THE INFLUENCE OF MELATONIN, CAFFEINE, AND BRIGHT LIGHT ON HUMAN CIRCADIAN PHYSIOLOGY

by

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ABSTRACT

Tina M. Burke (Department of Integrative Physiology)

The Influence of Melatonin, Caffeine, and Bright Light on Human Circadian Physiology

Thesis directed by Associate Professor Kenneth P. Wright, Jr.

The intrinsic circadian timing system allows for the temporal organization of many physiological and behavioral events across the 24h light-dark cycle. Both photic (light) and non-photic (e.g., exogenous melatonin) stimuli have been shown to influence the timing of the mammalian circadian system. Furthermore, in non-humans, there is a growing body of evidence to suggest that the integration of photic and non-photic stimuli may induce greater circadian adaptation than either stimulus alone. Understanding how photic and non-photic stimuli influence the human circadian system will help determine the most effective and efficient ways to shift the human circadian clock. Therefore, the aims of this dissertation were to: 1) determine if morning bright light exposure and evening exogenous melatonin administration would produce a circadian phase advance that would be integrated by the human circadian system to produce a greater shift than either stimulus alone; 2) determine whether morning caffeine administration induces a circadian phase advance of the human circadian clock; 3) determine if evening caffeine administration induces a circadian phase delay of the human circadian clock; and 4) determine if evening bright light exposure and evening caffeine administration produces a circadian phase delay that would be integrated by the human circadian system to produce a greater shift than either stimulus alone. The results indicate: 1) morning bright light combined with late afternoon exogenous melatonin induces a greater phase advance of the human circadian clock than either

treatment alone; 2) preliminary evidence that caffeine may be able to phase advance the human circadian clock; and 3) exposure to evening caffeine induces a circadian phase delay of the human circadian clock; and 4) exposure to evening bright light combined with evening caffeine does not induce a greater phase shift than either alone. These findings suggest that photic and some non-photic time cues alone and in combination can phase shift the human circadian system. Findings demonstrate for the first time the ability of evening caffeine administration to produce a circadian phase delay in humans, and suggest that morning caffeine administration may attenuate the drift in circadian phase under dim light conditions. These findings have implications for the treatment of circadian sleep disorders and circadian misalignment.

DEDICATION

This dissertation is dedicated to my family and friends for their unconditional love, support, and mentorship that has helped me to achieve my goals personally and professionally. I especially would like to thank my partner Tim Srenaski for his unwavering patience and unconditional love throughout this process.

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CHAPTER 1

RESETTING INTERNAL BIOLOGICAL TIME: THE INFLUENCE OF PHOTIC AND NON-PHOTIC STIMULI ON CIRCADIAN PHYSIOLOGY

Tina M. Burke

Introduction

The adaptation of internal biological timing to the external environment is a key feature of the circadian system and is important for the adjustment to rapid travel across time zones (jet lag) and the effective treatment of circadian sleep disorders. Photic (light) and non-photic (e.g., exogenous melatonin) stimuli have been shown to independently shift the timing of the mammalian circadian system. Furthermore, there is a growing body of evidence to suggest that the integration of photic and non-photic stimuli may induce greater circadian adaptation than either stimulus alone. Understanding how zeitgebers (German for time giver) influence the circadian system will help determine the most effective and efficient ways to shift the human circadian clock. This paper addresses what is known about three modulators used to shift the circadian timing system: light, exogenous melatonin, and caffeine administration. This comprehensive review begins with an introduction to the physiology of the circadian timing system, and then continues to explore how the circadian system is affected by external stimuli (photic and non-photic) before concluding with a detailed discussion regarding the clinical relevance of shifting the human circadian clock.

Circadian physiology

The suprachiasmatic nucleus (SCN)

The suprachiasmatic nucleus (SCN) is the neural substrate associated with the internal biological timing system in mammals (Czeisler & Wright, Jr., 1999; Inouye & Kawamura, 1979; Moore & Eichler, 1972). The SCN are located bilaterally in the basal portion of the anterior hypothalamus superior to the optic chiasm. Each nuclei contains approximately 8,000-10,000 tightly packed neurons (van den Pol, 1980). Research findings have clearly demonstrated that the

SCN is the site of the master circadian clock. The master circadian clock is responsible for the generation of circadian rhythms in mammals (Czeisler & Wright, Jr., 1999; Inouye & Kawamura, 1979; Moore & Eichler, 1972) and facilitates the synchronization of internal biological time with the environment (Rusak, 1977) to influence physiological (Moore & Eichler, 1972) and behavioral processes (Stephan & Zucker, 1972). Lesions to the SCN in animal models have been shown to produce arrhythmicity in behavioral and physiological measures. These models provide evidence that the SCN is the site of the master circadian clock (Eastman, Mistlberger, & Rechtschaffen, 1984; Edgar, Dement, & Fuller, 1993; Ibuka & Kawamura, 1975). Providing further evidence, circadian rhythmicity in wheel-running activity can be restored following transplantation of a donor fetal SCN into the third ventricle of the lesioned animal (Sawaki, Nihonmatsu, & Kawamura, 1984). The resulting length of the intrinsic circadian period (cycle length) in wheel-running activity of the lesioned animal is restored to the period of the donor (Ralph, Foster, Davis, & Menaker, 1990).

Molecular mechanisms

Individual SCN neurons have been shown to intrinsically oscillate at a near-24h rhythm (Welsh, Logothetis, Meister, & Reppert, 1995). The molecular mechanism facilitating the near-24h oscillation is produced by a gene transcription-translation negative feedback loop (Hastings & Herzog, 2004; Pace-Schott & Hobson, 2002; Reppert & Weaver, 2001; Shearman *et al.*, 2000) that is able to be modulated by photic and non-photic input (Castillo, Hochstetler, Tavernier, Jr., Greene, & Bult-Ito, 2004; Hamada, Antle, & Silver, 2004; Mendoza, Dardente, Escobar, Pevet, & Challet, 2004). The intracellular oscillation is due to the accumulation of protein products of clock genes that are active during the circadian day and are able to suppress their own transcription (Hastings & Herzog, 2004).

In mammals, the circadian clock genes, *Clock* (Circadian locomotor output cycles kaput) (Vitaterna *et al.*, 1994) and *Bmal1* (Brain muscle arnt like factor) drive transcription. In the transcription-translation feedback loop, the Clock/Bmal1 protein dimer promotes the transcription of *Period* (*Per* 1, 2 and 3) and *Cryptochrome* (*Cry* 1 and 2) genes. Transcription of *Per* and *Cry* occur when CLOCK/BMAL1 bind to the E-box promoter sequence. PER and CRY proteins dimerize in the cytoplasm and translocate to the nucleus. Phosphorylation of PER/CRY in the cytoplasm by Casein kinase Iε (CKIε) can slow translocation of PER/CRY to the nucleus. PER/CRY in the nucleus inhibit CLOCK/BMAL1 activity resulting in the decrease of *Per* and *Cry* transcription (Albrecht, 2004; Ko & Takahashi, 2006). CLOCK/BMAL1 also activate transcription of nuclear orphan receptor genes, reverse erythroblastosis virus α (Rev-Erbaα), and retinoic acid receptor-related orphan receptor α (Rorα), which inhibit and activate BMAL1 expression respectively (Guillaumond, Dardente, Giguere, & Cermakian, 2005; Preitner *et al.*, 2002; Sato *et al.*, 2004).

The negative feedback loop is thought to be involved in changing ion permeability and membrane potential of the cell to influence the firing rate of individual SCN neurons (Colwell, 2000). The electrical output of individual SCN neurons have peak firing rates during the biological day (a term used in diurnal species to represents internal circadian time associated with the promotion of wakefulness and associated processes) in vitro (Green & Gillette, 1982) and in vivo (Yamazaki, Kerbeshian, Hocker, Block, & Menaker, 1998). Individual SCN neurons are also influenced by intercellular signaling from surrounding SCN neurons which can result in differential excitation and inhibition. The release of gamma aminobytyric acid (GABA) by the surrounding neurons has an excitatory effect during the day and an inhibitory effect during the night which is thought to be attributed to the circadian variation in chloride ion permeability (

Colwell, 1997; Wagner, Castel, Gainer, & Yarom, 1997). Though an individual neuron may oscillate differently than surrounding neurons, the collective rate of firing of these neurons is highly predictive of the behavioral period (Herzog, Aton, Numano, Sakaki, & Tei, 2004; Liu, Weaver, Strogatz, & Reppert, 1997).

Neural pathways

Light is the dominant zeitgeber for the human circadian clock. The primary input of photic information to the SCN is through the retino-hypothalamic tract (RHT) (Levine, Weiss, Rosenwasser, & Miselis, 1991; Moore, Speh, & Card, 1995; Moore & Eichler, 1972). The RHT is a collection of intrinsically photosensitive retinal ganglion cell (ipRGC) axons that are thought to mediate non-image forming (NIF) responses to light (Berson, Dunn, & Takao, 2002). The ipRGCs contain the photopigment melanopsin (Hattar, Liao, Takao, Berson, & Yau, 2002) and are thought to be the primary contributors to circadian photoreception. Light is also detected by rods and cones which are synaptically connected to ipRGCs (Wong, Dunn, Graham, & Berson, 2007; Revell & Skene, 2007). The exact combination of photoreceptors and their respective contributions remain unknown. NIF responses modulate physiology and behaviors such as entrainment to the environmental light-dark cycle (Czeisler *et al.*, 1989; Duffy & Wright, Jr., 2005), the pupillary light reflex (Lucas *et al.*, 2003), and sleep and wakefulness (Altimus *et al.*, 2008; Tsai *et al.*, 2009).

The SCN receives photic information directly from the retina via the RHT (Levine *et al.*, 1991; Moore & Eichler, 1972; Moore *et al.*, 1995) and indirectly from the intergeniculate leaflet (IGL) of the thalamus (Moga & Moore, 1997) and midbrain raphe nuclei (Meyer-Bernstein & Morin, 1996; Moga & Moore, 1997). The IGL (Moga & Moore, 1997) and midbrain raphe nuclei (Meyer-Bernstein & Morin, 1996; Moga & Moore, 1997) are also thought to project non-

photic information to the SCN. The SCN afferents are able to modify the firing rates of neurons in the SCN to synchronize the master clock to the external light-dark cycle (Colwell, 1997).

The major efferent projections from the SCN include pathways that influence multiple physiological responses (e.g. synthesis and release of melatonin from the pineal gland, cortisol from the adrenal glands, and thermoregulatory processes in the preoptic area of the hypothalamus) (Figure 1).

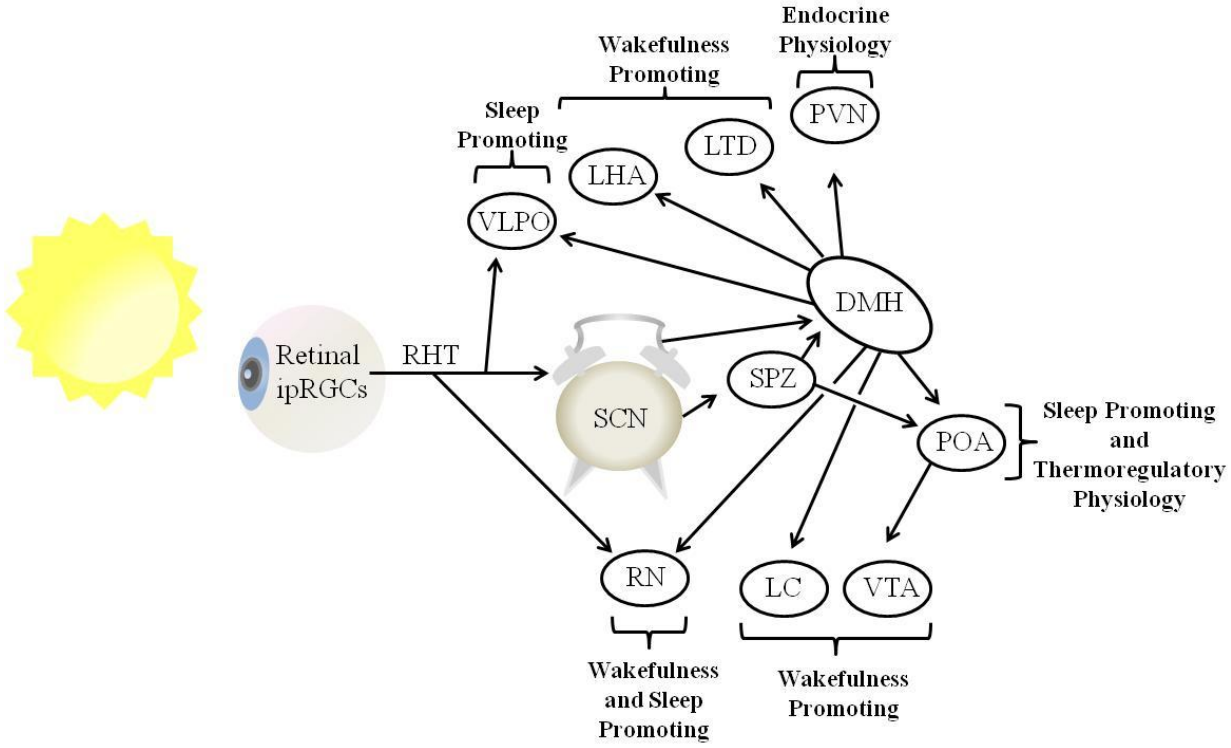

Figure 1. Schematic diagram illustrating major efferent pathways of the suprachiasmatic nuclei (SCN). The primary SCN afferents are direct projections from intrinsically photosensitive retinal ganglion cells (ipRGCs). The SCN projects directly to the dorsomedial hypothalamus (DMH), as well as indirectly to the DMH via the subparaventricular zone (SPZ). Projections from the DMH include major brain stem and hypothalamic arousal centers including: Brain stem raphe nucleus (RN), locus coerelus (LC), lateral dorsal tegmentum (LDT), and ventral tegmental area (VTA), as well as the lateral hypothalamic area (LHA). Dorsal SPZ efferents to the medial preoptic area (MPO) part of the the pre-optic area (POA) are integral for circadian regulation of body temperature. The DMH also projects to the sleep active ventrolateral preoptic nucleus (VLPO) part of the POA and the paraventricular nucleus of the hypothalamus (PVN) involved in hormone secretion. Direct projections from the RHT to the RN and VLPO may influence sleep and wakefulness promoting.

