MELANOPSIN GENE VARIANTS AND INDIVIDUAL DIFFERENCES IN SLEEP CHARACTERISTICS

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Light has a significant influence on sleep behaviors by influencing the circadian regulation of sleep-wake rhythms, the sleep homeostat, and through direct alerting effects. Melanopsin, the circadian photopigment discovered within intrinsically-photosensitive retinal ganglion cells (ipRGCs), detects environmental irradiance levels and is needed to process non-visual light information. Although a growing animal literature demonstrates the importance of melanopsin for sleep-wake timing and behavioral responses to light, little is known about its role in human sleep regulation. Preliminary evidence in healthy adults suggests that the P10L variant in the melanopsin gene (*OPN4*) interacts with day length to predict variations in self-reported sleep time (Roecklein et al. 2012). The present study aimed to replicate these findings in a larger sample while also using actigraphy to behaviorally quantify sleep characteristics. We hypothesized that variation in *OPN4* single nucleotide polymorphisms (SNPs; P10L and I394T) would be associated with differences in sleep timing and sleep duration as a function of day length. Participants were healthy, midlife community volunteers (*N*= 377; mean age 42.43 ± 7.37 years old, 50.4% Female; 100% Non-Hispanic Caucasian). Participants wore actigraphy monitors over a course of 7 nights. Day length on the first date of actigraphy assessment was determined by the U.S. Naval Observatory (2010). Linear regression analyses revealed that P10L and I394T were both not significantly associated with the timing of sleep onset, wake onset or sleep duration. There was no main effect of day length or an interaction effect between day

length and either polymorphism. Our findings suggest there may be no association between these genetic variants and behavioral sleep timing in a largely healthy population. Given insufficient power in the present study, however, and the different sleep assessment used in comparison to previous reports, it remains to be determined if a functional impact of these polymorphisms exists.

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PREFACE

List of Abbreviations

ED	electronic diary
EEG	electroencephalogram
IGL	intergeniculate leaflet
ipRGCs	intrinsically-photosensitive retinal ganglion cells
I394T	rs1079610, a single nucleotide polymorphism in the melanopsin gene
LD	linkage disequilibrium
MDD	major depressive disorder
NIF	non-image forming pathway
OPN4	melanopsin gene
$OPN4^{-/-}$	melanopsin gene knockout
PLR	pupillary light reflex
PSG	polysomnography
P10L	rs2675703, a single nucleotide polymorphism in the melanopsin gene
SAD	seasonal affective disorder
SC	superior colliculus
SCN	suprachiasmatic nucleus

SNPs	single nucleotide polymorphisms
SPZ	subparaventricular zone
VLPO	ventrolateral preoptic area

1.0 INTRODUCTION

Sleep disturbances have been linked to numerous chronic health illnesses, psychiatric disorders, diminished overall functioning, and all-cause morbidity (Foley, Ancoli-Israel, Britz, & Walsh, 2004; Ford & Cooper-Patrick, 2001; Meisinger, Heier, & Loewel, 2005; Nilsson, Nilsson, Hedblad, & Berglund, 2001). Although there is a large body of evidence supporting the increased risk of various health diseases associated with sleep disturbances, the biological mechanisms underlying disrupted sleep processes remain elusive. Efforts are needed to first understand the pathways contributing to naturally occurring variability in sleep timing and length within healthy populations.

The environmental 24-hour light-dark cycle is fundamental for coordinating the biological and physiological rhythms of processes such as the sleep-wake cycle. Non-visual light information is sent from the retina to various brain structures including regions involved in sleep and circadian regulation (Figure 1). The model shown in Figure 1 is simplified for our purposes, while more complex neural interactions involved in sleep behaviors are reviewed elsewhere (see Pace-Schott & Hobson, 2002; Saper, Scammell, & Lu, 2005; Hubbard et al., 2013). As shown in Figure 1, light information is sent through the retinohypothalamic tract or the non-image forming (NIF) pathway to hypothalamic nuclei such as the suprachiasmatic nucleus (SCN; the central clock) and intergeniculate leaflet (IGL) to entrain circadian rhythms. The ipRGCs both indirectly (via the SCN) and directly project to the ventrolateral preoptic area (VLPO). Other brain regions

that have direct projections from ipRGCs include the lateral hypothalamus and subparaventricular zone (SPZ). These nuclei are connected to ascending and descending neural pathways that together maintain wake and sleep states (Saper, Chou, & Scammell, 2001). For instance, the SCN projects to the pineal gland and coordinates the release of melatonin, a sleep-promoting hormone that is secreted in darkness. In addition, activation of neurons in the VLPO inhibits the arousal-promoting neurons that elicit wakefulness. Retinal projections also lead to the superior colliculus (SC) of the midbrain, a region important for saccadic eye movements and implicated in certain light influenced sleep behaviors (described in detail below). Originating from retinal projection pathways, the influence of light on sleep behaviors can be divided into three types of pathways: 1) circadian photoentrainment involving retinal projections to the SCN, 2) pathways independent of the circadian clock that influence the homeostatic sleep drive, and 3) direct retinal projections to various sleep-promoting regions that influence alertness. Given recent advances in knowledge about the pathways mediating non-visual light responses, studying a component of the non-visual light pathway may improve our understanding of the circadian and light-related neurobiology of normal and abnormal sleep regulation.

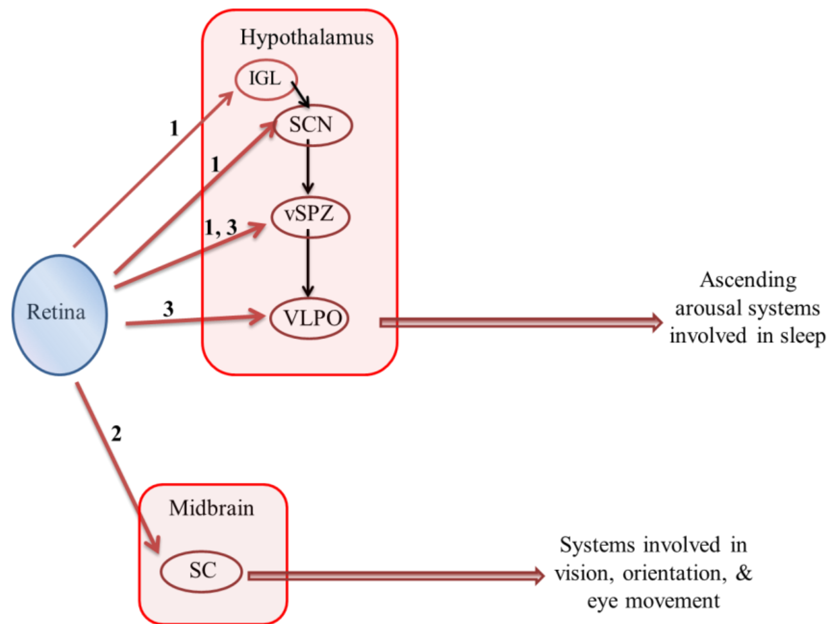

Figure 1. Schematic of Non-Visual Light Input Pathways that influence sleep behaviors.

The depicted pathways are simplified to demonstrate that melanopsin may play a role in several pathways.1= Circadian Pathways; 2= Circadian-Independent Pathways; 3 = Direct projects to sleep-promoting brain regions. IGL = intergeniculate leaflet; SCN = suprachiasmatic nucleus; vSPZ = ventral subparaventricular zone; VLPO = ventrolateral preoptic area; SC = superior colliculus.

