such as amphetamine, fenfluramine and other phenylethylamine derivatives. The endorphins however, slowly stepped onto the scene.

Early work revealed that naloxone reduced food intake in prior food deprived rats, possibly due to a blockade of the endogenous opioid system and food-deprivation resulted in a decrease in hypothalamic β-endorphin concentrations (Gambert *et al*, 1980). It was also observed that the pituitary glands of obese mice and rats contained more β-endorphin than those of lean littermates (Gambert *et al* 1980). This led to speculation that the release of excess endorphin might be responsible for overeating in obese animals.

Injection of naloxone into the hypothalamic nuclei and the globus pallidus results in a reduction in the feeding response (Gosnell *et al*, 1986), suggesting a possible modulation of the feeding response by the opioid system at this level.

Naloxone may also reduce feeding in humans. Atkinson (1984) reported that naloxone (15 mg) reduced food intake in 8 out of 14 obese humans, but did not affect food intake in 5 out of 5 lean humans.

Drinking and Water Balance

Maickel *et al* (1977) first observed that naloxone and naltrexone reduced the ingestion of water by thirsty rats. This antidipsogenic

effect of the opioid antagonists was speculated to be mediated through specific opioid receptors, although no clear-cut data is available to point to one opioid receptor as opposed to another being responsible for this effect.

It is known that morphine has antidiuretic effects, and recent evidence implicates β-endorphin in this reportedly μ-receptor mediated opioid action (Rodgers and Cooper, 1988).

Although an exact mechanism for this action has not been elucidated, it has been suggested that the opioids function to facilitate mechanisms that minimize water loss.

Sexual Behaviour

Male heroin addicts commonly show symptoms of sexual dysfunction (Cushman, 1981) and morphine abstinence syndrome is associated with erection, spontaneous ejaculation and orgasm (Cushman and Dole, 1973).

Most studies on the role of the endorphins in sexual behaviour has been carried out on male rats, however, some investigations have been carried out on females.

Administration of β-endorphin (1 μg intraventricularly) results in an inhibition of mounting behaviour in rats, with a resultant reduction in the number of intromissions (Meyerson and Terenius, 1977). This inhibitory effect of the opioid on copulatory behaviour is prevented by the opioid antagonist naltrexone. Higher doses of endorphin (6 μg) caused a complete cessation of copulatory behaviour in rats.

The occurrence of lordosis (concave back flexion, neck extension and lateral tail deviation) is considered to be related to the degree of sexual receptivity in female rats. Intracerebroventricular (icv) administration of β-endorphin suppresses lordosis in rats, probably via an action on μ-opioid receptors (Sirinathsinghji, 1984).

Systemic administration of naloxone was observed to increase

copulatory behaviour in otherwise sexually inactive male rats (Gessa *et al*, 1979) probably suggesting that sexual impotence in rats may in part be due to endorphinergic hyperactivity in the CNS.

These findings are important in that possible therapeutic value might be obtained from the use of opioid agonists and antagonists to modulate sexual hyperactivity or impotence in man.

Social Interaction and Attachment

The young of many mammalian species call persistently when separated from their mothers. Centrally administered α-, β-, and γ-endorphins were observed to reduce the calling of chicks, with β-endorphin having the most potent effect, suggesting that this opioid influences separation-induced distress (Benton and Brain, 1988).

The young mammal also has a strong urge to frolic with its littermates, and naloxone decreases this playful activity (Beatty and Costello, 1982).

Social isolation is known to decrease opioid receptor populations in many brain regions (Benton and Brain, 1988). Group housed rats show an increased tendency to jump and suffer from diarrhoea on precipitation of the morphine withdrawal syndrome with naloxone when compared to rats housed in isolation.

A predominant characteristsic of autistic children is a failure to seek company. High brain opioid activity has been suggested to be responsible for this effect (Panskepp, 1979).

Imbalances in the opioid system may thus be suggested to be responsible for some emotional disorders.

Learning and Memory

Central administration of β-endorphin was found to impair performance in rats tested in assays for memory. Also, this peptide impairs the development of shock-induced fighting in rats and slowed down the extinction of the pole jumping avoidance response

(Benton and Brain, 1988).

Endogenous release of β-endorphin also occurs when rats are exposed to a novel environment, but no such increase occurs on subsequent exposure to the same environment. Stein and Belluzi (1978) suggested that activation of the opioid system after a learning procedure may facilitate memory consolidation.

Exploration and Activity

Katz (1980) reported that the Straub tail response (typical sign of opioid activation) could be elicited by a variety of environmental manipulations including stress and exploration. The Straub tail response occurred in conjunction with exploratory activity and was not present during normal locomotor activity. This effect was reduced by naloxone.

Pharmacological blockade of opioid receptors (with naloxone) interferes with the initial expression of exploratory activity. Grevert and Goldstein (1977) observed that naloxone (10 mg/kg) increases the latency for mice to enter a novel dark environment significantly, suggesting an opioid-exploratory activity link.

Naloxone (0.5 - 2 mg/kg) decreased motor activity in some autistic children, as well as improved their clinical status - suggesting, once again, a role for the endogenous opioid system in some aspect of autism.

Hormones

The endogenous opioid peptides are possibly involved in neuroendocrine function. β-endorphin administered icv to rats stimulates growth hormone (GH) and prolactin release (Schmauss and Emrich, 1988) and naloxone decreases plasma levels of these hormones (Grandison and Guidotti, 1977).

In man, naloxone was observed to significantly elevate serum cortisol, ACTH, LH and follicle stimulating hormone, with no effect on basal GH and prolactin levels.

Activation of different receptor subtypes have been suggested to be involved in the opioid-modulated regulation of pituitary hormone release.

Shock and Trauma

Plasma β-endorphin levels are increased significantly after shock and may probably contribute to shock-induced hypotension. This effect can be reversed by naloxone (Toro and Way, 1983)

A role for the opioids in modulating microcirculation in hamsters has been suggested (Akil, 1984). The beneficial action of naloxone is possibly due to the blockade of the opioid peptide-induced circulatory vasodilation, thus allowing the blood pressure to increase.

Opioid antagonists could thus play a beneficial role in the clinical management of shock.

ANALGESIA

The opioid drugs and their analogues are perhaps the most potent analgesic agents yet discovered. The conservatism in our search for newer analgesic agents is made more obvious by the fact that the synthetic opioid analgetics still exhibit a spectrum of pharmacological and clinical actions quite similar to that of morphine, of which the most unwanted side-effects (tolerance, dependence, addiction liability) still prevail. The agonist-antagonist synthetic agents (eg buprenorphine) have found some clinical use, but are still not the optimum agents for pain relief.

This could, perhaps, be due to a lack of knowledge on the exact nature of the pain controlling mechanisms. Much has been discovered regarding the nature of the pain controlling mechanisms since the discovery of the endogenous opioid peptides, but still, the knowledge obtained thus far is incomplete.

Various experimental assays have been designed in laboratories to measure the analgesic activity of drugs (see table 3). These methods have enjoyed different degrees of popularity, depending on the type of compound being assayed for analgesic activity, and in some cases the cost effectiveness of the design.

TABLE 3 : DESCRIPTION OF VARIOUS ANALGESIC ASSAYS

Assay	Stimulus	Response
Rat tail flick	Thermal	SP
Mouse tail flick	Thermal	SP
Rat tail immersion	Thermal	SP
Rat hot plate	Thermal	CM
Mouse writhing	Chemical irritation	CM
Rat air writhing	Physical irritation	CM
Guinea pig EDTA test	Chemical irritation	V
Mouse tail clamp	Mechanical pressure	CM
Rat inflamed paw	Mechanical pressure	CM
Rabbit tooth pulp	Electrical	CM
Monkey shock titration	Electrical	LP
Rat shock titration	Electrical	V
Dog Bradykinin	Pain mediator	V +CM
Mouse Ultrasonic	Ultrasonic	CM

Adapted from Lednicer, 1982

SP = spinal reflex, CM = complex motor, V = vocalization, LP = lever press

Historically, two main theories existed to explain the sensation of pain: the *specific theory* and the *intensive theory* (Lednicer, 1982).

SPECIFIC THEORY	INTENSIVE THEORY
Pain is transmitted from peripheral pain receptors to a pain centre in the brain	Pain results from an over-stimulation of other sensory modalities such as touch, sight and sound
∇	∇
Pain is a separate sensory modality	Pain is a sensation attributed to a central summation of sensory input

Melzack and Wall (1965) later proposed a new theory in an attempt to unify these two theories - the *gate control theory.*

Very, simply, this theory suggests that pain impulses are conveyed by specific groups of nerve fibres. The activity of these fibres are modified by other fibres conveying non-noxious impulses until a critical level of stimulus intensity is reached. At this point the noxious stimuli are projected rostrally, and pain is perceived.

This theory thus allows for peripheral specificity, central summation and psychological alteration of the pain experience.

Peripheral Pain Pathways

Pain sensitive receptors, termed nociceptors, appear as free nerve endings in the periphery. Small diameter slow conducting ($A\delta$) and unmyelinated (C) fibres that can be stimulated by thermal, chemical and/or mechanical cutaneous stimulation are found peripherally. Not all $A\delta$ and C fibres are nociceptive in nature (deStevens 1965, Lednicer, 1982).

Aδ fibre activation produces a sharp rapid pain sensation, whereas C fibre activation evokes a dull, chronic pain - described by some investigators as a *first pain* and a *second pain*.

It has also been observed that stimulation of large diameter pain fibres (Aα and Aβ) also evokes pain sensation in man (Willer *et al*, 1980) and this has been suggested to be responsible for the persistence of pain in certain diseases.

Spinal Pain Pathways

The first site of synapse of the peripheral pain perceiving neurons is the dorsal horn of the spinal chord, the general anatomy and physiology of which have been described and are still accepted (Rexed, 1954; Wall, 1967). The dorsal horn has been differentiated into six distinct laminae on a functional basis (Wall, 1967).

The substantia gelatinosa (SG) in laminae 11 contains many neurons. Substance P, enkephalin, dynorphin, cholesystokinin, and neurotensin are some of the peptides found in these neurons (Gibson *et al*, 1981; Hunt *et al*, 1981), as well as a large population of GABA-ergic neurons which may possibly control the primary afferent fibres presynaptically (Basbaum *et al*, 1981).

Substance P (SP), the most suitable candidate for neurotransmission for primary afferent nociceptive fibres, is found in high concentrations in the superficial laminae of the dorsal horn (Takahashi and Otsuka, 1975).

Opioid analgesics have been observed to produce analgesia as well as inhibit noxious dorsal horn neuronal activity in normal as well as spinalized animals (LeBars *et al*, 1975). This spinal modulation of the analgesic response by the opioid drugs (fig1.3) has been proposed as a presynaptic inhibitory process in which small interneurons containing enkephalins inhibit both the activity and release of SP from the small diameter afferent fibres (Jessel and Iverson, 1977).

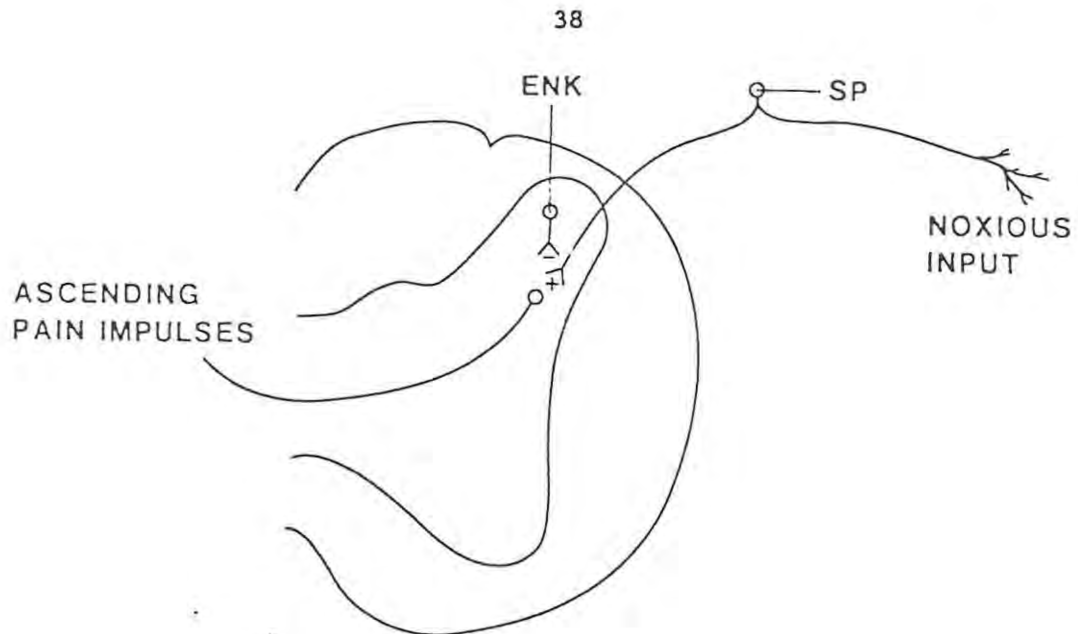


Fig 1.3: Presynaptic inhibition of Substance P (SP) containing primary afferents by enkephalinergic (ENK) interneurons.

Proposed by Jessel and Iversen, (1977).

Ascending Pain Pathways

The most prominent pathway via which noxious information travels to the supraspinal pain perceiving areas is the spinothalamic tract, which in higher animals consists of the neospinothalamic tract and the paleospinothalamic tract (see fig1.4).

The neospinothalamic tract consists primarily of rapidly conducting myelinated fibres. These fibres first synapse with cell bodies in the thalamus (Mehler, 1969).

The paleospinothalamic tract follows a similar pathway, but synapses at various levels of the brain stem reticular core and innervates diffuse supraspinal structures (Mehler, 1969)

Other ascending pathways including the spinoreticular tract, the dorsal columns, the spinocervical tract and the propriospinal tract have also been suggested to play partial or supporting roles in conveying noxious information to higher centres in the CNS (Lednicer, 1982).

Supraspinal Pain Pathways

Most of the neospinothalamic, spinocervical and postsynaptic dorsal column fibres terminate in the ventrobasal thalamus, which relays information to the somatosensory cortex (Lednicer, 1982).

The posterior thalamus also acts as a nociceptive relay station, and electrical stimulation of this area produces pain in humans (Hassler and Riechert, 1959).

The nociceptive information received by the medial thalamus (MT) arises mainly from the paleospinothalamic tract, and lesions in the area of the MT have been used to alleviate pain in man (Lednicer, 1982).

The brain stem reticular formation is densely innervated by spinoreticular and paleospinothalamic fibres and has efferent connections to the thalamus. The nucleus reticularis gigantocellularis is excited by and responds to noxious somatic stimuli (Mohrland and Gebhart, 1980). Enkephalinergic terminals and opioid receptor binding sites have been identified in the mesencephalic reticular formation (Atweh and Kuhar, 1977) providing good evidence that opioid-induced analgesia may partly arise from a possible supraspinal inhibition of noxious stimuli.

Endogenous opioid peptides and opioid receptor binding sites have also been identified in the cerebral cortex (Pert and Snyder, 1983). Although it has been suggested that the somatosensory cortex is unlikely to be a pain modulating centre (Lednicer, 1982), it could very well be involved in integrating noxious information with other somatosensory information.

Descending Pain Pathways

The first region to be implicated in the modulation of pain was the periaqueductal gray (PAG) - (Reynolds, 1969), yet its intrinsic circuitry is still largely unknown.

Focal stimulation of the PAG produces profound analgesia in many

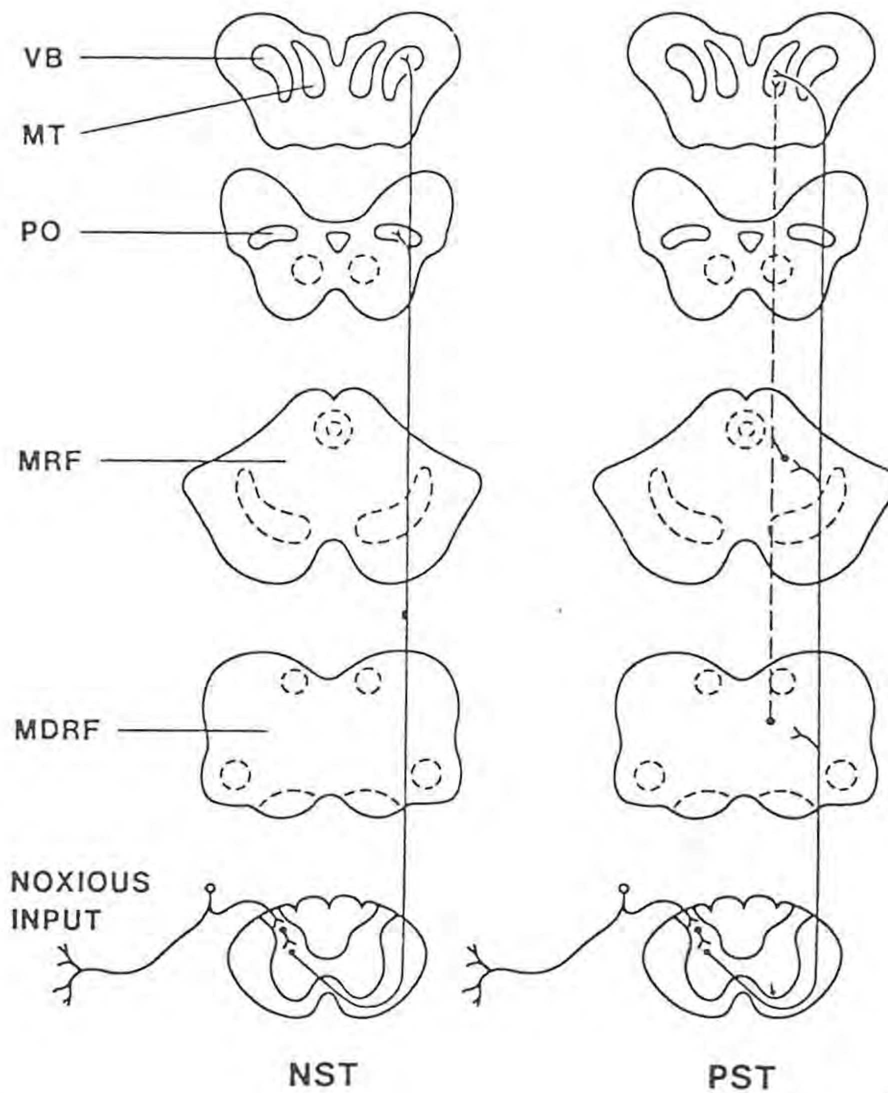


Fig 1.4: The two divisions of the spinothalamic tract: the neospinothalamic tract (NST) and the paleospinothalamic tract (PST). MDRF: medullary reticular formation, MRF: mesencephalic reticular formation, PO: posterior thalamus, MT: medial thalamus, VB: ventrobasal thalamus.

Adapted from Lednicer, 1982.

species, including man (Hosobuchi, 1979). In order to initiate descending pain control mechanisms, excitation of the PAG output neuron is required (Basbaum and Fields, 1984). Nicoll *et al* (1980) have have suggested that opiate actions inhibit target neuron activity, and it could thus be suggested that these drugs do not act directly on the PAG output neuron. However, a vast number of enkephalinergic neurons have been observed presynaptic to dendrites in the PAG (Moss and Basbaum, 1983), and the analgesia produced by activation of the output neuron could be attributed to an inhibition of inhibitory interneurons by this or other opioid peptides.

The PAG efferrent fibres project to the nucleus raphe magnus (NRM), the axonal fibres of which project to the dorsal horn of the spinal chord via the dorsolateral funiculus (Basbaum and Fields, 1984). Many peptidergic neurons have been demonstrated in the NRM, including enkephalin, somatostatin, and substance P neurons (Basbaum and Fields, 1984). Two putative neurotransmitters can possibly co-exist in the same neuron (Hokfelt *et al* 1980) and in the NRM serotonin and SP coexist in the raphespinal neurons, suggesting that SP may act as a neuromodulator. A reduction of the serotonin tone in the NRM or the PAG leads to an inhibition of the analgesic effects of stimulation in these areas, suggested by the ability of serotonin antagonists to block this stimulation produced analgesia (Satoh *et al*, 1980).

Microinjection of the NA α-adrenoreceptor antagonist, phentolamine, into the NRM produces a methysergide (administered intrathecally) reversible hypoalgesia (Hammond *et al*, 1980), suggesting the possible presence of a tonic NA-mediated inhibition of the 5HT neurons in this bulbar nucleus.

A possible role for pain modulation by cholinergic agonists has been surmised by the ability of iontophoresis of ACh, and injection of carbachol, into the NRM to produce analgesia (Basbaum and Fields, 1984). The role of the locus coeruleus (LC) in the modulation of nociception is not entirely clear. Morphine is known to inhibit LC neuronal activity (Aghajanian, 1978), suggesting that this inhibition may possibly accompany analgesia.

Thus it can be seen from the above that there are many sites within the CNS and the periphery at which nociceptive information may be modulated.

Although it has been a matter of controversy, evidence is accumulating that the neurotransmitter at the supraspinal site of synapse for ascending pain fibres is the undecapeptide Substance P (Jessel *et al*, 1979). It could thus be assumed that inhibition of the neurotransmitter function of Substance P would yield analgesic effects. In fact, capsaicin, a pungent compound derived from red pepper, causes a long-lasting depletion of SP with a resultant analgesia (Yaksh *et al*, 1979). This compound is, however, toxic to sensory nerves (Nagy *et al*, 1980) and is thus not therapeutically useful. In this regard it has been speculated that less potent Substance P depletors or the development of SP receptor blockers may yield important clinical advances especially in the treatment of chronic pain.

Although the exact neural and neurochemical mechanisms of the pain pathway have not been fully elucidated, our knowledge and understanding of the physiology of pain modulation has increased significantly since the time when Melzack and Wall (1965) proposed the *gate control theory* of pain. One important observation of this theory however, was the presage that descending modulatory controls over the activity of spinal chord pain transmission neurons existed.

Several endogenous systems may possibly modulate nociceptive input (Basbaum and Fields, 1984; Mayer and Price, 1976; Watkins and Mayer, 1982) and neurotransmitters and hormones involved in the neuronal circuitries underlying analgesic mechanisms are being elucidated. These endogenous systems may be activated by many environmental stimuli with varying degrees of complexity.

Since the initial description by Reynolds (1969) of the phenomenon of stimulation produced analgesia, a wide variety of environmental factors have been found to elicit analgesia in rodents (Table 4).

TABLE 4 : DESCRIPTION OF VARIOUS ENVIRONMENTAL FACTORS ELICITING ANALGESIA IN RODENTS

Brain stimulation	Body pinch	Centrifugal rotation
2-Deoxy-D-glucose	Insulin	Classical Conditioning
Copulation (males)	Novelty	Electroconvulsive shock
Food deprivation	Irradiation	Restraint/ immobilization
Social conflict	Predation	Vaginal stimulation
"Stress" odours	Tail pinch	Social isolation
Heat exposure	Tail pressure	Centrifugal rotation

Adapted from Rodgers and Randall, 1988

Different environmental manipulations can selectively activate opiate as well as non-opiate analgesia systems (Watkins and Meyer, 1982). A complex picture of at least four endogenous pain inhibitory systems (doubly dissociated on neural/hormonal and opiate/nonopiate dimensions) is beginning to emerge and the diverse analgesia producing environmental stimuli can be categorised into these inhibitory systems (see table 5). The opioid-sensitive forms of stress-induced analgesia can be differentiated from the non-opioid forms depending on certain factors. These include cross-tolerance to morphine, antagonism by naloxone, and determination of opioid peptide release after stress exposure.

TABLE 5 : STIMULI ACTIVATING ENDOGENOUS PAIN INHIBITORY SYSTEMS

Neural-opioid	Hormonal opioid
Brief front paw shock Conditioning to footshock Systemic morphine Intracerebral morphine Intrathecal morphine	Acupuncture Prolonged shock to all four paws. Immobilization
Neural non-opioid	**Hormonal non-opioid**
Brief hind paw shock Brief four paw shock 2-Deoxy-D-glucose	Cold water swims Insulin
Unknown opioid	**Unknown non-opioid**
Transcutaneous nerve stimulation (TNS) - low frequency, high intensity	TNS - high frequency, low intensity Hypnosis

Modified from Watkins and Mayer (1982)

The pituitary-adrenal axis plays an important role in the adaptive response to stress (Selye, 1974). Terman *et al*, (1984) observed that hypophysectomy attenuates only the opioid form of stress-induced analgesia. Removal of the pituitary gland, however, also compromises the activity of the adrenal glands - which are known to secrete enkephalin-like peptides in response to stress. Terman *et al*, (1984) noted that adrenalectomy too blocked only the opioid

form of stress-induced analgesia, probably due to the loss of the enkephalin-like peptides from the adrenal medulla.

One of the most commonly used stressors, restraint, involves both somatic and emotional components and is also associated with analgesic mechanisms. The technique involves painlessly forcing an animal into a defined posture, so making it very difficult for the animal to perform normal body and limb movements.

Altered levels of β-endorphin immunoreactivity in the brain, blood and pituitary gland and opioid-like alterations in animal behaviour after exposure to restraint immobilization suggests that this process activates the endogenous opioid system (Porro and Carli, 1988).

Rats restrained in a plexiglass chamber show a significant analgesia on the hot plate 30 minutes later (Amir and Amit, 1978) possibly mediated by endogenous opioids. Pretreatment with naltrexone (1 mg/kg bw) before immobilization is able to prevent the onset of immobilization-stress-induced analgesia (Kelly and Franklin, 1985). Vaswani *et al* (1988) have observed changes in opioid peptide levels in the CNS and the plasma, suggesting an activation of this system in cold swim stress. In human beings, it was observed that cold stress (submerging foot in ice cold water for as long as it was bearable) significantly increased the pain threshold to an aversive electric shock (Jungkunz *et al*, 1983).

Bhatacharya *et al* (1978) observed a time-related (1 - 4 hrs) increase in tail flick latencies in completely immobilized rats, and showed that serotonin and prostaglandins might also mediate this effect. Kelly and Franklin (1985) observed that pre-loading a rat with valine or tyrosine (and thus inhibiting tryptophan uptake across the blood brain barrier) reduces immobilization-induced analgesia, suggesting that serotonin is involved in this mechanism.

The muscarinic antagonist, scopolamine, reduces only the opioid form of analgesia, whereas methylscopolamine (peripherally active) has no such effect (Terman *et al*, 1984) further implicating an interaction between the central cholinergic and opioid systems in intermittent footshock-induced analgesia.

The cerebral renin-angiotensin system has been implicated in immobilization-induced analgesia (Haulica *et al*,1986) and is suggested to be mediated by the release of endogenous opioids and, possibly, pineal gland activation. Food-deprived mice immobilized for 15 minutes show a stress-induced increase in salt intake, and this effect is reversed by naloxone (0.01 mg, 1 mg, and 0.1mg/kg bw) in a dose dependent manner (Kuta *et al*, 1984). These investigators suggest that stressors activating the endogenous opioid system lead to an increase in salt intake.

A variety of stressful stimuli are known to activate noradrenergic neurons in many brain regions (hypothalamus, amygdala and thalamus) in animals (Tanaka *et al*, 1983). Endogenous opioid peptides are also released in these regions, which are closely related to emotionality. Tanaka *et al* (1983) have observed that morphine (6mg/kg bw sc) attenuates stress induced increases in NA release in these brain regions in rats. They also observed (1988) that naloxone 5mg/kg bw sc) enhances stress induced increases in NA in the early periods of immobilization stress. They suggest that this is due to a blockade of endogenous opioid receptors by naloxone thus preventing the activity of endogenous opioid peptides released on exposure to stress (Porro and Carli, 1988).

Using *in vivo* autoradiography, it was observed that significant alterations in opioid levels in the brain occurred in stressful conditions, suggesting a release of the endogenous opioids (Seeger *et al*, 1984) Also, it was found that opioid receptors exist on NA-containing nerve terminals (Llorens *et al*, 1978) and that stimulation of these opioid receptors results in an inhibition of NA release (Arbilla and Langer, 1978). Abercrombie and Jacobs (1988) have observed a stress-induced increase in the noradrenergic activity of locus coeruleus neurons in cats, and this effect was potentiated by naloxone (1mg/kg bw iv) administration. This dose of naloxone also increased signs of distress in restrained cats.

Immobilization has been reported to selectively alter DA synthesis and release in certain brain regions (DeSouza and Van Loon, 1986). Repeated immobilization enhances apomorphine-induced stereotyped behaviour in mice, possibly due to a stress induced modification of

sensitivity of DA-ergic activity.

Acute restraint stress has been observed to increase ACh release, as well as enhance high affinity choline uptake in the hippocampus and the frontal cortex (Gilad *et al*, 1983).

Serotonin turnover, manifested by 5-HIAA levels, as well as tryptophan levels in the brain, are increased after acute immobilization (Curzon *et al*, 1972). This effect is abolished with repeated immobilization (Kennet *et al*, 1985).

Tonic immobility (TI) is another manifestation of exposure to restraint stress, and can be induced by a short period of restraint in the supine and inverted position (Porro and Carli, 1988). Analgesia is known to occur during TI . Morphine potentiates the duration of TI, and this effect can be abolished by naloxone (Peters and Hughes, 1978). Stress is also known to facilitate opioid-induced catalepsy in the rat (Katz, 1980).

Serotonin may modulate the mechanisms involved in the control of the duration of TI (Farabollini *et al*, 1984) and conflicting evidence exists for a role for ACh. Pineal melatonin has also been observed to increase the duration of tonic immobility in chickens at low doses (0.4 mg/kg) - (Hennig *et al*, 1980a). Administration of methysergide completely abolishes this effect on melatonin. These investigators (1980b) suggest that melatonin exerts this effect by a direct action on serotoninergic mechanisms in the CNS, and that methysergide acts to reduce the duration of tonic immobility by inhibiting the conversion of 5HT to its metabolites, one of which is melatonin.

STRESS

Restraint and/or immobilization has been used for many years to investigate physiological, pathological and pharmacological effects of stress. Scientists from a wide variety of disciplines study the stress syndrome and its implications for human health and disease.

The very diversity of the topic of stress becomes evident in the difficulty of finding an apt definition for it. The term stress was first used in the field of physics describing strain, force or pressure applied to a system. It was first used as a medically-related term by Selye, who noticed that a wide range of physical ailments had common symptoms; such as muscular weakness, body pain, elevated blood pressure and a lethargic attitude (Selye, 1974). He called this the "syndrome of just being sick."

He defined stress as the "non-specific response of the body to any demand made upon it" and this definition includes a physical as well as a psychological component.

For any stimulus to evoke a stress response, it must first be received by the sensory receptors of the parasympathetic nervous system (PNS). These receptors then send impulses toward the brain, and once in the CNS they diverge from the main ascending pathways to the neocortex where an interpretive analysis of the stimulus is undertaken. These interpretations are then transferred to the limbic system (Everly and Rosenfeld, 1981).If they are perceived as aversive, or challenging, emotional and physiological arousal will result.

The pituitary and adrenal glands are important in the physiological responses to stress. In acute stress situations, the adrenal glands provide a protective action, and adrenalectomy increases the severity of the response to stress (Brodie and Hanson, 1960; Selye; 1946). Stressful stimuli activate sensory centres in the brain, and via mediation by the hypothalamus, ACTH is released by the pituitary into the bloodstream. The adrenal glands respond to the stress-induced release of ACTH by secreting corticosteroids. These hormones then mobilize energy stores, and make high energy glucose available to the body to combat the stress.

The General Adaptation Syndrome (GAS) has been described by Selye (1946). It is a triphasic phenomenon, the first of which is the *alarm* phase in which the body is exposed to the stressor and responds by activating its defense mechanisms. The pituitary-adrenal axis is activated as the body attempts to combat the shock of the stressor. This is followed by the *stage of resistance*, in which the body tries to maintain a homeostatic balance in the presence of the stressor. A stage is reached when all the "adaptive energy" is depleted, and the body enters the stage of *exhaustion* (Schaffer 1982).

The stress response may result from the activation of one or more of the following mechanisms :

1) Neural innervation via SNS or PNS
2) Fight or flight response
3) GAS

These mechanisms are described in fig (1.5).

Chronic elicitation of the stress response can result in end organ dysfunctions and pathologies that can manifest themselves as structural changes in the end organ tissues. Various stress-related disorders are known to occur in man. The precise nature and mechanism/s of the human stress reponse have yet to be defined conclusively.

Various end organs may be affected by psychophysiological arousal, depending on (according to Lachman, 1982) :

1) Predisposition of the organ due to genetic factors.
2) The intensity and duration of involvement of the organ in response to arousal in these conditions

Some of the most commonly encountered stress-related diseases are:

1) GIT disorders eg. peptic ulcer and ulcerative colitis. The pathogenesis of stress ulceration seems to involve a combination of emotional and genetic factors, however, the specific causal mechanisms involved in peptic ulceration are not clearly known.

2) Respiratory disorders - such as allergy are aggravated by stressful situations. Hyperventilation is also a manifestation of acute stress. Periodic bursts of hyperventilation that are not diagnosed at an early stage, later present as various vague symptoms including nausea, aches and pains, vomiting, and chest pains (Everly and Rosenfeld, 1981).

3) Cardiovascular disorders such as hypertension and migraine are potentiated by stress. The cardiovascular system is thought to be the prime target end-organ for stress.

4) Tension headaches and lower back pain - these are common symptoms of stress-related musculoskeletal disorders - and occur as a result of prolonged contraction of the muscles of the back, and the head and neck area.

5) Skin disorders such as acne, eczema, urticaria and psoriasis are common stress-related disorders.

6) Thymus involution and lymphopaenia have also been associated with stress.

7) Various psyhcological disturbances have been associated with chronic stress, the most common of which is anxiety.

The desire for instant relief from various ailments often leads man to a pharmacological solution. Various pharmacological agents are used to treat the pathological conditions related to stress. These treatments are reserved for chronic elicitations of the stress response. The drugs used include:

1) The barbiturates, in small doses, to counter restlessness and

agitation.

2) The benzodiazepines - to counter stress-related anxiety, inhibition and isolation.

3) Antihistamines are sometimes used for their sedative side-effects to counter stress-induced insomnia.

4) β-adrenergic receptor blockers - to counter peripheral autonomic symptoms. These drugs are not officially appproved for this particular use.

5) Tricyclic antidepressants - for stress-related depression.

6) Phenothiazines - for individuals in whom anxiety forms part of a more hyperactive, distractible, and disorganized picture.

Pharmacological treatment can also be used to treat other manifestations of the stress response, eg analgesics for lower back pain and tension headache, and antacids for gastric ulcers.

The obvious solution would be to actually treat the cause of the stress before it reaches pathological proportions. Relaxation techniques, meditation, breathing exercises and neuromuscular relaxation exercises are useful techniques that can be used clinically. Recommendations about dietary requirements are also important.

A holistic approach to the treatment of stress is perhaps the best clinical solution, so as to ensure psychological as well as physiological therapy.

Gastric ulceration is a very common manifestation of exposure to excessive stress (Kleiman *et al*, 1988). Acute gastric lesions occur in patients with severe burns, intracranial lesions, myocardial infarctions, and after surgery (Rhodes, 1972; Kleiman *et al*, 1988).

Stress (restraint, immobilization) is a simple, rapid, effective, non-surgical means of inducing gastric erosions in the experimental animal (Brodie and Hanson, 1960). When subjected to abnormally high

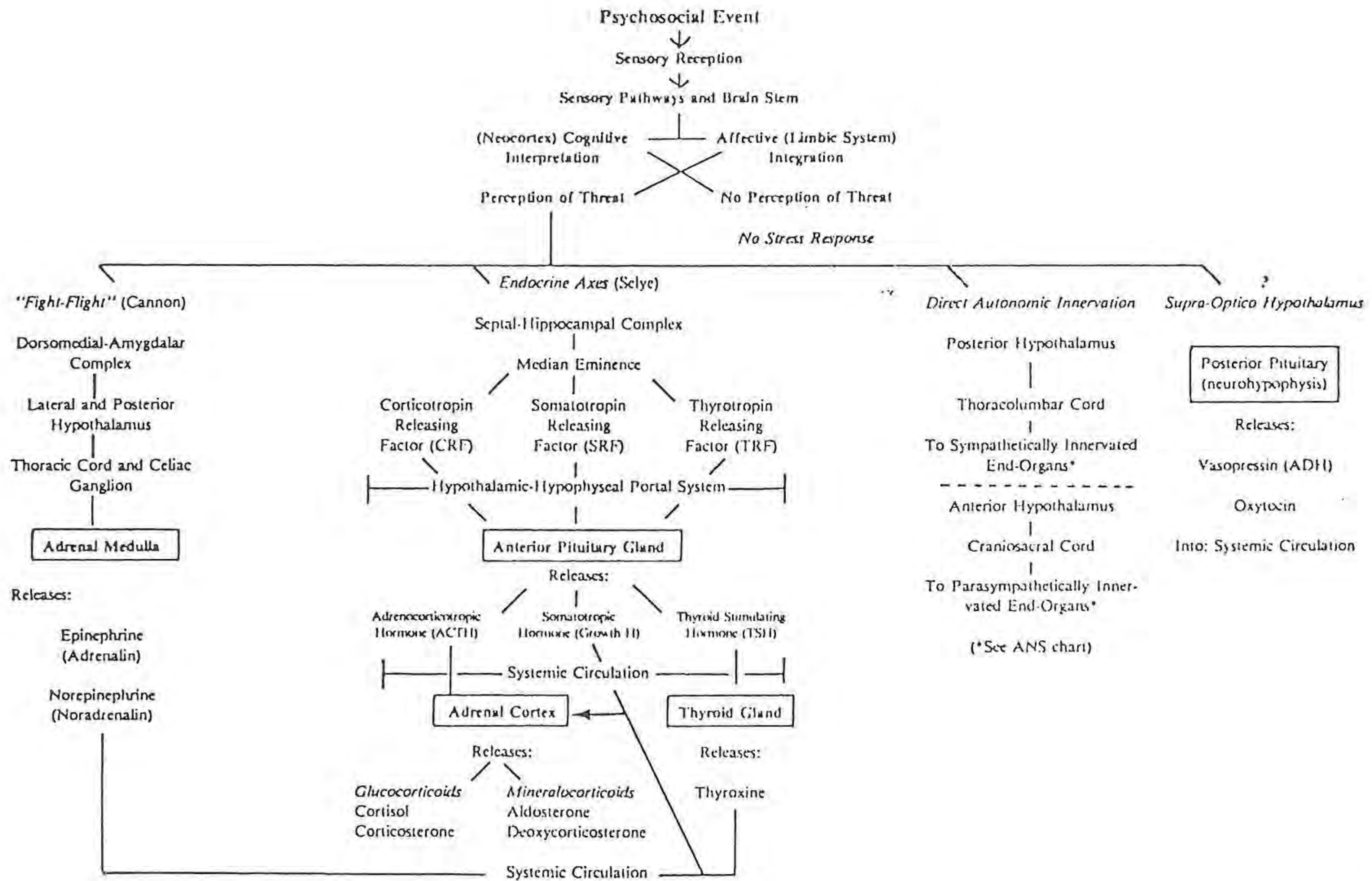


Fig 1.5: A model of potential pathways for stress reactivity to psychosocial stimuli.

