TEMPORAL AND CIRCADIAN EFFECTS OF MELATONIN ACTION ON NEUROTRANSMITTERS IN MALE SYRIAN HAMSTERS

by

Nancy Anna Marie Alexiuk

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the degree of Doctor of Philosophy

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BY

NANCY ANNA MARIE ALEXIUK

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Dedicated to the memory

of my dear father, Steve.

To Mary, my mother, and to

Maralyn - for always being there.

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ABSTRACT

This study was designed to examine temporal and circadian effects of melatonin action on the rate-limiting enzymes of catecholamine and serotonin synthesis in male Syrian hamsters and to determine whether these effects were dependent on changes in serum testosterone. Melatonin-induced alterations in monoamines and GABA were also examined. The **in situ** activities of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) were determined by measuring accumulations of L-DOPA and 5-HTP, following administration of the aromatic L-amino acid decarboxylase inhibitor, NSD-1015. Precursors, neurotransmitters and metabolites were separated and assayed via HPLC-EC.

Significantly reduced dopamine (DA) levels in the pituitary neurointermediate lobe (NIL) and median eminence (ME)/arcuate region of the mediobasal hypothalamus (MBH) were demonstrated as early as 5 weeks after daily late afternoon melatonin injections. Melatonin decreased MBH and NIL DA over a 24 hour period following 9.5 weeks. Reduced norepinephrine (NE) concentrations were shown after 1 week (MBH) and 3 weeks (NIL) of melatonin administration - while at 9.5 weeks, the elevation in MBH NE reached significance 15.5 hours following the last melatonin injection.

Melatonin-induced inhibition of TH activity was demonstrated as early as 1 week (MBH) and 3 weeks (NIL) - while 9.5 weeks of treatment resulted in a highly significant increase in MBH TH activity over a 24 hour period ($F = 57.8$). Data showing a melatonin-induced elevation in hypothalamic TPH activity after 5 and 9.5

weeks suggest that serotonin synthesis is subject to regulation by melatonin. Melatonin also reduced MBH GABA concentrations after 9.5 weeks.

Melatonin treatment produced a highly significant increase in MBH TH activity of gonadectomized (GX) hamsters - concomitantly with marked reductions in MBH ($p < 0.001$) and NIL ($p < 0.01$) DA levels after 9.5 weeks. These data provide strong evidence for melatonin's role in the regulation of tuberoinfundibular DA turnover. Significant effects of the indole were also demonstrated in other regions, suggesting that melatonin's effects on monoaminergic systems are not limited to the MBH. Since all hamsters in this GX study were castrated, the effects of melatonin on TH activity, DA and serotonin were not secondary to a melatonin-induced reduction in circulating levels of testosterone.

LIST OF ABBREVIATIONS

3,4-dihydroxybenzeneacetic acid (DOPAC)

3,4-dihydroxyphenylalanine (L-DOPA)

3,5,3'-triiodothyronine (T3)

5-hydroxyindoleacetic acid (5-HIAA)

5-hydroxyindole-O-methyl transferase (5-HIOMT)

5-hydroxytryptophan (5-HTP)

Alpha-methyl-para-tyrosine (α-MPT)

Analysis of variance (ANOVA)

Aromatic-L-amino acid decarboxylase (AAAD)

Central nervous system (CNS)

Dopamine (DA)

Follicle stimulating hormone (FSH)

Gamma-aminobutyric acid (GABA)

Growth hormone (GH)

High performance liquid chromatography with electrochemical detection (HPLC-EC)

Homovanillic acid (HVA)

Luteinizing hormone (LH)

Luteinizing hormone releasing hormone (LHRH)

Medial preoptic area (MPOA)

Median eminence (ME)

Mediobasal or medial basal hypothalamus (MBH)

Mediobasal hypothalamus/median eminence (MBH/ME)

Norepinephrine or noradrenaline (NE)

Not significant (N.S. or NS)

Neurointermediate lobe of pituitary (NIL)

Nuclei; Nucleus (n.)

Paraventricular nuclei (PVN)

Serotonin (5-HT)

Superior cervical ganglia (SCG)

Suprachiasmatic nucleus (SCN)

Thyroid releasing hormone (TRH)

Thyroid stimulating hormone - thyrotropin (TSH)

Thyroxine (T4)

Tryptophan hydroxylase (TPH)

Tuberoinfundibular dopamine (TIDA)

Tuberohypophyseal (Tuberohypophysial) dopamine (THDA)

Tyrosine hydroxylase (TH)

1. INTRODUCTION - LITERATURE REVIEW

1.1 ISOLATION OF MELATONIN

The existence and putative physiological significance of pineal indole substances have been the subjects of speculation since antiquity - and the time of Herophilas of Alexandria. However, the actual discovery and synthesis of the principle pineal product, melatonin, did not occur until the twentieth century. It was in 1917 that McCord and Allen demonstrated that the administration of bovine pineal extracts to amphibians induced marked contraction of melanin granules in tadpole melanophores (McCord and Allen, 1917). The causal factor involved in this phenomenon, however, was not determined until the late 1950's. It was then, that the dermatologist Aaron Lerner (using a similar assay), isolated and named this potent skin-lightening indoleamine, melatonin - N-acetyl-5-methoxytryptamine (Lerner et al., 1958; 1959).

Soon after the identification of melatonin, Wurtman and collaborators, hypothesized that the neuroendocrine effects of the pineal gland were due to the release and action of melatonin. This was based on the discovery that melatonin inhibited estrous cyclicity when administered to rats; and that the indole was synthesized and secreted at a rate inversely proportional to environmental lighting (Wurtman et al., 1963; Chu et al., 1964; Wurtman and Axelrod, 1965). Melatonin - the mysterious 'hormone of darkness'- (Reiter, 1991a; Utiger, 1992), has recently stimulated controversy within the scientific community as to its actual functional

status. Presently, the fundamental question of whether melatonin behaves mechanistically like a classical hormone, a neurotransmitter, a neuromodulator - or like a combination of all three - remains to be elucidated.

1.2 FUNCTION OF THE PINEAL GLAND - A HISTORICAL PERSPECTIVE

Centuries ago, the Ancient Greeks and Rene Descartes (Descartes, 1662) regarded the human pineal gland with dignity and deference by referring to it as 'the seat of the soul'. This unpaired brain organ has also been described as the 'third eye', the 'cervical body', 'konareion' (the pine cone) and the 'appendix of the brain' (see Bhatnagar, 1990). A tap or valve function - controlling the flow of 'spirits' from the third to fourth brain ventricles - was attributed to the pineal by Herophilas (325-280 B.C.). Galen (129-199 A.D.) later disputed this tap analogy of the gland and suggested that it had a function which parallels that of lymphatic tissue (Miles and Grey, 1988).

Unfortunately, this little gland in the centre of the mammalian brain has also acquired notoriety from those who have described it as nothing more than a vestigial organ. Recently, the pineal gland has gained acceptance and recognition as a functioning neuroendocrine transducer which converts information about daylength into the nocturnal release of melatonin. The pineal gland effectively serves as an interface between the exogenous photic environment and the internal milieu of the body (Rosenstein and Cardinali, 1990; Reiter, 1991a).

1.3 SECRETION OF MELATONIN

The major blood supply to the pineal gland consists of branches of the posterior cerebral arteries - the posterior choroidal arteries (Le Gros Clark, 1939). The primary sources of pineal drainage are represented by the great cerebral vein of Galen, the internal cerebral veins and the straight sinus. It is generally accepted that the gland's major secretory product, melatonin, is released directly into the pineal's perivascular spaces before entering the venous component of the circulatory system. It has been hypothesized, however, that the cerebral spinal fluid may represent a secondary source of transport of the indole (Reiter, 1990).

1.4 SOURCES OF MELATONIN

Quay first reported that the pineal glands of rats were characterized by a distinct 24 hour (hr) cycle (Quay, 1963; 1964). It is now well documented that melatonin is synthesized and released during the dark phase of the 24-hr light/dark cycle by the pineal gland in virtually all vertebrate classes studied (Axelrod, 1974; Cardinali, 1981; Underwood and Goldman, 1987; Krause and Dubocovich, 1990).

The discovery that circulating levels of melatonin are undetectable following pinealectomy (Vorkapic et al., 1987) suggests that the pineal gland represents the major source of serum concentrations of the indole. However, there is some controversy over secondary sources of its production. Extrapineal sites of melatonin synthesis have been attributed to a variety of regions including: rodent harderian glands (Cardinali and Wurtman, 1972; Reiter et al., 1983a), human erythrocytes

(Vollrath, 1981), extraorbital lacrimal glands (Mhatre et al., 1988), chick and rat gut (Huether et al., 1992), peripheral nerves and rabbit platelets (Launay et al., 1982). However, no other structure has received more attention as an alternate source of melatonin production than the retina (Pang and Allen, 1986; Redburn and Mitchell, 1986; Wiechmann, 1986; Nowak et al., 1989; Olcese and Moller, 1989; Zawilska and Nowak, 1992).

The evidence for the presence and synthesis of retinal melatonin has not been unequivocally demonstrated in all vertebrate classes - with the exception of Aves (birds) (Hamm and Menaker, 1980; Reppert and Sagar, 1983; Zawilska and Iuvone, 1989; Zawilska, 1992). However, reports suggest that it is also found in the retinas of several mammalian species including the rat (Yu et al., 1981; Redburn and Mitchell, 1986; 1989), the squirrel (Reiter et al., 1981a), the cow (Hall et al., 1985), the rabbit (Nowak, 1987) and the human (Leino, 1984). In the retina, melatonin is considered to be a neuromodulator (Cahill and Besharse, 1989) that is synthesized locally - and is released in a paracrine manner. Therefore, retinal melatonin does not contribute to circulating levels of the hormone. Investigators (Zawilska and Nowak, 1992; Wiechmann and Graft, 1993) suggest that the indole is synthesized in retinal photoreceptors. This line of reasoning is based on the detection of immunoreactive melatonin in the retinas of a number of vertebrate species (Wiechmann and Hollyfield, 1989) including the rat (Reiter et al., 1983a) and human (Wiechmann and Hollyfield, 1987). Since the enzyme system for melatonin synthesis can be localized to the retina, this provides indirect evidence for a secondary source

of the indole (other than the pineal gland itself). The presence of the enzymes serotonin (5-HT) N-acetyl transferase (Hamm and Menaker, 1980; Besharse and Iuvone, 1983; Iuvone and Besharse, 1983; Besharse et al., 1984) and hydroxyindole-O-methyl transferase (HIOMT) (Cardinali and Rosner, 1971; Cardinali and Wurtman, 1972; Eichler and Moore, 1975; Wiechmann et al., 1985) in the retina has been documented by several investigators.

1.5 INNERVATION OF THE PINEAL GLAND

The mammalian pineal gland is innervated primarily via peripheral postganglionic sympathetic fibers derived from the superior cervical ganglia (Kappers, 1960). A cholinergic parasympathetic innervation (Kenny, 1961; Kappers and Schade, 1965; Romijn, 1973) of the pineal (when it exists), does not appear to play a role of major significance - in terms of the synthesis of melatonin (Finocchiaro et al., 1990).

The neuroanatomical pathway regulating the secretion of pineal melatonin begins in the retina. External light or dark stimuli initiate the process of melatonin suppression or synthesis, via activation of retinal photoreceptors. This exogenous energy is converted into neural information (electrical or chemical impulses) that is relayed via the retinohypothalamic tract (Moore and Lenn, 1972) to the ipsilateral and contralateral suprachiasmatic nuclei (SCN) of the anterior hypothalamus. These fibers then synapse in the paraventricular nuclei (PVN). The pathway continues into the lateral hypothalamus and via the medial forebrain bundle (Moore, 1978)

proceeds to the tegmentum and to the intermediolateral cell column of the thoracic cord. Pre-ganglionic fibers synapse on the superior cervical ganglia (SCG) while post-ganglionic noradrenergic sympathetic fibers terminate on pinealocytes of the pineal gland. A secondary pathway is the geniculohypothalamic tract which includes synaptic connections with the geniculate bodies (Pickard et al., 1987). This particular pathway may play a role in circadian rhythm entrainment (Moore and Card, 1985).

During the darkness of night, action potentials arriving at the SCG cause the release of norepinephrine (NE) (or noradrenaline) from noradrenergic sympathetic nerve terminals. Synaptic NE stimulates alpha-1 and beta-1 adrenergic receptors on pinealocyte membranes. The concept of concomitant activation of alpha and beta receptors in order to initiate melatonin production has been described by some investigators as the "AND" gate mechanism (Cena et al., 1991). There is some evidence for the existence of a 24-hr rhythm in the density and sensitivity of these receptors (Pangerl et al., 1990). The enzyme, adenyl cyclase, is activated following stimulation of beta-1 adrenergic receptors which are coupled to G-stimulatory proteins (Spiegel, 1988). Increased cyclic AMP, as well as protein synthesis for 5-HT N-acetyl transferase, the rate-limiting enzyme in melatonin synthesis, occur following adrenergic stimulation. Although the elevation of pineal cyclic GMP has also been demonstrated following adrenergic receptor activation, the functional importance of this occurrence is still under investigation (Klein, 1985; Ho et al., 1987; Chik and Ho, 1989).

1.6 BIOSYNTHESIS OF MELATONIN

The biosynthetic pathway for melatonin commences with the amino acid precursor, tryptophan, which is actively taken up by pinealocytes of the pineal gland (Axelrod et al., 1969). Tryptophan is hydroxylated via the rate-limiting enzyme, tryptophan hydroxylase (TPH), and the reduced pteridine cofactor, to form 5-hydroxytryptophan (5-HTP) (Sitaram and Lees, 1978; Cooper et al., 1991). 5-HTP undergoes decarboxylation via the aromatic L-amino acid decarboxylase (AAAD) enzyme and pyridoxal phosphate as a cofactor. This process results in the synthesis of the putative indoleamine neurotransmitter, serotonin (5-HT).

During the dark phase of the 24-hr light/dark cycle, noradrenergic stimulation of alpha-1 and beta-1 adrenergic receptors on pinealocyte cell membranes causes a very rapid elevation in cyclic AMP and protein synthesis (Klein and Weller, 1972; Romero et al., 1975). Pineal 5-HT then undergoes N-acetylation via the rate-limiting enzyme 5-HT N-acetyl transferase and an acetyl donor, acetylcoenzyme A, to form N-acetyl-5-hydroxytryptamine. During the dark phase, an O-methylation reaction occurs which requires S-adenosyl methionine as a methyl donor. The enzyme 5-hydroxyindole-O-methyl transferase (5-HIOMT) is the enzyme that completes the final step in melatonin biosynthesis (Axelrod, and Weissbach, 1960; 1961) and results in the production of N-acetyl-5-methoxytryptamine (refer to Fig. A1) or melatonin (Ebadi, 1984).

In the vertebrate retina, the activity of the enzyme 5-HT N-acetyl-transferase varies with alterations in light intensity. The amplitude of its rhythm increases during

the dark phase of the 24-hr light/dark cycle as exemplified by chick (Hamm and Menaker, 1980), Xenopus (Besharse and Iuvone, 1983) and rat retinas (Redburn and Mitchell, 1986). This rhythm apparently mirrors that which occurs within the pineal gland.

1.7 RELEASE, HALF-LIFE AND METABOLISM

Pineal melatonin is thought to be released episodically into the bloodstream via diffusion (Reiter, 1991b). The indole is considered to be largely bound to albumin when circulating (Cardinali et al., 1972; Cardinali, 1981). Presently, there is little or no evidence for the existence of a long term storage mechanism for melatonin (Reiter, 1989; Krause and Dubocovich, 1990; Reiter, 1991b); pinealocytes appear to contain few secretory or storage vesicles. Melatonin is a small, highly lipophilic, nonpolar, nonionized molecule - which enables it to diffuse easily through all cell membranes and fluid compartments in the body. This indole has been reported in human saliva (Vakkuri, 1985), in rabbit anterior eye chamber fluid (Yu et al., 1990), in human ovarian follicular fluid (Brzezinski et al., 1987), in male seminal fluid (Bornman et al., 1989), as well as in calf cerebral spinal fluid (Hedlund et al., 1976). Melatonin readily crosses the blood-brain barrier to enter the CNS (Reppert et al., 1979; Young et al., 1984). Therefore, it is not surprising that investigators continue to look for this indole's site of action here and hypothesize about its effects on brain function.

At the acrophase or zenith of the serum melatonin circadian rhythm, this

indole usually reaches concentrations in the picomolar/low nanomolar range in the blood (Arendt, 1988a; 1988b). For example, in humans, plasma levels of melatonin in the daytime are extremely low (5 picograms/ml of blood); while the nighttime peaks of the indole reportedly range from 20 to 140 picograms/ml (Lewy and Markey, 1978).

Interspecies variability of melatonin's half-life for elimination has proven to be relatively low. The half-life for melatonin in the Rhesus monkey has been demonstrated to be approximately 30 minutes (Reppert et al., 1979). In humans, de-Leiva and collaborators (1990) found no significant sex differences in the half-life of melatonin. In females, the half-life for the indole was in the 19 minute range; while in males it was reported to be around 15 minutes (de-Leiva et al., 1990). In the adult rat, cold melatonin infusions had a half-life of 17 minutes; while 23.2 minutes was the half-life reported for radioactive melatonin infusions (Gibbs and Vriend, 1981). This was similar to the half-life of 28 minutes demonstrated with the melatonin agonist, 6-chloromelatonin, in the rat (Clemens et al., 1980). In the Syrian hamster, Brown and collaborators (1985) have reported two components of melatonin's half-life for elimination; one (the alpha phase representing tissue distribution) was in the 17 minute range (Brown et al., 1985). The other half-life (the beta phase representing metabolism) approximated 25 hours in length (Brown et al., 1985).

In the liver of humans and rodents, melatonin is metabolized to 6-hydroxymelatonin conjugates (Kopin et al., 1961). Sulphate and glucuronide

conjugation results in 6-sulphatoxymelatonin, as a primary urinary metabolite (Fellenberg et al., 1981). In other cases, melatonin has been reported to undergo demethylation to produce N-acetylserotonin (Leone and Silman, 1984) or a hydroxylation reaction which results in 2-hydroxymelatonin (Vakkuri et al., 1987). In the CNS, metabolism of melatonin can occur via ring-splitting of the indole to produce kynurenamine derivatives - the major brain metabolite is believed to be N-acetyl-5-methoxy-kynurenamine (Hirata et al., 1974). Conversion of melatonin to water-soluble metabolites facilitates the elimination process from the body, via the kidneys.

1.8 TYPES OF MELATONIN CIRCADIAN RHYTHMS

The pineal derived melatonin circadian rhythm parallels that of plasma melatonin (Wilkinson et al., 1977; Arendt, 1988a; 1988b; Reiter, 1990) as well as that of melatonin metabolites in urine of most mammalian species (Iuvone and Besharse, 1983; Arendt, 1988a; 1988b; Reiter, 1990) - with the exception of the pig (Reiter et al., 1987). In mammals, there is a characteristic increase in melatonin synthesized and secreted during the dark phase - followed by a marked reduction (and an eventual non-detectability) of the indole at the onset of daylight (Axelrod, 1974; Wetterburg, 1978; Arendt, 1985a). Like other circadian rhythms (Wirz-Justice, 1987), the melatonin rhythm is endogenously generated and genetically determined. It is approximately 24 hours in duration and is regulated by the SCN of the anterior

hypothalamus (Moore and Klein, 1974; Vaughan, 1986; Klein et al., 1991). The SCN is considered to be the master endogenous circadian pacemaker (or oscillator) in mammalian species (Stetson and Watson-Whitmyre, 1976; Moore, 1983). The melatonin circadian rhythm is synchronized to an external time cue (or zeitgeber) - the daily 24-hr light/dark cycle.

The nocturnal serum melatonin rhythm generally follows three consistent patterns which tend to be species-specific (Reiter, 1987; 1991b; 1991c). The first (Type A), which represents a discrete delayed peak that occurs in the late dark phase, typifies the rhythms of the Syrian hamster, the mongolian gerbil and the house mouse. This pattern does not appear to be particularly common. The second melatonin circadian rhythm (Type B) is characterized by an acrophase that occurs in the centre of the dark period and is found in the albino rat, ground squirrels, the eastern chipmunk, the Turkish hamster and the human. The third melatonin circadian rhythm (Type C) is square in shape, due to a very rapid increase in sustained serum melatonin concentrations. It typifies the rhythms of the white-footed mouse, the cotton rat, the Djungarian hamster, the domestic sheep and the cat (Reiter, 1987; 1991b; 1991c).

The particular characteristics of the melatonin rhythm depend on rather specific variables. These include the following: the species, the time of the year (especially in Northern temperate or polar latitudes) and how rapidly melatonin levels rise prior to reaching an acrophase. Hence, in regions with dramatic seasonal changes in photoperiod, in temperature, and in available food resources (such as in

temperate or polar latitudes), the duration of the melatonin rhythm would be considerably longer in the winter months, than during the summer (Reiter, 1991a; 1991b). In equatorial areas, the duration of the circadian melatonin rhythm should theoretically change little, since the photoperiod approximates 12 hours throughout the course of the year.

1.9 PHYSIOLOGICAL ROLES FOR MELATONIN

1.9.1 Reproductive Effects

A major physiological role that has been attributed to alterations in the nocturnal melatonin signal is its ability to induce changes in seasonal (circannual) rhythms of reproductive, behavioral, and neuroendocrine function (Reiter, 1980; Goldman and Darrow, 1983; Bittman and Karsch, 1984; Karsch et al., 1984; Pevet, 1988; Bartness and Goldman, 1989). The interval between dawn and dusk - or photoperiod - varies depending on the time of year or season. This is especially characteristic of Northern temperate regions and polar latitudes (Arendt, 1988a; 1988b). Changes in photoperiod (and its accompanying melatonin signal or rhythm) can elicit alterations in physiological and behavioral parameters related to seasonal breeding, hibernation, migration, growth, fat deposition, coat color and pigmentation (Hoffman, 1981; Arendt, 1985a; 1988a; Darrow and Goldman, 1985; Tamarkin et al., 1985). Periods of gestation are timed to coincide with optimal geoclimatic conditions that will maximize offspring survival rates. Seasonal breeders may be of the short-day breeding variety which include sheep, deer and goats; long-day breeders are

exemplified by ferrets, horses and hamsters.

Photoperiodic changes, light deprivation (blindness) or daily administration of melatonin, can induce marked changes in the neuroendocrine axis of a seasonal breeding species. The endocrine effects of these various treatments include alterations in the secretion of gonadotrophs, thyrotrophs and lactotrophs as well as in serum levels of the gonadal steroid hormones. There are changes that occur in the size of the gonads and accessory organs. Photoperiodic variations - or a melatonin administration regime that attempts to mimic the physiological effects of a particular photoperiod - can either initiate or inhibit the onset of seasonal breeding. Dramatic sexual or aggressive behavioral changes are associated with the periods of reproductive competence/incompetence (Kripke et al., 1987).

It was Tamarkin and collaborators who first reported that properly timed injections of melatonin can duplicate the effects of a natural photoperiod (Tamarkin et al., 1976a). In the Syrian hamster (Mesocricetus auratus) - a long-day breeder and the animal model utilized in this study - short photoperiod exposure (less than 12.5 hours of light a day), or daily administration of melatonin late in the light period for several weeks (Reiter et al., 1976; Tamarkin et al., 1976a) produce marked changes in various parameters of reproductive function. These 'antigonadotrophic' effects of melatonin have been demonstrated in many studies (Hoffmann, 1974; Reiter and Johnson, 1974; Reiter et al., 1974; 1975; Tamarkin et al., 1976b; Reiter, 1980). In the male of the species, 8-10 weeks of melatonin administration (or short-photoperiod exposure) results in testicular involution (decreased testes weight) and

arrested spermatogenesis (Hoffman and Reiter, 1965; Gaston and Menaker, 1967). Low temperatures can increase the rate of testicular atrophy (Pevet et al., 1989); whereas a marked elevation in temperature can retard this process (Li et al., 1987).

This period of infertility, which is characterized by gonadal atrophy in the male Syrian hamster, is associated with specific hormonal changes. Both melatonin injections (or exposure to short-days) produce significant reductions in circulating levels of testosterone, LH, FSH, prolactin, T3, T4 and TSH (Reiter and Johnson, 1974; Tamarkin et al., 1976b; Vriend and Reiter, 1977; Reiter, 1980; Goldman et al., 1981; Vriend, 1983a; 1983b; Krajnak et al., 1994a). Elevation in hypothalamic LHRH (Jackson et al., 1984) also occurs; suggesting a suppression of its release. The fact that animals subjected to pinealectomy or superior cervical ganglionectomy no longer respond to short photoperiod (or melatonin) has provided strong evidence that these photoperiodic phenomena are pineal-mediated (Reiter and Hester, 1966; Reiter, 1968; Reiter, 1972a).

In the female hamster, daily administration of melatonin late in the light period (Tamarkin et al., 1976a) or short photoperiod exposure (Reiter and Hester, 1966; Trakulrungsi et al., 1979; Stetson and Hamilton, 1981) results in an anovulatory or anestrous condition similar to that initiated by light deprivation or blinding (Vaughan et al., 1982). The estrous cycles of females exposed to an inhibitory melatonin signal, characteristically cease in the diestrus II stage of the four day cycle (Vriend et al., 1987). During this anovulatory state, the uterus and vagina atrophy (Vriend et al., 1987), ovarian preantral/antral follicular and corpora lutea

development becomes arrested and the interstitial ovarian tissue hypertrophies (Silavin and Greenwald, 1982). Gonadotropin levels surge daily instead of every four days as in the normal cycling hamster (Bast and Greenwald, 1974; Bridges and Goldman, 1975). Reductions in serum estradiol (Seegal and Goldman, 1975), prolactin, (Bast and Greenwald, 1974) T3, T4 (Vriend et al., 1982; Petterborg and Rudeen, 1989) TSH (Vriend et al., 1982) and abnormal progesterone surges (Trakulrungsi et al., 1979; Jorgenson and Schwartz, 1987) also characterize the female Syrian hamster's response to melatonin treatment or short photoperiod exposure.

This period of conspicuous reproductive inactivity in both male and female Syrian hamsters is termed reproductive 'quiescence' (Hoffman and Reiter, 1965; Berdtson and Desjardins, 1974). After an extended period of time in this quiescent state (approximately 20-22 weeks), the Syrian hamster will no longer respond to the inhibitory melatonin signal associated with short photoperiod exposure or daily melatonin injections. At this time, the size and functional capacity of the reproductive organs regenerate - a process known as 'recrudescence' (Reiter, 1972b; 1980). This long day breeder will paradoxically enter a period which is characterized by the rejuvenation of full reproductive competence and marked by an insensitivity to the inhibitory melatonin signal (short daylength, light deprivation, or daily late afternoon melatonin injections). During this time, the hamster brain and neuroendocrine system are considered to be experiencing a phenomenon known as 'photorefractoriness' (Reiter, 1972b; Bittman and Zucker, 1981). In order to

terminate this refractory status, the animal must be exposed to a critical length (approximately 10 weeks) of a stimulatory photoperiod (long daylength) and its accompanying melatonin signal (Reiter, 1972b; Bittman, 1978; Reiter et al., 1979).

A similar refractory response in hamsters (Reiter et al., 1977) has been induced via administration of constant infusions of melatonin (or subcutaneously deposited melatonin reservoirs). This has led to the interpretation that continuously available melatonin in the Syrian hamster, has the capacity to down-regulate or desensitize its own receptor sites - resulting in a response that is identical to long photoperiod exposure (Chen et al., 1980; Reiter, 1980). A marked circadian sensitivity to melatonin in the Syrian hamster (or a melatonin-induced modulation of the density of melatonin-binding sites - Zisapel et al., 1988 [rat]), is reflected in the fact that only injections of the indole late in the light period (of a 14L/10D photoperiod) can induce pronounced antigonadotrophic effects and a status of reproductive incompetence (Tamarkin et al., 1976a; 1976b; Reiter et al., 1976; Chen et al., 1980). There appears to be a conspicuous circadian and seasonal (circannual) 'window' of sensitivity of the hamster neuroendocrine system to the action of melatonin (Reiter, 1987).

1.9.2 Circadian Effects

The second major physiological role that has been postulated for melatonin is based on evidence which demonstrates the indole's ability to regulate biological rhythms and to maintain circadian rhythm stability (Armstrong et al., 1986;

Armstrong, 1989a; 1989b; Cassone, 1990; Cassone et al., 1993). As previously described, the SCN is considered to be the major circadian pacemaker which generates circadian rhythms including the production and release of melatonin from the pineal gland (Moore and Klein, 1974; Takahashi and Menaker, 1979; Takahashi and Zatz, 1982; Moore, 1983; Berria et al., 1988; Ralph et al., 1990). Although it has been well established for several years, that the pineal gland is involved in the regulation of circadian rhythms of locomotor activity in lizards and birds, (Underwood, 1977; 1981; Gwinner, 1978; Takahashi and Menaker, 1979; 1982) such evidence was initially lacking in mammalian species (Cassone, 1990). This interpretation was based on data showing that some mammals had little change in their activity rhythms following pinealectomy (Aschoff et al., 1982; Cassone, 1990). However, subsequent investigations have demonstrated that administration of physiological doses (picomolar) of melatonin have the capacity to synchronize (or entrain) free-running locomotor activity rhythms of the Djungarian hamster (Cassone, 1990; Darrow and Doyle, 1990). This rhythm entrainment was also reported in Syrian hamster fetuses exposed to injections of melatonin (Davis and Mannion, 1988). These studies provided credence to the interpretation that the mammalian pineal gland and its principal secretory product, melatonin, are involved in circadian rhythm entrainment (Armstrong, 1989a; 1989b; Golombek and Cardinali, 1993) to the light/dark cycle.

Many other studies have verified melatonin's ability to entrain free-running locomotor rhythms in a number of vertebrate species including: the rat (Redman et

al., 1983; Armstrong and Redman, 1985; Cassone et al., 1986a; 1986b; Chesworth et al., 1987; Redman and Armstrong, 1988; Thomas and Armstrong, 1988; Cassone, 1992), the Syrian hamster fetus (Davis and Mannion, 1988), the bird (Gwinner and Benzinger, 1978; Gwinner, 1978; Menaker et al., 1981; Chabot and Menaker, 1992) and the iguanid lizard (Underwood and Harless, 1985; Underwood and Goldman, 1987; Underwood, 1989). Melatonin administration has been able to entrain feeding (Menaker et al., 1978; Chabot and Menaker, 1992) drinking (Cassone et al., 1988) and body temperature rhythms (Armstrong et al., 1986) as well as the sleep-wake cycle (Arendt, 1985a; 1985b; Arendt et al., 1984; 1985; 1986; 1987; 1988b; 1988c; Sack and Lewy, 1988; Petrie et al., 1989; Dahlitz et al., 1991; Palm et al., 1991).

Endocrine rhythms influenced by treatment with the indole include melatonin-induced synchronization of cortisol and the melatonin rhythm itself in humans (Mallo et al., 1988; Sack and Lewy, 1988). A melatonin-induced effect on the generation of circadian rhythms of other hormones such as human prolactin and growth hormone (Waldhauser et al., 1987; Petterborg et al., 1991) has also been documented. Niles and collaborators (1979a) reported an entrainment of rat rhythms of TSH and testosterone to the light/dark cycle via melatonin treatment (Niles, 1979a). Another group of investigators demonstrated that pinealectomy in rats abolished the circadian rhythm of corticosterone, and disrupted T3, T4 and testosterone rhythms (Zwirska-Korczala et al., 1991). However, administration of melatonin to pinealectomized rats reportedly restored the circadian rhythm of corticosterone and suppressed the circadian rhythmicity of T3, T4 and testosterone (Zwirska-Korczala et al., 1991).

Vaughan and collaborators have studied circadian rhythms of gonadotropins, prolactin and thyroid hormones in the Syrian hamster (Vaughan et al., 1994). The number of time points examined over a 24 hour period, the age of the animals used in the study and the genetic or strain differences (intraspecies variability) were highlighted as issues of relevance which contributed to variations in the results derived from a number of laboratories (Vaughan et al., 1994).

The capability of exogenously administered melatonin to synchronize its own circadian rhythm is of paramount importance in a temporal context. It has been suggested that the endogenous melatonin rhythm provides two distinct types of messages to the organism in question. Since melatonin has been described accurately as a biochemical indicator of the environmental state of darkness (chemical expression of darkness), it therefore is believed to dictate valuable time-of-day (clock) information (Reiter, 1993). Alterations in the duration of the melatonin rhythm also reflects seasonal or time-of-year (calendar) data to the organism (Reiter, 1993). The fact that exogenously administered melatonin can modify seasonal rhythms has been useful in animal experiments involving seasonal breeders or in the manipulation of seasonal breeders for agricultural/commercial purposes (Arendt et al., 1983; Martinet and Allain, 1985).

Melatonin's ability to synchronize human circadian rhythms has been important in the treatment of several clinical conditions. Travellers can alleviate the disconcerting symptoms of jet-lag via properly-timed administration of the indole (Arendt et al., 1986; 1987; Arendt et al., 1988; Petrie et al., 1989; Lino et al., 1993).

Melatonin has also been used to effectively treat 'shift-workers syndrome' (Czeisler et al., 1983) as well as 'delayed sleep-phase syndrome' (Dahlitz et al., 1991) - possibly through the indole's ability to synchronize or entrain the sleep-wake cycle (Arendt, 1988b; Arendt et al., 1988; Palm et al., 1991). Investigators have suggested that melatonin administration in humans accentuates the amplitude and acrophase of an age-related deficiency in the melatonin rhythm (Iguchi et al., 1982). An alternate hypothesis is that melatonin alters the phase and timing of the sleep-wake cycle of the central endogenous circadian system (Arendt, 1987). Theoretically - in the future - optimally timed administration of melatonin, light and temperature manipulations may all be useful in the treatment of a variety of clinical conditions involving the desynchronization of biological rhythms.

1.10 SUPPRESSION OF MELATONIN SECRETION

The phenomenon of light suppression of melatonin secretion is well-recognized in pineal literature. The intensities of light used to elicit melatonin-suppression are variable and often species-specific (Lewy et al., 1980; Lynch et al., 1981; Brainard et al., 1984; Arendt and Ravault, 1988). In the albino rat, for example, a mere 0.0005 microwatts per centimeter squared (Webb et al., 1985) is necessary to ensure melatonin suppression; whereas in the Richardson's ground squirrel, approximately 1850 microwatts per centimeter squared are required (Reiter et al., 1983b). A much greater intensity of light (2500 lux), however, is needed to suppress the secretion of melatonin in most humans (Lewy et al., 1980).

Certain drugs, such as the beta-blockers propanolol and atenolol (Vaughan, 1976; Hanssen et al., 1977; Cowen et al., 1983), alpha-1 adrenergic antagonists and the alpha-2 agonist, clonidine, (Lewy et al., 1986; Checkley and Palazidou, 1988) can also inhibit the production and output of pineal melatonin. Some investigators suggest that there is a suppressive effect of electromagnetic fields on melatonin secretion (Reiter, 1991c).

1.11 MELATONIN BINDING SITES

Initially, the quest for locating physiologically relevant melatonin receptor sites was impeded by a vast assortment of technical obstacles and a paucity of essential pharmacological tools (Krause & Dubocovich, 1991). Pioneering studies utilized tritiated melatonin as a ligand (Cardinali et al., 1978; 1979; Cohen et al., 1978; Niles et al., 1979b; Niles, 1987) - and generally demonstrated melatonin binding in various regions of the brain and body including bovine hypothalamus, cerebral and cerebellar cortex (Cardinali et al., 1978; 1979; Vacas and Cardinali, 1979), rat hippocampus (Niles et al., 1979b) and hamster gonadal tissue (Cohen et al., 1978). However, this particular tissue binding approach and its methodology have been criticized for creating inconsistent and irreproducible results (Stankov and Reiter, 1990; Stankov et al., 1991a; Kennaway and Hugel, 1992). Stankov and Reiter (1990) argued that tritiated melatonin ligands have a 'relatively low specific activity, (comparable to iodinated ligands)' and suggested that this prevented the 'detection of high affinity binding sites with low receptor density' (Stankov and Reiter, 1990). Kennaway and

Hugel (1992) have argued that this situation may have occurred due to the inferior quality and the 'instability of commercially available ligand' (Cardinali et al., 1985). It should be clarified (at this point), that although melatonin 'binding' and 'receptor' sites are used interchangeably (Stankov et al., 1991a), evidence for a definitive physiological melatonin receptor site in many mammalian species has not yet been established. Laduron (1984) has stated that "when one starts any binding study, one must keep in mind that there is no equivalence between a binding site and a receptor site; numerous ligands label sites not related to receptors."

Two techniques have been utilized to characterize melatonin binding sites: in vitro ligand receptor binding and the more anatomically precise method - autoradiography. Although the use of the tritiated melatonin ligand initially had provided conflicting results (Stankov et al., 1991a; Kennaway and Hugel, 1992), the subsequent development and synthesis of halogenated melatonin analogues or agonists offered a greater potential for defining a true physiological melatonin receptor in more discrete brain regions. Clemens and colleagues, synthesized the first analogues of melatonin - 6-chloromelatonin and 6-fluoromelatonin - in 1980. The most potent of the melatonin agonists developed (Vakkuri et al., 1984a; 1984b), however, is considered to be 2-iodomelatonin. It has been hypothesized (from several studies) that the presence of N-acetyl and 5-methoxy substituents of the indole nucleus are of paramount importance for the induction of the biological activity and binding affinity in these melatonin analogues (Dubocovich, 1988; Stankov and Reiter, 1990; Stankov et al., 1991a). Halogenation in the C-2 or C-6 positions

appears to accentuate these particular characteristics (Clemens et al., 1980; Weaver et al., 1988a; 1988b; Stankov et al., 1991a; Duranti et al., 1992). Duranti and colleagues (1992), who developed 2-bromomelatonin, suggest that the enhanced binding affinity of halogenated melatonin could be due to the induction of an increase in the biological activity of the analogue (ie. accentuated inhibition of cyclic AMP activity; Niles and Hashemi, 1990). Perhaps the halogenation process is able to prolong the half-life, or increase the degree of lipophilicity displayed by the analogue in comparison to an endogenous source of melatonin (Duranti et al., 1992). Whatever the case may be, the development of the radioligand I-125-2-iodomelatonin (Vakkuri et al., 1984a; 1984b) facilitated a relative explosion in melatonin binding studies.

Recently, it was demonstrated that the indole nucleus is not absolutely essential in the activation of the melatonin receptor or binding site. Howell and colleagues (1991), reported that naphthalene can replace the indole nucleus to produce an agonist with comparable biological activity to melatonin, itself. The biological activity of this melatonin analogue, N-[2(7-methoxy-1-naphthalenyl)ethyl]acetamide, has been demonstrated in the chicken retina (Dubocovich et al., 1993). Other pharmacological agents with mixed agonist/antagonist potential have been reported (Stankov et al., 1991). A recently described group of compounds, known as the amidotetralines (Copinga et al., 1993), have been demonstrated to inhibit Ca2+ dependent release of retinal DA (Dubocovich et al., 1992; 1993) similarly to the action of melatonin.

In general, the greatest density of melatonin binding sites have been found in two regions: the SCN and the median eminence (ME)/pars tuberalis (adenohypophysis) region of the hypothalamic/pituitary complex. These melatonin binding sites meet the criteria for receptor identification (Burt, 1978; Cooper et al., 1991) since they have been demonstrated to be saturable, to be high-affinity in nature and to be of reversible kinetics - by a number of investigators (Vanecek et al, 1987; Dubocovich, 1988; Reppert et al, 1988; Weaver et al., 1989; Morgan et al., 1989; Laitinen et al., 1990). Since the binding sites exhibited dissociation constants (Kds) in the picomolar/nanomolar range - which is comparable to circulating levels of endogenous melatonin - high-affinity binding status was attained.

The SCN of the anterior hypothalamus was considered the region most likely to be the primary site of action for melatonin - many years before studies provided evidence to support this hypothesis. This, of course, is not particularly surprising since the SCN's putative role as a circadian pacemaker was already established. Evidence was rapidly accumulating to support a role for the pineal gland and melatonin in circadian function. Early studies demonstrated high-affinity melatonin binding sites in the SCN of the rat (Vanecek et al., 1987; Laitinen et al., 1989; Laitinen and Saavedra, 1990) and the visual SCN of the chicken (Dubocovich and Takahashi, 1987). Melatonin binding sites have been localized in the SCN of a number of species across several vertebrate classes including: humans (Reppert et al., 1988; Weaver et al., 1993), Syrian hamsters (Weaver et al., 1988a; 1988b; Williams et al., 1989) and Djungarian hamsters (Duncan et al., 1989), white-footed

mice (Weaver et al., 1989), lizards (Rivkees et al., 1989a), chickens (Rivkees et al., 1989b; Stehle, 1990; Brooks and Cassone, 1992), rabbits, horses and sheep (Stankov et al., 1991b). Scientists, who have related melatonin binding sites to its putative biological role, suggest that the occurrence of melatonin receptors in the SCN may be associated with its ability to synchronize circadian rhythms (Arendt, 1988a; 1988b; Reppert et al., 1988; Cassone, 1990; Krause and Dubocovich, 1991).

Initial binding studies performed in the rat (Vanecek et al., 1987) identified possible melatonin 'receptor' sites in the region located at the base of the mediobasal hypothalamus, known as the median eminence (ME) (Vanacek and Jansky, 1989). Melatonin binding was also reported in the ME/arcuate nucleus region of the fetal hamster (Weaver et al., 1988a; 1988b).

However, it was subsequently discovered that the greatest melatonin binding, in actuality, occurred in a structure that wraps around the pituitary stalk (Gross, 1984) called the pars tuberalis; a component of the adenohypophysis (Williams and Morgan, 1988; Williams, 1989b). The ventral surface of the pars tuberalis is in close proximity to the pars distalis; while dorsally the pars tuberalis is adjacent to (and perhaps overlapping) the ME (Morgan et al., 1994). Hence, it has been argued, that it is extremely difficult to differentiate between the ME and the pars tuberalis. However, some researchers claim that this has been accomplished in the rat (Williams, 1989), in the Syrian (Williams et al., 1989) and in the Djungarian hamsters (Duncan et al., 1989). In fact, to further support this, the pars tuberalis was identified as the region with the greatest melatonin binding in a species with a much

larger brain - that of the short-day breeding sheep (Morgan et al., 1989). However, it should be clarified that the existence of marked species differences has not been an unusual occurrence in these binding studies. Although the predominance of melatonin binding sites characterized within the pars tuberalis is a phenomenon that has been demonstrated in several seasonal and non-seasonal breeders, this had not been proven to be the case for the human pars tuberalis (Weaver et al., 1993) - until recently (Reppert et al., 1994). Perhaps this paucity of melatonin binding sites (or receptors?) found within the pars tuberalis of the non-seasonal breeding human was related to a difference in reproductive responsiveness to photoperiodic phenomena - which dictates the regulation of the neuroendocrine axis in the seasonal breeder (Reppert et al., 1988; Weaver et al., 1993). Since melatonin binding sites have been identified in the pars tuberalis of a relatively close primate relative - the seasonally breeding Rhesus monkey (Weaver et al., 1993) - this provided further support for the concept of melatonin's regulation of neuroendocrine function via activation of 'receptor' sites in this region (Williams and Morgan, 1988).

Certain investigators (Krause and Dubocovich, 1991) have concluded that the melatonin binding found in the ME/pars tuberalis is related to this indole's recognized reproductive and neuroendocrine (ie. antigonadotrophic and antithyrotrophic) effects. Recent studies by Malpaux and colleagues (1993; 1994), however, have raised some intriguing questions about the importance of melatonin binding sites specifically found in the pars tuberalis. Melatonin microimplants placed directly in the MBH (including the arcuate and ventromedial nuclei) significantly

modified LH (increased) and prolactin (decreased) secretion in the short-day breeding ewe (Malpaux et al., 1993). These investigators suggested that the MBH and adjacent regions (but not the pars tuberalis) may represent the major site at which melatonin acts to mediate seasonal reproduction (Malpaux et al., 1993). In a subsequent study, Malpaux and colleagues (1994) concluded that melatonin binding sites in the pars tuberalis do not regulate the photoperiodic effects of melatonin on gonadotropin (LH) and prolactin secretion in the ewe (Malpaux et al., 1994). Hence, the controversy regarding the meaning of melatonin binding site or 'receptor' continues.