Findings from animal models have demonstrated projections from the SCN to the subparaventricular hypothalamic nucleus (SPZ) and the dorsomedial hypothalamic nucleus (DMH) (Leak & Moore, 2001; Watts, Swanson, & Sanchez-Watts, 1987; Watts & Swanson, 1987; Watts, 1991). The DMH also receives indirect input from the SPZ (Leak & Moore, 2001). The SPZ and DMH both have projections to areas involved in sleep/wake regulation as well as other physiological processes (Chou *et al.*, 2003; Aston-Jones, Chen, Zhu, & Oshinsky, 2001). For example, the circadian regulation of body temperature is regulated by the efferent projection of the SPZ to the medial preoptic area (MPO) (Saper, Lu, Chou, & Gooley, 2005). The DMH has projections to arousal centers in the brain stem. These arousal centers influence the locus coeruleus (LC), ventral tegmental area (VTA), and the raphe nucleus (RN). The DMH also has projections to nearby hypothalamic nuclei important for the regulation of sleep and wakefulness which include the ventrolateral preoptic area (VLPO), the lateral hypothalamus (LH), and the paraventricular nucleus (PVN). The DMH efferents to the PVN are specifically associated with the circadian regulation of endocrine function (e.g. cortisol and melatonin secretion). Corticosteroid release is mediated by corticotropin releasing hormone (CRH) into circulation via PVN stimulation (Chou *et al.*, 2003). CRH promotes adrenocorticotropic hormone (ACTH) release by the anterior pituitary and acts on the adrenal cortex to produce the rhythmic secretion of corticosteroids. There is also evidence of a polysynaptic pathway from the SCN to the adrenal cortex via projections from the SCN to the PVN which then projects to the intermediolateral column (IML) of the spinal cord that mediates adrenal activity directly (Buijs *et al.*, 1999).

Melatonin release is mediated by direct and indirect pathways from the SCN to the PVN. The PVN facilitates the rhythmic production of melatonin in the pineal gland (Moore, 1995). Stimulation of sympathetic neurons in the PVN facilitates glutamate, oxytocin, and vasopressin

release onto the cholinergic preganglionic IML of the spinal cord. The IML preganglionic neurons project to the noradrenergic postganglionic fibers of the superior cervical ganglion, which project to the pineal gland (Teclemariam-Mesbah, Ter Horst, Postema, Wortel, & Buijs, 1999; Moore, 1995). Norepinephrine binds primarily to β-adrenergic receptors located on the pineal cell membranes and initiates increases in intracellular cyclic adenosine monophosphate (cAMP), which in turn increases arylalkylamin N-acetyltransferase (AANAT), the rate limiting enzyme for melatonin synthesis. AANAT is also able to be phosphorylated by cAMP-dependent protein kinase A (PKA) to permit continual synthesis of melatonin (Schomerus & Korf, 2005).

As stated earlier, during the biological day, SCN neural firing rates are high and this acts to inhibit melatonin production by decreasing sympathetic activity, turning off norepinephrine stimulation of β-adrenergic receptors resulting in no AANAT expression and therefore decreased melatonin synthesis. During the biological night (a term used in diurnal species to represent internal circadian time associated with the promotion of sleep and associated processes), when the SCN output decreases, inhibition of melatonin is removed causing an increase in melatonin levels by increasing sympathetic activity (Mitler, Hajdukovic, Shafor, Hahn, & Kripke, 1987). In both diurnal and nocturnal species, high circulating melatonin levels occur during darkness (Arendt, 1988). Once melatonin is released by the pineal, melatonin circulates though the blood stream to provide information to the brain and peripheral tissues about internal biological time (Reiter, 1993; Cardinali & Pevet, 1998). Light exposure input from the RHT to the SCN during the evening reduces melatonin synthesis by increasing the inhibitory output of the SCN, allowing for a mechanism by which the modulation and entrainment to the environmental light-dark cycle occurs (Lewy, Wehr, Goodwin, Newsome, & Markey, 1980).

Markers of circadian phase

In humans, the internal circadian clock in the SCN cannot be directly measured. Therefore, physiological and neuroendocrine outputs of the clock (e.g., body temperature and melatonin, respectively) are assessed and used as markers of the circadian clock. These markers can be used to assess the phase (time within a cycle that an event occurs), period (cycle length), and amplitude (magnitude between the mesor and the peak of the rhythm) of the circadian rhythm. However, many factors can influence markers of the internal circadian clock which can mask the true rhythm (e.g. posture, activity, food intake, and light) (Nathan, Jeyaseelan, Burrows, & Norman, 1998; Wever, 1985; Minors & Waterhouse, 1984). A common technique used to assess circadian rhythms, while controlling for masking effects, is the constant routine protocol. The constant routine protocol (Czeisler *et al.*, 1985) is able to minimize environmental and behavioral effects by maintaining constant wakefulness and posture, limiting behavioral activity, providing isocaloric mini meals, and keeping dim light conditions.

Temperature

Core body temperature (CBT) is regulated by the preoptic area and receives input from the SCN (Cagnacci, Krauchi, Wirz-Justice, & Volpe, 1997). CBT can be assessed through tympanic temperature, rectal thermisters (Krauchi, Cajochen, Pache, Flammer, & Wirz-Justice, 2006), or through an ingested pill that transmits internal temperature to a recording device (Markwald, Lee-Chiong, Burke, Snider, & Wright, Jr., 2010). In general, when tested under controlled constant routine conditions, internal temperature peaks prior to habitual bedtime. Sleep is then initiated when CBT begins to decline (Campbell & Broughton, 1994) and CBT reaches its nadir approximately two hours prior to an individual's habitual waketime (Krauchi *et al.*, 2006).

Melatonin

The hormone melatonin, produced by the pineal gland, is one of the most commonly used markers of the human circadian system (Lewy, Cutler, & Sack, 1999). Melatonin is an indoleamine primarily synthesized in the pineal gland. The pinealocytes convert tryptophan from the circulation to serotonin through the process of hydroxylation and decarboxylation. Serotonin is then converted via N-acetyl transferase to N-acetyl-serotonin and can then be methylated into melatonin (Macchi & Bruce, 2004).

As mentioned in the previous section, during darkness, similar to diurnal species, nocturnal species maintain high circulating melatonin levels (Arendt, 1988). This conservation among species suggests the importance of melatonin to indicate environmental nighttime (Reiter, 1993). In humans, sleep is initiated in the evening following an increase in melatonin levels while wakefulness occurs in the morning following a decrease in melatonin levels. The melatonin acrophase (timing of the melatonin peak) typically occurs during the biological night when sleep is taking place (Benloucif *et al.*, 2005; Voultsios, Kennaway, & Dawson, 1997). Melatonin can be reliably measured in the plasma and saliva (Voultsios *et al.*, 1997), however, melatonin levels measured in plasma are higher than melatonin levels measured in saliva. During the melatonin acrophase (the peak of the rhythm) plasma melatonin levels are approximately 80-100pg/ml while daytime levels are below 10pg/ml (Lewy *et al.*, 1999).

To determine the phase of melatonin, sampling should occur in dim light (Lewy *et al.*, 1980), as light exposure during the biological nighttime can suppress endogenous melatonin production. The dim light melatonin onset (DLMO), and more recently the dim light melatonin offset (DLMOff), and the melatonin midpoint, have been used as markers of circadian phase. The DLMO occurs when melatonin levels surpass a predetermined threshold and the DLMOff is

the point in time where melatonin levels are subthreshold (Lewy *et al.*, 1999). In order to measure melatonin accurately as a marker for circadian phase, melatonin should be sampled under controlled conditions due to effects on melatonin secretion (Lewy, Wehr, & Goodwin, 1980; Nathan *et al.*, 1998). Though there are multiple markers of circadian phase, melatonin, when compared to temperature and cortisol has been reported to provide a more accurate measurement of circadian phase (Klerman, Gershengorn, Duffy, & Kronauer, 2002).

Phase shifting

Phase response curves, phase advance, and phase delay

Phase response curves (PRC) depict the direction and magnitude in phase shifts of the circadian system by a specific synchronizing stimulus (e.g. light, melatonin). The circadian system can be phase shifted in a delayed (shifting the clock to a later time) or advanced (shifting the clock to an earlier time) direction. The degree or magnitude that the circadian clock will be shifted is dependent upon the strength, time and duration of the stimulus. It has also been reported in a rodent model that the intrinsic circadian period may influence the phase resetting response.

There are two types of PRC shapes, Type 1 and Type 0 phase resetting (Winfree, 1970), with Type 1 phase resetting being the more common of the two. Type 1 phase resetting is characterized by phase shifts that are smaller than Type 0 and exhibit relative continuity between phase delays and advances with a cross over near the temperature minimum, whereas Type 0 phase resetting exhibits large abrupt shifts between advances or delays near the temperature minimum.

Circadian entrainment

As noted, photic (light) stimuli is the primary environmental synchronizer of the circadian system in humans. Factors such as prior light exposure (Hebert, Martin, Lee, & Eastman, 2002), timing of exposure (Lewy *et al.*, 1998; Khalsa, Jewett, Cajochen, & Czeisler, 2003; Minors, Waterhouse, & Wirz-Justice, 1991), temporal pattern of exposure (e.g., continuous versus intermittent light exposure) (Burgess, Revell, & Eastman, 2008; Gronfier, Wright, Jr., Kronauer, Jewett, & Czeisler, 2004; Rimmer *et al.*, 2000), and wavelength (Brainard *et al.*, 2001; Lucas *et al.*, 2003) can influence the changes mediated by light. Exposure to the environmental light-dark cycle influences the intrinsic timing of the circadian system (Wright, Jr., Hughes, Kronauer, Dijk, & Czeisler, 2001; Wright, Jr., Hull, Hughes, Ronda, & Czeisler, 2006) and the neurobiological systems modulated by the SCN. The light-dark cycle regulates physiology and behavior through both neural and hormonal mechanisms modulating circadian rhythms. Light influences neuroendocrine (e.g., melatonin and cortisol) and physiological (e.g., body temperature) markers of the circadian clock. In humans, the melatonin acrophase (timing of the melatonin peak) and the body temperature minimum typically occur during the biological night, when sleep is taking place. The influence of light can have phase shifting effects on physiology and behavior. Entrainment is the ability of the internal circadian system to synchronize to an external period of a synchronizing stimulus or T cycle (e.g., the 24h light-dark cycle). In general, the average period, or tau (τ), of the internal circadian system in humans is slightly longer than 24h (Czeisler *et al.*, 1999; Duffy *et al.*, 2011; Wright, Jr. *et al.*, 2001; Wyatt, Ritz-De Cecco, Czeisler, & Dijk, 1999). Due to the longer circadian period in most individuals the range of daily adjustments needed to entrain or synchronize to the 24h day requires a daily phase advance of the circadian system whereas some individuals with an intrinsic period shorter

than 24 hours requires a daily phase delay in the shift of the circadian system (Duffy & Wright, Jr., 2005). Compiled data from Duffy and Wright (2005) suggest the average daily adjustment for entrainment in young adults whose period was assessed using a forced desynchrony protocol ranged from a daily 12min delay to a 19min advance. This relationship is called the phase angle of entrainment. Differences in the phase angle of entrainment between individuals may be due in part to the individual differences of the intrinsic circadian period (Wright, Jr., Gronfier, Duffy, & Czeisler, 2005). Inability to entrain to the environmental time (e.g., due to shift work, jet lag, circadian sleep disorders) can result in adverse psychological, neurobehavioral, and physiological consequences (Barger, Lockley, Rajaratnam, & Landrigan, 2009; Knutsson, Akerstedt, Jonsson, & Orthgomer, 1986; Scheer, Hilton, Mantzoros, & Shea, 2009; Srivastava, 2011). The phase of an intrinsic rhythm, in general, maintains a consistent relative relationship to the external, environmental period (Pittendrigh & Daan, 1976).

Photic stimuli

The influence of light on circadian physiology

In addition to the acute effects, light also has a phase shifting effect on physiology and behavior. Shifting of the circadian clock is characterized by shifts in the physiological and neuroendocrine markers. PRCs to light appear to be relatively conserved across species (Czeisler & Wright, Jr., 1999; Murphy & Campbell, 1996). Exposure to light in the evening elicits a delay in the circadian clock (phase delay) and conversely exposure to morning light elicits an advancing (phase advance) of the internal clock (Figure 2).

