THE ROLE OF MELATONIN IN HUMAN THERMOREGULATION AND SLEEP

by

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Index

Abstract

A putative role for the pineal hormone melatonin in the regulation of normal sleep has often been suggested. This is based primarily on the coincidence of nocturnal melatonin production with normal sleep and that daytime administration is associated with soporific effects. A significant suppression of daytime core body temperature after melatonin administration has also led to suggestions that thermoregulatory effects may mediate the soporific effects of melatonin.

However, the majority of previous studies have utilised melatonin administration protocols that produce high peak hormone levels or short durations in the circulation. Therefore, the aim of studies in this thesis was to determine the effects of melatonin on sleepiness and body temperatures under conditions that better approximated the endogenous melatonin profile. First, an examination of the effects of endogenous melatonin onset was undertaken both in the presence and absence of normal sleep. Next, two melatonin administration studies attempted to reproduce endogenous levels of melatonin during the day with long or short durations. Finally, the potential for beneficial soporific effects of a nocturnal melatonin pulse was assessed in elderly chronic insomniacs.

The results of the first study suggest that there are few, if any effects on sleepiness and thermoregulation at endogenous melatonin onset. The daytime melatonin administration studies provide evidence that both peak level and rate of onset of melatonin interact to determine the acute affects of melatonin administration. This knowledge could lead to a more effective use of melatonin as a therapy for sleep disturbance. In elderly insomniacs, sustained

supraphysiological melatonin administration was able to significantly lower rectal core and hand skin temperatures. However, this change in thermoregulatory output was achieved without any concurrent changes in sleep quality or architecture. Therefore, an age-related increase in nocturnal body temperature is unlikely to be related to concurrent increases in sleep disturbance, they may rather arise directly from decreased responsiveness to endogenous melatonin production. It is also clear that there is no simple relationship between melatonin and its acute effects on temperature and sleepiness, which are influenced by factors including time of administration, age, dose and rate of onset in the circulation.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this thesis, when deposited in the University Library, being made available for loan and photocopying.

Signed ..... Date 21/5/98.....

Acknowledgements & Dedication

I would like to thank those at The Centre for Sleep Research and the Department of Obstetrics and Gynaecology who have helped me accomplish this work.

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Above all, I would like to congratulate my wife Lisa for completing her thesis and thank both her and our families for help when it was most needed.

I would like to dedicate this work to the memory of Ayrton Senna da Silva, whose accidental death coincided with the beginning of my research career - *Ayrton, nossos pensamentos é sempre com você*

"We knocked the bastard off"

Sir Edmund Hillary upon descending from Mt. Everest, May 1953.

"Sometimes I try to beat other people's achievements but on many occasions I find it's better to beat my own achievements. That can give me more satisfaction"

Ayrton Senna, shortly before his tragic accident at Imola, May 1994.

Errata

Page 4, line 9. The word "*ectotherms*" should read "*endotherms*".

Page 4, line 10. The word "*endotherms*" should read "*ectotherms*".

Page 4, line 13. The word "ectotherms" should read "endotherms".

Page 4, line 14. The word "endotherms" should read "ectotherms".

Page 12, line 18. Delete the word "of".

Page 25, line 22. Insert after "Tc" the text "(core temperature)".

Page 29, line 10. Replace the word "retina" with "SCN".

Page 40, line 23. Delete the word "remain".

Page 52, line 2. At the end of the first sentence, the following text should be added, "Therefore, inclusion criteria were nocturnal awakenings on 3 or more nights per week lasting longer than 30 minutes, or several awakenings lasting at least five minutes each summing to 40 minutes. Subjects were considered chronic sleep maintenance insomniacs if they met either of these criteria based on their average sleep log data."

Page 61, line 3. Replace the word "Rechtschaffen" with "Dement".

Page 171, line 10. Insert the following citation: "Dawson, A. and King, V. Thyroidectomy does not affect the daily or free-running rhythms of plasma melatonin in European starlings. *J.Biol.Rhythms,* 1994, **9**, 137-144."

Page 191, line 15. Insert the following citation: "Soszynski, P., Zgliczynski, S., and Pucilowska, J. The circadian rhythm of melatonin in hypothyroidism and hyperthyroidism. *Acta Endocrinol.Copenh.*, 1988, **119**, 240-244.

Chapter 1

1.1 The Concept of Homeostasis

Over a century ago the French physiologist Claude Bernard [1813-1878] wrote, "The constancy of the internal environment is the condition for free and independent life" (Bernard, 1859; reprinted in Satinoff, 1980). Bernard recognised that a constant "milieu interieur" or internal environment enabled an organism to live free of environmental constraints imposed by, for example, environmental temperature or food availability. Bernard's statement illustrates the principle that was later termed homeostasis by Walter B. Cannon in his landmark book, "The Wisdom of the Body" (Cannon, 1932). Cannon described homeostasis as "the various physiologic arrangements which serve to restore the normal state, once it has been disturbed".

Cannon therefore perceived not only that the internal environment was continually disturbed but that there was active regulation to achieve a median or "normal" state. This was the first indication that homeostasis, like many other regulatory mechanisms, employs the principle of negative feedback. Typically, a negative feedback system detects deviations from a reference level and triggers compensatory changes that continue until the desired state is achieved. The cells and physiological systems of multicellular organisms typically rely on a constant environment for continuing function; thus, it is not surprising that many regulatory mechanisms have evolved to maintain the internal environment. Homeostasis perhaps represents the highest level of sophistication and complexity of these regulatory mechanisms.

Claude Bernard identified four essential elements required by the internal environment "for the exercise of free life", as water, oxygen, 'reserves' of chemical substances and heat (Bernard, 1859; reprinted in Satinoff, 1980). These elements, he noted, were also those that are essential for sustaining life in unicellular and other simple organisms.

1.2 The History of Thermology

The study of thermal behaviour in living things can be traced back to the origins of civilisation. According to Benziger, pre-Hippocratic Greek medicine had already associated heat with life and health (Benziger, 1969), notions that Hippocrates refined in "On Ancient Medicine" (cited in Bruck, 1978). For example, the Greeks were the first to link elevated body temperatures (or fever), with disease. "The Book of Prognostics" further detailed the practice of temperature measurement of body parts in diagnosing disorders and in the prediction of disease outcomes (Bruck, 1978). From ancient medicine through to the 19th Century, health was commonly perceived as a balance of elements, one of which was heat. Thus the significance of heat (or more correctly, thermoregulation) to health has long been acknowledged.

As a complement to the development of thermology was the development of biothermometry, described as "the technology that facilitates the quantitative assessment of temperature of biological systems" (Anbar, 1994a). The first device that allowed changes in temperature to be observed, was Galileo Galilei's 'thermoscope', invented c. 1592. The first thermometer, basically a thermoscope calibrated with graded marks to the temperatures of ice water and a candle flame, was in clinical use by 1611. The development of the thermometer has

been attributed to the physician Santorio Sanctorius, who used it to show temperature variations in the mouth between healthy and ill patients (Hammel, 1968). The implementation in the mid-19th Century of routine temperature measurement in clinical medicine is generally attributed to Bärensprung, Traute and Wunderlich.

For almost 400 years, the use of contact thermometry in science, medicine and other fields has been almost universal. Recently however, there has been some deliberation as to whether contact thermometry is the most reliable and accurate method of temperature assessment, particularly in research and clinical settings (Anbar, 1994a). In this case, the development of telethermometry, using detection of emitted and radiated energy to measure surface temperature has been proposed as a solution. At present however, this technology remains prohibitively expensive and ineffective over large surface areas (ie. greater than a few cm^2). For the time being therefore, telethermometry will likely remain not as a replacement but a useful complement to contact thermometry for clinical and research use.

1.3 Thermoregulation

In the last 30 years there have been numerous publications on different aspects of thermoregulation (for example, Benziger, 1969; Bruck, 1978; Cabanac, 1975; Clark, 1981; Hammel, 1968; Hardy, 1961; Hensel, 1981a; Parmeggiani, 1987; Reinberg and Smolensky, 1983). This introduction is not intended to cover all of the several thousand papers estimated to have been published in the area of thermal physiology (see bibliometric studies by Refinetti, 1989; Refinetti, 1990). Rather, it is intended to summarise some of the major topics in human

thermoregulation and the possible interactions between melatonin, thermoregulation and sleep.

1.3.1 Classification of thermoregulatory responses

The classical element of "heat", or temperature, is one of the most apparent and easily measured internal factors that are subject to homeostatic control. The capacity for thermoregulation is not constant however, but varies widely between species. The typical contemporary classification of thermoregulatory responses includes two dichotomous sets. Simply stated, animals that maintain a body temperature above that of the environment are called *ectotherms*; those that are unable to do so, are *endotherms*. When an animal is able to maintain a constant body temperature despite environmental variations in temperature, they are said to be *homeotherms*; those that cannot, are *poikilotherms*. While there are exceptions and the distinction may not always be clear, ectotherms are typically homeothermic while endotherms are usually poikilothermic.

Recently, one group has speculated that there is no fundamental difference between ectotherms and endotherms, except varying ability to maintain thermal homeostasis (Varghese and Pati, 1996). The authors' review of the existing literature leads them to suggest that a spectrum exists in thermoregulatory control. This model, removes the need for classification of thermoregulatory responses, however it is not yet clear whether this model can satisfactorily cover the responses of all or most species that regulate body temperature. Therefore, in this review the contemporary classification of thermoregulatory responses will be used where necessary.

1.3.2 The importance of thermoregulation

Thermoregulation is known to be essential for life, as cellular functions are typically sensitive to fluctuations in temperature and can only continue within a narrow range. For example, even moderate elevations of body temperature can cause nerve malfunction and irreversible protein damage. Cannon recognised that temperature extremes could be damaging or even fatal to humans by observing outcomes in patients exhibiting acute alcohol and anaesthetic-related falls in core temperature to 24°C and fevers up to 43°C (Cannon, 1932).

For poikilotherms to avoid death at extreme temperatures, they must adjust their activity or move to an area with more hospitable ambient conditions to achieve temperature regulation. The 'heliothermic' reptiles are examples of animals that thermoregulate by alternating between sun and shade. This behavioural thermoregulation under some conditions restricts poikilotherms from living the "free life" that Bernard ascribes to homeotherms. For example, heliotherms will forsake thermoregulation while defending territory or avoiding predation (Templeton, 1970) during which time their body temperature may vary by more than 20°C.

The suggestion that homeothermy confers a selective advantage in terms of allowing an animal to remain active in cold conditions, was perhaps stated best by Cannon who wrote, "cold-blooded animals, therefore, having the temperature of their surroundings; can act with alacrity only when the weather is warm; the warm-blooded, which maintains a fairly fixed high temperature in spite of external cold, can act quickly at all times" (Cannon, 1932).

1.4 Aspects of Human Temperature Regulation

The actual temperature maintained by warm-blooded animals varies between species and less significantly between individuals. In humans, the mean oral temperature is generally considered as 37°C. Temperature however differs depending on where in or on the body it is measured. This is illustrated by the typical temperature gradient between the core (highest), trunk, limbs and extremities (lowest). This relationship holds across a range of ambient temperatures, but the difference between warmest and coolest body parts may decline from >10 degrees at an ambient temperature of 23°C, to <3 degrees when the environment is 35°C (Hardy and DuBois, 1932). The temperature of the body's "core", generally regarded as the abdominal and thoracic organs, the central nervous system and the skeletal muscles, is considered to be the temperature that is homeostatically regulated. As an example of this and of the efficiency of human thermoregulation, Hammel stated that naked subjects could maintain core temperature at "about" 37°C under environmental conditions ranging from 15 - 54°C (Hammel, 1968).

1.4.1 Thermoregulation as heat balance

Homeostatic control of body temperature is achieved by balancing heat production (thermogenesis), heat conservation and heat loss (thermolysis). Specific central and peripheral nervous structures constantly monitor temperature and attempt to keep heat input balanced against heat output. Thermoregulation must be a dynamic process, as heat balance is consistently disturbed by internal and external factors not related to thermoregulation. The most notable internal factors are exercise-induced and (following food ingestion)

digestive increases in heat production (internal disturbances). The most important external factor affecting thermoregulation is the ambient temperature, which affects the rate of heat loss or gain between the environment and the body.

1.5 Effectors of Thermoregulation

1.5.1 Heat input

Heat input to the body consists of internal heat production and heat gain from the environment, when conditions are warmer than body temperature. The internal production of heat occurs by the oxidative breakdown of ingested food, by basic metabolic processes and by the activity of muscle and adipose tissues. As stated above, the majority of heat production however normally results from skeletal muscle activity, but internal heat production can also be effected by other means, such as thermogenesis by adipose tissue.

1.5.1.1 Shivering thermogenesis

Shivering is an autonomic response to cold that results from activation of skeletal muscle activity. Shivering muscles contract at a rate of 10-20 times per second, thereby increasing internal heat production or thermogenesis by 2-5 fold over basal levels. Shivering is very efficient, with all activity converted to heat as no external work is done. The increase in heat production not only warms the body but slows conductance of heat from the core to the skin, thus slowing the rate of heat loss from the core to the skin (but not from the skin to the environment). Cold also results in a general increase in motor activity which is not entirely voluntary, as these behaviours may share some common neural pathways. For

example, foot stamping and hand rubbing are common behaviours in cold weather, which may be effective in local or general heat production.

1.5.1.2 Non-shivering thermogenesis

In the cold, heat production can be increased by greater metabolism of adipose tissue (called non-shivering thermogenesis). Experimentally, chronic cold exposure increases metabolic heat production independent of muscular contraction, appearing to be mediated instead by elevated endocrine activity. In humans, this appears to be most important in infancy (Risbourg et al., 1991) as the shivering response does not develop early. Whether non-shivering thermogenesis plays a role in adults remains controversial, with some evidence that the ability to produce heat in this way may disappear after early stages of development (Balmagiya and Rozovski, 1983). In birds however, non-shivering thermogenesis appears to be functional even in adult animals (Saarela and Heldmaier, 1987). The release of catecholamines and perhaps thyroid hormone increases the metabolic rate of adipose tissue and appears to mediate the activation of non-shivering thermogenesis (Leduc, 1976; Swanson, 1998).

Infants have deposits of a particular type of adipose, brown fat, which has a high rate of metabolism and efficiently converts stored energy into heat. The function of brown fat in thermogenesis, as distinct from that of white fat as a reserve of energy, had been clarified by the late 1970's (McCance and Widdowson, 1977). The increases in energy expenditure during non-shivering thermogenesis appear to be mediated by the sympathetic nervous system, which causes activation of heat production in brown adipose tissue (Stock and Rothwell, 1986). While this group stated that, "hormones such as glucagon, thyroid, melatonin, TSH,

endorphins and sex hormones are implicated in one way or another in the regulation of energy balance and the control of thermogenesis", the precise role of these hormones in temperature regulation however, remains unclear.

1.5.2 Heat output

As human body temperature is typically higher than the ambient temperature, the control of heat loss is perhaps one of the most critical aspects of thermoregulatory physiology. The exchange of heat between the body and environment occurs by passive loss down a thermal gradient (ie. from warm to cool). Critically important for heat loss are the skin and the circulatory system. Skin is the largest organ of the body and contains hair follicles, sweat glands, adipose tissue and extensive vascular networks, all of which participate in the regulation of temperature. The circulation of blood transfers heat around the body as well as having well-defined roles in nutrient and waste transport. For example, for the body to lose heat, local blood flow in the skin typically increases resulting in an increased thermal gradient between the skin and ambient air, promoting dissipation of heat. The suggestion that the control of blood flow through cutaneous vessels plays a role in body temperature homeostasis is ascribed to Claude Bernard.

1.5.2.1 Convective, conductive or radiative heat loss

The actual transfer of heat occurs by convection, conduction or radiation, processes sometimes called collectively "insensible heat loss". Convection describes the movement of a liquid (such as air) along a thermal gradient. Heat loss by conduction occurs when objects at different temperatures come into contact. Radiation is the transfer of heat between objects that are not in contact.

The amount of heat lost via the skin is altered by central regulation of blood flow from the tissues of the core to the skin. Normally 3 to 4% of cardiac output flows through the skin (Johnson et al., 1986). In response to ambient heat stress however, vasodilation of cutaneous vessels can allow more than a tenfold increase in skin perfusion (Houdas and Ring, 1982a). This warms the skin, which then loses heat by the processes of convection, conduction and radiation. Conversely, when heat loss needs to be minimised (ie. cold stress), cutaneous blood flow is restricted by extreme vasoconstriction until almost no blood flows (Johnson et al., 1986). The blood which would normally flow through the superficial vessels is shunted to the *venae concomitantes*, the deep veins insulated by subcutaneous fat (Rowell, 1983) and adjacent to arteries. Heat from the arterial circulation transfers to the venous circulation by countercurrent exchange and is carried back into the body without reaching the skin. In this way, heat is "held" in the core. The rate of blood flow through the skin has been shown to vary by several orders of magnitude in order to effect skin temperature changes (Bongard and Bounameaux, 1993).

As was stated above, heat balance is a dynamic process and this is reflected in the rhythmic nature of cutaneous temperature changes. Measurement typically reveals an oscillation in skin temperature, with an amplitude and frequency determined by local microcirculation (Rhodin, 1981). This oscillation therefore occurs due to an alternating vasoconstriction and vasodilation of arterioles and is indicative of a typical homeostatic process.

While blood flow is controlled by the autonomic nervous system, the loss of heat from the skin to the environment is not under physiological control and occurs

faster in colder conditions (when heat loss may not be favourable to thermoregulation). If the ambient temperature is higher than body temperature, absorption of heat by the body can occur and add to the heat "load". In this situation, the only mechanism of heat loss available is evaporation of sweat from the skin.

1.5.2.2 Increased evaporative heat loss due to sweating or panting

Homeotherms typically lose heat by continuous evaporation of water from the mucous membranes of the respiratory tract. This insensible water loss occurs continuously, and in humans up to 50 ml per hour can be lost (Ganong, 1987). Some animals, including humans, also lose heat by an active process called perspiration or sweating, which is under autonomic nervous control. The efficiency of both sweating and insensible water loss as heat loss mechanisms vary proportionately with the humidity of the environment. For example, sweat secretion rates may increase from negligible loss in the cold, up to 1600 ml per hour in hot ambient conditions (Ganong, 1987)

Some mammals pant, or use shallow and rapid breathing to increase the evaporation of water from the respiratory tract and thereby increase heat loss. Panting and sweating are more effective at ambient temperatures approaching body temperature, whereas at lower temperatures radiation contributes more to heat loss.

1.5.2.3 Decreased heat loss

Heat loss in cold ambient conditions can be slowed dramatically in some mammals and birds, by a process called horripilation. Typically, the piloerector muscles stiffen hair follicles or feathers and trap air close to the skin. The air

becomes warm, insulates the skin and prevents dramatic heat loss to the environment. In animals (such as humans) without fur or feathers, cold-induced horripilation is inefficient and results in characteristic but effectively useless "goose bumps".

1.5.3 Thermoregulation as a function of ambient temperature

The effectors that are employed are known to be functionally dependent on the ambient temperature (Anbar, 1994b). As an example, animals in cold environments will typically regulate body temperature by changing heat production, with heat loss mechanisms operating at a minimal level. Conversely, in warmer environments metabolic heat production appears to be maintained at a minimum and heat loss mechanisms are employed in the regulation of body temperature. Animals can employ behavioural strategies however, to enhance the efficiency of heat loss or conservation and thereby minimise or prevent 'load' on the autonomic thermoregulatory system.

1.5.4 Behavioural thermoregulation: strategies to alter heat loss or gain

While both Bernard and Cannon were concerned almost exclusively with the physiological and chemical regulators of homeostasis, several researchers later investigated of the role of behaviour as an important contributor to temperature regulation (Kinder, 1927 and Richter, 1936; cited in Richter, 1943). These two groups reported that hypophysectomy in rats, "seriously disturbed" physiological heat-regulating mechanisms and increased nest-building activity up to 5-fold compared with control animals. The withdrawal of nest building paper from one hypophysectomised rat resulted in death after one month presumably from hypothermia, as body temperature was 15 °F below normal at the time of death.

Experimentally, the disruption of the thermoregulatory system in these animals was found to result in attempts by the animal to restore internal temperature. In this way, behaviour was shown as an important part of thermoregulation in both cold- and warm-blooded animals. Thermoregulation can therefore be seen to be an integration of both behavioural and physiological responses to achieve optimum body temperature.

In cold ambient temperatures, behavioural thermoregulation generally takes one of three forms:

(a) ambient temperature selection (ie. conscious or instinctive choice of a warmer location, if available),

(b) increased (voluntary) activity to produce heat in the muscles,

(c) increased nesting, covering or clothing (depending on the species).

In warm environments, behavioural strategies for thermoregulation are typically opposite, ie. avoidance of warm locations or direct sunlight, decreasing activity, and reducing the amount of covering or clothing worn.

Experimentally, Cabanac and colleagues demonstrated behavioural thermoregulation by allowing dogs to select their preferred environmental conditions (Cabanac, 1975). It was found that the dogs usually selected thermoneutral or slightly warmer conditions. When given hot water to drink, core temperature increased slightly and the dogs selected colder ambient temperatures, but returned to the warmer conditions when core temperature had returned to normal (for similar experiments, see review in Hensel, 1981a).

As to the relative contributions to overall thermoregulation by autonomic and behavioural components, this has not yet been clearly defined. In the

experiments above for example, some of Cabanac's dogs were observed to 'supplement' behavioural thermoregulation by shivering or panting, while others utilised thermoregulatory behaviour to 'spare' autonomic responses. Under extreme conditions such as exercise, autonomic responses clearly prevail, but homeotherms are thought to rely much of the time on complex behavioural responses to thermal challenges in preference to autonomic responses (Hensel, 1981a).

1.6 Autonomic Control of Temperature Regulation

The regulation of temperature is predominantly under involuntary or autonomic nervous control. There are rare exceptions however, where certain individuals have learnt to consciously control autonomic body functions, including the control of body temperature. From a functional perspective, central regulatory centres integrate signals from both peripheral and central temperature-sensitive cells. Any required physiological effector responses are then activated by both neural and humoral means. Both autonomic and behavioural responses are activated in this manner, although it is thought that autonomic and behavioural thermoregulation may be independently controlled (Blatteis, 1980; Glotzbach and Heller, 1989)

1.6.1 The preoptic / anterior hypothalamus and set point temperature

The neural regulation of temperature relies on central integration of information. In particular, the preoptic area of the anterior hypothalamus (POAH) has long been regarded as the main centre for thermoregulatory control (Boulant, 1980; Glotzbach and Heller, 1994; Houdas and Ring, 1982b; Magoun et al., 1938). The inputs from central temperature-sensitive cells (including within the POAH

itself) are processed with reference to a postulated "set point" of temperature determined by the POAH, which represents the optimum local temperature for the organism under the prevailing conditions. Peripheral and ambient temperatures, as well as pyrogens and other endogenous factors are able to alter these conditions.

The notion of a set point is a traditional thermoregulatory concept, defined as "the value of a regulated variable which a healthy organism tends to stabilise by the process of regulation" (Cabanac and Simon, 1987). The set point therefore acts as a thermostat, which can activate or inhibit effector neurons to produce heat loss, heat retention or heat production responses. Unlike a physical thermostat however, the set point is a complex and variable value that accounts for internal and external factors (Kobayashi, 1988).

The POAH acts as a locus of thermoregulatory control over effectors of the thermoregulatory system (Boulant, 1994), by integrating feedback information from hypothalamic thermosensitive cells. These thermosensitive neurons can be classified as either warm- or cold-sensitive, depending on whether increasing or decreasing temperature elicits a change in their respective firing rates. The POAH also receives input from feed-forward signals arising in afferent projections from cutaneous, spinal and body core thermoreceptors (Glotzbach and Heller, 1989; Hellon, 1970; Wit and Wang, 1968). The importance of the POAH is evident for example, where experimental lesions or local anaesthesia of the POAH have been shown to prevent animals from thermoregulating effectively in both cold and warm ambient conditions (eg. Satinoff, 1974). In reptiles and

fish, POAH lesions predominantly impair behavioural attempts to regulate body temperature (Berk and Heath, 1975; Nelson and Prosser, 1979).

It has been known for almost 25 years that thermal stimulation applied locally at the hypothalamus will produce thermoregulatory responses in various species (for historical review, see Bligh, 1973). Experiments pioneered by Hammel, Hardy and colleagues used implanted, water-perfused hypothalamic thermodes to induce local heating or cooling of the POAH, and allowed simultaneous measurement of physiological responses. Studies in several species have shown that POAH warming elicits panting, sweating, increased skin blood flow and heat loss behaviours (Krönert and Pleschka, 1976; Smiles et al., 1976; Pleschka et al., 1979). Conversely, localised POAH cooling elicits shivering, non-shivering thermogenesis and heat conservation behaviours such as nesting (reviewed in Boulant, 1980; Boulant and Dean, 1986; Hensel, 1981b; Satinoff, 1974).

Implanted thermode experiments also allowed the detection of the threshold (set point) temperature, above which heat production remains at basal levels and below which heat production increases proportionally with cooling (Glotzbach and Heller, 1989). It has been found that set point temperature is not constant, but changes depending on arousal state and perhaps most importantly, time of day (Tayefeh et al., 1998). From this perspective, circadian (L. *circa* - near, *diem* - day) changes in hypothalamic sensitivity may underlie at least part of the daily rhythm in body temperature.

1.6.2 The posterior hypothalamus

An important finding of the POAH thermal stimulation studies was that both autonomic and behavioural responses could be elicited by changing POAH temperature. In contrast, thermal stimulation of the posterior hypothalamus leads predominantly to changes in behavioural thermoregulation (reviewed in Blatteis, 1980; Hensel, 1981b). These reviews also detail studies employing lesions of the preoptic area, which have been shown to impair autonomic but not behavioural thermoregulation in animals. The results suggest that impaired autonomic regulation can be compensated by altered behaviour, most likely under the direct control of the posterior hypothalamus.

The interrelation between autonomic and behavioural thermoregulation is illustrated schematically in Figure 1.1, overleaf.

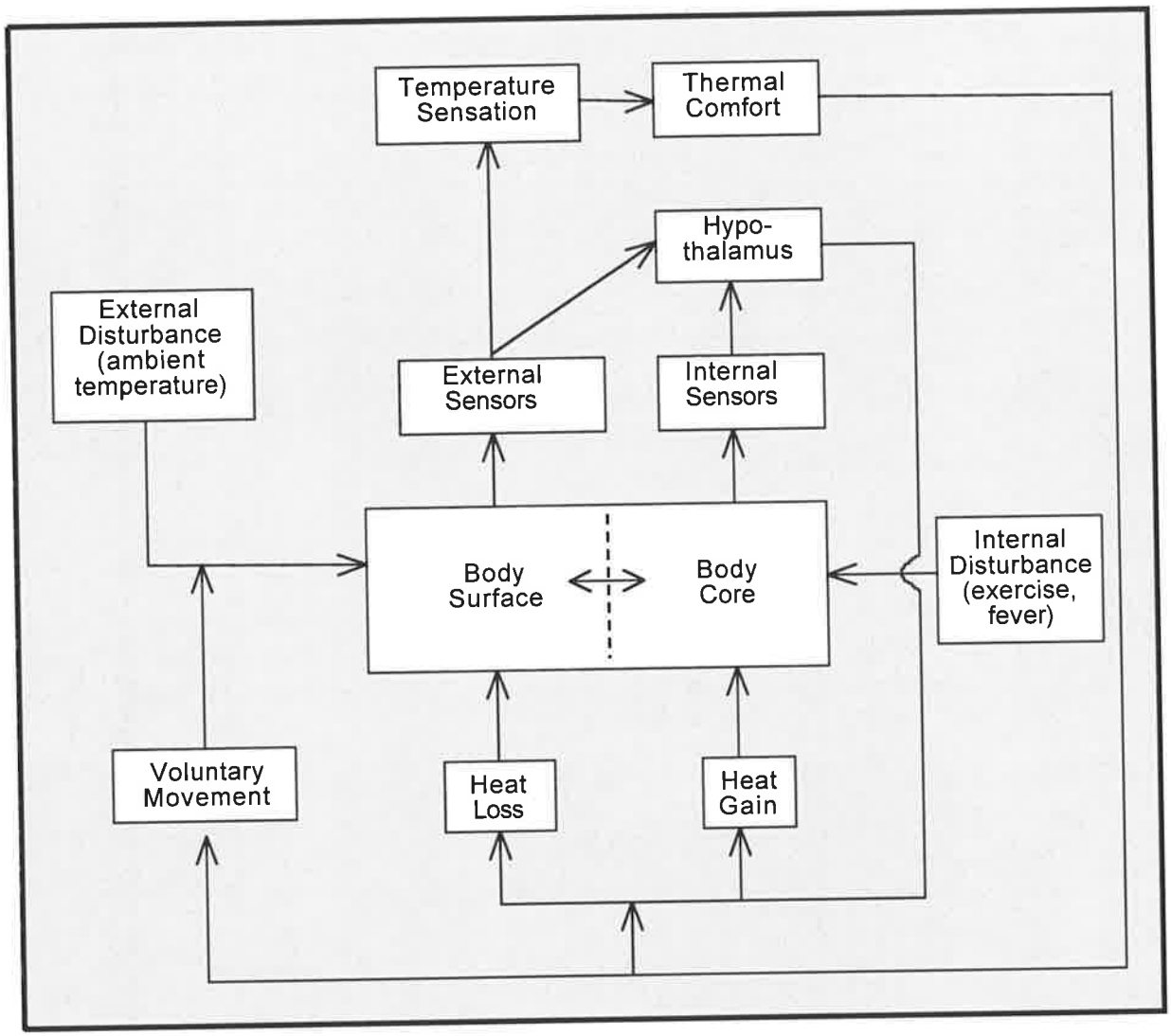

Figure 1.1

Schematic diagram of interactions in human autonomic and behavioural thermoregulation (adapted from Hensel, 1981b). Changes in activity, behaviour and environment selection can alter heat loads on the body and thereby regulate body temperature. The autonomic nervous system can in turn influence behaviour and thereby thermoregulation.

1.6.3 The suprachiasmatic nucleus

Both warm and cold-sensitive neurons are found in a paired structure in the hypothalamus called the suprachiasmatic nucleus (SCN). The mammalian SCN has a well-described role as a physiological pacemaker that entrains the animal to its environment. For example, lesions of the SCN can completely impair rhythms of activity in many species (for review, see Refinetti and Menaker, 1992). Exactly how the SCN is coupled to its many outputs however is not currently well understood. Neural efferents from the SCN which terminate in other parts of the hypothalamus (Reinberg and Smolensky, 1983; Silver and LeSauter, 1993; Watts, 1991), suggest a mechanism by which the SCN may regulate other hypothalamic functions. Efferents of the SCN to other hypothalamic areas have been shown to project in highest numbers the paraventricular dorsomedial nucleus or PVN/DMH (Stephan et al., 1981; Watts et al., 1987; Watts and Swanson, 1987; Watts, 1991). This area of the hypothalamus has been suggested as a mediator of neuroendoocrine and autonomic control, particularly in the control of adrenal glucocorticoids (Swanson, 1989; cited in Kalsbeek et al., 1992)

In 1982, Satinoff and coworkers found that medial preoptic lesions in rats disturbed not only thermoregulatory homeostasis but also the normal daily rhythm in core temperature (Satinoff et al., 1982). Observations like these have lead to speculation of a strong functional relationship between the thermoregulatory and the circadian systems. It has been proposed that the functional connection between hypothalamic centres including the SCN may allow control of a postulated circadian rhythm in temperature set point (Reinberg

and Smolensky, 1983). However, the accumulated evidence is at best, equivocal and inconclusive (reviewed in Refinetti and Menaker, 1992). Recently, one group has shown experimentally that SCN lesions do not affect homeostatic thermoregulation in rats (Wachulec et al., 1997). While this apparently precludes the SCN from a significant role in body temperature homeostasis, the SCN has a clearly defined role in the regulation of core temperature changes across the day (see section 1.7).

Taking a different view, Moore recently postulated a model of neural organisation which suggests that thermoregulation may be only one of many hypothalamic homeostatic functions directed by the SCN (Moore, 1996; Moore, 1997). According to this model, the SCN via neural or neuroendocrine signals monitors autonomic factors including hormonal feedback (melatonin) and sensory inputs from the limbic system (affective state), reticular formation (behaviour) and hypothalamus (internal milieu). Integration of all the afferent information allows altered SCN efferent control of the hypothalamic homeostatic functions, such as sleep propensity (Nakao et al., 1995a; Nakao et al., 1995b). This model of circadian organisation may therefore account for some level of control by the SCN of both adaptive and predictive homeostatic changes, in order to provide a selective advantage that may vary between species (see Refinetti and Menaker, 1992; Reinberg and Smolensky, 1983). Overall, it is clear that the circadian pacemaker plays a complex role in the regulation of many physiological systems, regardless of the ultimate hierarchy in which it participates.

1.7 Circadian Changes in Body Temperature

Two of the pioneers of circadian physiology, Jürgen Aschoff and Rütger Wever suggested almost 35 years ago that, "Rhythmicity is a ubiquitous biological phenomenon. Like homeostasis, it is one of the basic manifestations of living systems" (Aschoff and Wever, 1962). This rhythmicity can be demonstrated by continuous measurement of body temperature. Under normal conditions, the body temperature repeats approximately every 24 hours (a nycthemeral rhythm). In unmasking conditions (ie. where activity, food intake and other behaviours are controlled for and there is no exposure to external time cues), human body temperature rhythms are maintained with a period of 24-26 hours (Aschoff, 1955; Aschoff, 1979; Wever, 1986). This endogenous "free run" period suggests that an internal pacemaker generates the rhythm of core temperature. Nycthemeral rhythms that are endogenously driven are classified as circadian rhythms. Simpson and Galbraith (1906), who observed an apparently unaltered CRT in a monkey under constant lighting provided the first indication of the endogenous nature of the circadian rhythm of temperature (CRT).

While thermoregulation is typically an adaptive process, reacting to restore optimal temperature despite internal and external disturbances, circadian changes in temperature suggest there is a predictive component also. That is, changes in temperature occurring at particular times of the day provide a time keeping mechanism for the body, allowing certain activities (eg. sleep) to take place at the optimum phase of the circadian cycle. Predictive homeostasis of body temperature (ie. the CRT) has been suggested to provide a selective advantage toward the conservation of energy (Refinetti and Menaker, 1992). As

an example, effective thermoregulation has been demonstrated to require precise internal synchronisation of the circadian timekeeping system (Fuller et al., 1978). This group showed that squirrel monkeys exposed to cold whilst free running, suffered from greater hypothermia than control animals synchronised to environmental (light-dark) time cues.

The measured rhythm in temperature originates from two main influences, a circadian 'pacemaker' and masking activities (Wever, 1979). As mentioned in the previous section, the paired suprachiasmatic nuclei (SCN) are typically regarded as the pacemaker of the circadian system, which determines the oscillation and period of circadian rhythms in the body. However, when the SCN are destroyed in squirrel monkeys, the CRT has been shown to persist (Moore-Ede et al., 1983), suggesting that some pacemaker function for thermoregulation may reside outside of the SCN. It has been postulated however, that this and similar results have been found only where the SCN are not completely destroyed (Refinetti and Menaker, 1992) and some functional pathways still exist. This hypothesis is further supported by 'forced-desynchrony' protocols using human subjects, which impose a schedule shorter or longer than 24-hrs to which the circadian system cannot be entrained. Eventually, the CRT and sleep/wake activity begin to cycle with different periods (for example, Czeisler et al., 1986; Eastman and Miescke, 1990). These experiments have verified the existence of separate pacemakers for sleep and/or temperature whose outputs are normally coincident due to environmental entrainment (eg. light). While the location of the CRT pacemaker has not been verified within the SCN, it may be different between species (Johansson et al., 1991).

