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Original Article

ORIGINAL ARTICLE

Melanopsin-dependent phototransduction is impaired in delayed sleep–wake phase disorder and sighted non–24-hour sleep–wake rhythm disorder

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Abstract

Study Objectives: The circadian system must perform daily adjustments to align sleep–wake and other physiologic rhythms with the environmental light–dark cycle: This is mediated primarily through melanopsin containing intrinsically photosensitive retinal ganglion cells. Individuals with delayed sleep–wake phase disorder (DSWPD) exhibit a delay in sleep–wake timing relative to the average population, while those with sighted non–24-hour sleep–wake rhythm disorder (N24SWD) exhibit progressive delays. An inability to maintain appropriate entrainment is a characteristic of both disorders. In this study, we test the hypothesis that individuals with DSWPD exhibit alteration in melanopsin-dependent retinal photo-transduction as measured with the postillumination pupil response (PIPR).

Methods: Twenty-one control and 29 participants with DSWPD were recruited from the community and clinic. Of the 29 DSWPD participants, 17 reported a history of N24SWD. A pupillometer was used to measure the PIPR in response to a bright 30-second blue or red-light stimulus. The PIPR was calculated as the difference in average pupil diameter at baseline and 10–40 seconds after light stimulus offset.

Results: The PIPR was significantly reduced in the DSWPD group when compared with the control group (1.26 ± 1.11 mm vs 2.05 ± 1.04 mm, $p < 0.05$, *t*-test). The PIPR was significantly reduced in the sighted N24SWD subgroup when compared with individuals with the history of only DSWPD (0.88 ± 0.58 mm vs 1.82 ± 1.44 mm, $p < 0.05$, analysis of variance [ANOVA]) or controls (0.88 ± 0.58 mm vs 2.05 ± 1.04 mm, $p < 0.01$, ANOVA).

Conclusions: These results indicate that reduced melanopsin-dependent retinal photo-transduction may be a novel mechanism involved in the development of DSWPD and sighted N24SWD.

Statement of Significance

Patients with delayed sleep–wake phase (DSWPD) and non–24-hour sleep–wake disorder (N24SWD) exhibit sleep–wake patterns that are misaligned with the light–dark environment. The underlying cause of these disorders remains unclear. The aim of this study was to test the hypothesis that patients with DSWPD have impaired melanopsin-dependent phototransduction when compared with controls. Using pupillometry to measure the postillumination pupil response (PIPR), a physiological marker of melanopsin function, we demonstrated that individuals with DSWPD and those with DSWPD and a history of sighted N24SWD exhibit a reduced PIPR compared to control subjects. These data support our hypothesis that some individuals with severe DSWPD and sighted N24SWD have impaired melanopsin-dependent phototransduction, which may explain their inability to appropriately entrain with the environment.

Key words: circadian; melanopsin; delayed sleep–wake phase disorder; non–24-hour sleep–wake rhythm disorder

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Introduction

The circadian system serves to coordinate physiology and behavior with the 24-hour physical environment. In mammals, the primary circadian pacemaker is located in the suprachiasmatic nucleus (SCN) in the hypothalamus [1]. The SCN receives environmental signals to maintain entrainment with the surrounding environment and coordinates the 24-hour rhythms of behavior and physiology within the individual. There is increasing evidence that an inability to maintain appropriate alignment between an individual's circadian timing and the environment, referred to broadly as a circadian rhythm sleep–wake disorder (CRSWD) can have significant negative health consequences. This was first demonstrated with shift workers, who have previously been demonstrated to have an increased risk for malignancy [2] and cardiometabolic disease [3] independent of total sleep time. However, more recently, it has been demonstrated that later sleep–wake timing is associated with an elevated body mass index [4], poorer glycemic control in diabetes [5], and an increased risk for all-cause mortality [6]. Despite the growing evidence for health risks associated with delayed sleep–wake timing we do not currently have a clear understanding of what factors predispose an individual to develop this pattern. However, potential causes include genetic, behavioral, and environmental factors.

Further insight into the potential causes of circadian misalignment can be gained by studying the clinical manifestation of delayed sleep–wake phase disorder (DSWPD), which is the most common CRSWD. In DSWPD, an individual's habitual sleep and wake times are delayed by at least 2 hours compared to the average population, resulting in complaints of both insomnia and excessive sleepiness when trying to follow a traditional schedule [7]. Along with the health impacts noted above, the personal and societal impacts of this disorder can be significant, with many of these individuals finding it difficult to maintain steady employment and social engagements due to their sleep disorder. While the underlying cause of DSWPD is unknown, several potential factors have been identified, and the disorder appears to be heterogeneous. Behavioral factors certainly play a role in many individuals, with up to 43% of patients presenting with clinical symptoms of DSWPD, but exhibiting normal timing of melatonin profiles [8, 9], suggesting the designations of circadian (delayed dim light melatonin onset [DLMO] and sleep–wake timing) and noncircadian (normal DLMO but delayed sleep–wake timing) subtypes of DSWPD. In patients with circadian DSWPD, genetic studies have demonstrated that these individuals often exhibit a prolonged circadian period [10], associated with mutations in the *CRY1* [11] and *PER2* [12] genes. Finally, in addition to the above factors, one of the strongest signals that maintains circadian entrainment is light, with light at specific circadian times serving to either advance or delay the internal clock [13–15]. As such, one theory regarding the pathophysiology of DSWPD is that these individuals have an altered circadian response to light. Previous studies have demonstrated a hypersensitivity to evening light in individuals with DSWPD, which may promote greater daily phase delays [16, 17]. However, it is also possible that some of these individuals are less sensitive to the circadian effects of light during the early morning, a time when light normally advances the circadian clock. Weak daily advancing signals can result in either a later phase angle of entrainment of the sleep–wake rhythm with respect to the environmental light dark cycle, or a tendency to gradually progress later each day, resulting in a non-24 pattern. Of note, a subset of individuals with DSWPD have been reported to develop symptoms of non–24-hour sleep–wake disorder (N24SWD) in which individuals exhibit progressive daily delays of sleep–wake rhythms (Figure 1). This disorder was first described in blind individuals who lack light perception, but is increasingly being identified in sighted individuals who may initially present with symptoms of DSWPD [18, 19], often with later and longer sleep times, and longer circadian periods than average [20]. These data suggest that sighted N24SWD may represent an extreme phenotype of DSWPD, which is more vulnerable to nonentrainment, possibly through impaired light perception.