Adapted from Everly and Rosenfeld, 1981.

physiological demands, acute superficial inflammatory lesions may occur in the proximal (acid/pepsin) area of the stomach (Kleiman *et al*, 1988). This technique is often used by researchers in the field of gastrointestinal drugs to screen new compounds.

Various restraining techniques are used to induce gastric stress lesions. These include wrapping the animal in plaster of Paris, enclosing it in an window screen envelope, reducing the cage volume, and using special metal tube cylinders (Pare and Glavin, 1988) over varying time spans.

The rapid production of experimental ulcers is facilitated by a pre-stress starvation period (Brodie and Hanson, 1960) and exposure to cold (Senay and Levine, 1967). Both stressors reduce the duration of restraint required to induce ulcers.

Various physiological and biochemical changes (central and peripheral) occur in response to stress. These include changes in gastric secretion and motility, increases in heart rate and blood pressure (Vogel, 1987) elevated plasma corticosterone/cortisol, fatty acids and glucose levels (Pare and Glavin, 1988) and accelerated NA and DA turnover in many brain regions (Glavin *et al*, 1983). Beattie (1978) observed that cold and restraint stress increases the volume of urinary output (with increased K^+ and urea excretion) and plasma urea in rats. Marked increases in corticosterone and thyroxine levels, and a reduction in insulin levels were noted, as well as leucopaenia and lowering of blood pH.

The exact reasons for stress ulcer formation have not been adequately explained. Various factors contribute to the maintenance of the physical integrity of the gastric mucosa and protect it from the adverse effects of back-diffused Hydrogen ions. These include adequate mucosal blood flow (Hase and Moss,1973), an adherent mucous layer (Kleiman *et al*, 1988), mucosal bicarbonate secretion (Kleiman *et al*, 1988) and the presence of prostaglandins (Szabo and Szelenyi, 1987).

The prostaglandins (PG's) are widely distributed im mammalian tissue and body fluids. They have been found in, amongst other

areas, the kidneys, eyes, spleen, lungs, thymus, adipose tissue, thyroid gland, uterus, adrenal glands, and in the stomach (Curtis-Prior, 1976). PG's have been proposed to have cytoprotective properties - protecting the gastric mucosa without altering gastric acidity, by promoting mucosal repair (Jacobsen, 1986; Curtis-Prior, 1976). Exogenous administration of PG's protect the gastric mucosa of rats and humans against the effects of aspirin, ethanol and acidic solutions (Robert, 1979).

The beneficial mechanism of action of the PG's in mucosal protection appears to be multifactorial, including increased mucous production, increased mucosal cell integrity (Robert, 1979) and increased mucosal perfusion (Whittle, 1977).

The origins of gastric stress erosions can be attributed to many causes. The removal of mucous from the gastric mucosa is followed by the development of lesions, providing evidence for the beneficial role played by mucous in gastric cytoprotection (Turnberg, 1985). The integrity of the epithelial cells also plays a role in the resistance of the mucosa to various injurious agents - with rapid cell regeneration strengthening the mucosal barrier (Svanes *et al* 1982).

Among other possible factors is the observation that a causative relationship exists between the mucosal microcirculation of the stomach and the formation of lesions (Hase and Scarborough, 1973). Gastric mucosal blood flow is essential for the maintenance of the integrity of the gastric mucosal barrier. It has been suggested that the extent of injury to the gastric mucosa is directly related to the severity of the ischemia (Kleiman *et al*, 1988). It has been shown that the rat stomach generally has the same vascular arrangement as that of the human stomach (Arabehety *et al*, 1959). These investigators postulated that one of the initial direct responses to stress was a generalized mucosal ischemia, becoming localized as the stress persists. Hase and Moss (1973) explained that this localized mucosal ischemia gives rise to early signs of microvascular breakage in the stomach wall. Also, the protective capacities of the surface epithelium and the mucous layer in the ischemic region could be altered, making it more vulnerable to the erosive action of gastric juice thus leading to the initial damage

of the gastric mucosal epithelium.

These investigators (Hase and Moss, 1973) suggested that the microcirculatory pattern becomes more irregular as the stress persists, with smaller lesions showing little haemorrhage, and larger lesions being surrounded by engorged mucosa and showing severe haemorrhage.

In the pathogenesis of stress ulceration, the hypersecretion of acid is not as important as the way in which the stomach deals with the back diffused hydrogen ions (Kleiman *et al*, 1988). Generally, it is accepted that mucosal ischemia is the primary factor leading to gastric ulceration (Moody and Cheung, 1976; Skillman and Silen, 1976). An efficient blood flow is essential to dispose of back-diffused hydrogen ions - which release histamine from mast cells and stimulates acid and pepsin secretion.

Antoon and Gregg (1976) found that ulcer induction in rats exposed to restraint and cold stress was accompanied by a marked drop in core temperature. They also observed that the incidence of ulceration could be greatly reduced by preventing hypothermia. They observed that when body temperature was maintained at 38°C, a striking reduction in gastric stress ulceration occurred. However, rats still developed ulcers, and it was concluded that although hypothermia was not a causative agent in the production of stress ulcers, it contributed to its formation.

Various experiments carried out by Natelson *et al* (1984) provided further evidence to support the conclusion that hypothermia was correllative and not causative of gastric stress ulceration.

Although the pathogenesis of stress ulceration is not completely understood, the primary factors involved in such damage to the gastric mucosa are localized mucosal ischemia and an elevated localized hydrogen ion concentration.

In 1977, Guilleman *et al* noted that β-endorphin and ACTH are secreted concommitantly by the pituitary gland in conditions of acute stress. This stimulated much research to determine a role for the endogenous opioid system in stress and it has since been

implicated in many of the biochemical, physiological, and behavioural responses to stress (Adams, 1987; Rodgers and Cooper, 1988). Lishmanov *et al* (1987) have shown that a 4-hour period of stress in rats causes an elevation of β-endorphin levels in the blood. Other hormones characteristic of the *alarm* phase were also present. They also reported that preliminary adaptation of animals to short term stresses led to an increase in basal β-endorphin levels in the plasma. Subsequent exposure of these animals to stress prevented the rise in β-endorphin and weakened other hormonal responses to stress. They concluded that the opioid peptides may have an important role to play in the adaptation to stress.

Exposure to stress has been associated with increased brain levels of opioid peptides (Madden *et al*, 1977) and with a decrease in the number of opioid binding sites in the brain. The latter finding could be due to the presence of competing endogenous ligands (Chance *et al*, 1977) or to receptor down regulation from prolonged exposure of receptors to elevated levels of endogenous ligands (Hnatowich *et al*, 1986).

Enkephalin-like materials are also stored together with catecholamines in adrenal medullary chromaffin cells, and both may be released simultaneously in response to stress (Viveros *et al*, 1979).

Considerable research has been undertaken to determine the role of the opioid drugs in gastric stress pathology, and there are many conflicting reports in the literature on this topic.

Arrigo-Reina and Ferri (1980) showed that exposure of rats to stressful stimuli (restraint, cold [4°C] for 5 hrs) lead to the production of gastric stress ulceration. This effect was exacerbated by naloxone (5mg/kg bw sc). Prior treatment of rats with 10 mg/kg bw morphine showed a significant inhibition of lesion formation. They also observed that FK 33-824, an enkephalin analogue, prevented this stress-induced gastric damage. An interesting observation was the prevention of gastric damage by morphine methyliodide, a compound which does not cross the blood brain barrier, suggesting a possible peripheral action of the

opioids in gastric stress pathology.

Glavin (1985) also observed that various doses of morphine inhibited lesion formation in rats exposed to stress. The opioid antagonist naloxone exacerbated lesion formation, suggesting that the endogenous opioid peptides released under stressful conditions may act to relieve the pathological effects of stress on the gastric mucosa. Glavin *et al* (1986) also observed that chronic treatment with naloxone augmented stress-induced gastric ulceration, and morphine-dependent rats stressed during spontaneous or naloxone precipitated withdrawal presented with severe ulceration as compared to controls.

Similar protective effects of opioids on stress-induced gastric ulceration have been observed by other investigators (Ray *et al*,1988; Arrigo-Reina and Ferri, 1980; Del Tacca *et al*, 1987; Ho *et al*, 1984).

Neuropharmacological data have indicated a significant correllation between activity in the central nucleus of the amygdala and the regulation of gastric mucosal integrity in stress situations (Henke, 1982; 1985). Ray *et al* (1988) have shown that intra-amygdalar injections of endorphins and enkephalins protect against stress ulceration and this effect is antagonized by intra-amygdalar administration of naloxone. They suggest that opioidergic neurons in the central nucleus of the amygdala are involved in the regulation of gastric mucosal integrity in stress, and that sigma and possibly μ-opioid receptors are important in the mediation of this effect.

Conversely, other investigators have observed that opioid drugs exacerbate, and naloxone inhibits lesion formation (Dai and Chan, 1983; Del Tacca *et al*, 1987; Morley *et al*, 1982; Feldman *et al*; 1980).

Other investigators (Canfield and Spencer, 1981) noted no effect of morphine or naloxone on gastric stress ulceration.

The contradiction in the data obtained thus far may arise from differences in drug dose, the type of stress employed, the

chronicity of the stressful challenge, the route of drug administration, and other factors in the experimental design.

Prima facie evidence for an involvement of the pineal gland in stress exists, although the precise nature of its involvement has yet to be elucidated.

Although publications concerned with pineal-adrenocortical interactions appear infrequently, many reports support the view that the pineal gland possibly participates in the regulation of the hypothalamo-pituitary-adrenal axis (HPAA) in rats (Vaughan *et al*, 1972; Herman and Porter, 1980; DeFronzo and Roth, 1972). Studies indicate that the pineal gland may possibly modulate adrenal steroidogenesis under normal and stress conditions. Kinson and colleagues (1967) suggested that the pineal gland has an inhibitory effect on the adrenal cortex. Dickson and Hasty (1972) suggested that the pineal gland secretes a substance that blocks the synthesis and release of ACTH at the level of the hypothalamo-pituitary axis, exerting an inhibitory influence on the adrenal glands. Other investigators suggest that the pineal gland exerts a pituitary mediated inhibition of adrenal activity (DeFronzo and Roth, 1972), and others provide evidence for a possible direct inhibitory action of the pineal gland on the adrenals (Giordano *et al*, 1970).

Ogle and Kitay (1976) have suggested that the pineal gland may directly or indirectly affect ACTH secretion, via oestradiol. They concluded that the pineal gland, together with the ovaries, plays a role in adrenal steroidogenesis.

In vitro studies (Porter and Heiman, 1977) show that a crude aqueous pineal extract inhibits the ACTH-induced release of corticosterone from adrenal cells in culture medium. Porter and Heiman proposed that the aqueous pineal extract contains some substance that may act directly on the adrenal glands to interfere by a yet unknown mechanism with ACTH mediated corticosterone release. They suggest that this substance is not melatonin. These investigators (Heiman and Porter, 1980) later observed that addition of increasing concentrations of ACTH to the culture media did not reverse the inhibitory effect of the pineal extract on

corticosterone release by the adrenal cells. The interaction of ACTH with its receptor is known to activate adenylate cyclase (Garren *et al*, 1971), resulting in a cAMP mediated release of corticosterone from adrenal cells. Heiman and Porter (1980) showed that the addition of dibutyryl cAMP to the culture medium also stimulated corticosterone release; and the pineal extract inhibited this effect. They thus suggested that the active constituent of the crude extract does not compete with ACTH for the same receptor site.

Mehdi and Sandor (1977) explored the effects of melatonin *in vitro* on the biosynthesis of corticosteroids using bovine adrenal cortex. They observed that melatonin significantly (33 - 63%) inhibited the the transformation of radiolabelled progesterone to cortisol, possibly by inhibiting 17- and 21-hydroxylation by adrenal microsomes. Kinetic studies carried out by these investigators seem to support a possible non-competitive inhibition by melatonin of 17- and 21-hydroxylases in bovine adrenal glands.

Subcutaneous and intra-hypothalamic implantation of melatonin results in a significant adrenal atrophy, and a reduction in plasma corticosterone levels in rats (Motta *et al*, 1968). Blinding of rats (with a resultant stimulation of pineal biosynthetic activity) abolishes the normal plasma corticosterone levels (Jacobs, 1974) and this effect can be reversed by pinealectomy. Nir *et al* (1971) have shown that the pineal gland exerts an inhibitory influence on adrenocortical function in rats, and pinealectomy results in increased plasma corticosterone levels. These investigators suggest that an antagonism exists between the pineal gland and the adrenals. However, this evidence is contradictory, and other workers (Niles *et al*, 1977; Gromova *et al*, 1967) have observed a stimulatory effect of melatonin on adrenal activity. Possible reasons for the variation in the results between the various investigators could be differences in the environmental conditions to which the rats were subjected and the time at which the experiments were undertaken.

Rivest *et al*, (1979) studied the effects of the pineal gland on the reactivity of the adrenal cortex to stress in rabbits. The pineal stalk of the rabbits was sectioned (with a resultant sectioning of

the vein of Galen). Presumably, the adrenergic innervation via the nervi conarii was left intact. Six days after the operation, the rabbits were subjected to cold stress (7°C) for two hours. Blood samples were collected through an implanted jugular cannula during cold exposure and for two hours thereafter, and plasma corticosteroid levels were continuously assayed. Exposure to cold resulted in an enhanced release of circulating glucocorticoids, the levels slowly returning to normal on return to the warmer environment. A greater and earlier increase in plasma corticoids was noted in pineal stalk-sectioned rats compared to sham-operated rats. These findings are interesting in that they may reflect an important role for pineal attachment to the diencephalon, and indicate that the endocrine capabilities of the pineal gland may be disrupted by pineal stalk interruption in the rabbit. Further studies on other animals might reveal a possible important role for an intact pineal stalk in the normal functioning of the pineal gland.

Although it has generally been accepted that the role of the pineal gland in stress is mainly anti-stressogenic (Reiter, 1982), controversy surrounds the actual effects of stress on pineal gland biosynthetic activity.

Exposure to stress may increase tryptophan uptake by the pineal gland (Singh *et al*, 1979) and increase pineal adrenaline concentrations (Saavedra *et al*, 1982).

During acute stress, the catecholamines released into the circulation can increase the the activity of pineal NAT in rats (Lynch *et al*, 1973; Parfitt and Klein, 1976), suggesting a possible activation of melatonin synthesis in stress. Vaughan *et al* (1978) measured NAT activity in ether stressed rats, and observed a four fold increase in pineal NAT activity in the light phase. Lynch *et al* (1973) showed that, in rats, melatonin content and NAT activity in the pineal gland increase rapidly in response to physical immobilization and insulin induced hypoglycaemia. In humans however, an intravenous challenge with insulin did not alter the melatonin pattern (Coetzee and Theron, 1988). Lynch *et al* (1973) also observed that destruction of the sympathetic innervation to the pineal gland does not abolish the effect of stress on NAT

activity and pineal melatonin content. They suggested that a possible explanation for this effect could be an action of circulating catecholamines released during stress on supersensitive pineal cells. An interesting observation made by these investigators was that propanolol could block the stress-induced increase in NAT activity, but had no effect on the increased melatonin concentration. It could be suggested that factors influencing pineal blood flow and other intracellular functions may impair melatonin secretion from the pineal gland.

Parfitt and Klein (1976), however, observed only very small increases in stress-induced pineal NAT activity in rats and suggested that the pineal gland has a built in protective mechanism against non-transsynaptic adrenergic stimulation. They propose that the pineal nerve endings act as a sponge to remove catecholamines arising from other areas (eg adrenal glands) and the neuronal uptake mechanisms facilitate the maintenance of a substimulatory concentration of NA in the perivascular space. Thus the pineal gland is protected from stimulation by catecholamines released during stress.

Recent work on the effect of various stressors (cold, hind leg saline injection) on pineal biosynthetic activity has provided paradoxical results. It has been observed that cold (2°C) exposure during the light phase induced a slight but significant ($p < 0.05$) increase in pineal NAT activity without affecting pineal melatonin levels (Tannenbaum *et al*, 1988). Nighttime exposure (2 hrs) to the same stressor caused a 50% reduction in pineal NAT activity without affecting melatonin content. Administration of 1.5 ml of saline into the hind leg of rats led to a depression in pineal melatonin production (Joshi *et al*, 1986), this effect possibly being mediated by the release of corticosterone from the adrenal glands (Troiani *et al*, 1988). These findings are in conflict with many other studies (Lynch *et al*, 1973; Illernova, 1976; Champney *et al*, 1985; Seggie *et al*, 1985; Tannenbaum *et al*, 1987) suggesting an activation of pineal gland activity in response to stressful stimuli. Possible reasons for these differences in findings could be related to the timing of the experiments and the nature of the stressor (mild or intense).

Forced swimming was observed to increase the release of melatonin

from the pineal gland. It has been suggested that this effect could either be a stress response, or a specific response to some aspect of swimming - e.g. exercise (Troiani, 1989). Exercise in itself is recognised as a stressor (Everly and Rosenfeld; 1981). An interesting study was carried out by Oosthuizen (1983) on the effects of physical stress (step climbing at a rate of twelve steps per minute for 240 minutes including rest sessions) on, amongst other parameters, plasma melatonin levels in mineworkers. A steady increase in plasma melatonin was noted until 30 minutes after commencing exercise. No further increase was noted after this time point of melatonin determination, and plasma melatonin levels plateaued until the end of the exercise session (240 minutes). One hour after the exercise session, plasma melatonin levels had returned to normal. Oosthuizen (1983) suggested that melatonin could, together with cortisol and prolactin, possibly be recognized as a stress hormone.

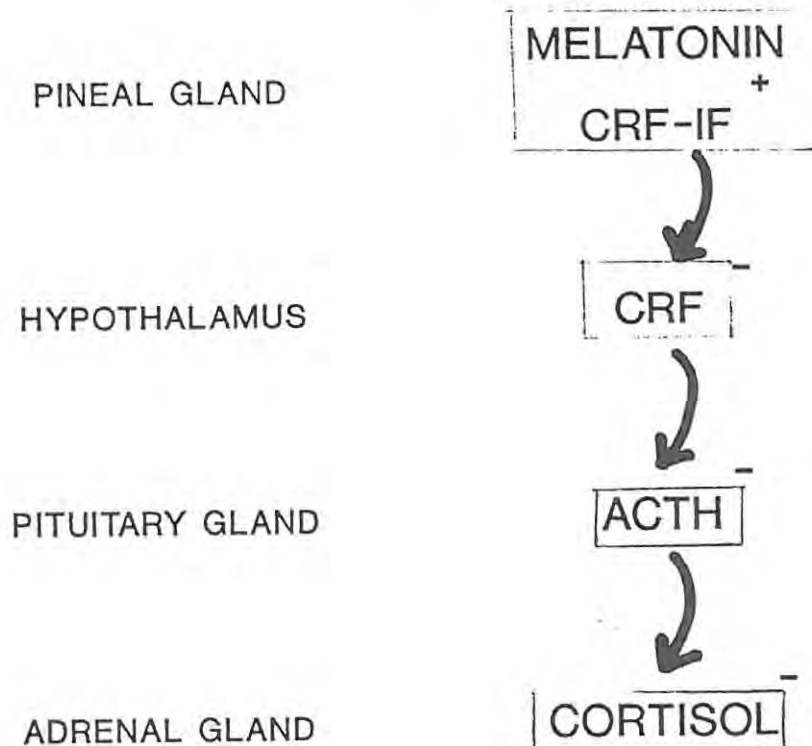
It has been suggested that a relationship exists between the pineal gland and the pituitary adrenal axis in health, endocrine and psychiatric conditions (Wetterberg, 1983). Some patients suffering from Cushing's disease and depressive disorders have disturbed melatonin levels (Carrol, 1982). It has been proposed that low nocturnal melatonin levels may be a genetic trait marker for vulnerability to depression (Wetterberg, 1983). This investigator also suggested that the pineal gland may secrete a factor together with melatonin, inhibiting the release of corticotropin-releasing factor from the hypothalamus (see fig 1.6).

It has previously been suggested that the pineal gland may be involved in feedback inhibition of the hypothalamo-pituitary-axis in stress, due to the ability of melatonin to reverse pinealectomy-induced adrenal hypertrophy (Reiter, 1982).

Exposure to stressful stimuli could trigger neuroendocrine and behavioural compensatory mechanisms to return the body to normality. It could be proposed that the pineal gland reacts to stressful stimuli by releasing substances that facilitate the maintenance of a homeostatic balance in the body. It's generally accepted anti-stressogenic effect, and the previously described efficient uptake mechanism (Parfitt and Klein, 1976) of its

sympathetic neurons, are possible mechanisms whereby the pineal gland may maintain its integrity as a precise photoperiodic responding system.

Fig 1.6: Possible relationship between the pineal gland and the hypothalamic-adrenal axis.



Adapted from Wetterberg, (1983).

CRF = Corticotropin releasing factor; CRF IF = corticotropin releasing factor inhibiting factor; ACTH = adrenocorticotropic hormone.

3 The Pineal Gland and The Opioid System

The pineal gland has been the subject of many investigations for the past few decades, and is a useful experimental model for the monitoring of circadian rhythms.

Research on the relationship between the pineal gland and the opioid system is a comparatively new field of study, and recent progress in this direction suggests that a necessary balance between these two systems of neuromodulation could be important for the maintenance of psychophysical health (Reiter, 1987).

Various investigators are now working on the possible links between these two systems, and it has been suggested that the pineal gland is necessary for opioid substances to exert some of their effects.

To give further credence to an interaction between these two systems, enkephalin-like peptides (Cherdchu *et al*, 1988) and β-endorphin-like immunoreactivity is present in the pineal gland (Vuolteenhao *et al*, 1980) - suggesting that the pineal gland may be regulated in part by specific opioid peptides.

Pineal-opioid research may be regarded to be in its stage of infancy, and as such, no vast scope of literature is available on this subject. The following review will attempt to describe some of the research being undertaken in this field.

The pineal gland and melatonin can affect analgesic activity in rodents. Lakin *et al* (1981) have shown that pinealectomy abolishes the nocturnal increase in morphine-induced analgesia. Winters *et al* (1983) suggested that the diel rhythm in morphine and ketamine-induced analgesia could be due to the diurnal rhythm in melatonin synthesis, and proposed that melatonin triggers the release of pituitary peptides. Sugden (1983) observed analgesic effects in mice.

An age-dependent reduction in the nocturnal peak of serum and pineal melatonin has been observed in rodents (Reiter, 1987). Kavaliers *et al* (1983) undertook a study to determine the effects of ageing on the diel rhythms of murine analgesia and to determine

if the pineal gland mediates these changes. They observed an age-related decline in the rhythm of morphine analgesia in mice, and these effects were modulated by the pineal gland. These investigators suggest that age related declines in pineal activity could affect and disrupt various other neuroendocrine transmitter systems that possibly affect opioid activity.

Substantial evidence exists for opioid involvement in feeding behaviour. Administration of opioids stimulate food intake in many animals (Rodgers and Cooper, 1988). Kavaliers *et al* (1984) observed that morphine increases the nighttime ingestion of food, and this effect is depressed but treatments that inhibit nocturnal pineal gland activity (brief light pulse or benserazide (50 mg/kg) treatment). These findings suggest a link between the pineal gland and the opioid system in the modulation of feeding behaviour.

Lowenstein *et al* (1984) observed that naloxone (10 mg/kg) reduced the nocturnal increase in rat pineal melatonin concentrations *in vivo*. They thus suggested that an opioid synapse modulates the effect of light on pineal activity.

Kumar *et al* (1982) noted that pinealectomy eliminates the daily fluctuation in met-enkephalin levels in the preoptic area of the hypothalamus. Using electrophysiological techniques, Dafny and Burks (1976) noted that morphine affects the amplitude of sensory-evoked potentials in the pineal body and several brain nuclei. These investigators suggested that morphine may interact with neuronal 5HT associated with sympathetic nerves innervating the pineal gland (Bertler *et al*,1964) or at other neural sites interacting with the pineal gland. They also suggested (Burks and Dafny, 1977) that this influence of morphine on the pineal body could influence pineal melatonin, and thereby exert effects on the hypothalamus. These investigators are thus of the opinion that the pineal gland may determine some of the actions of morphine on the hypothalamus.

Esposti *et al* (1988) have suggested that an important relationship could possibly exist between the pineal gland and the opioid system in the modulation of several psycho-neuroendocrine functions.

Melatonin is reported to influence several neurotransmitters, including enhancement of brain 5HT and GABA content (Anton Tay *et al*, 1970) and an inhibition of DA release (Zisapel and Laudon, 1982). Esposti *et al* (1988) undertook an investigation to determine whether melatonin influences the activity of the opioid system. Melatonin and naloxone were administered to healthy volunteers and serum levels of GH and LH were measured. The results showed that naloxone induces an increase in LH secretion, and this effect is reversed by melatonin administration. Also, melatonin stimulated GH secretion, and naloxone completely inhibited this effect. Naloxone administered alone had no effect on GH release. These observations suggest that melatonin's endocrine stimulating action on GH secretion requires opioid modulation; and that this pineal hormone may be involved in the opioid modulation of gonadotropin release.

It could therefore be proposed that some of the actions of melatonin may require at least in part an interaction of the opioid system, and also, it is likely that the opioid peptides require a participation of the pineal gland through a modulation of melatonin release to manifest some of their actions.

Lissoni *et al* (1986) demonstrated an increase in melatonin levels after administration of FK 33-854 - a met-enkephalin analogue - to healthy, normal human subjects. Naloxone partially blocked this stimulatory effect on melatonin secretion. These investigators also noted that melatonin administration (0.2 mg/kg bw at 14h00) induces a significant decrease in plasma β-endorphin levels. They thus suggested that a feedback mechanism with a stimulatory action of the opioids on the pineal gland, and an inhibitory effect of the pineal gland on the opioid system could possibly exist.

In vitro studies however, have suggested that melatonin may have a stimulatory effect on opioid peptide release from anterior pituitary cells (Abou-Samra *et al*, 1985).

Work done in the laboratories of Fraschini *et al* (cited in Reiter, 1987) on heroin drug addicts showed that a "street dose" of heroin (0.5 g iv) induced a significant decrease in melatonin serum levels, whereas a remarkable increase in serum melatonin levels was noted after the administration of naloxone (0.4 mg iv) to these

addicts. Administration of methadone (10mg iv) to these naloxone treated addicts gradually brought melatonin levels back to normal serum values.

In normal healthy patients, methadone (10 mg iv) had no significant effect on melatonin serum levels, however, a significant decrease was noted in addict patients. To the contrary, it was found that another opioid, FK 33-854 (0.3 mg/50 ml infusion) provoked an increase in melatonin levels in healthy patients, having no effect at all on addict patients.

These investigators then examined the effects of melatonin as maintenance therapy . Groups of patients who had been drug addicts for 4 - 5 years and were being maintained on approximately 2.5 g/day "street" heroin, were injected either with naloxone (o.4 mg iv) or an intramusclar dose of melatonin (40 mg) prior to naloxone administration at 9h00 (two groups) and 15h00 (another two groups). Melatonin did not seem to block the naloxone-induced abstinence syndrome at either time point in heroin addicts.

It was thus concluded that chronic administration of opioid substances may in some way induce changes in pineal function, and thus alter a possible equilibrium existing between these two systems. The clinical usefulness of melatonin therapy for opioid drug addicts requires further research.

Reports have indicated that both the pineal gland (Maestroni *et al*, 1987; Jankovich *et al*, 1970) and the opioid system (Fischer and Falke, 1984) may regulate cell proliferation and immune responses; and both systems are involved in the control of tumor growth (Buswell, 1975; Lewis *et al*, 1983). Esposti *et al* (1988) carried out studies on groups of patients suffering from early or advanced cancer in order to investigate the relationship between the pineal gland and the opioid system in human neoplasms. They observed that metastatic patients had significantly lower β-endorphin:melatonin ratios than that observed in normal patients. However, no difference in this ratio was observed in controls and cancer patients having no metastatic lesions.

They also observed (1988a) that melatonin levels in healthy human

beings were increased after FK 33-854 administration (0.3 mg/20 ml iv infusion. In patients with cancer, however, FK 33-854 induced increases in serum melatonin were noted in only 4 cases, while 5 cancer patients showed no change in melatonin levels and another 4 patients showed decreased melatonin levels.

They thus suggest that an altered pineal-opioid relationship might prevail in patients with cancer, and suggest that these alterations could represent a risk factor for the development of metastases in cancer patients. It would therefore appear that a balanced relationship between the pineal gland and the opioid system could possibly result in some anti-neoplastic activity; and that a correlation exists between an altered pineal-opioid relationship and the clinical course of cancer.

Maestroni and colleagues (1987) have proposed that lymphocyte products act as messengers to the CNS, due to the ability of antigens to induce rapid hormonal responses early after injection into mice. They observed that the circadian release of melatonin by the pineal gland modulates immune reactivity; and that exogenous melatonin(10 mg/kg) had a powerful immunoaugmenting effect in mice. Furthermore, these investigators also observed that melatonin (2 mg/kg bw) fully antagonized the immunosuppressive effects of corticosterone and partially antagonized that of cyclophosphamide in mice.

Opioid receptors have been localized on leukocytes, and opioid peptides may possibly modulate immune functions (Fischer and Falke, 1984). With the proposed functional link between the opioid system and the pineal gland (Reiter, 1987) in mind, Maestroni *et al* (1987) investigated the effects of melatonin on the immune system in mice treated with naltrexone (7 mg/kg bw). They observed that the opioid antagonist had no effect on the primary antigen response (in this case sheep red blood cells were used) when administered alone, but induced a complete antagonism of melatonin's immuno-augmenting effect. This suggested that melatonin influences humoral and cellular immune responses by activating the endogenous opioid system; and that no naltrexone-sensitive, opioidergic tone normally controls antibody production.

These findings are of importance if one notes that low levels of melatonin have been associated with affective disorders, in which an increased incidence of infection, autoimmunity and cancer has been reported (Maestroni, 1977).

Also, adrenal corticosteroids have been reported to mediate the negative influences exerted by stress on the immune system. During stress, opioid peptides are secreted concomitantly with Adrenocorticotopic Hormone (Guilleman *et al*, 1977) and melatonin production is enhanced (Lynch *et al*, 1977).

These findings support a connection between the pineal gland and the neuroendocrine mechanisms mediating the effects of stress. Maestroni *et al* (1988) observed that the exogenous administration of melatonin (20 mg/kg sc for 4 days) counteracts the effects of stress on thymus weight. Naltrexone (1 mg/kg) administered simultaneously, antagonized this response. These investigators thus concluded that melatonin may have the physiological role of restoring the ability of an "exhausted" opioid system to co-ordinate the stress response.

From the foregoing discussion, it would seem apparent that a relationship could exist between the pineal gland and the opioid system. This study was thus undertaken to observe the effects of the opioid agents on the pineal gland *in vitro*, and to observe if a possible relationship exists between the pineal gland and the opioid system in the modulation of the stress response and nociception.

CHAPTER TWO

PINEAL ORGAN CULTURE STUDIES

2.1 INTRODUCTION

The mammalian pineal gland is highly vascularized, and, second only to the kidney, is the most richly perfused organ. Various drugs are capable of affecting pineal indole synthesis and metabolism, and 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 manipulations *in vitro.*

The organ culture system is one such technique. This technique originated in the laboratories of Strangeways and Fell in 1926, and has been adapted and modified to facilitate keeping an organ, or parts of an organ, alive *in vitro,* (Trowell, 1959) as well as preventing growth and redifferentiation of cells.

Many researchers have successfully developed organ culture techniques for the pineal gland. (Chan and Ebadi,1981; Wainright and Wainright, 1981, Alphs *et al*, 1980, Rowe and Parr, 1979, Klein and Notides, 1969, Axelrod *et al*, 1969, Shein and Wurtman, 1969). The viability of rat pineal glands can be maintained for up to 6 days if they are incubated at 37°C in a suitably aerated culture medium (Klein, 1969).

Most techniques involve incubation of the pineal gland in the presence of a radioactive precursor (tryptophan, 5 hydroxy tryptophan or serotonin) in a suitable culture medium. The pineal gland takes up the radioactive precursor, incorporates it as part of its normal biosynthetic pathway, synthesizes radioactive metabolites and releases them into the culture medium. The metabolites are then separated and quantitated using, amongst other methods, bi-dimensional thin layer chromatography and liquid scintillometry.

The following sections will describe the organ culture system used, the separation of indoles using bi-dimensional thin layer

chromatography, the measurement of the radioactive [^{14}C] serotonin metabolites formed, and the use of the system to determine the effects of drug manipulations.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals and Reagents

BGJb medium (Fitton- Jackson modification) was obtained from Gibco Europe. Radioactive 5-Hydroxy[side-chain-2-^{14}C] tryptamine creatinine sulphate, (specific activity 57mCi/mmol) was purchased from Amersham International, Weir Organisation, South Africa. The antibiotics benzyl penicillin and streptomycin were purchased from Hoechst S.A., and amphoterecin B from Squibb Laboratories, South Africa. All the standard indoleamines, melatonin, NAS and hydroxy and methoxy tryptophols were purchased from Sigma Chemical Co., USA.

Aluminium backed thin layer chromatography plates ($60F_{254}$ coated with silica gel 0.2mm) were purchased from Merck.

2.2.2 Animals

Inbred male rats of the Wistar strain were used in all experiments. Female rats were not included in the experiments because of the variation in [^{14}C] serotonin metabolism noted in female rats in different stages of the estrus cycle (Daya, 1976). The male rats weighed between 200-300g at the time of sacrifice. They were housed four or five per cage in a well ventilated room with an automatically regulated lighting cycle of LD 12:12.(lights on 6h00-18h00) Rats were allowed access to a standard diet and water *ad libitum.*

Rats were killed by neck fracture and then decapitated. A blade of a sharp pair of scissors was inserted into the foramen magnum and the skull was cut laterally on both sides to the area just behind the orbit. A clean 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. A curved forceps was then inserted into this groove, and the pineal gland easily removed. Once perfected, the entire process took no longer than 30 seconds.

2.2.3 Organ Culture

The pineal glands were rapidly removed after decapitation. Adhering tissue was removed and the pineal stalk, if present, was excised. The glands were individually placed in sterile culture tubes containing 52 μl culture medium. The culture medium used was a Fitton-Jackson modification of the BGJb medium (see table 2.1 for list of contents and composition) supplemented with the following antibiotics: Benzyl penicillin 0.06mg/ml, Streptomycin sulphate o.1mg/ml, and Amphoterecin B 2.5 ug/ml. An 8ul aliquot of the precursor subtrate(side,chain-2-[^{14}C] serotonin creatinine sulphate, specific activity 57mCi/mmol) was added to each tube. The pineal glands floated freely in this medium. The tubes were aerated with carbogen (95% CO_2/5% O_2) until saturated, and then tightly sealed with parafilm. The pineal glands were then incubated in the dark at 37°C in a Forma-Scientifica 3028 incubator for varying lengths of time as required for the experiment being carried out. Blanks containing no tissue were simultaneously incubated.

Table 2.1: COMPOSITION OF THE FITTON-JACKSON MODIFICATION OF THE BGJb MEDIUM USED FOR PINEAL GLAND ORGAN CULTURE STUDIES.

CONTENTS	CONCENTRATION IN mg/L
Amino Acids	
L-Alanine	250.00
L-Arginine	175.00
L-Aspartic Acid	150.00
L-Cysteine HCl	90.00
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
DL-Valine	65.00
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	0.40

Vitamin B_2	0.04
Inorganic salts	
Dihydroden 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
Other Components	
Calcium Lactate	555.00
Glucose	10 000.00
Phenol Red	20.00
Sodium Acetate	50.00
Supplements	
Benzyl Penicillin	200 mg/L
Streptomycin Sulphate	100 units/ml
Amphoterecin B	2.5 μg/ml

2.2.4 Bi-dimensionsional TLC Analysis of Pineal Indoles

After the necessary incubation time, the incubation was terminated by removing the pineal gland from the culture medium. A 10 μl aliquot of the culture medium was spotted onto a TLC plate (silica gel $60F_{254}$, 10x10 cm, Merck). A 10 μl solution of non-radioactive pineal indole standards (see figure 2.1) was then spotted on top of the culture medium spot, and these served to localise the various radioactive spots after development. To make up the standard solution, 1mg of each of the 8 standard indoles used was dissolved in 5ml of absolute ethanol, resulting in a final concentration of 2ug/10ul of each standard. Earlier methods suggested the addition of ascorbic acid into the standard solution to protect those substances sensitive to oxidation from metabolic breakdown (Klein and Notides, 1969; Daya, 1976). However, ascorbic acid is known to catalyse

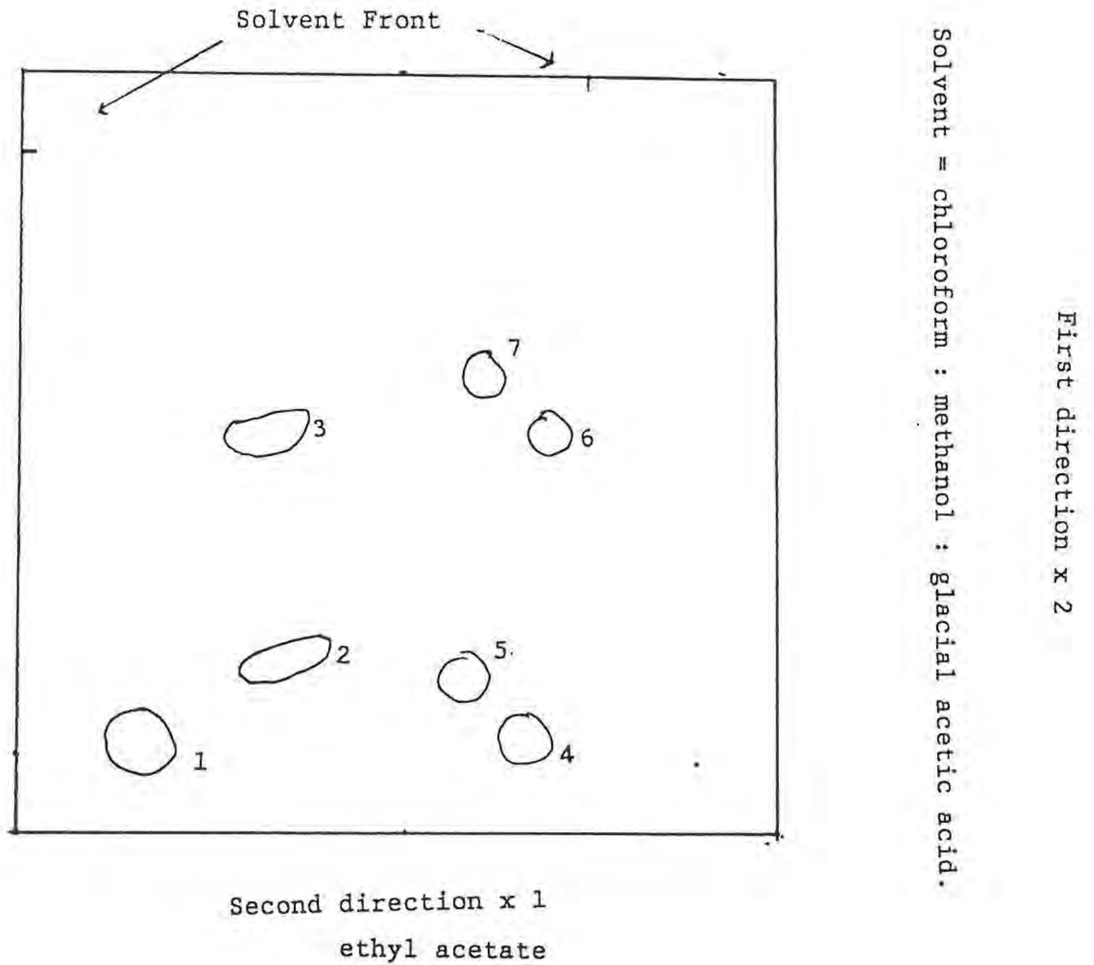
aromatic hydroxylation (Udenfriend *et al*, 1954), and its protective capacity in this regard is questionable. When stored in an amber vial at -20°C, no degradation of the standard solution was noticeable 3 months after preparation. Thus the antioxidant was not used in this study. A gentle steady stream of nitrogen was always used to dry the spots, to prevent atmospheric degradation of the metabolites. The spots were always less than 0.5cm in diameter.

