The effect of food access schedule and diet composition on the rhythmicity of serum melatonin and pineal N-acetyltransferase activity in rats.

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ABSTRACT

Melatonin is a hormone secreted by the pineal gland, which is known to modulate biological rhythms in mammals. This study investigated the effect of food access schedule and dietary composition on serum melatonin and pineal NAT activity in adult male Wistar rats. These rats were maintained on a 12:12 h light:dark schedule with lights on at 0800h. The rats were randomly assigned to two dietary groups. A group was simultaneously fed a protein-rich and carbohydrate-rich granulated diet and the other group fed granulated rat chow. Each dietary group was further divided based on dietary feeding schedules. Animals were fed between 0800-1600 h or fed *ad libitum*. The study revealed that protein intake of rats fed the dietary choice was lower with the restricted access than in the free access. In rats fed dietary choice, the nocturnal melatonin levels and pineal NAT activity were significantly lower under the restricted access feeding when compared to the *ad libitum* feeding schedule. This was not observed in rats fed single chow diet. In conclusion our data demonstrate that food composition does affect the nocturnal synthesis of melatonin as well as the activity of the enzyme NAT. This could be via dietary intake of tryptophan, which is a precursor melatonin synthesis in the pineal gland.
La mélatonine, hormone sécrétée par la glande pinéale, est connue pour son effet modulateur pour les rythmes biologiques chez les mammifères. Dans la présente étude, l’effet de l’accès à la nourriture et de la composition des diètes sur la mélatonine sérique et sur l’activité NAT dans la glande pinéale ont été étudiés chez des rats. Des rats mâles Wistar adultes ont été assignés à un cycle lumière obscurité 12 :12h avec un début de lumière à 0800 heures. Les animaux ont été divisés en deux groupes alimentaires : un groupe a été nourri au chow et l’autre groupe a été nourri au choix entre des diètes riches en protéine et en sucre. Chaque groupe alimentaire a ensuite été divisé sur la base de la cédule d’accès alimentaire soit un accès ad libitum soit un accès entre 0800 et 1600 heures. Les résultats obtenus ont montré que dans les conditions du choix alimentaire, les rats ayant un accès restreint ont ingéré moins de protéines que les rats ayant un libre accès. La comparaison entre les sous-groupes de rats nourris au choix alimentaire a montré que les rats soumis à l’accès restreint ont subi une diminution significative des taux de mélatonine sérique et de l’activité de l’enzyme NAT par rapport aux rats soumis à l’accès libre. Aucun changement n’a été observé chez les rats nourris avec la diète chow. En conclusion, notre étude a montré que la composition des diètes pourrait affecter la synthèse nocturne de la mélatonine ainsi que l’activité NAT. L’ingestion de tryptophane, le précurseur de la mélatonine pourrait être le mécanisme impliqué.
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_I can do all things through Christ who gives me strength_

{Philippians 4: 13}
CONTRIBUTION OF AUTHORS

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**Food Access Schedule and Diet Composition Alter Rhythmicity of Serum Melatonin and Pineal NAT Activity.**

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LIST OF ABBREVIATIONS

ADD: Addition/Calculation test
aMT6s: 6-sulphatoxymelatonin
ANOVA: analysis of variance
C: choice diet
Ci/mmol: curie per millimole
CSF: cerebrospinal fluid
DSST: Digit Symbol Substitution Test
DST: dexamethasone suppression test
FA: free food access
GnRH: gonadotrophin releasing hormone
g: gram
h: hour
HCl: hydrochloric acid
HIOMT: hydroxyindole-O-methyltransferase
Hz: hertz
5-HI AA: 5-hydroxyindoleacetic acid
5-HT: 5-hydroxytryptamine or serotonin
ICU: Intensive Care Unit
KSS: Karolinska Sleepiness Scale
M: molar
mg: milligram
min: minute
ml: milliliter
mM: millimolar
n: number
NAT: N-acetyltransferase
NE: norepinephrine
nmol: nanomole
pg/ml: picogram per milliter
pg: picogram
PRM: probed recall memory test
PVN: paraventricular nuclei
PVT: psychomotor vigilance task
RA: restricted access
RIA: radioimmunoassay
S: single diet
SCN: suprachiasmatic nuclei
SEM: standard error of mean
SIGH-SAD: structured interview guide for the hamilton depression rating scale—seasonal affective disorder version
6-SMT: 6-sulphatoxymelatonin
μl: microliter
μT: micro Tesla
Chapter 1

INTRODUCTION

Melatonin, referred to as the pineal gland’s chief hormone, is an indole hormone that is synthesized and secreted mainly by the pineal gland in most animal species. In the production of melatonin, N-acetyltransferase (NAT) is one of the precursor enzymes needed for its synthesis. Melatonin was not discovered until the late 1950's. The discovery was quite unexpected and occurred when an American dermatologist, Aaron Lerner and his coworkers tried to isolate and identify the frog lightening factor present in beef pineal as N-acetyl-5-methoxytryptamine. Lerner named the compound melatonin. Mela was given because it lightens the cells producing the melanin pigment and tonin because melatonin is a derivative of serotonin (Lerner et al, 1958). In the decades of research that followed, series of investigations have conclusively documented melatonin rhythm in the pineal gland of a variety of non-mammalian and mammalian vertebrates including man (Arendt, 1984; Binkley, 1981; Reiter, 1983). In the human brain, the pineal gland occupies a central location between two cerebral hemispheres and in front of the cerebellum at the posterodorsal area (Yu et al, 1993). In rats, the pineal gland is much smaller than the human pineal, most of it being located just below the surface of the skull (Brainard et al, 1999).

Melatonin production however, is not only limited to the pineal gland. There have been reports of melatonin synthesis in extra-pineal tissues such as the retina and extraorbital lacrimal gland (Mhatre et al, 1988; Reiter, 1991b), the Harderian gland and the enterochromaffin cells of the gastrointestinal tract (Bubenik et al, 1978; Raikhlin et al, 1975). Though production in these
tissues is cyclic, it is not considered a significant contributor to the nighttime increase in melatonin levels (Stokkan et al, 1991).

There are a number of documented factors that are seen to affect the melatonin rhythmicity. These include photoperiod (Mayeda et al, 1998; Reiter, 1991b), electromagnetic fields (Selmaoui and Touitou, 1995), sleep-wake cycle (Peder et al, 1989), pathological states (Bartsch and Bartsch, 1999) and hormonal states (Reiter and Vaughan, 1991). Food consumption, which also occurs rhythmically, has been shown to have an effect on melatonin secretion as well (Challet et al, 1997). Literature though seems to be relatively lacking on what role feeding schedule and food composition might play in melatonin secretion. Moreover, in none of the previous studies, has macronutrient selection been used as a component of research.

Thus the hypothesis of the present study was that the secretory pattern of melatonin levels and NAT activity will be altered by food access schedule and by dietary feeding paradigm in rats. This hypothesis was tested by randomly assigning male Wistar rats to two dietary groups. A group was simultaneously fed a protein-rich and carbohydrate-rich granulated diet and the other group fed granulated rat chow. Each dietary group was further divided based on dietary feeding schedules. Animals were fed between 0800-1600 h or fed ad libitum.

The objectives of the present study were to study (1) the effect of restricted food availability schedule on the nocturnal pattern of NAT activity and melatonin levels, and (2) the influence of dietary composition on these parameters. These objectives were achieved by measuring food intake of selected rats from the two subgroups in order to: (1) calculate total intake and intakes from chow diet, protein rich and carbohydrate rich diet and absolute intakes from protein, carbohydrate and tryptophan, (2) compare the above parameters to the secretory levels of melatonin and NAT activity measured every 2 h of the dark phase starting at 2000 h.
Additionally, body weight measurements were made daily and compared between the dietary groups and sub-groups on specified feeding schedules.

The results of this study may help enlighten us about the relationship between food intake and melatonin secretion.
Chapter 2  
LITERATURE REVIEW  

2.1 Melatonin Biosynthesis

Tryptophan is the substrate of melatonin synthesis. Tryptophan is delivered to the pineal gland by systemic vascular supply then it is moved into pinealocytes by an amino acid transport mechanism. Tryptophan is then hydroxylated in position 5 to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase. 5-hydroxytryptophan is decarboxylated to 5-hydroxytryptamine (5-HT or serotonin) by the enzyme 5- hydroxytryptophan decarboxylase. Serotonin is then acylated by the enzyme N-acetyltransferase (NAT). This results in the formation of N-acetylserotonin, which in the presence of the enzyme hydroxyindole-O-methyltransferase (HIOMT), is converted to melatonin (Figure A1a). Melatonin, once produced, escapes rapidly into the blood circulation (Craft, 1993).

2.1.1 Neural Pathway

The increase in melatonin synthesis in the pineal observed at night is stimulated by electrical signals originating from neurons in the suprachiasmatic nuclei (SCN) of the hypothalamus (Moore and Klein, 1974). These neurons receive input from the eyes and send impulse via the hypothalamic paraventricular nuclei (PVN) and the spinal cord to the superior cervical ganglia (SCG) of the sympathetic nervous system (Figure A1b). These fibres terminate adjacent to pinealocytes. The post-synaptic nerve endings release the neurotransmitter norepinephrine (NE) into the synaptic cleft. After its release, NE acts on the post-synaptic adrenergic receptors in the pinealocytes membrane. NE being a mixed beta and alpha adrenergic receptor agonist, binds to the alpha and beta adrenergic receptors on the pinealocyte membrane
and triggers a series of intracellular responses. The activated beta receptor stimulates guanine nucleoside binding protein (G-protein) leading to the activation of adenylate cyclase (Spiegel, 1990). ATP is hydrolyzed and the level of cAMP rises, this leads to the enhancement of a cAMP dependent protein kinase leading to the activation of mRNA expression for the synthesis of the NAT enzyme protein (Klein, 1993). It is this enzyme that is responsible for the formation of N-acetyl serotonin, which in the presence of HIOMT, is converted to melatonin.

In rats, pineal melatonin synthesis and secretion depends on the pineal gland being stimulated by NE (Zawilska and Nowak, 1999). The rodent retina-pineal pathway produces melatonin rhythms similar to that in humans, having a melatonin peak at night and low levels through the day (Nowak and Zawilska, 1998).

