UNDERSTANDING CIRCADIAN PHYSIOLOGY IN EARLY CHILDHOOD: THE ROLE OF NAPPING AND LIGHT AT NIGHT

by

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ABSTRACT

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Understanding Circadian Physiology in Early Childhood: The Role of Napping and Light at Night

Thesis directed by Assistant Professor Monique K. LeBourgeois

The circadian clock, localized to the suprachiasmatic nucleus of the anterior hypothalamus, is responsible for regulating rhythms in behavior and physiology including the timing and secretion of the hormone melatonin. Light is the strongest environmental input to the circadian system. Specifically, light at night can delay the timing of the melatonin rhythm and suppress secretion of this sleep-promoting hormone.

Although data on factors that influence the biological clock of adolescents and adults is expanding, very little is known about circadian physiology in the early childhood years. The collective aim of this dissertation was to assess the influence of several modifiable factors on the circadian physiology of young children. In study 1, sleep and circadian timing were compared between napping and non-napping toddlers. Napping toddlers had significantly later bedtimes, sleep onset times and circadian phases than non-napping toddlers. These differences in circadian timing are likely mediated by the later bedtimes of nappers, which facilitate light exposure later in the evening thereby delaying the clock. The purpose of study 2 was to quantify the magnitude of light-induced melatonin suppression in response to evening light exposure in preschoolers. Children experienced ~90% melatonin suppression in response to a 1 hour long bright light stimulus before bedtime, an effect that persisted up to 50 minutes

after the light stimulus was terminated. In study 3, we examined children's evening light exposure in association with circadian timing. The amount of light children were exposed to during the 2 hours before bedtime predicted variance in circadian timing over and above bedtime alone in our sample of young children. The findings of this dissertation demonstrate the robust sensitivity of the circadian system of young children to light at night and represent important first steps in understanding fundamental aspects of circadian physiology during the early years of life.

DEDICATION

I would like to dedicate this dissertation to my family and friends who have supported me throughout my graduate career. Specifically, I would like to dedicate this to my parents who both made many sacrifices to allow me to get the education that I received and for their continuous encouragement as I pursued my doctorate. I wouldn't have been able to get to this far without them.

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CHAPTER 1

CIRCADIAN PHASE IN HUMANS: AGE-RELATED DIFFERENCES AND THE INFLUENCE OF LIGHT

Lameese D. Akacem

Introduction

The internal circadian clock of humans is responsible for coordinating daily behavior and physiology, including the timing of sleep and wakefulness and the rhythm of the hormone melatonin (Moore 1992, Reppert and Weaver 2002, Kalsbeek, Palm et al. 2006). Interactions between children's social and familial environments (e.g., preschool, daycare, dual-working parent households) and their internal biological timing may not always be conducive to sleep. About 30% of toddlers and preschool children reportedly do not get enough sleep (Foundation 2004) and up to 60% experience evening sleep disturbances, including bedtime resistance and difficulties falling asleep (Beltramini and Hertzig 1983, Lozoff, Wolf et al. 1985, Bruni, Lo Reto et al. 2000). Such sleep problems often track into late childhood and adolescence and are associated with poor health and developmental outcomes (e.g., mood disorders, attentional/cognitive problems, obesity) (Kataria, Swanson et al. 1987, Zuckerman, Stevenson et al. 1987, Gregory, Caspi et al. 2005, Al Mamun, Lawlor et al. 2007, Friedman, Corley et al. 2009, Wong, Brower et al. 2010). The prevalence of sleep problems may in part be due to misalignment between intrinsic biological timing and environmental demands. Our recently published data indicate that children who are put to bed by their parents at a time too close to their evening rise in melatonin experience increased bedtime resistance and take longer to fall asleep at night (LeBourgeois, Wright et al. 2013). We have also reported wide inter-individual variability in circadian phase (~3.5 h) as measured by dim light melatonin onset (DLMO) time in a sample of toddlers (LeBourgeois, Carskadon et al. 2013). Because melatonin onset signals an individual's

physiological readiness for sleep, our results beg for a better understanding of modifiable factors that influence variability in circadian phase in early childhood. Such data are essential to uncovering novel intervention and prevention targets for early childhood sleep problems.

Light is the most salient environmental time cue to the circadian system (Czeisler, Richardson et al. 1981, Duffy and Wright 2005), which is "gated" by sleep timing. Sleep patterns change across development, with an increase in nighttime sleep consolidation and a gradual decline in sleep duration in the first two decades of life (Iglowstein, Jenni et al. 2003). As such, age-related changes in sleep likely play an important role in the observed maturational shifts in circadian physiology (Dijk, Duffy et al. 2000, Carskadon, Acebo et al. 2004). Whether the timing of the circadian clock changes across early childhood and if light exposure accounts for such shifts remain unknown. The influence of light is of particular significance in addressing these questions because most children are developing in the context of media-saturated social environments. Many parents report placing a television in their child's bedroom to help them fall asleep at night (Vandewater, Rideout et al. 2007), and in the past 2 years, the use of mobile media devices among young children has more than doubled (Media and Rideout 2011). The extent to which light from these sources influences the biological timing of young children has not been studied. Given the high prevalence of sleep problems in early childhood and their long-term consequences, understanding the role of light via electronic media in determining circadian phase is critical.

This comprehensive review will describe age-related changes in the timing of the clock, with a strong focus on light as the primary zeitgeber of the circadian system. I will

i) discuss the two-process model of sleep regulation, a fundamental framework for understanding the timing, duration, and intensity of sleep; ii) summarize the fundamental aspects of circadian physiology; iii) review published data on age-related changes in the circadian system; and iv) discuss how light influences the timing of the circadian system.

Two-Process Model of Sleep Regulation

The Two-Process Model of Sleep Regulation was proposed by Alexander Borbély in 1982 and posits that the timing, duration, and intensity of sleep are influenced by two physiological processes: a sleep homeostatic process dependent on the amount of prior wakefulness (Process S) and a 24 h clock-like circadian process (Process C) (Borbely 1982). These two independent processes interact to promote wakefulness during the day and sleep during the biological night. Although the following literature review will focus primarily on the circadian process, both processes are important in the consolidation of sleep and wakefulness.

The physiological marker of sleep homeostasis is electroencephalogram (EEG) slow wave activity (SWA; 0.5-4.5 Hz) in non-rapid eye movement (NREM) sleep (Borbely 1982, Finelli, Baumann et al. 2000, Achermann and Borbely 2003). SWA declines during sleep, with the highest amount occurring at the beginning of the sleep episode. The time course of SWA during sleep is modulated by the NREM-rapid eye movement (REM) sleep cycle (Achermann 2004). A well-established literature

indicates that longer durations of wakefulness are associated with greater amounts of SWA during subsequent sleep (Borbély, Baumann et al. 1981, Dijk, Brunner et al. 1990). Conversely, daytime sleep dissipates sleep propensity and is associated with less SWA during the succeeding nighttime sleep episode (Werth, Dijk et al. 1996, Achermann and Borbely 2003, Lassonde, Achermann et al. 2013).

Unlike Process S, which has no anatomical locus, the suprachiasmatic nucleus (SCN) of the anterior hypothalamus is responsible for coordinating the circadian system as represented by Process C (Dibner, Schibler et al. 2010). Levels of Process C vary with a period of ~24 h and correspond to the promotion of varying degrees of wakefulness across the day (Akerstedt and Gillberg 1981, Borbely 1982). The shape of the curve corresponds to inversed sleep propensity levels during sleep deprivation or the core body temperature rhythm (Akerstedt and Froberg 1976). The circadian system plays a key role in opposing the build up of homeostatic sleep pressure across the day and maintaining sleep during the latter half of the night after the majority of sleep propensity has been dissipated (Borbely 1982).

The Circadian System

History

The field of circadian research is thought to have started with a very simple experiment performed by French astronomer Jean Jacques d'Ortous De Marian in 1729. De Mairan observed that a Mimosa plant opened its leaves during the day when exposed to sunlight and closed its leaves during the dark period. In order to test whether this rhythmic pattern persisted in constant darkness, De Mairan placed the

Mimosa plant in a light-proof cupboard. Indeed, the rhythmic opening and closing of the Mimosa leaves persisted even in the absence of light, suggesting that an endogenous factor was responsible for driving the observed rhythm. He noted, “The Mimosa feels the sun without in the least being able to see it” (De Marian 1729). More than 250 years later, these endogenous, near 24 h oscillations, known as circadian rhythms, have been documented in organisms ranging from cyanobacteria to humans (Aschoff 1965, Aschoff and Wever 1976, Kondo, Strayer et al. 1993).

Fundamentals of Circadian Physiology

Circadian (“circa,” approximately; “dies,” day) rhythms are endogenously generated biological rhythms that exhibit an approximately 24 h cycle known as circadian period (Reppert and Weaver 2002). Characteristic features of circadian rhythms include that they are (a) self-sustained and occur in the absence of environmental time cues, (b) entrainable to environmental cycles (e.g. the 24 h solar day), and (c) temperature-compensated (i.e. length of oscillation remains constant with varying temperatures) (Edery 2000). Circadian rhythms are governed by the bilateral SCN of the hypothalamus (Ralph, Foster et al. 1990, Dibner, Schibler et al. 2010). In humans, each nuclei of the SCN is made up of a tight network of ~10,000 GABAergic neurons (Moore and Speh 1993). The cells of the SCN are organized into a dorsal shell of neurons containing vasopressin (AVP) and a ventral core containing vasoactive intestinal polypeptide (VIP) expressing neurons (Abrahamson and Moore 2001). Electrophysiological studies have demonstrated that the near 24 h rhythm exhibited by the SCN is driven at the level of individual neurons within this nucleus (Welsh,

Logothetis et al. 1995). Both GABA and VIP, acting through A-type and VIP receptor subtype 2 (VPAC2) receptors, respectively synchronize the autonomous neurons of the SCN (Liu and Reppert 2000, Harmar, Marston et al. 2002).

Although findings from many studies indicated that the SCN was required for circadian rhythmicity in mammals (Moore and Eichler 1972, Stephan and Zucker 1972, Ibuka and Kawamura 1975, Eastman, Mistlberger et al. 1984), whether circadian rhythms actually originated from this nucleus was unknown until a fundamental study in 1990 (Ralph, Foster et al. 1990). Using two strains of hamsters with a circadian period mutation of either 22 h (heterozygote) or 20 h (homozygote), Ralph and colleagues performed a series of SCN transplant experiments. Following surgical removal of their own SCN, hamsters were given a mutant SCN transplant. Once rhythmic activity was restored, transplant recipients exhibited the circadian period of the donor SCN, thereby, demonstrating the central role of this nucleus in driving overt circadian rhythms (Ralph, Foster et al. 1990).

The circadian system is organized in a hierarchical fashion and is composed of a central clock (the SCN) and peripheral clocks located throughout the body, including the liver (Akhtar, Reddy et al. 2002), kidney (Stow and Gumz 2011), heart (Davidson, London et al. 2005) and lungs (Yamamoto, Nakahata et al. 2004). This central pacemaker is responsible for synchronizing peripheral clocks located throughout the body via direct and indirect pathways. Rest/activity cycles driven by the SCN indirectly influence peripheral clocks by ‘gating’ feeding times (Schibler, Ripperger et al. 2003). Activity also influences body temperature and thus can help entrain peripheral oscillators (Schibler and Sassone-Corsi 2002). Direct entrainment of peripheral clocks

occurs via neuronal and hormonal outputs of the SCN, as well as body temperature rhythms driven by central clock (Brown, Zumbrunn et al. 2002, Buijs, van Eden et al. 2003). Synchronization of the SCN with peripheral clocks is important for maintaining coordinated physiology throughout the body. In fact, when the SCN is lesioned in rodents, circadian gene expression in peripheral organs and tissues is abolished (Akhtar, Reddy et al. 2002, Reppert and Weaver 2002). Following a SCN graft, peripheral oscillators resume cycles corresponding to the new SCN rather than their previous oscillations thus demonstrating the strong role of the central clock in coordinating peripheral oscillators (Hastings, Reddy et al. 2003, Sujino, Masumoto et al. 2003). Furthermore, when the SCN and peripheral tissues from a rodent are isolated, oscillations continue for more than 30 days in the SCN but last no longer than a week without a medium change in peripheral tissues (Yamazaki, Numano et al. 2000, Yamazaki, Straume et al. 2002, Yamazaki and Takahashi 2005). This evidence suggests that peripheral clocks are damped oscillators in contrast with the central clock, which maintains a robust rhythm when isolated.

The 24 h rhythmicity of the master circadian clock is regulated at the molecular level (Reppert and Weaver 2002, Ko and Takahashi 2006). A collection of genes in the neurons of the SCN are involved in integrated positive and negative transcriptional feedback loops. In mammals, these genes include the period (Per1, Per2, Per3) and cryptochrome (Cry1 and Cry2) genes (Ko and Takahashi 2006). In the primary feedback loop, positive activators aryl hydrocarbon receptor nuclear translocator-like (BMAL1) and circadian locomotor output cycles kaput (CLOCK) bind to their E-box promoters to activate transcription of Per and Cry genes (Gekakis, Staknis et al. 1998,

Kume, Zylka et al. 1999, Reppert and Weaver 2002, Hastings, Reddy et al. 2003, Ko and Takahashi 2006). Per and Cry mRNA are then transported to the cytosol for translation into protein by ribosomes (Hastings, Reddy et al. 2003). Once synthesized into their respective proteins, PER and CRY heterodimerize and translocate back to the nucleus and inhibit their own transcription by interacting with BMAL1 and CLOCK thereby closing the negative feedback loop (Kume, Zylka et al. 1999, Shearman, Sriram et al. 2000, Vielhaber, Eide et al. 2000, Sato, Yamada et al. 2006, Takahashi, Hong et al. 2008). During the night, these dimers are degraded by casein kinase (Akashi, Tsuchiya et al. 2002, Eide, Vielhaber et al. 2002, Gallego and Virshup 2007). Eventually inhibition of BMAL1 and CLOCK is removed and transcriptional activation starts over (Akashi, Tsuchiya et al. 2002, Hastings, Reddy et al. 2003).

In addition to activating the transcription of Per and Cry, BMAL1 and CLOCK also drive the transcription of orphan nuclear receptors reverse erythroblastosis virus alpha (Rev-Erbα) and retinoic acid receptor-related orphan receptor alpha (Rorα) (Preitner, Damiola et al. 2002, Hastings, Reddy et al. 2003, Triqueneaux, Thenot et al. 2004, Akashi and Takumi 2005). Once translated, REV-ERBα and RORα proteins enter the nucleus where they compete for binding sites on the retinoic acid-related orphan receptor response elements (ROREs) on the Bmal1 promoter (Guillaumond, Dardente et al. 2005). Binding of RORα and REV-ERBα to ROREs activates and represses Bmal1 transcription respectively (Guillaumond, Dardente et al. 2005). One cycle of these oscillatory changes in gene expression takes approximately 24 h and dictates the exact length of the circadian period (Ko and Takahashi 2006) .

Although the molecular clock machinery is based on transcription-translation feedback loops, recent groundbreaking work from O'Neill and colleagues demonstrated that transcription is not necessary for cellular circadian rhythms in humans (O'Neill and Reddy 2011). In fact, circadian rhythms were observed in isolated anucleate red blood cells. This study found that red blood cells keep time with near 24 h oscillations in peroxiredoxin, a cellular antioxidant protein. Rhythms in peroxiredoxin were entrainable to external time cues and temperature compensated, both hallmark features of circadian driven oscillations. Circadian rhythms in the redox cycle of peroxiredoxin have now been demonstrated in all domains of life and may be in part responsible for the evolution of circadian rhythms (Edgar, Green et al. 2012).

Circadian Phase Markers

In humans, it is impossible to study the physiology of the SCN directly, thus, examining the outputs of the clock is key to understanding the timing of the central clock (Duffy and Wright 2005). Measurements of circadian physiology include period, defined as the time it takes for one full oscillation to occur, and phase, which represents the state of an oscillation at a specific time. Outputs of the circadian clock include the timing of sleep and wakefulness, melatonin, cortisol and core body temperature. The rhythms of melatonin, cortisol and core body temperature are most often measured to assess the timing of the clock in humans and will be discussed below (Lewy, Cutler et al. 1999, Klerman, Gershengorn et al. 2002, Refinetti 2010).

Melatonin

The rhythm of melatonin secretion is regulated by the SCN of the hypothalamus via a multi-synaptic pathway (Moore and Klein 1974). From the SCN, GABAergic neurons project to the paraventricular nucleus (PVN) of the hypothalamus. Neurons from the PVN travel inferiorly to the intermediolateral (IML) column of the spinal cord. From here, sympathetic neurons project to the superior cervical ganglion (SCG). Finally, the SCG projects postganglionic sympathetic nerve fibers to the pineal gland which secretes melatonin (Moore and Klein 1974, Kalsbeek, Palm et al. 2006). In humans, melatonin levels begin to rise in the evening, peak during the middle of the night and return to baseline levels during the day (Arendt 2005).