The circadian response to light has been demonstrated to be mediated by melanopsin containing intrinsically photosensitive retinal ganglion cells (ipRGCs), which send projections through the retinohypothalamic tract to the SCN [21, 22]. Melanopsin is hypothesized to be important for producing full pupil constriction at high irradiance as well as for maintaining sustained pupil constriction in response to light [23]. While melanopsin activity cannot be directly assessed in humans, it can indirectly be measured by quantifying pupillary constriction and re-dilation in response to specific light stimuli as a surrogate marker of melanopsin function. In a clinical population, studies

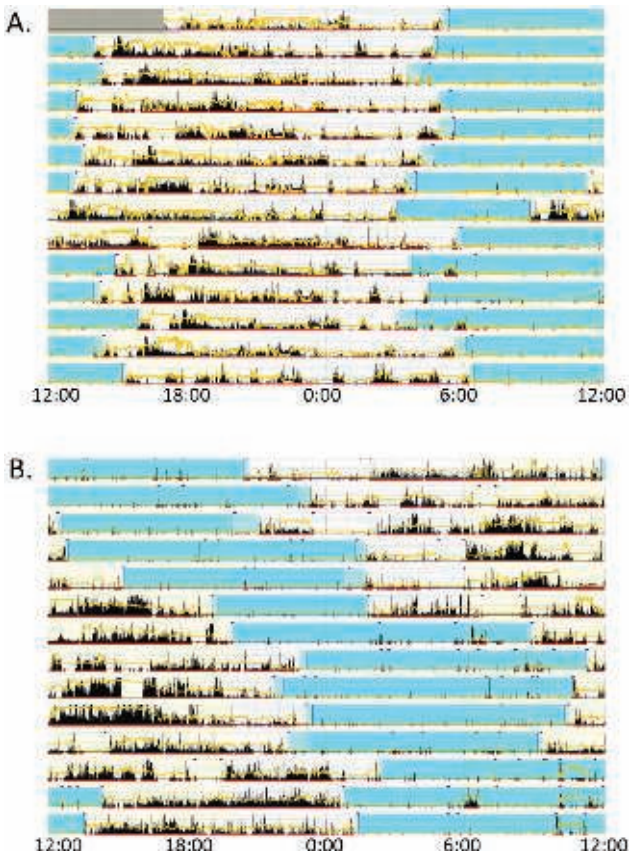

Figure 1. Examples of DSWPD and N24SWD. Representative actigraphy data from individuals with (A) DSWPD and (B) N24SWD. Each line represents 24 hours, with relative activity counts indicated in black, light exposure indicated by the yellow line, and rest intervals indicated with the blue rectangles. The individual with DSWPD exhibits an average sleep onset of 04:30, and sleep offset of 13:30, while the individual with N24SWD exhibits progressive daily delays in sleep onset and offset.

have demonstrated that the melanopsin-driven pupillary response is primarily mediated by high-intensity short wavelength (blue) light, while the rod response is mediated by low-intensity blue light, and the cone response is best demonstrated with high-intensity red light administered on a background of blue light, to suppress the rod and melanopsin contributions [24]. Melanopsin function can be quantified using the postillumination pupil response (PIPR), a measure of the ability to maintain sustained contraction of the pupil following removal of a light stimulus [25].

The aim of this study is to test the hypothesis that melanopsin-dependent retinal photo-transduction, as measured by the PIPR is altered in DSWPD, when compared with individuals with conventional sleep–wake timing, and that these differences are more pronounced in individuals who exhibit an overlap of DSWPD and N24SWD phenotypes.

Methods

Participants

Twenty-nine individuals with DSWPD and 21 controls (mid-sleep of 3:00–5:00) were recruited from the community and the Northwestern Medicine Sleep Disorders clinic. Inclusion criteria for DSWPD subjects included a self-reported mid-sleep point of 6:00 or later and a clinical history consistent with the ICSD-3 diagnostic criteria for DSWPD. Seventeen of the subjects classified with DSWPD had mixed clinical history, where sometimes they would exhibit rest–activity patterns consistent with DSWPD, while at other times they would follow a non–24-hour schedule. Controls had an intermediate self-reported mid-sleep point of 3:00–5:00, confirmed with 2 weeks of actigraphy. Subjects were excluded for the following reasons: (1) Prior eye surgery or use of eye drops known to affect pupillary response; (2) retinal or optic nerve disease; (3) use of medications with known sympathetic or parasympathetic effects; (4) use of medications known to affect melatonin secretion (e.g. β-blockers, NSAIDs); (5) sleep disorders other than DSWPD/N24SWD as assessed by history and the STOP and Pittsburgh Sleep Quality Index; (6) history of, or concurrent unstable or serious medical illness; and (7) shift work or self-imposed irregular sleep schedule within the past 6 months.

