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# UNIVERSITÉ D'AMSTERDAM

# UNIVERSITÉ DE BÂLE

*ÉCOLE DOCTORALE Sciences de la Vie et de la Santé (ED 414)*

**Institut des Neurosciences Cellulaires et Intégratives (CNRS UPR 3212)**

# THÈSE EN COTUTELLE présentée par :


## Wisse Pier VAN DER MEIJDEN


soutenue le : **19 septembre 2018**

Pour obtenir le grade de : **Docteur de l'université de Strasbourg**
**&**
**Docteur de l'université d'Amsterdam**

Discipline/ Spécialité : ***Sciences de la vie / Neurosciences***

# Lumière, physiologie et comportement

## Des effets dans l'obscurité dépendants de la couleur de lumière préalablement administrée

**THÈSE dirigée par :**

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# Left in the dark

Wavelength-dependent post-illumination effects
on human physiology and behavior

Wisse Pier van der Meijden

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# Left in the dark

## Wavelength-dependent post-illumination effects on human physiology and behavior

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ter verkrijging van de graad van doctor aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus
prof. dr. ir. K.I.J. Maex

ten overstaan van een door het College voor Promoties ingestelde commissie,
in het openbaar te verdedigen in de Aula der Universiteit
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Dit proefschrift is tot stand gekomen in het kader van het NeuroTime programma, een Erasmus Mundus Joint Doctorate, met als doel het behalen van een gezamenlijk doctoraat. Het proefschrift is voorbereid in de Faculteit der Geneeskunde, van de Universiteit van Amsterdam; en in het *Institut des Neurosciences Cellulaires et Intégratives* van de *Université de Strasbourg* en in het *Zentrum für Chronobiologie der Universitäre Psychiatrische Kliniken Basel* (UPK) van de *Universität Basel*.

This thesis has been written within the framework of the NeuroTime program, an Erasmus Mundus Joint Doctorate, with the purpose of obtaining a joint doctorate degree. The thesis was prepared in: the Faculty of Medicine at the University of Amsterdam; and in the *Institut des Neurosciences Cellulaires et Intégratives* of the *Université de Strasbourg* and in the Centre for Chronobiology, Psychiatric Hospital of the University of Basel (UPK), at the University of Basel.

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## Contents

# CHAPTER 1

## Introduction

## General introduction

Environmental light does not only allow us to use visual information, but also critically drives human physiology and behavior. Key players in these non-image forming effects of light are intrinsically photosensitive retinal ganglion cells (ipRGCs), which directly transduce information on environmental light intensity from the retina to downstream areas in the brain[1,2]. These specific retinal ganglion cells not only integrate information on environmental light from extrinsic rod-cone input, but are also able to intrinsically detect light levels through the expression of the photopigment melanopsin[3]. This photopigment is maximally sensitive to short-wavelength light in the blue part of the spectrum (460-480 nm)[4]. As a result of widespread projections to the brain, the downstream output of ipRGCs drives numerous physiological and behavioral downstream pathways including circuitries involved in autonomic control and both the acute as well as the rhythmic expression of sleep[5,6].

The ipRGC-driven effects of light on the autonomic nervous system are most explicitly expressed by the pupillary light reflex[7]. This reflex is mediated through direct ipRGC projections to the olivary pretectal nucleus in the brainstem (i.e., the center for pupil size control[8]). Most characteristics of this reflex are the result of a joint activation of rods, cones, and melanopsin[9]. Interestingly, there is one specific feature of the pupillary light reflex that is almost entirely driven by the intrinsic melanopsin-signaling circuitry. Following light *offset*, ipRGCs repolarize considerably slower than rods and cones[10], and these slow kinetics result in a remaining downstream output, causing pupil constriction to be sustained even beyond light offset[11]. The strength of this so-called *post-illumination* pupil response (PIPR) is highly dependent on the wavelength of the illumination. The strongest PIPR occurs after exposure to light with a wavelength around melanopsin peak sensitivity.

Post-illumination changes were also found to be wavelength-dependent in other physiological dimensions. Heart rate and core body temperature remained increased after exposure to blue light, but not green light, in the period preceding bedtime[12]. The increased core body temperature after blue light was maintained even beyond sleep onset[13]. Blue light exposure close to bedtime moreover induced changes in brain activity preceding and during sleep[13,14]. Wavelength-dependent differences in post-illumination effects on brain activity were not only found during the night, but also during daytime[15]. Time of day may moreover differentially modulate the post-illumination effects of different wavelengths of light. For example, the post-illumination effects of blue versus green light exposure on alertness are differentially modulated by time of the day[16]. Immediately following daytime blue light exposure, alertness was higher with similar sleepiness levels, while after nighttime blue illumination, alertness was similar and sleepiness was lower. These acute post-illumination effects on alertness and sleep are thought to be mediated through projections from ipRGCs to several key brain areas for arousal regulation[17], including the ventrolateral preoptic nucleus, which is one of the few

nuclei that shows higher activity during sleep than during wakefulness[18].

In addition to their mediation of the acute effects of light on sleep and arousal, ipRGCs also mediate the effects of light on in the 24-hour rhythmic modulation of sleep propensity. The central pacemaker for physiological and behavioral rhythms, including the sleep-wake cycle[19,20], is the suprachiasmatic nucleus in the hypothalamus[21]. The so-called circadian rhythms have an intrinsic periodicity of approximately 24 hours. In order to be in phase with a day on earth, circadian rhythms are synchronized to an exact 24-hour cycle using environmental information, with the light-dark cycle as the most important cue[22]. This circadian photoentrainment is mediated by ipRGCs, as the orchestrating suprachiasmatic nucleus is one of the main targets of their downstream projections[5,6]. Intriguingly, timing of the sleep-wake schedule and its sensitivity to circadian photoentrainment varies considerably between individuals[14]. Relative to the light-dark cycle, the sleep phase is advanced in 'early birds', while delayed in 'night owls'. It has been suggested that these individual differences could involve differential responses to light[23,24], which may arise from melanopsin polymorphisms[25,26].



## Scope

The present thesis focuses on individual differences in ipRGC-driven phototransduction. More specifically, we assessed the non-image forming effects of light on autonomic control, alertness, and sleep. We addressed individual differences in the specific functionality of the melanopsin-signaling circuitry, quantified by the PIPR after blue light, and whether this interindividual functional variation may be involved in individual differences in the timing of sleep. In addition to individual differences in responses following blue light exposure, the scope of this thesis also includes functional changes after red illumination. Previous studies on post-illumination effects mainly focused on the photoinduced effects after blue light around melanopsin peak sensitivity. Red illumination hereby only served as a reference, assuming no non-image forming changes following light with such a long wavelength. However, evidence for post-illumination modulations after long-wavelength light is growing[27-29]. Recent work showed that the intrinsic firing frequency of melanopsin-containing cells can be promptly suppressed after discontinuation of long-wavelength light exposure, both in ipRGCs isolated from extrinsic input[30] as well as in non-retinal cells with heterologous melanopsin expression[31]. In view of their widespread downstream projections throughout the brain, the possibility that exposure to red light may subsequently suppress the intrinsic firing rate of ipRGCs assessed in darkness, would have several functional consequences. We assessed acute post-illumination effects by estimating wavelength-dependent changes in autonomic control, in alertness performance and effort fighting sleep, and in sleep propensity. In addition, the specific relationship between autonomic control and sleep propensity was examined in more detail.

## Outline

In the **second chapter,** we present a method for estimating the specific functionality of an individual's intrinsic melanopsin-dependent phototransduction circuitry. We assessed the robustness of a newly designed pupillometry paradigm, which aimed to provide a stable, trait-like PIPR after blue light. We estimated the within-subject test-retest reliability in order to evaluate whether the method is useful to investigate individual differences in case-control and intervention studies. In addition, we evaluated the method's sensitivity to potential confounders, which are of relevance for planning experimental and clinical evaluations. We compared morning and afternoon assessments in order to check whether the outcome parameters were similar across office hours. Light exposure history was evaluated in order to estimate the sensitivity of the method to either the absence or presence of environmental light preceding the assessment. We moreover assessed the effect of body position in order to test whether the method could be not only applied in an upright posture but also in a supine position, which is typical for functional magnetic resonance imaging[15].

The aforementioned method involves pharmacological pupil dilation, which is not only a burden for the participants but also requires ophthalmological expertise. Given that the functionality of the intrinsic melanopsin-signaling phototransduction circuitry is also highly relevant to researchers without ophthalmologic medical training, the **third chapter** proposes a method for quantifying the PIPR after blue light without mydriatics. We assessed the reliability of this alternative PIPR assessment method and compared its performance with the method introduced in the second chapter. In addition, we here investigate whether the estimates were robust across seasons[32]. Taken together, in chapter two and three we aim to establish the PIPR after blue light as a biomarker for the functionality of an individual's intrinsic melanopsin-dependent phototransduction circuitry.

In the **fourth chapter,** we examine whether individual differences in sleep timing are associated with interindividual variation in the functionality of the melanopsin-driven phototransduction circuitry. We estimated functionality of the melanopsin-signaling circuitry from the PIPR after blue light and assessed sleep timing in three ways: using a chronotype questionnaire, a sleep diary, and actigraphy. The study population consisted of healthy adolescents and young adults, i.e. the age range with the highest vulnerability to a delayed sleep phase[33,34].

The **fifth chapter** shifts focus from post-illumination effects of blue light to post-illumination effects of red light. We assessed effects on pupil diameter, response speed, heart rate, and sleep propensity in order to provide a multidimensional picture of the functional alterations following daytime red light exposure. In order to put the post-illumination effects of red light exposure into perspective, we also examined the post-illumination changes in these functional dimensions following blue and green light exposure.

The **sixth chapter** zooms in on the relationship between sleep propensity and autonomic control of heart rate. Increased sleepiness is associated with a decreased resting state pupil diameter indicating a lower sympathetic drive[35]. From a cardiac perspective less sympathetic activity is expressed by a reduced heart rate[36]. Fluctuations in pupil diameter and heart rate were moreover found to be synchronized[37]. Accordingly, pupil diameter and heart rate were both found to be decreased in the sleep-permissive supine body position[38]. However, sleepiness levels and the sympathovagal balance have also been reported to change in opposite directions[39]. All these studies were performed in healthy volunteers. To evaluate the association under more extreme conditions, we investigate the association between sleepiness and cardiac autonomic control in narcolepsy with cataplexy, a sleep disorder characterized by excessive daytime sleep propensity[40].

In the **seventh chapter,** we provide a summary and discussion of the findings of the preceding chapters. We moreover discuss possible applications resulting from the outcomes and indicate future research directions.

# CHAPTER 2

## Post-illumination pupil response after blue light: reliability of optimized melanopsin-based phototransduction assessment

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## Abstract

Melanopsin-containing retinal ganglion cells have recently been shown highly relevant to the non-image forming effects of light, through their direct projections on brain circuits that regulate alertness, mood, and circadian rhythms. A quantitative assessment of the functionality of the melanopsin-signaling pathway could be highly relevant in order to mechanistically understand individual differences in the effects of light on these regulatory systems. We here propose and validate a reliable quantification of the melanopsin-dependent post-illumination pupil response (PIPR) after blue light, and evaluated its sensitivity to dark adaptation, time of day, body posture, and light exposure history. Pupil diameter of the left eye was continuously measured during a series of light exposures to the right eye, of which the pupil was dilated using tropicamide 0.5%. The light exposure paradigm consisted of the following five consecutive blocks of five minutes: baseline dark; monochromatic red light (peak wavelength: 630 nm, luminance: 375 cd/$m^2$) to maximize the effect of subsequent blue light; dark; monochromatic blue light (peak wavelength: 470 nm, luminance: 375 cd/$m^2$); and post-blue dark. PIPR was quantified as the difference between baseline dark pupil diameter and post-blue dark pupil diameter (PIPR-mm). In addition, a relative PIPR was calculated by dividing PIPR by baseline pupil diameter (PIPR-%). In total 54 PIPR assessments were obtained in 25 healthy young adults (10 males, mean age ± SD: 26.9 ± 4.0 yr). From repeated measurements on two consecutive days in 15 of the 25 participants (6 males, mean age ± SD: 27.8 ± 4.3 yr) test-retest reliability of both PIPR outcome parameters was calculated. In the presence of considerable between-subject differences, both outcome parameters had very high test-retest reliability: Cronbach's $\alpha > .90$ and intraclass correlation coefficient > .85. In 12 of the 25 participants (6 males, mean age ± SD: 26.5 ± 3.6 yr) we examined the potential confounding effects of dark adaptation, time of the day (morning vs. afternoon), body posture (upright vs. supine position), and 24-h environmental light history on the PIPR assessment. Mixed effect regression models were used to analyze these possible confounders. A supine position caused larger PIPR-mm ($\beta = 0.29$ mm, SE = 0.10, $p = 0.01$) and PIPR-% ($\beta = 4.34$ %, SE = 1.69, $p = 0.02$), which was due to an increase in baseline dark pupil diameter; this finding is of relevance for studies requiring a supine posture, as in functional magnetic resonance imaging, constant routine protocols and bed-ridden patients. There were no effects of dark adaptation, time of day, and light history. In conclusion, the presented method provides a reliable and robust assessment of the PIPR to allow for studies on individual differences in melanopsin-based phototransduction and effects of interventions.

## Introduction



Light reaching the retina of the eyes does not only provide the brain with images of the environment, but also generates several non-image forming effects. These include constriction of the pupil, changes in the arousal level of the brain, and entrainment of the biological clock of the brain to the environmental 24-hour light-dark cycle. The observation that this circadian photoentrainment was preserved in some blind individuals[41] as well as in mice lacking rods and cones[42,43] has led to the discovery of an entirely new photoreceptor system. Indeed, we now know that a small subset of retinal ganglion cells express an opsin/vitamin A-based photopigment, called melanopsin. This photopigment is maximally sensitive to short wavelengths (peak sensitivity ~ 480 nm) and renders these cells intrinsically photosensitive[1,4]. Intrinsically photosensitive retinal ganglion cells (ipRGCs) were demonstrated to be strongly involved in the mentioned non-image forming effects of light on pupil diameter[7], enhancement of mood and alertness[44], and modulation of circadian rhythms[2]. Given these strong and important effects, a quantitative assessment of the functionality of the melanopsin-signaling pathway could be highly relevant in order to mechanistically understand individual differences in the effects of light on the regulation of mood, alertness and circadian rhythms.

The pupillary light reflex may provide the most feasible non-invasive method to assess functionality of the melanopsin-signaling pathway. The reflex is mediated through direct connections between ipRGCs and the Olivary Pretectal Nucleus[5], the nucleus that controls pupil size[8]. However, this reflex is not exclusively driven by the melanopsin-signaling pathway, but also highly dependent on the input from rods and cones[9]. Still, there is one characteristic of the pupillary light reflex that is specific to the melanopsin-signaling pathway. In contrast to rods and cones, ipRGCs show a delayed repolarization after light offset, resulting in a sustained pupil constriction. This phenomenon has been dubbed 'post-illumination pupil response' (PIPR)[10]. The PIPR that can be recorded following exposure to bright blue light is almost entirely attributable to ipRGC activity[11,45] and can therefore be used to estimate functioning of the melanopsin signaling pathway[46]. Several studies suggest that the processing of light by the ipRGC may be altered in disorders including diabetes type II[47], neuroretinal visual loss[48], glaucoma[49], and seasonal mood disorder[50]. In order to be of value in case-control, intervention, and mechanistic studies, it is of great importance to assess the PIPR according to a maximally reliable standardized protocol. Test-retest reliability of PIPR assessment has previously been evaluated for two other specific protocols and was rated as moderate to high[51,52]. The light stimuli in these two paradigms were of short duration (i.e., 400 ms and 20 s). In view of the characteristic low sensitivity and slow kinetics of ipRGCs, however, longer stimulus duration allows for more specific assessment of the melanopsin-signaling pathway[1,53]. Accordingly, previous animal work showed that phase shifts in circadian rhythms were larger with a 300-s light stimulus compared to light stimuli with a shorter duration[54]. In addition, these circadian phase shift effects did not grow any further with extending the light stimulus beyond 300

s. Others explained this saturation effect by showing that light adaptation of ipRGCs was completed after 300 s of light exposure[55]. We therefore here propose a both feasible and reliable PIPR assessment protocol using prolonged light exposure with a duration of 300 s.

We first assessed the within-subject between-day test-retest reliability. Because the pupil response to light is dependent on many inputs, in part originating from the autonomic nervous system[56], we moreover addressed sensitivity of our PIPR assessment protocol to four possible confounders: 1) Dark adaptation, to check whether the eyes were dark adapted and pupil diameter was stabilized prior to the light exposure (PIPR is quantified relative to pre-exposure pupil diameter); 2) Time of day (i.e., morning vs. afternoon), to test whether the protocol provides similar estimates across office hours; 3) Body posture, to check whether the test could be applied in both upright and supine position (i.e., application in bed-ridden participants and in magnetic resonance imaging environments[15]); 4) Environmental light history, to confirm that the outcome measures were unaffected by previous light exposure, which is of relevance with respect to planning of experimental and clinical evaluations. Previous studies[55,57-59] showed an effect of short-term light history on pupil response. We here add to these findings by assessing also long-term effects of prior light exposure (i.e., from 24-h prior to the test).

## Methods

To evaluate and validate our PIPR protocol, two experiments were performed, using similar light exposure and pupillometry procedures. The aim of the first experiment was to estimate the within-subject, between-day test-retest reliability. The second experiment aimed to evaluate possible effects of dark adaptation, time of day, body posture, and environmental light history on the outcomes of the PIPR protocol.

### Participants

In total 25 healthy young adults were recruited by advertisement and word of mouth. These 25 participants were distributed over the two experiments as follows: 2 of the 25 of participated in both experiments, 13 of the 25 in experiment 1 only, and 10 of the 25 solely in experiment 2. All participants were in good health, free of medication, non-smoking, and had neither sleep complaints nor a history of ocular pathology, as indicated by the Duke Structured Interview for Sleep Disorders[60]. All participants worked regular office hours and did not travel across time zones for at least a month prior to participation. Results from the Munich Chronotype Questionnaire (MCTQ) showed that none of the participants was an extreme chronotype (mean mid-sleep on free days ± SD: 5:05 AM ± 1:05) and all in the center part of the normative distribution for the age range of our participants (5:00 AM ± 1:23)[61].

According to Nagel anomaloscope tests none of the participants suffered from color vision deficiency. Participants received oral and written information on the study, signed informed consent before study participation, and did not receive any incentive. The study was approved by the Medical Ethical Committee of the VU University Medical Center Amsterdam (protocol NL43319.029.13) and adhered to the tenets of the Declaration of Helsinki.



**Light exposure protocol**

Our light exposure protocol was designed to obtain a prolonged steady-state PIPR after blue light, with a high signal-to-noise ratio[58]. In order to obtain maximal stimulation of the melanopsin-based phototransduction system, the pupil of the right eye was dilated using 0.5% tropicamide[62]. In accordance with previous work[57,58], the right eye was first pre-exposed to five minutes of bright monochromatic red light (LED Cree C503B-RAS, Durham, NC, USA; peak wavelength (full width half maximum): 630 (20) nm; luminance: 375 cd/m$^2$) in order to maximize the PIPR after blue light (supplemental material, Figure S1). The right eye was subsequently exposed to 5 minutes of bright monochromatic blue light (LED Cree C503B-BAN, Durham, NC, USA; peak wavelength (full width half maximum): 470 (20) nm; luminance: 375 cd/m$^2$). Wavelength and luminance of the light stimuli were calibrated using a spectrometer (AvaSpec-3648-USB2, Avantes, Apeldoorn, the Netherlands). Both monochromatic lights were transmitted through a diffuser and presented in free view. There were 5-minute blocks of darkness before (here labeled 'baseline'), between, and after ('post blue') the light blocks (Figure 1).