Cortisol

The circadian pacemaker also drives the rhythm of cortisol, a hormone secreted by the adrenal cortex in response to adrenocorticotropic hormone (ACTH) from the pituitary gland (Kalsbeek, Palm et al. 2006). Secretion of cortisol is not only under circadian control via the stimulation of neuroendocrine neurons that control ACTH release but also via a multi-synaptic neural output pathway from the SCN to the adrenal gland (Buijs, Wortel et al. 1999, Kalsbeek, Palm et al. 2006). Cortisol secretion peaks in the morning upon awakening and reaches its nadir in the late evening. It is thought that the morning increase in cortisol is adaptive in nature in order to prepare humans for the stressors of the upcoming day (Powell and Schlotz 2012).

Core Body Temperature

Core body temperature (CBT) also follows a circadian rhythm. In humans, CBT peaks during the daytime and reaches its lowest point in the middle of the night (Refinetti 2010). In fact, the CBT minimum usually aligns with midpoint of the night sleep period in entrained individuals (Roenneberg 2012). Furthermore, the CBT minimum is an important phase maker for appropriately timing phase-shifting light treatments (Khalsa, Jewett et al. 2003).

Circadian Protocols

In order to ensure accurate measurements of circadian rhythms, researchers have devised a number of protocols that control for exogenous factors that could potentially influence these rhythms. In a constant routine protocol, any periodic changes in the environment that could potentially mask or obscure a circadian rhythm are minimized or completely eliminated (Duffy and Dijk 2002, Duffy and Wright 2005). Such a routine requires that subjects maintain constant wakefulness in order to eliminate the influence of sleep on measurable outputs (i.e. core body temperature, cortisol) (Minors, Waterhouse et al. 1994). Subjects remain in dim-light to avoid the alerting and melatonin suppressive effects of light and maintain constant posture in controlled temperature conditions so as not to influence thermoregulatory physiology (Lewy, Wehr et al. 1980, Minors and Waterhouse 1989). Additionally subjects are provided with evenly distributed isocaloric snacks throughout the duration of the protocol to control for the influence of feeding on circadian rhythms (Duffy and Dijk 2002, Duffy and Wright 2005, Wirz-Justice 2007). Once these controls are

implemented, researchers are able to accurately measure clock output rhythms including melatonin, cortisol and core body temperature.

Developed by Nathaniel Kleitman, the forced desynchrony protocol allows researchers to tease apart rhythms that are due to behavioral factors (i.e. sleep/wake, feeding etc.) and rhythms that are circadian in origin (Dijk and Czeisler 1994). During this protocol, subjects are placed on a short or longer day length (e.g. 20 or 28 h) (Carskadon, Labyak et al. 1999, Dijk, Duffy et al. 1999, Wirz-Justice 2007). The circadian system is unable to entrain to these extreme light/dark conditions and subjects exhibit their endogenous circadian rhythms. Behavioral influences, including sleep/wake patterns, feeding, and activity, are equally distributed across all circadian phases (Wirz-Justice 2007). This protocol allows for an accurate measurement of intrinsic circadian period.

Another protocol used to assess circadian rhythms is an ultra-short sleep/wake cycle. In this type of approach, periods of sleep and wakefulness in dim light alternate in an ultradian manner. Patterns of sleep and wakefulness vary and have ranged from 20-minute cycles (13 minutes awake, 7 minutes asleep) to 180-minute cycles (120 minutes awake, 60 minutes asleep) (Weitzman, Nogeire et al. 1974, Lavie and Scherson 1981). These cycles can be repeated for up to 10 days (Weitzman, Nogeire et al. 1974). Ultra-short sleep wake cycles have an advantage over constant routine protocols in that there is a smaller accumulation of sleep debt (Kline, Durstine et al. 2010). Additionally, instead of completely eliminating potential masking effects of sleep and activity, these confounders are evenly distributed across all cycles (Kline, Durstine et al. 2010).

The intensive protocols described above require spending days to weeks in the laboratory, thus, they are not feasible for assessing circadian parameters in young children. Instead, by collecting one evening of saliva samples in dim light conditions, the phase of the melatonin rhythm can be determined (Lewy and Sack 1989). In this assessment, subjects are placed in dim light conditions to avoid the suppressive effects of light on melatonin secretion and maintain constant posture during sample collection (Lewy, Wehr et al. 1980, Deacon and Arendt 1994). Melatonin onset can be calculated as the time at which salivary melatonin concentrations pass and remain above 4 pg/mL and represents the most reliable phase marker of the internal clock (Klerman, Gershengorn et al. 2002, Benloucif, Guico et al. 2005, LeBourgeois, Carskadon et al. 2013). Because melatonin phase is the most feasible indicator of the timing of the circadian clock that we can obtain in a population of young children, this literature review will focus on how various factors (i.e. age and light) influence the timing of circadian phase in humans.

Age-Related Changes in Circadian Phase

Changes in Circadian Phase Across the Lifespan

An inverted U-shape relationship exists between circadian phase and age across the lifespan. A recent study from our laboratory found that average DLMO was 19:29 in a sample of toddlers (LeBourgeois, Carskadon et al. 2013). However, a large gap exists in the literature on circadian timing between the ages of 3 and 9 years. Cross sectional studies of older children and young adolescents found an average DLMO of

20:28 in a sample of 9-12 year olds and 20:41 in 13-16 year olds during the academic year (Crowley, Acebo et al. 2006). Melatonin onset continues to delay in adults aged 22-39 years with an average time of 22:51 (Kawinska, Dumont et al. 2005). DLMO time begins to advance in the middle age years to 21:32 and becomes even earlier in those over the age of 65 years with an average DLMO of 21:04 (Gooneratne, Metlay et al. 2003, Kawinska, Dumont et al. 2005).

Although no published data on circadian phase between the ages of 3 and 9 years exist, recent work in our laboratory found differences in DLMO based on napping status. Toddlers who napped had a later circadian phase than children who had naturally stopped napping (Akacem, Simpkin et al. In Preparation). Given that many studies have established a decline in napping frequency across the early childhood years (Weissbluth 1995, Thorleifsdottir, Bjornsson et al. 2002, Iglowstein, Jenni et al. 2003, Crosby, LeBourgeois et al. 2005), it is possible that circadian phase may concurrently advance with the decline in napping frequency and then delay in the school age years and adolescence. A longitudinal study of circadian phase across early childhood is necessary to test this hypothesis.

Adolescent Phase Delay

Adolescence represents a period of pronounced changes in the timing of the internal circadian clock (Crowley, Acebo et al. 2007). A delay in bedtimes during adolescence has been reported in several countries across 6 continents including the U.S.A. (Carskadon 1990), Canada (Laberge, Petit et al. 2001), Poland (Szymczak, Jasinska et al. 1993), Belgium (Van den Bulck 2004), Australia (Henschel and Lack

1987), Finland (Saarenpaa-Heikkila, Rintahaka et al. 1995), Taiwan (Gau and Soong 2003), South Africa (Reid, Maldonado et al. 2002), and Brazil (Andrade, Benedito-Silva et al. 1993). Many researchers have attributed the observed shift to increased school-work and extracurricular activities associated with high school (Crowley, Acebo et al. 2007); however, findings from Andrade and colleagues demonstrated a biological basis for this trend such that later sleep times in the adolescents were associated with more mature pubertal development (Andrade, Benedito-Silva et al. 1993). Furthermore 6th grade girls who rated themselves as more physically mature also self-reported a more evening circadian phase preference (Carskadon, Vieira et al. 1993). Additionally, the timing of circadian phase as measured by the melatonin rhythm was positively correlated with Tanner stage (Carskadon, Acebo et al. 1997, Carskadon, Acebo et al. 2004, Taylor, Jenni et al. 2005). Research across mammalian species demonstrate a circadian delay during puberty indicating that this phenomenon is likely driven by an endogenous biological change and not extrinsic factors (Weinert and Waterhouse 1999, Golub, Takeuchi et al. 2002, Neuman, Gothilf et al. 2005, Hummer, Jechura et al. 2007, McGinnis, Lumia et al. 2007, Hagenauer, Perryman et al. 2009). Several physiological features are thought to contribute to this developmental phase delay, including changes in light sensitivity, the homeostatic sleep system, and circadian period length (Carskadon, Acebo et al. 2004, Hagenauer, Perryman et al. 2009).

Although the hypothesis that light sensitivity changes during adolescent maturation has been tested in several studies, the developmental trajectory is still unclear (Crowley, Acebo et al. 2007, Hagenauer, Perryman et al. 2009, Carskadon 2011). Specifically, it has been proposed that the amplitude of the phase response

curve changes such that mature adolescents may be less sensitive to the phase advancing effects of morning light and/or more sensitive to delaying evening light exposure. Preliminary findings show that older adolescents are significantly less sensitive to morning dim light exposure (15 lux) as measured by melatonin suppression than younger adolescents, however no difference in sensitivity was observed in response to late evening light (Carskadon, Acebo et al. 2002) Additionally, adolescents showed a phase delay after exposure to 1 h of short wavelength light upon awakening in the morning offering further support to the hypothesis that adolescents may be less sensitive to phase advancing morning light (Crowley and Carskadon 2010). Longitudinal work on human adolescents is necessary to examine if light sensitivity does in fact change as a function of age during this period.

Another hypothesis posits that the observed phase delay in adolescence is due to developmental changes in the build-up of homeostatic sleep pressure, or Process S (Carskadon, Acebo et al. 2004, Crowley, Acebo et al. 2007, Hagenauer, Perryman et al. 2009, Carskadon 2011). Findings from a 36 h total sleep deprivation study revealed that homeostatic sleep drive as measured by SWA builds up slower in post-pubertal adolescents than pre-pubertal adolescents (Jenni, Achermann et al. 2005). Another study measured sleep tendency using sleep latency tests (speed of falling asleep) following 14.5 h, 16.5 h and 18.5 h of wakefulness and found that more mature adolescents (as measured by Tanner stage) took longer to fall asleep during the sleep latency tests (Taylor, Jenni et al. 2005). These data suggest that in comparison to pre-pubertal children, fully mature adolescents are able to sustain greater amounts of

wakefulness due to a slower build up in homeostatic sleep pressure, thereby permitting later sleep times.

The third most common hypothesis postulates that the observed phase delay in adolescence is due to a longer circadian period (Carskadon, Labyak et al. 1999, Carskadon, Acebo et al. 2004, Hagenauer, Perryman et al. 2009). Data from the Carskadon laboratory found a circadian period of 24.27 h in a sample of adolescents, which is significantly longer than the 24.18 h period length observed in adults (Carskadon, Labyak et al. 1999, Czeisler, Duffy et al. 1999). According to entrainment theory, a longer circadian period would delay the output rhythms of the clock and thereby delay circadian phase (Roenneberg, Daan et al. 2003). However, continuous light exposure has been found to lengthen circadian period in at least five species who also exhibit a phase delay during puberty (Martinez Jr 1972, Pittendrigh and Daan 1976, Tokura and Aschoff 1978, Summer, Ferraro et al. 1984, Lee and Labyak 1997, Hagenauer, Perryman et al. 2009). Thus, the phase delay may be in part due to changes in sensitivity to continuous light and not circadian period per se (Hagenauer, Perryman et al. 2009). A study manipulating continuous light exposure in adolescents is necessary to investigate this possibility. Taken together, the observed developmental changes in the homeostatic system coupled with a delay in circadian timing and changes in light sensitivity result in a strong drive to stay up later in the adolescent years.

Phase Advance in Older Adults

An earlier timing of the circadian clock in older adult subjects is well established (Richardson, Carskadon et al. 1982, Sherman, Wysham et al. 1985, Duffy, Dijk et al. 1998, Dijk and Duffy 1999, Yoon, Kripke et al. 2003). The mechanism for the observed phase advance during older age is unclear and could possibly be attributed to light exposure gated by earlier sleep times, phase-dependent changes in light sensitivity (discussed below), or age-related changes in circadian period (Dijk, Duffy et al. 2000, Cajochen, Munch et al. 2006). Published findings are inconsistent with regard to how circadian period changes with advancing age, if at all (Weitzman, Moline et al. 1982, Van Gool and Mirmiran 1986, Davis and Viswanathan 1998, Czeisler, Duffy et al. 1999). Numerous animal studies and one human study reported a shorter circadian period with aging; however, such changes were relatively small (Pittendrigh and Daan 1974, Weitzman, Moline et al. 1982, Morin 1988). A study by Czeisler and colleagues comparing younger and older adults did not observe a significant difference in circadian period (Czeisler, Duffy et al. 1999). Animal studies confirm this finding, including one that tracked circadian period in Syrian hamsters across their lifespan (Davis and Viswanathan 1998). In all, these studies suggest that although circadian phase advances, circadian period does not change reliably across adulthood, but further human longitudinal work is necessary to best answer this research question (Dijk, Duffy et al. 2000).

Light and Circadian Phase

One important characteristic of the circadian system is the ability of light to influence the period of its oscillation (Czeisler and Wright 1999, Roenneberg, Kantermann et al. 2013). The human circadian system is extremely sensitive to light. For example, one study showed that as little as 12 lux was able to phase shift the clock in controlled laboratory settings (Duffy and Wright 2005). Additionally, light exposure causes an immediate suppression of melatonin secretion (Lewy, Wehr et al. 1980). Entrainment is the process by which the intrinsic circadian period conforms to earth's 24 h day with a stable phase relationship between the endogenous circadian period and the light/dark cycle (Roenneberg and Foster 1997, Johnson, Elliott et al. 2003). Exogenous factors that can entrain the circadian clock are called 'zeitgebers' or time cues, the strongest of which is light (Czeisler, Richardson et al. 1981, Duffy and Wright 2005, Roenneberg, Kantermann et al. 2013). Non-photic time cues, including feeding behavior, caffeine, and exercise may also influence the circadian clock to a lesser extent (Castillo, Hochstetler et al. 2004, Atkinson, Edwards et al. 2007, Burke, Markwald et al. 2012). Two proposed mechanisms of entrainment exist: discrete (nonparametric) entrainment and continuous (parametric) entrainment, which differ in how light influences the clock (tonic vs. phasic effects) (Johnson, Elliott et al. 2003). Human entrainment to the light/dark cycle may occur as a result of both discrete and continuous mechanisms.

Discrete Entrainment

Colin Pittendrigh first proposed the model of discrete entrainment (Pittendrigh 1967). The proposed mechanism for discrete entrainment is that light pulses

administered at different circadian phases result in a phase shift that is equal to the difference between the endogenous circadian period and the period of the environmental light/dark cycle. This model was first demonstrated in Drosophilia, with the entrainment of eclosion rhythms in response to brief light pulses administered at different phases (Pittendrigh 1967). Discrete entrainment is based on knowledge of the free running period and the phase response curve (PRC) (Johnson, Elliott et al. 2003). PRCs developed for zeitgebers such as light form the foundation of this model of entrainment and will be discussed in detail below (Minors, Waterhouse et al. 1991, Khalsa, Jewett et al. 2003).

Continuous Entrainment

The model of continuous entrainment was proposed by Jurgen Aschoff and posits that light exposure has a continuous influence on the clock (Johnson, Elliott et al. 2003). In continuous entrainment the angular velocity of the free running period changes with exposure to differing light intensities for a relatively long duration (i.e., a photoperiod) and allows the circadian clock to change its period to match that of the environment (Johnson, Elliott et al. 2003). The continuous entrainment model is based on the observation that varying intensities of continuous light exposure influenced the length of the free running period, such that the clock runs faster in light and slower in darkness for nocturnal animals. This is known as Aschoff's Rule (DeCoursey 1973).

Circadian Photoreception

The mechanisms of circadian photoreception have only been recently understood in humans. Initially, it was believed that the visual rod and cone photoreceptors were responsible for entraining the circadian clock to the external light/dark cycle. The first attempt at testing this hypothesis involved the comparison of the circadian response to light in mice with and without rod photoreceptors (Foster, Provencio et al. 1991). By measuring locomotor rhythms following the administration of a light pulse, researchers found that mice lacking rods exhibited comparable phase shifts to mice with intact rods (Foster, Provencio et al. 1991). Freedman and colleagues proceeded to investigate whether strains of mice lacking cones and or both rods and cones were able to entrain to light (Freedman, Lucas et al. 1999). This study found that both strains of mice maintained normal circadian phase shifting abilities to light following removal of these photoreceptors. However, circadian entrainment was completely abolished after removing the eyes, suggesting that another photoreceptor within the eye is primarily responsible for transmitting light information to the circadian clock (Freedman, Lucas et al. 1999).

