AN INVESTIGATION INTO DOPAMINE-MELATONIN INTERACTIONS IN THE RAT CORPUS STRIATUM AND PINEAL GLAND: A POSSIBLE PINEAL-STRIATAL AXIS

THESIS

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Clinton Shane Boyd

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Abstract

Dysfunction of central dopaminergic systems has been implicated in neuroendocrine, neurodegenerative and psychiatric disorders. Monoamine oxidase and catechol-O-methyltransferase represent the key catabolic enzymes of dopamine, terminating neurotransmission following synaptic release of this catecholamine. Thus, both enzymes have been associated with the pathology of dopaminergic systems and represent therapeutic targets of enormous clinical importance. Some neuroendocrine and circadian effects of melatonin have been attributed to an antidopamimetic effect of this pineal hormone in the hypothalamus and pituitary. Furthermore, both melatonin and dopamine modulate the behavioural output of the mesencephalic dopaminergic pathways of the basal ganglia, including movement disorders. However, the biochemical basis for the tonic inhibitory effect of melatonin in the nigro-striatal pathway has been poorly delineated. Thus, this study determined whether melatonin influences dopaminergic function in the corpus striatum of the Wistar rat by modulating monoamine oxidase and catechol-O-methyltransferase activity. Reciprocally, the putative existence of an intrapineal dopaminergic system was investigated by determining the effect of selective dopaminergic agents, R-(-)-apomorphine, haloperidol and dopamine, on indole metabolism of the pineal gland. The akinetic state of drug-induced catalepsy was employed as an animal model of Parkinson's disease to probe the neurotransmitter systems involved in the behavioural effects of melatonin.

Indole metabolism was a reliable indicator of state-dependent metabolic fluxes in pineal gland function. These included a robust diurnal and seasonal variation in N-acetylserotonin and melatonin biosynthesis, and photoperiod- and drug-induced alterations of metabolism. The predominant changes could be attributed to an effect on serotonin N-acetyltransferase activity and/or the melatonin/5-methoxytryptophol ratio. Pineal 5-methoxyindole biosynthesis was determined primarily by the bioavailability of the corresponding 5-hydroxyindole and its affinity for hydroxyindole-O-methyltransferase. Evidence was found for the negative feedback or paracrine control of pineal indole metabolism by melatonin. A high inter-individual variability was observed in the biosynthesis of N-acetylserotonin and melatonin biosynthesis, and the weight of the pineal glands. Accordingly, the rats could be classified as either high or low capacity producers of these two indoles. R-(-)-apomorphine and dopamine *in vitro*, but not acute haloperidol *in vivo*, had dose- and phase-dependent effects on pineal indole metabolism. The predominant effect was a suppression of the scotophase-dependent induction of N-acetylserotonin and melatonin biosynthesis by dopamine and R-(-)-apomorphine. It is postulated that these agonists inhibited nocturnal N-acetyltransferase activity via postsynaptic pineal D_2 or D_2-like

receptors. The observed modulatory nature of the intrapineal dopaminergic system suggests that dopamine may be involved in the long-term regulation of pineal indole biosynthesis.

Several lines of evidence are presented that the activity of striatal monoamine oxidase A and catechol-O-methyltransferase, represented predominantly by the soluble isoform, is state-dependent and regulated *in vivo* by endogenous melatonin. Firstly, both enzymes showed a day-night variation in activity. Secondly, acute and subchronic administration and photoperiod manipulation studies indicated that both exogenous and endogenous melatonin inhibited each enzyme in a chronotypic fashion, with a more robust effect against catechol-O-methyltransferase. The intensity of the *in vivo* effects was critically dependent on the dose, duration, route and the phase-timing of administration during the light:dark cycle, and the length of the exposure to constant light. Melatonin *in vitro* had no effect on basal or Mg^{2+}-induced catechol-O-methyltransferase activity. Thus, it is proposed that the *in vivo* effects of the hormone can be attributed to a time-dependent change in the amount of active molecules of this enzyme. In contrast, melatonin and numerous other endogenous indolic compounds were found to be reversible inhibitors of striatal monoamine oxidase A *in vitro*. Structure-activity modeling revealed that the 5-methoxy moiety on the indole nucleus and substitution of the free primary amine of these compounds were the principal determinants of the potency and time-dependency of inhibition. Thus melatonin most likely has a direct inhibitory effect *in vivo* at the level of the active site of monoamine oxidase A.

Exogenous melatonin alone had no cataleptogenic potential whereas a variety of behavioural responses were observed following intraperitoneal administration of γ-hydroxybutyrate. The latter responses were state-dependent with day-night variations in intensity. Furthermore, γ-hydroxybutyrate stimulated melatonin biosynthesis during the photophase both *in vitro* and *in vivo*. These results point to a possible involvement of melatonin in the behavioural and neurochemical effects of γ-hydroxybutyrate.

Thus the general conclusion is that dopamine and melatonin display functional antagonism at the level of the pineal gland and corpus striatum of the Wistar rats. Therefore melatonin may be an important homeostatic modulator of dopaminergic neurotransmission throughout the central nervous system. Furthermore, the putative existence of a functional pineal-striatal axis would greatly strengthen the argument for a holistic concept of brain homeostasis. The ability of endogenous melatonin to regulate monoamine oxidase A and catechol-O-methyltransferase may represent an alternative strategy for the treatment of disorders associated with these enzymes.

"no vestige of a beginning, -no prospect of an end"
James Hutton (1788)

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"The seat of the passions is not the heart ...
There is a little gland in the brain where the
soul exercises its functions more especially
than in other regions ... This gland is regarded
as the principal seat of the soul."
René Descartes (1649)

TABLE OF CONTENTS

List of Abbreviations

3MT	3-methoxytyramine
5HIAA	5-hydroxyindole acetic acid
5HIAL	5-hydroxyindoleacetaldehyde
5HT	5-hydroxytryptamine (serotonin)
5HTOH	5-hydroxytryptophol
5MIAA	5-methoxyindole acetic acid
5MIAL	5-methoxyindoleacetaldehyde
5MT	5-methoxytryptamine
5MTOH	5-methoxtryptophol
6OHDA	6-hydroxydopamine
AD	adrenaline
ANOVA	analysis of variance
APO	apomorphine
APUD	Amine Precursor Uptake and Decarboxylation system
BSA	bovine serum albumin
bwt.	body weight
BZ	benzodiazepine
CaM	calmodulin
cAMP	adenosine 3′:5′-cyclic monophosphate
CAT	Central African Time
CER	constant environment room
cGMP	guanosine 3′:5′-cyclic monophosphate
CNS	central nervous system
COMT	catechol-O-methyltransferase
CPZ	chlorpromazine
CSF	cerebrospinal fluid
CVO	circumventricular organ
DA	dopamine
DBH	dopamine-β-hydroxylase
DMABA	dimethylaminobenzaldehyde
DMSO	dimethylsulphoxide

DOPAC	3,4-dihydroxyphenylacetic acid
DPM	disintegration per minute
FAD	flavin adenine dinucleotide
G-protein	guanine nucleotide binding protein
GABA	gamma-aminobutyrate
GHB	gamma-hydroxybutyrate
HAL	haloperidol
HIOMT	hydroxyindole-O-methyltransferase
HPLC	high performance liquid chromatography
HVA	homovanillic acid
i.p.	intraperitoneal
L-Dopa	3,4-dihydroxy-L-phenylalanine
MAO	monoamine oxidase
MAOP	monoamine oxidase products
MB-COMT	membrane-bound COMT
MBH	medial basal hypothalamus
MEL	melatonin
MPP^+	1-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NA	noradrenaline
NAD	nicotinamide adenine dinucleotide
NAS	N-acetylserotonin
NAT	(serotonin) N-acetyltransferase
NIL	neurointermediate lobe
NO	nitric oxide
QA	quinolinic acid
R.I.M.A	reversible inhibitor of MAO-A
REM	rapid eye movement
RER	rough endoplasmic reticulum
RIA	radioimmunoassay
S-COMT	soluble COMT
s.c.	subcutaneous
SAD	seasonal affective disorder
SAH	S-adenosylhomocysteine

SAIMR	South African Institute of Medical Research
SAM	S-adenosyl-L-methionine
SAR	structure-activity relationships
SCG	superior cervical ganglion
SCN	suprachiasmatic nucleus
SD	standard deviation
SEM	standard error of mean
SLA	spontaneous locomotor activity
SNS	sympathetic nervous system
TAT	tyrosine aminotransferase
TH	tyrosine hydroxylase
THDA	tuberohypophyseal dopaminergic pathway
TIDA	tuberoinfundibular dopaminergic pathway
TLC	thin layer chromatography
UK	United Kingdom
USA	United States of America
UV	ultraviolet

LIST OF TABLES AND FIGURES

Chapter 1

Literature Review

1.1 Introduction

The 1990s were declared the "Decade of the Brain" in recognition of the accelerating impact of brain-related disorders on society and national economies. As the average life-span of more affluent populations increases in response to modern medicine, age-related neurodegenerative diseases are becoming more common, especially the terribly debilitating Parkinson's and Alzheimer's disease. In further response to the modern lifestyle, stress- and anxiety-induced affective disorders are becoming widespread, with depression as the most ubiquitous psychiatric malady of the 1990s. This has resulted in a growing public awareness and concern regarding mental health. This can be attested to by the fact that numerous neuropharmaceutical agents have become household words and a part of the normal contents of the average medicine cabinet.

Neuroscience represents a multidisciplinary approach uniting scientists of disparate fields in an attempt to unravel the mysteries and workings of the brain. A final goal has been to explain complex neural problems such as consciousness, self-awareness and memory. Research has ranged from extreme reductionism on one hand, to treating the brain as a "black box" on the other, where only the inputs and outputs or emergent properties of the brain are considered. Now as the "Decade of the Brain" closes and the new millennium approaches, some researchers have expressed a concern that neuroscience has become stagnant and has failed to deliver an unified concept of brain function, especially of consciousness. However, this pessimistic view and preoccupation with the neural version of the "theory of everything", should not detract from a key concept that has developed in the field of neuroscience: brain homeostasis.

Typically, brain function has been delineated from an understanding of brain dysfunction. Now there is a growing appreciation that brain disorders or diseases are functional entities in their own right, and not merely abnormal versions of normal brain functioning. Neural systems are in permanent dynamic flux and their plasticity and ability to self-adjust is well-known. They can respond to perturbations by establishing a new steady-state or equilibrium, and even take over functions normally assigned to other neuronal circuits, following damage to the latter. This

concept was eloquently demonstrated for encephalitic Parkinsonism patients in the Oliver Sacks book *Awakenings* (1973). Virally-induced damage to the basal ganglia resulted in an inability to process sensory information into a motor response that was appropriate for real-time conditions. Closer inspection, however, revealed that these patients had different perceptions of space and time that helped to create and shape their own virtual reality. For example, the inability to initiate movement in a plain hospital corridor was overcome by painting white and black blocks on the floor. Inappropriate processing of sensory information is a hallmark of what is termed "disorders of impulse control", and includes schizophrenia and other affective disorders. Impulse control and motor co-ordination are regulated by the dopaminergic limbic system and nigro-striatal pathway of the basal ganglia, respectively. Behaviour represents an obvious manifestation of brain function. Thus the abnormal behavioural repertoires associated with dysfunction of the basal ganglia are one reason why this brain system is a fascinating and topical area of research.

The neuronal circuits of the basal ganglia involved in impulse control also represent an ideal system for investigating the importance of homeostasis or balance in brain function. Dopamine (DA) is the key neurotransmitter of the mesolimbic and nigro-striatal pathways (Graybiel, 1990). Most disorders of impulse control are associated with a dysfunction or imbalance of dopaminergic function, for example schizophrenia and Parkinson's disease. In a highly simplified model, schizophrenia and Parkinson's can be viewed as the opposite manifestations of a "DA see-saw". Parkinson's disease is characterized by hypoactivity of the dopaminergic neurons, whereas schizophrenia is a hyperactive state (Goldstein and Deutch, 1992; Greenfield, 1992). The modern therapeutic approach is thus to rectify this imbalance with the aid of selective dopaminergic agents. The DA precursor 3,4-dihydrox-L-phenylalanine (L-Dopa) and selective DA receptor agonists are used in the treatment of Parkinson's disease, whereas the antipsychotic drugs used in the treatment of schizophrenia are DA antagonists (neuroleptics). However, chronic administration of these agents can over-correct the imbalance, swinging the "see-saw" in the opposite direction. For example, neuroleptic-induced Parkinsonism and other movement disorders are common in treated schizophrenic patients. In turn, L-Dopa can induce hallucinations and dyskinesias in Parkinson's patients that are reminiscent of the psychotic episodes of schizophrenia (Jankovic, 1995). These drug-induced side-effects represent the major cause of poor patient compliance with the current therapeutic approach.

Even local neuronal circuits, such as those involved in impulse control, constitute a complex interaction between several pathways employing a number of different neurotransmitter systems. It is naïve to believe that therapeutic agents will only target the specific neuronal circuit in question or that a complex disease state can be cured by selectively targeting only one neurotransmitter. A similar argument can be used against the "pill-popping" philosophy of treating disorders such as depression or eating disorders. Rather a holistic view is needed to appreciate the new homeostatic state of a specific neuronal system following a perturbation. The contribution of each component of the neural system to the new equilibrium must be ascertained.

A holistic view of brain function cannot ignore the growing awareness of the role of the pineal gland in homeostatic control of the body and brain. Historically, the 17th century philosopher Rene Descartes, who helped shape the Western paradigm of thinking, supported and extended Plato's proposal that the pineal is the seat of the soul (Miles and Philbrick, 1988). There has also been a movement to equate the pineal gland with the "third eye" of Hindu religion, which is believed to represent "the seventh chakra" or the gateway to full spiritual enlightenment. Although too esoteric for scientific objectivity, this way of thinking appears to have preempted the current awareness of the pineal. Initially, science saw the pineal gland as a vestigial organ with no function. It was only after the isolation of melatonin (5-methoxy-N-acetyltryptamine; MEL) from the bovine pineal gland by Lerner *et al.* (1958) that the current explosion in pineal research began. The following literature review will clearly show that MEL is an important neuromodulator of several key neurotransmitters and thus influences numerous brain functions. Neuroscientists, or pineal researchers at least, have now gone as far to state that the pineal may indeed be a "master gland" or the principal homeostatic regulator. One apparent mechanism involves an effect of MEL on well-defined neuronal circuits and assemblages, such as the hypothalamic-pituitary-adrenal axis. Hypothalamic DA appears to be the key point at which MEL regulates the latter axis. This raises the question of whether MEL may be a key component of homeostatic regulation of dopaminergic function of the basal ganglia and thus disorders of impulse control. Indeed the corpus striatum of the basal ganglia has the richest dopaminergic innervation of any brain region (Moore and Bloom, 1978).

Thus it is hypothesized that a functional interaction may exist between the pineal gland of the rat and the corpus striatum, i.e. a pineal-striatal axis. A reciprocal or feedback relationship may exist between pineal MEL and DA. Briefly, it is postulated that a dopaminergic system may modulate

pineal function and, in turn, MEL may modulate dopaminergic activity and behavioural output of the rat corpus striatum.

The main objectives of the present thesis can be summarized as follows:

(i) To determine the effect of dopaminergic agents on pineal indole metabolism.

(ii) To determine the effect of MEL on striatal dopaminergic function at the level of the enzymes monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT).

(iii) To determine the interaction of MEL and dopaminergic agents on behaviour employing catalepsy as a model of Parkinson's disease.

1.2 The Pineal Gland

The rat pineal complex, or epiphysis cerebri, consists of the superficial pineal gland and the deep pineal (Reiter, 1989; Zhang *et al.*, 1991). The superficial pineal gland is located on the surface of the brain at the junction of the cerebellum and cerebral hemispheres in close association with the third ventricular region. The deep pineal is located in the diencephalon on the dorsal part of the brain stem between the habenular complex and posterior commissural areas. The two components are connected via a pineal stalk or peduncle (Reiter, 1989; Zhang *et al.*, 1991). Unless otherwise specified, the term pineal gland will be used to refer to the superficial pineal. The word "pineal" is derived from the Latin *pinealis, pinea* meaning pine cone, as this is the shape resembled by the human pineal gland. The term epiphysis means "what is grown on something" (Erlich and Apuzzo, 1985).

The pineal is an endocrine gland ideally situated anatomically to receive, integrate and compare information from both the external environment and the internal physiological milieu. By transducing photoperiodic information into a hormonal signal, the pineal plays an integral role in the temporal organization of numerous metabolic, physiological and behavioural processes. For this reason, the pineal has been defined as a "neuroendocrine transducer" (Axelrod, 1974) with the indoleamine MEL as the principal hormone secretion (Erlich and Apuzzo, 1985).

The mammalian pineal gland itself is not photoreceptive. In these animals, the pineal is connected to the phototransducing ganglion cells of both retinas through a specific polyneuronal pathway (see Figure 1.1). An independent retinohypothalamic tract connects these ganglion cells to the

paraventricular nucleus and suprachiasmatic nucleus (SCN) of the anterior hypothalamus. From here, the fibres run through the brainstem and the medial forebrain bundle, terminating on pre-ganglionic sympathetic neurons in the intermediolateral cell columns of upper thoracic cord. Efferent axons pass in the cephalid direction via the sympathetic trunk to synapse in the superior cervical ganglia (SCG). Post-ganglionic fibres of the sympathetic nervous system (SNS), known as the nervi conarii, enter the cranial cavity via the internal carotid plexus and terminate primarily on the perivascular processes of the pinealocytes. In this way, the parenchymal cells of mammalian pineals are unusual in that they receive direct sympathetic innervation, mediating pineal responses to environmental lighting (Erlich and Apuzzo, 1985; Moore, 1993).

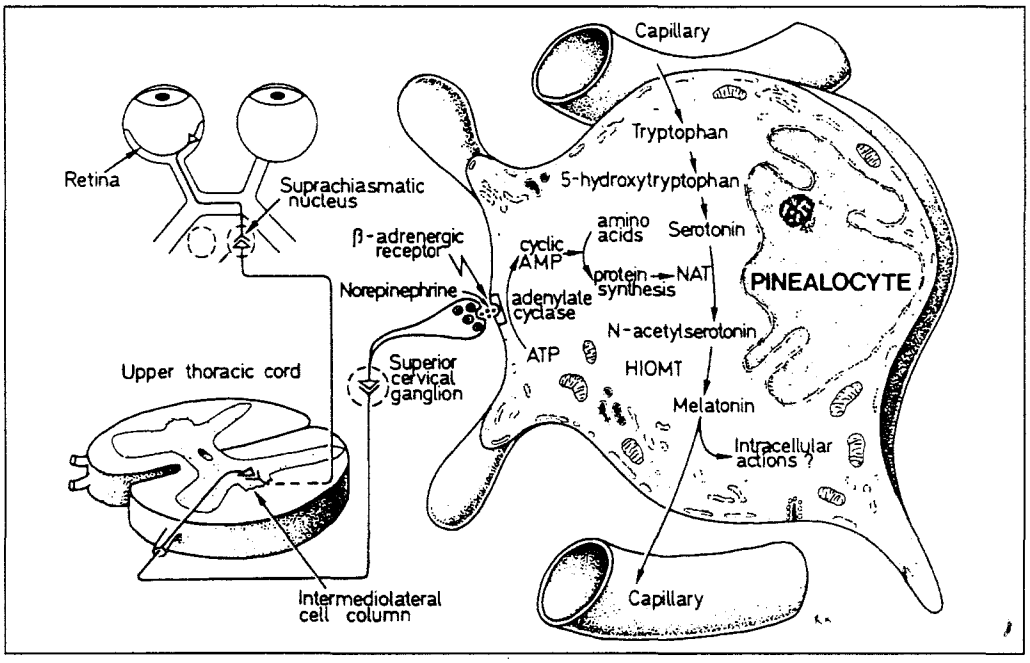


Figure 1.1: Diagrammatic representation of the multi-synaptic connection between the retina and a pinealocyte of the pineal gland. The pineal indole biosynthesis pathway is depicted in the pinealocyte (Reproduced from Reiter, 1988).

The hypothalamic SCN represents the site of the endogenous "biological clock" or internal "zeitgeber". The SCN is an intrinsic pacemaker, which generates a free-running rhythm of pineal MEL synthesis with a periodicity of slightly greater than 24h (*ca.* 25 hrs). Light acts as an entraining agent to readjust the clock to a period of 24 hrs (i.e. a circadian rhythm) in response to daily and seasonal shifts in the photoperiod. Light holds the SCN under tonic inhibition thereby

reducing circadian output. The onset of darkness (i.e. the absence of light) results in the disinhibition of the SCN triggering a surge in the synthesis and release of MEL at night (Armstrong, 1989; Reiter, 1989; Moore, 1993). For this reason, MEL has been called the "chemical expression of darkness" (Reiter, 1991). By means of MEL, the pineal can provide information about both the absolute daylength and whether the photoperiod is increasing or decreasing. Numerous animals, described as photoperiodic, utilize the seasonal change in daylength as an environmental cue to regulate circannual fluctuations in internal physiology, especially reproduction. After pinealectomy, most mammals are either totally aseasonal or revert to inherent free-running annual cycles. Indeed, the two main functions attributed to MEL include:
(a) The timing of the annual rhythm in reproductive function in seasonally breeding animals. MEL has both pro- and anti-reproductive effects, depending on the stage of the circannual rhythm, and also influences pubertal development (Reiter, 1991; Waldhauser *et al.*, 1993).
(b) Circadian entrainment. MEL acts as an entraining agent capable of gating and synchronizing the daily rhythms of other circadian oscillators, especially the SCN. In this way, MEL is responsible for phase-shifting numerous biochemical, physiological and behavioural processes to the prevailing light:dark cycle (Reiter, 1991).

The pineal gland is also one of eight extra-blood-brain structures collectively named the circumventricular organs (CVOs) in mammals (Johnson and Gross, 1993). The other members include the subfornical organ, organum vasculosum of the lamina terminalis, median eminence, pituitary intermediate lobe, pituitary neural lobe, subcommissural organ and area postrema. These are small midline structures bordering the third and fourth ventricles, generally displaying sensory and neuroendocrine characteristics. Seven of the eight CVOs, including the pineal gland, are blood-brain-barrier-deficient regions, although they have extraordinarily high capillary density (high vascularization). The typical tight junctions between endothelial cells lining the capillaries are lacking, resulting in fenestrated vessels, and the glial cells which are normally tightly wrapped around the vessels abluminally are more loosely opposed, resulting in relatively large perivascular spaces. These morphological features are very favourable for high permeability fluid-brain exchange, allowing even large and polar substances to readily move between blood and perivascular spaces. CVOs also possess specialized ependymal cells called tanycytes, which have multiple processes that contact the cerebrospinal fluid (CSF) and others that extend into the CVO body. Thus CVOs have cellular contacts with two fluid phases - blood and CSF. Furthermore all appear to have neural connections with strategic nuclei, as discussed above for the pineal gland (Johnson and Gross, 1993). It must be noted that these physical properties of the pineal have

important implications for pharmacological manipulation studies. Firstly, the pineal gland can potentially be influenced by peripheral neurochemical agents that cannot enter the central nervous system (CNS). Secondly, the high degree of vascularization could increase contact time due to accumulation of the agent.

It has also been proposed that the pineal meets all the criteria to be classified as a part of the APUD (Amine Precursor Uptake and Decarboxylation) system (Leong and Matthews, 1979; Erlich and Apuzzo, 1985). The latter represents a diffuse neuroendocrine system of widely dispersed, apparently unconnected series of glandular structures linked by a common embryological derivation from the neural crest and secretion of closely similar functional (hormonal) products. Some other members include pituitary corticotrophic cells, pituitary melanotrophic cells, pancreatic islet cells, thyroid C cells, argyrophilic G cells of the stomach and intestinal enterochromaffin cells. Hormonal secretory products of these tissue would then include insulin, melanocyte-stimulating hormone and thyrotropin.

Thus reciprocal interactions may exist between the pineal and other members of the CVO and APUD systems. In this way, the pineal may represent a "master gland", facilitating co-ordination between diverse structures both peripherally and centrally. Such a diffuse network would expand the possible regulatory aspects of the pineal and MEL.

1.3 Pineal Indole Biosynthesis

The functional endocrine cells of the pineal gland are known as pinealocytes (Reiter, 1989). These parenchymal cells possess one to several processes that terminate near capillaries or in proximity to nerve endings present in the perivascular spaces. The nerve endings are predominantly of the sympathetic postganglionic fibres originating from the SCG. Connective tissue septae divide groups of pinealocytes into cords and lobules or follicles. The pinealocytes are the hormone producing cells of the pineal producing a number of biologically active compounds in addition to MEL, including proteins and neuropeptides. Of current importance, the pinealocyte is also the cellular site for the biosynthesis of MEL and other indoles (Figure 1.1). Pineal indole metabolism is very similar to that seen in the brain as summarized in Figure 1.2. The shaded area in the latter figure depicts those biosynthetic steps that are unique to the pineal and certain other tissues and will be discussed below. Due to some species-related differences in the regulation of pineal indole and MEL biosynthesis, the following description will be limited mostly to the rat (Reiter, 1989).

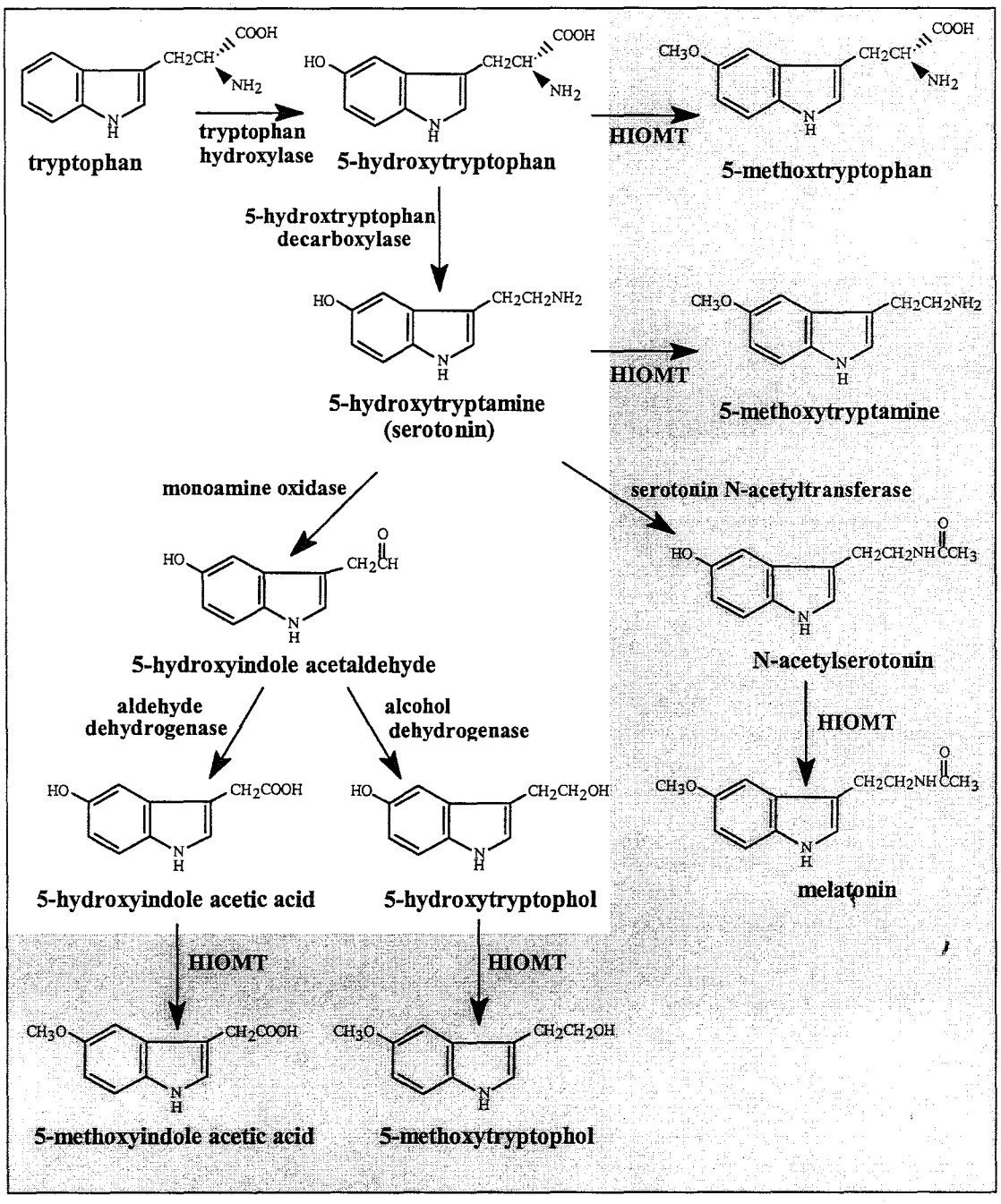


Figure 1.2: Indole metabolism in the rat brain and pineal gland.

Circulating plasma L-tryptophan is taken up by pinealocytes and hydroxylated to 5-hydroxytryptophan by tryptophan hydroxylase. The enzyme is typically the rate-limiting step in serotonin (5-hydroxytryptamine; 5HT) synthesis (Naoi *et al.*, 1994). However, the low levels of tryptophan in the pineal suggest that this enzyme may normally be unsaturated and thus substrate bioavailability would determine the rate of hydroxylation (Reiter, 1989). Tryptophan hydroxylase shows a clear diurnal rhythm, with peak activity in the dark-phase and decreasing towards the end of the dark-phase (Reiter, 1989). 5-Hydroxytryptophan is in turn decarboxylated by 5-hydroxytryptophan decarboxylase (aromatic L-amino acid decarboxylase; L-Dopa decarboxylase) to produce the indoleamine 5HT. It appears that there are two distinct pools of 5HT within the pinealocyte, a vesicular and a cytoplasmic pool (Racké *et al.*, 1991). The vesicular pool includes, in part, the storage of 5HT in dense-core vesicles (Juillard and Colin, 1979), where the indoleamine is protected against degradation by MAO. There appears to be preferential release of newly synthesized cytosolic 5HT, whereas the vesicular store does not appear to be released via a exocytotic secretion mechanism, but rather an efflux dependent on a permanent *de novo* synthesis of 5HT. Released 5HT can be taken up by the nerve terminals of the SNS innervating the pineal gland (Axelrod, 1974; Juillard and Collin, 1979). Indeed pineal SNS nerve terminals contain more 5HT than noradrenaline (NA), but there is no evidence for co-release of 5HT and NA from these nerve terminals upon activation of the neuronal tract during the dark-phase.

The newly synthesized 5HT undergoes a three-fold metabolic fate. The main route involves oxidative deamination of 5HT by MAO, which is highly concentrated in the pineal gland (Muller and Da Lage, 1977; Juillard and Collin, 1979). The deaminated intermediate 5-hydroxyindole acetaldehyde (5HIAL) is unstable and either oxidized to 5-hydroxyindole acetic acid (5HIAA) or reduced to 5-hydroxytryptophol (5HTOH) by aldehyde dehydrogenase and alcohol dehydrogenase respectively. The relative production of 5HIAA and 5HTOH is largely dependent on the prevailing NAD^+/NADH ratio (Wainwright, 1977). Catabolism by MAO is believed to occur largely within the cytosolic compartment of pinealocytes (Muller and Da Lage, 1977; Juillard and Collin, 1979), such that most of the 5HIAA efflux (unlike 5HT) appears to arise from a single compartment within the pinealocytes (Racké *et al.*, 1991). However this may be debatable considering that MAO is present within the pinealocytes, interstitial cells of the pineal and within in the SNS nerve terminals. Indeed it is reported that the pineal tissue itself contains 30% of measurable MAO activity, whereas the SNS nerve terminals account for 70%. Thus considering that released 5HT is taken up by these nerve terminals, it is feasible that this represents the main site of oxidative deamination.

A second metabolic route involves the conversion of 5HT to N-acetylserotonin (NAS) by the enzyme serotonin-N-acetyltransferase (NAT) during the scotophase. This represents the rate-limiting enzyme in the biosynthesis of MEL. The vesicular 5HT, protected against deamination, is preferentially utilized (Racké *et al.*, 1991). Daytime 5HT content is crucial for determining the amount of NAS synthesized. Thus, although tryptophan hydroxylase activity is high at night, conversion of 5-hydroxytryptophan to 5HT by the decarboxylase enzyme is highest during the light-phase. This explains the inverse relationship of pineal 5HT and MEL levels, with high levels of 5HT during the photophase. The synthesized 5HT is only converted to NAS the following night, explaining why the synthesis of MEL from tryptophan can be expected to cycle through 36 hrs (Reiter, 1989).

Another important pineal enzyme is hydroxyindole-O-methyltransferase (HIOMT). This cytosolic enzyme is the most abundant protein in the pineal, representing about 2-4% of the total soluble protein (Jackson and Lovenberg, 1971). This enzyme catalyses the O-methylation of 5-hydroxyindoles to their corresponding 5-methoxyindoles utilizing S-adenosyl-L-methionine (SAM) as the methyl donor. In other words, 5HT is converted to 5-methoxytryptamine (5MT), NAS to MEL, 5HIAA to 5-methoxyindole acetic acid (5MIAA), and 5HTOH to 5-methoxytryptophol (5MTOH) [Reiter, 1989; Morton, 1990]. In addition to the pineal, the retinas, harderian glands, extra-orbital lacrimal glands, erythrocytes, mononuclear leucocytes and various areas of the gastrointestinal tract also contain HIOMT and thus are capable of synthesizing MEL (Reiter, 1989; Finocchiaro *et al.*, 1991). It is still disputed whether pineal HIOMT activity is also under noradrenergic control and circadian in nature with peak activity during the scotophase as seen for NAT.

The circadian rhythmicity in pineal indole biosynthesis and MEL secretion is determined primarily by the noradrenergic secretory drive (Deguchi and Axelrod, 1972a; Axelrod, 1974; Reiter, 1991). NA is stored in the postganglionic sympathetic nerve terminal innervating the pineal gland and is strictly released only at night. The production of NA is controlled by tyrosine hydroxylase (TH) in the SNS nerve terminals, the activity of which increases at night (Craft *et al.*, 1984). NA release is sustained for the whole duration of the scotophase, which is determined by the prevailing photoperiod. It is believed that light via the retina is responsible for the inhibition of SCN metabolic function and that the onset of darkness (i.e. the absence of light) is responsible for disinhibition, allowing flow of action potential. For this reason MEL biosynthesis by pinealocytes occurs primarily during darkness (Reiter, 1991)

The postsynaptic signal transduction events following NA release are mediated via β- and α-adrenergic receptors located on the pinealocyte membrane (Deguchi and Axelrod, 1972a; Reiter, 1991). Stimulation of these receptors results in a cascade of specific chemical events leading to enhanced MEL biosynthesis. The cyclic nucleotide adenosine 3′:5′-cyclic monophosphate (cAMP) is the essential intracellular second messenger. Both receptor subtypes are coupled, via a stimulatory guanine nucleotide binding protein (G protein), to the enzyme adenylate cyclase that converts adenosine-5′-triphosphate (ATP) to cAMP. β-Adrenergic receptor stimulation is the primary and essential signal leading to a rise in pineal cAMP during the scotophase. Stimulation of the α-adrenergic receptors alone has no effect, but potentiates cAMP accumulation when co-stimulated with the β-receptor. This dual receptor regulation of cAMP translates into a similar pattern of receptor-induced stimulation (by β-receptors) and augmentation (by α-receptors) of the activity of NAT and MEL production (Klein *et al.*, 1983).

The increase in cAMP levels is responsible for the induction of NAT activity during the dark-phase by increasing mRNA synthesis and translation of *de novo* protein. The timing and magnitude of NAT induction following the onset of darkness appears to be species-specific. In particular, a very robust induction of NAT activity and MEL biosynthesis is seen in the rat pineal. These differences in NAT induction characteristics may explain the different patterns of MEL production between species. Three patterns have been provisionally defined: Type A = discrete peak in the late scotophase (e.g. Syrian hamster, Mongolian gerbil, House mouse); Type B = peak near mid-scotophase (e.g. albino rat, Richardson's ground squirrel, human); Type C = prolonged peak during majority of the scotophase (e.g. white-foot mouse, domestic cat, sheep). Regardless of the pattern involved, there are some basic similarities: the magnitude of the nocturnal MEL peak in blood is comparable in all species so far investigated, regardless of magnitude of NAT activity; the peak always occurs in the dark-phase, irrespective of the locomotor activity pattern i.e. diurnal *vs.* nocturnal species; the duration of the MEL peak is proportional to the duration of the dark-phase, i.e. the light:dark ratio or photoperiod (Reiter, 1988). In other words, the duration of β-adrenergic stimulation determines the length of the MEL peak and not its magnitude (Simmoneaux *et al.*, 1989). The drop in MEL production near the end of the dark-phase is probably due to down-regulation of adrenergic receptors.

1.4 Melatonin

Although some important physiological effects have been attributed to 5MT and 5MTOH, MEL is regarded as the principal hormonal secretory product and "chemical transducer" of the pineal gland. For this reason, further discussion will be limited to the pleiotropic nature of this simple indoleamine. It can be argued that since the pineal 5HT content is 100-fold greater than that in the brain, the MEL rhythm must be of some considerable physiological importance.

1.4.1 Distribution and Metabolic Fate

The secretion of pineal MEL shows a surge during the scotophase. Apparently, MEL is not stored in the pinealocytes, but is rapidly secreted as a direct consequence of its increased biosynthesis at night (Reiter, 1991; Follenius *et al.*, 1995). MEL is primarily released into the vascular system via the confluens sinuum or into the CSF via the cisterna magna (Chan *et al.*, 1990). Due to its hydrophobic nature, the secretion of MEL is believed to involve simple passive diffusion. However, there is some tentative evidence for an active transport system (e.g. Mauviard *et al.*, 1991). Two distinct secretory modes have been characterized in several species, including man. Episodic secretion, characterized by ultradian pulses, is superimposed on the circadian pattern of tonic basal secretion (Chan *et al.*, 1990; Follenius *et al.*, 1995).

Due to its rapid release, plasma and body fluid MEL levels parallel its pineal production rate (Reiter, 1986; Reiter, 1988; Reiter, 1990). By virtue of its lipophilic nature, MEL can penetrate the blood-brain barrier and cellular membranes (Vitte *et al.*, 1988). For this reason, every body fluid has proven positive for MEL, including cerebrospinal fluid, saliva, ovarian follicular fluid, male seminal fluid, fluid of the anterior chamber of the eye, milk and amniotic fluid (Reiter, 1988). Thus every cell in the body can be informed of the current photoperiodic state via the hormone MEL. In this sense, the pineal gland serves as the "eyes" of all organs. Researchers are now of the opinion that no organ or tissue may escape the influence of MEL (Reiter, 1988). Uptake of MEL into these fluids may be mostly via passive diffusion or in some cases an active uptake mechanism. There is evidence that the uptake of MEL into the brain appears to be both saturable and region-specific, suggesting the existence of active transport systems (Anton-Tay *et al.*, 1988; Vitte *et al.*, 1988).

On a day-to-day basis under specific photoperiodic conditions, the circadian MEL rhythm is very reproducible and is thus a reliable index of pineal MEL synthesis (Reiter, 1988). However, in mammals, the ratio of trough to peak MEL levels may vary between species. By convention, a doubling of the basal daytime level is considered a physiologically relevant change (Reiter, 1988).

The main catabolic route of MEL occurs peripherally in the liver. Hepatic microsomal enzymes catalyze the 6-hydroxylation of MEL to produce 6-hydroxymelatonin (Kopin *et al.*, 1961). Phenobarbital, which induces microsomal enzymes, significantly lowers plasma and brain levels of systemically administered ^{3}H-MEL (Wurtman *et al.*, 1968a). 6-Hydroxymelatonin is subsequently conjugated with sulphuric or glucuronic acids and excreted (Kveder *et al.*, 1961). This rapid route of metabolism is responsible for the short half-life of MEL. In the CNS, MEL is typically degraded to N-acetyl-5-methoxykynurenamine through cleavage of the indole ring (Hirata *et al.*, 1974). Other minor catabolic routes are also evident. For example, the enzyme aryl acylamidase can deacetylate MEL to 5MT or NAS to 5HT (Finocchiaro *et al.*, 1991) and in rats, MEL can be demethylated to NAS (Leone and Silman, 1984).

1.4.2 MELATONIN RECEPTORS

The advent of the selective and highly potent radioligand [^{125}I]-iodomelatonin (Laudon and Zisapel, 1986) resulted in the identification and characterization of two types of high affinity MEL binding sites. These were designated MEL_{1A} and MEL_{1B} (Dubocovich, 1988). Subsequently, molecular biology has confirmed the existence of these two receptors and a third subtype, designated MEL_{1C}, in several vertebrate species (Reppert *et al.*, 1995a and 1995b). There is also a preliminary indication of a MEL_{1D} receptor (Shiu *et al.*, 1996). All the MEL receptors belong to the superfamily of G-protein coupled receptors (Stankov and Reiter, 1990; Morgan *et al.*, 1994). The aforementioned MEL receptor subtypes are associated with the plasma membrane, but high affinity MEL receptors have also been identified in the nucleus in association with large amounts of MEL (Acuña Castroviejo *et al.*, 1994). This nuclear MEL receptor was subsequently found to repress expression of the 5-lipoxygenase gene through association with the orphan receptor RZR (Carlberg and Wiesenberg, 1995).

Radioligand binding studies, autoradiography and *in situ* hybridization have confirmed a wide distribution of MEL receptor subtypes throughout the periphery and CNS. The MEL_{1A} receptor is predominantly found in the hypophyseal pars tuberalis, with moderate expression in the SCN and

a restricted distribution elsewhere in the brain and body. This subtype is believed to mediate the circadian and some of the reproductive effects of MEL. Other brain regions that express the MEL_{1A} subtype include the median eminence, hypothalamus, medulla pons, hippocampus, cerebellum, parietal cortex, striatum, amygdala and medial basal hypothalamus. Peripheral tissues include the arteries, harderian gland, adrenal gland, heart and lungs, gastrointestinal tract, kidney, testes, ovary, uterus, mammary glands, liver and dermal melanophores (Morgan *et al.*, 1994; Reppert *et al.*, 1994; Reppert *et al.*, 1995a). The MEL_{1B} subtype is very abundant in the mammalian retina and shows very low expression in the brain (Reppert *et al.*, 1995a).

An important feature of MEL receptors is that, in most tissues, they exhibit a circadian rhythm in density. This most likely represents the basis for the chronotypic responsiveness of numerous tissue biochemical and physiological responses to MEL. Pinealectomy and MEL administration are capable of phase-shifting this day-night rhythm and also alter the density of the receptors (Stankov and Reiter, 1990; Morgan *et al.*, 1994). Characterization of the different MEL receptor subtypes has led to the design of selective and potent agonists and antagonists, including S-20098, luzindole and ML-23 (Dubocovich, 1988; Zisapel and Laudon, 1987; Cajochen *et al.*, 1997). These and other synthetic ligands will circumvent the problems associated with using an endogenous ligand, such as MEL. They are thus likely to be useful chronobiotics in circadian research and in delineating the receptor-mediated effects of MEL.

1.4.3 MELATONIN AND SIGNAL TRANSDUCTION

An understanding of the signal transduction events underlying the receptor-mediated effects of MEL may help clarify the cellular mode of action of the hormone. In this regard the cyclic nucleotides cAMP and guanosine 3′:5′-cyclic monophosphate (cGMP) appear to be the most important second messengers. Both the MEL_{1A} and MEL_{1B} receptor subtypes are negatively coupled to adenylate cyclase through a pertussis toxin-sensitive G_i protein (Carlson *et al.*, 1989; Reppert *et al.*, 1995). Characteristically, activation of these receptors results in an inhibition of adenylate cyclase and a reduction in cAMP accumulation in all tissues expressing the receptors, including the hypothalamus, pituitary and retina (Vacas *et al.*, 1981; Iuvone and Gan, 1994; Vanecek, 1995). In contrast, MEL *in vitro* potently enhanced cGMP accumulation in the medial basal hypothalamus of rats (Vacas *et al.*, 1981). This is consistent with the ability of MEL to induce both central and peripheral guanylate cyclase activity (Vesely, 1981). Indeed it is proposed that the inhibitory effect of MEL on Ca^{2+} entry into neurons may be related to its effect on intracellular cGMP levels (Vanecek, 1995). The intracellular levels of cAMP and cGMP of several

brain regions exhibit circadian rhythms that are endogenous by nature (Nagayama, 1993). Typically, the nucleus accumbens, SCN and amygdala show such a rhythm for both cyclic nucleotides, whereas the olfactory tuberculum and caudate-putamen only have day-night variations in cGMP levels. A more recent study has confirmed the existence in circadian fluctuations of cAMP content in the SCN and anterior hypothalamus (Yamazaki *et al.*, 1994). Preliminary studies have also found a circadian rhythm in adenylate cyclase and phosphodiesterase activity of some brain regions (Nagayama, 1993)

Several other minor, but potentially important, signal transduction systems have been associated with MEL. Vanecek and Vollrath (1990) have presented evidence that, in the anterior pituitary of the rat at least, diacylglycerol and arachidonic acid may be second messengers of MEL, also through a pertussis toxin-sensitive mechanism. Cardinali *et al.* (1980) reported that nanomolar concentrations of MEL suppress prostaglandin E_2 release from the medial basal hypothalamus. Finally, MEL was found to stimulate phosphatidyl inositol turnover in the chicken brain (Popova and Dubocovich, 1995).

Besides events mediated by nuclear and plasma membrane receptors, MEL also exerts direct cytosolic effects that could potentially modify enzyme activity or the signal transduction events of other receptor systems. The amphiphatic nature of MEL allows it to cross all morphophysiological barriers and to enter every cell and all subcellular compartments, including the nucleus (Acuña Castroviejo *et al.*, 1994). Thus the potential targets of MEL are numerous. Through its action as a direct antagonist of calmodulin (CaM), MEL can also inhibit several key neuronal enzymes that display Ca^{2+}/CaM dependent activities. These include Ca^{2+}/CaM-kinase II, cAMP phosphodiesterase and Ca^{2+}/Mg^{2+} ATPase (Benítez-King *et al.*, 1996). In particular, Ca^{2+}/CaM-kinase II is abundant in the nervous system where it regulates neurotransmitter synthesis and release, axonal transport, and receptor mobilization.

Pozo *et al.* (1994) found that physiological concentrations of MEL inhibited the activity of nitric oxide synthase in rat cerebellum *in vitro* and *in vivo*. The enzyme product, nitric oxide (NO), is believed to be a diffusable secondary messenger capable of retrograde transmission. NO has been shown to play an important role in memory process of hippocampal long term potentiation. In addition, this effect of MEL on NO synthase was blocked by CaM, consistent with the ability of MEL to bind to this protein. Finally, much current investigation has focused on the powerful antioxidant capacity of MEL. This hormone is a potent scavenger of the cytotoxic hydroxyl

radicals and other reactive oxygen species, and influences numerous enzymes and factors associated with the intracellular antioxidative defense system, such as glutathione (Reiter, 1995). In summary, MEL is a ubiquitous hormone displaying a distinct functional pleiotropy and multiple sites of action.

1.4.4 Evidence of Melatonin in Dopaminergic Systems

The uptake and accumulation of plasma MEL has been observed in several important dopaminergic areas of the rat and human brain. These regions include the caudate nucleus, substantia nigra, nucleus accumbens, amygdala, arcuate nucleus of the hypothalamus, habenular complex and the hippocampus (Kopp *et al.*, 1980; Sequela *et al*, 1982; Zisapel and Laudon, 1982; Vitte *et al.*, 1988).

The rat striatum expresses a high density of high affinity ^{125}I-MEL binding sites. However, unlike other brain regions, a day-night variation in the density and affinity of these sites was not evident (Laudon *et al.*, 1988; Zisapel *et al.*, 1988). For this reason, any receptor-mediated effect of MEL in the striatum is not likely to be chronotypic or display refractory periods. To add to the paradox, striatal ^{125}I-MEL binding sites are also insensitive to the age-related decrease in MEL receptor density seen in other brain regions, especially the hypothalamus (Laudon *et al.*, 1988). This has led to the following statement by Zisapel *et al.* (1988): "The role of the striatal ^{125}I-MEL-binding sites is still an enigma." Ten years later, this statement still rings true.

1.5 Evidence for Dopaminergic Modulation of the Pineal Gland

Numerous neurotransmitter systems modify pineal function through a plethora of receptor types that have been identified on membrane preparations of pineal glands (Cardinali *et al.*, 1987). These include, *inter alia*, adrenergic, cholinergic, serotonergic and opioidergic receptors, and binding sites for gamma-aminobutyric acid (GABA). In contrast, evidence for the modulation of pineal and MEL function by dopaminergic systems is rather sparse and fragmented. An attempt to compile all such relevant information will be made presently.

A circadian rhythmicity in TH activity and DA and NA content, with peak timing in the dark-phase, has been well established in the pineal gland (Craft *et al.*, 1984; Abreu *et al.*, 1987; Hermes *et al.*, 1994; Miguez *et al.*, 1995). This increase in catecholamine synthesis is temporally

correlated with the pineal noradrenergic secretory drive. To reiterate, the nocturnal surge in MEL synthesis is dependent on increased release of presynaptic NA vesicular stores during the dark-phase. Of particular importance is the observation that the DA turnover rate, which also peaks in the dark-phase, shows a more apparent and robust unimodal rhythm than the NA turnover rate (Craft *et al.*, 1984; Hermes *et al.*, 1994). This is a consequence of the fact that DA serves as a precursor of NA during catecholamine biosynthesis. The synthesis of DA and NA both require the rate-limiting enzyme TH, whereas dopamine-β-hydroxylase (DBH) catalyses the conversion of DA to NA. A scheme of catecholamine biosynthesis is shown in Figure 1.3. Thus the day-night variation observed for pineal DA and TH is typically seen as a consequence of the rhythmicity in the NA secretory drive.

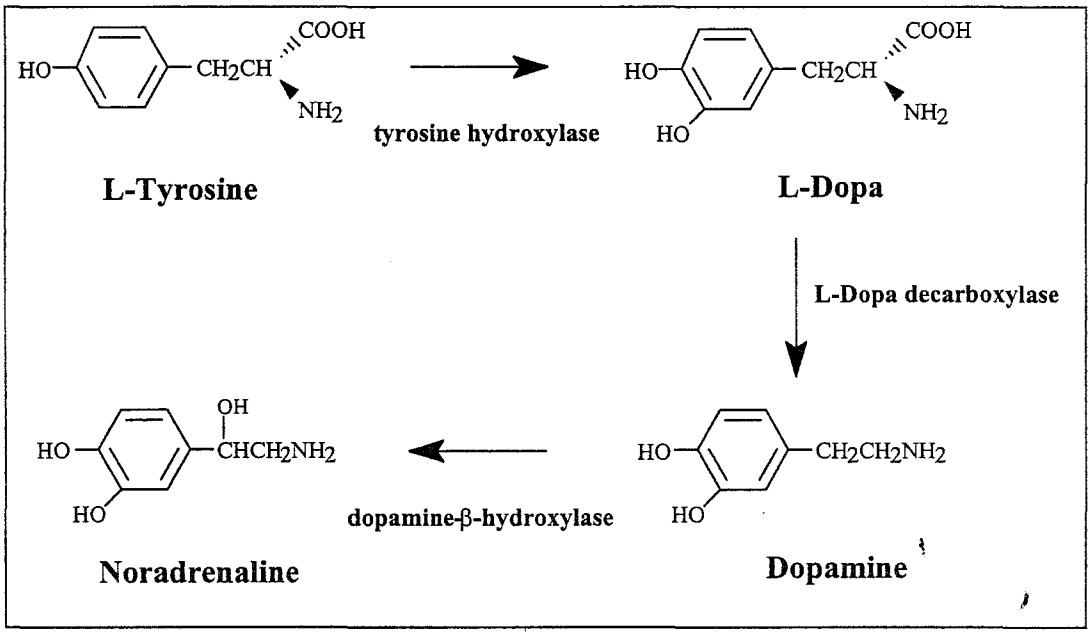


Figure 1.3: Biosynthesis of the catecholamines DA and NA.

For this reason, the existence of an independent dopaminergic system within the pineal has to be experimentally delineated from the noradrenergic system before pineal DA can be seen as a neurotransmitter in its own right. Indeed, the failure of earlier studies can be attributed to their inability to successfully distinguish between the two neurotransmitter systems due to lack of specificity of agents employed. For example, both L-Dopa and amphetamine were found to induce pineal NAT activity, in synergism with NA (Deguchi and Axelrod, 1972a; Altar *et al.*, 1981). L-

Dopa is a precursor of both DA and NA, and amphetamine is a releasing agent of the vesicular stores of both monoamines. Thus the observed induction may have been mediated by either catecholamine or both.

Initially, attention will be paid to immunohistochemical studies with antibodies directed against the catecholamine-synthesizing enzymes TH and DBH. Synthesis of DA requires only TH, whereas synthesis of NA would require the presence of both enzymes. Several studies (Jin *et al.*, 1988, Shiotani *et al.*, 1989, Hermes *et al.*, 1994) have confirmed the existence of TH-positive/DBH-negative immunoreactive neuron-like cell bodies in the pineal gland of adult golden and Djungarian hamsters, but not of rats and gerbils. These cells were unaffected by bilateral superior cervical ganglionectomy, whereas TH-positive/DBH-positive (noradrenergic) neurons were clearly reduced, as expected. Thus it was proposed that these TH-positive/DBH-negative cells are dopaminergic neurons, intrinsic to the pineal gland. They exhibited close proximity to pinealocytes cell bodies and processes, though no typical synapses were seen. It remains unclear whether these cells have axons projecting outside the pineal gland. Intrapineal neurons are generally considered to be uncommon (Shiotani *et al.*, 1989).

In another study on the Wistar rat, TH-immunoreactive fibres, but not cell bodies, were found in the superficial pineal, pineal stalk, deep pineal and the habenular complex and posterior commissures. Following bilateral superior cervical ganglionectomy, TH-fibres were completely abolished except in the deep pineal, pineal stalk and rostral part of the superficial pineal (Zhang *et al.*, 1991). This would suggest an extra-sympathetic origin of these fibres. Indeed a similar pattern of TH-immunoreactivity was found to remain after incubating intact rat pineal glands for 72 hrs (Hernández *et al.*, 1994). Prolonged incubation degenerates the presynaptic noradrenergic nerve terminals (Klein *et al.*, 1983), without damaging the pinealocytes (Santana *et al.*, 1994). Additionally, bilateral superior cervical ganglionectomy and 72 hr incubation only caused a partial reduction in TH activity, whereas NA content was reduced by 99% (Schwarzchild and Zigmond, 1989; Hernández *et al.*, 1994; Santana *et al.*, 1994). On the otherhand, dispersion of pinealocytes completely abolishes both TH-immunoreactivity and TH activity (Hernández *et al.*, 1994). TH-positive/DBH-negative immunoreactive nerve fibres, but not cell bodies, have also been identified in the porcine pineal (Kaleczyc *et al.*, 1994). All these studies point to the existence of fibres of extra-sympathetic origin.

It is well-established that the mammalian gland, in addition to direct projections from the SCG, is also innervated by central nerve fibres via the pineal stalk. These pinealopetal fibres originate from several forebrain and midbrain structures and peripheral ganglia. These include the habenular complex, posterior commissures, the lateral geniculate body, stria medullaris thalami, the periventricular gray of the mesencephalon, and the pterygopalatine and trigeminal ganglia (Møller and Korf, 1983; Matsuura *et al.*, 1994). Thus it is proposed that in the rat (and porcine) pineal gland some of these fibres are dopaminergic in nature with cell bodies located outside the pineal gland. This is in contrast to the hamster, where the putative dopaminergic neurons appear to be intrinsic to the pineal.

It now remains to try and clarify the nature and function of these dopaminergic fibres of the rat pineal gland. For example, following bilateral superior cervical ganglionectomy, the remaining TH activity did not exhibit the typical circadian rhythmicity seen in the intact pineal, with a peak in the dark-phase (Hernández *et al.*, 1994). This would suggest that the pineal dopaminergic system is not influenced by photoperiod.

Govitrapong *et al.* (1989) found that DA *in vitro* had bi-phasic effects on the NAT activity of rat pineal glands. As it was unstated, it is presumed that this study was performed during the light-phase. At a concentration of 0.1 μM, DA inhibited, and at concentrations between 0.1 μM and 10.0 μM had no effect, whereas 100 μM stimulated basal NAT activity. The latter response was partially reversed by the β-adrenergic antagonist, propranolol and supports the finding of an earlier study. DA (30 μM) doubled the *in vitro* synthesis of [^{14}C]MEL and [^{14}C]5HT from [^{14}C]tryptophan by rat pineals (Axelrod *et al.*, 1969). Govitrapong *et al.* (1989) also reported that relatively high concentrations of the selective D_2 agonists bromocriptine and LY-171555 *in vitro* prevented NA-induced NAT activity, an effect blocked by preincubation with the D_2 antagonists domperidone and haloperidol (HAL). Acute *in vivo* administration of HAL and the atypical neuroleptic sulpiride increased both pineal NAT activity and MEL content. However, chronic administration of bromocriptine to rats failed to alter basal pineal NAT activity. These results suggest that the stimulatory effects of high DA concentrations were mediated by the β-adrenergic receptors, whereas the inhibitory effects were independently mediated by putative postsynaptic D_2 receptors. The latter also appeared amenable to down-regulation (Govitrapong *et al.*, 1989). This would appear to confirm an earlier finding by Govitrapong *et al.* (1984). Radioligand binding studies with [^{3}H]spiroperidol revealed specific low and high affinity sites on synaptic membranes

of bovine pineal glands. The former site showed relatively high affinity for HAL, cis-flupenthixol, chlorpromazine (CPZ), domperidone and (+)-butaclamol, but low affinity for (±)-sulpiride, DA and apomorphine (APO). These authors argue that this pharmacological profile is consistent with the D_2 receptor subtype.

The D_2 receptor is negatively coupled to adenylate cyclase (Niznik, 1987; Strange, 1987). Ligand-induced activation of this receptor results in an inhibition of adenylate cyclase and, thus, decreased cAMP levels. Govitrapong *et al.* (1989) propose that this is consistent with the inhibitory effects of DA observed at low concentrations of the catecholamine. Stimulation of postsynaptic D_2 receptors located on pinealocytes will result in a reduction of cAMP-dependent activity of pineal NAT.

It is unfortunate, however, that Govitrapong *et al.* (1989) did not use a more selective D_2 radioligand, as the concomitant high-affinity binding of [^{3}H]spiroperidol to putative 5HT receptors undermined the conclusiveness of these results. Furthermore, both bromocriptine and quinpirole have weak agonist activity at α-receptors. Zawilska and Iuvone (1990) have strongly contended that the ability of bromocriptine and quinpirole to suppress the nocturnal increase in NAT activity and MEL content of chicken pineals are mediated by α_2-receptors, and not by D_2 receptors. This may simply reflect species-specific differences in the receptor-mediated regulation of pineal function or a possible problem with the interpretation of the results of Govitrapong *et al.* (1984 and 1989). Indeed the reported effects of bromocriptine and quinpirole (Govitrapong *et al.*, 1989) occurred at relatively higher concentrations than expected for D_2 receptors. In another study DA (30 μM), but not APO (30 μM) increased phosphatidyl inositol turnover in the rat pineal, an effect clearly mediated by α-adrenergic receptors (Nijjar *et al.*, 1980). In contrast, D_2 receptors are also coupled to an inhibition of phosphatidyl- inositol turnover (Niznik, 1987; Strange, 1987).

Further clarification can be sought through pharmacological studies with other dopaminergic agents. Particular focus will be placed on the effect of D_2 receptor antagonists, commonly referred to as antipsychotic drugs or neuroleptics, on pineal function.

Generally, acute administration of HAL, (±)-sulpiride and CPZ to rats has been reported to dramatically increase the MEL content of pineal glands during both the light- and dark-phases (Gaffori *et al.*, 1983; Govitrapong *et al.*, 1989; Srinivasan, 1989; Ozaki *et al.*, 1976). HAL and

sulpiride also induce a concomitant increase in pineal NAT activity during the light-phase, an effect consistent with their ability to antagonize the inhibitory effect of DA, putatively mediated by postsynaptic D_2 receptors (Govitrapong *et al.*, 1989). This profile is consistent with the *in vitro* effects of CPZ and HAL on the metabolism of [^{14}C]tryptophan by rat pineal glands cultured during the dark-phase (Nir and Hirschmann, 1983). HAL increased the synthesis of [^{14}C]MEL, [^{14}C]5HT, decreased [^{14}C]5HIAA levels and had no effect on [^{14}C]NAS. This suggests that HAL may have increased HIOMT activity without an effect on NAT activity, and inhibited MAO activity. The increase in [^{14}C]5HT levels would be consistent with an inhibition of MAO, but perhaps also a potentiation of tryptophan hydroxylase activity or tryptophan uptake. In turn, CPZ increased both [^{14}C]NAS and [^{14}C]MEL and decreased [^{14}C]5HIAA and [^{14}C]5HTOH without influencing [^{14}C]5HT levels. Thus CPZ may have stimulated either NAT or HIOMT activity or both, and inhibited MAO activity. The effects of CPZ were potentiated by NA *in vitro*, consistent with the known ability of CPZ to enhance NA release from presynaptic nerve terminals. In contrast, HAL differentially reversed or potentiated the effects of NA *in vitro* (Nir and Hirschmann, 1983). However, Wakabayashi *et al.* (1989) demonstrated a dose-dependent decrease in the MEL content of rat pineals during the dark-phase following acute administration of either HAL or CPZ. The pineal content of 5HT and NAS were unaffected, which is consistent with an inhibition of HIOMT and not NAT activity by the neuroleptics.

Certain DA antagonists can also influence pineal function and MEL levels by direct, non-receptor-mediated mechanisms. HAL and CPZ, but not fluphenazine, *in vitro* dose-dependently inhibited both crude and purified HIOMT activity, isolated from bovine pineal glands (Hartley *et al.*, 1972; Cremer-Bartels *et al.*, 1983). On this basis, these neuroleptics would be expected to decrease pineal MEL synthesis as reported by Wakabayashi *et al.* (1989), but contested by Nir and Hirschmann (1983). It is possible that there are species-specific differences in pineal HIOMT, resulting in a differential susceptibility to direct inhibition by certain neuroleptics.

Ultimately plasma and brain MEL levels, and thereby the function of this indole, are determined by both synthesis and metabolism. Acute administration of the phenothiazine neuroleptics CPZ, promethazine or promazine dose-dependently and time-dependently increased the plasma and tissue half-life of exogenous [^{3}H]MEL, following intravenous, but not intraventricular administration of the indole (Wurtman *et al.*, 1968a). Furthermore CPZ *in vitro* dose-dependently inhibited the catabolism of [^{3}H]MEL by liver slices (Wurtman *et al.*, 1968a). This suggests that phenothiazines increase plasma MEL levels by inhibiting the catabolism of MEL in the liver by

microsomal enzymes, and not via an effect on pineal MEL synthesis. Indeed hepatic hydroxylation to 6-hydroxymelatonin is the main route for peripheral metabolism of MEL (Kopin *et al.,* 1961). Ozaki *et al.* (1976) have confirmed that the administration of CPZ increases the plasma half-life of both exogenous nonlabelled MEL, administered intravenously, and endogenous MEL present in plasma and the pineal gland during the light-phase. These authors propose that the increase in pineal MEL is consistent with an additional effect of chlorpromazine on MEL synthesis. Interestingly, they attribute this to an antagonism of pineal α-receptors, thereby increasing NAT activity (Ozaki *et al.*, 1976).

Similar results have been found in human beings. Chronic administration of CPZ dose-dependently elevated serum, but not CSF, MEL levels in psychiatric patients, whereas the antipsychotic drugs flupenthixol and fluphenazine had no effect (Smith *et al.*, 1979). Furthermore, the phenothiazine derivative pipotiazine failed to alter the amplitude, waveform or timing of plasma MEL levels when administered to schizophrenic patients (Loloum *et al.*, 1993). These studies clearly indicate that an inhibitory effect on MEL catabolism in the liver is not a general characteristic of neuroleptics, but shows specific structure-activity relationships that can be partially classified according to the class of antipsychotic drug.

Finally, strong evidence for dopaminergic control of MEL function is also seen at the level of other components of the retinohypothalamic tract, including the photosensory retina and SCN, and other types of pineals. Unlike mammalian species, the pineal gland of a poikilothermic animal, such as the rainbow trout, is photoreceptive. A circadian intrapineal dopaminergic system has been characterized in the trout pineal. Inhibitory effects of DA on MEL release and neural activity of this gland appear to be mediated by D_1 and D_2 receptors, respectively (Martin and Meissl, 1992). An endogenous MEL-generating system, similar to that of the pineal gland, is also present in the chick and mammalian retina (Dubocovich, 1988; Zawilska and Iuvone, 1990). NAT activity and MEL biosynthesis peak during the scotophase. Several circadian oscillators appear to be operating in the mammalian retina and can be entrained directly by the light:dark cycle (Tosini and Menaker, 1996). In turn, the retinal amacrine cells are dopaminergic in nature (Dubocovich, 1983). In contrast to a suppression of retinal NAT activity, a light stimulus induces TH activity and thus DA biosynthesis (Iuvone *et al.*, 1978). Retinal DA D_4 receptors mediate the ability of DA and agonists to inhibit NAT activity through a negative effect on the cAMP cascade (Dubocovich, 1988; Zawilska, 1994; Zawilska *et al.*, 1995). Thus it has been proposed that, within the retina at least, DA is the chemical expression of the onset of the photophase. Finally, a dopaminergic

system is also operative within the mammalian SCN. For example, D_1 and D_2 agonists influence the period and amplitude of the rest-activity cycle mediated by the SCN (Yamada and Martin-Iverson, 1991). In particular, D_1 receptor-mediated events are very robust in the fetal SCN, but photic control may be transferred to a glutamatergic system with ontogenic development of the retinohypothalamic tract (Weaver *et al.*, 1992; Viswanathan *et al.*, 1994).

1.6 Evidence for Dopamine-Melatonin Interactions in the CNS

1.6.1 DOPAMINERGIC SYSTEMS

Dopaminergic innervation in the brain is widespread and diffuse. This is consistent with the modulatory nature of DA, behaving more like a hormone than a neurotransmitter. This is consistent with DA acting on metabotropic receptors rather than iontotropic receptors, the latter being responsible for rapid and direct excitatory or inhibitory effects on cells (Cohen and Servan-Schreiber, 1993). DA represents more than 50% of the total catecholamine content of the CNS of most mammals with highest levels being found in the caudoputamen, nucleus accumbens and tuberculum olfactorium. The main dopaminergic pathways are found in the basal ganglia (Moore and Bloom, 1978).

The basal ganglia are subcortical structures that are involved in the initiation and co-ordination of movement. The two main mesencephalic dopaminergic pathways arise from cell bodies in the midbrain. The nigro-striatal (or mesostriatal) pathway originates from the pars compacta zone (A9 cell group) of the substantia nigra and terminates in the caudoputamen. Indeed the neostriatum receives the richest dopaminergic innervation of any brain structure. It must be pointed out that the term neostriatum refers to the caudate and putamen in primates. In the case of rodents, the term corpus striatum encompasses the caudate, putamen and an additional subcortical area, the globus pallidus. The mesolimbic pathway originates in the ventral tegmental area (A10 cell group) and sends axons to the nucleus accumbens, lateral septal nucleus and amygdala. In turn, the olfactory tubercle is targeted by both the mesostriatal and mesolimbic pathways. In addition to modulating locomotion, the mesolimbic pathway also controls emotive behaviour, such as grooming and sniffing. Thus the mesolimbic pathway is often referred to as the reward system of the brain and has been implicated in affective disorders. A second system, the mesocortical pathway, also originates in the A10 cell group and projects to parts of the cerebral cortex, including the cyru cinguli and entorhinal and prefrontal cortices. The entire ascending forebrain

projection of the mesencephalic dopaminergic system makes up the mesotelencephalon (Moore and Bloom, 1978).

Input structures of the basal ganglia, including the caudoputamen receive projections from the frontal, parietal and temporal lobes of the cerebral cortex. In turn, output of the basal ganglia is sent via the thalamus to motor areas of the frontal and prefrontal cortices and visual areas of the inferotemporal cortex. Thus, in summary, the basal ganglia "funnels" information from diverse areas of the cortex in order to direct motor output (Middleton and Strick , 1996). This "funneling" process arises through a functional interaction between glutamatergic, GABAergic and dopaminergic pathways and local cholinergic interneurons (Graybiel, 1990). Of particular importance is the high expression of D_1 and D_2 DA receptors in the striatum, with a predominance of the D_1 subtype (Camps *et al*, 1990). GABAergic interneurons in the striatum and globus pallidus send descending projections with nerve terminals located in both the pars compacta and pars reticulata zones of the substantia nigra. This is known as the striato-nigral pathway. Input to the striatum from the cortex activates this pathway, inducing the release of GABA in the pars reticulata zone. GABA receptors are located on the cell bodies or dendrites of dopaminergic neurons in the pars reticulata zone, and in the corpus striatum. Activation of the GABA receptors decreases the firing rate of the dopaminergic neurons and thus inhibits DA release. In this way, the substantia nigra pars reticulata, and thus the output to the thalamus, is held under tonic inhibition by the cortical inputs.

The nigro-striatal pathway serves to reverse the inhibitory output of the pars reticulata zone and thereby initiates movement by enhancing impulse flow to the subthalamic nucleus. This is achieved by the release of DA in the corpus striatum, which results in an inhibition of the GABAergic neurons via an effect on the cholinergic interneurons (Graybiel, 1990). The release of dendritic DA from nigral neurons also plays a crucial modulatory role on the activity of the nigro-striatal pathway and the pars reticulata neurons. The striatal outputs are segregated with respect to D_1- and D_2-dependent behaviours. This occurs as a result of the differential distribution of these subtypes in the striatum and substantial nigral zones, especially with respect to postsynaptic and presynaptic location in relation to the dopaminergic neurons (Graybiel, 1990; Camps *et al*, 1990). It is proposed that postsynaptic D_1 receptors mediate the direct stimulation of striatonigral GABAergic neurons, which co-express substance P. Whereas inhibitory D_2 receptors located on striatal GABAergic neurons form an indirect pathway. The latter neurons express enkephalin and project to the globus pallidus (Graybiel, 1990; Camps *et al*, 1990).

Another interesting feature of the nigro-striatal pathway is that the dopaminergic fibres show a slow rate of spontaneous firing. For this reason, the release of DA at presynaptic nerve terminals is generally steady and uniform, and not quantal like acetylcholine. An increase in the firing rate of these neurons is only seen after pharmacological manipulation. In contrast, the mesocortical dopaminergic neurons have an unusually high rate of firing, similar to the retina (Tam and Roth, 1997).

Two other very important pathways, mediating the neuroendocrine effects of DA, are the tuberoinfundibular (TIDA) and tuberohypohyseal (THDA) dopaminergic pathways. Both originate in the arcuate nucleus (A12 cell group) of the medial basal hypothalamus (MBH) sending projections to the neurointermediate lobe (NIL) of the posterior pituitary and the median eminence (Moore and Bloom, 1978). DA released from the median eminence enters the hypophyseal portal system and is transported to the anterior pituitary.

1.6.2 NEUROENDOCRINOLOGY

Of all the main dopaminergic systems, it will become apparent that the strongest evidence for DA-MEL interactions is seen at the level of the hypothalamic-pituitary axis. This is consistent with the fact that this axis mediates the main antigonadotrophic and counter antigonadotrophic effects of MEL (Reiter, 1989; Reiter, 1995). Although species-related differences are apparent, both MEL and DA are known to modulate the synthesis and release of numerous hormones in the rodent. These include prolactin, somatostatin, growth hormone, thyroid-stimulating hormone, follicle-stimulating hormone, gonadotropin-releasing hormone, luteinzing hormone, oxytocin and vassopressin. Furthermore, both MEL and DA have been implicated in the pathogenesis of numerous neuroendocrine and chronobiological disorders associated with the hypothalamic-pituitary axis (Sandyk, 1990a). Whether this is a causal or a coincidental relationship would make for interesting research.

A direct interaction between MEL and DA is believed to exist, particularly, at the level of prolactin. DA is a well known prolactin-inhibiting factor. The release of DA from the TIDA and THDA systems results in an inhibition of the synthesis and release of prolactin. In turn, MEL appears to be responsible for the circadian rhythm of plasma prolactin levels, evident as an afternoon surge in the release of the hormone. Pinealectomy and *in vivo* administration studies

suggest that MEL synchronizes the prolactin surge through modulation of the dopaminergic systems (Mai and Pan, 1995; Steger *et al.,* 1995; Alexiuk *et al.*, 1996).