**Pupillometry**

Participants had little to no experience with ophthalmological tests and were naïve to the experimental paradigm (i.e., there was no rehearsal trial). They were placed in front of a custom-made infrared pupillometry set-up built around a printed circuit board charge coupled device camera (Sony d2463r, Sony Electronics Inc., San Diego, CA, USA). Participants were asked to focus on a fixation target, integrated in the set-up in front of their left eye. This fixation target was projected at infinity to prevent accommodation. The eyes were separated by a septum. The pupil diameter of the left eye was measured throughout the entire protocol using infrared radiation. The pupil was illuminated from the lateral side with an 880 nm infrared radiation emitting diode, in such a way that the pupil appeared as a black disk in a bright iris on the camera. Visible radiation was blocked by a Wratten 87 gelatin filter (Kodak, Rochester, NY, USA). The images were digitized at 25 Hz with a USB frame grabber (Grabby, Terratec, Alsdorf, Germany) and analyzed real time with custom software written in C++ using the OpenCV image analysis library (Itseez, Nizhny Novgorod, Russia). Missing data points (e.g., due to eye blinks) were interpolated using nearest neighbors interpolation. Pupil diameter was assessed continuously from the baseline block until the post-blue block. During baseline darkness, the pupil diameter remained stable over the entire block. During post-blue darkness we observed in several assessments that during the first minute after

light offset the pupil first dilated to an intermediate level before it constricted again to a level at which the constriction was sustained (Figure 1). Similar pupil behavior has been shown previously after cessation of bright light stimuli with durations of 2 minutes[63] and 3 minutes[64]. These complicated dynamics are a result of the interaction between the image forming and non-image forming photoreceptors after light offset[10,11]. Another between-trial variation was observed during the final minute of the post-blue block: in most trials, the pupil constriction was maintained over the entire minute, but in some trials the pupil already started redilating towards baseline size within this minute. In view of these observed inter-assessment differences in pupil dynamics, the first and last minute of the post-blue block were excluded from the analysis: we used the averaged pupil diameter over minutes 2 to 4 of the post-blue block. In order to optimize the comparison between the baseline and post-blue dark block we decided to use equal metrics for both blocks. Accordingly, we calculated the averaged pupil diameter over minutes 2 to 4 of the baseline block. From the baseline and post-blue pupil diameter, we calculated two PIPR outcome parameters[49,50].

1. PIPR-mm = baseline pupil diameter – post-blue pupil diameter
2. PIPR-% = 100 * PIPR-mm / baseline pupil diameter

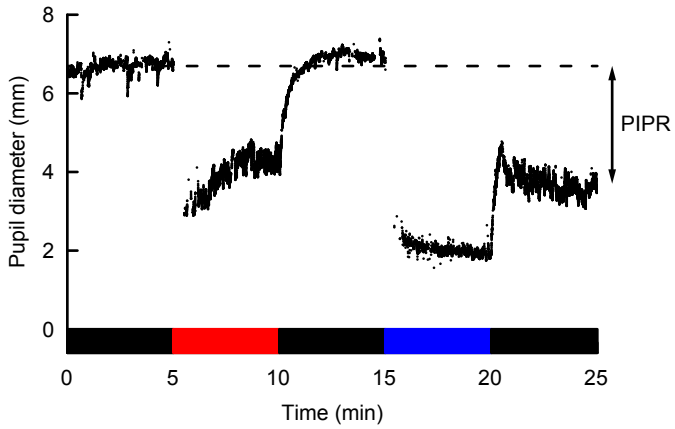


**Figure 1.** Example of the change of pupil diameter in the left eye throughout the light exposure protocol. The bar in the bottom indicates when the right eye was exposed to light; black = darkness, red = monochromatic red light, blue = monochromatic blue light. The dashed line represents mean pupil diameter during the first dark block (i.e., baseline pupil diameter). Post-Illumination Pupil Response (PIPR) indicates the difference between baseline pupil diameter and the mean pupil diameter during the last dark block (i.e., post-blue pupil diameter) and can be expressed either as the absolute difference in mm (PIPR-mm, here 2.99 mm) or the percentage change from baseline (PIPR-%, here 44.7 %).

**Experiment 1**

***Procedures***

Fifteen participants (6 males, 9 females, mean age ± SD: 27.8 ± 4.3 yr) underwent the PIPR assessment in upright position twice on consecutive days. Both tests within one participant were at the same time of day. Between participants this time point ranged from 09:00 AM to 16:30 PM. Measurements were performed using the same set-up at two different locations: ten participants (5 males, 5 females, mean age ± SD: 26.1 ± 3.3 yr) were assessed at the Netherlands Institute for Neuroscience in Amsterdam and five (1 male, 4 females, mean age ± SD: 31.2 ± 4.1 yr) at PsyQ Expertise Center Adult ADHD in The Hague.



***Statistical analysis***

Mixed effect regression models were used to assess whether between-subject differences in PIPR-mm and PIPR-% were confounded by the time point of measurement and the assessment location. Mixed effect models are optimally suited to account for nested data structures. The data were structured in a 2-level hierarchy: PIPR outcome parameters were measured in two assessments that were nested in fifteen participants. Time point and location were included in the model as regressors. The significance of their estimated effects was evaluated using the Wald test and Likelihood-ratio tests were performed to compare models[65].

Cronbach's α was calculated to examine the within-subject reliability of the PIPR assessment[66]. Values of Cronbach's α > .90 are considered as satisfactory for clinical application[67]. To examine test-retest reliability, we computed the two-way random effects single measures intraclass correlation coefficient (ICC) for absolute agreement[68]. Bland-Altman plots were made to visually inspect test-retest reliability[69]. Data processing was conducted using MATLAB (Version R2013A, The MathWork Inc, Natick, MA, USA). Statistical analyses were conducted using the software packages 'lme4'[70], 'cocron'[71], and 'ICC'[72] for R (Version 3.1.1, R Foundation for Statistical Computing, Vienna, Austria).

**Experiment 2**

***Procedures***

Twelve participants (6 males, 6 females, mean age ± SD: 26.5 ± 3.6 yr) underwent the PIPR assessment twice with 3 days between assessments. An RGB multiband light sensor (Dimesimeter, Rensselaer Polytechnic Institute, Troy, NY, USA)[73], integrated in a brooch, was worn to assess environmental light spectrum and intensity exposure history over 24 h prior to the start of each assessment. The ability of the light sensor to measure light in multiple bands enabled us to take the composition of the light into account. The separate output values from the red, green, and blue band were weighted, based on the sensitivity of the ipRGC network for each bandwidth, and integrated into a single light exposure parameter, which was quantified as: irradiance, spectrally weighted for effects on circadian rhythm (weighted $W/m^2$)[74].

To investigate effects of dark adaptation duration and stabilization of baseline pupil diameter, the start of the light exposure protocol was delayed by either 0, 5, or 10 minutes in darkness (0 $cd/m^2$), randomly assigned to the different participants, resulting in dark adaptation durations of 5, 10 or 15 minutes. If the eyes were sufficiently adapted to the dark environment within 5 minutes, baseline pupil diameter would not change with further extension of the dark exposure duration. Otherwise, ongoing dark adaptation would result in a larger baseline pupil diameter with increasing delay.

To investigate potential time-of-day effects of the PIPR outcomes during office hours, one trial started in the morning (09:00 AM) and the other in the afternoon (01:00 PM). To assess the effect of body posture on the PIPR outcomes, one assessment was performed in upright (i.e. sitting) and the other in supine position. In supine position, the pupillometry set-up was placed above the participant's head in such a way that the angle and distance relative to the eyes were similar to the upright position. The orders of posture and time of day were counterbalanced across participants. At the start of each trial, participants were placed in the upright or supine position. Room lights were dimmed for 30 minutes (0.5 $cd/m^2$) and subsequently the light exposure protocol was commenced.

**Statistical analysis**

Mixed effect regression models were used to analyze the repeated assessments of PIPR-mm and PIPR-%, and the effects of dark adaptation, time of day, posture and light history. To dissect pre- and post-exposure effects, the same models were performed on baseline and post-blue pupil diameter. The assessed data represented a 2-level hierarchy: variables were measured in two assessments that were nested in twelve participants. Dark adaptation duration, time of day, posture, and 24-h environmental light history were added to the model as regressors. All analyses were performed using the R-package 'lme4'.

## Results

### Experiment 1

Interindividual differences in PIPR outcome measures were confounded neither by time point of measurement (PIPR-mm: β = 0.09, SE = 0.10, *P* = 0.37; PIPR-%: β = 0.9, SE = 1.0, *P* = 0.38) nor by assessment location (PIPR-mm: β = 0.49, SE = 0.52, *P* = 0.36; PIPR-%: β = 7.8, SE = 5.0, *P* = 0.15).



Cronbach's α was larger than .90 for both PIPR-mm and PIPR-% (Table 1). The ICC point estimations for PIPR-mm and PIPR-% were both >.85, which indicates almost perfect test-retest reliability. Bland-Altman plots for both parameters indicate almost zero bias between the two assessments (Figure 2).

**Table 1.** *PIPR outcome parameters from session 1 and 2 and test-retest reliability outcomes.*

| | PIPR-mm | PIPR-% |
|---|---|---|
| **Session 1 - Mean ± SD [range]** | 2.88 ± 1.01 [1.14–4.49] | 46.9 ± 10.4 [22.9–59.2] |
| **Session 2 - Mean ± SD [range]** | 2.81 ± 0.88 [1.48–4.10] | 47.0 ± 9.0 [32.6–59.3] |
| **Cronbach's α (95% confidence interval)** | 0.95 (0.84–0.98) | 0.93 (0.78–0.98) |
| **ICC (95% confidence interval)** | 0.90 (0.74–0.96) | 0.87 (0.67–0.95) |
| **Bland-Altman bias (95% limits of agreement)** | 0.08 (-0.78 to 0.94) | -0.11 (-10.3 to 10.1) |

PIPR, Post-Illumination Pupil Response; ICC, Intraclass Correlation Coefficient.

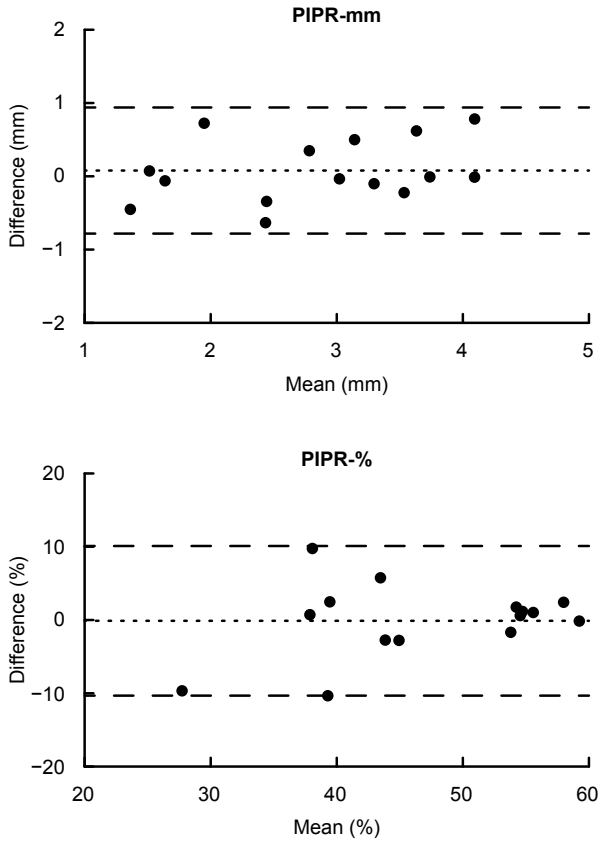


**Figure 2.** *Bland-Altman plots for PIPR-mm and PIPR-%. Differences between the two measurements on two consecutive days (i.e., day 1 minus day 2) are plotted against the mean of the two measurements. The bias (dotted line) is the mean difference between all measurements on the first day and all measurements on the second day. The 95% limits of agreement (dashed lines) define the range between which 95% of the differences between measurements by the two devices will lie. The smaller the limits of agreement, the better the agreement between two measurements.*

**Experiment 2**

***Dark adaptation duration***

PIPR-mm was smaller ($P$ = 0.04), and PIPR-% had a tendency to be smaller ($P$ = 0.09), with increasing dark adaptation duration prior to the first light exposure (Table 2). This was caused by a decrease in baseline pupil diameter with increasing dark adaptation duration prior to the start of the light exposure protocol ($P$ = 0.02). Post-blue pupil diameter was not affected by dark adaptation duration ($P$ = 0.99).

***Time of day***

No differences in PIPR-mm ($P$ = 0.11) and PIPR-% ($P$ = 0.22) were found between the morning and afternoon assessments (Figure 3).

***Body posture***

PIPR-mm ($P$ = 0.01) and PIPR-% ($P$ = 0.02) were larger during assessments in a supine posture relative to an upright posture. The difference was explained by a larger baseline pupil diameter during the supine posture ($P$ = 0.007), whereas the post-blue pupil diameter did not change with posture ($P$ = 0.81).

***Environmental light history***

The 24-h mean environmental spectrally weighted irradiance per subject was on average 0.16 (SD = 0.03) weighted W/m$^2$. Previous environmental light history did not affect PIPR-mm ($P$ = 0.72) and PIPR-% ($P$ = 0.75).

**Table 2.** *Estimates of the effects of dark adaptation duration, time of day, body posture, and environmental light history on PIPR outcome parameters and baseline and post-blue pupil diameter.*

| | PIPR-mm | PIPR-% | Baseline pupil diameter (mm) | Post-blue pupil diameter (mm) |
|---|---|---|---|---|
| **Intercept** | 1.79 ± 0.32*** | 38.97 ± 5.11*** | 4.62 ± 0.32*** | 2.93 ± 0.33*** |
| **Dark adaptation duration (/hour)** | -1.96 ± 0.87* | -27.91 ± 14.98* | -1.98 ± 0.72* | 0.02 ± 1.00 |
| **Time of day (morning vs. afternoon)** | 0.17 ± 0.10 | 2.22 ± 1.74 | 0.17 ± 0.08 | -0.01 ± 0.12 |
| **Body posture (supine vs. upright)** | 0.29 ± 0.10* | 4.34 ± 1.69* | 0.26 ± 0.08** | -0.03 ± 0.11 |
| **Environmental light history (weighted W/m$^2$)** | 0.51 ± 1.40 | -7.89 ± 23.92 | 1.54 ± 1.17 | 0.43 ± 1.59 |

Mean values ± SE are displayed. PIPR, Post-Illumination Pupil Response. * $P$ < 0.05; ** $P$ < 0.01; *** $P$ < 0.001.

## Discussion

The aim of the present study was to present and validate a PIPR protocol with high robustness and reliability to assess interindividual differences in the melanopsin-signaling pathway. The two proposed outcome parameters both showed a Cronbach's $\alpha > .90$ and an ICC > .85, indicating a very high reliability. PIPR outcome parameter estimates were larger in the supine position, due to its dilating effect on the pupil diameter during baseline darkness. Outcome measures were not affected by dark adaptation, time of day, and environmental light history.

Single measure reliability of PIPR assessment using monochromatic short-wavelength light (~ 470 nm) was evaluated in two previous studies. One study reported an ICC of 0.80 when using 20-s light stimulation (300 cd/m$^2$) twice with 3 minutes between sessions[51]. In the other study PIPR was induced by exposing specific retinal areas to a 400-ms light stimulus (400 cd/m$^2$)[52]. All areas were stimulated three times each with several minutes between sessions. The ICC was 0.84 for full-field and 0.87 for central-field stimulation. These PIPR protocols with relative short durations may be more user-friendly. Others moreover showed that comparable stimulus durations were sufficient to detect group differences[47,49,50]. Light stimuli of longer duration, as we evaluated here, however allow for a more specific targeting of the melanopsin-signaling pathway; ipRGCs are less photosensitive and have a slower photoresponse than rods and cones[1,53]. In addition, the brief stimuli applied in previous work evoked a transient PIPR (i.e., the post-exposure pupil diameter returned to baseline already during the assessment), while the prolonged stimulus in our protocol induced a PIPR that remained stable during the entire 5-minute post-blue assessment interval. This high PIPR robustness in our protocol may explain the high ICC values of 0.90 for PIPR-mm and 0.87 for PIPR-% with a relatively large interval of 24 hours between sessions. Although our protocol takes longer to complete than previously proposed protocols, and may thus be slightly more difficult to accomplish in clinical settings, this duration seems necessary to obtain a robust assessment that is not sensitive to the onset and offset variability discussed in the introduction section[10,11,63,64]. We would even suggest that it could be interesting for future studies, to extend the post-blue assessment interval and quantify the time course of normalization of the pupil diameter, as a further probe to individual differences in ipRGCs kinetics[59]. An example of such a normalization curve is provided in the supplemental material (Figure S2).

Both PIPR-mm and PIPR-% met the test-retest criteria for clinical use[67], and the potential of our PIPR protocol as a diagnostic tool was furthermore expressed by the consistency of these outcome parameters assessed at different regular office hours. Caution is however needed when performing the test outside these hours, since previous studies showed that PIPR was altered during the evening and night as a result of a modulation of melanopsin-based phototransduction[75-77]. We found that both PIPR outcome measures were dependent

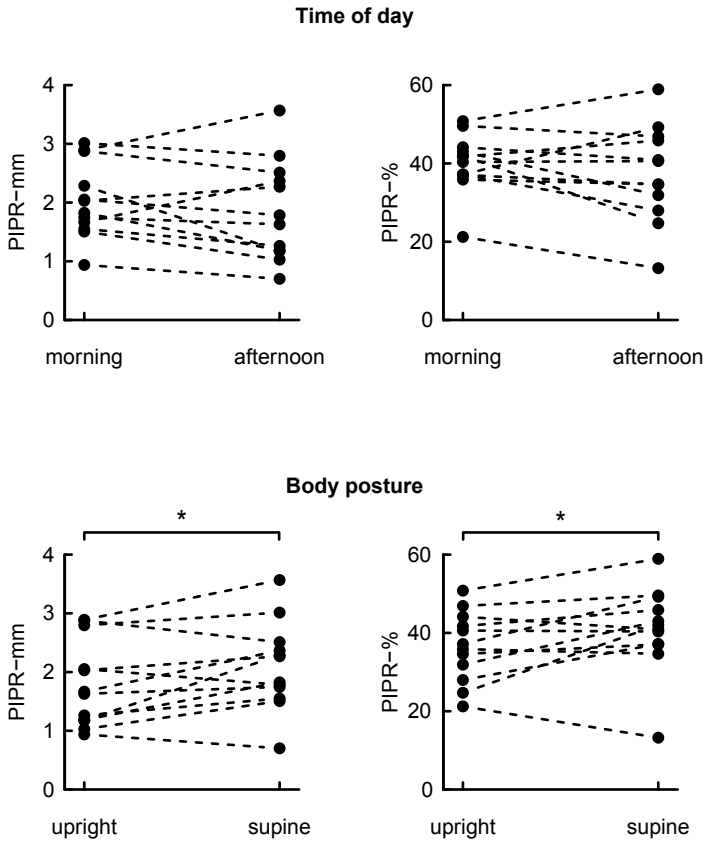


**Figure 3.** *Effects of the factors time of day and body posture on the outcome parameters PIPR-mm and PIPR-%. In the plots for both time of day (top row) as well as body posture (bottom row) each dashed line connects the two repeated measures within a participant to visualize the difference between the two conditions. Horizontal lines with an asterisk (*) above a plot indicate a significant effect of the factor on the outcome parameter (P < 0.05).*

on body posture, secondary to the effect of posture on the baseline pupil diameter during darkness. This should be taken into account when performing future research on the effects of light when subjects have to maintain a supine position, such as in magnetic resonance imaging studies[15]. Previous work found a smaller pupil diameter in a supine position than in a sitting position, in agreement with cardiovascular studies showing less sympathetic activation in a supine position[38]. We found the opposite, and consider that this indication of increased sympathetic activity could be caused by 'fighting against sleep'[78]. Such seemingly paradoxical changes have also been reported in EEG beta-power, which indexes central nervous system activation[39]. Because of the dependency of PIPR measures on baseline pupil diameter it is recommended to reporting them as well in any PIPR study.