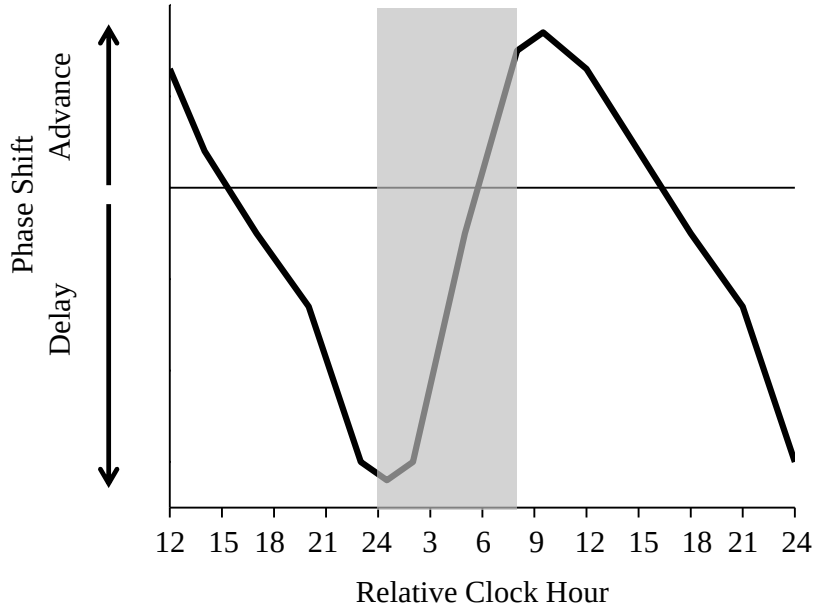

Figure 2. The PRC to a single 6.7h bright light exposure at 9,500lux. In general, evening bright light exposure near habitual bedtime produces the largest phase delay and morning bright light exposure near habitual waketime produces the largest phase advance. The shaded area represents habitual sleep time in an individual with a habitual bedtime of midnight

As most humans have an intrinsic period of slightly longer than 24h (Czeisler *et al.*, 1999; Duffy *et al.*, 2011; Wright, Jr. *et al.*, 2001; Wyatt *et al.*, 1999), light in the morning, which falls in the advancing portion of the PRC, will phase advance the human period to maintain synchronization with the environmental 24h day. The PRC to a single bright light pulse of 9,500lux for 6.7h produces the largest phase delay when exposure occurs near habitual bedtime (approximately 1-3h following the DLMO) and the largest phase advance occurs near the habitual wake time (approximately 10h following the DLMO) (Khalsa *et al.*, 2003). In general, the brighter the light the larger the phase shift (Boivin, Duffy, Kronauer, & Czeisler, 1996), although there is a non-linear saturating function for the phase shifting effect of light (Zeitzer, Dijk, Kronauer, Brown, & Czeisler, 2000). The influence of light on circadian phase can also be influenced by wavelength of light (Lockley, Brainard, & Czeisler, 2003), prior light history

(Hebert *et al.*, 2002; Jasser, Hanifin, Rollag, & Brainard, 2006; Smith, Schoen, & Czeisler, 2004), and even the absence of light (Buxton, L'Hermite-Baleriaux, Turek, & Van Cauter, 2000). When bright light exposure occurs during the biological daytime and the timing of sleep is held constant, the degree to which the circadian phase is shifted is reduced (Dumont & Carrier, 1997). Bright light (>100,000lux similar to mid-day looking at a bright blue sky) is able to shift the phase of the circadian clock. Lux is a measure of illumination to indicate the intensity of light. One lux is equivalent to the light of one candle one meter away from the eye. Though bright light can shift the circadian clock, light levels as low as ~12lux have been able to shift the circadian clock (Duffy & Wright, Jr., 2005; Wright, Jr. & Czeisler, 2002; Zeitzer, Kronauer, & Czeisler, 1997).

Figure 2 shows phase shifting response to a single pulse of light; however, intermittent exposures to bright light have also been reported to phase shift the human circadian clock (Gronfier *et al.*, 2004). In a study by Rimmer *et al.* (2000) , as little as ~5.3min intermittent bright light exposure (~10,000lux, similar to light intensity at sunrise or sunset) ~1.5h after the core body temperature minimum, at 25min intervals for 5h, was able to produce a phase advance of the human circadian clock to a similar degree as a continuous bright light exposure for 5h. Intermittent bright light exposure also had similar phase advancing effects with 25min (31% total light exposure) and 90min (63% total light exposure) intervals when compared to the continuous light exposure condition. Findings from these studies suggest that there is a nonlinear relationship between relative duration of light exposure and the phase shifting effect. These data suggest that individuals receiving bright light during the most sensitive periods of circadian phase resetting (e.g. medical residents, shift-workers) may be at a higher risk for circadian disruption.

The spectral composition of light stimuli in humans, similar to non-humans (Pohl, 1999; McGuire, Rand, & Wurtman, 1973), influences the circadian clock timing. The circadian clock in humans has been found to be most sensitive to short wavelength (blue portion of the visual light spectrum) light (Lockley *et al.*, 2003; Revell & Eastman, 2005; Warman, Dijk, Warman, Arendt, & Skene, 2003; Wright, Lack, & Kennaway, 2004). For example, a study by Warman and colleagues (2003) reported dim blue light produced similar phase advances when compared to bright polychromatic white light. When comparing monochromatic blue light to polychromatic white light, there was a significantly enhanced circadian response (light-induced melatonin suppression) to polychromatic light (Brainard *et al.*, 1985; Revell & Skene, 2007). These findings indicate that in polychromatic light, the presence of additional wavelengths present may be contributing to the greater light-induced melatonin suppression. Recent findings suggest that using blue-enriched polychromatic light is more effective at phase delaying or phase advancing the circadian clock than white polychromatic light of a lower correlated color temperature (CCT) (Gooley *et al.*, 2010; Smith & Eastman Smith, 2009; Revell, & Eastman, 2009).

Light induced regulation of circadian clock genes is thought to be driving the molecular mechanism associated with the shifting of circadian phase. Findings from a study by Miyake and colleagues (2000) indicate that a 30min bright light (1000lux) pulse induces elevations in *Per1* and *Per2* (weakly) mRNA in the SCN of rats. In a study conducted by Paul and colleagues (2004), transduction of light to the pineal was blocked with a neurotoxin that specifically blocked sodium-dependent action potentials while light input to the clock genes *Per1* and *Per2* in the SCN was maintained. They also found a loss of acute melatonin suppression while still eliciting a behavioral phase shift in circadian phase (Paul *et al.*, 2004). Findings from these

studies suggest the molecular mechanism of how light influences the shifting of the circadian clock through circadian clock genes.

Non-photic stimuli

Mainly animal models have explored the effects of non-photic synchronizers and their influence on the ability of the circadian clock to phase shift. These models have included synchronizers such as exogenous melatonin, exercise, sleep deprivation, external temperature changes, and social interactions (Challet, Turek, Laute, & Van Reeth, 2001; Hastings, Duffield, Smith, Maywood, & Ebling, 1998; Mistlberger, 1992; Mrosovsky, 1996). Non-photic stimuli such as melatonin (Hack, Lockley, Arendt, & Skene, 2003; Lewy, Ahmed, Jackson, & Sack, 1992; Lockley *et al.*, 2000) and exercise (Baehr *et al.*, 2003; Barger, Wright, Jr., Hughes, & Czeisler, 2004; Miyazaki, Hashimoto, Masubuchi, Honma, & Honma, 2001) have also been explored in human models. Exercise, when compared to photic stimuli, produces relatively small phase shifts (Youngstedt, Kripke, & Elliott, 2002).

Melatonin

Immediate release melatonin (2mg) reaches peak levels occurring within 30-75min after administration (Mandel, 2002) with an average half-life of 3-7h (Kaplan *et al.*, 1997). Exogenous melatonin administration was first reported in humans to shift the circadian clock by Arendt and colleagues (1985). Similar to light, exogenous melatonin can shift circadian timing according to a PRC (Figure 3) (Burgess, Revell, Molina, & Eastman, 2010; Burgess *et al.*, 2008; Lewy *et al.*, 1998).

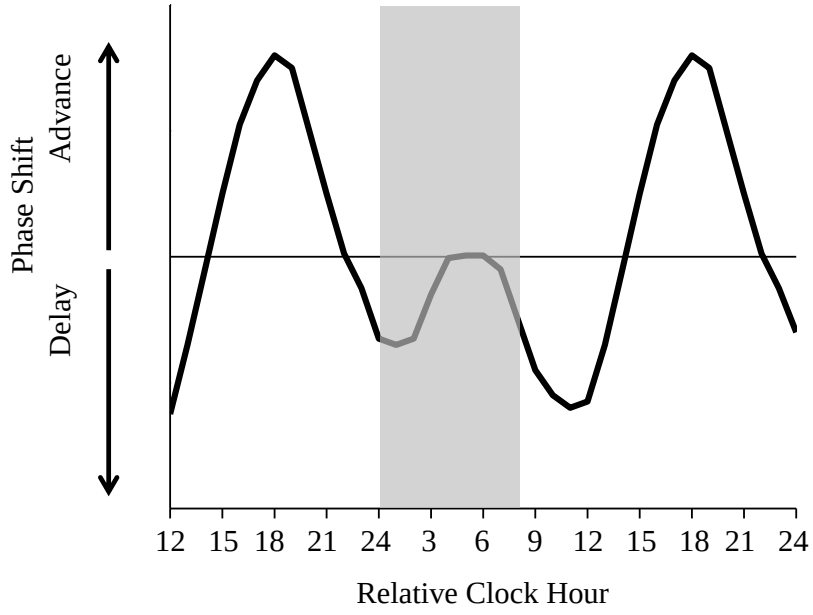

Figure 3. The PRC to 3-5mg of exogenous melatonin. In general, melatonin administration in the late afternoon/ early evening produces the largest phase advance and administration in the morning produces the largest phase delay. The shaded area represents habitual sleep time in an individual with a habitual bedtime of midnight.

Lewy and colleagues (1998) reported phase shifting responses of exogenous melatonin as illustrated in a melatonin phase response curve after administering 0.5mg exogenous melatonin in an outpatient research trial over 4 consecutive days. When exogenous melatonin was administered in the morning, the endogenous melatonin rhythm was delayed. Exogenous melatonin administered in the late afternoon/early evening was able to advance the endogenous melatonin rhythm. The PRC of light compared to PRC of melatonin is approximately 12h out of phase (Lewy *et al.*, 1998). The shape of the melatonin PRC has since been replicated with different methods of administration (e.g., I.V administration (Zaidan *et al.*, 1994)) and dose (Burgess *et al.*, 2010). A recent study by Burgess and colleagues replicated and expanded upon the previously generated PRCs of exogenous melatonin by comparing a single daily dose of either 0.5mg or 3mg for 3 consecutive days. On average, the magnitude of the phase shifts are

similar, although the lower dose peak phase advances and phase delays occur approximately 2h later than those of the higher dose. These findings suggest the most sensitive time of administration of exogenous melatonin for maximal phase advances and phase delays for lower dosages is later than for higher dosages.

Exogenous melatonin has been reported to induce phase advancing and phase delaying effects in core body temperature rhythms (Deacon & Arendt, 1995; Krauchi, Cajochen, Mori, Graw, & Wirz-Justice, 1997). The phase advancing effects have also been shown in the endogenous melatonin rhythm (Mallo *et al.*, 1988). In fact, administration of exogenous melatonin has been shown to phase advance the endogenous melatonin rhythm in a dose dependent manner (Deacon & Arendt, 1995). When controlling for light exposure, exogenous melatonin (5mg) in the evening advanced the endogenous melatonin rhythm (Wirz-Justice *et al.*, 2004), but was unable to induce a phase delay when administered in the morning (Wirz-Justice, Werth, Renz, Muller, & Krauchi, 2002).

Exogenous melatonin during the biological day when endogenous levels are low has hypnotic effects. Melatonin has been shown to affect subjective sleepiness (Dollins, Zhdanova, Wurtman, Lynch, & Deng, 1994) and impair performance when administration is in the early morning/late afternoon (Dollins *et al.*, 1994; Rogers, Phan, Kennaway, & Dawson, 1998; Graw *et al.*, 2001).

Melatonin induced regulation of circadian clock genes has recently been the focus of molecular mechanisms associated with the shifting of circadian phase. Membrane bound melatonin receptors in mammals have been identified in many areas of the body; however, melatonin receptors located on the SCN have been a prime target for the phase shifting mechanism behind exogenous melatonin. Findings from a study by Poirel *et al.* that assessed the

expression of clock genes (*Per* 1-3, *Bmal*1, *Cry* 1, *AVP* mRNA) after melatonin injection, suggest that there is no immediate change in expression ; however, there are significant increases in expression 24 hours later (*Per* 1,3, *Bmal*1, *AVP* mRNA) (Poirel *et al.*, 2003). A following study assessd the influence of melatonin injection on nuclear orphan receptors (*Rev-Erbα*, *Rorα*, and *Rorβ*) associated with *Bmal*1 expression. Results showed a phase advance in expression of *Rev-Erbα* mRNA (Agez, Laurent, Pevet, Masson-Pevet, & Gauer, 2007). Data from these animal models taken together with the clock gene expression observed with photic stimuli suggest that there may be differential clock gene regulation that is dependent upon the synchronizing stimuli.

Caffeine

Caffeine is a methylxanthine and an adenosine antagonist that is widely used to promote wakefulness. Adenosine is a sleep promoting molecule that accumulates in the brain during the biological day, dissipates with sleep, and is considered a marker of sleep pressure. Additionally, there is evidence that adenosine binds to adenosine receptors on the pineal gland and can increase melatonin levels (Gharib *et al.*, 1989). Therefore, caffeine is thought to influence wakefulness via its action as an adenosine antagonist in several brain areas. Caffeine is most commonly consumed in coffee, tea, soft drinks, and chocolate and an individual's sensitivity to caffeine can be influenced by a number of factors (e.g. age, weight, prior caffeine use, genetic variation in adenosine metabolism). Caffeine, when administered in pill form, is rapidly absorbed in the gastrointestinal track with peak levels occurring within 30-40 min (Liguori, Hughes, & Grass, 1997; Mumford *et al.*, 1996). The half-life of caffeine is on average 5-6h (Brachtel & Richter, 1992). During waking, caffeine has been suggested to decrease the accumulation of sleep propensity. However, caffeine can have a negative impact on sleep quality (e.g. decreased total sleep time, increased sleep fragmentation, increased sleep onset latency and

wake after sleep onset) (Bonnet & Arand, 1992; Carrier *et al.*, 2007; Lieberman, Tharion, Shukitt-Hale, Speckman, & Tulley, 2002).