Melatonin binding sites have been demonstrated in a number of other structures throughout the brain (Stankov et al., 1991a). Binding of melatonin to the paraventricular nucleus of the thalamus (Duncan et al., 1989; Williams et al., 1989; Bittman and Weaver, 1990) may be involved in the regulation of body rhythms possibly via a relay to various limbic structures. Binding of the indole in the area postrema demonstrated in the rat (Vanecek, 1988; Laitinen and Saavedra, 1990) and the white-footed mouse (Weaver et al., 1990), may be involved in the regulation of autonomic functions and blood pressure modulation (Borison, 1989; Stankov et al., 1991a).

Melatonin's functional biological role in response to binding in the retina (Dubocovich and Takahashi, 1987; Blazynski and Dubocovich, 1991) has been associated with dark adaptive responses in the eye. These include: disc shedding and modulation of phagocytosis in photoreceptor outer segments in chicks (Ogino et al.,

1983; McCormack and Burnside, 1991) and frogs (Besharse and Dunis, 1983), cone retinomotor movement in Xenopus laevis (Pierce and Besharse, 1985), the green sun fish (Dearry and Burnside, 1985) and the Midas chiclid (McCormack and Burnside, 1991) as well as melanosome aggregation in retinal pigmented epithelium in guinea pigs (Pang and Yew, 1979). Accumulating evidence suggests that dopamine (DA) is synthesized during the light period and regulates light-adaptive responses in the retina. Retinal melatonin, on the other hand, increases in the dark phase of the light/dark cycle. Thus, melatonin and DA appear to have an antagonistic relationship in the retina (Zawilska and Nowak, 1991).

Other regions of melatonin binding reported were in various brain structures of mammalian species including: the hypothalamic preoptic area (Williams et al., 1989), anterior and tuberal medial basal hypothalamus (Bittman and Weaver, 1990), arcuate n. (Weaver et al., 1988), ventral medial n. (Williams et al., 1988; Stankov et al., 1991b), lateral and periventricular hypothalamus (Deveson et al., 1990; Stankov et al., 1991b). Additional structures with the presence of melatonin binding are: hippocampus (Bittman and Weaver, 1990; Pickering and Niles, 1990), subiculum (Bittman and Weaver, 1990), amygdala (Stankov et al., 1991b), frontal and occipital cortex (Stankov et al., 1991b), striatum (Chavez et al., 1991), olfactory bulbs (Duncan et al., 1988), pons (Bittman and Weaver, 1990), ventral tegmental area of midbrain (Williams et al., 1989), nucleus accumbens, septum (Deveson et al., 1992), cingulate gyrus (Stankov et al., 1991b) pineal gland (Weaver et al., 1988), lateral and medial habenular n. of the epithalamus (Weaver et al., 1989; Williams et al., 1989; Stankov

et al., 1991b). Many of these regions of melatonin binding are considered to be low-affinity sites. Some of them may prove to have important neuropharmacological potential in the future.

Although efficacious and potent melatonin agonists have been synthesized in recent years, this has not been the case with the list of putative melatonin antagonists. Perhaps the most well-known of these drugs is 2-benzyl-N-acetyltryptamine (Luzindole) developed by Dubocovich, 1987; 1988b; and its later modification - 5-methoxyluzindole (Duncan et al., 1990). Luzindole, although demonstrating significant efficacy in certain experiments (ie. antagonism of melatonin-induced inhibition of DA release - Dubocovich, 1988b), was found to be rather ineffective in tests for melatonin inhibition of forskolin-stimulated cyclic AMP synthesis in the pars tuberalis region of sheep (Howell and Morgan, 1990). Other potential melatonin antagonists include N-(3,5-dinitrophenyl)-5-methoxytryptamine (ML23), (Laudon et al., 1988; Nordio et al., 1989), N-acetyl serotonin (Heward and Hadley, 1975), N-acetyl-tryptamine (Dubocovich, 1984; Mason and Brooks, 1988), prazosin (Niles et al., 1987a), 6-methoxy-2-benzoxazolinone (Yuwiler and Winters, 1985) and 5-methoxy-indole-N-methyl-3-propioamide (Askam et al., 1985). The major difficulty encountered with the use of the drugs N-acetyl-tryptamine (Mason and Brooks, 1988), N-acetyl serotonin (Heward and Hadley, 1975) and prazosin (α1-adrenoceptor antagonist - Niles et al. 1987a) was their mixed agonist/antagonist activity. These drugs, in fact, have been shown to be effective only for the particular cellular purpose for which they were designed and presently no melatonin antagonist

has been synthesized that can reverse all the biological effects of melatonin. Recently, a novel melatonin antagonist, 4-phenyl-acetamidotetraline (Dubocovich et al., 1992) has been under investigation. However, the potency and efficacy of this drug, to this date, remains to be proven. Hence, the evidence for the existence of a true melatonin antagonist presently, remains tenuous (Stankov et al., 1991a), at best.

Morgan and colleagues (1994), have emphasized the two major problems which could be responsible for impeding the identification of a true melatonin receptor (Morgan et al., 1994). The first, addresses the issue of isolation of the gene which encodes the melatonin receptor. Recent data have demonstrated some advances in this area of research (Reppert et al., 1994); however, the melatonin receptor gene has been cloned only in the human and sheep. The second problem impeding the quest for understanding of the site of action of melatonin, is the continuing frustration in the process of identification of a potent and efficacious melatonin antagonist (Morgan et al., 1994).

As previously described, the criteria for reliable receptor identification requires binding sites to be: saturable, of high-affinity and of reversible kinetics (Burt, 1978, Cooper et al., 1991). In contrast to most other investigators in this field, Kennaway and Hugel (1992) have suggested that some melatonin binding sites have a rather 'unique pharmacological profile'. The low-affinity sites that have been demonstrated in whole rat and hamster brain (Duncan et al., 1988; 1989; Pickering and Niles, 1990), have nanomolar affinity as well as rapid association and dissociation

times (reversible kinetics) reflecting an 'inappropriate pharmacology' (Kennaway and Hugel, 1992). However, the high-affinity (picomolar) melatonin binding sites of 'appropriate pharmacology' (Kennaway and Hugel, 1992) have been shown to have slow dissociation and association rates (irreversible kinetics) in a number of studies (Vanecek, 1988; Rivkees et al., 1989; 1989a) in which the areas of binding are more restricted to discrete regions within the SCN and ME/pars tuberalis (Kennaway and Hugel, 1992). These investigators then raise doubts and question whether melatonin does, in fact, act through a classical membrane bound receptor and/or through a conventional second messenger system. Recent studies reporting the localization of melatonin in the cytosolic fraction (Menendez-Pelaez and Reiter, 1993) as well as the localization of nuclear melatonin binding (Menendez-Pelaez et al., 1993) lend credence to the interpretation that melatonin may not be acting simply through a membrane bound receptor. It has been argued that the site of action for melatonin must be determined before it can definitively be evaluated as a classical hormone (Kennaway and Hugel, 1992).

1.12 MECHANISM OF MELATONIN ACTION

Reiter has proposed three hypotheses to explain melatonin's mechanism of action (Reiter, 1987b; 1991b; 1991c). The most popular of the three has been defined as the "duration hypothesis". Photoperiodic length, characterized as the time intervening between dawn and dusk - undergoes marked seasonal changes - most notably in Northern temperate regions. In order to ensure the efficacy of melatonin's

action, the Syrian hamster requires exposure to a particular minimum length of dark period (< 12.5 hours of light) or melatonin injections late in the light period of a 14L/10D light/dark cycle. Grosse and colleagues (1993) provided strong support for the duration hypothesis by demonstrating the importance of the length of sustained exposure to melatonin in pinealectomized Syrian hamsters (Grosse et al., 1993) in relation to the light period.

The second theory is called the internal coincidence model (Reiter, 1987b; 1991b; 1991c). In this particular perspective, elevated levels of melatonin must occur concomitantly with a crucial window of melatonin receptor sensitivity (internal coincidence). According to this model, if these two factors are desynchronized, melatonin's physiological effects will not be expressed. This is important in the context of the down-regulation or subsensitization of melatonin receptors that occurs with constant infusions of melatonin, early morning melatonin injections or melatonin treatment after a long duration of time (20-22 weeks) (Reiter, 1980).

The third theory is the "amplitude hypothesis" which suggests that melatonin requires a certain acrophase to ensure that it produces its physiological effects (Reiter, 1987b). One might also speculate as to the importance of other geoclimatic variables (ie. environmental temperature changes) influencing the efficacy of melatonin (Li et al., 1987; Pevet et al., 1989).

1.12.1 Cellular or Post-synaptic Events

Although the site(s) and mechanism(s) for melatonin's action continue(s) to

remain elusive, accumulating evidence is pointing in certain directions. The major question to be addressed is whether melatonin acts through a classical membrane bound receptor to modify presynaptic and/or postsynaptic neuronal functions. The fact that melatonin is characteristically highly lipophilic, raises the issue of whether the indole readily diffuses into cells to act on cytosolic or nuclear cell membranes. Perhaps, in order to adequately explain the diversity of melatonin's actions, one might conclude that the indole has the unique capacity to do both (Reiter, 1993).

Melatonin has been demonstrated to alter the electrical and/or the metabolic activity (neuronal firing rates) in various brain regions (Pazo, 1979; Cassone et al., 1987; 1988; Shibata et al., 1989; Stehle et al., 1989; Cassone, 1990; Krause and Dubocovich, 1990; Naranjo-Rodriguez et al., 1991; Duranti et al., 1992; Rusak and Yu, 1993) - suggesting the modulation of impulse flow as the indole's possible mechanism of action. This raises the issue of whether melatonin produces its effects on cell polarization through the activation of its own membrane-bound receptor. An alternate hypothesis is that melatonin may be controlling neuronal firing rates through a modulatory effect on other receptors (ie. GABA 'A' receptor site) which directly regulate ion channels (Coloma and Niles, 1988; Rosenstein et al., 1989; Niles and Peace, 1990; Golombek and Cardinali, 1993).

The concept that melatonin binding to its receptor could be responsible for indirectly controlling ion channels via inactivation of cyclic nucleotide second messenger systems has been widely investigated since the work of Abe and colleagues (1969). Numerous studies have demonstrated melatonin-induced alterations in

second messenger systems: cyclic AMP (Vacas et al., 1981; 1984; Carlson et al., 1989; Morgan et al., 1989; Vanecek and Vollrath, 1989; 1990), cyclic GMP (Vanecek, 1991), arachidonic acid (Vanecek, 1990; Vanecek and Vollrath, 1990; Vanecek, 1991) and CA2+ (Zisapel and Laudon, 1983; Dubocovich 1983; 1985). The majority of these studies have documented the capacity of the indole to inhibit cyclic AMP synthesis (Vacas et al., 1981; Vanecek and Vollrath, 1990; Daniolos et al., 1990; Hazlerigg et al., 1991) through activation of a G-inhibitory protein - and inactivation of the enzyme adenyl cyclase (Carlson et al., 1989; Rivkees et al., 1989a; Niles and Hashemi, 1990a; 1990b; Niles et al., 1991; Hazlerigg et al., 1993). Niles and Hashemi (1990a; 1990b), reported that picomolar concentrations of melatonin (via activation of high-affinity melatonin binding sites) inhibited forskolin-stimulated adenylate cyclase in the male Syrian hamster hypothalamus. On the other hand, these investigators also suggested that low-affinity melatonin binding sites may be activating adenylate cyclase (or positively coupled to the enzyme) in this species (Niles and Hashemi, 1990a). Melatonin's activation of specific binding sites - in terms of its putative mechanism of action - may, therefore be dose-dependent (Niles and Hashemi, 1990a).

Although this effect on adenyl cyclase and second messenger systems was found with administration of very low concentrations of melatonin (picomolar range) in many of these studies, it has been argued that there are inconsistencies in the work that utilized whole cell cultures in contrast to broken cell homogenates (Kennaway and Hugel, 1992). Melatonin-induced inhibition of cyclic AMP occurred only in the

former case and not the latter - again raising the question of whether this indole acts through a classical membrane bound receptor.

Melatonin-induced modulation of calcium-dependent DA release in both the rabbit retina (Dubocovich, 1983) and in various brain regions including the rat hypothalamus (Zisapel et al., 1982; Zisapel and Laudon, 1983) have been demonstrated by several investigators. At least one group has found evidence for binding of tritiated melatonin to calmodulin; suggesting the interpretation that melatonin-calmodulin binding modulates intracellular CA2+ functions (Benitez-King et al., 1990; 1993; Huerto-Delgadillo et al., 1994).

An alternate hypothesis suggests that melatonin - because of its characteristically high lipophilicity - has the capacity to diffuse directly into the cell nucleus and alter DNA, RNA, and protein synthesis (in other words, gene expression) (Reiter, 1993). Certain studies have, in fact, demonstrated that melatonin is capable of modifying c-fos expression in the SCN (Kilduff et al., 1992), while others have provided convincing evidence for nuclear melatonin binding (Menendez-Pelaez et al., 1993). Investigators have suggested that melatonin could be acting through either membrane or cytosolic/nuclear receptors and perhaps, both (Reiter, 1993). Some even question the logic in seeking classical membrane receptors for such a highly lipophilic substance - comparing melatonin's mechanism of action to that of the steroid hormones - rather than to that of the more hydrophilic protein and peptide hormones (Benitez-King and Anton-Tay, 1993; Reiter, 1993). The evidence that melatonin has capabilities as a hydroxyl radical

scavenger (Poeggeler et al., 1993; 1994; Reiter et al., 1993a; Tan et al., 1993) seems to suggest a mechanism of action for melatonin that is independent of a membrane-bound receptor (Reiter, 1993), however, this does not disprove that melatonin may also produce some its physiological effects via pre or postsynaptic membrane binding.

1.13 NEUROTRANSMITTER REGULATION OF HORMONES

Modulation of the hypothalamo-hypophyseal-gonadal axis is a well-recognized physiological effect of melatonin or photoperiodic changes. The major mechanism through which the indole facilitates this occurrence is believed to be via a melatonin-induced modification of the firing rates of the hypothalamic gonadotropin-releasing hormone pulse generator (Pickard and Silverman, 1979; Karsch et al., 1984; Robinson et al., 1986; Robinson, 1987; Robinson and Karsch, 1988). The melatonin or short-photoperiod induced reduction that occurs in serum LH and FSH could be related to an elevation in hypothalamic LHRH stores (Jackson et al., 1984; Steger et al., 1986) and a suppression of LHRH secretion (Pickard and Silverman, 1979; Steger et al., 1983; Jackson et al., 1984). A hypothalamic site of action has been proposed for the photoperiodic effects of melatonin on reproductive function (Reiter et al., 1981; Glass and Lynch, 1982).

An alternate hypothesis is that melatonin acts primarily outside the brain in the pars tuberalis of the adenohypophysis. This structure (which wraps around the pituitary stalk - Gross, 1984) was hypothesized to release an unidentified substance in a paracrine manner upon the terminals of the ME or into the primary portal

plexus (Stankov et al., 1991a; Morgan et al., 1994). This chemical was believed to act secondarily upon the releasing hormones (ie. LHRH) of the arcuate region of the MBH (Stankov et al., 1991a; Nakazawa et al., 1991; Morgan, 1994). A variation of this hypothesis is that melatonin binds to its 'receptors' in the pars tuberalis and directly modulates LH release from this region - which in turn alters LHRH secretion from the terminals in the ME (Nakazawa et al., 1991).

The exact physiological process whereby melatonin modifies the responses of the gonadotropin-releasing hormone pulsatile generator remains elusive. It is hypothesized that the indoleamine alters the activity/metabolism of hypothalamic and extrahypothalamic neurotransmitters which regulate adenohypophyseal hormone secretion. Steger and Bartke (1991) have suggested that the effects of short-photoperiod exposure are initially evident on brain neurotransmitter systems (of the male Syrian hamster). Secondly, serum hormone (prolactin, LH, FSH) levels decrease. Finally, testicular atrophy occurs (Steger and Bartke, 1991).

It has been well-documented in numerous investigations that NE is the major neurotransmitter involved in the stimulation of gonadotropin (LH and FSH) release (Weiner and Ganong, 1978; Meites and Sonntag, 1981; Kalra and Kalra, 1983; Steger et al., 1985a). Administration of melatonin agonists to the female rat, have been shown to inhibit LH release - and consequently, ovulation (Clemens et al., 1980). Melatonin could thus be modulating LHRH release through its effects on NE.

Both the monoamines and the amino acid transmitter gamma-aminobutyric acid (GABA), have been shown to be involved in the regulation of the

adenohypophyseal hormone secretion including the gonadotropins, prolactin, thyrotropin and GH (Vijayan and McCann, 1978; Racagni et al., 1982; Casanueva et al., 1984). It has been argued that the serotoninergic system has an inhibitory effect upon LH and FSH release (Schneider and McCann, 1970; Weiner and Ganong, 1978; Meites and Sonntag, 1981). These transmitters may be playing a contributory role in the hypothesized melatonin-induced alterations in LHRH and gonadotropin release mechanisms (facilitation or activation). A role for DA in the modulation of gonadotropin secretion currently remains disputed (Ben-Jonathan, 1985).

Dopamine (DA; or prolactin-inhibitory factor), is believed to be the principal neurotransmitter involved in the inhibition of prolactin release (Gudelsky, 1981; Tuomisto and Mannisto, 1985; Ben-Jonathan, 1985). Serotonin (5-HT) may also be involved in modulating prolactin secretion (Clemems et al., 1978; Weiner and Ganong, 1978; Meites and Sonntag, 1981; Van de Kar et al., 1989). Administration of 5-HT agonists have been shown to increase prolactin release, whereas, 5-HT antagonists inhibit the hormone's secretion (Korden et al., 1973; Lawson and Gala, 1978). NE has been found to have both facilitory and inhibitory effects on prolactin release (Meites and Sonntag, 1981; Ben-Jonathan et al., 1989).

Changes in TRH and the opioid peptides may also be affecting prolactin release (Ben-Jonathan et al., 1989). A modulatory effect for melatonin on TRH secretion has been hypothesized (Vriend and Wilber, 1983). The inhibitory effects of melatonin injections on thyroid hormones (including T3, T4 and TSH) have been demonstrated in several studies (Vriend and Reiter, 1977; Vriend et al., 1982;

Vriend, 1983a; 1983b; Vriend and Steiner, 1988). Various transmitters including NE (Krulich et al., 1977), 5-HT (Krulich et al., 1979; Smythe et al., 1982a; 1982b), DA (Foord et al., 1980; Ben-Jonathan, 1985) and GABA (Vijayan and McCann, 1978) have been implicated in the regulation of thyrotropin (TSH) secretion.

It should be noted that short-day-induced reductions in serum prolactin levels have been reported as early as 1 week after initiation of the short photoperiod (Steger et al., 1994 Abs.) and 4 weeks for LH and FSH (Steger and Bartke, 1991). It has been suggested that the most fundamental factor modulating short photoperiodic alterations in reproductive function are changing serum levels of prolactin (Vaughan et al., 1994). Administration of prolactin to short-day hamsters has been shown to retard the rate of testicular regression, to stimulate testicular function and to alter feedback sensitivity (Bartke et al., 1975; Matt et al., 1984). Bex and colleagues (1978) demonstrated that injections of prolactin to testicular-atrophic hamsters promoted the regeneration of the testes and testosterone synthesis in addition to elevating the number of testicular LH receptors (Bex et al., 1978).

1.14 EFFECTS OF MELATONIN AND SHORT PHOTOPERIOD ON MONOAMINERGIC NEUROTRANSMITTERS

It has been predicted that the neuroendocrine and neurochemical effects of late afternoon melatonin administration for several weeks are similar to those of short photoperiod exposure (Reiter, 1980; Steger et al., 1985a). This relationship has

been clearly demonstrated with short photoperiod and melatonin-induced endocrine changes in the Syrian hamster (Tamarkin et al., 1976a: Reiter et al., 1976). However, the neurochemical similarities between these two treatments have not been demonstrated.

A logical hypothesis that follows for melatonin's mechanism of action predicts that the indole modulates the activity and metabolism of neurotransmitters that are regulating hypothalamic and pituitary hormone secretion (Alexiuk and Vriend, 1991; Alexiuk and Vriend, 1993b). As previously described, melatonin injections or short photoperiod exposure for several weeks reduce circulating levels of gonadotrophs, thyrotrophs, prolactin and gonadal steroids. Evidence is now accumulating that an inhibitory melatonin signal (derived from short photoperiod exposure or melatonin administration) may be influencing the synthesis and metabolism of hypothalamic neurotransmitters which regulate hormone release.

Earlier studies demonstrated that short photoperiod exposure decreased DA concentrations in the Syrian hamster hypothalamus after 10 weeks of treatment (Steger et al., 1982; Steger et al., 1984). After 11.4 weeks, short photoperiod exposure reduced DA in the more specific regions of the hamster - the MBH and the MPOA/SCN (Steger et al., 1985b). Benson (1987) demonstrated significantly decreased DA levels in the MBH/ME of the Syrian hamster after 9 and 12 weeks of short photoperiod exposure. Krajnak and colleagues (Krajnak et al., 1992 Abs; 1994a) also found a short photoperiod-induced reduction in tuberoinfundibular DA (TIDA) after 12 weeks.

Short-photoperiod exposure was reported to reduce hypothalamic DA turnover (Steger et al., 1982) including the ME and MBH (Steger et al., 1985b; 1986; Steger and Bartke, 1991) and the MBH/ME (Benson, 1987) using the alpha-methyl-para-tyrosine (α-MPT) method after several weeks of treatment.

A melatonin-induced reduction in DA levels was documented in the ME/arcuate region and the posterior pituitary of female Syrian hamsters after 10 weeks of daily late afternoon injections of the indole (Alexiuk and Vriend, 1991). Melatonin markedly decreased MBH DA accumulation after administration of pargyline (Alexiuk and Vriend, 1991) in both intact and ovariectomized hamsters.

Although a melatonin-induced inhibition of DA release has been well-documented in the retina (Dubocovich, 1983; 1985; Nowak, 1988) presently, no studies have demonstrated effects of short photoperiod or melatonin on TIDA release.

Evidence is accumulating from several laboratories, suggesting that both short photoperiod exposure (Steger et al., 1986; Benson, 1987; Steger and Bartke, 1991) and melatonin administration (Fang and Dubocovich, 1990; Alexiuk and Vriend, 1991) significantly affect the noradrenergic system of the ME/arcuate region of the MBH. The effects of melatonin or short photoperiod exposure on NE concentrations in the MBH, however, shows less consistency in the results. Two groups of investigators (Steger et al., 1985b; 1986; Benson, 1987; Steger and Bartke, 1991) have found short photoperiod-induced inhibition of NE turnover (using the α-MPT method) in the ME and MBH/ME region after several weeks of treatment. Vriend

and colleagues reported a melatonin-induced decrease in NE turnover in the MBH (Vriend et al., 1990) - also via the α-MPT technique. Reductions in the accumulation of NE after pargyline administration were demonstrated in the ME/arcuate region (Alexiuk and Vriend, 1991) as well as in the amygdala and in the pontine brainstem (Alexiuk and Vriend, 1993b) after 10 weeks of melatonin administration.

Although it is clear that melatonin has marked effects on the catecholamine system, it remains unclear whether these are direct ones or whether they are secondary to melatonin-induced effects on other neurons. The hypothesis that melatonin is acting through the serotoninergic system originated from studies showing that acute melatonin administration increased the concentrations of hypothalamic and midbrain serotonin (Anton-Tay et al., 1968). Short photoperiod exposure elevated serotonin synthesis in the MBH (Steger et al., 1990) of the male Syrian hamster. Melatonin treatment both decreased and increased 5-HT accumulation after pargyline in the amygdala and in the pontine brainstem, respectively, of the female hamster (Alexiuk and Vriend, 1993b). Significant effects on serotoninergic metabolism in the hamster hypothalamus has been documented in several studies utilizing a diversity of treatments including orbital enucleation (Vriend, 1989), short photoperiod exposure (Benson, 1987) and melatonin administration (Vriend et al., 1990; Vriend, 1991; Alexiuk and Vriend, 1991; 1993b). Melatonin treatment for 10 weeks elevated 5-HIAA levels in the both the ME/arcuate region and the striatum (Alexiuk and Vriend, 1991; 1993b). Melatonin administration (for 10 weeks) also

increased the 5-HIAA/5-HT ratio in the hypothalamus (Vriend, 1989), the hypothalamus and brainstem (Vriend et al., 1990; Vriend, 1991). However, melatonin decreased this ratio in the amygdala (Alexiuk and Vriend, 1993b).

1.15 EFFECTS OF MELATONIN ON GABA

Early studies by Anton-Tay (Anton-Tay et al., 1971; Anton-Tay, 1974) showing the acute dose-dependent effects of melatonin on GABA concentrations, suggested the hypothesis that the primary neurochemical effects of this indole was mediated via the major CNS inhibitory neurons - the GABAergic system. The question arises as to whether melatonin's effects on monoaminergic transmitters are direct ones or are secondary to melatonin-induced effects on GABA. Subsequent studies have found significant effects of melatonin on GABAergic activity offering accumulating support for this hypothesis. A melatonin-induced increase in GABA turnover (Rosenstein and Cardinali, 1986) - possibly via an increase in glutamic acid decarboxylase (the rate-limiting enzyme in GABA synthesis) activity (Rosenstein et al., 1989) - has been reported. Melatonin has been shown to augment chloride ion influx by potentiating a GABA-induced increase of chloride ion uptake (Rosenstein et al., 1989). Melatonin has the capacity to increase benzodiazepine (Niles et al., 1987b), GABA (Acuna-Castroviejo et al., 1986) and GABA "A" agonist binding (Coloma and Niles, 1988) to GABA "A" receptor sites. The indole has also been shown to inhibit binding of a chloride channel blocker to its GABA "A' site (Niles and Peace, 1990).

The motivation to study the GABAergic system was originally derived from

an urgency to understand the mechanism of action of the major groups of sedative/hypnotic drugs - the benzodiazepines and barbiturates. Since melatonin has been reported to have a similar psychopharmacological potential as this particular group of pharmaceutical agents (Brown and Niles., 1982; DeFeudis, 1983; Sugden, 1983; Niles et al., 1987), the question arose as to whether this indole presents with an identical mechanism and/or site of action.

Melatonin's sedative and hypnotic properties in both humans and animal models have been documented for years (Marczynski et al., 1964; Holmes and Sugden, 1982;1983; Lieberman et al., 1984; Wurtman and Lieberman, 1985; Waldhauser et al., 1990; Macfarlene et al., 1991; Zhdanova et al., 1994). Similar to the action of the major sedative/hypnotic drugs, melatonin treatment has been demonstrated to have anxiolytic (anxiety-relieving) effects (Neville and McNaughton, 1986; Guardiola Lemaitre et al., 1992; Golombek et al., 1993; Pierrefiche et al., 1993) in several behavioral paradigms. Melatonin has also been reported to have anticonvulsive effects, analogous to the major sedative/hypnotic drugs. Administration of melatonin in a particular regime has been reported to have effective anticonvulsive properties in a variety of seizure models including: the human (Anton-Tay et al., 1971), the baboon (Brailowsky, 1976), the rat (Izumi et al., 1973; Albertson et al., 1981; Sugden, 1983), the mouse (Sugden, 1983), the gerbil (Champney and Champney, 1992), and the Syrian hamster (Golombek et al., 1992). Although a melatonin-induced effect on GABA in the production of melatonin's anticonvulsive effects has been postulated (Anton-Tay et al., 1971; Albertson et al.,

1981), some evidence suggests that a kynurenamine metabolite of melatonin (N-acetyl-5-methoxykynurenamine) could be involved (Kennaway and Hugel, 1992). It has been reported that melatonin is non-toxic and non-addictive - even when administered in large pharmacological doses (Vaughan, 1984; Arendt et al., 1985; Reppert et al., 1988).

Since the site of action of most of the benzodiazepines and barbiturates is considered to be the GABA "A" receptor, this raised the issue of whether melatonin also acts through GABA receptors. In addition to the similarities of melatonin's psychopharmacological effects to those of the major sedative and hypnotic drugs, the indole also has been reported to compete for and increase benzodiazepine binding at the GABA "A" receptor site. This has raised the audacious question of whether melatonin represents an endogenous diazepam. Several studies have addressed this issue by examining whether the benzodiazepine antagonist (flumazenil) could reverse or decrease melatonin-induced behavioral effects. This was shown to be true in several experiments, on melatonin-induced effects on locomotor activity (Golombek et al., 1991), anticonvulsant abilities (Golombek et al., 1992), anxiolytic properties (Golombek et al., 1993; Pierrefiche et al., 1993), and melatonin-induced effects on the reentrainment of circadian rhythms (Golombek and Cardinali, 1993) - hence providing some evidence for this hypothesis. However, since it has not been consistently replicated (Niles and Peace, 1990; Niles and Hashemi, 1990b) in all documented studies (and continues to be complicated by the existence of GABA-independent benzodiazepine binding sites) the issue remains inconclusive.

1.16 CLINICAL EFFECTS OF MELATONIN

Melatonin has been implicated in the etiology and/or pathophysiology of a number of clinical conditions - many of which are associated with reproductive and endocrine physiology (Reiter, 1991b; 1991c). Hypothalamic amenorrhea, delayed or advanced puberty, hypogonadotropic hypogonadism, late luteal phase dysphoric disorder, anovulation and oligospermia/aspermia have all been hypothesized to be causally related to alterations in pineal functions and/or changes in the output of melatonin secretion (Reiter, 1991b; 1991c). Increases in the amplitude of the nocturnal melatonin rhythm has been demonstrated in hypothalamic amenorrhea (Berga et al., 1988; Brzezinski et al., 1988), anorexia nervosa (Tortosa et al., 1989), late luteal phase dysphoric disorder (Wirz-Justice and Arendt, 1979) and hypogonadotropic hypogonadism (Puig-Domingo et al., 1992). Decreases in the melatonin rhythm have been associated with certain depressive subtypes of mood disorders (Wetterberg et al., 1990) as well as with paranoid schizophrenia (Monteleone et al., 1992). However, the issue of whether changes in melatonin rhythm amplitude is causally related to the pathophysiology of these conditions remains to be determined.

It has also been suggested that melatonin may be regulating hormone responsive tumor growth (oncostatic actions) perhaps via a modulation of the immune system (Maestroni et al., 1986). In addition to this, melatonin is thought to have extensive antioxidant abilities (Reiter et al., 1993b; Tan et al., 1993), is considered to be a 'free radical scavenger' (Reiter et al., 1993a; Tan et al., 1993) and

to possess antiaging potential (Armstrong and Redman, 1991; Reiter et al., 1993a).

Another clinical area in which melatonin has received considerable attention includes the study of the indole's role in the disorders of circadian or biological rhythms. Melatonin and its relationship to delayed sleep phase syndrome, jet lag, shift workers syndrome and various mood disorders - especially seasonal affective disorder (SAD) - have all been well-documented in the melatonin literature (Rosenthal et al., 1984). This particular subtype of mood disorder is characterized by an elevated sensitivity to changes in environmental lighting and temperature. Hence, the frequency of this disorder is much greater in higher latitudes (temperate or polar areas) in comparison to that of equatorial regions. Patients with SAD express atypical vegetative symptomatology; characterized by hypersomnia, fatigue, hyperphagia, carbohydrate craving and weight gain - which can include manifestations of clinical depression. Some researchers attribute these atypical symptoms to a "medial hypothalamus syndrome" involving altered alpha 2-noradrenergic and serotoninergic mechanisms (Krauchi and Wirz-Justice, 1987; 1988). It is argued that the two indoleamines, melatonin and serotonin, as well as corticotropin-releasing factor are causally involved in SAD (Wehr, 1992). Individuals with SAD may be experiencing a melatonin-induced cluster of symptoms (Sack et al., 1987). Phototherapy (bright light treatment) is often administered at an intensity which suppresses the synthesis and secretion of melatonin (Lewy et al., 1980). Light therapy (in combination with pharmacological intervention) has been deemed an efficacious treatment for individuals suffering from SAD (Rosenthal et al., 1985) -

also called "winter depression". Melatonin's role in the pathophysiology of mood disorders, in general, remains under continuing investigation (Sack and Lewy, 1988; Oren, 1991; Wehr, 1992).

Many studies have demonstrated melatonin's sedative/hypnotic effects (Zhdanova et al., 1994) and its capacity to treat insomnia (Waldhauser et al., 1990) and delayed sleep-phase syndrome (Dahlitz et al., 1991). Its unique ability in the process of reentrainment of disturbed circadian (biological) rhythms - which are characteristic of individuals who suffer from jet lag (Arendt, 1987) and shift workers syndrome (Czeisler et al., 1983) - attests to melatonin's potential future as an efficacious pharmaceutical agent.

1.17 OBJECTIVES OF THE PRESENT INVESTIGATION

Previous studies have suggested that melatonin is producing its marked neuroendocrine effects by modulating the synthesis/release and/or metabolism of neurotransmitters that regulate hypothalamic and pituitary hormone secretion (Alexiuk and Vriend, 1991; 1993b). The hypothesis that melatonin modifies the activity of the rate-limiting enzymes in catecholamine and serotonin (5-HT) synthesis, is derived from evidence demonstrating that melatonin significantly influences brain monoaminergic neurotransmitter systems (Alexiuk and Vriend, 1991; 1993b). The major overall objective of the present investigation is to provide information on the temporal sequence (time course) and circadian (24 hour) effects of melatonin action in the male Syrian hamster.

The first objective was to determine the temporal effects of daily late afternoon melatonin injections on the concentrations of monoamines and metabolites in the ME/arcuate region of the MBH.

The second objective was to ascertain whether melatonin influences monoaminergic neurotransmitters outside of the MBH.

A third objective was to determine whether melatonin administration influences the **in situ** activity of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH); the rate-limiting enzymes in catecholamine and 5-HT synthesis, respectively.

The fourth objective was to ascertain whether the effects of melatonin on enzyme activity (TH; TPH) and transmitter concentrations are restricted to a particular phase of the light/dark cycle.

A fifth objective was to determine whether melatonin influences the concentrations of the excitatory and inhibitory amino acid neurotransmitters (ie. glutamate and GABA); and to ascertain whether these effects occur at a specific time point over the 24-hr light/dark cycle.

A final objective was to determine whether the effects of melatonin in the ME/arcuate region of the MBH and the posterior pituitary are independent of melatonin-induced changes in circulating levels of gonadal steroids.

2. METHODS AND MATERIALS

2.1 EXPERIMENT ONE: TIME COURSE FOR MELATONIN ON MONOAMINE CONCENTRATIONS IN THE MBH

The purpose of this experiment was to determine the temporal (time course) effects of daily late afternoon administration of melatonin on the tissue concentrations of monoamines and metabolites in the median eminence (ME)/arcuate region of the medial basal hypothalamus (MBH) of male Syrian hamsters. This experiment was designed to demonstrate the effects of melatonin on catecholamines in animals not treated with NSD-1015.

2.1.1 Animals

Twenty, nine-week old male Syrian (golden) hamsters (strain LAK:LVG, Charles River, St. Constance, Quebec), were maintained under controlled lighting (14L/10D) and temperature (22° ± 2° C) conditions. Food and water was provided ad libitum.

2.1.2 Experimental Design

In this experiment, 4 groups of animals (N = 5), received daily sc injections of either 0.1 ml of physiological saline or 25 micrograms (25 μg) of melatonin (N-acetyl-5-methoxytryptamine) in 0.1 ml of physiological saline; 1-2 hours prior to lights

out. Hamsters were sacrificed via decapitation after three and six weeks of treatment. They received no additional drugs prior to sacrifice. The brains were removed and immediately frozen. From the MBH, a 1 millimeter (mm) deep slice was dissected and a 2 mm circular punch of tissue containing the ME and arcuate nucleus was taken. The mean weight of MBH samples was 3.15 mg.

2.1.3 Monoamine Determination

Tissue samples remained frozen until they were processed for high performance liquid chromatography with electrochemical detection (HPLC-EC). At this time, the tissue samples were homogenized in 0.1 N perchloric buffer. The concentrations of monoamines and metabolites were determined via HPLC-EC. All of these electrochemically active neurochemicals were separated using an HPLC system that consisted of a Beckman solvent delivery system (Model 114M), an Altex injector (Model 210A), and a 10 cm C-18 reverse-phase column (Chromatography Sciences Co., Canada). The electrochemical detector (ESA Model 5100A) was equipped with a high sensitivity cell (ESA Model 5011). The mobile phase (running buffer) consisted of 60 mM sodium acetate, 122 nM EDTA, 763 nM octane sulphonate, 7% methanol. The mobile phase was brought to a pH of 4.25 with glacial acetic acid.

2.1.4 Statistical Significance

The data was subjected to analysis of variance (2-way ANOVA).

ANOVA (treatment x week) was used to analyze the data on the concentrations of catecholamines. The data were then subjected to Student's t tests. Statistical significance was considered as a p value of less than 0.05. The following levels of significance were distinguished: $p < 0.05$; $p < 0.01$; $p < 0.001$.

2.2 EXPERIMENT TWO: TIME COURSE FOR NSD-1015

The present study was designed to test whether 3,4-dihydroxyphenylalamine (L-DOPA) and 5-hydroxytryptophan (5-HTP) increased linearly with time after administration of the aromatic L-amino acid decarboxylase inhibitor (AAAD), NSD-1015 (m-hydroxybenzylhydrazine), Sigma; 100 mg/kg (Demarest and Moore, 1980). Another purpose of this experiment was to determine the optimal time of sacrifice of the male Syrian hamster, following the administration of the drug.

2.2.1 Animals

Thirty-six nine week old male Syrian (golden) hamsters were used in this experiment (strain LAK:LVG, Charles River, St. Constance, Quebec). Hamsters were maintained under controlled lighting (14L/10D) and temperature (22° ± 2° C) conditions - and were provided with food (Teklad rodent diet) and water ad libitum.

2.2.2 Experimental Design

The thirty-six Syrian hamsters were divided into two groups of eighteen (18). Each of these 18 was further divided into two groups containing nine (9) animals.

Two of the groups of 9 received ip injections of either 0.1 ml of physiological saline or the AAAD inhibitor, NSD-1015 (m-hydroxybenzylhydrazine, Sigma; 100 mg/kg) - thirty (30) minutes prior to sacrifice. The remaining two groups of 9 were administered ip saline or NSD-1015 (100 mg/kg) - sixty (60) minutes prior to sacrifice.

All animals were killed via decapitation during the light period. Their brains were removed and immediately frozen on dry ice. In order to facilitate removal of the brain regions specified, the brains were partially thawed. A 1 mm deep dissection of the MBH was made from which a 2 mm deep circular punch of tissue (containing the ME and arcuate nucleus) - was removed. The mean weight was 3.13 mg. A punch of the caudate nucleus (striatum) - with a mean weight of 3.42 mg - was also dissected at this time; in preparation for HPLC-EC processing (high-performance liquid chromatography with electrochemical detection). All tissue samples remained frozen until they were ready for processing via HPLC. At this time, the tissues were homogenized and then centrifuged at 12,000 g for 7 minutes.

2.2.3 Monoamine Determination

Since NSD-1015 is an aromatic L-amino decarboxylase inhibitor, the catecholamine and serotonin precursors - L-DOPA and 5-HTP - will accumulate after administration of this drug. The determination of these neurochemicals is a measurement of the **in situ** activity of the rate-limiting enzymes (refer to Fig. A1 and Fig. A2) in catecholamine and serotonin synthesis - tyrosine hydroxylase (TH) and

tryptophan hydroxylase (TPH), respectively (Carlsson et al., 1972). Concentrations of precursors (L-DOPA, 5-HTP) were separated and assayed via HPLC-EC. The tissue levels of monoamines dopamine - 4-(2-aminoethyl)-1,2-benzenediol (DA), norepinephrine - 4-(2-amino-1-hydroxyethyl)-1,2-benzenediol (NE), serotonin - 3-(2-aminoethyl)-1 H indole-5 ol (5-HT), and metabolites homovanillic acid (HVA), 3,4 dihydroxybenzeneacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA) remaining after NSD-1015 administration were also determined via HPLC-EC. The lower limit of detectability for the catecholamines was ≤ 25 pg; for HVA and for 5-HT, the limit of detectability was ≤ 50 pg. The detector was set at an oxidative potential of D1 = +0.04; guard cell = +0.04; D2 = +0.30. A Shimadzu integrator (Model C-R3A) was used to record and integrate peak areas and to calculate the concentrations of monoamines, precursors and metabolites. The mobile phase (running buffer) consisted of 0.05 M sodium phosphate monobasic, 1.2 mM heptanesulphonic acid, 0.2 mM EDTA and 3% methanol. The mobile phase was brought up to a pH of 3.50 with phosphoric acid.

2.2.4 Statistical Analysis

All of the data was subjected to two-way analysis of variance (ANOVA). ANOVA (treatment x time of sacrifice) was utilized to analyze the data, the concentrations of precursors, monoamines and metabolites. The data was then subjected to Student's t-tests. Statistical significance was considered as a p value of less than 0.05. The following levels of significance were distinguished: $p < 0.05$; p

< 0.01; $p < 0.001$; $p < 0.0001$.

2.3 EXPERIMENT THREE: TEMPORAL SEQUENCE OF MELATONIN ON TH AND TPH ACTIVITY

The purpose of this experiment was to determine the temporal effects of daily melatonin injections on the **in situ** activities of TH and TPH in selected regions of the Syrian hamster brain. The experiment was also designed to relate the effects of melatonin on enzyme activity to changes in monoamine and metabolite concentrations following NSD-1015 administration. Several regions (posterior pituitary (NIL), ME/arcuate of MBH, striatum, amygdala, pons, midbrain) were examined for comparison purposes.

2.3.1 Animals

Forty-eight (48) nine-week old male (golden) hamsters (strain Lak:LVG, Charles River, St. Constance, Quebec) were used in this first experiment - a time course study. The hamsters were maintained under controlled lighting and temperature conditions (22° ± 2° C). The photoperiod began at 0400 hours and ended when lights were turned off at 1800 hours. The light intensity at the level of the cage was approximately 200 footcandles. Food (Teklad rodent diet) and water were provided ad lib and the hamsters were housed 4 per cage.

2.3.2 Experimental Design

The hamsters were acclimatized to laboratory conditions for approximately one week. The forty-eight hamsters were divided into three groups of sixteen (16). Each of these three groups were then further divided into two groups of eight (8). The first group of hamsters received daily sc injections of 0.1 ml of physiological saline; while the remaining eight animals were treated with daily injections of 25 micrograms (μg) of melatonin (N-acetyl-5-methoxytryptamine) in 0.1 ml of saline. The injections were administered between 1600 and 1700 hours.

Following daily treatments for one (1) week, all hamsters from the first group (both saline and melatonin-treated), received an ip injection of an aromatic-L-amino-acid decarboxylase inhibitor, NSD-1015 (m-hydroxybenzylhydrazine, Sigma) - 100 mg/kg - forty minutes before sacrifice. After three (3) weeks of treatment with saline or melatonin, all sixteen animals from the second group were injected with NSD-1015 and following forty minutes were sacrificed via decapitation. This was repeated also for the third group of hamsters after five (5) weeks of saline or melatonin treatment. All animals were sacrificed between 1100 and 1300 hours - approximately 18-20 hours following the last saline or melatonin injection. The posterior pituitaries (neurointermediate lobes) were removed and separated from the anterior lobes - then immediately frozen on dry ice. From the MBH, a 1 mm deep slice was dissected and a circular punch (2 mm diameter) containing the ME and arcuate nucleus were removed. The mean weight of MBH samples from this experiment was 2.97 mg. A punch of the striatum containing the caudate nucleus

(mean weight = 4.09 mg) was also removed. A region of the temporal lobe containing the amygdala was dissected - with a mean weight of 20.46 mg. The brainstem was also dissected and divided into the pons (mean weight = 132.10 mg) and the midbrain (mean weight = 70.32 mg). Regions outside the MBH were examined in order to ascertain whether melatonin had extrahypothalamic effects on TH and TPH activity. Testicular weights were determined at this time in order to verify the physiological effectiveness of melatonin (Tamarkin et al., 1976a).