1.6.3 BIOCHEMISTRY AND PHARMACOLOGY

Evidence for day-night variations in DA function, particularly circadian rhythmicity, and modification by photoperiod manipulation, pinealectomy and MEL administration are taken as being possible indicators of modulation by MEL. Brain DA function shows considerable dynamic fluctuations through the light-dark cycle. In fact, several brain regions that do show diurnal variations in DA function are innervated by efferent projections from the SCN, e.g. the hypothalamic paraventricular nucleus which regulates the hypothalomo-pituitary-adrenocortical axis, and the medial preoptic area which contains thermosensitive neurons responding to local brain temperature (Ozaki *et al.*, 1993). Conflict, however, arises in the nature of the temporal characteristics of this flux. This dispute may be attributed to several factors, including: whole brain *vs.* region-specific determinations; strain- and species-specific differences, particularly diurnal *vs.* nocturnal species; ontogenic development and aging; and, finally, methodological differences. In this review, consideration will be paid mostly to brain region-specific studies, particularly the main dopaminergic systems of the cortex, hypothalamus and basal ganglia. Whole brain determinations will tend to mask or average any differences in the temporal characteristics of DA function between specific regions of the brain. In addition, attention will be focused mostly on the brain of rodents, especially rats.

1.6.3.1 Levels of Dopamine and Metabolites

Early studies typically observed a unimodal rhythm of DA content in the striatum of nocturnal species such as rats, hamsters and gerbils. The content peaked in the mid-scotophase and exhibited a trough in the light-phase (Bobillier and Mouret, 1971; Friedman and Piepho, 1979; Matsumoto *et al.*, 1981; Basharan and Radha, 1984). The unimodal DA rhythm in the rat hypothalamus, cortex and striatum also exhibited age-dependent phase characteristics: peak amplitude in the photophase for young (21 day old) rats, but a dark-phase peak in adults (Basharan and Radha, 1984).

More recently, Pietilä *et al.* (1995) confirmed a unimodal rhythm in the mouse striatum. The content of DA and 3-methoxytyramine (3MT), an O-methylated metabolite of DA, showed a synchronous peak in the scotophase and a trough in the early photophase. The other DA metabolites, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), did not

exhibit any day-night variation. Schade *et al.* (1993a and 1993b) demonstrated more complex time-dependent variation in DA content in the rat striatum, substantia nigra and nucleus accumbens of rats. A normal circadian rhythm was coupled to additional ultradian (5-6 and 10-12 hr) and infradian (7-10 day) rhythms. The most robust peak occurred at mid-photophase for all three regions. Of additional interest, this DA rhythm was found to be out-of-phase with a similar rhythm in the neuropeptide cholecystokinin, which is known to co-exist with DA in these brain areas and displays neuroleptic properties. On the otherhand, Ozaki *et al.* (1993) failed to observe day-night variations in DA content of the caudate nucleus, substantia nigra, nucleus accumbens and ventral tegmental area of the golden hamster.

An analysis of extracellular levels of DA and its metabolites by *in vivo* microdialysis may help point to the origin of the flux of DA function. O'Neill and Fillenz (1985) found a robust circadian rhythmicity in extracellular HVA levels in the frontal cortex, striatum and nucleus accumbens of rats. Peak levels were found in the scotophase and correlated with peak motor activity in these nocturnal animals. In another study, the extracellular concentrations of DA and the metabolites DOPAC and HVA increased significantly at night in the dorsolateral caudate nucleus of rats (Paulson and Robinson, 1994). Whereas in the nucleus accumbens only DA metabolites, and not DA, exhibited circadian variation with a dark-phase peak. A lack of day-night variation in extracellular levels of DA in the nucleus accumbens of rats was confirmed by Piazza *et al.* (1996).

It must be noted that the observed flux of DA is a consequence of complex functional interactions between synthesis, storage, release and degradation. For this reason the *in vivo* turnover rate of DA is accepted as an ideal index of the functional state of the neuron. It represents the overall rate at which the whole amine store of a given tissue is replaced, and is not necessarily identical with biosynthetic rates. This index is measured following inhibition of either catecholamine synthesis or degradation.

Kempf *et al.* (1982) found distinct strain-specific differences in striatal DA turnover rates of C57 and BALB mice. Only the C57 strain exhibited distinct day-night variations in striatal DA turnover, with a peak in the dark-phase, and the rhythm was found to persist under conditions of constant light. Unfortunately, limited studies have assessed DA turnover rates in rats under a normal light-dark cycle. Lemmer and Berger (1978a) reported a bimodal rhythm of DA turnover in the whole brain of rats, with peaks in the late-photophase and early scotophase. In contrast, DA turnover in the lateral ventricular fluid of Rhesus monkeys peaks in the photophase (Perlow *et al.*,

1977). This correlates with the time of greatest arousal (activity) in this diurnal species. Lastly, a circadian rhythmicity in the DA turnover rate of the median eminence has been well-established for the rat TIDA pathway. The turnover rate is high during the early-photophase and low by late-photophase (Mai and Pan, 1995). This contrasts with the finding of Alexiuk *et al.* (1996) that the DA content of the MBH peaked in the dark-phase, whereas no rhythm was observed in the NIL of the pituitary.

In conclusion, the main dopaminergic pathways all show day-night fluctuations in DA activity as reflected by DA turnover and concentrations of DA and its metabolites. However, the phase characteristics of this rhythm are distinctly region-specific. There is strong agreement that, in nocturnal species, the dopaminergic activity of the striatum, substantia nigra, nucleus accumbens and ventral tegmental area increases with the onset of the scotophase. The initiation and control of locomotor activity and behaviour is mediated by these subcortical structures of the basal ganglia (Graybiel, 1990).

It is thus argued that the parallel increase in dopaminergic activity of these areas and spontaneous locomotor activity (SLA) over the light-dark transition is to be expected in nocturnally active species. In other words they are considered to be mutually inclusive (Lemmer and Berger, 1978b; O'Neill and Fillenz, 1985; Pietilä *et al.*, 1995; Piazza *et al*, 1996). Indeed the strain-specific differences in striatal DA turnover of C57 and BALB mice discussed above were also manifested in differences in motor activity. A circadian rhythmicity in wheel-running activity, with peak activity correlating with peak DA turnover in the dark-phase, was observed in both strains. However, both rhythms persisted under constant light conditions in the C57 mice only (Kempf *et al.*, 1982). Dopaminergic activity of the nucleus accumbens also appears to further increase on initiation of behavioural activities such as drinking and eating (Piazza *et al.*, 1996). Furthermore, rats can be divided into high and low respondents on the basis of novelty-seeking behaviour. High respondent rats show higher than average exploratory behavior, SLA and DA levels (Piazza *et al.*, 1996). However, Paulson and Robinson (1996) have argued strongly that the regulation of SLA *per se* is not a primary function of these dopaminergic pathways. They found a positive, but weak, correlation between DA neurotransmission in the dorsolateral caudate nucleus (as assessed by *in vivo* microdialysis) and SLA of freely-behaving rats. An even weaker correlation existed between activity and DA function in the nucleus accumbens.

Photoperiodic manipulation has marked effects on DA metabolism of several brain regions. This is particularly true for the THDA and TIDA pathways, which mediate the neuroendocrine effects of DA. Both Bobillier and Mouret (1971) and Friedman and Piepho (1979) and found that although reversal of the photoperiod significantly enhanced striatal DA levels of rats, the unimodal rhythm of DA still peaked in the subjective dark-phase. This would suggest that this rhythm is exogenous, most likely entrained by light and related to motor activity. Steger *et al.* (1995) investigated changes in DA turnover following the transfer of golden hamsters from long photoperiod to short photoperiod conditions. Both short- and long-term exposure to the shortened daylength enhanced DA turnover of the NIL of the posterior pituitary, without an effect on the steady-state DA content. Concomitantly, there was a decreased DA turnover in the hypothalamus and median eminence without an effect in the anterior pituitary. This NIL-associated increase in DA turnover was correlated with a short photoperiod-induced reduction in plasma prolactin. In a similar study, hypothalamic DA turnover was decreased in the MBH, but increased in the medial preoptic-suprachiasmatic nuclei. These effects were reversed by pinealectomy, indicating that MEL may mediate the effects of photoperiodism on hypothalamic DA (Steger *et al.*, 1984). This would then suggest that the various dopaminergic systems are independently and differentially phase-shifted by MEL, if at all.

Indeed, the circadian rhythmicity of tuberoinfundibular dopaminergic activity and the prolactin surge is abolished by bilateral superior cervical ganglionectomy (Mai and Pan, 1995), further suggesting a role for MEL. However, Shieh and Pan (1995) have demonstrated an endogenous cholinergic system that reverses the rhythm in DA activity and attenuates the afternoon surge in prolactin. It is unclear whether this cholinergic system acts independently of MEL or the SCN.

In other studies, short-term pinealectomy was found to have a minor or no effect on cortical DA levels of male rats during the light-phase (Sugden and Morris, 1979; Niles *et al.*, 1983), though there was some evidence for a gender-dependent effect (Sugden and Morris, 1979). However chronic blinding has been shown to decrease striatal DA levels in both sham-operated and pinealectomized male rats (Niles *et al.*, 1983). The known ability of pinealectomy to induce kindled seizures in rats was not associated with changes to DA content or turnover of several brain regions (Philo and Reiter, 1978; Stockmeier and Blask, 1986).

Changes induced by photoperiod manipulation and pinealectomy only offer indirect, but suggestive, evidence for modulation by MEL. Cotzias *et al.* (1971) found that a large single

intraperitoneal (i.p.) dose of MEL (400 mg/kg) to mice failed to increase whole brain levels of DA or to prevent the increase induced by L-Dopa. This suggests that MEL does not decrease the uptake of L-Dopa or act as a L-Dopa decarboxylase inhibitor. On the other hand, administration of smaller doses of MEL (250 μg/day, i.p.) over 5 days resulted in a significant rise in whole brain DA levels in rats exposed daily to a novel environment (Datta and King , 1979). Wendel *et al.* (1974) demonstrated that intra-arterial (250 μg/kg) and intracisternal (40 μg/kg) administration of MEL at mid light-phase almost doubled whole brain DA levels of male rats. It must be noted that i.p. administration of MEL results in rapid conversion of MEL to 6-hydroxymelatonin in the liver (Kopin *et al.*, 1961). This will minimize the efficacy of the administered MEL, whereas intracisternal injection would circumvent the blood-brain barrier and peripheral metabolism.

Several studies investigated the effect of chronic subcutaneous (s.c.) administration of MEL (25 μg/animal) to golden hamsters during the late photophase on the DA content of the median eminence/arcuate region of the MBH, and the NIL (Alexiuk and Vriend, 1991; Alexiuk and Vriend, 1993; Alexiuk *et al.*, 1996). The DA content of both areas was consistently reduced in castrated males, ovariectomized females, and sham-operated and control animals of both sexes. This indicates that the effect of MEL was not modified by or dependent on gonadal steroids. However, the MEL-induced reduction in amine content was more marked and consistent following 10 weeks administration of MEL, compared to 1, 3 or 5 weeks administration (Alexiuk and Vriend, 1993; Alexiuk *et al.*, 1996). Following the longer administration period, MEL abolished the circadian rhythm seen in the DA content of the MBH. It must be noted that these results contradict the short photoperiod-induced increase in DA content of the NIL reported by Steger *et al.* (1995). The latter authors argue that MEL should basically mimic short-photoperiodism and contend that the results of Alexiuk and Vriend (1993) are due to methodological differences.

Intranigral administration of ng quantities of MEL to mice dose-dependently decreased striatal dopaminergic function as reflected in an increased DA content and a reduced DOPAC/DA ratio (Bradbury *et al.*, 1985). Identical results were obtained following bilateral injection of MEL into the nucleus accumbens. Functionally, these biochemical changes were associated with significant changes in SLA and behaviour (see section 1.6.4). Both the behavioural and biochemical effects of intranigral MEL administration were partially reversed by sulpiride, a selective D_2 receptor antagonist. This suggests that MEL may have a modulatory effect on presynaptic DA autoreceptors located on dopaminergic cell bodies in the substantia nigra. However, the partial

reversal by sulpiride suggests a possible interaction of MEL with other neurotransmitter systems, for example 5HT and opioids, as reported in the nucleus accumbens (Gaffori and Van Ree, 1985a and 1995b).

The above discussion has established the existence and phase characteristics of temporal variation in DA levels and turnover rates in several brain regions. Evidence has also been presented for a region-specific modulation by MEL. It will now be determined whether MEL modulates monoamine synthesis, metabolism or induces changes in the rate of release of DA or a combination of these processes.

1.6.3.2 Dopamine Release

The day-night variation in the tissue content or extracellular levels of DA and its metabolites discussed above is most commonly attributed by researchers to a rhythm in DA release. Following the release of presynaptic DA stores, the catecholamine is postsynaptically O-methylated to 3MT. DA can also be oxidatively deaminated to DOPAC either postsynaptically or presynaptically following re-uptake into the presynaptic nerve terminal. For this reason, extracellular levels of both HVA and 3MT, but in particular 3MT, are taken as reliable indicators of DA release (Kehr, 1976).

In vitro superfusion studies have consistently shown that picomolar concentrations of MEL inhibit the Ca^{2+}-dependent release of ^{3}H-DA evoked by electrical field stimulation and K^{+}-induced depolarization, but not spontaneous Ca^{2+}-independent release (Zisapel and Laudon, 1982; Zisapel *et al.*, 1982; Dubocovich, 1983, 1984 and 1988; Zisapel and Laudon, 1983; Nowak, 1988). However, the aforementioned inhibitory effect appears to be distinctly region-specific. It was observed in the following areas: hypothalamic preoptic area, median and posterior hypothalamus, ventral hippocampus and medulla pons of female rats in oestrus (Zisapel *et al.*, 1982), and retinas of the rabbit (Dubocovich, 1983; Nowak, 1988) and chicken (Dubocovich, 1984). Within the hypothalamus, the greatest effect was seen in the preoptic area including the SCN (Zisapel *et al.*, 1982). No effect was found in the dorsal hippocampus, cerebellum, cerebral cortex and the striatum of rats (Zisapel *et al.*, 1982). More recently, MEL has also been shown to regulate endogenous DA release in the amphibian retina (Boatright, 1994). Furthermore MEL *in vivo* dose-dependently inhibited the amphetamine-induced increase in extracellular DA levels, an indication of release, in the anterior hypothalamus of male rats, which incorporates the SCN (Exposito *et al.*, 1995).

Radioisotopic studies with $^{45}Ca^{2+}$ and a calcium ionophore have further clarified that MEL exerts this effect by reducing maximal presynaptic Ca^{2+} entry following a depolarization stimulus (Zisapel and Laudon, 1983; Vacas *et al.*, 1984). The ability of MEL to inhibit ^{3}H-DA release is also definitely mediated by specific MEL receptors (Dubocovich, 1983, 1984 and 1988; Zisapel and Laudon, 1987). Thus it is proposed that these receptors are located presynaptically and either directly coupled to presynaptic Ca^{2+} channels or regulate these channels through a sequelae of signal transduction events.

The predominance of this effect of MEL in the hypothalamus is consistent with the well-established anti-gonadal, entraining and neuroendocrine effects of the hormone. However the distribution reported above is more region-specific than the distribution of high affinity MEL receptors. In particular, the apparent lack of effect of MEL on DA release in the striatum is paradoxical and requires due consideration.

The striatum receives the richest dopaminergic innervation and expresses high affinity MEL receptors (Moore and Bloom, 1978). *In vivo* microdialysis studies of the rat striatum have demonstrated a distinct and robust circadian rhythm in the release of endogenous DA, with peak release correlating with increased motor activity in the dark-phase (O'Neill and Fillenz, 1985; Pietilä *et al.*, 1995; Piazza *et al*, 1996; Paulson and Robinson, 1996). Furthermore, this spontaneous rhythm is modified by the oestrus cycle in female rats (Dluzen and Ramirez, 1985). Here it must be noted that the original study of Zisapel *et al.* (1982) used female rats in oestrus during the early photophase. A subsequent study with female rats revealed that the inhibition of ^{3}H-DA release by MEL in the hypothalamic preoptic area was dependent on the stage of the oestrus cycle (Zisapel *et al.*, 1983). The greatest effect was seen at the oestrus stage and the weakest at the diestrous stage. In male rats the inhibitory effect of MEL was constant, but weaker in all hypothalamic areas (Zisapel *et al.*, 1983). This is consistent with the finding that the sex steroid estradiol alone can modify MEL-induced inhibition of hypothalamic DA release (Zisapel, 1987). However the apparent lack of an *in vitro* inhibitory effect of MEL on striatal ^{3}H-DA release during the photophase has been confirmed in both male and female rabbits (Dubocovich, 1983; Nowak, 1988).

Zisapel *et al.* (1985) also found that the effect of MEL on hypothalamic ^{3}H-DA release was circadian in nature with inhibition maximal in the early light-phase and minimal in the late light-phase. This parallels the circadian rhythmicity of hypothalamic MEL receptors and coincides with

the chronotypic counter-antigonadotrophic response to MEL administration. All the above studies, including those on the striatum, were performed during the light-phase. Therefore it is possible that the previous studies missed the "circadian window" of maximal effect in the striatum. DA turnover and release peaks in the photophase within the hypothalamus, but within the striatum it is maximal in the scotophase. In other words, peak DA release in the striatum coincides with the timing of the nocturnal surge in plasma MEL levels. This in itself would tend to support the results that MEL does not inhibit striatal DA release. Apparently there is no circadian rhythm in the density of striatal MEL receptors (Laudon *et al.*, 1988; Zisapel *et al.*, 1988), indicating a possible lack of chronotypic and state-dependent responsiveness to MEL. Thus the striatal MEL receptor may represent a different subtype, be functionally coupled to an alternative signal transduction system, other than the Ca^{2+} channel, or be located postsynaptically rather than presynaptically. Presynaptic DA release is regulated by multiple types of Ca^{2+} channels (Turner *et al.*, 1993). Perhaps different channels regulate release in the hypothalamus and striatum.

However, MEL may inhibit DA release in the substantia nigra and nucleus accumbens. The reduced DOPAC/DA ratio reported in these areas of mice following direct application of MEL is consistent with an inhibition of DA release (Bradbury *et al.*, 1985). Indeed, unlike the striatum, there is no indication for increased DA release *in vivo* in the nucleus accumbens during the dark-phase (Paulson and Robinson, 1994; Piazza *et al.*, 1996). This may indicate inhibition by endogenous MEL.

1.6.3.3 Dopamine Uptake

Re-uptake by the presynaptic DA transporter represents the main route by which the synaptic function of DA is terminated. In preliminary *in vitro* studies, MEL inhibited DA uptake in the rat hypothalamus (Cardinali *et al.*, 1975; Zisapel and Laudon, 1982). This effect was noncompetitive and may be non-specific in that the synaptosomal uptake of 5HT, NA and glutamate was also impaired (Cardinali *et al.*, 1975). A parallel study confirmed that pinealectomy, superior cervical ganglionectomy and *in vivo* MEL administration modified hypothalamic DA uptake (Cardinali, 1975).

Morisette and Dipado (1993) found that striatal DA uptake sites vary with sex and the oestrus cycle. Additionally, the circadian nature of DA uptake in adrenal chromaffin cells was found to be dependent on a circadian rhythmicity in the surge of glucocorticoid release from the adrenal

cortex (Hirano *et al.*, 1995). Both these studies confirm the role of steroids in the observed day-night variations in dopaminergic function discussed above.

1.6.3.4 Dopamine Receptors

Several studies have shown distinct day-night variations in [^{3}H]spiroperidol binding in the rat striatum and forebrain, which has been attributed to the D_2 receptor subtype (Naber *et al.*, 1981; Wirz-Justice *et al.*, 1981; Nagayama, 1993). In all cases, the rhythm was at the level of the density of binding sites (B_{max}) and not affinity (K_D) for the radioligand. Generally, a bimodal rhythm was observed with peak binding approximating mid-light and mid-dark phases. However, phase characteristics (including wave-form, phase (peak timing), amplitude and 24 hr mean binding) were observed to be tissue-specific within the same animal and varied with age (Jenni-Eiermann *et al.*, 1985), season (Naber *et al.*, 1981), and between strains of rat (Jenni-Eiermann *et al*, 1986). This day-night variation in binding also persisted under conditions of constant darkness, indicating that the rhythm is most likely free-running and endogenous (Naber *et al.*, 1981).

1.6.3.5 Dopamine Biosynthesis

(i) Tyrosine hydroxylase and Tyrosine aminotransferase

TH is the key regulatory enzyme in catecholamine biosynthesis (Ribeiro *et al.*, 1992). It catalyses the hydroxylation of L-tyrosine to L-Dopa, the rate determining step (See Figure 1.3). The extent to which L-tyrosine bioavailability is a crucial determinant of the synthetic rate is dependent on the physiological state of the neuron. Normally, TH is believed to be saturated with L-tyrosine with substrate supply only being limiting under conditions of enhanced neuronal activity (Tam and Roth, 1997). Plasma and brain tyrosine levels do show daily variations, which in mice show strain-specific phase characteristics (Calcet-Veys *et al.*, 1978).

However, less than 2% of the free, circulating L-tyrosine is utilized for brain catecholamine biosynthesis. The majority of the amino acid is degraded to p-hydroxyphenylpyruvate in the liver by the cytosolic enzyme tyrosine aminotransferase (TAT) [Zigmond and Wilson, 1973]. A mitochondrial form of the latter enzyme is also homogeneously distributed in the brain, including the striatum. Considering the high capacity of TAT for L-tyrosine, the catabolism of this substrate may be important for catecholamine biosynthesis. In other words, the metabolic fate of L-tyrosine, and thus DA, may depend on the relative activity of TH and TAT. In this regard, a circadian rhythmicity in the activity of both enzymes has been characterized.

Cytosolic TAT exhibits a robust unimodal rhythm in the rat liver, kidney and thymus, with peak activity at early scotophase (Zigmond and Wilson, 1973; Scalabrino *et al.*, 1979). This rhythm was not affected by pinealectomy (Scalabrino *et al.*, 1979). No day-night variation in mitochondrial TAT was seen in the whole brain (Zigmond and Wilson, 1973). Cahill and Ehret (1981), however, observed a unimodal rhythm with a peak in the late photophase in a brain area defined as the "brain stem". The latter comprised the medulla oblongata, pons, thalamus and the midbrain, excluding the striatum. In contrast, "brain stem" TH activity exhibited a bimodal rhythm. Peak activity occurred at mid-photophase and mid-scotophase, with a trough at the light-dark transition. The effects of α-methyl-p-tyrosine, a TH inhibitor, were chronotypic, only decreasing enzyme activity at the dark-light transition (Cahill and Ehret, 1981). Interestingly, the rhythms of TH and TAT activity in the "brain stem" are out-of-phase by about 180°. This fits expectation with regards to the regulation of catecholamine biosynthesis.

Natali *et al.* (1980) found strain-specific differences in the day-night variations of TH activity in the locus coeruleus, the major noradrenergic centre of the brain. A unimodal rhythm was found for all three inbred strains of mice, but with differences in the peak timing. Further studies with F_1 hybrids clearly suggest that TH activity and catecholamine biosynthesis are genetically controlled.

Alexiuk *et al.* (1996) found that *in situ* TH activity of the MBH, but not the NIL, exhibited a circadian rhythm in sham-operated and castrated mice. Peak activity occurred in the dark-phase. In an initial study, chronic administration of MEL (25 μg/animal) to male golden hamsters during the late photophase appeared to have time-independent effects on *in situ* TH activity of the NIL (Alexiuk and Vriend, 1993). Enzyme activity was reduced after 3 weeks of MEL administration, but no different to controls after 5 weeks. In a subsequent study, chronic administration of MEL for 9 weeks increased *in situ* TH activity of the MBH, but not the NIL (Alexiuk *et al.*, 1996). The effect in the MBH was not chronotypic in that MEL increased TH activity over the whole 24 hr period. As discussed above, this increase in synthetic rate was associated with a MEL-induced decrease in DA content in these brain areas. The authors propose that this reflects a stimulatory effect of MEL on DA release. They further argue that this is consistent with the ability of MEL to inhibit the afternoon prolactin surge, as DA is a well known prolactin-inhibiting factor. However, these results contradict those published by Steger *et al.* (1995) and Zisapel *et al.* (1982) discussed above.

It is important to note that several studies have found that the peak TH activity does not necessarily correlate with peak DA content or turnover in most brain regions investigated (Cahill and Ehret, 1981; Alexiuk and Vriend, 1993; Alexiuk *et al.*, 1996; Lemmer and Berger, 1978a). This is because other parameters, including release, other enzymes and cofactors, may themselves be circadian in nature, contributing to the overall rhythm in DA.

Both pineal and retinal TH activity show a robust unimodal rhythm. Pineal TH exhibits peak activity in the dark-phase (Craft *et al.*, 1984; Abreu *et al.*, 1987), whereas retinal TH activity is low and constant during darkness with a rapid increase with the onset of the light phase (Iuvone *et al.*, 1978). This phasing of TH is perfectly correlated with peak NA and DA synthesis and turnover in the pineal gland and amacrine cells of the retina, respectively.

(ii) Pyridoxal phosphokinase

Administration of MEL (i.p.) to female Wistar rats induced a very significant dose- and time-dependent increase in the activity of pyridoxal phosphokinase, but *in vitro* MEL had no effect on the enzyme. This enzyme also exhibited peak activity at mid-dark phase, paralleling peak MEL levels (Antón-Tay *et al.*, 1970). Thus MEL may enhance the *de novo* synthesis of the enzyme. Pyridoxal phosphokinase catalyses the formation of pyridoxal-5′-phosphate, which is an essential co-factor of numerous decarboxylase and transaminases. The nonspecific enzyme aromatic L-amino acid decarboxylase is necessary for catecholamine and indoleamine biosynthesis and glutamate decarboxylase is required for GABA biosynthesis. On the other hand, a transaminase like tyrosine aminotransferase will influence the bioavailability of L-tyrosine for catecholamine biosynthesis. Thus through an effect on pyridoxal phosphokinase, MEL may indirectly exert both a positive or negative effect on neurotransmitter synthesis, including DA.

(iii) Aromatic-L-amino acid decarboxylase

Exposure of male Syrian hamsters to a short photoperiod (i.e. decreased daylength) was found to reduce aromatic-L-amino-acid decarboxylase immunostaining in the arcuate nucleus and median eminence (Krajnak and Nunez, 1996). This would suggest an inhibitory effect of MEL on the amount of enzyme present.

1.6.3.6 Dopamine Catabolism

In mammals, catecholamines such as DA are metabolized by three distinct enzymes: COMT, MAO and phenolsulphotransferase. MAO and COMT represent the main two catabolic enzymes influencing dopaminergic neurotransmission (Napolitano *et al.*, 1995). The catabolic pathway of DA by MAO and COMT is shown in Figure 1.4.

(i) Phenolsulfotransferase

Although phenolsulfotransferase represents a minor degradative pathway, relative to MAO and COMT, it has been suggested to account for 15% of total DA metabolism in the brain (Marazziti *et al.*, 1995). This enzyme catalyses the transfer of sulphate from 3'-phosphoadenosine-5'-phosphosulphate to exogenous and endogenous phenols, including catecholamine neurotransmitters and their metabolites. The thermolabile isoform in particular displays substrate preference for DA. A distinct seasonality has been reported in platelets for this isoform with peak activity in summer and a definite reduction with decreasing daylength (Marazziti *et al.*, 1995). It remains to be determined whether there is day-night variation in activity and possible modulation by MEL.

(ii) Monoamine oxidase

Unless the specific isoform is designated, e.g. MAO-A, the term MAO will be used to designate that a combination of the two isoforms was assayed. Distinct day-night variations exist in brain MAO activity (Olcese and Devlaming, 1979; Chevillard *et al.*, 1981; Bhaskaran and Radha, 1984; Nagayama, 1993). However phase characteristics of this rhythm were dependent on brain region, age and MAO isoform investigated. Bhaskaran and Radha (1984) investigated age-dependent changes in the rhythm of brain MAO in male albino rats using a substrate common to both MAO-A and -B isoforms. In very young (21 day old) rats, combined MAO activity peaked roughly at mid-light phase in the cortex, cerebellum and medulla oblongata, but at approximately mid dark-phase in the hypothalamus, midbrain and striatum. In contrast, for rats older than 21 days, combined MAO activity consistently peaked in the early light phase in the cortex, cerebellum, medulla oblongata, hypothalamus, midbrain and striatum. These results clearly indicate the age-dependent increases in MAO activity of different brain regions with full synchronization of the rhythm by 3 months of age. The ontogeny of MAO-A and -B progress at different developmental rates (Lewinsohn *et al.*, 1980; Garrick and Murphy, 1982) and thus synchronization of the rhythm reported in this study may result from both isoforms reaching adult levels. Bhaskaran and Radha (1984) concomitantly investigated rhythms in DA levels as discussed above. Of importance here, is

the observation that in adult rats the circadian rhythmicity of MAO and DA levels were out of phase, with the latter peaking in the dark-phase.

With more specific attention to the MAO isoform, Chevillard *et al.* (1981) reported that within the locus coeruleus of the brainstem, MAO-A peaked 7 hrs after the onset of dark and 3 hrs after dark for MAO-B. Although the isoforms differed in the exact timing of peak activity, both occurred in the dark-phase.

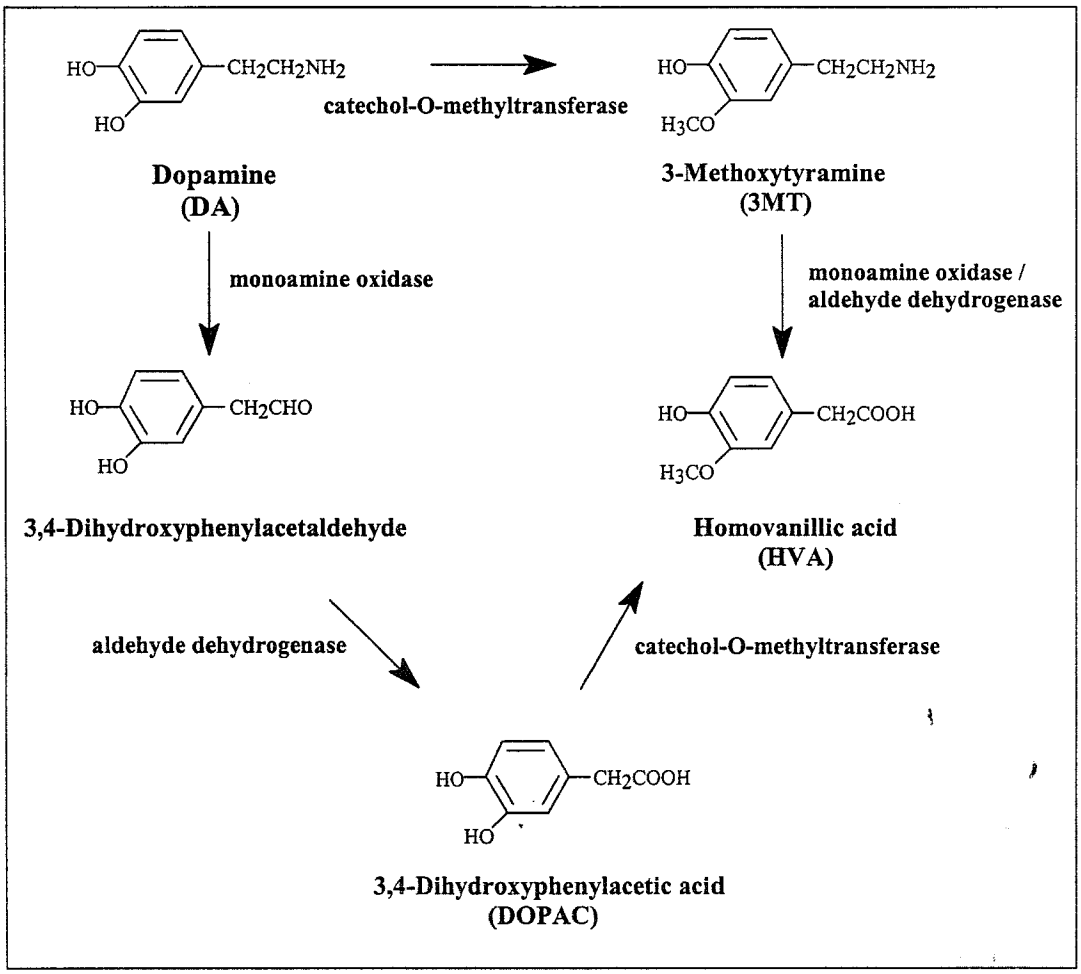


Figure 1.4: Catabolism of DA in the corpus striatum by MAO and COMT.

Urry and Ellis (1975) found that the effect of photoperiod manipulation and pinealectomy on rat brain MAO activity, presumably MAO-A, was region-specific. MAO activity of the whole hypothalamus was unaffected by photoperiod, but increased by pinealectomy. Pituitary MAO

activity was increased by constant light and pinealectomy and decreased by constant darkness. In addition, MEL *in vitro* was a weak but dose-dependent inhibitor of MAO activity regardless of brain region. These results all suggest that endogenous MEL may inhibit MAO activity *in vivo*. In confirmation, a significant daily variation in MAO activity was observed in the hypothalamic basal medial lobe of the goldfish exposed to a long, but not a short photoperiod (Olcese and Devlaming, 1979). Indeed MAO activity was depressed by a short photoperiod, whereas pinealectomy only increased activity in fish exposed to a short photoperiod.

Esquifino *et al.* (1994) demonstrated the chronotypic effects of MEL administration on adrenal medullary MAO and COMT activity. Male Syrian hamsters received chronic administration of MEL (25 μg/animal) during the early morning (08h00) or later afternoon (16h00) for 60 days and were sacrificed during the early light-phase. Adrenal medullary MAO was significantly reduced by MEL administration at both time periods with a greater effect in the 16h00-treated animals compared to the 08h00-treated animals

There is also evidence of an ontogenic effect of MEL administration on MAO activity. Neonatal administration of a single dose of MEL to neonatal male rats on postnatal day 5 increased hypothalamic MAO-A at day 30 and 45, but decreased activity on day 60 of age, whereas MAO-B was only increased at day 45. These changes were related to changes in plasma prolactin and luteinizing hormone levels (Moreno *et al.*, 1992). Thus neonatal MEL administration, through its effect on hypothalamic MAO activity may induce earlier sexual maturation in male rats.

(iii) Catechol-O-methyltransferase

In the study by Esquifino *et al.* (1994) described above, adrenal medullary COMT activity was significantly reduced by MEL administration at both time periods with a greater effect in the 16h00-treated animals compared to the 08h00-treated animals. Thus the inhibitory effect of MEL *in vivo* on COMT activity would appear to be chronotypic.

1.6.4 ELECTROPHYSIOLOGICAL STUDIES

The anti-motor and anticonvulsant properties of MEL are attributed to a hyperpolarization of neuronal membranes. Administration or iontophoretic application of MEL results in the inhibition of spontaneous and evoked neuronal activity of several important brain nuclei, including the rat suprachiasmatic nucleus amygdala, reticular formation and striatum (Stehle *et al.*, 1989; Naranjo-Rodriguez *et al.*, 1991; Rusak and Yu, 1993; Escames *et al.*, 1996). In turn, pinealectomy, through

depolarization, induces paroxysmal outbursts of slow wave, high amplitude kindled seizures in several rodent species, which may lead to death by tonic-clonic convulsions (Philo and Reiter, 1978; Stockmeier and Blask, 1986). MEL administration can counteract the resultant cerebral hyperexcitability and reduce the convulsive threshold in animals and humans (Antón-Tay, 1974; Golombek *et al.*, 1992). Thus MEL may be beneficial in the treatment of temporal lobe epilepsy (Antón-Tay, 1974).

Several mechanisms have been proposed to explain how MEL can suppress neuronal electrical activity. These include modulation of the inhibitory GABA/benzodiazepine complex (Acuña Castroviejo *et al.*, 1986a and 1986b) and altering Cl^- and Na^+ influx via an effect on the neuronal Na^+/K^+ - ATPase pump (Rosenstein *et al.*, 1989; Acuña Castroviejo *et al.*, 1992).

A paradoxical pineal-dependent effect of MEL on the electrophysiology of the rat striatum has been reported (Castillo Romero *et al.*, 1993 and 1995). In sham-operated rats, MEL typically inhibited the spontaneous firing rate of responsive striatal neurons. In pinealectomized rats, MEL had biphasic effects, but was predominantly excitatory. Pinealectomy alone had no effect on the mean firing rate. Subsequent studies showed that the inhibitory effects of MEL are potentiated by the pineal peptide arginine^8-vasotocin. This dependence on other components (e.g. intact pineal gland) is consistent with the role of MEL as a neuromodulator. Alone MEL may have opposite or weak effects. Further studies also observed an age-related decrease in the electrophysiological responsiveness of rat striatal neurons to the MEL administration (Castillo Romero *et al.*, 1995). It is unfortunate that the authors did not determine identity of the striatal neurons investigated. Furthermore it would also be informative to compare these results to a study performed in the dark-phase, when a dissociation between the circadian rhythms of plasma MEL and striatal MEL receptors may exist. In another study, acute MEL administration at low doses increased electrical activity of the caudate nucleus, whereas a high dose was inhibitory (Naranjo-Rodriguez *et al.*, 1991).

1.6.5 BEHAVIOUR

The mesencephalic dopaminergic systems of the basal ganglia are intimately involved in the affective, motivational and motor components of behavioural processes (see section 1.6.1). In particular the striatum is involved in modulating behavioural subprograms, such as learning, avoidance behaviour and food-hoarding behaviour.

1.6.5.1 Dopamine and Behaviour

In a nocturnal species such as the rat, the period of activity occurs with the onset of the scotophase, whereas rest is associated with the photophase. This is true for SLA, eating and drinking, and exploratory, novelty-induced and self-stimulation behaviour. As discussed in section 1.6.2.1, the onset of activity is strongly correlated with an increase in nigro-striatal and mesolimbic dopaminergic activity. This is evident in the increase in DA release and extracellular levels of DA, 3MT and HVA in the striatum and nucleus accumbens (Kempf *et al.*, 1982; Lemmer and Berger, 1978b; O'Neill and Fillenz, 1985; Paulson and Robinson, 1996; Piazza *et al.*, 1996; Pietilä *et al.*, 1995).

Removal of an entraining agent such as light results in free-running activity rhythms. Following long-term exposure to constant darkness, clear onsets and offsets of activity phases are still evident with peak activity in the acrophase (subjective night). In contrast, distinct activity phases are no longer apparent under constant light and the amplitude of acrophase activity is decreased (Yamada and Martin-Iverson, 1991). The phase characteristics of the activity-rest cycle can also be modulated by DA. Disorganization of the circadian rhythm with dopaminergic agents indicates that independent components of the rhythm are differentially controlled by DA receptor subtypes, possibly in the SCN. Chronic administration of D_2 agonists increases only the amplitude of activity under conditions of constant darkness or light. In contrast, D_1 agonists had no effect on amplitude, but prolonged the period length of the activity rhythm under constant darkness (Yamada and Martin-Iverson, 1991). The DA releasing agents amphetamine and metamphetamine increase the amplitude and period of activity under an entrained lighting schedule of 12L:12D (Honma *et al.*, 1985; Martin-Iverson and Iversen, 1989). This is consistent with an indirect effect on both D_1 and D_2 receptors.

At this point it must be noted that MAO inhibitors have potent effects on the phase characteristics of the activity-rest cycle. For example, administration of the MAO-A selective inhibitor clorgyline delays the onset and offset of activity, increases the intrinsic period of the rhythm and slows the rate of re-entrainment of the activity-rest cycle to a shifted light-dark-cycle (Duncan *et al.*, 1988). These effects are likely to be related to the ability of clorgyline to phase-shift the day-night variations of DA, NA and 5HT in various discrete brain regions (Ozaki *et al.*, 1993).

It is well known that acute, subchronic and chronic administration of dopaminergic agents, neuroleptics in particular, induces severe movement disorders ranging from bradykinesia and akinesia to dyskinesia. Some examples are Parkinsonism, akathisia, neuroleptic malignant syndrome and tardive dyskinesias. The latter comprise dystonia, tics, stereotypy, tremor, myoclonus and chorea (Jankovic, 1995). These side-effects represent a major source of poor compliance to the use of DA agonists and antagonists in the treatment of Parkinson's disease and schizophrenia.

1.6.5.2 Melatonin and Behaviour

Both endogenous and exogenous MEL or MEL agonists can entrain and synchronize the circadian rhythmicity of locomotor activity and behaviour in a wide-range of animal species. The latter include lizards, birds, fish, amphibians and mammals, including humans (Redman *et al*, 1983; Armstrong, 1989; Underwood, 1990).

Of particular importance here is the observation that in all species studied MEL levels consistently peak in the dark-phase regardless of whether the species is diurnal or nocturnal. Thus the timing of peak MEL levels may be either positively or negatively correlated with the species-specific activity-rest rhythm. As one suggestion, MEL may serve as an endogenous signal of the onset of darkness, that, through a "gating mechanism", times the switch from behavioural quiescence to activity or vice versa (Redman *et al.*, 1983; Armstrong, 1989). This limitation of a behavioural effect of MEL to a critical point in the circadian cycle arises through changes in responsiveness of certain biochemical parameters, such as receptors, to MEL. This is consistent with the modulatory role of MEL, potentiating or attenuating the prevailing "tonus".

MEL can also have a direct effect on behaviour independent of its role as a "zeitgeber". For example, the sleep-inducing or soporific effects of MEL are well-known. Oral administration of MEL or the MEL agonist S-20098 dose-dependently increased sedation, sleep duration, the duration of rapid eye movement (REM) sleep and decreased latency of sleep-onset in humans (Dollins *et al.*, 1994; Cajochen *et al.*, 1997). Pinealectomy tends to increase SLA, wheel-running activity and exploratory behaviour in rats, whereas MEL administration has an inhibitory effect (Armstrong, 1989; Chuang and Lin, 1994). Reiss *et al.* (1967) reported that rats could be divided into high and low spontaneous runners on the basis of treadwheel activity. Furthermore, the cell density of the pineal glands was markedly greater for the slow runners.

Direct administration of ng quantities of MEL into the substantia nigra and bilaterally into the nucleus accumbens of mice both result in dose-dependent decreases in SLA and rearing (Bradbury *et al.*, 1985). Intra-accumbenal application also resulted in an increase in emotive behaviours such as grooming and sniffing. These behavioural changes were associated with a reduction in dopaminergic transmission in both the striatal and limbic regions. Only the intranigral effects of MEL were partially reversed by the selective D_2 antagonist sulpiride. Identical behavioural responses following bilateral administration of MEL into the nucleus accumbens have been reported by Gaffori and Van Ree (1985a and 1985b) and Durlach-Misteli and Van Ree (1992). In these studies, however, the effects of MEL were blocked by 5HT, antidepressants of various classes and by β-endorphin-(10-16), but not by the DA antagonists HAL or sulpiride. The behavioural effects of intra-accumbenal administration of APO are dose-dependent. Low doses induce hypolocomotion whereas high doses induce hyperlocomotion (Kendler *et al.*, 1982). These effects are mediated by presynaptic and postsynaptic DA receptors respectively. The hyperlocomotion induced by APO in the nucleus accumbens was only blocked by HAL and sulpiride (Gaffori and Van Ree, 1985a and 1985b). However only chronic administration of antidepressants results in a potentiation of APO-induced hyperlocomotion and antagonism of APO-induced hypolocomotion in the nucleus accumbens (Durlach-Misteli and Van Ree, 1992). This is believed to arise through the known ability of antidepressants to induce presynaptic DA receptor subsensitivity and postsynaptic DA receptor supersensitivity.

This suggests that the behavioural effects of MEL are mediated by DA in the nigro-striatal pathway and additionally by serotoninergic and/or opioidergic systems in the mesolimbic system. Furthermore MEL and APO in the nucleus accumbens may mediate exploratory behaviour through independent systems.

1.6.5.3 Melatonin and Movement Disorders

The effects of MEL on behaviour can also be investigated after creating animal behavioural models of dopaminergic function. In other words, the tone of the relevant pathways is primed by pharmacological manipulation with dopaminergic agents. For example, catalepsy is a drug-induced akinetic state reflecting dopaminergic hypofunction. A single dose of MEL (1 mg/animal i.p.) was found to markedly reduce reserpine-induced catalepsy in intact female rats during the late photophase. This effect of MEL was potentiated by nalbuphine, an opiate agonist, and partially reversed by naloxone, an opiate antagonist (Sandyk and Mukherjee, 1989). In a parallel study, naloxone only prolonged reserpine-induced catalepsy in pinealectomized rats when the

animals were pretreated with MEL, and not when administered alone (Sandyk and Fisher, 1989a). The latter authors proposed that these results indicate a mutual inclusiveness or co-dependence of MEL and the striatal opioid system on motor behaviour. This is consistent with the known cataleptogenic ability of the endogenous opiate β-endorphin-(10-16) which is also capable of antagonizing the behavioural effects of MEL in the nucleus accumbens (Gaffori and Van Ree, 1985b). Unfortunately, Sandyk and Fisher (1989a) failed to make the necessary comparison of reserpine-induced catalepsy and its modulation by MEL administration between pinealectomized and sham-operated rats.

The Ungerstedt model of rotational behaviour is routinely used as an animal model of Parkinson's disease and dyskinesias (Ungerstedt, 1971). Selective unilateral lesioning of the nigro-striatal pathway with the dopaminergic neurotoxin 6-hydroxydopamine (6OHDA) results in a supersensitive DA receptor model. DA agonists are characterized by their ability to induce contralateral turning (rotational) behaviour in lesioned rats by stimulating the supersensitive receptors in the denervated striatum. Typically the mixed D_1/D_2 agonist APO is used at a dose sufficient to stimulate postsynaptic receptors. An additional model involves the selective unilateral lesioning of the corpus striatum with the neurotoxin quinolinic acid (QA) [Schwarcz *et al*, 1983]. This results in normosensitive DA receptors, with APO inducing ipsilateral rotational behaviour. It is used as an animal model of Huntington's chorea, a genetic movement disorder characterized by hyperkinetic choreiform movements.

In an early model of rotational behaviour or adventitious turning, Cotzias *et al* (1971) produced a partial lesion of the right caudate nucleus of male mice by physical ablation, causing the animals to turn towards the side of the lesion. Administration of MEL dose-dependently inhibited L-Dopa- and APO-induced dyskinesias in both intact and lesioned mice. This suggests a possible effect of MEL on postsynaptic DA receptors. Burton (1989) and Burton *et al.* (1991) observed that prior administration of MEL significantly reduced APO-induced stereotypy, hyperlocomotion and rotational behaviour in both 6OHDA- and QA-lesioned rats. MEL alone had no effect in the lesioned animals. Exposure of rats to bright light during the dark-phase increased these behaviours, again suggesting that endogenous MEL can modify APO-induced behaviour. Paradoxically, MEL also antagonized the ability of sulpiride to block APO-induced behaviour. This is of interest considering that Bradbury *et al.* (1985) showed that sulpiride, in turn, partially reversed the inhibition of SLA by intra-nigral MEL administration. This lends further support to the proposal that within the nigro-striatal pathway MEL exerts effects on the dopaminergic

system. Alone MEL has an "antagonistic" effect, but in the presence of D_2 antagonist displays "agonistic" effects.

More recently, the results of Burton *et al.* (1991) have been confirmed and extended by Tenn and Niles (1995). Both MEL and the central-type benzodiazepine agonist clonazepam dose-dependently suppressed APO-induced rotational behaviour in 6-OHDA-lesioned rats. In addition, MEL decreased turning induced by the D_1 agonist SKF38393 and the D_2 agonist quinpirole, but was more potent against the D_1 agonist. Furthermore, both the central-type benzodiazepine antagonist, flumazenil, and the $GABA_A$ receptor antagonist, bicuculline, attenuated the above effect of MEL (Tenn and Niles, 1995).

Pinealectomy has also been shown to have marked, but differential effects on movement disorders. For example, pinealectomy failed to alter the time-course or intensity of APO-induced stereotypy in non-lesioned rats (Arushanyan and Ovanesov, 1986), but increased amphetamine-induced stereotypy and lessened HAL-induced catalepsy (Arushanyan *et al*, 1992). Also, pinealectomy markedly increased the incidence and severity of HAL-induced oro-facial dyskinesias in male rats (Sandyk and Fisher, 1989b). Subsequent MEL administration only caused a nonsignificant reduction, which the authors attribute to the lack of a functional opioidergic system. Indeed, MEL does augment the antidyskinetic effects of naloxone in HAL-induced oro-facial dyskinesias in the rat (Sandyk and Fisher, 1989c).

These studies clearly indicate that MEL exerts antidopaminergic effects on behaviour and can modulate the motor side-effects of neuroleptics and other dopaminergic agents. These behavioural effects would appear to be differentially facilitated by several neuronal systems. These include the dopaminergic, serotonergic and opioidergic systems of the basal ganglia and the inhibitory striatal GABAergic system through central-type benzodiazepine (BZ) receptor. Studies with MEL analogs and various indoles suggest the above effects of MEL are receptor-mediated (Tenn and Niles, 1995). It is unclear whether this reflects an involvement of specific MEL receptors or a direct allosteric modulation of BZ receptors by MEL. Central-type benzodiazepine (BZ) receptors are linked to $GABA_A$ receptors in the CNS, the $BZ/GABA_A$ receptor complex. Benzodiazepines and related ligands act as allosteric effectors of GABA binding, thereby increasing the frequency at which the associated chloride channel is opened. The resultant hyperpolarization effectively inhibits DA release in the striatum and nucleus accumbens. Furthermore the mechanism underlying the apparent differential interaction of MEL with D_1 and D_2 pathways is unclear. There

is a higher density of D_1 than D_2 receptors on striatal neurons (Camps *et al*, 1990), and the latter may also express BZ/$GABA_A$ receptor complexes or MEL receptors. The mechanism may lie at the level of a common signal transduction system, such as adenylate cyclase and cAMP or phosphoinositol turnover.

1.6.5.4 Melatonin and Psychiatric Disorders

Both DA and MEL have been implicated in numerous psychiatric disorders, including several affective disorders (Erlich and Apuzzo, 1985). Studies on manic-depression have consistently found an association with a marked reduction in the amplitude of plasma MEL, a failure to exhibit a normal circadian secretory rhythm, phase-advances in the secretory onset and a super-sensitivity to light-induced suppression of the output of MEL (Miles and Philbrick, 1988; Waldhauser *et al.*, 1993). This has led to the formal categorization of hypomelatoninemia or the "low melatonin syndrome" in this affective disorder.

There is also considerable indirect or anecdotal evidence for a role of MEL in winter seasonal affective disorder (SAD) and the major depression syndrome. SAD is characterized by regularly occurring autumn and winter depressions, alternating with nondepressed periods in spring and summer (Partonen, 1994). This suggests a strong causal relationship with seasonal changes in the prevailing photoperiod (light:dark cycle), with diminished light exposure in winter. Indeed phototherapy, by employing controlled light exposure regimes, does improve the clinical picture of SAD and other forms of depression (Lewy *et al.*, 1987; Miles and Philbrick, 1988; Waldhauser *et al.*, 1993). This suggests that a suppression or phase-shifting of pineal MEL biosynthesis may be involved in the therapeutic efficacy of light. Several biochemical, behavioural and clinical studies seem to confirm that MEL may worsen depressive symptoms. Firstly, chronic administration of lithium and antidepressants, such as desmethylimipramine, induces a suppression of pineal NAT activity and MEL biosynthesis (Miles and Philbrick, 1988). Secondly, the MEL antagonist luzindole shows antidepressant activity in the behavioural despair test (or Porsolt's swim test), which is counteracted by MEL itself (Dubocovich *et al.*, 1990).

In its initial form, the DA hypothesis of schizophrenia proposed that the pathogenesis of the disease arose through the hyperactivity of central dopaminergic systems. More recent studies have led to a revision of this simple hypothesis. Regionally restricted changes in the dopaminergic systems of the basal ganglia towards either hyperactivity or hypoactivity appear to be the causative factors. The mesolimbic and mesocortical DA pathways and the prefrontal cortex appear

to be particularly prone. There is also a growing recognition that other neurotransmitters, including 5HT and glutamate, may contribute to the disease pathology (Goldstein and Deutch, 1992; Cohen and Servan-Schreiber, 1993). Historically, extracts of the pineal gland have been used to treat several mental illnesses including schizophrenia (Miles and Philbrick, 1988). Modern biochemical approaches have indeed implicated the pineal gland, HIOMT activity and MEL production in a subgroup of chronic or paranoid schizophrenic or schizoaffective patients (Sandyk and Kay, 1990). The nocturnal surge in plasma MEL levels is dramatically phase-shifted, suppressed or even absent in drug-free subjects (Robinson *et al.*, 1991; Monteleone *et al.*, 1992; Rao *et al.*, 1994). This particular group is characterized by a well-defined symptomology, including ventricular enlargement, negative symptoms, impaired cognitive function, increased susceptibility to drug-induced movement disorders, and poor response to neuroleptic treatment (Sandyk and Kay, 1990). The lower efficacy of D_2 antagonists suggests that excess dopaminergic activity is less prominent in this subtype of schizophrenia, as indicated by Goldstein and Deutch (1992). This reduction in nocturnal MEL is interesting in the light that several D_2 antagonists are known to increase the plasma levels of the hormone, as discussed in section 1.5 (Wurtman *et al.*, 1968a; Ozaki *et al.*, 1976; Smith *et al.*, 1979; Loloum *et al.*, 1993).

The "transmethylation hypothesis" has made several come-backs as a contending etiology for schizophrenia (Miles and Philbrick, 1988). In its original form, this hypothesis proposed that schizophrenia is caused by the build-up of abnormal O- or N-methylated metabolites in the brain, several of which are related the hallucinogenic β-carbolines. A more recent contention states that the pathogenesis may be related to an abnormal ratio of para- to meta-O-methylated products due to abnormal SAM-dependent transmethylation reactions (Da Prada *et al.*, 1994). In this regard, it is interesting that MEL and other 5-methoxyindoles represent O-methylated products of HIOMT.

1.6.5.5 Melatonin and Parkinson's Disease

Parkinson's disease is one of the most common age-related neurodegenerative diseases. The pathogenesis is characterized by the progressive cell death of the dopaminergic neurons of the pars compacta zone of the substantia nigra. This results in a parallel loss of DA content in the caudoputamen (Greenfield, 1992; Naoi and Maruyama, 1993). Sandyk (1990a) provides a thorough review and hypothesis of the current evidence for a role of the pineal gland and MEL in Parkinson's disease. Most of the evidence is indirect or anecdotal and based on the purported antidopamimetic effects of MEL seen in biochemical and behavioural studies. In addition, it is interesting that most, if not all, of the chronobiological and neuroendocrine disturbances observed

in Parkinson's patients are known to be regulated by both DA and MEL. It is also argued that the progressive nature of the disease is paralleled by the well-established age-dependent decrease in MEL biosynthesis and plasma levels. In this regard, one hypothesis claims that the DA neuronal cell death is a consequence of oxidative stress by free radicals (Greenfield, 1992; Naoi and Maruyama, 1993) and the potent radical scavenging and antioxidative capacity of MEL is well known (Reiter *et al.*, 1995).

An additional hallmark of Parkinson's disease pathology is a deficiency of specialized APUD cells in the hypothalamus (Sandyk, 1990a). It has already been discussed in section 1.2 that the pineal gland also represents a part of the APUD system, and may actually be the "master gland" (Leong and Matthews, 1979). Sandyk *et al.* (1992) reported that magnetic fields are therapeutic in the management of Parkinson's disease, effectively reducing the clinical dose of L-Dopa required. This in turn lessened the incidence and severity of L-Dopa-induced dyskinesias or motor fluctuations. The authors attributed this to the known ability of magnetic fields to suppress pineal MEL biosynthesis (Reiter and Richardson, 1992). In another intriguing hypothesis, Sandyk (1990b) proposed that the spontaneous blink rate may reflect the status of central dopaminergic function and its modulation by MEL. For example, the lack of the blink reflex in Parkinson's patients is postulated to reflect the hypodopaminergic state of the basal ganglia, whereas the paroxysmal blinking seen in psychotic episodes of schizophrenia is associated with a hyperdopaminergic state. Blink rate has indeed been shown to alter the activity of the neostriatum (White *et al.*, 1994) and pupil size regulates the threshold of light-induced suppression of pineal MEL levels (Gaddy *et al.*, 1993). This hypothesis elegantly demonstrates the importance of balance in dopaminergic function and the potential role of MEL in maintaining homeostasis.

It must be noted that the above hypotheses are all theoretical and do not allow for the formation of one central argument. Some require that Parkinson's disease be associated with elevated MEL levels, whereas others necessitate a "low melatonin syndrome" as seen in schizophrenia. Only limited studies have actually investigated the status of MEL or the pineal in Parkinson's disease. Fertl *et al.* (1993) found a normal circadian rhythm and nocturnal amplitude in plasma MEL levels of *de novo* Parkinson's patients that could be phase-advanced by L-Dopa therapy. The finding of Antón-Tay (1974) that 1.2 g/day of MEL improved the symptoms of Parkinson's patients has not been confirmed by other studies (Sandyk, 1990a). In fact, the behavioural studies of Burton *et al.* (1991) and Tenn and Niles (1995) discussed above suggest that MEL would worsen the clinical picture.

1.7 Monoamine oxidase

The enzyme monoamine oxidase [monoamine:oxygen oxidoreductase (deaminating) (flavin-containing); E.C. 1.4.3.4; MAO] is an intrinsic flavoprotein of the outer mitochondrial membrane (Abell *et al.*, 1994; Singer and Ramsay, 1995). Utilizing molecular oxygen as an electron acceptor, MAO catalyses the oxidative deamination of endogenous, dietary and xenobiotic amines to their corresponding aldehydes both in the CNS and periphery. A generalized reaction scheme for MAO is shown below:

$$RCH_2NR_aR_b + O_2 + H_2O \rightarrow RCHO + NHR_aR_b + H_2O_2$$

The substrate may be a primary, secondary or tertiary amine and the substituents R_a and R_b may be either hydrogen or methyl groups. Typically, deamination of a primary amine produces an unstable imine intermediate which spontaneously hydrolyses to give the corresponding aldehyde and ammonia (Abell *et al.*, 1994; Singer and Ramsay, 1995). MAO is also the prime generator of hydrogen peroxide, which can be converted to cytotoxic hydroxyl radicals in the presence of transition metals by the Fenton reaction. MAO is also responsible for the bioactivation of selective dopaminergic neurotoxins such as isoquinolines and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6OHDA (Naoi and Maruyama, 1993).

Endogenous biogenic amines include the neuroactive and vasoactive monoamine neurotransmitters DA, NA, adrenaline (AD) and 5HT, and the dietary or trace amines, namely, β-phenethylamine, tyramine and octopamine. Thus the physiological functions of MAO can be summarized as follows (Luque *et al.*, 1995):

(i) maintenance of low cytosolic amine concentrations in monoaminergic neurons and other cells (e.g. glia) in order to enhance uptake of monoamines by respective transporters following synaptic release of the said monoamine. In other words, presynaptic MAO activity is essential for terminating synaptic signal of a neurotransmitter by enhancing presynaptic uptake.

(ii) prevent accumulation of substrates that could interfere with the uptake, storage and release of neurotransmitters.

1.7.1 MAO ISOFORMS

MAO occurs as two well-characterized and pharmacological distinct protein isoforms, designated MAO-A and MAO-B (Weyler *et al.*, 1990; Singer and Ramsay, 1995). This original classification is based on substrate specificity and sensitivity to low concentrations of the irreversible acetylenic inhibitors pargyline, clorgyline and L-deprenyl (Johnston, 1968; Yang and Neff, 1973; Fowler *et al.*, 1978). MAO-A preferentially deaminates 5HT, NA and AD and is selectively inactivated by clorgyline. MAO-B preferentially catabolizes β-phenylethylamine and benzylamine and is selectively inhibited by L-deprenyl. The amines tyramine, tryptamine, telemethylhistamine, kynuramine and, more importantly, DA are common substrates for both isoforms.

It is important to note that the aforementioned substrate specificity's are not absolute, but show important tissue- and species-specific variations (Fowler *et al.*, 1978; Garrick and Murphy, 1982; Weyler *et al.*, 1990). For example, although purified MAO-B from bovine liver preferentially deaminates [^{14}C]β-phenethylamine, [^{14}C]5HT can be utilized as a substrate to a lesser extent (Chen *et al.*, 1987). In contrast, in the rat brain 5HT is exclusively deaminated by MAO-A (Fowler *et al.*, 1978; Garrick and Murphy, 1982). Furthermore, species-variations are seen in the inhibitory potency of oxadiazolone and oxadiazothione derivatives towards MAO-B, suggesting differences in the structure of the substrate binding site (Singer and Ramsay, 1995). Ultimately, substrate selectivity is dependent upon the K_{cat}/K_m ratio for each isoenzyme (Palfreyman *et al.*, 1987). These discrepancies have lead to the suggestion that there may be other MAO isoforms, requiring revision of this, perhaps simplistic, binary classification. Thus it is proposed that a consideration of inhibitor sensitivity is more reliable and consistent than substrate specificity for isoform characterization.

1.7.2 PROTEIN STRUCTURE

The above binary classification of MAO isoforms was confirmed when the cDNA-derived amino acid sequences (primary structure) were obtained for human liver MAO-A and MAO-B (Bach *et al*, 1988). Each isoform is a distinct homodimeric protein, but they differ in physical (e.g. molecular size), biochemical and immunological properties (Chen *et al*, 1987; Weyler *et al.*, 1990; Abell *et al.*, 1994; Singer and Ramsay, 1995).

It is proposed that the active site of MAO is composed of two domains, one housing the obligatory flavin adenine dinucleotide (FAD) prosthetic group, and the other comprising the substrate

binding site. The flavin binding site is situated in the carboxyl terminus of the polypeptide. In each isoform, this site is associated with the conserved pentapeptide, Ser-Gly-Gly-Cys-Tyr, producing the covalently bound 8-S-cysteinyl FAD. The AMP binding site is situated in the N-terminus, displaying sequence identity with the AMP sites from other flavoproteins (Singer and Ramsay, 1995). Unfortunately, precise knowledge regarding the amino acid sequence and catalytic nature of the active site and the substrate binding site remains unclear. Preliminary studies with photoaffinity labels such as 4-fluoro-3-nitrophenylazide (Chen *et al.*, 1987; Hsu and Shih, 1988) showed clear differences in the degree of photo-dependent inactivation of MAO-A and MAO-B. This provided strong evidence for the existence of conformational or structural differences in the active sites of the isoforms. The results further suggested that the substrate and inhibitor selectivity between MAO-A and -B can be attributed to differences in the substrate binding site and not the FAD binding site (Chen *et al.*, 1987). This can be further delineated once labeled probes that bind preferentially or exclusively to the substrate binding site have been found. Further, it is known that cysteine residues are the source of thiol groups essential for the catalytic activity of both isoforms. Site-directed mutagenesis revealed that human liver MAO-A and -B catalytic activity were completely dependent on one and two cysteine residues, respectively, in addition to a cysteine residue associated with the FAD moiety (Wu *et al.*, 1993). Although it is unclear whether these essential cysteines are involved in forming the active site or an appropriate enzyme conformation, this illustrates a further structural difference between MAO-A and -B. Hydropathy plots indicate a greater hydrophobicity in human MAO-B than -A, which may explain why apolar amines are better substrates for MAO-B (Singer and Ramsay, 1995). Until the 3-dimensional shape of the tertiary and quaternary structures of the isoforms can be obtained from X-ray crystallographic data, SAR studies will remain limited.

Little is known regarding the biosynthesis, post-translational modification and transport of the MAO enzyme. An uncleavable targeting sequence in the 29 residues of the COOH-terminus is known to be responsible for insertion into the outer mitochondrial membrane (Singer and Ramsay, 1995).

1.7.3 Molecular Biology

MAO-A and -B are encoded by two distinct cytoplasmic genes and the cDNA for both isoforms has been cloned from tissue of several species, including the livers of humans and rats (Bach *et al.*, 1988; Ito *et al.*, 1988). There is enormous conservation of predicted amino acid sequence

identity between isoforms and species, especially for the FAD binding domain. In humans, both genes are located on the short arm of chromosome X (Bach *et al.*, 1988; Ozelius *et al.*, 1988; Lan *et al.*, 1989; Grimbsy *et al.*, 1991). The human isoforms share 73% nucleotide sequence identity and exhibit identical intron-exon organization (Bach *et al.*, 1988; Grimbsy *et al.*, 1991), suggesting that they probably arose from duplication of an ancestral gene. The core promoters of both genes show major differences in organization and activity (Zhu *et al.*, 1992; Zhu *et al.*, 1994; Ekblom *et al.*, 1996). The human MAO-A gene is under the regulation of a bi-directional promoter and transcriptional activity is chiefly governed by Sp1-like transcription factors. The MAO-B gene is regulated by Sp1-like and other unidentified transcription factors.

Chen *et al.* (1994) cloned a novel MAO cDNA from the trout liver and Zhang and McIntire (1996) reported the cloning and sequencing of a copper-containing, topa quinone-containing monoamine oxidase from human placenta. It is highly probable that molecular biology will expose further novel MAO isoforms and thus help clarify the pharmacological and biochemical debate (Johnston, 1968; Knoll and Magyar, 1972; Garrick and Murphy, 1982).

Several DNA polymorphisms have been characterized for both isoforms (Ozelius *et al.*, 1988; Hotamisligil and Breakefield, 1991; Grimbsy *et al.*, 1992; Girmen *et al.*, 1992; Kurth *et al.*, 1993). In the case of MAO-A, three polymorphisms which do not affect the amino acid sequence of the MAO-A protein are significantly associated with enzyme activity (Hotamisligil and Breakefield, 1991). For example, the EcoRV polymorphism arises from a nucleotide substitution in the third base of a codon located within the non-coding regulatory region of the MAO-A gene. Since differences in MAO activity arise from differences in amount of MAO enzyme, and not catalytic properties of the enzyme, EcoRV controls the amount of enzyme translated. Segregation analyses have confirmed that MAO-A activity is regulated by a single major locus, the MAO gene itself, and represents a stable characteristic of an individual with a high heritability factor (Rice *et al.*, 1984). Furthermore, Schalling *et al.* (1987) found clear evidence of low, intermediate, and high platelet MAO activity in human subjects, with an apparent relation to psychopathology. In the case of MAO-B, no correlation has yet been found between specific alleles/polymorphisms, e.g. the intronic MaeIII polymorphism (Kurth *et al.*, 1993), and activity levels (Girmen *et al.*, 1992; Grimbsy *et al.*, 1992; Ho *et al.*, 1994).

1.7.4 TISSUE DISTRIBUTION

MAO is distributed throughout the CNS and periphery. The ratio of MAO-A to MAO-B expression is distinctly cell-, tissue- and species-specific and shows ontogenic development. This differential expression of A and B isoforms in CNS and peripheral tissues may be regulated independently by tissue-specific factors (Zhu *et al.*, 1992; Zhu *et al.*, 1994; Ekblom *et al.*, 1996). MAO-A is expressed before MAO-B in fetal brains (Lewinsohn *et al.*, 1980), but MAO-B activity is higher than MAO-A in the adult human brain (Garrick and Murphy, 1982). Good agreement in the tissue distribution of MAO-A and -B in several species (including rats, monkeys and humans) was seen following quantification of enzyme activity, protein levels and mRNA expression by a variety of techniques. Typically, MAO-A is expressed in catecholaminergic neurons, with highest levels in adrenergic and noradrenergic neurons, and moderate to low expression in dopaminergic neurons. Thus the noradrenergic system, particularly the locus coeruleus, is the main component of MAO-A in the rat, guinea pig, Mongolian gerbil, monkey and human brain (Grimsby *et al*, 1990; Luque *et al.*, 1995; Jahng *et al.*, 1997). MAO-B distribution is more discrete, with highest expression in non-neuronal glial cells (astrocytes), circumventricular organs (e.g. pineal organ), serotonergic (particularly the raphe nuclei) and histaminergic neurons (Levitt *et al.*, 1982; Westlund *et al*, 1985; Jahng *et al.*, 1997). Human placenta express only MAO-A, whereas platelets and lymphocytes express predominantly MAO-B (Garrick and Murphy, 1982; Hsu *et al*, 1988; Grimsby *et al*, 1990).

The abundance of MAO-B, rather than MAO-A, in serotonergic neurons is paradoxical considering that 5HT is preferentially catabolized by MAO-A. A good case in point is the pineal gland, which contains 50 to 60 times more 5HT than the rest of the brain. The pineal expresses both MAO isoforms, although MAO-B is distinctly the predominant isoenzyme. Nonetheless, oxidative deamination represents the main metabolic fate of 5HT. A model has been proposed that MAO-A is responsible for the normal *in vivo* deamination of 5HT, whereas MAO-B only metabolizes this indoleamine under conditions of low or inhibited MAO-A activity (Luque *et al.*, 1995). Further, serotonergic neurons may contain a non-vesicular pool of 5HT requiring low levels of MAO-A activity to be functional. Serotonergic MAO-B may play a scavenging role by catabolizing trace amines (e.g. tryptamine) and other neurotransmitters (e.g. DA), and xenobiotics that could interfere with 5HT uptake, storage and metabolism. Indeed tryptamine is closely associated with 5HT pathways, possesses a high turnover rate and is known to elicit LSD-like hallucinations and amphetamine-like motor activation (Luque *et al.*, 1995).

1.7.5 Relation to Dopamine Metabolism

Although DA is a common substrate of MAO-A and -B, the relative abundance of MAO-A in dopaminergic systems is strongly debated. Protein and activity studies indicate that the ratio of MAO-A to MAO-B in the rat striatum is 3:1, whereas for the guinea pig and human striatum the ratio is 1:3, favouring MAO-B (Juorio *et al.*, 1994). In addition, Juorio *et al.* (1994) reported a strong level of expression of MAO-A mRNA in the guinea-pig nigro-striatal pathway. On the other hand, Luque *et al.* (1995) found that although MAO-A mRNA and protein is present in the rat striatum, MAO-B was more abundant. In contrast, only MAO-A mRNA was expressed in the rat substantia nigra pars compacta zone (Luque *et al.*, 1995; Jahng *et al.*, 1997). This supports the finding that mouse striatal MAO-B is localized to postsynaptic cholinergic interneurons, particularly in the dorsal caudate putamen, which receives input from the ventral pars compacta zone (Nakamura *et al.*, 1995). In the case of humans, MAO-B is present in the caudate nucleus, whereas neither MAO-A nor -B are found in the substantia nigra (Naoi and Maruyama, 1993).

It is proposed that MAO- A mRNA is translated in cell bodies of the substantia nigra and the protein is conveyed by axonal transport to the presynaptic nerve terminal and inserted into the outer mitochondrial membrane, explaining the relative distribution of MAO-A mRNA and protein. In contrast, MAO-B mRNA and protein are abundant in the cell body of post-synaptic cholinergic interneurons in the striatum. Indeed it is well established that in the rodent brain, released DA is principally metabolized by MAO-A following neuronal reuptake under physiological conditions. Minor amounts of DA will be deaminated by glial MAO-A and -B following diffusion of the neurotransmitter away from the synapse (Berry *et al.*, 1994; Juorio *et al.*, 1994; Luque *et al.*, 1995). In the case of the primate brain, MAO-B may be the neuronally important isoform for DA metabolism (Garrick and Murphy, 1982; Grimsby *et al*, 1990; Juorio *et al.*, 1994).

1.7.6 Pathology and Clinical Significance

MAO has been implicated in numerous brain diseases and psychiatric disorders. This is attested to by the vast amount of literature published on this topic, but the following only represents a brief overview of the material. Although human platelets contain exclusively MAO-B and MAO-A is of primary importance for the metabolism of central 5HT, there is a strong positive correlation between central serotonergic turnover (e.g. 5HIAA levels in CSF) and platelet MAO-B activity. For this reason, platelet MAO activity has been suggested as a trait-dependent indicator of

vulnerability to psychopathology, which implies a stability of the enzyme in an individual irrespective of changes in mood, season and symptoms (Schalling *et al.*, 1987).

Low platelet and lymphocyte MAO-B activity has been found in chronic and paranoid schizophrenic patients (Wyatt *et al*, 1980; Zureick and Meltzer, 1988). The influence of the MAO-A EcoRV polymorphism has also been reported (Hotamisligil and Breakefield, 1991). Low platelet MAO activity and low 5HT turnover have also been associated with eating disorders, impulsivity, novelty seeking, substance abuse, aggression, borderline personality, antisocial and recurrent suicidal behaviour and type II alcoholism (Sullivan *et al.*, 1979; Moss *et al*, 1990; Verkes *et al*, 1996). Ekblom *et al.* (1996) found that ethanol caused a 2-fold increase in MAO-B gene expression and activity. This effect may be cell-type specific and involve ethanol-induced changes in the binding of the transcription factor to the core promoter of the gene. Point mutations in the MAO-A gene have also been associated with impulsive aggression in some men of a Dutch family (Brunner *et al.*, 1993). In this regard, transgenic mice lacking the MAO-A gene show abnormal levels of 5HT and NA and aggressive behaviour (Cases *et al.*, 1995). Furthermore, the loss of both the MAO-A and MAO-B genes has been implicated in the severe mental retardation of some patients with Norrie disease (Lan *et al.*, 1989).