We found that PIPR was not affected by 24-h environmental light history. This indicates that correction for previous light exposure is not necessary, which simplifies the implementation of PIPR assessment. To our knowledge, we are the first to assess the effects of 24-hr light history on PIPR. Previous work on short-term light history did show ipRGC modulation by previous light exposure: 5 minutes of prior long-wavelength light increased the pupil response to blue light, while prior short-wavelength light blunted this response[57,58]. The lack of effect of prior light exposure on our outcome measures should not be interpreted as absence of effects of light history; rather, it indicates that our approach is robust to differences in light history.

We found a decline in baseline pupil diameter with increasing dark adaptation duration prior to the start of the light exposure protocol indicating that the eyes were adapted to the dark using 5 minutes of darkness. If dark adaptation would be incomplete, the pupil would be growing instead of declining with an extension of the baseline dark period. The enhancement of pupil constriction over time spent in the dark may be caused by increasing sleepiness[35], but this effect is not expected to be large enough to be able to mask a possible effect of uncompleted dark adaptation. We therefore assume that five minutes of dark pre-exposure is both required and adequate for PIPR assessment.

The high reliability of our PIPR assessment protocol renders it a sensitive tool for research on group differences in human ipRGC functioning and ipRGC modulation in response to intervention, allowing for acceptable sample sizes. In view of ipRGC projections to the biological clock and brain areas involved in sleep-wake regulation[5], it would be interesting to assess the association between PIPR and interindividual differences in circadian phase. Interesting patient populations for future PIPR research include not only patients with ophthalmological diseases but also patients with disorders associated with sleep and alertness complaints (e.g., insomnia, narcolepsy, mood disorders and attention deficit/hyperactivity disorder[79]). A possible limitation of our study could be that we used a period of only 5 minutes between red and blue light exposure, which may not have been sufficient to completely exclude rod and cone interference on the priming effect of red light[58]. We however felt that a dark period of 5 minutes ruled out most rod and cone interference while preserving feasibility of the test. In future studies it may be interesting to investigate whether the PIPR after blue light assessment can be further enhanced by increasing the intermediate dark period or, alternatively, by using other priming light exposures. Accordingly, others showed that using low light levels the sustained pupil constriction after light offset was enhanced by using intermittent green light instead of continuous stimulation, which was explained by increased cone activation[59]. We however do not expect that implementing intermittent light stimuli in our paradigm would further increase the PIPR outcome parameters: the intensity and duration of our light stimulus are expected to already saturate ipRGCs[55]. Whereas in our protocol acute effects of cones are likely minimal, because we excluded the first minute after light offset, it could be most interesting to add complementary paradigms using intermittent light to dissect ipRGC activity

from rods and cones to the pupillary light reflex. In future studies it is therefore interesting to include multiple light exposure protocols in order to make a multivariate fingerprint of rod, cone, and ipRGC involvement in the PIPR.



Experiment 1 was performed at two different environments by different experimenters. We observed no systematic differences when comparing data from both settings, which supports the applicability of our PIPR paradigm at multiple sites. Experiment 2 was limited by the lack of objective measurements of circadian rhythms (e.g., melatonin levels[80]). Individual differences in sleep-wake rhythm may lead to different sleepiness levels, which may be a potential bias in the assessment of the time-of-day effects on pupil diameter[35]. In view of the absence of extreme chronotypes in our population, however, we expect circadian sleep-wake timing to be similar in all participants. Another limitation of experiment 2 was that during the 30-minute habituation period prior to the start of the light exposure protocol, room lights were set at a very dimmed level instead of switching them completely off. This small amount of light was maintained in order to prevent participants from falling asleep, especially in supine condition[81]. Complete darkness may have been preferred in order to maximize repolarization of rods and cones in order to increase their response to the light exposure protocol. Although the dim light exposure was standardized, it may have biased the pupillary light reflex as a result of variance in membrane potentials of rods and cones[9]. The contribution of rods and cones to the pupillary light reflex is particularly substantial during stimulation and immediately after stimulus offset[10,11]. We however included pupil diameter measures from 1 minute after light offset and therefore do not expect that our PIPR measures were affected by using dim light during the habituation period instead of darkness. A limitation of both experiments in this study was that we only included young healthy adults. Future studies can use the proposed assessment protocol to evaluate the reliability of the proposed PIPR assessment protocol in clinical and in pediatric or older populations. In conclusion, the present protocol can reliably quantify the PIPR outcomes to evaluate ipRGC functioning in case-control and intervention studies.

## Acknowledgements

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## Supplemental material

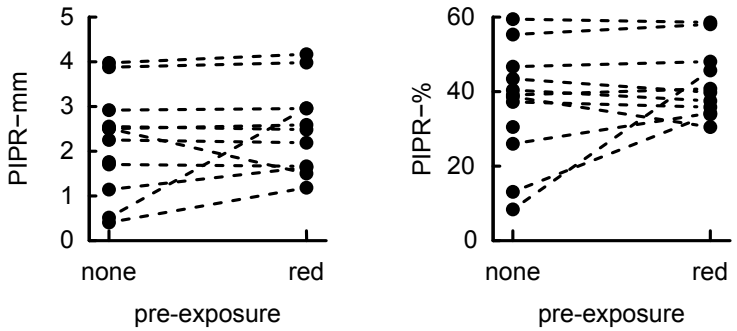


**Figure S1.** *PIPR-mm and PIPR-% with and without pre-exposure to bright red light. The PIPR after blue light was measured twice in twelve young healthy individuals (6 males, 6 females, mean age ± SD: 24.6 ± 2.1 yr). Ten participants did not participate in either of the two experiments described in the manuscript, while the other two participated in experiment 1. The participants were exposed to two light exposure conditions: one with pre-exposure to red light and one without pre-exposure. In the red light pre-exposure condition the light exposure protocol was as described in the manuscript (i.e., dark, red, dark, blue, dark), while in the no pre-exposure condition the first dark block and the red block were omitted from that protocol (i.e., dark, blue, dark). Prior to the start of both light exposure paradigms, the participants were exposed to 30 minutes of dim light (0.5 cd/m$^2$). PIPR-mm and PIPR-% for both conditions were calculated using the methods described in the manuscript. In the plots, each dot represents one measurement and the dashed lines connect the two repeated measures within a participant to visualize the difference between the two conditions. One trial in the red light pre-exposure condition was left out, because of insufficient reliable data points (i.e., our algorithm provided negative PIPR values). Both PIPR outcome measures seemed somewhat higher with red light pre-exposure than without, but mixed effect regression models showed that the differences did not reach significance (PIPR-mm: β = 0.28, SE = 0.24, P = 0.27; PIPR-%: β = 5.3, SE = 3.7, P = 0.18). This is probably due a ceiling effect: other measures were already taken to increase the PIPR towards maximum values (i.e., using light sources with high luminance and dilating the pupil of the exposed eye). Despite the PIPR increase after red light pre-exposure being non-significant, considerations of robustness (e.g., in case of a different protocol or submaximal exposure) and previous work give enough reason to recommend pre-exposure with red light in order to guarantee a maximal PIPR.*

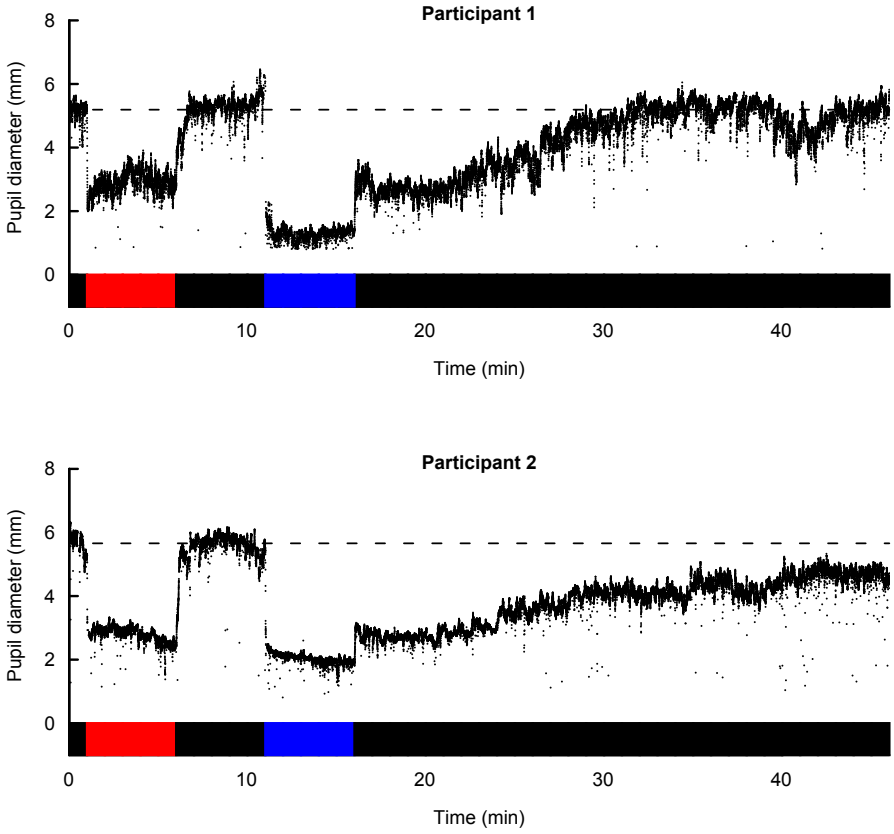


**Figure S2.** *Example of the change of pupil diameter beyond the light exposure protocol. Two healthy young adult males were exposed to the light exposure protocol as described in the manuscript with the baseline dark period reduced to 1 minute. The dashed line represents mean pupil diameter during this baseline dark period. Post-blue dark was extended to 30 minutes to monitor the normalization of pupil diameter after light offset. Interestingly, in participant 1 we observe pupil diameter reaching baseline size after approximately 15 minutes of post-blue darkness, while in participant 2 redilation to baseline diameter is not completed within the 30-minute dark interval after blue light offset. This large difference in pupil redilation time between the two participants indicates that for future studies it may be interesting to extend the post-blue dark period to allow for quantification of the time course of pupil redilation in order to further examine individual differences in ipRGCs kinetics.*

# CHAPTER 3

## Individual differences in the post-illumination pupil response to blue light: assessment without mydriatics

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*Biology 2016; 5:34*

## Abstract

Melanopsin-containing retinal ganglion cells play an important role in the non-image forming effects of light, through their direct projections on brain circuits involved in circadian rhythms, mood and alertness. Individual differences in the functionality of the melanopsin-signaling circuitry can be reliably quantified using the maximum post-illumination pupil response (PIPR) after blue light. Previous protocols for acquiring PIPR relied on the use of mydriatics to dilate the light-exposed eye. However, pharmacological pupil dilation is uncomfortable for the participants and requires ophthalmological expertise. Hence, we here investigated whether an individual's maximum PIPR can be validly obtained in a protocol that does not use mydriatics but rather increases the intensity of the light stimulus. In 18 participants (5 males, mean age ± SD: 34.6 ± 13.6 years) we evaluated the PIPR after exposure to intensified blue light (550 $\mu W/cm^2$) provided to an undilated dynamic pupil. The test-retest reliability of the primary PIPR outcome parameter was very high, both between day-to-day assessments (intraclass correlation coefficient (ICC) = 0.85), as well as between winter and summer assessments (ICC = 0.83). Compared to the PIPR obtained with the use of mydriatics and 160-$\mu W/cm^2$ blue light exposure, the method with intensified light without mydriatics showed almost zero bias according to Bland-Altman plots and had moderate to strong reliability (ICC = 0.67). In conclusion, for PIPR assessments, increasing the light intensity is a feasible and reliable alternative to pupil dilation to relieve the participant's burden and to allow for performance outside the ophthalmological clinic.

## Introduction

The suprachiasmatic nucleus (SCN) in the hypothalamus encompasses the endogenous biological clock, which drives physiological, endocrine, and behavioral rhythms with a periodicity of about 24 h[21]. The daily environmental light-dark cycle serves as the main synchronizer to entrain these so-called circadian rhythms to an exact 24-h cycle in order to remain in phase with the 24-h period of a day on Earth[22]. Intrinsically photosensitive retinal ganglion cells (ipRGCs) play a key role in this circadian photoentrainment[1,2]. ipRGCs express the photopigment melanopsin which enables them to intrinsically decode ambient light levels[1,4]. Melanopsin is maximally responsive to short-wavelength (i.e., blue) light with peak sensitivity between 460–480 nm. In addition, ipRGCs receive extrinsic input from rods and cones[10,82]. Through direct connections between ipRGCs and the brain, the integrated intrinsic and extrinsic information on environmental light is transferred from the retina to downstream non-image forming brain regions, with the SCN as one of the main targets[83].



Besides circadian photoentrainment, ipRGCs are also involved in the regulation of mood[84], alertness[44], pineal melatonin production[85], and the pupillary light reflex (PLR)[10]. The contribution of ipRGCs to the PLR is mediated through direct projections onto the olivary pretectal nucleus (OPN; i.e., the brain region for pupil size control[8]). The involvement of ipRGCs in the PLR allows for a non-invasive method to measure functionality of the intrinsic melanopsin-based signaling pathway[5]. More specifically, the characteristic sustained contraction of the pupil after blue light can be almost entirely assigned to the melanopsin-driven delay in ipRGC repolarization after light offset[11,45]. This phenomenon, known as the post-illumination pupil response (PIPR)[83], can thus be used to estimate the functionality of the intrinsic melanopsin-signaling circuitry[11].

Accordingly, we have previously developed a reliable and robust method to assess individual differences in the maximum PIPR[86] and showed its relevance for circadian entrainment[87]. The assessment procedures include pharmacological pupil dilation in the illuminated eye[62], which is highly uncomfortable for the participants[88]. The mydriatics that are used for pupil dilation, like tropicamide, phenylephrine, and cyclopentolate, entail several possible side effects including a dry mouth, blurred vision, stinging, and sensitivity of the eyes to light[89]. In addition, dilation of the pupil may persist up until 24 h after administration causing prolonged reduced sight, which is often experienced as unpleasant and impedes tasks like driving and reading[90]. A more serious risk is that pharmacological pupil dilation may lead to closed-angle glaucoma[91]. Ophthalmological expertise is therefore indispensable for the use of mydriatics. This proficiency is usually present in eye research. However, given the myriad of ipRGC-driven downstream photoregulatory functions, PIPR assessments are also highly relevant for non-ophthalmic research on sleep[87,92,93], cognition[17], alertness[16,44], mood[94], and emotion[95], where ophthalmological expertise to safely apply pupil dilation may be insufficient. Taken

together, to relieve the participant's burden and to allow for a wide application of safe PIPR measurements, it would be highly desirable to have a protocol that does not rely on the use of mydriatics while preserving the high reliability and robustness of the assessments.

Previous studies have presented reliable PIPR measurements without pharmacological mydriasis[51,52]. The PIPR in these previous studies was only transient, however, while we here aimed to elicit a more melanopsin-specific maximum prolonged PIPR. Considering that the PIPR increases with increasing light intensity[46,96-98], the aim of the present study was to examine whether an individual's maximum PIPR can be validly assessed without mydriatics by instead increasing the intensity of the light stimulus[11]. We therefore examined the within-subject between-day test-retest reliability of an alternative protocol without mydriatics with increased light intensity and compared this to the previously found very high reliability of the protocol with mydriatics[86]. In a previous different protocol to assess the PIPR, outcome measures showed variability across seasons[32]. We therefore evaluated whether our protocol without mydriatics might result in a more stable trait-like biomarker, robust across seasons, by assessing the test-retest reliability across the winter and summer.

## Materials and methods

### Participants

A total of 18 volunteers (5 males, mean age ± SD [range]: 34.6 ± 13.6 [22–69] years) were enrolled. Volunteers were assessed at two different locations with different experimenters and the same equipment: the Netherlands Institute for Neuroscience in Amsterdam (12 participants, 5 males, mean age ± SD: 35.8 ± 15.5 years) and PsyQ Expertise Center Adult ADHD in The Hague (6 participants, 0 males, mean age ± SD: 32.3 ± 9.4 years). All participants were informed about the procedure, signed informed consent, and did not receive any incentive. None of the participants had a self-claimed history of ocular pathology and none reported to use any medication known to influence the PLR. According to Nagel anomaloscope tests, none of the participants had a color vision deficiency. The study was approved by the Medical Ethical Committee of the VU University Medical Center Amsterdam (protocol NL43319.029.13) and adhered to the tenets of the Declaration of Helsinki.

### Pupillometry

The previously established pupillometry procedures have been described elsewhere[86]. In brief, the right eye was exposed to an illumination protocol, while the left eye was continuously recorded using a custom-made infrared pupillometry set-up. The surface of the light source was 16 × 10 cm and the distance between the illuminated eye and the light stimulus was 5 cm. The light exposure protocol consisted of the following five 5-min intervals:

baseline dark, monochromatic red light (635 nm) to maximize the pupil response after blue light, dark[58], monochromatic blue light (465 nm), and post-blue dark (Figure 1). These longer stimulus durations allow for more specific assessment of the melanopsin-signaling pathway because of the low sensitivity and slow kinetics of ipRGCs[1,53]. Previous research showed that light adaption of ipRGCs was completed after 5 min of light exposure, probably saturating the ipRGCs response[55]. From the baseline and post-blue pupil diameter, two PIPR outcome parameters were calculated[49,50]: the (1) PIPR-mm and the (2) PIPR-%.



1. PIPR-mm = baseline pupil diameter – post-blue pupil diameter
2. PIPR-% = 100 * PIPR-mm / baseline pupil diameter

We previously showed that these PIPR measurements have very high within-subject test-retest reliability[86].

The previously established protocol to assess the PIPR involved a blue light stimulus with an irradiance level of 160 μW/cm$^2$ and the pupil of the right eye was dilated with the use of mydriatics, tropicamide 0.5% (160My+). The duration and intensity of the blue light stimulus[54,55], in combination with the pharmacological pupil dilation in the illuminated eye[62], were expected to saturate the melanopsin-driven photoresponse, resulting in a maximum PIPR. In order to examine whether the application of mydriatics substantially adds to eliciting the maximum PIPR this saturating PIPR assessment condition was compared to the condition with the same blue stimulus of 160 μW/cm$^2$ but with the pupil in its natural undilated state (160My−). In order to assess whether increasing the intensity of the light stimulus may compensate for the possible reduced PIPR in the absence of pharmacological mydriasis the previously used saturating PIPR assessment condition was also compared to the condition in which the light intensity was increased, 550 μW/cm$^2$ during blue light and the illuminated pupil was kept in its natural undilated state (550My−). In view of comments from participants in a previous pilot study, reporting major discomfort during exposure to a stimulus of such intensity with tropicamide application, we refrained from assessing the PIPR after intensified blue light with pharmacological mydriasis. Light intensities were calibrated using a spectrometer (AvaSpec-3648-USB2, Avantes, Apeldoorn, the Netherlands). Since the light stimuli activate all photoreceptors, we estimated an illuminance value for each specific photoreceptor[99] for each light stimulus (Table 1).

**Table 1.** *Illuminance and luminance levels of the blue and red light during the previously established light conditions and the intensified light conditions.*

| Parameter | Previously used blue light | Intensified blue light | Previously used red light | Intensified red light |
|---|---|---|---|---|
| **Peak wavelength (nm) (full width half maximum)** | 465 (20) | 465 (20) | 635 (20) | 635 (20) |
| **Irradiance (μW/cm²)** | 158.75 | 548.96 | 178.16 | 473.34 |
| **Log photon flux (1/cm²/s)** | 14.57 | 15.11 | 14.75 | 15.18 |
| **Illuminance (photopic lux)** | 120.91 | 421.13 | 327.03 | 843.35 |
| **Luminance (cd/m²)** | 375 | 1300 | 375 | 1000 |
| ***Photoreceptor illuminance*** | | | | |
| **S cone (cyanopic lux)** | 1131.92 | 3889.54 | 12.25 | 61.76 |
| **Melanopsin (melanopic lux)** | 1060.86 | 3680.33 | 8.21 | 36.84 |
| **Rod (rhodopic lux)** | 751.94 | 2608.91 | 12.78 | 43.59 |
| **M cone (chloropic lux)** | 378.40 | 1315.53 | 114.67 | 297.14 |
| **L cone (erythropic lux)** | 198.81 | 691.60 | 419.58 | 1085.07 |

S cone, short-wavelength cone; M cone, mid-wavelength cone; L cone, long-wavelength cone.