1.1 INFLUENCE OF LIGHT ON SLEEP

1.1.1 Circadian

The first pathway through which light input influences sleep is through the circadian coordination of sleep-wake behaviors. Circadian rhythms are physiological and behavioral processes, such as the sleep-wake cycle, that demonstrate oscillations of roughly 24 hours and are coordinated primarily by the central clock located in the suprachiasmatic nuclei (SCN; Klein, Moore, & Reeper, 1991). Although circadian regulated behaviors have self-sustaining properties

such that there remains a rhythm when isolated from environmental stimuli, these rhythms deviate from the regular 24 hour cycle (Aschoff, 1978). The solar light-dark cycle serves as the primary environmental cue, known as a zeitgeber that entrains the central clock (Aschoff, 1978) and consequently contributes in part to human sleep and wake timing. The two-process model of sleep (Borbély, 1982) posits that the sleep-wake cycle is regulated by both circadian factors and a homeostatic drive (i.e. buildup of sleep debt). Specifically, sleep duration is regulated by an interaction between the homeostatic and circadian factors while sleep timing is thought to be a function of circadian peak in arousal that may be modified by accumulated sleep debt (Dijk, Duff, Riel, Shanahan, & Czeisler, 2000; Dijk & Czeisler, 1995). Given that light is the primary zeitgeber that entrains the clock coordination of circadian rhythms, a disruption in light pathways to the central clock may disrupt the phase relationship between the light-dark cycle and the sleep cycle (Pittendrigh & Daan, 1976).

1.1.2 Direct Pathway

Light cues also affect sleep behavior in an acute and direct manner that does not involve influencing the SCN or circadian coordination. Nocturnal animals, including various species of rodents used in animal model studies, will decrease activity and sleep when exposed to light and are awoken by darkness (Benca et al., 1998). In contrast, humans and other diurnal animals experience an alerting effect of light and will sleep during the solar night. Although it is not clear what distinguishes these inverse effects of light on nocturnal versus diurnal animals, the light-dark transition cue appears to initiate a “flip” in the switch between activity and sleep (see Hubbard et al., 2013).

The circadian rhythm of sleep was eliminated in animals with bilateral lesions in the SCN (Borbély, 1978; Eastman et al., 1984), but sleep and wake behaviors were retained in response to light stimuli and indicates a circadian-independent effect of light. Benca et al (1995) studied nocturnal rats in a laboratory protocol with constant light and dark pulses, a manipulation that disrupts the 24 hour cycle of circadian rhythms, and found that brief light exposure acutely suppressed motor activity and enhanced sleep whereas dark promoted wakefulness (Benca, Gilliland, & Obermeyer, 1995; see Saper, et al., 2005). Direct manipulations of light exposure and destruction of the central clock in animal models suggest that light asserts a direct influence on mammalian sleep and wakefulness.

Laboratory protocols in humans have similarly revealed a direct response to light. Unlike nocturnal animals, humans exhibit an alerting response to light and a soporific response to darkness. In humans, broad-spectrum white light exposure has been shown to induce acute, dose-dependent alerting effects such as decreases in reaction time (Lockley et al., 2006). Following light exposure, participants have demonstrated decreased electroencephalogram (EEG) measured delta-wave power density, which suggests a possible lower amount of sleep pressure build-up (see Badia, Myers, Boecker, Culpepper, & Harsh, 1991; Burgess, Sletten, Savic, Gilbert, & Dawson, 2001; Cajochen, Zeitzer, Czeisler, & Dijk, 2000; Campbell & Dawson, 1990; Daurat et al., 1993; Lockley et al., 2006). More so, sleep deprived participants reported decreased subjective sleepiness after being exposed to light (Lockley et al. 2006). In conjunction with animal studies, the human literature lends evidence to an additional mechanism whereby light not only serves as an essential zeitgeber for circadian entrainment but also initiates an acute alerting effect that may impact different components of sleep regulation.

1.2 MELANOPSIN: A CIRCADIAN PHOTOPIGMENT

A growing body of literature suggests that the effect of light on sleep is mediated in part by melanopsin (see Dijk & Archer, 2009). In the primary visual pathway, the ganglion cells in the retina that project to the brain depend on classical photoreceptors (rods and cones) to provide information used in visual image formation. In contrast, the non-image forming (NIF) pathway consists of the classical rod and cone cells as well as another class of ganglion cells that express melanopsin (Provencio et al., 2000). The latter cells, known as intrinsically photosensitive retinal ganglion cells (ipRGCs), encode light intensity levels for non-visual functions (Berson, Dunn, & Takao, 2002).

In addition to being directly photosensitive, ipRGCs receive input from rod and cone networks (Dacey et al., 2005) and relay light information from both the rod-cone system and melanopsin system to modulate sleep (Altimus et al., 2008). Clinically blind individuals have been shown to retain some physiological responses to light exposure (e.g. suppression of melatonin levels; Czeisler et al., 1995). In addition, researchers recently found that a blind individual had diminished pupillary light reflex (PLR) at low irradiance but sustained PLR in response to high-irradiance light when compared to normal sited individuals (Gooley et al., 2012), which confirms rod and cone visual photoreceptors are not necessary for some NIF light responses. Guler et al (2008) more specifically investigated the role of ipRGCs in mice by altering the mouse melanopsin gene such that there was a reduction in melanopsin proteins expressed and consequently a decrease in ipRGCs. The animals retained pattern vision but had significantly impaired circadian photoentrainment and pupillary light reflexes (Guler et al., 2008). Taken together, ipRGCs are important for NIF functions separate from vision including sleep-related light responses.

Melanopsin drives ipRGC photosensitivity (Berson et al., 2002; Gooley et al., 2003; Gamlin et al., 2007; see Berson, 2007). Melanopsin is a G-protein coupled receptor and is in the opsin class (Provencio et al., 2000; Provencio et al., 1998). In the dark, melanopsin binds to 11-*cis* retinaldehyde (Walker et al., 2008). Similar to other opsins, melanopsin uses 11-*cis* retinal as a light-sensing ligand or chromophore. Irradiance exposure leads the 11-*cis* retinal to absorb a photon of light and photoisomerize to all-*trans* retinal, which triggers a conformational change in melanopsin (Walker et al., 2008). This conformation change initiates a signaling cascade and ultimately, to the depolarization of ipRGCs (as reviewed in Hatori & Panda, 2010; Benarroch 2011; Hankins 2008).

Given that ipRGCs play a critical role in NIF light responses and that melanopsin is needed to activate these cells, melanopsin is therefore important for sleep-related NIF functions. Studies in mice have shown a deficiency in melanopsin, rod, and cone cells leads to a complete loss of circadian photoentrainment, pupillary light reflexes, and acute light effects on sleep onset (Hattar et al., 2003; Lupi, Oster, Thompson, & Foster, 2008; Panda, et al., 2003). This evidence demonstrates that both the visual and non-visual systems are needed to drive NIF responses. Evidence suggesting that melanopsin plays a critical role in the non-visual system highlights its importance in both the circadian and non-circadian light influences on sleep.

1.2.1 Melanopsin and Circadian Responses to Light

It was originally believed that light effects on circadian rhythms and other NIF responses were mediated by the classical photoreceptors. However, mice deficient of rods and cones still demonstrated melatonin suppression and circadian phase-shifting responses to light exposure

(Freedman et al., 1999). It later became evident that an alternate photoreceptive system involving melanopsin (Provencio et al., 2000) drives circadian photoentrainment.