### 2.1.2 The Pineal Gland and N-acetyltransferase

In humans, the pineal is located near the anatomical centre of the brain: it lies in the immediate vicinity of the post boundary of the third ventricle and occupies the depression between the superior collicule of the mesencephalon. The pineal gland is highly cellular and consists of pinealocytes producing both the indolamines and peptide/proteins and neuroglial cells (Vollrath, 1984). It is on average of equal size in males and females though there are individual variations (Reiter, 1998). Functionally, the important innervations of the pineal gland are sympathetic in nature (Vollrath, 1984).

Documented investigations of the pineal gland span 2200 years. Erasistratus and Herophilus gave the first descriptions about 300 BC. Both believed that the pineal gland functioned as a tap between the third and fourth ventricle. The name ‘pineal’ was given by Galen because of its resemblance (in humans) to a pinecone (Bergstrom and Hakanson, 1998). Through the years the pineal gland has acquired various descriptive terms such as, ‘sphincter to control
the flow of thought’, ‘the seat of the soul’, ‘a third eye’ and also, a ‘neuroendocrine transducer’ (Ebadi, 1984). These names were all given to help depict how researchers actually viewed the pineal gland in relation to its function.

With the discovery of melatonin in 1958-59 came subsequent findings of pineal precursor enzymes essential for melatonin synthesis. NAT is one of the four enzymes necessary for melatonin synthesis, the others being tryptophan hydroxylase, 5-hydroxytryptophan decarboxylase and HIOMT. Similar to the production pattern of melatonin, the activities of NAT and HIOMT rise and fall in inverse proportion to the intensity of the environmental light. NAT however has demonstrated the closest reciprocal relationship. NAT requires a coenzyme A as a cofactor to N-acetylate its substrate. The induction of NAT activity requires protein synthesis, and is also a cAMP Ca$^{2+}$ dependent process (Reiter, 1991b; Zawilska and Nowak, 1992). The major control of NAT activity, thus melatonin production comes from the postganglionic sympathetic fibres innervating the pineal gland (Nowak and Zawilska, 1998). NAT is considered to be the rate-limiting enzyme in pineal conversion of serotonin to melatonin (Barassin et al, 1999). The quantity of melatonin that is formed during darkness seems in many cases to be related to the activity of this enzyme (Ebadi, 1984). It has been observed that when there is no nocturnal increase in NAT, there is as well, no nocturnal nighttime rise in levels of pineal melatonin (Reiter, 1991a). It is thus safe to say that measuring NAT along with melatonin levels not only aids in providing good indication of the synthesis of melatonin but becomes an able detector of its presence.

2.2 Factors Affecting Melatonin Secretion

Melatonin secretion is affected by a number of factors namely, photoperiod, electromagnetic field, sleep-wake cycle and food intake.
2.2.1 Photoperiod

Light is known to be the primary regulator of the circadian production of melatonin. Both daylight and bright artificial light suppress nighttime melatonin (Lewy et al, 1980). When the effects of indirect light on melatonin levels and propranolol (a β receptor agonist) were studied in normal human subjects, Mayeda et al (1998) observed that melatonin levels obtained with indirect lighting protocol showed that light of 500 lux significantly suppressed nocturnal melatonin. This suppression was seen to be dose related between 300 and 2000 lux. Sitting in dim light did not seem to have any significant effect on melatonin suppression. In another study that investigated the effects of polarized vs nonpolarized light on melatonin in healthy humans, Brainard et al (2000) exposed subjects to both polarized and nonpolarized light of four different light intensities (20, 40, 80 and 3200 lux) as well as to a control dark exposure. Each evening the subjects were exposed to 120 minutes dark exposure (0000-0200 h) followed by a 90 minutes light exposure (0200-0330 h). The results showed that at each illuminance, both polarized and nonpolarized light elicited significant suppression of plasma melatonin compared to control exposures. Furthermore, there were no significant differences observed between the effects of polarized light and nonpolarized light at any illuminance.

In mammals, the retinas are known to be the specific group of cells capable of responding to light (Reiter and Lerchl, 1993). Light coming through the retina, reaches the SCN through a non-visual pathway called the retinohypothalamic tract (Czeisler et al, 1995). Light inhibitory effects of pineal melatonin in mammals is achieved by suppressing the electrical activity of the SCN of the hypothalamus, which during darkness signal the release of the neurotransmitter NE from the postganglionic sympathetic neurons that innervate the pineal gland (Zatz, 1981). This is followed by events in the pineal gland, which culminate in the increased production of melatonin.
during darkness (Klein et al, 1981). Since the altering of the light and dark periods synchronizes the 24 h rhythm of melatonin production and secretion, it follows that this rhythm is thrown into disorder when light is introduced during periods when the mammals are not usually exposed to light, such as during the dark period. When light is introduced at these times, it tends to alter the phasing of the essential circadian signals and results in noticeable physiological consequences. This is seen in people who work at night and people traveling across many time zones. Such people, exposed to light at unusual times during their circadian cycle, tend to have their cyclic production of melatonin disrupted.

It also has been shown that the ability of light to influence nighttime melatonin production is intensity and wavelength dependent, and this varies among species. The melatonin levels of diurnal active species are less sensitive to light inhibition than those of nocturnally active rodents (Reiter and Peters, 1984; Zawilska and Nowak, 1999). In humans, complete inhibition of pineal requires 2500 lux for 1 hour while partial inhibition needs 100-300 lux for 30 minutes (Brainard et al, 1993). In rats, as little as 1 second light impulse at night inhibits melatonin production in the pineal gland (Nurnberger, 1985). With regards to the effects of light wavelength, studies in the Syrian Hamster have shown that the light most inhibitory to the pineal gland at night is in the blue range of the spectrum (Brainard et al, 1984). In rats, Cardinali and colleagues (1972) found that the green wavelengths maximally suppressed the conversion of N-acetylserotonin to melatonin. Both red and yellow wavelengths though, have not been shown to reduce nocturnal pineal NAT activity or melatonin levels (Reiter and Lerchl, 1993). This forms the basis in pineal studies for use of red light to substitute darkness.
2.2.2 Electromagnetic fields

A number of studies have demonstrated that manipulating the earth’s magnetic field, results in the pineal gland’s altered capacity to synthesize melatonin. Selmaoui and Touitou (1995) studied the effects of both duration and intensity of magnetic fields as it affects serum melatonin and pineal enzyme activities. The study showed that in 5-week old male Wistar rats, long-term exposure to a magnetic field (10μT and 100μT) for 18 hours per day during 30 days significantly depressed the nocturnal peak of serum melatonin concentration and pineal NAT activity. A short-term exposure of 12 hours, had an effect only at the highest intensity used (100μT). In other studies, exposure to either a pulsed directed current or an extremely low frequency and magnetic field also caused a reduction in melatonin and/or NAT levels in rodents. Kato et al (1993) found that when 11-18 week-old Wistar-King male rats were exposed to a 50-Hz rotating magnetic field for 6 weeks, there was a significant decrease in pineal melatonin production. Lerchl et al (1990) found decreased pineal NAT activity in adult AMES mice and male Sprague Dawley rats when exposed to an artificial magnetic field generated by Helmholtz coils, for a one-hour period.

In studies with larger animals and in human studies, the electromagnetic induced reduction of melatonin often seen in smaller animals, have often shown opposite results (Graham et al, 1997; Lee et al, 1993). Some authors attribute this to the substantial physiological difference between these species (Brainard et al, 1999). In a study performed in 32 men age 20 to 30 years, the possible effects of acute exposure (9 hours) to 50-Hz linearly polarized magnetic field (10μT) on pineal function was investigated (Selmaoui et al, 1996). This study reported that acute exposure to 50Hz magnetic fields of 10μT had no effect on both urinary and serum
melatonin levels. The researchers suggested that it could be possible that chronic exposure or higher intensity of magnetic fields affect melatonin secretion or its circadian rhythmicity or both, in humans.

It still remains unclear how magnetic fields act on the pineal. Magnetic fields may alter the electrical activity of pinealocytes or their ability to produce the pineal hormone melatonin or both (Semm et al, 1980). And since other studies (Rudolph et al, 1988) demonstrated that exposure to static magnetic fields depresses cAMP, it may also be possible that the decrease of NAT activity is related to the decrease in cAMP.

2.2.3 Sleep-wake cycle

The alterations of melatonin secretion, which include a reduction in amplitude and phase shift that have been seen in individuals with jet lag and shift workers, demonstrate the relationship melatonin secretion has with the sleep-wake cycle. In castrated Wistar rats, ages ranging from 3 months to 4 months, Peder et al (1989) demonstrated that 4 days of rapid eye movement (REM) sleep deprivation elevated early morning pineal melatonin content by a factor of 2. This was consistent with findings that showed melatonin levels in humans decreased during REM sleep (Luboshitzky et al, 1999)

Another human study showed a greater than two fold increase in melatonin plasma levels during the night following 3 hours of sleep deprivation (Salin-Pascual, 1988). When melatonin secretion was examined in night shift workers, Waldhauser et al (1986) showed that in 5 men working normal hours from 0900 h to 1700 h, melatonin peak was detected at 0400 h. In contrast, 2 bakers working from 1900 h to 0400 h having sleep periods from 0600 h to 1400 h exhibited advanced melatonin rhythm with peaks recorded at 1400 h and 2000 h. From these studies, it is
evident that there is a correlation between the amplitude of a nocturnal melatonin peak and sleep schedule. Further investigations into the relationship between the sleep-wake cycle and melatonin are providing insights for treatment of the disoriented feeling associated with traveling across different time zones, shift work, as well as for patients suffering from insomnia (Cole et al., 1992; Folkard and Arendt, 1993; Webb and Puig-Dumingo, 1995).

2.2.4 Food intake

From the studies that have been conducted regarding melatonin and food intake, it is clear that food intake is among the factors that might affect melatonin secretion, though its mechanism as of yet remains unclear.