The order of the three conditions was randomized with 1 to 7 days between measurements. Full recovery of tropicamide application generally takes place within 6 h. In some cases, however the effects may last up until 24 h after administration[90]. PIPR assessments within a participant were therefore separated by at least 24 h. All sessions were performed between 9:30 a.m. and 4:00 p.m. in the months February and March 2016. To avoid time-of-day effects[75], the timing of the assessments was consistent within participants. All participants were invited to return to the lab on two consecutive days in the months July and August 2016 in order to assess the test-retest reliability of the 550My− protocol. The summer assessments were performed at the same time of day as the winter assessments. In order to assess the long-term cross-seasonal test-retest reliability between the winter and summer season, the PIPR outcome parameters of the first summer sessions were compared to the winter assessments.

### Statistical analysis

For both PIPR-mm and PIPR-%, differences between 160My+ and 160My− and differences between 160My+ and 550My− were visually inspected using Bland-Altman plots[69]. To evaluate whether 160My+ and 160My− procedures yield similar PIPR results a two-way random effect single measurement intraclass correlation coefficient (ICC) for absolute agreement was calculated[68]. Additional ICC values were calculated to examine reliability in pupil diameter during the baseline and post-blue intervals between 160My+ and 160My−. To compare differences in PIPR outcome measures, baseline and post-blue pupil diameter between 160My+ and 550My− the same ICC analyses were performed as in the comparison between 160My+ and 160My−. Bland-Altman plots and ICC analyses of the same outcome parameters were also used to evaluate the day-to-day test-retest reliability of 550My−

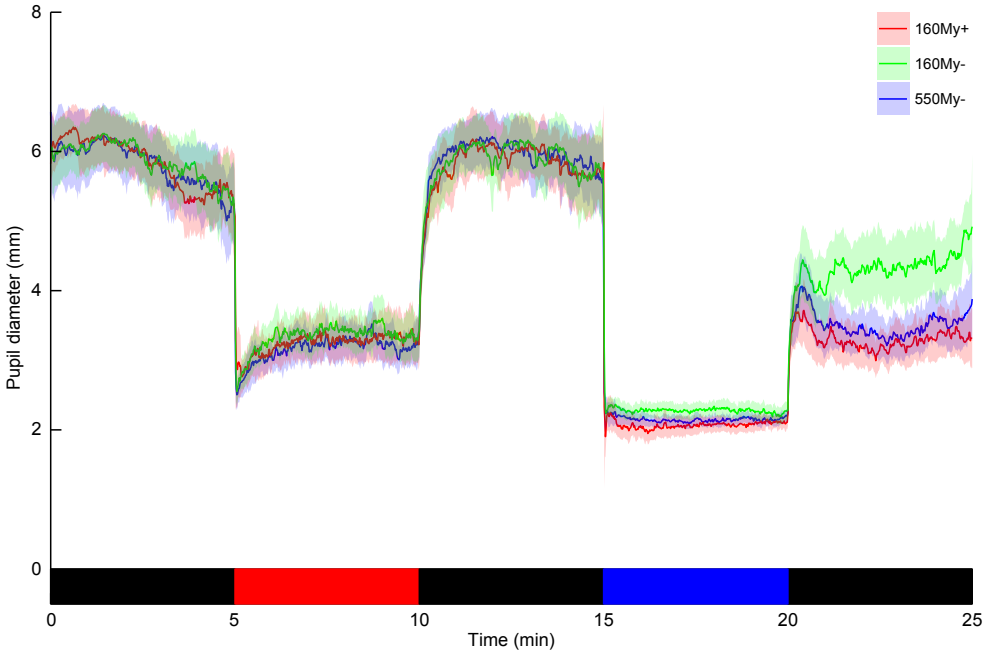


**Figure 1.** *The change in pupil diameter of the left eye throughout the light exposure protocol in three different conditions. The traces represent the population mean pupil diameter, with the semi-transparent areas indicating the 95%-confidence interval, for each of the three conditions (red trace: the condition including 160 µW/cm² blue light with the use of mydriatics (160My+), green trace: the condition including 160 µW/cm² blue light with the pupil in its natural state (160My−), blue trace: the condition including 550 µW/cm² blue light with the pupil in its natural state (550My−)). The bottom bar indicates the light exposure sequence, which was equal for all three condition (black = dark, red = monochromatic red light and blue = monochromatic blue light).*

protocol and to examine the seasonal effects on this condition. Data processing and analyses were conducted in R (version 3.2.1, R Foundation for Statistical Computing, Vienna, Austria), using the software packages 'ICC'[72], 'MethComp'[100], and 'cocron'[71].

## Results

The values for baseline and post-blue pupil diameter, PIPR-mm and PIPR-%, are shown in Table 2. Retinal illuminance during blue light was estimated based on the mean pupil diameter during blue light to be 18850 Td in 160My+, 1545 Td in 160My−, and 4897 Td in 550My−.

Bland-Altman bias indicated low agreement between 160My+ and 160My− for the PIPR-mm and PIPR-%. In addition, there was low reliability between the PIPR outcome parameters in these conditions, as indicated by the ICC (PIPR-mm: −0.12; PIPR-%: −0.27) (Table 3). Negative ICC estimates indicate within-subject variability exceeding between-subject variability, and thus a very poor ICC[68]. There was also a low agreement between 160My+ and 160My− for the post-blue pupil diameter (ICC = 0.03).

The Bland-Altman plots indicated almost zero bias between 160My+ and 550My− for PIPR-mm and PIPR-% (Figure 2) indicating high agreement. When comparing the condition 160My+ to the condition 550My−, the ICC values indicate a moderate to strong agreement for the PIPR outcome parameters (PIPR-mm: ICC = 0.67; PIPR-%: ICC = 0.58). The ICC of 0.77 indicated a strong agreement between these conditions for the post-blue pupil diameter, which was confirmed by an almost-zero Bland-Altman bias. During baseline dark, there was an almost perfect agreement between 160My+ and 160My− (ICC = 0.88) and between 160My + and 550My− (ICC = 0.92). Bland-Altman biases also indicated this high similarity.

**Table 2.** *Outcome parameters of the pupil measurement from the three different conditions.*

| | PIPR-mm | PIPR-% | Baseline pupil diameter (mm) | Post-blue pupil diameter (mm) |
|---|---|---|---|---|
| **160My+** | 2.68 ± 0.57 [1.44-3.83] | 45.23 ± 6.61 [29.89-55.08] | 5.91 ± 0.81 [4.04-7.22] | 3.23 ± 0.53 [2.20-4.35] |
| **160My−** | 1.65 ± 0.80 [0.51-3.30] | 27.40 ± 12.34 [8.22-55.49] | 5.97 ± 0.79 [4.08-7.25] | 4.33 ± 0.88 [2.65-5.96] |
| **550My−** | 2.54 ± 0.66 [1.41-3.51] | 42.27 ± 8.32 [22.99-54.03] | 5.96 ± 0.81 [4.09-7.51] | 3.43 ± 0.59 [2.36-4.71] |

Mean values ± SD [range] are displayed. Baseline and post-blue pupil diameter represent the mean pupil diameter during middle three minutes of the respective 5-min interval. PIPR, Post-Illumination Pupil Response; 160My+, 160-µW/cm$^2$ blue light with mydriatics; 160My−, 160 µW/cm$^2$ blue light with natural pupil; 550My−, 550-µW/cm$^2$ blue light with natural pupil.

Fifteen (5 males, mean age ± SD (range): 36.4 ± 14.3 (23–70) years) out of the 18 participants returned to the lab for two consecutive summer assessments, one day apart. Bland-Altman plots indicated almost zero bias between the outcome measures of the 550My− protocol for PIPR-mm and PIPR-% (Figure 3), indicating high agreement. The ICC values for the PIPR outcome parameters moreover indicated a very high day-to-day test-retest reliability (PIPR-mm: ICC = 0.85; PIPR-%: ICC = 0.87) (Table 4).

**Table 3.** *Reliability and agreement of the two PIPR outcome parameters and the baseline and post-blue pupil diameter between the condition including mydriatics and the two conditions without mydriatics.*

| | PIPR-mm | PIPR-% | Baseline pupil diameter | Post-blue pupil diameter |
|---|---|---|---|---|
| ***160My+ – 160My–*** | | | | |
| **ICC (95% confidence interval)** | -0.12 (-0.54 to 0.35) | -0.27 (-0.64 to 0.21) | 0.88 (0.71-0.95) | 0.03 (-0.42 to 0.47) |
| **Bland-Altman bias (95% limits of agreement)** | 1.04 (-0.54 to 2.62) | 17.83 (-5.36 to 41.01) | -0.06 (-0.84 to 0.73) | -1.10 (-2.40 to 0.91) |
| ***160My+ – 550My–*** | | | | |
| **ICC (95% confidence interval)** | 0.67 (0.32-0.86) | 0.58 (0.19-0.82) | 0.92 (0.80-0.97) | 0.77 (0.50-0.91) |
| **Bland-Altman bias (95% limits of agreement)** | 0.15 (-0.84 to 1.14) | 2.96 (-9.97 to 15.89) | -0.05 (-0.72 to 0.62) | -0.20 (-0.87 to 0.48) |

PIPR, Post-Illumination Pupil Response; ICC, Intraclass Correlation Coefficient; 160My+, 160-µW/cm$^2$ blue light with mydriatics; 160My–, 160-µW/cm$^2$ blue light with natural pupil; 550My–, 550-µW/cm$^2$ blue light with natural pupil.

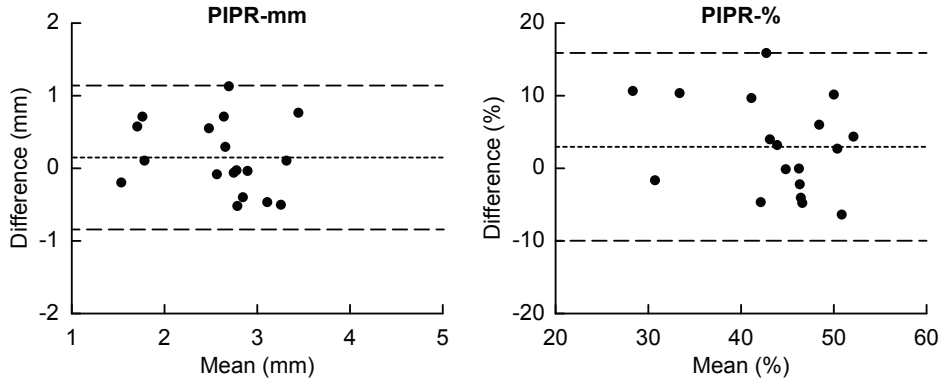


**Figure 2.** *Bland-Altman plots for PIPR-mm (left panel) and PIPR-% (right panel) between 160-µW/cm$^2$ blue light with mydriatics (160My+) and 550-µW/cm$^2$ blue light with natural pupil (550My–). Differences between the two conditions (i.e., 160My+ minus 550My–) are plotted against the mean of the two measurements. The dotted line represents the bias, i.e., the mean difference between all measurements of 160My+ and 550My–. The dashed lines are the 95% limits of agreement: 95% of the differences between the conditions lies between these lines. The smaller the limits of agreement, the better the agreement between the two measurements.*

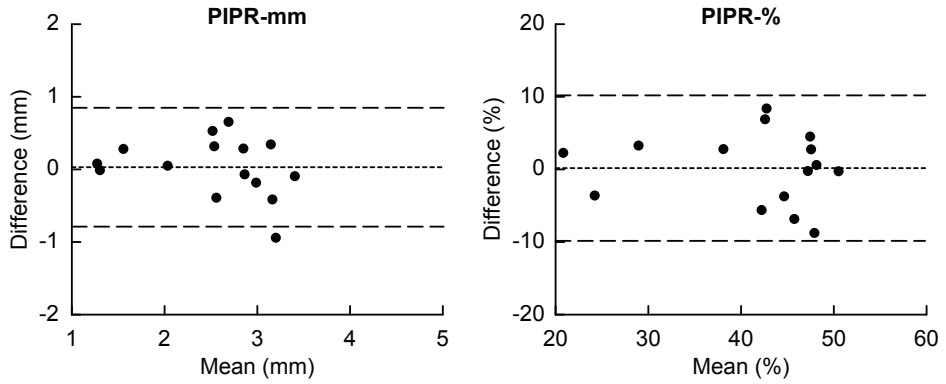


**Figure 3.** *Bland-Altman plots for PIPR-mm (left panel) and PIPR-% (right panel) between the two consecutive summer assessments using the protocol with intensified blue light (550 µW/cm²) with a natural pupil (550My–). Differences between the two measurements (i.e., summer session 1 minus summer session 2) are plotted against the mean of the two measurements. The dotted line represents the bias (i.e., the mean difference between all measurements of the first session and all measurements of the second session). The dashed lines are the 95% limits of agreement.*

**Table 4.** *PIPR outcome parameters and test-retest reliability outcomes of the summer assessments using the condition with intensified blue light with a natural pupil.*

| | **PIPR-mm** | **PIPR-%** |
|---|---|---|
| **Summer session 1 - Mean ± SD [range]** | 2.55 ± 0.67 [1.30–3.36] | 41.34 ± 9.30 [21.97–50.39] |
| **Summer session 2 - Mean ± SD [range]** | 2.52 ± 0.77 [1.23–3.67] | 41.61 ± 9.85 [19.69–52.28] |
| ***Summer session 1 – summer session 2*** | | |
| **ICC (95% confidence interval)** | 0.85 (0.62–0.95) | 0.87 (0.67–0.95) |
| **Bland-Altman bias (95% limits of agreement)** | 0.03 (-0.79 to 0.85) | 0.18 (-9.84 to 10.20) |
| ***Summer session 1 – winter session*** | | |
| **ICC (95% confidence interval)** | 0.83 (0.57–0.94) | 0.80 (0.51–0.93) |
| **Bland-Altman bias (95% limits of agreement)** | 0.05 (-0.78 to 0.89) | -0.01 (-11.82 to 11.80) |

PIPR, Post-Illumination Pupil Response; ICC, Intraclass Correlation Coefficient.

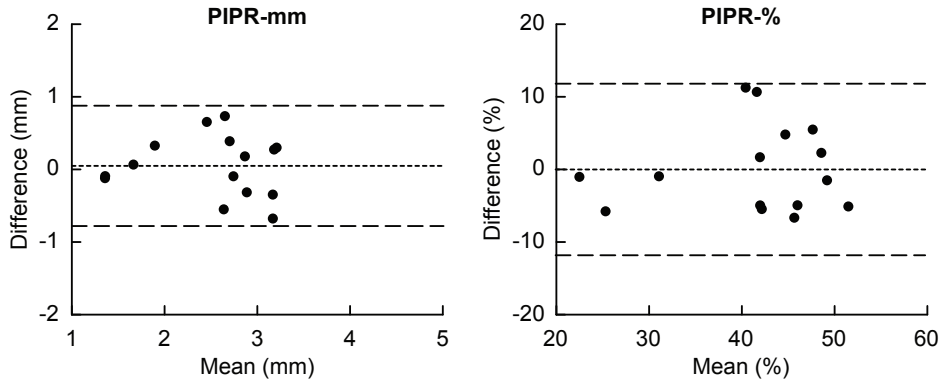


**Figure 4.** *Bland-Altman plots for PIPR-mm (left panel) and PIPR-% (right panel) between the first summer session and the winter assessment using the protocol with intensified blue light (550 µW/cm²) with a natural pupil (550My–). Differences between the two measurements (i.e., summer minus winter) are plotted against the mean of the two measurements. The dotted line represents the bias, i.e., the mean difference between all measurements of the summer and the winter.*

The Bland-Altman plots also indicated almost zero bias between 550My– winter and 550My– summer measurements for PIPR-mm and PIPR-% (Figure 4), indicating high agreement. Again, the ICC values indicated a very high reliability for the PIPR outcome parameters (PIPR-mm: ICC = 0.83; PIPR-%: ICC = 0.80).

## Discussion

In order to allow for a wide application of assessing the functionality of the melanopsin-signaling circuitry, we here examined whether an individual's maximum PIPR can be validly assessed without mydriatics by instead increasing the intensity of the light exposure. In our previously established paradigm, with mydriatics, the 5-min duration of the blue light stimulus was expected to saturate the ipRGCs [54,55,86], leading to a maximum PIPR. Shorter blue light stimuli durations of milliseconds to a few seconds, as used in previous studies, may propose less discomfort for the participant but only elicit a transient PIPR (i.e., the post-exposure pupil diameter returns to baseline already during the assessment)[46,51,52,97]. On the contrary, our use of high intensity light stimuli of longer duration, tailored to the slow photoresponse and low sensitivity of ipRGCs, allows for more specific targeting of melanopsin-signaling pathway. Furthermore, in a previous shorter protocol to assess the PIPR, outcome measures showed variability across seasons[32]. Our longer protocol may result in a more trait-like biomarker that is stable across the winter and summer.

The previously established blue light stimulus with an irradiance of 160 µW/cm$^2$, evoked a reduced PIPR when the pupil was kept in its natural dynamic state compared to the condition with the use of mydriatics. This indicates that dilation of the pupil or intensifying the light is required to evoke a maximum PIPR. The retinal illuminance during blue light was over four times higher for the previously established protocol with mydriatics compared to the condition with intensified blue light without mydriatics. In spite of this fourfold difference, the PIPR did not systematically differ between these conditions. This suggests that during both conditions, the ipRGCs response was saturated and a maximum PIPR was generated, since there was no further increase in PIPR even though the blue-light retinal illuminance was higher in the previously established protocol. The moderate to strong agreement between the previously established 160 µW/cm$^2$ blue light with mydriatics protocol and the alternative protocol with intensified blue light with a natural pupil indicates that both approaches are valid for eliciting the maximum PIPR. However, there can be individual variation between the results obtained with these two approaches, indicating that they should not be used interchangeably.

The within-subject test-retest of the protocol with intensified blue light with a natural pupil showed a very high agreement for both PIPR-mm and PIPR-%, approaching the reliability of our formerly established protocol with 160-µW/cm$^2$ blue light and mydriatics (PIPR-mm: ICC = 0.90; PIPR-%: ICC = 0.87). This indicates that although the pupil is dynamic and the amount of light reaching the retina has a larger variation in the protocol with intensified blue light without mydriatics, this does not seem to affect the reliability of this protocol, suggesting saturation of the response at a certain intensity.

Previous research showed that the post-illumination contraction amplitude following blue light was larger in summer compared to winter[32]. However, there was only a difference between summer and winter for photopically adapted eyes. Our protocol with intensified blue light and a natural pupil showed no effect of season on the PIPR outcome parameters. We showed a very high agreement between the winter and summer measurements. Our protocol did not include an adaption period and provided the light stimulus for a much longer duration (i.e., 5 min instead of 1 s). The high reliability of our protocol with intensified blue light and a natural pupil, between days and even between seasons, provides it as a sensitive and robust possible biomarker to study trait-like individual differences in ipRGCs functioning.

In patients with retinal diseases pharmacological pupil dilation may not be without risk since dilation can lead to a rise in intraocular pressure[91]. With ipRGCs affected in diverse optic nerve and retinal diseases such as glaucoma[49,101], age-related macular degeneration[102], retinitis pigmentosa[103], and diabetes[47], PIPR measurements without dilation may provide a useful diagnostic tool. Excluding pharmacological dilation of the pupil from PIPR assessments is not only beneficial for ophthalmic patients, but also for individuals without eye-related

complaints. Since pharmacological pupil dilation may be uncomfortable for participants and can even be dangerous when driving [104]. With the current reliable PIPR assessment protocol a maximum PIPR can be evoked without pupil dilation making the protocol accessible for non-ophthalmic research fields, such as sleep[87,92,93], cognition[17], alertness[16,44], mood[94], and emotion[95] research. In addition, considering the important role ipRGCs play in circadian rhythms through their direct projections to the SCN, our current protocol can be easily applied in chronobiological studies.