The phase shifting effects of caffeine have been reported by measuring electrical activity of SCN slices in vitro (Diaz-Munoz *et al.*, 1999). The influence caffeine has on the SCN was reported to be due to an increase in intracellular calcium via stimulation of ryanodine receptors (RyR), as caffeine is a RyR agonist. A recent study by Pheffer and colleagues (2009) compared mice with impaired clock gene expression to wild type mice. They reported that *RyR* expression is activated by CLOCK/BMAL and inhibited by mCRY1. Using two-photon microscopy, they describe decreased calcium in response to caffeine in the BMAL1 impaired mice compared to wild type (Pfeffer *et al.*, 2009).

Another potential mechanism for the phase shifting effects of caffeine is via adenosine receptors located on pineal cells (Sarda, Gharib, Reynaud, Ou, & Pacheco, 1989). Caffeine, an adenosine antagonist, was able to block adenosine induced melatonin increases in the rat pineal (Babey, Palmour, & Young, 1994). A similar finding was presented by Wright and colleagues (1997) in humans. They reported that the administration of 200mg of caffeine was able to reduce melatonin levels (Wright, Jr. *et al.*, 1997). Adenosine is also associated with brain regulatory centers that facilitate sleep/wake behavior. It has been shown to increase in the basal forebrain and brain stem with increasing time awake and this increase can be blocked by caffeine (Shearman *et al.*, 2000; Shibui, Uchiyama, & Okawa, 1999). Future studies conducting a systematic examination on the influence of caffeine will be needed to determine if caffeine itself or in combination with other synchronizing stimuli is able to shift the human circadian clock.

Combination of photic and non-photic stimuli

The integration of photic and non-photic stimuli in data from animal models suggests that influence of the combination is summed, such that the combination is larger than the individual effects, but the phase shifting effects do not necessarily equal a direct sum of the contributing parts (Benloucif, Masana, Yun, & Dubocovich, 1999; Lall & Biello, 2002; Yannielli & Harrington, 2004). Wirz-Justice and colleagues (2004) found that when photic and non-photic stimuli were combined, in healthy young men, the combination of an evening single pulse of bright light (timed to induce a phase delay) and a single dose of exogenous melatonin (timed to induce a phase advance) lead to no change in salivary melatonin phase as compared to the placebo group (dim light plus placebo pill). Findings from this study, similar to the animal models, support the idea that photic and non-photic stimuli are integrated as well in humans. To what degree each of these stimuli contributes to the phase shifting effects in humans still remains unknown.

A previous study by Wright and colleagues looked at the combination of bright light exposure and caffeine and found that salivary melatonin levels decreased and tympanic temperature increased to a greater extent than in either condition alone (Wright, Jr. *et al.*, 1997; Wright, Jr., Myers, Plenzler, Drake, & Badia, 2000). Babkoff and colleagues (2002) replicated the findings by Wright and colleagues. The study looked at the influence of caffeine and a single pulse of light during simulated shift work and found that individually light and caffeine were able to decrease salivary melatonin levels (42% and 21.3% respectively) and the combination of bright light and caffeine decreased salivary melatonin levels to 47.4% (Babkoff *et al.*, 2002). Further research is needed to determine if caffeine has phase shifting properties and if so, what

the most sensitive timing of caffeine administration would be to produce desired phase shifting effects in humans.

Summary

This literature review has covered research on how photic and non-photic information influence the circadian system and the potential mechanisms by which this occurs. Briefly, light exposure regulates transcriptional/translational regulatory feedback loops of the circadian clock, located in the SCN. Changes in gene expression are able to regulate the oscillatory activity of the SCN, shifting the endogenous rhythm to be synchronized to the 24h light-dark cycle. Light exposure suppresses melatonin synthesis by inhibiting the sympathetic stimulation of the pineal gland. Circulating melatonin is able to feedback to the SCN due to the presence of melatonin receptors on the SCN. Light stimuli in the early or late biological night are able to facilitate a phase delay or phase advance, respectively in the endogenous melatonin rhythm. The administration of endogenous melatonin is also able to phase shift the circadian clock. Exogenous melatonin that is given when endogenous levels are low (i.e. early in the biological night or in the early morning) can advance and delay the endogenous melatonin rhythm respectively. Caffeine, a common stimulant, is thought to also influence the SCN. Caffeine is an adenosine antagonist, and is thought to influence the SCN by regulation of melatonin in the pineal gland. The administration of caffeine in the evening is able to reduce endogenous melatonin levels. Phase shifting effects of caffeine on human circadian rhythms still remain unexplored. There is a need for more properly controlled studies to assess the integration of photic and non-photic stimuli and elucidate the relative contributions of the stimuli as well as to expand the types of stimuli associated with shifting the circadian clock. Finally, understanding

the integration of photic and non-photic stimuli will provide more informed and efficient recommendations for clinicians in the treatment of circadian rhythm sleep disorders.

Dissertation aims

The aim of this dissertorial work was to understand how photic and non-photic stimuli influence the human circadian system. Specifically, the aims of this dissertation were to: 1) determine if morning bright light exposure and evening exogenous melatonin administration would produce a circadian phase advance that would be integrated by the human circadian system to produce a greater shift than either stimulus alone; 2) determine whether caffeine administration induces a circadian phase advance of the human circadian clock; 3) determine if evening caffeine administration induces a circadian phase delay of the human circadian clock; and 4) determine if evening bright light exposure and evening caffeine administration produces a circadian phase delay that would be integrated by the human circadian system to produce a greater shift than either stimulus alone. The following hypotheses were tested: 1) morning bright light exposure and evening exogenous melatonin administration would produce a circadian phase advance that would be integrated by the human circadian system to produce a greater shift than either stimulus alone; 2) caffeine administration would induce a circadian phase advance of the human circadian clock; 3) evening caffeine administration would induce a circadian phase delay of the human circadian clock; and 4) evening bright light exposure and evening caffeine administration would produce a circadian phase delay that would be integrated by the human circadian system to produce a greater shift than either stimulus alone.

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CHAPTER 2

ADVANCING THE HUMAN CIRCADIAN CLOCK WITH THE INTEGRATION OF PHOTIC AND NON-PHOTIC PHASE SHIFTING STIMULI

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Non-technical summary

Shifting the internal body clock is needed to adapt human physiology and behavior to jet travel and for the treatment of circadian sleep disorders. Both photic and non-photic time cues have been shown to influence the timing of the body clock with light being the dominant environmental synchronizer. We found that morning bright light exposure combined with early evening exogenous melatonin administration shifted the internal body clock earlier than did either stimulus alone. Findings also suggest that caffeine can influence the human body clock. Understanding how photic and non-photic stimuli influence the human circadian system will help determine the most effective and efficient ways to shift the human circadian clock.

Abstract

Photic and non-photic stimuli have both been shown to shift the phase of the human circadian clock. We sought to extend the understanding of how photic and non-photic time cues are integrated by the human circadian system by assessing the phase advancing effect of evening exogenous melatonin, alone and in combination with morning bright light exposure. We also sought to determine whether morning caffeine administration induces a circadian phase advance. In two experiments we studied 47 participants. In experiment 1, 36 participants aged 22±4y (mean ± SD), and in experiment 2, 11 participants aged 22±3y (mean ± SD), completed an ~12 daylong protocol. The effects of dim light (~1.9lux)-placebo, dim light-melatonin (5mg), bright light (~3000lux)-placebo, and bright light-melatonin in experiment 1 and of dim light-caffeine (2.9mg/kg) in experiment 2, on circadian phase was assessed by the change in the salivary dim light melatonin onset (DLMO) prior to and following treatment under constant routine conditions. Melatonin or placebo was administered 5.75h prior to habitual bedtime and 3h of bright light exposure started 1h prior to habitual waketime. Caffeine was administered 1h prior to habitual waketime. Our findings show that morning bright light combined with early evening exogenous melatonin induces a greater phase advance of the human circadian clock than either treatment alone. Findings also provide preliminary evidence that caffeine may be able to phase advance the human circadian clock. These findings have implications for the adaptation to jet travel and for the treatment of circadian sleep disorders.

Introduction

The mammalian master circadian clock is located in the suprachiasmatic nucelus (SCN) of the hypothalamus (Moore & Eichler, 1972; Swaab, Fliers, & Partiman, 1985). The SCN provides environmental and biological timing information to the rest of the body so that physiology and behavior are coordinated for optimal functioning relative to the time of day (DeCoursey, Walker, & Smith, 2000; Pittendrigh, 1993). The SCN receives input about environmental time through photic pathways via rod, cone and melanopsin photoreceptors in the retinae (Chen, Badea, & Hattar, 2011; Guler *et al.*, 2008). The SCN also receive input about behavioral and physiological state through non-photic pathways (e.g., serotonergic input from the raphe nucleus; melatonin from the pineal gland) (Challet & Pevet, 2003; Hastings *et al.*, 1992; Maywood & Mrosovsky, 2001; Novak, Ehlen, & Albers, 2008). Misalignment between environmental time and internal biological timing (e.g., shift work, jet lag, circadian sleep disorders) can result in adverse psychological, neurobehavioral, and physiological consequences (Barger, Lockley, Rajaratnam, & Landrigan, 2009; Knutsson, Akerstedt, Jonsson, & Orthgomer, 1986; Scheer, Hilton, Mantzoros, & Shea, 2009; Srivastava, 2011). In humans, photic and non-photic stimuli have both been used to phase shift the human circadian clock however, there is limited information about the integration of phase shifting stimuli by the circadian clock in humans. Findings from research in non-humans suggest the combination of photic and non-photic stimuli interact to increase or attenuate the magnitude of a circadian phase shifts (Benloucif, Masana, Yun, & Dubocovich, 1999; Lall & Biello, 2002; Lall & Biello, 2003; Smart & Biello, 2001; Yannielli & Harrington, 2004). In humans, Wirz-Justice *et al.* (2004) examined the combination of exogenous melatonin, timed to advance the circadian clock, and bright light exposure, timed to delay the circadian clock, and found an additive interaction such that the

combination resulted in no phase shift relative to the control condition. These findings suggest that photic and non-photic stimuli may be integrated by the human circadian system. We hypothesized that photic and non-photic information are integrated by the circadian timing system in humans and that combinations of properly timed stimuli will induce a greater phase shifting response than individual stimuli. Such potential integration of photic and non-photic stimuli by the human circadian clock has important implications for the treatment of circadian sleep disorders and for rapid circadian adaptation to jet lag and shift work schedules.

Although light is the primary synchronizer of the SCN to the external environment (Czeisler & Wright, Jr., 1999; Duffy & Wright, Jr., 2005), non-photic stimuli, such as activity, exercise, restricted food availability, and exogenous melatonin have also been shown to shift the timing of the mammalian circadian system (Krauchi, Cajochen, Mori, Graw, & Wirz-Justice, 1997; Mrosovsky, 1996; Youngstedt, Kripke, & Elliott, 2002; Stephan, Swann, & Sisk, 1979). In humans, both light and melatonin have been found to phase shift the circadian system and in the majority of studies conducted to date, subjects were exposed to multiple days of light exposure or melatonin administration Burgess, Crowley, Gazda, Fogg, & Eastman, 2003;Burgess, Revell, & Eastman, 2008; Burgess, Revell, Molina, & Eastman, 2010; Crowley, Lee, Tseng, Fogg, & Eastman, 2003; Duffy & Czeisler, 2009; Burgess, Crowley, Gazda, Fogg, & Eastman, 2003; Rajaratnam, Cohen, & Rogers N.L, 2009; Sack *et al.*, 2007a). Fewer investigations have examined the influence of one session of light exposure or one dose of melatonin (Buresová, Dvoráková, Zvolsky, & Illnerová, 1991; Deacon & Arendt, 1996; Gronfier, Wright, Jr., Kronauer, Jewett, & Czeisler, 2004; Khalsa, Jewett, Cajochen, & Czeisler, 2003; Wright, Jr. & Czeisler, 2002; Wirz-Justice *et al.*, 2004). Nonetheless, findings in general are consistent in showing that bright light exposure in the evening produces large phase delays and bright light

exposure in the early morning produces large phase advances. Nearly 12 hours out of phase with the phase response curve (PRC) to light (Lewy *et al.*, 1998), melatonin administration in the late afternoon/early evening produces a large phase advance and administration in the early morning following habitual wake time produces a large phase delay (Burgess *et al.*, 2010). Few studies have directly compared the phase resetting response to one day of light exposure and one day of melatonin administration (Paul *et al.*, 2011).

We conducted two experiments using controlled circadian protocols to evaluate the phase resetting response of the human circadian clock to photic and non-photic stimuli. In the first experiment, the influence of one session of bright light exposure and one dose of exogenous melatonin, alone and in combination, were examined. This first experiment was based upon previous research in humans showing that light and melatonin alone can phase shift circadian rhythms (Buresová *et al.*, 1991; Dawson, Lack, & Morris, 1993; Deacon & Arendt, 1995; Honma & Honma, 1988; Kennaway *et al.*, 1987; 1993; Khalsa *et al.*, 2003; Krauchi *et al.*, 1997; Mallo *et al.*, 1988; Minors, Waterhouse, & Wirz-Justice, 1991; Sack, Lewy, & Hoban, 1987; Van Cauter *et al.*, Wirz-Justice *et al.*, 2004; Wirz-Justice, Werth, Renz, Muller, & Krauchi, 2002) and findings from animal and human studies showing that melatonin can attenuate light induced phase shifts (Cagnacci, Soldani, & Yen, 1997; Deacon & Arendt, 1996; Mrosovsky, 1996; Wirz-Justice *et al.*, 2004). The first experiment tested the hypothesis that the circadian phase advance induced by the combination of morning bright light exposure and late afternoon exogenous melatonin administration would be integrated by the human circadian system and would induce a greater phase shift than either stimulus alone. Four experimental conditions were compared: dim light-placebo (DLP), dim light-melatonin (DLM), bright light-placebo (BLP) and bright light-melatonin (BLM). Timing of photic and non-photic stimuli was based on light

and melatonin PRCs and was selected to induce large phase shifts. Specifically, to achieve a large phase advance, a single 3h light exposure with an intensity of ~3,000lux was applied in the early morning (Khalsa *et al.*, 2003; Zeitzer, Dijk, Kronauer, Brown, & Czeisler, 2000) and a single 5mg dose of melatonin was administered in the evening (Burgess *et al.*, 2010; Lewy *et al.*, 1998).