Chik et al. (1987) investigated the effect of food restriction on 24 h serum and rat pineal melatonin in 200-300g male Wistar rats. The authors showed that restricting rats to 50% of rat chow for 1 week had no effect on serum or pineal melatonin. However, three weeks of restricted feeding caused a decreased nocturnal pineal melatonin content, with an accompanying increase in amplitude and duration in the nocturnal rise in serum melatonin. Another study using one hundred and eighty 20-day old Sprague-Dawley male rats, examined the influence of protein-calorie malnutrition on circadian rhythmicity of pineal melatonin (Herbert and Reiter, 1981). The control animals were fed a standard laboratory diet containing 27% protein (source not mentioned), 59% starch, 10% vegetable oil and 4% salt mixture USP XIV. The protein-calorie malnourished rats were fed a low protein diet containing 8% protein (source not mentioned), 78% starch, 10% vegetable oil and 4% salt mixture USP XIV. The two isocaloric diets were administered for 30 consecutive days. The light cycle was 14 h light:10 h dark with lights on at 0500 h. The study showed that although the general pattern of melatonin secretion was similar in
both control and protein-calorie malnourished group, significantly less melatonin was found from 0700 h-1900 h in the pineal gland of rats that were protein-calorie malnourished. Ishibashi et al (1966) demonstrated that pinealectomy increased feed consumption in Sprague-Dawley female rats. Rojdmark and Wetterberg (1989) demonstrated in humans that nocturnal serum melatonin level decreased by 19±3% (P<0.002) in 19 healthy adult male volunteers that had been subjected to a 2 day fast. They also showed that supplementation with small oral doses of glucose during the fast, restored the reduced melatonin secretion to normal.

A study by Challet et al (1997) investigated the possible effects of timed caloric restriction on the light-dark synchronization of four biological rhythms, namely those of melatonin, body temperature, locomotor activity and corticosterone in 8-10 week old male Wistar rats. Rats adapted to the photoperiod of 12 h light:12 h dark (lights on at 0800 h) were fasted for three days and thereafter at 1000 h given 50% of their usual mean daily food intake of chow pellets during baseline. Food was restricted for a period of 1 month in one group and 2 months in the other. With respect to melatonin secretion, this study showed that restricting food for a period of 2 months resulted in a 2 h phase advance of the pineal melatonin rhythm. However restricting food for 1 month advanced the nocturnal onset of pineal melatonin but not its decline. This indicated a larger width of pineal melatonin peak compared to that obtained in the controls fed ad libitum. The study further suggested that timed caloric restriction is a potent phase-shifting agent interacting with the light-dark cycle time cues which may thus affect the internal synchronization of the circadian system.

In a recent study, Kalsbeek et al (2000) tested the accelerating potency of restricted feeding on re-entrainment of the melatonin rhythm. In this study, the researchers used 45 male Wistar rats and adapted 16 of the animals to restricted feeding with 2 h food access at the
projected time of a new dark onset. This 8 h phase advance was also introduced to 15 animals fed *ad libitum* while other animals were fed *ad libitum* without any phase advancement introduced. Melatonin measurement was made using a transpineal microdialysis probe. The results of this study showed that in light-dark entrained conditions, restricted feeding did not produce any significant changes in nocturnal melatonin profile as compared to *ad libitum*. However, after an 8 h phase advance, animals on the restricted feeding schedule resynchronized more slowly to the new light-dark cycle than *ad libitum* fed animals. The researchers suggested that prior entrainment to a nonphotic stimulus such as restricted feeding may be counteractive by "phase locking" the circadian oscillator through a process of associative learning and thus hinder resynchronization that may take place after a phase shift.

### 2.3 Melatonin Rhythms and Health

#### 2.3.1 Aging

Melatonin, along with its rate-limiting enzyme NAT, are subject to change during development and aging (Klein and Lines, 1969; Reiter *et al*, 1980). Studies with rats have demonstrated that by 3 weeks of age, the highest melatonin and enzyme levels in the pineal gland are present. After that, melatonin and HIOMT concentrations in the pineal drop rapidly until 8 weeks of age. Melatonin concentration declines rather moderately from then on until old age (Klein and Lines, 1969; Pang *et al*, 1984). Stokkan *et al* (1991) proposed that the pineal gland through the secretion of melatonin, may be seen as a possible neuroendocrine mediator between food restriction and some disorders that are age-related. These investigators demonstrated that by restricting the food intake of 172 male Fischer rats, either 3 or 28 months old and being fed Purina chow diet fortified with vitamins, this retarded the aging process of the
pineal gland, giving the pineal gland of older rats a youthful appearance. The authors proposed that food restriction, which noticeably increases life span and reduces age-related physiological deterioration and diseases seen in many animals, might mediate some of its effect through pineal activity that is sustained in old age.

In humans, Touitou and colleagues (1981) looked at 24-hour mean plasma level in elderly subjects aged 62-91 years. These levels were seen to be half of those in young men averaging about 24 years of age. Similarly, Iguchi (1981) found that the nocturnal rise of melatonin was significantly reduced in elderly subjects average age 84.8 years when comparing them to younger men with an average of age of 24 years. Another study by Liu et al, (1999) looked at melatonin levels in the cerebrospinal (CSF) fluid of 85 patients with Alzheimer’s disease (AD). These subjects were mean of 75±1.1 years. Eighty-two age matched subjects without primary neurological or psychiatric disease with mean of 76±1.4 years were also examined. In this study the authors showed that in old control subjects (>80 years of age), CSF melatonin levels were half of those observed in control subjects 41-80 years of age. In AD patients, these levels were only 1/5 of those in control subjects.

In a study conducted in women, Okatani et al (2000) determined melatonin levels in premenopausal and postmenopausal subjects. The authors showed from 17 to 45 years, the nocturnal melatonin secretion in premenopausal women moderately declined but increased during the period from 46 to 58 years. Among postmenopausal women, a steep age related decline in nocturnal melatonin secretion was observed for up to 15 years postmenopause. This was followed by an extremely gradual decline thereafter. The authors found a significant negative correlation between the peak serum melatonin concentration and the serum 17 beta-estradiol concentration in premenopausal women aged 40-50 years and observed that melatonin
levels were suppressed when postmenopausal women were given daily oral administration of conjugated estrogen (0.625 mg). The authors related transient elevated nocturnal melatonin secretion during menopause to the existence of a low estrogen environment.

One of the suggested roles for the pineal gland and melatonin in aging is melatonin's role as a potent free radical scavenger (Reiter, 1995). Melatonin thus directly works to protect neurons from the attacks of oxygen radical. As one ages, it is proposed that there is an accumulation of free radical damage with eventual death of human cells (Harman, 1992). So with aging melatonin is lost, and the organism suffers progressively more oxidative damage from free radicals. This degree of damage accelerates as melatonin reduction becomes more and more severe with aging (Reiter, 1995).

2.3.2 Development and child studies

Throughout an individual's lifetime there is remarkable marked change in the rhythm of melatonin (Reiter, 1998). In humans diurnal melatonin rhythm is expressed in pregnant women's blood throughout gestation with serum concentration much higher in the latter part of pregnancy than the earlier part (Kivela, 1991). Although melatonin is detectable in the foetus during the first week after birth, it is secreted in low daytime levels (Davis, 1997). In rats the rhythm of the activity of NAT begins earlier (2-4 days postnatal). In humans, full term newborn infants develop a melatonin rhythm between the ninth and twelfth week after birth (Kennaway et al, 1992).

Melatonin levels have been measured in a number of clinical disorders in children. In Sudden Infant Death syndrome (SID) infants, Sturner et al (1990) observed a significantly lower mean of melatonin concentrations in the CSF of infants when compared to non-SID
controls. Munoz Hoyos et al (2001) assessed the existence of a possible nocturnal ultradian rhythm (frequency 20 h) of melatonin in 28 paediatric patients (12 males and 16 females) mean aged 9.08±2.2 years. Plasma melatonin was sampled every 30 minutes between 2100 h and 0900 h. The results showed that the pattern of melatonin levels was related to the cause of growth delay in children studied, although the means of nocturnal concentration of melatonin were similar in all children. In children with genetic low height, there was found high melatonin levels between 0430 h and 0630 h and low levels between 2200 h and 2400 h. The constitutional growth delay group showed higher melatonin values than in other groups, while the partial growth hormone deficit group was characterized by lower melatonin levels than in the other groups tested. The authors concluded that the study proved that children exhibit an ultradian rhythm of melatonin like that in adults. However it is not clear whether the pattern of melatonin production is required for its biological actions as the existence of irregular pulses may reflect endocrine influences at this age and/or the immaturity of the intrinsic pulse generator.

In another study involving 13 boys with fragile X, aged between 4.7 and 11 years and 8 normal control boys aged between 6.1 and 11 years, Gould et al (2000) found elevated levels of saliva melatonin across the circadian cycle in fragile X individuals. Nocturnal melatonin production which was expressed as both peak level and area under the concentration time-curve taken between 2000 h and 0800 h, was found to be significantly larger in fragile X boys compared to controls. The mean minimum daytime melatonin levels were additionally found to be significantly higher in the fragile X boys than in control subjects. One of the mechanisms for these elevated levels goes to the reason of melatonin receptor malfunctioning. The authors suggested that the diminished receptor activation might lead to the overproduction of melatonin as a compensatory response.
2.3.3 Coronary artery disease

Sakotnik et al (1999), in a study examining the nighttime melatonin production in patients with coronary heart disease, demonstrated that there was indeed a decrease in melatonin production in such patients and that beta blockers did not further suppress melatonin production. These results were confirmed by a recent study by Girotti and colleagues (2000). In assessing whether a correlation existed between melatonin production and uncharacterized coronary heart disease, the researchers found from urine samples of 24 healthy subjects (mean age: 63 ±13 years), 32 patients with chronic stable coronary disease (62 ± 11 years) and 27 patients with unstable angina (62 ± 12 years) that urinary 6-sulphatoxymelatonin excretion was significantly lower in patients with unstable angina compared to healthy subjects or patients with stable angina. The level of 6-sulphatoxymelatonin in patients treated with beta-adrenoceptor blockers did not seem to differ significantly in coronary patients not receiving beta-blockers. The results thus revealed that patients with coronary disease had a low melatonin production rate, which was greatly decreased in those with higher risk of cardiac infarction and/or death.

2.3.4 Eating disorders

Although few studies have reported unaltered pineal melatonin secretions in women with anorexia nervosa (Kennedy et al, 1990), several have found higher levels of both diurnal and nocturnal mean plasma melatonin concentrations in patients with untreated anorexia nervosa with differences observed in the peak for nocturnal melatonin secretions (Arendt et al, 1992; Ferrari et al, 1990). It is hypothesized that these increased levels are related to hypothalamic hypogonadism seen in anorexia nervosa patients (Pacchierotti et al, 2001). Authors suggest that
in anorexia nervosa a primary disregulation of the hypothalamic control of gonadotrophin releasing hormone (GnRH) activity may lead to both enhanced melatonin synthesis and secretion, which comes as a result of the impaired gonadal steroid input to the pineal (Pacchierotti et al, 2001).

