A STUDY OF THE EFFECTS OF THE PINDEAL HORMONE, MELATONIN, ON DOPAMINERGIC TRANSMISSION IN THE CENTRAL NERVOUS SYSTEM OF RATS

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ABSTRACT

Dopamine mechanisms in the central nervous system are important in the control of both normal and abnormal motor function. The recent observations in both animal and human studies, that melatonin, the principal hormone of the pineal gland, may have a role in the control of movement and the pathophysiology of movement disorders, have given rise to the concept that melatonin may have a modulatory influence on central dopaminergic neurotransmission. This study makes use of three animal behavioural models as well as a biochemical model of central dopaminergic function to further investigate the concept.

Results from studies using the biochemical model, which investigated the effect of melatonin on dopamine and apomorphine stimulation of dopamine-sensitive adenylate cyclase, suggest that melatonin is neither a competitive antagonist nor agonist at the D1 receptor level, although the possibility of physiological stimulation or antagonism is not excluded.

In behavioural studies, prior melatonin administration (1 and 10 mg/kg (BM) ip) inhibited apomorphine induced stereotypy and locomotor activity in normal rats, and apomorphine-induced rotational behaviour in 6-hydroxydopamine and quinolinic acid lesioned rats. The possibility that these results may have physiological significance is borne out by the observation that, under environmental lighting conditions that are associated with raised endogenous melatonin levels, apomorphine-induced stereotypy and locomotor activity is attenuated.

The general conclusion is that melatonin has an inhibitory influence on central nervous system dopaminergic function, suggesting therefore, that the pineal gland and melatonin may have a role in the pathophysiology and treatment of movement and behavioural disorders associated with dopaminergic dysfunction.
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ABBREVIATIONS USED

AAR  active avoidance response
ACh  acetyl choline
ADC  adenylate cyclase
ALAA aromatic-L-amino acid
APO  apomorphine
ATP  adenosine triphosphate
AVT  arginine vasotocin
BM   body mass
BSA  bovine serum albumin
cAMP 3':5' cyclic adenosine monophosphate
cGMP 3':5' cyclic guanosine monophosphate
COMT catechol-O-methyl transferase
CSF  cerebrospinal fluid
DA   dopamine
DOPA 3,4-dihydroxyphenylalanine
DOPAC 3,4-dihydroxyphenylacetic acid
EEG  electroencephalogram
EGTA ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid
Fig. figure
GABA γ-aminobutyric acid
GH   growth hormone
Glut. glutamate
5HIAA 5-hydroxyindoleacetic acid
5HIAL 5-hydroxyindoleacetaldehyde
HIOMT hydroxyindole-O-methyl transferase
6H-MEL 6-hydroxymelatonin
5HT  serotonin
5HTOH 5-hydroxytryptophol
5HTP  5-hydroxytryptophan
HVA  homovanillic acid
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<td>ip</td>
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<td>iv</td>
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<td>LH</td>
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CHAPTER 1
LITERATURE REVIEW

1.1 INTRODUCTION

Although the existence of the pineal gland has been recognised for many centuries, until 30 years ago it was considered to be a functional vestige - "a remnant of the third eye". Only subsequent to the isolation and characterization of melatonin - a secretion of the pineal gland, by Lerner and his co-workers, in 1958, has the functional significance of this enigmatic gland begun to be unravelled. Today the pineal gland is recognised as an active, functioning neuroendocrine organ - an integral and important component of the neuroendocrine system.

Since the effects of the pineal tend to be modulatory rather than primary, that is, to change the timing, amplitude or intensity of response, rather than to initiate such responses, "it is no longer a matter of what organs or organ systems (the pineal and) melatonin influences; but rather it is a question of whether any escape its influence" [Reiter (1988)].

Many animal and human studies have suggested that the pineal gland, by secreting melatonin, may play a vital role in several behavioural processes and be involved in the pathophysiology of movement disorders [Reviews - Datta and King (1980) and Sandyk (1988)]. The results of such studies have also led to the conclusion that the effects of melatonin on brain and behaviour are inseparable from the available concentrations of central neurotransmitters such as γ amino butyric acid, serotonin, dopamine and noradrenaline, and more specifically to the concept that melatonin "has a modulatory influence on dopaminergic activity in
The involvement of the central catecholamine - dopamine, in the mediation of motor behaviours and movement and in the pathophysiology of movement disorders such as Parkinsonism, tardive dyskinesias and Huntington's disease has long been recognised. Changes in dopamine neurotransmission bring about profound changes in behaviour ranging from akinesia to hyperactive stereotyped behaviour. Dopamine agonists have a firmly established role in the treatment of Parkinson's disease and may be of value in the therapy of tardive dyskinesias, while the use of dopamine antagonists have a long history in the treatment of Huntington's disease and in schizophrenia.

The exact mechanism of dopamine's action is not fully understood and the situation is complicated by the known existence of two different dopamine receptor mechanisms, termed D₁ and D₂; the differential roles for the major central dopamine systems; and the known relevance of various other neurotransmitter systems in the control of normal and abnormal motor function. Ungerstedt (1979) concludes that "there is little basis for the assumption that we will ever be able to formulate a global theory for the role of dopamine neurotransmission in the control of behaviour".

Therefore, although the involvement of dopamine in the control of normal and abnormal motor behaviours is well established, the lack of clarity on its exact mechanism of action permits the concept of an involvement of the pineal gland and melatonin, in a modulatory capacity.

This study makes use of a biochemical model of dopaminergic activity - the dopamine-sensitive adenylate cyclase model, together with three behavioural models of central dopaminergic activity - apomorphine induced stereotypy, apomorphine induced locomotor activity and the "Ungerstedt" rotational model - to investigate the effects of melatonin on the central nervous system and more
specifically, on nigrostriatal dopaminergic neurotransmission in rats. It is an attempt to further elucidate the possible modulatory role of melatonin on central dopaminergic function and the possible involvement of the pineal gland in the pathophysiology of movement disorders. An established modulatory role for the pineal gland and melatonin could lead to an improved understanding of both melatoninergic and dopaminergic central activity and their involvement in normal and abnormal motor behaviours.
1.2 THE PINEAL GLAND AND MELATONIN

1.2.1 The Pineal Gland

The rat pineal gland, or the epiphysis cerebri, is located between the cerebral cortex and the cerebellum, proximate to the third ventricle and is connected to the diencephalon by a stalk or peduncle. It is a solid organ, formed with characteristic cells called "pinealocytes" and also contains glial cells called "astrocytes".

Typically, the pinealocytes have one or more processes that terminate near capillaries. The capillary network within the pineal gland is very dense, reflective of a profuse blood supply to the organ [Reiter (1989)]. The pinealocyte processes lie adjacent to nerve endings in the perivascular spaces. The nerve connections are considered to be of sympathetic origin, with their cell bodies in the superior cervical ganglia (SCG) [Kappers (1965)]. Photosensory information involved in the regulation of the pineal is conveyed to the gland via a complex polynervous pathway that begins at the eye, synapses in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, follows a multisynaptic pathway through the brainstem and the upper thoracic cord, leaving with the preganglionic fibers to the SCG [Cardinali (1981), Reiter (1988)].

The innervation of the pineal gland appears to be critical to its activity, and sympathetic denervation of the gland via destruction of the SCG destroys the ability of the gland to synthesize pineal hormones [Datta and King (1980)]. The pineal gland has been described by Wurtman et al (1963) as a "neuroendocrine transducer" whose hormonal output depends upon, or is mediated by, the light message reaching the pineal through its nerve supply.

The proposed neural connections between the eyes and the
pinealocyte, the hormone producing cell, in the pineal gland in mammals is presented diagrammatically in Fig. 1.1.

Although the activity of the afferent sympathetic neurons appears to be the major driving force of the pineal gland, the gland can receive other hormonal effects through its blood supply. However, hormonal effects have been found to be subsidiary to, and modulated by, the sympathetic neuronal effects [Cardinali (1981) and Datta and King (1980)].

The pineal contains, synthesizes and secretes a number of biologically active compounds. These include the methoxyindoles - such as melatonin (5-methoxy-N-acetyltryptamine); 5-methoxytryptophol; 5-methoxytryptamine and 5-methoxyindole-3-carboxyaldehyde, and a large number of protein and peptide compounds [Review - Pevet (1983)]. Melatonin (MEL) is generally considered to be the principal pineal hormone, although the other compounds may also prove to be of biological importance [Reiter (1989)].
FIGURE 1.1 Diagrammatic representation of the proposed neural connections between the eyes and a pinealocyte in the pineal gland.

The synthesis of MEL is shown in the pinealocyte. Interruption of the neural connections between the suprachiasmatic nucleus and the pineal eliminates cyclic MEL production. [Reiter (1988)].
1.2.2 The Chemical Structure, Biosynthesis and Metabolism of Melatonin

In 1917 McCord and Allen reported that amphibians fed with pineal extracts exhibited skin lightening, but were unable to specify the cause. The active blanching agent was later isolated and identified by Lerner et al. (1958) as 5-methoxy-N-acetyltryptamine and given the name melatonin. MEL is synthesized and secreted by the pineal gland of all vertebrate species examined [Cardinali (1981)]. The pathways of MEL biosynthesis, its chemical structure and metabolism are shown in Fig. 2.1.

The pathways in the biosynthesis of MEL are well understood. The production of MEL in the pinealocytes begins with the uptake of tryptophan from the bloodstream. Within the pinealocytes, tryptophan is hydroxylated by the enzyme tryptophan hydroxylase to 5-hydroxytryptophan, and it is suggested that the rate at which this occurs depends on the availability of tryptophan [Reiter (1989)]. Once formed, 5-hydroxytryptophan is decarboxylated in the presence of the enzyme aromatic L-amino acid decarboxylase (dopa decarboxylase) and forms the biogenic amine 5-hydroxytryptamine (serotonin). Serotonin concentrations in the pineal gland are very high and exceed those in any other organ in the body [Reiter (1989)]. Serotonin then undergoes a three way transformation: (1) some of the serotonin is metabolized by monoamine oxidase (MAO) to 5-hydroxyindole acetaldehyde, an unstable intermediate, that is either oxidised to 5-hydroxyindole acetic acid, or reduced to 5-hydroxytryptophol; (2) serotonin can be acted on directly by hydroxyindole-O-methyltransferase (HIOMT) to form 5-methoxytryptamine, also considered to be a biologically active pineal hormone; (3) a major pathway for the metabolism of serotonin is its conversion to MEL, via N-acetylation by N-acetyltransferase (NAT), to yield N-acetylserotonin. N-acetylserotonin is then O-methylated by the enzyme HIOMT to form MEL. S-adenosylmethionine serves as the methyl donor.
FIGURE 12 Schematic representation of the biosynthesis, chemical structure and metabolism of MEL.

[Modified from Cardinali (1981) and Reiter (1989)].

ABBREVIATIONS:

TP: Tryptophan
5HTP: 5-Hydroxytryptophan
5HT: Serotonin
NAS: N-Acetylserotonin
MEL: Melatonin
6H-MEL: 6-Hydroxymelatonin
5HIAL: 5-Hydroxyacetaldehyde
5HTOH: 5-Hydroxytryptophol
5MTOH: 5-Methoxytryptophol
5HIAA: 5-Hydroxyindoleacetic acid
5MIAA: 5-Methoxyindoleacetic acid
5MTP: 5-Methoxytryptophol
5MT: 5-Methoxytryptamine
ALAA: Aromatic L-amino acid
NAT: N-Acetyltransferase
MAO: Monoamine oxidase
HIOMT: Hydroxyindole-O-methyltransferase
NA-NF-MK: N-Acetyl-N2-Formyl-
5-Methoxykynurenamine
5-MEKA: 5-Methoxykynurenamine
MEL synthesis in mammals is not exclusively restricted to the pineal gland. It appears to be produced in the retina, in the Harderian gland, in extraorbital lacrimal glands, in the intestine and possibly even in red blood cells. It is, however, a common and possibly accurate assumption, that the MEL concentration in the blood is a consequence almost exclusively of pineal MEL synthesis and release. [Reviews - Cardinali (1981) and Reiter (1989)].

MEL appears to be secreted by the pineal gland by simple diffusion, no active mechanism of secretion has yet been described. Although the matter remains to be conclusively resolved, it is generally accepted that the blood of the pineal capillaries, rather than the cerebro-spinal-fluid (CSF) is the primary site of MEL secretion [Cardinali et al (1983)]. MEL has a half life of about 20 mins in the blood of rats [Gibbs and Vriend (1981)]. The major portion of MEL in blood is bound to plasma protein [Pardridge and Mietus (1980)], predominantly to the high-capacity, low-affinity binding sites on albumin [Cardinali et al (1972)]. Plasma protein binding does not impede MEL transport into the brain, since albumin-bound MEL is readily transported through the blood brain barrier [Pardridge and Mietus (1980)].

The primary site for the metabolism of MEL in experimental animals is the liver [Wurtman et al (1968)]. The major MEL metabolite appears to be 6-hydroxymelatonin, which is conjugated to sulphate and to a lesser extent glucuronide derivatives, which are excreted in the urine [Kveder and McIsaac (1961)]. More recently, N-acetylserotonin has been identified as a MEL metabolite in the urine [Young et al (1985)], suggesting that MEL is demethylated back to its precursor, implying the existence of a complex feedback mechanism controlling MEL synthesis.

MEL is also actively metabolized in the brain, being readily oxidised to N-acetyl-5-methoxykynurenamine via N-acetyl-N-formyl-5-methoxykynurenamine [Review - Cardinali (1981)].
MEL production in the pineal gland is cyclical in both diurnally and nocturnally active mammalian species. The highest pineal MEL production occurs during the dark period, with minimal synthesis occurring during the day. It is the light, perceived by the eyes that is essential for controlling pineal MEL production, via the neuronal connections outlined in Fig. 1.1 [Reiter (1988)]. It is widely accepted that the elevated production of pineal MEL during the dark period is due primarily to the release of NA (NA) from the postganglionic nerve terminals within the pineal gland. NA acts on β-adrenergic receptors on the pinealocyte membrane to increase cAMP production, promoting the activity of NAT and eventually MEL synthesis. α-adrenoreceptors also appear to be operative in the process, in that they are capable of potentiating the β-adrenergic stimulation of NAT [Sugden et al (1984)].

The circadian noradrenaline release is possibly mediated by a "biological clock", which is located in or near the SCN of the hypothalamus [Axelrod (1978)]. The darkness associated increase in MEL production appears to be a common feature in virtually all mammals "under a variety of photoperiodic conditions", and "on a day to day basis is highly reproducible in a given individual" [Reiter (1988)]. The MEL rhythm persists in complete darkness or in blinded animals, but can be suppressed by maintaining animals in continuous light [Klein and Weller (1970)] and therefore appears to be endogenous, dependent on the sympathetic neural input and subject to modification by environmental lighting.

The duration of raised MEL levels in animals is a consequence of the length of the night to which the animal is exposed, thus longer nights translate into longer time periods of raised MEL. Since the length of the nights is a function of the seasons of the year, the changing MEL signal serves to synchronize the physiology of the animal with the appropriate seasons of the year [Reiter (1988)].

Although the primary input for controlling the amplitude and length of the MEL production cycle is light, it appears that the levels of
other hormones, such as estrogens, progesterones, glucocorticoids and prostaglandins are also important "in the full expression of the final response" [Cardinali and Vacas (1987)].

Environmental stimuli other than light may also be involved in the regulation of pineal MEL synthesis. Lynch and co-workers (1973) and Sampson (1975) demonstrated that MEL synthesis may be activated by stress and hypoglycaemia. Ambient temperature has also been implicated, leading to the suggestion that the pineal gland may be involved in thermoregulation [Ralph et al (1979)].

1.2.3 Sites and mechanism of action of melatonin within the central nervous system

The principal locus of MEL activity is still a matter of controversy. Most investigators agree, however, that the central nervous system is probably the major site of MEL action [Anton Tay (1974); Cardinali (1981); Sugden (1983) and Smith (1985)].

The existence of a saturable uptake mechanism for MEL in the brain was demonstrated by Cardinali et al (1979), who showed that the intracisternal injection of excess amounts of non-labelled MEL to rats resulted in a significant decrease in the subsequent brain uptake of radioactive MEL. Subsequent binding studies made use of [³H]-MEL, to demonstrate MEL binding sites in crude membrane fractions of bovine medial basal hypothalamus and occipital and cerebral cortex. MEL binding activity in these studies was undetectable in the amygdala, pons or striatum. Also in 1979, Niles and co-workers demonstrated the presence of high affinity binding sites for MEL in cytosol fractions of hypothalamus, hippocampus and striatum, suggesting that in the brain, MEL binding may be associated with cytosol receptors.

Binding studies with [³H]-MEL proved, however, to be irreproducible and thus could not be extended [Cardinali P. cited in Zisapel et al
Zisapel et al., suggest that this is possibly due to the relatively fast dissociation of [3H]-MEL from the receptor and to masking by nonspecific binding.

Laudon and Zisapel (1986) introduced the use of a potent MEL analog [2-125I]iodo-melatonin (125I-iodo-MEL) as a specific probe for MEL binding sites. 125I-iodo-MEL binding sites have been identified in the synaptosomal preparations of hypothalamus, striatum, medulla pons, hippocampus, cortex and amygdala of both the male and female rat [Laudon and Zisapel (1987) and Zisapel et al (1988)]. Furthermore Zisapel et al (1988) demonstrated a distinct diurnal variation in binding site density (not affinity) in the hypothalamus, but not in the striatum.

125I-iodo-MEL binding sites have also been identified in the SCN and the small part of the choroid plexus at the caudal end of the fourth ventricle in the brain, and outside of the central nervous system in retina and anterior pituitary [Vaneček (1988)].

It is possible that the daily variations in MEL binding sites in the hypothalamus may be a consequence of the daily cyclical changes in the endogenous content of MEL, or down regulation of MEL-binding sites by elevated nocturnal MEL levels. However the regional specificity (eg hypothalamus, but not striatum) of the rhythms and the evidence that the binding site density declines 5-7 hours before the circulating MEL level reportedly reaches its nocturnal peak, contradicts this possibility [Zisapel et al (1988)]. The diurnal variations in the density of MEL-binding sites at specific brain regions may, however, underlie the physiological response of the neuroendocrine system to MEL.

The MEL binding sites identified with 125I-iodo-MEL fulfill the requirements for classification as receptors. Binding to these sites is specific, saturable, time-dependent and reversible, with an apparent dissociation constant of MEL binding, as judged by its ability to compete for 125I-iodo-MEL binding sites, in the micromolar
range [Zisapel (1988)].

The presence of the MEL binding sites in the hypothalamus appear to have physiological relevance, since a number of studies have implicated the hypothalamus as the main site of MEL's neuroendocrine activities. Zisapel et al (1988) however, conclude that "the role of the striatal $^{125}$Iodo-MEL binding sites is still an enigma."

Although the identification of MEL receptors assists in an understanding of the molecular basis of MEL action, the subcellular and biochemical mechanisms affected by MEL in the brain are not yet clearly characterized. Various relevant brain functions and components are affected by treatment with MEL. These include protein synthesis, serotonin and GABA whole brain concentrations, neurotransmitter synthesis, uptake, reuptake and release, axonal transport, tubulin levels and prostaglandin and neurohormone release [Review - Cardinali (1981)]

Many investigators have suggested that the behavioural effects of MEL administration may be due to changes in brain serotonin concentration [Review Reiter (1977)]. These suggestions are based for the main part on a report by Anton Tay et al (1968) that intraperitoneal (ip) administration of MEL (500 μg/kg (BM)) results in elevated whole brain serotonin concentrations. Sugden (1983), following the demonstration that a dose of MEL which clearly had a sedative/hypnotic effect (20 mg/kg (BM) ip) failed to alter whole brain serotonin or 5-hydroxyindoleacetic acid, suggested that the mechanism of action of MEL in behavioural effects is not due to an increase in brain serotonin, although more subtle changes in serotonergic neurotransmission may be involved.

MEL does not appear to be a competitor for serotonin binding to the serotonin receptor [Bennet et al (1975)]. It does, however, impair serotonin synaptosomal reuptake in a noncompetitive and relatively nonspecific manner (it also impairs NA, DA and glutamate
MEL administration in doses of 50\(\mu\)g/kg to 250\(\mu\)g/kg have been shown to increase GABA concentrations in the hypothalamus and cerebral cortex of the rat brain, 2 hours after injection [Anton Tay (1971)].

It has been suggested that the action of MEL on brain GABA and serotonin is mediated by its effect on brain pyridoxal phosphokinase activity. Pyridoxal phosphokinase activity in brains of rats treated ip with 200\(\mu\)g/kg MEL showed an almost six-fold increase, 45 min after MEL injection, and remained high even after 180 min [Anton Tay et al. (1970)]. Both MEL and pyridoxal phosphokinase activities peak at midnight, six hours after the onset of the dark phase. Pyridoxal phosphokinase catalyzes the formation of pyridoxal phosphate - a prosthetic group of the enzyme aromatic L-amino acid decarboxylase and of glutamic acid decarboxylase, the synthesizing enzymes of serotonin and GABA respectively. Pyridoxal phosphokinase is also a factor in the synthesis of other catecholamines such as DA and NA, the synthesis of which may also be modified by MEL.

Coloma and Niles (1988) have demonstrated that MEL is capable of enhancing the \textit{in vitro} binding of GABA in the rat brain. A similar \textit{in vivo} enhancement of low-affinity GABA binding in the central nervous system has also been observed following chronic administration of MEL to rats [Niles et al. (1987)]. This suggests that the psychopharmacological effects of MEL may be due to modulation of central GABA function, similar to that previously described for benzodiazepines and barbiturates [DeFeudis (1983)].

MEL competes for benzodiazepine binding sites in rats with an affinity of about 400 \(\mu\)M [Coloma and Niles (1988)], which is in excess of brain and circulating levels of MEL [Marangos et al. (1981)]. This would indicate that only the pharmacological effects of MEL involve benzodiazepine binding sites. On the basis of the
observation that nanomolar concentrations of MEL are able to alter GABA binding, Coloma and Niles (1988) suggest that high affinity and physiologically relevant MEL binding sites are involved in modulating GABAergic function.

Coloma and Niles further propose that the mechanism underlying MEL's enhancement of GABA binding is an increase in phospholipid methylation, this however remains to be demonstrated.

Datta and King (1978) demonstrated that MEL treatment (250 μg/rat/day ip) over 5 days leads to a significant rise in whole brain DA levels in rats exposed daily to a novel environment. This increase in DA was accompanied by a decrease in novelty induced defecation and a decrease in plasma 11-hydroxycorticosterone concentrations. These investigators suggested therefore that the rise in brain DA after MEL administration may be mediated by the rise in pyridoxal kinase activity [Anton Tay et al (1970)], and that MEL acts via an increase in DA, to inhibit the pituitary adrenal response to stress. This would indicate that a possible mechanism of MEL's central activity is to increase whole brain DA concentrations. These studies of Datta and King are far from conclusive and the results should be treated with caution, since whole brain neurotransmitter concentrations are not a very good indicator of the neurotransmitter activity centrally. The increase in brain catecholamine levels may be the result of decreased release, with a resultant decrease in activity, rather than increased synthesis.

Picomolar concentrations of MEL inhibit the calcium ion dependent release of DA from the retina [Dubocovich (1983)], the ventral hippocampus, medulla pons, preoptic area and the median and posterior hypothalamus [Zisapel et al (1982)]. No inhibitory effect of MEL on DA release was observed in the cerebral cortex, cerebellum, dorsal hippocampus and striatum. The distribution of MEL effects on DA release is more region-specific than the distribution of MEL binding sites, since high affinity binding sites have also been detected in the cerebellar cortex, amygdala
striatum and medulla pons.

In an attempt to explain this discrepancy, Zisapel et al (1982) suggest the following possibilities:
(1) MEL may affect neurosecretion of other neurotransmitters, differentially localized within the brain.
(2) MEL may also inhibit the re-uptake of DA, thus reducing the net effect of MEL on the amount of radioactive DA released in regions where the re-uptake mechanism is specifically inhibited. The effect of MEL on DA release would therefore be "masked".
MEL was demonstrated to impair DA reuptake but this was in a noncompetitive, nonspecific manner [Cardinali et al (1975)] Further testing would be necessary to validate these proposals.

The regional distribution of MEL effectivity on DA release corresponds very well with the distribution of sites relating to the antigenadal activity of MEL [Zisapel (1988)].

The modification in cyclic nucleotide synthesis is another effect of MEL which exhibits kinetic properties and structure activity relationships, compatible with the interaction with MEL receptors [Cardinali et al (1983)].

Evidence published in 1974 by Ortega et al [cited in Anton-Tay (1974)] indicates that MEL modifies the activity of brain adenylate cyclase. Brain 3':5'cyclic adenosine monophosphate (cAMP) levels were assayed by radioimmunoassay after administration of MEL (ip) to rats. The effects of MEL on cAMP were region-specific with the midbrain showing a 31% fall in cAMP levels, in contrast to the cerebellum which showed an increase in 57%; while concentrations in the cerebral cortex remained unchanged. Maximal changes in cAMP levels were detected 10 min after MEL administration and the decrease in the midbrain was apparent for 40 min. MEL has also been demonstrated to decrease medial basal hypothalamic cAMP accumulation [Vacas et al (1981)]
Anton Tay (1974) suggests that the changes in brain cAMP observed after MEL administration imply that MEL acts on at least two different kinds of receptors, located in the midbrain and cerebellum.

Effects of physiological concentrations of MEL on cGMP synthesis have also been demonstrated in vitro. Vessely (1981) found that MEL (10⁻⁹M) increased guanylate cyclase activity 2-3 fold in rat adenohypophysis, testis, ovary, thyroid and liver. A significant increase in cGMP in medial basal hypothalamus was also induced by MEL (10⁻⁸M or higher) [Vacas et al (1981)].

The relationship of these cAMP and cGMP changes, mediated by MEL, to the mechanism of the MEL activity-response effect has not yet been established. The function of cAMP is generally that of a second messenger, transducing specific neurotransmitter actions onto target cells, while cGMP has been implicated in physiological processes at synapses in both peripheral and central nervous system tissue.

Other biochemical activities of MEL have also been described, including the ability to depress prostaglandin synthesis and release [Cardinali (1981)], the ability to regulate the secretion of melanocyte-stimulating hormone (MSH) and melanocyte-stimulating hormone inhibiting factor-I (MIF-I) [Datta and King (1979)]. It has also been hypothesized that MEL affects microtubule- or microfilament-dependent processes in living organisms and that MEL may affect contractile protein dependent processes in the brain [Cardinali (1981)].

There is no single, well defined mechanism to describe MEL activity in the brain - the suggestion can only be made that MEL is capable of modulating various neurotransmitter systems, in many areas of the brain. The effects of MEL on brain do, however, appear to be inseparable from the available concentrations of central putative neurotransmitters (for example GABA, DA, serotonin and NA) [Datta
1.2.4 The brain and behavioural effects of the pineal gland and melatonin

The pineal gland and MEL have a widespread influence on a variety of physiological functions. It has been suggested that the function of all other endocrine organs may be modulated by the secretion of pineal melatonin and that "any physiological event that has a 24 hour or seasonal rhythmic aspect should be suspected of being influenced by the MEL cycle" [Reiter (1989)].

Although not discussed here, reports have been published on the effects of MEL administration on the adrenal glands, on both glucocorticoid and aldosterone synthesis and release, on the thyroid gland, on growth hormone, and on MSH and MIF-I [Review - Skene (1979)].

Data supporting the role of the pineal gland and MEL in the modulation of the hypothalamic-pituitary-gonadal axis is vast, suggesting an important role for the pineal gland in the control of reproduction, particularly in seasonal animals [Reiter (1980 and 1981)]. Cardinali et al (1983) suggests that a number of aspects of the hypothalamic-pituitary-gonadal axis are depressed by MEL administration in hamsters, voles, mice, gerbils, rats, sheep, steer, dogs, rabbits, hares, monkeys and weasels [Review- Cardinali (1981)]. The anatomical sites implicated in MEL antagonadal activity appear to be primarily central, but gonadal tissue also appears to be involved [Cardinali et al (1983)]. The endocrine effects of exogenous MEL in humans are not well investigated and conclusions drawn are not definitive, although MEL has been shown to cause a reduction in serum lutenizing-hormone (LH), and have dramatic effects on growth hormone (GH) production [Review - Reiter (1989)].
Not only are the endocrine effects of MEL possibly mediated by the central nervous system, but the administration of MEL also produces distinct effects on EEG activity, sleep and behaviour.

1.2.4.1 Sleep and electrophysiological changes

The rise in MEL during the dark period concomitant with human sleep suggests an influence of the pineal gland on sleep and rest. Somnolence and sedation are not an uncommon occurrence after MEL administration. MEL administered orally, intranasally, or by the iv route, in a variety of pharmacological doses, to healthy human subjects and Parkinsonian patients, resulted in sensations of "well being and moderate elation", relaxation, contentment and tranquilization [Anton Tay et al (1971) and Cramer (1974)].

Anton Tay et al. (1971) demonstrated that 1,25 mg/kg iv administration of MEL resulted in an increase in both the percentage of and amplitude in EEG alpha rhythms, accompanied with an increase of rapid eye movement (REM) sleep. Cramer et al., reported that MEL (50 mg iv) appeared to be a potent inducer of sleep. However, the MEL-induced sleep, behaviourally as well as by its polygraphic (EEG) pattern, resembled nocturnal sleep, with no significant changes in percentage and frequency of REM sleep or in dream activity. An increase in degree of synchronization was found during deep and REM sleep.