As there are no published studies on caffeine and phase shifting in humans, the second experiment was designed to be exploratory in nature and tested whether caffeine administration could induce a circadian phase advance of the human circadian clock since in non-humans, caffeine has been shown to influence circadian physiology. Specifically, caffeine has been shown to phase shift the electrical activity rhythm of the SCN under in vitro conditions (Diaz-Munoz *et al.*, 1999; Ding *et al.*, 1998). Caffeine has also been shown to influence c-*fos* expression in the SCN (Shearman & Weaver, 2001). Furthermore, in humans, caffeine has been shown to acutely reduce endogenous melatonin (Babkoff, French, Whitmore, & Sutherlin, 2002; Wright, Jr., Badia, Myers, Plenzler, & Hakel, 1997; Wright, Jr., Myers, Plenzler, Drake, & Badia, 2000), suggesting caffeine may influence human circadian phase. We tested the hypothesis that administration of 2.9mg/kg caffeine in the early morning would induce a circadian phase advance.

Methods

Ethical approval

Study procedures were approved by the Institutional Review Board at the University of Colorado at Boulder and the Scientific Advisory and Review Committee of the Colorado Clinical and Translational Sciences Institute. Subjects gave written informed consent and were

compensated for their participation. The protocol was in accordance with the standards set by the latest revision of the *Declaration of Helsinki*.

Subject characteristics

We studied a total of 48 subjects in two experiments. Thirty-six young healthy subjects (18 females) aged 22.0 ± 3.8 years, BMI 22.3 ± 2.1 (mean ± SD) participated in experiment 1. Eleven young healthy subjects (5 females) aged 21.6 ± 2.5 years, BMI 22.1 ± 2.1 (mean ± SD) participated in experiment 2. Prior to the study, subjects underwent detailed health screening. Health of subjects was determined by medical evaluation at the Clinical and Translation Research Center (CTRC) and Sleep and Chronobiology Laboratory at the University of Colorado at Boulder based on physical exam, blood chemistries, clinical electrocardiography, and medical, psychiatric and sleep histories. Exclusion criteria included: known medical, psychiatric, or sleep disorders, abnormal blood chemistries, illicit drug or nicotine use; habitual sleep duration <7h or >9h, medication use (exception for oral contraceptives), body mass index outside the range of 18.5 to 27, shift work or living below the altitude 1600m in the year prior, and travel across more than one time zone in the three weeks prior to in-laboratory procedures.

Subjects maintained a regular ~8h sleep-wakefulness schedule based on their habitual sleep and waketimes for one week prior to the in-laboratory study. Regular sleep-wakefulness schedules were verified via sleep logs, time-stamped voice-recorder of bed and waketimes, and wrist actigraphy recordings (Actiwatch-L, Minimitter, Bend, OR). Subjects were proscribed to refrain from over-the-counter medications, supplements, and caffeine for two weeks prior, naps one week prior, exercise three days prior, and alcohol two days prior to the in-laboratory protocol. Subject's self-reported compliance with the above requests. Additionally, upon admission to the in-laboratory protocol, urine toxicology and an alcohol breath test (Lifeloc

Technologies Model FC10, Wheat Ridge, CO) were performed and female subjects were given a pregnancy test to verify absence of pregnancy. Five of the female subjects were taking oral contraceptives.

In-laboratory protocol

Subjects were studied individually in specially designed sleep and circadian research suites that provided an environment free of external time cues for 5 calendar days. Following seven days of ambulatory monitoring as described above, subjects arrived at the Sleep and Chronobiology Laboratory ~4h prior to habitual sleep time for the in-laboratory protocol (Figure 1). All protocol events such as meal times, pill administration, light exposure, and sleep opportunities were scheduled relative to the subject's habitual wake time. Ambient temperature was maintained in thermoneutral range (~22.2 °C) and except for the bright light exposure conditions, lighting in the angle of gaze was maintained at dim levels (~1.9lux; ~0.6Watts/m^2) during scheduled wakefulness and darkness during scheduled sleep opportunities. Day 8 consisted of a habituation period followed by an 8h nighttime sleep opportunity. Days 9-10 consisted of a 28h modified constant routine (CR1) (Duffy & Wright, Jr., 2005). The constant routine protocol is used to estimate circadian phase while controlling for the influence of environmental and behavioral influences. Subject's maintained wakefulness while being exposed to dim light under bed rest conditions with the head of the bed raised to ~35° and were provided hourly snacks to distribute nutrition and fluid intake over the wakefulness period. Non-photic stimuli were administered on Day 9 for caffeine (experiment 2) and Day 10 for melatonin (experiment 1). Photic stimuli exposure was Day 10-11. This was followed by a second 19h constant routine (CR2) on Day 11 to reassess circadian phase.

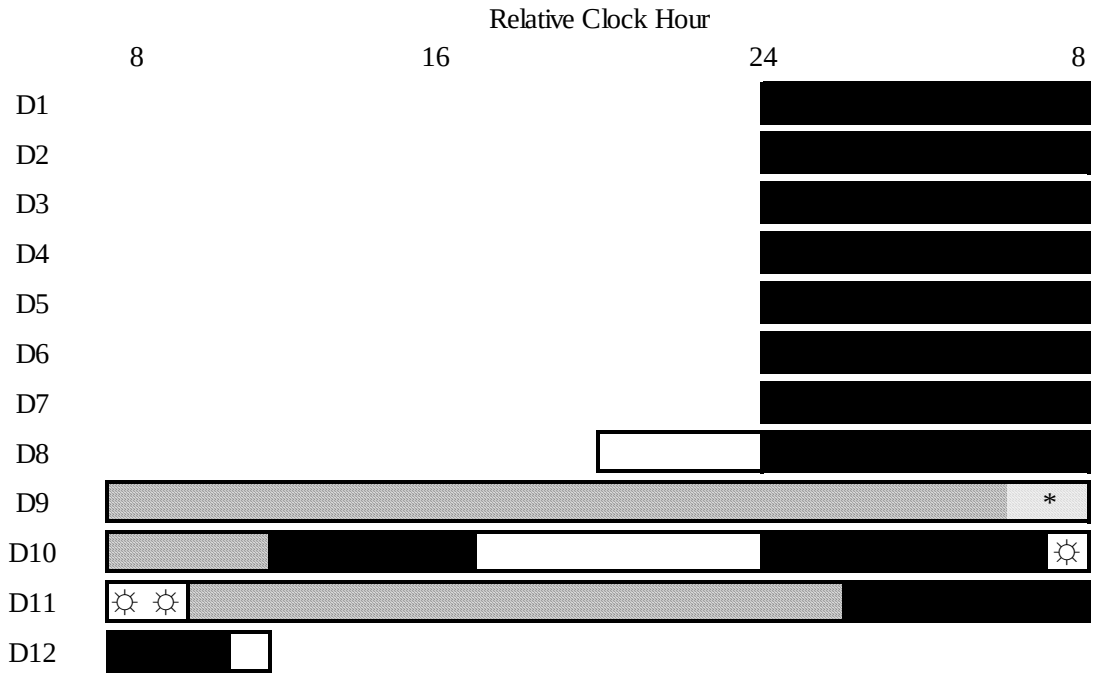

Figure 1. ~12 daylong phase shifting protocol. Schematic of in-laboratory protocol**.** Example in-laboratory protocol for a subject with a habitual bedtime at midnight. Black bars represent scheduled sleep opportunities and the hatched bars represent constant routines. The protocol consisted of 2 constant routines which were used to estimate circadian phase. In experiment 1, subjects received melatonin or rice-powder filled placebo (◊) ~5.75h prior to habitual bedtime and in experiment 2, caffeine (*) was administered 23h following habitual waketime. For both experiment 1 and 2, 3h exposure to bright or dim light (☼) beginning 1h prior to habitual waketime.

Experimental conditions

Non-photic stimuli

Pill administration was double-blind. Implementation of pill allocation was performed by the CTRC pharmacist who provided pills identical in appearance. Pills consisted of five capsules of either immediate release melatonin (5mg total dose; Life Extension Foundation, Inc.), caffeine (2.9mg/kg total dose; Gallipot, Inc, Mendota Heights, MN), or identical looking capsules of rice flour placebo. The allocation sequence was concealed until interventions were assigned and data were prepared for statistical analysis.

Photic stimuli

Subjects were exposed to either 3h of continuous bright light or dim light exposure beginning 1h prior to habitual waketime. Commercially available ceiling mounted fluorescent lamps (Sylvania Octron 32W T8 bulb) provided broad spectrum white light exposure, similar to natural, midday, daylight (6500-K color temperature). During the light exposure, subjects were under the direct supervision of research assistants who remained in the suite to ensure that the intended intensity of illumination was achieved. Subjects maintained constant posture while alternating between fixing their gaze on a target for 6min or free gaze for 6min. Average light intensities during the fixed gaze were 2984 ± 367lux (~7Watts/m^2) for the bright light conditions and ~1.9 ± 0.4lux (~0.6Watts/m^2) for the dim light conditions.

Experiment 1 - melatonin and bright light

Subjects were randomly assigned to one of four conditions; dim light-placebo (DLP), dim light-melatonin (DLM), bright light-placebo (BLP), bright light-melatonin (BLM). Pill administration of either placebo or 5mg melatonin was scheduled in the late afternoon ~5.75h prior to habitual bedtime.

Experiment 2 - caffeine

Subjects received caffeine (2.9mg/kg) during CR1 at 23h following habitual waketime. Caffeine (DLC) was administered in dim light (~1.9lux; ~0.6Watts/m^2).

Experiments 1 and 2 were tested concurrently and subjects were informed that they could receive melatonin, caffeine or placebo, and therefore the DLP condition from experiment 1 was used as the control condition for the exploratory experiment 2.

Circadian phase assessment and analysis

Salivary melatonin was collected every 30min at night and every 60min during the day (hours 3-10 of habitual wakefulness) of each constant routine. Collected saliva was centrifuged and then frozen at -80°C until assayed. Salivary melatonin concentration was determined by ELISA assay according to manufacturer instructions (IBL International, Hamburg, Germany). Circadian phase shifts were determined by the change in the timing of the salivary dim-light melatonin onset (DLMO) between CR1 and CR2. The salivary DLMO was defined as the linearly interpolated time point when melatonin levels exceeded and remained two standard deviations above the stable baseline mean (Benloucif *et al.*, 2005; Lewy, Cutler, & Sack, 1999). Circadian phase shifts for the four experimental conditions in experiment 1 were analyzed with a mixed model ANOVA assigning drug and light conditions as fixed factors. Planned one-tailed independent t-tests were used to determine significance for our directional hypotheses: Specifically we hypothesized: 1) that melatonin and bright light experimental conditions would induce significant phase advance shifts compared to the dim light-placebo control condition, which was expected to show a delay drift due to the on average longer than 24h circadian period (Czeisler *et al.*, 1999; Duffy *et al.*, 2011; Wright, Jr., Hughes, Kronauer, Dijk, & Czeisler, 2001; Wyatt, Ritz-De Cecco, Czeisler, & Dijk, 1999); 2) that bright light-placebo would induce a significant phase advance shift compared to dim-light melatonin; and 3) that bright light-melatonin would induce a significant phase advance shift compared to dim light–melatonin and bright light-placebo. Circadian phase shifts for the caffeine condition in experiment 2 were analyzed with a t-test for independent samples compared to the dim light-placebo condition. We also analyzed changes in DLMO from a zero baseline using a non-planned two-tailed single sample t-test. In addition to the above statistical tests, effect sizes (Cohen *d′*) were calculated to

determine the size of phase resetting effects. Standard interpretations of effect size were used (Cohen, 1988) (small, d'=0.2; moderate, d'=0.5; large, d'=0.8). To test whether the phase angle of entrainment was similar among individuals, the DLMO to bedtime phase angle of entrainment was calculated. Paired t-tests were used to assess differences among individuals.

Results

Experiment 1 - melatonin and bright light

Figure 2 shows circadian phase shifts for individual subjects and the condition means for the melatonin and light conditions. The phase angle of entrainment was not significantly different among individuals. Significant main effects for bright light $F(1, 32) = 14.04$, $p < 0.001$ and for melatonin $F(1,32) = 10.58$, $p < 0.01$ were observed for circadian phase shifts. The interaction effect between light exposure and pill condition was not significant $F(1,32) = 0.29$, $p= 0.60$. We found a mean phase delay in the dim light-placebo (DLP) condition of ~26min. Dim light-melatonin (DLM), bright light-placebo (BLP), and bright light-melatonin (BLM) significantly advanced circadian phase compared to DLP ($p < 0.05$) with large effect sizes, d'= 1.40, 1.53, and 2.81, respectively. There were no statistical differences found between DLM and BLP ($p = 0.37$) conditions with a small effect size, d'= 0.16. The largest phase advance shift was found for the bright light-melatonin (BLM) condition, which induced a phase shift significantly larger than DLM and BLP ($p < 0.05$) with large effect sizes , d'= 1.12 and 0.93, respectively.

