NOTE TO USERS

The Rhythmic Expression of Circadian Clock Genes in Human Peripheral Blood Mononuclear Cells: Investigating the Functional Clock in Circulating White Blood Cells.

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DEDICATION

To my parents, Franklyn and Tommie, with love.

ACKNOWLEDGMENTS

I am supported by brilliant people throughout my academic and personal life. Though I can't name them all here, I hope that they'll each accept my unending gratitude.

My supervisors, Diane Boivin and Nicolas Cermakian, provided me with the many tools I needed throughout each step of this process. They've been very generous in their mentorship and I'm grateful to them both. I also thank my advisory committee for their guidance through this process and my mentor, Joseph Rochford, for his steady and valuable insight.

The students and staff of the Centre for Study and Treatment of Circadian Rhythms, past and present, are an essential part of the work that went into this thesis. Park Cho was there at the start of these projects and it was he who inspired me to look at these clocks in the first place. The students and staff in the Laboratory of Molecular Chronobiology, particularly Valérie Bélanger and Sylvain Charbonneau, patiently helped me set up the molecular experiments.

My mother and father, Tommie and Franklyn, are the embodiment of grace and humility. I can afford the luxury of studying for the very love of it because of sacrifices that they've made. I may never understand how I came to be so fortunate as to be the recipient of the unconditional love and support that they and my formidable little sister Yolande have shown me. I am, in every day, grateful for the pleasure of their company. I thank my grandmother Mathilda for her loving and unbridled enthusiasm for my success.

My godfather, Leo Bertley, succumbed to cancer in the weeks before this thesis was completed. He was an extraordinary individual, and it was my privilege to have learned from him both within and without a formal classroom.

ABSTRACT

The rhythmic expression of an autoregulatory loop of circadian clock genes underlies the intrinsic circadian rhythmicity in the central circadian pacemaker of the suprachiasmatic nucleus (SCN). Clock genes are also known to be rhythmically expressed outside the SCN and outside the brain. While these peripheral circadian oscillators are functional, their expression is chiefly coordinated by the central circadian pacemaker of the SCN. This thesis presents the first evidence of a functional circadian oscillator in human peripheral blood mononuclear cells (PBMCs). Measuring levels of RNA transcripts in PBMCs sampled throughout specific behavioural protocols permitted the characterization of these peripheral circadian oscillators in humans. The expression of clock genes *HPER1*, *HPER2*, and *HPER3* peaks early after the time of typical awakening and demonstrates a significant circadian rhythmicity in PBMCs sampled from healthy young men that persists in time isolation and under the constant behavioural conditions of a constant routine (CR). The functional circadian oscillator in human PBMCs is also observable in the presence of the sleep/wake cycle. Using frequent sampling over 72 hours, it was determined that the patterns of *HPER1* and *HPER2* expression are comparable when sampled in the presence of a habitual sleep/wake cycle or during a CR. The expression of *HBMAL1* in PBMCs was more variable under different behavioural conditions. The pattern of light and darkness exposure including bright light during night shifts, shielding from morning light and sleep in darkened quarters can induce adaptive phase delays in the endogenous circadian rhythms of cortisol secretion in night shift workers. In the presence of such a light intervention, the cortisol rhythm in night shift workers re-assumes an appropriate temporal alignment with the shifted sleep/wake schedule and cortisol levels peak near the shifted time of awakening. Following nine days of simulated night shift work, *HPER1* and *HPER2* expression in PBMCs is aligned to the shifted sleep/wake schedule of a typical night shift worker in the presence of a comparable light/darkness intervention. This thesis will consider the implications of a functional clock in human white blood cells in light of the demonstration of a functional and shiftable circadian oscillator in human PBMCs.

RÉSUMÉ

L'expression des gènes de l'horloge est à la base de la rythmicité intrinsèque de l'horloge circadienne central du noyau suprachiasmatique (NSC). Les gènes de l'horloge sont aussi exprimés de façon rythmique dans les tissus à l'extérieur du NSC et du cerveau. Ces oscillateurs circadiens périphériques sont fonctionnels, mais leur expression est principalement coordonnée par l'horloge du NSC. Cette thèse identifie pour la première fois un oscillateur circadien fonctionnel dans les cellules mononucléaires du sang périphérique (CMSP) chez l'humain. L'analyse des niveaux d'ARN des gènes de l'horloge dans des CMSP prélevées au cours de protocoles comportementaux a permis la caractérisation de ces oscillateurs circadiens périphériques. Les niveaux d'ARN des gènes *HPER1*, *HPER2*, et *HPER3* mesurés dans les CMSP prélevées de jeunes hommes sains démontrent une rythmicité circadienne significative qui persiste même sous les conditions contrôlées de la routine constante (RC). Dans ces conditions, les niveaux de *HPER1*, *HPER2* et *HPER3* présentent un maximum après l'heure habituelle du réveil. L'oscillation des gènes de l'horloge dans les CMSP humains peut aussi être observée au cour d'un cycle éveil/sommeil typique. L'utilisation de prélèvements sanguins fréquents sur une période ininterrompue de 72 heures a permis de déterminer que les rythmes d'expression de *HPER1* et *HPER2* en présence d'un cycle éveil/sommeil habituel sont similaires aux rythmes observés durant une RC. L'expression de *HBMAL1* dans les CMSP est plus variable sous ces conditions expérimentales. L'horaire d'exposition à la lumière et à l'obscurité, incluant l'utilisation de la lumière vive durant les quarts de nuit, l'exposition à de faibles niveaux de luminosité le matin et le maintien d'un horaire de sommeil en obscurité, peut permettre la resynchronization des rythmes endogènes des travailleurs de nuit. En présence de cette intervention, le rythme de cortisol des travailleurs de nuit reprend un alignement temporel approprié et atteint son maximum après l'heure du réveil. Après neuf jours de travail de nuit simulé, les rythmes d'expression de *HPER1* et *HPER2* dans les CMSP sont alignées à l'horaire éveil/sommeil décalé quand cette intervention est appliquée. Cette thèse se conclut sur une discussion des implications d'une telle horloge fonctionnelle dans les globules blancs.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
BMAL1	Brain and muscle arnt-like 1 protein
CR	Constant routine
CRY1	Cryptochrome 1 protein
CRY2	Cryptochrome 2 protein
CRH	Corticotropin-releasing hormone
DD	Dark/dark: constant dim light schedule
DEC1	Deleted in esophageal cancer 1 protein
DNA	Deoxyribonucleic acid
EEG	Electroencephalogram
HBMAL1	Human brain and muscle arnt-like 1 RNA
HPER1	Human period homolog 1 RNA
HPER2	Human period homolog 2 RNA
HPER3	Human period homolog 3 RNA
LD	Light/dark cycle
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PER1	Period homolog 1 protein
PER2	Period homolog 2 protein
PER3	Period homolog 3 protein
PVN	Paraventricular nucleus of the hypothalamus
REM	Rapid-eye movement
REV-ERB	Orphan nuclear receptor, group D protein
RHT	Retino-hypothalamic tract
RNA	Ribonucleic acid
ROR	Retinoic acid related orphan receptor protein
SCN	Suprachiasmatic nucleus
TSH	Thyroid stimulating hormone

CONTRIBUTION OF AUTHORS

Chapter 2:

Diane B. Boivin, Francine O. James, Aibin Wu, Park F. Cho-Park, Huabao Xiong, and Zhong S. Sun. *Circadian clock genes oscillate in human peripheral blood mononuclear cells.* Blood (2003) 102:4143-5.

Behavioural experiments in time isolation were designed by Diane B. Boivin and performed under her supervision at the Centre for Study and Treatment of Circadian Rhythms. Together with the students and research staff of the Centre for Study and Treatment of Circadian Rhythms, I performed whole blood sampling during the time isolation study. Park F. Cho, an undergraduate student and research assistant isolated peripheral blood mononuclear cells (PBMCs) from whole blood samples and performed RNA extractions. All experiments pertaining to the real-time PCR quantification of clock gene expression, including oligonucleotide design, were performed by Aibin Wu and Huabao Xiong under the supervision of Zhong S. Sun, at the Weill Medical School of Cornell University. I performed the analyses that appear in the paper and prepared the manuscript for publication.

Chapter 3:

Francine O. James, Diane B. Boivin, Sylvain Charbonneau, Valérie Bélanger, Nicolas Cermakian. *Expression of clock genes in human peripheral blood mononuclear cells throughout the sleep/wake and circadian cycles.* Submitted to the Am J Physiol- Regul Integr Comp Physiol, September 2006.

Experiments in time isolation were performed at the Centre for Study and Treatment of Circadian Rhythms under the supervision of Diane B. Boivin. Extraction of RNA and quantitative real-time PCR was performed in the Laboratory of Molecular Chronobiology under the supervision of Nicolas Cermakian. Sylvain Charbonneau assisted me in the design and testing of oligonucleotides and RNA extractions. Valérie Bélanger participated in the set-up of the RNA extraction and real-time quantitative PCR techniques. Together with the students and research staff of the Centre for Study and Treatment of Circadian Rhythms, I performed whole blood sampling and PBMC isolations and was the project leader for these studies. I also performed all real-time PCR experiments. I performed all the analyses that appear in the paper and prepared the manuscript for publication.

Chapter 4:

Francine O. James, Claire-Dominique Walker, Diane B. Boivin. ***Controlled exposure to light and darkness realigns the salivary cortisol rhythm in night shift workers.*** Chronobiol Int. 2004;21(6):961-72.

Behavioural experiments were conducted at the Centre for Study and Treatment of Circadian Rhythms under the supervision of Diane B. Boivin. Analysis of salivary cortisol concentration was performed in the laboratory of Claire-Dominique Walker at the Douglas Hospital Research Centre. Together with students and research staff of the Centre for Study and Treatment of Circadian Rhythms, I performed sampling during laboratory and ambulatory portions of the study and was the project leader for these studies. I performed all the analyses that appear in the paper and prepared the manuscript.

Chapter 5:

Francine O. James, Nicolas Cermakian , Diane B. Boivin. ***Entrainment of circadian rhythms of melatonin, cortisol and clock gene expression to simulated night shift work.*** Submitted to the J Clin Endocrinol Metab, September 2006.

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ORIGINAL CONTRIBUTION TO KNOWLEDGE

Chapter 2:

Diane B. Boivin, Francine O. James, Aibin Wu, Park F. Cho-Park, Huabao Xiong, and Zhong S. Sun. ***Circadian clock genes oscillate in human peripheral blood mononuclear cells.*** Blood (2003) 102:4143-5.

Where peripheral circadian oscillators had been identified in a number of studies in non-human organisms, the present experiment measures clock gene expression in peripheral blood mononuclear cells (PBMCs) sampled from healthy young men under tightly controlled behavioural and environmental conditions. The results of this investigation establish the presence of a functional peripheral circadian oscillators in human PBMCs.

Chapter 3:

Francine O. James, Diane B. Boivin, Sylvain Charbonneau, Valérie Bélanger, Nicolas Cermakian. ***Expression of clock genes in human peripheral blood mononuclear cells throughout the sleep/wake and circadian cycles.*** Submitted to the Am J Physiol- Regul Integr Comp Physiol, September 2006.

The results of this investigation provide the first evidence of the nature of clock gene expression in PBMCs sampled from healthy adults under both constant behavioural conditions and in the presence of a sleep/wake cycle. It also demonstrates the feasibility of evaluating clock gene expression in a human peripheral circadian oscillator over a sustained period (3 days).

Chapter 4:

Francine O. James, Claire-Dominique Walker, Diane B. Boivin. ***Controlled exposure to light and darkness realigns the salivary cortisol rhythm in night shift workers.*** Chronobiol Int. 2004;21(6):961-72.

Night shift workers often demonstrate hormonal rhythms that are maladapted to the work schedule, despite several consecutive shifts worked and many years of night work experience. The results presented in this investigation demonstrate that an appropriate pattern of light/darkness exposure throughout the day significantly influences circadian adaptation to night shift work in real-life: workers receiving an appropriate light/darkness intervention display salivary cortisol rhythms that are more appropriately aligned to the shifted sleep/wake schedule than workers who did not receive the complete intervention.

Chapter 5:

Francine O. James, Nicolas Cermakian , Diane B. Boivin. *Entrainment of circadian rhythms of melatonin, cortisol and clock gene expression to simulated night shift work.* Submitted to the J Clin Endocrinol Metab, September 2006.

The results of this study indicate that following a period on a night shift work schedule, the expression of clock gene expression in PBMCs becomes adapted to the shifted sleep/wake schedule in the presence of a light/darkness schedule that is known to adapt circadian rhythms of plasma melatonin and cortisol concentration. No known study had previously investigated the entrainment of peripheral clocks to a shifted sleep/wake schedule.

PREFACE

In this manuscript-based thesis, each chapter represents a manuscript that has been submitted for publication or published in a peer-reviewed journal. The bibliography for each manuscript appears at the end of each chapter. A preface to each chapter is intended to outline the logical progression through the experimental hypotheses. The complete reference list for the Introduction (Chapter 1) and the General Discussion (Chapter 6) appears in the bibliography at the end of the thesis.

CHAPTER 1

Introduction

"From life's outset, the major environmental cycles of day, tide, month, and year have confronted natural selection with windows of opportunity and hazard that recur with precisely predictable frequency;...Such programs offer the clear advantage of anticipatory preparation for predictably recurrent conditions"

- Colin Pittendrigh, 1993 [1]

1.1 Circadian rhythm generation in rodents and humans

1.1.1 A central endogenous pacemaker coordinates circadian rhythms

The term "circadian" was first conceptualized in 1959 by Franz Halberg subsequent to his observation of an unexplained variability in mouse whole blood cell numbers sampled at multiple times of day [2, 3]. This term, from the Latin *circa* (about) and *dies* (day) encompassed the reliable recurrence of events with an approximate 24-hour period, and would eventually come to describe the stable and precise daily rhythmicity that had been previously observed in the physiology of a number of different species. Daily growth lines on fossilized corals are an indication of a predictable daily reoccurrence in the organism [4]. The ancient Greek sculptor Androsthenes (b. *circa* 440 BC) noted day/night differences in the opening of the leaves of the tamarind tree [2] which foreshadowed de Mairan's 18th century observations on day/night differences in the leaves of the heliotrope which persisted even when the plant was kept away from sunlight [5]. An endogenous clock has been proposed as a functional entity in a wide range of organisms from prokaryotes to humans [4, 6]. It has been suggested that synchronizing behavioural and physiological processes to naturally occurring rhythmicity in the environment makes the organism able to efficiently respond to predictable changes in the geophysical environment, and thus more fit from an evolutionary perspective [1, 4, 6]. For example, ground squirrels and chipmunks with ablated circadian clocks living under naturalistic conditions are more likely to become prey, possibly resulting from heightened activity at inappropriate times of day [7, 8].

1.1.1a The SCN in non-human mammals

The early 1970s provided some of the first evidence that the suprachiasmatic nucleus- a bilateral cluster of cells on either side of the third ventricle in the anterior

hypothalamus [2]- was the physical structure responsible for the generation of endogenous circadian rhythmicity. Electrolytic lesions of the suprachiasmatic nucleii (SCN) of rats resulted in the ablation of circadian rhythms of corticosterone and of drinking and activity –results that were not observed with enucleation, lesions in the medial preoptic area, or lesions rostral or caudal to the SCN [9, 10]. Subsequent experiments in non-human animals came to establish the SCN of the anterior hypothalamus as the structure responsible for generating observable endogenous circadian rhythmicity [11, 12]. Transplantation of suspensions of foetal SCN tissue is sufficient to restore circadian organization to the locomotor activity of behaviourally arrhythmic SCN-lesioned hamsters in the period of the donor animal [13-16].

Studies based on recordings *in vivo* [17], in organotypic slices [18] or in single cells [19] have revealed that the SCN demonstrates an intrinsic circadian rhythmicity in its electrical activity that is distinct from adjacent hypothalamic structures. The measured uptake of radioactive deoxyglucose in the SCN of experimental rats suggested day/night differences in the metabolic activity of the neurons of the SCN [20] such that in nocturnal, crepuscular and diurnal mammals, peak glucose utilization occurred during daylight hours [21]. The firing rates of individual neurons recorded in rat SCN slices or in dispersed neurons demonstrate a range of periods in their firing activity (e.g. 16-26 hours [22]). However, the majority of cells have an approximate 24-hour period such that the mean period of firing rates also approximates 24 hours [22, 23].

1.1.1b The SCN in humans

While the identification of the human SCN was initially controversial, *post-mortem* investigations on hypothalamic tissue resulted in the identification of the SCN structure in humans [24] with an organization that is analogous to what has been observed in rats [25]. Reports of altered sleep/wake patterns following aneurysm near the SCN [26] and altered temperature rhythmicity following tumour involving the ventral hypothalamus [27] are consistent with the role of the SCN in the generation of circadian rhythmicity in humans [28].

1.1.2 The expression of SCN-driven rhythms in humans

In humans, the SCN consists of a structure of approximately 45,000 cells [29] and current technology limits our ability to ethically make *in vivo*, real-time assessments of SCN function. Thus, observed physiological variables, ultimately controlled by the

circadian pacemaker, are accepted surrogates for estimating the phase of the endogenous biological clock.

1.1.2a Core body temperature

The rectally-recorded core body temperature rhythm is a well characterized circadian rhythm in humans, which persists even when humans live in time isolation (in a research laboratory room or in a cave, for example) over extended periods of time [30-32]. The variation of the endogenous temperature rhythm is quasi-sinusoidal, with a progressive elevation of core body temperature in the morning, a maximum a few hours before typical bedtime, and a progressive descent in the early night to reach a minimum 1-2 hours before the habitual time of waking [33-36] (**Figure 1.1**). Electrolytic lesion of the SCN can ablate circadian rhythmicity in recorded core body temperature [37, 38]. The SCN may exert control over the core body temperature rhythm via thermoregulatory centers in preoptic hypothalamic areas [39, 40], although more studies are needed to clarify the precise anatomic relationship that contributes to this circadian rhythm. In the broader sense, overall thermoregulation as it is influenced by the regulation of heat production [34] and distal vasodilation [41], is also is under some circadian regulation [33, 42]. The endogenous core body temperature rhythm is easily masked by the effects of daily activity, postural changes and sleep, where the observed amplitude would be greater than the endogenously generated component [43, 44]. Mathematical paradigms have been discussed for unmasking the body temperature rhythm (e.g. [45, 46]). However, to date the endogenous core body temperature is arguably most accurately measured under the conditions of the constant routine (CR) since putative masking effects such as the thermoregulatory response to activity or sleep, for example, are not evenly distributed throughout the circadian cycle [47-49]. As discussed in the subsequent chapters, the CR protocol conducted in time isolation requires that the experimental subject maintains wakefulness in a semi-recumbent posture and remains under dim light conditions for extended periods [50-52]. Meals are replaced by frequent nutritionally-balanced snacks. Thus, these conditions are designed to limit and more evenly distribute the factors that could evoke perturbations in measured endogenous rhythms such as body temperature.

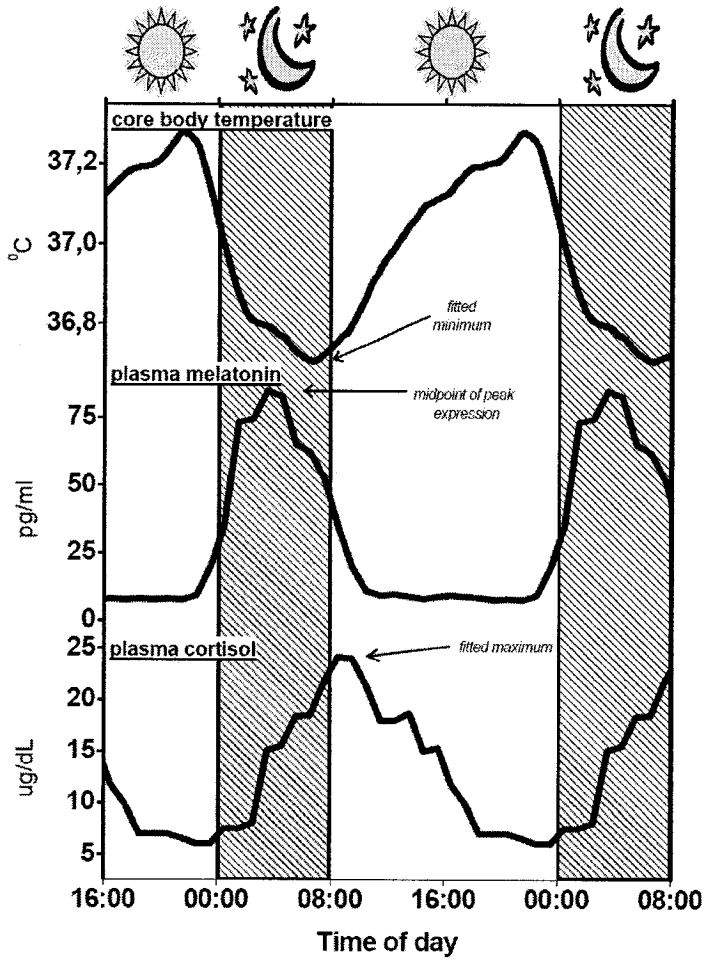

Figure 1.1. Markers of endogenous circadian rhythmicity observed in humans. The typical daily variation in circadian rhythms of core body temperature (upper panel), plasma melatonin concentration (middle panel), and plasma cortisol concentration (bottom panel) are given in their habitual relationship to the sleep/wake cycle. Circadian phase of the temperature rhythm may be described as the time of the fitted minimum, which normally occurs in the last hours of the sleep episode. Circadian phase of the melatonin rhythm may be described as the time of midpoint of peak concentration, which typically occurs in the middle of the habitual sleep episode (hatched area). Circadian phase of the cortisol rhythm may be described as the time of the fitted maximum, which occurs in the early hours of the wake period under entrained conditions.

1.1.2b Hormonal rhythms

Melatonin

Well-described among hormones that display circadian rhythmicity is the melatonin rhythm. The production of melatonin is controlled by the SCN via a neuronal pathway to the paraventricular nucleus (PVN). The PVN is an essential relay in the regulation of the melatonin rhythm, and its neurons project to the superior cervical ganglion which innervates the pineal gland [53, 54]. The variation of plasma melatonin concentration is a bell-shaped curve when plotted against time of day, where the peak of melatonin concentration typically occurs in the middle of the night and daytime levels are very low [55] (**Figure 1.1**). Lesion of the SCN results in a loss of melatonin rhythmicity at intermediate levels suggesting that the normal melatonin rhythm is the result of a daytime inhibitory and nighttime stimulatory net input from the SCN [56]. Descriptions of the melatonin rhythm are often used as more reliable markers of circadian phase since it is less influenced by daily activity than the core body temperature rhythm [57]. Since light can rapidly suppress melatonin production in humans [58], this rhythm is also reliably assessed under dim light conditions such as in a CR procedure [50].

Cortisol

The daily variation in plasma cortisol levels also demonstrates a strong circadian component. Indeed, electrolytic lesion to the SCN abrogates the circadian rhythm in plasma corticosterone in rats [59]. The rhythm typically has its trough in the early hours of the nocturnal sleep episode and peaks at the end of the sleep/darkness episode in the morning [55, 60, 61] (**Figure 1.1**). The human SCN has direct projections to the PVN which secretes corticotrophin releasing hormone (CRH), leading to the release of adrenocorticotropic hormone (ACTH) by the anterior pituitary and ultimately resulting in cortisol release by the adrenals [62]. The PVN may also send autonomic projections to the adrenal that also act to control the cortisol rhythm [25, 62]. The endogenous cortisol and ACTH rhythms may be altered by early awakening or disrupted sleep [63] and experimental sleep loss may raise mean cortisol levels over days [61, 64], although this is not a consistent observation [65, 66]. The cortisol secretory rate appears to be inversely correlated with the appearance of slow-wave sleep in the electroencephalogram (EEG) [67, 68]. Despite the strong circadian component in its

regulation, the cortisol rhythm may also be affected by factors such as posture in the time of measurement [69], and the timing of meal intake [70].

1.1.2c Observable rhythms in multiple systems

In a similar fashion, a number of the overt rhythms of a variety of physiological and behavioural variables demonstrate an endogenous circadian component when measured under controlled conditions or in time isolation. Circadian rhythms in subjective and EEG-estimated alertness [71-74], neurobehavioural output [75], mood [76], learning [77], electrolyte excretion [78], metabolic rate and respiratory control [79], white blood cell numbers in peripheral blood [80-82], cardiovascular and hepatic function [2] are consistent with an influence of the endogenous pacemaker in multiple systems.

The relative contribution of circadian and sleep/wake dependant regulation may influence the expression of circadian rhythmicity in hormonal rhythms to varying extents. Growth hormone secretion, for example, is mainly influenced by the time of sleep occurrence in the day and the presence of slow wave sleep in the EEG [83, 84] and demonstrates only a relatively minor circadian component [85]. By comparison, melatonin and cortisol are mainly influenced by circadian processes. The repeated measurement of multiple circadian rhythms under controlled behavioural conditions such as the CR, is a useful tool in the study of human circadian rhythmicity and this is the approach that is favoured in the experiments described in the subsequent chapters.

1.1.2d The sleep/wake cycle

Lesion of the SCN in nocturnal and diurnal mammals results in a loss of sleep/wake rhythmicity [86], which is consistent with a role for the SCN in the consolidation of sleep and waking at fairly predictable times of day. The results of laboratory experiments support models of the consolidation of human sleep as the result of a complex interaction including homeostatic and circadian components [72, 87-91]. The circadian phase at which sleep is initiated, and the time passed since a previous sleep episode interact to regulate the sleep episode length [91-93]. Thus, the fraction of the sleep episode scored as wakefulness increases throughout the sleep episode [94, 95]. Sleep parameters measurable with polysomnography such as sleep propensity, sleep latency, sleep efficiency, the fraction of the sleep episode scored as rapid eye movement (REM) stage sleep, REM sleep latency, and the fraction of sigma activity (12-15 Hz, i.e. sleep spindles) in non-REM sleep co-vary with circadian rhythms of core body temperature or

plasma melatonin concentration [73, 91, 93-98]. While sleep is typically initiated shortly after to the temperature maximum, on the falling limb of the core body temperature rhythm [92], initiating sleep close to the temperature nadir results in abbreviated sleep lengths [92, 95, 99, 100].

1.1.3 Light is the primary synchronizer of endogenous circadian rhythms

Arguably, it is not sufficient that an endogenous biological clock simply beats out a rhythm for the expression of physiological rhythms. An endogenous circadian pacemaker that confers a true advantage to an organism in its environment must also be able to respond to its environment, particularly with respect to the cues or *zeitgebers* which indicate the time or duration of the day.