Although the SCN does not depend solely on melanopsin for light input, melanopsin contributes significantly to circadian light responses (Ruby et al., 2002). Melanopsin gene knockout (*OPN4*$^{-/-}$) mice retained the capacity to entrain to light-dark cycles (Panda et al., 2002), presumably through light input from rod and cone RGCs. However, *OPN4*$^{-/-}$ mice that were exposed to brief light pulses demonstrated circadian phase-shift responses that were significantly lower in magnitude relative to control mice (Panda et al., 2002; Ruby et al., 2002). In contrast, rod and cone-deficient mice continued to demonstrate circadian photoentrainment (Barnard et al., 2004) with peak sensitivity at a light spectrum matching that of ipRGCs. Taken together, evidence suggests that although melanopsin signaling is not the sole factor involved in circadian photoentrainment, it is an important component.

The influence of melanopsin on circadian sleep regulation can be explained in part by the melanopsin-containing retinal projections to brain structures involved in circadian photoentrainment. Melanopsin-expressing RGCs have been shown to project to the SCN and the ventral SPZ (vSPZ; Gooley et al., 2001; Gooley et al., 2003). Rats with lesions in the vSPZ demonstrated significant reductions in circadian regulation of sleep and locomotor activity (Lu et al., 2001). Given the direct projections of ipRGCs to both the SCN and vSPZ, it is possible that melanopsin influences sleep rhythms via this circadian pathway to both brain structures (Gooley, Lu, Fischer, & Saper, 2003).

Melanopsin is not intrinsic to the functioning of the central clock itself (Do & Yau, 2010), but a deficiency in melanopsin may disrupt necessary phototransduction to the SCN and subsequently disturb the cascade of biological processes involved in sleep regulation. At the

molecular level, the clock mechanism is comprised of a network of transcriptional-translational feedback loops that drive 24-hour expression patterns of core clock genes (Reppert & Weaver, 2002). Clock genes are transcriptional regulators that produce proteins necessary to generate circadian rhythms within individual cells (Takahashi, 2004). In humans, various clock gene polymorphisms have been shown to correlate significantly with extreme diurnal preference, delayed sleep phase syndrome, insomnia, waking performance and early morning executive functions (Archer et al., 2003; Groeger et al., 2008; Jones et al., 1999; Viola et al., 2007). However, the significance of these candidate clock genes remains inconclusive given inconsistent findings and non-replication (Robilliard et al., 2002). Although a large body of literature continues to examine the association of clock genes with sleep characteristics, further efforts are needed to investigate the possible influence of genetic variation in components of other key pathways such as the melanopsin gene in the NIF pathway.

1.2.2 Direct Light Responses Mediated by Melanopsin: Nocturnal Rodent Models

Aside from its role in circadian photoentrainment, the melanopsin system is an important component of acute, direct light effects on sleep behavior. When exposed to light, wild type mice demonstrated acute sleep induction while $OPN4^{-/-}$ mice failed to show the expected sleep response even at bright irradiances (Lupi et al., 2008). However, rod and cone-deficient mice demonstrated light-induced sleep responses were similar to wild type mice, suggesting that melanopsin alone is able to mediate acute light effects on sleep independent of the classical photoreceptors at least in rodless-coneless mice. In intact rodents, it is predicted that melanopsin also mediates acute light responses. More so, melanopsin-expressing ipRGCs have been shown

to project to sleep-promoting centers separate from the circadian clock such as the VLPO and the SC (Hannibal & Fahrenkrug, 2004; Gooley et al., 2003). A light-induced c-Fos immunoreactivity study showed that *OPN4*$^{-/-}$ mice had attenuated, but not absent, light-induced SCN activity (Tsai et al., 2009), whereas there was a significant reduction in light-induced activity in the VLPO region and the SC (Lupi et al., 2008). These findings indicate that melanopsin may be needed for light-induced activation of these brain regions. The VLPO is a sleep-activating region; neurons from the VLPO region inhibit arousal systems in the brain that promote wakefulness (Saper et al., 2001). Likewise, the SC has been implicated in the light regulation of sleep (Miller, Obermeyer, Behan, & Benca, 1998), although direct projections from melanopsin-containing RGCs to the SC are sparse (Gooley et al., 2003). Taken together, these findings suggest a rodent pathway involving direct ipRGC projections to sleep regulating brain regions, in which melanopsin mediates light effects on sleep behaviors independent of the circadian pathway.

1.3 INVESTIGATING THE ROLE OF MELANOPSIN IN HUMANS

The aforementioned evidence that implicates melanopsin in sleep regulation is primarily based on findings from animal studies. However, the SCN is recognized as the primary central regulator of the clock network in most mammals including humans. Likewise, melanopsin expression is similarly localized to the retina in rodents, primates, and humans (Provencio et al., 2000). The melanopsin system appears to be conserved in mammals evolutionarily (see Berson, 2003). A group of intrinsically photosensitive melanopsin-expressing ganglion cells exists in primates (Dacey et al., 2005) while studies on human behavioral responses to light (discussed in detail below) suggest that this non-visual pathway is likewise conserved in humans (e.g. Gooley

et al., 2010; Lockley et al., 2006). For example, melanopsin has been shown to drive the photoresponse of ipRGCs (i.e. sustained pupillary light reflex) in both rodent and human samples (Berson et al., 2002; Gooley et al., 2003; Gamlin et al., 2007). Hence, inferences about the role of melanopsin in sleep and alertness based on rodent studies can be translated to humans.

Although there is a paucity of literature in humans, emerging evidence suggests melanopsin plays a similar role in the human NIF pathway as it does in animals. While rodent models enable the use of genetic knockout methods and ablation technology to isolate and study the role of melanopsin, alternate methodological designs are required in human studies. Melanopsin cells demonstrate peak sensitivity to light at 480 nm wavelengths (blue light) in humans, leading to the use of specific light irradiances and wavelengths to derive inferences about melanopsin functioning in humans (e.g., Lockley et al., 2003; Gooley et al., 2010).

In humans, physiological responses to light involved in sleep-wake regulation, such as circadian phase resetting and suppression of melatonin, are most sensitive to short-wavelength light (Brainard et al., 2001; Cajochen et al., 2005; Lockley et al., 2003). In a study exposing individuals to 6.5 constant hours of green (~555 nm) versus blue (~460nm) light, it was shown that blue light elicited a nearly constant suppression of melatonin (Gooley et al., 2010). In contrast, green light exposure initially elicited similar responses but after the first quarter (about 1.5 hours), melatonin suppression lifted exponentially. These findings suggest that melanopsin photoreceptors provide light information continuously across long-term light exposure while cone photoreceptors, which show peak sensitivity to green light, are only involved in temporary suppression of melatonin lasting about one hour (Gooley, et al., 2010). Continuous phototransduction mediated by melanopsin may explain why a blind individual with no detectable rod or cone function demonstrated a constant level of melatonin suppression when

exposed to short wavelength light (~460 nm), but no melatonin suppression during long wavelength light exposure (Zaidi et al., 2007). Another study demonstrated that 6.5 hours of constant exposure to blue light more effectively decreased sleepiness and increased alertness relative to green light (Lockley et al., 2006). Given the peak sensitivity of melanopsin cells to blue light, humans appear to rely on melanopsin and the ipRGC pathway for light influences on sleep-wake regulation (Hankins & Lucas, 2002; Lucas et al., 2003).

