Dawn, light at night and the clock

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DAWN, LIGHT AT NIGHT AND THE CLOCK

Impact on Human Alertness, Performance and Physiology

university of groningen

The research in this thesis was carried out at the Department of Chronobiology, University of Groningen, The Netherlands.
The research was supported by the University of Groningen, the 6th Framework Project EUCLOCK (No. 018741), Philips Lighting, Philips Consumer Lifestyle, Bühlmann laboratories and STW (No. 12187).
This thesis was printed with financial support from the University of Groningen Library and the Graduate School of Science Groningen.

Lay-out and figures: Stichting Kalamiteit, Dokkum
Cover design: Pico van de Werken
Printed by: Gildeprint Drukkerijen, Enschede

ISBN: 978-90-367-6653-1
ISBN: 978-90-367-6654-8 (electronic version)

RIJKSUNIVERSITEIT GRONINGEN

DAWN, LIGHT AT NIGHT AND THE CLOCK
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Proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
vrijdag 6 december 2013
om 14.30 uur

door

Maan van de Werken

geboren op 14 januari 1978
te Dantumadeel

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Voor mijn lieve schatjes Rolo en Ivora.

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CHAPTER

INTRODUCTION AND DISCUSSION

Maan van de Werken

Selection pressures for a clock

Our Solar System was formed about 4.6 billion years ago, with our planet Earth approximately 4.5 billion years ago. The Earth is the third nearest planet to the sun and it rotates around the sun in a close to circular orbit. One rotation of the Earth around the sun makes one calendar year of about 365 days, and one rotation around its axis makes a day of 24 hours. For over 4 billion years, the most predictable changes on Earth are the transitions from day to night and from night to day. In its early history, the Earth was exposed to high dosages of light, including damaging UV light since no ozone had been formed in the atmosphere (Cockell & Horneck, 2001). The first forms of life on Earth made use of light to generate biomass (through photosynthesis) and dealt with this UV-challenge by evolving adequate protective and repair mechanisms. Both the advantageous and the detrimental effects of light created selection pressures to regulate biological activities in response to light and darkness; 'masking', but also a selective pressure to anticipate these exquisitely predictable changes; 'timing'. This latter selective advantage has resulted in the development of biological clocks, molecular clockworks able to keep time independent of external cues, allowing to anticipate predictable/cyclic changes in the environment. This selective advantage is demonstrated experimentally in cyanobacteria, one of the first organisms that evolved on Earth. Mutations in the KaiC gene generate bacteria with deviating intrinsic periods of their clock, and these mutants outcompete wild-types in mixed cultures when the extrinsic light-dark cycle matches the intrinsic period of the mutant (Ouyang et al., 1998). Under constant light conditions an intact molecular clock even has an apparent selective disadvantage (Woelfle et al., 2004).

Clocks

Biological clocks are found in organisms as diverse as cyanobacteria, fruit flies, rodents and humans (Dunlap, 1999). The basic properties that make a clock are the ability to self-sustain rhythmicity under constant conditions, and the ability to become entrained to relevant external cues, 'Zeitgebers', to synchronize the internal timing mechanism with the external cycle. While the clock needs to be sensitive to the relevant Zeitgeber, it should not be sensitive to other influences, such as environmental temperature (Pittendrigh, 1960; Roenneberg & Merrow, 2005). This insensitivity to temperature fluctuations is a special

feature for clock cells since normally reaction rates of molecular processes speed up with higher temperatures. This has to be avoided in clocks to avoid clock speed being affected by fluctuations in temperature of the organism itself or its environment. Initial investigations into the molecular machinery that underlies these abilities have pointed to the importance of transcription translation feedback loops (TTFLs) (Dunlap, 1999). These loops consist of the transcription and translation of a 'clock' protein that negatively feeds back on its own transcription via interlocked loops with other 'clock' proteins (Lakin-Thomas, 2006). The delay in this feedback is fundamental to generate the rhythmic expression of such a 'clock' protein. In many model organisms mutations in 'clock' proteins result in deviations in rhythmic behaviour and TTFLs were therefore considered to be responsible for the molecular basis of keeping time (Baylies et al., 1987; Van der Horst et al., 1999). This view changed when cyanobacteria were shown to exhibit rhythmic KaiC (a 'clock' protein) phosphorylation in the dark when transcription is absent (Tomita et al., 2005) and when three isolated proteins (plus ATP) from cyanobacteria (KaiA, KaiB and KaiC) were shown to generate rhythmic oscillations in KaiC phosphorylation in a test tube (Nakajima et al., 2005). This demonstrated that TTFLs are not always necessary for rhythmic behaviour. Recently, self-sustained rhythmic behaviour was also demonstrated to occur for the protein peroxiredoxin in human red blood cells that lack a nucleus and therefore do not perform transcription (O'Neill & Reddy, 2011).

On the level of the whole organism the ability to self-sustain rhythmicity with a period close to 24 h (circa-dian) can be observed in for example rhythmic behavioural and physiological changes when they are kept void of the external cues. These cues entrain the endogenous rhythm of an organism to the external 24 h cycle and in their absence, for example in constant darkness, the endogenous rhythm is revealed and free-runs (Chapter 5; Aschoff, 1965). Explants of cells or tissues also readily show self-sustained rhythms in a dish (Stokkan et al., 2001; Welsh et al., 2004; Yamazaki et al., 2000). These rhythms are especially clear in explants from the suprachiasmatic nucleus (SCN) (Bos & Mirmiran, 1990; Yamaguchi et al., 2003), an organ located in the brain specialized to keep time. The SCN acts as a master clock and synchronizes other brain areas and peripheral tissues to regulate physiology and behaviour according to time (Buijs & Kalsbeek, 2001; Dibner et al., 2010). Via entrainment of the endogenously rhythm of the SCN to external cues the SCN

can predict external time. Hereby the SCN offers mammals to live their lives highly tuned to external time and this allows them to do the 'right thing at the right time'. The transitions between night and day are the most stable reference points for the time of day. Hence, it is not surprising that light is the main Zeitgeber for many organisms, including humans.

Light entering the body

There are several ways via which light can affect an organism. The physical properties of light can be damaging (UV damaging DNA and molecules) or its energy can be utilized, for example to transform useful compounds (for example vitamin D formation), or to produce biomass (photosynthesis). Alternatively light can be perceived, and used for vision or as a direct environmental cue (non-image forming responses to light). In perception, light enters an organism's neuronal circuitry once it hits photoreceptors that 'translate' photonic exposure into neuronal action potentials. In the retina of the eye, specialized cells are present that perceive and integrate light information via the expression of specialized photopigments. Rods, cones and a subset of retinal ganglion cells all express photopigments that capture photons and transform the light signal into neuronal action potentials. The photopigments in these cells are sensitive to different parts of the light spectrum (Hatori & Panda, 2010). A specific group of retinal ganglion cells is directly photoreceptive due to their expression of the photopigment melanopsin (Bailes & Lucas, 2010; Berson et al., 2002; Hattar et al., 2002), and these cells are especially relevant for circadian biology. These so-called intrinsically photosensitive retinal ganglion cells (ipRGCs) are most sensitive to the blue part of the light spectrum, and they also receive input from rods and cones (Hatori & Panda, 2010). IpRGCs are the main photoreceptive cells needed for photoentrainment, since photoentrainment is lost after selective lesion of these cells (Hatori et al., 2008) and also after knockout of the melanopsin gene (Hattar et al., 2003). IpRGCs project to the SCN via the rentinohypothalamic tract (Hannibal, 2002), that follows the optic nerves and innervates the SCN at the optic chiasm (where the optic nerves cross). Neurotransmitters released at the end of this tract directly activate SCN cells (Hannibal, 2002). In this way, the SCN is entrained daily to local time. Electrical activity of the SCN peaks during the day, in both day- and night-active animals (Challet, 2007). The SCN entrains, gates, stimulates and/or

inhibits other brain areas, which in turn affect other parts of the body (Dibner et al., 2010).

Direct effects of light

Besides the SCN playing an important role in orchestrating synchrony within bodily processes, it also gates, in a phase dependent manner, the effects of light on specific brain and peripheral areas. Light can only directly affect physiological systems if there are neuronal connections with light receptors, often but not always guided via the SCN. Only certain parts of, and processes in, the body are directly influenced by light (e.g. masking). Light for example directly inhibits the nightly production of melatonin in the pineal gland (Chapter 1; Brainard et al., 2001; Lewy et al., 1980; Thapan et al., 2001), but it also directly stimulates the production of cortisol in the adrenal gland at the beginning of the subjective day/active period (Scheer & Buijs, 1999). Bodily processes that are influenced directly by light are subject to relatively high fluctuations in stimulatory and/or inhibiting input (possibly dependent upon circadian phase), resulting from the presence and intensity of light exposure. Especially cells that show relatively low signal transduction strength (see Individual Differences section below) are predicted to be influenced strongly by light, rendering individuals with low excitability cells more susceptible to fluctuations in light exposure (Chapter 3).

Light not only influences physiology, but also has direct effects on behaviour. Especially short-wavelength ('blue') light has been shown to increase alertness (Revell et al., 2006) and subjective activation (Chapter 2) and to decrease subjective sleepiness (Cajochen et al., 2005). Another example of a direct effect of light is the accelerated dissipation of sleep inertia by artificial dawn simulation; light exposure gradually increasing in the morning before waking up (Chapter 4). Although morning light commonly induces phase advances, no phase-shifting response to artificial dawn was detected in a complementary study performed by Giménez et al. (2010). The dawn simulation effects (Chapter 4; Giménez et al. 2010) are thus likely attributable to a direct activating effect by light.

Effects of light differ between species, for example different species occupy different temporal niches. Accordingly, light either signals the animal's active or inactive period, in diurnal and nocturnal animals respectively (Challet, 2007). The effects of light on physiology and behaviour therefore depend

on the species and its neuronal organization (Castillo-Ruiz & Nunez, 2007; Kalsbeek et al., 2012). Most experimental studies in circadian biology in mammals are performed with nocturnal rodents. The responses to light are often extrapolated to diurnal mammals as if these responses are simply opposite to the responses reported in these nocturnal models. This is based on the possibly misleading assumption that the physiology regulating differences between night- and day- active animals only changes the sign of the response, emphasizing the importance of translating knowledge about the effects of light and circadian biology obtained in animal studies to humans.

Light at 'unnatural' times in humans

In a natural situation, only daylight activates the SCN resulting in a clear representation of daytime. Since the discovery of electricity, artificial lighting provides light at unexpected phases of the clock and also allows humans to be active during the dark phase. In most cases light is used at times corresponding to personal preferred wake time. Therefore most people do not choose to expose themselves to light or to be active in the middle of the night because at that time they prefer to sleep. Consequently, the use of artificial light at 'unnatural' times is relatively limited and this leaves circadian physiology and behaviour relatively unaffected. Yet, the use of artificial light does create a discrepancy with natural photoperiodic cues that regulate seasonal rhythmicity. Short winter days are elongated by the use of artificial lighting more so than long summer days (Giménez et al., 2013). Other differences between timing of light exposure and activity in comparison to internal circadian phase result from flying east or west, also termed jet lag, and from working at night. In the Western World many people travel using transcontinental flights, and about 15-30% of the working population in the industrialized countries regularly perform work at night (Boivin et al., 2007; Haus & Smolensky, 2006).

Health and the clock

Shift work and repeated travel between time zones have been associated with increased risk of disease. Shift work is associated with higher risks of cancer (Schernhammer & Schulmeister, 2004; Straif et al., 2007; Viswanathan & Schernhammer, 2009) with increased risk of breast cancer in nurses (Schernhammer et al., 2001), obesity (Karlsson et al., 2001), diabetes (Karlsson et al., 2001; Morikawa et al., 2005), cardiovascular disease (Bøggild

& Knutsson, 1999; Hublin et al., 2010; Knutsson et al., 1986; Vyas et al., 2012), and depression (Glass & Fujimoto, 1994; Scott et al., 1997). Also pilots and cabin personnel suffer from increased risks of cancer (Rafnsson et al., 2000; 2001; Reynolds et al., 2002). The association of this risk with the number of time zones travelled suggests the involvement of circadian rhythm disturbances in addition to the possible involvement of cosmic radiation and life style (Rafnsson et al., 2000). Light and activity out of phase with the clock and repeated re-entrainment, shifting of the clock, all have been proposed as possible mechanisms underlying these higher risks of disease and mortality. Animal studies indeed suggest a causal involvement of light at night and repeated shifting of the clock. Light at night increases the growth rate of tumours (Blask et al., 2005; Cos et al., 2006) and forced shift work results in obesity, but only when feeding is also allowed outside of the natural active phase (Arble et al., 2010; Salgado-Delgado et al., 2010). Repeated phase-shifting accelerates mortality in mice (Davidson et al., 2006; Penev et al., 1998). Interestingly, even the association with depression-like behaviour is apparent as a consequence of light provided at night in a diurnal rodent: the Nile grass rat (Fonken et al., 2012). The hypotheses suggesting that phase shifting or light at night underlie the higher risks of disease found in shift work and flight personnel are distinct but are also not mutually exclusive. On a more mechanistic level phase-shifting may cause circadian dysregulation between the rhythm of the SCN and peripheral rhythms in other tissues, possibly contributing to the observed higher risks of disease (Haus & Smolensky, 2006). On the other hand, phase shifts will be associated with light (and activity) at the beginning and/or at the end of the dark phase and hence the two are difficult to disentangle.

Melatonin

One hormone thought to be responsible for conferring the rhythm of the SCN to the rest of the body is melatonin. Melatonin is often referred to as 'the hands of the clock' and it shows a highly robust intrinsic rhythm in humans (Chapter 5). Administration of melatonin can entrain activity rhythms in humans and rodents. Blind individuals can be entrained by timed dosages of melatonin (Lockley et al., 2000; Sack et al., 2000) and rodents and monkeys in constant conditions can also be entrained by melatonin administration (Cassone et al., 1986; Masuda & Zhdanova, 2010). Peripheral tissues express

melatonin receptors (Ekmekcioglu, 2006) and are influenced or entrained by melatonin accordingly (Pévet & Challet, 2011). Although it is also sometimes referred to as 'the sleep hormone', this is based on the coincidence of melatonin release and sleep in diurnal species, such as humans. Causal associations with feelings of sleepiness and lower vigilance were only reported during daytime after melatonin administration likely resulting in blood concentrations in a pharmacological range (Cajochen et al., 1996; Graw et al., 2001; Kräuchi et al., 2006). In this thesis we do not find any associations between physiological melatonin concentrations and subjective ratings of activation or sleepiness at night (Chapter 2), contrary to results of Cajochen et al. (2000), but in line with the conclusions in Rüger et al. (2005). Yet, melatonin administration in the evening does reduce time needed to fall asleep and it increases sleep duration as concluded from a meta-analysis, but only slightly so, by 4 minutes and 13 minutes respectively (Brzezinski et al., 2005).

Melatonin is produced in the pineal gland in the brain and its rhythmic synthesis is regulated directly by the SCN. During daytime the SCN inhibits melatonin production by inhibiting the firing rate of the paraventricular nucleus (PVN) that is located in the hypothalamus. At night the SCN relieves its inhibition of the PVN, which then stimulates the pineal gland via a multi-synaptic pathway. At the end of this pathway the neurotransmitter noradrenaline is released, which activates β_1-adrenoceptors of the pineal cells (Simonneaux & Ribelayga, 2003). Binding of noradrenaline to these G protein-coupled receptors activates the adenylyl cyclase (AC) – cyclic-AMP (cAMP) – protein kinase A (PKA) signalling pathway leading to phosphorylation of Arylalkylamine N-acetyltransferase (AANAT), the rate limiting enzyme in melatonin synthesis. Phosphorylated AANAT forms a regulatory complex with 14-3-3- proteins, which activates the AANAT enzyme but also prevents its degradation via proteasomal proteolysis (Klein, 2006). AANAT phosphorylation is a crucial step in the synthesis of melatonin and it is reduced in response to light exposure. Light suppresses nightly melatonin production in a dose- and duration- dependent manner (Brainard et al., 2001; Chang et al., 2012; Thapan et al., 2001). Light inhibits, via the SCN, the stimulatory input of the PVN most likely at the level of the pineal gland (Chapter 3; Kalsbeek et al., 1999). Reduced noradrenergic stimulation of β_1-adrenoceptors of the pineal cells will decrease AC – cAMP – PKA stimulation and increase AANAT proteasomal proteolytic degradation. The drop in melatonin synthesis upon

light exposure is almost immediately measurable in saliva.
Light information responsible for this suppression is transduced via ipRGCs to the SCN and hence melatonin is most effectively suppressed with light of relatively short wavelengths (blue light) (Chapter 2). Melatonin suppression thus provides a direct response to light at night and probably phase shifts peripheral rhythms, but also alters the daily melatonin profile, a cue of photoperiod that is implicated in the regulation of seasonal rhythmicity (Arendt, 1998). It is not surprising that this important hormone in circadian regulation has been hypothesized to be involved in the negative effects of light at night (Flynn-Evans et al., 2009; Schernhammer & Schulmeister, 2004; Straif et al., 2007; Viswanathan & Schernhammer, 2009). Experimentally, melatonin has been shown to repress growth of human breast cancer xenografts (Blask et al., 2005) suggesting direct protective roles of melatonin. These include anti-proliferative effects (Blask, 1984; Blask et al., 1999), antioxidant effects (Blask et al., 2002; Brzezinski, 1997), immunostimulatory effects (Brzezinski, 1997; Panzer & Viljoen, 1997) and modulation of the expression of the tumour suppressor gene p53 (Mediavilla et al., 1999). Melatonin may also play a crucial role in the associations of shift-work with diabetes, hypothesized to be generated by disrupted rhythms in peripheral tissues (Staels, 2006). A possible mechanism could be direct disruption of insulin secretion rhythms, as isolated pancreatic tissue of rats can be shifted by melatonin (Peschke & Peschke, 1998). Knockout of the melatonin receptor in mice induces insulin resistance (Contreras-Alcantara et al., 2010; Mühlbauer et al., 2009). Therefore the health of shift workers may benefit when melatonin suppression by light at night is prevented. In this thesis the use of short-wavelength attenuated polychromatic white light ('yellow light') in a simulated shift work setting is shown to result in very limited melatonin suppression, as measured in saliva and urine, without substantial declines in alertness or performance (Chapter 2).

Cortisol

Another hormone that shows pronounced circadian rhythmicity is cortisol. In humans, cortisol starts to rise during the second half of the night and highest levels are reached in the early morning where after a slow decline results in lowest levels in the evening (Edwards et al., 2001; Wüst et al., 2000). Waking up in particular results in large increases in cortisol. This is termed the awakening cortisol response and is characterized by a pronounced peak in production

typically 30-40 min after waking up, which returns back to baseline levels after approximately 60-90 min (Chapter 4; Edwards et al., 2001; Hucklebridge et al., 2005; Pruessner et al., 1997; Wüst et al., 2000). Cortisol is released in response to stressors and prepares the body to either fight or flee the stressor. For example stress stimulates gluconeogenesis, the release of glucose from the liver and increases blood pressure (Kemeny, 2003). The awakening cortisol response is therefore also hypothesized to contribute to the dissipation of sleep inertia, the period of reduced alertness and reduced consciousness, directly after waking up (Clow et al., 2010). Yet, in Chapter 4 it is reported that an alarm clock, which simulates dawn by slowly increasing light intensity, reduced sleep inertia complaints, without a clear effect on cortisol. Also, subjective ratings of activation appeared to be negatively associated with absolute levels of cortisol, possibly also arguing against a direct relationship between the awakening cortisol response and sleep inertia dissipation.

Cortisol levels do not only increase upon awakening and in response to stress, but also readily in response to food intake and light exposure (Gibson et al., 1999; Gonzalez-Bono et al., 2002; Scheer & Buijs, 1999). Negative effects of long-term stress are implicated to increase risk of disease and cortisol specifically has been linked to obesity (Rosmond et al., 1998), cardiovascular disease (Girod & Brotman, 2004; Whitworth et al., 2005), and depression (Cowen, 2010). Interestingly these health problems are also more experienced by shift workers (Bøggild & Knutsson, 1999; Glass & Fujimoto, 1994; Hublin et al., 2010; Karlsson et al., 2001; Knutsson et al., 1986; Morikawa et al., 2005; Scott et al., 1997; Vyas et al., 2012). Shift workers have also been shown to have higher long-term levels of cortisol, as, for instance, shown from hair sampling (Manenschijn et al., 2011). Abnormally low or high levels of cortisol can also impact on cognitive functioning, possibly reducing adequate decision making (Belanoff et al., 2001). The relationship between cortisol and cognitive functioning may be one of the factors contributing to the higher rate of occupational accidents in shift work (Folkard & Lombardi, 2006; Suzuki et al., 2004) and major accidents have been linked to faulty decisions in a shift work setting, for example the Chernobyl and 3-mile island nuclear disasters, the Exxon Valdez oil tanker grounding and the explosion of the space shuttle Challenger (Harrington, 2001).

Cortisol is released by the adrenal glands upon activation by adrenocorticotropic hormone (ACTH) produced in the anterior pituitary

upon stimulation by the PVN via corticotrophin releasing hormone (CRH). This system comprises the hypothalamic-pituitary-adrenal axis (HPA) (Kalsbeek et al., 2012). The circadian rhythm in cortisol is generated by dual control of the adrenal cortex (Engeland & Arnhold, 2005). Direct neuronal innervation of the adrenals by the SCN generates a circadian rhythm in the sensitivity of the adrenal to ACTH, thereby orchestrating rhythmic cortisol release. ACTH is also rhythmically released and this has been interpreted as a direct action of the central clock to produce a set amount of ACTH (Cascio et al., 1987; Engeland & Arnhold, 2005). However, the study in Chapter 5 suggests that this response is likely due to the central clock modulating the sensitivity to release ACTH upon stimulation by CRH, or to sensitivity to produce CRH in the first place. In that study (Chapter 5) we find using a forced desynchrony protocol, in which clock and masking components can be separated for their effects on behaviour and physiology, cortisol levels are predicted closely by a multiplication model of the masking and circadian component. This model fits with physiological regulation of cortisol release via modulation of sensitivity by the clock to produce cortisol in response to a stimulus. This means that the cortisol response to a certain stimulus will drastically vary in magnitude dependent on circadian phase. Shift workers operating at the peak of the circadian rhythm in stress sensitivity are predicted to be subjected to abnormally high levels of cortisol. This may generate future health risks and may reduce performance. Health and performance of shift workers may therefore improve when stress is reduced at times when the clock produces high stress sensitivity (at 8:42 sensitivity to produce cortisol is highest for average chronotypes, Chapter 5).

Skin temperature

Another profound change throughout the body is the distribution of heat within the body resulting from dynamic changes in core versus proximal and distal skin temperature. Core temperature is low at night and is preceded by vasodilation in hands and feet resulting in higher distal skin temperatures (Kräuchi et al., 2000). This process has a strong circadian component (Gradisar & Lack, 2004). These changes can be characterized using a distal-to-proximal skin temperature gradient (DPG) which is correlated to subjective sleepiness (Kräuchi et al., 1997; 2004; Rubinstein & Sessler, 1990), subjective activation (Chapter 2) and vigilance (Raymann & Van Someren, 2007). Preceding

nocturnal sleep, distal skin temperatures increase (Kräuchi et al., 1999; 2000) and this relationship has a causal component. Time needed to fall asleep is reduced when extremities are experimentally warmed (Raymann et al., 2005; 2007). Melatonin is suggested to regulate changes in vasodilation and indeed melatonin administration results in increases in skin temperature and decreases in core temperature (Kräuchi et al., 1997; 2006). Contrary to these findings, differences in melatonin measured during a night under dim and under full-spectrum light were not related to skin temperature differences between these nights (Chapter 2), questioning direct causal involvement of melatonin in regulating the level of vasodilation resulting in changes in skin temperature. A possible explanation for these contradictory results could be that the experiment described in Chapter 2 was performed at night while the experiments by Kräuchi et al. (1997, 2006) were performed during daytime. In the process of waking up, skin temperatures decline and during this period of sleep inertia it is linked to the dissipation of sleepiness and decrements in activation (Chapter 4; Kräuchi et al., 2004). Interestingly in this thesis strong and consistent relationships between skin temperature and subjective ratings of activation and sleepiness are reported, both in the morning during waking up (Chapter 4) and while staying awake at night (Chapter 2). This in contrast with the lack of associations between subjective activation and sleepiness with melatonin and between subjective sleepiness and cortisol (Chapter 2; Chapter 4). This may suggest that changes in blood flow dynamics are among the strongest determinants of activation and sleepiness (Van Someren, 2000). Indeed, skin temperature is also likely causally involved since cooling of the skin increases vigilance (Fronczek et al., 2008). The concordant experimental effect of artificial dawn simulation on skin temperature and subjective ratings of activation and sleepiness (Chapter 4) also suggest such a causal relationship.

Individual differences

Examples of between individual differences in biology are manifold, as they are also in chronobiology. One of the most characteristic examples is the preferred timing of sleep and activity in the day. Some individuals prefer to get up and go to bed early, whereas others are late types and prefer to stay up late and to sleep in (Horne & Östberg, 1976; Roenneberg et al., 2003) and these differences have a heritable component (Klei et al., 2005; Vink et al., 2001). Late chronotype is associated with higher risk of depressive mood (Hidalgo et

al., 2009), cardiovascular disease and diabetes (Merikanto et al., 2013). Shifts in activity patterns may contribute to these relationships, either directly or indirectly via effects on sleep. These shifts in activity patterns can arise due to social or work obligations because early types stay up too late or late types get up too early with respect to their internal phase. This creates a difference in activity patterns between free days and workdays, termed social jetlag. The degree of social jetlag is linked to smoking habits (Wittmann et al., 2006) and obesity (Roenneberg et al., 2012). In a sense, individuals suffering from social jetlag may be viewed as undergoing repeated shift work and may also suffer from the associated health problems, and hence may also benefit from possible interventions that may improve health of shift workers.

Another striking level of individual variation was encountered in the study presented in Chapter 3. Melatonin suppression differed markedly between individuals in response to a similar level of light stimulation. Because melatonin is hypothesized to play a role in the health deficits of shift workers, a molecular basis of these individual differences was sought. From part of the subjects enrolled in Chapter 2, we also collected skin fibroblasts to test the sensitivity of a part of the molecular signalling cascade that is also involved in melatonin suppression, namely the AC – cAMP – PKA pathway. Light exposure at night reduces noradrenergic stimulation of β_1-adrenoceptors of the pineal cells (Simonneaux & Ribelayga, 2003), causing a reduced stimulation of the intra-pineal cell AC – cAMP – PKA pathway, resulting in reduced melatonin synthesis. This latter intracellular signalling pathway was stimulated in fibroblasts obtained from subjects in order to determine the degree of excitability of this pathway. In the same subjects, the degree of melatonin suppression was investigated in response to light. Intriguingly *in vivo* melatonin suppression showed a strong negative correlation with *in vitro* cellular excitability suggesting that part of the between-individual differences in melatonin suppression is caused by differences in either AC or PKA (Chapter 3). In addition, these results show that fibroblasts can be used as a model for processes regulated in the human brain, such as melatonin suppression.

A prediction on individual sensitivity for melatonin suppression would be highly relevant within the context of shift work and health.

Improving human performance and health around the clock

Knowledge about circadian biology and the effects of light on the body opens

exciting avenues to improve human performance and health. In this thesis we show that light stimulation in the morning, through dawn simulation, reduces sleep inertia complaints (Chapter 4). Many people suffer from sleep inertia, reduced alertness and consciousness immediately after waking up, which may last for over an hour. Melatonin suppression by light has been implicated in the development of health deficits related to shift work. By using knowledge about the spectral sensitivity of the non-image forming light sensitive system providing input to the circadian system, health of shift workers may improve. In Chapter 2, we show that the use of short-wavelength attenuated polychromatic white light during work at night allowed for an undisturbed melatonin profile without large repercussions for performance, although the relatively high distal skin temperature may be a physiological indication that vigilance might be affected negatively. Individuals involved in night or shift work, likely differ in their susceptibility to the negative health effects of light at unnatural times. For example, individuals with low melatonin suppression under a considerable amount of lighting may be less susceptible. Insight in the mechanisms underlying variation in melatonin suppression may help shift workers and may even have implications for the treatment of individuals suffering from seasonal affective disorder (Chapter 3). By linking *in vivo* melatonin suppression to the excitability of a specific pathway in fibroblasts obtained from the same subjects, the first steps are taken in understanding the physiology underlying variation in human melatonin suppression. This knowledge may facilitate a better understanding of the molecular background of individual differences in the sensitivity to light, or even to detrimental effects of working night shifts in general. Not only light at night, but also performing activities at an 'unnatural' circadian phase may result in abnormal physiological responses that may underlie health problems prevalent in shift workers. Using a forced desynchrony protocol we show that cortisol production, a marker of stress, has a strong circadian component. Most importantly this circadian component influences cortisol production in a multiplicative fashion. Therefore activities such as eating or psychological stress are predicted to result in extremely low or extremely high levels of cortisol, dependent on circadian phase (Chapter 5). Dysregulation of cortisol levels may also underlie part of the health problems incurred by shift workers as cortisol is implicated in many health problems similar to those that shift workers suffer from. Shift workers may benefit from limiting stress during times when their circadian stress sensitivity is highest.