In bulimic individuals, melatonin levels were shown to increase only during daylight hours in which bulimic behaviours are more frequent (Wurtman and Wurtman, 1989). At night, melatonin levels remain normal except in bulimic patients with comorbid depression where the melatonin peak was flattened (Kennedy, 1994). A study by Mortola et al (1993) though showed that in bulimic, albeit amenorrheic patients, there was no significant decrease of nocturnal melatonin secretion.

2.3.5 Psychiatric disorders and depression

Several authors have reported altered circadian rhythms of the neuroendocrine system in depressive disorders (Coplan et al, 2000; Posener et al, 2000) and investigators have theorized that these dysfunctions could be based on an altered melatonin rhythm, since this hormone regulates some endogenous rhythms (Pacchierotti et al, 2001).

In a study conducted in schizophrenics Monteleone et al (1997) found a decreased nocturnal secretion of melatonin in 9 drug-free schizophrenics when compared to controls. They also observed that though chronic treatment with antipsychotic drugs significantly improved psychotic symptomatology, it did not result in a change in the secretory pattern of melatonin. The data goes to show that though biosynthetic activity of the pineal gland may be impaired in chronic schizophrenia, successful treatment with antipsychotic drugs does not produce changes in melatonin production.
In a study by Beck-Friis et al (1985) the authors used 32 acutely ill inpatients (18 males and 14 females) mean aged 43±1.9 years and 33 healthy subjects mean aged 40±2.2 years to investigate the relationship between melatonin and some clinical variables in patients with major depressive orders and healthy controls. Serum samples for cortisol and melatonin assays were drawn every 4th hour during the first day and every 2nd hour during the first night. The dexamethasone suppression test (DST) was done with oral administration of 1 mg dexamethasone at 2200 h on the second night. Serum cortisol samples were drawn at 0800 h, 1600 h and 2200 h on the third day, and the maximum (MT_max) nocturnal melatonin level was defined as the highest level obtained between 2000 h and 0800 h. In the study, 53% of the patients did not suppress cortisol below 200 nmol/l at 0800 h, 1600 h of 2200 h after the dexamethasone administration, thus fulfilling their criterion for abnormal DST. The study results showed that depressed patients with abnormal response to oral dexamethasone administration had lower MT_max levels (0.19±0.03 nmol/l) than depressed patients with normal response to oral dexamethasone (0.30±0.02 nmol/l) and controls (0.30±0.03 nmol/l). Loss of parents in early ages has been seen to be of importance in inducing depressive behavior (Beck-Friis et al, 1985). In this study, it was observed that patients who reported parental loss before 17 years of age had nocturnal melatonin levels that were below normal. This significantly differed from the patients with no parental loss. The study also showed that patients who reported suicide attempts had a significantly higher mean MT_max levels than those without reported suicide attempts. Indeed the pineal gland of patients of violent suicide has been shown to exhibit non-specific morphological changes, such as pinealocytes in a lobular disposition and irregular dissemination of astrocytes. It is hypothesized that the pineal gland in such patients makes an attempt to compensate in presuicide period and this results in an increase in melatonin secretion. With regards to depressed
patients, the authors suggested the possibility of a low melatonin syndrome and proposed that this is characterized by low nocturnal melatonin, abnormal dexamethasone suppression test, disturbed 24h rhythm of cortisol and less pronounced daily rhythm, and annual cyclic variation in depressive symptomology.

Seasonal affective disorder (SAD) is an affective illness with recurrent depression episodes in winter or fall, which is remitted by the following spring or summer (Rosenthal et al, 1984). Alterations of endogenous rhythms including melatonin have been reported in such patients (Terman et al, 1987). The importance of the circadian rhythm of melatonin in the determination of SAD is evident by the treatment of choice for these patients being light therapy, which acts by inducing a phase advance of the circadian rhythms. This was demonstrated in a study by Terman et al (2001) who investigated a possible mechanism of action for the antidepressant response to light phase advances of the circadian clock. Participants in this study included 42 research volunteers aged 21 to 56 years (39.2 ±9.3 years), with 29 women (69%) and 13 men (31%). Within a crossover design, 21 subjects first received morning light and then evening light (MIE2), and 21 received treatment in the opposite order (EIM2). After a minimum 2-week baseline interval that verified a current depressive episode, subjects received 10 to 14 days of treatment (30 minutes per day) in both periods. The lighting device produced 10 000 lux, 2700°K fluorescent illumination through a 28 x 61-cm diffusing screen. Melatonin was sampled at intervals in the 2 protocols. Raters who were blinded to the treatment, administered to subjects the 29-item Structured Interview Guide for the Hamilton Depression Rating Scale–Seasonal Affective Disorder Version (SIGH-SAD) at baseline and after both treatment periods. This was performed on the same day melatonin was sampled. The results of the study showed that the morning light produced phase advances of the melatonin rhythm, while evening light, produced
delays. The magnitude of these advances the authors suggested depended on the interval between melatonin onset and light exposure, or circadian time (morning, 7.5 to 11 hours; evening, 1.5 to 3 hours). The study also revealed that though later evening light brought larger delays, advances were larger with earlier morning light. And though similar depression ratings were observed with light at either time of day, response to morning light increased with the size of phase advances, up to 2.7 hours regardless of baseline phase position. There was no such correlation observed for evening light. The authors recommended that to elicit an antidepressant effect of light, light therapy of 10 000 lux should be administered for 30 minutes. This should be scheduled in circadian rather than clock time, about 8.5 hours after melatonin onset or 2.5 hours after the sleep midpoint.

Other documented psychiatric disorders reporting impairment of melatonin rhythm/levels include bipolar disorder (Kennedy et al, 1996; Nurnberger et al, 2000), suicidal behavior (Maes et al, 1996; Stanley and Brown, 1988) and obsessive-compulsive disorder (Monteleone et al, 1994).

### 2.3.6 Sleep and sleep disorders

Sleep being a restorative process, has been shown to be controlled by the interaction of an output of the circadian pacemaker, presumably located in the SCN (Cajochen et al, 1999). The pineal hormone melatonin is considered to play a major role in the circadian regulation of sleep and thus melatonin’s irregularity in rhythm is consequently observed when sleep is altered or deprived in various ways.

In a study by Shilo et al (1999), the authors investigated the melatonin secretory pattern in patients in the Intensive Care Unit (ICU). These patients suffered various forms of sleep
deprivation associated with the nature of clinical activities, which were related to patient care in these units. The results showed that the patients lacked normal sleep behaviour for the entire study period. Analysis of actigraphy, used to assess sleep, suggested patients did not sleep during the entire study period (72 h) except for very short naps lasting up to one hour. Urine samples were collected every 3 h for 24 h from these 21 adults (14 from ICU and 6 control subjects) and analysed for melatonin metabolite 6-sulphatoxymelatonin (6-SMT). The results showed 6-SMT excretory pattern was abnormal in all ICU patients. It was also observed that the expected nocturnal rise of melatonin in 12 of 14 patients was absent.

The rhythm of melatonin has seen to be impaired in jet-lag maladaptation (Waterhouse et al. 1998) and in individuals with desynchronized sleep-wake cycle. Wyatt et al. (1999) studied the interaction of homeostatic and circadian processes in the regulation of waking neurobehavioral functions and sleep. In this 27-day protocol, six subjects, one female and five male (age 19-27 years, mean 23.2 years) were scheduled to 15-24 repetitions of a 20-h rest/activity cycle. This resulted in desynchrony between the sleep-wake cycle and the circadian rhythms of body temperature and melatonin; the forced desynchrony segment was similar to one traveling eastward four time zones per day, having bedtimes and wake times advanced by 4 h per cycle. In the subject's living area and bathroom, ambient lighting was ~15 lux (angle of gaze) during wake episodes and <0.03 lux during sleep episodes. Hourly blood samples were taken for the analysis of melatonin. Neurobehavioural functioning was assessed by administering Probed Recall Memory test (PRM); Psychomotor Vigilance Task (PVT); Addition/Calculation test (ADD); Digit Symbol Substitution Test (DSST) at 2 hour intervals, and at 30 minute intervals beginning 30 minutes after scheduled awakening subjects were rated on the Karolinska Sleepiness Scale (KSS). The study results showed that even with only 13 h 20 minutes of scheduled wakefulness
that preceded each sleep episode, there was still significant homeostatic and circadian modulation of sleep structure. The highest sleep efficiency took place in sleep episodes bracketing the melatonin maximum and core body temperature minimum. The authors observed however, that at these same circadian phases and toward the end of each wake episode, there was a maximal impairment of neurobehavioral functioning seen across all subjective and objective measures. The study results the authors say make clear the importance of the sleep/wake homeostatic system, the endogenous circadian system, and their interaction in the regulation of sleep and neurobehavioral functions in healthy young adults.

Other sleep related disorders/conditions studied that have exhibited altered melatonin rhythms include: shift work syndrome (Lewy et al, 1996), insomnia (Armstrong, 1999; Portaluppi, et al 1994) and delayed sleep phase syndrome (Shibui et al, 1999).

2.3.7 Cancer

Since Lapin and Frowein (1981) first documented a decrease in pineal melatonin levels in rats with growing undifferentiated (Yoshida) tumours, there has been growing interest in studying the changes in circulating levels of melatonin in oncolological patients, and how this could be used as a potential diagnostic and/ or prognostic marker for neoplastic disease (Blask, 1993).

In a study with untreated breast cancer patients, Bartsch et al (1981) monitored melatonin levels in the urine samples collected from these patients. Their study revealed that there was a 30% depression of the 24 h excretion of melatonin. This was accompanied by a phase delay of the circadian peak of urinary melatonin excretion, which showed higher levels in the morning than at night. During the afternoon (1400 h-1800 h) and night (2200 h-0600 h) intervals, the
excretion of melatonin was seen to be 50% lower and became statistically significant when cancer patients with an advanced primary tumour (greater than stage II) were considered.