Three years later, a fundamental study by Berson and colleagues discovered a group of intrinsically photosensitive retinal ganglion cells (ipRGCs) whose axons form part of the retinohypothalamic tract (Berson, Dunn et al. 2002). It was later discovered that these cells contained the photoreceptor melanopsin (Provencio, Rollag et al. 2002). Located in the ganglion cell layer of the retina, these ipRGCs integrate light information from rods and cones with their intrinsic light response and transmit this integrated input to discrete brain regions. For example, melanopsin containing ipRGCs contribute to accessory visual functions including circadian photoentrainment through its projections

to the SCN and the pupillary light reflex (PLR) with projections to the olivary pretectal nucleus (OPN) (Hattar, Liao et al. 2002, Schmidt, Do et al. 2011).

Although melanopsin plays an important role in mediating circadian responses to light, findings from animal studies indicate that this photoreceptor is not solely responsible for these effects. In fact, melanopsin-deficient mice maintained the ability to photoentrain and phase-shift but these responses were attenuated by about 40% (Ruby, Brennan et al. 2002). If rods, cones, and melanopsin are eliminated, all non-image forming responses to light are abolished (Hattar, Lucas et al. 2003). These findings suggest that rods, cones, and melanopsin act together to mediate circadian responses to light (Menaker 2003).

Light Timing

Many studies have established that the timing of light exposure is important in phase shifting the clock. Specifically, humans are most sensitive to light during the biological night (Zeitzer, Dijk et al. 2000). In order to understand how a light stimulus presented at various circadian phases influences the timing of the clock, the construction of a PRC is necessary. A PRC depicts the magnitude and direction of a phase shift that occurs in response to a light stimulus given at different circadian times (Johnson, Elliott et al. 2003). The first PRC to light was created by Honma and Honma in 1988; however, this PRC did not have a prominent phase delay portion and light pulses were not evenly distributed across the circadian cycle (Honma 1988). Future attempts at creating a PRC had similar issues including lack of data and/or well-controlled methodology (Minors et al. 1991; Jewett 1994). In 2003, Khalsa and

colleagues constructed a comprehensive PRC to light with evenly spaced light stimuli (6.7 h of 10,000 lux) across circadian phases using highly controlled methodology (Khalsa, Jewett et al. 2003). This light PRC dictates that light exposure occurring in the early biological night prior to the core body temperature minimum will result in a delay in the timing of circadian phase. Conversely, light exposure that occurs towards the end of the biological night and after the core body temperature minimum will result in a phase advance. A rapid transition occurs between the phase delay and phase advance portions of the response curve at the time of the core body temperature minimum. The transition between these two portions of the PRC during the biological day is gradual, and no strong evidence exists for a mid-day dead-zone in the human PRC to light (Jewett, Rimmer et al. 1997, Khalsa, Jewett et al. 2003).

Mechanistically, it is hypothesized that the phase shifts to light occur due changes at the molecular level. Work from Agostino and colleagues demonstrate a strong role for the post-transcriptional modifier casein kinase in photic entrainment (Agostino, Plano et al. 2008, Agostino, Harrington et al. 2009). These studies observed a heightened sensitivity to light when casein kinase is mutated or pharmacologically inhibited (Agostino, Plano et al. 2008, Agostino, Harrington et al. 2009). It is believed that casein kinase, which exhibits a circadian rhythm peaking at the beginning of the biological night, controls photic input from the retinohypothalamic tract to the central clock by modulating glutamate NMDA receptors on the SCN (Chergui, Svenningsson et al. 2005). Thus, the actions of casein kinase may contribute to the observed heightened sensitivity to light during the biological night. Furthermore, changes in Per1 and Per2 gene expression have been reported in response to light exposure

(Shigeyoshi, Taguchi et al. 1997, Albrecht, Zheng et al. 2001). Light exposure early in the night induces expression of both Per1 and Per2, and light exposure in the morning results in the expression of only Per1, which is thought to mediate phase delays and advances respectively (Shigeyoshi, Taguchi et al. 1997, Albrecht, Zheng et al. 2001). One study found that mice lacking the Per2 gene were not able to phase delay, and mice lacking the Per1 gene were not able to phase advance, thus providing further evidence for a key role of these genes in resetting the internal clock at the molecular level (Albrecht, Zheng et al. 2001, Yan, Hochstetler et al. 2003).

The development of a PRC to light has significant clinical utility. Light therapies have applied this PRC to treat circadian rhythm sleep disorders (CRSD), including shift work disorder, jet lag disorder, and advanced and delayed sleep phase disorder (Gooley 2008). CRSDs are characterized by a misalignment between circadian phase and the sleep/wake cycle (Gooley 2008). Thus, by using the PRC to light, appropriately timed light exposure can help shift circadian phase to the desired time.

Type 0 and Type 1 Phase Response Curves

Two types of qualitatively different PRCs exist based on the strength of the resetting stimulus. These are differentiated by the magnitude of phase shifts that occur in response to light. Type 0 PRCs are characterized by large phase shifts (usually greater than 6 h) and exhibit a discontinuous transition between the phase advance and delay portions of the PRC. A Type 0 PRC has been demonstrated in humans using a 3 cycle bright light stimulus (Czeisler, Kronauer et al. 1989). Conversely, Type 1 PRCs are characterized by smaller phase shifts (usually less than 6 h) and display a

continuous transition between the two portions of the PRC (Johnson, Elliott et al. 2003). Type 0 and Type 1 PRCs are named after the slope of their phase transition curve, which plots phase prior to and after a light treatment (Johnson, Elliott et al. 2003).

Light Intensity

Data from numerous studies indicate that the circadian clock of humans responds to light in an intensity-dependent manner (Boivin, Duffy et al. 1994, Boivin, Duffy et al. 1996, Zeitzer, Dijk et al. 2000, Zeitzer, Khalsa et al. 2005, Duffy, Zeitzer et al. 2007). For example, one study by Zeitzer and colleagues exposed subjects to 3 consecutive days of 5 h light stimuli of 0.03, 12, 180, 600, 1,260, or 9,500 lux 1.5 h after the core body temperature minimum. Findings indicated that phase advances occur in an intensity dependent-manner (Zeitzer, Khalsa et al. 2005). A similar study demonstrated the same relationship between light intensity and phase shifts in the early biological night (i.e. inducing phase delays) (Zeitzer, Dijk et al. 2000). Both studies demonstrate that the circadian clock responds to increasing light intensities during the biological night in a logistical fashion such that half-maximal phase shifts can be achieved with $1/10^{th}$ of maximal light exposure. The response to increasing light intensities appears to saturate after about 10,000 lux (Zeitzer, Dijk et al. 2000, Zeitzer, Khalsa et al. 2005).

The response to light intensity has also been investigated as a function of age. Although the maximum observed phase shift to a 6.5 h stimulus of light was equivalent among older and younger subjects, older subjects were less sensitive to low to moderate intensities of light (between ~50-1,000 lux) during the phase delay portion of

the PRC (Duffy, Zeitzer et al. 2007). Rather than an attenuated circadian sensitivity to light, these findings have been attributed to an increase in lens absorption and a decrease in pupil diameter that occurs with age (Charman 2003, Duffy, Zeitzer et al. 2007). These changes in lens absorbency and pupil diameter effectively decreases the amount of light reaching the retina and may explain the observed decreased sensitivity to moderate light intensities. These findings have important implications for prescribing light treatments to the elderly.

Light Duration

The most intriguing studies examining the influence of light duration on phase shifting compared the effects of intermittent light exposure to continuous light exposure. One study by Gronfier and colleagues exposed subjects to one of three light treatments during the phase delay of the portion of the PRC: continuous bright light (~9,500 lux) for 6.5 h, 15 minute bright light pulses (~9,500 lux) separated by 60 minutes of dim light over the course of 6.5 h, or 6.5 h of dim light (<1 lux) (Gronfier, Wright et al. 2004). Although subjects in the intermittent light treatment group were only exposed to 23% of the duration of light received by those in the continuous treatment group, no significant difference in phase shifts was apparent between the two groups. Findings from this study suggest that intermittent light is more effective per minute than continuous light exposure (Gronfier, Wright et al. 2004). A similar study in which subjects were exposed to either intermittent light or continuous light found that intermittent light exposure elicited phase shifts similar in magnitude to the continuous light exposure treatment (Rimmer, Boivin et al. 2000). Furthermore, work from Zeitzer and colleagues

demonstrated that 60 millisecond flashes of light administered during the first half of the biological night was able to phase shift the melatonin rhythm (Zeitzer, Ruby et al. 2011). Together, findings from these studies demonstrate that the human circadian system is sensitive to intermittent pulses of light and suggest a role for brief light exposures in human circadian entrainment (Rimmer, Boivin et al. 2000, Gronfier, Wright et al. 2004, Duffy and Wright 2005). The human circadian response to continuous light exposure is such that increases in light duration result in greater phase shifts. In fact, the human circadian system is more sensitive to increasing light duration than intensity (Dewan, Benloucif et al. 2011). These findings have important implications for light therapy and may facilitate light treatments for patients with CRSDs.

Spectral Sensitivity to Light

It is well established that the human circadian system is most sensitive to blue short wavelength light. This spectral sensitivity is consistent with the sensitivity of melanopsin photoreceptors (482-484 nm) contained within iPRGCs that communicate directly with the SCN (Hattar, Liao et al. 2002, Lockley and Gooley 2006). Circadian responses to light, including melatonin suppression, phase shifting, and pupillary constriction show increased sensitivity to shorter wavelengths of light (Lockley and Gooley 2006). One study established an action spectrum for melatonin suppression using different wavelengths of light and found shorter wavelengths of light were most effective at suppressing melatonin (Thapan, Arendt et al. 2001). Human phase shifting responses are also more sensitive to short wavelength light. A study comparing an exposure of 8 lux of short wavelength light to 12,000 lux of bright white light found that

the short wavelength light produced similar phase shifts as bright light that contained 185 times more photons (Warman, Dijk et al. 2003). Pupillary constriction in blind individuals is also most sensitive to short wavelengths of light (Zaidi, Hull et al. 2007). Although a well-established literature indicates that circadian responses are most sensitive to blue light, the irradiance of light is also an important factor to consider. Work from Gooley and colleagues demonstrated that green light (555 nm) was actually more effective at phase shifting than blue light at low irradiances (Gooley, Rajaratnam et al. 2010). Thus, it is important to take into account the spectral distribution as well as the irradiance of a light stimulus, as both can significantly alter the observed circadian response.

Circadian Adaptation to Prior Light/Dark Exposure

Studies on humans have demonstrated the ability of prior photic history to influence the sensitivity of the circadian clock. One study compared light sensitivity as measured by melatonin suppression in subjects after a week of dim light exposure or bright light exposure. Findings from this study indicate recent photic history influences light sensitivity such that prior dim light exposure increases the sensitivity of the circadian system to light (Hebert, Martin et al. 2002). Work from Chang and colleagues is consistent with these findings such that subjects exposed to dim light as opposed to room light experienced increased melatonin suppression following a 90 lux light stimulus (Chang, Scheer et al. 2011). Another study compared melatonin suppression after a stimulus of 6.5 h of 200 lux following 3 days of exposure to 0.5 lux or 200 lux. Subjects who were exposed to 0.5 lux prior to the experimental light stimulus had

greater levels of melatonin suppression compared to those who had a photic history of 200 lux (Smith, Schoen et al. 2004). Higher levels of light exposure therefore desensitize the clock to subsequent light stimuli, and vice versa.

Summary

This comprehensive review discussed known changes in circadian timing across the lifespan. Furthermore, this review summarized the literature pertaining to the effects of light on the timing of the circadian clock. Our current understanding of factors that influence circadian phase in early childhood is extremely limited. Longitudinal studies and experimental manipulations of light in young children are necessary to close this wide knowledge gap. Although age and light are two distinct factors that can influence circadian phase, they may interact with each other to affect the timing of the clock. Although it is well established that light influences the timing of circadian phase, light sensitivity has been demonstrated to change as a function of age (Charman 2003, Herljevic, Middleton et al. 2005, Duffy, Zeitzer et al. 2007). Furthermore, the timing of sleep, which is in part gated by the circadian system, also gates daily light exposure which can in turn influence circadian phase. Sleep timing has also been demonstrated to change across the lifespan with age, which may indirectly influence the timing of the clock via gating daily light exposure. Thus, as demonstrated in Figure 1, the way in which age and light influence circadian phase may result from a complex interaction.

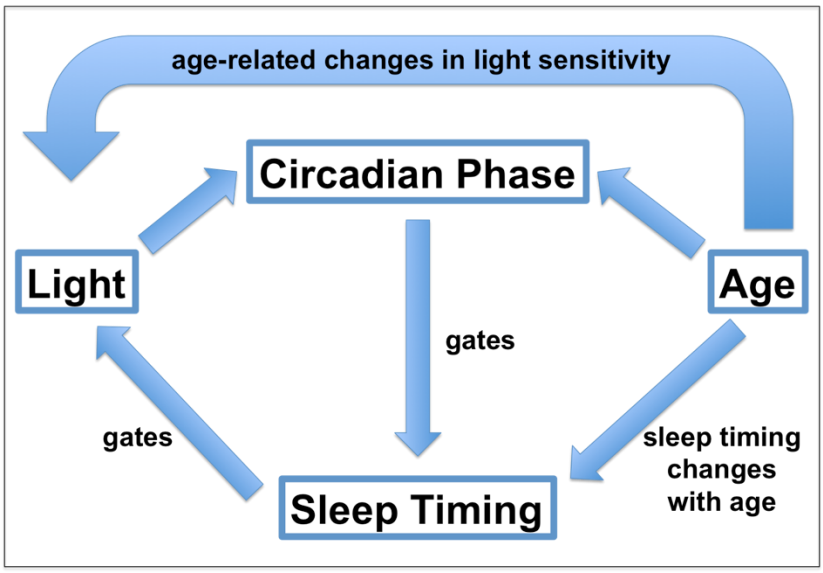

Figure 1. Model for how age and light may influence circadian phase. Age-related changes in light sensitivity may influence how light affects the clock. Additionally changes in sleep timing with age may gate light exposure at different times, which can influence the timing of circadian phase. These complex interactions must be examined in early childhood.

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CHAPTER 2

THE TIMING OF THE CIRCADIAN CLOCK AND SLEEP DIFFER BETWEEN NAPPING AND NON-NAPPING TODDLERS

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Abstract

The timing of the internal circadian clock shows large inter-individual variability across the lifespan. Although the sleep-wakefulness pattern of most toddlers includes an afternoon nap, the association between napping and circadian phase in early childhood remains unexplored. This study examined differences in circadian phase and sleep between napping and non-napping toddlers. Data were collected on 20 toddlers (34.2±2.0 months; 12 females; 15 nappers). Children followed their habitual napping and non-napping sleep schedules (monitored with actigraphy) for 5 days before an in-home salivary dim light melatonin onset (DLMO) assessment. On average, napping children fell asleep during their nap opportunities on 3.6±1.2 of the 5 days before the DLMO assessment. For these napping children, melatonin onset time was 38 min later ($p=0.044$; $d=0.93$), actigraphically-estimated bedtime was 43 min later ($p=0.014$; $d=1.24$), sleep onset time was 59 min later ($p=0.006$; $d=1.46$), and sleep onset latency was 16 min longer ($p=0.030$; $d=1.03$) than those not napping. Midsleep and wake time did not differ by napping status. No difference was observed in the bedtime, sleep onset, or midsleep phase relationships with DLMO; however, the wake time phase difference was 47 min smaller for napping toddlers ($p=0.029$; $d=1.23$). On average, nappers had 69 min shorter nighttime sleep durations ($p=0.006$; $d=1.47$) and spent 49 min less time in bed ($p=0.019$; $d=1.16$) than non-nappers. Number of days napping was correlated with melatonin onset time ($r=0.49$; $p=0.014$). Our findings indicate that napping influences individual variability in melatonin onset time in early childhood. The delayed bedtimes of napping toddlers likely permits light exposure later in the evening, thereby delaying the timing of the clock and sleep. Whether the early developmental

trajectory of circadian phase involves an advance associated with the decline in napping is a question necessitating longitudinal data as children transition from a biphasic to monophasic sleep-wakefulness pattern.

Introduction

Early childhood is a time of significant changes in the duration and timing of sleep (Beltramini and Hertzig 1983, Lozoff, Wolf et al. 1985, Bruni, Lo Reto et al. 2000, Iglowstein, Jenni et al. 2003, Acebo, Sadeh et al. 2005). Total 24 h sleep time declines in the early years of life, which is primarily due to a gradual reduction in napping frequency and duration (Iglowstein, Jenni et al. 2003). Although almost all 2-year-olds meet part of their sleep need by napping, longitudinal and cross-sectional data indicate cultural differences in the age at which children consolidate sleep into one nocturnal episode. For example, about 7% of Swiss and Icelandic children are still napping at least one day per week at the age of 5 years, which differs from reports of white (60%) and black (90%) children raised in the United States (Thorleifsdottir, Bjornsson et al. 2002, Iglowstein, Jenni et al. 2003, Crosby, LeBourgeois et al. 2005). The sleep changes observed across early childhood likely result from complex interactions of developing intrinsic bioregulatory sleep processes and extrinsic factors, including daycare and preschool schedules, parental preferences, and family demands (Weissbluth 1995, Jenni and O'Connor 2005, Jenni and LeBourgeois 2006, Ward, Gay et al. 2008).

