REGULATION OF PINEAL MELATONIN PRODUCTION BY SEX HORMONES

By

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THIS THESIS IS DEDICATED

TO MY MOTHER MRS. JIAN-RONG LI

AND MY FATHER MR. NAI-KUNG YIE

IN APPRECIATION OF

THEIR CONSTANT LOVE AND ENCOURAGEMENT

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Abstract

The effect of sex hormones on pineal melatonin production has been examined. The diurnal profile in urinary aMT6s excretion, which is believed to represent pineal melatonin production and 24 hour sex hormone excretion was determined by RIA in male and female rats at age of 3 weeks, 2 months, 8 months, 14 months and 20 months. A concomitant decrease in metabolite excretion corrected for body weight was found with increasing age together with a highly significant sex difference in aMT6s output at 3 weeks, 2 months and 8 months of age. Increase of body weight with age is an important factor responsible for the age-related alteration while the difference in sex hormone milieu between male and female animals could be one explanation for the sex difference in aMT6s excretion in younger rats.

The effect of castration on the diurnal profile in urinary aMT6s excretion was observed in both male and female rats at 3 weeks, 2 months and 8 months of age. Castration caused a fall in urinary aMT6s excretion in 3 week old female rats but increased the metabolite output in both 2 month and 8 month old female rats. In male rats castration reduced aMT6s excretion at 2 months of age but there was no significant effect at 3 weeks or 8 months of age. In addition, urinary aMT6s excretion varied with the stages of the rat oestrous cycle; the lowest excretion was observed at proestrus corresponding with the highest 24 hour urinary oestradiol excretion.

Sex hormone treatment was performed on 2 month old castrated male and female rats. Both testosterone treatment on male castrated rats and oestradiol on female castrated rats abolished the effects of castration on aMT6s excretion. Administration of oestradiol to male rats and testosterone to female rats decreased and increased urinary aMT6s excretion, respectively.

Castration had no significant effect on the half life of melatonin elimination from plasma either in 2 month old male and female rats or 6 month old male rats.

The effect of castration and sex hormone treatment on the pineal response to isoproterenol (ISO) was also investigated in 2 month old male and female rats. Castration increased aMT6s excretion in female rats but reduced aMT6s output in male animals in both time-course and dose-dependence studies. Changes in serum and pineal melatonin response to ISO with castration and sex hormone treatment were examined by an Elisa. Consistently, castration in female rats increased pineal and serum melatonin responses to ISO and oestradiol treatment to castrated female rats blocked the elevation, whereas in male rats castration decreased the responses and testosterone treatment to castrated male rats abolished the reduction.

The effect of sex hormones on pineal beta-adrenergic receptors were studied in 2 month old rats. The beta-adrenergic receptor binding was determined by using ^{3}H-dihydroalprenolol (DHA) in single pineal

glands. Castration in female rats increased the receptor density compared with oestradiol treated and sham-operated animals. By contrast, the receptor concentration in castrated male rats was lower than testosterone treated and sham-operated male animals.

The results of the present study demonstrate that sex hormones are involved in the regulation of pineal melatonin production; in 2 month old rats, oestradiol has an inhibitory while testosterone exerts a stimulating effect. The effects do not result from the alteration of circulating melatonin metabolism. One of the mechanisms is that sex hormones alter the pineal response to adrenergic stimulation and act on the pineal beta-adrenergic receptors.

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CHAPTER I

INTRODUCTION

The effects of the pineal gland and its hormone melatonin on mammalian reproductive function have been extensively studied (Arendt, 1986; Bittman, 1984; Brzezinski & Wurtman, 1988; Cassone, 1990a; Reiter, 1980; Stetson & Watson-Whitmyre, 1984). A reasonable hypothesis that pineal melatonin production may be, in turn, regulated by sex hormones has also been long postulated (Cardinali, 1980, 1984; Cardinali et al, 1987; Preslock, 1977; 1984). However, published data about sex hormonal regulation of pineal melatonin production are sometimes contradictory. The mechanisms of the hormonal feedback at the molecular level are also poorly understood. In this introduction reproductive effects of melatonin, underlying mechanisms of these effects, the biochemistry of melatonin synthesis and metabolism as well as the neural control of pineal melatonin production will all be briefly described. A more detailed review will be focused upon previous work on sex hormonal regulation of pineal melatonin production. Based upon the literature review the present study was designed to understand whether sex hormones have significant effects on pineal melatonin production and what are the possible underlying mechanisms.

1.1. Reproductive Effect of Melatonin

The pineal gland has been related to a large body of physiologic functions both in animals and human beings including the control of gonadal, adrenal and thyroid function, sleep, body temperature regulation and various biological rhythms (Mattews & Seamark, 1981; Reiter, 1981). Among these, physiological effects of the pineal

on the reproductive system have been intensively investigated. Although a number of indoles and peptides have been proposed to be instrumental in pineal effects on reproductive function, melatonin is generally regarded as the primary product from the pineal gland affecting gonadal function in a variety of species (Vaughan, 1981). The following sections will review the effects of melatonin on reproductive function in seasonal, nonseasonal animals and humans.

Effect of Melatonin on Gonadal Function in Seasonal Animals

Some seasonal breeding animals such as the hamster mate during the spring and summer and are infertile during the fall and winter; others such as the sheep display an opposite pattern. The patterns of reproduction in those seasonal breeding animals normally reflect the selective pressures invoked on each species. In other words, animals who are subjected to yearly fluctuations in ambient photoperiod, temperature, precipitation and food availability give birth at a time of year conducive to survival of parents and offspring. The pineal is now believed to be an essential component of the neuroendocrine system in the regulation of photoperiod response. Pineal melatonin production exhibits a circadian pattern with high levels during the dark phase under the dark:light cycle. The duration and amplitude of the nocturnal rise in seasonal breeders are synchronized with the prevailing dark:light environment as perceived by the retina and thus, with day-length changes throughout the year. These changes in the production pattern convey information about day-length from neural

components of the circadian system to control reproductive functions (Arendt, 1986; Bittman, 1984; Stetson & Watson-Whitmyre, 1984).

Indeed, the antigonadal effect of short photoperiod (the fall and winter) in the hamster is prevented by pinealectomy (Reiter, 1966); while gonadal inhibition can be achieved during long days (the spring and summer) by daily injections of melatonin (Stetson & Watson-Whitmyre, 1976). Later experiments showed that the time at which melatonin is administered relative to the prevailing light-dark schedule is very important since melatonin administration in late afternoon induces gonadal effects but is ineffective in the morning (Goldman, 1984; Tamarkin et al, 1976). In sheep, a short-day breeder, the time of the year at which reproductive activity occurs is also synchronized by the number of hours per 24 hour day that the pineal secretes melatonin (Bittman et al, 1983, 1985). However, melatonin in this species is apparently progonadal. Changes in the duration and amplitude of the nocturnal rise in melatonin secretion is as critical to melatonin 's role in sheep as it is in hamster (Foster et al,1986).

Reproductive Effect of Melatonin in Non-seasonal Animals

Although earlier reports of the antigonadal effects of melatonin were concerned primarily with rats (Alonso et al, 1978; Koning & Rega, 1978; Wurtman et al, 1963), the role of the pineal and its hormone is less well defined in this species. Rats are not

considered markedly photoperiodic by most investigators. However, there is evidence for some photoperiodism in the rat. Blinding produces a slight reduction in the size of rat testes (Reiter, 1968) and slows the growth rate of the ovaries (Reiter & Ellison, 1970); pinealectomy reverses the effects of blinding. However, the effects are much less than those in seasonal rodents such as hamster (Reiter, 1968). Hence, the reproductive system in rats has been considered to be relatively insensitive to pineal regulation.

Injection of large doses of melatonin in female rats completely inhibits ovulation and prevents luteinizing hormone (LH) release (Ying & Greep, 1973) and low doses suppress the ability of continuous light to accelerate ovarian growth and to cause persistent oestrus (Chu et al, 1964). In male rats, melatonin treatment decreases testis weight, spermatogenesis and plasma testosterone levels (Kinson & Peat, 1971; Konig & Rega, 1978; Mas et al, 1979). However, the inhibitory influence on the reproductive axis was more marked in young animals. Therefore, an age-dependent responsiveness of the reproductive system to melatonin with a pronounced reaction in prepubertal animals was proposed. It was further demonstrated that the responsiveness to exogenous melatonin was highly dependent upon the time at which it is given during the light: dark cycle (Lang et al, 1984) or upon the phase of the animal's oestrus cycle (Ying & Greep, 1973). Melatonin has been also shown to exert an inhibitory effect in

vitro on rat testicular (Ellis, 1972; Kano & Miyachi, 1976; Ng & Lo, 1988) or rabbit ovarian steroidogenesis (Younglai, 1979).

Effect of Melatonin on Human Reproduction

Since pineal tumours were associated with precocious puberty one century ago, the relationship between the pineal gland and gonadal function in humans has been recognized. However, the role of melatonin in human reproduction is still poorly understood. Previous published works focused on the role of melatonin in puberty and on correlations between the menstrual cycle and changes in serum melatonin levels. Most of these studies examined large subject populations throughout childhood and observed decreases in serum and urinary melatonin with puberty (Arendt, 1978; Gupta et al, 1983; Silman et al, 1979; Waldhauser et al, 1984; Wetterberg et al, 1976) although no significant correlation between melatonin secretion and sexual maturation was found by other workers (Ehrenkranz et al, 1982; Lenko et al, 1982). Based upon these findings a hypothesis that a gradual and prolonged decrease in pineal melatonin production at the onset of puberty might trigger the onset of pubescence has been proposed (Brzezinski & Wurtman, 1988). With determination of melatonin in serum and urinary melatonin concentration, it was found that melatonin levels changed with the cycle; it rises during the luteal phase and falls before ovulation (Hariharasubramanian et al, 1986; Webley & Leindenberger, 1986). Others demonstrated that the melatonin diurnal profile did not change significantly during the normal menstrual cycle

(Brzezinski et al, 1988). However, an elevated plasma melatonin levels in women with hypothalamic amenorrhea were reported in two recent studies (Berga et al, 1988; Brzezinski et al, 1988).

Interestingly, recent evidence has appeared that melatonin is present in human seminal plasma (Bornman et al, 1989; Oosthuzein et al, 1986; Yie et al, 1991) and follicular fluid (Brzezinski et al, 1987; Ronnberg et al, 1990; Webley et al, 1988) in relatively high concentrations. Melatonin levels were found to have a weak but significant correlation with sperm density and progressive motility (Oosthuzein et al, 1986; Yie et al, 1991) although this finding has been disputed (Bornman et al, 1989). Significant circadian and annual variations in melatonin concentration in follicular fluid and its high concentration suggest that melatonin could have a role in human reproduction.

Possible Mechanisms

The target sites and mechanisms of action of melatonin are only beginning to be identified. Since melatonin action seems to be a time signal through its circadian production pattern, a neural site for melatonin action has been strongly suggested. Several observations have indicated that melatonin most likely acts at the hypothalamic level, probably interfering with GnRH secretion (Lang et al,1983). It is well demonstrated in the ewe that high-frequency LH pulses are found in short-day (oestrus) and lower frequency LH but higher amplitude pulses in long-day (Karsch et al,

1984). In the rat melatonin treatment during the juvenile and pubertal periods decreased hypothalamic GnRH levels (Lang, 1986). It is thus assumed that melatonin acts, directly or indirectly, on the so called GnRH pulse generator (Arendt, 1986). Further evidence supporting this hypothesis is that specific melatonin binding sites have been identified by using ^{3}H-melatonin or ^{125}I-iodomelatonin (IMEL) in many specific regions of the central nervous system, primarily in the hypothalamus (Table 1) although there is a striking species difference in distribution (Morgan & Williams, 1989).

However, the hypothalamic neural circuits involved in the control of GnRH release are complex. At present it is unclear how melatonin exerts its action on the GnRH pulse generator through its putative receptors. Moreover, the pituitary has been also proposed to be an important target in melatonin control of reproductive function since administration of melatonin both in vivo and in vitro suppressed pituitary response to GnRH (Martin et al, 1977; 1980). It also reduces pituitary LH and follicular stimulating hormone (FSH) contents (Lang, 1986). Specific melatonin binding sites have been detected in the pituitary (Morgan & Williams, 1989). A recent in vitro study indicates that 33melatonin acts on its receptors in the pituitary to inhibit LH release, thereby stimulating the release of GnRH from the median eminence. Thus there is a negative short-loop feedback of LH to inhibit basal GnRH release (Nakazama et al, 1991). Together with

these results it seems that the reduction of weight of gonads and plasma sex hormone level by melatonin may be the result of decreased stimulation of the hypothalamic-pituitary axis.

However, melatonin could also exert a direct effect on the gonads. There is reported evidence for ^{3}H-melatonin binding in cytoplasmic fraction in gonads (Cohen et al, 1978) although this report was not corroborated by Vanecek (1988) using IMEL. Nevertheless, melatonin, as described above, is present in human preovulatory follicular fluid, it stimulated follicular secretion of progesterone in vivo and in vitro in humans (Webley et al, 1988) and monkeys (Webley & Heran, 1987) but blocked the stimulation of rabbit ovarian follicles by hCG (YoungLai, 1979). There also is evidence that melatonin inhibits rat testicular steroidogenesis in vitro (Ellis, 1972; Kano & Miyachi, 1976; Ng & Lo, 1988) and there is a significant negative correlation between melatonin concentrations in seminal plasm and sperm motility in humans (Oosthuizen et al, 1986; Yie et al, 1991), but no specific binding of melatonin has been detected in the human testes to date.

1.2 Biochemistry of Melatonin

Pineal Melatonin Biosynthesis

The biosynthetic pathway of pineal melatonin has been established (Fig.1 a) (Sugden, 1989). The indole amino acid tryptophan is the

Table 1

Central 2-^{125}I-melatonin Binding Sites Identified by in vitro Autoradiography

Laboratory rat	Hamster	Sheep	Human
	(Syrian)		
Suprachiasmatic nucleus (SCN)	SCN	SCN	SCN
Area postrema (AP)	Preoptic area (POA)	Hippocampus	
Pars tuberalis (PT)	Anterior paraventricular nucleus of the thalamus (APVT)	PT	
	Laternal habenular nucleus (LHN)		
	Habenular commissure		
	Deep pineal		
	Ventromedial nucleus (VMN)		
	Ventral tegmental area (VTA)		
	PT		
	Median eminence		
Median eminence	Anterior pituitary		
Choroid plexus			
Anterior pituitary			
	(Djungarian)		
	SCN		
	Median eminence		
	Medial habenular nucleus		
	Septum		
	Pituitary		
	(Djungarian – foetal)		
	SCN		
	Median eminence		
	Arcuate nucleus region		
	Pineal		
	Anterior pituitary		
	Preoptic area		

The data summerized from Morgan & Williams: Experientia 45: 955-965, 1989.

precursor of melatonin. Once taken up in the pinealocyte, this amino acid is converted into 5-hydroxytryptophan catalyzed by tryptophan hydroxylase (TH). 5-hydroxytryptamine (serotonin) is the product of the next step in the pathway achieved by the action of aromatic L-amino acid decarboxylase (dopa-decarboxylase). Serotonin in the pineal has a complex fate, but a major pathway for the metabolism of serotonin is its conversion to melatonin. Two enzymes achieve this conversion, N-acetyltransferase (NAT), which converts serotonin to N-acetylserotonin and hydroxyindole-O-methyltransferase (HIOMT), which catalyzes the transfer of a methyl group from S-adenosylmethionine (SAM) to the 5 hydroxy position of N-acetylserotonin. The product of the last reaction is melatonin (5-methyl-N-acetyltryptamine).

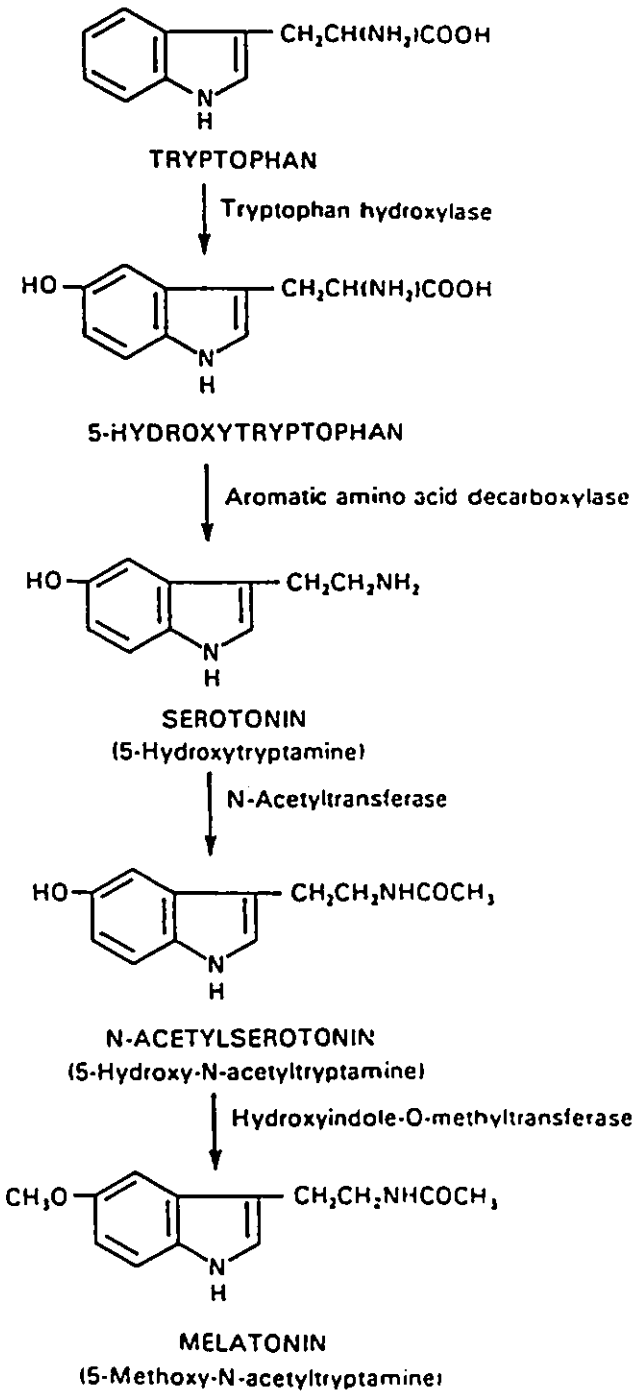
The production of melatonin within the pinealocyte requires the uptake of the tryptophan from the circulation. However, the role that the blood titre of tryptophan may play in determining melatonin production has not been resolved (Sugden, 1989). Pinealocytes possess all the enzymes that are required for melatonin synthesis. The action of TH requires the presence of O^2, ferrous ion and a reduced pteridine co-factor (Lovenberg et al, 1968) and this enzyme in the pineal is normally unsaturated (Reiter, 1989) suggesting that the rate of this enzyme activity depends upon the availability of tryptophan. Although dopa decarboxylase is a widely distributed enzyme, its activity in the pinealocytes is very high (Quay, 1974) so that serotonin

concentrations in the pineal exceed those of any other organ in the body. In addition, the pineal has a higher turnover rate for serotonin. This is due to metabolism of the pineal serotonin metabolized by monoamine oxidase (MAO) to 5-hydroxyindole acetaldehyde (Hakanson & Owman, 1965; 1966) or by HIOMT to 5-methyltryptamine (Prozialeck et al, 1978) rather than to melatonin. NAT is also a widely distributed enzyme but in the pineal it is the key enzyme in the control of the circadian rhythm of melatonin synthesis (Deguchi, 1975; Ellison et al, 1972). Unlike other enzymes, HIOMT is restricted to only a few organs such as the retina and the harderian gland (Cardinali & Wurtman, 1972) in addition to being present in the pineal.

Secretion

Unlike most transmitter and hormone systems, there are no specific storage or secretion mechanisms for melatonin. Once melatonin is formed in the pineal gland, it is immediately released by simple diffusion (Reiter, 1989). Therefore, it is not surprising that regulation mechanisms for melatonin levels are directed at the level of synthesis. Whether melatonin is primarily secreted into the blood stream or the cerebrospinal fluid remains controversial, but it does cross the blood brain barrier easily (Anton-Tay & Wurtman, 1969). It has been clearly demonstrated that circulating melatonin is mainly secreted from the pineal gland (Lewy et al, 1980).

Fig.1 (a). Biosynthesis of Melatonin



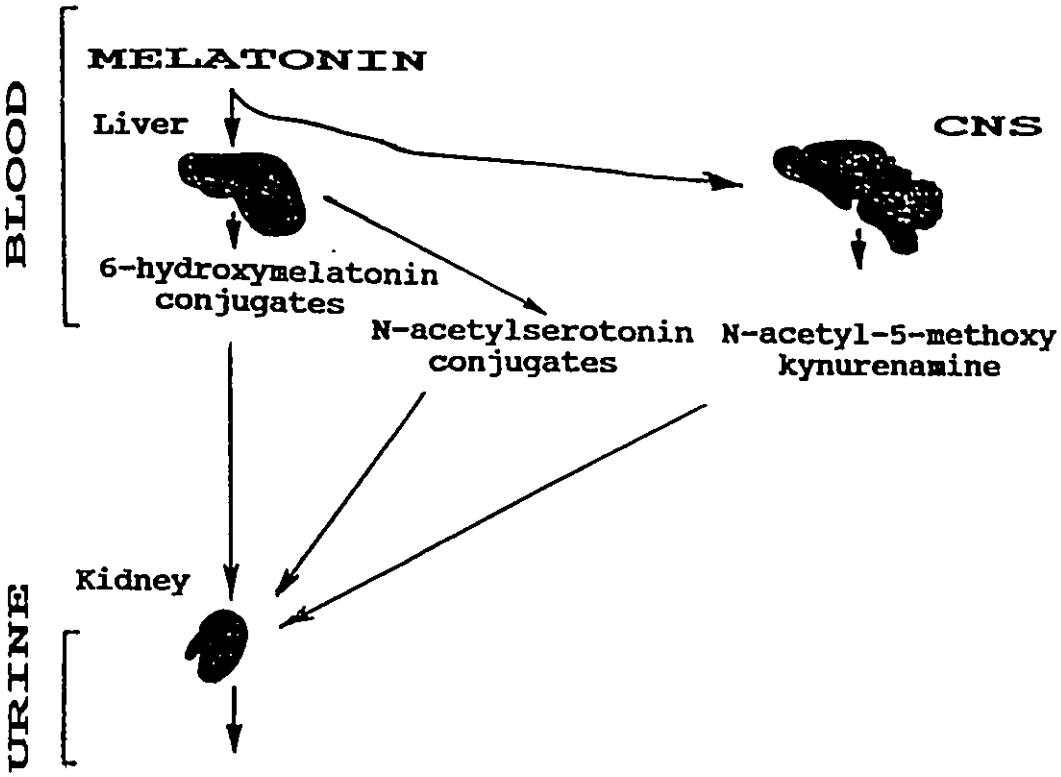
Distribution

Most melatonin in the plasma is bound to proteins especially albumin (Cardinali et al, 1972). Melatonin bound to albumin may have limited excretion via the kidney; usually less than 1% of exogenously administered melatonin escapes into the urine in unmetabolized form (Jones et al, 1969). Although administered melatonin could be taken up by a variety of tissues, a greater uptake was demonstrated in some specific tissues such as brain and ovaries (Quay, 1974), supporting the concept that these are target tissues for melatonin.

Metabolism (Fig.1b)

Melatonin is metabolized in at least two sites: the liver and the brain (Vaughan, 1984). In animals melatonin is rapidly metabolized in the liver to 6-hydroxymelatonin which is conjugated to sulfuric acid and excreted in urine (Kopin et al, 1961; Kveder & McIsaac, 1961). A small amount of melatonin in humans is converted to N-acetylserotonin in the liver and appears in the blood as a sulfate or glucuronide conjugate and excreted in the urine (Young et al, 1985). Melatonin taken up by the CNS is converted in part to N-acetyl-5-methoxykynurenamine, which, along with the hepatic metabolite of melatonin is excreted in urine (Reiter, 1990)

Fig.1 (b). Metabolism of Melatonin



1.3. Regulation of Pineal Melatonin Production

Neural Control

The most striking features of pineal melatonin production are its diurnal variation and relationship to environmental light. Under diurnal lighting conditions, melatonin concentrations in all species so far investigated are elevated during the dark phase of the light/dark cycle (Waldhauser & Wurtman, 1983). The melatonin rhythm persists in darkness and is suppressed by continuous illumination (Ralph et al, 1971). It is well established that the daily rhythm of melatonin production exhibited by the pineal gland is generated by an endogenous circadian clock in the suprachiasmatic nuclei (SCN) of the hypothalamus (Cassone, 1990b) which communicates to the pineal via a complex pathway with many components, e.g. the neuronal circuit retina-retinohypothalamic tract-suprachiasmatic nucleus-periventricular hypothalamus-internal carotid nerves-nervi conarii-pineal gland (Cardinali & Vacas, 1987). This pathway seems to be the primary neural control of pineal melatonin production (Cardinali, 1984). Rats subjected to bilateral superior cervical ganglionectomy (SCGx), ganglion decentralization or section of the internal carotid nerves exhibited an absence of the nighttime rise of melatonin (Moor, 1978; Wurtman et al, 1964), whereas the rise could be induced by electrical stimulation of the sympathetic trunk (Bowers & Zigmond, 1982). As shown in Fig.2, the retinal perception of darkness

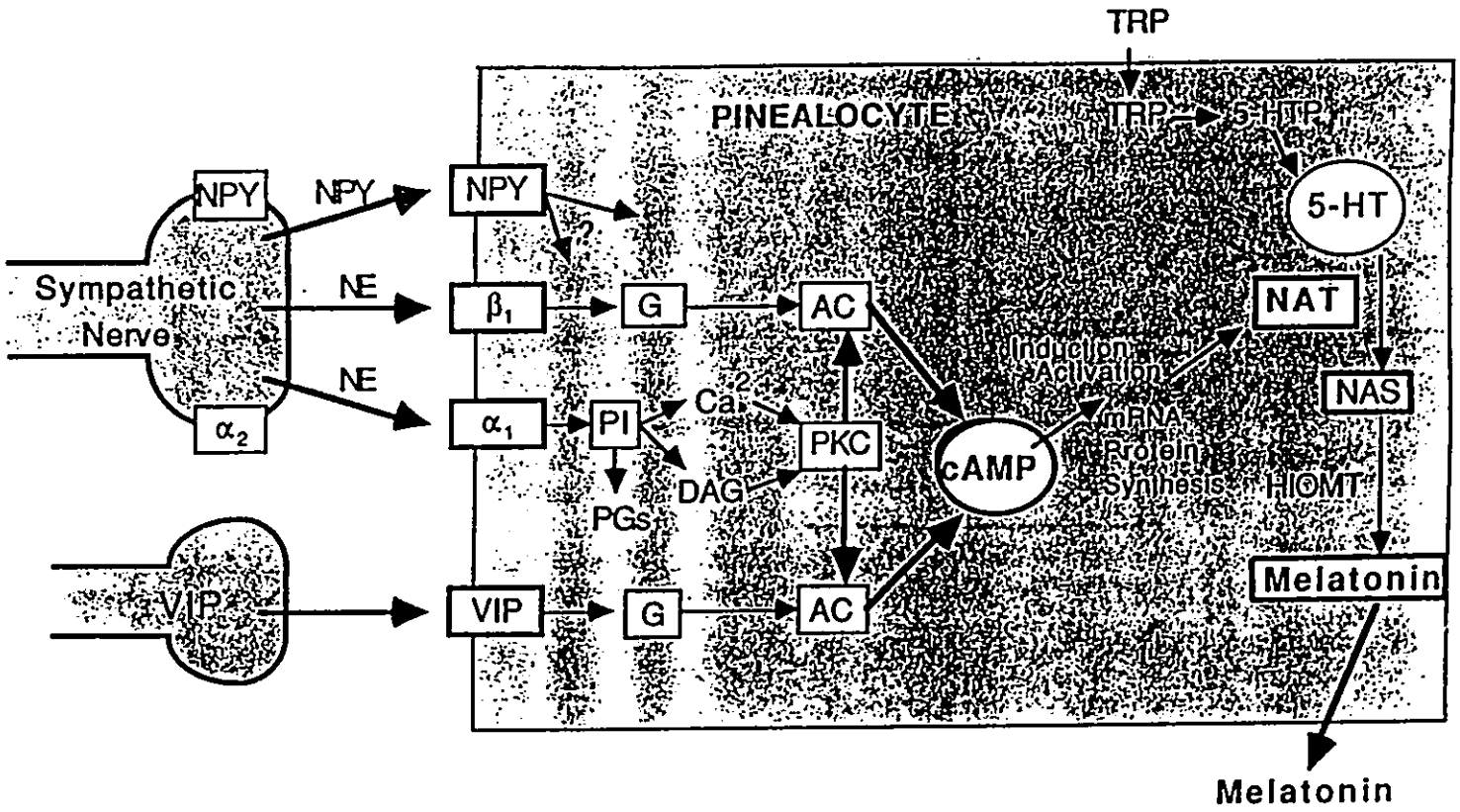


Fig. 2. *A model of receptors and second messengers underlying neural control of melatonin production in the rat pinealocyte. Induction and activation of* N-acetyltransferase *(NAT), the rate-limiting enzyme for melatonin synthesis, result from increases in cAMP. β_1-Adrenoceptors and vasoactive intestinal peptide (VIP) receptors independently stimulate cAMP production. The stimulation produced by either receptor is potentiated by α_1-adrenoceptors that are coupled to the phosphoinositide (PI) system. The α_1-adrenoceptors appear to cause potentiation of adenylate cyclase via calcium (Ca^{2+})-phospholipid-dependent protein kinase C (PKC) which is first activated by diacylglycerol (DAG) and Ca^{2+}. Neuropeptide Y (NPY) also appears to stimulate NAT activity, but it is not clear whether it activates adenylate cyclase (AC) directly or via potentiation of the β_1-adrenergic effect. Alpha$_2$-adrenoceptors and NPY receptors located presynaptically mediate feedback inhibition of sympathetic output. Other abbreviations: G, regulatory G protein; HIOMT, hydroxyindole-O-methyltransferase; 5-HTP, 5-hydroxytryptophan; NAS,* N-acetylserotonin; *NE, norepinephrine; PGs, prostaglandins; TRP, tryptophan.*

stimulates the sympathetic fibres innervating the pineal gland to release norepinephrine (NE) which acts upon beta-1-adrenergic receptors on the pinealocytes, which in turn stimulate cAMP production. cAMP triggers the induction as well as activation of NAT activity. Simultaneously, NE stimulates pinealocyte alpha-1-adrenergic receptor response through actions of the phosphoinositide system particularly protein kinase C (Sugden et al, 1985). Sympathetic fibres innervating the pineal have recently been shown to contain neuropeptide Y (NPY) (Shiotani et al, 1986) which also appears to enhance melatonin production (Reuss & Schroder, 1987; Vacas et al, 1987a). In addition to sympathetic nervous innervation, the pineal receives direct innervation from central neurons that send their fibres via the pineal stalk (Cardinali & Vacas, 1987). The functions and transmitters of these nerves are not well established. It was proposed that a number of transmitters, possibly released from the central pinealopetal fibres, may also have a role in fine-tuning melatonin production. GABA (and benzodiazepines), dopamine and glutamate may inhibit melatonin production, whereas VIP, opioids and delta-sleep-inducing peptide may stimulate this process. Receptor binding sites for these transmitters have also been demonstrated in the pinealocytes (Ebadi & Govitrapong, 1986).

Hormonal Modification

The possible involvement of gonadal steroids in pineal melatonin production has usually been examined by 1) removing the gonads; 2)

looking for a correlation between reproductive events and changes in melatonin in pineal, serum and urine; 3) treatment with exogenous sex steroids and 4) testing the effects of gonadal steroids on melatonin production by pineal organ culture. Using these approaches, a number of reports have been published during the past 30 years in a variety of species. The evidence is reviewed as follows:

Effects of Castration: In the early studies the effects of castration upon melatonin-forming enzyme activities were examined. Whether SNAT activity is affected by castration remains unresolved because of contradictory results (Cardinali & Vacas, 1978; Daya & Potgieter, 1982; Illnerova, 1975; Shivers & Yochim, 1979). Pineal HIOMT activity was markedly increased after castration in female rats operated during their prepubertal period (Alexander et al, 1970). In contrast, an inhibitory effect of castration in female adult rats was observed by two other reports (Daya & Potgieter, 1982; Wallen & Yochim, 1974). The discrepancy probably is due to neglect of the stage of the rat oestrous cycle since pineal HIOMT activity in ovariectomized rats was less than that in control cyclic rats at oestrus but higher than that at proestrus (Cardinali et al, 1974 b). A subsequent study dealing with changes in melatonin levels following castration reported that castration augmented nocturnal melatonin concentration in serum but not in the pineal gland of rats two months after surgery (Ozaki et al, 1978). Recent evidence showed that pineal melatonin response to adrenergic

stimulation in ovariectomized rats was blocked by oestradiol or progesterone (Moujir et al, 1990). In ewes, serum melatonin concentrations were greater in the ovariectomized group than those in controls, especially 16 days after surgery (Arendt et al, 1983). In male rats, castration decreased HIOMT activity (Nagle et al, 1974; Daya & Potgieter, 1982) as well as the pineal melatonin levels (Hernandez et al, 1990).