More recently, Bartsch et al (1997) performed a similar study in patients with primary mammary cancer to confirm earlier findings with breast cancer patients. This study showed that among patients with primary mammary cancer, nocturnal urinary excretion of 6-sulphatoxymelatonin (aMT6s) was significantly depressed by 48%. In addition, when breast cancer patients were subdivided according to the stages of their tumour growth, a clear inverse relationship was found between melatonin and tumour size as patients with T2 and T3 tumours showed respectively a 40% and 71% significant depression. In contrast to these studies, Skene et al (1990) reported in breast cancer patients that the overall 24 h excretion of aMT6s as well as the amplitude of its nighttime rise were significantly attenuated in women with breast cancer compared to the benign tumour group.

In other forms of cancer, studies have shown a reduction in circulating melatonin in patients with gastric and colorectal cancer (Colombo et al, 1991; Khoory and Stemme, 1988), and in patients with stomach and colon cancer Kvetnoi and Levin (1987) reported depressed levels of urinary excretory levels of melatonin.

2.4 Biological Rhythms

2.4.1 Melatonin and the circadian system

Melatonin is said to be the marker of circadian rhythms (Barassin et al, 1999). Pineal melatonin is secreted in a circadian pattern with high levels in the dark period. Since the cyclic nocturnal increase of melatonin levels is proportional to the length of nights in the light-dark cycle, melatonin thus conveys a photoperiodic message and functions in an organism as an
2.4.2 Feeding and biological rhythms

In mammals, circadian rhythms which are mainly driven by the SCN are entrained by the light-dark cycle. This is made possible by the light entrainable oscillator located in the SCN (Miler et al, 1996). However the circadian system can be affected by stimuli other than light, which in some cases may be through the oscillators outside the SCN (Davidson and Stephan, 1998). Richter (1922) first reported that rats fed daily-restricted meals exhibited a marked increase in wheel running during the hours that shortly preceded meal times. This is known as anticipatory activity. Associated with this are behavioural and physiological variables that
change both their circadian phase and shift in relation to the feeding schedule. This has been demonstrated in a number of studies, one of which is that by Diaz-Munoz et al (2000). In this study, rats were put on 2 feeding schedules, controls fed *ad libitum* and rats exposed to daily-restricted feeding schedule. These rats were fed from 1200 h-1400 h for 3 weeks after which they were randomly killed at 0900 h, 1000 h, 1100 h, 1200 h, 1400 h or 1800 h to obtain blood samples. The authors observed that in restricted rats there was a significant increase in corticosterone before food presentation, a decrease in insulin during hours before food presentation (from 0900 h to 1200 h), which increased after feeding. There was also an increase in glucagon levels at all time points, and when compared with controls, the increase in glucagon levels was significant and 2-fold before food access. Also observed was an increase of free fatty acids from (1100 h to 1200 h) when compared with controls, this rise however declined after feeding, whereas liver glycogen was depleted before feeding and recovered after ingestion. The latter two results were in line with previous results (Escobar *et al*, 1998) and thus confirmed the efficacy of the procedure the authors used to induce the expression of feeding entrained oscillators.

Studies have shown that rhythms of body temperature and blood corticosterone levels in rats and mice persists under constant lighting conditions (Gibbs, 1976; Halberg, 1960). However, both rhythms are readily altered by daily feeding schedules and show anticipatory increases preceding feeding time. As food becomes available, corticosterone levels generally start to decrease (Holloway *et al*, 1979; Moberg *et al*, 1975; Morimoto *et al* 1977) whereas body temperature continues to rise during feeding but decreases shortly after (Krieger, 1974; Nelson *et al*, 1975).
Food restriction seems to also affect liver functions. Stokkan et al. (2001) investigated the effect of restricted feeding on rhythmicity in the liver. The researchers used a transgenic rat model whose tissue expressed luciferase in vitro to monitor the rhythmic expression of this clock gene. This was done by recording light emission from the tissue in vitro. The study showed that the restricted feeding regimen, when food was made available only for 4 hours during the day of a 12h light:12h dark cycle, was able to shift the circadian clock in the liver by 10 hours within 2 days. After 7 days of 8 hour of restricted feeding, liver rhythmicity was shifted by 12 hours. The authors suggest that the peripheral circadian oscillators like those found in the liver may be coupled to the SCN and may take place primarily through rhythmic behaviour, such as feeding. Other daily biological rhythms in rats and mice that have been affected in their expression by restricted feeding are insulin secretion (Vuguin et al., 2001), water intake (Mittal et al., 2000), locomotor activity (Challet et al., 1997; Yi et al., 1995) and lung rhythmicity (Stokkan et al., 2001).

2.4.3 Feeding Patterns and Macronutrient Selection

It has been duly reported in the literature that food selection involves different temporal patterns of brain parameters (Thibault, 1992). In this study Thibault (1992) investigated the relationship between feeding rats a single diet (20% casein) versus choice between two diets (0% and 60% casein diet) and central idoleamines and catecholamines. The study revealed that in animals adapted to the choice-diet pattern, the 5-HIAA/5-HT ratio in the brain striatum and 5-HT levels in brain piriform lobe were significantly higher than those found in rats adapted to the single-diet pattern. The catecholamine levels in these animals showed that animals offered a single diet displayed a significantly higher dihydroxyphenylacetic (DOPAC)/dopamine (DA)
ratio in brain piriform lobe, higher noradrenaline (NA)/dopamine (DA) ratio in the hypothalamus and higher NA levels in the raphe nuclei when compared with animals adapted to the choice-diet paradigm. The results of the study suggest that diet paradigm does influence both serotonergic and catecholaminergic systems in rats.

Thibault and Booth (1999) suggested that the mechanisms that control the selection of macronutrients include sight and sound, smell, irritance, taste and touch. They added that behavioural requirements are involved, which regard food choice directed at particular nutrients. One of these requirements is that foods chosen by the eater, at least in that past, are that which improve nutritional status, especially with regards to the nutrient in question. Foods must also somehow convey information to the eater about that particular nutrient. Animals must thus learn to recognise in a particular foodstuff, a pre/oral feature that produces an after effect. This then sends signals to the brain distinctive for the macronutrient that is present in large amounts in that particular food. The authors further suggest that in order for animals to acquire an appetite that is nutrient specific, they must build up a representation of the food-sensed identity. They must also have a representation of the changes in the body’s nutritional state, which is controlled by ingestion.

Research in rat's feeding patterns has resulted in a number of theories that may help to explain the basis of rats' feeding behaviour. Rats seem to exhibit circadian rhythm in their eating pattern (Rosenwasser et al, 1981). Being nocturnal animals, rats are active during the night and are found to have a typical pattern of nocturnal feeding with greater than 70% of food intake occurring during the dark phase (Zucker, 1971). Studies in rat feeding patterns have suggested that there exists not only appetite for calories but also appetite for protein and carbohydrate, two of the energy producing macronutrients (Wurtman et al, 1983; Musten et al, 1974). DiBattista
investigated specific appetites for protein and carbohydrate by allowing mice and rats time-restricted access to either protein or carbohydrate, and it was demonstrated that during restriction phase, animals that were protein-restricted showed substantial selective increases in protein intake during the one-hour periods when protein was made available. These animals consumed 40-45% of their normal daily protein intake during this phase of the experiment. In contrast, carbohydrate restricted rats did not demonstrate any evidence for the development of carbohydrate appetite. The author explained the possibility that one-hour duration of the carbohydrate availability period in the study might have masked the development of a genuine carbohydrate appetite, adding that if this appetite did develop it might have been for a short period of time (e.g. 15 to 30 minutes).

The appetite for carbohydrate was observed however in an earlier study by Wurtman et al (1983). In their study, the authors observed that when animals restricted to a ketogenic diet containing only protein and fat were allowed to choose between 25% and 75% dextrin diets, they responded by choosing more of their food from the high carbohydrates during the first 30 minutes of feeding, such that their total carbohydrate intake was significantly higher than that consumed by the controls. In the same study, in which animals were designated to a receive a carbohydrate premeal 1 hour earlier than meal time or mixed nutrient, and they observed that rats eating the carbohydrate premeal subsequently ate as much total food as the mixed nutrients controls however, these rats consumed significantly less carbohydrate. The authors suggested that animals are able to regulate their carbohydrate intake in one meal in response to the amount carbohydrate consumed in previous meals. They further added that this behaviour brings evidence that distinct mechanisms regulate appetites not only for total calories but for carbohydrates as well.
The fact that macronutrient selection in rodents influence subsequent feeding choices by these animals is further demonstrated in a recent study by White et al (2000). In this study 38 young and 38 older male Sprague Dawley rats were used to study the effects age might have on the feeding response to moderately low protein diet. Rats were given 20% casein diet for 1 week after which one group continued to receive the control 20% casein diet. The other two diet groups received one of two isocaloric low protein diets (10% casein). In one of these diets the caloric difference due to the low protein was compensated by the addition of carbohydrate and for the other low protein diet, caloric difference was compensated by the addition of fat. After 12 days of consuming these diets, the young rats had greater feeding response to 10% casein diets than did the older rats and this response occurred regardless of whether fat or carbohydrate compensated for the caloric differences due to decreased protein. Young rats have higher protein requirements than older rats, with the two extremes ranging between 15 % to 5% protein. Thus in the study, the authors suggest that 10% dietary casein was well above the protein requirement of older rats, thus leading to no increase in food intake. On the other hand, the 10% dietary casein may have been close to the protein requirements in the younger rats resulting in an increase in food intake. The authors proposed that protein regulated food intake is maximized when the level of dietary protein is at or around that of the protein requirement of the animal, and that increased food intake associated with moderately low dietary protein may be in fact related to the reduction of a compound, whose production is associated with the level of amino acid deamination.

Research has also shown that animals regulate protein and energy intake in relation to daily need and rats exhibit specific pattern of food intake and macronutrient selection during different periods of the night. A study by Tempel et al (1989) using 24 adult male Sprague Dawely rats, examined on a free feeding self-selection paradigm with three macronutrient diets
namely a carbohydrate (28% dextrin, 28% cornstarch and 37% sucrose), a protein (93% casein) and a fat diet (86% lard). The study showed shifts in nutrient selection patterns over time. During the early dark period animals were seen to have a preference for the carbohydrate meal relative to fat and protein, accounting for 35% of the animal’s total 24-hr carbohydrate intake, while the final 3 hours of the dark phase was characterized by a generally equal preference for each of the 3 macronutrients. However, just before the onset of light, the animals displayed a dietary preference by selecting more fat and protein than carbohydrates. This study is in accordance with that of Shor-Posner et al (1991) and both suggest food ingested during the first half of the dark phase is used to fulfill immediate energy requirements and to promote lipogenesis, while late in the night nutrient and energy stores are thus replenished. Feeding then becomes anticipatory and is geared towards storage, and subsequent utilization of nutrients during the light cycle.