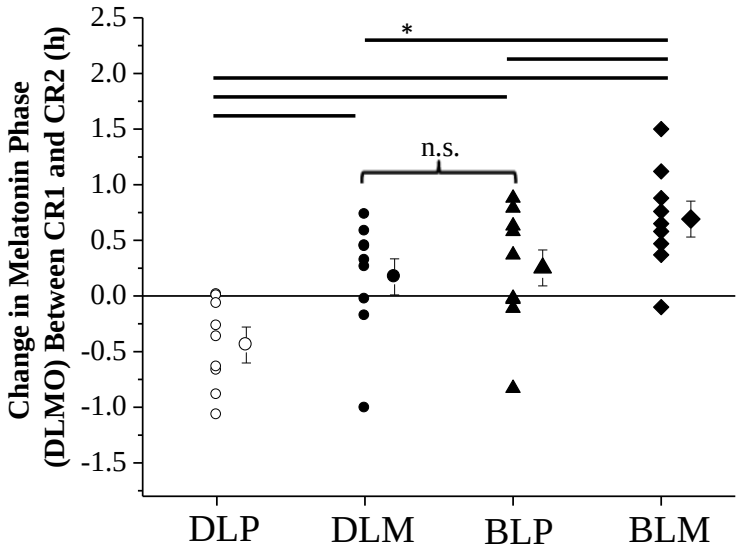

Figure 2. Phase resetting response to bright light and melatonin. On the y-axis, phase advances are plotted as positive values and phase delays are plotted as negative values with a value of zero representing no phase shift. Plotted are phase shifts for individual subjects within each condition as well as the condition mean±SEM. DLP dim light-placebo, DLM dim light-melatonin, BLP bright light-placebo, BLM bright light-melatonin. Lines represent significant effects between conditions at either end of the line ($p<0.05$). On average, DLM, BLP and BLM induced ~37min, ~41min and ~68min phase advances relative to DLP, respectively.

Experiment 2 – caffeine

Figure 3 shows the average phase shift for the DLP control condition and the dim light-caffeine (DLC) condition. The phase angle of entrainment was not significantly different among individuals. The main effect for caffeine was not significant $t(18) = 1.42$, $p = 0.09$, with a moderate effect size, $d' = 0.67$. However, the drift in DLMO for the DLP condition was significantly greater than zero, single sample t-test, $t(8) = -3.33$, $p = 0.01$, whereas the drift in DLMO for the DLC condition was not statistically different from zero $t(11) = -1.14$, $p = 0.26$.

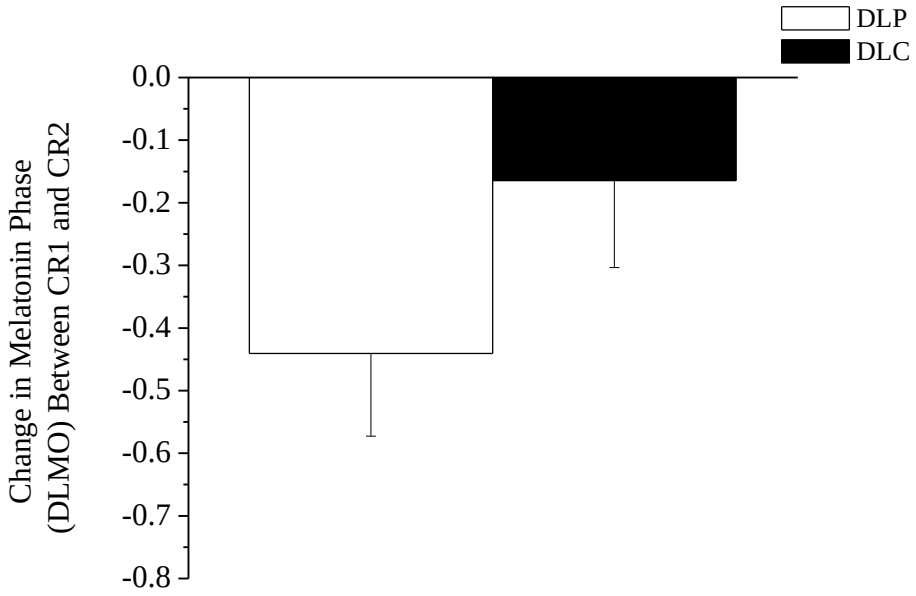

Figure 3. Phase resetting response to caffeine. Plotted are the average phase delays mean±SEM for caffeine and placebo. DLP dim light-placebo, DLC dim light-caffeine.

Discussion

Consistent with our hypotheses, the current findings indicate that a single exposure to 3h of morning bright light combined with a single 5mg dose of evening exogenous melatonin can be combined to induce a greater circadian phase advance of the human circadian clock than either treatment alone. Our findings also reveal that under controlled dim light conditions, a single administration of 5mg melatonin is able to induce a comparable phase advance to that induced by a single 3h exposure to ~3000lux bright light. Caffeine also appears to have some influence on the human circadian clock. These findings have important implications for understanding fundamental physiological principals of the human circadian timing system and for the treatment of circadian sleep disorders (e.g., adapting to eastward travel and treatment of delayed sleep phase) (Sack *et al.*, 2007a; Sack *et al.*, 2007b). Specifically, our findings suggest that photic and

non-photic treatments are integrated by the human circadian clock and reveal that the combination of properly timed light exposure and melatonin administration may provide an effective means of advancing the human circadian clock. The finding that photic and non-photic stimuli are integrated by the human circadian time keeping system also highlights the need to consider patients' light exposure when using melatonin to shift the circadian clock. It appears that light may impede (Wirz-Justice *et al.*, 2004) or facilitate the desired phase shift induced by melatonin depending on the biological timing of exposure.

The finding that the dim light placebo condition showed an average phase delay is consistent with previous findings of an average delay drift in phase of the human circadian clock in an environment absent of time cues (Duffy & Wright, Jr., 2005; Gronfier *et al.*, 2004; Wright, Jr. & Czeisler, 2002), which is likely due to the longer than average circadian period in humans (Czeisler *et al.*, 1999; Duffy *et al.*, 2011; Wright, Jr. *et al.*, 2001; Wyatt *et al.*, 1999). Individual differences in the observed drift and circadian phase resetting responses to melatonin and bright light stimuli are also likely driven by individual differences in circadian period (Czeisler *et al.*, 1999; Duffy *et al.*, 2011; Gronfier *et al.*, 2004; Wright, Jr. *et al.*, 2001; Wright, Jr. & Czeisler, 2002).

In the present study, melatonin administration was timed to induce maximal phase advance shift based on the three pulse phase response curve for 3mg melatonin (Burgess *et al.*, 2008). More recently, a three pulse phase response curve has been published comparing a 3mg dosage of melatonin to 0.5mg of melatonin (Burgess *et al.*, 2010) suggesting that a lower dosage of melatonin is just as effective at phase shifting the human circadian clock as the higher dose if timed appropriately. Specifically, 0.5 mg of melatonin had a similar magnitude of phase shifts as compared to 3mg of melatonin ~ 2h later (Burgess *et al.*, 2010). Light exposure was timed on the

PRC to induce a maximal phase advance shift (Khalsa *et al.*, 2003) and light intensity was on the asymptotic portion of the luminance intensity curve (Zeitzer *et al.*, 2000).

Large effect sizes of all conditions relative to placebo suggest the strong efficacy of shifting the human circadian clock. The phase shifts induced are also physiologically and clinically meaningful. For example, the average human requires a daily phase advance shift of the human circadian clock to entrain or synchronize it to the 24h day (Czeisler *et al.*, 1999; Duffy & Wright, Jr., 2005; Wright, Jr. *et al.*, 2001; Wyatt *et al.*, 1999). Based on the range of circadian periods observed in healthy sighted humans (Duffy *et al.*, 2011), the current light and melatonin treatments would be sufficient to entrain to the 24h day all sighted subjects tested to date who show a longer than 24h circadian period.

There have been several studies aimed at exploring the ability of a single administration of exogenous melatonin to induce a circadian phase advance. For example, results from a study by Deacon and Arendt (1995) found that a phase shift response to a single administration of immediate release melatonin was dose dependent with mean phase advances of plasma melatonin onset of 24, 42, and 84min, relative to placebo for 0.05, 0.5, and 5mg doses, respectively. In recent studies by Paul *et al.* (2010; 2011), a single administration of either 3mg immediate release, 3mg sustained release (SR) or a combined 1mg immediate release with 2mg sustained release melatonin preparations induced similar phase advances between ~26-43min as compared to placebo. Single administration of 5mg immediate release melatonin, has previously been found to produce an ~44min (Krauchi, Cajochen, & Wirz-Justice, 1997) phase advance relative to a pooled placebo. Our finding of ~37min phase advance following the single administration of 5mg immediate release melatonin relative to placebo is consistent with the latter findings.

There have also been studies aimed at exploring the ability of a single exposure to light to induce a circadian phase advance (Buresová *et al.,* 1991; Dawson *et al.,* 1993;Khalsa *et al.,* 2003; Paul *et al.,* 2011). Our finding of ~41min phase advance following single exposure to ~3,000lux broad spectrum white light relative to placebo has a decreased phase advance relative to results from a study by Paul *et al.* (2011) which found an ~ 63min phase advance to a single exposure of 350lux green light (500nm) exposure compared to <10lux dim light room light.

Relative to placebo, the magnitude of the response for exogenous melatonin and bright light exposure were similar for the 5mg melatonin and the ~3,000lux light exposure. This result suggests that melatonin may possibly be as effective as strictly controlled bright light exposure to shift the human circadian clock. Using exogenous melatonin as an alternative to strictly controlled bright light exposure may be more efficient in real world applications, as strictly controlled bright light exposure to elicit phase shifts can be more of a challenge for individual comfort and compliance.

Findings from studies of non-humans indicate that non-photic time cues can facilitate entrainment to shifted light dark cycles (Mrosovsky & Salmon, 1987) such as those induced by jet lag and shift work. The current study results indicate that combined treatments may also facilitate circadian adaptation in humans to these schedules. Two previous studies looking at the combination of photic and non-photic time cues reported; 1) that five days of exposure to intermittent bright light (5,000lux) in a gradually delaying light-dark cycle induced large and significant phase delay shifts and that the addition of 1.8mg sustained release melatonin did not facilitate the phase resetting response (Crowley *et al.,* 2003); and 2) that three days of exposure to intermittent bright light (5,000lux) in a gradually advancing light-dark cycle combined with either 0.5mg or 3.0mg melatonin induced relatively large and significant phase advance shifts as

compared to a dim light-placebo condition (Revell *et al.*, 2006). The latter study did not include bright light alone or dim light-melatonin alone conditions, thus it is unknown whether the combination of the treatments tested in that study would be more effective than either alone. However, given the number of treatment days, exposure to light alone may be sufficient to induce large phase shifts. Findings from a recent study by Paul *et al.* (2011) show that afternoon administration of sustained released 3mg melatonin and morning exposure to moderate levels of green light (500nm), combined with an advanced 13.5h sleep opportunity, induced a significant phase advance shift relative to green light alone and to a dim light-placebo control. Green light alone did not induce a significant phase shift relative to dim light-placebo, whereas melatonin alone induced a significant phase advance relative to dim light-placebo, but not green light alone. Green light exposure was timed 1h earlier in the combined condition than in the green light alone condition and could have contributed to their findings. Nonetheless, these findings are consistent with our findings of the effectiveness of combined treatments of photic and non-photic time cues for phase shifting the human circadian clock. Findings from Crowley *et al*. (2003) also show that multiple days of exposure to bright light are more effective than multiple days of administration of melatonin. As light is considered the strongest environmental time cue for the human circadian clock, addition of melatonin when using multiple days of treatment may not provide additional benefit assuming that light exposure is properly timed.

Findings from experiment 2 provide preliminary evidence that caffeine may influence the circadian system in humans. Caffeine administered in the early morning hours appears to influence circadian phase by reducing delay drift observed due to the on average longer than 24h period of the human circadian clock (Duffy *et al.*, 2011; Wright, Jr. & Czeisler, 2002). Although the difference between the dim light-placebo and the dim light-caffeine conditions was not

significant, these findings suggest that morning caffeine use could contribute to an advance of the human circadian clock as the dim light-caffeine group was significantly different than zero. The 2.9mg/kg dose used in the current study is equivalent to 200mg dose of caffeine or the amount of caffeine in a double espresso for a 68kg (150lb) person. The moderate effect size for caffeine appears smaller than that of the observed large effect size for the melatonin condition in experiment 1. Additional research is needed to follow-up on these preliminary findings for caffeine and test different timings and doses of caffeine to determine effects of caffeine on the human circadian timing system. Whether caffeine can phase delay the human circadian clock remains to be tested.

In summary, when advancing the human circadian clock is desired (e.g., adapting to eastward travel and treatment of delayed sleep phase), the combination of bright light exposure in the early morning and exogenous melatonin in the evening would provide the greatest phase shift than either stimulus alone. Findings also provide preliminary evidence that caffeine may be able to influence the human circadian clock. These findings have implications for the adaptation to jet travel and for the treatment of circadian sleep disorders and to facilitate the daily entrainment to the 24h light-dark cycle.