Changes of Pineal Melatonin Production during the Oestrous or Menstrual Cycle: Much information is available regarding the changes of pineal melatonin production during the oestrous or menstrual cycle in a wide variety of species including man. In rats a number of pineal constituents and enzyme activities related to melatonin synthesis were shown to vary as a function of the stage of the oestrous cycle including NE-induced increase of adenylate cyclase activity (Weiss & Crayton, 1970), cyclic AMP levels (Davis, 1978), HIOMT activity (Cardinali et al, 1974 b; Daya & Potgieter, 1982; Wallen & Yochim, 1974; Wurtman et al, 1965) and 5-HT and melatonin contents (Johnson et al, 1982; Moujir et al, 1990; Quay, 1974). Most of those studies demonstrated that these pineal constituents were significantly decreased at the proestrus when oestradiol levels were maximum. In addition, melatonin in rat urine was also shown to be the lowest during the proestrous stage (Ozaki et al, 1978).

However, the peak pineal melatonin levels occurring at 8 h after

the onset of the darkness were similar in magnitude on each day of the oestrus cycle in the female hamster (Rollag et al, 1979). Concurrently, the oestrous cycle of ewes did not modify plasma melatonin (Arendt et al, 1978; Rollag et al, 1978).

In women, one early study (Wetterberg et al, 1976) using a single early morning time point observed that plasma melatonin levels were the lowest at the time of ovulation, increased during luteal phase and peaked at the time of menstruation. This was confirmed by subsequent studies measuring 24 hour mean serum melatonin (Birau, 1986) or urinary melatonin levels (Penny, 1982). By contrast, a recent report using frequent blood sampling showed that the circadian rhythm in plasma melatonin was remarkably stable throughout the menstrual cycle; no significant preovulatory decrease in melatonin values was evident (Brzezinski et al, 1987). This was consistent with the observation that nocturnal urinary excretion of melatonin was not decreased at the time of ovulation (Brun et al, 1987). However, in amenorrheic women, both peak amplitude and duration of melatonin secretion were significantly higher than those in normal women (Berga et al, 1988; Brzezinski et al, 1988).

Although there are wide discrepancies among the above findings concerning a possible relationship between changes in oestradiol and melatonin levels during the menstrual cycle, it is consistently reported that melatonin secretion increases during the luteal phase

of the menstrual cycle. One early report showed that plasma melatonin rose to a peak coinciding with the mid-luteal progesterone peak (Arendt et al, 1978). Two recent studies measuring plasma secretion in frequent blood samples (Haribarasubramamian et al, 1986; Webley et al, 1984) further demonstrated this relationship. Urinary melatonin excretion was also found to be significantly higher during the luteal phase than that during the follicular phase (Brun et al, 1987).

The foregoing data suggest species difference in the hormonal regulation of pineal melatonin production between seasonal and non-seasonal breeders.

Changes of Melatonin during Pregnancy: Although systematic and detailed studies of pineal function during pregnancy are rare, the available evidence indicates that changes in pineal and plasma melatonin levels in pregnancy take place in a number of species. When the concentrations of melatonin in maternal peripheral plasma in human were measured during late pregnancy, the mean levels were higher during both term and pre-term labour (Mitchell et al, 1979). Pang et al (1984 b) also reported that plasma concentration was high in week 1-24, low in week 24-26 and then high again in week 36-42.

Fluctuations of the pineal melatonin content in the rat during different stages of pregnancy have been demonstrated (Pang et al,

1986). High pineal melatonin contents from about 36 h after mating were followed by generally lower levels in later periods of pregnancy. Pseudopregnant rats were reported to have a different diurnal pattern of HIOMT activity although there was no deviation in its mean rate (Yochim & Wallen, 1974).

Effect of Administration of Sex Hormones: Wurtman et al (1965) administered a single dose (10 µg/day) of oestradiol benzoate (EB) to mature, cyclic female rats and reported a significant decrease in the activity of HIOMT in the pineal gland. Subsequently, Alexander et al (1970) reported that administration of 50 µg/day EB abolished a stimulating effect of prepubertal ovariectomy on pineal HIOMT activity. However, in another study daily injections of 20 µg EB to ovariectomized female rats stimulated pineal HIOMT activity (Houssay & Barcelo, 1972). It was suggested that there might be a dose-dependent response of pineal HIOMT activity to oestradiol since low doses of oestradiol (0.01-1 µg/day) restored HIOMT to normal in ovariectomized rats, while higher doses (10 µg/day) decreased this enzyme activity (Daya & Potgieter, 1982).

Administration of progesterone was ineffective in reversing the norepinephrine-induced increases in pineal adenylate cyclase activity in rats (Weiss & Crayton, 1970). Houssay and Barcelo (1972) reported that progesterone (200 µg/day) decreased pineal HIOMT activity. Thus, a dose-dependent responsiveness of pineal HIOMT to progesterone was also postulated since progesterone (100

μg/day) slightly inhibited pineal HIOMT in ovariectomized rats while a dose of 10 μg/day increased this enzyme activity (Daya & Potgieter, 1982).

In Nagle's report testosterone propionate (0.1-1.0 mg/day) restored HIOMT activity in the pineal of male rats to that of intact controls, while higher doses of 5 mg/day administered to castrates decreased activity compared to controls (Nagle et al, 1974). This observation was confirmed by Daya and Potgieter (1982) who reported that testosterone (0.01- 1 mg/day) restored pineal HIOMT activity in castrated male rats to that of intact controls, while 10 mg/day decreased pineal HIOMT activity in castrates.

Plasma melatonin levels in normal women taking contraceptive pills were found to be no different from nonusers of the pill in one investigation (Beck-Friis et al, 1984), but levels were elevated in other studies (Brun et al, 1987; Tapp et al, 1980; Webley et al, 1984; Webley & Leindenberger,1986).

Effect of Gonadal Steroids in vitro: Evidence of the effect of sex hormones upon pineal melatonin production also comes from in vitro pineal gland culture studies although the results were also conflicting. Mizobe & Kurokawa (1976) reported that oestradiol significantly increased the HIOMT activity within 2 h after addition into cultures of the pineal glands obtained from ovariectomized rats; the extent of the increase was dose-dependent

within the concentration ranges from 0.1-15 nM, being increased by 80% at 15 nM. Enhancement of the enzyme activity was blocked by clomiphene citrate. This result differed from the report by Daya and Potgieter (1985) in which there was depression of ^{14}C serotonin conversion to melatonin in the rat pineal gland by 10^{-8} M oestradiol, but is analogous to the observation that 10^{-8} to 10^{-5} M oestradiol increased melatonin production in rat and guinea-pig pineal explants (Cardinali et al, 1987).

Progesterone alone, in vitro, inhibited the release of melatonin from the pineal gland of adult ovariectomized rats in one study (Wilkinson & Arendt, 1978), while no effect of progesterone at concentrations of 10^{-9} to 10^{-5} incubated with rat pineal explants was reported in another study (Cardinali et al, 1987).

Testosterone at 10^{-8} M concentration increased melatonin production in the pineal gland of male rats in vitro (Daya & Potgieter, 1985) whereas a significant decrease of rat pineal melatonin content in vitro was observed with 10^{-5} M of testosterone in spite oflittle or no effect upon the pineal melatonin content at 10^{-9} to 10^{-7} M of testosterone (Cardinali et al, 1987).

Effect of Gonadotropins and Prolactin: The experiments describing changes in pineal function following castration or administration of gonadal steroids obviously cannot exclude the possibility that the changes observed are due to changes in gonadotropin and

prolactin following the treatments. With administration of gonadotropins to castrated or hypophysectomized and injection of GnRH to castrated rats in vivo (Cardinali et al, 1976 a) or by observation of the effect of gonadotropins and prolactin in vitro on pineal organ culture (Cardinali et al, 1987; Vacas et al, 1987 b), it has been demonstrated that anterior pituitary hormones may play a substantial role in changing the amplitude of the daily melatonin rhythm as a function of the endocrine status. Moreover, recent data showed significant correlations between plasma melatonin concentrations and gonadotropins and prolactin in women (Fernandez et al, 1990). Receptors for prolactin in the pineal gland have been reported (Cardinali, 1983), but no information exists as yet on the occurrence of high affinity binding sites with the characteristics of receptors for gonadotropins in the pinealocytes.

Mechanisms of the Sex Hormonal Modulation: Two possible sites of sex hormonal regulation of pineal melatonin have been proposed, e.g. endocrine-endocrine and endocrine-neural transduction (Cardinali, 1984; Cardinali & Vacas, 1987). Endocrine-endocrine transduction may take place in the pineal gland since pineal receptors for sex hormones including oestrogen, androgens and progesterone have been identified in several mammals (Cardinali et al, 1974 a; Cardinali, 1983; Moeller et al, 1984; Stumpf & Sar, 1979; Vacas et al, 1979 b). The characteristics of the sex hormone receptors in the pineal gland were shown to be similar to

those in typical sex hormone targets (Cardinali, 1980; 1984). As in the uterus, hypothalamus and adenohypophysis, concentrations of the receptors and dynamic changes were regulated by sex hormones (Cardinali, 1977; Nagle et al, 1973). These data strongly suggest that the pineal gland is a target for sex hormones. Sex hormones have been shown to affect a variety of aspects of pineal function including RNA and protein synthesis (Cardinali et al, 1974 b; Mizobe & Kurokawa, 1976; 1978), serotonin and NE turnover rates (Cardinali et al, 1975 b; Vacas & Cardinali, 1979 a), beta-adrenergic receptor density (Foldes et al, 1983) and pineal HIOMT activity (Cardinali, 1980; Preslock, 1984). However, there is no conclusive evidence that those effects were achieved through the sex hormone-receptor mechanism in the pineal. Considerable progress has been made in the past few years in our understanding of the mechanisms of sex hormone action in classic sex hormone target tissues (King, 1988). Purified receptors and clonal genes have been achieved for androgen, oestrogen and progesterone receptors, which open up prospects for future investigations. Clearly if the actions of sex hormone receptors in the pineal gland were known, endocrine-endocrine mechanisms in pineal melatonin production could be clarified.

The concept of endocrine-neural transduction implies a primary site of action of the sex hormones on the neurons innervating the pineal gland (Cardinali, 1979). The SCG are feasible sites of action for sex hormone effects on the pineal melatonin production. Both

oestrogen and androgen receptors are detected in rat and bovine SCG (Cardinali, 1979; 1980). Oestradiol injection decreased tyrosine hydroxylase (TH) and dopamine-beta-hydroxylase (DBH) and testosterone treatment modified SCG NE synthesis in both newborn and adult rats. The mechanism by which sex hormones may affect these parameters is not well established.

Interestingly, pineal oestrogen and androgen receptors are under neural control, as shown by the depression or abolition of binding sites after pineal denervation. This phenomenon has subsequently been demonstrated in several other areas of the brain (Carrillo & Sheridan, 1980; Nock et al, 1981; Thompson et al, 1983).

From the above it is clear that the mechanisms of sex hormonal regulation of pineal melatonin production are complex. Sex hormones may affect melatonin synthesis indirectly by modification of the neural control mechanisms and directly by affecting the melatonin synthetic pathway in the pinealocytes. Both approaches are not mutually exclusive.

1.4. Melatonin Assays

Since most of the physiological functions of the pineal gland are mediated by its hormone melatonin, investigations of endocrine functions of the pineal heavily rely on the quantitative estimation of melatonin. This putative pineal hormone has been quantified

using various techniques including fluorometry (Miller & Maickel, 1970), bioassay (Lynch et al, 1975), gas chromatography-mass spectrometer (GC-MS) (Koslow, 1974), GC-MS with negative chemical ionization (GCMS-NCl) (Lewy & Markey, 1978), high pressure liquid chromatography (HPLC) with fluorometric detection (Anderson et al, 1982), radioimmunoassay (RIA) (Arendt et al, 1975; Pang et al, 1977) and enzyme ligand immunoassay (Elisa) (Ferrua & Massey, 1985). Each approach exploits a different biological, chemical or physical property of melatonin. In fluorometry melatonin reacts with o-phthalaldehyde (OPT) to yield highly fluorescent compounds under conditions of heat and strong mineral acid. This measurement requires lengthy separation procedures and suffers from a lack of specificity and sensitivity. The bioassay of melatonin is carried out by observing its ability to induce melanin aggregation in dermal melanophores of larval rana pipiens (Ralph & Lynch, 1970). This assay requires animal breeding facilities and is very tedious. GCMS requires expensive equipment and lacks convenience while HPLC is limited to the pineal for quantification of melatonin because of lack of sensitivity. RIA offers by far the greatest efficiency for a large number of samples, is not expensive and affords routine analysis. Except for GCMS-NCl RIA gives the greatest sensitivity. Moreover, the sensitivity was improved by using ^{125}I-labelled melatonin as tracer (Vakkuri et al, 1984) instead of ^{3}H-melatonin. Another improvement in the immune assay is the development of Elisa (Ferrua & Massey, 1985). This method is more convenient and comparable with results of RIA. The

conjugate is more stable and more convenient than radiolabelled melatonin and there is no problem with disposal of radioactivity. It also eliminates or decreases the need for centrifugation.

In the investigation of pineal function frequent sampling is needed because the circadian rhythm of pineal melatonin production is the most characteristic feature of the hormone and the possible physiological functions of the pineal depend upon both the duration and amplitude of melatonin production as described above. In clinical practice frequent blood sampling is difficult. It is also impossible to measure the serum melatonin profile in the individual animal such as laboratory rat. 6-sulphatoxymelatonin (aMT6s) is the major metabolite of melatonin in the urine as described in section 1.2. A RIA for the metabolite has been developed both for human and rat (Bojkowski et al, 1987; Brown et al, 1991). In the human RIA, a direct correlation between serum melatonin and urinary aMT6s excretion has been confirmed (Arendt et al, 1985; Bojkowski et al, 1987; Fellenberg et al, 1980; Tetsuo et al, 1981). The assay of urinary aMT6s excretion has also been presented as an index of pineal melatonin production in rat (Brown et al, 1991). Therefore, this new technique provides an useful tool in investigation of pineal functions.

1.5. Rationale

As reviewed above, the pineal melatonin production is under control

by sex hormones. However, the effects have not been well characterized because of a variety of factors. Because melatonin has an inhibitory effect on rat gonads and since the pineal gland is considered to be a part of the neuroendocrine reproductive axis, it is reasonable to postulate that testosterone and oestradiol are involved in the hormonal regulation of pineal melatonin synthesis and release in rats. In order to test these hypotheses it is necessary to determine effects of castration and sex hormone replacement on the melatonin diurnal profile in vivo rather than on pineal HIOMT activity. In addition, the age, sex, endocrine status of animals and the time and dose of administered gonadal steroids administrated should be considered.

The sites and mechanisms of sex hormonal regulation of pineal melatonin production are still not well understood, although receptors for sex hormones have been detected in both the pineal and SCG. It is likely that sex hormones act on pineal melatonin forming enzyme activities or modify the neural control mechanisms. Effects of sex hormones on pineal HIOMT but not SNAT activity have been documented. However, the modification of pineal adrenergic mechanisms by sex steroids is not well characterized. Therefore, the present study is designed to answer following the questions:

1. Is there any sex difference in the melatonin production profile in rats; do oestradiol and testosterone exert different effects on pineal melatonin production?

2. Does castration cause significant effects on pineal melatonin production in rats ?
3. Is the alteration induced by castration in male rats different from that in female animals?
4. Are the effects of castration age-dependent?
5. Does sex hormone treatment abolish castration-induced effects?
6. Does castration also have effects on metabolism of circulating melatonin, if urinary aMT6s excretion diurnal profile was used as an index?
7. Do sex hormones affect the pineal response to adrenergic stimulation? If so, do these effects correspond to those on melatonin production profile?
8. Are pineal beta-adrenergic receptors the site for sex hormone actions if they alter the pineal response to adrenergic stimulation?

Hence, five experiments are performed as follows:

Experiment 1: Observation of effect of age and sex on urinary aMT6s excretion in rats.

Experiment 2: Investigation of castration effects on diurnal profile of urinary aMT6s excretion in three age groups of both male and female rats. Further sex hormone treatment will be focused on the one age group which was most affected by castration.

Experiment 3: Observation of the effect of castration on melatonin

metabolism in the rat.

Experiment 4: Study of the alteration of the pineal response to adrenergic stimulation by sex hormones.

Experiment 5: Observation of the effect of sex hormones on pineal beta-adrenergic receptors.

CHAPTER II

AGE-ASSOCIATED CHANGES AND SEX DIFFERENCE IN DIURNAL RHYTHM OF URINARY aMT6s EXCRETION IN RATS

2.1 Abstract

The diurnal rhythm of 6-sulphatoxymelatonin (aMT6s) excretion has been determined in male and female rats at 3 weeks, 2 months, 8 months, 14 months and 20 months of age. All animals have a pronounced diurnal pattern of aMT6s excretion under a 12 hour dark: 12 hour light cycle. A significant increase in aMT6s excretion associated with age from 3 weeks to 14 months is observed and the excretion decreases at 20 months. However, there is a highly significant correlation between aMT6s excretion and body weight ($r = 0.73$ for female rats and $r = 0.74$ for male rats; p values are all less than 0.001). Thus, a concomitant decrease in aMT6s excretion associated with increasing age occurs when body weight is taken into consideration. There is also a highly significant sex difference in aMT6s excretion at 3 weeks, 2 months and 8 months of age; the output is higher in males. Urinary testosterone excretion in male rats and oestradiol in female rats increase from 3 weeks old to 8 months and decrease at older ages. These data suggest that increase of body weight from 3 week old to 14 month old is an important factor in the age-related alteration. The sex differences in aMT6s excretion in younger rats may be associated with their sex hormonal milieu.

2.2 Introduction

A number of reports have appeared suggesting that there are marked changes in melatonin synthesis and secretion with growth and aging in both animals and humans (Murakami et al, 1989; Pang et al, 1984 a; Reiter et al, 1980; 1981; Tang & Pang, 1988; Waldhauser et al, 1988), although sex differences in pineal function have not been studied in detail. In the rat, pineal and serum melatonin levels have been reported to increase with age during development (Murakami et al, 1989; Tang & Pang, 1988) and decrease with the aging process (Pang et al, 1984 a; Reiter et al, 1980).

Melatonin production and release are, in all species examined including the rat (Binkley, 1984), greatest during the dark period and fall precipitously with the onset of the light phase. Moreover, melatonin secretion changes with the light dark cycle (Ho et al, 1984) and is relatively resistant to other influences (Chik et al, 1987 a; Ho et al, 1985). The magnitude and timing of melatonin secretion are important to its possible physiological functions. Thus,it is essential to define the characteristics of its secretion profile. However, since previous reports of melatonin levels in rats did not provide a full 24 h profile (Pang et al, 1984 a; Reiter et al, 1980; Tang & Pang, 1988), a description of the precise nature of variation was not possible.

Reportedly, circulating melatonin is rapidly metabolized

predominantly to sulphate conjugated 6-hydroxymelatonin, which is excreted into urine (Kopin et al, 1961; Kveder & McIsaac, 1961). We have recently validated a highly specific and sensitive radioimmunoassay for measuring 6-sulphatoxymelatonin (aMT6s) in rat urine (Brown et al, 1991). Using this technique, we demonstrated that normal adult rats have a pronounced diurnal pattern of the metabolite excretion; its excretion can be suppressed by light and it is derived almost entirely from pineal gland melatonin. In this study, therefore, we investigated the diurnal variation of urinary aMT6s excretion in both male and female rats of five age groups.

2.3 Materials and Methods

Animals

Male and female Sprague-Dawley rats were used in this experiment. The rats were one of five ages: 3-week-old, 2-month-old, 8-month-old, 14-month-old and 20-month-old. In each group, there were 4 male and 6 female animals, respectively. Upon arrival the animals were singly housed in metabolic cages under a light dark cycle of 12:12. Lighting was provided by cool white fluorescent tubes controlled by an automatic timer which gave an average illuminance of 110 $\mu W/cm^2$ as measured by an Opiken radiometer with a broad spectrum head and an infrared filter. Red photo-safe bulbs were used to allow urine collection during darkness. All data are shown as relative subjective day in which lights are turned on at

24:00. Food and tap water were supplied ad libitum.

Urine Collection

Urine samples from individual animals were collected in 50 ml plastic beakers every three hours throughout a 24 hour period (In order to collect urine samples completely, the cage bottom was rinsed with distilled water in later experiments). Sample volumes were measured after centrifugation at 5000 x g for 20 min. The resulting supernatant fluid was frozen and stored at -20° C prior to assay.

Assays

6-sulphatoxymelatonin: Urinary aMT6s concentration was assayed by radioimmunoassay using antiserum (CID-S-375) raised in sheep and iodinated aMT6s as tracer, which is based on the human assay of Bojkowski et al (1987). Briefly, following dilution 500-1000 times of urine with the tricine buffer (tricine (Sigma) 17.9 g/L, NaCl (BDH) 9.0 g/L, gelatin (Sigma) 1.0 g/L and thimerosal (Sigma) 0.1 g/L, pH 5.5), a 500 µl aliquot was incubated with 0.1 ml anti-aMT6s serum (1:15,000) and 50 µl ^{125}I-aMT6s (5,000 cpm) at 4°C overnight. Bound and free aMT6s were separated by incubation of 0.1 ml 1% charcoal-dextran (Sigma) solution at 4°C for 20 min followed by centrifugation at 4,000 g for 15 min. This assay shows good parallelism and recovery, a sensitivity of 1 pg per tube, and a mid-range of 22 pg per tube (Brown et al, 1991).

Samples purified on HPLC give the identical values after correction for recovery (Brown et al, 1991). Intra-assay coefficients of variation were 5.71% at 11.07 ng/ml and 3.66% at 45.54 ng/ml; Inter-assay coefficients of variation were 8.78% and 7.03% at 11.07 ng/ml and 45.45 ng/ml, respectively. The assessment of within and between CVs was performed using the method of Rodbard (1974).

Oestradiol and Testosterone: Urinary testosterone excretion in male rats and oestradiol in female rats were determined by RIAs. The RIAs used an anti-oestradiol antiserum donated by Dr. YoungLai of McMaster University (YoungLai et al, 1985) and a highly specific testosterone antiserum produced in this Lab (Bubenik et al, 1975). ^{3}H-oestradiol (SA = 91 Ci/mmol) and ^{3}H-Testosterone (SA = 89 Ci/mmol) purchased from New England Nuclear Corp. USA were utilized as tracers. The assay of sex steroid hormones in human urine was developed by us (Yie et al, 1986; Yie et al 1989) and other investigators (Cekan et al, 1985). In the present study the technique was used to measure oestradiol and testosterone concentrations in rat urine. The assays showed good parallelism (Fig.3) and recovery (Table 2). Intra-assay and inter-assay coefficients of variation were 7.1% and 9.9% at 242 pg/ml for oestradiol assay and 3.9% and 6.4% at 2.4 ng/ml for testosterone assay, respectively. Regression analysis of the results with and without prior organic solvent extraction yield a line, $Y = -52.1 + 0.79\ X$, $r = 0.90$ for oestradiol and $Y = -0.11 + 0.58\ X$, $r = 0.94$ for testosterone) (Fig 4). The measurements of urinary oestradiol

and testosterone were used to determine changes in the internal gonadal steroid milieu with age.

Statistical Analysis

Body weight and urinary sex hormone excretion were analyzed by one-way analysis of variation. Correlations between body weight and total 24h excretion of urinary aMT6s were analyzed by linear regression analysis. aMT6s diurnal profiles were statistically analyzed using a MANOVA followed by an one-way analysis of variance and multiple range test Duncan or a t test. Data points are expressed as mean $\pm$ standard errors.

2.4 Results

Highly significant phasic variations in aMT6s excretion were consistently observed ($p < 0.001$) in all age groups, whether urinary aMT6s excretion was expressed as absolute (ng/3 hours) or relative (ng/3 hours/100 g body weight) values. Fig.5. presents the absolute values of aMT6s excretion profile in the five age groups. There were significant differences in aMT6s excretion among these groups ($F = 12.6$, $df = 4,15$, $p = 0.0001$ for male rats and $F = 12.6$, $df = 4,25$, $p = 0.0001$ for female rats) indicating a aMT6s excretion increase with age in both male and female rats. However, a significant interaction of group by time ($F = 3.26$, $df = 28,105$, $p = 0.0001$ for male rats and $F = 74.26$, $df = 28,175$, $p = 0.0001$ for female rats) was also observed. Multiple range test

showed that aMT6s excretion gradually increased from 3 weeks to 14 months at almost all nocturnal time points and decreased at 20 months in both male and female animals.

Because body weight also increased with age, particularly in male rats (Table 3), and there was a highly significant correlation between total 24 hour excretion of urinary aMT6s and body weight ($Y = 35.1 + 0.476 X$, $r = 0.73$ for female rats and $Y = 134.4 + 0.479 X$, $r = 0.74$ for male rats) (Fig.6), body weight seemed to have a pronounced effect on aMT6s output. When the body weight was taken into consideration, a significant decrease in aMT6s excretion relative to body weight occurred from 3 weeks to 20 month old animals. In female rats,on repeated measures MANOVA,there was a significant difference among the five age groups in aMT6s excretion levels ($F = 4.87$, $df = 4,25$, $p = 0.005$) and a significant interaction of group by time ($F = 2.76$, $df = 28,175$, $p = 0.0001$). Multiple range test revealed that 3 week old rats exhibited higher levels in nocturnal aMT6s excretion at 18-21 hr than their older counterparts (Fig.7). In male rats, repeated measures MANOVA also showed significant difference in relative aMT6s excretion levels ($F = 14.05$, $df = 4,15$ $p = 0.0001$) and interaction of age by time ($F = 4.75$, $df = 28,105$, $p = 0.0001$). Comparing each time point across age by multiple range test, aMT6s excretion in 3 week old rats was higher at 15-18, 18-21 and 21-24 h, whereas lower excretion of aMT6s was found in 14 month and 20 month old animals (Fig.7).

aMT6s excretion (ng/3 hours/100 g body weight) was compared between male and female rats of five age groups. Significantly higher values in male than female animals were detected for 3 week, 2 month and 8 month age groups, but the interaction of sex by time was also significantly different in the 3 week old group and 8 month old rats ($p < 0.001$). There was no significant sex difference in aMT6s excretion for either 14 month or 20 month old rats. T test in the younger rats indicated that at most nocturnal time points throughout the lighting cycle male rats had a higher aMT6s excretion than females, although there was no significant difference in aMT6s excretion at 18-21 hr during the dark period in immature rats.

24 h excretion of urinary testosterone per body weight in male rats or oestradiol in female rats varied with age; highest values were detected in 3 week, 2 month and 8 month old rats (Fig.8).

2.5 Discussion

The results presented in this study indicate that absolute values (ng/3 hours) of urinary aMT6s excretion increase gradually from 3 weeks to 14 months and then decrease at 20 months of age in both male and female rats (Fig.5). However,urinary aMT6s excretion per 100 g body weight declines gradually from 3 weeks to 20 months (Fig.7). These findings are in close agreement with previous

reports about pineal weight in rats (Tait et al, 1969), rat pineal hydroxyindole-O-methyltransferase (HIOMT) activity (Klein & Lines, 1968) and aMT6s output in humans (Testsuo et al, 1982; Rager et al, 1989). Our data on absolute aMT6s output closely parallel findings by Pang et al (1990) showing that both mid dark pineal melatonin content and melatonin in the confluens sinuum of the rat increased during development to 60 days and were significantly lower at 510 days. Moreover, results on relative aMT6s excretion also agree well with findings that mid dark serum melatonin in the rats increased with development reaching a peak on days 16 and 17 and thereafter falling to > 510 days (Pang et al, 1990). In that study serum melatonin levels were found to correlate well both with plasma melatonin in confluens sinuum of the rat corrected for body weight to head weight ratio and also with pineal melatonin content corrected for body weight. These findings indicate that during development absolute aMT6s excretion reflects absolute pineal melatonin content and also that weight corrected aMT6s output mirrors changes in blood melatonin levels and in weight corrected pineal melatonin content. The significant positive correlation between total aMT6s excretion and body weight both in male and female rats, together with very similar regression of aMT6s and body weight between sexes (Fig.6), provide further evidence that the increase of body weight from 3 weeks to 20 months in rats is an important factor in the age related alteration in aMT6s output. A body weight independent decrease of aMT6s excretion at 20 months of both male and female rats agrees with other reports and

indicates that the rate of secretion from the pineal decreases at that age (Pang et al, 1984 a; Reiter et al, 1980).

Other factors that must be considered in the age-dependent alteration in aMT6s excretion include changes in pineal beta-adrenergic receptor density and in nocturnal pineal serotonin N-acetyltransferase (SNAT) activity. However, a decrease in pineal beta-adrenergic receptor density with age was reported by Weiss and his colleagues (Weiss et al, 1979), but was not corroborated by Dax and Sugden (1988). No significant change in SNAT activity with age was noted by one study (Dax & Sugden, 1988) but a small reduction was observed in 29 month old rats (Reiter et al, 1981). A decline in pineal HIOMT activity with age was consistently detected (Dax & Sugden, 1988; Klein et al, 1968; Tait et al, 1969). However, HIOMT is not rate limiting in pineal melatonin synthesis. It is unlikely that the reduction in pineal melatonin production in aged rats is due to alterations in pineal HIOMT activity.

The significant sex difference in urinary aMT6s excretion from 3 week to 8 month age groups obtained in this study is in contradiction with the general view that the sex of animals has no significant impact on pineal melatonin production (Reiter, 1984). Moreover, as discussed above, a sexual difference in age-associated aMT6s excretion per body weight is also observed. However the effect of sex on pineal function has not been investigated in

detail to date. Wakabayashi & Shimada (1986) removed pineal gland from rats during the light phase and found no difference in pineal melatonin content between sexes. However, sex differences in pineal morphology have been reported (Blumfield & Tapp, 1970).

A number of studies have demonstrated that melatonin has a regulatory effect on gonadotropins (Reiter, 1980) and that melatonin synthesis and secretion, in turn, may be regulated by sex hormones and gonadotropins (Cardinali, 1980; 1984; Cardinali & Vacas, 1987; Preslock, 1977; 1984). There are pronounced sex differences in the hormonal milieu and in regulatory mechanisms between male and female rats from early development to old age (Frederick et al, 1988; Ojeda & Urbanski, 1988). For example, plasma gonadotropin levels in female rats are higher than those in males during prepuberty (Dohler & Wuttke, 1975). Plasma gonadotropin secretion is subjected to cyclic regulation in females and to tonic control in males during adulthood. Female rats may reach their infertility stage (around 8 months) with high levels of gonadotropin earlier than males (Frederick et al, 1988). In the present study, relative urinary testosterone in male rats and oestradiol in female rats were high from 3 weeks to 8 months but then reached low levels by 14 months. At 3 weeks, 2 months and 8 months when sex hormone output was high significant sex differences were seen in aMT6s but disappeared at older ages. Assuming that the metabolic rate of these hormones do not change drastically with age, then hormone levels corrected for body weight will be closely

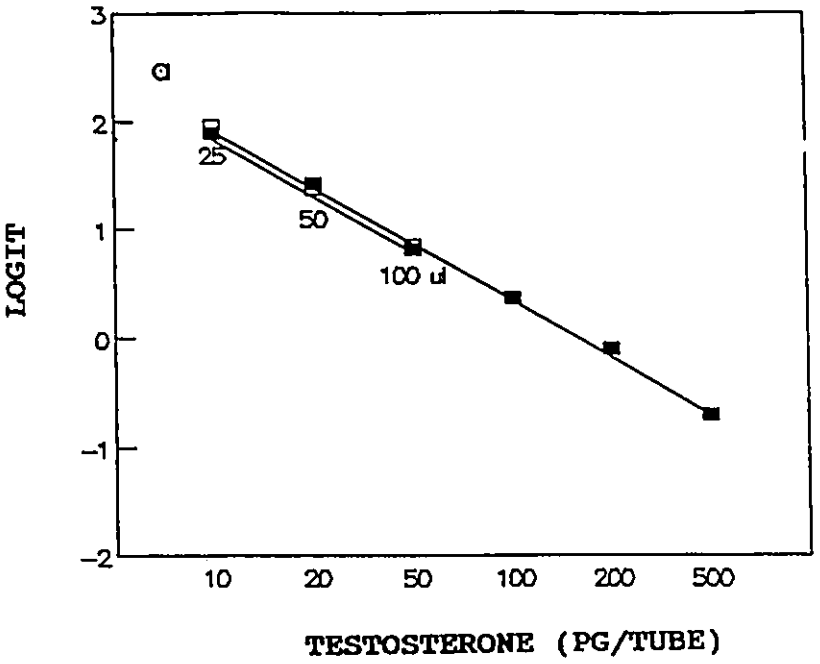
related to the circulating levels which would influence the pineal. Furthermore, recent reports indicated that oestradiol blocked the isoproterenol-induced elevation of pineal melatonin levels in castrated adult female rats while testosterone abolished an inhibitory effect of castration on pineal melatonin content in adult male rats (Harnendez et al, 1990; Moujir et al, 1990). These data suggest that oestradiol may have an inhibitory but that testosterone plays a stimulating role in the hormonal regulation of pineal melatonin secretion, although previous reports on the effects of sex steroids on pineal function are sometimes contradictory.