The inversion of the sleep-waking pattern, so that sleep is diurnal is not accompanied by an increase in MEL, although the nocturnal rise in MEL was attenuated [Vaughan et al (1979)]. In sleep-deprived subjects exposed to constant light, the nighttime rise in MEL not only persists, but increases and in the postdeprivation sleep recovery period, MEL levels return to those of predeprivation baseline values [Akerstedt (1979)]. This increase in MEL during the sleep deprivation period suggests a role for MEL in the induction of sleep, but not in sleep mechanisms [Armstrong et al. (1982)].
Armstrong et al (1982) concluded that "MEL contributes to conditions underlying the sleep state rather than being an integral part of sleep mechanisms perse".

There have been a number of studies on the effects of MEL and the influence of the pineal gland on sleep, in animals. These studies are complicated by the fact that many laboratory animals such as rodents, are nocturnal, thus the existence of predominantly diurnal sleep with a nocturnal increase in MEL synthesis is paradoxical.

MEL potentiates hexobarbital induced sleeping time in mice [Barchas (1967) and potentiates pentobarbitone induced sleep in male rats, in a dose related manner [Martini and Fioretti (1971)]. Sugden (1983) present studies that confirm MEL's sedative/hypnotic effect in rodents, and demonstrate MEL potentiation of pentobarbitone induced sleep in both mice and rats, after various routes of administration (ip, iv and oral). The sedative dose of MEL (20mg/kg ip) was low, in comparison to the dose demonstrated to have analgesic and anticonvulsant activity (200mg/kg ip).

MEL administration also induces sleep-like EEG patterns in mice, rabbits and cats [Armstrong et al (1982)].

The mechanism of MEL's sedative or hypnotic activity is uncertain. However, the sleep potentiating and inducing activity of MEL has been ascribed to:

1. MEL's ability to increase brain serotonin concentrations (discussed in Sec.1.2.3).
2. MEL's ability to interact with benzodiazepine receptors [Holmes and Sugden (1982)] - or rather, MEL's ability to enhance GABA receptor binding [Sec 1.2.3 and Coloma and Niles (1988)].
3. the possible release of arginine vasotocin (AVT) (a neuropeptide implicated in the sleep process), by MEL [Reiter (1989)].
4. Modulation of the parathyroid gland, involved in the regulation of serum calcium [Reiter and Morgan (1972)].
Despite the uncertainty about its mechanism of action, one of the functions of MEL may be to "contribute to a state of relaxation and drowsiness assisting entry into sleep and a state of tranquility disposed towards maintaining undisturbed sleep throughout the night" [Armstrong et al (1982)]. MEL may therefore prove useful in the treatment of insomnias [Sugden (1983)] and in the study and treatment of other sleep disturbances, particularly those occurring in relation to shift work and travel across longitudes [Wetterberg (1982)] and those related to emotional disorders [Cramer et al (1974)]. Armstrong et al (1982) also suggests that the whole range of human sleep disorders, that is insomnia, hypersomnia and narcolepsy, should be screened for "aberrant pineal influences".

1.2.4.2 Avoidance behaviour

The study of avoidance behaviours is a commonly used method to evaluate depressant drug activity, and since neuroleptic and hypnotic drugs exert different effects on conditioned avoidance behaviour, the studies allow for some degree of differentiation between depressant drugs.

Skene (1979) demonstrated that MEL's inhibitory effect on a learned avoidance response is similar to the effect of chlorpromazine - a phenothiazine neuroleptic.

Martini and Fioretti (1971) and Kovacs (1974) reported that MEL administration facilitated the extinction of an active avoidance response (AAR), although MEL had little or no effect during the acquisition of the AAR. Datta and King (1980) suggest that the effects of MEL on retention of the AAR are a result of reduced activity of the animals, but only in the absence of the stressor (shock), which was present during the acquisition phase. MEL treatment (250 mg/rat) also facilitates the extinction of learned passive shock avoidance and decreases defecation induced by this aversive situation [Datta and King (1977)].
These investigations of avoidance behaviour in rats suggest that MEL can reduce certain behavioural aspects of emotional stress [Datta and King (1980)].

1.2.4.3 Spontaneous locomotor activity (SLA)

Studies on the effects of MEL administration on locomotor activity have yielded varied and often contradictory results. The picture is further complicated by the often conflicting results obtained in studies of locomotor activity in pinealectomized rats.

Wong and Whiteside (1968) demonstrated that MEL administration to food deprived rats (10 μg/rat daily) decreased activity (measured in terms of running on an activity wheel), for the first five days of testing. These results supported those obtained by Reiss et al (1963), who demonstrated that pinealectomy in neonatal rats resulted in increased treadwheel activity, measured 12 weeks after surgery. Neonatal and postpubertal pinealectomy of rats was also shown to increase open field and activity cage activity respectively [Sampson and Bigelow (1971) and Karppanen et al (1973)].

Reiss et al (1967) demonstrated microscopically that the pineal glands of male rats classified as "slow runners" exhibited a greater pineal cell density than the "fast runners", suggesting greater MEL production in "slow runners" than in "fast runners".

MEL also reduces SLA in mice. Motor activity measurements carried out during the day, when spontaneous activity of mice is low, revealed that MEL injection (ip) to mice produced a dose-dependent (20-100 mg/kg) inhibition of activity between 5 and 25 min after injection [Sugden (1983)].

Results such as these suggested that pineal functioning, and in particular MEL, decreases the total locomotor activity in rodents. However, several other reports have indicated that melatonin does
not affect the locomotor activity of rats and that pinealectomy may even result in decreased activity.

MEL administration in doses ranging from 50-200 \( \mu \text{g/rat} \) for periods between 3 and 14 days, to normal, pinealectomized and hypophysectomized rats, failed to affect activity measured in the open field or in activity cages [Sampson and Bigelow (1971), Kastin et al (1973) and Kovacs et al (1974)].

Neonatal and postpubertal rat pinealectomy has also been shown to have no effect on locomotor activity in both food deprived and normal rats [Remley et al (1969), Quay (1968 and 1970a), Relkin (1970a and 1970b) and Kovacs et al (1974)]. Kincl et al (1970) demonstrated reduced motor activity, as measured in activity cages, 13 weeks after neonatal pinealectomy.

More recent studies involving the administration of MEL to more discrete areas of the brain have shown reduced SLA. The intranigral injection of 0.01 and 0.1 \( \mu \text{g} \) of MEL showed a 50% and 83% reduction in SLA respectively [Bradbury et al (1985)]. Bilateral injections of MEL into the nucleus accumbens also induced a dose-related decrease of locomotor activity [Gaffori and Van Ree (1985)]. The most effective dose appeared to be 10ng, the smallest effective dose being 0.1ng and a dose of 1000 ng having no influence on the behaviour of the rats. In both these studies the decrease in SLA was accompanied by an increase in grooming and sniffing, interpreted by Gaffori and Van Ree (1985) as an increase in emotivity. In the substantia nigral studies the inhibition of SLA by MEL was partially reversed by sulpiride (a \( \text{D}_2 \) selective antagonist), suggesting that MEL may also influence \( \text{D}_2 \) receptors (possibly autoreceptors). This was supported by results in the same study indicating a simultaneous reduction in DA "function in striatal and limbic regions". However, a component of MEL's action was unaffected by sulpiride and Bradbury and co-workers suggest that MEL may also act on serotonergic transmission, since serotonin is known to be intimately involved with the release of nigral
dendritic DA and may have a role in the nigro-striatal pathway [Williams and Davies (1983)].

Following the demonstration in their study that MEL's effect on SLA was not antagonized by pretreatment with sulpiride or haloperidol, but by serotonin and various antidepressant drugs injected into the nucleus accumbens, Gaffori and Ree proposed that serotonin, rather than dopamine, is involved in the MEL induced behavioural changes. They also suggest that the serotonin-melatonin interactions are functionally important in the nucleus accumbens.

It is therefore apparent that MEL does not exert a simple effect on locomotor activity. Coupled to the observation that rats are nocturnal animals, exhibiting highest locomotor activity at night, when MEL secretion is highest, it can be suggested that MEL does not exert direct control over locomotor activity. MEL may act via dopaminergic and serotonergic systems in the substantia nigra and nucleus accumbens, to modulate locomotor activity.
1.2.5 Clinical studies and possible therapeutically beneficial effects of melatonin administration.

1.2.5.1 Normal Subjects

The most common effects of MEL administration to normal, healthy volunteers, seems to be sedation, accompanied by a "sensation of well being and moderate elation". The effects of MEL on sleep are covered in Sec.1.2.4.1. In general, subjects exhibited no abnormal behaviours and no side effects of melatonin have been described, except for mild headache and abdominal cramps, even following doses as high as 6.6g, administered orally for 35 days [Wurtman et al (1983)].

The administration of MEL to normal volunteers subjected to travel over longitudes, resulted in the rapid synchronization of shifted MEL rhythms to normal diurnal rhythms [Desir et al (1980)], suggesting that MEL may be useful in treatment of the condition known as "jet lag" [Reiter (1988)].

1.2.5.2 Neurological disorders

1.2.5.2.1 Epilepsy

The treatment of three epileptic patients with MEL resulted in the same well-being and elation seen in normal subjects, together with decreased temporal lobe electrical activity and transient suppression of paroxysmal activity [Anton-Tay et al (1971)]. A further study of the effects of MEL on epilepsy, in a group of six patients with intractable epilepsy of various aetologies, confirmed the results of the first study. In all six patients, a reduction in spiking activity was observed with decreased frequency of seizures during the treatment [Anton-Tay (1974)]. Wurtman et al (1985) suggest that, although the influence of MEL on epilepsy is not clear, it justifies further exploration.
1.2.5.2.2 Parkinson's Disease

The administration of MEL to two Parkinsonian patients (1.2g daily for 4 weeks) resulted in an improvement in their overall clinical picture [Anton-Tay (1971)]. After the second week, both patients reported a general feeling of well-being, associated with an improvement in the performance of daily tasks, and by the end of treatment rigidity and tremor improved. In both patients, placebo substitution led to overall deterioration. This study was later extended and confirmed in two groups of patients, one consisting of five patients to whom MEL (1.2g daily) was given orally for 4 weeks in an open trial and a second group to which the same dose was given in a random, double-blind, cross-over trial [Anton-Tay (1974)]. At the end of the fourth week all patients showed amelioration in their clinical picture, with a progressive deterioration two weeks after the end of the trial. In both these studies, previous medication was stopped 24 hours prior to starting MEL treatment.

However, the beneficial effects of MEL in Parkinson's Disease have not been confirmed by other authors.

Following the demonstration that the administration of MSH (β-MSH) to patients with Parkinson's disease darkened their skin and aggravated their tremor, Cotzias et al (1971a) administered MEL - an agent that antagonizes the "darkening of the skin" and several other actions of MSH, to a patient with Parkinsonism. It diminished tremor and caused sedation but had no effect on other clinical features. The same group of investigators then conducted a single blind study in 11 Parkinsonian patients who received either MEL alone or MEL plus levodopa (L-dopa) [Papavasiliou et al (1972)]. In doses ranging between 3 and 6.6 g/day MEL did not affect the signs of Parkinsonism or the antiparkinson effects of L-dopa.

Shaw et al (1973) also reported that doses of MEL up to 1g, were ineffective in modifying the clinical picture in a group of four Parkinsonian patients, in whom MEL was well tolerated, with
transitory sedation the only noticeable effect.

Anton Tay (1974) suggests that "these contradictory results are probably due to differences in method of (MEL) administration; since MEL is practically insoluble in biological fluids, it must be given in an appropriate vehicle in order to be absorbed by the gastrointestinal tract".

Further studies are necessary to evaluate the data presented here and to further evaluate the possible effects of MEL in Parkinson's disease.

1.2.5.2.3 Tardive Dyskinesias

Sandyk (1988) proposed that the pineal gland and MEL may be involved in the pathophysiology of neuroleptic-induced tardive dyskinesias and that MEL may be protective against the disorder. This hypothesis is based on the observation that pinealectomy markedly enhanced the incidence and severity of orofacial dyskinetic movements in haloperidol treated rats, as compared to haloperidol treated non-pinealectomized rats [Sandyk and Fisher (1988). Moreover, ip administration of MEL attenuated the dyskinetic movements in the pinealectomized rats, suggesting that the development of orofacial dyskinesias was a result of a reduction in MEL secretion.

The observation that the incidence of the disorder is higher in depressed patients in whom nocturnal MEL secretion is reduced [Brown et al (1985) and Hamra et al (1983)], further advances the concept that the development of the disorder is enhanced by reduced MEL secretion.

The mechanisms by which MEL exerts this antidyskinetic effect requires further elucidation. Sandyk and Fisher (1988) suggest that the mechanism could be via attenuation of serotonergic, GABAergic or dopaminergic pathways, or even through its antigonadotrophic
MEL is, however, not the only pineal factor implicated in exerting antidyskinetic effects. The melanocyte inhibiting factor, L-propyl-L-leucylglycinamide (PLG), administered to humans, attenuates the severity of the disorder, while in rats PLG, administered concurrently with haloperidol, blocked the enhancement of radioligand binding to DA receptors [Review - Sandyk and Fisher (1988)]. Chiu et al (1981) postulate that a distinct receptor for PLG is functionally coupled to the neuroleptic-DA-receptor complex and the activation of this putative receptor is responsible for the observed "desensitizing" effects of PLG on striatal DA receptors.

1.2.5.2.4 Huntington's Chorea

In a single study involving 2 patients with Huntington's Chorea - an "autosomal-dominant" inherited movement disorder associated with degeneration of neurons in the basal ganglia [Coyle and Schwarcz (1976)], Carman et al (1976) reported that MEL administration resulted in depression and psychomotor retardation.

1.2.5.3 Psychiatric disorders

1.2.5.3.1. Schizophrenia

The possibility of a relationship between the pineal gland and schizophrenia was suggested as early as 1954 by Woolley and Shaw. In 1961 McIsaac et al. demonstrated that MEL, in vitro, can be cyclo-dehydrated to 10-methoxyharmalan, a serotonin antagonist with significant effects on behaviour. They suggested that 10-methoxyharmalan may be an endogenously produced alkaloid, involved in the pathophysiology of psychotic states.

Jones et al (1969) administered MEL to three schizophrenic and two normal subjects and found a slightly different pattern of excretion
of MEL metabolites in the schizophrenic subjects compared to the normal subjects. The schizophrenic patients appeared to excrete a higher percentage of an unidentified acidic metabolite, with a slightly lower excretion of 6-hydroxymelatonin - indicating a possible altered metabolism of MEL in schizophrenic patients.

Based on the "transmethylation theory" of schizophrenia, which suggests that abnormal biochemical processes are involved in the aetiology of the disorder [Osmond and Smythies (1952) and Kety (1965)], Greiner (1970) suggested that HIOMT, a methylating enzyme, may be congenitally defective in schizophrenic patients. HIOMT is a methylating enzyme involved in the biosynthesis of MEL and Greiner suggests that if HIOMT is inhibited or defective, MEL synthesis would be reduced in favour of the production of a harmalan alkaloid, as suggested by McIsaac (1961). This hallucinogenic alkaloid would be responsible for the psychotic picture in schizophrenia and the decreased MEL may be associated with the diffuse melanosis described by Greiner in schizophrenic patients. Greiner concedes that there are "many if's" in his theory, since the hypothesis is based on the assumptions:
(1) that the harmalan alkaloid is synthesized in vivo,
(2) that the HIOMT enzyme is defective in schizophrenics and
(3) that the melanosis seen in schizophrenic patients and which is potentiated by chlorpromazine is related to a skin lightening effect of MEL.
He does, however, propose that the pineal gland may be the locus of defect in schizophrenic patients.

In more recent postmortem studies, HIOMT has been shown to be elevated in the pineal glands of one group of psychotic individuals, while another group showed normal values [Smith et al (1977) and Smith et al (1981)].

Animal studies have tended to suggest that neuroleptic drugs inhibit pineal HIOMT activity. Naylor and Olley (1969) reported that following the administration of the potent neuroleptic,
haloperidol, to rats, the drug is found in higher concentrations in
the pineal gland than in any other part of the brain. Hartley et al
(1972) showed that haloperidol and fluphenazine inhibit the in
vitro formation of MEL by inhibition of HIOMT. In addition, Hartley
and Smith (1973) demonstrated that psychotomimetic agents such as
lysergide, mescaline and amphetamine activate HIOMT in vivo.

Watson and Madden (1977) suggest that it would be useful to study
the CSF of schizophrenics for O-methylated monoamines and MEL. If
MEL and the methylated compounds are methylated by a common enzyme
(HIOMT), parallel changes in their endogenous levels would be
expected and the administration of neuroleptic agents to such
patients may yield a dose-related decrease in both MEL and
methylated monoamines.

Normal circadian rhythms of MEL have been reported in
schizophrenics [Wetterberg (1978) and Hanssen et al (1980)]. However,
Ferrier et al (1982a and 1982b) reported reduced midnight levels of
MEL in schizophrenic patients, coupled with raised cortisol levels,
demonstrating a low MEL/cortisol (M/C) ratio.

Although limited, clinical studies to evaluate the therapeutic
value of MEL in the treatment of schizophrenia have yielded poor
results. The administration of MEL (iv) to 2 schizophrenics caused
a "florid exacerbation of symptoms" [Young and Silman (1982)].
Lerner and Nordlund (1978) noted an exacerbation of clinical
symptoms in some cases, and no effect in others.

Several investigators have, however, reported that the
administration of a MEL-free aqueous pineal extract (containing a
peptide of approximately 1000 daltons) to chronically medicated
schizophrenic patients has resulted in a moderate, but variable,
improvement in their clinical profiles [Alteschule (1957), Eldred et
al (1960) and Bigelow (1974)].

In summary, it appears that the pineal gland may be involved in the
pathophysiology of schizophrenia, and may be one of the sites of activity of neuroleptic drugs such as haloperidol and fluphenazine. Although MEL biosynthesis or metabolism may be involved in the pathophysiology of the disorder, it appears that a pineal peptide may be of more therapeutic value in the treatment of the disorder.

1.2.3.5.3 Mania and depression

Studies on MEL rhythms or patterns and levels in depression have yielded conflicting results. Normal MEL basal levels and rhythms have been described in depressed patients and have been maintained not only in the testing state, but also in sleep deprivation and constant light [Wetterberg (1978) and Jimerson et al (1977)].

Other investigators have, however, demonstrated significant changes. MEL levels appear to correlate with mood. The general trend in studies reviewed by Young and Silman (1982), appears to be: low MEL levels in depression, high levels in mania, and scattered levels in bipolar manic depression.

In a double-blind crossover by Carman et al (1976), using both psychotically and nonpsychotically depressed patients, MEL was administered in varying amounts either orally or by iv infusion. During treatment, depression ratings increased and were associated with moderate to severe insomnia and anorexia. These investigators suggest that although MEL was clearly not therapeutic in depression, it might be useful in the treatment of manic patients, or as a mild anti-anxiety agent.

1.2.6 Summary

Armstrong et al (1982) state that "considering the multitude of biochemical, physiological, endocrinological and behavioural changes attributable to the pineal, it is unlikely to have so many specific functions" and they suggest that the function of the
pineal gland must be at a higher level of integration. This suggestion, coupled with others, such as the proposals that the pineal gland acts as a "regulator of regulators" [Reiter and Hester (1966) cited in Armstrong et al (1982)] and that MEL acts as a "central inhibitory modulator", "neuroendocrine transducer" or "biological clock" [Datta and King (1980)], supports the hypothesis proposed by Armstrong et al (1982), that the pineal is a "synchronizer of regulators", with environmental lighting acting as the main Zeitgeber (entraining agent or synchronizer).

The pineal gland is ideally placed, anatomically, to collect, integrate and compare information from both extracranial sources and intracranial sites. The regulatory function of the pineal gland on the body as a whole (including brain) appears to be by MEL release (although other pineal secretions and peptides may be involved).

From the data presented, it would appear that MEL acts by binding to specific MEL receptors, to induce functional changes in other neurotransmitter processes, responsible for the effects ascribed to MEL.

Apart from the already well established role of MEL as a modulator of the hypothalmic-pituitary-gonadal axis, and therefore its possible involvement in the reproductive cycle, MEL may well prove to be an important modulator of behaviour.
1.3 CENTRAL NERVOUS SYSTEM DOPAMINERGIC NEUROTRANSMISSION

1.3.1 The chemical structure, biosynthesis and metabolism of dopamine.

DA (3-hydroxytyramine) is biosynthesised in a sequence of enzymatic reactions which lead to DA, NA, and adrenaline synthesis. Not only is DA synthesized in dopaminergic neuron terminals, but it is also synthesized in adrenergic neurons and in chromaffin cells of the adrenal medulla, since DA is the immediate metabolic precursor of NA and adrenaline in the pathway of catecholamine synthesis.

The major precursor in the synthesis of these catecholamines is the amino acid L-tyrosine, derived from the breakdown of dietary or endogenous proteins [Fuller (1981)]. L-tyrosine is hydroxylated by a specific enzyme, tyrosine hydroxylase, present within catecholamine forming neurons in the brain and peripheral nervous system, to 3,4-dihydroxyphenylalanine (dopa). Tyrosine hydroxylase also accepts L-phenylalanine as a substrate, which may in some circumstances play an important role as a catecholamine precursor [Bagchi and Smith (1977)]. Tyrosine hydroxylase is also the rate limiting enzyme involved in the biosynthesis of catecholamines [Roth et al (1978)].

Dopa is decarboxylated by the nonspecific enzyme, aromatic L-amino acid decarboxylase (dopa decarboxylase), to form DA. Dopa decarboxylase is present in most tissues in high concentrations and does not play a limiting role in catecholamine biosynthesis, unless its activity is substantially inhibited.

In dopaminergic neurons, tyrosine hydroxylase and dopa decarboxylase are the only synthesizing enzymes present. Adrenergic neurons, however, contain the enzyme dopamine β-hydroxylase, responsible for hydroxylating DA to NA. The final enzyme in the
catecholamine biosynthetic pathway, NA N-methyltransferase, is present only in neurons and in adrenal-medullary cells that synthesize adrenaline as their neurotransmitter, and hormone, respectively.

DA is present in other catecholamine-synthesizing cells besides dopaminergic neurons. In sympathetic adrenergic neurons, DA constitutes about 10% of the total catecholamine and is thought to be present as a reserve of precursor for NA synthesis. In all neurons in which it is present, DA is formed in the cytoplasm and taken up by an active process into, and bound by, organelles termed storage vesicles.

Following stimulation of dopaminergic neurons, DA is released by exocytosis from the storage vesicles into the synaptic cleft, by a calcium-ion dependent process. In the synaptic cleft, DA is capable of activating DA receptor sites both post- and/or pre-synaptically. It is also subject to re-uptake by a presynaptic uptake mechanism or it may be enzymatically metabolized by the enzyme catechol-O-methyltransferase (COMT) to 3-methoxytyramine. DA and its 3-methoxy metabolite are also metabolized intraneuronally by monoamine oxidase (MAO), a mitochondrial enzyme, to the major DA metabolite, homovanillic acid (HVA), which is excreted in the urine [Daftary and Wang (1976)]. MAO occurs in multiple forms, present in differing amounts in various cells, organs and tissues. A simple classification suggests two types, MAO A and B. In the rat DA is thought to oxidised by type A MAO [Fuller (1981)].

The chemical structure, biosynthesis and metabolism of DA, and its role in the biosynthetic pathway of the other catecholamines, NA and adrenaline, is presented in Fig. 1.3.
FIGURE 13 Schematic representation of the pathway of catecholamine biosynthesis and the metabolism of DA.

[Modified from Hornykiewicz (1966) and Bowman and Rand (1980)]
1.3.2 Dopaminergic pathways in the central nervous system

Biochemical and histological studies, together with localized brain lesions, led to the observation that DA in the striatum is localized within the terminals of neurons originating in the pars compacta zone of the substantia nigra (designated as A8 and A9 cell body areas in the rat brain) [Anden et al (1964) and Ungerstedt (1971)]. The neurons originating in the A8 and A9 cell bodies of the substantia nigra and terminating in the striatum form the nigrostriatal pathway, considered to be the major dopaminergic neuronal system in the brain [Moore and Kelly (1978)]. The nigrostriatal system, however, forms only a part of the mesotelencephalic system, which encompasses the entire ascending forebrain projection of the mesencephalic dopamine neuron system.

The mesotelencephalic system is divided into two major subsystems: (1) the mesostriatal system, comprises the nigrostriatal system (2) the mesocortical system, which includes the mesolimbic system and the mesocortical system, both these systems comprising neurons extending from the A10 cell bodies, contained primarily in the interpeduncular nucleus and within the decussation of the superior cerebellar peduncle and the ventral tegmental area, with terminals in the limbic forebrain regions - (the nucleus accumbens; olfactory tubercle; the lateral septal nucleus and the amygdala) and in specific regions in the cerebral cortex - (the cinguli; entorhinal cortex and the prefrontal cortex), respectively.

Other well characterized central dopaminergic systems include: (1) the tuberoinfundibular systems, which have cell bodies in the arcuate nucleus (A12) and terminate in the external layer of the median eminence and the pituitary gland. (2) the incertohypothalamic systems, which have cell bodies located in anterior and posterior hypothalamic sites (A11, A13), the neurons of which are thought to constitute a short intradiencephalic DA system of possible importance in the
regulation of neuroendocrine functions in the hypothalamus. The A14 cell group, the fibers of which are thought to extend to the periventricular, preoptic, suprachiasmatic preoptic nucleus, anterior hypothalamic area and the most caudal portion of the lateral septal nucleus, is also considered to be part of this system. [Lindvall and Björklund (1978) and Jacobowitz (1978)]

Some of these major dopaminergic pathways (particularly the mesostriatal, mesocortical and mesolimbic systems), in the sagittal section, are schematically represented in Fig. 1.4.

The nigrostriatal, mesolimbic and mesocortical systems appear to be the central dopaminergic pathways involved in normal motor control and linked to the behavioural pathophysiology of a number of neurological and psychiatric disorders [Costall and Naylor (1979)].

FIGURE 1.4 Schematic representation of sagittal projections of nigrostriatal, mesolimbic, mesocortical and tuberinfundibular dopaminergic pathways in the rat brain.

Shaded areas indicate major DA nerve terminal areas. [Modified from Ungerstedt (1971) and Richelson (1981)].
1.3.3 Dopamine receptors

1.3.3.1 Receptor subtypes

Early biochemical, pharmacological and physiological studies yielded results indicative of multiple DA receptor subtypes in nervous tissue [Kebabian and Calne (1979)].

The presence of at least two DA receptor subtypes involved in the regulation of mammalian behaviour was suggested by Cools and van Rossum (1976). These subtypes were designated DA excitation-mediating (DAe) and DA inhibition-mediating (DAi), on the basis of their similarity to DA receptors mediating excitation and inhibition of ganglion cells in snail Helix aspersa neurons. In 1980, Cools and van Rossum substantiated their original concept of DAe and DAi receptor subtypes, "including the predicted correlation with the anatomical, histochemical, biochemical and functional features of the distinct neuronal structures in which they occur".

On the basis of neurochemical considerations, Kebabian (1978) also suggested the existence of two DA receptor subtypes, which he designated α-dopaminergic and β-dopaminergic receptors, and later termed D₁ and D₂ receptors, respectively. D₁ receptors were defined as those receptors coupled to the DA mediated stimulation of adenylate cyclase, and D₂ receptors as those not having an adenylate cyclase linkage [Kebabian and Calne (1979)].

Biochemical and pharmacological studies of the renovascular and cardiovascular systems have also suggested the existence of two distinct DA receptor subtypes, DA-1 receptors are positively linked to adenylate cyclase and DA-2 receptors are not linked to the enzyme [Goldberg and Kohli (1983)].

Subsequent studies, in particular - radioligand binding studies, led to the suggestion of up to four DA receptor subtypes, designated D₁-D₄ [Seeman (1980)].
However, this confused situation with regard to DA receptor subtypes and their terminology appears to have been resolved by the hypothesis of two distinct categories of DA receptors, designated as $D_1$ and $D_2$ receptors, and categorised by biochemical and pharmacological criteria [Kebabian et al (1986) and Waddington (1986)].

The "quadruplet heterogeneity" has, for the most part, been resolved by the demonstration that both $D_1$ and $D_2$ receptors exist in two interconvertible affinity states - high and low [Leff and Creese (1983a), Hamblin et al (1984), Seeman et al (1985)]. The $D_3$ receptor is recognised as being $D_1^{\text{high}}$, and the $D_4$ receptor as $D_2^{\text{high}}$. A summary of this concept is presented schematically in Fig. 1.5.

**FIGURE 1.5** Schematic summary of the nomenclature and dissociation constants of brain $D_1$ and $D_2$ receptors.

The $K_D$ values are for DA and spiperone. $D_1^{\text{high}}$ was formerly $D_3$ and $D_2^{\text{high}}$ was formerly $D_4$. [Seeman et al (1985)].
It has also been recognised, and accepted, that the peripheral DA1 and DA2 receptors are very similar, if not identical, to the D₁ and D₂ receptors respectively [Kebabian et al (1986)].

With regards the Cools and Van Rossum concept of D₁e and D₁i receptors, Offermeier and Van Rooyen (1982) presented findings to suggest that the D₁i receptor may be linked to adenylate cyclase and therefore "have certain characteristics in common with the D₃ receptor". They also proposed that since the post-synaptic D₁e-receptors in the rat nucleus accumbens, which mediate increases in locomotor activity, are not linked to adenylate cyclase, they "may therefore be classified as D₂-receptors."