1.3.1 Melanopsin Gene (*OPN4*) Association Studies

Evidence from *OPN4* association studies suggests that melanopsin plays a role in human sleep regulation. In an initial effort to link *OPN4* variants to human biological and physiological responses to light, Roecklein et al. (2009) examined *OPN4* polymorphisms in 120 individuals with seasonal affective disorder (SAD) and 90 healthy controls. The authors reported that individuals homozygous for the minor T allele at P10L (Sherry et al., 2001) had a 5.6 times increased risk of SAD. The effect size was medium (d = 0.46 using the Arcsine test) and larger than the expected small effect size for any one gene in a disorder of complex genetics such as SAD. All individuals with the TT genotype were in the SAD group. Although the P10L SNP contributed to a SAD diagnosis in only 5% of cases, this may represent a subset within a heterogeneous disorder. A specific subphenotype may be characterized by related conditions caused in part by deficiencies in the non-visual light pathway involving melanopsin. These findings suggest that *OPN4* variants may be associated with differences in circadian, sleep and behavioral responses to light.

Given that seasonal affective disorder is thought to originate in part from circadian and light response abnormalities, it was hypothesized that *OPN4* variants are also associated with

differences in sleep behaviors in healthy individuals. In a subsequent study, Roecklein et al. (2012) tested the association of *OPN4* variants (P10L, I394T, and rs2014084) with individual sleep characteristics in a sample of healthy adults with no psychiatric or cardiometabolic illness at the time of assessment. Day length on the date of assessment, as determined by the U.S. Naval Observatory (2010), was included as an independent variable and tested in interaction with genotype. Age, gender, and depressive symptoms were included as covariates. Individuals with the P10L TT genotype had earlier sleep onset ($p < .05$) and also reported earlier chronotypes (i.e. morning preference for activity and sleep timing) when assessed on shorter days compared to individuals assessed on longer days. I394T and rs2014084 were not associated with sleep onset. None of the SNPs were associated with differences in wake onset or sleep duration.

Based on this preliminary report, P10L may interact with day length to influence sleep onset and preferred sleep-activity times in healthy individuals in the absence of depressive psychopathology. This study was the first to not only examine *OPN4* as a candidate gene associated with human sleep characteristics but also to investigate a possible gene by environment (i.e. photoperiod) interaction. One interpretation of these results is that P10L TT individuals are less receptive to the low light levels during shorter days and that melanopsin plays a role in shifting circadian phase and sleep timing. That the TT genotyped individuals had relatively earlier sleep onset times on short days but no difference in wake onset may explain the lack of difference in sleep duration across day lengths. The influence of other zeitgebers aside from light may contribute to the null association between P10L and sleep onset time. For example, work schedules may have served as social cues that led individuals to wake up at a determined time. If individuals woke up earlier than preferred, it is possible that they built up greater sleep debt over the day, which in turn could have induced earlier sleep times. Lastly, it is

important to note that sleep timing and duration were measured through the PSQI, a self-report questionnaire providing estimated times for average bed and wake time across the last month (Buysse, Reynolds, Monk, Berman, & Kupfer, 1989). The use of only self-reported sleep times may have presented potential confounds from recall bias and other limitations of self-report questionnaires (see below). Given that this study was the first to examine the association of *OPN4* variants and sleep characteristics, further studies are needed to replicate these findings with alternate measures of sleep timing in order to strengthen interpretation of these results.

1.4 USE OF ACTIGRAPHY FOR BEHAVIORAL SLEEP MEASURES

The present study utilized actigraphy to measure sleep timing and duration while balancing participant demand and ecological validity. Actigraphy, the use of accelerometer-based watch-like devices to monitor activity, is used in sleep research to infer sleep patterns from rest and activity counts (Sadeh & Acebo, 2002). Polysomnography (PSG) is used as the gold standard in sleep research to track sleep timing, study sleep architecture, and diagnose sleep disorders. Polysomnography (PSGinvolves a multi-parametric, comprehensive test of various biophysiological processes that occur when an individual sleeps. Although thorough, PSG methods are expensive and typically capture individual sleep outcomes over brief time periods. In contrast, actigraphy is an ambulatory device that is less intrusive for participants and enables researchers to infer sleep-wake patterns over multiple nights while participants reside in their home environment.

1.4.1 Validity of actigraphically determined sleep characteristics

Studies comparing actigraph and PSG sleep measurements support the use of actigraphy in place of PSG measures for some sleep characteristics. We report here two methods of evaluating the reliability and validity of actigraphy: (1) agreement rates of PSG and actigraphy, and (2) the ability of actigraphy to detect sleep parameters in comparison to self-reported questionnaires (Ancoli-Israel et al., 2003).

Various studies comparing actigraphy and PSG in healthy, non-sleep disordered subjects yielded >90% agreement rates (minute-by-minute agreement between the two methods) in distinguishing sleep and wake states, which suggests actigraphy can reliably determine when individuals are asleep (Sadeh, Hauri, Kripke, & Lavie, 1995). Reports for the validity of actigraphy vary in consistency based on the sleep variables of interest. A study on healthy individuals demonstrated that actigraphy was highly correlated with PSG for total sleep time (r = .79-.94, Jean-Louis et al., 2001). In a recent study on healthy young adults, t-test comparisons between two different brand activity monitors (Basic Mini-Motionlogger® and Actiwatch®) and PSG revealed that both actigraphs had similar reports on wake time, sleep efficiency, and total sleep time when compared to the PSG measures (Tonetti et al., 2008).

While sleep questionnaires and diaries are often used in studies as the only measure of sleep variables given their ease of use, self-report measures alone are not consistently correlated with PSG or actigraphy. For instance, the Pittsburgh Sleep Quality Index (PSQI) has been shown to correlate with psychological symptoms and sleep disturbances recorded in daily sleep diaries, but not with actigraphy or PSG (Buysse et al., 2008). In a sample of healthy middle-aged adults (n=669), self-report and actigraphy-determined sleep duration were only modestly correlated (r=

0.45; Lauderdale et al., 2008). Specifically, sleep duration was on average .80 hours longer when measured by subjective report in comparison to actigraphy. In contrast, evidence suggests that actigraphy has reasonable validity and reliability when compared to PSG in assessing sleep timing and duration in healthy individuals with average or good sleep quality (see Sadeh, 2011) and is therefore a suitable method to quantify the sleep variables of interest in the current study.

1.5 STUDY AIMS

Here, we aimed to reexamine the relationship between variants in *OPN4* and sleep timing as well as duration, using additional participants added to the initial study population, while improving upon some of the previous methodological limitations (Roecklein et al., 2012). Actigraphy was used as a behavioral measure of individual sleep and activity timing, and a larger sample size was also utilized for this replication.

The current study implemented a hypothesis-driven approach based on a single candidate gene rather than a genome wide, exploratory approach. We hypothesized that variations in *OPN4* SNPs would be associated with differences in sleep onset and sleep duration in interaction with day length. Although Roecklein et al. (2012) previously reported no significant relationship between I394T and sleep characteristics, we included this polymorphism because both the P10L (rs2675703) and I394T SNPs (rs1079610) are missense mutations, coding region polymorphisms that result in a non-synonymous amino acid substitution in the melanopsin protein (NCBI database; Sherry 2001). We predicted that both SNPs would impact the functioning of melanopsin and thereby, via disruption in the light pathways, would affect sleep time and sleep duration.