The ubiquitous occurrence and gradual decline of napping in early childhood provides a rich developmental context for examining questions about sleep regulation. For example, does napping contribute to the large inter-individual variability in circadian timing observed in humans across the lifespan? How does napping influence interactions between the homeostatic and circadian processes governing sleep timing

and duration? As first proposed by Borbély, the homeostatic process dictates that sleep propensity builds with increasing time awake and dissipates during periods of sleep (Borbély 1982). Sleep electroencephalography (EEG) findings from adults and toddlers indicate reduced nocturnal nighttime sleep drive as a function of daytime napping (e.g., longer sleep onset latency, decreased slow wave activity in non-rapid eye movement sleep, 0.75 – 4.5 Hz) (Werth, Dijk et al. 1996, Achermann and Borbely 2003, Lassonde, Achermann et al. 2013). Additionally, evidence linking naps, nighttime sleep, and the homeostatic process in children is inferred from studies using parent-reports or actigraphy suggesting that preschoolers who nap longer during the day are more likely to sleep less the following night (Koch, Soussignan et al. 1984, Tikotzky and Sadeh 2001, Acebo, Sadeh et al. 2005). Although these nap-dependent results are in line with predictions made by the two-process model of sleep regulation, they speak only to the influence of napping on nighttime sleep homeostasis.

Little is known about associations between napping and the circadian timing system in early childhood. An established literature indicates that the timing of the circadian clock is influenced by environmental cues such as light and promotes alertness across the day (being highest in the early evening) (Borbély 1982, Czeisler 1995). Circadian phase is highly variable in humans (Gooneratne, Metlay et al. 2003, Kawinska, Dumont et al. 2005, Crowley, Acebo et al. 2006, LeBourgeois, Carskadon et al. 2013, Crowley, Van Reen et al. 2014). Even in habitually napping toddlers, we have reported a range of ~3.5 h in melatonin onset time (LeBourgeois, Carskadon et al. 2013). Later circadian timing is consistently associated with delayed bedtimes, sleep onset times, midsleep times, and wake times (Martin and Eastman 2002, Burgess, Savic et al. 2003, Burgess

and Eastman 2005, Crowley, Acebo et al. 2006, LeBourgeois, Carskadon et al. 2013, Wright, McHill et al. 2013). Based upon the circadian phase dependent response to light in adults, morning light exposure shifts the circadian clock to an earlier time, whereas evening light exposure delays the timing of the circadian pacemaker (Khalsa, Jewett et al. 2003). In the context of known nap-dependent differences in evening sleep timing, we propose that napping indirectly influences the timing of the circadian clock via later bedtimes and the "gating" of light exposure.

In this study, we obtained a reliable marker of circadian phase, dim light melatonin onset (DLMO), and actigraphic estimates of sleep in a sample of healthy napping and non-napping 30- to 36-month-olds. Because napping has been shown to dissipate the waking homeostatic sleep drive, we expected nap status differences in a number of sleep variables. Specifically, we hypothesized that napping toddlers would have later sleep timing (i.e., bedtime, sleep onset time, midsleep time), longer sleep onset latencies (interval from bedtime to sleep onset time), and shorter nighttime sleep durations than those who did not nap. Additionally, we explored group differences in morning wake time. In terms of the circadian process, we hypothesized that napping in comparison to non-napping toddlers would have later circadian phases. Our multi-day sleep actigraphy protocol also allowed us to test the hypothesis that nap frequency (i.e., number of days napping during the 5 days before DLMO), duration, and timing would be positively correlated with melatonin onset. Finally, we explored associations between napping and phase differences (i.e., interval between DLMO and 5-day average of bedtimes, sleep onset times, midsleep times, wake times).

Methods

Families in Providence, RI were recruited through flyers and laboratory website advertisements and at community events. All participants were healthy, normally developing toddlers with no sleep or behavioral problems. Exclusion criteria included the following: regular co-sleeping, variable sleep schedules of >2 hours between weekdays and weekends, travel across >2 time zones within 3 months of the study, regular use of medications influencing the sleep and circadian systems, diagnosed sleep problems, developmental disabilities, neurological/metabolic disorders, chronic medical conditions, lead poisoning, head injury involving loss of consciousness, conceptual age of ≤37 weeks or >42 weeks, low birth weight (<5.5 lb.) or first degree family history of narcolepsy, psychosis, or bipolar disorder. The Institutional Review Board at Brown University approved this study. Approval number: FWA00004460. All parents signed an informed consent form approved by the Brown University Institutional Review Board. Families were compensated with $50 cash, and children received a $50 United States Savings Bond upon completion of the study.

Participants

Participants were 20 healthy children (11 females; 18 Caucasian, 1 African-American, 1 mixed-race) ages 30- to 36-months (34.2±2.0). Six toddlers attended daycare (4 full time; 2 part-time), 5 received in-home care by an extended family member or non-family member, and 9 were cared for exclusively by their parents. We initially categorized children as napping or non-napping based upon parental report of

children's habitual sleep patterns. We then verified napping status with data from actigraphy and sleep diaries during the 5 days before the DLMO assessment. Napping children (n=15) had a biphasic sleep pattern (i.e., one daily nap opportunity and a nighttime sleep episode) and were defined as those who fell asleep during their daily nap opportunity ≥1 of the 5 days (20%) before their DLMO assessment, whereas non-napping children (n=5) were those who slept only at night. All subjects awakened spontaneously in the morning. The napping children in this analysis represent a subset of a larger sample for which DLMO, sleep timing, and phase difference data were described (LeBourgeois, Carskadon et al. 2013) and were also included in a previous publication of associations between circadian parameters and nighttime settling difficulties in toddlers (LeBourgeois, Wright et al. 2013).

Protocol

During study days 1-5, children followed their habitual sleep schedules and researchers performed several in-home training visits to ensure participants could provide adequate saliva samples for the DLMO assessment. On the sixth day, children participated in an in-home DLMO assessment performed by researchers. During the protocol, caffeine and medications affecting the sleep, circadian, and arousal systems were avoided. Researchers performed daily tracking of children's sleep patterns and study compliance through email or telephone. Children were studied only during the fall, winter, and spring (September-May), and data were not collected within 1-week following transitions to or from daylight saving time.

Measures

Sleep Diary

Parents completed a daily 26-item sleep diary throughout the study. Diary entries were used to confirm actigraphic recordings.

Actigraphically-Estimated Sleep Variables

Participants wore an actigraph (model AW64; MiniMitter Company, Bend, OR, USA) on their non-dominant wrist throughout the study to provide continuous recordings of estimated sleep-wakefulness states from motor activity. Parents/caregivers pressed an event marker at the time they expected their child to try to fall asleep (lights-out) and the time their child awakened from sleep for nap and nighttime episodes. Actigraphs were downloaded on Day 6, and the actograms were inspected by researchers and reviewed with parents to verify that times from event markers were consistent with sleep diaries and/or daily call-in/email reports. This approach shows high concordance ($r=0.88$, $p<0.001$) between sleep onset latency as measured by polysomnorgraphy and by the actigraphy in healthy preschool children (Craven 2012). Standard laboratory methods for scoring actigraphy data have been previously published (Acebo, Sadeh et al. 2005, Berger, Miller et al. 2012, LeBourgeois, Carskadon et al. 2013, Simpkin, Jenni et al. 2014). Average estimates from actigraphy were computed within individual subjects across study days 1-5 for the following variables: bedtime (lights-off time), sleep onset time, wake time (sleep end time), sleep duration (interval between sleep onset and sleep end), and sleep onset latency (interval between bedtime and sleep

onset time). These mean values were used to compute the respective phase differences relative to DLMO time. Due to non-compliance, actigraphy data were not available for one child (a non-napper); in this case, we used daily diary data to compute napping, nighttime sleep, and phase difference variables.

Salivary Dim Light Melatonin Onset (DLMO)

Details regarding the saliva collection protocol have been previously published (LeBourgeois, Carskadon et al. 2013). Briefly, children provided saliva samples (~2 mL) in dim-light (<10 lux) every 30 min for 6 h up to an hour past their average bedtime (12 samples total). Saliva was collected by having children chew on a braided dental cotton roll (Henry Schein Inc., Denver, Pennsylvania, USA) for 1-2 min. Light levels were measured and recorded for each sample using a lux meter held approximately 5 cm adjacent to the child's eye and directed in the angle of gaze (Extech Instruments, Spring Hill, Florida, USA). Samples were immediately centrifuged (LabEssentials, Inc. Monroe, Georgia, USA), refrigerated on-site, and then frozen at the laboratory (-20˚C) within 12 h. Assays were performed at the Bradley Hospital Sleep and Chronobiology Laboratory (Providence, RI, USA) using radioimmunoassay (ALPCO Diagnostics, Salem, New Hampshire, USA), minimum detection limit 0.2 pg/mL.

Data Processing and Analysis

We used previously published methods to establish circadian phase and to compute phase differences (Crowley, Acebo et al. 2006, LeBourgeois, Carskadon et al. 2013). DLMO was calculated as the first interpolated time point above 4 pg/mL, with at

least one subsequent sample remaining above this threshold. Phase differences were computed as the time interval between DLMO and average 5-day actigraphic estimates of bedtime, sleep onset time, midsleep time, and wake time.

Statistical analyses were performed with SPSS Statistics 21.0 (IBM Corp. Armonk, NY, USA). Summary measures are presented as M ± SD. Pearson and Spearman correlations were used to assess associations between continuous and ordinal variables, respectively. Independent t-tests were performed to investigate differences in circadian and sleep variables between napping and non-napping children. Effect size in SD units was computed for sleep and circadian M comparisons [$d=(M_{sample1}- M_{sample2})/SD_{pooled}$]. An effect size of 0.20 is considered small, 0.50 is considered medium, and ≥0.80 is considered large (Cohen 1988).

Results

Descriptive data and statistical comparisons for circadian and actigraphic sleep variables are presented in Table 1. Napping children fell asleep during their daily nap opportunities on 3.6±0.8 of the 5 days before the DLMO assessment. On average, the DLMO time of nappers was 38 min later than non-nappers (p=0.044, d=0.93). Napping toddlers also spent 48 min less time in bed (p=0.019, d=1.16) and slept an average of 69 min less at night (p=0.006, d=1.47); however, total 24 h sleep duration did not differ between groups (p=0.156). Nappers also had 43 min later bedtimes (p=0.001, d=1.24) and fell asleep an average of 59 min later (sleep onset time; p=0.006, d=1.46) than non-nappers. On average, napping children took 16 min longer to fall asleep after bedtime

(sleep onset latency) than those not napping (p=0.030, d=1.03). Morning wake times did not differ by napping status (p=0.575). Together, this sleep pattern resulted in a non-significant trend toward earlier sleep timing in non-napping than napping toddlers, as reflected in the midpoint of sleep (p=0.058). Although the bedtime, sleep onset, and midsleep phase differences did not differ based on napping status, the average wake time phase difference was narrower in napping than non-napping toddlers (p=0.029, d=1.23). The number of days children napped in the 5 days before the DLMO assessment was correlated with melatonin onset time, such that children who napped more often had later circadian phases (r=0.49; p=0.014; see Fig. 1). Contrary to our hypotheses, neither nap duration nor nap midpoint time in napping subjects was associated with melatonin onset time (r=0.007, p=0.490; r=-0.36, p=0.091, respectively).

	Napping	Non-Napping	Statistics		
	(n = 15)	(n = 5)	*t*	*d*	*p*
Circadian Variables					
Dim Light Melatonin Onset Time	19:48 ± 0:42	19:10 ± 0:33	1.81	0.93	0.044
Bedtime Phase Difference (min)§	30.4 ± 33.9	25.0 ± 41.1	-0.29	0.15	0.773
Sleep Onset Phase Difference (min) §	63.9 ± 32.5	42.0 ± 42.0	-1.22	0.63	0.240
Midsleep Phase Difference (min) §	367.0 ± 28.6	379.6 ± 25.3	-0.87	0.45	0.394
Wake Time Phase Difference (min) §	670.1 ± 39.9	717.1 ± 33.1	-2.37	1.23	0.029
Sleep Variables					
Bedtime	20:18 ± 0:36	19:35 ± 0:30	2.42	1.24	0.014
Sleep Onset Time	20:51 ± 0:43	19:52 ± 0:30	2.84	1.46	0.006
Midsleep Time	1:54 ± 0:30	1:30 ± 0:23	1.65	0.85	0.058
Wake Time§	6:58 ± 0:30	7:07 ± 0:41	-0.57	0.30	0.575
Sleep Onset Latency (min)	33.0 ± 16.6	17.1 ± 10.2	-2.01	1.03	0.030
Nighttime Time in Bed (min)	656.9 ± 38.9	705.6 ± 51.9	-2.24	1.16	0.019
Nighttime Sleep Duration (min)	606.3 ± 43.9	675.0 ± 56.2	-2.84	1.47	0.006
24-h Sleep Duration (min) §	708.9 ± 40.2	675.0 ± 56.2	1.48	0.76	0.156
Days Napping (of 5)	3.6 ± 1.2	-	-	-	-
Nap Midpoint Time	14:43 ± 0:46	-	-	-	-
Nap Duration (min)	102.6 ± 20.1	-	-	-	-

Statistics are shown for independent t-tests (napping versus non-napping participants).
§ indicates a two-tailed test.

Table 1. Descriptive statistics (Mean ± SD) for circadian and actigraphic sleep measures for napping (n=15) and non-napping (n=5) toddlers.

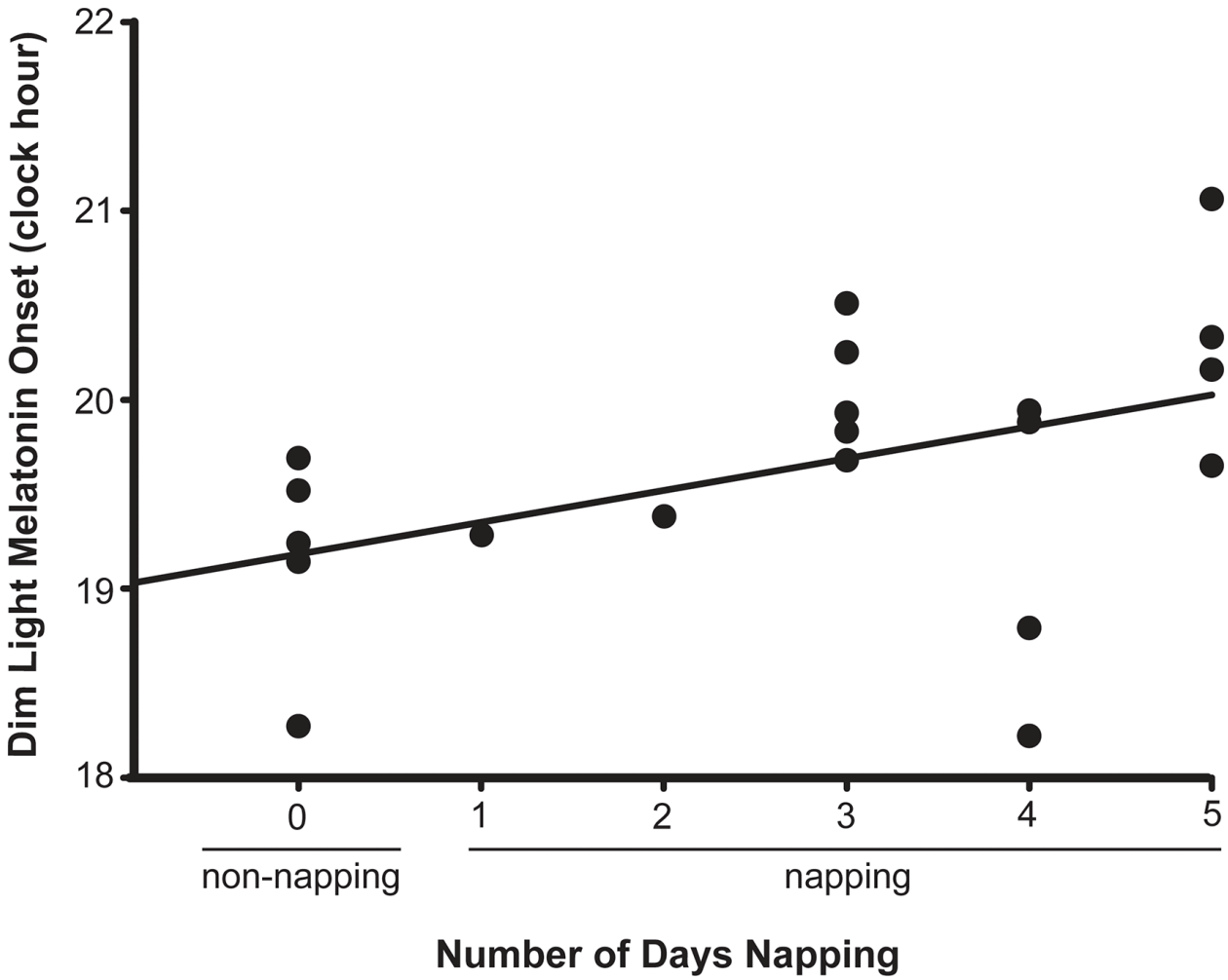

Figure 1. Scatterplot showing the association between napping and circadian phase in toddlers (n = 20). Number of days that toddlers napped during the 5-days preceding the dim light melatonin onset (DLMO) assessment was estimated with actigraphy (r = 0.49, p = 0.014).