Procedures

Upon giving written informed consent, participants were given questionnaires including the Pittsburgh Sleep Quality Index (PSQI) [26] to evaluate for sleep difficulties, the Munich Chronotype Questionnaire (MCTQ) [27] to determine circadian preference, the Epworth Sleepiness Scale (ESS) [28] to evaluate for excessive sleepiness and the STOP questionnaire to screen for presence of obstructive sleep apnea [29]. Eligible subjects underwent a basic eye examination and medical and sleep history interview, including questioning about a history of sleep–wake patterns consistent with N24SWD for all subjects with DSWPD. Participants were then sent home with a Spectrum Actigraph (Philips/Respironics) and sleep log to collect wrist activity and light levels for 2 weeks. All participants returned to the sleep lab after 2 weeks, and if actigraphy data met inclusion criteria, they then completed pupillometry testing.

Due to the rare nature of this disorder, a subset of pupillometry data (17 subjects) were obtained from clinic subjects who had been diagnosed with DSWPD at the Northwestern Circadian Medicine clinic and underwent pupillometry testing as part of their routine clinic visit. These subjects all completed a detailed medical and sleep history interview, as well as a physical exam, completed by one of two physicians (S.M.A. or P.C.Z.). Actigraphy data concurrent to the time of pupillometry testing were available from eight of those subjects. The other nine subjects did not complete actigraphy at the time of pupillometry primarily either due to the cost of testing, or because they had completed this testing with another physician. As these pupillometry data were obtained retrospectively, the PSQI and STOP questionnaire were not obtained from these subjects. The MCTQ and ESS were obtained as part of the routine clinical evaluation of these subjects.

The Institutional Review Board at Northwestern University approved this study. Before study participation, written informed consent was obtained from all participants who were prospectively enrolled in the study. IRB approval was also obtained to conduct a retrospective chart review of patients who had previously had pupillometry performed as part of their routine clinical evaluation.

For all participants, pupillometry testing was completed between 2015 and 2019.

Pupillometry

All subjects were placed in a dark room for 5 minutes to allow pupils to dark adapt. The PIPR was then determined using the Neuroptics DP-2000 Pupillometer (Irvine, CA). Baseline pupil diameter was measured for 10 seconds; then, subjects were presented with a full-field blue LED (467.7 nm, 398 lux, 1.5 log W/m^2) light stimulus for 30 seconds followed by pupil diameter recording for 40 seconds. Testing was then repeated with a full-field red LED (632.9 nm, 3 lux, –2.0 log W/m^2) light stimulus (Figure 2) to assess non–melanopsin-driven pupillary responses. Participants underwent 2 minutes of dark readaptation between stimuli. All stimuli were presented to the left eye, and the consensual pupil diameter measurements were recorded from the right eye. Sampling frequency was 30 Hz.

Data were cleaned to remove blinks and artifacts; then, the PIPR was calculated using MATLAB software. Baseline pupil diameter was determined based on the average pupil diameter during the 10-second baseline recording obtained prior to the light stimulus. The sustained pupil diameter was calculated based on the average pupil diameter recorded over 30 seconds, beginning 10 seconds after the offset of the light stimulus. PIPR (mm) = [baseline pupil diameter mm – sustained pupil diameter mm]. Percent from baseline = [((baseline pupil diameter – sustained pupil diameter)/baseline pupil diameter) × 100].

Actigraphy

Wrist activity monitoring was completed using an Actiwatch Spectrum watch (Philips Respironics, Bend, OR) worn on the nondominant wrist. Subjects were instructed to wear the activity monitor for 2 weeks, keeping the face of the watch uncovered at all times. Data were collected in 30-second epochs. Subjects were instructed to press the marker button on the watch at bedtime and wake time, and were also instructed to maintain a sleep diary throughout the duration of the activity monitoring. Wrist activity data were analyzed using the