Protein measurements of tissues were carried out using a Bio-Rad Protein Assay Kit (Bio-Rad).

2.3.3 Monoamine Determination

All tissue samples remained frozen until they were processed for high performance liquid chromatography with electrochemical detection (HPLC-EC). At this time the tissues were homogenized in the running buffer containing the internal standard dihydroxybenzylamine (DHBA 10 ng/ml) and ascorbic acid (1 μg/ml buffer) - and centrifuged at 12,000 g for 7 min. The precursors L-DOPA and 5-HTP, the monoamines dopamine - (DA), norepinephrine - (NE) and serotonin - (5-HT) were separated and assayed via HPLC-EC. The acid metabolites of the monoamines: HVA, DOPAC and 5-HIAA were also measured simultaneously.

The enzymes tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) are considered to be rate-limiting in catecholamine (Levitt et al., 1965; Masserano and Weiner, 1983) and 5-HT synthesis (Ashcroft et al., 1965) respectively - under normal

conditions.

Extracts of the posterior pituitary, the ME/arcuate region of the MBH and the caudate n. were separated using the HPLC system. The detector was set at a reduction potential of D1 = +0.04; guard cell = +0.40; D2 = -0.34. The mobile phase (running buffer) consisted of 0.05 M sodium phosphate monobasic, 1.2 mM heptanesulphonic acid, 0.2 mM EDTA, and 3% methanol. The mobile phase was brought to a pH of 3.00 with phosphoric acid. Extracts of the midbrain, the pons and the amygdala were also separated using the HPLC system. The detector was set at an oxidative potential of D1 = +0.02; guard cell = +0.03; D2 = +0.35. The mobile phase (running buffer) consisted of 0.05 M sodium phosphate monobasic, 1.2 mM heptanesulphonic acid, 0.2 mM EDTA, and 4% methanol. The mobile phase was brought to a pH of 2.77 with phosphoric acid.

2.3.4 Statistical Analysis

All the data was subjected to two-way analysis of variance (ANOVA). ANOVA (treatment x week) was utilized to analyze the data on the L-DOPA and 5-HTP accumulation after administration of NSD-1015 as well as the concentrations of monoamines and metabolites. The data were then subjected to Student's t-tests. Statistical significance was considered as a p value of less than 0.05. The following levels of significance were distinguished: $p < 0.05$; $p < 0.01$; $p < 0.001$.

2.4 EXPERIMENT FOUR: CIRCADIAN (24 HOUR) EFFECTS OF MELATONIN

The secretion of melatonin is circadian in nature with a release pattern that is synchronized to the daily light/dark cycle. Therefore the effects of this indole on the synthesis/release (turnover) and concentrations of catecholamines (DA, NE), 5-HT and amino acid transmitters over a 24 hour period may be important. The purpose of this experiment was to determine the circadian (24-hr) effects of longterm daily melatonin injections (9.5 weeks) on TH and TPH activities, and to relate this to tissue concentrations of catecholamines and 5-HT (and their metabolites) in the ME/arcuate region of the MBH. The posterior pituitary (NIL) and striatum (caudate n.) were also examined for comparison purposes and to provide information on different dopaminergic systems (tuberoinfundibular, tuberohypophyseal and nigrostriatal). This experiment also examined the 24-hr effects of melatonin on amino acid transmitters as well as on the monoamines.

2.4.1 Animals

Sixty-four (64) nine-week old male Syrian hamsters (strain Lak:LVG, Charles River, St. Constance, Quebec) were used in this experiment. The hamsters were maintained under controlled lighting and temperature conditions (22° ± 2° C). The photoperiod began at 0400 hours (4 AM) and ended when lights were turned off at 1800 hours (6 PM). Food (Teklad rodent diet) and water were provided ad lib and the hamsters were housed 4 per cage.

2.4.2 Experimental Design

In this fourth experiment, 64 male Syrian hamsters were divided into 2 groups. Thirty-two hamsters received daily sc injections of physiological saline while the remaining animals were administered 25 μg of melatonin (N-acetyl-5-methoxytryptamine) in 0.1 ml of saline. Following nine and a half (9.5) weeks of treatment, hamsters were divided into 4 groups (N = 8) representing 4 different times of sacrifice - 6 hours apart. Sacrifice times were at 2 PM (1400 hrs); 8 PM (2000 hrs); 2 AM (0200 hrs); and 8 AM (0800 hrs). Animals from the 8 PM and 2 AM groups were killed during the dark phase (under very dim red light). Forty minutes prior to the time of sacrifice, each animal received an ip injection of NSD-1015, in order to measure the accumulation of L-DOPA, an indication of the **in situ** activity of the rate-limiting enzyme of catecholamine synthesis, TH, (Levitt et al., 1965). This procedure also was used to measure 5-HTP accumulation after NSD-1015 in order to determine the activity of TPH.

The brains were frozen on dry ice upon removal. The posterior pituitaries (neurointermediate lobes) were also removed and immediately frozen. At the time of dissection, the brains were partially thawed. A 1 mm deep slice of the MBH was dissected and from this piece of tissue, a circular punch (2 mm diameter) containing the ME and arcuate nucleus was removed. The mean weight of these tissue samples was 2.56 mg. The mean weight of the striatal punches (caudate nucleus) was 4.27 mg. All tissue samples remained frozen until they were processed for high-

performance liquid chromatography with electrochemical detection (HPLC-EC). At the time, the tissues were homogenized in the running buffer containing the internal standard dihydroxybenzylamine (DHBA 10 ng/ml) and ascorbic acid (1 μg/ml buffer) - and centrifuged at 12,000 g for 7 min. The precursors L-DOPA and 5-HTP, the monoamines DA, NE and 5-HT, as well as the acid metabolites of DA (HVA, DOPAC) and the 5-HT metabolite, (5-HIAA) were separated and assayed via HPLC-EC.

The posterior pituitary, the MBH and the striatum were separated using an HPLC system that consisted of a Beckman solvent delivery system (Model 114M), and an Altex injector (Model 210A), and a 10 cm C-18 reverse-phase column (Chromatography Sciences Co., Canada). The electrochemical detector (ESA Model 5100A) was equipped with a high sensitivity cell (ESA Model 5011). The detector was set at a reduction potential of D1 = +0.04 volts; guard cell = +0.40; D2 = -0.34. A Shimadzu integrator (Model C-R3A) was used to record and integrate peak areas and calculate the concentrations of monoamines, precursors and metabolites. The mobile phase (running buffer) consisted of 0.05 M sodium phosphate monobasic, 1.2 mM heptanesulphonic acid, 0.2 mM EDTA, and 3% methanol. The mobile phase was brought to a pH of 2.96-3.00 with phosphoric acid.

Protein measurements of posterior pituitary extracts were carried out using the Bio-Rad Protein Assay Kit (BIO-RAD).

2.4.3 Measurement of Amino Acid Neurotransmitters

For measuring the amino acids, the identical HPLC system with slight modifications was utilized. The 10 cm C-18 reverse-phase column (short) was replaced with a (long) 25 cm C-18 reverse-phase column (Beckman). An o-phthaldialdehyde (OPA) reagent solution was utilized for precolumn derivatization of the amino acids (1.5 minutes prior to sample injection). The electrochemical detector was set at an oxidative potential; D1 = +0.01; guard cell = +0.0; D2 = +0.35. The mobile phase (running buffer) consisted of 0.1 M sodium phosphate dibasic, 4% acetonitrile, and 25% methanol. The mobile phase was brought to a pH of 6.70 with phosphoric acid. The flow rate was modified to 0.90 ml/min. rather that the usual rate of 1.00 ml/min.

2.4.4 Statistical Analysis

All of the data was subjected to two-way analysis of variance (ANOVA). ANOVA (treatment x time of sacrifice) was utilized to analyze the data: precursors L-DOPA and 5-HTP, monoamines DA, NE and 5-HT, metabolites HVA, DOPAC and 5-HIAA as well as the amino acids. The data were then subjected to Student's t-tests. Statistical significance was considered as a p value of less than 0.05. The following level of significance were distinguished: $p < 0.05$; $p < 0.01$; $p < 0.001$; $p < 0.0001$.

2.5 EXPERIMENT FIVE: GONADECTOMY STUDY

This experiment was designed primarily to ascertain whether the effects of daily melatonin injections for 9.5 weeks are direct ones or are secondary to melatonin-induced decreases in circulating levels of testosterone.

2.5.1 Animals

Twenty-four nine week old male Syrian hamsters (strain Lak; LVG, Charles River, St. Constance, Quebec) were used in this study (weighing in at approximately 110-120 grams). The animals were maintained under controlled lighting and temperature conditions (22° ± 2° C) as described previously. The photoperiod began at 0400 hr and ended when the lights were turned off at 1800 hrs.

Sodium pentobarbital (50 mg/kg) was used for anesthesia prior to surgery. All hamsters were gonadectomized (castrated) via a midline linea alba incision (about 2 cm in length). Testes were removed from the abdominal cavity - following dissection from their respective fat pad attachments. The testicular arteries were tied off in order to prevent internal bleeding. The rectus abdominus muscle was sutured and the overlying skin stapled securely shut. Banamine (2.5 mg/kg) was utilized as an analgesic/anti-inflammatory agent post-surgically.

2.5.2 Experimental Design

Following a week of surgical recovery, the animals were divided into two groups of twelve (N = 12). One group received daily sc injections of 0.1 ml of physiological saline; while the remaining animals were administered daily injections of 25 μg of melatonin (N-acetyl-5-methoxytryptamine) in 0.1 ml of saline (as in similar protocols previously described). All animals received either treatment between 1600 and 1700 hrs. Following 67 days (9.5) weeks of treatment, all hamsters received an ip injection of the aromatic-L-amino-acid decarboxylase inhibitor, NSD-1015 (m-hydroxybenzylhydrazine, Sigma) - 100 mg/kg - 40 minutes prior to sacrifice. All animals were sacrificed between 12 AM (0000) and 2 AM (0200) hrs; in the middle of the dark phase.

All brains were frozen on dry ice upon removal. The posterior pituitaries (containing both the neural and intermediate lobes) were dissected at the time of sacrifice. At a later time, the brains were partially thawed in preparation for dissection of the MBH. A 1 mm deep slice of the MBH was dissected; from this a circular punch of tissue (2 mm diameter) containing the ME and arcuate nucleus was removed. All tissue samples remained frozen until they were processed for HPLC-EC. At this time, the tissues were homogenized in the running buffer containing the internal standard, DHBA, (10 ng/ml) and ascorbic acid (1 μg/ml buffer). The samples were then centrifuged at 12,000 g for 7 min. The precursors L-DOPA and 5-HTP; the monoamines, DA, NE and 5-HT; as well as the metabolites HVA, DOPAC and 5-HIAA were separated and assayed via HPLC-EC (depending on the

brain region).

The precursors, monoamines and metabolites were separated and assayed using the HPLC system. The detector was set at a reduction potential of D1 = +0.04 volts; guard cell = +0.40; D2 = -0.34 (posterior pituitary) or -0.35 for the ME/arcuate region. The mobile phase (running buffer) consisted of 0.05 M sodium phosphate monobasic, 1.2 mM heptanesulphonic acid. 0.2 mM EDTA, and 3% methanol. The mobile phase was brought to a pH of 3.00 (for the posterior pituitary) and 2.99 (for the ME/arcuate region) with phosphoric acid.

Protein measurements of posterior pituitary extracts were carried out using a Bio-Rad Protein Assay Kit (Bio-Rad).

2.5.3 Statistical Analysis

All data was subjected to one-way analysis of variance (ANOVA) and Student's t-tests. Statistical significance was considered as a p value of less than 0.05. The following levels of significance were distinguished: $p < 0.05$; $p < 0.01$; $p < 0.001$.

3. RESULTS

3.1 EXPERIMENT ONE: TIME COURSE FOR MELATONIN ON MONOAMINE CONCENTRATIONS IN THE MBH

3.1.1 Dopamine (DA) Levels in the ME/Arcuate Region of the MBH

Melatonin decreased DA concentrations at both 3 weeks ($t = 2.39$; $p < 0.05$; Fig. 1) and 6 weeks of treatment ($t = 2.46$ $p < 0.05$). This melatonin-induced effect was highly significant ($F = 11.70$; $p < 0.01$; Fig. 1) as detected by ANOVA. No interaction effects were observed.

3.1.2 Norepinephrine (NE) Levels in the MBH

Melatonin treatment significantly increased NE concentrations ($F = 7.86$; $p < 0.025$; Fig. 2). There was also an interaction in the ANOVA showing that this melatonin-induced effect on NE was demonstrated in animals treated for 6 weeks only ($F = 5.28$; $p < 0.05$; $t = 3.15$; $p < 0.05$).

3.1.3 DOPAC Levels in the MBH

Melatonin significantly decreased the concentrations of the DA metabolite, DOPAC, ($F = 11.60$; $p < 0.01$; Fig. 3) in the ME/arcuate region. These melatonin-induced reductions in DOPAC levels were significant at both 3 weeks ($t = 2.76$; p

< 0.05) and 6 weeks ($t = 2.70$; $p < 0.05$) of treatment. A significant time-effect detected by the ANOVA ($F = 9.86$; $p < 0.01$) demonstrated that the overall concentrations of DOPAC (in both saline and melatonin-treated animals) was greater after 6 weeks of treatment (ie. in older animals).

3.1.4 Figures for Experiment One

Fig. 1. Effects of melatonin administration on DA concentrations (after 3 and 6 weeks) in the median eminence (ME)/arcuate region of the mediobasal hypothalamus (MBH) in male Syrian hamsters. Data points represent Mean ± SE. *$p < 0.05$ compared to saline-treated controls.

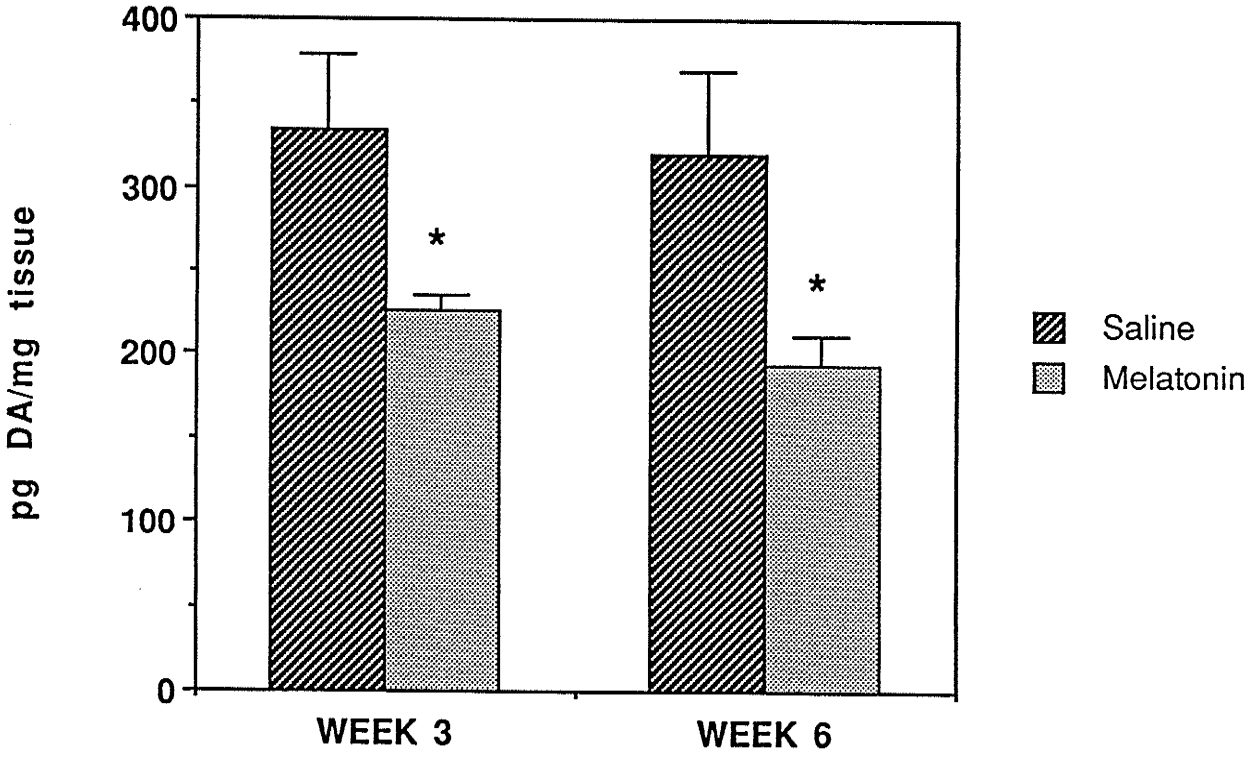

400
300
200
100
0
pg DA/mg tissue
*
*
WEEK 3
WEEK 6
Saline
Melatonin

Fig. 2. Effects of melatonin administration on NE concentrations (after 3 and 6 weeks) in the ME/arcuate region of the MBH in male Syrian hamsters. Data points represent Mean ± SE. *$p < 0.05$ compared to saline-treated controls.

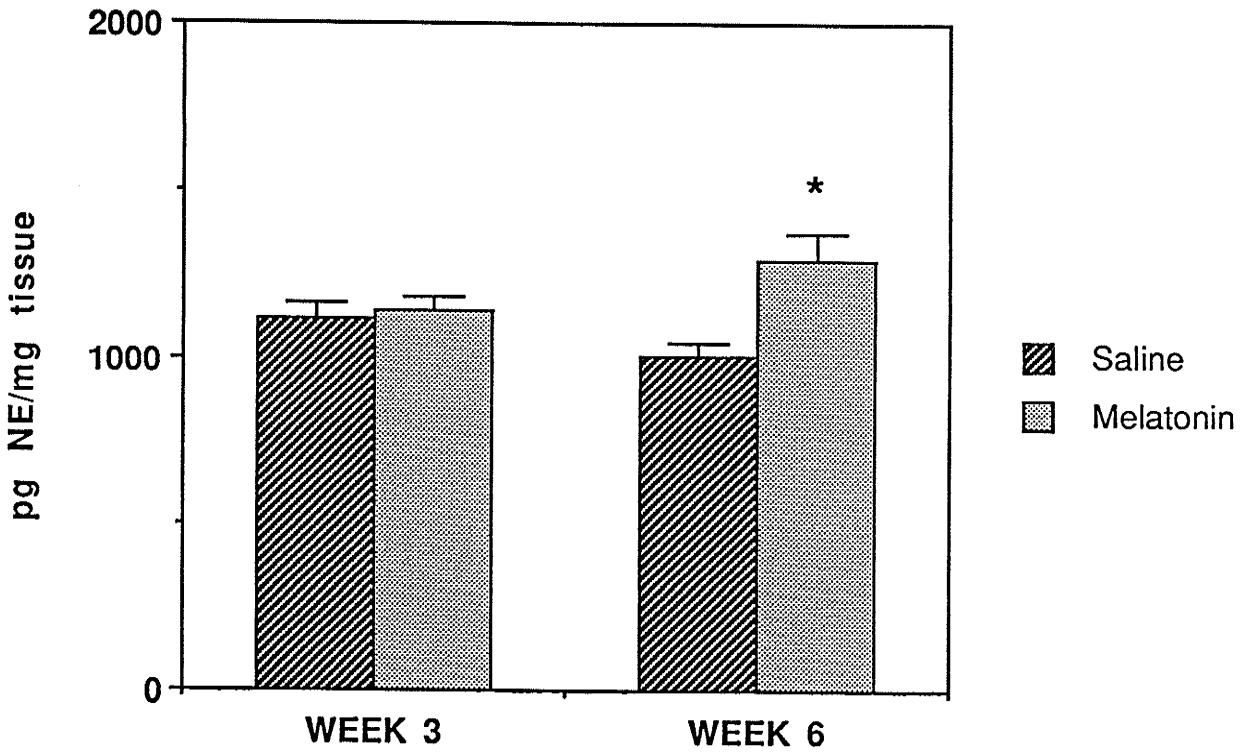
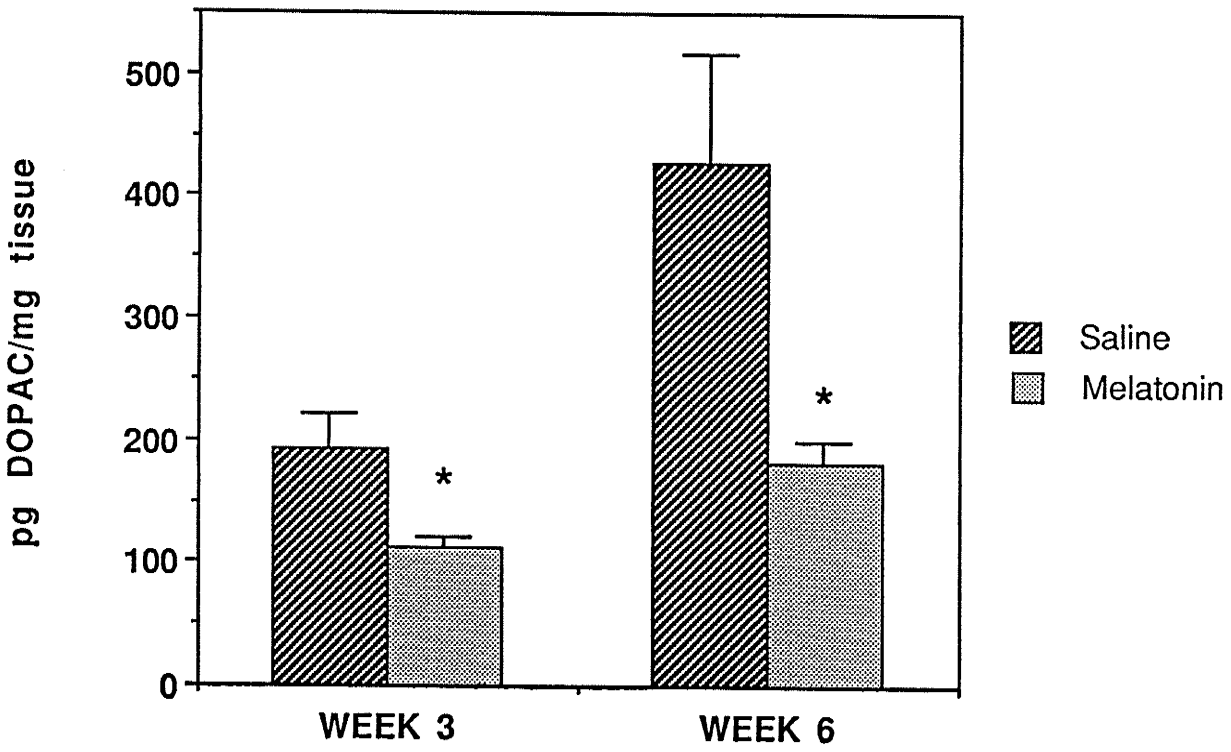

2000
1000
0
pg NE/mg tissue
*
WEEK 3
WEEK 6
Saline
Melatonin

Fig. 3. Effects of melatonin administration on DOPAC concentrations (after 3 and 6 weeks) in the ME/arcuate region of the MBH of male Syrian hamsters. Data points represent Mean ± SE. *$p < 0.05$ compared to saline-treated controls.


pg DOPAC/mg tissue
500
400
300
200
100
0
*
*
WEEK 3
WEEK 6
Saline
Melatonin

3.2 EXPERIMENT TWO: TIME COURSE FOR NSD-1015

3.2.1 L-DOPA and 5-HTP Accumulation after NSD-1015 in the ME/Arcuate Region of the MBH

Fig. 4 and Fig. 5 illustrate the time course for the accumulation of L-DOPA and 5-HTP (following 30 or 60 minutes) after administration of the aromatic L-amino acid decarboxylase inhibitor, NSD-1015, in the ME/arcuate region. Concentrations of the DA precursor, L-DOPA, increased with time until 30 minutes following NSD-1015 administration (Fig. 4); and less rapidly until 60 minutes. Concentrations of L-DOPA were significantly higher in animals sacrificed 60 minutes following administration of NSD-1015 ($t = 3.17$; $p < 0.01$; Table 1). The accumulation of the serotonin (5-HT) precursor, 5-HTP, increased linearly with time until 60 minutes after NSD-1015 administration (Fig. 5) in the MBH. Concentrations of 5-HTP were highly significantly increased in animals sacrificed 60 minutes following administration of NSD-1015 ($t = 7.69$; $p < 0.001$; Table 2). As expected, L-DOPA and 5-HTP concentrations were not detectable via HPLC-EC in animals not treated with NSD-1015 (saline-treated controls).

3.2.2 Effects of NSD-1015 on Monoamines and Metabolites in the ME/arcuate Region of the MBH

No significant differences on DA concentrations could be detected between

saline and NSD-1015-treated animals ($F = 2.00$; N.S.; Table 1) in the MBH. As expected, animals treated with NSD-1015, had a highly significant overall reduction in the DA metabolite, HVA ($F = 108.69$; $p < 0.0001$; Table 1) compared to saline-treated controls. This decrease in ME/arcuate HVA of NSD-1015-treated animals occurred at both 30 ($t = 8.19$; $p < 0.001$) and at 60 minutes ($t = 6.81$; $p < 0.001$) post treatment. Levels of DOPAC were not detectable via HPLC in the ME/arcuate region of the MBH, 30 or 60 minutes following administration of the drug NSD-1015.

ANOVA detected a significant NSD-1015-induced reduction in the concentration of serotonin (5-HT) ($F = 9.84$; $p < 0.01$; Table 2). An interaction in the ANOVA suggested that this effect was more prominent 30 minutes post administration of the drug ($F = 7.42$; $p < 0.025$). NSD-1015 significantly decreased concentrations of 5-HT after 30 minutes of drug treatment ($t = 4.29$; $p < 0.01$). Concentrations of the 5-HT metabolite, 5-HIAA, were highly significantly decreased by NSD-1015 administration as detected by ANOVA ($F = 198.45$; $p < 0.0001$; Table 2) compared to saline-treated controls. This reduction in 5-HIAA concentrations in NSD-1015-treated hamsters was demonstrated at both 30 ($t = 11.06$; $p < 0.001$) and 60 minutes ($t = 9.41$; $p < 0.001$).

An interaction in the ANOVA demonstrated a significant NSD-1015 induced time-specific effect on NE concentrations, 60 minutes following administration of the drug ($F = 8.61$; $p < 0.01$; Table 1). NSD-1015 elevated NE levels compared to saline-treated controls, 60 minutes following drug adminstration ($t = 2.43$; $p < 0.05$; Table 1).

3.2.3 L-DOPA and 5-HTP Accumulation after NSD-1015 in the Striatum

The accumulation of L-DOPA (after NSD-1015) in the striatum increased with time until 30 minutes; and less rapidly until 60 minutes - following administration of the drug NSD-1015 (Fig. 6). The increase in L-DOPA accumulation between 30 and 60 minutes after sacrifice, however was not significant ($t = 0.59$; N.S.; Table 3). As expected, L-DOPA concentrations were not detectable via HPLC in saline-treated controls. The accumulation of 5-HTP (after NSD-1015) in the striatum increased linearly with time following treatment with NSD-1015 (Fig. 7). Concentrations of 5-HTP were significantly increased in animals sacrificed 60 minutes following the NSD-1015 injection ($t = 3.06$; $p < 0.01$; Table 4) compared to animals killed 30 minutes post drug treatment. 5-HTP concentrations were not detected in animals not treated with NSD-1015.

3.2.4 Effects of NSD-1015 on Monoamines and Metabolites in the Striatum

NSD-1015 significantly decreased tissue DA levels as detected via ANOVA ($F = 8.83$; $p < 0.01$; Table 3). NSD-1015 decreased DA concentrations 30 minutes following drug treatment ($t = 2.26$; $p < 0.05$; Table 3) compared to saline-treated controls. A significant effect of time after injection was also demonstrated ($F = 4.01$; $p < 0.05$). ANOVA demonstrated a highly significant overall decrease in HVA concentrations following administration of the drug, NSD-1015 ($F = 74.50$; $p < 0.0001$; Table 3). Concentrations of HVA were reduced in NSD-1015-treated hamsters at both 30 ($t = 4.92$; $p < 0.001$; Table 3) and 60 minutes ($t = 6.97$; $p <$

0.001; Table 3) compared to saline-injected controls. An interaction detected via ANOVA ($F = 10.17$; $p < 0.001$) shows that this effect was more predominant 60 minutes after treatment. A highly significant NSD-1015-induced decrease in the concentrations of DOPAC were detected by ANOVA ($F = 234.58$; $p < 0.0001$; Table 3). The levels of DOPAC was reduced in NSD-1015-treated animals at both 30 ($t = 9.37$; $p < 0.0001$; Table 3) and 60 minutes ($t = 13.51$; $p < 0.0001$; Table 3) compared to saline-injected controls. A significant time effect was also detected by ANOVA ($F = 7.78$; $p < 0.01$).

Concentrations of 5-HT were not detected in the striatum via HPLC, in either saline or NSD-1015-treated animals. Concentrations of the 5-HT metabolite, 5-HIAA, were significantly decreased by NSD-1015 ($F = 123.13$; $p < 0.0001$; Table 4), compared to saline-treated controls, as detected by ANOVA. Levels of 5-HIAA were significantly reduced by NSD-1015 at both 30 minutes ($t = 5.69$; $p < 0.001$; Table 4) and 60 minutes ($t = 9.86$; $p < 0.0001$) following injection of the drug. An interaction detected by the ANOVA ($F = 11.29$; $p < 0.01$) showed that this effect was greater after 60 minutes.

3.2.5 Figures and Tables for Experiment Two

Fig. 4. The effects on L-DOPA accumulation in the median eminence (ME)/arcuate region of the mediobasal hypothalamus (MBH) after administration of the aromatic L-amino acid decarboxylase inhibitor, NSD-1015, for 30 or for 60 minutes in male Syrian hamsters.

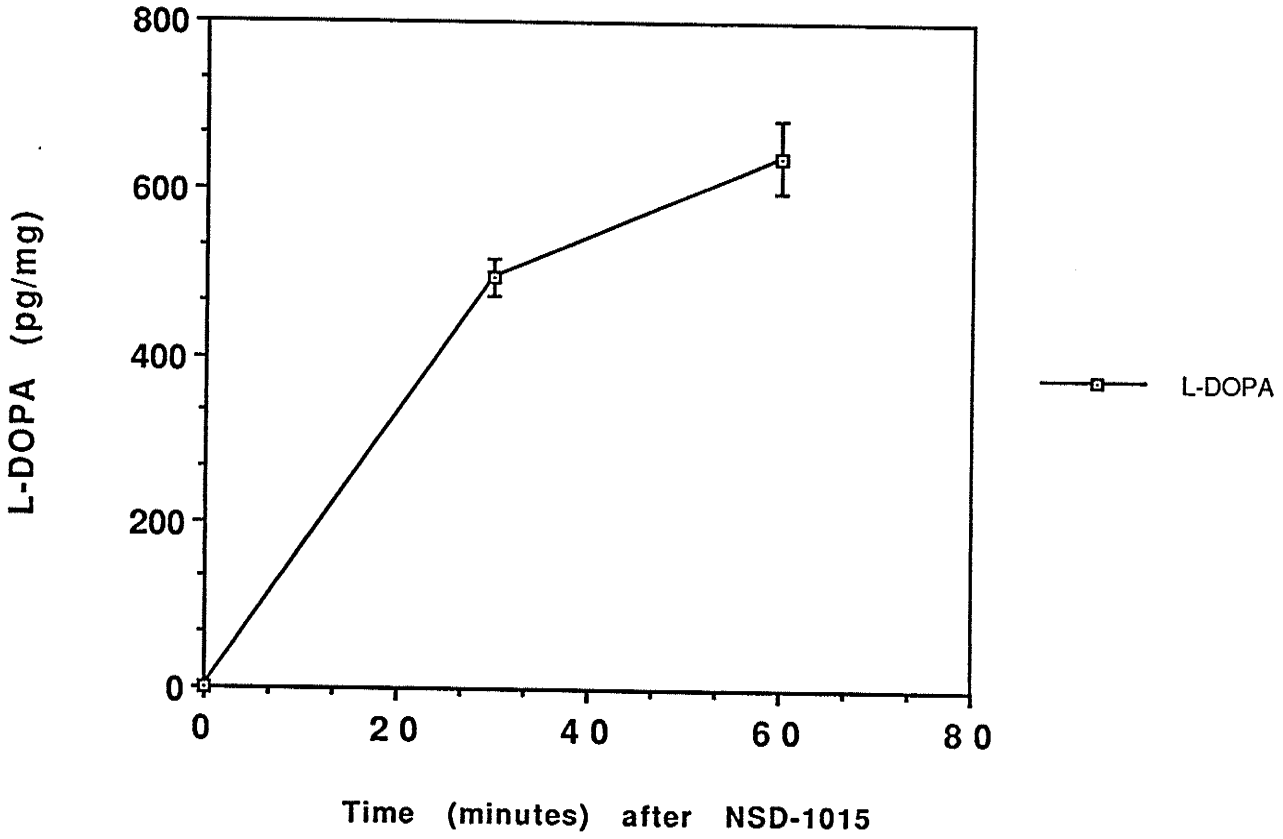

800
600
400
200
0
L-DOPA (pg/mg)
0
20
40
60
80
Time (minutes) after NSD-1015
L-DOPA

Fig. 5. The effects on 5-HTP accumulation in the ME/arcuate region of the MBH after administration of NSD-1015, for 30 and 60 minutes in male Syrian hamsters.

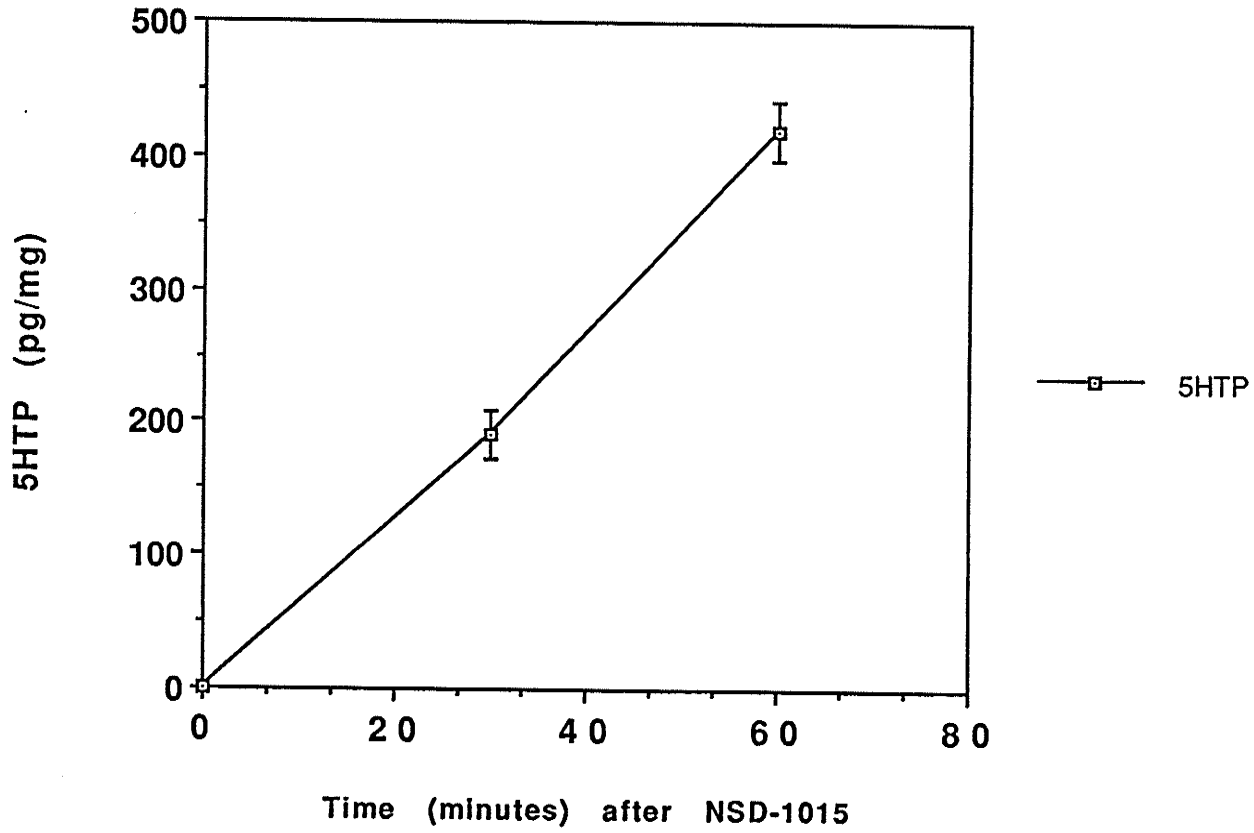

500
400
300
200
100
0
5HTP (pg/mg)
0
20
40
60
80
Time (minutes) after NSD-1015
5HTP

Fig. 6. The effects on L-DOPA accumulation in the striatum after administration of NSD-1015, for 30 and 60 minutes in male Syrian hamsters.

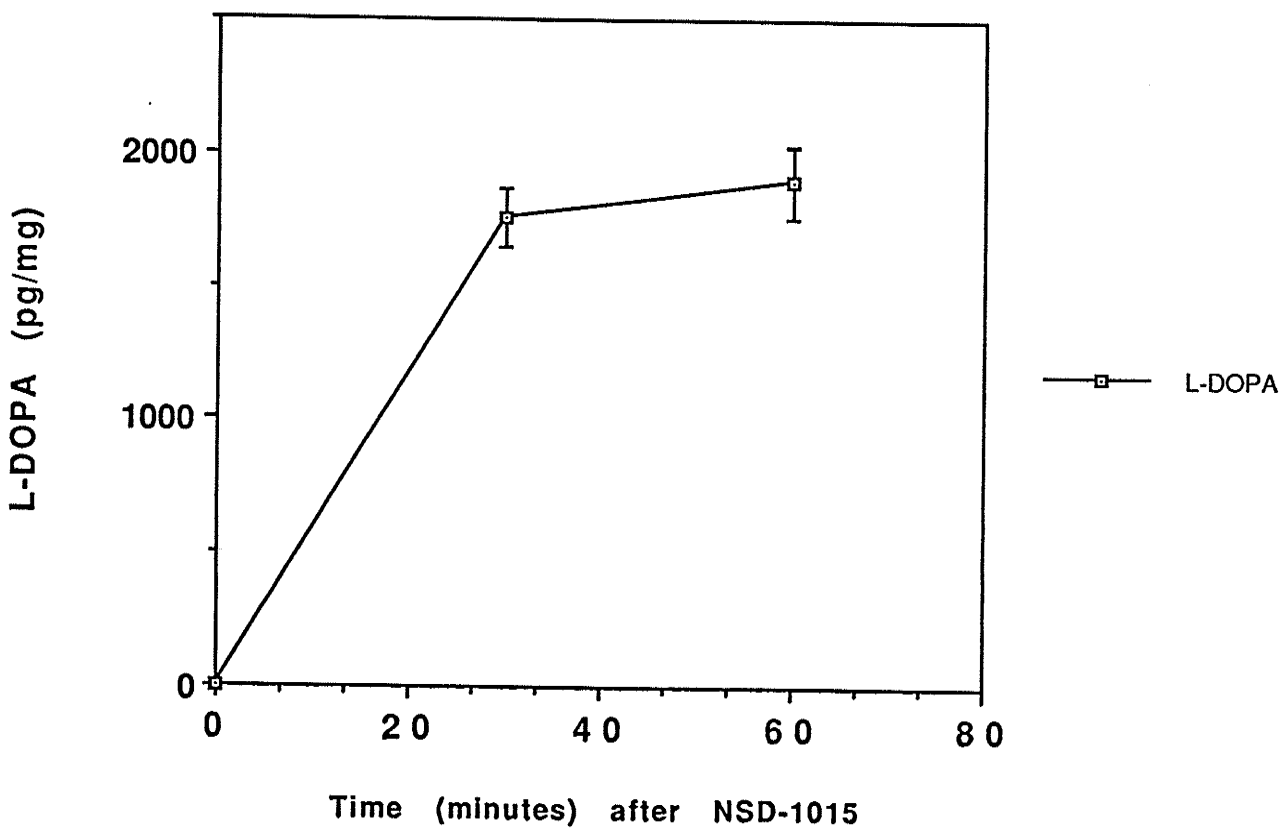

L-DOPA (pg/mg)
2000
1000
0
0
20
40
60
80
Time (minutes) after NSD-1015
L-DOPA

Fig. 7. The effects on 5-HTP accumulation in the striatum after administration of NSD-1015, for 30 and 60 minutes in male Syrian hamsters.

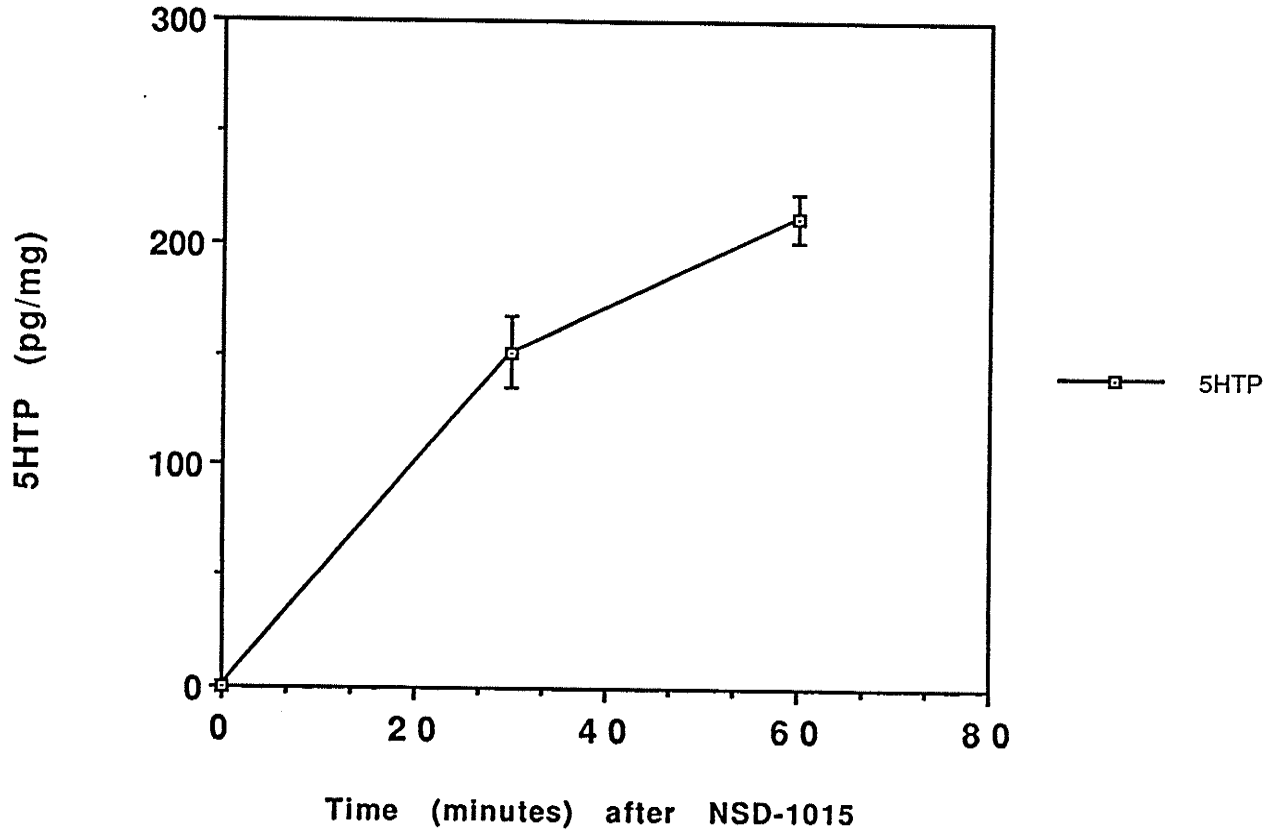

300
200
100
0
5HTP (pg/mg)
0
20
40
60
80
Time (minutes) after NSD-1015
5HTP

TABLE 1. Effects of NSD-1015 administration on L-DOPA, DA, NE and HVA concentrations in the ME/arcuate region

Treatment	L-DOPA accumulation (pg/mg ± S.E.)	DA concentrations (pg/mg ± S.E.)	HVA concentrations (pg/mg ± S.E)	NE concentrations (pg/mg ± S.E.)
NSD-1015 IP; 30 min. before sacrifice	494 ± 22	671 ± 141	43 ± 5***	1767 ± 116
Saline-controls	not detectable	812 ± 102	109 ± 7	2051 ± 105
NSD-1015 IP; 60 min. before sacrifice	641 ± 44**	694 ± 72	34 ± 3***	2218 ± 144*
Saline-controls	not detectable	1006 ± 220	88 ± 7	1705 ± 15

*$p < 0.05$
**$p < 0.01$ (compared to 30 minutes)
***$p < 0.001$ (compared to saline-treated controls)
Data are expressed as Mean ± S.E.