With the advent of the identification of D₁ and D₂ selective agonists and antagonists, it was recognised that some, but not all, D₂ receptors can mediate the inhibition of adenylate cyclase activity; that is, certain D₂ receptors are negatively-linked to the adenylate cyclase enzyme [Onali et al (1985) and Cooper et al (1985)].

There is now a general acceptance of the "two receptor hypothesis", with D₁ receptors being classified as those DA receptors which mediate DA stimulated adenylate cyclase activity, and D₂ receptors being either unassociated with, or negatively linked to, the adenylate cyclase enzyme [Kebabian et al (1986) and Waddington (1986)].

1.3.3.2 **Localization of brain DA receptors**

1.3.3.2.1 **D₁ receptors**

D₁ receptor sites in the brain have been identified by biochemical assays, by radiolabeled ligand binding assays and by quantitative autoradiography [Kebabian et al (1986)].
The biochemical assay tests for the presence or absence of DA-sensitive adenylate cyclase activity within the central nervous system, and therefore is useful for identifying the presence of D₁ receptors, but does not give any idea of receptor densities. Using this approach, the D₁ receptor has been located in the caudate-putamen, nucleus accumbens, olfactory tubercle, substantia nigra pars reticulata and cortex of the rat brain [Review - Kebabian et al (1986)].

Binding assays, using a radioactive ligand selective for D₁ receptors, have led to the suggestion that the caudate putamen, olfactory tubercle and nucleus accumbens are enriched with D₁ receptor sites, while the brain stem and cerebellum are almost devoid of them [Schulz et al (1985)].

Specific ligand ([³H]-SCH 23390, [³H]-SKF 835666) binding sites corresponding to the D₁ receptor, have been visualized in thin tissue sections with quantitative autoradiography, and have been demonstrated in high concentrations in the caudate putamen, nucleus accumbens, olfactory tubercle, substantia nigra pars reticulata, medial amygdaloid nucleus and VI layer of the frontoparietal cortex [Dawson et al (1985) and Boyson et al (1985)].

Seeman (1980) reviewed evidence of several lesion studies and suggested that D₁ receptor sites are not found on DA-containing neurons, but are located on neurons postsynaptic to the DA cells.

1.3.3.2.2 D₂ receptors

Using ligand binding studies, quantitative autoradiography and D₂ antagonist based photoaffinity labels, D₂ receptor sites have been identified in various brain regions including the striatum, olfactory tubercle, hypothalamus, nucleus accumbens, ventral tegmental area (the A10 cell origin of the mesolimbic pathways), chemosensitive trigger zone, the carotid body and discrete
localities in the cerebral frontal cortex in accordance with the localization of the DA nerve terminals of axons arising from extrastriatal sites [Review - Seeman (1980)]. Low concentrations have also been identified in the substantia nigra [Kebabian et al (1986)].

D₂ binding sites have been identified both pre- and postsynaptically in the mesostriatal system [Review - Stoof and Kebabian (1984)]. D₂ postsynaptic binding sites are located on cholinergic interneurons in the striatum. D₂ presynaptic binding sites designated "autoreceptors" have been identified on the DA neuron terminal endings within the striatum, as well as on the DA cell processes (soma and dendrites) within the substantia nigra [Reviews - Seeman (1980) and Stoof and Kebabian (1984)]. These sites modulate the "turnover of DA". Activation of these sites with DA or APO results in less DA being released, as well as less DA being synthesized by tyrosine hydroxylase [Seeman (1980)]. D₂ autoreceptors also regulate the electrical activity of the dopaminergic nigro-neostriatal neurons, with stimulation of the autoreceptors resulting in depression of the spontaneous electrical activity of the neurons [Stoof and Kebabian (1984)].

1.3.3.3 DA receptor agonists and antagonists

These include both selective and non selective D₁ and D₂ selective agonists and antagonists, examples of which, are presented in Table 1.1. D₁ receptor-selective drugs include the benzapine analogs, SKF 38393 and SCH 23390, which stimulate or block the D₁ receptor respectively. LY 171555 - an ergoline derivative, LY 141865 and RU 24213 are examples of preferential agonists, and sulpiride - a benzamide, is a preferential antagonist at the D₂ receptor [Robertson and Robertson (1987) Waddington (1986)].

The most well known non-selective agonist is apomorphine (APO), while most neuroleptic drugs - for example phenothiazines and butyrophenones, antagonise stimulation of both D₁ and D₂ receptors.
TABLE 1.1 Examples of selective and non-selective DA receptor agonists and antagonists

<table>
<thead>
<tr>
<th>AGONIST</th>
<th>ANTAGONIST</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D₁ SELECTIVE</strong></td>
<td></td>
</tr>
<tr>
<td>SKF 38393</td>
<td>SCH 23390</td>
</tr>
<tr>
<td>(2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzapine)</td>
<td>7-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzapine-7-ol</td>
</tr>
<tr>
<td><strong>D₂ SELECTIVE</strong></td>
<td></td>
</tr>
<tr>
<td>RU 24213</td>
<td>SULPIRIDE</td>
</tr>
<tr>
<td>N-n-propyl-N-phenylethyl-p(3-hydroxy phenyl) ethylamine</td>
<td>N-(1-ethyl-2-pyrrolidinylmethyl) -2-methoxy-5-sulphamoyl benzamide</td>
</tr>
<tr>
<td><strong>NON-SELECTIVE</strong></td>
<td></td>
</tr>
<tr>
<td>APOMORPHINE</td>
<td>HALOPERIDOL</td>
</tr>
<tr>
<td></td>
<td>4-[4-(4-Chlorophenyl)-4-hydroxypiperidino]-4'-fluorobutyrophenone</td>
</tr>
</tbody>
</table>
1.3.3.4 D1:D2 Receptor Interactions

Although DA receptors have been separated into two distinct classes the specific roles that the receptors play in the expression of DA stimulated effects, especially behaviour, has not been clearly defined.

Previous studies have tended to suggest that D2 receptors were the principal mediators of the effect of DA on the central nervous system, especially motor behaviours, while the D1 receptor was dismissed as the "receptor in search of a function" [Kebabian et al (1986), Waddington (1986) and Koller and Herbster (1988)].

The use of selective D1 and D2 agonists and antagonists in biochemical, physiological and behavioural studies, has led to recognition of the fact that both receptor subtypes play a role in the mediation of dopaminergic effects and have further led to suggestions of the possibility of a functional interaction between D1 and D2 receptor subtypes [Braun and Chase (1986) and Barone et al (1986)].

D1 and D2 receptor stimulation appears to produce opposite effects on a number of biochemical parameters, especially in the striatum [Waddington (1986) and Robertson and Robertson (1987)]. For example in superfused neostriatum slices, the D1 agonist - SKF 38393 induces cAMP efflux, in accordance with its ability to stimulate the adenylate cyclase activity, whereas the D2 agonist - LY 141865, inhibits SKF 38393 release of cAMP [Stoof and Kebabian (1981)]. The D1 receptor antagonist reduces the in vivo increase in striatal DA metabolites elicited by D2 receptor blockade and also attenuates the in vitro release of tritiated acetylcholine from striatal slices induced by D2 antagonists [Saller and Salama (1986)]. D1 and D2 activation also seem to have opposing biochemical actions on the release of striatal tritiated-GABA and the release of the polypeptide cholecystokinin from dopaminergic neuronal terminals.
Paradoxically, behavioural studies (discussed more fully in Sec. 1.3.4) indicate that the systemic administration of $D_1$ and $D_2$ agonists can produce similar, additive or synergistic effects on a number of DA-related behaviours [Robertson and Robertson (1987)]. For example, the combined administration of SKF 38393 and LY141865 can restore locomotor activity in reserpinized mice, while neither drug alone has this ability [Gershanik et al (1983)]. In studies of rotational behaviour in rats with 6-hydroxydopamine induced lesions of the nigro-striatal pathway, both $D_1$ and $D_2$ agonists caused contralateral rotation [Gower and Marriott (1982) and Gershanik (1983)]. In rats with kainic or quinolinic acid induced lesions of the striatum, the $D_2$ agonist - LY 171555, but not SKF 38393, induced rotational behaviour, but given together, SKF 38393 increased the turning induced by LY 171555, in a dose dependent fashion [Barone et al (1986)]. SKF 38393 had a similar effect on APO induced stereotypy in normal rats. SKF 38393 did not induce stereotypy on its own, but enhanced APO induced stereotypy in a dose dependent manner. Administered independently, SKF 38393 and LY 171555 elicited dose dependent increases in complex motor behaviours such as grooming and locomotion, while typically stereotyped behaviours were observed only when these agents were combined [Braun and Chase (1986)].

In support of the behavioural studies, electrophysiological studies indicate that the concurrent stimulation of $D_1$ and $D_2$ receptors has synergistic effects on neuronal activity in the globus pallidus and the substantia nigra pars reticulata [Carlson et al (1987) and Weick and Walters (1987)].

The following two observations give support to the suggestion that it is only at higher levels of integrative dopaminergic activity that $D_1$ receptor activation or inhibition influences $D_2$ mediated processes [Waddington (1986)]:

(1) In vitro binding studies failed to show that SCH 23390
occupation of D₁ receptors attenuates the binding of a D₂ agonist to D₂ receptor [Review – Waddington (1986), and (2) recent in vivo electrophysiological studies by Sasa and co-workers [cited in Waddington (1986)] examining the effects of SCH 23390 on D₂-mediated responses confirmed that SCH 23390 failed to attenuate the physiological response of a D₂ receptor to a D₂ agonist.

Waddington also suggests that in some instances D₁ and D₂ receptor systems may exert an influence over what is ultimately a common efferent system for the expression of behaviours and speculates that D₂ systems select a mode of expression of behaviour and D₁ systems influence the intensity of expression of the mode selected.

Herrera-Marschitz and Ungerstedt (1984a and 1984b) suggest that striatal output systems are segregated with respect to D₁ and D₂ dependent behaviours, while Fletcher and Starr (1987) suggest that the functional dichotomy of striatal D₁ and D₂ dependent behaviours occurs at the level of the substantia nigra, rather than the striatum.

The possibility also exists that the D₁ and D₂ receptors involved in the regulation of movement may be found in different brain regions. This possibility is supported by the fact that DA neurons in the substantia nigra pars compacta zone (SNC) are bi-directional, terminating in the striatum and in the substantia nigra pars reticulata (SNR), with DA released in the SNR apparently acting on D₁ receptors, while DA released in the striatum appears to act on D₂ receptors (Fig. 1.6) [Robertson and Robertson (1987)].

Robertson and Robertson have proposed a model which integrates the data obtained from behavioural, anatomical, biochemical and electrophysiological studies, in an attempt to explain the ability of concurrent D₁/D₂ receptor stimulation to increase the intensity of expression of a number of behaviours, especially rotational behaviour, in rats with unilateral lesions of the nigro-striatal DA
The striatum contains a high density of both D₁ and D₂ receptors. In the SNC, the density of D₁ receptors is high, while the D₂ receptor density is relatively lower. The SNR is supplied predominantly with D₁ receptors located presynaptically on GABA-ergic neuron terminals.

The model holds that since DA can be released from dendrites from the SNC into the SNR, as well as into the striatum, the D₁/D₂ synergism results from the concurrent activation of D₂ receptors in the striatum and D₁ receptors in the SNR, and the dendritic release of DA plays a crucial modulatory role over the activity of the striato-nigral pathway. Within the SNR, DA acting via D₁ receptors enhances the release of GABA from its synaptic terminals — increasing the inhibitory influence of this pathway over the SNR. In the striatum it appears that D₂, rather than D₁, receptor stimulation is of greater functional consequence and D₂ stimulation results in activation of the striato-nigral pathway.

D₁ and D₂ agonists would then be expected to have a synergistic action in the expression of behaviours, since D₂ receptors in the striatum and D₁ receptors in the SNR are concurrently activated, resulting in the facilitation of GABA release from the striato-nigral pathway. Robertson and Robertson suggest that the output of the striatum is initiated by D₂ agonist activity and that D₁ agonists can be seen as "gating" the output at the level of the SNR. Although this model still requires further electrophysiological and biochemical validation, it provides an explanation for the discrepancy between results obtained in biochemical and behavioural studies of D₁ and D₂ receptor interactions, and provides a feasible explanation for the involvement of both receptor subtypes in the expression of DA-related behaviours.

Jackson et al (1987) suggest, however, that the synergism between D₁
and D₂ receptors discussed by Robertson and Robertson may also derive from the mesolimbic DA areas, and in particular the nucleus accumbens, rather than solely from the striatum and SNR.

It appears, therefore, that the D₁:D₂ receptor interaction in the expression of DA related behaviours is an interaction of selective DA receptors, located on different neurons, in various areas of the brain including, the mesolimbic and mesostriatal DA pathways.

In general, the physiological functions attributed to D₁ receptors are: maintenance of motor functions in the central nervous system and stimulation of parathyroid hormone release and relaxation in vascular smooth muscle; and those attributed to D₂ receptors are: maintenance of behavioural and motor functions in the central nervous system, inhibition of the release of α-MSH and prolactin from the anterior pituitary gland, emesis via the area postrema and inhibition of the release of NA from the sympathetic nervous system [Govitrapong et al (1989)].
D_1 receptors appear to be located postsynaptically on interneurons of the striatum, on DA cell bodies in the SNC and presynaptically on GABAergic fibers in the SNR. D_2 receptors are also located postsynaptically on the intrinsic neurons of the striatum, and on DA cell bodies in the SNR. D_2 autoreceptors are located presynaptically on dopaminergic neurons of the striatum, and are also thought to be located presynaptically on glutamergic fibers of the corticostriatal pathway.
1.3.4 Behavioural and motor effects of central nervous system DA receptor stimulation.

Until the last decade, the study of central nervous system behavioural and motor responses has been limited by the number of drugs available for such studies. Amphetamine - the catecholamine releasing drug, and apomorphine (APO) - a nonselective DA agonist, were the only agonists available, while typical neuroleptics such as phenothiazines and butyrophenones were the DA antagonists used. Although these relatively non-selective DA agonists provide a basis for the study of DA induced behaviours, the introduction of newer and more selective DA agonists and antagonists has aided in furthering the understanding of the functional basis of DA induced behaviours [Sec.1.3.3.3].

In rats, the systemic administration of APO and amphetamine induces well recognised behavioural responses, which include sniffing, grooming, licking, rearing and gnawing. These effects were believed to result from the stimulation of postsynaptic DA receptors (directly in the case of APO, and indirectly with amphetamine), in the corpus striatum [Van Ree and Wolterink (1981) and Maj et al (1972)]. The newer DA antagonists and agonists, together with the introduction of sophisticated stereotaxic techniques which have allowed the deposition of agonists and antagonists into specific brain regions, has allowed for recognition of the involvement of other cerebral DA systems and receptors in the control of DA related behaviours [Costall and Naylor (1979)].

1.3.4.1 Stereotypy

The mixed acting DA agonist, APO, is an agent that induces stereotyped behaviour, which may be defined as "behaviour which is increasingly perseverant and confined to fewer and fewer classes of
behavioural response, as the dose effect increases" [Lyon and Randrup (1972)]. In the rat, the classes of behavioural response generally include sniffing and head-movements at lower doses of the drug, with licking and gnawing becoming predominant at higher dose levels [Sahakian and Robbins (1975)]. The mechanism by which increased DA stimulation gives rise to stereotyped behaviour may relate to the role of DA neurotransmission in sensory-motor integration [Cools (1973) cited in Ungerstedt (1979), and Lyons and Robbins (1975)].

APO-induced stereotypy has generally been thought of as the result of stimulation of DA receptors in the corpus striatum [Maj et al (1972)] and as a D₂ receptor effect [Creese et al (1983)]. However, the ability of selective D₁ antagonists to modify D₂ agonist-induced stereotyped behaviour prompted the suggestion that D₁ agonists would also be capable of enhancing such responses [Waddington (1986)]. Although compulsive, fixated stereotyped behaviour, is a typical response to APO, Pugh et al (1985), could not induce such a response, even with high doses of the selective D₂ agonist RU 24213. However, prior administration of the D₁ agonist, SKF 38393, resulted in threshold doses of RU 24213 inducing a greater and more compulsive stereotypy response. The D₁ agonist alone did not induce stereotyped behaviour, although grooming was evident, suggesting that its combination with RU 24213 was synergistic rather than additive [Waddington (1986)].

Using LY 171555 and SKF 38393, Braun and Chase (1986) presented results which also failed to support the view that the classical DA agonist induced stereotype behaviours are predominantly D₂ mediated, and suggested that a functional interaction between D₁ and D₂ receptors is involved in the generation of these behaviours.

White et al (1988) presented results which support the hypothesis that D₁ and D₂ receptors function in a synergistic manner to regulate the intensity of stereotyped behaviour induced by DA agonists, and further suggest that the interaction takes the form
of "an absolute requirement" of D₁ receptor stimulation for postsynaptic D₂ receptor-mediated behaviours. This relationship, however, does not appear to be reciprocal, since D₂ receptor stimulation does not appear to be necessary for the grooming response elicited by SKF 38393.

In the rat, the intrastriatal injection of DA induces a stereotype behavioural response, while DA injected into the nucleus accumbens, induces a hyperactivity, with stereotypy contributing only a minor response [Review - Costa and Naylor (1979)]. Ungerstedt (1979) suggests that the gnawing/biting/licking stereotypy is a function of DA activity in the striatum, while the olfactory tubercle may also be involved in the continuous movements of the head and sniffing.

The conclusion can thus be drawn that DA agonist induced stereotypy in rats is the expression of a functional D₁:D₂ receptor stimulation interaction in the mesostriatal system, although the olfactory tubercle may be involved.

1.3.4.2 Grooming

The D₁ selective agonist - SKF 38393, administered in doses ranging from 4 to 64 mg/kg, produced a dose dependent increase in overall and in intensive grooming behaviour [Braun and Chase (1986)]. This response has also been described by other authors [Molloy and Waddington (1984), Koller and Herbster (1988) and White et al (1988)]. This behaviour is characterized by rigorous or robust grooming of the trunk or flank, with the snout.

This response is antagonised by D₁ specific antagonists and D₂ receptor stimulation is not necessary for the induction of the behaviour [Molloy and Waddington (1984) and White et al (1988)].
1.3.4.3 Locomotor activity

Although conflicting results have been published [Ljungberg and Ungerstedt (1977a)], increased locomotor activity in the rat is generally considered to be one of the manifestations of DA agonist induced behaviours.

Koller and Herbster (1988), demonstrated an inhibition of locomotor activity by low doses of APD, related to the stimulation of DA autoreceptors, while high doses of APD stimulated postsynaptic receptors, resulting in an increase in locomotion. SKF 38393 enhanced the stimulatory, but not the inhibitory, effect of APD on locomotor activity. However, SKF 38393 alone did not alter locomotor activity.

When administered alone, the D₂ agonist - LY 171555, induced locomotor activity in a dose dependent fashion throughout a range of doses (0.375 - 24 mg/kg) [Braun and Chase (1986)]. This response was, however, inhibited in animals in which endogenous DA had been depleted by pretreatment with α-methyl-para-tyrosine (AMPT), and could be restored by concurrent administration of the D₁ receptor agonist - SKF 38393. This suggests that the induction of locomotion by stimulation of D₂ receptors requires concurrent activation of D₁ receptors.

Both the mesostriatal and mesolimbic DA systems appear to be involved in the expression of locomotor activity [Costall and Naylor (1979)].

1.3.4.4 Rotation

Rats have a normal asymmetry in nigrostriatal function. DA agonists potentiate the dopaminergic asymmetry and induce rotational behaviour - "rotation appears to be the ultimate consequence of a consistent and persistent side preference" [Glick and Cox (1978)].
The expression of turning behaviour in rats with quinolinic acid induced striatal lesions appears to depend on the concurrent activation of D₁ and D₂ receptors [Braun et al (1986)]. The receptors in this model are normosensitive, in comparison to supersensitive receptors in rats lesioned with 6-hydroxydopamine (Discussed in Sec 5.1 and 5.3.2). In this normosensitive receptor model, both D₁ and D₂ receptors appear to contribute to rotational behaviour. Barone et al (1986) suggests that D₁ receptor stimulation provides a "tonic" background activation, which allows the "phasic" component of D₂ stimulation to become effective, thus suggesting a functional D₁:D₂ receptor interaction in the expression of rotational behaviour in rats with normosensitive receptors.

1.3.4.5 Perioral movements

DA agonists induce characteristic perioral movements in rats. These behaviours are characterized by vacuous chewing movements, licking and biting.

Licking or biting can be induced in both control and reserpine-pretreated rats by pergolide (a selective D₂ agonist) and APO, while SKF 38393 induces marked licking and biting only after reserpine pretreatment [Arnt (1985)]. Thus selective D₁ or D₂ receptor stimulation induces oral activity in reserpinized rats, which is blocked by selective antagonists respectively, or by mixed acting D₁/D₂ antagonists. These results are in contrast to results obtained by Rosengarten et al (1983), where perioral movements in naive rats were shown to be induced by a D₁ agonist and by a D₂ antagonist, suggesting opposite D₁/D₂ receptor function. The results presented by Rosengarten et al also appear to be in contrast to other D₁ and D₂ behavioural effects, since, in general, these two receptor subtypes act in a synergistic manner to control DA-related behaviours.
1.3.5 DA and its role in disease states

Since both the D₁ and D₂ receptor systems have been implicated in the maintenance of motor functions in the central nervous system, it would follow that central nervous system dopaminergic pathways are involved in the pathophysiology of movement disorders. The involvement of DA systems in the pathophysiology of neuropsychiatric diseases and abnormal motor behaviours has long been recognised and drugs whose primary activity is the stimulation or inhibition of DA receptors have been extensively used in the treatment of such disorders as Parkinson's disease, schizophrenia and Huntington's disease.

1.3.5.1 Parkinson's disease

Parkinsonism is a pathophysiological state or clinical syndrome reflecting a striatal DA deficiency, which may result from "degeneration of the dopaminergic nigrostriatal neuronal system, pharmacological depletion of striatal DA, blockade of striatal DA receptors or loss of the striatal neurons bearing DA receptors" [Duvoisin (1986)]. Although generally idiopathic in nature, Parkinsonism is often manifest after carbon monoxide or manganese poisoning, tumours of the basal ganglia, cerebral traumas, certain viral infections, some forms of encephalitis and as a side effect of chronic therapy with neuroleptics [Schmidt (1981) and Duvoisin (1986)].

In idiopathic Parkinson's disease, massive cell death occurs in the substantia nigra. This is accompanied by a degeneration of the dopaminergic nigrostriatal pathway and a depletion of striatal DA. It is not clear whether the degenerative process is accompanied by an increase in post-synaptic DA receptor function, since biochemical and radioligand binding studies have yielded inconsistent results [Review - Riederer et al (1984)]. Riederer and co-workers propose a theory which suggests that an initial increase in DA receptors may accompany early neuronal degeneration and may
compensate for the loss of DA function, but that in the latter stages of the disease there is a loss of DA receptors.

The onset of the disease usually occurs in middle or late life and begins with tremors in the fingers and hands, followed by limb stiffness and general slowing of motor activities, eventually manifesting in a triad of tremor, bradykinesia and rigidity. In the latter stages, the patient is immobilized by profound akinesia and rigidity [Schmidt (1981) and Duvoisin (1986)]. The symptoms of Parkinson's disease occur typically after a loss of about 70% of striatal DA [Riederer et al (1984)]. Riederer and co-workers suggest that this reflects the nature of the regulatory mechanisms, which serve to keep the functional activity of DA within normal limits and include presynaptic DA autoreceptors, the nigro-striatal GABAergic pathway, as well as increases in post-synaptic receptors.

Parkinson symptoms were first treated with neurosurgical procedures and anticholinergic agents, which block the overactivity of cholinergic interneurons, normally inhibited by DA [Schmidt (1981)]. Since characterization of the striatal DA deficit associated with the disease, DA agonists have become the mainstay of therapy, although anticholinergic drugs are still employed.

Treatment with DA itself is not feasible, due to its rapid metabolism and inability to cross the blood brain barrier. Administration of a noncatechol precursor of DA - L-dopa circumvents the difficulty with central nervous system permeability and results in a marked improvement of Parkinsonian symptoms. Combination of L-dopa with a peripheral decarboxylase inhibitor such as carbidopa or benzerazide, which inhibits peripheral metabolism of L-dopa, permits more L-dopa to reach the brain and reduces peripheral catecholamine stimulation. It also allows for a reduction in the therapeutically effective dose of L-dopa, resulting in a significant decrease in the incidence and intensity of untoward effects, although such untoward effects are not eliminated [Schmidt (1981). Combined L-dopa and decarboxylase
inhibitor treatment is the primary therapy for Parkinson's disease [Muenter (1986) and Riederer et al (1984)]. This drug combination provides 50-100% improvement in most patients, in contrast to the lesser degree of improvement usually seen in response to anticholinergic and other agents [Muenter (1986)].

Inhibition of DA catabolism is another means of extending L-dopa's antiparkinsonian effect. In humans oxidative deamination of DA is selectively carried out by MAO-B and deprenyl. A MAO-B inhibitor has been used in some studies, as an adjunct to L-Dopa [Lewitt (1984)]. Used alone, deprenyl has very little anti-parkinsonian effect but it may be useful for producing a "smoother", more sustained, response from each dose of L-dopa [Sandler and Stern (1982)].

The only other DA agonist in use in the treatment of Parkinson's disease is bromocriptine. Although the direct stimulation of postsynaptic receptors and mainly D2 receptors appears to be the major pharmacological effect of this drug, the action of bromocriptine is possibly more complex than a purely agonist effect at post-synaptic DA receptors [Schmidt (1981) and Riederer et al (1984)]. Bromocriptine therapy has generally been less effective than carbidopa/L-dopa therapy, although combined with carbidopa/L-dopa therapy, it appears to have synergistic therapeutic value [Schmidt (1981)]. At low doses, bromocriptine appears to block DA synthesis via effects on presynaptic sites and also acts on receptors to other transmitters [Riederer et al (1984)], and it may be some of these actions which account for its synergistic action in L-dopa/carbidopa therapy. Bromocriptine can, however, act independently of a presynaptic neuron, while L-dopa requires relatively intact presynaptic function for its decarboxylation to DA and subsequent storage and release [Riederer et al (1984)] and thus bromocriptine is relatively more effective in the later stages of the disease, when degeneration of the striatal dopaminergic terminals is well advanced.
In animal experimentation, the D₁ receptor is now recognised as having a functional role in the control of motor functions (Sec. 1.3.3.4 and 1.3.4) and it has even been suggested that a combination of D₁ and D₂ agonists may represent optimal drug treatment for Parkinson's disease [Koller and Herbster (1988)]. The administration of D₁ agonists, such as SKF 38393 as an adjunct to bromocriptine and L-dopa therapy in Parkinson's disease, as well as the effectiveness of other mixed acting (D₁/D₂) DA receptor agonists, is under investigation.

Other drugs employed in the treatment of the disease include the anticholinergic agents such as biperidin, trihexyphenidyl and diphenhydramine, and an antiviral agent - amantidine. Amantadine activity in the treatment of Parkinson's has been ascribed to its ability to stimulate DA and NA receptors, increase the electrically stimulated release of DA, inhibit serotonin reuptake and binding and its possible ability to stimulate serotonin release [Schmidt (1981)].

Current treatment of Parkinson's disease is aimed at treating the symptoms of the disease (the lack of DA). The pathology of the disease (the loss of neurons and nigral cells) progresses even "in the face of successful treatment of the physical symptoms" [Schmidt (1981)]. However, until the pathogenesis of this disorder is understood, the treatment of the disease will remain largely symptomatic [Schmidt (1981) and Lewitt (1986)].

Although the mesostriatal dopaminergic system appears to be the primary system implicated in the pathophysiology of the disease, other dopaminergic systems, as well as other neurotransmitter and neuropeptide systems may be involved. Marked decreases in DA levels and altered DA metabolism has also been identified in the mesolimbic system and particularly the nucleus accumbens in postmortems of Parkinsonian patients [Price et al (1978)]. The loss of neuro-transmitter is not confined to the DA system. A hypofunction of serotonergic systems is implied by the observation
of abnormally low serotonin concentrations in brain tissue taken postmortem from Parkinsonian patients and low NA concentrations have also been reported [Schmidt (1981) and Riederer et al (1984)]. The presence of GABA, taurine, cholecystokinin, substance-P and enkephalins in the basal ganglia has also led to the suggestion that these neuroactive substances may be of importance in the pathophysiology of the disease [Schmidt (1981) and Lewitt (1986)]. Neuromelanin, a pigmented substance found in many neurons in the substantia nigra and responsible for its darker colour, also appears to be of importance to the pathophysiology of the disease. Neuromelanin containing neurons appear to be particularly vulnerable to the neurogenerative process of the disease and, significantly, Parkinson's disease is characterized by depигmentiation of the substantia nigra [Hirsch et al (1988) and Langston (1988)].

1.3.5.2 Huntington's Disease

The symptoms of Huntington's disease or chorea, an "autosomal-dominant" hereditary movement disorder, result from degeneration of neurons primarily in the basal ganglia [Coyle and Schwartz (1976) and Fann and Wheless (1981)]. The primary neurological features in Huntington's disease include the presence of normal dopaminergic systems associated with reduced availability of GABA-containing inhibitory neurons and degeneration of cholinergic fibers in the basal ganglia and in particular the striatum [Fann and Wheless (1981)].