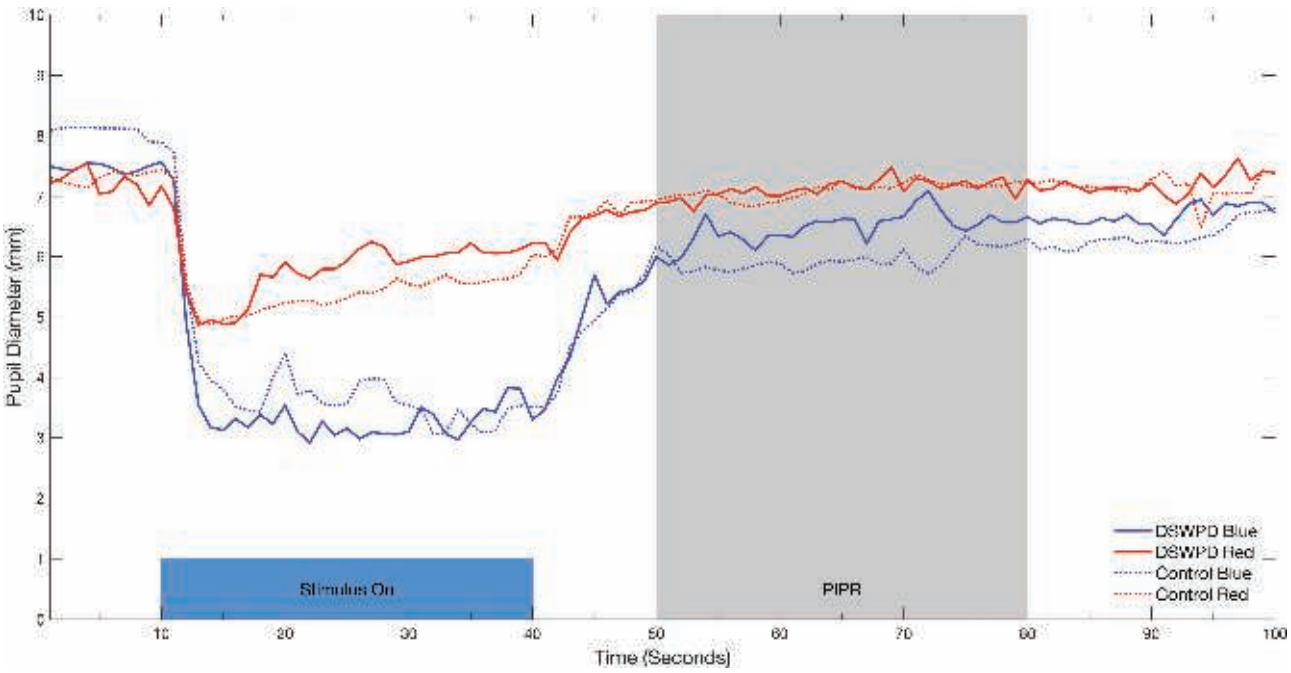

Figure 2. Pupillometry. Example pupillometry tracing from a control subject (dashed line) and a subject with DSWPD (solid line) demonstrating a more rapid return to baseline pupil diameter following the blue light stimulus in the subject with DSWPD compared to the control subject.

Philips Actiware software (Version 6.0.8, Philips Respironics). Actiwatch-recorded sleep variables were calculated using the period between self-reported bed time to wake time (time in bed) from the marker data. If marker data were missing or noted to be inaccurate, sleep diaries were used. The following variables were determined: sleep start (sleep onset), sleep end (actual wake time), and actual sleep time (total sleep time). Sleep start was defined as the beginning of the first 10 minutes period in which no more than one epoch was scored as "mobile." Sleep end was defined as the end of the last 10 minutes period in which no more than one epoch was scored as "immobile." Actual sleep time was defined as the amount of time between sleep start and sleep end, that was scored as "sleep."

Data analysis

Unpaired *t*-tests were used to examine differences in PIPR between control and DSWPD subjects. One-way analysis of variance (ANOVA) with Tukey post hoc analysis was used to examine differences between control, DSWPD with N24SWD, and DSWPD without N24SWD. Receiver operating characteristic (ROC) curves were generated to determine the clinical utility of the PIPR in distinguishing patients with DSWPD and N24SWD from healthy controls. All analyses were conducted using the GraphPad Prism 8.4.2 software (GraphPad Software, San Diego, CA).

Results

Twenty-nine subjects with DSWPD were recruited from the community and the Northwestern Circadian Medicine Clinic, and 21 healthy controls with an intermediate sleep–wake pattern were recruited from the community. The average age was 25.0 ± 4.0 (range 19–36) for the controls and 27.9 ± 10.8 (range 16–54) for the DSWPD. Eleven DSWPD and 15 control subjects were female. Actigraphy data prior to testing were available for 16 of the DSWPD subjects and 20 of the control subjects. One subject in the DSWPD category exhibited a non–24-hour pattern while undergoing actigraphy testing, so their data were excluded from this analysis, as sleep onset and offset progressively moved later throughout the recording. The final analysis of actigraphy data was conducted on 15 DSWPD and 20 control subjects. Actigraphy determined average sleep onset (3:30 ± 1:52 [range 0:34–6:00] vs 23:41 ± 0:38 [range 22:30–0:45]) and sleep offset (12:10 ± 1:41 [range 8:46–14:23] vs 7:45 ± 0:31 [range 6:46–8:48]) were significantly later in the subjects with DSWPD compared to controls ($p < 0.0001$), while total sleep time was not significantly different (7:17 ± 1:27 [range 5:35–10:45] vs. 6:56 ± 0:39 [range 5:26–8:09], $p = 0.48$). The mid-sleep time was significantly later in the DSWPD subjects compared to controls (7:49 ± 1:36 [range 5:03–10:11] vs 3:43 ± 0:30 [range 2:48–4:44], $p < 0.0001$; Table 1).

There were no significant differences between baseline and sustained pupil diameter in response to blue (baseline (7.85 ± 1.46 mm [range 4.96–10.88 mm] vs 8.02 ± 0.98 mm [range 5.93–10.02 mm], $p = 0.66$; sustained (6.59 ± 1.22 mm [range 3.97–9.68 mm] vs 5.96 ± 1.24 mm [range 3.75–8.32 mm], $p = 0.08$) or red light (baseline [7.43 ± 1.15 mm [range 5.11–9.37 mm] vs 7.36 ± 1.12 mm [range 5.21–8.92 mm], $p = 0.90$; sustained (7.31 ± 1.25 mm [range 4.98–10.70 mm] vs 7.07 ± 1.00 mm [range 4.45 to 8.63 mm], $p = 0.46$) in subjects with DSWPD compared to controls (Table 2).

The PIPR mm (1.26 ± 1.11 mm [range –0.27 to 5.46 mm) vs 2.05 ± 1.04 mm [range 0.7–4.61 mm], $p < 0.05$) and PIPR % (15% ± 11% [range –4% to 50%] vs 25% ± 12% [range 9%–46%], $p < 0.01$) for blue light were significantly reduced for subjects with DSWPD