Together these results indicate that making relatively small changes to the shift worker's environment has the potential for considerable gains in their health. Benefits from a biological clock are especially reaped when living in tune with the external light dark cycle. However, social and/or work related obligations may cause misalignment of our internal timing with our actual behaviour and exposure to light. Investigating under which conditions individual preferences and actual behaviour are ideally combined is becoming more and more important now that these two factors are diverging in an increasing number of people.

CHAPTER

2

SHORT-WAVELENGTH ATTENUATED POLYCHROMATIC WHITE LIGHT DURING WORK AT NIGHT: LIMITED MELATONIN SUPPRESSION WITHOUT SUBSTANTIAL DECLINE OF ALERTNESS

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Chronobiology International (2013) 30:843-854.

Abstract

Exposure to light at night increases alertness, but light at night (especially short-wavelength light) also disrupts nocturnal physiology. Such disruption is thought to underlie medical problems for which shift workers have increased risk. In 33 male subjects we investigated whether short-wavelength attenuated polychromatic white light (< 530 nm filtered out), at night preserves dim light melatonin levels and whether it induces similar skin temperature, alertness, and performance levels as under full-spectrum light. All 33 subjects participated in random order during three nights (at least 1 wk apart) either under dim light (3 lux), short-wavelength attenuated polychromatic white light (193 lux), or full-spectrum light (256 lux). Hourly saliva samples for melatonin analysis were collected along with continuous measurements of skin temperature. Subjective sleepiness and activation were assessed via repeated questionnaires and performance was assessed by the accuracy and speed of an addition task. Our results show that short-wavelength attenuated polychromatic white light only marginally (6%) suppressed salivary melatonin. Average distal-to-proximal skin temperature gradient (DPG) and its pattern over time remained similar under short-wavelength attenuated polychromatic white light compared with dim light. Subjects performed equally well on an addition task under short-wavelength attenuated polychromatic white light compared with full-spectrum light. Although subjective ratings of activation were lower under short-wavelength attenuated polychromatic white light compared with full-spectrum light, subjective sleepiness was not increased. Short-wavelength attenuated polychromatic white light at night has some advantages over bright light. It hardly suppresses melatonin concentrations, whereas performance is similar to the bright light condition. Yet, alertness is slightly reduced as compared with bright light, and DPG shows similarity to the dim light condition, which is a physiological sign of reduced alertness. Short-wavelength attenuated polychromatic white light might therefore not be advisable in work settings that require high levels of alertness.

Introduction

In industrialized countries, about 15-30% of the working population is involved in some kind of permanent night and rotating shift work (Boivin et al., 2007; Haus & Smolensky, 2006). This means that many people are exposed to light and to darkness at atypical biological times because their work and subsequent sleep are not synchronized with the natural light-dark cycle. Sleeping and working out of phase with the body's circadian rhythms for longer periods can cause (chronic) fatigue and affect performance and cognitive functions (Costa, 1996; Härmä et al., 2002; Wright et al., 2006). Moreover shift work has been associated with health problems including cardiovascular disease, impaired glucose and lipid metabolism, type 2 diabetes, gastrointestinal discomfort such as stomach ache or diarrhea, reproductive difficulties, and cancer (Haus & Smolensky, 2006; Jasser et al., 2006; Mahoney, 2010; Morikawa et al., 2005; Rüger & Scheer, 2009; Straif et al., 2007; Viswanathan & Schernhammer, 2009).

The medical problems encountered by shift workers are thought to result from disturbed physiological rhythms, circadian misalignment and sleep dept (Rajaratnam & Arendt, 2001; Rüger & Scheer, 2009; Scheer et al., 2009; Van Cauter et al., 1997). Being exposed to light at night shifts the phase of the central master clock in the brain (suprachiasmatic nucleus [SCN]), which is responsible for synchronizing endogenous circadian rhythms, such as the melatonin rhythm, and the rhythm of peripheral clocks. Light, and especially short-wavelength light, the blue part of the light spectrum, entrains circadian rhythms in humans (Lockley et al., 2003; Revell & Eastman, 2005; Rüger et al., 2012; Warman et al., 2003), but also exerts an acute suppressing effect upon melatonin production (Brainard et al., 2001; Thapan et al., 2001). In addition, short-wavelength light suppresses sleepiness (Cajochen et al., 2005) and increases alertness (Revell et al., 2006) effectively, which is beneficial for performance at night (Chellappa et al., 2011). These specific effects of short-wavelength light are attributed to non-visual photoreceptive input by specific retinal ganglion cells towards the SCN (Gooley et al., 2003; Hattar et al., 2002). These cells express a specific photopigment, melanopsin, which has a peak absorbance around 480 nm with an approximate upper boundary at 540 nm (Dacey et al., 2005; Walker et al., 2008).

Long-term suppression of melatonin production is thought to play an

important role in increased prevalence of cancer in shift workers (Flynn-Evans et al., 2009; Schernhammer & Schulmeister, 2004; Straif et al., 2007; Viswanathan & Schernhammer, 2009). Direct protective roles of melatonin against cancer development have been proposed: antiproliferative effects (Blask, 1984; Blask et al., 1999), antioxidant effects (Blask et al., 2002; Brzezinski, 1997), immunostimulatory effects (Brzezinski, 1997; Panzer & Viljoen, 1997), and modulation of the expression of the tumour suppressor gene p53 (Mediavilla et al., 1999). Shift work has also been associated with increased prevalence of diabetes and the metabolic syndrome (Karlsson et al., 2001; Morikawa et al., 2005). This is thought to be caused by disrupted physiological rhythms in peripheral tissues (Staels, 2006). Melatonin may play a crucial role here as well, for melatonin has been shown to shift insulin secretion rhythms of isolated pancreatic tissue of rats (Peschke & Peschke, 1998).

In our 24-h society it becomes more and more important to unravel the biological mechanisms that are involved in the development of health problems in shift workers and to investigate different countermeasures. One of such measures, the use of short-wavelength attenuated polychromatic white light, was investigated in the present study. From an economical point of view, it seems beneficial to expose shift workers to full-spectrum light at night to increase alertness and improve performance at night. On the other hand, if working under full-spectrum light at night is detrimental for health on the long term, alternatives such as short-wavelength attenuated polychromatic white light should be subject of investigation. To assess the magnitude of possible decrements in performance under short-wavelength attenuated polychromatic white light, we included an addition task and measures of subjective sleepiness, subjective activation, and skin temperature during prolonged wakefulness while working at night. Skin temperature is linked to sleepiness (Kräuchi et al., 1997, 2004) and vigilance (Raymann & Van Someren, 2007). Sleep initiation is preceded by a rise in distal skin temperature (Kräuchi et al., 1999, 2000) and experimentally warming extremities reduces the time needed to fall asleep in healthy subjects (Raymann et al., 2005, 2007). During night sleep, distal skin temperatures remain high and core body temperature drops (Kräuchi et al., 2000) and this process has a strong circadian component (Gradisar & Lack, 2004). Upon waking up, skin temperature declines and during this stage lower skin temperatures are linked to decreased sleepiness and increased activation (Kräuchi et al., 2004; Van de Werken et al., 2010). Indeed cooling instead of

warming of the skin increased vigilance in narcolepsy patients (Fronczek et al., 2008). The physiological functions of skin temperature dynamics during day and night, other than those associated with sleep, are not well known compared with our understanding of the actions of melatonin. However a disturbance in the regular dynamics of thermal regulation may also have negative effects on health, unknown to us thus far.

In the present study we studied 33 male subjects in three conditions in three separate nights (at least 1 wk apart in randomized order): a dim light condition, a short-wavelength attenuated polychromatic white light condition, and a full-spectrum light condition. We investigated the effects of these light conditions on subjective ratings of sleepiness and activation, performance on an addition task, melatonin levels, and the distal-to-proximal skin temperature gradient (DPG) (Kräuchi et al., 2000; Rubinstein & Sessler, 1990).

Materials and Methods

Subject Characteristics

Subjects (33 healthy males, mean age [±SD] 22.6 ± 2.2 yr) did not have sleep disorders (Pittsburgh Sleep Quality Index < 6; Buysse et al., 1989), somatic diseases, depressed mood (Beck Depression Inventory-II < 8; Beck et al., 1996, 2002), chronic diseases, colour blindness (Ishihara test, Clark, 1924), or visual impairment (assessed by general health questionnaire). Subjects did not use medication (assessed by general health questionnaire). Neither did they work in night shifts or travelled across more than one time zone respectively 3 and 1 mo preceding the study. Subjects had an average chronotype for their gender and age (mean midsleep on free days [±SD] 5:45 h ± 43 min, with a mean sleep onset [±SD] at 01:24 h ± 58 min and a mean sleep offset [±SD] at 10:05 h ± 49 min, rated on the Munich Chronotype Questionnaire; Roenneberg et al., 2003). Subjects did not report excessive intake of caffeinated drinks (average cups a day [±SD] 2 ± 2) or alcohol (average glasses a week [±SD] 8 ± 5), were non-smoking and did not use drugs (assessed by general health questionnaire). The study protocol was approved by the Medical Ethics Committee of the University Medical Center of Groningen, The Netherlands, and conformed to international ethical standards (Portaluppi et al., 2008). All subjects gave written informed consent and were financially compensated for their participation.

Experimental Design

In this paper the results of two similar studies were combined. These studies differed in a single aspect: in Study I subjects stayed together with other participants in one room and in Study II all subjects stayed in individual rooms. The studies consisted of three nights from 21:00 till 7:00 h in the laboratory, with a minimum of 1 wk between successive measurements (see Figure 1). The first study in which 17 subjects participated was carried out between October 10 and November 22, 2008, and the second study in which 16 subjects participated was carried out between June 19 and August 7, 2010. Only in Study I the experiment continued the subsequent day at home until 23:00 h, during which urine was collected for 6-sulfatoxymelatonin (aMT6s) analysis. During the first 2 h of each night, subjects stayed in dim light (< 5 lux). In the first hour (21:00 – 22:00 h), subjects were oriented to study the protocol (data not included, except for salivary melatonin concentration). During the second hour (22:00 – 23:00 h), baseline data were collected. In random order, from 23:00 till 7:00 h subjects stayed in one of the following three conditions: (1) dim light (horizontal light intensity [±SD] 1 ± 1 lux, vertical light intensity at the level of the eye [±SD] 3 ± 2 lux); (2) short-wavelength attenuated polychromatic white light (horizontal light intensity [±SD] 438 ± 101 lux, vertical light intensity at the level of the eye [±SD] 193 ± 108 lux; short-wavelength [< 530 nm] reducing foil [heat shrinkable tubing; WLF-NR400-4-SP; Tyco Electronics, UK] melted around a Philips TL-D 36W/830, aimed to reduce non-visual photoreception via melanopsin, total irradiance from 420 to 530 nm = 0.0 W/m^2); or (3) full-spectrum light (horizontal light intensity [±SD] 625 ± 151 lux, vertical light intensity at the level of the eye [±SD] 256 ± 131 lux, total irradiance from 420 to 530 nm = 0.14 W/m^2; Philips TL-D 36W/830); see Figure 2 for the spectral composition of the lights. Subjects were placed in the same location in the room in all three conditions to reduce within individual variation in light intensities and stayed seated behind their computers as far as possible. Subjects were not allowed to sleep at any time during the experiment. Hourly snacks of 100 calories and 130 mL water (room temperature) were supplied. Computer screens and dim lights were covered with a colour filter (E-colour +, 105 Orange; Rosco Laboratories, London, UK) to decrease short-wavelength light transmission.

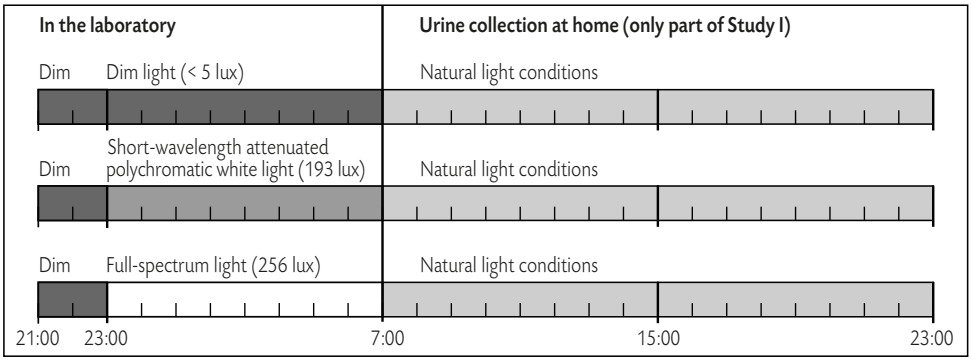


Figure 1: Overview of the experimental design; for the experimental details refer to the Experimental Design section (Methods).

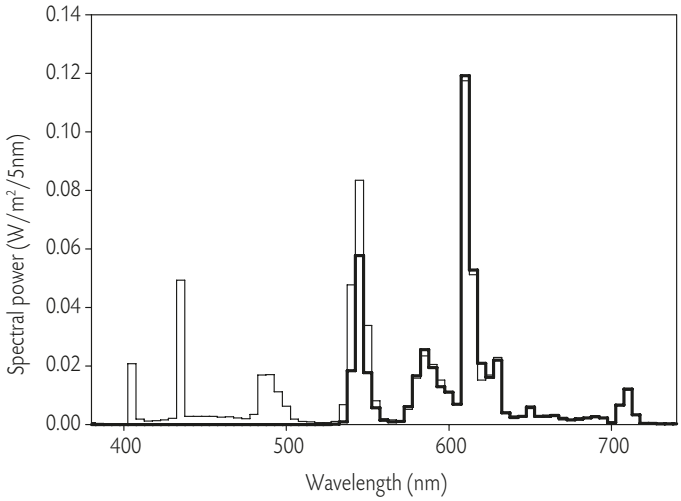


Figure 2: Spectral power distribution. Short-wavelength attenuated polychromatic white light (thick line) was obtained by melting a yellow filter (see Materials and Methods for specifications) on top of a 830 TL tube (3000K), which was also used in the full-spectrum light condition (thin line).

Measurements

Salivary Melatonin

Each hour (from 21:30 till 6:30 h), saliva samples were collected using cotton swabs (Salivettes; Sarstedt, Etten-Leur, The Netherlands). Melatonin concentration was assessed by radioimmunoassay (RK-DSM; Bühlmann Laboratories, Alere Health, Tilburg, The Netherlands). Each sample was analysed once and all samples from each individual were analysed together within the same assay. Analytical sensitivity: 0.2 pg mL^{-1}; intra- and inter-assay coefficients of variability: 7.8% (mean 14.5 pg mL^{-1}) and 13.8% (mean 17.6 pg mL^{-1}), respectively. Two baseline measurements were carried out in each condition in dim light (at 21:30 and 22:30 h).

Urinary 6-Sulfatoxymelatonin (aMT6s)

Just before the experimental light condition was set in the first study (at 23:00 h), subjects were asked to empty their bladder. From here on, total 24-h urine production was collected at three 8-h intervals. Urine from the first interval (23:00 – 7:00 h) was collected during the experiment in the laboratory. Urine from the second (7:00 – 15:00 h) and third (15:00 – 23:00 h) intervals was collected at home. aMT6s concentration was assessed by enzyme-linked immunosorbent assay (ELISA; EK-M6S; Bühlmann Laboratories, Alere Health, Tilburg, The Netherlands). Each sample from one individual was analysed within the same assay. Analytical sensitivity: 0.14 ng mL^{-1}; intra- and inter-assay coefficients of variability: 10.6% (mean 25.3 ng mL^{-1}) and 21.2% (mean 26.1 ng mL^{-1}), respectively. Total aMT6s excretion was calculated by multiplying concentration and volume.

Distal-to-Proximal Skin Temperature Gradient

Skin temperatures were recorded throughout the night at a rate of one sample per minute. Recording ended immediately after the last testing periods at 7:00 h. Skin temperature was measured using Ibuttons (DS1922L; Maxim Integrated Products, Sunnyvale, CA, USA; resolution 0.0625 °C; for further technical details and validation of the use of Ibuttons to measure skin temperature, see Van Marken Lichtenbelt et al., 2006) that were placed on the base of left and right middle fingers (distal skin temperature) and left and right infraclavicular regions (proximal skin temperature). The DPG was calculated as distal minus proximal skin temperature.

Subjective Ratings of Sleepiness and Activation

Subjective ratings of sleepiness (Karolinska Sleepiness Scale; Åkerstedt & Gillberg, 1990) and activation (two factors of the Thayer adjective checklist; Thayer, 1967) were determined hourly.

Addition Task

Every hour, subjects made as many correct additions as possible within a 3-min interval using a custom-made computer test. All additions consisted of two random numbers of two digits each.

Statistical Analysis

The differences over time of salivary melatonin, urinary aMT6s, subjective ratings of sleepiness, activation, and the addition task were tested with a repeated-measures analysis of variance (ANOVA) with one within factor (time) and one within factor (condition). The percentage of melatonin suppression is calculated by comparing the area under the curve in the dim light condition with the area under the curve in the short-wavelength attenuated polychromatic white light and full-spectrum light condition over the whole night (from 23:30 till 6:30 h). Percentage of correct additions was tested non-parametrically (k related samples) with a Friedman test. Baseline measurements were tested separately, to check whether already at this moment a difference could be observed between the three conditions. Study was included as a between-subject factor and was maintained in the model if it contributed significantly (either as main effect or in interaction with condition or with condition × time), which was only the case for salivary melatonin and subjective activation. We detected that in Study I absolute salivary melatonin levels were slightly lower than in Study II across the three conditions ($P = 0.02$). Within the analysis on subjective ratings of activation, we detected an interaction between study and condition ($P = 0.03$). These results, also taking into account that in exploring the effects of study we carried out multiple tests for which we did not correct P values upwards, indicate that there are no strong differences between these two data sets that confound our interpretations.

Skin temperature outliers (defined as quartile + [2 × interquartile range]) were deleted and interpolated. This resulted in data loss at the end of the night because data could not be interpolated there, resulting in a maximum of 12-min data reduction to which all subjects were truncated. For the analysis of

the resulting DPG, we used mixed-effect regression analysis using MLwiN software (Centre for Multilevel Modelling, Institute of Education, London, UK). The analyses take the hierarchical structure of the design into account, in our case the temperature measurements *i* that were nested within participants *j*, once more nested within condition *k*, and lastly nested within study *l* (Twisk, 2003). Because the sampling frequency of skin temperature was high (once per minute), we also corrected for auto-correlated residual errors by including an auto-correlation function that was optimized manually to fit the data best into the models. To control for pseudo-replication optimally, we not only included random intercepts but also random coefficients (Schielzeth & Forstmeier, 2009) for linear time, sqrt(time), condition, and the interactions including condition. The following model equation was used to fit the data:

$$\begin{aligned}\text{Temperature}_{ijkl} = {} & \beta_{0jkl} + \beta_{1j} \times \text{Condition}_{ijkl} + \beta_{2j} \times \text{Linear\_time}_{ijkl} + \beta_{3j} \times \\ & \text{Sqrt(time)}_{ijkl} + \beta_{4j} \times \text{Condition} \times \text{Linear\_time}_{ijkl} + \beta_{5j} \times \\ & \text{Condition} \times \text{Sqrt(time)}_{ijkl} + \beta_{6} \times \text{Ambient\_Temperature}_{ijkl}\end{aligned}$$

where β_0 represents the model intercept, β_1 the deviation from the intercept (at 23:00 h) of each condition (Condition), β_2 and β_3 together represent the non-linear time course of temperature during the night (Linear_time indicates the time since 23:00 h, Sqrt(time) indicates the square root of linear time), β_4 the effect of condition on the linear component of the time course, β_5 the effect of condition on the non-linear time component, and β_6 the effect of ambient temperature during the night. Maximum likelihood was used to estimate the regression coefficients of which the statistical significance was evaluated using z-tests. To test whether the average DPG across the night differed between conditions, we implemented repeated-measures procedures outlined above with DPG residuals corrected for room temperature as the dependent variable.
A Pearson correlation was used to show the relationship between the change in melatonin and the change in DPG with the change in sleepiness and activation between the dim light and short-wavelength attenuated polychromatic white light conditions and between the dim light and the full-spectrum light conditions. These values were first z-transformed as follows: the difference of each individual measurement between conditions (from 23:00 to 6:30 h) was first averaged per subject and then averaged for all subjects, the overall average was subtracted from the average per subject and then divided by the SD of the

overall average for all subjects.
For graphical purposes only, salivary melatonin was normalized to the maximum value of the best-fitted melatonin curve (Van Someren & Nagtegaal, 2007) in the dim light condition, and the number of correct additions were normalized as follows: all samples per subject were divided by the average of all samples over the dim light condition per subject (indicated by the dotted line in the graph) (from 23:00 to 6:00 h) and then multiplied by 100%.
Values are described as average ± SEM. All tests were two tailed with a 0.05 level of significance.

Results

Salivary Melatonin

At baseline (21:30 and 22:30 h), before initiating the three light conditions, salivary melatonin values were similar ($F_{2,30} = 0.95$, non-significant [NS]) between conditions. In the dim light and short-wavelength attenuated polychromatic white light conditions, melatonin remained high throughout the night, whereas within the full-spectrum light condition, melatonin was suppressed compared with dim light and short-wavelength attenuated polychromatic white light (Figure 3; dim light: $F_{1,31} = 32.69$, $P < 0.001$, short-wavelength attenuated polychromatic white light: $F_{1,31} = 27.62$, $P < 0.001$; interaction with time, dim light: $F_{7,25} = 12.15$, $P < 0.001$, short-wavelength attenuated polychromatic white light: $F_{7,25} = 3.13$, $P < 0.05$). Within the short-wavelength attenuated polychromatic white light condition, melatonin was significantly suppressed relative to dim light ($F_{1,31} = 5.27$, $P < 0.05$; interaction with time, $F_{7,25} = 1.51$, NS), but suppression was small (6 ± 4%) compared with the suppression in the full-spectrum light condition (45 ± 6%).

Urinary 6-Sulfatoxymelatonin (aMT6S)

Melatonin production as measured by aMT6s excretion showed significantly different patterns between conditions over the 24-h period (Figure 4; $F_{4,13} = 10.12$, $P = 0.001$). Post hoc tests revealed that the difference in pattern over time was caused by a significantly lower aMT6s concentration in the full-spectrum light condition compared with both the dim light and short-wavelength attenuated polychromatic white light conditions in the interval

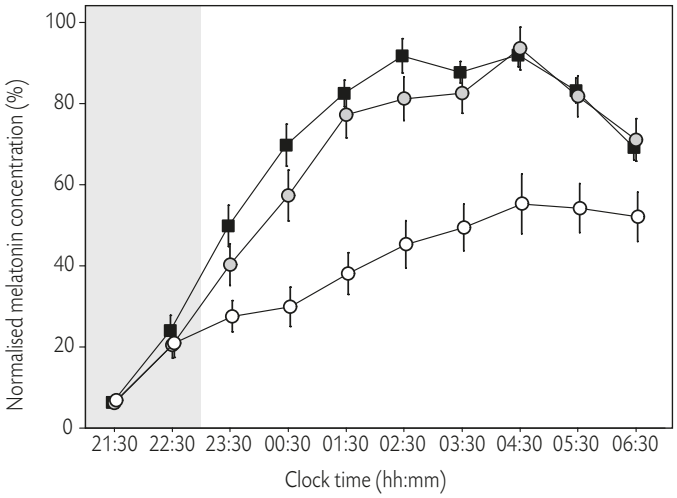


Figure 3: Salivary melatonin concentration. Mean (±SEM) melatonin concentration in the dim light (black squares), short-wavelength attenuated polychromatic white light (grey circles), and full-spectrum light (white circles) conditions (n = 33).
The first two samples of each condition (at 21:30 and 22:30 h) were taken in dim light.

23:00 – 7:00 h (dim light: $F_{1,16} = 19.47$, $P < 0.001$; short-wavelength attenuated polychromatic white light: $F_{1,16} = 39.05$, $P < 0.001$). At the same interval, a trend was observed suggesting lowered melatonin in the short-wavelength attenuated polychromatic white light compared with the dim light condition ($F_{1,16} = 4.17$, $P = 0.06$), which fits the salivary melatonin data. That the differences in aMT6s concentrations are found only at this first interval and not in the second interval, which reflects early morning melatonin levels, suggests that the largest differences in melatonin levels between light conditions are found in the middle of the night. This pattern fits with the salivary melatonin data as well.

Distal-to-Proximal Skin Temperature Gradient

With respect to DPG, the three conditions did not differ at the start of the experiment (P values ≥ 0.5). DPG rose in the dim light and short-wavelength

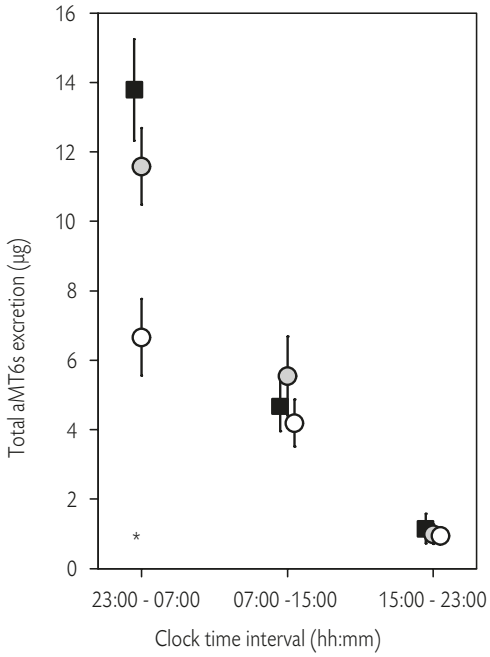


Figure 4: Urinary 6-sulfatoxymelatonin (aMT6s). Mean (±SEM) of total aMT6s excretion in the dim light (black squares), short-wavelength attenuated polychromatic white light (grey circles), and full-spectrum light (white circles) conditions (n = 17) during the experiment in the laboratory (first interval: 23:00 – 7:00 h) and during the subsequent day at home where subjects were exposed to their own lights (second interval: 07:00 – 15:00 h and third interval: 15:00 – 23:00 h). Note that only in Study I the experiment continued the subsequent day at home (n = 17).

attenuated polychromatic white light conditions (Figure 5), with a similar trajectory over the whole night (β estimate ± error: Condition × Linear_time = 0.03 ± 0.17 °C h^{-1}, NS; Condition × Sqrt(time) = -0.12 ± 4.19 °C h^{-1}, NS). The rise in DPG was not observed in the full-spectrum light condition. This resulted in significantly different trajectories of DPG in the full-spectrum light condition compared with the dim light and short-wavelength attenuated polychromatic white light conditions (vs. dim light: Condition × Linear_time = 0.51 ± 0.17 °C h^{-1}, $P < 0.005$, Condition × Sqrt(time) = -11.75 ± 4.26 °C h^{-1}, $P < 0.01$; vs. short-wavelength attenuated polychromatic white light: Condition × Linear_time = 0.48 ± 0.18 °C h^{-1}, $P < 0.01$, Condition × Sqrt(time) = -11.62 ± 4.59 °C h^{-1}, $P < 0.05$) and a significantly lower average DPG in the experimental night under full-spectrum light (dim light: $F_{1,32} = 15.39$, $P < 0.001$; short-wavelength attenuated polychromatic white light: $F_{1,32} = 5.30$, $P < 0.05$) compared with dim light and short-wavelength attenuated polychromatic white light (which were not significantly different, $F_{1,32} = 2.27$, NS).