TABLE 2. Effects of NSD-1015 administration on 5-HTP, 5-HT and 5-HIAA in the median eminence/arcuate region of male Syrian hamsters

Treatment	5-HTP accumulation (pg/mg ± S.E.)	5-HIAA levels (pg/mg ± S.E.)	5-HT levels (pg/mg ± S.E.)
NSD-1015 (30 min.)	190 ± 19	108 ± 8***	1461 ± 132**
Saline-controls (30 min.)	not detectable	323 ± 21	2276 ± 130
NSD-1015 (60 min.)	420 ± 22α	85 ± 4***	1773 ± 136
Saline-controls (60 min.)	not detectable	288 ± 24	1830 ± 137

α $p < 0.001$ (compared to 30 minutes)
**$p < 0.01$
***$p < 0.001$ (compared to saline-treated controls)
Data are expressed as Mean ± S.E.

TABLE 3. Effects of NSD-1015 on L-DOPA, DA and metabolites in the striatum of male Syrian hamsters

Treatment	L-DOPA levels (pg/mg ± S.E.)	Dopamine levels (pg/mg ± S.E.)	HVA levels (pg/mg ± S.E.)	DOPAC levels (pg/mg ± S.E.)
NSD-1015 (30 min. before sacrifice)	1755 ± 113	7946 ± 542*	787 ± 45***	505 ± 41***
Saline-controls (30 min.)	not detectable	10,006 ± 754	1138 ± 56	1604 ± 116
NSD-1015 (60 min. before sacrifice)	1893 ± 135	6759 ± 743	483 ± 65***	284 ± 34***
Saline-controls (60 min.)	not detectable	8579 ± 529	1245 ± 88	1418 ± 77

*$p < 0.05$
***$p < 0.001$ (compared to saline-treated controls)
Data are expressed as Mean ± S.E.

TABLE 4. Effects of NSD-1015 administration on 5-HTP, and 5-HIAA in the striatum of male Syrian hamsters

Treatment	5-HTP accumulation (pg/mg ± S.E.)	5-HIAA levels (pg/mg ± S.E.)
NSD-1015 (30 min. before sacrifice)	151 ± 16	217 ± 17***
Saline-controls (30 min.)	not detectable	346 ± 14
NSD-1015 (60 min. before sacrifice)	212 ± 11**	142 ± 15***
Saline-controls (60 min.)	not detectable	383 ± 19

**$p < 0.01$ (compared to 30 minutes)
***$p < 0.001$ (compared to saline-treated controls)
Data are expressed as Mean ± S.E.

3.3 EXPERIMENT THREE: TEMPORAL SEQUENCE OF MELATONIN ON TH AND TPH ACTIVITY

3.3.1 L-DOPA Accumulation in the Neurointermediate Lobe (NIL)

Melatonin treatment was demonstrated to produce an overall inhibitory effect on the accumulation of the catecholamine precursor, L-DOPA, after administration of NSD-1015 ($F = 10.18$; $p < 0.01$; Fig. 8). The greatest inhibition of TH activity (as reflected by the decreases found in the accumulation of L-DOPA after NSD-1015), was observed in those animals treated with melatonin for 3 weeks ($t = 4.396$; $p < 0.001$). This was substantiated by the interaction effect detected by ANOVA ($F = 5.82$; $p < 0.01$). No significant effect was observed at 5 weeks. Significant time effects were also demonstrated ($F = 16.74$; $p < 0.001$).

3.3.2 DA Content in the NIL

Melatonin injections significantly decreased DA content after NSD-1015 administration in the posterior pituitary ($F = 9.42$; $p < 0.01$; Fig. 9). This effect was demonstrated at 3 weeks and significant at 5 weeks of treatment ($t = 3.22$; $p < 0.01$).

3.3.3 NE Content in the NIL

Melatonin treatment significantly decreased NE content but according to ANOVA this effect was time-dependent ($F = 3.55$; $p < 0.05$; Fig. 10). The greatest inhibition of NE was evident during week 3 ($t = 2.70$; $p < 0.05$). No significant decrease was observed at 5 weeks in the neurointermediate lobe.

3.3.4 Testes Weight

As expected after several weeks of melatonin administration (or short-photoperiod exposure), following 5 weeks of melatonin treatment, there was evidence of testes involution. Melatonin administration significantly decreased testes weight at 5 weeks of treatment ($t = 3.12$; $p < 0.01$; Fig. 11). There were no significant differences in testes weight after 3 weeks of treatment.

3.3.5 L-DOPA Accumulation in ME/Arcuate Region of the MBH

Melatonin administration had a significant ($F = 3.91$; $p < 0.05$; Fig. 12) inhibitory effect (75.67% controls) on TH activity (as demonstrated via decreases found in the accumulation of L-DOPA following administration of NSD-1015) after 1 week of melatonin treatment ($t = 2.63$; $p < 0.05$; Fig. 12) in the ME/arcuate region of the MBH. This effect was no longer significant at 3 or 5 weeks of melatonin treatment (Fig. 12). These data were expressed as pg/micrograms (μg) protein.

3.3.6 DA Levels in the MBH

ANOVA demonstrated a melatonin-induced decrease in DA concentrations ($F = 5.03$; $p < 0.05$; Fig. 13) that was significant in the ME/arcuate region after 5 weeks of treatment ($t = 2.22$; $p < 0.05$; Fig. 13).

3.3.7 NE Levels in the MBH

No significant overall effect of melatonin on ME/arcuate NE could be detected by ANOVA ($F = 0.99$; N.S.); melatonin significantly decreased NE levels in this region after 1 week of treatment ($t = 2.78$; $p < 0.05$; Fig. 14). This melatonin-induced effect occurred concomitantly with decreases in L-DOPA accumulation at 1 week. A significant effect of time was also detected via ANOVA ($F = 6.36$; $p < 0.05$).

3.3.8 5-HTP Accumulation in the MBH

A melatonin-induced increase in 5-HTP accumulation after NSD-1015 in the ME/arcuate region, was detected via 2-way ANOVA ($F = 6.30$; $p < 0.025$; Fig. 15). This melatonin-induced elevation in 5-HTP was even more pronounced when the data was expressed in pg/μg protein ($F = 7.39$; $p < 0.01$; Fig. 16). After 5 weeks of melatonin administration, this indoleamine increased 5-HTP accumulation after NSD-1015 ($t = 2.24$; $p < 0.05$; Fig. 15; pg/μg protein - $t = 2.43$; $p < 0.05$; Fig. 16).

3.3.9 5-HT Levels in the MBH

No significant effect of melatonin on 5-HT could be detected in this region ($F = 1.29$; N.S.; Table 5).

3.3.10 5-HIAA Levels in the MBH

No significant melatonin-induced effects on 5-HIAA were demonstrated in this region ($F = 3.96$; N.S.; Table 5).

3.3.11 Temporal Effects of Melatonin in the Striatum

No significant temporal effects of melatonin treatment on TH ($F = 2.61$; N.S.; Table 6) or TPH activity ($F = 0.10$; N.S.; Table 7) could be detected by ANOVA. No significant effects of melatonin could be demonstrated on the neurotransmitters DA ($F = 0.28$; N.S.; Table 6), NE ($F = 0.17$; N.S.; Table 6), or metabolites DOPAC ($F = 1.18$; N.S.; Table 6), HVA ($F = 0.66$; N.S.; Table 6) and 5-HIAA ($F = 0.49$; N.S.; Table 7) in this region by ANOVA. No significant effects of melatonin could be detected by ANOVA when the data was expressed as pg/μg protein.

3.3.12 Temporal Effects of Melatonin in the Amygdala:

3.3.13 NE Levels in the Amygdala

An interaction effect detected via ANOVA demonstrated significant effects of melatonin treatment - this was dependent on the duration of administration ($F = 3.93$; $p < 0.05$). Melatonin treatment for 5 weeks, significantly increased NE concentrations in the amygdala ($t = 3.09$; $p < 0.01$; Fig. 17).

No significant effects of melatonin on L-DOPA ($F = 0.69$; Table 8), 5-HTP ($F = 0.02$; Table 8) DA ($F = 0.06$; Table 9), HVA ($F = 0.41$; Table 9), DOPAC ($F = 1.31$; Table 9), 5-HT ($F = 1.16$; Table 10) or 5-HIAA ($F = 0.02$; Table 10) could be detected via ANOVA in the amygdala.

3.3.14 Temporal Effects of Melatonin in the Pons:

3.3.15 L-DOPA Accumulation, DA and Metabolite Levels (DOPAC) in the Pons

No significant effects of melatonin could be detected by ANOVA on L-DOPA ($F = 0.20$; Table 11), DA ($F = 1.50$; Table 12) or DOPAC ($F = 1.55$; Table 12) in the pontine brainstem.

3.3.16 5-HTP Accumulation in the Pons

No significant effects of melatonin on TPH activity could be detected by ANOVA in this region ($F = 0.12$; N.S.; Table 11).

3.3.17 5-HT Levels in the Pons

An overall significant melatonin-induced increase in 5-HT concentrations was detected by ANOVA in the pontine brainstem ($F = 5.76$; $p < 0.025$; Fig. 18). Melatonin treatment for 5 weeks elevated 5-HT levels in this region, in comparison to saline treated controls ($t = 3.05$; $p < 0.01$; Fig. 18).

3.3.18 5-HIAA Levels in the Pons

An interaction in the ANOVA demonstrated that melatonin's effects on 5-HIAA concentrations in the pons depended on the duration of treatment ($F = 3.64$; $p < 0.05$). The levels of this 5-HT acid metabolite were significantly decreased following 1 week of melatonin treatment ($t = 2.86$; $p < 0.05$; Fig. 19).

3.3.19 5-HIAA/5-HT Ratio in the Pons

An interaction in the ANOVA detected a melatonin-induced decrease in the ratio of 5-HIAA/5-HT in the pontine brainstem ($F = 3.22$; $p < 0.05$; Fig. 20). Melatonin decreased the 5-HIAA/5-HT ratio after 1 week of treatment in this region ($t = 2.31$; $p < 0.05$; Fig. 20).

3.3.20 NE Levels in the Pons

Although no significant effect of melatonin on NE concentrations could be demonstrated in the pons, a significant effect of time on NE ($F = 5.03$; $p < 0.025$; Table 12) was detected by ANOVA.

3.3.21 Temporal Effects of Melatonin on the Midbrain

No significant effects of melatonin could be detected on TH ($F = 2.63$; N.S.; Table 13) or TPH activity ($F = 1.47$; N.S.; Table 13) in the midbrain. No significant effects on the transmitter concentrations of DA ($F = 0.51$; N.S.; Table 14), NE ($F = 0.84$; N.S.; Table 14) or 5-HT ($F = 0.02$; N.S.; Table 15) could be demonstrated via ANOVA in this region. No significant effects of melatonin on metabolites HVA ($F = 0.11$; N.S.; Table 14) DOPAC ($F = 1.62$; N.S.; Table 14), 5-HIAA ($F = 0.73$; N.S.; Table 15) could be detected by ANOVA. There was also no effect of melatonin on the 5-HIAA/5-HT ratio in the midbrain ($F = 0.87$; N.S.; Table 16).

Significant time effects on midbrain 5-HTP accumulation after NSD-1015 were detected by ANOVA ($F = 5.83$; $p < 0.01$; Table 13), as well as 5-HT levels ($F = 3.43$; $p < 0.05$; Table 15) and 5-HIAA ($F = 4.86$; $p < 0.05$; Table 15) in this region. A significant effect of time was also demonstrated for DA in the midbrain ($F = 5.84$; $p < 0.01$; Table 14). Similar results were demonstrated when the data was expressed

as pg/μg protein.

3.3.22 Figures and Tables for Experiment Three

Fig. 8. Effects of melatonin (for 1, 3 and 5 weeks) on TH activity (L-DOPA accumulation after NSD-1015 administration) in the posterior pituitary (neurointermediate lobe) of male Syrian hamsters. Data points represent Mean ± SE. ***$p < 0.001$ compared to saline-treated controls.

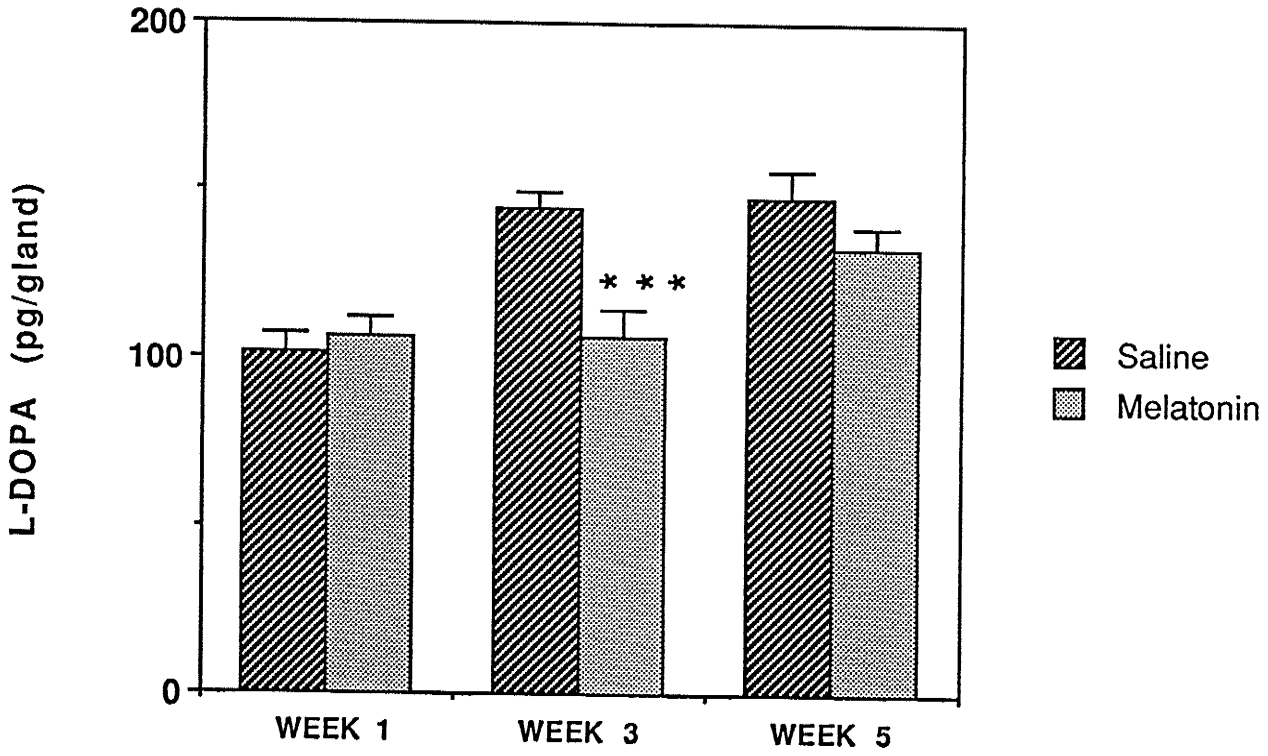

200
100
0
L-DOPA (pg/gland)
* * *
WEEK 1
WEEK 3
WEEK 5
Saline
Melatonin

Fig. 9. Effects of melatonin (for 1, 3 and 5 weeks) on DA content in the posterior pituitary (neurointermediate lobe) of male Syrian hamsters. Data points represent Mean ± SE. **$p < 0.01$ compared to saline-treated controls.

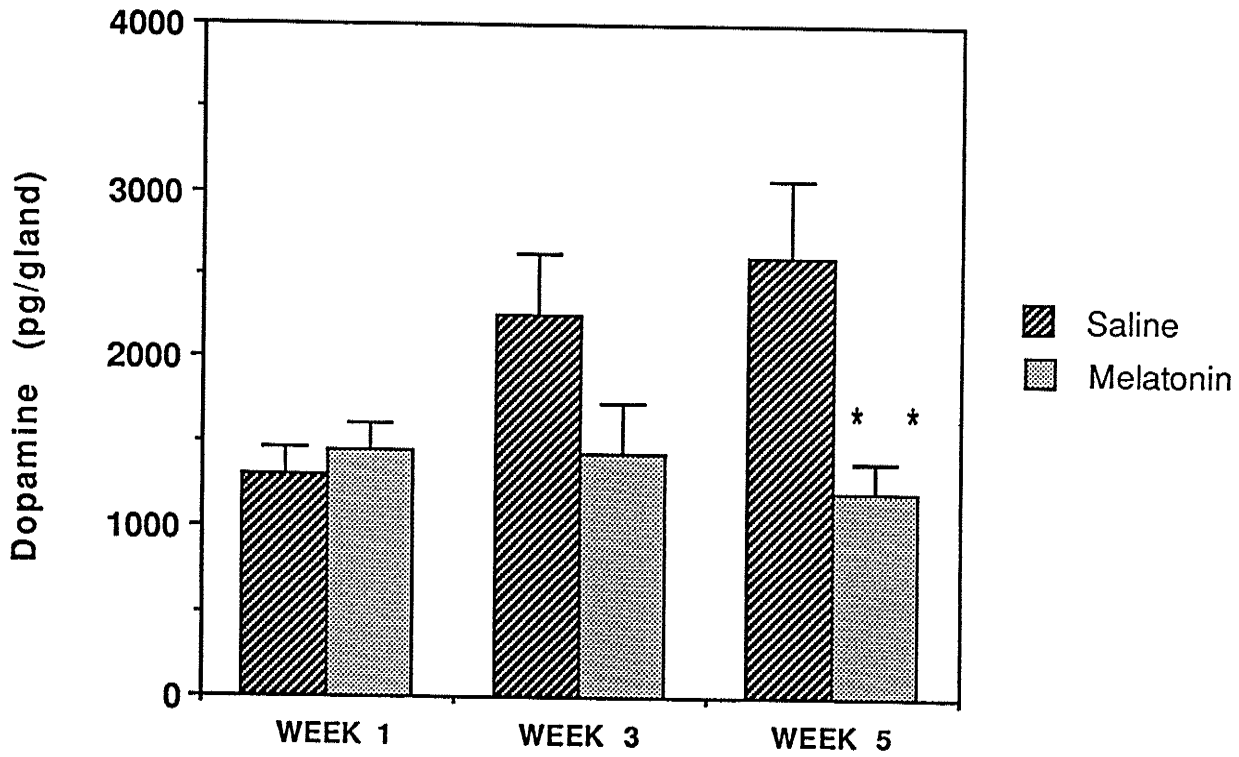

4000
3000
2000
1000
0
Dopamine (pg/gland)
WEEK 1
WEEK 3
WEEK 5
*
*
Saline
Melatonin

Fig. 10. Effects of melatonin (for 1, 3 and 5 weeks) on NE content in the posterior pituitary (neurointermediate lobe) of male Syrian hamsters. Data points represent Mean ± SE. *$p < 0.05$ compared to saline-treated controls.

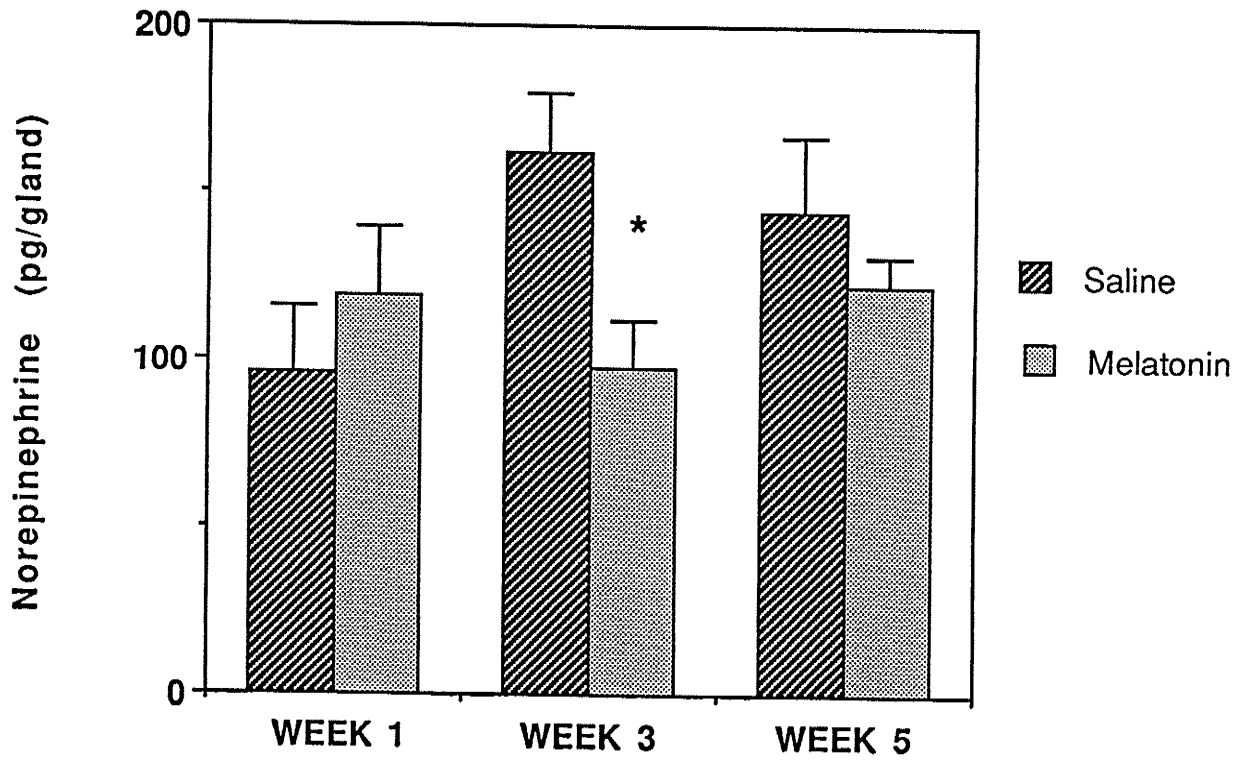

Norepinephrine (pg/gland)
200
100
0
*
WEEK 1
WEEK 3
WEEK 5
Saline
Melatonin

Fig. 11. Temporal effects of melatonin administration on testes weight of male Syrian hamsters. Data points represent Mean ± SE. $^{**}p < 0.01$ compared to saline-treated controls.

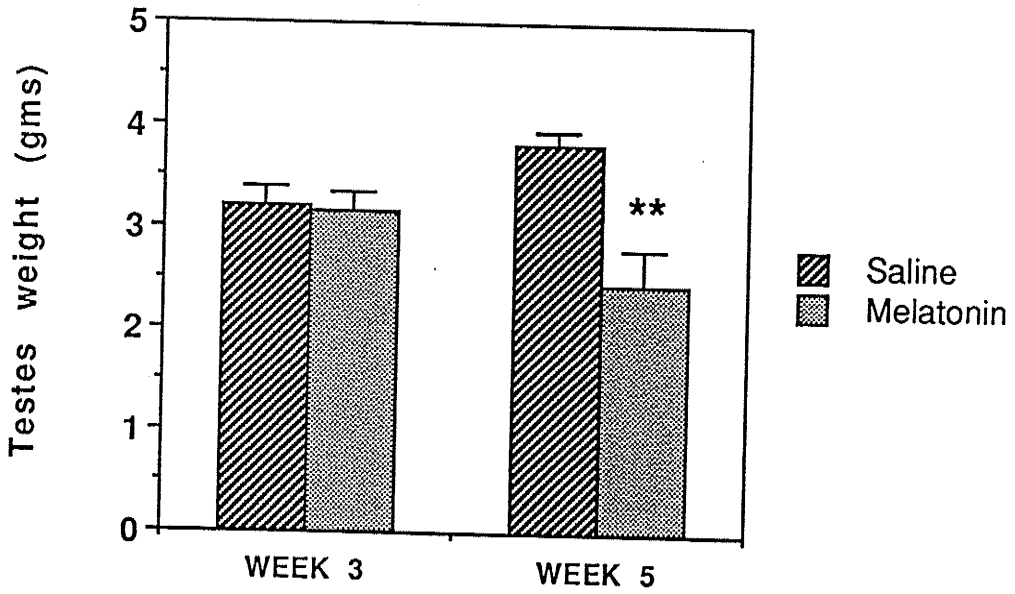

Testes weight (gms)
5
4
3
2
1
0
**
WEEK 3
WEEK 5
Saline
Melatonin

Fig. 12. Effects of melatonin (for 1, 3 and 5 weeks) on TH activity (L-DOPA accumulation after NSD-1015 administration) in the ME/arcuate region of the MBH in male Syrian hamsters. Data points represent Mean ± SE. *$p < 0.05$ compared to saline-treated controls.

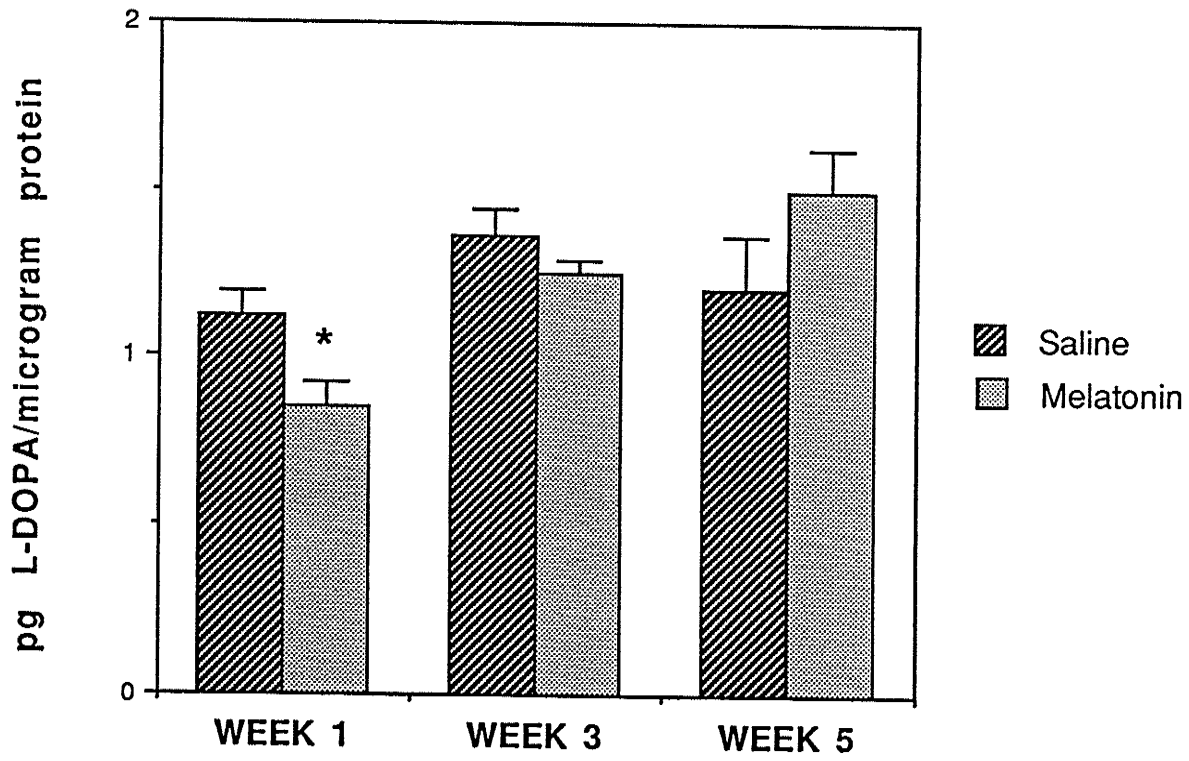

pg L-DOPA/microgram protein
2
1
0
*
WEEK 1
WEEK 3
WEEK 5
Saline
Melatonin

Fig. 13. Effects of melatonin administration (for 1, 3 and 5 weeks) on DA concentrations in the ME/arcuate region of the MBH of NSD-1015 treated hamsters. Data points represent Mean ± SE. *$p < 0.05$ compared to saline-treated controls.

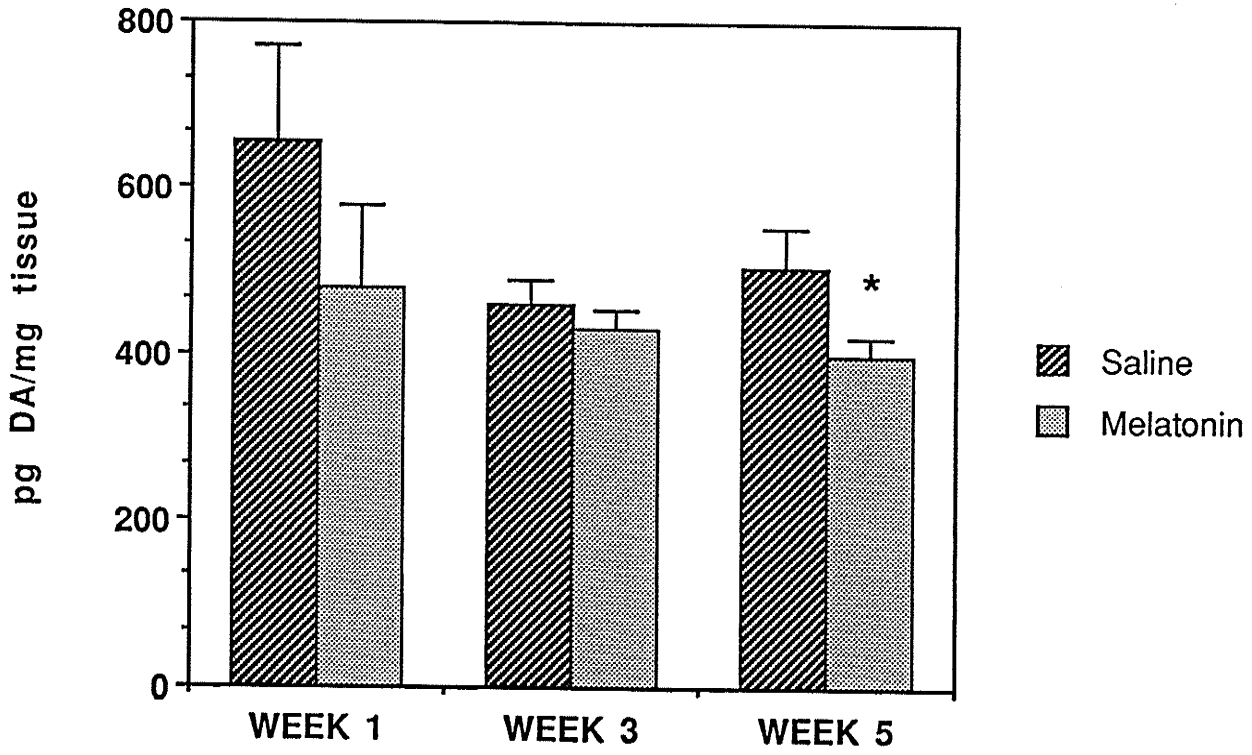

800
600
400
200
0
pg DA/mg tissue
WEEK 1
WEEK 3
WEEK 5
*
Saline
Melatonin

Fig. 14. Effects of melatonin (for 1, 3 and 5 weeks) on NE concentrations in the ME/arcuate region of the MBH of NSD-1015 treated hamsters. Data points represent Mean ± SE. *$p < 0.05$ compared to saline-treated controls.

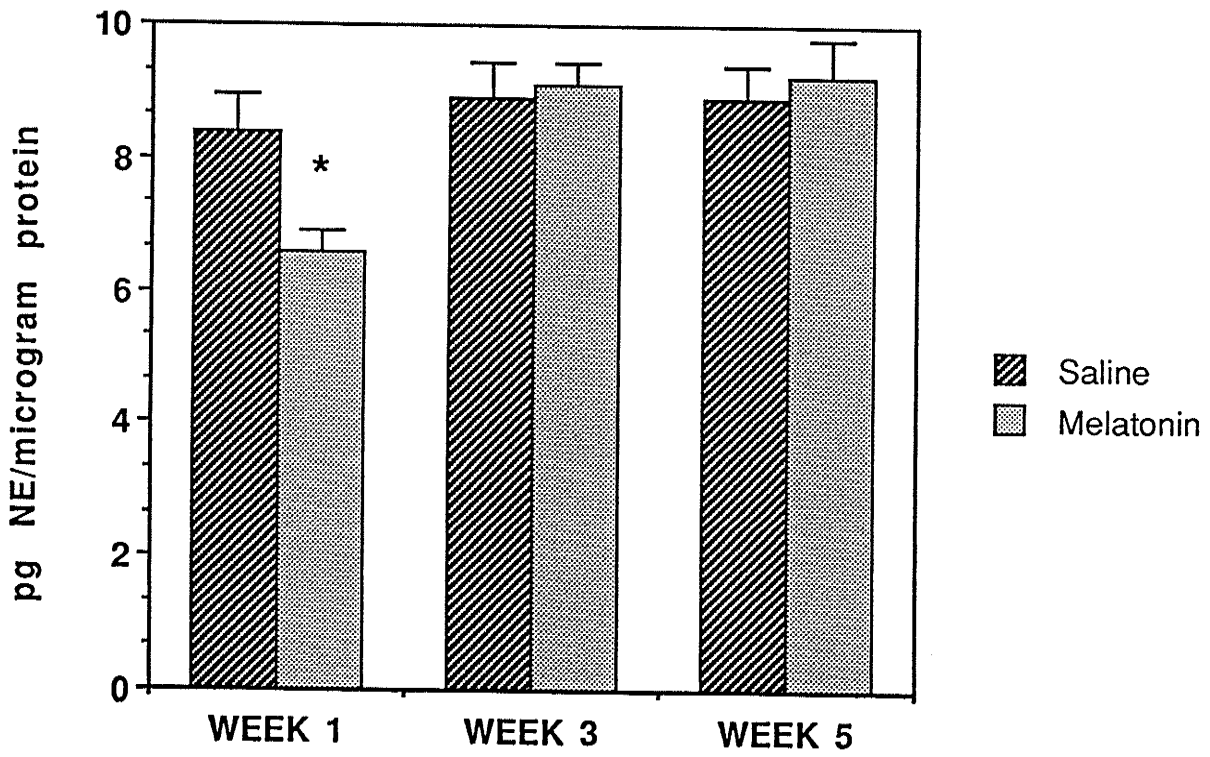

pg NE/microgram protein
10
8
6
4
2
0
*
WEEK 1
WEEK 3
WEEK 5
Saline
Melatonin

Fig. 15. Effects of melatonin (for 3 and 5 weeks) on TPH activity (5-HTP accumulation after NSD-1015 administration) in the ME/arcuate region of the MBH in male Syrian hamsters. Data points represent Mean ± SE. *$p < 0.05$ compared to saline-treated controls.

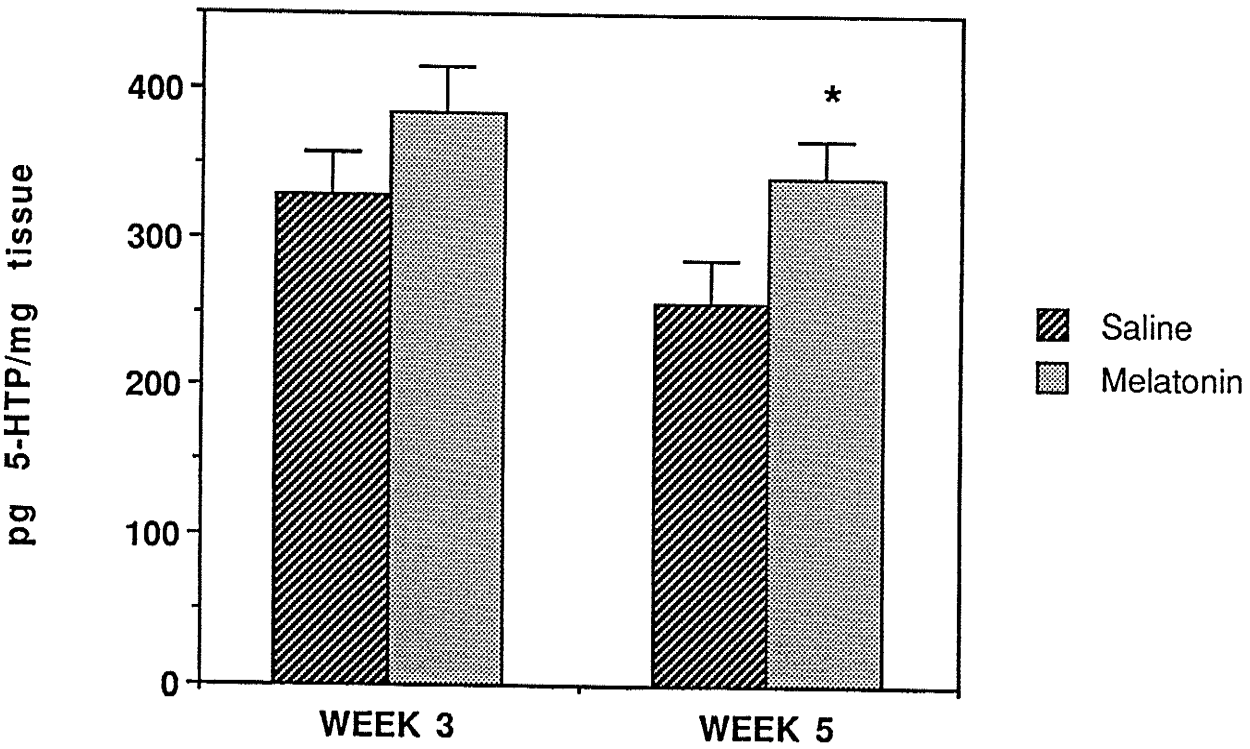

pg 5-HTP/mg tissue
400
300
200
100
0
*
WEEK 3
WEEK 5
Saline
Melatonin

Fig. 16. Effects of melatonin (for 3 and 5 weeks) on 5-HTP accumulation (pg/μg protein) after NSD-1015 administration in MBH of male Syrian hamsters. Data points represent Mean ± SE. *$p < 0.05$ compared to saline-treated controls.

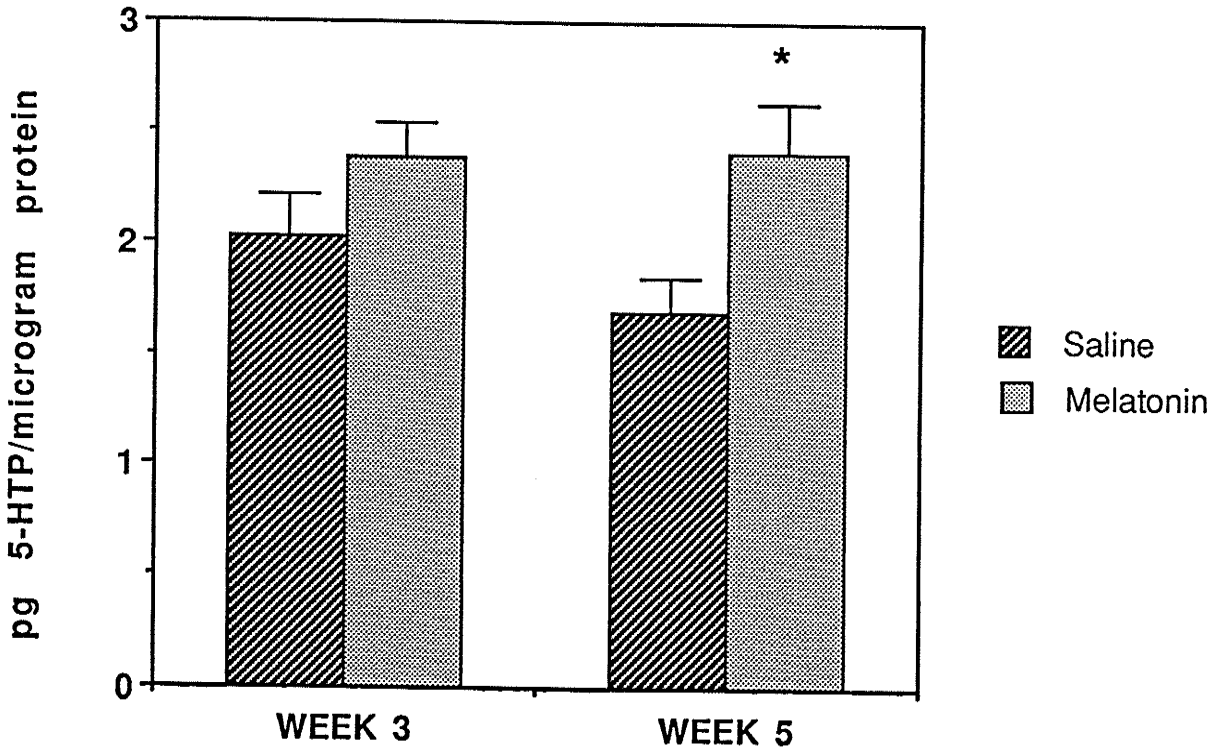

pg 5-HTP/microgram protein
3
2
1
0
*
WEEK 3
WEEK 5
Saline
Melatonin

Fig. 17. Temporal effect of melatonin on NE levels in the amygdala of male Syrian hamsters. Data points represent Mean ± SE. **$p < 0.01$ compared to saline-treated controls.