Table 1. Demographics of all study participants

Characteristic	Control (*n* = 21)	DSWPD (*n* = 29)	*P*
Age (years)	25.0 ± 4.0 (19–36)	27.9 ± 10.8 (16–54)	0.31
Gender (M:F)	6:15	18:11	<0.05
Horne-Ostberg	56.8 ± 5.2 (46–66)	27.0 ± 6.4 (16–35) (*n* = 9)	<0.0001
PSQI	2.3 ± 1.4 (0–5)	6.8 ± 3.0 (3–14) (*n* = 9)	<0.001
ESS	5.0 ± 2.0 (0–9)	4.8 ± 3.4 (0–12) (*n*=24)	0.79
Average sleep onset (hh:mm)	23:41 ± 0:38 (22:30–0:45)	3:30 ± 1:52 (0:34–6:00) (*n* = 15)	<0.0001
Average sleep offset (hh:mm)	7:45 ± 0:31 (6:46–8:48)	12:10 ± 1:41 (8:46–14:23) (*n* = 15)	<0.0001
Average total sleep time (hh:mm)	6:56 ± 0:39 (5:26–8:09)	7:17 ± 1:27 (5:35–10:45) (*n* = 15)	0.48
Average mid-sleep (hh:mm)	3:43 ± 0:30 (2:48–4:44)	7:49 ± 1:36 (5:03–10:11) (*n* = 15)	<0.0001
Pupillometry testing (time-since-wake)	6:24 ± 2:36 (0:43–9:35)	2:56 ± 2:41 (0:01–8:48)	<0.01

Some of the measures were not available for all subjects. In those cases, the total number of subjects used for the analysis is listed in parentheses after the value. All numbers indicate mean ± standard deviation, with range presented in parentheses.

Table 2. Pupillometry measures in all participants

Measure	Control (*n* = 21)	DSWPD (*n* = 29)	*P*
Baseline diameter (blue) (mm)	8.02 ± 0.98 (5.93–10.02)	7.85 ± 1.46 (4.96–10.88)	0.66
Sustained diameter (blue) (mm)	5.96 ± 1.24 (3.75–8.32)	6.59 ± 1.22 (3.97–9.68)	0.08
PIPR mm (blue)	2.05 ± 1.04 (0.7–4.61)	1.27 ± 1.11 (–0.27 to 5.46)	<0.05
PIPR % (blue)	26% ± 12% (9%–46%)	15% ± 11% (–4% to 50%)	<0.01
Baseline diameter (red) (mm)	7.36 ± 1.12 (5.21–8.92)	7.43 ± 1.15 (5.11–9.37)	0.90
Sustained diameter (red) (mm)	7.07 ± 1.00 (4.45–8.63)	7.31 ± 1.25 (4.98–10.70)	0.46
PIPR mm (red)	0.30 ± 0.57 (–1.33 to 1.63)	0.12 ± 0.75 (–2.54 to 1.2)	0.35
PIPR % (red)	4% ± 8% (–23% to 18%)	–1% ± 11% (–31% to 10%)	0.15

All numbers indicate mean ± standard deviation, with range presented in parentheses.

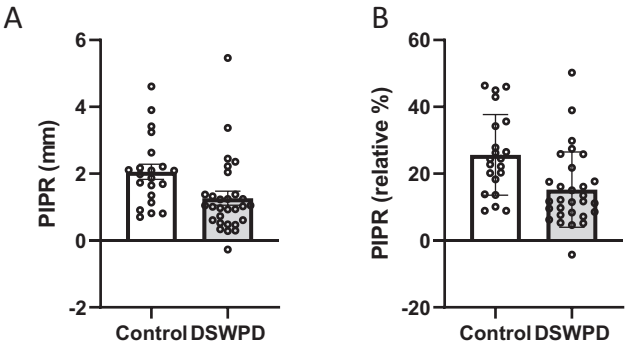

Figure 3. The PIPR in controls and subjects with DSWPD. The average PIPR is significantly reduced in subjects with DSWPD when compared with controls presented as absolute mm (A, $p < 0.05$, unpaired *t*-test) or relative to baseline pupil size (B, $p < 0.01$). Error bars represent SD, and dots indicate individual data points.

compared with controls (Table 2; Figure 3). These differences remained significant after including age and sex as covariates. There was no significant difference between groups when comparing the PIPR mm (0.12 ± 0.75 mm [range –2.54 to 1.2 mm] vs 0.30 ± 0.57 mm [range –1.33 to 1.63 mm], $p = 0.35$) or PIPR % (–1% ± 11% [range –31% to 10%] vs 4% ± 8% [range –22% to 18%], $p = 0.15$) in response to red light. Pupillometry measurements were conducted at an average of 4:53 ± 3:11 hours after wake.

Out of the 29 subjects classified as having DSWPD, 17 of those subjects reported a clinical history of exhibiting a non–24-hour sleep–wake pattern at some point in their life. Individuals with DSWPD who also had a history of symptoms of N24SWD exhibited a later mid-point of sleep timing (8:22 ± 1:22 vs 6:54 ± 1:37, $p < 0.001$) when compared with those who only had symptoms of DSWPD (Table 3). PIPR mm (0.88 ± 0.60 mm [range –0.27 to 5.46 mm] vs 1.82 ± 1.44 mm [range 0.28–5.46 mm] vs 2.05 ± 1.04 mm [range 0.7–4.61 mm], $p < 0.01$) and PIPR % (12% ± 8% [range –4% to 26%] vs 20% ± 14% [range 5%–50%] vs 26% ± 12% [range 9%–46%], $p < 0.01$) were significantly reduced in response to blue light in DSWPD subjects with a history of symptoms of N24SWD when compared with those who only had symptoms of DSWPD or controls (Table 4; Figure 4). These differences remained significant after including age and sex as covariates.

An ROC curve was constructed for PIPR % to blue light for controls and DSWPD subjects with a history of N24SWD (Figure 5). Using Youden's index, a PIPR % cutoff of <18% provides 88% sensitivity and 76% specificity for distinguishing control individuals from DSWPD subjects with a history of N24SWD (AUC 0.85 [0.006], $p < 0.001$).

Actigraphy data were available for 15 of the DSWPD participants and 20 control participants to allow for calculation of the mid-sleep point. There is a moderate negative correlation between the PIPR % and mid-sleep point ($r = –0.54$, $p < 0.001$; Figure 6).