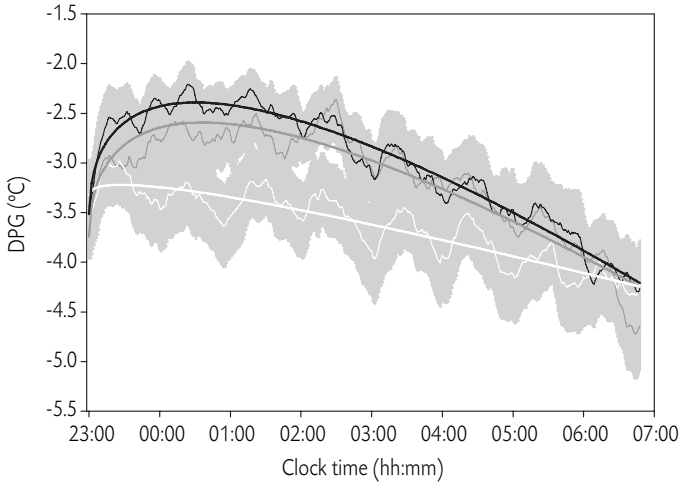


Figure 5: Distal-to-proximal skin temperature gradient. Thin lines represent the mean (±SEM) DPG values and thick lines represent the best fit through the raw data; in dim light (black lines), short-wavelength attenuated polychromatic white light (grey lines), and full-spectrum light (white lines) conditions (n = 33). Hourly oscillations in all conditions are visible and these are attributable to the hourly tests the subjects performed (see Methods).

Subjective Sleepiness and Activation

At baseline (22:00 h), subjective sleepiness did not differ significantly between conditions ($F_{2,31} = 0.82$, NS) nor did subjective activation ($F_{2,30} = 0.42$, NS). Sleepiness increased significantly during the course of the night (Figure 6A; $F_{7,26} = 18.07$, $P < 0.001$), but no significant differences were found between the three conditions (condition: $F_{2,31} = 2.77$, NS). Sleepiness showed a significantly different pattern over time between the three conditions (condition × time: $F_{14,19} = 2.73$, $P < 0.05$; this was not solely attributable to the small spike at 3:00 h in the dim light condition; without these data in the analysis the interaction remained significant). Activation decreased significantly during the night (Figure 6B; $F_{7,25} = 35.94$, $P < 0.001$), with a similar pattern over time between the three conditions ($F_{14,18} = 1.95$, NS). No significant difference was found between the dim light and short-wavelength attenuated polychromatic white light conditions

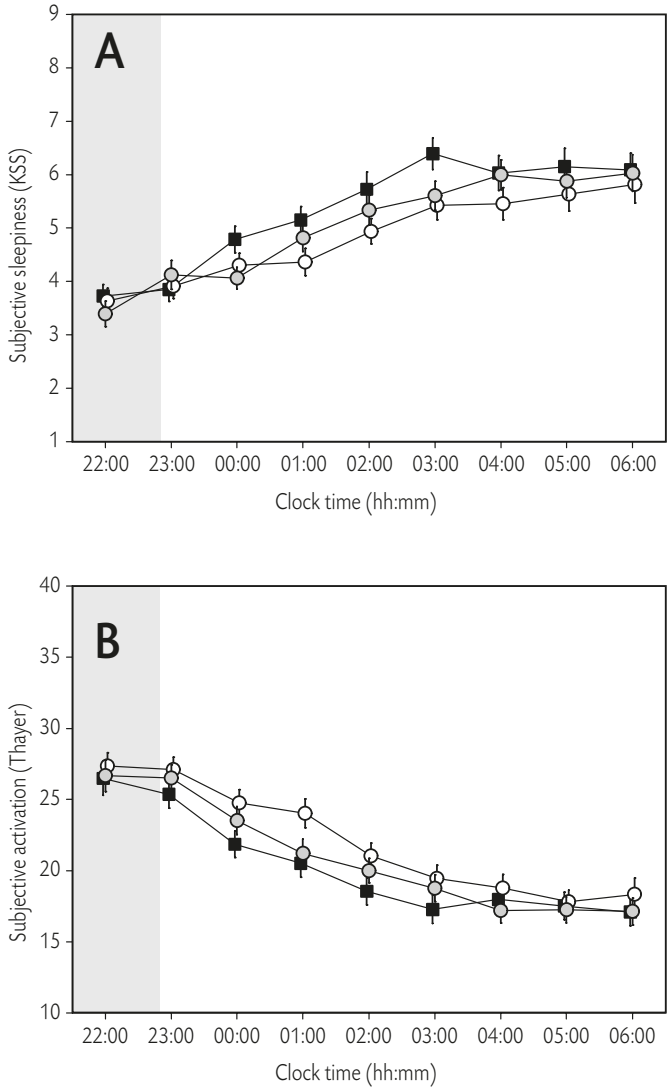


Figure 6: Subjective sleepiness and activation. (A) Mean (±SEM) subjective ratings of sleepiness (Karolinska Sleepiness Scale, 1 = lowest and 9 = highest sleepiness) and (B) mean (±SEM) subjective ratings of activation (Thayer adjective checklist, 10 = lowest and 40 = highest activation) in the dim light (black squares), short-wavelength attenuated polychromatic white light (grey circles), and full-spectrum light (white circles) conditions (n = 33). Baseline measurements were performed at 22:00 h in dim light.

($F_{1,31}$ = 1.43, NS). However, both conditions differed significantly from the full-spectrum light condition (dim light: $F_{1,31}$ = 5.54, P < 0.05; short-wavelength attenuated polychromatic white light: $F_{1,31}$ = 6.33, P < 0.05).

Addition Task

During the baseline measurement (22:00 h), no significant differences between conditions were found in the number of correct additions obtained during a 3-min interval by the subjects (dim light: 36 ± 2; short-wavelength attenuated polychromatic white light: 37 ± 2; full-spectrum light: 38 ± 2; $F_{2,31}$ = 0.62, NS) nor in the percentages of additions that were correct (dim light: 95%, short-wavelength attenuated polychromatic white light: 95%, full-spectrum light: 96%; χ^2 = 1.27, NS). During the experimental periods, subjects performed equally well in each lighting condition in terms of the fraction of correctly made additions (average percentage correct additions, dim light: 96%, short-wavelength attenuated polychromatic white light: 96%, full-spectrum light:

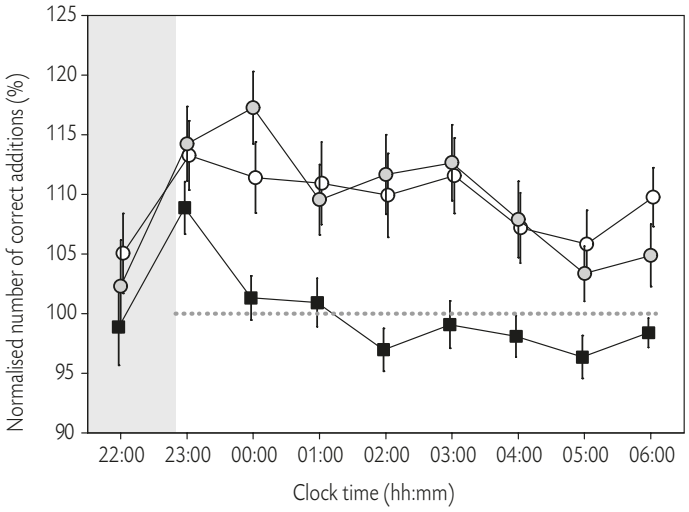


Figure 7: Number of correct additions. Mean (±SEM) number of correct additions in the dim light (black squares), short-wavelength attenuated polychromatic white light (grey circles), and full-spectrum light (white circles) conditions (n = 33). The number of correct additions made during a 3-min time interval were normalized within subjects relative to their dim light average from 23:00 up to 6:00 h inclusive (indicated by the dotted line). Baseline measurements were done at 22:00 h in dim light.

96%; $\chi^2 = 1.70$, NS). The total number of correct additions made (Figure 7) was higher in the short-wavelength attenuated polychromatic white light (on average 40 ± 2; $F_{1,32} = 18.30$, $P < 0.001$) and in the full-spectrum light (on average 40 ± 2; $F_{1,32} = 15.87$, $P < 0.001$) conditions compared with the dim light condition (on average 36 ± 2), whereas no significant difference was found between the short-wavelength attenuated polychromatic white light and full-spectrum light conditions ($F_{1,32} = 0.01$, NS). This suggests that subjects perform better under full-spectrum light and short-wavelength attenuated polychromatic white light conditions because they work faster at a similar error rate compared with the dim light condition. Conditions differed in their pattern over time ($F_{14,19} = 2.34$, $P < 0.05$), during which the number of correct additions on average decreased (time: $F_{7,26} = 6.92$, $P < 0.001$).

Relationships Between Melatonin, DPG, Sleepiness, and Activation

The difference in melatonin between the dim light condition and the short-wavelength attenuated polychromatic white light condition or full-spectrum light condition did not correlate with the difference in sleepiness or activation between these conditions (dim light vs. short-wavelength attenuated polychromatic white light: sleepiness $r = 0.094$, NS, activation $r = -0.185$, NS; dim vs. full-spectrum light: sleepiness: $r = 0.179$, NS, activation: $r = -0.074$, NS; see Supplementary Material). The difference in sleepiness or activation between the dim light and short-wavelength attenuated polychromatic white light conditions did not correlate with the difference in DPG between these conditions (sleepiness: $r = 0.263$, NS; activation: $r = -0.045$, NS). However, between the dim light and full-spectrum light conditions, the correlations between the difference in subjective ratings and difference in DPG were significant (sleepiness: $r = 0.437$, $P < 0.05$; activation: $r = -0.401$, $P < 0.05$), indicating that with increasing DPG people felt more sleepy and less subjectively activated. The degree of melatonin suppression under short-wavelength attenuated polychromatic white light or full-spectrum light did not correlate to the difference of DPG under dim light with short-wavelength attenuated polychromatic white light or full-spectrum light (short-wavelength attenuated polychromatic white light: $r = -0.182$, NS; full-spectrum light: $r = 0.105$, NS).

Discussion

This study shows that when healthy male individuals are exposed to short-wavelength attenuated polychromatic white light at night, both salivary melatonin profile and average salivary melatonin concentration are only marginally, but significantly, affected (on average 6 ± 4% suppression) compared with the same situation in dim light. As expected, a clear suppression of melatonin was observed under full-spectrum light (on average 45 ± 6% compared with dim light). Similar results were found for urinary aMT6s concentration measured in a subsample of our subjects. Two earlier studies (Kayumov et al., 2005; Rahman et al., 2011) also found some melatonin suppression when short-wavelengths were filtered out, but not significantly so. This is surprising because both these studies used short-wavelength attenuated light conditions that can be predicted to result in higher melatonin suppression compared with our study; in the study of Rahman et al. less of the spectrum was filtered out (filters used at the level of the eye: 0% transmission < 480 nm) and higher light intensities were used (439 lux in angle of gaze) than in our study (193 lux at the level of the eye) and Kayumov et al. also used higher light intensities (800 lux), but with a similar filtering of the spectrum (< 530 nm, using goggles with filters). With a larger sample size than both these studies (33 subjects in the current study vs. 12 and 19 subjects in Rahman et al. and Kayumov et al., respectively), we now detect a significant but only small suppression of melatonin (6%) under short-wavelength attenuated polychromatic white light. This effect could be due to some activation at the edge (around 550 nm; see Figure 2) of the action spectrum of melatonin suppression (Brainard et al., 2001; Thapan et al., 2001) by the short-wavelength attenuated polychromatic white light. This could mean that melanopsin expressing retinal ganglion cells are responsible for the suppression of melatonin we observe and/or that the visual system of rods and cones is contributing. Connections of rods and cones with the intrinsically photosensitive retinal ganglion cells (ipRGCs) are presumed to be responsible for rod/cone input towards the SCN (Belenky et al., 2003; Hatori & Panda, 2010; Wong et al., 2007). Indeed at relatively low intensities, 'green' light (555 nm) can suppress melatonin as effectively as 'blue' light (460 nm), but this effect is progressively lost with increasing duration of light exposure (measured up to 6.5 h) (Gooley et al., 2010). This fits with the melatonin suppression by short-wavelength

attenuated polychromatic white light that we observed during the first 4 – 5 h, but not at the end of the protocol (Figure 3). That the suppression of melatonin in our short-wavelength attenuated polychromatic white light condition did not reach the degree of melatonin suppression in the full-spectrum light condition as for 'green' light in Gooley et al. (2010) suggests that light across only a range of wavelengths can elicit this combined response. In support of this, high intensity 'red' light (630 nm) was not able to induce melatonin suppression in humans (Hanifin et al., 2006). Physiologically, this could mean that both cones and ipRCGs have to be excited to obtain signal transduction towards the SCN, limiting these responses to the wavelengths at which both these photoreceptors are excited, namely the 'blue' towards 'green' light. The 'green' proportion in the spectrum of the short-wavelength attenuated polychromatic white light condition we used (Figure 2) was probably not sufficient to elicit this response fully. Possibly illustrative of how small differences in the amount of 'blue' light filtered can effect study outcomes, a study that used orange lenses glasses, which filtered out more wavelengths (< 540 nm) than our study, reported no melatonin suppression compared with a grey lenses glasses condition (14 subjects; Sasseville et al., 2006). On average, even a slight non-significant increase was found. In this study, even higher light intensities (1300 lux at the eye level) were used than the two other studies on melatonin suppression under short-wavelength attenuated light (Kayumov et al., 2005; Rahman et al., 2011), including our study. The two separate studies that were combined in this paper were performed in groups or in isolation, and also in different seasons, namely winter and summer. That we find across these two studies consistent effects of short-wavelength attenuated polychromatic white light strengthens the potential general applicability of short-wavelength attenuated polychromatic white light. The degree of melatonin suppression in response to full-spectrum light has been shown to depend upon prior light history (Hébert et al., 2002; Higuchi et al., 2007; Smith et al., 2004). However in comparing the two studies (i.e., seasons) included here we do not detect significantly different levels of melatonin suppression (no interaction effect of condition × study, or condition × time × study, see also the Statistical Analysis section).

Subjective sleepiness showed the characteristic increase during the night, which is the result of increased homeostatic sleep pressure due to prolonged wakefulness in combination with a high circadian pressure for sleep at the end

of the night (Dijk & Edgar, 1999). Subjects, however, did not feel significantly sleepier under dim light or short-wavelength attenuated polychromatic white light compared with full-spectrum light. The average full-spectrum light intensities (256 lux) that we used are around the inflection point of the S-shaped dose-response curve of light intensity against sleepiness (Cajochen et al., 2000). Indoors, these light intensities are often used and are therefore relevant to a potential shift work application. Effects of light of these intensities may not show consistent results across all parameters considered, because different physiological responses can have differently shaped dose-responses to light. This may be why we do detect an effect on melatonin suppression, but no effect on sleepiness. This already suggests, and correlational analysis confirmed this, that under the light intensities used, there is no association between sleepiness and (physiological) melatonin concentrations *at night*. This is contrary to earlier results from Cajochen et al. (1996, 2000), but in line with the conclusion of Rüger et al. (2005). Interestingly, higher DPG was significantly associated with both increased sleepiness and lowered activation, in concordance with earlier experimental work (melatonin administration) carried out during the day (Kräuchi et al., 2006).

Short-wavelength attenuated polychromatic white light negatively affected activation contrary to earlier reports (Kayumov et al., 2005; Rahman et al., 2011). Also skin temperature, a physiological parameter linked to sleepiness and vigilance, was similar in the short-wavelength attenuated polychromatic white light condition and the dim light condition. These two results do suggest a possible decrement in overall performance, vigilance, and efficiency, although performance on an addition task was not negatively affected by short-wavelength attenuated polychromatic white light. If exposure to full-spectrum light at night is indeed involved in the causation of the detrimental effects of shift work on health, short-wavelength attenuated polychromatic white light at night has the potential to improve health in shift work. Note that in our study we only included relatively young healthy males and that direct extrapolation towards a more variable shift work workforce with a wider range of ages and including females will require further study. Our results also indicate that short-wavelength attenuated polychromatic white light may not be applicable in all settings. Especially when safety of others or of the shift worker is at stake, implementation of a severe reduction of short-wavelength light might not be advisable.

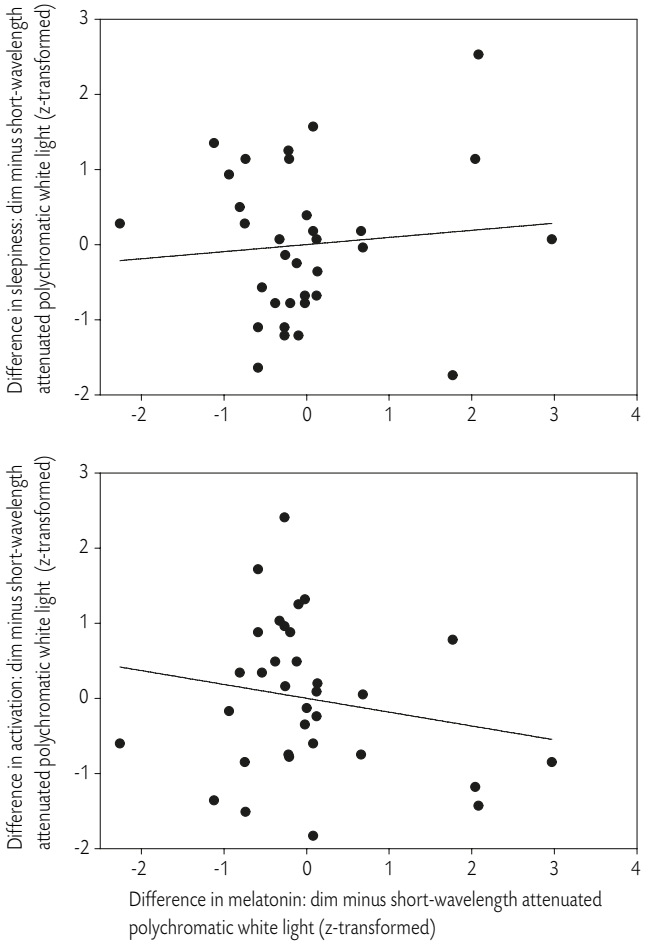
Acknowledgements

We thank the subject volunteers for their participation, Luc Schlangen and Peter van der Burgt (Philips Lighting) for providing the lamps and short-wavelength reducing foil used in this study, and Lotte van Nierop, Wolter Stam, Vincent Hulst and Joop Luider for practical assistance.

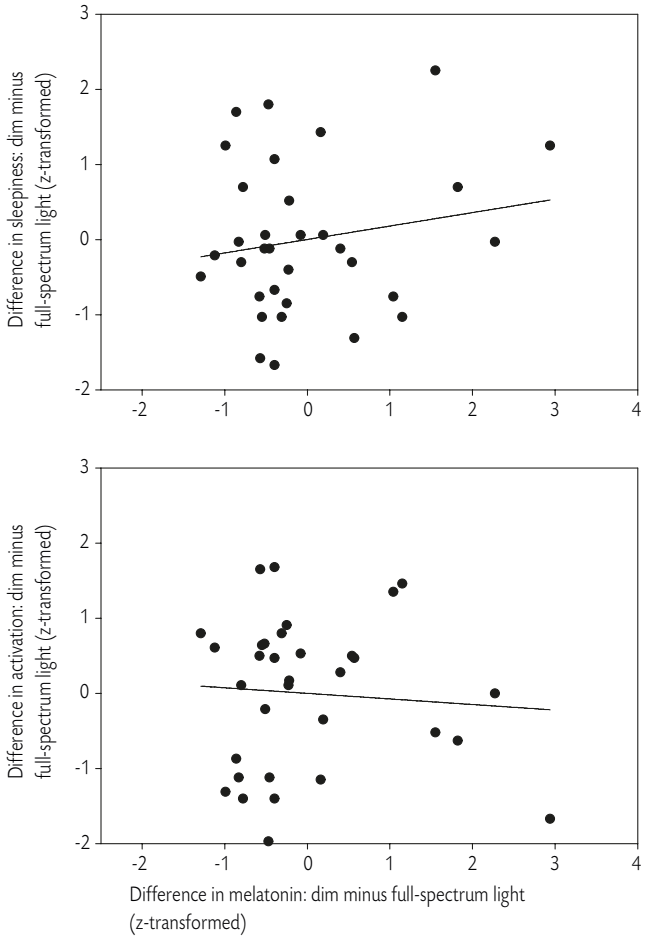
Declaration of interest

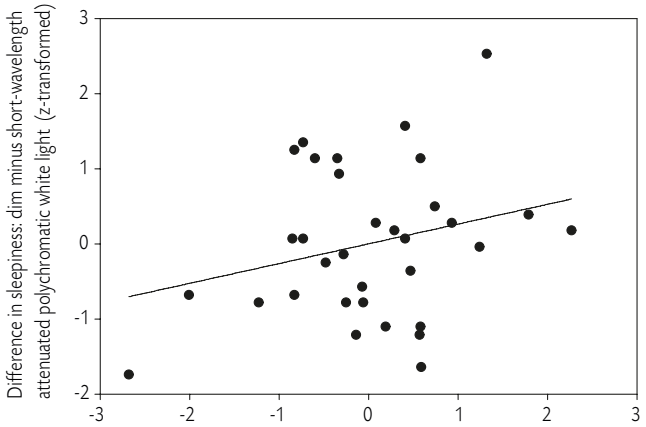
Financial support was obtained from the 6th European Framework project EUCLOCK (018741).
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

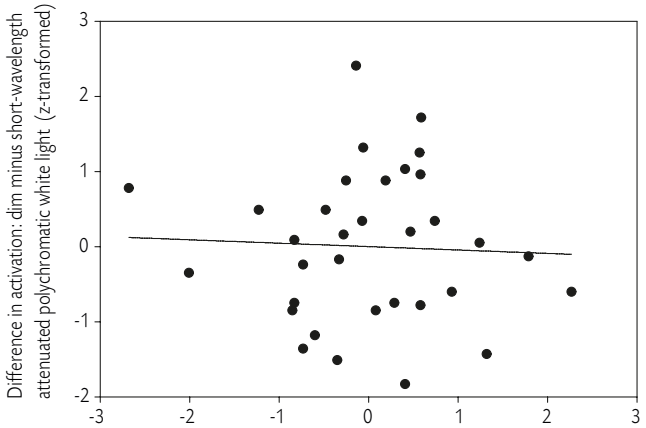
Supplementary Material

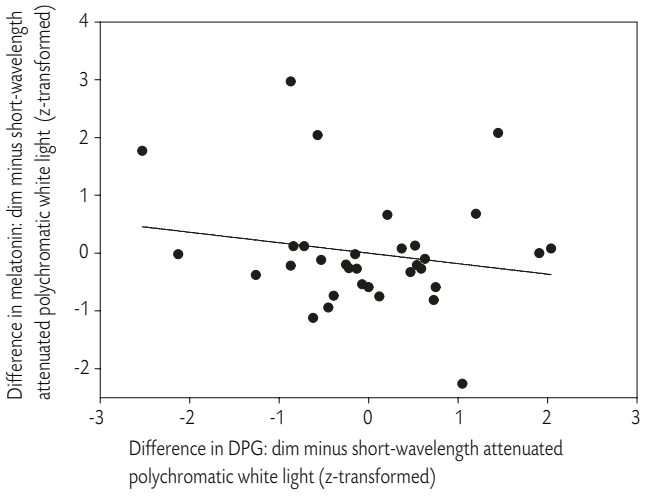


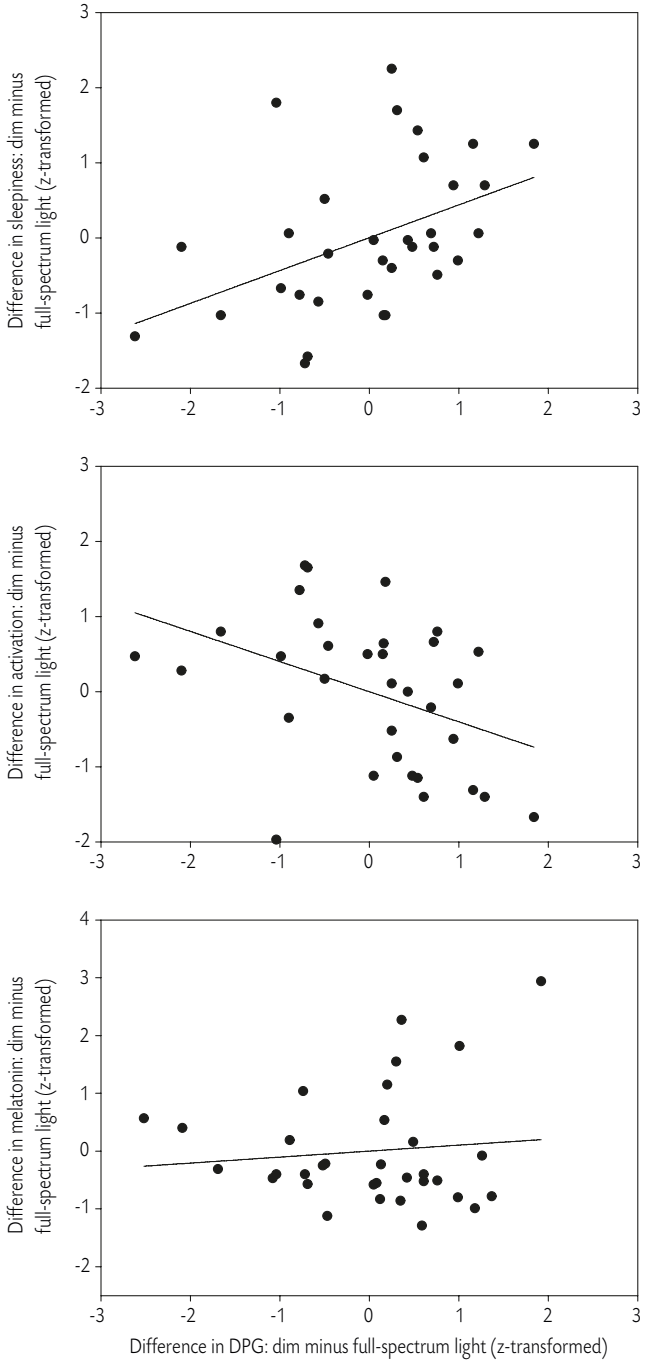
Supplementary Figures: Melatonin, distal-to-proximal skin temperature gradient (DPG), sleepiness and activation values obtained in the dim light condition were subtracted by the values obtained in either the short-wavelength attenuated polychromatic white light condition or the full-spectrum light condition, then averaged within subject, and subsequently z-transformed. Higher values indicate that higher melatonin, DPG, sleepiness and activation values were obtained in the dim light condition compared with the short-wavelength attenuated polychromatic white light or full-spectrum light condition. Lower values indicate that higher melatonin, DPG, sleepiness or activation values were obtained in either the short-wavelength attenuated polychromatic white light condition or the full-spectrum light condition compared to the dim light condition.