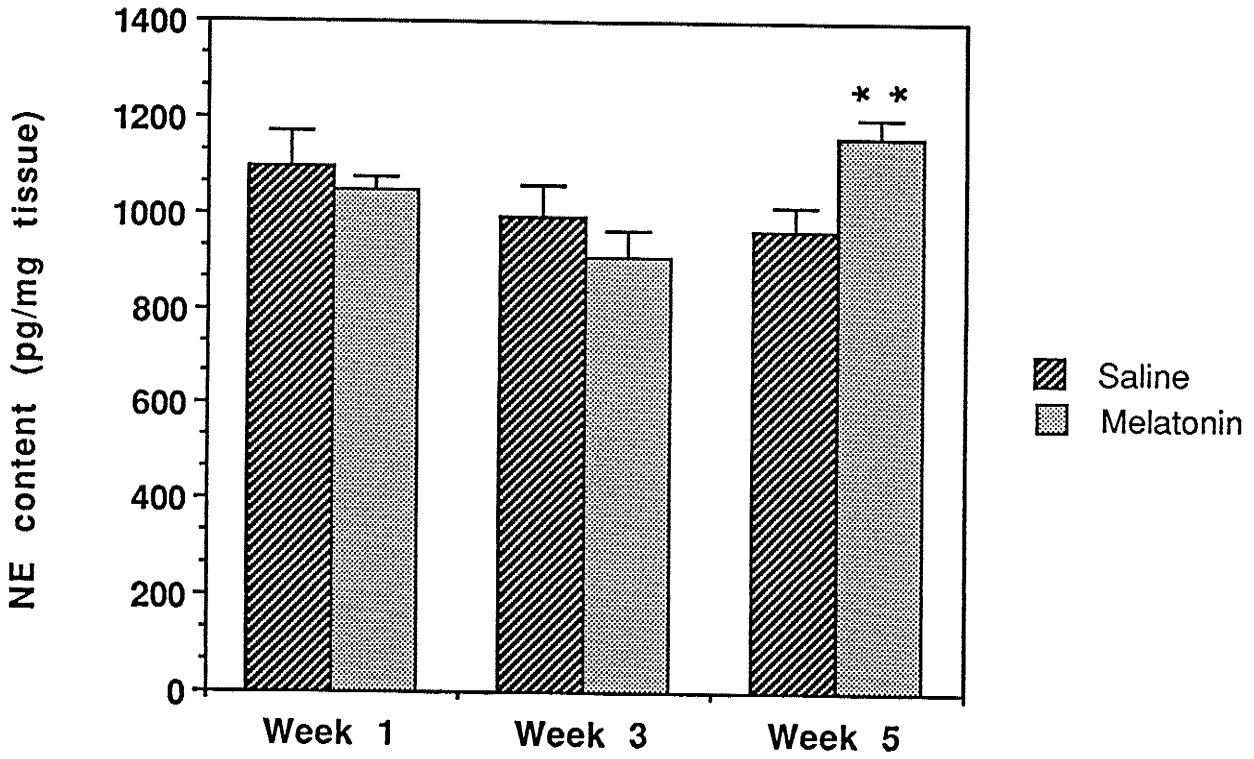

NE content (pg/mg tissue)
1400
1200
1000
800
600
400
200
0
* *
Week 1
Week 3
Week 5
Saline
Melatonin

Fig. 18. Temporal effects of melatonin on 5-HT levels (after NSD-1015) in the pons of male Syrian hamsters. Data points represent Mean ± SE. **p < 0.01 compared to saline-treated controls.

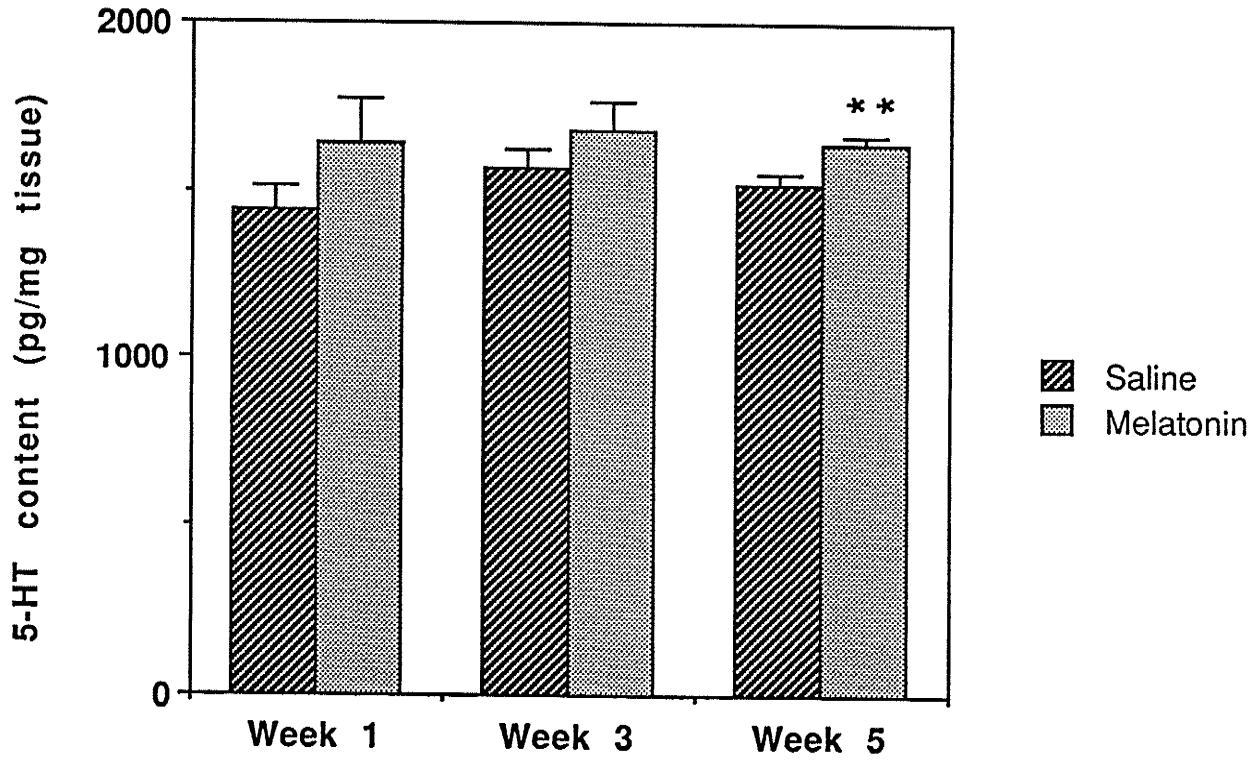

2000
1000
0
5-HT content (pg/mg tissue)
* *
Week 1
Week 3
Week 5
Saline
Melatonin

Fig. 19. Temporal effects of melatonin on 5-HIAA concentrations in the pons of male Syrian hamsters. Data points represent Mean ± SE. *$p < 0.05$ compared to saline-treated controls.

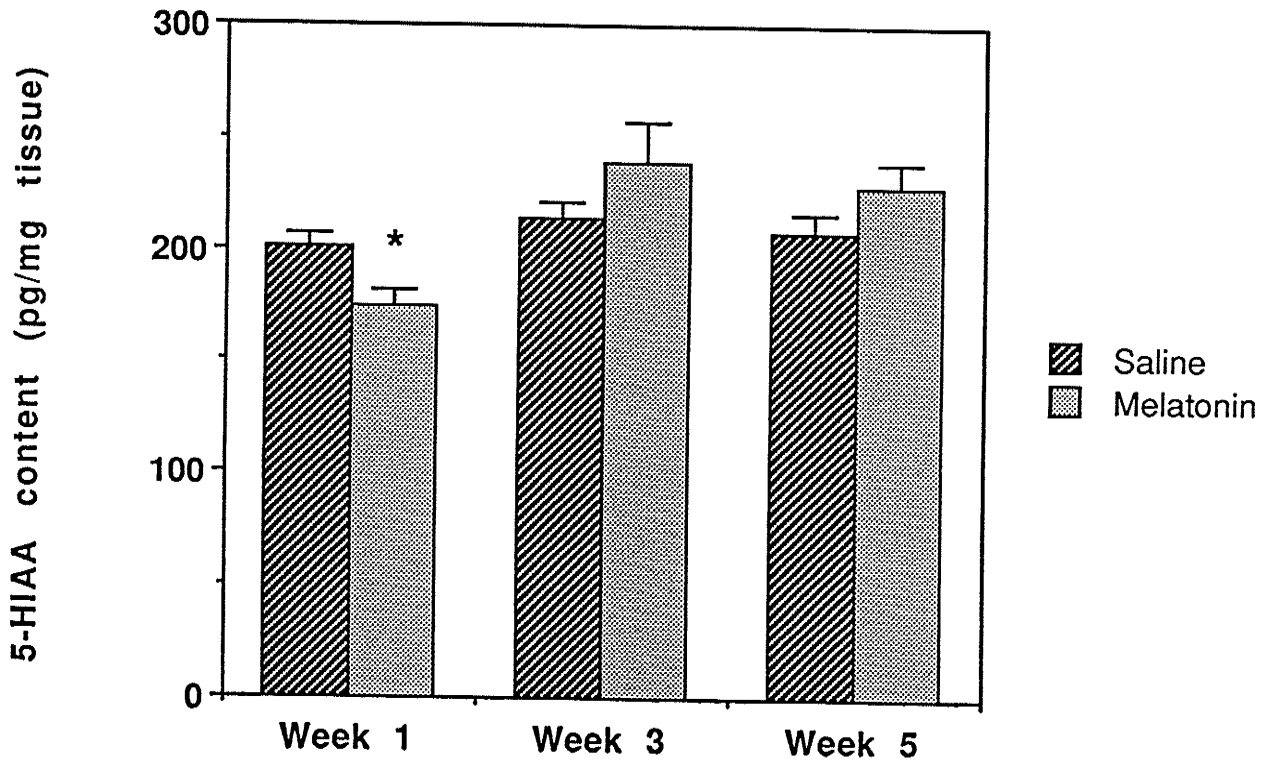

5-HIAA content (pg/mg tissue)
300
200
100
0
*
Week 1
Week 3
Week 5
Saline
Melatonin

Fig. 20. Temporal effects of melatonin on 5-HIAA/5-HT ratio (x 10,000) in the pons of male Syrian hamsters. Data points represent Mean ± SE. *$p < 0.05$ compared to saline-treated controls.

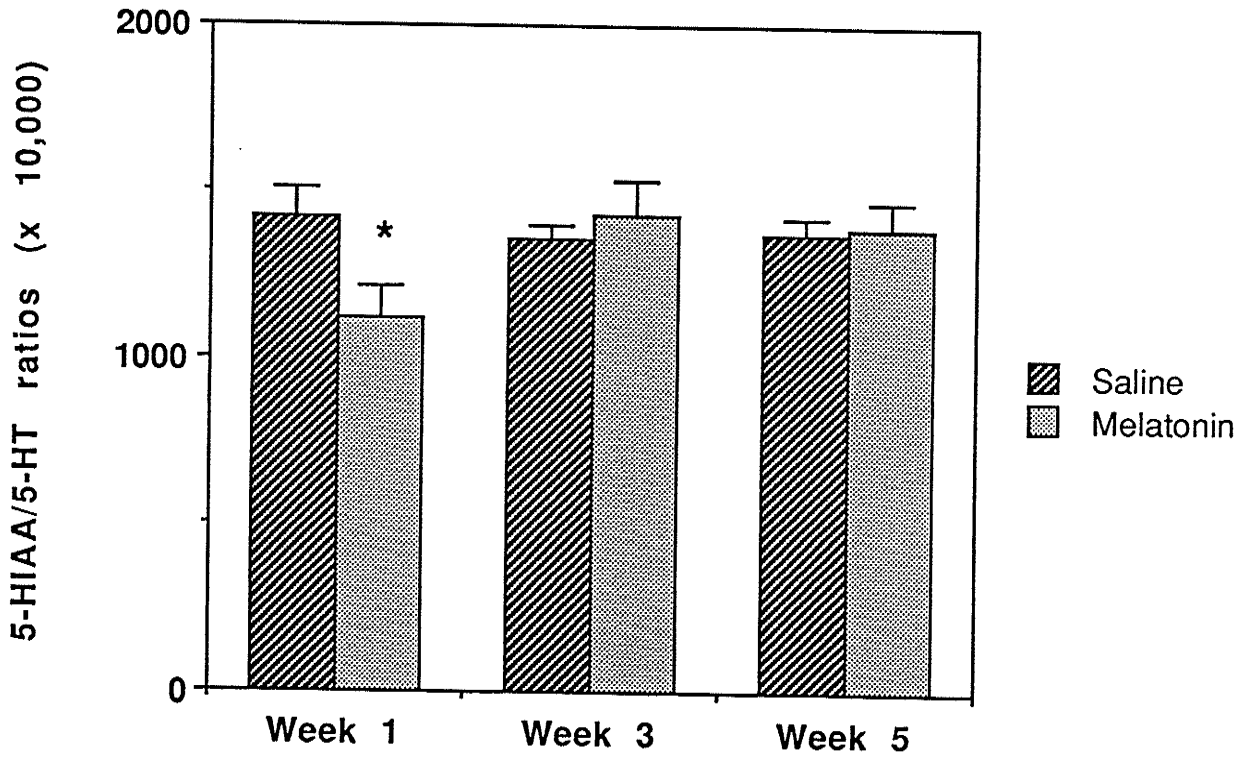

2000
1000
0
5-HIAA/5-HT ratios (x 10,000)
*
Week 1
Week 3
Week 5
Saline
Melatonin

TABLE 5. Temporal effects of melatonin administration on 5-HT and 5-HIAA levels in the ME/arcuate region of male Syrian hamsters

Treatment	5-HT levels (pg/mg tissue ± S.E.)	5-HIAA levels (pg/mg tissue ± S.E.)
Saline (3 weeks)	407 ± 16	136 ± 6
Melatonin (3 weeks)	420 ± 24	151 ± 6
Saline (5 weeks)	365 ± 34	129 ± 13
Melatonin (5 weeks)	406 ± 21	144 ± 6

Data are expressed as Mean ± S.E.

TABLE 6. Temporal effects of melatonin on catecholamine and metabolite levels in the striatum

Treatment	NE (pg/mg ± S.E.)	L-DOPA (pg/mg ± S.E.)	DA (pg/mg ± S.E.)	HVA (pg/mg ± S.E.)	DOPAC (pg/mg ± S.E.)
Saline (1 week)	132.0 ± 14.0	1678.4 ± 102.4	11553.2 ± 434.0	766.0 ± 42.4	365.2 ± 16.4
Melatonin (1 week)	147.6 ± 48.4	1491.6 ± 85.2	10795.2 ± 702.8	705.6 ± 44.4	325.0 ± 27.6
Saline (3 weeks)	105.2 ± 15.2	1698.0 ± 97.2	11908.0 ± 359.6	866.0 ± 58.0	364.6 ± 29.6
Melatonin (3 weeks)	116.4 ± 31.6	1490.4 ± 124.8	11627.6 ± 549.2	901.2 ± 54.8	383.2 ± 42.0
Saline (5 weeks)	88.4 ± 22.4	1747.2 ± 120.8	11269.2 ± 845.2	843.6 ± 85.6	347.6 ± 31.6
Melatonin (5 weeks)	72.4 ± 9.6	1828.4 ± 106.0	13056 ± 406.4	996.4 ± 62.4	458 ± 45.2

Data are expressed as Mean ± S.E.

TABLE 7. Concentrations of precursor (5-HTP) and metabolite (5-HIAA) of serotonin in striatum

Treatment	5-HTP accumulation after NSD-1015 (pg/mg tissue ± S.E.)	5-HIAA levels (pg/mg tissue ± S.E.)
Saline (1 week)	189.7 ± 12.6	695.0 ± 36.1
Melatonin (1 week)	189.0 ± 18.6	726.3 ± 32.8
Saline (3 weeks)	212.0 ± 14.8	229.5 ± 13.2
Melatonin (3 weeks)	196.5 ± 25.3	205.5 ± 23.6
Saline (5 weeks)	198.5 ± 22.4	213.5 ± 18.1
Melatonin (5 weeks)	229.6 ± 15.9	257.1 ± 14.1

Data are expressed as Mean ± S.E.

TABLE 8. Temporal effects of melatonin on TH and TPH activity in the amygdala

Treatment	Accumulation of L-DOPA (pg/mg ± S.E.) after NSD-1015 administration	Accumulation of 5-HTP (pg/mg ± S.E.) after NSD-1015 administration
Saline (1 week)	325.0 ± 40.3	259.3 ± 13.2
Melatonin (1 week)	373.5 ± 54.0	307.7 ± 11.7
Saline (3 weeks)	356.0 ± 30.2	324.0 ± 8.2
Melatonin (3 weeks)	268.5 ± 36.1	301.3 ± 8.3
Saline (5 weeks)	354.0 ± 31.3	331.3 ± 10.7
Melatonin (5 weeks)	317.5 ± 29.0	299.0 ± 10.6

Data are expressed as Mean ± S.E.

TABLE 9. Temporal effects of melatonin on DA, HVA and DOPAC in the amygdala

Treatment	DA levels (pg/mg ± S.E.)	HVA levels (pg/mg ± S.E.)	DOPAC levels (pg/mg ± S.E.)
Saline (1 week)	1631.0 ± 279.6	76.3 ± 11.4	136.9 ± 18.1
Melatonin (1 week)	2093.3 ± 350.4	118.6 ± 16.5	156.8 ± 18.3
Saline (3 weeks)	2006.8 ± 368.1	115.8 ± 31.0	132.5 ± 11.4
Melatonin (3 weeks)	1632.3 ± 373.4	121.5 ± 30.3	144.0 ± 17.0
Saline (5 weeks)	2024.6 ± 440.2	104.9 ± 19.9	102.3 ± 20.0
Melatonin (5 weeks)	2158.0 ± 312.3	92.0 ± 15.3	128.4 ± 24.6

Data are expressed as Mean ± S.E.

TABLE 10. Temporal effects of melatonin on 5-HT and 5-HIAA levels in the amygdala

Treatment	5-HT levels (pg/mg tissue ± S.E.)	5-HIAA levels (pg/mg tissue ± S.E.)
Saline (1 week)	1961 ± 161	323 ± 65
Melatonin (1 week)	2101 ± 162	280 ± 15
Saline (3 weeks)	2235 ± 129	279 ± 13
Melatonin (3 weeks)	2307 ± 138	306 ± 38
Saline (5 weeks)	2267 ± 161	281 ± 13
Melatonin (5 weeks)	2439 ± 117	286 ± 15

Data are expressed as Mean ± S.E.

TABLE 11. Temporal effects of melatonin on TPH and TH activity in the pontine brainstem

Treatment	5-HTP accumulation (pg/mg ± S.E.) after NSD-1015 administration	L-DOPA accumulation (pg/mg ± S.E.) after NSD-1015 administration
Saline (1 week)	225.3 ± 17.2	110.0 ± 5.4
Melatonin (1 week)	209.5 ± 8.8	112.8 ± 4.6
Saline (3 weeks)	228.5 ± 14.3	110.2 ± 6.0
Melatonin (3 weeks)	235.3 ± 10.2	104.0 ± 8.0
Saline (5 weeks)	263.8 ± 17.6	109.3 ± 8.8
Melatonin (5 weeks)	259.0 ± 20.4	105.6 ± 6.2

Data are expressed as Mean ± S.E.

TABLE 12. Temporal effects of melatonin on catecholamines and metabolites in pontine brainstem

Treatment	DA levels (pg/mg ± S.E.)	DOPAC levels (pg/mg ± S.E.)	NE levels (pg/mg ± S.E.)
Saline (1 week)	47.1 ± 8.1	34.0 ± 5.7	719.8 ± 21.6
Melatonin (1 week)	26.7 ± 6.4	35.4 ± 4.9	751.5 ± 38.2
Saline (3 weeks)	46.0 ± 4.2	40.3 ± 4.8	653.3 ± 17.0
Melatonin (3 weeks)	39.1 ± 5.2	46.6 ± 5.3	650.0 ± 22.3
Saline (5 weeks)	39.0 ± 6.3	29.3 ± 3.9	699.3 ± 28.8
Melatonin (5 weeks)	48.0 ± 5.4	36.9 ± 6.0	698.2 ± 25.4

Data are expressed as Mean ± S.E.

TABLE 13. Temporal effects of melatonin on TH and TPH activity in midbrain

Treatment	L-DOPA accumulation (pg/mg tissue ± S.E.) after NSD-1015	5-HTP accumulation (pg/mg tissue ± S.E.) after NSD-1015
Saline (1 week)	154.3 ± 8.8	336.3 ± 21.8
Melatonin (1 week)	143.5 ± 8.6	328.5 ± 16.5
Saline (3 weeks)	152.9 ± 6.6	365.7 ± 21.5
Melatonin (3 weeks)	135.7 ± 11.2	292.3 ± 11.8*
Saline (5 weeks)	173.5 ± 11.8	377.0 ± 25.1
Melatonin (5 weeks)	157.6 ± 12.9	400.0 ± 16.5

*$p < 0.05$ (compared to saline-treated controls)
Data are expressed as Mean ± S.E.

TABLE 14. Temporal effects of melatonin on catecholamine and metabolite concentrations in midbrain

Treatment	NE levels (pg/mg tissue ± S.E.)	DA levels (pg/mg tissue ± S.E.)	HVA levels (pg/mg tissue ± S.E.)	DOPAC levels (pg/mg tissue ± S.E.)
Saline (1 week)	668.8 ± 40.8	335.8 ± 26.4	41.1 ± 8.2	93.5 ± 6.9
Melatonin (1 week)	734.0 ± 53.4	310.8 ± 18.4	58.6 ± 19.2	94.3 ± 7.8
Saline (3 weeks)	784.6 ± 28.5	243.1 ± 23.1	41.7 ± 5.2	74.0 ± 9.1
Melatonin (3 weeks)	749.4 ± 43.6	231.1 ± 25.9	32.3 ± 4.1	91.7 ± 4.4
Saline (5 weeks)	714.3 ± 28.8	343.5 ± 28.6	38.0 ± 4.2	93.5 ± 6.5
Melatonin (5 weeks)	775.5 ± 37.9	326.0 ± 40.5	37.3 ± 5.4	94.3 ± 7.1

Data are expressed as Mean ± S.E.

TABLE 15. Temporal effects of melatonin on 5-HT and 5-HIAA concentrations in the midbrain

Treatment	5-HT levels (pg/mg tissue ± S.E.)	5-HIAA levels (pg/mg tissue ± S.E.)
Saline (1 week)	1501.0 ± 68.5	259.0 ± 10.9
Melatonin (1 week)	1541.3 ± 85.0	239.8 ± 8.5
Saline (3 weeks)	1678.9 ± 72.1	283.7 ± 15.8
Melatonin (3 weeks)	1698.7 ± 112.3	277.7 ± 27.1
Saline (5 weeks)	1685.8 ± 33.9	270.5 ± 11.7
Melatonin (5 weeks)	1652.2 ± 47.9	309.8 ± 16.0

Data are expressed as Mean ± S.E.

TABLE 16. Temporal effects of melatonin on 5-HIAA/5-HT ratio (x 1,000) in the midbrain

Treatment	5-HIAA/5-HT ratio in saline-treated hamsters (Mean ± S.E.)	5-HIAA/5-HT ratio in melatonin-treated hamsters (Mean ± S.E.)
1 Week	174.8 ± 9.1	159.2 ± 11.2
3 Weeks	164.4 ± 7.5	175.4 ± 15.5
5 Weeks	160.7 ± 7.2	188.0 ± 9.3*

*$p < 0.05$ (compared to saline-treated controls)

3.4 EXPERIMENT FOUR: CIRCADIAN (24 HOUR) EFFECTS OF MELATONIN

3.4.1 NIL (Posterior Pituitary):

3.4.2 L-DOPA Accumulation in the NIL

No significant effects of melatonin administration on TH activity ($F = 0.75$; N.S.; Table 17), could be detected in the NIL by ANOVA, after 9.5 weeks of treatment. This was also true when the data was expressed as pg/μg protein. Again, no significant effect of melatonin on L-DOPA accumulation after NSD-1015 administration could be shown by ANOVA ($F = 0.05$; N.S.; Table 17).

3.4.3 DA Content in the NIL

An overall significant melatonin-induced decrease in posterior pituitary DA content was detected via ANOVA ($F = 6.04$; $p < 0.05$; Table 18). This melatonin-induced reduction in pituitary DA reached significance during the 2 PM sacrifice ($t = 2.71$; $p < 0.05$; Table 18). This effect of melatonin was even more significant when the data was expressed as pg/μg protein ($F = 8.18$; $p < 0.01$; Fig. 21). Again, during the 2 PM time period, a significant melatonin-induced reduction in DA concentrations was shown via t-test ($t = 2.68$; $p < 0.05$; Fig. 21).

3.4.4 L-DOPA/DA ratio in the NIL

An interaction in the ANOVA demonstrated an effect of melatonin at a particular time of sacrifice ($F = 2.90$; $p < 0.05$; Table 19). A significant effect of time of day was also detected via ANOVA ($F = 5.14$; $p < 0.01$). No significant effects of melatonin were detected by Student's t-tests (2 PM: $t = 2.27$, N.S.; 8 PM: $t = 1.27$, N.S.; 2 AM: $t = 0.16$, N.S.; 8 AM: $t = 0.45$, N.S.).

3.4.5 NE Content in the NIL

No significant effects of melatonin administration on posterior pituitary NE concentrations could be detected by ANOVA ($F = 0.21$; N.S.; Table 18) after 9.5 weeks of treatment.

3.4.6 Testes Weight

As demonstrated in previous investigations, melatonin treatment for 9.5 weeks in the present study was found to significantly decrease testes weight ($t = 11.79$; $p < 0.001$; Table 20).

3.4.7 Body Weight

The body weight of these male hamsters treated with melatonin for 9.5 weeks was not significantly different from those treated with saline ($F = 3.31$; $t = 1.819$; N.S.; Table 20).

3.4.8 Water Intake

After 8.5 weeks of daily injections, water intake levels were assessed in all animals. Melatonin-treated hamsters were shown to have a significant decrease in daily water intake ($F = 13.94$; $p < 0.001$; Fig. 22) compared to saline-treated controls.

3.4.9 ME/Arcuate Region of the MBH:

3.4.10 L-DOPA Accumulation in ME/Arcuate Region of the MBH

Melatonin treatment for 9.5 weeks, produced a highly significant overall increase in the activity of TH ($F = 57.8$; $p < 0.0001$; Fig. 23) in the ME/arcuate region of the MBH. These melatonin-induced elevations (as reflected by increases in the accumulation of L-DOPA) were observed consistently over a 24-hr period - at all points of sacrifice (2 PM: $t = 4.86$, $p < 0.001$; 8 PM: $t = 2.39$, $p < 0.05$; 2 AM: $t = 3.27$, $p < 0.01$; 8 AM: $t = 4.61$, $p < 0.001$). Melatonin increased TH activity to 149% of controls at 2 PM; to 121% of saline-treated animals at 8 PM; to 133% of controls at 2 AM; and to 167% of saline-treated animals at 8 AM. No significant effects of time were demonstrated, nor were there any significant interaction effects detected in the ANOVA.

3.4.11 DA Levels in the MBH

After 9.5 weeks of treatment, melatonin produced a highly significant overall

decrease in DA concentrations (remaining after NSD-1015 administration) in the ME/arcuate region of the MBH, according to ANOVA ($F = 19.19$; $p < 0.001$; Fig. 24). Reduced DA levels were highly significant at the 2 AM sacrifice ($t = 5.23$; $p < 0.001$). Melatonin decreased DA concentrations to 73% of controls at 2 PM; to 79% of saline-treated animals at 8 PM; to 48% of controls at 2 AM; and to 69% of saline-treated animals at 8 AM. ANOVA also demonstrated a significant time effect ($F = 2.97$; $p < 0.05$). Evidence existed for a significant nighttime rise in DA (at 9.5 weeks) in saline-treated animals (as much as 30%) in the MBH, that produced a conspicuous circadian or 24 hr rhythm. DA levels at 2 PM and at 8 PM of these controls, were significantly lower than DA concentrations at 2 AM; ($t = 2.21$; $p < 0.05$; $t = 2.47$; $p < 0.05$) respectively. Melatonin treatment was demonstrated to decrease both the amplitude and acrophase of this daily DA rhythm - there was no evidence for a 24-hr rhythm in DA levels in melatonin-treated hamsters.

3.4.12 L-DOPA/DA ratio in the MBH

Melatonin treatment for 9.5 weeks induced a highly significant overall increase in the ratio of L-DOPA/DA ratio in the ME/arcuate region ($F = 71.04$; $p < 0.0001$; Table 21) as detected by ANOVA. These melatonin-induced elevations in the L-DOPA/DA ratios were significant at all time points of sacrifice (2 PM: $t = 4.39$, $p < 0.001$; 8 PM: $t = 2.37$, $p < 0.05$; 2 AM: $t = 6.53$, $p < 0.001$; 8 AM: $t = 4.66$, $p < 0.001$). A significant effect of time of sacrifice was also detected via ANOVA ($F = 3.99$; $p < 0.05$).

3.4.13 NE Levels in the MBH

After 9.5 weeks of melatonin treatment, ANOVA detected a overall significant melatonin-induced increase in NE concentrations ($F = 5.61$; $p < 0.025$; Fig. 25). The greatest elevation in NE levels were evident during the 8 AM sacrifice ($t = 2.44$; $p < 0.05$). No time effects were detected by ANOVA, therefore no 24-hr rhythm was apparent in NE in either controls or melatonin-treated animals.

3.4.14 HVA Levels in the MBH

Although there were no significant effects of melatonin detected by ANOVA, on homovanillic acid (HVA) concentrations, there was a highly significant effect of time of sacrifice demonstrated ($F = 10.80$; $p < 0.001$; Fig. 26). A definite 24-hr rhythm for HVA was found with evidence of a melatonin-induced phase-shifting of this rhythm (delay).

3.4.15 HVA/DA Ratio in the MBH

An overall significant melatonin-induced increase in the ratio of HVA/DA was detected by ANOVA ($F = 6.43$; $p < 0.025$; Fig. 27). Melatonin significantly decreased the ratio during this period ($t = 2.38$; $p < 0.05$). A significant time effect was also demonstrated ($F = 6.54$; $p < 0.001$).

3.4.16 DOPAC Levels in the MBH

No significant effect of melatonin administration on DOPAC concentrations

(after NSD-1015 administration) could be detected by ANOVA ($F = 0.42$; N.S.; Table 22).

3.4.17 5-HTP Accumulation in the MBH

An overall melatonin-induced increase in the activity of TPH of the MBH was detected via ANOVA ($F = 5.29$; $p < 0.05$; Fig. 28). The melatonin-induced elevation in 5-HTP (after NSD-1015) was demonstrated during the 8 AM time period of sacrifice ($t = 2.41$; $p < 0.05$; Fig. 28).

3.4.18 5-HT Levels in the MBH

An interaction in the ANOVA demonstrated a melatonin-induced elevation in the levels of 5-HT that was dependent on the time of day ($F = 2.80$; $p < 0.05$; Fig. 29). Melatonin treatment for 9.5 weeks significantly elevated 5-HT during the 2 AM sacrifice time period ($t = 2.40$; $p < 0.05$; Fig. 29).

3.4.19 5-HIAA Levels in the MBH

A significant effect of time of sacrifice on 5-HIAA concentrations was detected via ANOVA ($F = 10.21$; $p < 0.001$). An interaction effect was also demonstrated by ANOVA suggesting that melatonin increased 5-HIAA at a certain time of day ($F = 5.28$; $p < 0.01$; Table 22). Student's t-test did, in fact, show that melatonin increased 5-HIAA concentrations during the 8 AM sacrifice ($t = 2.61$; $p < 0.05$).

3.4.20 Amino Acid Neurotransmitters:

3.4.21 GABA Levels in the ME/Arcuate Region of the MBH

Melatonin administration for 9.5 weeks induced an overall highly significant decrease in gamma-aminobutyric acid (GABA) concentrations in the ME/arcuate region - as detected by ANOVA ($F = 16.33$; $p < 0.001$; Fig. 30). A significant effect of time of sacrifice was also shown ($F = 7.09$; $p < 0.001$). This melatonin-induced decrease in GABA levels was significantly different from saline-treated controls during the 2 PM time period of sacrifice ($t = 2.90$; $p < 0.05$; Fig. 30) as well as the 8 PM time period ($t = 2.79$; $p < 0.05$; Fig. 30).

3.4.22 Glutamate (Glutamic acid) Levels in the ME/Arcuate Region

An overall significant melatonin-induced decrease in the concentrations of the GABA precursor, glutamate, in the ME/arcuate region, was detected by ANOVA ($F = 5.86$; $p < 0.025$; Fig. 31). A significant effect of time of sacrifice was also demonstrated ($F = 35.95$; $p < 0.001$; Fig. 31).

3.4.23 Glutamine Levels in the ME/Arcuate Region

Melatonin treatment for 9.5 weeks was shown to reduce the concentrations of glutamine, as demonstrated via ANOVA ($F = 8.12$; $p < 0.01$; Fig. 32). There was also a significant time effect also detected by ANOVA ($F = 14.21$; $p < 0.001$). The melatonin-induced decrease in glutamine was significantly different from saline-

treated controls during the 2 PM time period of sacrifice ($t = 3.37$; $p < 0.01$; Fig. 32).

3.4.24 Effects of Melatonin on Glycine, Serine and Alanine in the ME/Arcuate Region

No significant effects of melatonin treatment ($F = 0.07$: N.S.; Table 23) or time of sacrifice ($F = 1.94$; N.S.) on glycine levels were detected by ANOVA. No significant effects of melatonin ($F = 0.13$; N.S.; Table 23) on serine were detected by ANOVA. An effect of time of sacrifice, however, was demonstrated ($F = 5.65$; $p < 0.01$; Table 24). No significant effects of melatonin administration on alanine ($F = 1.86$: N.S.; Table 25) were detected by ANOVA. A significant effect of time of sacrifice, however, was demonstrated ($F = 5.11$; $p < 0.01$).

3.4.25 Circadian Effects of Melatonin in the Striatum:

3.4.26 L-DOPA Accumulation in the Striatum

Melatonin treatment for 9.5 weeks significantly increased the activity of striatal TH as detected by ANOVA ($F = 4.63$; $p < 0.05$; Fig. 33). Melatonin elevated L-DOPA (after NSD-1015) greatest during the 8 PM time of decapitation ($t = 3.70$; $p < 0.01$). There was also a significant time effect detected by ANOVA ($F = 3.73$; $p < 0.05$).

3.4.27 DA Levels in the Striatum

Although there were no significant effects of melatonin on DA detected by ANOVA ($F = 0.30$; N.S.), there was a significant effect of the indole on DA levels found during the 8 PM sacrifice ($t = 2.30$; $p < 0.05$; Fig. 34). Melatonin treatment increased DA (concomitantly with elevated L-DOPA) in the striatum.

3.4.28 L-DOPA/DA Ratio in the Striatum

No significant effects of melatonin on the L-DOPA/DA ratio could be detected via ANOVA in the striatum ($F = 3.93$; N.S.; Table 26). However, there was a significant effect of time of day on the ratio demonstrated by ANOVA ($F = 10.56$; $p < 0.01$).

3.4.29 HVA Levels in the Striatum

Although no overall significant effects of melatonin on HVA concentrations could be detected by ANOVA ($F = 0.78$; N.S.; Table 27), a significant effect of time of sacrifice was demonstrated ($F = 10.29$; $p < 0.001$).

3.4.30 DOPAC Levels in the Striatum

ANOVA detected no significant effect of melatonin treatment on DOPAC concentrations in this region ($F = 0.26$; N.S.; Table 27). However, there was a statistically significant effect of time of sacrifice ($F = 5.65$; $p < 0.01$).

3.4.31 NE Levels in the Striatum

No significant effect of melatonin administration on NE in the striatum could be detected by ANOVA ($F = 0.02$; N.S.; Table 28).

3.4.32 5-HTP Accumulation in the Striatum

Although no overall significant effect of melatonin treatment could be detected via ANOVA ($F = 3.67$; N.S.; Fig. 35), a significant elevation in 5-HTP was demonstrated during the 2 PM sacrifice by t-test ($t = 2.16$; $p < 0.05$; Fig. 35).

3.4.33 5-HT Levels in the Striatum

An overall significant effect of melatonin administration on striatal 5-HT concentrations was detected by ANOVA ($F = 9.22$; $p < 0.01$; Fig. 36). The indole significantly increased 5-HT at all four times of sacrifice. Melatonin elevated 5-HT concentrations to 114% of controls at 2 PM; to 136% of saline-treated animals at 8 PM; to 110% of controls at 2 AM and to 103% of saline-treated animals at 8 AM. Melatonin's elevating effect on 5-HT levels was shown to be highly significant during the nighttime 8 PM sacrifice ($t = 4.34$; $p < 0.001$; Fig. 36).

3.4.34 5-HIAA Levels in the Striatum

Although no significant effect of melatonin could be detected by ANOVA ($F = 1.14$; N.S.; Table 29), there was a significant effect of time of sacrifice demonstrated ($F = 5.44$; $p < 0.01$; Table 29). Daytime (2 PM) concentrations of 5-

HIAA were decreased compared to other times of sacrifice.

3.4.35 Figures and Tables for Experiment Four

Fig. 21. Twenty-four hour effects of saline and melatonin injections for 9.5 weeks on DA concentrations in the neurointermediate lobe (posterior pituitary) following NSD-1015 administration in male Syrian hamsters. Dark bar indicates the 10 hr dark period of the daily light/dark cycle (6 PM - 4 AM). *$p < 0.05$ compared to saline-treated controls.

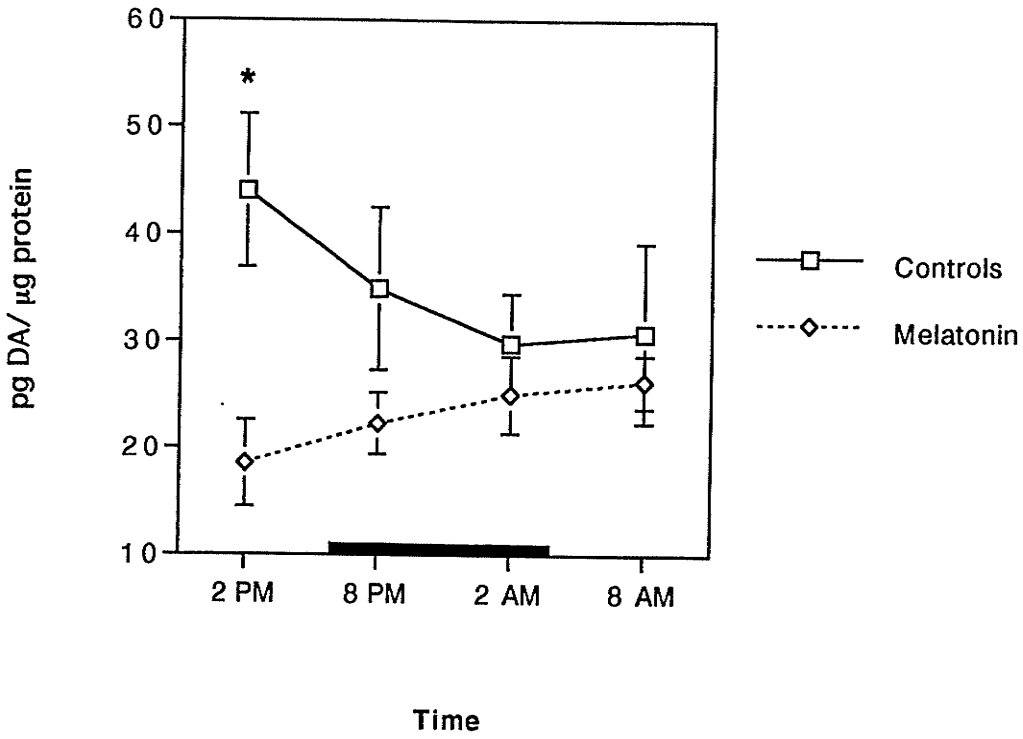

60
50
40
30
20
10
*
pg DA/ µg protein
2 PM
8 PM
2 AM
8 AM
Time
Controls
Melatonin

Fig. 22. Effects of melatonin administration for 8.5 weeks on water intake in male Syrian hamsters. Data points represent Mean ± SE. **$p < 0.01$ compared to saline-treated controls.

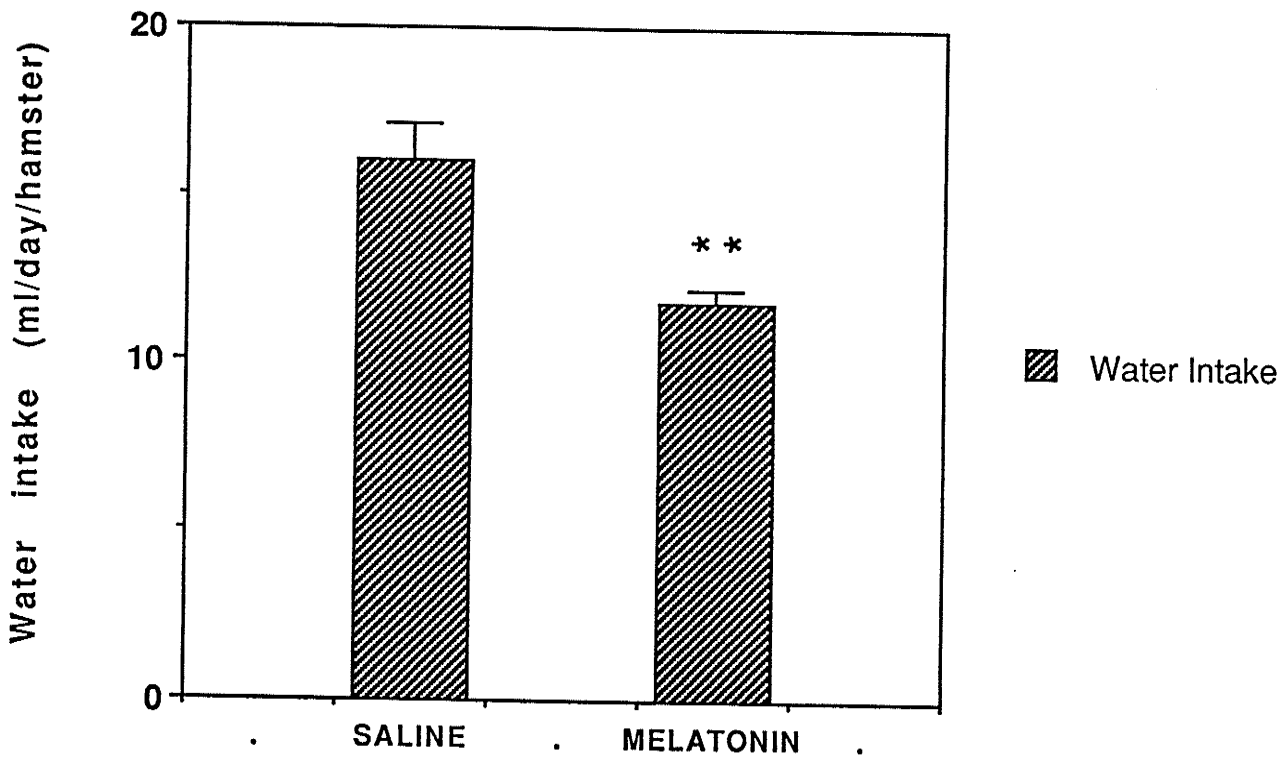

Water intake (ml/day/hamster)
20
10
0
* *
SALINE
MELATONIN
Water Intake

Fig. 23. Twenty-four hour effects of saline and melatonin injections for 9.5 weeks on the in situ activity of tyrosine hydroxylase (L-DOPA accumulation after NSD-1015), in the median eminence (ME)/arcuate region of the mediobasal hypothalamus (MBH) in male Syrian hamsters ($F = 57.8$). Dark bar indicates the 10 hr dark period of the daily light/dark cycle (6 PM - 4 AM). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to saline-treated controls.