2.4.3.1 Tryptophan intake and brain neurotransmitter levels

Several studies over the years have shown that function of the brain is indeed influenced by diet (Fernstrom, 2000). This truth is evident in studies regarding dietary tryptophan as it relates to level of brain neurotransmitters.

In a study by Ashley and Curzon (1981), 80 Sprague Dawley rats were used to investigate the relationship between plasma and brain tryptophan concentration and brain 5-HT metabolism. In this study, 72 rats were fed 1.45% tryptophan diet *ad libitum* while the remaining 8 were fed non hydrolysed casein diets. After a period of 6 days those on the 1.45% tryptophan diet were divided into 2 groups of equal size, one group continued on this diet, the other was fed a 0.4% tryptophan diet. The biochemical data showed at the end of the experiment that in rats fed a low tryptophan diet, brain tryptophan, brain 5-HT, and 5-hydroxyindoleacetic acid (5-HIAA)
were rapidly depleted. And after one day, significant decrements of plasma tryptophan were related to proportional decrements of brain 5-hydroxyindole concentrations.

When White et al (1988) investigated the effects dietary tryptophan might have on macronutrient selection, the researchers used 24 thirty-day-old male Sprague Dawley rats and administered one group a diet containing no tryptophan, another group received 2% tryptophan as 2% tryptophan by weight of each macronutrient source, while the last group received tryptophan as 2% of calories for each macronutrient in the experiment. It was observed that protein intake increased to 30% for the tryptophan-calorie group and decreased to 20% for the non tryptophan group. Carbohydrate consumption by the non tryptophan group did not change, but rats in both experimental groups decreased their intake of carbohydrate. The results revealed that the rats receiving tryptophan showed significantly higher hypothalamic 5-HIAA levels. The authors suggested that this increased concentration might reflect increased serotonin metabolism since the ratio of 5-HIAA to 5-HT was significantly higher in the hypothalamus of these animals.

Tryptophan intake also affected brain neurotransmitter levels in broiler chickens. Rosebrough (1996) assigned 7-28 day old broiler chickens to nine treatment diets. These diets consisted of 3 levels of crude proteins (120, 210 or 16.7g/kg) arranged in a 3x3 factorial design. The chickens were put on a 12 h light-dark cycle (lights on at 0600 h), treatments were randomly assigned and both feed and water were provided ad libitum. The results showed that although both the dietary crude protein and its interaction with tryptophan had little effect on 5-HIAA levels in the brain, supplemental dietary tryptophan increased both 5-HIAA and 5-HT while both crude protein and tryptophan were seen to decrease the 5-HT: 5-HIAA ratio. The authors affirmed that altered levels of brain neurotransmitters in fact accompanied changes in feed intake which were caused by different levels of both crude protein and tryptophan.
Infused tryptophan can affect brain melatonin levels. This was demonstrated in a study by Ouichou and Pevet (1992). Adult male Wistar rats were raised under a photoperiod schedule of 12 h light: 12 h dark cycle and received food and water *ad libitum*. These animals were sacrificed at the beginning of their light period. On analysis of the pineal gland following a 25-min tryptophan (10^-4 M) stimulation, tryptophan was shown to give a dose dependant effect on pineal melatonin release. Tryptophan infusion produced an immediate increase in melatonin release, reaching its maximum after a period of 40 minutes. Furthermore, this increase in response was not significantly reduced by pre-treating pineal with 10^-5 M phenanthroline (PHE), an aminopeptidase inhibitor.

There are various theories put forth in literature attempting to explain the variables affecting brain tryptophan levels. These include: plasma tryptophan concentration (Fernstrom and Wurtman, 1971), tryptophan binding to albumin (Bloxam *et al*, 1980), plasma concentration of large neutral amino acids competing with tryptophan for transport into the brain (Fernstrom *et al*, 1973) and the changes of the kinetic characteristics of the transport system (James *et al*, 1978). And so, though apparent that neurotransmitters of the brain are indeed influenced by diet, the means by which tryptophan influences brain tryptophan and 5-HT levels still seems controversial (Ashley and Curzon, 1981).
Chapter 3

MANUSCRIPT

Food Access Schedule and Diet Composition Alter Rhythmicity of Serum Melatonin and Pineal NAT Activity.

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Food Access Schedule and Diet Composition Alter Rhythmicity of Serum Melatonin and Pineal NAT Activity.


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SELMAOUI, B., A. OGUINE AND L. THIBAULT. Food access schedule and diet composition alter rhythmicity of serum melatonin and pineal NAT activity. PHYSIOL BEHAV 000-000.-

This study investigated the effect of dietary composition and food access schedule on the rhythmicity of serum melatonin and pineal N-acetyltransferase (NAT) activity. Wistar rats maintained on a 12:12h light: dark cycle were assigned to two dietary groups: a group fed rat chow and a group fed a choice between a protein-rich and a carbohydrate-rich diet. Each dietary group was further divided based on feeding schedule, with food available between 0800 and 1600 h or ad lib access to food. Regardless of dietary condition, total food and carbohydrate intake of rats having free access to food was higher than under the restricted food access schedule. Protein intake of rats fed the dietary choice was lower with the restricted access than in the free access. In rats fed the dietary choice, melatonin levels and NAT activity were significantly decreased with restricted access compared to free access. Such results were not found in rats offered restricted chow. This study suggests that the rhythms of melatonin secretion and NAT activity can be altered by dietary composition.
**Key words:** Circadian rhythm, Rats, Protein-rich diet, Carbohydrate-rich diet, Restricted food access, Free food access.

**INTRODUCTION**

Melatonin, the main hormone secreted by the pineal gland, plays a role in circadian and seasonal rhythms of several physiological and behavioral processes (Malpaux et al., 1999; Reiter, 1995; Webb and Puig-Domingo, 1995), and is stimulated by darkness and inhibited by light (Lewy et al., 1980). Entrainment of melatonin secretion by the light-dark cycle occurs through the retino-hypothalamic projection to the suprachiasmatic nuclei (Klein and Moore, 1979; Meijer and Rietveld, 1989; Rusak and Zucker, 1979). Melatonin secretion is also affected by numerous conditions such as jet lag (Fevre-Montange et al., 1981), depression (Claustrat, 1984), sleep disturbances (Portaluppi et al., 1994) and active schizophrenia (Monteleone et al., 1992). Although the most powerful synchronizer of melatonin secretion is the light-dark cycle, there is some evidence that the daily schedule of food availability might be another important external synchronizer for the circadian system. Indeed, in rodents daily restricted food access schedules resulted in marked phase shift of the circadian rhythmicity of many biological variables. For example, in most mammalian species timed food restriction entrained the circadian rhythm of plasma corticosterone (Broocks et al., 1990; Kato et al., 1980; Krieger and Hauser, 1978), body temperature (Murphy et al., 1993, Challet et al., 1997) and locomotor activity (Broocks et al., 1991; Challet et al., 1997; Stephan, 1986, Yi and Stephan, 1995). In addition, it has been reported in rats that food restriction decreased the amplitude or phase-advanced pineal melatonin rhythm (Challet et al., 1997; Chick et al., 1987), but increased serum melatonin concentrations (Chick et al., 1987). Although food restriction seems to have an effect on the circadian system, it remains
unclear whether it is the access schedule or food restriction that is the most influential. So far, no studies have reported the effect that food access schedule may have on the rhythmicity of melatonin and pineal NAT activity. Because melatonin is considered as a marker of circadian rhythmicity, the purpose of the present study was to investigate in rats the effect of restricting food access during daytime on nocturnal variations of melatonin and its rate-limiting enzyme NAT. In addition, rats offered a choice among macronutrient rich diets exhibit circadian rhythm in their preference for carbohydrate and protein (Thibault and Booth, 1999). Moreover, tryptophan is an essential amino acid which is hydroxylated then decarboxylated to produce serotonin, which is itself converted into melatonin. In the light of the circadian variations in macronutrient intake and the importance of tryptophan in melatonin biosynthesis, it was thus worthy to also investigate the effect that dietary composition may have on melatonin and NAT activity by providing animals a single chow diet or a choice between a carbohydrate-rich and a protein-rich diet.

MATERIALS AND METHODS

Animals and Diets
One-hundred and sixty-eight male Wistar rats weighing 175-185 g (Charles Rivers, St-Constant, Québec) were housed individually in a room with controlled temperature (23 ± 2°C) and humidity (55 ± 5%), under a 12:12 h light: dark cycle with fluorescent lights on at 0800 h. Rats were randomly divided into 2 dietary groups of 84 animals each. One group was fed a simultaneous choice between isocaloric carbohydrate-rich (47.2% dextrin, 31.4% corn starch, 10% solid vegetable shortening, 5% alphacel, 5.2% salt mixture, 1.1% vitamin mixture, 0.1% choline chloride) and protein-rich (78.6% casein, 10% solid vegetable shortening, 5% alphacel,
5.2% salt mixture, 1.1% vitamin mixture, 0.1% choline chloride) diets. The diet ingredients were purchased from ICN Biomedicals Inc. (Aurora, OH, USA). Diets were presented in granular form. Granular diets were prepared fresh daily by adding a fixed amount of water to the powdered diet (25 ml water to 100 g of the carbohydrate-rich diet and 50 ml water to 100 g of protein-rich diet) and diets were left to dry overnight in order to evaporate added water (Mok and Thibault, 1999). The second group of animals was fed a single diet made of ground standard chow (Purina # 5075: 18.1% crude protein, 4.5% crude fat, 3.4% crude fiber, 57.3% nitrogen free extract; the remaining components mainly consisted of mineral mixture and vitamin mixture) purchased from Charles Rivers (St- Constant, Québec). Tap water was supplied ad lib.

**Experimental procedure**

Each dietary group (choice and single) was divided into two sub-groups (n = 42 each) based on feeding schedule: a group was given a restricted access to food between 0800 and 1600 h (daytime) and the other group was fed ad lib. Rats with restricted food access and those fed ad lib were kept in separate rooms. Rats’ body weights were measured at 0800 h every other day. After two weeks adaptation to the environment, the diets and the feeding schedule, rats from each dietary group (12 food restricted and 12 ad lib) were randomly selected for daily food intake and body weight measurements for 8 days. Food intake was corrected for spillage. Carbohydrate and protein ingestions were calculated according to the percentage of these components in diets. At the end of the 21 days experimental period, all rats were killed under dim red light every two hours (12 restricted and 12 ad lib from both dietary groups at each time point) of the nocturnal cycle starting at 2000 h until 0800 h. Trunk blood was collected into test tubes without anticoagulant, centrifuged and serum was stored at -70°C until analysis of
melatonin. The pineal gland was quickly removed under dim red light and stored at \(-70^\circ\text{C}\) until analysis of NAT activity.