1.7.1 Circadian rhythm of thermoregulation

There is a circadian rhythm in core body temperature with a daily amplitude of about 1-1.5 °C, which is related to an underlying rhythm in thermoregulation. However, the functions of both the circadian system in regulation of body temperature (Kittrell, 1991) and the POAH in its homeostatic control (Kobayashi, 1988) are still unclear. Available evidence would suggest that in homeotherms, the stable circadian variation in body temperature results from both homeostatic and circadian regulatory components. Interestingly, while ectotherms regulate their body temperature primarily by behavioural means, some recent work with lizards has revealed that in both a light:dark cycle and constant dim light, a circadian rhythm in core temperature is also maintained (Tosini and Menaker, 1995). Clearly then, the daily variation in core temperature has important physiological sequelae across both endothermic and ectothermic species.

As changes in body temperature can only arise from variations in heat production, heat loss or both, it is important to identify which of these thermoregulatory mechanisms, under the influence of homeostasis and circadian variation may be responsible for the CRT. In a review of the CRT literature by Refinetti and Menaker (1992), it was suggested that variations in heat loss are primarily responsible for the CRT, as body temperature retains its rhythmicity even when heat production is constant (Fuller et al., 1985). However, under controlled constant-routine conditions designed to unmask the basal temperature rhythm, heat production has also been shown to have a significant circadian rhythm with a peak between 1130-1200 h (Kräuchi and Wirz-Justice, 1994). In a recent review, Cagnacci and colleagues (1997) suggest that rhythms of both heat

production and loss, with near-identical amplitudes and a constant phase relationship across the day, are responsible for generating the CRT. This evidence is based on studies by Kräuchi and Wirz-Justice (1994) and also earlier observations by Aschoff (1983). Whereas both these studies measured thermoregulatory changes in humans during constant bed-rest, subjects in the Aschoff study were not prevented from sleeping. However, the overall relationship between rhythms in heat loss and production did not appear to be different when the masking influence of sleep was removed by the constant routine conditions in the Kräuchi study. Specifically, the circadian rhythm of heat loss evaluated by distal (hand and foot) temperature, was found to be delayed relative to both the rhythms in heat production and core temperature (Kräuchi and Wirz-Justice, 1994). Therefore in this study at least, the circadian variation in core body temperature has been attributed to endogenous rhythms in both heat production and heat loss.

1.8 Sleep

Sleep has been defined as "a reversible behavioural state of perceptual disengagement from and unresponsiveness to the environment" (Carskadon and Dement, 1989). Sleep normally proceeds through alternating cycles of rapid eye movement (REM) sleep and non-REM sleep, the functions of which have been speculated upon but not confirmed. It remains a mystery precisely why we need regular sleep, but it is clear that sleep deprivation can be detrimental or even fatal. Interestingly, chronic sleep deprivation in the rat reliably results in death associated with disordered thermoregulation (Landis et al., 1992; Prete et al., 1991; Rechtschaffen et al., 1989). This and similar research has led to a

suggestion that sleep plays a critical role in energy conservation. For example, the effects of sleep deprivation on the brain are inclined toward decreased energy metabolism, primarily in the hypothalamus and other regions associated with control of thermoregulation, endocrine regulation, and sleep (Everson et al., 1994).

1.8.1 Temperature and sleep

Previous studies have suggested that sleep has an evoked effect on core body temperature in humans, observed as a significant decline in core body temperature after sleep onset (Alfoldi et al., 1990; Barrett et al., 1993; Campbell and Broughton, 1994; Gillberg and Åkerstedt, 1982; Lack and Lushington, 1996; Obal, Jr., 1984; Zulley et al., 1981). However, in the absence of sleep, a significant decline in core temperature both in entrained and free-running conditions, still occurs due to an underlying circadian variation (Gillberg and Åkerstedt, 1982). Experimentally, a fall in core temperature around sleep onset appears to be due to increased peripheral heat loss and a decline in the production of metabolic heat. In laboratory studies these body temperature changes are manifested by increased peripheral skin blood flow, increased sweating (Anbar, 1994b; Kräuchi and Wirz-Justice, 1994; Satoh et al., 1965) and decreased heart rate (Kräuchi and Wirz-Justice, 1994). As may be expected, the opposite changes to those observed at sleep onset are generally observed at the termination of the sleep period, ie. increased metabolic heat production, decreased skin blood flow and increased Tc (Anbar, 1994b; Kräuchi and Wirz-Justice, 1994; Weitzman et al., 1979).

These experiments support the hypothesis that set-point temperature is lowered during sleep, independent of circadian temperature changes (Gillberg and Åkerstedt, 1982). The peak and subsequent drop in body temperature in the late evening may, however, 'gate' the optimal timing of sleep onset (Tzischinsky et al., 1993). This can be seen in terms of a superimposition of circadian and sleep-evoked temperature decline, resulting in maximal heat loss. However, this association between the circadian-driven nocturnal downturn in core temperature and increased sleep propensity does not prove a causal link exists between them.

1.8.2 Thermoregulation during NREM and REM sleep

The POAH appears to control not only thermoregulation but also participates in non-rapid eye movement (NREM) sleep regulation. In animals, lesions of the POAH suppress heat loss responses and NREM sleep quality (McGinty and Szymusiak, 1990). Local POAH warming induces peripheral vasodilation and increases the proportion of sleep spent in NREM stages (Glotzbach and Heller, 1989; Glotzbach and Heller, 1984). Humans exposed to elevations in core temperature prior to sleep, for instance by warm baths or exercise, exhibit increased propensity for the deep (or slow wave) stages of NREM sleep (Horne and Moore, 1985; Jordan et al., 1990). The increase in NREM sleep following body heating is generally interpreted as a thermoregulatory compensation as NREM is normally associated with declining brain and body temperature.

The importance of hypothalamic control of normal sleep has been known since clinical observations made early this century (cited in Sterman and Shouse, 1985). It has only been recently discovered however, that a group of

hypothalamic neurons may act as a "switch" for sleep. The ventrolateral preoptic area or VLPO in rats (Sherin et al., 1996), rabbits and cats (Suntsova, 1994) has been shown to be very active during sleep initiation. Patients with injuries of the VLPO typically experience significant insomnia (Sherin et al., 1996), suggesting that the VLPO may have a similar role in the regulation of sleep in humans as in animals.

The co-localisation of control centres for sleep, neuroendocrine function and thermoregulation in the hypothalamus suggests that there may be a functional, as well as structural relationship, in the control of these functions. This is supported by lesion and stimulation studies implicating thermosensitive neurons in the POAH in the control of NREM sleep (McGinty and Szymusiak, 1990). The firing rate of warm-sensitive neurons increases with temperature and those in the POAH have been shown to fire more often at natural sleep onset (Hays et al., 1995; Alam et al., 1994). In rats, the medial preoptic area of the hypothalamus at least partially regulates both sleep and body temperatures (Ramesh and Mohan-Kumar, 1995). It may be however, that separate neuronal groups are involved, since NMDA-lesions or induction of sleep by administration of adrenergic agonists to the medial preoptic area were not associated with simultaneous changes in body temperature (Mohan-Kumar et al., 1995; Ramesh and Mohan-Kumar, 1995). Taken together, evidence from these experiments supports the hypothesis that NREM sleep is at least partially under control of the POAH as a thermoregulatory effector, with body cooling resulting as the functional end point (Obal, 1984).

Unlike NREM, the normal homeostatic control of temperature appears to be inhibited or even totally suppressed during rapid eye movement (REM) sleep in many homeotherms including humans (Parmeggiani, 1977; Parmeggiani, 1987). Unlike changes in temperature during NREM sleep however, it is not yet clear what role the suppression of thermoregulation associated with REM sleep may play in homeotherms. If necessary processes are conserved through evolution, then the characteristic thermoregulatory events associated with sleep can be assumed to play some yet unknown role in the body. For example, the changes in temperature regulation with sleep may support a homeostatic thermoregulatory mechanism or decrease the sustained exposure of heat-sensitive cells to the relatively higher body temperatures associated with wakefulness (McGinty and Szymusiak, 1990).

1.9 Melatonin

Since the discovery that the secretion of the pineal hormone melatonin was elevated at the time people normally sleep, there has been considerable interest in the possible physiological role of melatonin in regulating sleep. Currently, there is some evidence that melatonin, as an output of the circadian system, may play a role in regulation of many circadian rhythms, however in the following section, discussion will be limited to the potential role of melatonin in regulation of body temperature and sleep/wake behaviour.

Melatonin was first identified and purified in the late 1950's, by Lerner and colleagues (Lerner et al., 1958). Under the influence of external time cues or zeitgebers (Germ. *Zeit* - time, *geber* - giver), the SCN of the hypothalamus acts as a neural pacemaker driving the pineal production of melatonin, as well as

other indoles. Two recent reviews give a detailed summary of the putative mechanisms by which mammalian (Illnerová and Sumová, 1997) and human (Shanahan et al., 1997) melatonin rhythms are entrained by environmental light. In normal entrained conditions, melatonin is secreted with a characteristic circadian secretory profile (Ebadi, 1984; Reiter, 1986), with low diurnal and high nocturnal levels.

The SCN receives afferent stimuli from the retina, directly via the retino-hypothalamic tract and indirectly via the geniculo-hypothalamic tract and via the retino-raphe-hypothalamic tract (Rusak et al., 1993). Other afferent pathways to the retina such as from the olivary and posterior pretectal nuclei, have been shown to exist in the rat using both anterograde and retrograde tracing techniques (Mikkelsen and Vrang, 1994). While it is possible that the pretectal area receives input from the retina (Scalia, 1972), detailed treatment of this and other entrainment pathways may be beyond the scope of this introduction.

Neural output from the SCN is carried to the paraventricular nucleus and then to the superior cervical ganglion. Noradrenaline released at the postganglionic nerve terminal binds primarily to ß-adrenergic receptors on the membrane of the pineal cells, triggering a biochemical cascade resulting in production of melatonin within the pinealocytes. Secreted melatonin is thought to diffuse passively down a concentration gradient into the cerebrospinal fluid, the blood and its filtrates (eg. saliva and urine).

1.9.1 The physiological role of melatonin

It appears that the general function of melatonin is to transduce photoperiodic information (ie. day/night length) to an organism based upon the entrainment of

the SCN to the light/dark cycle. The control of melatonin synthesis by the SCN therefore allows humoral control of internal physiology in relation to predictable daily changes. This biological timing function of melatonin appears to be relatively conserved from unicells to invertebrates and vertebrates, including humans (see reviews by Cassone et al., 1993; Cassone and Natesan, 1997). One specific case where melatonin regulates biological timing and one of the closest temporal associations in humans, is that with the circadian rhythm of core temperature (Cagnacci et al., 1992).

1.9.2 Melatonin as a thermoregulatory neurohormone

There is considerable although indirect evidence indicating that melatonin is involved in the regulation of body temperature in animals and man. Two convincing lines of evidence linking melatonin and thermoregulation are (1) that pinealectomised animals typically have compromised thermoregulation when exposed to thermal stress (John et al., 1978; George, 1982), and (2) there are circadian influences on thermoregulation in some animals (see section 1.7), probably mediated by melatonin (Heller et al., 1983).

In some animals, daily administration of melatonin can lead to effects consistent with a seasonal change in day length. As endogenous melatonin production is thought to relay photoperiod information from the pineal as a hormonal signal, melatonin administration will alter the organism's subjective photoperiod. In the case of field mice, 50 micrograms of melatonin/day leads to reproductive regression, some bouts of torpor and shedding to a winter pelt (Heath and Lynch, 1981), consistent with a longer dark period. While an annual variation in melatonin (longer nocturnal duration in winter) clearly has reproductive sequelae

in some species, it may also relate to temperature regulation. For example, some birds (John et al., 1978; Binkley, 1974) and mammals (Beasley and Leon, 1986) have been shown to have lower average body temperatures during winter than spring and summer, when the amplitude and duration of melatonin production is decreased. While there is some suggestion that melatonin and temperature may co-vary on an annual scale in humans (Hofman et al., 1995; Wehr et al., 1993), there is more conclusive data that melatonin is involved in circadian changes in thermoregulation.

1.9.2.1 Melatonin and the circadian rhythm of core temperature

In the last decade, a substantial amount of research has focussed on the potential for melatonin to mediate circadian changes in human core temperature (eg. Badia et al., 1992; Myers et al., 1992). It has been suggested that the nocturnal secretion of melatonin is directly responsible for the nocturnal decline in core temperature (see review by Myers, 1995). In normally entrained subjects (and one phase-shifted shift worker), the timing of melatonin production has been shown to significantly correlate with the circadian rhythm of core temperature (Cagnacci et al., 1992). While this correlation does not provide direct evidence of a link between rhythms of core temperature and endogenous melatonin production, converging evidence is provided by experimental studies of daytime melatonin administration and nocturnal melatonin suppression.

1.9.2.2 Experimental evidence from melatonin studies

It is generally accepted that oral daytime doses of melatonin between 1-5 mg, are associated with an immediate 0.2-0.3 °C suppression of the normal increase in core temperature (Cagnacci et al., 1992; Dollins et al., 1993; Dawson et al.,

1996; Hughes and Badia, 1997; Reid et al., 1996). Experimental studies using suppression of nocturnal melatonin production typically support the hypothesis of active involvement of melatonin in thermoregulation. Following the discovery that endogenous melatonin could be experimentally suppressed by bright light of greater than 2,500 lux intensity (Lewy et al., 1980), it was found that nocturnal bright light significantly increased core temperature by 0.3-0.4 °C (Badia et al., 1990; Campbell and Dawson, 1990; Myers and Badia, 1993). Similar results have been obtained with pharmacological suppression of endogenous melatonin (Cagnacci et al., 1992; van den Heuvel et al., 1997). As the total change in the human core temperature rhythm across 24 hours is between 1-1.5 °C, melatonin may therefore account for around 40% of the circadian variation in (masked) body temperature in follicular phase women (Cagnacci et al., 1992) and in men (Strassman et al., 1991). Notably, the methods used to suppress nocturnal melatonin production are not likely to be specific. That is, β-blockers have well-defined antihypertensive, bradycardiac and other cardiovascular effects, while bright light may increase alertness independent of effects on melatonin production. Nevertheless, the effects of melatonin suppression are generally opposite to those of melatonin administration during the day.

Perhaps the most obvious effect of daytime melatonin administration in both humans and some animals is an increase in sleep propensity (eg. Hughes and Badia, 1997; Reid et al., 1996). However, a 0.2-0.3 °C attenuation of the normal daytime increase in core temperature is also evident, leading to the suggestion that melatonin may mediate sleep-wake behaviour by a direct action on the thermoregulatory system (Dawson and Encel, 1993; detailed in section 1.9.4

below). As stated previously however, that changes in temperature and sleep are concomitant with elevated melatonin levels does not provide evidence of a causal relationship between them. For example, aged women may be relatively resistant (ie. show less hypothermia) in response to large daytime doses of melatonin (Cagnacci et al., 1995; Lushington et al., 1998). However, in the Lushington study the sleep propensity enhancing effect of melatonin was not different to that observed in a previous study in our laboratory, which used young adults in an identical protocol (Reid et al., 1996). Together these results support the suggestion that melatonin and temperature may merely be different "hands of the (same) circadian clock" (Myers, 1995). In this context, exogenous melatonin during the day would decrease core temperature indirectly by influencing SCN activity.

The SCN may also be necessary for mediating other circadian effects of melatonin. For example, rats free-running in constant darkness can be re-entrained to a circadian schedule by daily melatonin injections (Armstrong et al., 1986). Entrainment in this study was however shown to be SCN-dependent as bilateral lesions to the SCN prevented entrainment by melatonin administration (Bartness and Wade, 1985; Cassone et al., 1993). Myers has suggested that determining whether the melatonin-evoked decrease in daytime core temperature persists in the absence of the SCN would confirm the necessity of the SCN in the melatonin response (Myers, 1995). To date, this experiment has apparently not been carried out.

If an endogenous role of the SCN is to differentially drive the circadian rhythms of core temperature (or thermoregulation) and melatonin secretion, it could be

argued that in studies using light to suppress nocturnal melatonin that functional connections between the SCN and the POAH directly mediate changes in core temperature. However, exogenous melatonin has been shown to reverse both light (Strassman et al., 1991) and β-blocker mediated suppression of nocturnal melatonin (van den Heuvel et al., 1997). Unfortunately, it is not clear in these studies whether melatonin is acting directly to affect core temperature and sleep propensity, or indirectly at the level of the SCN according to the above model.

1.9.3 Proposed mechanisms for temperature effects of melatonin

Currently, several possible mechanisms may explain the effects of administered melatonin on core temperature. The first is that melatonin directly elicits core hypothermic effects (Cagnacci et al., 1992; Cagnacci et al., 1993; Dawson and Encel, 1993; Dawson et al., 1996). This effect may arise directly by melatonin acting on specific receptors centrally (Saarela and Reiter, 1994) or indirectly in the periphery (Badia et al., 1992). Evidence supporting both central and peripheral effects of melatonin has been provided by the localisation of specific melatonin receptors. However, whether receptor occupation in different tissues always leads to a functional change in cellular activity is not yet clear. Alternately, melatonin may interact with other endogenous compounds involved in thermoregulation or sleep.

1.9.3.1 Central melatonin receptors

A pre-requisite to understanding the physiological mechanisms of action of melatonin is the identification of the target sites where the hormone may act. The radioligand 2-$^{[125]}$I-iodomelatonin has been used extensively to localise binding sites in both the brain and peripheral tissues. In general these binding

sites have been found to be high affinity, with Kd in the low picomolar range, and selective for structural analogues of melatonin. An early investigation of melatonin's molecular actions was carried out by White and colleagues (White et al., 1987). Using amphibian dermal melanosome preparations, this group found that a regulatory protein with homology to a mammalian protein mediated melatonin's binding. Mammalian cells use this related protein to mediate the action of hormones that inhibit adenylate cyclase through a cell surface receptor. An early autoradiographic study localised putative melatonin receptors predominantly in the median eminence (implicated in seasonal/reproductive regulation) and the SCN (Vanecek et al., 1987). This latter finding lead to a suggestion that endogenous melatonin could feedback on the circadian pacemaker.

Soon after, Dubocovich isolated two distinct melatonin binding sites; ML1 from chicken brain and ML2 from hamster tissues (Dubocovich, 1988) and Weaver's group found high affinity melatonin binding sites in hamster brain (Weaver et al., 1988). Competitive 2-$^{[125]}$I-iodomelatonin binding in this study was observed in the median eminence/arcuate nucleus, pars tuberalis, suprachiasmatic nucleus, pineal gland, anterior pituitary and preoptic area in the foetal brain. Other authors have confirmed these findings (eg. Krause and Dubocovich, 1990; Reppert et al., 1988). There is also a low density of melatonin binding sites spread diffusely throughout the brain. In decreasing order of abundance in humans, melatonin receptors detected by in-situ hybridisation are found in the cerebellum, occipital cortex, parietal cortex, temporal cortex, thalamus, frontal

cortex and hippocampus (Mazzucchelli et al., 1996). Whether melatonin has a role in any or all of these areas is yet to be determined.

More recently, mammalian melatonin receptors have been isolated, purified and cloned (Ebisawa et al., 1994; Reppert et al., 1994). Until recently there had been some contention as to whether there are multiple distinct types of receptors (Morgan et al., 1994), however it now appears that in mammals melatonin receptors are orthologs of a single melatonin receptor class (Reppert, 1997). The melatonin receptor family has been classified within the G-protein coupled receptor "superfamily" and appear to be sites at which melatonin exerts physiological effects (Dubocovich, 1995; Reppert et al., 1996). For example, the melatonin receptors located within the SCN are presumed to mediate some of the circadian effects of melatonin (Reppert, 1997).

1.9.3.2 Peripheral melatonin receptors

The ability of melatonin to affect the tone of both brain and peripheral arteries involved in thermoregulation (Krause et al., 1995; Viswanathan et al., 1990; Viswanathan et al., 1993), would suggest that melatonin may modify body temperatures directly. Melatonin has been shown to enhance both noradrenergic (Krause et al., 1995) and electrically-evoked (Ting et al., 1997) constriction of isolated rat tail arteries. In addition to this peripheral effect, melatonin directly constricts rat cerebral arteries in vitro (Geary et al., 1997), as shown by decreased luminal diameter of rat middle cerebral artery segments. In this way, thermal homeostasis can be altered throughout the whole animal. For example, altered blood flow to the skin from peripheral arteries may change heat loss or heat conservation (Krause et al., 1995; Viswanathan et al., 1990). The reduction

in cerebral blood flow observed in rats after a 10 ng subcutaneous melatonin injection (Capsoni et al., 1995), may in turn cause changes in central thermosensitive neuronal discharge (Boulant, 1994). In humans, melatonin would appear to have similar modulatory effects, as administration is associated with decreased cerebral arterial blood flow and increased peripheral heat loss (reviewed in Cagnacci, 1996).

In rats, a melatonin-mediated constriction of peripheral arteries could be expected to elevate core temperature, which is supported by the results of Padmavathamma and Joshi (1994). In this study, single injections of melatonin (25 μg) induced acute hyperthermia during the subjective day, but not at night when melatonin is produced endogenously. Interestingly, the hyperthermic effects of melatonin were slightly blunted by adrenalectomy and prevented by thyroidectomy, suggesting that in rats these organs may be involved in the thermoregulatory responses to melatonin, although the dose investigated was supraphysiological. Furthermore, the effects of large doses (3 mg/kg) of both melatonin and the melatonin agonist S-20098 in rats injected intraperitoneally during the first 6 hr of the light period, showed no effect on brain temperature (Tobler et al., 1994). Together, these results suggest that rats, as a nocturnally active animal, may have at least partially different responses to melatonin than humans (ie. core hyperthermia vs. hypothermia). However, there is some early evidence that daytime administration of 100 μg/kg melatonin and other indoles in rats may actually evoke core hypothermia of over 2°C (Morton, 1987). It is difficult to interpret this result as temperature changes in rats of this amplitude

are normally only associated with cholinomimetic administration or similar disturbances to the thermoregulatory system.

While the changes in core and brain temperatures in response to melatonin may not currently be clear across different species, overall it appears that melatonin may mediate changes within a species that are appropriate to a particular time of day. For example, the onset of melatonin production in diurnal species may precipitate a downturn in core temperature and increase sleep propensity, while in nocturnal species the opposite is likely to occur.

1.9.3.3 Other putative effects of melatonin

Some actions of melatonin may be independent of (melatonin) receptors, due to its lipophilicity allowing the molecule to enter all cells in the body (Menendez-Pelaez et al., 1993), or an interaction with other endogenous compounds or their receptors.

1.9.3.3.1 Cytoskeletal structure

It has been suggested that melatonin has effects on cellular processes that involve rearrangement of cytoskeletal structure (Benitez-King et al., 1990). This group reported a specific interaction between melatonin and calmodulin, an acidic polypeptide with four calcium ion (Ca^{++}) binding domains (Benitez-King and Anton-Tay, 1993; Benitez-King et al., 1993). The Ca^{++}-calmodulin complex typically activates intracellular enzymes in many mammals, invertebrates and plants. The high affinity of melatonin for calmodulin suggests that intracellular cell activity can be modulated at physiological levels of melatonin by regulation of intracellular activity. Calmodulin is known to interact with and regulate many

enzymes and cellular processes, including myosin kinases, phosphodiesterase, phospholipase A_2, Ca^{++}-ATPase, NAD kinase, guanylate cyclase, phosphorylation, neurontransmitter release, cellular secretion, microtubule disassembly and gap junction permeability (Cheung, 1980). Therefore, it can be seen that the rhythmic levels of melatonin circulating in body fluids may potentially "regulate the regulators" of cell activity. The authors themselves suggest that, "since both calmodulin and melatonin are phylogenetically well preserved compounds, their interaction may represent a primary mechanism for both the regulation and the synchronization of cell physiology".

1.9.3.3.2 Mimicking the effects of endogenous compounds

Most studies have consistently shown that there are few endocrine effects of acute melatonin administration (ie. for 1-4 days). The majority of studies report no significant effects of melatonin on plasma levels of markers of pituitary or adrenal function (see Dawson and Encel, 1993). There are equivocal reports of acute melatonin affecting prolactin and decreasing growth hormone secretion, both of which are normally secreted during the first half of the sleep period (for example, Arendt et al., 1985; Petterborg et al., 1991). However, whether this is related to a neuroendocrine role of melatonin, or an indirect effect mediated by changes in sleep architecture is not known. A further prospect, is an interaction between melatonin and substances known to have soporific or thermoregulatory effects such as thyroid hormones, serotonin and other neurotransmitters. In this section, we will however only deal with substances that affect body temperature, as it is not yet clear whether sleep-regulating effects are always accompanied by changes in body temperature. In addition, while a large number of putative

interactions between melatonin and other compounds have been suggested, only the most likely based on available evidence are covered here.

Immunomodulators

There is some preliminary evidence that the effects of melatonin on temperature may be mediated by an interaction with immunomodulating molecules (Badia et al., 1992). For example, changes in melatonin levels may regulate production of prostaglandins, which are known to have an enormous range of effects in the body, including actions on both immune function, thermoregulation and sleepiness. In the pineal gland, melatonin, prostaglandins and noradrenaline (norepinenphrine) appear to be involved in a inhibitory feedback control loop (Cardinali et al., 1982; Cardinali and Ritta, 1983). Pineal tissue in particular appears to release prostaglandins, especially PGE_2, when cultured with noradrenaline. PGE_2 in turn augments melatonin release by cultured pinealocytes, but inhibits noradrenaline release from pre-ganglionic synapses.

However, based on in vitro experiments it is unclear whether melatonin is sufficiently potent to suppress prostaglandin synthesis, like the structurally similar molecule indomethacin (Kelly et al., 1984). This group showed that a metabolite of melatonin formed in the brain, N-acetyl-5-methoxy kynurenamine, was a potent inhibitor of prostaglandin synthesis. It has since been shown that melatonin administered in vivo can inhibit biosynthesis of prostaglandins and other products of cyclo-oxygenase (Pawlikowski et al., 1984; Franchi et al., 1987). While it appears that melatonin can exert some effects via inhibition of hypothalamic prostaglandin synthesis, these effects remain have not been completely characterised.

Gamma-aminobutyric acid (GABA)

It has been suggested that melatonin may exert some physiological effects by interaction with the GABA-ergic chloride channel complex (Golombek et al., 1996), where the benzodiazepine drugs are known to at least partially exert their hypnotic effects. In addition, GABA administration in rats at doses from 250-1000 mg/kg produces a dose-dependent decrease in core temperature (Minano et al., 1987). Thus, extreme supraphysiological concentrations of melatonin are required in animals to show any effect on the GABA system (Sack et al., 1997). Furthermore, a recent study in humans has shown that a benzodiazepine receptor antagonist does not block either the soporific or core hypothermic effects of 3 mg melatonin administration (Nave et al., 1996). Therefore, if melatonin does alter GABA channel function it is unlikely that this occurs by direct occupation of the receptor. It is also currently unclear what the physiological sequelae of modulation of GABA receptor function would be if indeed melatonin does act there.

Thyroid hormones

Based on extensive research in animals, it has been suggested that a pineal-thyroid negative feedback loop may regulate the growth and secretion of thyroid cells. Chronic melatonin administration has typically been found to suppress thyroid cell growth and secretion in a variety of species, including rats and hamsters (Csaba and Richter, 1975; Singh and Turner, 1972; Soszynski et al., 1988; Vriend et al., 1982) and amphibians (Wright et al., 1996). Furthermore, a study by Padmavathamma and Joshi (1994) suggests that melatonin may mediate thermoregulatory effects in rats by an interaction with the thyroid and to

a lesser extent, with the adrenal gland. In this study, a 25 μg injection of melatonin increased core temperature in rats during the photophase (light period), and the effect was suppressed by adrenalectomy and eliminated by thyroidectomy (Padmavathamma and Joshi, 1994). It is not clear whether a different adaptation to melatonin by nocturnally active species (eg. rats), precludes a similar thyroid-related mechanism in diurnal species, such as humans.

While it appears that acute hypo- or hyperthyroidism has no significant affect on nocturnal melatonin production in rats (Bauer et al., 1989) and birds (Dawson and King, 1994), chronic changes have not yet been investigated. In humans, it is equivocal whether thyroid disease is associated with altered melatonin production. There is clinical evidence that hypothyroidism is associated with increased nocturnal melatonin production (Rojdmark et al., 1991), but in other studies there is no evidence of any alteration in endogenous melatonin production with either hypo- or hyperthyroidism (Dawson and King, 1994; Soszynski et al., 1988).

Serotonin

Communication between neuronal systems or individual neurons in the brain is conducted by the release of neuroactive substances into synapses. Neuroactive substances that are particularly relevant in understanding the potential interactions of melatonin, are those that may be involved in the control of temperature regulation. In this regard, substances produced in the hypothalamus are the most likely mediators of "melatonin-like" effects.

It has long been acknowledged that the monoamine substances adrenaline, noradrenaline and serotonin (5-hydroxytryptamine or 5-HT) play a major role in hypothalamic control of body temperature. Studies beginning in the early 1960's have demonstrated that direct injection of these substances into the cerebral ventricles or anterior hypothalamus alter body temperature (reviewed in Cremer and Bligh, 1969). Typically, injection of adrenaline and noradrenaline into the rostral hypothalamus of cats, dogs or monkeys causes an acute hypothermic effect on core temperature and inhibition of shivering, whereas serotonin typically evokes core hypothermia and shivering (Feldberg et al., 1967).

Serotonin, a biochemical precursor of melatonin, is widely distributed in the brain including the SCN and pineal gland. While serotonin can be obtained exogenously from the diet in some fruits, nuts and other foods, serotonin is also synthesised in situ from tryptophan. Serotonin is produced in many mammalian cells, including neurons, blood platelets and mast cells and in the gastrointestinal mucosa. By analogy to the catecholamines (dopamine, noradrenaline and adrenaline), the indoleamines (serotonin, n-acetylserotonin and melatonin) form a synthetic sequence and may have independent roles as neurotransmitters or hormones (Brown et al., 1984). The functions of serotonin are numerous, including control of appetite, sleep, memory and learning, temperature regulation, mood, behaviour, cardiovascular function, muscle contraction, endocrine regulation and depression.

The indirect elevation of free cytosolic serotonin (5-HT) in the rat by administration of tryptophan or 5-HT-releasing drugs causes a dose- and time-dependent elevation of circulating melatonin levels both during the day and night

(Huether et al., 1993). In this study, inhibitors of the enzyme monoamine oxidase, that metabolises serotonin, also increased free 5-HT and melatonin production. The authors thus claim that some of the effects of indirectly acting 5-HT receptor agonists, such as on sleep architecture, may be mediated by the elevation of circulating melatonin. This may not hold true in humans however, as a recent study investigating the effects on endogenous melatonin production found no effect on melatonin levels after oral administration at 2000 h of indirect 5-HT agonists or a 5-HT_{1A} receptor partial agonist (Nathan et al., 1996). In this case, the authors suggest that dosing limitations imposed by human experimentation may have prevented sufficient 5-HT release to affect the pool of 5-HT available for conversion to melatonin. However, the 5-HT_{1A} receptor partial agonist ipsapirone has been shown to significantly decrease core temperature in healthy adults (Rammsayer et al., 1993). In this study, a similar decrease in core temperature was observed in subjects after exposure to an ambient temperature of 5°C. It is possible given the structural similarity between serotonin and melatonin, that melatonin may actually exert core hypothermic effects in humans by mimicking the effects of serotonin acting at 5-HT_{1A} receptors, however there is currently no direct evidence for this hypothesis.

A recent study by Eison and co-workers (1995) supports the suggestion that melatonin may interact with other serotonin receptors (5-HT_{2A}) in the central nervous system. Melatonin administration to rats in vivo blocked the behavioural effects (increased head shaking) induced by a 5-HT_{2A} receptor-mediated agonist, and in vitro, inhibited biochemical events (phosphoinositide hydrolysis) mediated by activation of the same receptor. Activation of 5-HT_2 receptors has been

implicated in the control of sleep in humans including yawning behaviour (Sandyk, 1996). Ritanserin, a selective 5-HT_{2A} receptor antagonist, increases the duration of delta or slow wave sleep in both rats and humans. This effect is more pronounced during the light period when melatonin plasma levels are low, an effect which is interpreted as a direct suppression of the sleep effects of ritanserin by melatonin (Sandyk, 1996). While the precise role of both melatonin and also serotonin in sleep and temperature regulation is not clear, these experimental studies together suggest that melatonin and its precursor may both participate in a wide range of regulatory activities, including sleep, thermoregulation and behaviour. In some instances, however, the actions of these related neurohormones are not analogous. For example, it appears that melatonin and serotonin may have opposite effects *outside* the CNS, such as on contractility in isolated perfused rat intestine (Bubenik, 1986). In addition, it is not yet clear why serotoninergic agents have similar effects on sleep and thermoregulation in both rats and humans, unlike melatonin.

Summary

In the previous section, some potential interactions between melatonin and some endogenous neuroactive substances were discussed. However, no clear picture exists concerning how melatonin may exert its acute physiological effects via these putative mechanisms. Whether melatonin plays a role in endocrine function or interacts with other endogenous substances, current evidence suggests that the acute thermoregulatory and soporific effects are not likely to be indirect effects mediated by these potential interactions. Following absorption, the onset of the acute effects of melatonin is extremely rapid, often appearing

within minutes and lasting up to several hours. Furthermore, common oral doses of melatonin that produce significant thermoregulatory and soporific effects (ie. 1-5 mg) exert little or no acute changes in endocrine function.

1.9.4 Integrating the effects of melatonin: soporific effects

One proposed physiologic function of the daily change in body temperature is to achieve changes in sleep/wake behaviour. However, nearly all early human and animal studies typically administered doses of melatonin that would have elevated plasma levels to at least several orders of magnitude above normal "physiological" levels observed during the night (ie. 40-80 pg/ml; Arendt, 1986). Following daytime administration of oral melatonin doses that elevate plasma melatonin into the supraphysiological range, acute hypothermic and soporific effects are observed concurrently (Cagnacci et al., 1992; Dollins et al., 1994; Reid et al., 1996). Therefore, the soporific effects of melatonin administration were widely presumed to be a side effect of the high plasma levels achieved, particularly at doses of 5 mg or more. However, melatonin has been shown to exert significant increases in self-rated fatigue at night at oral doses of 2 mg given at 1700 h (Arendt et al., 1984). Despite a large first-pass effect whereby melatonin is metabolised directly, oral doses of even 0.1-0.3 mg may still elevate melatonin levels in bodily fluids transiently above the normal physiological range for healthy young adults at night (Dawson et al., 1996; Zhdanova et al., 1996). Therefore, despite a large variability in achieved melatonin levels after oral administration, these effects may not be representative of the role of melatonin endogenously.

An alternate mechanism by which melatonin may participate in the physiological regulation of sleep is by determining the phase of circadian rhythms of sleep and sleepiness (Dijk and Cajochen, 1997; Redman and Armstrong, 1988; Tzischinsky et al., 1993; Wurtman and Lieberman, 1985; Wurtman, 1986). According to this hypothesis, the timing of melatonin secretion determines an optimal window or gate for the initiation and maintenance of sleep ("(Shochat et al., 1997; Tzischinsky et al., 1993). If this is the case, then the covariance of body temperature and sleep rhythms may be merely coincidental. Support for this hypothesis is provided by observations that, under certain circumstances, the circadian rhythms of sleep/wake and temperature can become dissociated (eg. Dijk and Czeisler, 1994; Gander et al., 1985). Furthermore, in blind individuals not entrained to a 24-hr rhythm (ie. free-running due to a lack of photic input to the SCN), a tight association appears to exist between sleep propensity and endogenous melatonin rhythms. Nakagawa and colleagues (1992) studied a middle-aged blind man and observed that the subject's sleep propensity and core temperature rhythms remained in synchrony, despite free running against the entraining influences of the light/dark cycle and a normal sleep-wake schedule.