Based on preliminary findings, it was predicted that P10L would be associated with differences in sleep onset. Specifically, we expected individuals with the TT genotype assessed during periods of shorter day length would demonstrate earlier sleep onset relative to those assessed during periods of longer day length, and relative to other genotype groups. Given that individuals in the sample were all adult community residents who work at least part-time, work schedules were expected to serve as social *zeitgebers* (Ehlers et al., 1988) that influence wake time. Hence, we predicted that wake onset would not significantly differ between genotype groups or across different day lengths. Given that sleep duration is thought to be regulated by an interaction between homeostatic (sleep debt) and circadian factors (Czeisler & Dijk, 1995; Dijk et al., 1999) and the role of melanopsin in circadian entrainment, it was predicted that variations in *OPN4* would be associated with differences in sleep duration in interaction with day length. Despite null results in the preliminary study, we hypothesized that when assessed during shorter days individuals with the P10L TT genotype would demonstrate longer sleep duration relative to the other genotype groups. Given the null I394T results in the previous study, we predicted that I394T would not be associated with the sleep outcomes but exploratory analyses were conducted to further examine these relationships.

2.0 RESEARCH METHODS AND DESIGN

The current study relied on ambulatory activity monitors to assess individual sleep characteristics. Participants wore an activity monitor at all times for seven days. A research associate made five phone calls to participants during the monitoring period to address equipment concerns and review instructions for all procedures. The start day of activity monitoring was used to determine the day length at time of assessment. Day length was recorded by the U.S. Naval Observatory (2010) and was used as a measure of environmental dawn and dusk timing.

2.1 PARTICIPANTS

Participants were non-Hispanic Caucasian adults (30-54 years old, N = 401) from the greater Pittsburgh community who volunteered for the Adult Health and Behavior II (AHAB II) project registry. Exclusion criteria included the use of antidepressant medications or psychoactive drugs that might alter their responses to questionnaires or interview measures, a history of major neurological disorders, schizophrenia, and/or psychotic illness. Participants who met DSM-IV criteria for a current mood disorder were also excluded from analyses but those who reported any history of a mood disorder were included (N = 42, 10%).

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2.2 MATERIALS AND ASSESSMENTS

2.2.1 Actigraphy

Participants wore an Actiwatch-16 activity monitor (Bend, OR: Philips Electronics) on their non-dominant wrist for a 7-night monitoring period. Processing and scoring of the data was performed using validated algorithms provided in the Actiware software (v5.59; Bend, OR: Philips Respironics). In congruence with most actigraphy analyses, acceleration data were stored using 1-minute epochs (Ancholi-Israel et al., 2003). Participants were instructed to wear the Actiwatch continuously, day and night, and were informed that there was no need to remove it in the shower.

The following measures were quantified: sleep timing (sleep onset and wake onset) and sleep duration. Given that the current study participants were healthy, middle aged individuals, wake threshold was set at the standardized medium threshold of 40 activity counts per epoch (Actiware v5.59). Sleep onset was determined as the beginning time point of a nighttime period lasting at least 10 consecutive minutes with activity counts lower than the wake threshold. Wake onset, the end point of the sleep interval, was designated at the time point followed by least 10 consecutive minutes with activity counts greater than the wake threshold. Sleep duration was calculated as the total time between sleep onset and wake onset. Daytime rest periods meeting the aforementioned criteria were not included in the present study.

2.2.2 Mood Disorder Diagnosis

Individuals who met DSM-IV criteria for Major Depressive Disorder, Dysthymic Disorder, or Bipolar I or II Disorder (APA, 2000) were excluded from analyses. Participants underwent the Mini International Neuropsychiatric Interview (MINI; Sheehan et al., 1998) for diagnosis of DSM-based Axis I disorders. The MINI was developed to allow administration by non-clinically-trained interviewers and assesses for current presence of 17 disorders based on DSM-IV diagnostic criteria. In a validation study of the MINI (*N*= 370; 308 psychiatric patients and 62 controls) it was found that the kappa coefficient for concordance between MINI and Structured Clinician Interview for DSM-IV Patient Version(SCID-P; Spitzer et al., 1992) diagnosis was .84 for major depressive disorder (MDD; Sheehan et al., 1998). Overall, the MINI demonstrated high sensitivity for detecting any current diagnosis with only the kappa coefficient for current drug dependence falling below .5 (Sheehan et al., 1998). Sensitivity for MDD was .96. The specificity of the MINI in comparison to the SCID-P for all disorders was good (range: .86 to 1.00), with .88 for MDD. In the total sample, test-retest reliability was acceptable for MDD (kappa coefficient: .87).

2.2.3 Depression Symptoms

Given that sleep disturbances may be symptoms of depression, depressive symptoms were included as covariates in the analyses. Subjects completed the Beck Depression Inventory – 2nd Edition (BDI-II) and the Center for Epidemiologic Studies - Depression Scale (CES-D; Beck, Steer, & Brown, 1996; Radloff, 1977). Both questionnaires were included because the BDI-II is the most widely used measure of the severity of depressive symptoms, while the CES-D was

designed to assess past week frequency of depressive symptoms. To avoid confounding sleep problems and depression symptoms, the total scores of each questionnaire minus the sleep items were used (e.g., Franzen et al., 2010).

The BDI-II is a 21-item measure of depressive symptom severity, providing four severity options for each of the 21 items for participants to select (Beck et al., 1996). The BDI-II has demonstrated good test-retest reliability and convergent validity (Beck et al., 1996). The CES-D is a 20-item self-report depression symptom scale developed by the Center for Epidemiologic Studies (Privitera, Moynihan, Tang, & Khan, 2010). Participants report how often in the past week they have experienced each of 20 depression symptoms. Studies using five psychiatric populations and a community sample show that the CES-D is valid as a screening tool to detect depression symptoms, and can be used to measure change in symptom severity over time (Weissman, Sholomskas, Pottenger, Prusoff, & Locke, 1977).

2.2.4 Other Covariates

Self-reported age and gender were also included as covariates in the statistical models. Analyses based on sleep time are especially important to adjust for age due to age-related changes in sleep duration and timing (Roenneberg et al., 2007; Dijk et al., 2000)._Gender was included as a covariate given evidence that women tend to sleep longer than men (Lindberg et al., 1997).

2.2.5 DNA Collection and Genotyping

DNA was collected from participants through blood samples. The region of *OPN4* containing the containing the P10L and I394T SNPs were amplified using polymerase chain reaction methods.

Fluorescence polarization (FP; Chen et al., 1999) was then be used to determine was used to genotype the sample.

2.3 DATA ANALYSIS

Three different models of inheritance (additive, recessive, and dominant allele effects) were examined for both P10L and I394T genotypes. For each model, hierarchical linear regression analyses were conducted. In the first step, age, gender, CES-D and BDI-II scores (minus sleep items) were entered as covariates. Next, genotype and day length were entered as predictors to test for main effects of these variables. Finally, an interaction term derived from genotype*day length was entered to investigate whether differences in photoperiod would have equivalent influences on sleep characteristics across genotype groups.

As detailed below, the categorical genotype groups were coded differently for each analysis dependent upon the model of inheritance. Day length was entered as a continuous variable. An interaction term was calculated as the product of day length and the genotype variables. Dependent variables included sleep onset, sleep offset, and sleep duration, which were all entered as continuous variables.