Although chorea, or mental deterioration, may be the presenting feature, subtle personality and behavioural changes are nearly always the earliest manifestation. Involuntary movements, normally most conspicuous in the face and upper limbs, develop insidiously. As the disease progresses, dementia becomes more profound and chorea more "grotesque". In a fully established syndrome, patients are "radically regressed" and the body "writhes, twists and turns"
in incessant, uncontrollable movement" [Fann and Wheless (1981)].

The most effective agents in the treatment of chorea are those which induce Parkinsonism, that is drugs which reduce DA function in the striatum and include agents which decrease striatal DA content (reserpine), block DA receptors (phenothiazines and butyrophenones) and drugs which enhance cholinergic activity (physostigmine and deanol). Therapy with GABA and certain of its analogues such as muscimol has also proved successful [Barbeau (1978)]. Current treatment approaches are, however, palliative rather than curative.

Although the disorder is characterized by a basal ganglia deficit in GABA and acetyl choline, dopaminergic antagonists are thought to be of value in the treatment of the disorder, since DA usually exerts an inhibitory effect on striatal cholinergic interneurons [Karczmar and Dun (1978)], and GABAergic striato-nigral neurons [Baldessarini and Tarsy (1978)].

1.3.5.3 Schizophrenia

The observation that high doses of amphetamine can induce a paranoid psychosis similar to paranoid schizophrenia in nonschizophrenic patients, coupled to the acute blockade of DA receptors by antipsychotic agents, has led to the formulation of the "DA hypothesis" of schizophrenia [Van Kammen and Bunney (1981)]. The hypothesis holds that "some (or perhaps all) of the symptoms of schizophrenia arise from excessive activity of dopaminergic mechanisms, and the effects of this excess (and thus the symptoms of schizophrenia) can be reduced by the dopamine receptor blockade induced by neuroleptic drugs" [Crow et al (1978)]. Crow et al suggest that there are two separate and logically distinct hypotheses - the "dopamine overactivity" hypothesis of schizophrenia, and the "dopamine receptor blockade" hypothesis of the antipsychotic effect.
Substantial objections to the "dopamine overactivity" have been raised and are summarised as follows by Crow et al (1978):

1. CSF studies of inhibition of the elimination of the DA metabolite homovanillic acid (HVA) by probenecid, have yielded no evidence of increased DA turnover in schizophrenic subjects, moreover it has been suggested that DA turnover decreases with increasing severity of the disease.

2. Prolactin secretion is not decreased in unmedicated acute and chronic schizophrenic patients, which would be expected if there is increased tuberoinfundibular release.

3. Schizophrenic illness can occur in patients with long standing Parkinson's disease, and DA in schizophrenic subjects is as depleted in mesolimbic regions as in the corpus striatum in Parkinson's disease.

In studies involving HVA and cAMP levels in the CSF, Kato et al (1982) failed to demonstrate dopaminergic system hypersensitivity, but suggested that, in schizophrenia, a regulation in balance of DA activity in the mesostriatal and mesocortical systems is shifted, to assist activation on the postsynaptic function of the mesocortical system.

By contrast, a number of receptor assay studies have suggested that DA receptors are increased in about two-thirds of schizophrenic subjects studied [References – Crow (1980)], and at present the only change found consistently in the postmortem studies of the schizophrenic brain is the increase in DA receptor number.

Although not effective in all schizophrenics, neuroleptic agents such as phenothiazines, thioxanthines, and butyrophenones remain the most effective tools in the treatment of schizophrenia. DA receptor blockade (D₁ and D₂) is a significant, and possibly the "sole" component in the therapeutic effect of neuroleptics. The therapeutic effect of these drugs, however, does not appear to be a direct effect of DA receptor blockade, since "benefit follows blockade of the DA receptors only after an interval of at least two
weeks” [Crow et al (1978) and Crow (1980)]. Crow (1980) suggests that the blockade of receptors may be necessary in order to permit “some other change to occur”. In agreement with this concept, Van Kammen and Bunney (1982) suggested that neuroleptics induce clinical “stability” or "normalization" through an initial blockade of DA receptors and that this stability may persist with some patients, even after neuroleptic withdrawal.

1.3.5.4 **Tardive dyskinesia**

Tardive dyskinesia is a late-onset extrapyrimidal side effect of neuroleptic therapy. It is a motor disturbance characterized by abnormal, involuntary, persistent movements of the tongue, lips, facial and sometimes trunk muscles [Klawans (1973)].

The current hypothesis finding general acceptance for the pathogenesis of tardive dyskinesia, is the development of supersensitivity of inhibitory DA receptors on cholinergic interneurons of the corpus striatum [Ehrensing (1978)]. It has been proposed that DA receptor supersensitivity in the striatum leads to decreased acetyl choline at the cholinergic receptors of the GABA neurons, which in turn leads to decreased activity of the GABA neurons and decreased release of GABA in the substantia nigra and at excitatory neurons which project to the thalamus. The disinhibition of the neurons projecting to the thalamus lead to the hyperkinetic syndrome of tardive dyskinesia [Richelson (1981)].

Although DA is strongly implicated in tardive dyskinesia, it is unlikely that it is the only neurotransmitter responsible for the disorder, and it has been suggested that cholinergic hypofunction may also be involved [Richelson (1981) and Fann and Wheless (1981)]. Abnormalities in GABAergic [Tarsy and Baldessarini (1977)], serotoninergic [Kozell et al (1987)] and opioidergic [Sandyk (1986)] neuronal systems have also been implicated in the pathophysiology of the disorder.
Paradoxically, neuroleptics, the drugs which cause the manifestations of tardive dyskinesia, are also capable of ameliorating the symptoms of the disorder. However, since the disorder is frequently irreversible, an increase in neuroleptic dose does not appear to be an advisable method of treatment, although it is usually effective [Baldessarini and Tarsy (1978)]. Drug treatment of the disorder has not proved very successful, although DA-depleting agents such as reserpine and tetrabenazine have been used [Fann and Wheless (1981)].

1.3.6 Summary

Since DA was found biochemically in the brain [Carlson et al (1958) and Montagu (1957)] and its intraneuronal localization first demonstrated histochemically [Fuxe (1965)], the recognised function of DA in the brain has shifted from that of a precursor for NA, to that of a neurotransmitter in its own right [Creese et al (1983)].

Dopaminergic pathways in the brain have a recognised role in the maintenance and regulation of motor function. Although the nigrostriatal pathway is the major dopaminergic neuronal system in the brain associated with the control of motor function, the role and importance of the mesolimbic and mesocortical systems is now being recognised [Costall and Naylor (1979)].

The association of disturbances of dopaminergic neurotransmission with neurological and psychiatric disorders has further emphasized the important role of this neurotransmitter in normal brain function. Dopaminergic agonists have a firmly established role in the treatment of Parkinson's disease and may also be of value in the treatment of tardive dyskinesia, while DA antagonists are the mainstay of therapy in schizophrenia and Huntington's chorea [Creese et al (1983)].
For many years the D₂ receptors were recognised as the principal mediators of the effect of DA on motor behaviours and recognised as being responsible for the beneficial effect of DA in Parkinson's disease, while D₁ receptors were receptors without a functional role. The role of D₁ receptors and the functional D₁/D₂ receptor interaction in the mediation of normal and abnormal motor behaviours is now being realised [Koller and Herbster (1988)].

The dopaminergic system does not operate in isolation and the importance of other neurotransmitter systems and their functional interaction with the dopaminergic system in the control of motor behaviours is becoming established. Noradrenergic, serotonergic, GABAergic and cholinergic systems all have an important role in the modulation of dopaminergic function, both in normal and abnormal motor behaviours [Antelman and Caggiula (1977), Pycock et al (1978) and Karczmar and Dun (1978)]. Recognition and characterization of such functional interactions provides the possibility for treating neurological and psychiatric disorders primarily related to dopaminergic dysfunction, with agents other than the classic dopaminergic drugs.
1.4 Melatonin: A possible modulator of central dopaminergic neurotransmission?

It has been indicated that, of all the potential action sites for pineal hormones, the "primary candidate" is the brain [Aldegunde et al (1985)]. Since its discovery as the principal 5-methoxyindole of the pineal gland, MEL has been implicated in the regulation of numerous brain functions, including various influences on motor behaviour.

Recent animal and human studies have led to the suggestion that MEL may be involved in the regulation of movement and in the pathophysiology of movement disorders, while DA seems to have a major role in the control of both normal and abnormal motor behaviour.

In 1971, Cotzias and co-workers demonstrated that the administration of MEL to intact mice blocked adventitious turning, induced by L-dopa and in mice receiving L-dopa after a lesion in the right caudate nucleus, MEL blocked turning to the side of the lesion and induced running to the left. This effect could be repeated with APO, when MEL was administered 5 minutes prior to APO administration and not concurrently. Although the mechanism of this interaction was not described, Cotzias et al suggested that "competition of neurotransmitters for neuronal receptors does not provide an adequate explanation" [Cotzias et al (1971b)].

More recently, MEL has been shown to regulate forebrain dopaminergic functions in the rat. Bradbury and co-workers (1985) demonstrated inhibition of behavioural locomotor activity following intranigral injection of MEL. This effect was accompanied by reduced DA "function" in the striatal and limbic systems, as determined by increased DA content and a reduced DOPAC/DA ratio, suggesting a reduction in the release of DA in these brain areas.
These effects were partially reversed by the D₂ selective antagonist, sulpiride. Bradbury et al suggested that ascending DA systems projecting from the substantia nigra were possibly regulated by an action on DA autoreceptors, and that MEL may also influence DA autoreceptors. However, since sulpiride was not capable of inhibiting a large component of MEL's action, they suggest that MEL may also act on serotonergic receptors and thereby regulate dopaminergic activity in the nigro-striatal pathway [Bradbury et al (1985)].

MEL has also been demonstrated to inhibit the release of DA from the rat hypothalamus [Zisapel and Laudon (1983)], and from rabbit retina [Dubocovich (1983)], however, no inhibition of DA release has been observed in the cerebral cortex, cerebellum, dorsal hippocampus or in the striatum. High affinity binding sites for ¹²⁵Iodo-MEL have, however, been identified in the striatum, as well as the hippocampus, hypothalamus, cortex and amygdala of both the male and female rat [Laudon and Zisapel (1987)]. The hypothalamic binding sites appear to correlate with MEL's neuroendocrine's activity but, as previously stated, Zisapel et al (1988) conclude that the role of the striatal ¹²⁵Iodo-MEL binding sites is still an enigma.

DA receptors have been demonstrated in the pineal gland [Erlich and Apuzzo (1985)], and Govitrapong and co-workers (1989) have suggested that pineal gland D₂ receptor sites may have an independent functional role in the regulation of MEL synthesis. Rats treated with haloperidol have been found to have higher concentrations of the drug in the pineal gland than in other brain regions [Naylor and Olley (1969)]. Acute administration of haloperidol and sulpiride has been demonstrated to increase pineal MEL levels and the activity of NAT [Smith et al (1979) and Govitrapong et al (1989)].

MEL has thought to be of relevance to Parkinson's disease, the movement disorder characterized primarily by "striatal DA
deficiency", (Reviewed in Secs. 1.2.5.2.2. and 1.3.5.1) and to schizophrenia - a disorder possibly associated with DA hyperactivity (Reviewed in Secs. 1.2.5.3.1 and 1.3.5.3). MEL's antidyskinetic activity and the role of the pineal gland has begun to be recognised in neuroleptic induced tardive dyskinesia, a disorder associated with a supersensitivity of DA receptors (Secs. 1.2.5.2.3 and 1.3.5.4). MEL has also been demonstrated to cause "psychomotor retardation" when administered to patients with Huntington's chorea, the movement disorder characterized by "incessant and uncontrollable movement", and primarily treated with DA antagonists (Secs. 1.2.5.2.4 and 1.3.5.2).

Although DA pathways are thought to be central in the control of motor behaviour and in abnormal movement disorders, the role of other transmitter systems such as noradrenergic, serotonergic, GABA'ergic and cholinergic systems, has also been recognised. MEL's activity in the brain has often been ascribed to its action on some of these other neurotransmitter pathways and agents, particularly GABA and serotonin (Sec. 1.2.3).

The results of all these studies has led to the proposal that MEL has a modulatory influence on central nervous system dopaminergic activity, particularly in the nigrostriatal system [Bradbury et al (1985) and Sandyk (1988)], and that MEL and the pineal gland may be involved in the pathophysiology of DA-related movement disorders. The presence of DA receptors in the pineal gland would also suggest a mutual interaction between MEL and striatal DA neurons.
CHAPTER 2

CYCLIC AMP STUDIES IN THE CORPUS STRIATUM

2.1 INTRODUCTION

Measurement of DA sensitive adenylate cyclase (ADC) activity in the corpus striatum, the most prominent dopaminergically innervated area of the rat brain [Fuxe (1965)], is an accepted model for studies of DA D₁ receptor activity in the central nervous system [Woodruff et al (1977)]. In this study, the DA sensitive ADC model is used to investigate the possibility that MEL may modulate DA function at the level of the D₁ DA receptor.

Striatal homogenates and partially sliced striata of male Wistar rats were incubated with DA, APO and or MEL in various concentrations and combinations. Drug activity was assessed by assaying the resultant cAMP levels, which are a good indicator of D₁ linked ADC activity [Kebabian and Calne (1979)].

Various methods have been used to assess striatal cAMP [Karobath and Leitch (1974), Kebabian et al (1972), Forn et al (1974)]. The rapid, simple procedure developed by Brown et al (1971) and used by investigators such as Kebabian et al (1972) was chosen in this study. The assay method was chosen due to its low cost [Modipane et al (1985)], adequate sensitivity [Kebabian et al (1972)], and specificity [Brown et al (1971)].

2.2 MATERIALS AND METHODS

2.2.1 Animals

Male albino rats of the Wistar strain weighing between 200 - 300g
were used. They were housed in a temperature controlled environment (21-23 °C), on a regulated 12 hour light : 12 hour dark cycle (lights on 06h00 : lights off 18h00). The rats had access to food and water \emph{ad libitum}.

2.2.2 Buffer Media

Various homogenizing, preincubation, incubation and assay buffers were prepared as follows:

\textbf{Medium A} : (Striatum homogenizing buffer) 2mM tris-(hydroxy-methyl) aminomethane-maleate [Tris-maleate] buffer pH 7.4 with 2mM ethylene glycol-bis-(β-aminoethylether)-N,N'-tetraacetic acid (EGTA).

\textbf{Medium B} : (Striatum homogenate preincubation and incubation buffer) 80mM Tris-maleate buffer pH 7.4 with 2mM magnesium sulphate; 10mM theophylline and 0.2mM EGTA.

\textbf{Medium C} : (Striatal slices preincubation buffer) Krebs Ringer Bicarbonate buffer. Composition in mM : NaCl 124; KCl 5; NaHCO\textsubscript{3} 26; CaCl\textsubscript{2} 0.8; MgCl\textsubscript{2} 1.3; KH\textsubscript{2}PO\textsubscript{4} 0.4 and glucose 10. pH7.4.

\textbf{Medium D} : (Striatal slices incubation buffer) Krebs Ringer Bicarbonate Buffer with 10mM theophylline.

\textbf{Medium E} : (cAMP Assay buffer) 50mM Tris-HCl pH7.5 with 8mM theophylline and 6mM mercaptoethanol.

\textbf{Medium F} : (cAMP Binding Protein Preparation buffer) 50mM Tris-HCl with 0.25mM sucrose, 25mM potassium chloride and 5mM magnesium chloride.

2.2.3 Experimental Procedure

The rats were killed by decapitation between 08h30 and 10h30, their
brains were rapidly removed, in a cold room and placed in ice cold Medium E. Corpora striata were dissected out according to the method of Kebabian et al (1972). The brain was hemisected along the midline and the lateral ventricle of one cerebral hemisphere was opened, with a medial incision superior to the corpus callosum. The caudate nucleus was separated from the internal capsule and rapidly removed. This procedure was then repeated on the contralateral hemisphere.

The striatal homogenates were treated by a modification of the methods of Kebabian et al (1972) and Clement-Cormier et al (1975) and the slices by a combination of the methods of Forn et al (1974) and Wilkening et al (1975). The methods, described briefly here, are laid out in Table 2.1.

Striatal homogenates:
Dissected striata were homogenized with 20 strokes in a hand held all-glass homogenizer in 25 vol of ice cold medium A. 50μl aliquots of the homogenate were preincubated in 0.45ml of medium B in both the presence or absence of test drugs (MEL, APO and or DA) and equilibrated with Carbogen (O₂ 95% and CO₂ 5%). Preincubation took place in glass tubes for 20min at 0°C. Following preincubation the reaction was initiated by the addition of ATP (Boehringer Mannheim) to a final concentration of 0.5mM to the preincubation mixture. The tubes were incubated with shaking at 30°C for 3min, transferred to a boiling water bath for 3 min in order to terminate the reaction, and then centrifuged at low speeds, sufficient to sediment the denatured protein. 50μl aliquots of the supernatant solution were assayed for cAMP content by the saturation binding protein assay developed by Brown et al (1971) and described fully in Sec. 2.2.4. Protein content was determined by a modification of the Folin Lowry method [Lowry et al (1951), Miller (1959)], described fully in Sec. 2.2.4. All data was expressed as pmol cAMP formed/mg protein/3min incubation period.
Striatal slices:
10-12 partial slices were made into intact dissected striata with a blade. The striata were individually preincubated in medium C (1ml per 50mg wet tissue) for 75min, with shaking, at 37°C, with 2 changes in medium. 0,2ml of the preincubation medium, with approximately 15mg of tissue, were added to tubes containing 10mM theophylline and test drugs (MEL and or DA and or APO), to a final volume of 0,3ml. The reaction was initiated by the addition of ATP to a final concentration of 0,5mM. Incubation took place for 5 minutes at 37°C, with shaking. The tissue was then rapidly homogenized in the all-glass hand held homogeniser and kept in water bath at 90°C for 10 minutes in order to terminate the reaction.

cAMP and protein content were assayed as for homogenates and cAMP was expressed as pmol cAMP/mg protein /5min incubation period.

2.2.3.1 Discussion on experimental procedure:
Maximal basal and DA (100μM) stimulated ADC activity was observed at 30°C in homogenate samples (Fig.2.1) and at 37°C in intact striatal samples (Fig.2.2). Clement-Cormier et al (1975) demonstrated that stimulation of ADC was observed over a range of pH values (6.5 - 10), with maximum percentage stimulation between pH 7 and 8. A pH of 7.4 was thus used in all experiments described here.

Preincubation of freshly prepared striatal homogenates for 30 min at 0°C (prior to the addition of ATP and incubation at 30°C) decreased basal ADC activity, without decreasing the absolute increase in cAMP formation in the presence of DA [Clement-Cormier et al (1975)]. The percentage increase in ADC activity was, as a result, larger after preincubation at 0°C and therefore such an incubation period was employed for homogenate samples in all experiments.
In sliced striatal samples, Wilkening and Makman (1975) demonstrated that modification of the method of Forn et al (1974), by extension of the preincubation time at 37°C to 75 min, also lowers basal ADC activity with a resultant increase in percentage stimulation of enzyme activity by DA.

Clement-Cormier et al (1975) demonstrated that under standard assay conditions, ADC activity of homogenates is proportional to protein concentration, up to 0.25 mg protein/ml final volume, the highest concentration studied, and is proportional to reaction time for up to 5 min, the longest time studied. cAMP content is therefore expressed in all results as pmol cAMP per mg protein per 3 or 5 min incubation period.
| **TABLE 2.1 Protocol for treatment of striatal homogenates and slices.** |
| :---: | :---: | :---: |
| **STRIATAL HOMOGENATES** | **STRIATAL SLICES** |
| **LITERATURE REFERENCES.** | **Forn et al 1974.** |
| | **Wilkening et al 1975.** |
| **TISSUE PREPARATION.** | **10-12 partial slices into intact tissue with a blade.** |
| Homogenization in 25vol med.A. | |
| **PREINCUBATION.** | 1ml per 50mg wet tissue of med.C, without test drugs, for 75min at 37°C |
| 50µl homogenate in 0,5ml med.B. with test drugs, for 20min at 0°C. | 1ml per 50mg wet tissue of med.C, without test drugs, for 75min at 37°C |
| **INCUBATION / REACTION.** | 2ml aliquots of preincubation medium with approx 15mg of tissue were added to tubes containing 10mM theophylline with test drugs to a final vol. of 0,3ml. ATP was added to a final conc. of 0,5mM. 5min at 37°C with shaking. |
| Addition of ATP to a final concentration of 0,5mM. 3min at 30°C with shaking. | 2ml aliquots of preincubation medium with approx 15mg of tissue were added to tubes containing 10mM theophylline with test drugs to a final vol. of 0,3ml. ATP was added to a final conc. of 0,5mM. 5min at 37°C with shaking. |
| **REACTION TERMINATION.** | Homogenization of tissue followed by 10min at 95°C. |
| 3min at 95°C. | 50ml aliquots were assayed for cAMP content by the saturation binding protein assay method of Brown et al (1971) and for protein content by the method of Lowry et al (1951). |
FIGURE 2.1 Effect of temperature on ADC activity of homogenized rat caudate nuclei in the absence or presence of 20μM DA.

Each point represents the mean of 4 samples ± the standard error of the mean (SEM).
FIGURE 2.2 Effect of temperature on ADC activity of sliced rat caudate nucleii in the absence or the presence of 20μM DA.

Each point represents the mean of 4 samples ± the standard error of the mean (SEM).
2.2.4 cAMP Assay

2.2.4.1 Introduction


A simple adrenal extract is prepared and used as the source of binding protein. Tissue samples are incubated in medium E, with binding protein and tritiated cAMP ([³H]-cAMP). The endogenous cAMP and [³H]-cAMP compete for the cAMP binding sites on the binding protein. Charcoal is employed to separate bound from free labelled-cAMP. Bound cAMP is then counted and, using a standard cAMP curve which is generated for each assay, the endogenous cAMP of the tissue sample is determined.

2.2.4.2 Preparation of Binding Protein

Bovine adrenal glands were collected, as soon as possible after slaughter from the Grahamstown abattoir and transported in medium A, on ice, to the laboratory. They were stripped of fat and the adrenal cortices were homogenised in a mixer emulsifier (Ultra-Turrax) with 1,5vol of medium A. The homogenate was centrifuged at 2000g for 5min and the supernatant was respun at 5000g for 15 minutes. All centrifugation was done at 4°C. The final supernatant was aliquoted into 0,5ml fractions and stored at -20°C. A separate portion was thawed and diluted with the assay buffer (medium E) for each assay.
2.2.4.3 Binding Protein Dilution Curves

The sensitivity attainable using a saturation assay method is dependent on a number of factors: on the affinity constant of the binding reagent for its specific ligand, on the specific activity of the tracer available, and on the experimental errors inherent in the manipulative procedures employed—specifically, the statistical error of counting, the experimental error resulting from the pipetting of reagents and misclassification of bound and free nucleotide in the separation procedure. These factors are dependent on the reagent concentrations, which ultimately determines the assay sensitivity [Brown et al (1972)]. Binding protein dilution curves were set up for each new preparation of binding protein, in order to determine the optimal dilution to be used in subsequent assays [Daya (1985)].

Serial dilutions of binding protein in assay buffer (medium C) were prepared and the protocol reflected in Table 2.2 was followed, together with the general method for cAMP assay explained in Sec. 2.2.4.6. As is indicated in Table 2.2, a curve was set up for dilution with respect to both zero concentrations of cAMP and a standard concentration of cAMP of 8pmol/assay tube. Each dilution was assayed for radioactivity in duplicate. The radioactivity (CPM) was plotted against the dilution.

The results obtained from the dilution curves (Fig.2.3) suggested that a 1:3 dilution with assay buffer provided adequate binding and radioactive counts. It also provided sufficient difference in counts between a zero dose of cAMP and a standard concentration of 8pmol cAMP per assay tube. Dilution curves were established for each batch of binding protein prepared, since it was highly probable that different batches would have different protein concentrations. The dilution curve was reproducible for all batches tested. In all subsequent cAMP assays, a dilution factor of 1:3 for the binding protein was used.
TABLE 2.2 Binding Protein Dilution Curve Protocol

<table>
<thead>
<tr>
<th></th>
<th>0 dose (µl)</th>
<th>Std. dose (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[³H]-cAMP</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Standard cAMP</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>Diluted binding</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>reagent</td>
<td>200</td>
<td>150</td>
</tr>
<tr>
<td>Buffer (Medium E)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Total Volume</td>
<td>350</td>
<td>350</td>
</tr>
</tbody>
</table>

FIGURE 2.3 cAMP Binding Protein Dilution Curve

Each point represents the mean of duplicate samples ± SEM.
2.2.4.4 Tritium labelled cAMP: ([³H]-cAMP)

[8-³H]Adenosine 3',5'-cyclic phosphate ammonium salt ([³H]-cAMP), purchased from Amersham (England), had a specific activity of 30 Curies/mmol and a radioactive concentration of 1mCi/ml. It was diluted to a concentration of 0.16µCi/ml in 50% ethanol-water and stored in 6ml portions at -20°C until use.

2.2.4.5 Standard cAMP

Standard cAMP (Boehringer Mannheim) was serially diluted in assay buffer (medium E) for each assay, to yield concentrations ranging between 1-8 pmol/assay tube. A standard cAMP calibration curve was set up for each assay.

2.2.4.6 Assay Procedure

The assay was carried out in 1.5ml Eppendorff microcentrifuge tubes. Each assay tube contained either known amounts of cAMP (0-8 pmol/assay tube) standard or 50 µl of sample, 100 µl of 1:3 dilution of binding protein and assay buffer (medium E), to a final volume of 350 µl. The incubation tubes were gently agitated and then held on ice at 4°C for 100 minutes. After the incubation period, separation of the free from bound cAMP was effected by the addition to the tubes of a stirred suspension of 10% w/v charcoal (Activated Charcoal G.R.- Merck) in medium E, containing 2% Bovine serum albumin (Sigma). The tubes were briefly vortexed and centrifuged at 1200g for 15 minutes. Although temperatures at 4°C could not be maintained, attempts were made, such as pre-cooling the rotor, to keep the temperature as low as possible during centrifugation. Centrifugation at room temperature results in response curves that are slightly displaced to the normal curve [Brown et al 1972]. A 100 µl aliquot of the supernatant was added to 3ml of scintillation cocktail (Beckman HP/b Ready-Solv Premixed Cocktail), briefly
agitated and then counted for radioactivity in a Beckman Liquid Scintillation Counter. Calibration curves were plotted in terms of standard concentration of nucleotide (pmol) against the $C_0/C_x$ ratio (obtained by dividing the radioactivity (CPM) of the zero standard ($C_0$) by the radioactivity (CPM) of the higher standards ($C_x$) after subtraction of the blank values from both.) Blank values were derived from control tubes, set up to check the efficiency of the separation step and nonspecific binding in the assay. In the control tubes, the binding reagent was replaced by 100 µl buffer. Additional controls were set up with some assays, in order to determine the standard amounts of radioactivity (total CPMS) introduced into the incubation mixtures, and to allow for calculation of maximum binding. The amounts of cAMP in unknown samples were determined with reference to the standard curve in each assay.
## TABLE 2.3 cAMP Assay Protocol

Table shows microlitre quantities of reagents used in the cAMP determination method of Brown et al (1971).

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Blank</th>
<th>Zero Dose</th>
<th>Std. Doses</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer.</td>
<td>-</td>
<td>300</td>
<td>200</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Std. cAMP.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>Sample.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>[^3H]-cAMP</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Diluted protein.</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

**Incubation:** 100 minutes at 4°C

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Blank</th>
<th>Zero Dose</th>
<th>Std. Doses</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charcoal slurry.</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

**Centrifugation:** 15min, 14000g at low temperatures

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Blank</th>
<th>Zero Dose</th>
<th>Std. Doses</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant.</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Add to 3ml scintillation cocktail and count radioactivity.
2.2.4.7 Results

2.2.4.7.1 Standard cAMP Curve

The standard response curve for cAMP can be plotted as percentage total tracer ([3H]-cAMP) bound, (CPM std/CPM total), as a function of unlabelled standard cAMP concentration (pmol/tube) [Brown et al (1972)]. A typical plot is presented in Fig. 2.4.

However, as was indicated in Sec. 2.2.4.6, standard response or calibration curves were plotted in terms of standard concentration of nucleotide (pmol/tube) against the C_o/C_x ratio.

\[
C_o = \text{CPM zero standard} - \text{CPM blank} \\
C_x = \text{CPM standard} - \text{CPM blank}
\]

This transformation yielded a linear standard curve between 0 pmol and 8 pmol cAMP, such as the example presented in Fig. 2.5 (linear regression analysis; \(r^2 = 0.998\)).

The assay had a mean overall interassay coefficient of variation (C.V.) calculated from 10 separate standard curves of 7.92 % (Table 2.4). The mean and standard deviations of the 10 standard curve (C_o/C_x) values are presented, for each concentration of cAMP used in the standard curves, in Fig 2.6.

The detection limit of the assay (defined as the concentration of cAMP resulting in a response twice the standard deviation away from the value obtained for the zero standard [Skene (1986)]) was 0,513 pmol/tube.

2.2.4.7.2 Interference by non-cAMP material.

The cAMP assay was checked for the presence of non-cAMP interfering substances. Quadruplicate tissue samples (prepared as in Table 2.1)
were incubated with phosphodiesterase (Sigma) for 1 hour at 37°C to destroy cAMP. Samples incubated without phosphodiesterase (PDE) acted as controls. Samples incubated with PDE yielded cAMP values of 0.40 ± 0.09 (mean ± std) pmol/tube, or 2.72 ± 1.68 pmol/mg protein and controls yielded values of 1.06 ± 0.25 pmol/tube, or 7.18 ± 1.68 pmol/mg protein. These results therefore indicate the presence of some substance or substances which interfere with [3H]-cAMP binding to the binding protein. However, these low levels, with respect to the detection limit of the assay (0.513 pmol/tube), are negligible. The levels were also significantly lower than the cAMP levels of the control samples, incubated without PDE (p<0.005, Student's t-test).