A limitation of the study is the unequal gender distribution, there were more females included than males, which may have confounded our PIPR results. Accordingly, some parameters of the PLR were shown to be mildly affected by gender[105]. Future studies should therefore use a larger sample with a more even gender distribution to examine the effects of gender. Furthermore, future studies should examine whether the proposed protocol without pupil dilation is applicable in patients suffering from eye diseases and whether it may be used for diagnosing abnormalities in the melanopsin-signaling circuitry.

## Conclusion

The aim of the present study was to evaluate an accessible PIPR assessment protocol without mydriatics to relieve the participant's burden and to allow for a wide application of safe PIPR measurements. This protocol was an adaption of an established reliable pupillometry paradigm, which was designed to evoke a maximum PIPR in order to estimate functionality of the intrinsic melanopsin-dependent circuitry [86]. In spite of its reliability, its reliance on mydriatics may impede widespread use of the protocol. We therefore omitted pharmacological pupil dilation and instead increased the intensity of the light stimuli. For most participants this adapted protocol, without mydriatics, showed reasonable agreement with the previous protocol, with mydriatics, in PIPR outcome measures and there was no bias between the two methods. In addition, the protocol without mydriatics showed very high test-retest reliability for the PIPR outcome parameters, both between consecutive days as well as across seasons. In conclusion, the method presented here can be used to reliably quantify trait-like individual biomarkers of the functionality of the intrinsic melanopsin-signaling circuitry.

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views only of the author, and the Commission cannot be held responsible for any use which may be made of the information contained therein. This work was supported by Project NeuroSIPE 10738, of the Dutch Technology Foundation STW, which is part of the Netherlands Organization for Scientific Research (NWO) and partly funded by the Ministry of Economic Affairs, Agriculture and Innovation.

# CHAPTER 4

## Individual differences in sleep timing relate to melanopsin-based phototransduction in healthy adolescents and young adults

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## Abstract

Individual differences in sleep timing have been widely recognized and are of particular relevance in adolescents and young adults who often show mild to severely delayed sleep. The biological mechanisms underlying the between-subject variance remain to be determined. Recent human genetics studies showed an association between sleep timing and melanopsin gene variation, but support for functional effects on downstream pathways and behavior was not demonstrated before. We therefore investigated the association between the autonomic (i.e., pupil diameter) and behavioral (i.e., sleep timing) readouts of two different downstream brain areas, both affected by the same melanopsin-dependent retinal phototransduction: the olivary pretectal nucleus (OPN) and the suprachiasmatic nucleus (SCN). Our study population included 71 healthy individuals within an age range with known vulnerability to a delayed sleep phase (16.8-35.7 yr, 37 males, 34 females). Pupillometry was performed to estimate functionality of the intrinsic melanopsin-signaling circuitry based on the OPN-mediated post-illumination pupil response (PIPR) to blue light. Sleep timing was quantified by estimating the SCN-mediated mid-sleep timing in three different ways in parallel: using a chronotype questionnaire, a sleep diary, and actigraphy. All three measures consistently showed that those individuals with a later mid-sleep timing had a more pronounced PIPR ($0.03 < P < 0.05$), indicating a stronger blue-light responsiveness of the intrinsic melanopsin-based phototransduction circuitry. In conclusion, trait-like individual differences in the melanopsin phototransduction circuitry contribute to individual differences in sleep timing. Blue-light-sensitive young individuals are more prone to delayed sleep.

## Introduction

The suprachiasmatic nucleus (SCN) in the hypothalamus is the central pacemaker for endocrine, physiological, and behavioral rhythms[21]. The rhythms orchestrated by this endogenous clock are characterized by a periodicity of approximately 24 h and are therefore dubbed 'circadian rhythms' (circa = about, dies = day)[106]. In order to be in phase with the environment (i.e., a day on Earth), however, these circadian rhythms need to be synchronized to an exact 24-h cycle. The most important environmental information used for this entrainment is the light-dark cycle[22]. Key players in this so called circadian photoentrainment are the intrinsically photosensitive retinal ganglion cells (ipRGCs)[1,107]: a small subset of retinal ganglion cells expressing the photopigment melanopsin (peak sensitivity 460-480 nm), which renders them intrinsically photosensitive[2,4]. The ipRGCs integrate their intrinsic photoresponse with the extrinsic input from the classic rod and cone phototransduction pathways[10]. As a result of direct connections between ipRGCs and the brain, the integrated information on environmental light is directly transduced from the retina to downstream areas, with the SCN as one of the main targets[83]. From an evolutionary perspective, ambient light solely consisted of sunlight, but since the introduction of electric light, the 24-h exposure profile is increasingly becoming a mixture of solar and artificial light[108] Findings from previous studies suggest that this 'light pollution' affects photoentrainment of the biological clock and may shift circadian rhythms[109,110].

The direction of photoinduced circadian shifts is dependent on the diurnal profile of solar and artificial light exposure according to a so-called phase response curve[111]. Morning bright light advances circadian phase[112], whereas bright light exposure in the evening causes a phase delay[113]. Classic studies on circadian rhythms identified circadian phase changes from shifts in the core body temperature minimum or the dim light onset of the sleep-promoting hormone melatonin[114,115]. Photoinduced shifts were also found in timing of the sleep-wake cycle[116], which is the most explicitly expressed behavioral circadian rhythm[19,20]. Interestingly, the phase shifting effects of light on sleep-wake timing show a considerable interindividual variation[14]. Whereas entrainment in 'early birds' is more likely to result in a sleep-wake schedule that is advanced relative to the environmental light-dark cycle, the entrainment of 'night owls' is more likely to result in a sleep-wake schedule that is relatively delayed. These individual differences in internal-external phase relationships are thought to result, in part, from polymorphisms in core clock genes[117-119]. Recent findings moreover suggest that the individual differences may involve as well polymorphisms in genes of the phototransduction circuitry[25,26]. Human genetics studies found an association between sleep timing and melanopsin gene variation. Previous work also showed chronotype-dependent differential responses to light[23,24]. It remains to be evaluated however whether such individual differences actually involve a specific functionality of the melanopsin phototransduction circuitry.

We here address this question, by assessing the association of individual differences in a pupillary response that is independent from the inputs from rods and cones and specific to intrinsic melanopsin-dependent phototransduction effects on the downstream olivary pretectal nucleus (OPN), with those in three different markers of sleep timing involving the SCN[120]. We focus on healthy adolescents and young adults, a population in which a mild to severely delayed sleep timing is highly prevalent[33,34]. Functionality of the intrinsic melanopsin-driven phototransduction circuitry is assessed from the post-illumination pupil response (PIPR) after bright blue light (i.e., the sustained pupil constriction), which indicates the strength of the photoresponsiveness of the intrinsic melanopsin-signaling phototransduction circuitry[11]. Individual differences in PIPR are correlated with sleep timing assessed in three ways: using the Munich Chronotype Questionnaire (MCTQ)[121], the Consensus Sleep Diary[122], and actigraphy[123].

## Methods

### Participants

We included participants with an age between 16 and 36 yr. Other inclusion criteria were self-acclaimed health and no use of medication, as indicated by the sleepregistry.nl[124] implementation of the health-related questions of the Duke Structured Interview for Sleep Disorders[60]. The sleepregistry.nl implementation includes questions on current and past health issues according to the 10 categories of the International Classification of Diseases. Shift workers were excluded. Nagel anomaloscope tests were used to exclude participants suffering from color vision deficiency[125]. Participants received oral and written information on the study and signed informed consent before participation. The study was approved by the Medical Ethical Committee of the VU University Medical Center Amsterdam.

Our study population included 37 males and 34 females with a mean age ± standard deviation [range] of 22.7 ± 5.0 [16.8 – 35.7] yr, and was composed of participants included in three different studies. As part of these studies, all 71 participants underwent a PIPR assessment, and completed the MCTQ to obtain a measure for habitual sleep timing. Recent sleep timing was measured in 56 of the 71 participants using a sleep diary and actigraphy: in 29 participants immediately following the PIPR assessment and in 27 participants one month prior to the PIPR assessment.

### Post-illumination pupil response

Most characteristics of the pupillary light reflex are determined by the combined activation of rods, cones, and melanopsin[9]. Conversely, the PIPR is a feature of the pupillary light reflex that is almost entirely driven by melanopsin activation, with only limited contribution of rod

and cone activity, and can therefore be used to quantify the functionality of the intrinsic melanopsin-signaling system[11]. Details of the pupillometry paradigm for PIPR assessment are described elsewhere[86]. In brief, using a custom-made infrared pupillometry setup, the pupil diameter of the left eye was measured while the dilated (tropicamide 0.5%) pupil of the right eye underwent a light exposure protocol. This protocol contained five consecutive 5-min blocks: baseline dark; monochromatic red light (peak wavelength (full width half maximum): 630 (20) nm, luminance: 375 cd/m$^2$) to maximize the effect of subsequent blue light[58]; dark; monochromatic blue light (peak wavelength (full width half maximum): 470 (20) nm, luminance: 375 cd/m$^2$); and post-blue dark. We calculated the PIPR by subtracting pupil diameter during post-blue dark from pupil diameter during baseline dark[49,126]. All PIPR assessments were performed between 8:30 and 17:00 to avoid the evening and nighttime modulation of the melanopsin-based phototransduction circuitry[75]. We previously showed that this outcome parameter is sensitive to individual differences and has very high within-subject test-retest reliability[86].



**Habitual sleep timing: Munich Chronotype Questionnaire**

From the MCTQ we obtained habitual lights-out time, sleep onset latency, and final wake up time, separately for work days and free days. Sleep onset time was calculated by subtracting sleep onset latency from lights out time[121]. Sleep duration was calculated as the duration between sleep onset time and final wake up time and mid-sleep as the midpoint between them.

**Recent sleep timing: Consensus Sleep Diary**

As a second measure of sleep timing, 56 participants kept a sleep diary for 1 w. From the sleep diaries we obtained lights out time, sleep onset latency, and final wake up time. For each of the 7 days, sleep onset time, mid-sleep time, and sleep duration were calculated as previously described.

**Recent sleep timing: Actigraphy**

To obtain an objective estimate of sleep timing, 56 participants wore an actigraph during the week they kept a sleep diary. Participants were equipped with either a traditional actigraph (Philips Actiwatch Spectrum, Philips Respironics, Murrysville, PA, USA) or a microelectromechanical accelerometer (Move II, Movisens GmbH, Karlsruhe, Germany). We previously showed almost perfect agreement between these two types of actigraphs in discriminating between sleep and wake[127]. Sleep onset time and final wake up time were automatically estimated using a detection algorithm[128] implemented in Matlab (Version 2014A, The Mathworks Inc., Natick, MA, USA). The algorithm searches for these two parameters between the lights out time and final wake up time as obtained from the sleep diary (https://github.com/btlindert/actant-1). Mid-sleep and sleep duration were calculated as previously described.

**Statistical analysis**

Mixed effect regression models were used to estimate how individual differences in the PIPR predicted individual differences in sleep timing. Mid-sleep was used as a the main marker for sleep timing[129]. Ancillary analyses with sleep onset time and final wake up time as outcome measures were run to determine whether mid-sleep shifts arose from changes in sleep onset time, wake up time or both. Finally, it was evaluated whether individual differences in the PIPR predicted individual differences in sleep duration.

The data from all three measurement techniques represented two-level hierarchies. For each participant, the MCTQ provided two sleep timing measures: one for work days and one for free days. The sleep diaries and actigraphy provided seven sleep timing measures: five for work days and two for free days. Type of day (work day versus free day) was included as within-subject time-varying covariate, dummy coded as 0 for work days and 1 for free days. Age and sex were initially included as possible confounding covariates[34]. Sex was dummy coded as 0 for females and 1 for males. Covariates were selected for inclusion in the final model using backward elimination and comparing models with likelihood-ratio tests. The PIPR and age variable were centered around the grand mean to optimize interpretation of the model's intercept and slope parameters[130]. Q-Q plots were made for all models to evaluate the assumption of a normal distribution of the residuals. Mixed-effect regression models were conducted using the 'lme4' package for R (Version 3.1.1, R Foundation for Statistical Computing, Vienna, Austria). The significance of the effects of the independent variables on the sleep timing variables was evaluated using t-tests with the denominator degrees of freedom based on Satterthwaite approximation.

## Results

Underscoring the value of multiple outcome measures, sleep timing derived from the MCTQ shared no more than 48.3 % of its variance with the variance in mid-sleep timing estimated from sleep diaries and 51.2 % of its variance with the variance in mid-sleep timing estimated from actigraphy. The mid-sleep timing estimates from sleep diaries and actigraphy shared 94.5 % of their interindividual variance.

Regression analyses for all three measures consistently indicated that people with more pronounced PIPR showed a later mid-sleep timing (MCTQ: P = 0.03; sleep diary: P = 0.046; actigraphy: P = 0.04) (Figure 1). The association between the magnitude of the PIPR and mid-sleep timing arose from later sleep onset (MCTQ: P = 0.01; sleep diary: P = 0.02; actigraphy: P = 0.04) rather than a delay in wake up time (MCTQ: P = 0.15; sleep diary: P = 0.30; actigraphy: P = 0.17) (Table 1). MCTQ data indicated that individuals with a more pronounced PIPR had a

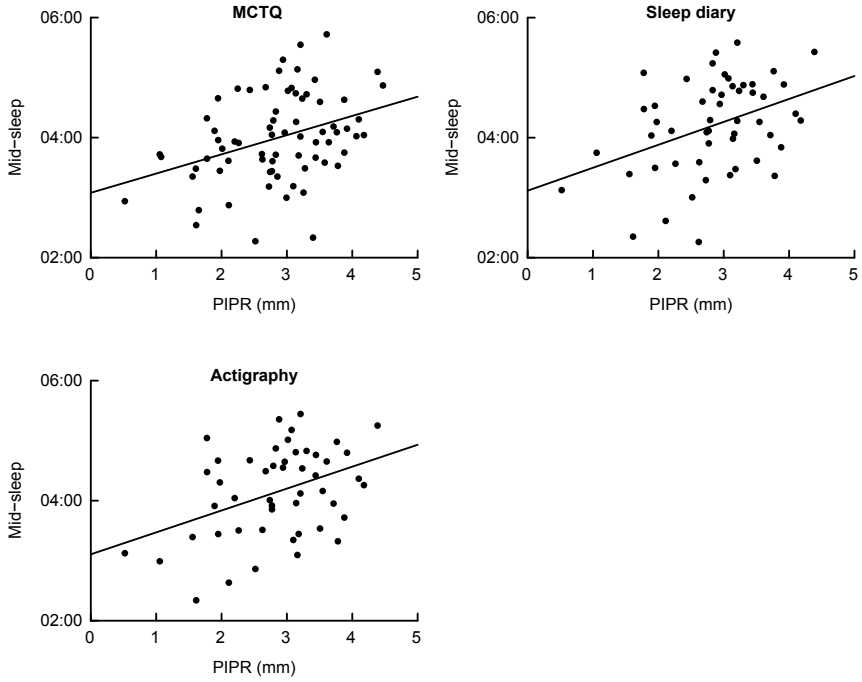


**Figure 1.** *The relationship between post-illumination pupil response (PIPR) and mid-sleep timing. Mid-sleep was obtained using the Munich Chronotype Questionnaire (MCTQ; top left panel), the Consensus Sleep Diary (sleep diary; top right panel), and actigraphy (bottom panel). Each dot represents the weekly average of mid-sleep per participant. For the MCTQ the average mid-sleep for 1 w was calculated using the following formula: [ (5 * mid-sleep during work days + 2 * mid-sleep during free days) / 7 ]. The lines indicate the association between PIPR and mid-sleep timing as obtained from MCTQ (r = 0.36), sleep diaries (r = 0.39), and actigraphy (r = 0.40).*

shorter sleep duration (P = 0.04), but this association was not confirmed using sleep duration estimates from sleep diaries (P = 0.12) or actigraphy recordings (P = 0.25).

The association of individual differences in PIPR magnitude and sleep timing was not secondary to confounding by common effects of age or sex. Sex did not affect either PIPR magnitude (P = 0.82) or any of the three mid-sleep variables (0.19 < P < 0.51). Whereas PIPR magnitude decreased with increasing age (p = 0.03) and mid-sleep timing on all three measures became earlier with increasing age ($6 \cdot 10^{-4} < P < 0.01$), these changes did not confound the association between PIPR and sleep timing: the inclusion of age as a covariate in the linear regression models did not eliminate the association between PIPR and sleep timing. Type of day explained a considerable part of the within-subject variance in mid-sleep timing (MCTQ: 78.2 %; sleep diary: 41.0 %; actigraphy: 43.1 %). Inclusion of type of day as a covariate therefore improved estimation of individual sleep timing and allowed for a more

**Table 1.** *Model estimates of the effects of PIPR, age, and type of day on sleep timing.*

| | Mid-sleep | Sleep onset time | Wake up time | Sleep duration |
|---|---|---|---|---|
| ***Intercept*** | | | | |
| **MCTQ** | 03:30 ± 00:06*** | 23:46 ± 00:07 | 07:14 ± 00:06*** | 07:28 ± 00:06*** |
| **Sleep diary** | 03:45 ± 00:06*** | 00:07 ± 00:07 | 07:22 ± 00:06*** | 07:15 ± 00:07*** |
| **Actigraphy** | 03:40 ± 00:06*** | 00:10 ± 00:08 | 07:10 ± 00:06*** | 06:59 ± 00:07*** |
| ***PIPR (mm)*** | | | | |
| **MCTQ** | 00:14 ± 00:06* | 00:20 ± 00:08* | 00:08 ± 00:06 | -00:12 ± 00:06* |
| **Sleep diary** | 00:15 ± 00:07* | 00:22 ± 00:09* | 00:09 ± 00:08 | -00:14 ± 00:09 |
| **Actigraphy** | 00:16 ± 00:07* | 00:21 ± 00:10* | 00:10 ± 00:07 | -00:10 ± 00:09 |
| ***Age (yr · $10^{-1}$)*** | | | | |
| **MCTQ** | -00:38 ± 00:11*** | -00:25 ± 00:14 | -00:50 ± 00:09*** | -00:25 ± 00:10* |
| **Sleep diary** | -00:40 ± 00:14** | -00:12 ± 00:17 | -01:07 ± 00:15*** | -00:56 ± 00:16*** |
| **Actigraphy** | -00:37 ± 00:14* | -00:08 ± 00:19 | -01:05 ± 00:14*** | -00:57 ± 00:17*** |
| ***Type of day (free vs. work)*** | | | | |
| **MCTQ** | 01:43 ± 00:06*** | 01:07 ± 00:06*** | 02:20 ± 00:08*** | 01:14 ± 00:07*** |
| **Sleep diary** | 01:35 ± 00:07*** | 01:25 ± 00:08*** | 01:46 ± 00:08*** | 00:20 ± 00:09* |
| **Actigraphy** | 01:38 ± 00:07*** | 01:21 ± 00:08*** | 01:54 ± 00:08*** | 00:33 ± 00:09*** |

Mean values ± standard error are displayed. *P < 0.05; **P < 0.01; ***P < 0.001. Each estimate was obtained from the Munich Chronotype Questionnaire (MCTQ; top row), the Consensus Sleep Diary (sleep diary; center row), and actigraphy (bottom row). The intercept reflects the population mean during work days and is referenced to midnight (00:00). PIPR, post-illumination pupil response.

reliable evaluation of the relationship between individual differences in sleep timing and interindividual variation in PIPR (see Figure S1 in supplemental material for the relationship between PIPR and mid-sleep during free days[131]). Predictably, the effect of type of day on mid-sleep timing was significant for all three measures: mid-sleep during free days was later than during work days (all: $P < 1 \cdot 10^{-15}$).

## Discussion

The aim of the current study was to assess the relationship between interindividual variation in functionality of the intrinsic melanopsin-dependent circuitry and individual differences in the timing of sleep in a healthy young population with known vulnerability to a delayed sleep phase. We found a significant association showing that individuals with a stronger blue-light responsiveness of the intrinsic melanopsin-based phototransduction circuitry slept at a later phase of the 24-h light-dark cycle.