Many studies in the last decade have investigated the hypnotic effects of exogenous melatonin as potentially resulting from circadian phase-shifting effects (Arendt, 1994; Dahlitz et al., 1991; Folkard et al., 1990; Lewy et al., 1992; Palm et al., 1991; Sack et al., 1991; Deacon and Arendt, 1994). Depending on the timing of administration, melatonin has been reported to variously advance or delay the circadian system (Lewy et al., 1992; Zaidan et al., 1994), presumably by acting on the SCN (Cagnacci, 1996; Gillette and McArthur, 1996; McArthur et

al., 1997). The phase-response curve (PRC) to melatonin (Lewy et al., 1992; Zaidan et al., 1994), suggests that markers of circadian phase (eg. core temperature minima or endogenous melatonin onset) advance when melatonin is given late in the subjective day and delay when melatonin is given in the morning. Recently, Arendt proposed that melatonin may also phase-shift the timing of sleep, based on data from her own lab (unpublished data cited in Arendt, 1997).

Although the phase-shifting effects of melatonin have improved sleep in subjects whose sleep schedules are desynchronised from environmental timecues, such as the blind (Lewy and Sack, 1992), this effect is considered to be separate from the more acute hypnotic effects of melatonin. This is supported by a study in which melatonin administered to humans produced acute hypnotic effects without concurrent shifts in the circadian system (Dawson et al., 1995). In this study, subjects adapting to a simulated night shift showed no difference in melatonin onset (as a phase marker) when they received 2 mg of melatonin orally at 0800 h and then 1 mg at 1100 and 1400 h, compared with placebo. Appropriately timed melatonin administration has been shown to phase-shift both endogenous melatonin production and core temperature as markers of circadian phase, but the effect was measured over 24 hours (ie. subsequent circadian cycle) after melatonin administration (Deacon et al., 1994). However, it is also reasonable in this case that the temperature response to melatonin may be an acute index of circadian responsivity to melatonin. The suggestion that the acute hypnotic effects of melatonin are not likely to be related to phase-shifts of the circadian system, is reinforced by the observation that it may take several days and

reinforcing signals (ie. appropriate timing of administration) before melatonin-induced phase shifts are apparent.

1.10 Rationale for Studies

Almost 40 years after melatonin was first identified we are still unsure of the physiological role of melatonin and exactly how melatonin evokes its effects. Whether or not melatonin has an endogenous role in the "physiological concert" (Cagnacci et al., 1997) of temperature regulation has been investigated using indirect experimental approaches for over 30 years.

It is generally agreed that administration of melatonin to humans during the day, when endogenous production is low, typically decreases core temperature and increases sleepiness. A significant body of indirect research suggests that melatonin may be involved in temperature homeostasis and that these effects of melatonin underlie the sleep-inducing effects of melatonin. However, it may also be the case that melatonin's acute effects on temperature and sleep propensity are not causally related. Despite the discovery of melatonin receptors in many tissues in the body, the lack of melatonin antagonists available for research use has to date prevented a precise characterisation of melatonin's mechanism(s) of action. Furthermore, most melatonin administration studies have used doses and routes of administration that result in inappropriate circulating levels and/or durations of the hormone.

In the first experiment in this thesis (Chapter 3), the changes in core and peripheral temperatures and subjective sleepiness were investigated around both endogenous melatonin onset and normal nocturnal sleep. It was proposed that in addition to a sleep-evoked decrease in core temperature, that a decline in core

temperature would be evident before sleep onset. The postulated function of this pre-sleep shift in thermal homeostasis is to facilitate the normal initiation of sleep. It was further hypothesised that the normal nocturnal production of melatonin may mediate the postulated pre-sleep change in core temperature.

In the next series of experiments (Chapters 4-5), melatonin was administered at a range of doses during the day to young adults. In the study in Chapter 4, it was hypothesised that a prolonged melatonin infusion that elevated plasma melatonin into the nocturnal physiological range would not significantly change core temperature or subjective sleepiness measures. Furthermore, these acute effects were expected to be observed only when melatonin levels were elevated into the supraphysiological range. In the following study, we proposed that a daytime injection of melatonin that elevated plasma levels into the nocturnal physiological range, with a short duration of effect than the infusion protocol, would similarly not affect core temperature nor subjective sleepiness.

Finally, we investigated the potential therapeutic effects of melatonin administration at supraphysiological levels, in a group of elderly chronic sleep maintenance insomniacs. It was proposed that a sustained administration of melatonin, using a transbuccal patch, would significantly improve the sleep of elderly insomniacs. In addition, it was hypothesised that any improvement in sleep would be accompanied by a significant decrease in nocturnal core temperature.

In summary, this thesis aims to examine the relationships between melatonin, body temperature and sleep propensity using protocols that more closely mimic the physiological profile of the hormone.

Chapter 2

2.1 Ethics and Subject Recruitment

All studies in this thesis were approved by The Queen Elizabeth Hospital Ethics of Human Research Committee using guidelines established by the National Health and Medical Research Council of Australia. Potential subjects responded to media advertisements or notices on University boards. Subjects were aged between 18-30 years for studies in Chapters 3-5 and aged above 55 years in Chapter 6.

In each case, a meeting was arranged with each respondent where the experimental protocol was explained and a subject information sheet was given (see Appendix A for subject information sheets). In addition, potential subjects completed the required screening questionnaires, including a General Health Questionnaire (Appendix B) and a 1 or 2-week sleep diary (Appendix C). In Chapters 3 and 6, both sleep diary and an overnight polysomnographic (PSG) assessment before the study proper confirmed subjects' sleep status. All subjects completing the screening requirements for each study and after reading the relevant information sheet were asked to sign a consent form (Appendix D). The consent form confirmed subjects' volunteer status in the experiments and was understood to mean that subjects understood their rights and obligations in the study.

In Chapter 6, the subject recruitment period also included 7 days of continuous actigraphy (a validated measure of sleep quality), complete daytime blood analysis and clinical interview by a staff psychologist. All subjects selected for the study in Chapter 6 satisfied the International Classification of Sleep Disorders or ISCD criteria for psychophysiological insomnia (1990) and the Waters et al.

(1993) criteria for sleep maintenance insomnia, with a chronic history (at least 6 months) of poor sleep. Subjects who had a sleep history consistent with either Advanced or Delayed Sleep Phase Syndrome were excluded from participating.

Potential subjects in all studies that were smokers, had current health, psychiatric or sleep problems were also excluded from participating. Furthermore, all subjects were required to be free of medication known to affect thermoregulation, sleep or melatonin production (eg. aspirin, β-blockers) for at least one month prior to entering the study. For the 24 hours before each session subjects also abstained from alcohol and caffeine.

Where subjects participated in non-consecutive experimental conditions (ie. studies in Chapter 3-5), they were asked to comply with their typical pattern of sleep-wake activity when not in the laboratory.

2.2 Methodological Considerations

2.2.1 Subject room conditions

In all studies, subjects were housed in single sound-proofed rooms. Studies in Chapters 3, 4 and 5 were conducted with room temperature set at 25 °C (range ± 1°C) and light levels maintained below 50 lux. Subjects were not allowed any other covering (eg. bed clothes) except in Chapter 6, where subjects were housed in rooms without temperature control and therefore were allowed to sleep with as much covering as required. Light levels in each room were checked before the study with a Sekonic DigiLite L-318 lux meter (Sekonic Co. Ltd., Tokyo, Japan).

In all studies, subjects wore a T-shirt and shorts, or equivalent clothing.

2.2.2 Menstrual phase

Female subjects in Chapters 4 and 5 were studied during the follicular phase of their menstrual cycles, which has previously been shown as the phase of the cycle when women are most sensitive to daytime melatonin (Cagnacci et al., 1996). All subjects in Chapter 6 were post-menopausal.

2.2.3 Double-blinding and randomisation

In all studies, subjects were assigned to active treatments and control conditions using a counter-balanced design with complete randomisation of the first condition for each subject. In Chapters 3, 4 and 5, all experimenters except the principal investigator were blinded to the treatment that subjects received. Subjects were always blinded to experimental treatments.

To ensure double blinding and prevent bias in these studies, data were independently coded and entered into spreadsheets by an investigator with no prior knowledge of the protocol, treatments or codes. In Chapter 6, all investigators and subjects were blinded to the treatments, with the blind held by Laboratoire 3M Sante in France until completion of the study.

2.2.4 Melatonin administration

2.2.4.1 Aqueous solutions

Melatonin solutions for infusion (Chapter 4) and injection (Chapter 5) were prepared by The Queen Elizabeth Hospital Pharmacy Production Unit within 72 hours of the start of each session and refrigerated until required. Each infusion solution was covered by black plastic before and during the study to protect the melatonin from possible degradation by light and to ensure blinding of the subjects to the treatment.

Melatonin (Sigma Aldrich Pty. Ltd., Castle Hill, New South Wales, Australia) was supplied as solid pharmaceutical grade (99.9% pure) in 5g amounts. The general method used by the Pharmacy Production Unit was, under sterile conditions, to weigh out the melatonin, dissolve in ethanol, then dilute in 0.9% saline solution to the required concentration for each condition. Solutions were then stored at 4 °C in 500ml saline infusion bags or 1ml single-use syringes as required.

2.3 Melatonin Radioimmunoassay

2.3.1 Plasma melatonin assay procedure

The RIA used was initially developed by Kennaway and colleagues for the measurement of plasma melatonin in sheep plasma samples. The assay protocol for use with human plasma was conducted according to the instructions in kits from Bühlmann Laboratories AG (Allschwil, Switzerland). This assay utilises the Kennaway G280 antibody (Earl et al., 1985; Kennaway et al., 1982) which has been used in both tritiated $^{[3]}$H-melatonin and $^{[125]}$I-2-iodomelatonin based radioimmunoassays (Vaughan, 1993). The present plasma assay uses [^{125}I]-2-iodomelatonin as a ligand as it has been shown that the higher specific activity compared with tritiated melatonin results in greater sensitivity of the assay (Rollag and Niswender, 1976; Fraser et al., 1983; English et al., 1993).

Plasma samples obtained in studies in this thesis were stored at -20°C for up to 3 months before being assayed. Melatonin in the plasma (including standards, negative controls and quality controls) was extracted using small C_{18} reverse-phase columns (Bühlmann). The columns were placed in 10x125 mm glass tubes and sequentially washed by centrifugation at 1000 rpm for 1 minute using 2 x 1 ml of methanol followed by 2 x 1 ml of water.

Standards (4.3 - 430 pM) and quality control samples were prepared by reconstituting the supplied melatonin in 5 ml 0.1 M phosphate buffered saline, pH 7.4, containing 0.5% bovine serum albumin. Upon thawing, plasma samples were centrifuged for 10 minutes at 2000 rpm and 0.5 ml of each sample was loaded onto a separate column. Standards and quality controls (1 ml) were also loaded onto washed columns by pipette. Following centrifugation at 1000 rpm, the columns were again sequentially centrifuged at 1600 rpm for 1 minute, using 2 x 1 ml 10% methanol in water and then 1 ml n-hexane.

The columns were finally placed in clean borosilicate tubes and melatonin was eluted by centrifugation at 1000 rpm with 1 ml methanol. The solvent was evaporated from the tubes using a heating block held at 37°C and a gentle stream of air directed into the tubes. The extract was reconstituted with 1 ml buffer and vortexed, allowed to equilibrate at room temperature for 30 minutes and 400 µl was transferred to a new borosilicate tube. Next, 100 µl of $^{[125]}$I-2-iodomelatonin tracer and 100 µl of G280 antibody were added to the tubes, the content vortexed and incubated at 4°C overnight. The next day, 25 µl of cellulose immobilised second antibody (Sac-Cel donkey anti-sheep/goat) was added to the tubes and incubated for 30 minutes at 4°C. To all tubes, 1 ml of ice cold water was added and then they were immediately centrifuged at 4000 rpm for 15 minutes. The supernatant was decanted and the tubes blotted on absorbent paper. The pellets containing the bound melatonin were counted in a LKB 1272 Clinigamma counter and the amount of melatonin calculated using the RIACALC program.

Where required, the assay for samples containing high melatonin levels was modified so that a 1:1000 dilution of sample was assayed. This was achieved by

decreasing the amount of sample added onto extraction columns from 500 μl to 50 μl and then performing 2 serial dilutions (1:10) of the reconstituted sample in buffer, before adding antibody or tracer. Samples from individual subjects (both control and experimental conditions) were assayed together.

2.3.2 Saliva melatonin assay procedure

Melatonin was first detected in saliva by Vakkuri, who used a chloroform extraction technique to show that melatonin had a circadian profile much like those previously observed in plasma (Vakkuri et al., 1985). Vakkuri's suggestion that melatonin diffused into saliva from the plasma has been supported by observations that salivary melatonin levels typically reach about a third of those in plasma (Mcintyre et al., 1987; Nowak et al., 1987; Voultsios et al., 1997).

The direct assay for salivary melatonin (Voultsios et al., 1997) employed for studies in Chapters 3-5 was similar to the plasma assay detailed above, except that no extraction step was included. Standards (8.6 - 860 pM) and quality control samples were prepared by reconstituting supplied melatonin in 2.5 ml of charcoal-stripped saliva. Upon thawing, saliva samples were centrifuged for 10 minutes at 2000 rpm and 200 μl of each sample (and 200 μl of the standards) were transferred into clean borosilicate tubes. Next, 200 μl of $^{[125]}$I-2-iodomelatonin tracer and 200 μl of G280 antibody were added to the tubes, which were vortexed and incubated at 4°C overnight. The next day, 25 μl second antibody (Sac-Cel donkey anti-sheep/goat) was added to the tubes and incubated for 30 minutes at 4°C. One ml of ice cold water was then added to each tube followed immediately by centrifugation at 4000 rpm for 15 minutes. The supernatant was decanted and tubes were blotted on absorbent paper. The pellets containing bound melatonin were counted in the dried tubes using a LKB

1272 Clinigamma counter and the amount of melatonin was calculated using the RIACALC program.

Where required, the assay for samples containing high melatonin levels was modified so that a 1:100 dilution of sample was assayed. This was achieved by serial 1:10 dilutions of samples in buffer, before adding antibody or tracer. Samples from individual subjects (both control and experimental conditions) were assayed together.

2.3.3 6-sulphatoxymelatonin in urine

The major metabolite of melatonin in urine, 6-sulphatoxymelatonin (aMT.6S) correlates well with plasma melatonin (Arendt et al., 1985a). The Circadian Physiology Laboratory in the Dept. of Obstetrics and Gynaecology, University of Adelaide, assayed the urine samples collected in the study in Chapter 6. The concentration of aMT.6S in the urine samples was determined using the radioimmunoassay of Aldhous and Arendt (1988) without any modifications. Urine samples obtained in the study in Chapter 6 were stored at -20°C for up to 3 months before being assayed. The assays were completed using reagents obtained from Stockgrand Ltd., Surrey, UK.

Sample duplicates were diluted 1:50 in tricine buffer, made from 17.9g tricine, 9.0g sodium chloride, 1.0g gelatin, 0.2mg sodium azide and 1.0L distilled water. A 50μl aliquot of diluted sample, standard or quality control sample was then added to 550μl tricine buffer using a Hamilton Digital Diluter (Reno, NA). aMT.6S antibody (100μl diluted 1:360,000) was added to all tubes excluding totals and non-specific binding (NSB) duplicates, followed by 100μl of $^{[125]}$I-aMT.6S (approx.

4000cpm/tube). All tubes were then multivortexed for 20 seconds and incubated for 14-16 hours at 4°C.

To separate bound and free aMT.6S, 100μl of charcoal solution (1.0g charcoal, 50ml tricine buffer) was added and the tubes vortexed in duplicate. Samples were immediately centrifuged at 4°C and 4000rpm for 15 minutes. The supernatant was decanted and the tubes were blotted with tissue paper. A Multigamma 1261 Counter (Wallac, Oy, Finland) counted the charcoal pellets for 60 seconds each. Samples from individual subjects (both control and experimental conditions) were assayed together.

2.4 Temperature Measurement

2.4.1 Thermistors

Various single-use, sterile thermistors were utilised for temperature measurements. These included:

(a) skin temperature thermistors for external use on the hands, feet and forehead (YSI-4499E, Yellow Springs Instruments, OH)

(b) thermistors for use in the auditory canal adjacent to the tympanic membrane (Sheridan Catheter Corp., Argyle, NY) and

(c) core temperature thermistors for measurement ≥10 cm into the rectum (Steri-Probe 491B, Cincinnati Sub-Zero Products Inc., Cincinnati, OH).

The YSI-4000 series and compatible thermistors (ie. Sheridan and Steri-Probe) have a resistance of 2252 Ω at 25°C and are interchangeable at the ± 0.1°C level of accuracy. Skin and tympanic temperature probes were fixed and reinforced where necessary with thin, porous surgical tape.

2.4.2 Mini-logger system

In Chapters 3, 4 and 6, all thermistors were connected to Mini-logger ambulatory data recorders (Mini Mitter, Sun River, OR), that allowed simultaneous sampling, storage and display of temperature data at 2-minute intervals in each experimental session. The Mini-loggers are factory-calibrated for use with YSI-4000 series and compatible thermistors. In this configuration, the Mini-logger measurements have an accuracy of ± 0.2°C and precision of ± 0.04°C over the range from 31-42°C.

2.4.3 Strawberry Tree system

In Chapter 5, all thermistors were connected to a custom data collection, display and storage system comprising Workbench for Windows software and hardware supplied by Strawberry Tree, Inc. (Sunnyvale, CA). Temperatures were sampled at 2-minute intervals throughout the study.

The Strawberry Tree system was supplied and installed by Automated Process Control Systems (Stepney, South Australia) for use with YSI-4000 series and compatible thermistors. Calibration conducted before installation determined preset digital values for each channel, which are stored permanently on a control computer. Accuracy and precision of temperature measurements over the range 10-40 °C using compatible thermistors was determined to be ± 0.05°C and ± 0.001°C, respectively.

2.4.4 Temperature data treatment

In Chapters 4-6, raw temperature data collected at 2 minute intervals were "binned" into 30 minute averages for each subject. Similarly, temperature data in Chapter 3 were binned into 10 minute averages. The choice of 10 or 30 minute

temperature data bins was made in each case by determining which gave the best compromise between reducing inter-individual variance and the discrimination to accurately detect when small changes (<0.2°C) in temperatures may occur. In each case, in order to detect small changes in temperature, averaging the 2 minute data into longer periods allowed us to decrease data variability and increase statistical power sufficiently, while retaining a reasonable ability to discriminate significant changes over time.

Generally, the binned temperature data was expressed relative to the time at which the treatment commenced (eg. injection, infusion, sleep onset), however in Chapter 6 temperature data collection did not start until after the treatment had been applied (see section 6.4).

2.5 Polysomnography

In Chapter 3, an 8-channel polysomnographic (PSG) recording was used to determine the time of sleep onset from lights-out. In Chapter 6, both sleep onset latency and overnight sleep recordings were measured with an 8-channel PSG. These recordings were made using a SAC 847 Sleep Analyzing Computer (Oxford Instruments, Oxford, UK). PSG data were displayed online and stored on optical disk for subsequent manual scoring and analysis.

Each PSG recording in Chapters 3 and 6 included recordings of the electroencephalogram (EEG), eye movements (electrooculogram or EOG), facial muscle activity (electromyogram or EMG), the electrocardiogram (ECG) and oximetry (blood oxygenation) on one index finger. However, only the EEG, EOG and EMG channels were routinely analysed for sleep recordings. The additional channels were used to verify that no occult sleep or other medical disorders were present during screening or the study proper.

2.5.1 Electrode application

In adherence to the International 10/20 System of electrode placement, all subjects undergoing PSG had their heads measured to allow consistent placement between individuals. According to Carskadon and Rechtschaffen (1994), the 10/20 placement system describes the positioning of electrodes at intervals of 10 or 20 percent of the total distance between landmarks. The landmarks of the system are the nasion, inion and preauricular points of the left and right ears.

EEG channels are bipolar, that is they measure the voltage difference between two separate electrodes (called a reference pair). In studies in this thesis, two EEG channels were recorded, C3/A2 and O2/A1. C3 is the central electrode placement site over the left hemisphere and A2 is a reference placement over the mastoid behind the right ear. C3/A2 therefore indicates the reference pair used for the recording of the primary EEG channel. A secondary EEG channel, O2/A1, used a right occipital electrode placement (O2) referenced to the left mastoid region (A1).

After measurements of each head for electrode placement, the hair was separated and the scalp cleaned using a coarse-grained solution (Omni Prep, D.O. Weaver & Co., Aurora, CO) rubbed on with a cotton-tipped applicator. Grass E5GH gold-plated silver EEG electrodes (Grass Instrument Co., Quincy, MA) were attached to the scalp using collodion (Surgicon Systems, Marden SA, Australia) dried with a gentle stream of air. The cup of each electrode was then filled using a blunt needle and syringe filled with ECI electrode gel (Surgicon Systems, Marden SA, Australia).

Eye movement electrodes were placed above and below the right and left outer canthus (ROC and LOC, respectively). The upper electrodes were referenced to the lower electrodes on the opposite side. Chin EMG was recorded by placing 2 electrodes over the chin (or under the chin if subjects had facial hair), about 3 cm apart. These positions are referred to as the mentalis (or submentalis) placements. Both EOG and EMG electrodes were attached in the same way as ECG electrodes, with the addition of a short piece of porous surgical tape over the electrode cups to secure them in place.

A self-adhesive electrode recorded the ECG over the right clavicle referenced to another over the lower left thorax. These electrodes were secured using porous surgical tape. Oximetry was recorded by an Biox 3740 pulse oximeter (Ohmeda, Louisville, CO) interfaced with the SAC computer via a DC channel input.

2.5.2 Manual sleep scoring

The standard criteria for identifying and scoring sleep stages in normal young subjects from PSG data were published in 1968 by Rechtschaffen and Kales. In general terms, the computer-scored PSG data stored by the SAC computers were re-analysed by two independent manual scorers. PSG data were presented in 30-second epochs that were each assigned a sleep stage (or score) that best characterised the predominant activity during that interval. The precise criteria used for scoring sleep onset and distinguishing sleep stages is described in detail by Rechtschaffen and Kales (1968) and summarised more recently by Carskadon and Rechtschaffen (1994). If a discrepancy was detected between the independent scorers for an epoch, then a third scorer re-analysed the entire record and the median score for each epoch was adopted.

2.6 Subjective Sleepiness

Subjective measures of sleepiness were determined using an adapted visual analog scale self-report (Carskadon & Dement, 1985), called the Linear Sleepiness Rating or LSR. The test consisted of a single sheet of paper on which is printed a 10 cm horizontal, marked 'very wide awake' and 'very sleepy' at the left and right extremes, respectively. Subjects were asked to draw a line through the horizontal line on each sheet, in order to assess "..how sleepy you are". LSR scores were measured in millimetres from the left extreme, so that a range of scores between 0 and 100 were obtained, higher LSR scores indicating greater sleep propensity.

Chapter 3

3.1 Introduction

In many previous studies, a significant decline in core body temperature after sleep onset has been observed (Alfoldi et al., 1990; Barrett et al., 1993; Campbell and Broughton, 1994; Gillberg and Åkerstedt, 1982; Lack and Lushington, 1996; Obal, 1984; Zulley et al., 1981). This is consistent with the suggestion that sleep has an evoked effect on core body temperature in humans (eg. Barrett et al., 1993). Furthermore, the relationship between temperature and sleep propensity is strengthened by observations that the circadian rhythm in core body temperature is inversely correlated with changes in sleep duration or propensity (Campbell and Broughton, 1994; Czeisler et al., 1980; Lack and Lushington, 1996; Parmeggiani, 1987; Zulley et al., 1981). The results of these and similar studies suggest that the nocturnal decline in core body temperature may be involved in the regulation of normal sleep onset and maintenance (Campbell and Broughton, 1994; Campbell and Dawson, 1992; Lack and Lushington, 1996; Murphy and Campbell, 1997).

The exact time course of changes in thermoregulatory balance in relation to nocturnal sleep onset is not currently well defined. Early studies of thermoregulation and sleep indicated that sleep onset was associated with both increased heat loss and reduced heat production (Geschickter et al., 1966; Kreider et al., 1958). Similar changes in thermoregulatory balance have also been recently reported to occur in the absence of sleep (Barrett et al., 1993; Kräuchi and Wirz-Justice, 1994), suggesting that they may be driven by an output of the circadian system and not by changes in sleep/wake status.

This relationship is shown for example, by the observation that in individuals entrained to a normal light/dark cycle, the pineal hormone melatonin typically shows a circadian profile with low diurnal and high nocturnal levels (Vaughan et al., 1976). Since the first observations that the secretion of melatonin is elevated at the time people normally sleep (Lynch et al., 1975), there has been considerable interest in the possible physiological role of melatonin in regulating sleep. Several reports have since correlated the nocturnal increase in melatonin production with increased sleep propensity (Åkerstedt et al., 1979; Zhdanova et al., 1996), which has been described as the opening of a nocturnal "sleep gate" (Tzischinsky et al., 1993).

One mechanism whereby melatonin may mediate changes in sleep propensity is via acute thermoregulatory effects (Brown, 1994; Cagnacci, 1996; Dawson and Encel, 1993; Dollins et al., 1994; Hughes and Badia, 1997; Reid et al., 1996; van den Heuvel et al., 1997). For instance, several studies have reported an acute dose-related decrease in daytime core temperature after oral administration of melatonin (Dawson et al., 1996; Dollins et al., 1994). This core hypothermia is also associated with increased sleep propensity (Dollins et al., 1994). If the nocturnal onset of melatonin production does mediate increased sleepiness by a downturn in core temperature, then this should be apparent before normal sleep onset, which reportedly occurs several hours after melatonin onset (Cagnacci et al., 1992; Deacon et al., 1994). However, research using rats entrained to a cycle of daytime sleep and nighttime activity has shown that brain temperature typically decreases after sleep onset (Alfoldi et al., 1990; Obal et al., 1983). As sleep onset in these animals occurs shortly

after melatonin offset, it is not yet clear whether endogenous melatonin may directly be involved in temperature changes associated with sleep.

Despite the finding by Haskell and colleagues that peripheral skin temperature in humans increases during the first hour after sleep onset (Haskell et al., 1981), there is not yet a comprehensive picture of changes in human body temperatures around sleep onset. Thus, the present study investigated the changes in melatonin production, core and peripheral temperatures and subjective sleepiness around the time of normal nocturnal sleep. These measures before habitual sleep onset were compared with values at the same time from a control night during which subjects' sleep was delayed (but were otherwise under the same conditions).

3.2 Methods

3.2.1 Subjects

Fourteen healthy males, aged between 19 and 27 years (mean ± S.E.M. = 22.1 ± 0.6 years) gave informed consent to participate in the study.

3.2.2 Experimental protocol

Subjects spent three non-consecutive nights in the laboratory within two weeks. The first night allowed the subjects to habituate to the laboratory environment, experimental measures and their sleeping conditions. The second and third experimental nights were assigned using a counter-balanced crossover design. Subjects reported to the laboratory at 1700 h on each night. Between 1730 and 1830 h, a standard montage of polysomnographic (PSG) electrodes were

attached to the subjects' face and scalp and a standard hospital meal was consumed.

Before 1900 h, skin temperature thermistors were attached on the dominant hand and foot using supplied adhesive pads and reinforced with thin, porous surgical tape. In addition, tympanic and rectal thermistors were inserted. Thermistors were connected to a Mini-logger ambulatory recorder sampling at two-minute intervals. To minimise effects of activity on body temperature measures, subjects lay in bed in a single room from 1900 h until the next morning. Before lights out, subjects were allowed to read, watch television or engage in any other sedentary activity as long as they remained supine.

Lights-out time on the habituation and one experimental night was self-selected (this night was designated as the Habitual Sleep condition). Subjects were asked to request lights out whenever they wished to initiate sleep, at which time PSG recordings commenced. As a control for the Habitual Sleep condition, subjects were instructed to stay awake on another night until all measures were completed after 0100 h (Delayed Sleep condition). This typically took between 5-15 mins for each subject. From lights-out on all nights, an 8-channel PSG recording was used to record the time of sleep onset.

In all conditions, half-hourly saliva samples were collected in 5ml tubes while subjects chewed on a small piece of paraffin film for 2 minutes. Samples were collected from 1900 h to the time of lights out. Immediately after collection, saliva was frozen and later assayed for melatonin using a direct radioimmunoassay (Voultsios et al., 1997). In addition, subjects completed a new Linear Sleepiness Rating (LSR) immediately after each saliva sample.

Saliva and LSR measures were taken until the time of lights out in each condition.

Each sleep onset time was subsequently determined from polysomnographic records using standard sleep scoring criteria (Rechtschaffen and Kales, 1968). Two-minute temperature samples for each subject were averaged into 10-minute bins and then expressed relative to the temperature at sleep onset in the Habitual Sleep condition (time zero). Ten minute temperature data bins were selected to give the best compromise between reducing inter-individual variance and the discrimination to accurately detect when changes in temperatures may occur. Temperature data for the period from -60 minutes to 0 minutes were compared using repeated measures Analysis of Variance (ANOVA), with two within-subject factors (condition and time). Planned Means Comparisons were performed to detect whether significant changes in temperature occurred between conditions preceding PSG-defined sleep onset. Rectal core temperature data was also analysed as a rate of change curve (degrees C/10 minute interval) in the Habitual Sleep condition. The time at which core temperature declined at its maximum rate (MROD) was compared with the time of sleep onset to determine the MROD to sleep onset interval.

Melatonin levels in saliva were expressed relative to a mean baseline level of 3 samples taken between, 1900 and 2000 h. The criterion for determining dim light melatonin onset (DLMO) was the time when melatonin levels were greater than 2 standard deviations above the baseline level. In a recent study by our lab, this method for determining nocturnal melatonin onset was validated as more suitable than traditional threshold methods (Voultsios et al., 1997). Both

subjective sleepiness and melatonin data were analysed using repeated measures ANOVA with planned means comparisons where required.

3.3 Results

3.3.1 Sleep onsets and latencies

Mean sleep onsets (± SEM) occurred at 2340 h ± 13 mins during habituation, 2344 h ± 8 mins during the Habitual Sleep condition and 0129 h ± 4 mins during the Delayed Sleep condition. Sleep onset during the habituation and Habitual Sleep conditions did not differ significantly from mean habitual sleep onsets recorded in the pre-study 2 week sleep diaries (2323 h ± 28 mins). When expressed relative to the time of sleep onset in the Habitual Sleep condition (time zero), sleep onset in the Delayed Sleep condition occurred at 105 ± 9 mins.

Sleep onset latency was taken as the time from lights out until the appearance of the first epoch of stage 2 sleep. Sleep latency in the Habitual Sleep condition was 22.1 ± 4.8 minutes, compared with 16.4 ± 3.2 minutes in the Delayed Sleep condition. A paired t test revealed no significant difference in sleep onset latency between Habitual and Delayed Sleep conditions.

3.3.2 Rectal core temperature

Figure 3.1 shows the mean rectal temperature data for all subjects against clock time. When expressed relative to Habitual Sleep onset, repeated measures ANOVA indicated significant decreases in rectal core temperature over time in the Habitual Sleep ($p<0.01$) but not the Delayed Sleep condition (Figure 3.2).

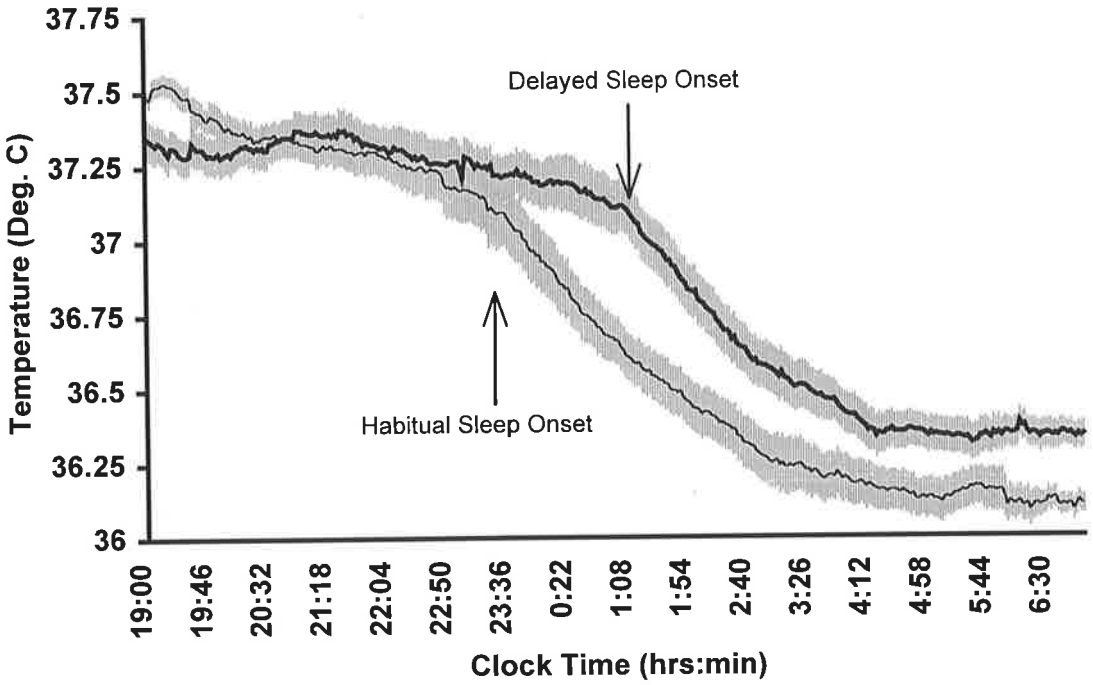

Figure 3.1

Mean core temperature data (± S.E.M.) in 2-minute intervals between 1900 h and 0700 h. The arrows indicate approximate times of respective sleep onsets in the Habitual Sleep condition (—) and the Delayed Sleep condition (—). Core temperature in the delayed sleep condition was maintained at a higher level than the Habitual Sleep condition until at least 0700 h.

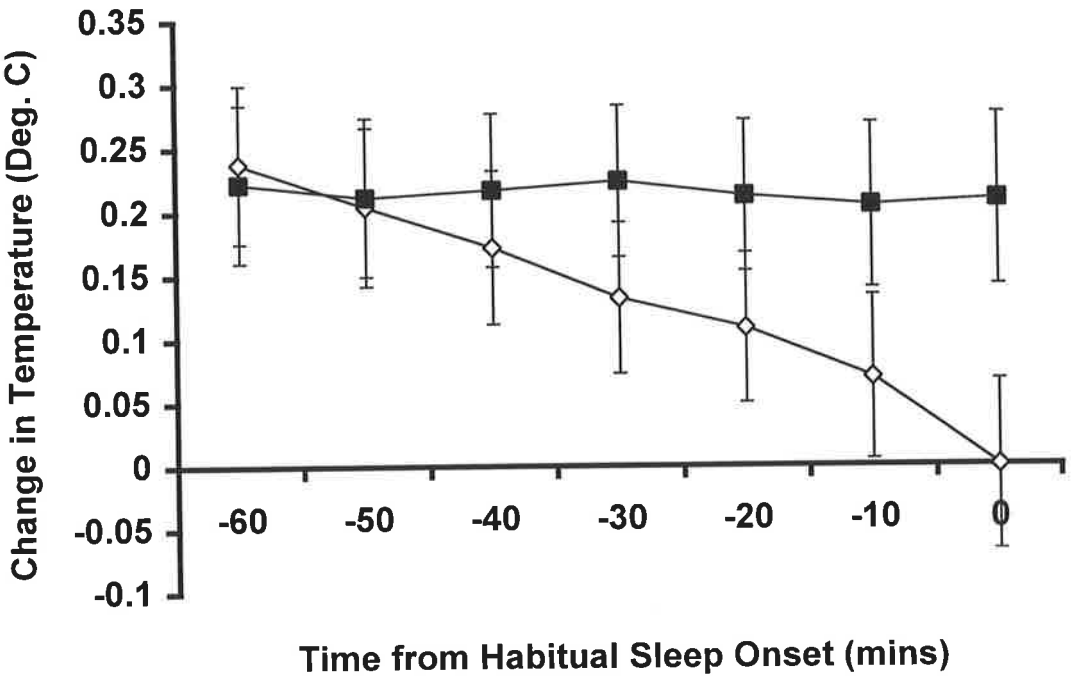

Figure 3.2

Mean core temperature plot of time courses in rectal temperature. Rectal temperature for the Habitual Sleep condition (◊) and the Delayed Sleep condition (■) were binned into 10-minute intervals and expressed relative to the temperature at sleep onset (time zero) in the Habitual Sleep condition. Therefore, in this and following figures, moving vertically along the x-axis compares data from individuals at the same clock time. The decline in rectal temperature was attenuated in the Delayed Sleep condition and there was a significant difference between conditions during the 20 minutes before habitual sleep onset ($p<0.01$). Data shown as mean ± SEM for n=14 subjects.