Test drugs DA and MEL present in tissue sample homogenates were also checked for interference with the cAMP assay. Standard concentrations of inactive cAMP were assayed in the presence and absence of the test drugs. Test drugs in assay buffer (medium E) were added, to a final concentration of 50μM, to assay tubes containing standard amounts of cAMP. The presence of the test drugs had no effect on cAMP binding, since test sample cAMP levels did not differ significantly from control samples (free from test drugs) cAMP levels. Thus indicating that the presence of test drugs, DA and MEL in tissue samples would not interfere with cAMP determination.
FIGURE 2.4 Standard response curve for cAMP:
Percentage of total tracer ([3H]-cAMP) bound as a function of unlabelled standard cAMP concentration.
FIGURE 2.5  Standard response curve for cAMP
Relating the response ratio $C_0/C_x$ to unlabelled standard cAMP concentration.
TABLE 2.4 Interassay Coefficient of Variation (C.V.) for cAMP Assay Standard Curves:

<table>
<thead>
<tr>
<th>cAMP (pmol/tube)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>2.8</td>
</tr>
<tr>
<td>0.5</td>
<td>5.5</td>
</tr>
<tr>
<td>1.0</td>
<td>4.6</td>
</tr>
<tr>
<td>2.0</td>
<td>9.9</td>
</tr>
<tr>
<td>4.0</td>
<td>13.7</td>
</tr>
<tr>
<td>8.0</td>
<td>11.0</td>
</tr>
</tbody>
</table>

C.V. was calculated from 10 standard curves.

FIGURE 2.6 The means of multiple standard cAMP curves as a function of standard cAMP concentrations.

Each point represents the mean ± the standard deviation of 10 cAMP standard curves.
2.2.5 **Protein determination**

2.2.5.1 **Introduction**

Since ADC activity of corpus striatum homogenates is proportional to protein concentration [Clement-Cormier *et al* (1975)], or proportional to the size of the corpus striatum portion tested in slices [Forn *et al* (1974)], it was necessary to express all cAMP assay results per mg protein (Sec. 2.2.3.1).

Protein content in samples, or rather, relative protein content with respect to bovine serum albumin (BSA) standards, was determined by a modification of the method described by Lowry *et al* (1951).

2.2.5.2 **Experimental procedure**

2.2.5.2.1 **Stock reagents**

1. 1% Copper sulphate (CuSO₄) solution.
2. 2% Sodium potassium tartrate solution (NaK tartrate).
3. 2% Sodium carbonate in 0.1 N Sodium hydroxide (Soln. A).

2.2.5.2.2 **Working solutions:**

Prepared on day of use:
1. Equal volumes of stock reagents 1 and 2 to yield a 0.5% CuSO₄ in 1% NaK tartrate solution (Soln. B).
2. Copper reagent (Soln. C):
   Mix 50ml of Soln. A with 1ml Soln. B.
3. Folin reagent:
   Dilute commercial Folin-Ciocalteu reagent with an equal volume of distilled water.
4. Bovine serum albumin (BSA) (0.2mg/ml):
A 2mg/ml BSA soln. is prepared in distilled water which is then diluted to 0.2mg/ml with distilled water (Crystallised and lyophilized BSA obtained from Sigma was used.)

2.2.5.2.3 Assay procedure

1. Serial dilutions of the diluted BSA solution were made in distilled water, to yield duplicate samples containing 200, 150, 100, 50 and zero μg/ml BSA.
2. Duplicate test samples were prepared.
3. 1ml of Soln.C. was added to 1ml of standard, or test sample, mixed thoroughly and allowed to stand at room temperature (20°C) for 10 min.
4. 100μl of Folin reagent was added with immediate mixing.
5. After 30 min, absorbance was read against the blank at 750nm.
6. A protein standard curve was established by plotting the log of absorbance vs the log of the protein concentration of standard BSA samples, from which relative protein content of test samples could be rapidly calculated.

2.2.5.3 Typical standard curve

Bates and MacAllister (1974) and Stauffer (1975) have shown that the nonlinear Lowry protein standard curve is transformed into a linear function by plotting the log of absorbance vs the log of the protein concentration, which permits rapid and objective protein calculation of test samples.

An example of such a curve is presented in Fig.2.7. Linear regression analysis yielded an r² value of 0.99. The y axis intercept (m) in this example was -2.9 and the slope (c) 0.9. Relative protein concentration of test samples could then be readily calculated from the equation:

\[ \log(\text{prot}) = (\log(\text{Abs}) - c)/m \]
Where prot. and Abs. refer to the protein concentration and absorbance of the test sample.

**FIGURE 2.7** Log-log plot of the standard curve for 50-200\(\mu\)g of BSA.

Duplicate results are indicated for each standard BSA concentration and the curve is the best fit curve (Linear regression analysis \(r^2=0.99\)).
The effect of MEL on the DA and APO stimulation of rat caudate nucleus ADC activity.

2.3.1 Introduction

The dose response effect of increasing doses of DA, MEL and APO on rat caudate nucleus ADC activity was established. Following the establishment of such controls, the effect of various concentrations of MEL, on DA and APO stimulation of ADC, was determined.

Experiments were carried out according to the procedure described fully in Sec. 2.2.3.

The stimulation of ADC was studied in both homogenized striatal tissue and partially intact sliced striata. The homogenized tissue was homogenized in hypotonic medium in order to disrupt compartments enclosed by cellular and subcellular membranes [Karobath and Leitch (1974)]. Such homogenization, enables demonstration of the effect of test drugs at an intracellular level, free from effects on amine uptake mechanisms which might influenced in the more complex sliced tissue.

2.3.2 Results

2.3.2.1 Effect of increasing doses of DA on ADC activity in both homogenized and sliced rat corpora striata.

DA stimulated ADC in both homogenized and sliced striata, in a dose dependent manner (Fig. 2.8 and Fig. 2.9).

In homogenized striatal samples:
Baseline ADC activity was found to be 6.96 ± 1.02 pmol cAMP/ mg
protein/3 min incubation period. Half maximal stimulation occurred with a 4μM concentration of DA and maximal stimulation (an increase of 18.53 ± 1.8 pmol cAMP/ mg protein / 3 min over basal levels) with a concentration of 12μM DA (Fig 2.8).

In sliced striatal samples:
Basal ADC activity was 5.32 ± 0.8 pmol cAMP / mg protein / 5 min incubation period. Half maximal stimulation also occurred with 4μM DA. Maximal stimulation (11.87 ± 1.68 pmol cAMP/mg protein / 5 min above basal levels) occurred with 10μM DA (Fig. 2.9). All results are the mean ± the standard deviation of 4 samples.

2.3.2.2 Effect of increasing doses of MEL on ADC activity in both homogenized and sliced rat corpora striata.
MEL, up to a concentration of 100μM, had no significant effect on ADC basal activity. MEL failed to significantly increase or decrease basal cAMP levels in either homogenized or sliced striatal samples (Fig. 2.8 and Fig 2.9).

2.3.2.3 Effect of increasing doses of APO on ADC activity in homogenized rat corpora striata.
APO was examined for possible effects on ADC in homogenized samples. In low concentrations, APO caused a dose dependent increase in ADC activity (Fig 2.10). Maximal stimulation (10.62 ± 1.03 pmol cAMP/mg protein/3 min above basal levels) occurred at a concentration of 10μM and half maximal stimulation occurred at a concentration of about 6μM. A dose dependent decrease in percentage stimulation was evidenced at concentrations higher than 10μM, with an increase of only 3.8 ± 0.28 pmol/mg/3min at an APO concentration of 20μM and no significant stimulation above basal levels with a concentration of 100μM. Kebabian et al (1972) demonstrated that concentrations of APO higher than 100μM inhibited basal activity.
FIGURE 2.8 The effect of various concentrations of DA and MEL on ADC activity in rat striatal homogenate.

Each point represents the mean ± the standard deviation of 4 samples.
FIGURE 2.9 The effect of various concentrations of DA and MEL on ADC activity in rat striatal slices.

Each point represents the mean ± the standard deviation of 4 samples.
FIGURE 2.10 The effect of various concentrations of APO on ADC activity in rat striatal homogenate.

Each point represents the mean ± the standard deviation of 4 samples.
2.3.2.4 Effect of various doses of MEL on DA stimulated ADC activity in homogenized rat corpora striata.

The effect of MEL (100μM) was determined on half maximally, and maximally, DA stimulated ADC activity, in homogenized striatal samples (DA concentrations of 4 and 20 μM respectively). MEL concentrations of 4 and 10 μM were also investigated, for their effects on the stimulation of ADC activity by a DA concentration of 10 μM.

Data was statistically analysed using one way analysis of variance followed by Scheffe's post hoc test for multiple comparisons.

Results presented in Fig.2.11 indicate that MEL (at all of the concentrations tested) had no significant effect on the stimulation of ADC by various concentrations of DA, in striatal tissue homogenate.

2.3.2.5 Effect of various doses of MEL on DA stimulated ADC activity in sliced rat corpora striata.

The experiments outlined in Sec. 2.3.2.4 were repeated in sliced striatal samples and data was analysed statistically in the same manner.

As with homogenate samples, results presented in Fig. 2.12 indicate that, at the concentrations tested, MEL failed to have any significant effect on the DA stimulation of ADC activity in rat striatal samples.
FIGURE 2.11  The effect of various concentrations of MEL on DA stimulation of ADC activity in rat striatal homogenate.

Each point represents the mean ± the standard deviation of 4 samples.
FIGURE 2.12 The effect of various concentrations of MEL on DA stimulation of ADC activity in rat striatal slices.

Each point represents the mean ± the standard deviation of 4 samples.
2.3.2.6 Effect of various doses of MEL on APO stimulated ADC activity in homogenized rat corpora striata.

The effects of MEL (1 μM) on APO (1, 10, and 100 μM), and MEL (10 μM) on APO (4, 10, and 20 μM) induced stimulation of ADC activity were investigated.

Data was statistically analysed as in Sec. 2.3.2.4.

At the concentration combinations tested, MEL was shown to have no significant effect on the stimulation of ADC activity in homogenized rat corpus striatum by APO (Fig. 2.13 and Fig. 2.14).

2.3.3 Discussion

It has generally been accepted that the striatal ADC enzyme system is a useful model for studies of DA receptors in the brain [Miller and Iversen (1974)], and more specifically for studies of D₁ receptor activity [Kebabian and Calne (1979)].

Although it was initially thought that the D₂ DA receptor was not coupled to ADC [Kebabian and Calne (1979)], later studies have shown that the D₂ receptor may be negatively coupled to ADC in rat striata [Stoof and Kebabian (1981), Onali et al (1984)]. When DA activation of D₁ sites is impaired by SCH 23390, a specific D₁ blocker, DA causes inhibition of basal ADC activity [Onali et al (1984)]. Results presented by Onali et al (1985) suggest that D₁ stimulatory and D₂ inhibitory receptors may co-exist on the same striatal neurons and interact by modulating the response of a common ADC system. The D₁ receptor/ADC intercation is mediated by a stimulatory guanine nucleotide binding protein (N₁), and the D₂ receptor/ADC interaction is mediated by an inhibitory guanine nucleotide binding protein (N₂) [Review - Creese (1985)]. It would, however, appear that the assay conditions employed in the present
studies favour stimulation of ADC, over inhibition, and that the effect of DA at the D₁ receptor is the predominant effect. [Cooper et al 1985].

In general, drugs with a high neuroleptic activity have been shown to be potent inhibitors of DA-sensitive ADC [Karobath and Leitch (1974)]. Although the relative affinities for D₁ receptor sites do not correlate well with the therapeutic efficacy of such drugs [Miller and Iversen (1974)], the striatal ADC enzyme system has been shown to be useful as a preliminary screening procedure for neuroleptic activity of drugs. There are, however, selective D₂ receptor antagonists, such as sulpiride and molindone, which are effective neuroleptic agents, although they do not inhibit DA-sensitive ADC [Review – Kebabian and Calne (1979)].

The results presented in this study thus indicate:
1. If MEL does have a role on dopaminergic transmission in the corpus striatum of the brain, it does not appear to be at the level of the D₁ receptor. This model, however, does not exclude the possibility that MEL may physiologically antagonise D₁ receptor mediated mechanisms.
2. MEL does not, in this preliminary screening procedure, show promise as a neuroleptic agent.
FIGURE 2.13 The effect of 1μM MEL on APO stimulation of ADC activity in rat striatal homogenate.

Each point represents the mean ± the standard deviation of 4 samples.
FIGURE 2.14 The effect of 10µM MEL on APO stimulation of ADC activity in rat striatal homogenate.

Each point represents the mean ± the standard deviation of 4 samples.
CHAPTER 3
STEREOTYPE BEHAVIOURAL STUDIES

3.1 INTRODUCTION

The study of behaviours induced by dopaminergic agents which act centrally, is an accepted and classical means of investigating the pharmacology of the dopamine system and pathophysiology of human neuropsychiatric diseases, for which these behaviours serve as animal models [Braun et al (1986)].

The behavioural studies which have found wide use in the analysis of differential dopamine function are the studies of the induction or antagonism of stereotype behaviour.

DA agonists, including amphetamine and APO, induce a behavioural syndrome of stereotyped behaviour in all mammalian species, including man. Stereotypy can be defined as the apparently purposeless repetition of an invariant sequence of movements, confined to fewer and fewer classes of response as the dose effect increases [Sahakian and Robbins (1975), Fray et al (1980) and Braun and Chase (1986)]. In the laboratory rat, these behaviours are often manifested as stereotyped sniffing, repetitive head movements, locomotor activity and rearing at low doses, with stereotyped licking or gnawing evident at higher dose levels or with chronic administration.

APO induced stereotypy in rats is considered to be the result of direct stimulation of DA receptors in the corpus striatum [Maj et al (1972)] and recent findings such as those presented by White et al (1988) support the hypothesis that D₁ and D₂ receptors function in a synergistic manner to regulate the intensity and form of
stereotyped behaviour produced by DA agonists.

Drug induced, unconditioned behaviour is very difficult to measure quantitatively and reliably; objective and precise measurement of behaviours such as licking, grooming, gnawing and sniffing are difficult to obtain and thus it is difficult to evaluate the intensity of stereotypy. In an attempt to overcome this problem, rating scales have been used [Creese and Iversen (1973), Ellinwood and Balster (1974), Sahakian et al (1975)] where particular behavioural patterns are rated according to intensity of stereotypy.

Useful as rating scales are, and have been, in behavioural pharmacology, they have a number of disadvantages:

1. Intensification of stereotypy is usually inferred by the transition from one class of response (e.g. sniffing) to another (e.g. licking). It has, however, been shown that the commonly assumed intensity relationship between different behavioural patterns in APO induced behaviour does not appear to be correct [Ljungberg and Ungerstedt (1977a)].

2. Ratings, even if made blind to drug treatment or dosage, are still subjective, requiring the observer not only to judge whether a particular response has occurred, but also to assess its stereotypic nature, which can easily lead to confusion.

3. This type of rating may lead to a loss of information about the actual responses of the animal, since a single rating category may include different classes of response (e.g. gnawing and licking) percieved to have similar degrees of stereotypy.

4. Rating scales are not suitable for the assessment of behaviours induced by drugs that are selective for a particular DA receptor subtype ie. D₁ or D₂ receptors [Cameron et al (1988)].

In the present studies behaviour is assessed in terms of a rating scale, adaptated from the rating scale of Creese and Iversen (1973), but this is coupled with the use of a method developed by Fray et al (1980), in which behavioural scoring is defined by the
presence or absence of different response categories or target behaviors, but not by the occurrence of stereotypy (called here after - the occurrence observation method). This method has the advantages of requiring a rater of minimal behavioural rating sophistication, as well as allowing a precise statement about the effect of a drug or drugs on each behavioural category.

These two methods are used to investigate the effect of intraperitoneally (ip) administered MEL on the induction of stereotype behaviours by APO, thereby investigating the hypothesis that MEL may modulate central nervous system dopaminergic activity.

3.2 MATERIALS AND METHODS

3.2.1 Animals

All animals used in these studies were male albino rats of the Wistar strain, weighing approximately 225-275g at time of testing. Rats were housed eight per cage and maintained on food and water ad libitum. They were maintained in a light regulated environment, with a 12/12 hr light/dark cycle (06h00 on and 18h00 off).

3.2.2 Apparatus

All testing took place in observation boxes, which are black painted aluminium boxes measuring 500 mm by 500 mm square by 200 mm high. The boxes are mounted on 800 mm high bases. The floor of each box comprises an array of nine 60 mm diameter holes and the box is covered with a wire grid. The holes in the floor of the boxes provide an environmental stimulus to which the rats may react, with head dipping into the holes being a measure of exploratory behaviour [Bossier and Simon (1962)]. The hole count or head dipping is however only of indirect value in the analysis of APO induced behaviours which are usually characterised by a lack of head dipping [Ljungeberg and Ungerstedt (1977)]. The holes do,
however, serve an important function in that their edges provide opportunity for the rats to gnaw [Lungberg and Ungerstedt (1977a)]. The wire grid covering the cages also provide an object for the rats to gnaw. Four identical observation boxes were used simultaneously. A video camera was used to film the rat behaviour.

Ventilation fans in the test room provided background noise of 65-70 dB, which masked extraneous sounds. Illumination level immediately above the cages was approximately 1800 lux.

3.2.3 Procedure

For 3 consecutive days prior to testing, each rat was placed in an observation box for a 2 hr period of habituation. On the day of testing, each rat was removed from its home cage and habituated to the observation box for a 25 min period prior to drug treatment. Following the habituation period, it was removed, immediately injected with MEL or the MEL injection vehicle, and returned to the observation box. 5 mins later, it was once again removed and injected with APO or the APO injection vehicle. It was returned to the box for a 1 hr test session.

Testing was conducted during the first 4 hrs of the light cycle - normally between 08h00 and 12h00.

At each 10 min period of the 1 hr test session, each rat was videoed for 1 min. The videos were later viewed by observers denied knowledge of the treatment received by the animals. During the second 10 sec period of the 1 min test periods filmed, each rat was observed for the occurrence of all the behavioural response categories listed in Table 3.1 and the presence or absence of the categories was recorded on a score sheet, as presented in Table 3.2. It should be noted that any combination of the response categories could be exhibited by an animal during a single 10 sec observation period. Following each 10 sec observation period the rats were also
scored for stereotypy according to a 0 - 6 rating scale adapted from that of Creese and Iversen (1973) and presented in Table 3.3. The two lowest ratings:

0. Asleep or stationary and
1. Active, were intended for undrugged or control animals, that is for animals exhibiting normal behaviour. The other categories theoretically describe increasing intensities of stereotyped behaviour:

2. Predominantly active ie. locomotion, with bursts of sniffing or rearing;
3. Stereotyped behaviour over a wide area of the cage, eg sniffing along a fixed path in the cage;
4. Stereotyped sniffing or rearing maintained in one location in the cage;
5. Stereotyped behaviour in one location in the cage with bursts of gnawing or licking of the cage;
6. Continual gnawing or licking of the cage.

The best method of obtaining a complete description of behaviour would be continuous observation of each rat. However, Fray et al (1980) suggest that even at low doses, short observations every 10 min are sufficient to detect drug-induced behavioural changes.
<table>
<thead>
<tr>
<th>RESPONSE CATEGORY</th>
<th>DEFINITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Still (Still)</td>
<td>Inactive or asleep, with an occasional sniff.</td>
</tr>
<tr>
<td>Locomotion (Loco)</td>
<td>Active, normal walking around the cage, involving all four limbs.</td>
</tr>
<tr>
<td>Rearing (Rear)</td>
<td>Both front feet off the cage floor, animal supporting itself on rear paws.</td>
</tr>
<tr>
<td>Sniffing (Sniff)</td>
<td>Sniffing, head down with nose in contact with cage surface for more than 3 sec.</td>
</tr>
<tr>
<td>Licking (Lick)</td>
<td>Licking of the cage surface for more than 3 sec.</td>
</tr>
<tr>
<td>Gnawing (Gnaw)</td>
<td>Gnawing the cage or cage lid for more than 3 sec.</td>
</tr>
<tr>
<td>Head Down (Head)</td>
<td>Animal standing, walking or running, with its nose below horizontal, for more than 5 sec.</td>
</tr>
<tr>
<td>Swaying (Sway)</td>
<td>Rhythmic swaying movements of the animal's head, for more than 3 sec.</td>
</tr>
<tr>
<td>Grooming (Groom)</td>
<td>Animal licking paws and washing face or body for more than 3 sec.</td>
</tr>
<tr>
<td>Nose Poking (Nose)</td>
<td>Dipping the nose into one of the holes on the cage floor for more than 3 sec.</td>
</tr>
</tbody>
</table>
TABLE 3.2 Typical scoring sheet used by observers to note presence or absence of categories of behaviour listed in Table 3.1.

<table>
<thead>
<tr>
<th>SCORING SHEET FOR BEHAVIOURAL ANALYSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAT NO.      DATE       CODE NO.</td>
</tr>
<tr>
<td>0 10 20 30 40 50 50 50 50</td>
</tr>
<tr>
<td>STILL        LOCO        REAR</td>
</tr>
<tr>
<td>SNIFF        LICK        GNAW</td>
</tr>
<tr>
<td>HEAD DOWN    SWAY        GROOM</td>
</tr>
<tr>
<td>NOSE POKE    SCORE</td>
</tr>
</tbody>
</table>

TABLE 3.3 The rating scale used to score the intensity of stereotype behaviour.

<table>
<thead>
<tr>
<th>SCORING STEREOTYPE BEHAVIOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - Asleep or stationary</td>
</tr>
<tr>
<td>1 - Active</td>
</tr>
<tr>
<td>2 - Predominantly active with bursts of sniffing or rearing</td>
</tr>
<tr>
<td>3 - Stereotype behaviour over a wide area of the cage</td>
</tr>
<tr>
<td>4 - Sniffing or rearing maintained in one location in the cage</td>
</tr>
<tr>
<td>5 - Stereotype behaviour in one location with bursts of gnawing or licking</td>
</tr>
<tr>
<td>6 - Continual gnawing or licking</td>
</tr>
</tbody>
</table>
3.2.4 Drugs and dosage

Firstly, a control group of rats was tested, to establish normal or undrugged behaviour. Rats were injected intra-peritonially (ip) with the vehicle in which the MEL injections were prepared, at a dose of 0.4ml/kg body mass (BM) and 5 min later with saline containing 1mg/ml ascorbic acid, at a dose of 0.8ml/kg (BM) administered sub-cutaneously (sc) in the flank.

The behavioural response to APO was established with 0.5; 1; 2 and 5 mg/kg (BM) APO. APO was dissolved in saline and 1mg/ml ascorbic acid was added as antioxidant. All APO injections were given sc. in the flank.

The effects of 1 and 10 mg/kg (BM) MEL on APO response were established for each of above doses of APO. MEL injections were prepared in the following vehicle and administered ip.

Melatonin (2.5mg/ml) in vehicle
- Melatonin 25mg
- Benzyl alcohol 200μl
- Anhydrous citric acid 25mg
- Tween 80 1ml
- Water for injection to 10ml

Sample sizes varied between 8 and 20 rats per group. Rats were reused in testing sessions, in a random manner, after 2 week drug-free "wash out" periods.

3.2.5 Data analysis

Since the scores obtained in both methods used to rate behaviour constitute neither an interval nor a ratio scale of measurement [Robbins (1977), Fray (1980)], statistical analysis of the results requires nonparametric testing, which tests for differences in contingency tables. Contingency tables were set up for both methods.
of recording behaviour, and the degree of heterogeneity in each table was then calculated by a likelihood ratio analysis method, the "information statistic" [Kullback (1968)]. This test is analogous to the better-known chi-squared test, but has the advantages that it is not hampered by the constraint of small cell frequencies and that it lends itself readily to decomposition into independent components which can be identified with submatrices of data, and that it can be partitioned to locate the sources of statistical significance in a dose response determination [Robbins (1977) and Fray et al (1980)].

1. Analysis of data in the occurrence observation method:

According to the method of Fray et al (1980):

For each response category, at each dosage combination, and for each 10 min interval, the number of rats out of the total sample group showing, as well as those not showing, a particular category of behaviour was tabulated, as in the example given in Table 3.4. Heterogeneity was calculated in each table as 

$$2I = 2\sum_{ij} [N_i \ln(N/E)]$$

where N is each observation in the table, E is the expected value (row total x column total/grand total) and i and j are the numbers of rows and columns, respectively. 2I is distributed as chi-squared with \((i-1)(j-1)\) degrees of freedom (df). Each of the six time intervals (10, 20, 30, 40, 50 and 60 min) for each behavioural response category were analysed independently and the six values for 2I were then added, to give a total 2I for each category. The category totals were added to yield a grand total 2I. The df were also added, to give a total df value for each category, which were added to provide a grand total df. Only if the grand total 2I was significant (using chi-squared tables, \(p < 0.05\) was considered acceptable), were the totals for each category inspected, and if one of these categories was significant, then each time interval within that category was examined. If a time interval was found to be significant, then contrasts or comparisons were made between doses using a method of planned contrasts. The lowest dose was compared to the next dose and if they were not significantly different, they were added together and substituted for the second
dose. If the first two doses were different, the second dose was compared with the third dose and so the process was repeated, until the last dose was reached. This method only allowed for comparison of neighbouring doses or neighbouring groups of doses. Neighbouring doses could be grouped such that there were no differences between members of a group, but neighbouring groups were significantly different. Five percent significant levels were used throughout.

2. Analysis of data derived from the rating scale method:
According to the method of Robbins (1977), median rating scores for each rat were cast into a matrix form, reflecting the frequency distribution of the 6 scale categories, for each drug treatment, as in the example given in Table 3.5. The contingency tables were then analysed by the same method as that described for the analysis of the occurrence observation method.

Since two observers were used in the scoring of behaviours, it was necessary to determine interobserver reliability. This was done by a method described by Cameron et al (1988). Both observers rated eight rats simultaneously, using score sheets as presented in Table 3.2. The two recording sheets for each rat were compared at each interval, to determine the number of occasions on which (1) the observers agreed on an occurrence (AO), (2) disagreed on an occurrence (DO), (3) agreed on a nonoccurrence (AN), and (4) disagreed on a nonoccurrence (DN). In order to avoid artificially inflated reliability estimates, if behaviours occurred on fewer than 50% of the intervals, interobserver reliability was calculated using agreement about the occurrence of a behaviour, and the converse was true if behaviours were at a higher frequency than 50% of the intervals. Using the following formulae, interobserver agreement (IA) was calculated:

1. If incidence of behaviour was less than 50% 
   \[ IA = \frac{AO}{(AO + DO)} \times 100 \]

2. If incidence of behaviour was greater than 50% 
   \[ IA = \frac{AN}{(AN + DN)} \times 100 \]

An IA value of greater than 80% was considered acceptable.
TABLE 3.4 Example of contingency table for the effect of APO on sniffing 10 min post-injection.

<table>
<thead>
<tr>
<th>APO DOSE (mg/kg BM)</th>
<th>NO. OF RATS SNIFFING</th>
<th>NO. OF RATS NOT SNIFFING</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>0.5</td>
<td>6</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td><strong>TOTALS</strong></td>
<td><strong>52</strong></td>
<td><strong>14</strong></td>
<td><strong>66</strong></td>
</tr>
</tbody>
</table>

TABLE 3.5 Example of contingency table showing the effect of APO on frequency of rats per rating scale 50 min post-injection.

<table>
<thead>
<tr>
<th>APO DOSE (mg/kg BM)</th>
<th>Stereotype rating scale - categories</th>
<th>ROW TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 1 2 3 4 5 6</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>14 4 1 0 1 0 0</td>
<td>20</td>
</tr>
<tr>
<td>0.5</td>
<td>8 0 0 0 0 0 0</td>
<td>8</td>
</tr>
<tr>
<td>1</td>
<td>2 0 2 1 5 0 0</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>1 0 1 9 4 0 1</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>1 0 0 0 0 0 12</td>
<td>12</td>
</tr>
<tr>
<td><strong>TOTALS</strong></td>
<td><strong>25</strong> 4 4 10 10 0 13 <strong>66</strong></td>
<td></td>
</tr>
</tbody>
</table>
3.3 RESULTS AND DISCUSSION

3.3.1 Interobserver reliability

As was discussed in Sec.3.2.5, the use of two observers to observe and rate behaviour made it necessary to determine interobserver reliability. For 8 rats observed simultaneously by both the observers the interobserver agreement (IA) was found to be 93.6%. Interobserver reliability was therefore considered to be acceptable (ie above 80%).

3.3.2 Apomorphine dose response

The effect of APO on unconditioned behaviours was determined using both the response occurrence observation method and the stereotypy rating scales described in Sec 3.2.3. These results were necessary to serve as a control from which to study the effects of MEL on behaviours elicited by APO. APO produced both dose and time dependent changes among the various behavioural response categories, as well as in the stereotype behavioural scores.

3.3.2.1 Stereotypy ratings

The ratings obtained using the adapted Creese and Iversen (1973) stereotypy rating scale followed a dose response relationship for APO, as presented in Fig. 3.1.