Previous studies showed that individual differences in the habitual timing of sleep are related to the magnitude of the effect of light exposure on acute melatonin levels. One study reported that two individuals with early habitual bedtimes lacked the melatonin-suppressing effect of light[24]. In contrast, another study showed enhanced light-induced melatonin

suppression in individuals with very late habitual bedtimes due to delayed sleep phase disorder[23]. Interestingly, others showed that individuals with an increased photoinduced delay of melatonin secretion had a stronger PIPR[132]. These endocrine findings are in line with our results on behavioral circadian measures indicating that an increased PIPR strength is associated with a larger photoinduced delay of sleep timing. For future studies, it would therefore be interesting to integrate endocrine and behavioral circadian measures and relate them to the PIPR to more specifically investigate to what extent individual phase differences are related to individual differences at the very earliest stage of circadian photoentrainment (i.e. the melanopsin-expressing ipRGCs in the retina). If a robust association is demonstrated, it is tempting to imagine the feasibility of estimating an individual's circadian phase from the PIPR in combination with one's 24-h light exposure profile. This would be a major advantage, because such measures may be less costly than dim light melatonin onset assessment (i.e., one of the most reliable markers of the phase of the circadian pacemaker in the SCN)[86,133].



A limitation of our study was that our study design does not allow for a causal interpretation of the association between individual differences in sleep timing and interindividual variation in functionality of the intrinsic melanopsin-based phototransduction circuitry. However, we consider it more likely that an individual's melanopsin-based phototransduction circuitry affects habitual sleep, rather than that an individual night affects the PIPR, because we have previously shown that the PIPR magnitude is highly replicable across multiple assessments within subjects[86], whereas sleep timing is quite variable over subsequent nights[134]. An adequate estimate of habitual sleep timing requires averaging 1 w of data to overcome most of the day-to-day variance[134], as employed in the current study. A limitation of the study was that information on the participant's use of alcohol, health food supplements, or recreational drugs was incomplete. The intake of such substances may alter the pupil response[135] and sleep timing[136] and thus have increased the unexplained variance in our data. We did obtain the number of alcohol consumptions during the week in which the participants filled out the sleep diary and wore an actigraph. We found no association between alcohol intake and mid-sleep during work days ($P = 0.35$), indicating that the possible confounding effect of substance use on our results would be only small, if present. Another limitation of our study was that the PIPR assessment only allows for a quantification of the functionality of the intrinsic melanopsin-based phototransduction circuitry and not for estimating functionality of the extrinsic rod and cone pathways. For future studies, it may therefore be interesting to include functional measures on both the intrinsic as well as the extrinsic circuitry in order to get a more complete picture of the individual differences in ipRGC functionality. Another possible limitation of our study was that we did not measure light history. Differences in habitual environmental light exposure may contribute to interindividual variation in sleep timing[111]. However, whereas a different way of assessing the PIPR is sensitive to one's light history[58], the measure we here applied is more robust and does not seem to be confounded by it[86].

We have previously shown that the PIPR, assessed in the way presented here, may be a very reliable biomarker with considerable interindividual differences, yet marginal sensitivity to environmental and behavioral changes. We therefore consider it likely that these individual differences in functionality of the intrinsic melanopsin-dependent circuitry result from the natural variation in the melanopsin gene. Indeed, melanopsin-driven characteristics of pupillary light reflex have previously been associated with melanopsin polymorphisms[137]. Interestingly, other studies reported that melanopsin gene polymorphisms were also associated with the timing of sleep[25,26]. Future studies should ideally combine assessment of melanopsin polymorphisms, PIPR, dim light melatonin onset, and sleep timing in order to elucidate the mechanisms underlying their associations and to assess the value of the PIPR assessment to understand individual differences in circadian regulation.

## Acknowledgements

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## Supplemental material

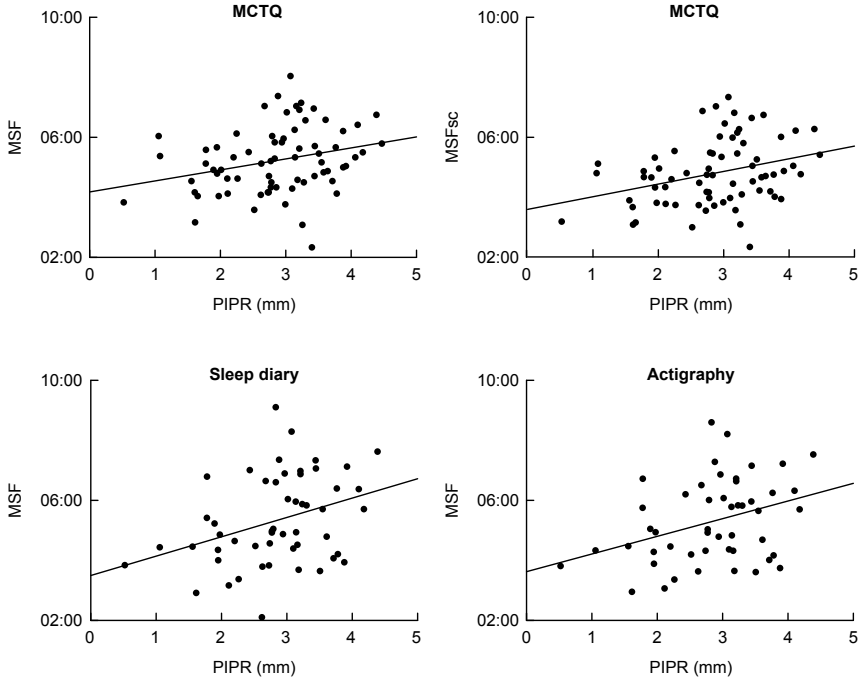


**Figure S1.** *The relationship between post-illumination pupil response (PIPR) and mid-sleep timing during free days (MSF). Each dot represents the PIPR and mid-sleep during free days for a participant. From the Munich Chronotype Questionnaire (MCTQ) we obtained one observation of mid-sleep timing during free days for each participant (top left panel). We corrected this value for mid-sleep during free days for the sleep debt accumulated during work days (MSFsc; top right panel). For the Consensus Sleep Diary (sleep diary; bottom left panel) and actigraphy (bottom right panel) the individual mid-sleep during free days was calculated by averaging mid-sleep during the 2 free days of the measurement week for each participant. The lines indicate the association between PIPR and mid-sleep timing during free days as obtained from the MCTQ (MSF: r = 0.27; MSFsc: r = 0.32), sleep diaries (r = 0.35), and actigraphy (r = 0.35).*

# CHAPTER 5

## Sustained effects of prior red light on pupil diameter and vigilance during subsequent darkness

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## Abstract

Intrinsically photosensitive retinal ganglion cells (ipRGCs) are critically involved in the effects of environmental light on physiology and behavior, including pupil size, vigilance and sleep. Previous work showed that these non-image forming effects can last long beyond discontinuation of short-wavelength light exposure. The possible functional effects after switching off long-wavelength light, however, have been insufficiently characterized. Previous patch clamp studies on mouse retinas showing prompt silencing of intrinsic firing of ipRGCs after discontinuation of long-wavelength light exposure led us to hypothesize downstream functional consequences, expressed by increased pupil diameter and reduced vigilance. In a series of controlled experiments in healthy adult volunteers, we showed that 5 minutes of intense red light emitted entailed, during subsequent darkness: (1) sustained pupil dilation, (2) slower response speed in a vigilance task in spite of electrocardiographic indices of increased effort, and (3) increased electroencephalography-derived sleep propensity. The consistent functional effects are compatible with sustained post-illumination suppression of ipRGC firing rate. Our findings suggest the possibility of using red light as a nightcap.

## Introduction

Environmental light drives many aspects of human physiology and behavior, including pupil diameter and both the rhythmic and acute expression of vigilance and sleep. These non-image forming effects of light critically involve intrinsically photosensitive retinal ganglion cells (ipRGCs) that directly transduce information on environmental light intensity from the retina to downstream non-image forming brain areas[2,4]. Unlike regular retinal ganglion cells, ipRGCs not only relay extrinsic input from the classical rod and cone phototransduction pathways, but are also light sensitive themselves through their expression of melanopsin. This photopigment, maximally sensitive to short-wavelength (blue) light, enables ipRGCs to intrinsically encode environmental light intensity by increasing their firing rate[1]. In the absence of light, ipRGCs can spontaneously fire at a low rate[1,53,138,139]. During light exposure, their firing frequency increases considerably. Interestingly, ipRGCs can maintain the photoinduced increase in firing rate even beyond light exposure[1], resulting in a persistent increased input to downstream regulatory circuitries, with functional consequences[10,53]. For example, sustained post-illumination activation of the olivary pretectal nucleus (OPN) by ipRGCs leads to the sustained pupillary constriction that is known as the post-illumination pupil response (PIPR). The PIPR particularly emerges following short-wavelength light around melanopsin peak sensitivity[11].



Intriguingly, patch clamp experiments on in vitro mouse retinas with blocked synaptic transmission showed that the sustained ipRGC photoresponse induced after short-wavelength light is acutely suppressed after subsequent exposure to long-wavelength (orange) light, which was explained by melanopsin acting in a tristable equilibrium of one signaling and two silent states[30]. Biochemical experiments showed that short-wavelength light exposure shifts the balance from the silent melanopsin ground state (containing 11-*cis* retinal) towards the signaling metamelanopsin state (all-*trans* retinal). Conversely, upon exposure to long-wavelength light this balance is dominated by extramelanopsin (7-*cis* retinal)[140], which is considered as a specific silent state and is therefore likely be involved the extinction of ipRGC signaling following long-wavelength light[30,141].

In contrast to the well-recognized post-illumination effects of short-wavelength light, possible intrinsic sustained effects after exposure to long-wavelength light have remained insufficiently explored, neither with respect to the intrinsic firing rate of ipRGCs, nor with respect to the resulting functional consequences for downstream regulatory circuitries[27,29]. Previous studies on the PIPR after blue light presumed no post-illumination effects of red light and considered it an inert control condition[46,51,142]. However, a closer examination of the results of some studies on PIPR suggests that the pupil diameter could increase even *beyond baseline* after cessation of a red light stimulus[143,144]. These observations open up the intriguing possibility of a reduction in spontaneous firing of ipRGCs during darkness following long-wavelength

illumination relative to darkness at baseline, with consequently an attenuated input to the downstream OPN. A first aim of our studies was to test this hypothesis by systematically quantifying the pupil diameter during darkness following exposure to red light.

Given that ipRGCs also project to several key brain areas for vigilance regulation[17], such a hypothesized attenuated ipRGC output during darkness following long-wavelength light could not only result in pupil dilation but also in a vigilance reduction. A specific vigilance-related brain structure that is more responsive to long-wavelength (green) light than blue light is the ventrolateral preoptic area (VLPO)[145], which plays a pivotal role in the transitions between wakefulness and sleep[146]. Indeed, as compared to exposure to darkness, two hours of evening exposure to green light - with a wavelength that is considered to generate rather silent extramelanopsin rather than signaling metamelanopsin[30] - appeared to accelerate the decrease of core body temperature that is known to be conductive to sleep[13]. Moreover, slow wave activity seemed to be higher during the first two hours of sleep and to decline faster during the first half of the night, which could be interpreted as sleep with a highly efficient dissipation of sleep pressure[147]. A second aim of our studies was therefore to systematically evaluate the effect of exposure to red light on sleep propensity during subsequent darkness. We hypothesized that ipRGC-silencing following exposure to light of a longer wavelength could facilitate sleep[28].

Given these aims, it is important to consider the light intensity range required to expect post-illumination changes after long-wavelength light. In view of the low sensitivity of melanopsin to long-wavelength light and the slow kinetics of the intrinsic ipRGC response[1], the intensity and duration of the long-wavelength stimuli used in previous studies may have been insufficient to induce substantial post-illumination effects. We therefore here assess the acute functional consequences following prolonged exposure to light of an intensity that can be expected to generate maximum ipRGC-driven functional responses[148]. We here report multiple experiments in healthy young adults, to evaluate three different downstream functional indicators of the hypothesized ipRGC resting-state firing suppression after discontinuation of long-wavelength light[30]: pupil diameter, psychomotor vigilance, and sleep propensity. We expected a larger pupil diameter, reduced vigilance, and increased sleep propensity.

## Methods

### Participants

In total 40 young adults participated (experiment 1: 6 males, 6 females, mean age ± SD: 24.7 ± 2.1 yr; experiment 2: 6 males, 6 females, mean age ± SD = 26.5 ± 3.6 yr; experiment 3: 6 males, 10 females, mean age ± SD = 24.4 ± 4.2 yr). All participants were in good health,

free of medication, and had neither sleep complaints nor a history of ocular pathology. The protocol was approved by the Medical Ethical Committee of the VU University Medical Center Amsterdam (protocol NL43319.029.13) and all participants gave their written informed consent.

**Experiment 1**

The participants underwent two daytime light exposure paradigms in a randomized order, separated by 3 days, each consisting of three consecutive 5-min blocks: 1) darkness, red light (peak wavelength (full width half maximum): 635 (20) nm), darkness; and 2) darkness, blue light (465 (20) nm), darkness (Figure 1A). During the dark blocks, we assessed pupil diameter, which is the most recognized readout to estimate ipRGC functioning[149], for 3 minutes (i.e., minutes 2-4). Simultaneously with the pupil diameter assessments, the participants performed an auditory version of the psychomotor vigilance task (PVT), which is the most commonly used validated reaction time task to assess sustained attention[150]. In order to assess the functional consequences following long-wavelength light, we estimated the changes in pupil diameter and PVT performance in complete darkness from before to after the red and the blue light exposure.



**Experiment 2**

In preparation of experiment 3, that aimed to evaluate post-illumination effects of long wavelength light using a multiple sleep latency procedure designed to evaluate sleep propensity across the day, we first systematically evaluated whether the effects found in experiment 1 were consistent across the day and across supine and upright postures. Moreover, experiment 2 addressed whether a possible reduction in response speed after long wavelength light involved slower processing or less effort, by simultaneous electrocardiographic assessment of the R-R interval[151]. The participants underwent 4 light sequences, each randomly selected from 3 different exposure paradigms consisting of 7 consecutive 5-minute blocks (Figure 2A). During one block, the participants were exposed to red light and during a later block to blue light. Five minutes of prior bright light exposure persistently desensitizes the subsequent photoresponse of the intrinsic ipRGC phototransduction circuitry[55]. Given that melanopsin already possesses low sensitivity to red light, while it is maximally sensitive to blue light[1], the red light block was always earlier in the sequence than the blue light block in order to maintain sufficient melanopsin sensitivity to both wavelengths for eliciting functional responses. All other blocks were kept dark. To allow for mixed effect model-disentangling of wavelength effects from time-on-task effects[152], the timing of the light exposure blocks was systematically shifted across the three sequences (Figure S2).

**Experiment 3**

The third experiment assessed whether the wavelength of prior light exposure affected sleep propensity during subsequent darkness, as indicated by the ease of transitioning from

wake to sleep. Prior to multiple sleep opportunities in an extended all-day adaptation of the multiple sleep latency test[153], the participants were exposed to 5 minutes of either darkness, red (peak wavelength (full width half maximum): 630 (20) nm), blue (460 (20) nm) or green (520 (30) nm) light. The latter mid-wavelength was added in experiment 3 in order to obtain a more complete picture of the wavelength dependency of the post-illumination effects. The participants received each exposure twice, randomly distributed over 8 blocks across 1 day. Each block commenced with 40 minutes of dim light, followed by 5 minutes of darkness and 5 minutes of exposure, after which they tried to fall asleep as fast as possible within 30 minutes of complete darkness.

**Light exposure**

In the first and second experiment, we illuminated the left eye with a dilated pupil (tropicamide 0.5%) in free view using red and blue light-emitting diodes (red: C503B-RAS, Cree, Durham, NC, USA; blue: C503B-BAN, Cree, Durham, NC, USA) that were integrated a light panel (16 x 10 cm) at 5 cm from the illuminated eye. In the third experiment we applied the light exposure to both eyes with a natural pupil using a light panel (53 x 31 cm) containing RGB light-emitting diodes (WS2812B, Luxalight B.V., Eindhoven, the Netherlands), that was placed in front of the participant at 57 cm from the eyes (Table S1).

**Pupil diameter**

Pupil diameter was continuously measured during the entire light exposure paradigm using a custom-made infrared pupillometry set-up that was validated and previously described in more detail[86]. Each light exposure paradigm was preceded by 30 minutes of dim light. All analyses used the pupil diameter averaged over the middle three minutes for each of the 5-minute blocks of darkness.

**Vigilance and effort**

Vigilance was assessed using a 3-minute auditory PVT[154,155] implemented on an Arduino Mega microcontroller (https://www.arduino.cc/). The PVT commenced at the onset of the second minute of each 5-minute non-illumination block, overlapping with the interval of pupil diameter averaging. The participants had to press a response button as fast as possible when a continuous 880-Hz tone was played. When the button was pressed, the tone stopped and the response latency was recorded by the microcontroller. The inter-stimulus interval quasi-randomly varied between 1 and 10 seconds. Averages were calculated over the inverse of the response latency to obtain a normally distributed measure of response speed without compromising statistical power[156].

In experiment 2, electrocardiography (Shield-ECG/EMG, Olimex, Plovdiv, Bulgaria) was co-recorded during PVT performance in order to obtain an optimal objective estimate of mental effort from the R-R interval averaged over the three minutes[151]. The R-R interval was quantified

as the period between the peaks of two consecutive R waves in the electrocardiogram, which were automatically detected using the Pan-Tomkins algorithm[157] implemented in Matlab (Version 2014a, The Mathworks Inc., Natick, MA, USA).

**Sleep propensity**

Using electroencephalography (Geodesic EEG system 300, Electrical Geodesics, Inc, Eugene, USA) and standardized sleep scoring procedures[158], sleep onset was defined as three consecutive 30-s epochs of light sleep (i.e., stage 1) or one 30-s epoch of deeper sleep (i.e., stage 2 or slow wave sleep)[153]. Sleep onset latencies have the same skewed distribution as response latencies in the PVT, which we verified to likewise be best reciprocally transformed, resulting in a normally distributed measure of sleep propensity.



**Statistical analysis**

Data were analyzed with mixed-effect regression models (Supplemental experimental procedures) using the 'lme4' package for R (Version 3.2.3, R Foundation for Statistical Computing, Vienna, Austria).

## Results

**Pupil dilation and reduced vigilance during darkness after red light**

In experiment 1 we showed that the average pupil diameter was larger during darkness after prior red light (6.24 ± 0.28 mm, P = 0.02; Figure 1C; Table S2) than the diameter during darkness preceding the light exposure (mean ± SEM: 5.67 ± 0.22 mm; Figure 1B). In agreement with previous work[11], the average pupil diameter was smaller after blue light exposure (3.52 ± 0.24 mm, P < 0.001; Figure 1D). There was a non-significant trend of slowing of PVT response speed from before (4.12 ± 0.12 $s^{-1}$) to after red light exposure (4.05 ± 0.16 $s^{-1}$, P = 0.10), but there was no change after blue light (4.18 ± 0.13 $s^{-1}$, P = 0.17; Figure S1).

The results from experiment 2 replicated the functional changes after long-wavelength light seen in experiment 1, with even more pronounced effect, possibly because of a lower contribution of between-day variance in experiment 2 than in experiment 1. Compared to baseline darkness (pupil diameter mean ± SEM: 4.66 ± 0.10 mm; response speed adjusted for time-on-task: 4.11 ± 0.07 $s^{-1}$), we again showed pupil dilation (5.34 ± 0.12 mm, P < 0.001; Figure 2B) and slower response speed (4.01 ± 0.12 $s^{-1}$, P = 0.004; Figure 2C) in darkness following red light (Table S3). Interestingly, response speed slowed in spite of increased mental effort as indicated by a shorter R-R interval (918 ± 20 vs. 927 ± 11 ms, P = 0.04; Figure 2D). In darkness after blue light we found the well-documented sustained pupil constriction (2.95 ± 0.08 mm, P < 0.001), but no change in response speed (4.11 ± 0.12 $s^{-1}$, P = 0.96) and

mental effort (927 ± 17 ms, P = 0.93). All post-illumination effects were consistent across the day and across upright and supine posture (all P > 0.12), thus meeting the conditions for a subsequent multiple sleep latency study.