There was also a significant main effect of condition on rectal temperature ($p<0.01$). Compared with the Delayed Sleep condition temperature which stayed relatively stable for the hour before and after habitual sleep onset, a significant decline in rectal temperature was observed from 20 minutes prior until sleep onset in the Habitual Sleep condition ($p<0.01$). In the hour before habitual sleep onset, the mean difference in core temperature between conditions was 0.06 ± 0.02 °C. The mean difference in core temperature in the hour following sleep onset was 0.36 ± 0.03 °C.

In the Habitual Sleep condition, there was a significant effect of time on the rate of change in core temperature ($p<0.05$; Figure 3.3). The maximum rate of decline (MROD) in the rate of change curve was -0.069 ± 0.021 °C/10 minutes and occurred during the 10 minutes centred on the time of habitual sleep onset. In the hour before sleep onset, the average MROD was less than in the hour after sleep onset (-0.030 ± 0.004 °C/10 mins vs. -0.053 ± 0.003 °C/10 mins, respectively).

The distribution of individual MROD in rectal temperature (Habitual Sleep condition) to sleep onset intervals is shown in Figure 3.4. The MROD occurred after sleep onset in 64.3 % (9/14) of subjects, at sleep onset in 14.3 % (2/14) of subjects and before sleep onset in 21.4 % (3/14) of subjects. MROD occurred at an average of 56.4 minutes after habitual sleep onset (95% C.I. = 53.1 mins, range =-50 to 270 mins).

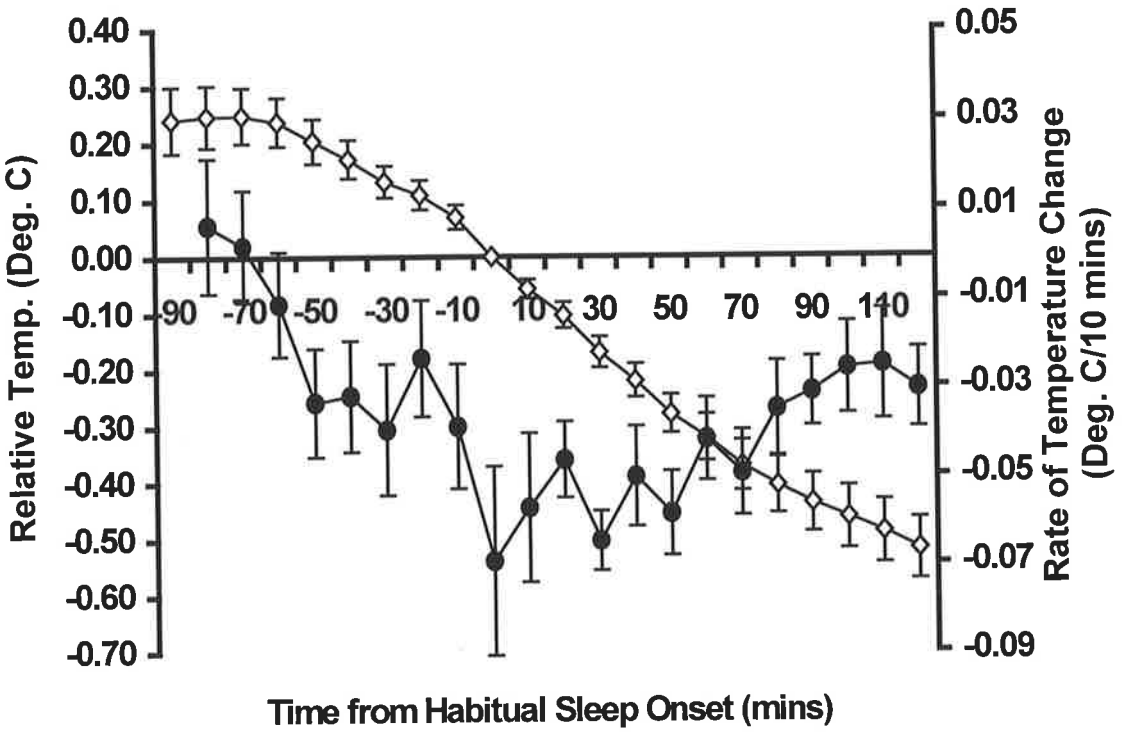

Figure 3.3

Relative rectal temperature and rate of change curves for the Habitual Sleep condition. The rate of change of rectal temperature data (●) for each subject was calculated from the relative temperature data (◊) for the period –90 to 120 minutes (primary y-axis). The maximum rate of decline (MROD) in the rate of change curve occurred at the time of habitual sleep onset (time zero) and is plotted on the secondary y-axis. Data shown as mean ± SEM for n=14 subjects.

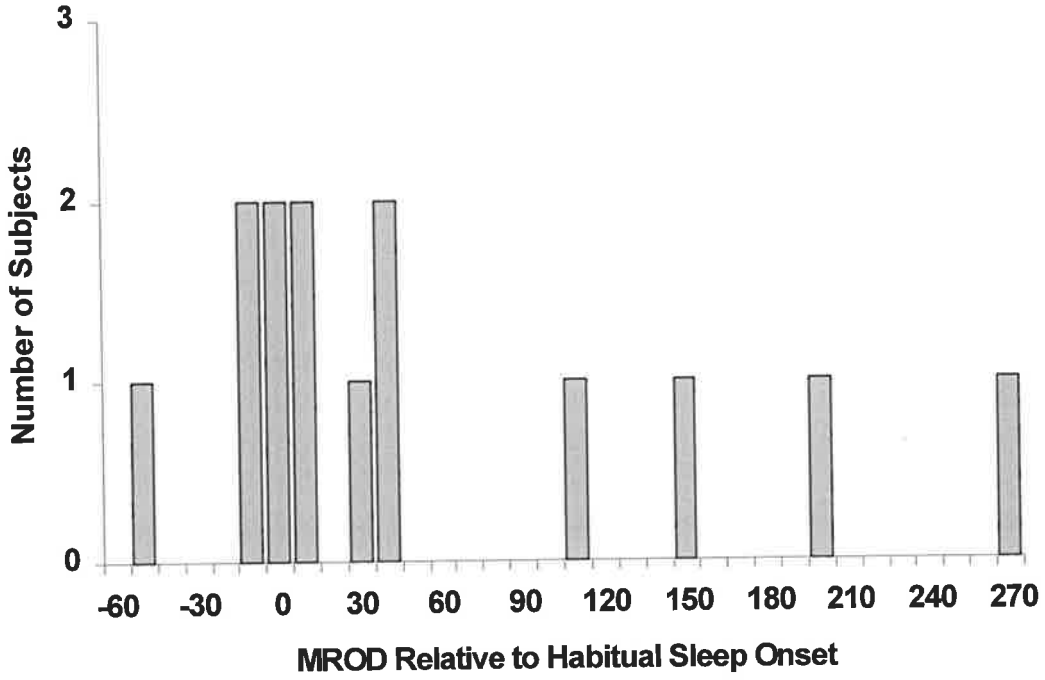

Figure 3.4

The distribution of individual intervals from the maximum rate of decline (MROD) in rectal temperature to the time of habitual sleep onset. Only data for the Habitual Sleep condition is shown (mean ± S.E.M. for n=14 subjects).

3.3.3 Hand skin temperature

Repeated measures ANOVA revealed a significant increase in relative hand temperature across time in both conditions ($p<0.05$), and a main effect of condition ($p<0.05$). Planned means comparisons indicated that hand temperature in the Habitual Sleep condition (Figure 3.5) was significantly higher than in the Delayed Sleep condition, between -50 minutes and habitual sleep onset ($p<0.01$). The mean difference in hand temperature between conditions over this period was 0.59 ± 0.05 °C, compared with 1.01 ± 0.06 °C in the hour following sleep onset.

3.3.4 Foot skin temperature

For 7 subjects, foot temperature data was excluded from analysis due to temperature probes becoming dislodged after sleep onset on one or more nights. Nevertheless, foot temperature for the remaining subjects (n=7) increased significantly over time in the Habitual Sleep condition ($p<0.01$) but not the Delayed Sleep condition (see Figure 3.6). There was a main effect of condition ($p<0.05$), and planned means comparisons indicated that foot temperature increased significantly at sleep onset ($p<0.01$). The mean difference in foot temperature between conditions in the hours before and after habitual sleep onset were 0.08 ± 0.18 °C and 2.76 ± 0.13 °C, respectively

3.3.5 Tympanic Temperature

There were no significant main or interaction effects on tympanic temperature. There was a significant amount of variation in tympanic temperature measures both between subjects and within subjects across conditions.

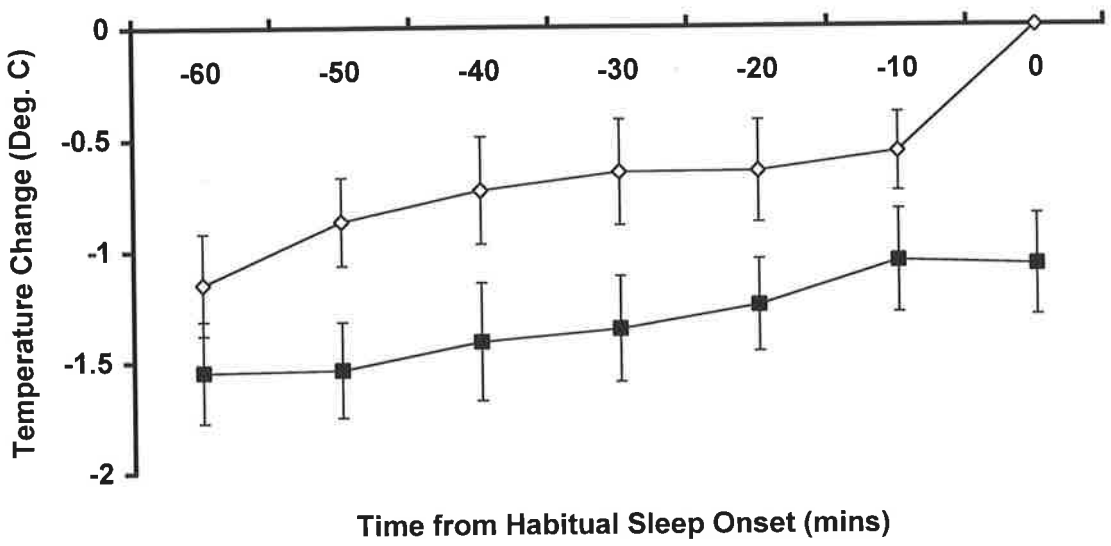

Figure 3.5

Time course of change in hand skin temperature before habitual sleep onset. Hand temperature for the Habitual Sleep condition (◊) and the Delayed Sleep condition (■) in 10-minute intervals are expressed relative to the temperature at sleep onset (time zero) in the Habitual Sleep condition. The increase in hand temperature was attenuated in the Delayed Sleep condition and there was a significant difference between conditions from -50 minutes before until habitual sleep onset ($p<0.01$). Data shown as mean ± S.E.M. for n=14 subjects.

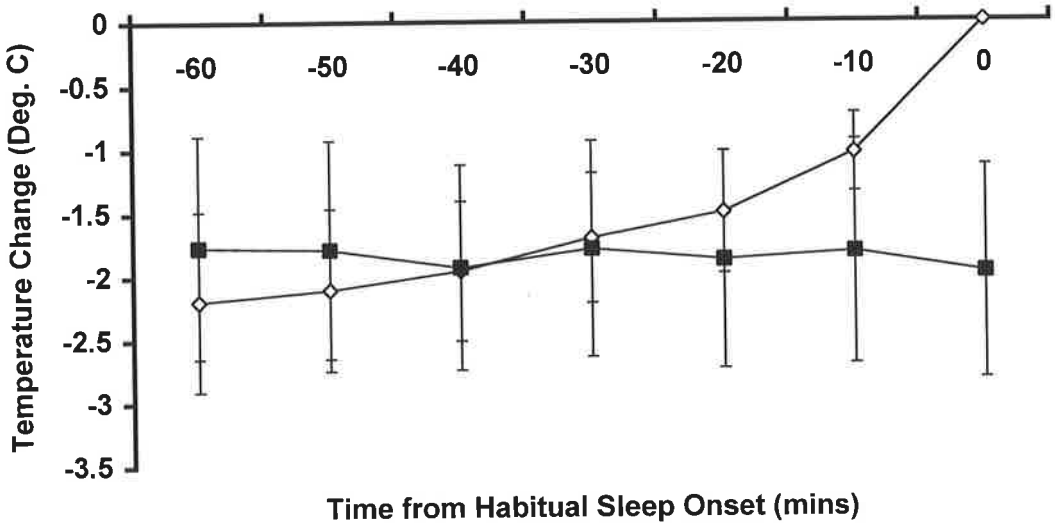

Figure 3.6

Time course of change in foot skin temperature for the hour before habitual sleep onset. Foot temperature for the Habitual Sleep condition (◊) and the Delayed Sleep condition (■) in 10-minute intervals are expressed relative to the temperature at sleep onset (time zero) in the Habitual Sleep condition. There were no significant differences between conditions until the 10 minute interval surrounding habitual sleep onset ($p<0.01$). Data shown as mean ± S.E.M. for n=7 subjects (see text).

3.3.6 Subjective sleepiness

A significant increase in linear sleepiness ratings occurred over time in both conditions ($p<0.01$), however, the increase in LSR scores was less rapid in the Delayed Sleep condition. ANOVA revealed a significant main effect of condition on subjective sleepiness ($p<0.05$). Figure 3.7 shows that sleepiness in the Habitual Sleep condition diverged from LSR scores in the Delayed Sleep condition and was significantly higher in the 150 minutes prior to sleep onset ($p<0.01$). Notably, LSR scores for both conditions reached similar levels of sleepiness in the last test administered before the respective times of sleep onset. That is, the LSR score for the Habitual Sleep condition at –30 mins was 67 ± 7 units, compared with that for the Delayed Sleep condition at 90 mins of 73 ± 7 units. The mean difference in LSR scores between conditions in the 150 minutes preceding sleep onset was 17.9 ± 1.7 units.

3.3.7 Dim Light Melatonin Onset (DLMO)

The mean saliva melatonin levels and number of subjects exceeding the onset criterion in each period in the Delayed Sleep condition are shown in Figure 3.8. When the DLMO criterion was applied to saliva melatonin data from the habituation and Habitual Sleep conditions, reliable dim light onset could not be determined for >90% of subjects prior to sleep (data not shown) and were therefore not included in analysis. The average of individual DLMO in the Delayed Sleep condition occurred at 0010 h $\pm$ 15 mins, while mean saliva melatonin significantly increased above baseline levels (mean from 1900-2000 h) from 2400 h onwards ($p<0.05$).

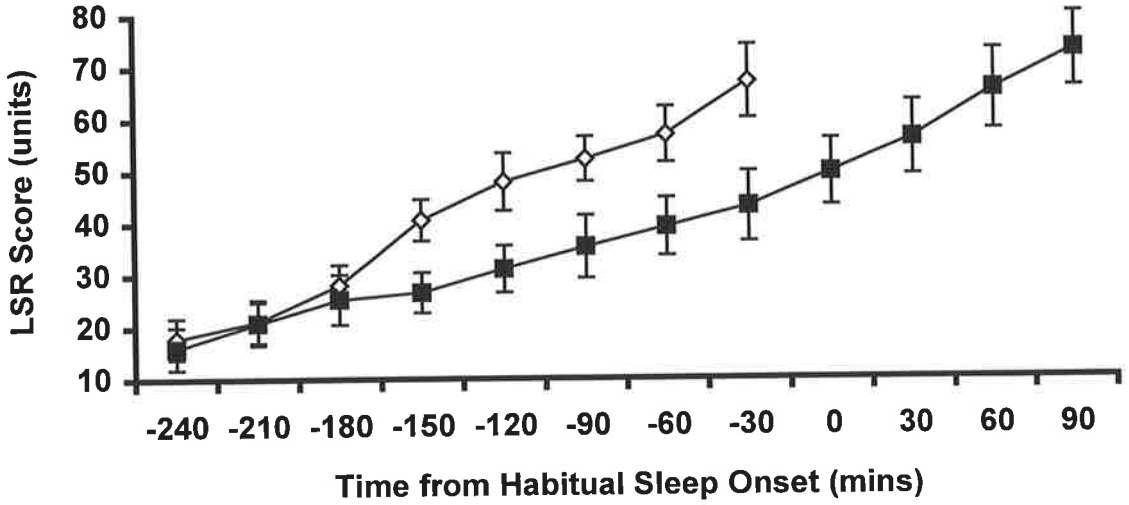

Figure 3.7

Time course of subjective sleepiness scores in 30-minute intervals. The LSR scores (mean ± S.E.M., n=14) are expressed relative to the time of sleep onset in the Habitual Sleep condition. The increase in subjective sleepiness in the Habitual Sleep condition (◊), was attenuated in the Delayed Sleep condition (■) and there was a significant difference between conditions from -150 minutes until the time of habitual sleep onset ($p<0.05$).

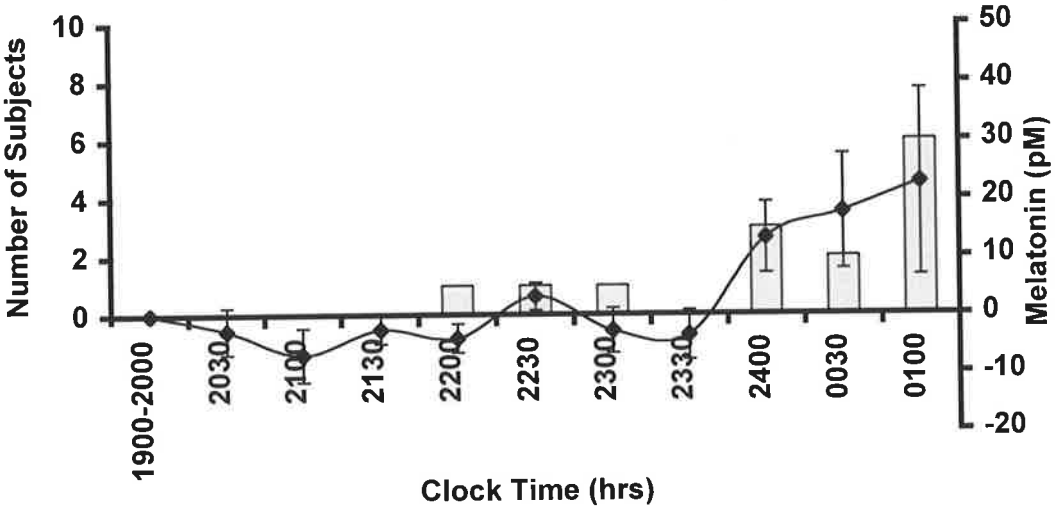

Figure 3.8

Plot of melatonin levels in saliva taken at 30-minute intervals during the Delayed Sleep condition (primary y-axis). Melatonin data (◆) is expressed relative to the baseline period, 1900-2000 h. The number of subjects exceeding DLMO criteria in each time period (see text for explanation), is superimposed on the saliva melatonin data (filled bars, secondary y-axis). There was a significant difference between the baseline melatonin and group melatonin values between 2400 and 0100 h ($p<0.01$). Data shown as mean ± S.E.M. for n=14 subjects.

3.4 Discussion

This study aimed to investigate the changes in peripheral and core temperatures around the time of nocturnal dim light melatonin onset (DLMO). As DLMO and sleep onset apparently occur close together, a secondary aim of the study was to assess the sleep-evoked contribution to thermoregulatory changes both around habitual sleep onset and when delayed by up to two hours.

The current study found that a significant increase in hand skin temperature and a significant decrease in rectal core temperature *preceded* the onset of sleep. In addition, a significant increase in subjective sleepiness was observed 100 minutes before significant thermoregulatory changes were apparent. No DLMO could be reliably detected in the Habitual Sleep condition and saliva samples in the Delayed Sleep condition support the finding that most subjects' melatonin onset occurred after habitual sleep onset. Furthermore, the changes in body temperature and sleepiness before habitual sleep onset were significantly attenuated when subjects' sleep was delayed by almost 2 hours.

The present results suggest that a downturn in rectal temperature before sleep may not be related directly to the statistical onset of melatonin measured in saliva. Furthermore, they may indicate that awareness of an impending sleep opportunity may lead to preparatory behaviour consistent with increases in subjective sleepiness. Whether or not the processes involved in mediating sleep propensity and body temperature changes before sleep onset are linked is not yet clear.

As significant changes in body temperature and sleepiness were observed before subjects initiated sleep (Habitual Sleep condition), but not at the same clock time in the Delayed Sleep condition, the observed changes may be at least partially independent from circadian influences. As habitual sleep onset is likely to coincide with changes in circadian output, such as the onset of melatonin secretion, it was a possibility that any observed changes in the Habitual Sleep condition were not due directly to the initiation of sleep. However, the present study suggests that the observed changes in body temperatures and sleepiness are associated with the process of initiating sleep, since the changes were attenuated when lights out was delayed and subjects were awake at the same clock time in the Delayed Sleep condition. Furthermore, mean DLMO determined using saliva samples occurred almost an hour after mean sleep onset in the Habitual Sleep condition. As melatonin in saliva is a reliable indicator of plasma melatonin levels (Miles et al., 1985; Mcintyre et al., 1987; Vakkuri, 1985; Voultsios et al., 1997), the current results suggest that nocturnal melatonin onset is an unlikely mediator of the pre-sleep changes in body temperature and subjective sleepiness. Similar support is given by the report of Claustrat and colleagues (1986). In this study, a lack of correlation between the nocturnal pattern of melatonin secretion and nocturnal sleep stages in young men was observed. Based on these results, the authors did not favour the suggestion that there is a direct relationship between melatonin secretion and the architecture of the sleep-wake cycle.

These results generally do not agree with the common view that nocturnal melatonin onset occurs 2-3 hours before sleep onset in normally entrained

individuals (eg. Cagnacci et al., 1992; Deacon et al., 1994). Even allowing for a delay in saliva melatonin relative to circulating plasma levels of up to 40 minutes (Voultsios et al., 1997), the DLMO data in the present study clearly indicate that mean melatonin onset (0010 ± 0015 h) occurred at or after habitual sleep onset (2342 ± 0008 h). It is possible that the young male subjects (mostly university students) in this study were relatively phase delayed relative to subjects in previous studies. However, our screening procedures confirmed that all subjects were entrained to a regular sleep/wake cycle and it appeared that the experimental protocol did not shift their habitual sleep onset time or subsequent sleep quality. The good agreement between self-reported and PSG-determined sleep onset in the laboratory also suggests that subjects complied with instructions and accurately recorded their sleep patterns. Nevertheless, this study represents the first study concomitantly measuring pre-sleep changes in body temperatures, sleepiness and melatonin production under unmasking conditions. While the results of this study would suggest significant changes in body temperature that occur before sleep are not related to the onset of melatonin production, this result is yet to be replicated.

The decline in core body temperature in relation to sleep onset in the present study parallels the findings of previous research by Magnussen and colleagues between 1939-1953 (citations 2621-2625 in Kleitman, 1987). This series of studies consistently found a sudden rise in foot temperature and gradual decrease in rectal temperature heralded the onset of sleep. Magnussen interpreted these thermoregulatory changes as a sign of "vegetative preparedness for sleep". The present results also are in agreement with those

of Campbell and colleagues (Campbell and Broughton, 1994; Murphy and Campbell, 1997). Both of these studies accepted that the maximum rate of decline (MROD) in core body temperature occurred both before the decision to retire was made and before sleep onset. The present study found a significant decline in core body temperature occurred at 20 minutes prior to sleep initiation, compared to a relatively stable core temperature in the Delayed Sleep condition. In the present study however, the average MROD was found to occur 56.4 minutes after habitual sleep onset reflecting the sleep-evoked effect on core temperature. Furthermore, mean differences in rectal, hand and foot temperature between conditions, were always greater in the first hour after habitual sleep onset than in the hour prior. These results suggest that while temperature changes are evident before sleep onset, the amplitude of pre-sleep effects is less than the evoked effects of sleep on body temperatures.

The core body temperature decline before sleep, and not the rate of change in rectal temperature, may therefore act as a physiological signal and impetus for sleep onset and a promoter of subsequent sleep maintenance. In fact, Campbell and colleagues proposed a similar hypothesis in their previous studies (Campbell and Broughton, 1994; Murphy and Campbell, 1997). This hypothesis is generally supported by the results of a study by van Vianen and colleagues of body temperature changes before and after sleep onset in poor sleepers (van Vianen et al., 1993). Although a control group of good sleepers was not included, this group found a 0.23°C decline in core temperature occurred between "bed in" and the occurrence of maximum delta EEG activity. Most of this decline was attributed to posture change from upright to supine

and decreased motor activity, while only 0.05°C or less was attributed to sleep onset itself and "a weak circadian trend".

It is suggested that differences between the present and previous studies may have arisen due to the different protocols used (habitual sleep versus sleep during disentrainment), including the precise instructions given to subjects before sleep onset.

The observation in the present study of discrete thermoregulatory changes prior to sleep onset challenges the traditional view that emphasises circadian and sleep-evoked effects as mediators of nocturnal temperature changes. The results of the present study suggest that non-circadian, non-evoked temperature changes may precede EEG-defined sleep onset. Anticipation of sleep may trigger a series of physiological events including changes in thermoregulation and sleep propensity. As subjects were not prevented from knowing the time of lights-out, which was either self-determined (in the Habitual Sleep condition) or predetermined (Delayed Sleep), this may have facilitated subjects' anticipation of sleep onset and the subsequent changes to temperature and self-rated sleepiness. These results are generally complemented by the finding of Aschoff et al. (1974), who showed that anticipation of an impending forced awakening during the night increases core temperature. This "Heizaffekt" or heating affect (see Aschoff et al., 1974, for historical review) on core temperature typically reflects increased vigilance or anticipation of some future event. In the present study, it is possible that the awareness or anticipation of sleep onset can have the opposite, cooling effect on core temperature and thereby facilitate sleep.

It is hypothesised that this anticipatory cooling process occurring before traditional PSG-defined sleep-onset may be a mediator of the "fluffing of the physiological pillow" (Campbell and Broughton, 1994). If this hypothesis proves correct, then heat loss at the periphery may potentially gate the time taken to fall asleep. Future studies may be useful to ascertain precisely how awareness of sleep time may affect the process of sleep initiation. If this turns out to be true, it may account for observed differences in circadian period under free-run conditions due to instructional differences, such as allowing subjects to nap when they feel sleepy.

If the heat loss at the periphery does mediate pre-sleep changes in core temperature, the observation that heat loss capacity declines with age (Stuttgen and Ott, 1990), may explain the age-related decrease reported in sleep propensity and sleep quality (eg. Myers and Badia, 1995; Webb and Campbell, 1980). It is interesting to speculate as to how sleep anticipation may alter physiological functions, such as the output of the thermoregulatory system. While the mechanisms by which thermoregulatory changes affect sleep propensity are currently unknown, it is possible that a common central locus may co-ordinate both thermoregulation and sleep, and involve both physiological and behavioural inputs.

If the decline in core temperature before habitual sleep does determine the quality of subsequent sleep bouts, the results of the present study suggest that the change in core temperature may be achieved by heat loss at the periphery. As body temperature homeostasis is achieved by balancing heat gain and heat loss, either decreased heat production or increased heat loss can mediate a

decrease in core temperature. As heat production at night is typically basal (Kräuchi and Wirz-Justice, 1994), the increase in peripheral skin temperatures before sleep onset indicates that increased peripheral heat loss mediates decreased core body temperature and in turn may facilitate the initiation of sleep. Pioneering studies by Geschickter et al., (1966) and Kreider et al., (1958) found that generally, heat loss increased and heat production decreased around sleep onset and were a likely cause of the nocturnal decline in core body temperature. The present study found an increase in heat loss both before and after sleep onset as indicated by significantly increased hand and foot temperatures. Although a significant difference between conditions was not evident in foot temperature until sleep onset, the greater surface area of the feet and legs may dissipate a physiologically important amount of heat with a non-significant temperature change, compared with the hands and arms. It may therefore be useful in future studies to have temperature measurements at more sites, to gain a clearer picture of changes in body temperatures.

Nevertheless, the changes in peripheral skin temperature in the present study are supported by the findings of Haskell et al., (1981), who investigated thermoregulation during sleep in humans exposed to heat and cold and found transient increases in peripheral skin temperature following sleep onset. Unfortunately, there were no skin temperature measurements reported before lights out in this study.

One possible limit to interpretation of the current results is that subjects were in relative darkness during the sleep onset latency period (approx. 21 minutes) between lights out and PSG-defined sleep onset in the Habitual Sleep

condition. It is possible that the change from dim light to dark, as compared with continuing dim light (<50 lux) in the Delayed Sleep condition at the same time, may have had some effect on the experimental measures. While this explanation could especially account for the change in rectal temperature, it is thought to be unlikely as significant changes in sleep propensity and hand temperature occurred well before lights out. Furthermore, there is no evidence that the dim lighting before lights out could affect the human circadian system or melatonin onset and was therefore anticipated to be ineffective in changing body temperature or sleepiness. However, the design of future experiments could be improved by accounting for this limitation of the current protocol.

In conclusion, a significant increase in peripheral skin temperature is concomitant with a decline in core body temperature that precedes the initiation of sleep. Additionally, these temperature changes appear to be independent of changes in temperature evoked by the circadian system (particularly melatonin onset) and sleep itself. The findings of the present study support the hypothesis that significant changes in body temperature contribute to the facilitation of sleep onset. While the mechanisms by which thermoregulatory changes affect sleep propensity are currently unknown, a common central locus may coordinate both thermoregulation and sleep. A better understanding of the regulation of sleep may lead to better treatment of human sleep disorders that address the underlying aetiology rather than just the symptoms of sleep disturbance.

Chapter 4

4.1 Introduction

In one of the first reported studies of melatonin administration to humans, doses of 1.25-25.0 mg/kg injected intravenously at 4pm for five days resulted in decreased brain electrical activity consistent with increased sleepiness and induction of sleep 15 to 20 minutes after administration (Anton-Tay et al., 1971). The reported hypnotic effects in this study were said to be stronger at the higher melatonin doses. Interestingly, the subjects were woken easily after 45 minutes with no adverse or hangover effects, suggesting melatonin has a relatively short half-life and a mode of action different to traditional sedatives.

From the early 1980's, studies typically used lower doses of daytime melatonin to better examine the "physiological" effects of the hormone. Lerner and colleagues found significant increases in subjective sleepiness in subjects ingesting 0.25-1 gram of melatonin (Lerner and Nordlund, 1978; Nordlund and Lerner, 1977). However, even oral melatonin doses as low as 2 mg (given daily at 1700 hours for 4 weeks), significantly increased subsequent self-rated fatigue scores in 12 subjects (Arendt et al., 1984). These results indicated that the soporific effects of melatonin might be apparent at much lower doses than used in previous research. However, large inter-individual variations in first pass metabolism of oral melatonin (Aldhous et al., 1985; Lane and Moss, 1985) and the associated variability in circulating supraphysiological melatonin levels may limit the interpretation of results from previous studies.

Despite nearly 20 years of research, human studies have been unable to clearly define whether melatonin has a physiological role in regulating normal nocturnal

sleepiness. The aim of this study therefore, was to examine the effects of physiological melatonin levels on body temperatures and subjective sleepiness in young adults. Melatonin was infused at various rates with the aim of reproducing the salient features (ie. level and duration) of nocturnal physiological melatonin production, as well as maintaining supraphysiological levels during the day, when endogenous melatonin production is low.

4.2 Methods

4.2.1 Subjects

Twenty-four subjects (12 male, 12 female) aged 19-28 years gave informed consent and volunteered to attend the laboratory for 2 non-consecutive experimental sessions. The subjects' mean age (± S.E.M.) was 24.1 ± 0.8 years.

4.2.2 Experimental protocol

Each session of the study was conducted between 0700-1900 h. Upon presentation at 0700 h, subjects were cannulated in each forearm by medical staff. Between 0700-0800 h, subjects were fitted with a montage of thermistors for the measurement of body temperature on the back of the non-dominant hand, in the middle of the forehead, adjacent to the tympanic membrane in the ear of the non-dominant side and 10 cm into the rectum.

Between 0800-1900 h, subjects lay quietly awake on a bed and could watch TV or read, but were not allowed to move from the supine position except for short toilet trips as required (where possible, subjects were asked to used a bed pan). Subjects were fed a standard hospital meal between 1230-1300 h and 1730-1800 h.

At 1000 h in each session, a 500ml infusion bag connected to a digital volumetric pump (IVAC Space-Saver 597, Phoenix Medical, Adelaide, South Australia) was connected to the cannula on the subject's dominant arm. In one condition subjects received 0.9% saline and in the other, melatonin dissolved in 0.9% saline. The order of presentation of treatments was counterbalanced for all subjects. Each subject was randomly assigned to one of three groups with the constraint that each group consisted of 4 males and 4 females and the resulting groups received infusions at a different dose rate. The melatonin doses were administered according to the body weight of subjects at entry into the study, using the following dose rates: Low (0.04 μg/hr/kg body weight), Medium (0.08 μg/hr/kg) and High (8.0 μg/hr/kg). Saline infusions were delivered at the same rate as melatonin to ensure subjects received equal infusion volumes across conditions.

The cannula on each subject's non-dominant side was used to sample 10 ml of blood at 0700, 1000, 1300, 1630 and 1900 h in each session. Blood samples were stored in heparinised tubes for up to 2 h at 4°C and then centrifuged at 1500 rpm for 10 mins. Plasma was prepared and frozen at -20 °C for later assay. One subject in the Low dose group had severely haemolysed blood samples in one session, which were not assayed.

Saliva was collected while subjects chewed on a small piece of paraffin film for 2 minutes at hourly intervals between 0700-1000 h and 1700-1900 h and half-hourly intervals during the infusion period (ie. 1030-1630 h). The saliva samples were stored at -20 °C for later assay (see Chapter 2). Coinciding with each

saliva collection, subjects were presented with a Linear Sleepiness Rating (LSR) sheet.

Melatonin levels across time were compared using two-way repeated measures ANOVA with both within groups (Saline vs. Melatonin) and between groups (Low vs. Medium vs. High) factors. Subjective sleepiness scores were expressed as a difference from the Saline condition scores. Temperature data across each session were binned into 30 minute averages and expressed relative to a baseline average obtained between 0800 and 1000 h. Relative sleepiness and body temperature measures both within and between treatment groups were then compared using two-way repeated measures ANOVA. Fisher's protected least squares difference (PLSD) tests were performed where required to determine where significance occurred.

4.3 Results

4.3.1 Plasma melatonin

Plasma melatonin levels in each condition at the start of the study (0700 h) were significantly elevated but decreased to basal daytime levels (range = 5-22 pM) by the start of the infusion period (1000 h). Repeated measures ANOVA revealed a significant interaction for condition by dose, such that plasma melatonin increased significantly above Saline condition levels in all dose groups ($p<0.05$) and mean melatonin levels were significantly different from each other ($p<0.05$). Mean plasma levels (average of samples 1300-1630 h) were elevated to 125 $\pm$ 32 pM and 232 $\pm$ 38 pM following infusion of melatonin at the Low and Medium dose rates, respectively. The High dose melatonin infusion resulted in levels of the hormone that were greater than 100-fold higher than in the Medium dose

condition (34.5 ± 12.4 nM). Mean melatonin levels across the same period following Saline infusion were 12 ± 2 pM, 14 ± 3 pM and 9 ± 3 pM, for the Low, Medium and High dose groups respectively. Plasma profiles of melatonin in each condition are shown in Figure 4.1.