In this study, we estimated melatonin synthesis and secretion by measuring its urinary metabolite rather than serum levels. Although a close correlation between these two compounds has been demonstrated in humans (Bojkowski et al, 1987), the possibility that melatonin metabolism is altered with age and sex cannot be excluded in the present study. Furthermore, it is possible that melatonin metabolism is influenced by steroids. Nevertheless, in this study, the use of repeated measurement of urinary aMT6s excretion in the same individual animal permits a description of the precise nature of diurnal profile.

In summary, the results of the present study confirm the age-related changes in the diurnal profile of pineal melatonin production in rats. The increase of body weight from 3 weeks to

14 months of age is an important factor for the alteration in weight related aMT6s excretion. The present study also reveals a sex difference in pineal melatonin production in the younger rats, which may result from the difference in sex hormonal milieu.

Fig.3. Displacement curves obtained with different quantities of authentic testosterone (a) and oestradiol (b) standards (■) and parallel displacement with different aliquots of rat urine (□) were observed using anti-oestradiol (1:2,000) and anti-testosterone (1:2,000) , as well as tritium labelled testosterone and oestradiol, respectively.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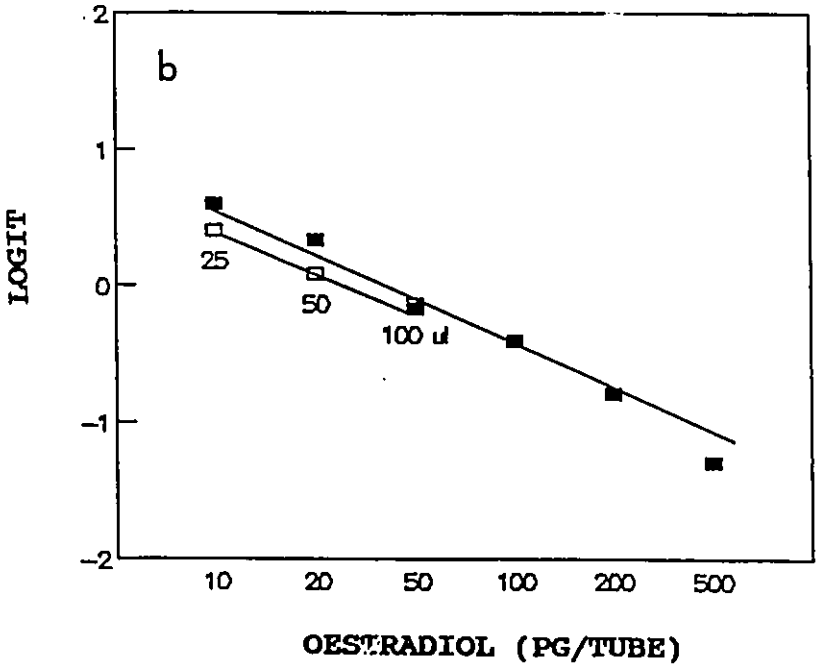
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OESTRADIOL (PG/TUBE)

Table 2

Analytical Recovery of Urinary Testosterone in Rats

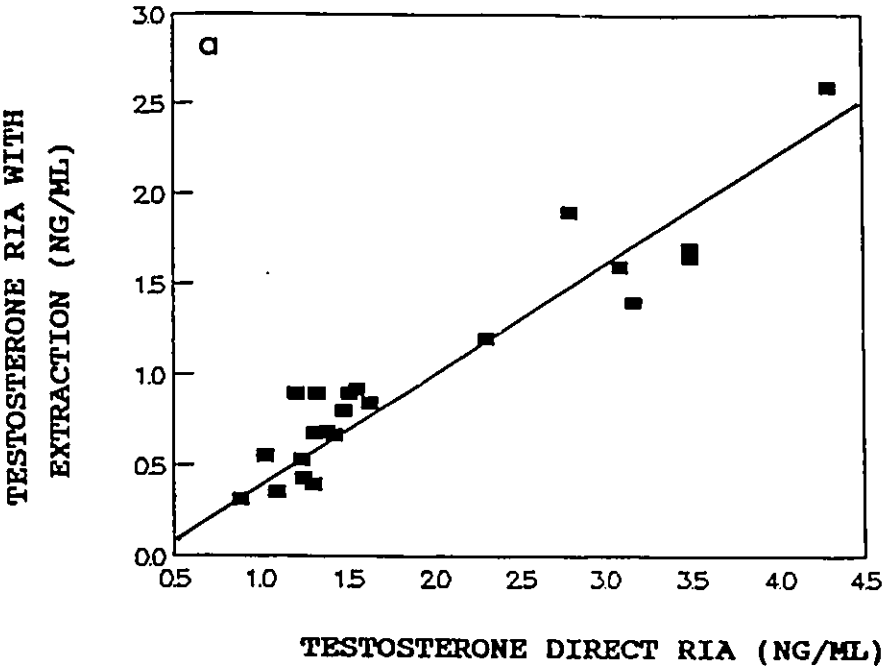
Assay	Assayed Value pg/tube	Expected Value pg/tube	Recovery%
Extracted Assay	7.8	7.8	
	24.2	27.8	87
	38.8	57.8	67.9
	96.	107.8	89.1
mean ± SE			81.1 ± 12.1
Direct Assay	23.5	23.5	
	40.6	43.5	93.3
	62.4	73.5	84.9
	113.5	123.5	91.9
mean ± SE			90.3 ± 4.5

Table 3

Analytical Recovery of Urinary Oestradiol in Rats

Assay	Assayed Value pg/tube	Expected Value pg/tube	Recovery
Extracted Assay	13.5	13.5	
	33.1	33.5	98.8
	51.3	63.5	80.9
	78.7	113.5	69.3
mean ± SE			83.0 ± 14.9
Direct Assay	2.3	2.3	
	22.3	22.3	100
	52.1	52.3	99.6
	106.9	102.3	104.5
mean ± SE			101.4 ± 2.7

Fig.4. Regression analysis of rat urinary testosterone (a) and oestradiol (b) assayed by the RIAs with (Y) and without (X) an organic solvent-extraction step.


a
TESTOSTERONE RIA WITH EXTRACTION (NG/ML)
TESTOSTERONE DIRECT RIA (NG/ML)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5

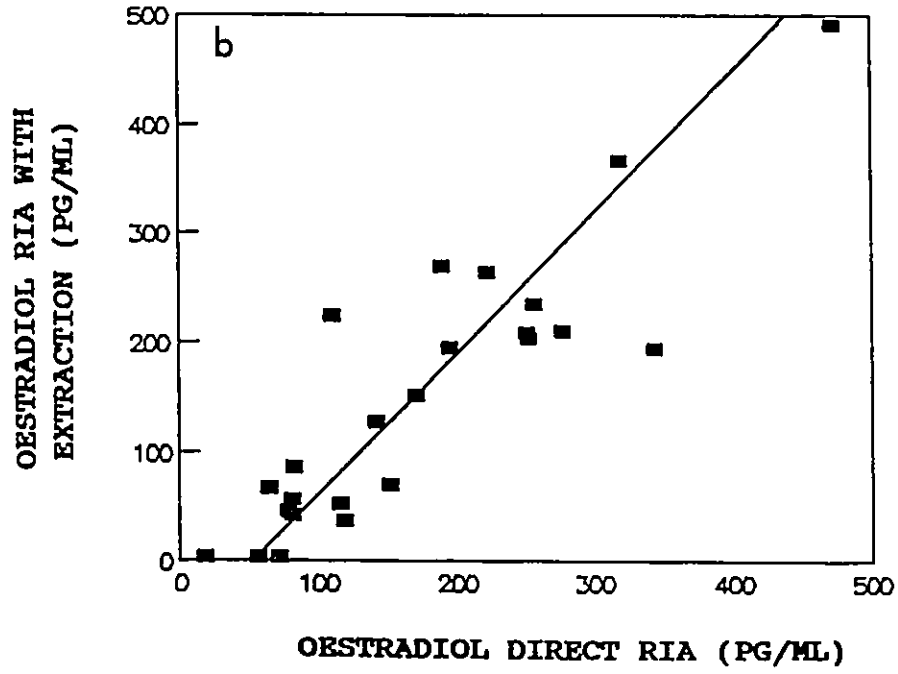

b
OESTRADIOL RIA WITH EXTRACTION (PG/ML)
OESTRADIOL DIRECT RIA (PG/ML)
0
100
200
300
400
500

Table 4

Body Weight (g) in the Rats

Age	Male* Mean ± SE	Female** Mean ± SE
3 weeks	55.0 ± 1.8	65.0 ± 2.2
2 months	250.0 ± 2.5	191.6 ± 3.1
8 months	353.0 ± 4.2	410.0 ± 16.3
14 months	720.0 ± 7.3	430.0 ± 32.4
20 months	820.0 ± 18.2	480.0 ± 37.1

* Body weight significantly increases from 3 weeks to 20 months.

** There are significant increases in body weight from 3 weeks to 8 months, but no differences among 8 months to 20 months.

Fig.5. Diurnal variation of absolute(ng/ 3 h) aMT6s excretion in urine in Sprague-Dawley male (■) and female (□) at 3 weeks (a), 2 months (b), 8 months (c), 14 months (d) and 20 months (e) of age. The animals were kept under a 12:12 LD cycle. Urinary samples were collected every three hours throughout the cycle. Each point represents the mean ± standard errors. The black bar represents the daily period of darkness.

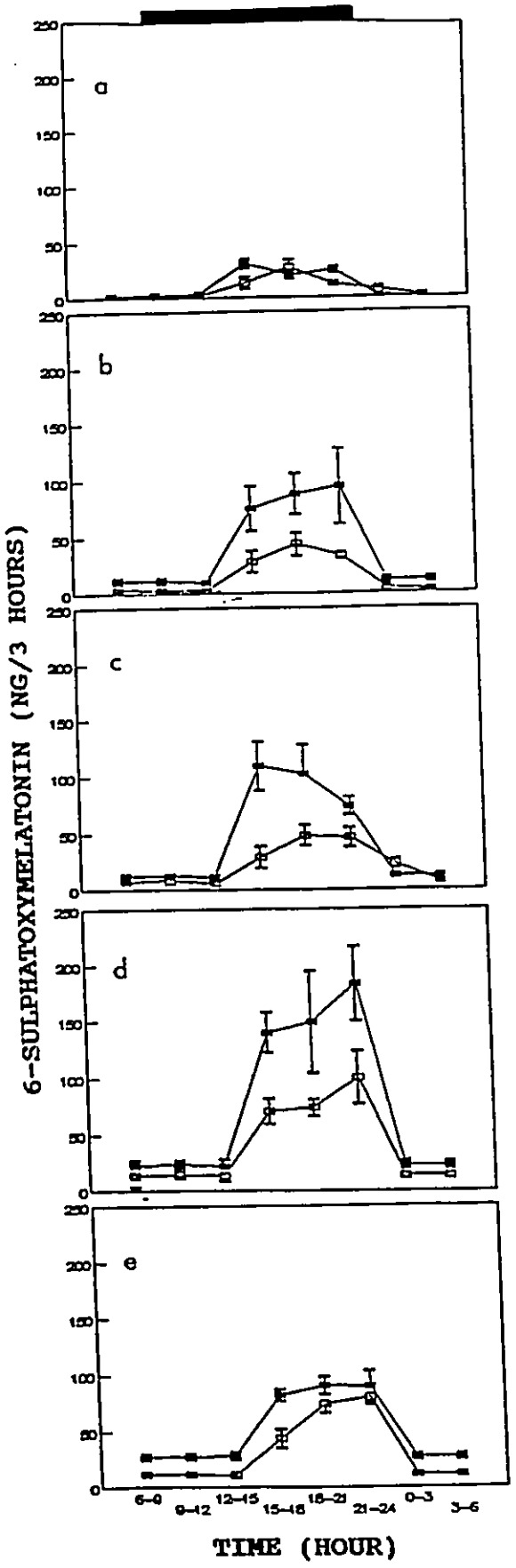

a
b
c
d
e
6-SULPHATOXYMELATONIN (NG/3 HOURS)
0
6-9
9-12
12-15
15-18
18-21
21-24
0-3
3-6
TIME (HOUR)

Fig.6. Correlation between body weight and total 24 h excretion of urinary aMT6s in Sprague-Dawley male (▲) and female (■) rats. Regression equations were Y = 35.1 + 0.476 X, r = 0.73, n = 30, p = 0.001 for female and Y = 134.4 + 0.479 X, r = 0.74, n = 20, p = 0.001 for male rats.

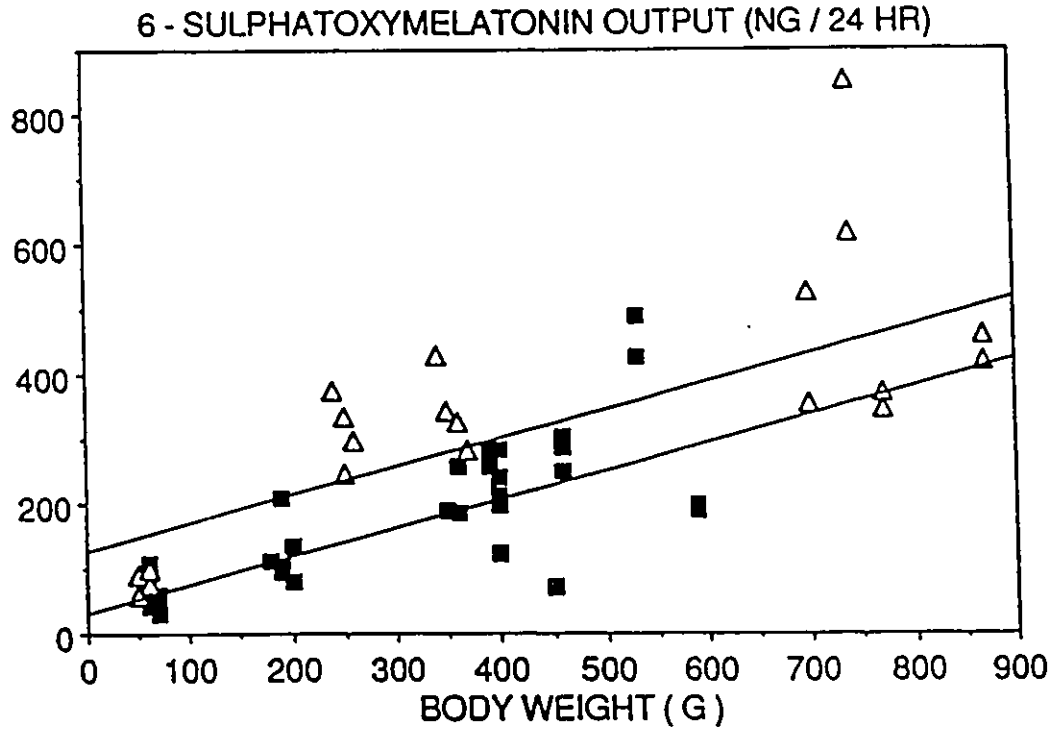


Fig.7. Relative (ng/3 hour/100 g body weight) aMT6s excretion in urine in Sprague-Dawley male (■) and female (□) rats of 3 week (a), 2 month (b), 8 month (c), 14 month (d) and 20 month (e) age groups. Values at each 3 hour time point are mean ± standard errors. The black bar on the time axis represents the dark period during 12 hr and 12 hr lighting cycle.

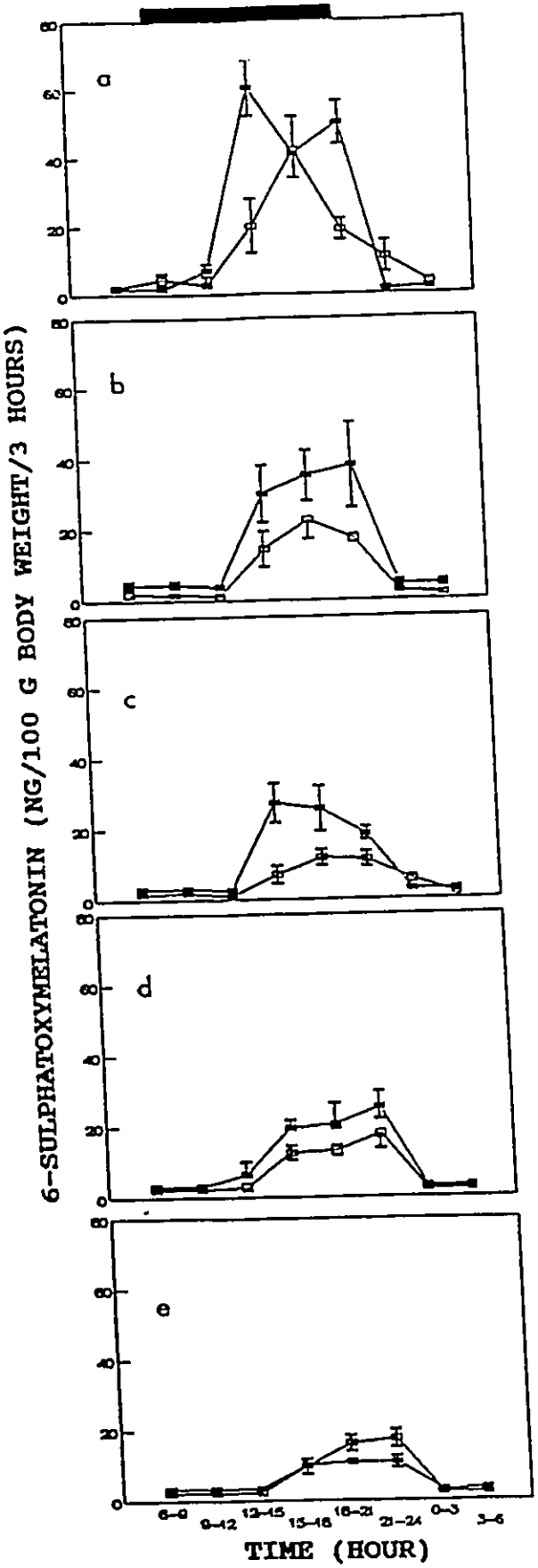
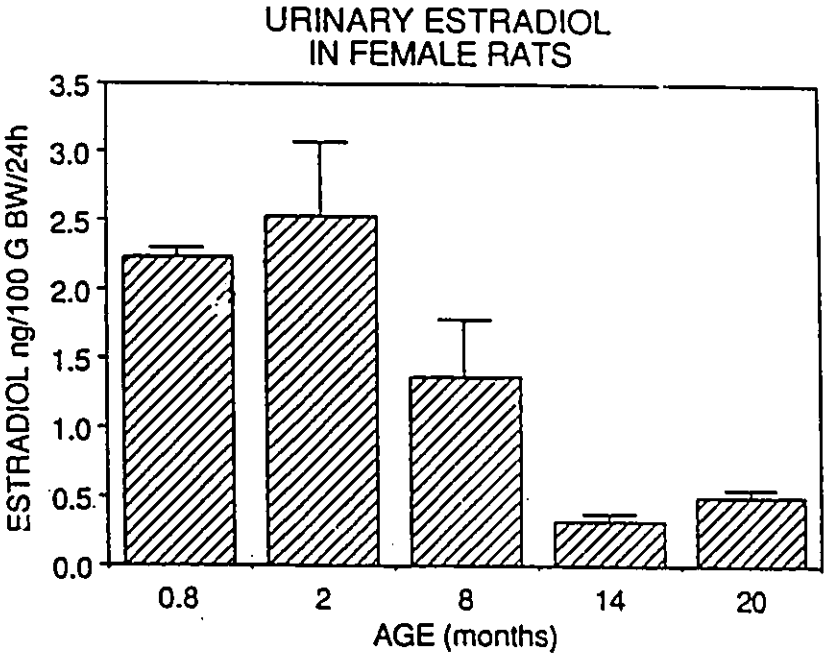
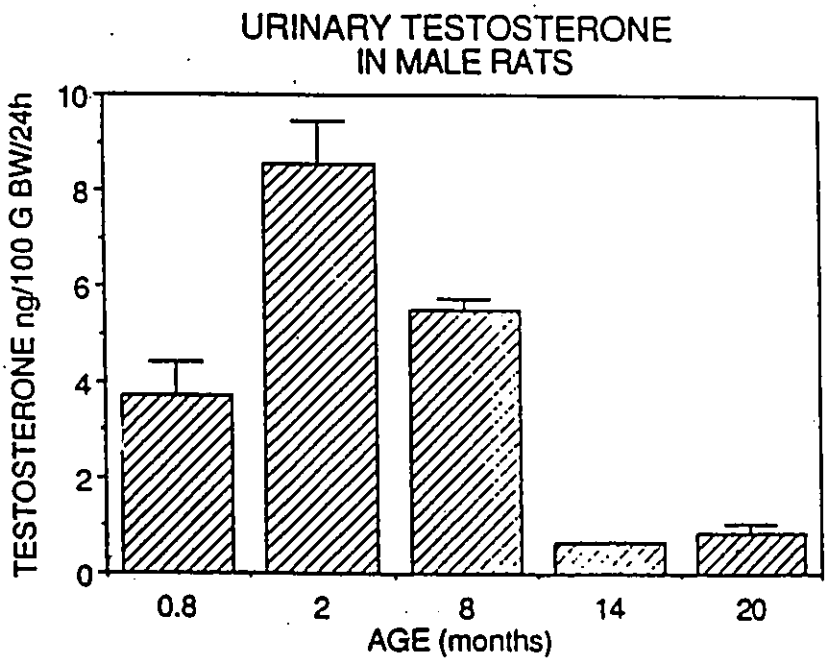

a
b
c
d
e
6-SULPHATOXYMELATONIN (NG/100 G BODY WEIGHT/3 HOURS)
0
6-9
9-12
12-15
15-18
18-21
21-24
0-3
3-6
TIME (HOUR)

Fig.8. Age-associated variation of 24 hour excretion of urinary testosterone in the male rats and oestradiol in the female rats. Each bar represents mean ± standard errors.


URINARY ESTRADIOL
IN FEMALE RATS
ESTRADIOL ng/100 G BW/24h
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.8
2
8
14
20
AGE (months)


URINARY TESTOSTERONE
IN MALE RATS
TESTOSTERONE ng/100 G BW/24h
10
8
6
4
2
0
0.8
2
8
14
20
AGE (months)

CHAPTER III

EFFECT OF CASTRATION AND SEX HORMONE TREATMENT ON DIURNAL PROFILE OF URINARY aMT6s EXCRETION IN RATS

3.1 Abstract

The effect of castration and sex hormone treatment on 6-sulphatoxymelatonin (aMT6s) excretion, the major metabolite of melatonin in urine, was determined by radioimmunoassay in both male and female rats. In 3 week old female rats castration caused a fall in aMT6s excretion while ovariectomized rats had a higher aMT6s excretion than their sham-operated controls at both 2 months and 8 months of age. aMT6s excretion varied with stage of the oestrous cycle in 2 month old female rats with the lowest value during proestrus which corresponded to the highest level of oestradiol. In 2 month old male rats castration induced a decrease of aMT6s excretion, but no significant effect of castration on aMT6s excretion was found in either 3 week or 8 month old male rats. Administration of oestradiol benzoate (EB) to castrated female rats and testosterone propionate (TP) to castrated male animals was performed at 2 months of age; they abolished the effects of castration in both male and female rats. Furthermore, administration of EB to 2 month old male rats and TP to female rats decreased and increased aMT6s excretion, respectively. These data clearly indicate that gonadal steroids have a marked influence on aMT6s excretion; in 2 month old rats oestradiol has an inhibitory while testosterone has a stimulating effect.

3.2 Introduction

The role of the pineal gland in controlling mammalian reproduction, mainly through melatonin, is well established (Brzezinski & Wurtman, 1988; Reiter, 1980). In seasonal breeders, such as sheep, the time of the year at which reproductive activity occurs is synchronized by the number of 24 hours per day that the pineal gland secretes melatonin (Bittman et al, 1985). In non-seasonal breeders, like laboratory rodents, melatonin has been reported to inhibit reproductive function including sexual maturation (Lang et al, 1984), timing of ovulation (McCormark & Sridaran, 1978), gonadal steroidogenesis (Ellis, 1972; Kano & Miyachi, 1976; Ng & Lo, 1988) and sexual behaviour (Mitchell & Yochim, 1970). On the other hand, increasing evidence indicates that pineal melatonin production, in turn, might be regulated by the internal hormonal milieu, primarily by gonadal steroids (Cardinali, 1980; 1984; Cardinali & Vacas, 1987; Preslock, 1977; 1984). However, results concerning effects of sex hormones on pineal functions observed by previous studies were inconsistent.

The investigation of pineal function to date still heavily relies on measurement of melatonin content in pineal gland and serum. Pineal and serum melatonin concentrations show a diurnal rhythm with high levels during the dark period and low values during the light phase in all mammals studied. Because both the magnitude and timing of melatonin production are important to its possible

physiological functions, it is more valuable to determine its production profile. A sensitive and specific RIA for 6-sulphatoxymelatonin (aMT6s), the major metabolite of melatonin, in rat urine was developed recently in this lab. It has been demonstrated that there is a marked diurnal pattern of aMT6s excretion similar to that of blood melatonin in adult male rats and that urinary aMT6s derives almost entirely from the pineal gland (Brown et al, 1991). Thus, this technique seems ideal for investigation of the regulation of melatonin production by gonadal steroids in rat.

Because melatonin may exert an inhibitory effect on the gonadal axis in rats (Brzezinski & Wurtman, 1988; Reiter, 1980) and the significant sex difference in urinary aMT6s excretion was found in younger rats (chapter II), it may be assumed that sex hormones, in turn, regulate pineal melatonin production; probably oestradiol and testosterone exerting an opposite effect in this species. In order to test this hypothesis, three series of experiments were designed to investigate 1) effect of castration upon aMT6s excretion in both male and female rats at 3 weeks, 2 months and 8 months of age; 2) variation of aMT6s excretion during the oestrous cycle in 2 month old animals and 3) effects of sex hormone treatment on aMT6s excretion in rats at 2 months of age.

3.3 Materials and Methods

Animals

In the first series of experiments, 3 week, 2 month and 8 month old male and female Sprague-Dawlay rats were used. Each experimental age group consisted of 4 male or 6 female animals. The rats were maintained at a temperature of 21° C in a cabinet with a 12 hour light/12 hour dark cycle (LD 12:12). Lighting was provided by cool white fluorescent illuminance of 110 $\mu W/cm^2$. Red photo-safe bulbs were used to allow urine collection during darkness. Food and tap water were supplied ad lib.

2 month old female rats were used in the second series of experiments and both male and female rats at 2 months of age in the third series. Each experimental group in these series of experiments consisted of 6 animals. All housing conditions were the same as described in the first series of experiments.

Castration

Rogarsetic and Atravet were used to achieve general anaesthesia. Under sterile conditions the ovaries were removed through a midline incision into the abdomen and the testes were cut off from an incision in the scrotum. The protocol for sham operation was identical except for actual removal of the ovaries or testes.

Urine Collection

Urine samples were collected in 50 ml plastic beakers from individual animals housed in metabolic cages. At each collection the cage bottom was rinsed with 20 ml distilled water and the volume was measured. The rinsed sample was then centrifuged at 5000 x g for 20 minutes and an aliquot of the supernatant was stored at -20°C prior to assay.

In the first series of experiment, urine collection was performed every 2 hours in male rats or 3 hours in female rats throughout 24 hours after 7 days of recovery from castration and sham operation. In the second series of experiment daily 24 hour oestradiol excretion was determined to monitor oestrous cycles and urine sampling was done at 3 hour intervals over 4 successive days. In the third series of experiment urine samples were collected every 3 hours over a 24 hour period on the 7th day after castration or sham-operation. Then, the ovariectomized rats were administered intramuscularly (i.m) oestradiol benzoate (EB) (50 μg/kg body weight/day) and orchiectomized rats i.m testosterone propionate (TP) in sesame oil (200 μg/kg body weight/day) or vehicle to both castrated control and sham-operated animals for 5 days. The injections were carried out at the middle of the dark phase. Then, urine samples were collected every 3 hours throughout 24 hours. The urine sampling was also performed on the 7th day post the sex hormone treatment. In order to further confirm whether testosterone has a stimulating while oestradiol has an inhibitory

effect on aMT6s excretion in 2 month old rats, EB (50 μg/kg body weight/day) in sesame oil was injected i.m to male rats and TP (200 μg/kg body weight/day) to female rats or vehicle at the middle of the dark phase for 5 days, respectively; and then urine samples were collected at 3 hour intervals during 24 hours.

Assays

6-sulphatoxymelatonin: Urinary aMT6s concentration was assayed by radioimmunoassay as described in chapter II. Within assay coefficient of variation (CV) is 5.7% for a quality control sample of 11.1 ng/ml and between assay CV is 8.8% (N=33). For a sample of 45.5 ng/ml within and between assay CVs are 3.7% and 7.9%, respectively. The assessment of within and between cv was done using the method of Rodbard (1974). All samples collected on the same day from castrated and sham animals in one experimental group under a given lighting cycle were measured in one assay.

Oestradiol and Testosterone: Oestradiol levels in 24 hr urine pool of rats were estimated by radioimmunoassay as described in chapter II. The mean intra-assay and inter-assay coefficients of variation were 7.11% and 9.90% for oestradiol assay , and 3.00% and 6.40% for testosterone assay, respectively. The measurements of oestradiol and testosterone excretion were used to determine the internal gonadal steroid milieu after castration and sex hormone treatment.

Statistical Analysis

Twenty four hour rhythm data between castrated and sham-operated animals, during the oestrous cycle and between castrated rats with sex hormone treatment and sham-operated controls were analyzed by MANOVA followed by a t-test or one way analysis of variance and multiple range test Duncan as described below. Values showed in tables and figures present mean $\pm$ standard errors. All data are also shown as relative subjective day in which light is turned on at 24:00.

Results

Because there is a highly significant correlation between body weight and aMT6s excretion in rats (Chapter II), all data on aMT6s output in this study were presented as ng/100 g body weight/2 or 3 hour. There was a significant effect of light on aMT6s in both castrated and sham-operated animals; all showed elevated nocturnal output.

Effect of Castration on aMT6s Excretion

Female Rats (Fig.9): Urinary aMT6s contents of the ovariectomized 3 week old rats were significantly lower than those of sham-operated animals ($F = 6.75$, $df = 1,10$, $p = 0.027$) and a significant interaction of group by time was also found ($F = 5.20$, $df = 7,70$, $p < 0.001$). Results analyzed by t test showed that at one time point (15-18 h) during the dark phase the ovariectomized

animals had significantly lower aMT6s excretion than controls. However, aMT6s excretion was significantly increased after ovariectomy both in 2 month ($F = 22.4$, $df = 1,10$, $p = 0.001$) and 8 month ($F = 14.86$, $df = 1,10$, $p = 0.003$) old animals. A significant interaction of group by time was also detected in both age groups ($F = 10.66$, $df = 7,70$, $p < 0.0001$ for 2 month old rats and $F = 3.01$, $df = 7,70$, $p = 0.008$ for 8 month old animals, respectively). During the dark period two time points (15-18 h and 21-24 h) in 2 month old rats and one time point (15-18 h) in 8 month old rats were significantly increased in ovariectomized as compared with sham-operated rats (on t test).

Male Rats (Fig.10): Orchiectomized 2 month old rats had lower aMT6s levels than sham-operated animals ($F = 8.6$, $df = 1,6$, $p = 0.026$) and a significant interaction of group by time ($F = 3.01$, $df = 9,54$, $p = 0.006$). Orchiectomized animals had significantly lower values in aMT6s excretion at 16-18 h and 20-22 h during the dark phase than controls. Orchiectomy had no significant effect either on aMT6s level or on the interaction of group by time in male rats at 3 weeks and 8 months of age.