As actigraphy and salivary melatonin data were not available for all subjects, it was not possible to determine the exact circadian time at which pupillometry was performed for each subject; however, this was approximated for all subjects based on the difference between wake time on the day of testing, either obtained from actigraphy or self-report, and wall clock time of pupillometry testing. Pupillometry was performed closer to wake time in N24 (2:27 ± 2:59 hours) and DSWPD (3:36 ± 2:06 hours) compared to control subjects (6:24 ± 2:36 hours). However, differences in PIPR measures in response to blue light between the three groups remained significant after adding time-since-wake of pupillometry as a covariate.

Table 3. Demographics of all study participants, broken down into DSWPD with and without a history of N24SWD features

Characteristic	Control (*n* = 21)	DSWPD (without N24SWD features) (*n* = 12)	DSWPD (with N24SWD features) (*n* = 17)	*P*
Age (years)	25.0 ± 4.0 (19–36)	29.7 ± 12.3 (18–54)	26.6 ± 10.2 (16–50)	0.34
Gender (M:F)	6:15	6:6	12:5	
Horne-Ostberg	56.8 ± 5.2 (46–66)	26.2 ± 7.4* (16–35) (*n* = 6)	28.7 ± 4.6* (26–34) (*n* = 3)	0.0001
PSQI	2.3 ± 1.4 (0–5)	6.8 ± 3.4* (3–14) (*n* = 6)	6.7 ± 0.6* (6–7) (*n* = 3)	0.0001
ESS	5.0 ± 2.0 (0–9)	5.1 ± 3.2 (0–11) (*n* = 10)	4.4 ± 4.2 (0–12) (*n* = 14)	0.79
Average sleep onset (hh:mm)	23:41 ± 0:38 (22:30–0:45)	3:05 ± 1:14* (1:53–5:00) (*n* = 6)	3:45 ± 2:12* (0:34–6:39) (*n* = 9)	<0.0001
Average sleep offset (hh:mm)	7:45 ± 0:31 (6:46–8:48)	10:49 ± 1:55* (8:46–13:40) (*n* = 6)	12:58 ± 0:53* (11:49–14:23) (*n* = 9)	<0.0001
Average total sleep time (hh:mm)	6:56 ± 0:39 (5:26–8:09)	6:41 ± 0:00 (5:58–7:38) (*n* = 6)	7:36 ± 1:40 (5:35–10:45) (*n* = 9)	0.08
Average mid-sleep (hh:mm)	3:43 ± 0:30 (2:48–4:44)	6:54 ± 1:37* (5:49–9:20) (*n* = 6)	8:22 ± 1:22* (6:41–10:11) (*n* = 9)	<0.0001
Pupillometry testing (time-since-wake)	6:24 ± 2:36 (0:43–9:35)	3:36 ± 2:06* (0:37–7:30)	2:27 ± 2:59* (0:01–8:48)	<0.05

Some of the measures were not available for all subjects. In those cases, the total number of subjects used for the analysis is listed in parentheses below the value.
All numbers indicate mean ± standard deviation, with range presented in parentheses.
* indicates $p < 0.05$ when compared with controls.

Table 4. Pupillometry measures broken down by DSWPD with and without N24SWD features

Measure	Control (*n* = 21)	DSWPD (without N24SWD features) (*n* = 12)	DSWPD (with N24SWD features) (*n* = 17)	*P*
Baseline diameter (blue) (mm)	8.02 ± 0.98 (5.93–10.02)	8.57 ± 1.39* (5.47–10.88)	7.35 ± 1.32 (4.96–9.48)	<0.05
Sustained diameter (blue) (mm)	5.96 ± 1.24 (3.75–8.32)	6.74 ± 1.34 (5.18–9.68)	6.48 ± 1.15 (3.97–8.03)	0.19
PIPR mm (blue)	2.05 ± 1.04* (0.7–4.61)	1.82 ± 1.44* (0.28–5.46)	0.88 ± 0.60 (–0.27 to 2.45)	<0.01
PIPR % (blue)	25% ± 12%* (9%–46%)	20% ± 14%* (5%–50%)	12% ± 8% (–4% to 26%)	<0.01
Baseline diameter (red) (mm)	7.36 ± 1.12 (5.21–8.92)	7.76 ± 0.92 (5.76–8.78)	7.19 ± 1.27 (5.11–9.37)	0.41
Sustained diameter (red) (mm)	7.07 ± 1.00 (4.45–8.63)	7.38 ± 0.95 (5.61–8.78)	7.25 ± 1.45 (4.98–10.70)	0.73
PIPR mm (red)	0.30 ± 0.57 (–1.33 to 1.63)	0.37 ± 0.48 (–0.08 to 1.2)	–0.06 ± 0.86 (–2.54 to 0.8)	0.15
PIPR % (red)	4% ± 8% (–23% to 18%)	5% ± 6% (–1% to 15%)	–1% ± 11% (–31% to 10%)	0.17

All numbers indicate mean ± standard deviation, with range presented in parentheses.
* indicates $p < 0.05$ when compared with DSWPD with N24SWD features.

Discussion

Our results demonstrate that subjects with DSWPD have a significantly reduced PIPR in response to short wave length (blue) light when compared with control subjects, and this is further reduced when only analyzing those subjects with DSWPD and a history of N24SWD. As the PIPR in response to blue light represents a measure of melanopsin-dependent retinal photo-transduction, this suggests that impaired function of the melanopsin containing retinal ganglion cells, which are known to transduce light signals to the SCN, plays a role in the pathogenesis of DSWPD. Furthermore, this difference appears to be more pronounced in sighted individuals who exhibit clinical features of both DSWPD and N24SWD, suggesting that this phenotype may result in part due to decreased sensitivity to entraining light signals.