Difference in sleepiness: dim minus full-spectrum light (z-transformed)
3
2
1
0
-1
-2
-2
-1
0
1
2
3
4
Difference in activation: dim minus full-spectrum light (z-transformed)
3
2
1
0
-1
-2
-2
-1
0
1
2
3
4
Difference in melatonin: dim minus full-spectrum light (z-transformed)


Difference in sleepiness: dim minus short-wavelength attenuated polychromatic white light (z-transformed)
3
2
1
0
-1
-2
-3
-2
-1
0
1
2
3


Difference in activation: dim minus short-wavelength attenuated polychromatic white light (z-transformed)
3
2
1
0
-1
-2
-3
-2
-1
0
1
2
3


Difference in melatonin: dim minus short-wavelength attenuated polychromatic white light (z-transformed)
4
3
2
1
0
-1
-2
-3
-2
-1
0
1
2
3
Difference in DPG: dim minus short-wavelength attenuated polychromatic white light (z-transformed)


Difference in sleepiness: dim minus full-spectrum light (z-transformed)
Difference in activation: dim minus full-spectrum light (z-transformed)
Difference in melatonin: dim minus full-spectrum light (z-transformed)
Difference in DPG: dim minus full-spectrum light (z-transformed)

CHAPTER

3

MELATONIN SUPPRESSION BY LIGHT IS NEGATIVELY CORRELATED TO CREB SIGNALLING IN FIBROBLASTS; IMPLICATIONS FOR SEASONAL AFFECTIVE DISORDER

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Manuscript

Abstract

All cells in the body share the same genetic make-up. Therefore characteristics of individual cells in a certain tissue can resemble characteristics of cells in other tissues. In this study we show that the efficacy of melatonin suppression by light *in vivo* is negatively correlated to the excitability of the adenylyl cyclase (AC) – cyclic adenosine monophosphate (cAMP) – protein kinase A (PKA) pathway in cultured fibroblast cells. We reason that fibroblasts can be used as a model for brain-regulated melatonin suppression: melatonin suppression by light results from inhibition, via the SCN, of the PVN signal that eventually stimulates melatonin synthesis by the release of noradrenaline at the pineal cell. With higher light intensities lower noradrenergic stimulation reaches the pineal cells with lower melatonin synthesis as a consequence. If an individual has easily excitable melatonin producing cells, these cells will still produce melatonin upon a reduced noradrenergic signal. Hence, the suppression of melatonin by light will be limited. This observation can have consequences for the effects of light in patients suffering from seasonal affective disorder (SAD). SAD patients show higher levels of melatonin suppression by light than healthy controls. Those data have been interpreted to reveal high photosensitivity in SAD patients. Yet, depressive symptoms in SAD patients are reduced by increased light exposure (light therapy), suggesting sub-sensitivity to light. A novel hypothesis on cellular excitability solves this apparent contradiction. Serotonin production increases by light stimulation. An individual with high cellular excitability will produce more serotonin during daytime than a subject with low cellular excitability. High serotonin is predicted to combat depression. The same high cellular excitability will yield low melatonin suppression by light at night. Therefore the relationship between melatonin responses to light and depression may thus come about via a pleiotropic effect of cellular excitability.

Introduction

Human behavioural timing (Roenneberg et al., 2007) and human circadian physiology (Burgess & Fogg, 2008) show large individual variation. Some of this variability is associated with prevalence of disease. For instance, sleep duration has been linked to mortality risk (Gallicchio & Kalesan, 2009; Kripke et al., 2002), and chronotype to obesity risk (Roenneberg et al., 2012) and the risk to develop type 2 diabetes (Merikanto et al., 2013). In mental illness, the large suppression by light of melatonin production (Nathan et al., 1999; Thompson et al., 1990) and circadian phase shifting (Lewy et al., 2007) have been linked to depressive symptoms. Understanding the mechanistic basis for inter-individual differences in these processes is hampered by the impossibility of investigating living human brain tissue. Fibroblasts are one of the few human cell types available for sampling in subjects and their properties in culture have already been shown to be associated with timing of behaviour (Pagani et al., 2010). Although the 'master clock' controlling mammalian diurnal behaviour is located in the suprachiasmatic nucleus (SCN) in the brain (Ralph et al., 1990), its mechanism is cell-autonomous and conserved in most cells of the body (Ripperger & Brown, 2010). One of the best-studied rhythmic processes regulated by the human circadian clock, which is easily measurable *in vivo*, is melatonin concentration. This hormone is secreted at night, is rapidly suppressed by light, and is thought to signal time to the rest of the body (Arendt, 2006). Investigating individual differences in the melatonin suppression and/or synthesis pathway in human pineal gland tissue is impossible, but a large part of this signalling pathway is also functional in fibroblasts. To begin to unravel the mechanistic basis of individual differences in melatonin suppression by light, we investigated whether the excitability of a similar pathway in fibroblasts (*in vitro*) is correlated to melatonin suppression in saliva in the same subjects.

The SCN controls the circadian rhythm in melatonin synthesis. During daytime the SCN inhibits melatonin production by inhibiting the firing rate of the paraventricular nucleus (PVN). At night this inhibition is relieved. Signals from the PVN then stimulate the pineal gland via a multi-synaptic pathway. At the end of this pathway noradrenaline is excreted, which activates β_1-adrenoceptors of the pineal cells (Simonneaux & Ribelayga, 2003). These receptors are coupled to a G-protein and binding activates adenylyl cyclase

(AC) (Sunahara et al., 1996). This enzyme catalyses the synthesis of the second messenger cyclic adenosine monophosphate (cAMP), which via the activation of protein kinase A (PKA) inhibits the lysis of the rate limiting enzyme serotonin-n-acetyltransferase (AANAT) thereby increasing melatonin synthesis (Ackermann & Stehle, 2006; Simonneaux & Ribelayga, 2003). Light at night inhibits this stimulatory signal coming from the PVN due to GABA-ergic inhibition of the PVN by the SCN (Kalsbeek et al., 1999; Simonneaux & Ribelayga, 2003). This causes a reduction in AC activity and consequently reduced melatonin synthesis.
We investigated the excitability of the AC – cAMP – PKA signalling pathway in fibroblasts, using forskolin. Forskolin binds, beyond the receptor, directly to the transmembrane enzyme AC. It has been shown to increase melatonin in a dose dependent manner in cultured pineal glands of the Syrian hamster (Santana et al., 1988). Direct stimulation of AC by forskolin causes an increase in intracellular cAMP (Sunahara et al., 1996; Zhang et al., 1997). cAMP activates PKA, which then phosphorylates and thereby activates cAMP response element binding (CREB) factor inside the nucleus. CREB is a member of the transcription activators family and is implicated in many pathways, including the circadian clock, light signalling, hormone synthesis, and depression (Blendy, 2006; Ginty et al., 1993; Lonze & Ginty, 2002). AC stimulation ultimately leads to the expression of different genes through binding of the phospho-CREB complex to gene promoter binding sites (Brindle & Montminy, 1992; Sands & Palmer, 2008). We investigated the pharmacodynamics of the AC – cAMP – PKA signalling pathway leading to the upstream phosphorylation of CREB in fibroblasts (*in vitro*) of the same subjects that were investigated for their degree of salivary melatonin suppression by light.

Materials and Methods

In vivo

Subject Characteristics

16 subjects participated in the study. Data from 2 subjects could not be included due to a failure in culturing their fibroblasts. The remaining 14 healthy males (mean age [± SD] 21.9 ± 2.0 y) did not suffer from sleep disorders

(Pittsburgh Sleep Quality Index < 6; Buysse et al., 1989), somatic diseases, depressed mood (Beck Depression Inventory-II < 8; Beck et al., 1996, 2002), chronic diseases, colour blindness (Ishihara test; Clark, 1924), or visual impairment and did not use medication (general health questionnaire). They were recruited using advertisements. None of them had worked in night shifts or had travelled across more than 1 time zone respectively 3 and 1 month(s) preceding the study. They lived regular lives without smoking or drug, alcohol, or caffeine problems. The subjects had an average chronotype (gender and age corrected mean midsleep on free days [±SD] 5:44 ± 44 min, rated through the Munich Chronotype Questionnaire [Roenneberg et al., 2003]). The Medical Ethics Committee of the University Medical Center Groningen approved the study and all subjects gave written informed consent.

Experimental Design

The study consisted of 2 nights in the lab between June 19 and August 7, 2010, the Netherlands. The two nights were separated by a minimum of 1 and a maximum of 2 weeks. All subjects stayed in individual rooms. After 2 hours of dim light (< 5 lux) exposure, subjects were randomly assigned to one of two conditions: dim light or full-spectrum light (Philips TL-D36W/830 mounted on the ceiling, horizontal 627 ± 16 lux, vertical 164 ± 33 lux) from 23:00 till 7:00 h. During the second night in the lab, subjects were exposed to the alternative light condition. Subjects stayed awake during the whole night and performed computer tests (the screen was covered with a colour filter to block blue light transmission, E-colour +, 105 Orange; Rosco Laboratories, London, UK) and completed questionnaires, which are not subject of this paper (Van de Werken et al., 2013). They received hourly snacks of 100 Calories and 130 ml water. Each hour (from 21:30 till 6:30 h) saliva samples were collected for melatonin analysis, using cotton swabs (Salivettes®, Sarstedt B.V. Etten-Leur, The Netherlands). Melatonin concentration was quantified by radioimmunoassay (RK- DSM; Bühlmann Laboratories, Alere Health, Tilburg, The Netherlands). All samples from one individual were analysed within the same assay. Analytical sensitivity: 0.2 pg/ml. Intra- and inter-assay variations: 7.78% and 7.90%, respectively. Melatonin results are shown from the experimental period only (saliva taken from 23:30 till 6:30 h).

In Vitro

Two 2 mm dermal punch biopsies were taken from each subject. These biopsies were shipped to the Institute of Pharmacology, Zurich, Switzerland, within 48 h. As described previously (Brown et al., 2005), human primary fibroblasts were isolated from skin biopsies of these subjects by 4 - 7 h digestion of the tissues in 2 ml of DMEM containing 10% FBS, 1% Penicillin/Streptomycin, 1% Gentamycin, 0.325 W.U./ml Liberase Blendzyme3.

A novel cell - based reporter system was used for the measurement of the transcriptional activation of CREB in these cells. This system features a reporter construct coding the firefly luciferase downstream of a synthetic promoter which is joined to five tandem repeats of GAL4 binding elements. A separate transcriptional activator construct contains a coding sequence for activation domain of CREB fused with the DNA binding domain of yeast trans – activator GAL4. In addition to the reporter and activator construct, a control normalizing lentiviral construct coding the secreted alkaline phosphatase (SEAP) was included. A lentiviral version of this reporter system was produced by the restriction cloning of the coding sequences of activator plasmids (pFA2 - CREB) and reporter plasmids (pFR-Luc) into the pENTR4 gateway entry vector (Invitrogen) between attL1 and attL2 sites. Ultimately, Gateway® LR Clonase® II reaction was used to recombine and transfer inserts from entry vectors into a 3'LTR (long terminal repeats) of the promoter-less lentiviral destination vector containing either Hygromycin (pLDEST-CREB-Hygro) or Puromycin (pLDEST-FR-Luc-Puro) selection marker. All viruses were produced and concentrated as described in Cepko, 2001.

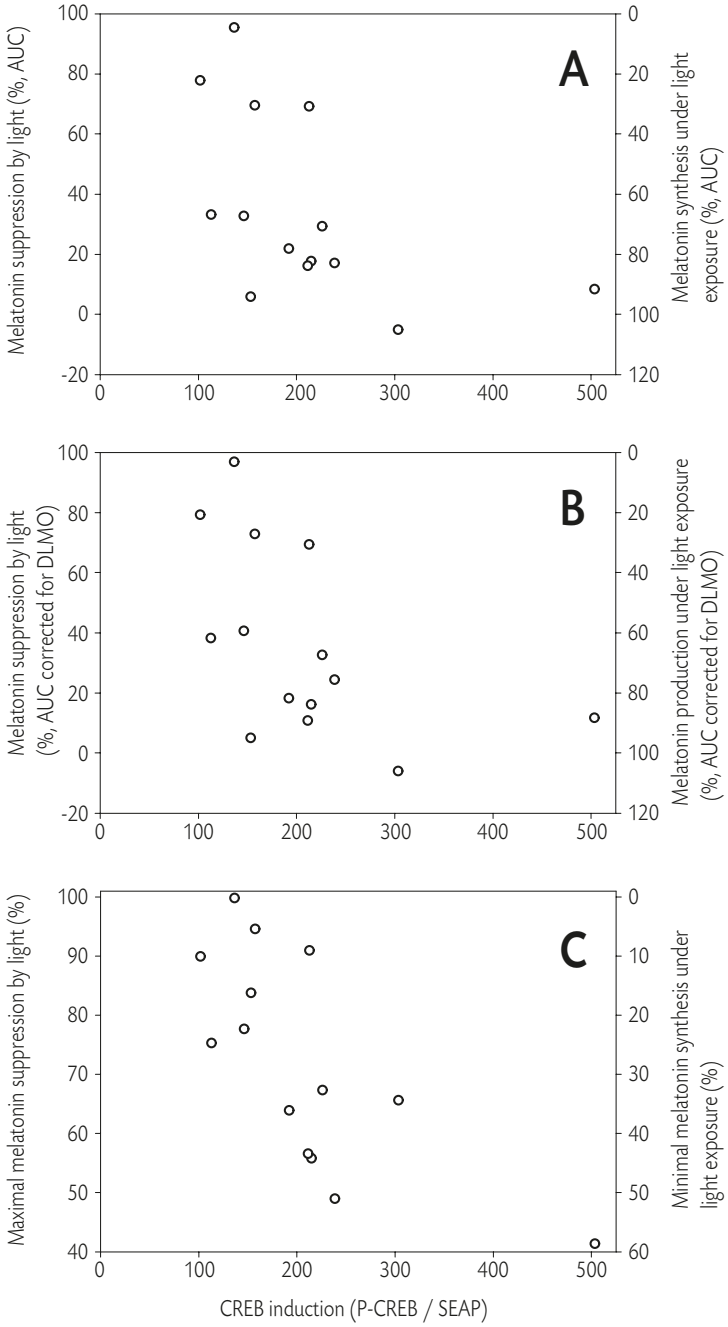
Subsequently, semiconfluent plates of primary fibroblasts were initially transduced and selected for the lentiviral luciferase reporter construct carrying Puromycin resistance (pLDEST-FRLuc-Puro), and then additionally cotransfected with the activator (pLDEST-CREB-Hygro) and normalizing lentivirus vector (pLDEST CMV-SEAP Hygro). Prior to bioluminescence measurement of the transcriptional activation, aliquots of the medium taken from the fully confluent samples were used to detect levels of secreted alkaline phosphatase by Phospha-Light™ Secreted Alkaline Phosphatase Reporter Gene Assay System (Cat. N. #T1015). Ultimately, DMEM medium high glucose, w/o phenol red, 10% KnockOut™ Serum Replacement, 1% Penicilin/

Streptomycin, 1% Gentamycin, 0.1 mM Luciferin was used to measure basal levels of the light emission during 20 - 30 min. Subsequently, pathway specific drug stimulus was added to the tested samples (5 μM forskolin for CREB activation) and the signal was recorded over the 24 - 48 hours.
A single experiment included samples measured in four replicates. Each sample was normalized by dividing the activator activity (maximal photon count value of CREB induction) by the control reporter activity (photon counts corresponding to SEAP values). Replicate samples were then averaged and the same calculation was done for all tests.

Statistical Analysis

There are multiple ways of calculating melatonin suppression. We employed three different ways, which correct for inter-individual differences in general level of melatonin production (Burgess & Fogg, 2008), for example caused by differences in the size of the pineal gland (Gómez Brunet et al., 2002; Nölte et al., 2009). This was done because we wanted to relate individual responsiveness to light *in vivo* to sensitivity at the cellular level, rather than investigating individual differences in melatonin production.
Melatonin suppression was determined from 23:30 till 6:30 h by dividing the area under the curve (AUC) in full-spectrum light by the AUC in dim light (method 1). In some subjects the dim light melatonin onset (DLMO) occurred after lights were turned on. To correct for a small underestimation in melatonin suppression due to this, we calculated the AUC starting from DLMO in those subjects. The total time over which the AUC was calculated in all subjects was truncated to 5 hours and 26 min, determined by the one subject with the latest DLMO, which was at 1:04 h (method 2). Additionally we also investigated the maximal suppression in response to full-spectrum light disregarding phase. This approach is conservative in not assuming a similar phasing of melatonin production in the dim and full-spectrum condition. Starting from lights on or from DLMO (if a subject's DLMO was after lights on), the minimal melatonin value measured in the full-spectrum light condition was divided by the maximum melatonin value in the dim condition (method 3). Spearman correlations were used ($\alpha = 0.05$) to correlate the *in vivo* measurements and *in vitro* measurements.

Results


A
Melatonin suppression by light (%, AUC)
Melatonin synthesis under light exposure (%, AUC)
B
Melatonin suppression by light (%, AUC corrected for DLMO)
Melatonin production under light exposure (%, AUC corrected for DLMO)
C
Maximal melatonin suppression by light (%)
Minimal melatonin synthesis under light exposure (%)
CREB induction (P-CREB / SEAP)

Subjects showed large individual differences both in the suppressive effect of light on melatonin concentration and in CREB induction in their fibroblasts in response to forskolin (melatonin suppression ranges: method 1 = -5% to 95%, method 2 = -6% to 97%, method 3 = 41% to 100%; CREB induction range: 102 to 503 P-CREB/SEAP, see Figure 1). We found that melatonin suppression and CREB induction were significantly negatively correlated (method 1: r_s = -0.666, P = 0.009; method 2: r_s = -0.600, P = 0.023; method 3: r_s = -0.688, P = 0.007).

Discussion

A positive correlation between melatonin suppression and CREB induction might be expected because both depend on a similar signal-transduction pathway (AC – cAMP – PKA). Note however, that light at night does not actively decrease melatonin synthesis; it reduces the stimulatory noradrenergic signal that allows the pineal cell to produce melatonin, thereby reducing the inhibition of AANAT degradation, which finally leads to a decrease in melatonin synthesis (Simonneaux & Ribelayga, 2003). Therefore, based on excitability arguments, a positive correlation is expected between the percentages of remaining melatonin synthesis upon light exposure with CREB induction. This is similar to expecting a negative correlation between CREB induction and melatonin suppression, in accordance with the data. Our results therefore show that within an individual there is a similar degree of signal transduction strength in response to a stimulus both in their fibroblasts and in the neural circuitry regulating melatonin suppression. The data reveal that there

Figure 1: CREB expression in fibroblasts predicts melatonin suppression in human subjects. Correlation between melatonin suppression (left y axis) or melatonin synthesis (right y axis) under full-spectrum light exposure with CREB induction in response to forskolin in fibroblasts from the same subjects. A) The area under the melatonin concentration curve under full-spectrum light divided by het area under the curve under dim light is plotted in relation to CREB induction (method 1, r_s = -0.666, P = 0.009). B) Same as in A, except that the areas under the curve were corrected for DLMO (method 2, r_s = -0.600, P = 0.023). C) The minimal melatonin concentration value under full-spectrum light divided by the maximal melatonin value under dim light is plotted in relation to CREB induction (method 3, r_s = -0.688, P = 0.007). Removal of the possible outlier on the right of the graphs still resulted in significant correlations in all 3 methods.

is large variation in this response between individuals. The physiological basis of this variation likely involves mechanisms beyond the receptor, since forskolin directly activates AC.

Based on this finding we hypothesize that individuals differ in their degree of 'cellular excitability'. Melatonin synthesis under light exposure, expressed as a percentage of maximal melatonin synthesis in dim light (thereby independent of pineal size), represents an indication of the degree of cellular excitability (Figure 2A). Individuals with relatively low cellular excitability show relatively higher degrees of light-induced melatonin suppression than individuals with relatively high cellular excitability (Figure 2B). This suggests a larger dependency upon the noradrenergic stimulus towards melatonin synthesis in subjects with low cellular excitability. Apparent differences in light sensitivity measured by melatonin suppression are predicted to be at least partly caused by differences in the intracellular response (excitability) to a stimulus, a trait inherent to the cell and to the individual (and similar at the central and peripheral levels). From this we have reasoned towards a general mechanism that explains apparent contradictions in the understanding of the relationship between melatonin suppression and depressive symptoms. Melatonin suppression is generally interpreted as a measure of non-visual light sensitivity (Nathan et al., 1999; Smith et al., 2004; Thompson et al., 1990) and is found to be higher in seasonal affective disorder and bipolar disorder compared to healthy controls, suggesting supersensitivity to light (Lewy et al., 1985; Nathan et al., 1999; Thompson et al., 1990). In contrast, patients benefit from light therapy treatment (Golden et al., 2005) and thus seem sub-sensitive to light.

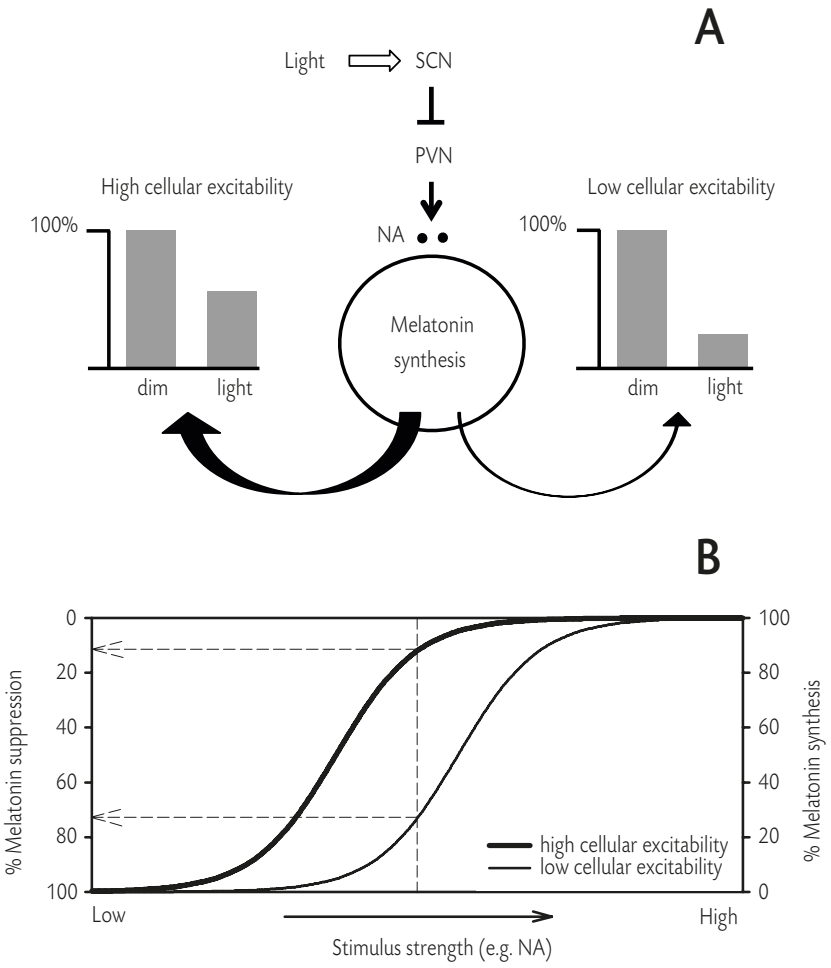


Figure 2: % Melatonin suppression determined by degree of cellular excitability. This figure illustrates the hypothetical relationship between extracellular stimulus strength (e.g. noradrenaline [NA]), which depends upon light intensity, and the resulting melatonin suppression and remaining synthesis. A different relationship is expected between SAD patients and healthy controls. A) Light at night causes the SCN to inhibit the PVN, resulting in reduced NA release in the pineal gland. A certain amount of NA release is expected when being exposed to moderate light intensities. Compared to maximal melatonin production under dim light (= 100%), a lower percentage of melatonin suppression by light is expected in high cellular excitability than in low cellular excitability. B) In moderate light intensities (indicated by the vertical dashed line) the difference in melatonin suppression between degrees of cellular excitability is most pronounced. Subjects with relatively high cellular excitability (indicated by the thick line) are less dependent upon stimulus strength to reach their maximum in melatonin synthesis. Whereas subjects with relatively low cellular excitability (indicated by the thin line) are more dependent upon extracellular stimulus strength in order to reach maximal synthesis.

Hypothesis

Seasonal affective disorder (SAD) is characterized by recurrent episodes of major depression occurring with a seasonal pattern. In winter SAD the depressed state occurs during fall and winter with remission in spring and summer (Rosenthal et al., 1984). Both serotonin and noradrenaline are involved in the aetiology of depression. Therapy is commonly based on enhancement of serotonergic and/or noradrenergic neurotransmission via a variety of mechanisms that ultimately lead to an increase in monoamine concentration in the synaptic cleft (Kandel et al., 2000; Schloss & Henn, 2004). Particularly Serotonin Reuptake Inhibitors (SSRI's) (Lam et al., 2006), but also noradrenaline reuptake inhibitors (Hilger et al., 2001), have been shown to be effective in the treatment of SAD.

The effectiveness of light therapy has been established in many mood disorders (Golden et al., 2005) and is the preferred treatment in SAD; depressive symptoms improve already after one week of morning light exposure (Meesters et al., 1993) and the treatment has only minor adverse events (Lam et al., 2006; Meesters & Letsch, 1998). Interestingly, the effectiveness of bright-light treatment in SAD depends on the availability of serotonin and noradrenaline as depletion of tryptophan or catecholamine abolished the effect of light therapy on mood (Neumeister et al., 1998). Higher amounts of light exposure have been linked to higher serotonin synthesis rates in humans (Lambert et al., 2002) and similar effects have been shown experimentally in hamsters (Ferraro & Steger, 1990). Light has been shown to directly increase the discharge rate in a subpopulation of raphe neurons, part of the serotonergic system (Mosko & Jacobs, 1974). Electrical stimulation of serotonergic neurons caused an increase in the activation of tryptophan hydroxylase (the rate limiting enzyme in serotonin synthesis) and in the release of serotonin into the synaptic cleft via exocytosis (Boadle-Biber, 1993; Boadle-Biber et al., 1986). Besides that, there is a direct neural projection between the retina and the raphe nucleus, suggesting that light may directly increase serotonin production via this connection (Fite & Janusonis, 2001; Foote et al., 1978; Reuss & Fuchs, 2000; Shen & Semba, 1994).

Because depression is linked to reduced brain serotonin levels and is relieved by light therapy, these findings suggest that SAD patients might be less photoreceptive and hence need more light to combat winter depression.

The opposite however has been shown to be the case: SAD patients show supersensitive responses to light in winter as measured by melatonin suppression (Nathan et al., 1999; Thompson et al., 1990). A history of low light intensity exposure has been shown to increase melatonin suppression (Hébert et al., 2002; Higuchi et al., 2007; Smith et al., 2004). However SAD patients and healthy controls have similar bright light exposure in winter (Graw et al., 1999; Guillemette et al., 1998).

An explanation for the discrepancy in apparent light sensitivity differences could lie in the fact that light at night *inhibits* melatonin synthesis (Kalsbeek et al., 1999; Simonneaux & Ribelayga, 2003) whereas light during daytime *stimulates* serotonin synthesis and exocytosis, thereby increasing serotonergic neurotransmission. If a cell is not very responsive to the various stimuli it receives (low cellular excitability), an increase in strength of one specific stimulus can be determinative in reaching a certain threshold in synthesis capacity required for optimal functioning. Reduction of this stimulus (for example by light at night in the case of melatonin suppression) will lead to a relatively large drop in synthesis capacity (seen as relatively high melatonin suppression). Serotonergic neurotransmission could be insufficient in individuals with low cellular excitability, especially under reduced light exposure during fall and winter. Due to the stimulating effects of light, the required threshold levels are reached in summer. The increase in serotonin upon light treatment has been shown to be significantly higher (twice as high) in patients with major depression than in healthy controls, suggesting a larger dependency upon light in serotonergic neurotransmission (Rao et al., 1992). Although healthy individuals also show reduced serotonin levels in winter (Carlsson et al., 1980), they may not depend upon extra light to reach the threshold levels required to remain free of depressive symptoms, because their cells are more responsive to stimulation. Depressed patients are therefore predicted to show reduced levels of cellular excitability, at least during their depressed state. Indeed fibroblasts from patients diagnosed with major depression have a blunted β-adrenoceptor mediated PKA response leading to significantly reduced CREB induction compared to healthy controls (Manier et al., 2000). Many studies support the view that post-receptor disturbances in G protein-linked cAMP signalling play a major role in the pathophysiology of mood disorders (Chang et al., 2001; Perez et al., 2000). Different isoforms of proteins within this route may modulate signal transduction strength, as is

suggested for AC (Defer et al., 2000). Different levels of cellular excitability are also predicted to result in differences in absolute levels of melatonin production, however such differences are likely swamped by individual differences in pineal size (Nölte et al., 2009). The combination of lower light exposure in winter and low cellular excitability can explain why serotonergic neurotransmission is affected in SAD patients and why light therapy is beneficial. The combination of reduced noradrenergic stimulus input to the pineal gland caused by light exposure at night and low cellular excitability can explain large drops in melatonin synthesis (high melatonin suppression) in SAD. Light therapy may compensate for lowered cellular excitability, stimulating serotonergic neurotransmission, thereby combatting depression. We hypothesise therefore that the relationship between melatonin suppression and depressive disorders is caused by a pleiotropic effect of cellular excitability on depression and melatonin production rather than a causal relationship.

Acknowledgements

We thank the subject volunteers for their participation and Wolter Stam, Vincent Hulst and Joop Luider for practical assistance. Financial support was obtained from the sixth European Framework project EUCLOCK (018741).