For both P10L and I394T, the primary analyses estimated an additive model of inheritance. Genotype was treated as three separate categories (i.e., CC, CT, TT) for both SNPS. The overall model was used to predict whether there was a dose-dependent effect of genotype such that each additional minor allele accounted for additional variance in the dependent variable. Dummy coding was used in order to test for significant differences between genotype

groups in comparison to the reference groups, defined as the homozygous minor allele group (i.e., TT for P10L; CC for I394T).

A recessive model of inheritance was estimated for both SNPs in order to examine whether a genotype effect is only present in individuals with a homozygous minor allele genotype. Genotype was entered as a dichotomous variable, with the heterozygous and homozogyous major allele genotypes grouped together (i.e., P10L: TT=0, CT & CC=1). Given that past studies have found only the TT homozygous genotype to be associated with SAD as well as earlier sleep onset (Roecklein et al., 2009; Roecklein et al., 2012), it is possible that the P10L SNP may have a recessive effect.

Lastly, a dominant model of inheritance was estimated for both SNPs in order to examine whether any presence of a minor allele, regardless of whether there is one or two, would have an effect on sleep characteristics. Genotype was entered as a dichotomous variable, with both homozygous minor allele and heterozygous allele coded as 1 and the homozygous major allele coded as 0 (i.e., P10L: CT & TT= 0, CC=1). Although evidence suggests a recessive effect for P10L, the functional consequences of the variant remain unknown and therefore a dominant model was also estimated.

A double-loci risk score analysis was also conducted in order to examine whether a combination of both P10L and I394T variants would have a cumulative effect on the sleep characteristics of interest. It is possible that the predicted sleep variations are associated with polymorphisms in several loci, each with small effect sizes. The risk score was calculated as the sum of minor alleles an individual carries from both SNPs. For example, an individual who is homozygous for the T allele at P10L (TT= 2 minor alleles) and heterozygous for the C allele

(CT= 1 minor allele) at I394T would have a risk score of 3. Risk score was entered as a continuous predictor variable into the hierarchical regression model.

3.0 RESULTS

3.1 PARTICIPANT CHARACTERISTICS

A total of 401 non-Hispanic Caucasian participants were recruited in the AHAB-II registry. Of these participants, 42 met criteria for a past mood disorder but none met criteria for a current depressive episode and were therefore included in analyses. Due to technical difficulties with the actiwatch or error with data import, actigraphy data was not available for some participants (n = 15) which left a total of 386. Six participants were missing CES-D total scores, while several participant samples (see below) failed to genotype for either the P10L or I394I locus and were therefore excluded from analyses (see below). A total of 377 participants (42.43 ± 7.37 years old, 50.4% Female) were included in the present study. Although only 374 were available for the P10L analyses, 377 participants were included in the I394I analyses in order to maximize the available data. Table 1 reports the means and standard deviations in the sample for all dependent variables of interest. Multivariate ANOVAs revealed that within both P10L and I394T SNPs there were no genotype differences (i.e., CC vs CT vs TT) between age, depressive symptoms, or day length during assessment (p's >.05). There were no significant differences in gender

frequencies between genotype groups in either P10L (X^2 = .45, p=.80) or I394T (X^2 = 4.86, p = .09).

Table 1. Participant Characteristics

	Mean (SD) or %	Minimum	Maximum
Years of Education	17.22 (2.79)	10	24
Family Income	20.2% ≥110k	<10k	>185k
BMI	26.38 (4.92)	17.5	49.6
Day Length	12.61 (1.89) hrs	9.3 hrs	15.07 hrs
Sleep Onset	11:37 PM (1 hr 8 mins)	9:10 PM	4:31 AM
Wake Onset	6:28 AM (1 hr 5 mins)	3:33 AM	10:33 AM
Sleep Duration	6 hrs 51 mins (51 mins)	4 hrs 22 mins	10 hrs 27 mins
CES-D Total	8.37 (7.99)	0	45
CES-D*	7.65 (7.64)	0	42
BDI-II Total	4.33 (4.50)	0	29
BDI-II*	3.98 (4.29)	0	27
PSQI Total	4.88 (2.58)	0	16

Note. *Values shown are based on questionnaire scores minus sleep-related items. BMI= body mass index; CES-D= Center for Epidemiologic Studies Depression Scale (Radloff, 1977); BDI-II= Beck Depression Inventory – 2nd Edition (Beck, Steer, & Brown, 1996); PSQI= Pittsburgh Sleep Quality Index (Buysse, et al., 1989).

3.2 GENOTYPING

Failure rates for genotyping were less than 1.5% (P10L, n=6 (1.5%) failed to genotype, I394T n=3 (.7%) failed to genotype). Therefore, the total sample size for P10L analyses was n= 395 and for I394T n= 398. Table 2 reports the number of individuals within each genotype groups. The distribution of P10L and I394T genotypes satisfied Hardy-Weinberg equilibrium as determined using a goodness of fit chi-square test (X2 < 1, p > .05). Haploview (Barrett et al., 2005) was

used to calculate linkage between the two OPN4 SNPs. P10L and I394T showed strong pairwise linkage disequilibrium (LD), indicated by a D` value of 1 (CI: .85-1.0). More so, the logarithm of odds (LOD) score was 11.11 which indicates a high degree of confidence in the obtained D` value. Lastly, the Haploview results included a r2 value of 0.084. Although the r2 coefficient is surprisingly low zero between the two SNPs, the high D` and LOD scores indicate a high likelihood that the two SNPs are in LD and are inherited together.

Table 2. Genotype frequencies (n, %)

	Genotypes		
	CC	CT	TT
P10L (rs2675703)	310 (77.3%)	80 (20.0%)	5 (1.2%)
I394T (rs1079610)	56 (14.0%)	198 (49.4%)	144 (35.9%)

Note. P10L *n*=395 (6 failed to genotype), I394T *n*=398 (3 failed to genotype). A total of 377 participants had complete actigraphy and genotyped data.

3.3 P10L

As shown in Tables 3-5, the additive model including age, sex, depression scores, P10L genotype, day length, and P10L x day length interaction was not significant for predicting sleep onset, wake onset, or sleep duration (p's > .05). There were no significant main effects of genotype, day length, or interaction effect of P10L x day length (p's >.05). Likewise, the dominant and recessive models of genetic influence revealed no significant genotype, day length, or interaction effect (Tables 3-5; p's > .05).

Table 3. Sleep Onset is not associated with P10L across all models of inheritance

	Model of Inheritance					
	Additive		Dominant		Recessive	
Predictor	ΔR^2	β	ΔR^2	β	ΔR^2	β
Step 1	0.06*		0.06*		0.06*	
Covariates						
Step 2	0.01		0.01		0.00	
P10L		CC: -0.10 CT: -0.16		-0.05		0.03
Day Length		0.05		0.06		0.05
Step 3	0.00		0.00		0.00	
Day Length x P10L		CC: -0.13 CT: -0.10		-0.03		0.02
Total R^2	0.07		0.06		0.06	

Note. *$p < .001$, two-tailed. Values shown in Step 2 and 3 of the additive model are based on a dummy coded term for P10L genotype groups. Reference group is TT.