Linear regression analysis of subjects' body weight against average plasma melatonin levels for the period 1300-1630 h, revealed no significant correlations. Regression coefficients for each condition were r=0.46 (p=0.30), r=0.47 (p=0.23) and r=0.16 (p=0.71) for Low, Medium and High, respectively.

4.3.2 Saliva melatonin

At the start of the study, saliva melatonin levels in each condition were elevated but also decreased to basal daytime levels (range = 18-45 pM), before commencement of the infusions. Repeated measures ANOVA did not detect any significant differences between melatonin levels following Low dose melatonin infusion compared with Saline control levels. Mean saliva melatonin levels (average of samples from 1030-1630 h) were 51 ± 12 pM during the Low melatonin infusion compared to 33 ± 11 pM during the Saline infusion. However, there were significant increases in saliva melatonin following Medium and High melatonin infusions compared with levels following the corresponding Saline infusions ($p<0.05$; Figure 4.2). Saliva melatonin levels were elevated to 65 ± 13 pM following infusion of melatonin at the Medium dose rate compared with levels during saline infusion of 27 ± 9 pM. The High dose melatonin infusion resulted in saliva levels of melatonin approximately 20 times greater than that observed with Medium infusion. Mean saliva melatonin during High dose infusion was 576 ± 67 pM compared with levels during saline control infusion of 28 ± 4 pM.

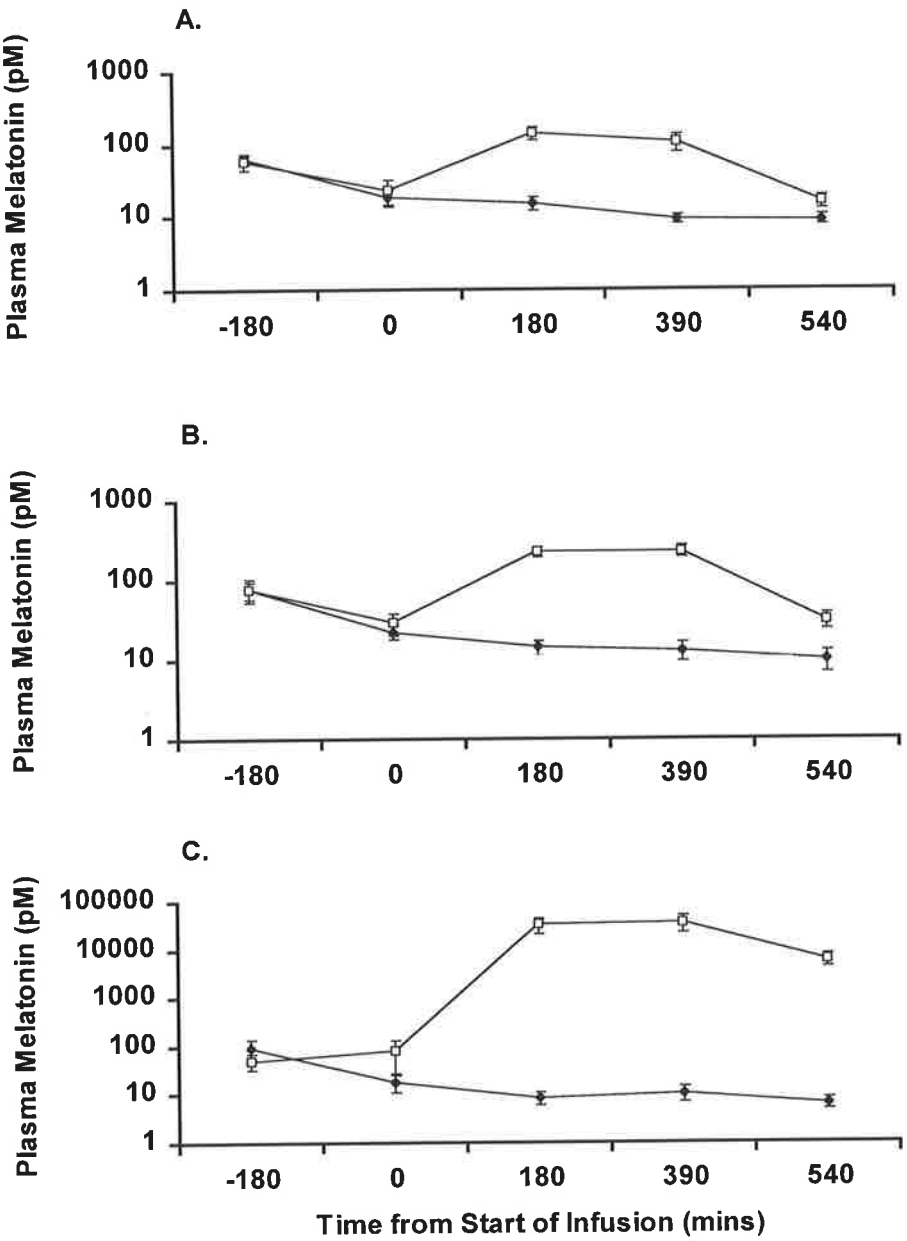

Figure 4.1

Plot of plasma melatonin levels (mean ± S.E.M.) for each dose group: A. Low, B. Medium and C. High infusion dose rates. Data plotted on log scale as open squares (□) for melatonin and filled diamonds (◆) for saline condition. Data was omitted for one subject in the Low dose group (see text). Plasma melatonin increased significantly from 1300-1630 h following melatonin infusion at all dose rates compared with the saline control conditions ($p<0.05$). Plasma melatonin at 1900 h in the High melatonin condition remained significantly greater than in the saline condition due to the high peak level achieved during the infusion.

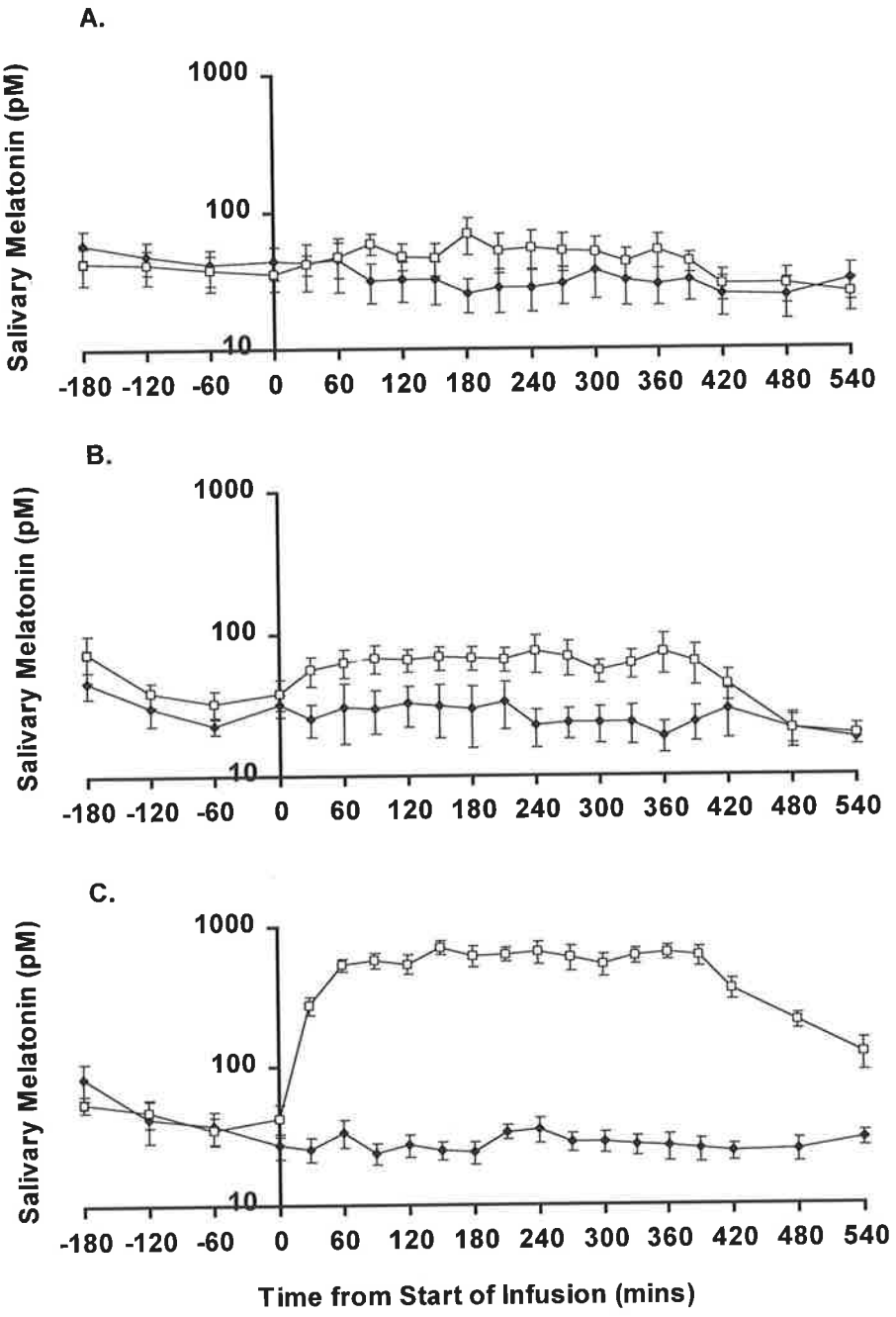

Figure 4.2

Saliva melatonin levels (mean ± S.E.M.) for each dose group: A. Low, B. Medium and C. High infusion dose rates. Data plotted on log scale as open squares (□) for melatonin and filled diamonds (◆) for saline condition. Saliva melatonin levels did not increase significantly above levels in the saline condition with Low melatonin infusion. There were significant increases in saliva melatonin from 1030-1630 h at the Medium rate and from 1030-1900 h at the High dose rate (both $p<0.05$).

4.3.3 Saliva to plasma melatonin ratio

Analysis of the ratio of saliva to plasma (S:P) melatonin levels, as a proportional measure of free to bound melatonin in the body, was performed at both 1300 and 1630 h with data from the Medium and High melatonin dose groups. The ratio was not calculated for the Low dose group, as saliva levels did not increase above saline control levels. The proportion of melatonin in saliva relative to that in plasma, at both 1300 and 1630 h, decreased significantly with increasing doses of melatonin ($p<0.05$). The ratio at 1300 h was 10-fold lower in the High group (3.5 $\pm$ 0.9 %) than in the Medium dose group (35.1 $\pm$ 10.0 %). At 1630 h, a similar pattern of lower S:P ratios with increasing melatonin dose was observed, where the Medium dose ratio was 33.1 $\pm$ 13.0 % and the High dose ratio was 3.5 $\pm$ 0.9 %.

4.3.4 Core temperature - rectal and tympanic measures

Mean rectal core temperatures in all conditions increased significantly across the day ($p<0.05$). For analysis, data in each condition were also expressed relative to the temperatures at the start of the infusion (ie. 1000 h). Rectal core temperature showed a significant main effect of condition by time after High dose melatonin infusion ($p<0.05$). The daytime increase in core temperature was significantly attenuated relative to the saline condition within 30 minutes of the start of the infusion and remained lower until 540 mins (ie. 1030-1900 h; $p<0.05$ for all time groups). The mean relative decrease in rectal temperature during the infusion or between 1030-1630 h was 0.17 $\pm$ 0.01°C. The rectal temperature data are shown in Figure 4.3. There were no significant effects of condition or time in tympanic temperature.

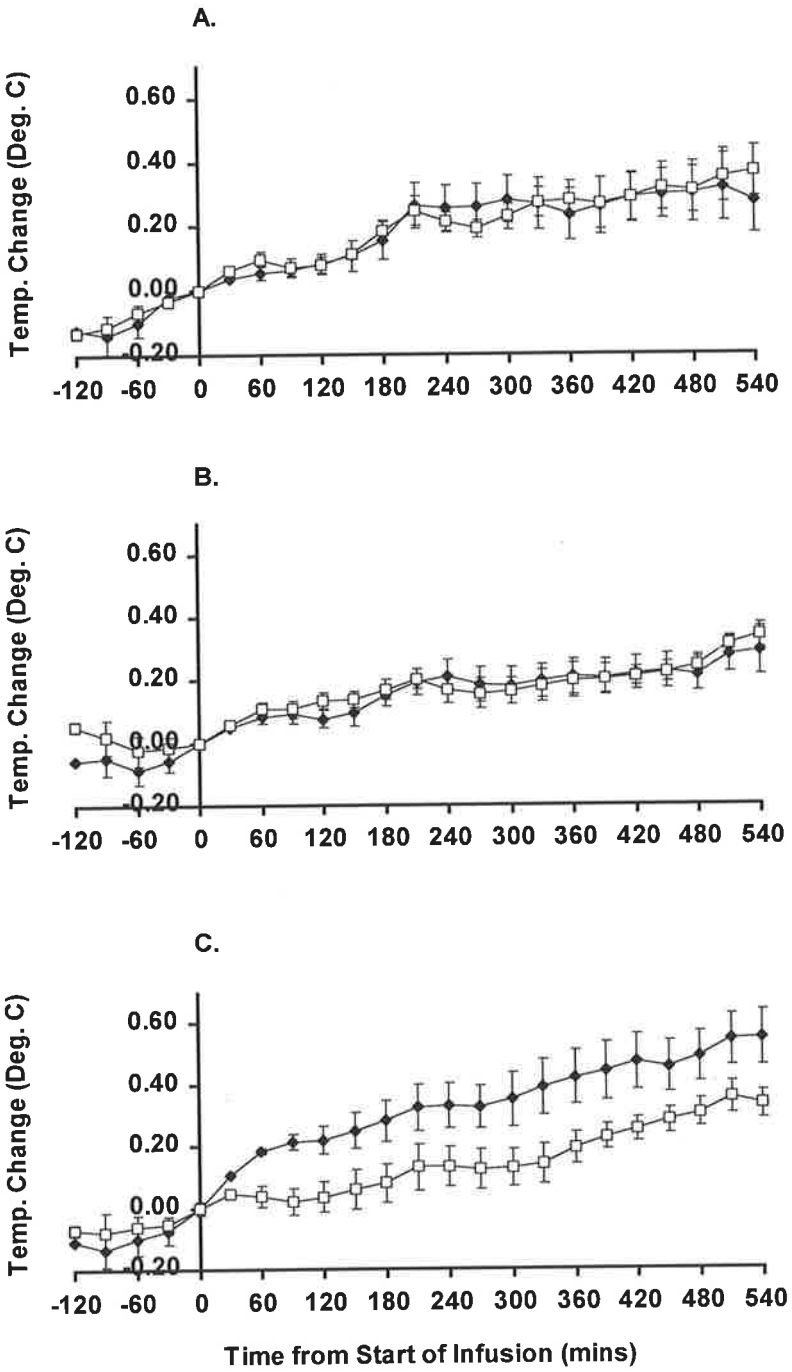

Figure 4.3

Change in relative rectal core temperature for all infusion doses (A. Low, B. Medium and C. High). Data for the saline (◆) and melatonin conditions (□) were expressed relative to the temperature at 1000 h (start of the infusion). Melatonin significantly decreased core temperature between 30-540 mins from the start of the infusion, but only at the highest dose ($p<0.05$).

4.3.5 Skin temperature measures

Hand skin temperature increased significantly and earlier than in the saline condition for the 2 hour period immediately following the start of the High dose melatonin infusion ($p<0.05$). That is, hand temperature was significantly elevated by High dose melatonin within 30 minutes of the start of the infusion and remained elevated relative to the saline condition until 180 mins. During this period, hand temperature was elevated by an average of $0.64 \pm 0.29°C$ relative to the saline condition. However, there were no significant differences in hand temperature in the Low or Medium dose groups, or at other times in the High dose condition (Figure 4.4). There were however non-significant trends (p values ranging from 0.1-0.2) for increased hand temperature for the 2 hours after the end of the High dose infusion (ie. between 390-480 mins inclusive).

There were no significant effects of condition or time in measures of forehead skin temperature.

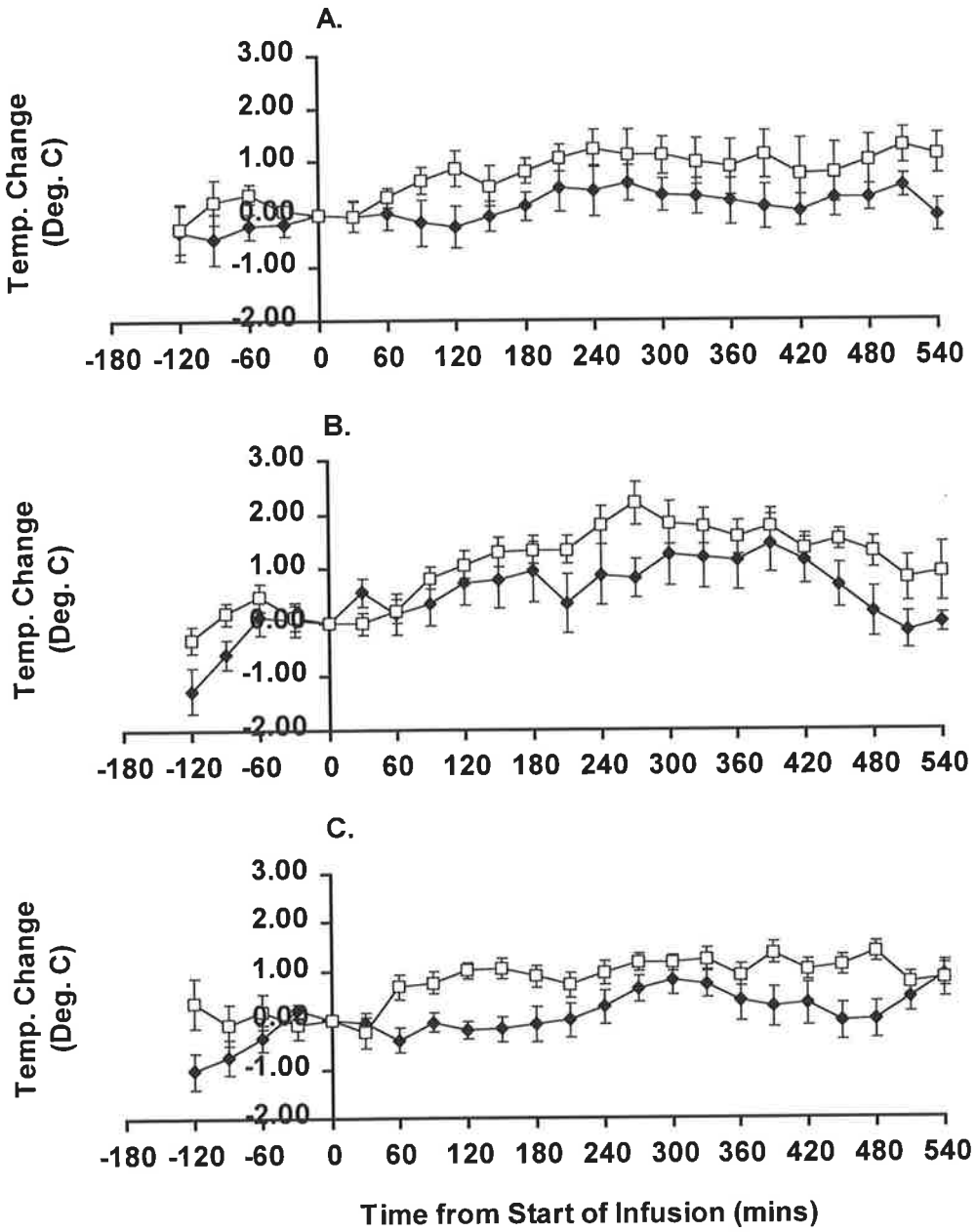

Figure 4.4

Hand temperature plots for each dose group: A. Low, B. Medium and C. High. Data were shown as open squares (□) for melatonin and filled diamonds (◆) for saline conditions. Data (mean ± S.E.M.) were expressed relative to mean temperature in the Pre-Infusion period (0800-1000 h). A significant difference between melatonin and saline conditions occurred only at the High melatonin dose rate ($p<0.05$), where hand temperature increased significantly between 1000-1200 h relative to the saline condition ($p<0.05$).

4.3.6 Regression analyses

Separate regression analyses were performed with (1) mean plasma melatonin and rectal temperature between 1300-1630 h and (2) mean saliva melatonin and rectal temperature between 1030-1630 h. There were significant correlations between changes in temperature with both plasma ($r=-0.41$, $p<0.05$) and saliva melatonin ($r=-0.43$, $p<0.05$) as independent variables. The logarithmic regression equations that best fit the data were $y=0.14-0.08 \log (x)$ and $y=0.12-0.10 \log (x)$, for plasma and saliva respectively. Figure 4.5 shows that the core temperature changes tend to be greatest with increasing levels of plasma melatonin. However, the regression line plotted on a log scale appears to be linear and therefore may not clearly show that core temperature changes plateau at the supraphysiological levels of melatonin achieved with High dose infusion.

4.3.7 Subjective sleepiness

Subjective sleepiness did not change significantly across time in any dose group (see Figure 4.6) and was not significantly affected by melatonin infusion at the Low or Medium dose rates. However, melatonin infused at the High dose rate significantly increased subjective sleepiness during the 120 minute period following the start of the infusion (ie. between 1030-1200 h; $p<0.05$). The mean increase in LSR scores relative to Saline during this period of the High dose infusion was 17.4 ± 4.3 units. During the same period of the Low and Medium dose infusions, subjective sleepiness scores were (not significantly) lower than in the corresponding saline control condition, at -6.6 ± 1.0 and -6.2 ± 2.4 units respectively.

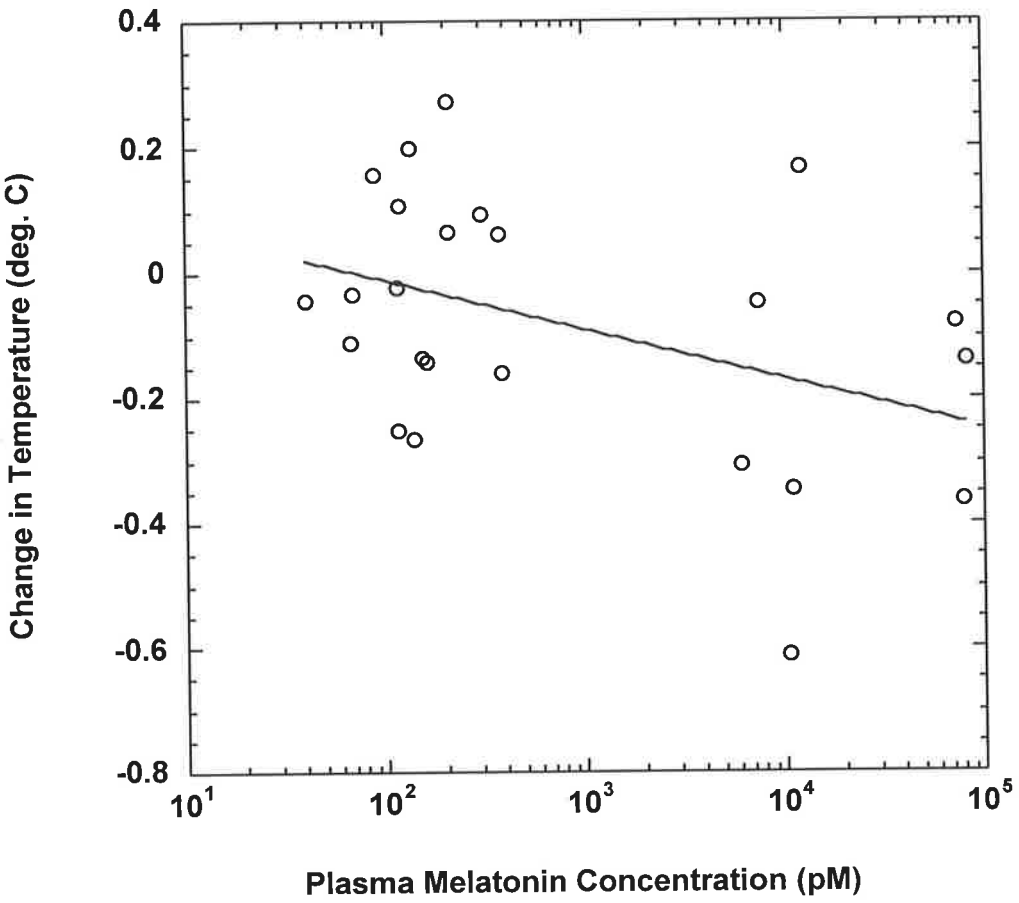

Figure 4.5

Scatter plot on log scale of mean plasma melatonin level (between 1300-1630 h) against the relative change in rectal core temperature over the same period. Regression analysis showed a log curve (equation $y = 0.15 - 0.08 \log x$) fit the data points significantly ($p<0.05$) with a correlation coefficient of $r=-0.41$.

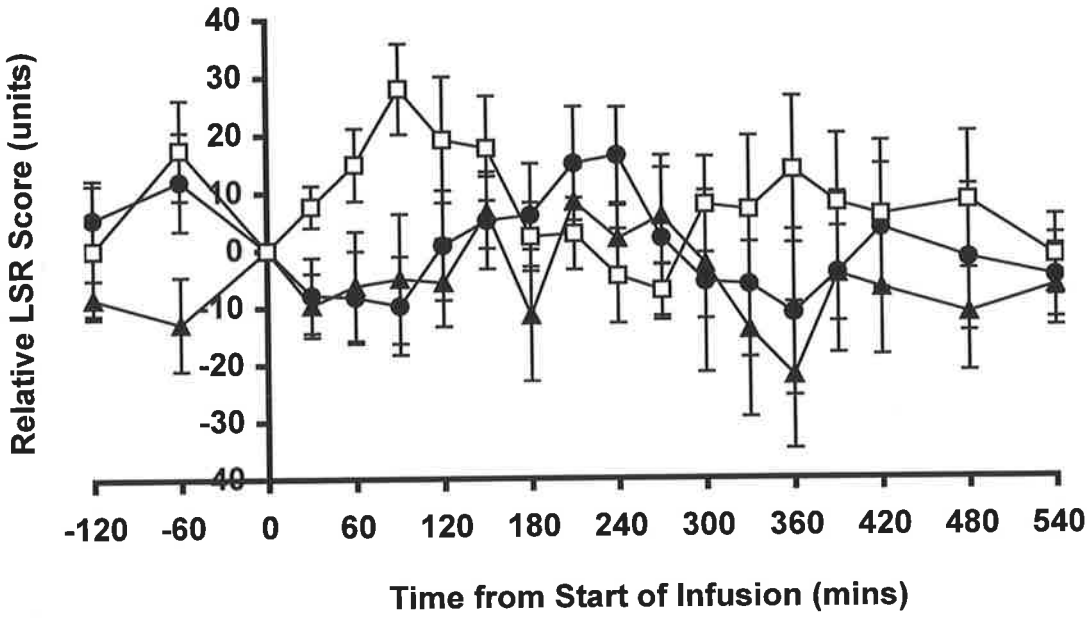

Figure 4.6

Linear Sleepiness Rating (LSR) scores expressed as both a difference between conditions (melatonin - saline) and relative to the start of infusion at 1000 h (mean ± S.E.M.). The X-axis labels are printed below graph for clarity. Relative LSR scores are shown as filled triangles (▲) for Low, filled circles (O) for Medium and open squares (□) for the High dose rate. Subjective sleepiness increased significantly ($p<0.05$) for the first 120 minutes only during High dose melatonin infusion.

4.4 Discussion

The present study examined the effects of a prolonged daytime infusion of melatonin on temperature and sleepiness in healthy young adults. Low and Medium melatonin infusion rates raised the daytime levels of plasma and saliva melatonin into the nocturnal physiological range observed in young healthy adults (for example, Brzezinski, 1997; Voultsios et al., 1997; Webley et al., 1985; Zhdanova et al., 1996). However, these dose rates had no significant effects on core or peripheral temperatures or subjective sleepiness. Infusion at the High dose rate (100-fold higher than the Medium dose) induced a significant attenuation of the daytime increase in rectal temperature, observed within 30 mins of the start of the infusion. A mean difference of 0.17 ± 0.01°C in rectal temperature was maintained during the infusion and it remained lower than temperature in the saline control condition for at least 2.5 hours after the infusion. Hand skin temperature during the High dose infusion was increased significantly at an earlier time than in the saline condition. During the first 2 hours after the start of the High melatonin infusion, hand temperature was 0.64 ± 0.29°C higher than in the saline condition. In addition, melatonin infused at the High dose rate significantly elevated subjective sleepiness for the first 120 minutes of the infusion period. Together, the results suggest that melatonin must be elevated into the range from high normal to supraphysiological levels during the day in order to elicit core hypothermic and soporific effects.

It follows that the effects on thermoregulation and sleep observed with daytime oral melatonin administration, where levels are elevated into the supraphysiological range, may not be the same as the physiological response to

the endogenous nocturnal rise of melatonin. Most previous studies examining the soporific and core hypothermic effects of exogenous melatonin have typically administered oral melatonin capsules (eg. Arendt et al., 1984; Cagnacci et al., 1992; Cagnacci et al., 1993; Dawson et al., 1996; Dollins et al., 1994; Reid et al., 1996). These studies are confounded by large inter-individual variations in first pass metabolism and the rapid elimination half-life of oral melatonin (Aldhous et al., 1985; Dawson et al., 1996; Lane and Moss, 1985; Waldhauser et al., 1990), resulting in an early peak and rapid decline, as well as achieved circulating melatonin levels that vary up to 300-fold. While the area under the curve (AUC) following oral administration of 0.1-0.3 mg melatonin may be quantitatively similar to the AUC of endogenous nocturnal melatonin (Dollins et al., 1994), it has been shown that plasma concentrations following ingestion of melatonin doses of 0.5 mg or greater peak at supraphysiological levels (Dawson et al., 1996). It is also possible that there are time of day differences in melatonin pharmacokinetics, although the results are unclear from the few studies administering identical doses at different times of the day (Zhdanova et al., 1996). Together, these confounds have limited the interpretation of results from previous studies concerning the physiological effects of exogenous daytime melatonin.

In attempting to assess the physiological role of melatonin, not only appropriate level but also the duration of the elevated melatonin levels may be important in signalling to various brain or peripheral effector systems. If this is the case, then oral melatonin administration studies have one general drawback: increasing the dose will increase duration but result in circulating levels far above those observed endogenously and may evoke pharmacological effects rather than

mimic physiological responses to the hormone. To achieve daytime circulating plasma concentrations at or above the equivalent levels observed at night in healthy young adults, rapid-release doses of 1-5 mg are typically used. Even slow-release preparations of equivalent doses produce supraphysiological levels in the distribution phase following administration (Garfinkel et al., 1995; Haimov and Lavie, 1995; Haimov et al., 1995). In the present study, infusing melatonin achieved constant circulating levels during the day that were similar to both normal nocturnal levels (Low and Medium dose rates) and those achieved immediately after oral administration of 4-5 mg melatonin (High dose rate).

Interestingly, controlling administered dose by weight in this study did not noticeably reduce the variability in achieved melatonin levels compared with oral administration. This suggests that the pharmacokinetics of melatonin may not be strictly related to body weight or related variables such as blood volume. It is possible then that melatonin metabolism will introduce more variability than does the normal range of physical characteristics in healthy young adults. Furthermore, it is not apparent whether "fast" or "slow" metabolism of melatonin has any physiological consequences in terms of the thermoregulatory, sleep or circadian systems.

Previous research has indicated that 1-5 mg of oral melatonin can significantly lower core body temperature and increase sleepiness when administered to young adults during the day (Cagnacci et al., 1992; Dollins et al., 1994; Reid et al., 1996). The present data suggest that melatonin maintained at supraphysiological levels during the day, but not physiological levels, attenuates the daytime increase in core temperature, by an average 0.17 °C. The change in

core temperature was at least partially achieved by an acute increase in peripheral heat loss, as inferred from an increase in hand skin temperature during the first 2 hours following the start of the High melatonin dose infusion. However, the relationship between changes in body temperatures may not be a straightforward one, as melatonin maintained a significant difference in rectal temperature, but not hand temperature, until the end of the study. Subjective sleepiness, which was elevated by High melatonin for 2 hours from the start of the infusion, also decreased to control levels despite continuing high melatonin levels. These results suggest that hand temperature and sleepiness may respond to the onset of melatonin during an infusion, but not be affected by the duration.

No significant changes in temperature of the forehead or tympanic membrane occurred at any melatonin dose, suggesting that if melatonin actually exerts discernible effects at these sites, these measures may be relatively insensitive to the small temperature changes observed with melatonin administration. It would therefore appear that the effects on core body temperature are dose-related, and do not appear until circulating levels reach a threshold that is above the physiological range. With the present data it is not possible to clearly define what the melatonin threshold for significant core temperature effects is and what factors it may be influenced by.

It has been argued recently (Zhdanova et al., 1996), that melatonin administered prior to endogenous onset is unlikely to achieve acute changes in body temperature and sleep propensity by an immediate advance of the circadian pacemaker. The authors suggest that a phase shift of up to 10 hours would be

required, if this "shift hypothesis" were to explain the soporific and thermoregulatory effects of daytime melatonin administration. However, the phase-response curve for melatonin administration suggests that melatonin is unable to shift the pacemaker by more than 1-2 hours per day (Zaidan et al., 1994) and therefore does not support this explanation. Furthermore, if melatonin is acting acutely at the level of the circadian pacemaker, the results in this Chapter suggest it may do so only at supraphysiological levels. Taken together, this evidence supports a direct effect of melatonin on sleep and temperature control centres, yet it is likely that these effects represent a pharmacological and not physiological role of the hormone.

The present results are supported by previous studies which have reported soporific and hypothermic effects of daytime melatonin administration that resulted in supraphysiological blood levels, but not at doses that increased melatonin levels into the nocturnal physiological range (Dollins et al., 1994; Zhdanova et al., 1995). In a recent study by our group (Dawson et al., 1996), it was apparent that even at oral daytime doses of 0.1 and 0.5 mg, which produced supraphysiological peak (± S.D.) plasma melatonin levels in 8 subjects of 536 ± 421 pM and 3054 ± 3022 pM respectively, melatonin did not produce significant changes in core temperature. It remains a possibility that the absence of thermoregulatory effects, when melatonin levels are increased into the nocturnal range may reflect a time-of-day effect in responsiveness to melatonin. If this were the case, it would seem that the sensitivity to melatonin is lowered during the day and thus large, supraphysiological doses are required to elicit an effect on temperature and sleepiness. While this has yet to be systematically studied,

there is some evidence that evening melatonin administration at doses of 0.3-1.0 mg can significantly increase sleep propensity (Zhdanova et al., 1996). Further, a melatonin infusion at night that restored physiological levels of the hormone can reverse the core hyperthermic effects of light-induced suppression of endogenous melatonin (Strassman et al., 1991). It remains to be seen whether exogenous melatonin administered in the presence of endogenous melatonin production at night can exert additional effects on body temperature regulation and thus indicate whether the thermoregulatory and soporific effects of melatonin are saturated at physiological levels. This is particularly important given that nocturnal melatonin administration has been suggested as a potential therapy for age-related sleep disturbance (Dawson and Encel, 1993).