Parkinson's disease is also associated with low platelet MAO-B activity, whereas there is evidence for very high levels of MAO-B in plaque-associated astrocytes in the brains of Alzheimer's patients. (Williams *et al*, 1991; Saura *et al.*, 1994). Studies have failed to find an association between the intronic MaeIII polymorphism of MAO-B and Parkinson's disease (Kurth *et al.*, 1993; Ho *et al.*, 1994).

1.8 Catechol-O-methyltransferase

Catechol-O-methyltransferase (S-adenosyl-L-methionine; catechol-O-methyltransferase; EC 2.1.1.6; COMT) is an important phase II metabolic enzyme involved in transmethylation reactions. In the presence of Mg^{2+} ions, COMT catalyses the SAM-dependent O-methylation of a variety of exogenous and endogenous catechols, including catecholamine neurotransmitters, catechol steroids (e.g. estrogen) and xenobiotics, including APO (Axelrod, 1966). Thus COMT has a broad substrate specificity, but with a strict requirement for the catechol functionality. The mechanism involves the transfer of methyl group from SAM to primarily the *m*-hydroxyl group

(position 3) of catechols, although a minor proportion of the 4-O-methylated product is also produced. In turn, S-adenosyl-homocysteine (SAH) is formed as the demethylated co-product. Important catecholamine substrates include DA, NA and AD, and the precursor L-Dopa. Thus the physiological functions of COMT can be summarized as follows (Tilgmann and Ulmanen, 1996):
(i) inactivation of neurotransmitters following synaptic release. For example, extraneuronal or interstitial DA (3-hydroxytyramine) in the synaptic cleft is O-methylated to 3-methoxytyramine (3MT). Thus the extracellular levels 3MT are often used as an index of DA release (Kehr, 1976).
(ii) role as a barrier enzyme regulating the access of catechols to biological compartments. For example, COMT associated with the blood-brain-barrier can limit the uptake of xenobiotics into the CNS.
(iii) regulation of catecholestrogens and catabolism of xenobiotics, especially in the liver, including toxic and potentially carcinogenic compounds.

1.8.1 COMT Isoforms and Subcellular Distribution

COMT occurs as two distinct monomeric protein isoforms, which differ in molecular mass: soluble COMT (S-COMT) [24KD], and membrane-bound COMT (MB-COMT) [28KD] (Grossman *et al.*, 1985; Karhunen *et al.*, 1994; Tilgmann and Ulmanen, 1996). The MB-COMT isoform contains the S-isoform and an additional hydrophobic N-terminal anchor segment. MB-COMT is an integral membrane protein with most of the protein (including the catalytic domain) orientated towards cytoplasmic side of membrane (Ulmanen and Lundström, 1991). Cell fractionation (Tilgmann *et al.*, 1992) and immunohistochemical studies (Lundström *et al.*, 1995) confirm the localization of MB-COMT to plasma membranes and rough endoplasmic reticula (RER). In contrast, S-COMT is distinctly cytosolic (Karhunen *et al.*, 1995a and 1995b; Lundström *et al.*, 1995) and is the more abundant isoform. Even though MB-COMT represents a minor proportion of total enzyme, it is considered the neuronal and functionally important isoform (Tenhunen and Ulmanen, 1993). MB-COMT has a several-fold higher affinity for substrates than S-COMT, and is capable of metabolizing low concentrations of catecholamines (Rivett *et al.*, 1983; Lotta *et al.*, 1995; Borges *et al.*, 1998).

There is some evidence for species- and tissue-specific heterogeneity of S-COMT, suggesting the existence of isoenzymes. Multiple forms of S-COMT have been isolated that differ in primary structure, molecular mass, isoelectric points and number of cysteine residues, especially those associated with the active site (Lotta *et al.*, 1995; Tilgmann and Ulmanen, 1996).

The cloning and expression of the COMT gene (see below) in a variety of vector systems has allowed production of large quantities of recombinant proteins of both isoforms (Malherbe *et al.*, 1992; Tilgmann *et al.*, 1992). This, coupled to successful purification protocols, has resulted in the crystallization and resolution of the atomic structure of S-COMT (Tilgmann and Ulmanen, 1996). This has allowed accurate determination of the kinetic parameters and substrate selectivity of S-COMT. Clarification of the structure and kinetic reaction mechanism will ultimately lead to the design of more potent and clinically useful COMT inhibitors.

1.8.2 MOLECULAR BIOLOGY

The cDNAs and genes for rat and human S- and MB-COMT have been cloned and characterized (Salminen *et al.*, 1990; Bertocci *et al.*, 1991; Tenhunen *et al.*, 1993 and 1994; Lundström *et al.*, 1995). Only one gene directs the synthesis of both S- and MB-COMT proteins in rats and humans. Two distinct promoters and initiation codons regulate the translation of two mRNAs from the same gene. A proximal promoter (P1) gives rise to S-COMT mRNA (1.3 kb transcript) and a distal promoter (P2) gives rise to MB-COMT mRNA (1.5 kb transcript) [Tenhunen and Ulmanen, 1993; Tenhunen *et al.*, 1993 and 1994]. Further characterization of the proximal promoter has helped to explain the tissue-specific regulation of S-COMT gene expression in mammalian tissues (Tenhunen, 1996).

Human COMT activity is inherited as an autosomal co-dominant trait (McLeod *et al.*, 1994). There are numerous polymorphic variants of the gene. The most common polymorphism results in high, low and intermediate levels of COMT activity and influences enzyme stability (Aksoy *et al.*, 1993; Kunugi *et al.*, 1997). Photoaffinity and Western blot analysis of human hepatic COMT reveals three proteins of different isoelectric points, but the same molecular mass, regardless of phenotypic category (Aksoy *et al.*, 1993). This is consistent with the existence of S-COMT isoenzymes, but implies that COMT genetic polymorphism must be reflected at some other level of the protein structure, e.g. the active site. The frequency of each COMT allele also shows distinct ethnic differences, but no variations between gender. For example, an analysis was performed on erythrocyte COMT activity of American whites and blacks (McLeod *et al.*, 1994). Caucasians are 25% homozygous for the low activity allele, 50% heterozygous for intermediate activity, and 25% have the high activity allele. In contrast, Afro-Americans have a higher frequency of the high-activity allele. In another study, a Saami (Lapps) population was found to have lower erythrocyte COMT activity than a non-Saami Norwegian population. This is also the

first report of a population having a lower activity level than a Caucasian population (Klemetsdal *et al.*, 1994).

1.8.3 TISSUE DISTRIBUTION

COMT is an ubiquitous enzyme, widely distributed peripherally and in the CNS. Good agreement on the distribution of the enzyme has been obtained from determination of activity levels, immunohistochemistry, and mRNA levels. Peripherally, strong expression is seen in mammalian placenta, erythrocytes, thyroid, adrenal cortex, liver, kidney, stomach, duodenum, ileum and pancreas, with highest S-COMT activity found in the liver and kidney (Karhunen *et al.*, 1994; Karhunen *et al.*, 1995; Tilgmann and Ulmanen, 1996).

In the CNS, COMT activity is spread relatively evenly and exhibits both a neuronal and extraneuronal distribution, without following the distribution of catecholamines and catecholamine synthesizing enzymes (Rivett *et al.*, 1983; Kaakola *et al.*, 1987; Karhunen, *et al.*, 1994; Karhunen *et al.*, 1995a and 1995b; Lundström *et al.*, 1995). Using a polyclonal antibody to both isoforms, weak to moderate COMT expression was found in the molecular layer of the cerebellum, neostriatum, parietal cortex, pituitary glands, and scattered small neurons in spinal sensory ganglia. In these CNS regions, COMT-immunoreactivity is associated with the cytoplasm, large tubular structures and near the plasma membranes of dendritic processes and spines. Typically, COMT was distributed in or around the synapse. Thus COMT is clearly located post-synaptically, with no evidence for its presence in neurons presynaptic to the dendritic processes (Karhunen *et al.*, 1995a and 1995b). The post-synaptic neuronal localization of the neuronal high-affinity MB-COMT has also been confirmed by lesion studies (Kaakola *et al.*, 1987; Naudon *et al.*, 1992). In the case of non-neuronal glial cells, strong COMT expression is seen in the cytoplasm of type-1 and type-2 astrocytes and oligodendrocytes (Karhunen *et al.*, 1995a and 1995b). The strongest COMT-immunoreactivity in the CNS is observed in the choroid plexus, leptomeninges, ependymal cells lining the lateral and third ventricles, astroglial end feet around capillaries and tanycytes in the median eminence (Karhunen *et al.*, 1995a). This is consistent with the proposal that COMT serves a barrier function in the blood-brain-barrier. In summary, on the basis of the distribution reported here, the inactivation of catecholamines by COMT may occur primarily in post-synaptic dendritic and/or astrocytic processes around synapses and capillaries.

1.8.4 PATHOLOGY AND CLINICAL SIGNIFICANCE

Inhibitors of COMT are of enormous clinical potential in the treatment disorders associated with aberrant catecholamine function and metabolism. This is particularly true for Parkinson's disease, for several reasons (Da Prada *et al.*, 1994). Firstly, the progressive loss in DA levels can be retarded by inhibiting the O-methylation of the catecholamine. Secondly, the movement disorders and "on-off" fluctuations observed with L-Dopa therapy may be minimized by enhancing the bioavailability and delivery of L-Dopa to the CNS. Peripherally, COMT is responsible for converting L-Dopa to 3-O-methyldopa, which also competes for L-Dopa uptake into the brain. Finally, COMT inhibitors, such as tolcapone and entacapone, have "SAM-sparing effects", making more SAM available for other transmethylation reactions (Da Prada *et al.*, 1994).

COMT may also be important in certain psychiatric disorders. An abnormal meta/para ratio of O-methylated products and abnormal SAM-dependent transmethylation reactions have been implicated in several disorders (Da Prada *et al.*, 1994). The possible association of COMT gene polymorphism and brain diseases is also being actively investigated. Initial studies have found an association with alcoholism, aggression and antisocial behaviour, bipolar affective disorder, and possibly with Parkinson's disease, but not with schizophrenia (Lachman *et al.*, 1996; Kunugi *et al.*, 1997; Strous *et al.*, 1997).

1.9 Gamma-hydroxybutyrate

1.9.1 STATUS AS A NEUROTRANSMITTER

γ-Hydroxybutyrate (GHB) is an endogenous short chain 4-carbon fatty acid found in the brain of mammals (Roth and Giarman, 1970). It is derived *in vivo* from the metabolism of the inhibitory neurotransmitter GABA and 1,4-butanediol, and thus represents a structural analog of GABA (Snead *et al.*, 1989) Reciprocally, GHB may be converted back to GABA (Vayer *et al.*, 1985). Although endogenous levels are very limited, there are several lines of argument that GHB may play a role as a neuromodulator and even a neurotransmitter (Vayer *et al.*, 1988; Cash, 1994). There is a discrete subcellular anatomical distribution for GHB and its synthesizing enzymes (Rumigny *et al.*, 1981; Snead, 1987; Vayer *et al.*, 1988), with highest CNS levels occurring in the hypothalamus and substantia nigra. Peripherally, highest levels are found in the kidney, heart, skeletal muscle and brown fat. There is evidence of a Ca^{2+}-dependent release mechanism (Maitre *et al.*, 1983) and a Na^{+}-dependent uptake mechanism (Hechler *et al.*, 1985). Specific GHB binding

sites, unrelated to GABA receptors, have been identified. There is a good correlation between GHB distribution and that of [^{3}H]GHB binding sites, except the substantia nigra, which is relatively poor in specific GHB binding sites (Benavides *et al.*, 1982; Maitre *et al.*, 1983; Hecher *et al.*, 1992). Maximal [^{3}H]GHB binding is found in the hippocampus and cerebral cortex.

1.9.2 Behavioural and Biochemical Effects

Endogenous GHB is believed to serve as a relatively specific, state-dependent regulator of central DA neurons, particularly of the basal ganglia. Numerous biochemical studies conducted *in vivo*, *ex vivo* and *in vitro* have confirmed that GHB and its prodrug gamma-butyrolactone suppresses the spontaneous firing rate (impulse flow) of midbrain DA neurons (Walters and Roth, 1976; Alter *et al.*, 1984; Howard and Feigenbaum, 1997). This results in an increase in brain DA levels through a concomitant inhibition of DA release and increase in the activity of presynaptic TH activity.

Consistent with an inhibition of DA release, GHB is known to induce antidopamimetic behavioural responses, including akinesia, hypokinesia, sedation, catalepsy, loss of the righting reflex, absence-like seizures, hypoventilation and anaesthesia (Cott and Engel, 1977; Ellinwood *et al.*, 1983; Tunnicliff, 1992; Howard and Feigenbaum, 1997). Moreover, GHB inhibits the DA-release dependent behaviours induced by indirect DA agonists irrespective of whether they are impulse-independent (e.g. d-amphetamine) or impulse-dependent (e.g. methylphenidate). In turn, the effects of GHB can be reversed by drugs stimulating DA release (Cott and Engel, 1970; Ellinwood *et al.*, 1980).

More recent studies have actually shown that the dose-dependent effects of GHB are bi-phasic (Engberg and Nissbrandt, 1993; Howard and Feigenbaum, 1997). High doses of GHB ($\geq$ 400 mg/kg) inhibited the spontaneous firing rate of substantia nigral DA neurons and regularized the firing rhythm. In contrast, low doses of GHB ($<$ 200 mg/kg) increased the spontaneous firing rate, regularized the firing rhythm and converted burst firing neurons to non-burst firing (Engberg and Nissbrandt, 1993). The low doses of GHB employed in the latter study are more in line with endogenous GHB levels in the substantia nigra (Vayer *et al.*, 1988). This suggests that endogenous GHB may actually have stimulatory effects and pacemaker potential in midbrain DA systems. This may help explain why some authors found a stimulation of central DA release following GHB administration (e.g. Diana *et al.*, 1991; Howard and Feigenbaum, 1997).

Initially it was believed that GHB had a direct effect on DA release. However, very strong evidence now exists that the central effects of GHB, including the inhibition of nigro-striatal function, is due to agonistic activity at the $GABA_B$ receptor (Waldmeier, 1991; Xie and Smart, 1992; Engberg and Nissbrandt, 1993; Williams *et al.*, 1995; Snead, 1996). Indeed the effects of GHB are consistently blocked by the $GABA_B$ receptor antagonist CGP 35348 and mimicked by the selective $GABA_B$ agonist baclofen. In contrast, GHB has no effect at the $GABA_A$ /benzodiazepine receptor complex (Serra *et al.*, 1991). Inhibitory GABAergic nerve terminals terminate in both the pars compacta and pars reticulata zones of the substantia nigra. $GABA_B$ receptors are located on DA cell bodies in both areas and are known to regularize the firing pattern and to prevent burst firing of these nigral neurons (Engberg and Nissbrandt, 1993). In contrast, specific [^{3}H]GHB binding sites are relatively sparse in the substantia nigra (Benavides *et al.*, 1982; Hecher *et al.*, 1992). It has even been proposed that the [^{3}H]GHB binding site and the $GABA_B$ receptor are one and the same (Snead, 1996). However, this is unlikely considering that a selective GHB antagonist has been designed (Maitre *et al.*, 1990; Waldmeier, 1991). Finally, it must be noted that GHB may exert its effects on a system downstream and common to the $GABA_B$ receptor, without a direct effect on the latter receptor. Some evidence has also been found for an interaction of GHB with opioidergic systems (Feigenbaum and Howard, 1997).

Chapter 2

Experimental Procedures

2.1 Animals and Housing Conditions

All studies were performed using adult male albino rats of the Wistar strain obtained as an outbred colony from a single breeder, the South African Institute for Medical Research (SAIMR, Johannesburg). Unless otherwise stated, all animals were *ca.* 3 months old with body weights between 200 and 300 g. Rats were maintained in a windowless, well-ventilated constant environment room (CER) under an automated 12L:12D light-dark cycle (lights on at 06h00, Central African Time [CAT]). Artificial lighting was provided by standard cool-white fluorescent bulbs (75 W) with an intensity of illumination of 250 $\mu W/cm^2$. Ambient temperature was regulated at 22 ± 1 °C.

Animals were housed 5 or 6 per cage to minimize stress (Gambardella *et al.*, 1994). Standard laboratory chow and tap water were available *ad libitum*. Cage cleaning and feeding were performed randomly, and only in the photophase, to avoid induction of secondary exogenous rhythms. All animals were given a one week acclimatization period prior to experimental manipulation. Every effort was made to minimize stress to the rats, including experimental handling time. The housing conditions and all experimental protocols were approved by the Rhodes University Ethics Committee for Animal Research.

Experimental manipulation and decapitation in the scotophase were performed under a dim red photographic safety light (wavelength > 620 nm; intensity = 0.8 $\mu W/cm^2$). Red light does not influence pineal indole biosynthesis (Cardinali *et al.*, 1972; Reiter, 1985).

2.2 Tissue Preparation

Rats were sacrificed at designated times by cervical dislocation and decapitation. Using a pair of scissors, an incision was made through the bone from the foramen magnum to near the orbit of each eye. The top of the skull and the meninges were removed. The pineal gland was rapidly excised from the meninges with a sterile microforceps and immediately explanted for organ culture studies. The

whole brain was removed, placed on a chilled glass plate on crushed ice, and rinsed in ice-cold saline [0.9% (w/v) NaCl]. The cerebellum and corpus callosum were removed and the corpus striatum exposed by separating the cerebral hemispheres. Both the right and left corpora striata were microdissected by undercutting the paleostriatum, and pooled. Following a rinse in ice-cold 0.32 M sucrose containing 0.1% (v/v) dimethylsulphoxide (DMSO) as a cryoprotectant, the striata were slowly and uniformly frozen in the vapour-phase of liquid N_2. Samples were stored at -70 °C until further use.

2.3 Crude Enzyme Preparation

The following protocol was used to prepare crude fractions of MAO and COMT from the same tissue source by differential centrifugation and subcellular fractionation.

The pooled striata were slowly thawed on ice and suspended in ice-cold 0.32 M sucrose to give a 5% (w/v) total homogenate, based on the original fresh wet weight of the tissue. This suspension was manually homogenized on ice with a fixed number of strokes using a glass-tephlon homogenizer. The total homogenate was then centrifuged at 2000 rpm for 10 min at 4 °C (Selecta Mixtasel benchtop centrifuge). The supernatant (S_1) was centrifuged at 16500 rpm for 30 min at 4 °C (Beckman L-70 Ultracentrifuge). The resultant supernatant (S_2) represents the cytosolic/microsomal fraction. The sedimented pellets P_1 and P_2 were resuspended in 0.32 M sucrose by gentle agitation and re-homogenization to give a 5% (w/v) nuclear/cell debris fraction and a 4% (w/v) mitochondrial/lysosomal fraction, respectively. For each fraction the % (w/v) was based on the original fresh wet weight of the striata. The nuclear/cell debris fraction was only used in the case of subcellular enzyme distribution studies. All fractions were stored at -20 °C until use.

2.4 Protein Determination

Protein concentrations were determined by modification of the Folin-Lowry assay (Lowry *et al.*, 1951), relative to bovine serum albumin (BSA) as the standard protein. Determinations were performed in triplicate and the mean value used in subsequent calculations. A protein standard curve is presented in Figure 2.1.

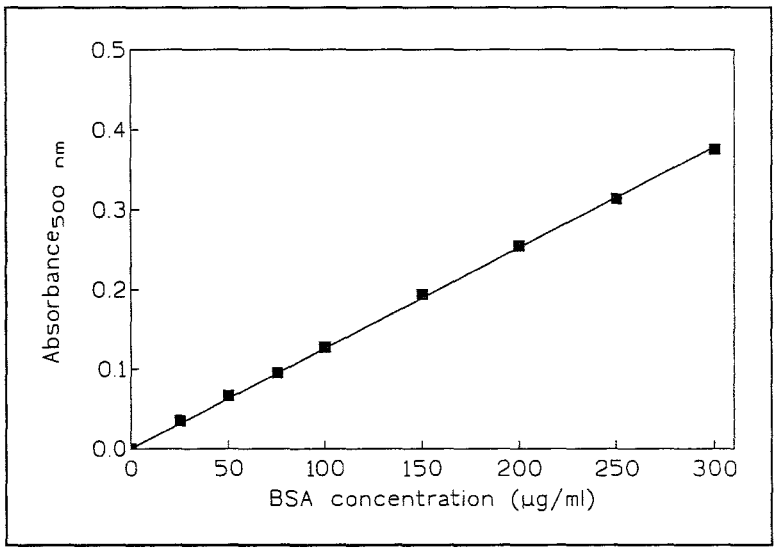


Figure 2.1: Protein standard curve with BSA as the standard protein. (Data represents mean ± SEM (n = 5); $r^2 = 0.9995$ $P < 0.0001$)

2.5 Liquid Scintillation Spectroscopy

2.5.1 CHEMICALS AND REAGENTS

Emulsifier Scintillator Plus™ scintillation cocktail and 8 ml capacity scintillation vials were purchased from Packard Instrument Company (Netherlands).

2.5.2 EXPERIMENTAL PROCEDURE

Only [^{14}C]-labeled radioisotopes were used for experimental work. This allowed quantification of β-emissions by liquid scintillation spectroscopy in a Beckman LS2800 scintillation counter at a counting efficiency of 96% for the full ^{14}C window (0-1000). Samples were added to 8 ml scintillation vials containing 3 ml scintillation fluid and counted. Quench correction was automated by the external channel ratios method and the data converted to disintegrations per minute (DPM).

2.6 Behavioural Studies: Catalepsy

2.6.1 INTRODUCTION

The ability of DA agonists and antagonists to alter DA-mediated behaviour arises primarily from an effect on dopaminergic striatal, limbic and cortical projections (Moore and Bloom, 1978; Jankovic, 1995). A behavioural model was required to help delineate the potential antidopamimetic effects of MEL.

Catalepsy is a drug-induced akinetic state, believed to reflect altered striatal function (Sanberg *et al.*, 1988). It is commonly used as a rodent model of drug-induced Parkinsonism. By definition catalepsy represents the ability of an animal to maintain an abnormal posture for prolonged periods of time. Most commonly used cataleptogenic drugs include the monoamine depletor reserpine and neuroleptics such as HAL.

Typically, catalepsy is assessed by the duration of immobility after placing the animal in an abnormal posture (Yurek and Randall, 1985). This has led to a wide diversity of tests, restricted mostly to the imagination of the researcher and the physical possibility of the abnormal posture. Examples of exotic tests include the "four-cork test" and "Buddha position". This enormous variation in test design does not facilitate the comparison of the cataleptogenic potential and intensity of drugs between laboratories. This has led to the design of the Standardized Horizontal Bar Test (Sanberg *et al.*, 1988), which was routinely employed in this study to test the cataleptogenic potential of MEL and other drugs.

2.6.2 METHODOLOGY

(i) Testing Environment

The catalepsy apparatus comprised a 1 cm diameter wooden bar supported 9 cm above the floor of a testing arena. The testing arenas consisted of the plastic cages (35 x 35 x 26 cm) used to house the animals. The cages were placed on bench tops and the wire-floor removed. Animals were tested individually with the walls of the cages preventing visual contact between adjacent test subjects. All experiments were performed in sound-attenuated CERs. Testing in the dark-phase was performed under red photographic safety lights. Every precaution was taken to minimize handling-induced stress in the animals.

(ii) Testing Procedure

Following a 5 min habituation period in the arena, both the forepaws of an animal were placed on the bar while the hindpaws remained firmly on the floor. The height of the bar (9 cm) was matched to the average size of the animals to minimize discomfiture of the upright posture. Animals were placed on the bar by neck-pinching induced immobilization. The time taken to remove either of the forepaws from the bar was manually timed with a stop-watch and constituted the catalepsy score in seconds (s). A maximum "cut-off" time of 300 s was employed. The final catalepsy score per animals represents the mean ± SEM of three separate trial sessions (n = 3). The presence of catalepsy was occasionally confirmed by the righting reflex test. The latter involves placing the animal on its back and recording the time taken to right itself.

2.7 Data and Statistical Analysis

All raw data were converted to a mean ± SD or SEM, with the sample size (n) specified for each sample group. Statistical comparison of mean values was achieved by the parametric, unpaired Student *t*-test or by the nonparametric, ordinary one-way analysis of variance (ANOVA). In the case of parametric tests, the data was continuous with a normal distribution around the mean. The Student *t*-test was used to compare two mean values within a treatment group (e.g. control *vs.* experimental data) and generated a two-tailed P-value. More complex interactions between multiple (i.e. more than two) mean values were analysed by ANOVA, generating a P-value by the F-test. Main interactions of the ANOVA were further analysed by the Bonferroni multiple comparisons test. Where specified, the less stringent Student-Newman-Keuls was also employed as a post test for comparison. Significant events were then confirmed by the Student *t*-test. In all cases, a $P < 0.05$ was accepted as being significant.

Simple linear regression was performed by the least squares method, generating a correlation co-efficient (r), r^2, and a P-value. The P-value determines whether the slope is significantly different to zero. Non-linear regressions were performed by plotting the data as a rectangular hyperbola (binding isotherm) or as a sigmoidal curve. An r^2 value was generated as a measure of "goodness-of-fit". A F-test to compare two non-linear curves was used to determine whether the data fitted a single or multiple binding site model.

Chapter 3

Pineal Organ Culture Studies: Characterization and Optimization

3.1 Introduction

The robust diurnal rhythm in pineal indole metabolism is a reliable index of the functional state of the pineal gland (Drijhout *et al.*, 1996). The most direct and popular method of assessing indole biosynthesis in circadian research is at the level of the pineal itself. Organ culturing offers a more physiological assessment of tissue biochemistry than homogenate preparations. The large size of most organs in fact necessitates the use of tissue slices. In contrast, the accessibility and small size of the rat pineal gland is convenient for easy culturing in an intact state. Thus the organ culture system would allow for the pharmacological and biochemical manipulation of pineal indole biosynthesis free from hormonal, neuronal and other complicating influences of the *in vivo* milieu.

This has led to the development of a radiometric technique capable of monitoring the *in situ* biosynthesis of [^{14}C]indole products from a radiolabeled precursor by a single pineal (Wurtman *et al.*, 1968b; Klein and Notides, 1969; Klein and Rowe, 1970; Daya and Potgieter, 1982; Morton, 1990). Essential biosynthetic enzymes remain stable and active for at least 48 hrs in culture. Degeneration of presynaptic sympathetic elements is evident after 48 hrs, but the pinealocytes apparently remain morphologically sound (Klein *et al.*, 1983; Santana *et al.*, 1994).

3.1.1 Principle of the Pineal Organ Culture Technique

Pineal glands in culture readily take up an exogenous radiolabeled precursor and synthesize [^{14}C]indoles during a 24 hr incubation period. Approximately 98% of the [^{14}C]indole products are secreted into the culture medium, apparently by passive diffusion (Klein and Rowe, 1970; Morton, 1990). The relative amounts of each indole are similar in the pineal and medium. Thus the culture medium is considered a good reflection of pineal indole biosynthesis (Klein and Notides, 1969; Morton, 1990). Previously, multiple organ solvent extraction systems were required to separate, identify and quantify the [^{14}C]indole products (e.g. Wurtman *et al.*, 1968b; Axelrod *et al.*, 1969). A better approach is to analyze the culture medium directly by thin layer chromatography (TLC), without the need for prior sample treatment.

The profile and number of [^{14}C]indoles formed is dependent on the nature of the chosen radiolabeled substrate, for example [^{14}C]tryptophan *vs.* [^{14}C]5HT (Olivieri *et al.*, 1990; Morton, 1990). This in turn determines the nature of the TLC system to be employed. In the present studies, [^{14}C]5HT was employed as the exogenous substrate. The metabolic products derived from 5HT in the pineal are depicted in Figure 1.2 and include 5MT, NAS, 5HIAA, 5HTOH, MEL, 5MIAA and 5MTOH. This necessitates use of the bi-dimensional TLC system of Klein and Notides (1969) and Morton (1990), employing two distinct mobile phases. A typical chromatogram is shown in Figure 3.1. Excellent resolution is achieved for the following indoles: NAS, 5HIAA, 5HTOH, MEL, 5MIAA and 5MTOH. A disadvantage is that both 5HT and 5MT remain at the origin. An additional TLC system would be required to separate these two indoles. Screening of the remaining TLC plate revealed no radioactivity that could be attributed to an unidentified [^{14}C]indole product (Morton, 1990).

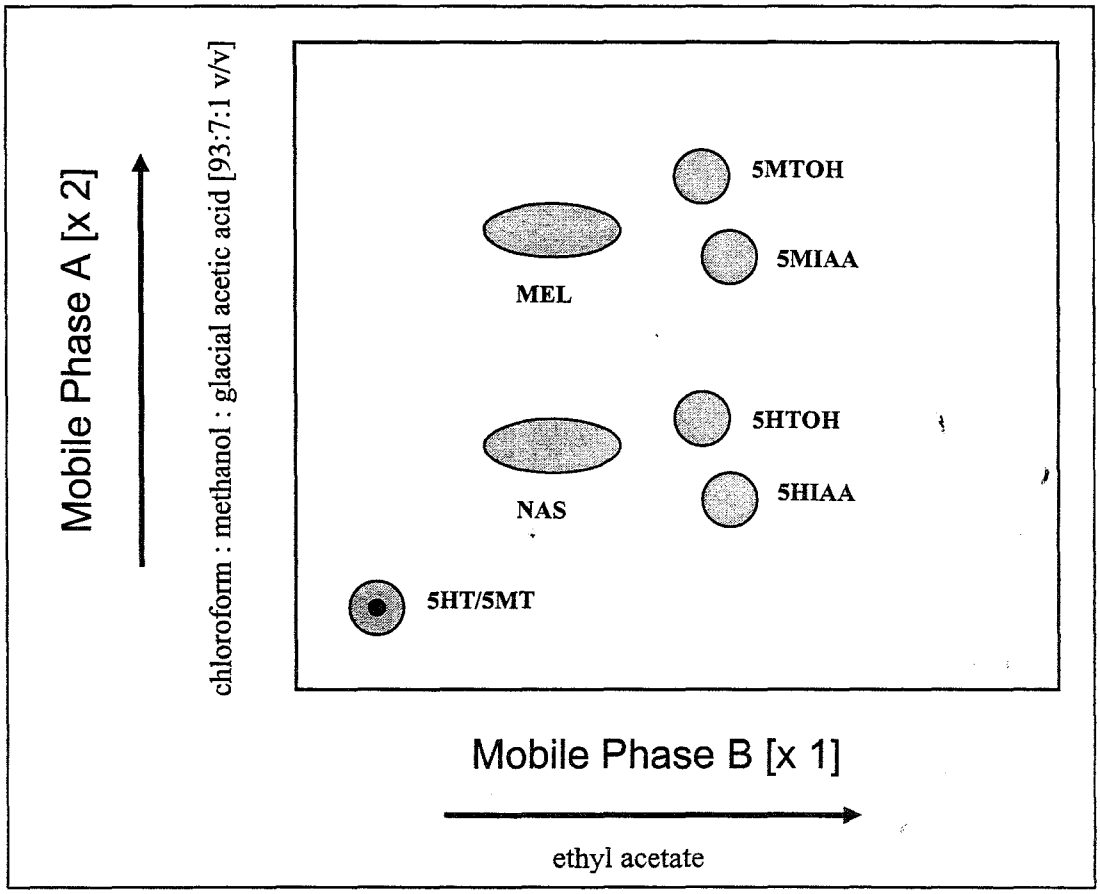


Figure 3.1: Schematic of a typical bi-dimensional thin layer chromatogram of the pineal indoles tested. (● : origin).

The isolated [^{14}C]indoles are then quantified by liquid scintillation spectroscopy. Thus a combination of the radiometric pineal organ system with TLC analysis represents a reliable, reproducible and very sensitive technique. It is capable of separating and quantifying trace amounts of [^{14}C]indoles (≈1 pmol/10 µl culture medium).

It must, however, be stressed that this radiometric technique determines the *in situ* biosynthesis of exogenous [^{14}C]indoles and not endogenous indoles. The concentration of intermediates derived from endogenous substrates is supposedly negligible compared to those derived from the radiolabeled precursor (Morton, 1990). In other words, the radioactive products represent an approximation of the absolute quantities of metabolites formed. However, it is possible that the [^{14}C]indole biosynthetic profile may be influenced by differential compartmentalization of the radioisotopic and non-radioisotopic (cold) substrates and their competition for the same metabolic pathway. A more modern approach is to determine the pineal content of endogenous indoles by high performance liquid chromatography (HPLC) coupled with fluorometric and electrochemical detection (Chin, 1990). Unfortunately, practical and cost considerations prohibited the use of this technology.

Thus the main objective of this chapter was to determine whether the radiometric organ culture technique can reliably monitor the functional state of the pineal gland. The robust difference between the basal and induced NAS and MEL biosynthesis during the photophase and scotophase, respectively, represent two such ideal and opposite states. In the process, this allowed for characterization of untreated rat pineal indole biosynthesis prior to the studies with dopaminergic agents.

3.2 Materials and Methodology

3.2.1 Chemicals and Reagents

The radiochemical 5-hydroxy-(side-chain-2-^{14}C)tryptamine [5HT] creatinine sulphate (specific activity 56 mCi/mmol) was purchased from Amersham International [Amersham, United Kingdom (UK)]. The synthetic indoles 5HT, 5MT, 5HIAA, 5HTOH, NAS, MEL, 5MIAA and 5MTOH were obtained from Sigma Chemical Company [United States of America (USA)]. 4-Dimethylaminobenzaldehyde (DMABA) was also obtained from Sigma; aluminum TLC plates pre-coated with silica gel 60 F_{254} (0.2 mm thickness) from Merck (Darmstadt, Germany), BGJb culture medium (Fitton-Jackson Modification) from GibcoBRL Life Technologies (Paisley, Scotland), HPLC grade ethyl acetate and methanol from LAB-SCAN Analytical Sciences

(Saarchem), HiPerSolv® chloroform for HPLC from BDH Laboratory Supplies (Poole, England) and analytical grade glacial acetic acid from uniVAR® (Saarchem, SA). All other chemicals and reagents were purchased from local commercial sources and were of the highest purity available.

3.2.2 ANIMALS

Male Wistar rats were maintained as described in section 2.1. For all studies, the animals were acclimatized for 1 week to the prevailing light-dark cycle prior to commencement of the experiments.

3.2.3 TISSUE PREPARATION

Intact pineals were rapidly excised as described in section 2.2 and immediately explanted. An attempt was made to remove the pineal stalk under a light microscope prior to culturing.

3.2.4 EXPERIMENTAL PROCEDURE

The organ culture technique and TLC analysis must be performed under subdued light to minimize photo-oxidation of the indolic compounds. For scotophase studies, the removal of the pineal gland was performed under a dim red photographic safety light.

(i) Organ Culture

Prior to the experiment, the BGJb culture medium (Fitton-Jackson Modification) was fortified with µg quantities of the antibiotics sodium benzylpenicillin (Novopen), streptomycin sulphate and amphotericin B (Fungazone®) under sterile conditions.

The pineal glands were placed individually in sterile Kimble glass culture tubes (borosilicate 12 mm x 75 mm) containing 52 µl culture medium. The reaction was initiated by adding 8 µl of [^{14}C]5HT (0.4 µCi/tube). The atmosphere within the culture tubes was then saturated with carbogen [95% O_2:5% CO_2 (v/v)], the tubes immediately sealed and incubated in darkness at 37 °C for 24 hrs. Following the incubation period, the pineals were removed and the culture medium stored at -20 °C until further analysis by TLC. It must be noted that the default total reaction volume is 60 µl and the final concentration of [^{14}C]5HT was ± 120 µM. A control blank was obtained by omitting the pineal gland.

(ii) TLC Analysis of Indoles

A standard solution of non-radiolabeled ("cold") indoles was freshly prepared in 1 ml 95% (v/v) ethanol containing 1% (w/v) ascorbic acid to minimize oxidation and was stored in the dark at 4 °C until use. This solution contained 4 µg of each of the following synthetic indoles: 5HT, 5MT, 5HIAA, 5HTOH, NAS, MEL, 5MIAA and 5MTOH.

A 10 µl aliquot of the sample culture medium was loaded onto a 10 x 10 cm TLC plate at a demarcated origin. The spot was dried with a gentle stream of nitrogen to minimize auto-oxidation of the indoles. To ensure good resolution of the indoles, the spot should ideally be no more than 4-5 mm in diameter. A 10 µl aliquot of the standard indole solution was also spotted onto the origin and again dried with nitrogen. The plates were then developed in saturated TLC tanks according to the scheme outlined in Figure 3.1. First the plates were run twice in the same direction in a mobile phase of chloroform:methanol:glacial acetic acid [93:7:1 % (v/v)]. The solvent front was allowed to move ± 9 cm. The plates were then developed once in ethyl acetate at 90° to the first direction until the solvent front had moved about ± 6 cm. After each run the plates must be thoroughly dried with nitrogen.

The location of each indole was visualized by spraying the plate with van Urk's reagent (1g DMABA dissolved in 50 ml of 25% v/v HCl, followed by the addition of 50 ml of 95% v/v ethanol). The plates were dried in a 60 °C oven for 5 min to allow colour development of the indole spots. With time, all the plated indoles become a blue-green colour. Once dry, each spot was cut out and the associated silica scraped into a scintillation vial containing 1 ml absolute ethanol. The vials were vigorously shaken for 20 min to facilitate extraction of the indoles into the ethanol. The relative amount of each [^{14}C]indole was quantified by liquid scintillation spectroscopy as described in section 2.5.

3.2.5 EXPERIMENTAL DESIGN

Preliminary studies indicated a large inter-individual variation in pineal indole biosynthesis and the possibility of annual fluctuations, in addition to the expected day-night variations. The following experiments represent steps to delineate and control the sources of variation in an attempt to fully characterize and optimize the organ culture technique.

(i) Seasonal Variations

The months of the year were broadly divided into two equal groups, representing "summer" and "winter" months of the southern hemisphere. The six summer months included mid-September to mid-March. The six winter months included mid-March to mid-September. The *in situ* biosynthesis of [^{14}C]indoles by pineals cultured at mid-photophase (12h00) was determined throughout the summer (n = 25) and winter months (n = 25). The monthly data within each group was then pooled and statistically compared.

(ii) Day-Night Variations

A comparison of *in situ* biosynthesis of pineal [^{14}C]indoles during the photophase (n = 25) and scotophase (n = 25) was performed in the winter months of mid-March to mid-September. Pineals were explanted and cultured at mid-photophase (12h00) or mid-scotophase (24h00). Experiments were performed throughout the winter months and pooled for statistical analysis.

(iii) Inter-Individual Variation in [^{14}C]NAS and [^{14}C]MEL Biosynthesis

The previous studies revealed large inter-individual variation in indole biosynthesis. This was particularly true for the biosynthesis of [^{14}C]NAS and [^{14}C]MEL. It was also noted that some pineal glands were visibly larger to the naked eye than others. Furthermore, within a given group of all animals, body weight appeared to be a potential determinant of the observed biosynthetic variability. Therefore the potential inter-individual variation in [^{14}C]NAS and [^{14}C]MEL biosynthesis was investigated in the winter months of mid-March to mid-September for both the photophase and scotophase.

All rats weighed approximately 250 g at the time of being assigned to groups (n = 5/group). Animals were left free to interact, feed and drink for at least two weeks. Groups of rats were then randomly sacrificed throughout the winter period at either mid-photophase (12h00) [n = 50] or mid-scotophase (24h00) [n = 50]. Body weight was recorded just prior to sacrifice. Following incubation, the pineal glands were washed in saline and weighed twice on a micro-balance. Good consistency was found for each weighing attempt and an average pineal weight was recorded. For both the photophase and scotophase studies, the [^{14}C]NAS and [^{14}C]MEL data were pooled separately for analysis. In each case, evidence for high and low production of the indole was determined using the nonparametric One Sample Runs Test which divides the data around the sample median. This allowed for the construction of a High-Low plot of each indole. The potential correlations between body weight, pineal weight, [^{14}C]NAS and [^{14}C]MEL were determined by simple regression analysis.

3.2.6 DATA AND STATISTICAL ANALYSIS

Each TLC plate was assessed for quality of separation and % recovery of total radioactivity. The latter was calculated by comparing the total plated radioactivity (sum of all [^{14}C]indoles) to the expected total radioactivity present in 10 µl medium (after correction for quenching). Generally, 98-100% recovery was obtained for the blank (lacking a pineal) and samples. Radioactivity in the blank is attributable to non-metabolized [^{14}C]5HT. A comparison of blank values at zero-time and following a 24 hr incubation period confirmed that there was no apparent non-enzymatic conversion of [^{14}C]5HT. There was no evidence for radioactivity above background counts in the rest of the plate, excluding the origin.

To improve the reliability of the TLC system, each sample (for all pineal organ culture studies) was plated in duplicate, and sometimes triplicate. Data was only used for analysis if there was good resolution of the indoles. Typically, the consistency between consecutive plates was excellent. Thus the data for each [^{14}C]indole of an individual sample was pooled to produce a single mean value, expressed as DPM/10 µl. These individual means were then used to calculate a group mean ± SEM. The sample size (n) depends on the study. Group means were statistically compared by the Student *t*-test.

It must be reiterated that the present TLC system is incapable of separating 5HT and 5MT. Both of these indoles remain at the origin (Figure 3.1). Further analysis by an alternative TLC system revealed that only trace amounts of 5MT are produced by O-methylation of 5HT under the present conditions (data not shown). Practically, this did not warrant the complication of an additional experimental step. For this reason, data obtained from the origin of the present TLC design is referred to as 5HT and not "5HT+5MT". Besides quantification of the [^{14}C]indoles shown in Figure 3.1, the following metabolic indices were introduced to aid in data analysis: total methylation, total monoamine oxidase products (MAOP), 5HT/MAOP ratio and the 5HIAA/5HTOH ratio. Total methylation is the sum of the 5-methoxyindoles [^{14}C]MEL, [^{14}C]5MTOH and [^{14}C]5MIAA and represents an estimation of O-methylation and thus HIOMT activity in the pineal. This index does not include 5MT. Total MAOP is the sum of the deaminated indoles [^{14}C]5HIAA and [^{14}C]5HTOH. 5HT/MAOP is the ratio of [^{14}C]5HT levels to total MAOP. In combination with total MAOP, this ratio is an indication of pineal MAO activity. The production of [^{14}C]5HIAA and [^{14}C]5HTOH is largely dependent on the ratio of NAD^+/NADH and typically shows a value of ± 2.0 in the rat pineal. Thus the 5HIAA/5HTOH ratio can be used as an indicator of the oxidative state and, thus, the health of the pineal gland.

3.3 Results

(i) Seasonal Variations

Evidence for seasonal variation in the photophase biosynthesis of pineal [^{14}C]indoles is presented in Figure 3.2. Compared to the winter months, there was a substantial decrease in [^{14}C]NAS and [^{14}C]MEL biosynthesis in summer ($P < 0.001$ in each case). Conversely, there was a slight increase in [^{14}C]5MIAA ($P < 0.05$) and a large elevation in [^{14}C]5MTOH levels ($P < 0.001$). This translated into a small increase in total methylation in summer ($P < 0.05$) [Figure 3.2A]. There was no difference in the levels of [^{14}C]5HT, [^{14}C]5HIAA, [^{14}C]5HTOH and total MAOP between the winter and summer months (Figure 3.2B). It must be noted that the compilation of monthly data into two groups tended to dampen the observed trends. For example, the altered 5-methoxyindole biosynthesis and decreased [^{14}C]NAS levels were more pronounced around mid-summer (December) when compared to mid-winter (June). Furthermore, the data was notably more variable at this time, especially with regards to [^{14}C]5HT, [^{14}C]5HIAA, [^{14}C]5HTOH and total MAOP.

(ii) Day-Night Variations

A marked difference was observed in the *in situ* biosynthesis of 5-hydroxyindoles and 5-methoxyindoles by pineals cultured at mid-photophase and mid-scotophase (Figure 3.3). There was a dramatic induction in the biosynthesis of [^{14}C]NAS and [^{14}C]MEL during the scotophase ($P < 0.001$ in each case). This was associated with a significant and substantial decrease in [^{14}C]5MIAA and [^{14}C]5MTOH levels, but an increase in total methylation ($P < 0.001$ in all cases) [Figure 3.3A]. [^{14}C]5HT levels were elevated in the scotophase, whereas [^{14}C]5HIAA, [^{14}C]5HTOH and total MAOP were reduced ($P < 0.001$ in all cases). This was further reflected in an increased 5HT/MAOP ratio ($P < 0.001$) [Figure 3.3B].

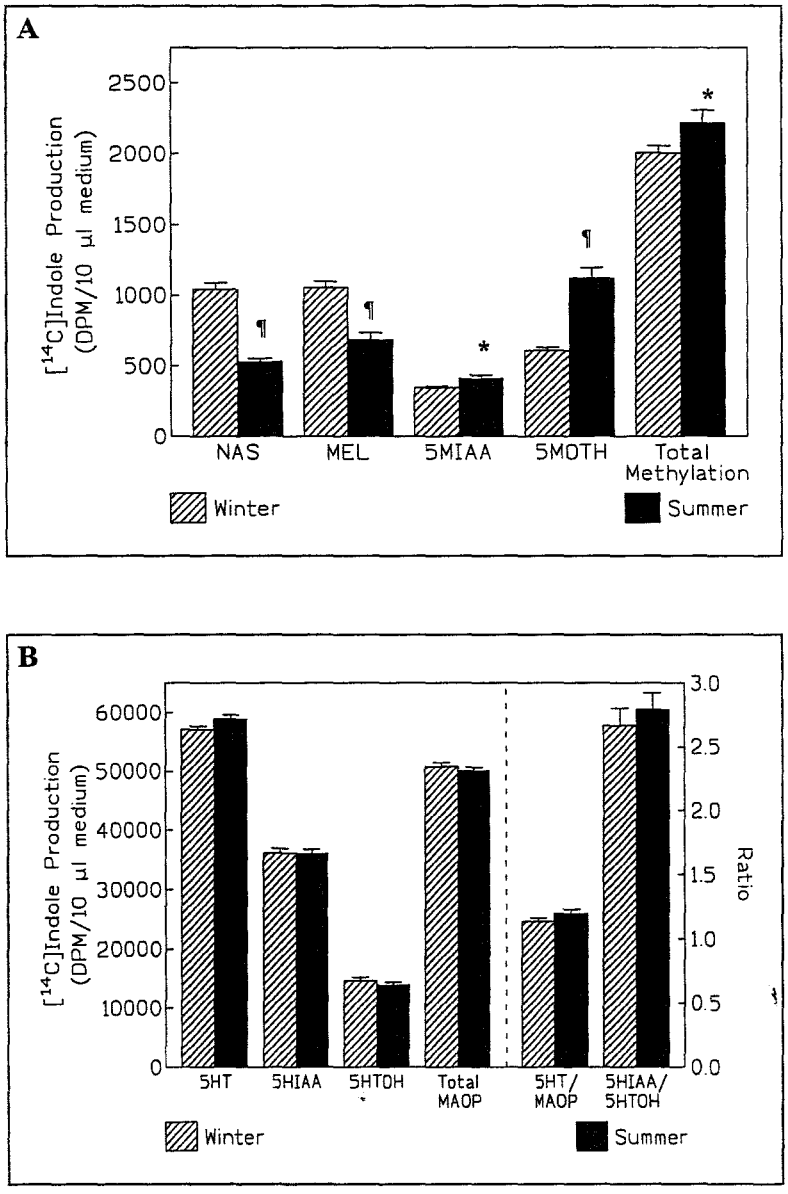


Figure 3.2: Seasonal variation in the *in situ* biosynthesis of [^{14}C]indoles by pineal glands cultured at mid-photophase [12h00]. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 25); P-values (compared to winter): * < 0.05; ¶ < 0.001]

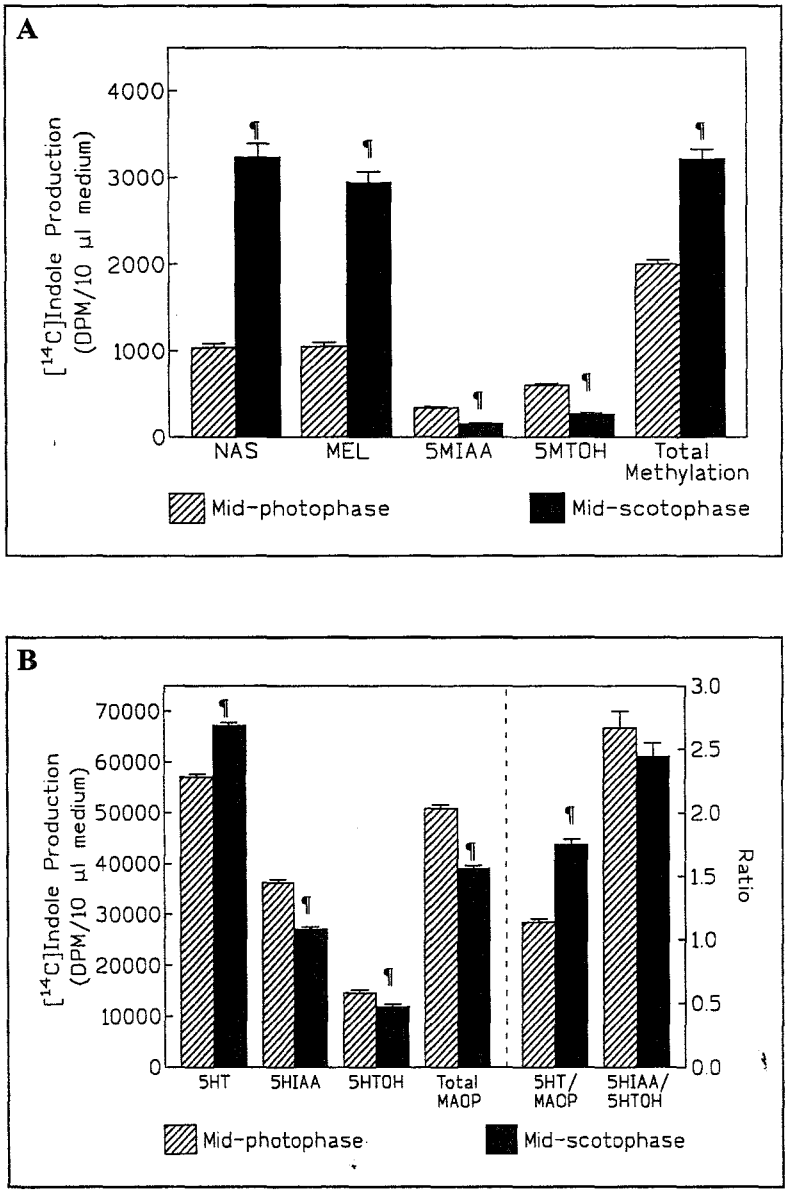


Figure 3.3: Day-night variation in the *in situ* biosynthesis of [^{14}C]indoles by cultured pineal glands. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 25); P-values (compared to mid-photophase): ¶ < 0.001]

(iii) Inter-Individual Variation in [^{14}C]NAS and [^{14}C]MEL Biosynthesis

The inter-individual variation in the biosynthesis of [^{14}C]NAS and [^{14}C]MEL during the winter photophase and scotophase is evident in Figures 3.4 and 3.5 respectively. The data range, median and mean ± SEM for each condition, expressed as DPM/10 µl medium, is summarized below: (a) photophase [^{14}C]NAS: range = 422.08 to 1526.83; median = 917.221; mean = 954.81 ± 39.5984 [Figure 3.4A]; (b) photophase [^{14}C]MEL: range = 524.01 to 1877.26; median = 911.351; mean = 980.70 ± 41.059 [Figure 3.4B]; (c) scotophase [^{14}C]NAS: range = 1279.21 to 5230.87; median = 2989.826; mean = 2970.039 ± 139.776 [Figure 3.5A]; (d) scotophase [^{14}C]MEL: range = 1203.57 to 4196.35; median = 2757.454; mean = 2757.542 ± 104.393 [Figure 3.5B].

In all cases the [^{14}C]indole biosynthetic data could be significantly divided into two distinct groups around the median (One Sample Runs Test: $P < 0.0001$) or the mean ± SEM (data not shown). These two groups were designated the high and low production groups. It must be noted that the median consistently falls within the range of the mean ± SEM for each data group confirming the significance of the finding.

A large variation was seen in the weight of the destalked pineal glands, ranging from 0.64 to 1.87 mg (Figure 3.6). There was no relationship between pineal weight and [^{14}C]NAS levels (Figure 3.6A; correlation co-efficient, $r = -0.0963$; $P = 0.5824$) or [^{14}C]MEL levels (Figure 3.6B; correlation co-efficient, $r = -0.0158$; $P = 0.9284$). There was a significant, but weak correlation between pineal weight and body weight (Figure 3.6A; correlation co-efficient, $r = 0.4627$; $P = 0.0051$). In some cases, the latter relationship was substantially stronger (e.g. $r > 0.70$) when the data from groups of co-habiting animals ($n = 5$) was analysed in isolation, rather than being pooled.

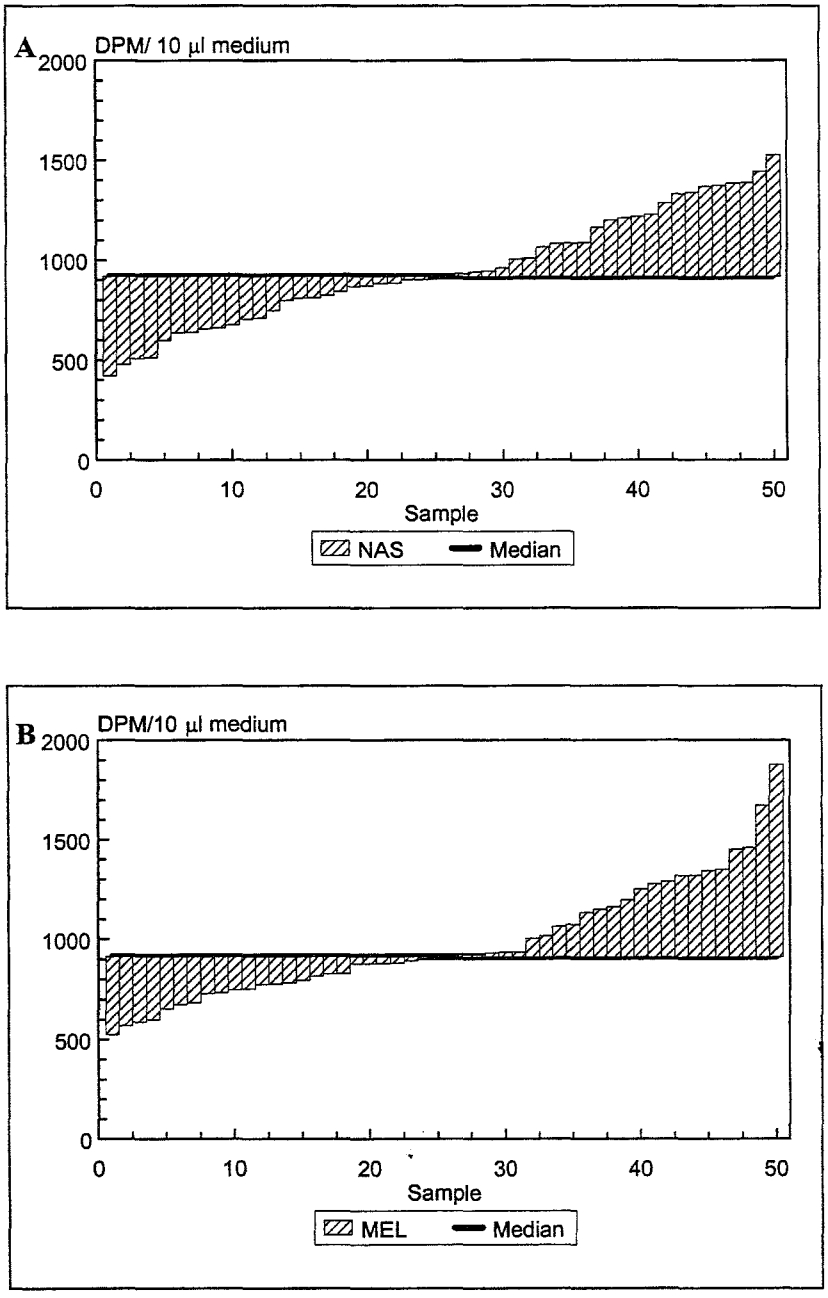


Figure 3.4: High-Low plots showing inter-individual variability in NAS (A) and MEL (B) biosynthesis in the photophase of winter months.

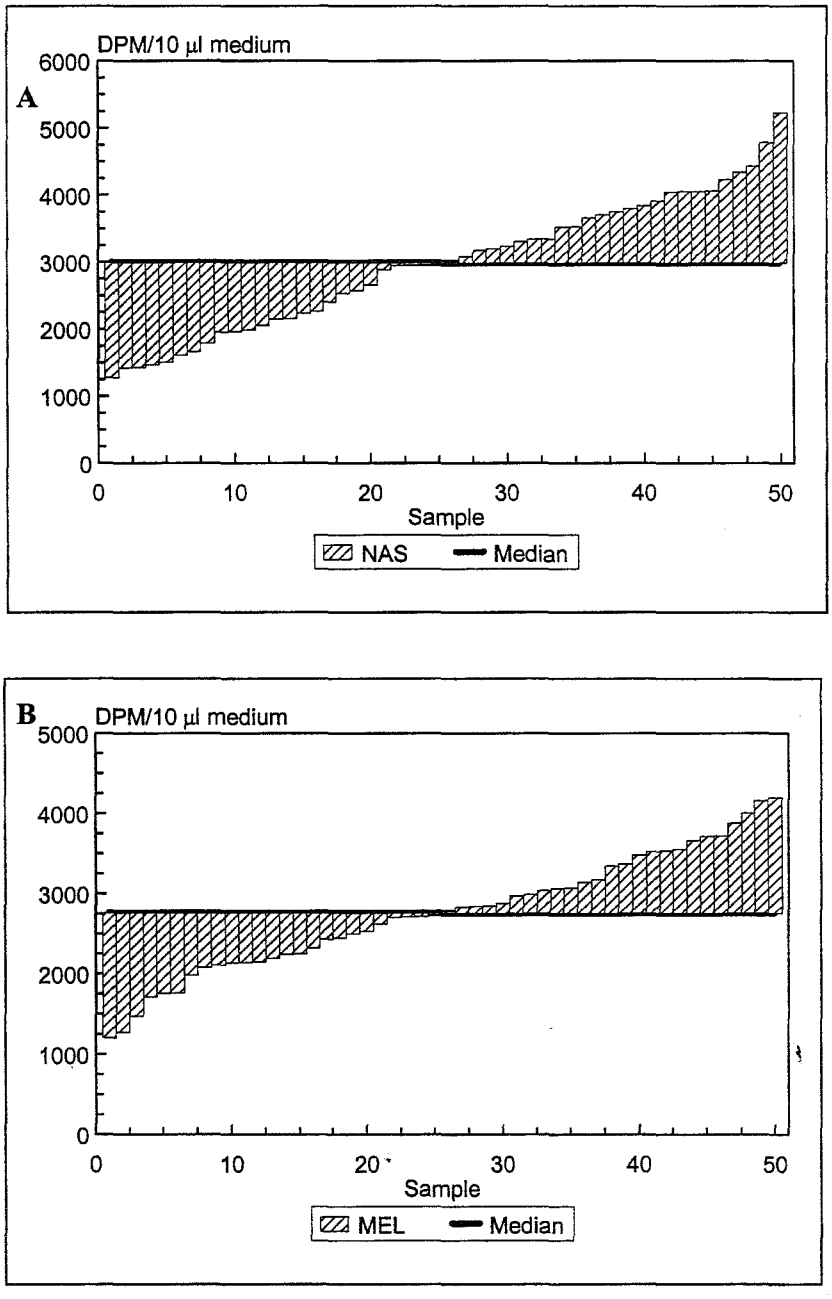


Figure 3.5: High-Low plots showing inter-individual variability in NAS (A) and MEL (B) biosynthesis in the scotophase of winter months.

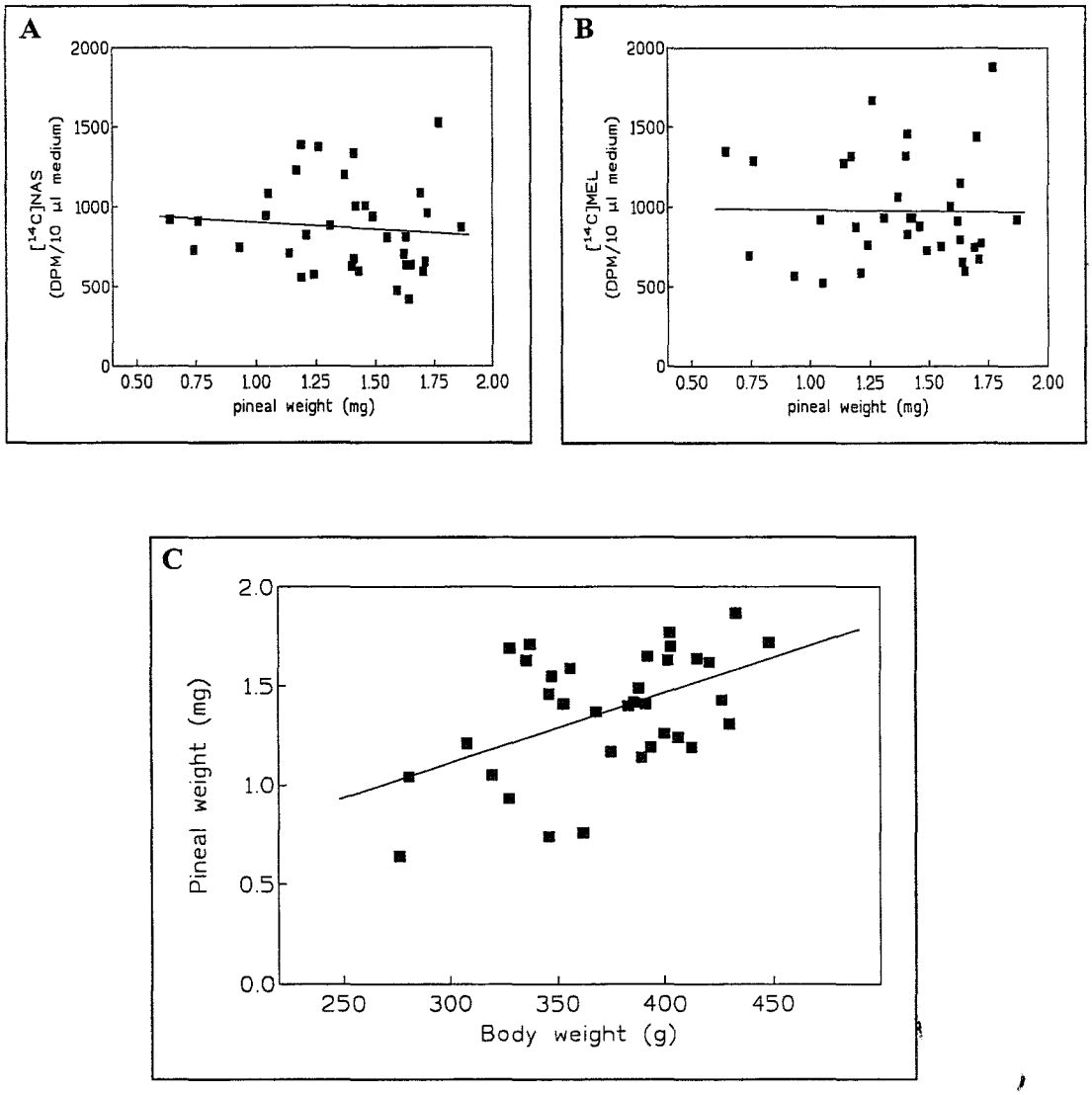


Figure 3.6: Simple regression analysis of pliotophase data (n = 35) during winter months. (A) [^{14}C]NAS biosynthesis *vs.* pineal weight. (B) [^{14}C]MEL biosynthesis *vs.* pineal weight. (C) pineal weight *vs.* body weight.

3.4 Discussion and Conclusion

The present results confirm that cultured rat pineal glands are capable of taking up exogenous [^{14}C]5HT and synthesizing the various [^{14}C]indoles *in situ* from the radiolabeled precursor during a 24 hr incubation period. Furthermore, the radiometric organ culture technique is capable of detecting fluxes in the metabolic state of the pineal, namely photophase *vs.* scotophase, and, paradoxically, winter *vs.* summer months. The maintenance of laboratory animals under the stable conditions of a CE room and a regulated light:dark cycle constitutes an environment very different to natural conditions. In the absence of environmental cues such as temperature and changing daylength, the rats would be expected to be aseasonal. However, there is widespread agreement that the MEL rhythm under these artificial conditions is still similar to that seen in the field (Brainard *et al.*, 1982), confirming the present evidence for seasonality in pineal indole metabolism..

The indole biosynthetic profile observed during the photophase and scotophase was consistent with that routinely reported for the rat pineal gland (Wurtman *et al.*, 1968b; Juillard and Collin, 1979; Daya and Potgieter, 1982; Olivieri *et al.*, 1990; Morton, 1990). During both phases, the deaminated metabolites of [^{14}C]5HT were the major products, with [^{14}C]5HIAA as the predominant metabolite followed by [^{14}C]5HTOH. In contrast, the major metabolite of cultured chick pineal glands is [^{14}C]5HIAA followed by [^{14}C]5MIAA with negligible amounts of [^{14}C]5HTOH being detected. In the case of the pigeon pineal, [^{14}C]5HTOH is the major metabolite followed by [^{14}C]5HIAA (Wainwright, 1977). Thus in the rat, chick and pigeon pineals, deamination by MAO represents the main metabolic fate of [^{14}C]5HT, but the ratio of [^{14}C]5HIAA:[^{14}C]5HTOH differs between the species. This is consistent with the abundance of MAO in the pineal gland (Muller and Da Lage, 1977; Juillard and Collin, 1979). The difference in the [^{14}C]5HIAA:[^{14}C]5HTOH ratio may reflect the relative prevalence of alcohol dehydrogenase and aldehyde dehydrogenase in the pineal or differences in the NAD^+/NADH ratio (Wainwright, 1977). In contrast to 5HT, MEL represents the major metabolite when tryptophan is used as the radiolabeled substrate (Wainwright, 1977; Olivieri *et al.*, 1990). The use of tryptophan also allows for the monitoring of tryptophan hydroxylase and L-Dopa decarboxylase activity. For these reasons, it is typically considered more physiological to use tryptophan than 5HT as the substrate (Klein and Notides, 1969; Morton, 1990).

In comparison to the photophase, three main differences were seen in the indole biosynthetic profile during the scotophase:

(i) An induction of [^{14}C]NAS and [^{14}C]MEL production.

(ii) An increase in total methylation and a change in the relative contribution of each [^{14}C]5-methoxyindole.

(iii) A decrease in [^{14}C]5HIAA and [^{14}C]5HTOH production, indicative of a reduction in pineal MAO activity.

The increase in [^{14}C]NAS and [^{14}C]MEL production correctly reflects the well-established induction of pineal NAT activity during the scotophase due to activation of the noradrenergic secretory drive (Deguchi and Axelrod, 1972a; Axelrod, 1974; Reiter, 1991). The increase in [^{14}C]MEL biosynthesis may also be a consequence of an increase in HIOMT activity at night. As shown in Figures 1.2 and 3.1, every 5-hydroxyindole can be O-methylated to a corresponding 5-methoxyindole by HIOMT. The biosynthesis of the 5-methoxyindoles is dependent on three factors: (i) the activity of HIOMT; (ii) the bioavailability of each 5-hydroxyindole; (iii) the affinity of each 5-hydroxyindole for HIOMT. Overall methylation is described by the following theoretical equation: Total methylation = $\Sigma(C_i.A_i)$; where C_i = concentration of the 5-hydroxyindole and A_i = affinity of the 5-hydroxyindole for HIOMT. This results in the following expression for pineal O-methylation: Total methylation = 0.6868 [NAS] + 0.2151 [5HTOH] + 0.0341 [5HIAA] + 0.0640 [5HT] (Morton, 1990). This shows that HIOMT has a preference for NAS, the product of NAT activity. Thus an increase in NAS levels could result in increased MEL levels without an elevation of HIOMT activity. In addition, this equation indicates why 5MTOH is more abundant than 5MIAA, second to MEL. Strong linear relationships were found between each 5-hydroxyindole and its 5-methoxyindole, and between actual and theoretical methylation for cultured rat pineal glands (Morton, 1990). In the present studies, the total methylation index does not include [^{14}C]5MT and thus it is only an approximation of total pineal O-methylation. The equation indicates that on the basis of affinity for HIOMT the amount of [^{14}C]5MT produced would be comparable to that of [^{14}C]5MIAA, which was only present in trace amounts compared to [^{14}C]5HT. Nonetheless, this index did succeed in highlighting state-dependent alterations in O-methylation and the relative contribution of each [^{14}C]5-methoxyindole. In the case of the present scotophase studies, the increase in [^{14}C]MEL levels and decrease in [^{14}C]5MIAA and [^{14}C]5MTOH levels supports the bioavailability argument. However, the change in the 5-methoxyindole profile still translates into a significant elevation in total methylation. The latter is highly suggestive of an increase in pineal HIOMT activity during the dark-phase, although the existence of a diurnal rhythm in the activity of this enzyme has been strongly debated (Reiter, 1991).

Several papers have suggested that pineal MAO activity, in addition to NAT, may be under noradrenergic control (Axelrod *et al.*, 1969; Olivieri *et al.*, 1990). However, it is more likely that the apparent decrease in MAO observed during the scotophase is a consequence of differential uptake of exogenous [^{14}C]5HT and its competition with endogenous 5HT and NA. There are two distinct pools of 5HT within the pinealocyte, a vesicular pool and a cytoplasmic pool (Racké *et al.*, 1991). During the photophase, the high 5HT content of the pineal is associated with the vesicular pool, where it is protected from deamination by MAO. At night, this pool is preferentially utilized for the synthesis of NAS. *In vitro* studies with isolated bovine pinealocyte suspensions have demonstrated the existence of two independent uptake systems for ^{3}H-5HT: a high affinity/low capacity system and a low affinity/high capacity system (Ducis and DiStefano, 1980). The first system is a temperature- and sodium-dependent carrier mediated system highly specific for 5HT, showing a high affinity with a K_m of 200-700 nM. The second system is a non-specific temperature-dependent system, showing a low affinity for 5HT ($K_m \approx 200$ μM). Although the second system is most probably not of major physiological importance, it may be responsible for the uptake of 5HT into the SNS nerve terminals innervating the pineal gland (Juillard and Collin, 1979). In the current radiometric organ culture technique, [^{14}C]5HT was added to the culture medium at a final concentration of ≈ 120 μM. Thus both transport systems are likely to have been operative during culturing.

The pinealocyte contains 30% of measurable pineal MAO activity, whereas the SNS nerve terminals contain 70% (Racké *et al.*, 1991). Furthermore, NA is also a substrate of MAO (Garrick and Murphy, 1982). Thus it is proposed that following uptake into the pinealocytes and the SNS nerve terminals, [^{14}C]5HT has to compete with 5HT and NA respectively for deamination by MAO. During the photophase, NA levels are low and the access of [^{14}C]5HT to the vesicular pools is limited as they are saturated with endogenous 5HT. Thus [^{14}C]5HT is preferentially metabolized by MAO, resulting in high [^{14}C]5HIAA and [^{14}C]5HTOH production. Conversely, during the scotophase, the increased NA levels out-compete exogenous [^{14}C]5HT for deamination by MAO, and the radioisotope also has increased accessibility to the vesicular stores.

A comparison of the summer and winter studies showed that pineal [^{14}C]indole biosynthesis also displays seasonal variations. It is arguable that the decrease in [^{14}C]NAS and [^{14}C]MEL biosynthesis reported here for the summer months mimics that which would be seen in the wild in response to increased daylength. The decrease in [^{14}C]MEL was associated with a dramatic increase in [^{14}C]5MTOH levels, and a slight increase in [^{14}C]5MIAA synthesis and total methylation. This profile is very similar to that reported by Balemans *et al.* (1979), who found that the MEL/5MTOH ratio, but not total methylation, shifted during the year in rat pineal glands. In

their analysis, the ratio was inverted in spring and autumn months. The authors proposed that the observed profile was due to a differential preference or affinity of HIOMT for endogenous substrates, as argued by Morton (1990) and discussed above. In other words, this would suggest a seasonal rhythm in "the production of methylated hydroxyindoles" rather than HIOMT activity (Balemans *et al.*, 1979). Furthermore, the seasonality was attributed to an influence of endogenous pteridines, which have been shown to regulate retinal and pineal HIOMT activity (Cremer-Bartels *et al.*, 1983). The current results also suggest that the basal NAT activity was lower during the summer months. It is speculated that the pineal may be sensitive to fluctuations in geomagnetic fields, from which the gland would not be effectively shielded in a CER. The ability of magnetic fields to suppress pineal MEL biosynthesis has been demonstrated by Reiter and Richardson (1992).