A bi-dimensional system was then used to develop the plates. The plates were first placed in a thin layer chromatography tank at room temperature in chloroform:methanol:glacial acetic acid (97:3:1), and the solvent front was allowed to run 9cm up the plate. The plate was then removed, quickly dried under nitrogen,and returned to the same developing tank and run in the same direction again. After drying under nitrogen, the plate was developed in ethyl acetate at right angles to the first run. The solvent front was allowed to run 7cm up the plate. The plate was then dried under nitrogen. Once developed, the plates could not be stored, and exposure to air was kept at a minimum to prevent oxidation of the sensitive indoles.

The plates were then sprayed with Van Urk's reagent (1g of 4-dimethylaminobenzaldehyde dissolved in 50ml of 25% hydrochloric acid, to which 50ml ethanol is added) so as to visualize the spots. The entire procedure took approximately two hours. Test plates were always run prior to spotting so as to ensure that the system was working well.

The spots were then marked,and carefully cut off the aluminium backed TLC plates. They were immediately placed in a scintillation vial containing 1ml of absolute ethanol to solubilise the indoles. After 1 hour, 3ml of scintillation cocktail (Beckman) was added to each vial. The vials were vortexed for 20 seconds each, and placed in a Beckman LS 3150 T scintillation counter. The radioactivity in dpm's was counted for 5 minutes per vial. The ESCR (external standard channel ratio) was used establish the quenching curve.

Fig 2.1: Illustration of a chromatogram after indole seperation using bi-dimensional thin layer chromatography.



1 = hydroxytryptophol and methoxytryptophol

2 = N-acetylserotonin

3 = melatonin

4 = hydroxyindoleacetic acid

5 = hydroxytryptophol

6 = methoxyindole acetic acid

7 = methoxytryptophol

2.2.5 Statistical Analysis of Results

All data was statistically analyzed using oneway analysis of variance followed by Scheffe's test for multiple range comparisons, or the Students-*t*-test. Results are expressed as means ± SEM.

2.3 RESULTS

The solvent system used for the separation of the indoles (chloroform:methanol:acetic acid, 7:3:1 followed ethyl acetate) was found to be very efficient.

After the plate had been developed twice in the first solvent, all the standards were not separated from each other. Melatonin and 5-MIAA appeared as a single spot, as was also observed (under u.v. light) for NAS and 5-HTOH. Development in the second solvent separated these compounds (see fig 2.1). Good separation of the metabolites was obtained in all cases. The original starting spot gave the highest counts, because serotonin and methoxytryptamine did not migrate from this position (see Figure 2.1).

2.4 DISCUSSION

The TLC method described here has been used in our laboratory previously (Daya, 1976; Skene, 1985), and is the original method developed by Klein and Notides (1969). Other workers have attempted to use various other solvent systems (Skene,1985; Morton,1982,), and the above method has been found to give good separation of all the standards (Daya, 1976; Skene,1985). In view of its relative success, this method was thus adopted for use in all future experiments.

2.5 ADAPTABILITY OF THIS ORGAN CULTURE SYSTEM FOR USE WITH OTHER TISSUE OR GLANDS PRODUCING MELATONIN.

2.5.1 Introduction

The pineal gland is not the only site of melatonin synthesis in the body. Immunohistochemical techniques have been used to localise other nonpineal sites of melatonin synthesis (Bubenik *et al*, 1977). Thus far, these studies support the localization of melatonin in the intestine, retina and the Harderian gland (Bubenik *et al*, 1977).

2.5.1.1 The Retina

The isolated retina has been suggested as a model of the CNS (Nowak, 1988) and could thus serve as a useful tool for tissue experimentation. All the major neurotransmitters found in the brain and the spinal chord have also been found in the retina, with the exception of NA (Bonting, 1976).

The features that make the retina an attractive model for *in vitro* experimentation include :

1) Ease of dissection: The retina is localised extracranially, and is strong enough to remain intact throughout the dissecton procedure.

2) Viability: The retina maintains nearly all of its physiological activity for several hours after dissection. It receives its nutrients by diffusion from capillary networks on either surface.

3) Drug treatments: These can be introduced directly onto the intact retinal cells *in vitro*.

Nowak (1976) has explained that various pharmacological applications used for studying synaptic mechanisms in brain tissue may be applied to retina equally well. This is due to the similarity between the retinal and brain neurotransmitter systems, eg the dopaminergic, cholinergic, GABA-ergic, serotoninergic and glycinergic systems.

Cardinali and Rosner, (1971), were the first investigators to localize the enzyme HIOMT in the retina of the rat, and thus provided the initial evidence that melatonin was produced by the retina of mammals. They suggested that further investigations were necessary to determine the significance of the biosynthesis of melatonin by the retina.

Dubocovich (1983) observed that melatonin potently inhibited the stimulated release of dopamine from the retina, possibly acting via specific melatonin receptors. It has thus been suggested that the retina could also be used as a model tissue for investigations into the nature of melatonin receptors, as well as to test for specific melatonin antagonists.

2.5.1.2 The Harderian Gland

The exact function of the Harderian gland in mammals has yet to be determined.

The Harderian gland is one of the extrapineal sites of melatonin synthesis (Cardinali and Wurtman, 1972), and is thought to contribute to the plasma concentrations of melatonin observed in certain pinealectomized animals.

NAS is the immediate precursor of melatonin within the pineal gland, and is also synthesized in the Harderian gland. The localisation of the enzyme HIOMT is highly limited in mammals. It is found in high concentrations in the pineal gland and in substantially lower concentrations in the retina, intestine and

Harderian gland (Reiter, 1981).

2.5.2 Experiments

The organ culture system described in this section is a reliable method to use for the incubation of pineal glands so as to keep them viable for long periods.

Experiments were undertaken to evaluate the organ culture system described here, for its ability to keep other melatonin-synthesizing tissue viable.

2.5.3 Materials and Methods

2.5.3.1 Bovine retina

Bovine eyes were removed from sockets and were transported in a buffered solution on ice from the local abbatoir to the laboratory. Retinas were then carefully removed from the eyeball and stripped of all adhering tissue. Pieces of retina were weighed, and then individually transferred to culture tubes containing the incubation medium as previously described (section 2.2.3).

The incubation medium was then gassed with carbogen (95% CO_2: 5% O_2) and the culture tubes tightly sealed. The retinas floated freely in the incubation medium, which was then incubated for 8 hrs as previously described (section 2.2.3).

The incubation was terminated by removing the retina from the culture tube. A 10 µl aliquot of culture medium was then spotted onto a TLC plate and assayed as previously described (section 2.2.4).

2.5.3.2 Harderian glands

The Harderian gland is found in the cavity just below the lower eyelid in the rat (see fig 2.2). Initially, localization of the Harderian gland proved difficult. Once localised, it was easily

removed by slitting the sides of the eye, inserting a forceps into the lower cavity, and drawing the gland out on the forceps. The pieces were weighed, and individually transferred to culture tubes containing the incubation medium (see section 2.2.2).

The Harderian glands were then treated as previously described (Section 2.2.3). After an 8 hr incubation time, the reaction was stopped by removing the pineal gland, and the culture medium (10 μl) was assayed as described earlier (Section 2.2.4).

In all the experiments described here, blanks containing no tissue were simultaneously incubated.

2.5.4 Results

The following table (2.2) expresses the results obtained indicating that the bovine retina is capable of producing ^{14}C-melatonin from $^{14}[C]$-serotonin in the culture medium.

TABLE 2.2 INDOLE PRODUCTION BY BOVINE RETINA

NAS	1146.56 ± 332.73
MEL	313.03 ± 50.70
HIAA	11802.20 ± 1987.83
HTOH	1188.04 ± 551.12
MIAA + MTOH	250.03 ± 25.83

All results are expressed as total ^{14}C-serotonin metabolised in dpm's/ 10 μl incubation medium/ retina/ 8hr incubation. (means ± SEM)

The Harderian gland of the rat also synthesized melatonin via the uptake of radioactive ^{14}C-serotonin from the culture medium. The following table describes the results obtained from this experiment.

TABLE 2.3 INDOLE PRODUCTION BY THE HARDERIAN GLAND IN VITRO

NAS	2170.55 ± 336.73
MEL	309.01 ± 50.74
HIAA	10807.10 ± 1987.83
HTOH	1188.04 ± 551.12
MIAA + MTOH	250.03 ± 25.83

All results are expressed as total ^{14}C-serotonin metabolised in dpm's / 10 µl culture medium/ mg wet tissue/8 hr incubation. (means ± SEM)

The levels of indoles produced from the ^{14}C-serotonin in the culture medium were much lower than those produced by the pineal gland (see table 2.4).

2.5.5 Discussion

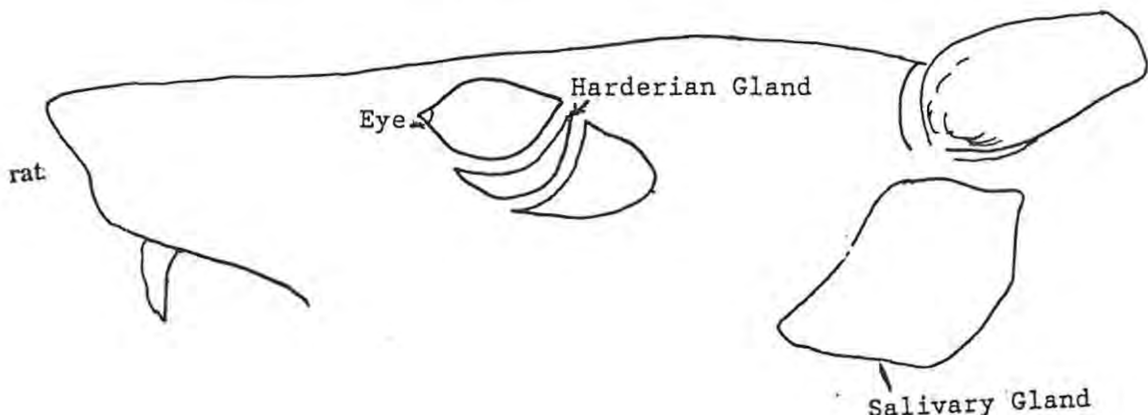
The results obtained from these experiments verify previous reports indicating the existence of extra-pineal sites of melatonin production.

The results showing a presence of melatonin in the bovine retina indicate that this tissue has the ability to produce melatonin *in situ*. It is therefore possible to suggest that this tissue has the ability to synthesize melatonin rather than obtaining it from the circulating pool of melatonin (in plasma) originating from the pineal gland. The Harderian gland was also shown to produce melatonin via the same pathway.

The organ culture system used in our laboratory was thus found to be adequate for the measurement of indole production at sites other than the pineal gland. The indole levels measured in the pineal gland were, however, much higher than those measured in the retina or the Harderian gland (See table 2.4).

The ease of dissection and the ability to adequately stimulate the melatonin biosynthetic pathway in the pineal gland, as well as the high levels of indoles produced by the pineal gland in organ culture (see table 2.4), indicated that the pineal gland was the most suitable melatonin-synthesizing system to be investigated.

Fig 2.2: Diagram showing a rat Harderian Gland.



2.6 EXPERIMENTS

2.6.1 EXPERIMENT 1 : ANALYSIS OF THE [^{14}C] INDOLE LEVELS IN THE PINEAL GLAND AND THE CULTURE MEDIUM.

2.6.1.1 Introduction

The pineal gland releases its hormone melatonin on production, possibly indicating that it is unable to store the melatonin produced. Thus, the amount of radioactive metabolites in the culture medium of a pineal organ culture system would necessarily have to be greater than that within the pineal gland itself.

Some investigators have assayed the culture medium, (Daya,1976, Morton,1982) while others have assayed a mixture of culture medium and pineal homogenate, (Balemans *et al,* 1979) or pineal homogenate only (Voisin *et al,* 1983).

This experiment was thus undertaken to compare the levels of the pineal [^{14}C] indoles present in the pineal gland itself with those present in the culture medium after an 8 hour incubation time.

2.6.1.2 Materials and Methods

Pineal glands (n = 4) were removed and cultured as previously described. (Sections 2.2.1 and 2.2.2) After incubation, (24 hrs), a 10 μl aliquot of culture medium was removed from each tube, spotted onto a TLC plate and treated as described. (Section 2.2.4)

The pineal glands were removed and homogenised individually in 60 μl of culture medium. A 10 μl aliquot of the culture medium was spotted onto a TLC plate and separated and quantitated as above. (Section 2.2.4)

2.6.1.3 Results

The results obtained from this experiment are shown in table 2.4. Analysis of these results shows that most of the indoles formed from the uptake of [^{14}C] serotonin are released into the culture medium, with very little being present in the pineal gland itself.

Table 2.4 COMPARISON OF THE INDOLE LEVELS IN THE CULTURE MEDIUM AND THE PINEAL GLAND AFTER A 24 HR INCUBATION PERIOD.

	CULTURE MEDIUM	PINEAL GLAND
NAS	4421.42 ± 795.19	194.01 ± 7.009
MEL	2296.53 ± 415.77	216.01 ± 35.27
HIAA	18743.6 ± 5468.17	3720.29 ± 226.46
HTOH	5042.55 ± 1239.31	1781.49 ± 82.06
MIAA	100.24 ± 6.10	19.24 ± 7.89
MTOH	57.80 ± 3.15	7.01 ± 5.66

All results are expressed as Total ^{14}C-serotonin metabolised in dpm's/ 10 μl culture medium/pineal gland /24hr incubation (means ± SEM.)

2.6.1.4 Discussion

The results show that most of the radioactive indoles that are synthesized by the pineal gland are released into the culture medium. Using a solvent extraction method, Wurtman *et al,* observed that the concentration of radioactive indoles remaining in the pineal gland after a 48hr incubation time was negligible,indicating that almost all of the compounds formed by the pineal gland are released into the culture medium during incubation. This was supported by Klein and Rowe (1970), who observed that 95% of the melatonin synthesised by the pineal gland after 24 hrs incubation in organ culture is released into the culture medium.

In all the experiments carried out in this study, the culture medium was analysed for its contents of [^{14}C]-indoles produced by the pineal gland.

2.6.2 EXPERIMENT 2 : THE EFFECT OF ISOPRENALINE ON PINEAL GLAND INDOLE PRODUCTION IN ORGAN CULTURE.

2.6.2.1 Introduction

The pineal gland is innervated by postganglionic sympathetic neurons arising from the superior cervical ganglia, releasing NA in the dark phase which activates the β_1 adrenergic receptors. Stimulation of the β receptor in turn activates adenylate cyclase, and via cyclic-AMP induces SNAT, thus activating the melatonin biosynthetic pathway (Axelrod, 1974).

The pineal gland thus is invariably susceptible to the actions of other β_1 adrenergic receptor agonists, eg isoprenaline. Wurtman *et al* (1968) have shown that the exposure of rat pineal glands to NA in organ culture causes a significant increase in melatonin production.

The following experiment was carried out to determine the concentration of isoprenaline at which the pineal gland is able to maximally stimulate melatonin production.

2.6.2.2 Materials and Methods

Rats were killed by neck fracture and then decapitated. Pineal glands (n = 4) were removed as previously described (section 2.2.2).

Pineal glands were then incubated for 8 hours in culture medium containing 1 μM, 10 μM, and 100 μM (-)-isoprenaline HCl (Sigma) and treated as before (section 2.2.3). After incubation, pineal glands were removed and 10 μl aliquots of culture medium were analysed as previously described (section 2.2.4).

2.6.2.3 Results

Figure 2.2 describes the results obtained from the above experiment. After an 8 hour incubation period in the presence of 1-isoprenaline HCl, an increase in the amount of N-acetylated products formed was noted. This increase was not significantly different to control values when the pineal glands were incubated in the presence of 1 μM isoprenaline. However, incubation in 10 μM and 100 μM of the β-adrenergic agonist led to a significant increase in the total amount of NAS + melatonin formed when compared to control values. Maximal stimulation was noted at the 10 μM concentration, this increase not being significantly different to that at the 100 μM concentration of isoprenaline.

2.6.2.4 Discussion

Adrenergic β-receptor agonists such as isoprenaline are known to increase the synthesis of pineal NAS and melatonin, (Axelrod *et al*, 1969), principally by stimulating cyclic AMP production via the activation of adenylate cyclase (Axelrod, 1974). In a 24 hour profile on the effect of isoprenaline on [^{14}C]-serotonin metabolism by the pineal gland in organ culture, Banoo *et al* (1987) noted significant stimulation of melatonin and NAS production by the pineal gland within 4 hrs of incubation, at a concentration of 10 μM isoprenaline.

These workers also observed that after 16hrs of incubation, NAS and melatonin production were approximately 10-fold greater in isoprenaline treated pineals (10 μM) than in untreated controls.

Figure 2.2 shows maximal stimulation of pineal NAS and melatonin production when incubated in the presence of 10 μM isoprenaline. This confirms that 10 μM is the optimum concentration of isoprenaline in which to incubate the pineal glands so as to achieve maximal stimulation of pineal NAS and melatonin production.

Although an 8 hour incubation period is not optimal (Banoo *et al*, 1987), significant (6-fold) stimulation of methoxyindole production is observed after this time.

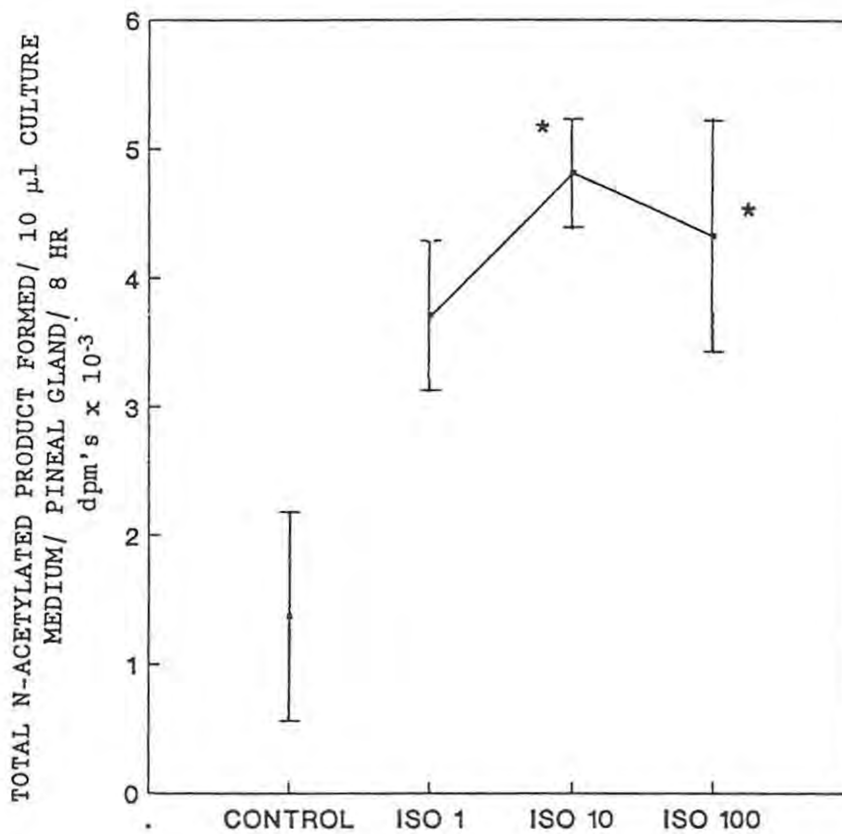


Fig 2.2: Isoprenaline (10^{-5} M and 10^{-4} M)accelerates the production of [^{14}C]-NAS and [^{14}C]-melatonin from [^{14}C-serotonin by the pineal gland *in vitro.*

n = 5, $p < o.05$

* = significantly different to control

The lack of further increases in methoxyindole production above the 10 μM concentration of isoprenaline is probably due to the fact that the 10 μM concentration is sufficient to stimulate the β_1-adrenergic receptors maximally, and addition of more agonist (as in the 100 μM concentration) will not further stimulate the pineal gland to produce more N-acetylated products.

In all subsequent studies where stimulation of pineal indole production was necessary, 10 μM isoprenaline was therefore added to the culture medium, and incubation time was usually kept to 8 hours.

2.6.3 EXPERIMENT 3 : THE EFFECT OF MORPHINE AND METHADONE ON PINEAL GLAND INDOLE METABOLISM IN VITRO.

2.6.3.1 Introduction

The pineal gland is implicated in the modulation of at least some of the actions of the opiates (Reiter, 1987). Dafny and Burks (1977), using electrophysiological studies, suggested that the pineal gland may be involved in the determination of the action of morphine on the hypothalamus.

Recent reports also suggest that opioid substances are involved in the modulation of melatonin release by the pineal gland. In a clinical study on the relationship between the pineal gland and the opioid system, Lissoni *et al* (1986) showed that administration of FK 33-824, a met enkephalin analogue, resulted in an increase of circulating melatonin levels. Lakin *et al* (1981) have suggested that the pineal gland modulates the enhanced nocturnal analgesia seen in mice after treatment with morphine. Morphine administration has also been found to reduce the nocturnal activity of SNAT (Zatz and Browenstein, 1979) in rats. Small doses of morphine (1-3 mg/kg bw ip) have been observed to increase plasma melatonin concentrations in the rat, suggestive of an activation of the melatonin biosynthetic pathway in the pineal gland (Esposti *et al*, 1984).

The mechanism of this action has yet to be elucidated. It is known that the development of tolerance and dependence to morphine in the rat is associated with a concomitant acceleration of serotonin synthesis in the brain (Way *et al*, (1968)). Theoretically, a direct or indirect action of morphine on serotonin synthesis could be postulated. Morphine could modify the activity of tryptophan hydroxylase and aromatic l-amino acid decarboxylase, which transform tryptophan to serotonin (direct), or (indirectly) alter the physiological activity or excitability of serotonin containing neurons.

Tryptophan hydroxylase and aromatic l-amino acid decarboxylase (enzymes catalyzing serotonin synthesis) are found in the pineal gland of the rat, and do not appear to differ from these same enzymes in the brain (Shein *et al*, 1970). Thus if morphine affects serotonin metabolism in the pineal gland, it could also affect the production of other pineal substances.

The following experiments were carried out in order to determine whether the opiates were (in any way) capable of modifying the production of indoles by the pineal gland *in vitro.*

2.6.3.2 Materials and Methods

Male rats were killed by neck fracture, and pineal glands (n=5) were removed as previously described (section 2.2.2), and incubated in culture medium containing 10 μM morphine HCl, 10 μM methadone HCl, or culture medium only, for 8 hours. The incubation was terminated by removing the pineal glands from the culture medium, and an aliquot of the culture medium was analyzed as previously described (section 2.2.4).

2.6.3.3 Results

The results obtained from this experiment indicate that the opiates have no effect on [^{14}C] serotonin metabolism by the pineal gland *in vitro,* after an 8 hour incubation period.

The indoleamine concentrations do not differ significantly between the treatment and the control groups.(See figures 2.3 to 2.8)

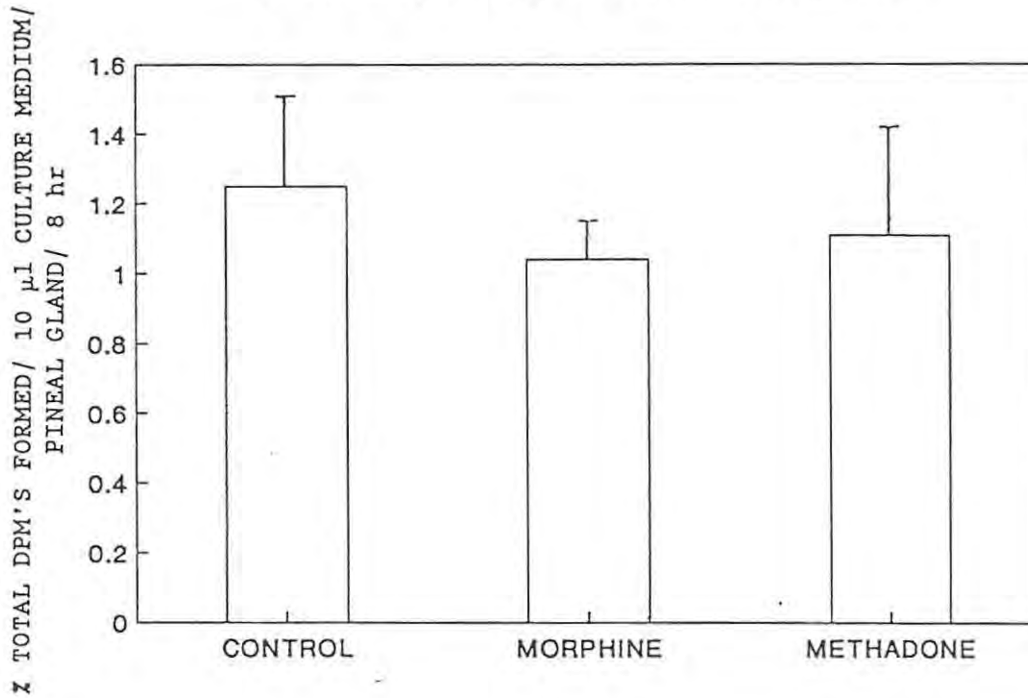


Fig 2.3: The amount of [^{14}C]-NAS released into the culture medium by the pineal gland after an 8 hr incubation time in the presence of opioids is not significantly different to that of the control.

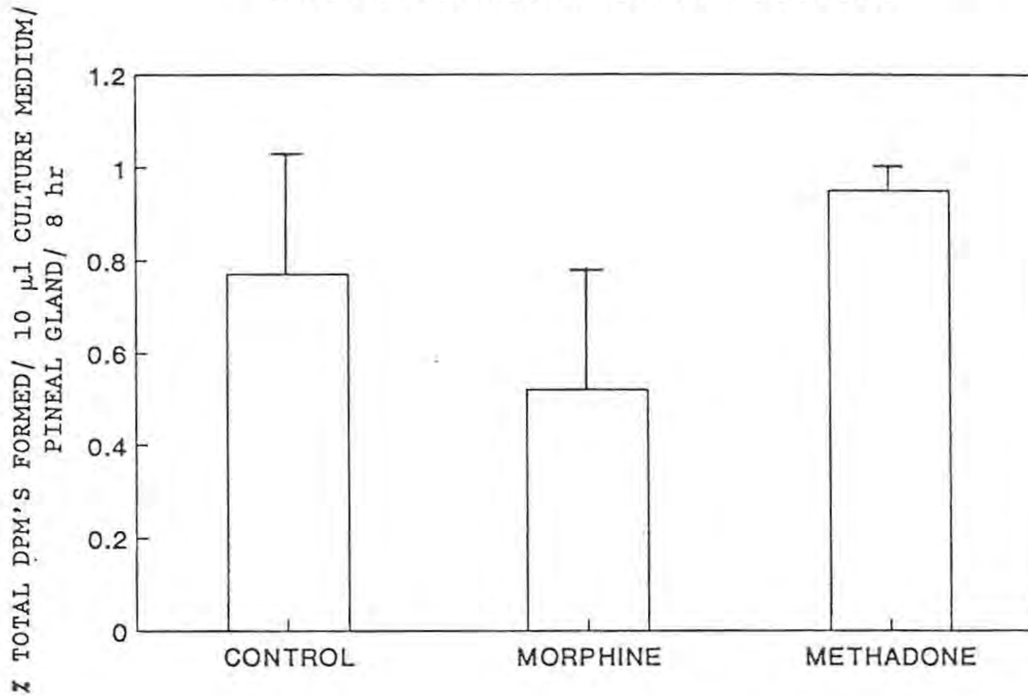


Fig 2.4: The amount of [^{14}C]-MEL released into the culture medium by the pineal gland after an 8 hr incubation time in the presence of opioids is not significantly different to that of the control.

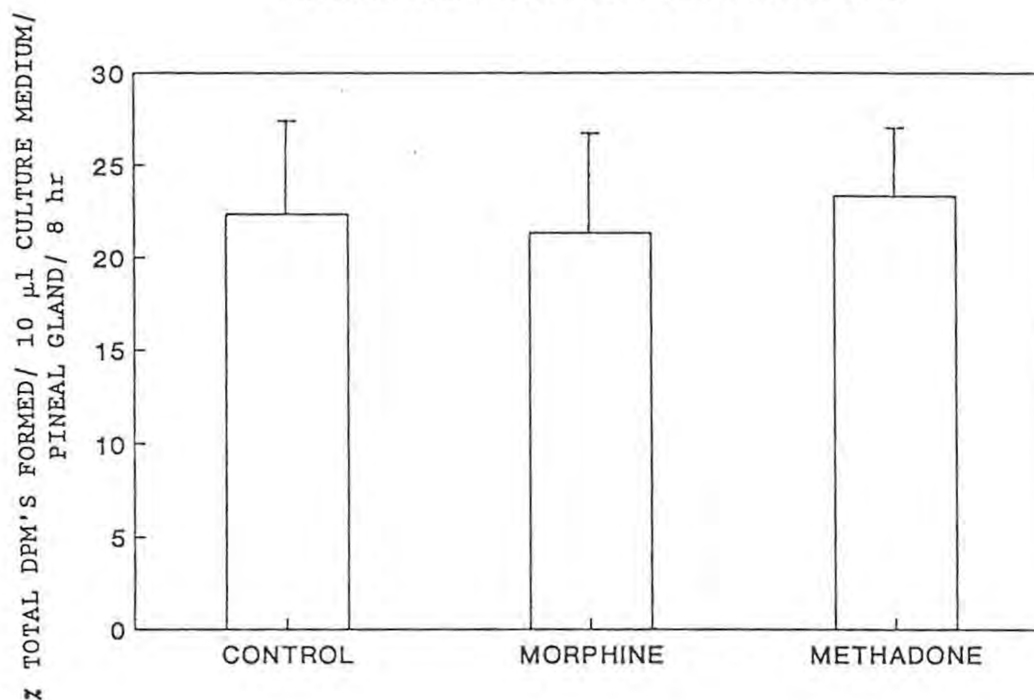


Fig 2.5: The amount of[^{14}C]- HIAA released into the culture medium by the pineal gland after an 8 hr incubation time in the presence of opioids is not significantly different to that of the control.

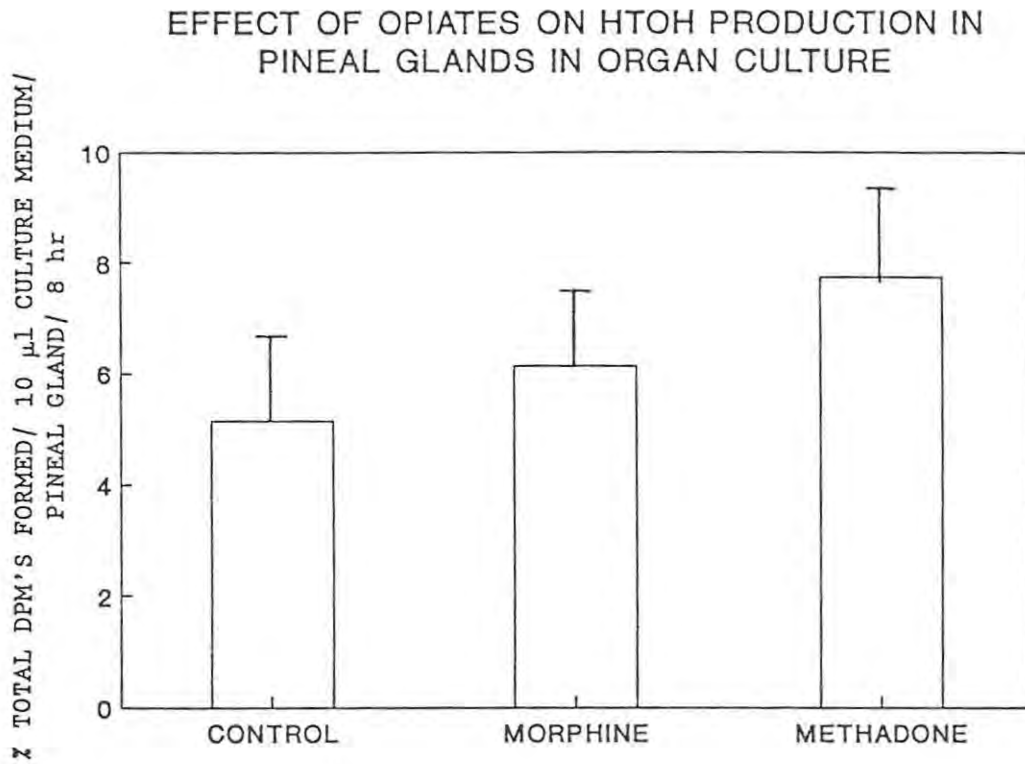


Fig 2.6: The amount of [^{14}C]-HTOH released into the culture medium by the pineal gland after an 8 hr incubation time in the presence of opioids is not significantly different to that of the control.

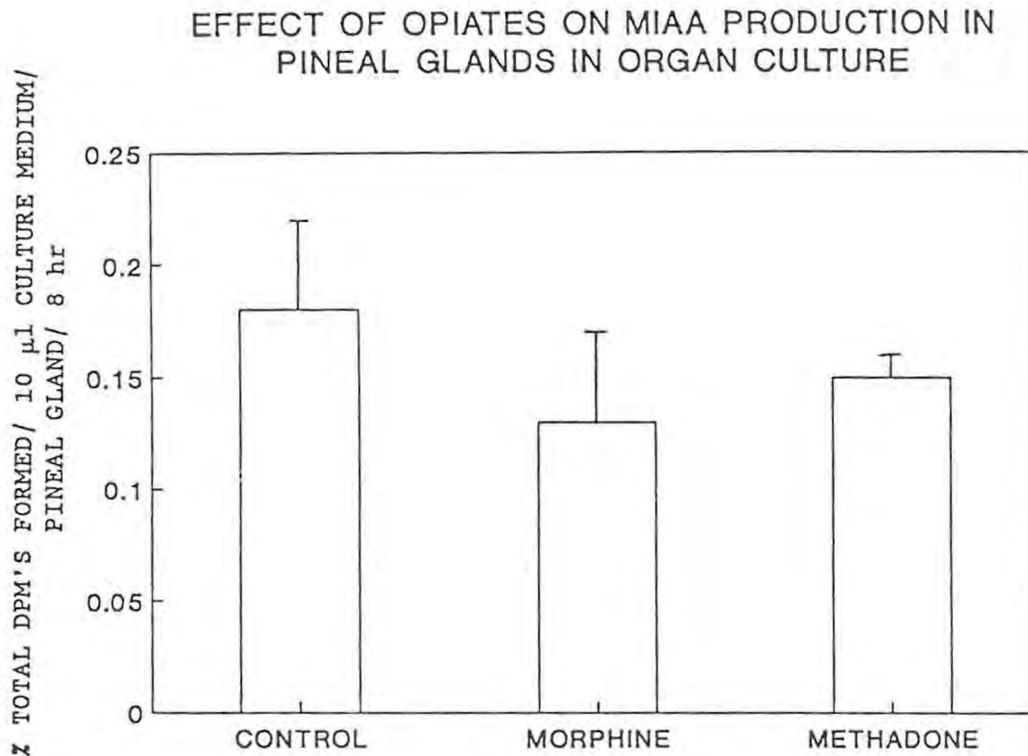


Fig 2.7: The amount of [^{14}C]-HIAA released into the culture medium by the pineal gland after an 8 hr incubation time in the presence of opioids is not significantly different to that of the control.

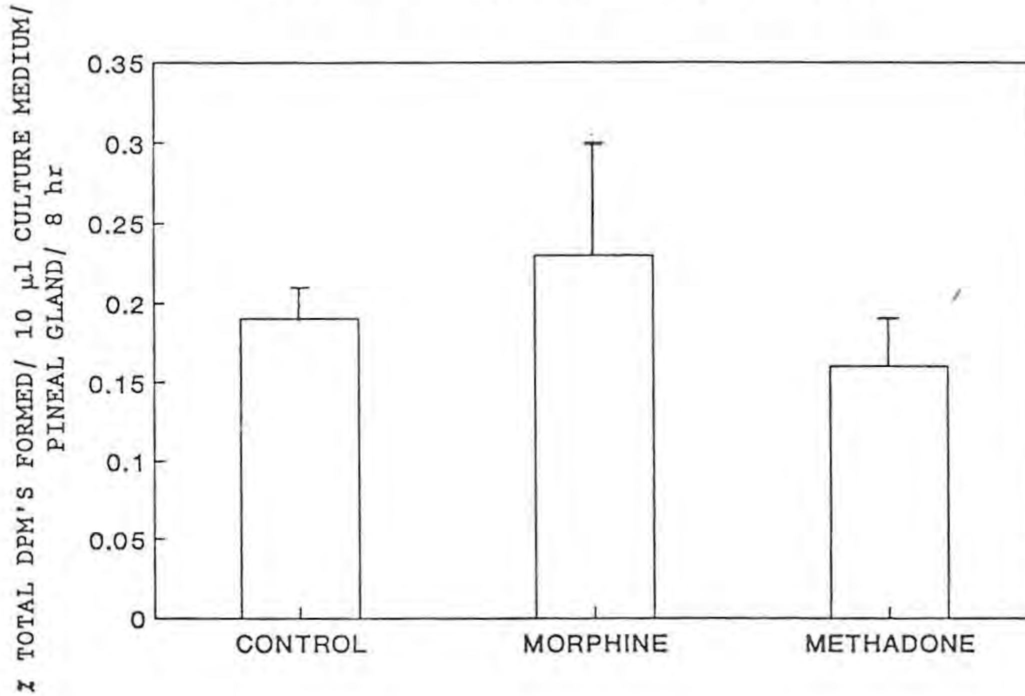


Fig 2.8: The amount of [^{14}C]-MTOH released into the culture medium by the pineal gland after an 8 hr incubation time in the presence of opioids is not significantly different to that of the control.