Table 4. Wake Onset is not associated with P10L across all models of inheritance

	Model of Inheritance					
	Additive		Dominant		Recessive	
Predictor	ΔR^2	β	ΔR^2	β	ΔR^2	β
Step 1	0.05*		0.05*		0.05*	
Covariates						
Step 2	0.01		0.01		0.00	
P10L		CC: -0.03 CT: -0.12		-0.08		0.01
Day Length		-0.01		-0.01		-0.01
Step 3	0.00		0.00		0.00	
Day Length x P10L		CC: 0.34 CT: 0.13		-0.05		-0.04
Total R^2	0.06		0.06		0.05	

Note. *$p \leq .001$, two-tailed. Values shown in Step 2 and 3 of the additive model are based on a dummy coded term for P10L genotype groups. Reference group is TT.

Table 5. Sleep Duration is not associated with P10L across all models of inheritance

	Model of Inheritance					
	Additive		Dominant		Recessive	
Predictor	ΔR^2	β	ΔR^2	β	ΔR^2	β
Step 1	0.05*		0.05*		0.05*	
Covariates						
Step 2	0.01		0.01		0.01	
P10L		CC: 0.09		-0.03		-0.02
		CT: 0.06				
Day Length		-0.08		-0.08		-0.08
Step 3	0.00		0.00		0.00	
Day Length		CC: 0.59		-0.03		-0.07
x P10L		CT: 0.29				
Total R^2	0.06		0.06		0.06	

Note. $^*p \leq .001$, two-tailed. Values shown in Step 2 and 3 of the additive model are based on a dummy coded term for P10L genotype groups. Reference group is TT.

3.4 I394T

As shown in Tables 6-8, the overall additive model including age, sex, depression scores, I394T genotype, day length, and I394T x day length interaction was not significant for predicting sleep onset, wake onset, or sleep duration (p's > .05). There were no significant main effects or interaction effect of I394T and day length (p's >.05). Null findings persisted in both the dominant and recessive models of genetic influence (p's >.05)

Table 6. Sleep Onset is not associated with I394T across all models of inheritance

	Model of Inheritance					
	Additive		Dominant		Recessive	
Predictor	ΔR^2	β	ΔR^2	β	ΔR^2	β
Step 1	0.05*		0.05*		0.05*	
Covariates						
Step 2	0.00		0.00		0.00	
I394T		CT: 0.01		-0.01		-0.01
		TT: 0.01				0.06
Day Length		0.06		0.06		
Step 3	0.00		0.00		0.00	
Day Length		CT: 0.00		0.00		0.00
x I394T		TT: -0.00				
Total R^2	0.06		0.06		0.06	

Note. *$p < .001$. Values shown in Step 2 and 3 of the additive model are based on dummy coded terms for I394T genotype groups. Reference group is CC.

Table 7. Wake Onset is not associated with I394T across all models of inheritance

	Model of Inheritance					
	Additive		Dominant		Recessive	
Predictor	ΔR^2	β	ΔR^2	β	ΔR^2	β
Step 1	0.05*		0.05*		0.05*	
Covariates						
Step 2	0.00		0.00		0.00	
I394T		CT: 0.08		0.02		-0.04
		TT: 0.04				
Day Length		-0.01		-0.01		-0.01
Step 3	0.00		0.00		0.00	
Day Length		CT: -0.05		0.07		0.04
x I394T		TT: -0.08				
Total R^2	0.05		0.05		0.05	

Note. *$p < .05$. Values shown in Step 2 and 3 of the additive model are based on dummy coded terms for I394T genotype groups. Reference group is CC.

Table 8. Sleep Duration is not associated with I394T across all models of inheritance

	Model of Inheritance					
	Additive		Dominant		Recessive	
Predictor	ΔR^2	β	ΔR^2	β	ΔR^2	β
Step 1	0.05*		0.05*		0.05*	
Covariates						
Step 2	0.01		0.01		0.01	
I394T		CT: 0.09 TT: 0.03		0.03		-0.04
Day Length		-0.09		-0.09		-0.09
Step 3	0.00		0.00		0.00	
Day Length x I394T		CT: -0.06 TT: -0.10		0.08		0.05
Total R^2	0.07		0.06		0.06	

Note. **p* ≤ .001. Values shown in Step 2 and 3 of the additive model are based on dummy coded terms for I394T genotype groups. Reference group is CC.

3.5 RISK SCORE

The aggregate risk score did not significantly associate with differences in the sleep outcome variables (p's > .05; Table 9). There was no significant interaction effect of risk score x day length (p >.05).

Table 9. Cumulative risk score not associated with sleep onset, wake onset, or sleep duration

	Outcome Variable					
	Sleep Onset		Wake Onset		Sleep Duration	
Predictor	ΔR^2	β	ΔR^2	β	ΔR^2	β
Step 1	0.06*		0.05*		0.05*	
Covariates						
Step 2	0.00		0.00		0.01	
Risk Score		-.04		-0.06		-0.02
Day Length		.05		-0.01		-0.08
Step 3	0.00		0.00		0.00	
Risk Score x Day Length		-0.01		0.03		0.05
Total R^2	0.06		0.05		0.06	

Note. $^*p \leq .001$.

3.6 VARIABILITY IN NUMBER OF NIGHTS MEASURED

Although all participants were instructed to wear the actiwatches for 7 nights, there was a range of collection nights among participants. Results, however, remained nonsignificant after a) exclusion of individuals who had less than 7 nights of data and b) the inclusion of number of monitoring nights as a covariate in all regression models (p's > .05).

3.7 SELF-REPORTED SLEEP TIMES

Exploratory analyses were conducted post-hoc to examine self-reported sleep information. On the first 4 days of the 7-day actigraphy monitoring period, participants were instructed to indicate via an electronic diary (ED) the times they went to bed and woke up. Wake onset was determined by the time at which the participant logged onto the ED device and pressed a button

indicating they had awoken. After this entry, participants were prompted to indicate the time at which they fell asleep the night before. This latter entry was used to determine sleep onset. Sleep duration was determined as the time elapsed between sleep onset and wake onset. Moderate correlations were observed between ED-based and actigraphy-derived sleep outcomes (r's= .67-.74). P10L, I394T, the risk score, and day length were not significantly associated with the self-reported sleep variables (data not shown; p's >.05).

3.8 POST-HOC POWER ANALYSIS

After genotype frequencies were obtained, post-hoc power analyses were conducted using the QUANTO 1.1 software package (Gauderman & Morrison, 2006). The sample sizes of 377 and 374 were used for the P10L and I394T analyses, respectively. Table 10 reports the statistical power calculated from the obtained effect sizes across each model of inheritance. The current study was insufficiently powered to detect a possible main effect of P10L, I394T or a genotype by day length interaction effect on sleep outcomes across each model. The unique variance explained by the P10L x day length effect on sleep onset, the sleep outcome previously linked to P10L (Roecklein et al., 2012), was very small in both the additive and recessive models (f2= .020 and .016, respectively). Hence, it is possible that the null effects found in the current report are due to insufficient power.