An APO dose of 0.5 mg/kg (BM) only induced significant stereotype behaviour up to 10 min post-injection, whereas 1 mg/kg (BM) induced significant stereotypy up to 50 min post-injection. 2 and 5 mg/kg (BM) induced significant stereotypy during the entire test period, with the 5 mg/kg (BM) dose effect becoming maximal 40 min post injection time and being maintained at this level (the ceiling level of the scale) until the end of the test period.
3.3.2.2 Behavioural response categories occurrence observations:

APO produced both dose and time dependent changes among the various behavioural response categories (Fig 3.2(a)-(g)). Some behaviours, such as swaying, grooming and nose-poking were only observed very infrequently and APO failed to show any significant effect on these behaviours. They are therefore not presented in the results or discussed any further.

The results presented in Fig. 3.2(a) show that, during the entire test period, there was a general dose dependent decrease in the percentage of rats exhibiting the category of behavioural response - stillness as defined in Table 3.1. Doses of APO of 1, 2 and 5 mg/kg (BM) resulted in a significant decrease, if not total abolition of stillness. However, the lowest dose of APO tested (0.5 mg/kg (BM)) failed to cause a significant decrease in the percentage of rats exhibiting stillness and at the end of the test period significantly increased the percentage of rats exhibiting stillness with respect to saline treated rats. These results would therefore imply a dose dependent increase in general activity in other behavioral response categories by APO at doses above 0.5 mg/kg (BM).

APO 0.5 mg/kg (BM) failed to induce significant locomotion in subjects, whereas the higher doses tested elicited significant locomotion up to 50 min post-injection time (Fig. 3.2(b)).

Percentage sniffing, rearing, licking and gnawing responses were all increased at different threshold doses and at different time intervals (Fig.3.2(c,d,f and g)). Sniffing was increased at 1 mg/kg (BM) from the initial test period (10 min post-injection time). From 30 min until the end of the test period, sniffing was less evident at the highest dose 5mg/kg (BM), possibly being replaced by the gnawing and licking responses. Gnawing and licking responses were only evident with the highest dose tested, licking becoming significant 30 min post-injection time and gnawing at 40 min.
Rearing was also only evident at the highest dose tested and was only significant during two test periods (20 and 30 min post injection time). Although other workers found the "head down" response to be a good indicator of drug effect [Fray et al 1980], in these studies it was only significantly elevated by 2mg/kg (BM) 30 and 40 min post-injection and at 5 mg/kg (BM) 40 and 60 min post injection time and therefore does not appear to be a good indicator of drug activity (Fig. 3.2(e)).

3.3.2.3. Discussion

The results presented here are in general agreement with other authors [Fray et al (1980), Cameron et al (1988), Ljungberg and Ungerstedt (1977a)], in that the DA agonist APO was shown to increase the occurrence of certain unconditioned behaviours such as sniffing, gnawing and licking. It does, however, appear that factors such as species, strain, dose parameters and environmental design affect the specific dose response profiles.

Ljungberg and Ungerstedt (1977a) demonstrated two different patterns of response with clear-cut differences in time course between them. The two patterns: locomotion and sniffing (LS) and gnawing (G), with LS predominating in the earlier phases and G in the latter phases of the test period, could be manipulated by such factors as route of administration and absorption rate of APO - the ascorbic acid content of the injection vehicle being found to be a critical variable. Although the differences between locomotion, sniffing and gnawing were not as clear cut in the present study, at the highest dose tested (5mg/kg (BM)) and in the latter stages of the test period, sniffing and locomotion did become less significant as licking and gnawing responses became evident. These results differ from those of Fray et al (1980) who found that the G behavioural element was evident concomitantly with the LS element throughout the test period and not only in the latter stages. Cage design has been suggested as a possible reason for such
discrepancies in results [Fray et al (1980), Ljungberg and Ungerstedt (1977b)]. Wire cages, such as those employed by Fray et al (1980), allow the rat the freedom to perambulate and gnaw simultaneously and continuously, whereas the observation boxes used in the present study and those used by Ljungberg and Ungerstedt (1977) provide less opportunity for simultaneous gnawing and locomotion.

Although both methods of behavioural analysis employed in these studies have limitations and shortcomings (such as those discussed above and in Sec. 3.1), the results obtained for the dose effect of APO by both methods still provide important controls against which the effect of MEL administration on APO elicited behaviours can be studied.

Bearing in mind the shortcomings of the stereotypy rating method (discussed in full in Sec 3.1), the results obtained provide some idea of the intensity of stereotypy elicited at various concentrations of APO. The results obtained with the occurrence observation method, however, presents a detailed description of the gross behavioural effects elicited by APO. The profile presented clearly indicates that the different behavioural responses were elicited at different threshold doses and were also affected by temporal factors.
FIGURE 3.1 Median stereotypy scores obtained with varying doses of APO at various post-injection time intervals.

Each point represents the median stereotypy score of rats (8 ≤ N ≥ 12) at each dose of APO (0; 0.5; 1; 2; 5 mg/kg BM). The boxes above the doses indicate the statistical significance (p < 0.05) of dose effects at the particular time intervals. For example, at the 50 min time interval results can be interpreted as follows: 0 and 0.5 mg/kg (BM) doses are not significantly different from one another and neither are the 1 and 2 mg/kg (BM) doses. However these two groups 0 and 0.5, and, 1 and 2 are significantly different from each other and the group 1 and 2 is significantly different from the 5mg/kg (BM) dose.
FIGURE 3.2(a) - 3.2(g) Effects of varying doses of APO on behavioural response categories defined in Table 3.1

Each point represents the percentage of rats (8 ≤ N ≥ 20) at each dose of APO (0; 0.5; 1; 2; 5 mg/kg (BM)) exhibiting a particular category of behaviour at each 10 min interval of the 60 min test period. The boxes above the doses indicate the statistical significance of dose effects at each particular time interval (the explanation being the same as outlined in the legend to Fig. 3.1 and in Sec. 3.2.5). "% Rats" on the y-axis represents the percentage of rats exhibiting the particular behavioural response.

FIGURE 3.2(a) Effect of varying doses of APO on the behavioural response category - Still.
FIGURE 3.2(b) Effect of varying doses of APO on the behavioural response category - Locomotion.

FIGURE 3.2(c) Effect of varying doses of APO on the behavioural response category - Sniffing.
FIGURE 3.2(d) Effect of varying doses of APO on the behavioural response category - Rearing.

FIGURE 3.2(e) Effect of varying doses of APO on the behavioural response category - Head Down.
FIGURE 3.2(f) Effect of varying doses of APO on the behavioural response category - Licking.

FIGURE 3.2(g) Effect of varying doses of APO on the behavioural response category - Gnawing.
3.3.3 The effect of MEL administration on the APO induced
behavioural response.

These results are presented in two ways -
1. The effect of MEL 1mg/kg (BM) or 10mg/kg (BM) on the
behavioural response induced by increasing doses of
APO (0; 0,5; 1; 2; and 5 mg/kg (BM)).
2. The effect of increasing doses of MEL (1 and 10 mg/kg
(BM)) on the behavioural response elicited by each test
dose of APO (0; 0,5; 1; 2 or 5 mg/kg (BM)).

3.3.3.1 The effect of MEL administration on the behavioural
response induced by increasing doses of APO.

The results presented in Fig. 3.3 (median stereotypy rating
scores) and in Fig. 3.4 (occurrence observation behavioural
categories response) are obtained in the presence of 1mg/kg (BM)
MEL, and in Fig. 3.5 and 3.6, in the presence of 10mg/kg (BM) MEL,
administered ip 5 min prior to APO administration.

Considering the stereotypy rating scores:

In the presence of 1mg/kg (BM) MEL (Fig 3.3), APO at a dose of 0.5
mg/kg (BM) failed to have any statistically significant effect on
stereotypy at any time interval, and APO 1 mg/kg (BM) significantly
increased stereotypy scores during the first test period. 1 mg/kg
(BM) APO significantly increased the scores up to 40 min post
injection, whereas 5 mg/kg (BM) increased scores throughout the
test period.

In the presence of 10mg/kg (BM) MEL (Fig 3.5), only 5mg/kg (BM) APO
significantly increased stereotypy scores and this was only up to
40 min post-injection time.

These results suggest that MEL, at the doses tested, does not
totally inhibit the induction of stereotype behaviour by APO, but rather in a dose dependent manner increases the APO dose threshold at which stereotypy is induced.

Considering the behavioural response categories:

In the presence of 1 mg/kg (BM) MEL, APO 1 mg/kg (BM) only significantly decreased the percentage of rats maintaining "stillness", during the first 10 min test interval, unlike in the absence of MEL where it decreased "stillness" throughout the test period. APO 2 mg/kg (BM) decreased the percentage of rats in this category up to 50 min, and 5 mg/kg (BM) continued to decrease the percentage throughout the test period (Fig. 3.4(a)). Only 5mg/kg (BM) APO showed any significant decrease in the percentage of rats showing "stillness" in the presence of 10 mg/kg (BM) MEL and this was significant up to 50 min post-injection time (Fig 3.6(a)).

Significantly, the percentage of rats which showed locomotion in the presence of MEL 1mg/kg (BM) (Fig.3.4(b)), was only increased by doses of 2mg/kg (BM) APO, 30 and 40 min post-injection time, and by 5mg/kg (BM) 30, 40 and 50 min post injection time. In the absence of MEL, there was a dose response increase in locomotion with doses of APO above 1mg/kg (BM), up to 50 min post injection time. In the presence of 10mg/kg (BM) MEL locomotion is increased by 1 and 2 mg/kg (BM) APO at 20 min, and by 5mg/kg (BM) APO at 10, 20 and 50 min (Fig.3.6(b)).

In the presence of 1mg/kg MEL, the percentage sniffing response was increased by doses of APO above 1mg/kg (BM) from the first 10 min test interval but at 40 min the increase induced by even the highest dose of APO was not significant. At 50 min, only the highest dose of APO, resulted in a significant increase and at 60 min there was a dose dependent increase with doses of 1mg/kg (BM) and above (Fig 3.4( c)). Only the highest dose of APO tested elicited any significant increase in percentage sniffing in the presence of 10mg/kg (BM) MEL (Fig 3.6(c)). This is in contrast with
the results obtained in the absence of MEL, where maximum percentage sniffing after 20 min was evidenced with 2mg/kg (BM) APO, and not with 5mg/kg (BM) APO, which showed a significantly lower percentage sniffing.

All doses of APO failed to increase the percentage of rats, rearing, licking and gnawing, in the presence of either 1 or 10mg/kg (BM) MEL (Fig 3.4(d,f,g) and Fig 3.6(d,f)).

From these results, it would appear that MEL increases, in a dose dependent manner, the threshold doses of APO at which behaviours as sniffing, and locomotion appear, confirming the results obtained from the use of a stereotypy rating scale. At the doses tested, MEL, however, almost entirely inhibits the induction of behaviours by APO associated with intense stereotypy, such as gnawing and licking.
FIGURE 3.3 Median stereotypy scores obtained with varying doses of APO in the presence of MEL 1mg/kg (BM) at various post-injection time intervals.

The boxes above the doses indicate the statistical significance ($p < 0.05$) of dose effects at the particular time intervals. Each point represents the median stereotypy of rats ($8 \leq N \geq 12$) at each dose of APO (0; 0.5; 1; 2; 5 mg/kg (BM)).
FIGURE 3.4(a)-3.4(g) Effects of varying doses of APO on behavioural response categories defined in Table 3.1 in the presence of MEL 1mg/kg (BM) administered ip 5 min prior to APO injection.

Each point represents the percentage of rats (8 ≤ N ≥ 12) at each dose of APO (0; 0.5; 1; 2; 5 mg/kg (BM)) exhibiting a particular category of behaviour at each 10 min interval of the 60 min test period. The boxes above the doses indicate the statistical significance of dose effects at each particular time intervals (the explanation being the same as outlined in the legend to Fig. 3.1 and in Sec. 3.2.5)

FIGURE 3.4(a) Effect of varying doses of APO following prior MEL 1mg/kg (BM) administration on the behavioural response category - Stillness.
FIGURE 3.4(b) Effect of varying doses of APO following prior MEL 1mg/kg (BM) administration on the behavioural response category - Locomotion.

FIGURE 3.4(c) Effect of varying doses of APO following prior MEL 1mg/kg (BM) administration on the behavioural response category - Sniffing.
FIGURE 3.4(d) Effect of varying doses of APO following prior MEL 1mg/kg (BM) administration on the behavioural response category - Rearing.

FIGURE 3.4(e) Effect of varying doses of APO following prior MEL 1mg/kg (BM) administration, on the behavioural response category - Head Down.
FIGURE 3.4(d) Effect of varying doses of APO following prior MEL 1mg/kg (BM) administration on the behavioural response category - Licking.

FIGURE 3.4(g) Effect of varying doses of APO following prior MEL 1mg/kg (BM) administration on the behavioural response category - Gnawing.
FIGURE 35 Median stereotypy scores obtained with varying doses of APO in the presence of MEL 10mg/kg (BM) at various post-injection time intervals.

The boxes above the doses indicate the statistical significance (p < 0.05) of dose effects at the particular time intervals. Each point represents the median stereotypy of rats (8 ≤ N ≥ 12) at each dose of APO (0; 0.5; 1; 2; 5 mg/kg (BM)).
FIGURE 3.6(a) Effects of varying doses of APO on behavioural response categories defined in Table 3.1 in the presence of MEL 10mg/kg (BM) administered ip 5 min prior to APO injection.

Each point represents the percentage of rats (8 ≤ N ≥ 12) at each dose of APO (0; 0.5; 1; 2; 5 mg/kg (BM)) exhibiting a particular category of behaviour at each 10 min interval of the 60 min test period. The boxes above the doses indicate the statistical significance of dose effects at each particular time interval (the explanation being the same as outlined in the legend to Fig. 3.1 and in Sec. 3.2.5)

FIGURE 3.6(a) Effect of varying doses of APO following prior MEL 10mg/kg (BM) administration on the behavioural response category - Still.
**FIGURE 3.6(b)** Effect of varying doses of APO following prior MEL 10mg/kg (BM) administration on the behavioural response category - Locomotion.

**FIGURE 3.6(c)** Effect of varying doses of APO following prior MEL 10mg/kg (BM) administration on the behavioural response category - Sniffing.
FIGURE 3.6(d) Effect of varying doses of APO following prior MEL 10mg/kg (BM) administration on the behavioural response category - Rearing.

FIGURE 3.6(e) Effect of varying doses of APO following prior MEL 10mg/kg (BM) administration on the behavioural response category - Head Down.
FIGURE 3.6(1) Effect of varying doses of APO following prior MEL 10mg/kg (BM) administration on the behavioural response categories - Licking and Gnawing.
3.3.3.2 The effect of administration of two doses of MEL on the behavioural response induced by varying doses of APO.

In all tests, 1 or 10mg/kg (BM) MEL was administered ip 5 min prior to APO administration. The results of these doses of MEL on the behavioural responses elicited by APO, 1; 2 and 5 mg/kg (BM) are presented in Figs. 3.7; 3.8 and 3.9 respectively. Only the behavioural response categories at each APO dosage level which were significantly affected by MEL are presented in these figures. Using both the rating scales and the occurrence observation methods of behavioural analysis, MEL had no significant effect on the "normal" activity or behaviour - that is, behaviour of the "undrugged" rat. It also had no effect on the behavioural response elicited by APO 0.5mg/kg (BM). These results are therefore not presented graphically.

Considering the stereotypy rating scores: The median stereotypy scores elicited by 1; 2 and 5 mg/kg (BM) APO was significantly decreased by both doses of MEL, but at most test intervals the decrease was not significantly dose dependent, although MEL did show some dose dependent reduction in stereotypy during the first thirty minutes of the APO 2mg/kg (BM) test period (Fig. 3.8(a)). At the highest test dose of APO (5mg/kg (BM)), the scores were significantly decreased by MEL, but still remained at 2 or above, thus indicating that stereotype behaviour was not totally abolished, but the intensity of the stereotype behaviours observed were reduced (Fig. 3.9(a)).

These results thus indicate that at the doses tested, MEL administration 5 min prior to the administration of APO significantly reduces the stereotype behavioural response normally elicited by APO.

Considering the occurrence observation of behavioural response categories: The only behavioural responses elicited by APO 1mg/kg (BM) that
were affected by MEL administration were stillness and sniffing (Fig. 3.8(b) and (c)). At both dosage levels, MEL significantly increased the percentage of rats remaining inactive after APO administration at the 30; 40 and 50 min test intervals. MEL reduced the percentage of rats sniffing at the 20; 30 and 40 min test intervals. It is of interest to note that MEL did not reduce the APO 1mg/kg (BM) increase in percentage of rats showing locomotion (see Fig. 3.2(b)).

MEL did, however, significantly reduce the increase in locomotion elicited by APO 2mg/kg (BM)(Fig 3.8(b)), in addition to reducing the percentage sniffing response (Fig 3.8(c)). With both these responses, there was a dose-dependent effect shown by MEL, up to the 40 min test interval. These results are in close agreement with those obtained from stereotypy rating scores.

The percentage gnawing and licking responses normally induced by APO 5mg/kg (BM) (Fig 3.2(f) and (g)) were significantly reduced by both doses of MEL, at all time intervals during which these behaviours were normally evidenced (Fig 3.9(d) and (e)). It is interesting to note, however, that at the 40 and 50 min test intervals percentage sniffing was increased significantly by both dosage levels of MEL (Fig 3.9(c)). This appears to confirm the earlier suggestion (Sec. 3.3.2.3) that in these studies the behavioural responses elicited by APO follows a pattern whereby with increased APO dose, and in the latter stages of the 60 min test period, percentage locomotion and sniffing become reduced as licking and gnawing responses become evident. This would seem to suggest that intensification of stereotypy in these studies is accompanied by the transition from one response (eg. sniffing) to another response (eg. licking or gnawing). By decreasing the observed occurrence of gnawing and licking while increasing that of sniffing, MEL appears to decrease the intensity of stereotypy, rather than inhibiting the induction of stereotype behaviour by APO.
FIGURE 3.7(a)-3.7(c) The effect of two doses of prior administration of MEL on the unconditioned behaviours induced by and the stereotypy score obtained with the administration of APO 1mg/kg (BM).

The boxes above the doses indicate the statistical significance (p < 0.05) of dose effects at the particular time intervals. Each point represents either the median stereotypy score or the percentage of rats (8 ≤ N ≥ 12) exhibiting a particular category of behaviour induced with APO 1 mg/kg (BM) at each 10 min interval of the 60 min test period, in the presence of MEL 0; 1 and 10 mg/kg (BM).

FIGURE 3.7(a) Median stereotypy scores obtained with APO 1 mg/kg (BM) in the presence of two doses of MEL.
FIGURE 3.7(b) Effect of two doses of MEL on the behavioural response category - Still induced by APO 1mg/kg BM.

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FIGURE 3.7(c) Effect of two doses of MEL on the behavioural response category - Sniff induced by APO 1mg/kg BM.
FIGURE 3.8(a)-3.8(d) The effect of two doses of MEL on the unconditioned behaviours induced by and the stereotypy score obtained with the administration of APO 2mg/kg (BM).

The boxes above the doses indicate the statistical significance (p < 0.05) of dose effects at the particular time intervals. Each point represents either the median stereotypy score or the percentage of rats (8 ≤ N ≥ 12) exhibiting a particular category of behaviour induced with APO 2 mg/kg (BM) at each 10 min interval of the 60 min test period, in the presence of MEL 0; 1 and 10 mg/kg (BM).

FIGURE 3.8(a) Median stereotypy scores obtained with APO 2 mg/kg (BM) in the presence of two doses of MEL.
FIGURE 3.8(b) Effect of two doses of MEL on the behavioural response category - Still induced by APO 2mg/kg BM.

FIGURE 3.8(c) Effect of two doses of MEL on the behavioural response category - Sniff induced by APO 2mg/kg BM.
FIGURE 3.8(d) Effect of two doses of MEL on the behavioural response category - Locomotion induced by APO 2mg/kg BM.
FIGURE 3.9(a)-3.9(d) The effect of two doses of MEL on the unconditioned behaviours induced by and the stereotypy score obtained with the administration of APO 5mg/kg (BM).

The boxes above the doses indicate the statistical significance (p < 0.05) of dose effects at the particular time intervals. Each point represents either the median stereotypy score or the percentage of rats (8 ≤ N ≥ 12) exhibiting a particular category of behaviour induced with APO 5 mg/kg (BM) at each 10 min interval of the 60 min test period, in the presence of MEL 0; 1 and 10 mg/kg (BM).

FIGURE 3.9(a) Median stereotypy scores obtained with APO 5 mg/kg (BM) in the presence of two doses of MEL.
FIGURE 3.9(b) Effect of two doses of MEL on the behavioural response category - Still induced by APO 5mg/kg BM.

FIGURE 3.9(c) Effect of two doses of MEL on the behavioural response category - Sniff induced by APO 5mg/kg BM.
FIGURE 3.9(d) Effect of two doses of MEL on the behavioural response category - Lick induced by APO 5mg/kg BM.

FIGURE 3.9(e) Effect of two doses of MEL on the behavioural response category - Gnaw induced by APO 5mg/kg BM.
3.3.4 The effects of light on APO induced behaviours.

Although there are great interspecies variations, in all mammalian species examined, pineal melatonin production and levels are maximal during the daily period of darkness [Reiter (1988)]. In the albino rat maintained under a regulated light dark cycle, peak pineal MEL levels are usually reached 4-5 hrs after the onset of darkness, with a gradual sustained drop thereafter, such that daytime levels of melatonin are achieved at about the time of "lights on" [Johnson et al (1982)]. In such rats, maintained under laboratory photoperiods, abrupt exposure to light during the dark period, when MEL levels are high, leads to a sudden and steep decline in the MEL content of the pineal gland [Rollag et al (1980) and Brainard et al (1982)]. The lowest irradiance of white light found to be capable of suppressing the pineal content of MEL in the albino rat is 0.0005 μW/cm², (normal room lighting being about 100-200 μW/cm²) [Webb et al (1985)]. In mammalian species, the half-time (t½) for the drop in MEL has been shown to be as short as 2 min in the cotton rat [Thiele et al (1983)], but generally about 8 min [Reiter et al (1983) and Rollag et al (1980)]. The drop in pineal MEL content appears to be an all-or-nothing phenomenon, and once the inhibition of MEL synthesis is initiated, it appears to go to completion before it can be reactivated [Reiter (1985)]. It does however appear that the reduction in pineal MEL is dependent on the wavelength of light, and a red light source (635-770nm) has been shown to be ineffective in reducing pineal MEL [Reiter (1983)].

Since the 24hr cycle of plasma MEL in mammals is eliminated following pinealectomy [Arendt (1986)], it is commonly assumed that the melatonin rhythm in the blood is a consequence almost exclusively of the rhythmic pattern of pineal MEL production and release [Reiter (1988)]. Once synthesised by the pineal gland, MEL is rapidly released, primarily, if not exclusively, into the vascular system [Reiter (1988)]. Since MEL in the blood of rats only has a half life of about 20 min [Gibbs and Vriend (1981)], it
appears reasonable to assume that all the above effects of light on pineal MEL production would be closely followed by similar effects on blood MEL levels, and thus also on brain MEL levels, since MEL in the blood is transported across the blood brain barrier [Pardridge et al (1980)].

In order to investigate a possible effect of endogenous MEL on central dopaminergic function, the effects of APO administration 5 hrs after the onset of darkness, on induced behaviours, was studied. The results of such observations were compared with the observation of rats which had been exposed to white light 5 hrs after the onset of the dark period. From all the factors discussed above, it was assumed that blood MEL levels in rats exposed to light would be significantly lower compared to those maintained in the dark.

All rats were maintained on a strictly regulated light cycle prior to testing. Observations in the dark were performed under low intensity red light. The rats which had been exposed to the light were also observed in darkness, since illumination of the test environment can be a powerful determinant of the magnitude of drug effect [Kinnard et al (1966)]. The rats were exposed to light for a 20 min period, rehabituated to the dark for 10 min, after which APO was administered and the 60 min behavioural test period begun.

The results obtained in this study are presented in Figs 3.10(a) - 3.10(d). The dose of APO tested (2mg/kg (BM)) only induced significant percentage increases in the sniffing and locomotion behavioural response categories in the dark. Exposure to light significantly increased the percentage of rats showing APO-induced sniffing during the first 40 min of the test period as compared to the rats not exposed to light (Fig 3.10(c)). Light also significantly increased APO-induced locomotion, but this was only at the 40 min test period, with a significant increase in locomotion being induced by darkness at the 10 min test interval. The percentage of rats exhibiting stillness was significantly
reduced by light at the 20, 30 and 40 min test intervals, thus indicating a general increase in activity as the result of exposure to light.

The median stereotypy score was significantly higher during the first 30 min of the test period in rats exposed to light as compared to respect to those retained in the dark.

These results indicate that, at the dose tested, APO induced behaviours are significantly increased during the middle of the dark period, when rats are exposed to light for 20 min. This is with the exception of locomotion which is decreased during the first 10 mins but significantly increased thereafter.

Since it was assumed that blood MEL levels in the rat retained in the dark would be higher than levels in the rats exposed to light, these results suggest that endogenous MEL may modulate the behavioural response of the central dopaminergic nervous system to APO. These results are in agreement with those obtained with exogenously administered MEL (Sec 3.3.3), where MEL decreased the behavioural response to APO. It is also of interest to note that APO induced behaviours during the dark period appear to be reduced in comparison to those induced by the same dose of APO administered 4-5 hours after the onset of the light period (Figs 3.1 and 3.2). During the light phase of the cycle, APO showed sniffing in almost 100% of the rats tested for almost the entire 60 min test period and median stereotypy score was between 3,5 and 4. These differences have, however, to be treated with caution, since test conditions (such as test environment illumination) were not the same.

These results are far from conclusive, since it would obviously be necessary to demonstrate the effects of light on the MEL levels in these rats under the specific test conditions used. It would also be necessary to rule out the effects of light on other neurotransmitter or hormonal systems in the rat, which may also
affect APO induced behaviours. However, coupled to the results obtained with exogenous MEL, it does appear that MEL reduces the behavioral response to the DA agonist APO, and thus may have a modulating function on central nervous system dopaminergic neurotransmission.
FIGURE 3.10(a)-3.10(d) The effect of exposure to light on the unconditioned behaviours induced by and the stereotypy score obtained with the administration of APO 2mg/kg (BM).

The boxes above the doses indicate the statistical significance (p < 0.05) of light conditions at the particular time intervals. Each point represents either the median stereotypy score or the percentage of rats (N = 12) exhibiting a particular category of behaviour induced with APO 2 mg/kg (BM) at each 10 min interval of the 60 min test period. L indicates rats exposed to light prior to testing and D represents rats retained in the dark.

FIGURE 3.10(a) Effect of prior exposure to light on the median stereotypy scores obtained with APO 2 mg/kg (BM).
FIGURE 3.10(b) Effect of prior exposure to light on the behavioural response category - Still, induced by APO 2mg/kg BM.

FIGURE 3.10(c) Effect of prior exposure to light on the behavioural response category - Sniff, induced by APO 2mg/kg BM.
FIGURE 3.10(d) Effect of prior exposure to light on the 
behavioural response category - Locomotion, induced by APO 
2mg/kg BM.
CHAPTER 4

LOCOMOTOR ACTIVITY STUDIES

4.1 INTRODUCTION

In the laboratory rat, increased motility, or locomotor activity, is one of the manifestations of DA agonist induced stereotype behaviour (Sec. 3.1). In general, unconditioned stereotype behaviours are very difficult to measure quantitatively and reliably, however locomotor activity is the exception. Locomotor activity is generally regarded as horizontal or lateral forward directed movements of the animal, such as walking or running and has for many years been mechanically and quantitatively measured [Review - Kinnard and Watzman (1966)]. Some of these methods include the use of: 1. photocell cages, 2. running wheels, 3. stabilimeters and jiggle cages, and 4. tilt cages [Review - Robbins (1977)].

Although many modifications of the photocell activity cage have been described in the literature, it has been used extensively as a "drug screening device", to determine the effects of psychoactive drugs on the activity of small animals [Kinnard and Watzman (1966)]. It operates on a photocell system, in which interruptions of one or more light beams produced by lateral movements of animals are converted to electrical impulses, which are transferred to digital or computerized counters. Locomotor activity of the animal is thus reflected as the number of light beam interruptions during a specified time period.

Maj et al (1972) demonstrated that photocell measured APO induced activity is a useful method for studying the role of dopaminergic mechanisms in motility. This study makes use of photocell cage measurement of locomotor activity to further investigate the
possible modulatory effects of MEL on central dopaminergic neurotransmission.

Locomotor activity was one of the criteria on which stereotype behavioural scores were allocated in Chapter 3 (Sec. 3.2.3), and was one of the behavioural response categories marked as present or absent in the occurrence observation method (Sec. 3.2.3). Robbins (1977) suggests that it is a "useful research strategy" to obtain and compare results using different modes of measurement in separate experiments, and that different methods of measuring activity may reflect different aspects of that activity. The results obtained in this study are thus compared with the results obtained in Chapter 3, where locomotor activity was assessed by a more subjective method of direct observation, in the occurrence observation method.

4.2 MATERIALS AND EXPERIMENTAL PROCEDURE

4.2.1. Animals

In all locomotor activity studies, male rats of the Wistar strain, with masses between 200 and 250g at time of testing, were used. Female rats were not used, since the activity of female rats fluctuates as a result of the estrus cycle [Robbins (1977)]. The use of male rats also eliminated the possibility of an interaction between administered MEL and the estrus cycle.

Prior to testing, rats were housed six to a cage, in a temperature controlled environment (21-23°C), on a regulated 12 hour light : 12 hour dark cycle). The rats had access to food and water ad libitum.