Conclusions

We examined differences in sleep and circadian parameters between healthy, normally developing napping and non-napping toddlers using actigraphy as a measure of sleep and DLMO time as a marker of circadian phase. As hypothesized, we found that napping toddlers had later bedtimes, sleep onset times, and circadian timing than non-napping toddlers. Although we observed a non-significant trend towards a later midsleep time in nappers ($p=0.058$) than non-nappers, we did not find a nap-related difference in morning wake times. Nappers also took longer to fall asleep and slept less at night as compared to their non-napping peers; however, total 24-h sleep time was similar. Although we found no nap status differences in the bedtime, sleep onset, and midsleep time relative to DLMO, nappers did show a more narrow range of wake times relative to DLMO than non-nappers. Moreover, toddlers who napped more often had later circadian phases; however, nap duration and nap timing were not associated with melatonin onset time.

In this study, evening actigraphically-estimated sleep timing was delayed in nappers compared to non-nappers, which is consistent with published observational data (Sakashita and Fukuda 1995, Komada, Asaoka et al. 2012). In one study, bedtimes were reportedly later in napping than non-napping preschoolers (Komada, Asaoka et al. 2012), and in another, nursery school children who were required to take an afternoon nap had bedtimes that were 30 minutes later than those of the same age who did not nap (Sakashita and Fukuda 1995). These findings indicate that as children stop napping, caregivers may adjust bedtimes based upon their children's total sleep

need, readiness for sleep, and/or signs of evening sleepiness. Additionally, data from previous reports indicate that daytime naps reduce subsequent night homeostatic sleep pressure, which provides a underlying mechanism for our results of later sleep onset times, longer sleep onset latencies, and shorter nighttime sleep durations in napping than non-napping toddlers (Werth, Dijk et al. 1996, Achermann and Borbely 2003, Lassonde, Achermann et al. 2013). Our finding of no significant nap-related difference in 24 h sleep time (~30 min; p=0.156; see Table 1) may suggest stability in overall sleep need as children transition from a biphasic to a monophasic sleep pattern; however, this interpretation must be taken with caution due to our small sample size.

Our findings of circadian phase differences between napping and non-napping children demonstrate that napping habits may contribute to the observed large inter-individual variability in the timing of the clock in early childhood (LeBourgeois, Carskadon et al. 2013). Consistent with our hypothesis, we found that napping in comparison to non-napping toddlers had later melatonin onset times, which may in part be mediated by differences in bedtimes and sleep onset times. Delayed sleep times not only facilitate exposure to more indoor electrical lighting, but may also create a later window of time to use media and electronic devices. This is especially significant given the media-saturated context in which children are developing and recent data showing an increase in mobile electronic media use among young children (Common Sense Media Website 2013). Furthermore, recent experimental data indicate that the timing, duration, and intensity of light produced by electronics are important considerations in the context of understanding the timing of the circadian clock (Cajochen, Frey et al. 2011, Chang, Aeschbach et al. 2012, Wood, Rea et al. 2013, Van der Lely, Frey et al.

2014) and evening arousal levels (Cajochen 2007, Cajochen, Frey et al. 2011). Although a detailed description of the phase shifting effects of light on circadian rhythms is not yet available for children, we would expect evening light exposure to delay the timing of the biological clock based upon established adult data (Khalsa, Jewett et al. 2003). It is important to note that in this study, the morning wake times of napping and non-napping toddlers were similar. Burgess and colleagues found that with a set wake time, individuals with late bedtimes had later DLMOs compared to individuals with early bedtimes, suggesting that a later bedtime facilitates a phase delay of the clock (Burgess and Eastman 2004). Indeed, in our recent work on napping toddlers, we showed that sleep timing and DLMO were positively correlated, such that later bedtimes, sleep onset times, midsleep times, and wake times were associated with a later timing of evening melatonin onset (LeBourgeois, Carskadon et al. 2013).

Although the adolescent circadian phase delay is well established, earlier developmental changes in the timing of the clock are unknown (Carskadon, Acebo et al. 2004, Crowley, Acebo et al. 2007). In our previously published work, we found an average DLMO time of 19:29 in a sample of 45 napping toddlers, whereas a DLMO of 20:42 was reported in 9 year-old children who were assessed during the academic year (LeBourgeois, Carskadon et al. 2013, Crowley, Van Reen et al. 2014). This age-related phase delay continues later into the second decade of life, with mature adolescents exhibiting an average DLMO of 21:53 (Crowley, Van Reen et al. 2014). Although the current literature points to a delay in circadian phase between early and late childhood, findings from this study suggest that circadian phase may first advance as children begin to drop their naps. We not only found that non-nappers had ~35 min earlier

melatonin onset times than nappers, but also that napping less frequently was associated with earlier DLMOs. In sum, these results suggest a phase advance in the timing of the circadian clock may occur as children give up their naps; however, longitudinal data of circadian phase during the transition from a biphasic to monophasic sleep pattern are needed to address this important ontogenetic research question.

The cessation of napping typically unfolds gradually during early childhood. Observational findings indicate that naps become less frequent (days per week), shorter, and occur at later times of the day with increasing age (Dales 1941, Weissbluth 1995, Iglowstein, Jenni et al. 2003, Pierpoint, Achermann et al. 2014). As hypothesized, we found that napping frequency was positively correlated with melatonin onset time. Napping more often across a week may have cumulative effects on melatonin onset via associated later bedtimes, which gate evening light exposure. Because experimental data in adults indicate that morning naps delay circadian phase, evening naps advance the clock, and afternoon naps have no influence on melatonin onset time (Buxton, L'Hermite-Baleriaux et al. 2000), we hypothesized that nap timing would be associated with circadian phase. We also expected children taking longer naps to have later melatonin onset times due to greater dissipation of sleep propensity during the daytime nap episode (Werth, Dijk et al. 1996, Achermann and Borbely 2003, Lassonde, Achermann et al. 2013). Neither relationship was supported by our findings, which may have been due to little variability in nap timing and nap duration. That is, in our sample of 30- to 36-month-olds, naps occurred in the afternoon, almost 12 hours after nocturnal midpoint of sleep, a time at which we would expect the clock to be less sensitive to phase shifts. Similarly, nap duration was on average about 100 minutes,

with a narrow range (~83 minutes). Assessing the influence of multiple nap dimensions on the circadian clock will necessitate longitudinal data that provide more sensitive trajectories of shifts in napping patterns across early childhood.

Phase differences provide an approach to quantifying associations of the sleep and circadian systems. In this study, we did not observe nap status differences in the bedtime, sleep onset, or midsleep phase differences. Napping children, however, had longer sleep onset latencies than those who had given up their naps. Together, this pattern of findings highlights the interaction between the homeostatic and circadian processes in determining sleep propensity and sleep timing. In regularly napping toddlers, more narrow bedtime phase differences are associated with longer sleep onset latencies (LeBourgeois, Wright et al. 2013), indicating the importance of the circadian timing of sleep when evening levels of homeostatic sleep pressure are more-or-less similar. Our current analysis comparing napping and non-napping children showed that children were being put to bed at the same circadian time, regardless of their napping status. Thus, although the interval between melatonin onset and bedtime is important in determining sleep onset latency in napping children, it may make a smaller contribution once children give up their naps. At this developmental transition, the build up of homeostatic sleep pressure may play a stronger role in promoting levels of evening sleepiness than the circadian timing of sleep. Additionally, we found napping status differences in the wake time phase difference, which can be explained by the earlier DLMO times observed and longer nocturnal sleep in the non-nappers and the similar wake times between napping and non-napping children. Although our methods did not permit measurement of the length of the biological night, our data suggest that

napping children may wake up at an earlier circadian time compared to their non-napping peers. Whether this nap-related pattern influences variability in sleep inertia, alertness, cognitive performance, or mood in the morning hours is an important future research direction with social and academic implications.

Shifting from a napping to a non-napping sleep schedule is not always a smooth developmental transition. Many toddlers and preschoolers naturally drop their naps; however, about one-third are forced to stop napping due to parental preferences and preschool or daycare schedules (Weissbluth 1995). The compulsory nighttime consolidation of sleep may have advantages and disadvantages for parents and children. For example, recent data indicate that the transition to full-day kindergarten influences the sleep patterns of children, including a sharp decline in weekday napping, an advance in nocturnal sleep timing, and parent-reports of fewer difficulties going to bed and falling asleep (Cairns and Harsh 2013). Moreover, our finding of longer sleep onset latencies in napping children suggests that removing daytime naps may be beneficial to children who exhibit evening settling difficulties (e.g., prolonged sleep onset, bedtime resistance), which are reportedly reasons for compulsory consolidation by ~30% of U.S. parents (Weissbluth 1995). On the other hand, certain children who are required to stop napping may not be able to adapt to associated increases in homeostatic load and may suffer from daytime sleepiness, poor daytime functioning, or parasomnias such as night terrors (Dahl 1996, Mason and Pack 2005, Hartman and LeBourgeois 2011, Berger, Miller et al. 2012, Miller, Seifer et al. 2014). For instance, one study reported a nap-dependent improvement on recall tests in habitual but not non-habitual napping preschoolers (Kurdziel, Duclos et al. 2013). Furthermore, our

previously published results indicate that taking away one nap of ~90 minutes impairs toddlers' emotion processing and self-regulation strategies (Berger, Miller et al. 2012, Miller, Seifer et al. 2014). Based upon these nap-dependent functional data, individual differences in the biologically optimal time of nighttime sleep consolidation likely exist. The development of different strategies for phasing out naps that maintain their cognitive and emotional benefits as well as reduce associated evening settling difficulties represents an important area of future research.

We interpret our findings of napping-associated differences in evening sleep timing, sleep onset latency, and circadian phase in the social context in which children develop. For example, findings from a number of studies indicate that the age at which the majority of children stop napping varies across cultures. In Iceland, the proportion of children who nap decreases early in life, with ~90% napping at age 2 years and only ~22% napping at age 3 years (Thorleifsdottir, Bjornsson et al. 2002). In Japan and Switzerland, the proportion of children who nap is ~85% at 2 years and ~50% by the age 3 years (Iglowstein, Jenni et al. 2003, Komada, Asaoka et al. 2012). In contrast, napping cessation occurs on average at a later age in children raised in the United States and varies across ethnicities. In one Chicago-based longitudinal study, ~70% of 3-year-olds and ~30% of 4-year-olds were reported by their parents to nap (Weissbluth 1995). Furthermore, another study of children living in Southern Mississippi found that the majority of white children were no longer napping between ages 5 and 6 years, while the transition to exclusive nighttime sleep in black children occurred about one year later (Crosby, LeBourgeois et al. 2005). Institutional demands such as those put in place by early childcare settings can also influence the napping habits of young children

(Jenni and O'Connor 2005). Daycares and preschools may or may not enforce a “quiet time” or nap period, and the duration of nap opportunities may be developmentally inappropriate or occur at a time too late in the day with respect to the bedtimes parents select for their children. A recent emphasis on early academic performance and school readiness has also resulted in a number of early childhood centers replacing naptime with learning activities (Daniel and Lewin 2005, Jenni and LeBourgeois 2006). As more than 70% of American children between the ages 2 and 5 years attend center-based care, institutional napping policies impact a significant proportion of U.S. children (U.S. Department of Health and Human Services Website 2008). Thus, the context in which children develop (e.g., culture, daycare, preschool, home care) plays an important social role in the development of sleep patterns, the emergence of nighttime settling difficulties, and observed inter-individual variability in the timing of evening sleep patterns and melatonin onset times.

Although this study represents an important step in understanding sleep behavior and circadian physiology in early childhood, it has several limitations. Our sample size was small with 15 napping children and 5 non-napping children. In general, this ratio reflects the proportion of napping and non-napping toddlers; however, a larger sample size would strengthen our conclusions. Furthermore, we enrolled only healthy, good-sleeping toddlers. Considering the prevalence of behavioral sleep problems in childhood, our findings may not be reflective of the general population. Finally, our analytic approach permitted comparisons solely between nappers and non-nappers, which limits the interpretation of more time-sensitive, developmental trajectories of sleep

patterns and the timing of the circadian system as a function of multiple nap dimensions (i.e., duration, timing, frequency).

In summary, our results suggest that napping in toddlerhood is not only associated with later evening sleep timing, shorter nighttime sleep duration, and longer sleep onset latencies, but also later timing of the internal circadian clock. Given the increase in media exposure in childhood, as well as the importance of light in determining melatonin phase, future experimental and naturalistic studies examining the influence of light exposure on the circadian system of toddlers and preschoolers are warranted. Time-sensitive, longitudinal research assessing concurrent changes in the sleep and circadian systems and daytime functioning during the transition from a biphasic to monophasic sleeping pattern is also needed to understand the fundamental question of the optimal timing of nighttime sleep consolidation in early childhood.

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CHAPTER 3

LIGHT-INDUCED MELATONIN SUPPRESSION IN PRESCHOOL-AGE CHILDREN

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Key terms: light, melatonin suppression, circadian, children, preschool

Abstract

Although the light-induced melatonin suppression response is well characterized in adults, studies examining the dynamics of this effect in early childhood are scarce. The purpose of this study was to quantify the magnitude of evening light-induced melatonin suppression in preschool-age children. Ten healthy children (7 females; 4.3±1.1 years) participated in a 7-day protocol. On days 1-5, children followed a strict sleep schedule. On day 6, children entered a dim-light environment (<15 lux) for 1-hour before providing salivary samples every 20- to 30-minutes from the afternoon until 50-minutes after scheduled bedtime. On day 7, subjects remained in dim-light conditions until 1-hour before bedtime, at which time they were exposed to a bright-light stimulus (~1,000 lux) for 1-hour and then re-entered dim-light conditions. Saliva samples were obtained before, during, and after bright-light exposure and were time-anchored to samples taken the previous evening. We found robust melatonin suppression (87.6±10.0%) in response to the bright-light stimulus. Melatonin levels remained attenuated for 50-minutes after termination of the light stimulus ($p<0.008$). Furthermore, fifty minutes after light exposure, melatonin levels did not return to 50% of those observed in the dim-light condition. Our findings demonstrate a robust light-induced melatonin suppression response in young children. These findings have implications for understanding the role of evening light exposure in the development of evening settling difficulties and may serve as experimental evidence to support recommendations regarding light exposure and sleep hygiene practices in early childhood.

Introduction

The human circadian clock, localized to the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, is responsible for driving rhythms in behavior and physiology, including the sleep-wakefulness cycle and the rhythm of the pineal hormone melatonin (Ralph, Foster et al. 1990, Kalsbeek, Palm et al. 2006). The circadian clock receives direct environmental light input from retinal photoreceptors via the retinohypothalamic tract (Moore and Card 1985, Johnson, Moore et al. 1988). Light exposure in turn influences circadian timing and acutely suppresses melatonin secretion (Lewy, Wehr et al. 1980). The human circadian system is sensitive to light: as little as 12 lux produces significant changes in the timing of the clock (Duffy and Wright 2005) and as little as ~50-130 lux induces melatonin suppression (Zeitzer, Dijk et al. 2000).

Age-related changes in ophthalmological features may modulate the clock's sensitivity to light. For example, cross sectional data show that young children have lenses that are more transparent and pupils that are larger than adults (Weale 1985, Yang, Thompson et al. 2002, Charman 2003). Also, one study comparing pupil size between school-age children and their parents in moderate bright light found that children had larger pupil diameters than their parents (Higuchi, Nagafuchi et al. 2014). Together, higher lens transparency and greater pupil size facilitate increased retinal illumination, which likely results in a stronger signal to the SCN in younger children than adults (Charman 2003, Turner and Mainster 2008).

Understanding of circadian sensitivity in school-age children, adolescents, and adults is rapidly increasing; however, a large gap in the literature exists in the early

childhood years. To our knowledge, school-age children are the youngest age at which the melatonin suppression effects of light have been investigated (Higuchi, Nagafuchi et al. 2014). Findings from this study indicated that school-age children were almost twice as sensitive to the melatonin suppression effects of evening moderate bright light exposure (~580 lux) (Higuchi, Nagafuchi et al. 2014). Additionally, based upon an objective measure of pubertal development (Tanner 1962), Crowley and colleagues found that that pre-pubertal adolescents (Tanner stage 1-3) are more sensitive to the suppressive effects of evening light on melatonin than post-pubertal adolescents (Tanner stage 4-5) (Crowley, Cain et al. 2015). Another study comparing melatonin suppression in response to short-wavelength light found that younger adults experienced greater melatonin suppression than older adults (Herljevic, Middleton et al. 2005). Together, the current literature suggests a trend for greater sensitivity to the melatonin suppression effects of light early in life and a decrease in sensitivity with age. Here, we extend the work of others by quantifying the degree to which melatonin is suppressed in response to bright light before bedtime in a sample of healthy preschool-age children.