The biosynthetic capacity and secretory output of pineal MEL is known to be highly variable in rodents. Besides distinct species-specific variations, there are large differences between strains and breeding stocks of a given species. For example, pigmented rats (e.g. the Wistar strain) appear to have larger pineal sizes, 5HT and MEL contents, and NAT and HIOMT activities than pigmented rats (Vollrath *et al.*, 1989). In other words, the activity of the pineal seemed to be positively correlated to the size of the gland, at least between strains. In the present studies, there was a large variation in the production of [^{14}C]NAS and [^{14}C]MEL during both the photophase and scotophase within the same strain of rat, namely the Wistar strain. Extensive inter-individual variability has also been observed in the synthesis, secretion and excretion of MEL by humans. The latter subjects could subsequently be divided into distinct groups of high and low MEL secretors/excretors (Follenius *et al.*, 1995). Statistical analysis of the current data suggests that Wistar rats can similarly be divided into a high and low producers on the basis of [^{14}C]NAS and [^{14}C]MEL biosynthetic capacity.

Multiple variables are likely to be responsible for the observed inter-individual variability in indole biosynthesis. As a first approximation, a relationship between pineal size and MEL production would appear to be an obvious candidate. The mammalian pineal is highly variable in gross-anatomic structure and size. In rodents, the volume of the pineal can differ by 300-fold between species. Furthermore, the pineal weight of male Sprague-Dawley rats can vary considerably from 0.4 to 1.85 mg (Vollrath *et al.*, 1989). A similar range in pineal weight was found for adult male Wistar rats in the current study. However, there was no relationship between pineal weight and [^{14}C]NAS or [^{14}C]MEL production during the winter photophase. It is possible that the density of the pinealocytes, the MEL synthesizing and secretory cells, within the pineal

may be more important than the absolute weight of the gland. Reiss *et al.* (1967) did report that rat pineal glands could be divided into two distinct groups on the basis of pineal cell density.

The circadian rhythm in pineal MEL production is the most reliable hormonal rhythm in vertebrates (Drijhout *et al.*, 1996). Irrespective of the inter-individual variability in humans, the rhythm and amplitude of MEL is highly robust and stable within a given individual (Follenius *et al.*, 1995). Thus the variability in MEL biosynthesis may reflect individual variations in the genetic control of the pineal noradrenergic secretory drive and/or the activity of the two rate-limiting enzymes, NAT and HIOMT (Ebihara *et al.*, 1986). An understanding and consideration of this variability may help explain the high inter-individual variability observed in certain brain functions and behavioural responses known to be modulated by MEL.

Alternatively, the variability may be due to environmental factors such as handling-induced stress and social interactions between the animals. Stress is known to induce pineal MEL synthesis in rats (Oxenkrug and McIntyre, 1985), but in the present studies all attempts were made to standardize the handling of the animals. Interestingly, there was a trend towards a positive correlation between pineal weight and body weight for the sample group as a whole. When the analysis was extended to animals that were housed together, the strength of the latter correlation was significantly stronger for certain groups. Vollrath *et al.* (1989) found that within a strain of rat there was no correlation between pineal protein content and body size, but between strains these two parameters showed a positive correlation during both the day and night. A preliminary working hypothesis proposed here is that this phenomenon may be related to the dominance hierarchical structure within the cage. All animals weighed approximately the same (250 g) when they were first housed together. It is thus proposed that the resultant difference in body weight with time could be due to differential access to food and water, which in turn is determined by the dominance of an animal. Reciprocally, the heavier body weight of the rat will reinforce its position in the hierarchical structure. This model is consistent with the fact that MEL regulates growth hormone secretion, appetite and body metabolism (Reiter, 1989). Interestingly, Kozlova *et al.* (1996) reported that brain MAO activity of male rats varied with the social dominance status of the animal.

In summary, the *in situ* radiometric organ culture technique coupled to TLC analysis does allow for the sensitive and reproducible quantification of pineal [^{14}C]indole biosynthesis. The reliability of the technique as an index of the functional state of the pineal was confirmed by the day-night and seasonal studies. At the same time, the successful identification of potential sources of

variation will allow for improved experimental design to further minimize variation and optimize the technique. For example, pineal organ culture studies should be performed in winter months. However, the apparent day-night variation in pineal MAO activity does highlight a potential problem with using exogenous [^{14}C]5HT rather than quantifying endogenous indole content of the pineal gland.

Furthermore, two other problems may exist with the static nature of the pineal organ culture technique. Firstly, the viability of the cultured pineal cells must be considered. The outer cells may be healthy, but more central cells may suffer from hypoxic injury and thus be metabolically different. Alternatively, the outer cells are more likely to be prone to physical damage. Sampling of the culture medium also poses problems. For example, there is the possibility that the build up of indoles in the non-renewed medium around the pineal may induce end-product feedback inhibition of indole biosynthesis (Trentini *et al.*, 1982; Morton, 1990; Yanez. and Meissl, 1995). Under *in vivo* conditions, MEL is continuously drained away from the pineal gland by blood flow of the venous sinus. Furthermore, the organ culture technique does not allow for the study of variations in indole biosynthesis and MEL secretion over short periods. This has led to the argument that the medium content of MEL is not a reliable index of pineal MEL biosynthesis due to the lack of change in rate of MEL release. Both these problems could be overcome by time-dependent sampling, but this is time-consuming and adds further complications. Alternatively, endogenous indole content of the pineal should be assessed, or their release determined by *in vivo* microdialysis (Drijhout *et al.*, 1996).

Chapter 4

The Effect of Dopaminergic Agents on Pineal Indole Biosynthesis

4.1 Introduction

Immunohistochemical studies have suggested that the nature of the mammalian pineal dopaminergic system is species-specific. This has been extensively reviewed in section 1.5. In summary, the pineals of Syrian and Djungarian hamsters contain intrinsic dopaminergic neurons (Jin *et al.*, 1988, Shiotani *et al.*, 1989; Hermes *et al.*, 1994). In contrast, rat, gerbil and porcine pineal glands appear to be innervated by dopaminergic fibres arising outside the gland, mostly from central structures such as the habenula complex and the posterior commissure (Zhang *et al.*, 1991; Kaleczyc *et al.*, 1994). However, the function of the dopaminergic system in each case is unclear, particularly since it does not appear to be influenced by photoperiod.

The biochemical and pharmacological evidence for an independent dopaminergic system within the rat pineal is often conflicting and contentious. The lack of a systematic experimental approach has resulted in a mass of confusing data that is difficult to unravel. Mostly, there has been a lack of consideration for dose-dependent and state-dependent effects, poor specificity of neurochemical agents and species- or strain-specific variations. For example, Govitrapong *et al.* (1984) presented evidence for a D_2 receptor in the bovine pineal gland. However, the effects of DA and D_2-selective agonists and antagonists on NAT activity and MEL biosynthesis were investigated on rat pineals (Govitrapong *et al.*, 1989). In addition, the latter study investigated the effect of DA on basal NAT activity, but the effect of D_2 agonists on NA-induced NAT activity. Likewise, numerous studies, including the latter two, also failed to state whether the experiments were performed during the photophase or the scotophase. Furthermore, the neurochemical agents employed must be able to delineate between noradrenergic and dopaminergic systems. The noradrenergic secretory drive of the pineal is well characterized (Deguchi and Axelrod, 1972a; Axelrod, 1974; Reiter, 1991), whereas DA may simply serve as a precursor of NA or be a neurotransmitter in its own right. In either case, the enzymes TH and L-Dopa decarboxylase are common to the biosynthesis of both DA and NA. The similarity between pineal indoleamine and catecholamine biosynthesis complicates the picture even further (see Figures 1.2 and 1.3 for comparison).

The main objective of this chapter was to clarify the presence and nature of a dopaminergic system capable of modulating indole biosynthesis in the pineal glands of adult male Wistar rats. To this end, the dopaminergic agents DA, APO and HAL were employed (Figure 4.1).

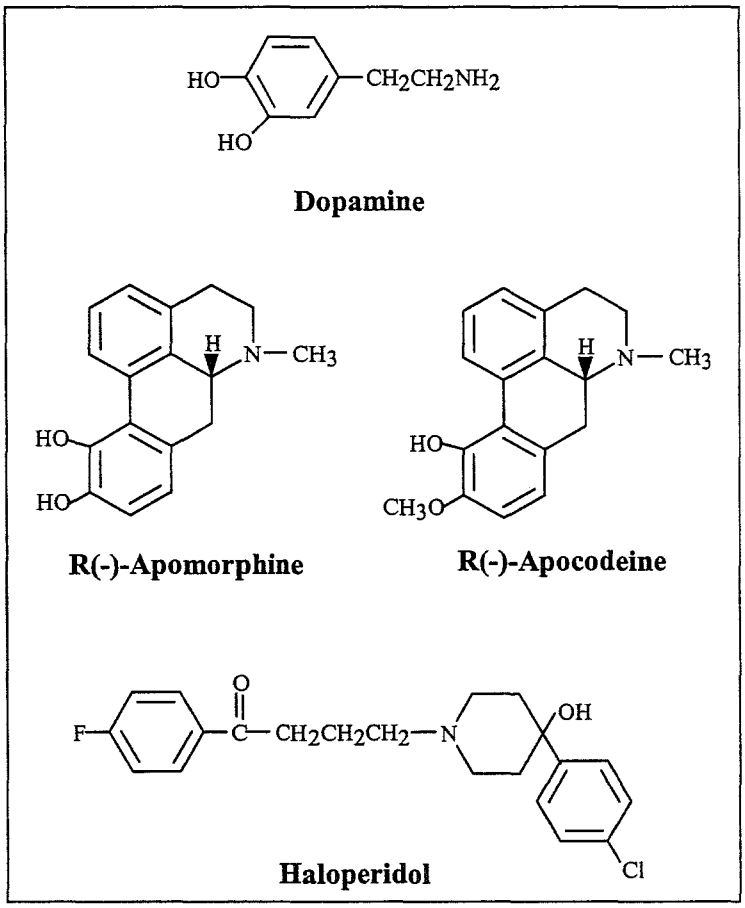


Figure 4.1: Structures of some dopaminergic agents.

Particular attention was paid to possible dose-dependent and chronotypic effects. DA is the endogenous ligand for the DA receptor, regardless of the receptor subtype. The synthetic drug APO occurs as two stereoisomers, R-(-)-APO and S-(+)-APO. R-(-)-APO is generally defined as a mixed D_1/D_2 agonist. More specifically, it is a partial agonist or antagonist at D_1 receptors, but a potent full agonist with high intrinsic activity (nanomolar potency) at the D_2 receptor (Kebabian and Calne, 1979; Niznik, 1987; Strange, 1987). In contrast, S-(+)-APO is pharmacologically a full DA antagonist. The R-(-)-stereoisomer was employed for these studies. HAL is an antipsychotic of the butyrophenone class. This drug is a typical neuroleptic and acts as a relatively specific

antagonist at both presynaptic (autoreceptor) and postsynaptic D_2 receptors (Kendler *et al.*, 1982; Creese *et al.*, 1984; Lidsky and Banerjee, 1993). Furthermore, the radioligand ^{3}H-HAL has been shown to only label D_2 receptors (Creese *et al.*, 1984). The main neurochemical effects and the therapeutic efficacy of HAL in affective disorders such as schizophrenia is directly related to its blockade of the postsynaptic D_2 receptor.

4.2 Materials and Methodology

4.2.1 CHEMICALS AND REAGENTS

The chemical inventory for the pineal organ culture technique is listed in section 3.2.1. DA HCl, R-(-)-APO HCl and HAL were purchased from Sigma.

4.2.2 ANIMALS

Adult male Wistar rats (200-300 g) were housed for 1 week prior to the studies, as previously described (see section 2.1). Animals were randomly sacrificed by cervical dislocation at either 12h00 (mid-photophase) or 24h00 (mid-scotophase), depending on the study. All scotophase studies were performed under a red photographic safety light. Unless otherwise specified, all experiments were performed in the winter months of mid-March to mid-September.

4.2.3 PINEAL ORGAN CULTURE TECHNIQUE

The standard organ culture technique and TLC analysis of [^{14}C]indoles was performed as described in 3.2.4. Following excision of the pineal gland, an attempt was made to rapidly remove the pineal stalk prior to incubation.

(i) *In Vitro* Studies

Fresh working solutions of DA HCl and R-(-)-APO HCl were prepared in culture medium containing 1 mg/ml ascorbate as an antioxidant. The solutions were stored in brown borosilicate bottles in the dark until use to reduce auto-oxidation.

Pineals were randomly placed in individual Kimble tubes. The test drugs or vehicle were added in a volume of 10 µl to give the desired final concentration of the agent. Pineal indole biosynthesis was initiated by the addition of [^{14}C]5HT immediately thereafter. Thus, for all practical purposes, there was no preincuabtion period with the test drug. The final total volume of all samples was 70 µl for the *in vitro* studies, giving a final concentration $\approx$ 100 µM [^{14}C]5HT.

(ii) *In Vivo* Haloperidol Administration

The acute administration of HAL was performed at two doses: 2.5 mg/kg and 5.0 mg/kg body weight (bwt). In both cases, the vehicle comprised: 5% (v/v) benzyl alcohol, 30% (v/v) propylene glycol, 65% (v/v) deionised H_2O. A single dose of HAL or vehicle was administered i.p. 90 min prior to sacrifice at 12h00. The behavioural responses of the animals were recorded prior to sacrifice. Pineals were rapidly transferred to the corresponding pre-labeled Kimble tubes. The final total volume of all samples was 60 µl for the *in vivo* studies, giving a final concentration ≈ 120 µM [^{14}C]5HT.

4.2.4 Data and Statistical Analysis

Data is expressed as DPM/10 µl medium and graphically represented as mean ± SEM. The sample size (n) for each study is recorded in the figure captions. Mean experimental values were statistically compared to their respective control mean values by the Student *t*-test.

4.3 Results

4.3.1 Dopamine Organ Culture Studies

(i) Photophase

At final concentrations of 100 nM, 1 µM and 10 µM, DA *in vitro* had no significant effect on the *in situ* biosynthesis of any [^{14}C]indole by the pineal glands explanted and cultured during the mid-photophase (12h00) [Figures 4.2, 4.3, and 4.4]. In the presence of 1 µM DA, a slight decrease in [^{14}C]5HT levels (P = 0.0836) was accompanied by a small, but nonsignificant increase in [^{14}C]5HIAA levels (P = 0.1302) and total MAOP (P = 0.085) [Figure 4.3B]. This would suggest a possible effect on MAO activity, but there was no similar trend with 10 µM DA. It must also be noted that the 1 µM DA study was performed in early summer (February). This is evident in the lower [^{14}C]MEL and [^{14}C]5MIAA levels and higher [^{14}C]5MTOH levels obtained for the control samples (Figure 4.3A) as discussed in section 3.4 (see Figure 3.2). Furthermore, it has been argued that intra- and inter-assay variation appears to be higher for studies performed in summer months, and thus the putative effects of 1 µM DA on MAO may be an artifact.

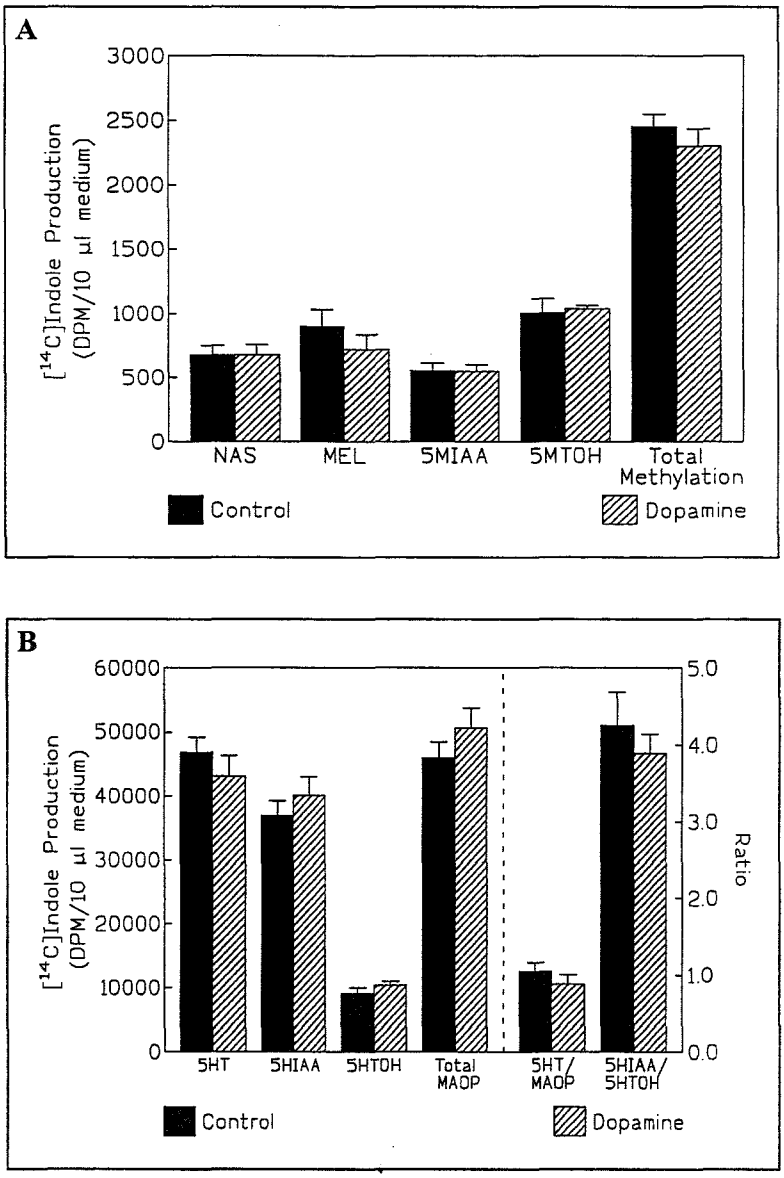
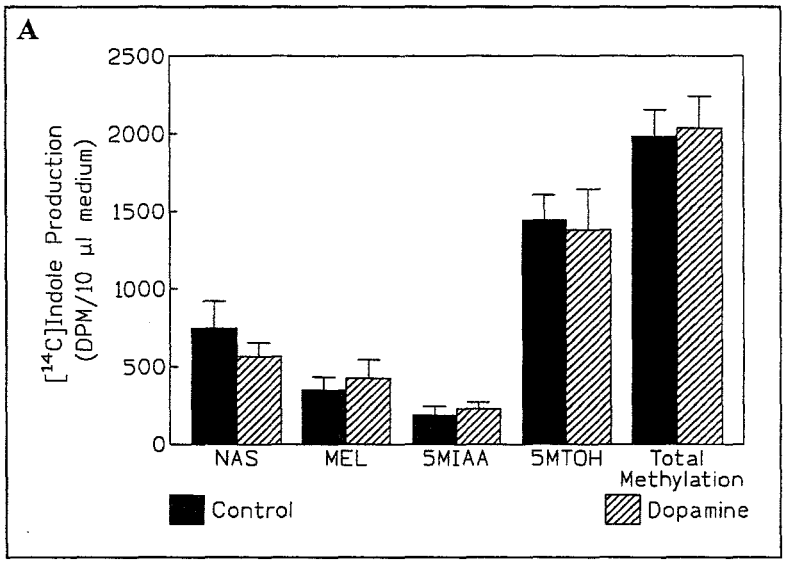


Figure 4.2: The *in vitro* effect of DA HCl (100 nM) on the *in situ* biosynthesis of [^{14}C]indoles by rat pineal glands cultured during the mid-photophase [12h00]. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 5)]



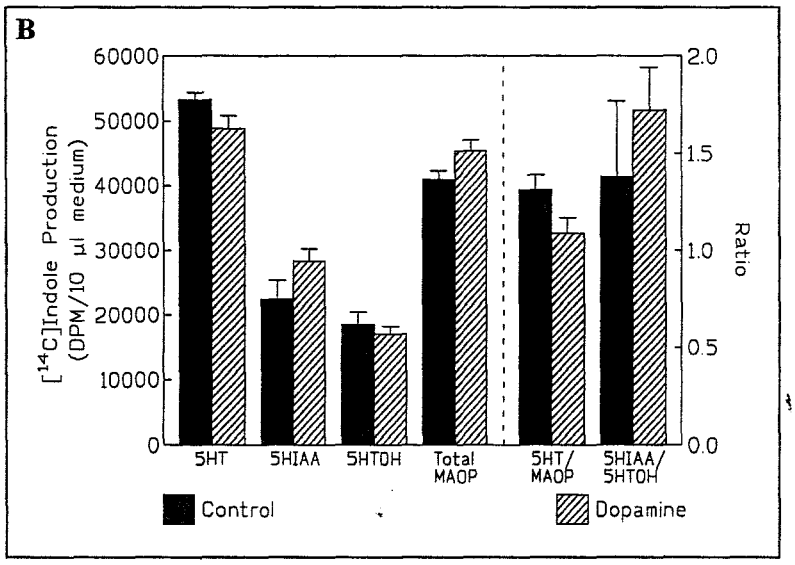
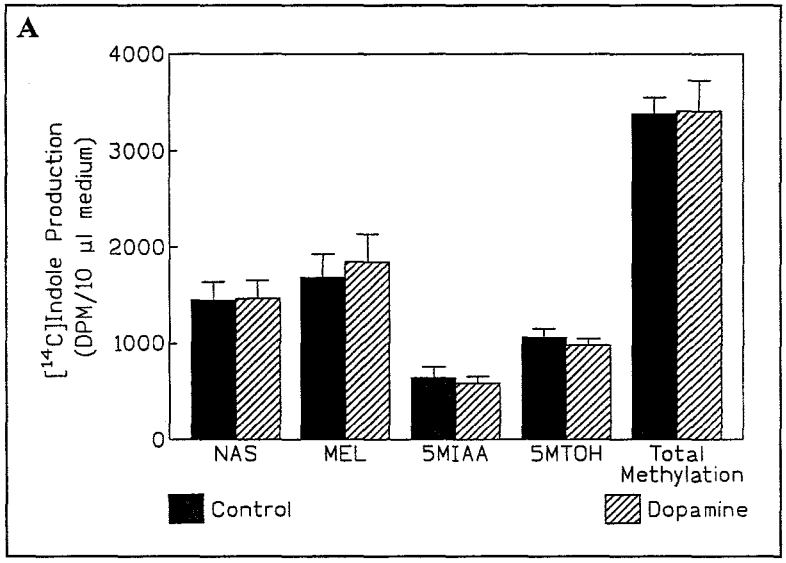


Figure 4.3: The *in vitro* effect of DA HCl (1 µM) on the *in situ* biosynthesis of [^{14}C]indoles by rat pineal glands cultured during the mid-photophase [12h00]. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 5)]



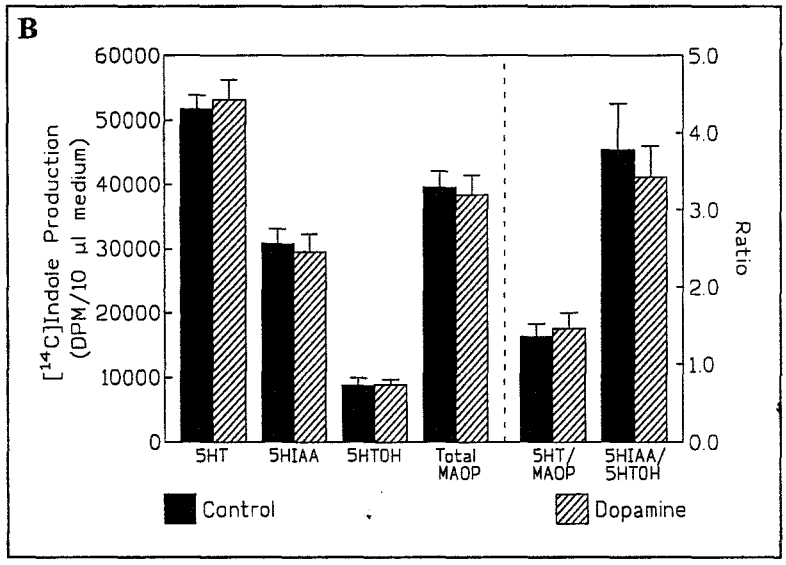


Figure 4.4: The *in vitro* effect of DA HCl (10 µM) on the *in situ* biosynthesis of [^{14}C]indoles by rat pineal glands cultured during the mid-photophase [12h00]. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 5)]

(ii) Scotophase

Typically, the scotophase studies revealed two distinct responses of the *in situ* biosynthesis of pineal [^{14}C]indoles to 10 μM DA *in vitro*. Results representative of each response are presented and discussed below. In the first study (Figure 4.5), DA significantly and potently decreased [^{14}C]NAS levels ($P < 0.001$) without any effect on the other 5-hydroxyindoles. DA also affected a decrease in [^{14}C]MEL levels ($P < 0.05$). This was accompanied by an almost 2-fold increase in both [^{14}C]5MIAA and [^{14}C]5MTOH levels ($P < 0.05$), such that there was no significant change in total methylation. The results of the second study are graphically represented in Figure 4.10, and discussed below in combination with an APO (10 μM) study.

4.3.2 APOMORPHINE ORGAN CULTURE STUDIES

Unless stated otherwise, the term R-(-)-APO will be replaced by the abbreviation APO.

(i) Photophase

Typically, two responses to APO (500 nM) *in vitro* were observed on the *in situ* biosynthesis of [^{14}C]indoles by pineal glands explanted and cultured at mid-photophase (12h00). Results representative of each response are presented below.

In the first case, APO significantly increased [^{14}C]NAS levels ($P < 0.05$), without any effect on the 5-methoxyindoles and total methylation (Figure 4.6A). APO may have influenced MAO activity as evidenced by a slight, but significant decrease in [^{14}C]5HIAA levels ($P < 0.05$), but no effect on [^{14}C]5HTOH levels (Figure 4.6B). This is further reflected in almost significant changes in total MAOP ($P = 0.077$), the 5HT/MAOP ratio ($P = 0.077$) and the 5HIAA/5HTOH ratio ($P = 0.078$) [Figure 4.6B].

In the second type of response, APO had no effect on [^{14}C]NAS levels, but significantly increased [^{14}C]MEL levels ($P < 0.01$) and total methylation ($P < 0.01$), without any effect on the other 5-methoxyindoles (Figure 4.7A). Again, APO may have decreased MAO activity as reflected in an almost significant decrease in total MAOP ($P = 0.0690$), without a change in the levels of the two respective [^{14}C]deaminated indoles or the 5HT/MAOP and 5HIAA/5HTOH ratios (Figure 4.7B)

In contrast, 10 μM APO *in vitro* had no significant effect on the *in situ* biosynthesis of any pineal [^{14}C]indole (Figure 4.8).

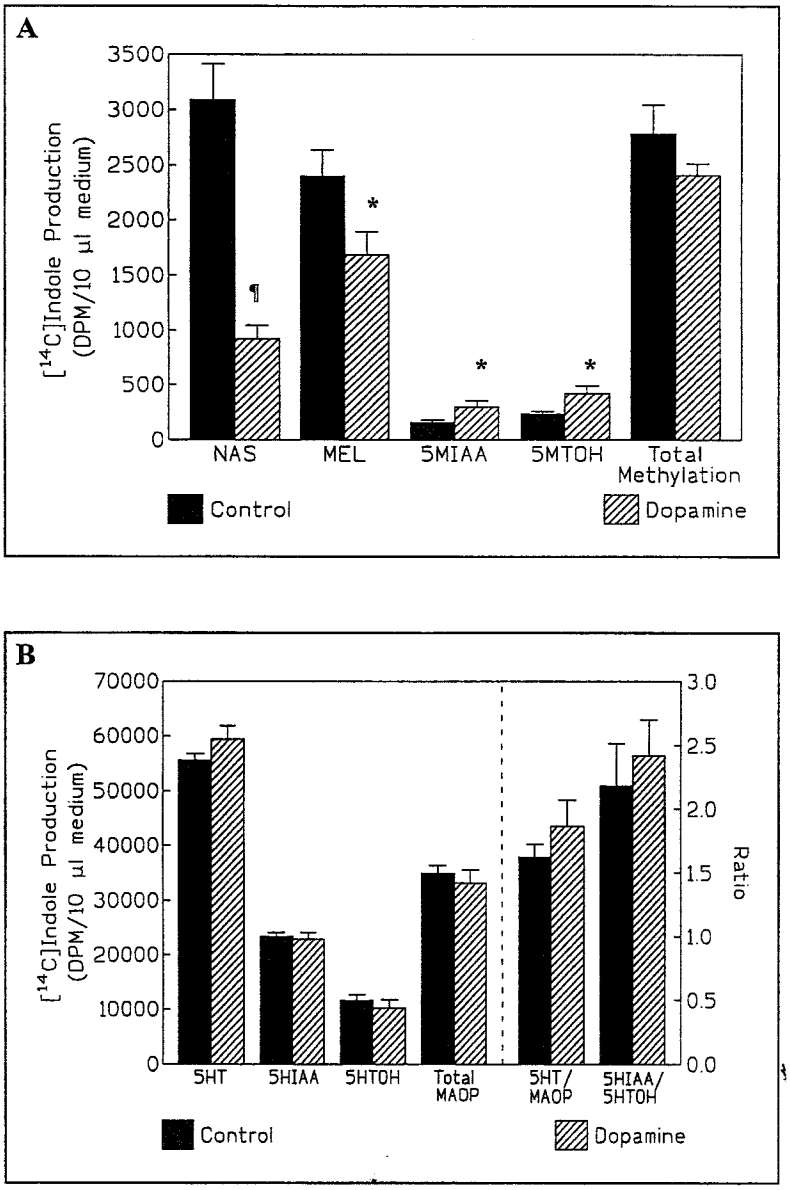


Figure 4.5: The *in vitro* effect of DA HCl (10 µM) on the *in situ* biosynthesis of [^{14}C]indoles by rat pineal glands cultured during the mid-scotophase [24h00]. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 6); P-values (compared to controls): * < 0.05, ¶ < 0.001]

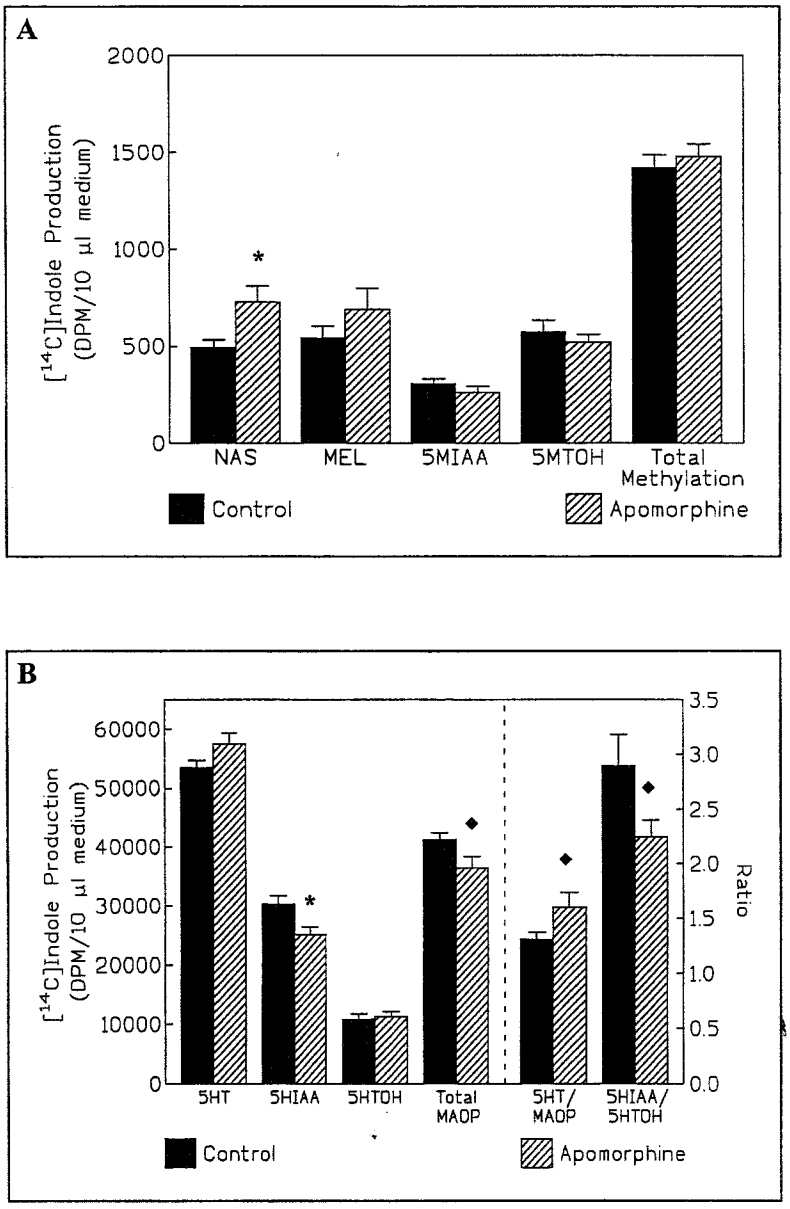


Figure 4.6: The *in vitro* effect of APO HCl (500 nM) on the *in situ* biosynthesis of [^{14}C]indoles by rat pineal glands cultured during the mid-photophase [12h00]. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 5); P-values (compared to controls): * < 0.05, ◆ ≈ 0.077]

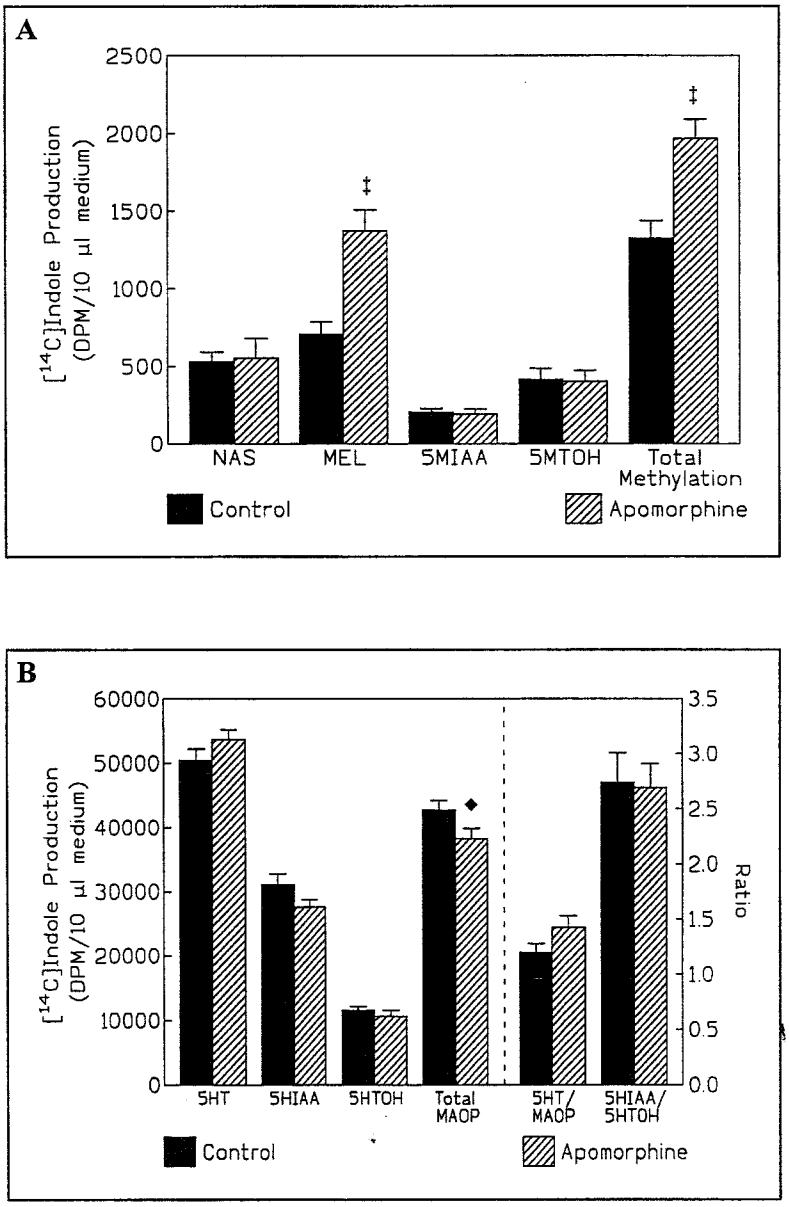


Figure 4.7: The *in vitro* effect of APO HCl (500 nM) on the *in situ* biosynthesis of [^{14}C]indoles by rat pineal glands cultured during the mid-photophase [12h00]. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 5); P-value (compared to control): ‡ < 0.01, ◆ = 0.069]

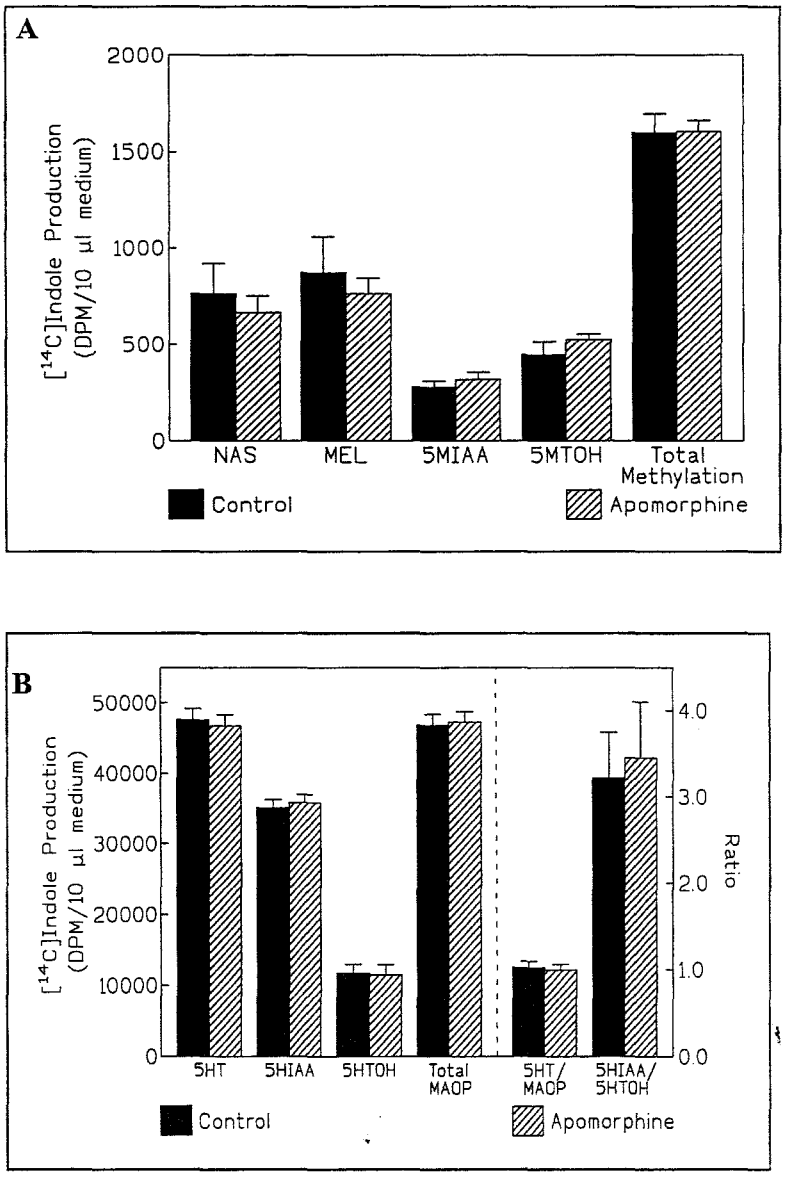


Figure 4.8: The *in vitro* effect of APO HCl (10 µM) on the *in situ* biosynthesis of [^{14}C]indoles by rat pineal glands cultured during the mid-photophase [12h00]. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 5)]

(ii) Scotophase

Studies performed during the dark-phase showed clear dose-dependent effects of APO *in vitro*. At 500 nM APO had no significant effect on the *in situ* biosynthesis of any [^{14}C]indole (Figure 4.9), whereas the effects of 10 μM APO, performed in combination with the second DA (10 μM) study, were very significant (Figure 4.10).

APO (10 μM) significantly decreased [^{14}C]NAS ($P < 0.001$), [^{14}C]MEL ($P < 0.001$), [^{14}C]5HT ($P < 0.001$) levels , and the 5HT/MAOP ratio ($P < 0.001$). APO significantly increased the levels of [^{14}C]5HIAA ($P < 0.001$), [^{14}C]5HTOH ($P < 0.01$), total MAOP ($P < 0.001$) and [^{14}C]5MTOH ($P < 0.05$). There was no effect on [^{14}C]5MIAA levels or the 5HIAA/5HTOH ratio. There was a slight, but significant decrease in total methylation ($P < 0.001$) [Figure 4.10].

DA (10 μM) affected a similar profile of [^{14}C]indole biosynthesis. There was a significant decrease in [^{14}C]NAS and [^{14}C]MEL biosynthesis ($P < 0.05$ in each case), whereas the levels of [^{14}C]5MIAA and [^{14}C]5MTOH were slightly, but significantly elevated ($P < 0.05$). Total methylation was slightly decreased with marginal significance ($P = 0.0457$) [Figure 4.10A]. DA potently and significantly increased the levels of [^{14}C]5HIAA ($P < 0.001$), [^{14}C]5HTOH ($P < 0.05$) and total MAOP ($P < 0.001$), but decreased [^{14}C]5HT levels ($P < 0.001$) and the 5HT/MAOP ratio ($P < 0.001$) [Figure 4.10B].

However, APO (10 μM) had more potent effects on 5-hydroxyindole biosynthesis than DA (10 μM) [$P < 0.05$], whereas the ligands were equipotent against 5-methoxyindoles. Finally, it must be noted that the control data for all the above scotophase studies (Figure 4.5, 4.9, and 4.10) are consistent with the values and profile of night-time pineal [^{14}C]indole biosynthesis reported in section 3.3 (see Figure 3.3).

4.3.3 *IN VIVO* HALOPERIDOL ORGAN CULTURE STUDIES

Acute administration of either 2.5 mg/kg or 5.0 mg/kg bwt HAL (i.p., single dose) during the photophase had no significant effect on *in situ* [^{14}C]indole biosynthesis by pineal glands explanted and cultured at mid-photophase (Figures 4.11 and 4.12).

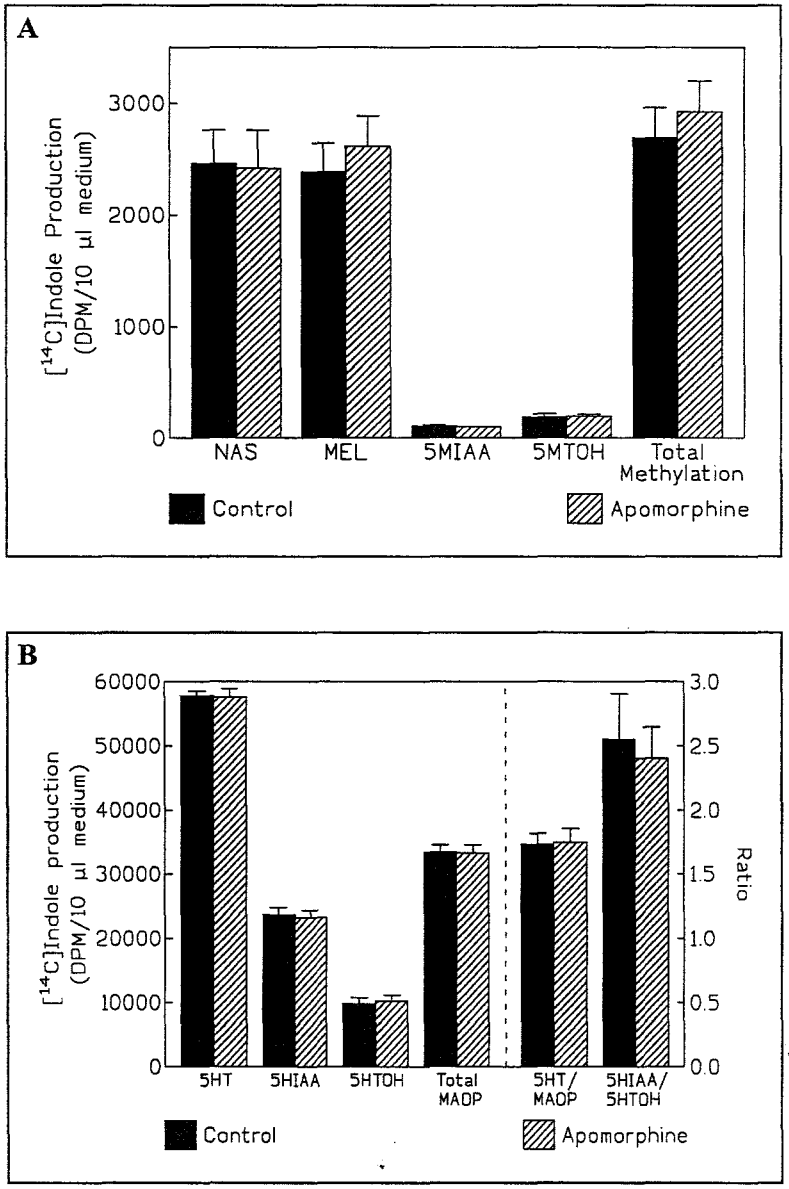


Figure 4.9: The *in vitro* effect of APO HCl (500 nM) on the *in situ* biosynthesis of [^{14}C]indoles by rat pineal glands cultured during the mid-scotophase [24h00]. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 5)]

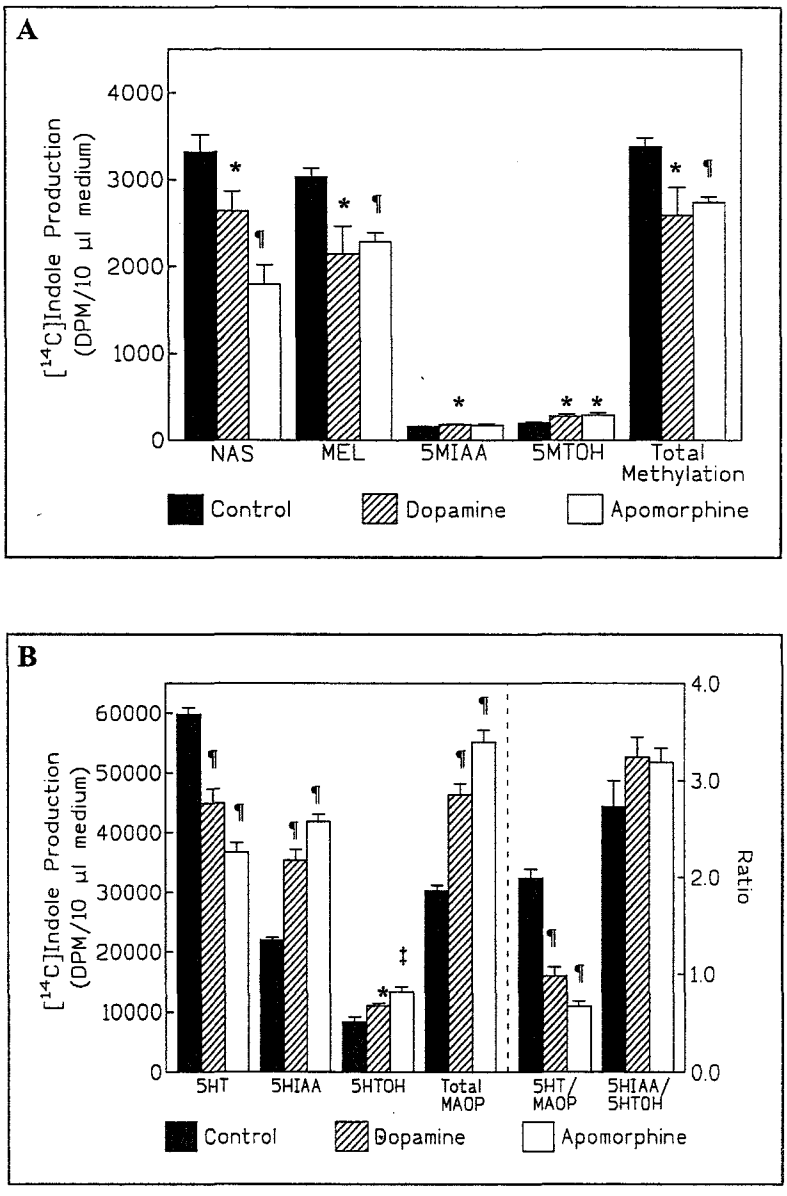


Figure 4.10: The *in vitro* effect of DA HCl (10 µM) and APO HCl (10 µM) on the *in situ* biosynthesis of [^{14}C]indoles by rat pineal glands cultured during the mid-scotophase [24h00]. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 5); P-values (compared to control): * < 0.05, ‡ < 0.01, ¶ < 0.001]

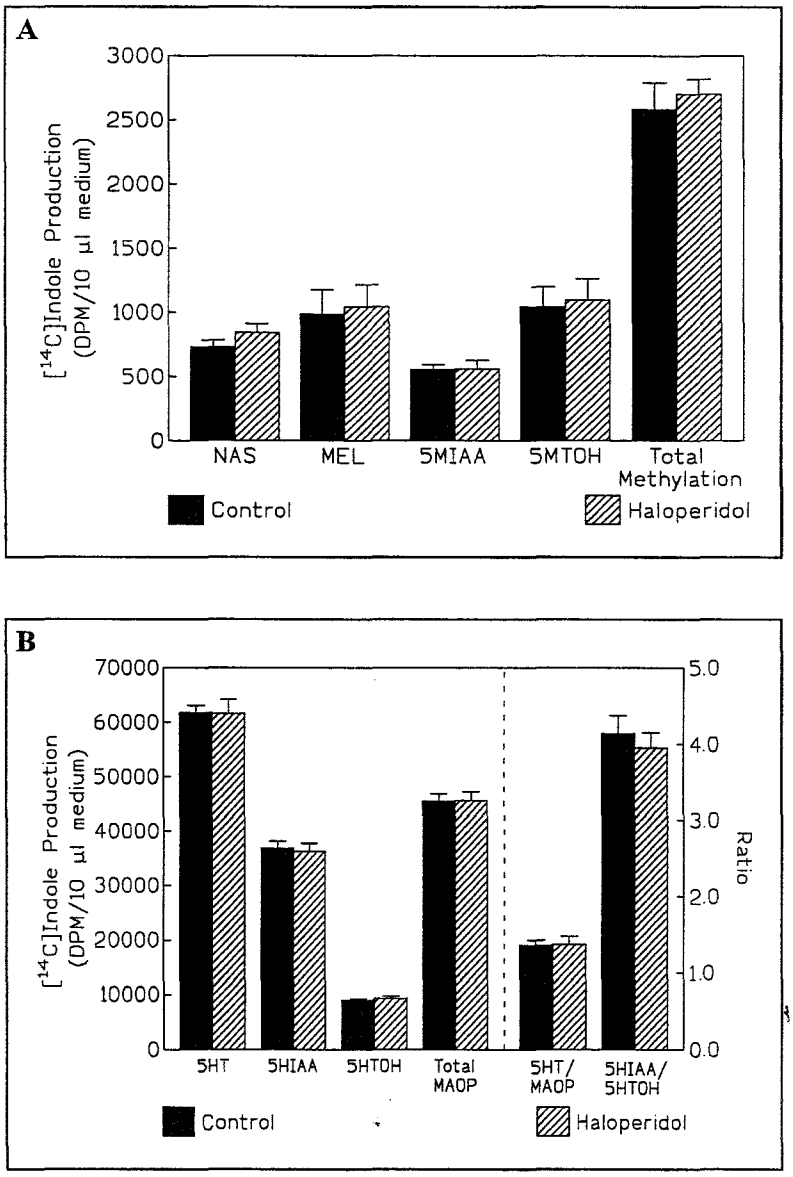


Figure 4.11: The effect of acute administration of HAL (2.5 mg/kg i.p., single dose) at mid-photophase (12h00) on the *in situ* biosynthesis of [^{14}C]indoles by cultured rat pineal glands. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 5)]

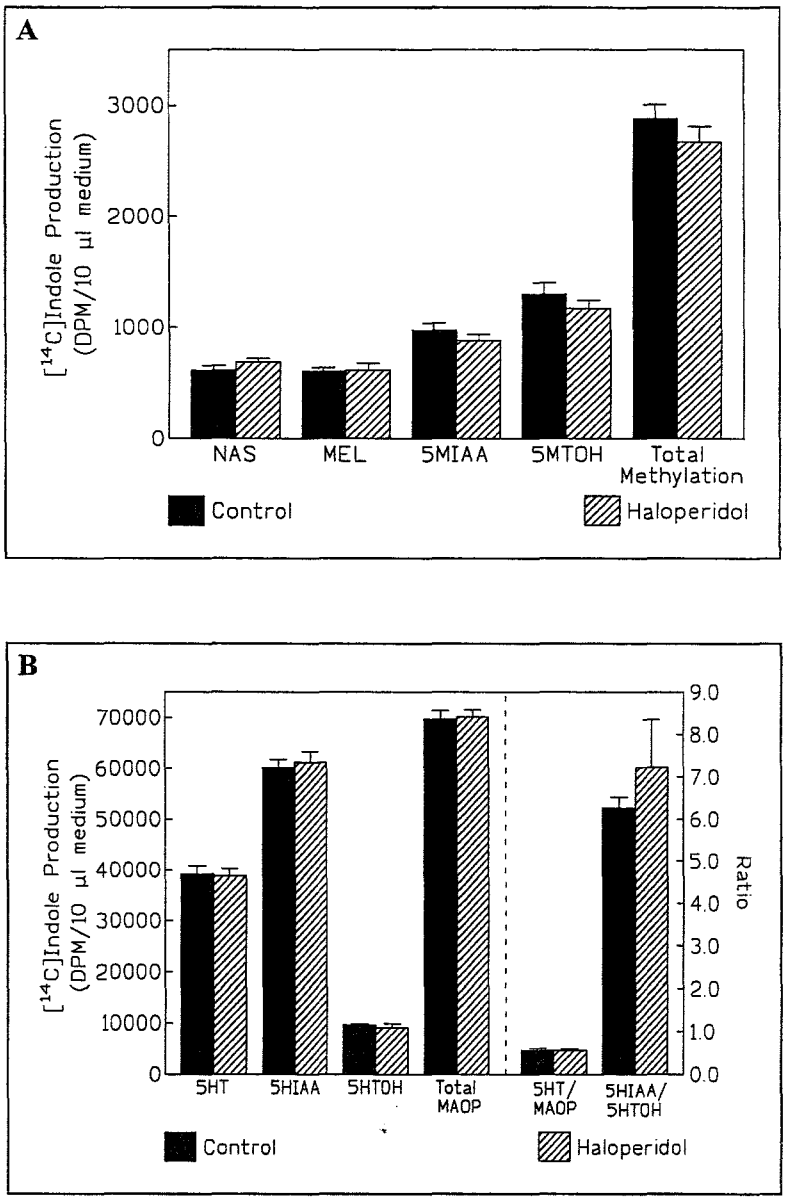


Figure 4.12: The effect of acute administration of HAL (5.0 mg/kg i.p., single dose) at mid-photophase (12h00) on the *in situ* biosynthesis of [^{14}C]indoles by cultured rat pineal glands. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 5)]

4.4 Discussion and Conclusion

The results presented here provide strong pharmacological evidence that the dopaminergic agents DA and APO can modulate the biosynthesis of [^{14}C]indole by the pineal glands of male Wistar rats. Both drugs exerted complex dose-dependent effects, which were chronotypic and state-dependent in nature. The complexity of the current nomenclature for DA receptors makes it difficult to assign these effects to a specific receptor subtype.

DA receptors belong to the superfamily of G-protein coupled receptors, containing seven transmembrane domains. In 1979, Kebabian and Calne proposed the existence of two DA receptors, namely the D_1 and D_2 receptors. The D_1 receptor is functionally coupled to the stimulation of adenylate cyclase activity and thus tissue cAMP levels. The D_2 receptor is negatively coupled to adenylate cyclase and also mediates the inhibition of phosphatidylinositol turnover, the activation of K^+ channels and the inhibition of Ca^{2+} channel activity (Kebabian and Calne, 1979; Strange, 1993). Subsequently, a multitude of other DA receptor subtypes have been cloned and pharmacologically and biochemically defined. This has led to a revision of the original classification and the formation of the D_1 and D_2 subfamilies. The D_1 subfamily comprises the classic D_1 receptor and the D_5 subtype. The D_2 subfamily contains two isoforms of the original D_2 receptor, D_{2L} and D_{2S}, and the D_3 and D_4 subtypes (Sibley and Monsma, 1992; Strange, 1993). Further studies are likely to reveal the existence of even more novel DA receptor subtypes. The original D_1 and D_2 receptors still show the highest expression in the CNS and appear to be the most important neuronal subtypes.

Typically, DA shows nanomolar affinity for the D_2-like receptors, but micromolar affinity for the D_1-like receptors. The R-(-)-stereoisomer of APO is a partial agonist or antagonist at D_1-like receptors, but a full agonist at D_2-like receptors. The receptor subtypes differ further in affinity for agonists within a subfamily. For example, the D_3 has a 20-fold higher affinity for DA than the D_1 or D_2 subtypes. In turn, R-(-)-APO shows higher potency at D_3 and D_4 subtypes ($K_D \approx 5$-20 nM) compared to the D_2 receptor ($K_D \approx 350$ nM). Evidence also exists that DA receptor subtypes can exist in different conformational states, designated the high- and low-affinity states. For example, R-(-)-APO shows K_D values ≈ 5 nM and ≈ 350 nM, respectively, for the high- and low- affinity states of the D_2 receptor (Kebabian and Calne, 1979; Creese *et al.*, 1984; Sibley and Monsma, 1992; Strange, 1993). The interconversion between each state is likely to be dependent on factors such as the presence of endogenous ligands. R-(-)-APO is also more potent at pre- than post-

synaptic DA receptors, which again may represent different receptor subtypes or conformational states of the D_2 receptor. This is consistent with the proposal that the D_3 subtype is an autoreceptor (Creese *et al.*, 1984; Sibley and Monsma, 1992; Strange, 1993). The differential effect and potency of APO at pre- and postsynaptic receptors is also evident in the dose-dependent behavioural responses to the DA agonist. A high dose of APO induces hyperlocomotion, stereotypy and exploratory behaviour via an effect on postsynaptic receptors. A low dose of APO induces hypolocomotion and suppresses behaviour via activation of presynaptic DA autoreceptors (Kendler *et al.*, 1982). Thus, with respect to the rat pineal gland, the number of different DA subtypes present, their conformational state and location (presynaptic *vs.* postsynaptic) are important variables in interpreting the present results.

Radioligand binding studies by Govitrapong *et al.* (1984) suggested the presence of a D_2 receptor in the bovine pineal gland. Pharmacological studies with more selective D_2 agonists and antagonists appeared to extend this finding to pineals of Sprague-Dawley rats (Govitrapong *et al.*, 1989). It is thus probable that if DA receptors are present in the pineal of Wistar rats it will be the D_2 subtype. Nonetheless the final DA concentration was ranged from 100 nM to 10 μM to cover, at least theoretically, the affinity of all DA receptor subtypes for this catecholamine. Higher concentrations of DA (>> 10 μM) were not employed to prevent non-specific stimulatory effects at the pineal β-adrenergic receptors and interference of pineal MAO activity. More specific probing of the D_2 receptor was achieved with R-(-)-APO. The experiments with the latter were based around a final concentration of 500 nM as this concentration covers the range consistently cited as the K_D, IC_{50} or EC_{50} value of this agonist for the different subtypes and affinity states of the D_2 subfamily (Kebabian and Calne, 1979; Niznik, 1987; Strange, 1987). R-(-)-APO will only act as an agonist at D_1 receptors at micromolar concentrations and in the absence of an endogenous ligand, such as DA. In the presence of the latter, R-(-)-APO will antagonize D_1 receptor-mediated events. The abbreviation APO will now be used instead of R-(-)-APO.

The drug-induced changes to [^{14}C]NAS and [^{14}C]MEL biosynthesis observed during both the photophase and scotophase studies can be explained by one or more of the following events:

(a) stimulation or inhibition of NAT activity.

(b) changes in the availability of [^{14}C]NAS as a substrate for O-methylation by HIOMT, without an effect on HIOMT itself.

(c) time-delay between the N-acetylation and O-methylation events.

(d) stimulation or inhibition of MAO activity.

These events are not necessarily mutually exclusive, but the activity of NAT is seen as the pre-determining step in the present studies. The synthesis of 5-methoxyindoles is not only dependent on the activity of the O-methylating enzyme HIOMT, but also on the availability and affinity of the corresponding 5-hydroxyindoles as substrates for HIOMT (Morton, 1990). As postulated in section 3.4, a change in the relative proportion of each 5-methoxyindole to total methylation, without a change in the absolute value of this index itself, is seen as being indicative of this phenomenon. Although the results can be parsimoniously explained without postulating a drug-induced increase in HIOMT, such an increase cannot be completely ruled out. The observed biosynthetic profile can also be influenced by MAO activity. For example, by reducing the activity of this enzyme, more [^{14}C]5HT will be available for N-acetylation to [^{14}C]NAS and thus O-methylation to [^{14}C]MEL.

During the photophase, DA HCl (100 nM, 1 μM and 10 μM) *in vitro* did not stimulate the *in situ* biosynthesis of any [^{14}C]indole by the cultured pineal glands as would be seen for the β-agonist NA. The basal nature of pineal indole biosynthesis during the daytime should and did preclude the possibility of seeing an inhibitory effect of DA. In contrast, APO (500 nM) *in vitro* stimulated the *in situ* synthesis of either [^{14}C]NAS or [^{14}C]MEL during the photophase, depending on the study. In both cases, this is attributed to either a stimulation of NAT, an inhibition of MAO, or both. The significance of the contribution of MAO, however, differed between the two studies. Furthermore the magnitude of the effect on [^{14}C]NAS or [^{14}C]MEL levels is considerably greater than that seen on [^{14}C]5HIAA and [^{14}C]5HTOH levels, especially for the second study. This would suggest that a stimulation of NAT was the predominating and more robust effect. It is proposed that in the first study (Figure 4.6), the increased [^{14}C]NAS levels had not yet translated into increased MEL levels, whereas this conversion is complete in the second study (Figure 4.7). The increase in total methylation, but not in the levels of [^{14}C]5MTOH and [^{14}C]5MIAA in the latter study, supports the contention that APO did not induce a general increase in HIOMT activity. The lack of effect of 10 μM APO undermines the dose-dependency of the above profile, but may be due to opposing effects of APO on multiple receptor subtypes at this higher concentration.

In the scotophase studies, equimolar DA and APO (10 μM) markedly reduced the *in situ* production of [^{14}C]NAS and [^{14}C]MEL. Unlike the photophase studies, APO was dose-dependent in that no effect was seen at a concentration of 500 nM. For both ligands, the observed profile of pineal [^{14}C]indole biosynthesis can be explained by either an inhibition of NAT or a stimulation of MAO activity, or a complex combination of both. In the first DA study (Figure 4.5), an inhibition

of NAT was the pre-determining step with no contribution by MAO. There was no need to postulate an effect on HIOMT, as outlined above. This is consistent with reports that DA has no significant *in vitro* effect on the activity of crude bovine pineal HIOMT (Hartley *et al.,* 1972; Hartley and Smith, 1973).

In contrast, the profile seen with APO (10 μM) and the second DA study (Figure 4.10) is complicated by very significant drug-induced increases in the apparent activity of pineal MAO. By increasing MAO activity, more [^{14}C]5HT is deaminated to [^{14}C]5HIAA and [^{14}C]5HTOH. This should decrease the amount of [^{14}C]5HT available for N-acetylation to [^{14}C]NAS with a concomitant decrease in [^{14}C]MEL levels. Similarly, an increase in the respective O-methylation of [^{14}C]5HIAA and [^{14}C]5HTOH to [^{14}C]5MIAA and [^{14}C]5MTOH should be evident. Since this profile is similar to that seen following inhibition of NAT, it is difficult to delineate the relative contribution of each enzyme. However, results from the first DA scotophase study show that an inhibition of NAT activity is clearly implicated. Furthermore the decrease in total methylation indicates that the increase in [^{14}C]5MIAA and [^{14}C]5MTOH levels did not compensate for the decrease in [^{14}C]MEL levels. It is therefore argued that this implies that the decrease in [^{14}C]MEL biosynthesis is too robust to be simply explained by a stimulation of MAO activity.

Evidence has been presented earlier (section 3.3) that apparent pineal MAO activity is lower in the scotophase compared to the photophase. Both DA and APO reversed this dark-phase associated trend. DA is a substrate for both MAO-A and –B (Fowler *et al.*, 1978; Garrick and Murphy, 1982) and thus may compete with [^{14}C]5HT for deamination. However, this should be evident as decreased rather than increased [^{14}C]5HIAA and [^{14}C]5HTOH levels, and total MAOP, and no such effect of DA was seen in the photophase studies. Furthermore, the fact that APO, which is not a MAO substrate, also increased the levels of these 5-hydroxyindoles suggests that this may indeed be a DA receptor-mediated event. Previous studies have also reported that NA and isoproterenol, a selective β-adrenergic agonist, may modulate pineal MAO activity via β-adrenergic receptors (Axelrod *et al.*, 1969, Olivieri *et al.*, 1990).

The fact that APO (10 μM) was more potent than an equivalent concentration of DA on the scotophase biosynthesis of [^{14}C]5-hydroxyindoles may involve several factors. Two possibilities include:

(i) the respective affinities of the receptor subtype or conformational state for DA and APO.

(ii) differences in the degree of metabolic inactivation of each ligand.

DA is catabolized by both MAO and COMT (Napolitano *et al.*, 1995) and none of the subsequent products are pharmacologically active at DA receptors. In contrast, APO is O-methylated by COMT to form apocodeine (Axelrod, 1966), which retains agonistic activity at the DA receptor.

The present pharmacological profile of APO and DA and their ability to inhibit NAT and MAO activity is consistent with the presence of a D_2 or D_2-like receptor in the pineal gland. To reiterate, pineal NAT activity is induced during the scotophase by a nocturnal surge in the activation of β-adrenergic receptors by NA released from presynaptic SNS nerve terminals. The postsynaptic β-adrenergic receptors are located on pinealocytes and coupled, via G-proteins, to the stimulation of adenylate cyclase. The concomitant surge in intracellular cAMP levels is responsible for the induction and activation of NAT (Reiter, 1991). D_2 receptors are negatively coupled to adenylate cyclase. If the putative D_2 receptors were located post-synaptically, ligand-induced activation could antagonize the NA-induced increase in cAMP levels and thus NAT activity. Alternatively, D_2 receptors are also known to be located on sympathetic nerve terminals where they mediate the inhibition of NA release (Niznik, 1987; Strange, 1987). Thus the putative D_2 receptors may be located pre-synaptically on the SNS nerve terminals innervating the pineal gland. NA is a MAO substrate and 70% of pineal MAO activity is associated with the noradrenergic nerve terminals innervating the pineal (Racké *et al.*, 1991). Thus an inhibition of NA release would explain both the reduction in NAS and MEL biosynthesis and the increase in apparent MAO activity during the scotophase. However, it is likely that most NA has already been released by mid-scotophase *in vivo*, prior to the removal of the pineal and the addition of DA and APO during the culture period.

Zawilska and Iuvone (1990) have strongly contended that the suppression of the nocturnal increase in NAT activity and MEL content of chicken pineals by the D_2-selective agonists bromocriptine and quinpirole was mediated by α_2-adrenergic receptors, and not D_2 receptors. This is in contrast to Govitrapong *et al.* (1989) who attributed similar effects of these ligands in the rat pineal to the D_2 receptor. Bromocriptine, quinpirole and DA are known to act on α_2-adrenergic receptors at high concentrations. This may simply reflect species differences, but Govitrapong *et al.* (1989) did note that higher than expected concentrations of bromocriptine and quinpirole were required to exert the observed effects. Whether DA at a concentration of 10 μM acted on α_2-adrenergic receptors in the present studies is unclear. It is unfortunate that lower concentrations of DA were not additionally tested in the scotophase studies. Nonetheless, APO does not display affinity for α_2- or β-receptors. APO potently attenuated the nocturnal rise in NAT activity and MEL content in chicken retinas via stimulation of D_2 receptors, with no effect in chicken pineal

glands (Zawilska and Iuvone, 1990). The results obtained in this study, especially for APO, tend to confirm the conclusions of Govitrapong *et al.* (1989), i.e. the presence of a postsynaptic D_2 receptor in the rat pineal. The problems associated with bromocriptine and quinpirole in the latter study may have been related to the exact nature of the D_2-like receptor subtype. However, the dose-dependent variation in response to APO during the photophase and scotophase may involve a state-dependent effect at the level of the D_1 receptor. R-(-)-APO is a partial agonist at the D_1 receptor (Kebabian and Calne, 1979). In other words, in the absence of DA, APO will act as an agonist, but as a full antagonist in the presence of DA. The activity of dopaminergic neurons and thus the release of DA is greater at night in nocturnal animals such as the rat (Paulson and Robinson, 1996). Therefore it is plausible that the state-dependent presence or absence of DA may influence the activity of APO at pineal D_1 receptors. Alternatively, the same day-night cycle in dopaminergic activity may be reflected in a variation in the sensitivity or conformational state of the D_2 receptor. For example, the ability of a low dose of APO to induce yawning via an effect on the D_2 autoreceptor is more pronounced during the dark-phase than the light phase (Nasello *et al.*, 1995).

DA also may have numerous other cytosolic/nonreceptor-mediated effects. DA *in vitro* has been shown to inhibit both tryptophan hydroxylase and TH by complexing with the essential Fe^{3+} co-factor (Naoi *et al.*, 1994; Ribeiro *et al.*, 1992). However, under the present experimental design, these effects are unlikely to be important.

The nature of DA receptor subtypes can also be probed with selective antagonists. HAL is a relatively selective antagonist of the D_2 receptor (Creese *et al.*, 1984; Lidsky and Banerjee, 1993). Unfortunately, HAL appears to have both cytosolic and receptor-mediated effects on pineal function that may, in addition, be species-specific. HAL administration to rats is generally reported to increase pineal NAT activity and the endogenous MEL content during the both the light- and dark-phases (Gaffori *et al.*, 1983; Govitrapong *et al.*, 1989; Srinivasan, 1989). Only Wakabayashi *et al.* (1989) have reported a decrease in rat pineal MEL content during the dark-phase. All these studies administered acute HAL (single dose), but differed in the dosage and post-administration interval. A stimulatory effect on pineal MEL is commonly attributed to the ability of HAL to block the inhibitory effects of DA on NAT activity via the D_2 receptor. On the otherhand, a reduction in MEL levels would be consistent with the inhibitory effect of HAL *in vitro* on crude and purified bovine HIOMT (Hartley *et al.*, 1972; Cremer-Bartels *et al.*, 1983). To further complicate the story, HAL (10 μM) *in vitro* was found to alter [^{14}C]indole biosynthesis by

rat pineals during the scotophase in a manner consistent with an inhibition of MAO and the activation of HIOMT activity (Nir and Hirschmann, 1983). This included an increase in [^{14}C]MEL levels. In the present study, HAL (2.5 mg/kg or 5 mg/kg i.p.) administered during the photophase had no effect on the biosynthesis of any radiolabelled indole by pineal glands cultured at mid-photophase, including [^{14}C]NAS, [^{14}C]MEL and the MAO products [^{14}C]5HIAA and [^{14}C]5HTOH. The doses employed here are comparable or greater than those used in the aforementioned studies. Furthermore, brain levels of HAL are reported to be maximal 60 min after administration (Naylor and Olley, 1969), consistent with the 90 min post-administration interval used here. Administration of HAL by the i.p. route results in extensive first-pass metabolism of the drug by the liver. HAL is rapidly metabolized by hepatic microsomal enzymes to reduced HAL, which is frequently present in the tissues of HAL-treated subjects at a much higher concentration than HAL itself. This metabolite may be reoxidized to HAL *in vivo*. It must be noted, however, that reduced HAL shows preferential activity at σ (sigma) receptors over D_2 receptors (Bowen *et al.*, 1990).