CHAPTER

4

EFFECTS OF ARTIFICIAL DAWN ON SLEEP INTERTIA, SKIN TEMPERATURE, AND THE AWAKENING CORTISOL RESPONSE

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Journal of Sleep Research (2010) 19: 425-435.

Abstract

The effect of artificial dawn during the last 30 min of sleep on subsequent dissipation of sleep inertia was investigated, including possible involvement of cortisol and thermoregulatory processes. Sixteen healthy subjects who reported difficulty with waking up participated in random order in a control and an artificial dawn night. Sleep inertia severity was measured by subjective ratings of sleepiness and activation, and by performance on an addition and a reaction time task measured at 1, 15, 30, 45, 60, and 90 min after waking up at habitual wake up time at workdays. At all intervals, saliva samples were collected for cortisol analysis. Sleep electroencephalogram was recorded during the 30 min prior to waking up; core body temperature and skin temperatures were recorded continuously until 90 min after waking up. Subjective sleepiness was significantly decreased and subjective activation increased after waking up in the artificial dawn condition as compared with control in which lights were turned on at waking up. These effects can be explained by effects of artificial dawn on skin temperature and amount of wakefulness during the 30 min prior to the alarm. Artificial dawn accelerated the decline in skin temperature and in the distal-to-proximal skin temperature gradient after getting up. No significant effects of artificial dawn on performance, core body temperature, and cortisol were found. These results suggest that the physiology underlying the positive effects of artificial dawn on the dissipation of sleep inertia involves light sleep and an accelerated skin temperature decline after awakening.

Introduction

During the period immediately after waking up people may suffer from confusion, disorientation, sleepiness and grogginess, and cognitive and physical performances may not be optimal. This transitory process is called sleep inertia (Dinges, 1990; Kleitman, 1963; Tassi & Muzet, 2000). The severity and duration of sleep inertia vary due to variations in sleep architecture, sleep stage upon awakening, and circadian phase (Scheer et al., 2008; Tassi & Muzet, 2000). Under natural situations sleep inertia generates risks, in particular when performance immediately upon awakening must be high, for example when participating in traffic (Dinges & Kribbs, 1991; Seminara & Shavelson, 1969). It is of great interest for individuals and for society to understand the processes involved in waking up and to test methods to reduce sleep inertia. One of these methods is tested in this study, which experimentally addresses the effects of artificial dawn prior to waking up.

Sleep inertia is seen upon awakening from various sleep durations (Brooks & Lack, 2006; Jewett et al., 1999), and at all times of day and night (Naitoh et al., 1993; Wilkinson & Stretton, 1971). Immediately after waking up, sleep inertia complaints are largest (Seminara & Shavelson, 1969). In the first hour after awakening, subjective alertness and cognitive performance rapidly improve, and sleep inertia slowly dissipates in an asymptotic manner (Jewett et al., 1999). The severity of sleep inertia is mainly assessed by its intensity and duration (Tassi & Muzet, 2000). Reported durations of sleep inertia vary from several minutes (Wilkinson & Stretton, 1971) up to several hours (Naitoh, 1981). This large variation can be a consequence of the applied types of tests, for example, with high and low cognitive load, or the methods of analysis (Achermann et al., 1995; Ferrara & De Gennaro, 2000; Jewett et al., 1999; Muzet et al., 1995). Sleep inertia is influenced by preceding sleep and can be quite severe. After 8 h of normal sleep, the effects of sleep inertia are reported to be modest and short-lived (Achermann et al., 1995; Jewett et al., 1999), nevertheless cognitive performance immediately after waking up is worse than after a night of total sleep deprivation (Wertz et al., 2006).

Especially late chronotypes suffer from sleep inertia on a daily basis (Roenneberg et al., 2003); because of the demands of society they show a large discrepancy between obligatory and preferred timing of sleep (Horne & Östberg, 1976; Roenneberg et al., 2003; Zavada et al., 2005), resulting in

so-called social jetlag (Wittmann et al., 2006). The increased severity of sleep inertia of late chronotypes compared with early chronotypes may originate from three sources. First, their circadian phase in the morning is not optimal for high performance. Second, sleep duration during workdays is short because of late sleep onset and early wake up. Recovery sleep after partial or total sleep deprivation is known to increase the severity of sleep inertia (Balkin & Badia, 1988), possibly due to increased amounts of slow-wave sleep (SWS; deep sleep) or to an increased chance of waking up from SWS (Dinges, 1990). Third, being sleepy in the morning motivates to get out of bed as late as possible, shifting the interval of severe sleep inertia to a period with high performance demands like commuting.

In this study, we test if artificial dawn reduces the severity of sleep inertia in people who report having difficulty getting up in the morning on workdays. We also investigate possible physiological correlates of the induced effects.

The process of waking up in the morning coincides with a wide range of physiological changes, among which changes in electroencephalogram (EEG) spectrum (Tassi et al., 2006), thermoregulatory changes (Kräuchi et al., 2004) and changes in cortisol level (Aschoff, 1978). These changes might be associated with the dissipation of sleep inertia. Changes in the distal-to-proximal skin temperature gradient (DPG) have been shown to correlate with sleepiness immediately after waking up (Kräuchi et al., 2004). Cortisol starts to increase during the second half of the night and is typically characterized by peak levels shortly after awakening (Edwards et al., 2001; Wüst et al., 2000). Waking up during the peak of the cortisol rhythm results in a short-lasting further elevation of the cortisol level (Edwards et al., 2001; Hucklebridge et al., 2005; Wüst et al., 2000); this elevation is called the awakening cortisol response. Elevated plasma cortisol levels during the second half of the night have been associated with an increase in stage 1 sleep, movements, and wakefulness, supporting an awakening effect of cortisol (Born et al., 1986; Fehm et al., 1986). This was confirmed experimentally by direct administration of cortisol during sleep (Born et al., 1989). Whether the awakening cortisol response is related to sleep inertia complaints is not known.

Light plays an important role in alerting the body (Cajochen et al., 2000; Rüger et al., 2003), both during the day and during the night (Phipps-Nelson et al., 2003; Rüger et al., 2006). Furthermore, bright light after waking up in the morning has been shown to increase cortisol levels in healthy humans while

light at other times of the day does not (Leproult et al., 2001; Rüger et al., 2006; Scheer & Buijs, 1999).
Few studies test the effects of a dawn light signal in the early morning on physiological and psychological parameters. Dawn simulation that started during sleep increased the awakening cortisol response (Thorn et al., 2004) and improved sleep quality (Leppämäki et al., 2003) in healthy individuals. A dawn signal has been shown to decrease depressive symptoms in seasonal affective disorders (SAD; Avery et al., 1993; 2001; Terman et al., 1989) and has been shown to be effective in decreasing sleep inertia in patients with SAD and subsyndromal SAD (Avery et al., 2002; Norden & Avery, 1993). Dawn-dusk simulation light therapy has been shown to improve sleep quality and to advance sleep timing in demented elderly (Fontana Gasio et al., 2003).
This lab study involved two conditions in which only the dawn period during the last 30 min of sleep was manipulated. A condition with artificial dawn during sleep and light at waking up was compared with a condition with light at waking up only. The purpose of this experiment was to investigate the effect of artificial dawn during sleep on sleep inertia and physiological processes in people who regularly have to wake up earlier than desired. Three aspects were investigated: sleep architecture during the stimulus; body (core and skin) temperature regulation; and cortisol production immediately after waking up, in relation to sleepiness, activation, stress, and performance measures.

Materials and Methods

Subjects

Subjects were recruited by advertisements in public places and at the University of Groningen. Sixteen healthy subjects (eight men and eight women, mean age [±SD] 22.8 ± 4.6 years) were selected, based on the following criteria: subjects had to be between 18- and 36-years old and live a regular life that consisted of at least four working days a week. On these days, they had to report that they need at least 60 min to fully wake up in the morning (rated on the Munich Chronotype Questionnaire [MCTQ]; Roenneberg et al., 2003). This selection resulted in relatively late chronotypes: mean midsleep on free days (MSF) (±SD) 5:53 ± 64 min (range from the same age categories, mean MSF 4:25 – 5:23 h, Dutch population; Zavada et al., 2005), who also had a relatively large

'social jetlag' mean (±SD), 1.88 ± 0.82 h (Wittmann et al., 2006). To obtain a marker for circadian phase, MSF corrected for sleep deficit accumulated over the workweek (Roenneberg et al., 2007) was calculated: mean MSFsc (±SD) 5:23 ± 64 min. Other subject characteristics (mean ± SD) were: timing of lights off on workdays 23:48 ± 53 min; timing of alarm on workdays 7:53 ± 54 min; time needed to fully wake up on workdays 1.75 ± 0.87 h; phase angle between MSFsc and start of artificial dawn 1.99 ± 0.76 h (range 0.77–3.43 h). Subjects were healthy, did not suffer from (winter) depression (Beck Depression Inventory-II, Dutch version, BDI-II_NL ≤ 8; Beck et al., 1996, 2002) or sleep disorders, and did not use medication including sedatives, except for oral contraceptives (three women), NuvaRing® (Organon, Oss, The Netherlands) (one woman), hormone spiral (one woman) and copper spiral (one woman). Shift workers and persons who had experienced transmeridian flights within the last month were excluded from the study. All subjects were born and raised in the Netherlands so that they were fully able to understand the Dutch questionnaires. All subjects gave written informed consent and were paid for their participation. The experimental protocol was approved by the Medical Ethics Committee of the University Medical Center Groningen.
Subjects were asked to keep a sleep-wake schedule for workdays during the 7 days at home prior to participation, and to take no naps during the experimental days. In most cases, subjects would participate on the same day of the week in the control and artificial dawn condition (maximum difference 2 days). Drinking coffee or alcohol on the days of participation was not allowed. All subjects reported to have kept to this regime.

Experimental Design

Subjects came to the human time isolation facility of the Department of Chronobiology at the University of Groningen on two occasions (control condition and experimental condition; Figure 1), consisting of two nights each. Subjects were free to go home during the day in between. Subjects stayed in individual living and bedrooms with no information about time of day. All rooms were completely dark, without windows. The living room was lit by ceiling lighting resulting in an intensity of 300 lux measured at eye level in the direction of the computer screen. During all four nights of the experiment subjects slept according to their habitual sleep time on workdays.
Condition order was randomized, and there was a minimum of 1 week between

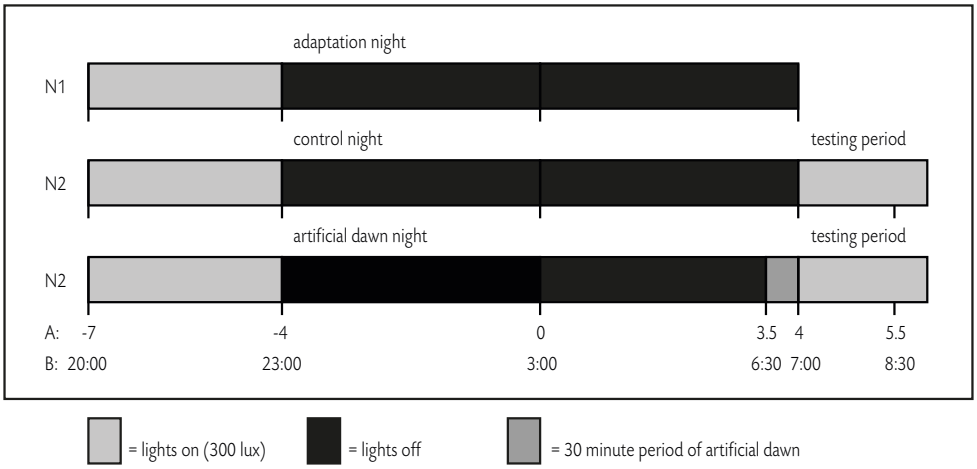

Figure 1: Scheme of the experimental design. Subjects arrived 3 h before habitual bedtime. All tests and questionnaires were made twice during this period to get familiar to them. The first night (N1) served as an adaptation night. The second night (N2) was either the control night or the experimental night, which ended in a 30-min period of artificial dawn. The first test session started 1 min after waking up, the last test session started 90 min after waking up. (A) Relative time to habitual midsleep on workdays (h). (B) Accompanying example if habitual sleep time on workdays is from 23:00 to 07:00 h.

conditions. The first night of each condition served as an adaptation night, which was not followed by a testing period in the morning, and subjects were allowed to leave after breakfast. The second night was either the control or experimental night, followed by a testing period of 90 min after which subjects were allowed to leave. Adaptation and control nights did not have a period of artificial dawn prior to wake up, instead, simultaneously with the audible alarm, the light was switched on with an intensity of 300 lux, measured at eye level in the direction of the Wake-up Light at 40 cm distance (the Wake-up Light is modified in such a way that no period of artificial dawn preceded the alarm; Philips DAP B.V., Drachten, The Netherlands). The experimental night was concluded with a 30-min period of artificial dawn, in which the light increased (up to the maximum of 300 lux) before the alarm and remained on after the audible alarm (normal Wake-up Light; Figure 5). Two Wake-up Lights were used, placed on either side of the bed to make sure that the subjects were exposed to the light. The Wake-up Light uses a 100-W light bulb (HalogenA Pro, Philips Lighting, Eindhoven, The Netherlands). Each night was ended by an audible alarm at the habitual wake up time, which made a ticking sound. At

the same moment, a researcher entered the room to make sure that the subjects got out of bed immediately. They subsequently walked to their individual living room and stayed seated behind the computer for the 90-min testing period. They were allowed to visit the toilet once preferably between 15 and 30 min after the audible alarm. All recordings were carried out between 16 January and 15 March 2007.

Measurements

Sleep Inertia: Subjective Ratings and Performance

All questionnaires and performance tasks were practiced twice on each of the four evenings in the lab. Subjective ratings of sleepiness (Karolinska Sleepiness Scale [KSS]; Åkerstedt & Gillberg 1990) were obtained at 1, 15, 30, 45, 60, and 90 min after the alarm. Ratings on the KSS range from 1 to 9, with 1 meaning very alert and 9 meaning very sleepy. Subjective ratings of activation and stress (two factors of the Thayer adjective checklist; Thayer 1967), with ratings ranging from 10 = minimal up to 40 = maximal, were obtained 1, 30, 60, and 90 min after the alarm. At 1, 30, 60, and 90 min after the alarm two performance tasks were conducted. The first one was an addition task in which subjects were asked to make as many correct additions as possible within a 3-min period. All additions consisted of two numbers of two digits. The second task was a simple reaction time task in which during 2 min, 30 stimuli were presented with varying intervals. Subjects had to respond by pressing the spacebar as quickly as possible. For the calculation of the average reaction time, lapses (response time > 500 ms) and anticipatory responses (response < 150 ms) were excluded.

Body Temperature

Core body and skin temperatures were recorded throughout the night at a rate of one sample per minute. Recording ended immediately after the alarm following the adaptation nights, and after the last testing periods following the control and experimental night. Core body temperature was measured continuously by a rectal probe and recorded with the online wireless recording Puck Temperature Telemetry system (Ambulatory Monitoring, Ardsley, NY, USA). Skin temperature was measured using Ibuttons (DS1922L, Maxim Integrated Products, Sunnyvale, CA, USA; resolution 0.0625 °C; for validation, see Van Marken Lichtenbelt et al., 2006) that were placed on

11 locations: both hands (ventral part of left and right wrist) and both feet (inner part of left and right foot, just below the ankle bone); left and right infraclavicular region; inner part of left and right thigh; inner part of left and right calf; and one on the sternum. For the analysis distal skin temperature was calculated by averaging the skin temperature of both hands and feet, and proximal skin temperature was calculated as the average temperature of the left and right thigh, left and right infraclavicular region, and sternum using the following formula (see Mitchell & Wyndham, 1969):

$$\text{proximal skin temperature} = \frac{\text{average thigh} + \left(\frac{\text{average infraclavicular region+ sternum}}{2}\right)}{2}$$

The data from the calf were excluded in calculating proximal skin temperature because they appeared to represent intermediate values between those from distal and proximal locations. The DPG was calculated as the difference between distal minus proximal skin temperatures.
Only the last 30 min of sleep until 90 min after the alarm will be compared between conditions for core body and skin temperature. One male subject had to be excluded from the analysis of skin temperature because data of some skin temperature locations were missing.

Cortisol

Saliva samples for cortisol analysis were taken at 1, 15, 30, 45, 60, and 90 min after the alarm, using Salivettes® with a cotton swab (Sarstedt B.V., Etten-Leur, The Netherlands). During the 90 min eating and drinking (other than water) were not allowed. A coated tube radioimmunoassay cortisol kit was used to determine cortisol levels (Spectria, Orion Diagnostica, Espoo, Finland). Each series from one individual was analysed within the same assay; sensitivity: 0.19 nmol L^{-1} (lower limit); intra- and inter-assay variations: 3.9 and 6.7%, respectively.

Sleep EEG

Sleep EEGs were recorded during all four nights. EEG derivations consisted of C3-A2, Fz-A1, in addition to two electrooculogram (eye movements) and two

electromyogram (EMG; muscle tone) electrodes. The EEG recordings were low-pass filtered at 30 Hz (24 dB oct^{-1}) and digitized at a sample rate of 128 Hz. Sleep stages were visually scored on 30-s epochs according to the criteria defined by Rechtschaffen & Kales (1968). For the present purpose, only the last half hours of sleep in the control condition and the artificial dawn condition were analysed.

Statistical Analysis

The differences over time of subjective ratings of sleepiness, activation, stress and performance on an addition and reaction time task were tested with a repeated-measures ANOVA, with two within factors (time and condition). The first measurement after waking up was tested separately to check whether already at this moment a difference could be observed between the control and artificial dawn condition. Similar statistics was used to test the pattern of cortisol concentration over time.

To determine the effects of light condition on core body, distal skin, proximal skin and DPG temperature profiles, mixed effect regression analysis (also known as hierarchical or multilevel analysis) were applied using MLwiN software (Centre for Multilevel Modelling, Institute of Education, London, UK). These analyses take into account the interdependency of the data points inherent to the hierarchical structure of the design, in our case the 1-min interval sequential temperature measurements i that were nested within days j, once more nested within participants k (Twisk, 2003). Moreover, the software package allows for the definition of an autocorrelated residual error data structure, which cannot be neglected in frequently sampled temperature values. After observation of the temperature curves and based on our hypothesis, the following model equation was used to fit the data:

$$\begin{aligned}\text{Temperature}_{ijk} = {} & \beta_{0ijk} + \beta_1 \times \text{Dawn}_{ijk} + \beta_2 \times \text{tpost}_{ijk} + \beta_3 \times \text{Sqrt(tpost)}_{ijk} + \\ & \beta_4 \times \text{Dawn} \times \text{tpost}_{ijk} + \beta_5 \times \text{Wakefulness}_{ijk} + \\ & \beta_6 \times \text{Wakefulness} \times \text{tpost}_{ijk}\end{aligned}$$

where β_0 represents the model intercept, β_1 the main effect of the artificial dawn condition (Dawn) as present from the start of dawn signal to the end of the 90-min post alarm period, β_2 and β_3 together represent the non-linear time course of the decline in temperature after getting up (tpost indicates the time since

getting up), β_4 the linearly increasing difference between the artificial dawn and control temperature time courses after getting up, β_5 the main effect of the amount of wakefulness during the 30 min prior to the audible alarm, and β_6 the effect of wakefulness prior to the alarm on the rate of decline of temperature after getting up. Parameters β_1 describe the main (time-independent) effect of artificial dawn, and β_4 its accelerating effect on the decline in temperatures after getting up. The autocorrelation of residual errors was included in the model as an exponentially decaying function of the time interval between successive temperature measurements. Maximum likelihood was used to estimate the regression coefficients, which were tested for significance with the *z*-test.

To determine if cortisol concentration during the period of artificial dawn is already influenced, the difference in cortisol concentration at 1 min after waking up was analysed first, using a paired samples *t*-test. The effect of artificial dawn over time was analysed using repeated-measures ANOVA (both the whole 90 min and the first 30 min based on the results found by Thorn et al., 2004). The highest peak during the 90 min after waking up was analysed using a paired samples *t*-test and the difference in the timing of the highest peak in cortisol concentration was analysed using a 'sign' test. For graphical purposes only (Figure 4), cortisol samples were normalized as follows: all samples per subject were divided by the average of all samples over both conditions of that subject and then multiplied by 100%.

The differences between conditions for percentages of sleep stages, sleep efficiency, first arousal, accumulation of wakefulness, and final wake up time were tested with the Wilcoxon matched-pairs signed rank test, and the difference in sleep stage on final awakening with a chi-square test.

A mixed effect multiple regression analysis (MLwiN software) was used to determine whether sleepiness and activation scores could be predicted (other than by the time since alarm, dawn and their interaction) by the physiological parameters 'momentary temperature' (core, distal, proximal, DPG), 'momentary cortisol' and 'wake/sleep stage duration during the final 30 min prior to the alarm' (movement time, W, S1-4, rapid eye movement [REM]). To determine if circadian phase could explain sleepiness and activation scores, the phase angle between MSFsc and start of artificial dawn was added to the model. To investigate whether artificial dawn had a differential effect on sleepiness and activation depending on circadian phase, the interaction between dawn and circadian phase was tested. By the use of the -2 × loglikelihood the most

parsimonious model was selected using backward selection. Ancillary analyses using forward selection led to identical results. The significance of regression coefficients was tested with the z-test. Values are described as average ± SEM. All tests are performed with $\alpha = 0.05$, two-tailed.

Results

Subjective Ratings and Performance

Sleepiness (KSS) was highest shortly after waking up and decreased significantly during the following 90 min (Figure 2A; $F_{5,11} = 14.11$, $P < 0.001$), whereas subjective ratings of activation (Thayer-activation) showed the opposite pattern (Figure 2B; $F_{3,13} = 21.03$, $P < 0.001$). Significantly lower levels of sleepiness ($F_{1,15} = 4.58$, $P < 0.05$) and higher levels of subjective activity ($F_{1,15} = 7.58$, $P < 0.02$) were found in the 90 min after artificial dawn compared with the same period in the control condition. At the first time point after waking up, neither sleepiness nor activation differed significantly between the artificial dawn and control condition (sleepiness: $F_{1,15} = 0.32$, NS; activation: $F_{1,15} = 3.09$, NS). There was no significant interaction between condition and time, neither for sleepiness ($F_{5,11} = 1.44$, NS) nor for activation ($F_{3,13} = 0.79$, NS), meaning that there was no significant deceleration/acceleration of sleepiness and activation after waking up between conditions. The subjective stress levels (Thayer-stress) were very low (data not shown) and there was no significant difference in pattern over time ($F_{3,13} = 3.33$, NS). No significant main effect ($F_{1,15} = 3.07$, NS) or interaction effect ($F_{3,13} = 1.63$, NS) over time was found between conditions. The first stress rating after waking up did not differ between the artificial dawn and control condition ($F_{1,15} = 0.08$, NS).

Performance was measured by addition and simple reaction time tasks. On the addition task, both the number of correct additions and the total number of additions increased over the 90-min wake time after the audible alarm. The number of correct additions at 1 min after waking up increased from 35.6 ± 2.3 to 43.2 ± 2.0 at 90 min after waking up ($F_{3,13} = 9.21$, $P < 0.01$), and the total number of additions increased from 37.0 ± 2.3 to 44.3 ± 2.1 ($F_{3,13} = 12.73$, $P < 0.001$) with no significant differences between conditions (correct additions: $F_{1,15} = 0.11$, NS; total additions: $F_{1,15} = 0.16$, NS) and no significant interaction between condition and time (correct additions: $F_{3,13} = 1.55$, NS; total additions: $F_{3,13} = 1.09$, NS). At the first time point after waking up, neither

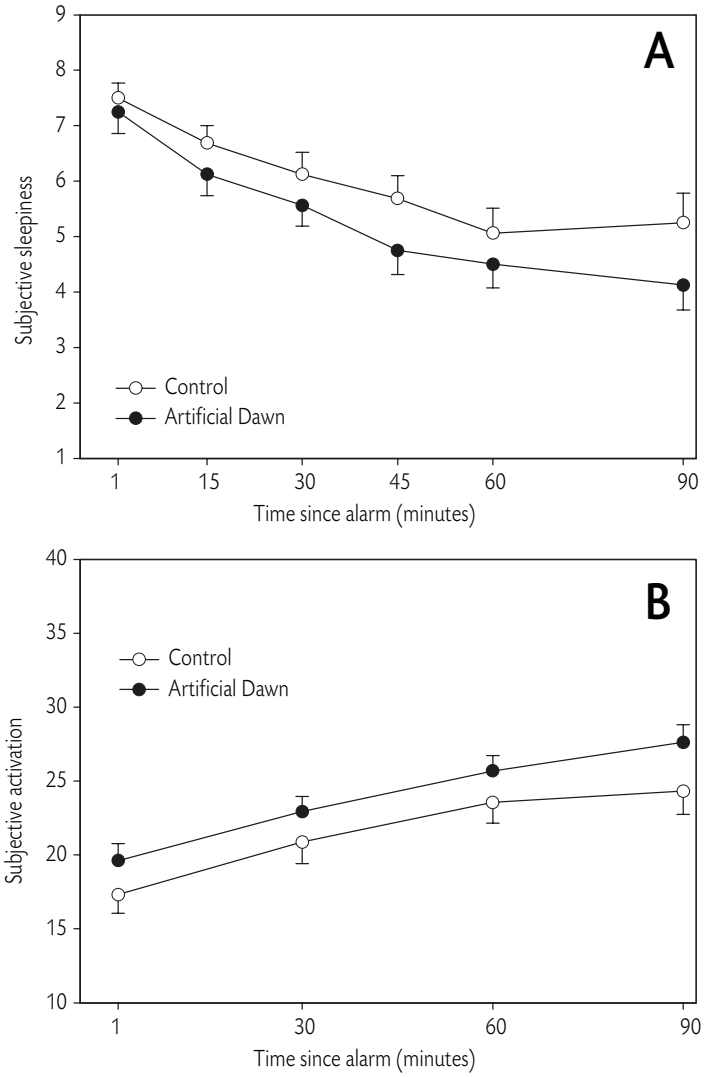

Figure 2: Average (±SEM) subjective ratings in the control (open symbols) and artificial dawn conditions (closed symbols) during the 90 min after the audible alarm for: (a) Sleepiness (KSS, 1 = low and 9 = high sleepiness); (b) Activation (Thayer adjective checklist, 10 = low and 40 = high activation) (*n* = 16).

number of correct additions nor total number of additions differed significantly between the artificial dawn and control condition (correct additions: $F_{3,13}$ = 0.55, NS; total additions: $F_{1,15}$ = 0.49, NS).
On the simple reaction time task, a significant reduction in reaction time over time was found (average reaction time 1 min after waking up: 270.5 ± 5.0 ms, decreasing to 258.4 ± 3.7 ms at 90 min after waking up, $F_{3,13}$ = 8.08, P < 0.01), but no significant differences were found between conditions ($F_{1,15}$ = 1.37, NS), nor was there a significant interaction effect between condition and time ($F_{3,13}$ = 0.66, NS). The first measurement after waking up did not differ between the artificial dawn and control condition ($F_{1,15}$ = 0.00, NS).

Skin and Core Body Temperatures

As shown in Figure 3, the artificial dawn condition did not significantly affect temperatures while still in bed, but did accelerate the decline in distal and proximal skin temperature, and their difference, after getting up. The mixed effect regression model fitted the time course of the temperature measures well, and confirmed that there were no significant main effects of artificial dawn on distal temperature (β_1 estimate = -0.06 ± 0.15 °C, NS), proximal temperature (-0.15 ± 0.13 °C, NS), DPG (0.10 ± 0.14 °C, NS); or core body temperature (-0.11 ± 0.07 °C, NS). In contrast, artificial dawn significantly accelerated the decline in skin temperatures after getting up, most strongly so for distal temperature (β_4 estimate = -0.36 ± 0.04 °C h^{-1}, P < 0.0001), less prominent but still significant for proximal temperature (-0.07 ± 0.03 °C h^{-1}, P < 0.03), and strong as well for DPG (-0.29 ± 0.03 °C h^{-1}, P < 0.0001). The increase in core body temperature after getting up was not significantly accelerated by artificial dawn temperature (β_4 estimate = 0.003 ± 0.007 °C h^{-1}, P = 0.68).