Whereas melatonin has previously been thought to diffuse passively into all tissues and compartments in the body, the ratio of saliva to plasma melatonin was not constant across the achieved plasma melatonin concentrations. The reported ratio of saliva to plasma (S:P) melatonin is approximately 0.30 (Vakkuri et al., 1985; Voultsios et al., 1997) and this was confirmed in the present study with the Medium infusion rate. At the highest plasma concentrations of melatonin, S:P ratio was as low as 0.04. This finding supports the results of Laakso and colleagues (Laakso et al., 1990), who found that the proportion of endogenous melatonin found in saliva decreases with increasing plasma levels. The present results support the suggestion that there is a limit in the rate of diffusion of free melatonin from blood to saliva, possibly due to the saturation of melatonin binding by blood-borne proteins above physiological melatonin levels (Voultsios et al., 1997). This conclusion was drawn from measurements of saliva

melatonin concentrations with and without saliva flow stimulation. If melatonin binding is saturated at high plasma concentrations, then the precision of estimates of plasma melatonin derived from saliva is likely to decrease significantly with increasing doses of melatonin.

Finally, it may be possible to affect temperature and sleepiness differently by changing the profile of administered melatonin (eg. by bolus melatonin injection, transbuccal administration, and nasal insufflation) at a constant melatonin level. It is possible that the shape of the melatonin signal (ie. rapid or slow onset) as well as the dose, timing and duration may be important determinants of the effects of melatonin in the body (Reiter, 1987). Despite some evidence therefore that endogenous melatonin may not play a significant role in the regulation of nocturnal body temperature and sleep propensity changes, it has demonstrated utility in the treatment of jet-lag, some sleep disorders and as an entraining signal for the circadian system (Arendt et al., 1987; Armstrong, 1991; Sack et al., 1995). Future studies should aim to investigate not only the role melatonin can play both endogenously and therapeutically, but also the mechanisms by which the hormone achieves these effects.

Chapter 5

5.1 Introduction

From the early 1960's a considerable number of studies began experimentally administering melatonin to both animals (Arutyanyan et al., 1964; Barchas, 1968; Hishikawa et al., 1969; Marczynski et al., 1964; Wurtman and Axelrod, 1966) and humans (Anton-Tay et al., 1971; Cramer et al., 1974; Lerner et al., 1960; Shaw et al., 1973; Smythe and Lazarus, 1974). With regard to a possible regulatory role in thermoregulation or sleep, the majority of recent studies administering melatonin to human subjects have utilised oral preparations of 0.1 to 100 mg, given during the day or evening (Arendt et al., 1984; Cagnacci et al., 1992; Cagnacci et al., 1995; Cajochen et al., 1996; Dawson et al., 1996; Dollins et al., 1994; Hughes and Badia, 1997; Krauchi et al., 1997; Mishima et al., 1997; Nave et al., 1996; Reid et al., 1996; Zhdanova et al., 1995; Zhdanova et al., 1996). However, only four of these studies included low melatonin doses (ie. 0.1-0.3 mg) that elevated melatonin levels close to nocturnal physiological levels (Dawson et al., 1996; Dollins et al., 1994; Zhdanova et al., 1995; Zhdanova et al., 1996).

Administration of melatonin has typically been performed during the day as endogenous production is lowest during the daylight hours (Arendt et al., 1977). While oral daytime dosing has some obvious advantages in research and clinical settings, several authors have suggested that mimicking the endogenous nocturnal melatonin plasma profile may better reveal the physiological effects of melatonin (Dawson and Encel, 1993; Dijk and Cajochen, 1997; Strassman et al., 1987; Zaidan et al., 1994).

The results of the study in Chapter 4 showed that a prolonged daytime infusion of melatonin, with a slow (1-2 h) onset to peak plasma levels, had significant effects on temperature and subjective sleepiness only at supraphysiological levels. In this Chapter, the aim was to examine the effects on body temperatures and subjective sleepiness of elevating melatonin levels during the day into the nocturnal physiological range. As opposed to the steady infusion in Chapter 4, we aimed to achieve a rapid systemic onset and short latency to peak melatonin levels using an intravenous bolus injection.

5.2 Methods

5.2.1 Subjects

Eight subjects aged 20-27 years gave informed consent and volunteered to participate.

5.2.2 Experimental protocol

Subjects attended the laboratory for 4 non-consecutive bed-rest sessions between 0800-1500 h. Before 0800 h, subjects had an intravenous cannula placed by medical staff in the antecubital vein of the non-dominant forearm. Subjects were also fitted with a montage of thermistors for the measurement of body temperature on the back of each hand, the instep of both feet and 10 cm into the rectum.

Between 0800-1500 h, subjects lay quietly awake on a bed and could watch TV or read, but were not allowed to move from the supine position except for short toilet trips if required (where possible, subjects used a bed pan). Subjects were

fed a standard hospital meal between 1230-1300 h and were allowed drinking water whenever requested.

At 1000 h in each session, a 1 ml sterile intravenous injection was administered in the antecubital vein of the dominant (un-cannulated) arm. Subjects received in counterbalanced order 0.9% saline and melatonin (Sigma Aldrich Pty. Ltd., Castle Hill, New South wales, Australia) dissolved in 0.9% saline at three doses (3 μg, 10 μg and 30 μg).

The intravenous cannula was used to sample 10 ml of blood hourly from 0800-1500 h, with additional samples at 1015 and 1030 h. Blood samples were stored in heparinised tubes for up to 2 h at 4°C and then centrifuged at 1500 rpm for 10 mins. Plasma was prepared and then frozen at -20°C for later assay. Saliva samples were taken immediately after each plasma sample, using a non-treated Salivette (Sarstedt, Disposable Products, Adelaide, South Australia). The Salivettes were centrifuged at 1500 rpm for 10 mins, the upper portion discarded and the lower portion containing saliva was capped and stored at -20°C for later assay. The concentration of melatonin in 500 μl plasma and 200 μl saliva samples was determined using the Bühlmann melatonin radioimmunoassay kit (Bühlmann Laboratories AG Allschwil, Switzerland); see Chapter 2 for details.

Coinciding with each saliva collection, subjects were presented with a Linear Sleepiness Rating (LSR) sheet for measurement of introspective sleepiness.

Melatonin levels across doses and time were compared using repeated measures ANOVA. The average hand and foot temperature measures (across both limbs) were collapsed into 30 minute bins and expressed relative to the temperature at 1000 h in each condition. Subjective sleepiness scores were also

expressed relative to 1000 h. Temperature and sleepiness data between 1015 and 1500 h were then analysed using repeated measures ANOVA, with two within subjects factors (dose and time). Planned means comparisons were performed where required, determining where statistically significant differences occurred.

5.3 Results

5.3.1 Plasma Melatonin

Plasma melatonin levels analysed between 1015 and 1500 h showed a non-significant trend to decrease over time, from 16.8 ± 4.0 pM (mean ± S.E.M.) at 1015 h to 8.7 ± 4.0 pM at 1500 h. Following melatonin administration, peak plasma melatonin levels occurred at 15 mins after injection at all melatonin doses (see Figure 5.1). Peak melatonin levels reached 63.5 ± 20.1 pM, 150.0 ± 25.7 pM and 569.1 ± 110.8 pM for the 3, 10 and 30 μg doses respectively, compared to 16.8 ± 4.0 pM following saline. There was a significant effect of dose on plasma melatonin concentration ($p<0.05$).

Planned comparisons showed that plasma melatonin levels were significantly higher than in the saline condition following injection of 10 and 30 μg doses ($p<0.05$), but not after 3 μg melatonin. The 10 μg dose produced mean plasma melatonin levels across the experimental session that were significantly higher than the 3 μg dose ($p<0.05$), but significantly lower than the 30 μg melatonin dose ($p<0.05$). Plasma melatonin remained elevated above levels in the saline condition up to and including 60 and 120 mins after injection of 10 μg and 30 μg melatonin, respectively.

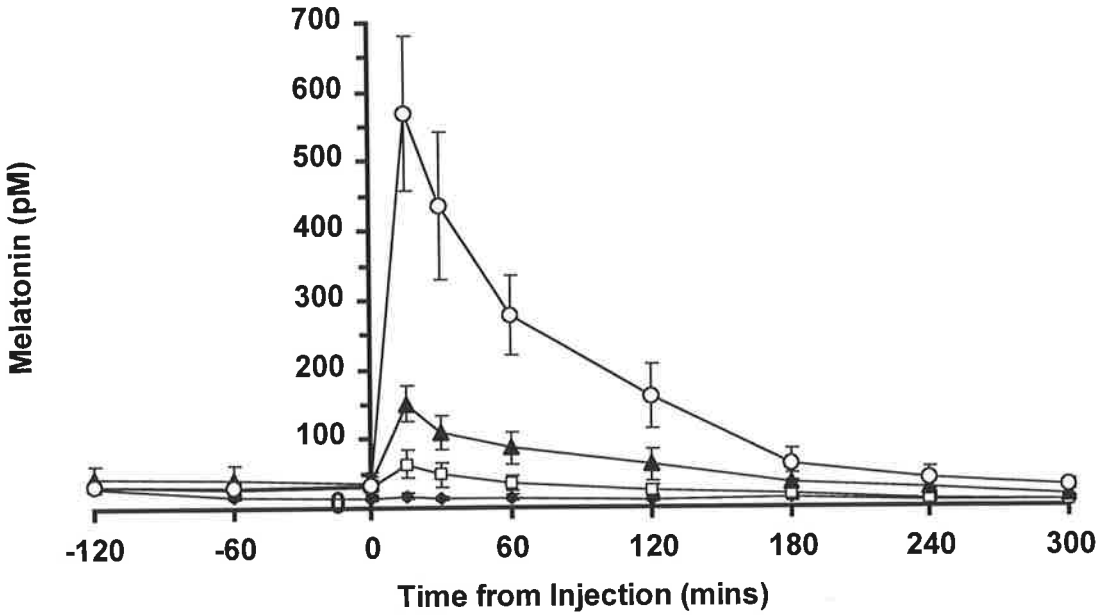

Figure 5.1

Plot of plasma melatonin levels (mean ± S.E.M.) for each dose group. Data shown on linear scale as filled diamonds (◆) for saline, open squares (□) for 3 μg melatonin, filled triangles (▲) for 10 μg melatonin and open circles (○) for 30 μg melatonin. Plasma melatonin increased significantly compared with the saline condition ($p<0.05$) following injection at 1000 h (Time=0) of 10 and 30 μg, but not 3 μg. Plasma melatonin remained significantly elevated above levels in the saline condition until 60 mins and 120 mins after injection of 10 μg and 30 μg melatonin, respectively.

Peak melatonin levels in plasma did not correlate significantly with either body weight or BMI of subjects. Regression analyses conducted on each melatonin dose separately against weight yielded (non-significant) correlation coefficients of r=0.38 (3 μg), r=0.06 (10 μg) and r=0.04 (30 μg). Regression analysis of peak plasma melatonin against BMI gave (non-significant) correlation coefficients of r=0.43, r=0.10 and r=0.18 for the 3, 10 and 30 μg melatonin doses, respectively.

5.3.2 Saliva Melatonin

Saliva melatonin levels following injection of the saline vehicle decreased non-significantly across the day, from 22.3 ± 4.3 pM at 0800 h, to 12.0 ± 4.1 pM at 1500 h. Repeated measures ANOVA revealed a significant effect of melatonin dose on saliva melatonin levels analysed between 1015 - 1500 h ($p<0.05$). Saliva melatonin levels increased significantly above those following saline injection at both the 10 μg and 30 μg melatonin doses ($p<0.05$, see Figure 5.2). Mean saliva melatonin levels in the 3 μg melatonin condition were not significantly different to those following saline injection. Peak saliva melatonin levels occurred at 15 mins after melatonin injection in each condition and were 42.2 ± 6.1 pM (3 μg), 136.1 ± 41.8 pM (10 μg) and 321.4 ± 63.6 pM (30 μg), compared to vehicle injection levels at the same time (1015 h) of 13.1 ± 2.9 pM. The 10 μg dose produced mean saliva melatonin levels between 1015-1500 h that were significantly higher than the 3 μg dose ($p<0.05$), but significantly lower than the 30 μg melatonin dose ($p<0.05$). Saliva melatonin remained significantly elevated above levels in the saline condition until 60 mins after injection at both 10 μg and 30 μg doses.

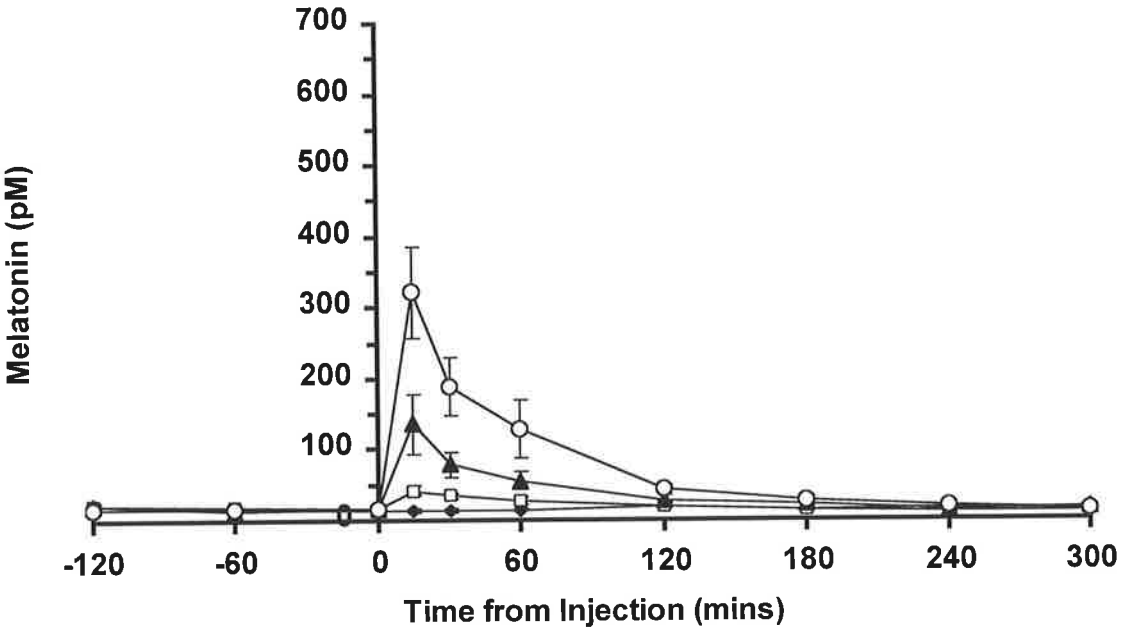

Figure 5.2

Plot of saliva melatonin levels (mean ± S.E.M.) for each dose group. Data shown on linear scale as filled diamonds (◆) for saline, open squares (□) for 3 μg melatonin, filled triangles (▲) for 10 μg melatonin and open circles (○) for 30 μg melatonin. Saliva melatonin increased significantly compared with the saline condition ($p<0.05$) following injection of 10 and 30 μg doses. Saliva melatonin remained significantly elevated above levels in the saline condition until 60 mins after injection of both 10 μg and 30 μg melatonin.

5.3.3 Rectal core temperature

Figure 5.3 shows the changes in rectal temperature following injection with melatonin or saline vehicle. Melatonin at 1000 h had a significant main effect on core temperature ($p<0.05$). Injection of both 10 μg and 30 μg melatonin significantly attenuated the normal daytime increase in rectal temperature ($p<0.05$); the 3 μg dose did not significantly change core temperature. Rectal temperature following 10 μg and 30 μg melatonin administration remained significantly lower than following saline vehicle administration for at least 300 minutes after injection ($p<0.05$). The changes in rectal temperature between 15 - 300 minutes after injection (ie. 1015-1500 h) were -0.16 ± 0.04 °C (10 μg) and -0.18 ± 0.04 °C (30 μg), relative to the saline condition. While there was no significant effect of 3μg melatonin, the relative temperature change between 15-300 minutes after injection was -0.11 ± 0.03 °C.

Regression analysis indicated that peak plasma melatonin levels across conditions were highly correlated with the mean relative change in rectal temperature ($r=0.95$, $p<0.0001$). A log regression with equation $y = 0.12 - 0.12 \log (x)$ best fit to the data (Figure 5.4).

5.3.4 Hand skin temperature

There was a significant effect of melatonin dose ($p<0.05$; Figure 5.5). ANOVA revealed that hand temperature increased significantly relative to injection of saline after administration of 3, 10 and 30 μg melatonin doses ($p<0.05$ for each dose). Planned comparisons revealed no significant differences between the mean hand temperature changes at different melatonin doses; 0.72 ± 0.12 °C (3 μg), 0.95 ± 0.15 °C (10 μg), and 0.65 ± 0.11 °C (30 μg).

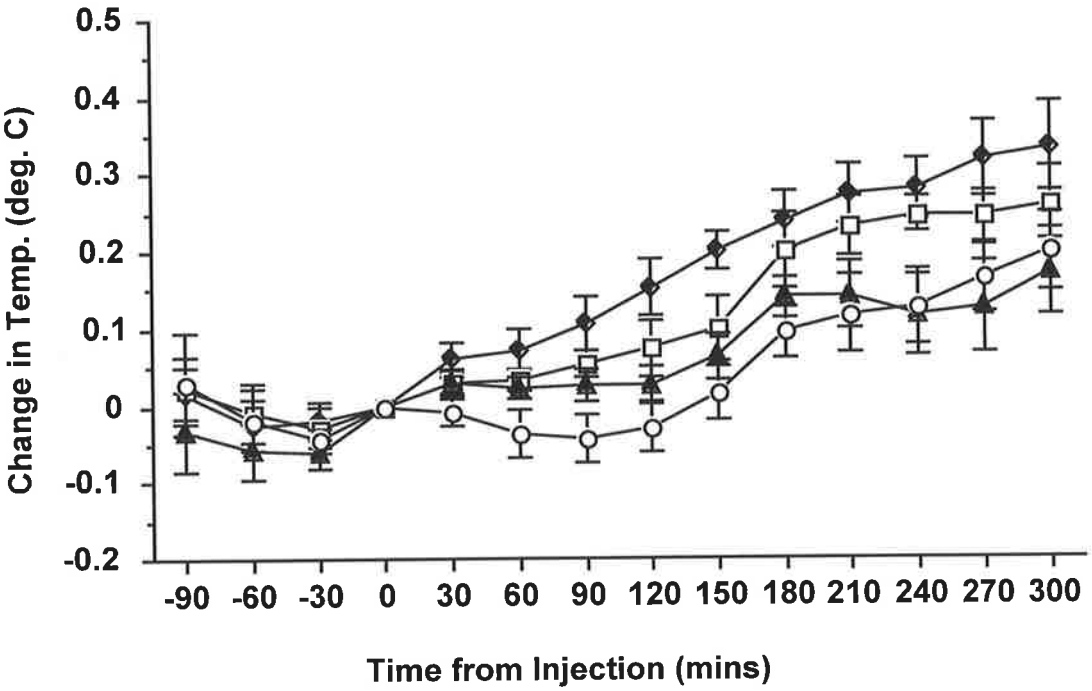

Figure 5.3

Relative rectal core temperature (mean ± S.E.M.) for all conditions. Data is expressed relative to the temperature at 1000 h in each condition and shown as filled diamonds (◆) for saline, open squares (□) for 3 μg melatonin, filled triangles (▲) for 10 μg melatonin and open circles (○) for 30 μg melatonin.

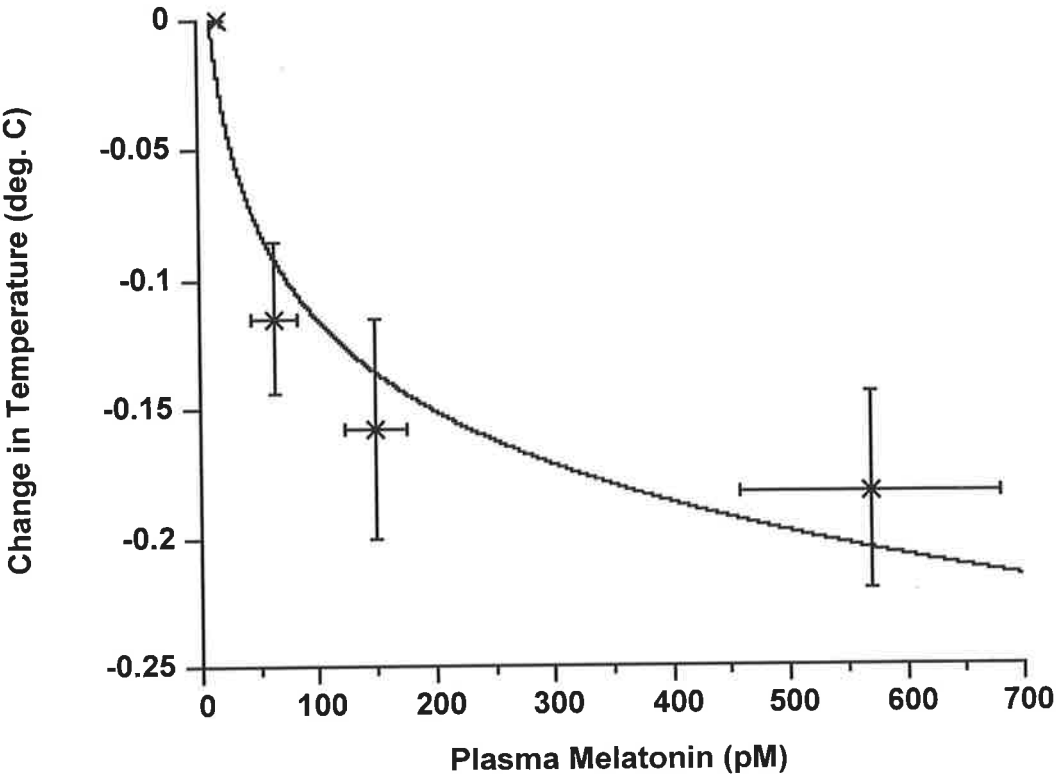

Figure 5.4

Group plot of peak plasma melatonin level in each condition (linear scale) against the mean change in rectal core temperature relative to the saline condition, over the period 1015-1500 h. Data expressed for saline and 3 melatonin dose groups as mean with S.E.M. error bars on both x- and y-axes.

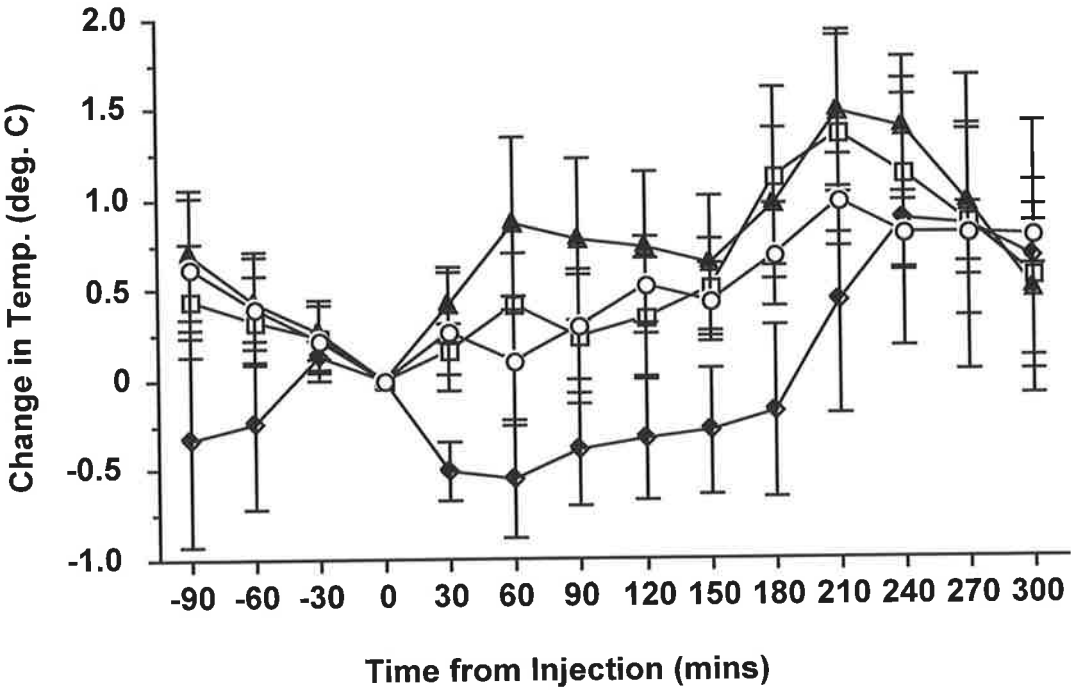

Figure 5.5

Timecourse of hand skin temperature (mean ± S.E.M.) for all conditions. Data is expressed relative to the temperature at 1000 h in each condition and shown as filled diamonds (◆) for saline, open squares (□) for 3 μg melatonin, filled triangles (▲) for 10 μg melatonin and open circles (○) for 30 μg melatonin.

5.3.5 Foot skin temperature

Foot temperature did not change significantly across time nor were measures significantly affected by melatonin injections at any dose.

5.3.6 Subjective sleepiness

There was a slight trend for subjective sleepiness to change with condition ($p=0.10$), with peak differences occurring at 15 minutes after injection of melatonin or saline. Mean relative LSR scores at 1015 h (coinciding with peak melatonin) showed a trend to increase with melatonin dose (Figure 5.6). The relative LSR scores at 1015 h in each condition were -7.1 ± 2.8 (saline), -0.3 ± 3.1 (3 μg), 5.6 ± 2.3 (10 μg) and 11.4 ± 4.2 (30 μg).

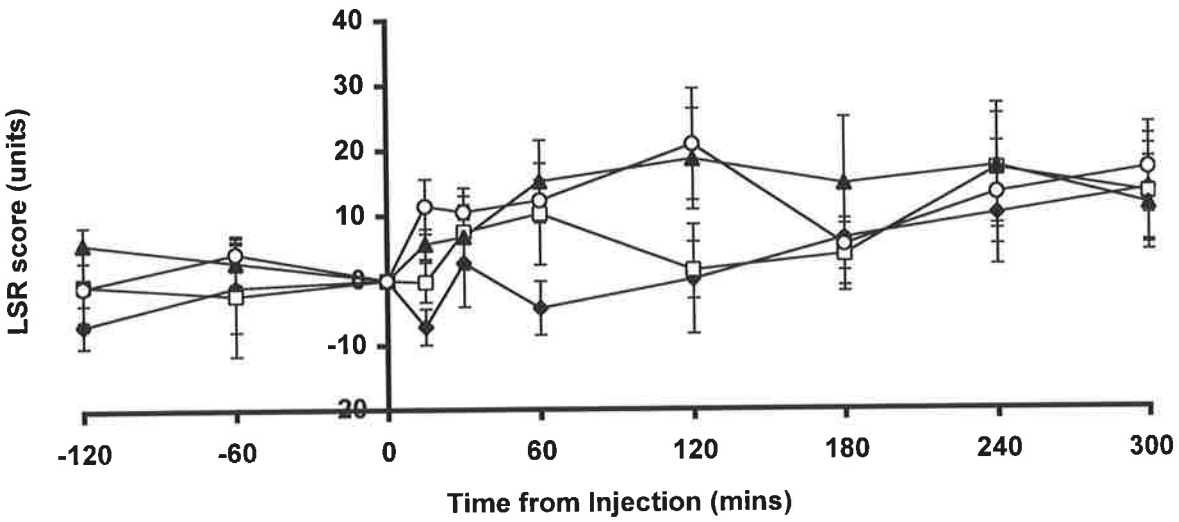

Figure 5.6

Linear sleepiness rating scores (mean ± S.E.M.) for all conditions, expressed relative to scores at 1000 h. Data are shown as filled diamonds (◆) for saline, open squares (□) for 3 μg melatonin, filled triangles (▲) for 10 μg melatonin and open circles (○) for 30 μg melatonin. There were no significant main effects or interactions however there was a trend for increased sleepiness with dose, particularly at 15 mins post-injection.

5.4 Discussion

The data show that a single daytime intravenous injection of melatonin at a dose between 10-30 μg can significantly suppress rectal temperature for at least 300 minutes and increase hand temperature for up to 180 minutes. Temperature effects persisted longer than the statistical elevation of melatonin in plasma and saliva, which lasted between 60-120 minutes (however, mean melatonin did not return to levels numerically equivalent to the saline condition until 120-180 minutes after injection). Melatonin injected at 10-30 μg produced peak melatonin levels at 15 minutes after injection, which were found to be within the nocturnal physiological range in plasma and saliva for healthy adults measured in our laboratory (Voultsios et al., 1997). It appeared that rectal and hand temperature effects approached a plateau after 10-30 μg of melatonin.

The effects of melatonin injection in the current study were similar to those in the Chapter 4 study using prolonged melatonin infusion at supraphysiological levels. The maximum change in core temperature in the present study (-0.18 ± 0.04°C), achieved by mimicking physiological levels of melatonin, is similar to that observed in Chapter 4 following High dose infusion (-0.17 ± 0.01°C). While the corresponding plasma melatonin levels which produced the rectal temperature changes were approximately 70-fold higher in Chapter 4 (34.5 ± 12.4 nM), compared with the peak after a 30 μg injection (569.1 ± 110.8 pM), it is not clear where the threshold for physiological effects occurred in the infusion study. While it appears that the rectal temperature effects of melatonin administration are maximal within the normal physiological range of endogenous melatonin production, this is yet to be conclusively demonstrated. It is also unclear whether

the threshold for significant acute effects changes depending upon the rate of onset of melatonin in the circulation. The intravenous infusion protocol allows precise control over dose delivery and therefore would be ideal in future experiments to characterise how thresholds for physiological effects of melatonin may change with dose and rate of onset. Comparing the present results with those of Chapter 4, it is apparent that the duration of elevated melatonin in the plasma has less bearing on the effect on body temperature. This is demonstrated by both short (injection) and long melatonin durations (infusion) suppressing daytime core temperature for at least 1-2 hours after plasma melatonin levels return to normal daytime values. Taken together, the results of both studies suggest however that both rate of onset and achieved level are determinants of the acute body temperature response to daytime melatonin administration.

In the present study, significant effects on self-rated sleepiness were not apparent at any melatonin dose administered. This result represents the first clear demonstration that melatonin administration can significantly affect body temperature without any change in sleep propensity measures. Previous studies reporting effects of melatonin on both sleepiness and body temperature have typically used supraphysiological oral doses (eg. Cagnacci et al., 1992; Dollins et al., 1993; Reid et al., 1996), suggesting that the soporific effects of melatonin may be apparent only at circulating levels above those normally produced during the night. For example, one study reported significantly shortened latency to sleep onset 2-4 hours after 0.3 mg oral melatonin at 2100 h, at which time physiological levels of melatonin in serum were measured (Zhdanova et al.,

1996). In this study however, it is likely that circulating melatonin levels reached supraphysiological levels shortly after administration. This group found similar results with 0.3 mg and 1.0 mg oral melatonin doses given at 6,8 and 9pm (Zhdanova et al., 1995). One limit to interpretation of the current results however, is the use of subjective sleepiness ratings. A more precise and objective measure of sleep propensity than those used in the present studies (Chapters 4 and 5) is the Multiple Sleep Latency Test with polysomnography (Carskadon and Dement, 1987). As a non-significant trend for increased sleep propensity following melatonin was observed in this Chapter, it may be the case that a rapid onset of melatonin to physiological levels following injection may produce both soporific and thermoregulatory effects commonly associated with large daytime oral doses (>1 mg).

Overall however, the current results raise some doubt as to whether soporific effects always accompany thermoregulatory effects of melatonin administration, and therefore suggest that endogenous melatonin may not play a role in mediating nocturnal increases in sleep propensity. Alternatively, it has been previously suggested (Lieberman, 1986) that melatonin may participate in the physiological regulation of sleep by determining the phase of circadian rhythms of sleep and sleepiness (Redman et al., 1983; Tzischinsky et al., 1993; Wurtman and Lieberman, 1985). By precluding a direct relationship between physiological melatonin levels and increased sleep propensity, the present results may provide support for a regulatory role of melatonin via its chronobiotic or entraining effects on the circadian system (eg. Dawson and Armstrong, 1996).

While the precise mechanism of melatonin's acute effects remains unclear, the results in this study suggest that the alteration in core temperature following daytime melatonin injection is most likely achieved by increased peripheral heat loss. According to current models of thermoregulation, temperature homeostasis is achieved by balancing heat production and heat loss. In order for melatonin to reduce core temperature therefore, either heat loss at the periphery has to increase (as suggested by an increase in peripheral skin temperature) or heat production by metabolism must decrease. Of course, both of these may occur and it is not clear from the present results what relative contribution if any, changes in heat production make to the acute response to melatonin administration. In addition, in the present study the increase in hand temperature suggests that heat loss was not equally distributed over the extremities (ie. hands versus feet). Whether or not these functional changes have any significance to the mechanism or site of effect of exogenous melatonin is unclear.

The reported ratio of saliva to plasma (S:P) melatonin is approximately 0.30 (Vakkuri et al., 1985; Voultsios et al., 1997), however this was generally lower than the range of values obtained in the present study. At the highest plasma concentrations of melatonin, the S:P ratio only reached as low as 0.5. In general however, this finding supports the results of Laakso and colleagues (1990), who found that the proportion of endogenous melatonin found in saliva decreased with increasing plasma levels. It is possible that the rapid onset and elimination of melatonin following intravenous injection results in altered binding kinetics as reflected by a higher than expected ratio of free melatonin in the saliva compared to melatonin in the plasma. On the other hand, due to a smaller difference

between peak melatonin levels compared to those in the saline condition, the relatively high background levels of saliva melatonin may artificially truncate ratios.

In conclusion, the present results suggest that raising the levels of melatonin into the nocturnal physiological range by intravenous bolus significantly suppressed the normal daytime increase in core temperature. This thermoregulatory effect appears to be maximal within a range of melatonin levels in plasma achieved during the night. A slight but non-significant trend for increased subjective sleepiness was observed at the highest bolus dose. Based on the results of the previous (infusion) study, no significant temperature or sleepiness effects were predicted for nocturnal physiological melatonin levels in this study. It therefore seems that the rate of onset of melatonin in the circulation may alter the threshold for acute physiological effects. Furthermore, one interpretation of the present results is that the typical soporific effects observed after daytime oral doses of 0.3 mg or more reflect a pharmacological side effect of the hormone, rather than mimicking the normal physiological action. It is likely however, that endogenous melatonin plays a role in entraining the timing of sleepiness and sleep episodes.

Future studies administering melatonin should aim to control the rate of increase as well as peak level and possibly duration when assessing its effects. Furthermore, it is unclear whether the soporific and thermoregulatory effects of melatonin are dissociable using objective measures, and under what conditions of dose (ie. achieved level), rate of onset and time of day.

Chapter 6

6.1 Introduction

The elderly commonly suffer from sleeping difficulties, confirmed by laboratory studies as difficulty in initiating sleep and increased fragmentation of the nocturnal sleep period (Carskadon et al., 1980; Carskadon et al., 1982; Miles and Dement, 1980; Monane, 1992; Roth, 1993; Webb and Campbell, 1980). Despite an unclear aetiology, age-related sleep disturbance has been suggested to result from decreased endogenous melatonin production and an attenuated decline in nocturnal core temperature (for reviews, see Cagnacci, 1996; Dawson and Encel, 1993). As daytime administration of ≥0.3 mg oral melatonin typically has soporific and core hypothermic effects (Cagnacci et al., 1992; Dawson et al., 1996; Dollins et al., 1994; Hughes and Badia, 1997; Reid et al., 1996; Zhdanova et al., 1995), it has often been suggested that exogenous "replacement" of attenuated endogenous production may lower nocturnal core temperature and improve sleep quality in elderly insomniacs.

Several studies have in fact reported successful improvement in the quality and duration of nocturnal sleep in older insomniacs by administration of oral melatonin in the evening at doses from 2 mg (Garfinkel et al., 1995; Haimov et al., 1995b) to 75 mg (Macfarlane et al., 1991).

However, not all evidence supports a beneficial effect of melatonin replacement on sleep architecture in insomniacs. Studies in adult chronic insomniacs reported no improvement following evening melatonin administration of 1-5 mg (Ellis et al., 1996; James et al., 1990). A similar lack of effect on sleep architecture is seen in healthy young subjects, despite some decrease in sleep

onset latency indicating an acute increase in sleep propensity (Zhdanova et al., 1996). The discrepancy may relate to the different aetiologies underlying the insomnias observed in these previous studies and whether or not a decrease in endogenous melatonin plays a role in mediating the sleep disturbance in various subject groups.