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CHAPTER 3

EVENING CAFFEINE PHASE DELAYS THE HUMAN CIRCADIAN CLOCK

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Abstract

Shifting of the human circadian clock is a necessary component of the treatment of circadian related sleep disorders and rapid adaptation to jet travel. Photic and non-photic stimuli have both been shown to shift the human circadian clock. Further, recent findings suggest that the combination of photic and non-photic stimuli (exogenous melatonin) are integrated by the human circadian system in an additive fashion. Caffeine, a commonly ingested stimulant, has previously been shown in non-human models to influence circadian phase. We sought to extend the understanding of how photic and non-photic time cues are integrated to influence the human circadian clock by assessing the phase delaying effect of caffeine, alone or in combination with bright light exposure. For each randomly assigned intervention [dim light-placebo (DLP), dim light-caffeine (DLC), bright light-placebo (BLP), and bright light-caffeine (BLC)], five subjects completed a sensitive, within-subject, ~ 49 daylong phase shifting protocol. Subjects received either caffeine (2.9mg/kg) or placebo in a double blind fashion, 3h prior to habitual bedtime with 3h of either bright (~3000lux) or dim (~1.9lux) light exposure at their habitual bedtime. For all four interventions, circadian phase was assessed during a constant routine protocol using salivary dim light melatonin onset (DLMO pre- and post-intervention). We observed average phase delays of: DLP, 0.27±0.34h, DLC, 0.93±0.37h (mean±SEM), BLP, 1.71±0.30h, and BLC, 2.02±0.47h. DLC alone was able to significantly phase delay subjects as compared to DLP. DLC, BLP, and BLC were all significantly different than DLP. Caffeine could be used as a non-photic therapeutic agent to facilitate the treatment of circadian related sleep disorders and rapid adaptation to jet travel.

Introduction

Caffeine is one of the most commonly used drugs in the world (Fredholm, 2011) and is often used to improve alertness and mood, especially when sleepy (Bonnet, Gomez, Wirth, & Arand, 1995; Penetar *et al.*, 1993). However, there are negative effects of caffeine use since it also promotes brain arousal and disrupts sleep (Alford, Bhatti, Leigh, Jamieson, & Hindmarch, 1996; Karacan *et al.*, 1976; Roehrs & Roth, 2008). Although the wakefulness promoting and sleep disrupting effects of caffeine are well established, the effect of caffeine on the human circadian timekeeping system is unknown. The circadian system is a key regulator of the timing of sleep and wakefulness, as well as other physiological and behavioral processes across the 24h day (Gillette & Sejnowski, 2005). Understanding how caffeine can influence the circadian system has implications for the treatment of circadian related disorders (e.g., delayed sleep phase, shift work disorder) and jet lag.

The mammalian master circadian clock is located in the suprachiasmatic nucleus (SCN), of the hypothalamus (Swaab, Fliers, & Partiman, 1985; Moore & Eichler, 1972). The SCN is able to facilitate synchrony of internal biological timing to external environmental timing, such that physiology and behavior are coordinated for optimal functioning relative to the time of day (DeCoursey, Walker, & Smith, 2000; Pittendrigh, 1993). Light is the primary environmental synchronizing stimulus to the SCN (Czeisler & Wright, Jr., 1999; Duffy & Wright, Jr., 2005). Non-photic stimuli such as activity, exercise, restricted food availability, and exogenous melatonin have also been shown to shift the timing of the master circadian clock (Krauchi, Cajochen, Mori, Graw, & Wirz-Justice, 1997; Mrosovsky, 1996; Stephan, Swann, & Sisk, 1979; Youngstedt, Kripke, & Elliott, 2002). Desynchrony between internal biological time and external environmental time can result in negative health consequences (Barger, Lockley, Rajaratnam, &

landrigan, 2009; Knutsson, Akerstedt, Jonsson, & Orthgomer, 1986; Scheer, Hilton, Mantzoros, & Shea, 2009; Srivastava, 2011). Recent findings suggest that the combination of photic (bright light exposure) and non-photic stimuli (exogenous melatonin) are integrated by the human circadian system in an additive fashion. Wirtz-Justice *et al.* (2004) reported that the combination of exogenous melatonin (timed to advance the circadian clock) and bright light exposure (timed to delay the circadian clock) resulted in no phase shift relative to the control condition. We sought to determine if photic and non-photic information are integrated by the circadian timing system in humans such that the combination of properly timed photic and non-photic stimuli would induce a greater phase shifting response than the individual stimuli alone.

Caffeine, an adenosine antagonist, has been shown to influence circadian physiology in humans by attenuating the decline in tympanic temperature and suppressing endogenous melatonin in healthy men (Wright, Jr., Badia, Myers, Plenzler, & Hakel, 1997) and women not taking oral contraceptives (Wright, Jr., Myers, Plenzler, Drake, & Badia, 2000). Although there have been limited human studies assessing the influence of caffeine on circadian physiology, there have been studies conducted in non-humans assessing the influence of caffeine on the circadian timing system.

In these non-human studies, caffeine has been reported to phase shift the electrical activity rhythm of the SCN (Chen & van den Pol, 1997; Hallworth, Cato, Colbert, & Rea, 2002; Sigworth & Rea, 2003) and adenosine has been shown to attenuate light induced phase shifts (Elliott, Todd, & Rea, 2001; Watanabe *et al.*, 1996).

This study aimed to examine the influence of caffeine on phase shifting the human circadian clock. Specifically, using a within-subjects design, we tested the hypothesis that: 1) caffeine would be able to influence the circadian system; and 2) the combination of a single 3h

light exposure of ~ 3,000lux and a single 2.9mg/kg dose of caffeine administered in the evening would phase delay the human circadian clock greater than either stimulus alone.

Methods

Subjects

Five healthy subjects (3 females) aged 23.8 ± 3.1y, BMI 23.9 ± 0.9 (mean ± SD) participated in a ~49 daylong phase shifting protocol. Health of subjects was determined by medical evaluation at the Clinical and Translational Research Center (CTRC) at the University of Colorado at Boulder based on physical exam, blood chemistries, clinical electrocardiography, medical and psychiatric histories. Exclusion criteria included: known medical, psychiatric, or sleep disorder, illicit drug or current nicotine use, habitual sleep duration <7h or >9h, medication use, body mass index outside the range of <18.5 or >27, shift work or living below the altitude 1600m in the year prior, and travel across more than one time zone in the 3 weeks prior to in-laboratory procedures.

Subjects maintained a regular ~8h sleep-wakefulness schedule based on their individual habitual sleep and waketimes for a minimum of one week prior to study (Figure 1). Regular sleep-wakefulness schedules were verified via sleep logs, time-stamped voice-recorder of bed and waketimes, and wrist actigraphy (Actiwatch-L, Minimitter-Respironics, Bend, OR). Subjects were proscribed to refrain from usage of over the counter medications, supplements, and caffeine for two weeks prior and to refrain from naps one week prior to the first in-laboratory visit and for the duration of the entire study. Subjects were also asked to refrain from exercise for three days prior and alcohol for two days prior to all in-laboratory visits. Subject's self-reported compliance with the above requests and urine toxicology and an alcohol breath test

were performed upon admission to each in-laboratory visit. Female subjects were also given a pregnancy test to verify that they were not pregnant upon admission. Study procedures were approved by the Institutional Review Board at the University of Colorado at Boulder and the Scientific Advisory and Review Committee of the Colorado Clinical and Translational Sciences Institute. Subjects gave written informed consent and were compensated for their participation. The protocol was in accordance with the standards set by the latest revision of the *Declaration of Helsinki*.

In-laboratory protocol

A randomized, within-subject design was used to study subjects in four interventions: dim light-placebo (DLP), dim light-caffeine (DLC), bright light-placebo (BLP), bright light-caffeine (BLC). Subjects were studied individually in a research suite in the Sleep and Chronobiology Laboratory at the University of Colorado at Boulder. Each laboratory visit lasted ~5.25 days. Laboratory visits were scheduled with a minimum of ~1 week of home monitoring between visits depending upon subject availability. Subjects arrived at the laboratory ~6h prior to their habitual bedtime (Figure 1). All protocol events such as meal times, bathroom breaks, cognitive testing and sleep opportunities were scheduled relative to a subject's habitual waketime.

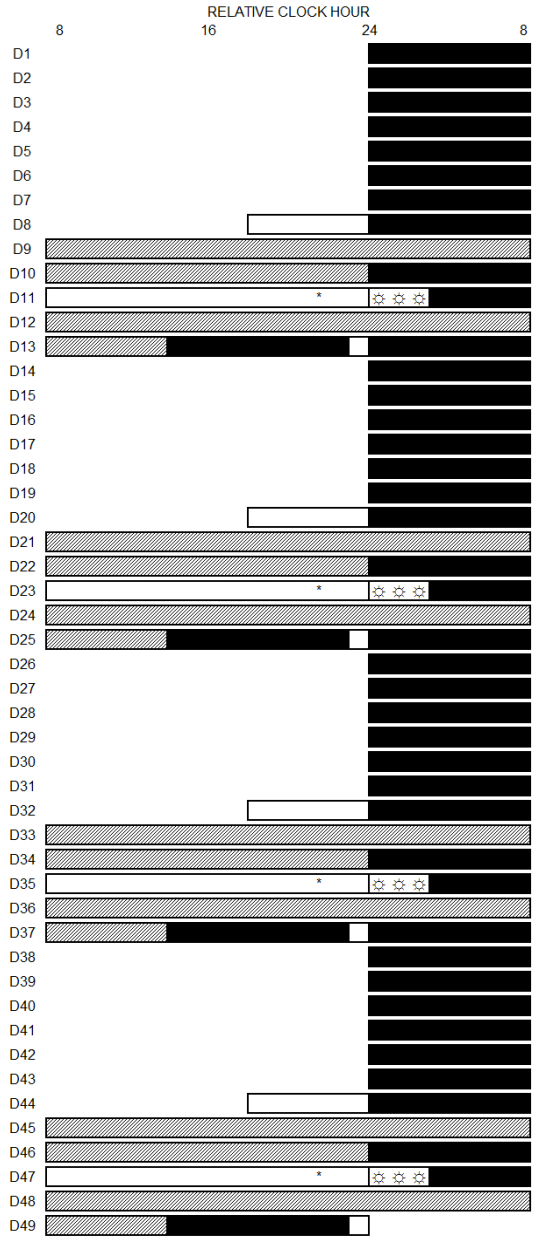

Figure 1. ~49 daylong phase shifting protocol. Following 7 days ambulatory monitoring, participants remained in an environment free of time cues under thermoneutral (~22.2 °C), dim light conditions (< 1.9 lux) during scheduled wakefulness and darkness during scheduled sleep (black bars). Day 8 included an 8h nighttime sleep opportunity. Days 9-10 consisted of a 40h modified constant routine (diagonal bars). On day 11 participants received either caffeine or rice-powder filled placebo double-blind (*) 3h prior to habitual bedtime and 3h exposure to bright or dim light (☼) beginning at habitual bedtime. Days 12-13 consisted of a second constant routine of 30h. Lab procedures were repeated four times across 49 days (D1-49). Relative clock hour shown. Actual times dependent upon participant's habitual bed and waketimes.

Experimental Interventions

Caffeine

Caffeine was administered double-blind. Implementation of pill allocation was performed by the CTRC pharmacist who provided pills identical in appearance. The allocation sequence was concealed until interventions were assigned and data prepared for statistical analysis. Pills were 5 capsules containing rice flour placebo or caffeine (2.9mg/kg total dose; Gallipot, Inc, Mendota Heights MN), equivalent to approximately 200mg of caffeine for a 150lb adult (68kg) or equivalent to a double espresso. The administration of caffeine, 3h prior to habitual bedtime, was designed to induce a phase delay of the human circadian clock as caffeine promotes arousal and has been shown to acutely suppress or delay melatonin onset (Wright, Jr. *et al.*, 1997; Wright, Jr. *et al.*, 2000).

Photic stimuli

Subjects were exposed to either 3h continuous bright light or dim light exposure beginning at habitual bedtime. Ceiling mounted fluorescent lamps (Sylvania Octron 32W T8 bulb) provided broad spectrum white light exposure, similar to daylight exposure (6500-K color temperature). During the light exposure, subjects were under the direct supervision of research assistants to ensure that they achieved the intended intensity of illumination. Subjects maintained constant semi-recumbent posture while alternating between fixing their gaze on a target for 6min or free gaze for 6min. Average light intensities during the fixed gaze were 2985 ± 388lux (~7Watts/m^2) for the bright light interventions and 1.9 ± 0.4lux (~0.6Watts/m^2) for the dim light interventions. Light exposure was timed to induce a maximal phase delay shift based on existing phase and luminance response curves (Khalsa, Jewett, Cajochen, & Czeisler, 2003; Zeitzer, Dijk, Kronauer, Brown, & Czeisler, 2000).

Circadian phase assessment and analysis

A modified constant routine protocol was used to assess changes in circadian melatonin phase to minimize masking effects (Czeisler & Wright, Jr., 1999). Saliva for melatonin assessment was collected every 30-60min and frozen at -80°C until assayed with ELISA (IBL International, Hamburg, Germany). Phase shifts were determined as change in timing of salivary dim-light melatonin onset (DLMO) (Paul *et al.*, 2011) between constant routines. The salivary DLMO was defined as the linearly interpolated time point when melatonin levels exceeded and remained two standard deviations above the stable baseline mean (Benloucif *et al.*, 2005; Lewy, Cutler, & Sack, 1999). Data were analyzed with repeated measures ANOVA and planned comparisons, with Bonferroni correction for multiple comparisons, using one-tailed Dunnett's test for hypothesis driven comparisons versus DLP control. Caffeine and bright light group comparisons were performed with dependent t-tests. In addition to the above statistical tests, effect sizes (Cohen d') were calculated to determine the size of phase resetting effects. Standard interpretations of effect size were used (Cohen, 1988) (small, d'=0.2; moderate, d'=0.5; large, d'=0.8). To test whether the phase angle of entrainment was similar among individuals, the DLMO to bedtime phase angle of entrainment was calculated and paired t-tests were used to assess differences among individuals.