Materials and Methods

Participants

This study included 10 healthy children (7 females; 9 Caucasian, 1 mixed race) aged 4.3±1.1 years (*M*±*SD*). Families were recruited from the Boulder, CO area through posted flyers, a laboratory database of former study participants, and a database of

local residents who expressed interested in having their children participate in research studies through the University of Colorado Boulder.

Parents of 14 children completed a telephone screening interview and online surveys to assess inclusion/exclusion criteria. Of these, 12 children were enrolled and 11 completed the study. One child did not exhibit melatonin suppression during the light exposure and was excluded. Thus, 10 children are included in the present analysis. Exclusion criteria included parental report of any of the following: behavioral/emotional problem, chronic illness, metabolic disorder, serious infectious illness, neurological disorder, or developmental disorder; a reported habitual sleep schedule differing by >2 hours between weekdays and weekends; travel >2 times zones within 2 months of the study; medication use influencing the sleep or circadian systems; family history of a sleep or psychiatric disorder; light sensitizing medication use in the 1 year before the study (reviewed by a physician); or diagnosed visual impairment (e.g. color blindness, impaired pupillary reaction to light). Parents provided written informed consent, as approved by the Institutional Review Board at the University of Colorado Boulder. Families were compensated $150 for completing the study.

Protocol

Children participated in a 7-day protocol (**Figure 1**) in July-September 2015.

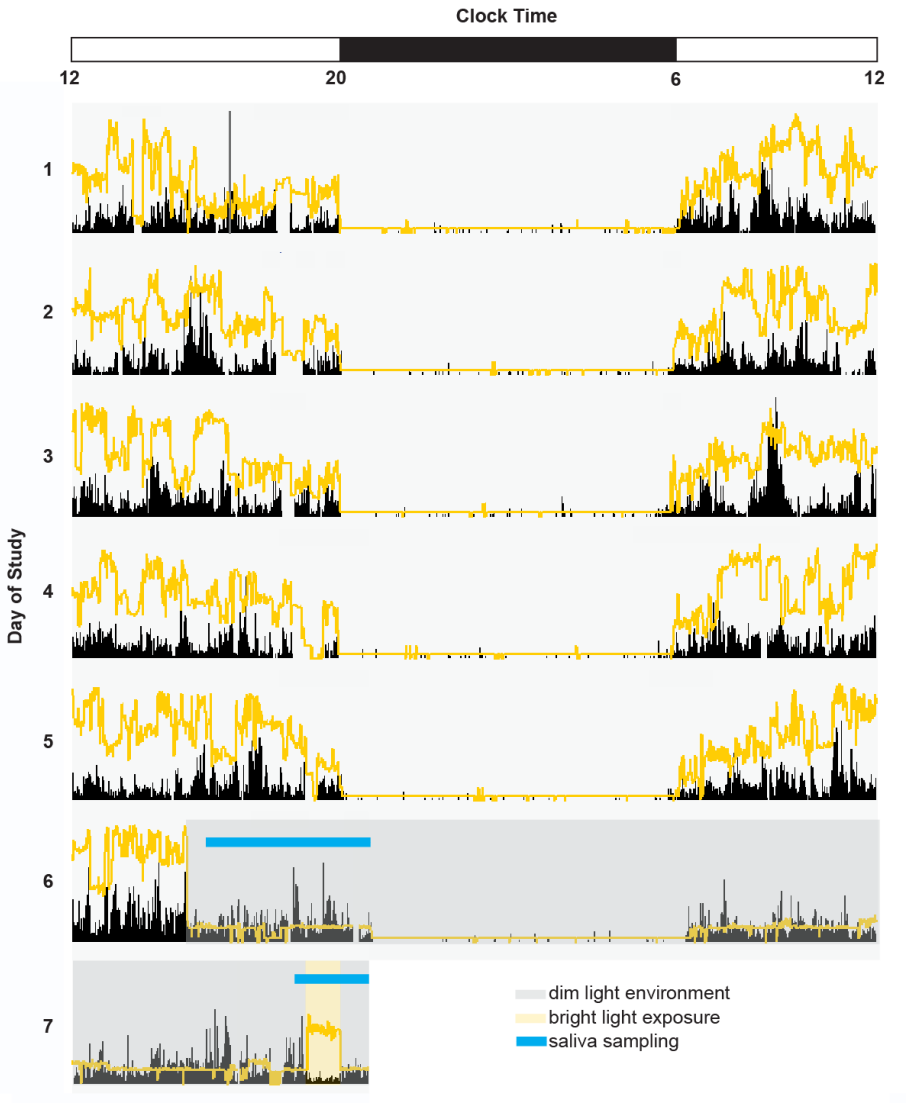

Figure 1. Experimental Protocol for One Participant
Actogram for a 4-year-old participant wearing an Actiwatch Spectrum. Black tick marks represent activity and yellow line represents light exposure in lux (scale .1 lux – 200,000 lux). Clock hour is indicated on the x-axis and day of study on the y-axis. The dark bar on the x axis represents this individual subject's sleep interval (time in bed; 20:00-6:00). On days 1-5, children followed a strict sleep/wake schedule. On day 6, children entered dim-light conditions (<15 lux; denoted by gray shading) 1 hour before the start of saliva sampling, where they remained until bright light exposure (~1,000 lux; denoted by yellow shading) on day 7. Subjects returned to dim-light conditions for 50 minutes following the bright light stimulus. Times of saliva sampling are denoted by the blue line on days 6 and 7.

On days 1-5, subjects followed a strict sleep/wakefulness schedule and refrained from caffeine and medications in the 96 hours before data collection. Researchers contacted parents daily to confirm sleep schedule compliance. Researchers also made several in-home visits during this interval to train subjects on providing saliva samples. Subjects wore an actigraph (AW Spectrum, Phillips/Respironics, Pittsburg, PA, USA) throughout the study to obtain an objective measure of sleep timing. Parents reported their child's sleep timing with a daily diary (Akacem, Simpkin et al. 2015), which was used to facilitate actigraphy data scoring as detailed in our previous publication (LeBourgeois, Carskadon et al. 2013). For one child, we replaced actigraphic measures with sleep diary report data due to non-compliance with wearing the actigraph.

On the afternoon of day 6, children participated in the dim-light condition. An in-home dim-light environment of <15 lux was created by covering windows with black plastic and using low wattage bulbs and dimmer switches to manipulate existing light sources. Subjects entered dim-light conditions 1-hour before the first saliva sample. They remained seated for 5-minutes and did not eat or drink for 15-minutes before each saliva sample. After eating, subjects chewed on a dry dental cotton roll and/or rinsed with water to remove any food debris from their mouth at least 15-minutes before the next saliva sample. Saliva samples were collected by having subjects chew on a dry dental cotton roll (Henry Schein Inc., Denver, PA, USA) for ~2-minutes to produce at least ≥2mL of saliva. Light readings were taken in children's angle of gaze at each saliva sample using a lux meter held approximately 5 cm from their face (Extech Instruments, Spring Hill, FL, USA). Samples were centrifuged (LabEssentials Inc.,

Monroe, GA, USA), refrigerated on site, and then frozen within 1-hour of the assessment (-80° C).

The first saliva sample occurred 3 hours and 20 minutes before scheduled bedtime. The first 5 samples on this evening were taken 30-minutes apart. During the hour before scheduled bedtime, samples were taken every 20-minutes (samples 6, 7, and 8). Two more samples were taken after scheduled bedtime (30-minutes apart; samples 9 and 10). After the last saliva sample on this evening, subjects were put to bed by their parents. Dim-light conditions were maintained the next day.

On day 7, researchers arrived at the subject's home just before scheduled wake time and stayed with the subject for the day to ensure that dim-light conditions were maintained within the home. Twenty minutes before the start of the light exposure children provided one saliva sample in dim-light (occurred at the same time as sample 5 the previous night). During the hour before habitual bedtime, subjects were exposed to bright light for 1 hour via a 'light table' made from a plastic storage bin (Sterilite, Townsend, MA, USA). A light box was placed inside the bin (Phillips Original Bright Light HF3301, Andover, MA, USA; Phillips 55-Watt Natural 5000K Energy Saver Compact Fluorescent Light Bulb, Andover, MA, USA) and was covered with a neutral density filter with 51.2% transmission (LEE Filters 209 .3ND, Burbank, CA, USA) to attenuate the intensity of the light box and provide ~1,000 lux of light at the subject's angle of gaze. The top of the light table was made from a clear sheet of polycarbonate (Sabic Innovative Plastics, Pittsfield, MA, USA). Light measurements were taken at the subject's angle of gaze every 10 minutes throughout the light exposure. Children were exposed to an average of 1,033±158 lux during the experimental light stimulus.

Researchers played with subjects at the light table to ensure their angle of gaze was downwards toward the light source. Activities at the light table included coloring on clear overhead sheets and playing with open magnetic tiles to maximize the subject's light exposure. Saliva samples were obtained 10-, 30-, and 50-minutes after the start of the light exposure (time anchored to samples 6, 7, and 8 taken on day 6). Following the light exposure, subjects re-entered dim-light conditions (<15 lux) and provided 2 more saliva samples occurring 20- and 50-minutes following the light exposure (time anchored to samples 9 and 10 from day 6).

The intensity of the chosen light stimulus was based upon the adult illuminance response curve which dictates ~1,000 lux induces a near saturating melatonin suppression response (Zeitzer, Dijk et al. 2000). This intensity of light is also relatively similar to the average amount of light preschool-age children living in Colorado are exposed to in the 2-hours before bedtime (710.1±1418.2 lux) (Akacem, Wright et al. 2016. Under Review).

Data Analysis and Statistics

Percent melatonin suppression was computed using area under the curve (AUC; trapezoidal method; MATLAB, MathWorks, Natick, MA, USA). Specifically, area under the curve was calculated for the duration of the bright light stimulus (AUC_{BL}) and compared to AUC at the same clock times in the dim-light condition on day 6 (AUC_{DL}). Melatonin suppression was computed using the following equation: $[1\text{-}AUC_{BL}/AUC_{DL}] \times 100$ (Gooley, Chamberlain et al. 2011). Melatonin levels were compared between conditions using a paired samples t-test with a Bonferroni correction ($p<0.008$; SPSS

Statistics, IBM Corp., Armonk, NY, USA). All paired t-tests were one-tailed with the exception of the comparison of melatonin levels before the start of the light exposure which was a two-tailed. Dim-light melatonin onset (DLMO) was computed as the clock time salivary melatonin levels passed 4 pg/mL in the dim-light condition. One subject's melatonin levels did not pass 4 pg/mL, and thus, we were unable to determine the timing of DLMO for this subject. Hence, average DLMO timing presented here reflects a sample size of 9 participants.

Results

Average bedtime during the 5 days before the start of the experimental protocol was 20:27±00:17. The timing of the dim-light melatonin onset (DLMO), a marker of the beginning of the biological night, was 19:47±00:34 during the dim-light condition (n=9; see Data Analysis and Statistics). The time interval between the start of the bright light stimulus and DLMO ranged from ~70 minutes before to ~12 minutes after the DLMO (20.1±29.1 minutes before DLMO). Melatonin levels 20 minutes before the start of the light exposure were not significantly different between conditions (p=0.65). Average melatonin suppression across the light exposure was 87.6±10.0%. Bright light suppressed melatonin levels at 10-minutes (p=0.004, d=0.70), 30 minutes (p=0.003, d=1.56), and 50-minutes after the start of the light exposure (p=0.001, d=1.86). Melatonin levels remained significantly lower at 20-minutes (p<0.001, d=2.07) and 50-minutes following the end of the bright light exposure condition (p<0.001, d=1.43) compared to the dim-light condition (**Figure 2**).

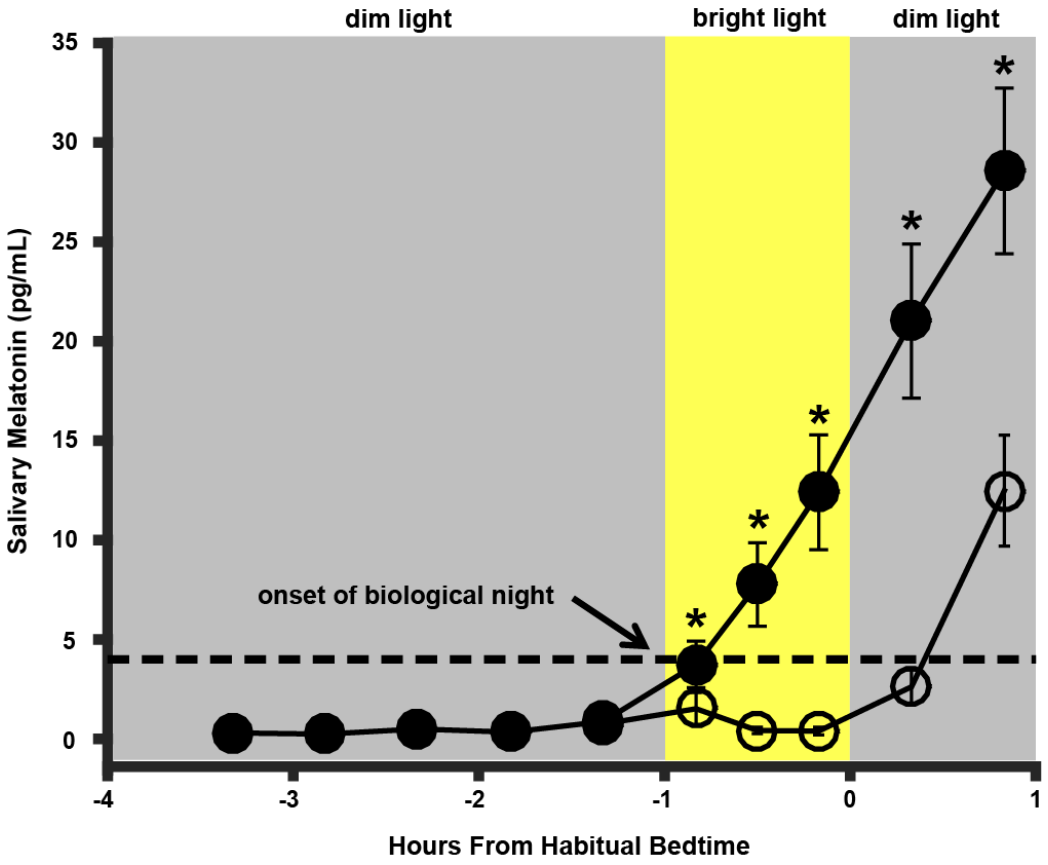

Figure 2. Melatonin suppression
Average melatonin profile in the dim-light exposure night and bright-light exposure night. Error bars represent standard error. Mean melatonin suppression was 87.6%±10.0% (*M*±*SD*). Melatonin levels in samples taken 1-h 20-minutes before the start of bright light exposure were not significantly different between conditions (p=0.65). Melatonin levels were significantly lower in samples taken at 10-, 30- and 50-minutes after the start of bright light exposure in the bright light versus the dim-light condition (* p<0.008; Bonferroni correction). Compared to the dim-light condition, melatonin levels in the bright light condition remained lower 20- and 50-minutes after children returned to dim-light.

Furthermore, at 50-minutes after light exposure, melatonin levels did not return to 50% of those observed in the dim-light condition for 7/10 children.

Discussion

Our findings demonstrate that preschool-age children are highly sensitive to the melatonin suppressing effects of evening light exposure. We observed ~90% melatonin suppression in response to 1 hour of bright light exposure before bedtime in a sample of healthy preschool children. Additionally, the effects of evening bright light exposure persisted at least 50 minutes following termination of the light stimulus such that melatonin levels remained suppressed and did not return to 50% of those observed in the dim-light condition for 7/10 children.

Given the circadian clock and melatonin's role in the regulation and promotion of sleep, our findings may have important implications for understanding the etiology of evening sleep problems in early childhood. Approximately 30% of young children experience difficulties transitioning from wakefulness to sleep, including bedtime resistance and sleep onset delay (Beltramini and Hertzig 1983, Lozoff, Wolf et al. 1985, Bruni, Lo Reto et al. 2000). It is well known that melatonin is responsible for preparing the body for sleep (Cajochen, Krauchi et al. 2003). Thus, the robust and sustained suppression (at least 50-minutes) we observed in response to evening bright light exposure before bedtime likely impairs young children's success in falling asleep. Our findings may be especially important in the context of an abrupt transition from relatively bright indoor light before bedtime to dim/dark conditions at lights-off that may be part of

children's bedtime routine. Follow-up studies examining both the relationship between light-induced melatonin suppression and nighttime settling as well as the dynamics and duration of the melatonin recovery process following light exposure in young children are necessary.