The observation of sedation, ptosis and catalepsy in the experimental, but not the control animals, was consistent with the known behavioural responses of HAL (Kendler *et al.*, 1982; Sanberg *et al.*, 1988; Lidsky and Banerjee, 1993), and indicates that sufficient HAL must have penetrated the CNS. In fact, due to the absence of a blood-brain-barrier around the highly vascularized pineal, hydrophobic HAL has been shown to accumulate primarily in the pineal gland of the rat following systemic administration (Naylor and Olley, 1969). Due to its hydrophobic nature, HAL would be expected to enter the pinealocytes and SNS nerve terminals innervating the gland. The study of Gaffori *et al.* (1983) showed a marked increase in pineal MEL content after the s.c. administration of only 300 ng HAL/rat. This all leads to the conclusion that the observed lack of effect here was not due to problems associated with the dosage and pharmacokinetics of HAL or the nature of the vehicle. However, the problem may be related to the fact that the radiometric method employed in the present study quantified the synthesis of exogenous [^{14}C]indoles released into the culture medium and not endogenous pineal indole content as in previous investigations. Only the *in vitro* HAL study of Nir and Hirschmann (1983) employed a similar *in situ* pineal organ culture technique.

Actually, the lack of effect of HAL in the present study is consistent with the lack of effect of DA on basal pineal [^{14}C]indole biosynthesis during the photophase. As a DA receptor antagonist, HAL can block the effects of DA agonists, such as DA or APO, but does not have any intrinsic activity itself at the receptor. Theoretically then, HAL would not be expected to have an apparent effect on

daytime basal indole biosynthesis, in the absence of an agonist. This would, however, suggest that, if there is a dopaminergic system intrinsic to the rat pineal, the endogenous agonist DA is not responsible for maintaining the basal or photophase level of indole biosynthesis. Furthermore, the present studies cannot exclude the possibility that HAL may effect the hepatic metabolism of plasma MEL, independent of an effect on biosynthesis as seen for CPZ (Wurtman *et al.*, 1968a).

In summary, the dopaminergic agents DA and APO appear to have state-dependent effects on the indole biosynthesis by pineal glands of Wistar rats at the level of the NAT and MAO enzymes. It is argued that these preliminary studies indicate that these effects are mediated by postsynaptic D_2-like receptors. It would have been informative to determine the effect of DA and APO on NA-stimulated NAT activity during the photophase, in addition to basal activity of the enzyme. More specific agonists and antagonists, immunocytochemistry and molecular biology techniques (e.g. mRNA probes) need to be employed to elucidate the exact nature of the receptor subtype.

It is also not impossible to determine from the present investigations whether this putative dopaminergic system is intrinsic to the pineal, consists of dopaminergic fibres arising outside the pineal, or is restricted simply to DA receptors expressed on pinealocytes. Further electrophysiological, biochemical and enzyme studies should help to characterize the nature of the dopaminergic system. Another possible experimental approach would be to re-perform the above experiments with DA and APO using pineal glands lacking noradrenergic input. This can be achieved *in situ* by creating a dispersion of pinealocytes or by culturing the pineals for 48-72 hrs prior to the addition of the agonists (Hernández *et al.*, 1994; Santana *et al.*, 1994).

Regulation of pineal indole biosynthesis appears to be highly species-specific. With further regards to human beings, the very limited number of studies that have been performed would suggest a lack of dopaminergic control of pineal function. Neither the DA agonists APO and quinpirole nor the antagonist sulpiride had an effect on pineal MEL synthesis or secretion (Zimmermann *et al.*, 1994). In addition, L-Dopa has no effect on the amplitude of plasma MEL in healthy human subjects (Wetterberg, 1978) and Parkinson's patients (Fertl *et al.*, 1993), but does induce phase-advancement of MEL secretion in the latter. This would suggest that the results presented here for the rat pineal gland most likely cannot be extended to the human pineal gland.

Chapter 5

Monoamine oxidase: Characterization and Optimization

5.1 Introduction

MAO occurs in the mammalian brain as two isoforms, designated MAO-A and MAO-B (Singer and Ramsay, 1995). Endogenous DA is primarily metabolized by MAO-A in the rodent brain (Berry *et al.*, 1994; Juorio *et al.*, 1994; Luque *et al.*, 1995). Furthermore, enzyme activity, protein, and mRNA studies have shown that the latter isoform is abundant in the rat corpus striatum, apparently localized in presynaptic dopaminergic nerve terminals (Juorio *et al.*, 1994; Luque *et al.*, 1995; Jahng *et al.*, 1997). Thus the MAO-A activity of the rat corpus striatum was quantified by an *in vitro* micro-radioenzymatic assay modified from Urry *et al.* (1972), Fowler *et al.* (1979) and Parvez and Parvez (1973).

5.1.1 Principle of MAO-A Assay

The principle of the assay is based on a direct aqueous-organic solvent extraction system to separate the radiolabeled reactants and products. The reaction scheme is outlined in Figure 5.1. Briefly, in the presence of molecular oxygen, the radioisotopic substrate [^{14}C]5HT is deaminated by MAO-A to produce [^{14}C]5HIAL. Acidification of the reaction mixture with HCl terminates the enzyme reaction and protonates the free amino group of [^{14}C]5HT. This allows for the efficient separation of [^{14}C]5HIAL from the charged [^{14}C]5HT by extracting the aldehyde with ethyl acetate. The analyte is then quantified by liquid scintillation spectroscopy.

In the presence of the enzymes alcohol dehydrogenase and aldehyde dehydrogenase, 5HIAL can be further reduced to 5HTOH or oxidized to 5HIAA respectively. The relative production of 5HTOH and 5HIAA is dependent on the prevailing NADH/NAD$^+$ ratio (i.e. oxidative state) of the tissue. Both these additional products can be extracted with ethyl acetate following acidification of the reaction mixture. Alcohol dehydrogenase is a cytosolic enzyme, whereas aldehyde dehydrogenase can occur as several isoenzymes that differ in subcellular distribution, including the cytosol, mitochondria and microsomes (Kennedy and Tipton, 1990). Thus, the actual identity of the extracted products will differ with the nature of the tissue preparation. For this reason, the

term total MAOP formation was used to describe MAO activity, and refers to the absolute amount of radiolabeled product, irrespective of the nature or number of the products extracted from the reaction mixture.

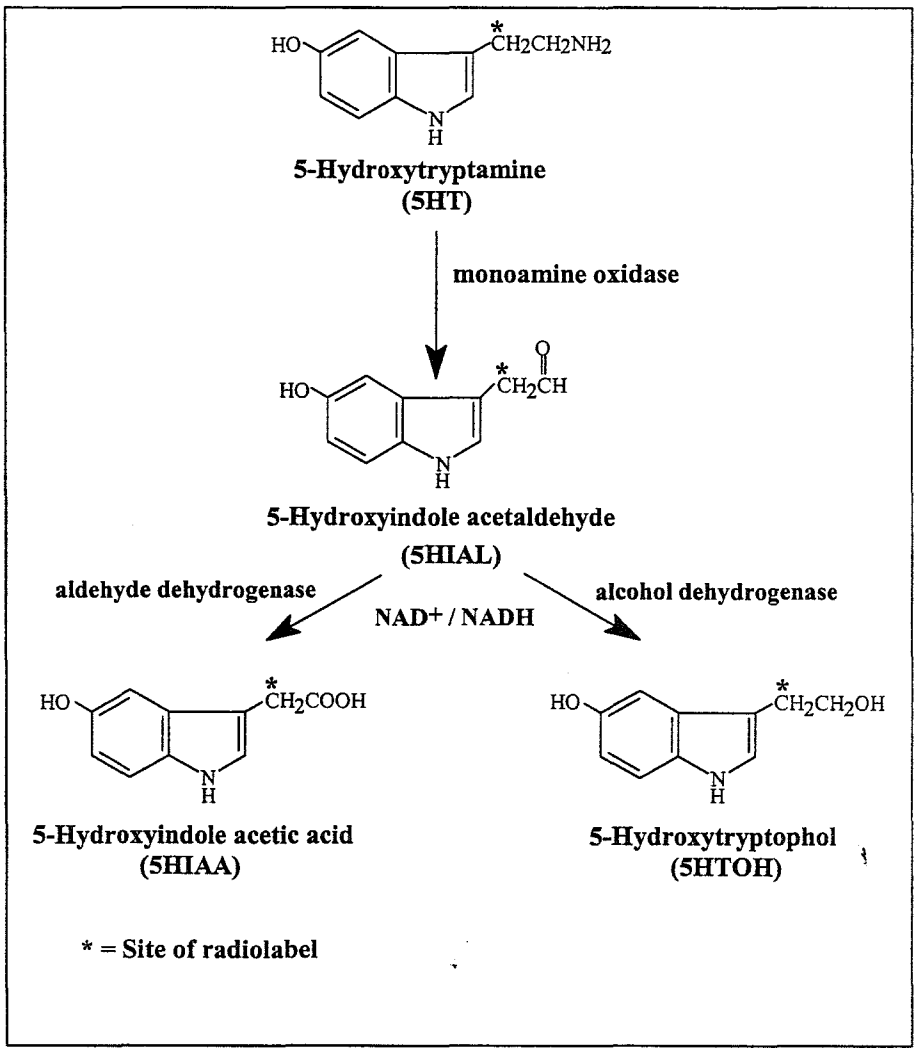


Figure 5.1: Reaction scheme for the *in vitro* radioenzymatic MAO-A assay.

More modern spectrophotometric and fluorometric assay systems have been designed (Zhou *et al.*, 1996; Holt *et al.*, 1997), but these too have several disadvantages. These include complexity of design (e.g. the need for coupled-reactions), lack of isoform specificity and need for a relatively pure sample, necessitating a clean-up step (e.g. deproteinization, removal of endogenous substrates). In contrast, the radioenzymatic assay is very sensitive, isoform-specific, reproducible

and can be used on crude homogenates. For these reasons, the radiometric method still remains the most commonly used assay for MAO-A and -B. The cost of the radiochemicals is the main disadvantage associated with this assay protocol. The applicability of the radioenzymatic assay to the analysis of MAO-A activity of the rat corpus striatum was assessed in this chapter. Of particular importance was the flexibility of the assay for comparison between *in vitro* and *in vivo* studies, and thus the ability to detect MAO activity in very small quantities of tissue sample. This required full characterization of the MAO enzyme, verification of the presence of MAO-A in the rat striatum, and optimization of the assay conditions. An attempt was also made to minimize the cost without sacrificing reliability.

5.2 Materials and Methodology

5.2.1 Chemicals and Reagents

The radiochemical 5-hydroxy-(side-chain-2-^{14}C)tryptamine [5HT] creatinine sulphate (specific activity 56 mCi/mmol) was purchased from Amersham International (Amersham, UK). 5HT creatinine sulphate was purchased from Sigma and HPLC grade ethyl acetate from LAB-SCAN Analytical Sciences (Dublin, Ireland). Clorgyline HCl and pargyline HCl were purchased from Sigma, and L-deprenyl HCl [R-(-)-deprenyl; selegiline HCl] from Research Biochemicals International (RBI). All other chemicals and reagents were purchased from local commercial sources and were of the highest purity available. All buffers and reagents were prepared in MilliQ water.

5.2.2 Tissue Preparation and Storage

Unless otherwise stated, all MAO optimization studies were performed on tissue samples prepared from the striata of animals sacrificed at mid-photophase (12h00). The striata were pooled, dissected and stored as described in section 2.2. Stability studies performed on the mitochondrial/lysosomal fraction in 0.32 M sucrose indicated that MAO activity must be assayed within 72 hrs of storage at -20 °C. Whole tissue can be stored at -20 °C for several months.

5.2.3 Assay Reaction Mixture

All reagents were prepared in the assay buffer, 0.1 M potassium phosphate buffer (pH 7.4). A 50 mM Tris-HCl buffer (pH 7.4) was found to inhibit MAO-A activity as previously reported (Fowler *et al.*, 1978; Salach and Detmer, 1979). A standard reaction mixture, with a total volume of 100

µl, comprised: 45 µl assay buffer, 50 µl enzyme fraction, and substrate solution. By default, the substrate solution consisted of 5 µl [^{14}C]5HT working solution (0.025 µCi/assay tube), giving a final concentration of ± 4.5 µM [^{14}C]5HT. This was designated "hot" or "[^{14}C]5HT" substrate activity. In certain experiments, parallel samples were prepared containing, in addition, non-radioisotopic 5HT at a final concentration of 150 µM. This was designated "[^{14}C]5HT+5HT" substrate activity. For further comparison, the ratio of "[^{14}C]5HT" activity to "[^{14}C]5HT+5HT" activity was also calculated.

5.2.4 Assay Procedure

Briefly, all components, except [^{14}C]5HT, were prepared in a 1.5 ml Eppendorf vial on ice in the order listed above. Following saturation with carbogen (95% O_2:5% CO_2 v/v), the reaction mixture was preincubated for 5 min in a 37 °C water bath, with gentle shaking, to allow thermal equilibration. The reaction was initiated by the addition of 5 µl [^{14}C]5HT working solution and incubated at 37 °C for 30 min. The reaction was terminated by the addition of 60 µl ice-cold 2 N HCl. After 15 min, this was followed by the addition of 1 ml ethyl acetate and vortexed for 60 s. Samples were then centrifuged at 3000 rpm for 10 min (Selecta Mixtasel benchtop centrifuge) and 0.8 ml of the solvent extracted and quantified by liquid scintillation spectroscopy. Zero-time blanks were prepared by the addition of HCl prior to [^{14}C]5HT.

5.2.5 Experimental Design

The composition of the reaction mixture represents optimum values that were experimentally determined as described below. Typically, one component (the dependent variable) was allowed to vary, while the independent variables were fixed at the optimum values. The volume of assay buffer was adjusted to compensate for any changes in the composition of the reaction mixture. By necessity, each experiment is presented in a logical linear fashion, but the design of one experiment was dependent on the results of several other experiments.

(i) Subcellular Distribution Studies

The following subcellular fractions were prepared as described in section 2.3: 5% (w/v) total homogenate, 5% (w/v) nuclei/cell debris, 4% (w/v) mitochondria/lysosomes and 5% (w/v) cytosol/microsomes. MAO activity and specific activity of each fraction was determined using 50 µl aliquots with [^{14}C]5HT (0.025 µCi) as the sole substrate.

(ii) Linearity with Enzyme Content

MAO activity was determined for 10, 20, 30, 40, 50 and 60 µl aliquots of a 4% (w/v), 5% (w/v) and 10% (w/v) mitochondrial/lysosomal fraction. Activity was determined with [^{14}C]5HT (0.025 µCi; 4.5 µM) as the sole substrate, and in the presence of 150 µM 5HT. The ratio of "[^{14}C]5HT" activity to "[^{14}C]5HT+5HT" activity was also calculated for each aliquot.

(iii) Linearity with Assay Incubation Time

The time-dependency of MAO specific activity was determined by terminating the assay reaction at fixed time intervals of 0, 5, 10, 20, 30, 45 and 60 min. Activity was determined with [^{14}C]5HT (0.025 µCi; 4.5 µM) alone or in conjunction with cold 5HT at a final concentration of 150 µM. The ratio of "[^{14}C]5HT" activity to "[^{14}C]5HT+5HT" activity was calculated for each time point.

(iv) Kinetic Analysis: [^{14}C]5HT

The substrate kinetics of MAO for [^{14}C]5HT was determined by initiating the assay reaction with 0.00625, 0.0125, 0.025, 0.05, 0.125 or 0.25 µCi of the radioligand. This gave approximate final concentrations of 1.125, 2.25, 4.5, 9.0, 22.5 and 45.0 µM [^{14}C]5HT respectively. Parallel studies were performed in the presence of cold 5HT at a final concentration of 150 µM. The ratio of "[^{14}C]5HT" activity to "[^{14}C]5HT+5HT" activity was calculated for each [^{14}C]5HT concentration. The activity data obtained in the presence of 150 µM 5HT was further analysed by the Hanes-Wolf plot (S/V vs. S) where the substrate (S) is the [^{14}C]5HT concentration (µM) and V is the enzyme activity (DPM/30 min). The x-intercept of this plot represents the apparent K_m value.

(v) Kinetic Analysis: 5HT

The substrate kinetics of MAO were further investigated by competing a fixed concentration of [^{14}C]5HT (0.025 µCi; 4.5 µM) with varying final concentrations of non-radioisotopic 5HT (1, 5, 25, 50, 100, 200 and 400 µM). Time-dependency of "inhibition" was determined by preincubating the enzyme with 5HT for T = 5 min and T = 60 min prior to the addition of [^{14}C]5HT. The 5 min preincubation period is equivalent to the normal thermal equilibration period. An inhibition curve and the Dixon plot were employed to determine an IC_{50} value for 5HT (see section 5.2.6). An IC_{50} value represents that concentration of inhibitor that produces 50% inhibition.

(vi) Inhibition by Pargyline, L-Deprenyl and Clorgyline

In vitro inhibition profiles were generated for these irreversible MAO inhibitors by varying the final concentrations of each as follows: pargyline, 10^{-3} to 10^{-9} M; L-deprenyl, 10^{-3} to 10^{-9} M; and clorgyline, 10^{-3} to 10^{-22} M. [^{14}C]5HT (0.025 μCi; 4.5 μM) was employed as the sole substrate. The preincubation period with each inhibitor was limited to the thermal equilibration period of 5 min. IC_{50} values were determined from the respective inhibition curves and Dixon plots.

5.2.6 Data and Statistical Analysis

The DPM data was normalized for 1 ml of the extraction solvent and corrected for the zero-time blank value. MAO-A activity is represented by total [^{14}C]MAOP and the units DPM/30 min. Specific activity has the units DPM/30 min/100 μg protein. Data is graphically presented as mean ± SEM with the sample size (n) depending on the experiment. An inhibition curve was constructed by plotting % inhibition *vs.* log molar (M) concentration of the inhibitor (I). The mean data for each inhibitor concentration was converted to a % inhibition with respect to the control data. The linear Dixon plot involves plotting 1/V *vs.* I, where the x-intercept represents the IC_{50} value (Dixon, 1972). For this purpose, the reciprocal of the mean value, (DPM/30 min/100 μg protein)$^{-1}$, was calculated. Generally, the data that fell within the linear section of the inhibition curve was used to construct the Dixon plot. Linear and non-linear regressions were performed according to section 2.7.

5.3 Results

(i) Subcellular Distribution Studies

The subcellular distribution of MAO-A activity is presented in Figure 5.2. The majority of MAO-A activity and specific activity was observed in the mitochondrial/lysosomal fraction, with comparable activity levels found only in the total homogenate. Only trace amounts of activity were detected in the nuclear/cell debris and negligible activity in the cytosolic/microsomal fractions. The crude mitochondrial/lysosomal fraction represents a "purer" enzyme preparation than the total homogenate, giving a more accurate reflection of MAO-A specific activity.

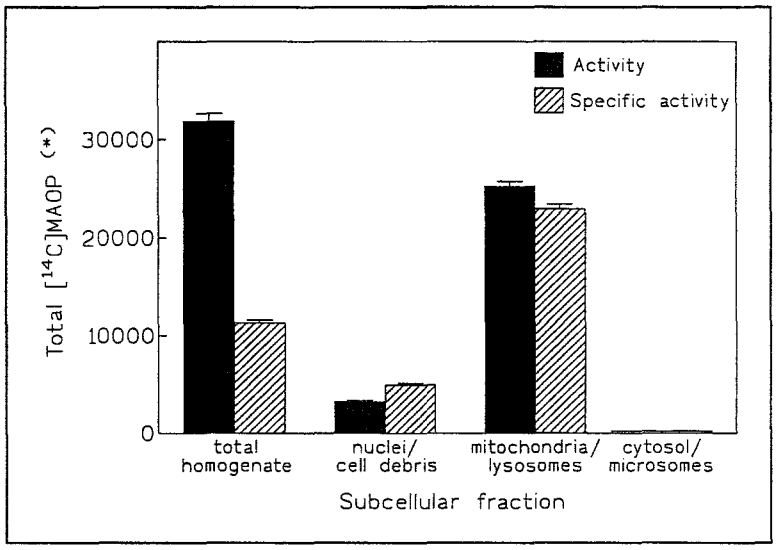


Figure 5.2: Subcellular distribution of striatal MAO-A activity. [Data represents mean ± SEM (n = 5); *: Activity = DPM/30 min, Specific activity = DPM/30 min/100 μg protein]

(ii) Linearity with Enzyme Content

Preliminary results found a non-linear relationship between homogenate volume and MAO-A activity for the 10% (w/v) and 5% (w/v) mitochondrial/lysosomal fractions (r^2 = 0.996 and r^2 = 0.996 respectively) in the presence of 4.5 μM [^{14}C]5HT (data not shown). In the case of the 4% (w/v) fraction (Figure 5.3), linear responses were obtained for 4.5 μM [^{14}C]5HT alone (r^2 = 0.9990,P < 0.0001) and in the presence of 150 μM 5HT (r^2 = 0.9984, P < 0.0001). The ratio of "[^{14}C]5HT" activity to "[^{14}C]5HT+5HT" activity remained constant at *ca.* 2.0 for each homogenate aliquot (Figure 5.3 Insert). A 50 μl aliquot of a 4% (w/v) mitochondrial/lysosomal fraction was chosen as the optimum for all subsequent MAO-A assays.

(iii) Linearity with Assay Incubation Time

The relationship between MAO-A activity and assay reaction time is shown in Figure 5.4. Activity with [^{14}C]5HT alone was linear over the first 30 min (r^2 = 0.983, P = 0.0009), but tapered off by 60 min (non-linear r^2 = 0.992). In the presence of 150 μM 5HT, activity remained linear for 45 min (r^2 = 0.967, P = 0.0004) and then also tapered off by 60 min (non-linear r^2 = 0.996). A similar pattern is seen in the ratio of "[^{14}C]5HT" activity to "[^{14}C]5HT+5HT" activity (Figure 5.4 Insert). A ratio ≈ 2.0 is seen for the first 30 min, but then it drops below 1.7 at 45 and 60 min.

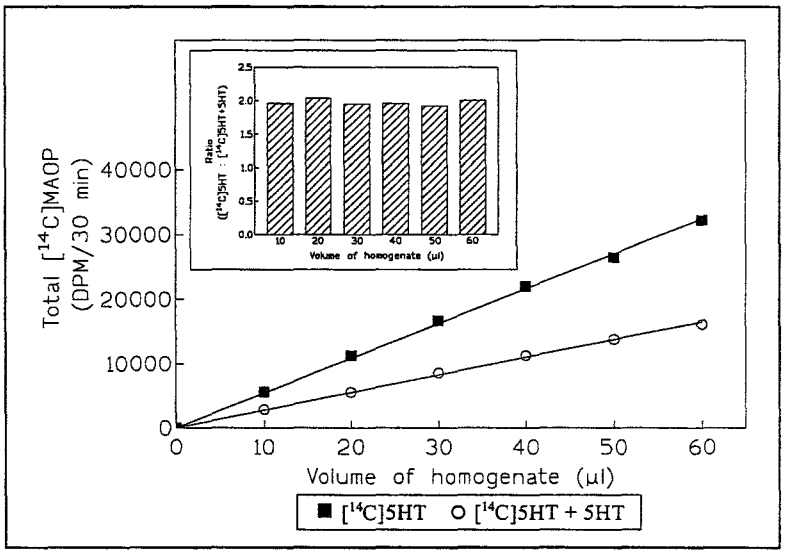


Figure 5.3: MAO-A activity as a function of enzyme concentration. ***Insert*****: Ratio of "[^{14}C]5HT" activity to "[^{14}C]5HT+5HT" activity.** [Data represents mean ± SEM (n = 5)]

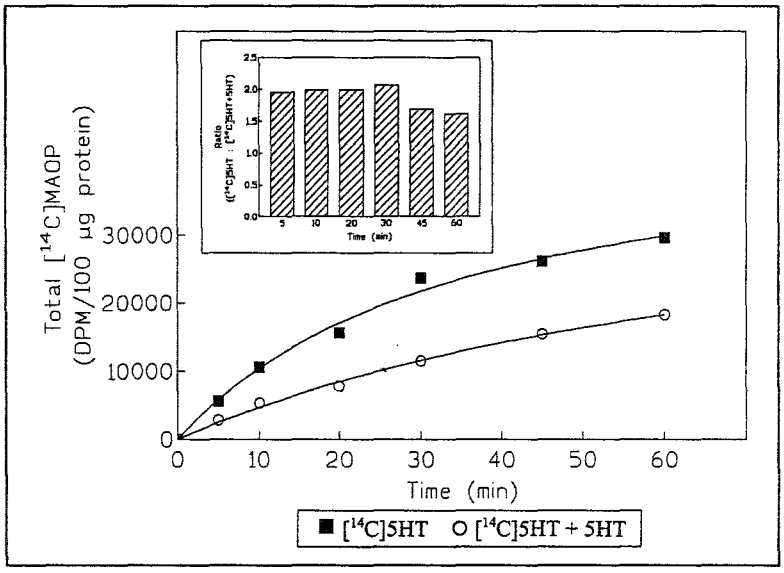


Figure 5.4: MAO-A activity as a function of assay incubation time. [Data represents mean ± SEM (n = 5)]

(iv) Kinetic Analysis: [^{14}C]5HT

The relationship between MAO-A activity and substrate concentration is shown in Figure 5.5A. Activity with [^{14}C]5HT alone was linear over the concentration range of 0.0-45.0 μM (r^2 = 0.999, $P < 0.0001$). In the presence of 150 μM 5HT, activity remained linear over this range (r^2 = 0.987, $P < 0.0001$), but also gave a perfect non-linear fit (r^2 = 1.00). This deviation is further reflected in the ratio of "[^{14}C]5HT" activity to "[^{14}C]5HT+5HT" activity (Figure 5.5B). Initially, the ratio is constant at ± 2.0 for 1.125 to 9.0 μM [^{14}C]5HT, but then progressively increases. The Hanes-Wolf plot for the "[^{14}C]5HT+5HT" activity data is shown in Figure 5.5C. A linear plot was generated (r^2 = 0.983, P = 0.0001), with an apparent $K_m \approx 100$ μM obtained from the x-intercept.

(v) Kinetic Analysis: 5HT

Non-radioisotopic 5HT *in vitro* dose-dependently competed for MAO activity, in a time-independent manner (Figure 5.6). For the T = 5 min data, an IC_{50} value ≈ 135 μM was obtained from the sigmoidal plot (r^2 = 0.992) and ≈ 130 μM from the Dixon plot (Figure 5.6 Insert; r^2 = 0.9975, $P < 0.0001$). For the T = 60 min data, an IC_{50} value ≈ 140 μM was obtained from the sigmoidal plot (r^2 = 0.991) and ≈ 125 μM from the Dixon plot (Figure 5.6 Insert; r^2 = 0.9916, $P < 0.0001$). Since there were no significant differences in these values, an average $IC_{50} \approx 135$ μM was calculated.

(vi) Inhibition by Pargyline, L-Deprenyl and Clorgyline

The structures of pargyline, L-deprenyl and clorgyline are shown in Figure 5.7. Inhibition profiles for each inhibitor are presented in Figure 5.8. Monophasic sigmoidal curves were obtained for each inhibitor. For pargyline, an $IC_{50} \approx 0.8$ μM was generated from the sigmoidal curve (r^2 = 0.999) and an $IC_{50} \approx 0.45$ μM was obtained from the Dixon plot (Figure 5.9A; r^2 = 0.9986, $P < 0.0001$). For L-deprenyl, an $IC_{50} \approx 1.8$ μM was generated from the sigmoidal curve (r^2 = 1.000) and an $IC_{50} \approx 1.0$ μM was obtained from the Dixon plot (Figure 5.9A; r^2 = 0.9980, $P < 0.0001$). In comparison, the inhibition curve for clorgyline was markedly shifted to the left. The sigmoidal plot generated an $IC_{50} \approx 6.5 \times 10^{-20}$ M (r^2 = 0.973) and a similar value, $IC_{50} \approx 7.8 \times 10^{-20}$ M, was calculated from the Dixon plot (Figure 5.9B; r^2 = 0.9992, $P < 0.0001$). This potent inhibition by clorgyline was repeatedly confirmed in several independent attempts.

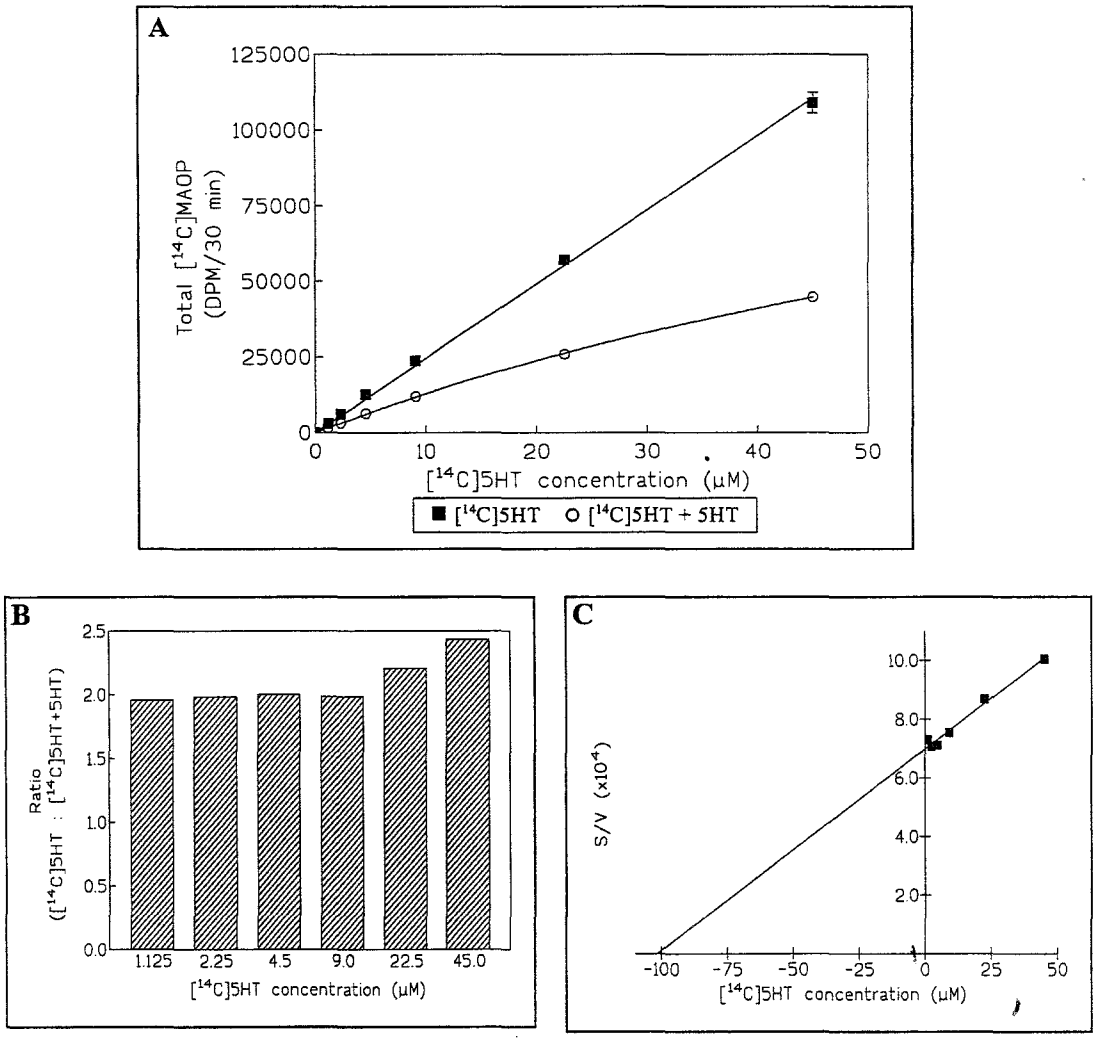


Figure 5.5: MAO-A activity as a function of [^{14}C]5HT concentration. (A) Activity determined in the presence and absence of 150 μM 5HT. (B) Ratio of "[^{14}C]5HT" activity to "[^{14}C]5HT+5HT" activity. (C) Hanes-Wolf plot for "[^{14}C]5HT+5HT" activity. [Data represents mean ± SEM (n = 4)]

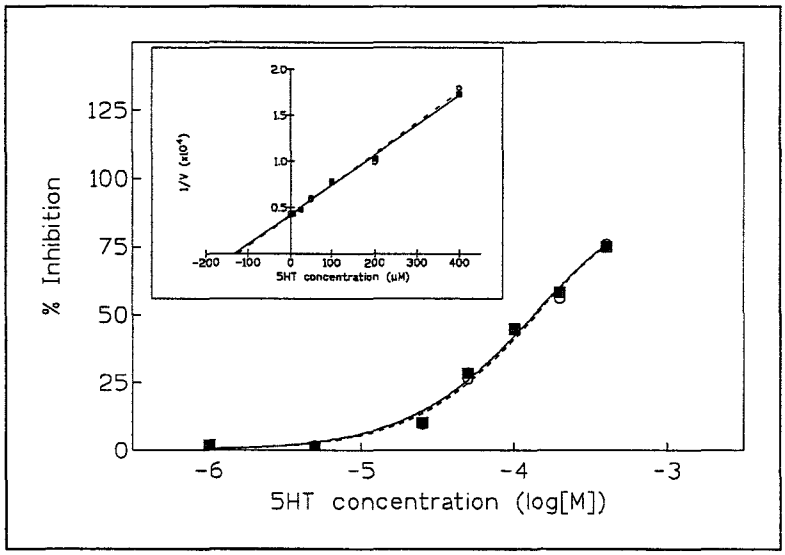


Figure 5.6: The effect of varying concentrations of non-radioisotopic 5HT on MAO-A activity. *Insert*: Dixon plot

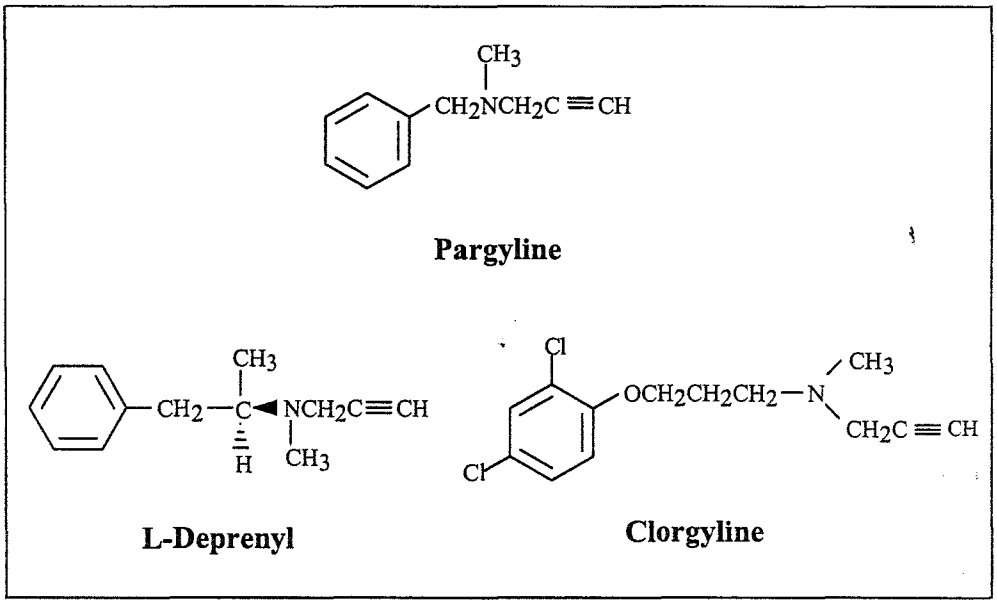


Figure 5.7: Structures of pargyline, L-deprenyl and clorgyline.

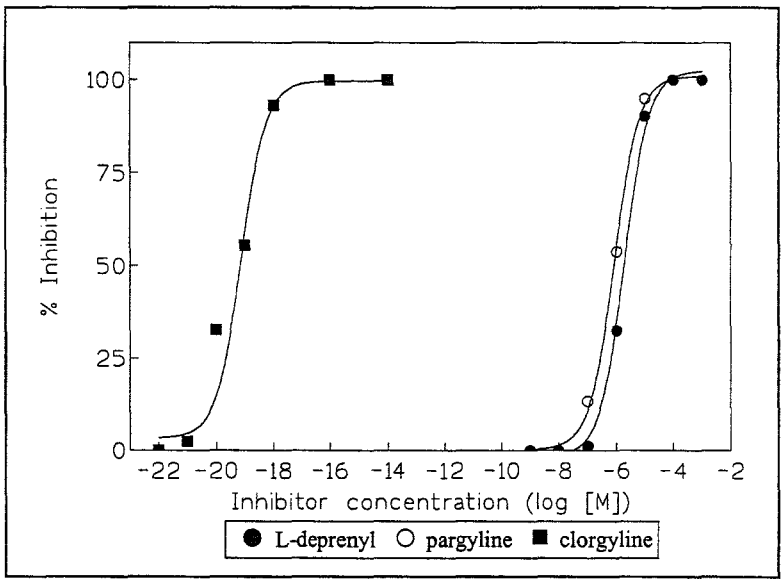


Figure 5.8: Inhibition profiles of selective irreversible MAO inhibitors.

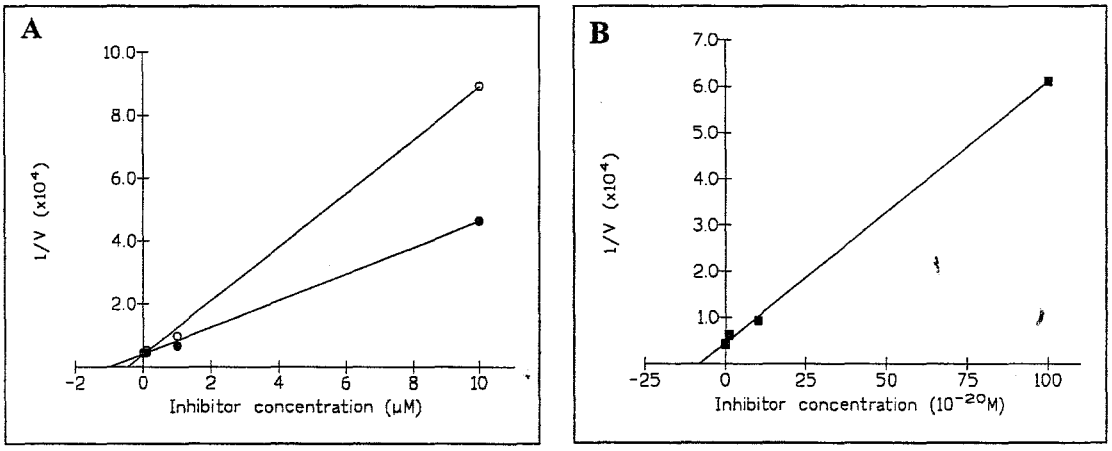


Figure 5.9: Dixon plots of irreversible inhibitors. (A) pargyline and L-deprenyl. (B) clorgyline.

5.4 Discussion and Conclusion

The results of the subcellular fractionation study clearly indicate that the majority of MAO specific activity was present in the mitochondrial/lysosomal fraction. Only trace amounts of activity were observed in the nuclei/cell debris fraction. This profile is consistent with the well established fact that both MAO-A and -B are integral proteins of the outer mitochondrial membrane (Abell *et al.*, 1994; Singer and Ramsay, 1995). Obata *et al.* (1994) purportedly purified a cytosolic MAO isoform from the rat liver by countercurrent chromatography. This isoform had properties more like MAO-B than MAO-A in terms of substrate specificity and sensitivity to clorgyline inhibition. In the present study, negligible activity was observed in the cytosolic/microsomal fraction. However, it must be noted that MAO-B activity was not assayed.

The similar activity obtained in the total homogenate and the mitochondrial/lysosomal fraction is indicative of the efficiency of the homogenization-fractionation process with no apparent loss of important co-factors *inter alia*. Furthermore, this simple, but rapid protocol provides an adequate and consistent source of MAO, with minimal enzyme loss and variability between striatal samples. MAO is tightly bound to the mitochondria and its release and solubilization (e.g. by sonication and detergents) can result in considerable loss of activity and alteration of kinetic properties. This has important implications for comparison between samples, especially with regard to the *in vivo* studies. Thus all further studies were performed on a 4% (w/v) mitochondrial/lysosomal fraction, which gives a truer reflection of MAO specific activity. Use of mitochondria also removes or minimises the interference from the coupled enzymes alcohol dehydrogenase and aldehyde dehydrogenase (see Figure 5.1).

The relative proportion of MAO-A and MAO-B protein and mRNA in the striatum and substantia nigra is a matter of contentious debate, and appears to be species-specific. It has been strongly argued in section 1.7.5 that in the rat striatum, presynaptic MAO-A is the key isoform with regards to the *in vivo* deamination of 5HT and DA following reuptake of these neurotransmitters. For this reason, the *in vitro* radioenzymatic assay was designed to quantify MAO-A activity. Subsequent studies were performed to characterize and confirm the nature of the MAO isoform being assayed. A linear relationship was observed between [^{14}C]5HT and MAO activity without evidence of saturation. Cost limitations and the specific activity of the radiochemical batch did not allow the use of final [^{14}C]5HT concentrations above 45.0 μM. This prevented the calculation of an apparent K_m value for [^{14}C]5HT under the present conditions. However, in the presence of

150 μM non-radioisotopic 5HT, an apparent $K_m \approx 100$ μM was determined. Similarly, an $IC_{50} \approx$ 135 μM was obtained for the inhibition of MAO activity by 5HT when [^{14}C]5HT was fixed at a concentration of 4.5 μM. These values (100 and 135 μM) fall within the range of published K_m values (100-170 μM) of both crude and purified MAO-A for 5HT and [^{14}C]5HT (Garrick and Murphy, 1982; Wu *et al.*, 1993).

Although 5HT is preferentially deaminated by MAO-A, it has been shown that MAO-B can metabolize 5HT in certain species and tissues, especially when MAO-A activity is low or deficient (Fowler *et al.*, 1978; Garrick and Murphy, 1982; Cases *et al.*, 1995; Luque *et al.*, 1995). However, MAO-A is abundant in the rat corpus striatum (Juorio *et al.*, 1994; Luque *et al.*, 1995; Jahng *et al.*, 1997) and it has been conclusively shown that 5HT is exclusively metabolized by MAO-A in the rat brain (Fowler *et al.*, 1978; Garrick and Murphy, 1982). Ultimately, inhibitor sensitivity is considered the most reliable and consistent trait for isoform characterization than substrate specificity.

Pargyline, clorgyline and L-deprenyl (selegiline) represent enzyme-activated/mechanism-based ("suicide") inhibitors of MAO. Pargyline is a propynylamine, whereas clorgyline and L-deprenyl are propargylamine derivatives. All are acetylene-containing compounds that covalently attach to N-5 of the flavin moiety causing irreversible inhibition (Palfreyman *et al.*, 1987). The target enzyme is effectively titrated with the duration of inhibition dependent on the turnover rate (half-life) of the enzyme molecule. Thus the observed IC_{50} value is dependent on the amount of enzyme present (Ackermann and Potter, 1949). Sensitivity to these inhibitors formed the basis of the original classification of MAO-A and MAO-B (Johnston, 1968; Yang and Neff, 1973; Fowler *et al.*, 1978). Clorgyline is the most selective MAO-A inhibitor known so far, with inhibition still apparent at very low concentrations, e.g. 10^{-11} to 10^{-13} M. L-Deprenyl is highly selective for MAO-B. Pargyline, on the otherhand, is non-selective and equipotent against both isoforms, although some researchers claim that pargyline is slightly more selective for MAO-B (e.g. Knoll and Magyar, 1972; Wu *et al.*, 1993).

Two important features must be noted for the inhibition profiles obtained for these irreversible inhibitors in the present study. Firstly, clorgyline was substantially more potent than pargyline and L-deprenyl, which were roughly equipotent. Secondly, in each case a monophasic fit was obtained, whereas a biphasic titration curve is indicative of the presence of both isoforms (Johnston, 1968; Fowler *et al.*, 1978; Salach and Detmer, 1979). Since IC_{50} values of irreversible

inhibitors are dependent on the amount of enzyme present, the current inhibitory potencies of these inhibitors cannot be compared to published values. What is important, is the relative position of each inhibition curve. On this basis, it can be confidently stated that with 5HT as the substrate, only one MAO isoform was assayed, namely MAO-A. Since these inhibitors effectively titrate the enzyme, they can be used to quantify the amount of MAO protein present in the homogenate. However, unlike pargyline and L-deprenyl, clorgyline does not bind stoichiometrically with the enzyme due to nonspecific binding of the inhibitor (Salach and Detmer, 1979). This only allows for an estimation of the quantity of MAO-A. Nonetheless, the inhibition of MAO-A by very low concentrations of clorgyline reported here (10^{-14} to 10^{-20} M) indicates that very small quantities of the enzyme are reliably detected by the assay. This confirms the micro-radioisotopic nature of the assay.

For cost purposes, a fixed final concentration of 4.5 μM [^{14}C]5HT was routinely employed. Although this concentration is markedly sub-saturating, it was more than sufficient considering the micro-nature of the tissue samples. At this concentration, the assay was also extremely reproducible and met the required criteria of linearity with enzyme content and reaction time. This is consistent with the fact that MAO-A activity is related to the amount of enzyme present, and not due to different catalytic properties of molecules (Costa *et al.*, 1980; Fowler and Wiberg, 1980). It is important to note that the ratio of "[^{14}C]5HT" activity to "[^{14}C]5HT+5HT" activity remained constant at *ca.* 2.0 for those assay conditions that were chosen as being optimum, namely: 50 μl homogenate, 4.5 μM [^{14}C]5HT and 30 min reaction time. Deviations in the ratio reflected changes in the relative percentage contribution of the 4.5 μM [^{14}C]5HT to the overall substrate concentration. For example, a prolonged reaction time (> 30 min) decreased the ratio indicating that [^{14}C]5HT became progressively more limiting with time.

In summary, the current characterization of the striatal MAO isoform under investigation met all the necessary and published criteria for MAO-A: appropriate subcellular and tissue distribution, substrate specificity and inhibitor sensitivity. The radioenzymatic assay was very reproducible - evident in the low data variability - and extremely sensitive, well-suited to the small tissue samples to be employed for *in vivo* studies. It would have been informative to determine the amount of MAO-B activity present in the rat striatum under identical conditions by using [^{14}C]β-phenethylamine as the selective substrate (Yang and Neff, 1973). An additional precautionary step would have been to perform the MAO-A assay in the presence of a very low concentration of L-deprenyl to block any interference from MAO-B. This would definitely be necessary if the current

assay method was extended to the striatum of the guinea pig or human. In the latter species, MAO-B, and not MAO-A, is the dominant isoform in the striatum and primarily involved in the *in vivo* deamination of DA (Garrick and Murphy, 1982; Luque *et al.*, 1995)

Chapter 6

Inhibition of MAO-A by Indoles

6.1 Introduction

MAO is intimately involved in CNS neurotransmission, blood pressure regulation and detoxification. MAO has also been implicated in psychiatric, neurodegenerative and cardiovascular disorders, senescence, and the prolongation of life (Schalling *et al.*, 1987; Weyler *et al.*, 1990; Abell *et al.*, 1994; Singer and Ramsay, 1995). This has been reviewed in section 1.7.6. Thus the search for novel MAO inhibitors represents a hot spot of pharmaceutical research, especially as antidepressants and antihypertensive agents. The first generation of MAO inhibitors were based on the hydrazine class of drugs, including iproniazid. Unfortunately, these drugs are long-lasting irreversible MAO inhibitors with hepatotoxic properties. The new wave of research has lead to a plethora of potent drugs from a wide range of chemical classes, including esoteric compounds such as the aminoalkylsilanes and organogermanium compounds (Singer and Ramsay, 1995). This multitude of compounds can be divided into three main types of inhibitors:

(i) time-dependent irreversible inhibitors, most of which are mechanism-based "suicide" inhibitors.

(ii) reversible inhibitors at the substrate site (competitive inhibitors).

(iii) reversible inhibitors, which are not purely competitive.

The modern biochemical approach for the development of clinically useful drugs is based on the search for isoform-selective, reversible MAO inhibitors (Priest *et al.*, 1995). Some reversible MAO-A selective inhibitors include moclobemide, brofaromine, Ro 41-1049 and toloxatone. Some reversible MAO-B selective inhibitors include Ro 16-6491, Ro 19-6327 and oxadiazolone and oxadiazothione derivatives (Palfreyman *et al.*, 1987).

Other important classes of inhibitors are based on the indole nucleus, a nitrogen-containing heterocycle. Indeed the indoleamines tryptamine and 5HT are non-selective and selective substrates of MAO-A, respectively (Fowler *et al.*, 1978; Garrick and Murphy, 1982). Some representative indole-based MAO inhibitors are shown in Figure 6.1. Indole itself shows moderate potency, with slight selectivity for MAO-B (Medvedev *et al.*, 1995). β-carboline alkaloids have long been known as competitive, reversible and short-acting inhibitors of MAO both *in vitro* and *in vivo* (Udenfriend *et al.*, 1958; Kim *et al.*, 1997). These compounds are heterocyclic, dehydrogenated derivatives of tryptophan and tryptamine arising through ring-embedding of the

side-chain (Kim *et al.*, 1997). Fully aromatic β-carbolines and dihydro-derivatives include the hallucinogenic harmala alkaloids such as harman, harmine, harmaline and numerous others. They represent very potent inhibitors of both MAO isoforms, but with higher affinity for MAO-A. Pinoline, a tetrahydro-β-carboline derivative, is also a well known, but weak MAO inhibitor (Ho *et al.*, 1968).

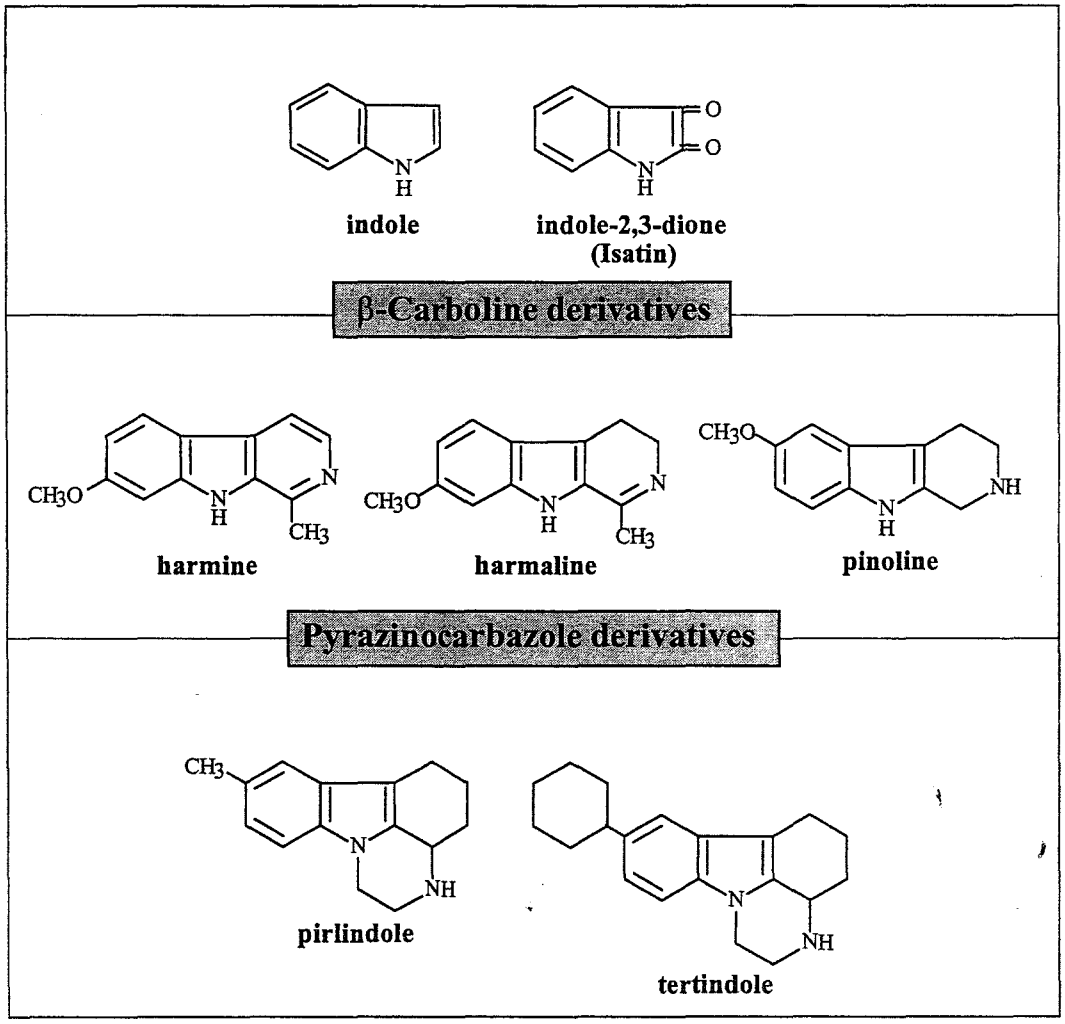


Figure 6.1: A selection of indole-based MAO inhibitors.

A more complex class of indolic chemicals is represented by the pyrazinocarbazole derivatives. Both pirlindole and tertindole display antidepressant activity and are potent, selective and reversible inhibitors of MAO-A (Medvedev *et al.*, 1994).

Numerous β-carbolines, including harman and norharman, are endogenous to the mammalian brain and various peripheral organs (Kim *et al.*, 1997). A stress- and anxiety-induced substance, designated tribulin, was found in the mammalian brain, and rat urine and heart (Yuwiler, 1990). Tribulin is known to inhibit MAO activity and benzodiazepine binding. Subsequently, it was determined that a major component of tribulin is indole-2,3-dione (isatin), which has been shown to be a very selective and potent inhibitor of MAO-B (Medvedev *et al.*, 1995). Obata *et al.* (1994) also isolated an, as yet unidentified, endogenous MAO inhibitor from the rat liver cytosol.

Thus several indolic compounds may be endogenous MAO inhibitors, having important regulatory effects on neurotransmission, especially during stress and anxiety, as in the case of isatin. Endogenous, reversible MAO inhibitors in the brain may represent a clinically important therapeutic avenue. For example, an ability to manipulate the levels of naturally occurring compounds may reduce the side-effect profiles and time- and dose-related fluctuations in efficacy typically associated with pharmaceutical drugs. In this regard, the numerous pineal indolic compounds may represent an important source of endogenous MAO inhibitors. All these indoles are more closely related structurally to the MAO substrates tryptamine and 5HT than the other indole derivatives discussed above. Furthermore, they represent a diverse array of structures, including the existence of a corresponding 5-methoxyindole for each 5-hydroxyindole (Figure 6.2). This represents a valuable opportunity to probe the nature of the active site of MAO by performing structure-activity relationship (SAR) studies. Thus the main objective of this chapter was to screen the compounds listed in Figure 6.2 as potential inhibitors of MAO-A *in vitro*.

6.2 Materials and Methodology

6.2.1 Chemicals and Reagents

The chemicals and reagents for the MAO-A radioenzymatic assay are described in section 5.2.1. Tryptamine HCl, NAS, 5HTOH, 5HIAA, 5MT, MEL, 5MTOH and 5MIAA were purchased from Sigma.

6.2.2 Tissue Preparation

The corpora striata were removed at mid-photophase (12h00) and stored as described in section 2.2. The striata were pooled and all studies were performed on a 4% (w/v) mitochondrial/lysosomal fraction prepared as described in section 2.3. The protein concentration of each enzyme batch was maintained at 2.0 mg/ml.

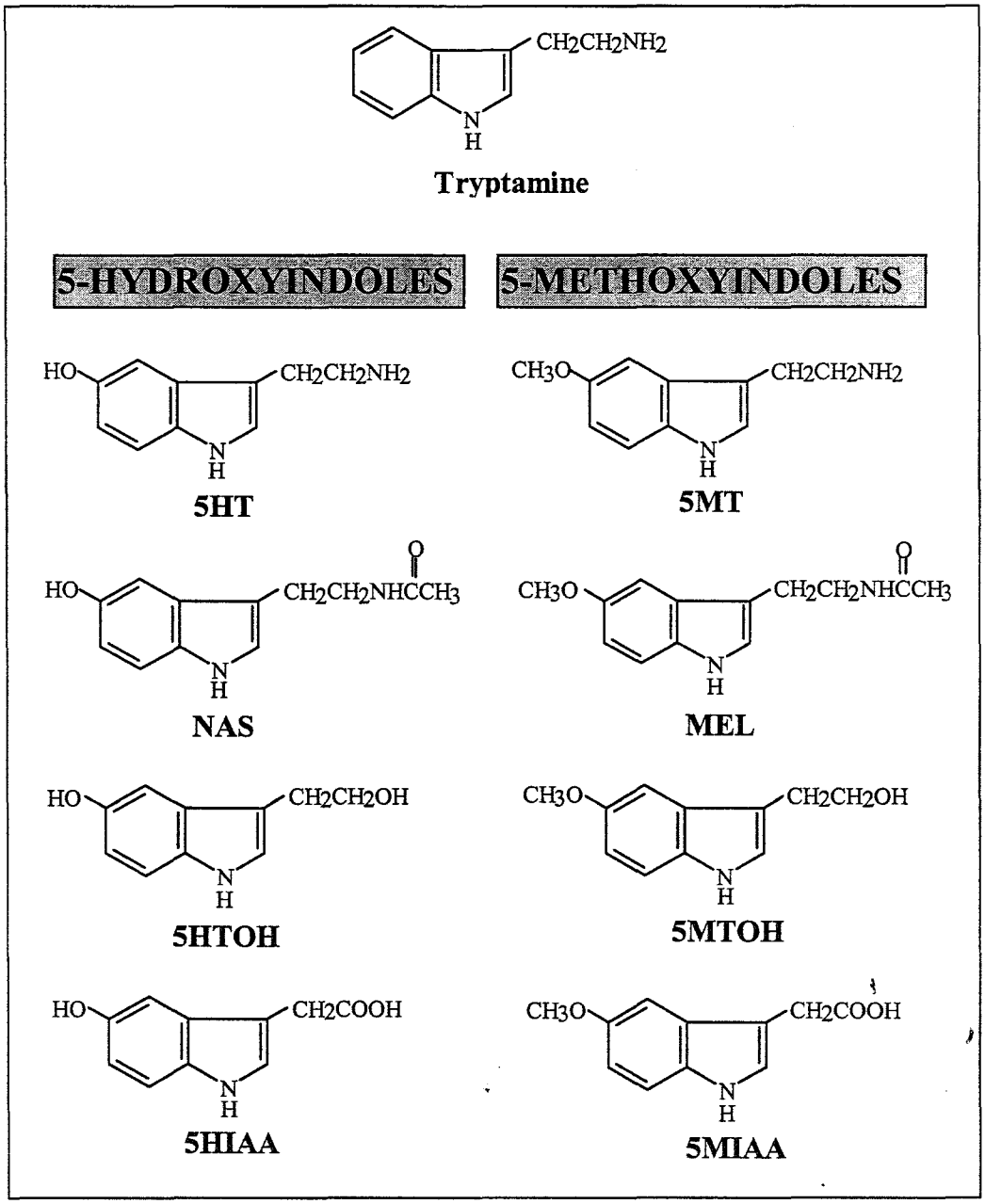


Figure 6.2: Structural comparison of tryptamine and pineal 5-hydroxyindoles and 5-methoxyindoles.

6.2.3 MAO-A ASSAY

The radiometric MAO-A assay was performed as described in 5.2.3 and 5.2.4. The assay components and conditions were based on the optimum values experimentally determined in section 5.3. Activity was quantified with [^{14}C]5HT (0.025 μCi; 4.5 μM) as the sole substrate.

(i) Inhibition Profiles

The following indoles were tested *in vitro* for their inhibitory potential against MAO-A: tryptamine HCl, 5HTOH, 5HIAA, NAS, MEL, 5MT, 5MTOH and 5MIAA. Each indole was tested over a range of final concentrations to produce an inhibition curve. An IC_{50} value was calculated for each indole from the inhibition curve and corresponding Dixon plot.

Working solutions of the 5-methoxyindoles were prepared in buffered ethanol. The MAO enzyme was exposed to a maximum of 0.2% (v/v) final concentration of ethanol. At this level, the ethanol had no effect on control MAO-A activity (data not shown). For purposes of accuracy, control activity in all studies reported here refers to the vehicle-treated enzyme.

(ii) Reversibility

Reversibility of inhibition was determined by the Ackermann-Potter Dilution Method (Ackermann and Potter, 1949). Briefly, MAO-A activity was determined for varying concentrations of enzyme (homogenate) in the presence of the vehicle (control) or a fixed concentration of the test drug. A range of homogenate volumes known to correlate with MAO-A activity in a linear fashion was used (see section 5.3). Typically, a concentration of indole corresponding to its calculated IC_{50} value (see results in section 6.3) was tested. A 5 min preincubation period was employed in all cases. In the presence of the inhibitor, a linear response that passes through the origin is indicative of a reversible inhibitor. For an irreversible inhibitor, the linear plot passes through the x-axis prior to the origin with the magnitude of the x-intercept depending on the inhibitor concentration.

(iii) Time-Dependency

The time-dependency of inhibition was determined by preincubating the enzyme with the test indole for T = 5 min and T = 60 min at 37 °C prior to the addition of [^{14}C]5HT. The 5 min preincubation period is equivalent to the normal thermal equilibration period. Inhibition profiles and Dixon plots were plotted for both time periods to allow comparison of the IC_{50} values.

6.2.4 DATA AND STATISTICAL ANALYSIS

MAO-A activity is expressed in the units DPM/30 min. The raw data was converted to a mean ± SEM (n = 5). The mean data was then converted for the inhibition curves and Dixon plots as described in section 5.2.6. In the case of the 5-methoxyindoles 5MT, MEL and 5MTOH, the vehicle-treated data served as the control for calculation of % inhibition. The data for 5HIAA and 5MIAA was graphically presented in bar graphs as the mean ± SEM. Linear and non-linear regressions were performed as summarised in section 2.7. In all cases, the non-linear regressions were monophasic, indicating that the data was consistent with a single site model.

6.3 Results

(i) Tryptamine HCl

Tryptamine *in vitro* dose- and time-dependently reduced MAO-A activity (Figure 6.3). In the case of the T = 5 min data, an IC_{50} value ≈ 20 μM was obtained from the sigmoidal plot (r^2 = 0.997) and the Dixon plot (Figure 6.3 Insert; r^2 = 0.9985, P < 0.0001). Paradoxically, a 2-fold decrease in inhibitory potency was seen for T = 60 min, with an IC_{50} value ≈ 40 μM calculated from the sigmoidal plot (r^2 = 0.990) and the Dixon plot (Figure 6.3 Insert; r^2 = 0.984, P < 0.0001). A comparison of the inhibition curves shows that for T = 60 min, less inhibition is seen at the lower concentrations of tryptamine (5-100 μM) with recovery and similar potencies at higher concentrations (200-600 μM). The reversibility of inhibition by tryptamine (25 μM), following a 5 min preincubation period, is shown in Figure 6.11A. Data for both the control (r^2 = 0.9961, P < 0.0001) and tryptamine (r^2 = 0.9865, P < 0.0001) gave a linear response that passed through the origin, indicative of reversible inhibition.

(ii) 5HTOH

5HTOH *in vitro* dose-dependently inhibited MAO-A activity, in a time-independent manner (Figure 6.4). An IC_{50} value ≈ 280 μM was obtained for both the T = 5 min (r^2 = 0.988) and the T = 60 min (r^2 = 0.996) data from the sigmoidal plots. The Dixon plots (Figure 6.4 Insert) generated approximate IC_{50} values of 240 μM for T = 5 min (r^2 = 0.9958, P = 0.0021) and 215 μM for T = 60 min (r^2 = 0.9911, P = 0.0045). The latter IC_{50} values were not significantly different and thus an average IC_{50} value ≈ 250 μM was calculated. The reversibility of inhibition by 5HTOH (250 μM) is shown in Figure 6.11B. Data for both the control (r^2 = 0.9915, P < 0.0001) and 5HTOH (r^2 = 0.9974, P < 0.0001) gave a linear response that passed through the origin, indicative of reversible inhibition.

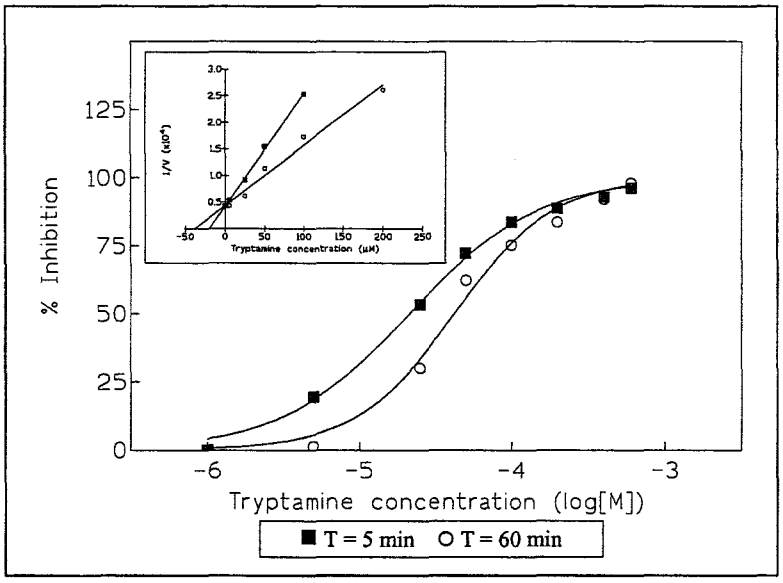


Figure 6.3: Time-dependency of inhibition of MAO-A by tryptamine. *Insert*: Dixon plot.

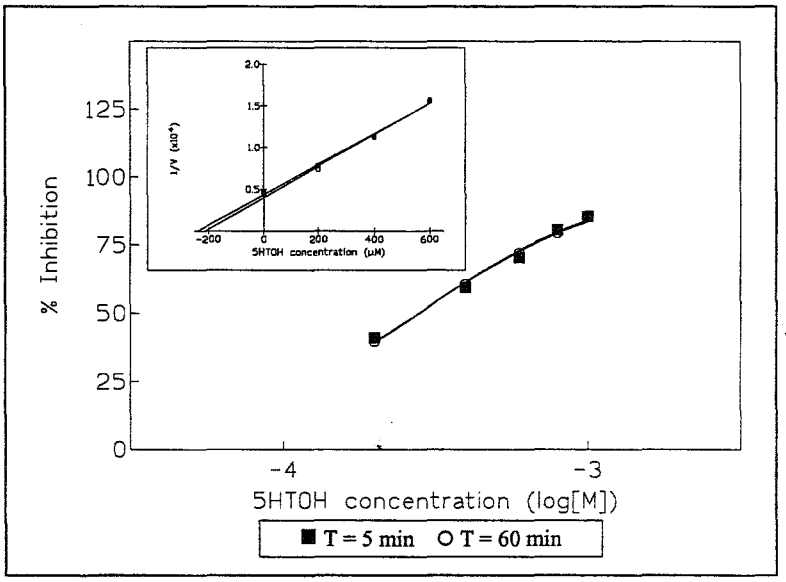


Figure 6.4: Time-dependency of inhibition of MAO-A by 5HTOH. *Insert*: Dixon plot.

(iii) 5HIAA

The activity data for the 5HIAA inhibition study is shown in Figure 6.5. 5HIAA was a very weak, time-independent inhibitor of MAO-A *in vitro*. A maximum of 12-15% inhibition was observed at 1 mM for both T = 5 and 60 min. The reversibility of inhibition was not determined due to the weak inhibitory potency of 5HIAA.

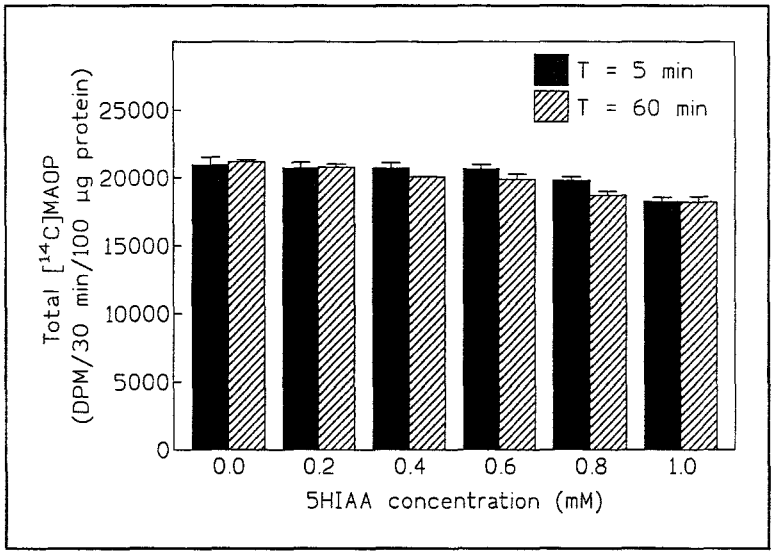


Figure 6.5: Time-dependency of inhibition of MAO-A by 5HIAA. [Data represents mean ± SEM (n = 5)]

(iv) NAS

The *in vitro* inhibition profile for NAS is presented in Figure 6.6. NAS is a weak, time-independent inhibitor of MAO-A. The sigmoidal plots generated an IC_{50} value ≈ 850 µM for T = 5 min data (r^2 = 0.995) and 885 µM for T = 60 min data (r^2 = 0.990). There were insufficient data points either side of the 50% inhibition point to construct reliable Dixon plots. The slight difference in IC_{50} values was not significant and thus an average IC_{50} value ≈ 870 µM was accepted. The reversibility of inhibition by NAS (800 µM) is shown in Figure 6.11C. Data for both the control (r^2 = 0.9973, P < 0.0001) and NAS (r^2 = 0.9971, P < 0.0001) gave a linear response that passed through the origin, indicative of reversible inhibition.

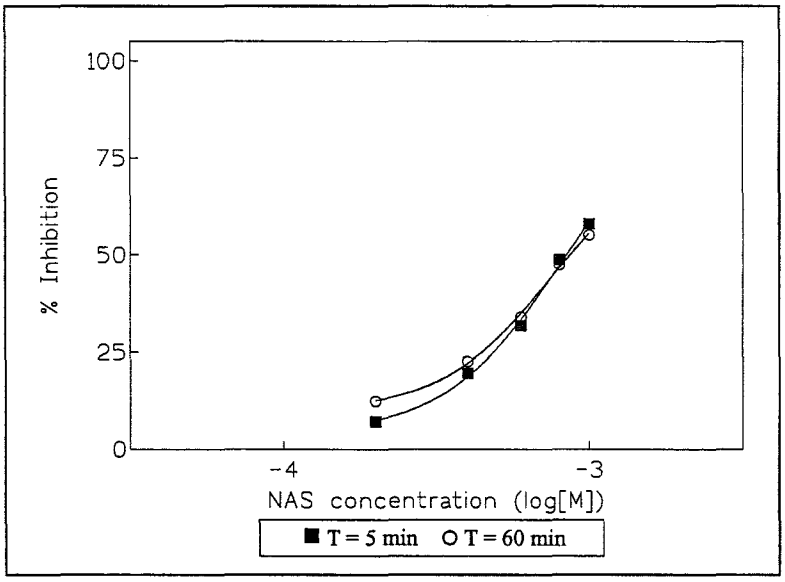


Figure 6.6: Time-dependency of inhibition of MAO-A by NAS.

(v) MEL

MEL *in vitro* dose-dependently inhibited MAO-A activity, in a time-independent fashion (Figure 6.7). An $IC_{50} \approx 190$ μM was generated from the sigmoidal plots for both T = 5 min ($r^2 = 0.997$) and T = 60 min ($r^2 = 0.991$). This value was confirmed by the Dixon plots (Figure 6.7 Insert) for both T = 5 min ($r^2 = 0.9965$, $P < 0.0001$) and T = 60 min ($r^2 = 0.9891$, $P = 0.0005$). The reversibility of inhibition by MEL (200 μM) is shown in Figure 6.11D. Data for both the control ($r^2 = 0.9987$, $P < 0.0001$) and MEL ($r^2 = 0.9990$, $P < 0.0001$) gave a linear response that passed through the origin, indicative of reversible inhibition.

(vi) 5MT

5MT potently reduced MAO-A activity *in vitro*, in a dose- and time-dependent manner (Figure 6.8). For the T = 5 min data, an IC_{50} value ≈ 1 μM was obtained from the sigmoidal plot ($r^2 = 0.971$) and the Dixon plot (Figure 6.8 Insert; $r^2 = 0.9997$, $P = 0.0124$). Paradoxically, a 10-fold decrease in inhibitory potency was seen for T = 60 min, with an IC_{50} value ≈ 14 μM calculated from the sigmoidal plot ($r^2 = 0.990$) and the Dixon plot (Figure 6.8 Insert; $r^2 = 0.9905$, $P < 0.0001$). A comparison of the inhibition curves shows that for T = 60 min, less inhibition is seen at the lower concentrations of 5MT (0.5-50 μM) with recovery and similar potencies at higher concentrations (100 and 200 μM). The reversibility of inhibition by 5MT (25 μM),

following a 5 min preincubation period, is shown in Figure 6.11E. Data for both the control (r^2 = 0.9993, P < 0.0001) and 5MT (r^2 = 0.9966, P < 0.0001) gave a linear response that passed through the origin, indicative of reversible inhibition.