Awakening Cortisol Response

Cortisol concentration 1 min after the alarm did not differ significantly between the control and artificial dawn condition (control: 10.4 ± 1.3 nmol L^{-1}; dawn: 12.1 ± 1.5 nmol L^{-1}, t = -1.30, NS). The concentration changed significantly over the 90 min after waking up ($F_{5,11}$ = 10.97, P = 0.001), with the highest concentration on average 34.7 ± 1.8 min after the alarm in the control condition and 30.9 ± 2.9 min after the alarm in the artificial dawn condition (sign test, NS; Figure 4). Artificial dawn did not affect the peak concentration (control: 24.3 ± 2.2 nmol L^{-1}, dawn: 24.5 ± 1.9 nmol L^{-1}, t = -0.13, NS). No

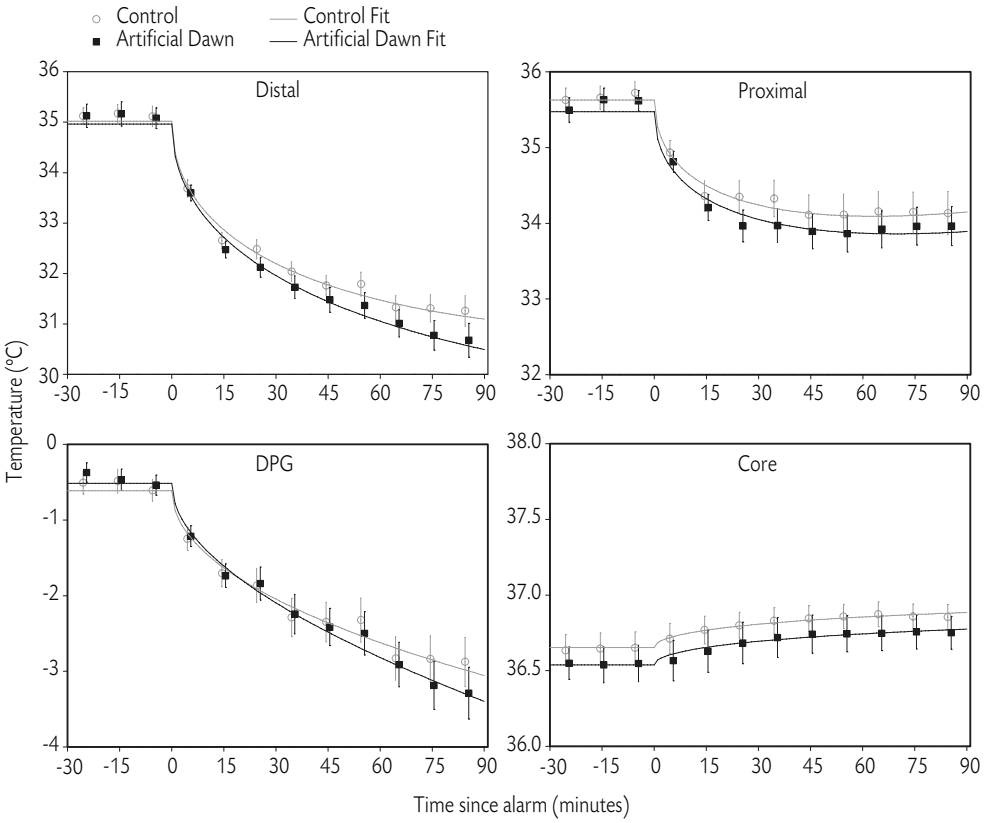

Figure 3: Average (±SEM) patterns of distal and proximal skin temperatures, of distal-to-proximal skin temperature gradient (DPG), and of core body temperature starting 30 min prior to the audible alarm (at 0) until 90 min after the alarm in the control (circles, open symbols) and the artificial dawn (squares, closed symbols) conditions. The lines represent the best-fitted curve through the data points (n = 15).

significant main effect of artificial dawn ($F_{1,15}$ = 1.86, NS) nor a significant artificial dawn by time interaction effect ($F_{5,11}$ = 1.99, NS) was observed over the 90 min in the amount of cortisol. The main effects of artificial dawn and the interaction effects with time during the first 30 min after waking up were tested separately. This was performed based on our hypothesis that the major changes in cortisol could be expected until the peak in the awakening cortisol response was reached after approximately 30 min (see also Thorn et al., 2004). No significant effects of artificial dawn on cortisol during the first 30 min were found (main effect: $F_{1,15}$ = 2.12, NS; interaction: $F_{2,14}$ = 1.82, NS).

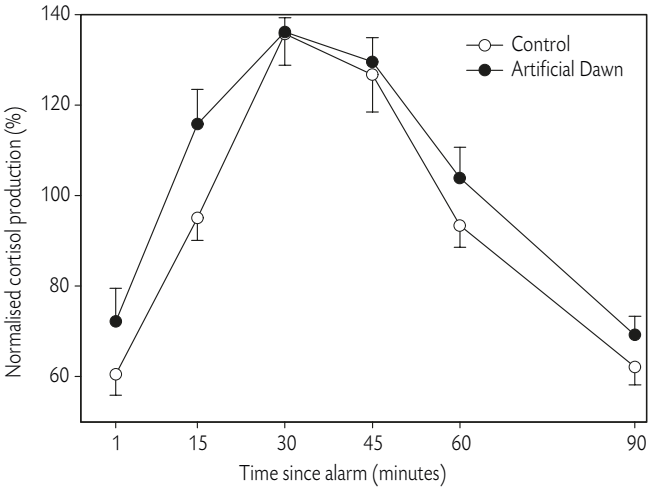

Figure 4: The average (±SEM) course of cortisol during the control (open symbols) and artificial dawn (closed symbols) conditions at 1, 15, 30, 45, 60, and 90 min after the alarm. Cortisol levels were normalised with respect to the average cortisol level (of both conditions) of each subject (n = 16).

Sleep EEG

No significant differences between both conditions were found in the percentages of each sleep stage during the last 30 min prior to the audible alarm (Table 1). A non-significant trend towards more wakefulness was seen in the artificial dawn condition compared with control (Z = -1.81, P = 0.07). Sleep efficiency (percentages of stages 2 + 3 + 4 + REM in the 30 min) was 78.1 ± 4.7 and 69.8 ± 4.8% in the control and the artificial dawn conditions, respectively (Z = -1.57, NS).

From the EEG recordings, the onset time of the first arousal after the start of artificial dawn and the onset time of the last arousal not followed by sleep was scored. An arousal was defined as a switch to either wakefulness, stage 1 or movement time. The timing of the last arousal without subsequent sleep, by definition, is wake up time. The first arousal within the 30 min prior to the audible alarm occurred on average 6.5 ± 1.8 min and 7.7 ± 1.9 min after the start of the 30-min period in the control and artificial dawn conditions, respectively (Z = -0.41, NS). In the artificial dawn condition, this corresponded with a light intensity of 1.8 lux measured at the pillow, facing the Wake-up Light. Between 5 and 10 min after the start of the 30-min period, the accumulation of wakefulness started to differ between conditions. In this period, light intensity in the experimental condition increased from 0.4 to 4.5 lux. Only between 10.5 and 14.5 min after the start of the 30-min period the cumulative amount of arousals in the artificial dawn condition reached significance compared with

Table 1: Average percentages of each sleep stage (±SEM) during the last 30 min prior to the audible alarm in the control and artificial dawn conditions (n = 16).

Sleep stage (%)	Control	Artificial dawn	Z	P
Stage 1	10.3 ± 2.2	12.3 ± 3.1	-0.57	NS
Stage 2	36.5 ± 8.4	40.5 ± 10.1	-0.17	NS
Stage 3	0.1 ± 0.1	0.1 ± 0.0	0.00	NS
Stage 4	1.4 ± 1.4	0.0 ± 0.0	-1.00	NS
Wakefulness	10.3 ± 3.9	16.7 ± 4.2	-1.81	0.07
REM sleep	40.2 ± 9.0	29.2 ± 7.3	-0.83	NS
Movement time	1.3 ± 0.4	1.3 ± 0.3	0.00	NS

NS, not significant; REM, rapid eye movement.

the control condition (two-tailed P-values between 0.04 and 0.09). The light intensity increased during this period from 5.4 to 17.8 lux. Most subjects fell asleep again after short periods of wakefulness between 10.5 and 14.5 min after the start of dawn. The final wake up time differed significantly between conditions (Z = -2.59, P = 0.01), but the difference was small: 2.2 ± 0.9 min before the alarm in the control; and 4.7 ± 1.1 min before the alarm in the artificial dawn condition (Figure 5).

Sleep stage on final awakening did not differ between conditions (control: eight subjects REM sleep and eight subjects Stage 2; artificial dawn: seven subjects REM sleep and nine subjects Stage 2 (chi-square, NS).

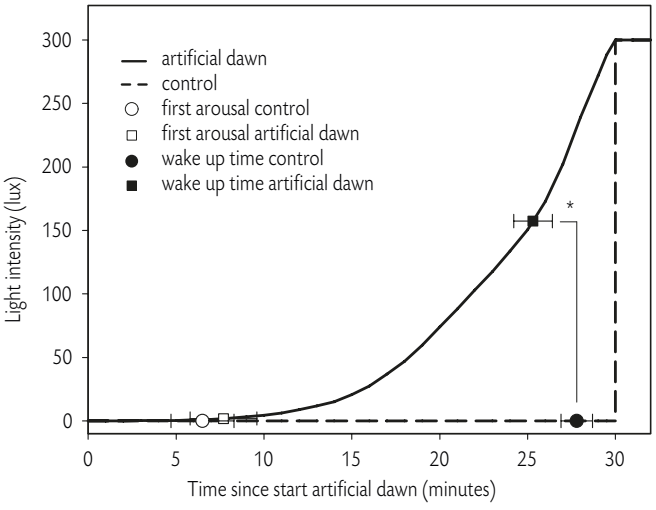

Figure 5: Rise in light intensity (lux) of the Wake-up Light (solid line) starting 30 min prior to the audible alarm (at 30). In the control condition (dashed line) the light was switched on at the audible alarm. Mean (±SEM) first arousal in the control (white circle) and the artificial dawn (white square), and mean (±SEM) wake up time in the control (black circle) and the artificial dawn (black square).

Parameters Explaining Subjective Ratings

To test what physiological parameters contributed to the significant differences in sleepiness and activation between the artificial dawn condition and the control condition, a mixed effect multiple regression analysis was performed (see Section 'Materials and methods' for procedure).
Sleepiness could best be explained by the following model:

$$\text{Sleepiness}_{ijk} = \beta_{0ijk} + \beta_1 \times \text{Distal-temperature}_{ijk} + \beta_2 \times \text{Wakefulness}_{ijk} + \beta_3 \times \text{Sqrt(tpost)}_{ijk}$$

Sleepiness was positively related to momentary distal skin temperature (β_1 estimate = 0.31 ± 0.10 unit of sleepiness $^{\circ}C^{-1}$, $P < 0.01$); negatively to the amount of wakefulness in the half hour prior to the alarm (β_2 estimate = -0.16 ± 0.05 unit of sleepiness min^{-1}, $P < 0.01$); and to sqrt(tpost) (-0.14 ± 0.05 unit of sleepiness min^{-1}, $P < 0.01$).
Activation could best be explained by the following model equation:

$$\text{Activation}_{ijk} = \beta_{0ijk} + \beta_1 \times \text{Distal-temperature}_{ijk} + \beta_2 \times \text{Wakefulness}_{ijk} + \beta_3 \times \text{Cortisol}_{ijk}$$

Activation was negatively related to the momentary distal skin temperature (β_1 estimate = -1.77 ± 0.13 unit of activation $^{\circ}C^{-1}$, $P < 0.01$) and to the momentary cortisol concentration (-0.07 ± 0.04 unit of activation per nmol L^{-1}, $P < 0.05$), and positively related to amount of wakefulness prior to waking up (0.50 ± 0.15 unit of activation min^{-1}, $P < 0.01$).

Discussion

In this study, we confirmed that sleep inertia (subjective ratings on sleepiness and activation, and performance) is most severe immediately after waking up and decreases during the following 90 min (Achermann et al., 1995; Jewett et al., 1999; Wertz et al., 2006). When exposed to 30 min of artificial dawn prior to the alarm, subjects felt less sleepy and more active during the 90 min after waking up compared with the 90 min in the control condition. These differences between conditions in sleepiness and activation scores were not apparent in the first minute after waking up. This may be due to either statistical power or indicate a fundamental property of the waking up process itself. Obviously the repeatedly lower sleepiness and higher activation values during the 90-min waking period after artificial dawn compared with the same period in the control condition resulted in a significant main effect, but the variance in the data could be too high and the number of subjects too low to be able to find a significant difference at only one time point (immediately after waking up). On the contrary, 'the waking up process' may take some time and may only become apparent after waking up.

For the purpose of the study, subjects were selected who substantially suffer from sleep inertia. They reported to require more than 1 h before feeling fully awake on workdays (Roenneberg et al., 2003). Chronotype evaluations (Zavada et al., 2005) revealed that late chronotypes are overrepresented in the sample of selected subjects, and that their sleep on workdays is much earlier than on free days. MSF as obtained by the MCTQ has been shown to correlate to Morningness-Eveningness Questionnaire (MEQ) scores (Zavada et al., 2005), and both midsleep time and MEQ score have been shown to correlate to dim-light melatonin onset (Martin & Eastman, 2002) and to other physiological circadian phase markers like body temperature and cortisol (Bailey & Heitkemper, 2001). Subjects within the present study differed in circadian phase as determined by their MSF corrected for sleep deficits accumulated over the workweek (MSFsc; Roenneberg et al., 2007). The start of artificial dawn and waking up time was in accordance to habitual wake up time on workdays. Because of these reasons, there was some interindividual difference in circadian timing of artificial dawn exposure and waking up. The phase angle between MSFsc and start of artificial dawn and subsequent waking up could, however, not explain sleep inertia severity; neither did it explain the effect of artificial

dawn. This is interesting because circadian phase is a major factor for the non-visual effects of light exposure. It suggests that the artificial dawn in this study does not necessarily interact with the underlying circadian system but rather has an acute effect upon physiological processes around the moment of waking up irrespective of its timing within the present (narrow) range of circadian phases. In this study, it is shown that changes in skin blood flow and associated changes in skin temperature parallel the sleep inertia process; similar to how these thermoregulatory processes are correlated to the evening increase of sleepiness and the initiation of sleep after lights off in the evening. Sleep is typically initiated when heat loss is maximal and usually occurs during the circadian peak of skin temperature (Kräuchi, 2007; Van Someren, 2004) that is the major cause of the nocturnal decline in core body temperature rhythm (Campbell & Broughton, 1994; Kräuchi, 2007; Van Someren et al., 2002; Zulley et al., 1981). In the morning, the opposite occurs when heat production is dominant over heat loss, resulting in an increase in core body temperature (Kräuchi, 2007). In this study, subjects changed from supine to upright position after waking up. This has caused strong masking effects in body temperature on top of the waking up process during both the control and artificial dawn conditions. Nevertheless, artificial dawn prior to waking up did result in an accelerated decline in distal and proximal skin temperatures after getting up, which was also reflected in an accelerated decline in the DPG. This suggests that vasoconstriction of skin blood vessels develops faster after being exposed to artificial dawn prior to waking up compared with the control condition. This could be explained by direct activation of the sympathetic nervous system by light (Saito et al., 1996; Scheer et al., 1999).

Fluctuations in distal skin temperature are always larger than fluctuations in proximal skin temperature (Kräuchi, 2007). Therefore, it is not surprising that in this study the absolute effect of artificial dawn is larger in distal skin than proximal skin regions. Another explanation could be that artificial dawn interacts with the peripheral vasoconstriction caused by the change in body position and that this effect is stronger in distal skin regions.

During the 30 min of artificial dawn, there is little difference in visually scored sleep stages compared with the last 30 min of sleep in the control condition. The average timing of the first arousal (either wakefulness, stage 1 or movement time) did not differ between the artificial dawn and control condition. Therefore, the relatively low light intensity in the artificial dawn condition

during the first 10 min, in which these arousals occurred, did not wake up the subjects. However, during the following 5 min the accumulation of arousals was steeper in the artificial dawn condition than in the control condition. This period also coincides with a substantial increase in light intensity (from 5.4 to 17.8 lux). Short arousals (periods of artefacts over EMG + EEG) occur regularly during the whole sleep period (Dijk et al., 1987; Gordijn et al., 1999), and most of them are not noticed by the person at all. Only some appear to be associated with conscious awareness of being awake. It is not known whether subjects were consciously aware of the light during an arousal and whether this has contributed to the observed changes after waking up. By asking the subjects afterwards, they reported to have noticed the difference between the artificial dawn and control conditions. It is also unknown whether subjects opened their eyes during an arousal. Only about 5% of light intensity comes through the eyelids (Ando & Kripke, 1996), and if subjects opened their eyes they were thus exposed to a much higher light intensity.

Abrupt awakenings are reported to worsen sleep inertia complaints (Dinges, 1990; Dinges et al., 1981). A gradual way of waking up, induced by artificial dawn, could thus reduce sleep inertia complaints.

Indeed in our regression analysis the amount of wakefulness during the 30 min prior to the alarm contributed both to the decrease in sleepiness and to the increase in activation after waking up. In addition to the amount of wakefulness prior to the alarm, the decrease in distal skin temperature after waking up contributed strongly to the model. Our data confirm the close functional relationship between the dissipation of sleepiness and skin temperature, as was previously reported in relation to DPG (Kräuchi et al., 2004). It is interesting that in our study distal skin temperature rather than DPG or proximal skin temperature added significantly to the model explaining sleepiness. Little is known about the association of the regulation of skin blood flow and the regulation of alertness upon waking up from sleep. Typically, a 10-min nap does not induce the adverse effects of sleep inertia upon awakening, and this may be because of insufficient time for thermoregulatory changes to occur (Brooks & Lack, 2006). Further experiments are required to determine whether skin temperature changes after waking up and the dissipation of sleep inertia are causally related, as has been shown to be feasible using mild skin temperature manipulation while obtaining objective vigilance measures during daytime (Fronczek et al., 2008; Raymann & Van Someren, 2007). This study is the first

to show that manipulation of sleep inertia by artificial dawn coincides with effects on skin temperature.

Interestingly, but also unexpectedly, the effect of the awakening cortisol response as included in the model on activation appeared to be negative. This negative relationship suggests that an increased awakening cortisol response has a detrimental effect on the dissipation of sleep inertia. The exact function of the awakening cortisol response is as yet unknown. Although cortisol does show consistent responses to stress (Kemeny, 2003), the morning increase in cortisol was not associated to any detectable psychological stress in our study. Previously, light after waking up has been shown to increase cortisol production (Scheer & Buijs, 1999), and one study showed a significantly higher awakening cortisol response with the use of a dawn waking up system (Thorn et al., 2004). In this study, we did not find an effect of the dawn signal on cortisol. The discrepancy between our study and the study of Thorn et al. (2004) can be explained by differences between the control conditions used in both experiments. In the study of Thorn et al. (2004) artificial dawn was tested against a normal alarm clock (with no light). In our study light was turned on in the control condition, together with the audible alarm at waking up. This was performed to test whether dawn itself, and not the light exposure after waking up, induced a reduction of sleep inertia and an increase in cortisol. It is possible that the light exposure immediately after the alarm in both conditions may have increased cortisol to such an extent that a possible additional effect of artificial dawn on cortisol could not be detected.

Conclusion

People who require a large amount of time after awakening before feeling fully alert can reduce their symptoms of sleep inertia and the time until feeling fully awake by exposing themselves to an artificial dawn signal. Skin temperatures showed an accelerated decline after awakening in the artificial dawn condition. A multiple regression analysis revealed that the physiological background explaining the positive effects of artificial dawn on the dissipation of sleep inertia involves light sleep and an accelerated distal skin temperature decline after awakening.

Acknowledgements

We thank the subject volunteers for their participation, Kurt Kräuchi (Psychiatric University Clinics Basel) for his advice on our skin temperature protocol, and Mirre Simons and Martijn Hessels for their general help and advice.
Financial support was obtained from Philips DAP B.V., CoC Vitality Care, Drachten, The Netherlands and the sixth European Framework project EUCLOCK (018741).

CHAPTER

5

THE BIOLOGICAL CLOCK MODULATES THE HUMAN CORTISOL RESPONSE IN A MULTIPLICATIVE FASHION

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Submitted

Abstract

Human cortisol levels follow a clear circadian rhythm (Czeisler et al., 1999; Linkowski et al., 1993). We investigated the contribution of alternation of sleep and wakefulness and the circadian clock, using forced desynchrony. Cortisol levels were best described by a multiplication of a circadian and a wake-time component. The human cortisol response is modulated by circadian phase. Exposure to stress at an unnatural phase, as in shift work (Boivin et al., 2007), is predicted to result in abnormal cortisol levels. Health of shift workers may therefore improve when stress is reduced at times when the clock produces high stress sensitivity.

Introduction

Biological clocks regulate physiology and behavior. When external timing and internal timing are in sync, circadian regulation of our physiology remains largely unnoticed. Yet, when we fly across time zones or are up at night, this circadian regulation becomes apparent quickly and impacts negatively on well-being, performance and potentially health (Gillberg et al., 1994; Vyas et al., 2012; Waterhouse et al., 2007). Experimentally we can investigate such circadian regulation without the confounding effects of actual activity by gradually shifting the activity cycle across the internal period; a forced desynchrony protocol (Czeisler et al., 1999; Dijk & Czeisler, 1995; Hiddinga et al., 1997; Koorengevel et al., 2002). Here we apply forced desynchrony in nine subjects to investigate circadian cortisol regulation. Cortisol is a central hormone in our body regulating a wide array of bodily functions and responses to stressors. Moreover long-term elevation of cortisol levels has been linked to major diseases, e.g. obesity (Rosmond et al., 1998) and cardiovascular disease (Girod & Brotman, 2004; Whitworth et al., 2005). We discuss the relevance of circadian cortisol regulation for shift workers and test two specific theoretical models, an additive and a multiplicative relationship between the masking and circadian component.

Materials and methods

Subject Characteristics

Nine healthy subjects (5 males, 4 females; mean age [±SD] 22.7 ± 2.1 yr) did not have sleep disorders (Pittsburgh Sleep Quality Index < 6; Buysse et al., 1989), somatic diseases, depressed mood (Beck Depression Inventory-II < 8; Beck et al., 1996), chronic diseases, colour blindness (Ishihara test; Clark, 1924), or visual impairment (assessed by general health questionnaire). Subjects did not use medication on a regular basis during 3 months preceding the study (assessed by general health questionnaire). All women used hormonal contraception. Subjects did not work in night shifts or travelled across more than one time zone respectively 3 months and 2 weeks preceding the study. Subjects had an average chronotype for their age and gender (mean midsleep on free days [±SD] 4:56 h ± 40 min with a mean sleep onset [±SD] at 0:30 h ± 46 min and a mean sleep offset [±SD] at 9:21 h ± 44 min, rated on the Munich Chronotype Questionnaire (Roenneberg et al., 2003). Subjects

reported that their lives contained at least five days a week with regular daytime activity and sleep exclusively at night. Subjects did not smoke or use drugs and did not report excessive intake of alcohol or caffeine. The study protocol was approved by the Medical Ethics Committee of the University Medical Center Groningen, the Netherlands, and conformed to international ethical standards (Portaluppi et al., 2008). All subjects gave written informed consent. They were financially compensated for their participation.

Experimental Procedures

The study was carried out from March 28 till May 17, 2009. Subjects remained in a time isolation facility in individual rooms for one week. No information about time of day (no clocks or sunlight) was present. During the 4 days directly prior to the study, subjects were instructed to follow a regular sleep-wake cycle at home (bedtime: ~00:00 h, wake-up time: ~08:00 h). The actual interval with forced desynchrony was preceded by an extra night in the lab to get familiar with the setup and tests and questionnaires (data not shown). Additionally, from 19:00 till 01:00 h hourly (in dim light < 10 lux), and from 1:00 till 9:00 h (in darkness, subjects went to bed from 00:00 – 9:00 h) every two hours saliva samples were collected for melatonin analysis, to determine circadian phase. After this first night subjects were free to go home but returned to the lab the same evening. The tests and questionnaires were again practiced by the subjects after which they had a normal 8.5 h night of sleep (from 00:00 – 08:30 h). The forced desynchrony protocol started upon waking up. Subjects were subjected to a sleep-wake cycle of 20 h; 13.5 h in dim-light (<10 lux) and 6.5 h in darkness. During the dark intervals subjects were instructed to try to sleep. During the dim-light intervals subjects needed to stay awake and were monitored by the experimenters accordingly. Each day had the same temporal structure. Because of this strict structuring we only analysed the cortisol data starting after the first night of sleep within the forced desynchrony protocol. The dim-light interval started by switching on the lights (< 10 lux) simultaneously with the alarm. Subjects had to get up immediately. They completed questionnaires and performed several tests (e.g. reaction time and addition tests, data not shown here). They collected saliva samples for cortisol and melatonin analysis at 1, 15, 30, 45, 60 and 90 min after the alarm. For the remainder of the subjective day, subjective ratings of sleepiness and activity, ratings on a food preference questionnaire and performance tasks were

collected every two hours (data not shown). Saliva samples for melatonin and cortisol analysis were collected and tympanum (eardrum) temperature was measured every hour (the latter data is not shown). Subjects had breakfast, lunch and dinner and took a shower according to a strict time schedule, which was respectively 1:45, 5:35, 9:35 and 3:35 h after waking up. The last subjective day of the experiment lasted 15.5 h (till 00:00 h). Saliva collections from 23:00 till 1:00 h hourly, and from 1:00 – 9:00 h every two hours were only used for melatonin but not cortisol analysis. The experiment ended after subjects had a normal night of sleep from 00:00 – 9:00 h.

Measurements

Saliva samples were collected for melatonin and cortisol analysis using two separate salivettes prior to every questionnaire/test session: 1, 15, 30, 45, 60 and 90 min after waking up using cotton swabs (Salivettes®, Sarstedt B.V. Etten-Leur, the Netherlands). From here on saliva samples were collected hourly for the remainder of the subjective day. Eating and drinking was not allowed during the 90 min after waking up and 30 min prior to sampling during the remainder of the day. Subjects were to remain seated behind their desk at least 10 min prior to every sample taken.

After saliva was collected it was immediately stored at -20°C. Melatonin and cortisol concentrations were assessed by radioimmunoassay (Melatonin: RK-DSM; Bühlmann Laboratories, Alere Health, Tilburg, the Netherlands and Cortisol: Spectria, Orion Diagnostica, Mediphos, Renkum, the Netherlands). Each sample was analysed once and all samples from each individual were analysed together within the same assay. Melatonin: analytical sensitivity: 0.2 pg/ml; intra- and inter-assay variations: 7.8% (mean 14.5 pg/ml) and 4.4% (mean 15.6 pg/ml), respectively. Cortisol: analytical sensitivity: 0.44 nmol L^{-1} (lower limit); intra- and inter-assay variations: 5.1% (mean 23.7 nmol L^{-1}) and 9.2% (mean 23.7 nmol L^{-1}), respectively. All individual samples fell beyond the lower limit of analysis negating the problem of non-linearity of metrics used in forced desynchrony analysis (Achermann, 1999; Jewett & Kronauer, 1999). Both salivary melatonin and salivary cortisol levels have been demonstrated to correlate strongly with circulating blood levels, e.g. (Putignano et al., 2001; Voultsios et al., 1997).

Statistical analysis

The choices for a wake-time related component and a circadian component were iteratively optimised per subject as follows. First the overall average cortisol concentration across the 108 data points was calculated. As a first step, it was assumed that, during wakefulness, the masking-component was constant over time, and equal to the calculated average. The masking-component was estimated in this way on a per-minute basis. Similarly, the raw cortisol data across the 120 h of the experiment were linearly interpolated during the wake-intervals. The intervals of sleep were considered missing data. The circadian component was subsequently estimated by division (or subtraction, depending on the model) of the raw data by the thus defined wake-component and plotting the result at the appropriate circadian time, which was defined on the basis of the estimated tau (circadian period) from the individual's melatonin data. This provided several results per circadian minute. Those results were averaged to yield an estimate of the circadian profile.