1.1.3a Shifting the circadian pacemaker

In animals, including humans, the pattern of light exposure is the synchronizer that exerts the most powerful influence on the endogenous circadian pacemaker [58, 101-105]. Retinal ganglion cells and the SCN are directly connected via the retinohypothalamic tract, which projects to the SCN [106]. In addition to suppressing nocturnal melatonin production [58], the pattern of light exposure can be planned to rapidly reset the circadian pacemaker to different phases.

A "phase response curve" [101, 107-109] describes the response of the endogenous circadian pacemaker to light stimuli applied at different times of internal day (or, "circadian phases"). In practical terms, it was found that light exposure centered before the minimum of the core body temperature cycle (around 06:00) could result in predictable shifts of core body temperature, melatonin and cortisol rhythms to later times (phase delay) [101, 109-111]. In comparison, a light application centered after the temperature minimum could shift the core body temperature, melatonin and cortisol rhythms towards earlier times (phase advance) [109, 112]. A light pulse of strong enough intensity centered close to the core body temperature minimum could reduce the rhythm amplitude and, if applied repeatedly over several cycles, could elicit larger phase shifts than those centered further away from the temperature minimum [101, 103, 109]. Light applied during the middle of the subjective day may still exert minimal phase shifts [113]. Simply advancing or delaying the sleep/wake schedule on a background of dim light (10-15 lux) is not sufficient to elicit significant circadian phase shifts and observable

rhythms continue to oscillate with the slightly longer-than-24-hour endogenous circadian period [96].

The intensity of the light stimulus is also relevant for its effect on the endogenous circadian pacemaker. The dose-response relationship between the intensity of the light stimulus and its resetting effect on the circadian pacemaker is non-linear [102, 104, 105]. Thus, while it was initially held that the circadian pacemaker would only respond to bright light (> 2,500 lux), it was subsequently found that the biological pacemaker was keenly sensitive to light and that resetting and melatonin suppression could also occur with simple indoor room light exposure [104, 105, 114-116]. This interpretation is consistent with a study from this laboratory, which revealed that the timing of indoor room light of ~380 lux is sufficient to promote 5-h phase advances of the plasma melatonin and core body temperature rhythms, as measured with a CR following a simulated Montreal-London voyage [117] (**Figure 1.2**).

There is increasing evidence to suggest that the integration of light information by the SCN is sophisticated. Three cycles of a 5-hour exposure to bright light (7,000-10,000 lux) produce phase shifts of different direction and magnitude depending on the light intensity preceding and following the exposure period- even when the bright light pulses are all centered at the same circadian phase [101]. The history of light exposure is similarly relevant where the magnitude of light–induced phase advances and delays of the dim light melatonin onset is smaller when subjects maintain a short 6-hour sleep/darkness episode than when the main sleep/darkness period lasts 9 hours [118, 119]. In a similar manner, light-induced melatonin suppression is more effective when the light pulse is preceded by [120] or presented on a background of [121] dim white light compared to brighter light intensities. Additionally, the circadian pacemaker integrates light information in such a way that bright light maintains an effect on the circadian pacemaker when the light pulse is intermittently interrupted by darkness. Delays in the core body temperature and plasma melatonin phase caused by a 5-hour bright light pulse timed before the core body temperature minimum are comparable to those observed when lights are turned off for ~44 minutes of each 90-minute segment of the light exposure period [122, 123].

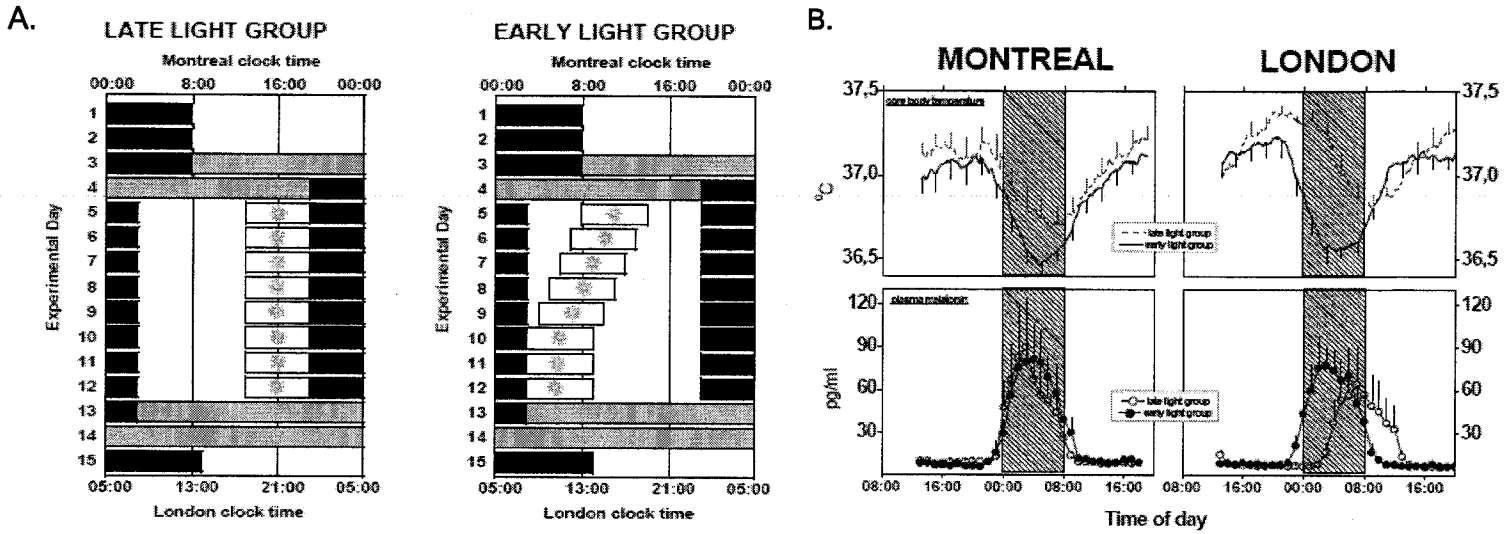

Figure 1.2: An appropriately timed exposure to indoor room light can improve circadian adaptation to simulated jet-lag. (A) The experimental protocol, for late and early light treatment groups, as reported [117]. Upon their admission to the laboratory, subjects maintained their habitual sleep/darkness times (black bars) until sleep times were advanced by 5 hours as of experimental day 5 to simulate a Montreal-London voyage. The London-based sleep schedule was maintained for 7 days, while participants received a daily 6-hour exposure to indoor room light (sun symbols, mean +/- SD: 379+/-10 lux) timed just before bed (late light group) or on an advancing schedule (early light group). Circadian phase was estimated during constant routines (grey bars) performed before and after one week on the shifted sleep/wake schedule. (B) At the start of the study, both experimental groups demonstrate temperature and melatonin rhythms that are appropriately aligned to the Montreal sleep/wake schedule. Typical sleep times on each schedule are shown as hatched areas. Following one week of simulated jet lag (London), the endogenous rhythms remained maladapted in the late light group (dashed lines; open symbols) while an appropriate temporal alignment is regained in subjects on the advancing early light group schedule (solid lines; closed symbols). Figure created from results reported in [117].

1.1.3b Entraining the circadian pacemaker

The effect of light on the human circadian pacemaker is an essential part of the biological role of the central circadian pacemaker. Experimental observations have revealed that the period of the endogenous circadian pacemaker (as estimated with core body temperature, melatonin, or cortisol rhythms) is approximately 24.2 hours [73, 124]. This pacemaker, freed from environmental time cues, would then free-run and physiological rhythms expressed with the endogenous period would become desynchronized with 24-hour day/night cycles. Such a situation has been observed in some blind individuals. For example, the plasma cortisol rhythm in one blind woman free runs with a period between 24 and 24.5 hours as sampled repeatedly during a study spanning 5 days [125]. In another individual, the melatonin rhythm cycles with the endogenous period and occurs at a slightly later time in every subsequent 24-hour day [126]. The core body temperature rhythm of another blind subject free-runs with a period of about 24.22 hours despite the maintenance of a 24-hour schedule [47]. A clock that gradually drifts out of time with the environment poses a problem. Part of the function of photic regulation of the SCN is the phenomenon of entrainment, by which the alignment of the circadian pacemaker is constantly reset to the 24-hour geophysical day [127].

1.1.3c Spectral sensitivity of the circadian pacemaker

Recent evidence has demonstrated that conscious light perception is not necessary to elicit the response of the circadian system to light. While certain individuals lacking conscious visual perception will demonstrate circadian rhythms that are not entrained by the pattern of light/darkness exposure and free-run with the endogenous period, others can demonstrate melatonin suppression with bright light stimulus [128]. Mice that have neither rods nor cones still demonstrate melatonin suppression or phase shifts in locomotor activity following light pulse; a response that is eliminated when the animal is enucleated [129, 130]. The focus of a number of investigations has been the identification of the retinal photopigment that is responsible for the light response of the SCN. Melanopsin-containing retinal ganglion cells project to brain regions involved in non-visual light responses including the SCN [131-133]. Melanopsin-null mice have a reduced, though not abolished, locomotor activity phase shift response to light pulses [134]. However, melanopsin-deficient animals lacking rod and cone function have activity rhythms that do not entrain to the light/darkness schedule [132, 135].

In the rat, melanopsin-bearing retinal ganglion cells demonstrate an intrinsic sensitivity for light of 484 nm [136] although it has been demonstrated that melanopsin expressed in COS-1 cells reconstituted with 11-cis-retinal is most efficiently activated by light of 420-440 nm [137]. In the hamster, monochromatic light of 515 or 476 nm more efficiently induces phase shifts than light of 574 nm [138]. In mice lacking rods and cones, a locomotor activity phase-shifting response is most efficiently generated using light of about 481 nm [135].

In humans, plasma melatonin suppression is most efficiently mediated with light of wavelengths in the 446-477 nm range [139, 140]. This spectral sensitivity is consistent with the identification of melanopsin in human retinal ganglion cells of eyes harvested *post-mortem* [141]. Light of wavelengths below 500 nm more effectively suppress melatonin than light of longer wavelengths, even when the irradiance dose is kept equal [42, 142]. Lenses equipped with filters that block wavelengths below 540 nm are sufficient to counteract the melatonin suppression that would have been incurred by a bright full-spectrum light provided at night [143, 144]. Similarly, shorter wavelength light (e.g. in the violet/blue visible light range) can more efficiently induce significantly larger phase shifts in the temperature and melatonin rhythms than light of longer wavelengths (e.g. in the green visible red light range) [145, 146]. Therefore, the evidence is consistent with the observation that light perception by the circadian system is mainly mediated by non-rod non-cone photoreceptors with sensitivity for wavelengths in the blue visible light range.

1.1.3d Other synchronisers of the circadian pacemaker

Other cues can reset the oscillation of the circadian pacemaker. Certain individuals lacking both conscious visual and circadian light perception with endogenous periods close to 24 hours can be entrained to a 24-hour sleep/wake schedule [147]. Exercise has been proposed as one of the non-photic signals that can entrain circadian rhythms in animals [148, 149]. Indeed, appropriately-timed exercise may induce larger phase delays [150, 151] or phase advances of the plasma melatonin rhythm [49] than those induced by shifts of the sleep/wake schedule on an indoor room light background alone.

There is evidence to suggest that other non-photic cues such as food intake [152], auditory stimuli [153] or exogenous melatonin [154] can significantly affect endogenous circadian rhythms in humans. The effect of these factors on resetting is still, in large part, being established since light exposure was not always carefully controlled

in several early studies [155]. Yet, the influence of non-photic synchronisers on the central circadian pacemaker cannot be completely dismissed.

1.2 Molecular bases of circadian rhythmicity

1.2.1 A functional molecular clock underlies SCN function

The characteristic endogenous rhythmic activity of the neurons of the SCN is dependant on the expression of so-called circadian clock genes. The molecular components of the circadian clock are now known to include, but are not limited to, *Clock* [156] and *Bmal1* [157], three *Period* genes (*Per 1, 2, 3*) [158, 159], and two cryptochrome genes (*Cry 1, 2*) [160] (**Figure 1.3**). The expression of these genes is organized in a series of auto-regulatory loops, with both positive and negative limbs, in a manner that is somewhat conserved throughout evolution [161-163]. In mammals, CLOCK and BMAL1 proteins are transcription factors that dimerize and promote the expression of the *Per* and *Cry* genes as well as clock-controlled genes that are the output of the pacemaker (e.g. neuropeptides [164]). The PER and CRY proteins, in turn, associate in the cytoplasm and act to repress their own transcription in the nucleus [160, 165, 166]. The CLOCK:BMAL1 protein dimer is also thought to promote the transcription of orphan nuclear receptors *Rev-erb* (α and β) and *Ror* (α and β), which in turn modulate the activity at the *Bmal1* promoter [167-170]. Specifically, the REV-ERB protein binds the retinoic acid response element in the *Bmal1* promoter to repress its transcription [167, 171], while ROR activates *Bmal1* transcription [168, 170, 171]. This is such that within the SCN, molecular loops are expressed with the endogenous circadian period of the animal, and individual clock genes demonstrate a circadian rhythm in the levels of transcripts in the cell. In the rodent SCN, the expression of *Per* transcripts peaks in the mid to late part of the light period, *Cry* expression peaks towards the end of the light period and *Bmal1* transcript expression peaks in the middle of the dark phase. Interestingly, this temporal organization is maintained relative to the light/dark cycle in diurnal rodents [172].

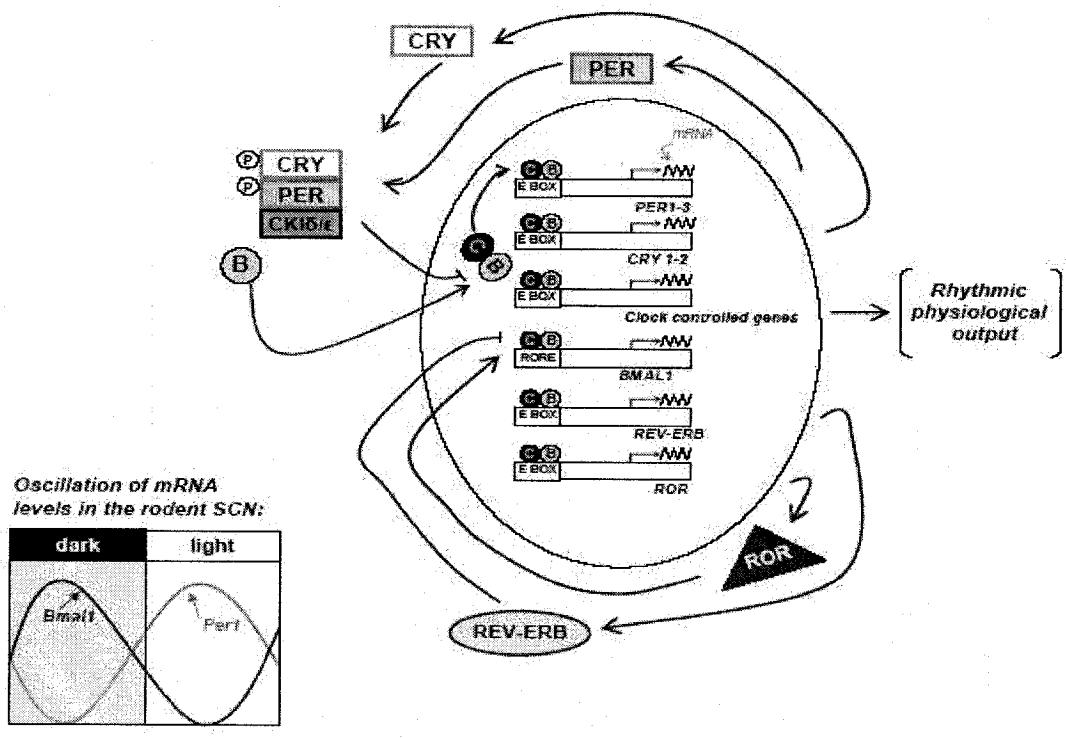

Figure 1.3. The molecular clock. The transcription of clock genes *Per1*, *Per2*, *Per3* and *Cry1*, *Cry2* is induced at their respective promoter E boxes by the CLOCK:BMAL1 protein dimer (C:B symbol). The activity of PER and CRY proteins is modulated post-translationally by phosphorylation and ubiquitination. In this manner, the casein kinases I (δ and ε) play an important role in the regulation of the molecular clock. The PER and CRY protein products then enter the nucleus and negatively regulate their own transcription by acting on the CLOCK:BMAL1 dimer. CLOCK:BMAL1 also regulates the rhythmic transcription of clock controlled genes (e.g. vasopressin production), which may be specifically related to the cell's function. In another arm of the regulatory loop, the activity at the response element *Bmal1* promoter is modulated by retinoic acid-related orphan nuclear receptor response elements (RORE). Specifically, the retinoic acid-related orphan nuclear receptors (RORs) positively modulate activity at the *Bmal1* promoter. The orphan nuclear receptors REV-ERBs repress *Bmal1* transcription by acting at the RORE of the *Bmal1* promoter. **Inset**: The regulatory loops result in a 24-hour rhythmicity in the observed levels of clock gene transcripts. Clock genes *Per1* and *Bmal1* demonstrate rhythms that are in anti-phase to each other.

As a result of the dependence of the SCN on a functional molecular clock, deleting or mutating to the components of the molecular clock may incur observable effects on circadian rhythms. Mice bearing mutations in the *Per1* gene have reduced periods of locomotor activity [158, 159, 173]. *Bmal1* mutant animals are arrhythmic [157] and *Per2* mutant animals display short periods in locomotor activity and become arrhythmic under constant conditions [158, 174]. Animals with a *Clock* mutation demonstrate long periods under constant darkness (DD) conditions and altered clock gene expression rhythms in the SCN [164, 175]. One of the first circadian mutants serendipitously identified was the *tau* hamster mutant which displayed a short period of locomotor activity of approximately 20 hours [176]. The *tau* hamster encodes a mutant casein kinase (Iε) that is deficient in its ability to phosphorylate the PER proteins, thereby profoundly altering the regulation of the period of the molecular clock, and ultimately the period of recorded locomotor activity [177]. Analogous to what had been observed in SCN-lesioned animals, transplanting foetal SCN grafts of genetic clock mutants confers the behavioural phenotype of the donor in the partial rescue of locomotor activity rhythms [178]. Transplanting SCN to genetic mutant animals rescues locomotor activity and clock gene expression in the period of the donor animal [179].

Similar to what is observed in animals with mutations of clock genes, certain aspects of human sleep preference and disorders have also been associated with circadian clock genes. Familial advanced sleep phase disorder is characterized by advanced circadian phase and sleep times [180] and is associated with nucleotide substitutions in the *CKIδ* and *HPER2* genes. These substitutions result in a reduced period in transgenic mice [181, 182] although this appears to be genetically heterogeneous [183]. A haplotype of *HPER3* has been associated with the delayed sleep phase disorder in a Japanese population [184]. The N408 allele of *CKIε* is a marker for other circadian rhythm sleep/wake disorders such as delayed sleep phase disorder and the non-24-hour sleep/wake disorder based on samples drawn from ~135 patients [185]. Similarly, diurnal preference has been associated with polymorphisms in *HCLOCK* [186, 187], *HPER2* [188] and *HPER3* [189, 190].

1.2.2 Functional circadian clocks are also present outside the SCN

A rhythmic expression of components of the molecular clock has also been demonstrated in non-SCN brain regions and non-neuronal tissues. Temporarily exposing fibroblasts to media rich in horse serum (in a so-called "serum-shock" experiment) can

induce the rhythmic expression of *Per1* and *Per2* transcripts [191]. It would then appear that the molecular machinery in non-SCN tissue is functionally similar to that in the SCN. In the SCN, just as in peripheral tissues including the heart, eye and lung in rodents, *Per2* and *Bmal1* mRNA levels peak in anti-phase to each other [192-194]. *Per* transcripts oscillate in neuronal extra-SCN and in non-neuronal tissues with peaks occurring several hours after that observed in SCN culture [191, 195-203].

In an analogy to SCN graft studies, Pando et al [204] established a method of transplanting mouse embryo fibroblasts within a collagen pellet to a new host. By varying the genotype of the graft and the host, they determined that in the presence of a host with a functional clock, grafted fibroblasts will display the characteristics of peripheral oscillators of the host, such that a host-derived signal may rescue some phenotypes in the graft. The abolition of diurnal differences in *Per* expression in peripheral tissues following SCN ablation [205] supports this view. SCN-lesioned mice do not demonstrate day/night differences in mRNA levels of *Per1*, *Per2* or *Bmal1* in the liver or kidney, until their blood stream is physically connected to SCN intact animals whereupon the differences in clock gene expression are restored [206]. Similarly, fibroblasts plated in the presence of SCN cells demonstrate a coherent rhythm in their clock gene expression that is delayed relative to that of SCN cells, and that is lost when they are plated in the absence of SCN neurons [207].

Cell cultures derived from tissues of SCN and extra-SCN origin can demonstrate rhythmic expression of *Per* transcripts, which may be particularly robust in certain tissues (such as the pituitary or the liver) [195, 208]. That clock gene expression in non-SCN tissue could not be observed over sustained periods was thought to signify that the SCN was necessary for the sustained function of these peripheral clocks.

Recent evidence has served to challenge the notion that the function of peripheral oscillators is singularly dependant on the central circadian pacemaker. Rat-1 fibroblasts transfected with a luciferase reporter fused to the *Bmal1* or *Per2* genes revealed that individual cells demonstrate independant expression of clock gene-driven luminescence, with periods that averaged close to 24 hours but that varied between ~19 and 30 hours [209]. Contrary to the dampening in amplitude that was thought to occur, rhythmicity in these cells became synchronised to each other with serum shock, and then gradually desynchronized from each other [209]. Increasing time in culture accounted for a loss in mean estimated amplitude [209]. Similarly, individual fibroblasts

demonstrate circadian rhythms in clock gene expression when a fluorescent reporter is fused to the *rev-erbα* gene [210]. The rhythmicity in the expression of a modified clock protein can be maintained over several cycles in multiple tissues even when the peripheral organs are harvested from SCN-lesioned animals [211]. Instead, the phase of peak PER2:LUCIFERASE expression is desynchronized between many explants from SCN-lesioned mice leading to an apparent loss of rhythmicity within the tissue [211]. Similarly, fluorescence reporting associated with *Bmal1* transcription has been used to demonstrate sustained rhythmic activity in the liver over 3 weeks [200]. In culture, the expression of a reporter fused to the *Bmal1* gene is out of phase in individual fibroblasts and this accounts for the loss in mean amplitude over time [210]. This evidence is consistent with the truly functional nature of peripheral circadian oscillators. The SCN is currently proposed as the mechanism by which the cells constituting peripheral clocks are synchronized.

Proposed mechanisms for the mediation of SCN direction have included diffusible factors from the SCN [212], adrenergic innervation [213], signalling via glucocorticoid receptors [214] or hormone-induced interaction with retinoid receptors [215]. Interestingly, although dominance may be ultimately relegated to the SCN, the nature and mode of control of a diffused factor on the expression of peripheral clock genes may be tissue-specific [202, 215]. For example, transplanting the SCN from a genetic mutant hamster results in the ablation or restoration of clock gene rhythms in certain tissues but not in others [179]. The existence of functional circadian oscillators in the SCN and in the periphery does not preclude the importance of the relationship between them.

1.2.3 Identifying human peripheral circadian oscillators

Zanello and colleagues first showed the expression of clock genes in the human periphery by detecting *HCLOCK* and *HPER1* transcripts and proteins in human keratinocytes, fibroblasts and melanocytes [216]. Biopsied colonic crypts also contain *HPER1* and *HPER2* mRNA and their protein products [217]. Just like non-human tissue culture, human tissue culture can demonstrate rhythmic clock gene activity. Neuroblastoma cells transfected with an *HPER1* promoter-luciferase reporter exhibit promoter show continued rhythmic oscillation during two cycles following stimulation with serum-rich medium [218].

Table 1.1 Expression of clock genes in human peripheral tissues.

Abbreviations: PBMCs- Peripheral blood mononuclear cells, LD- Light/Darkness cycle; CR- constant routine)

Year *(Ref)*	Tissue	Conditions	Genes	Observations
Assessment of rhythmicity under LD or CR conditions:				
2001 *[219]*	Oral mucosa, skin	~ 4-hour sampling for 24-hours	*HPER1, HBMAL1, HCRY1, HTIM, HCLOCK*	Significant circadian rhythmicity in *HPER1*, *HBMAL1*, *HCRY1* transcript levels. Greater variability, lower amplitude in skin
2002 *[220]*	PBMCs	Sampling at 09:00 and 21:00	*HCLOCK, HPER2*	Morning/evening differences in *HPER2* expression
2003 *[Ch. 2]*	PBMC	~2-hour sampling during 35-hour CR	*HPER1-3, HDEC1*	Significant circadian rhythmicity in transcript levels sustained under constant conditions
2004 *[221]*	Mono and polymorpho-nuclear cells	~2-hour sampling during 26-hour modified CR	*HPER1*	Significant circadian rhythmicity in transcript levels in both cell subpopulations. Secondary peak in *HPER1* expression in polymorphonuclear cells
2005 *[222]*	PBMCs	~4-hour sampling during 24 hours	*HPER2, HBMAL1, HREV-ERB*	Peaks in *HPER2* and *HBMAL1* expression segregate into morning and late molecular chronotypes
2006 *[Ch. 3]*	PBMC	-2 hour sampling during 72 hours	*HPER1,HPER2, HBMAL1*	Comparable circadian rhythmicity in *HPER1*, *HPER2* transcript levels in habitual sleep/wake cycle and under constant conditions
Culture of human biopsies				
2005 *[223]*	Skin, hair root, keratinocytes, monocytes	Primary cell culture	*HBMAL1* luciferase reporter	Inter-tissue variability in amplitude of clock gene expression; inter-individual variability in period of expression
Following an intervention				
2005 *[224]*	Whole blood cells	Patient with non-24 hour sleep/wake syndrome	*HPER1-3, HCLOCK, HBMAL1*	Combined therapy including bright light (2,500-3,000 lux), exercise, melatonin, methyl B_{12},, methylphenidate hydrochloride alters clock gene expression
2005 *[225]*	PBMCs	Sampling before and after 24-hour blue light exposure	*HBMAL1, HCRY1*	Inter-individual variability in measured levels
2006 *[Ch. 5]*	PBMCs	Simulated night shift work with light intervention	*HPER1,HPER2, HBMAL1*	Re-alignment in melatonin, cortisol and clock gene expression after nine days

Serum shock performed in human fibroblasts from different cell lines (lung, myeloid, epithelioid) resulted in an immediate increase in *HPER1*, *HPER2* transcripts with an intermediate increase in *HBMAL1* [226]. *HPER1* transcription is induced in autopsied human bronchial epithelia cells in culture stimulated by fenoterol and procaterol (β-adrenoreceptor agonists) for 1h [227]. Dexamethasone stimulates *HPER1* expression in human bronchial epithelial cells and in PBMCs *in vitro* [228]. The serum shock response of human tissues in culture is also evident in the induction of *HPER2* oscillation in vascular smooth muscle cells or the phase shift in this rhythm with retinoic acid [215].