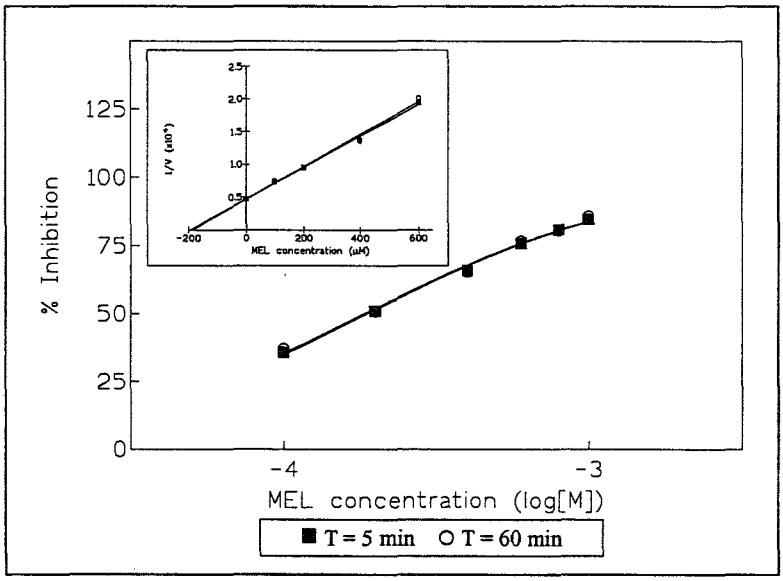


Figure 6.7: Time-dependency of inhibition of MAO-A by MEL. *Insert*: Dixon plot.

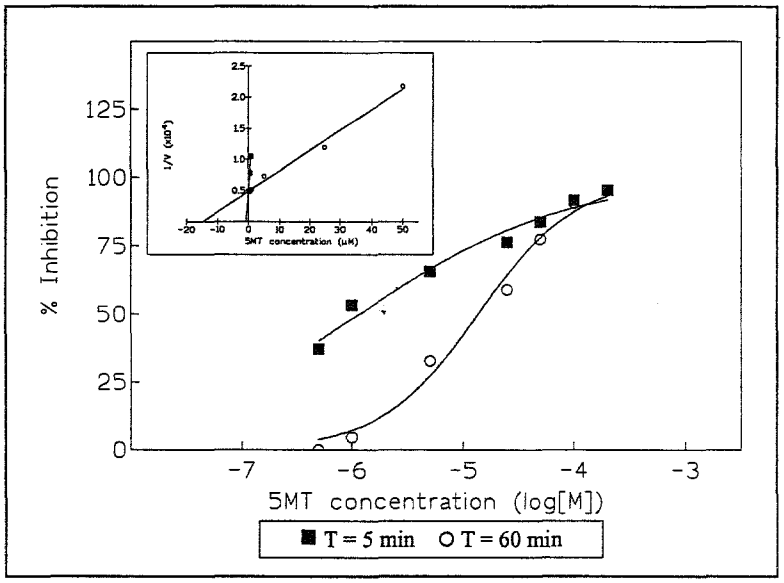


Figure 6.8: Time-dependency of inhibition of MAO-A by 5MT. *Insert*: Dixon plot.

(vii) 5MTOH

The *in vitro* inhibition profile for 5MTOH is presented in Figure 6.9. 5MTOH is a potent dose- and time-dependent inhibitor of MAO-A. For the T = 5 min data, an IC_{50} value ≈ 24 μM was obtained from the sigmoidal plot (r^2 = 0.9960) and the Dixon plot (Figure 6.9 Insert; r^2 = 0.9991, P < 0.0001). For the T = 60 min, an IC_{50} value ≈ 14 μM was calculated from the sigmoidal plot (r^2 = 0.992) and the Dixon plot (Figure 6.9 Insert; r^2 = 0.9928, P = 0.0003). A comparison of the inhibition curves shows that 5MTOH was more potent at T = 60 min than T = 5 min at all concentrations tested except 1 μM. The reversibility of inhibition by 5MTOH (25 μM), following a 5 min preincubation period, is shown in Figure 6.11F. Data for both the control (r^2 = 0.997, P < 0.0001) and 5MTOH (r^2 = 0.9951, P < 0.0001) gave a linear response that passed through the origin, indicative of reversible inhibition.

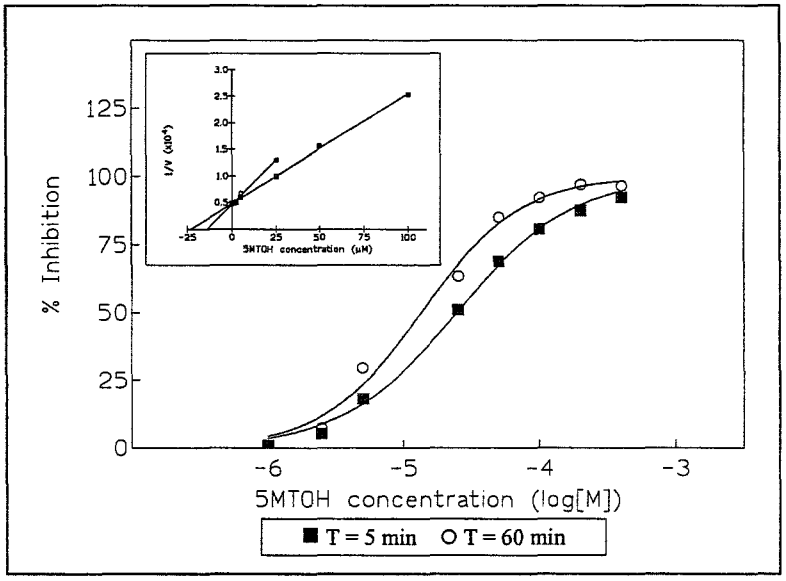


Figure 6.9: Time-dependency of inhibition of MAO-A by 5MTOH. *Insert*: Dixon plot.

(viii) 5MIAA

The activity data for the 5MIAA inhibition study is shown in Figure 6.10. 5MIAA was a very weak, time-independent inhibitor of MAO-A *in vitro*. A maximum of 20% inhibition was observed at 1 mM for both T = 5 and 60 min. The reversibility of inhibition was not determined due to the weak inhibitory potency of 5MIAA.

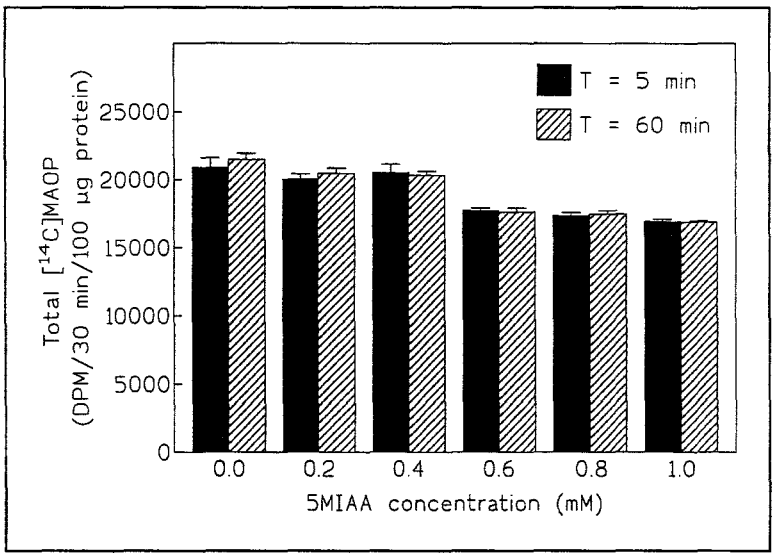


Figure 6.10: Time-dependency of inhibition of MAO-A by 5MIAA. [Data represents mean ± SEM (n=5)]

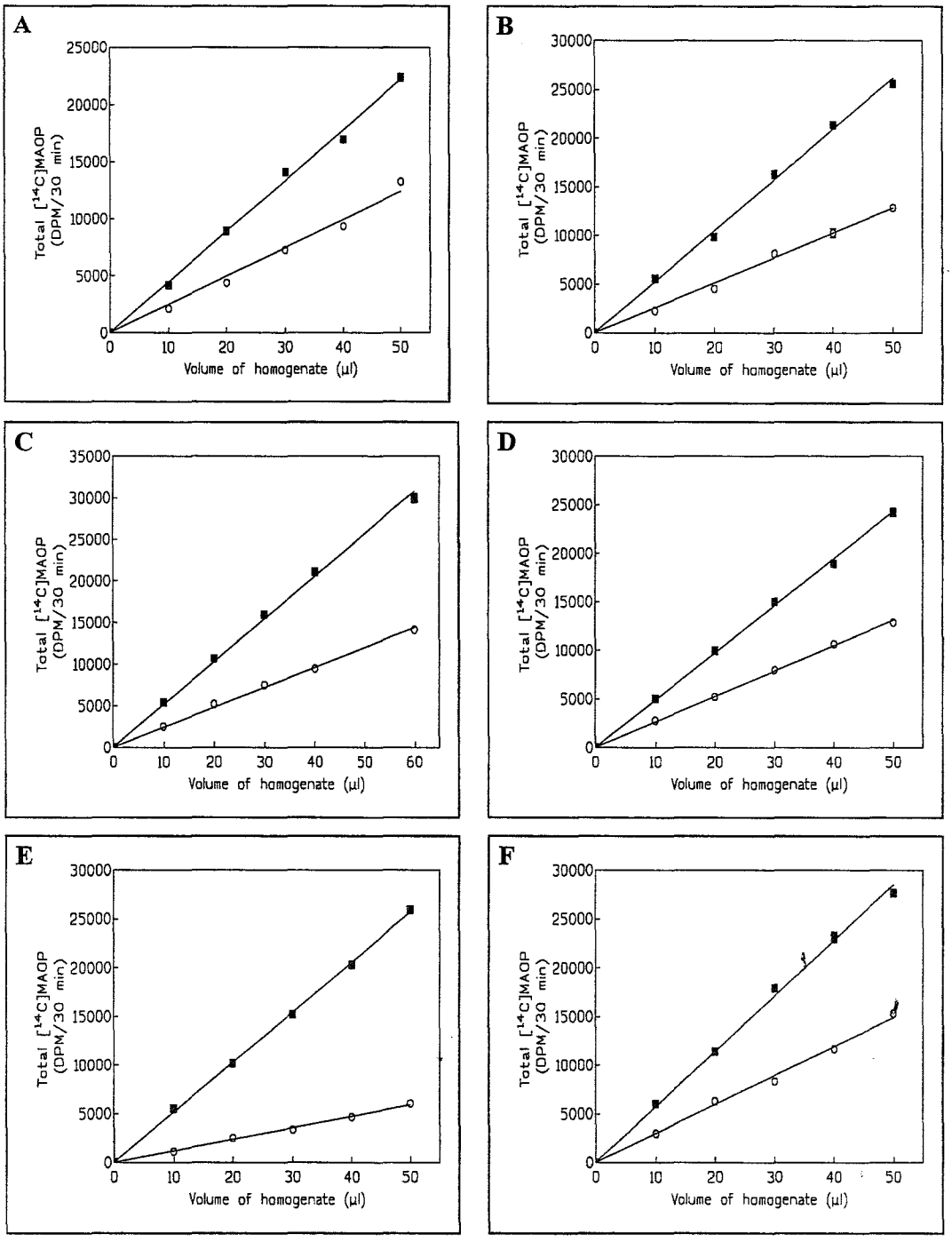
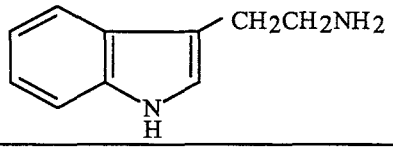
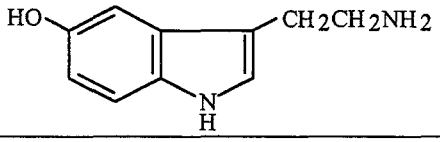
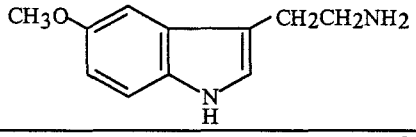
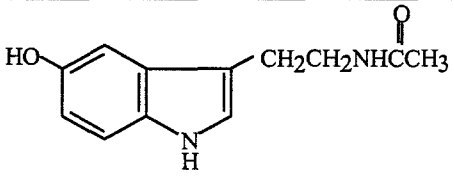
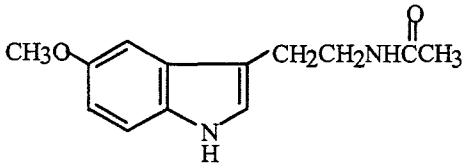
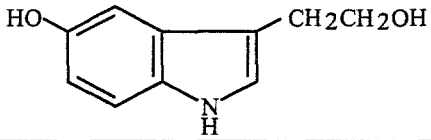
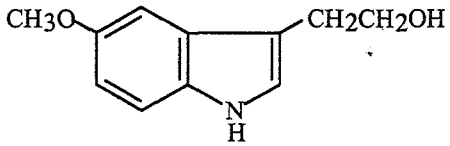
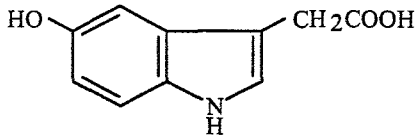
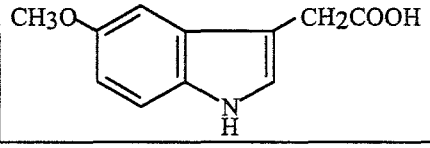


Figure 6.11: Reversibility of inhibition by indolic compounds. [A] Tryptamine (25 µM). [B] 5HTOH (250 µM). [C] NAS (800 µM). [D] MEL (200 µM). [E] 5MT (25 µM). [F] 5MTOH (25 µM). [Data represents mean ± SEM (n = 5); ■ : control, O : test drug]

Table 6.1: Summary of Inhibition Data for Indolic Compounds.

Name	Structure	IC_{50} (μM)		Reversibility
		T = 5 min	T = 60 min	
Tryptamine		20	40	YES
5HT		135	135	YES
5MT		1	14	YES
NAS		870	870	YES
MEL		190	190	YES
5HTOH		250	250	YES
5MTOH		24	14	YES
5HIAA		> 1000	> 1000	nd[1]
5MIAA		> 1000	> 1000	nd

[1]: not determined (see text)

6.4 Discussion and Conclusion

These studies clearly indicate that several indoles, endogenous to the brain and pineal, are capable of inhibiting MAO-A *in vitro* in a reversible fashion. The potency and time-dependency of each indole tested is summarized in Table 6.1, including non-radioisotopic or cold 5HT. Results for 5HT were obtained from section 5.3. Although 5HT and tryptamine are MAO-A substrates, by competing with [^{14}C]5HT they effectively act as competitive inhibitors. Comparing the inhibitory potencies of the indoles with respect to 5HT allowed for SAR studies. The rank order of potency was as follows: 5MT >> 5MTOH > tryptamine >> 5HT > MEL > 5HTOH >> NAS > 5HIAA ≈ 5MIAA.

The active site of MAO is proposed to comprise two domains, the substrate binding site and a pocket housing the FAD moiety (Singer and Ramsay, 1995). The exact nature of the substrate binding site is still elusive, but it appears that the 3-dimensional conformational shape and the stereoelectronic and steric requirements of the catalytic sites of MAO-A and -B differ. For example, the fact that MAO-B favours more hydrophobic substrates is supported by hydropathy plots of the protein (Singer and Ramsay, 1995). One approach for modeling is the recognition that 5HT receptors and MAO-A share 5HT as a selective ligand. In both cases, 5HT must be cationic for binding, by protonation of the free amino group (NH_3^+) at physiological pH. Computational modeling and site-directed mutagenesis of the $5HT_{2A}$ has led to the identification of key amino acids for the binding of 5HT and structural analogues (Sealfon *et al.*, 1997; Wang *et al.*, 1993). Serine residues co-ordinate with hydroxyl groups and the indolic N-9. An aspartate residue acts as a counterion, interacting with the protonated amine, which is reinforced by a further serine residue. Similar residues may be required for the positioning of 5HT in the substrate binding site of MAO-A (Figure 6.12).

Computer-modeling of MAO substrates and inhibitors reveals that a common arrangement includes an aromatic ring with an amino group located in the ring plane. The distance between the center of the nitrogen and the ring is between 0.5 and 0.55 nm (Medvedev *et al.*, 1995). This is consistent with the structure of tryptamine and 5HT. More specific to this thesis, structure-activity modeling with several types of reversible indole-based compounds has also contributed some basic rules to understanding the MAO active site. Indole itself has moderate inhibitory potency (IC_{50} value ≈ 100-200 μM) and is two-fold more selective for MAO-B than -A (Medvedev *et al.*, 1995). Typically substitution on the indole ring enhances inhibitory potency, with isoform selectivity depending on the nature of the substituent.

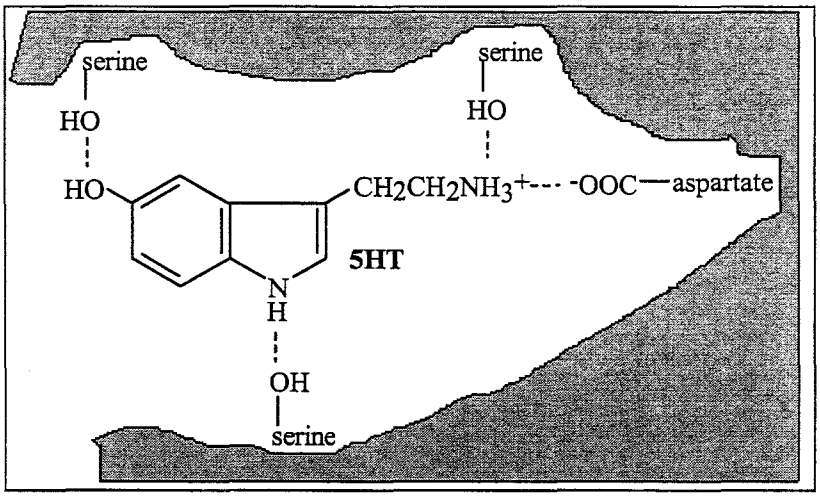


Figure 6.12: Proposed model for the substrate binding site of MAO-A.

Insertion of oxo group(s) into the indole ring, particularly at the 3-position, increases potency and selectivity towards MAO-B and not MAO-A. For example, indole-2,3-dione (isatin) > oxindole > indole. In contrast, 5-hydroxyisatin is a potent (IC_{50} value $\approx$ 8 μM) and selective inhibitor of MAO-A. Additionally, increased potency and selectivity for MAO-A required co-planar substituents at C2 of the indole ring, whereas MAO-B inhibitors require electron-donating groups in the same position (Medvedev *et al.*, 1995). Substitution of the indole ring typically produces time-independent, reversible inhibitors of micromolar potency. In contrast, ring embedding of the primary amine, as seen in β-carbolines or the pyrazinocarbazole derivatives, produces competitive, reversible inhibitors of nanomolar or low micromolar potency (Udenfriend *et al.*, 1958; Ho *et al.*, 1968; Medvedev *et al.*, 1994, Kim *et al.*, 1997).

Fully aromatic β-carbolines and dihydro-derivatives are very potent MAO inhibitors with IC_{50} or K_i values ranging from 5 nM to 5 μM. In contrast, α-carbolines are relatively inactive and tetrahydro-β-carbolines are several-fold less potent than the fully aromatic compounds. The potency of these harmala alkaloids is attributed to that fact that they contain elements of the substrate tryptamine substituted in the α-indole position and at the side chain nitrogen (Udenfriend *et al.*, 1958; Ho *et al.*, 1968). Subsequent studies on purified enzyme indicate that β-carbolines inhibit both MAO-A and -B, but are more selective for MAO-A by at least two orders of magnitude (Kim *et al.*, 1997).

Typically, substitution in the benzene ring or at C-2 in the pyridine ring does not alter degree of inhibition significantly. The ring-embedded N-2 must be basic to ensure inhibition and thus acetylation of this group decreases inhibition. N-methylation of β-carbolines and dihydro-derivatives has no effect on inhibitory potency, but in general 1-methyl and 7-methoxy substituents increased potency (Kim *et al.*, 1997). In contrast, N-methylation of N-9 in the indole ring of tetrahydro-β-carboline produced a potent and competitive inhibitor of MAO-A. This is attributed to increased binding to the enzyme by van der Waals forces and hydrophobic bonding (Ho *et al.*, 1968). Thus harmine, 2-methylharminium, 2,9-dimethylharminium and harmaline were the most potent inhibitors of purified MAO-A with K_i values in the low nanomolar range. Cationic 2,9-dimethyl derivatives are also good inhibitors, in keeping with the importance of the positive charge in binding to MAO-A (Kim *et al.*, 1997).

The micromolar range of inhibitory potencies of the indoles tested here are consistent with that seen for substitutions on the indole nucleus without ring-embedding of the free amine. Only 5MT, 5MTOH and tryptamine approached the potency of ring-embedded indoles. Certain parallels can be drawn with the results obtained in the present study. The following represents the main features arising from consideration of the SAR of the tested indoles :

(i) tryptamine was more potent than 5HT.

(ii) 5-methoxyindoles were more potent inhibitors than their corresponding 5-hydroxyindoles: 5MT > 5HT, MEL > NAS, and 5MTOH > 5HTOH

(iii) acetylation of the free amino group in the side-chain decreases inhibitory potency, possibly by steric hindrance.

(iv) substitution of the free amino group with an alcohol group (CH_2OH) enhanced inhibitory potency, whereas a carboxyl group (COOH) markedly reduced potency.

Tryptamine is a non-selective substrate, common to both MAO-A and -B (Fowler *et al.*, 1978; Garrick and Murphy, 1982). 5-Hydroxylation of tryptamine to 5HT produces a shift in selectivity toward MAO-A, similar to that seen for 5-hydroxyisatin. It is has been proposed that 5-hydroxylation of tryptamine or isatin induces a more favourable fit in the active site. Alternatively, if the indole nucleus binds to an electron-poor locus of the enzyme in a charge transfer complex, an electron-donating group such as an hydroxyl may enhance binding (Ho *et al.*, 1968; Medvedev *et al.*, 1994). On the basis of the 5HT receptor model, however, the 5′-hydroxyl group of 5HT would be expected to interact with an electron rich area, namely a serine residue. In the present study, tryptamine was more potent than 5HT and O-methylation of the 5′-hydroxyl group to produce 5-methoxyindoles increased inhibitory potency. For example, 5MT > 5HT >

tryptamine. This would suggest that inhibitory potency is related to the degree of hydrophobicity. This creates the problem that MAO-B is known to preferentially deaminate apolar substrates. The results presented in section 5.3 established that the characteristics of the present MAO enzyme are consistent with MAO-A. These studies were performed on a crude enzyme preparation with MAO still inserted in the mitochondrial membrane. One suggestion would be that this environment may have differentially influenced the availability of the substrates.

However, studies with purified MAO-A have confirmed that methoxy substituents can increase the inhibitory potency of β-carbolines by several-fold, depending on the position of the substituent. Pinoline, a 6-methoxy-1,2,3,4-tetrahydro-β-carboline, is a very weak inhibitor (Ho *et al.*, 1968). The 6-methoxy group of harmala alkaloids is equivalent to the 5-methoxy group of the indoles tested here. In contrast, 6-methoxylated and 7-methoxylated β-carbolines and dihydro-β-carbolines are potent inhibitors, although the 7-methoxylated derivatives are more potent. Thus site-specific methylation also increased inhibitory potency (Kim *et al.*, 1997). The bulky and hydrophobic pyrazinocarbazole derivatives pirlindole and tertindole are also very potent and selective inhibitors of MAO-A. Typically, they have IC_{50} values in the nanomolar range for MAO-A and in the micromolar range for MAO-B (Medvedev *et al.*, 1994). Thus it is proposed the increased potency of 5-methoxyindoles for striatal MAO-A may be attributed to one or more of the following factors: van der Waals forces and hydrophobic binding; higher electron-donating potential of the 5-methoxy group compared to the 5-hydroxy group; and finally, an improved induced fit of the molecule in the active site due to the bulkier 5-methoxy group.

The other SAR noted in this study are consistent with the $5HT_{2A}$ receptor model (Sealfon *et al.*, 1997; Wang *et al.*, 1993). Acetylation of the free amine will interfere with co-ordination with the aspartate and serine residues due to steric hindrance. The alcohol group of 5HTOH can still interact with the serine residue that normally interacts with the free amine. In contrast, repulsion will occur between the anionic carboxyl groups of 5HIAA and the aspartate residue, both deprotonated at physiological pH. The results for 5HTOH suggest that this deaminated indole product may control MAO-A *in vivo* by feedback inhibition. 5HTOH is normally the minor reaction product of MAO-A, compared to 5HIAA, and was a weak, reversible inhibitor *in vitro*. However, a change in the oxidative state (NAD^+/NADH ratio) of the tissue micro-environment or relative activities of alcohol dehydrogenase and aldehyde dehydrogenase may favour greater production of 5HTOH.

Ultimately, SAR modeling for MAO will be limited until the 3-dimensional conformation of the substrate binding site can be ascertained from X-ray crystallography data on the purified enzyme (Singer and Ramsay, 1995). Presently, photoaffinity labeling and site-directed mutagenesis has indicated that important structural differences must exist between the substrate binding sites of MAO-A and MAO-B (Chen *et al.*, 1987; Hsu and Shih, 1988; Wu *et al.*, 1993). In this regard, it would have been informative to determine the IC_{50} of each pineal indole for MAO-B using [^{14}C]phenethylamine as the substrate. A comparison with the present IC_{50} values for MAO-A would give an idea of the selectivity of an indolic inhibitor for each MAO isoform. Future studies should also aim to determine the inhibition dissociation constant (K_i), rather than the IC_{50} values, and the nature of the inhibition (e.g. competitive, noncompetitive, uncompetitive or mixed competitive inhibition). In the present setup, the use of a substrate concentration considerably lower than the K_m value would allow the detection of competition inhibition, but cannot establish for certain the nature of inhibition. Competitive inhibitors do not display time-dependency of inhibition. Thus, with regards to the nature of inhibition, the present results can only say for certain that 5MTOH was not a competitive inhibitor.

Unfortunately, the lower substrate concentration utilized in the present study does not permit comparison of the present IC_{50} values with those of other indolic inhibitors of MAO, such as the indole nucleus, isatin or β-carbolines. For monosubstrate reactions, the IC_{50} values of a competitive inhibitor depend on substrate concentration and can only be compared when calculated under identical assay conditions. Furthermore, the IC_{50} value can only be correlated with K_i when the substrate concentration is several-fold higher than the K_m. The same argument also applies for noncompetitive and uncompetitive inhibitors: the IC_{50} value is only independent of substrate concentration when the latter is in excess of K_m (Cheng and Prusoff, 1973). To circumvent this problem it would have been ideal to test a known indolic inhibitor of MAO, such as harmine, under the identical assay conditions employed here.

During the time-dependency studies with 5MT and tryptamine, it was noted that the IC_{50} values were higher after 60 min preincubation compared to 5 min. This is indicative of less inhibition with time and this was particularly apparent at low concentrations. A time-dependent inhibitor would typically show greater inhibition following a prolonged preincubation, as seen for 5MTOH. Preincubation means that the test drug was added prior to the substrate, namely [^{14}C]5HT. Tryptamine is also a substrate, and thus can be metabolized by MAO-A during the preincubation period. This decreases the ability of tryptamine to subsequently compete with [^{14}C]5HT, especially at low concentrations of the test indole. A similar pattern was not seen with cold 5HT, due to the

high IC_{50} value of this indoleamine. Thus if the presence of this temporal pattern can be used to delineate substrates, it is proposed, on the basis of these preliminary findings, that 5MT may be a novel substrate of MAO.

5MT is simply an O-methylated derivative of 5HT with a primary amine freely accessible for oxidative deamination. Due to the structural similarity to 5HT, a similar reaction scheme is postulated here for 5MT (Figure 6.13). Briefly, it is proposed that oxidative deamination of 5MT produces 5-methoxyindoleacetaldehyde (5MIAL). This intermediate is then further oxidized to 5MIAA or reduced to 5MTOH respectively by aldehyde dehydrogenase or alcohol dehydrogenase. This scheme would require that the two latter coupled enzymes can utilize 5MIAL as a substrate. If conclusive proof can be obtained that 5MT is a novel MAO substrate, this has some important implications. Firstly, it strengthens the above argument that 5-methoxylation may enhance the affinity of both indole substrates and inhibitors for MAO-A. Secondly, if the proposed reaction is accepted, catabolism of 5MT may explain a novel and alternative biosynthetic origin of 5MIAA and 5MTOH in the pineal gland. 5MIAA and 5MTOH are currently believed to arise through the O-methylation of 5HIAA and 5HTOH, respectively, by HIOMT. Oxidative deamination of 5MT by MAO may represent an alternative source of these indoles. The relative production of 5MIAA and 5MTOH by HIOMT and MAO may then also depend on the affinity of 5HIAA and 5HTOH for HIOMT *vs.* the affinity of 5MT for MAO.

The presence of several potential MAO inhibitors in the pineal is likely to have implications for the modeling of pineal indole biosynthesis. As discussed in section 3.4, one disadvantage of the *in situ* radiometric pineal organ culture technique is the build up of radiolabeled indoles in the culture medium. Some evidence has been presented for a negative feedback control of pineal indole biosynthesis by the secreted indoles, especially at the level of HIOMT (Trentini *et al.*, 1982; Morton, 1990; Yanez and Meissl, 1995). The current results suggest that MAO activity may represent a further target of paracrine control of the pineal gland. Thus it is interesting that the nocturnal elevation in the pineal levels of MEL, shown here to be a MAO-A inhibitor, is correlated with decreased MAO activity in the pineal during the scotophase compared to the photophase when MEL biosynthesis is basal (see Figure 3.3). The endogenous MAO-A inhibitor Tribulin is also postulated to be responsible for the increases in MEL biosynthesis associated with stress or anxiety (Oxenkrug and McIntyre, 1985).

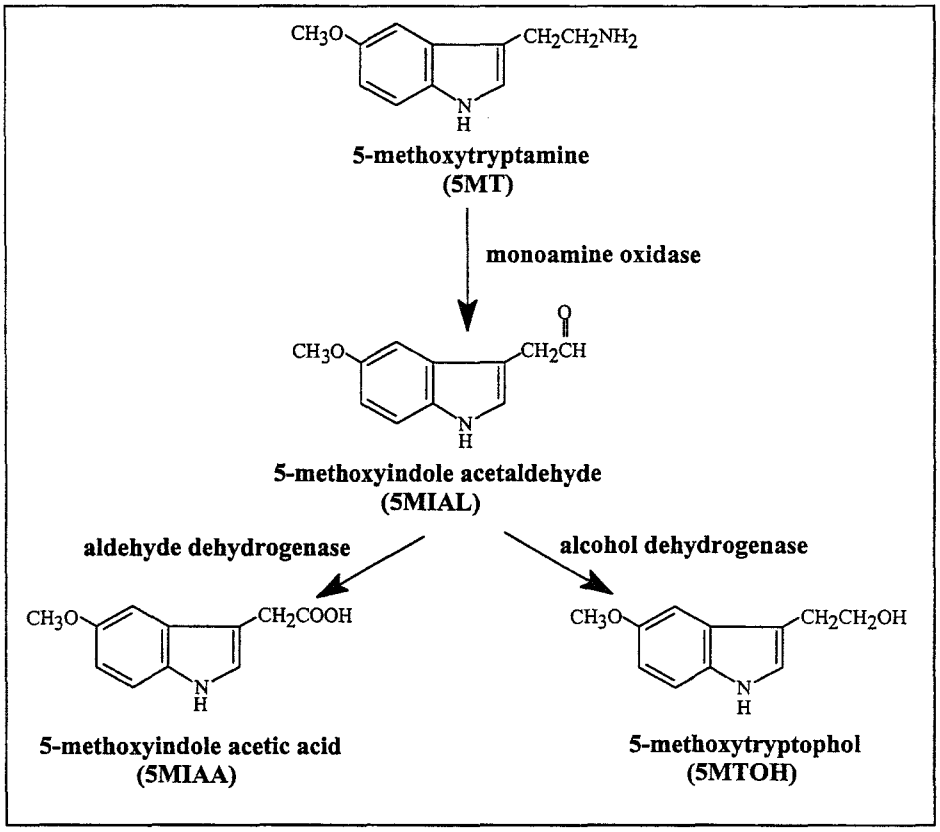


Figure 6.13: Proposed reaction scheme for 5MT as a MAO substrate.

In summary, MEL is a time-independent, reversible inhibitor of MAO-A *in vitro*, although a relatively weak one. The extension of this finding to endogenous MEL could have enormous consequences for understanding the neurochemical effects of this hormone. For example, MAO-A *in vivo* selectively deaminates 5HT and NA (Johnston, 1968; Garrick and Murphy, 1982). For this reason, the new generation of antidepressants are based on a class of reversible inhibitors of MAO-A (known as R.I.M.As) that include moclobemide (Aurorix®) and brofaromine. On the basis of the present results, MEL could be assigned to this class of antidepressants. However, phototherapy and studies with MEL antagonists and other classes of antidepressants (e.g. NA or 5HT selective reuptake inhibitors) suggest that MEL may actually worsen the clinical picture of depression (Lewy *et al.*, 1987; Miles and Philbrick, 1988; Dubocovich *et al.*, 1990; Waldhauser *et al.*, 1993).

Future studies also need to ascertain whether MEL can inhibit MAO-B. In primates, including humans, DA is selectively metabolized by MAO-B in the basal ganglia rather than MAO-A, unlike the rat brain (Garrick and Murphy, 1982; Grimsby *et al*, 1990; Naoi and Maruyama, 1993; Juorio *et al.*, 1994). MAO-B activity is one of the most important factors involved in the nigro-striatal cell death associated with Parkinson's disease (Naoi and Maruyama, 1993). Firstly, by catabolizing DA, the enzyme contributes to the progressive loss of this catecholamine, which is a hallmark of the disease pathogenesis. Secondly, MAO is the prime generator of hydrogen peroxide in the basal ganglia, which can be converted to cytotoxic hydroxyl radicals in the presence of transition metals by the non-enzymatic Fenton reaction. Ferrous iron is abundant in the substantia nigra pars compacta and thus a current hypothesis attributes the selective neurodegeneration of these DA neurons to oxidative stress induced by toxic radicals (Greenfield, 1992). Surviving neurons are believed to compensate for the neuronal death by increasing the release of DA, which has an extra-synaptic distribution. This excess DA is then further catabolized or auto-oxidized in the presence of hydrogen peroxide to the selective dopaminergic neurotoxin 6OHDA. Similarly, MAO-B, in particular, is responsible for the bioactivation of several other DA-selective neurotoxins, including MPTP and isoquinolines (Greenfield, 1992; Naoi and Maruyama, 1993). MAO-B converts MPTP to 1-methyl-4-phenylpyridinium ion (MPP^+), the true toxic species of the neurotoxin, which induces nigro-striatal cell death through a radical mechanism. In turn, MPP^+ and several analogs are reversible, competitive product inhibitors of both MAO-A and MAO-B (Singer and Ramsay, 1995).

The postulated role of MEL as a neuroprotectant in neurodegenerative diseases such as Parkinson's disease has mostly been attributed to the radical scavenging and anti-oxidative properties of the hormone (Reiter *et al.*, 1995). For example, Acuña Castroviejo *et al.* (1996) found that MEL is protective against MPTP-induced striatal and hippocampal lesions and the authors contributed this to MEL's ability to scavenge hydroxyl radicals. Alternatively, it is equally feasible that this effect was due to an inhibition of MAO, and thus the bioactivation of MPTP, by MEL. The MAO-B selective inhibitor L-deprenyl has been shown to be an effective therapeutic agent in Parkinson's disease (Singer and Ramsay, 1995). The neuroprotective effects of this inhibitor have been attributed to a slowing of the progressive loss of DA and a reduced production of reactive oxygen species and 6OHDA. In the light of the present studies, a similar argument could be made for MEL.

Chapter 7

Catechol-*O*-methyltransferase: Characterization and Optimization

7.1 Introduction

COMT activity of the rat corpus striatum was quantified by an *in vitro* micro-radioenzymatic assay modified from McCaman (1965), Parvez and Parvez (1973) and Zürcher and Da Prada (1982).

7.1.1 Principle of COMT Assay

The principle of the assay is based on a direct aqueous-organic solvent extraction system to separate the radiolabeled reactants and products. The reaction scheme is outlined in Figure 7.1. Briefly, using [^{14}C]SAM as the methyl donor, the exogenous substrate DA is O-methylated by Mg^{2+}-dependent COMT to produce [^{14}C]3MT and the co-product S-adenosylhomocysteine (SAH). Alkalization of the reaction mixture with borate buffer (pH 10.0) terminates the enzyme reaction and deprotonates the free amino group of 3MT. Thus [^{14}C]3MT becomes nonpolar, whereas [^{14}C]SAM remains charged. This allows for efficient extraction of [^{14}C]3MT with toluene:isoamyl alcohol (3:2 v/v), with minimal extraction of [^{14}C]SAM. The analyte is then quantified by liquid scintillation spectroscopy. Although [^{14}C]3MT is the predominant product, other minor O-methylated products may also be formed (e.g. 4-methoxytyramine) and extracted. Furthermore the presence of [^{14}C]3MT in the analyte was not verified. Thus the term [^{14}C]O-methylated product formation was employed to describe COMT activity.

Several HPLC techniques, coupled to ultraviolet (UV), fluorometric, radiochemical or electrochemical detectors, and gas chromatography techniques have been employed more recently for the assay of COMT activity (Tilgmann and Ulmanen, 1996). These techniques have improved the sensitivity and specificity of analysis, allowing the simultaneous quantification of substrates and all O-methylated products. However, these techniques can only be applied to relatively pure samples following a clean-up step (e.g. deproteinization, removal of endogenous catechols). Furthermore, in the case of electrochemical detectors, chemical treatment of the O-methylated products may be required to allow detection (Tilgmann and Ulmanen, 1996). The limit of sensitivity for HPLC techniques is adequate for analysis of COMT activity from tissue which

contain large amounts of the enzyme (e.g. the liver), but is often deficient for the low activity of COMT in the brain. Thus the increased sensitivity of radiochemistry and the ease of solvent extraction was opted for the analysis of striatal COMT activity. Furthermore, the use of DA as the exogenous substrate allows for the modeling of the potential fate of endogenous DA in the corpus striatum.

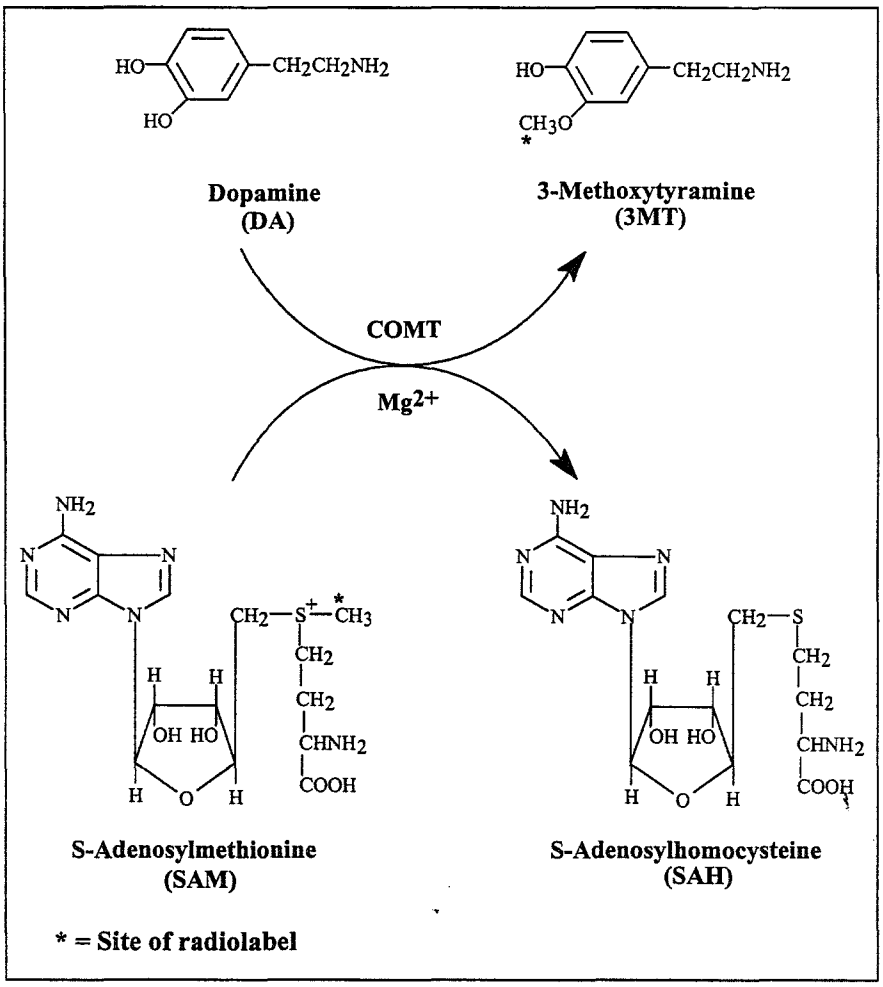


Figure 7.1: Reaction scheme for the *in vitro* radioenzymatic COMT assay.

This chapter assessed the applicability of the radioenzymatic assay described here to the analysis of COMT activity of the rat corpus striatum. Of particular importance was the flexibility of the assay for comparison between *in vitro* and *in vivo* studies performed on small quantities of tissue sample. This required full characterization of the COMT enzyme and optimization of the assay design. The ability of MEL to modulate striatal COMT activity *in vitro* was also investigated.

7.2 Materials and Methodology

7.2.1 Chemicals and Reagents

The radiochemical S-adenosyl-L-[methyl-^{14}C]methionine (SAM; specific activity 56 mCi/mmol) was obtained from Amersham International (Amersham, UK). DA HCl and MEL were purchased from Sigma. Analytical grade toluene and isoamyl alcohol, and $MgCl_2 \cdot 6H_2O$ were obtained from UniVar® (Saarchem-Holpro Analytic, South Africa), SAM hydrogen sulphate from Boehringer Mannheim, and 3,5-dinitrocatechol (OR-486) from RBI (USA). All other chemicals and reagents were purchased from local commercial sources and were of the highest purity available. All buffers and reagents were prepared in MilliQ water.

7.2.2 Tissue Preparation and Storage

Unless otherwise stated, all COMT optimization studies were performed on tissue samples prepared from the striata of animals sacrificed at mid-photophase (12h00). The striata were dissected, pooled and stored as described in section 2.2. Stability studies showed that whole tissue or the cytosolic/microsomal fraction in 0.32 M sucrose can be stored at -20 °C or -70 °C for several months with minimal loss of COMT activity. This is consistent with the fact that the presence of reducing agents, such as cysteines, markedly stabilize the COMT enzyme during purification (Tilgmann and Ulmanen, 1996).

7.2.3 Assay Reaction Mixture

All reagents were prepared in the assay buffer, 50 mM Tris-HCl (pH 7.9). This buffer was chosen as it exhibits negligible binding of Mg^{2+}. A standard reaction mixture, with a total volume of 500 µl, comprised in the following order: 145 µl assay buffer, 50 µl $MgCl_2$ (100 mM stock solution), 100 µl DA (5 mM stock solution), 200µl enzyme fraction, and 5 µl [^{14}C]SAM stock solution (0.025 µCi / assay tube). This gives a final concentration of ± 893 nM (0.45 nmoles) for [^{14}C]SAM.

7.2.4 Assay Procedure

Briefly, all components, except [^{14}C]SAM, were prepared in a glass extraction vial on ice in the order listed above. The reaction mixture was then preincubated for 10 min in a 37 °C water bath, with gentle shaking, to allow thermal equilibration. The reaction was initiated by the addition of 5 µl [^{14}C]SAM and incubated at 37 °C for 60 min. The reaction was terminated by addition of

500 µl ice-cold 50 mM borate-KCl buffer (pH 10). After 15 min, this was followed by the addition of 3 ml toluene:isoamyl alcohol [3:2 v/v] and vortexed for 60 s. Samples were then centrifuged at 3000 rpm for 10 min [Selecta Mixtasel benchtop centrifuge] and 2.4 ml of the solvent extracted and quantified by liquid scintillation spectroscopy. A zero-time blank was created by adding the borate buffer prior to the [^{14}C]SAM. A further control involved determining activity in the absence of exogenous substrate and was designated as "no substrate" activity.

7.2.5 Experimental Design

The composition of the reaction mixture represents optimum values that were experimentally determined as described below. Typically, one component (the dependent variable) was allowed to vary, while the independent variables were fixed at the optimum values. The volume of assay buffer was adjusted to compensate for any changes in the composition of the reaction mixture. By necessity, each experiment is presented in a logical linear fashion, but the design of one experiment was dependent on the results of several other experiments.

(i) Subcellular Distribution Studies

The following subcellular fractions were prepared as described in section 2.3: 5% (w/v) total homogenate, 5% (w/v) nuclei/cell debris, 4% (w/v) mitochondria/lysosomes and 5% (w/v) cytosol/microsomes. COMT activity and specific activity of each fraction was determined using 200 µl aliquots under conditions of maximal induction (1 mM DA and 10 mM $MgCl_2$). All subsequent experiments were performed on the 5% (w/v) cytosolic/microsomal fraction.

(ii) Linearity with Enzyme Content

COMT activity was determined for 50, 100, 150, 200 and 250 µl aliquots of a 5% (w/v) cytosolic/microsomal fraction.

(iii) Linearity with Assay Incubation Time

The time-dependency of COMT specific activity was determined by terminating the assay reaction at fixed time intervals of 0, 15, 30, 45, 60, 75 and 90 min.

(iv) Kinetic Analysis: Dopamine

Substrate kinetics of COMT were determined by varying DA to give final concentrations of 0.05, 0.1, 0.5, 1.0, 2.0, 3.0 and 5.0 mM. The Michaelis-Menten kinetics were further analysed by the Hanes-Wolf plot (S/V *vs.* S), where the substrate (S) is the DA concentration (mM) and V is the enzyme activity (DPM/60 min/500 μg protein).

(v) Mg^{2+}- dependency

The induction of COMT activity by Mg^{2+} was investigated by varying $MgCl_2$ to give final concentrations of 0.0, 0.5, 1.0, 2.0, 5.0, 10.0, 15.0, 20.0 and 30.0 mM.

(vi) Kinetic Analysis: SAM

Co-factor kinetics were analyzed through competition between non-radioisotopic SAM and [^{14}C]SAM. The final concentration of cold SAM was varied from 10^{-3} to 10^{-4} M and the concentration of [^{14}C]SAM fixed at 893 nM.

(vii) Inhibition by OR486

The inhibitory potency of OR486 was determined *in vitro* with respect to a near-saturating concentration of DA (1 mM). An inhibition profile was generated by varying the final concentration of OR486 from 10^{-3} to 10^{-10} M. The preincubation period with OR486 was limited to the thermal equilibration period of 5 min.

(viii) Inhibition by Melatonin

The potential *in vitro* inhibitory effect of MEL on basal and Mg^{2+}-induced COMT activity was determined with respect to a sub-saturating concentration of DA (200 μM). This concentration approximates the apparent K_m value ≈ 185 μM calculated from Figure 7.5. $MgCl_2$ was present at a final saturating concentration of 10 mM to allow maximal induction. Basal activity was determined in the absence of exogenous $MgCl_2$. A stock solution of MEL was prepared in assay buffer and ethanol such that the highest final concentration of ethanol in the assay reaction mixture was 0.2% (v/v). MEL was added to give final concentrations of 0.25, 0.50, 0.75 and 1.0 mM. Control activity was determined in the presence of 0.2% (v/v) ethanol. MEL was preincubated with the enzyme for 30 min prior to the initiation of the reaction.

7.2.6 DATA AND STATISTICAL ANALYSIS

The DPM data was normalised for 3 ml of the extraction solvent and corrected for the "no substrate" activity. COMT activity is expressed as [^{14}C]O-Methylated product formation and is represented by the units DPM/60 min. Specific activity is expressed as DPM/60 min/500 μg protein. Data is graphically presented as mean ± SEM with the sample size (n) depending on the experiment. IC_{50} values were calculated from inhibition curves and Dixon plots as outlined in section 5.2.6. Data was converted to the reciprocal of each mean value, (DPM/60 min/500 μg protein)$^{-1}$, for the Dixon plot. Linear and non-linear regressions were performed as described in section 2.7.

7.3 Results

(i) Subcellular Distribution Studies

The subcellular distribution of COMT activity is presented in Figure 7.2. The majority of COMT activity and specific activity was observed in the cytosolic/microsomal fraction, with comparable levels found only in the total homogenate. Only trace amounts of activity were detected in the nuclear/cell debris and mitochondrial/lysosomal fractions. The crude cytosolic/microsomal fraction represents a "purer" enzyme preparation than the total homogenate, giving a truer reflection of COMT specific activity. The 5% (w/v) cytosolic/microsomal fraction was used for all subsequent COMT assays.

(ii) Linearity with Enzyme Content

Figure 7.3 shows a linear relationship (r^2 = 0.9915, P < 0.0001) between COMT activity and homogenate volume over the range tested for a 5% (w/v) cytosolic/microsomal fraction. An aliquot of 200 μl was chosen as the standard homogenate volume for all subsequent studies.

(iii) Linearity with Assay Incubation Time

The time-dependency of COMT specific activity (Figure 7.4) was linear for the first 45 min of the assay reaction (r^2 = 0.973, P = 0.0135) and tapered off by 60 min (non-linear r^2 = 0.997). A plateau was observed between 60 and 75 min, with a substantial decrease in activity by 90 min. A fixed-time of 60 min was chosen as the standard reaction time for all COMT assays.

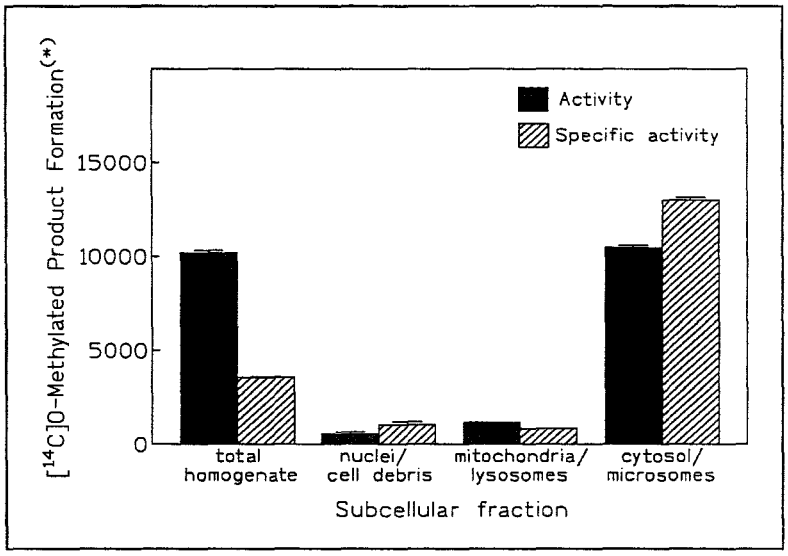


Figure 7.2: Subcellular distribution of striatal COMT activity. [Data represents mean ± SEM (n = 4); *: Activity = DPM/60 min, Specific activity = DPM/60 min/500 μg protein]

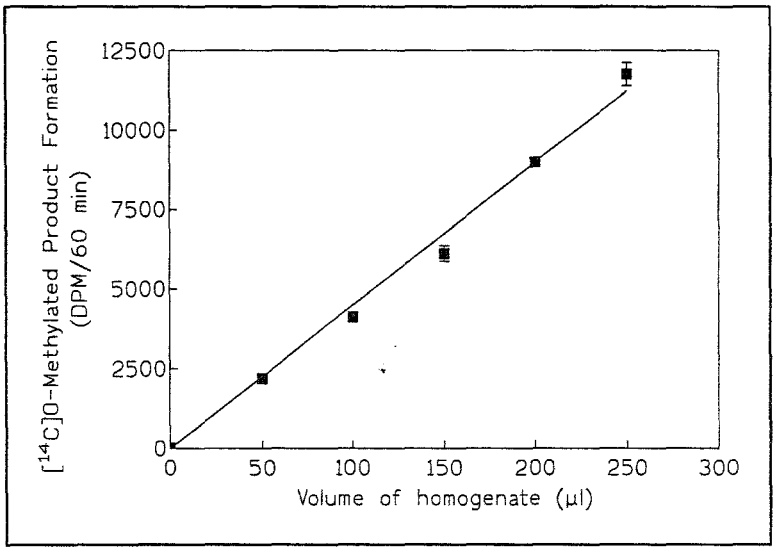


Figure 7.3: COMT activity as a function of enzyme content. [Data represents mean ± SEM (n = 4)]

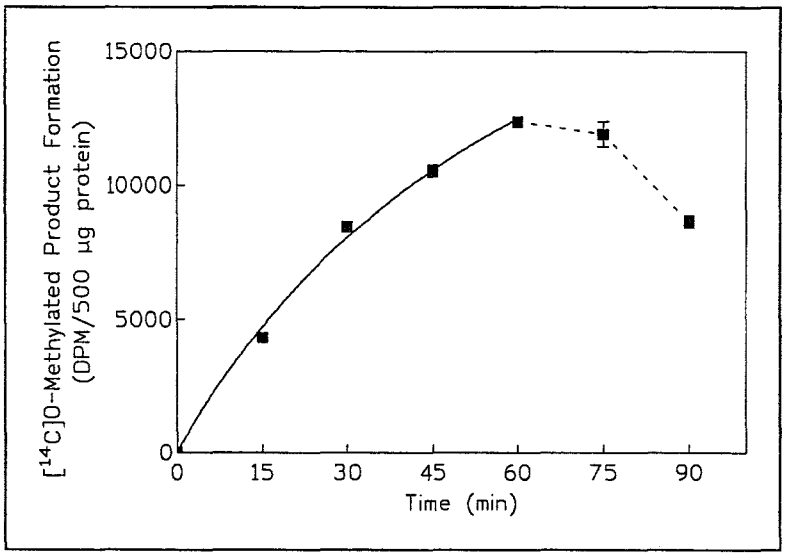


Figure 7.4: COMT activity as a function of assay incubation time. [Data represents mean ± SEM (n = 4)]

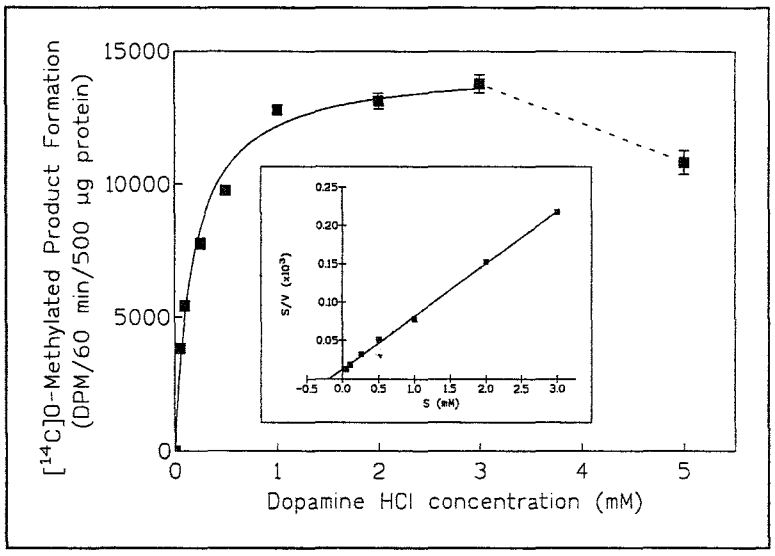


Figure 7.5: COMT activity as a function of DA concentration. ***Insert*****: Hanes-Wolf plot.** [Data represents mean ± SEM (n = 4)]

(iv) Kinetic Analysis: Dopamine

The relationship between DA concentration and COMT specific activity is presented in Figure 7.5. A classic monophasic binding isotherm ($r^2 = 0.988$), consistent with a single class of sites, was obtained. Near-saturating conditions were obtained between 1 and 3 mM DA, with a substantial decrease in activity seen at 5 mM DA. Kinetic analysis of the saturation curve and the Hanes-Wolf plot (Figure 7.5 Insert; $r^2 = 0.999$, $P < 0.0001$) produced an apparent K_m value $\approx$ 185 μM. Unless otherwise stated, a near-saturating concentration of 1 mM DA was employed as the standard substrate condition.

(v) Mg^{2+}- dependency

Some minimal activity with 1 mM DA was observed in the absence of exogenous $MgCl_2$. This was attributed to basal, Mg^{2+}-independent activity or due to induction by trace amounts of endogenous Mg^{2+} present in the crude enzyme fraction. Data was accordingly corrected for this "background" activity. A biphasic response to Mg^{2+} concentration was observed (Figure 7.6). Initially, the "dose-response" curve increased non-linearly ($r^2 = 0.994$) in a monophasic fashion with maximal induction occurring at 10 and 15 mM $MgCl_2$. Higher concentrations of $MgCl_2$ (20 and 30 mM) resulted in inhibition of COMT activity. A saturating concentration of 10 mM $MgCl_2$ was used for all COMT assays to allow maximal induction.

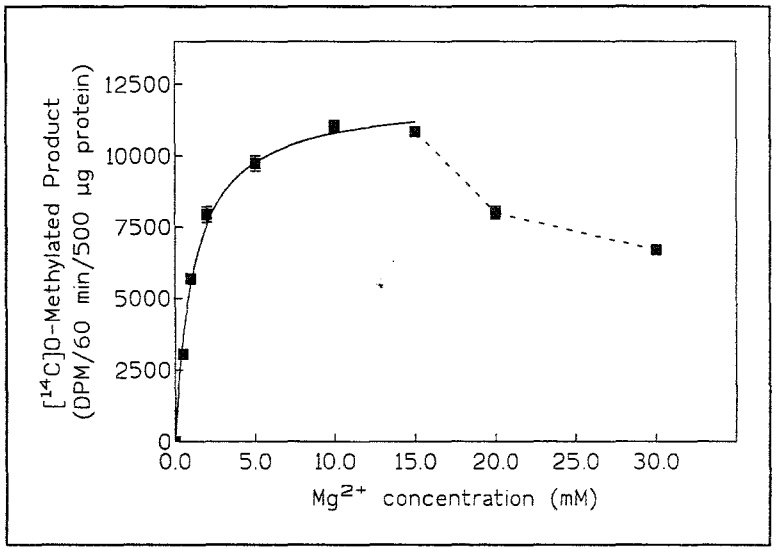


Figure 7.6: Mg^{2+}-dependency of COMT activity. [Data represents mean ± SEM (n = 4); Data was corrected for basal activity in the absence of Mg^{2+}]

(vi) Kinetic Analysis: SAM

Competition by non-radioisotopic SAM was evident as a monophasic sigmoidal curve (r^2 = 0.997), consistent with a single class of binding sites (Figure 7.7). This generated an IC_{50} value ≈ 4.5 μM. Similarly, the Dixon plot (Figure 7.7 Insert; r^2 = 0.9676, P = 0.0025) produced an IC_{50} value ≈ 7 μM.

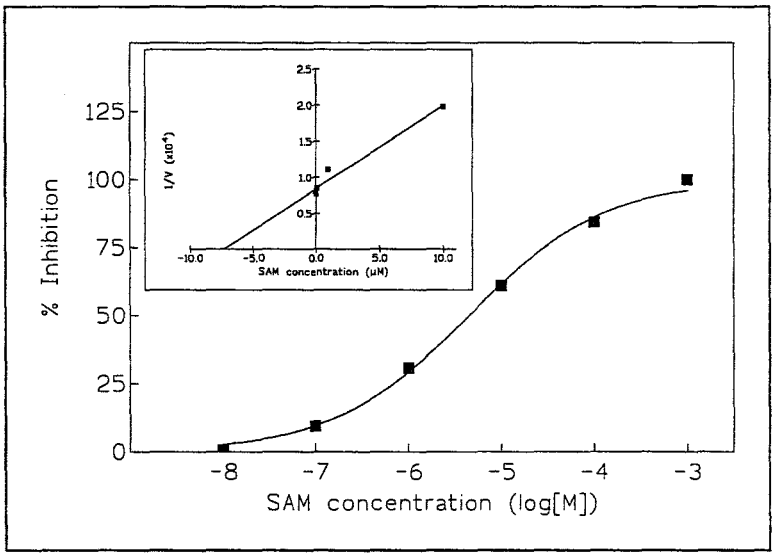


Figure 7.7: The effect of varying concentrations of non-radioisotopic SAM on COMT activity. *Insert*: Dixon Plot.

(vii) Inhibition by OR486

The structure of OR486 (3,5-dintrocatechol) is shown in Figure 7.8. An *in vitro* inhibition profile for OR486 is presented in Figure 7.9. A typical monophasic sigmoidal curve (r^2 = 0.994) was obtained, consistent with a single class of sites. Analysis of the sigmoidal curve and the Dixon plot (Figure 7.9 Insert; r^2 = 0.9999, P < 0.0001) generated an IC_{50} value ≈ 35-40 nM.

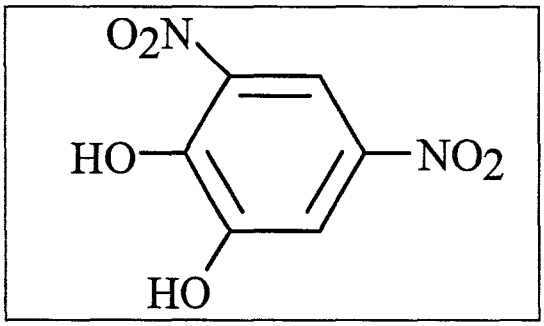


Figure 7.8: Structure of OR486 (3,5-dintrocatechol).

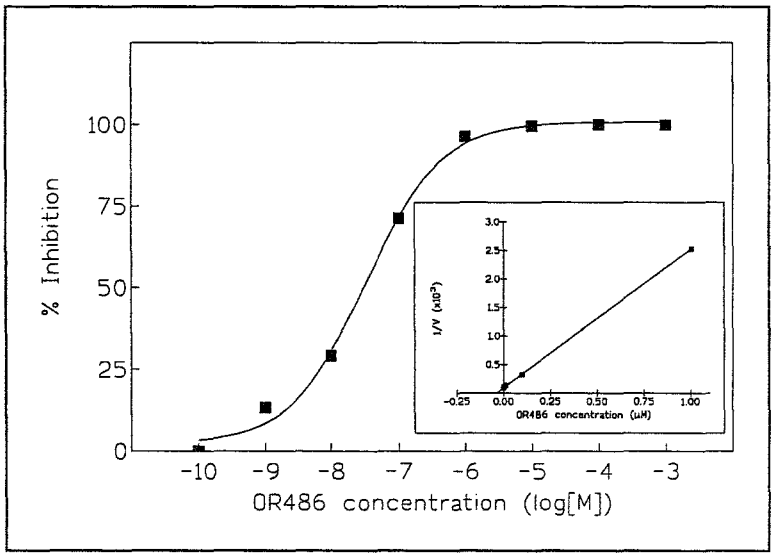


Figure 7.9: Inhibition of COMT by OR486. *Insert*: Dixon Plot. [Data represents mean ± SEM (n = 5)]

(viii) Inhibition by Melatonin

MEL (0.0 – 1.0 mM) *in vitro* had no effect on basal (Mg^{2+}-independent) or Mg^{2+}-induced COMT activity (Figure 7.10) with respect to subsaturating DA (200 μM), following a 30 min preincubation period. Since control activity was not sensitive to 0.2% (v/v) ethanol (data shown), the control is depicted as the vehicle-treated activity.

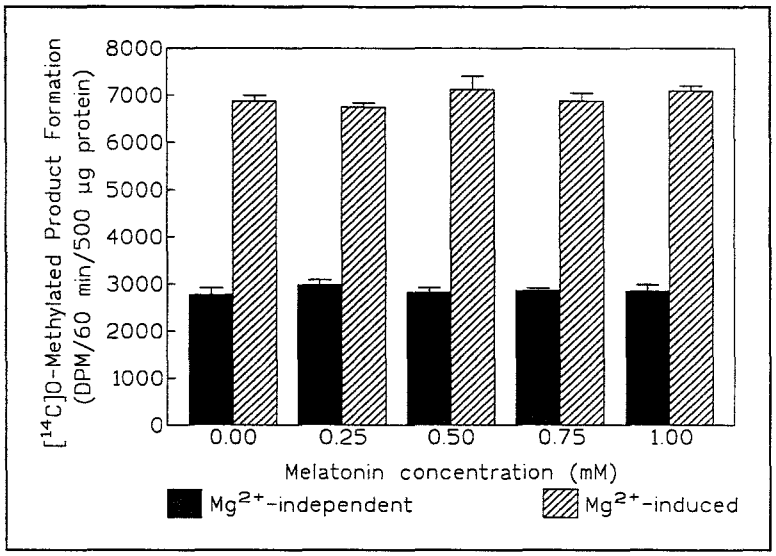


Figure 7.10: The *in vitro* effect of MEL on basal and Mg^{2+}-induced COMT activity. [Data represents mean ± SEM (n = 5)]

7.4 Discussion and Conclusion

Cell fractionation and immunohistochemical studies of the rat brain have clearly show that MB-COMT is localized to plasma membranes and/or RER, whereas S-COMT is distinctly cytosolic (Tilgmann *et al.*, 1992; Karhunen *et al.*, 1995a and 1995b; Lundström *et al.*, 1995). The results of the subcellular fractionation study presented here (Figure 7.2) are consistent with this profile. The majority of COMT activity was found in the cytosolic/microsomal fraction following differential centrifugation. This fraction contains the soluble fraction arising from the cytosol, including soluble proteins and low density organelles such as plasma membranes and RER. Thus it is proposed that this fraction contains both MB- and S-COMT. The similar activity obtained in the total homogenate and the cytosol/microsomal fraction is indicative of the efficiency of the homogenization and fractionation process with no apparent loss of important co-factors *inter alia*. The trace amounts of activity seen in the nuclear/cell debris and mitochondrial/lysosomal fractions probably represent "contaminants". Grossman *et al.* (1985) claimed that COMT was associated with the outer mitochondrial membrane, but this has been negated by a more sophisticated fractionation study (Tilgmann *et al.*, 1992). Some immunoreactivity to MB-COMT was also

observed along the outer mitochondrial and nuclear membranes by Karhunen *et al.* (1995a; 1995b), but the authors argue that this may simply reflect diffusion of the reactive product.
All further studies were performed on a 5% (w/v) cytosol/microsomal fraction, which gives a truer reflection of specific activity. Subsequently, it must be noted that COMT activity will refer to a combination of both MB- and S-COMT, without reference to the relative contribution of each isoform. MB-COMT is believed to be the neuronally important isoform. However, preliminary studies (data not shown) showed very low activity of MB-COMT in the microsomal fraction relative to the amount of tissue employed. For this reason, it was believed that the additional isolation steps that would be required were not warranted. This simple, but rapid homogenization-fractionation protocol provides an adequate and consistent source of both COMT isoforms, with minimal enzyme loss and variability between striatal samples. This is important for comparison between samples, especially with regards to *in vivo* studies.

The *in vitro* micro-radiometric assay designed and employed here conforms to the following criteria: specificity; linearity with striatal enzyme content; linearity with reaction time; and saturable kinetics with respect to substrate and co-factors. The results also confirm that the enzyme being assayed in the cytosolic/microsomal fraction is COMT. Kinetic analysis of the substrate DA revealed a $K_m \approx 185$ μM. Reportedly, MB-COMT has a several-fold higher affinity for substrates than S-COMT (Rivett *et al.*, 1983; Lotta *et al.*, 1995; Borges *et al.*, 1998). MB-COMT is capable of metabolizing low concentrations of DA and thus is considered the neuronal and functionally important isoform in the striatum (Tenhunen and Ulmanen, 1993). The present study with DA produced a monophasic Michaelis-Menten plot (Figure 7.5) consistent with a single class of sites. It is possible that the concentration range employed did not allow for resolution of the differential affinity of the two COMT isoforms for DA, masking the contribution of MB-COMT. However, MB-COMT is known to represent a minor proportion of the total enzyme. Indeed, as stated above, preliminary studies found very low COMT activity in the microsomal fraction of striatal tissue. Thus the present K_m value for DA is attributed to S-COMT. This also indicates that the latter isoform is abundant in the striatum and may actually be important in regulating dopaminergic neurotransmission. The IC_{50} value for cold SAM (*ca.* 5-7 μM) is consistent with the finding that a final concentration of 300-500 μM SAM is typically saturating for COMT (Byrne and Tipton, 1996; Borges *et al.*, 1998). Although the fixed concentration of [^{14}C]SAM (*ca.* 890 nM) employed was sub-saturating, the results obtained here indicate this concentration was not limiting due to the low activity of striatal COMT.

A decrease in COMT activity was observed after a prolonged reaction time and at high DA concentrations (5 mM). Possible reasons could be substrate inhibition, end-product inhibition or time-dependent inhibition by $MgCl_2$. Borges *et al.* (1998) reported that normetanephrine, the O-methylated product of NA, did not influence liver COMT activity with AD as the substrate. This in itself does not exclude the possibility that 3MT may inhibit striatal COMT with DA as the substrate. However, Borges *et al.* (1998) did observe that the demethylated product SAH inhibited the enzyme with respect to both SAM and DA.

Finally, the enzyme was potently inhibited by the selective COMT inhibitor, OR486. Catechols with electron-withdrawing substituents, particularly NO_2 groups, represent a second generation of more potent (nanomolar range) and highly selective COMT inhibitors (Byrne and Tipton, 1996). In particular, OR486 is a potent competitive inhibitor of COMT displaying tight-binding kinetics. OR486 is reported to inhibit recombinant human S-COMT and MB-COMT *in vitro* with K_i values of ± 8 and ± 24 nM respectively (Byrne and Tipton, 1996). In the present study, OR486 dose-dependently inhibited Mg^{2+}-induced COMT activity, with respect to DA, in a monophasic manner with an $IC_{50} \approx 40$ nM. The small discrepancy between this value and the published K_i values can be attributed to the following: the use of crude enzyme fraction containing both COMT isoforms; the use of near-saturating concentration of substrate, DA; and the calculation of an IC_{50} value rather than K_i. Furthermore, the low resolution of the OR486 concentration range possibly did not allow for separation of the effects on S- and MB-COMT, explaining the monophasic response observed. Under the specified conditions, MEL *in vitro* had no direct effect on basal or Mg^{2+}-induced striatal COMT activity, neither inhibitory nor stimulatory. Preincubation with MEL for 30 min excludes the possibility that MEL had time-dependent inhibitory or stimulatory effects.

In conclusion, the radioenzymatic assay employed here is sensitive, reliable, extremely reproducible, and easy to use for small quantities of striatal tissue. The assay shows that substantial Mg^{2+}-dependent COMT activity is present in the corpus striatum of the rats, which can be successfully isolated and detected. Characterization of COMT and optimization of the assay allowed for adaptation of this protocol for *in vivo* studies. Of further importance for the *in vivo* studies is that both MAO-A and COMT activity can be assayed in the same striatal sample.

Chapter 8

In Vivo Melatonin Administration Studies

8.1 Introduction

The potential modulation of striatal dopaminerigc function and behavioural output by MEL *in vivo* can be investigated by several strategies. These include assessment of circadian rhythmicity, photoperiod or light manipulation, chronotypic *in vivo* MEL administration, and pinealectomy. Results presented in sections 6.3 and 7.3 have shown that MEL is a reversible, time-independent inhibitor of striatal MAO-A activity *in vitro*, but does not have a direct *in vitro* effect on Mg^{2+}-dependent COMT activity. The potential role of MEL as an endogenous MAO-A inhibitor, as seen for the indolic derivative isatin (Yuwiler, 1990), is a strong motive for determining if MEL also regulates striatal MAO *in vivo*. Furthermore, MEL *in vivo* may have effects on COMT not seen in the *in vitro* situation.

A few previous studies have shown that the *in vivo* effect of MEL on MAO and COMT is highly tissue-specific (Urry and Ellis, 1975; Esquifino *et al.*, 1994). To date, no studies have been extended to the corpus striatum. Thus this chapter ascertained the ability of MEL to regulate striatal MAO-A and COMT by two strategies: circadian rhythmicity studies, and administration of exogenous MEL. Plasma and tissue MEL levels are circadian in nature, with peak amplitude coinciding with the scotophase (Reiter, 1991). Thus day-night variations in enzyme activity may reflect an influence of the prevailing MEL concentration. Likewise, the phase-dependent level of endogenous MEL can be altered by administering the hormone.