2.6.3.4 Discussion

The results obtained are indicative of a lack of any direct effect of morphine and methadone on [^{14}C]-serotonin metabolism in the pineal gland *in vitro*.

These results suggest that if the opiates modify the action of the pineal gland by altering the production of any of the pineal indoles, this effect is unlikely to be a direct one.

These results are supported by the work of Shein *et al* (1970), who have provided evidence indicating that morphine (10^{-5}M and 10^{-4}M) does not affect the *in vitro* synthesis of ^{14}C-melatonin by the pineal gland. They have also shown that the synthesis of ^{14}C-serotonin and ^{14}C-protein from ^{14}C-tryptophan is not affected by opiate treatment *in vitro*.

Fevre-Montange *et al* (1984) observed that the peptide Des-Tyr-ö-endorphin does affect the content of melatonin in the pineal gland *in vitro*, also indicative of the absence of a direct effect of the endorphin on pineal melatonin production.

These results also rule out the possibility that the opiates exert a post-synaptic effect on [^{14}C]-serotonin metabolism by the pineal gland *in vitro*.

2.6.4 EXPERIMENT 4 : A 24 HOUR TIME PROFILE ON THE EFFECT OF OPIATES ON ^{14}[C] SEROTONIN METABOLISM BY THE PINEAL GLAND IN ORGAN CULTURE.

2.6.4.1 Introduction

An organ culture system can remain viable for up to six days when incubated at 37°C in a suitably aerated culture medium (Klein, 1969).

The results of the previous experiment (exp 3) indicated that 10 μM Morphine HCl did not affect pineal gland indole synthesis after an 8 hour incubation period.

This experiment was thus carried out to determine whether the opiates would induce changes in ^{14}C-serotonin metabolism during an incubation period exceeding 8 hours.

2.6.4.2 Materials and Methods

Male rats (200-250g) were killed by neck fracture between 10h00 and 11h00. Pineal glands (n=5) were rapidly removed as previously described (section 2.2.2). They were then incubated separately (as described in section 2.2.3) in culture medium containing 10 μM morphine HCl or 10 μM methadone, or in culture medium only (control group). An additional tube containing the complete incubation mixture without the pineal gland was also incubated at the same time and was used to replenish culture tubes after sampling. The morphine HCl solution was freshly made up in culture medium on the day of experimentation.

Samples of culture medium (10 μl) were removed at t = 4hrs, 8hrs, 12hrs, and 24hrs from each tube. Each tube was immediately replenished with 10 μl culture medium from the additional tube.

The aliquots of culture medium were then separated and analysed as previously described (section 2.2.4).

2.6.4.2 Results

The following set of figures (fig 2.9 and fig 2.10) describes the results obtained from this experiment. For the sake of clarity, the standard errors of the mean have not been included.

The results clearly indicate that morphine and methadone have no direct effect on pineal gland indole metabolism over a 24 hr time period. None of the indoles assayed in the presence of morphine or methadone showed any significant difference in levels to that of the control group.

2.6.4.3 Discussion

The results obtained in this experiment verify those of experiment 3.

From these results, it could also be concluded that morphine and methadone do not directly modify the synthesis or the activity of the enzymes regulating melatonin synthesis eg SNAT *in vitro*. This is manifested by the inability of these opioids to modify melatonin production *in vitro* as seen from the above experiment.

2.6.5 EXPERIMENT 5 : THE EFFECT OF OPIATES ON ISOPRENALINE STIMULATION OF PINEAL GLANDS IN ORGAN CULTURE.

2.6.5.1 Introduction

The post-ganglionic sympathetic neurons innervating the pineal gland release the neurotransmitter noradrenaline onto the pineal β_1-adrenergic receptor, stimulating the production of melatonin.

The addition of the synthetic β-adrenergic agonist isoprenaline to pineal glands in organ culture is known to accelerate indole production (see section 2.5.2), via stimulation of the pineal β-receptor followed by an activation of cAMP (Axelrod, 1974).

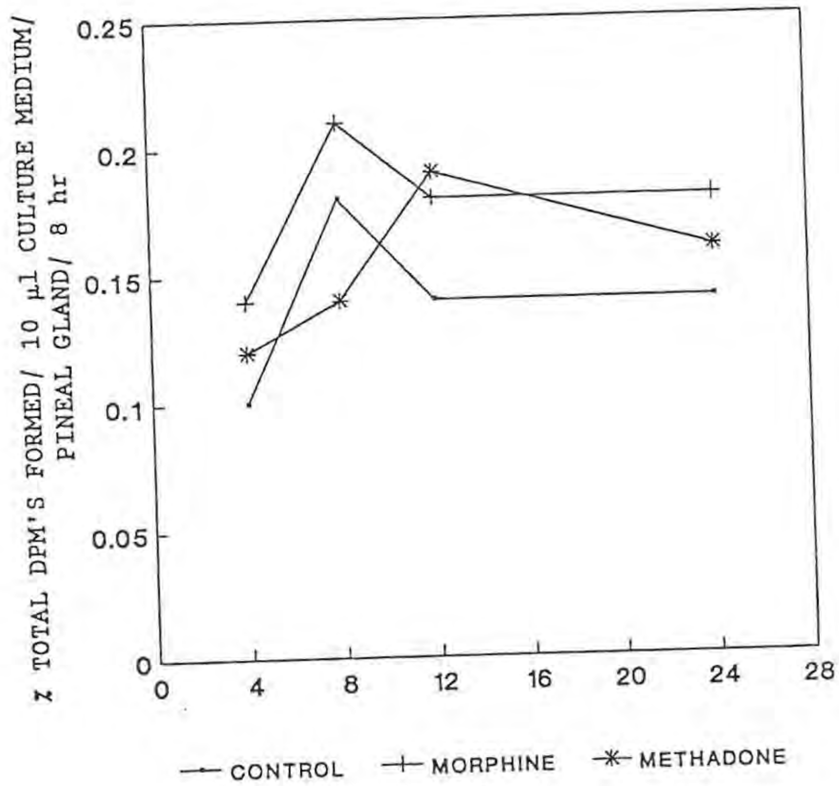


Fig 2.9: Pineal glands were incubated in an organ culture system in the presence or absence of opioids, and the culture medium was assayed at various time intervals for indole production. The opioids do not significantly alter the levels of [^{14}C]-MEL formed from ^{14}C-serotonin by the pineal gland.

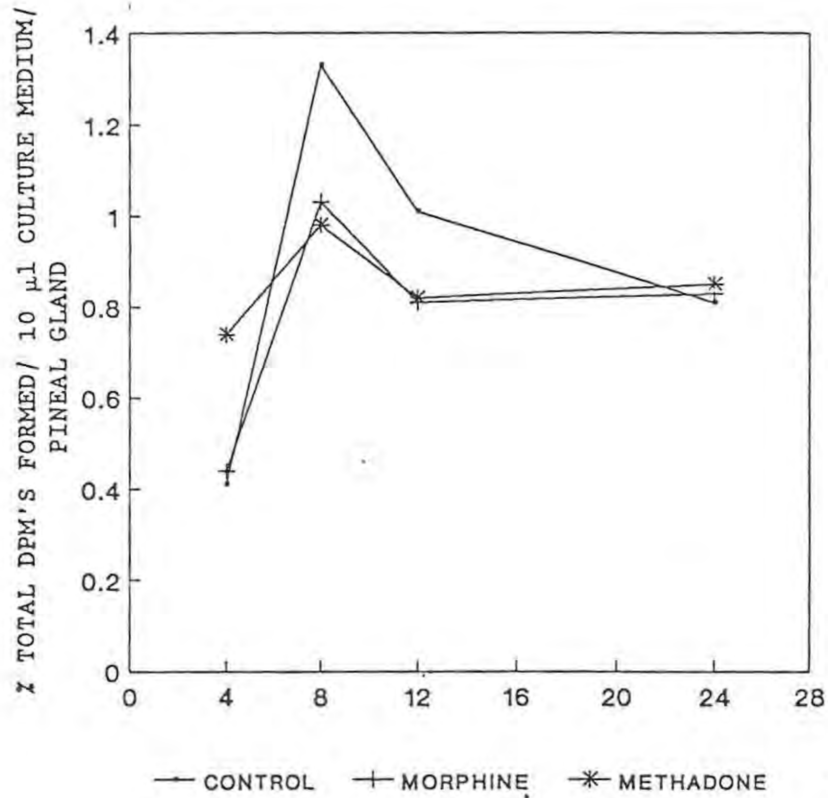


Fig 2.10: Pineal glands were incubated in an organ culture system in the presence or absence of opioids, and the culture medium was assayed at various time intervals for indole production. The opioids do not significantly alter the levels of [^{14}C]-NAS formed from ^{14}C-serotonin by the pineal gland.

Opiates may modulate the activity of adenylate cyclase in the brain (Collier and Roy, 1974). It has been observed that the opiates inhibit dopamine-stimulated cAMP levels in the striatum and the whole brain (Minneman, 1977; Wilkening *et al*, 1976; Motonatsu *et al*, 1977; Havemann and Kuschinsky, 1978) as well as NA-stimulated cAMP levels in the cortex and the cerebellum (Tsang *et al*, 1978).

Opioid peptides can pre-synaptically modulate the release of neurotransmitters (Barker *et al*, 1978). Arbilla and Langer (1978) have shown that opioid agonists inhibit NA release.

The following experiment was undertaken to examine whether the concomitant addition of morphine and methadone to the culture medium would modify the response of the pineal gland to isoprenaline.

2.6.5.2 Materials and methods

Male wistar rats were killed by neck fracture and the pineal glands were removed as previously described. (Section 2.2.2)

The pineal glands (n=5) were then individually incubated in culture medium only, culture medium containing 10 μM (-)-isoprenaline, or culture medium containing 10 μM l-isoprenaline plus morphine HCl (10 μM) or methadone HCl (10 μM) and incubated for 8 hours as previously described (section 2.2.3).

2.6.5.3 Results

As was previously observed, isoprenaline (10 μM) accelerated pineal gland indole production. [(Total dpm's NAS + MEL formed/pineal gland = 1168.29 ± 134.33 (control) and 4818.10 ± 358.44 (isoprenaline stimulated)].

The results obtained from this experiment indicate that neither morphine nor methadone modifies the response of the pineal gland to isoprenaline. The levels of the various indoles produced in each group do not differ significantly from each other (see table 2.5).

TABLE 2.5 THE EFFECT OF THE OPIATES ON ISOPRENALINE STIMULATED PINEAL GLANDS IN ORGAN CULTURE.

	% TOTAL ^{14}C-SEROTONIN METABOLIZED/10 µl CULTURE MEDIUM/PINEAL GLAND			
	CONTROL	MORPHINE	METHADONE	
NAS	2.21 ± 0.31	1.97 ± 0.19	2.90 ± 1.13	NS
MEL	2.42 ± 0.02	2.60 ± 0.32	1.75 ± 0.25	NS
HIAA	17.88 ± 0.89	20.19 ± 2.74	14.79 ± 2.04	NS
HTOH	11.17 ± 2.74	11.51 ± 1.10	7.78 ± 0.71	NS
MIAA	0.12 ± 0.09	0.17 ± 0.04	0.15 ± 0.02	NS
MTOH	0.33 ± 0.006	0.38 ± 0.07	0.54 ± 0.22	NS

*NS = not significantly different to controls.

Pineal glands were incubated in the presence of isoprenaline only, or with the concomitant addition of morphine (10 µM) or methadone (10 µM) for 8 hours. Results are expressed as (means ± SEM).

2.6.5.4 Discussion

The results obtained in this experiment are supported by the work of Shein *et al*, (1970). These investigators observed that morphine did not modify the NA-induced stimulation of ^{14}[C]-melatonin synthesis, and also had no effect on the incorporation of ^{14}[C]-tryptophan into ^{14}[C]-protein.

The data obtained once again provide evidence for a lack of a direct effect of the opiates on the pineal gland.

2.6.6 EXPERIMENT 6 : THE EFFECT OF NALOXONE ON PINEAL GLAND INDOLE PRODUCTION IN ORGAN CULTURE.

2.6.6.1 Introduction

Lowenstein *et al*, (1984) carried out experiments to investigate the effects of opioid antagonists on the pineal gland. They observed that s.c. administration of 10mg/kg bw naloxone to rats resulted in a reduction of the nocturnal peak of melatonin in the pineal gland.

Esposti *et al*, (1988b) showed a dose dependent increase in melatonin production after administration of 1, 1.5 and 2 mg/kg bw morphine to rats at 4.00 pm. This effect was blocked by pretreatment (15 minutes) with 1mg/kg bw naloxone.

The following experiments were carried out to observe whether the effect of naloxone on pineal gland indole production was mediated via a direct mechanism, suggesting an intrinsic opioid effect in the pineal gland.

2.6.6.2 Materials and Methods

Naloxone HCl (Sigma) was freshly prepared in culture medium on the day of the experiment.

2.6.6.2(a) Rats were killed by neck fracture and pineal glands removed as previously described (section 2.2.2). and individually incubated in culture medium only, or in culture medium containing 10 μM naloxone and incubated for 8 hrs as previously described (section 2.2.3).

2.6.6.2(b) Pineal glands were incubated in culture medium only, culture medium containing 10 μM l-isoprenaline, or culture medium containing 10 μM l-isoprenaline as well as 10 μM naloxone for 8 hours as previously described (section 2.2.3).

After incubation, 10 μl aliquots of culture medium were removed and separated and quantitated as previously described (Section 2.2.4).

2.6.6.3 Results

The results indicate that naloxone has no effect on indole production by the pineal gland. The levels of all the indoles assayed in this experiment do not differ significantly between the treatment groups (fig 2.12 and fig 2.13). Isoprenaline stimulation of pineal indole production was observed in 2.6.2.6b (see fig 2.11). However, naloxone does not modify the response of the pineal gland to l-isoprenaline (see table 2.6).

22.6.6.4 Discussion

The results obtained from these experiments indicate that naloxone does not modify the activity of the pineal gland *in vitro*, as manifested by indole production.

These findings are supported by the work of Lowenstein *et al* (1984), who have shown that naloxone does not modify the content of melatonin in the rat pineal gland *in vitro*.

It has also been observed that naloxone has no effect on the release of pre-loaded tritiated NA from the rat pineal gland when release is induced using a depolarising (K^+) stimulus (Lowenstein *et al*, 1984).

It could thus be suggested that opioid receptor blockade has no effect on the production of ^{14}[C]-indoles from ^{14}[C]-serotonin by the rat pineal gland in organ culture.

TABLE 2.6: THE EFFECT OF NALOXONE ON ISOPRENALINE STIMULATED PINEAL GLANDS IN ORGAN CULTURE

	% TOTAL ^{14}C-SEROTONIN METABOLISED /10 μl CULTURE MEDIUM/PINEAL GLAND/8HR	
	CONTROL	NALOXONE
NAS	2.21 ± 0.31	1.93 ± 0.48
MEL	1.41 ± 0.02	1.56 ± 0.12
HIAA	11.16 ± 2.74	14.27 ± 4.708
HTOH	7.51 ± 0.89	11.72 ± 5.22
MIAA	0.12 ± 0.09	0.18 ± 0.01
MTOH	0.3308 ± 0.006	0.34 ± 0.0576

Pineal glands were incubated in the presence of isoprenaline (10 μM) only, or with isoprenaline and naloxone (10 μM).

Results show that naloxone treatment has no significant effect on isoprenaline treated pineal glands in organ culture.

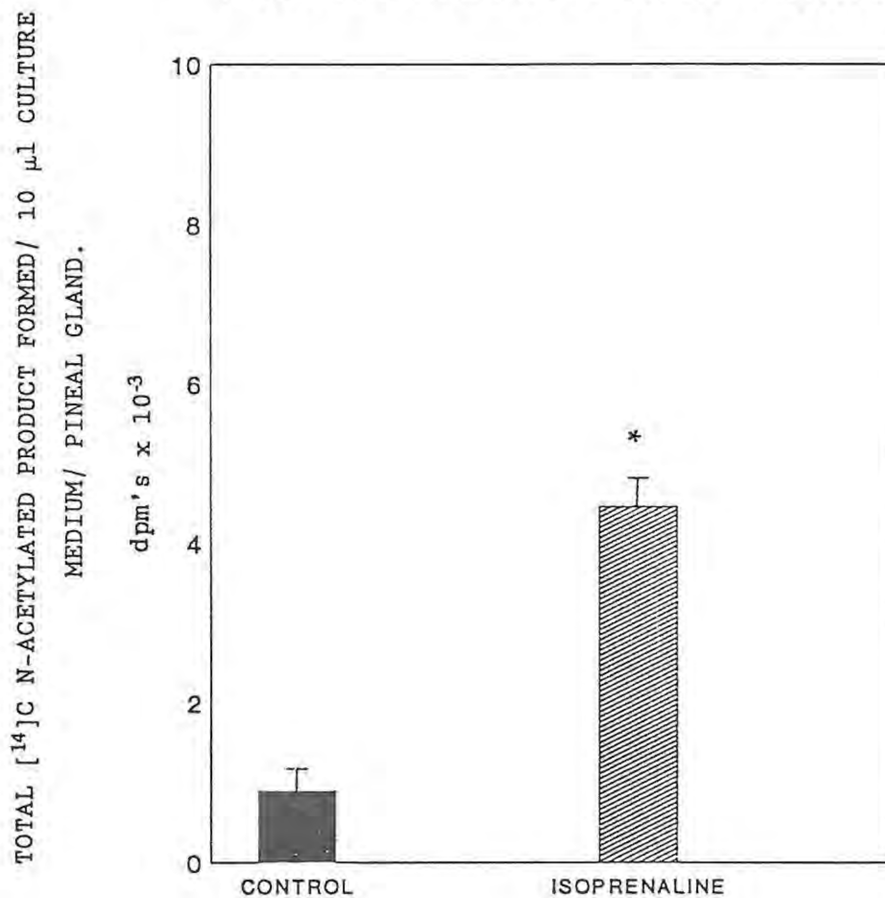


Fig 2.11: Isoprenaline stimulates the production of N-acetylated products (NAS + MEL) from [^{14}-C]-serotonin by the pineal gland *in vitro.*

$n = 5$, $p < 0.005$

* = significantly different to control

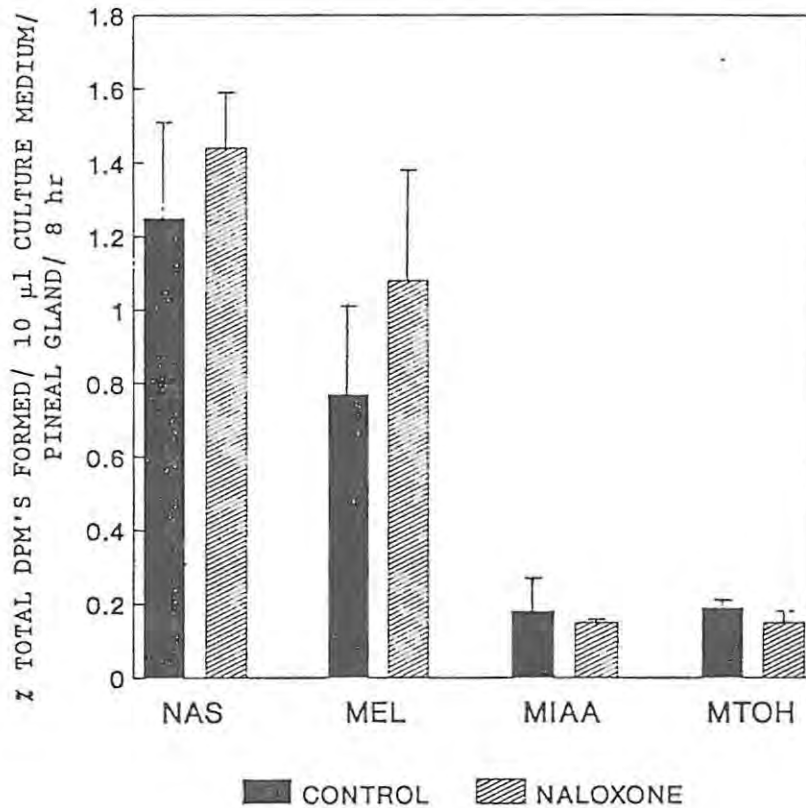


Fig 2.12: Pineal glands (n = 5) were incubated in the organ culture system described in the presence or absence of 10^{-5} M naloxone for 8 hours. Naloxone did not alter [^{14}C]-indole production significantly when compared to controls.

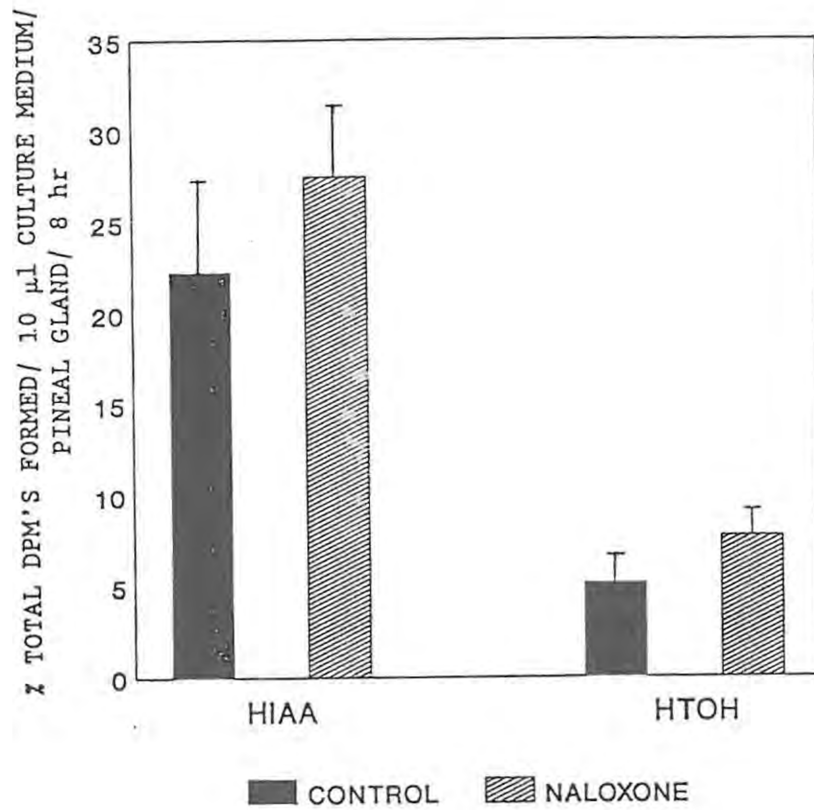


Fig 2.13: Pineal glands (n = 5) were incubated in the organ culture system described in the presence or absence of 10^{-5} M naloxone for 8 hours. Naloxone did not alter [^{14}C]-indole production significantly when compared to controls.

2.6.7 EXPERIMENT 8: A 24 HOUR PROFILE ON THE EFFECT OF NALOXONE ON ^{14}[C] SEROTONIN METABOLISM BY THE PINEAL GLAND IN VITRO

2.6.7.1 Introduction

The results from the previous experiment indicate that naloxone does not modify the response of the pineal gland to isoprenaline stimulation *in vitro*. Naloxone also failed to significantly alter the basal response of the pineal gland in this organ culture system after an 8 hr incubation period.

This experiment was thus undertaken to examine the effects of naloxone on the pineal gland in organ culture over a 24 hr time period.

2.6.7.2 Materials and Methods

Male Wistar rats were killed by neck fracture and pineal glands were rapidly removed as previously described (section 2.2.2).

Pineal glands were then incubated individually in culture medium only, or in culture medium containing 10 μM naloxone HCl. An additional tube containing the complete incubation mixture excluding a pineal gland was simultaneously incubated, and served to replenish the culture tubes after sampling.

Aliquots (10 μl) of culture medium were removed at t = 4hrs, 8hrs, 12hrs, and 24hrs from each tube, which was immediately replenished with 10 μl incubation mixture.

The samples were then spotted onto TLC plates and separated and the ^{14}C-indoles were quantitated as previously described.

2.6.7.3 Results

The 24 hour time profile on the effect of naloxone on pineal gland indole production showed that naloxone had no significant effect on the levels of the indoles assayed in this experiment.

No significant alteration in pineal indole levels was observed at any time point at which the culture medium was assayed.

Figures 2.14 and 2.15 describe the results obtained in this experiment graphically.

2.6.7.4 Discussion

From the results obtained in this study, it can be concluded that naloxone does not in any way modify the production of any of the indoles produced by the pineal gland assayed in this organ culture system.

It would thus appear that this specific opiate antagonist does not have any direct effect on ^{14}C-serotonin metabolism by the pineal gland *in vitro*.

It could thus be concluded that any action of the opiates or the opiate antagonists on the pineal gland would necessarily occur at a pre-synaptic level, proximal to the pineal gland itself.

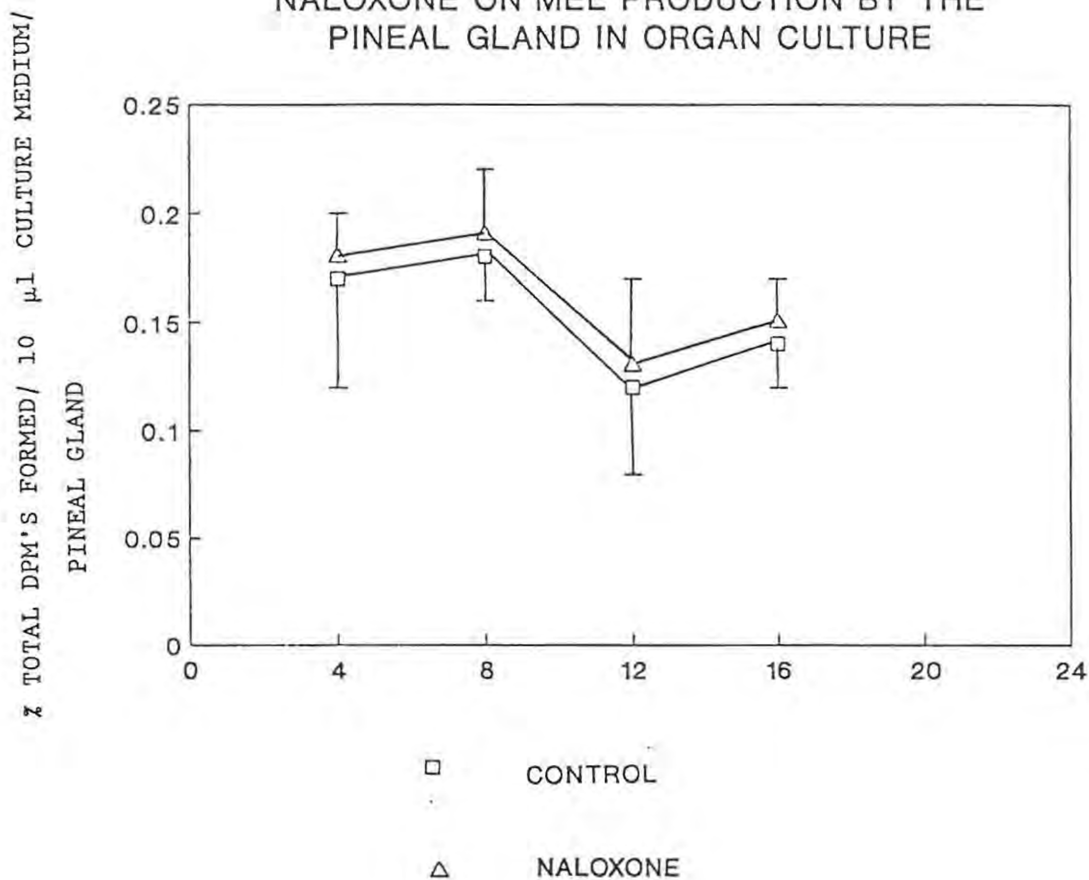


Fig 2.14: Pineal glands were incubated in an organ culture system in the presence or absence of naloxone, and the culture medium was assayed at various time intervals for indole production. Naloxone does not significantly alter the levels of $[^{14}C]$-MEL formed from ^{14}C-serotonin by the pineal gland.

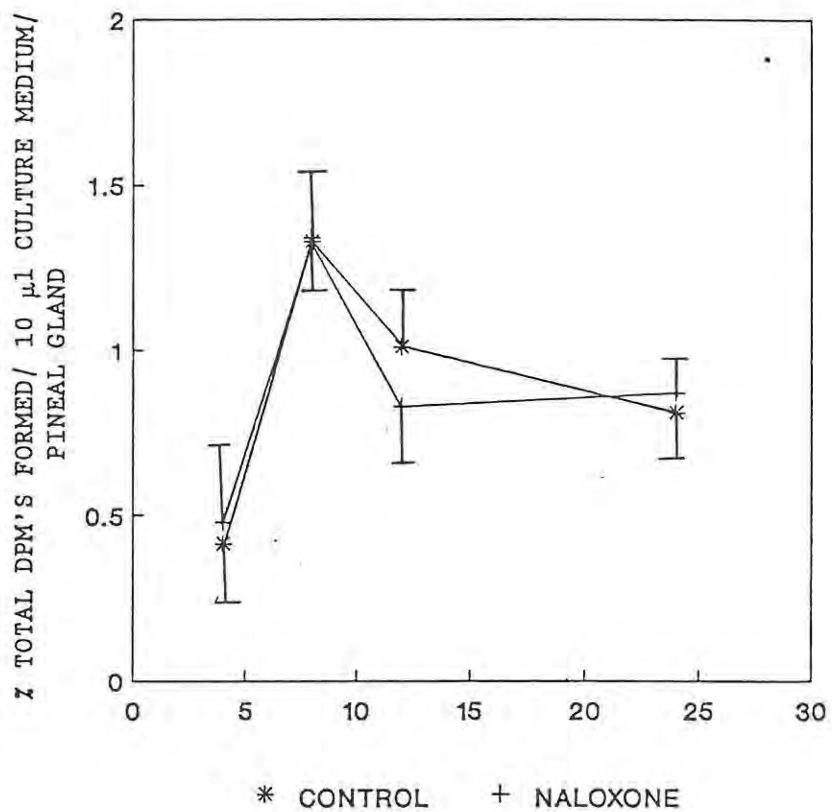


Fig 2.15: Pineal glands were incubated in an organ culture system in the presence or absence of naloxone, and the culture medium was assayed at various time intervals for indole production. Naloxone does not significantly alter the levels of [^{14}C]-NAS formed from ^{14}C-serotonin by the pineal gland.

2.7 Conclusion

Many publications have suggested a role for the opioid system in the functioning of the pineal gland (Zatz and Browenstein, 1979, Esposti *et al*, 1988b, Lowenstein *et al*, 1984). It has also been suggested that the pineal gland is necessary for at least some of the actions of the opioid system (Reiter, 1986). Accordingly, it would appear that an inter-relationship between these two systems does exist.

Subcutaneous administration of small doses of morphine have been found to increase plasma melatonin concentrations (Esposti *et al*, 1988b) while the administration of large doses of morphine (20mg/kg bw ip) results in a temporary reduction of the SNAT nocturnal activity.

Lowenstein *et al*, (1984) found that the opioid antagonist naloxone depressed the nocturnal rise of melatonin in rats and thus suggested that at least one opioid synapse is involved in the conveyance of light information to pineal gland.

These workers also found that naloxone (10^{-4} to 10^{-6} M) did not affect the *in vitro* synthesis of melatonin in organ culture.

The presence of immunoreactive β-endorphin in extracts of rat pineal glands (Vuolteenhao, 1980) and the enkepalin-like immunoreactivity observed in neurons of the human pineal gland (Moore and Siboney, 1988) suggest that the effects of the opiates on the pineal gland may be exerted at both a peripheral and a central level.

The experiments described in this chapter show that the opiates do not have a direct effect on the pineal gland *in vitro*. Naloxone also failed to significantly alter the response of the pineal gland as manifested by indole production in this organ culture system. These observations are supported by those of other workers (Shein *et al*, 1970, Lowenstein *et al*, 1984, Fevre-Montagne *et al*, 1984).

The stimulatory effect of the opioids on melatonin secretion by the pineal gland observed by other workers (Esposti *et al*, 1984, Lissoni *et al*, 1986) could have resulted either from a direct effect via an action on specific opioid receptors in the pineal gland, or from an indirect effect on neurons afferent to the pineal gland itself.

The results of this study clearly indicate a lack of a direct effect of the opioids and the opioid antagonist naloxone on the pineal gland *in vitro*.

These observations would thus favour the explanation that a central or an indirectly-acting peripheral opioid synapse is involved in the manifestation of the *in vivo* effects of the opioids on the pineal gland.

CHAPTER THREE

STRESS

3.1 INTRODUCTION

Man and animals are constantly exposed to stressful events that can lead to biochemical, behavioural, physiological and/or pathological changes that affect their homeostasis.

The diversity of the topic of stress is reflected in the difficulty of finding an adequate definition for it. Diverse conditions can be described as being stressful, eg pain, noise, heat, isolation, fear, and it would seem as if stress is merely an intensification of what would normally be a tolerable experience.

The Selyean concept of stress is perhaps the most apt description. Dr Hans Selye defines stress as being "the nonspecific response of the body to any demand made upon it." This demand, created by the stressor on the body, initiates adaptive functions for readjustment, and for the re-establishment of normalcy (Selye, 1974).

The pineal gland secretes a hormone, melatonin, first identified in 1957 by Lerner and co-workers (Lerner *et al*, (1958). It has since been implicated in the functional activities of many organs, eg the brain, adrenal glands, gonads, thyroid gland and smooth muscle (minireview, Minneman and Wurtman, 1984).

Many studies have shown that stressful stimuli alter pineal melatonin production (Lynch *et al*, 1973, Tannenbaum *et al*, 1988, Vaughan *et al*, 1978). Miline (1980) proposed that the pineal gland exerts an inhibitory influence on the adrenal-hypothalamo-pituitary axis, while Romijn (review, 1978) suggested that the pineal gland acts as a tranquilizing organ.

In 1977, Guilleman *et al* observed that in rats, β-endorphin and ACTH are secreted simultaneously by the pituitary gland in conditions of

acute stress. This stirred much interest as to the role of the endorphins in stress. Many studies have since examined the role of opiates and opiate antagonists in stress, (Dai *et al*, 1983, Ferri *et al* 1983, Del Tacca *et al*, 1987, Glavin *et al*, 1986) and contradiction exists as to their exact function.

Recently, Maestroni *etal* (1988) reported that the administration of melatonin to rats could counter the effects of acute stress on thymus weight. They also observed that this action of melatonin was exerted via an opioidergic mechanism, suggesting a link between the melatonin and the opioidergic system in the manifestation of thymus involution due to stress exposure.

The following study was thus undertaken to examine a possible role for melatonin as an anti-stress hormone, and to investigate whether melatonin acts via an opioidergic mechanism.

Restraint and immobilization are techniques that have been commonly used to induce stress in laboratory animals. One of the common peripheral changes indicative of exposure to stress is gastric ulceration. Pare and Glavin (1986) have extensively reviewed various restraining techniques and found that, after starving rats for 24 hours, prone restraint on a board, coupled with exposure to cold (4-7°C) for three hours, resulted in a 100% incidence of gastric stress ulceration. This was in accordance with the findings of Senay and Levine, (1967) who concluded that the combination of stressors (cold and restraint) acted synergistically to rapidly produce gastric ulceration in starved rats.

The following sections will describe the development of the stress procedure used in this study, the pinealectomy technique, and its application to this study.

3.2 MATERIALS AND METHODS

3.2.1 Drugs

Melatonin, naloxone HCl and indomethacin were purchased from Sigma Chemical Co., USA. Morphine HCl was obtained from Labethica, South Africa. Diazepam was obtained from Roche, South Africa. All drug

solutions were freshly prepared on the day of experimentation.

3.2.2 Animals

Female rats of the Wistar strain (180-250 g) housed five per cage were used in all experiments. Animals were maintained in an automatically regulated lighting cycle of LD 12:12 with lights on at 06h00. They were allowed free access to a standard diet and tap water prior to experimentation.

3.2.3 Stress

The following method of stress induction was found to be the most suitable.

3.2.3.1 Starvation

Rats were deprived of food for 24 hours prior to restraint, but allowed access to water *ad libitum*. Coprophagy was prevented by using cages with steel grid bases, elevated from a receptacle in which faeces accumulated.

3.2.3.2 Stress Induction

Experiments carried out in the dark phase (three hours after lights off - 21h00) were facilitated by the use of a dim photosafe red light source. Daytime experiments commenced three hours after lights on. (09h00)

After the starvation period, animals were lightly anaesthetized with ether and injected with treatment drugs or control vehicles (see experiments). They were then restrained in the supine position, ventral surface upwards, on wooden boards using adhesive plaster. By this time the rats had recovered from ether anaesthesia and were immediately transported to a cold room (4-7°C) where they remained for the duration of the stress procedure. After two hours, the rats were removed from the cold room and sacrificed by neck fracture. Stomachs were rapidly dissected out, cut along the greater curvature, rinsed in distilled water and quickly pinned onto polystyrene boards.

3.2.3.3 Examination of lesions

Stomachs were then examined for gastric ulceration by an observer unaware of treatment conditions. Total length of ulceration to the nearest 0.1 mm was measured using vernier calipers. Another original method for measurement of the extent of gastric ulceration was also developed. The total stomach area as well as the area/s of ulceration were mapped out onto graduated transparencies. The area of ulceration as a percentage of total ulceration in mm^2 was calculated. Due to the variability in the sizes of the stomachs, it was thought that this method would give a more accurate description of the results.

3.2.4 Oral administration of drugs

Oral dose syringes were made to administer drugs to rats. The equipment consisted of 5 ml syringes, to which a long (21 gauge) needle (0.80 x 38 mm)had been attached. The tip of the needle was cut away and blunted, and a small bubble was blown around this area with an epoxy-wood glue. After the glue had hardened, the needle was slightly curved so as to facilitate oral dosing. All syringes were tested to see if they were functioning effectively. At the time of the experiment, the syringe was filled with control vehicle or drug suspension, and dipped in a dilute sugar solution. The rat was held behind the head near the area of the neck, so that its mouth was partly opened. The syringe was then gently inserted into the mouth and the rat was fed with the contents of the syringe.