**Serum melatonin assay**

Duplicate 100 µl serum samples were assayed for melatonin by a modification of the RIA method described by Fraser *et al* (1983), using a \(^{125}\text{I}\)-melatonin tracer (Ravault *et al*, 1984). The intra- and inter-assay coefficients of variation were 9% and 13% (n =10) for 130 pg/ml. The sensitivity of the assay is 5 pg/ml. Radioactivity was measured in a gamma counter (LKB Wallac, 1282 compugamma CS, Fisher Scientific, Montreal, Québec).

**NAT assay**

Each pineal gland was homogenized in 100 µl of 0.1 M sodium phosphate buffer (pH 6.8). Fifty µl of this homogenate was mixed with 50 µl of sodium phosphate buffer containing 0.5 mM \(^{3}\text{H}\) acetylcoenzyme A and 10 mM tryptamine, and NAT activity was determined by the radioenzymatic assay method of Deguchi and Axelrod (1972). The reaction was carried out for 20 min at 37°C, and was stopped by the addition of 1 ml of chloroform at \(+4^\circ\text{C}\). The N-acetyltryptamine produced was extracted with chloroform and its radioactivity was measured by liquid scintillation spectrometry with a beta counter (Wallac, 1414 liquid scintillation counter, WinSpectral™, Fisher Scientific, Montréal, Québec).

**Chemicals and reagents**

For NAT assay, tryptamine HCl, acetylcoenzyme A, and acetylcoenzyme A (acetyl-\(^{3}\text{H}\)), with a specific activity 12 Ci/mmol were purchased from ICN (Québec, Canada). Melatonin was
purchased from ICN (Québec, Canada), melatonin 2 \([^{125}\text{I}}(50 \, \mu\text{Ci})\) was purchased from NEN (Québec, Canada), low melatonin rats’ serum was bought from Stockgrand Ltd. (Surrey, England) and rabbit antiserum was provided from INRA (Nouzilly, France).

**Statistical analysis**

Results are expressed as means ± SEM. Data were analyzed with a repeated-measures 2-way ANOVA (SuperANOVA v1.11). The main effects of food access schedule, diet paradigm and their interaction were tested. Scheffe’s post-hoc analysis was used. The cosinor method (Halberg et al, 1967) was used to verify and characterize the circadian rhythm of the dependent variables. The amplitude is the measure of one half of the extent of the rhythmic change estimated by the mathematical model e.g. cosine curve. Acrophase is the measure of timing of a rhythm in relation to a defined reference timepoint selected e.g. midnight, and represents the crest time of the cosine curve best fitting to the data and expressed here in hours and minutes. Mesor is the midline-estimating statistic of rhythm, that is value midway between the highest and the lowest values of the (cosine) function best fitting to the data. The Bingham’s test for the cosinor method was used to compare the acrophases of the circadian rhythm melatonin and NAT activity. Differences were considered to be statistically significant at p < 0.05.

**RESULTS**

**Body Weight**

Body weight variations of rats fed a single diet or a choice between a carbohydrate-rich and protein-rich diet ad lib or under a restricted food access schedule are presented in Figure A2a. In the free food access schedule condition, rats gained weight regularly from the first to the last
Body weight increased in ad lib dietary groups, from 178.48 ± 0.99 to 320.81 ± 5.83 g (choice) corresponding to 80 ± 3% weight gain and from 180.55 ± 1.13 to 340.88 ± 2.72 g (single) (89 ± 1% weight gain). In the restricted food access schedule, rats given a dietary choice did not show any great variation in the mean of their body weight (from 177.93 ± 1.05 to 181.62 ± 7.83 g) (2 ± 4% weight gain). However, rats that given a restricted access to the single diet showed a slight decrease in their body weight during the first 3 days (from 179.67 ± 1.17 to 176.74 ± 1.23 g) then started to increase regularly to reach a value of 279.98 ± 2.02 g) (56 ± 1% weight gain). In all rats (n = 168), the main effect of diet composition was significant [F (1, 1640) = 722, p = 0.0001]. In addition, all rats in the free food access condition fed a single diet or a dietary choice had mean body weights significantly higher than those in the restricted food access condition [F (1, 1640) = 3057, p = 0.0001]. The interaction between diet and food access schedule was also significant [F (1, 1640) = 196, p = 0.0001]. With restricted access, body weight of rats fed the single diet was significantly higher (p = 0.0001) than that of rats fed choice.

Rats which were randomly selected for daily food intake and body weight measurement during the last 8 days of experimentation (12 food restricted and 12 ad lib per dietary group) have shown similar daily mean weight gains of 6.1 ± 0.3 g (single ad lib), 6.6 ± 0.2 g (single restricted) and 6.1 ± 0.5 g (choice ad lib), with the exception of rats having a restricted access to dietary choice that gained only 1.84 ± 0.9 g daily.

**Total food, protein and carbohydrate intakes**

Total food intake and carbohydrate and protein intakes of rats fed a single diet or a choice between a carbohydrate-rich and a protein-rich diet placed under an ad lib or a restricted food
access schedule are presented in Figures A3a and A3b. A significant interaction was found between diet composition and access schedule for total food intake [F (1, 352) = 30, p = 0.0001], protein ingestion [F (1, 352) = 144, p = 0.0001] and carbohydrate ingestion [F (1, 352) = 8.3, p = 0.004]. In both single and dietary choice groups, total food intake of animals having a restricted access to food was significantly lower than that of freely feeding animals (p = 0.0001). However, in both free and restricted access conditions, total intake of rats fed the single diet was significantly higher than that of rats fed a choice (free: p = 0.0004; restricted: p = 0.0001). For carbohydrate intake, similarly to total food intake, comparing the different groups of rats showed that with both access schedules, carbohydrate was more ingested with the single diet than with choice (p = 0.0001). However, rats fed either the single diet or the dietary choice ate less carbohydrate in restricted access condition than in free access condition (p = 0.0001). Regarding protein ingestion, a significant interaction was observed between diet and food access schedule (p = 0.0001), with rats given a dietary choice ad lib eating more protein than under the restricted access (p = 0.0001). Rats fed the single diet ate protein similarly in either ad lib or restricted access conditions.

Serum melatonin levels and pineal NAT activity

In all groups, nocturnal serum melatonin levels varied from low values at 2000 h, raising to reach their maximum level at around 0200 h-0400 h, and then decreasing to low values at 0800 h (Figures A4a and A4b). The activity of NAT, the rate limiting enzyme in the synthesis of melatonin, showed similar variations (Figures A4a and A4b). For melatonin, statistical analysis has shown a significant interaction between diet composition and food access schedule [F (1, 140) = 28.2, p = 0.0001]. In rats fed a choice between a carbohydrate rich and a protein rich diet,
serum melatonin levels were significantly lower in the restricted food access than in the free food access condition \((p = 0.0001)\). No such difference was observed in rats fed the single diet. In the free food access condition, serum melatonin levels were significantly higher in rats fed a dietary choice than in rats fed a single diet \((p = 0.0001)\). But when comparing groups of restricted rats, serum melatonin concentrations of animals fed chow were similar to those of rats fed the dietary choice. For NAT, a difference was detected only between restricted and ad lib groups of rats fed the dietary choice. Indeed, NAT activity was significantly higher in rats having a free access to food than in restricted ones \(F(1, 138) = 8.14, p = 0.005\). In order to examine whether the nocturnal peak of melatonin level and NAT activity was shifted or not, the cosinor method was used to verify and characterize the nocturnal variation of the variables in the 12h period (Table A3). The results showed that the acrophases of melatonin were around 0500-0600 h in all groups, whereas those of NAT were about 0700 h. Neither melatonin nor NAT acrophases differed when rat groups were compared by using Bingham’s test.

**DISCUSSION**

The present study showed that serum melatonin levels and NAT activity were significantly decreased in rats fed a dietary choice with restricted access during daytime compared to ad lib access. Regardless of dietary condition, total food and carbohydrate intakes of rats having free access to food were higher than those of rats under the restricted food access schedule. In contrast, protein intake of rats fed the dietary choice was lower with the restricted access schedule compared to free access, whereas protein was ingested similarly in groups fed the single chow diet. The difference in body weight gain found between rats fed freely and those under a restricted access schedule was essentially due to the difference in the quantities of food.
consumed. Indeed, rats given an 8 h restricted access to a single diet have ingested approximately 83% of the food ingested over 24 h by animals having free access to food. With dietary choice, rats under a restricted access schedule have ingested around 64% of the food ingested in free access condition.

The adjustment of rats' intake when food availability was restricted to an 8 h period during daytime is the consequence of food deprivation reinforcing anticipatory hunger (White et al 2000). This control of the amount eaten was better acquired with the single chow diet than with the dietary choice. This suggests that rats fed a familiar diet (chow) can learn more efficiently to consume greater quantities of the diet presented during a restricted period of food availability (8 h) to cover their energy needs during the deprivation period (the remaining 16 h) in comparison to rats fed a less familiar dietary choice.

In the present study, food access limited to daytime failed to affect the nocturnal rhythmicity of serum melatonin and pineal NAT activity in rats fed a single chow diet. Studies that have reported effects such as phase advance of the pineal melatonin rhythm (Challet et al, 1997) or changes in the amplitude of the 24 h pineal and serum melatonin profile (Chik et al, 1987) have been using chronic food restriction by offering rats 50% of usual chow consumed at the beginning of the light period. The discrepancy between these former studies and our work could be related to the degree of food restriction, which corresponded to 17% of chow consumed in free feeding conditions in the present study. Rats self-restricted to a small extent as food was freely available during the limited daytime access. This suggests that food access schedule per se has no effect on both nocturnal secretion of melatonin and NAT activity when rats are offered a single chow diet.
In contrast, in rats offered a dietary choice between a carbohydrate-rich and a protein-rich diet nocturnal levels of serum melatonin were significantly lower and NAT activity significantly decreased with restricted food access when compared to free access. The difference in the results between single diet and dietary choice could be related to the more important self-restriction with restricted access to dietary choice (36% of usual choice consumed in ad lib condition versus 17% of usual chow consumed). In addition, rats given a dietary choice in a restricted access ingested less protein compared to ad lib fed rats, whereas protein ingestion was similar in rat fed the single chow diet irrespective to food access schedule.