It should be noted that in this study, we are using the PIPR as a surrogate marker to test the function of the melanopsin containing retinal ganglion cells; however, we are not directly assessing the ability of these cells to provide a circadian entraining light signal. The PIPR is mediated by a projection from the ipRGCs to the olivary pretectal nucleus in the midbrain. The circadian entraining response is mediated by projections from the iPRGCs, through the retinohypothalamic tract, synapsing at the SCN. In rodents, the pupillary light reflex and the circadian entraining response, while both representing melanopsin-dependent pathways, are mediated by different subsets of melanopsin containing ipRGCs; this has not yet been demonstrated in humans [30]. Further studies are still needed to demonstrate whether our observed differences in the PIPR in these individuals translate into functional differences in the circadian response to light, as seen with melatonin suppression or phase shifting.

However, despite the above limitations of the PIPR, there is previously published evidence for an association between pupillary light responses and circadian responses to light. A recent study by van der Meijden et al. evaluated the PIPR in healthy individuals without DSWPD, who had a range of mid-sleep times between 2:00 and 6:00, measured with sleep logs and actigraphy. In this study, they found a correlation between later sleep–wake times and an increased PIPR [31]. It should be noted that if we only look at our control subjects, who like the participants in van der Meijden et al., have a mid-sleep time between 2:00 and 6:00 we see a similar (though nonsignificant) positive association between later sleep–wake timing and increased PIPR. Thus, in healthy individuals, later sleep–wake timing may be related to greater sensitivity to light in the evening, which could result in greater phase delays in response to evening light. Similarly, McGlashan et al. demonstrated that individuals with DSWPD (average bedtime of 1:07) appeared to be hypersensitive to daytime bright light (2–8 hours after wake) when compared with controls, exhibiting a faster constriction velocity in response to bright white light [32]. In contrast, our results demonstrate a reduced sensitivity to daytime light in the participants with DSWPD, with our testing occurring at a similar time in relation to sleep–wake timing (1–9 hours after wake). A key difference between our study and van der Meijeden and McGlashan's studies

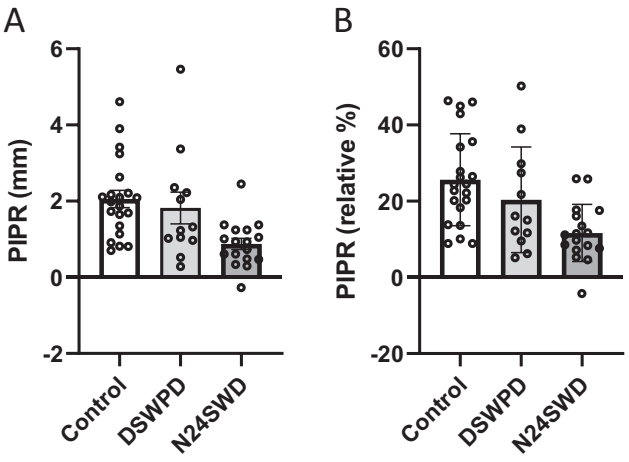

Figure 4. The PIPR in control, DSWPD, and N24SWD subjects. (A) The PIPR measured in mm in participants with DSWPD who also exhibit N24SWD features is significantly reduced when compared with controls ($p < 0.01$), or participants who only have DSWPD ($p < 0.05$). (B) The PIPR % in participants with DSWPD who also exhibit N24SWD features is significantly reduced when compared with controls ($p < 0.001$), or participants who only have DSWPD ($p < 0.05$). One-way ANOVA, with Tukey post hoc analysis. Error bars represent SD, and dots indicate individual data points.

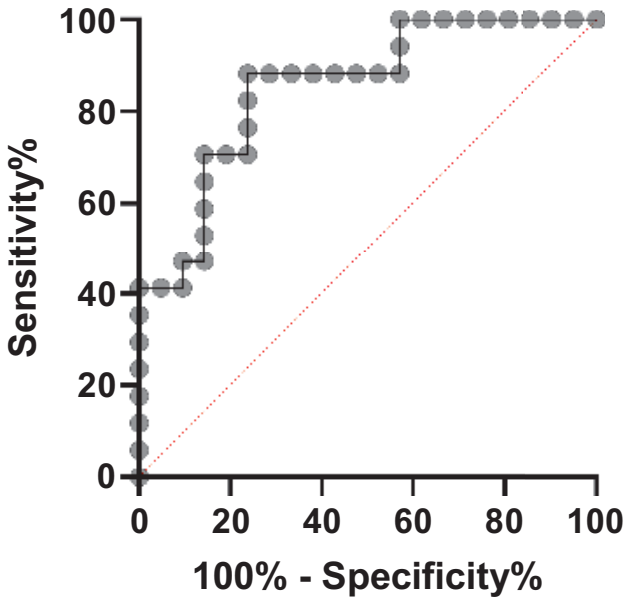

Figure 5. ROC curve demonstrating the sensitivity and specificity of the PIPR % in control and N24SWD subjects. Using a cutoff of <18%, the PIPR % to blue light has 88% sensitivity and 76% specificity for distinguishing control participants from those with N24SWD. AUC 0.85 (0.06), $p < 0.001$.

is that our subjects exhibited much later sleep–wake timing (average mid-sleep of 7:49 and sleep onset of 3:43, compared to a mid-sleep range of 2:00–6:00 and an average sleep onset of 1:07, respectively). The extremely delayed phenotype may represent a subtype with a different pathophysiological mechanism, compared to the milder delays in sleep–wake timing that can be observed in the general population. The results of our study suggest that in addition to the previously identified circadian and noncircadian phenotypes of DSWPD, within circadian DSWPD, there may be at least two distinct populations of delayed individuals that can be distinguished though testing of melanopsin function. The first group, as demonstrated by van der Meijeden and McGlashan's studies, are hypersensitive to the delaying effects of evening light, but presumably are still able to also respond to the advancing effects of morning light, resulting in overall slightly later sleep–wake patterns. Our study has potentially identified a separate population of individuals who appear to have decreased melanopsin-dependent retinal phototransduction, with significantly later sleep–wake timing, as well as a tendency to develop a non–24-hour sleep–wake pattern. These individuals, who possess normal image-forming vision, appear to have impaired non–image-forming vision, clinically appearing more like the visually blind individuals who are unable to appropriately entrain to environmental light. In light of these findings, it will be important for future studies of DSWPD to include these extreme phenotypes for comparison.