In addition to the acute suppression of melatonin we observed in the current study, a delay in circadian timing may be another pathway by which evening light exposure can contribute to nighttime sleep problems in young children. Although the current study was not designed to assess changes in circadian timing in response to an experimental light stimulus, this represents an important area of future investigation. Currently, phase response curves to light that predict both the magnitude and direction of phase shifts in response to light presented at various biological times have only been published for adults (Khalsa, Jewett et al. 2003), although data in adolescents is underway (Crowley and Eastman 2012). Because the magnitude of the circadian response to light may differ in young children, well-controlled studies on the phase shifting effects of light in this age group are necessary.

Beyond promoting sleep, the pineal hormone melatonin plays an important role in overall health and normal physiological functioning (Pandi-Perumal, Trakht et al. 2008). Melatonin is the body's internal signal of biological nighttime and has been implicated in a variety of essential physiological processes, including thermoregulation (Saarela and Reiter 1994), blood pressure (Simko and Paulis 2007), and glucose homeostasis (la Fleur, Kalsbeek et al. 2001, Owino, Contreras-Alcantara et al. 2016). Additionally, melatonin functions as an antioxidant in the body by interacting with intracellular components (Reiter, Tan et al. 1999). An established literature in adults demonstrates

that inappropriately timed light exposure during the biological night can suppress melatonin and disrupt normal physiological functioning that may contribute to a variety of negative health outcomes including obesity (Fonken, Workman et al. 2010), diabetes (Qian, Block et al. 2013) and cancer (Schernhammer and Schulmeister 2004). Because the preschool years are a sensitive and vulnerable window in human development (Center on the Developing Child 2010), future research should investigate the role of evening light exposure in health and disease in early life.

To contextualize the brightness of the experimental light stimulus used in this study, typical indoor lighting is <200 lux (Wright, McHill et al. 2013), while bright indoor lighting can range from 300-1,000 lux (Crowley, Molina et al. 2015). Although the 1,000 lux stimulus administered in this study would be similar to a brightly-lit home environment, it is not comparable to the intensity of light emitted from electronic devices (~30-50 lux; (Chang, Aeschbach et al. 2015)). Still, our findings of robust melatonin suppression in this age group call for an understanding of the effects of light-emitting electronic devices on the circadian physiology of young children. The amount of time young children spend using mobile electronic devices has tripled in recent years (Common Sense Media 2011), and 90% of parents report that their children use electronic media devices before the age of 2 years (American Academy of Pediatrics 2011). Often electronic device use in this young age group occurs in the hour before bedtime and is therefore, incorporated into a child's bedtime routine (Vandewater, Rideout et al. 2007). Findings from our laboratory indicate that the natural increase of endogenous melatonin in well-controlled dim-light conditions occurs ~50-minutes before bedtime (i.e. bedtime phase angle; interval between melatonin onset and bedtime) in

young children (LeBourgeois, Carskadon et al. 2013). Thus, the 1-hour before bedtime may represent a sensitive window during which the circadian system is sensitive to perturbations (i.e. suppression of melatonin and phase delays) (Khalsa, Jewett et al. 2003). Recently published data demonstrate that adults are sensitive to the effects of electronic media use in the 1-hour before bedtime (Chang, Aeschbach et al. 2015). Specifically, electronic device use before bedtime suppressed melatonin, delayed the timing of melatonin onset, delayed REM sleep onset and even negatively affected next day alertness. Considering that the screens of most electronic devices peak in the short wavelengths and lens transparency differences across age are most prominent at shorter wavelengths (Charman 2003), we expect that young children are more sensitive to the effects of light emitted from these sources. The almost ubiquitous nature of electronic media use in this young age group supports the critical need for studies assessing the effect of evening electronic media use on melatonin levels, circadian timing, and subsequent sleep and alertness early childhood.

The limitations of this study are worth noting. First, we enrolled a small sample size from a relatively narrow age range. Additional research with a larger sample of children is necessary to establish a more accurate estimate of light-induced melatonin suppression effects, and longitudinal data are critical to determine whether such a response decreases with age. Second, the experimental light stimulus was anchored to the child's bedtime and not intrinsic circadian timing. Thus, subjects were exposed to the bright light stimulus at various circadian times. Future research should consider individual circadian timing when delivering a light stimulus. Lastly, this study included both napping and non-napping preschoolers, who based upon the 2-process model of

sleep regulation (Werth, Dijk et al. 1996) and our recent findings (Lassonde, Rusterholz et al. 2016. Under Review), likely had different levels of accumulated sleep pressure at bedtime. For this reason, we were not able to asses the effects of evening bright light exposure on subsequent sleep parameters (e.g. sleep onset latency, bedtime resistance), suggesting the need for studies of independent samples of napping and non-napping preschool-age children.

In summary, these data demonstrate a robust and sustained response of the circadian system of preschool-age children to evening bright light exposure. Our approach and findings represent a first step in understanding the dynamics of the circadian response to light in early childhood and suggest a potential role of evening light exposure in the etiology of evening sleep disturbances. Because multiple dimensions of sleep health (Buysse 2014) can influence developmental trajectories across the lifespan, studies aimed at fully understanding the characteristics and sensitivity of the circadian and subsequent sleep response to light in this age group are warranted.

Author Contributions

LDA obtained funding for this study; LDA, KPW, and MKL designed the study; LDA collected and analyzed data with support from KPW and MKL; all authors wrote and approved the final manuscript.

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CHAPTER 4

EVENING LIGHT EXPOSURE INFUENCES CIRCADIAN TIMING IN PRESCHOOL-AGE CHILDREN: A FIELD STUDY

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Key Words: light, bedtime, circadian phase, melatonin, DLMO, preschool, children

Abstract

Light exposure and sleep timing are two factors that influence the inter-individual variability in the timing of the human circadian clock. The aim of this study was to quantify the degree to which evening light exposure predicts variance in circadian timing over and above bedtime alone in preschool children. Participants were 21 children ages 4.5-5.0 years (4.7 ± 0.2 years; 9 females). Children followed their typical sleep schedules for 4 days during which time they wore a wrist actigraph to assess sleep timing and a pendant light meter to measure minute-by-minute illuminance levels in lux. On the 5th day, children participated in an in-home dim-light melatonin onset (DLMO) assessment. Light exposure in the 2 hours before bedtime was averaged and aggregated across the 4 nights preceding the DLMO assessment. Mean DLMO and bedtime were 19:22 ± 01:04 and 20:07 ± 00:46, respectively. Average evening light exposure was 710.1 ± 1418.2 lux. Children with later bedtimes (lights-off time) had more delayed melatonin onset times (r=0.61, p=0.002). Evening light exposure was not independently associated with DLMO (r=0.32, p=0.08); however, a partial correlation between evening light exposure and DLMO when controlling for bedtime yielded a positive correlation (r=0.46, p=0.02). Bedtime explained 37.3% of the variance in the timing of DLMO, and evening light exposure accounted for an additional 13.3% of the variance. These findings represent an important step in understanding factors that influence circadian phase in preschool-age children and have implications for understanding a modifiable pathway that may underlie the development of a late sleep phenotype and evening settling problems in early childhood.

Introduction

The secretion of the hormone melatonin is under circadian control via a multi-synaptic pathway between the suprachiasmatic nucleus (SCN) and the pineal gland (Moore and Klein 1974, Kalsbeek, Palm et al. 2006). In humans, the rhythm of melatonin is the most robust marker for assessing the timing of the internal biological clock (Klerman, Gershengorn et al. 2002, Benloucif, Guico et al. 2005). Although understanding of the circadian timing system in adolescents and adults is rapidly increasing (Gooneratne, Metlay et al. 2003, Kawinska, Dumont et al. 2005, Crowley, Acebo et al. 2006, Crowley, Van Reen et al. 2014), basic fundamental knowledge of the circadian system in early childhood remains relatively scarce. Consistent with findings in adolescents and adults, our recent study revealed large inter-individual variability in the circadian phase of children ages 2.5-3.0 years (~3.5 hours) (LeBourgeois, Carskadon et al. 2013). Although light exposure and sleep timing may contribute to this observed variability in circadian phase, little is known about such links in early childhood.

Light is the most powerful input to the circadian timing system and can alter many aspects of its physiology including the clock's timing (Czeisler, Richardson et al. 1981, Khalsa, Jewett et al. 2003, Duffy and Wright 2005). Specifically, light exposure near habitual bedtime induces a phase delay in an intensity-dependent manner (Zeitzer, Dijk et al. 2000). This non-linear relationship is such that room light (~100 lux) produces half the phase shift achieved with bright light (~9,000 lux) (Zeitzer, Dijk et al. 2000). Studies in adults experimentally manipulating the intensity of light in the hours before bedtime further demonstrate the effects of evening indoor light on circadian timing. For

example, a field study that experimentally manipulated light exposure in the 4 hours before bedtime found that adult subjects had a later circadian phase after a week of maximizing home lighting in the hours before bedtime (~65 lux) compared to a week of dim light (~3 lux) exposure before bedtime (Burgess and Molina 2014). Furthermore, another study in adults found that room lighting (<200 lux) in the 8 hours before bedtime suppressed melatonin levels and shortened melatonin duration compared to exposure to dim light (<3 lux) during this sensitive time window (Gooley, Chamberlain et al. 2011). Together, these findings demonstrate that even exposure to typical indoor light intensity (<200 lux) before bedtime can have profound effects on the circadian timing system of humans.

Although light is the strongest signal to the biological clock, sleep timing can indirectly influence circadian phase by 'gating' light exposure. Through this gating of light exposure, sleep timing in part determines the light/dark cycle experienced by humans. Therefore, later bedtimes allow light exposure later in the evening resulting in a delay in circadian timing. In a relatively large sample of toddlers, we observed a moderate positive relationship between sleep timing and circadian phase, such that later bedtimes were associated with later circadian timing (LeBourgeois, Carskadon et al. 2013). This association has been replicated across the lifespan in both observational and experimental studies (Martin and Eastman 2002, Burgess, Savic et al. 2003, Burgess and Eastman 2004, Burgess and Eastman 2005, Burgess and Eastman 2006, Crowley, Acebo et al. 2006, Wright, McHill et al. 2013). In order to demonstrate the effect of bedtime on circadian timing, one study manipulated the bedtimes of participants while keeping a fixed wake time. This study found a delay in the melatonin

rhythm after adult subjects had a late compared to an early bedtime (Burgess and Eastman 2004). These findings indicate that in addition to the intensity of light exposure in the hours before bed, sleep timing also impacts the circadian phase of humans.

Considering the wide variability in circadian timing during early childhood and the influence of ordinary room light on the circadian system of humans, we examined the degree to which light exposure before bedtime impacts the timing of the clock in a field setting. We focus on evening light exposure and bedtime in the present analysis because evening settling problems (i.e., bedtime resistance, delayed sleep onset) are highly prevalent in the preschool years (Beltramini and Hertzig 1983, Lozoff, Wolf et al. 1985, Bruni, Lo Reto et al. 2000) and because these factors may promote the development of a late sleep phenotype in early childhood. They are also both modifiable intervention targets that can be altered to shift circadian timing if desired. The aim of this observational study was to assess the role of evening light exposure and bedtime in influencing the circadian timing of young children using hierarchical linear regression. We hypothesized that evening light exposure would predict variance in circadian phase over and above bedtime alone in our sample of preschool-age children.

Materials and Methods

Participants

Twenty-one healthy children (9 females; 20 Caucasian, 1 Multi-Racial) ages 4.5-5.0 years (4.7 ± 0.2 years) participated in the study. Families were recruited from Boulder, CO, USA and immediate surrounding areas via flyers and at community events. Subject

screening and exclusionary criteria are described in detail in LeBourgeois et al. 2013 (LeBourgeois, Carskadon et al. 2013). Briefly, all children were healthy and normally developing and did not regularly use medications that influence sleep or the circadian system. Parents signed an informed consent form approved by the Institutional Review Board at the University of Colorado Boulder. Families received monetary compensation for completing the study.

Protocol

Children participated in a 5-day protocol, where they followed their typical sleep schedule at home for 4 days. Researchers made in-home visits during this interval to train children on providing saliva samples. On the 5th day of the study, subjects participated in a dim light melatonin onset (DLMO) assessment (described below). Children refrained from the consumption of caffeine and medications throughout the duration of the study, and researchers contacted parents daily to ensure compliance with study protocol. Data were collected between September and May excluding the week following daylight saving time changes. Five children completed the study in the Fall, 11 in the Winter months, and 6 in the Spring.

Measures

Actigraphy and Sleep Diary

Subjects wore a wrist actigraph (Actiwatch Spectrum, Phillips Respironics, Pittsburg, PA, USA) on their non-dominant wrist throughout the duration of the study to

objectively assess the timing of sleep and wakefulness via motor activity. We used our standard methods for reviewing and scoring actigraphy data, as detailed in our previous publications (Berger, Miller et al. 2012, LeBourgeois, Carskadon et al. 2013). Parents completed a daily sleep diary throughout the study in which they recorded information on their child's sleep timing and times the actigraph was not worn (Akacem, Simpkin et al. 2015). We also made daily contact with parents via telephone, text, or email to ensure compliance with study rules.

Ambient Light Exposure

Participants wore a pendant light meter (Dimesimeter, Lighting Research Center, Troy, NY, USA) around their neck during periods of wakefulness for the 4 days before the circadian phase assessment. The device collects lux levels in 1-minute epochs. Parents put the pendant light meter over all their child's clothing facing forward. The device was attached upon awakening in the morning and removed during sleep (i.e. naps and at bedtime) and for baths. If the device was not being worn, parents were instructed to have the device face up in the same room (e.g. nightstand for periods of sleep, bathroom counter for bath time) as near as possible to the child. Parents filled out a daily light meter diary that documented whether or not the device was removed during waking hours and if so where the device was during that time. Researchers confirmed that the child was wearing the device through daily contacts.

Salivary DLMO Assessment

On the 5th day of the study, children participated in an in-home salivary DLMO assessment (LeBourgeois, Carskadon et al. 2013). Researchers created dim light conditions within the home by covering windows with black plastic and using dimmer switches and low wattage bulbs on existing light sources. Children entered dim light conditions of <10 lux 1 hour before the first saliva sample. Children provided saliva samples every 30 minutes for 6 hours ending 1 hour after the average bedtime of the 4 preceding days. Saliva samples were obtained by having subjects chew on a dental cotton stick (Henry Schein Inc., Denver, PA, USA) for ~2 minutes to produce at least 2 mL of saliva. Researchers helped children rinse and clean their mouth as needed after eating at least 15 minutes before obtaining a saliva sample. Children sat still for 5 minutes before each saliva sample to control for the effects of posture on melatonin levels. Lux levels were measured in the child's angle of gaze at the time of each saliva sample (Extech Instruments, Spring Hill, FL, USA). Saliva samples were centrifuged (LabEssentials Inc., Monroe, GA, USA) and refrigerated in the child's home. Following completion of the assessment, samples were immediately transported to the laboratory where they were frozen (-20 C). Radioimmunoassay was used to quantify the amount of melatonin in each saliva sample (ALPCO Diagnostics, Salem, NH, USA). Melatonin assays were performed at Solid Phase Inc. (Portland, ME, USA) and had a minimum detection of 0.2 pg/mL. The intra-assay coefficient was 4.1% and the interassay coefficient was 6.6%.

Data Processing and Analysis

DLMO was computed as the time at which salivary melatonin levels surpassed a 4 pg/mL threshold using linear interpolation (Deacon and Arendt 1994, Carskadon, Acebo et al. 1997, LeBourgeois, Carskadon et al. 2013, Simpkin, Jenni et al. 2014, Akacem, Simpkin et al. 2015). Phase angles were computed as the time interval between average actigraphy scored sleep times (4 days before the circadian phase assessment) and DLMO. For the present analysis, we averaged light exposure in the 2 hours before actigraphy scored bedtime and aggregated the data across the 4 nights before the circadian phase assessment. Lux levels in the 2 hours before bedtime were averaged using MATLAB (MathWorks, Natick, MA, USA) and were log transformed due to a positively skewed distribution.

One tailed Pearson correlations were used to (a) confirm the relationship between bedtime and DLMO observed in our previous work (LeBourgeois, Carskadon et al. 2013) and (b) assess associations between evening light exposure and DLMO and bedtime. A partial correlation was performed between evening light exposure and DLMO while controlling for bedtime, and a hierarchical linear regression was used to quantify how much variance in DLMO was predicted by bedtime (step 1) and evening light exposure (step 2). All statistical analyses were performed in SPSS Statistics (IBM Corp., Armonk, NY, USA). All descriptive statistics are presented as M $\pm$ SD.