3.2.5 Pinealectomy

3.2.5.1 Anaesthesia

Initially, phenobarbitone (Sagatal^{R}) in a dose of 70mg/kg bw was used to induce anaesthesia. The use of this drug posed the following serious problems:-

1) Failure to induce sufficient anaesthesia: This effect varied from day to day and from morning to afternoon.

2) Long recovery times: Rats took between three to four hours to recover from the anaesthetic. Respiratory depression would also set in after the operation, and animals had to be resuscitated.

Due to the lack of success and the high mortality rate, the use of this anaesthetic was discontinued.

Ether anaesthesia was then attempted. Rats were placed in an ether saturated dessicator until they fell asleep. They were then removed from the dessicator and placed on the stereotaxic apparatus. An ether saturated cottonwool strip was placed at the tooth bar of the apparatus, covering the area of the nose and mouth of the rat, so as to maintain anaesthesia.

The use of ether as an anaesthetic had many advantages. During surgery, anaesthesia could be controlled to maintain sufficient respiration by removing the cottonwool strip from the nose and mouth area. Also, a faint pink colour of the paws and the tail of the rat gave a reasonable indication of good respiration. A cyanosis of the limbs indicated that the administration of the anaesthetic had to be discontinued.

3.2.5.2 Surgery

Previously, the pinealectomy procedure used by other workers in our laboratory was found to be very problematic. The method adopted was based on the stereotaxic clamping of the rat. A small hole was drilled over the pineal area, and pinealectomy was attempted; or else entry was gained lateral to the large sinuses (transverse and saggital) covering the pineal and fine tweezers were used to grip the pineal stalk. These operations were unsuccessful because of the high mortality rate of rats due to excessive bleeding, and failure of most rats to recover from anaesthetic (Midlane, 1974, Paton, personal communication).

The method used for all pinealectomies in this study was that of Hoffman and Reiter (1974). A dental drill was fitted with a special drill bit made at Rhodes University according to the specifications of Hoffman and Reiter (1974). It consisted of a long shaft with

serrated edges. An adjustable collar fitted with Allen keys was attached to the shaft, and was used to vary the extent to which the bone of the skull could be penetrated, so as to prevent damage to the underlying brain tissue (see figure 3.1).

Rats were anaesthetised with ether, and immediately, the head of the animal was rigidly mounted onto the stereotaxic apparatus (see figure 3.2) so that it was temporarily immobilized.

The fur around the skull region was then rapidly shaved off. A neat antero-posterior cut was made along the midline of the skull, from the area slightly behind the eyes to just beyond the base of the skull. Alcohol swabs were used to wipe the area clean. The underlying tissue was carefully parted using a sharp scalpel, to prevent entanglement with the drill and the collar.

The lambda axis, above the transverse and saggital sinuses was thus exposed. The drill was centered on this axis, and a disc shaped hole was drilled into the skull to the desired depth. The bone was carefully removed so as to prevent excessive bleeding. A fine forceps was inserted into the junction of the two sinuses, and the pineal gland was rapidly removed. The gland was easily discernible, being paler than the rest of the tissue and approximately the size of a pinhead.

The invasive nature of the procedure resulted in bleeding, which was eased by quickly replacing the bone disc into its original position, and applying gentle pressure on it using an alcohol saturated cottonwool swab. The skin flaps were then pulled together and held with a forceps. The wound was then deftly sutured.

Occasionally, shallow or gasping breathing would be noted during or near the end of the operation. The rat was then resuscitated using a small animal respirator.

3.2.5.3 Recovery

The rat was placed on a warm base near a thermostatically controlled oscillating heater. Recovery time from the anaesthetic ranged between ten and twenty minutes.

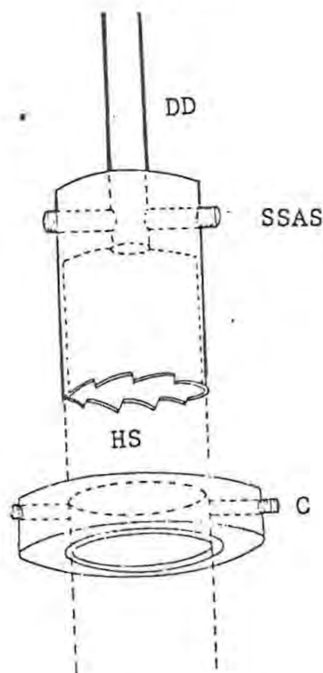
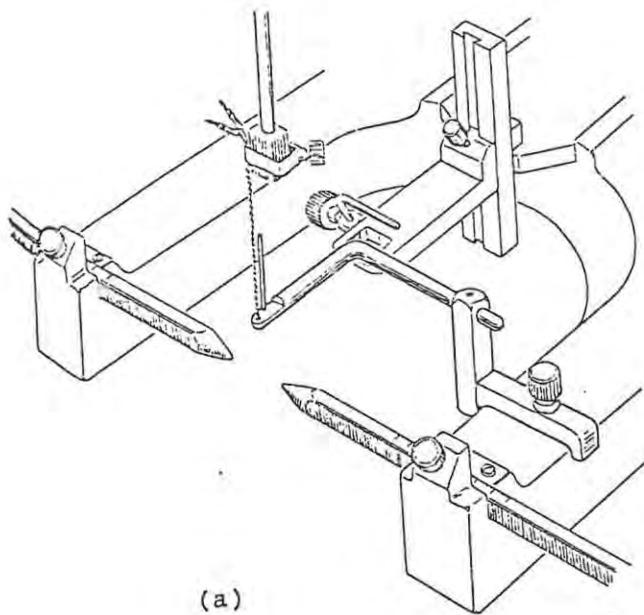


Fig 3.1: Diagram of the drill complex used for pinealectomy. C: collar, DD: dental drill, HS: hollow shaft, SSAS: stainless steel Allen screw.



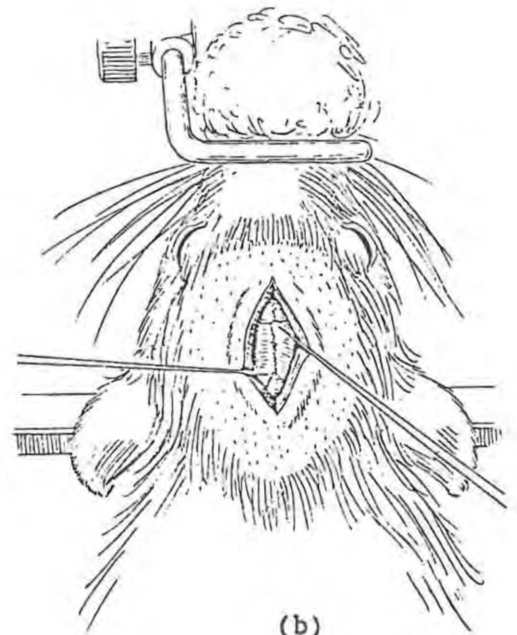


Fig 3.2: Diagram of the stereotaxic apparatus used for pinealectomy (a) and illustration of a rat mounted on the stereotaxic apparatus (b).

From Skinner, (1971)

As soon as the rats regained consciousness they were placed in cages in a warm environment for a few hours.Some rats would resume eating and drinking behaviour within 30 minutes of the operation. They were then housed in an animal room with an automatically regulated lighting cycle of LD 12:12 (lights on at 06h00). They were allowed access to a standard diet *ad libitum*. The rats were housed individually for no less than six days after the operation, to prevent injury to the operated area.

3.2.5.4 Sham operations

Sham operations were carried out in a similar manner. After removal of the bone disc of the skull (see section 3.2.5.2 above), the pineal gland was lightly touched without it being displaced. The bone disc was replaced, the wound sutured, and the animal was allowed to recuperate as described above.

Rats were used in experiments after a minimum of two weeks post-operation.

3.2.5.5 Confirmation of pinealectomy.

After being used in experiments, rats were killed by neck fracture, decapitated, and the brains were examined to check for the absence of a pineal gland, so as to ensure the success of the pinealectomy. On two occasions, the pineal glands removed during surgery were incubated in the organ culture system described previously (see chapter 2). The culture medium was analysed to check for indole production, and the presence of high levels of indoles when compared to blank samples indicated that the organ removed was indeed a pineal gland. The organ culture technique is, however, very expensive, and as such this technique was not used to confirm any further pinealectomies.

3.2.6 SNAT Assay

3.2.6.1 Preparation of solutions

a) Buffer Solutions

1) The phosphate buffer was prepared as follows:

Solution A: 908 mg of KH_2PO_4 was dissolved in 100 ml distilled water.

Solution B: 1.19 g of $Na_2HPO_4.2H_2O$ was dissolved in 100 ml distilled water.

Solution C: 50.8 ml of solution A and 49.2 ml of solution B was mixed together to give a phosphate buffer with pH=6.8.

2) The borate buffer was prepared as follows:

Solution A: 620 mg of H_3BO_3 and 746 mg of KCl was dissolved in 100 ml distilled water.

Solution B: 50 ml Of Solution A was then adjusted to pH 10 using a 0.1 N NaOH (0.4 g in 100ml distilled water) solution.

b) Working solution

A working solution containing, per sample, 5.6 mM tryptamine HCl, 800 μM acetyl coA, and 40 nCi [^3H] acetyl coA in a final volume of 10 μl 0.05 M chilled phosphate buffer (ph 6.8) was prepared immediately before the assay was undertaken. The working solution was kept chilled in a vial placed on a crushed-ice bed.

3.2.6.2 Assay

The assay procedure used for the determination of SNAT activity is the one originally described by Deguchi and Axelrod (1972) and further modified by Champney, *et al*, (1984).

The principle of the assay is based on the N-acetylation of a substrate, tryptamine HCl, by the endogenous SNAT in the pineal gland. The [^3H] Acetyl Coenzyme A acts as the acetyl donor, transferring the acetyl group to tryptamine to form the radiolabelled product, [^3H] N-Acetyl tryptamine. The enzyme SNAT is

able to utilize tryptamine far more readily than the natural substrate 5-hydroxy-tryptamine (Morton, 1982) and tryptamine was thus used as the substrate in this assay.

The radiolabelled product thus formed can be extracted into an organic solvent, and the radioactivity measured using liquid scintillometry. The amount of radioactive N-acetyltryptamine formed/time is then used as a measure of SNAT activity.

Rats (n=4) were killed by neck fracture. After decapitation, the pineal glands were rapidly removed as previously described (Section 2.2.2). Removal of the pineal glands in the dark proved to be very difficult. After various attempts, the technique was perfected to ensure rapid removal of the pineal gland in the dark. The glands were then individually homogenized in 100 μl chilled phosphate buffer (pH 6.8).

The assay was carried out in 1500 μl eppendorf microfuge tubes. 10 μl of the homogenate of each pineal gland was transferred to the bottom of separate cold microfuge tubes (placed on an ice bed). An aliquot (10 μl) of the working solution was then pipetted onto the side of each tube. The surface tension was adequate to keep the homogenate at the bottom of the tube separate from the working solution on the side. The tubes were tapped on the workbench, quickly vortexed using a rotamixer (Hook and Tucker deluxe model), and tapped again to ensure combination of the homogenate and the working solution. The tubes were then incubated for 20 minutes at 37 C in a shaking water bath. Blank incubations were performed in which only the enzyme (SNAT in pineal homogenate) was omitted from the assay.

The reaction was terminated by adding 100 μl of chilled borate buffer (pH 10) to each tube. The radioactive [^{3}H] NAT formed was then extracted into an organic solvent. 1 ml of chilled chloroform was added and each tube was capped tightly. The tubes were then shaken for 5 minutes using a Griffin flask shaker to facilitate the extraction of the radioactive NAT. The tubes were then centrifuged for 30 seconds in a microfuge. The supernatant was aspirated using a pasteur pipette connected to a suction flask in which the radioactive waste was collected. Chilled borate buffer (100 μl) was

added to each tube to wash out any contamination. Once again, the tubes were shaken for 5 minutes as before, centrifuged for 30 seconds and the supernatant aspirated as described above. Aliquots of chloroform (500 μl) were then transferred to separate scintillation vials and evaporated until completely dry.

Beckman HP/b scintillation cocktail (3 ml) was added to each vial. The vials were capped tightly, vortexed for 20 seconds each, and the radioactivity was counted for 5 minutes per sample on a Beckman LS 2800 liquid scintillation counter.

3.2.6.3 Calculation of SNAT activity

The following equation was used to measure the SNAT activity/pineal gland/hr:

$$\frac{(DPM_{sample} - DPM_{blank}) \times a \times b \times 2 \times 3}{DPM_{total}} = \frac{\text{nmol SNAT}}{\text{pineal} \times \text{hour}}$$

where a = fraction of pineal gland assayed (1 for whole, 2 for half pineal, 5 for 1/10 th of a pineal, etc)

b = nMoles of acetyl Coenzyme A per sample (*hot + cold)

2 = factor for counting only half the chloroform used for extraction

3 = factor for incubating the sample for 20 minutes only.

3.2.7 Locomotor activity

3.2.7.1 Apparatus:

Locomotor activity was recorded by an improved version of the photocell activity cage (Physics Department, Rhodes University). The activity of the animals is measured in a black-painted aluminium box measuring 500 mm square by 200 mm high. The box (cage) is mounted on an 800 mm high base; the base carries the sensors and associated electronics.

Activity is monitored by recording the interruption of beams of

infra red (i-r) light by the bodies of the animals. The light sources for the detection of locomotor activity comprise 8 high power (243 mW/steradian) narrow beam (4 deg. half-angle) type ME7124 i-r light emitting diodes situated equidistantly (100 mm apart) in two of the cage walls, opposite to which are placed 8 MT2 broad band phototransistors. The i-r sources eliminate the use of bright visible light sources usually employed in photocell activity cages and which is said to provide a mildly stressful situation (Sampson, 1975). The cages may also be used under conditions of normal ambient lighting or in the dark.

In addition to the eight i-r beams which detect locomotor activity the cages are also equipped with a further 6 beams below the cage floors to measure exploratory behaviour. These beams (3 originating from each of 2 adjacent walls) are broken if a rat peers through any of an array of 9 60 mm diameter holes in the cage floor. Such beam-breaks are detected when a tank is interrogated and these breaks are separately recorded as a measure of exploratory behaviour.

The beams are so positioned that if a rat blocks a beam a rat moving in the vicinity will break an adjacent beam. Moreover a rat which obstructs a beam for an extended period is counted only once since the computer only records **changes** in the status of the beams.

When a rat interrupts an i-r beam the voltage level at the collector of the phototransistor rises and this rise is detected by an analog voltage comparator. The comparator delivers a logic signal (beam intact/beam broken) to one of an array of buffers. These buffers may be interrogated by the data-logger computer and the current beam status recorded. A number of cages may share a common data bus and be individually interrogated by the computer.

The data logging program first invites the user to test the cages. The cages carry monitor lamps to indicate the state of indiviual beams. The computer assists the user by providing an audible acknowledgement (beep) whenever a beam is broken or restored during testing.

The program then asks the user for information relating to the

experiment (name, date, identification etc.) with the computer supplying default value for missing answers. Some of this information is used by the program; all of the information supplied is incorporated in the data file's 'header' record and may be used at a later time to identify the data-set (see figure 3.3).

Fig 3.3: Description of a header record.

Header,user's name,run id.,filt factor,filename,date,day,filtperiod,nr of tanks
Header,Razeeya,control with vehicle,a:control1.rat,890831,wednesday, 0 , 3 ,

A problem discovered while using these cages was that of "nuisance counts" resulting from a rat grazing a beam and causing many rapid interruptions. This problem is addressed by the data-logging program which, if a beam is broken, can be instructed to only record the break if it is sustained for a certain period. The computer asks the user to indicate a 'Filter Factor' which is then multiplied by a predetermined constant to yield the required period for which breaks must be sustained. Typically a constant of 0,1 is used; a filter factor of 2 would then require that all breaks be sustained for 0,2 seconds before they are eligible for recording.

Data are recorded on disk at user selectable intervals and appear as ASCII records of "upper" and "lower" breaks together with the time at which the record was made and that identification code of the tank which supplied the data.

The program may be interrupted at any time, whereupon a menu appears which allows the user to view the presently logged data records, modify various operational parameters (record length, filter factor), resume data logging at the next-but-one record or terminate the session in a variety of ways.

3.2.7.2 Animals

Preliminary investigations showed that placing three rats in each cage gave results that were within a reasonable SEM. Thus the locomotor activity of three rats was used as one reading, and 18 rats were used to give six readings (n=6).

3.3 Results

The results from the various stress experiments carried out in this study indicate that the method used produced a 100% incidence of gastric stress ulceration in control rats. The starved rats, stressed according to this procedure, all developed many clearly discernible, measurable ulcers in the time prescribed.

The technique used to measure the area of ulceration as a percentage of the total stomach area did not produce any superior information to that measuring the total length of ulceration in mm (see figure 3.4). This method was thus used occasionally only for comparison to confirm results obtained with the total length of ulceration method of measurement.

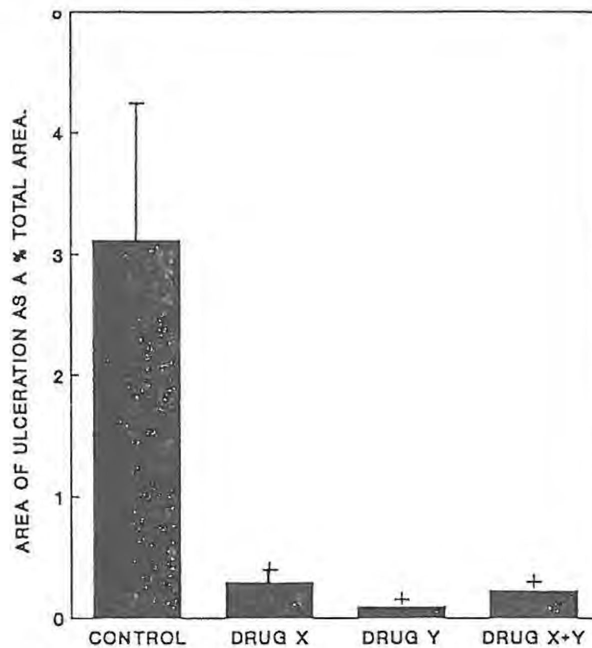
3.4 Discussion

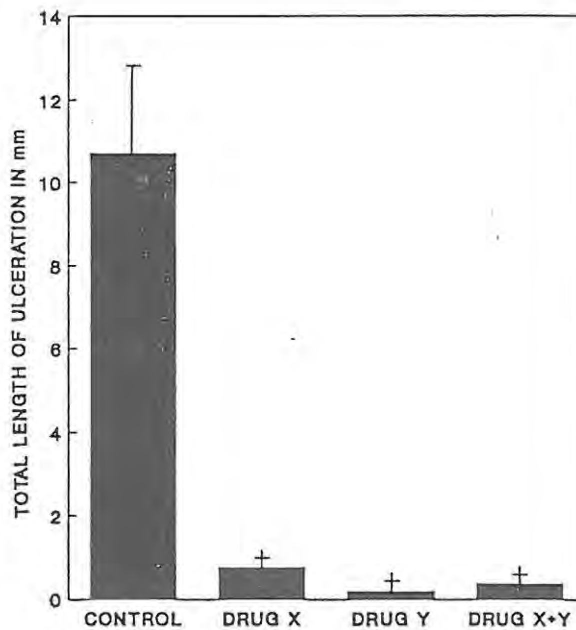
In 1936, Selye reported that exposure of rats to various nonspecific stressors led to the development of acute gastric ulcers. Initially, he observed the that tying up of a rats legs and wrapping them up tightly in a towel for long hours produced these lesions. Since then, many new and effective techniques have evolved, and these have been extensively reviewed (Brodie, 1971; Pare and Glavin, 1986).

The technique described by Senay and Levine (1967) has been successfully adapted and modified for use in our laboratory. A 100% incidence of gastric ulceration was noted in all rats stressed, indicating that this is in fact a very efficient method.

The use of ether as an anaesthetic was useful because of the ease of administration, lack of post-operative complications, and a good

Fig 3.4: COMPARISON OF THE TOTAL LENGTH OF ULCERATION METHOD, AND THE AREA OF ULCERATION AS A PERCENTAGE OF TOTAL AREA METHOD, OF ULCER MEASUREMENT





The area of ulceration method does not give any superior information to the total length method of ulcer measurement. The latter method was adopted for use in all experiments.

recovery rate.

The pinealectomy procedure of Hoffman and Reiter was very effective. Many rats survived the surgery, and removal of the pineal gland was relatively simple.

The methods described above were thus used in all experiments. Although starvation in itself is a mild stressor, the term stress as regards the experimental work will be used to describe the cold and restraint-immobilization procedure.

3.5 PRELIMINARY INVESTIGATIONS TO OPTIMIZE THE STRESS-INDUCTION PROCEDURE.

3.5.1 Introduction

The nature of this work called for the use of a very aversive technique, and as such, preliminary work was very necessary to adopt a method that would reduce the exposure of the animals to the stressful stimuli to an absolute minimum.

Several criteria had to be met in order to assess the validity and efficacy of a reasonable model of stress induction leading to gastric ulceration, and at the same time minimizing the exposure of the animals to the stressful stimuli. The criteria included:

1) A minimization of the pre-stress starvation period,
2) A minimal exposure to the major stressors, and
3) The production of true ulcers.

The following experiments were carried out in an attempt to optimize the stress ulcer-induction technique.

3.5.2 Materials and Methods

3.5.2 a) Rats (n=4) were starved for 12 hours and 24 hours respectively. They were then killed by neck fracture and stomachs were rapidly dissected out and examined for gastric ulceration.

3.5.2.b) Rats (n=2) were starved for 24 hours and subjected to the stress procedure as described in section 3.2.3 for a duration of 1.5, 2, 2.5 and 3 hours at cold temperature (4-7°C) respectively. They were then killed by neck fracture, and stomachs were examined for the presence of ulceration.

3.5.3 Results

Examination of the stomachs from experiment 3.5.2.a showed a total absence of gastric lesions at both time periods. However, at t=12 hours, the rats still had some food remaining in the stomach.

The rats exposed to restraint-immobilization stress for two hours in experiment 3.5.2.b. developed many clearly recognizable, measurable ulcers (average total length of ulceration = 9.2mm) that could easily be discerned from petechiae and small surface erosions. The rats exposed to the major stressors for 1.5 hours developed few ulcers. (Average total length of ulceration = 3.6mm)

Both rats tested for the 3 hour period died before the stress exposure time had expired.

3.5.4 Discussion

It is now recognized that experimental ulcers are more reliably produced if the gut of the animal is "primed" by gastric emptying due to a period of food deprivation prior to the induction of restraint stress (Pare and Glavin, 1986).

The previous use of long periods of starvation (48-72 hours) often produced ulcers located only in the rumen of the rat stomach, indicative of starvation and not of exposure to stress (Pare and Temple, 1973).

A total lack of ulceration was noted after both the starvation periods used in this experiment. No rumenal ulceration, indicative of starvation,was present. However, after the 12 hour time period, the presence of food in some stomachs was noted. Food-deprived rats require a shorter duration of exposure to stress before gastric ulcers are manifested (Buchel and Gallaire, cited in Pare and

Glavin, 1986) when compared to fed rats, indicating that the presence of food in the stomach inhibits the production of lesions to some extent. Thus a 24 hour starvation period was used in all experiments.

It is well known that exposure to multiple stressors produces a high incidence of gastric ulceration within a short period of time (Dai and Chan, 1983). The presence of many ulcers after two hours of exposure to restraint-immobilization and cold temperatures indicated that this was the minimum time required for exposure to the major stressors.

Thus the optimum conditions that had to be used (which spared the animal any further discomfort) were a pre-stress starvation period of 24 hrs, followed by 2 hours exposure to restraint-immobilization and cold (4-7°C).

3.6 EXPERIMENTS

3.6.1. EXPERIMENT 1: THE EFFECT OF STRESS ON PINEAL SNAT ACTIVITY

3.6.1.1. Introduction

A transsynaptic adrenergic mechanism controls the circadian rhythm in the activity of pineal SNAT, the enzyme regulating large changes in melatonin production.

One of the responses to stress is the release of catecholamines from nerve endings. The pineal gland is an end organ of the sympathetic nervous system, and may thus exhibit changes in the secretion of melatonin in response to certain stressors.It has been observed that catecholamines released into the circulation during acute exposure (2-2.5 hrs) to stress, can increase pineal SNAT activity (Lynch *et al,* 1973). Exposure to certain stressors can elicit a four fold increase in SNAT activity (Vaughan *et al*, 1978).

This experiment was undertaken to examine the effects of stress on pineal SNAT activity.

3.6.1.2 Materials and methods

Groups of rats were either maintained on their normal diet or starved for 24 hours prior to experimentation. At 21h00, one group of starved rats (n=4) was exposed to cold and restraint immobilization stress as described earlier (section 3.2.3) for 2 hours. At 23h00 the stressed rats, as well as one group (n=5) of starved rats and one group of fed (n=5) rats were killed by neck fracture and then decapitated. The pineal glands were rapidly removed (see chapter2) and assayed for SNAT activity according to the method of Champney *et al* (1984) (see section 3.2.6).

3.6.1.3 Results

Fig 3.5 describes the results obtained in this experiment. Starved rats show a slight but non-significant increase in SNAT activity as compared to control rats. Restraint immobilization and cold stress induced an almost four fold increase in SNAT activity. ($F(df=12) = 4.32$, $p < 0.05$).

3.6.1.4 Discussion

The results from this study support the possibility of an activation of the melatonin biosynthetic pathway on exposure to stress.

Different stressors seem to elicit different responses as regards pineal SNAT activity and melatonin synthesis.

During the light phase, insulin induced hypoglycaemia and restraint immobilization have been shown to increase pineal SNAT activity. In contrast, subjecting rats to hind leg saline injection at night (Joshi *et al*, 1986) or forced swimming (Troiani *et al*, 1988) results in a depression of melatonin synthesis.

Histological studies (Miline, 1979) have suggested an increase in pinealocyte activity in response to stress, indicative of a possible increase in melatonin synthesis and release.

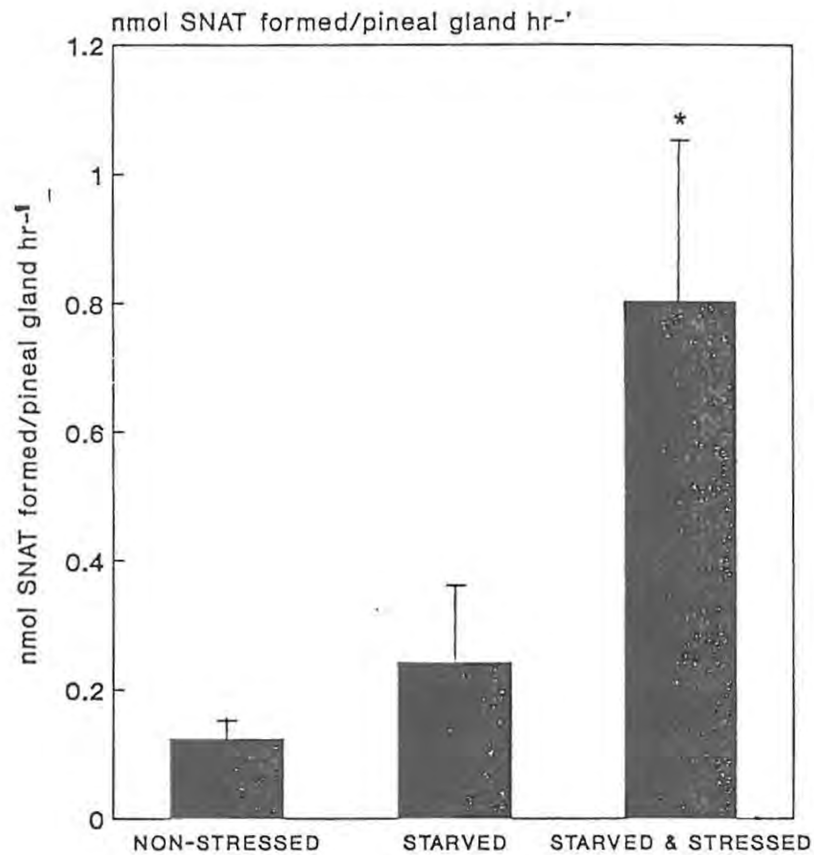


Fig 3.5: SNAT activity was measured in starved, starved and stressed, and unstressed rats. Stressed rats show an approximately four-fold increase in SNAT activity as compared to controls.

* = significantly different to control.

$F(df=12) = 4.32$, $p < 0.05$.

A wide variety of acute stressors increases melatonin synthesis in the rat, and it could be suggested that the general response of the pineal gland to stress results in an increase in pineal activity.

3.6.2 EXPERIMENT 2: THE EFFECT OF MELATONIN ON THE PRODUCTION OF GASTRIC STRESS-INDUCED LESIONS IN RATS.

3.6.2.1 Introduction

Various stressful stimuli alter pineal melatonin production (Lynch *et al*, 1973, Tannenbaum *et al*, 1988, Vaughan *et al*, 1978). The exact role of the pineal hormone melatonin in stress is not clear. Studies have shown that exposure to stress can increase pineal SNAT activity, suggesting a possible activation of the melatonin biosynthetic pathway in response to certain aversive stimuli. (Vaughan *et al*, 1978). It is thus conceivable that melatonin could act as an anti-stress hormone.

One of the peripheral consequences of stress exposure in rats is gastric ulceration (Ader, 1964). The following study was undertaken to examine the effect of melatonin on stress-induced gastric ulceration.

3.6.2.2 Materials and Methods

Rats (n = 5) were starved as previously described and lightly anaesthetised with ether. They were then injected with melatonin (100 μg, 250 μg, and 500 μg/kg bw ip) or melatonin vehicle only (control).

The melatonin was freshly prepared by dissolving in a base containing 2% benzyl alcohol and 0.025% w/v citric acid to which 10% tween (polysorbate 80) was added. The injection was then made up to volume using distilled water.

The rats were then exposed to stressors (cold and restraint) for two hours, killed by neck fracture, and stomachs were examined for lesion production. (Section 3.2.3)

3.6.2.3 Results

Figure 3.6 summarizes the gastric stress ulcer data obtained from this experiment. At the low doses used in this study, melatonin was observed to induce a dose dependent inhibition of gastric stress ulceration. ($F(df=19) = 6.73$, $p < 0.01$). A 500 μg dose of melatonin was shown to induce an 88.79% ± 0.04% inhibition of lesion production.

3.6.2.4 Discussion

The restraint-immobilization and cold stress technique have been used to evaluate the efficacy of anti-ulcer compounds, (Szelenyi *et al*, 1986; Hanson and Brodie, 1960) as well as certain anti-stress pharmacological agents (Doteuchi and Costa, 1975; Hanson and Brodie, 1960).

In this experiment melatonin significantly inhibits the production of gastric stress-induced lesions. Due to the nature of this study, the effect of melatonin could be indicative either of an anti-stress effect at a central (CNS) or peripheral (adrenal glands) level, or of a protective effect at the level of the gastro-intestinal tract.

The presence of melatonin in the gastro-intestinal tract has been noted (Bubenik *et al*, 1976), with higher concentrations being found in the stomach and the duodenum than in other parts of the gut. These authors suggested that melatonin could participate in some aspect of intestinal physiology. Recent studies have shown that pre-incubation of rat stomachs with melatonin *in vitro* prevents ethanol induced gastric ulceration, possibly via a serotoninergic mechanism (Cho *et al*, 1988).

The effect of melatonin noted here, albeit an anti-stress effect or an anti-ulcerogenic effect, may reflect a physiological function of endogenous melatonin.

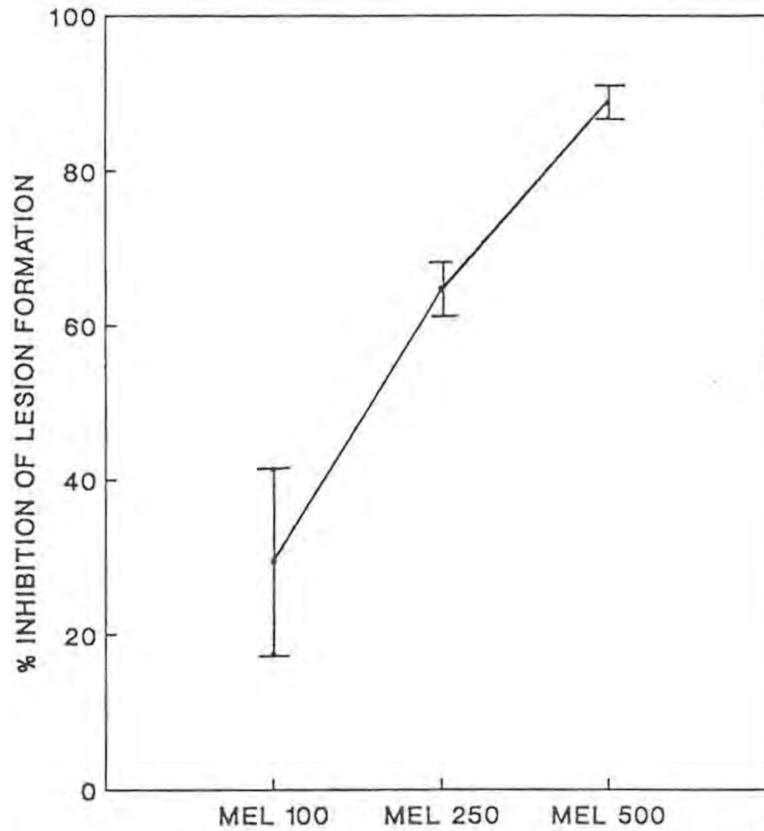


Fig 3.6: Melatonin (100 μg, 250 μg, and 500 μg/kg bw ip) was administered to rats immediately before the induction of stress. Melatonin induces a dose-dependent reduction in lesion formation at low doses.

$F(df=19) = 6.73$, $p < 0.01$.

3.6.3 EXPERIMENT 3 : INVESTIGATION INTO A POSSIBLE CIRCADIAN RESPONSE OF WISTAR RATS TO STRESS.

3.6.3.1 Introduction

Pineal melatonin synthesis is known to undergo a circadian rhythm, with highest concentrations being produced during the dark phase of the light dark cycle (Axelrod, 1974). The previous study showed that administration of exogenous melatonin produced a dose dependent reduction in gastric ulceration. The possibility thus exists that the high endogenous melatonin levels present during the dark phase of the LD cycle could facilitate a reduction in gastric stress ulceration as compared to daytime exposure to stress.

The following experiment was carried out to determine whether exposure of rats to stress in the dark phase produced any significantly different results when compared to light phase controls.

3.6.3.2 Materials and Methods

Female Wistar rats (n=10) were starved for 24 hours as described above (3.2.3). At 21h00 they were subjected to the stress procedure as described (section 3.2.3) for a period of 2 hours. A dim red photosafe light source was used to facilitate handling of the animals during stress induction.

Exposure of the rats to light during the dark phase immediately suppresses melatonin synthesis, and as such care had to be taken to prevent stray light from entering the experimental area, which, except for the doorway, was totally sealed off.

3.6.3.3 Results

Interestingly, the results indicate that exposure to stress at night leads to a reduced incidence of gastric stress lesions (see fig 3.7). Daytime experiments (n=25) indicated the presence of many more ulcers as compared to rats stressed at night under exactly the

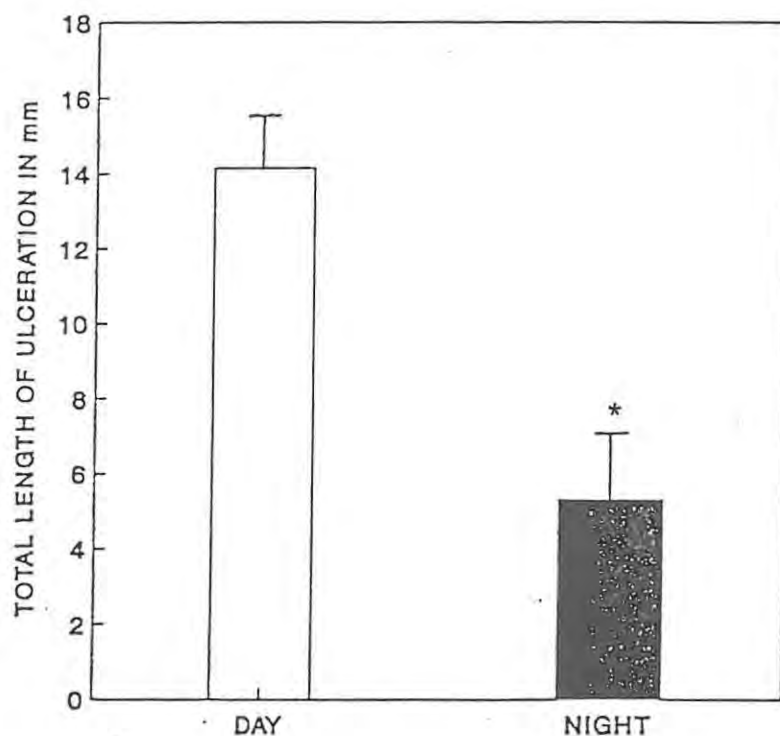


Fig 3.7: Exposure of rats to stressful stimuli in the dark phase leads to a significant reduction in lesion formation as compared to light phase controls.

$t = 3.46$, $p < 0.001$.

* = significantly different to control

same conditions, in the absence of light. ($t = 3.46$, $p < 0.001$)

3.6.3.4 Discussion

The rats stressed at night show a reduced susceptibility to gastric ulceration when endogenous melatonin levels are high. It could be implied that this high level of melatonin present in the rat during the dark phase is probably responsible for the reduced effects of stress as manifested by gastric ulceration observed in these rats.

However, various other rhythms persist in the body, and, although the possibility does exist, the results from this experiment cannot at this stage be directly attributed to the increased circulating levels of melatonin present at night. To clarify this, the following experiment was performed.

3.6.4 EXPERIMENT 4: THE EFFECT OF PINEALECTOMY ON THE NIGHTTIME RESPONSE TO STRESS.

3.6.4.1 Introduction

The exact role of the pineal gland in stress is not clear. Research has indicated that stress induces an increase in activity in the pineal gland (Miline, 1980).

It has been shown that certain progressive structural changes occur in the pineal gland on chronic exposure to low temperature (cold stress) and irradiation (800 r) stress (Miline 1980). These include hypertrophy and hyperplasia of the pinealocytes, hypertrophy of the Golgi zone, and an abundance of free ribosomes.

Miline (1980) thus proposed that the pineal gland plays a protective role in the maintenance and fundamental stabilization of homeostasis under stress induced conditions.

From the previous experiments it could be suggested that melatonin, and possibly the pineal gland, are responsible for the amelioration of the effects of stress as manifested by gastric ulceration.