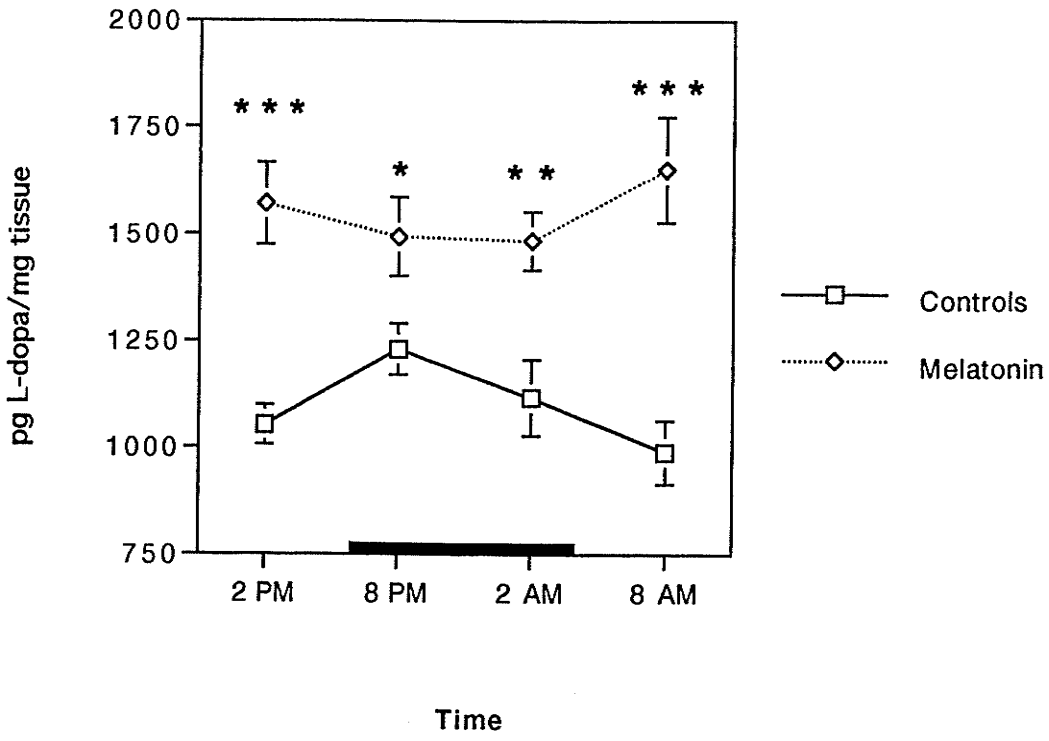

2000
1750
1500
1250
1000
750
pg L-dopa/mg tissue
* * *
*
* *
* * *
2 PM
8 PM
2 AM
8 AM
Time
Controls
Melatonin

Fig. 24. Twenty-four hour effects of saline and melatonin injections for 9.5 weeks on dopamine (DA) concentrations in the ME/arcuate region of the MBH (following NSD-1015 administration) in male Syrian hamsters ($F = 19.19$; $p < 0.001$). Dark bar indicates the 10 hr dark period of the daily light/dark cycle (6 PM - 4 AM). ***$p < 0.001$ compared to melatonin-treated animals.

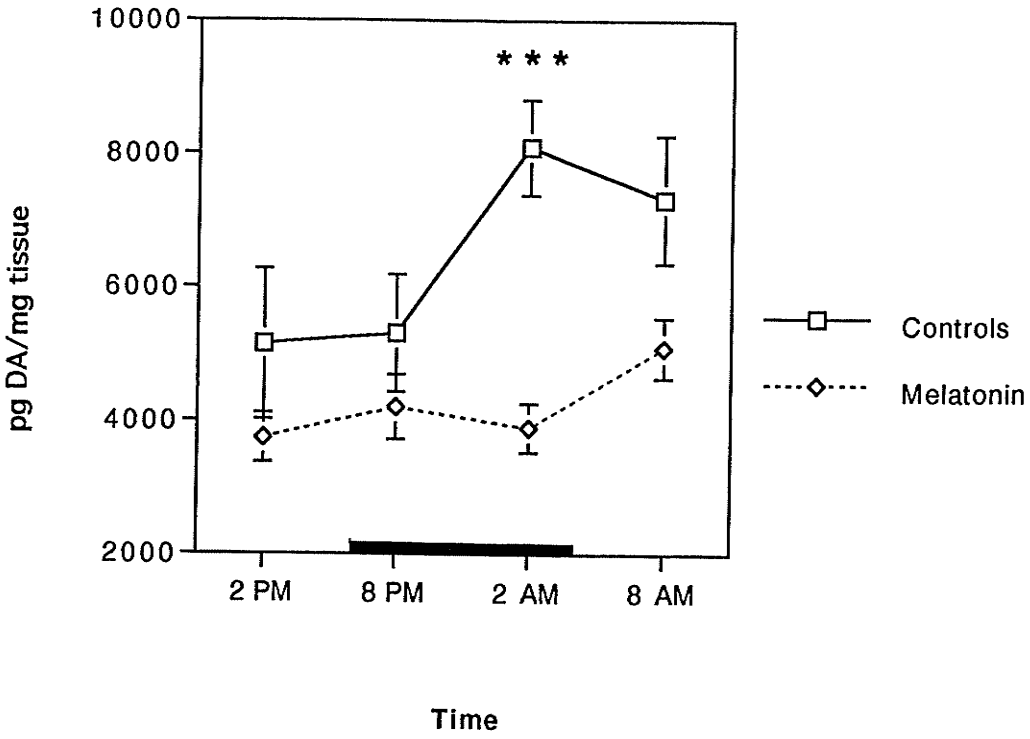

10000
8000
6000
4000
2000
pg DA/mg tissue
* * *
2 PM
8 PM
2 AM
8 AM
Time
Controls
Melatonin

Fig. 25. Twenty-four hour effects of saline and melatonin injections for 9.5 weeks on norepinephrine (NE) concentrations in the ME/arcuate region of the MBH (following NSD-1015 administration) in male Syrian hamsters. Dark bar indicates the 10 hr dark period of the daily light/dark cycle (6 PM - 4 AM). *$p < 0.05$ compared to saline-treated controls.

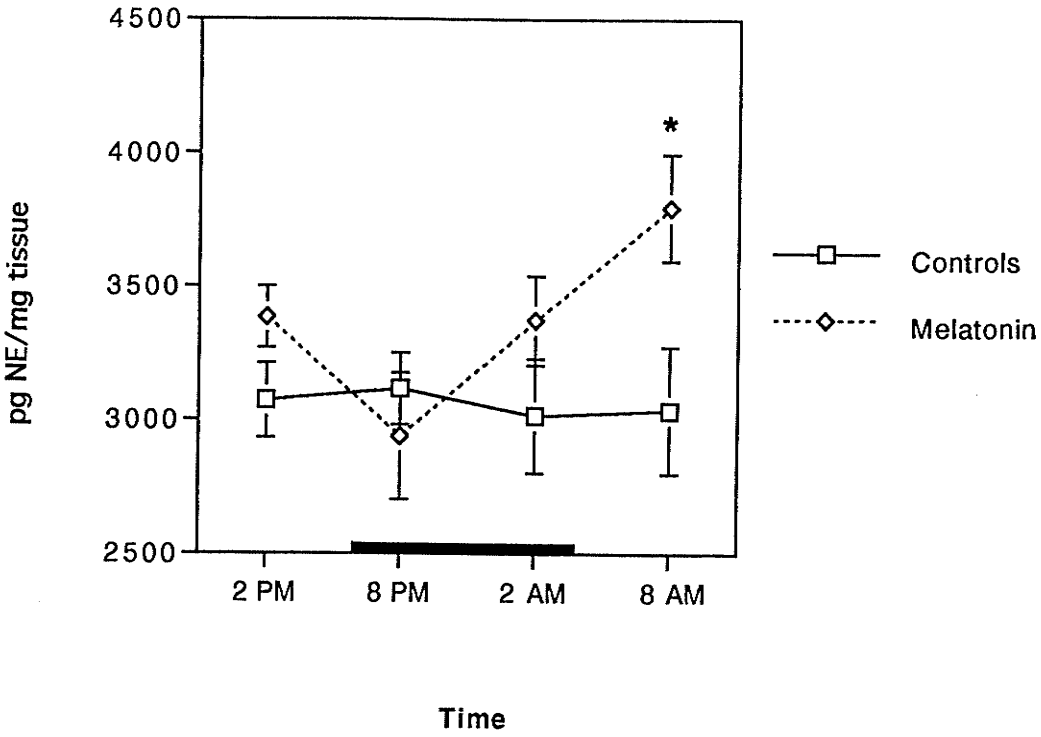

4500
4000
3500
3000
2500
pg NE/mg tissue
*
2 PM
8 PM
2 AM
8 AM
Time
Controls
Melatonin

Fig. 26. Twenty-four hour effects of saline and melatonin injections for 9.5 weeks on HVA concentrations in the ME/arcuate region of the MBH (following NSD-1015 administration) in male Syrian hamsters. Dark bar indicates the 10 hr dark period of the daily light/dark cycle (6 PM - 4 AM).

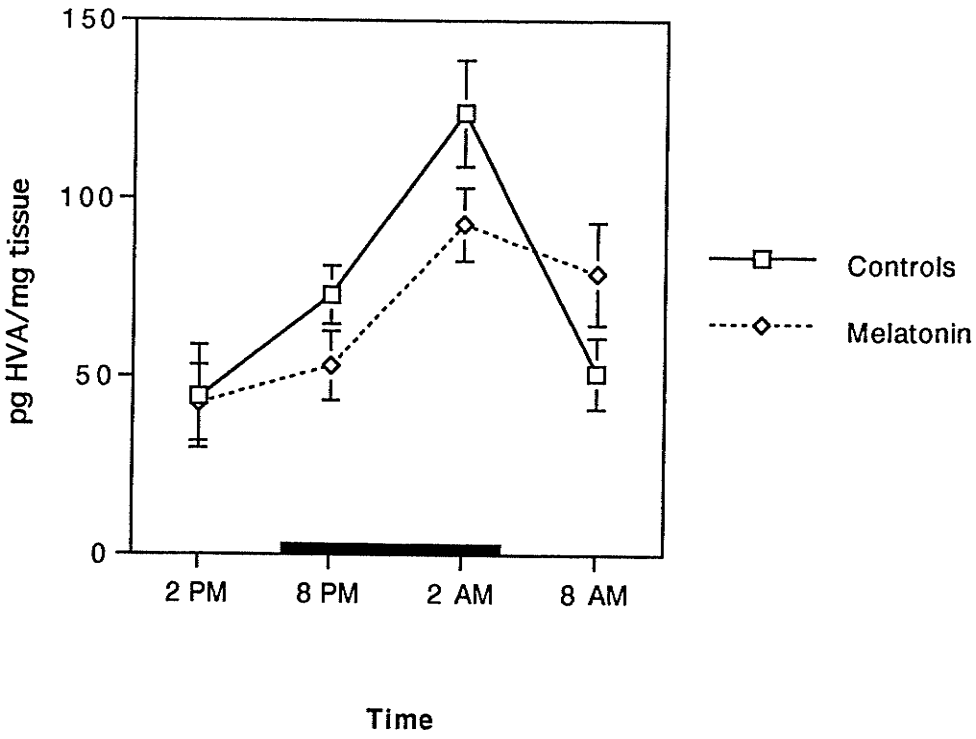

150
100
50
0
pg HVA/mg tissue
2 PM
8 PM
2 AM
8 AM
Time
Controls
Melatonin

Fig. 27. Twenty-four hour effects of saline and melatonin administration for 9.5 weeks on the ratio of HVA/DA (x 10,000), in the ME/arcuate region of the MBH in male Syrian hamsters. Dark bar indicates the 10 hr dark period of the light/dark cycle (6 PM - 4 AM). *$p < 0.05$ compared to saline-treated controls.

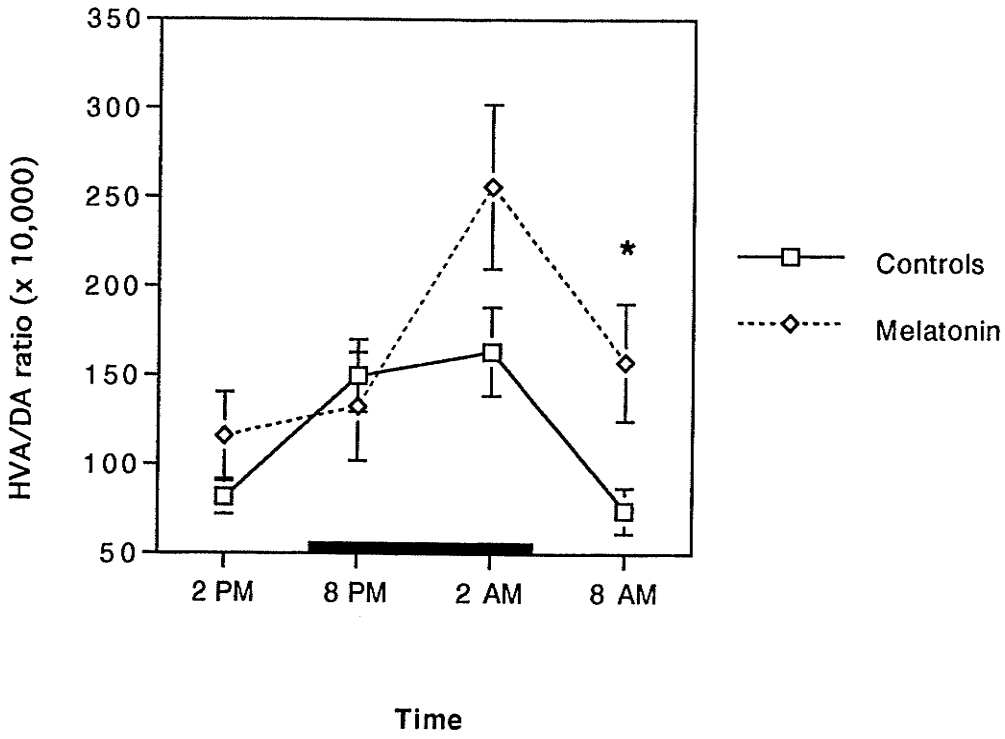

350
300
250
200
150
100
50
HVA/DA ratio (x 10,000)
2 PM
8 PM
2 AM
8 AM
*
Time
Controls
Melatonin

Fig. 28. Circadian (24-hr) effects of 9.5 weeks of melatonin injections on TPH activity (5-HTP accumulation after NSD-1015 administration) in the ME/arcuate region of the MBH. Dark bar indicates the 10 hr dark period of the light/dark cycle (6 PM - 4 AM). Data points represent Mean ± SE. *$p < 0.05$ compared to saline-treated controls.

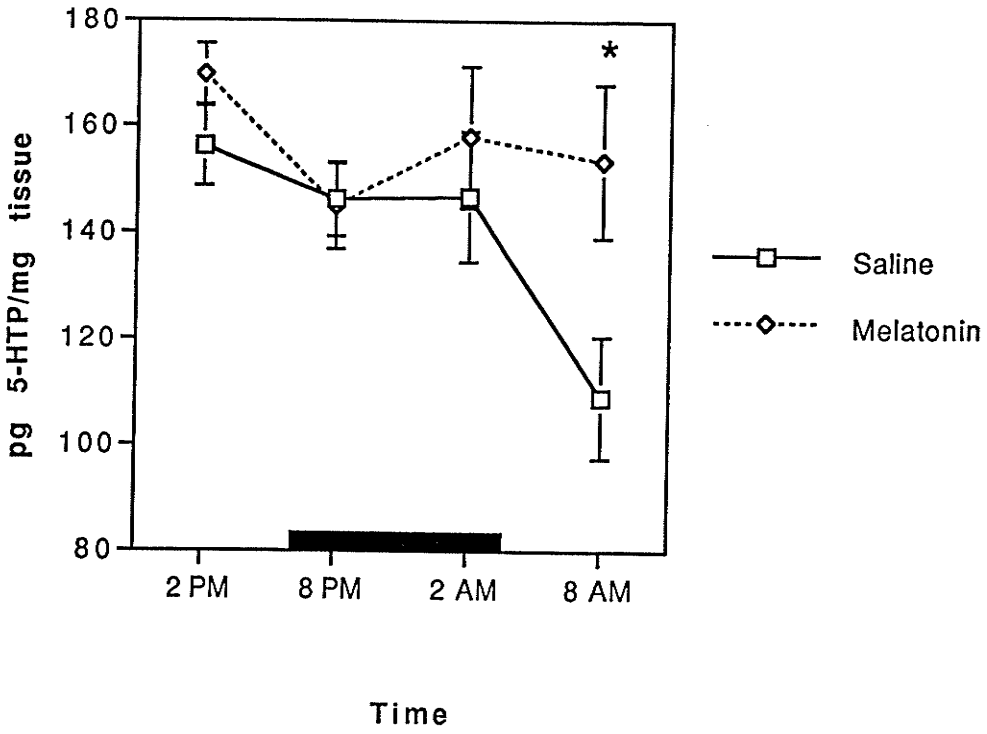

180
160
140
120
100
80
pg 5-HTP/mg tissue
*
2 PM
8 PM
2 AM
8 AM
Time
Saline
Melatonin

Fig. 29. Circadian (24-hr) effects of 9.5 weeks of melatonin injections on 5-HT concentrations in the ME/arcuate region of the MBH. Dark bar indicates the 10 hr dark period of the light/dark cycle (6 PM - 4 AM). Data points represent Mean ± SE. *$p < 0.05$ compared to saline-treated controls.

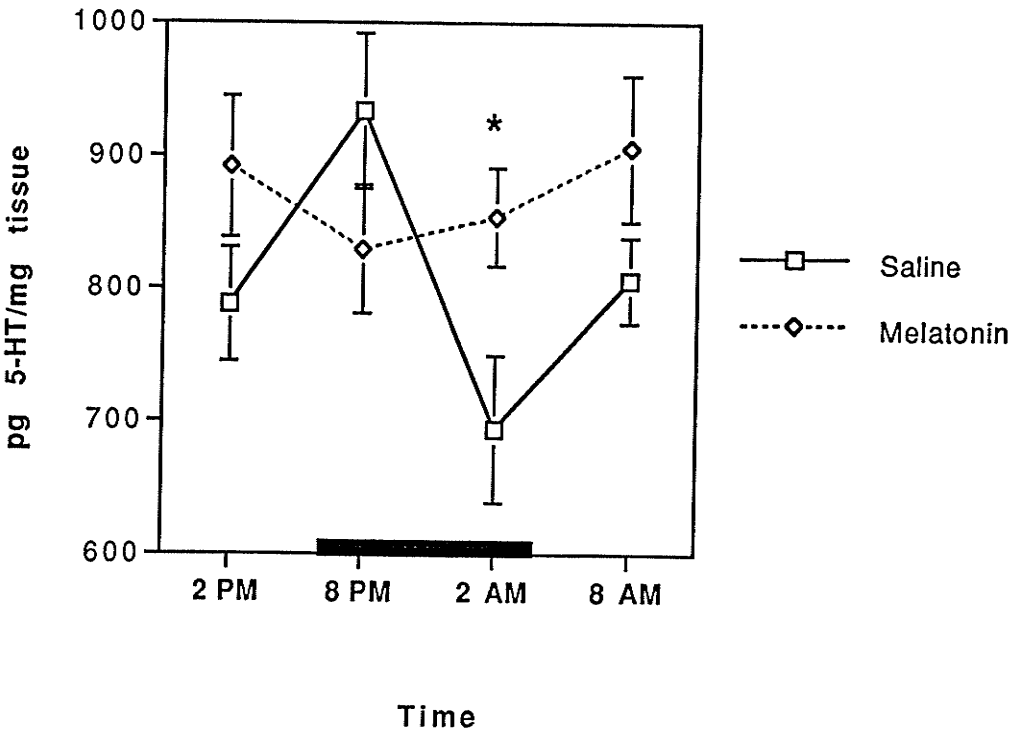

1000
900
800
700
600
pg 5-HT/mg tissue
*
2 PM
8 PM
2 AM
8 AM
Time
Saline
Melatonin

Fig. 30. Circadian (24-hr) effects of 9.5 weeks of melatonin injections on GABA concentrations in the ME/arcuate region of the MBH. Dark bar indicates the 10 hr dark period of the daily light/dark cycle (6 PM - 4 AM). Data points represent Mean ± SE. *$p < 0.05$ compared to melatonin-treated animals.

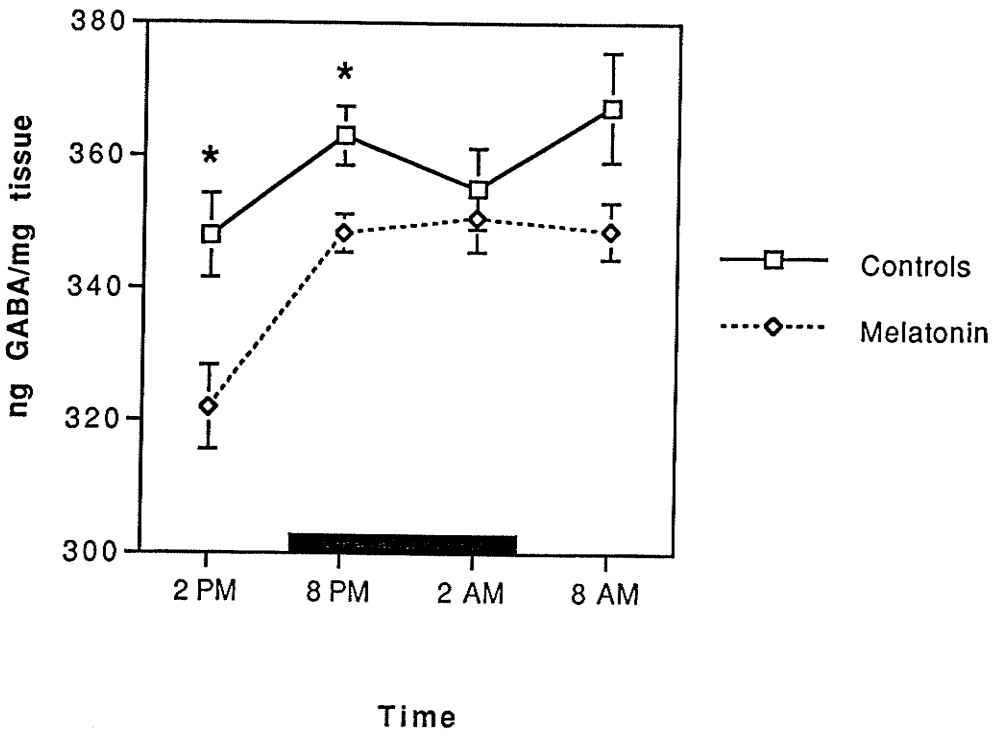

380
360
340
320
300
ng GABA/mg tissue
*
*
2 PM
8 PM
2 AM
8 AM
Time
Controls
Melatonin

Fig. 31. Circadian (24-hr) effects of 9.5 weeks of melatonin injections on glutamate concentrations in the ME/arcuate region of the MBH. Dark bar indicates the 10 hr dark period of the daily light/dark cycle (6 PM - 4 AM). Data points represent Mean ± SE.

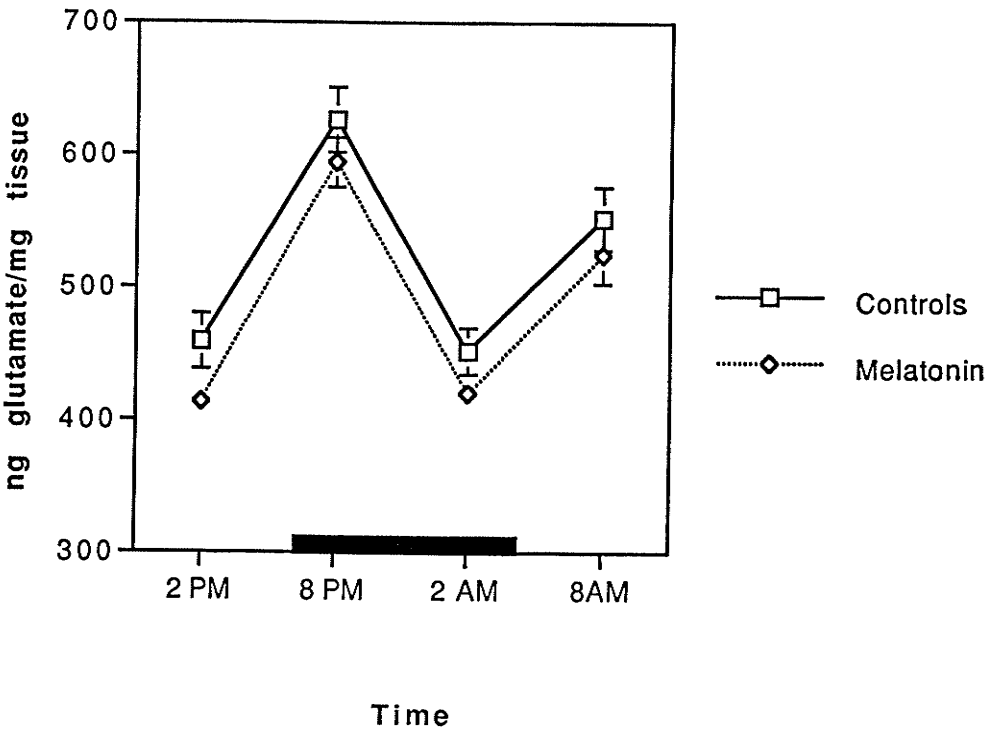

700
600
500
400
300
ng glutamate/mg tissue
2 PM
8 PM
2 AM
8AM
Time
Controls
Melatonin

Fig. 32. Circadian (24-hr) effects of 9.5 weeks of melatonin injections on glutamine concentrations in the ME/arcuate region of the MBH. Dark bar indicates the 10 hr dark period of the daily light/dark cycle (6 PM - 4 AM). Data points represent Mean ± SE. **$p < 0.01$ compared to melatonin-treated animals.

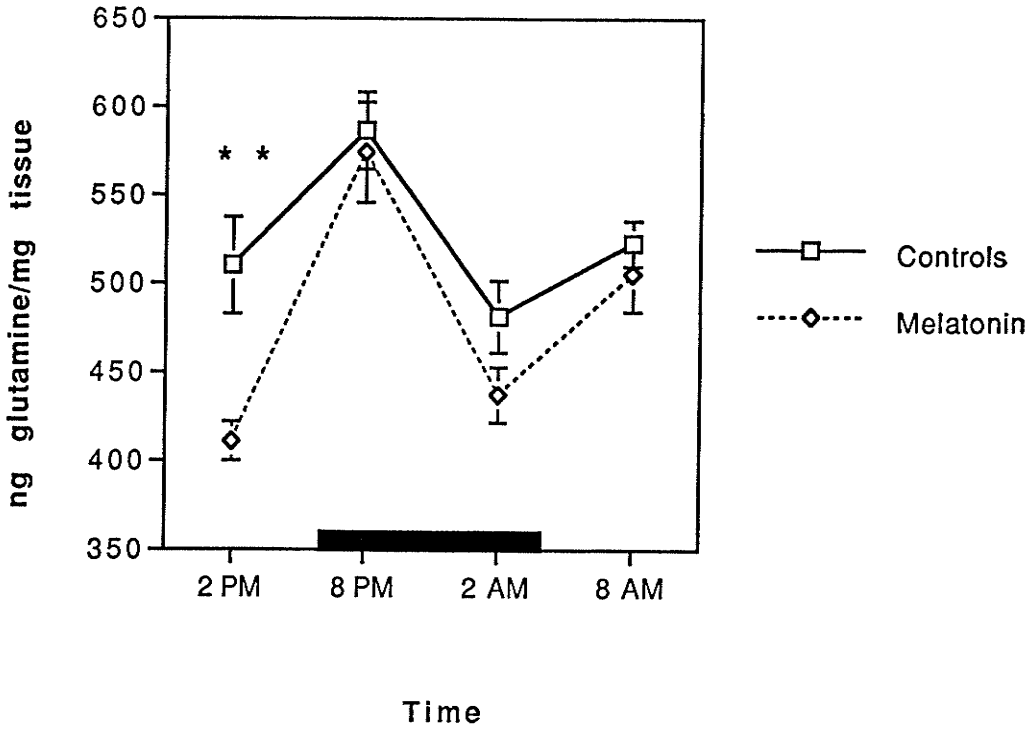

650
600
550
500
450
400
350
ng glutamine/mg tissue
* *
2 PM
8 PM
2 AM
8 AM
Time
Controls
Melatonin

Fig. 33. Circadian (24-hr) effects of saline and melatonin injections for 9.5 weeks on TH activity (L-DOPA accumulation after NSD-1015), in the striatum of male Syrian hamsters. Dark bar indicates the 10 hr dark period of the daily light/dark cycle (6 PM - 4 AM). $^{**}p < 0.01$ compared to saline-treated controls.

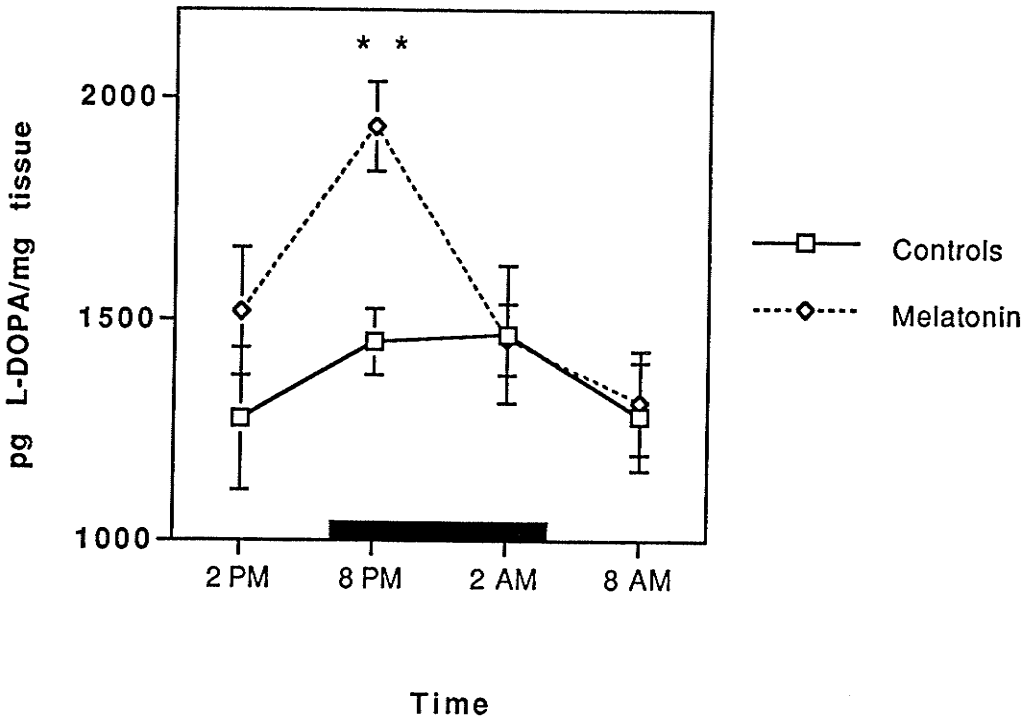

* *
2000
1500
1000
pg L-DOPA/mg tissue
2 PM
8 PM
2 AM
8 AM
Time
Controls
Melatonin

Fig. 34. Twenty-four hour effects of saline and melatonin injections for 9.5 weeks on DA concentrations in the striatum (following NSD-1015 administration) in male Syrian hamsters. Dark bar indicates the 10 hr dark period of the daily light/dark cycle (6 PM - 4 AM). *$p < 0.05$ compared to saline-treated controls.

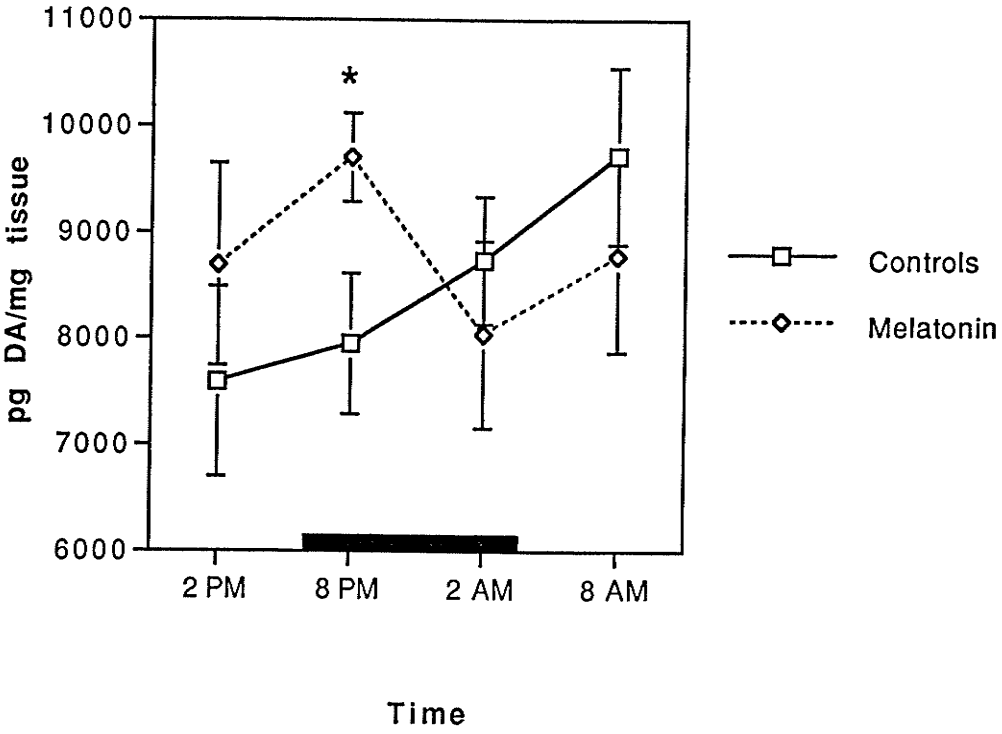

11000
10000
9000
8000
7000
6000
pg DA/mg tissue
*
2 PM
8 PM
2 AM
8 AM
Time
Controls
Melatonin

Fig. 35. Circadian (24-hr) effects of 9.5 weeks of melatonin injections on TPH activity (5-HTP accumulation after NSD-1015 administration) in the striatum. Dark bar indicates the 10 hr dark period of the daily light/dark cycle (6 PM - 4 AM). Data points represent Mean ± SE. *p < 0.05 compared to saline-treated controls.

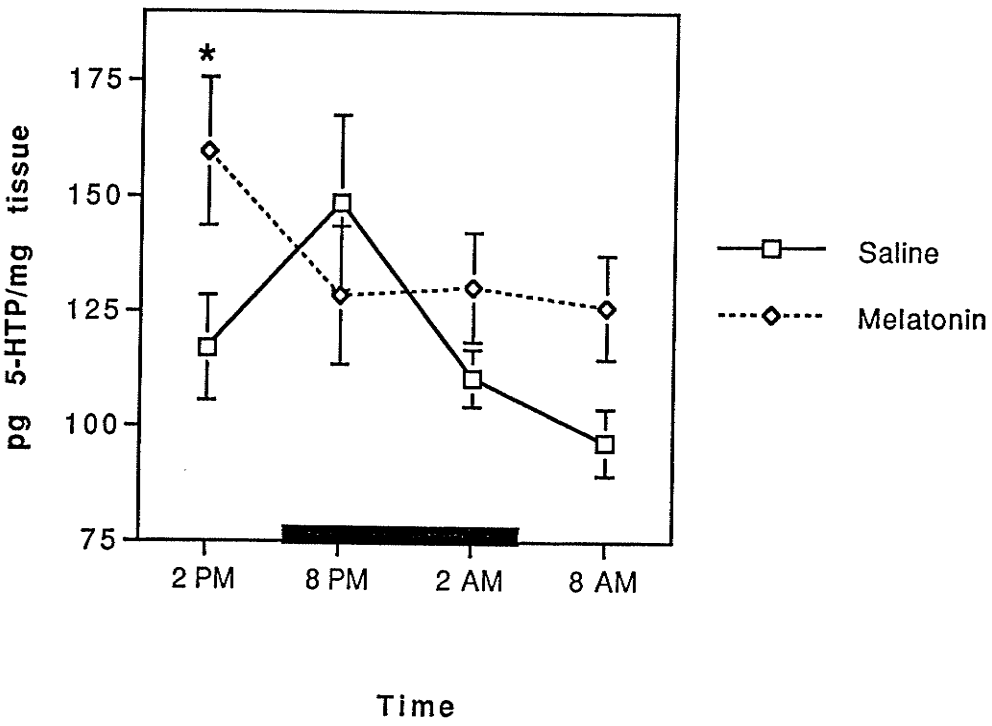

*
175
150
125
100
75
pg 5-HTP/mg tissue
2 PM
8 PM
2 AM
8 AM
Time
Saline
Melatonin

Fig. 36. Circadian (24-hr) effects of 9.5 weeks of melatonin injections on 5-HT levels in the striatum. Dark bar indicates the 10 hr dark period of the daily light/dark cycle (6 PM - 4 AM). Data points represent Mean ± SE. ***$p < 0.001$ compared to saline-treated controls.

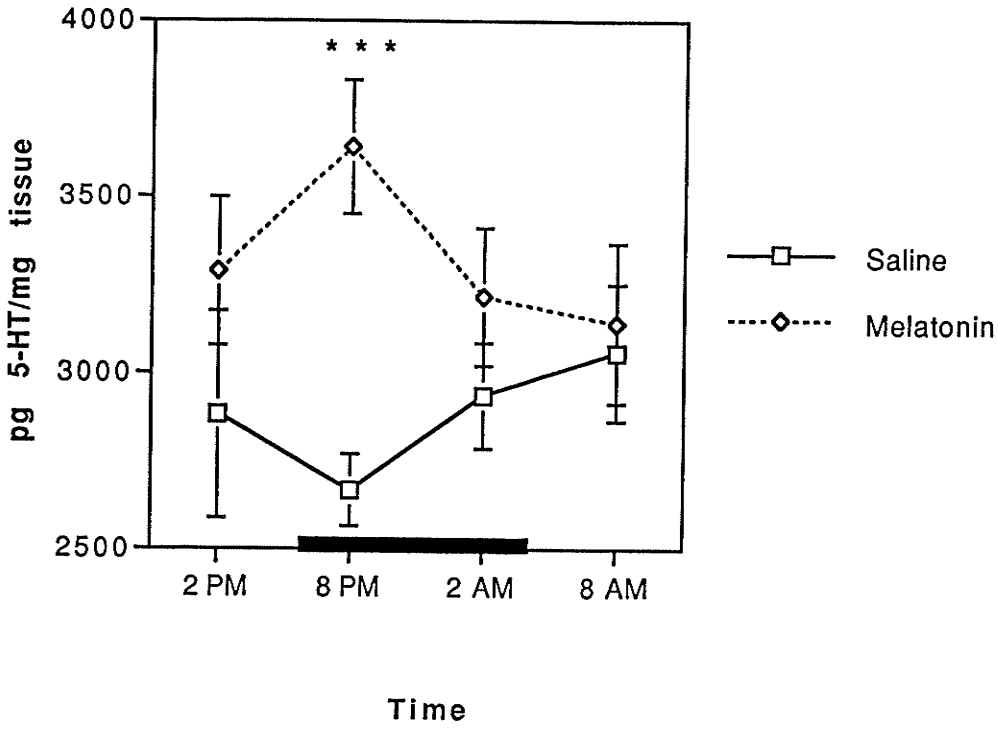

4000
3500
3000
2500
pg 5-HT/mg tissue
* * *
2 PM
8 PM
2 AM
8 AM
Time
Saline
Melatonin

TABLE 17. Circadian (24 hour) effects of melatonin administration on TH activity in the posterior pituitary of male Syrian hamsters

Time of Sacrifice	L-DOPA accumulation after NSD-1015 (pg/gland) Mean ± S.E.	L-DOPA accumulation after NSD-1015 (pg L-DOPA/μg protein) Mean ± S.E.
2 PM	223.6 ± 46.5 (Sal)	5.12 ± 1.26 (Sal)
2 PM	250.0 ± 23.8 (Mel)	5.34 ± 1.39 (Mel)
8 PM	177.1 ± 24.8 (Sal)	2.87 ± 0.45 (Sal)
8 PM	180.0 ± 23.6 (Mel)	2.79 ± 0.37 (Mel)
2 AM	177.9 ± 18.1 (Sal)	3.06 ± 0.40 (Sal)
2 AM	231.9 ± 44.2 (Mel)	4.10 ± 0.93 (Mel)
8 AM	230.6 ± 56.7 (Sal)	4.25 ± 1.26 (Sal)
8 AM	239.2 ± 32.7 (Mel)	3.64 ± 0.23 (Mel)

Saline-treated (Sal)
Melatonin-treated (Mel)

TABLE 18. Circadian (24 hour) effects of melatonin on catecholamine content in the posterior pituitary of male Syrian hamsters

Time of Sacrifice	Dopamine content (pg/gland) Mean ± S.E.	Norepinephrine content (pg/gland) Mean ± S.E.
2 PM	2025 ± 327 (Saline)	110 ± 20 (Saline)
2 PM	901 ± 80* (Melatonin)	101 ± 34 (Melatonin)
8 PM	2081 ± 381 (Saline)	129 ± 32 (Saline)
8 PM	1419 ± 153 (Melatonin)	105 ± 34 (Melatonin)
2 AM	1656 ± 239 (Saline)	134 ± 50 (Saline)
2 AM	1424 ± 156 (Melatonin)	90 ± 22 (Melatonin)
8 AM	1707 ± 374 (Saline)	121 ± 38 (Saline)
8 AM	1771 ± 190 (Melatonin)	149 ± 58 (Melatonin)

*$p < 0.05$ (compared to saline-treated controls)

TABLE 19. Circadian effects of melatonin on L-DOPA/DA ratio in the posterior pituitary (neurointermediate lobe)

Time of Sacrifice	L-DOPA/DA Ratio (x 10,000) in saline-treated hamsters (Mean ± S.E.)	L-DOPA/DA Ratio (x 10,000) in melatonin-treated hamsters (Mean ± S.E.)
2 PM	1615 ± 511	3315 ± 539
8 PM	971 ± 173	1470 ± 318
2 AM	1726 ± 548	1631 ± 198
8 AM	1372 ± 170	1266 ± 165

TABLE 20. Testes and body weights of male Syrian hamsters in circadian study

Treatment	Mean Testes Weight ± S.E. (grams)	Mean Body Weight ± S.E. (grams)
Saline	3.95 ± 0.07	206 ± 4
Melatonin	1.28 ± 0.21***	217 ± 5

***$p < 0.001$ (compared to saline-treated controls)

Table 21. Circadian effects of melatonin on the L-DOPA/DA ratio in the ME/arcuate region of male Syrian hamsters

Time of Sacrifice	L-DOPA/DA Ratio (x 10,000) in saline-treated hamsters (Mean ± S.E.)	L-DOPA/DA Ratio (x 10,000) in melatonin-treated hamsters (Mean ± S.E.)
2 PM	2488 ± 318	4351 ± 281***
8 PM	2536 ± 218	3855 ± 514*
2 AM	1509 ± 213	3986 ± 314***
8 AM	1463 ± 280	3375 ± 295***

*$p < 0.05$
***$p < 0.001$ (compared to saline-treated controls)

TABLE 22. Circadian (24 hour) effects of melatonin administration on monoamine metabolites in the ME/arcuate region of male Syrian hamsters

Time of Sacrifice	DOPAC levels (pg/mg tissue) Mean ± S.E.	5-HIAA levels (pg/mg tissue) Mean ± S.E.
2 PM	99 ± 40 (Sal)	63 ± 8 (Sal)
2 PM	84 ± 15 (Mel)	58 ± 4 (Mel)
8 PM	159 ± 30 (Sal)	92 ± 9 (Sal)
8 PM	132 ± 34 (Mel)	78 ± 5 (Mel)
2 AM	214 ± 35 (Sal)	117 ± 9 (Sal)
2 AM	180 ± 40 (Mel)	108 ± 7 (Mel)
8 AM	76 ± 15 (Sal)	74 ± 10 (Sal)
8 AM	223 ± 66 (Mel)	129 ± 19* (Mel)

*$p < 0.05$ (compared to saline-treated controls)

TABLE 23. Circadian (24 hour) effects of melatonin on glycine concentrations in the ME/arcuate region of male Syrian hamsters

Time of Sacrifice	Glycine levels (ng/mg tissue ± S.E.) in saline-treated hamsters	Glycine levels (ng/mg tissue ± S.E.) in melatonin-treated hamsters
2 PM	141.19 ± 8.38	140.31 ± 9.03
8 PM	150.42 ± 4.95	167.42 ± 11.78
2 AM	147.62 ± 4.27	148.52 ± 5.82
8 AM	153.56 ± 4.72	142.39 ± 6.34

Data are expressed as Mean ± S.E.

TABLE 24. Circadian (24 hour) effects of melatonin on serine concentrations in the ME/arcuate region of male Syrian hamsters

Time of Sacrifice	Serine levels (ng/mg tissue ± S.E.) in saline-treated hamsters	Serine levels (ng/mg tissue ± S.E.) in melatonin-treated hamsters
2 PM	67.98 ± 3.52	65.66 ± 4.16
8 PM	79.02 ± 4.93	84.89 ± 7.20
2 AM	66.57 ± 2.72	66.81 ± 0.69
8 AM	80.17 ± 3.76	71.87 ± 3.41

Data are expressed as Mean ± S.E.