Results

Mean DLMO occurred at 19:22 $\pm$ 01:04. Average bedtime was 20:07 $\pm$ 00:46, and mean light exposure in the 2 hours before bedtime across the 4 evenings before

the circadian phase assessment was 710.1 ± 1418.2 lux. Bedtime was positively correlated with the timing of circadian phase (r=0.61, p=0.002; Figure 1A). Light exposure before bedtime was not independently correlated with DLMO (r=0.32, p=0.08) or bedtime (r=-0.07, p=0.38); however, a partial correlation between evening light exposure and DLMO while holding bedtime constant yielded a significant positive correlation (r=0.46, p=0.02; Figure 1B).

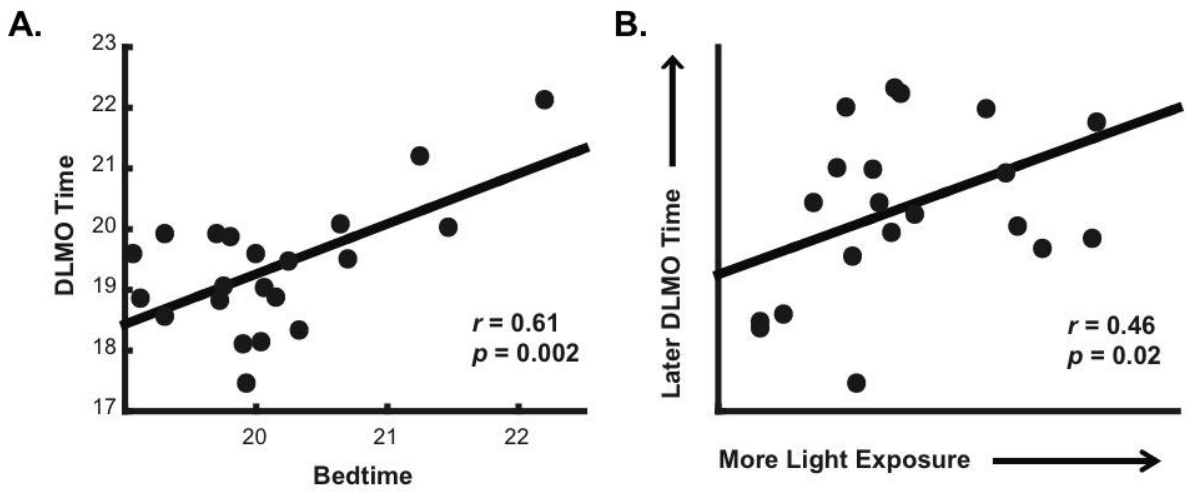

Figure 1. Scatterplots of the association between bedtime and DLMO (A) and of evening light exposure and DLMO after controlling for bedtime (B). Lines show the slope of the regression line.

In a hierarchical linear regression, bedtime explained 37.3% of the variance in the timing of DLMO. Adding evening light exposure to the model accounted for 13.3% more variance in DLMO in addition to bedtime (Table 1 and Figure 2).

Step	Factor	R^2	R^2 Change	F Change	Sig. F Change
1	Bedtime	0.37	0.37	11.3	0.003
2	Evening Light Exposure	0.51	0.13	4.9	0.041

Table 1. Results of the hierarchical linear regression.

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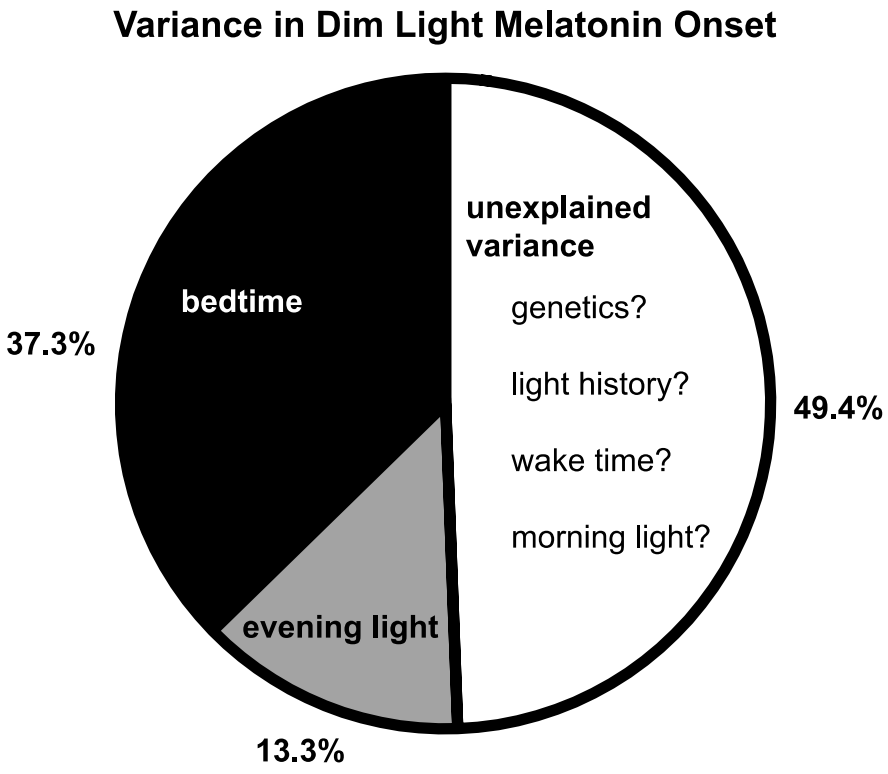

Figure 2. Visual depiction of variance in circadian timing predicted by bedtime and evening light exposure (n=21; ages 4-5 years). Forty-nine percent of the variance in dim light melatonin onset (DLMO) timing was unaccounted for but may be due to additional factors like genetics, light history, wake time and morning light.

Discussion

The results of this field study indicate that the amount of light preschool-age children are exposed to in the 2 hours before bedtime predicts variance in circadian phase over and above bedtime alone. Consistent with our previous findings (LeBourgeois, Carskadon et al. 2013), we observed a positive association between bedtime and DLMO such that later bedtimes were associated with later circadian phases. After controlling for bedtime, evening light exposure was associated with DLMO. That is, bedtime predicted 37.3% of the variance in DLMO; however, after adding evening light exposure into the model, we accounted for a total of 50.6% (13.3% more) of the variance in circadian timing. To our knowledge, this is the first observational study to examine evening light exposure in young children in association with circadian phase.

Our findings have implications for understanding how late evening sleep timing may develop and persist during the early childhood years. Evening light exposure and late bedtimes delay the timing of the clock (Zeitzer, Dijk et al. 2000, Khalsa, Jewett et al. 2003, Burgess and Eastman 2004), thus, chronic exposure to light during the evening hours and late bedtimes together may interact to facilitate the development of a late sleep phenotype early in life. This type of sleep pattern is a significant risk factor for a host of negative health outcomes, including bedtime sleep problems, chronic medical conditions (e.g. obesity, diabetes, depression), poor cognitive function, and mood disorders (Levandovski, Dantas et al. 2011, Spruyt, Molfese et al. 2011, Roenneberg, Allebrandt et al. 2012, Foster, Peirson et al. 2013, Genzel, Ahrberg et al. 2013,

LeBourgeois, Wright et al. 2013, Reutrakul, Hood et al. 2013, Chan, Lam et al. 2014, Miller, Kaciroti et al. 2014, Miller, Lumeng et al. 2015). Although early childhood is a sensitive period when poor sleep patterns commonly emerge (Zuckerman, Stevenson et al. 1987, Gregory and O'Connor 2002), evening light exposure and bedtime are both modifiable factors. Thus, experimental studies that promote an understanding of the effects of evening light exposure and bedtime on circadian timing are a necessary for clinicians to make evidence based recommendations regarding light at night and sleep timing for young children.

In addition to delaying the timing of the clock, evening light exposure also causes an immediate suppression of the hormone melatonin (Lewy, Wehr et al. 1980). Naturally, melatonin levels begin to rise in the evening hours before bedtime; however, exposure to light in the early biological night prevents the secretion of this hormone. Considering the diverse role of melatonin in physiological processes such as glucose homeostasis (la Fleur, Kalsbeek et al. 2001, Owino, Contreras-Alcantara et al. 2016), thermoregulation (Saarela and Reiter 1994), blood pressure (Simko and Paulis 2007) and sleep promotion (Zhdanova, Lynch et al. 1997), chronic suppression of the hormone melatonin likely has negative consequences for overall health and well-being.

Understanding the effects of evening light on the circadian system in the early years of life is especially important because young children are proposed to be more sensitive to the circadian effects of light than adults. The clear crystalline lens and large pupil size of young children may render them more sensitive to the effects of evening light than adults (Charman 2003, Turner and Mainster 2008). One recent study comparing melatonin suppression in response to light at night found that the percentage

of melatonin suppression in school-age children was almost twice that of the adults (Higuchi, Nagafuchi et al. 2014). Furthermore, work from our laboratory has shown that 1 hour of bright light exposure (~1,000 lux) in the hour before bedtime induces ~90% suppression of melatonin in preschool age children (Akacem, Wright et al. 2016). The melatonin levels of these preschoolers remained attenuated for up to 50 minutes after this light stimulus ended, thus, demonstrating the robust effects of evening light in this young age group. Together, these findings are important in the context of our previously published data showing that toddlers with later circadian phases exhibited longer sleep onset latencies and more bedtime resistance (LeBourgeois, Wright et al. 2013). By influencing both the timing and levels of melatonin, we believe that evening light exposure may impair a child's physiological readiness for sleep (i.e. settling down after lights-off) and their ability to fall asleep. The high prevalence (~30%) of evening sleep problems following lights-off time in early childhood (Beltramini and Hertzig 1983, Lozoff, Wolf et al. 1985, Bruni, Lo Reto et al. 2000) suggests a critical need for evidence-based recommendations on evening light exposure for children.

Light exposure at night is emerging as a serious health concern (Schernhammer and Schulmeister 2004, Stevens, Brainard et al. 2013, Cho, Ryu et al. 2015). Before electrical lighting, the light exposure of humans was limited to the hours between dusk and dawn. With the introduction of electrical lighting and light-emitting electronic devices, humans now construct their own light/dark cycle and extend exposure to light far beyond dusk. This pattern of light/dark exposure influences the melatonin rhythm, which in turn communicates photoperiod length to the rest of the body (Wehr, Moul et al. 1993, Wehr 1998). Thus, not only does light exposure at night delay the clock and

suppress melatonin, constantly extending light exposure past dusk deceives the body into maintaining a perpetual summer-like photoperiod. These constructed photoperiods effectively decouple human circadian physiology from the natural 24-hour light dark cycle (Czeisler 2013). A recent study investigating the impact of a constructed light/dark environment on circadian physiology found that subjects had a later circadian phase after a week living in a constructed light environment (i.e. freely able to control lights on/lights off times) compared to after a week of camping when subjects were strictly exposed to natural light. Additionally, this study found that subjects were exposed to significantly more light after sunset following a week of living in the real world compared to camping (Wright, McHill et al. 2013). Studies are needed to understand the effects of living in a chronically extended photoperiod in the early years of life on future health and development..

Certain limitations of the present study are worth noting. First, we studied a relatively small sample size. A larger sample of children would provide the opportunity to more accurately estimate the amount of variance predicted by bedtime and evening light exposure, and studying children longitudinally may provide key insights in developmental changes in evening light sensitivity. Additionally, mean light exposure in the present sample (710.1 lux) is much higher than what has been published in adults (e.g. 40-617 lux; (Burgess and Eastman 2004, Scheuermaier, Laffan et al. 2010, Santhi, Thorne et al. 2012). Larger scale studies are necessary to determine whether this relatively high average is representative of evening light exposure for this young age group in this geographical location. Furthermore, only healthy preschoolers were included in this study, thus our findings may not be generalizable to the general

population. Future studies should examine light exposure in relationship with circadian timing in a large community or population based sample.

In summary, this study represents an important step in understanding factors that contribute to the observed wide variability in circadian timing during early childhood. Our findings indicate that evening light exposure is important in predicting a significant portion of the observed variability in circadian timing in this age group. Future research should examine additional factors that may account for the unexplained variance including genetics, morning light exposure, wake time, and prior light history. Evidence based recommendations for evening light exposure are critical for promoting healthy sleep and development across early childhood and beyond.

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Conflicts of Interest

The authors have none to declare.

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CHAPTER 5

CONCLUSION

Lameese D. Akacem

Summary of Results

The purpose of this dissertation was to understand factors that influence the circadian physiology of young children. Although data from a well-established literature exists on the circadian system of adults (Czeisler and Wright 1999, Duffy and Wright 2005, Duffy and Czeisler 2009), very little is known about factors that influence circadian physiology in the early childhood years. This dissertation focused on the role of napping and evening light exposure in influencing the circadian clock of young children.

Firstly, it was unknown how napping habits are associated with circadian and sleep timing in early childhood. Due to the dissipation in homeostatic sleep pressure that occurs in a daytime nap (Achermann and Borbely 2003, Lassonde, Achermann et al. 2013), we hypothesized that children who habitually napped would have later bedtimes and sleep onset times and longer sleep onset latencies than children who did not nap. Additionally, we expected that children who napped would have later circadian phases due to later bedtimes of nappers gating light exposure later in the evening (Khalsa, Jewett et al. 2003). We explored differences in wake time and phase angles of entrainment between the two groups. Our findings were consistent with our hypotheses such that napping toddlers had later bedtimes and sleep onset times and longer sleep onset latencies but had similar wake times than children who did not nap. Children who napped also had later circadian phases and wider wake time phase angles than children who did not nap. We believe our finding of later circadian timing in nappers is

mediated by the later bedtimes of toddlers, which facilitate light exposure later into the evening thereby delaying the timing of their clock.

Secondly, to our knowledge the melatonin suppression response to evening bright light had not yet been characterized in young children. Thus we aimed to quantify the magnitude of the melatonin suppression response to evening light in preschooler-age children. In this study we exposed children to bright light (~1,000 lux) for 1 hour before their typical bedtime. Salivary melatonin levels were compared between a dim light control condition and the bright light condition. We observed ~90% melatonin suppression in response to evening bright light, an effect that persisted 50 minutes after the the light stimulus was terminated.

Finally, it was unknown how the light young children are exposed to in the hours before bedtime affects circadian timing. Therefore, we collected observational data on the amount of light preschoolers were exposed to in the 2 hours before bedtime and examined whether light exposure in this time interval before bedtime explained variance in circadian timing over and above bedtime alone. Considering the strong effect of light on the circadian system, we hypothesized that evening light exposure would predict additional variance in DLMO than just bedtime alone. We found that bedtime predicted 37.3% of the variance in DLMO and the amount of light young children were exposed to in the hours before bedtime accounted for an additional 13.3% variance in the timing of circadian phase.

Collectively, the findings of this dissertation demonstrate how napping can influence the timing of the clock and the robust sensitivity of the circadian system of young children to light. These findings have implications for understanding potential

developmental changes in circadian timing across early childhood as children drop their naps. Additionally, these findings represent an important step in understanding how evening light influences the circadian physiology of young children and have implications for understanding the etiology of sleep problems in this age group.

Future Directions

Several future directions would extend the work of this dissertation. First, longitudinal studies indicate that the prevalence of napping decreases across early childhood (Thorleifsdottir, Bjornsson et al. 2002, Iglowstein, Jenni et al. 2003). Given that we observed an earlier circadian phase in a cross-sectional sample of non-napping toddlers, we hypothesize that as children shift from a biphasic to a monophasic sleep pattern across early childhood, there will be a concurrent advance in circadian timing. However, a longitudinal study across early childhood is necessary to determine whether or not this phase shift occurs as children stop napping.

Another future direction is to understand how circadian sensitivity to light changes across early childhood. Considering the increase in lens transparency and decrease in pupil size that occurs with age (Charman 2003, Turner and Mainster 2008), we hypothesize that the melatonin suppression response to evening light will decrease across the early childhood years; however, a longitudinal within-subject design study across early childhood is necessary to test this hypothesis.

The robust effects of evening light exposure on the circadian physiology of young children observed in these dissertation studies demonstrate the critical need for

experimental studies that promote an understanding of how varying intensities of evening light influence both circadian timing and melatonin suppression. Studies of this nature would allow researchers and clinicians to make evidence-based recommendations for evening light exposure to promote healthy sleep hygiene habits in early childhood and beyond. Furthermore, electronic media use is a common occurrence in the hours before bed and is often incorporated into bedtime routines (Vandewater, Rideout et al. 2007). In fact, mobile media use (e.g. iPads) has more than tripled among young children in recent years (Common Sense Media 2011). Recent findings in adults demonstrate the profound effects of electronic device use before bed on sleep onset latency, sleep architecture and melatonin levels and circadian timing (Chang, Aeschbach et al. 2015). Thus, experimental studies on electronic device use in the hours before bedtime in this young age group are necessary in order understand how these devices influence circadian timing and subsequent sleep.

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