Several parameters need to be carefully considered in the experimental design when administering a drug *in vivo*, and this is especially true for MEL. Potential parameters include dose, route of injection (e.g. i.p. *vs.* s.c.), choice of vehicle, duration (e.g. acute, subchronic, chronic) and chronotypic effects. Chronotypism means that there is a temporal variation in the sensitivity or responsiveness of a neural substrate to a drug (Cahill and Ehret, 1981). Numerous effects of MEL show this critical dependence on the timing of the light:dark cycle. Reiter (1987) proposed two hypotheses to explain how the MEL information is decoded by target tissues. The "Duration Hypothesis" states that the duration of the nocturnal MEL signal is important, as it is proportional to the length of the scotophase. The "Internal Coincidence Hypothesis" is more informative as it

can explain the chronotypism of MEL's action. A response is only observed when the peak of MEL coincides with a "circadian window" of responsiveness to MEL. In part, this responsiveness is most likely due to variations in the density of the high-affinity MEL receptors. For example, MEL administration during the late photophase, but not the early photophase, results in gonadal regression (Tamarkin *et al.*, 1976; Reiter, 1988). A lack of response, defined as a "refractory period", would then represent a lack of coincidence of between the MEL message and the window of responsiveness. Furthermore, the duration of MEL administration is also an important variable determining the responsiveness to the hormone. For example, Tamarkin *et al.* (1976) and Moreno *et al.* (1992) report that the anti-reproductive effect of MEL is only seen after 9 weeks administration.

In this study, the plasma and tissue levels of endogenous MEL were not measured. Therefore it was not possible to ascertain whether exogenous MEL administration altered the amplitude of endogenous MEL levels and/or induced a phase-shift in the endogenous MEL rhythm. For this reason, the *in situ* organ culture technique was employed to assess the functional state of the pineal, and thus by inference, the state of endogenous MEL synthesis following MEL administration during the photophase and scotophase. Furthermore, this will allow determination of the potential of MEL to exert feedback control on its own biosynthesis by the pineal.

Numerous reports have implicated MEL in the pathology of movement disorders. The main consensus from behavioural models of DA dysfunction is that MEL exerts an anti-dopamimetic effect in the basal ganglia (Cotzias *et al.*, 1971; Burton *et al.*, 1991; Tenn and Niles, 1995). Also, the fact that APO-induced yawning is more pronounced at night is suggestive of a synergistic role for MEL in this behavioural paradigm(Nasello *et al.*, 1995). Diverse biochemical pathways are postulated to mediate the effects of MEL on DA-mediated behaviours, including, *inter alia*, the serotonergic, dopaminergic, opioidergic and GABAergic systems (Bradbury *et al.*, 1985; Gaffori and Van Ree, 1985a and 1985b; Sandyk and Fisher, 1989c; Tenn and Niles, 1995). Catalepsy is a drug-induced akinetic state commonly used as a rodent model of drug-induced Parkinsonism (Sanberg *et al.*, 1988). This state is induced by neurochemical agents that typically exert antidopamimetic effects, such as reserpine and HAL. Thus catalepsy was employed as a simple test to determine whether the proposed antidopamimetic action of MEL is manifested behaviourally. Ultimately, the behavioural responses to MEL were correlated with the effect of the hormone on pineal indole biosynthesis and striatal MAO-A and COMT activity.

8.2 Materials and Methodology

8.2.1 Chemicals and Reagents

MEL was purchased from Sigma. The chemicals and reagents for the pineal organ culture technique, MAO-A assay and COMT assay are listed in section 3.2.1, 5.2.1 and 7.2.1 respectively.

8.2.2 Animals

Male Wistar rats were housed as previously described in section 2.1. For all studies, the animals were acclimatized for 1 week to the prevailing light-dark cycle prior to commencement of the experiments.

For the *in vivo* studies, 48 rats were randomly assigned to either a "vehicle-treated" (n = 6) or a "MEL-treated" (n = 6) group prior to habituation. One of each treatment group was further assigned to the following "zeitgeber" (circadian) time periods: 08h00 (early photophase), 12h00 (mid-photophase), 16h00 (late photophase) and 24h00 (mid-scotophase).

8.2.3 Melatonin Administration

MEL was prepared in the following vehicle: 2% (v/v) ethanol, 4% (v/v) Tween 80 and 94% (v/v) deionised water. The animals of each time group were administered i.p. with either MEL (1 mg/kg bwt) or an equivalent volume of vehicle once daily for 4 days. Animals were sacrificed 30 min after the last administration on day 4 at the times indicated for the respective groups. Scotophase studies, including behavioural testing, were performed under dim red light.

8.2.4 Behavioural Studies: Catalepsy

The cataleptogenic potential of MEL was only assessed on those animals assigned to the mid-photophase (12h00) and mid-scotophase (24h00) groups. This represents each phase of the normal rest-activity cycle and should allow a more robust comparison of the state-dependent behavioural activity of these nocturnal animals. Catalepsy was tested by the Standardized Horizontal Bar Test described in section 2.6.

8.2.5 PINEAL ORGAN CULTURE STUDIES

The standard organ culture technique and TLC analysis of [^{14}C]indoles was performed as outlined in section 3.2.4. Following excision of the pineal glands, an attempt was made to rapidly remove the pineal stalk prior to incubation.

(i) *In Vivo* Studies

The effect of MEL administration on *in situ* pineal [^{14}C]indole biosynthesis was only determined for the mid-photophase (12h00) and mid-scotophase (24h00) groups. Pineals were rapidly transferred to the corresponding pre-labeled Kimble tubes. The final total volume of all samples was 60 µl for the *in vivo* studies, giving a final concentration ≈ 120 µM [^{14}C]5HT.

(ii) *In Vitro* Study

The *in vitro* effect of MEL (350 µM) on [^{14}C]indole biosynthesis was determined using pineal glands explanted and cultured at mid-photophase (12h00) from untreated animals. The pineals were randomly placed in individual Kimble tubes. MEL (n = 6) or the culture medium-ethanol vehicle (n = 6) was added in a volume of 10 µl to give the desired final concentration and 0.135% (v/v) ethanol, respectively. [^{14}C]Indole biosynthesis was initiated by the addition of [^{14}C]5HT immediately thereafter. Thus, for all practical purposes, there was no preincuabtion period with MEL. The final total volume of all samples was 70 µl for the *in vitro* studies, giving a final concentration ≈ 100 µM [^{14}C]5HT.

8.2.6 ENZYME ASSAYS

8.2.6.1 Tissue Preparation

MAO-A and COMT activity was determined for the vehicle- and MEL-treated groups of all the designated time periods. The corpora striata were removed and stored as described in section 2.2. Crude preparations for the both enzymes were obtained from the same tissue source by the subcellular fractionation protocol described in section 2.3.

8.2.6.2 Protein Determination

For all *in vivo* studies, statistical analysis was performed on enzyme specific activities. Thus the protein concentration of each enzyme preparation was determined in triplicate according to the procedure in section 2.4.

8.2.6.3 MAO-A Assay

All studies were performed on the 4% (w/v) mitochondrial/lysosomal fraction. The assay components and conditions were based on the optimum values experimentally determined in section 5.3. MAO-A activity was determined with [^{14}C]5HT (0.025 μCi; 4.5 μM) as the sole substrate, and in the presence of 150 μM 5HT. The ratio of "[^{14}C]5HT" activity to "[^{14}C]5HT+5HT" activity was also calculated for each sample.

8.2.6.4 COMT Assay

All studies were performed on the 5% (w/v) cytosolic/microsomal fraction. The assay components and conditions were based on the optimum values experimentally determined in section 7.3. It must be stressed that for *in vivo* studies, COMT activity was determined under conditions of maximal induction by $MgCl_2$ (10 mM) and a near-saturating concentration of DA HCl (1 mM).

8.2.7 Data and Statistical Analysis

For the catalepsy and enzyme studies, the significance of time-related or "between-group" variance for each treatment protocol was determined by ANOVA and Bonferroni's multiple comparisons test. For all studies, the mean values of vehicle- and MEL-treated samples within a specific data group were statistically compared by the Student *t*-test.

8.3 Results

8.3.1 Behavioural Studies

The cataleptogenic potential of subchronic administration of MEL during the photophase and the scotophase are shown in Figure 8.1A and 8.1B respectively. Following a post-administration interval of 20 min, MEL failed to induce catalepsy during either phase on any of the four days in comparison to the vehicle-treated data. The baseline scores prior to administration (0 min) for both treatment groups were relatively uniform on each day. There was a progressive increase in the variability of catalepsy scores following administration (20 min) of both the vehicle and MEL, especially in the scotophase studies. At no stage, however, is the behavioural response of sufficient intensity to be indicative of catalepsy. Rather, this may be explained by fear-induced immobilization due to repeated handling of the animals and neck-pinching. It is must be noted that the latter is actually of low magnitude, bearing in mind the scale of the data.

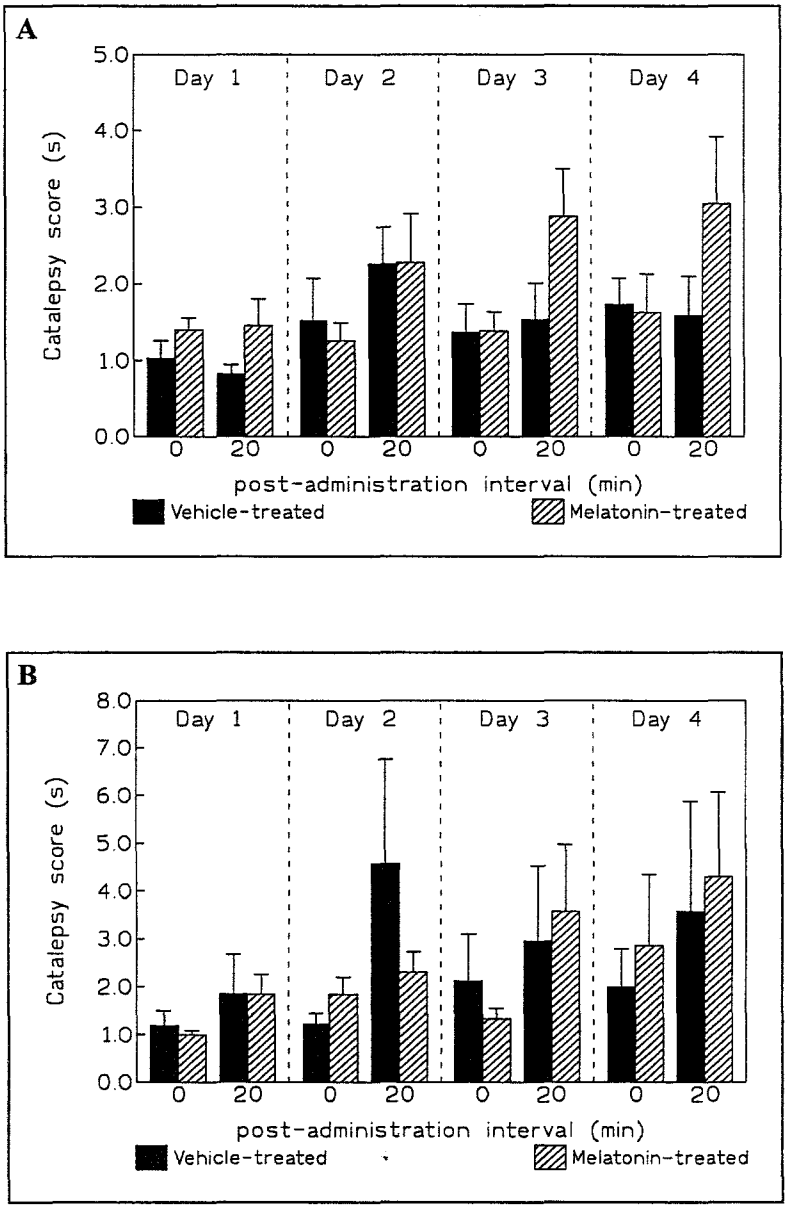


Figure 8.1: Cataleptogenic potential of MEL administered during the mid-photophase (A) and mid-scotophase (B). [Data represents mean ± SEM (n = 6)]

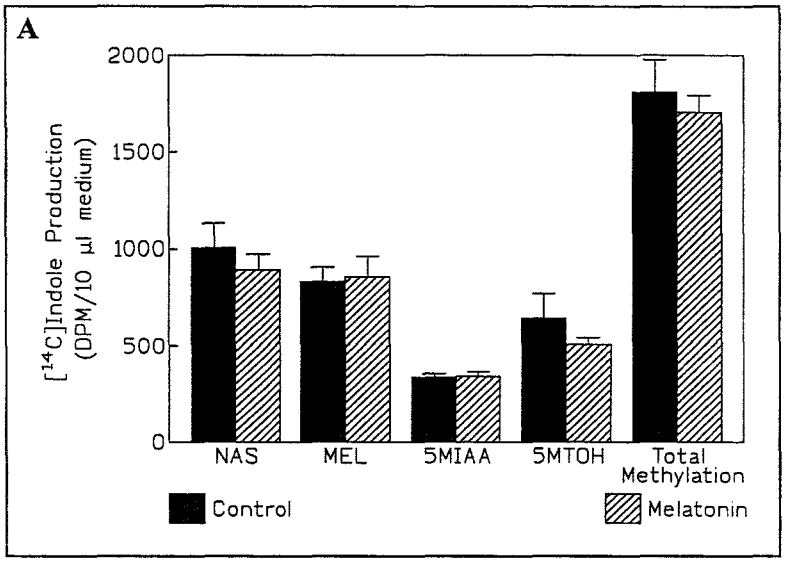
8.3.2 PINEAL ORGAN CULTURE STUDIES

(i) *In Vivo* Studies

MEL administration (1 mg/kg i.p., once daily for 4 days) at mid-photophase [12h00] or mid-scotophase [24h00] had no significant effect on the *in situ* biosynthesis of any exogenous [^{14}C]indole by the pineal glands cultured at the respective time periods (Figure 8.2 and 8.3). A comparison of the control values for the photophase and scotophase studies confirms the day-night variations in the indole biosynthetic profile described in section 3.3.

(ii) *In Vitro* Studies

The *in vitro* effect of MEL (350 μM) on the *in situ* biosynthesis of pineal [^{14}C]indoles is presented in Figure 8.4. The vehicle [0.135% (v/v) ethanol] did not influence biosynthesis (data not shown), and thus the effects of MEL are compared to the vehicle-treated data. MEL had no effect on [^{14}C]NAS levels, but significantly decreased the levels of [^{14}C]5MIAA, [^{14}C]5MTOH and total methylation ($P < 0.01$ in all cases). A parallel small decrease in [^{14}C]MEL biosynthesis was almost significant ($P = 0.0576$) [Figure 8.4A]. MEL also significantly increased [^{14}C]5HT levels ($P < 0.001$) and decreased [^{14}C]5HIAA levels ($P < 0.01$). There was a further trend towards decreased [^{14}C]5HTOH biosynthesis ($P = 0.1185$), such that total MAOP was significantly decreased ($P < 0.001$) and the 5HT/MAOP ratio was significantly increased ($P < 0.01$) [Figure 8.4B].



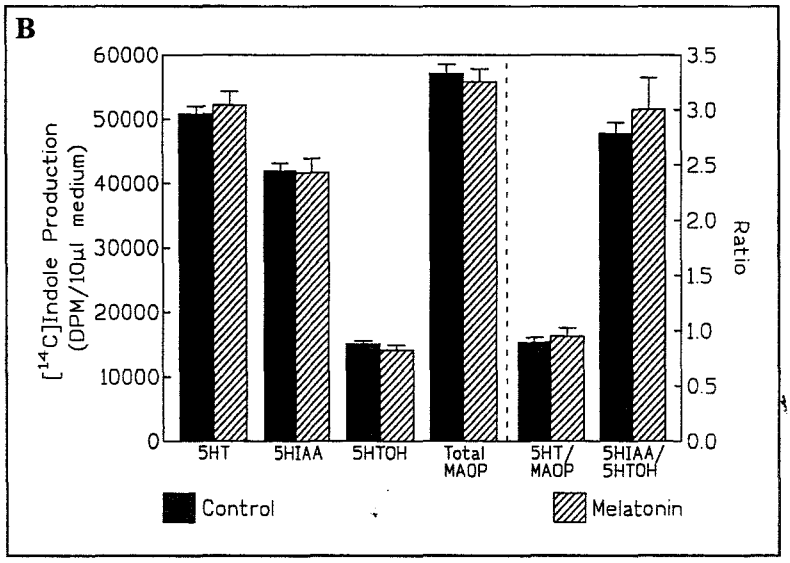


Figure 8.2: The effect of subchronic administration of MEL (1 mg/kg i.p., for 4 days) during the photophase on the *in situ* biosynthesis of [^{14}C]indoles by rat pineal glands cultured at mid-photophase [12h00]. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 6)]

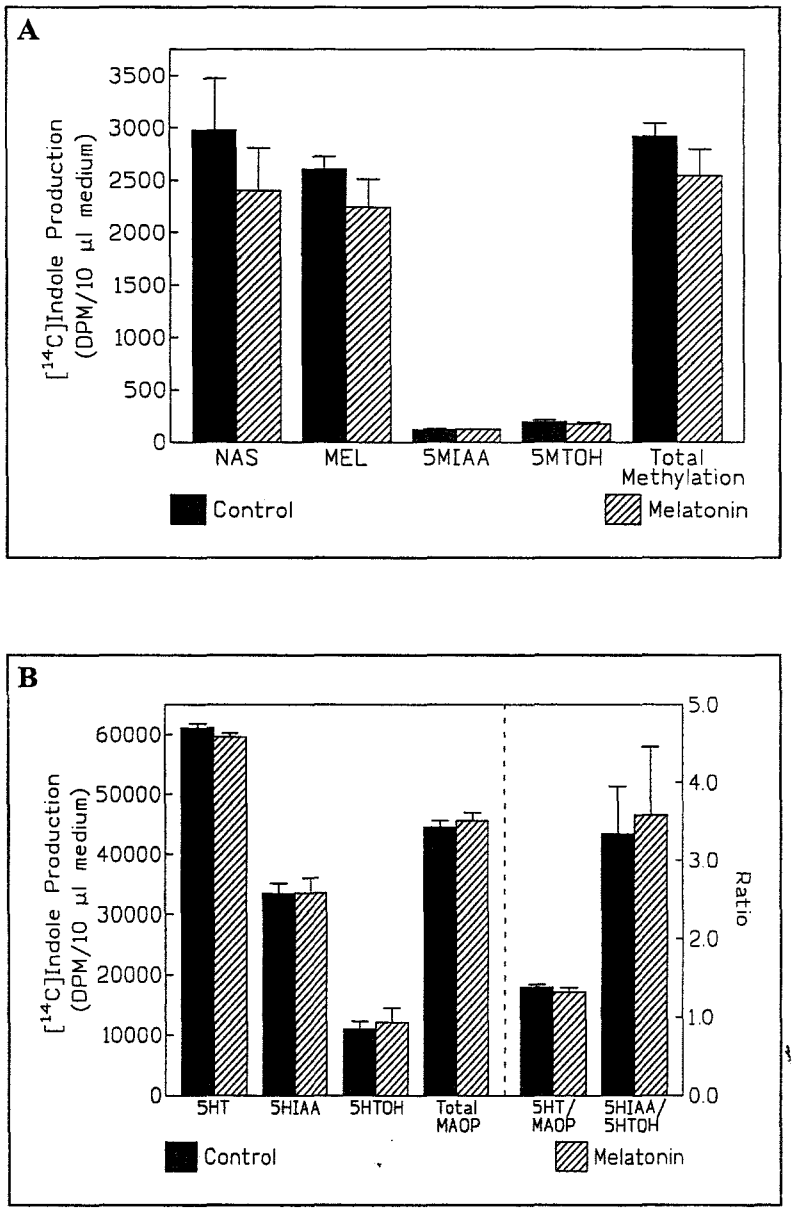


Figure 8.3: The effect of subchronic administration of MEL (1 mg/kg i.p., for 4 days) during the scotophase on the *in situ* biosynthesis of [^{14}C]indoles by rat pineal glands cultured at mid-scotophase [24h00]. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 6)]

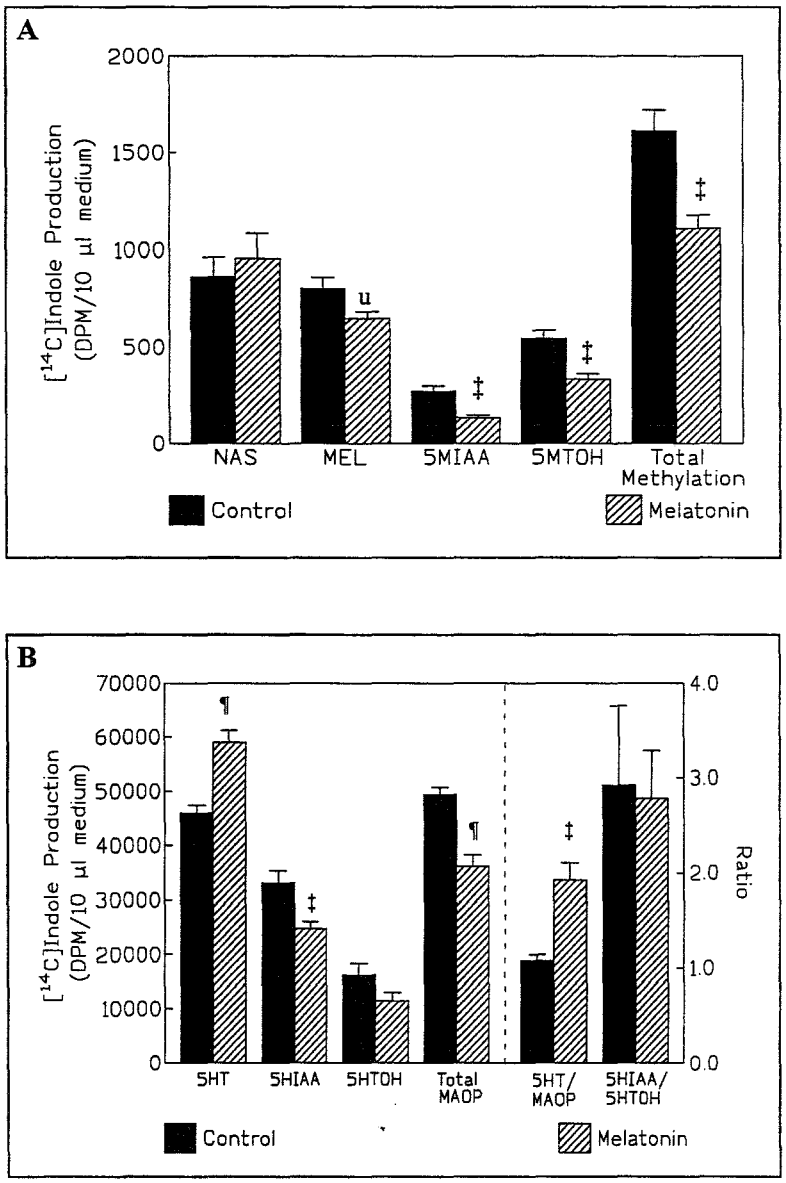


Figure 8.4: The *in vitro* effect of MEL (350 µM) on the *in situ* biosynthesis of [^{14}C]indoles by rat pineal glands cultured during the mid-photophase (12h00). (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 6); P-values (compared to controls): ◆ = 0.0576, ‡ < 0.01, ¶ < 0.001]

8.3.3 ENZYME ASSAYS

(i) MAO-A

There were no time-related differences in MAO-A activity as determined in the presence of [^{14}C]5HT alone (Figure 8.5A) or in combination with 5HT (Figure 8.5B) for both vehicle-treated and MEL-treated samples. The "[^{14}C]5HT:[^{14}C]5HT+5HT" ratio (Figure 8.5C) did show significant time-related variations during the light:dark cycle for the vehicle-treated (ANOVA: $P < 0.01$, $F = 5.719$) and MEL-treated samples (ANOVA: $P < 0.05$, $F = 3.201$). For the vehicle-treated samples, the ratio at 24h00 was significantly increased compared to that seen at 08h00, 12h00 and 16h00 (Bonferroni: $P < 0.05$, $P < 0.05$ and $P < 0.01$ respectively). No differences in the ratio were observed between the 08h00-, 12h00- and 16h00-groups. For the MEL-treated samples, the ratio at 24h00 was significantly increased only when compared to the 16h00-group (Bonferroni: $P < 0.05$). No differences in the ratio were observed between the 08h00-, 12h00- and 16h00-groups. Within each time group, MEL had no effect on "[^{14}C]5HT" activity, "[^{14}C]5HT+5HT" activity or the ratio in comparison to the vehicle-treated values.

(ii) COMT

Very significant time-related variations in COMT activity (Figure 8.6) were observed during the light:dark cycle for both vehicle-treated (ANOVA: $P < 0.001$, $F = 50.75$) and MEL-treated samples (ANOVA: $P < 0.001$, $F = 78.647$). For both treatment groups, activity at 08h00 was substantially lower than that at 12h00 and 16h00, whereas 24h00 activity was markedly elevated compared to all three of the other time groups (Bonferroni: $P < 0.001$ in all cases). No significant differences were observed between the 12h00- and 16h00-groups. Within each time group, MEL administration significantly decreased COMT activity of the 08h00-group only (Student *t*-test: $P < 0.001$).

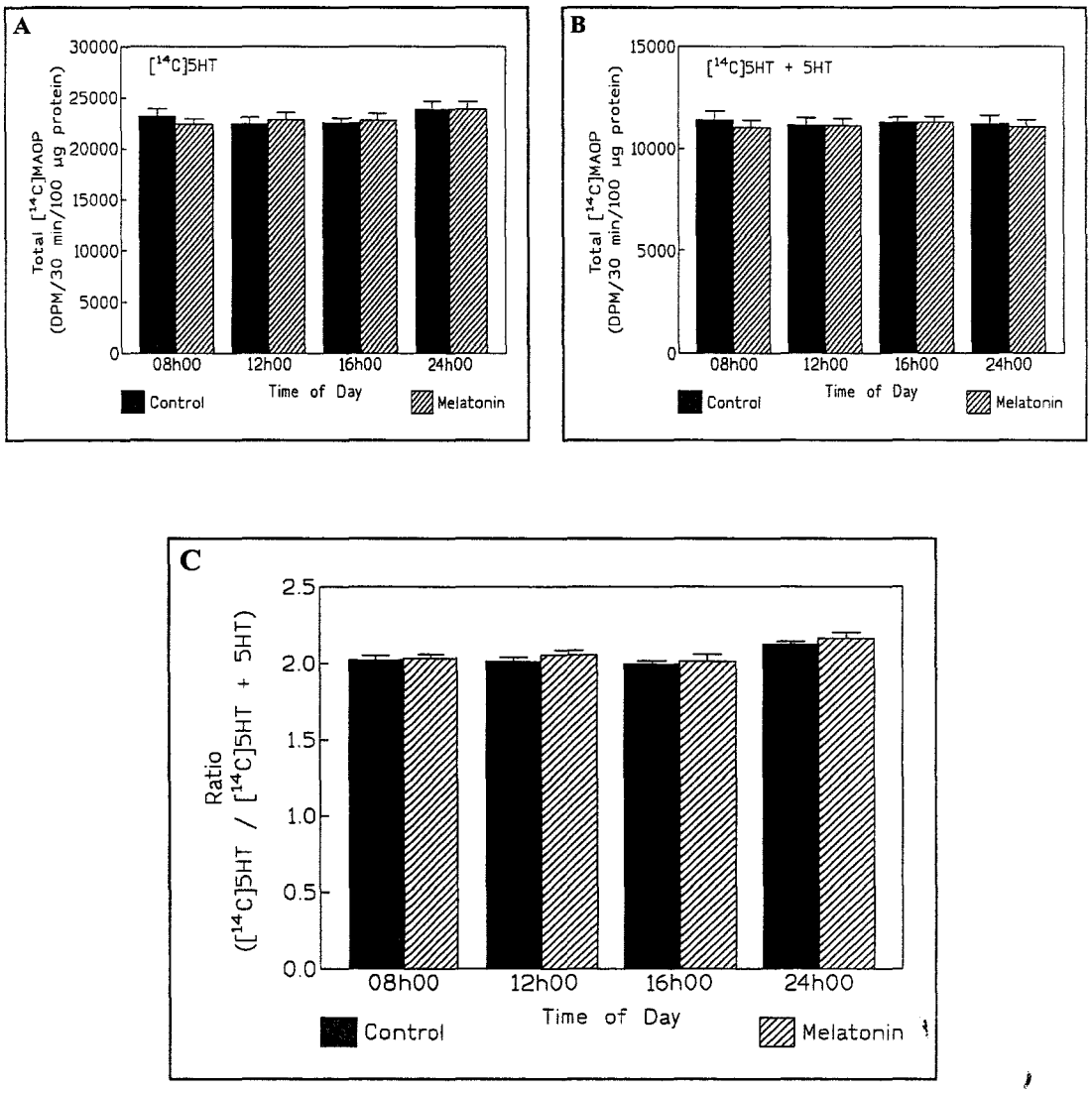


Figure 8.5: Day-night variation of striatal MAO-A activity and the chronotypic effect of *in vivo* MEL administration. (A) Activity with [^{14}C]5HT. (B) Activity with [^{14}C]5HT+5HT. (C) Ratio of activity with [^{14}C]5HT to [^{14}C]5HT+5HT. [Data represents mean ± SEM (n = 6)]

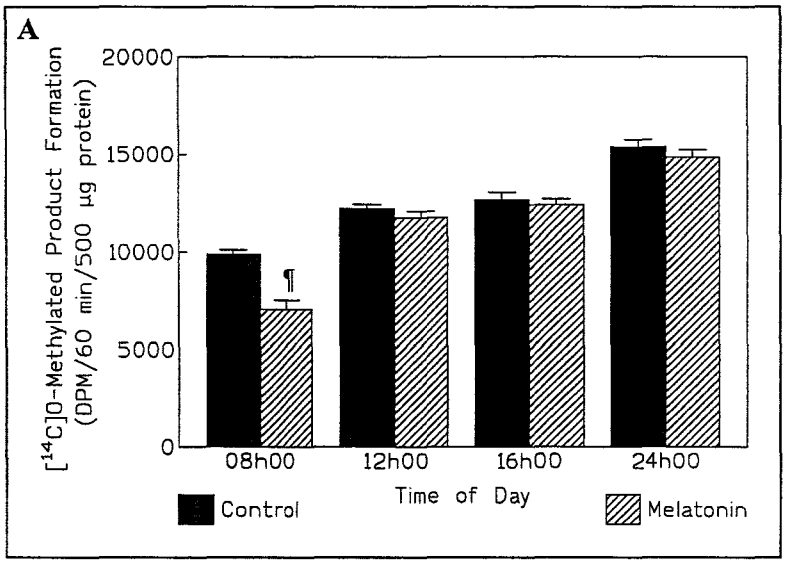


Figure 8.6: Day-night variation of striatal COMT activity and the chronotypic effect of *in vivo* MEL administration. [Data represents mean ± SEM (n = 6); ¶ : P < 0.001 (compared to 08h00-control)]

8.4 Discussion and Conclusion

The average concentration of MEL in brain tissue falls within the picomolar to low nanomolar range. Subcutaneous administration of MEL at a dose of 25 µg/kg bwt results in a physiological brain concentration of 1-3 nM (Castillo-Romero *et al.*, 1992). For this reason, it is often argued that administration of MEL at a dose such as 1 mg/kg will result in supraphysiological plasma and tissue levels of MEL and any subsequent effects may simply be pharmacological artifacts. However, there is evidence for tissue-specific and brain region-specific uptake and accumulation of circulating MEL (Anton-Tay *et al.*, 1988; Vitte *et al.*, 1988). For example, the rat hypothalamus contains 1 µM MEL (Zisapel and Laudon, 1982). In contrast, very low amounts of the hormone are typically found in the nucleus accumbens, striatum and substantia nigra (Kopp *et al.*, 1980; Seguela *et al.*, 1982). In other words, the physiological concentration of MEL required for a CNS effect may not necessarily reflect the prevailing plasma concentration. The route of administration must also be considered. Intraperitoneal administration of MEL results in a single large bolus of the hormone, which is rapidly degraded by first-pass metabolism in the liver (Kopin *et al.*, 1961). The effective concentration of MEL reaching the brain will depend on the degree of metabolism

and the subsequent pharmacokinetics of transport and uptake. The short half-life of MEL (± 20 min) is the main factor determining the duration of the "spike" in plasma levels. In contrast, s.c. administration bypasses hepatic metabolism and results in a gradual, but uniform uptake of MEL, which enhances tissue levels of the hormone. Thus it could be argued that a supraphysiological dose may be necessary when MEL is administered by the i.p. route. It must be also noted that the duration of MEL administration may be an important variable (Tamarkin *et al.*, 1976; Moreno *et al.*, 1992).

In the present studies, it was not possible to determine the effect of exogenous MEL administration on endogenous plasma MEL levels, by RIA for example. Therefore any effect of exogenous MEL may either be a direct physiological effect independent of its action as the internal zeitgeber time signal, or arise through an induction of a phase-shift in the circadian rhythm of endogenous MEL. *In situ* indole biosynthesis was thus employed as an index of the functional state of the pineal following MEL administration. This further served to investigate the claim that MEL may exert feedback control on pineal function in a chronotypic fashion (Trentini *et al.*, 1982; Yanez and Meissl, 1995; Drijhout *et al.*, 1996). For example, MEL (50 μg/animal for 28 days) blocked or shifted the rhythm in endogenous pineal 5HT when administered to rats at mid-photophase, but not at late photophase, just prior to the onset of darkness (Fiske and Huppert, 1968).

Following the current administration regime, exogenous MEL had no effect on pineal [^{14}C]indole biosynthesis during the photophase or scotophase. This included a lack of effect on pineal MAO activity. In contrast, MEL (350 μM) *in vitro* had a significant effect on pineal indole biosynthesis in a manner consistent with an inhibitory effect on pineal HIOMT and MAO activity. This discrepancy between the *in vitro* and *in vivo* studies may be due to the dose of MEL administered, the vehicle, and the subsequent pharmacokinetics and degree of metabolic inactivation. Hartley and Smith (1973) reported that MEL (120 μM) *in vitro* had no effect on crude bovine pineal HIOMT activity. However, the decrease in 5-methoxyindole biosynthesis and total methylation observed here, without a concomitant reduction in NAS biosynthesis, is consistent with an inhibition on HIOMT and not NAT. Metabolically, MEL is a product of HIOMT activity and thus product inhibition may be evident at high endogenous concentrations of the indole, as supplied here exogenously.

The inhibitory effect of MEL *in vitro* on striatal MAO activity has been previously described in section 6.3. It must be noted that the striatal studies represented *in vitro* conditions on a crude mitochondrial preparation where tissue and cellular integrity has been disrupted. Thus this effect of MEL may not be physiological. The pineal gland is rich in MAO activity (Juillard and Collin, 1979) and the intact pineal organ would represent a good physiological system for determining the *in situ* effects of MEL on MAO activity (Schwarzchild and Zigmond, 1989). The change in [^{14}C]5-hydroxyindole biosynthesis was indeed consistent with an inhibition of MAO by MEL, but the effect was weaker than expected. From the striatal studies, 350 μM MEL typically induced ± 60% inhibition, but only ± 25% inhibition was observed in the *in situ* organ culture assay. Several factors may explain this discrepancy. These include, *inter alia*: bioavailability of the exogenous MEL in the intact gland due to diffusion kinetics and intracellular compartmentalization; predominance of MAO-B rather than MAO-A in the pineal; catabolism of MEL; and competition between endogenous 5HT and exogenous [^{14}C]5HT, especially in the noradrenergic nerve terminals.

It is important to note that the MEL-induced changes in 5-methoxyindole biosynthesis can also be explained by an inhibition of pineal MAO activity, for two reasons. Firstly, 5HIAA and 5HTOH are O-methylated by HIOMT to 5MIAA and 5MTOH respectively (see Figure 1.2). Thus a decrease in the bioavailability of the two deaminated 5-hydroxyindoles, affected through inhibition of MAO, should be reflected in a subsequent decrease in the biosynthesis of the corresponding 5-methoxyindoles. Secondly, it has been tentatively hypothesized (in section 6.4) that 5MT may be a novel substrate of MAO with 5MIAA and 5MTOH as the deaminated products (see Figure 6.13). Thus inhibition of pineal MAO could directly result in decreased levels of 5MIAA and 5MTOH, irrespective of O-methylation. Both of these alternative proposals help explain why these two 5-methoxyindoles were affected more than [^{14}C]MEL. The results of this *in situ* study confirm previous studies that MEL and other indoles may exert negative feedback or paracrine control over pineal function (Trentini *et al.*, 1982; Yanez and Meissl., 1995; Drijhout *et al.*, 1996).

Reportedly, brain MAO activity displays distinct day-night variations, but the phase characteristics are dependent on enzyme isoform, brain region and age (Chevillard *et al.*, 1981; Bhaskaran and Radha, 1984). With respect to the corpus striatum of adult rats, Bhaskaran and Radha (1984) found that MAO activity peaked in the early photophase. Esquifino *et al.* (1994) reported that vehicle-treated MAO activity of the rat adrenal medulla was higher during the late (16h00) than the early photophase (08h00). It must be noted that the latter two studies were not isoform specific as a

substrate common to both MAO isoforms was employed, namely kynuramine (Bhaskaran and Radha, 1984) and tyramine (Esquifino *et al.,* 1994). In the present study, there was no obvious day-night variation in striatal MAO-A. Only the "[^{14}C]5HT:[^{14}C]5HT+5HT" activity ratio showed a small, but significant increase at 24h00 compared to the other time periods for both the vehicle-treated samples. This may represent an assay artifact, but a similar trend was observed for the MEL treated samples and in follow-up experiments (see chapter 9).

Under the optimum assay conditions, experimentally determined and presented in section 5.3, the "[^{14}C]5HT:[^{14}C]5HT+5HT" activity ratio was constant at *ca.* 2.0. This suggests that the deviation in the ratio at 24h00 may have arisen due to changes in the kinetic properties, such as substrate affinity, of the MAO enzyme. Indeed preliminary studies did find that striatal MAO-A isolated during the scotophase showed altered sensitivity to inhibition by the pineal indoles compared to that seen in the photophase (data not shown). This may represent changes in the catalytic properties of the enzyme or the presence of "modifiers" in the striatum at night. However, MAO-A activity is considered to be related to the amount of enzyme present, and not due to different catalytic properties of the enzyme molecules (Costa *et al.*, 1980; Fowler and Wiberg, 1980). This suggests interference by a "modifier" and endogenous MEL itself represents a likely candidate. Plasma and tissue levels of the hormone are very low during the photophase and increase dramatically at night (Reiter, 1991). MEL is ubiquitously distributed intracellularly, being present in plasma membranes, cytosol, mitochondria and the nucleus (Acuña Castroviejo *et al.*, 1994; Reiter, 1995). Although MAO activity was determined on mitochondrial preparations, it is thus possible that MEL was present in amounts dependent on the phase of the light:dark cycle.

It is proposed that the lack of an obvious day-night variation in striatal MAO-A in this study compared to that of Bhaskaran and Radha (1984) is a matter of experimental design. In both cases, MAO activity was determined *ex vivo*. A circadian nature of MAO activity could arise through several factors, including fluctuations in the amount of functional MAO protein (e.g. variation in gene expression of MAO), substrate bioavailability, or the circadian nature of MEL or other "modifiers". Firstly, Bhaskaran and Radha (1984) assayed non-specific MAO activity, whereas this study assayed MAO-A. Secondly, they prepared a total homogenate of the striatal tissue, in contrast to the mitochondrial preparation used here. Thus the day-night variation observed by Bhaskaran and Radha (1984) may be attributed to MAO-B and/or a circadian variation in certain cytosolic components, including endogenous substrates and "modifiers". For example, endogenous MAO substrates, such as DA, are high at night, but low during the photophase following deamination in the striatum (Paulson and Robinson, 1994). Thus there would be less competition between kynuramine

and endogenous substrates in the early photophase, giving an apparent increase in MAO activity at this time as determined by the spectrophotometric method of Bhaskaran and Radha (1984). In contrast, use of a mitochondrial fraction should result in the removal of endogenous substrates and other cytosolic factors, but not necessarily MEL. In this light, the change in the "[^{14}C]5HT:[^{14}C]5HT+5HT" activity ratio at mid-scotophase may be very significant.

Limited studies have investigated the effect of MEL administration on MAO activity. Esquifino *et al.* (1994) found that chronic administration of MEL (25 μg/animal) for 60 days had chronotypic effects on adrenal medullary MAO activity in male Syrian hamsters. MAO activity was reduced following MEL administration during the early (08h00) and late photophase (16h00), but to a greater extent in the 16h00-group. The effect of MEL on MAO activity may also be dependent on the developmental stage of the animal. Neonatal administration of a single dose of MEL to male rats on postnatal day 5 increased hypothalamic MAO-A at day 30 and 45, but decreased activity on day 60 of age, whereas MAO-B was only increased at day 45 (Moreno *et al.*, 1992). With regard to the basal ganglia, bilateral injection of ng quantities of MEL into the substantia nigra and the nucleus accumbens dose-dependently increased DA content and reduced the DOPAC/DA ratio (Bradbury *et al.,* 1985). These results could reflect an inhibition of DA release or an inhibition of MAO. However, to this author's knowledge, no direct information is available regarding the effect of MEL on MAO of the corpus striatum. In the present study, short-term administration of exogenous MEL failed to effect striatal MAO-A activity or the "[^{14}C]5HT:[^{14}C]5HT+5HT" ratio at any of the time periods, including the vehicle-treated increase in the latter at 24h00.

It is possible that the lack of day-night variation and an effect of MEL on striatal MAO is related to the nature of the *in vitro* assay and the experimental protocol (e.g. dose, duration of administration etc.). Analysis of tissue levels of DA, DOPAC and HVA may reveal changes in the *in vivo* activity of striatal MAO-A not observed here. Furthermore, the time periods chosen may represent a "refractory period" and thus it would have been informative to investigate MAO activity at shorter time intervals, especially between 24h00 and 08h00. Ultimately, a possible effect of MEL on striatal MAO-A cannot be excluded until all possible combinations of the administration parameters have been investigated.

A search of the literature indicates that little to no research has been performed on the possible interaction of MEL and COMT, particularly in the corpus striatum. In the present study, strong evidence was found for day-night variations of striatal COMT in the rat and for chronotypic modulation by MEL *in vivo*. Activity was highest at mid-scotophase and lowest in the early

photophase with a plateau evident at other times of the photophase. Due to the resolution of the time intervals, it was not possible to determine the timing of peak activity or to infer the actual waveform nature of the rhythm, i.e. whether it was circadian with a period of *ca.* 24 hrs. The dark-phase increase in COMT activity may be part of the general state-dependent increase in dopaminergic activity associated with the nocturnal activity of rats. The increase in DA release during the scotophase results in a concomitant elevation in extracellular 3MT levels (Paulson and Robinson, 1994). 3MT is formed from the 0-methylation of DA by COMT and is a reliable index of DA release (Kehr, 1976). It could be argued that COMT activity would increase at night due to increased availability of substrate. However the present *ex vivo* assay employed a fixed amount of exogenous substrate and the apparent COMT activity was corrected for any background activity due to the presence of endogenous substrate. This suggests that the actual amount or proportion of active COMT enzyme molecules may increase at night.

It must be noted that the apparent peak of striatal COMT activity coincides with the nocturnal surge in MEL plasma levels, whereas the trough activity is temporally correlated with the return of plasma MEL basal daytime values. This would suggest an enhancing effect of MEL on COMT activity. However, an inhibitory effect of MEL administration was only observed on 08h00-COMT activity. In other words, a reversal of basal MEL levels during the early photophase back to that seen in the scotophase would appear to potentiate a decrease in COMT activity. Alternatively, exogenous MEL may have phase-shifted the rhythm in COMT activity. On the basis of this preliminary evidence, it is hypothesized that the time-lag between peak plasma MEL levels and maximal inhibitory effect on COMT activity may arise through an effect of MEL at the level of COMT gene expression or post-translational modification. A time lag should be apparent before an alteration in gene transcription is observed at the mRNA and protein level. This may require a consideration of the turnover rate and half-life of mRNA transcripts and protein molecules. The onset of darkness may trigger an increase in COMT activity, whereas a progressive increase in the sensitivity to MEL may act as a signal to begin reducing the contribution of the enzyme. It would be informative to compare the present results to an assessment of *in vivo* COMT activity by analyzing tissue levels of 3MT and HVA, and COMT mRNA. MEL may also influence the CNS bioavailability of the essential cofactor, SAM, through an effect on SAM synthase or SAM decarboxylase. The latter enzyme exhibits a circadian activity rhythm in the liver, kidney and thymus. Pinealectomy failed to phase-shift the rhythm in any of the tissues, but increased the amplitude of enzyme activity in the kidney (Scalabrino *et al.*, 1979). An inhibitory effect of MEL *in vivo* on COMT is supported by Esquifino *et al.* (1994). Chronic administration of MEL (25 μg/animal for 60 days) reduced adrenal medullary COMT activity in

male Syrian hamsters during both the early (08h00) and late photophase (16h00), but to a greater extent in the 16h00-group. No difference was observed between vehicle-treated activity at 08h00 and 16h00. This indicates that the chronotypism of MEL towards COMT and the phase characteristics of the enzyme are likely to be tissue-specific. It is possible that a more chronic administration of MEL in the present study may have revealed an alternative pattern in striatal COMT activity.

The biochemical effects of MEL reported here, namely pineal indole biosynthesis, striatal COMT and MAO-A, did not appear to translate into a behavioural response. Although numerous models of behaviour assign an antidopamimetic role to MEL (Cotzias *et al.*, 1971; Burton, 1989; Tenn and Niles, 1995), the pineal hormone failed to induce catalepsy under the present experimental conditions. The MEL catalepsy studies were intentionally performed at mid-photophase and mid-scotophase in order to represent the two robust phases of the rest-activity cycle. It is possible that these times coincided with "refractory" periods. The switch from quiescence to activity at the onset of darkness or *vice versa* at the onset of the photophase may represent more sensitive "circadian windows" to the behavioural effects of MEL (Redman *et al.*, 1983; Armstrong, 1989). In a follow-up study (data not shown), the chronic administration of a more physiological dose of MEL (25 μg/animal s.c., once daily) for a week during the photophase also failed to induce catalepsy. It must be noted that in contrast to the current research, previous studies investigated MEL in animal models of movement disorders, in which the "tonus" of dopaminergic systems was challenged with neurochemical agents. For example, selective lesioning of the nigro-striatal pathway with 6OHDA produces supersensitive DA receptors. In contrast, the cataleptogenic potential of MEL was currently determined on an intact, steady-state dopaminergic system. Indeed this lack of an effect is actually in line with the modulatory or tonic nature of the hormone. MEL may either potentiate or attenuate the direction of the prevailing "tonus" without initiating the change itself.

In summary, short-term administration of exogenous MEL modulated both striatal MAO-A and COMT activity with a clear and marked inhibitory effect in the case of COMT. It is very likely that a more chronic administration paradigm would have induced more robust changes in both enzymes. The time-of-day studies suggest that endogenous MEL may also regulate both enzymes, particularly COMT. In the following chapter, manipulation of the prevailing photoperiod will be employed to further investigate the role of endogenous MEL.

Chapter 9

Photoperiod Manipulation Studies

9.1 Introduction

Light is the most important environmental factor regulating the pineal noradrenergic secretory drive and MEL production (Reiter, 1988; Reiter, 1991). For a given latitude, the annual variation in daylength (photoperiod) is a much more accurate predictor of season than ambient temperature. The light:dark cycle is thus the most robust rhythm to which life is synchronized as reflected in the circadian rhythmicity of both diurnal and nocturnal species. Photoperiodism of reproduction, body weight, metabolism and coat colour are common to seasonally breeding animals. Under natural conditions, most mammals are photoperiodic to varying degrees, with the degree of photoperiodism increasing progressively with higher latitudes. With regards to typical laboratory animals, the golden or Syrian hamster is fully photoperiodic, whereas the rat is only partially seasonal (Reiter, 1988).

The rhythm in SCN metabolic function and pineal NAT activity, and thus MEL biosynthesis, is intrinsically free-running with a period of *ca.* 25 hrs. For this reason, the latter are designated as endogenous rhythms (Armstrong, 1989; Moore, 1993; Reiter, 1991). Photic stimulation via the retino-hypothalamic tract influences the SCN and thus pineal gland biochemistry in two ways: (1) it entrains the endogenous circadian rhythm in MEL production to the ambient light:dark cycle or daylength; (2) exposure to light during the scotophase rapidly suppresses the nocturnal NAT activity and MEL production. Even brief exposure to low intensity lighting (< 100 lux, or 5-20 $\mu W/cm^2$) at this time is sufficient to cause a precipitous decline in pineal MEL synthesis and release, and a parallel decline in plasma and tissue MEL levels. This is a direct consequence of a rapid inactivation of NAT activity (Reiter, 1988; Deguchi and Axelrod, 1972b). For purposes of comparison, normal room light has an intensity of 50-100 $\mu W/cm^2$ (100-400 lux). Single pulses of light during the scotophase are also capable of phase-shifting the MEL circadian rhythm.

Although some species-related differences are seen, the threshold for light-induced suppression is qualitatively similar between all mammals, including humans (Gaddy *et al.*, 1993; Boivin *et al.*, 1996). It has also been reported that the spontaneous blink rate and pupil size are important parameters in determining the critical threshold of light-induced suppression of nocturnal MEL

biosynthesis (Gaddy *et al.*, 1993; Sandyk, 1990b). Closer examination actually reveals that specific wavelengths of light (or the electromagnetic spectrum) have differential and species-related effects on pineal, retinal and harderian MEL biosynthesis (Cardinali *et al.*, 1972; Reiter, 1985). For example, Reiter (1985) reported that violet and yellow light are most effective in nocturnal animals, whereas blue-green and red light have the greatest effect in diurnal animals. More specifically, green light has a maximal effect on rat pineal indole biosynthesis, whereas red is incapable of suppressing nocturnal NAT activity (Cardinali *et al.*, 1972).

In all the studies presented so far in this thesis, the animals have been housed under a 12L:12D cycle with the onset of light at 06h00. This lighting schedule extends the scotophase symmetrically around midnight with 6 hrs before and 6 hrs after this peak. Under these conditions, a robust day-night rhythm was observed in striatal COMT with some indication for a similar pattern in MAO activity. Furthermore the administration of exogenous MEL had chronotypic effects on both enzymes. The question remains whether endogenous MEL can have similar effects, and thus entrain striatal dopaminergic function at the level of MAO and COMT. Considering the effects of photic stimulation, the contribution of endogenous MEL can be investigated by photoperiodic manipulation. For example, animals can be maintained under a long photoperiod (e.g. 14L:10D) or a short photoperiod (e.g. 10L:14D) to differentially phase-shift the chronotypism in sensitivity to MEL (Miguez *et al.*, 1995). Alternatively, the circadian oscillator can be reverted to a free-running rhythm by removing the entraining light signal. This results in a disorganization of the circadian and circannual control of behavioural, biochemical and physiological processes, and animals may become aseasonal. To achieve this, the rats can be housed under constant light (i.e. 24LL) or constant darkness (24DD). If a biochemical parameter still exhibits a rhythm under constant darkness, irrespective of its phase characteristics, it is said to be endogenous and independent of the light:dark cycle as seen for pineal function. This further implies that modulation by MEL must be a direct effect, independent of its role as an entraining agent or "Zeitgeber". In turn, constant light results in a complete suppression of nocturnal pineal MEL biosynthesis and thus plasma levels. The absence of light is required for the resurgence of MEL biosynthesis at night and thus the suppression is permanent for the duration of the constant illumination.

One strategy to truly determine the role of MEL *in vivo* would be to suppress the synthesis of the hormone and remove it from circulation. Thus the main objective of this chapter was to determine the effect of housing animals under constant light or constant dark on pineal indole biosynthesis

and striatal MAO-A and COMT activity. It must be stressed that light was supplied by a full spectrum cool-white fluorescent tube with very little long-wave emission and no UV radiation. This light source delivers a total radiant energy level of 250 $\mu W/cm^2$, considerably in excess of the 0.0005 $\mu W/cm^2$ of white light required to suppress pineal NAT activity and MEL biosynthesis in the Wistar rat (Reiter, 1988).

9.2 Materials and Methodology

9.2.1 Chemicals and Reagents

MEL was purchased from Sigma. The chemicals and reagents for the pineal organ culture technique, MAO-A assay and COMT assay are listed in section 3.2.1, 5.2.1 and 7.2.1 respectively.

9.2.2 Animals and Photoperiod Manipulation

Male Wistar rats were housed as described in section 2.1. Initially, the animals were randomly assigned to groups and acclimatized for 1 week to a normal 12L:12D (LD) lighting schedule (lights on 06h00) prior to photoperiod manipulation.

(i) Protocol 1

Rats were maintained under the following lighting conditions for 7 consecutive days: LD, constant light (24LL; n = 8) or constant darkness (24DD; n = 7). Two groups of rats were maintained under the LD conditions, representing the photophase (LD-DT; n = 7) and the scotophase (LD-NT; n = 7) respectively. Animals were sacrificed at 12h00 for the LD-DT, 24LL and 24DD groups, and at 24h00 for the LD-NT group.

(ii) Protocol 2

In the second photoperiod study, animals were subjected to the following lighting conditions for 21 consecutive days: LD (n = 5) and constant light (24LL). The latter comprised a "vehicle-treated" (n = 5) and "MEL-treated" group (n = 5), designated 24LL and LL-MEL respectively. MEL was administered as described below. The animals of all three groups were sacrificed on day 21 at 16h00 (late photophase), 24 hrs after the last administration.

9.2.3 MELATONIN ADMINISTRATION

For protocol 2, MEL was prepared in the following vehicle: 2% (v/v) ethanol, 4% (v/v) Tween 80 and 94% (v/v) deionised water. Animals received either MEL (25 μg s.c.) or an equivalent volume of vehicle at 16h00 once daily for 20 days.

9.2.4 PINEAL ORGAN CULTURE STUDIES

The standard organ culture technique and TLC analysis of [^{14}C]indoles was performed as described in section 3.2.4. Following excision of the pineal glands, an attempt was made to rapidly remove the pineal stalk prior to incubation. Pineals were rapidly transferred to the corresponding pre-labeled Kimble tubes. The final total volume of all samples was 60 μl for the *in vivo* studies, giving a final concentration ≈ 120 μM [^{14}C]5HT.

9.2.5 ENZYME ASSAYS

9.2.5.1 Tissue Preparation

The MAO-A and COMT activity of each experimental group was determined. The corpora striata were removed and stored as described in section 2.2. Crude preparations of both enzymes were obtained from the same tissue source by the subcellular fractionation protocol outlined in section 2.3.

9.2.5.2 Protein Determination

For all *in vivo* studies, statistical analysis was performed on enzyme specific activities. Thus the protein concentration of each enzyme preparation was determined in triplicate as described in section 2.4. The mean protein concentrations of each experimental group were not significantly different (data not shown).

9.2.5.3 MAO-A Assay

All studies were performed on the 4% (w/v) mitochondrial/lysosomal fraction. The assay components and conditions were based on the optimum values experimentally determined in section 5.3. MAO-A activity was determined with [^{14}C]5HT (0.025 μCi; 4.5 μM) as the sole substrate, and in the presence of 150 μM 5HT. The ratio of "[^{14}C]5HT" activity to "[^{14}C]5HT+5HT" activity was also calculated for each sample.

9.2.5.4 COMT Assay

All studies were performed on the 5% (w/v) cytosolic/microsomal fraction. The assay components and conditions were based on the optimum values experimentally determined in section 7.3. It must be stressed that for these *in vivo* studies, COMT activity was determined under conditions of maximal induction by $MgCl_2$ (10 mM) and a near-saturating concentration of DA HCl (1 mM).

9.2.6 Data and Statistical Analysis

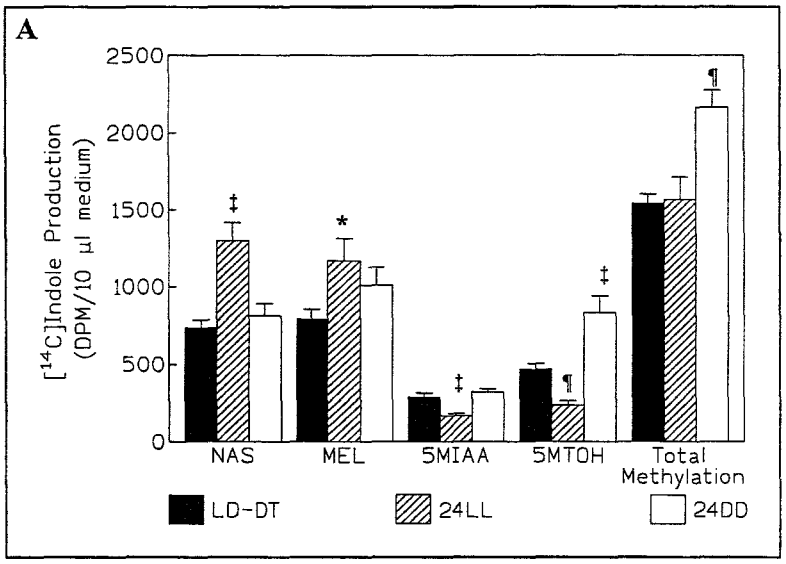
All data are presented as mean ± SEM. The sample size (n) depends on the experimental group and is indicated in the respective figure captions. Striatal MAO-A and COMT are expressed as specific activity in the units DPM/30 min/100 μg protein and DPM/60 min/500 μg protein respectively. The significance of group-dependent variation in MAO and COMT activity was determined by ANOVA followed by Bonferroni's post test. Where specified, the less stringent Student-Newman-Keuls test was also employed. In addition, the mean data of the enzyme and pineal studies was statistically compared by the unpaired Student *t*-test. A two-tailed P-value was generated with respect to either the LD-DT group (protocol 1) or the LD group (protocol 2) as the control data. Only the latter Student *t*-test P-values were graphically presented.

9.3 Results

9.3.1 Pineal Organ Culture Studies

(i) Protocol 1

The effects of 24LL and 24DD for 7 days on *in situ* pineal indole biosynthesis in comparison to LD-DT conditions are shown in Figure 9.1. The P-values in the text below were generated by the Student *t*-test. 24LL markedly increased the levels of [^{14}C]NAS ($P < 0.01$) and [^{14}C]MEL ($P < 0.05$). There was a concomitant decrease in the biosynthesis of [^{14}C]5MIAA ($P<0.01$) and [^{14}C]5MTOH ($P < 0.001$) without a change in total methylation (Figure 9.1A). Under conditions of 24DD, there was a similar, but marginally insignificant increase in [^{14}C]MEL levels ($P = 0.1267$). In addition, there was a substantial elevation in the biosynthesis of [^{14}C]5MTOH ($P < 0.01$) and total methylation ($P < 0.001$) [Figure 9.1A]. Neither 24LL nor 24DD caused a change in the levels of [^{14}C]5HT, [^{14}C]5HIAA, [^{14}C]5HTOH or total MAOP (Figure 9.1B). Pineal indole biosynthesis was not determined for the LD-NT group.



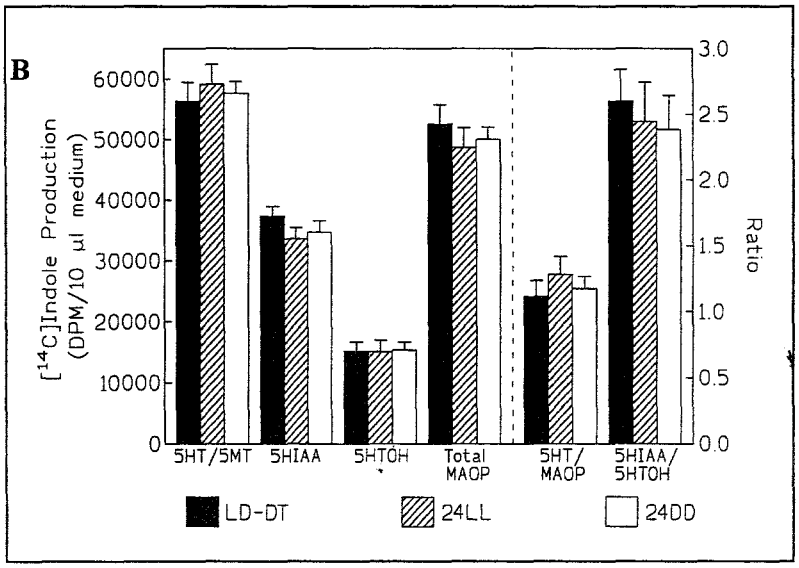


Figure 9.1: The effect of photoperiodic manipulation on the *in situ* biosynthesis of [^{14}C]indoles by rat pineal glands cultured during the mid-photophase (12h00). (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM; LD: normal 12L:12D cycle (n = 7), 24LL: constant light (n = 8), 24DD: constant darkness (n = 7); P-values (compared to LD-group): * < 0.05, ‡ < 0.01, ¶ < 0.001]

(ii) Protocol 2

The effect of long-term exposure to 24LL and MEL administration (LL-MEL) on *in situ* pineal indole biosynthesis in comparison to LD conditions are shown in Figure 9.2. The P-values in the text below were generated by the Student *t*-test. 24LL for 21 days induced a 3- to 4-fold increase in [^{14}C]NAS biosynthesis ($P < 0.01$) with a parallel, but smaller increase in [^{14}C]MEL levels ($P < 0.01$). Conversely, the levels of [^{14}C]5MIAA and [^{14}C]5MTOH were substantially reduced ($P < 0.01$ in both cases) without a change in total methylation (Figure 9.2A). An identical pattern was seen in the LL-MEL group: [^{14}C]NAS ($P < 0.01$), [^{14}C]MEL ($P < 0.05$), [^{14}C]5MIAA ($P < 0.01$) and [^{14}C]5MTOH ($P < 0.01$) [Figure 9.2A]. There was no change in the levels of [^{14}C]5HT, [^{14}C]5HIAA, [^{14}C]5HTOH or total MAOP for the 24LL and LL-MEL groups (Figure 9.2B). Only the 5HIAA/5HTOH ratio was elevated in these two groups compared to the LD group, but this was not significant due to greater degree of variability in the data for 24LL and LL-MEL. The similar profile between the 24LL and LL-MEL groups indicates that MEL administration failed to modulate the effects of constant light.

9.3.2 Enzyme Studies

9.3.2.1 MAO-A

(i) Protocol 1

Striatal MAO-A specific activity of the LD-DT, LD-NT, 24LL, and 24DD groups is presented in Figure 9.3. There was no evidence for variation in MAO activity between the different groups as assessed in the presence of [^{14}C]5HT alone or in combination with 5HT. The "[^{14}C]5HT:[^{14}C]5HT+5HT" activity ratio did show significant group-dependent variations (ANOVA: $P < 0.001$, $F = 9.699$). The ratio of the LD-NT group was significantly increased compared to that seen for LD-DT (Bonferroni and Student *t*-test: $P < 0.05$), 24LL (Bonferroni and Student *t*-test: $P < 0.01$) and 24DD (Bonferroni and Student *t*-test: $P < 0.05$). The ratio of the 24LL group was also significantly lower than seen for the LD-DT and 24DD groups (Student *t*-test: $P < 0.05$ in each case).

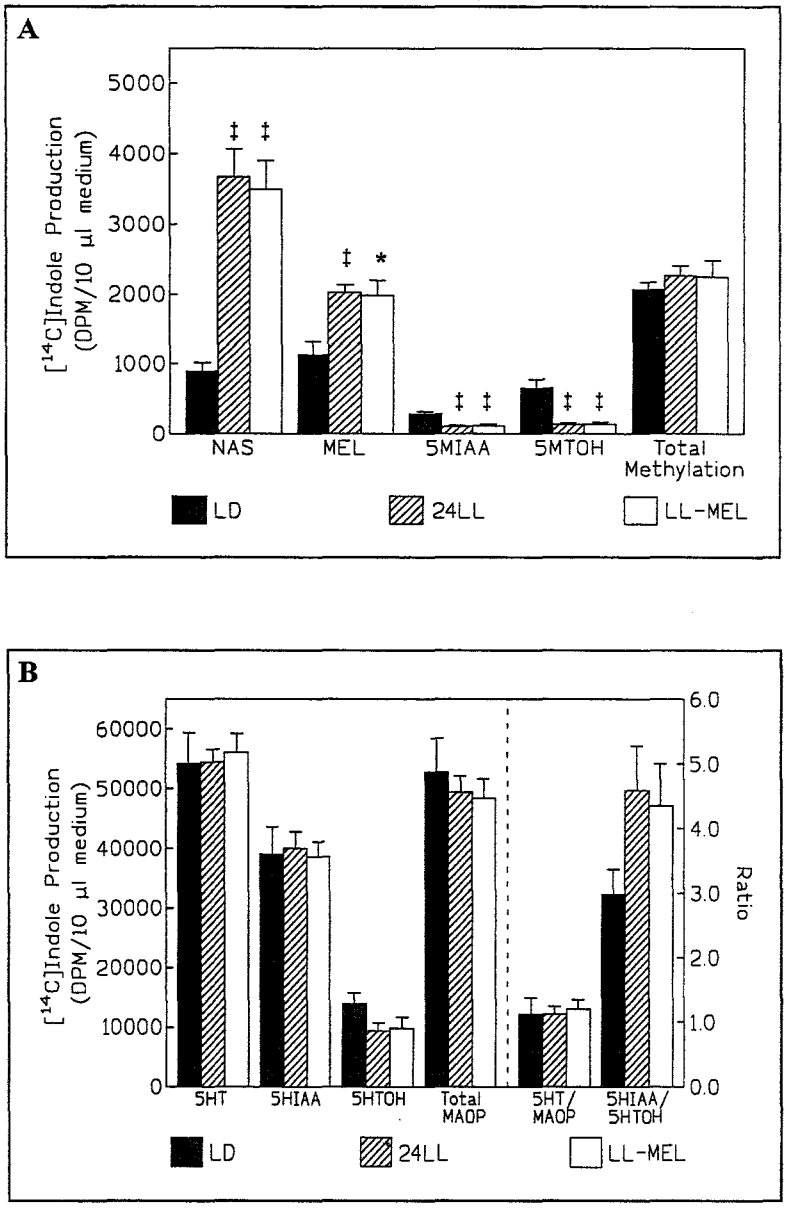


Figure 9.2: The effect of photoperiodic manipulation and late-afternoon MEL administration on the *in situ* biosynthesis of [^{14}C]indoles by rat pineal glands cultured during the late photophase. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 5); LD = normal lighting schedule; 24LL = constant light: vehicle-treated; LL-MEL = constant-light: MEL-treated; P-values (compared to LD-group): * < 0.05, ‡ < 0.01]

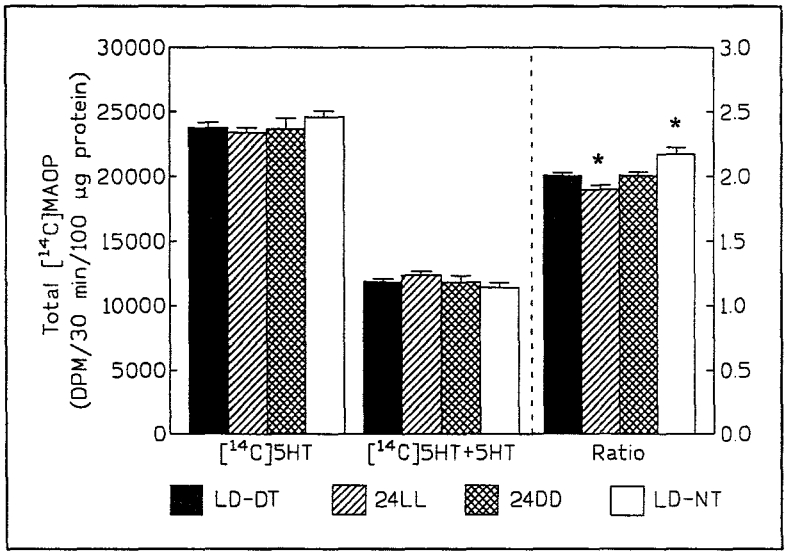


Figure 9.3: Day-night variation in striatal MAO-A activity and the effect of photoperiod manipulation. [Data represents mean ± SEM; LD-DT: mid-photophase of 12L:12D cycle (n = 7), 24LL: constant light (n = 8), 24DD: constant darkness (n = 7), LD-NT: mid-scotophase of 12L:12D cycle (n = 7); Ratio = [^{14}C]5HT:[^{14}C]5HT+5HT; P-values (compared to LD-DT): * < 0.05]

(ii) Protocol 2

Figure 9.4 shows the striatal MAO-A specific activity of the LD, 24LL and LL-MEL groups. Activity in the presence of [^{14}C]5HT alone showed no differences between the groups, whereas there was variation in combination with cold 5HT (ANOVA: $P < 0.05$; $F = 4.901$). The "[^{14}C]5HT+5HT" activity of the 24LL group was slightly, but significantly elevated compared to LD (Bonferroni and Student *t*-test: $P < 0.01$). There was also a marginal trend towards a decrease in "[^{14}C]5HT+5HT" activity of the LL-MEL group compared to 24LL (Student *t*-test: $P = 0.0502$). LL-MEL was not significantly different to LD. The "[^{14}C]5HT:[^{14}C]5HT+5HT" activity ratio also showed considerable group-dependent variation (ANOVA: $P < 0.01$; $F = 12.299$). The ratio of 24LL was significantly lower than for LD (Bonferroni and Student *t*-test: $P < 0.01$) and LL-MEL (Bonferroni: $P < 0.01$; Student *t*-test: $P < 0.05$). The LL-MEL ratio was marginally lower than for LD (Student *t*-test: $P = 0.0655$). This indicates that the present MEL administration protocol can reverse the effect of long-term light exposure on striatal MAO-A.

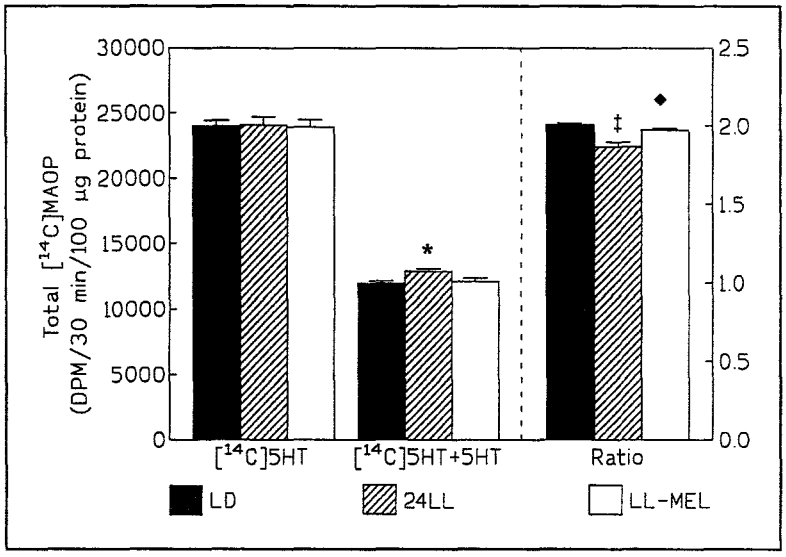


Figure 9.4: The effect of photoperiod manipulation and late-afternoon MEL administration on striatal MAO-A activity of the rat corpus striatum. [Data represents mean ± SEM (n=5); LD = normal lighting schedule; 24LL = constant light: vehicle-treated; LL-MEL = constant-light: MEL-treated; P-values (compared to LD): ◆ = 0.0655; * < 0.05; ‡ < 0.01]

9.3.2.2 COMT

(i) Protocol 1

Striatal COMT specific activity of the LD-DT, LD-NT, 24LL, and 24DD groups is presented in Figure 9.5. There was considerable variation in COMT activity between these groups (ANOVA: $P < 0.01$; $F = 6.260$). Activity of the LD-NT group was significantly increased compared to the LD-DT (Bonferroni: $P < 0.05$; Student *t*-test: $P < 0.01$) and 24DD groups (Bonferroni and Student *t*-test: $P < 0.01$). There was also a trend towards increased COMT activity in the 24LL group compared to the LD-DT (Student *t*-test: $P = 0.0672$) and 24DD groups (Student-Newman-Keuls and Student *t*-test: $P < 0.05$). There was no significant difference between the LD-DT and 24DD groups.