TABLE 25. Circadian (24 hour) effects of melatonin on alanine concentrations in the ME/arcuate region of male Syrian hamsters

Time of Sacrifice	Alanine levels (ng/mg tissue ± S.E.) in saline-treated hamsters	Alanine levels (ng/mg tissue ± S.E.) in melatonin-treated hamsters
2 PM	51.56 ± 2.99	45.62 ± 3.01
8 PM	64.62 ± 3.45	68.62 ± 8.24
2 AM	61.80 ± 5.64	52.89 ± 4.02
8 AM	56.97 ± 4.23	49.32 ± 3.02

Data are expressed as Mean ± S.E.

TABLE 26. Circadian effects of melatonin on L-DOPA/DA ratio in the striatum of male Syrian hamsters

Time of Sacrifice	L-DOPA/DA Ratio (x 10,000) in saline-treated hamsters (Mean ± S.E.)	L-DOPA/DA Ratio (x 10,000) in melatonin-treated hamsters (Mean ± S.E.)
2 PM	1693 ± 106	1772 ± 51
8 PM	1909 ± 117	1995 ± 62
2 AM	1727 ± 86	1902 ± 141
8 AM	1332 ± 87	1530 ± 94

TABLE 27. Circadian effects of melatonin on DA metabolites in the striatum of male Syrian hamsters

Time of Sacrifice	HVA levels (pg/mg ± S.E.) in saline-treated hamsters	HVA levels (pg/mg ± S.E.) in melatonin-treated hamsters	DOPAC levels (pg/mg ± S.E.) in saline-treated hamsters	DOPAC levels (pg/mg ± S.E.) in melatonin-treated hamsters
2 PM	434.0 ± 67.8	437.5 ± 63.7	196.5 ± 32.5	183.0 ± 25.0
8 PM	702.0 ± 84.4	871.1 ± 80.7	314.5 ± 44.9	332.9 ± 35.2
2 AM	746.5 ± 58.0	818.5 ± 115.9	321.5 ± 42.6	286.0 ± 50.1
8 AM	834.0 ± 67.0	787.0 ± 78.0	373.5 ± 43.9	342.0 ± 63.0

Data are expressed as Mean ± S.E.

TABLE 28. Circadian (24 hour) effects of melatonin on NE concentrations in the striatum of male Syrian hamsters

Time of Sacrifice	NE levels (pg/mg tissue ± S.E.)
2 PM	101 ± 24 (Saline)
2 PM	94 ± 12 (Melatonin)
8 PM	133 ± 37 (Saline)
8 PM	115 ± 23 (Melatonin)
2 AM	98 ± 28 (Saline)
2 AM	112 ± 11 (Melatonin)
8 AM	121 ± 21 (Saline)
8 AM	123 ± 19 (Melatonin)

Data are expressed as Mean ± S.E.

TABLE 29. Circadian effects of melatonin treatment on 5-HIAA concentrations in striatum

Time of Sacrifice	5-HIAA levels (pg/mg tissue ± S.E.) in saline-treated animals	5-HIAA levels (pg/mg tissue ± S.E.) in melatonin-treated animals
2 PM	77 ± 9	80 ± 8
8 PM	102 ± 11	101 ± 9
2 AM	101 ± 8	107 ± 5
8 AM	103 ± 6	119 ± 8

Data are expressed as Mean ± S.E.

3.5 EXPERIMENT FIVE: GONADECTOMY STUDY

3.5.1 Posterior Pituitary (Neurointermediate Lobe - NIL):

3.5.2 L-DOPA Accumulation in the NIL

Melatonin treatment for 9.5 weeks in gonadectomized (GX) hamsters had no significant effect on the accumulation of L-DOPA following cessation of catecholamine synthesis (after administration of NSD-1015) as detected by one-way ANOVA or t-test ($F = 1.08$; $t = 1.04$; N.S.; Table 30).

In concordance with the data above, melatonin treatment had no significant effect on TH activity (accumulation of L-DOPA) when L-DOPA values were expressed as pg/μg protein ($F = 0.08$; $t = 0.28$; N.S.; Table 31).

3.5.3 DA Content in the NIL

As similarly demonstrated in a previous investigation with intact hamsters, melatonin administration for 9.5 weeks in GX animals (after NSD-1015) significantly decreased DA content of the NIL ($F = 5.02$; $p < 0.05$; Table 30) as detected by ANOVA. This occurred at 0-2 AM during the dark phase of the daily light/dark cycle ($t = 2.24$; $p < 0.05$).

This was confirmed and demonstrated to be even more significant when expressed in pg/μg protein. Melatonin treatment significantly decreased DA levels compared to saline-treated controls ($F = 8.89$; $p < 0.01$; Fig. 37) as detected by

ANOVA and t-test ($t = 2.98$; $p < 0.01$; Fig. 37). Melatonin reduced DA content of the NIL to 59% of saline-treated controls.

3.5.4 L-DOPA/DA Ratio in the NIL

Melatonin treatment for 9.5 weeks resulted in a significant increase in the L-DOPA/DA ratio in the NIL of the pituitary of castrated hamsters ($F = 13.10$; $p < 0.01$; Table 32) as detected by ANOVA. This occurred at 0-2 AM during the dark phase of the light/dark cycle ($t = 3.62$; $p < 0.01$; Table 32).

3.5.5 NE Content in the NIL

Melatonin administration for 9.5 weeks in GX hamsters did not significantly influence NE content (after NSD-1015) in the NIL ($F = 1.12$; $t = 1.06$; N.S.; Table 30) according to ANOVA and the Student's t-test.

Similar findings were demonstrated when pituitary NE was expressed in pg/μg protein ($F = 1.14$; $t = 1.07$; N.S.; Table 31).

3.5.6 ME/Arcuate Region of the MBH:

3.5.7 L-DOPA Accumulation in the ME/Arcuate region of the MBH

Daily injections of melatonin in castrated hamsters for 9.5 weeks produced a highly significant increase in the activity of TH of the ME/arcuate region ($F = 16.30$; $p < 0.001$; Fig. 38) as detected by ANOVA. The elevation in L-DOPA accumulation

(after NSD-1015) was highly significant at this 0-2 AM time of sacrifice ($t = 4.04$; $p < 0.001$; Fig. 38) in this area. Melatonin increased TH activity to 149% of saline-treated controls at this time.

3.5.8 DA Levels in the MBH

Compared to saline-treated GX controls, melatonin administration for 9.5 weeks in GX hamsters produced a highly significant reduction in DA concentrations in the ME/arcuate region ($F = 18.15$; $p < 0.001$; Fig. 39) as detected by ANOVA. This melatonin-induced decrease in DA occurred during the 0-2 AM time of sacrifice ($t = 4.26$; $p < 0.001$; Fig. 39), concomitantly with the significant elevation in L-DOPA. Melatonin reduced DA concentrations to 52% of saline-treated controls at this time.

3.5.9 L-DOPA/DA Ratio in the MBH

Melatonin treatment for 9.5 weeks resulted in a highly significant increase in the L-DOPA/DA ratio in the MBH of castrated Syrian hamsters ($F = 33.40$; $p < 0.001$; Table 33) as detected by ANOVA. This melatonin-induced elevation in the ratio occurred during the dark phase of the 24 hr light/dark cycle ($t = 5.78$; $p < 0.001$; Table 33).

3.5.10 NE Levels in the MBH

No significant effects of melatonin could be detected by ANOVA ($F = 0.05$;

N.S.; Table 34) or via Student's t-test (t = 0.22; N.S.; Table 34) on NE levels of the ME/arcuate region of GX male hamsters during this 0-2 AM time of sacrifice.

3.5.11 HVA Levels in the MBH

Although concentrations of HVA were theoretically decreased by cessation of synthesis following administration of NSD-1015, levels of HVA were still detectable via the HPLC system in the present study. Administration of melatonin in GX hamsters was associated with a significant decrease in ME/arcuate concentrations of this acid metabolite (HVA), compared to saline-treated controls ($F = 6.84$; $p < 0.025$; Fig. 40), as demonstrated by ANOVA. This melatonin-induced decrease occurred during the 0-2 AM time of sacrifice ($t = 2.62$; $p < 0.05$; Fig. 40).

3.5.12 HVA/DA Ratio in the MBH

No significant effects of melatonin were demonstrated on the HVA/DA ratio as detected by ANOVA ($F = 1.02$; N.S.; Table 35) or t-test ($t = 1.01$; N.S.; Table 35) during this 0-2 AM time period of the dark phase of the daily light/dark cycle.

3.5.13 DOPAC Levels in the MBH

No significant effects of melatonin administration was demonstrated on DOPAC concentrations in this region ($F = 0.13$; $t = 0.36$; N.S.; Table 34) in the present experiment.

3.5.14 5-HTP Accumulation in the MBH

No significant effects of melatonin on the accumulation of 5-HTP (following the administration NSD-1015) could be detected by ANOVA ($F = 2.05$; N.S.; Table 36) in the ME/arcuate region of GX hamsters. This lack of significance in the activity of TPH occurred during the 0-2 AM sacrifice ($t = 1.43$; N.S.).

3.5.15 5-HT Levels in the MBH

Melatonin treatment significantly increased the concentrations of 5-HT in GX hamsters in the ME/arcuate region as detected by one-way ANOVA ($F = 8.84$; $p < 0.01$; Fig. 41). This melatonin-induced elevation in MBH 5-HT occurred during the 0-2 AM phase of the daily light/dark cycle ($t = 2.97$; $p < 0.01$; Fig. 41). Melatonin increased 5-HT levels to 120% of saline-treated controls at this time.

3.5.16 5-HIAA Levels in the MBH

No significant effects of melatonin treatment could be detected by ANOVA or t-test on the concentrations of the 5-HT metabolite, 5-HIAA, in this region ($F = 0.40$; $t = 0.63$; N.S.; Table 36).

3.5.17 Figures and Tables for Experiment Five

Fig. 37. Effects of melatonin injections for 9.5 weeks on DA content in the posterior pituitary (neurointermediate lobe) of castrated (GX) Syrian hamsters. Data points represent Mean ± SE. **$p < 0.01$ compared to saline-treated controls.

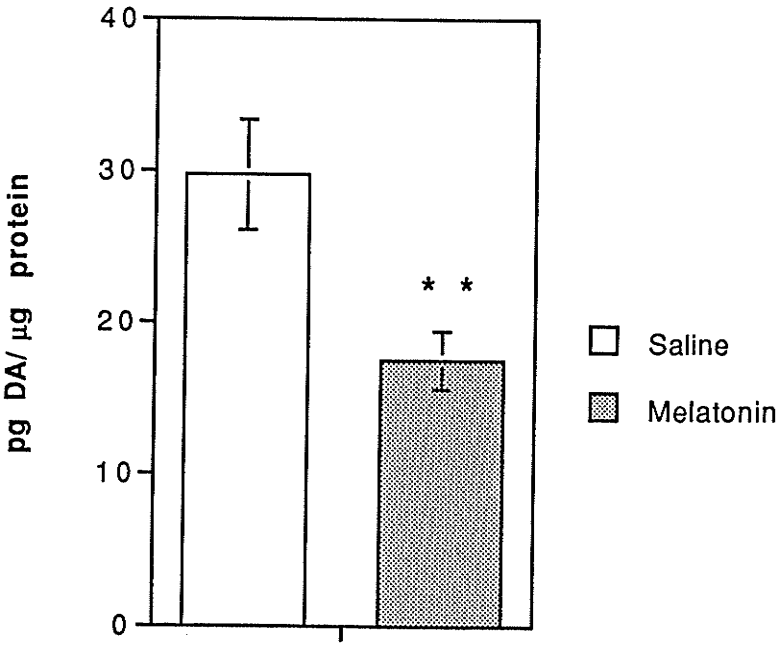

40
30
20
10
0
pg DA/ µg protein
* *
Saline
Melatonin

Fig. 38. Effects of melatonin injections for 9.5 weeks on TH activity (L-DOPA accumulation after NSD-1015 administration) in the ME/arcuate region of the MBH in castrated (GX) Syrian hamsters. Data points represent Mean ± SE. ***$p < 0.001$ compared to saline-treated controls.

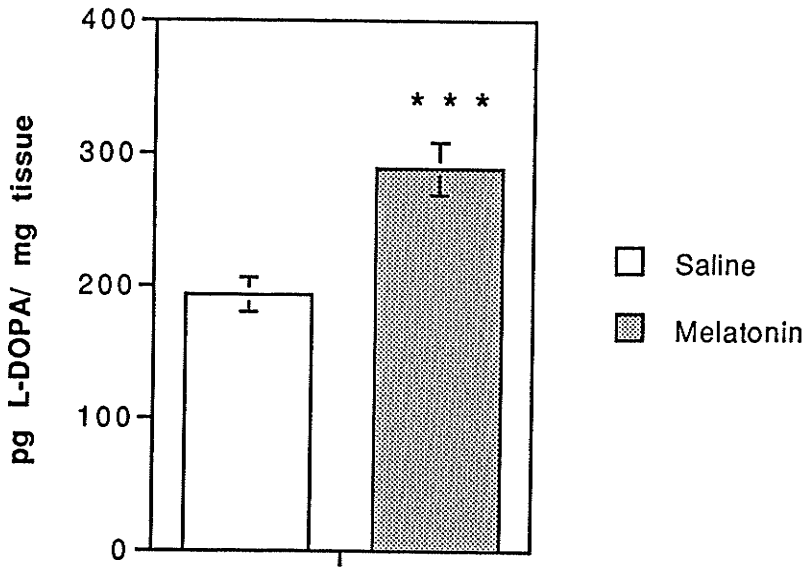

400
300
200
100
0
pg L-DOPA/ mg tissue
* * *
Saline
Melatonin

Fig. 39. Effects of melatonin injections for 9.5 weeks on DA concentrations in the ME/arcuate region of the MBH in castrated (GX) Syrian hamsters. Data points represent Mean ± SE. ***$p < 0.001$ compared to saline-treated controls.

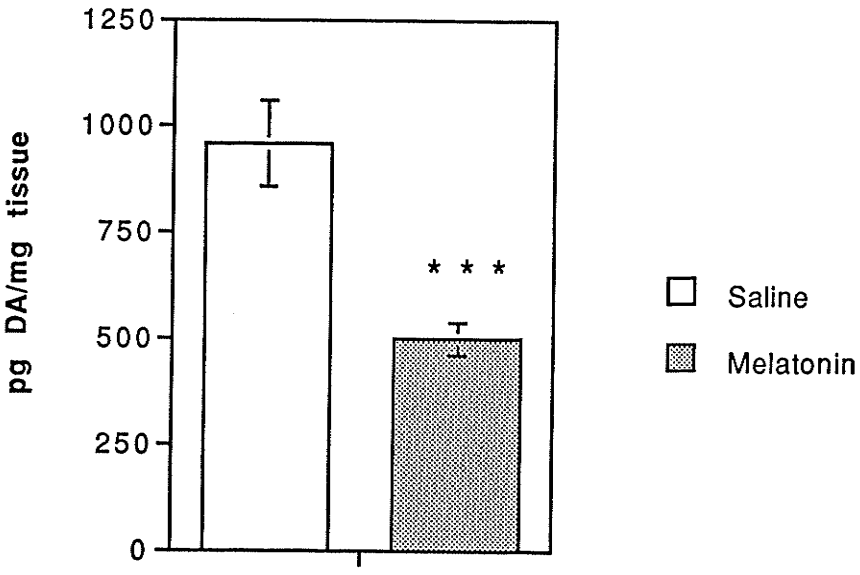

1250
1000
750
500
250
0
pg DA/mg tissue
* * *
Saline
Melatonin

Fig. 40. Effects of melatonin administration for 9.5 weeks on HVA concentrations in the ME/arcuate region of the MBH in castrated (GX) Syrian hamsters. Data points represent Mean ± SE. *$p < 0.05$ compared to saline-treated controls.

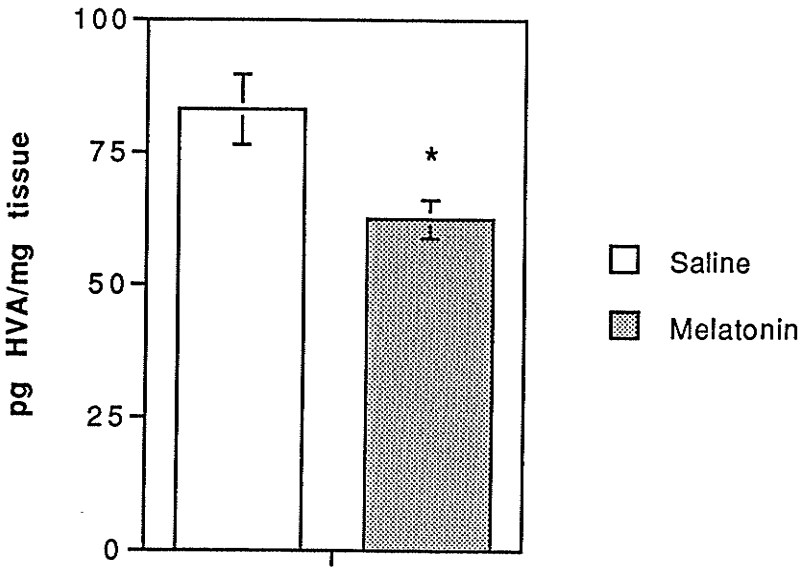

100
75
50
25
0
pg HVA/mg tissue
*
Saline
Melatonin

Fig. 41 Effects of melatonin administration for 9.5 weeks on 5-HT concentrations in the ME/arcuate region of the MBH in castrated (GX) Syrian hamsters. Data points represent Mean ± SE. **$p < 0.01$ compared to saline-treated controls.

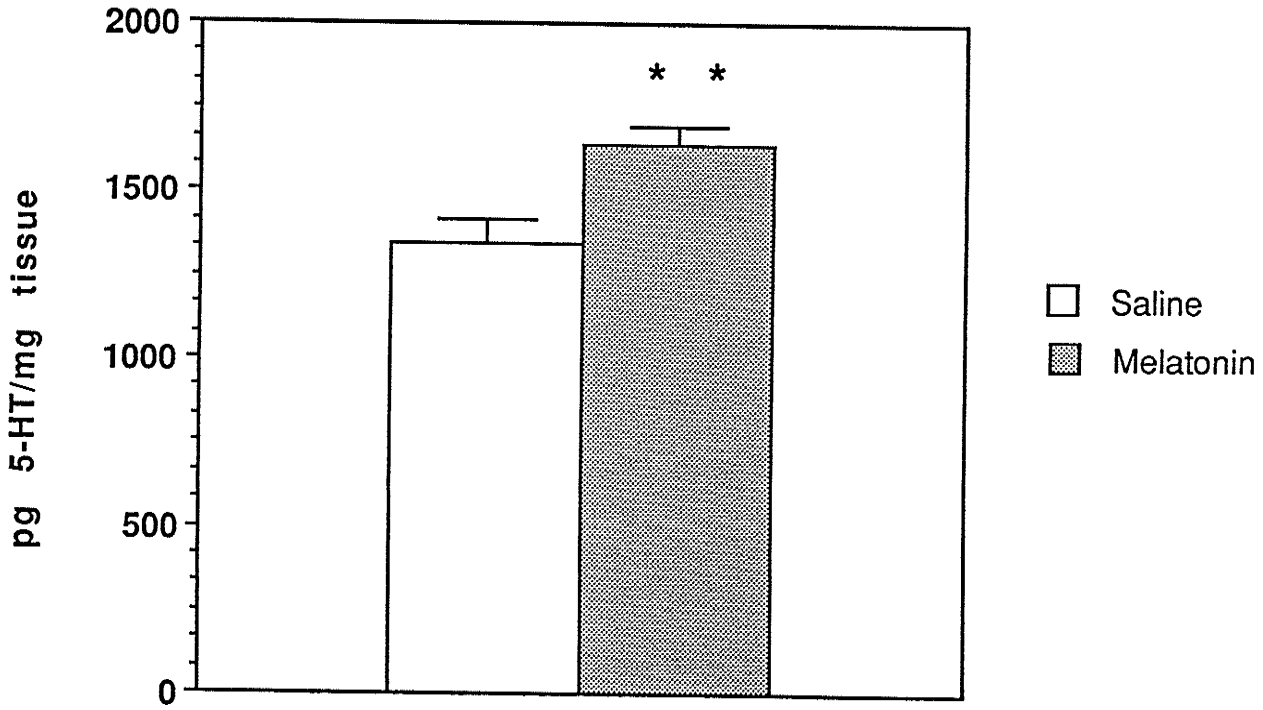
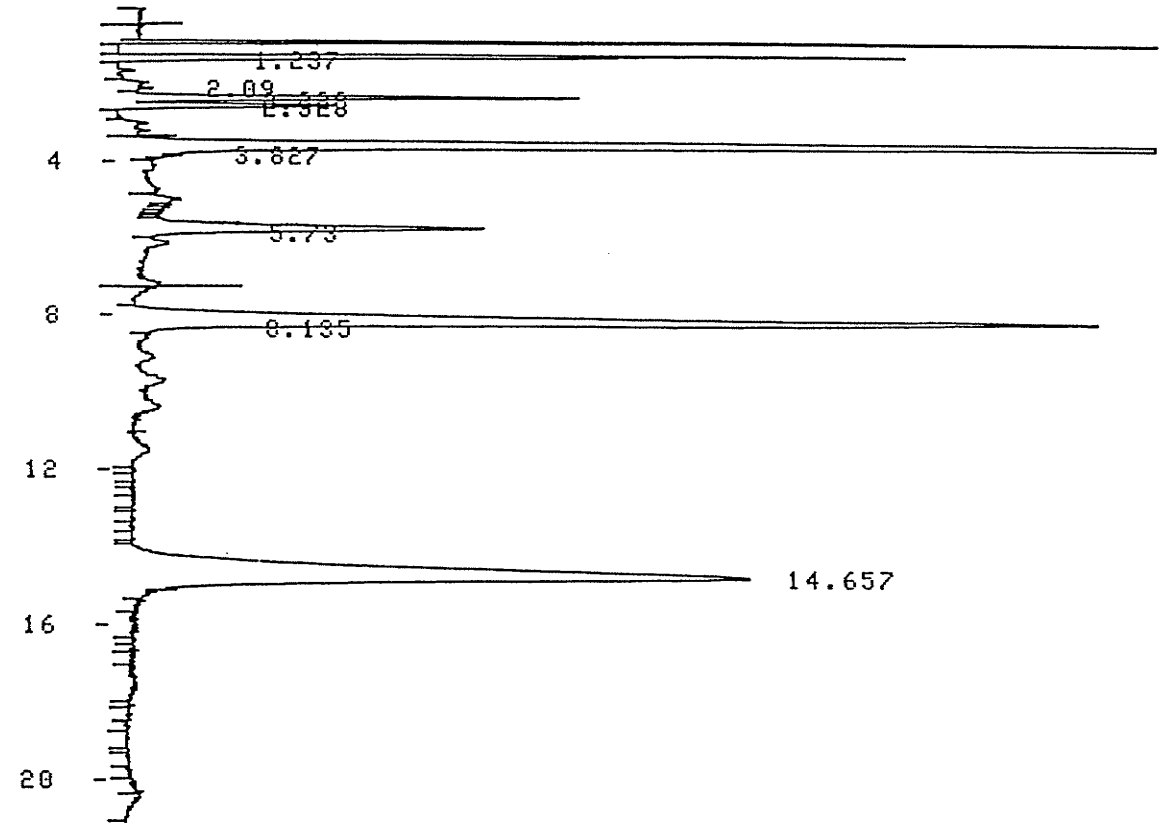

2000
1500
1000
500
0
pg 5-HT/mg tissue
* *
Saline
Melatonin

Fig. 42 Typical HPLC chromatograms for the ME/arcuate region of the MBH of saline-treated (top chromatogram) and melatonin-treated castrated hamsters (bottom chromatogram) after 9.5 weeks. Note increased L-DOPA accumulation (time = 5.7 minutes) and decreased dopamine (time = 14.6 minutes). Internal standard (time = 8.1 minutes) is DHBA.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8.135
14.657
4
8
12
16
20

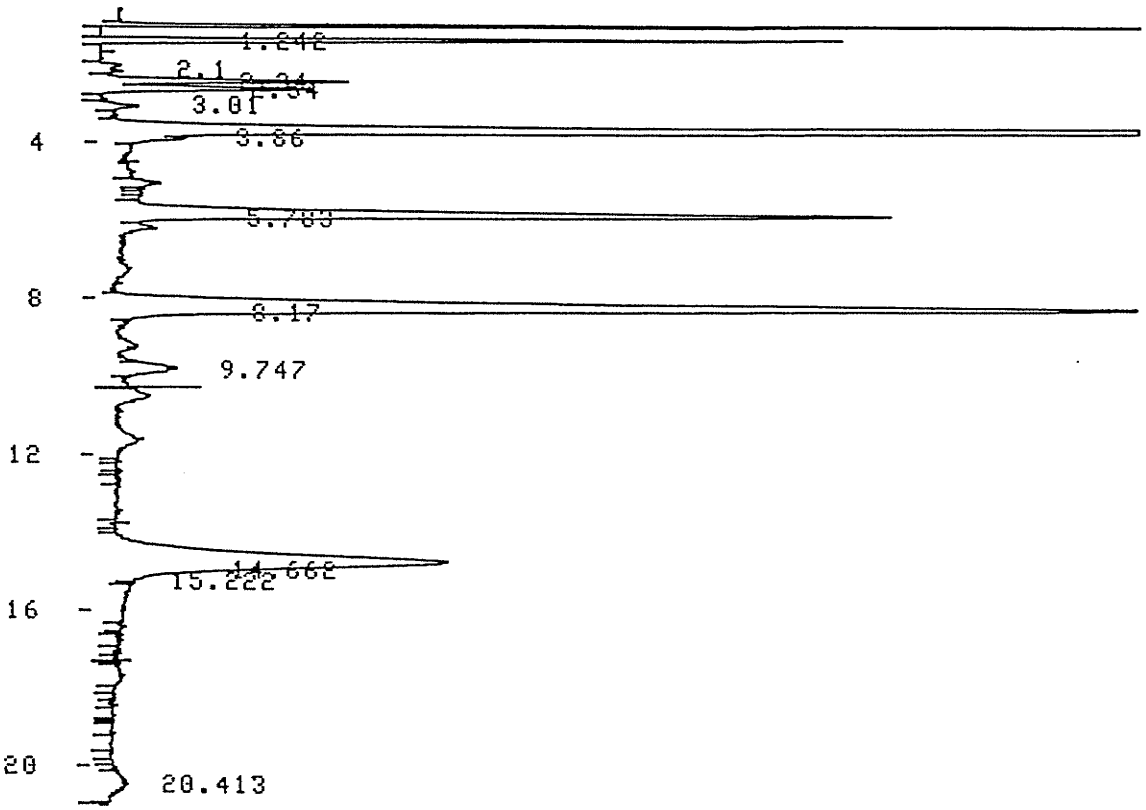
1.242
3.01
3.86
5.783
8.17
9.747
15.222
20.413
4
8
12
16
20

TABLE 30. Effects of melatonin on TH activity and catecholamine content in the posterior pituitary of castrated Syrian hamsters

Treatment	L-DOPA accumulation after NSD-1015 (pg/gland) Mean ± S.E.	Dopamine content (pg/gland) Mean ± S.E.	Norepinephrine content (pg/gland) Mean ± S.E.
Saline	133.8 ± 5.3	1800.4 ± 211.1	110.9 ± 12.5
Melatonin	163.4 ± 28.0	1194.6 ± 169.0*	95.0 ± 8.2

*$p < 0.05$ (compared to saline-treated controls)

TABLE 31. Effects of melatonin on TH activity and NE content in the posterior pituitary of castrated male hamsters

Treatment	L-DOPA accumulation after NSD-1015 (pg/μg protein) Mean ± S.E.	NE content after NSD-1015 (pg/μg protein) Mean ± S.E.
Saline	2.20 ± 0.13	1.77 ± 0.21
Melatonin	2.30 ± 0.34	1.49 ± 0.15

TABLE 32. Effects of melatonin on L-DOPA/DA ratio in the posterior pituitary of castrated Syrian hamsters

Treatment	L-DOPA/DA ratio (x 10,000) ± S.E.
Saline	845 ± 83
Melatonin	1446 ± 140**

**$p < 0.01$ (compared to saline-treated controls); Data are expressed as Mean ± S.E.

TABLE 33. Effects of melatonin on the L-DOPA/DA ratio in the ME/arcuate region of castrated Syrian hamsters

Treatment	L-DOPA/DA ratio (x 10,000) Mean ± S.E.
Saline-treated hamsters	2423 ± 434
Melatonin-treated hamsters	5996 ± 441***

***$p < 0.001$ (compared to saline-treated controls)

TABLE 34. Effects of melatonin on NE and DOPAC concentrations in ME/arcuate region of castrated male Syrian hamsters

Treatment	NE levels (pg/mg ± S.E.)	DOPAC levels (pg/mg ± S.E.)
Saline	1276 ± 76	9 ± 1
Melatonin	1301 ± 85	10 ± 2

Data are expressed as Mean ± S.E.

TABLE 35. Effects of melatonin on the HVA/DA ratio in the ME/arcuate region of castrated (GX) hamsters

Treatment	HVA/DA ratio (x 10,000) ± S.E.
Saline	1026 ± 190
Melatonin	1279 ± 158

Data are expressed as Mean ± S.E.

TABLE 36. Effects of melatonin treatment on TPH activity and 5-HIAA concentrations in ME/arcuate region of GX male Syrian hamsters

Treatment	5-HTP accumulation after NSD-1015 (pg/mg tissue ± S.E.)	5-HIAA levels in ME/arcuate region (pg/mg tissue ± S.E.)
Saline	289 ± 21	125 ± 10
Melatonin	336 ± 25	133 ± 7

Data are expressed as Mean ± S.E.

4. DISCUSSION

4.1 EXPERIMENT ONE: TIME COURSE OF MELATONIN ON MONOAMINE CONCENTRATIONS IN THE MBH

Experiment 1 was designed to determine the effects of melatonin treatment on catecholamine levels of the MBH. Melatonin administration for 3 and 6 weeks significantly decreased DA concentrations in the ME/arcuate region of the MBH of male Syrian hamsters (Fig. 1). This study demonstrates that these reductions in ME/arcuate DA levels were exclusively due to the effects of melatonin.

DOPAC concentrations of the ME/arcuate region was also significantly reduced in melatonin-treated animals after both 3 and 6 weeks of treatment (Fig. 3). Since the decrease in DA occurred concomitantly with reduced DOPAC, the present data suggests that the melatonin-induced reduction in ME/arcuate DA levels was not due to an increase in DA catabolism (metabolism). Decreased DOPAC content was previously demonstrated in the posterior pituitary of animals treated with melatonin for 10 weeks (Alexiuk and Vriend, 1991).

Some investigators have interpreted a reduction in ME DOPAC concentrations to be reflective of changes (decreases) in tuberoinfundibular dopaminergic neuronal activity (Lookingland et al., 1987; Roth, 1987; Krajnak et al., 1994b). Krajnak and collaborators (1994a) found no differences in ME DOPAC levels in male hamsters exposed to long or short days for 12 weeks. However, it

should be noted that (unlike the present experiment) these studies were not all performed in Syrian hamsters injected daily with melatonin nor did their tissues sampled contain the arcuate region.

It has been suggested that some of the neurochemical and endocrine effects of melatonin administration are similar to those produced by short-photoperiod exposure (Reiter, 1980). Previous studies utilizing melatonin treatment for 10 weeks in female hamsters showed significant melatonin-induced reductions in DA levels in the ME/arcuate region and the posterior pituitary (Alexiuk and Vriend, 1991). Short-photoperiod induced reductions in DA concentrations have been demonstrated in several publications including: after 11 and 10 weeks treatment respectively, in the hypothalamus (Steger et al., 1982; 1984); following 11.4 weeks in the MBH and MPOA-SCN (Steger et al., 1985b); after 9 and 12 weeks in the MBH/ME (Benson, 1987) and at 12 weeks short-photoperiod exposure in the ME (Krajnak et al., 1992 Abs.; Krajnak et al., 1994a). Nunez and investigators showed a decrease in DA concentrations as early as 28 days (4 weeks) after beginning experimental exposure of male hamsters to short days (Nunez et al., 1994 Abs.). Thus the present results suggest that short photoperiod and melatonin injections have similar effects on DA neurons of the MBH.

NE levels of the ME/arcuate region (Fig. 2) was increased after 6 weeks of melatonin treatment in the present experiment. Investigators have demonstrated short-photoperiod induced increases in hypothalamic NE after 15 and 17 weeks of treatment (Steger et al., 1982) in the Syrian hamster. Steger and colleagues, on the

other hand, also showed a short-photoperiod decrease in ME NE concentrations following 11.42 weeks (Steger et al., 1985b). Benson (1987), found that exposure of male hamsters to short days elevated NE levels in the MBH/ME region as early as 3 weeks. However, after longterm exposure, short-photoperiod reduced NE in the same region (Benson, 1987). The effects of melatonin or short photoperiod on tissue levels of NE (elevations or reductions) appear to be dependent on the duration of treatment and the specific regions examined.

Previous investigations have demonstrated that melatonin (the principal substance studied in this thesis) has significant effects on both catecholamine and 5-HT concentrations and metabolism (Alexiuk and Vriend, 1990 Abs.; Vriend et al., 1990; Alexiuk, 1991 - MSC Thesis; Alexiuk and Vriend, 1991; 1993b). The hypothesis that melatonin alters the activity of TH was first proposed in the early 1990's (Alexiuk and Vriend, 1991). The present studies provide information on the effects of melatonin on 5-HT and catecholamine turnover.

4.2 EXPERIMENT TWO: TIME COURSE OF NSD-1015

The NSD-1015 method facilitated the examination of the temporal and circadian effects of melatonin administration on the **in situ** activity of the rate-limiting enzymes tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH), monoamine concentrations and turnover.

The methodology of administering hydrazine compounds in order to inhibit

the activity of the AAAD enzyme has been implemented for several decades (Porter et al., 1962; Hempel and Mannl, 1968; Cegrell et al., 1970; Bedard et al., 1971; Carlsson et al., 1972; Carlsson and Lindqvist, 1973; Demarest et al., 1979; Demarest and Moore, 1980). In recent years, the drug NSD-1015 has been described as an important pharmacological agent that can be used to measure the **in situ** activity of TH - the rate-limiting enzyme in catecholamine synthesis (Nagatsu et al., 1964; Levitt et al., 1965). In actuality, this AAAD inhibitor has been used to facilitate the estimation of the activities of TH and TPH simultaneously (Carlsson et al., 1972), since the enzyme AAAD is apparently identical in both catecholamine and 5-HT synthetic pathways respectively (Cooper et al., 1991).

The precursors L-DOPA and 5-HTP are not detectable via HPLC, under normal conditions; when the AAAD enzyme is not inhibited by NSD-1015 (Cooper et al, 1991). The high activity of the AAAD enzyme, which causes it to turn over so quickly, may explain why endogenous levels of L-DOPA and 5-HTP are not detectable (without pharmacological intervention) (Cooper et al., 1991). However, administration of NSD-1015 at a particular time prior to animal sacrifice, causes L-DOPA and 5-HTP to accumulate (Carlsson et al., 1972; Carlsson and Lindqvist, 1973), and allows for their electrochemical detection via HPLC.

Other methods have been utilized to measure brain monoamine turnover or synthesis. Investigators who have demonstrated the effects of short-photoperiod exposure on catecholamine synthesis (Steger et al., 1982; 1985b; 1986; Benson, 1987; Steger and Bartke, 1991), have used an alternate method for measuring DA and NE

turnover. They administered the drug α-methyl-para-tyrosine (α-MPT), a TH inhibitor; then calculated the turnover rate from the exponential decline curve (or depletion) of the neurotransmitter. One of the problems with this method is that an assumption of the existence of steady-state conditions has been made by the investigators throughout the course of these experiments. However, one should not presume that steady-state conditions are maintained in long term short photoperiod investigations or those using melatonin injections for several weeks. An experiment performed by Fang and Dubocovich (1990), who administered acute injections of melatonin agonists, may have utilized the α-MPT method more appropriately.

The results of this second experiment demonstrated that the optimal time for administration of NSD-1015 in this particular species (Syrian hamster) was approximately forty (40) minutes prior to the time of expected sacrifice. This was ascertained via an examination of the linearity of both L-DOPA and 5-HTP concentrations (of the ME/arcuate region of the MBH and of the striatum) 0, 30 or 60 minutes following the administration of NSD-1015. The data showed that concentrations of L-DOPA of the MBH (Fig. 4) and of the striatum (Fig. 6) increased with time until 30 minutes. Hypothalamic 5-HTP concentrations, however, continued to increase linearly with time after NSD-1015 administration - peaking at 60 minutes (Fig. 5; Fig. 7) especially in the ME/arcuate region. The evidence in this particular experiment, which originally proposed to measure both TH and TPH activities concurrently, suggested that 40 minutes was the most optimal and feasible time to accomplish both goals simultaneously in the Syrian hamster. Therefore,

following this NSD-1015 time course study, 40 minutes was selected and consistently utilized in the various experiments comprising this thesis.

As expected, NSD-1015 administration resulted in highly significant reductions in the tissue levels of metabolites (HVA, DOPAC, 5-HIAA) in both the ME/arcuate region as well as in the striatum (Tables 1; 2; 3). It might also be expected that the drug would decrease concentrations of both the catecholamines and 5-HT, since only vesicular transmitter (old and stored) would remain following NSD-1015 administration. Although the drug did significantly reduce ME/arcuate 5-HT levels (Table 2) and nigrostriatal DA concentrations (Tables 3), evidence for this was not as predominant as that of the NSD-1015-induced decrease in monoamine metabolites. Cessation of synthesis was confirmed by the detectability of both L-DOPA and 5-HTP in tissue samples of the drug-treated animals; while these precursors could not be measured in saline-treated hamsters. The precursors L-DOPA and 5-HTP are normally immeasurable by HPLC without the implementation and administration of decarboxylase inhibitors (Cooper et al., 1991).

Although NSD-1015 is primarily used to inhibit the AAAD enzyme, minor monoamine oxidase (MAO) inhibiting capacities of NSD-1015 have also been reported (Carlsson et al., 1972). Studies which have addressed the issue of the MAO inhibiting ability of the drug have been characteristically in vitro experiments.

4.3 EXPERIMENT THREE: TEMPORAL SEQUENCE OF MELATONIN (with NSD-1015)

Previous studies have suggested that administration of melatonin alters catecholamine levels and synthesis in the MBH and posterior pituitary (Alexiuk and Vriend, 1990 Abs.; Alexiuk and Vriend, 1991). The hypothesis that melatonin may be affecting the activity of the rate-limiting enzyme of catecholamine synthesis, TH, was also postulated, at that time (Alexiuk and Vriend, 1991). Four dopaminergic systems are examined in this experiment - the tuberohypophyseal (THDA) (posterior pituitary - NIL), the tuberoinfundibular (TIDA) (median eminence), the nigrostriatal (caudate nucleus; striatum) and the mesolimbic (amygdala). Also, the cell bodies of the major monoaminergic neurotransmitters (DA, NE and 5-HT) are represented by the midbrain and pontine brainstem.

4.3.1 Neurointermediate Lobe (Posterior Pituitary)

The present study is the first to show significant temporal effects of daily late afternoon administration of melatonin on the **in situ** activity of TH in the neurointermediate lobe (NIL) of the pituitary. The present results have demonstrated that melatonin treatment for 3 weeks induced highly significant decreases in the activity of TH of the NIL and this was associated with a reduction in the content of NE. However, these melatonin-induced effects were no longer significant after 5 weeks (Alexiuk and Vriend, 1993a; Alexiuk et al., 1993 Abs.). DA

content of the pituitary NIL, on the other hand, was progressively decreased by melatonin; and reached significance at 5 weeks of treatment. This melatonin-induced reduction in THDA levels is consistent with the data of Experiment 1 showing a decreased ME/arcuate DA caused by 6 weeks of melatonin treatment (Fig. 1). The current study provides data suggesting that melatonin may be a regulator of catecholamine synthesis/release and metabolism in the NIL.

The occurrence in the present investigation, of a concomitant decrease in the accumulation of L-DOPA ($p < 0.001$) and NE content ($P < 0.05$) in the NIL of the pituitary after 3 weeks of melatonin treatment, is consistent with the interpretation that melatonin inhibited TH activity in noradrenergic neurons that enter this lobe. Noradrenergic fibers of the posterior pituitary (NIL) have been demonstrated to include those with central as well as those with peripheral (derived from sympathetic neurons of the superior cervical ganglia) origin (Alper et al., 1980). The lack of a significant melatonin-induced inhibition of TH activity or of a decrease in NE content after 5 weeks of melatonin administration raises the question of whether compensatory mechanisms that serve to enhance TH activity had been activated before this time. On the other hand, the present data also suggest that the temporal effects of melatonin on NE and DA turnover (and on TH activity) may be different - and perhaps in opposite directions.

This temporal sequence study (Exp. 3), has provided strong evidence that melatonin administration progressively decreased DA levels (remaining after inhibition of synthesis) of the posterior pituitary (Fig. 9). After 5 weeks of daily late

afternoon melatonin treatment, the DA content of the NIL was reduced to less than 50% of saline-treated controls ($p < 0.01$) - concomitantly with a melatonin-induced decrease in testicular weights ($p < 0.01$). The detection of testicular atrophy at 5 weeks of treatment (Fig. 11) verified the physiological effectiveness of melatonin (Reiter, 1980).

The lack of a significant effect of melatonin on the **in situ** activity of pituitary TH after 5 weeks, occurred at a period when differences in DA content of control and of melatonin-treated hamsters were most significant. These data suggest that the melatonin-induced decrease in DA stores in the NIL were not due to an inhibition of DA synthesis in THDA axons. Since all of the animals in this study were treated with the decarboxylase inhibitor, NSD-1015, before sacrifice, the tissue DA content measured would not include newly synthesized DA. The present data raise the issue of whether melatonin treatment decreased NE turnover, and increased DA turnover concurrently.

The NIL is composed of two distinct dopaminergic systems. The perikarya of the tuberohypophyseal (THDA) system are predominately localized in the rostral portion of the arcuate nucleus and terminate in the neural and intermediate lobes of the pituitary (Bjorklund et al., 1973). Dopaminergic neurons originating from the arcuate nuclei and terminating in the neural lobe of the pituitary, lack autoreceptor regulation of DA synthesis and release (DA receptor mediated modulatory mechanisms). These axons of the THDA system resemble tuberoinfundibular (TIDA) neurons in this respect. However, THDA neurons terminating in the

intermediate lobe do respond to D2 autoreceptor activation and possess a reuptake mechanism (Munemura et al., 1980); therefore they have a greater similarity to the nigrostriatal and mesolimbic dopaminergic systems (Lookingland et al., 1985; Koulu et al., 1989; Cooper et al., 1991). Although Lookingland and colleagues (1985) have reported finding higher levels of DA in the intermediate lobe than in the neural lobe of the rat pituitary, these investigators demonstrated no significant differences in turnover rates. Their data suggested that in the rat, the basal activity of THDA are similar in both areas (Lookingland et al., 1985). This is important in the context of the present study since it is very difficult to separate the neural and intermediate lobes of the pituitary in the Syrian hamster.