Using human skin biopsies and oral mucosa punches obtained under local anaesthesia at 4-hour intervals, Bjarnason and colleagues have ascertained that *HPER1*, *HCRY1*, and *HBMAL1* all oscillate rhythmically in human peripheral tissues [219]. More precisely, in oral mucosa they identified a peak expression of *HPER1* early in the active (light) period, a peak of *HCRY1* later in the active period and a peak of *HBMAL1* at almost 12 hours from that of *HPER1.* In skin, the time of peak *HBMAL1* expression was similar, while the expression of *HPER1* occurred later. More recently, Takata and colleagues [220] have described the expression of HPER2 in human peripheral blood mononuclear cells (PBMCs) at two time points during the day. They determined in a group of 9 healthy volunteers that the expression of *HPER2* in PBMCs at 09:00 was significantly greater than that determined at 21:00 hours. Using a modified CR, Kusanagi and colleagues confirmed the results discussed in Chapter 2 of this thesis by sampling white blood cells and determined *HPER1* expression in mononuclear and polymorphonuclear white blood cell subsets [221]. Teboul and colleagues have shown that a significant circadian variation in the expression of *HREV-ERBα*, *HBMAL1* and *HPER2* mRNA in human PBMCs using sampling in medical residents during their habitual sleep/wake schedule with the exception of being briefly awakened for night samples [222]. Studies on human clock gene expression (with an emphasis on clocks in circulating blood cells) are summarized in **Table 1.1**.

1.2.4 Synchronizing peripheral circadian oscillators

Shifts in the light/dark cycle resynchronize peripheral oscillators more slowly than the SCN and phase shifts in peripheral tissues may follow shifts observed in the SCN by several days [195, 208, 229, 230]. As a result, desynchronization may exist between tissues or between the SCN and peripheral clocks following a shift in the light/darkness

schedule. This presents an interesting concept in its similarity to the desynchronization that exists between hormonal and behavioural rhythms following transmeridian flight or shift work. It remains unclear whether the light responsiveness of clock gene expression occurs in the SCN only. Thirty minutes of exposure to 2,000 lux white light can induce *Per1* expression in the adrenals of transgenic mice [231]. However, this induction is abrogated in SCN lesioned animals which suggests that the responsiveness of at least some peripheral tissues is SCN-dependant.

Very little evidence on the effect of light on human peripheral clocks is available. *HCRY1* and *HBMAL1* expression in PBMCs sampled from neonates before and after 24 hours of blue light exposure with covered eyes suggests a slight decrease in the transcription of *HBMAL1* and an increase in *HCRY1* transcription [225]. However, this experiment reported only two time points, and the experimental results include a reduction in melatonin levels that suggest that a change in melatonin secretion (either suppression or a phase shift) was induced by light [225]. Thus, interpretation of these results is difficult. It was also recently demonstrated that levels of *HPER2* transcripts in oral mucosa are greater in the presence of a nocturnal blue light pulse (460 nm) than when participants were exposed to green light (555 nm) or darkness in the same period [232].

In experiments with nocturnal rodents, it has been demonstrated that the restriction of food-availability to the light period results in a shift in the liver expression of *Per1,2,3, Cry1* and clock-controlled genes [233-235]. Observed shifts in peripheral tissues such as the liver or kidney may be modulated by the presence of glucocorticoids since phase shifts occur more rapidly in adrenalectomized animals and animals lacking functional glucocorticoid receptors [236]. Daytime restricted feeding also results in changes in *Per1* and *Per2* transcripts and PER2 protein expression in brain areas including the cortex, hippocampus and limbic forebrain [237, 238]. Importantly, the restricted feeding regimen in these experiments elicited no phase shifts in SCN clock gene expression. Considering the mechanisms by which the SCN may contribute to the coordination of peripheral clocks, it is noteworthy that an artificial environmental temperature cycle, designed to oppose the light and darkness phase (with warmer temperatures during darkness and cooler temperatures during the light period) can shift the expression of *Per2* and *Cry1* in mouse liver, without any significant effect on their expression in the SCN [239]. These results are consistent with the hypothesis that

peripheral oscillators may become synchronized by inputs from the SCN (as suggested by the effect of light to which the SCN would be sensitive [232]) or by the direct effect of non-photic synchronizers.

Krauchi and colleagues have examined the effects of early and late carbohydrate-rich meals on circadian phase of core-body temperature, heart rate and salivary melatonin [152]. While they observe significant differences between groups in the core body temperature and heart rate rhythms in the intervention group, no differences were observed in the time of onset of melatonin and it remains difficult to discern which elements of these changes may be accounted for by masking effects of meals administered immediately before the evaluation. Nevertheless, these results do not preclude the possibility that non-photic cues such as food intake may also influence peripheral oscillators in humans.

1.3 Shift work: A case of circadian misalignment

Humans may be unique in their capacity and desire to deliberately counteract the endogenous circadian clock and sleep at atypical circadian phases. This is the case for night shift workers who remain affected by a circadian misalignment with their atypical sleep/wake schedule. This may not be without consequences: night shift work has been associated with an increased risk for cardiovascular pathology [240, 241], the development of insulin-resistance markers (i.e. hyperglycaemeia, hypertension, hypertriglyceridemia) [242] and gastrointestinal illnesses [240, 243], cancer [244-246], pregnancy complications [247] or high numbers of events for which they were hospitalised or required medical consultations [248].

The number of shift workers who reveal appropriate circadian phase shifts in the absence of a circadian intervention are in the minority. In most individuals, circadian adjustment of cortisol and melatonin circadian rhythms remains incomplete [249-253]. This is particularly true on the first night shift worked in which endogenous core body temperature and endocrine rhythms continue to display their habitual entrainment to a day-oriented schedule. Maladaptation to the night shift may persist despite consecutive shifts worked: nurses working two consecutive night shifts with no intervention demonstrate persistently day-oriented rhythms of plasma cortisol and urinary excretion rate of 6-sulfatoxymelatonin [254] and night guards continue to demonstrate high nighttime plasma melatonin levels and elevated plasma cortisol towards the end of the night shift despite a week of consecutive night shifts [250].

A number of studies have applied the phase and dose response curves to light to promote circadian adaptation to night shift work. Initial laboratory studies demonstrated that an appropriately timed bright light stimulus (5,000-12,000 lux) could induce a phase shift of the circadian pacemaker to adjust to the schedule of a typical night worker [255, 256]. Light exposure planned in conjunction with times of light avoidance (with the use of sunglasses or a stable sleep/darkness schedule) is also associated with appropriate circadian phase shifts in simulated night shift work and jet-lag [257, 258]. Bright light of 1,000-4,000 lux presented during three simulated night shifts in the laboratory results in a 6.25-hour delay in the core body temperature rhythm in middle-aged (40-60 years) subjects [259]. Medium intensity light of ~1,230 lux for six simulated night shifts is sufficient to shift the core body temperature minima into the daytime sleep/darkness period [260]. Gradually delaying bright light exposure together with the sleep/darkness period that follows it results in delays in the core body temperature and acrophase of 6-suphatoxymelatonin that are comparable to the shift in the sleep/wake cycle [261]. As suggested by the sensitivity of the human circadian pacemaker, intermittent exposure to bright light (5,000 lux) during simulated night shift work such that one third of each hour is spent in indoor room light (of 500 lux) was sufficient to delay temperature minima into the daytime sleep period [262]. Similarly, the proportion of subjects demonstrating large delays in the temperature rhythm was larger when either a 3 or 6-hour bright light pulse was used compared to when participants remained in room light throughout the simulated night shift [263].

In a manner that is consistent with the effect of light on the endogenous circadian pacemaker, the pattern of light and darkness spontaneously selected by night shift workers can be associated with adaptive phase delays that permit the alignment of physiological rhythms with the shifted sleep/wake cycle. Specifically, the times selected for sleep are important in determining the magnitude of phase shifts induced by night time bright light exposure [264]. Nurses who demonstrate appropriate delays in 6-sulphatoxymelatonin secretion were more likely to go to bed in the morning and consequently avoid daylight at times of day that could counteract circadian adaptation [265]. Similarly, night shift workers who maintain stable bedtimes (thereby controlling the pattern of light/darkness exposure throughout the day) are more likely to demonstrate adapted salivary cortisol rhythms [253]: workers who avoided morning bright light exposure had further delayed circadian rhythms of cortisol and melatonin

[251]. A number of studies have stressed the importance of protection from morning light on the commute home [264, 266] since bright light exposure at that time could enforce the maintenance of a day-oriented circadian alignment. Field studies on the night shift worker population have been of fundamental importance in demonstrating the efficacy of bright light in the workplace and the importance of the pattern of light/darkness exposure for adaptation to atypical work schedules [265, 267, 268]. Using various combinations of sunglasses, darkened sleep quarters and bright light during night shifts results in longer daytime sleep and improved performance and vigilance measures during simulated and real night shifts [269, 270].

Together, night shift work and the use of the light/dark schedule to shift the central circadian pacemaker in the SCN provide a useful and practical background on which to explore the expression of a human peripheral circadian oscillator when challenged to adapt. Shifted sleep/wake and light/dark schedules can provide useful paradigms to explore the relationship of central and peripheral circadian oscillators in humans. An abrupt change in the sleep/wake cycle may reveal differences in the resetting capacity of peripheral oscillators relative to the central circadian pacemaker of the SCN. Establishing the existence of a functional circadian oscillator in the human periphery then provides a novel context in which the consequences of an internal desynchronization between oscillators can be explored.

1.4 Thesis rationale and goals

At the time that these experiments were started, very little information existed on the expression of peripheral circadian oscillators in humans. The experiments described in this thesis test the general hypothesis that an observable rhythmic expression of circadian clock genes exists in the human periphery. Specifically, clock gene expression in white blood cells isolated from serial blood samples was measured. These peripheral blood mononuclear cells (PBMCs) include monocytes and lymphocytes but not polymorphonuclear cells [271]. There is a demonstrated circadian rhythm in the numbers of PBMCs with a peak in cell numbers during the habitual sleep episode [81, 82]. These cells are easily isolated from whole blood on a density gradient and thus provide an ideal means by which to sample the clock throughout a circadian cycle. The experiments are designed to first measure the expression of clock genes under constant conditions and in the presence of a standard sleep/wake cycle, and to establish the relationship of these

peripheral oscillators with the SCN-driven rhythms of core body temperature and plasma melatonin secretion. As described for the initial identification of endogenous circadian rhythmicity in humans, it was expected that the presence of a functional peripheral circadian oscillator in PBMCs *in vivo* would manifest itself as a rhythm detectable under different behavioural and environmental conditions. This study had not yet been carried out in a putative peripheral circadian oscillator in humans. The resetting capacity of peripheral circadian oscillators in PBMCs was also then tested in a night shift work simulation with a bright light intervention expected to realign SCN-driven rhythms. To my knowledge, this is the first time that peripheral clock gene expression has been measured in humans following a shifted sleep/wake schedule. Thus the novel hypotheses behind the work presented here are:

I. **A significant circadian expression of circadian clock genes exists in human PBMCs.** This significant circadian expression of clock genes could be detected under constant behavioural and dim light conditions when the masking effect of sleep, activity and meal intake is minimized. The expression of clock genes in the peripheral oscillator of PBMCs would demonstrate a stable relationship with the endogenous melatonin circadian rhythm as detected in plasma. Chapter 2 (*Circadian clock genes oscillate in human peripheral blood mononuclear cells*) describes the expression of *HPER1*, *HPER2*, *HPER3* and *HDEC1* detected in PBMCs at ~120-minute intervals during a 35-hour constant routine procedure.

II. **The significant circadian expression of circadian clock genes in human PBMCs could be measured in the presence of a habitual sleep/wake cycle.** Clock gene expression would maintain a stable relationship with SCN-driven rhythms of plasma melatonin and cortisol secretion. Chapter 3 (*Expression of clock genes in human peripheral blood mononuclear cells throughout the sleep/wake and circadian cycles*) compares the expression of *HPER1*, *HPER2* and *HBMAL1* quantified under both constant routine conditions and in the presence of a habitual sleep/wake cycle.

III. **The peripheral oscillator in PBMCs could become realigned to a shifted sleep/wake schedule in the presence of a light intervention known to shift endogenous circadian rhythms of melatonin and cortisol.** Chapter 4 (*Controlled*

exposure to light and darkness realigns the salivary cortisol rhythm in night shift workers) describes the effect of a light intervention designed to significantly delay the SCN-driven endogenous cortisol rhythm. Chapter 5 (*Entrainment of circadian rhythms of melatonin, cortisol and clock gene expression to simulated night shift work*) describes a laboratory experiment of simulated night shift work, with a light intervention. The expression of *HPER1*, *HPER2* and *HBMAL1* transcripts is quantified in cells sampled at the beginning, the middle and the end of nine days on a night shift worker's schedule.

CHAPTER 2

Circadian clock genes oscillate in human peripheral blood mononuclear cells.

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2.1 Preface

At the time that this investigation was initiated, the presence of a circadian oscillator had been established in oral mucosa and skin (12). Testing *HPER2* expression in PBMCs sampled in the morning and the evening, suggested a diurnal difference in clock gene expression, but could not establish whether a circadian rhythm was present (13). The object of the present investigation was to perform repeated measurements of clock gene expression in PBMCs sampled from research participants living under constant conditions to test the hypothesis that a significant circadian expression of circadian clock genes exists in human PBMCs.

2.2 Abstract

In mammals, it is well documented that observable circadian rhythms are controlled by a central oscillator that is organized in transcriptional and translational feedback loops involving several clock genes. Although recent studies have demonstrated that clock genes oscillate in many peripheral tissues, their characteristics in the human immune system remain unknown. The present study investigates whether circadian clock genes function in human peripheral blood mononuclear cells. On the basis of studies derived from 3 human subjects under controlled conditions, circadian clock genes *HPER1*, *HPER2*, *HPER3*, and *HDEC1* are expressed in a circadian manner in human peripheral blood mononuclear cells (PBMCs), with the peak level occurring during the habitual time of activity. The demonstration of functional circadian machinery in human PBMCs suggests that peripheral blood cells may be useful for the investigation of human circadian rhythms and their associated disorders.

2.3 Introduction

In humans, as in other animal species, most physiological and behavioural functions are expressed rhythmically across days and nights. These daily rhythms, referred to as circadian, are controlled by self-sustained biologic oscillators. In mammals, primary neuronal cultures as well as ablation and transplantation studies indicate that the central component of this complex oscillatory system resides in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (1,2). Molecular components of the circadian oscillator in the mouse have been found to involve transcriptional-translational feedback loops of at least 10 genes, namely, *mPer1-3*, *mCry1-2*, *Clock*, *BMal1*, *Tau* (casein kinase Iε), *NPAS2*, and *Dec1-2*. Of these, *mPer1-2*, *mCry1-2*, and *Dec1-2* are negative regulators, whereas *BMal1* and *Clock/NPAS2* are positive regulators (3-5). In addition,

Tau (casein kinase Iε) binds and phosphorylates period proteins (PERs), thereby posttranscriptionally regulating their stability.

Studies from rodents have shown that the molecular clock mechanisms oscillate not only in the central nervous system, but also in peripheral organs, such as the liver, kidney, and fibroblast cells (6). Given that the many immune parameters, such as cytokine synthesis and white blood cell counts, fluctuate in a circadian fashion (7), we hypothesized that a veritable circadian expression of functional clock components may be observed in human peripheral blood mononuclear cells (PBMCs) and, therefore, may play certain roles in controlling the immune circadian physiology.

In this brief report, we present the expression profile of circadian clock genes *HPER1*, *HPER2*, *HPER3*, and *HDEC1* in human PBMCs from 3 human subjects studied in a time-free environment. As human blood is widely used in clinical settings, our approach may not only provide a useful tool to elucidate the molecular mechanisms of human circadian rhythms, but also prompt the investigation of new means to diagnose and treat sleep/wake cycle and mood disturbances associated with jet lag, shift work, and a variety of medical and psychiatric disorders.

2.4 Study design

Three physically and mentally healthy young men, aged 20, 22, and 27 years, were recruited from the community. Each provided an informed consent in accordance with the guidelines of the Douglas Hospital Research Ethics Board (Montreal, QC, Canada). For 3 weeks prior to their admission, subjects kept regular sleep times, restricted to a single 8-hour nocturnal sleep episode. This procedure served to ensure that the circadian pacemaker was well stabilized relative to the sleep/wake schedule.

Each subject was studied individually in a time-free, light-controlled laboratory room for at least 5 consecutive days. After 3 baseline days on their habitual schedule, subjects underwent a 35-hour constant routine procedure designed to unmask the endogenous circadian oscillation of physiological parameters. This procedure consists of a regimen of enforced waking, in a semirecumbent posture, under very dim light conditions (approximately 4 lux), with minimal levels of activity and with nutritional intake divided into hourly snacks. Indeed, the expression of physiologic markers known to be controlled by the circadian pacemaker may be confounded by levels of light exposure, meals, or postural changes. Thus, sampling for approximately 1.5 days under these

conditions allows us to investigate at least one complete circadian cycle with limited masking of the endogenous circadian expression.

Plasma melatonin concentration was determined by means of commercially available radioimmunoassays (Stockgrand, Surrey, United Kingdom). Every 120 minutes, PBMCs were isolated from heparinized blood samples by means of Histopaque-1077 (Sigma, St Louis, MO) gradient centrifugation, washed, and subsequently frozen at 80°C. The total RNA of each sample was extracted by means of Trizol reagent, and the cDNA was generated with Superscript II reverse transcriptase. We performed a TaqMan quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) to determine the level of *HPER1*, *HPER2*, *HPER3*, and *HDEC1* expression relative to the housekeeping gene *HCDK4* by means of the standard protocol described by Applied Biosystems (ABI, Foster City, CA).(9)

On the basis of the published sequences,(10) the primer sets for *HPER1*, *HPER2*, *HPER3*, *HDEC1*, and *HCDK*4 are designed as follows:

HPER1 forward primer,

5'-TTCCTGACGGGCCGAAT-3';

HPER1 reverse primer,

5'-CGCTTGCAACGCAGCA-3';

HPER1 TaqMan probe,

5'-FAM-TCTACATTTCGGAGCAGGCAGCCG-Tamra-3';

HPER2 forward primer,

5'-CCACGAGAATGAAATCCGCT-3';

HPER2 reverse primer,

5'-CCCGCACCTTGACCAGG-3';

HPER2 TaqMan probe,

5'-FAM-CCACCCCTTCCGCATGACGC-Tamra-3';

HPER3 forward primer, 5'-TTTCCTAATGTCGCCGAAGAG-3';

HPER3 reverse primer,

5'-CCTGGTATGTCATGAGAATGCG-3';

HPER3 TaqMan probe,

5'-FAM-ATCTGGAGAATGATACGGCAGACACCTGA-Tamra-3';

HDEC1 forward primer,

5'-GAGAATCGGAGAAGGGCGAC-3';

HDEC1 reverse primer,
5'-GCGTCCGTGGTCACTTTTG-3';
HDEC1 TaqMan probe,
5'-FAM-TGCGCAGTGAGCAGCCGTGC-Tamra-3';
HCDK4 forward primer,
5'-GAGGCGACTGGAGGCTTTT-3';
HCDK4 reverse primer,
5'-GGATGTGGCACAGACGTCC-3';
HCDK4 TaqMan probe,
5'-FAMAGCATCCCAATGTTGTCCGGCTGA-Tamra-3'.

A dual-harmonic regression model was used on individual curves for the expression of each gene with the use of a period search from 23.91 to 24.45 hours (11). As reported previously (12), a 12/24-hour composite model explains a greater amount of variance in the data. The circadian variation of transcriptional expression was considered significant if the 95% confidence interval (CI) describing the amplitude of the first harmonic did not include the zero axis. We also performed cross-correlation analyses between the variation in the expression of clock genes and that of the plasma melatonin secretion, a reliable circadian marker.

2.5 Results and discussion

Regression analyses indicated that transcripts of all clock genes studied displayed a daily rhythm that was significantly different from zero with the exception of the expression of *HDEC1* for subjects S13 and S20 (**Table 2.1**). Peak clock gene expression was observed mostly during the usual time of activity and light exposure. Transcript levels of all 4 genes were found to correlate positively and significantly with the secretion of plasma melatonin, a reliable circadian marker. Peak clock gene expression followed the peak of melatonin concentration by a maximum of 9 hours (**Figure 2.1**). These results are consistent with animal studies since the oscillation of clock genes in peripheral tissues often lags several hours behind that of the SCN of the central nervous system.

Our findings of a significant oscillation of *HPER* mRNA levels across circadian phases in PBMCs of human subjects are consistent with the results of a prior study (12) conducted under ambulatory conditions on oral mucosa and skin biopsies. One study also revealed that the expression of *HPER2* in PBMCs is significantly different in the

morning and the evening.(13) The enforcement of constant conditions in the present study implies that the significant circadian oscillation we observe is not explained by postural changes, alteration in exposure to light, food intake, or the stress induced by needle insertion or biopsy punches. The use of the constant routine procedure also reveals that the oscillation of clock genes in PBMCs is present even in the absence of sleep/darkness episodes, an observation that supports its endogenous nature. While interindividual variability may account for the reduced regression amplitude observed for *HDEC1* transcripts in 2 subjects, it is possible that the period of awakening associated with the constant routine may have influenced our observations. Further investigation into the expression of clock genes under a variety of conditions will elucidate the influence of sleep and waking on clock gene expression in humans.

Although limited to a few subjects, our results support the presence of a significant and cyclic transcription of clock genes in peripheral PBMCs of healthy controls. They also demonstrate the feasibility of studying PBMCs as an accessible surrogate for the identification of rhythmic clock gene expression in humans. Experimental tools using this technique could thus be refined in humans to investigate the effects of various drugs on the sleep/wake cycle and endogenous circadian rhythms. Future studies should clarify the time relationship of clock gene expression in the SCN and PBMCs in lower mammals and extend these findings to humans.

Table 2.1

Expression of clock gene mRNA in human PBMCs based on dual-harmonic regression analysis

Gene and subject	Amplitude	95% CI for amplitude	Time of fitted maximum
HPER1			
S12	5.000	3.155 – 6.884	09:26
S13	12.389	6.433 – 18.344	09:06
S20	7.408	2.638 – 12.178	12:23
HPER2			
S12	2.029	0.672 – 3.387	12:28
S13	2.379	1.805 – 2.953	06:43
S20	0.955	0.375 – 1.536	12:35
HPER3			
S12	2.639	1.377 – 3.901	12:28
S13	2.584	0.182 – 4.986	06:23
S20	3.665	0.174 – 7.157	12:19
HDEC1			
S12	22.566	7.598 – 37.533	06:19
S13	2.773	-0.203 - 5.75	09:22
S20	1.723	-0.399 - 3.845	12:19

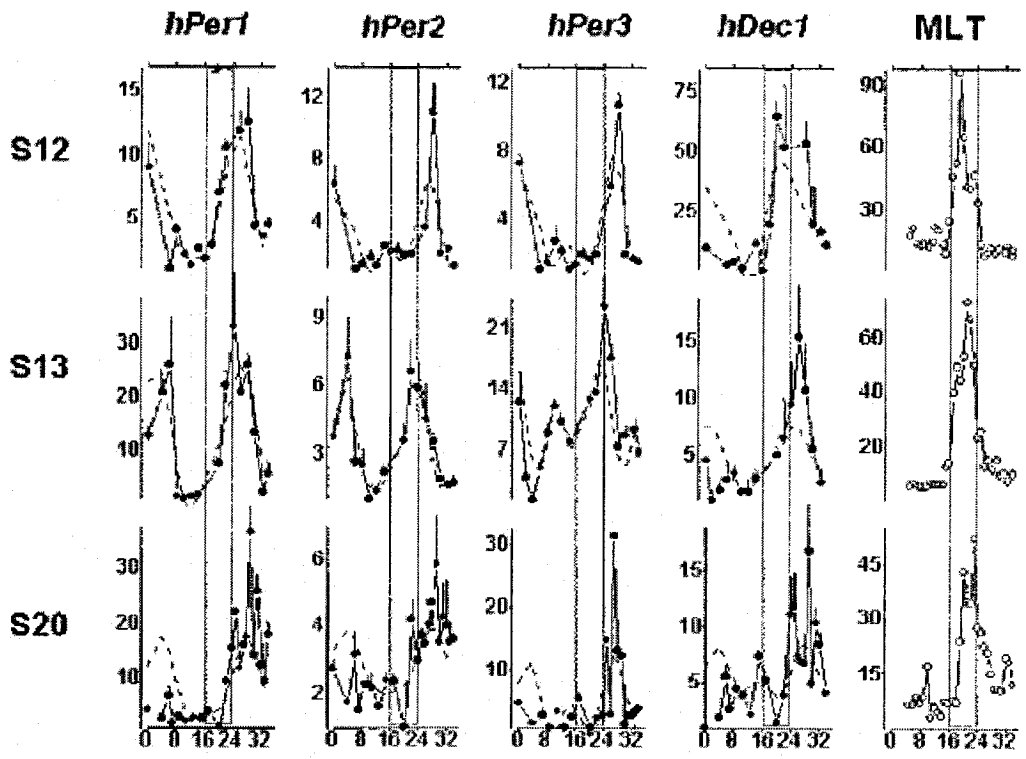

Figure 2.1

Circadian variation of clock gene mRNAtranscripts in human PBMCs. Transcript expression of *HPER1*, *HPER2*, *HPER3*, and *HDEC1* for each subject is shown with a solid line and the dual-harmonic regression with a dotted line. The y-axis represents the relative intensity of mRNA expression. The value of the lowest mRNA expression is designated 1, and the levels of mRNA expression at all other time points are calibrated to this value. Error bars indicate the standard deviation on the basis of the mRNA samples assayed in triplicates. The x-axis indicates the time, in hours, under the constant-routine procedure. In the melatonin (MLT) panel, the y-axis represents plasma melatonin concentration (picograms per milligram) in each subject. For the purposes of illustration, times where subjects are habitually asleep are projected as open rectangles (□).