Results

Figure 2 shows the mean phase shifts for the caffeine and light interventions. A significant main effect for light exposure $F(1,4)=193.8$ $p<.001$ was observed. The main effect for caffeine, and the interaction effect between light exposure and caffeine, was not significant.

Planned comparisons show that DLP induced a mean phase delay of ~16min. DLC, BLP, and BLC significantly delayed circadian phase compared to DLP ($p < 0.01$) with large effect sizes, $d' = 2.08$, 5.05, and 4.82, respectively. BLC delayed melatonin phase more than DLC ($p < 0.01$) with a large effect size, $d' = 2.92$. No significant differences were observed between DLC and BLP (non-significant trend p=0.07) or between BLP and BLC (p=0.27) however both effect sizes for the difference in shift were considered large, $d' = 2.62$, 0.90, respectively. The current phase shifting protocol permitted control for the influence of individual differences in circadian period (Czeisler *et al.*, 1999; Duffy *et al.*, 2011) on the phase shifting response, as each participant was exposed to all interventions. Prior to interventions, circadian DLMO phase were similar (p=0.41): DLP -1.7±0.9h (mean±SD; 95% CI: -2.8 to -0.6h); DLC -1.5±0.6h (95% CI: -2.2 to -0.7h); BLP -2.3±1.2h (95% CI: -3.8 to -0.8h); and, BLC -2.1±0.9h (95% CI: -3.2 to -0.9h).

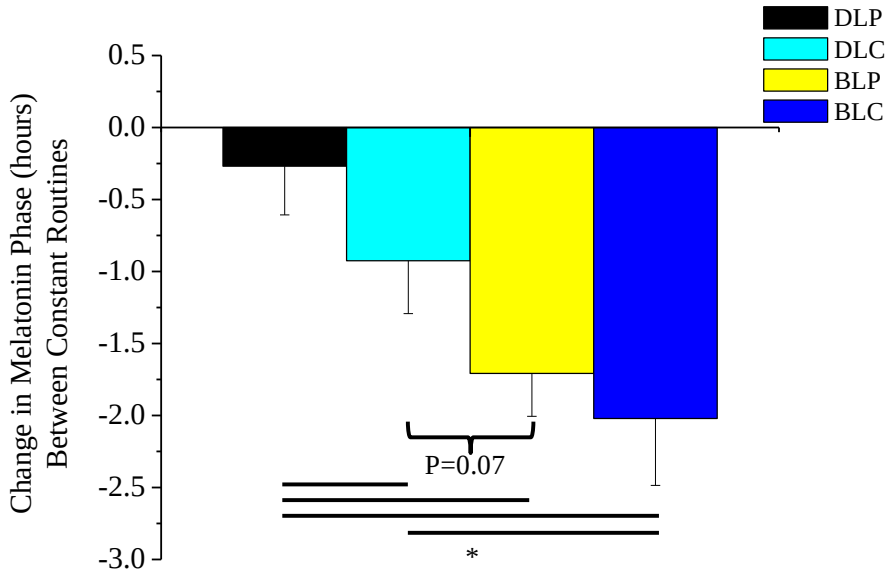

Figure 2. Phase resetting response to bright light and caffeine. Mean circadian phase shifts for groups DLP, DLC, BLP and BLC are plotted. Error bars represent SEM. Delays are denoted as negative numbers. On average, the circadian phase of the dim light melatonin onset delayed in the DLP condition, consistent with drift in phase due to the longer than average intrinsic circadian period in humans when exposed to dim light (Czeisler *et al.*, 1999; Duffy *et al.*, 2011).

Caffeine (DLC) induced a phase delay that was ~40min larger than the drift observed in DLP, whereas BLP and BLC induced phase delays of ~86 and 105min more than DLP.

Discussion

This study sought to determine the ability of caffeine and bright light, alone and in combination, to phase delay the human circadian system. We specifically hypothesized that caffeine would induce a significant phase delay shift of the human circadian timing system and that the phase delay induced by a combination of bright light exposure and caffeine administration would be integrated by the human circadian timing system and thus induce a greater phase shift than that of either treatment alone. As predicted, caffeine significantly phase delayed the human circadian clock and the combination of bright light and caffeine produced a greater phase delay than the dim light-placebo and dim light-caffeine alone. However, contrary to our hypothesis, the combination of bright light and caffeine did not produce a greater phase delay than bright light alone. The effect sizes for the bright light alone and bright light in combination with caffeine was a little over twice that of caffeine alone when compared to placebo. Specifically, we found that a single dose of caffeine, when taken 3h prior to habitual bedtime induced a significant phase delay relative to dim light-placebo. Continuous bright light exposure, at habitual bedtime lasting for 3h, also produced a significant phase delay relative to dim light-placebo. Though the phase delay in the dim light-caffeine intervention was not significantly different from that in the bright light-placebo intervention, there was a non-significant trend between their phase shifting abilities. The sum the phase delays for each intervention, relative to placebo, of caffeine (0.66h) and bright light (1.44h) added together (2.10h) is larger than the phase shift induced by the combination of the two interventions (1.75h). Thus, while caffeine may produce a significant phase delay, the human circadian system does not

appear to integrate these two stimuli in an additive manner, at least for the phase, caffeine dose and light duration and intensity tested.

Caffeine administration was timed to facilitate a phase delay from research findings that showed acute reductions in salivary melatonin levels in the early evening (Babkoff, French, Whitmore, & Sutherlin, 2002; Wright, Jr. *et al.*, 1997; Wright, Jr. *et al.*, 2000). The timing and intensity of bright light exposure was designed to produce a large phase delay based upon existing phase response curves and irradiance response curves to broad spectrum light (Khalsa *et al.*, 2003; Zeitzer *et al.*, 2000). Our study represents the first systematic examination of the phase delaying influence of caffeine administration in humans. Importantly, these results are not due to the inter-individual variability in the phase resetting response for each individual subject as this was a within-subjects design. There were no differences in the analysis of the pre-treatment DLMO prior to each intervention suggesting that the week separating each intervention was sufficient for subjects to return to near baseline.

Findings from non-human studies suggest that: 1) multiple pathways by which caffeine may influence circadian timing; and 2) that caffeine may enhance photic phase shifts. Caffeine may influence circadian timing by competitive binding at the adenosine receptor (Alanko, Heiskanen, Stenberg, & Porkka-Heiskanen, 2003). By blocking the adenosine receptors, caffeine facilitates the release of excitatory neurotransmitters and, at high doses, can influence calcium flow into cells (Fredholm, Battig, Holmen, Nehlig, & Zvartau, 1999). Caffeine may influence the circadian system by direct actions on the SCN and pineal or indirectly by influencing the afferents into the SCN. Results from a study by Diaz-Munoz *et al.* (1999) suggest that caffeine is able to shift the electrical activity of the SCN in vitro due to an increase in the calcium through stimulation of a ryanodine receptor. Caffeine has also been reported to induce the expression of

cFos in a fetal rat SCN (Shearman & Weaver, 2001). Both adenosine receptors (located on pineal cells) and adenosine agonists have been reported to increase melatonin release, whereas adenosine antagonists reduce melatonin release (Babey, Palmour, & Young, 1994; Falcon, Privat, & Ravault, 1997; Falcon, Van Camp, & Collin, 1995; Gharib *et al.*, 1989; Gharib, Delton, Lagarde, & Sarda, 1992; Nikodijevic & Klein, 1989; Sarda, Gharib, Reynaud, Ou, & Pacheco, 1989). Alterations in melatonin then feeds back onto the SCN, which contains melatonin receptors regulating subsequent melatonin levels. Caffeine has also been reported to block adenosine induced melatonin increases in the pineal (Babey *et al.*, 1994). Another mechanism by which caffeine may influence the circadian system is through action on the retinal ganglion cells. Adenosine has previously been reported to influence retinal function and transmission of photic information via the retinohypothalamic tract (RHT). Kvanta *et al.*. (1997) reported adenosine receptor mRNA was expressed in retinal ganglion cells which may include melanopsin and non-melanopsin retinal ganglion cells which project to the SCN. Adenosine administration has also been reported to attenuate phase shifts induce by photic input in a dose dependent manner (Elliott *et al.*, 2001; Watanabe *et al.*, 1996). This attenuation in photic input may be due to the altered RHT transmission and reduction of glutamate release onto the SCN (Chen & van den Pol, 1997; Hallworth *et al.*, 2002; Sigworth & Rea, 2003). Another potential mechanism of caffeine producing a phase shift is through serotonergic neurons. The SCN has been found to receive input from serotonergic neurons in the dorsal and medial raphe nucleus (Meyer-Bernstein & Morin, 1996; Moga & Moore, 1997). In a non-human study (Nehlig & Boyet, 2000), a low dose (1mg/kg) administration of caffeine increased glucose utilization in the medial and dorsal raphe nuclei supporting the hypothesis that the raphe nucleus is involved in the transmission of non-photic information.

Although it is possible that caffeine influences photic pathways to the SCN, we did not find a significant difference in phase with the combination of bright light and caffeine. The bright light stimulus used is on the upper asymptote of the irradiance response curve and thus lower light levels need to be tested in combination with caffeine to determine if caffeine can enhance photic phase shifts. In the current study, we only examined one circadian phase and one dose of caffeine administration. Thus, other times of administration and other doses of caffeine need to be tested to determine if there are phase and dose dependent effects of caffeine on human circadian physiology. In the current study, timing of caffeine was based upon previous studies that have shown caffeine to acutely suppress or delay melatonin onset (Wright, Jr. *et al.*, 2000; Wright, Jr. *et al.*, 1997). In the future, developing a phase response curve for caffeine may improve the timing of administration to produce a larger phase shifting response.

Our findings demonstrate that a moderate dose of caffeine induces a phase delay of the human circadian clock that is approximately half of the phase shift induced by evening exposure to bright light. Whether caffeine can phase advance the human circadian clock remains to be tested. The finding that caffeine can phase delay the human circadian clock indicates that caffeine could be used to facilitate phase delays to treat circadian disorders such as the treatment of advanced sleep phase syndrome, advanced circadian phase in older adults, and jet lag due to westward travel, entrainment of non-24h sleep-wake cycle and circadian adaptation of the human circadian clock to longer than 24h day lengths (e.g., the 24.65h day length of the planet Mars). However, consideration needs to be given to the effects of caffeine on subsequent sleep in these interventions as well as how habitual caffeine use in the evening, in addition to evening bright light exposure, may contribute to circadian sleep disorders (e.g., delayed sleep phase in adolescents and young adults).

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CHAPTER 4

CONCLUSIONS

Tina M. Burke

Summary of results

The purpose of this dissertation was to address the following deficiencies in our present state of knowledge about the influence of photic and non-photic timing stimuli on the human circadian system. First, it was unknown whether morning bright light exposure and evening exogenous melatonin administration would produce a circadian phase advance that would be integrated by the human circadian system to produce a greater shift than either stimulus alone. Therefore, we tested the hypothesis that the combination of morning bright light exposure (~3,000lux) and evening exogenous melatonin (5mg) would produce a phase advance that would be a greater phase shift than either stimulus alone. We found that morning bright light combined with early evening exogenous melatonin induces a significantly greater phase advance of the human circadian clock than either treatment alone.

Secondly, it was unknown whether caffeine administration induces a circadian phase advance of the human circadian clock. We tested the hypothesis that caffeine (2.9mg/kg) would induce a phase advance shift. We show preliminary evidence that caffeine may be able to phase advance the human circadian clock.

Finally, it was unknown if evening caffeine administration would induce a circadian phase delay of the human circadian clock, and if so, if evening bright light exposure and evening caffeine administration would produce a circadian phase delay that would be integrated by the human circadian system to produce a greater shift than either stimulus alone. Thus, we tested the hypothesis that caffeine administration would induce a circadian phase delay and that the combination of evening bright light and evening caffeine administration would produce a greater shift than either stimulus alone. We found that exposure to evening caffeine induces a circadian phase delay of the human circadian clock and, contrary to our hypothesis, that exposure to

evening bright light combined with evening caffeine does not induce a greater phase shift than either stimulus alone.

In summary, this dissertation aimed to improve our understanding of circadian physiology and how photic and non-photic stimuli are able to phase shift the human circadian clock. Specifically, when advancing the human circadian clock (e.g., adapting to eastward travel and treatment of delayed sleep phase) with combination of bright light exposure in the early morning and exogenous melatonin in the evening, the combination would provide a greater phase shift than either stimulus alone. Findings also provide preliminary evidence that caffeine may be able to phase advance the circadian clock. Delaying the human circadian clock (e.g., adapting to westward travel and perhaps treatment of advanced sleep phase) may be achievable with properly timed caffeine administration.

Future directions

Our findings have important implications for the treatment of circadian sleep disorders. The clinical use of non-photic stimuli (e.g., melatonin or caffeine) may provide an alternative to light treatment. Possible extensions of the research from this dissertation include the following. First, findings from previous research (Burgess, Revell, Molina, & Eastman, 2010) found that a smaller dose of exogenous melatonin (0.5mg) is able to facilitate an equivalent phase shift to that of a larger dose (3.0mg) as long as the timing for the smaller dose is timed properly should be evaluated. As we learn more about circadian physiology, and the ability of the human circadian clock to integrate both photic and non-photic stimuli, further studies are needed to assess if there is a dose- dependent relationship that influences the integration of stimuli. Second, with the finding that caffeine does influence the human circadian system, caffeine phase response curves

are needed to understand the most sensitive timing schedules of administration for potential clinical application resulting in desired phase advances and delays. Finally, our results suggest that since caffeine can influence the human circadian system, attention and future research should be directed towards how habitual evening caffeine use in today's 24h society along with increased evening light exposure may contribute to circadian sleep disorders that influence endogenous circadian timing.

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