It is also possible that age-related sleep disturbance arises from an attenuated response to endogenous melatonin (Cagnacci et al., 1995), in which case melatonin administration may be unlikely to improve sleep quality. Furthermore, different results may have been obtained in the studies cited above due to differences in administered oral doses and timing of administration. In order to address some of these discrepancies, we utilised a transbuccal patch developed by 3M Pharmaceuticals to sustain exogenous nocturnal melatonin for up to 12 hours in elderly sleep maintenance insomniacs. The transbuccal patch produces a profile of melatonin that is longer in duration and with a lower peak circulating level (Bénès et al., 1997), than can be achieved with standard or even sustained-release oral preparations.

6.2 Methods

6.2.1 Subjects

Nine females aged between 56 and 73 years (mean age ± S.E.M. = 66.5 ± 1.8 years) gave informed consent and attended the Centre for Sleep Research for a total of 8 nights. All subjects had chronic sleep-maintenance insomnia (details of subjects' recruitment and screening are given in Chapter 2).

6.2.2 Experimental protocol

The study was conducted in two sessions of 4 nights each, separated by a 3 day washout period. The beginning of each 4 night period thus began on the same day of the week for each subject. At 1900 hrs a patch containing either 0.5mg melatonin or placebo (Laboratoire 3M Sante, Pithiviers Cedex, France) was placed on the gum of subjects, in a randomised, double blind, crossover design. The mucoadhesive buccal patch was circular, with a surface area of 0.5 cm^2 and a thickness of 1.4mm. The patches were backed by an impermeable membrane to prevent washout and ingestion of melatonin. The patch was made from a blend of polyisobutylene and polyacrylic acid , with 0.5 mg melatonin in the active form. In all cases, the patches remained intact overnight with no reported discomfort or local inflammation.

Between 1900 and 2100 hrs on each night, a standard montage of electrodes for polysomnographic (PSG) recording were attached to the subject's face and scalp. Each night's PSG recordings were manually scored according to standard criteria (Rechtschaffen and Kales, 1968), with modified amplitude criteria for scoring the sleep of the elderly (Webb and Dreblow, 1982). In addition, skin temperature thermistors were attached to the forehead, as well as one non-dominant hand and one foot. Rectal thermistors were self-inserted to a depth of at least 10 cm.

Subjects lay in bed awake from 2100 h and could read or watch television until they chose to turn out the lights and then go to sleep. Rectal, hand, foot and forehead temperatures were monitored continuously from the time of lights out until terminal awakening, on all four nights in both experimental sessions.

Temperature data were later averaged into 30 minute periods for analyses and were available for all subjects from 2300-0600 h. Due to practical limitations in the laboratory, temperature data could not be collected for all subjects until at least the time of retiring to bed.

Small aliquots from total urine volumes produced between 1900-0700 h were frozen and later assayed to determine concentrations of the urinary melatonin metabolite 6-sulphatoxymelatonin (aMT.6S).

All temperature data were analysed using a repeated measures analysis of variance (ANOVA), with 3 within-subjects factors (condition, night and time). Sleep and aMT.6S data were analysed using repeated measures ANOVA with 2 within-subjects factors (condition and night). Where a significant main effect or interaction was detected, planned means comparisons were used to determine where significant differences occurred.

6.3 Results

6.3.1 6-sulphatoxymelatonin levels in urine

Melatonin administration significantly increased mean aMT.6S levels from an average of 37.4 $\pm$ 5.3 nmol/night in the placebo condition to 189.2 $\pm$ 16.4 nmol/night ($p<0.0001$) following the transbuccal administration of melatonin. There were no significant night-to-night differences in aMT.6S levels within either the placebo or melatonin conditions (Figure 6.1).

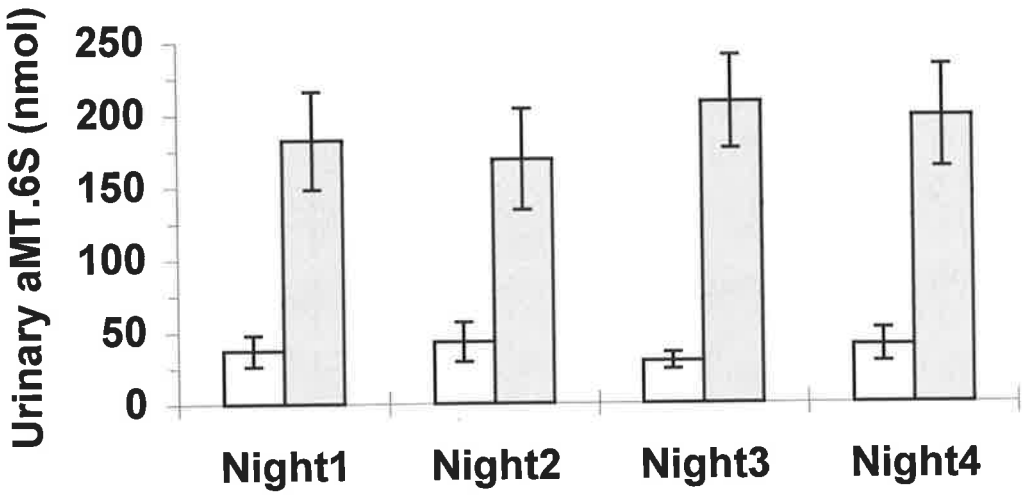

Figure 6.1

Graph of urinary 6-sulphatoxymelatonin production on each night. Data is shown as mean (± S.E.M.) for the placebo (unfilled columns) and melatonin (shaded columns) conditions. Melatonin significantly increased nocturnal aMT.6S production on each night of the study ($p<0.05$), however there were no night-to-night differences within each condition. Following melatonin treatment, aMT.6S levels were approximately 5 times the nocturnal physiological range in healthy adults.

6.3.2 Sleep Measures

Comparing sleep measures across nights revealed no significant night-to-night variability. Administration of transbuccal melatonin did not significantly alter any measured sleep variable when compared to the placebo condition. Data are summarised in Table 6.1 overleaf, but are not discussed in this thesis in detail, as part of this data is included in a Ph.D thesis in preparation (Kurt Lushington, Flinders University of South Australia).

6.3.3 Body temperatures

Figure 6.2 shows the changes in rectal core temperature across the night when averaged over the 4 nights of each condition. Melatonin administration produced a main effect on rectal temperature ($p<0.05$), which was decreased relative to mean rectal temperature in the placebo condition. Melatonin significantly decreased mean rectal temperature to 36.45 ± 0.03 °C from 36.55 ± 0.03 °C in the placebo condition. There were no significant interactions between condition and either treatment night or time of night.

Hand skin temperature also showed a main effect of condition ($p<0.05$). Hand temperature across time is shown in Figure 6.3 as an average of 4 nights for each condition. Melatonin significantly decreased hand temperature from an average of 34.58 ± 0.10°C during placebo treatment to 34.19 ± 0.10 °C. Hand temperature showed no significant interactions between condition, treatment night and time of night.

There were no significant main effects of condition, night or time in either foot or forehead temperature data.

Table 6.1 Summary of polysomnographic sleep data; mean (S.E.M.)

Variable, abbreviation (units)	Placebo	Melatonin	Significance between Conditions
Total Time in Bed, TIB (mins)	510.7 (5.1)	507.5 (6.3)	NS
Total Sleep Time, TST (mins)	360.6 (9.6)	353.9 (10.7)	NS
Sleep Period Time, SPT (mins)	464.5 (8.2)	455.5 (10.0)	NS
Sleep Efficiency, TST/TIB (%)	70.6 (1.8)	69.7 (1.8)	NS
Sleep Efficiency, TST/SPT (%)	77.6 (1.6)	77.7 (1.6)	NS
Sleep Onset Latency, SOL (mins)	19.8 (3.5)	18.2 (2.9)	NS
REM Onset Latency, ROL (mins)	74.6 (6.6)	78.4 (8.3)	NS
Time awake (% of SPT)	24.9 (1.7)	25.4 (1.7)	NS
Time in Stage 1 (% of SPT)	4.9 (0.5)	4.5 (0.4)	NS
Time in Stage 2 (% of SPT)	32.3 (1.9)	33.3 (1.6)	NS
Time in Stage 3 (% of SPT)	12.9 (0.9)	12.5 (0.8)	NS
Time in Stage 4 (% of SPT)	5.9 (0.9)	4.5 (0.9)	NS
Time in REM (% of SPT)	21.6 (0.9)	21.7 (1.0)	NS
Time in NREM (% of SPT)	57.8 (1.2)	58.1 (1.4)	NS
Time in Slow Wave Sleep (% of SPT)	18.7 (1.6)	17.7 (1.4)	NS
Sleep Stage Changes	127.2 (7.6)	117.3 (6.1)	NS

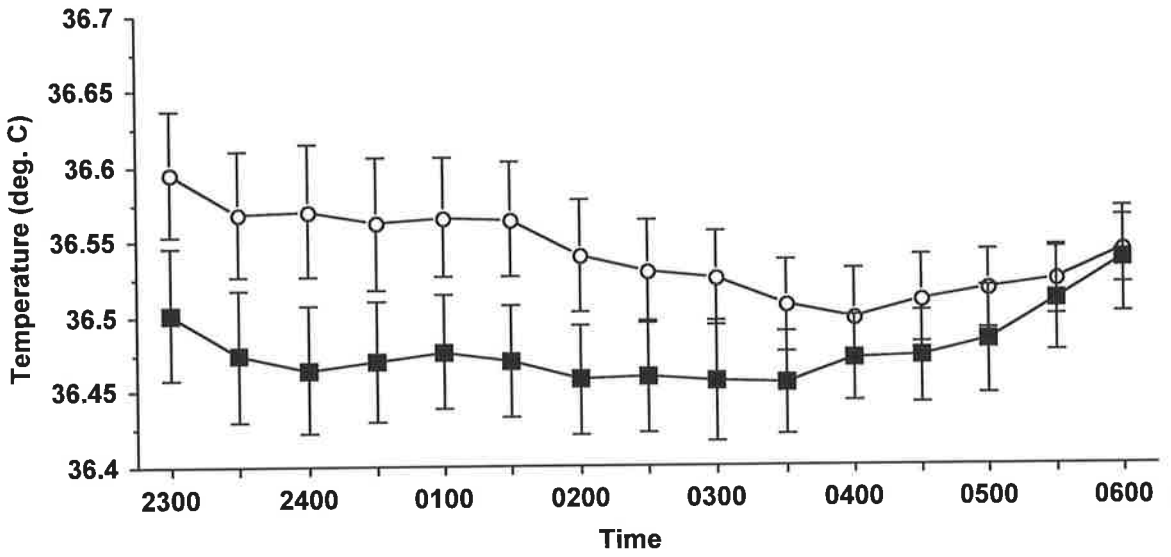

Figure 6.2

Rectal core temperature averaged over four nights for placebo and melatonin conditions (error bars represent 1 S.E.M.). Data shown as open circles (O) for placebo and filled squares (■) for the melatonin condition. Melatonin significantly decreased mean core temperature relative to the placebo condition ($p<0.05$). There were no significant night-to-night differences in core temperature within conditions.

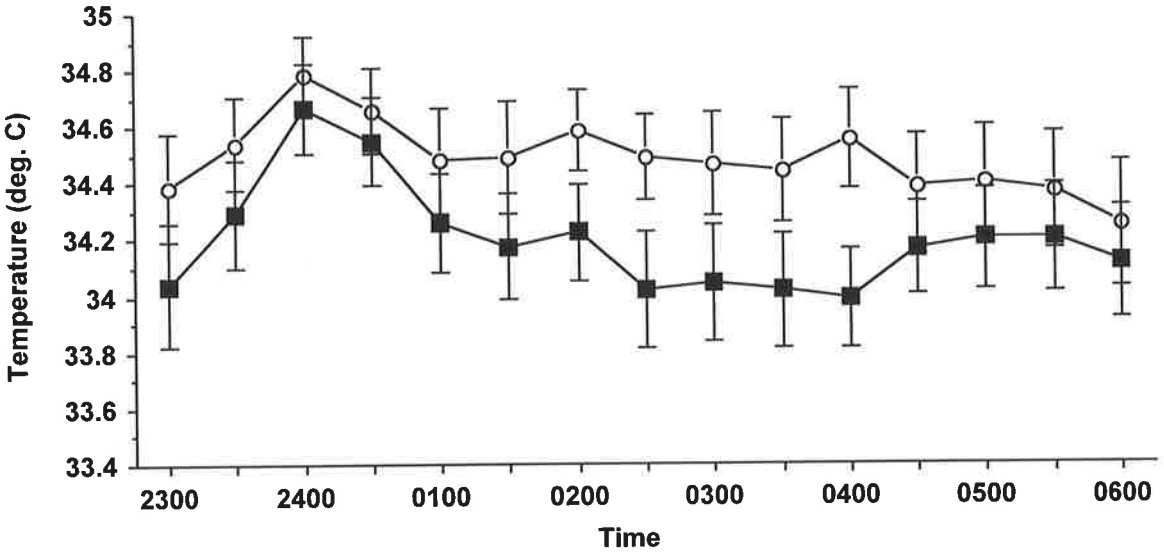

Figure 6.3

Hand skin temperature in each condition averaged over four nights. Data shown as open circles (O) for placebo and filled squares (■) for the melatonin condition. Mean (± S.E.M.) hand temperature in the melatonin condition was significantly lower than in the placebo condition ($p<0.05$). There were no significant night-to-night differences in hand temperature within conditions.

6.4 Discussion

In the present study, subjects were found to have melatonin metabolite levels within the nocturnal range for adults in our laboratory, at 37.4 ± 2.9 nmol/night. The 0.5 mg melatonin patch significantly increased nocturnal aMT.6S levels in the urine by approximately 5-fold, to 189.2 ± 8.7 nmol/night. The 3M transbuccal melatonin patch did not produce as much variability in achieved melatonin metabolite levels as has been observed with plasma melatonin following transdermal patch application (Haak and Gupta, 1992; cited in Dawson and Armstrong, 1996). The active treatment induced small but significant decreases in both rectal and peripheral hand temperatures averaged across 4 nights of study. It appears that changes in body temperature during melatonin administration did not result from alterations in any measured sleep variable. These measures included the timing and duration of sleep episodes, the duration of each sleep stage and wakefulness and the number of arousals and stage changes within the sleep period.

Previous studies have reported concomitant hypnotic and hypothermic effects of daytime or evening melatonin at supraphysiological levels but not physiological levels (Zhdanova et al., 1995; Dollins et al., 1994). However, the dose of melatonin administered (and hence the plasma levels achieved) as well as the time of day may determine the magnitude of acute effects. It is possible therefore, that the 0.5 mg transbuccal melatonin administered in the present study was insufficient to produce soporific effects and that these effects may be side effects of large supraphysiological levels of the hormone.

In Chapter 4, it was shown that a 6.5 h daytime infusion of melatonin, at supraphysiological but not physiological levels, significantly lowered rectal temperature and increased subjective sleepiness in healthy young adults. The present study partly supports the previous results, as supraphysiological levels of melatonin (inferred from metabolite levels in the urine) decreased both core and hand temperature. The transbuccal melatonin patch has a near square pulse delivery (Bénès et al., 1997), resulting in a plasma profile similar to that observed with the constant rate infusion in Chapter 4. Therefore, it is not clear from the present results whether the slow melatonin onset following transbuccal melatonin administration altered the threshold level for thermoregulatory and soporific effects of melatonin. In addition, it is possible that both the greater age of the subjects and nocturnal administration can alter the melatonin threshold. For example, the elderly may be more sensitive than young adults (and therefore show significant thermoregulatory effects closer to physiological levels of the hormone than younger adults), however this seems unlikely according to a few recent studies. The results of these studies suggest that the elderly may be less sensitive than young adults to exogenous oral melatonin, at both moderate (5 mg) supraphysiological doses (Lushington et al., 1998) and extreme (100 mg) doses (Cagnacci et al., 1995).

The present study therefore shows that sustained elevation of nocturnal melatonin to a level that produces about 5 times the normal excretion of metabolite, has no significant effects on sleep architecture in elderly sleep maintenance insomniacs. This suggests that the elderly insomniacs in the present study were either "melatonin resistant" or as suggested above, the

aetiology of their sleep disturbance may be unrelated to any changes in melatonin production. It is also a possibility that both may coexist in some individuals or other undefined aetiologies may exist. If melatonin resistance does underlie some age-related sleep disturbances, then new and novel treatments will be required in order to alleviate the insomnia in these individuals.

It would appear from the current results that age-related changes in the core temperature rhythm and sleep are not causally related, given the differential effect of supraphysiological melatonin levels on these measures. It follows that if melatonin has a direct physiological role in initiating and/or maintaining nocturnal sleep, it is probably not diminished by the modest decrease in endogenous production exhibited between young and elderly adults (Waldhauser and Steger, 1986).

The present results also partly supports the suggestion raised in Chapter 5, that the acute thermoregulatory effects of melatonin may be dissociable from its soporific effects. In the present circumstances, elevating melatonin levels across the night into the high physiological to supraphysiological range altered body temperatures but produced no effects on objectively measured sleep propensity or architecture. However, it has been suggested that a chronic melatonin-dosing regime (ie. >7 days) may be required to elicit significant sleep effects. For example, the studies reporting subjective improvements in elderly sleep (Garfinkel et al., 1995; Haimov et al., 1995b), have typically used dosing durations of 7 days or more. If these self-assessed improvements are reflected by physiological improvements in sleep quality, it is possible that the chronobiotic effects of melatonin (Dawson and Armstrong, 1996) will cause a cumulative and

beneficial phase-shift in the circadian pacemaker. According to the phase response curves for melatonin (Lewy et al., 1992; Lewy et al., 1995; Zaidan et al., 1994), several consecutive days of timed melatonin administration would be required to shift outputs of the circadian pacemaker (eg. body temperature, sleep) by even 3-4 hours. This is supported experimentally by the results of several studies in which the soporific effects of melatonin are observed only after several consecutive days of administration (Arendt et al., 1984; Lewy et al., 1992; MacFarlane et al., 1991).

In the present study, 4 consecutive nights of melatonin administration may not have produced a phase-shift of sufficient magnitude to affect sleep in our subjects, although this was not specifically tested. As melatonin was applied at 1900 h (typically before the endogenous nocturnal rise in melatonin), it is possible that core temperature began to decline earlier than during the placebo treatment due to endogenous production. This may have masked the underlying core temperature rhythm and counteracted any beneficial effect of melatonin on sleep, particularly in the second half of the night in this study. However, this assumes that endogenous melatonin exerts a regulatory role on sleep by thermoregulatory changes, which is not supported by the current results and those reported in Chapter 4.

Another interesting finding of the present study is that *both* core and peripheral skin (hand) temperature decreased with nocturnal melatonin administration. This suggests that nocturnal melatonin may be lowering core temperature by a decrease in heat production, unlike daytime melatonin in young adults which appears to increase heat loss at the periphery (see Chapter 3 discussion).

However, the time of day effects of melatonin on heat loss and heat production have not yet been investigated within specific age groups (eg. young adults). Therefore, it is not yet clear whether there is an age-related change in the effects of melatonin on thermoregulatory balance.

The present study failed to find any significant effect of melatonin on sleep architecture, which is generally supported by the results of a previous study by James et al. (1990). This paper reported an increased latency until the appearance of REM sleep after a 1mg, but not 5 mg oral melatonin dose in adult insomniacs. Therefore, it appears that the older subjects in our study may be no less sensitive to administered melatonin regarding nocturnal sleep effects, than younger adults. However, before concluding that exogenous melatonin has no significant effects on sleep architecture in elderly insomniacs, future studies should control for other factors that may contribute to a lack of repeatability between these studies. The most important of these factors appear to be age, dose, the precise experimental protocol and which sleep measures are recorded. In particular, the plasma levels at which the hypothermic and hypnotic effects emerge would appear to be an extremely critical issue. It may also be desirable to replicate the present study using a range of melatonin doses and/or longer administration periods.

Chapter 7

7.1 Summary

In the first study (Chapter 3), the concomitant effects on temperature and sleep propensity around the onset time of endogenous nocturnal melatonin production were investigated. The following three studies in this thesis investigated the effects on body temperatures and either subjective sleepiness (Chapters 4-5) or objectively-measured sleep architecture (Chapter 6), under conditions designed to mimic certain features of endogenous melatonin production. This was achieved using three different administration protocols in either young or poor sleeping elderly subjects. The general aim of these studies was to examine the relationships between melatonin, body temperature and sleepiness.

7.1.1 Chapter 3: Thermoregulation & sleepiness around melatonin onset

This study investigated the temporal relationship between changes in peripheral and core temperatures, nocturnal melatonin onset and habitual sleep onset. Interestingly, habitual sleep onset occurred before dim light melatonin onset in most subjects. However, increases in peripheral skin temperature and decreases in rectal core temperature significantly preceded the onset of sleep, by 50 and 20 minutes respectively. The first apparent change however occurred at an average of 150 minutes before sleep onset; a significant increase in self-reported sleepiness. Furthermore, the changes in body temperature and sleepiness before habitual sleep onset were significantly attenuated when subjects' sleep was delayed by almost 2 hours. This suggests that changes in thermoregulation and sleep propensity are part of the process of sleep initiation

and may have other influences apart from circadian changes (eg. melatonin onset) and sleep-evoked effects.

7.1.2 Chapter 4: Melatonin infusion, body temperatures and sleepiness

Melatonin infused at 0.04-0.08 μg/hr/kg raised the daytime plasma and saliva melatonin levels into the normal physiological range observed in young adults at night. Despite achieving physiological melatonin levels however, these dose rates did not significantly affect either core and peripheral temperatures or subjective sleepiness.

Prolonged melatonin infusion at a supraphysiological dose rate (ie. 8.0 μg/hr/kg) significantly suppressed the normal increase in daytime rectal temperature within 30 mins of the infusion onset. At this dose rate, a mean difference in rectal temperature of -0.17 ± 0.01 °C relative to the saline infusion control was maintained until at least 2.5 hours after the infusion, when the study ceased. Peripheral skin temperature (hand) increased for 2 hours after the start of the High dose melatonin infusion, by an average of 0.64 ± 0.29 °C above temperature in the saline condition. After the first two hours however, there were no discernible differences between conditions. Also associated with High dose melatonin for the first 2 hours of the infusion period was a significant increase in subjects' self-reported sleepiness, to a level equivalent to that observed in subjects in Chapter 3 between 2100-2130 h (before sleep onset).

7.1.3 Chapter 5: Melatonin injection, body temperatures and sleepiness

To investigate whether the rate of onset of melatonin could alter the thermoregulatory and soporific effects of melatonin, the study in Chapter 5 was

conducted using bolus intravenous melatonin injection. In Chapter 4, melatonin levels in saliva took between 60-90 minutes to reach a stable peak after commencement of a daytime infusion. The bolus melatonin doses used in the Chapter 5 study were designed to produce peak levels similar to that observed endogenously, but achieved much more rapidly than with the infusion.

A single intravenous injection of melatonin at doses of 10 and 30 μg significantly suppressed daytime rectal temperature for at least 300 minutes. Both these doses increased plasma and saliva melatonin levels to within the nocturnal physiological range in adults. Melatonin injection decreased rectal temperature by a maximum of -0.16 ± 0.04 °C (10 μg) and -0.18 ± 0.04 °C (30 μg), relative to temperature in the saline condition. Injections of 3, 10 and 30 μg all significantly increased hand temperature compared to saline for 180 minutes. The average increases in hand temperature over this period were 0.72 ± 0.12 °C (3μg), 0.95 ± 0.15 °C (10 μg), and 0.65 ± 0.11 °C (30 μg). These hand and core temperature effects persisted longer than the elevation of melatonin in plasma and saliva, which were significantly higher than saline condition levels for 60-120 minutes. Peak plasma and saliva melatonin levels typically occurred at the first sample (15 minutes after injection). In addition, it appeared from logarithmic regression analyses that mean changes in rectal and hand temperature were close to maximal following injection of melatonin doses between 10-30 μg.

7.1.4 Chapter 6: Transbuccal melatonin, body temperatures and sleep

The therapeutic potential of melatonin in elderly poor sleepers was investigated using an extended duration pulse of nocturnal melatonin. Due to the results in the infusion study in Chapter 4, a dose was selected that would elevate

melatonin levels above the normal physiological range, in order to improve the chance that any threshold for thermoregulatory or soporific effects was reached.

A 0.5 mg sustained release transbuccal melatonin patch elevated melatonin metabolite levels to approximately 5 times the nocturnal range for young adults. Despite small but significant decreases in both rectal core and peripheral hand temperature, four nights of melatonin administration did not significantly alter sleep architecture, latencies to sleep stages or any other standard sleep measure.

7.2 Conclusions

Melatonin administration has many reported actions, including synchronising biological rhythms in animals (Armstrong, 1989; Cassone et al., 1986; Yates and Herbert, 1976) and humans with disturbed circadian rhythmicity (Alvarez et al., 1992; Arendt et al., 1987; Folkard et al., 1990; Petrie et al., 1989; Sack et al., 1991). The strict covariance of circadian rhythms of melatonin production, sleep/wake and core temperature has lead to speculation that melatonin may regulate sleep propensity cycles by thermoregulatory effects (Cagnacci, 1996; Dawson and Encel, 1993; Strassman et al., 1991). This hypothesis is indirectly supported by daytime oral melatonin administration studies, which typically increase sleepiness (Arendt et al., 1984; Lerner and Nordlund, 1978; Lieberman et al., 1984) and suppress core temperature (Cagnacci et al., 1992; Dawson et al., 1996; Dollins et al., 1994; Hughes and Badia, 1997; Reid et al., 1996). In addition, nocturnal melatonin suppression by bright light or ß-adrenergic antagonists results in increased core temperature and decreased sleep propensity (Strassman et al., 1991; van den Heuvel et al., 1997). The

associations between altered thermoregulatory and soporific effects following exogenous melatonin administration or endogenous melatonin suppression however do not provide causal evidence of a link between them.

To better understand how melatonin may be beneficial in sleep or circadian rhythm disturbances requires assessment of the physiological role of the hormone. In past human administration studies, melatonin has mostly been given in large oral doses that do not produce either the peak level or duration of endogenous nocturnal melatonin production. However, several authors have suggested that conclusions drawn from exogenous melatonin administration studies may be strengthened if the profile of nocturnal melatonin production were better reproduced (Dawson and Encel, 1993; Strassman et al., 1991; Zaidan et al., 1994). In this thesis, melatonin was administered during the day in several administration protocols that allowed us to vary the peak circulating level, the duration of effect and the rate of melatonin onset. Nevertheless, it was apparent that the relationship between melatonin, body temperatures and sleep propensity is not a simple one. When daytime melatonin levels are elevated into the nocturnal physiological range in young adults, the appearance of body temperature and sleepiness changes are dependent upon how quickly melatonin levels are elevated. Furthermore, the present studies provide some support that the acute thermoregulatory effects of melatonin may appear at lower levels than soporific effects and that the latter may be unrelated to melatonin's endogenous role.

Following is a discussion of the most important issues raised by the experiments in this thesis. Firstly, the rate of onset of melatonin in the circulation appears to

influence whether thermoregulatory effects of melatonin are observed at physiological levels. This may represent a mechanism whereby the melatonin "threshold" level for acute effects can be regulated. Next, the thermoregulatory and soporific effects of daytime melatonin administration appear to occur at different plasma levels and may therefore be separable; suggesting that endogenous melatonin may not mediate sleep propensity via thermoregulatory changes. Whether the time of day of melatonin administration influences the acute changes observed is also discussed, with particular reference to the results in Chapter 6. Finally, some possible mechanisms by which melatonin may elicit thermoregulatory and soporific effects are briefly considered.

7.2.1 Rate of melatonin onset: A physiological mediator?

In a recent review, Lavie contends that of the variables introduced by previous melatonin administration studies, the time of melatonin administration appears to be most crucial in determining the soporific effects of the hormone (Lavie, 1997). However, the first two studies in this thesis clearly demonstrate that whether melatonin onset occurs quickly (injection) or slowly (infusion), to physiological peak levels, can also determine whether significant thermoregulatory effects are observed. In the absence of a defined mechanism linking melatonin with changes in body temperatures and sleep propensity, it is interesting to speculate how changes in rate of onset may alter the threshold for effects. There is currently no evidence for a change in the rate of endogenous melatonin onset between circadian or even seasonal cycles within individuals. In fact, it appears that the circadian rhythms in plasma melatonin (Coetzee et al., 1989; Voultsios et al., 1997) and urinary 6-sulphatoxymelatonin (Lushington et al., 1996) are

remarkably repeatable within individuals over several cycles. Therefore, it may be unlikely that changes in rate of melatonin onset represents a physiological mechanism by which the circadian system can regulate effects of melatonin. However, this point of view assumes that the melatonin target responsible for mediating the acute effects of exogenous melatonin, is within the same compartment (eg. circulation), that we are sampling melatonin from in the present studies. In addition, a high sampling rate in this same target tissue may very well mediate changes in the threshold for acute melatonin effects. This contention will however remain speculative until more is understood about how melatonin mediates changes in thermoregulation and sleepiness. For example, it has been suggested that different species may respond to different aspects of the "melatonin message", such as the duration of endogenous production or the coincidence of increased sensitivity of responsive tissues when melatonin is produced (Dawson and van den Heuvel, 1998; Reiter, 1987). In this context, it is possible that rate of endogenous melatonin onset may mediate some physiological responses.

In the light of the present results, it is possible that the soporific effects of melatonin administration are pharmacological side effects and not a physiological function of melatonin (cf. Dawson and Encel, 1993). The rapid onset to peak levels observed following bolus injection (ie. ≤15 mins), also represents a non-physiological circumstance. This is demonstrated by previous studies which show that peak melatonin levels in either plasma or saliva, may not occur for 4-6 hours after melatonin onset (eg. Bojkowski et al., 1987; Coetzee et al., 1989; Voultsios et al., 1997). However, as it is not definitely known whether melatonin

is excreted into the blood directly or via the CSF, it is difficult to speculate as to the levels and rate of increase of endogenous melatonin at the site where it exerts its acute effects.

The situation is further confused by the rate of increase in plasma following oral melatonin, which varies widely due to first pass metabolism (Aldhous et al., 1985; Lane and Moss, 1985) and therefore may exert widely varying effects between individuals. Whether this can result from the variability in achieved melatonin level or a possible broad range in rate of onset at the physiological site of action is not yet clear. Together with the present results that suggest the rate of onset is a critical factor in determining the effects of melatonin, it would therefore appear that oral melatonin administration might be a poor strategy for investigating the physiological effects of the hormone. This may be a critical problem with much of the research to date that has failed to control for differences in rate of onset of melatonin in the circulation (or other relevant compartment) following administration.

Nevertheless, as little is known about the toxicology and long-term safety of melatonin administration, using a rapid onset technique (eg. bolus injection of lower absolute amounts of melatonin) to lower the threshold for physiological effects may prove beneficial in future research and even clinical settings. A less invasive administration for melatonin with a rapid onset and low inter-individual variability may be the nasal insufflation method reported by Vollrath and colleagues (1981).

7.2.2 The melatonin threshold

In previous studies administering ≥ 1 mg oral melatonin during the day and recording core temperature, a significant attenuation of the normal increase in core temperature is typically observed (Cagnacci et al., 1992; Dollins et al., 1994; Reid et al., 1996). A few studies have reported no significant effect on core temperature with oral doses below 0.5 mg (Dawson et al., 1996; Dollins et al., 1994). It appears therefore that doses of at least 1-5 mg are required to produce consistent core temperature effects.

The lack of consistency may be due to a lack of systematic attempts in previous studies to control for subjects' body weight that could contribute to the variability in plasma levels following oral melatonin. However, this is not likely to be a major concern as the variability in melatonin levels in Chapter 4 was generally not less than that in Chapter 5, where subjects' doses were not administered according to body weight. Differences in melatonin pharmacokinetics between individuals would therefore seem to introduce more variability than body weight to achieved melatonin levels at a given dose.

The plasma levels of melatonin at oral doses greater than 0.1-0.3 mg elevate peak melatonin into the supraphysiological range (eg. Dawson et al., 1996). Therefore, any effects observed at such doses are likely to be pharmacological side effects or true physiological effects that plateau above physiological levels.

While it would appear therefore that thermoregulatory effects are elicited by and are probably maximal at physiological melatonin levels in Chapter 5, the results of the infusion study (Chapter 4) suggest that the threshold for melatonin effects on core temperature decreases with increasing rate of onset. Following

pharmacological melatonin infusion between 1000-1630 h, a plasma melatonin level greater than 232 ± 38 pM and equal to or less than 34.5 ± 12.4 nM was required to suppress daytime core temperature by an average of 0.17 ± 0.01 °C. Following intravenous injection at 1000 h, daytime core temperature was suppressed by 0.16 ± 0.04 °C at plasma melatonin levels of 150.0 ± 25.7 pM and by 0.18 ± 0.04 °C at 569.± 110.8 pM. It appears in these studies conducted under conditions of constant ambient temperature, lighting, posture and activity, that a maximum change in core temperature of approximately 0.20 °C can be achieved with even large pharmacological doses. While the threshold for significant thermoregulatory effects may be lower, detecting this limit depends also on the statistical power of the study in question. It is apparent that many factors, especially rate of onset, may alter the threshold for physiological effects. Given that the current studies were conducted during the day, it is possible that the threshold will change across the day also (see section 7.6.4, below). In the absence of melatonin antagonists for human research use, this could be tested by progressive suppression of nocturnal melatonin production, by bright light or adrenergic antagonists.

7.2.3 Are the effects of melatonin on temperature and sleepiness dissociable?

The studies in Chapters 4 and 5 support the contention that the thermoregulatory and soporific effects of melatonin administration may be dissociable, with increased sleepiness only observed at supraphysiological levels of melatonin. In previous studies, melatonin has demonstrated soporific effects only at oral doses of 0.1-0.3 mg or more (Dollins et al., 1994; Hughes and Badia, 1997; Reid et al., 1996; Zhdanova et al., 1995; Zhdanova et al., 1996). However, these effects are

generally subtle, typically including a shorter latency to sleep onset but not changes to subsequent architecture of sleep bouts. In addition, most studies have given doses that elevate peak melatonin levels above the nocturnal physiological range, such that both thermoregulatory and soporific effects are observed.

Using a self-rated sleepiness measure, the study in Chapter 5 found a slight, short-lived trend but no significant change in sleepiness across the physiological range of melatonin levels despite suppression of core temperature. However in Chapter 4, supraphysiological melatonin levels were associated with both increased sleepiness and suppressed core temperature. Therefore, temperature effects may plateau at lower plasma melatonin levels than soporific effects. While the power of these studies may have been relatively low, the potential separation of the acute effects of melatonin suggests that direct regulation of sleep propensity may not be a physiological role of endogenous melatonin production. At the very least, it appears that daytime melatonin levels elevated to within the nocturnal range has stronger effects on thermoregulation than on subjective sleepiness. The administration of melatonin to elderly insomniacs at night in Chapter 6 supports the hypothesis that the thermoregulatory effects and soporific effects of melatonin are separable, as significant effects on body temperature were achieved by sustained near-supraphysiological melatonin levels in the absence of any effects on nocturnal sleep architecture. It was not clear in this study however, what the exact rate of onset of melatonin in the circulation was using transbuccal administration. Furthermore, in young healthy adults no significant change in sleep propensity was observed near melatonin

onset measured in saliva (Chapter 3). In this study, greater changes in body temperatures and self-reported sleepiness were exerted by both "awareness" of one's approaching sleep time before sleep onset and evoked effects following sleep onset.