So how may diet composition affect the synthesis and/or the secretion of melatonin? In our study, undernutrition was not imposed on rats since they were provided with enough food and allowed to eat ad lib during the restricted access period. However, only rats self-selecting diet in restricted food access have ingested less protein than with free access, thereby suggesting that dietary protein could be involved in the synthesis of melatonin.

Dietary proteins provide the essential amino acid tryptophan. Tryptophan is the precursor of serotonin and melatonin in the pineal gland. An in vitro study with rat pineal gland have shown that tryptophan induces synthesis of serotonin, melatonin and 5-methoxytryptophol (Ouichou and Pevet, 1992). Other studies have also reported that in in vivo studies a similar effect of tryptophan on rat melatonin level has been shown (Young and Anderson, 1982; Benson et al, 1989). Therefore, it is conceivable that dietary intake of tryptophan might enhance the synthesis of serotonin in the pineal gland and thus of melatonin. In the present study, according to the
calculation of tryptophan in diet, rats in the free access schedule have ingested 3 times more tryptophan than rats with restricted access to dietary choice (0.12 ± 0.04 and 0.04 ± 0.03 g, respectively). However, the rat groups maintained on single diet have ingested similar amounts of tryptophan in both food access conditions [1.2 ± 0.14 (free access) and 1.02 ± 0.1 g (restricted access)]. This suggests that tryptophan might be a dietary factor of influence for melatonin synthesis.

In the free access animals, we observed that serum melatonin increased at night before pineal NAT activity increased. Illnerova and Sumova (1997) have also reported a rise in NAT some 3 h after lights off. Besides, NAT activity decreased in those same rats having low serum melatonin levels. The decrease in NAT activity may also have been related to the low protein intake since pineal protein is needed for synthesis of the enzyme. It has been reported that NAT activity is declined in starving rats (Welker and Vollrath, 1984). Therefore, alternatively, melatonin synthesis may have been indirectly suppressed through a reduced NAT activity in rats with a low protein intake.

In conclusion our study showed that dietary protein intake is a factor affecting the nocturnal rhythm of melatonin synthesis. The daytime food access schedule, however, seemed to have exerted a minor influence on the nocturnal rhythmicity of melatonin and NAT. Further studies are needed to ascertain the role played by protein intake in the synthesis of melatonin.
Chapter 4

GENERAL CONCLUSION

Factors that affect melatonin secretion and rhythmicity have been well documented in the literature. In this present study, a closer look was taken at one of these factors, namely “food”. It was thus investigated on how food access schedule and food composition in the form of a protein-rich diet, carbohydrate-rich diet and rat chow affects the secretion of nocturnal melatonin and NAT activity in the rat pineal gland. In summary, in this study it was observed that in animals simultaneously presented with a choice between a carbohydrate-rich and protein-rich diet and on a restricted food access and thus adapted to feeding schedules out of their norm, that the melatonin levels and NAT activity were significantly lowered. The food access schedule however, was not considered a factor contributing to this disparity. It was instead the amount of protein ingested by this subgroup that was noteworthy. The amount of protein ingested by these animals on the restricted access food schedule with choice diet, was seen to be much lower when compared to the free access group. Coupled with this was observed a significantly lower nocturnal melatonin production and NAT activity.

So does protein consumption in rats affect the synthesis of melatonin and pineal activity? This present study does suggest affirmatively by proposing that protein mediates this effect by providing the amino acid tryptophan, which is a precursor of serotonin and melatonin synthesis in the pineal gland. Indeed studies have shown that tryptophan made available in a diet or by transfusion does influence the brain serotonin and melatonin levels (Fadda et al 2000; Ouichou and Pevet, 1992). In the present study, rats on restricted access schedule ingested tryptophan in
the diet that were 3 times less than the rats on the free access schedule (Figure A3c). In the same group of rats, both the nocturnal melatonin levels and NAT activity were significantly lower when compared to the rats on the free access schedule.

Several studies have reported altered secretory rhythms of melatonin in patients with various clinical disorders such as insomnia (Armstrong, 1999), anorexia nervosa (Ferrari et al, 1990), schizophrenia (Monteleone et al, 1997) and cancer (Bartsch et al, 1997). Hence investigating the factors affecting melatonin's secretory pattern may prove advantageous, as these factors may provide useful tools in the therapeutic treatment of these disorders through their synchronizing effects. Melatonin also can be said to be a powerful chronobiotic, i.e a chemical substance therapeutically capable of re-entraining desynchronized circadian rhythms (Cagnacci, 1996). Light also is seen to perform the same function, as it is known to entrain certain physiological rhythms (Nowak and Zawilska, 1998). Light a powerful photic signal, is being used in treatment of various circadian disorders in patients who also exhibit altered melatonin rhythms (Dahlitz et al, 1991; Dawson and Encel, 1993). Food composition, if shown to be a non-photic signal performing in the similar fashion as light, may become useful in the evaluation and treatment of these various circadian disorders.

The pattern of melatonin secretion in rats is similar to that of man, that is high levels secreted always at night. This could mean that in some species melatonin levels are indicators of sleep such as in humans, while in other species e.g. rats, high melatonin levels indicate the activity period, that is when the animal is consuming most of its daily food intake. Another similarity exists in the retina-pineal pathway. And so since rats have the same melatonin secretory pattern as humans, as well as similar retina-pineal pathway, it is possible that the finding of this study may be beneficially applied to the human population.
In the present study a primary limitation could have been that of duration of restricted feeding, which was for only 3 weeks. When compared to other studies for instance, Challet et al (1997) only observed changes in melatonin rhythm after 1 and 2 months of food restriction. It is possible that certain aspects of melatonin production such as its pattern (phase advance or phase delay) or the nocturnal levels may become more evident with a longer duration of food restriction. A suggested approach to subsequent research would be to increase duration of restricted feeding coupled with administering an exclusive protein diet and then explore the outcome on melatonin secretion. This may perhaps help to make protein's effect on melatonin more apparent. This present study does form the basis of other research on food intake and melatonin, for example the possible effects of melatonin on food intake, but with regards to human subjects in general it is evident that further areas of investigation is indeed warranted.
**Table A1: Diet Composition**

**Dietary composition of protein-rich and carbohydrate-rich diets**

*(Dry weight, g/100g diet)*

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<th>Ingredients</th>
<th>Protein-rich</th>
<th>Carbohydrate-rich</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin-free casein</td>
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<tr>
<td>Vegetable Oil</td>
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<td>Dextrin</td>
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<td>Corn Starch</td>
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<tr>
<td>AIN76 Mineral Salt</td>
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<td>5.2</td>
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<tr>
<td>AIN76a VITAMIN MIX</td>
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<td>1.1</td>
</tr>
<tr>
<td>Alphacel</td>
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<td>5.0</td>
</tr>
<tr>
<td>Choline chloride</td>
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</table>
Table A2: Repeated 2-way ANOVA results. The main effects of diet composition and food access schedule and their interaction were tested.

<table>
<thead>
<tr>
<th>Dependent variables</th>
<th>Factors</th>
<th>df</th>
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<th>p-values</th>
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<tbody>
<tr>
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<td>Diet composition</td>
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<td>196</td>
<td>0.0001</td>
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<tr>
<td>Body weight</td>
<td>Access schedule</td>
<td>(1,352)</td>
<td>538</td>
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<td>24.59</td>
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<td>Access schedule</td>
<td>(1,352)</td>
<td>265</td>
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<td>Melatonin</td>
<td>Diet composition</td>
<td>(1,140)</td>
<td>9.18</td>
<td>0.003</td>
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<td>Access schedule</td>
<td>(1,140)</td>
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<td>Diet composition</td>
<td>(1,138)</td>
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<td>0.76</td>
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<td>Access schedule</td>
<td>(1,138)</td>
<td>8.14</td>
<td>0.005</td>
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<td>Diet by Access</td>
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<td>1.65</td>
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Table A3: Circadian rhythm parameters (Mean ±SEM) of melatonin and NAT in animals having free food access (FA) or restricted access (RA) and fed a choice (C) or a single (S) diet.

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<thead>
<tr>
<th>Dependent Variables</th>
<th>Group Categories</th>
<th>Mesor</th>
<th>Amplitude</th>
<th>Acrophase</th>
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<tr>
<td></td>
<td>FAS</td>
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<td>6h03±Oh52</td>
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<td>RAC</td>
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<td>FAS</td>
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<td>9.89±1.10</td>
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<td>7h12±Oh16</td>
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<td>8.50±0.55</td>
<td>7.34±0.70</td>
<td>7h07±Oh25</td>
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FIGURE A1a: MELATONIN BIOSYNTHESIS
Figure A1b: NEURAL PATHWAY OF MELATONIN PRODUCTION
Figure A2a: Rats body weight variation under free or restricted access to a choice between a carbohydrate-rich and a protein-rich diet or a single chow diet. Each value represents the mean ± SEM of 42 rats.
Figure A3a: Total food intake measured in rats maintained in free or restricted access conditions, and fed a choice between a carbohydrate-rich and a protein-rich diet or a single chow diet. Each value represents the mean ± SEM of 12 rats.
Figure A3b: Protein and carbohydrate intakes measured in rats maintained in free or restricted access conditions and offered a choice between a carbohydrate-rich and a protein-rich diet or a single chow diet. Each value represents the mean ± SEM of 12 rats.
**Figure A3c:** Tryptophan intake measured in rats maintained in free or restricted access conditions, and fed a choice between a carbohydrate-rich and a protein-rich diet or a single chow diet. Each value represents the mean ± SEM of 12 rats.
Figure A4a: Nocturnal variations of serum melatonin concentrations in rats having free or restricted food access to a choice between a carbohydrate-rich and a protein-rich diet or a single chow diet. Each time point represents the mean ± SEM of 6 rats.
Figure A4b: Nocturnal variations of pineal NAT activity in rats having free or restricted food access to a choice between a carbohydrate-rich and a protein-rich diet or a single chow diet. Each time point represents the mean ± SEM of 6 rats.
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