Table 10. Post-hoc power analyses based on observed effect sizes for predictors across each model of genetic influence

	Predictor	Model of genetic influence		
		Additive	Dominant	Recessive
P10L (*n*= 374)				
Sleep Onset	P10L	.05	.07	.05
	P10L x Day Length	.05	.07	.05
Wake Onset	P10L	.05	.09	.05
	P10L x Day Length	.05	.12	.05
Duration	P10L	.05	.06	.05
	P10L x Day Length	.05	.09	.07
I394T (*n*= 377)				
Sleep Onset	I394T	.05	.05	.05
	I394T x Day Length	.05	.05	.05
Wake Onset	I394T	.05	.05	.05
	I394T x Day Length	.05	.05	.05
Duration	I394T	.05	.05	.05
	I394T x Day Length	.05	.05	.05

**Note*: Values shown represent power (1- β error). Power analyses were conducted based on a α-level of .05 for a two-tailed test, sample size, minor allele frequency (MAF: P10L= .15; I394T= .34), and effect sizes obtained across each model of genetic influence.

4.0 DISCUSSION

The present study aimed to examine whether variants in OPN4 associate with naturally occurring differences in sleep timing and sleep duration among healthy, middle-aged adults. We did not find significant associations between either P10L, I394T, or a cumulative risk score and individual sleep characteristics. In the present sample, day length was not associated with any of the three sleep outcome variables nor did day length modify the effect of either SNP.

Based on previous findings (Roecklein et al., 2009), we had hypothesized that the P10L variant may have downstream impacts on the timing of sleep behavior and that this relationship would be modified by day length. Our inconsistent results may be in part related to differences in study samples. Roecklein et al. (2009) reported in a case-control study that the P10L TT genotype was related to risk for seasonal affective disorder (SAD), a mood disorder characterized by depressive episodes that occur in the fall and winter. In contrast, the current study excluded individuals who met criteria for depression at the time of assessment, and also statistically adjusted for sub-clinical depressive symptoms. Although the etiology of SAD remains unknown, individuals with this disorder are more likely to be phase delayed in their sleep cycles relative to healthy individuals (Lee et al., 2011). Hence the link between P10L and SAD may be driven in part by a more extreme range in sleep and activity phases that is underrepresented in the current healthy study sample.

The use of actigraphy in the current study to quantify sleep timing and duration was a strength in determining sleep characteristics. The previous report on sleep timing and OPN4 relied on subjective information from the PSQI (Roecklein et al., 2012). While actigraphy is not perfectly correlated with PSG measures, it is a reliable and valid method to infer sleep timing and provides the advantage of studying free-dwelling participants that captures behavioral rather than subjective-based estimates of sleep patterns (Ancoli-Isreal et al., 2003). One interpretation of our inconsistent results may be that the previous link between P10L and PSQI-based sleep onset was influenced in part by estimates confounded by poor sleep quality or undetected sleep disorders such as insomnia. In order to examine the possible influence of methodological differences, a replication was conducted with the present sample. Moderate correlations were observed between actigraphy-derived and PSQI-based sleep outcomes (r's= .42-.65). Neither P10L nor day length, however, were significantly associated with PSQI sleep or wake time (p's < 05). Our inconsistent findings may therefore not be fully explained by differences in sleep assessment.

Another explanation for the present null findings may be due to a relatively lower percentage of individuals with the P10L TT genotype in the present sample compared to the previous study (Roecklein et al., 2012). A post-hoc power analyses suggest that the present sample was not sufficient in size to detect a possible P10L by day length interaction effect. There were few subjects (n = 5, 1.3%) with a TT genotype relative to the total sample (N = 374), which provided limited variability in day length to capture potential interaction effects. While the overall sample size was larger in the present study when compared to the previous report (N = 323), the number of individuals with the P10L TT genotype remained the same (n = 5). In order to examine whether the null findings are in part due to insufficient power relative to the previous

report, analyses examining actigraphy-derived sleep measures were replicated in the previous sample. There were no significant associations between OPN4 variants and sleep characteristics when using actigraphy in the previous sample (p's >.05). It remains unclear whether the previously reported association between P10L and sleep onset was spurious. A future study sample enriched with P10L TT genotypes may help resolve the question of power and minor allele frequency with this specific locus.

Our current finding that I394I had no effect on any of the sleep phenotypes is in congruence with previous reports (Roecklein et al., 2009). In a recent study on Japanese students, Higuchi et al (2013) found an association between I394T and the pupillary light response (Higuchi et al., 2013). This report is the first to suggest the I394T melanopsin gene polymorphism has a functional role in non-image forming responses to light. The authors implemented a protocol, however, that exposed participants to light continuously for five minutes which greatly exceeds the time span used in other pupillary light response studies (e.g., 30-60 seconds; Hattar et al., 2003; Lucas et al., 2003; Roecklein et al., 2013) and may have confounded results. Limitations of this study and lack of replication thus far preclude a thorough understanding of the functional significance of I394T.

Several limitations in the present study include the use of average sleep and day length measurements without statistically adjusting for the possible influence of previous sleep and light exposure history. Artificial light use in modern society exposes individuals to light after sunset and can prolong wakefulness. Chang et al (2013) reported that the magnitude of an individual's alerting response to light depends not only on absolute light exposure but also on the history of light exposure. In a cross-over design, the participants were exposed to either 1 lux or 90 lux light stimuli when they were awake for three days preceding the experimental light exposure.

Alerting response was measured through subjective sleepiness ratings, reaction times to an auditory task, and EEG determined power density/delta waves. When participants in both groups were exposed to 6.5 hours of 90-lux light stimuli, participants who were previously exposed to brighter lights demonstrated a relatively smaller and shorter alerting response relative to those who were exposed to dim light. The possibility that sensitization may occur as a function of light history introduces a more complex model wherein a cross-sectional measure of day length may not fully capture the effect of disruptions in the non-image forming light pathway. Lastly, sleep duration and sleep time are influenced not only by the circadian clock but also by history of sleep. The present study characterized individuals based on an average measure of sleep time and duration but did not control for the possibility that accumulated sleep debt over the week may have affected bedtime and extended sleep duration. Accelerometers with lux detection features can be implemented in future studies in order to measure and statistically adjust for the possible confounding effects of both previous light exposure and sleep debt.

The present findings suggest that neither the P10L nor I394T melanopsin gene polymorphisms have a large effect on sleep timing, although it still remains possible the P10L TT genotype has an interaction effect with day length that we were underpowered to detect. Another approach that future studies can implement involves conducting a haplotype analysis. Haplotypes are specific combinations of alleles on a chromosome that tend to be inherited together in the population (Gelehrter, Collins, & Ginsburg, 1998). Single SNP association studies such as the current report assume the tested SNP is either itself a disease locus or is in high linkage disequilibrium (LD) with an unidentified disease locus. Commonly inherited or correlated alleles are usually in high LD with one another. Conducting only a SNP association analysis may not account for other significant loci that are in low LD with the SNPs of interest.

A haplotype analysis may identify potentially significant combinations of OPN4 alleles while eliminating the need to test for all possible combination of genotypes and thereby controls the degrees of freedom and increases statistical power (Clark, 2004). Given recent findings that CLOCK gene variants are linked with sleep duration (Allebrandt et al. 2010), future studies should also utilize a multi-locus risk score approach to investigate a possible cumulative effect of melanopsin and clock gene variants that may independently be too small to detect in single SNP association studies.

While a growing animal literature suggests melanopsin plays an integral role in circadian photoentrainment and downstream behavioral and physiological processes, there remains a limited understanding of its significance in humans. The current non-significant results warrant investigation of 1) other variations in the melanopsin gene upstream regulatory region, 2) other genes involved in the clock network that may impact circadian entrainment of the sleep-wake cycle, and 3) further study of the role of melanopsin in more proximal behavioral measures of non-image forming functions in humans.

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