4.2.2 Apparatus

Locomotor activity was monitored in three photocell activity cages constructed by the Physics Department, Rhodes University.
The cages, black painted aluminium boxes measuring 500mm square by 200mm high, are mounted on 800 mm high bases, which carry the sensors and associated electronics. Activity is monitored by computer recording of the interruptions of infra-red (i-r) light beams produced by lateral movements of the animals in the cages.

The light sources comprise eight high power (243 mW/steradian), narrow beam (4 deg. half-angle), type ME124 i-r light emitting diodes, situated equidistantly (100 mm apart), in two adjacent walls of the cages, opposite to which are placed eight type MT2 broad band phototransistors. The effect of the light beams on behaviour is reduced by the use of i-r beams, to which the retina of the rat is relatively insensitive [Robbins 1977]. The use of i-r light beams also permits the use of the cages under conditions of normal ambient lighting or in total dark.

The transmitter-receiver pairs are so positioned that if a rat breaks a beam, a second rat moving past will break another circuit and thus be counted. Moreover since the computer only measures changes in the status of the beams, a rat sitting in front of a beam is only counted once when it enters and once when it leaves the beam.

When a rat interrupts an i-r beam, the voltage level at 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 are interrogated by the data-logger computer and the current beam status recorded. The three cages share a common data bus and are individually interrogated by the computer. The computer interrogates each cage about 30 times every minute, limiting the maximum rate of counting to about 30 counts every minute per cage.

The data logging program provides opportunity for the user to pre-test the state of individual beams, and the integrity of the computer to record breaks in individual beams. The program also
allows input by the user of information such as name, date and drug treatments, which allows easy identification of the data-set.

In order to allow monitoring of gross movements, as opposed to fine movement, the data-logging program can be instructed to only record breaks which are sustained for a certain time period. Following preliminary testing, coupled with direct observation of animals in the test cages, in all subsequent studies the data-logging program was set in such a way that all breaks needed to be sustained for 0.2 secs before they were eligible for recording.

In addition to the eight i-r beams which detect locomotor activity, the cages are also designed to measure exploratory behaviour. An array of nine holes in the cage floor (60 mm in diameter and placed 110 mm apart) provide an environmental stimulus to which the rats may react, head dipping into the holes being a measure of such exploratory behaviour [Sec. 3.2.2 and Bossier and Simon (1962)]. A set of six beams below the cage floors (three originating from each of two adjacent walls), are placed in such a way that a rat dipping it's head into any of the holes will break a beam. These beam breaks are counted and recorded separately as exploratory behaviour. APO induced behaviours are, however, usually characterized by a lack of head dipping [Ljungberg and Ungerstedt (1977a)], and as was discussed in Sec. 3.2.2, the hole count or head dipping is therefore only of indirect value in the analysis of APO induced behaviours. Only the locomotor activity and not the head dipping or exploratory activity data was used in these studies.

Data is recorded on disk at user selected intervals (10 min intervals in all subsequent studies) and appears as ASCII records of "upper" breaks (locomotor activity) and "lower" breaks (exploratory activity), together with the time at which the records were made and the identification code of the cage which supplied the data.
4.2.3 Procedure

The testing procedure was very similar to that described in Sec. 3.2.3 for the observation and rating of stereotype behaviours. The habituation periods prior to and on the day of testing remained the same, as did the schedule for the test drugs or control injection administrations. Whereas the rats had been individually observed in the previous studies, after preliminary studies using the photocell cages, it was decided to monitor the activity of groups of three rats per photocell cage, and for each drug treatment the results of 9 groups of three rats were used. Preliminary experiments showed marked differences in the individual baseline activity and reactivity of rats to the same drug treatments, and thus large variances in activity counts. The combined recording of the activity of three rats per activity cage greatly reduced this variance in counts. Although there are only nine samples per drug treatment (N=9), their combined result in effect reflects the mean activity of 27 rats (9 samples of 3 rats per cage).

In order to minimise the number of rats required in these experiments, rats were re-used in testing sessions, in a random manner, following a minimum of a two week drug-free "washout" period.

The photocell cages were housed in a separate room. The background noise level produced by ventilation fans (necessary to control temperature and humidity in the room) was 65-70 dB and this masked extraneous noise. The illumination level immediately outside the photocell cages was approx 1800 lux.

In order to eliminate the possible effect of the biological variable of mass on locomotor activity, the combined mass of the three rats per activity cage was always between 670 and 680 grams. In all experiments, rats were subjected to a one hour test period during which locomotor activity was recorded by the computer every 10 minutes.
Since three activity cages were used, in order to overcome the possible effect of differences in sensitivity between the cages, the various levels of each experimental variable or drug treatment were tested in each cage.

4.2.3.1 **Experiment to determine "baseline" locomotor activity and the effects of environmental habituation on the baseline.**

Initially, it was necessary to assess and ascertain a consistent and reliable level of activity shown by animals after control treatments. The procedure outlined above (Sec.4.2.3) was followed with the rats being administered the MEL injection vehicle (ip) 5 min prior to injection sc with saline (with ascorbic acid 1mg/ml) following a 25 min habituation period in the activity cages. In order to determine the effect of the habituation period, the procedure was repeated in another nine groups of rats, without allowing the rats the 25 min habituation time. Rats were injected with MEL vehicle and returned to their home cages. Five minutes later, they were injected with saline and immediately placed in the activity cages and the recording of activity commenced.

4.2.3.2 **Experiment to determine the dose - activity-response with increasing doses of APO.**

The activity response to APO was established with doses of APO 0.5; 1; 2 and 5 mg/kg BM. APO was prepared in saline, with ascorbic acid 1mg/ml as anti-oxidant. All APO injections were administered sc, in the flank.

4.2.3.3 **Experiment to determine the effects of MEL administration on the APO activity response.**

The effect of MEL (1 and 10mg/kg) administration, 5 min prior to APO administration, was established on each of the above doses of
APO and on the baseline activity response determined in Sec. 4.2.3.1.

MEL was prepared in the same vehicle described in Sec. 3.2.4 and was administered via the ip route.

4.2.3.4 Experiment to determine the effect of DA antagonists sulpiride and haloperidol on APO induced locomotor activity.

As a comparison to the effects of MEL administration on APO induced locomotor activity, the effects of two DA antagonists, sulpiride (75 mg/kg (BM)) and haloperidol (5mg/kg (BM)) on the activity responses to APO (1mg/kg (BM)), in the presence and absence of MEL (1mg/kg (BM)), were determined.

Both sulpiride and haloperidol were administered in the same manner as MEL, ip, 5 min prior to APO administration, together with either MEL vehicle or MEL (1mg/kg (BM)). Sulpiride (Eglonyl® - Noristan) and haloperidol (Serenace® - Searle) ampoules for human use were used.

4.2.3.5 Experiment to determine the effects of exposure to light on APO induced activity in rats during the dark phase.

In an effort to determine the possible effects of endogenous MEL on central dopaminergic function, the effects of APO (1 mg/kg BM) on locomotor activity was determined 5½ hrs after the onset of darkness. The results of this study were compared to the locomotor activity data obtained from rats which had been exposed to white light for a 20 min period, 5 hrs after the onset of the dark period, re-habituated to the dark for 10 min and then administered APO (1mg/kg BM).

All rats were maintained on a strictly regulated lighting cycle
prior to testing and observations in the dark were performed under low intensity red light.

4.2.4 Statistical analysis

Robbins (1977) suggests that data such as frequency of photocell counts is best analyzed by parametric tests involving analysis of variance and Student-t test. In these studies, dose response effects involving more than two dosage levels, and time response effects, were analysed by use of analysis of variance with post hoc Scheffe multiple range comparison tests. Drug and dose effects involving only two comparisons were analysed using the Student-t test.

In the graphical presentation of results (Figs. 4.2 - 4.7), symbols such as *, • and I, are used to represent the statistical significance of results. In each graph, at each 10 min time interval, the results obtained for each dosage level or experimental variable are analysed with respect to all other dosage levels or experimental variables, using analysis of variance. A statistical difference is indicated by the absence or presence of one or more of the above symbols. The presence of the same symbol at two or more of the dosage levels, at any of the 10 min time intervals, indicates no significant difference between the response, at that time interval, to the dosages, whereas different symbols indicate a statistically significant difference in the response.

The same method is used to represent statistical analysis in all locomotor activity graphs in this chapter and, unless otherwise stated, a p value of p < 0.05 was considered as statistically significant.
4.3 RESULTS AND DISCUSSION

4.3.1. Baseline locomotor activity and the effects of habituation on the baseline.

Preliminary experiments, involving the monitoring of locomotor activity of one rat per activity cage, revealed marked differences in the individual baseline activity of individual rats. These individual differences were largely overcome by the use of three rats per activity cage.

A baseline of locomotor activity was obtained following a 20 min habituation period, with mean beam break counts ranging between 87 and 106 per 10 minutes over the one hour test period (Fig. 4.1). The mean counts at each of the 10 min time intervals were not significantly different with respect to each other.

Control activity measured without an habituation period decreased significantly with respect to time, after the first twenty minutes and again after thirty minutes. Baseline activity then stabilised for the last 30 minutes of the test period. For the first 30 minutes, baseline activity was significantly increased with respect to activity counts in rats which had been habituated to the cages. During the last 30 minutes, baseline activity of un-habituated rats did not differ significantly with that of habituated rats. (Fig. 4.1)

In all further experiments, prior to the testing period, rats were habituated to the cages for a total of 30 mins (25 min before and 5 min after MEL administration). The habituation period was important, since it allowed the rats time to explore the cages before the experimental drug was administered. The baseline level of activity was then reduced and consistent, allowing for a more reliable measurement of experimental drug induced locomotor activity.
FIGURE 4.1 The locomotor activity time-response curves of control rats with and without an habituation period.

Nine groups of rats (3 per group) were used for each experimental condition.

The effect of the two experimental conditions (with/without an habituation period), were analysed at each 10 min time period using the Student-t test (* p < 0.005).
4.3.2 The dose activity response with increasing doses of APO.

APO does not exert a simple effect on locomotor activity and conflicting reports on its effect have been published. Some investigators report that, depending on dose, APO induces an increase or decrease in locomotor activity, while other investigators report that it does not do so [Review - Ljungberg and Ungerstedt (1977a)]. Ljungberg and Ungerstedt suggest that "differences in terminology, experimental procedures", and the use of different types of "conventional non-descriptive activity boxes" account for the discrepancies between reports.

It was therefore necessary to obtain a full dose-response profile for APO in the activity cages used in these studies, before the effects of MEL on APO induced locomotor activity could be studied.

APO was shown to induce locomotor activity in a complex time-dose response manner. All the results are presented in Fig. 4.2. APO 0.5 mg/kg (BM) increased activity with respect to the control at the 20 and 30 min test intervals, while significantly reducing activity during the 50 min test interval. This inhibition of locomotion by APO 0.5 mg/kg (BM) at the 50 min test interval may be related to stimulation of DA autoreceptors, that are located presynaptically [Koller and Herbster (1988)].

At the other dosage levels tested, APO increased activity with respect to controls for at least the first 3 test intervals (10, 20 and 30 min). However, activity was maximally increased by a dose of 1 mg/kg (BM), which was significantly greater than the increase due to doses of 2 and 5 mg/kg (BM) during the first 4 test intervals. APO 1 and 2 mg/kg (BM) significantly increased activity with respect to controls throughout the one hour test period, whereas 5 mg/kg (BM) failed to significantly increase activity at the 40 and 50 min test intervals.

These results are in close agreement with the results obtained from
the direct occurrence observation method in Chapter 3 and presented in Fig. 3.2(b), although highest levels of locomotion in these results are seen with APO 1 mg/kg (BM), as opposed to 2 mg/kg (BM) in Chap. 3. The results obtained by the occurrence observation method merely reflect the number of rats observed to be exhibiting locomotion at the various 10 min time intervals, allowing for no continuous method of recording activity and no distinction between intensification or speed of locomotor activity. In the activity cages, it is presumed that an increase in intensification or speed of locomotion is accompanied by a corresponding increase in the number of beam breaks.

The low response in activity obtained with APO 5 mg/kg BM may be due to the induction of other stereotype behaviours, such as licking and gnawing evidenced in Chapter 3 (Fig. 3.2 (f) and (g)), with a concomitant reduction in locomotor or whole body activity. The design of the activity cages in this study does not allow for the simultaneous expression of locomotion and gnawing responses (Discussion - Sec. 3.3.2.3).
FIGURE 4.2 The locomotor activity time-response curves for increasing doses of APO.

\[ p < 0.0002 \text{ at all time intervals and points represent the means of samples } N=9 \pm \text{SEM}. \]
4.3.3 The effect of prior MEL administration on APO induced activity.

The results for the effect of prior MEL administration on control and APO induced locomotor activity are presented in Figs. 4.3 (a) - 4.3 (e).

MEL 1 and 10 mg/kg (BM) significantly reduced baseline activity during the first 10 min test period in a non-dose dependent manner, with both dosages decreasing activity to the same level. With the exception of the 40 min test interval, where MEL 1 mg/kg administration decreased activity, both doses of MEL failed to decrease baseline activity after the 10 min interval.

The influence of MEL and the pineal gland on locomotor activity of various animal species has been extensively investigated. Conflicting results, however, suggest a complex relationship between pineal activity, MEL and locomotor activity.

Early studies, investigating the effect of pinealectomy or exogenous MEL administration to rats, suggested that pineal function in the rat decreased baseline levels of motor activity [Reiss et al (1963), Wong and Whiteside (1968) and Karppanen et al (1973)]. Later contradictory results [Remley et al (1969), Relkin (1970a, 1970b), Quay (1965, 1968, 1970a), Kastin et al (1973) and Kovács et al (1974)], coupled with the fact that rats are nocturnal animals exhibiting highest locomotor activity at night, when pineal activity and in particular MEL secretion is highest, suggest that the pineal gland per se does not exert direct control over motor activity. It would, however, appear that MEL and the pineal gland have a modulatory control over locomotor activity, particularly as regards the activity of nocturnal animals in response to changing photoperiods. Kincl et al (1970) propose that the pineal gland acts as a "brake" in preventing rapid adjustments in motor activity of nocturnal animals to light changes. Quay (1970a), in response to similar observations, suggests that the
possibility should be considered that "other kinds of environmental disturbances, and not just light, may have central and circadian effects mediated by way of the pineal".

The results presented here suggest that MEL may have a small inhibitory effect on baseline locomotor activity during the first 10 minutes of the test period. The preliminary study to determine the baseline activity of rats not habituated to the test environment showed that it was during the fourth test period, between 30 and 40 min, that activity levels stabilised, reaching those of habituated rats (Fig. 4.1). Although there was no statistically significant difference in activity during the 10 min test intervals in rats habituated to the test environment for 30 min prior to testing, activity levels at 10 and 20 min were highest (Fig. 4.1). MEL administration decreased the initial raised activity levels, suggesting that MEL may be acting in a modulatory capacity to stabilise baseline activity of rats in the photocell activity cage test environment.

The results of MEL administration on APO induced activity are complex. Locomotor activity induced by all doses of APO tested was significantly decreased by MEL during some stage of the one hour test periods and by one or both of the doses of MEL tested (1 and or 10 mg/kg (BM)).

Only during the 20 min test interval, the interval in which APO 0.5 mg/kg (BM) induced activity was highest, was activity induced by APO 0.5 mg/kg (BM) administration decreased in a dose dependent manner by prior administration of MEL 1 and 10 mg/kg (BM) (Fig. 4.3(b)).

The administration of MEL (1 and 10 mg/kg (BM)) significantly inhibited the locomotor activity induced by APO 1mg/kg (BM) (Fig. 4.3(c)). During the 10 and 20 min test intervals, activity levels were most reduced with the administration of 1 mg/kg (BM) MEL. During the remainder of the test period there appeared to be no MEL
dose-dependent response, with both doses of MEL tested reducing the APO induced locomotor activity to the same extent.

MEL 10 mg/kg (BM) administration appears to more efficient than the administration of MEL 1 mg/kg (BM) in reducing the activityresponse of rats challenged with APO 2 and 5 mg/kg (BM) (Fig. 4.3(d) and (e)). The higher dose of MEL inhibited the induction of activity by doses of 2mg/kg (BM) APO during the 10, 20, 50 and 60 min test intervals, while the lower dose of MEL was only effective in the later stages of the test period, at 50 and 60 min. During the 30 and 40 min intervals, when the response to APO 2mg/kg was lowest, MEL had no effect on activity. MEL 10 mg/kg, inhibited the response to APO 5 mg/kg throughout the test period, while MEL 1mg/kg only slightly decreased the response during the last (60 min) test interval.

In Chapter 3, using the occurrence observation method, prior MEL administration only significant decreased the occurrence of APO 2mg/kg BM induced locomotion (Fig. 3.8 (d)). Using the occurrence observation method, the percentage of rats exhibiting locomotion was, however, highest with APO 2mg/kg (BM) administration (Fig. 3.2 (b)), as opposed to data obtained from photocell activity cages which indicated that APO 1mg/kg (BM) administration raised activity levels most (Fig. 4.2). In Chapter 3, it was suggested that MEL, rather than inhibiting APO induced stereotype activity, modified the intensity of the response. The results in this study, in which the "intensity" of the locomotor activity response was measured, as opposed to merely the presence or absence of the response (as in Chapter 3), may thus be viewed as support for the concept that MEL modulates the response of the central nervous system to APO induced activity.

These results further support the concept that MEL, and in particular the pineal gland, has a homeostatic role in modulating the effects of the central nervous system and more specifically dopaminergic neurotransmission to environmental disturbances.
FIGURES 4.3 (a)-(e) The effect of prior MEL (1 and 10mg/kg BM) administration on control and APO (0.5, 1, 2 and 5mg/kg (BM)) induced locomotor activity.

All points represent the mean ± SEM of N=9 groups of three rats. In all graphical presentations, at each 10 min test interval the results for all treatment schedules are analysed with respect to each other using analysis of variance and Scheffe multiple range tests. A statistical difference between groups at each 10 min test interval is indicated by the presence or absence of one or more symbols, as explained in Sec. 4.2.4. (p < 0.05).

FIGURE 4.3(a) The effect of MEL (1 and 10 mg/kg (BM)) administration on control or baseline locomotor activity.

* * *

The figure shows a graph with the following key:

- CONTROL
- MEL 1mg/kg
- MEL 10mg/kg

The x-axis represents time in minutes, ranging from 0:00 to 1:10. The y-axis represents the mean number of beam breaks, ranging from 40 to 160.
FIGURE 4.3(b) The effect of prior MEL (1 and 10 mg/kg (BM)) administration on APO 0.5mg/kg (BM) induced locomotor activity.
FIGURE 4.3(c) The effect of prior MEL (1 and 10 mg/kg (BM)) administration on APO 1 mg/kg (BM) induced locomotor activity.
FIGURE 4.3 (d) The effect of prior MEL (1 and 10 mg/kg (BM)) administration on APO 2 mg/kg (BM) induced locomotor activity.
FIGURE 4.3 (e) The effect of prior MEL (1 and 10 mg/kg (BM)) administration on APO 5 mg/kg (BM) induced locomotor activity.
4.3.4 The effect of DA antagonists, sulpiride and haloperidol, on the locomotor activity induced by administration of APO 1mg/kg (BM).

Early studies suggested that D₂ receptors were the principal mediators of dopaminergic behaviours [Reviews - Seeman (1980), Creese et al (1983) and Joyce (1983)] and the D₁ receptor was dismissed as a "receptor in search of a function" [Braun et al (1986)]. In more recent studies, the D₁ receptor agonist SKF 38393 has been shown to induce behaviours such as grooming, sniffing and locomotion [Molloy and Waddington (1984 and 1985)] and to enhance APO induced stereotypy [Koller and Herbster (1988)], in a dose-dependent manner. The selective D₁ antagonist SCH 23390 has been shown to inhibit behavioural responses to both APO and amphetamine administration [Mailman et al (1984) and Christensen et al (1984)] as well as behaviours induced by the selective D₂ agonist RU 24213 [Pugh et al (1985)]. Data such as this suggests that, underlying the expression of DA agonist related behaviours, may be a functional interaction between D₁ and D₂ receptor subtypes [Braun et al (1986) and Braun and Chase (1986)].

For comparative purposes, the effect of prior administration of DA antagonists, sulpiride and haloperidol, on APO induced locomotor activity was investigated. The combined effects of MEL 1 mg/kg (BM) and sulpiride and or haloperidol on APO induced activity was also determined.

Sulpiride is a D₂ selective antagonist, while haloperidol is a mixed acting antagonist at D₁ and D₂ receptors. The effects of single doses of sulpiride (75 mg/kg), haloperidol (5 mg/kg), and MEL (1 mg/kg) on the locomotor activity induced by APO (1 mg/kg) are presented in Fig. 4.4, and the effects of MEL (1 mg/kg) / haloperidol (5mg/kg) and MEL (1 mg/kg) / sulpiride (75 mg/kg) combinations on APO induced activity are presented in Fig. 4.5 and 4.6 respectively.
Haloperidol with its potent $D_1$ and $D_2$ activity had a marked effect on locomotor activity, inhibiting APO induced locomotion, and reducing activity to a level far below the baseline levels presented in Fig. 4.1. MEL administration had no effect on the haloperidol inhibition of APO induced activity (Fig. 4.5).

Sulpiride inhibited the induction of locomotion by APO, but did not reduce activity levels to the same extent as haloperidol. The inhibition of APO induced activity by sulpiride was only slightly greater than that due to MEL administration (Fig. 4.4). Co-administration of MEL 1mg/kg (BM) and sulpiride 75 mg/kg (BM) decreased the inhibition of APO induced activity evidenced with either drug alone during the first 20 min of the test period. Thereafter, the inhibition of activity was the same as that demonstrated with MEL, but less than that with sulpiride alone. It appears that MEL inhibits the inhibitory effects of sulpiride on APO induced activity.

The results of this study must be interpreted bearing in mind the limitations that only one dose of each of the test substances, haloperidol and sulpiride, was used and the results compared with the maximum inhibition seen with the doses of MEL used (1 and 10 mg/kg (BM)).

The results do, however, suggest that if the antagonistic activity of MEL is operative via a DA system or mechanism, either (1) MEL does not antagonise mechanisms mediated by both $D_1$ and $D_2$ receptors but only antagonises the activity of APO on either $D_1$ or $D_2$ receptor mediated mechanisms, or (2) MEL only weakly antagonises a system mediated by both DA receptor subtypes. It appears, however, that MEL's antagonistic activity is mediated via inhibition of $D_2$ receptor mediated mechanisms rather than those mediated by $D_1$ receptors, since MEL inhibited the $D_2$ receptor mediated inhibitory effect of sulpiride. The antagonist effect of MEL is possibly physiological, rather than a competitive antagonism at the DA receptor level.
Other neuromodulator systems and receptors, such as noradrenaline, serotonin, GABA and N-methyl-D-aspartate (NMDA), are known to influence locomotor activity [Swonger and Rech (1972), Svensson (1971), Carlsson and Carlsson (1989)], and this study does not exclude the possibility that MEL may be acting via one of these pathways to modify the APO induced locomotor response, rather than via a direct DA receptor mechanism.
FIGURE 4.4 The effect of sulpiride (75 mg/kg (BM)), haloperidol (5 mg/kg (BM)) and MEL (1 mg/kg (BM)) on APO (1 mg/kg (BM)) induced locomotor activity.

All points represent the mean ± SEM of N=9 groups of three rats. Drug treatments were analysed, with respect to each other, at each 10 min test interval, using analysis of variance and Scheffe multiple range testing. Statistically significant differences between groups is represented by the presence or absence of one or more symbols, as explained in Sec. 4.2.4. (p < 0.05).
FIGURE 4.5 The effect of prior administration of haloperidol (5 mg/kg (BM)) in combination with MEL (1 mg/kg (BM)) on APO (1 mg/kg (BM)) induced locomotor activity.

All points represent the mean ± SEM of N=9 groups of three rats. Drug treatments were analysed, with respect to each other, at each 10 min test interval, using analysis of variance and Scheffe multiple range testing. Statistically significant differences between groups is represented by the presence or absence of one or more symbols, as explained in Sec. 4.2.4. (p < 0.05)
FIGURE 4.6 The effect of prior administration of sulpiride (75 mg/kg (BM)) in combination with MEL (1 mg/kg (BM)) on APO (1 mg/kg (BM)) induced locomotor activity.

All points represent the mean ± SEM of N=9 groups of three rats. Drug treatments were analysed, with respect to each other, at each 10 min test interval, using analysis of variance and Scheffe multiple range testing. Statistically significant differences between groups is represented by the presence or absence of one or more symbols, as explained in Sec. 4.2.4. (p < 0.05).
4.3.5 The effect of exposure to light on locomotor activity induced by the administration of APO 1mg/kg (BM) during the dark phase.

APO induced locomotor activity is significantly higher in rats exposed to white light during the middle of the dark period in comparison to rats maintained in complete darkness throughout the dark period (Fig. 4.5). The baseline activity of rats exposed to and not exposed to light does not differ significantly.

This suggests that the results evidenced with exogenous MEL in this chapter are not purely pharmacological, but may have physiological significance. Since exposure to light during the middle of the dark phase results in a rapid reduction of raised night-time MEL levels [Rollag et al (1980), Brainard et al (1982)], rats maintained in the dark would have higher endogenous circulating MEL levels than rats exposed to light, suggesting that reduced endogenous MEL would be responsible for the higher locomotor activity levels induced by APO in rats exposed to light.

Although no direct comparison can be made since environmental illumination can alter the magnitude of drug response [Kinnard and Watzman (1966)], it is of interest to note that the locomotor response to APO 1mg/kg (BM) administration in rats exposed to light is comparable with that of rats tested during the light phase (Fig. 4.2), while the response of rats maintained in darkness is lower. Interpretation of these results is also subject to the restrictions discussed in Sec. 3.3.4, specifically, the effect of light on other neurotransmitter or hormonal systems has not been excluded and the effect of light on circulating MEL levels in these rats under these specific test conditions has not been demonstrated.

Bearing these restrictions in mind, the results obtained in this study support those obtained with exogenous MEL, giving impetus to the concept of MEL and the pineal gland as modulators of central dopaminergic neurotransmission.
FIGURE 4.7 The effect of exposure to light on the locomotor response to APO (1mg/kg (BM)) administration during the dark phase.

All points represent the mean ± SEM of N=9 groups of 3 rats. The effects of APO administration under both experimental conditions were analysed, with respect to each other and to the 2 groups of control rats, at each 10 min test interval, using analysis of variance and Scheffe multiple range testing. Statistically significant differences between groups is represented by the presence or absence of one or more symbols, as explained in Sec. 4.2.4. (p < 0.05).
CHAPTER 5

ROTATIONAL BEHAVIOURAL STUDIES

5.1 INTRODUCTION

An animal model, which has proved to be a useful tool for studying the effects of drugs on central dopaminergic neurons, was initially suggested by Anden et al (1966), and greatly popularized by Ungerstedt and Arbuthnot (1970), following the demonstration that it could be easily quantified.

This rotational method involves the unilateral lesion of the nigrostriatal system by administration of 6-hydroxy-dopamine (6-OHDA) into the DA cell group in the substantia nigra, or into the ascending bundle of DA axons. 6-OHDA is a neurotoxic chemical analogue of the catecholamines and results in degeneration of virtually all DA cell bodies and the entire nigrostriatal DA pathway within 48hrs [Ungerstedt et al (1973) and Ungerstedt (1971)]. This unilateral degeneration of the nigrostriatal system results in the development of receptor supersensitivity in the ipsilateral striatum [Ungerstedt (1971), Van Voigtlander and Moore (1973) and Costall and Naylor (1975)]. Such a concept is supported by behavioural [Mishra et al (1980)], receptor binding [Creese et al (1977) and Heikkila et al (1981)] and ionophoretic [Siggins et al (1974)] studies.

The administration of direct-acting DA receptor agonists, such as APO, to rats lesioned in this way results in contralateral circling behaviour [Review - Glick et al (1976)]. Indirectly acting DA agonists, such as amphetamine, which act by causing endogenous DA release from intact neurons, are most active on the unlesioned or intact side and result in ipsilateral rotation [Ungerstedt (1973)].
A dominance of DA receptor activity in the striatum on one side forces the rat to rotate towards the opposite side, and the rotational behaviour reflects a comparison between the effect of the drug on the innervated and denervated sides. The speed, duration and direction of rotation is a relative indication of the difference between the amount of DA activity on the two sides, and not just the effect on one side (Ungerstedt (1973) and Ungerstedt (1971)]. Costall et al (1983) suggest that a unilateral striatal lesion produced by 6-OHDA results in reduced functional dopaminergic activity in the opposing striatum, which facilitates the contralateral response initiated by supersensitive mechanisms in the denervated striatum.

APO induced turning behaviour in unilaterally lesioned rats can be manually [Glick et al (1976)] or automatically [Ungerstedt and Arbuthnott (1970)] recorded and quantified. Turning is expressed as the net total turns during a specified time period, which is obtained from the algebraic sum of the contralateral turns (positive) and the ipsilateral turns (negative) [Rouillard and Bédard (1988)].

This quantification of behaviour related to the function of the nigrostriatal dopaminergic system can be used to study several aspects of DA function in the striatum. Its primary use is in the evaluation of pre- or post-synaptically acting dopaminergic agonists or antagonists [Ungerstedt et al (1973)].