Sex Hormones in Urine: Table 5 shows urinary oestradiol and testosterone in female and male rats after castration and sham surgery in the three age groups. Significantly lower levels of oestradiol after ovariectomy and lower testosterone excretion after orchiectomy than sham-operated controls were consistently observed

in all animals except 3 week old male rats.

Variation in aMT6s Excretion with Oestrous Cycle

Fig.11. shows the oestrous pattern. There were significant effects of day ($F = 9.18$, $df = 3,15$, $p = 0.001$), time ($F = 12.97$, $df = 7,35$, $p < 0.001$) and interaction of day and time ($F = 10.29$, $df = 21,105$, $p < 0.001$). Nocturnal aMT6s excretion was lowest on the day of proestrus at a time when oestradiol was increased and highest on the day of metoestrus when oestradiol was lowest. On one way analysis of variance significantly increased aMT6s excretion was seen from 24 to 03 hours on the day of metoestrus and oestrus as compared to the other days ($F = 5.13$, $df = 1,8$, $p = 0.029$).

Sex Hormone Treatment

Fig.12 shows that aMT6s level was higher at 18-21 h in 2 month old ovariectomized rats than that in their sham-operated controls on the 7th day after the surgery. After injection with EB to the ovariectomized rats for 5 days there was no significant difference in aMT6s excretion between the EB treated ovariectomized and sham-operated animals, but the vehicle treated ovariectomized rats had higher aMT6s output than either of these two groups. Urinary aMT6s excretion in ovariectomized EB-treated rats was not significantly different from that in sham-operated animals on the 7th day post EB injection. In male rats orchiectomy decreased aMT6s excretion compared to sham-operated counterparts at 18-21 h and 21-24 h

(Fig.13). 5 days of testosterone treatment in orchiectomized animals increased their aMT6s excretion resulting in non-significant difference in the melatonin metabolite excretion between TP treated orchiectomized and sham-operated control animals, but aMT6s excretion in these two groups was higher than that in the vehicle treated orchiectomized group. The effect of orchiectomy on aMT6s excretion appeared again on the 7th day post testosterone treatment compared to sham-operation.

Administration of testosterone to ovariectomized rats induced a significant increase in aMT6s excretion at 15-18 h as compared with their vehicle treated castrated controls and testosterone administration to sham-operated female rats increased aMT6s excretion at 15-18 h and 21-24 h during the dark phase (Fig.14).

Fig.15. shows that oestradiol had no effect on aMT6s excretion when administered to orchiectomized animals compared to vehicle treated orchiectomized rats while aMT6s excretion in EB treated male sham-operated rats at 15-18 h and 21-24 h during the dark phase was lower than that in vehicle treated control male rats.

Table 6 and 7 show urinary testosterone and oestradiol excretion during the pericd of sex hormone treatment as described above.

3.5 Discussion

The present results indicate that sex hormones do not eliminate the primary photoperiodic variation in aMT6s excretion; under LD 12:12 there was a pronounced time effect in all animals whether castrated or sham-operated and whether or not they were treated with sex hormones or vehicle. However, the results clearly demonstrated that sex hormones do have a pronounced effect on the magnitude and timing of the diurnal profile in urinary aMT6s excretion in the rat.

Previous reports dealing with the effect of sex hormones on pineal hydroxyindole-O-methyltransferase (HIOMT) activity were inconsistent (Alexander et al, 1970; Cardinali et al, 1974 b; Daya & Potgieter, 1982; Houssay & Barcelo, 1972; Nagle et al, 1974; Wallen & Yochim, 1974; Wurtman et al, 1964), but HIOMT is not rate limiting in pineal melatonin production.

Serotonin N-acetyltransferase (SNAT), the key enzyme in melatonin synthesis, is generally thought not to be affected by gonadal steroids (Cardinali, 1980; Preslock, 1984). Neither ovariectomy or oestradiol administration altered the diurnal rhythm and isoproterenol (ISO)-induced activation of this enzyme in adult rats in vivo (Daya & Potgieter, 1982; Illnerrova, 1975). Oestradiol or pregnant mare serum gonadotropin (PMSG) treatment in immature female rats had no effect on norepinephrine (NE) induced SNAT

activity in vitro (Wilkinson & Arendt, 1978). Attempts to correlate rat SNAT activity with oestrous cycle stages had also been unsuccessful (Cardinali & Vacas, 1978; Shivers & Yochim, 1979). However, castration and testosterone treatment were reported to affect pineal SNAT activity in adult male rats in vivo (Daya & Potgieter, 1982). A pronounced effect on pineal SNAT sensitivity to beta-adrenergic challenge by both oestradiol and testosterone in 6 day old rats pineal organ culture was also reported (Yumiler, 1989).

Reasons for these contradictions are obscure. N-acetyltransferase activity exhibits a circadian rhythm in the pineal tissue similar to that of melatonin (Klein & Weller, 1970). It has been demonstrated that the rhythm of SNAT activity and melatonin synthesis in rats is mainly controlled by the photoperiod through a well known adrenergic mechanism (Binkley, 1983), while reproductive hormones only participate in expression of the final response (Cardinali & Vacas, 1987). Indeed, the present study shows that castration and/or sex hormone treatment did not completely eliminate the diurnal rhythm of aMT6s excretion. However, there were significant effects by sex hormones especially during the dark phase. Similarly, a significant variation of rat pineal melatonin content with the stages of the oestrous cycle was found when several time points, particularly during the nighttime, were examined (Johnson et al, 1982). No significant difference was found in pineal melatonin levels among ovariectomized, sex hormone

treated and control rats when a single time point was examined (Ozaki et al, 1978). Failure to detect a significant effect of sex hormone on pineal SNAT activity in the previous in vivo studies is probably due to single or non-frequent time points examined. Thus, it is extremely important to observe the 24 hour profile of pineal SNAT activity and melatonin production in investigation of hormonal modulation of pineal function.

The foregoing results demonstrated that oestradiol has an inhibitory while testosterone exerts a stimulating effect on pineal melatonin production in young adult rats. These correspond well with previous studies dealing with the effects of sex hormones on melatonin levels in urine, serum and the pineal gland (Hernandez et al, 1990; Johnson et al, 1982; Moujir et al, 1990; Ozaki et al, 1978).

Interestingly, our present results show that castration caused a reduction in aMT6s excretion in immature female rats while no significant effects were found in male rats at 3 weeks and 8 months of age. There are marked sex differences in sex hormone milieu between male and female rats from development to aging (Ojeda & Urbanski, 1988). Castration induced a significant difference in both oestradiol and testosterone excretion in the immature female rats but no significant changes in either hormone in immature male rats (Table 5). These may explain why aMT6s excretion was higher in ovariectomized immature female rats than their sham-operated

controls but not significantly different in immature male rats. However, further studies are needed to determine the characteristics of the effects of sex hormones on pineal melatonin production in immature rats. The reason why orchiectomy had no effect on aMT6s excretion in 8 month old male rats is obscure since there was a significant difference in testosterone excretion after castration. Further studies are also needed.

The mechanisms of sex hormonal regulation of pineal melatonin production are less understood. Effects on several pineal constituents relative to melatonin synthesis have been reported (Cardinali, 1980; Cardinali & Vacas, 1987). Recent evidence appeared to indicate that sex hormones can affect the pineal response to adrenergic stimulation (Moujir et al, 1990) suggesting that sex steroid action would be through modification of the adrenergic mechanisms. The present studies did not characterize or identify sites of these actions.

In summary, results represented in this study supported the hypothesis that gonadal steroids are involved in the regulation of pineal melatonin production in young adult rats and further suggest that oestrogen predominantly exerts an inhibitory but androgen plays a stimulating role in pineal melatonin production.

Table 5

Urinary oestradiol and testosterone excretion (ng/24 h) in male and female rats after castration and sham-operation.

Age (month)	Sex	Oestradiol		Testosterone	
		Castration	Sham	Castration	Sham
0.8	male	0.35+ ± 0.14++	0.54 ± 0.13	6.63 ± 1.30	7.87 ± 0.97
	female	0.24 ± 0.06	1.14* ± 0.25	0.35 ± 0.06	0.96* ± 0.20
2.0	male	0.81 ± 0.34	1.08 ± 0.19	2.91 ± 1.33	24.82* ± 3.52
	female	0.26 ± 0.04	4.57* ± 0.37	0.45 ± 0.06	1.43 ± 0.16
8.0	male	1.12 ± 0.14	0.90 ± 0.09	3.16 ± 1.02	22.32* ± 2.78
	female	0.62 ± 0.14	1.98* ± 0.36	0.60 ± 0.11	0.67 ± 0.31

*: Significant difference between castrated and sham-operated rats
+: mean; ++: standard error

Table 6

Urinary oestradiol and testosterone excretion (ng/24 h) in male and female rats of 2 month old after castration and hormone replacement

Treatment	Sex	Oestradiol			Testosterone		
		CAS	CAS+V	Sham	CAS	CAS+V	Sham
7 days after castration & sham	male	0.77 ± 0.25	-	0.55 ± 0.17	0.60 ± 0.13	-	9.16* ± 2.47
	female	0.83 ± 0.23	-	1.77* ± 0.05	0.27 ± 0.03	-	0.77 ± 0.27
hormone treatment	male	0.78 ± 0.27	0.27 ± 0.18	1.10 ± 0.47	11.27 ± 2.35	0.64 ± 0.13	17.00 ± 6.80
	female	4.05 ± 1.42	0.60 ± 0.27	3.08 ± 0.79	0.40 ± 0.04	0.58 ± 0.28	1.25* ± 0.14
7 days post hormone treatment	male	2.15 ± 0.53	-	1.18 ± 0.25	0.96 ± 0.15	-	12.74* ± 4.32
	female	0.75 ± 0.40	-	2.06* ± 0.34	0.21 ± 0.08	-	0.34 ± 0.12

CAS = castration; CAS + V = castration + vehicle;

*: Significant difference between castrated and sham-operated rats

Table 7

Urinary oestradiol and testosterone excretion (ng/24 h) after injection of oestradiol to male and testosterone to female rats

Treatment	Oestradiol		Testosterone	
	male	female	male	female
Castration	0.27 ± 0.18	0.60 ± 0.27	0.64 ± 0.13	0.58 ± 0.28
Castration + Hormone	5.47 ± 1.42	0.55 ± 0.64	0.82 ± 0.20	2.92 ± 0.66
Sham	0.78 ± 0.14	2.47 ± 0.67	11.41 ± 3.40	1.28 ± 0.58
Sham + Hormone	3.88 ± 0.79	1.97 ± 0.66	10.75 ± 1.90	4.21 ± 0.92

Fig.9. aMT6s excretion profile after castration (■) and sham-operation (□) in female rats at 3 weeks (a), 2 months (b) and 8 months (c) of age. The animals were kept under a 12:12 LD cycle. Urine samples were collected every three hours throughout the cycle. Each point represents the mean ± standard errors. The black bar represents the daily period of darkness. (*) indicates a significant difference between castrated and sham-operated rats at that time point.

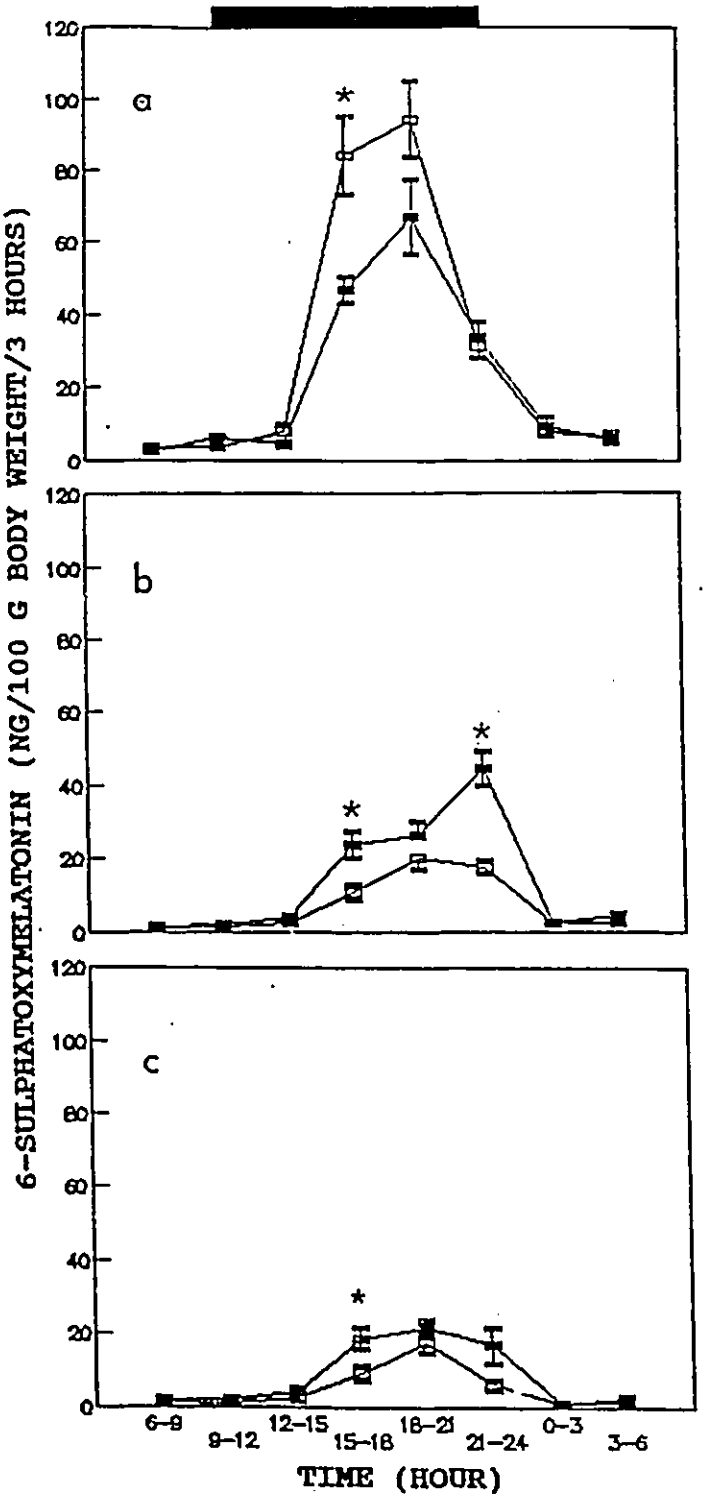

a
b
c
*
120
100
80
60
40
20
0
6-SULPHATOXYMELATONIN (NG/100 G BODY WEIGHT/3 HOURS)
6-9
9-12
12-15
15-18
18-21
21-24
0-3
3-6
TIME (HOUR)

Fig.10. aMT6s excretion profile after castration (■) or sham-operation (□) in male rats at 3 weeks (a), 2 months (b) and 8 months (c) of age. The animals were kept under a 12:12 LD cycle. Urine samples were collected every 2 hours throughout the cycle. Each point represents the mean ± standard error. The black bar represents the daily period of darkness. (*) represent a significant difference between castrated and sham-operated rats at that time point.

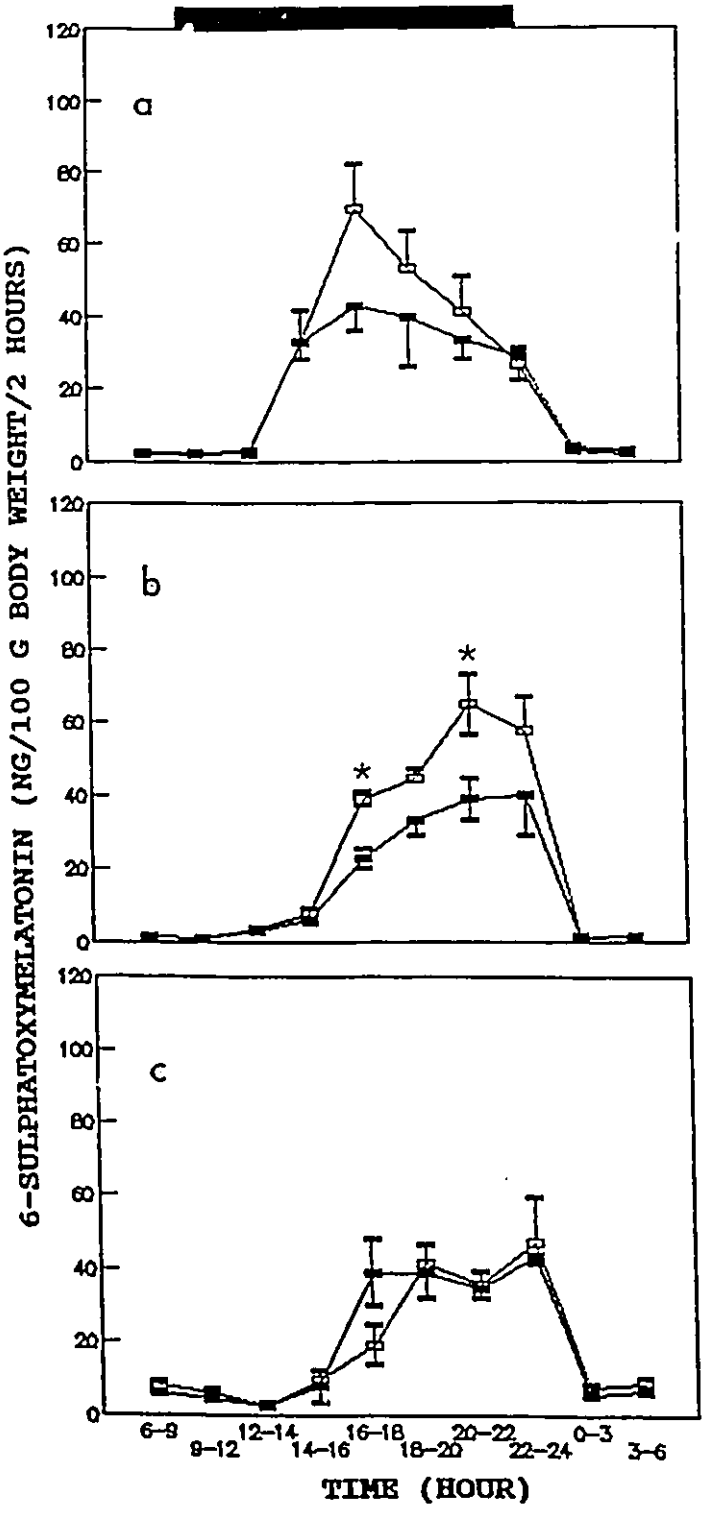
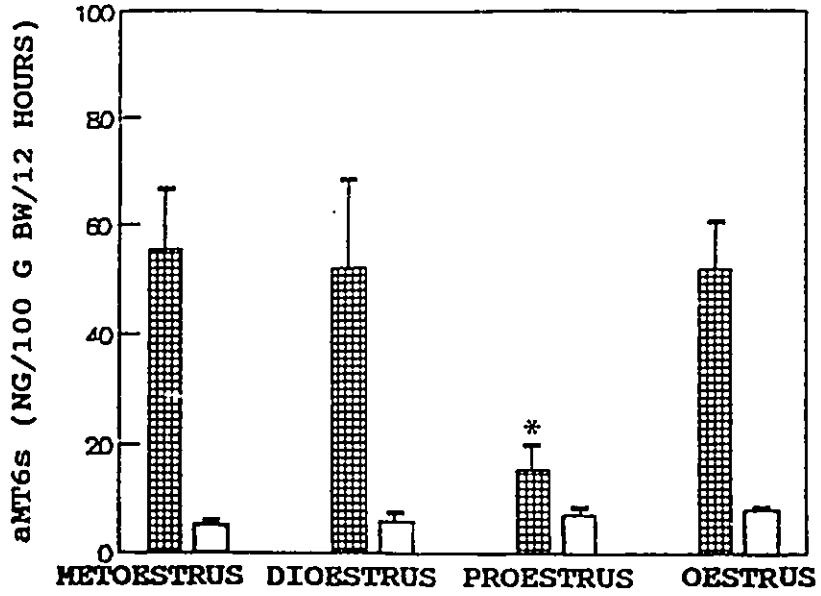

a
b
c
*
*
120
100
80
60
40
20
0
6-SULPHATOXYMELATONIN (NG/100 G BODY WEIGHT/2 HOURS)
6-9
9-12
12-14
14-16
16-18
18-20
20-22
22-24
0-3
3-6
TIME (HOUR)

Fig.11. Variation in aMT6s and oestradiol excretion during the oestrous cycle in 2 month old female rats. Pooled night (□) and day (□) values are shown for aMT6s. (*): $P < 0.05$


aMT6s (NG/100 G BW/12 HOURS)
100
80
60
40
20
0
*
METOESTRUS
DIOESTRUS
PROESTRUS
OESTRUS

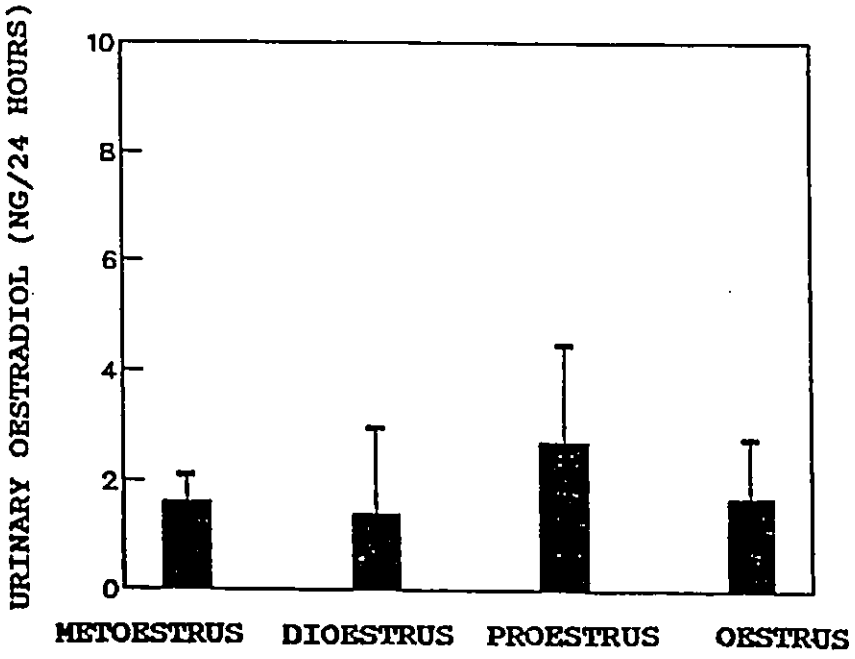

URINARY OESTRADIOL (NG/24 HOURS)
10
8
6
4
2
0
METOESTRUS
DIOESTRUS
PROESTRUS
OESTRUS

Fig.12. aMT6s excretion profile after castration (a), oestradiol treatment of castrated animals (b) and post hormone treatment (c) in female rats at 2 months of age. The animals were kept under a 12:12 LD cycle. Urinary samples were collected every 3 hours throughout the cycle. Each point represents the mean ± standard error. The black bar represents the daily period of darkness. (*) $P<0.05$

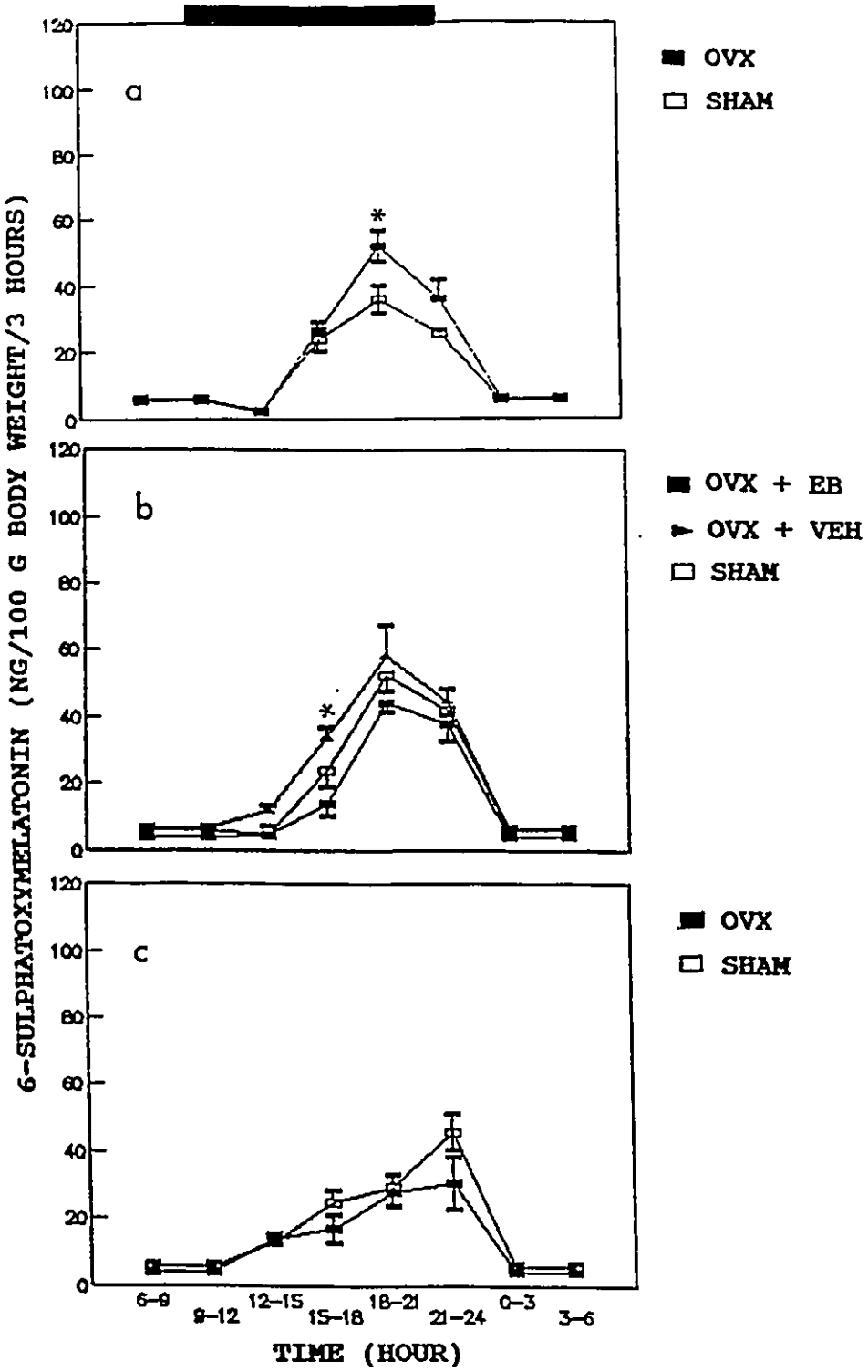

a
b
c
OVX
SHAM
OVX + EB
OVX + VEH
SHAM
OVX
SHAM
6-SULPHATOXYMELATONIN (NG/100 G BODY WEIGHT/3 HOURS)
0
20
40
60
80
100
120
*
6-9
9-12
12-15
15-18
18-21
21-24
0-3
3-6
TIME (HOUR)

Fig.13. aMT6s excretion profile after castration (a), testosterone treatment of castrated animals (b) and post hormone treatment (c) in male rats at 2 months of age. The animals were kept under a 12:12 LD cycle and urine samples were collected every 3 hours throughout the cycle. Each point represents the mean ± standard error. The black bar represent the daily darkness. (*) $P < 0.05$

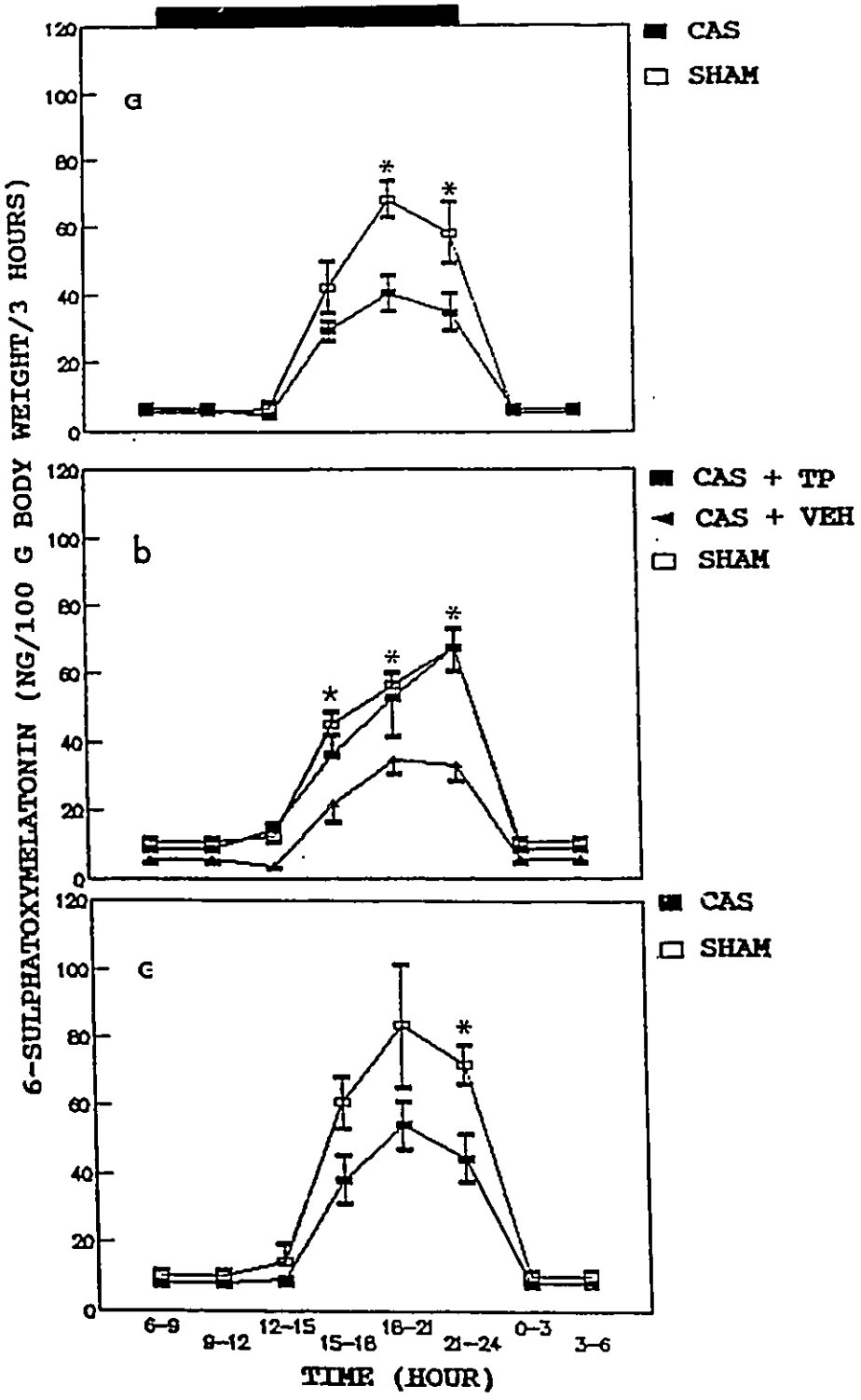

CAS
SHAM
a
CAS + TP
CAS + VEH
SHAM
b
CAS
SHAM
c
6-SULPHATOXYMELATONIN (NG/100 G BODY WEIGHT/3 HOURS)
0
20
40
60
80
100
120
6-9
9-12
12-15
15-18
18-21
21-24
0-3
3-6
TIME (HOUR)

Fig.14. aMT6s excretion profile after injection of testosterone in ovariectomized (a) and sham-operated female rats (b) at 2 months of age. The animals were housed under a 12:12 LD cycle and urine samples were collected in 3 hour interval through a day. Each point represents the mean ± standard error. The black bar present the daily darkness. (*): $P < 0.05$