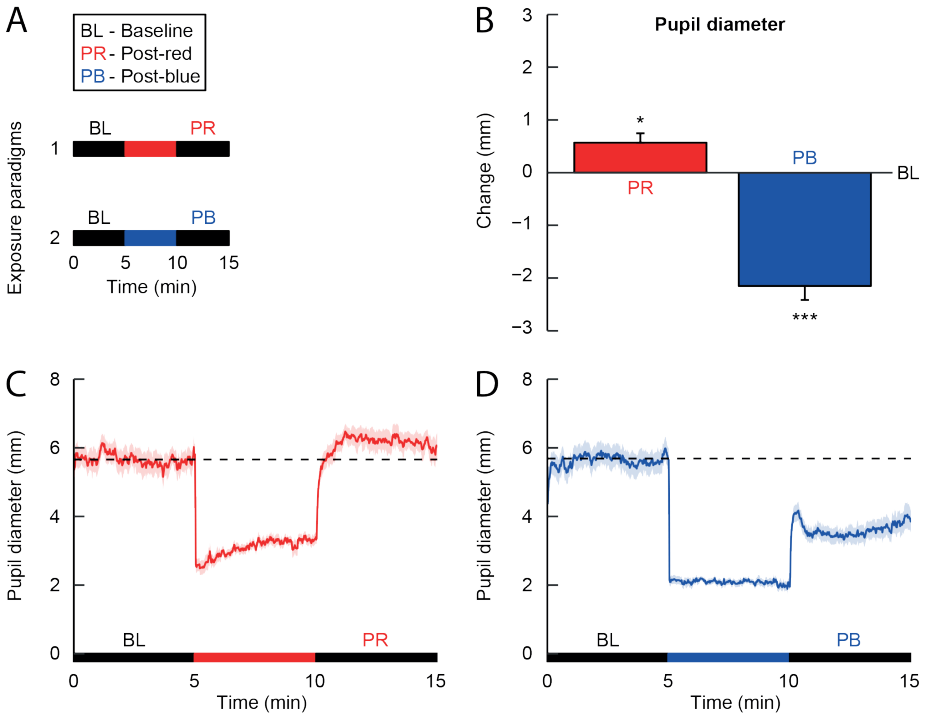


**Figure 1. Pupil diameter prior to, during, and after red and blue light (experiment 1).** *(A) The participants underwent two exposure paradigms on separate days, each consisting of 3 consecutive 5-minute blocks, as indicated by the bars: baseline darkness (BL); red or blue illumination; post-red (PR) or post-blue (PB) darkness. (B) The mean difference in pupil diameter of the middle 3 minutes of the post-illumination versus BL-blocks. The red bar on the left indicates the difference induced by red light exposure and the blue bar on the right the difference induced by blue light exposure. Error bars represent the within-subject standard error. Asterisks indicate within-subject differences in post-illumination changes induced after red versus BL and after blue versus BL (*P < 0.05, ***P < 0.001). (C-D) The pupil diameter trace averaged over the 12 participants for the red light (C) and blue light (D) exposure paradigm. Semi-transparent areas represent a range of ± one standard error. The dashed lines indicate the average pupil diameter during the BL-block prior to red or blue light exposure. Note that in comparison with the baseline dark block, there is not only the well-known smaller pupil diameter in the PB-block, but also a larger diameter in the PR-block.*

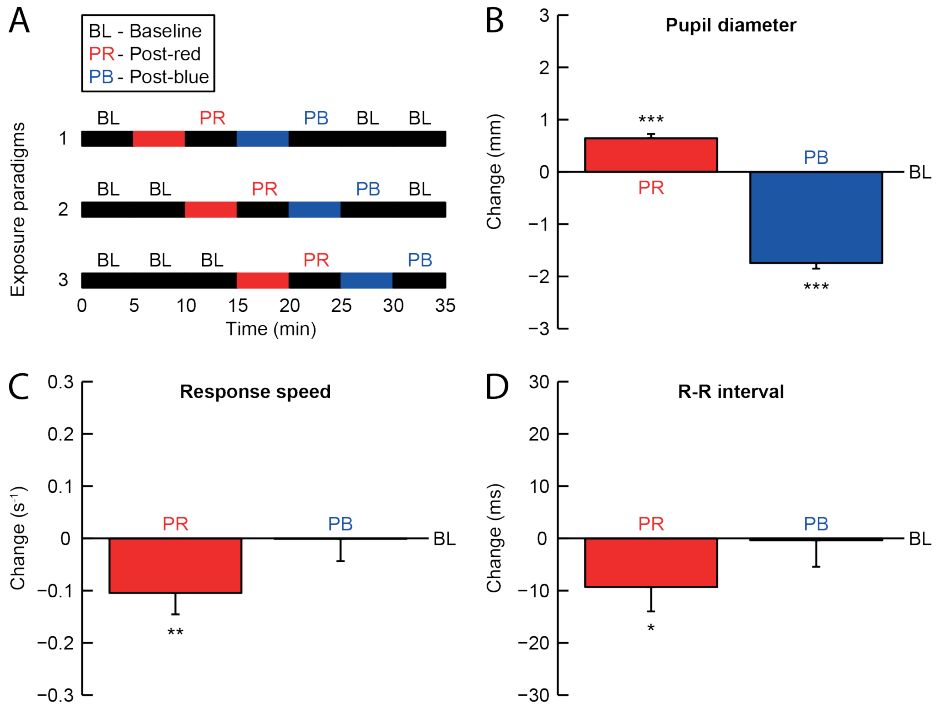


**Figure 2. Post-illumination changes in pupil diameter, vigilance, and mental effort measured during darkness after exposure to red and blue light (experiment 2).** *(A) To allow for mixed effect model-disentangling of wavelength effects from time-on-task effects, the timing of the light exposure blocks was systematically shifted across three paradigms, which were randomly distributed over the sample. (B-D) Vertical bar graphs show the post-red (PR) and post-blue (PB) changes, relative to the BL-blocks, for pupil diameter (B), response speed adjusted for time-on-task (C), and R-R interval, which is inversely associated with mental effort (D). Because the sustained pupil constriction after blue light remained even in the third consecutive BL-block, pupil data assessed during the BL-blocks after the PB-block were omitted from this figure. Error bars represent the within-subject standard error. Asterisks indicate within-subject differences between the post-illumination blocks and the baseline blocks (*P < 0.05, **P < 0.01, ***P < 0.001).*

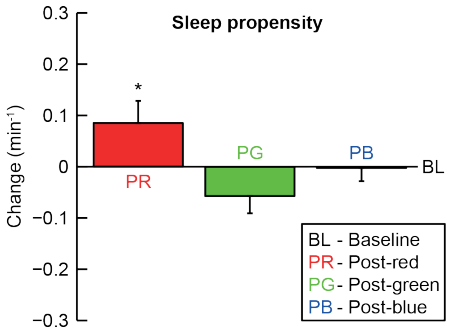


**Figure 3. Post-illumination changes in sleep propensity (sleep latency$^{-1}$) measured during darkness after exposure to red, green and blue light (experiment 3).** *The bars represent sleep propensity after red (left, PR), green (middle, PG) and blue (right, PB) light exposure relative to baseline sleep propensity (i.e. after prior exposure to darkness). The error bars represent the within-subject standard error. Asterisks indicate within-subject differences between the post-light trials and the post-dark trials (*P < 0.05).*

**Red light exposure increases subsequent sleep propensity**

The results from experiment 3 showed a stronger sleep propensity (i.e., the inverse of sleep latency) after 5-min red light (mean ± SEM: 0.41 ± 0.06 min$^{-1}$) than following 5-min darkness (0.33 ± 0.04 min$^{-1}$, P = 0.04; Figure 3; Table S4). Whereas long-wavelength light facilitated the subsequent transition to sleep in darkness, no significant effect was seen after blue (0.32 ± 0.03 min$^{-1}$, P = 0.95) or green light (0.27 ± 0.03 min$^{-1}$, P = 0.14).

## Discussion

Electrophysiological studies in mice showed that the persistently increased ipRGC firing rate, elicited by prior exposure to short-wavelength light, was immediately suppressed after long-wavelength light[30]. This silencing persisted after pharmacological blockage of input from rods and cones while preserving the intrinsic phototransduction circuitry, suggesting involvement of the intrinsic photosensitivity of melanopsin-expressing ipRGCs. The silencing property of long-wavelength light was demonstrated as well in non-retinal cells with heterologous melanopsin expression[31]. Cells started to fire when short-wavelength photons activated the melanopsin protein, which persisted after discontinuation of light exposure and could immediately be silenced by exposure to green-yellow light. It has been proposed that the long-wavelength light-induced silencing could involve changes in the equilibrium between three stable melanopsin isoforms[30,141]. Accordingly, a biochemical study showed that short-wavelength light exposure shifts the melanopsin balance from the ground state (containing 11-*cis* retinal) towards metamelanopsin (all-*trans* retinal), while a third distinct

melanopsin state (i.e., extramelanopsin (7-*cis* retinal)) was identified upon specific exposure to long-wavelength light[140]. It would therefore be interesting for future studies to investigate whether the post-red effects found in the present study may involve an accumulation of extramelanopsin.

In addition, the post-illumination effects of red light we found in our experiments may also involve post-illumination changes in the input that ipRGCs receive through the classical phototransduction pathways[159]. Acute bright red light hyperpolarizes long-wavelength cones and increases ipRGC firing rate[10], but cones adapt and start to slowly depolarize with sustained stimulation[160]. This light adapted state remains beyond light offset and may result in a post-illumination depolarization beyond the initial dark resting membrane potential that affects retinal output with a time constant comparable to the post-red pupil response we here observe[161]. An estimate of the relative contribution of the melanopsin-driven and classical phototransduction pathways cannot be made based on our experiments and needs to be determined in future studies.



However, the ipRGCs that connect to the OPN that controls the pupil diameter are characterized by a relatively high level of melanopsin in combination with only modest rod-cone input[162], suggesting that the PIPR is probably mainly melanopsin-driven. The post-red effects on vigilance and sleep propensity on the other hand do not necessarily involve the same population of ipRGCs that connect to the OPN. These ipRGCs may depend differently on melanopsin and classical photoreceptor inputs[163]. Such a differential functional response is in line with a recent study in nocturnal rodents, which showed a VLPO-dependent sleep induction in response to green but not to blue light, indicating that the different ipRGC-mediated neuronal pathways can have different spectral sensitivities[145]. Moreover, given that direct ipRGC projections to the VLPO are only sparse[6], the wavelength-dependent post-illumination effects on sleep propensity may also involve additional circuitries, including indirect pathways connecting retinal output to the VLPO[146].

In conclusion, the present study identified lasting effects of five minutes of exposure to long-wavelength light: sustained pupil dilation, slower response speed, and increased sleep propensity. The cross-modal consistency of these functional consequences may indicate common downstream effects originating in a suppressed ipRGC resting firing rate following long-wavelength illumination. Our findings advocate reevaluation of control conditions in research designs of studies on the effect of light. Several light therapy trials used red light as a placebo treatment. Although the intensity of these red light exposures was generally lower than in the present study, our findings suggest that these conditions may not necessarily be inert. Indeed, in our own previous work, daily red light may have increased subjective sleepiness[164]. An intriguing possibility suggested by our findings is that long-wavelength light prior to bedtime might have applied practical value to facilitate sleep onset. Future

studies should however first address whether the post-red effects on sleep propensity during daytime, as here demonstrated, can also be found prior to habitual bedtime. If so, long-wavelength light may serve as a nightcap and possibly be of use in the treatment of sleep-onset insomnia[165]. Given that the effect of prior light can endure beyond sleep onset[13], it seems moreover valuable to evaluate the effect of long-wavelength light prior to bedtime on subsequent sleep quality.

## Acknowledgements

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## Supplemental material

### Participants

Health, medication use, and the prevalence of sleep complaints and ophthalmological pathology were assessed using the Duke Structured Interview for Sleep Disorders[60]. According to Nagel anomaloscope tests none of the participants suffered from color vision deficiency. The Munich Chronotype Questionnaire (MCTQ) showed that none of the participants was an extreme chronotype (mean mid-sleep on free days ± SD: 05:18 PM ± 00:53)[61]. All participants worked regular office hours and did not travel across time zones for at least a month prior to participation. The participants were instructed to refrain from caffeine and alcohol intake from 04:00 PM on the day preceding each assessment. Daily sleep diaries[122] and actigraphy (Geneactiv, ActivInsights Ltd., Kimbolton, UK) were assessed to verify that participants kept to the sleep timing instructions[127].



### Experiment 1

The participants visited the lab twice, separated by 3 days. They were instructed to maintain regular and sufficient sleep from 3 days prior to each visit. Each visit commenced with 30 minutes of dim white light (< 3 lux) to standardize prior light exposure and increase ipRGC-driven responses to subsequent light[55], followed by the light exposure paradigm. The participants were exposed to each illumination once in a randomized order. Assessments were performed between 08:30 AM and 5:00 PM, because the post-illumination pupil response[75] and vigilance levels[166] may be different during the evening and night. The timing of both lab visits was kept constant within each participant.

### Experiment 2

The participants visited the lab twice, separated by 3 days, once at 9 AM and once at 1 PM. They received the instruction to maintain regular and sufficient sleep from 3 days prior to each lab visit. During each visit, the participants underwent 1 light exposure paradigm in upright and 1 in supine position. Time of day and posture were counterbalanced over the two visits and posture was moreover counterbalanced within each participant. Prior to each light exposure paradigm the participants were first put into the appropriate position, after which they remained in dim light (< 3 lux) for 30 minutes. The first light exposure paradigm of each visit was followed by 20 minutes of free movement in a room light environment (200 lux).

### Experiment 3

In a repeated measures design, sleep propensity can best be estimated from sleep onset latencies[153]. Participants were instructed to restrict their sleep during the night preceding the sleep propensity assessment: compared to their habitual sleep times, bedtime was 1 hour later and wake up time was earlier (i.e., at 06:00 AM). Sleep propensity was assessed using an adapted version of the multiple sleep latency test with 8 attempts to reach sleep onset across

1 day. These attempts were distributed over eight consecutive 81-minute blocks with the first block commencing at 09:00 AM.

Each block started with a standardized 10-minute break in dim light, which included a mandatory bathroom visit and consumption of an isocaloric snack (150 kcal), in order to avoid of effects of postural changes[81] and food intake[167] on sleep propensity. After this break, the participants were put in bed in a semi-supine position for 30 minutes, while allowed to read a book of their choice. The book was illuminated by a reading light with only limited light reaching the eyes. Subsequently, the reading light was switched off and the participants were in darkness for 5 minutes before the 5-minute exposure period (i.e., red, blue, green, or no light) started. Eye tracking (The Eye Tribe Tracker, The Eye Tribe, Copenhagen, Denmark) was performed to verify that the participants kept their eyes open and aimed their gaze at the light panel. Next, a 1-minute translation period in darkness commenced during which the bed was changed into a supine position and the participants adopted their habitual sleeping posture. After this translation lights remained off and the participants tried to fall asleep as fast as possible within an interval of 30 minutes. Upon online sleep onset detection, the participants were awoken, the dimmed room light was switched on, and the participants were asked to remain awake until the 30-minute interval was finished.

For further analysis, sleep stages were again scored offline in 30-s epochs by two experienced sleep technicians blind to the manipulations, with consensus reached in case of scoring differences. Given that standard sleep scoring procedures require at least half of the 30-s epoch to be spent in sleep for actually labelling this epoch as sleep[158], we estimated that sleep onset occurred at the midpoint of the first half of the sleep onset epoch. Where participants failed to reach sleep onset within 30 minutes, sleep onset latency was scored as 30 minutes[168]. We aimed to acquire a data set with 16 participants in a counterbalanced experimental design with each illumination condition applied twice in a fully randomized order. In total, we recruited 18 participants, of which 2 participants were excluded from the analysis: one participant did not comply with the instructed sleep restriction and offline sleep scoring revealed that another participant did not reach sleep onset in any of the blocks.

**Statistical analysis**

Mixed effect regression models were run to account for the hierarchical structure of the data from all three experiments. The data from experiment 1 were structured in a three-level hierarchy: 2 dark blocks were nested within each of 2 trials which in turn nested within each of 12 participants. Outcome parameters were pupil diameter and response speed. Post-red and post-blue covariates were included as dichotomous variables, which flagged the dark block following either red or blue light exposure (1 for the post-red or post-blue block and 0 for the baseline dark block).

The data from experiment 2 represented a four-level hierarchy: 12 participants came to the lab twice on 2 separate days and performed two trials per day (i.e., 1 trial in sitting position and 1 trial in lying position) with each trial consisting of 7 blocks (i.e., 5 dark blocks and 2 illumination blocks). Since we aimed to assess post-illumination changes in ipRGC performance during darkness, we included only the dark blocks in the analysis. The ipRGC-mediated readouts pupil diameter, response speed, and R-R interval were the outcome parameters. The post-red covariate was included as a dichotomous variable, which flagged the dark block immediately following red-light exposure (1 for the post-red block and 0 for the other dark blocks). The post-blue covariate was added to mark the dark blocks subsequent to the blue light. Since pupil diameter returned linearly to baseline within the three consecutive dark blocks after blue-light exposure, post-blue was included as a weighted variable in the model for pupil diameter (1 for the block immediately following blue light exposure, ⅔ for the second consecutive post-blue dark block, and ⅓ for the third consecutive post-blue dark block, and 0 for the dark blocks preceding the blue-light exposure). In the models for response speed and R-R interval the post-blue covariate was dummy coded with 1 for the dark block immediately following the blue-light exposure and 0 for the other dark blocks. We moreover added covariates for time of day (1 for afternoon and 0 for morning) and posture (1 for supine and 0 for upright). In the model for response speed we added block, ranging from 1 to 7, as a time-varying covariate in order to take the time-on-task effect on response speed into account. We compared between models with the inclusion of block as a first order polynomial, a second order polynomial or a square-root variable[152]. Likelihood ratio tests and visual inspection revealed that the time-on-task effect was best captured by the square root of the block covariate, which was therefore selected for inclusion in the model for response speed. Where a post-illumination effect was observed, we introduced the interaction between time of day and posture and the corresponding post-illumination variable in order to assess possible differences in post-illumination changes across the day and across body position. However, these interaction terms did not reach significance (all $P > 0.12$) and were therefore omitted from the models.

The data from experiment 3 were structured in a two-level hierarchy: the outcome parameter sleep propensity was estimated during 8 consecutive blocks, which were nested in 16 participants. Post-red, post-green, and post-blue were included as dummy variables (1 for the block containing either red, green or post light exposure and 0 for the block containing dark exposure). In line with the mixed model analysis of the time-on-task effect in experiment 2, we added block, ranging from 1 to 8, as a time-varying covariate in order to account for the time-of-day modulation of daytime sleep propensity[169]. We likewise compared between models with the inclusion of block as a first order polynomial, a second order polynomial or a square-root variable. Likelihood ratio tests and visual inspection revealed that the time-of-day effect was best captured by the second order polynomial of the block covariate, which was therefore selected for inclusion in the model for sleep propensity.