Melatonin levels during the light phase of the LD cycle are very low (Axelrod, 1974). At night, the release of NA from the postganglionic neurons onto the pineal β-receptor activates the melatonin biosynthetic pathway and hence leads to an increase in melatonin production (Axelrod, 1974).

This experiment was undertaken to observe the effects of pinealectomy on the nighttime response to stress.

3.6.4.2 Materials and Methods

Groups (n=6) of intact, sham-operated, and pinealectomized rats, were starved as previously described. They were exposed to stress for 2 hours at 21h00 (3 hours after lights off), sacrificed by neck fracture, and stomachs were examined for ulceration. (Section 3.2.3) The rats were decapitated, and brains were examined to ensure the success of the pinealectomy procedure.

3.6.4.3 Results

Figure 3.8 describes the results obtained from this experiment graphically.

Once again, a reduced incidence of gastric ulceration was observed in intact rats stressed in the dark phase. The total length of ulceration between the intact (3.42 ± 0.52) and sham operated (5.13 ± 1.17) rats did not vary significantly. Pinealectomised rats showed an increased incidence of ulceration, ($F(df=17) = 6.07$, $p < 0.01$) and the total length of ulceration (14.03 ± 2.68) observed in these rats is comparable to previous daytime controls (See figure 3.7).

All the pinealectomies were successful. On post mortem the rat brain appeared normal when compared to that of an unoperated rat, except for the absence of a pineal gland. No inflammation or presence of clotting was noted.

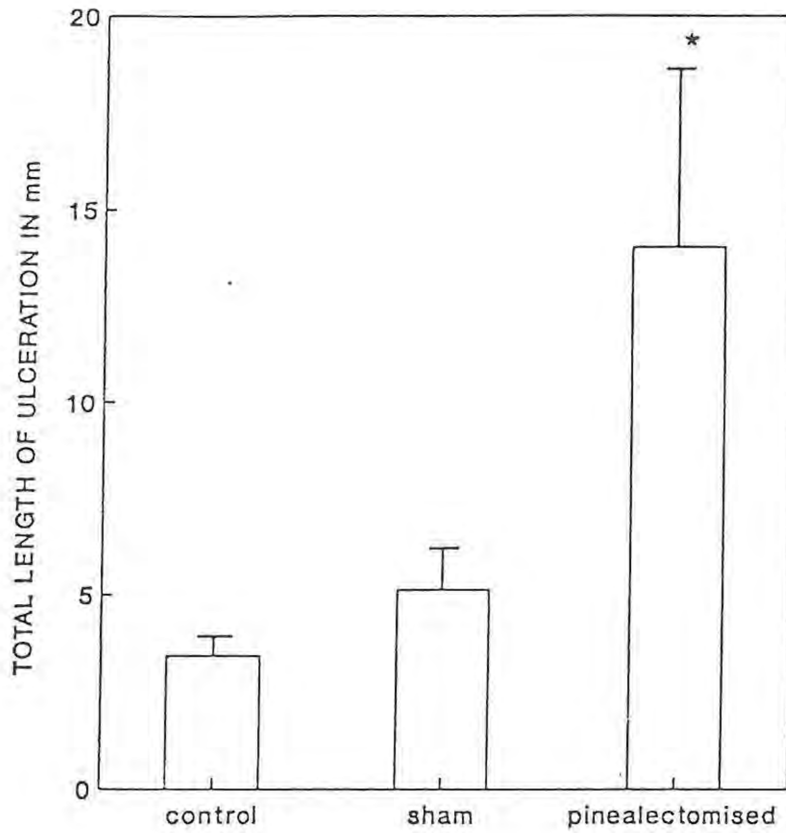


Fig 3.8: Intact, sham-operated and pinealectomized rats were exposed to stress in the dark phase. Pinealectomized rats present with a significant increase in gastric ulceration.

F(df=17), $p < 0.01$.

* = significantly different to control.

3.6.4.4 Discussion

Although the exact role of the pineal gland in the response to stress has not been elucidated, it has been suggested that the pineal gland is anti-stressogenic (Reiter, 1982)

Miline (1979) conducted various histological studies on the effects of stress on the pineal gland, and concluded that the pineal has a protective role in stress. The author has also obtained results indicative of a functional antagonism between the pineal gland and the hypophysis, and suggests that the pineal gland actively participates in the general adaptation syndrome and reacts to protect the organism against stress induced effects.

Rivest (cited in Romijn, 1978) suggested that the primary function of the pineal gland in stress is to control and regulate the physiological reactions of defence and adaptation in the general adaptation syndrome.

The results of this study indicate that the pineal gland is necessary for the protection of the organism against stress-induced gastric ulceration. The presence of a pineal gland protected the control group of rats against ulceration at night, and pinealectomy reversed this effect. It could thus be suggested that the pineal gland is responsible for the circadian resistance to stress-induced ulceration seen earlier (see fig 3.7).

The results from this experiment strengthen the hypothesis that the pineal gland plays a protective role in the response to stress. These observations are also suggestive of a possible anti-stress role for the pineal hormone melatonin.

3.6.5 EXPERIMENT 5: THE EFFECT OF MELATONIN ON STRESS INDUCED GASTRIC ULCERATION IN PINEALECTOMISED RATS.

3.6.5.1 Introduction

The previous experiments have indicated that pinealectomy

exacerbates the production of stress induced gastric ulceration (see fig 3.8), and that melatonin administration to intact rats in the light phase of the LD cycle inhibits lesion formation to a great extent (see fig 3.6)

The following experiment was undertaken to determine whether melatonin is one of the pineal substances responsible for the reduced incidence of lesion formation observed in intact rats in the dark phase of the LD cycle.

3.6.5.2 Materials and Methods

Groups (n=5) of pinealectomised rats were starved for 24 hours prior to experimentation. At 21h00, they were lightly anaesthetized with ether and injected with melatonin 250 μg, 500 μg, or 1 mg/kg b.w. or with melatonin vehicle only (control group), via the intraperitoneal route. They were then subjected to the stress procedure as previously described (Section 3.2.3). The rats were handled in the dark, in the light of a single dim photosafe red light source. After sacrifice, brains were examined to confirm the pinealectomy.

3.6.5.3 Results

Fig 3.9 indicates that the administration of small doses of melatonin to pinealectomised rats results in a dose dependent inhibition of gastric lesion formation ($F(df=19) = 6.82$, $p < 0.005$).

Once again, an increase in gastric ulceration, comparable to the daytime effects of stress on gastric lesion formation, is noted in pinealectomised rats.

A 100% success rate of the pinealectomies was attained. Once again, the rat brain appeared normal, and there was no visual indication of damaged or inflamed tissue.

3.6.5.4. Discussion

Miline (1979) noted that an aqueous extract of the pineal gland

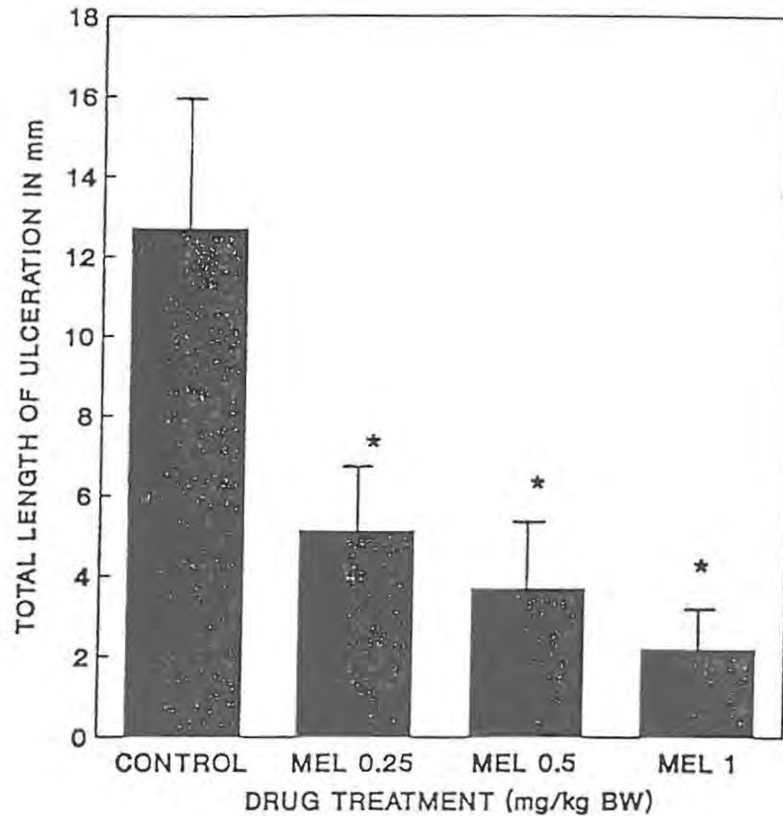


Fig 3.9: Pinealectomized rats were treated with melatonin vehicle, or with melatonin (0.25 mg, 0.5 mg, or 1mg/kg bw ip) prior to stress induction in the dark phase. Melatonin inhibits lesion formation in pinealectomized rats stressed in the scotophase, suggesting that it is probably the prime pineal substance responsible for the reduction in lesion formation noted in intact rats (see fig 3.8).

$F(df=19) = 6.82$, $p < 0.005$.

* = significantly different to control.

caused an inhibition in aggressiveness in the male species of the fish *Betta splendens* as well as an inhibition of aggressiveness and crowing in cocks of the Bantam breed. Changes in the morphodynamics of the thyroid gland, the adrenal gland, and the lymphatic gland after exposure to irradiation stress (800 r) were very mild in animals treated with this aqueous extract as compared to the strong reactions shown in animals not treated with this extract.

It has been suggested that the pineal gland modulates adrenal steroidogenesis in normal as well as stress conditions (Romijn, 1978). Mehdi and Sandor (1977) suggested that melatonin may be involved in a modulation of corticosteroidogenesis. Dickson and Hasty (1972) proposed that the pineal gland secretes a substance that acts at the level of the hypothalmo-pituitary axis to reduce the production and release of ACTH with a resultant inhibitory influence on the pineal gland.

Although the other pineal substances have not been excluded, it is evident from this study that melatonin is one of the important pineal substances responsible for the reduction of gastric lesion production observed in rats at night.

The exact mechanism of this action has yet to be elucidated.

3.6.6 EXPERIMENT 6 : THE EFFECT OF MELATONIN ON INDOMETHACIN INDUCED GASTRIC ULCERATION.

3.6.3.1 Introduction

Cardinali *et al* (1987) investigated the effect of prostaglandins (PG's) on the pineal gland. They found that α-adrenergic stimulation of pineal explants results in PGE_2 release in the rat pineal gland. Cardinali and Ritta (1983) also observed that the administration of PG synthesis inhibitors eg indomethacin, impaired the nocturnal rise in pineal melatonin synthesis and decreased NA-stimulated melatonin release. *In vitro* addition of PGE_2 increased rat pineal cAMP levels, SNAT activity, and melatonin release.

The high concentrations of PG's in the gastro-intestinal wall throughout the GIT suggest that these agents may play an important role in gastro-intestinal physiology. It has been shown that the E type PG's are capable of preventing the formation of gastric ulcers induced by cold stress (Kawarada *et al*, 1975) and a wide variety of non-steroidal anti-inflammatory drugs (NSAID's) such as aspirin and indomethacin (Lippman, 1974).

Melatonin is also found in the GIT, and although a reason for its presence here has not been elucidated, it has been suggested that the hormone may play a physiological role in the GIT.

3.6.3.2 Materials and Methods

An indomethacin suspension was prepared in 0.5% w/v carboxymethylcellulose for oral administration. Rats (n=5) were starved for 24 hours as previously described (section 3.2.3) and then fed with either 1) vehicle only, 2) indomethacin suspension (200mg/kg/ml) only, 3) or indomethacin suspension (200mg/kg/ml) followed by an ip injection of melatonin (1 mg/kg bw). Groups 1 and 2 were also injected ip with melatonin vehicle.

The rats were killed after 4 hours by neck fracture. Stomachs were dissected out, and examined for ulceration as previously described (section 3.2.3).

3.6.6.3 Results

Rats treated with vehicle only (group 1) presented with no gastric ulceration. Group 2 and group 3 had gastric lesions. These were distinguishable from stress lesions in that they were smaller, round and oval shaped ulcers, and they were clearly defined. Administration of mel (1mg/kg bw ip) did not affect lesion formation significantly (see fig 3.10).

3.6.3.4 Discussion

It is thought that the prostaglandins have "cytoprotective" properties that prevent mucosal injury (Roberts, 1979). The results

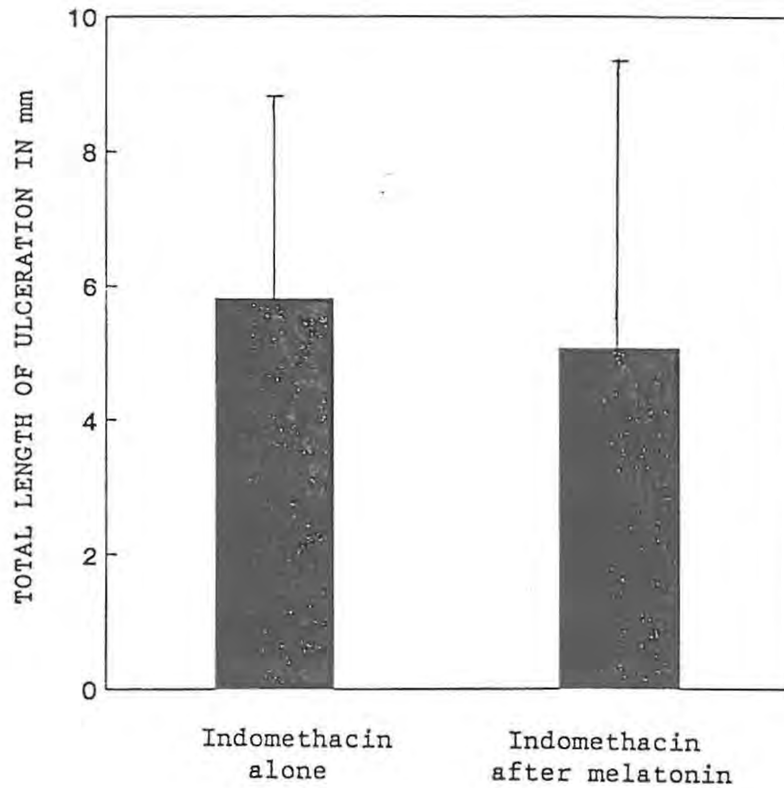


Fig 3.10: Rats were given an oral dose of indomethacin, and immediately injected with melatonin (1 mg/kg bw ip) or melatonin vehicle. Drug-induced gastric ulceration was measured 4 hours later. Melatonin could not reverse the effects of indomethacin on gastric ulceration.

of this experiment show that blockade of *de novo* synthesis of prostaglandins by the administration of a cyclo-oxygenase inhibitor (indomethacin) results in gastric ulceration. The inability of melatonin to counter this effect suggests that melatonin does not have a protective effect on drug-induced gastric ulceration, and does not act via the prostaglandin system.

3.6.7 EXPERIMENT 7: THE EFFECT OF VARIOUS DOSES OF MORPHINE ON STRESS INDUCED GASTRIC ULCERATION.

3.6.7.1 Introduction

Endogenous opioids have been implicated in many of the biochemical, physiological, and behavioural responses to stress (Adams, 1987).

In 1977, Guilleman *et al* showed that, in rats, β-endorphin and ACTH are secreted simultaneously by the pineal gland in response to stress. This stirred much interest as to the role of the opiates in stress. Many studies have since examined the role of the opiates and opiate antagonists in gastric stress pathology, and contradiction exists as to their exact function.

Much of the apparent conflict in the literature may be due to differences in the route of administration, the drug dose administered, the method of stress employed, and the chronicity of the challenge.

Some investigators have found that the opiate antagonist, naloxone, decreases stress ulcer formation (Dai and Chan, 1983; Del Tacca *et al*, 1987). Others have found that intraperitoneal or intracerebroventricular administration of opiates reduces gastric stress ulceration.

This experiment was undertaken to examine the effects of morphine on stress induced gastric lesions.

3.6.7.2 Materials and Methods

Rats (n=5) were starved for 24 hours, anaesthetized with ether, injected with morphine HCl (1 mg, 5 mg, or 10 mg/kg bw ip) or control vehicle (normal saline).

They were killed by neck fracture after 2 hours, and stomachs were examined for gastric ulceration (see section 3.2.3).

3.6.7.3 Results

Fig 3.11 shows that morphine induces a dose dependent inhibition of gastric ulceration at the doses used in this study. ($f(df=19) = 11.13$, $p < 0.0005$)

3.6.7.4 Discussion

Arrigo-Reina and Ferri (1980) showed that exposure of rats to stressful stimuli (restraint, cold [4°C for 5 hours]) led to the production of gastric lesions. Treatment of rats with 10mg/ kg bw morphine inhibited stress lesion formation to an extent that it was practically non-existent.

The results obtained in this study disagree with those of Ho *et al* (1984) who observed that morphine produced a naloxone reversible, dose dependent exacerbation of lesion production in pylorus ligated rats. Restraint stress ulceration and pylorus ligation induced ulceration have different etiologies, especially as pertains to the localisation of the gastric damage. Stress ulcers occur in the glandular region of the stomach, whereas pylorus ligation induced ulceration occur in the non-secreting portion of the stomach (rumen) - and the discrepancy in the results obtained could be indicative of a possibility that morphine and naloxone exert different effects when used with different ulcer inducing methods.

In fact, it has been shown by other investigators (Tanaka *et al*, 1983) that morphine accelerates brain NA turnover and core temperature in non-stressed rats, and the reverse occurs in stressed rats (ie reduced NA turnover and hypothermia).

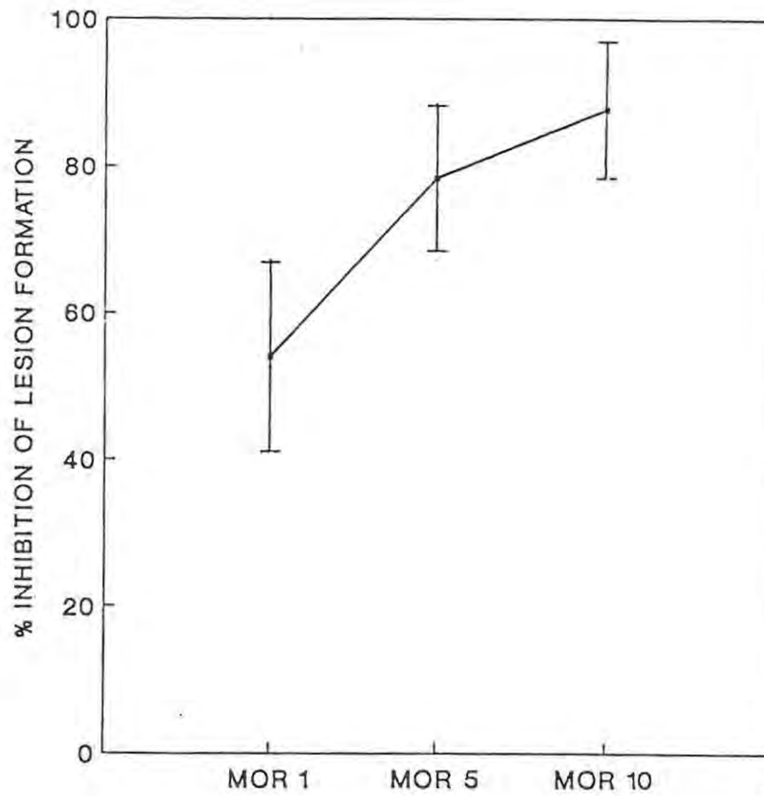


Fig 3.11: Rats were treated with morphine (1 mg, 10 mg, or 5 mg/kg bw ip) or saline vehicle prior to exposure to stress. Morphine induces a dose dependent inhibition of gastric stress lesion formation in rats, with the 10 mg/kg dose affording an almost 90% protection as compared to controls.

$F(df=19) = 11.13$, $p < 0.0005$)

The findings of this study show clearly that, under these conditions of stress, morphine significantly inhibits gastric stress ulceration in a dose dependent manner. These results are supported by the reports of other investigators (Glavin, 1985; Arrigo-Reina and Ferri, 1980; Glavin *et al*, 1986; Ferri *et al*, 1983) who observed similar effects of morphine on stress induced gastric ulceration.

These observations thus suggest a protective effect of morphine against gastric damage caused by exposure to stress.

3.6.8 EXPERIMENT 8 : THE EFFECT OF VARIOUS DOSES OF NALOXONE ON STRESS INDUCED GASTRIC ULCER FORMATION.

3.6.8.1 Introduction

The pituitary and adrenal glands play an important role in regulating the physiological responses to stress (Selye, 1974). β-endorphin and ACTH are derived from a common precursor molecule (Mains *et al*, 1977) and are released simultaneously in acute stress (Guilleman *et al*, 1977). Enkephalin-like material may also be stored with catecholamines in adrenal medullary chromaffin cells, and may be released simultaneously in response to stress (Viveros *et al*,1979). These observations suggest a possible role for the opioids in stress.

Contradictory evidence has been found on the effects of the opiate antagonist naloxone on gastric ulceration induced by stress.

Dai and Chan (1983) observed a significant reduction in gastric stress ulceration using high doses of naloxone (25 mg and 50 mg/kg bw), and suggest that the endogenous opioids may play a role in the causation of gastric stress ulceration. Del Tacca *et al* (1987) also observed a reduction in severity of ulceration in pylorus ligated rats using smaller (4 mg/kg bw ip) doses of naloxone.

Other workers have found evidence to the contrary (Glavin, 1985; Ferri *et al*, 1983; Glavin *et al*, 1986) showing an exacerbation of

gastric stress lesions by naloxone.

The following study was thus undertaken to observe the effects of various doses of naloxone on gastric stress ulceration.

3.6.8.2 Materials and Methods

Rats were starved for 24 hours, injected with naloxone (10 mg, 20 mg, or 30 mg/kg bw ip) or control vehicle (normal saline), and subjected to stress for 2 hours. (see section 3.2.3)

They were killed by neck fracture, stomachs were removed, and examined for gastric ulceration.

3.6.8.3 Results

The results from this study indicate that naloxone induces a dose dependent exacerbation of gastric ulceration, evident from the smallest dose of naloxone used in this study. ($F(df=19) = 30.21$, $p < 0.0001$) - see figure 3.12.

3.6.8.4 Discussion

The results observed in this study are supported by the reports of various other investigators (Arrigo-Reina and Ferri, 1980; Glavin, 1985; Ferii *et al*, 1983; Glavin *et al*, 1986).

Dai and Chan (1983), however, found that naloxone inhibits stress lesion formation. The experimental design used by these authors differs from this and other studies, in that the animals used in their study were allowed access to an 8% sucrose solution throughout the pre-stress starvation period and were exposed to restraint immobilization and cold for only 1 hour.

Glavin (1980) suggests that both these factors tend to reduce the incidence of gastric ulceration. Thus, using this procedure, the method of Dai and Chan (1983) may have been inadequate to determine the effects of naloxone on gastric stress pathology.

It has been suggested that the endogenous opioids may operate in a

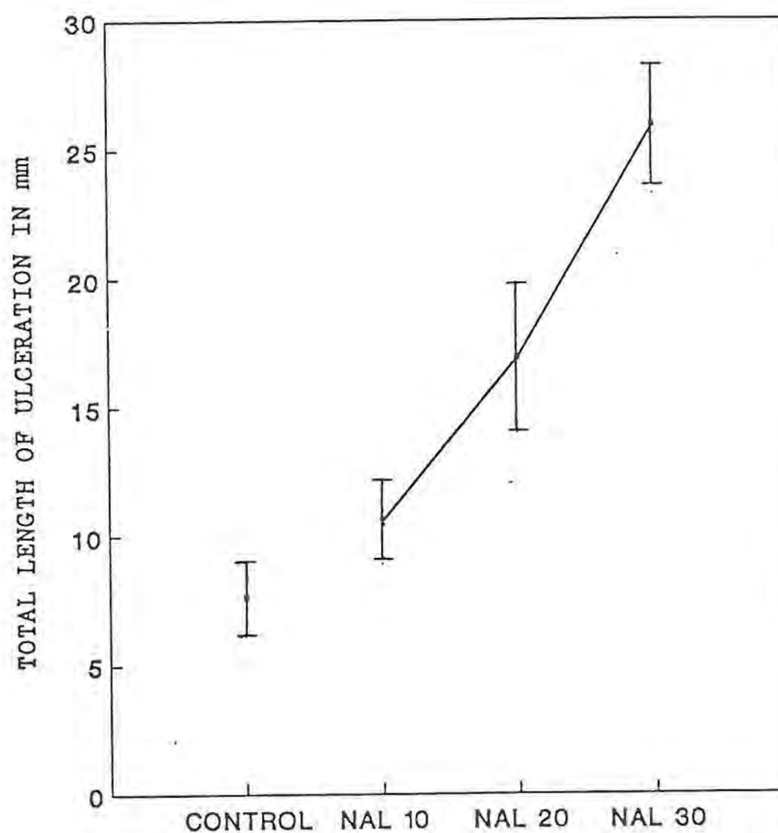


Fig 3.12: Naloxone (10 mg, 20 mg, and 30 mg/kg bw ip) or saline vehicle was administered to rats prior to exposure to stress. Naloxone induces a dose-dependent exacerbation of lesion formation.

F(df=19) = 30.21, $p < 0.0001$.

homeostatic system in the adaptive response of the organism to stressful stimuli (Arrigo-Reina and Ferri, 1980).

The results obtained from this study support their argument to some extent. The effects of the stress-induced release of β-endorphin (Guilleman *et al*, 1977) were possibly blocked by the opiate antagonist naloxone, with a resultant exacerbation in lesion production, suggesting a role for the endorphins in stress. However, this finding is based on the assumption that naloxone only exerts an opiate-antagonizing effect, and cannot exclude the possibility that this exacerbation of stress induced ulceration is due to a yet unknown possible pharmacological action of naloxone.

3.6.9 EXPERIMENT 9: THE EFFECT OF NALOXONE ON GASTRIC STRESS ULCERATION IN MORPHINE TREATED RATS.

3.6.9.1 Introduction

The previous experiments have shown that morphine induces a dose dependent inhibition in lesion formation (fig 3.11) and naloxone exacerbates lesion formation in a dose dependent manner (fig 3.12).

This experiment was undertaken to examine the effects of various doses of naloxone on stress ulceration induced in morphine treated rats.

3.6.9.2 Materials and Methods

Rats (n=5) were starved for 24 hours prior to experimentation. One group of rats was injected with control vehicle only (normal saline), another with morphine (5mg/kg bw ip) only, and the remaining groups with morphine 5mg/kg bw and naloxone (10 mg, 20 mg, or 30 mg/kg bw) prior to stress induction. After exposure to stressors, they were sacrificed by neck fracture and stomachs were examined for ulceration (see section 3.2.3)

3.6.9.3 Results

The results (fig 3.13) indicate that morphine reduces the incidence of lesion formation as compared to control rats. This effect of morphine was significantly antagonised by the 20 mg/kg and 30 mg/kg doses of naloxone. The administration of 10 mg/kg of naloxone had no significant exacerbating effect on morphine induced inhibition of lesion formation. Although naloxone exacerbated lesion formation at the higher doses, the total length of ulceration was reduced in the presence of morphine for both the 20 mg/kg (12.82 ± 3.62) and the 30 mg/kg (14.63 ± 3.26) doses when compared to the effect of 20 mg/kg (16.92 ± 2.86) and 30 mg/kg (25.91 ± 2.33) naloxone in the absence of morphine (see fig 3.12).

3.6.9.4 Discussion

The results obtained from this study indicate, once again, that morphine inhibits, and naloxone exacerbates, the production of stress induced gastric ulceration.

These observations are supported by other investigators (Arrigo-Reina and Ferri, 1980; Ferri *et al*, 1983; Glavin, 1985; Glavin *et al*, 1986) who also observed a naloxone reversible inhibition of lesion formation by morphine.

3.6.10 Experiment 10 : THE EFFECT OF MORPHINE AND MELATONIN ON STRESS INDUCED GASTRIC ULCERATION.

3.6.10.1 Introduction

The production of the pineal hormone, melatonin, is known to be altered by various stressful stimuli (Lynch *et al*, 1973; Vaughan *et al*, 1978; Tannenbaum *et al*, 1988; Troiani *et al*, 1988). Studies have shown an activation of the melatonin biosynthetic pathway on exposure to stressful stimuli in rats (exp 1, Vaughan *et al*, 1978; Lynch *et al*, 1973) suggesting a possible role for the pineal gland in stress.

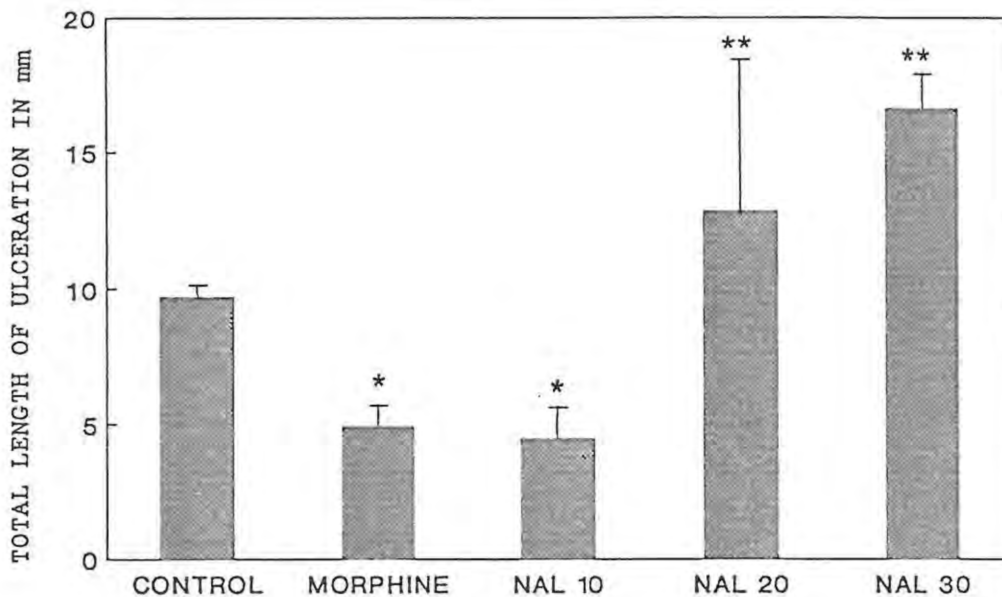


Fig 3.13: Naloxone (10 mg, 20 mg and 30 mg/kg bw ip) or saline was administered to morphine treated rats prior to stress-induction. Control rats were injected with saline only. Morphine inhibits lesion formation. Naloxone reverses the effect of morphine (5 mg/kg bw ip) on stress-induced gastric ulceration.

* = significantly different to control and naloxone (20 mg and 30 mg).

** = significantly different to control as well as morphine and naloxone (10 mg).

$F(df=24) = 3.57$, $p < 0.05$.

The opioidergic system is also known to attenuate the effects of stress in animals (Tanaka *et al*, 1985; Dai and Chan, 1983; Del Tacca *et al*, 1983; Selye, 1974; Glavin, 1985; Ferri *et al*, 1983; Glavin *et al*, 1986). A direct physiological mechanism for this action has not yet been elucidated.

It was recently observed that melatonin counteracts the effects of acute stress on thymus weight, acting via an opioidergic mechanism (Maestroni *et al*, 1988). This suggests a possible interaction between these two systems in the modulation of the stress response.

The following experiment was undertaken to examine the effects of a combination treatment of morphine and melatonin on gastric stress ulceration.

3.6.10.2 Materials and Methods

Rats (n=5) were starved for 24 hours prior to experimentation and they were injected with vehicle, morphine HCl (10mg/kg bw), mel (1mg/kg bw) or morphine (10 mg/kg) plus melatonin (1 mg/kg) prior to stress induction. The animals were sacrificed 2 hours later, and stomachs were examined for the presence of ulceration as described earlier (section 3.2.3).

3.6.10.3 Results

The results (fig 3.14) indicate, once again, that melatonin and morphine individually reduce the incidence of gastric stress ulcer formation in rats. A combination of these two treatments shows neither antagonism nor potentiation of this effect ($F(df=19) = 20.82$, $p < 0.0001$).

3.6.10.4 Discussion

Yet again, it is evident from this study that both morphine and melatonin have a protective action against gastric stress induced ulceration.

The exact locus of this action, be it a centrally mediated or a

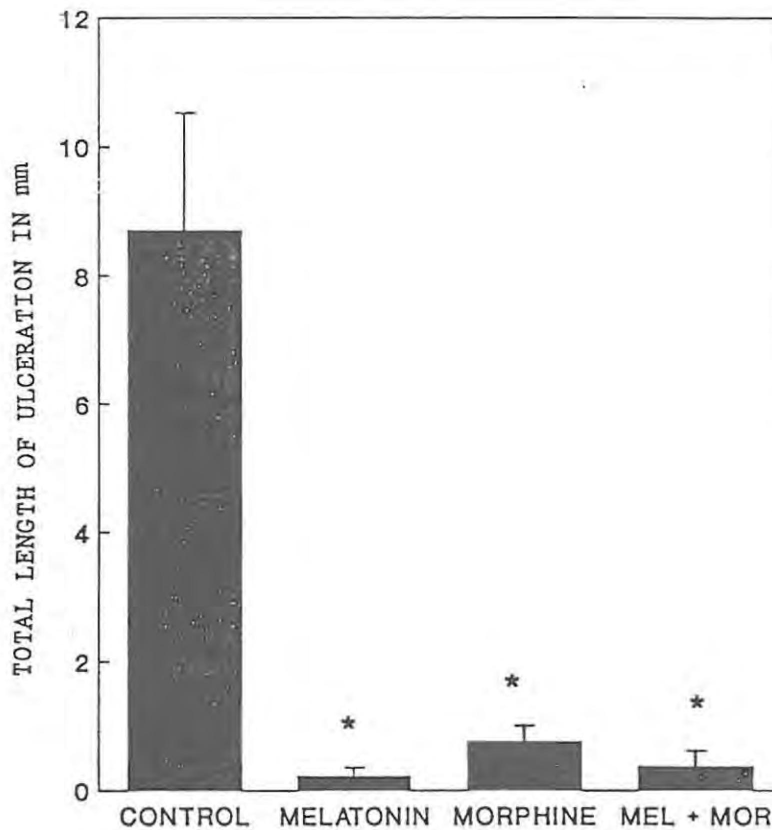


Fig 3.14: Rats were treated with saline vehicle, melatonin (1 mg/kg bw ip), morphine (10 mg/kg bw ip), or melatonin (1 mg/kg) plus morphine (10 mg/kg). Both morphine and melatonin significantly inhibit lesion formation and a combination treatment shows no antagonism of this effect.

$F(df=19) = 20.82$, $p < 0.0001$.

* = significantly different to control.

peripheral effect, has yet to be determined.

3.6.11 Experiment 11 : THE EFFECT OF NALOXONE ON MELATONIN INDUCED REDUCTION IN GASTRIC STRESS ULCERATION.

3.6.11.1 Introduction

The results from the previous experiment show that both morphine and melatonin reduce gastric stress ulceration. The question thus arises whether melatonin reduces gastric ulceration via an opioidergic mechanism. Alternatively, the noted effect could result from an action of morphine and melatonin via their own separate mechanisms. If melatonin is acting via an opioidergic mechanism, it would be expected that the opioid antagonist naloxone would counteract its stress-ulcer reducing activity.

This experiment was undertaken to elucidate the probability that melatonin was acting via an opioidergic mechanism to reduce gastric stress ulceration.

3.6.11.2 Materials and Methods

Rats (n=5) were starved for 24 hours as previously described (section 3.2.3). They were injected with control vehicle, melatonin 10mg/kg bw, naloxone 12.5 mg/kg bw, or melatonin 10 mg/kg bw plus naloxone (12.5 mg/kg bw), prior to stress induction.

2 hours later, they were killed by neck fracture, and stomachs were examined for ulceration.

3.6.11.3 Results

The results (fig 3.15) from this experiment indicate that:
1) naloxone exacerbates lesion formation; and
2) melatonin inhibits gastric stress lesion formation;
3) naloxone shows no antagonism of the inhibition of gastric stress ulceration by melatonin.

$(F(df=18) = 41.47, p < 0.0001)$

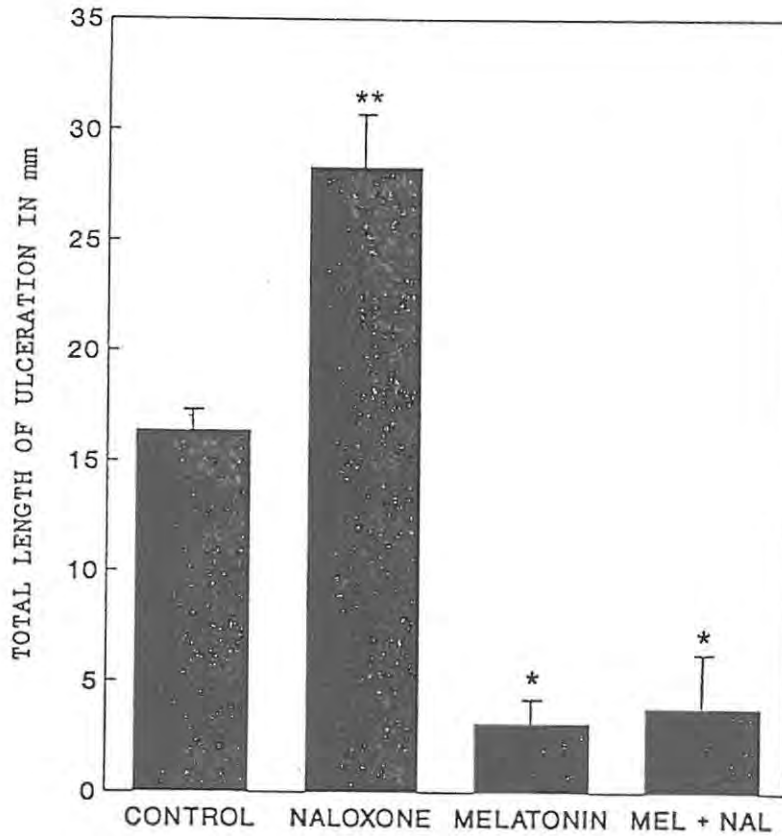


Fig 3.15: Rats were treated with saline, naloxone (12.5 mg/kg bw ip), melatonin (10 mg/kg bw ip), or melatonin (10 mg/kg) plus naloxone (10 mg/kg). Naloxone could not reverse the effects of melatonin on stress-induced gastric ulceration, suggesting that melatonin's action is not exerted via an opioidergic mechanism.