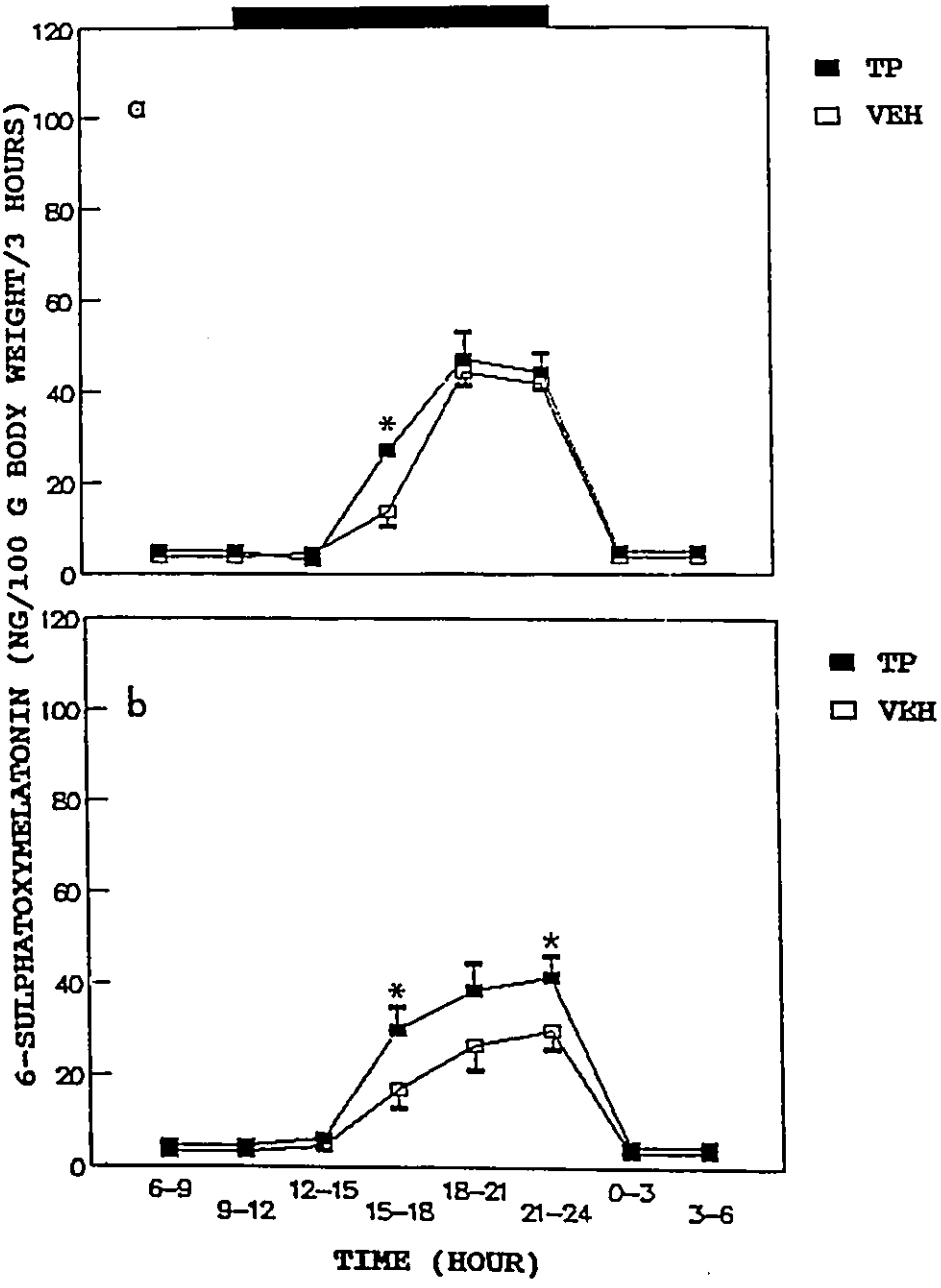
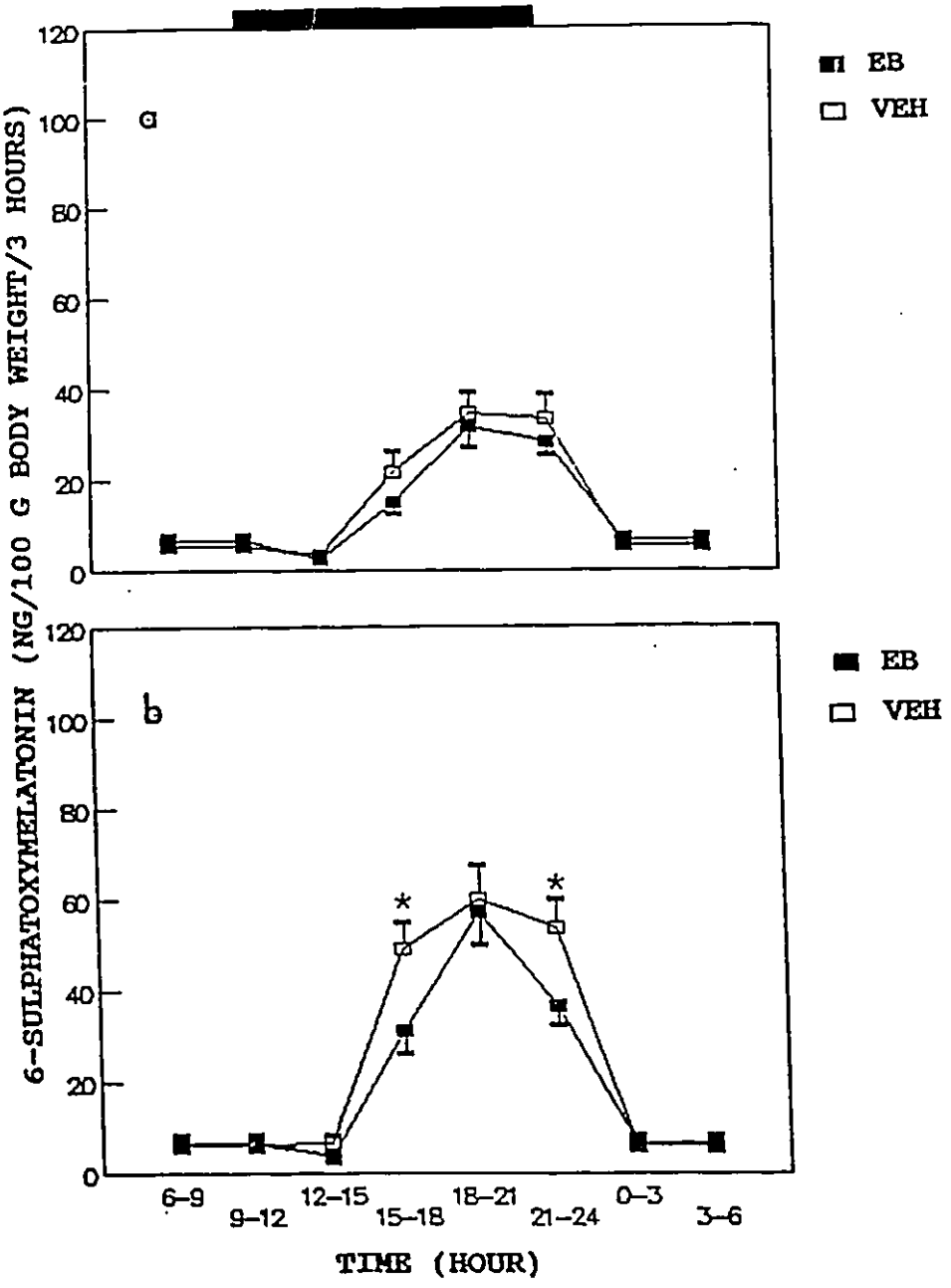

a
b
TP
VEH
TP
VEH
6-SULPHATOXYMELATONIN (NG/100 G BODY WEIGHT/3 HOURS)
0
20
40
60
80
100
120
*
6-9
9-12
12-15
15-18
18-21
21-24
0-3
3-6
TIME (HOUR)

Fig.15. aMT6s excretion profile after injection of oestradiol in orchiectomized (a) and sham control female rats (b) at 2 months of age. Experimental conditions and way data represented are the same as Fig.14.


a
b
EB
VEH
EB
VEH
120
100
80
60
40
20
0
6-SULPHATOXYMELATONIN (NG/100 G BODY WEIGHT/3 HOURS)
*
*
6-9
9-12
12-15
15-18
18-21
21-24
0-3
3-6
TIME (HOUR)

CHAPTER IV

EFFECT OF CASTRATION ON MELATONIN HALF LIFE IN RATS

4.1 Abstract

In order to determine whether effects of sex hormones on urinary aMT6s excretion in rats result from alterations in melatonin metabolism, investigation of effects of castration on the half life of elimination of plasma melatonin was carried out in 2 month old male and female rats and in 6 month old male rats. The animals were either castrated or sham-operated. ^{3}H-melatonin was infused into unanesthetized animals through an inferior vena cava catheter. Blood samples were withdrawn from an abdominal aorta catheter over the next 1 hour and radioactivity in the plasma samples was determined by liquid scintillation counting. The average half life of plasma melatonin ranged from 23.5 to 31.4 min among the sham-operated rats. Castration had no significant effect on the half life of circulating melatonin either in 2 month old male and female rats or 6 month old male rats. Therefore, the effects of sex hormones on urinary aMT6s excretion in young adult rats reflect their regulation of pineal melatonin production rather than effects on metabolism.

4.2 Introduction

The effects of castration and sex hormone treatment on the diurnal profile of urinary aMT6s excretion have been demonstrated in rats, especially at age of 2 months, in the last chapter. As reviewed in chapter I of this thesis, the major metabolic pathway of circulating melatonin in the rat is conversion to 6-hydroxymelatonin in the liver by microsomal enzymes (Kopin et al, 1961). This is followed by conjugation with sulphate or glucuronide and excretion mainly in the urine (Kveder & McIsaac, 1961). The changes in urinary aMT6s excretion profile by castration and/or sex hormone treatment may be explained by 1) sex hormones inducing an alteration in rate of pineal melatonin production; 2) sex hormones causing a change in rate of melatonin metabolism or 3) sex hormones altering rates of both pineal melatonin production and circulating melatonin metabolism. Indeed, it was reported that melatonin clearance rate changes in different periods of reproduction in women (Fernandez et al, 1988) and that melatonin metabolism in rat liver was changed with age from 3 days to one month in vitro (Weinberg et al, 1981). Therefore, the possibility cannot be excluded that castration and sex hormone treatment could affect melatonin metabolism resulting in changes in its metabolite excretion, although a close relationship between urinary aMT6s excretion and circulating melatonin level has been documented in rat (Brown et al, 1991) and humans (Bojkowski et al, 1987). In order to resolve this problem,

we compared the half life of melatonin in castrated rats with that in sham-operated control animals in 2 month old male and female and 6 month old male rats.

4.3 Materials and Methods

Animals

2 month old male and female and 6 month male Sprague-Dawley rats were used. The animals were divided into castrated and sham-operated groups; each experimental group consisted of 4 rats. They were individually housed in plastic cages in a cabinet with a 12:12 LD light cycle (110 $\mu W/cm^2$), maintained at 21° C and fed chow and tap water ad lib.

Ovariectomy and Orchiectomy

The surgical procedures were the same as those described in chapter III.

Blood Vessel Catheterizing

Two polyethylene catheters (Becton Dickinson, USA; I.D = 0.58 mm and O.D = 0.965 mm) were inserted as follows (Lestage et al, 1985): The animal was anaesthetized with Rogarsetic (Rogar/STB Inc, Canada; 63 mg/kg body weight) and Atravet (Ayerst Laboratories, Canada; 3.75 mg/kg body weight). Under sterile conditions the two catheters with vialon radiopaque catheters (Becton Dicknson, USA) were inserted into the inferior vena cava and abdomen aorta through

a middline incision into the abdomen. Both catheters, which were about 40 cm long, were tunnelled around the neck out through an incision in the skin over the skull. The incision was closed with wound chips and the catheters, filled with 8% heparin in saline, were fused. The surgical procedure took about 1 hour and the animals were placed under a 12:12 LD lighting cycle after they had recovered.

Infusion

Each animal was infused with ^{3}H-melatonin in saline (10 μCi/kg body weight) through the venous catheter after 1 to 2 days recovery from anesthesia. Blood samples were withdrawn through the abdominal aorta catheter at the 1st, 3rd, 5th, 10th, 15th, 20th, 25th, 30th, 40th, 50th and 60th min; 0.2 ml/time blood sample was drawn. The total volume of blood withdrawn during the experiment was about 5.5 ml.

Determination of radioactivity

The ^{3}H-melatonin was obtained from New England Corp. USA and had an initial specific activity of 85 Ci/mmol. Blood samples were allowed to clot at room temperature, centrifuged at 4000 x g at 4° C for 20 min and 50 μl volume of plasma was added into a vial containing 3 ml scintillation fluid cocktail (Beckman, USA). The total radioactivity in these samples was measured with a beta-counter (Beckman LS 7500, counting efficiency = 61%).

Analysis of ^{3}H-Melatonin by One-dimensional Thin Layer Chromatography (TLC)

In order to demonstrate that the radioactivity in blood samples was ^{3}H-melatonin, these samples from each experimental group were pooled and extracted by dichloromethane (Fisher Scientific, Canada) in a 1:5 ratio (CIDtech, 1988). The organic phase was then evaporated to dryness and the residue was resolved in 0.1 ml of methanol (Fisher Scientific). A 20 µl extracted sample was applied as a spot 2 mm from the bottom of 20 x 20 cm silica gel-coated TLC plate (Sigma), which had been scored to yield sections (5 cm wide). Fresh ^{3}H-melatonin was applied to one section of each sheet as a chromatographic reference standard. The plates were developed by ascending chromatography using a mixture of chloroform and ethyl acetate (30:120 v/v) for 80 min (Vakkuri et al, 1984). The sections of each plate were then divided into 13 segments. The silica gel from each 1 cm segment was eluted with methanol into scintillation vials. Scintillation fluid was added and counted on the beta-counter.

Statistical Analysis

The half life of melatonin was determined with a pharmacological programme in which the elimination half life of a drug was calculated according to equation $t_{1/2\beta} = 0.693/\beta$ (β = slope of disappearance curve of the drug from plasma) based upon a two - compartment open pharmacokinetic model (Greenblatt & Koch-Weser, 1975). Student t-test was performed for comparison of the half

life between castrated and sham-operated animals.

4.4 Results

The disappearance of ^{3}H-melatonin in each experimental group is shown in Fig.16. In all animals there is a rapid decrease in ^{3}H-melatonin concentration within 5 min after the infusion. Then the rate of the disappearance of the ^{3}H-melatonin was much slower. The half life of serum melatonin in each group is shown in Table 8. Castration had no significant effect on the half life either in 2 month old male and female rats or 6 month old male rats.

The TLCs of pools of the dichloromethanine extracts from each experiment group are shown in Fig 17. A single radioactivity peak is observed in all sample pools. Rf values were the same as the fresh ^{3}H-melatonin standard; around 0.44.

4.5 Discussion

The half life for elimination of plasma melatonin in rats has been estimated by a number of investigators with a range from 1-5 min (Kopin et al, 1961; Ozaki et al, 1976; Reppeart & Klain, 1978) to 17-23 min (Gibbs & Vriend, 1981). The large disparity could be caused by a variety of factors, such as the experimental procedure, dose of radio-labelled melatonin, age of animals and calculation methods. In our experiment the half life for elimination of

plasma melatonin is approximately 23.5 to 31.4 min in the sham-operated animals. This is similar to that reported by Gibbs and Vriend (1981). The surgical procedure used in the present study is similar to that adopted by Gibbs and Vriend (1981) with some modifications: 1) ^{3}H-melatonin was infused through the vein while blood was withdrawn from the aorta catheter because it is much easier for blood collection; 2) the infusion of ^{3}H-melatonin was carried out 1 to 2 days after animals recovered from anaesthesia so that interference by anaesthetizing drugs could be avoided (Mayer et al, 1980); 3) a physiological dose of ^{3}H-melatonin (10 μCi/kg ^{3}H-melatonin = 0.11 nmol/kg melatonin) was used in the experiment to avoid the possible effect of supra-physiological levels of exogenous melatonin on the accurate determination of its half life (Mayer et al, 1980); 4) with TLC, possible changes in ^{3}H-melatonin purity with time after the infusion and among animals was examined; 5) 5.5 ml of blood was collected during 1 hour test, which constituted a haemorrhage of about 13.8 -16.5 ml/kg; the metabolic condition of the animals would not be seriously affected according to results of Walsh et al (1980) and 6) a pharmacologic programme (Greenblatt & Koch-Weser, 1975) was used to calculate the half life. With these modifications, we think that our data may more accurately represent actual values.

The most important finding in the present study is that castration has no significant effect on melatonin half life either in 2 month old male and female rats or in 6 month old male rats. Ovariectomy

caused an increase in urinary aMT6s excretion while orchiectomy induced a decrease in the melatonin metabolite output in rats at 2 month of age (chapter III). No change in the half life of melatonin by castration suggests that the effects of sex hormones on urinary aMT6s excretion in this age group do not result from alteration of melatonin metabolism. Therefore, it can be concluded that effects of sex hormones on urinary aMT6s excretion are caused by changes in pineal melatonin production. It also strongly suggest that the measurement of urinary aMT6s can be an useful index in investigation of the regulation of pineal melatonin production by gonadal steroids.

In summary, castration does not induce significant alteration in the half life of elimination of plasma melatonin. The effects of sex hormones on urinary aMT6s excretion reflect their effects on pineal function. However, the mechanisms of these effects await further studies.

Fig.16. Disappearance of ^{3}H-melatonin from plasma in 2 month old male (a) and female (b) and 6 month old male rats (c) subjected to castration (■) and sham-operation (□). The animals were given 10 μCi/kg, i.v of ^{3}H-melatonin. Each point represents the mean plasma ^{3}H-melatonin concentration determined by the liquid scintillation counting of 4 rats at the indicated time after the administration of melatonin. Vertical bars represent the standard error of the mean.

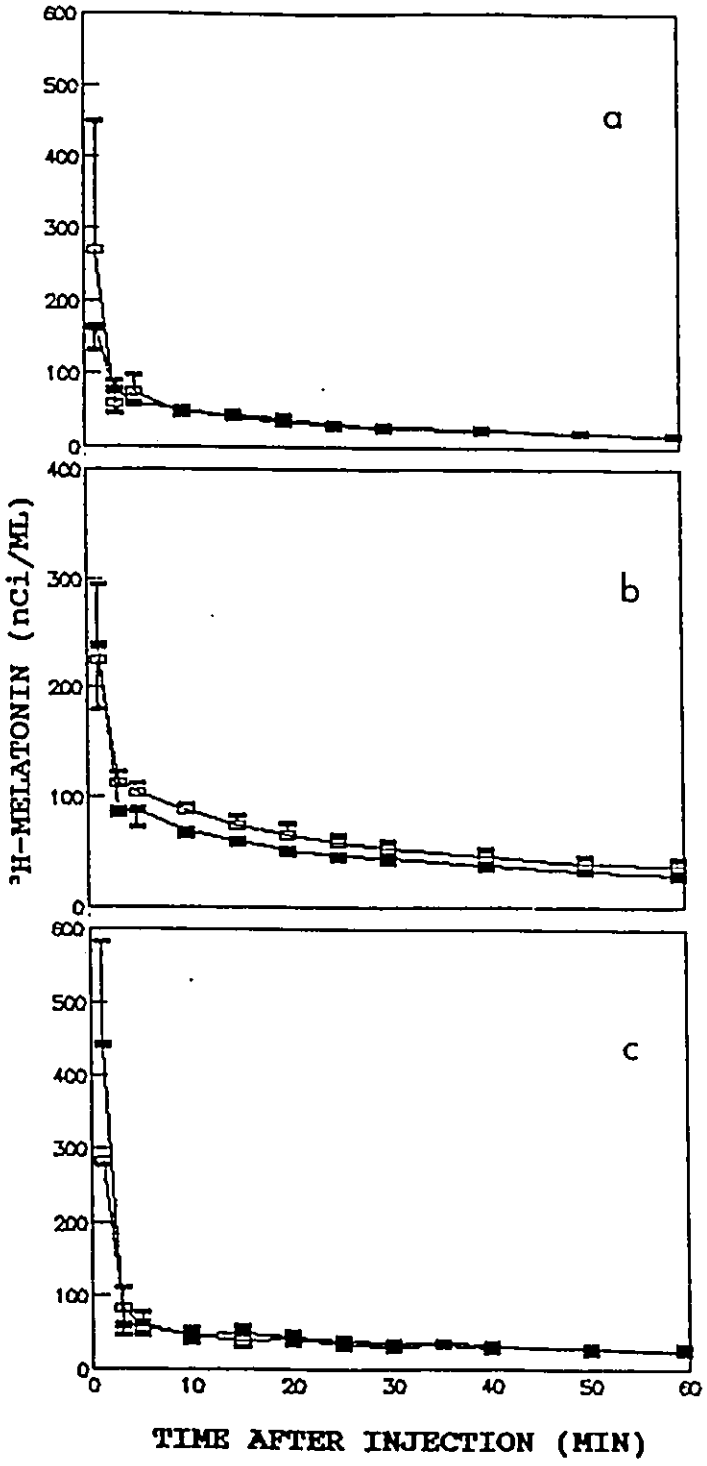

a
b
c
^{3}H-MELATONIN (nCi/ML)
TIME AFTER INJECTION (MIN)
0
100
200
300
400
500
600
10
20
30
40
50
60

Table 8

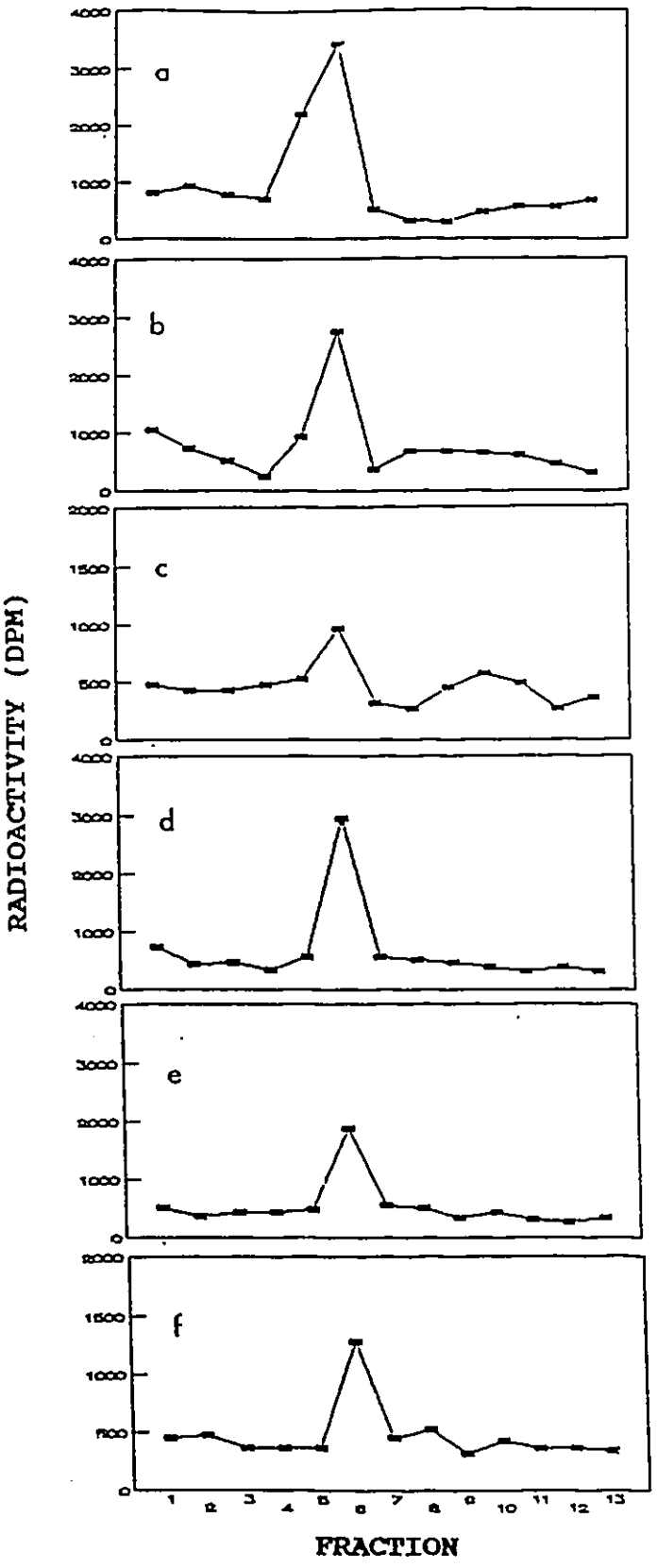
The Half Life (minutes) for Elimination of Plasma Melatonin in Rats after Castration and Sham-operation.

Age		Male	Female
2 month old	Castrated	24.8 ± 3.4*	28.6 ± 2.7
	Sham	23.5 ± 3.0	28.6 ± 1.3
6 month old	Castrated	30.1 ± 2.1	–
	Sham	31.4 ± 2.7	–

* mean ± SE (minutes)

The half life of melatonin was determined using a pharmacological programme (Greenblatt & Koch-Weser,1975) based upon the data shown in Fig 16 (a - c).

Fig.17. TLC of dichloromethane extracted plasma pools from each experimental group, i.e. 2 month old castrated (a) and sham-operated (b) male rats, 2 month old castrated (c) and sham-operated (d) female rats and 6 month old castrated (e) and sham-operated (f) male rats after injection of ^{3}H-melatonin. Thirteen 1 x 5 cm parallel sections of the gel were analyzed for radioactivity. The fraction number is the distance of the fraction from the origin. Similar chromatographic results were seen among the groups.


a
b
c
d
e
f
RADIOACTIVITY (DPM)
FRACTION

CHAPTER V

ALTERATION OF PINEAL RESPONSE TO ISOPROTERENOL BY SEX HORMONES IN RATS

5.1 Abstract

2 month old male and female rats were subjected to castration or sham-operation (N = 6 for each experimental group) and maintained under a 12:12 LD lighting cycle. The beta-adrenergic agonist, isoproterenol (ISO) was administered intra-peritoneally one hour after lights on. The urinary aMT6s excretion response to the injection was determined by RIA in animals individually kept in metabolic cages. In a time-course study, the animals were administered with saline or 1 mg/kg ISO and urine samples were collected every hour for seven hours after injection. In a dose-response study the animals were injected with various doses of ISO from 0 to 4 mg/kg and urine samples were collected for five hours post injection. Urinary aMT6s excretion response to ISO was higher in ovariectomized rats than that in sham-operated female animals. By contrast, orchiectomy reduced urinary aMT6s excretion response to ISO.

Changes in the serum and pineal melatonin responses to ISO by sex hormones were also studied in 2 month old male and female rats. Castrated, sex hormone-treated castrated and sham-operated animals (N = 6, for each group) kept under a 12:12 LD light cycle were injected with saline or 1 mg/kg ISO one hour after the onset of light. Pineal and blood samples were collected at 120 min post injection. Melatonin concentrations were determined by an Elisa. Consistently, ovariectomy increased the pineal and serum melatonin

responses to ISO and oestradiol blocked the elevation in ovariectomized rats, whereas orchiectomy decreased the response and testosterone treatment abolished the reduction. These results suggest that an oestradiol-induced inhibition and testosterone-caused stimulation of the pineal response to beta-adrenergic stimulation could be responsible for the effects of sex hormones on pineal melatonin production.

5.2 Introduction

As reported in Chapter III, sex hormones have a marked effect on urinary 6-sulphatoxymelatonin (aMT6s) excretion in rats; oestradiol inhibits while testosterone stimulates output of the melatonin metabolite in young adult rats. Since the effect does not result from alterations of melatonin metabolism (chapter IV), sex hormones seem to affect pineal melatonin synthesis and secretion. However, the question of the underlying mechanism of these effects remains unresolved.

It is well established that in the pineal gland melatonin is synthesized from serotonin through the activities of melatonin-forming enzymes, namely serotonin N-acetyltransferase (SNAT) and hydroxyindole-O-methyltransferase (HIOMT) (Axelrod & Weissbach, 1960; Weissbach et al, 1960). Of them, SNAT is the key enzyme in pineal melatonin synthesis and this enzyme is regulated by norepinephrine (NE), which is released from the sympathetic nerves that innervate the pineal gland, acting on pineal beta-adrenergic receptors (Kranse & Dubocovich, 1990; Sugden, 1989).

Existing evidence suggests that sex hormones may affect the activity of pineal HIOMT, but not pineal SNAT activity (Cardinal, 1980; Preslock, 1984). How sex hormones affect pineal HIOMT activity is unknown. On the other hand, the effect of sex hormones on this enzyme activity may not explain all mechanisms of

sex hormonal regulation of pineal melatonin production since the enzyme is not rate-limiting in melatonin synthesis. Sex hormones have been shown to affect several pineal constituents relative to melatonin synthesis both in vivo and in vitro. Those include NE turnover from pineal sympathetic endings (Cardinali, 1983) and in the pineal gland (Cardinali et al, 1975 b), pineal tryptophan hydroxylase (TH) activity (Moujir et al, 1990), serotonin content (Moujir et al, 1990) and metabolism (Vacas & Cardinali, 1979). Moreover, early and recent evidence has appeared indicating that sex hormones could induce changes in NE-induced increase of pineal adenylate cyclase activity (Weiss & Crayton, 1970) or cAMP levels (Davis, 1978) or elevation of pineal melatonin content by the beta-adrenergic agonist isoproterenol (ISO) (Hernandez et al, 1990; Moujir et al,1990). Activation of the beta-adrenergic receptors either through neural release of NE or injection of ISO induces an increase in pineal melatonin production (Axelrod et al, 1969; Brownstein et al,1973). Increased pineal melatonin will lead to higher circulating melatonin levels and urinary aMT6s excretion. According to recent evidence (Hernandez et al, 1990; Moujir et al, 1990) the effect of sex hormones on urinary aMT6s excretion in young adult rats could be the result of changes in the pineal response to adrenergic stimulation. Thus, two series of experiments were performed in the present study: 1) urinary aMT6s output response to stimulation by isoproterenol in castrated was compared with that in sham-operated 2 month old male and female rats and 2) a comparison of pineal and serum melatonin response to

injection with isoproterenol among castrated, sex hormone treated and sham-control rats at 2 months of age was carried out.

5.3 Materials and Methods

Animals

2 month old male and female Sprague-Dawley rats were used. In the first series of experiments, animals were divided into castrated and sham-operated groups; in the second series of experiments, castrated, sex hormone treatment and sham-control groups were divided. Each experimental group consisted of 6 animals. All animals were maintained in a cabinet with a temperature of 21°C under 12 hour light and 12 hour dark lighting cycle (12:12 LD). Lighting were provided by cool white fluorescent illuminance of 110 $\mu W/cm^2$. Food and tap water was supplied ad lib.

Castration

Ovariectomy/orchiectomy and sham-operation were performed as described in chapter III.

Urinary aMT6s Excretion Response to ISO

Time-course study: Both castrated and sham-operated animals were injected intraperitoneally (i.p) with saline as control or 1 mg/kg body weight of ISO (Sigma) dissolved in saline at one hour after lights on. The animals were held in metabolic cage individually for 7 hours. Urine samples were collected every hour from the

cage using the technique described in chapter III. The samples were stored at -20° C until assay.

Dose-dependent study: The animals were injected intraperitoneally (i.p) with different doses of ISO (0, 0.25, 0.5, 1 and 4 mg/kg body weight). The injection was also carried out one hour after lights on. The animals were kept individually in the metabolic cages and urine samples were collected for 5 hours after injection. The sample collection technique used was the same as above and samples were stored at -20°C prior to assay.

Urinary aMT6s RIA: Urinary aMT6s concentration was assayed by the radioimmunoassay using anti-6-sulphatoxymelatonin serum (CID-375) raised in sheep and iodinated aMT6s as tracer as previously described (chapters II & III).

Urinary Sex Hormone RIAs: Urinary oestradiol and testosterone excretion were measured by the RIAs as describe in Chapter II and III.

Pineal and Serum Melatonin Response to ISO.

Collection of serum and pineal gland: Oestradiol benzoate (EB) (50 μg/kg/day) and testosterone propionate (TP) (200 μg/kg/day) in sesame oil were administered intra-muscularly (i.m) to sex hormone treatment groups for 5 days, respectively. The animals were castrated 7 days before hormone treatment. Vehicle was injected

to castrated control and sham-operated groups. Injection of EB/TP or vehicle was carried out at the middle of the dark phase. Then, the animals were injected i.p. with either saline or 1 mg/kg body weight of ISO dissolved in saline one hour after lights on. Animals were killed by decapitation 120 min afterward and trunk blood was collected. The blood samples were allowed to clot at room temperature, centrifuged at 4000 x g for 20 min at 4°C and the serum was stored at -20°C until assay. The pineal glands were rapidly removed and stored at -70°C until assay.