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CHAPTER 3

Expression of clock genes in human peripheral blood mononuclear cells throughout the sleep/wake and circadian cycles.

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3.1 Preface

The previous experiment (Chapter 2) demonstrated that levels of clock gene transcripts oscillated in a circadian fashion in human PBMCs sampled under a constant routine. A functional circadian oscillator therefore exists in these cells. The present experiment develops and extends these observations. It was important to establish that measuring the expression of these clocks in individuals was possible over sustained periods. It was also important to describe the pattern of clock gene expression under entrained conditions and to compare it with data acquired under constant conditions. The experiments described in this chapter were designed to test the hypothesis that the significant circadian expression of clock genes in human PBMCs could be measured in the presence of a habitual sleep/wake cycle.

3.2 Abstract

Rhythmic expression of circadian clock genes in the neurons of the suprachiasmatic nucleus (SCN) is necessary for the manifestation of endogenous circadian rhythmicity in behaviour and physiology. Recent evidence demonstrating rhythmic clock gene expression in non-SCN tissues suggests that functional clocks exist outside the central circadian pacemaker of the brain. In this investigation, we evaluate the nature of an oscillator in peripheral blood mononuclear cells (PBMCs) by assessing clock gene expression throughout both a typical sleep/wake cycle (LD) and during a constant routine (CR). Six healthy men and women aged (mean ±SEM) 23.7 ±1.6 years participated in this 5-day investigation in temporal isolation. Core body temperature and plasma melatonin concentration were measured as markers of the central circadian pacemaker. The expression of *HPER1*, *HPER2*, and *HBMAL1* was quantified in PBMCs sampled throughout an uninterrupted 72-hour period. The core body temperature minimum and the midpoint of melatonin concentration measured during the CR occurred 2:17 ±0:20 and 3:24 ±0:09 hours before habitual awakening, respectively, and were well aligned to the sleep/wake cycle. *HPER1* and *HPER2* expression in PBMCs demonstrated significant circadian rhythmicity that peaked early after waketime and was comparable under LD and CR conditions. *HBMAL1* expression was more variable, and peaked in the middle of the wake period under LD conditions and during the habitual sleep period under CR conditions. Using sampling every 2 hours over 3 consecutive days, our results compare for the first time clock gene expression in a human peripheral oscillator under different sleep/wake conditions.

3.3 Introduction

The suprachiasmatic nucleus (SCN) of the anterior hypothalamus is the central circadian pacemaker and generates observable daily rhythmicity in physiology and behaviour. When humans live in a time-free environment, centrally-driven rhythms of core body temperature, melatonin and cortisol persist with a period of approximately 24 hours (18, 22, 35, 74, 75). The average ~24-hour rhythmicity in the firing activity of the neurons of the SCN (38, 71, 77) correlates with behavioural rhythmicity (45) and is thought to be the result of the expression of circadian clock genes in an autoregulatory feedback loop (19). In this molecular loop, core clock genes such as *Period* (*Per*) 1 and 2 and *Cryptochrome* (*Cry*) 1 and 2 are induced following the binding of a CLOCK:BMAL1 protein dimer to elements in their respective promoter sequences. PER and CRY proteins form dimers and enter the nucleus where they inhibit their own transcription. In addition to this, the REV-ERBα protein, whose gene transcription is also activated by CLOCK:BMAL1, enters the nucleus and represses the transcription of the *Bmal1* gene. A consequence of these feedback loops is the rhythmicity in messenger RNAs from many of these genes. For example, in the SCN of diurnal and nocturnal rodents *Per1* and *Per2* expression peaks during the subjective day (1, 5, 15, 19, 73, 82) while *Bmal1* expression peaks in anti-phase during the subjective night (15, 19, 60, 64).

The observation that circadian clock genes also rhythmically oscillate in cultured fibroblasts (7, 76) has led to the suggestion that functional circadian oscillators may exist outside the SCN. Indeed, rodent studies have confirmed the presence of circadian pacemakers in multiple physiological systems and rhythmic clock gene expression is present in, among other tissues, the liver (57, 81), the stomach (78), the lung (81), the bone marrow (20, 31), the vasculature (50, 55) and the adrenal glands (39, 58). Rhythmic clock gene expression has also been identified in non-SCN brain regions including the pineal (1), the olfactory bulb (3, 30) as well as regions of the forebrain (4, 42) and the hypothalamus (1). Lesion of the SCN results in the desynchronization in the cellular rhythms in peripheral oscillators (82) or an ablation of gene expression in certain tissues (4, 33). Thus, despite the presence of multiple oscillators, the SCN is still regarded as a master circadian pacemaker.

In an analogy to what has been observed in animal studies, clock gene products are present in human skin tissue culture (83), and rhythmic clock gene expression may be induced in cultured human vascular smooth muscle cells (40, 50), fibroblasts (53) and

neuroblastoma cell lines (48). To date, only a few human peripheral oscillators have been identified in vivo. *HPER1* and *HBMAL1* expression demonstrate significant circadian rhythmicity in the skin and oral mucosa sampled from healthy young men (8). *HPER2* expression in human peripheral blood mononuclear cells (PBMCs) displays day-night differences with two-point sampling under habitual sleep-wake conditions (68). In a prior study including three healthy young men studied under controlled conditions in a time-free environment, we have demonstrated a significant circadian expression of *HPER1* and *HPER2* in PBMCs (9). These results were confirmed by other studies conducted either in similar conditions (41) or during a regular sleep-wake cycle (69). Peripheral circadian oscillators in humans have the potential to become a valuable tool in the understanding of the circadian contribution to various medical disorders. To date, little information exists on the capacity for frequent sampling of peripheral clocks in PBMCs over extended periods. Moreover, little is known on the expression of peripheral oscillators under different behavioral conditions. In this investigation, clock gene expression was quantified in PBMCs sampled every two hours over three consecutive days under both habitual sleep/wake and constant routine conditions.

3.4 Materials and Methods

Subjects

Six healthy participants (four men aged ± SEM: 24.4 ± 2.4 years, and two women aged 22.5 ± 0.4 years) were empanelled in this investigation between January and August 2004 (**Table 3.1**). Potential candidates were recruited via ads placed in local newspapers, and selected candidates were remunerated for their participation in the study. This investigation was performed with the approbation of the Douglas Hospital Research Ethics Board. All participants provided informed consent prior to their empanelment in the study.

Inclusion/Exclusion Criteria

All candidates were verified to be in good physical health following a complete medical interview and examination including electrocardiogram and haematological screen. Calculated body mass indices (BMI) were all within normal ranges. Participants were drug-free, nonsmokers at the time of study and eliminated the intake of all alcoholic or caffeinated beverages in the three weeks preceding the start of the investigation. Candidates had no history of night work or transmeridian travel (across >2 time zones) in the 3 months prior to investigation.

Selected candidates had no evidence of psychopathology on structured interviews (Structured Clinical Interview for DSM-IV). They also had no history of sleep pathologies or disturbances, as confirmed following interview with a sleep disorders physician. Five participants scored as intermediate chronotypes and one participant scored as a morning type on the Horne-Ostberg morningness-eveningness questionnaire (37, **Table 3.1**). All reported good sleep quality with sleep latencies less than 15 min and the absence of nocturnal awakenings or premature morning awakenings.

Both of the female participants had no history of gynecological pathology and reported no pregnancies in the 6 months preceding the study. Menstrual cycles had a duration of 28 and 30 days with a maximum monthly variation of 3 days. Normal ovulation was confirmed by midluteal phase progesterone levels. The investigation was planned during the follicular phase of the menstrual cycle.

Study Protocol

The investigative protocol took place in the facilities of the Centre for Study and Treatment of Circadian Rhythms. Subjects were admitted to windowless, sound-proof and climate-controlled suites each equipped with a private bathroom and a section for meals and leisure. Subjects lived therein for the duration of the investigation without knowledge of time.

Study participants each maintained stable sleep/wake schedules for at least 14 days prior to their admission to the laboratory. The sleep/wake schedule included a single, nocturnal 8-hour sleep episode and naps were prohibited. In order to verify adherence to the sleep/wake schedule, participants were required to call the laboratory voicemail at bed and wake times in addition to completing sleep/wake logs. The sleep/wake schedule was also verified using wrist actigraphy (Actiwatch 64, MiniMitter, Bend, OR, USA) in the last week preceding the study start. The purpose of the imposed sleep/wake schedule was to stabilize the entrainment of the endogenous circadian pacemaker and limit the inter-individual variability in phase relationships between the endogenous circadian pacemaker and the sleep/wake schedule.

The laboratory investigation contained two separate behavioural conditions within the five-day laboratory study. Upon admission to the laboratory, participants maintained the sleep/wake schedules kept in the preparatory phase of the investigation (**Figure 3.1**). Specifically, the midpoint of 8-hour laboratory sleep episode corresponded with the midpoint of reported sleep/darkness times in the pre-study period. Ambient light intensity

measured in the angle of gaze with a research photometer (IL1400A, International Light, MA, U.S.A.) during wake periods was (mean ± SEM) 118 ± 8 lux and participants slept in darkness (~0.03 lux). This segment of the experiment was designed to evaluate central and peripheral clocks in the presence of a light/darkness schedule (LD). Upon awakening on experimental day three, participants maintained wakefulness throughout a 32-hour Constant Routine (CR) procedure (described below). A final, *ad libitum* sleep episode followed the CR. Equipment failure resulted in the interruption of blood sampling in subject S02. The participant subsequently returned to the laboratory to undergo a complete CR procedure, and the resulting data is discussed herein.

Constant Routine

Sleep, meal consumption and light exposure are among the conditions that may mask the endogenous expression of markers of the endogenous circadian pacemaker. The CR is a procedure designed to systematically limit potential masking effects on measured rhythmicity (21, 26, 52). Participants were required to maintain wakefulness throughout the CR procedure. Levels of activity were limited and participants maintained a semi-recumbent posture in bed throughout. Ambient light intensities remained constant and dim (mean ± SEM: 4 ± 0.4 lux), and meals were replaced by small, nutritionally-balanced hourly snacks. Daily caloric requirements, adjusted for reduced activity levels, were evaluated for each individual by a registered dietician and evenly distributed across snacks throughout the CR.

Sampling

Throughout the experiment, we sampled markers of both the central circadian pacemaker and the peripheral clock in PBMCs. For each research participant, we collected whole blood samples at regular intervals throughout a 72-hour period for the subsequent assay of plasma melatonin concentration and PBMC clock gene expression. Upon admission to the laboratory on experimental day 1, each participant had an indwelling catheter inserted in a forearm vein. Our blood sampling system included an intravenous extension which permitted frequent blood sampling for an extended duration without interruption of the participants' sleep. In between blood samples, an infusion of heparinized saline (7 IU/cc at 30cc/hr) was used to reduce the risk of clotting at the catheter insertion site. The volume drawn throughout all blood sampling periods did not exceed 610 mL. Basal plasma melatonin concentrations collected during habitual wake periods were not significantly altered throughout the 72-hour period (see individual

plasma melatonin results in supplementary figures) suggesting that important changes in total blood volume were unlikely.

Core body temperature, a marker of the central circadian pacemaker was continuously sampled via a 10-cm rectal sensor (Rectal-esophageal temperature probe, Cincinnati Sub Zero, Cincinnati, OH USA) and stored to a data acquisition system. For the assay of plasma melatonin concentration, another marker of the central circadian pacemaker, 2.5 mL whole blood samples were collected into K_2EDTA-coated Vacutainers (7.2 mg K_2EDTA, Becton Dickinson, Franklin Lakes, NJ, USA) every ~60 minutes, placed on ice for 15 minutes and centrifuged at 4^{0}C for 15 minutes. Plasma was stored at -80^{0}C until time of assay. Melatonin concentration was determined in duplicata using a radioimmunoassay kit (Stockgrand, Guilford, Surrey, UK), using a standard curve with a concentration range of 2.5- 250 pg/0.5 mL and a least detectable dose of 2.5 pg/mL.

Peripheral blood mononuclear cells were isolated from 10 mL whole blood samples drawn every ~120 minutes. Whole blood was collected into heparin-coated Vacutainers (86 USP units Sodium Heparin, Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged for 30 minutes on a density gradient (Histopaque-1077, Sigma-Aldrich Canada, Oakville, ON, Canada). As specified by the manufacturer, the cell layer retained for analyses excluded polymorphonuclear cells such as neutrophils, basophils and eosinophils and included mononuclear cells such as monocytes and lymphocytes. Isolated PBMCs were subsequently washed with a phosphate buffered saline solution (PBS) and stored at -80^{0}C in Trizol reagent (Invitrogen Canada, Burlington, ON, Canada). RNA was subsequently extracted and precipitated from each sample according to the manufacturer's instructions, and verified for its concentration on an agarose gel. Complementary DNA was reverse transcribed from extracted RNA using MultiScribe Reverse Transcriptase (TaqMan Reverse Transcription Reagents, Applied Biosystems, Foster City, CA, USA). Quantification of clock gene expression was performed by real-time PCR using SYBR Green chemistry (Applied Biosystems, Foster City, CA, USA) on an AB5700 cycler (Applied Biosystems, Foster City, CA, USA). The PCR program used included two minutes at 60^{0}C and 10 minutes at 950C followed by 40 cycles of 95^{0}C for 15 seconds and 60^{0}C for one minute. The expression of the clock genes *HPER1*, *HPER2*, *HBMAL1* were described relative to the expression of *HCDK4* (cyclin-dependent kinase 4) (9) using the following primers:

HPER1

Forward: 5'-TGGCTATCCACAAGAAGATTC-3'

Reverse: 5'-GGTCAAAGGGCTGGCCCG-3'

HPER2

Forward: 5'-GGCCATCCACAAAAAGATCCTGC-3'

Reverse: 5'-GAAACCGAATGGGAGAATAGTCG-3'

HBMAL1

Forward: 5'-GGCTCATAGATGCAAAAACTGG-3'

Reverse: 5'-CTCCAGAACATAATCGAGATGG-3'

HCDK4

Forward: 5'-ATCCCAATGTTGTCCGGCTG-3'

Reverse: 5'-TGATCTCCCGGTCAGTTCGG-3'

In order to avoid amplification of genomic DNA within extracted RNA samples, primer sets were designed to overlap junctions between successive exons, thereby conferring to the reaction a preference for the amplification of reverse transcribed cDNA. The concentration of primers used in reaction mixtures was adjusted for each primer such that the efficiency of amplification for each primer set was comparable over a range of possible cDNA concentrations. The specificity of the amplification with each primer set was ensured by the verification of the dissociation temperature of each reaction product. Each amplification reaction was performed in triplicate.

Statistical Analysis

The core body temperature phase for each individual was defined as the time of fitted minimum of temperature data collected under CR conditions. The first five hours of temperature data collected during the CR were removed for analysis to account for the possible masking effect of the preceding sleep episode. The temperature phase was determined following the application of a dual-harmonic regression model to the data (11) with parameters set for a search within two standard deviations of the expected period of the central circadian pacemaker (22) and without serial correlated noise. Due to the possible masking effects of a sleep/wake schedule on the expression of the endogenous temperature rhythm, core body temperature data collected during the LD subsegment of the investigation could not be reliably used as a marker of the central circadian pacemaker.

The phase of the plasma melatonin rhythm was defined as the midpoint between times when melatonin concentrations rose above and fell below 24-hour average concentrations (84). Typical indoor light intensities may be sufficient to mask the endogenous expression of the melatonin rhythm (85). Thus, melatonin data collected during the CR most reliably estimates the endogenous phase of the melatonin rhythm. Relative clock gene expression was determined from real-time PCR results expressed in cycles required to cross the detection threshold.

Fold change in gene expression of each clock gene was determined for each sample relative to *HCDK4* expression using the $2^{-\Delta\Delta C_T}$ calculation (47). Fold change in gene expression for each sample was then expressed as a proportion of maximum expression for each behavioural condition of the study (i.e. LD or CR). The maximum fold change in gene expression determined for each participant during LD and CR subsegments were compared using paired t-tests. In order to determine whether we could detect a significant circadian expression of clock genes in PBMCs, we performed a dual-harmonic regression on each individual's gene expression profile per experimental condition within a period search of 23-26 hours corresponding to the range of period found in the expression of *HBMAL1* in human fibroblasts (12). As described previously, we defined a statistically significant circadian oscillation where the 95% confidence interval for the amplitude of the expression did not include the zero value (9). These regression analyses also provided an estimate of the fitted amplitude of clock gene expression which represents the mean to trough difference in the first harmonic. Amplitude fits were compared between conditions using paired t-tests. The phase of clock gene expression in PBMCs was defined as the time of fitted maximum of expression as determined by dual-harmonic regression analyses. Circadian phase of clock gene expression during LD and CR were compared using paired t-tests.

To describe the relationship between circadian phase markers and the sleep-wake cycle, we calculated phase angles as: (Wake-time)-(Phase), where as described, circadian phase was defined as the time of fitted temperature minimum, midpoint of peak melatonin expression, and fitted maximum of *HPER1*, *HPER2*, and *HBMAL1*. Calculated phase angles during LD and CR were compared using paired t-tests.

In order to compare the relationship between the expression of circadian markers and the sleep/wake cycle, we calculated a profile of the expression of each circadian marker across a total of six wake or sleep 4-hour bins corresponding to the 24-hour day.

Individual data for clock gene expression, determined on a sample-by-sample basis, were first aligned to the time of habitual awakening, and data was assigned to one of four bins corresponding to the habitual wake period (W1-W4) or to one of two bins corresponding to the habitual sleep period (S1-S2). The selection of 4-hour analysis bins permitted us to analyse individual clock gene data in a meaningful way with respect to the sleep/wake cycle. Group means represent the average of individual subject means per bin and all participants are included in each 4-hour bin average. Expression profiles during CR and during LD were compared using two-factor ANOVA for repeated measures (factors: time of day and experimental segment).

Results are expressed as mean ± SEM.

3.5 Results

Participants maintained steady sleep/wake schedules in the pre-study period, such that mean sleep period lengths were 8:03 ± 0:01 hours. The longest and shortest average reported sleep period lengths were within five minutes of the indicated 8-hour sleep period length. Average bedtimes and waketimes in laboratory were 23:22 ± 0:09 and 07:22 ± 0:09, respectively.

All available data points for markers of central and peripheral circadian oscillators during the 3-day sampling period are shown for each individual in supplementary **Figures 3.S1-3.S6**. **Figure 3.2** illustrates the mean expression of circadian markers aligned to each individual's habitual waketime (assigned a relative clock hour of 08:00). The time of the fitted minimum of the core body temperature rhythm, as determined during the CR evaluation, occurred 2:16 ± 0:20 hours before habitual awakening (**Figure 3.2 A**). The midpoint of peak plasma melatonin concentration measured during the CR occurred 3:24 ± 0:09 hours before the time of habitual awakening (**Figure 3.2 B**).

Dual harmonic regression analyses performed on the expression of *HPER1* in PBMCs revealed a significant amplitude of oscillation in all cases during LD conditions (**Figure 3.2C**). Four of six individuals (S01, S02, S06, S07) demonstrated a significant circadian oscillation during CR. The average maximum fold change in *HPER1* expression in the group of participants was 3.5 ± 0.8 during LD conditions and 3.6 ± 0.7 during CR conditions ($p=0.8$). Dual harmonic regression analyses revealed that the fitted amplitude of *HPER1* expression was also comparable between LD and CR conditions ($p=0.2$). The time of fitted peak *HPER1* expression was comparable between conditions and occurred

2:36 ± 1:45 hours after awakening during the LD condition and 1:49 ± 2:11 hours after the time of projected awakening under CR conditions (p=0.8).

Four of the six participants (S01, S02, S03, S08) demonstrated a significant oscillation in *HPER2* expression in PBMCs during the LD subsegment of the study, while the amplitude of oscillation was statistically significant in all cases under CR conditions (**Figure 3.2 D**). The average maximum fold change in *HPER2* expression during LD and CR conditions were 2.8 ± 1.4 and 1.8 ± 0.2, respectively, and comparable between conditions (p=0.4). The fitted amplitude of *HPER2* expression was also comparable between LD and CR conditions (p=0.5). Under LD and CR conditions, peak *HPER2* expression in PBMCs occurred 1:55 ± 1:43 hours before awakening and 0:04 ± 1:22 hours after habitual awakening, respectively. These times were comparable between LD and CR conditions (p=0.3).

The circadian rhythmicity of *HBMAL1* expression in PBMCs was statistically significant in four of six subjects under both LD and CR conditions (**Figure 3.2 E**). Under LD conditions participants S01, S02, S07, and S08 demonstrated a significant circadian rhythmicity, while under CR conditions the amplitude of expression was significant in participants S02, S03, S06, and S08. The mean maximum fold change in *HBMAL1* expression was 2.5 ±1.4 during LD conditions and 1.6 ±0.1 during CR conditions and comparable between conditions (p=0.5). Similarly, the fitted amplitude of *HBMAL1* expression was comparable during LD and CR (p=0.8). Under LD conditions, the time of peak *HBMAL1* expression occurred 6:00 ± 2:27 hours after awakening, while under CR conditions, *HBMAL1* transcript levels peaked 2:08 ± 2:05 hours before habitual awakening. A trend towards a significant difference in calculated phase angles was observed between both conditions (p=0.06).

In order to compare the daily expression of circadian markers under LD and CR conditions, we calculated a profile of the expression of each marker across six wake or sleep bins corresponding to the 24-hour day (**Figure 3.3**). Core body temperature and plasma melatonin concentration varied significantly throughout the day (p<0.001 and p<0.001, respectively), while no significant difference was observed between LD and CR conditions (**Figure 3.3 A, B**). The expression of *HPER1* and *HPER2* in PBMCs also demonstrated a statistically significant variation over time (p=0.03, and p<0.01, respectively), where expression profiles were comparable during LD and CR (p=0.8 and p=0.7, respectively; **Figure 3.3 C, D**). The variation in *HBMAL1* expression over time

was not statistically significant (p=0.8) and no significant differences were detected between LD and CR conditions (p=0.2; **Figure 3.3** E).

3.6 Discussion

The inclusion of a 40-hour segment performed under normal sleep/wake conditions (LD) and a 32-hour segment performed under constant conditions (CR) within our blood sampling session demonstrates the possibility of assessing the function of peripheral circadian oscillators in human PBMCs with frequent sampling over an extended period. The sustained rhythmic expression of clock gene rhythmicity under constant conditions implies that the observed rhythmicity is not generated by behavioural or postural changes. The core body temperature and plasma melatonin rhythms measured under CR conditions confirmed a normal alignment of the central circadian pacemaker to the habitual sleep/wake schedule. As expected under conditions of normal entrainment, the core body temperature minimum occurred in the last hours of the habitual sleep period and the midpoint of peak plasma melatonin concentration occurred near the middle of the habitual sleep episode. Our observation of peak *HPER1* expression in PBMCs just after the time of habitual awakening is in accordance with previous observations that we have made under CR conditions (9) and with those of others made under modified CR conditions (41). The morning peak of *HPER2* expression in PBMCs is consistent with our prior observations under CR conditions (9) and those of others made in the presence of a sleep/wake cycle (68). The mean expression profile of *HBMAL1* in our individuals displays a less prominent rhythmicity than *HPER1* or *HPER2* (**Figure 3.2E**). While the alignment of *HPER1* and *HPER2* expression to the sleep/wake cycle were comparable under our LD and CR conditions, *HBMAL1* expression displayed a trend towards a difference in its expression under these conditions. Only during LD conditions did the mean daily variation in *HBMAL1* expression approximate a peak in anti-phase from peak *HPER1* and *HPER2* expression. The early morning fitted peak of *HBMAL1* expression under CR conditions and the low amplitude mean rhythm relative to the sleep/wake cycle suggest an inter-individual variability that obscures a coherent mean rhythm in *HBMAL1*.

Inter-subject variability in the expression of clock genes in PBMCs has previously been reported. Teboul and colleagues sampled *HPER2* and *HBMAL1* expression in PBMCs from whole blood sampled at 4-hour intervals for 24 hours (69). The inter-individual variability in times of peak *HBMAL1* expression was greater than that for

HPER2 expression, similar to what we have observed. They additionally noted that the times of peak clock gene expression segregate their experimental group into apparent molecular chronotypes. While we noted no such differences in our experimental group, the error values we calculated for the group mean phase of clock gene expression were larger than those calculated for the phase of core body temperature or plasma melatonin. Interestingly, when the period of *HBMAL1* expression is monitored in biopsied human fibroblasts, the intra-subject standard deviation is 8 times smaller than the inter-subject variability in the period of clock gene expression (12). The contribution of post-transcriptional events must also be considered. When *Per2* is constitutively expressed in mouse fibroblasts, PER2 protein may continue to display circadian rhythmicity (80). In the mouse liver, almost half of the rhythmically expressed proteins do not have correspondingly rhythmic RNA levels (61). Thus, post-transcriptional events may also significantly contribute to the function of peripheral oscillators in PBMCs.

Environmental changes may have specific effects on the function of peripheral oscillators. The feeding schedule, for example, can reset rodent peripheral oscillators independently of the SCN (24, 34, 65). Thus, although CR conditions are sufficient to reduce inter-individual variability in centrally-driven rhythms, they may have a negligible effect on peripheral oscillators in PBMCs. The factors that permit the SCN to coordinate peripheral rhythms are yet unknown, but may be specific to each peripheral oscillator (13, 32, 57, 79). A number of hormonal rhythms are driven by the central circadian pacemaker, and are potential signals for entrainment. There is evidence to suggest that melatonin is an immune modulator, where lymphocytes and PBMCs would exhibit transcription of genes encoding enzymes for melatonin synthesis (16, 27) as well as melatonin receptors (17). Glucocorticoids have also been associated with an immunomodulatory function and the trafficking of lymphocytes in peripheral blood (2, 28, 36). Glucocorticods can stimulate *HPER1* expression in human PBMCs *in vitro* and *in vivo* (14, 29). As we previously reported for PBMCs (9), observed rhythms of *HPER1* and *HPER2* are in stable relationships with melatonin, a centrally-driven circadian rhythm. Future investigation will be required to elucidate the specific relationship of these centrally-driven rhythms to the synchronization of peripheral clocks in PBMCs.