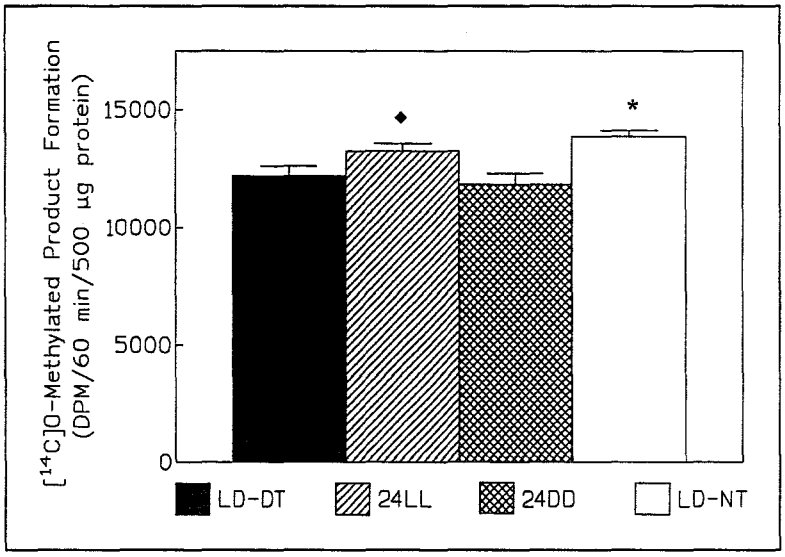


Figure 9.5: Day-night variation in striatal COMT activity and the effect of photoperiod manipulation. [Data represents mean ± SEM; LD-DT: mid-photophase of 12L:12D cycle (n = 7), 24LL: constant light (n = 8), 24DD: constant darkness (n = 7), LD-NT: mid-scotophase of 12L:12D cycle (n = 7); P-values (compared to LD-DT): ◆ = 0.0672; * < 0.05]

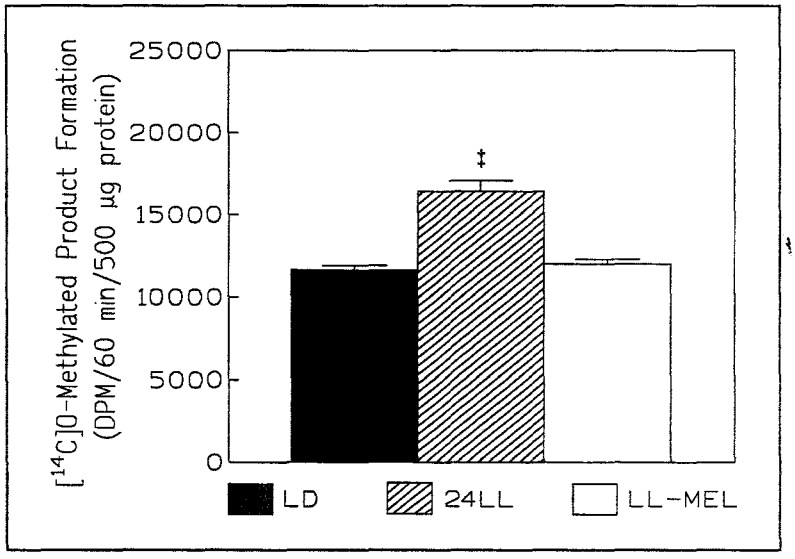


Figure 9.6: The effect of photoperiod manipulation and late-afternoon MEL administration on striatal COMT activity of the rat corpus striatum. [Data represents Mean ± SEM (n = 5); LD = normal lighting schedule; 24LL = constant light: vehicle-treated; LL-MEL = constant-light: MEL-treated; P-values (compared to LD): ‡ < 0.01]

(ii) Protocol 2

Striatal COMT specific activity of the LD, 24LL and LL-MEL groups is presented in Figure 9.6. There was considerable variation in COMT activity between these groups (ANOVA: $P < 0.001$; $F = 35.444$). The activity of the 24LL group was significantly greater than that of the LD (Bonferroni and Student *t*-test: $P < 0.001$) and LL-MEL groups (Bonferroni and Student *t*-test: $P < 0.001$). No significant difference in COMT activity was observed between the LD and LL-MEL. This indicates that MEL treatment potently reversed the effect of constant light on COMT activity to a level that was indistinguishable from control (LD) values.

9.4 Discussion and Conclusion

In situ [^{14}C]indole biosynthesis was employed as an index of the functional state of the pineal in the absence of information regarding the levels of endogenous MEL. The rhythm in pineal NAT activity and MEL production is endogenous, robust, and should persist unaltered in constant darkness (Miguez *et al.*, 1995). As an entraining agent, light makes small corrections to the phase characteristics of the rhythm, but MEL production consistently peaks during the scotophase, even in the absence of light. In the present study, however, constant darkness for 7 days resulted in a substantial change in the MEL/5MTOH ratio at mid-photophase. The synthesis of [^{14}C]5MTOH was dramatically enhanced without a change in [^{14}C]5MIAA and [^{14}C]MEL, resulting in a substantial increase in the apparent total methylation. Since there was no evidence for a change in NAT or MAO activity, the altered profile of methylation cannot be due to the bioavailability of 5-hydroxyindoles. Rather this profile must reflect an induction of HIOMT activity, but it is unclear why 5MTOH was preferentially increased. According to the O-methylation equation proposed by Morton (1990) [see section 3.4], NAS has the highest affinity for HIOMT and then 5HTOH. Thus MEL should be the preferential O-methylated product and not 5MTOH. Balemans *et al.* (1979) found that the MEL/5MTOH ratio in the rat pineal shifted with the season. The results presented in section 3.3 subsequently confirmed that the MEL/5MTOH ratio does appear to show photoperiod-dependent fluctuations, i.e. both circadian and seasonal. Balemans *et al.* (1979) attributed the change in pineal O-methylation to a pteridine-dependent regulation of HIOMT. Pteridines have been shown to potent inhibitors of pineal and retinal HIOMT (Cremer-Bartels *et al.*, 1983). Furthermore, several endogenous pterdines require photo-activation. Thus it is proposed that under conditions of constant darkness the synthesis of pterdines is reduced, disinhibiting the HIOMT enzyme.

The exposure of rats to constant light for 7 days or 21 days produced paradoxical effects on photophase pineal indole metabolism. Although the animals were sacrificed at mid-photophase and late photophase, respectively, the profile of [^{14}C]indole levels was identical, but the magnitude of the effect was more pronounced following the longer duration of illumination. In each case, the profile was consistent with a marked induction of NAT activity as evinced by the dramatic increase in [^{14}C]NAS. The increase in [^{14}C]MEL levels, but a decrease in [^{14}C]5MTOH and [^{14}C]5MIAA levels without a change in total methylation, is indicative of the relative bioavailability and affinity of each corresponding 5-hydroxyindole for HIOMT, without a change in the absolute activity of this enzyme (Morton, 1990). Cardinali *et al.* (1972) found that exposure of rats to continuous green light, the most effective wavelength of light, for 17 days resulted in an almost total loss of pineal HIOMT activity of Sprague-Dawley rats, and decreased the pineal weight. This is in contrast to the present study, where the extent of total methylation after 21 days of constant light is indistinguishable from that seen for the normal 12L:12D cycle, indicative of substantial HIOMT activity. This difference cannot be attributed to the nature or intensity of the light source used in the study. Common (cool-white) fluorescent light is reportedly more effective on pineal HIOMT than Vita-Lite, which simulates sea level solar radiation. This may be related to the fact that the cool-white light spectrum lacks erythemal UV radiation, which can transiently stimulate HIOMT activity (Cardinali *et al.*, 1972). However cool-white fluorescent tubes were also employed in the present study, and delivered a total radiant energy in excess of that used by Cardinali *et al.* (1972).

A reduction in sympathetic tone to the pineal causes a marked suppression of MEL biosynthesis through a rapid inactivation of pineal NAT activity. Sympathectomy can be produced by decentralization or bilateral ganglionectomy of the SCG, or physiologically with constant light conditions or exposure to light during the scotophase (Deguchi and Axelrod, 1972a and 1972b; Cantor *et al.*, 1981). These procedures increase the density or sensitivity of pineal β-receptors, which results in a superinduction of NAT and tryptophan hydroxylase in response to NA or other β-receptor agonists. Even a long photoperiod, rather than constant light, is sufficient to induce the aforementioned effects. Pineal indole biosynthesis during the photophase is basal and due to the lack of NA release rather than a lack of responsiveness of the β-adrenergic receptors. Constant light would maintain the post-ganglionic sympathetic nerve fibres of the pineal under permanent tonic inhibition through hyperpolarization. Furthermore, Abreu *et al.* (1987) reported that the activity of the NA-synthesizing enzymes TH and DBH of the rat pineal gland was completely abolished following constant illumination for three days. The subsequent reduction in the basal

synthesis and release of residual NA during the photophase may be sufficient to produce supersensitive β-receptors. It is further feasible that receptor sensitivity may increase with the duration of the constant light exposure, explaining the *ca.* 2-fold induction of NAT activity after 7 days, but a 4-fold increase after 21 days. However, the possibility of a chronotypic effect must be considered. Animals were sacrificed at mid-photophase and late photophase for the 7 day and 21 day study, respectively.

An alternative explanation involves the *in situ* radiometric nature of the organ culture technique, employing [^{14}C]5HT as the exogenous substrate. Although pineal L-Dopa decarboxylase activity peaks during the light-phase, the activity of pineal tryptophan hydroxylase, the rate determining enzyme in the synthesis of 5HT (Naoi *et al.*, 1994), is maximal during the dark-phase (Reiter, 1989). Thus chronic exposure to light may suppress tryptophan hydroxylase activity and 5HT synthesis. As a result, the vesicular stores of 5HT in the pinealocyte may be depleted, allowing the preferential N-acetylation of [^{14}C]5HT over 5HT. The lack of an effect of constant light on apparent pineal MAO activity further supports the notion that [^{14}C]5HT has been selectively taken up into the vesicular stores, rather than the cytoplasmic pool or into the presynaptic SNS nerve terminals (Juillard and Collin, 1979; Racké *et al.*, 1991). It must be reiterated that 70% of pineal MAO activity is present in the SNS nerve terminals and that NA is a substrate for MAO (Garrick and Murphy, 1982; Racké *et al.*, 1991). Thus a decrease in presynaptic NA levels induced by constant light would be expected to increase the degree of deamination of [^{14}C]5HT as described in section 3.4. The only possible effect on MAO activity, however, was a trend towards decreased [^{14}C]5HOTH synthesis after 21 days of constant light, which was also reflected in a more variable 5HIAA/5HTOH ratio, but not after 7 days of exposure. This lack of an obvious change in the apparent pineal MAO activity is a strong motivation for favouring an increase in the density or sensitivity of the β-receptors as the main mechanism behind the observed induction of [^{14}C]NAS and [^{14}C]MEL biosynthesis.

It must be noted that, regardless of the mechanism, the observed induction is more indicative of the functional state of the pineal at the time of removal than the extent of *in vivo* pineal MEL biosynthesis and plasma levels. With the current protocol, the explanted pineal is saturated *in situ* with exogenous [^{14}C]5HT making the superinduction of NAT activity apparent. *In vivo*, the tonic inhibition induced by light must be removed, the equivalent of adding substrate, before a rebound in MEL biosynthesis is possible. Ideally, another constant light group of rats should have been sacrificed at mid-scotophase for comparison. A suppression of nocturnal MEL synthesis under

conditions of constant light, but not constant darkness, would indicate that the organ culture technique is a reliable index of pineal function. The latter would also help interpret the mechanism operating behind the photophase constant light study.

The results also show that the chronic administration of MEL (25 μg/animal s.c.) for 20 days failed to alter the constant light-induced increase in [^{14}C]NAS and [^{14}C]MEL biosynthesis. This administration paradigm overcomes some of the problems that, arguably, may have been associated with the MEL administration studies presented in section 8.3. These include a more physiological dose and a longer duration of administration. In addition, the s.c. route delivers a more uniform and gradual release of MEL than the i.p. route, and largely bypasses hepatic metabolism. Nonetheless, in contrast to the *in vitro* study with MEL (see section 8.3), there is still no evidence for feedback regulation of pineal indole biosynthesis following *in vivo* administration of exogenous MEL, particularly at the level of MAO and HIOMT.

The photoperiod manipulation protocols also influenced both MAO-A and COMT activity of the corpus striatum, with a particularly robust effect on COMT activity. Exposure to constant darkness for 7 days had no effect on MAO-A activity at mid-photophase, but constant light caused a significant decrease in the ratio of "[^{14}C]5HT" activity to "[^{14}C]5HT+5HT" activity. Following 21 days of exposure, the decrease in the ratio was more pronounced and accompanied by an increase in "[^{14}C]5HT+5HT" activity. Subsequent MEL administration reversed the increase in "[^{14}C]5HT+5HT" activity and returned the ratio to a value that was almost indistinguishable to that seen for animals housed under a normal 12L:12D cycle. A parallel study confirmed that the "[^{14}C]5HT:[^{14}C]5HT+5HT" activity ratio is higher at mid-scotophase than at mid-photophase under a 12L:12D cycle as reported in section 8.3. These results further strengthen the argument (see section 8.4) that fluctuations in the ratio are potentially reliable indicators of changes in the activity or catalytic turnover rate of MAO-A. More specifically, both exogenous and endogenous MEL appear to be state-dependent modulators of the ratio. A decrease in the ratio appears to be associated with diminished endogenous levels of MEL (e.g. constant light), whereas an increased ratio is associated with high levels of MEL (e.g. during the scotophase, or following administration). Changes in the ratio are relative to a value that was calculated under the assay conditions specified in section 5.3. at mid-photophase for a normal 12L:12D cycle. The latter time period was also found to be a possible "refractory period" in the responsiveness of striatal MAO-A to MEL (see Figure 8.5). Thus deviations in the ratio appear to represent state-dependent shifts in the sensitivity of the enzyme to the pineal hormone.

It is difficult, however, to ascertain the reason behind the altered ratio with respect to the functioning of the enzyme. As discussed previously, MAO activity is most likely related to the amount of enzyme present (Costa *et al.*, 1980; Fowler and Wiberg, 1980), but changes in the catalytic properties of the enzyme cannot be excluded. For example, the presence of an endogenous inhibitor would be expected to alter the catalytic properties and thus the apparent activity. Under the conditions specified in section 5.2, both "[^{14}C]5HT" activity and "[^{14}C]5HT+5HT" activity increased with time, whereas the "[^{14}C]5HT:[^{14}C]5HT+5HT" ratio decreased after 30 min (see Figure 5.4). This decrease in the ratio can be attributed to the fact that the "[^{14}C]5HT" activity became progressively more non-linear as the amount of [^{14}C]5HT became limiting. By inference, a decreased ratio could also be equated with enhanced turnover of the substrate, i.e. increased enzyme activity. The relationship between MAO-A activity and enzyme content was determined in section 5.2 and is represented in Figure 5.3. The "[^{14}C]5HT:[^{14}C]5HT+5HT" ratio did not change over the range of mitochondrial fraction tested (10-60 µl). In other words, a decrease in enzyme content was not associated with an increase in the ratio. Furthermore a 20% increase in the amount of enzyme routinely used in the *in vivo* studies (50 *vs.* 60 µl) did not result in a decreased ratio. This implies that the change in MAO-A activity is not due to the amount of enzyme present, but due to the presence of a "modifier" such as MEL. In other words, the increase in the "[^{14}C]5HT: [^{14}C]5HT+5HT" ratio during the scotophase and following MEL administration is proposed to be a consequence of inhibition of the enzyme by MEL. The decreased ratio following exposure to constant light is consistent with the removal of MEL and thus disinhibition of the enzyme. This confirms the results of the previous *in vitro* and *in situ* studies (see sections 6.3 and 8.3 respectively) that MEL is a reversible inhibitor of MAO-A. More importantly, endogenous MEL would appear to regulate striatal MAO-A through an inhibition of the enzyme.

This conclusion is supported by previous studies. Urry and Ellis (1975) reported that MAO-A activity of the rat hypothalamus was unaffected by photoperiod, but increased by pinealectomy. In contrast, pituitary MAO was increased by constant light and pinealectomy, and decreased by constant darkness. Olcese and Devlaming (1979) reported that the MBH of the goldfish only exhibited a daily variation in MAO under a long, but not a short photoperiod. Furthermore, the MBH MAO activity was depressed by a short photoperiod, and pinealectomy increased the enzyme activity only when the fish were exposed to a short photoperiod. Thus the effect of endogenous MEL on MAO may be very tissue-specific, and the present study seems to be the first to report such an effect in the corpus striatum of the rat.

The results of photoperiodic manipulation of striatal COMT clearly suggest that both exogenous and endogenous MEL inhibit this enzyme, confirming the results presented in section 8.3. The extent of the constant light-induced increase in COMT activity was more pronounced after exposure for 21 days than for 7 days. It is believed that the time-dependency of this effect is related to the endogenous MEL levels and the mechanism behind MEL's inhibitory effect on COMT. A more chronic exposure would be expected to result in a more pronounced and complete suppression of plasma and tissue MEL levels. This is supported by the results discussed above that the pineal gland showed a greater degree of supersensitivity after 21 days than 7 days exposure. It was proposed in section 8.4 that MEL may influence the amount of active COMT enzymes, possibly at the level of gene expression or post-translational modification. Thus the longer MEL levels remain suppressed the longer the expression or modification of *de novo* COMT protein will be disinhibited. For example, 21 days may be more in line with the half-life or turnover rate of COMT mRNA and protein than 7 days. In a previous study (Figure 8.6), MEL administration at 16h00 (late photophase) had no effect on COMT activity. However, the latter study represented basal COMT activity under a normal 12L:12D cycle, whereas in the current study MEL reversed an induction of the COMT enzyme by constant light. This supports the contention that the replacement of the suppressed endogenous MEL with exogenous MEL maintains the inhibitory effect on COMT expression. The inability of constant dark exposure for 7 days to alter mid-photophase COMT activity is in line with the robust free-running rhythm of endogenous MEL synthesis. It further confirms that, under normal conditions, the mid-photophase represents a "refractory period" in responsiveness of the enzyme to MEL. Finally, a parallel study found that striatal COMT activity is higher at mid-scotophase of a 12L:12D cycle than at mid-photophase, confirming an earlier result (see Figure 8.6). This strengthens the argument put forward in section 8.4 to explain the apparent incongruity to the argument that MEL suppresses COMT activity.

Other methods of photoperiod manipulation could also be used to probe the nature and mechanism behind the regulation of striatal MAO-A and COMT by endogenous MEL. These include photoperiod reversal, exposure of dark-adapted animals to light or *vice versa*. It would also be informative to investigate the effect of constant dark and constant light on each enzyme at various times throughout the 24 hr cycle. This would indicate whether the day-night rhythm of each enzyme is endogenous or entrained by light. It is also possible that these lighting conditions may phase-shift the chronotypic sensitivity of MAO-A and COMT to MEL. The current studies were based on the principle that constant light exposure should suppress pineal MEL biosynthesis and plasma and tissue levels of the hormone. Although this assumption appeared to be validated by the

change in the functional state of pineal indole biosynthesis, the actual plasma levels of endogenous MEL were not determined. An alternative and more direct method to investigate the role of endogenous MEL would be to compare striatal MAO-A and COMT activity in sham-operated and pinealectomized rats.

Chapter 10

In Vivo γ-Hydroxybutyrate Administration Studies

10.1 Introduction

The predominant behavioural attribute of MEL is the entrainment of the circadian rhythmicity in locomotion and defined behavioural repertoires such as food-hoarding behaviour (Redman *et al.*, 1983; Armstrong, 1989; Underwood, 1990). As shown in section 8.3, subchronic administration of MEL during either the photophase or the scotophase failed to induce catalepsy, an akinetic state reflecting Parkinson's disease. At first, this appears paradoxical as catalepsy is a typical antidopamimetic behavioural response, and the evidence for an antidopamimetic effect of MEL has been extensively reviewed in section 1.6. MEL is a chronotypic modulator of dopaminergic function and DA-mediated behaviours, typically through tonic inhibition. Studies with animal models of DA dysfunction clearly show that the nature and intensity of the effect of MEL is dependent on the prevailing "tonus" of the midbrain dopaminergic pathways. The "tonus" can be differentially and selectively altered through pharmacological intervention with a variety of neurochemical agents (see section 1.6.5.3).

Several agents are capable of inducing catalepsy, but through different mechanisms (Sanberg, *et al*, 1988; Tunnicliff, 1992). These include reserpine, HAL and GHB. Reserpine is a nonspecific depletor of the presynaptic vesicular stores of DA, NA and 5HT. Specifically, the production of an akinetic state is attributed to the depletion of intraneuronal DA. HAL, a typical antipsychotic of the butyrophenone class, induces catalepsy through antagonism of postsynaptic D_2 receptors (Creese *et al.*, 1984; Lidsky and Banerjee, 1993). In contrast, GHB (Figure 10.1) suppresses the impulse flow and DA release of midbrain dopaminergic neurons (Walters and Roth, 1976; Engberg and Nissbrandt, 1993), primarily through an effect on inhibitory $GABA_B$ receptors (Engberg and Nissbrandt, 1993; Williams *et al.*, 1995; Snead, 1996). Thus a comparison of the ability of MEL to modulate the behaviour induced by each agent may give further information regarding the mode of action of MEL.

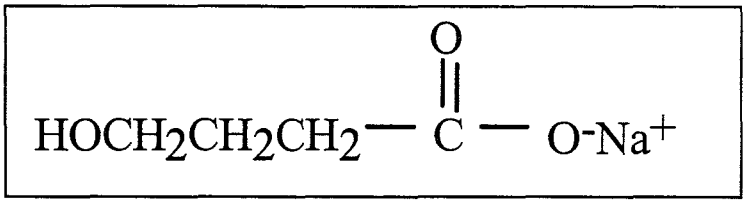


Figure 10.1: Structure of GHB monosodium salt.

MEL was reported to attenuate reserpine-induced catalepsy in female rats during the late photophase (Sandyk and Mukherjee, 1989; Sandyk and Fisher, 1989a). Pinealectomy was found to lessen HAL-induced catalepsy (Arushanyan *et al.*, 1992), but markedly increased the incidence and severity of HAL-induced oro-facial dyskinesia in male rats (Sandyk and Fisher, 1989b). Preliminary studies were performed by the present author to investigate the cataleptogenic potential of HAL and reserpine in adult male Wistar rats (data not shown). In the case of reserpine, a dose of 1 mg/kg bwt. was found to induce a catalepsy state that was far too robust for the present studies. Typically, the intensity of catalepsy in the reserpinized rats was in the order of 30 to 90 min, far in excess of the 180 s maximum cut-off allowed by the Standardized Horizontal Bar Test (Sanberg *et al.*, 1988; see section 2.6.2). This precluded the possibility of observing any effect of MEL. This was unfortunate, in that, unlike HAL, reserpine *in vivo* was found to have dramatic effects on pineal indole biosynthesis. In turn, HAL-induced catalepsy was highly variable, ranging in intensity from that seen for reserpine to mild sedation. Nonetheless, a preliminary study suggested that MEL pretreatment did not alter the intensity or frequency of HAL-induced catalepsy during the photophase.

To date no studies have investigated the effect of MEL administration and/or pinealectomy on GHB-induced catalepsy. The biochemistry of GHB has been reviewed in section 1.9. GHB represents a likely candidate in that it is a structural analogue of GABA (Vayer *et al.*, 1985). There is also strong evidence that it may be a neurotransmitter in its own right (Vayer *et al.*, 1988; Cash, 1994). The highest levels of endogenous GHB are found in the substantia nigra, although this area is sparse in specific [^{3}H]GHB binding sites (Hecher *et al.*, 1992). GHB inhibits both impulse-dependent and impulse-independent DA-mediated behavioural responses (Cott and Engel, 1977; Ellinwood *et al.*, 1983). In this way, GHB can induce sedation, catalepsy and anaesthesia (Tunnicliff, 1992).

Biochemically, several of the effects of MEL and GHB are mediated by similar neurotransmitter systems in the basal ganglia. GHB acts primarily through an effect on inhibitory $GABA_B$ receptors (Engberg and Nissbrandt, 1993; Williams *et al.*, 1995; Snead, 1996), but may also influence opioidergic systems (Feigenbaum and Howard, 1997). In turn, the effects of MEL on reserpine-induced catalepsy and HAL-induced oro-facial dyskinesias were attributed to a modulation of opioidergic systems (Sandyk and Fisher, 1989a; Sandyk and Fisher, 1989c). MEL also influences the central GABAergic/benzodiazepine system (Acuña Castroviejoy *et al.*, 1986a and 1986b; Rosenstein and Cardinali, 1986; Niles *et al.*, 1987; Coloma and Niles, 1988).

The prime object of this study was then to determine whether MEL can modulate GHB-induced behavioural responses. It was further investigated whether GHB has an effect on striatal MAO-A and COMT, as seen for MEL. Conversely, the possible reciprocal effect of GHB on pineal indole biosynthesis was also determined.

10.2 Materials and Methodology

10.2.1 Chemicals and Reagents

The chemical inventories for the pineal organ culture technique, MAO-A assay and COMT assay are listed in sections 3.2.1, 5.2.1 and 7.2.1 respectively. GHB monosodium salt was purchased from Sigma.

10.2.2 Animals

Male Wistar rats were housed as described in section 2.1. For all studies, the animals were acclimatized for 1 week to the prevailing light-dark cycle prior to commencement of the experiments.

10.2.3 Drug Preparation and Administration

GHB monosodium salt was prepared in saline (0.9% NaCl). In all studies, animals received a single dose of GHB (750 mg/kg bwt, i.p.) or an equivalent volume of the vehicle. MEL was prepared in the following vehicle: 2% (v/v) ethanol, 4% (v/v) Tween 80, 94% (v/v) deionised water.

10.2.4 BEHAVIOURAL TESTING

10.2.4.1 Testing Environment

Rats were habituated in individual testing arenas for 10 min prior to GHB administration. All testing was performed in a sound attenuated CER.

Preliminary observations revealed that the rats showed large inter-individual variation in behavioural responses to equivalent doses of GHB. Further observation led to the classification of the behavioural responses into three subjective, but distinct and robust states. These are defined below.

10.2.4.2 Categories

(i) Status A: Normal /Sedate

Following administration, these animals appeared, for all purposes, normal or showed mild sedation, evident as drowsiness. SLA was decreased to varying extents, if at all. The startle reflex appeared normal and the tail-pinch response was robust.

(ii) Status B: Cataleptic State

After a defined period following administration (see below), the GHB-treated animals were clearly cataleptic. Animals were lying flat on their stomachs with fore- and hind-limbs extended in spread-eagled posture. Ears were typically erect and eyes open with no evidence of spontaneous blink rate. The startle reflex in response to auditory stimuli was impaired and the tail-pinch response was delayed. The animals could be maintained in abnormal postures and the righting reflex was very delayed. Animals were aroused by more robust stimuli such as trying to move or handle them.

(iii) Status C: Anesthetic State

These animals exhibited the same behavioural responses as described for the "cataleptic state". However, there was no startle reflex or tail-pinch response, and the animals could be moved and handled without arousing them. In each experiment, the behavioural response of each animals was classified as above and the overall frequency of each behavioural state calculated

10.2.4.3 Time Parameters

In a further attempt to quantify the above behavioural responses, the time parameters were defined and measured as below.

(i) Onset Time

The time taken to enter either the "cataleptic" or "anesthetic" state as measured from the time of GHB administration [T = 0 min].

(ii) Duration

The time spent in "cataleptic" or "anaesthetic" state as measured from time of onset to first evidence of spontaneous recovery.

10.2.5 PINEAL ORGAN CULTURE STUDIES

The standard organ culture technique and TLC analysis of [^{14}C]indoles was performed as described in section 3.2.4. Following excision of the pineal glands, an attempt was made to rapidly remove the pineal stalk prior to incubation. These pineal studies were performed in the summer months of January and February.

(i) *In Vivo* Studies

The effect of GHB administration on photophase [^{14}C]indole biosynthesis was determined for pineal glands isolated and cultured at mid-photophase (12h00). Pineals were rapidly transferred to the corresponding pre-labeled Kimble tubes. The final total volume of all samples was 60 µl for *in vivo* studies, giving a final concentration ≈ 120 µM [^{14}C]5HT.

(ii) *In Vitro* Study

The *in vitro* effect of GHB (100 µM) on [^{14}C]indole biosynthesis was determined for pineal glands cultured at mid-photophase (12h00). Untreated pineals were randomly placed in individual Kimble tubes. GHB was prepared in culture medium and added in a volume of 10 µl to give the desired final concentration. [^{14}C]Indole biosynthesis was initiated by the addition of [^{14}C]5HT immediately thereafter. Thus, for all practical purposes, there was no preincubation period with GHB. The final total volume of all samples was 70 µl for *in vitro* studies, giving a final concentration ≈ 100 µM [^{14}C]5HT.

10.2.6 ENZYME ASSAYS

10.2.6.1 Tissue Preparation

The corpora striata were removed and stored as described in section 2.2. Enzyme preparations for the MAO-A and COMT assays were obtained from the same tissue source by the subcellular fractionation protocol described in section 2.3.

10.2.6.2 Protein Determination

For all *in vivo* studies, statistical analysis was performed on enzyme specific activities. Thus, the protein concentration of each enzyme preparation was determined in triplicate, as described in section 2.4. Mean protein concentrations did not differ significantly between the experimental groups for the MAO-A and COMT enzyme preparations (data not shown).

10.2.6.3 MAO-A Assay

All studies were performed on the 4% (w/v) mitochondrial/lysosomal fraction. The assay components and conditions were based on the optimum values experimentally determined in section 5.3. MAO-A activity was determined with [^{14}C]5HT (0.025 μCi; 4.5 μM) as the sole substrate, and in the presence of 150 μM 5HT. The ratio of "[^{14}C]5HT" activity to "[^{14}C]5HT+5HT" activity was also calculated for each sample.

10.2.6.4 COMT Assay

All studies were performed on the 5% (w/v) cytosolic/microsomal fraction. The assay components and conditions were based on the optimum values experimentally determined in section 7.3. It must be stressed that for *in vivo* studies, COMT activity was determined under conditions of maximal induction by $MgCl_2$ (10 mM) and a near-saturating concentration of DA HCl (1 mM).

10.2.7 EXPERIMENTAL DESIGN

(i) State-dependent behavioural effects of GHB

The behavioural effects of GHB were observed following administration at mid-photophase [12h00] (n = 22) or at mid-scotophase [24h00] (n = 21). The behavioural response of each animal was designated to one of three groups defined above, and where applicable the "onset time" and "duration" of the response was recorded.

(ii) Modulation of GHB-induced behaviour by melatonin

In an attempt to minimize the variation in behavioural responses, animals were screened in a preliminary study. Only rats (n = 28) which were completely anesthetized (i.e. Status C) by GHB (750 mg/kg i.p.) administration during the photophase were utilized for this study. These animals were randomly assigned to either the vehicle- (n = 14) or the MEL-pretreated group (n = 14) and allowed a 7 day "washout" period for recovery from the GHB treatment.

The animals then received a single dose of MEL (2 mg/kg bwt) or vehicle i.p. 5 min prior to GHB administration at 12h00. Behavioural responses were observed and quantified as above.

(iii) Effect of acute GHB administration on MAO-A, COMT and pineal function

Rats received a single dose of GHB (n = 6) or vehicle (n = 6) 20 min prior to sacrifice at mid-photophase (12h00).

(iv) *In vitro* effect of GHB on pineal function

The *in vitro* effect of GHB (100 μM) on pineal [^{14}C]indole biosynthesis was investigated according to the protocol described above. Animals were drug-free.

10.2.8 Data and Statistical Analysis

All data are graphically presented as mean ± SEM. The sample size (n) depends on the study. Statistical comparison of group mean values was performed by means of the Student *t*-test.

10.3 Results

10.3.1 Behavioural Studies

(i) Day-night variations in GHB-induced behavioural responses

Considerable inter-individual variation in the type and magnitude of behavioural responses to a fixed dose of GHB was observed in the rats. The GHB-treated animals could be consistently and reliably categorized into one of the three defined behavioural states. Vehicle-treated animals showed no obvious behavioural abnormalities. A comparison of the photophase and scotophase studies revealed no significant alteration in the frequency of each GHB-induced behavioural response (Figure 10.2). Significant state-dependent differences were seen in the onset time of catalepsy and anaesthesia, but not in the duration of these responses (Figure 10.3). The data

indicates that the rats were less susceptible to GHB treatment during the scotophase than the photophase ($P < 0.05$ for the "cataleptic" data; $P < 0.01$ for the combined "cataleptic and anaesthetic" data).

(ii) Modulation of GHB-induced behaviour by melatonin

Pretreatment with MEL failed to modulate GHB-induced behaviour with respect to the frequency of the three behavioural responses (Figure 10.4), or the onset time and duration of catalepsy and anaesthesia (Figure 10.5). It must be noted that all the animals used in this study were completely anesthetized by GHB in a preliminary screening experiment, but all three behavioural responses were induced by GHB in both the vehicle- and MEL-pretreated groups.

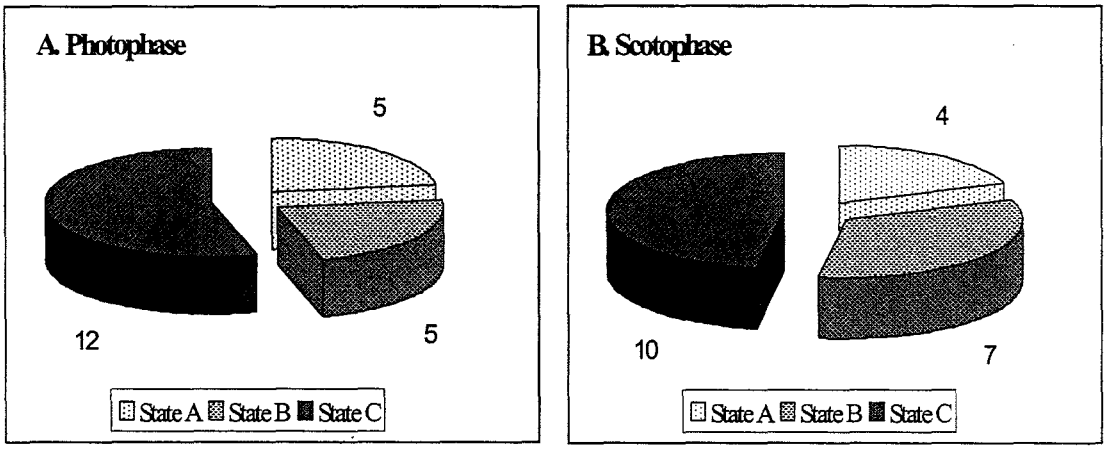


Figure 10.2: Frequency of GHB-induced behavioural states. (A) Photophase. (B) Scotophase. [State A: sedation; State B: catalepsy; State C: anaesthesia. See section 10.2.4.2 for explanation]

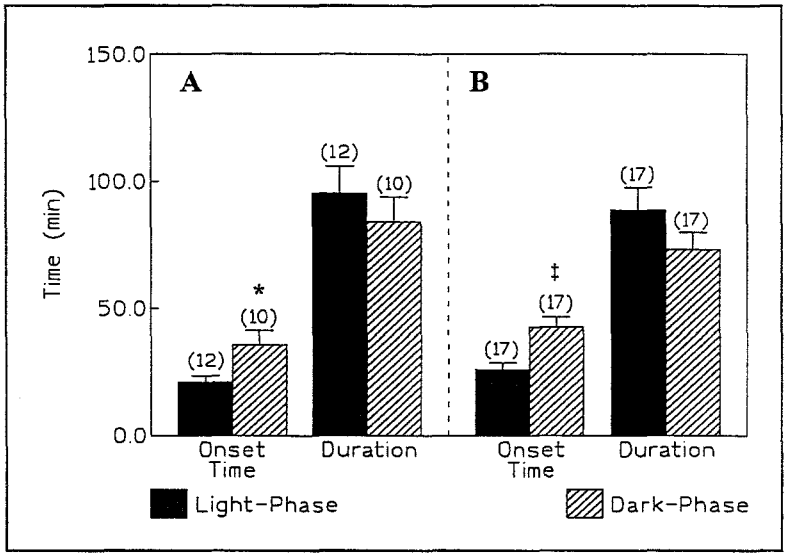


Figure 10.3: Comparison of time parameters of GHB-induced behavioural responses during the photo- and scotophase. (A) Cataleptic state. (B) Cataleptic and Anaesthetic states. [Data represents mean ± SEM; Numbers in parentheses refer to sample sizes; *: $P < 0.05$, ‡: $P < 0.01$]

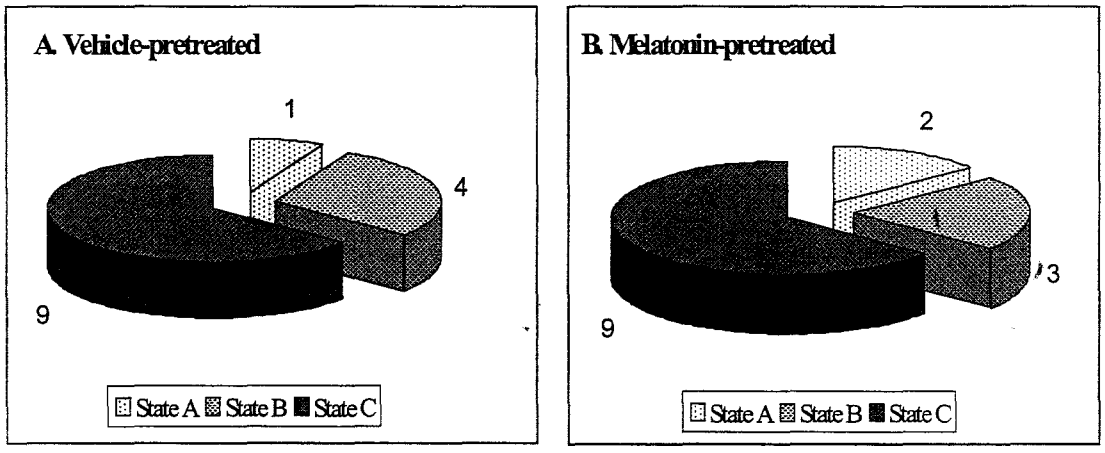


Figure 10.4: Frequency of GHB-induced behavioural states during the photophase: modulation by MEL. (A) vehicle-pretreated. (B) MEL-pretreated. [State A: sedation; State B: catalepsy; State C: anaesthesia. See section 10.2.4.2 for explanation]

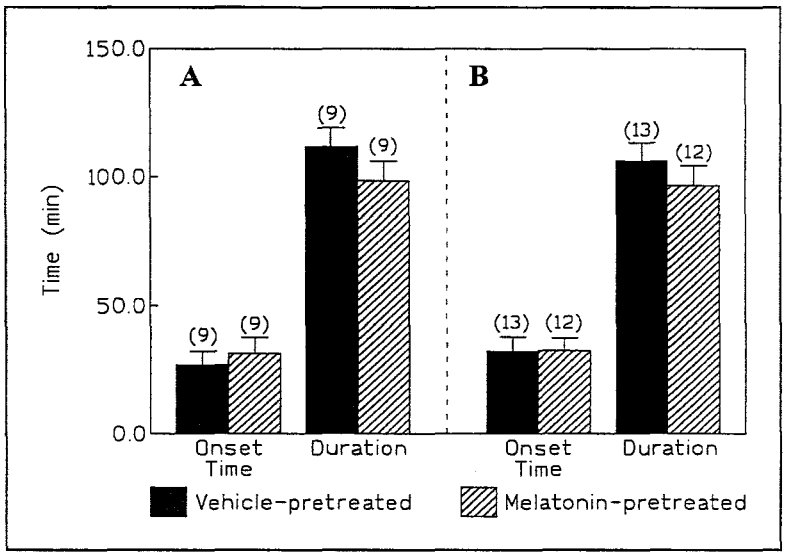


Figure 10.5: Comparison of time parameters of GHB-induced behavioural responses in vehicle- and MEL-pretreated animals during the photophase. (A) Cataleptic state. (B) Cataleptic and Anaesthetic states. [Data represents mean ± SEM; Numbers in parentheses refer to sample sizes]

10.3.2 Pineal Organ Culture Studies

(i) *In Vivo*

GHB administration resulted in a significant increase in [^{14}C]MEL synthesis and total methylation ($P < 0.05$ in each case), without an effect on any other 5-methoxyindole (Figure 10.6 A). There was no effect on the synthesis of any radiolabeled 5-hydroxyindole (Figure 10.6 B). The 5HIAA/5HTOH ratio showed higher than normal variability for both the GHB- and vehicle-treated samples. Furthermore, [^{14}C]5HT and [^{14}C]5HIAA levels, total MAOP and the 5HT/MAOP ratio of the GHB-treated samples also showed high variability. It is unclear whether this variability is an effect of the vehicle, GHB or the fact that the study was performed in February. However, the control and GHB-treated [^{14}C]NAS levels and control [^{14}C]MEL levels were similar to that normally seen for winter months, and not the lower levels expected for summer months (see section 3.3). This may be an effect of the saline vehicle.

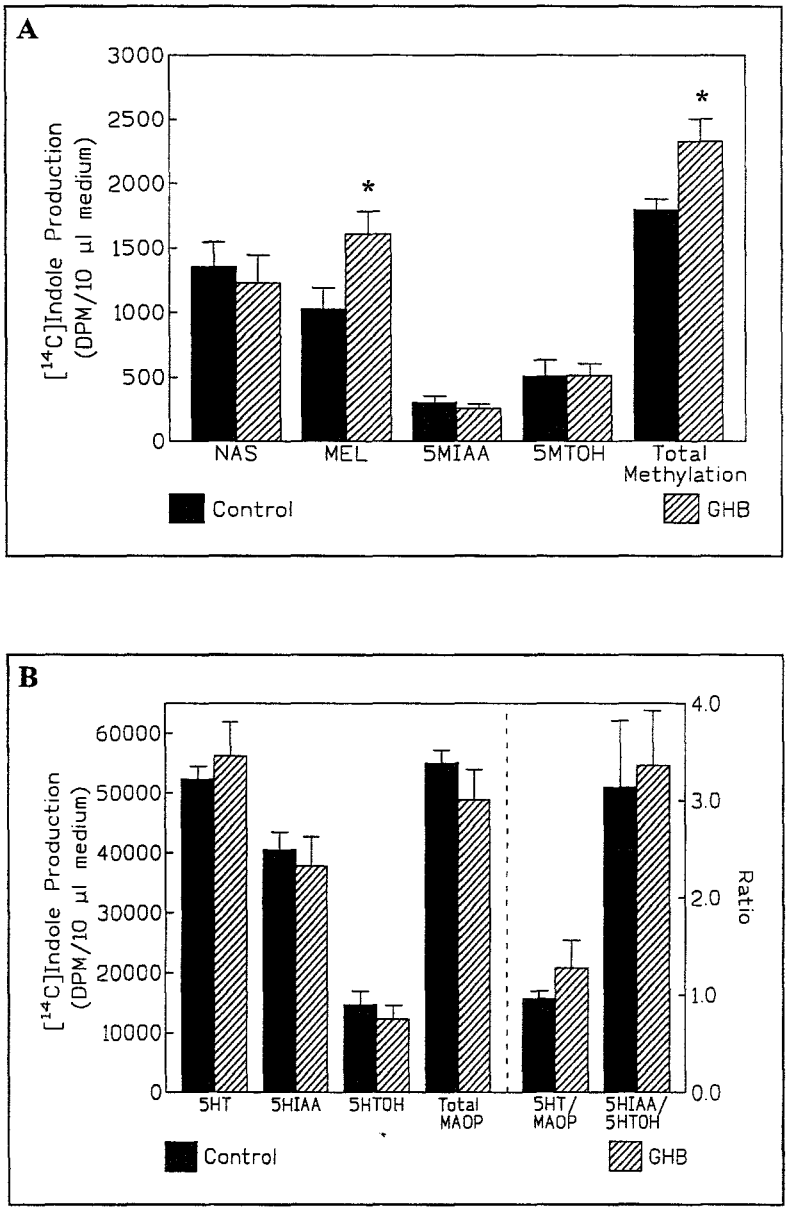


Figure 10.6: The effect of acute administration of GHB on the *in situ* biosynthesis of [^{14}C]indoles by rat pineal glands cultured during the mid-photophase [12h00]. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 6); P-values (compared to vehicle-treated controls): * < 0.05]

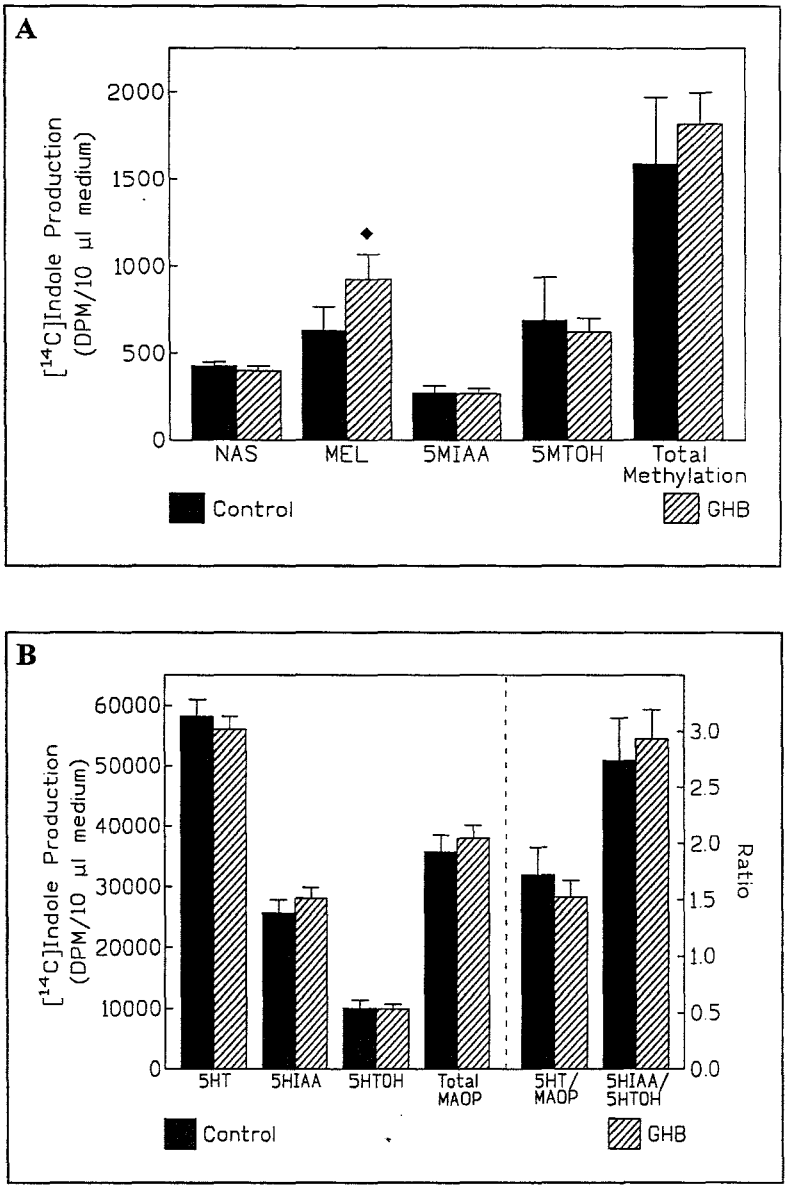


Figure 10.7: The *in vitro* effect of GHB (100 µM) on the *in situ* biosynthesis of [^{14}C]indole by rat pineal glands cultured during the mid-photophase [12h00]. (A) NAS and 5-methoxyindoles. (B) 5HT and deaminated indoles. [Data represents mean ± SEM (n = 6); P-value (compared to control): ◆ = 0.163]

(ii) *In Vitro*

GHB (100 μM) had no significant *in vitro* effect on the *in situ* biosynthesis of any [^{14}C]indole (Figure 10.7). There was a trend towards a GHB-induced increase in the synthesis of [^{14}C]MEL (P = 0.1633 compared to control; Figure 10.7 A). The low [^{14}C]NAS levels and high variability in 5-methoxyindole synthesis can be attributed to the fact that this study was performed in January (see section 3.3). This variability may explain the difficulty in delineating significance in the observed trends.

10.3.3 ENZYME STUDIES

(i) MAO-A

The effect of acute GHB administration on MAO-A activity of the corpus striatum is shown in Figure 10.8. MAO specific activity, with or without cold 5HT (150 μM), and the ratio of "[^{14}C]5HT" activity to "[^{14}C]5HT+5HT" activity were not significantly altered.

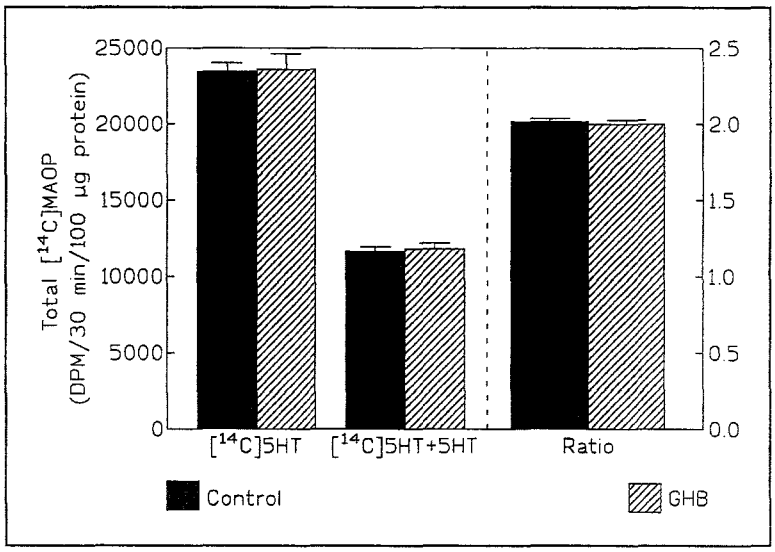


Figure 10.8: The effect of acute GHB administration on *in vitro* MAO-A activity of the rat corpus striatum during the mid-photophase (12h00). (Data represents mean ± SEM (n = 6); Ratio = [^{14}C]5HT:[^{14}C]5HT+5HT)

(ii) COMT

Acute GHB administration had no significant effect on COMT specific activity (Figure 10.9).

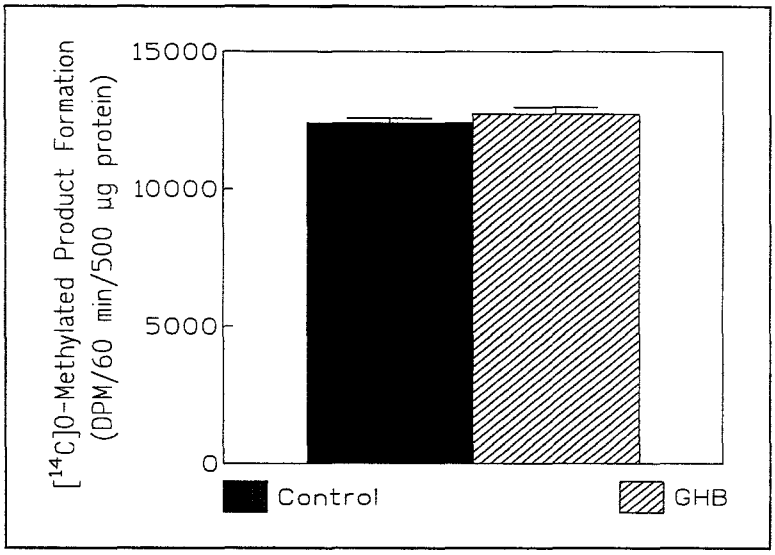


Figure 10.9: Effect of acute GHB administration on *in vitro* COMT activity of the rat corpus striatum during the mid-photophase (12h00). [Data represents mean ± SEM (n = 6)]

10.4 Discussion and Conclusion

In the present studies, a dose of GHB (750 mg/kg i.p.) known to cause anesthesia (Vickers, 1969; Walters and Roth, 1976; Tunnicliff, 1992) was routinely employed. Nonetheless, the responses of the animals were highly variable, ranging from mild sedation through catalepsy to complete anesthesia. Anesthesia, however, was the predominant effect at this high dose. This profile is consistent with the ability of GHB to induce behavioural responses indicative of dopaminergic hypofunction, including akinesia, catalepsy and sedation (Tunnicliff, 1992). In addition, subsequent challenges with GHB induced different behavioural responses in the same rat. This could not be attributed to an accumulative dose-effect. GHB is rapidly metabolized to carbon dioxide and water such that no drug is detectable in the urine 4-5 hrs after administration (Vickers, 1969). Thus GHB has no toxic effects on the liver, kidney or other organs. However, GHB may be converted back to GABA (Vayer *et al.*, 1985). For this reason, an extended "wash-out" period of 7 days between drug administrations was employed to allow normalization of brain function.

The observed variability may be related to the pharmacokinetics of GHB, which is not absorbed as rapidly and uniformly across the blood-brain barrier as its prodrug gamma-butyrolactone following i.p. administration (Walters and Roth, 1976). Alternatively, it may reflect individual differences in the activity state of the dopaminergic neurons. Neurophysiologically, GHB suppresses impulse flow from the substantia nigra to the corpus striatum with a concomitant inhibition of DA release (Walters and Roth, 1976; Alter *et al.*, 1984; Engberg and Nissbrandt, 1993; Howard and Feigenbaum, 1997). For this reason, the efficacy of a given dose of GHB may depend on the prevailing spontaneous neuronal firing rate. The activity of mesencephalic dopaminergic neurons is known to increase with locomotion, eating and drinking, and novelty seeking behaviour, and displays considerable inter-individual variation (Piazza *et al.*, 1996). This may explain the decreased sensitivity of rats to GHB-induced catalepsy and anesthesia in the scotophase compared to the photophase. Rats are nocturnal animals displaying increased SLA at the onset of darkness. This is paralleled by a rapid elevation in the firing rate of midbrain dopaminergic neurons and increased DA release (Piazza *et al.*, 1996; Paulson and Robinson, 1994). A delay in the ability of GHB to reduce extracellular DA to levels sufficient for the induction of catalepsy and anesthesia may then explain the prolonged "onset time" during the scotophase.

Plasma and tissue MEL levels also peak in the scotophase following a surge in secretion and biosynthesis of the pineal hormone at the onset of dark (Reiter, 1991). Thus it is possible that the decreased sensitivity to GHB during the scotophase may also be related to enhanced MEL levels at this time. There is a strong consensus that the central effects of GHB, including the inhibition of DA release and akinetic behaviours, relate mainly to the agonistic effect of the drug at $GABA_B$ receptors (Waldmeier, 1991; Xie and Smart, 1992; Engberg and Nissbrandt, 1993; Williams *et al.*, 1995; Snead, 1996), and an influence on opioidergic systems (Feigenbaum and Howard, 1997). MEL is reported to inhibit DA release in several brain regions, but, paradoxically, not the corpus striatum (Zisapel *et al.*, 1982; Dubocovich, 1983 and 1984; Nowak, 1988). The well-established ability of MEL to modulate behaviours indicative of DA dysfunction has been attributed to an effect on several neurotransmitter systems (see review in section 1.6.5.3). These include, *inter alia*, dopaminergic (Bradbury *et al.*, 1985; Burton *et al.*, 1991), serotonergic (Durlach-Misteli and Van Ree, 1992), opioidergic (Gaffori and Van Ree, 1985b) and GABAergic systems (Tenn and Niles, 1995). More specifically, the ability of MEL to antagonize APO-induced rotational behaviour in 6OHDA-lesioned rats appears to be facilitated by the striatal $GABA_A$/benzodiazepine receptor complex (Tenn and Niles, 1995). Although GHB has no effect on $GABA_A$ receptors

(Serra *et al.*, 1991), this does suggest that MEL and GHB may exert behavioural effects through a common denominator in the nigro-striatal pathway, namely the GABAergic system.

However, under the specified conditions, pretreatment with MEL, to mimic the scotophase, failed to modulate GHB-induced behaviour during the photophase. This does not preclude a role for endogenous MEL as other variables need to be considered, including the dose, route and time of administration of the hormone. Furthermore, earlier studies have clearly shown that the biochemical and behavioural effects of MEL are known to be chronotypic, depending on the responsiveness of the neuronal target. There was also no obvious relationship between the polymorphism in pineal [^{14}C]NAS and [^{14}C]MEL biosynthesis and the intensity of the behavioural response to GHB.

Conversely, the effect of GHB on *in situ* pineal [^{14}C]indole biosynthesis was also investigated. Following GHB administration, there was only a significant increase in [^{14}C]MEL biosynthesis and total methylation. This profile is difficult to interpret, but may arise through a stimulation of either NAT or HIOMT activity without an effect on MAO activity. The important point is that an effect on the biosynthesis and secretion of endogenous MEL may represent a part of the *modus operandi* of GHB. The existence of an intrapineal GABAergic system in the rat is well-established (Rosenstein *et al.*, 1990), but the stimulatory effect reported here is not consistent with agonistic activity of GHB at the inhibitory $GABA_B$ receptor. The existence of independent [^{3}H]GHB binding sites in the pineal gland has not yet been investigated. Alternatively, this effect of GHB may result from a reduction in endogenous DA release, disinhibiting NAT activity. An ability of DA to inhibit rat pineal NAT activity is implicit in the results reported in section 4.3, confirming a previous study by Govitrapong *et al.* (1989).

Strong immunohistochemical evidence has been presented that the rat pineal is innervated by dopaminergic fibres arising outside the gland, mostly from central structures including the habenula complex and the posterior commissure (Zhang *et al.*, 1991). It may be argued that GHB (100 μM) *in vitro* induced a trend towards increased [^{14}C]MEL biosynthesis. The lack of significance in the latter study may be related to the dose of GHB used *in vitro*, namely 100 μM. A more intensive dose-dependency study may have revealed more significant effects, paralleling the effective dose of the *in vivo* administration. Alternatively, the trend observed *in vitro* simply reflects variation in the sample data. This would then suggest that GHB, *in vivo,* may have inhibited presynaptic DA release in the pineal by suppressing the impulse flow of the

dopaminergic fibres arising outside the pineal, rather than a direct effect in the pineal itself. In this regard, GHB could be used to delineate the origin of these fibres. For example, one could check for an increase in pineal TH activity of TH-positive/DBH-negative immunoreactive fibres following the site-specific application of GHB. In particular, GHB may represent the ideal tool to determine if the pineal represents an output structure of the basal ganglia. This would strengthen the argument for a reciprocal relationship between the striatum and the pineal, i.e. the putative pineal-striatum axis.

Concomitant with a suppression of impulse flow and an inhibition of DA release, GHB induces a marked increase in the activity of TH and DA levels present within presynaptic dopaminergic nerve terminals (Walters and Roth, 1976). Indeed TH activity and DA levels are employed as presynaptic markers in the Walters-Roth model to investigate whether dopaminergic agents act at autoreceptors. Suppression of nigro-striatal impulse flow blocks postsynaptic feedback effects of the same drug arising through local neuronal loops, e.g. the striato-nigral pathway. For example, low doses of APO markedly attenuate the GHB-induced increase in TH activity and intracellular DA levels (Walters and Roth, 1976).

MAO-A and COMT are located pre- and post-synaptically in the rat corpus striatum. Thus the potential of these enzymes as biochemical markers for GHB studies, instead of TH, was investigated. The lack of effect of GHB administration on mid-photophase MAO-A or COMT activity suggests that these enzymes are not involved in the behavioural effects of the drug. This does not preclude possible dose- and state-dependent or chronotypic effects of GHB administration. It is important to note that both enzymes did not appear sensitive to the transient, but dramatic, flux in the steady-state dynamics of the neurons induced by GHB. Regardless of the GHB-induced increase in the levels of endogenous DA, a substrate common to both enzymes, there was no change in MAO-A and COMT activity determined *ex vivo*. In contrast, prolonged manipulation of endogenous MEL levels caused marked changes to the *ex vivo* activity of both enzymes (see sections 8.3 and 9.3). In future studies, the potential autoreceptor function of MEL in the striatum should by investigated by using the classic Walters-Roth model, in others words, to determine if MEL can modify the GHB induced increase in TH activity.

Chapter 11

Conclusions and Future Perspectives

The current results provide evidence suggestive of MEL-DA interactions in the CNS of the male Wistar rat at the level of the pineal gland and corpus striatum of the basal ganglia. The thesis objectives were achieved through two main experimental strategies. Firstly, dopaminergic agents were found to have an inhibitory effect on the induction of pineal MEL biosynthesis during the scotophase. Reciprocally, MEL exerted an inhibitory effect on striatal dopaminergic function at the level of COMT and MAO-A activity. In other words, the nature of the MEL-DA interaction would appear to be one of functional antagonism.

Previous research into the neuroendocrine and circadian effects of MEL revealed the antidopamimetic potential of this hormone in the hypothalamus and pituitary. Research on animal models of DA dysfunction also suggested that MEL exerted tonic inhibition on the behavioural output of the mesencephalic dopaminergic systems. However, the latter findings were backed up by limited biochemical studies. Furthermore, these behavioural effects of MEL could be equally attributed to an effect on numerous other neurotransmitter systems operative in the basal ganglia, besides the nigro-striatal and mesolimbic dopaminergic systems. To this author's knowledge, the present results represent the first evidence that MEL may be an endogenous and chronotypic regulator of striatal DA catabolic enzymes. This extension of an antidopamimetic effect of MEL to the corpus striatum supports the hypothesis that MEL may be a homeostatic modulator of dopaminergic neurotransmission throughout the CNS. In line with the existence of a pineal-hypothalamic axis, the present findings will help define the nature of a putative pineal-striatal axis. Such a holistic concept of brain homeostasis is necessary to delineate the diverse array of effects of MEL on brain neurochemistry and behaviour.

The robust diurnal rhythm in pineal indole metabolism is considered the most reliable indicator of the functional state of the pineal gland. The *in situ* nature of the present organ culture technique offered several advantages. These included the determination of indole biosynthesis by a single pineal under physiological conditions, but free from humoral or neural factors of the *in vivo* milieu. In other words, the effects of APO and DA could, theoretically, be investigated in isolation. The only potential disadvantage was the radiometric nature of the present technique.

Indole metabolism was quantified by sampling exogenous [^{14}C]indoles released into the culture medium, rather than the pineal content of endogenous indoles. Nonetheless, an assessment of [^{14}C]indole biosynthesis revealed that the radiometric technique was capable of accurately reflecting both the species-dependent profile of indole metabolism of the rat pineal gland, and state-dependent metabolic fluxes. More specifically, the present results confirmed that the activity of pineal NAT is the pivotal regulatory step associated with the day-night and seasonal variation in MEL biosynthesis. 5-Methoxyindole biosynthesis was primarily determined by the bioavailability and affinity of each corresponding 5-hydroxyindole for HIOMT, rather than the activity of the latter enzyme. In this regard, the MEL/5MTOH ratio appeared to be another important index. Both circadian and seasonal changes in photic stimulation resulted in a shift in this ratio. This may simply reflect a change in the relative synthesis of MEL and 5MTOH due to changes in NAT activity. Alternatively, the role of endogenous pteridines in the regulation of pineal HIOMT activity warrants further investigation.

It must be noted that a potential problem of the current radiometric technique involves the metabolic competition between exogenous [^{14}C]indoles and endogenous indoles. The latter was found to interfere with data analysis, especially with regard to the apparent pineal MAO activity. Generally, such complications were fairly noticeable and could be accounted for in data interpretation. It can also be argued that the awareness of this phenomenon may be informative, revealing aspects of pineal indole metabolism not observed in other techniques. For example, the present system revealed that MEL and other indoles may indeed exert negative feedback control on pineal indole biosynthesis. Evidence for such a paracrine control of the pineal by MEL has been strongly debated. Another case in point is that under certain experimental situations the metabolic state of pineal [^{14}C]indole metabolism quantified *in situ* is not directly correlated to the prevailing plasma level of MEL. For example, constant light exposure, which is known to suppress pineal MEL biosynthesis *in vivo* and thus the plasma levels of the hormone, potentiated [^{14}C]indole metabolism during culture. In future studies, these short-comings of the radiometric organ culture technique can be avoided by determining the pineal content of endogenous indoles by HPLC with dual electrochemical and fluorometric detection. Alternatively, the organ culture technique itself could be replaced by *in vivo* microdialysis with test drugs applied by iontophoresis. Finally, endogenous plasma and tissue levels of MEL should also be assessed directly by HPLC, RIA or ELISA. This will indicate whether an experimental situation altered pineal MEL biosynthesis, the distribution and metabolic fate of the hormone following secretion, or both.

High inter-individual variability in the biosynthesis of pineal [^{14}C]NAS and [^{14}C]MEL has also been a constant hall-mark of the radiometric organ culture technique. In an attempt to manage this variability, it was found that, like humans, Wistar rats can be divided into high and low producers of MEL. It is most likely that this polymorphism is inherent, arising through genetic regulation of key enzymes or the noradrenergic secretory drive. However environmental factors and social interactions cannot be ruled out, until a multifactorial analysis can be performed. There was no apparent correlation between the extent of [^{14}C]NAS and [^{14}C]MEL biosynthesis and the size of the pineal gland. In future studies, a relationship should be sought at the level of enzyme activities or the cell density of pinealocytes. This MEL polymorphism may have important functional implications, but none were apparent in the present studies. No obvious relationship was found between pineal [^{14}C]MEL production and striatal MAO-A and COMT activity, or behavioural responses of the animals to exogenous MEL or GHB administration. Practically, however, awareness of this polymorphism should assist in the optimization of experimental design.

The studies with APO and DA provided evidence that a phase-dependent dopaminergic system is operative in the pineal gland of the Wistar rat. Both these agonists caused a marked suppression of NAS and MEL biosynthesis during the scotophase, consistent with an inhibitory action on the nocturnal induction of pineal NAT activity. Pharmacologically, it is proposed that postsynaptic D_2 or D_2-like receptors, located on the pinealocyte plasma membrane, were responsible for mediating the effects of DA and APO. This finding clarifies and extends the studies of Govitrapong *et al.* (1984 and 1989). It remains likely that other DA receptor subtypes are also present in the pineal gland. There was also some indication that the functional or conformational state of pineal DA receptors may vary in a manner dependent on the phase of the light:dark cycle. The exact nature and location of these receptor subtypes can be probed with more selective D_2 agonists and antagonists, immunocytochemistry, and by *in situ* hybridization of mRNA transcripts. The mechanism behind these events should be investigated biochemically at the level of the NAT enzyme and the cAMP signal transduction system. For example, one could determine if DA and APO are capable of reversing the induction of NAT activity by NA or other β-agonists during the photophase.

The above D_2 receptor-mediated events provide a mechanism by which DA can control indole biosynthesis, but does not indicate the ultimate function of this neurotransmitter within the pineal. The functional nature of the dopaminergic system is likely to be closely associated with the morphology of the system. The pineal dopaminergic system of the rat is believed to be

independent of photoperiod and consists of fibres originating from central structures via the pineal stalk. In contrast, the dopaminergic amacrine cells of the retina are responsive to light and thus regulate the circadian rhythmicity of retinal NAT activity and MEL biosynthesis in a direct fashion. Determination of the origin and regulation of the pineal dopaminergic fibres should help delineate the function of this system. This could be achieved through electrophysiological studies, retrograde labeling experiments and the iontophoretic application of agents such as GHB. It would be of particular interest to determine if these fibres are associated with output structures of the basal ganglia. This would provide the closing feedback loop in the putative pineal-striatal axis. The indirect nature of the dopaminergic innervation is consistent with a modulatory role of DA within the pineal, in comparison to the direct inhibitory effect of DA in the retina. Therefore DA is likely to be involved in the long-term regulation or synchronization of pineal indole biosynthesis, consistent with a hormonal rather than a neuronal action. An appreciation of the ontogenic and evolutionary development of the retinohypothalamic-pineal tract may be help delineate the role of DA in this pathway. It is proposed here that the key may lie with the photo-responsiveness of each component of the tract. Thus, a comparison of dopaminergic function in photoreceptive (e.g. the trout) and non-photoreceptive (e.g. the rat) pineal glands is likely to be highly informative.

Dysfunction of dopaminergic systems has been implicated in neuroendocrine, neurodegenerative and psychiatric disorders. MAO and COMT are the key catabolic enzymes of DA, having a major influence on dopaminergic neurotransmission. Thus, it is of no surprise that both enzymes are associated with numerous pathologies and that inhibitors of these enzyme are of major clinical importance. The current thesis presented several lines of evidence that the activity of striatal MAO-A and COMT is state-dependent and inhibited *in vivo* by endogenous MEL in a chronotypic manner.

Both enzymes displayed day-night variations in activity and were modulated by experimental conditions that altered endogenous MEL levels. The latter included photoperiod manipulation and administration of exogenous MEL. The inhibitory effect of MEL was more robust against COMT than MAO-A, suggesting that COMT is the key enzyme in the regulation of DA catabolism by MEL. It is possible that a more robust effect on MAO-A would have been observed following changes in the timing, dose and duration of MEL administration or photoperiod manipulation. In fact, considering the functional pleiotropy and complexity of MEL, a possible effect of the

hormone on any biochemical parameter cannot be excluded until all possible parameters of an administration paradigm have been optimized.

Furthermore the present administration protocol cannot determine whether MEL had a direct inhibitory effect on both enzymes or phase-shifted the temporal characteristics of the activity pattern. Thus the influence of exogenous MEL and photoperiod on the enzyme activity should be re-assessed at more frequent time periods throughout the light:dark cycle. This will also give more information regarding the wave-form and period of the day-night variation in striatal MAO-A and COMT activity and indicate whether these rhythms are endogenous or exogenous (i.e. free-running or entrained by light or other circadian oscillators). Future studies should also compare the activity of each enzyme in sham-operated and pinealectomized neonatal and adult rats. Currently, the *in vivo* effect of MEL on MAO-A and COMT activity was determined *in vitro* by radioenzymatic assays. Additional information could be obtained by assessing the *in vivo* activity of these enzymes through quantification of DA and its deaminated and O-methylated metabolites by HPLC. This could be performed on striatal homogenates, or by real-time sampling of the extracellular fluid by *in vivo* microdialysis. Coupling of the latter with iontophoresis would allow for the site-specific application of MEL and determination of the optimum dose of the hormone.

An alternative, and the most likely, proposal for the differences in intensity of MEL on the two striatal enzymes is related to the mechanism of the hormone in each case. In the case of MAO-A, the profile of the *in vivo* effect of MEL was consistent with the finding that MEL is a reversible, time-independent inhibitor of MAO-A *in vitro*. In contrast, MEL *in vitro* had no effect on basal or Mg^{2+}-induced COMT activity. In other words, the reduction of COMT activity in the striatum was not due to a direct inhibitory effect of the hormone, but must be related to the amount of active enzyme present. Thus MEL may control the COMT enzyme at the level of gene expression, post-translational modification, compartmentalization, or stability and turnover of mRNA and protein molecules. Both the S- and MB-isoforms of COMT are derived from a common gene through two distinct promoters. Although MB-COMT is considered to be the neuronally important isoform, the present results suggest that abundant S-COMT is expressed in the corpus striatum and the latter appears to be the isoform regulated by MEL. Recent research has indicated that MEL influences transcriptional activation of certain genes through nuclear MEL receptors. An important research avenue would then be to determine if MEL can influence the promoter activity and expression of S-COMT. The proposal that the effect of MEL on MAO-A is cytosolic, but

receptor-mediated in the case of COMT, can be further investigated through the use of selective MEL receptor agonists and antagonists.

Besides MEL, several other pineal indoles were found to be reversible inhibitors of striatal MAO-A *in vitro*. Furthermore, the preliminary indication that 5MT may be a novel substrate of this enzyme needs to be verified. As a consequence, a re-assessment of the regulation of indole metabolic pathways within the brain and the pineal gland may be necessary. The latter includes the possible feedback regulation of MAO-A by product inhibition and an alternative biosynthetic pathway for pineal 5-methoxyindoles. The latter is of particular importance in that there is a growing recognition that 5MT and 5MTOH may also be important hormonal products of the pineal with potent CNS effects.

The 5-methoxy substituent on the indole nucleus appeared to be the principal determinant of the inhibitory potency of the pineal indolic compounds. This novel finding suggests the presence of an interesting peculiarity in the nature of the MAO-A active site. Further studies should be performed on purified MAO-A and MAO-B to calculate K_i values, rather than IC_{50} values, and to determine the type of inhibition and the isoform selectivity of inhibitory potency. This information and a comparison of the K_i values of the present pineal indoles with other endogenous MAO inhibitors, such as isatin and the β-carbolines, should provide useful data for the structure-activity modeling of the MAO substrate binding site. Subsequently, site-directed mutagenesis and recombinant protein technology could determine the exact nature and location of amino residues responsible for the enhanced inhibitory potency of the 5-methoxy moiety. Ultimately, this could lead to the design of potent reversible inhibitors of MAO-A and MAO-B. In particular, it may be advantageous to have therapeutic agents that are structural analogues of endogenous compounds.

Finally, the inability of MEL alone to induce catalepsy is also in line with the modulatory nature of this hormone in the nigro-striatal pathway. Catalepsy was employed as an animal model of Parkinson's disease and the Standardized Horizontal Bar Test was capable of reflecting the akinetic state induced by drugs such as reserpine, HAL and GHB. However, the flexibility and quantification of the Bar Test may be improved by using a rating scale or increasing the maximum "cut-off" time allowed. The studies with GHB suggest that this neurochemical will be a useful agent to delineate the neural circuitry behind the behavioural effects of MEL. It may be possible to overcome the variability in behavioural responses to GHB and the high frequency of

anaesthesia by lowering the dose (e.g. 400 mg/kg) and using the GHB prodrug, γ-butyrolactone. The Walters-Roth model should be used to investigate the potential effects of MEL on DA autoreceptor function and presynaptic TH activity in the nigro-striatal pathway. In this way, the modulatory nature of MEL on striatal DA catabolism may also be found to exist at the level of DA biosynthesis.

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"Excuse me while I kiss the sky"
Purple Haze, Jimi Hendrix (1967)