* = significantly different to control and naloxone.

** = significantly different to control and other treatment groups.

$F(df=19) = 41.47$, $p < 0.0001$.

3.6.11.4 Discussion

Recent observations have suggested a possible interaction between the pineal gland and the opioid system (Lakin *et al*, 1981; Esposti *et al*, 1986; Reiter, 1988; Lissoni *et al*, 1988)

Thymus involution, as well as gastric ulceration, is used as a marker for exposure to stress (Pare and Glavin, 1986). Maestroni *et al* (1988) recently observed that melatonin counteracts the effects of stress on thymus weight. This protective effect could be indicative of a possible anti-stress effect of melatonin.

These investigators, however, also observed that this effect of melatonin was exerted via an opioidergic mechanism. The results of the present study (fig 3.15) show that it is unlikely that melatonin does acts via an opioidergic mechanism to counter the effects of stress on gastric ulceration.

The study of Maestroni *et al* (1988) differs from the present study in that 1) thymus involution was used as a measure of exposure to stress; and 2) animals were restrained in 50 ml plastic tubes for 4 days from 10h00 in the morning. This method produced anxiety but not complete immobilization.

The application of stress in this manner could have had an immunosuppressive effect in the animals used in their study, resulting in thymus involution. Selye (1976) and Amkraut and Solomon (1974) have concluded that excessive stress can exert a generalized immunosuppressive effect - characterized by thymus involution.

It has been shown that β-endorphin modulates immune functions (Fischer and Falke, 1984). Maestroni *et al* have previously shown that inhibition of melatonin synthesis leads to a significant depression of humoral and cell-mediated immune responses in mice; and evening administration of melatonin antagonizes the immunosuppressive effect of corticosterone in mice (Maestroni *et al*, 1986). They also observed that this immunoaugmenting effect of melatonin was antagonized by naloxone (Maestroni *et al*, 1986).

It could perhaps therefore be suggested that the antagonism of thymus involution by melatonin noted by these workers is probably mediated via an immunological, and not an anti-stress mechanism.

From the above study (fig 3.15) it is clear that at the doses used here, naloxone does not antagonize the effect of melatonin on gastric ulceration, ruling out the possibility of an opiate mediation of melatonin's anti-stress effect.

The exact mechanism/s of action of melatonin and the pineal gland in stress have not yet been elucidated; and as such the clinical significance of these studies, as well as those of Maestroni *et al*, (1986) have yet to be clearly evaluated.

Taking into consideration that the opioid system is not responsible for melatonin's action in stress, the presence of GABA-ergic and benzodiazepine binding sites in the pineal gland suggests that GABA-ergic mechanisms may be involved in some aspect of pineal physiology (Cardinali, 1988) - and melatonin could exert some benzodiazepine-like psychopharmacological effects (Niles, 1988).

3.6.12 Experiment 12: EFFECT OF DIAZEPAM ON STRESS INDUCED GASTRIC LESIONS IN PINEALECTOMIZED RATS.

3.6.12.1 Introduction

The previous experiments carried out in this study have indicated that melatonin does not exert an anti-stress effect via an opioidergic mechanism. This experiment was undertaken to observe the effects of diazepam on gastric stress ulceration in pinealectomized rats.

Diazepam is an anxiolytic agent able to inhibit the nocturnal increase in SNAT activity (Zatz and Browenstein, 1979) possibly by an effect on the hypothalamus resulting in a reduced adrenergic stimulation of the pineal gland.

3.6.12.2 Materials and Methods

Intact and pinealectomized rats (n=5) were starved for 24 hours prior to experimentation. Intact rats were then injected with control vehicle, or diazepam 5 mg/kg bw, via the intraperitoneal route. The same procedure was undertaken using pinealectomized rats. They were stressed at 21h00 for two hours, killed by neck fracture, and examined for gastric ulceration (section 3.2.3).

3.6.12.3 Results

The results show that pinealectomized rats exhibit an increased incidence of lesion formation when compared to untreated controls. The inhibitory effect of diazepam on gastric stress ulceration is more prevalent in intact rats. Pinealectomized rats treated with diazepam show no significant difference in lesion production when compared to intact rats (see fig 3.16).

3.6.12.4 Discussion

Niles (1988) has suggested that the pineal hormone melatonin exhibits some of the psychopharmacological effects of the benzodiazepines. Pyridoxal phosphokinase is an enzyme that catalyzes the formation of pyridoxal phosphate in the brain (McCormick and Snell, 1959). Pyridoxal phosphate is essential for the synthesis of DA, 5-HT, and GABA (Lovenberg *et al*, 1962). Anton-Tay *et al* (1970) observed that ip administration of small doses of melatonin (0.25 mg/kg - 4 mg/kg) caused a significant dose dependent increase in pyridoxal phosphate activity in the brain. This action could affect various brain functions, especially serotoninergic and GABA-ergic mechanisms. Melatonin could thus alter the metabolic activity of the brain by increasing pyridoxal phosphate content.

Studies on rat pineal glands have indicated the presence of an exclusive population of peripheral-type benzodiazepine (BZP) receptors (Lowenstein *et al*, 1984), and pineal BZP binding decreased by 30-60% in SCGx rats. This suggests that intact sympathetic nerves are required for the benzodiazepines to manifest

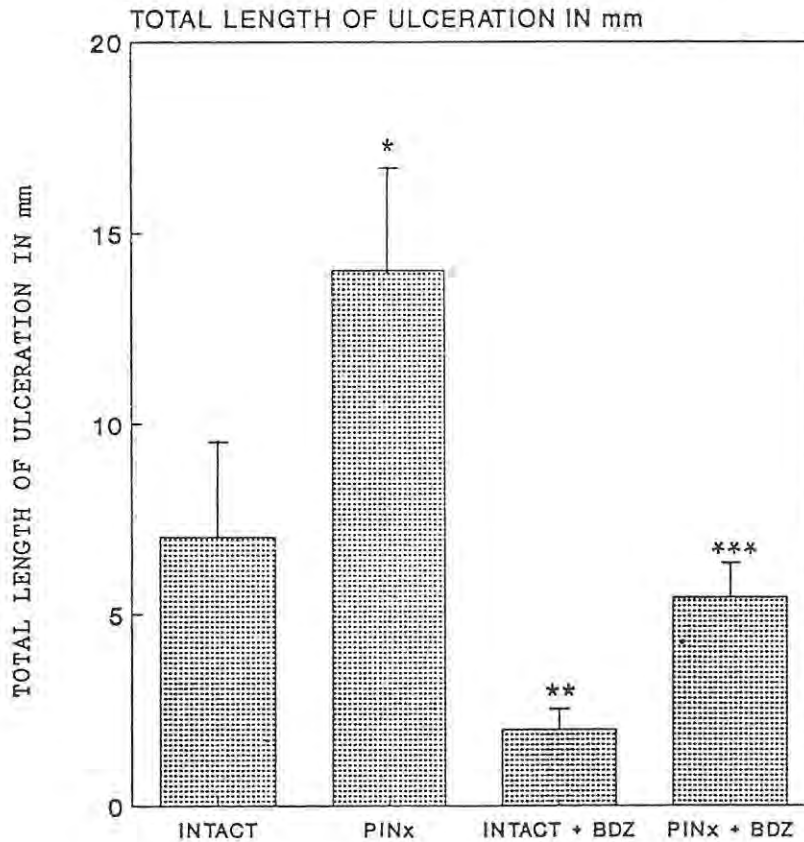


Fig 3.16: Intact and pinealectomized rats were treated with vehicle or diazepam (5 mg/kg bw) prior to the induction of stress in the dark phase. The inhibitory effect of diazepam on gastric stress ulceration in intact rats (***) is significantly different to that in pinealectomized rats (**).

$F(df=19) = 8.35$, $p < 0.01$.

a total effect on the pineal gland.

The results obtained in this study indicate that an intact pineal gland is necessary to modulate the protective effect of diazepam on gastric stress ulcer production.

It could thus be suggested that the pineal gland modulates the stress response via a GABA-ergic mechanism.

Further research in this direction did not fall within the scope of this study.

3.6.13 Experiment 13 : THE EFFECT OF ACTIVITY CYCLE ON STRESS INDUCED GASTRIC LESIONS IN RATS.

3.6.13.1 Introduction

It has been suggested by other investigators (Ader,1964; also cited in Pare and Glavin, 1986) that the relationship between the applied stressor and the animals activity level has to be considered when evaluating the response to stress.

An experiment carried out by Ader (1964) showed that restraining rats at the peak of their activity cycle is associated with lesion production, whereas restraint in the inactive period produced no ulceration. The investigator proposed that the perception of the restraint stress by the rat would be more intense if it were stressed as it was approaching its period of greatest activity, than it would be during relatively inactive periods.

The night-time experiments in this study were carried out at 21h00, 3 hrs after lights off (when SNAT activity is maximal). Results from these experiments showed a reduction in ulceration in rats stressed at this time than rats stressed during the day (see fig 3.7).

Rats are nocturnal animals, and an increase in activity would thus be expected in the scotophase. This experiment was thus undertaken

to compare the activity level of rats subjected to stress at 21h00 to the activity level at other time points.

3.6.13.2 Materials and Methods

Groups of rats (n=6, using 6 groups of 3 rats each = 18 rats) were placed in an activity monitor (described later) linked to a data processor for 48 hours. They were allowed access to food and water *ad libitum* so as to prevent any disturbances in activity due to starvation. Food boxes and water bottles were replenished at 06h00 daily. Activity levels were monitored every 20 minutes for 48 hours. Animals were maintained under the normal LD 12:12 cycle by manual regulation of the light switch.

3.6.13.3 Results

The results show a cyclic pattern of activity over a two day period; with peak activity during the dark phase of the LD cycle, and rats most active between 20h00 and 23h00 (see fig 3.17).

3.6.13.4 Discussion

From the above it has been ascertained that rats stressed at 21h00 were at the peak of their activity cycle, and yet showed a lower incidence of ulceration when compared to rats stressed at 09h00. These results differ from those of Ader (1964). The method of experimentation used by Ader has important differences when compared to this method:-

1) Lights were kept on throughout the experiment (2 weeks); and

2) Rats were stressed without a pre-restraint starvation period. Food was present in the stomachs of 8 out of 13 rats stressed at the time of low activity, and two out of 17 rats stressed during peak activity.

The presence of light could have altered many endogenous rhythms in the rat, and most definitely abolished the melatonin rhythm (Axelrod, 1964). It has been shown that an intact pineal gland (fig

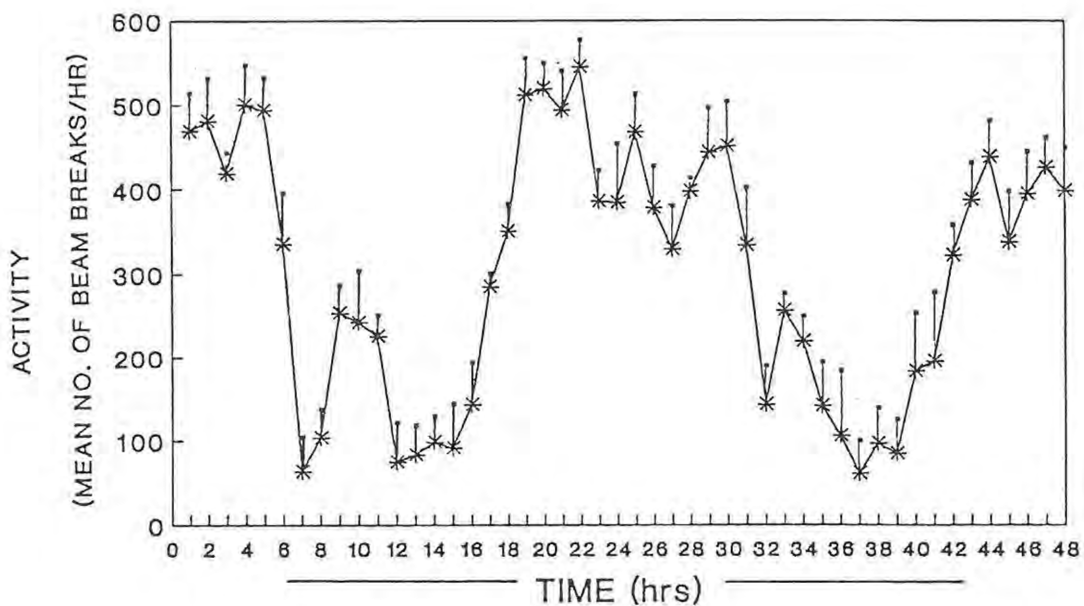


Fig 3.17: Rats show a cyclic pattern of activity over a two day period, with peak activity in the scotophase (——) when melatonin synthesis is high.

3.8), actively secreting melatonin (fig 3.9), protects against stress induced gastric ulceration. Rats stressed in the dark phase of a normal LD 12:12 cycle would therefore most probably present with less ulcers when compared to rats kept in a continuous lighting cycle. In the absence of the nocturnal increase in melatonin synthesis (as induced by constant lighting), it could possibly be suggested that the increase in gastric ulceration noted by Ader, (1964) could be a function of the state of activity of the rat.

The presence of food in the stomach protects against lesion formation (Pare and Glavin, 1986). 70.15 % of the rats stressed at the low activity period had food in their stomachs, and there is no indication of the level of protection afforded to rats by food that may have been present during the restraint period.

It could thus be suggested that the method used by Ader for determining the effect of activity level on gastric stress ulceration was not adequate.

It could be concluded from this study that the application of stress at the peak of the activity cycle is associated with a reduction in lesion formation.

CONCLUSION

The mind and body are so closely interwoven that it is difficult to perceive a physiological stressful event that does not initiate a somatic stressful one and vice versa.

Intense stress is associated with a variety of central and peripheral changes. These include gastric ulcers, hypertrophy of the adrenal glands, increased plasma levels of corticosterone, fatty acids and glucose, atrophy of the thymus and the spleen, hypothermia, and accelerated NA and DA turnover in the brain (review, Pare and Glavin, 1986).

The novel experimental work undertaken in this study indicates that the pineal gland is responsible for the circadian variation in

lesion formation observed in stressed Wistar rats at night and low doses (100 μg - 500 μg) of melatonin reduce gastric stress ulceration. This effect is not mediated via an opioidergic mechanism - and peliminary evidence exists for a possible modulation of this effect by a GABA-ergic mechanism.

The mode of action of melatonin in the prevention of lesion formation could be a cytoprotective one at the level of the GIT or a peripheral (adrenal glands) or central (CNS) anti-stress effect.

Further research could be carried out to determine the effect of melatonin on the stomach. Evidence already exists to show that melatonin pre-treatment protects against ethanol induced gastric ulceration in vitro (Cho *et al*, 1988), and work carried out in this study indicates that melatonin affords no protection against indomethacin induced gastric ulceration *in vivo.* Studies on the effect of melatonin on acid and mucus production, or the inhibition thereof, would be valuable in determining a possible cytoprotective action of this hormone.

Farrel (1960) was the first investigator to suggest that the pineal gland secretes a factor that is capable of inhibiting adrenal steroidogenesis. Earlier, other workers (Wurtman *et al*1959) noticed an increase in adrenal weight in female rats following pinealectomy. Dickson and Hasty (1972) suggested that the pineal gland secretes a substance which blocks the synthesis and release of ACTH at the hypothalamo-pituitary level, thus resulting in an inhibitory action upon the adrenal glands. Ogle and Kitay (1976) concluded that the pineal gland, together with the ovaries, plays a role in the modulation of adrenal steroidogenesis.

Anton-Tay (1970) observed that melatonin administration increases brain pyridoxal phosphate activity, a precursor necessary for the production of NA, DA and GABA (Lovenberg *et al*, 1962). He also found that melatonin increases brain serotonin concentrations.

It could thus be suggested that the pineal gland and melatonin modulate some aspect of the response to stress.

The search for new, safe and efficient drugs to improve the quality

of our lives and its expectancy is unending. The many side effects of prolonged stress, such as tension headaches, lower back pains, gastric ulceration, and the life-threatening hypertension, could very well lead to a decrease in productivity and *joi de vivre*.

Modern day therapy for the treatment of stress related disorders include the barbiturates and the benzodiazepines. Neither of these drugs is ideal.

Clinical investigations into the extent and mechanism of melatonin's action in stress could be invaluable. This substance could be a possible stress-reducing compound, and may very well provide us with a lead product for the development of a safer alternative for stress therapy.

CHAPTER FOUR

ANALGESIC TESTING

5.1 INTRODUCTION

Medical science is still involved in a relentless quest for newer drugs that alleviate pain; a syndrome with which every human being inevitably becomes familiar.

Pain is such a subjective and intimate experience that it is difficult to find a suitable definition for it. Lednicer (1982) described pain as being the "sensory phenomenon that may occur in response to noxious stimuli. This phenomenon has a physiological (magnitude of tissue damage, size, area) as well as a psychological (past experience, environmental setting, ethnic background) component (Lednicer, 1983)

The use of opium as an analgesic goes back in antiquity. The Sumerians may have used it more than 5500 years ago - and it is mentioned in the "Eber's Papyrus" (1500 B.C) - (deStevens, 1965). Some of the alkaloids derived from opium (eg morphine) are used clinically for the treatment of pain.

The pineal hormone, melatonin, has been shown to exhibit analgesic activity in mice (Lakin, 1981; Sugden, 1983) whereas pinealectomy interferes with the nocturnal response of mice to morphine-induced analgesia. These findings suggested a role for the pineal gland in the mechanisms of analgesia.

This study was undertaken to observe the effects of the opiates and the opiate antagonist, naloxone, on pain sensitivity; as well as to ascertain a possible role for the pineal gland in the opiate-modulated analgesic response.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Inbred female rats of the Wistar strain were used in all experiments. Rats were housed five per cage in an animal room under an automatically regulated lighting cycle of LD 12:12, with access to a standard diet and tap water *ad libitum*. It has been observed that ageing affects opioid analgesia (Kavaliers *et al* 1983) thus, per experiment , rats of similar age groups were always used.

4.2.2 Pinealectomy

Pinealectomies and sham operations were carried out as described earlier (chapter 3). Animals were used after a minimum of two weeks post-operation. Rats were killed by neck fracture after being used in experiments, and brains were examined for the absence of the pineal gland so as to confirm the success of the operation.

4.2.3 Analgesic Testing

Testing for analgesic activity in animals involves the presentation of a painful stimulus followed by an appropriate measurement of the animal's response to it.

Various stimuli (chemical, thermal, electrical, ultrasonic, mechanical) may be used to determine analgesic activity. The thermal method of analgesic testing is widely used, and a tail-immersion method was adopted for this study.

Animals were weighed and marked 3 days prior to testing; so as to diminish the effects of stress due to handling. The tail of each rat was marked annularly, 3 cm from the tip.

On the day of the experiment, the rat was injected with the test drug or vehicle as required. After the necessary lag time, the animal was placed in a plastic cylindrical tube, with tail emerging from one end. The tail was then quickly lowered to the 3cm mark

into a water bath set at 55°C (± 0.5°C). The time that elapsed before the tail jerked or twitched was then recorded as the response time by an observer unaware of treatment conditions. The response time was measured in seconds, to the nearest one hundredth of a second, using a stopwatch. The animal was removed from the restraining tube immediately after each test.

A cut-off time of 15 seconds was used to terminate the experiment so as to prevent tissue damage to the tail. Rats reaching the cut-off time were assumed to display total analgesia.

Experiments carried out in the dark phase were undertaken using a dim photosafe red light bulb to aid visibility.

4.2.4 Statistics

All data was statistically analysed using oneway analysis of variance followed by Scheffe's post hoc test for multiple range comparisons; or the Students-*t*-test.

4.3 EXPERIMENTS

4.3.1 EXPERIMENT 1 : DETERMINATION OF THE ANALGESIC EFFECT OF VARIOUS DOSES OF MORPHINE IN RATS.

4.3.1.1 Introduction

Opium contains more than 20 distinct alkaloids, one of which is morphine. Morphine was first isolated in 1806 by Serturner (Goodman *et al* , 1981) - and he named it after the Greek god of dreams, Morpheus.

Morphine is a potent analgesic agent, and although many new compounds producing analgesia have been synthesized, none of them have been found to be clinically superior to this opiate (Goodman *et al*, 1981). Morphine thus remains the standard drug against which new drugs with analgesic activity are tested.

The following experiment was undertaken to examine the analgesic effect of various doses of morphine in rats.

4.3.1.2 Materials and Methods

Rats (n=5) were injected with saline or morphine (10, 20, 30 mg/kg bw ip). They were then subjected to the tail immersion test at t = 0, 20, 40, 60, and 80 minutes; and tail flick responses were noted by an observer unaware of treatment conditions. Rats were removed from the restraining tube immediately after each determination.

4.3.1.3 Results

Results (fig 4.1) indicate that morphine exhibits good analgesic activity at all the doses used in this experiment. The reponse time for reaction to the noxious stimuli was significantly different from that of the control with all the doses of morphine used in this study, and at all time points excluding t = 0. The analgesic activity of morphine was dose-dependent. Rats treated with 30mg/kg bw morphine showed total analgesia at t = 40 minutes, and still exhibited significant analgesia at the last time point of testing (t = 80 minutes).

4.3.1.4 Discussion

The sensation of pain is a very necessary one for the prevention of damage to tissue and acts as a tell-tale sign of possible injury that may otherwise go unnoticed. Chronic pain, however, is debilitating. Morphine and its congeners are clinically useful drugs to counter chronic and intense pain.

This experiment shows that morphine exhibits good analgesia in rats at the doses used.

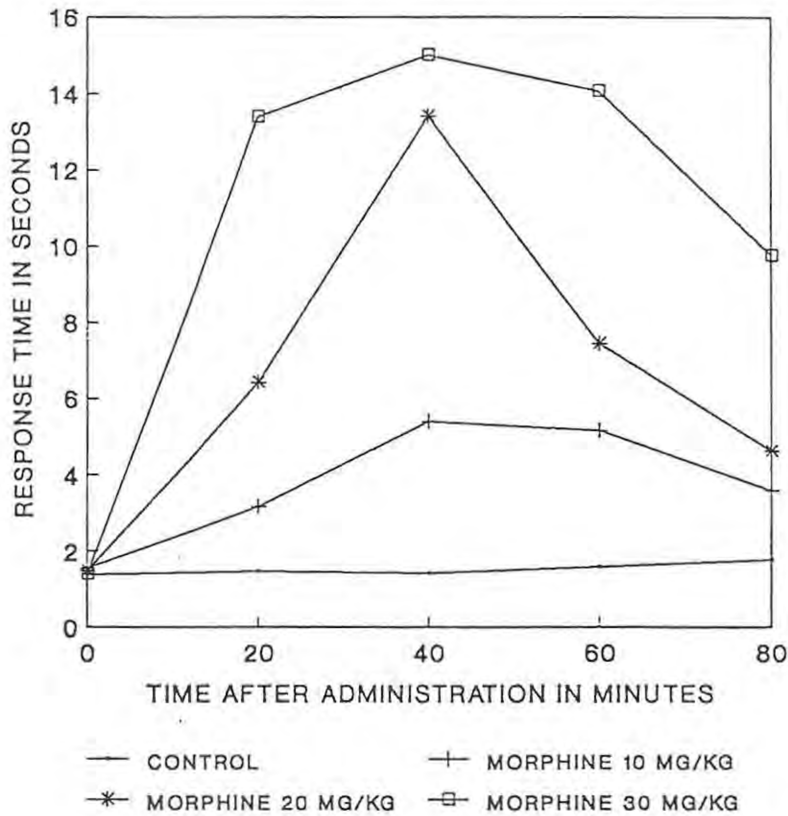


Fig 4.1: Rats (n = 5) were injected with morphine (10 mg, 20 mg, and 30 mg/kg bw ip) or saline vehicle and tested for response to noxious stimuli. Morphine exhibits a significant dose-dependent antinociceptive effect in rats.

4.2 EXPERIMENT 2 : DETERMINATION OF THE ENDOGENOUS RHYTHM OF ANALGESIA IN RATS

4.3.2.1 Introduction

Reynolds (1969) was the first investigator to report the presence of a potent analgesia following focal and electrical stimulation of the rat mid-brain. The presence of an endogenous pain modulating system was thus suggested.

Research in the early 1970's lead to the characterisation of opiate receptors (Pert and Snyder, 1973; Simon *et al*, 1973; Terenius *et al*, 1973), and spurred on the search for an endogenous opioid ligand, leading to the isolation of β-endorphin (Li and Ching, 1976).

The endogenous opiate system in mice and rats undergoes diurnal changes. Wesche and Frederickson (1979) have shown that the content of endorphin-enkephalin in mouse brain is greater in the afternoon than in the late morning. Intracerebroventicular injection of β-endorphin led to a dose related inhibition of the tail-flick and hot plate responses of mice to nociceptive stimuli (Loh, 1976).

Many physiological and biochemical parameters vary in a circadian manner in rodents (Turke and Campbell, 1979). This experiment was undertaken to observe the response of rats to noxious thermal stimuli in the mid-dark and mid-light phases of the LD cycle.

4.3.2.2 Materials and Methods

Rats (n=12) were tested for the response to painful stimuli as described earlier (section 4.2.3) at t = 12h00 (mid-light) and t = 24h00 (mid-dark).

4.3.2.3 Results

Figure 4.2 describes the results obtained from this experiment. An increase in the response time to painful stimuli is noted in the dark phase of the LD cycle as compared to that in the mid-light period.

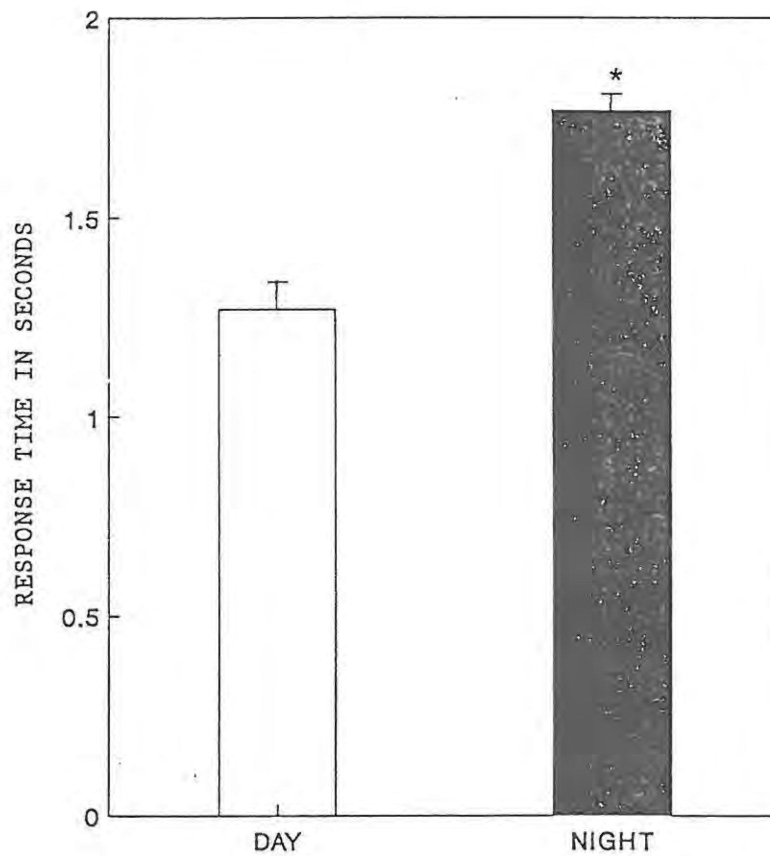


Fig 4.2: Rats were subjected to the tail immersion test at 12H00 and 24h00. An increased latency in the response to nociceptive stimuli was noted in the dark phase.

$n = 12$, $t = 5.74$, $p < 0.001$

* = significantly different to control

4.3.2.4 Discussion

Chronobiological studies in man and other mammals have shown that the metabolism of neurotransmitters such as NA (Perlow *et al*, 1978; Ziegler *et al*, 1976) and DA (Schering *et al*, 1978; Simon and George, 1975) undergo diurnal rhythmic changes.

Kerdelhue *et al* (1983) monitored β-endorphin concentrations in the anterior and intermediate lobes of the pituitary gland and some brain regions in the male rat over a 24 hr time period. They observed a circadian variation in the concentration of this peptide, with the highest levels present in the dark phase of the LD cycle.

Frederickson *et al*, also observed a circadian rhythm in the response of mice to noxious stimuli (hot plate), with highest jump latencies being present in the dark phase of the LD cycle.

The results of this study would thus support the existence of a time-dependent variation in analgesic activity, possibly due to a variation in the activity of β-endorphin with time.

4.3.3 EXPERIMENT 3 : EFFECT OF NALOXONE ON THE NOCTURNAL RESPONSE TO NOXIOUS STIMULI IN RATS

4.3.3.1 Introduction

Naloxone is a competitive antagonist at mu, sigma, kappa and delta opioid receptors (Goodman *et al*, 1981). Evidence for a physiological role of the endorphins and enkephalins as potential hormones and neurotransmitters is increasing (Frederickson, 1977) and it has been suggested that these peptides may function in nociception, neuroendocrine regulation, and may even influence mood and behaviour (Rodgers and Cooper, 1988).

The suggestion of a physiological role for these hormones would indicate that naloxone would have some direct pharmacological activity in naive animals, resulting from an antagonism of the

endogenous opioid system.

This experiment was undertaken to observe the effects of naloxone on the nocturnal response of rats to painful stimuli.

4.3.3.2 Materials and Methods

Rats (n=6) were injected with naloxone (10 mg/kg bw ip) or saline vehicle at 24h00. Fifteen minutes later, they were subjected to the tail immersion test described earlier (section 4.2.3).

4.3.3.3 Results

Figure 4.3 describes the results obtained from this experiment. Naloxone pre-treatment results in a reduced latency (response time) to painful stimuli as measured by the tail flick test.

4.3.3.4 Discussion

The results obtained from this experiment suggest that the endogenous opioids are responsible for the increased pain threshold noted in the dark phase (24h00). This is indicated by naloxone's antagonism of the delayed response to noxious stimuli (heat) noted during the dark phase of the LD cycle.

These observations support the suggestion of a role for the endogenous opioid peptides in the modulation of nociception.

4.3.4 EXPERIMENT 4 : DETERMINATION OF A POSSIBLE RHYTHM OF MORPHINE-INDUCED ANALGESIA IN RATS.

4.3.4.1 Introduction

It is now widely recognized that opiates and opioid peptides produce analgesia through an activation of opioid receptors located at both spinal and supra-spinal levels (Dubner *et al*, Basbaum and Fields, 1984).

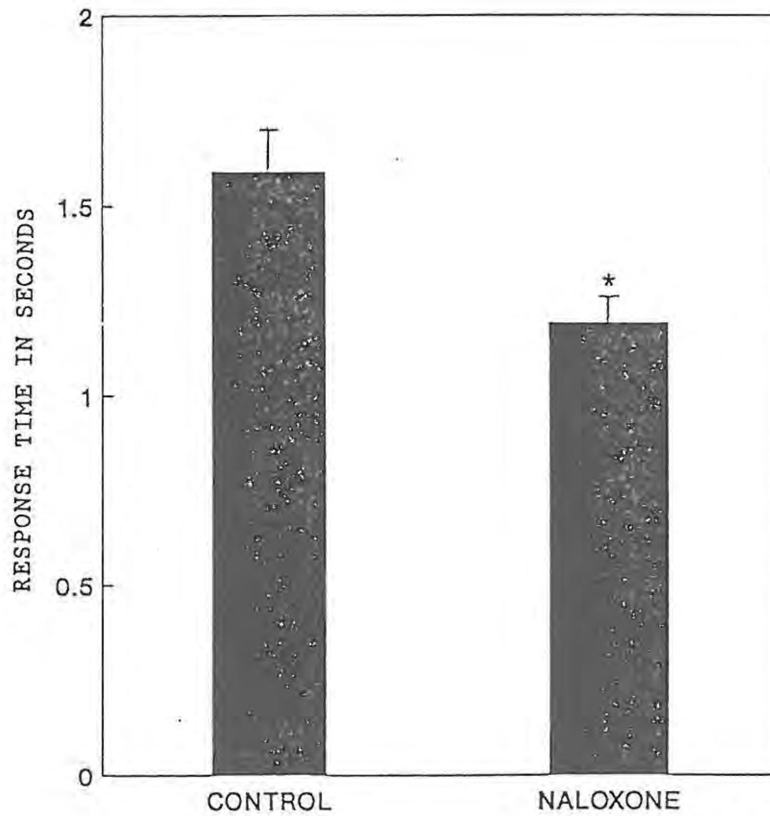


Fig 4.3: Naloxone (10 mg/kg bw ip) or saline vehicle was administered to rats in the dark phase prior to analgesic testing. The opioid antagonist was observed to reduce the response time of rats to noxious stimuli.

$n = 6$, $t = 2.91$, $p < 0.01$

* = significantly different to control

Loh *et al* (1976) observed that the administration of the opioid peptide β-endorphin directly into certain brain areas resulted in a potent analgesia. They found that β-endorphin, when compared to morphine sulphate on a molar basis, was 18-33 times more active as an analgesic agent.

The previous experiments suggest that the variation in the response to noxious stimuli observed in rats at the mid-dark and mid-light phases of the LD cycle is possibly due to a circadian variation in the the activity of β-endorphin. This experiment was undertaken to determine whether rats exhibited a variation in the susceptibility to morphine-induced analgesia at different time points (12h00 and 24h00) in the LD cycle.

4.3.4.2 Materials and Methods

Rats were treated with morphine HCl (10mg/kg bw ip) or saline vehicle at 12h00 (n=8) and 24h00 (n=10). Twenty minutes after drug administration, the rats were tested for analgesic response as described above (section 4.2.3).

4.3.4.3 Results

Figure 4.4 describes the results obtained in this experiment. Rats show an increased nocturnal analgesic response to noxious stimuli, as well as an increase in morphine-induced analgesia. ($F(df=35) = 62.46$, $p < 0.001$)

4.3.4.4 Discussion

Naber *et al* (1980) demonstrated that neurotransmitters as well as their specific receptors exhibit circadian rhythms. Although the receptor affinity remains unchanged, the number of binding sites of these receptors change significantly throughout the day.

Opioid peptide levels and pain sensitivity have been shown to undergo diurnal rhythms in mouse brain (Wesche and Frederickson, 1979), with higher levels and jump latencies observed at 15h00 than at 08h00.

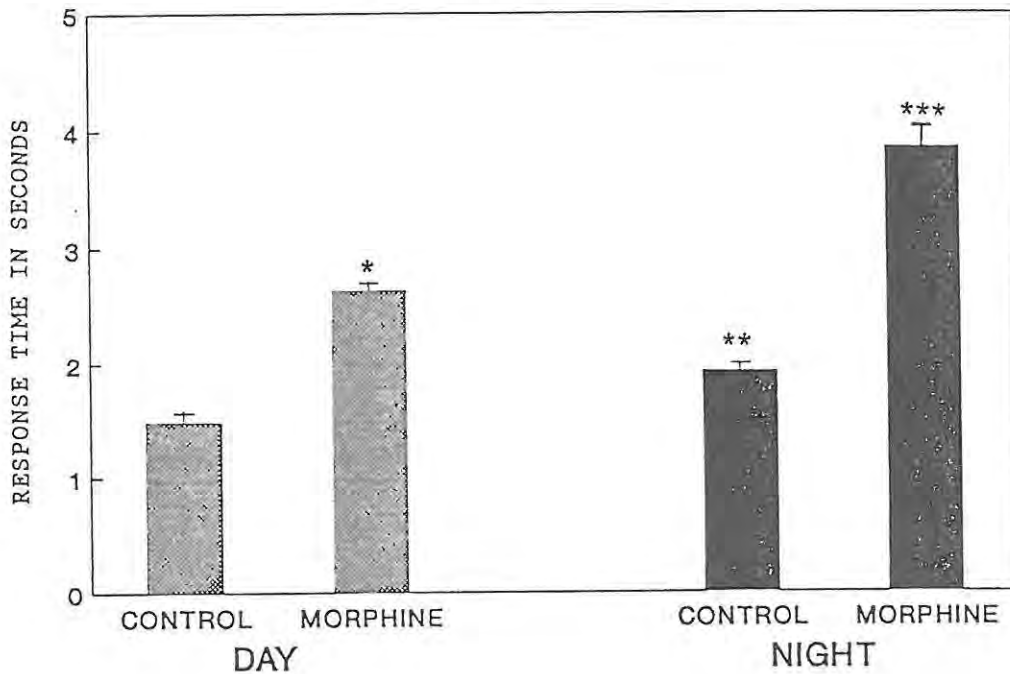


Fig 4.4: Rats were treated with morphine (10 mg/kg bw ip) or saline vehicle in the light (n = 8) and dark (n = 10) phase. An increase in the endogenous as well as morphine-induced response to the noxious thermal stimulus was noted in the dark phase.

$F(df=35) = 62.45$, $p < 0.001$

* = significantly different to all treatment groups

** = significantly different to all treatment groups

*** = significantly different to all treatment groups

Naber *et al*, (1980) investigated opioid receptor binding (using [^{3}H]-naloxone) in rat brain and found that these receptors undergo a circadian rhythm, with maximal binding in the first half of the dark phase.

The possible circadian activation of the endorphin system could thus be responsible for the increased analgesic effect of morphine noted in the dark phase (fig 5.4). Similar effects have been noted in mice (Lutsch and Morris, 1971; 1972) and other rodents (Pilcher *et al*, 1982; McGivern and Bernston, 1980; Rosenfeld and Rice, 1979), thus supporting these findings.

It has been suggested that a component of morphine-induced analgesia may be contributed via the release of endogenous opioids.

4.3.5 EXPERIMENT 5 : INVESTIGATION INTO THE CIRCADIAN RHYTHMS IN SUSCEPTIBILITY TO NOXIOUS STIMULI

4.3.5.1 Introduction

In 1967, Morris and Lutsch observed a quantitative variation in morphine-induced analgesia over a 24 hr time scale. These variations were evident in the heightened analgesia observed in the 12 hr dark period and the reduced levels in the 12 hr light period.

Pilcher *et al*, (1982) observed that the aversiveness (taste-conditioning) of naloxone during the dark phase was significantly different from that of the light phase; and that the analgesic potency of morphine varied within these phases.

Frederickson *et al*, (1977) observed diurnal rhythms in naloxone-induced hyperalgesia in mice. Similar effects were noted in rats by other investigators (Rosenfeld and Rice, 1979).

The previous experiments have indicated an increased sensitivity to morphine induced analgesia in the dark phase (fig 5.4). Naloxone was observed to reduce the increase in the response to painful stimuli noted in the scotophase.

This experiment was undertaken to observe the response of rats to noxious stimuli at various points in a 24 hr LD 12:12 cycle.