There are several limitations in these results. First, pupillometry was not performed with mydriatics to dilate the contralateral eye. However, it has recently been demonstrated that there is high test–retest reliability of the PIPR even when performed without mydriatics [33]. Second, pupillometry was not performed at the exact same circadian time for all subjects, with testing performed closer to wake time in subjects with DSWPD and N24SWD. However, all testing occurred within a range from 0 to 9 hours after habitual wake time, a window which has been demonstrated to be fairly stable for the PIPR [34] and differences between groups remained significant after adding time-since-wake of pupillometry testing as a covariate Third, the diagnosis of DSWPD was based on a detailed clinical interview, habitual self-reported sleep time, and confirmation with actigraphy (using the International Classification of Sleep Disorders criteria) and not confirmed by DLMO. Recent evidence indicates that almost half of the individuals with clinical features of DSWPD do not actually exhibit a delay of circadian biomarkers, suggesting the distinction between circadian and noncircadian subtypes of DSWPD [8]. It is possible that the greater variability observed in our subgroup of individuals with DSWPD without symptoms of N24SWD is a result of that group consisting of a mixed population of both circadian and noncircadian subtypes of DSWPD. In future studies, it will be important to obtain DLMO for all participants and complete pupillometry testing at the same circadian time for all subjects. Additional limitations include the small sample size, and that the results were not adjusted for additional confounding factors such as age. In addition, we were not able to fully match the control and DSWPD populations on all demographic factors. It should also be noted that our population of DSWPD subjects included a mix of prospective and retrospective clinical results. However, despite the potential heterogeneity of our clinical population, the response to blue light differed significantly between DSWPD and controls. While there is some overlap in PIPR results between groups, a threshold of <18% demonstrates a sensitivity and specificity of 88% and 76%, respectively for distinguishing control participants from those with DSWPD and sighted N24SWD. The PIPR may be a useful clinical tool for distinguishing these individuals from healthy controls. Finally, while these results represent the first stage in identifying a subset of severe DSWPD and sighted N24SWD who have reduced melanopic photo-transduction, further research is still necessary to demonstrated that these findings translate into an impaired circadian response to light.

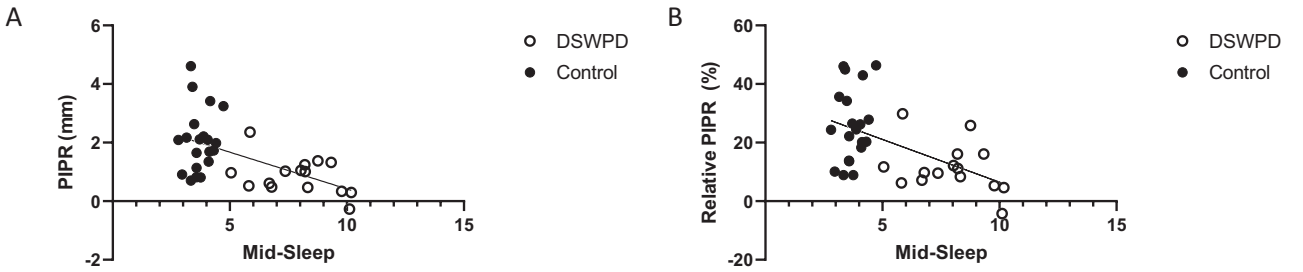

Figure 6. PIPR in relation to mid-sleep. Correlation between the PIPR in mm (A) and PIPR % (B). There is a moderate negative correlation between the PIPR % and mid-sleep point, based on actigraphy obtained prior to pupillometry testing ($r = -0.54$, $p < 0.001$).

In summary, our results provide evidence that reduced melanopic photo-transduction may be a novel mechanism for the alterations in circadian entrainment in individuals with DSWPD and sighted N24SWD. Furthermore, our findings identified a subset of individuals with delayed sleep–wake patterns that overlap with non-24 sleep–wake patterns, who appear to have intact image-forming vision, but impaired non–image-forming vision, suggested that they are in a sense "blind" to circadian light input. Further research is necessary to identify which component of the circadian photo-transduction pathway is responsible for the PIPR changes, whether this is due to differences in melanopsin, retinal ganglion cell function, or other components of this pathway.

Based on these results, we may need to re-evaluate treatment protocols, at least for this subset of patients with DSWPD and N24SWD overlap. Although the AASM clinical practice guidelines currently only recommend timed melatonin administration [35], timed morning bright light exposure is often used clinically as an additional resetting signal. Treatment of circadian rhythm disorders is often challenging, with inconsistent effectiveness, even in specialized sleep disorder centers. Understanding the mechanisms responsible for circadian rhythm disorder subtypes is crucial for the development of effective treatments. For example, it is possible that individuals with an impaired melanopsin response require either higher intensity light and/or manipulation of light spectral properties, or even a shift in focus to strengthening non–light-based timekeeping stimuli (such as timed feeding or structured physical/social activities) in order to realign or stably entrain circadian rhythms. These results both provide insight into the mechanisms underlying DSWPD and sighted N24SWD and development of treatments that can be personalized for the various circadian disorder phenotypes.

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Conflict of interest statement. None declared.

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