The per-minute interpolation of the data is necessary because the data were obtained at non-equidistant intervals, and because the period of the melatonin rhythms was not exactly equal to 24 h. The higher resolution of the analysis as compared to the resolution of the original data bears the risk of introducing oscillations in the results in a frequency range that is not included in the measurements. To avoid such artefacts, Fourier analysis was applied to the resulting circadian component. The 5 lowest frequency components (between about 1/24 h up to about 1/6 h) were used to reconstruct the circadian component. Higher frequencies were left out. The thus-defined circadian values were combined with the original masking-component (either by multiplication or by addition) and the results were compared with the original data by summing the squares of the differences. The sum of squares was minimized to improve the choice of the masking-component. This was done through stepwise increments or decrements per data point of the wake-component, first with large steps, later with smaller steps. The circadian component was calculated as explained above, and the resulting sum of squares was compared to the previous sum of squares. If the sum of squares decreased, the increment or decrement of the data-point was accepted as an improvement of the estimated wake-component, and the procedure was repeated, until no further improvements were obtained. The resulting curves of each individual and the average individual are plotted in Figure 2.

Results and Discussion

Nine healthy subjects (5 males, 4 females) were subjected to a forced desynchrony protocol, by imposing six successive sleep-wake cycles with a period of 20 h; 13.5 h waking in dim-light (< 10 lux) and 6.5 h sleeping in darkness. This resulted in free running rhythms in melatonin in all subjects, with a period of 1452 min ± 4.5 (mean ± SD, Table 1, Figure 1, Figure S1). The similarity of successive ~24 h melatonin cycles demonstrates that the imposed 20 h sleep-wake cycle has little effect on melatonin production. This is consistent with earlier studies which concluded that melatonin is a robust output signal of the circadian clock relatively insensitive to masking effects (Wyatt et al., 1999). Cortisol concentrations, in contrast, showed pronounced changes over time suggestive of interference between a circadian rhythm with a period similar to the melatonin rhythm and masking effects induced by the 13.5 h wake period (Figure 2). This interference between circadian phase and masking was especially apparent shortly after awakening (Figures 3 and 4).
The forced desynchrony protocol included 6 imposed sleep-wake cycles of 20 h, which equals 5 days of 24 h. Since the period of the melatonin rhythms turned out to be close to 24 h, the sleep-wake cycle stepped through the entire circadian cycle with -4 h intervals, providing a full set of phase combinations of the two rhythms. This allows for the mathematical separation of the masking and circadian components of cortisol production, which provides information about the underlying regulatory mechanisms responsible for cortisol production. Here we investigate two models for the interaction of the clock and masking: an additive and a multiplicative model. The additive model represents a physiological circadian system that yields specific levels of cortisol production by the mere addition of a circadian (~24 h) pattern in cortisol production and masking effects, such as induced by for example waking up triggering the awakening cortisol response (Clow et al., 2004). The masking effect and circadian effect are modelled in the additive model as resulting from independent pathways. The multiplicative model in contrast represents a system that responds to masking on the basis of a sensitivity that is controlled by circadian phase. In this model, if awakening occurs in the early morning, when circadian sensitivity is high, the cortisol response is proportionally higher than when awakening occurs in the evening, when circadian sensitivity is low.
The two components were estimated by an iterative procedure as explained in

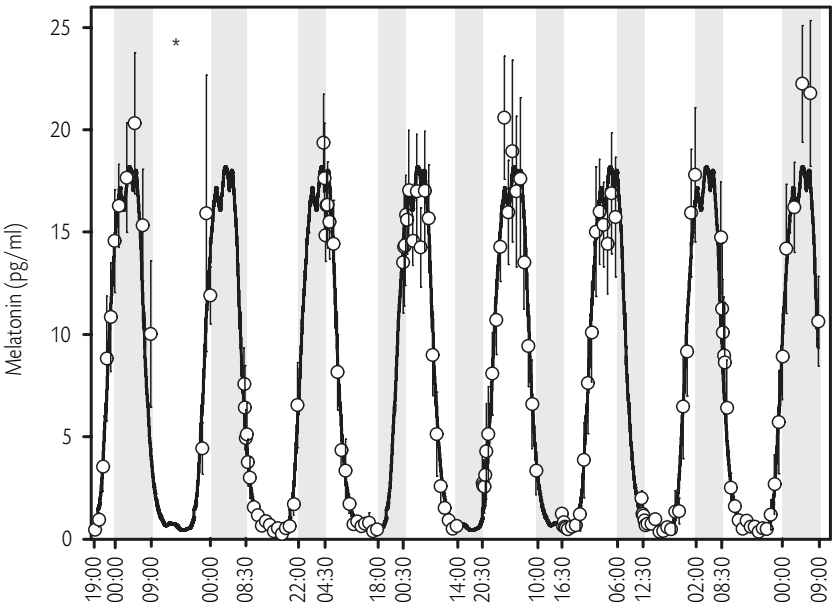


Figure 1: Mean (±SEM) melatonin concentrations of all 9 subjects are indicated by the white dots. See Figure S1 for the individual melatonin curves. Average tau (1452 min) of all 9 subjects is plotted by the black line. Grey areas indicate periods of darkness during which the subjects were instructed to sleep. White areas indicate periods of wakefulness (< 10 lux). First day: hourly saliva collection from 19:00 – 1:00 h and every two hours from 1:00 – 9:00 h. Subjects were instructed to sleep from 00:00 – 9:00 h. Upon waking up subjects were free to go home (indicated by the asterisk) but returned to the lab the same evening and 3 saliva samples were collected at 22:00 h, 23:00 h and 00:00 h. Subjects had a normal night of sleep from 0:00 – 8:30 h. The forced desynchrony protocol started upon waking up. The last subjective day was extended by an extra 2 hours (additional saliva collection at 23:00 h). Subjects were instructed to go to bed at 00:00 h, but saliva collection was continued at 1:00 h and from here on every two hours till 9:00 h. The experiment ended upon waking up.

the section Analysis Details. By examining how well the extracted masking and circadian components combine under the additive and multiplicative models to fit the raw data, we can test both models against each other. Model comparisons were performed on the basis of AIC differences (Akaike Information Criterion, AIC increases compared to the best model can be interpreted as follows, models differing ≤ 2 the alternative model still has considerable support, models which difference are ≥ 4 ≤ 7 the alternative model has considerably less support, and with AIC differences of over 10 the alternative model has essentially no support [Burnham & Anderson, 2004]). Table 1 provides the ΔAIC values of the two

models for all subjects. The differences demonstrate that the multiplicative model is preferred over the additive model in 8 out of 9 subjects. If we aggregate the data of all 9 subjects in order to study the average time course of cortisol, we find strong support for the multiplicative model (Table 1, Figure S2). That the multiplicative model is the strongly preferred model does not mean that the additive model does not fit the data well. The residual sum of squares over the 108 available data-points for this model is also relatively low. For the average subject the mean deviation between predicted and raw values of cortisol is

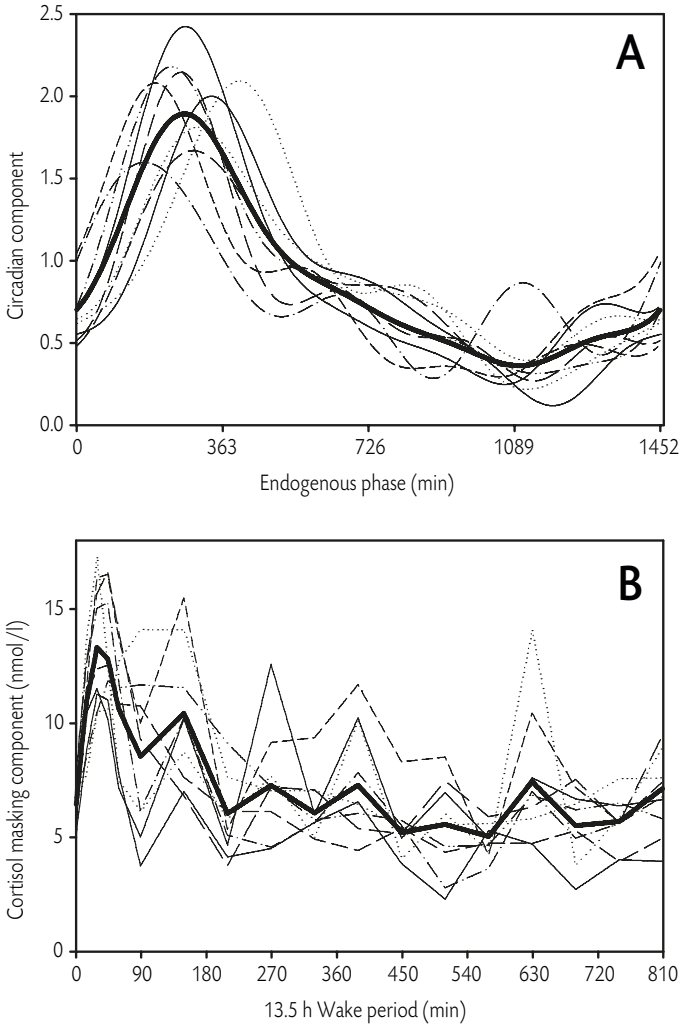


Figure 2: Circadian (A) and masking (B) component of cortisol regulation. The thin lines represent the estimated components per individual and the thick lines represents the estimated components across the average raw cortisol levels of the 9 subjects. Zero on the x-axis denotes the endogenous phase of the first cortisol measurement taken after the first night of short (6.5 h) sleep during the forced desynchrony experiment, corresponding to clock time 4:31 h.

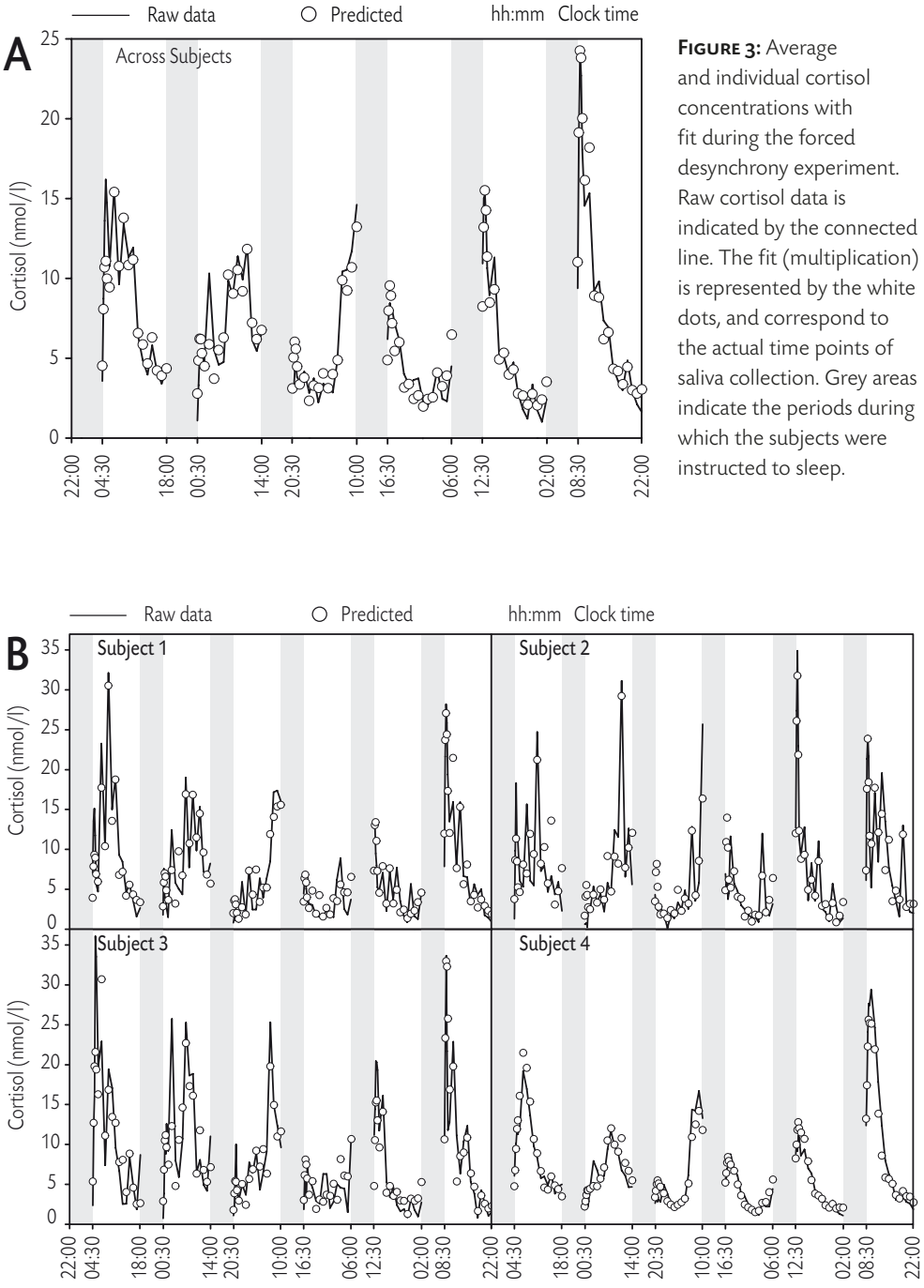
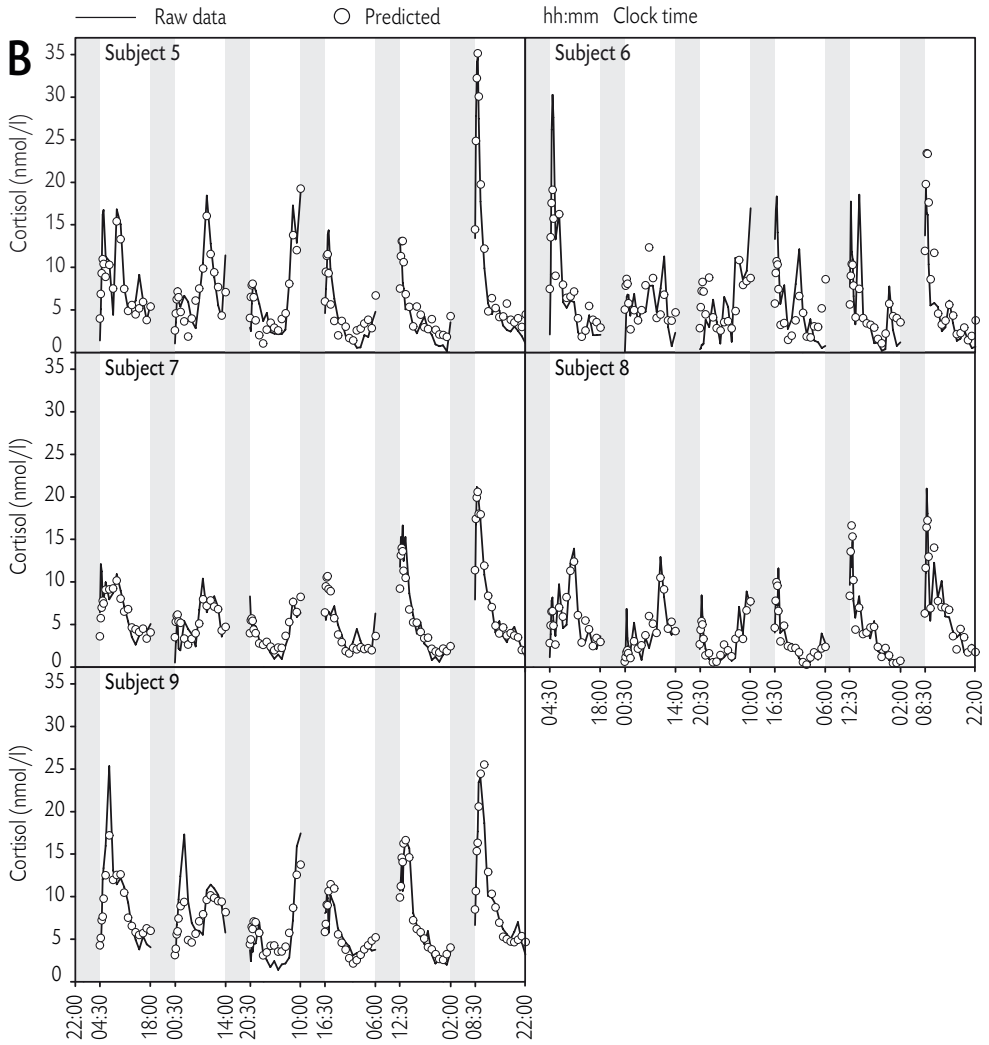


Figure 3: Average and individual cortisol concentrations with fit during the forced desynchrony experiment. Raw cortisol data is indicated by the connected line. The fit (multiplication) is represented by the white dots, and correspond to the actual time points of saliva collection. Grey areas indicate the periods during which the subjects were instructed to sleep.



1.31 nmol L^{-1} in the additive model vs. 1.01 nmol L^{-1} in the multiplicative model. We conclude that the cortisol concentrations are best fitted by a multiplication of a 'circadian' component and a 'wake' (masking) component across subjects (Figure 3A). Also for the individual subjects we find accurate fits (Figure 3B). The circadian modulation of the cortisol response shown in Figure 2A, runs from a minimum value of 0.36 to a maximum of 1.89, so there is a five-fold change in sensitivity across the circadian cycle, with peak sensitivity on average at 8:42 h under normal circumstances (during the forced desynchrony

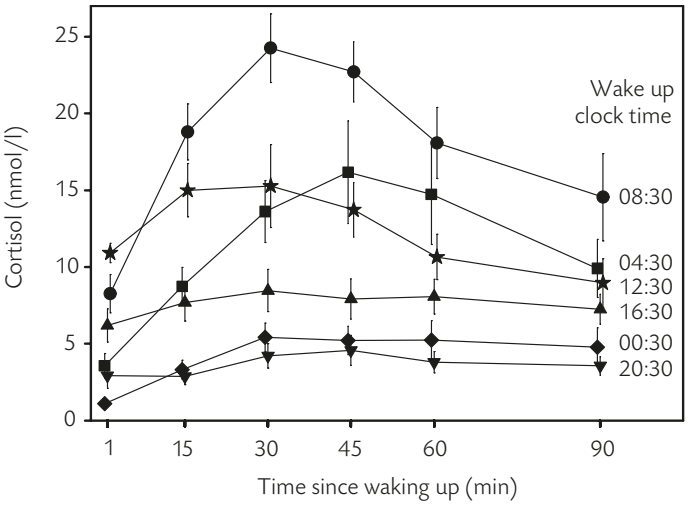


Figure 4: Awakening cortisol response at different circadian phases. Mean (±SEM) cortisol concentrations of all 9 subjects over the first 90 min after waking up at the different circadian phases during the forced desynchrony experiment. Wake up times represent actual clock time and occurred during the experiment in the following order: 4:30 h (squares), 00:30 h (diamond), 20:30 h (triangles down), 16:30 h (triangles up), 12:30 h (stars), 8:30 h (circles). Because the subjects' internal rhythms free ran within the forced desynchrony (see text), the corresponding clock times under natural circumstances are predicted at: 4:17 h (squares), 00:07 h (diamond), 19:57 h (triangles down), 15:47 h (triangles up), 11:37 h (stars), 7:27 h (circles), as calculated from the average subject's melatonin period.

experiment the cortisol peak occurred at 8:58 h clock time on day two; after ≈33 h of free run which caused an estimated 16 min delay of the melatonin rhythm). On top of that, the data show a decline in masking induced cortisol production across the subjective day. Maximum and minimum cortisol values of the average masking component were 13.32 nmol $L^{-1}$and 5.04 nmol L^{-1} respectively, showing a decline of a factor 2.6 (Figure 2B). This decline, however, is of a different nature as compared to the circadian decline: the circadian decline shows a difference in sensitivity of the system as a function of circadian phase, meaning that the same stimulus (e.g. waking up) when applied at a different phase will lead to a different response. The decline in masking across the waking interval, in contrast, results from changes in stimulation to release cortisol. For instance, waking up elicits a stronger cortisol response than having dinner. Note that these estimates are from an experimental setting in which subjects were well informed about the fact that the investigators

Table 1. Tau of the individual melatonin rhythms and the residual sum of squares (RSS) of the multiplication and addition model, with their corresponding Δ AIC.

Subject	Tau (min)	RSS Multiplication	RSS Addition	AIC multiplication – AIC addition
1	1449	563.32	883.45	-48.60
2	1447	1078.72	1818.70	-56.41
3	1449	1680.42	1749.92	-4.38
4	1457	254.36	565.52	-86.29
5	1452	524.36	996.14	-69.30
6	1454	1708.66	1768.90	-3.74
7	1447	337.54	509.93	-44.56
8	1454	266.46	335.44	-24.86
9	1460	500.13	499.98	0.03
Across all subjects	1452	181.98	307.74	-56.74

determined bed times. Subjects did not experience much stress. Specific effects of prominent stressors were not tested in this study. Yet, extrapolating our results to a more stressful situation, we expect cortisol responses to become extremely elevated at the peak circadian phase (on average at 8:42 h clock time under normal circumstances), and to be severely dampened at the nadir (around clock time 22:28 h under normal circumstances; during the forced desynchrony experiment the nadir occurred at 22:51 h clock time on day two; after ≈47 h of free run which caused an estimated 23 min delay of the melatonin rhythm). These effects can be especially relevant in a shift work environment where people are awake during a large part of their circadian peak in cortisol production. Under these conditions, work-related stress, light exposure (Scheer & Buijs, 1999), intake of protein (Gibson et al., 1999), and intake of glucose (Gonzalez-Bono et al., 2002) are predicted to trigger a much higher cortisol response than at other circadian phases. This may have consequences for accurate decision making since cortisol levels show an inverted U-shape relationship with memory consolidation and a negative relationship with memory retrieval (Belanoff et al., 2001). Indeed shift-work has been positively associated with occupational accidents (Folkard & Lombardi, 2006; Suzuki et al., 2004).

Insight in the circadian regulation of cortisol production is important for another reason. Cortisol has been linked to various health problems, including mental health issues; higher levels of cortisol have been associated with obesity

(Rosmond et al., 1998), cardiovascular disease (Girod & Brotman, 2004; Whitworth et al., 2005), and depression (Cowen, 2010). Moreover, high cortisol levels during pregnancy resulted in decreased cognitive abilities of the children born from these pregnancies (Bergman et al., 2010). Cortisol also regulates insulin secretion and is suggested to be responsible for the normal circadian pattern of glucose tolerance (Plat et al., 1996). Night shift-work is associated with elevated long-term cortisol release, as measured from hair samples (Manenschijn et al., 2011). Cortisol may therefore be a link to health problems experienced by shift-workers; they also experience higher risks of obesity (Karlsson et al., 2001), diabetes (Morikawa et al., 2005), cardiovascular disease (Bøggild & Knutsson, 1999; Vyas et al., 2012) and depression (Glass & Fujimoto, 1994; Scott et al., 1997). Health of shift-workers may improve when stress is reduced at times when the clock produces high stress sensitivity. Extreme early and late chronotypes (Roenneberg et al., 2003) suffer from social jetlag (Wittmann et al., 2006), the difference between their circadian timing and the social and working hours imposed by society. Waking up out of phase can have consequences for the height of the awakening cortisol response. The degree of social jetlag is associated with obesity (Roenneberg et al., 2012) and we predict that abnormal cortisol regulation may be a factor contributing to this relationship.

The circadian rhythm in glucocorticoids secretion/production results from a dual control of the adrenal cortex (Engeland & Arnhold, 2005). At the one hand increased adrenal sensitivity to adrenocorticotropic hormone (ACTH) is mediated via splanchnic neuronal innervation of the adrenal gland, which is controlled by the master clock, suprachiasmatic nucleus (SCN), via a polysynaptic connection (Engeland & Arnhold, 2005; Kalsbeek et al., 2012; Ulrich-Lai et al., 2006). Indeed administration of a set amount of ACTH results in different levels of cortisol release at different circadian phases in rats (Kaneko et al., 1981) and capuchin monkeys (Torres-Farfan et al., 2008). In humans, circadian modulation to adrenal sensitivity was not found at ACTH dosages in the physiological range (Dickstein et al., 1997), but was found with pharmacological dosages (Dickstein et al., 1991). Recent animal experiments also suggest SCN control via the splanchnic nerve of adrenal sensitivity to ACTH. Rats (in 11:11 LD) and golden hamsters (constant light) show splitting behaviour of their circadian activity and physiology, and also show dissociated rhythms of glucocorticoid release from ACTH, suggesting SCN

regulation of glucocorticoid release independent of ACTH (Lilley et al., 2012; Wotus et al., 2013). Moreover these rhythms are attenuated by disrupting the nerve connection between the SCN and the adrenal, i.e. splanchnicectomy (Ishida et al., 2005; Ulrich-Lai et al., 2006; Wotus et al., 2013). The other pathway towards circadian rhythms in ACTH has been interpreted to result from direct output from the SCN (Cascio et al., 1987; Engeland & Arnhold, 2005). If the SCN simply stimulates ACTH release in a direct manner, we expect the SCN to generate a set rhythm of ACTH release each circadian cycle. Variation in for example stress levels would add cortisol to this fixed circadian component. However, we find a multiplicative relationship between the masking and circadian component of cortisol regulation. This suggests that the SCN regulates sensitivity of the route (Kalsbeek et al., 2012) from masking/stress signals to ACTH. For example we predict that the amplitude of the circadian rhythm in ACTH release is higher under stressful conditions. Circadian modulation of the sensitivity of the stress-to-ACTH route generates larger amplitude differences in cortisol at higher stress levels, contrary to an additive model of cortisol regulation. These predictions require further testing in separate experiments. A multiplicative model also fits better to the reported synergy between the neuronal activation of the adrenal gland and ACTH (Bornstein et al., 2008). The multiplicative relationship between the masking and circadian component of cortisol regulation is in line with circadian modulation of the sensitivity to produce cortisol upon a stimulus.

Acknowledgments

We thank the subject volunteers for their participation and Vibeke Bruinenberg for her practical assistance during the weeks of data collection and Leon Steijvers for programming support. Bühlmann laboratories provided the direct saliva melatonin radioimmunoassay tests as part of their collaboration in the EUCLOCK project.

Declaration of interest

Financial support was obtained from the 6th European Framework project EUCLOCK (018741) and STW (12187). The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Supplementary Material

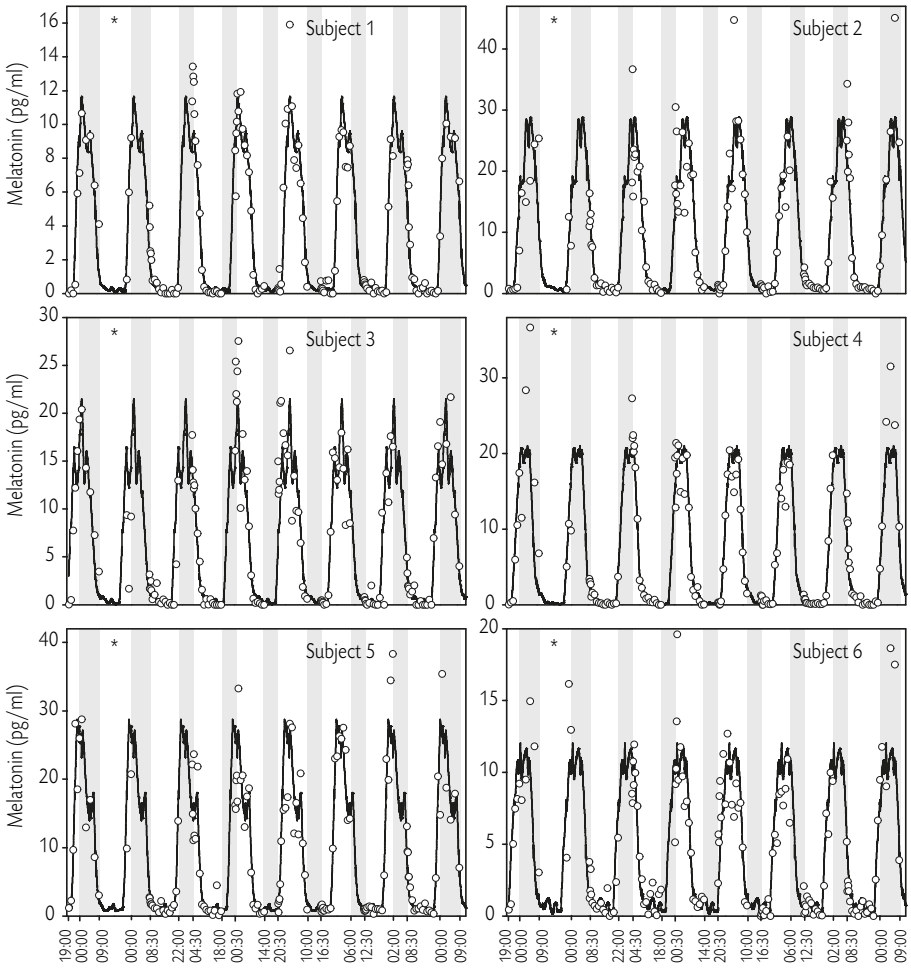


Figure S1: Individual melatonin profiles.
Individual melatonin concentrations are indicated by the white dots. Individual tau's (see Table 1) is plotted by the black line. Grey areas indicate periods of darkness during which the subjects were instructed to sleep. White areas indicate periods of wakefulness (< 10 lux). First day: hourly saliva collection from 19:00 – 1:00 h and every two hours from 1:00 – 9:00 h. Subjects were instructed to sleep from 00:00 – 9:00 h. Upon waking up subjects were free to go home (indicated by the asterisk) but returned to the lab the same evening and 3 saliva samples were collected at 22:00 h, 23:00 h and 00:00 h. Subjects had a normal night of sleep from 0:00 – 8:30 h. The forced desynchrony protocol started upon waking up. The last subjective day was extended by an extra 2 hours (additional saliva collection at 23:00 h). Subjects were instructed to go to bed at 00:00 h, but saliva collection was continued at 1:00 h and from here on every two hours till 9:00 h. The experiment ended upon waking up.