4.3.5.2 Materials and Methods

Groups of rats (n=5) were subjected to the analgesic test described above (section 4.2.3) at one of seven 4 hr intervals over a 24 hr time period, commencing at 16h00 on day 1 and ending at 16h00 on day 2. Animals were injected with saline, morphine HCl (10 mg/kg bw ip) or naloxone HCl (10mg/kg bw ip) 20 minutes and 15 minutes (in the case of naloxone) prior to analgesic testing. A total of 105 rats was used in this experiment.

4.3.5.3 Results

Fig 4.5 illustrates the results obtained from this experiment. A circadian rhythm in the analgesic activity of morphine and the hyperalgesic activity of naloxone, with peak analgesic activity in the scotophase is prevalent. A circadian rhythm of response to noxious stimuli is noted for saline treated controls, with peak response times in the dark phase, and lower reponses in the light phase.

4.3.5.4 Discussion

The results obtained from this study agree with those of other workers (Lutsch and Morris, 1971; 1972), who observed that morphine displays a quantitative variation in susceptibilty, characterized by peak analgesia in the scotophase in mice.

These investigators suggested that the increase in sensitivity to morphine in the scotophase could be due to a variability in the pain threshold or even to a variability in the quantity of morphine arriving at and reacting with the receptor site.

Wirz-Justice *et al*, (1982) and Naber *et al* (1981) have demonstrated a nocturnal increase in the binding of naloxone to opiate receptors in the brain, consistent with an increase in the number of binding sites.

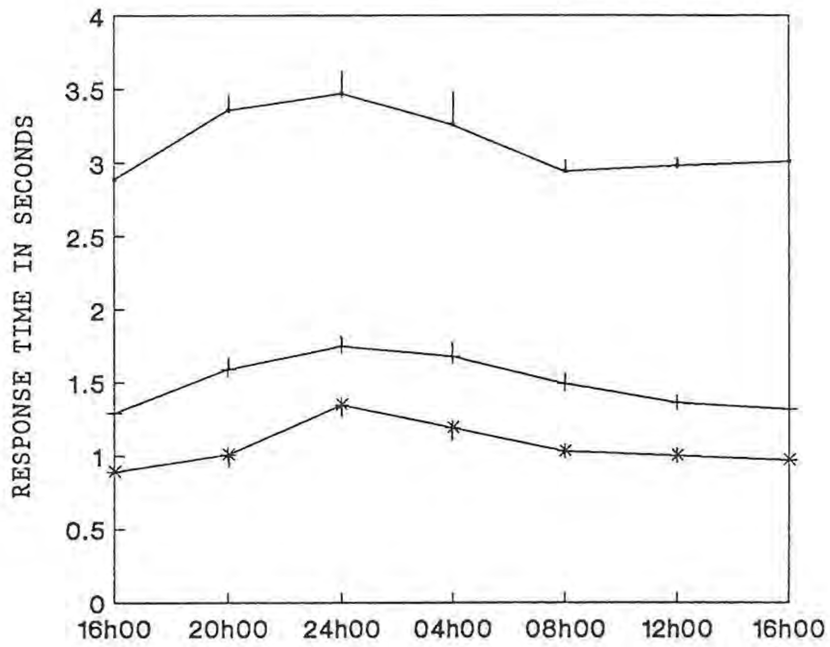


Fig 4.5: A rhythm in endogenous and morphine induced analgesia, as well as naloxone-induced hyperalgesia is observed.

Various workers have observed rhythms in analgesic responses over a time period. Frederickson *et al* (1979) demonstrated that the opiate antagonist naloxone was capable of blocking the endogenous increase in the analgesic response in the dark phase. These investigators suggested that the circadian rhythm of morphine-induced analgesia appears to be the result of an interaction between the endogenous fluctuation in pain sensitivity and the exogenously administered morphine.

Pilcher *et al*, (1982) also observed a rhythmic fluctuation in the sensitivity to pain in rats, and suggested that these changes arise as a result of rhythms in the activation of the endorphinergic system. These investigators, however, noted two peaks of reduced sensitivity to noxious thermal stimuli in rats over a single 24 hr LD cycle. One peak occurred in the light phase and the other more pronounced peak occurred in the dark phase. A rhythm with with one peak in the dark phase was noted when using the paw pinch method of analgesic testing. They thus concluded that the nociception of qualitatively different stimuli may be mediated by different endorphinergic systems. Another explanation for these findings could be that the thermal method of assaying analgesia is possibly more sensitive than the paw pinch procedure.

The observations from this study indicate that a circadian rhythm in the susceptibilty to pain as well as morphine-induced analgesia occurs in rats, and this endogenous rhythm can be blocked by the opiate antagonist naloxone.

4.3.6 EXPERIMENT 6 : EVALUATION OF MELATONIN AS A POSSIBLE ANALGESIC AGENT IN RATS

4.3.6.1 Introduction

It has been suggested that the pineal gland and melatonin have significant effects on opioid functions (Kavaliers *et al*, 1983; Reiter, 1987; Esposti *et al*,1988b). In the goldfish, it has been suggested that the thermoregulatory and behaviourial activating effects of β-endorphin may be mediated by the pineal gland

(Kavaliers *et al,* 1983a).

Electrophysiological studies carried out by Dafny and Burks (1977) suggested that the pineal gland may be involved in determining the actions of morphine on the hypothalamus.

A circadian variation in the sensitivity and response to pain (Frederickson *et al*, 1977; Rosenfeld and Rice, 1979), and the susceptibility to morphine-induced analgesia (Morris and Lutsch, 1969; Lutsch and Morris,1971; 1972) and melatonin synthesis by the pineal gland (Axelrod, 1974; Ozaki and Lynch, 1976), have been reported.

This experiment was undertaken to determine whether the pineal hormone melatonin displays analgesic activity in rats.

4.3.6.2 Materials and Methods

Rats (n=5) were injected with melatonin (30 mg, 60mg, or 90mg/kg bw ip) or melatonin vehicle (see section 3.2.1.) at 12h00. Tail-flick response time was measured at t = 0, 20, 40, 60, and 80 minutes as described earlier (section 4.2.3).

4.3.6.3 Results

The results of this experiment indicate that melatonin has significant analgesic activity noted at 40 minutes after drug administration (fig 4.6) - ($F(df=19) = 6.61$, $p < 0.01$. This analgesic effect of melatonin was not dose dependent at the doses used in this study.

4.3.6.4 Discussion

Melatonin is considered to be the major indoleamine hormone produced by the pineal gland. It has been implicated in the functional activities of many organs (Minneman and Wurtman, 1984).

The results from this study indicate that melatonin (30 - 90 mg/kg bw ip) administration to rats results in time-related changes in analgesic activity.

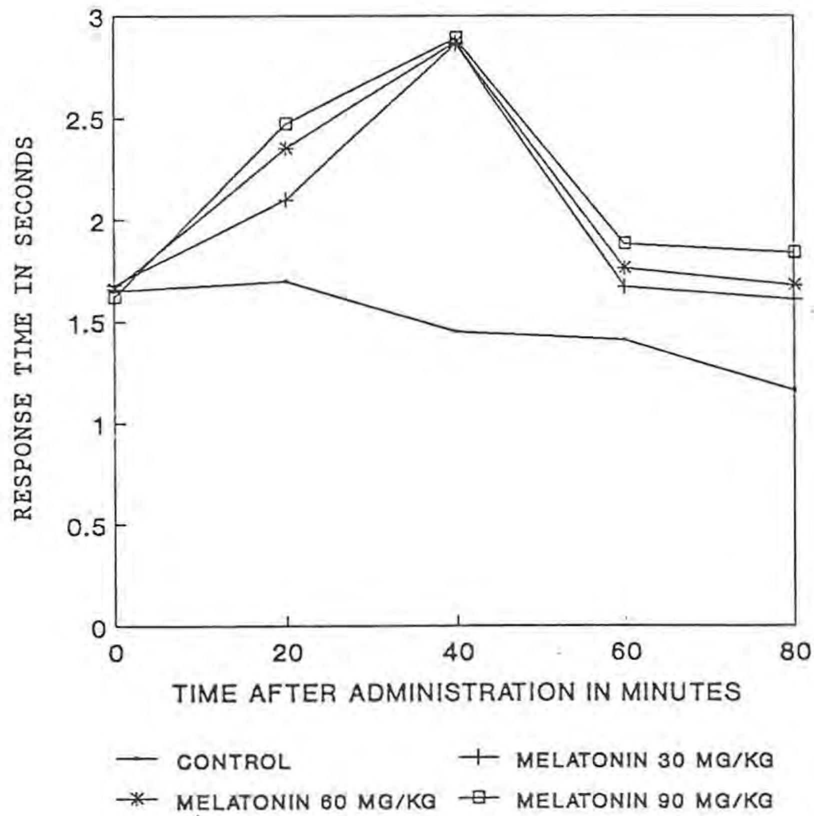


Fig 4.6: Rats were treated with melatonin (30 mg, 60 mg and 90 mg/kg bw ip) or melatonin vehicle and subjected to the tail immersion test. Although melatonin displays some analgesic activity, this effect is not dose-dependent.

Similar observations were made by Lakin *et al*, (1981) who showed that melatonin (30 - 90 mg/kg bw) administration produces a dose-dependent time-related analgesic effect in mice tested on the hot plate (55 ± 0.5°C).

Other investigators (Winters *et al*, 1981) suggested an analgesic role for melatonin in chicks.

Sugden (1983) carried out analgesic tests (hot plate and writhing tests) 30 minutes after melatonin administration to mice. Large doses of melatonin (200 mg/kg and 400 mg/kg bw) inhibited acetylcholine-(5 mg/kg bw) induced writhing and increased the reaction time on the hot plate (50 ± 0.5 °C) in mice, in a dose related manner.

These observations would thus suggest that melatonin has potential as an analgesic agent.

4.3.7 EXPERIMENT 7 : EFFECT OF CONSTANT LIGHTING ON THE NOCTURNAL SENSITIVITY TO MORPHINE INDUCED ANALGESIA IN RATS

4.3.7.1 Introduction

Melatonin synthesis undergoes a circadian rhythm in the pineal gland (Axelrod, 1974), with peak levels during the dark phase of a LD cycle. Data from the above experiments have shown that the analgesic response of rats treated with morphine is greater in the dark phase, when melatonin levels are high.

It has been suggested that the pineal release of melatonin is responsible for the diurnal cycle of analgesia induced by melatonin in chicks (Winters *et al*, 1980. Exposure to a continuous lighting regimen is known to suppress pineal melatonin synthesis (Wurtman *et al*, 1982). This experiment was thus carried out to observe the effects of an abolished melatonin rhythm on the nocturnal analgesic response in morphine-treated rats.

4.3.7.2 Materials and Methods

Rats (n=5) were either exposed to a continuous lighting regimen for three days or left under the normal constant lighting cycle of LD 12:12. On the day of the experiment, rats were injected with morphine HCl (10 mg/kg bw ip), 20 minutes prior to analgesic testing at 12h00 or 24h00. For the night-time experiments, rats were removed from the constant lighting cycle at 23h35.

4.3.7.3 Results

The findings of this experiment are illustrated in figure 4.7. The enhanced nocturnal analgesic response to morphine noticed in rats kept under the normal lighting cycle (LD 12:12) is abolished by exposure of rats to a constant lighting cycle.

5.3.7.4 Discussion

Information about environmental lighting is conveyed to the pineal gland via neuronal pathways from the retina (Klein and Moore, 1979). Lutsch and Morris (1972) have suggested that photic information is a dominant exogenous force synchronizing the morphine susceptibility pattern in mice. Their findings suggest that the analgesic pattern of morphine in the LD cycle was dependent on the lighting regimen. Alternating a 12 hr LD cycle, or reversal of a normal 12 hr LD cycle, always produced peak analgesia in the dark phase (when melatonin levels are high). In continuous light (when melatonin production is abolished), these workers showed no statistically significant differences in analgesic responses to morphine at various time points. It could be suggested that physiological doses of melatonin might be able to potentiate the analgesic activity of morphine.

The results of the present study are in agreement with these findings. Although other possibilities have not been excluded, it could be suggested that melatonin may enhance the morphine-induced analgesic response in rats.

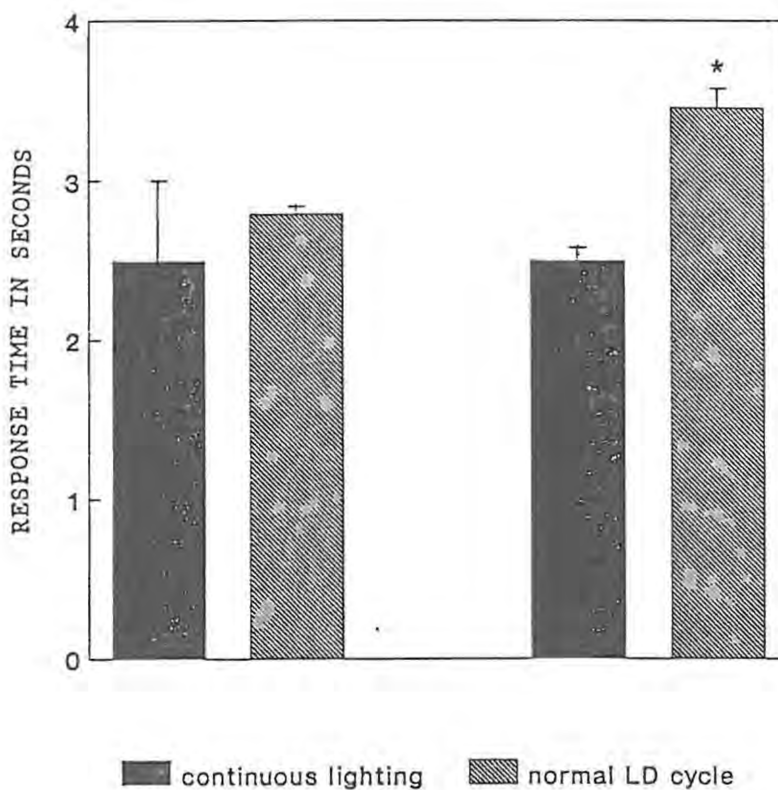


Fig 4.7: Rats were maintained in an normal LD 12:12 cycle, or under a continuous lighting cycle (LL) and subjected to the tail immersion test at 12H00 and 24H00. Only rats maintained under the normal lighting cycle showed an enhanced morphine (10 mg/kg bw ip)-induced nocturnal analgesic activity.

* = significantly different to all treatment groups.

$F(df=19) = 9.75$, $p < 0.001$.

4.3.8 EXPERIMENT 8 : EFFECT OF PINEALECTOMY ON MORPHINE-INDUCED ANALGESIA IN RATS

4.3.8.1 Introduction

The previous experiments have shown that a circadian rhythm in the response to noxious stimuli exists in naive and morphine-treated rats, with higher response times being noted in the dark phase of the LD cycle (fig 4.5). Also, the pineal hormone, melatonin, has some analgesic activity in rats (fig 4.6).

These findings initiated the following experiment, which was undertaken to determine whether the pineal gland played a role in the manifestation of the circadian changes in pain sensitivity and susceptibility to morphine.

4.3.8.2 Materials and Methods

Intact, sham operated and pinealectomized rats (n=5) were treated with morphine HCl (10mg/kg bw ip) at 12h00 and 24h00 and analgesic activity was determined twenty minutes later as described (section 4.2.3).

4.3.8.3 Results

The results (fig 4.8 and fig 4.9) from this study show that both intact and sham operated rats exhibit an increase in the endogenous and morphine-induced analgesic reponse in the dark phase (24h00). Pinealectomized rats, however, show a significant lowering of the response time to noxious thermal stimuli in the dark phase (24h00) when compared to intact and sham operated rats.

This effect was not noted in the photophase.

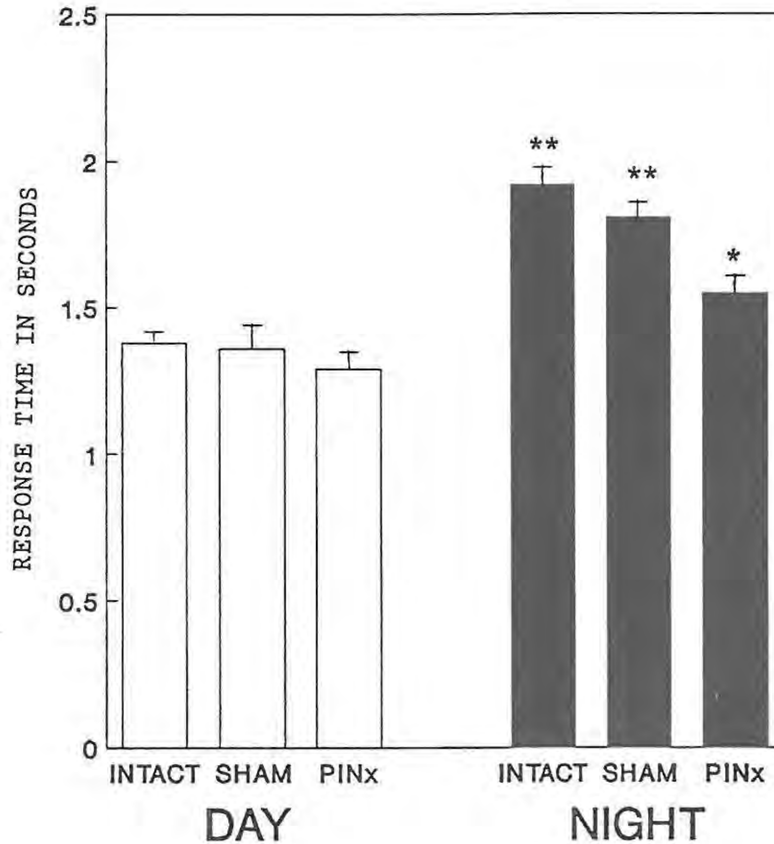


Fig 4.8: Intact, sham-operated and pinealectomized rats were subjected to the tail immersion test at 12H00 and 24H00. Pinealectomy causes a significant reduction in the endogenous response to noxious thermal stimuli in the dark phase. The response time in the dark phase remains unchanged.

F(df=29) = 49.50, p < 0.01

* = significantly different to nighttime controls and all daytime treatment groups.

** = significantly different to all other treatment groups.

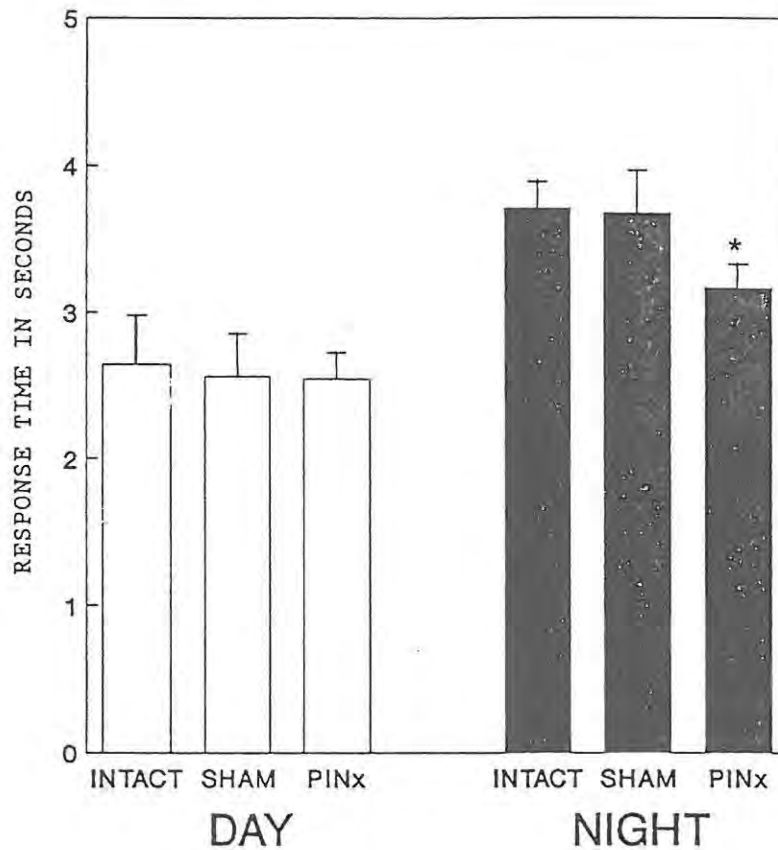


Fig 4.9: Intact, sham-operated and pinealectomized rats were treated with morphine (10 mg/kg bw ip) and subjected to the tail immersion test at 12H00 and 24H00. Pinealectomy cause a partial but significant reduction in morphine analgesia in the dark phase.

$F(df=29) = 49.56, \quad p < 0.001$

* = significantly different to all other treatment groups.

** = significantly different to all other treatment groups.

4.3.8.4 Discussion

In most rodent species studied, melatonin synthesis in the pineal gland and plasma levels of melatonin are greatest during darkness (Ozaki and Lynch, 1976), when animals have been reported to be most sensitive to morphine-induced analgesia (Lutsch and Morris,1971; 1972) and least sensitive to pain (Frederickson *et al*, 1977; Rosenfeld and Rice, 1979).

Kavaliers *et al* (1983), have proposed that the pineal gland is involved in the "fine-tuning" and integration of neural and endocrine activities that may affect opioid activity.

Kumar *et al* (1982) have observed that pinealectomy abolishes the daily fluctuations in met-enkephalin levels in the hypothalamus pre-optic area.

The findings of this experiment suggest that the pineal gland is an integral link in the expression of the circadian changes in the endogenous and morphine-induced response to noxious thermal stimuli. Removal of the pineal gland resulted in a loss of the dark phase augmentation of morphine analgesia, suggesting that this gland is involved in the manifestation of the circadian response to morphine in rats.

Lakin *et al* (1981) observed that pinealectomy completely abolished the rhythm of morphine analgesia in mice. The results of this study, however, indicate that pinealectomy induces only a partial but significant reduction in the nocturnal sensitivity to morphine-induced analgesia in rats.

4.3.9 EXPERIMENT 9 : EFFECT OF MELATONIN ON THE SENSITIVITY TO MORPHINE-INDUCED ANALGESIA IN PINEALECTOMIZED RATS.

4.3.9.1 Introduction

The results obtained from the previous experiments indicated that the pineal gland was necessary for the modulation of the increased nocturnal pain sensitivity noted in rats, as well as for the variation in morphine susceptibility.

Melatonin is considered to be the principal pineal hormone; and has many effects on the CNS (Quay *et al*, 1974; Reiter, 1977). This experiment was undertaken to examine whether the administration of melatonin to pinealectomized rats altered the morphine susceptibility in rats.

4.3.9.2 Materials and Methods

Two groups each of intact, sham-operated and pinealectomized rats were injected either with morphine (10 mg/kg bw ip) and melatonin vehicle, or morphine (10 mg/kg bw ip) and melatonin (5 mg/kg bw ip) twenty minutes prior to analgesic testing. At 24h00, rats were subjected to the tail immersion test described earlier.

4.3.9.2 Results

The results of this experiment indicate that melatonin administration results in a small but significant increase in the analgesic activity of morphine in pinealectomized rats (see figure 4.10).

4.3.9.3 Discussion

At the doses used in this study, melatonin was unable to fully reverse the effects of pinealectomy on the nocturnal susceptibility to morphine as measured using analgesic testing. A small but significant increase in response time was noted in melatonin-

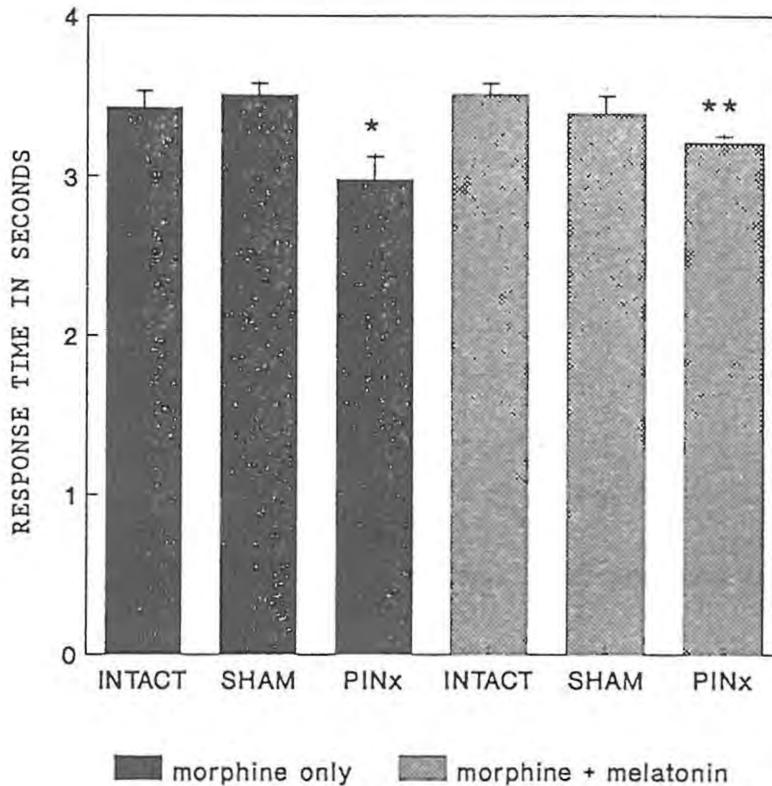


Fig 4.10: Intact, sham-operated and pinealectomized rats were treated with morphine (10 mg/kg bw ip) or melatonin (5 mg/kg bw ip) + morphine (10 mg/kg bw ip) and subjected to the tail immersion test at 24H00. Melatonin induced a slight but significant increase in the nocturnal analgesic activity of morphine. This dose of melatonin, however, could not fully reverse the effects of pinealectomy on the nocturnal analgesic activity of morphine.

$F(df=29) = 6.005, \quad p < 0.001$

* = significantly different to all other treatment groups.

** = significantly different to all other treatment groups.

treated rats as compared to control rats.

Winters *et al* (1983) found an analgesic effect of melatonin in chicks which, they suggested, appeared to be based on a melatonin triggered release of endogenous analgesic pituitary peptides. They suggested that pineal melatonin could be the basis for the diurnal rhythm of analgesia noted for morphine and ketamine. These investigators also observed that morphine, ketamine and melatonin analgesia is reversed by naloxone, and suggest a link between the pineal/pituitary glands and opioid receptors.

Lakin *et al*, (1981) also observed that melatonin-induced analgesia was blocked by naloxone; and suggested an opioid linked role for melatonin analgesia.

Sugden (1983) however, noted that the analgesic activity of melatonin was not reversed by naloxone, contrary to the findings of the above reports.

A problem arises in the design of these studies. Lakin *et al* (1981) and Sugden (1983) administered naloxone subsequent to melatonin injection. Naloxone has been shown to exert a hyperalgesic effect (Frederickson *et al*, 1977; Rosenfeld and Rice, 1979; Pilcher *et al*, 1982) and the reduction in the analgesic response to melatonin could be attributed to this effect.

From this study, it is clear that at the higher than physiological dose used in this experiment, melatonin is unable to fully reverse the effects of pinealectomy on the susceptibility to morphine. Various other peptides are released by the pineal gland (Cardinali and Vacas, 1988), and the possibility exists that one of these peptides may modulate the enhanced nocturnal analgesic activity in rats.

Although an activation of the endogenous opioid system by melatonin could be suggested, further research (including biochemical and physiological studies) would have to be initiated to determine the exact mode and site of action of this hormone.

4.3.10 EXPERIMENT 10 : EFFECT OF MELATONIN ON STRESS-INDUCED ANALGESIA.

4.3.10.1 Introduction

The endogenous opioid system plays an important role in analgesia (Akil *et al*, 1984). This system can be activated by a broad range of environmental stimuli, including stress (Guilleman *et al*, 1977).

One of the most common stressors used in animal experiments, restraint, involves both somatic and emotional components (Axelrod and Reisine, 1984) and is also associated with analgesia (Amir and Amit, 1978).

Immobilization in a plexiglass chamber has been found to increase the plasma concentration of β-endorphin in the rat (DeSouza and van Loon, 1985) and increases hot plate latencies in the mouse (Amir and Amit, 1978).

Winters *et al* (1983) have suggested that melatonin triggers the release of endogenous opioid peptides and thus exhibits analgesic activity. This experiment was undertaken to observe the effect of melatonin on immobilization-induced analgesia in rats.

4.3.10.2 Materials and Methods

Rats (n=5) were injected with melatonin vehicle, melatonin (5 mg/kg bw ip) or naloxone (10mg/kg bw ip) 20 minutes and 10 minutes (in the case of naloxone) prior to immobilization. Animals were restrained in the cylindrical tubes used for analgesic testing (see section 4.2.3) for fifteen minutes. At the end of the restraint period, they were subjected to the tail-flick test as described earlier (section 4.2.3).

4.3.10.3 Results

Results (fig 4.11) indicate that saline-treated rats exposed to immobilization for fifteen minutes show significant analgesia when

compared to unrestrained controls. Naloxone abolished this effect.

Melatonin was observed to significantly increase the tail flick response time when compared to saline treated controls.

4.3.10.4 Discussion

Many studies have reported the presence of analgesic activity after restraint (Amir and Amit, 1978; 1979; Appelbaum and Holtzman, 1984; 1985). Activation of the opioid system occurs in the course of immobilization stress (Guilleman *et al*, 1977), and is activation may also be characterized by the opioid-like alterations in animal behaviour (Porro and Carli, 1988).

Restraint has also been observed to potentiate morphine -induced analgesia in rats. Acute immobilization increases the pain threshold in the tail flick test, and increases the duration and potency of the analgesic effects of morphine and methadone.

Various systems, including DA-ergic, serotoninergic and NA-ergic mechanisms have been proposed to explain the increase in analgesic activity due to stress exposure (Porro and Carli, 1988). Kelly and Franklin (1985) have proposed that an increase in brain tryptophan may be a general mechanism for the effect of stress on sensitivity to pain.

The results from this study indicate that restraint induces an increase in analgesic activity, and that this effect is blocked by naloxone. Exposure to stress is known to increase pineal biosynthetic activity (See chapter 3).Melatonin administration was observed to potentiate the restraint-induced analgesia. This observation could indicate a possible activation of the opioid system in restraint by melatonin.

However, this is an indirect method of noting the effect of melatonin on the opioid system; and as such it cannot be conclusively assumed that melatonin exerts its stress-induced potentiation of the analgesic response via an opioid mechanism.

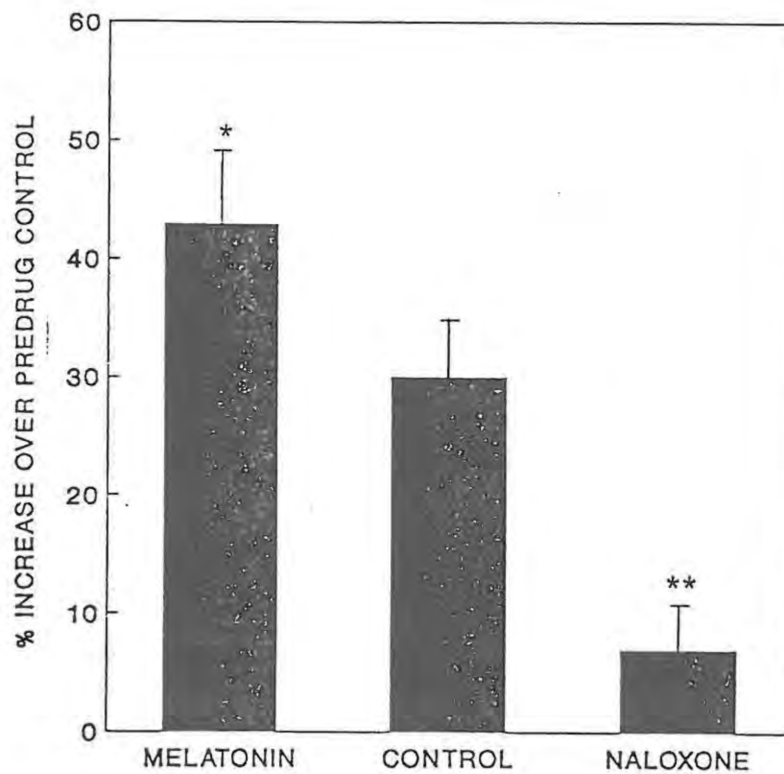


Fig 4.11: Melatonin potentiates the stress-induced increase in analgesic activity. Naloxone induces a reduction in the response time of rats subjected to noxious stimuli.

$F(df=14) = 14.35$, $p < 0.001$.

* = significantly different to control and naloxone

** = significantly different to control and melatonin.

No research in this field has yet been undertaken. Investigations into the actual mode of action of melatonin on the endogenous opioid system may help to explain a possible analgesic role for this hormone.

4.4 CONCLUSION

The pineal gland is a neuroendocrine transducer, capable of converting neural signals received from the retina in response to environmental lighting into a hormonal output (Axelrod, 1974).

The analgesic effect of morphine was shown to undergo a diurnal variation depending on the lighting regimen, suggesting that photoperiodicity has an important effect on morphine susceptibility in rats.

This study has also provided interesting results, suggesting that the pineal gland plays an important role in the endogenous as well as morphine-induced analgesic responses in rats.

Although some of the observations noted in this study suggest that melatonin may exert an effect in rats via an activation of the opioid system, these findings are inconclusive.

CHAPTER FIVE

CONCLUSION

This study investigated existing as well as new possible areas of interaction between the pineal gland and the opioid system.

Organ culture studies showed that the opioid agents do not affect [^{14}C]-serotonin metabolism by the pineal gland *in vitro.* This suggests that the opioids do not exert a direct action on the pineal gland. An indirect-acting opioid synapse may thus be responsible for the effects of opioids on the pineal gland observed by other investigators.

The opioid system was shown to play a possible role in stress. Using the restraint-immobilization stress technique, it was observed that morphine inhibits stress-induced gastric ulceration. Naloxone exacerbated this effect, as well as reversed the protective effects of morphine on stress-induced gastric ulceration. The pineal gland was shown to be necessary for the modulation of some aspect of stress-induced gastric pathology in rats. Its principal hormone, melatonin, could counter the effects of stress on gastric ulceration very effectively. This effect, however, was not exerted via an opioid mechanism. The pineal gland and melatonin thus possibly exert their effects via a different mechanism that has yet to be elucidated.

Pinealectomy was shown to abolish the diurnal rhythm of morphine-induced analgesia, suggesting that an intact pineal gland is necessary for this different diurnal sensitivity to the analgesic effects of morphine. Melatonin could only partly reverse this effect, and it could thus be suggested that other pineal factors may be involved in the mediation of this effect.

It would thus appear that the pineal gland may be necessary for the modulation of some of the effects of the opioid system. However, no clear cut evidence has been provided to infer that these effects of melatonin are exerted via the opioid system.

Melatonin could have many sites of action in its inhibition of stress-induced ulceration. These include possible peripheral effects such as local microcirculatory vasodilation, maintenance of an acid balance in the stomach, and maintenance of gastric mucosal integrity. Recent observations (Szabo, 1989) however, indirectly revoke this possibilty. Szabo (1989) has observed a circadian variation in chemically-induced gastric erosions in rats, with maximal lesions being present in the dark cycle. Melatonin levels are higher at night than during the day (Axelrod, 1974); and a possible cytoprotective effect of endogenous melatonin could be excluded.

A central effect of melatonin in stress could also be postulated. This pineal factor could possibly reduce emotionality in rats, or influence the stress-triggered activity of the hypothalamo-pituitary-adrenal axis to exert its protective effect on the gastric mucosa in stress.

Melatonin is known to influence brain concentrations of GABA, NA, DA, and serotonin (see chapter1), and any one of these neurotransmitters may be implicated in the actions of melatonin observed in this study.

During the past few decades, many investigators have studied the role of GABA in a variety of behavioural systems, and recent observations suggest that GABA exerts an analgesic effect that may differ from that of the opioids. Potent GABA agonists (e.g. muscimol) potentiate morphine-induced analgesia in the mouse and the rat (Biggio *et al*, 1977). It has been suggested that the elevation of cerebral GABA content has an anti-nociceptive effect in rats and mice, and that this effect is not opioid-like in nature (DeFeudis, 1982).

An explanation for some of the effects noted in this study could be surmised from the above discussion. Melatonin could possibly exert some of its effects via the GABA-ergic system. Administration of melatonin possibly increases brain GABA concentrations, and this effect could lead to an attenuation of the effects of stress on gastric ulceration (chapter 3) and an enhancement of the

antinociceptive response of rats subjected to stress (chapter 4).

It could be concluded that although a link between the pineal gland and the opioid system as observed in this study (chapter 4) may exist, a modulation of the noted effects by other systems (GABA-ergic, dopaminergic, noradrenergic, serotoninergic) cannot be excluded.

SUMMARY

CHAPTER1

A general review on the pineal gland, the opioid system, and possible interactions between these two systems has been presented, including an overview on stress and analgesia.

CHAPTER 2

The influence of opioids (methadone, morphine) and the opioid antagonist naloxone on [^{14}C]-serotonin metabolism by the pineal gland has been investigated, using an organ culture technique. Neither the opioids nor the opioid antagonist naloxone have any effect on [^{14}C]-serotonin metabolism by the pineal gland. The organ culture system described may be used to study melatonin production in tissue other than the pineal gland.

CHAPTER 3

The effects of melatonin, morphine and naloxone on stress-induced gastric ulceration has been studied. Melatonin and morphine significantly inhibit stress lesion formation in rats, whereas naloxone exacerbates this effect. Pinealectomized rats show a higher incidence of gastric lesions when compared to control rats, suggesting that the pineal gland is necessary for the modulation of some aspect of the stress response. This effect of melatonin is not exerted via an opiatergic mechanism.

CHAPTER 4

The response of rats to noxious stimuli after various manipulations (drug administration, pinealectomy, altered lighting cycle) has been investigated. A diurnal variation in the response of rats (treated with saline, morphine or naloxone) to noxious stimuli has been observed. Pinealectomy abolishes the nocturnal increase in morphine-induced analgesia, and melatonin only partly reverses this effect. Melatonin potentiates stress-induced analgesia in rats.

CHAPTER 5

The pineal gland and melatonin may be responsible for some of the actions of the opioid system. The principal pineal factor, melatonin, may have therapeutic use in the management of stress, and could serve as a lead product in the development of safer alternatives for stress therapy.

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PUBLICATIONS ARISING FROM THIS WORK.

1. Melatonin reduces stress-induced gastric ulceration in rats:

R. Khan, S. Burton, S. Morley, S.Daya and B. Potgieter. (1989) *Experientia, in press.*

2. The evaluation of melatonin as a possible anti-stress hormone:

R. Khan, S.Morley, S. Daya and B. Potgieter (1989); paper presented by B. Potgieter at the 1st Annual Congress on Therapy with Amino Acids and Amino Acid Analogues, at the University of Vienna, Austria - August 1989.

3. Evidence for a modulation of the stress response by the pineal gland:

R. Khan, S.Daya and B. Potgieter, (1989) *Experientia, awaiting publishing decision.*