It has been reported that melatonin is a potent inhibitor of DA release in selected tissues including the rabbit retina (Dubocovich, 1983) and the rat hypothalamus (Zisapel et al., 1982). The present data suggests that melatonin may be modulating DA release and/or turnover in THDA axons - assuming that melatonin has a similar mechanism of action in various brain regions. Alterations in neuronal firing rates is one mechanism whereby melatonin may be affecting the synthesis/release of neurotransmitters. Naranjo-Rodriguez and colleagues (1991), reported the effects of acute melatonin administration on spontaneous multiunit activity in various brain regions of the rat. They demonstrated that the most significant inhibitory changes were observed in the amygdala and the rostral hypothalamus - all doses of melatonin administered elicited a decrease in electrical activity in both of these areas. Other brain regions, especially the mesencephalic

reticular formation and the caudate nucleus, were shown to have an increase in impulse flow (electrical activity) in response to low doses of melatonin, while a decrease was demonstrated with higher doses (Naranjo-Rodriguez et al., 1991). The work of several investigators also demonstrated that melatonin changes the electrical activity in the SCN of the rat (Shibata et al., 1989; Stehle et al., 1989) and in that of the Syrian hamster (Rusak and Yu, 1993). If melatonin treatment alters neuronal firing in TIDA and THDA systems in the present study, it would be expected to change (increase or decrease) rates of DA synthesis and/or release.

The catecholamine, DA, is considered to be a major neurotransmitter that is involved in the regulation of the secretion of the hormones of the neural (Bridges et al., 1976; Holzbauer et al., 1978; Alper and Moore, 1982; Holzbauer et al., 1983) and intermediate (Tilders and Smelik, 1977; Farah et al., 1982; Holzbauer and Racke, 1985) lobes. However, a physiological role for a melatonin-induced decrease in THDA in the modulation of the hormones of the neural lobe of the posterior pituitary (oxytocin, vasopressin) or of the intermediate lobe (αMSH, ß-endorphin), remains to be determined.

THDA neurons are apparently responsive to alterations in sodium or osmoreceptor activation (Alper and Moore, 1982). The evidence that melatonin is involved in modulating vasopressin release, diuresis or water-intake has been reported by at least one group of researchers at present. Yasin and colleagues (1993), documented that melatonin inhibited (in a dose-dependent fashion) both basal and stimulated vasopressin and oxytocin release from the rat hypothalamus in

vitro. These investigators also suggested that melatonin may be altering water balance through modulation (inhibition) of vasopressin release (Yasin et al., 1993). It is interesting to speculate that melatonin's effects on DA and TH activity of the NIL could be causally related to changes in vasopressin and oxytocin secretion.

The present data supports the concept that DA axons of the A12 arcuate region of the hypothalamus are subject to regulation by melatonin. An alternative explanation for these data is that melatonin acts on neurons which synapse on dopaminergic axons distributed from the A12 arcuate region of the hypothalamus (ie. serotoninergic and/or GABAergic neurons).

4.3.2 Median Eminence/Arcuate Region

The present study provides data suggesting that melatonin may be a major regulator of catecholamine synthesis and release in both the NIL of the pituitary and the ME/arcuate region of the MBH. Since it is well-documented that THDA and TIDA neurons take origin from the arcuate nuclei of the MBH, the ME/arcuate region was the second area examined in order to determine the temporal effects of melatonin administration on TH activity and neurotransmitter concentrations. Even though the data of the ME/arcuate was not quite as striking as that of the NIL - there were some important similarities demonstrated between them.

Evidence for a melatonin-induced inhibition of TH activity in the ME/arcuate region was demonstrated after only 1 week of melatonin treatment - but not at 3 or 5 weeks. Congruent with the data of the NIL, this decrease in TH occurred

concomitantly with reductions in tissue levels of NE. The present data are consistent with the interpretation that inhibition in TH activity in melatonin-treated hamsters may predominantly reflect decreases in catecholamine synthesis in noradrenergic neurons. Investigators have demonstrated short-photoperiod induced decreases in NE turnover (using the α-MPT method) in various brain regions of the Syrian hamster. The following reductions in NE turnover were reported after: 11.4 weeks (ME and MBH) (Steger et al., 1985b), 12 weeks (ME and MPOA-SCN) (Steger et al., 1986) and 9 and 12 weeks (MBH/ME) (Benson, 1987) in Syrian hamsters exposed to short days. In fact, Steger and Bartke (1991) found a short photoperiod induced decrease in NE turnover (with α-MPT) at 4 and 8 weeks in the ME; and as early as 1, 2 and 4 weeks in the MBH and anterior hypothalamus. Vriend and collaborators (also using α-MPT) reported a melatonin-induced decrease in NE turnover in the MBH (Vriend et al., 1990). Daily late afternoon administration of melatonin for 10 weeks reduced NE accumulation after pargyline in the ME/arcuate region (Alexiuk and Vriend, 1991), in the amygdala and in the pons (Alexiuk and Vriend, 1993b) of ovariectomized hamsters. These reported studies are consistent with the present time course of melatonin action experiment which suggests that the indole is inhibiting TH activity and NE synthesis (turnover) early in the course of treatment.

A significant reduction of DA levels ($p < 0.05$) in the ME/arcuate region of melatonin-treated hamsters was demonstrated after 5 weeks of administration of the indole. This was consistent with the data of the NIL; melatonin also decreased DA

concentrations of the posterior pituitary at 5 weeks. The reduction in TIDA levels (similar to the melatonin-induced decrease in THDA concentrations) did not occur concomitantly with changes in TH activity. This suggests that the melatonin-induced decrease in DA stores of the ME/arcuate region after 5 weeks of treatment, were not due to an inhibition of TH activity nor to a reduction of synthesis in TIDA neurons.

This temporal sequence experiment has demonstrated the initial effects of melatonin treatment - not the changes that occur at the prototypic time used to ensure testicular regression - that is (8-10 weeks). However, a significant reduction in testes weight of melatonin-treated hamsters was demonstrated as early as 5 weeks (Fig. 11). This raises the question of the physiological significance of the melatonin-induced reduction of both TIDA and THDA levels at 5 weeks - concurrently with gonadal involution.

The issue of melatonin's effects on circulating levels of hormones may be of importance in the interpretation of the present data (Reiter, 1980). Changes in the amount of circulating prolactin reaching the ME/arcuate region reportedly could also influence DA turnover of the A12 axons. The concept of prolactin regulation of TIDA activity (and vice versa) was originally demonstrated by Hokfelt and Fuxe in 1972. For many years, it was believed that THDA neurons, on the other hand, were unresponsive to changes in prolactin. However, the hypothesis that THDA neurons may be involved in the regulation of prolactin secretion has existed for over a decade (Ben-Jonathan and Peters, 1982; Lookingland and Moore, 1985). A recent study has

also suggested that DA neurons may be regulating prolactin directly within the posterior pituitary itself (Steger et al., 1994 Abs.), however this data remains controversial at present.

One interpretation of the present study is that increased serum prolactin, could feedback on TIDA neurons to elevate TH activity (Moore, 1987). The present data of melatonin-induced reductions in TH activity at 1 week could be adequately accounted for, if prolactin levels were decreasing as early as 1 week of treatment. The initial melatonin-induced inhibition of ME/arcuate TH activity (after 1 week) could be explained by an attenuation of prolactin-induced positive feedback on TH (Tuomisto and Mannisto, 1985; Arbogast and Voogt, 1991) - if this occurred in dopaminergic neurons. Steger and colleagues (1994 Abs.) have demonstrated depressed serum levels of prolactin following only 1 week of short-photoperiod exposure and previously after 4 weeks of treatment (Steger and Bartke, 1991). However, this interpretation remains speculative, since it has not been ascertained whether the decrease in TH activity after 1 week of melatonin treatment, occurred in noradrenergic neurons rather than in TIDA terminals. In fact, the present data suggested that the melatonin-induced inhibition of MBH TH activity (at 1 week) could, in actuality, have occurred in NE-containing cells.

The present experiment demonstrated a significant effect of melatonin on TPH activity (5-HTP accumulation after NSD-1015) in the ME/arcuate region after 5 weeks of treatment (Fig. 15; 16). This melatonin-induced increase in TPH activity occurred concomitantly with reductions in TIDA and THDA concentrations. This

suggests the possibility that melatonin-initiated changes in DA levels may be related to melatonin-induced effects on serotoninergic fibers projecting to DA neurons. Serotoninergic terminals have been shown to synapse on catecholaminergic neurons in the ME/arcuate region (Kiss and Halasz, 1986) and could account for the present data. Melatonin-induced alterations in 5-HT metabolism have been demonstrated in several studies in the Syrian hamster (Vriend, 1991; Alexiuk and Vriend, 1991; 1993b). Similarly, Steger and colleagues found short-photoperiod induced (after 10 weeks) increases in the activity of MBH TPH using the NSD-1015 method (Steger et al., 1990).

An alternate hypothesis is that melatonin's major effect could be on GABAergic neurons which synapse on dopaminergic systems in the arcuate region (Tappaz et al., 1985). A melatonin-induced increase in the activity (or turnover) of GABAergic neurons could provide a major inhibitory or modulatory control.

4.3.3 Other Brain Regions

No significant temporal (1, 3 or 5 weeks) effects of melatonin administration could be demonstrated in the midbrain (which contains the cell bodies of the mesolimbic and nigrostriatal dopaminergic systems) or the caudate nucleus (which contains the terminals of the nigrostriatal dopaminergic system).

The fact that melatonin increased NE concentrations following 5 weeks of treatment in the amygdala (Fig. 17), however, suggests that the effects of the indole are not limited to the MBH (Alexiuk and Vriend. 1993b).

In the pontine brainstem, melatonin administration resulted in a overall elevation in 5-HT levels which reached significance after 5 weeks ($p < 0.01$) of treatment. A melatonin-induced decrease in 5-HT release, concurrent with no change in synthesis (TPH activity), is one interpretation that could explain the increase in intracellular pontine 5-HT concentrations in animals injected with the indole. Melatonin decreased 5-HIAA levels at 1 week treatment in the pons. The melatonin-induced reduction in the 5-HIAA/5-HT ratios at this time, suggests a decrease in the oxidative metabolism in brainstem 5-HT. A reduction in oxidative metabolism is often associated with reduced transmitter turnover (Smythe et al., 1982a; Kuhn et al., 1986), however, no significant effect of melatonin on TPH activity was demonstrated in this region.

The fact that significant melatonin-induced temporal effects were demonstrated in the pons and amygdala suggests that melatonin's action is not limited to the mediobasal hypothalamic/pituitary complex. The data showing significant effects of this indole on dopaminergic, noradrenergic and serotoninergic neurons in different brain regions also suggests that melatonin's effects are not restricted to one neurotransmitter system.

4.4 EXPERIMENT FOUR: CIRCADIAN STUDY

4.4.1 Posterior Pituitary (NIL) of Circadian Study

After 9.5 weeks of treatment, melatonin significantly decreased THDA content, with no significant effects on TH activity. This was consistent with the data of the previous temporal sequence study which showed melatonin-induced reductions in DA levels of the posterior pituitary after 5 weeks of treatment with no differences in TH activity between melatonin-treated animals and saline-injected controls (Alexiuk and Vriend, 1993a). In the current circadian study, the diurnal variation of DA content in the NIL of control hamsters is similar to that reported in the posterior pituitary of the rat (Barden et al., 1982). In both experiments, DA levels were highest during the light period (Fig. 21). Koulu and collaborators also found that DA levels of the intermediate and posterior lobes of the rat pituitary rose shortly after the onset of the light period (Koulu et al., 1989). The present data are consistent with the interpretation that melatonin disrupted a 24-hr rhythm in THDA content. No significant effect of melatonin upon tissue NE levels, however, could be detected after 9.5 weeks of treatment.

Data showing a highly significant melatonin-induced decrease in water intake after 8.5 weeks (Fig. 22) of treatment compared to saline-treated controls, again raises the question of the melatonin-induced modulation of release of hormones of the posterior pituitary (vasopressin, oxytocin). Effects of melatonin on vasopressin and oxytocin secretion have been demonstrated by at least one group of investigators

(Yasin et al., 1993). It has been suggested that DA of the NIL is the principle transmitter involved in the modulation of posterior pituitary hormone secretion (Bridges et al., 1976; Holzbauer et al., 1983; Holzbauer and Racke, 1985). However, a potent role for melatonin in the maintenance of water balance or blood pressure regulation has yet to be proven.

4.4.2 Median eminence/Arcuate region

The data of the present circadian experiment are the first (to my knowledge) to demonstrate highly significant effects of melatonin on TH activity in the hypothalamus of the male Syrian hamster. Nine and a half weeks of melatonin administration markedly increased this enzyme's activity in the ME/arcuate region of the MBH throughout the 24-hr light/dark cycle (Fig. 23). A comparison of the temporal sequence study and the present circadian experiment suggests that the melatonin-induced effects the activity of TH are changing over time. After 1 week, TH activity of the ME/arcuate region in melatonin-treated hamsters was demonstrated to be 76% of controls; while at 3 weeks, the activity of the enzyme in animals treated with the indole was 92% of saline-injected animals. The activity of MBH TH in melatonin-treated hamsters was shown to be 124% of controls after 5 weeks. Following 9.5 weeks, melatonin increased the activity of the enzyme to 149% - compared to saline-injected hamsters.

These highly significant melatonin-induced elevations in TH activity in the MBH, observed after 9.5 weeks of treatment, were associated with an overall

decrease in TIDA levels throughout the 24-hr period of measurement ($p < 0.001$). Consistent with the effect on DA observed in the NIL of the pituitary, melatonin administration produced an overall significant reduction in the concentrations of TIDA of the ME/arcuate region. Unlike the NIL (where reductions in THDA content did not occur concurrently with apparent changes in pituitary TH activity), the reductions in TIDA levels took place concomitantly with highly significant increases in TH activity. This could indicate important physiological differences between these tissues (MBH and NIL). The axons and terminals of the THDA neurons entering the NIL comprise two functional dopaminergic systems (Lookingland et al., 1985); specific fibers terminating in the intermediate lobe and those which enter the neural lobe. Compensatory mechanisms (autoreceptor stimulation and/or reuptake) may be activated in the more classical dopaminergic system represented by the intermediate lobe. Perhaps this could account for the differences in melatonin-induced effects on TH activity of the NIL and ME/arcuate region after 9.5 weeks. However, the fact that both THDA and TIDA neurons take origin from the same nucleus - the arcuate perikarya - suggests that melatonin may be producing its major effects in this region.

Since both THDA and TIDA neurons originate from cell bodies in the arcuate nuclei (adjacent to the ME) this structure may well represent a major physiological site of action for melatonin - even if this is not consistent with all of the high-affinity binding data presently published. Malpaux and colleagues (1993) have demonstrated that the administration of microimplants of melatonin into the arcuate nuclei of

sheep produced significant alterations in LH and prolactin release. These investigators concluded that the MBH (including the arcuate region) could represent an important site of action for melatonin in the regulation of seasonal reproductive and neuroendocrine function (Malpaux et al., 1993). A subsequent study provided additional evidence which suggests that melatonin binding sites in the pars tuberalis are not controlling the photoperiodic effects of melatonin on hormone (LH, prolactin) release (Malpaux et al., 1994). The reports of Malpaux and colleagues (1993; 1994) suggesting a site of action for melatonin within the arcuate region are consistent with the evidence presented in the current investigation - which supports the interpretation that the ME/arcuate region of the MBH represents an important site of melatonin action.

The highly significant melatonin-induced elevations in TH activity of the ME/arcuate region, which occurred concomitantly with marked overall reductions of TIDA concentrations, suggest the interpretation that melatonin increased DA turnover (synthesis/release) after 9.5 weeks of administration. No significant changes in DOPAC concentrations could be detected at this time suggesting that melatonin's effect on ME/arcuate DA levels were not due to an increase in DA metabolism. Therefore, a reduction in intracellular TIDA content would be consistent with an elevated rate of transmitter turnover (synthesis/release). This data is not congruent with the reports that short photoperiod exposure for 11.4 weeks (Steger et al., 1985b), 12 weeks (Steger et al., 1986), 9 and 12 weeks (Benson, 1987) or 8 weeks (Steger and Bartke, 1991) decreased DA turnover in the ME (Steger et al., 1985b; 1986; Steger

and Bartke, 1991) and the MBH/ME region (Benson, 1987) of the male Syrian hamster - using the α-MPT method. However, the differences in experimental protocol/methodology as well as the assumption of steady-state conditions made by these investigators, may have contributed to the incongruity with the conclusions of the present study.

Since all of the animals in the current circadian study were treated with AAAD inhibitor, NSD-1015, 40 minutes before sacrifice, TIDA concentrations would not reflect newly synthesized DA but rather intracellular DA remaining after inhibition of synthesis. The minor MAO-inhibiting capacity of NSD-1015 (Carlsson et al., 1972), may serve to impede the catabolism of this intracellular DA and to further stabilize TIDA levels. The concentrations of DA of the ME/arcuate region in the present study, could have been modified by different rates of synthesis and release, prior to the administration of NSD-1015. Hence, these data are consistent with the interpretation that 9.5 weeks of melatonin administration in intact male Syrian hamsters increased the rates of TIDA synthesis and release (turnover) concomitantly with marked testicular regression ($p < 0.001$). The question of whether these two events are causally related (directly or indirectly) is addressed in the final experiment of this thesis (Gonadectomy Experiment).

The suggestion that 9.5 weeks of melatonin treatment may be enhancing DA turnover in this circadian experiment, is supported by the data (Fig. 27) showing a melatonin-induced increase in the HVA/DA ratio. It has been suggested that the ratio of HVA/DA is an adequate indicator of DA turnover or metabolism. The

interpretation of enhanced release of DA (by melatonin) in the present experiment would be consistent with reports of decreased serum and pituitary prolactin in hamsters treated with melatonin for several weeks (Reiter, 1980; Goldman et al., 1981), since DA is a well-documented prolactin-inhibiting factor (Ben-Jonathan, 1985). It is generally accepted that important regulatory interactions between prolactin and TIDA occur (Moore, 1987).

The present study also provided evidence for a highly significant ($F = 71.04$) increase in the L-DOPA/DA ratio in the ME/arcuate region (Table 21) of melatonin-treated hamsters. This ratio is expressed in order to adjust (or correct) for differing amounts of DA in variable tissue punch dissections. The L-DOPA/DA ratio is used as an indication of DA synthesis rates per DA terminal (Altar et al., 1987; Molina-Holgado et al., 1994) or the 'fractional synthetic rate'. Since the L-DOPA/DA ratios were markedly elevated in melatonin-treated hamsters in the present circadian experiment, this provides further support for the interpretation that the indole increased DA turnover in the ME/arcuate region after 9.5 weeks.

The question of melatonin's mechanism of action in the present study is an important one. Previous investigators have demonstrated melatonin-induced alterations in neuronal firing rates in the various brain regions of the rat including the hypothalamus (Naranjo-Rodriguez et al., 1991) and in that of the Syrian hamster (Rusak and Yu, 1993). It is recognized that increased TH activity (catecholamine synthesis) can occur in response to an elevation in catecholaminergic neuronal activity (Masserano and Weiner, 1983). Melatonin-induced modulation of neuronal

impulse flow could represent one mechanism that can account for changes in TH activity (Weiner et al., 1978), catecholamine synthesis (and release), metabolism and intracellular neurotransmitter concentrations found in this fourth experiment.

An additional explanation of these data is that decreased end-product inhibition by catecholamines (Okuno and Fujisawa, 1991) enhanced TH activity in order to compensate for low intracellular content of DA (Masserano and Weiner, 1983). Regulation of TH activity in TIDA nerve terminals is apparently uncomplicated by mechanisms that are operative in other dopaminergic systems, since factors are released directly from the ME region into the hypothalamohypophyseal portal system rather than into a synaptic cleft. The uniqueness of the TIDA neurons is reflected in their lack of autoreceptors modulating synthesis and release as well as in their characteristic absence of a protein-regulated reuptake mechanism (Wolf and Roth, 1990). In light of the relative simplicity of the TIDA system, the reduced DA levels observed in the melatonin-treated hamsters of the present study, would be consistent with the interpretation of a melatonin-induced increase in TIDA turnover.

The data in Fig. 24 and Fig. 26 provide information on day/night differences in the amounts of DA and its acid metabolite, HVA, remaining after cessation of synthesis (via NSD-1015). In control hamsters, concentrations of both DA and HVA were significantly elevated during the dark phase of the daily light/dark cycle, suggesting the presence of daily variations in DA metabolism or release in the ME/arcuate region of the MBH. The data of Fig. 24 could be interpreted as

evidence that melatonin administration markedly reduced peak nighttime concentrations of DA. The greatest significance between DA levels of control and melatonin-treated hamsters was observed at 2 AM, 8 hours into the dark phase of the light/dark cycle. Other investigators have demonstrated that the highest concentrations of DA occurred during darkness in several brain regions of the rat (Owasoyo et al., 1979) including the hypothalamus (Bhaskaran and Radha, 1984). Together, the DA and HVA data suggest that melatonin injections may have altered the 24-hr pattern in DA metabolism by several hours. The DA variations may be an important reflection of the circadian sensitivity to melatonin administration; however, a cause-effect relationship to melatonin-induced changes in pituitary and in peripheral levels of hormones has not been established.

Melatonin-induced alterations in circulating hormones (Reiter, 1980; Alexiuk and Vriend, 1991), raises the question of the possible effects of changes in concentrations of these peripheral factors on TH activity and on DA metabolism. Increased serum prolactin, for example, could feedback on TIDA neurons to increase TH activity (Moore, 1987) and release DA (Gudelsky and Porter, 1980; Gudelsky, 1981). Normally, this prolactin-regulating mechanism of TIDA neuronal activity is believed to be characterized by a tight coupling of synthesis to release. Hence, dramatic changes in vesicular DA are not usually apparent because strong compensatory measures are implemented to prevent the occurrence of deficiencies in the transmitter stores (Roth et al., 1975). This, however, is not consistent with data showing that after several weeks of melatonin treatment, serum and pituitary

prolactin levels are decreased (as well as TIDA concentrations) compared to controls (Reiter, 1980; Alexiuk and Vriend, 1991).

Based on experiments performed in the rat (Kizer et al., 1974), low circulating levels of testosterone would be expected to increase TH activity in the ME. While melatonin treatment is known to reduce circulating levels of testosterone (Reiter et al., 1976; Reiter, 1980) in the hamster, this does not explain the concomitant melatonin-induced alterations in ME/arcuate TH activity and reductions in TIDA and THDA levels in the circadian study. The question of whether melatonin-induced testicular involution is related to these alterations in the TIDA and THDA systems is addressed directly in the results of the final GX experiment.

Hypothyroidism is another condition that theoretically could increase TH activity (Kizer et al., 1974). While melatonin administration does reduce circulating levels of T4 in the hamster (Vriend and Reiter, 1977) this, (like thyroidectomy), might be consistent with the elevations in TH found in the present circadian study.

The hypothesis that melatonin acts directly on catecholamine neurons has been implied throughout the design of the circadian study. However, an alternate interpretation of the data is that melatonin influences the activity of neurons that synapse on dopaminergic cells - characterized by axons which are distributed to the ME of the MBH and NIL of the pituitary (Vincent et al., 1982). The hypothalamic periventricular-arcuate region is an area in which GABAergic neurons do, in fact, synapse on TIDA neurons, providing a major inhibitory control (Tappaz et al., 1985). Thus, melatonin could modulate DA synthesis/release in TIDA neurons, indirectly,

by altering turnover and/or by activating GABA receptors (Coloma and Niles, 1988; Rosenstein et al., 1989; Rosenstein and Cardinali, 1986; 1990).

The data of the present investigation have demonstrated highly significant melatonin-induced reductions in GABA levels of the ME/arcuate region (Fig. 30). Concentrations of the GABA precursor (and excitatory transmitter) glutamate (Fig. 31) as well as MBH glutamine (Fig. 32) were also significantly decreased in melatonin-treated hamsters. The present data provides strong evidence for a role of melatonin in the regulation of GABAergic neurons of the MBH. This suggests that melatonin does, in fact, alter GABAergic function (turnover, release?) in the ME/arcuate region. GABA, it should be noted, influences the secretion of adenohypophyseal hormones including prolactin, GH, gonadotropins and thyrotropin (Vijayan and McCann, 1978; Racagni et al., 1982; Vincent et al., 1982; Casanueva et al., 1984).

Investigators have also suggested that GABAergic-dopaminergic interactions occur in the vertebrate retina (Zawilska and Nowak, 1992). Kazula and collaborators demonstrated that GABA plays a predominant role in the biosynthesis of DA and melatonin in the chick retina (Kazula et al., 1993). The GABA 'A' agonist, muscimol, was reported to inhibit DA synthesis during the light period; while a chloride channel blocker (picrotoxin) stimulated DA synthesis in the dark phase (Kazula et al., 1993).

Melatonin administration was shown to have significant effects on both GABA (Fig. 30) and DA (Fig. 24) neurons (of the MBH) in the present circadian study.

The fact that evidence is accumulating for GABAergic regulation of dopaminergic systems in the retina lends credence to the interpretation that melatonin's effects on TH activity (Fig. 23) and DA turnover in the ME/arcuate region could be secondary to the indole's effects on GABA (or vice versa).

While the current data provided evidence for a 24-hr variation in DA levels that were altered by melatonin treatments, there was no statistical evidence for diurnal changes in NE levels in the MBH of control hamsters after administration of the AAAD inhibitor, NSD-1015. Significant effects of time of day on NE concentrations in melatonin-treated hamsters (Fig. 25) suggested that intracellular levels of NE were related to the interval following the last injection of melatonin rather than to an endogenous 24-hr rhythm. NE concentrations increased to a maximum approximately 15.5 hours after the final late afternoon injection of the indole (concomitantly with elevated TH activity; Fig. 23). These data suggest that melatonin administration may have a pharmacological effect on noradrenergic neurons. Also, an overall melatonin-induced increase in NE levels (as demonstrated in the present study) could be consistent with the interpretation that melatonin decreased NE release. Modulation of NE release could regulate LHRH secretion in the ME/arcuate region.

Melatonin-induced changes in serotoninergic neurons synapsing on DA neurons (Kiss and Halasz, 1986) could also account for the present data. Melatonin-induced alterations in 5-HT metabolism has been demonstrated in several studies (Vriend, 1991; Alexiuk and Vriend, 1991; Alexiuk and Vriend, 1993b) in the hamster.

Although not quite as striking as the TH and DA data, the present study also provided evidence for a significant melatonin-induced elevation of TPH activity. This was demonstrated by a melatonin-induced increase in the accumulation of 5-HTP following administration of NSD-1015. This elevation in TPH activity reached significance at 8 AM (4 hours after the beginning of the light period), concomitantly with a melatonin-induced increase in 5-HIAA levels ($p < 0.05$). Daytime melatonin induced elevations in hypothalamic 5-HIAA concentrations have been previously documented in animals not treated with NSD-1015 (Vriend, 1991, Alexiuk and Vriend, 1991; Alexiuk and Vriend, 1993b). The data of the present circadian experiment suggest that late afternoon melatonin treatment is related to an increase in serotoninergic neuronal activity (synthesis and metabolism) at the commencement of the light period. The fact that melatonin increased intracellular 5-HT levels at 2 AM, compared to saline-treated controls, suggests that melatonin may be modulating 5-HT release. An increase in 5-HT concentrations is consistent with the interpretation of a melatonin-induced inhibition of nighttime 5-HT release. Cassone and collaborators reported a melatonin-induced increase in nighttime 5-HT levels in various brain regions of the chicken including: the hypothalamus, thalamus and retina (Cassone et al., 1983; 1986c). Chuang and colleagues documented a melatonin-induced decrease in hypothalamic 5-HT release in the rat (Chuang et al., 1993).

The present data suggests that the alterations in serotoninergic neuronal activity may related to the phase of the light/dark cycle. The concept that brain 5-HT levels are strongly influenced by exogenous (environmental) light or darkness has

been demonstrated by several investigators (Ferraro and Steger, 1990; Rao et al., 1990). The present data provide evidence that serotoninergic activity could be related to the state of 'internal' darkness generated by late afternoon melatonin administration.

4.4.3 Striatum

The nigrostriatal system is a more classical and complex dopaminergic system in nature. It is characterized by the presence of synthesis and release-modulating autoreceptors as well as a high-affinity protein reuptake mechanism. This particular dopaminergic system served as a excellent source of comparison for both the THDA and the TIDA neuronal systems in the present circadian experiment.

A melatonin-induced elevation in TH activity during the 8 PM time period occurred concurrently with increased DA concentrations (in melatonin-treated animals) suggesting an increased rate of DA synthesis. (No significant effects of melatonin on the DA metabolites DOPAC or HVA were detected in this region). This concomitant melatonin-induced elevation in both DA and its precursor L-DOPA, however, was no longer significant by 2 AM - indicating a possible circadian aspect to melatonin's effects of nigrostriatal TH activity. This may also be the result of the presence of a stronger coupling of synthesis/release rates to intracellular DA content in the nigrostriatal system. Hence, melatonin's effects on DA in the striatum may not be as prominent as that of the ME/arcuate region due to the activation of compensatory regulatory mechanisms which serve to maintain constant

intra/extracellular DA concentrations in the nigrostriatal dopaminergic system.

One interpretation of the present results (showing a concurrent elevation in TH activity and DA levels) is that the melatonin-induced increase in intracellular DA content at 8 PM, may have activated the process of end-product inhibition between the time period of 8 PM and 2 AM; resulting in a decrease in TH activity. A generally accepted neurochemical principle is the concept of reversible inactivation by the end-product - especially in the context of a regulatory mechanism for enzyme (TH) activity (Okuno and Fujisawa, 1991). Striatal DA may have reduced as well as stabilized TH activity (Okuno and Fujisawa, 1991). An increase in end-product inhibition by catechols typically activates the low affinity state of the enzyme TH. A decrease in the activity of the enzyme would therefore be a plausible result (at 2 AM), since high affinity forms of TH bind the cofactor tetrahydrobiopterin (BH4) more efficiently than the low affinity state. One conclusion can be made with confidence. The melatonin-induced effect on striatal TH activity is reflective of synthesis occurring in DA neurons since little NE can be detected within this region.

In addition, autoreceptor regulation of TH affinity states cannot be ruled out at this time. However, this must be approached with caution since melatonin-induced alterations in presynaptic and/or postsynaptic nigrostriatal DA receptor sensitivity (or density) has not presently been demonstrated.

The alterations that occur in TH activity and nigrostriatal DA turnover may be related to the last injection of melatonin relative to the time of sacrifice. Hence, animals killed at approximately 8 PM would have received the final administration

of the indole at 4-5 PM. Naranjo-Rodriguez and colleagues demonstrated acute effects of melatonin on electrical activity in the caudate nucleus; an increase in electrical activity occurred in response to low doses of melatonin in this region (Naranjo-Rodriguez et al., 1991). Perhaps, the elevation in TH activity and DA concentrations at 8 PM reflect an increase in neuronal activity in the striatum of melatonin-treated animals.

Owasoyo and colleagues (1979) have argued that the elevations that occurred in nighttime nigrostriatal (caudate n.) DA levels may be related to increased motor activity which characterize the activity rhythms of nocturnal rodent species. The present investigation demonstrating the 24-hr effects of melatonin administration on striatal DA would be consistent with the data and interpretation of Owasoyo and colleagues (1979). Significant melatonin-induced elevations in TH activity occurred concurrently with a nighttime (8 PM) rise in nigrostriatal DA levels of male Syrian hamsters. Similar to the rat - the Syrian hamster is also a nocturnally active species.

A melatonin-induced increase in striatal TPH activity occurring during the 2 PM time of sacrifice preceded the significant melatonin-induced elevation in 5-HT concentrations which was demonstrated at 8 PM. In fact, melatonin treatment increased 5-HT levels in the striatum throughout the 24-hr time period as detected by ANOVA (Fig. 36; $p < 0.01$). These data may be consistent with the interpretation that melatonin treatment decreased the release of 5-HT in the caudate n. of the Syrian hamster. A melatonin-induced decrease in striatal 5-HT release has been previously documented by Chuang and colleagues (1993) in the rat.

It is difficult to explain the significant effects of melatonin found in the striatum in the present study, since most of the high-affinity melatonin binding data points to the region of the hypothalamic/pituitary complex. Evidence for some melatonin binding in the striatum (caudate n.), however, has been demonstrated in at least one study in the rat (Chavez et al., 1991) - suggesting the possibility of a direct effect of the indole in this region.

4.5 EXPERIMENT FIVE: GONADECTOMY STUDY

4.5.1 Posterior Pituitary (NIL)

The present data demonstrates that melatonin treatment for 9.5 weeks in GX hamsters significantly decreased DA content of the NIL as it did in intact hamsters. This melatonin-induced reduction in DA levels occurred concomitantly with no significant change in the activity of pituitary TH. The present data suggest that this melatonin-induced decrease in intracellular DA of the NIL is not due to an inhibition of synthesis in THDA terminals. This interpretation was strengthened by the evidence showing that the L-DOPA/DA ratio (an indication of the fractional synthetic rate) was increased (Table 32) in melatonin-treated hamsters compared to saline-injected controls.

Melatonin treatment also had no detectable effects on NE concentrations (concurrently with no effect on TH). Similar results were obtained previously in the NIL of intact Syrian hamsters treated with melatonin for 9.5 weeks (Table 18). The

posterior pituitary data in this GX experiment is consistent with the interpretation that melatonin's effects on THDA concentrations are not secondary to melatonin-induced changes in circulating levels of testosterone.

4.5.2 Median Eminence/Arcuate Region

The circadian experiment demonstrated a highly significant decrease (Table 20; $p < 0.001$) in testes weight of intact hamsters after 9.5 weeks of melatonin treatment; an observable phenomenon which occurs concomitantly with marked reductions in serum testosterone levels (Reiter, 1980). It is well-documented that gonadal steroids affect hypothalamic neurotransmitter activity (McEwen, 1976; 1980; Gunnett et al., 1986; Toney et al., 1991). Decreased testosterone levels have been shown to increase ME TH activity in the rat (Kizer et al., 1974). Toney and collaborators (1991) also reported that elevated levels of testosterone inhibited the activity of TH in TIDA neurons (Toney et al., 1991) of the male rat. The present GX experiment has created a GX male Syrian hamster model in order to control for the possible effects of testosterone on catecholamine concentrations and TH activity.

Since the previous data (Alexiuk and Vriend, 1994) of the circadian experiment showed that the most significant effect of melatonin on DA levels ($p < 0.001$) occurred during the dark phase of the light/dark cycle, the animals in the present GX study were sacrificed at nighttime. Congruent with the data in intact animals, melatonin treatment significantly elevated ME/arcuate TH activity in GX male hamsters (Fig. 38; $p < 0.001$). This occurred concomitantly with a highly

significant melatonin-induced decrease in nighttime tissue DA (Fig. 39) levels (as was also previously shown in intact animals). Since all of the hamsters were castrated in this study, the effects of melatonin on TH activity and TIDA concentrations (see chromatograms in Fig. 42) could not be secondary to melatonin-induced changes in circulating levels of testosterone.

The present data would also be consistent with the interpretation that melatonin elevated DA (synthesis/release) turnover in the ME/arcuate region. This is supported by the evidence showing a highly significant ($F = 33.40$) increase in the L-DOPA/DA ratio in melatonin-treated hamsters compared to saline-injected controls (Table 33). A melatonin-induced increase in DA synthesis and release from TIDA neurons is one logical conclusion. It is also consistent with the data showing reduced serum prolactin levels after several weeks of melatonin treatment (Reiter, 1980) since DA is considered to be a major prolactin-inhibiting factor (Ben-Jonathan, 1985).

Hence, the data of this GX experiment replicate and confirm the predominant results that were previously demonstrated in the circadian investigation. In the present GX experiment, MBH concentrations of DA (remaining after cessation of synthesis), was significantly decreased in castrated melatonin-treated hamsters compared to saline-injected GX controls. In addition to markedly reducing nighttime DA levels, melatonin administration significantly decreased HVA concentrations (at this time of sacrifice) in the ME/arcuate region of GX hamsters. These data are consistent with the interpretation that melatonin treatment modified DA metabolism

during the dark phase of the 24-hr light/dark cycle.

Consistent with the data of the circadian experiment, melatonin treatment had no significant effect on NE concentrations in GX hamsters at this time of sacrifice.

Administration of melatonin in castrated hamsters significantly elevated nighttime 5-HT concentrations of the ME/arcuate region, compared to saline-treated controls. These data are congruent with that of the 2 AM time period in the circadian experiment when melatonin also significantly elevated MBH 5-HT levels. The GX investigation demonstrates a significant melatonin-induced elevation in 5-HT concentrations with no differences in TPH activity between melatonin-treated animals and saline-injected controls. These data are consistent with the interpretation that melatonin decreased the release of 5-HT during this time period (nighttime).

These data raise the question of whether the melatonin-induced effects on hypothalamic catecholamines are direct ones or whether they are secondary to melatonin-induced effects on ME/arcuate serotoninergic fibers synapsing on TH-containing neurons (Kiss and Halasz, 1986). Central administration of 5-HT in rats was reported to decrease TH activity in TIDA neurons (Mathiasen et al., 1992). Since 5-HT has been demonstrated to be involved in stimulation of prolactin-releasing factor (Clemens et al., 1978; Van de Kar and Bethea, 1982: Van de Kar et al., 1989), the interpretation of a melatonin-induced decrease in 5-HT release is consistent with the reduction that occurs in serum prolactin levels following several weeks of melatonin treatment (Reiter, 1980; Alexiuk and Vriend, 1991). A melatonin-induced decrease in hypothalamic 5-HT release has been demonstrated

by Chuang and colleagues (1993) in the rat and was suggested as an interpretation in a previous experiment performed in the female Syrian hamster (Alexiuk and Vriend, 1991).

The present study has provided unequivocal evidence that the highly significant effects of melatonin on ME/arcuate TH activity and DA concentrations are not secondary to a melatonin-induced reduction in serum levels of testosterone. However, the difficult question of whether the indole acts directly on MBH catecholaminergic neurons, remains to be determined. The fact that melatonin has been shown to have significant effects upon GABAergic neurons (Vriend and Alexiuk, 1995; in press) and 5-HT-containing cells of the ME/arcuate region, raises the issue of whether melatonin acts directly upon serotoninergic and/or GABAergic neurons which synapse on the TIDA system. Also, the question of the effects of melatonin-induced alterations in circulating thyroid hormones (Vriend and Reiter, 1977; Vriend, 1983; Vriend and Steiner, 1988) on ME/arcuate TH activity and TIDA levels has not yet been addressed. The complexity of melatonin's neuroendocrine and neurochemical effects are reflected in the fact that melatonin's action does not appear to be limited to just one hormone or to a single neurotransmitter system.

4.6 GENERAL DISCUSSION

Daily late afternoon melatonin administration resulted in decreased levels of DA in the MBH as early as 3 weeks of treatment. Following longterm injections of

the indole, DA concentrations were reduced in both the ME/arcuate region of the MBH and the NIL of the pituitary. The reduction in TIDA and THDA was associated with a marked increase in TH activity of the ME/arcuate region. This melatonin-induced elevation in TH activity was not due to changes in circulating levels of testosterone. The data suggest that melatonin increased DA turnover in neurons originating from the arcuate nuclei. The greatest effect of melatonin on TIDA turnover occurred during the dark phase of the daily light/dark cycle. This suggests that the TIDA system has a circadian sensitivity to melatonin.

Melatonin administration was associated with a decrease in MBH and NIL NE levels early in treatment. This melatonin-induced reduction in NE was related to an inhibition of TH activity at 1 and 3 weeks. However, melatonin increased NE levels in the MBH by 5 weeks of treatment. Although there was no circadian rhythm detected in MBH NE of saline-treated hamsters after 9.5 weeks, the increase in NE concentrations were related to the time of the last melatonin injection. This data suggests that melatonin may have an acute pharmacological effect on noradrenergic neurons.

Serotonin synthesis (TPH activity) of the MBH was increased as early as 5 weeks by melatonin treatment. At this time, 5-HT levels of the pons was also significantly elevated by melatonin. Melatonin increased daytime TPH activity in the MBH and striatum after 9.5 weeks. A significant melatonin-induced increase in nighttime MBH and striatal 5-HT concentrations was also observed. These data suggest that 5-HT synthesis and release are subject to regulation by melatonin in

various brain regions.

Decreased daytime GABA and glutamine concentrations of the MBH were observed after 9.5 weeks of melatonin treatment. The data suggest that melatonin modulates turnover in GABAergic neurons. Further experiments are necessary to determine whether the melatonin-induced changes in catecholamine turnover are secondary to alterations in firing of GABAergic neurons.

5. CONCLUSIONS

1. This is the first study to demonstrate the significant temporal effects of daily melatonin injections on the **in situ** activity of TH in the posterior pituitary (NIL) of the male Syrian hamster. Melatonin administration resulted in a highly significant inhibition of pituitary TH activity at 3 weeks of treatment and decreased TH activity at 1 week in the ME/arcuate region - concomitantly with reductions in NE levels.

2. This temporal sequence experiment suggests that the early decreases found in TH activity of melatonin-treated hamsters may be more reflective of catecholamine synthesis occurring in noradrenergic neurons, since the reductions in THDA and TIDA levels (after 5 weeks) did not occur concurrently with changes in axonal TH activity.

3. Significant melatonin-induced increases in MBH TPH activity after 5 and 9.5 weeks of treatment (and the melatonin-induced elevation in MBH 5-HT concentrations after 9.5 weeks) demonstrate that 5-HT synthesis and/or release are also subject to regulation by melatonin.

4. The current study is the first to demonstrate the highly significant effects of melatonin administration for 9.5 weeks on TH activity over a 24-hr period in the ME/arcuate region. This melatonin-induced elevation in TH activity occurred

concomitantly with a significant reduction in TIDA and THDA levels. These observations support the interpretation that longterm melatonin administration markedly increased TIDA synthesis/release (turnover) in the ME/arcuate region at this time.

5. Melatonin injections altered the daily rhythms of TIDA and THDA content.

6. The data show that the length of treatment and time of sacrifice are important factors in melatonin's action on noradrenergic neurons. This suggests that the indole may have an acute pharmacological effect on noradrenergic neurons.

7. Significant melatonin-induced increases in nighttime MBH 5-HT suggest that melatonin may be modulating release in serotoninergic neurons. Decreases in GABA, glutamate and glutamine levels raise the question of whether the melatonin-induced increase in TIDA turnover is a direct one or is secondary to melatonin-induced changes occurring in neurons that synapse on catecholamine-containing neurons (ie. serotoninergic and/or GABAergic).

8. The melatonin-induced increase in nighttime DA synthesis (TH activity) and in 5-HT levels of the striatum suggest that the indole's potent regulatory effects on brain dopaminergic and serotoninergic systems are not limited to the MBH.

9. Nine and half weeks of melatonin treatment significantly elevated MBH TH activity concomitantly with significant reductions in TIDA and THDA concentrations in castrated hamsters. Because these melatonin-induced effects occurred in GX hamsters as well as intact hamsters, the data of the present investigation provide strong evidence for a role of melatonin in the regulation of dopaminergic systems originating in the arcuate nucleus. Since all of the animals were castrated in this GX study, melatonin's significant effects on TH activity and tissue DA concentrations could not be secondary to melatonin-induced changes in circulating levels of testosterone.

10. The significant effects of melatonin on the catecholamines (DA, NE), 5-HT, GABA, glutamate and glutamine in the present study, demonstrate that this indole's action is not limited to a single neurotransmitter system.

11. The data of the present study suggest that the ME/arcuate region and NIL may be important physiological sites of melatonin action on monoaminergic (and amino acid) neurotransmitters.

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7. APPENDIX A

7.1 SYNTHETIC PATHWAYS

7.1.1 Figure A1: Indoleamine Synthetic Pathway

tryptophan::::::::::::::::::::> 5-HTP::::::::::::::::::::> serotonin
(1) (2)

::::::::::::::::::::> N-acetyl 5-hydroxytryptamine::::::::::::::::::::>
(3) (4)

N-acetyl 5-methoxytryptamine (melatonin)

Key:
(1) tryptophan hydroxylase

(2) aromatic L-amino acid decarboxylase

(3) serotonin N-acetyl transferase

(4) 5-hydroxyindole-O-methyl transferase

7.1.2 Figure A2: Catecholamine Synthetic Pathway

tyrosine::::::::::::::::::::> DOPA::::::::::::::::::::> dopamine
(1) (2)

::::::::::::::::::::> norepinephrine
(3)

Key:
(1) tyrosine hydroxylase

(2) aromatic L-amino acid decarboxylase

(3) dopamine ß-hydroxylase