Determination of melatonin: Serum and pineal melatonin concentrations was determined by a direct Elisa developed in our lab. Briefly, the anti-melatonin serum (CID-380W) raised in sheep diluted 1:1000 in coating buffer ($NaHCO_3$ (Fisher) 0.05 M, Na_2CO_3 (BDH) 0.05 M and thimerosal (Sigma) 0.1g/l, pH = 9.6) was used to coat immune micro-plates (Nunc, Denmark) at 4° C overnight. Then, the antibody coated plate was washed with a washing solution (NaCl (BDH) 15 mmol, thimerosal (Sigma) 0.1g/l and tween-20 (Sigma) 0.5 ml/l) three times. Melatonin standard (Sigma), ranging from 0.2 to 50 pg/well, melatonin alkaline phosphatase (AP) conjugate (20 ng/well, Helix Co.) and 50 μl serum or 5 μl supernatant of pineal gland homogenate were added in triplicates. 50 μl charcoal stripped rat serum was used to constitute the standard curve for serum melatonin measurement. The assay buffer was 0.1 M PBS (Na_2HPO_4 (BDH) 0.1 M, NaH_2PO_4 (BDH) 0.1 M, NaCl (BDH) 0.15 M, thimerosal (Sigma) 0.1 g/l and gelatin (Sigma) 1 g/l, pH = 7.4).

After overnight incubation at 4° C, the plates were washed three times with the washing solution. 150 µl/well of AP substrate (Sigma, 2 mg/ml) dissolved in glycine-NaOH buffer (glycine (Fisher) 0.1 M, NaOH (BDH) 0.1 M, $ZnCl_2$ (Caledon) 0.1 mM, $MgCl_2$ (BDH) 1 mM and Egg albumin (Sigma) 0.25 g/l) was added and incubated at room temperature overnight. The density of colour was determined by a micro-photometer at 405 nM.

Specificity of the anti-melatonin serum was excellent (Table 9). The assay has a sensitivity of 0.24 pg/well and average mid-range dosage of 3.51 pg/well. Within assay coefficient of variation (cv) is 6.4% for a quality control sample of 77.4 pg/ml and between assay cv is 7.6% (N=5). For a sample of 13.7 pg/ml within and between CVs are 11.4% and 14.4%, respectively. The assessment of within and between cv was done using the method of Rodbard (1974). The assay also showed good parallelism and recovery for both serum and pineal samples (Fig.18 and Table 10). Serum melatonin concentrations measured by the direct Elisa was compared with values obtained by a RIA using ^{3}H-melatonin as tracer (Pang et al, 1977) from the same samples. Linear regression analysis of the results yielded a line $Y = 1.84 + 0.98\ X$, $r = 0.91$, $n = 13$ (Fig.19). In order to further demonstrate the validation of the direct assay, comparison of assayed serum melatonin levels between the direct Elisa and the Elisa with prior dichloromethane extraction was performed. Linear regression analysis of the results also showed good correlation: $Y = 0.99 + 0.83\ X$, $r = 0.91$,

n = 38 (Fig.20).

Determination of Serum Sex Hormones: Serum testosterone in male rats and oestradiol in female rats were measured using an anti-oestradiol serum kindly donated by Dr. E Younglai of McMaster University (Younglai et al, 1985) and ^{3}H-oestradiol (New England Co.SA = 91 Ci/mmol) as a tracer and an anti-testosterone antiserum produced in this lab (Bubenik et al, 1975) and ^{3}H-testosterone (New England Co, SA = 89 Ci/mmol) as a tracer, respectively.

Statistic Analysis

All results are expressed as mean ± SE. The responses of urinary aMT6s output to ISO were statistically analyzed using a repeated measures MANOVA followed by one way analysis of variance with Duncan multiple range test or t-test as described below. One way analysis of variance with Duncan multiple range was performed for group comparisons of responses of melatonin in pineal gland and serum to ISO. Comparisons of urinary and serum sex hormone levels among castrated, sex-hormone replacement and sham-operated groups were also done by one-way analysis of variance. Correlations between serum melatonin levels measured by the Elisa and the RIA and between the Elisa with and without prior organic solvent extraction were analyzed by linear regression analysis.

5.4 Results

Urinary aMT6s Output Response to ISO

The time-course of the response of urinary aMT6s excretion to saline or 1 mg/kg body weight i.p ISO is shown in Fig 21. After injection of saline urinary aMT6s excretion was 0.29 ng/hr for female rats and 0.59 ng/hr for male rats; no marked changes occurred during the 7 hours post injection. No significant difference was found between castrated and sham-operated groups in either male or female rats after injection with saline. However, highly significant responses of urinary aMT6s excretion to ISO was observed in all experimental groups. In ovariectomized animals and their sham-operated controls aMT6s output significantly increased at 3 hours post injection, peaked at 4 hours and declined by 5 hours based on one way analysis of variance. There was a significant difference in urinary aMT6s excretion between ovariectomized and sham-operated rats ($F = 6.39$, $df = 1,10$, $p = 0.03$) with higher values in castrated animals. In male rats orchiectomy caused a different aMT6s response pattern from sham-operated controls ($F = 4.53$, $df = 6,60$, $p = 0.001$); i.e. orchiectomized animals only had a significantly higher value at 4 hours after injection of ISO while in sham-operated male rats an increase in aMT6s excretion began at 3 hours post injection, peaked at 4 hours and gradually declined until 7 hours (one way analysis of variance). Statistical analysis of these data showed that orchiectomy induced a decrease in aMT6s excretion response to ISO

(F = 9.98, df =1,10, p = 0.01). T-test revealed that at 2, 4 and 5 hours post injection orchiectomized rats had a lower aMT6s excretion than their controls.

The responses of urinary excretion to different doses of ISO injection are shown in Fig.22. In all animals aMT6s excretion responded to ISO doses from 0.25 to 4 mg/kg body weight doses in a dose-dependent fashion (p = 0.0001). Ovariectomized rats had a higher response than sham-operated female rats (F = 21.1, df = 1,10, p = 0.01) while orchiectomy decreased aMT6s excretion response compared with male sham-operated animals (F = 5.72, df = 1,10, p = 0.038). No significant interaction between group and ISO doses was detected in either male or female animals.

Serum and Pineal Melatonin Response to ISO

Serum and pineal melatonin levels after saline injection were significantly lower than those after to ISO (Fig.23 & 24). In female rats the highest serum (Fig.23 a) and pineal (Fig.23 b) melatonin responses to ISO were observed in the ovariectomized group (F = 20.3, df = 3, 20, p = 0.0001 for serum melatonin and F = 23.1, df = 3,20, p = 0.0001 for pineal melatonin). There were no significant difference in pineal and serum melatonin levels between oestradiol treated ovariectomized and sham-operated control animals. Sham-operated and testosterone treated orchiectomized rats had higher responses of serum (Fig 24 a) and pineal (Fig.24 b) melatonin than orchiectomized rats (F = 11.8, df = 3,20, p = 0.0001

for serum melatonin and $F = 14.4$, $df = 3,20$, $p = 0.0001$ for pineal melatonin). No significant difference in pineal and serum melatonin levels is found between sham-operated control and testosterone treated castrated rats.

Sex Hormones in Urine and Serum

In the first series of experiments urinary oestradiol output in ovariectomized animals was 0.75 ± 0.4 ng/24 hour (mean $\pm$ SE) while 2.06 ± 0.34 ng/ 24 hours was found in sham-operated female rats. Urinary testosterone excretions in orchiectomized and sham-operated male rats were 0.96 ± 0.15 ng/24 hours and 12.74 ± 4.32 ng/24 hours, respectively. In the second series of experiments serum oestradiol concentrations in ovariectomized animals was 1.39 ± 0.35 pg/ml (mean $\pm$ SE). In sham-operated female and oestradiol treated ovariectomized rats serum oestradiol levels were 8.2 ± 2.6 pg/ml and 145.8 ± 22.6 pg/ml, respectively. In orchiectomized animals serum testosterone concentration was 0.15 ± 0.02 ng/ml. Sham-operated male rats had 1.58 ± 0.61 ng/ml of serum testosterone and in testosterone treated orchiectomized animals serum testosterone concentration was 4.84 ± 0.2 ng/ml.

5.5 Discussion

The mechanisms of sex hormonal regulation of pineal melatonin production are not well known, although the effects of sex hormones on pineal function have been recognized for almost 30 years

(Cardinali, 1980; Preslock, 1984). It is established that pineal melatonin production is mainly controlled by the photoperiod through a well known noradrenergic mechanism (Abreu et al, 1987; Binkley, 1983). Removing the SCG or cutting the pre-ganglionic trunks of the SCG abolishes the nocturnal increase of melatonin secretion (Cardinali & Vacas, 1987; Preslock, 1984). Castration and/or sex hormone treatment did not abolish the primary photoperiodic variation of aMT6s excretion although the pattern or amplitude of the diurnal rhythm was changed (chapter III) suggesting that sex hormones may only act to modify the noradrenergic mechanism. Weiss & Crayton (1970) and Davis (1978) observed a reduction in response of pineal adenylate cyclase activity or levels of cAMP induced by NE during proestrus or after oestradiol administration in ovariectomized rats. Recently, two studies reported by Alonso's group showed that pineal melatonin responses to ISO were changed as a function of the stage of rat oestrous cycle, that oestradiol blocked the ISO-induced elevation of melatonin in ovariectomized rats and that castration impaired noradrenergic input in male rats (Hernandez et al, 1990; Moujir et al, 1990). The present results are in good agreement with those observations.

In the present study the time of injection of ISO was chosen to eliminate the influence of darkness. Rhythms in the density of pineal beta-adrenergic receptors have been previously reported in which a peak value occurs just prior to lights off (Wirz-Justice,

1987) or at the middle of the dark phase (Gonzalez-Brito et al, 1988; Weiland & Wise, 1989). Melatonin production also gradually increases after the onset of darkness (Binkley, 1983). Maximum responsiveness in the synthesis of melatonin to ISO stimulation can be obtained by injection at this time (Romero et al,1975 a). However, the effect of nocturnal activation of pineal function would not be eliminated. On the other hand, the light cycle would be changed if animals were kept under continuous lighting in order to eliminate nocturnal activation; in our case, 7 hours after light on was used in the time-course study and 5 hours in the dose-dependent study.

Since the measurement of urinary aMT6s excretion is believed to represent pineal melatonin production in both human (Bojkowski et al, 1987) and rats (Brown et al, 1991), presumably it can be also used as an index of the pineal response. Indeed, both castrated and sham-operated control animals had no response to saline injection. Post ISO stimulation, all animals had an elevation in urinary aMT6s output, which is similar to the response of pineal NAT activity (Illnerova & Vanecek, 1983; Romero et al, 1975 a; Zatz et al, 1978) and melatonin (Chik et al, 1987 b) to ISO. There was a delayed peak response and a slow decline of urinary aMT6s excretion compared to pineal and serum melatonin response to ISO injected just prior to lights off, which reached maximum at about 2 hours post injection (Chik et al, 1987 b). In order to have a consistent experimental condition, the same ISO injection time was

chosen for serum and pineal melatonin response to ISO as that of the aMT6s excretion response. ISO at 2 hours post injection increased pineal and serum melatonin concentrations in all animals compared with saline injection. The results of responsiveness in pineal and serum to ISO further strengthen the finding that urinary aMT6s can be used as an index of melatonin production and secretion.

The pineal melatonin response to ISO stimulation resides in the mechanisms regulating the various steps involved in the production of melatonin (Zatz et al, 1978). The present results suggest that sex hormones may affect pineal melatonin production through alteration of adrenergic mechanisms. However, the exact sites cannot be identified by this study. It could be acting on pineal beta-adrenergic receptors, pineal adenylate cyclase activity, melatonin forming enzyme activities etc. Hence, it would be of interest to determine which steps are affected by sex hormones.

In summary, we investigated the effect of sex hormones on the pineal response to beta-adrenergic stimulation in rats by observation of the response of urinary aMT6s, pineal and serum melatonin. The results showed that oestradiol reduced while testosterone stimulated the pineal response to beta-adrenergic stimulation. Oestradiol-induced sub-sensitivity and testosterone-induced super-sensitivity of pineal adrenergic systems may be one of the mechanisms of gonadal steroid regulation of pineal melatonin

production.

Table 9

Cross-reaction of various analogs of melatonin with melatonin antiserum raised in sheep (CID 380W) determined at 50% displacement of MT-pcb-AP conjugate.

Compound	Percent Cross-reaction
Melatonin	100
6-hydroxymelatonin	0.05
5-methoxytryptophol	<0.001
5-methoxytryptamine	0.001
N-acetylserotonin	0.0019
Serotonin	<0.001

Fig.18. Dose dependent inhibition of binding of melatonin standards and different aliquots of serum and pineal homogenate supernatant to melatonin antibody.

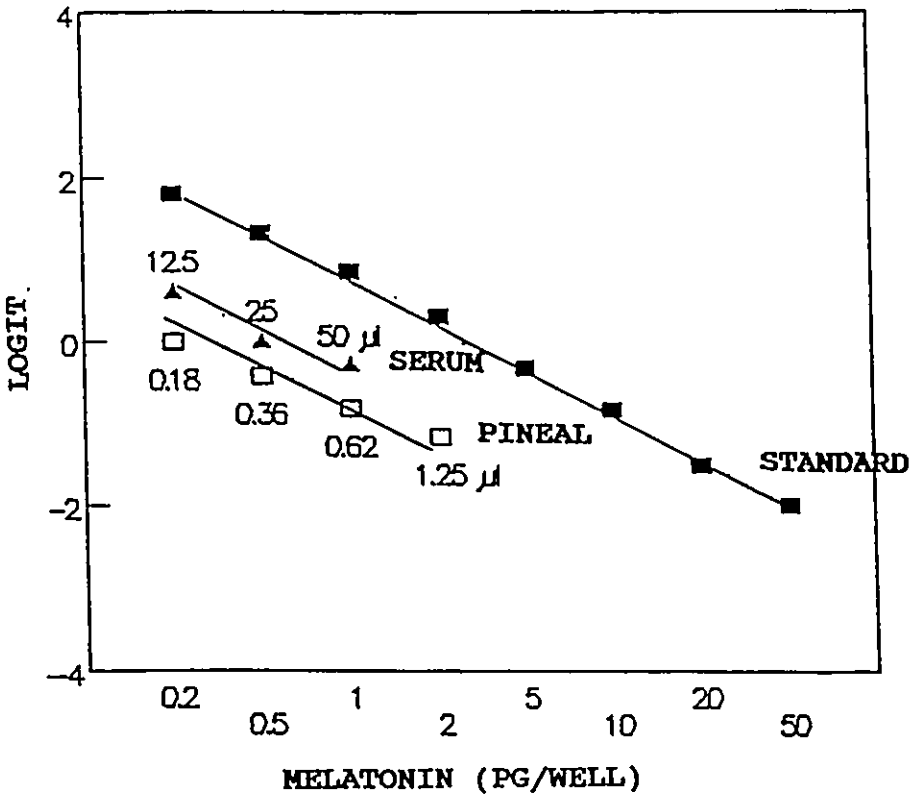


Table 10

Analytical recovery of melatonin in rat serum and pineal homogenate supernatant in Elisa.

Sample	Assayed Value pg/well	Expected Value pg/well	Recovery %
Serum Samples			
	1.58	1.58	
	2.58	2.58	100
	4.15	3.58	116
	6.75	6.58	103
	11.24	11.58	97
mean ± SE			103.2 ± 7.5
Pineal Homogenate (Diluted)			
	0.27	0.27	
	0.84	0.77	109
	1.2	1.27	94
	6.61	5.27	125
	10.6	10.27	103
mean ± SE			106.2 ± 11.8

Fig.19. Scattergram of melatonin concentration determined by RIA and Elisa for 13 rat serum samples.

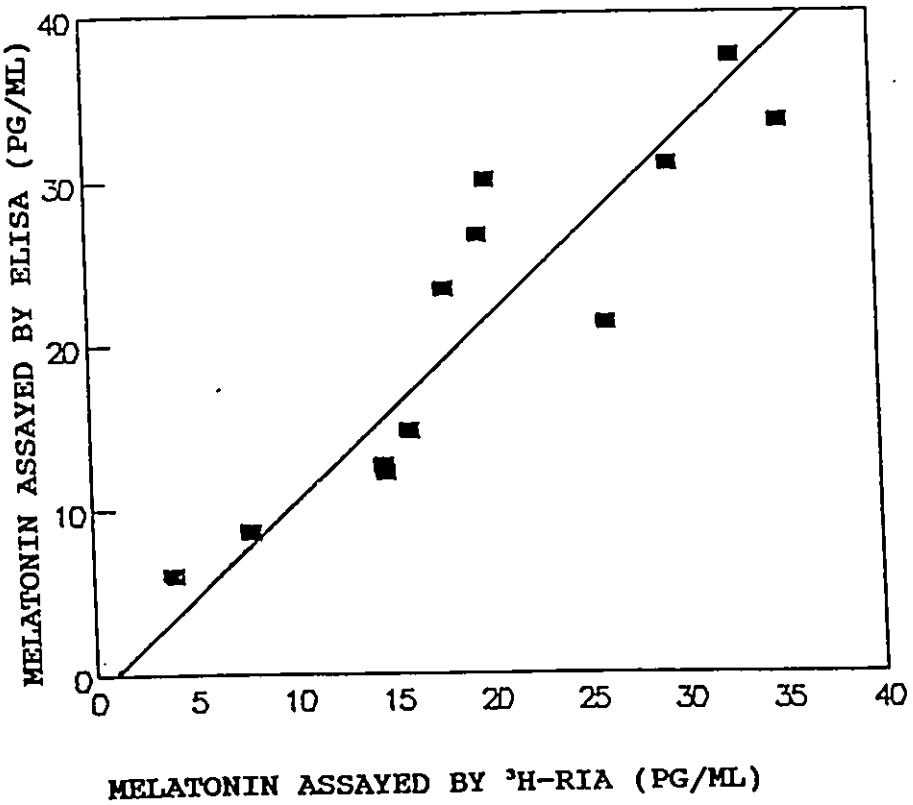


Fig.20. Relationship of melatonin concentrations in 36 rat samples as determined by the Elisa with (X) and without (Y) an organic solvent extraction step.

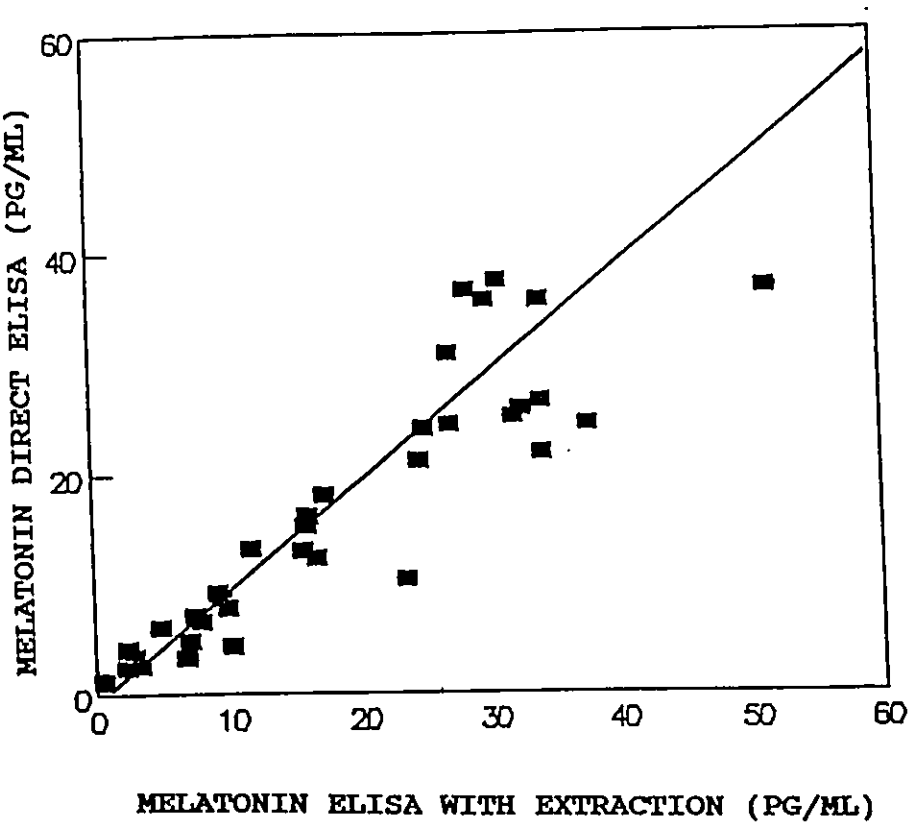


Fig.21. Time course of response of urinary aMT6s excretion to 1 mg/kg ISO (i.p) or saline in young adult male (a) and female (b) rats. Each point represents the mean ± SE, N = 6. (*): $p < 0.05$ sham-operated controls vs castrated ISO treated animals.

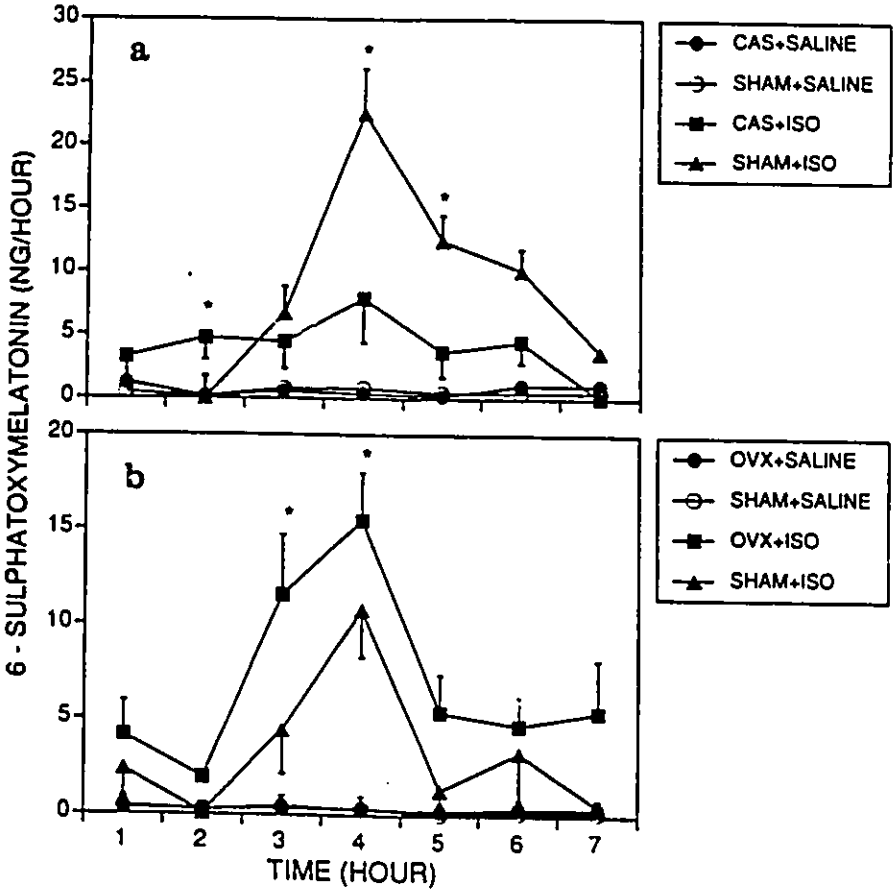

a
b
CAS+SALINE
SHAM+SALINE
CAS+ISO
SHAM+ISO
OVX+SALINE
SHAM+SALINE
OVX+ISO
SHAM+ISO
6 - SULPHATOXYMELATONIN (NG/HOUR)
TIME (HOUR)
0
5
10
15
20
25
30
1
2
3
4
5
6
7

Fig.22. Dose-dependent response of urinary aMT6s excretion 5 hours post ISO injection in young adult male (a) and female (b) rats. Each point represents the mean ± SE, N = 6. (*): $p < 0.05$ sham-operated control vs castrated animals.

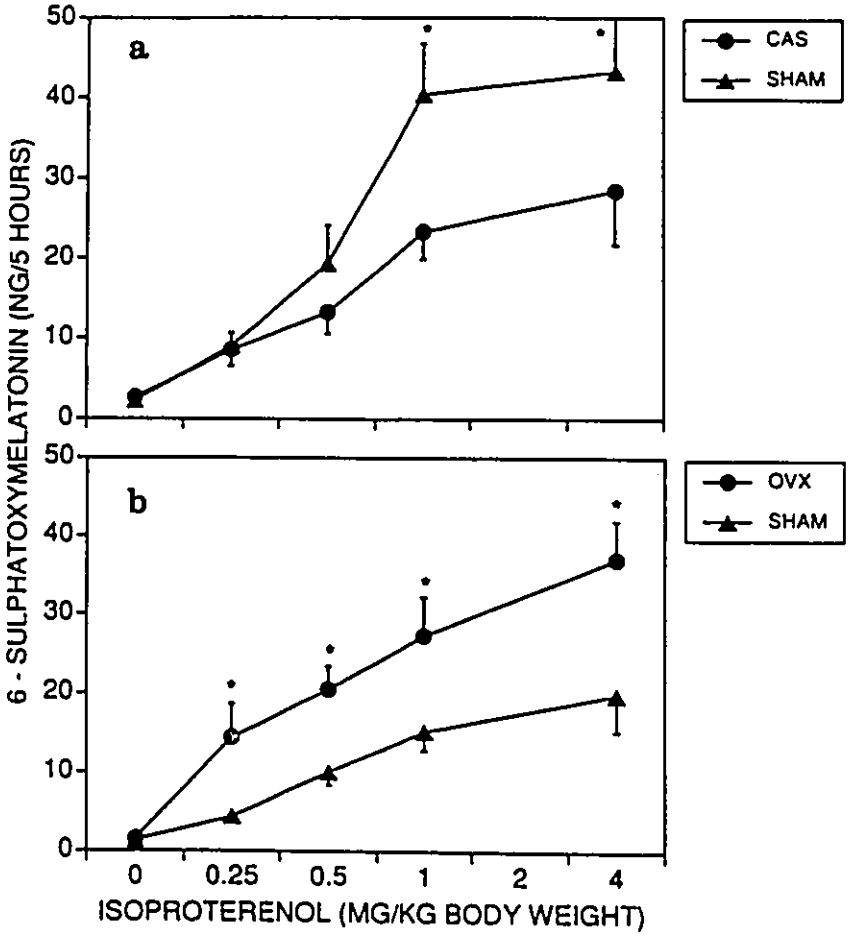

a
b
CAS
SHAM
OVX
SHAM
6 - SULPHATOXYMELATONIN (NG/5 HOURS)
0
10
20
30
40
50
0
0.25
0.5
1
2
4
ISOPROTERENOL (MG/KG BODY WEIGHT)

Fig.23. Mean serum (a) and pineal (b) melatonin levels at 2 hours after administration of 1 mg/kg ISO in ovariectomized, oestradiol treated ovariectomized and sham-operated female rats. One standard error of the mean is shown for each bar; N = 6 for each group. (*): $p < 0.05$ when ovariectomized rats are compared to oestradiol treated and sham-operated animals.

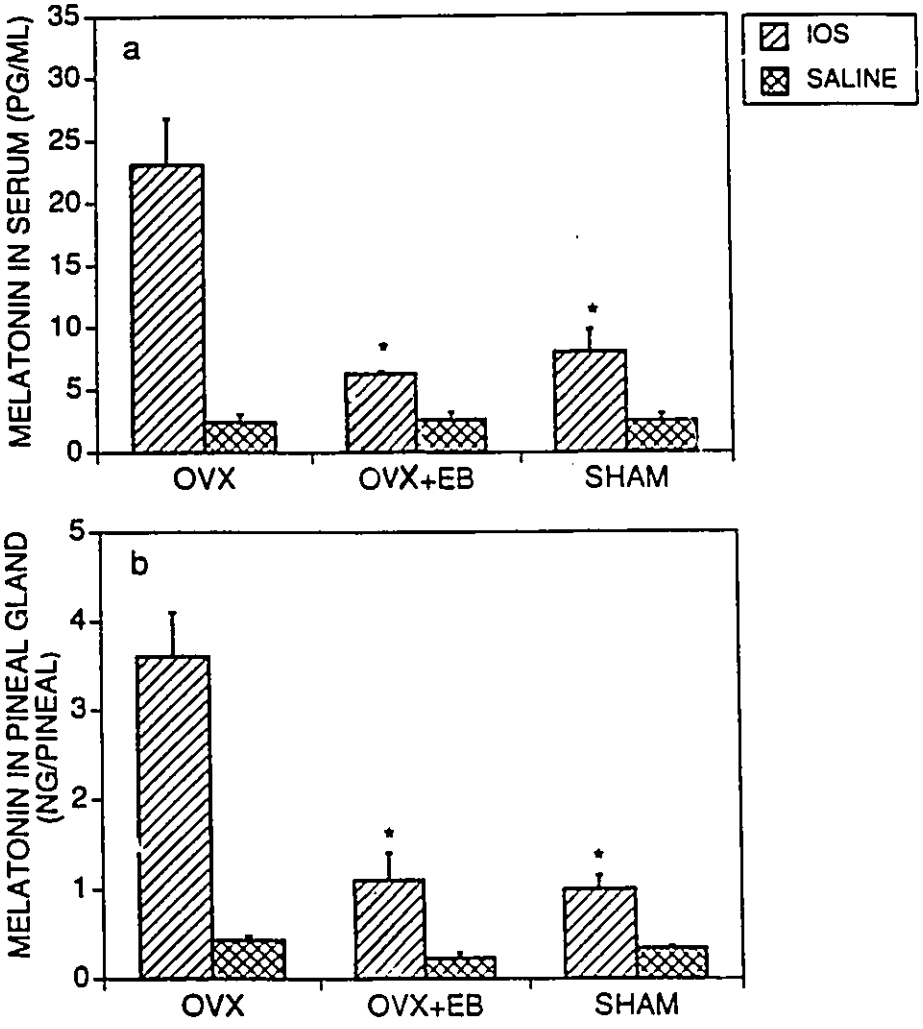
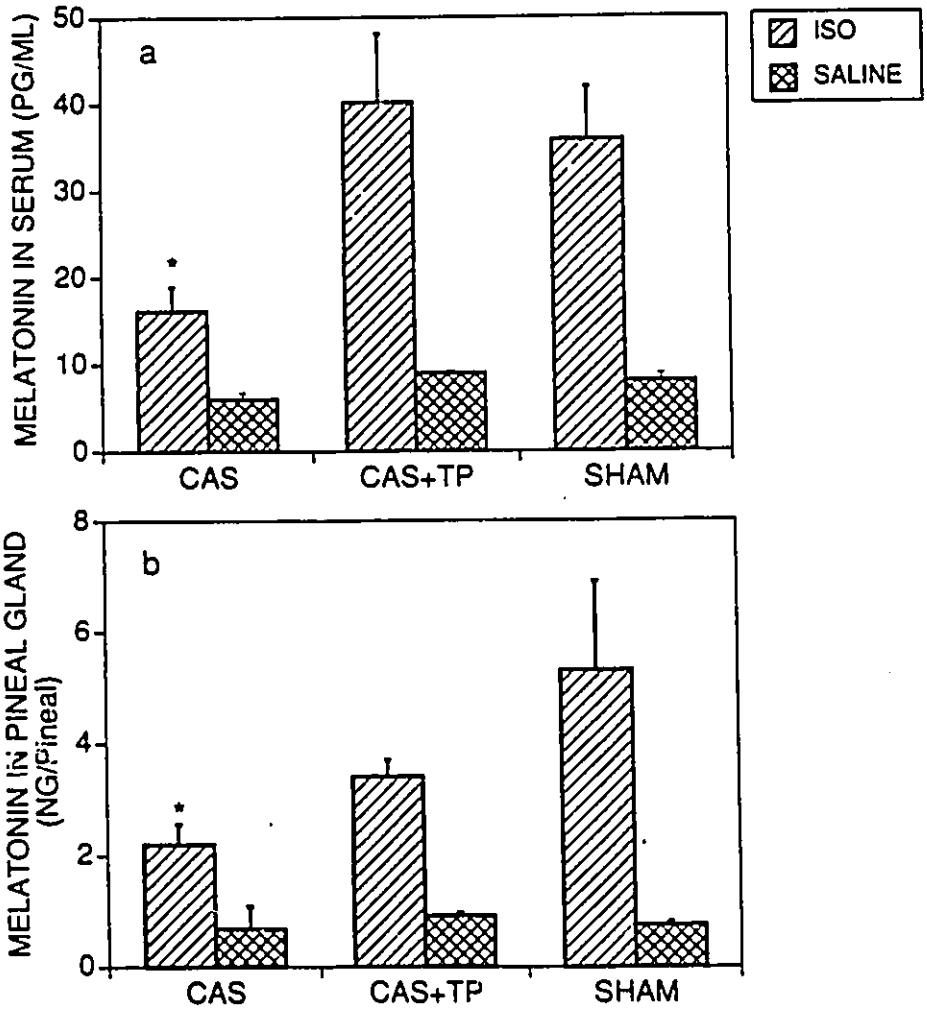

a
MELATONIN IN SERUM (PG/ML)
35
30
25
20
15
10
5
0
IOS
SALINE
OVX
OVX+EB
SHAM
b
MELATONIN IN PINEAL GLAND (NG/PINEAL)
5
4
3
2
1
0
OVX
OVX+EB
SHAM

Fig.24. Mean serum (a) and pineal (b) melatonin levels 2 hours after injection of 1 mg/kg ISO in orchiectomized, testosterone treated orchiectomized and sham-operated young adult male rats. The values are presented as mean $\pm$ SE; N = 6 for each group. (*): $p < 0.05$ when orchiectomized rats are compared to testosterone-treated and sham-operated animals.


a
MELATONIN IN SERUM (PG/ML)
50
40
30
20
10
0
CAS
CAS+TP
SHAM
ISO
SALINE
b
MELATONIN IN PINEAL GLAND (NG/Pineal)
8
6
4
2
0
CAS
CAS+TP
SHAM

CHAPTER VI

EFFECTS OF SEX HORMONES ON RAT PINEAL BETA-ADRENERGIC RECEPTORS

6.1 Abstract

The effects of sex hormones on pineal beta-adrenergic receptors were studied in 2 month old rats. Animals kept under a 12 : 12 LD lighting cycle were castrated or sham-operated. Sex hormones were administered to castrated animals and vehicle to castrated control and sham-operated groups; each group consisted of 6 animals. The pineal glands were collected one hour prior to the onset of the darkness and beta-adrenergic receptor binding was determined utilizing ^{3}H-dihydroalpranolol (^{3}H-DHA) in a single pineal gland. Ovariectomy increased the density of pineal beta-adrenergic receptors compared with oestradiol treated ovariectomized and sham-operated females rats. By contrast, orchiectomized rats had a lower concentration of the receptor than their sham-operated counterparts. Testosterone treatment to orchiectomized rats abolished the down-regulation by orchiectomy. No significant effect of sex hormones on K_d values of pineal beta-adrenergic receptors was found in either male or female rats. The results of this study demonstrated that one site of sex hormonal regulation of pineal melatonin production is through modulation of the density of pineal beta-adrenergic receptors.