Significant circadian rhythmicity has been observed in the numbers and relative proportions of white blood cell subsets. In particular, PBMCs were shown to peak in number during the sleep period (10, 51, 63, 70). By our method, the PBMCs include

lymphocytes and other mononuclear cells. However, each of these distinct white blood cell populations may display a unique rhythmicity in their cell numbers and proportions such that relative abundance of each cell subpopulation within sampled PBMCs may vary throughout the day (2, 10) or even across seasons (44). Our use of a non-rhythmic internal control gene in addition to the input of identical RNA amounts in each real-time PCR reaction controls for daily changes in total RNA resulting from diurnal or seasonal changes in total cell numbers. Nevertheless, our results point to the heterogeneity of the peripheral circadian oscillators in PBMCs, and the possibility that different immune cell subsets may have clocks that are individually regulated in a distinctive manner. Indeed, Kusanagi and colleagues demonstrated that although the rhythms of *HPER1* expression are comparable in human polymorphonuclear and mononuclear cells (41), polymorphonuclear cells demonstrated a secondary *HPER1* peak towards the end of the wake period that did not occur in the mononuclear population (41). Moreover, in PBMC tissue culture, the induction of *HPER1* by a systemic dose of corticosteroids is greater in monocytes than it is in lymphocytes, suggesting that the response of cell subpopulations to the cellular environment may not be the same (29).

Although it is likely that inter-individual differences play a role in the variability in PBMC clock gene expression, the mechanism is yet unclear. Glucocorticoids and sex hormones may interact in modulating the activity of the immune system (23). As lymphocytes and monocytes express estrogen receptors (43), the sex of the participants may have contributed to the variability in clock gene expression. However, of all the phase markers we analyzed, female participants in follicular phase differed from male participants only in the time of fitted peak of *HBMAL1* expression during the CR ($p=0.005$). The eight cases of non-significant circadian amplitude observed under both behavioral conditions were observed in a total of five individuals (including both female participants), suggesting that the regulation of clock genes in PBMCs is more complex than simple sex-based differences. Prior studies have shown that 24-hour mean core body temperature is comparable between men and women (6). Our selection of women in the follicular phase of the menstrual cycle was meant to limit sex-based inter-individual variability that could have occurred by including women taking oral contraceptives or in the luteal phase of their menstrual cycle (6). A non-significant amplitude in circadian expression of clock genes in PBMCs may not necessarily signify the absence of a functional molecular clock in individual cells (54, 72). Rather, a lack of

coordination between the clock gene expression in PBMC subpopulations may result in an apparent reduction in the amplitude of the observed rhythm. A greater understanding of the components that contribute to the organization of peripheral oscillators in PBMCs will significantly contribute to our ability to interpret peripheral clock gene expression in humans.

The results of microarray studies showed that most clock-controlled genes display rhythmicity in a tissue-specific manner (25, 49, 56, 59, 62, 66). This situation raises the possibility that clocks in peripheral tissues exert local control over a tissue's function while the central circadian pacemaker SCN orchestrates overall rhythmicity. A recent report showed that *Per2*-deficient mice have an increased survival rate as well as an altered NK cell-mediated cytokine response to LPS-induced endotoxic shock (46). Moreover, *Bmal1*-defficient mice demonstrate a deficiency in B lymphocyte development (67). These data indicate a functional role for the molecular clock in the immune system. Further study of clock genes and clock-controlled genes in human PBMCs and other tissues will provide important information on the associations between sleep/wake cycle disorders and overall health.

Subject	Sex	Age	Chronotype (37)[272]	Bedtime	Waketime	Month of Study
S01	M	19.6	Intermediate	23:25	07:25	January
S02	M	21.9	Morning	22:57	06:57	January/February*
S03	M	25.4	Intermediate	23:59	07:59	February
S06	F	22.9	Intermediate	23:32	07:32	March
S07	M	30.5	Intermediate	23:00	07:00	July
S08	F	22.0	Intermediate	23:24	07:24	August

Table 3.1

Study Participants. * This subject completed the experiment in two separate laboratory visits. See Methods for details.

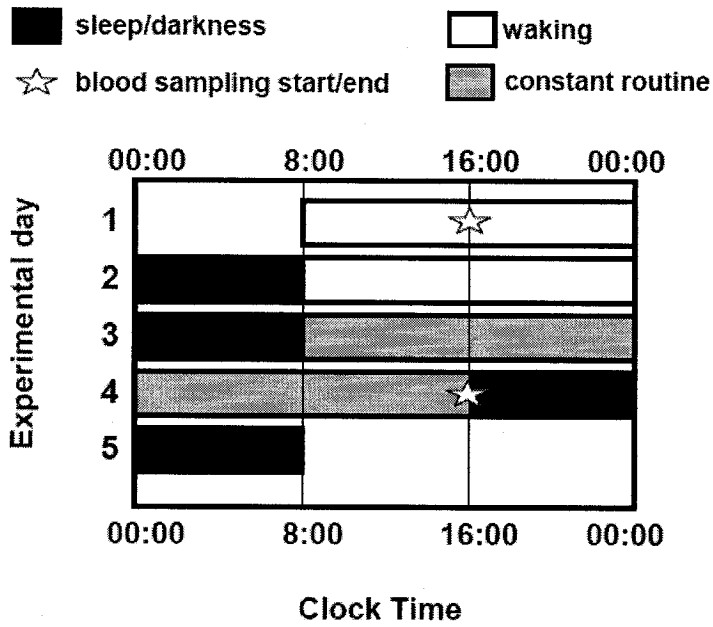


Figure 3.1

Experimental protocol. Blood samples, collected repeatedly over a continuous 72-hour period beginning on experimental day 1, were assayed for their melatonin concentration and for the expression of clock genes in PBMCs. At the start of the study, participants lived on their habitual sleep/wake schedule. Wake episodes were spent in normal indoor light intensities (mean ± SEM: 118± 8 lux), and sleep episodes took place in total darkness (~0.03 lux). A 32-hour Constant Routine (CR) procedure was initiated upon awakening on experimental day 3 (mean intensity ± SEM: 4 ± 0.4 lux; see Methods for details on the CR procedure).

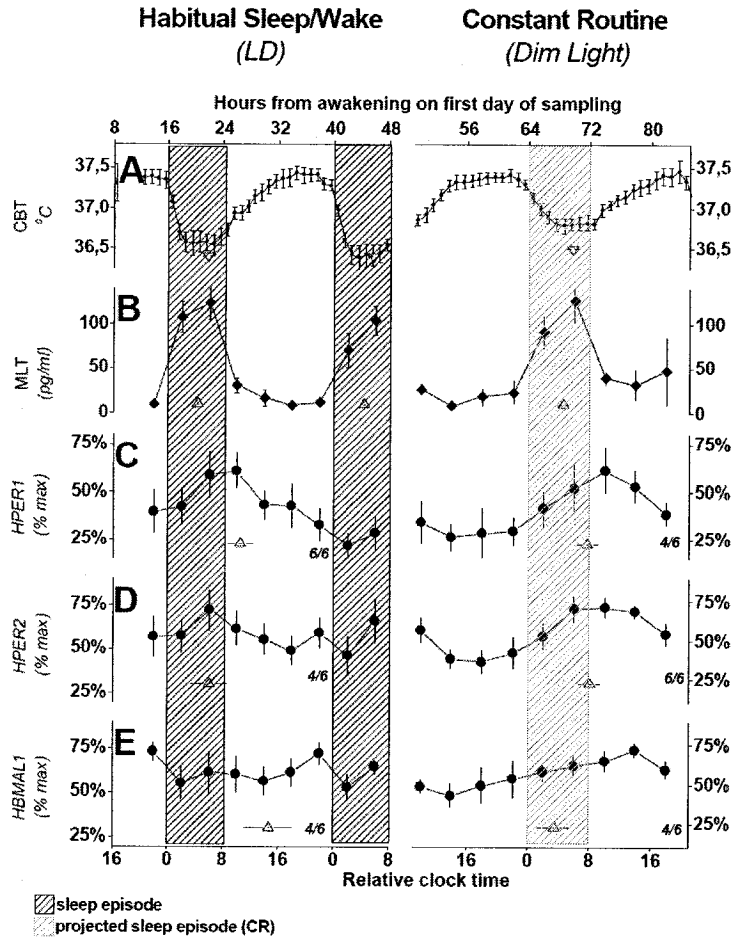


Figure 3.2

Circadian expression of central and peripheral markers for LD and CR experimental subsegments. Mean fitted core body temperature (CBT) minimum and midpoint of peak plasma melatonin (MLT) concentration are shown in panels A and B. Fitted maximum expression of *HPER1*, *HPER2* and *HBMAL1* in PBMCs are shown in panels C-E, respectively. Mean circadian phase for each marker is indicated within panels as a triangle. All error bars represent SEM. Although all subjects are included in the analyses, the fraction of the study group for whom a significant circadian amplitude in clock gene expression was detected is shown in panels C-E. Mean expression of circadian markers was calculated from individual subject data first aligned to habitual wake time. For visualisation purposes, bedtime and waketime are assigned the relative clock hours of 0 and 8, respectively.

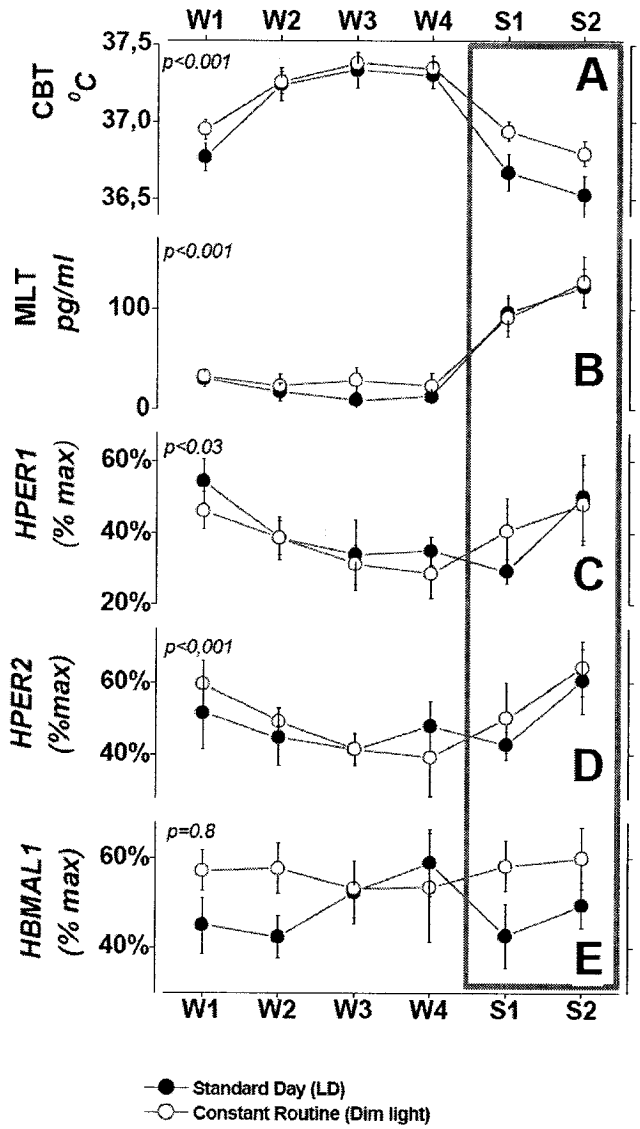
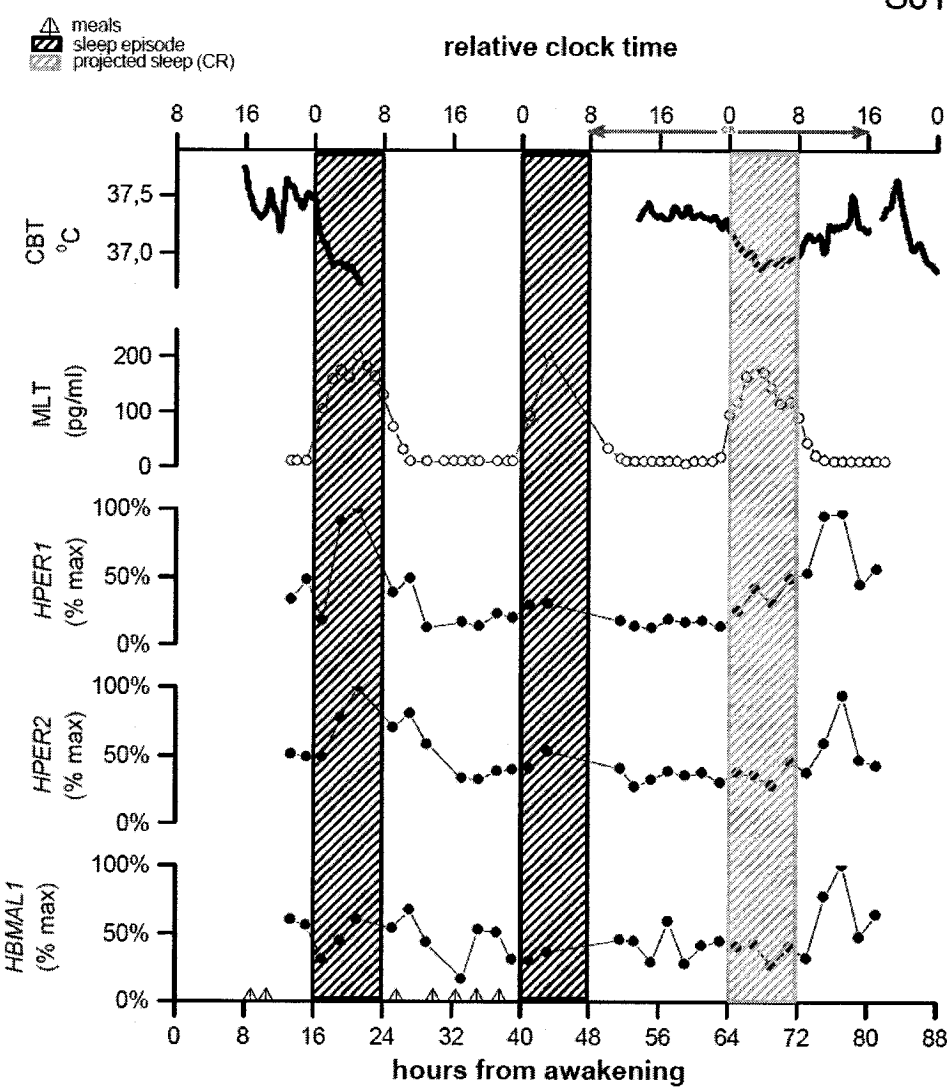


Figure 3.3

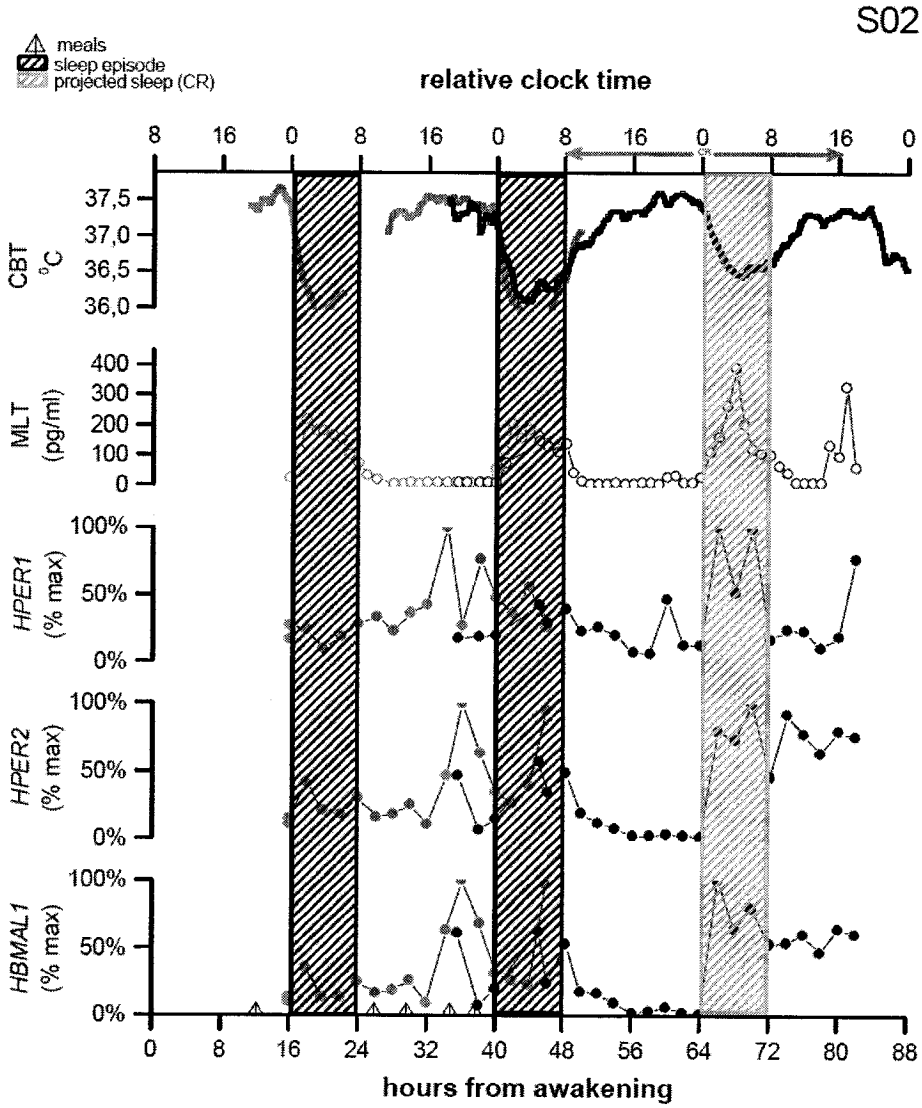
Daily profile expression of central and peripheral circadian markers. The 24-hour day is represented as 4-hour bins corresponding to habitual wake (W1 – W4) or sleep times (S1, S2) for all six participants. ANOVA for repeated measures revealed no significant differences between LD and CR conditions. P values for the time effect are shown in the upper left hand corner of each panel.

Figure 3.S1



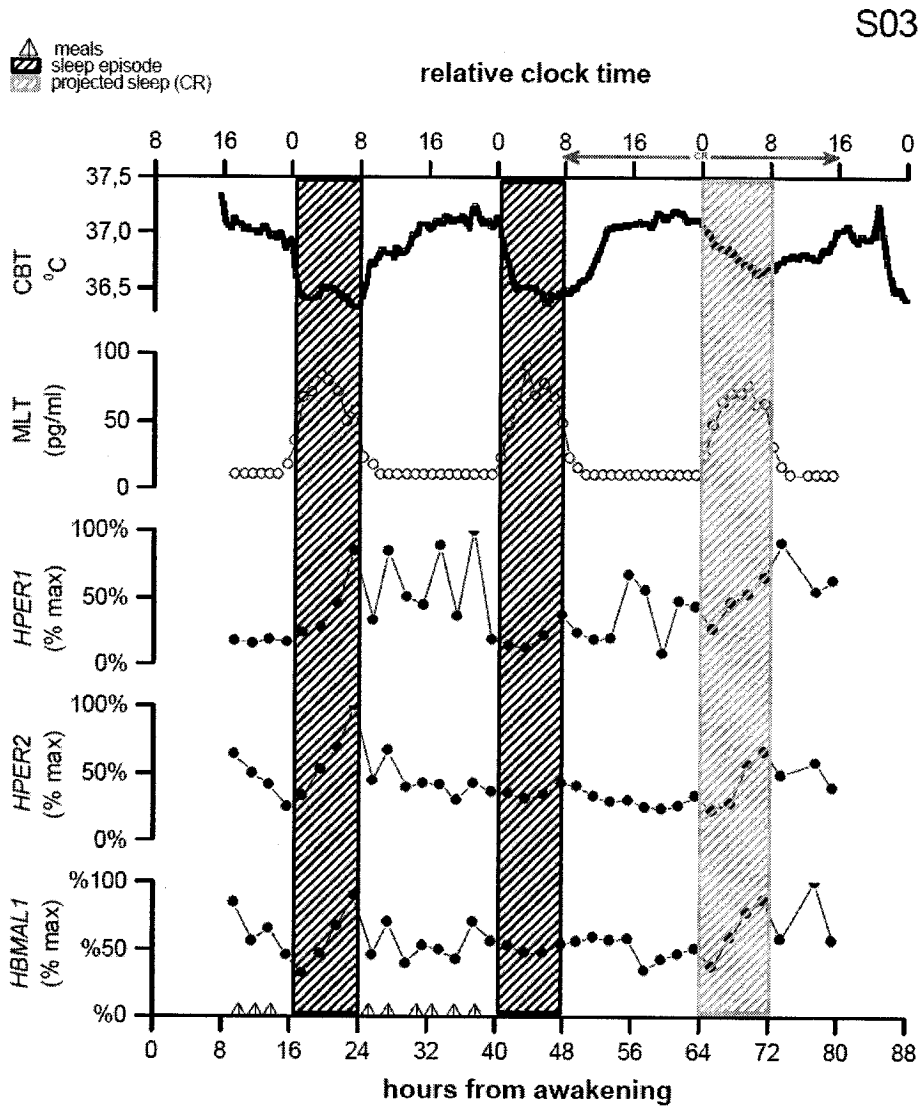
72 consecutive hours of repeated sampling data for male subject S01.
Genetic data expressed as proportion of maximum for the 72-hour segment.
Data is aligned to the time of typical awakeking, assigned the relative clock hour of 0800 hours.
The duration of the constant routine (CR) is indicated along the top horizontal axis.
Meal times during the standard day period (LD) are shown on the bottom horizontal axis.
Equipment failure during the LD portion of the investigation resulted in missing core body temperature data.

Figure 3.S2



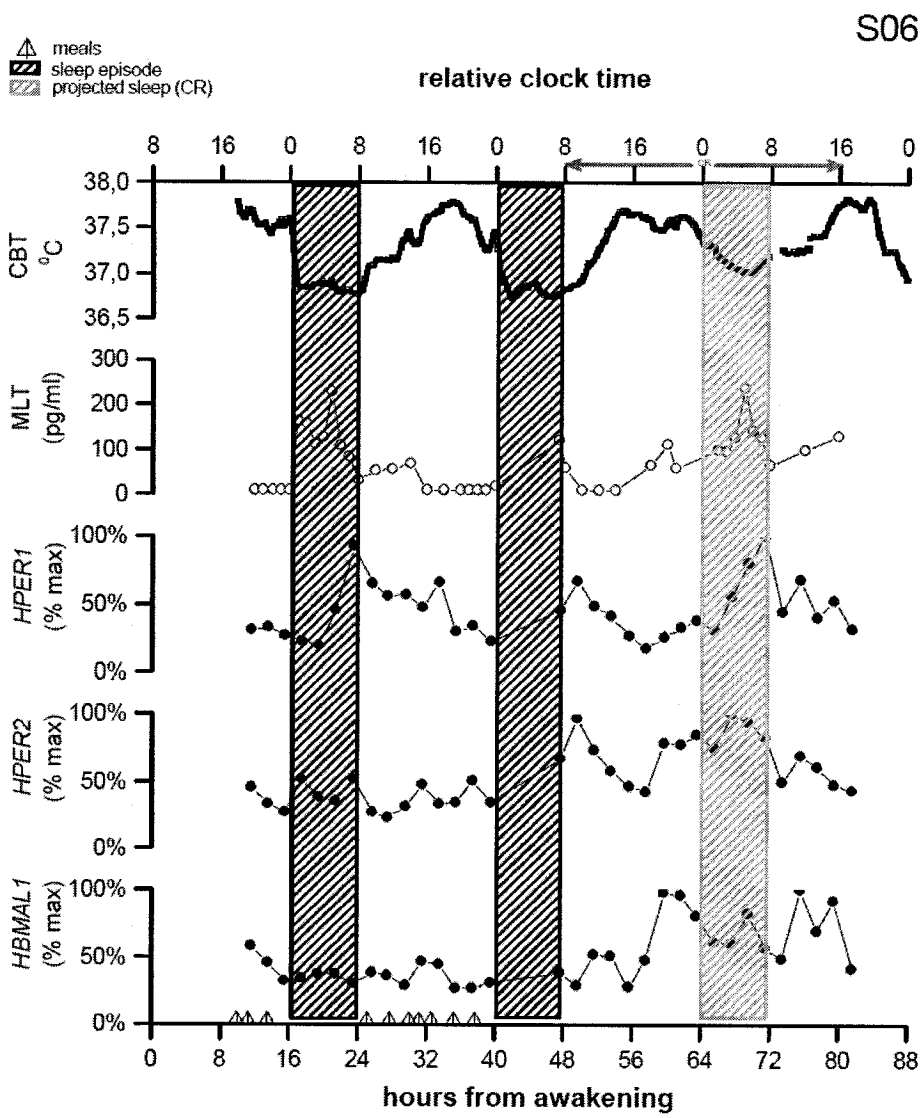
72 hours of repeated sampling data for male subject S02.
The 72-hour blood sampling session was interrupted in this subject.
Data from the first and second sampling sessions are indicated with gray and black symbols, respectively.
Genetic data expressed as proportion of maximum for each segment of the total 72-hour data train.
Data is aligned to the time of typical awakeking, assigned the relative clock hour of 0800 hours.
The duration of the constant routine (CR) is indicated along the top horizontal axis.
Meal times during the standard day period (LD) are shown on the bottom horizontal axis.
Equipment failure during certain portions of the investigation resulted in missing core body temperature data.