Useful as this model appears to be, it does have limitations which need to be considered:
1. Despite the relative simplicity of the rotatory response, variations in methodology, from laboratory to laboratory, may alter the kind and or magnitude of rotational data obtained [Glick et al (1976)]. Such variations include the exact site and method of 6-OHDA administration, the delay period before testing, the environment, in which testing takes place and the sex, strain and species of animals.
2. It would be incorrect to assume that the rotation in this model is due only to the nigrostriatal system and that DA agonists and antagonists act only on dopaminergic mechanisms in the area of the nigrostriatum. Ungerstedt (1971) showed that 6-OHDA administration to the substantia nigra also resulted in partial denervation of the olfactory tubercle and nucleus accumbens and results published by Kelly and Moore (1976) demonstrate that DA agonist induced rotation is markedly modified by concomitant activity at mesolimbic DA receptors.

3. Although it appears that the dopaminergic system is the primary system involved, rotation does not solely reflect the activity of a dopaminergic system. Other neurotransmitter pathways mediated by noradrenaline [Pycock et al (1975)], serotonin [Costall and Naylor (1975), Fleisher and Glick (1975)] acetylcholine [Glick et al (1974)] and GABA [Dray et al (1975)] appear to have modulatory roles, suggested by the fact that treatments which inhibit or damage these pathways enhance the rotatory effects of dopaminergic agonists.

4. A paradoxical "undrugged" contralateral rotation has been noted by several authors [Ungestedt (1971); Silverman and Ho (1981); Carey (1988)]. Brief exposure of unilaterally lesioned rats to an environment associated with APO induced contralateral rotation is capable of inducing contralateral rotation without drug treatment. This response has been shown to be latent [Silverman (1988)], developing two weeks after treatment and appears to be the property of direct but not indirect agonists [Silverman (1988)]. This response is thought to result from conditioned or associative learning [Burunat et al (1988)], with specificity for the environment in which the direct agonist had previously been administered. It has therefore been suggested that the use of APO to screen lesions results in a "changed experimental animal" [Silverman (1988)]. This paradoxical behaviour can be extinguished by regular handling of the experimental animals and through daily undrugged testing [Silverman (1988)].
Rouillard and Bédard (1988) and Herra Marschitz and Ungerstedt (1985) suggest that contralateral circling in this model may be mediated by differential actions on $D_1$ and $D_2$ receptor sites or mechanisms. In this supersensitive receptor model, denervation unmasks or allows some effect of $D_1$ and $D_2$ receptors not manifested in the intact structure [Rouillard and Bédard (1988)]. Either $D_1$ or $D_2$ agonists alone, are capable of inducing rotation, while selective antagonists alone are not capable of inhibiting the behavioural response elicited by stimulation of the complementary receptor. In a normosensitive receptor model, $D_1$ and $D_2$ receptor interaction is obligatory to induce a behavioural response which both $D_1$ and $D_2$ selective antagonists alone are highly potent in antagonizing [Braun et al 1986].

Notwithstanding these limitations, this model remains useful for screening potential dopamine agonists and antagonists and as a primary method for investigating central dopaminergic function. It was used in these studies to further investigate the concept that MEL may have a modulatory role on dopaminergic central nervous system function. It is, however, used in conjunction with a rotational behaviour - a normosensitive receptor model - the quinolinic acid (QA) lesioned rat.

In this second model, behavioural effects of DA receptor activation were studied in rats with unilateral striatal lesions induced by the excitotoxin QA. This toxin, which resembles kainic acid, destroys the striatal neurons, while sparing the axons projecting into the striatum [Schwarcz et al (1982)]. Two weeks after lesioning, administration of DA agonists such as APO results in an ipsilateral rotational response [Braun et al (1986)]. This response is attributed to an unbalanced stimulation of the two striata, without involving the phenomenon of DA receptor supersensitivity [Braun et al (1986)]. This model allows observation of dopaminergic actions and interactions at normosensitive receptors and the obligatory nature of the $D_1$ and $D_2$ receptor interaction is not uncoupled or unmasked as it is in the supersensitive receptor model - the 6-OHDA lesioned rat.
rat [Braun et al (1986)].

These two models of central dopaminergic activity are represented schematically in Fig. 5.1.(a) and (b).

**FIGURE 5.1(a) A schematic illustration of the 6-OHDA rotational model.**

A stereotaxic injection of 6-OHDA into the pars compacta zone of the substantia nigra induces a unilateral lesion that denervates the DA - innervated striatum unilaterally. APO administration induces contralateral rotation acting on postsynaptic DA receptors that become supersensitive after destruction of the DA innervation. Degeneration due to lesioning is indicated by shading. [Herrera-Marschitz and Ungerstedt (1984a)].
FIGURE 5.1(b) A schematic illustration of the QA rotational model.

A stereotaxic injection of QA into the left corpus striatum results in destruction of the striatum. APO administration results in ipsilateral rotation by acting on normosensitive receptors on the intact side. Degeneration due to lesioning is indicated by shading. (Modified from Herrera-Marschitz and Ungerstedt (1984a)).
5.2 MATERIALS AND EXPERIMENTAL PROCEDURES

5.2.1. Animals

Male Wistar rats, weighing 140 - 160 g at the time of the stereotaxic injections, were used in all experiments. At the time of subsequent rotation experiments, the rats had acquired a mass of 225 - 275 g. The animals were housed 4 in each cage prior to stereotaxic procedures and individually thereafter. They were maintained in a temperature controlled environment (21-23 °C), on a regulated 12 hour light : 12 hour dark light cycle. The rats had access to food and water ad libitum.

5.2.2 Surgical Procedures

5.2.2.1 Unilateral 6-OHDA-denervation

Rats were anaesthetized by ip administration of sodium pentobarbital (Sagatal® - May and Baker) 42 mg/kg (BM) and placed in a stereotaxic frame. The rat skull was orientated according to the König and Klippel stereotaxic atlas [König and Klippel (1963)]. After a sagittal cut in the skin of the skull, the bregma suture was located and, 4.6 mm posterior to the bregma and 2.0 mm lateral to the midline suture, a 2 mm diameter hole was drilled with an electrical trepan drill. Care was taken not to lesion the meninges. A Hamilton syringe with a cannula of diameter 0.3 mm was used to inject 8 μg of 6-OHDA-HCl (calculated as base, SIGMA) in 4 μl of ice cold saline (with 0.2% ascorbic acid as antioxidant) 7.4 mm below the brain surface. The injection was administered at a rate of 1 μl per minute and the cannula was left in situ for a further 3 min following drug injection, to allow for passive diffusion away from the cannula tip and to minimise spread into the injection tract. The cannula was then slowly removed and the scalp was closed with sutures. Animals were kept warm until recovery from anaesthesia.
5.2.2.2 Unilateral striatal QA lesion

In general, the method was the same as that outlined in Sec. 5.2.2.1. Rats received two stereotaxic injections of 1 μl of 150 nanomolar solution of QA (SIGMA), prepared in ice cold saline, into the left striatum (coordinates: 1.0 mm anterior from bregma, 2.3 mm lateral and 4.5 mm vertical, and 3.8 mm lateral at the bregma level and 4.5 mm vertical.)

5.2.2.3 Sham lesioned rats

Rats to be used as controls were subjected to the surgical procedures outlined in Secs. 5.2.2.1 and 5.2.2.2. However, stereotaxic injections into the brain regions in both cases were free of the drugs 6-OHDA and QA and comprised only the vehicle - saline and ascorbic acid.

5.2.3 Observation of rotational behaviour

Rotational behaviour was observed manually in a flat bottomed, circular plastic bowl (diameter: 800mm). An observer recorded the number of complete 360° turns, either wide or tight head to tail pivotal turns, made by the rats in a pre-specified time period. All animals were placed in the test environment for a 30 min habituation period prior to drug administration and observation of turning behaviour.

The total number of complete 360° turns was recorded, with contralateral turns being recorded as negative and ipsilateral turns as positive. Circling was expressed as net total turns, which was obtained from the algebraic sum of the contralateral and ipsilateral turns.
5.2.4 Behavioural evaluation or confirmation of lesioning

In order to select the successfully lesioned animals, rats were challenged with low doses of APO (0.5 mg/kg (BM)) two weeks after lesioning. Only rats which demonstrated vigorous (at least 3 turns per minute) and reliable turning were selected for further use in experiments. Successfully 6-OHDA lesioned rats showed contralateral rotation and successfully QA lesioned rats showed ipsilateral turning. Only about 60% of all rats lesioned showed acceptable turning behaviour when challenged with APO.

5.2.5 Preliminary rotational behaviour experiments

5.2.5.1 Determination of the time course for the development of turning behaviour in lesioned rats

A group of 12 rats were challenged, after 6-OHDA induced lesioning, on postoperative days 2, 6, 10, 20 and 60 with APO 1 mg/kg administered sc in the flank and observed for turning behaviour. Only data obtained from rats which developed a turning response were used as an indication of the time course for the development of turning behaviour.

The experiment was repeated with a group of QA lesioned rats.

5.2.5.2 Determination of the dose dependency of the APO induced turning response in lesioned rats.

A group of 6 rats which had been successfully screened for 6-OHDA induced lesioning two weeks post lesioning with APO 0.5 mg/kg (BM), was used to determine APO dose response turning behaviour. The rats were tested randomly (2 rats per dose per test day) at postoperative days 21, 28, and 35 for turning response to a
challenge with APO 1, 2 and 5 mg/kg (BM).

The same procedure was repeated with a group of QA lesioned rats.

5.2.5.3 Determination of the onset and duration of the APO induced turning response in lesioned rats.

Two groups of 4 rats - one group being 6-OHDA lesioned rats and the other QA lesioned rats, which had been successfully screened for turning behaviour 14 days post-operatively, were challenged with APO 1mg/kg (BM) and observed for rotational behaviour, 21 days post-operatively. Turning behaviour was recorded for each 2 minute test period and recording continued until turning had virtually ceased, usually about 60 min post injection time.

5.2.5.4 Determination of a possible undrugged "paradoxical" response.

A group of 5, 6-OHDA lesioned rats were challenged with a sc administered injection of saline 28 days post-operatively, 14 days after they were screened for turning behaviour with APO 1mg/kg (BM), and observed for any rotational behaviour.

5.2.6 Experiments to determine the effect of MEL administration on APO induced turning behaviour.

The effect of ip administered MEL (1 and 10 mg/kg (BM)), 5 min prior to challenge with APO 1 mg/kg (BM), on APO induced turning behaviour was observed, and recorded in groups of 5 and 7, 6-OHDA and QA lesioned rats respectively. Rotation was recorded as the net total turns (Sec. 5.2.3) during the 20 min period, beginning 5 min post APO administration. All rats were tested for response to APO 1 mg/kg 21 days after lesioning and the effect of MEL 1 and 10 mg/kg on this response was determined in a cross over study at 28 and 42
days after lesioning.

Rotational behaviour was observed in these experiments by an independent observer denied knowledge of drug treatment.

Control experiments included:
1. the effect of MEL on lesioned rats in the absence of APO
2. the effect of APO 1mg/kg (BM) on sham operated rats
3. the effect of APO 1mg/kg (BM) on unoperated rats.

5.2.7 Experiment to determine the effects of environmental lighting on APO induced rotation in 6-OHDA lesioned rats.

In order to investigate a possible effect of endogenous MEL on central dopaminergic function, the effects of APO (1 mg/kg (BM)) administration, 5½ hours after the onset of darkness, on rotational behaviour in 6-OHDA lesioned rats was studied (Refer to Sec. 3.3.4). The results of such observations were compared to the data obtained from rats which had been exposed to white light for a 20 min period, 5 hrs after the onset of the dark period, rehabituated to the dark for 10 min and then challenged with APO.

All rats were maintained on a strictly regulated light cycle prior to testing. Observations in the dark were performed under low intensity red light.

5.2.8 Confirmation of 6-OHDA induced lesioning.

At the conclusion of the experiments in Sec. 5.2.6, the rats lesioned with 6-OHDA were sacrificed by decapitation and the brains rapidly removed over ice. The striata were dissected from the brain tissue (as described in Sec. 2.2.3) and assayed for catecholamines using a high pressure liquid chromatograph coupled to an electrochemical detector. These assays were carried out at
Potchefstroom University for Christian Higher Education using the method described by Durand (1988).

The extent of DA depletion in the striatum on the lesioned side with respect to the striatum on the unlesioned side was found to range from 60 - 85 %.

5.2.9 Drugs

APO (SIGMA) was dissolved in saline and 1 mg/ml ascorbic acid was included as anti-oxidant. APO was always administered sc in the flank. MEL was prepared in a vehicle described in Sec. 3.2.4 and was always administered by the ip route.

5.2.10 Statistical analysis

Means and SEM were calculated and data was analysed using analysis of variance with Scheffe's post hoc test for multiple comparisons. A p value of, p < 0.05 was considered as statistically significant. The Student - t test was used when only 2 experimental groups were being compared.
5.3 RESULTS AND DISCUSSION

5.3.1 Results obtained from preliminary experiments

5.3.1.1 Time course for the development of turning behaviour in lesioned rats.

Results from preliminary experiments showed that in the 6-OHDA model, APO induced turning behaviour reached maximum response ten days after lesioning and remained at this level up until 60 days post lesioning (the last time of testing). At ten days, the individual responses varied greatly, and the SEM was large. By the fourteenth postoperative day the variation between individual responses was much smaller. (Fig 5.2)

Rats with unilaterally QA lesioned striata showed maximum turning behaviour at the first testing, two days post lesioning. This response also remained relatively constant up to 60 days after lesioning (Fig 5.2).

These results reflect the difference in these two models. In the 6-OHDA model, APO acts on a supersensitive receptor to induce rotation, and it is the development of this supersensitivity that requires a time lag after lesioning and neuron degeneration. APO, in the QA lesioned model, induces rotation by activity at normosensitive receptors, and the time lag is not evidenced.

Following this experiment, all lesioned rats were first screened for turning behaviour 14 days post-operatively. This allowed for a postoperative recovery period in both models and more specifically for the development of the turning response in the 6-OHDA lesioned model.
FIGURE 5.2 The time course in the development of turning behaviour induced by APO 1mg/kg (BM) in 6-OHDA and QA lesioned rats.

Each bar represents the mean ± SEM of samples of 5 rats in the 6-OHDA lesioned group and 6 rats in the QA lesioned group. (* p < 0.05; ANOVA)
5.3.1.2 Dose response of APO induced turning behaviour

Both 6-OHDA and QA lesioned rats showed a dose dependent response to challenges with increasing doses of APO (Table 4.1). 6-OHDA lesioned rats showed maximum turning when administered APO 1 mg/kg (BM), with a slightly decreased response to APO 2 and 5 mg/kg doses. In the QA lesioned rats, a maximum response was evidenced with 1 and 2 mg/kg doses and a slight decrease in response was also observed with the 5 mg/kg dose.

In all further experiments, rats were screened for turning activity with 0.5 mg/kg APO and the effects of MEL administration were determined on turning induced by APO 1 mg/kg (BM).

**TABLE 5.1 The rotational behaviour induced by increasing doses of APO.**

(N = 6 for each sample group and p < 0.05, ANOVA with Schffe multiple range test. * and ** represent significant differences with respect to other dosage levels in similarly lesioned rats.)

<table>
<thead>
<tr>
<th>DOSE OF APO mg/kg (BM)</th>
<th>6-OHDA LESIONED RATS</th>
<th>QA LESIONED RATS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean net turns</td>
<td>SEM</td>
</tr>
<tr>
<td>0.5</td>
<td>51.5</td>
<td>3.4</td>
</tr>
<tr>
<td>1</td>
<td>83.8 **</td>
<td>4.4</td>
</tr>
<tr>
<td>2</td>
<td>74.2 *</td>
<td>3.5</td>
</tr>
<tr>
<td>5</td>
<td>69.5 *</td>
<td>1.6</td>
</tr>
</tbody>
</table>
5.3.1.3 The onset and duration of APO induced turning response in lesioned rats.

The onset and duration of the APO induced turning responses was similar in rats lesioned by both methods. Onset of rotational behaviour occurred within 5-10 min of APO administration, with some very slow ipsilateral rotation being observed in both groups of rats during the first 5 min period. The duration of rotational effect was between 45 - 60 min with maximum intensity achieved within 10-20 min of onset. Thus, in all further experiments, rotational behaviour was observed and recorded for the 20 min period beginning 5 min after the time of APO administration.

4.3.1.4 Investigation of a possible "undrugged" or "paradoxical" conditioned response in 6-OHDA lesioned rats.

As was noted in Sec. 4.1, a possible conditioned rotational response may result as a consequence of screening turning behaviour with APO and it has been suggested that this response may result in a "changed" experimental animal [Silverman (1988)]. Results obtained in this experiment (Table 4.2) show that placement of animals, 14 days after screening for rotational behaviour with APO 0,5 mg/kg, in the test environment with or without administration of a saline injection, did not result in turning behaviour significantly different to that of sham operated or unoperated animals.
TABLE 4.2 The rotational behaviour observed in various groups of rats 14 days after screening with APO 0.5 mg/kg.

N=5 for each treatment group and p < 0.005; ANOVA with Schiff multiple range test. * represents a significant difference with respect to other treatment groups.

<table>
<thead>
<tr>
<th>RAT TREATMENT</th>
<th>Mean net turns</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unoperated</td>
<td>-0.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Sham operated</td>
<td>-0.2</td>
<td>1.4</td>
</tr>
<tr>
<td>6-OHDA Lesioned</td>
<td>2</td>
<td>0.8</td>
</tr>
<tr>
<td>+ Saline 1 ml/kg (BM)</td>
<td>2.8</td>
<td>0.9</td>
</tr>
<tr>
<td>+ APO 1 mg/kg (BM)</td>
<td>73.6*</td>
<td>4.3</td>
</tr>
</tbody>
</table>

5.3.2 The effect of MEL administration on APO induced rotational response.

At the doses tested, (1 and 10 mg/kg (BM)) ip administered MEL, significantly inhibited turning behaviour induced by APO 1mg/kg (BM) administration. MEL significantly reduced the net total turns, to a level that was not significantly different to that seen in sham lesioned and unoperated animals in 6-OHDA lesioned rats (Fig 5.3). In QA lesioned rats, although the turning response to APO was reduced, it still remained significantly greater than the response seen in controls (Fig. 5.4). In both models MEL administration alone failed to have any significant effect on the behaviour of lesioned animals.

At the doses tested, this effect of MEL was not shown to be significantly dose dependent, with both doses of MEL reducing turning behaviour to the same degree in both models.
These results thus indicate that MEL has a significant effect on striatal dopaminergic function, of which the APO induced turning behaviour in both these models acts as an index. Early speculation following results obtained with the 6-OHDA model suggested that MEL may be normalizes or compensates for the sensitivity of supersensitive DA receptors in the lesioned striatum. This was, in part, the reason for performing the experiment with the QA lesioned model, in which it was demonstrated that MEL antagonised turning behaviour induced by APO activity at normosensitive receptors, suggesting that MEL does not act by normalizing receptor sensitivity.

The effect of MEL in the QA lesioned model also suggests that MEL's activity in the 6-OHDA model is not only as a result of the uncoupling of an obligatory $D_1$ and $D_2$ receptor interaction normally present in animals with normosensitive DA receptors.

In the normosensitive receptor model, both $D_1$ and $D_2$ antagonists alone are capable of antagonizing the behavioural effects of DA agonists [Rouillard and Bédard (1988) and Braun et al (1986)]. Denervation in the 6-OHDA model induces a loss in ability of specific DA antagonists to antagonize completely the rotational behaviour induced by nonspecific DA agonists [Rouillard and Bédard (1988)]. The results obtained in this study would thus suggest that MEL's ability to block the APO induced turning response would not be a result of specific (either $D_1$ or $D_2$) DA receptor antagonism.

In discussion of these results, the limitations of these models, and more specifically the 6-OHDA model discussed in Sec. 4.1 cannot be overlooked. It should be stressed, that although the nigrostriatal dopaminergic system appears to be the primary system involved in the APO induced rotational response, the modifying role of other neurotransmitter systems, and in fact other cerebral regions, cannot be ignored. As was discussed in Sec 4.1, treatments which inhibit or damage other neurotransmitter pathways, for example noradrenergic, serotonergic, cholinergic and GABA-ergic,
modify the rotatory effects of dopaminergic agonists. These studies do not rule out the possibility that MEL may be acting via one of these pathways to modify the APO induced rotational response. They do, however, demonstrate that MEL modifies the behaviour response of the central nervous system to the DA agonist APO, and therefore support the concept that MEL may have a modulatory role on dopaminergic transmission in the central nervous system.

The 6-OHDA model is an accepted animal model of Parkinson's disease and is useful to screen anti-Parkinsonian drugs [Ungerstedt et al (1973)]. Antiparkinsonsonian agents are characterized by their ability to induce rotation in lesioned rats. MEL's ability to inhibit the induction of APO-induced turning suggests, therefore, that MEL may be involved in the pathophysiology of Parkinson's disease, and may be useful in the treatment of other disorders such as schizophrenia, Huntington's chorea and tardive dyskinesias, which are associated with DA receptor supersensitivity or DA overactivity. The QA lesioned rat, has been proposed as a model for Huntington's chorea [Schwarz et al (1982)], and significantly MEL also inhibited APO induced turning in these rats.
FIGURE 5.3 Turning behaviour evidenced by various groups of rats in the 6-OHDA lesioned model.

All bars represent the mean ± SEM of N=5 rats. (* p < 0.05 with respect to all other groups)
FIGURE 5.4 Turning behaviour evidenced by various groups of rats in the QA lesioned model.

All bars represent the mean ± SEM of N=7 rats. (* and ** p < 0.05 with respect to all other groups)
5.3.3 The effects of light on APO induced rotation in 6-OHDA lesioned rats.

The results presented in Fig. 5.5 indicate that exposure to light during the middle of the dark phase significantly increased the rotational response of lesioned rats to APO (1mg/kg (BM)).

Exposure to light during the middle of the dark phase rapidly reduces raised night-time MEL levels [Rollag et al (1980) and Brainard et al (1982)], suggesting that rats maintained in the dark would have raised endogenous MEL levels with respect to those exposed to light (Discussed in Sec. 3.3.4). The results in this study tend to suggest that endogenous MEL may modulate the rotational response of the central nervous system to APO in 6-OHDA lesioned rats. These results are in agreement with the results obtained for exogenously administered APO (Fig 5.3), although the decrease in rotation in this experiment is not as drastic.

It is also of interest to note that the rotational response of rats exposed to light compares with that of rats tested during the light phase (Fig 4.3) and rotation of rats maintained in darkness is reduced with respect to those tested during the light phase. This comparison and conclusion can, however, not be considered conclusive, since illumination of the test environment can be a powerful determinant of the magnitude of drug effect [Kinnard et al (1966)].

The 6-OHDA lesioned rat model can be considered to be an animal model of Parkinson's Disease, a syndrome in man which is linked to a biochemical deficiency of DA and a loss of cells in the substantia nigra [Ungerstedt et al 1973]. In addition to suggesting that MEL has a modulatory role on central dopaminergic neurotransmission, the results obtained in this study further suggest that the pineal gland and it's principal hormone MEL may be involved in the pathophysiology of Parkinson's Disease.
FIGURE 4.5 The effect of exposure to light on the APO (1mg/kg (BM)) induced rotational response in 6-OHDA lesioned rats, during the middle of the dark phase.

Bars represent the mean ± SEM of a sample N=5 rats. (* p < 0.05 Student t test)
Manipulation of dopaminergic mechanisms in laboratory animals produces significant changes in behaviour, which are not directly seen after modifying other neurotransmitter systems. Such behaviours are consistently reproducible and quantifiable and therefore serve as a useful basis on which to investigate the effects of other neurotransmitter functions [Pycock et al (1978)]. This study employed the systemic administration of APO, a DA agonist, with mixed (D_1 and D_2) receptor activity, to induce behaviours by which the effects of MEL could be studied.

Prior administration of MEL (1 and 10 mg/kg (BM) ip) inhibited APO induced stereotypy, locomotor activity, and rotational behaviour in both 6-OHDA and QA lesioned rats. Changes in environmental lighting, with its well recorded effect on endogenous MEL levels, were used in preliminary studies to test the possible physiological significance of the observed effects of administered MEL. The observation that both APO-induced stereotypy, and locomotor activity were increased following the exposure of rats to light, during the middle of the dark period, suggests that endogenous MEL also modifies APO induced behaviour.

The general conclusion of these behavioural studies is that MEL exerts an inhibitory influence on DA function as demonstrated by APO induced behaviours. Since, "the antagonism of apomorphine induced effects is generally considered as the the best animal-test to predict neuroleptic activity in man" [Puech et al (1978)], these studies also suggest that MEL may have therapeutic value as a neuroleptic agent.
The mechanism whereby, and site at which, MEL modifies these DA-dependent behaviours is not clear. Biochemically, at the level of the D₁ receptor, MEL had no demonstrable effect on DA and APO stimulated adenylate cyclase activity, and cAMP formation. However, this biochemical, in vitro test assays antagonism at the receptor level and excludes the possibility that MEL may physiologically antagonise D₁ receptor mediated mechanisms.

MEL's ability to antagonise sulpiride inhibition of APO-induced locomotor activity suggests that MEL antagonises D₂ receptor mediated mechanisms. Further behavioural and biochemical studies employing the newer selective D₁ and D₂ agonists and antagonists are necessary to characterize MEL's mechanism and site of action.

The results presented here support the hypothesis that MEL has an inhibitory modulating role on DA mechanisms in the central nervous system. This hypothesis may have application to the understanding of the pathophysiology and treatment of neurological and mental disorders, primarily related to DA dysfunction, such as Parkinson's disease, Huntington's chorea, schizophrenia, and tardive dyskinesia.
SUMMARY

CHAPTER 1:
The regulatory function of the pineal gland, ideally situated anatomically, to integrate and compare information from both extra- and intra-cranial sources, appears to be mediated through release of the principal pineal hormone, melatonin. Apart from the already well established neuroendocrine role of melatonin, animal and human studies have implicated melatonin as an important modulator of behaviour. Such studies have also led to the conclusion that the effects of melatonin on brain and behaviour are inseparable from the available concentrations of other neurotransmitters, and also to the concept that melatonin may have a modulatory influence on central nervous system dopaminergic neurotransmission.

Dopaminergic pathways in the brain, and especially the nigrostriatal pathway, have a recognised function in the control of both normal and abnormal behaviours. The exact mechanism of dopamine's activity is not fully understood and the situation is complicated by the known existence of two dopamine receptor subtypes, recognised differential roles for the major central dopamine systems, and the known relevance of other neurotransmitter systems in the control of behaviour.

Various other studies, investigating interactions between melatonin and dopamine-related mechanisms, have added further impetus to the concept that melatonin is capable of modulating central dopaminergic neurotransmission, and have also suggested that the pineal gland may be involved in the pathophysiology of dopamine-related movement disorders.
CHAPTER 2:
The striatal adenylate cyclase system, an accepted biochemical model for studies of D₁ receptor activity, was used in this study to investigate the possibility that melatonin may act at a dopamine, more specifically, a D₁ receptor level to modulate dopaminergic neurotransmission. The failure of melatonin to modulate dopamine and apomorphine stimulation of adenylate cyclase activity suggests that melatonin is not active at the D₁ receptor level, although these studies do not preclude the possibility of an in vivo, physiological interaction at the level of the D₁ receptor. This biochemical model is also a useful preliminary screening procedure for drug neuroleptic activity and therefore suggests that melatonin may not have useful neuroleptic activity.

CHAPTER 3:
The study of apomorphine induced stereotypy in rats is an accepted and classical model for studying central nervous system dopaminergic activity. Pretreatment of rats with melatonin prior to the administration of apomorphine, decreased the intensity of stereotypy induced by apomorphine and entirely inhibited the induction of behaviours associated with intense stereotypy, such as gnawing and licking. Studies carried out under various conditions of environmental lighting also tend to suggest that endogenous melatonin may attenuate the behavioural response of the central dopaminergic system to apomorphine administration. It therefore appears that both exogenous and endogenous melatonin have an inhibitory modulatory function on central nervous system dopaminergic neurotransmission.

CHAPTER 4:
Apomorphine-induced locomotor activity is another behavioural index of central dopaminergic function. In this study, photocell activity cages were used to determine the effects of prior administration of melatonin on apomorphine induced locomotion. Whilst melatonin had no effect on baseline activity of control rats, it did inhibit apomorphine induced locomotion. However, it did not reduce activity
to the same extent as sulpiride or haloperidol. Coadministration of melatonin and sulpiride reduced the latter's inhibitory effect on apomorphine-related activity. Melatonin had no effect on the haloperidol inhibition of apomorphine-induced locomotor activity.

Once again, studies carried out under various environmental lighting conditions, suggest that the effect of melatonin is not only related to exogenous administration, but may also be the result of endogenous melatonin. These studies further support the concept of a modulatory function for melatonin on central nervous system dopaminergic pathways, and add impetus to the proposal that it has an inhibitory modulatory role.

CHAPTER 5:
The inhibition by melatonin of apomorphine-induced rotational behaviour in both 6-hydroxydopamine and quinolinic acid lesioned rats further suggests that melatonin has a significant effect on striatal dopaminergic behaviour. The 6-hydroxydopamine and quinolinic acid lesioned rats are useful animal models of Parkinson's disease and Huntington's chorea, respectively, suggesting therefore that melatonin and the pineal gland may be involved in the pathophysiology of such dopamine-related movement disorders.

CHAPTER 6:
The results of these studies further support the concept that melatonin has a modulatory, or attenuating, function in the control of central nervous system dopaminergic neurotransmission and suggest that the pineal gland and melatonin may have an important role in the control of normal and abnormal motor behaviours.
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