6.2 Introduction

According to the results described in chapter V, oestradiol suppresses the beta-adrenergically induced activation of pineal melatonin production whereas testosterone elevates the pineal response. This is in good agreement with observations of an alteration in the responses of pineal adenylate cyclase activity and of pineal cAMP levels to norepinephrine (NE) (Davis, 1978; Weiss & Crayton, 1970) and of pineal melatonin content to ISO (Moujin et al, 1990).

Since the pineal response to adrenergic stimulation resides in the mechanisms regulating the various steps involved in the synthesis of melatonin (Zatz et al, 1978), it could be suspected that sex hormones interact with one or more of those steps; i.e. sex hormones could induce changes in the properties of the beta-adrenergic receptors, coupling of the receptor to adenylate cyclase or adenylate cyclase activity and the melatonin-forming enzyme activities. The present study was designed to investigate possible effects of sex hormones on pineal beta-adrenergic receptors.

The effect of oestradiol on pineal beta-adrenergic receptor in rats has been reported but the results were controversial. No significant effect of oestradiol on pineal beta-adrenergic receptors was reported by Vacas and coworkers (Vacas et al, 1980)

while Weiland and Wise (1989) demonstrated that oestradiol could induce a reduction in the diurnal profile of the pineal beta-receptor in rats. One report appeared showing an inhibitory effect of testosterone on pineal beta-receptors in rats (Vacas et al, 1982). Based upon our previous results (Chapter III & V), we assumed that oestradiol might have an inhibitory while testosterone a stimulating effect on pineal beta-adrenergic receptors in young adult rats. To test this hypothesis specific ^{3}H-dihydroalpraprolol (DHA) binding study was performed on the pineals obtained from castrated, sex hormone treated and sham-operated male and female rats at 2 months of age.

6.3 Materials and Methods

Animals

2 month old male and female Sprague-Dawley rats were maintained at 21°C in a cabinet with a diurnal lighting cycle (12 hour light and 12 hour dark). Light was provided by white fluorescent illuminance of 110 $\mu W/cm^2$. All rats had access to food and tap water ad libitum. The animals were castrated or sham-operated. The castrated animals were further divided into castrated control and castrated sex hormones-treatment groups. Each experimental group consisted of 6 animals.

Castration and Sex Hormone Treatment

Bilateral ovariectomy/orchiectomy and sham-operation were performed

under Rogarsetic (Rogar/STB Inc, Canada; 42 mg/kg body weight) and Atravet (Ayerst Laboratories, Canada; 2.5 mg/kg body weight) as described in chapter III. Oestradiol Benzoate (EB) (50 µg/kg/day) in sesame oil was administered intramuscularly (i.m) to castrated female rats and testosterone propionate (TP) (200 µg/kg/day, in sesame oil) i.m to male castrated rats and vehicle to castrated control and sham-operated groups for 5 days beginning 7 days after surgery. Injection of EB/TP or vehicle was performed at the middle of the dark phase.

Collection of Serum and Pineal gland

The rats were killed by decapitation one hour prior to the onset of darkness. The time was chosen because this is the time of peak pineal beta-adrenergic receptor density (Greenberg & Weiss, 1978; Reiter et al, 1982; Romero et al, 1975 b; Wirz-Justice, 1987). Pineals were rapidly removed, frozen and stored at -70° C until used. Trunk blood was collected for determination of oestradiol concentrations in female and testosterone levels in male rats. The blood samples were allowed to clot at room temperature, centrifuged at 4000 x g for 20 min at 4°C and serum collected was stored at -20° C prior to assays.

^{3}H-DHA Binding Assay

Single pineal glands were homogenized in 0.5 ml of ice-cold tris-HCl buffer (50 mM, pH = 7.4 at 4° C) containing Mg^{++} (10 mM) and EDTA (1 mM) (Dax & Sugden, 1988). A tri-R homogenizer (model-44)

was used (setting at 2.5 for 2 min). The pellets were centrifuged at 50,000 x g for 15 min, washed in the same buffer twice, and finally re-suspended in 0.5 ml of the buffer.

Pineal beta-adrenergic receptor binding was assayed by the method of Yocca & Friedman (1984). ^{3}H-DHA (New England Nuclear Cop, USA; SA = 107 Ci/mmol) was used as a ligand in the range of 0.4 to 5 nM. The concentrations of the ligand were chosen in such a way that the dissociation constant (K_d) of the ligand fell within the concentration range (Sethy et al, 1986). Five concentrations each in duplicate were used for the saturation isotherm in a single pineal assay.

^{3}H-DHA binding was routinely determined by incubating 0.3 ml aliquots of the above particulate suspension (representing around 10 µg protein of the pineal tissue) with 0.1 ml of the labelled-ligand and 0.1 ml of the buffer or (-) propranolol. The final incubation volume was 0.5 ml/tube. The samples were incubated for 40 min at room temperature (22° C). The incubation was terminated by filtering the sample under vacuum through a Whatman GF/B glass filter disc (2.4 cm in diameter). Each tube was rinsed twice with 4 ml of ice-cold regular tris buffer (tris base 6.06 g /l, 3N HCl 15 ml/l, pH = 7.4) which was also filtered and finally the filter disc was washed three times using 4 ml of the buffer for each wash. Total filtering time was about 10 sec. Then the filter paper was placed in a scintillation vial to which 3 ml of scintillation fluid

cocktail (Beckman) was added. The vials were capped and stood in dark at room temperature overnight and the total radioactivity in the discs was counted by a beta-counter (Beckman LS-7500; counting efficiency = 61%).

The extent of non-specific binding was defined from assay tubes which contained a large excess of (-) propranolol (10^{-4} M). Specific binding was defined as the total binding minus non-specific binding.

Protein was measured by the Lowry method (Lowry et al, 1951) with bovine serum albumin (BSA) as a standard. A single gland gave sufficient tissue to perform a five concentration saturation study in duplicate. In each binding assay pineal samples from each group were included; there were approximately 6 pineal glands per assay.

Serum Sex Hormone Assays

Serum testosterone levels in male rats and oestradiol in female animals were determined by RIAs as described in chapter V.

Statistical Analysis

Scatchard analysis (Scatchard, 1949) was used to determine K_d and maximum number of binding sites (Bmax) of pineal beta-adrenergic receptors. Statistical analysis of data, generated by the Scatchard plot, was done using one-way analysis of variance with

Duncan multiple range test. Comparisons of serum sex hormone levels among castrated, sex-hormone treatment and sham-operated groups were also done by one-way analysis of variance. All results are represented as mean ± SE.

6.4 Results

Tissue Linearity

The amount of ^{3}H-DHA bound by the filter in the absence of tissue was about 0.3 % of total radioactivity in the incubation tube and was the same in the presence and absence of 10^{-4} M (-) propranolol. This filter binding was part of the non-specific binding defined above. Pineal specific ^{3}H-DHA binding increases linearly with increasing pineal tissue over the range of 1 to 10 μg of homogenate protein per assay tube. At higher tissue protein concentrations the binding is less than linear (Fig.25). Thus, assays were routinely conducted with 5 - 10 μg of homogenate protein, which was well within the linear range.

Time Dependence

The specific binding of ^{3}H-DHA to the pineal homogenate was rapid, reaching equilibrium by approximately 20 to 40 min at room temperature. The half-maximal binding was attained at 10 to 15 min (fig.26).

Saturation of ^{3}H-DHA Binding

Specific binding of ^{3}H-DHA is saturable with a half maximal binding at about 1 nM (Fig.27 a). Non-specific binding increased linearly with increasing ^{3}H-DHA concentrations. Non-specific binding represented 11-32 % of total binding over the concentrations of ^{3}H-DHA used. Scatchard analysis of this saturation curve indicates a single binding component with an apparent dissociation constant of about 1.03 nM in a pineal homogenate pool (Fig.27 b). To test for cooperativity, we plotted these data according to the Hill equation (Chang et al, 1979) (Fig.27 c). A straight line is obtained with a Hill coefficient of 0.98, suggesting the absence of positive or negative cooperative effects within this concentration range.

Inhibition by (-) Propranolol

The specific binding was readily reversible by addition of (-) propranolol (10^{-12} to 10^{-2} M) (Fig.28 a). The IC_{50} value of inhibition of ^{3}H-DHA binding by (-) propranolol was 6.2 nM (Fig.28 b).

The effects of Sex Hormones on Pineal beta-Adrenergic Receptors

The pineal beta-adrenergic receptor bindings of different experimental groups are summarised in Table 13. No significant differences in K_d among experimental groups were detected in either male or female animals. An increase in pineal beta-adrenergic receptor density was found in ovariectomized rats compared with their sham-control and oestradiol treated ovariectomized rats (F =

6.62, df = 2,15, p = 0.009), but there was no difference between sham control and oestradiol treated animals. In male rats, sham-operated and testosterone treated orchiectomized animals had higher density of pineal beta-adrenergic receptor than orchiectomized rats ($F = 4.07$, df =2,15, $p = 0.04$), although no significant difference between sham and testosterone treated rats was found. Furthermore, to confirm the results, we pooled the pineal homogenate from each experimental group in male rats (pools of female rat pineal homogenate have been used for studies of experimental validation described above) to run more than five concentrations of ^{3}H-DHA. The results clearly showed that pineal beta-adrenergic receptor density was highest in testosterone-treated orchiectomized rats and lowest in orchiectomized animals (Fig.29-31). (However, the binding sites were lower than those average values in the single pineal binding study. Probably, it is because that the pineal homogenate pools were made from the single pineal samples which had been thawed and frozen twice during the experiment).

The protein contents in pineal glands are also shown in Table 11. There was no significant difference in pineal total protein among the experimental groups in either male or female rats.

Serum Sex Hormone Concentration

In ovariectomized animals the oestradiol concentration was 0.65 ± 0.01 pg/ml (mean ± SE) while levels of 6.1 ± 1.6 pg/ ml in sham-

operated and 164.8 ± 17.6 pg/ml in oestradiol-treated rats were found, respectively. Orchiectomized rats have lower serum testosterone levels (0.17 ± 0.01 ng/ml) than their sham-operated controls (2.26 ± 0.39 ng/ml and testosterone-treated orchiectomized rats (4.46 ± 0.3 ng/ml).

6.5 Discussion

Utilizing ^{3}H-DHA, pineal beta-adrenergic receptors in rats have been studied by a number of investigators (Ebadi & Govitrapong, 1986). However, the observations were done under different conditions. For example, the pineal glands were removed at 5 to 7 hours after lights on by some authors (Greenberg & Weiss, 1978) while the pineal collection was performed at the middle of the night by others (Yocca & Friedman, 1984). In some experiments the rats were housed under a 12/12 light/dark cycle (Greenberg & Weiss, 1978; Yocca & Friedman, 1984) while continuous light was provided to animals in another study (Moyer et al, 1980). Since diurnal rhythms in the density of beta-adrenergic receptors in the rat pineal gland have been demonstrated (Gonzalez-Brito et al, 1988; Romero et al, 1975 b; Weiland & Wise, 1989; Wirz-Justice, 1987), those results were not comparable. The Bmax values of pineal beta-adrenergic receptors in the sham-operated control animals in the present study are similar to those reported by Wirz-Justice (1987) under similar conditions. No clear evidence exists indicating that there is a diurnal variation in K_d values of pineal

beta-adrenergic receptors. The average K_d values (1.5 to 2.4 nM) observed in the sham-operated controls of the present study seem to be lower than those reported by others (4 - 5 nM reported by Greenberg & Weiss (1978) and Yocca & Friedman (1984) and 8 - 11 nM presented by Moyer et al (1980) and Vacas et al (1980; 1982)). The K_d values of a radiolabelled ligand bound to its receptor depend upon a variety of factors; one is the specific activity of the radiolabelled ligand used (Bennett & Yamamura, 1985). The SA (107 Ci/mmol) of ^{3}H-DHA used in the present study was much higher than those in previous reports (32-49 Ci/mmol). This may explain the difference in K_d values between us and others using the same assay method.

In this study we have demonstrated an reduction in the density of pineal beta-adrenergic receptors by oestradiol in young adult female rats. This is in good agreement with an observation by Weiland & Wise (1989) but contrary to that of Vacas et al (1980). The failure to observe a significant effect of oestradiol on pineal beta-adrenergic receptor density by Vacas et al was probably due to the use of a high concentration of pineal tissue (250-290 μg protein of pineal tissue per assay tube), in which ^{3}H-DHA binding is less than linear (fig.25). In addition, they removed the pineal glands 5 hours after lights on (the animals were kept under 12:12 LD lighting cycle), at which time the levels of pineal beta-adrenergic receptors would be low. At the middle of the light phase no effect of oestradiol on urinary aMT6s excretion (Chapter

III) or serum and pineal melatonin levels (Jonhson et al, 1982; Ozaki et al, 1978) were found. In the report of Weiland & Wise (1989) the effect of oestradiol on the beta-receptors during the light phase was also not as pronounced as that at the receptor peak time. Therefore, it is likely that the present results together with the finding by Weiland and Wise are valid. In addition, the foregoing finding is consistent with our previous observation of effects on urinary aMT6s excretion (Chapter III) as well as on the pineal response to adrenergic stimulation (Chapter V).

The stimulating effect of testosterone on pineal beta-adrenergic receptors in male rats in the present study is contrary to another study conducted by Vacas et al (1982), in which testosterone depressed the pineal beta-adrenergic receptor content in castrated rats. Testosterone was reported to affect pineal HIOMT activities in a dose-dependent way; low doses (0.01 - 1 mg/day) stimulated the enzyme activity while high doses (5 - 10 mg/day) decreased pineal HIOMT activity in castrates (Nagle et al, 1974; Daya & Potgieter, 1982). The inhibitory effect of testosterone in Vacas' report maybe resulted from the relatively high dose of testosterone used (5 mg/kg/day). Our previous results also consistently showed that testosterone exerts a stimulating effect on pineal melatonin production (chapter III) and elevates the pineal response to adrenergic stimulation (Chapter V) in a dose of 200 μg/kg/day; which are consistent with an increase in beta-adrenergic receptor density.

Although specific oestradiol and testosterone receptors have been identified in rat pineal gland (Cardinali et al,1974 a; 1975 a; Cardinali, 1977), the molecular mechanism of sex hormonal regulation of pineal beta-adrenergic receptor is completely unknown. It has been proposed that the mechanism of sex hormone action on beta-adrenergic receptors may be by affecting the transcription of the genes for the beta-adrenergic receptors, thus influencing the steady-state level of the receptor in cells (Stiles et al, 1984; Collins et al, 1989). However, this hypothesis has not been properly tested, although there was one report that orchiectomy and testosterone treatment affect the beta-2-adrenergic receptor mRNA in the rat ventral prostate (Collins et al, 1988).

On the other hand, sex hormones have been also shown to affect several pineal constituents including pineal morphology (Clementi et al, 1965; Ruiz-Navarro et al, 1982), lipid, nuclear acid and protein (Zweens, 1965; Nir et al, 1970; Cardinali, 1974 b; 1976 b; Mizobe & Kurokawa, 1978), NE and serotonin turnover rates (Cardinali et al, 1975 b; Vacas & Cardinali, 1979). The association between these effects and pineal melatonin production are unclear. However, these findings suggest that sex hormones may interact with multiple sites in pineal melatonin synthesis pathway. In addition, castration and sex hormone treatment can induce a large change in the hypothalamus-pituitary-gonadal axis. Gonadotropins and prolactin were also reported to have a pronounced influence on pineal melatonin production in vitro (Cardinali et al,

1987; Vacas et al, 1987 b) and a close correlation between serum melatonin and gonadotropins and prolactin was recently reported in women (Fernandez et al, 1990). We cannot rule out the possibility that the effect of gonadal steroids on pineal melatonin production could be due to changes in gonadotropins and prolactin induced by castration and sex hormone treatment. Further studies will be required to resolve these questions before a clear picture of the mechanisms of sex hormone regulation of pineal melatonin production is obtained.

In conclusion, we investigated the effect of sex hormones on pineal beta-adrenergic receptors in rats. The results showed that oestradiol induced a decrease while testosterone caused an increase in pineal beta-adrenergic receptor density. These observations are consistent with our previous findings that oestradiol inhibits while testosterone stimulates pineal melatonin production in rats, suggesting that both oestradiol and testosterone affect pineal beta-adrenergic receptors to regulate melatonin synthesis.

Fig.25. Binding of 1 nM ^{3}H-DHA (22° C, 40 min) as function of concentration of pineal tissue. Specific binding values were determined by the difference between total binding and non-specific binding as described in text.

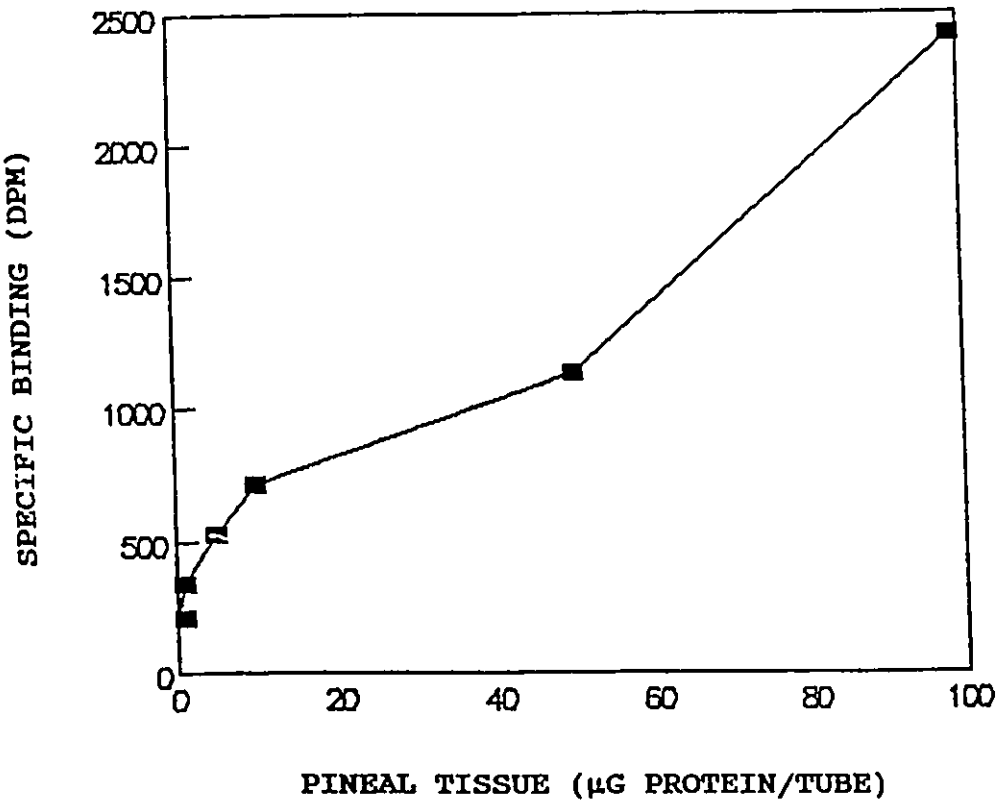


Fig.26. Time-dependent binding of 1 nM ^{3}H-DHA to pineal homogenate preparation measured at 22° C. Specific binding was determined as in Fig.25.

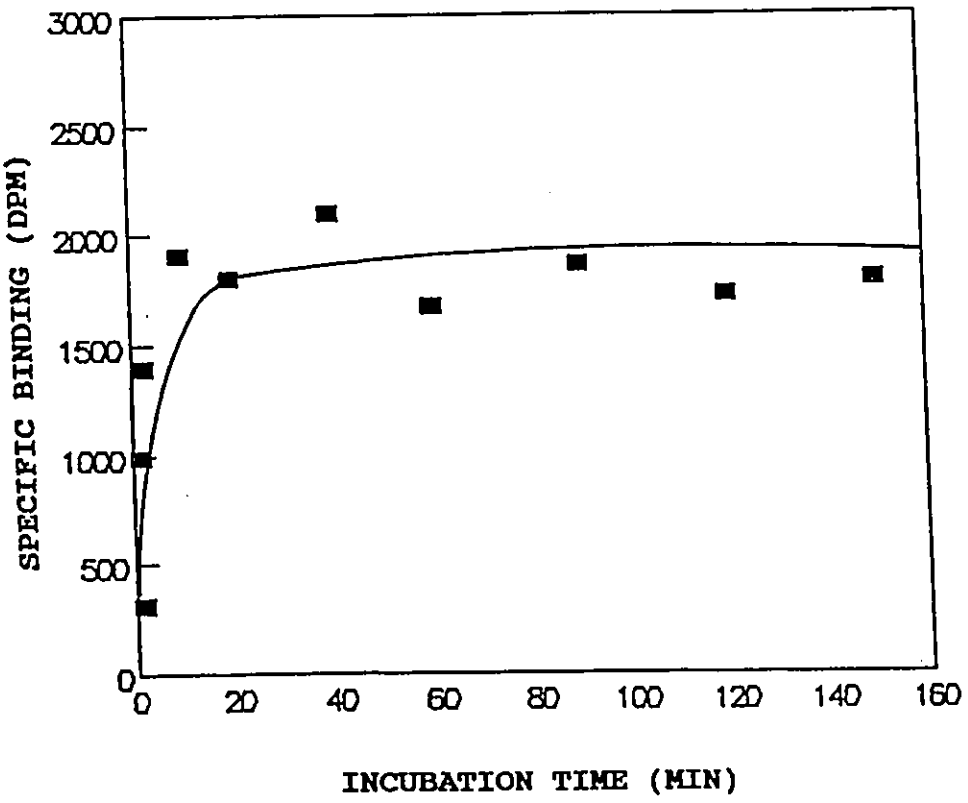
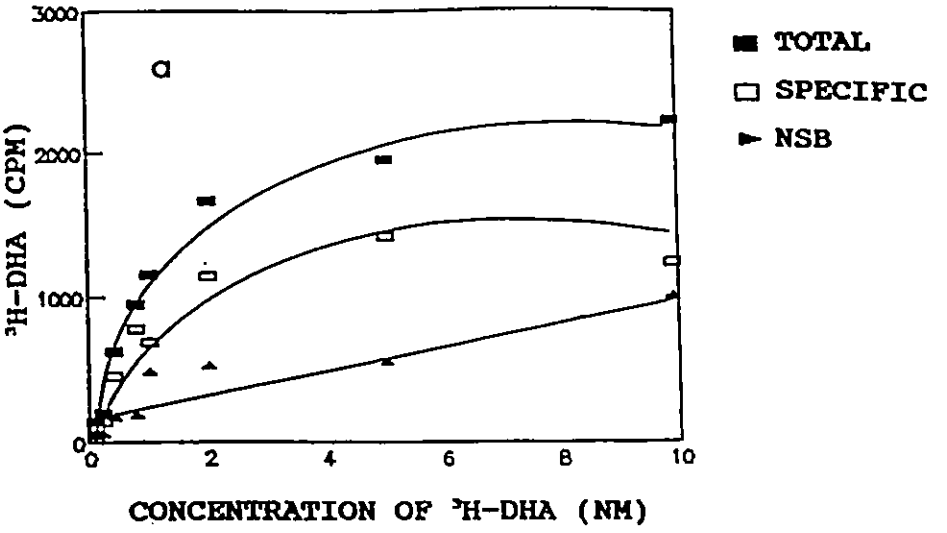
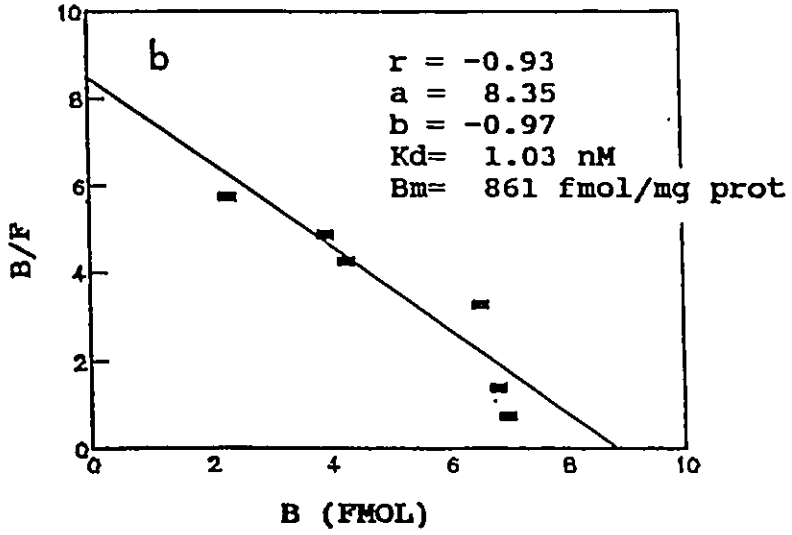


Fig.27. ^{3}H-DHA binding as a function of increasing concentrations of ^{3}H-DHA. The pineal homogenate pool (10 μg/assay tube) was assayed for ^{3}H-DHA binding using increasing concentrations of ^{3}H-DHA. (a): A direct plot of data, showing total binding, non-specific binding (with the addition of (-) propranolol 10^{-4} M) and specific binding. (b): Scatchard plot of the data indicating K_d =1.03 nM and Bmax = 861 fmol/mg protein. (c): Hill plot of the data indicating Hill coefficient = 0.98.


a
TOTAL
SPECIFIC
NSB
^{3}H-DHA (CPM)
CONCENTRATION OF ^{3}H-DHA (NM)


b
r = -0.93
a = 8.35
b = -0.97
Kd= 1.03 nM
Bm= 861 fmol/mg prot
B/F
B (FMOL)

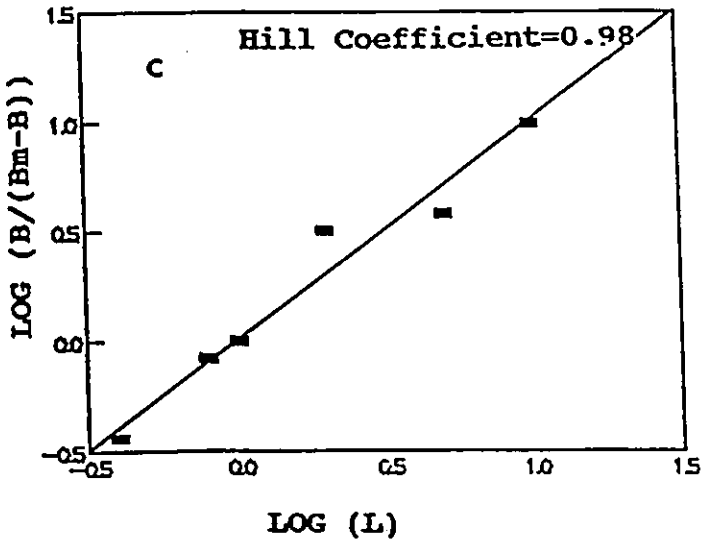
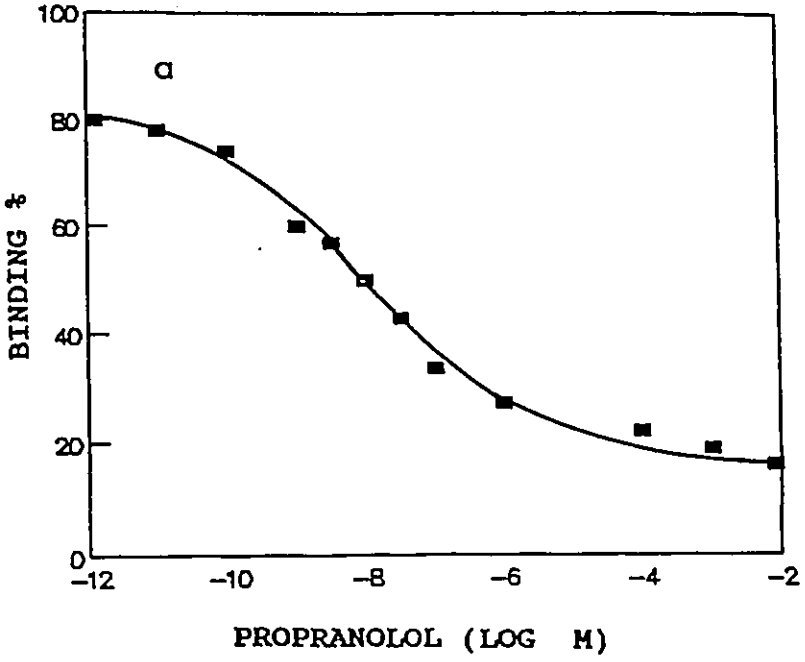

c
Hill Coefficient=0.98
LOG (B/(Bm-B))
LOG (L)

Fig.28. (a): The inhibition of ^{3}H-DHA binding was determined by addition of different concentrations of (-) propranolol. (b): The IC_{50} for (-) propranolol was calculated by utilizing logit-log plot of the data.


a
100
80
60
40
20
0
BINDING %
-12
-10
-8
-6
-4
-2
PROPRANOLOL (LOG M)

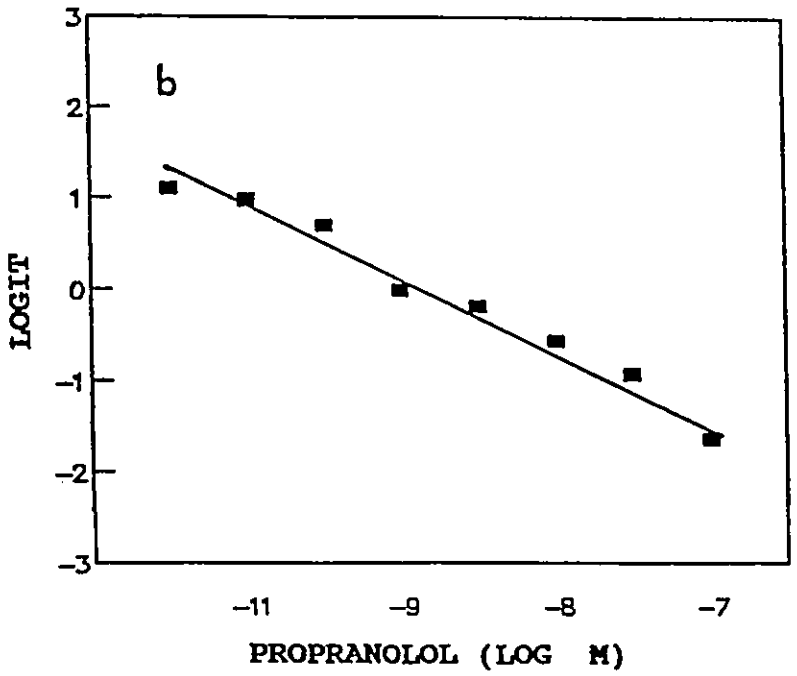

b
3
2
1
0
-1
-2
-3
LOGIT
-11
-9
-8
-7
PROPRANOLOL (LOG M)

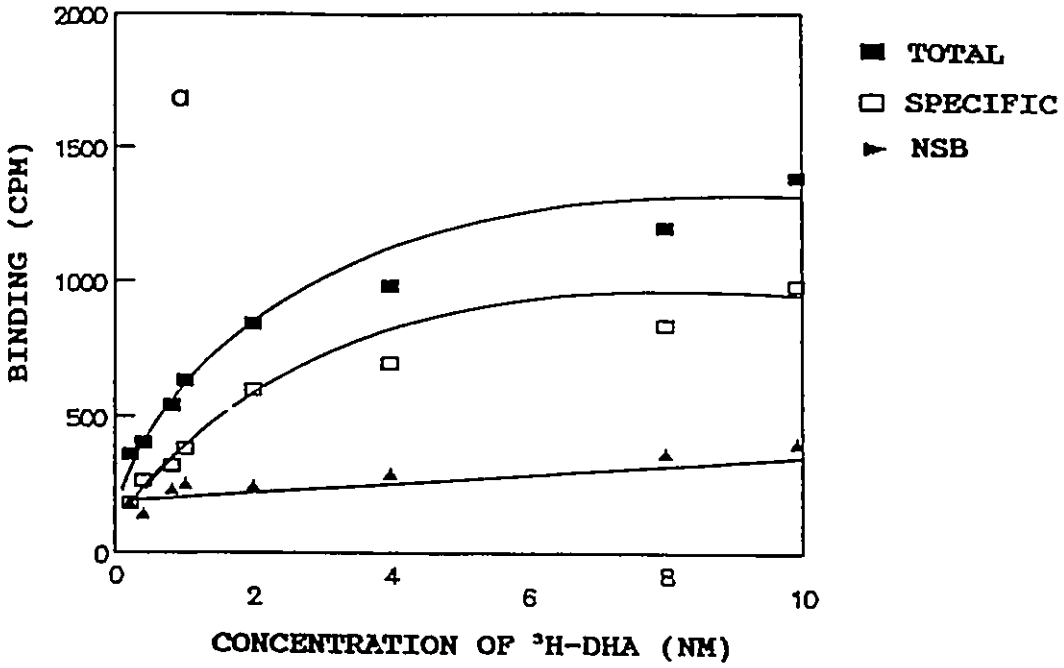
Table 11

Effect of sex hormones on rat pineal beta-adrenergic binding capacity

Treatment	Kd (nM)	Bmax (fmol/mg Prot)	Protein (μg/pineal)
Ovariectomy	2.08 ± 0.4	1875 ± 228*	147 ± 24
E_2-treatment	1.46 ± 0.2	1169 ± 107	147 ± 21
Sham (F)	2.01 ± 0.2	1198 ± 93	206 ± 37
Orchiectomy	1.01 ± 0.2	723 ± 97	165 ± 27
T-treatment	1.58 ± 0.2	1574 ± 338*	133 ± 19
Sham (M)	1.34 ± 0.1	1286 ± 122*	136 ± 20

Pineal ^{3}H-DHA binding in the study was carried out as described in the text. Bmax = fmol ^{3}H-DHA bound/mg protein; and K_d = dissociation constant. Each value is the mean ± SE of 6 animals. (*) : significant difference among experimental groups in male and female rats (one-way analysis of variance).