Nevertheless, the available evidence suggests that nocturnal melatonin suppression by bright light exposure (Strassman et al., 1991) and β-blocker administration (van den Heuvel et al., 1997) is associated with concomitant changes in sleep propensity and core temperature. Taken together, these and the current results support the suggestion that the relationship between melatonin, body temperatures and sleep propensity is not a simple one.

7.2.4 Time of day differences in melatonin response

It is possible that the time of melatonin administration will alter the effects of the hormone (reviewed in Lavie, 1997). From the literature, it is clear that nocturnal melatonin administration protocols require pharmacological doses to observe significant sleep effects. For example, Cramer decreased sleep latency in subjects following nighttime injection of 50 mg melatonin (Cramer et al., 1974). Similar results have been obtained by other researchers with oral doses of 75-80 mg (MacFarlane et al., 1991; Waldhauser et al., 1990). Lower but still supraphysiological oral doses (1-5 mg) at night typically do not affect sleep propensity or architecture in normal adults (James et al., 1987) or insomniac subjects (Ellis et al., 1996; James et al., 1990). During the evening, oral melatonin doses as low as 0.1-0.3 mg have been shown to significantly decrease latency to sleep when given at 1800, 2000 or 2100 h (Zhdanova et al., 1995). During the day, oral doses of 3 mg (Nave et al., 1996) to 5 mg (Reid et al., 1996)

are typically required in order to observe significant effects on sleep propensity or architecture. In these previous studies, melatonin was administered at 1200 h (Nave et al., 1996) and 1400 h (Reid et al., 1996) and increased sleep propensity was observed using polysomnographic measures.

The study that perhaps gives the best indication of time of day differences in response to melatonin administration, was that conducted by Tzischinsky and Lavie (1994). A 5 mg oral melatonin dose was administered at 1200, 1700, 1900 and 2100 hours to 18 young adults in a Latin Square protocol. Soporific effects were studied with the 7/13 ultrashort sleep-wake paradigm, which gives a 7 minute nap opportunity followed by 13 minutes of wakefulness. This 20 minute cycle is repeated for the study duration. Melatonin in this study significantly increased both objective and subjective measures of sleep propensity and decreased oral temperature. While the maximum effects at each time point were similar, the latency to maximum effect decreased linearly across the day from 220 minutes at 1200 hours to 60 minutes at 2100 hours. The authors interpreted these results as an indication that melatonin possesses a "time-dependent hypnotic effect" and that endogenous melatonin may therefore participate in sleep-wake regulation. The results of this study are therefore consistent with the hypothesis that sensitivity to melatonin may increase as the time of endogenous production approaches.

At this point, it is important to note that very few studies have administered melatonin at a time that has been verified as being after endogenous melatonin onset in the same subjects. This is important, as melatonin administration before endogenous onset may have different effects or none at all, compared with

administration after onset. Nevertheless, this possibility has not yet been systematically investigated. In the context of previous research then, it is not surprising that significant effects on sleep architecture were not observed in elderly sleep maintenance insomniacs after transbuccal melatonin (Chapter 6), particularly as maximum effects may have occurred before the sleep period began. However, as 0.5 mg transbuccal melatonin did elevate the melatonin metabolite aMT.6s to supraphysiological levels, a significant effect on core temperature might have been expected, if the thermoregulatory effects of melatonin were not saturated at physiological levels.

7.2.5 Some possible mechanisms for melatonin's acute effects

The current results do not provide direct evidence of the mechanism(s) by which melatonin exerts acute thermoregulatory and soporific effects. Based on the novel findings in this thesis however, some explanations for melatonin's effects may be more likely. Perhaps the most important of these explanations are whether acute effects of melatonin are mediated by the central nervous system or in the periphery, if melatonin mimics the actions of other endogenous compounds and whether the acute effects are manifestations of longer-term circadian effects.

7.2.5.1 Thermoregulation: Central or peripheral effects?

The relatively immediate drop in core temperature typically observed following ingestion of supraphysiological melatonin doses could potentially be achieved in several ways. Melatonin could act centrally to reset at a lower level the hypothalamic set-point temperature or it may be acting on peripheral vasculature to increase heat loss, leading to decreased core temperature. The evidence

from the study in Chapter 6 would suggest that, at least at night in a select group of elderly insomniacs, melatonin does not alter core temperature by acting in the periphery. In fact, the evidence suggests that nocturnal melatonin may actually be decreasing centrally mediated heat production as both peripheral skin and core temperatures decreased during melatonin treatment. However, this study was not performed on good-sleeping elderly or healthy younger adults as controls and therefore any conclusions drawn should be tentative. Furthermore, there is a possibility that both nocturnal administration and the supraphysiological dose used in this study led to different effects than typically observed with daytime melatonin administration.

These conclusions are supported by both animal and human studies, such as one early study which demonstrated that melatonin dilates isolated rabbit basilar artery (Shibata et al., 1989). However, this effect occurred only at high supraphysiological levels (10^{-5}-10^{-3} M) and is therefore unlikely to result from occupation of high-affinity melatonin receptors. This observation may relate to the possibility discussed in Chapter 1, that at high concentrations melatonin may bind at serotonin receptors or otherwise interact with endogenous compounds involved in temperature or sleep propensity regulation.

However, melatonin at nanomolar concentrations in rat caudal artery preparations has been shown to potentiate and prolong noradrenaline-mediated vasoconstriction (Viswanathan et al., 1990). While no studies to date have accounted for possible time of day effects, the vasodilatory and vasoconstrictive effects of melatonin apparent at physiological and supraphysiological levels respectively, provide a parallel for the more general systemic effects observed in

this thesis. That is, while the effects of administered melatonin within the physiological range may mimic the endogenous effects of melatonin, supraphysiological levels may have side-effects unrelated to its endogenous actions.

In the anterior cerebral artery of rats, melatonin activates high-affinity melatonin receptors and can directly constrict vessels at sub-nanomolar concentrations (Mahle et al., 1997). However, while melatonin-binding sites are present in human cerebral arteries, it is not yet clear whether melatonin can directly affect the tone of these vessels. However, daytime melatonin administration is associated with both decreased cerebral arterial blood flow and increased peripheral heat loss (Cagnacci, 1996). The physiological sequelae of either peripheral or cerebral vascular changes by melatonin are currently unclear and yet both may occur. There are likely to be inter-species differences in response to melatonin (Dawson and van den Heuvel, 1998) and it is envisaged that relating melatonin receptor occupation or other mechanisms with systemic effects will be technically difficult. Nevertheless, the present understanding is that both physiological and pharmacological effects of melatonin may relate to different mechanisms or pathways.

7.2.5.2 Soporific effects as manifestations of phase-shifts

Previous research has not typically supported the suggestion that melatonin's acute daytime effects relate to an immediate phase shift in the output of the SCN (Zhdanova et al., 1996). This evidence relies primarily on the observation that a maximum phase shift of 1-2 hours per day can be achieved with appropriately timed melatonin administration (Lewy et al., 1992; Zaidan et al., 1994). However,

ingestion of melatonin between 1000-1400 h typically increases sleep propensity and decreases core temperature, consistent with the output of the circadian system in the evening (eg. Dollins et al., 1993; Dollins et al., 1994; Hughes and Badia, 1997; Reid et al., 1996). As an immediate phase shift of up to 10 hours would therefore be required if this "shift hypothesis" of daytime melatonin effects is correct, it is unlikely that melatonin resets the circadian pacemaker by a sufficient magnitude to account for all of melatonin's acute effects.

Recently Sack and co-workers reviewed 2 potential SCN-mediated effects of melatonin that could explain the soporific effects of daytime melatonin at supraphysiological levels reported in many previous studies (Sack et al., 1997). The first of these consisted of acute phase shifting of the pacemaker, as discussed in the previous paragraph and in section 3.4. Secondly however, a mechanism was proposed whereby melatonin attenuates or antagonises an alerting signal coordinated at some level by the SCN. This hypothesis is based primarily on the demonstration of an alerting mechanism in squirrel monkeys, which is disrupted by SCN lesions (Edgar et al., 1993). This study found that daytime wake consolidation was significantly disrupted after SCN lesioning, suggesting that the pacemaker may drive the maintenance of wakefulness by opposing homeostatic sleep tendency during the subjective day. While no specific mechanism has been described whereby melatonin can oppose this SCN-dependent alerting signal, the model of Sack and colleagues may possibly explain a decrease in melatonin effects in the elderly. For example, it has been suggested that the alerting signal opposes a homeostatic process that increases with prior wakefulness (Edgar et al., 1993). In the elderly however, homeostatic

sleep drive has been reported to be less than in younger adults (Bliwise, 1993), and therefore an attenuation of the alerting signal by melatonin would theoretically have a greater impact in the young. These hypotheses are however, typically based on the observation of effects of melatonin at supraphysiological levels. As Sack notes in his recent review, "if pharmacological doses of melatonin are necessary to promote sleep, it is doubtful that the effect is confined to the normal role of endogenous melatonin" (Sack et al., 1997).

7.3 Future Directions

While the studies in this thesis have concentrated exclusively on the thermoregulatory and to a lesser extent the soporific effects of melatonin administration, it should be remembered that endogenous melatonin is also implicated as a neuroendocrine transducer of circadian, reproductive and immune functions. The present results indicate that a better understanding of the physiological role of melatonin may be gained by investigating how dose, rate of onset in the circulation, time of day and age affect the acute (thermoregulatory and soporific) effects of melatonin. Focussing on the acute effects induced by melatonin administration, it would appear that the most convenient way to achieve these aims would be to infuse melatonin, similar to the protocol in Chapter 3, and vary the rate of melatonin onset. The threshold for effects could be detected by choosing an endpoint (say, 0.15 deg. Tc suppression) and finding the melatonin level in individuals at which this endpoint is achieved at different melatonin infusion rates.

Unfortunately, the present studies do not clearly define the precise physiological role of melatonin concerning the regulation of body temperature and sleep. When antagonists and agonists become available for research use in humans, experimental evidence that more directly relates melatonin with physiological effects should be provided. Given the scarcity of studies investigating effects of physiologically relevant levels of melatonin and the suggestion in this thesis that the observed effects of oral daytime melatonin administration may be pharmacological side effects, the acute effects of melatonin administration remain unclear. In addition, in Chapters 3 and 4 physiological melatonin levels were only achieved during the day when it is possible that the sensitivity to melatonin was sub-maximal.

To remedy this situation, considerable basic research still needs to be carried out. Not the least of these studies would be an independent replication of the results herein. In a broader sense, it would seem prudent to investigate not only the acute thermoregulatory and soporific effects, but also the effects on the circadian, immune and reproductive systems. In relation to circadian regulation of sleep and temperature rhythms, it has been suggested in the past that melatonin mediates by chronobiotic effects (Cagnacci et al., 1992; Dijk and Cajochen, 1997; Lieberman, 1986; Redman et al., 1983; Refinetti and Menaker, 1992). If this is the case, it is possible that the acute effects of daytime supraphysiological melatonin administration represent nothing more than a disturbance to "circadian homeostasis".

The present results suggest that daytime melatonin with a rapid onset at physiological levels or nocturnal melatonin at high physiological levels or greater

at night, have significant thermoregulatory effects. This generally supports the hypothesis that the hormone plays a role in the circadian regulation of body temperature, although these results are compared across age, time of day and gender groups. Soporific effects of melatonin however, were not apparent except at steady supraphysiological levels, suggesting that regulation of sleep propensity may not be a physiological role of melatonin. Together, the results of this thesis challenge the idea that a simple relationship exists between melatonin, body temperature and sleep propensity in humans. Specifically, it has been suggested previously that melatonin, both endogenously and following exogenous administration, may exert soporific effects via changes in thermoregulatory balance. Regardless of the physiological interactions of melatonin, it may have some utility as an exogenous sleep-promoting substance and its chronobiotic effects may be of use in sleep disorders related to circadian rhythm disruption (eg. jet lag, shiftwork, and blindness). The potential therapeutic uses of melatonin however depend on the conclusive demonstration of the safety of chronic melatonin.

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Subject Information Sheet - Chapter 3

"The effects of sleep onset on body temperatures in humans"

As part of the ongoing research program in our lab, we are investigating the temperature changes throughout the body around the normal time of sleep initiation. Sleep is thought to play a role in the normal evening decline in core temperature, however temperature may already be declining before sleep onset and therefore may help to make you sleepy. If temperature is declining before sleep onset, we believe that a hormone produced in your brain at night, melatonin, may be the signal for temperature and sleepiness changes to occur. It is the relationship between melatonin (measured in your saliva before going to sleep), body temperature and sleep that we wish to examine in this study.

To assess whether you will be able to participate in this study, you will be required to complete a General Health Questionnaire and a 2-week sleep diary. If required, you will need to give consent to spend 3 separate nights in our lab. On 2 nights, you will be required to stay in bed from 1700 h and go to sleep normally whenever you feel sleepy. On one other night, you will have to stay in bed awake between 1700-0100 h, however you will be able to spend the remainder of the night asleep on this occasion.

On each night, you will have EEG electrodes attached to your face and scalp that will allow us to record your brain and facial muscle activity. These signals are fed to a sleep analysing computer and allow us to determine whether you are asleep or not. This is a non-invasive technique which will not hurt in any way, although some subjects report difficulty in getting to sleep on some occasions.

In addition, on each night you will need to insert a rectal temperature probe that measures core temperature. The probe is a thin piece of wire covered with sterile plastic. The probe is disposable and about 1mm in diameter. Should you experience any difficulty with the probe, one of the nursing staff will help you insert it. Some people have reported a small amount of irritation or a reflex urge to go to the toilet as they insert the probe. This feeling is normal and usually disappears after a few seconds. The thermistor will be attached to a small computerised recording device that will measure your temperature continuously.

In addition to the rectal probe, we will connect skin temperature probes to your hands, feet and the tympanic membrane in one ear, like a hearing aid. There should be little or no discomfort associated with these electrodes. Finally, we will be taking small saliva samples (3-4 ml) at half-hourly intervals. These will be used later to detect when your normal production of melatonin "switches on".

The first of your 3 nights in the lab is just to familiarise yourself with your sleeping environment and the testing procedures. On all three nights you will be wired up before 1900 h and will lie in your beds at 1900 h. On the second and third night, you will be required to either go to sleep when you feel sleepy or remain awake until after the last saliva sample and sleepiness measure is taken at 0100 h. The order in which you complete these conditions will be randomised, but you will know on each night what condition you are completing. While in bed, you may read, watch TV or videos, listen to music or any other quiet activity. You will be required to wear a T-shirt and shorts or equivalent clothing. There will be no bedclothes (ie. sheets, blankets) permitted, but the rooms are temperature controlled to 25 °C which should be comfortable.

You will be compensated for your participation, receiving $25 for each laboratory session. You will also receive a completion bonus of $45 for completing all parts of the study in the prescribed manner. You will be paid by cheque on completion of the study. In the future, we will be happy to tell you the results, and should you wish, forward you a copy of any publications resulting from the study.

We ask that you abstain from consuming alcohol or other drugs for the 24 hours prior to and including the experimental nights. Participation in this study is voluntary and if you wish, you may withdraw from the experiment at any stage with no explanation.

If you have any further questions concerning this protocol you may call the investigators on the following numbers (Drew Dawson (08) 8222 6755; Cameron van den Heuvel (08) 8222 6624). Should you wish to talk to an independent person not involved in the project, you can contact a representative of The Queen Elizabeth Hospital Ethics Committee, Paul Miller, on (08) 8222 6000.

Subject Information Sheet - Chapter 4

"The thermoregulatory effects of daytime melatonin infusion"

The purpose of this study is to help us to understand how and where a hormone called melatonin controls temperature and sleepiness. Melatonin is produced by the brain at night and is thought to play a role in regulating sleep and temperature. We believe that a better understanding of melatonin's role in temperature regulation may lead to more effective treatments for sleep disturbance in the elderly, who may suffer from low melatonin and high temperature at night. However, while we hope that this project will help us to develop such a treatment, it may not occur for many years or at all. This, unfortunately, is the nature of medical research.

The protocol will require you firstly to complete the General Health Questionnaire and a two-week sleep-wake diary in which you record the times at which you slept. If you are a suitable subject, you will be asked to spend two separate occasions in our lab, and abstain from alcohol or other drugs for 24 hours prior to and including each lab session.

On each occasion, you will need to attend the sleep lab and stay in bed awake from 0700 hrs until 1900 hrs, after inserting a sterile rectal probe that will measure your core body temperature. The probe consists of a thin piece of wire covered with plastic. The probe is disposable and is about 1 mm in diameter. You will be required to insert this to a depth of about 10 cm in your rectum at the start of the experimental session. Should you experience any difficulty one of the nursing staff will help you to insert the probe. Some people have reported a small amount of irritation or a reflex urge to go to the toilet as they insert the probe. This feeling is normal and usually disappears after a few seconds. The thermistor will be attached to a small computerised recording device that will measure your temperature continuously. In addition to the rectal probe, we will connect skin temperature probes to your forehead and one hand, while one will sit in your ear like a hearing aid. There should be little or no discomfort associated with these electrodes.

On both occasions, you will be connected to an intravenous drip that will infuse

melatonin or normal saline solution. The amount of the hormone that will be in your blood will be 4 - 8 ng/ml (up to 100 times the normal nighttime level for a young adult). You will not know on which occasion melatonin was infused, to prevent your response to the melatonin being biased by knowledge of what you have received. Trained medical staff with extensive experience in intravenous injections will connect this and another intravenous line on your opposite arm, from which we will take samples of blood every 30-60 minutes across the day. Finally, we will take small saliva samples and get you to rate how sleepy you feel (by drawing a line through a horizontal scale), coinciding with each blood collection.

Intravenous doses of melatonin with a total of up to 200 mg (many thousands of times greater than you will receive) have been given to many people in the past and there have not been any reported side effects. Nevertheless, should you experience any symptoms or discomfort you should let the investigators or other staff know immediately. Should you require any treatment a hospital doctor will treat you without cost to yourself.

You will be compensated for your participation in this experiment, receiving $50 for each laboratory session completed. You will also receive a bonus of $100 for completing all parts of the study in the prescribed manner (total = $200). This will be paid by cheque following the completion of the study. In the future, we will be happy to tell you the results, and should you wish, forward you a copy of any publications resulting from the study.

If you have any further questions concerning this protocol, you may call the investigators on the following telephone numbers. Should you wish to talk to an independent person not involved in the project, you can contact Paul Miller, Secretary, The Queen Elizabeth Hospital Ethics Committee on (08) 8222-6000.

Drew Dawson, Ph.D. Associate Professor, The Centre for Sleep Research

(08) 8222 6755

Cameron van den Heuvel

Ph.D. Student, Dept. of Obstetrics & Gynaecology, University of Adelaide

(08) 8222 6624

Subject Information Sheet - Chapter 5

"The thermoregulatory effects of daytime melatonin injection"

The purpose of this study is to help us to understand how and where a hormone called melatonin controls temperature and sleepiness. Melatonin is produced by the brain at night and is thought to play a role in regulating sleep and temperature. We believe that a better understanding of melatonin's role in temperature regulation may lead to more effective treatments for sleep disturbance in the elderly, who may suffer from low melatonin and high temperature at night. However, while we hope that this project will help us to develop such a treatment, it may not occur for many years or at all. This, unfortunately, is the nature of medical research.

The protocol will require you firstly to complete the General Health Questionnaire and a two-week sleep-wake diary in which you record the times at which you slept. If you are a suitable subject, you will be asked to spend four separate occasions in our lab. For 24 hours prior to and including each lab session, you will need to abstain from alcohol and other drugs.

On each occasion, you will need to attend the sleep lab and stay in bed awake from 0800 hrs until 1500 hrs, after inserting a sterile rectal probe that will measure your core body temperature. The probe consists of a thin piece of wire covered with plastic. The probe is disposable and is about 1 mm in diameter. You will be required to insert this to a depth of about 10 cm in your rectum at the start of the experimental session. Should you experience any difficulty one of the nursing staff will help you to insert the probe. Some people have reported a small amount of irritation or a reflex urge to go to the toilet as they insert the probe. This feeling is normal and usually disappears after a few seconds. The thermistor will be attached to a small computerised recording device that will measure your temperature continuously. In addition to the rectal probe, we will connect skin temperature probes to your forehead and one hand, while one will sit in your ear like a hearing aid. There should be little or no discomfort associated with these electrodes.

On all occasions, you will be given an intravenous injection at 1000 hrs that will

contain normal saline solution or 1 of 3 melatonin doses. The 3 melatonin doses injected will be 3, 10 and 30 μg (which will elevate peak melatonin in your blood to 1-2 times the normal night-time level for a young adult). You will receive all three doses and saline in a random order and will not know on which occasion melatonin was injected. This is to prevent your response to the treatment being biased by knowledge of what you have received. Trained medical staff with extensive experience will conduct the intravenous injections.

Intravenous doses of melatonin with a total of up to 200 mg (many thousands of times greater than you will receive) have been given to many people in the past and there have not been any reported side effects. Nevertheless, should you experience any symptoms or discomfort you should let the investigators or other staff know immediately. Should you require any treatment you will be treated by a hospital doctor without cost to yourself.

You will be compensated for your participation in this experiment, receiving $50 for each laboratory session completed. This will be paid by cheque following the completion of the study. In the future, we will be happy to tell you the results, and forward you a copy of any publications resulting from the study if required.

If you have any further questions concerning this protocol you may call the investigators on the telephone numbers below. Should you wish to talk to an independent person not involved in the project, you can contact Paul Miller, Secretary, The Queen Elizabeth Hospital Ethics Committee on (08) 8222-6000.

Drew Dawson, Ph.D.

Associate Professor, The Centre for Sleep Research

(08) 8222 6755

Cameron van den Heuvel

Ph.D. Student, Dept. of Obstetrics & Gynaecology, University of Adelaide

(08) 8222 6624

Subject Information Sheet - Chapter 6

"The effect of melatonin on temperature and sleep in elderly subjects"

The purpose of this study is to investigate whether a hormone produced normally in the brain, called melatonin, can improve the sleep of chronic insomniacs. Melatonin is produced mostly at night and when given to young adults during the day makes them sleepier. Thus, there is some association between melatonin and the regulation of sleep in the body. It has been suggested that chronic sleep disturbance in some older people may arise either by a decline in the production of melatonin with age, or by becoming less sensitive to the melatonin that is produced. By giving extra melatonin to older people with chronic sleep disturbance, we may be able to investigate some of the causes of age-related insomnia and perhaps even improve sleep in these subjects.

Before commencing this study we would like you to complete a General Health Questionnaire, a Sleep Questionnaire, a Sleep/Wake Diary for two weeks, and, if necessary, other psychological questionnaires and overnight sleep recording at the Repatriation General Hospital. The sleep diary, in which you record the times at which you slept and for how long you slept, must be completed each morning when you arise from bed. If you are a suitable subject, you will be required to spend two separate sessions in our lab. Each session will last 4 days and you need to be the laboratory at 6.30 PM on each night of the study. Please have your normal evening meal before you arrive and do not take any coffee, tea or alcohol whilst on the study. You will be required to stay in bed from 9pm to 7am, but you only have to go to sleep when you feel ready. If necessary, bring any aids that might help you sleep, for example pillows, reading material, radio etc. Breakfast will be provided prior to your departure on each morning.

At the start of each session, you will be required to insert a rectal probe (called a thermistor) that will measure your core body temperature. The probe consists of a thin piece of wire covered with plastic, about 1 mm in diameter and is disposable. Should you experience any difficulty one of the nursing staff or investigators will help you to insert the probe. The rectal probe will be attached to a small computerised recording device that will measure your temperature

every 2 minutes. In addition, you will be required to wear thermistors fastened to the back of one hand, one foot and your forehead. These will be for measuring skin temperature. On each night that you are in the lab, we will collect all your urine between 7PM and 7 AM.

On all nights in the lab, you will wear a small adhesive patch on the upper gum, which has been specially produced by 3M Pharmacetuicals. On one group of 4 nights you will receive only inactive placebo, and on the other a 0.5 mg dose of melatonin. The 0.5 mg melatonin patch will produce a level of melatonin in your blood at, or slightly above, the normal night-time level for a young adult. We do not tell you in which session you are being given the melatonin so that your response is not biased by knowing whether or not you have been given active treatment. In addition, we will not know ourselves whether you are being given melatonin or a placebo until after the study.

Melatonin doses of up to 200 mg or more have been given to many people in the past and there have not been any side effects reported. Nevertheless, should you experience any symptoms you should let the nursing staff or one of the investigators know immediately. The potential side effects with the use of melatonin patches are local irritation and may include redness and ulceration. Side effects with oral melatonin include hypotension and minor digestive troubles which are not expected to occur with the use of patches. Should you require any treatment you will be treated by a hospital doctor without cost.

You will be compensated for the inconvenience associated with your participation in this study. You will receive $400 for completing all parts of the study in the prescribed manner. You will be paid by cheque following the completion of the study. Following the study we will be happy to tell you the results, and should you wish, forward you a copy of any publications resulting from the study.

The purpose of this research study is to improve the quality of medical care however, your involvement may not be of any benefit to you.

If you are currently involved in another study please inform the attendant researcher.

Your involvement in the project is voluntary and will not affect your relationship

with your medical advisers in their management of your health. Your are free to withdraw from the project at any stage without prejudice for future treatment or studies.

There may be situations or circumstances that occur that require that the study be stopped or you, the subject, be withdrawn from the study.

Your subject records may be inspected for the purposes of source data auditing by authorised persons within the institution.

Results from the study will not be published so as to reveal your identity. Confidentiality will be maintained.

If you have any further questions concerning this protocol you may telephone the chief investigator on the number below. Should you wish to talk to someone not involved in the project you can contact Paul Miller of The Queen Elizabeth Hospital Ethics Committee, whose number is also located below:

Drew Dawson, Ph.D.

Associate Professor, The Centre for Sleep Research

(08) 8222 6755

Paul Miller

Secretary, The Queen Elizabeth Hospital Ethics Committee

(08) 8222 6624

General Health Questionnaire

1. Please indicate how frequently you experience the following, by circling the appropriate number:

	Almost Never	Quite Seldom	Quite Often	Almost Always
(a) How often is your appetite disturbed?	1	2	3	4
(b) How often do you have to watch what you eat to avoid stomach upsets?	1	2	3	4
(c) How often do you feel nauseous?	1	2	3	4
(d) How often do you suffer from heartburn or stomach-ache?	1	2	3	4
(e) How often do you complain of digestion difficulties? ?	1	2	3	4
(f) How often do you suffer from bloated stomach or flatulence?	1	2	3	4
(g) How often do you suffer from pain in your abdomen?	1	2	3	4
(h) How often do you suffer from constipation or diarrhoea?	1	2	3	4
(i) How often do you suffer from heart palpitations?	1	2	3	4
(j) How often do you suffer from aches and pains in your chest?	1	2	3	4
(k) How often do you suffer from dizziness?	1	2	3	4
(l) How often do you suffer from sudden rushes of blood to your head?	1	2	3	4
(m) Do you suffer from sudden rushes of blood to your head?	1	2	3	4
(n) How often have you been told that you have high blood pressure?	1	2	3	4
(o) Have you been aware of your heart beating irregularly?	1	2	3	4
(p) Do you suffer from swollen feet?	1	2	3	4
(q) How often do you feel "tight" in your chest?	1	2	3	4
(r) Do your gums bleed when you brush your teeth?	1	2	3	4

2. Have you ever suffered from any of the following (diagnosed by your doctor)?

	Yes	Never
(a) Chronic back pain	———	———
(b) Gastritis, duodenitis	———	———
(c) Gastric or duodenal ulcer	———	———
(d) Gall stones	———	———
(e) Colitis	———	———
(f) Sinusitis, tonsillitis	———	———
(g) Bronchial asthma	———	———
(h) Angina	———	———
(i) Heart attack (myocardial infarction)	———	———
(j) High blood pressure	———	———
(k) Cardiac arrhythmias	———	———
(l) Hypercholesterolaemia	———	———
(m) Diabetes	———	———
(n) Cystitis	———	———
(o) Kidney stones	———	———
(p) Eczema	———	———
(q) Chronic anxiety	———	———
(r) Depression	———	———
(s) Arthritis	———	———
(t) Haemorrhoids	———	———
(u) Varicose veins	———	———
(v) Anaemia	———	———
(w) Headaches	———	———
(x) Gingivitis	———	———
(y) Others	———	———

3. Have you ever taken any of the following medications for prolonged periods (more than three months)?

	Yes	Never
(a) Tranquilizers	———	———
(b) Sleeping tablets	———	———
(c) Anti-depressants	———	———
(d) Antacids	———	———
(e) Antispasmodics	———	———
(f) Laxatives	———	———
(g) Drugs to control high blood pressure	———	———
(h) Diuretics	———	———
(i) Heart medicines	———	———
(j) Vasodilators	———	———
(k) Bronchodilators	———	———
(l) Vitamins, tonics	———	———
(m) Pain killers	———	———
(n) Steroids	———	———
(o) Anti-inflammatory medicines	———	———
(p) Hormones (except contraceptive pills)	———	———
(q) Others	———	———

4. The following questions deal with how you have felt in general over the past few weeks. Please circle the most appropriate answer for each question. Concentrate on present and recent complaints, not those that you have had in the distant past.

Have you recently:

(a) been able to concentrate on what you are doing?	Better than usual	Same as usual	Less than usual	Much less than usual
(b) lost much sleep over worry?	Not at all	No more than usual	More than usual	Much more than usual
(c) felt that you are playing a useful part in things?	More so than usual	Same as usual	Less than usual	Much less than usual
(d) felt capable of making decisions about things?	More so than usual	Same as usual	Less than usual	Much less than usual
(e) felt constantly under strain?	Not at all	No more than usual	More than usual	Much more than usual
(f) felt you could not overcome your difficulties?	Not at all	No more than usual	More than usual	Much more than usual
(g) been able to enjoy your normal day to day activities	More so than usual	Same as usual	Less than usual	Much less than usual
(h) been able to face up to your problems?	More so than usual	Same as usual	Less than usual	Much less than usual
(i) been feeling unhappy and depressed?	Not at all	No more than usual	More than usual	Much more than usual
(j) been losing confidence in yourself?	Not at all	No more than usual	More than usual	Much more than usual
(k) been thinking of yourself as a worthless person?	Not at all	No more than usual	More than usual	Much more than usual
(l) been feeling reasonably happy all things considered?	More so than usual	About the same	Less so than usual	Much less than usual

5. Below are listed some descriptions of symptoms of anxiety.

Please indicate the degree to which you generally or typically experience the symptom when you are feeling anxious:

	Not at all		Somewhat		Very much so
(a) I perspire	1	2	3	4	5
(b) My heart beats faster	1	2	3	4	5
(c) I worry too much over something that doesn't really matter	1	2	3	4	5
(d) I feel jittery in my body	1	2	3	4	5
(e) I imagine terrifying scenes	1	2	3	4	5
(f) I get diarrhoea	1	2	3	4	5
(g) I can't keep anxiety provoking pictures out of my mind	1	2	3	4	5
(h) I feel tense in my stomach	1	2	3	4	5
(i) Some unimportant thought runs through my mind and bothers me	1	2	3	4	5
(j) I nervously pace	1	2	3	4	5
(k) I feel like I am losing out on things because I can't make up my mind soon enough	1	2	3	4	5
(l) I feel physically immobilised	1	2	3	4	5
(m) I can't keep anxiety provoking thoughts out of my mind	1	2	3	4	5
(n) I find it difficult to concentrate because of uncontrollable thoughts	1	2	3	4	5

Two Week Sleep Diary

The following information will help to familiarise you with the task of filling in the attached sleep diary. The diary consists of a record sheet, from which we gain information about your sleep/wake patterns over 2 weeks. It will provide us with information about how sleepy / alert you are feeling at certain times and how well you feel you have slept. We also wish to know the amount of sleep you estimate you have had over each 24-hour period.

The diary is set out in a manner that permits easy and quick recording. The diary should be commenced at midday (12 pm) on the day that the investigator has told you. Each row starts at midday and finishes at 11:59 am the following day. Therefore, each row is used to record 24 hours of activity and sleep. To fill in the diary requires a few things to be done when you get out of bed in the morning and just before you go to bed (at whatever time of day).

1) **The first step** involves recording every time you were **asleep** (not when you got in or out of bed). This includes **any** and **every** time you sleep during every 24 period. As shown in the example at the top of your diary, you indicate when you slept using a thick line, drawn from the beginning to the end of each sleep period. If you woke up during the sleep period, then you would draw a broken line with breaks at the times you were awake.

2) **The second step** requires you to rate how well you slept during **each** sleep period. Select a number from the following scale that best reflects how you slept and write it next to or above the thick black line you drew in (1) above:

1 - Very Well
2 - Well
3 - Average
4 - Poor
5 - Very Poor

3) **The third step** is to rate how sleepy / alert you feel just before you go to bed

each night ("PM" box under SSS heading) and as soon as you wake up in the morning ("AM" box). The scale you use is adapted from the Stanford Sleepiness Scale (SSS):

1 - Feeling active and vital. Alert and wide awake
2 - Functioning at a high level but not at peak. Able to concentrate
3 - Relaxed, awake, not at full alertness, responsive.
4 - A little foggy. Not at peak. Let down.
5 - Fogginess. Beginning to lose interest in remaining awake. Slowed down.
6 - Sleepiness. Prefer to be lying down. Fighting sleep. Woozy.
7 - Almost in reverie. Sleep onset soon. Lost struggle to remain awake.

4) Finally, first thing in the morning, please record how many hours of sleep you think you obtained during the previous 24 hour period. Accuracy is not important, your opinion is what counts here. The amount of sleep you think you got the previous day should be recorded in the "Hours of Sleep" box.

Name | Subject No.

			PM											AM											SSS		Hours of
			12	13	14	15	16	17	18	19	20	21	22	23	0	1	2	3	4	5	6	7	8	9	10	11			
Day	Date	Day of Week	MD	1	2	3	4	5	6	7	8	9	10	11	MN	1	2	3	4	5	6	7	8	9	10	11	PM	AM	Sleep
EXAMPLE		*Tuesday*																					*3*				*7*	*6*	*8*
1																													
2																													
3																													
4																													
5																													
6																													
7																													
8																													
9																													
10																													
11																													
12																													
13																													
14																													

Subject Consent Form

Institution: The Centre for Sleep Research, University of South Australia

See also Information Sheet attached

1. I _____ (please print) hereby consent to take part in the research project entitled:

2. I acknowledge that I have read the Information Sheet entitled:

3. I have had the project, so far as it affects me, fully explained to my satisfaction by the research worker. My consent is given freely.

4. Although I understand that the purpose of this research project is to improve the quality of medical care, it has also been explained that my involvement may not be of any benefit to me.

5. I have been given the opportunity to have a member of my family or a friend present while the project was explained to me.

6. I have been informed that, while information gained during the study may be published, I will not be identified and my personal results will not be divulged.

7. I understand that I am free to withdraw from the project at any time.

8. I am aware that I should retain a copy of this Consent Form, when completed, and the relevant Information Sheet.

9. I understand the statement concerning payment to me for taking part in the study, which is contained in the information sheet.

10. I confirm that I am over 18 years of age.

SIGNED _____ **DATE** _____

NAME OF WITNESS _____

SIGNED _____ **DATE** _____

I have described to _____ the nature of the procedures to be carried out. In my opinion, she/he understood the explanation.

SIGNED _____ **DATE** _____

List of Publications

Dawson, D. and van den Heuvel, C.J. Integrating the actions of melatonin on human physiology. *Ann.Med.*, 1998, **30**, 95-102.

van den Heuvel, C.J., Kennaway, D.J., and Dawson, D. Effects of daytime melatonin infusion in young adults. *Am.J.Physiol.*, (In Press).

van den Heuvel, C.J., Kennaway, D.J., and Dawson, D. The thermoregulatory and soporific effects of very low dose melatonin injection. *Am.J.Physiol.*, (Submitted).

van den Heuvel, C.J., Noone, J.T., and Dawson, D. Changes in sleepiness and temperature precede nocturnal sleep onset: Evidence from a polysomnographic study in young men. *J.Sleep Res.*, (In Press).