Figure 3.S3



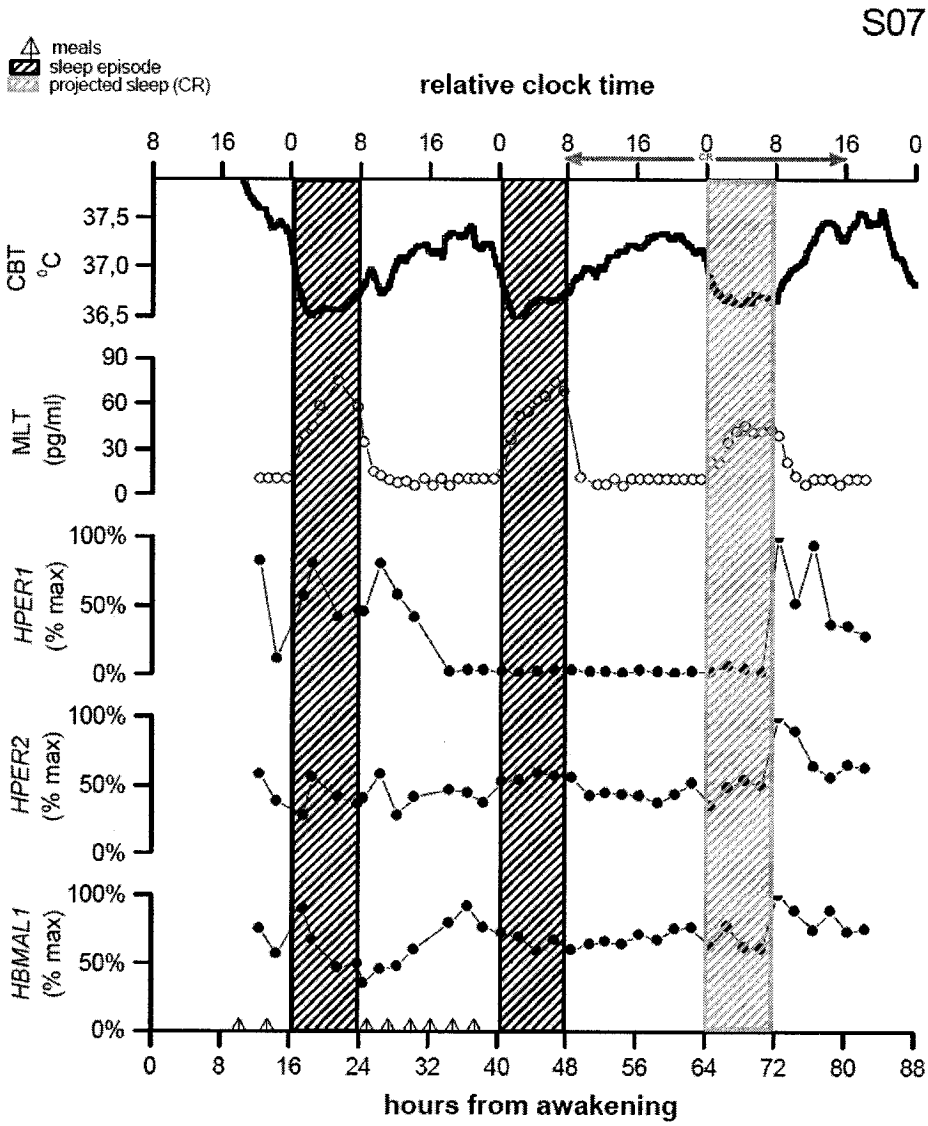
72 consecutive hours of repeated sampling data for male subject S03.
Genetic data expressed as proportion of maximum for the 72-hour segment.
Data is aligned to the time of typical awakeking, assigned the relative clock hour of 0800 hours.
The duration of the constant routine (CR) is indicated along the top horizontal axis.
Meal times during the standard day period (LD) are shown on the bottom horizontal axis.

Figure 3.S4



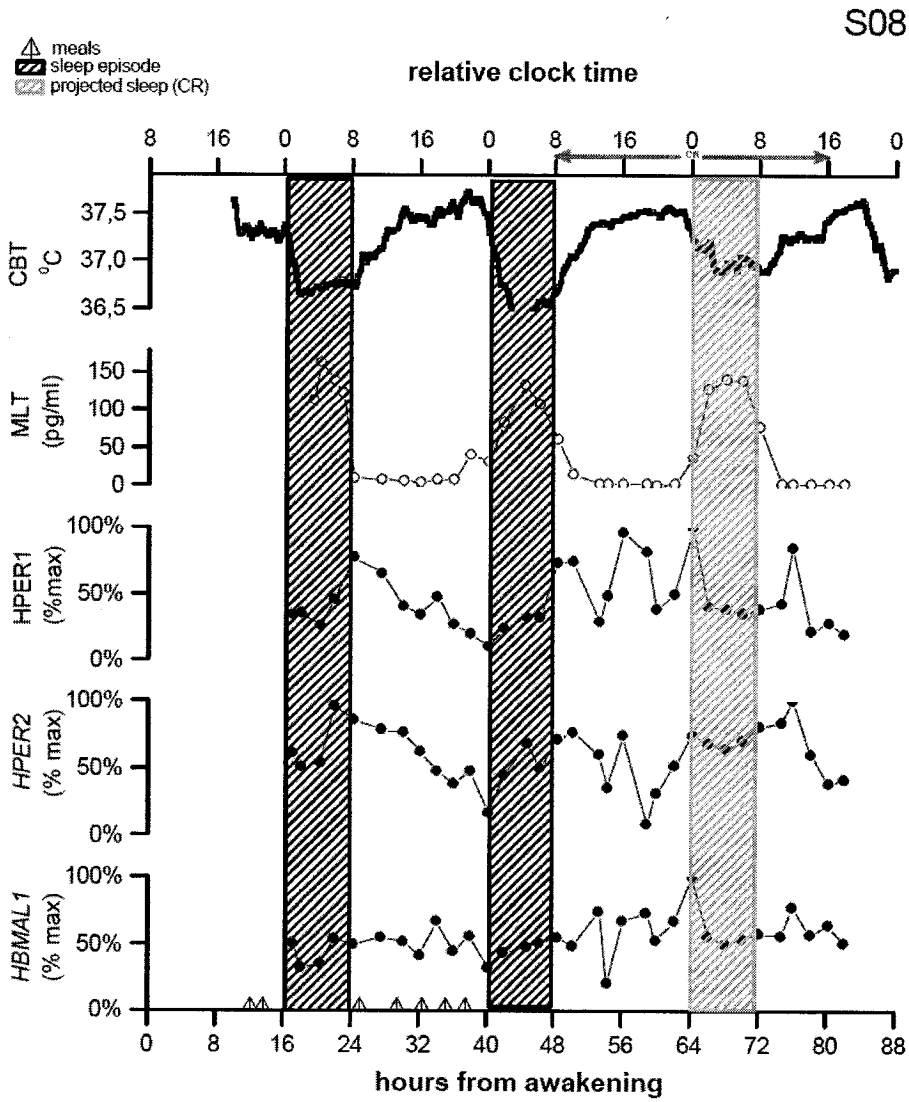
72 consecutive hours of repeated sampling data for female subject S06.
Genetic data expressed as proportion of maximum for the 72-hour segment.
Data is aligned to the time of typical awakeking, assigned the relative clock hour of 0800 hours.
The duration of the constant routine (CR) is indicated along the top horizontal axis.
Meal times during the standard day period (LD) are shown on the bottom horizontal axis.

Figure 3.S5



72 consecutive hours of repeated sampling data for male subject S07.
Genetic data expressed as proportion of maximum for the 72-hour segment.
Data is aligned to the time of typical awakeking, assigned the relative clock hour of 0800 hours.
The duration of the constant routine (CR) is indicated along the top horizontal axis.
Meal times during the standard day period (LD) are shown on the bottom horizontal axis.

Figure 3.S6



72 consecutive hours of repeated sampling data for female subject S08.
Genetic data expressed as proportion of maximum for the 72-hour segment.
Data is aligned to the time of typical awakeking, assigned the relative clock hour of 0800 hours.
The duration of the constant routine (CR) is indicated along the top horizontal axis.
Meal times during the standard day period (LD) are shown on the bottom horizontal axis.

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3.8 Acknowledgements

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CHAPTER 4

Controlled exposure to light and darkness realigns the salivary cortisol rhythm in night shift workers.

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4.1 Preface

The present experiment describes the manipulation of the pattern of light/darkness to phase delay and consequently realign the endogenous cortisol rhythm in night shift workers. This study included real night shift workers on their actual work schedules. Thus, principles established in laboratory experiments could be applied in the field. This investigation provides a demonstration of light-induced shifting of the central circadian pacemaker. As a prelude to the investigation of the effects of simulated night shift work on PBMC oscillators (Chapter 5), it is established here that a light/darkness intervention can promote the appropriate circadian alignment of the SCN-driven cortisol rhythms of night shift workers.

4.2 Abstract

The efficacy of a light/darkness intervention designed to promote circadian adaptation to night shift work was tested in this combined field and laboratory study. Six full-time night shift workers (mean age ± SD: 37.1 ± 8.1 years) were provided an intervention consisting of an intermittent exposure to full-spectrum bright white light (~2,000 lux) in the first 6 hours of their 8-hour shift, shielding from morning light with tinted lenses (neutral gray density, 15% visual light transmission), and regular sleep/darkness episodes in darkened quarters beginning 2 hours after the end of each shift. Five control group workers (41.1 ± 9.9 years) were observed in the presence of a regular sleep/darkness schedule only. Constant routines (CR) performed before and after a sequence of ~12 night shifts over 3 weeks revealed that treatment group workers displayed significant shifts in the time of peak cortisol expression and realignment of the rhythm with the night-oriented schedule. Smaller phase shifts suggesting an incomplete adaptation to the shift work schedule were observed in the control group. Our observations support careful control of the pattern of light and darkness exposure for the adaptation of physiological rhythms to night shift work.

4.3 Introduction

Despite years of night work experience, circadian maladaptation to night work may persist. Spontaneous adjustment of the core body temperature rhythm, and of melatonin, cortisol, thyroid stimulating hormone, or prolactin secretion to shifts in the rest-activity cycle are reported in a minority of shift workers (Sack et al., 1992; Roden et al., 1993; Koller et al., 1994; Weibel et al., 1997; Weibel and Brandenberger, 1998; Goh, 2000).

Appropriately-timed nocturnal bright light has proven an effective intervention to promote the realignment of endogenous rhythms of core body temperature, melatonin, and cortisol to a nocturnal reorientation of the work schedule in laboratory simulations (Czeisler et al., 1990; Dawson et al., 1993; Martin and Eastman, 1998; Horowitz et al., 2001; Crowley et al., 2003). Field studies have confirmed this finding, where the presence of higher light intensities during the night shift have been associated with circadian adaptation (Stewart et al., 1995; Gibbs et al., 2002; Boivin and James, 2002). It has been further demonstrated that control of the pattern of light and darkness exposure throughout the day, including limited exposure to bright light during the morning commute home, are important in the adjustment of circadian phase (Eastman et al., 1994; Buxton et al., 2000; Dumont et al., 2001; Crowley et al., 2003).

In a previous investigation (Boivin and James, 2002), we determined that a comprehensive light/darkness intervention including phototherapy in the workplace, shielding from bright morning light and the maintenance of a stable diurnal sleep/darkness period could promote circadian adaptation in nurses working full-time night shifts. In this report, we present an evaluation of the circadian rhythm of salivary cortisol collected in 11 of the 15 nurses participating in our previous study. Sampling was performed both before and after the period of night shift work under controlled laboratory conditions.

4.4 Methods

Subjects

Eleven nurses working full-time night shifts (at least 8 shifts /15 days) consented to this investigation designed to test the efficacy of a light/darkness intervention for the alignment of the circadian pacemaker with a schedule of night shifts. Night shifts were all of an 8-hour duration and started either at 23:30 or 00:00 hours. All workers were screened for medical and psychological conditions in an interview with a physician. None of the female subjects used birth control pills. The group of workers included 6 males (age ± SD: 40.7 ± 8.4 years) and 5 females (36.9 ±9.7 years) and was comprised of a subgroup of subjects for whom results have previously been reported (Boivin and James, 2002).

On average, workers drank 1.4 ± 1.1 cups of coffee and less than one serving of alcohol daily. Those who smoked (3 control group workers, 2 treatment group workers), consumed an average of 8.2 ±7.8 cigarettes daily. Alcohol consumption was prohibited

on work days and minimal (< 1 drink / day) on days off. Consumption of these products was comparable between groups. Informed consent was obtained prior to the experiment start in accordance with the procedures of the Douglas Hospital Research Ethics board, and the hospital sponsoring the participation of the nurses, where applicable.

Investigation Protocol

In order to establish a baseline condition, each worker was studied following a vacation period lasting at least 10 days. During this period, subjects lived on a day-oriented schedule, kept regular sleep times, and avoided daytime napping. Times in bed were noted in appropriate logs and were confirmed by wrist actigraphy monitoring (Actiwatch-64, or Actiwatch-L, Mini-Mitter, Bend, OR, USA) and daily telephone calls in the week prior to admission to the laboratory. Subsequent to the vacation period, workers were admitted to the laboratory for an initial evaluation of circadian phase. (**Figure 4.1**).

Circadian phase was assessed via the constant routine (CR) procedure designed to unmask the endogenous expression of the circadian system (Czeisler et al., 1986; Mills et al. 1978). In this laboratory procedure, subjects maintained wakefulness in a semi-recumbent posture for 36 hours. Light intensities within the laboratory suite, as measured in the angle of gaze (IL1400A, International Light, MA, U.S.A.), were dim (< 7 lux), and emitted from ceiling-mounted banks of full-spectrum cool-white fluorescent fixtures (4100 0K, TL80 F32T8/TL841 from, Philips, U.S.A., and Octron 800, F032/841 from Sylvania, U.S.A.) covered with lenses emitting less than 1% radiant energy up to 400 nanometres (K-S-H Uvalite Plus, K-S-H Inc., U.S.A.). Research assistants were present to assist the participant in staying awake.

Subsequent to the evaluation of circadian phase, all workers returned to their regular schedule of 11-12 night shifts over a 19-20 day period. The distribution of night shifts and days off varied according to the habitual practices of the place of employment. Workers were readmitted to the laboratory following the last night shift worked for a second 36-hour CR and the evaluation of circadian phase. The final CR was performed after an average of 4.7 ± 2.0 consecutive night shifts. The number of nights worked prior to the final CR was comparable between groups ($p=0.6$).

Light/darkness intervention

The 6 nurses (2 female, 4 male) studied in the treatment condition were provided an intervention designed to carefully control exposure to light and darkness throughout the

day. In the first 6 hours of each night shift, treatment group workers were intermittently exposed to full-spectrum bright, white light (mean intensity measured with a portable light meter in the angle of gaze ± SD: 2,590 ± 1,317 lux) via portable lamps (Sunbox Company, Gaithersburg, MD, USA) set up at the nursing station. Workers were instructed to remain under bright light as much as the workload permitted and to look into the lights as much as possible. In the final two hours of the night shift, the portable lights were shut off, and the workers remained in their habitual lighting environments (mean intensity ± SD: 104 ± 58 lux). Workers in the treatment group were also given a pair of goggles with neutral grey density lenses (Astrospec 3000 or Flashback both with SCT-gray lens tint, 15% visual light transmission, Uvex, Smithfield RI, U.S.A), and were instructed to wear them during the morning commute home. Control group workers (3 female, 2 male) received no bright light in the workplace (mean intensity ± SD: 131 ± 122 lux) and wore clear, UV-excluding goggles during the commute home (Astrospec 3000 or Flashback both with clear lens, 90% visual light transmission, Uxex Smithfield RI, U.S.A.). All workers were instructed to maintain stable sleep/darkness schedules including a single 8-hour diurnal sleep episode beginning 2 hours after the end of the night shift. Adherence to the sleep/darkness schedule was verified using sleep/wake logs, wrist actigraphy monitoring (Actiwatch-64, or Actiwatch-L, Mini-Mitter, Bend, OR, USA) and daily telephone calls to the laboratory at bed and wake times. In exceptional circumstances where the sleep/darkness period was abbreviated or interrupted (~12% of all diurnal sleep observations), workers wore darkened goggles throughout the period corresponding to the assigned sleep/darkness period and noted this in appropriate logs. Treatment group workers had their sleeping quarters deliberately darkened with opaque material covering the windows. It was observed, however, that control group workers also slept in dark bedrooms.

Measurements and analyses

During CRs, saliva samples destined for the assay of cortisol concentration were collected at 60-minute intervals and stored at –20°C until the time of assay. Salivary cortisol concentration was determined in duplicata via radioimmunoassay (Diagnostic Systems Laboratories, Webster, Texas). The kit had cross-reactivities of 100% for cortisol, 33% for prednisolone, 9.3% for corticosterone, and 1.4% for prednisone. Kit sensitivity was to 0.01 mg/dl. The salivary cortisol rhythm was characterised with the application of a single harmonic regression model to each subject's data. The amplitude

of the rhythm was defined as the mean to trough difference of this harmonic. Times of fitted maximum and fitted minimum were both used as circadian phase markers in our analyses. By convention, phase (ϕ) shifts in the cortisol rhythm were calculated as ϕ [initial] – ϕ [final], such that adaptive phase delays were expressed as negative values. In order to ascertain the alignment of the cortisol rhythm with the sleep/wake cycle, we calculated phase angles as the difference: (bedtime) – (time of fitted maximum of the cortisol rhythm). Thus, a negative phase angle would indicate that peak cortisol values were observed after bedtime. Sleep times for the analysis of data from the first CR were based on mean times in bed for the week prior to laboratory admission, scaled to an 8-hour length. In order to determine adaptation to the night shift work schedule, daily reported bedtimes for the ambulatory night-shift period were used for the calculation of phase angles in the final CR. Between and within group differences were analysed using non-parametric analyses. Analysis of reported sleep/darkness times revealed that the assigned schedule was well maintained.

Unless specified, all error values represent SEM.

4.5 Results

Baseline

Mean cortisol concentration during initial and final CRs is shown in **Figure 4.1** (lower panels). At the time of the initial CR, times of maximum and minimum cortisol concentration were comparable between groups (**Table 4.1**). The fitted amplitude of the cortisol rhythm was also comparable between groups. In the control and treatment groups, the peak of the cortisol rhythm occurred 8:32 ± 1:37 and 9:51 ± 0:18 hours after bedtime, respectively.

Final

Following the period of night shift work, the time of maximum cortisol concentration in the control group was shifted by -3:03 ± 2:07. The treatment group displayed a significantly larger shift of -11:04 ± 1:16 (P=0.02). Similarly, observed shifts in the time of minimum concentration were -3:41 ± 2:09 in the control group and -11:26 ± 1:26 in the treatment group, for a significant difference between groups (P= 0.03). The interval between the times of the maximum and minimum were comparable between groups for the initial and final CRs. Compared to the initial CR, a 39-minute reduction of this interval was observed in control group workers (P=0.08) compared to a 22-minute reduction in

treatment group workers (P=0.2). No differences between or within groups in 24-hour mean cortisol levels were detected.

Following the period of night shift work, peak cortisol concentration occurred 1:15 ± 2:10 after bedtimes in the control group, whereas peak levels of cortisol concentration occurred 11:38 ± 1:22 after bedtimes in the treatment group, for a significant difference between groups (P=0.009).

4.6 Discussion

Following a day-oriented vacation period, the shift workers empanelled in this investigation displayed cortisol rhythms well-adapted to day-oriented life. As expected, peak cortisol secretion occurred in the hours following the time of habitual awakening, and lowest concentrations were observed close to the onset of night time sleep (de Lacerda et al., 1973; Van Cauter, 1990). Following the period of night shift work, workers who remained in their habitual light environments displayed a misalignment between the endogenous circadian cortisol rhythm and their sleep/wake schedule. This resulted in higher cortisol concentration near diurnal bedtimes whereas minimal levels were detected near the start of the nocturnal active period. Conversely, workers who received the intervention displayed peak cortisol levels after awakening in the evening, prior to the start of their work shifts, and lowest levels in the morning close to their new bedtimes.

The phase angles between peak cortisol levels and bedtimes in the treatment group were comparable before and after 12 night shifts worked over a ~3-week period. This indicates a circadian re-entrainment with the shifted sleep and work schedule in this group. This result is consistent with the reentrainment of core body temperature and salivary melatonin rhythms in these subjects (mean shifts of -8:15 ± 1:08 and -10:33 ± 1:05, respectively). The smaller phase delay shift in the cortisol maxima of the control group (Table 1) suggests that a partial adaptation of this rhythm has occurred in the absence of the full intervention provided. Indeed, consistent phase shifts were detected in the core body temperature and salivary melatonin rhythms in these subjects (mean shifts of -2:46 ± 2:10 and -2:10 ± 2:38, respectively). This partial adaptation supports our prior interpretation (Boivin and James, 2002) that judicious timing of the schedule of sleep/darkness can induce adaptive phase delays of smaller magnitude compared to the three-tiered intervention of the treatment group.

The use of the constant routine procedure combined with a workplace intervention allowed us to accurately quantify circadian phase in nurses working nights.

The consistency of observed phase shifts between three reliable circadian markers assessed during CR reinforces our conclusion on the efficacy and usefulness of the intervention provided.

Weibel and colleagues previously reported on the rhythm of plasma cortisol in night shift workers (Weibel et al., 1996) sampled under controlled postural conditions. Their analyses revealed that shifts observed in the time of the acrophase were over twice the magnitude of changes in the timing of the quiescent period. Other groups have also reported on changes in the shape of the cortisol rhythm following an abrupt shift (Desir et al., 1981; Caufriez et al., 2002). In order to document changes in the shape of the cortisol secretion curve in the present study, we evaluated the interval between the peak and the nadir of salivary cortisol secretion for each subject (**Table 4.1**). No significant differences in this calculated interval were detected in either group of workers. However, it is possible that our sample size is insufficient to detect small modifications in the shape of the cortisol rhythm following night shifts. Interestingly, the night shift workers studied in the Weibel investigation had a wider variability in their sleep and wake times on work days than our workers. This, may have contributed to additional variability in the timing of peak and low cortisol secretion. In our study, workers of both groups went to bed two hours after the end of their night shifts and remained in darkness for comparable periods. Variability in the number of consecutive shifts worked immediately preceding the final CR (minimum 2 shifts, maximum 8 shifts) may also have contributed to some of the variability in the cortisol rhythm. Nevertheless, the mean number of consecutive shifts worked prior to the final CR was comparable between groups.

A lack of entrainment of melatonin and/or cortisol to a night-oriented schedule is reported in a number of studies despite a series of shifts (Sack et al., 1992; Roden et al., 1993; Koller et al., 1994; Costa et al., 1997; Hennig et al., 1998). The control group workers in this study demonstrated greater variability in the alignment of their cortisol rhythm with the sleepwake schedule than treatment group workers. This resulted in an apparent reduction in the mean amplitude of the cortisol rhythm in the control group following a work period including ~12 night shifts (**Figure 4.1**, lower panels). This change is secondary to the averaging of data points for illustrative purposes, since our analyses of individual curves revealed that circadian amplitude was maintained in both groups (**Table 4.1**).

The sampling interval used in the present study is large compared to the 10-20 minute sampling interval of plasma cortisol used in some prior studies (Weibel et al., 1996; Weibel and Brandenberger, 1998; Weibel and Brandenberger, 2002). It is thus possible that our longer sampling interval has introduced some variability in the reported results. Nevertheless, the cortisol data are consistent with those of salivary melatonin (sampled 1/hour) and core body temperature data (sampled 1/minute) also collected during CRs.

Populations of workers much like ours are described as having varying degrees of circadian adaptation, where some report no adaptation at all. The pattern of light exposure maintained by night shift workers who spontaneously adapt to a night shift work schedule are notable for the stability of sleep times (Roden et al., 1993), limited exposure to bright sunlight in the morning (Koller et al., 1994), brighter light during shift hours combined with sleep times in darkness (Dumont et al., 2001), and limited exposure to light throughout the day in Antarctic studies (Midwinter and Arendt, 1991). In particular, shift workers displaying adapted rhythms of salivary cortisol over a series of consecutive night shifts also reported stable morning sleep times (Hennig et al., 1998).

Circadian adaptation of the cortisol rhythm in shift workers is worthy of investigation for a number of reasons. The demonstration of reduced memory performance with pharmacological reduction of cortisol levels, and increased performance and cognitive efficiency in the presence of exogenous cortisol (Lupien et al., 2002), suggests that a misalignment in the cortisol rhythm resulting in lower levels during the waking episode could contribute to the observed reduction in performance in the workplace (Åkerstedt, 1995).

Indeed, the administration of exogenous glucocorticoids has resulted in reduced subjective ratings of fatigue in emergency room physicians on night shifts (Whitcomb et al., 2000) and increased objective measures of vigilance as assessed via polysomnography during repeated nap opportunities (Meixner et al., 2003). Thus, an appropriate alignment of peak cortisol levels with the start of the night shift (as achieved by the treatment group) seems to the worker's advantage.

As is well known, sleeping at inappropriate circadian phases as in shiftwork may result in an abbreviation of the sleep period (Foret and Benoit, 1974; Kurumatani et al., 1994; Rosekind et al., 1994; Sallinen et al., 2003). The accumulation of sleep debt over a number of consecutive night shifts worked, for example, may in turn result in elevated

basal cortisol levels (Leproult et al., 1997; Charloux et al., 2001). In our small sample of workers, we were unable to detect significant between group differences in mean salivary cortisol levels. The long-term consequences of a misalignment between the endogenous cortisol rhythm and the sleep/wake cycle are largely unknown, but could contribute to the increased risk of metabolic disorders, including diabetes, associated with shift work (Karlsson et al. 2001, Koller et al., 1978; Knutsson, 2003). This is particularly true in light of the hypothesis that cortisol may directly affect glucose metabolism (Van Cauter et al., 1992, 1997; Dinneen et al., 1993;). More specifically, the elevation of cortisol levels at inappropriate times of day results in profiles of insulin secretion and plasma glucose concentration that are consistent with a temporary insulin resistance (Lund et al., 2001 ; Plat et al., 1999).

In summary, the present study is in line with previous observations on the efficacy of careful control of light and darkness throughout the day as a practical means to promote reentrainment to a schedule of night work. Where persistent maladaptation to shiftwork may explain how working non-standard schedules can be predictive of the development of a chronic disorder (Shields, 2002), promoting the circadian adaptation of shift workers, or at least those on full-time night shifts, may be a worthy approach.