Fig.29. Specific ^{3}H-DHA binding in a pineal homogenate pool from orchiectomized rats. Specific binding was calculated from the difference between the total binding and non-specific binding as in Fig.27. A Scatchard analysis of the data gave K_d of 1.25 nM and Bmax of 478 fmol/mg protein.


a
TOTAL
SPECIFIC
NSB
BINDING (CPM)
2000
1500
1000
500
0
0
2
4
6
8
10
CONCENTRATION OF ³H-DHA (NM)

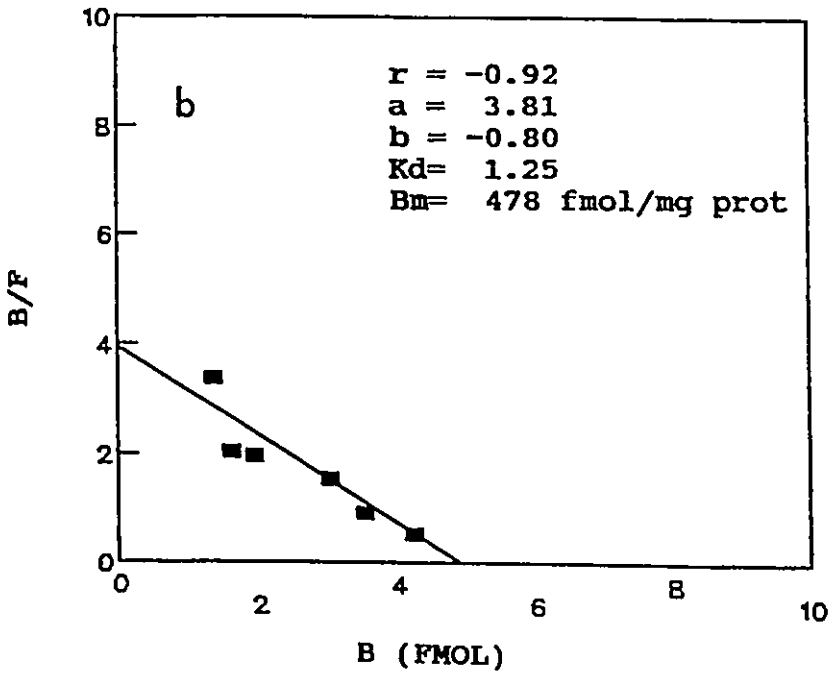
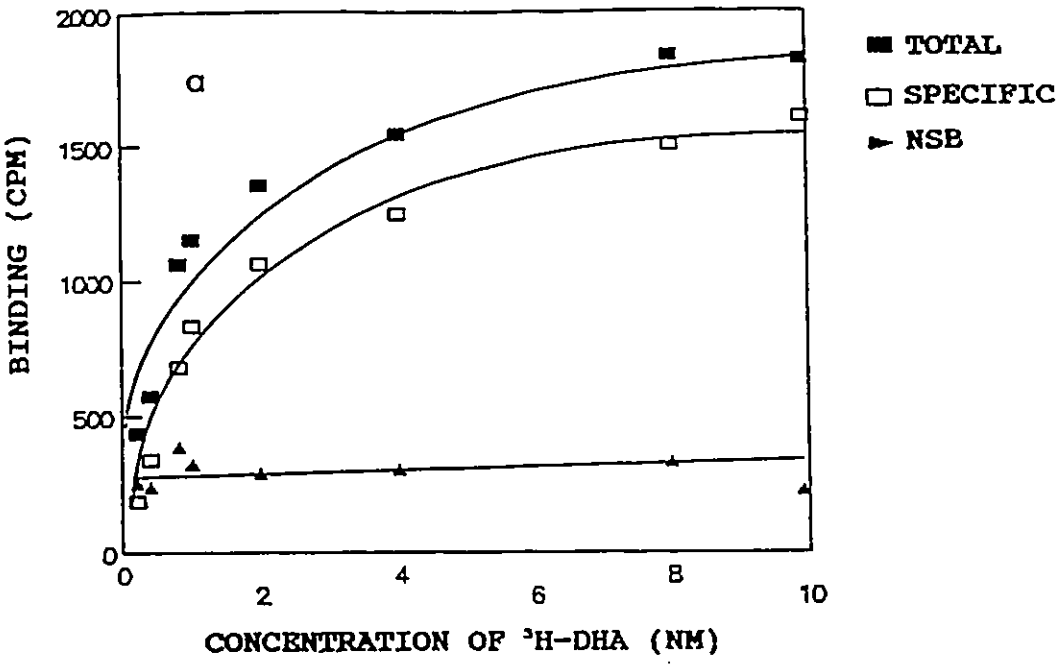

b
r = -0.92
a = 3.81
b = -0.80
Kd= 1.25
Bm= 478 fmol/mg prot
B/F
10
8
6
4
2
0
0
2
4
6
8
10
B (FMOL)

Fig.30. Specific ^{3}H-DHA binding in a pineal homogenate pool from testosterone treated orchiectomized rats. Scatchard analysis gave K_d of 1.75 nM and Bmax of 850 fmol/mg protein.


a
TOTAL
SPECIFIC
NSB
BINDING (CPM)
2000
1500
1000
500
0
0
2
4
6
8
10
CONCENTRATION OF ³H-DHA (NM)

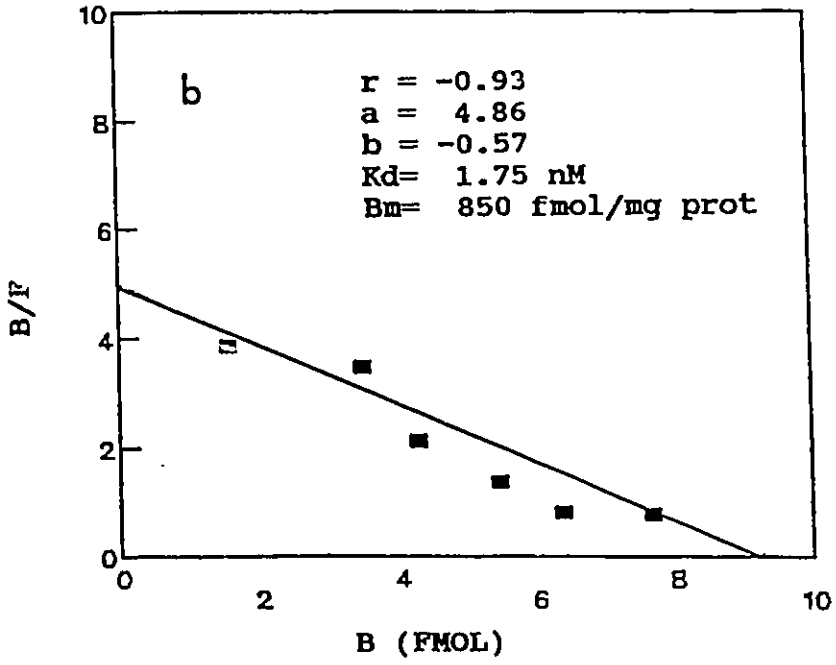
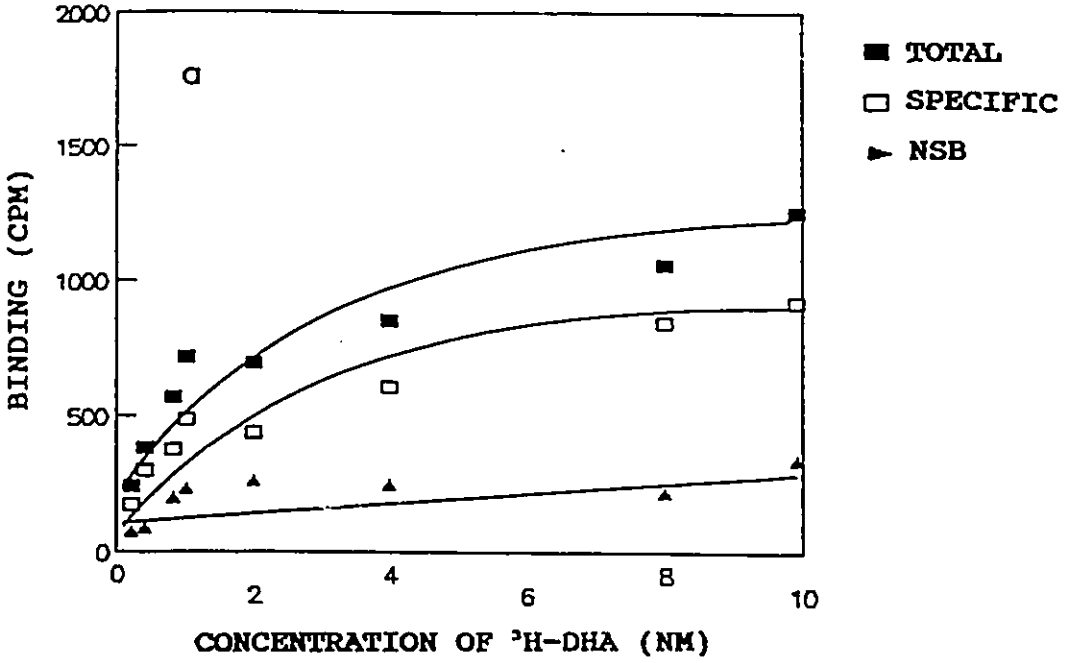
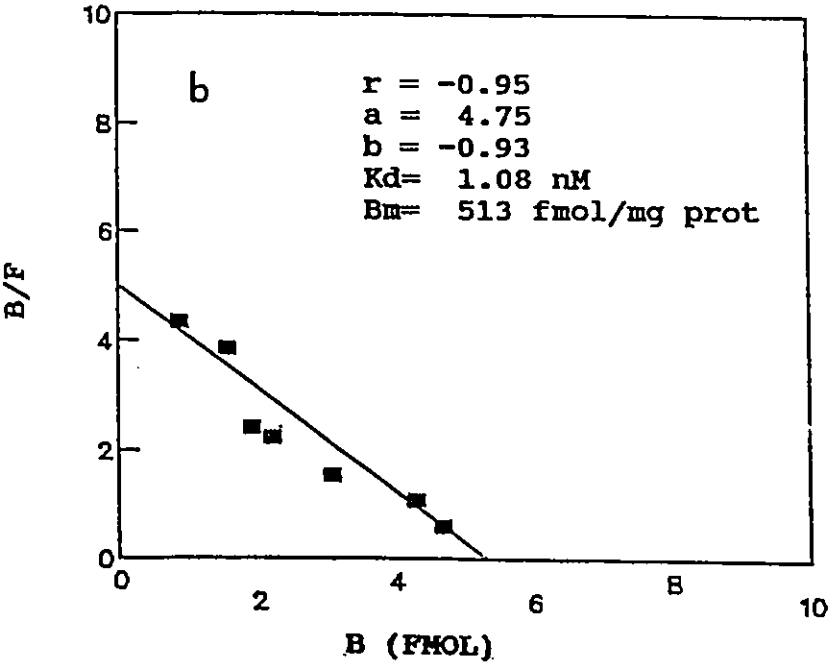

b
r = -0.93
a = 4.86
b = -0.57
Kd= 1.75 nM
Bm= 850 fmol/mg prot
B/F
10
8
6
4
2
0
0
2
4
6
8
10
B (FMOL)

Fig.31. Specific ^{3}H-DHA binding in a pineal homogenate pool from sham-operated control male rats. K_d = 1.08 nM and Bmax = 513 fmol/mg protein.


a
TOTAL
SPECIFIC
NSB
BINDING (CPM)
2000
1500
1000
500
0
0
2
4
6
8
10
CONCENTRATION OF ³H-DHA (NM)


b
r = -0.95
a = 4.75
b = -0.93
Kd= 1.08 nM
Bm= 513 fmol/mg prot
B/F
10
8
6
4
2
0
0
2
4
6
8
10
B (FMOL)

CHAPTER VII

GENERAL DISCUSSION

7.1 Characteristics of the Effects

The effects of sex hormones on pineal melatonin production in the rat were examined in the present study. Although the assumption that gonadal steroids play a role in the regulation of pineal melatonin synthesis and release has been long proposed (Cardinali, 1980; 1984; Cardinali & Vacas, 1987; Preslock, 1977; 1984), evidence of effects in the rat has been contradictory. A stimulating effect of oestradiol in vivo was suggested by two early studies in which daily injections of oestradiol benzoate (EB) into ovariectomized adult rats stimulated pineal hydroxyindole-O-methyltransferase (HIOMT) activity (Houssay & Barcelo, 1972) and ovariectomy decreased the enzyme activity (Daya & Potgieter, 1982). A stimulating effect in vitro on pineal HIOMT activity (Mizobe & Kurokawa, 1976) and melatonin content (Cardinali et al, 1987) was also reported. These results were contrary to those reported by other studies suggesting an overall inhibitory effect of oestradiol on pineal HIOMT activity (Wurtman et al, 1965; Alexander et al, 1970) and on melatonin levels in urine, serum and the pineal gland (Ozaki et al, 1978; Johnson et al, 1982; Moujir et al, 1990). Testosterone in vivo was reported to have a stimulating effect on pineal HIOMT activity (Nagle et al, 1974; Daya & Potgieter, 1982) and on the pineal melatonin content (Hernandez et al, 1990). Results presented by two in vitro studies were conflicting (Cardinali et al, 1987; Daya & Potgieter, 1985). Thus, the effects have not to date been well characterized.

These contradictory findings have been explained as due to several factors: 1) in some of the early studies the measurements were performed during the light portion of the daily photoperiod when pineal activity was low (Cardinali, 1980); 2) there was a dose-dependent effect of both oestradiol and testosterone, low doses had a stimulating influence while high doses inhibited pineal melatonin synthesis (Preslock, 1977; 1984); 3) the effect might be dependent upon age of animals since female rats castrated at 28 days of the age had significantly higher pineal HIOMT activity than un-operated controls while castration at 37 days of age had no effect on pineal HIOMT activity (Alexander et al, 1970); 4) pineal response to oestradiol might be changed with the stages of the rat oestrous cycle (Cardinali et al, 1974 b); 5) the effect of sex hormones in vivo is different from that in vitro (Yuwiler, 1985; 1989) and 6) as discussed in chapter III, use of only a single time point examination may be an important factor related to the previous negative or contrary studies.

Moreover, when carefully reviewing those data, it is clear that conflicting results were mainly raised in the early studies concerning pineal HIOMT activity. Pineal HIOMT activity is not considered to be rate limiting in melatonin production. It is also clear that HIOMT is only one step with which sex hormones can interact in the hormonal modulation of pineal melatonin production and thus, only changes in melatonin levels may represent the sum of the effects. When melatonin or aMT6s was examined the inhibitory

effect by oestradiol and the stimulating influence of testosterone in vivo were consistently observed by the present study (chapter III) and other studies (Hernandez et al 1990; Johnson et al, 1982; Moujir et al, 1990; Ozaki et al, 1978).

Obviously, effects of sex hormones in vitro can be different from those in vivo. On the one hand, neuroendocrine integration clearly occurs in pineal melatonin synthesis (Cardinali, 1984) since sex hormones act on both the SCG and the pineal (Cardinali, 1980) and pineal sex hormone receptors were under regulation by SCG activity (Cardinali, 1984; Cardinali & Vacas, 1987). Pineal sympathetic nerve degeneration occurs in vitro (Vacas et al, 1982) and hence, this integration is impaired. On the other hand, gonadotropins and prolactin have been shown to affect pineal melatonin production both in vivo (Urry et al, 1972) and in vitro (Cardinali et al, 1987; Vacas et al, 1987). Alteration of pineal melatonin production caused by castration and sex hormone treatment in vivo would be the result of co-operation of gonadal steroids and adenohypophysial hormones which are changed following the treatments.

Age-dependent effects of sex hormone on pineal melatonin were demonstrated in the present study; e.g. the sex difference in aMT6s excretion diurnal profile was only detected in rats at ages from 3 weeks to 8 months (chapter II) and castration induced different responses at 3 weeks, 2 months and 8 months (chapter III). The

results differ from the observation by Alexander et al (1970) in which castration in young adult female rats (castrated at 37 days of age and sacrificed at 53 days of age) had no effect on pineal HIOMT activity. However, most of the previous positive studies of castration, whether pineal HIOMT activity or melatonin level was examined, were carried out on adult rats.

In immature rats the gonads are relatively non-responsive to stimulation by gonadotropins and the hypothalamic-pituitary axis is also sub-sensitive to gonadal steroid feedback (Ojeda & Urbanski, 1988). Presumably this may also occur for the pineal response to sex hormones. If so, this may be another explanation for no significant effect of castration in 3 week old male rats (see discussion in chapter III). Since the timing of the onset of puberty of female rats is earlier than that of male animals (Ojeda & Urbanski, 1988), it would be expected that there is an effect of castration performed during the juvenile period of female rats (21 to 28 days of age (Lang, 1986) and observed 7 days later, although a stimulating effect of ovariectomy was observed (chapter III). In immature female rats there are extremely high levels of alpha-fetoprotein (AFP), which binds oestrogen avidly (Raymond et al, 1971) while testosterone and androstenedione are detected in relative high concentration (Andrews & Ojeda, 1981; Dohler et al, 1975; Mathews et al, 1987). The sex hormonal regulation of pineal melatonin production in immature female rats, if any, might be exerted by these androgens.

It should be very interesting to further observe the effect of sex hormones on pineal melatonin production during sexual development. Like humans, the initial mechanisms of the onset of puberty are not well established. A hypothesis that the gradual and constant decrease of pineal melatonin production might trigger the hypothalamic-pituitary-gonadal axis has been proposed (Brzezinski & Wurtman, 1988). However, a number of reports (Silman, 1991) including ours (chapter II) indicate that the melatonin production is relative constant from infancy onwards but body weight is increasing. Hence the circulating concentration of melatonin is dropping and puberty may result when it drops below a critical level (Silman, 1991). In addition, sex hormonal regulation may be also involved in this process, as we discussed in above paragraph. If so, the pineal response to gonadal steroids would be changed as a function of sexual development.

A stimulating effect of testosterone on pineal HIOMT activity was obtained at doses from 0.01 to 1.0 mg/day while testosterone at doses of 5 to 10 mg/day decreased the enzyme activity in male rats (Nagle et al, 1974; Daya & Potgieter, 1982). In vitro pineal melatonin content was decreased when a high concentration (10^{-5} M) of testosterone was added (Cardinali et al,1987). In this study a low dose (200 μg/kg/day) of testosterone has stimulating effects on aMT6s excretion, pineal response to adrenergic stimulation and pineal beta-adrenergic receptors (chapter III, V, VI). This dose produces a serum testosterone level of about twice the physiologic

level (chapter V & VI) suggesting that testosterone has a stimulating effect on pineal melatonin production in young adult rats at physiological doses. Oestradiol at doses of 0.01 to 1 μg/day stimulated pineal HIOMT activity while high doses (> 10 μg/day) decreased the enzyme activity in vivo (Daya & Potgieter, 1982). In most previous studies suggesting inhibitory effects of oestradiol relative high pharmacologic doses of the steroid were used (Moujir et al, 1990; Ozaki et al, 1978; Wurtman et al, 1964). However, melatonin in pineal, serum and urine (Johnson et al, 1982; Moujir et al, 1990; Ozaki et al, 1978) and urinary aMT6s excretion (present study) were lowest at rat proestrus. Oestradiol has both negative and positive feedback effects on the hypothalamic-pituitary axis. Conceivably, oestradiol also exerts both feedback controls on pineal function. In view of inhibitory effects of melatonin on the gonadal axis in rats, it is conceivable that there are inhibitory effects at proestrus and stimulating effects during the other stages of the rat oestrous cycle. Although an alteration of the pineal response to oestradiol with the oestrous cycle in rats has been suggested (Cardinali et al, 1974 b), better designed studies are needed to confirm this hypothesis.

Finally, we injected the sex hormones at the middle of the dark phase, a time at which high melatonin production occurs (Binkley, 1983; Klein et al, 1981); the time at which melatonin is administered relative to the light dark cycle is important to melatonin reproductive effects in rats (Lang et al, 1984). It is

possible that the sensitivity of the pineal response to sex hormones varies with the diurnal rhythm of pineal melatonin synthesis. If so, variation of sex hormone effects on pineal melatonin production may be also found because of different injection times. There was a report in which rats injected with testosterone propionate (TP) at the end of the dark period had a higher pineal protein synthesis than those injected at the middle of the light period (Nagle et al, 1975). Whether there is a time-dependency of pineal response to sex hormones awaits further investigation.

In conclusion, the results in the present study suggest that oestradiol has an inhibitory while testosterone has a stimulating effect on pineal melatonin production in adult rats in vivo. It should be further investigated whether the effects vary with age, hormone doses, the oestrous cycle and probably the time of hormone administration.

7.2 Sites of Action

It has been reported that sex steroids act on both the pinealocytes themselves and on pineal sympathetic nerves (Cardinali, 1984). Oestradiol injection decreased tyrosine hydroxylase activity and enhanced dopamine-beta-hydroxylase activity and NE uptake in SCG of rats (Cardinali, 1979). Testosterone treatment increases SCG tyrosine hydroxylase activity and NE uptake in both newborn and

adult rats (Cardinali et al, 1981). As discussed above, sex hormones dramatically affect pineal HIOMT activity in this species (Alexander et al, 1970; Daya & Potgieter, 1982; Wurtman et al, 1965). Moreover, the turnover rates of NE and 5-HT in the pineal have also been shown to change following sex hormone treatment of castrated rats (Cardinali et al, 1975 b; Vacas & Cardinali, 1979); the changes in pineal amine turnover may be explained partly by an alteration of monoamine oxidase (MAO) activity (Vacas & Cardinali, 1979). Recent evidence (Hernandez et al, 1990; Moujir et al, 1990) including ours (chapter V) on the pineal response to adrenergic stimulation corresponds well to previous findings that sex hormones influenced the NE-induced activation of adenylate cyclase (Weiss & Crayton, 1970) and pineal cAMP levels (Davis, 1978). It has also been demonstrated that oestradiol decreased pineal beta-adrenergic receptor density (Weiland & Wise, 1989; chapter VI) while testosterone increased the concentration of the receptors (chapter VI). Although it was generally thought that pineal SNAT activity was not affected by sex hormones, positive results were reported both in vivo (Daya & Potgieter, 1982) and in vitro (Yuwiler, 1989). Therefore, the accumulated evidence indicates that multiple sites of sex hormone action in pineal melatonin production exist, which are summarized in Fig.32.

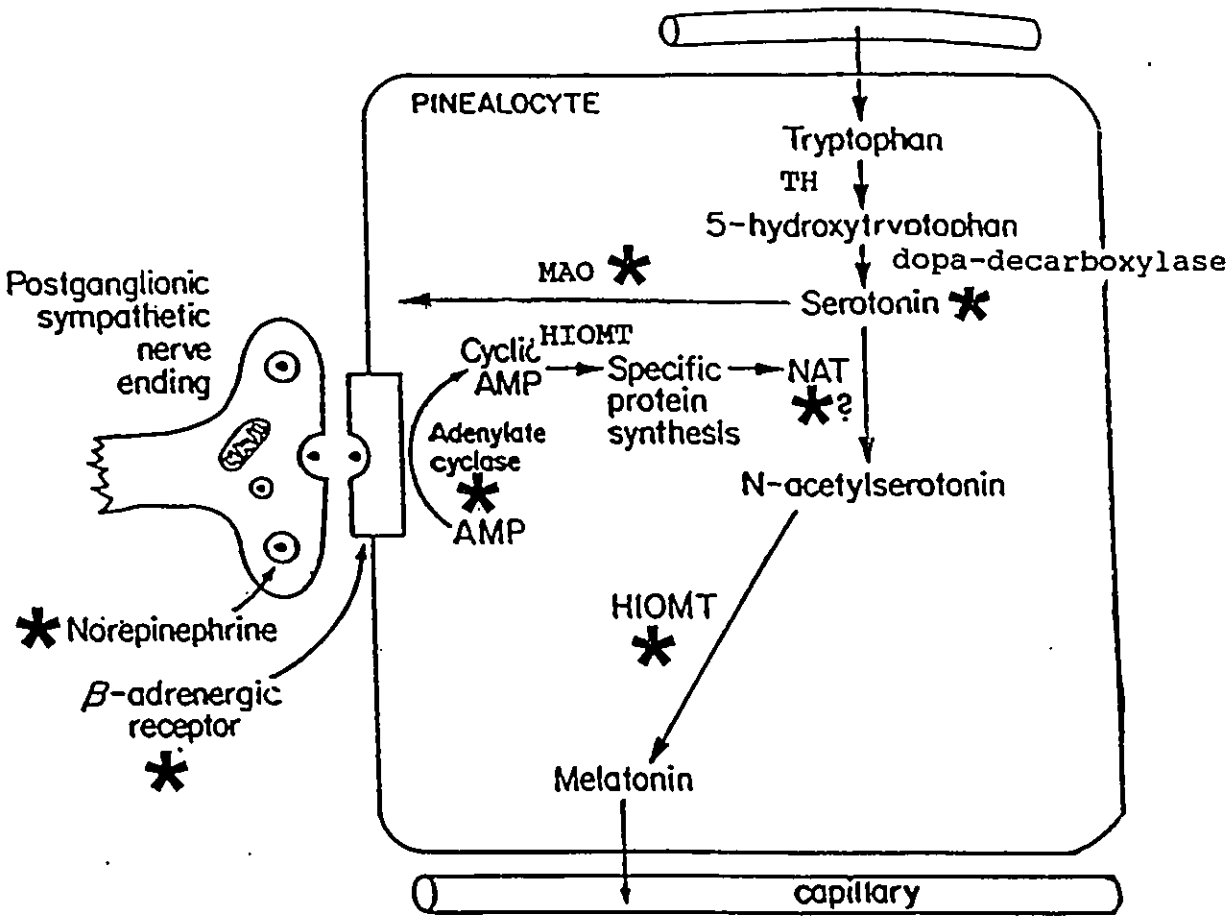
However, we still do not know which site (s) may be the key step in sex hormonal regulation of pineal melatonin production. In addition, pineal melatonin is also regulated by pineal alpha-

adrenergic receptors simultaneously activated by NE and other transmitters and their receptors in the pinealocytes (Cardinali & Vacas, 1987; Kranse & Dubocovich, 1990; Sugden, 1989). Whether those are also sites for sex hormone action has not been reported yet.

7.3 Mechanisms of Action

For both oestradiol (Cardinali et al, 1979) and testosterone (Cardinali et al, 1981), high affinity binding sites are detectable in rat SCG. In the pineal gland receptors for oestradiol (Cardinali et al, 1975 a; Cardinali, 1983; Stumpf & Sar, 1979) and testosterone (Cardinali et al, 1974 a) have been also identified. Using radioligand binding studies the characteristics of pineal oestradiol receptors are shown to be similar to those of the hypothalamus and adenohypophysis (Cardinali, 1977) and concentrations of oestradiol receptors varied concomitantly in pineal and uterus as a function of the stages of the oestrous cycle, attaining their minimum at the proestrus when plasma oestradiol level reached its maximum (Nagle et al, 1973). The pineal oestradiol and testosterone receptors are under neural control as shown by the depression or abolition of binding sites after pineal denervation (Cardinali, 1984). These data strongly suggest that the sex hormonal regulation of pineal melatonin

Fig.32. Possible sites (★) of sex hormone action on pineal melatonin production.



production is through their direct action on the pineal and the SCG, although detailed information on purification and isolation of these receptors from the rat pineal gland and SCG is lacking.

Most current evidence supports the general view that the mechanisms of action of gonadal steroids are best explained by selective changes in gene expression under the overall control of specific receptors. Regulation of sex hormone action at the levels of RNA processing, mRNA stabilization and enhanced translational efficiency has also been proposed (King, 1988: Parker, 1988). Several SCG and pineal constituents related to melatonin synthesis, as mentioned above, have been reported to be affected by sex hormones. The effects of sex hormones on pineal protein content (Nir et al, 1970) and protein and RNA synthesis (Cardinali et al, 1974 a; 1978; Mizobe & Kurokawa, 1978) were also reported. However, the molecular mechanisms of sex hormonal modification of melatonin forming enzymes and sympathetic signals are completely unknown. Recently several melatonin forming enzymes such as tryptophan hydroxylase (TH) (Darmon et al, 1986), SNAT (Namboodiri et al, 1987 a; 1987 b) and HIOMT (Sugden et al, 1986) have been purified from the rat pineal gland. cDNA clones encoding rat TH (Darmon et al, 1986) and bovine HIOMT (Ishida et al, 1987) have also been isolated from a rat pineal cDNA expression library and a bovine pineal cDNA library. Furthermore, the beta-adrenergic receptor gene has been cloned from other cells (Frielle et al, 1987). These outstanding successes in molecular aspects of pineal

melatonin synthesis may provide a vital impetus for future investigation on the molecular mechanisms of sex hormonal regulation of pineal melatonin production.

Since gonadotropins and prolactin have been shown to affect pineal melatonin production (Cardinali, 1980; Cardinali et al, 1987; Vacas et al, 1987 b) and receptors for prolactin were also detected in the pinealocytes (Cardinali, 1983), an indirect mechanism involving gonadotropin and prolactin changes following castration and sex hormone treatment should not be discounted. However, how these hormones alter melatonin forming enzymes and neural regulation mechanism awaits future studies.

Original Contributions

The findings that oestradiol has an inhibitory but testosterone exerts a stimulating effect on the diurnal rhythm in urinary aMT6s excretion, the pineal response to adrenergic stimulation and pineal beta-adrenergic receptors in young adult rats are original contributions to the area. However, two reports appeared from Alonso's group (Hernandez et al, 1990; Moujir et al, 1990) showed similar results to ours using different methods during the course of my experiments.

References

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