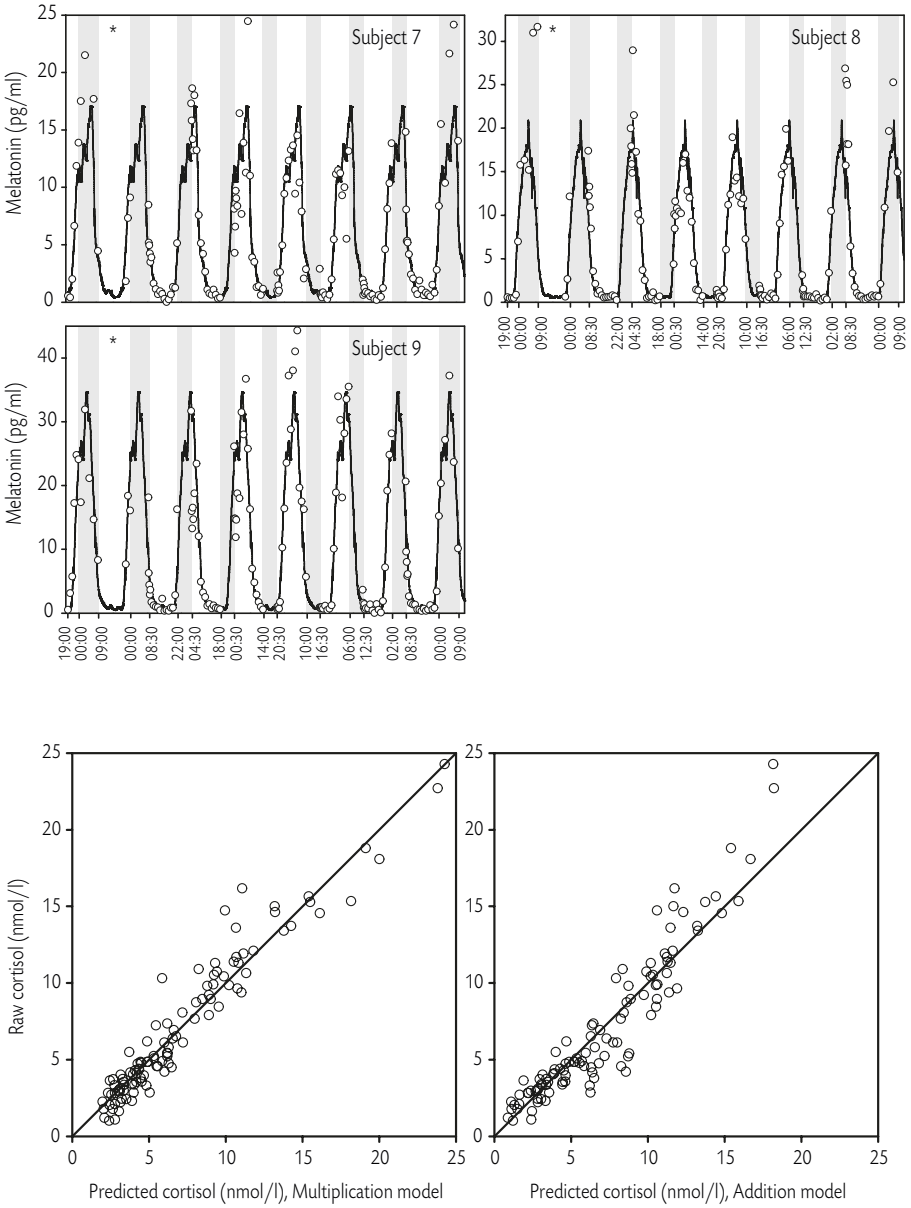


Figure S2: Model fits of the multiplicative and additive model.
Scatter plots of the predicted values of the multiplicative and the additive model against the raw data using the average cortisol levels across all subjects. The diagonal line is the x equals y line, at which predicted values are equal to the raw data.

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SAMENVATTING

Inleiding

In onze huidige 24 uur maatschappij zijn er steeds meer mensen wakker op tijdstippen waarop ons lichaam liever zou willen slapen. Werken tijdens de nacht, laat opblijven 's nachts voor een feest, vroeg opstaan in de ochtend voor werk, of door tijdzones vliegen, is veelvoorkomend gedrag dat in conflict staat met onze lichamelijke voorkeur voor slaap/waak verdeling over de 24 uur. Onze biologische klok in de hersenen, op tijd gezet door lichtinformatie van met name de zon, regelt onze 24 uurs ritmiek in fysiologie en gedrag. Het kan dus zijn dat je lichaam gereed gemaakt wordt om te slapen, maar het midden op de dag is omdat je net van Amsterdam naar New York gevlogen bent. Individuele verschillen in afstemming en periode van de biologische klok zorgen er voor dat mensen verschillen in de tijd dat zij het liefst slapen of opstaan. Dit maakt bepaalde mensen minder of meer geschikt voor werk op bepaalde tijdstippen van de dag. Niet iedereen zal werken in nachtdienst of in de vroege ochtend kunnen verdragen. Hierdoor zullen veel mensen kiezen voor werk dat uitgevoerd wordt op tijdstippen die het beste passen bij hun voorkeur voor de tijdstippen waarop zij wakker willen zijn.

Desalniettemin zijn er veel mensen die in nachtdiensten werken of doordeweeks vroeger op moeten staan dan zij zouden willen. Wakker zijn op momenten waarop men liever zou slapen kan gevolgen hebben voor de alertheid, iets wat de kans op fouten en op ongelukken verhoogt. Met behulp van licht kan de alertheid worden verhoogd. Met name blauw licht, licht met een korte golflengte, is effectief in het verhogen van een gevoel van activatie en het verminderen van slaperigheid. Vooral tijdens werken in de nacht kan licht een belangrijke rol spelen in het wakker en alert houden van mensen. Hierdoor kan de productiviteit hoger komen te liggen en vermindert de kans op ongelukken/fouten veroorzaakt door vermoeidheid.

Echter, blootstelling aan licht tijdens de nacht verstoort ook de nachtelijke fysiologie (zie hoofdstuk 2). 's Nachts is de temperatuur van de huid bijvoorbeeld hoger dan overdag. Een warme huid hangt samen met verhoogde gevoelens van slaperigheid en verminderde gevoelens van activiteit. Blootstelling aan licht tijdens de nacht verlaagt deze hogere huidtemperatuur en verhoogt de alertheid. Een ander voorbeeld van verstoring van de nachtelijke

fysiologie door licht is de aanmaak van melatonine. Melatonine is een hormoon dat alleen 's nachts wordt aangemaakt en mede hierdoor informatie geeft over de tijd van de dag aan de rest van het lichaam. Licht, met name blauw licht, tijdens de nacht onderdrukt de aanmaak van melatonine. Deze verstoring in de melatonine huishouding wordt in verband gebracht met een verhoogd risico op (onder andere) kanker, met name in mensen die *langdurig* in nachtdiensten werken. Er zijn grote verschillen tussen mensen in de mate waarin melatonine onderdrukt wordt door licht (zie hoofdstuk 3). Als we weten wie gevoelig zijn voor melatonine-onderdrukking onder invloed van licht kunnen we mogelijk beter vaststellen welke personen geschikter zijn voor nachtdienst. Het verminderen van de hoeveelheid blauw licht tijdens de nacht is ook een mogelijke interventie. 's Nachts wakker blijven onder geel/oranje licht blijkt het melatonine profiel inderdaad ongemoeid te laten (zie hoofdstuk 2).

Vroeg opstaan kan soms even moeilijk zijn als wakker blijven tijdens de nacht. Toch verschillen mensen hier sterk in, en kan 7 uur 's ochtends voor de een veel vroeger aanvoelen dan voor de ander. Bij de één is het lichaam zich al aan het voorbereiden op de wakkere periode en wordt snel een goed functioneringsniveau bereikt. Anderen hebben soms wel meer dan een uur nodig om zich volledig wakker te voelen in de ochtend (zie hoofdstuk 4). De mensen die 's ochtends moeite hebben met opstaan, hebben baat bij het zichzelf blootstellen aan licht in de ochtend, zelfs wanneer iemand op dat moment nog slaapt (hoofdstuk 4). Tijdens de ochtend, vlak voor het ontwaken begint het hormoon cortisol langzaam te stijgen. Direct na het ontwaken bereikt de cortisolconcentratie een piekwaarde. Deze ochtend cortisol piek geeft aan dat de wakkere periode van de dag is begonnen. De precieze rol van deze zogenaamde ochtend cortisol piek is nog niet helemaal duidelijk. De aanmaak van cortisol is een nauwkeurig samenspel tussen opdrachten gegeven door de biologische klok en stimulatie van buitenaf, zoals wakker worden, maar ook het ervaren van stress. Verhoogde spiegels van cortisol worden in verband gebracht met een scala aan ziektebeelden. Disregulatie van cortisol tijdens werken in de nacht zou dus mogelijk kunnen bijdragen aan de verhoogde gezondheidsrisico's van het werken in nachtdiensten (zie hoofdstuk 5).

Hoofdstuk 2

In de twee samengevoegde studies omschreven in dit hoofdstuk is onderzocht of werken in de nacht onder geel licht, waarbij blauw licht uit wit licht gefilterd wordt (*short-wavelength attenuated polychromatic white light*), de nachtelijke fysiologie minder verstoort dan werken onder volledig wit licht (*full-spectrum light*). De twee licht condities werden hiertoe vergeleken met een donkere controle conditie (*dim light*). Daarnaast is bekeken wat de gevolgen van deze licht condities zijn voor alertheid, slaperigheid en presteren op taken tijdens de nacht. De melatonine aanmaak bleek zo goed als onverstoord in geel licht: het betrof een onderdrukking van 6% vergeleken met de donkere controle tegenover 45% onderdrukking onder volledig wit licht. Ook de huidtemperatuur (*DPG*) was net zo warm in geel licht als in het donker, terwijl onder wit licht de huidtemperatuur aanzienlijk lager lag. Dus zowel melatonine als huidtemperatuur bleven met behulp van geel licht tijdens de nacht onverstoord. Een hoge huidtemperatuur kan echter gevolgen hebben voor de alertheid. In overeenstemming met eerder onderzoek vonden we namelijk dat er een samenhang is tussen huidtemperatuur en slaperigheid en alertheid. Hoe hoger de huidtemperatuur hoe slaperiger en minder alert iemand zich voelt. Interessant is dat deze samenhang niet werd gevonden tussen slaperigheid/alertheid en melatonine. Dit bevestigt het idee dat melatonine geen 'slaaphormoon' is, maar een hormoon dat de tijd van de dag aangeeft.

Hoofdstuk 3

Uit de studie omschreven in hoofdstuk 2 bleek dat er grote verschillen tussen mensen bestaan in de mate waarin hun melatonine onderdrukt wordt onder invloed van licht. In hoofdstuk 3 is bekeken in hoeverre deze verschillen tussen mensen op cellulair niveau verklaard kunnen worden. Melatonine wordt aangemaakt in cellen van de pijnappelklier in de hersenen, maar direct mechanistisch onderzoek aan deze cellen bij mensen is niet mogelijk. Echter omdat alle cellen in iemands lichaam gemaakt worden op basis van hetzelfde DNA, kan tot op zekere hoogte een andere lichaamscel vergeleken worden met een pijnappelkliercel. In deze studie is bekeken hoe de activiteit van melatonine producerende cellen in de pijnappelklier onder invloed van het onderdrukkende signaal van licht (vastgesteld door melatonine concentratie in

het speeksel) samenhangt met de mate waarin een huidcel opgekweekt vanuit een biopt geactiveerd kan worden, via dezelfde moleculaire route die ook in de pijnappelkliercel betrokken is bij melatonine onderdrukking. Hieruit bleek dat er een relatie bestaat tussen de kracht waarmee pijnappelkliercellen en huidcellen een opdracht verwerkten. De proefpersonen verschilden in de kracht waarmee een signaal op moleculair niveau verwerkt werd in hun huidcellen. Wanneer een persoon cellen bevatte die een opdracht krachtiger konden uitvoeren bleek deze persoon minder verstoord te raken door de remmende werking van licht op de nachtelijke melatonine productie. Melatonine aanmaak bleef dan relatief hoog ondanks blootstelling aan licht. Dit resultaat suggereert dat huidcellen gebruikt kunnen worden om processen in de hersenen op moleculair niveau te bestuderen. Mogelijk kan het ook worden gebruikt om informatie te verkrijgen over individuele verschillen in melatonine huishouding en misschien zelfs om te bepalen hoe geschikt een bepaald individu is om in nachtdienst te werken.

Hypothese

Het resultaat beschreven in hoofdstuk 3 heeft geleid tot het vormen een hypothese die een paradox in de werking van licht bij winterdepressie kan verklaren. Mensen met winterdepressie laten een relatief hoge mate van melatonine onderdrukking zien wanneer zij aan licht worden blootgesteld. Dit suggereert een hoge mate van gevoeligheid voor licht. Echter, juist het geven van *extra* licht, zoals bij licht therapie, is effectief in de behandeling van winterdepressie, hetgeen een verminderde gevoeligheid voor licht suggereert. Een verklaring hiervoor zou kunnen liggen in een verminderde gevoeligheid van lichaamscellen in mensen met winterdepressie. Lichtblootstelling tijdens de nacht zorgt ervoor dat de opdracht aan pijnappelkliercellen om melatonine aan te maken verminderd wordt. Wanneer iemand pijnappelkliercellen bevat die minder krachtig zijn, zal een vermindering in de opdracht een grote daling in productie van melatonine tot gevolg hebben. Licht therapie werkt waarschijnlijk via het hormoon serotonine. Serotonine-aanmaak kan verhoogd worden door extra licht. Wanneer zenuwcellen die serotonine aanmaken minder krachtig zijn, kan juist extra licht ervoor zorgen dat de aanmaak van serotonine verhoogd wordt. Dit kan dus mogelijk verklaren waarom mensen met winterdepressie *en* verhoogde melatonine suppressie laten zien *en* baat hebben bij licht om depressie tegen te gaan.

Hoofdstuk 4

Na het ontwaken met behulp van een wekker, zoals dat op werkdagen vaak het geval is, hebben veel mensen nog geruime tijd nodig om zich volledig wakker te voelen. In deze studie hebben proefpersonen deelgenomen die aangaven meer dan een uur nodig te hebben na het opstaan in de ochtend om zich volledig wakker te voelen. We onderzochten of deze periode verkort kon worden door iemand tijdens de laatste 30 minuten van de slaap aan kunstmatig opkomend ochtendlicht bloot te stellen (tot een maximale lichtintensiteit van 300 lux), door middel van de Philips Wake-up Light. Slaperigheid was lager en gevoel van activiteit hoger tijdens de 1,5 uur na het opstaan wanneer proefpersonen blootgesteld waren aan opkomend ochtendlicht vergeleken met geen licht tijdens de laatste 30 minuten van de slaap. Huidtemperatuur, gemeten rondom de torso (*Proximal*) en van handen en voeten (*Distal*), en het verschil hiertussen (Distal-to-proximal skin temperature gradient: *DPG*) daalde sneller na het opstaan bij gebruik van opkomend ochtendlicht tijdens de slaap. In het elektro-encefalogram (EEG) van proefpersonen onder opkomend ochtendlicht waren ook meer korte slaaponderbrekingen te zien mogelijk wijzend op een meer geleidelijke manier van wakker worden.
Net als in hoofdstuk 2, werd in deze studie een samenhang gevonden tussen huidtemperatuur en slaperigheid en gevoel van activiteit. Hoe lager de temperatuur hoe minder slaperig en hoe actiever de proefpersoon zich voelde. Al is bekend uit de literatuur dat licht in de ochtend de ochtend cortisol piek kan verhogen, werd er in deze studie geen hogere ochtend cortisol piek gemeten na het gebruik van gesimuleerd opkomend ochtendlicht. Dit kan komen omdat deze experimentele conditie vergeleken werd met een controle waarbij *na* het opstaan het licht ook aan was. Dit kan ervoor gezorgd hebben dat een eventueel effect van gesimuleerd ochtendlicht op de ochtend cortisol piek niet gemeten kon worden. Het is echter nog maar de vraag in hoeverre cortisol in de ochtend bijdraagt aan het proces van zich wakker *voelen*, cortisol liet namelijk een negatief verband zien met gevoelens van activiteit.

Hoofdstuk 5

In de studie omschreven in dit hoofdstuk werd onderzocht hoe de biologische klok de aanmaak van cortisol reguleert. Zoals beschreven in hoofdstuk 4 wordt

cortisol vlak na het wakker worden in de ochtend in hoge mate aangemaakt. Een piek in cortisol wordt gemiddeld 30 minuten na het wakker gemeten. De aanmaak van de ochtend cortisol piek wordt net als de voorkeur voor tijd van ontwaken gereguleerd door de biologische klok. Omdat de biologische klok ook de timing van slapen en waken bepaalt, is het moeilijk om de effecten van de biologische klok los te koppelen van de effecten die slaap of waak zouden kunnen hebben op cortisol spiegels gedurende de dag en nacht. Om enkel het effect van de biologische klok vast te kunnen stellen moet de slaap op verschillende fasen van de biologische klok plaatsvinden. Om dit voor elkaar te krijgen zonder dat een proefpersoon dit bemerkt, verblijven proefpersonen in een zogenaamde tijdvrije ruimte. In deze ruimte zijn geen indicatoren van de tijd van de dag aanwezig, er komt geen zonlicht binnen en het licht blijft gedimd. Zonder de invloed van licht tikt de biologische klok in zijn eigen tempo. Proefpersonen verbleven een week in deze gedimde tijdvrije ruimte en leefden niet op een 24 uur dag, maar op een dag van 20 uur. Zij sliepen 6,5 uur per 'nacht' en waren 13,5 uur wakker per 'dag'. Iedere 'dag' gingen zij dus 4 uur vroeger naar bed dan de dag ervoor terwijl de biologische klok de fysiologische processen in het lichaam gemiddeld nog steeds om de ongeveer 24 uur liet plaatsvinden. Hierdoor kon cortisol gemeten worden op verschillende fasen van de biologische klok, in combinatie met verschillende fasen van het slaap-waak ritme. Uit dit onderzoek komt duidelijk naar voren dat de ochtend cortisol piek het hoogst wordt na ontwaken in de natuurlijke ochtend. Bij ontwaken op ieder ander moment van de dag ligt de ochtend cortisol piek lager en verdwijnt zelfs helemaal bij ontwaken in de late middag en avond. Verder bleek dat de regulatie van cortisolaanmaak over de hele dag het beste verklaard kan worden met een model dat een vermenigvuldiging maakt van het effect van de biologische klok en effecten door het ontwaken en waken zelf. Dit past bij recent dierexperimenteel onderzoek waar gevonden is dat de gevoeligheid van de bijnierschors, die cortisol produceert, gereguleerd wordt door de biologische klok via een directe zenuwverbinding. Dit heeft belangrijke consequenties voor stress of ontwaken buiten de natuurlijke periode. Op sommige fasen van de biologische klok, in de 'natuurlijke' vroege ochtend, kan dit door deze mutiplicatieve relatie leiden tot extreme cortisol niveaus. Dit zou mogelijk verhoogde lange termijn cortisol spiegels van mensen in nachtdiensten kunnen verklaren en negatieve effecten van nachtdiensten op gezondheid zouden mogelijk verminderd kunnen worden als stress wordt vermeden op specifieke fasen van de biologische klok.

Conclusie

In dit proefschrift zijn effecten van licht op alertheid, prestatie en fysiologie onderzocht en is er gekeken hoe de biologische klok deze processen in mensen reguleert. Verschillende onderzoekingen zijn uitgevoerd om meer inzicht te verkrijgen in de effecten van lichtblootstelling tijdens de nacht en in de ochtend, hoe de effecten van licht verklaard kunnen worden op cellulair niveau, maar ook hoe fysiologische processen aangestuurd worden door de biologische klok wanneer deze geen informatie over tijd van de dag door middel van licht ontvangt. Kleine aanpassingen van bijvoorbeeld licht in onze werkomgeving (hoofdstuk 2), of in de huiselijke omgeving (hoofdstuk 4) en rekening houden met onze interne biologische klok (hoofdstuk 5) en individuele verschillen in aangrenzende fysiologie (hoofdstuk 3) bieden perspectief om in onze 24 uur maatschappij prestaties, welbevinden en gezondheid te verbeteren.

ACKNOWLEDGEMENTS

Domien, nooit zal ik het telefoongesprek vergeten waarin je me vertelde me aan te willen aanstellen op het project; het heeft mijn leven veranderd. Met het onzekere karakter waarmee ik aan het traject begon kon ik me geen betere begeleider toewensen. Je hebt me volledig de kans gegeven om me op mijn eigen tempo te ontplooien zodat ik zeker ben geworden over mijn eigen kunnen. Met iedere vraag kon ik bij je terecht. Ik heb zo veel geleerd van je scherpe inzicht in de wetenschap en ik zal onze regelmatige discussies missen. Ook in tijden van persoonlijk verdriet stond je voor me klaar en ik heb dit als zeer waardevol ervaren. Na de geboorte van Rolo vond je het goed dat ik langer dan gebruikelijk thuis ben gebleven. Ik weet dat dit een unieke kans was, zeker tijdens een AIO periode. Alles tezamen maakt dat ik me enorm thuis heb gevoeld in de Chronobiologie groep, mijn grote dank hiervoor.

Marijke, als ik aan jou denk, dan komt er een glimlach op mijn gezicht. Wat heb ik een leuke tijd met je gehad. Soms leek het alsof er nooit een eind zou komen aan het avontuur, de gezelligheid en alle congresbezoeken (waarbij we zeer kleine, en zeer luxe hotelkamers hebben gedeeld). Je positieve instelling en doorzettingsvermogen hebben de samenwerking met jou tot een groot plezier gemaakt. Je intensieve begeleiding en hulp tijdens de verschillende projecten waren onmisbaar. Ik mocht je 's nachts uit bed bellen en je hebt veel werk van mij overgenomen tijdens mijn zwangerschapsverlof. Wat promoveren vooral moeilijk maakt is het doorzetten op momenten dat er bergen met problemen op je pad lijken te liggen. Je hebt mij altijd geholpen op deze momenten. Mijn proefschrift is dan ook echt een resultaat van onze samenwerking.
Heel veel dank hiervoor.

Marina, what a great time we had! It was so much fun working with you, chatting about both science and personal stuff. EUCLOCK meetings and congresses were like small holidays with you, and sharing a room made it seem like a holiday even more. Thanks for listening to me practicing my conference presentations, for the scientific discussions, and for always being there to help me, even for three whole nights while staying in an, unfortunately, pretty cold room (sorry for that!)... And I also won't forget our Berlin talk. Forever Friends!

Bonnie, veel dank voor je intensieve hulp bij de vele analyses. Ik heb veel van je geleerd en het was altijd heel gezellig om met je samen te werken.

Menno, ik heb je goed leren kennen tijdens de EUCLOCK summer school in Matrahaza, Hongarije, waarbij ik je goede gevoel voor humor heb ontdekt. Wat heb ik toen veel gelachen. In de Chronobiologie groep heb ik veel gehad aan je wetenschappelijke inzichten.

Roelof, ik heb erg genoten van je gezellige karakter! Het is altijd leuk om met je te praten, over persoonlijke zaken en over onze gezamenlijke wetenschappelijke interesses. Ik hoop dat we nog regelmatig contact zullen hebben.

Serge, ik heb veel van je geleerd tijdens je aanwezigheid bij onze Show and Tell meetings, en in de gesprekken die wij hebben gehad over mijn onderzoek. Bedankt!

Martha, thank you for bringing molecular biology into the Chronobiology group. It has really helped me to view chronobiology differently, and this helped me to develop my ideas on the molecular regulation of melatonin suppression. Your door was always open and your enthusiasm and passion for women in science is stimulating. Thank you for your kindness and your very nice cakes.

I want to thank the reading committee, Steven Brown, Addie Johnson and Eus van Someren, for taking time to read my thesis. Steve, thank you for all your help, especially when the Philips grant deadline was close by and I lost track of my SAD hypothesis. Eus, ontzettend bedankt dat je me hebt laten zien hoe ik de temperatuur data het beste kon gaan analyseren, hierdoor heb ik je beter leren kennen, en er uiteindelijk een goede vriend bij gekregen.

Vanja, Luc en Peter, bedankt voor het leuke contact en al jullie hulp tijdens de afgelopen jaren, het was zeer verrijkend om met jullie te mogen samenwerken!

Voor alle gezelligheid, discussies, hulp en samenwerking wil ik bedanken Marian Comas, Ate Boerema, David Lenssen, Yaoyao Chen, Connie Madeti, Valeria Zonato, Vincent van der Vinne, Arjen Strijkstra, Violetta Pilorz , Maria Olmedo, Moniek Geerdink, Kim Gargar, Margien Raven, Jasper Bosman, Emma Wams, Tom Woelders, Sjaak Riede, Kees Mulder, Thomas Kantermann, Igor Hoveijn, Peter Meerlo, Rinaldo Bertossa, Joop Luider, Gerard Overkamp, Leon Steijvers, Anton Nolle, Jouke Franke, Margriet van der Pol, Janwillem Kocks,

Ludmila Cuninkova, Ybe Meesters, Stefan Knapen, Pauline Bollen, Leoni von Ristok, Marlies Hof en Pleuni Kraak.

Veel dank aan master studenten Michiel Schouten, Lotte van Nierop, Esi van der Zwan, Sanne Booij, Vincent Hulst en Wolter Stam voor de hulp bij het uitvoeren van de verschillende studies uit dit proefschrift. Zonder jullie hulp hadden we niet zulke intensieve experimenten kunnen uitvoeren.

Bedankt voor al jullie steun en begrip voor mijn afwezigheid tijdens de afgelopen drukke jaren èn voor de te gekke avonden uit lieve Mariken, Noor, Christel, Naomi, Hilde, Sonja (bedankt voor het wijzen op de vacature!), Inez, Chris, Kees, Sabina en David.

Lieve Agnes en Ditlev, Sereh en Jordi. Jullie zijn meer dan gewoon mijn schoonfamilie. Al vanaf het eerste begin voel ik mij enorm thuis bij jullie. Heel veel dank voor al jullie liefde en steun in de afgelopen 9 jaar alweer.

Lieve Ramon, Hélène, Anetta, Pico, Django, Ronin, Esker, Raven en Oma, bedankt voor de bijzondere momenten samen, de creativiteit, en het voorbeeld om nooit op te geven in moeilijke tijden en altijd te blijven dromen. Ramon, bedankt voor de prachtige vormgeving van mijn proefschrift. Lieve Pico, ik kan niet blijer zijn dan met een omslag van jouw hand.

Lieve kindjes van me, mijn Rolo & Ivora. Jullie zetten mij met beide benen op de grond. Als ik in tijden van stress en deadlines naar jullie blije gezichtjes kijk weet ik weer goed waar mijn leven echt om draait.

Lieve Mirre, ik heb zo veel aan je te danken. Door jou heb ik de rust gevonden die nodig is om na te denken en te kunnen presteren. Geen moeite was je te groot om mij te helpen wanneer ik vastliep. Ik hou ontzettend veel van je en voel me bovendien bevoorrecht je vrouw te zijn, je bent mijn grote voorbeeld.