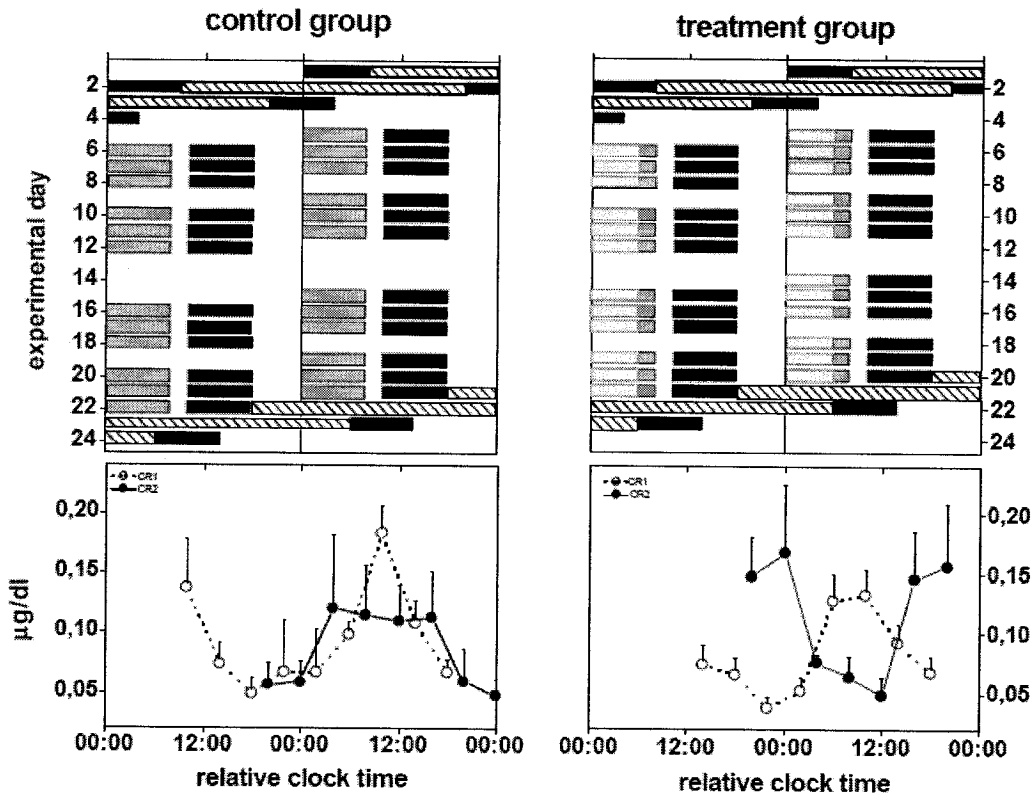

Figure 4.1

Experimental protocol and mean pattern of expression of salivary cortisol during CRs. Upper panels show the protocol for the experimental groups. Successive days are shown side-by-side and along the vertical axis. Sleep/darkness periods are indicated as black bars. Hatched bars indicate CR evaluations. Dark grey bars indicate periods of night shift work performed in habitual lighting. In the treatment group, intermittent exposure to bright light during the first 6 hours of night shifts is shown as light grey bars. In lower panels, mean salivary cortisol concentration per 4 hours is shown (±SEM) in both groups of workers for initial (CR1) and final (CR2) CRs. The times of fitted cortisol maxima relative to sleep/darkness episodes are shown for each group as solid triangles in upper panels. Mean cortisol concentration was averaged across groups based on hours of awakening into each constant routine. To facilitate visualization, workers were assigned relative clock times of awakening of 08:00 for the initial CR and 18:00 for the final CR.

	Initial CR	Final CR	P
Amplitude (°C)			
Control group	0.06 ± 0.01	0.07 ±0.02	0.5
Treatment group	0.05 ± 0.01	0.07 ± 0.02	0.3
P	0.7	0.8	
Time of fitted maximum			
Control group	08:00 ± 1:12	11:03 ± 02:02	0.1
Treatment group	10:13 ± 00:38	21:17 ± 01:21	0.03*
P	0.1	0.009*	
Time of fitted minimum			
Control group	19:52 ± 0:58	23:33 ± 2:05	0.1
Treatment group	21:48 ± 0:48	09:14 ± 1:23	0.03*
P	0.3	0.004*	
Phase angle calculated between bedtime and fitted maximum			
Control group	-8:32 ± 1:37	-1:15 ± 2:10	0.04*
Treatment group	-9:51 ± 0:18	-11:38 ± 1:22	0.2
P	0.8	0.009*	
Interval between maximum and minimum			
Control group	12:08 ± 0:16	11:29 ± 00:20	0.08
Treatment group	12:25 ± 0:17	12:03 ± 0:18	0.2
P	0.7	0.2	
24-hour mean concentration (µg/dl)			
Control group	0.08 ± 0.02	0.1 ± 0.03	0.3
Treatment group	0.07 ± 0.01	0.1 ± 0.02	0.07
P	1.0	0.4	

Table 4.1

Characteristics of endogenous salivary cortisol rhythm measured during CRs. For the calculation of phase angles, bedtimes in the first constant routine are based on mean sleep times during the preceding vacation period, scaled to an 8-hour length. Phase angles from the second constant routine are based on reported bedtimes during the night shift work period. By convention, negative phase angles indicate that the fitted cortisol maximum occurs after bedtime. Significant comparisons are marked with *.

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CHAPTER 5

Entrainment of circadian rhythms of melatonin, cortisol and clock gene expression to simulated night shift work.

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5.1 Preface

Having established the sustained circadian rhythmicity in the expression of clock genes in PBMCs (Chapters 2 & 3), the next test in the investigation was to determine whether these functional clocks could be shifted. In this case, the shift in the sleep/wake schedule simulates night shift work. Phase shifts in the expression of hormonal markers controlled by the SCN would be induced by judicious exposure to light and darkness, as observed in Chapter 4. It was hypothesized that the expression of peripheral oscillators in PBMCs could become realigned to a shifted sleep/wake schedule, in the presence of a light intervention that could shift endogenous rhythms of melatonin and cortisol.

5.2 Abstract

Context: Judicious light and darkness exposure throughout the day can promote the appropriate alignment of hormonal circadian rhythms to night shift work. The resynchronization of human peripheral circadian oscillators is largely unknown to date.

Objective: Evaluate clock gene expression in peripheral blood mononuclear cells (PBMCs) with respect to the simultaneous resetting of plasma melatonin and cortisol rhythms throughout simulated night shift work.

Participants: Five healthy candidates aged 18-30 years.

Design and Setting: Participants were placed on a 10-hour delayed sleep/wake schedule simulating nighttime "work" followed by a daytime sleep episode. Baseline, intermediate and final circadian evaluations were performed in the temporal isolation laboratory.

Intervention: Full-spectrum white light of (mean ±SEM) 6,036±326 lux during night shifts; dim light exposure after each night shift; maintenance of a single 8-hour sleep/darkness episode beginning two hours after the end of each night shift.

Outcome Measures: Circadian rhythms of plasma melatonin and cortisol. Circadian rhythms of expression of clock genes *HPER1*, *HPER2* and *HBMAL1* in PBMCs.

Results: Following nine days on the night schedule, hormonal rhythms were adapted to the shifted schedule. HPER1 and HPER2 expression in PBMCs displayed significant circadian rhythmicity, also appropriately aligned to the shifted sleep/wake schedule.

Conclusions: This is the first demonstration of the simultaneous entrainment of peripheral circadian oscillators in PBMCs and centrally-driven hormonal rhythms following a shifted schedule. The study of peripheral circadian oscillators has important implications for understanding the medical disorders affecting night shift workers.

5.3 Introduction

The rapid reorientation in the sleep/wake schedule brought about by night shift work results in a misalignment between the output of the endogenous circadian pacemaker and the shifted schedule. Night shift workers typically display hormonal circadian rhythms that are maladapted to a night-oriented schedule (1) . Light is the primary synchronizer of the endogenous circadian pacemaker of the suprachiasmatic nucleus (SCN), and careful control over light and darkness exposure throughout the day effectively promotes circadian adaptation of workers to night shifts (2, 3). The rhythmic expression of circadian clock genes required for the intrinsic rhythmicity of the SCN is also present in non-SCN tissue (4, 5), and the SCN coordinates the expression of cellular rhythmicity within peripheral tissues (6). Rhythmic clock gene expression has been observed in human oral mucosa and skin samples (7) and in peripheral blood mononuclear cells (PBMCs) (5, 8-10). Under constant conditions, the expression of core clock genes *HPER1* and *HPER2* displays a significant circadian oscillation in PBMCs with expression peaks a few hours after that of plasma melatonin concentration (10). The aim of this simulated night shift work experiment is to document the circadian alignment of clock gene expression in PBMCs when a light intervention that shifts plasma melatonin and cortisol rhythms is provided.

5.4 Methods

Four male and one female candidate, all healthy, non-smoking and drug-free (group mean age ±SD: 24.9 ±4.8 years) with normal BMI (23.4 ±1.6 kg/m2) gave informed consent to their participation in this study approved by the Douglas Hospital Research Ethics Board. Selected candidates had no history of night shift work or travel across time zones in the three months preceding the study. The female participant had regular menses with an average cycle duration of 32 days. The study took place within the follicular phase of her menstrual cycle.

For two weeks before the start of the investigation, subjects maintained a stable 8-hour night-time sleep/darkness period and took no naps during the day (**Table 5.S1**). Meal-times were standardized throughout the study and subjects took breakfast at 30 minutes after awakening, lunch at 5 hours after awakening, dinner at 11 hours after awakening and an evening snack at 13.5 hours after awakening.

The experimental protocol for the 12-day investigation is shown in **Figure 5.1**. After a baseline assessment on their habitual schedule (experimental days 1-2), subjects underwent a 9-day simulated night shift work procedure during which their sleep episode was delayed by 10 hours. Circadian phase was reevaluated after 4 simulated night shifts in the laboratory (on experimental days 5-6) and after additional 5 simulated night shifts at home (on experimental days 11-12). Evaluations were performed in a time-free laboratory and circadian rhythms of melatonin, cortisol and clock gene expression in PBMCs were determined from blood sampling via an indwelling catheter. Each 24-hour sampling period included a 16-hour constant posture (CP) period of limited activity where participants maintained a semi-recumbent posture in bed. Meals were replaced by hourly, nutritionally balanced snacks.

Circadian phase of the plasma melatonin rhythm was defined from hourly whole blood samples as the midpoint between the upward and downward crossing of the 24-hour average of plasma melatonin concentration (2). The phase of the cortisol rhythm was defined as the time of the fitted maximum of cortisol concentration, based on a single harmonic regression applied to hourly sampling data (3). RNA was extracted from PBMCs isolated from whole blood samples drawn every ~120 minutes (Extraction procedure detailed in supplementary material). Quantification of *HPER1*, *HPER2*, *HBMAL1* expression was determined relative to HCDK4 (10) expression using SYBR Green chemistry (Applied Biosystems, USA).

Fold change in gene expression of each clock gene for each sample was determined using the $2^{-\Delta\Delta C_T}$ calculation (11) and then expressed as a proportion of maximum expression for each blood sampling evaluation. A statistically significant circadian oscillation was observed where the 95% confidence interval for the amplitude of the expression estimated by a dual-harmonic regression analysis with a period search of 23-26 hours (12) did not include the zero value (10). The phase of clock gene expression in PBMCs was defined as the time of fitted maximum of expression. For each laboratory evaluation, we determined a profile of the expression of each circadian marker across a total of six wake or sleep bins corresponding to the 24-hour day. Two-factor ANOVA for repeated measures (time-of-day and sampling session) were performed on binned values.

Results are expressed as mean ± SEM.

5.5 Results

The midpoint of melatonin expression occurred 3:17 ±0:14, and 3:11 ±0:16 hours before waketime in the baseline and final evaluations, respectively (**Figure 5.1**). During these blood sampling sessions, the fitted maximum of cortisol occurred 3:07 ± 0:37, and 3:29 ± 0:40 hours after waketime, respectively. The alignment of melatonin and cortisol rhythms with the sleep/wake cycle were comparable in the baseline and final evaluations (t-test, p=0.7 and p=0.9, respectively). As observed in night shift workers receiving a similar intervention (2, 3), these markers displayed an appropriate alignment to the sleep/wake cycle in night and daytime sleep schedules (**Figure 5.2**).

During the baseline evaluation, the time of fitted maximum of *HPER1*, *HPER2* and *HBMAL1* occurred at 2:53 ± 3:16, 8:00 ± 2:35 and 10:14 ± 0:30 hours after waketime, respectively. At the end of nine days on the shifted sleep/wake schedule, the fitted peak of *HPER1* and *HPER2* expression occurred 5:35 ± 2:40 and 0:40 ±0:44 after waketime, respectively. The fitted peak of *HBMAL1* expression occurred 9:21 ± 4:20 before awakening. The alignment of *HPER1* and *HPER2* maxima to the sleep/wake schedule were comparable in the initial and final conditions (t-test, p=0.6 and p=0.2, respectively), although these phase angles were more variable than those calculated for hormonal rhythms (**Figure 5.S1**). The alignment of *HBMAL1* expression tended to differ in the initial and final conditions (p=0.07).

5.6 Discussion

This study has demonstrated an alignment of clock gene expression (particularly *HPER2*) to a shifted schedule in an experiment of light-induced phase shifts of the endogenous melatonin and cortisol rhythms. The time, duration, and intensity our light/darkness intervention were expected to promote rapid and large delays in the expression of the central circadian pacemaker (13). Changes in the profile of clock gene expression occurred as of 3 days on the night shift schedule such that a greater proportion of the expressed rhythms were of significant amplitudes and in their habitual relationships to the sleep/wake cycle after 9 days of simulated night shift work (**Figure 5.2**).

Inter-subject variability in *HPER1* and *HPER2* expression at baseline resulted in little rhythmicity in the group mean of gene expression (**Figure 5.2**). By the final evaluation, group means of *HPER1* and *HPER2* expression demonstrated clear rhythmicity throughout the day, and both reached maximal levels in the early hours of

the wake period. This observation is in line with what has previously been described for the pattern of *HPER1* and *HPER2* expression in PBMCs under constant conditions and in the presence of a habitual sleep/wake schedule (9, 10). Conversely, the group mean pattern of *HBMAL1* expression demonstrated rhythmic changes throughout the day at baseline and was more variable at the end of the experiment although the fitted peak of *HBMAL1* expression occurs late in the wake-period as we would expect (5).

Clock gene polymorphisms may contribute to diurnal preference and certain sleep/wake disorders (5) and an observed segregation in the times of peak *HPER2* expression in PBMCs has been attributed to the existence of molecular phenotypes that may in turn explain the heterogeneity in clock gene expression (8). Although none of our participants demonstrated extreme diurnal preferences, we cannot determine the extent to which these factors contributed to the variability we observed in the initial condition.

In rodents, the pattern of light exposure has been shown to affect the expression of clock genes in the SCN and in peripheral oscillators. However, the time required for phase shifts in extra-SCN oscillators may be longer and associated with a temporary internal desynchrony (14, 15). In jaundiced neonates with covered eyes, blue light therapy appears to reduce mean HBMAL1 expression in PBMCs when samples collected before and after light exposure are compared (16). While the result suggests that it may be possible to entrain oscillators in human PBMCs without the involvement of the SCN, significant inter-individual variability in the expression makes this interpretation tenuous. Blue light that suppresses plasma melatonin concentration was reported to induce *HPER2* expression in oral mucosa samples during a 2-hour exposure (17). The specific wavelength used led the authors to suggest that the effects on *HPER2* are mediated via the SCN. The factors by which the SCN coordinates clock gene expression in PBMCs are yet unknown. In our experiments, we tightly controlled behavioural and environmental conditions. However, unknown factors might still be present and reflected in our results. Interestingly, a number of experiments suggest that glucocorticoids may play a role in the communication between the SCN and peripheral oscillators *in vivo* (18). In humans, *HPER1* is induced in PBMCs sampled following prednisolone therapy (19). Here, we report a co-occurrence of the entrainment of the cortisol and clock gene expression rhythms. Future investigations are required to elucidate the nature of this relationship in PBMCs since the net effect of synchronizers acting on PBMC oscillators was variable rhythmicity at the first evaluation.

Changes in rodent feeding schedules may shift the oscillation of peripheral clocks without shifting SCN-driven rhythms and there may be an interaction between photic and non-photic resetting factors on peripheral clock gene expression (20). We cannot determine from our experiment the specific contributions of the shifted sleep/wake and meal schedule and of the light intervention on clock gene expression. It is tempting to suggest that in the presence of the light intervention, an SCN-directed entrainment signal was powerful enough to reduce inter-individual differences contributing to the variability observed in the baseline conditions and entrain clock gene expression to the shifted sleep/wake schedule. Future study will be required to partial out the nature of synchronizers of peripheral circadian oscillators, and to quantify their relative strength. It remains of interest that clock gene expression was in a conventional alignment relative to sleep times by the final evaluation.

The molecular clock is thought to interact with the regulation of the cell cycle and tumorigenesis (5). Moreover, cancerous cells may demonstrate abnormalities in circadian clock genes (5). In light of recent evidence associating increased risk of certain cancers with night shift work (5), our results demonstrate that circadian adaptation to night shift work may be advantageous on a fundamental level.

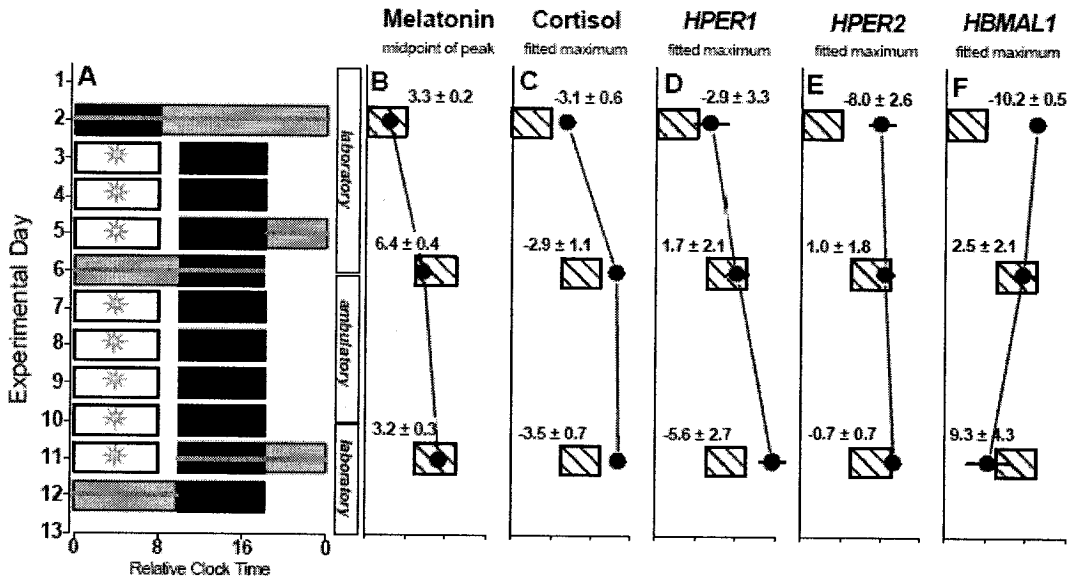

Figure 5.1

Experimental protocol and mean shifts in circadian markers. A: Participants remained in a time-free environment for 6 days. They maintained their habitual sleep/wake schedules for the first night in the laboratory and were placed on a schedule of simulated night work and a daytime sleep as of Experimental day 3. This schedule, delayed by 10 hours relative to habitual sleep/wake times, was maintained for the remainder of the 12-day study. On the day of laboratory admission participants remained in ordinary indoor room light (mean ±SD, in the angle of gaze 144±62 lux). Thereafter, ambient light levels during periods of wakefulness remained dim (6±3 lux) except when subjects were exposed to bright full-spectrum light (6,036±728 lux) during their 8-hour simulated night shifts (shown as open rectangles with symbol). Participants slept in darkness (~0.03 lux, shown as black rectangles). Subjects returned home in the evening of day 6. During the ambulatory segment of the investigation, full spectrum white light was provided by portable lamps equipped with fluorescent bulbs covered with an ultraviolet filter (Sunbox

Company, Gaithersburg, MD, U.S.A) throughout each 8-hour simulated night shift. Participants slept in their own darkened bedrooms, and reduced their exposure to morning light in the two hours preceding sleep/darkness by wearing sunglasses with 5% visual light transmission (The Litebook Company, Medicine Hat, AB). The maintenance of sleep/darkness schedules was verified by sleep/wake logs, regular telephone calls to the laboratory and wristactigraphy monitoring. Subjects returned to the laboratory on the evening of day 10 for the final circadian assessment. The expression of markers of the endogenous circadian pacemaker and of clock gene in PBMCs were measured by 24-hour blood sampling sessions performed before the start of simulated night shifts (experimental day 2), after three days on the shifted schedule (experimental days 5-6), and at the end of the shifted schedule (experimental days 11-12). Blood sampling periods are shown as horizontal lines overlaid on the protocol. Each sampling period included a 16-hour constant posture (CP) period shown as grey rectangles. For illustrative purposes, bedtimes for nighttime and daytime sleep episodes were assigned relative clock times of 00:00 and 10:00, respectively. All studies were performed in the months of July and August 2005. **B-F**: Mean phase of each marker is shown relative to night or daytime sleep periods (hatched boxes representing night and day sleep episodes). To demonstrate the relationship between the sleep/wake schedule and circadian phase, phase angles were calculated as: (waketime) – (circadian phase) for each individual. Thus, positive and negative phase angles indicate that the circadian marker occurred before and after waketime, respectively. Group mean phase angles are shown in decimal hours ±SEM. One subject did not participate in the final evaluation, thus results of this evaluation are given for n=4 subjects, while n=5 for the first two evaluations. See **Figure 5.S1** for individual results.

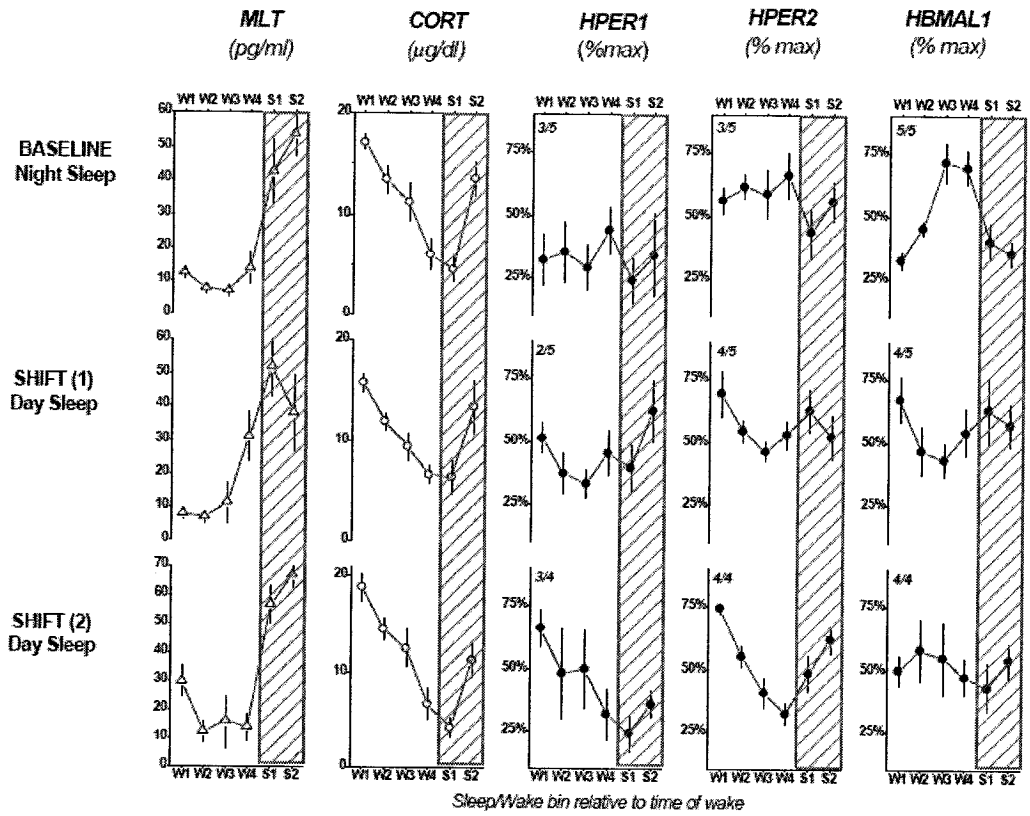

Figure 5.2

Expression of circadian markers throughout the sleep/wake cycle. Individual data was first aligned to the time of habitual awakening, and each data point was assigned to a 4-hour bin representing Wake (W1-W4) or Sleep (S1-S2) periods throughout the 24-hour day, and then averaged across all subjects. For each clock gene evaluation, the fraction of individuals for whom a significant circadian amplitude of expression was detected is shown in the upper left corner of each cell. One subject did not participate in the final evaluation, thus results for this evaluation are given for n=4 subjects. ANOVA for repeated measures performed on daily rhythms for all three blood sampling sessions revealed a significant time of day x blood sampling session interaction for melatonin ($F_{10,55}$=2.40, p=0.02), and a significant effect of time of day for cortisol ($F_{5,55}$=50.01, p<0.0001). No statistically significant effects or interactions were detected by ANOVA for *HPER1*. A significant time of day x blood sampling session interaction was detected for *HPER2* rhythms ($F_{10,55}$=2.99, p=0.004) where simple main effects analyses revealed a significant effect of time of day at the final blood sampling session (p=0.001). A

significant interaction was also detected for *HBMAL1* ($F_{10,55}$=3.34, p=0.002, respectively), where simple main effects analyses revealed a significant effect of time of day at the first blood sampling session (p=0.0006).

5.7 Supplementary materials

RNA Extraction and real-time PCR

Peripheral blood mononuclear cells were isolated from whole blood samples drawn every ~120 minutes on a density gradient (Histopaque-1077, Sigma-Aldrich Canada, Oakville, ON, Canada) and stored at -800C in Trizol reagent (Invitrogen Canada, Burlington, ON, Canada). RNA was extracted and reverse transcribed using MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA). Quantification of clock gene expression was performed by real-time PCR using SYBR Green chemistry (Applied Biosystems, Foster City, CA, USA). The expression of clock genes *HPER1*, *HPER2*, *HBMAL1* was described relative to the expression of HCDK4 using the following primers:

HPER1

Forward: 5'-TGGCTATCCACAAGAAGATTC-3'

Reverse: 5'-GGTCAAAGGGCTGGCCCG-3'

HPER2

Forward: 5'-GGCCATCCACAAAAAGATCCTGC-3'

Reverse: 5'-GAAACCGAATGGGAGAATAGTCG-3'

HBMAL1

Forward: 5'-GGCTCATAGATGCAAAAACTGG-3'

Reverse: 5'-CTCCAGAACATAATCGAGATGG-3'

HCDK4

Forward: 5'-ATCCCAATGTTGTCCGGCTG-3'

Reverse: 5'-TGATCTCCCGGTCAGTTCGG-3'

Sleep/Darkness schedule

During the screening phase preceding the start of the experiment, participants maintained stable sleep schedules including a single night-time sleep episode and restricting naps. Participants recorded times in and out of bed in sleep/wake logs and left voice messages with the laboratory to confirm these times. Sleep/wake schedules were also verified using wrist actigraphy. Mean reported times in bed are shown in Table S1. During the pre-study period, Mean sleep period lengths were within 10 minutes of the 8-hour target sleep/darkness period.

During the ambulatory segment of the investigation, participants maintained the night shift worker's sleep/wake schedule while at home. Times in and out of bed were

verified as in the pre-study segment of the investigation. During this ambulatory segment, participants reported sleep/darkness periods within 10 minutes of the 8-hour target period length.

	Baseline		Night shift schedule	
	Time in bed	Time out of bed	Time in bed	Time out of bed
T01	23:25	7:30	9:30	17:28
T02	22:01	6:04	8:05	16:05
T04	00:00	8:01	10:02	18:06
T05	23:26	7:29	9:29	17:36
T06	23:58	8:08	---	---

Table 5.S1

Mean times in and out of bed reported in sleep/wake logs for each participant. During the baseline period, subjects maintained a habitual day-oriented sleep-wake schedule prior to their admission to the laboratory. During the ambulatory phase of the investigation (experimental days 6-10), participants maintained a night-oriented schedule by remaining awake at night and sleeping during the day. Subject T06 did not participate in the last segment of the investigation (experimental days 6-12).

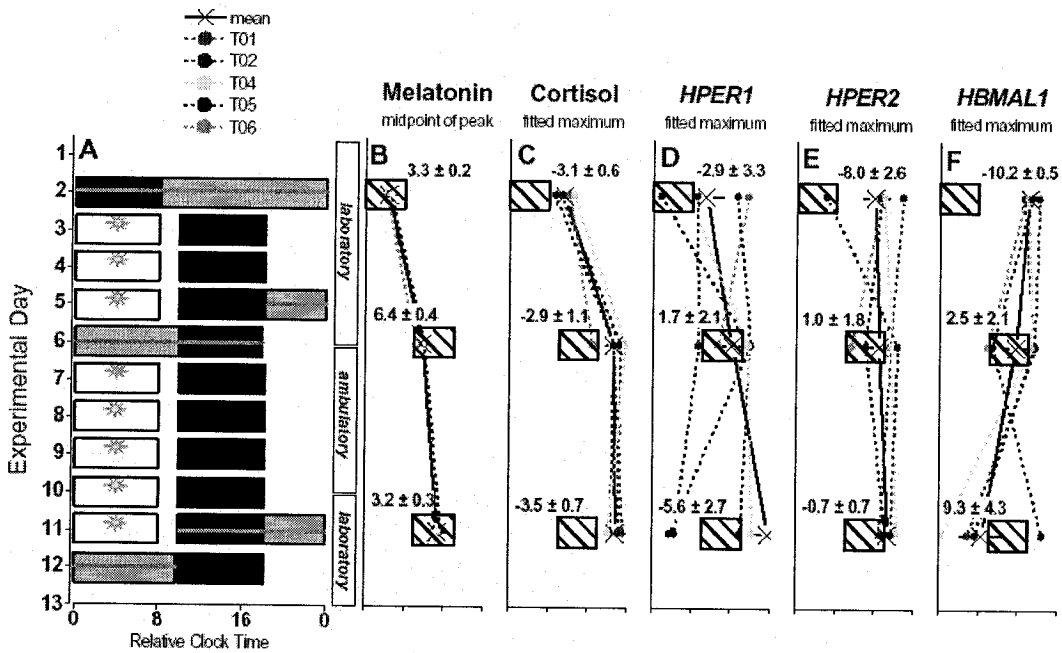

Figure 5.S1

Individual circadian phase markers during each blood sampling session of the experimental protocol. The experimental protocol is shown in panel A as in **Figure 5.1**. In panels B-F, phase markers for each individual are shown relative to night or daytime sleep periods (hatched boxes representing night and day sleep episodes). To demonstrate the relationship between the sleep/wake schedule and circadian phase, phase angles were calculated as: (waketime) – (circadian phase) for each individual. Individual data are shown in separate colors. Group mean phase angles are shown in decimal hours ±SEM. Subject T06 did not participate in the final evaluation, thus results of this evaluation are given for n=4 subjects, while n=5 for the first two evaluations.

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5.9 Acknowledgements

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CHAPTER 6

General Discussion

6.1 Characteristics and implications of an endogenous peripheral oscillator in PBMCs

The rhythmic and circadian expression of elements of the clock mechanism is taken to mean that the molecular loop that comprises the clock is functional. In recent years, evidence has been rapidly accumulating for the presence of rhythmic clock gene expression and a functional peripheral clock in a number of human tissues.

There was prior evidence to suggest that human fibroblast and skin cell culture could express clock gene mRNA or proteins [216], and that human vascular smooth muscle cells [215], fibroblasts [226] or neural cells [218] in culture could demonstrate an oscillation of clock genes following serum shock. However, none of these prior investigations used tissue harvested from humans at regular intervals. Thus, the presence of a functional clock could only be confirmed with repeated sampling throughout a circadian cycle. Bjarnason and colleagues used oral mucosa and skin sampling at 4-hour intervals for 24 hours and demonstrated that significant circadian rhythms in clock gene expression could be measured *in vivo* in these cells [219]. They were thus the first to identify a functional peripheral circadian oscillator in humans [219].

There was evidence to suggest the presence of diurnal changes in clock gene expression in the immune system. In rat mononuclear cells, *Per2* mRNA peaks at the beginning of the dark period [273]. A change in *HPER1* and *HPER2* expression was reported in human lymphocytes sampled before and after surgery. However, the aim of this report was to determine the effects of surgery on PBMC clock gene expression rather than to measure diurnal variation [274]. It was also previously known that in PBMCs sampled at 09:00 and 21:00, *HCLOCK* levels showed no significant difference while mean *HPER2* levels were higher in the morning sample [220]. In the present thesis, it was demonstrated with repeated sampling of PBMCs from whole blood over a 35-hour duration that *HPER1*, *HPER2* and *HBMAL1* levels oscillate in the presence of a standard sleep/wake cycle (Chapter 3). The persistence of a significant oscillation under controlled behavioural and constant dim light conditions (Chapters 2 and 3) confirms the endogenous nature of this circadian rhythm. From the experiments published so far on clock gene expression in the human circulating immune cells, the morning peak of

HPER1 is comparable in mononuclear cells and polymorphonuclear cells [221]. However, the circadian phase of *HPER2* and *HBMAL1* expression may be variable when sampled at 4-hour intervals from healthy adults in the presence of their habitual sleep/wake schedule [222].

To date, PBMC oscillators are also characterized by inter-individual variability in the patterns of clock gene expression. A number of specific factors may regulate clock gene expression in PBMCs, as discussed below (section 6.2). However, it is also possible that characteristics specific to an individual may significantly affect the presence of factors that would entrain peripheral clocks. The times of peak *HPER2* and *HBMAL1* expression during the sleep/wake cycle segregate into two groups which has led to the suggestion that molecular chronotypes may be defined based on PBMC clock gene expression [222]. This was not apparent from the investigations presented here, although significant inter-individual variability was present. Similarly, the polymorphisms that contribute to preferred diurnality [186-189] may also contribute to the variability in PBMC clock gene expression. The inclusion of participants of moderate diurnality in the present experiments makes it impossible to determine the extent of this influence.

Circadian rhythms are also shiftable by definition. A study in which *HCRY1* and *HBMAL1* are compared in PBMCs before and after 24-hours of phototherapy for jaundiced neonates, is difficult to interpret since the sampling frequency is insufficient to describe a circadian rhythm or reliably assess circadian phase [225]. Thus, there was no *a priori* evidence of the length of time required for light-induced phase shifts in human peripheral oscillators. The present investigations demonstrate that a change in the diurnal expression of clock genes in PBMCs can occur following a 9 days on shifted sleep/wake schedule. The synchronization that was observed suggests that a shift has occurred, although the mechanisms by which this shift was achieved remain unclear. Animal studies suggest that phase shifts in clock gene expression in the SCN precede those observed in peripheral tissues [195, 229]. However, stimuli such as feeding time can phase shift peripheral oscillators independently of the SCN [235]. Thus, the experimental paradigm presented in this thesis does not allow the distinction as to whether shifted peripheral rhythms are the result of bright light induced phase shifts of the SCN (as suggested by delays of cortisol and melatonin circadian rhythms) or other mechanisms such as the change in the sleep/wake and meal-timing schedules. Measuring clock gene expression in PBMCs in individuals who would undergo the same

experimental manipulation of the sleep/wake cycle but remain in constant dim light during wake periods could clarify this issue. Also relevant to this question, clock gene expression may be measured in the PBMCs of individuals lacking conscious and circadian vision in their naturalistic environment. As endogenous circadian phase drifts away from the 24-hour day, the peripheral oscillator will betray whether it remains in phase with the sleep/wake cycle or drifts in an out of phase with SCN-driven rhythms such as melatonin or cortisol secretion.

Intuitively, peripheral oscillators should serve to optimize the function of the peripheral tissues. Indeed, a tissue's function may be regulated by the molecular clock to a significant extent as measured in the number of rhythmic transcripts throughout the day in the liver [275], the heart [276], the adrenals [277] or in adipose tissue [278]. In support of this, ablating the SCN or changing the hormonal environment has different consequences for different tissues [179, 279-282]. Cells harvested from the femur-derived bone marrow of mice demonstrate differences in *Per1* and *Per2* expression based on the relative differentiation of the cell in the marrow [283-285]. In a similar fashion, peripheral oscillators in PBMCs may serve to optimize the function of PBMCs. It is possible that differences in clock gene expression confer some advantage to subsets of cells in their specific function. A secondary peak expression of *HPER1* in polymorphonuclear cells as observed under modified CR conditions is not present in mononuclear cells sampled simultaneously [221]. Similarly, the response of cultured human monocytes and lymphocytes to glucocorticoid stimulation is not the same [286]. Conversely, estimating clock gene expression from a more heterogeneous population (such as from the RNA extracted from non-separated whole blood cells) may result in mean circadian rhythms of lower amplitude [224].

In a functional sense, the clock in the immune system may contribute to the diurnal rhythmicity in cell numbers or even the variation in PBMC cellular subsets [80-82, 287-289]. It may also play a role in the function of white blood cells. Under constant darkness conditions and in the presence of 12h:12h light/darkness schedule, rat NK cells demonstrate a circadian rhythm in clock gene expression with *Per1* and *Per2* peaks occurring early in the subjective day and a *Bmal1* peak occurring in anti-phase [290, 291]. The rhythmic expression of the molecular clock components is associated with mRNA and protein for cytolytic factors essential to NK cell activity [290]. Using RNA interference to knock down rPER2 levels in an NK cell line results in a significant

reduction in detected granzyme B and perforin protein [291]. The expression of IFN-γ in response to a lipopolysaccharide challenge is reduced in *Per2* knockout mice compared to wild-type mice [292]. Similarly, the maturation of B lymphocytes is altered in *Bmal1* knockout mice [293]. *Bmal1* knockout mice demonstrate specific changes in the cell population of the immune system: the proportion of neutrophils and monocytes is significantly increased in 40-week old animals [294].

If the peripheral oscillators are specific to the tissue's function, then it may be advantageous that a peripheral oscillator can dissociate itself from the SCN. Following an abrupt shift in the light/darkness schedule, laboratory rodents demonstrate rhythmic *Per1* expression in the SCN that adapts more rapidly than *Per1* expression in the liver [195]. However, restricting food availability to a few hours during the light period can rapidly entrain peripheral clocks in the liver without affecting phase of clock gene expression in the SCN [233, 234]. It is known that clock gene expression in peripheral tissues may be directly related to functions such as fatty acid metabolism [278, 295] or phospholipid biosynthesis [296]. The rapid adaptation of a peripheral clock to a stimulus that is closely related to its function may therefore be useful to the organism. In this manner, the rapid response of the liver clock to a restricted feeding regimen [235] is consistent with the tissue's role in the response to food. Despite the advantage conferred by this organization, there may nevertheless be consequences to the internal desynchronization resulting from differences in the adaptation rates in central and peripheral clocks. Specifically, internal desynchronization may result in conflicting entrainment signals in peripheral clocks. Arguably, this may contribute to the chronic health consequences in populations prone to internal desynchronization such as night shift workers [240].

The present assays in humans principally discuss mRNA transcription as a surrogate for expression. The regulation of individual clock genes or clock controlled genes can differ between tissues in post-transcriptional modifications [297-299] and in the amplitude [279] or variability [209] of expression. Disruption of the CLOCK protein in the mouse SCN results in a reduction, but not an abrogation, in the amplitude of clock gene expression while locomotor rhythms remain robust [280]. In peripheral tissues, a similar disruption of CLOCK may have more severe consequences on the phase and amplitude of expression of specific clock genes in the liver clock, for example [280, 300]. DEC1 is known to bind the *Per1* promoter and regulate its expression [301, 302] but this

gene demonstrates little circadian rhythmicity in PBMCs under constant conditions (Chapter 2). *Dec1* expression in *Clock* mutant animals is arrhythmic in the heart while it maintains the circadian rhythm in its expression (although of reduced amplitude and phase delayed relative to wild type) in skeletal muscle [303]. Recent technical advances in the study of animals models, including RNA interference to knock down the translation of a specific gene transcript and measurement of clock gene-driven luminescence for multiple clock genes in a single tissue [200] may produce useful answers to the tissue-specific and clock gene specific regulation that occurs in peripheral oscillators.

6.2 Factors involved in the synchronization of human peripheral oscillators

The usefulness of assessing peripheral clock gene expression in PBMCs is limited by our understanding of the signals that entrain it. A circadian reporter construct including the mouse *Bmal1* promoter provided evidence for the oscillation of clock gene expression in autopsied skin fibroblasts, monocytes and keratinocytes and determined that the standard deviation in the estimated period is over 8 times higher between individuals than within several cultures from a single individual [223]. This would suggest that within an individual, there exists some mechanism by which peripheral clocks are coordinated. The variability in the clock gene expression [222], (Chapters 2, 3 & 5) may persist because the factors that contribute to the condition are not sufficiently reduced in the habitual sleep/wake cycle or under CR conditions. There is no circadian clock gene expression rhythm detected in horse PBMCs [304], which suggests that either no peripheral clock exists in horse PBMCs or, more likely, that under habitual light darkness sampling conditions, clock gene expression was not coordinated [304]. In the same animals, a functional circadian pacemaker was observed in adipose tissue [304]. Just as peripheral oscillators perform specific functions, they may also be coordinated in a specific way in different tissues.

A number of different factors contribute to the cellular environment of PBMCs: from regional blood flow [305] to cellular subtype trafficking [80]. SCN coordination of peripheral rhythms might be achieved via the rhythms it controls, such as cortisol or melatonin, both of which contribute to the peripheral blood environment [211]. Glucocorticoid rhythms can alter the expression of PBMCs in culture and *in vivo* [286]. Glucocorticoids may specifically affect the expression of molecular clocks in certain tissues more than others: adrenalectomy selectively abolishes PER2 rhythms in the central nucleus of the amygdala but not the basolateral amygdala or the dentate gyrus

[306]. Dexamethasone stimulates *Per1* expression in human bronchial epithelial cells and in PBMCs *in vitro*. [228]. Glucocorticoid receptors in the immune system are well identified [307, 308].

Melatonin also has specific properties in the immune system and is a reasonable means by which peripheral clocks may be entrained [309, 310]. PBMCs can synthesize their own melatonin under *in vitro* stimulation thus may also contribute to their own cellular environment [311, 312]. In sufficient doses *in vitro*, melatonin can increase T lymphocyte and NK cell activity and increase the transcription of cytokines in human monocytes [56]. Differences in clock gene expression are apparent in the adrenals of melatonin proficient and deficient mouse strains [313], and importantly it appears that any effect of melatonin is tissue-specific [314].

Alternatively, cellular clocks may be reset by mechanisms not driven by the SCN. In rat fibroblasts, signalling via pathways involving cAMP-dependant kinases, protein kinase C, Ca^{++} or glucocorticoids can induce *Per1* or *Per2* clock gene expression [315] and it appears that the amplitude and sustained rhythmicity of *Per2* expression depends on the signal used [316]. Further, the expression of genes for nuclear receptors, including glucocorticoid receptors, may be regulated in a circadian manner that is also tissue-specific [317], thereby presenting another level of regulation in which the expression of peripheral circadian oscillators may be controlled by a specific factor.

Just as hormonal rhythms are driven by the SCN to different extents, peripheral clocks may be more or less dependant on the SCN for their synchronization. It is more likely that photic and non-photic stimuli interact in producing the rhythmicity that is observed, much in the same way as mice on a time and calorie-restricted diet demonstrate larger light-induced phase advances in locomotor activity than mice fed *ad libitum* [318]. As suggested above, elements that are a part of the sleep/wake cycle may significantly affect the cellular environment of PBMCs and ultimately their clock expression.

6.3 A model for the regulation of clock gene expression in human PBMCs

Figure 6.1A presents a model for the factors that may influence the circadian rhythmicity in human PBMCs. This rhythmicity is the result of the effects of factors, which may include hormonal rhythms as well as yet unidentified tissue-specific blood-borne signals. The findings discussed in the present thesis show that under constant conditions and in the presence of a sleep/wake cycle, the resulting rhythmicity is observable.

A strong SCN stimulus may be induced in the presence of a powerful SCN synchronizer such as bright light (**Figure 6.1B**). This in turn may result in the synchronisation of peripheral oscillators and increased mean amplitude in the population of cells. Thus, just as a serum shock restores apparent rhythmicity in a group of cells despite the initial phase of desynchronized cells [210], a powerful light stimulus may invoke a powerful resetting stimulus in the periphery. This situation could explain the resynchronization in PBMC oscillators discussed in Chapter 5, in which a bright light stimulus was applied for 9 days. This relationship could be tested in an experiment that compares the effect of strong versus weak SCN entraining signals on readaptation to a shifted sleep/wake schedule. The rate of adaptation of PBMC oscillators may be compared in the presence of light stimuli versus a timed exercise regimen or sound cue, for example. The relative strength of the stimulus and the magnitude of the shift in the sleep/wake schedule may be varied to challenge the resetting capacity of peripheral oscillators. Depending on the length of the experiment and the number of blood sampling periods, it may be determined whether a weak SCN stimulus implies that it takes longer for peripheral rhythms to become synchronized to reveal an observable rhythmicity.

As discussed previously, a number of factors may contribute to the expression of clock genes in PBMCs. Blood-borne signals may differentially affect PBMC subpopulations and contribute to an increased variability and reduced amplitude of clock gene expression (**Figure 6.1C**). Differences in the presence of receptors in PBMC subtypes may also modulate the entrainment of peripheral clocks. Experiments in which primary PBMC cultures are maintained and studied may elucidate this relationship. The response of specific white blood cell subpopulations to glucocorticoids may be examined *in vitro*. If the glucocorticoid concentration applied to the cells is controlled to mimic the endogenous circadian rhythm, we may learn more about the relative contribution of endogenous cortisol levels to the regulation of PBMC molecular clocks.

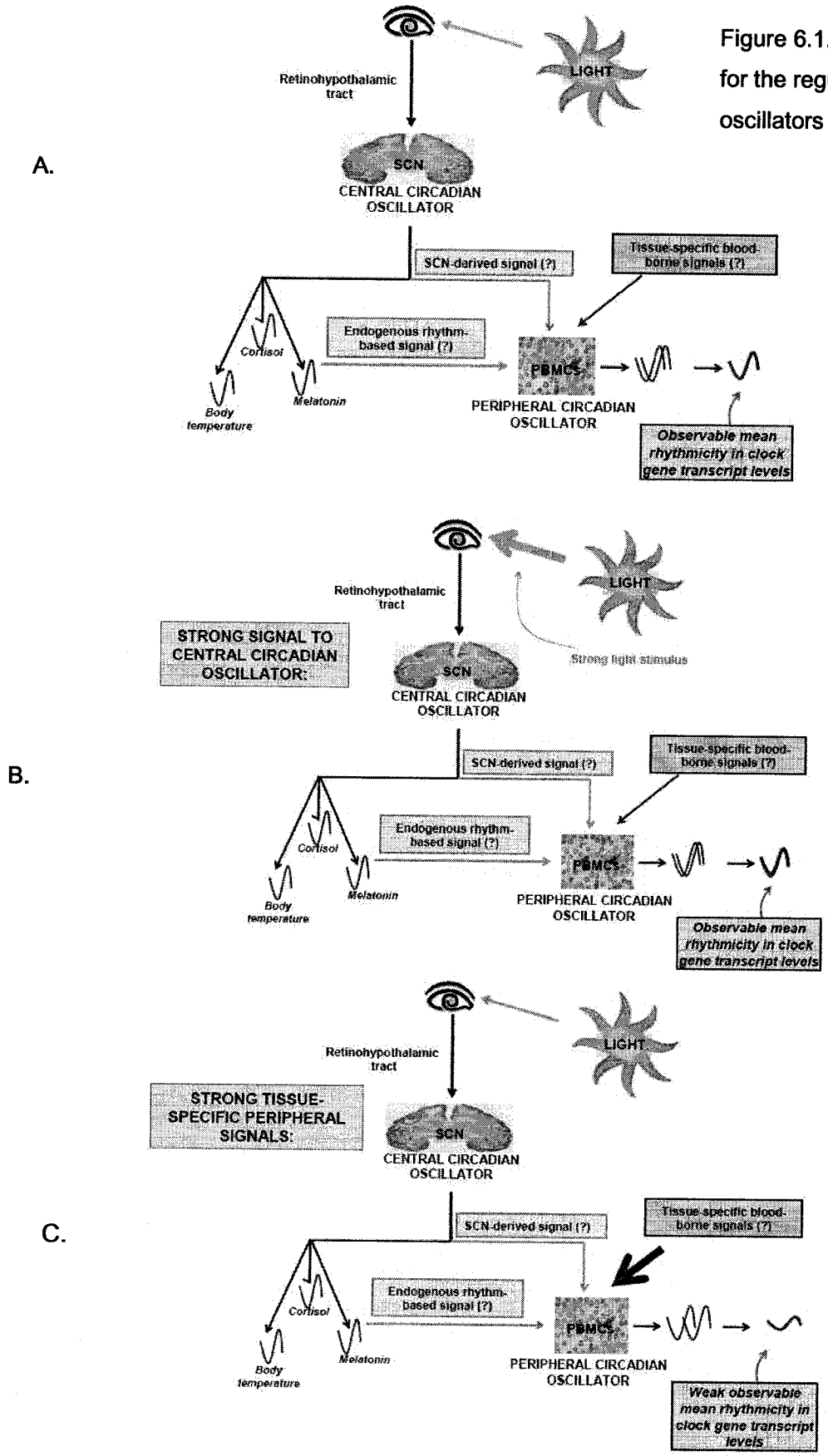

Figure 6.1. A proposed model for the regulation of peripheral oscillators in PBMCs

Further, experiments examining the presence of glucocorticoid receptors at different times of day in different cell populations may also significantly contribute to our understanding of this relationship. Finally, the contribution of inter-individual factors to the presence of non-specific signals is also of importance.

6.4 Outlook: the future of human peripheral clock study and the implications for human health

Considering the relationship of peripheral oscillators to tissue function, an easily accessible peripheral oscillator in humans, such as those in PBMCs, has potential use as a diagnostic tool to provide information on the tissue's function or its relationship to markers of the central circadian pacemaker. In an analogy to how cortisol and melatonin hormonal rhythms are accepted surrogates for estimating the activity of the SCN, measuring clock gene expression may be an additional indicator of tissue function proper. This may be considered in a number of ways. There is now evidence to demonstrate that peripheral oscillators become desynchronized in disease. Progressive stages of Alzheimer's disease is characterized by altered clock gene expression [319] and loss of rhythmicity in pineal and cerebrospinal fluid melatonin [320-322]. Cancerous tissue also demonstrates alterations in the pattern of HPER protein expression [323]. Thus, detecting desynchronized clock gene expression in human peripheral tissues may be a clinically-relevant indicator of disease severity. Indeed, differences in tumour clock gene expression relative to healthy cells may be useful in targeting chemotherapy [323]. One may, therefore, easily imagine how understanding the function of clocks in PBMCs may contribute to significant observations on susceptibility to infection, for example.

Sampling peripheral circadian oscillators may also be relevant in understanding the long-term effects of circadian misalignment such as in night shift work. The relative breast cancer risk posed by night shift work is thought to exceed that caused by well-known risks such as a diet high in fat or the use oral contraceptives, for example [244]. *HPER1* expression is thought to regulate the progression of the cell cycle and human colon cancer cell lines with knocked-down *HPER1* expression demonstrate a reduced apoptotic response following high dose irradiation [324]. In certain murine carcinoma cell lines, *Per2* over-expression reduces cell proliferation and increases apoptotic events [325]. The expression of peripheral clocks may therefore be an important mechanism by which cellular division and death are controlled as well as a valuable diagnostic tool.

Given that the phase of clock gene expression in whole blood cells may be altered in hypersomnia [326] and non-24 hour sleep/wake syndrome [224], it will be of interest to associate the expression of peripheral circadian oscillators with known sleep/wake disorders. It may also be of interest to investigate how circadian disturbances in psychiatric populations [327] affect peripheral clocks.

The present thesis contributes to the early stages of understanding human peripheral circadian oscillators. The potential benefits of this line of investigation may be significant. Exploring the expression of peripheral clocks in other human tissues may result in the characterization of a human peripheral oscillator that are less invasely sampled (e.g. keratinocytes [223]). This may be facilitated by improvements in PCR techniques that may allow the assessment of many clock genes in minute amounts of tissue. There may exist human peripheral oscillators that are more homogeneous than PBMCs with a variability of expression that is more easily controlled. This may provide a background on which significant advances may be made in our understanding of the regulation of human peripheral oscillators. It may even be possible to be able to gather useful information from a single sample of a peripheral clock. For example, introducing luminescent reporters in the sample and then evaluating the ability of specific agents to induce clock gene expression in the sample may be useful to better understand the state of the tissue.

For some time, the health consequences of night shift work have suggested that circadian disorganization could be a cause of physiological dysfunction- although the precise mechanism is yet unknown. In this application as in others, the continued exploration of peripheral oscillators in humans is likely to yield valuable observations at the interface between circadian clock function and proper function of the organism as a whole.

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APPENDIX A

Article reprints

Circadian clock genes oscillate in human peripheral blood mononuclear cells. Diane B. Boivin, Francine O. James, Aibin Wu, Park F. Cho-Park, Huabao Xiong and Zhong S. Sun. Blood (2003) 102:4143-5. © 2003 by The American Society of Hematology

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APPENDIX B

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APPENDIX C

Douglas Hospital Research Ethics Board approval for studies performed