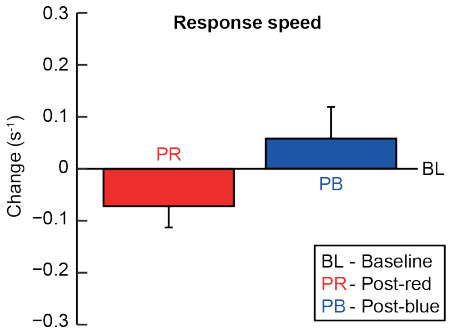


**Figure S1. Post-illumination changes in response speed after red and blue light.** *The bars indicate the mean difference in pupil diameter of the post-illumination block minus the baseline block (BL). The red bar indicates the difference from baseline following red light (PR) blue light (PB) exposure. The error bars represent the within-subject standard error.*

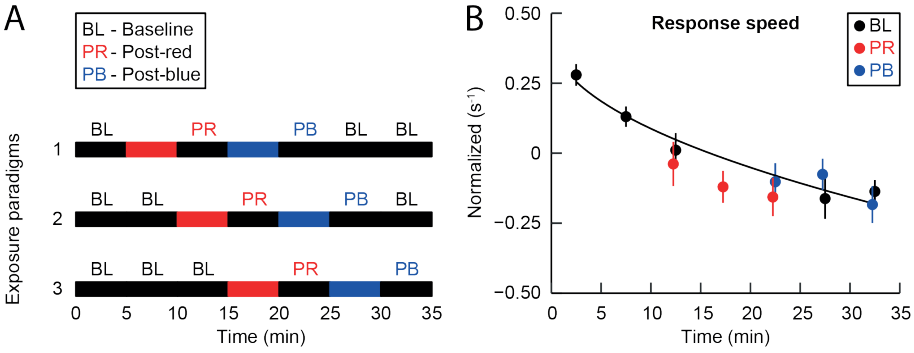


**Figure S2. The time-on-task effect on response speed.** *(A) To allow for mixed effect model-disentangling of wavelength effects from time-on-task effects, the timing of the light exposure blocks was systematically shifted across three paradigms, which were randomly distributed over the population. (B) The dots indicate the average response speed in each dark block for each type of block (black: baseline; red: post-red; blue: post-blue). The line represents the time-on-task effect and is quantified as the optimal square-root fit through the baseline values (QR decomposition: slope = -0.26 · √Block). Note that the lower response speed after red light exposure is not merely explained as a time-on-task effect. Whereas the post-blue response speed is around the estimated baseline curve, the post-red response speed is systematically below this curve. The data were normalized to the average over all dark blocks within each participant. The error bars indicate the within-subject standard error.*

**Table S1.** *Illumination characteristics.*

| | *Experiment 1 and 2* | | *Experiment 3* | | |
|---|---|---|---|---|---|
| | **Red light** | **Blue light** | **Red light** | **Green light** | **Blue light** |
| **Peak wavelength [FWHM] (nm)** | 635 [20] | 465 [20] | 630 [20] | 520 [30] | 460 [20] |
| **$Log_{10}$ photon flux ($1/cm^2/s$)** | 14.8 | 14.6 | 15.6 | 15.5 | 15.8 |
| **Irradiance ($\mu W/cm^2$)** | 178 | 159 | 1286 | 1108 | 2501 |
| **Photopic illuminance (photopic lux)** | 327 | 121 | 2514 | 5350 | 1574 |
| **S cone illuminance (cyanopic lux)** | 12 | 1132 | 11 | 297 | 20855 |
| **Melanopsin illuminance (melanopic lux)** | 8 | 1061 | 14 | 5575 | 15533 |
| **Rod illuminance (rhodopic lux)** | 13 | 752 | 62 | 6666 | 10859 |
| **M cone illuminance (chloropic lux)** | 115 | 378 | 892 | 5998 | 5260 |
| **L cone illuminance (erythropic lux)** | 420 | 199 | 3207 | 4441 | 2737 |

The characteristics were estimated from corneal spectral power distributions (AvaSpec-3648-USB2 spectrometer, Avantes, Apeldoorn, the Netherlands) and described according to standard procedures[99]. FWHM, full width at half maximum; S cone, short-wavelength cone; M cone, mid-wavelength cone; L cone, long-wavelength cone.

**Table S2.** *Mixed model estimates of the post-illumination effects on pupil diameter and response speed (experiment 1).*

| | **Pupil diameter (mm)** | **Response speed ($s^{-1}$)** |
|---|---|---|
| **Intercept** | 5.67 ± 0.25 | 4.12 ± 0.15 |
| **Post-red** | 0.57 ± 0.23* | -0.09 ± 0.05 |
| **Post-blue** | -2.15 ± 0.3*** | 0.07 ± 0.05 |

The table shows coefficients and the standard error of their estimate. *P < 0.05, ***P < 0.001.

**Table S3.** *Mixed model estimates of the post-illumination effects on pupil diameter and response speed, R-R interval (experiment 2).*

| | **Pupil diameter (mm)** | **Response speed ($s^{-1}$)** | **R-R interval (ms)** |
|---|---|---|---|
| **Intercept** | 4.75 ± 0.21 | 4.62 ± 0.23 | 877.3 ± 29.9 |
| **Post-red** | 0.65 ± 0.08*** | -0.10 ± 0.04** | -9.3 ± 4.4* |
| **Post-blue** | -1.72 ± 0.08*** | -0.002 ± 0.04 | -0.4 ± 4.4 |
| **Time of day (afternoon vs. morning)** | -0.003 ± 0.13 | -0.04 ± 0.06 | -23.1 ± 24.0 |
| **Posture (supine vs. upright)** | -0.13 ± 0.08 | 0.05 ± 0.05 | 102.1 ± 14.2*** |
| **√Block** | | -0.26 ± 0.03*** | |

The table shows coefficients and the standard error of their estimate. *P < 0.05, **P < 0.01, ***P < 0.001.

**Table S4.** *Mixed model estimates of the post-illumination effects on sleep propensity (experiment 3).*

| | **Sleep propensity ($min^{-1} \cdot 10^{-1}$)** |
|---|---|
| **Intercept** | 1.91 ± 0.73 |
| **Post-red** | 0.85 ± 0.41* |
| **Post-green** | -0.62 ± 0.42 |
| **Post-blue** | -0.03 ± 0.40 |
| **Block** | 0.91 ± 0.29** |
| **Block²** | -0.11 ± 0.03*** |

The table shows coefficients and the standard error of their estimate. *P < 0.05, **P < 0.01, ***P < 0.001.

# CHAPTER 6

## Time- and state-dependent analysis of autonomic control in narcolepsy: higher heart rate with normal heart rate variability independent of sleep fragmentation

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## Abstract

Narcolepsy with hypocretin deficiency is known to alter cardiovascular control during sleep, but its etiology is disputed. As cardiovascular control differs between sleep states, and narcolepsy affects sleep architecture, controlling for both duration and transitions of sleep states is necessary. We therefore aimed to assess heart rate and its variability in narcolepsy during sleep taking these factors into account. We included 12 medication-naïve patients with narcolepsy with cataplexy and hypocretin deficiency (11 males, 16-53 years) and 12 sex- and age-matched healthy controls (11 males, 19-55 years). All subjects underwent one-night ambulatory polysomnography recording. Cardiovascular parameters were calculated for each 30-s epoch. Heart rate was significantly higher in narcolepsy patients than in controls in all sleep states and during wakefulness prior to sleep. Groups did not differ in heart rate variability measures. The effects of sleep state duration on heart rate and its variability were similar between patients and controls. In conclusion, heart rate was consistently higher in narcoleptic patients than controls, independent of sleep stage and sleep fragmentation. A direct effect of hypocretin deficiency therefore seems probable.

## Introduction

Narcolepsy with cataplexy (NC) is a chronic disabling sleep disorder, with as core symptoms excessive daytime sleepiness, cataplexy, sleep paralysis, hypnagogic hallucinations, and a disturbed nocturnal sleep[40]. NC patients are more obese than healthy controls[170]. NC is associated with deficiency of the neuropeptide hypocretin[171], also called 'orexin'. Hypocretin is solely produced in the lateral hypothalamus and plays an important role in sleep-wake regulation[172]. Hypocretin also affects thermoregulatory and cardiovascular control[173]. Heart rate (HR) and blood pressure during sleep were altered in hypocretin-deficient rodents[174-176]. However, these findings are not consistent[177]: while one study reported a decreased blood pressure with an unaltered HR in hypocretin neuron-ablated transgenic rats[174], others found an elevated blood pressure along with an increased HR in transgenic and knock-out mice[175,176].



Human case-control studies in NC indicated that (1) HR during nocturnal sleep was continuously elevated[178-180] except for two studies[181,182]; (2) the normal nocturnal decrease in blood pressure was less marked[178,183]; (3) HR increased less in response to arousal[180] and leg movements[180,184].

Because arousal increase HR and blood pressure, and as sleep architecture in NC is highly fragmented, these cardiovascular changes may be explained through frequent arousals and sleep state changes in NC[178]. Alternatively, hypocretin deficiency itself may directly affect central autonomic control[179,185]. To better understand the role of the hypocretin system on HR control in NC, we aimed to dissect the effects of hypocretin deficiency per se and altered sleep architecture. We analyzed HR and HR variability (HRV) during nocturnal sleep in hypocretin-deficient NC patients and healthy controls taking duration and transitions of sleep stages into account.

## Methods

### Participants

Healthy controls and NC patients were selected from the Leiden University Medical Centre sleep study database. All participants had undergone a one-night ambulatory polysomnography (PSG). NC cases were included if they (1) fulfilled the International Classification for Sleep Disorders – second edition criteria (ICSD-2); (2) had no detectable level of hypocretin-I level in their cerebrospinal fluid (conforming to a level below 50 pg/ml); (3) had not used and were not using medication for narcolepsy symptoms at the time of the PSG. Each patient was matched for sex and age (+/- 5 years) to one healthy control. Controls were recruited through an advertisement in local newspapers[186,187]. None of the controls suffered from a sleeping

disorder or relevant psychiatric or medical conditions. Exclusion criteria for both groups included age below 16 years, a Body Mass Index (BMI) over 31, an apnea/hypopnea index higher than 10, the use of hypnotics or drugs affecting HR, a history of diabetes mellitus, and pregnancy. The study protocol was approved by the local medical ethics committee, and written informed consent was obtained from the healthy controls. According to Dutch law, no individual informed consent is required from patients if the data analyzed were obtained exclusively in the context of patient care, which was the case here.

**Polysomnography**

Ambulatory PSG was recorded according to standard procedures[188] using Somnologica (Version 5.1.1, Embla, CO, USA, sampling frequency: 256 Hz) in 8 cases and 12 controls. In the remaining 4 NC patients PSG was recorded using Polysmith (Version 5.0.22.0, Neurotronics, FL, USA, sampling frequency: 200 Hz). Sleep stages, leg movements, and apnoea/hypopnoea events were manually scored in 30-s epochs by experienced sleep technicians, according to the AASM manual for the scoring of sleep[158]. All PSG exams in NC patients were scored as part of diagnostic evaluation; technicians were therefore unaware of the final diagnosis. The technicians were unblinded when scoring PSG data in healthy controls. Only the nocturnal sleep period and quiet wakefulness (15 min of wakefulness prior to lights off) were selected for analysis.

**Electrocardiography and respiration**

A continuous wavelet transform analysis was implemented in Matlab (Version 13.1, Mathworks, MA, USA) to detect R-peaks in the electrocardiogram[189]. Prior to analysis a visual inspection of the quality of the signal was performed. A filter was used to exclude outliers, defined as values that differed more than 25 beats per minute from the previous or next sample.

The resulting time series of RR-intervals were used for further analysis. The mean HR of each epoch was calculated. HRV parameters were derived as follows. A frequency spectrum per epoch was calculated using a fast Fourier transform. The low frequency (LF; 0.04-0.15 Hz) and high frequency (HF; 0.15-0.4 Hz) power components were calculated from the power spectra. The HF component is considered to represent vagal activity, and the LF component to reflect sympathetically mediated baroreflex activity. The LF/HF ratio was computed to be used as a reflection of the sympathovagal balance[190]. The total power in the 0-0.4 Hz range was calculated. An increase in total power is generally related to a decline in sympathetic tone[191].

As the HF peak is largely determined by respiratory frequency, HF results can be influenced by differences in respiratory frequency (Figure 1). The sympathovagal balance was therefore also assessed in a second way, in which the individual respiratory frequency was used to define the

HF bandwidth. Respiratory frequency for each epoch was derived from thoracic respiratory band movements. The lower limit of the respiratory frequency power component ($HF_{RF}$) was calculated by 0.65*respiratory frequency (Hz) and the upper limit by 1.35*respiratory frequency (Hz)[192]. The LF/$HF_{RF}$ ratio was calculated.

**Sleep parameters**

Total sleep time, sleep efficiency, latency to sleep stage 1, rapid eye movement (REM) latency from sleep onset, and stage shift index (number of shifts between sleep stages per hour) were derived from the PSG. Relative duration of stages 1, 2, 3 (here labeled 'slow-wave sleep' (SWS)), and REM sleep were expressed as percentages of total sleep time. Event indices (i.e. number of events per hour sleep) were computed for apnea/hypopnea, leg movements (LM), and periodic LM (PLM).



**HR, HRV and respiratory frequency per sleep state**

HR, LF/HF ratio, LF/$HF_{RF}$ ratio, total power of HRV, and respiratory frequency were calculated for sleep stage 1 and 2, SWS, REM sleep, and quiet wakefulness. All epochs of sleep following onset of nocturnal sleep and prior to awakening in the morning and all epochs during quiet wakefulness were included in our analysis. To account for the autonomic effects of arousals, we identified all epochs that coincided with apnea or leg movements. In addition, we labeled an epoch with 'arousal transition' when this epoch followed a transition from SWS to sleep stage 2 or 1 and from sleep stage 2 to 1.

**Effects of sleep state duration on HR and HRV**

We first identified all 'periods' of a sleep stage: this is a series of consecutive epochs of a particular sleep stage. The duration of such periods will likely vary considerably within and between subjects. Including all epochs would result in a large number of epochs immediately following a state shift and a progressively lower number of epochs as the duration of the sleep stage becomes lower. To avoid this problem, we chose a specific duration for each sleep stage. The method chosen to define that period was based on the median duration of all periods of that stage. In view of expected differences in duration between cases and controls, we calculated the median duration separately for the NC and the control group. The shortest of both values was applied to both groups and used for analysis. Periods with a shorter duration than the median value were omitted from analysis, and periods with longer duration were included, but only the part until the defined cut-off value. This analysis could result in differences in numbers of periods that subjects contributed to the analysis, so the number of periods per subject was tabulated to check for any such bias. As only relative minor interindividual differences were seen in this number (supplemental material, Table S1), we used the group median duration for the analysis. For each period, the temporal order of epochs was recorded (first, second, third, etc.) and included as the variable 'epoch order' to assess the effects of sleep state duration.

### HR and HRV during sleep state transitions

Two types of sleep stage transitions were defined for this analysis: the first was the point between epochs where sleep stages changed from any NREM state (1, 2 or SWS) to REM sleep. To analyze these transitions, we compared HR and LF/HF ratio during all epochs within a period of 5 minutes before transition with all epochs within a period of 5 minutes after transition. The second type of change concerned transitions from any NREM state to the waking state; the analysis was similar except for the duration of the pre- and post-transition periods, which was set to two minutes. For both types of transitions, epochs were labeled as either pre- or post-transition; this factor was used in our analysis of sleep state transitions.

### Statistics

The Mann-Whitney U test was used to assess group differences in baseline characteristics and sleep parameters. Mixed effect regression models were used to analyze the effect of sleep state, sleep state duration, and transitions on cardiovascular parameters. NC, BMI, leg movements, apnea events, and arousal transitions were selected as fixed effects and participants as random effect. An interaction between NC and sleep state was added to the model to assess whether differences between cases and controls varied across sleep states. Random effect variances were computed per group and per sleep state. Likelihood ratio tests were performed to obtain the best model. For the analysis of the effects of sleep state duration, we added the fixed factor epoch order and its interaction with NC to the model. For the effect of sleep state transitions, the fixed factor pre- vs. post-transition and its interaction with NC were added to the model. P-values below 0.05 were considered significant. All analyses were performed using the software package R (Version 3.0.0, R).

## Results

### Participant characteristics and sleep parameters

No groups differences were found in mean age and BMI (Table 1). As expected, NC patients had a shorter REM latency and higher LM and PLM indices. Total sleep time, latency to sleep stage 1, apnea/hypopnea index, and arousal transitions index did not differ between groups. Sleep architecture in NC patients was characterized by a higher percentage of sleep stage 1, a higher stage shift index and a lower percentage of SWS. The relative duration of sleep stage 2 and REM sleep did not differ between groups.

### HR, HRV and respiratory frequency per sleep state

Overall we included an average total number of 860 epochs per participant. Cardiovascular parameters were significantly affected by sleep state, leg movements, apnea, and arousal transitions (Table 2). These variables were included as covariates in the main mixed effect

**Table 1.** *Participant characteristics and sleep parameters in patients with narcolepsy with cataplexy and healthy controls.*

| | NC patients | Healthy controls |
|---|---|---|
| **Number of participants (male/female)** | 12 (11/1) | 12 (11/1) |
| **Age (years)** | 28.3 ± 11.6 | 30.0 ± 12.6 |
| **BMI (kg/m²)** | 26.4 ± 3.3 | 24.3 ± 1.7 |
| **Total sleep time (min)** | 398.7 ± 70.8 | 445.9 ± 82.0 |
| **Sleep efficiency (%)** | 86.6 ± 10.5* | 95.7 ± 3.6 |
| **Sleep latency to sleep stage 1 (min)** | 6.8 ± 5.2 | 9.6 ± 9.9 |
| **REM sleep latency from sleep onset (min)** | 41.5 ± 38.6* | 82.0 ± 26.0 |
| **Sleep stage 1 (% total sleep time)** | 18.2 ± 5.5*** | 10.3 ± 4.4 |
| **Sleep stage 2 (% total sleep time)** | 44.8 ± 8.7 | 42.3 ± 6.8 |
| **Slow-wave sleep (% total sleep time)** | 16.0 ± 9.4* | 25.7 ± 5.3 |
| **REM sleep (% total sleep time)** | 21.1 ± 5.0 | 21.7 ± 4.5 |
| **Stage shift index (shifts/hour)** | 18.9 ± 4.1** | 14.1 ± 3.1 |
| **Apnea/hypopnea index (events/hour)** | 1.8 ± 2.2 | 2.2 ± 2.0 |
| **LM index (movements/hour)** | 20.3 ± 14.3*** | 1.4 ± 3.4 |
| **PLM index (movements/hour)** | 13.8 ± 17.1** | 0.5 ± 1.1 |
| **Arousal transitions index (transitions/hour)** | 4.6 ± 1.1 | 3.9 ± 0.8 |

Mean values ± standard deviation are displayed, except for the number of participants. NC, Narcolepsy with Cataplexy; BMI, Body Mass Index; REM, Rapid Eye Movement; LM, Leg Movements; PLM, Periodic Leg Movements. *P < 0.05; **P < 0.01; ***P < 0.001.



regression model. Age, BMI, and the interaction between NC and sleep state did not significantly influence HR or HRV and were omitted from the model. HR was significantly increased in NC patients, but LF/HF ratio and total power of HRV were similar between groups (Figure 1). The increase in HR in the narcolepsy group was seen in all sleep states and during wake. Respiratory frequency during sleep tended to be increased (0.05 < P < 0.1) in the NC group. The analysis of $LF/HF_{RF}$ ratio did not reveal significant differences between groups during any sleep stage or wake.

**Table 2**. *Effects of model coefficients on cardiovascular parameters.*

| | HR (bpm) | LF/HF ratio | Total power of HRV ($ms^2 \cdot 10^3$) |
|---|---|---|---|
| **Intercept (quiet wake)** | 71.10 ± 2.65 | 5.68 ± 0.75 | 44.73 ± 16.02 |
| **Sleep stage 1** | -17.03 ± 1.67*** | -1.79 ± 0.59** | -13.29 ± 15.11 |
| **Sleep stage 2** | -20.32 ± 1.60*** | -3.20 ± 0.54*** | -30.45 ± 15.16 |
| **Slow-wave sleep** | -18.66 ± 1.64*** | -3.59 ± 0.55*** | -39.63 ± 15.95* |
| **REM sleep** | -17.27 ± 1.64*** | -1.97 ± 0.63*** | -28.51 ± 15.26 |
| **NC** | 6.84 ± 3.00* | -0.39 ± 1.46 | 13.95 ± 13.37 |
| **Apnea** | 2.15 ± 0.23*** | 1.16 ± 0.17*** | 16.21 ± 2.17*** |
| **Leg movement** | 2.17 ± 0.13*** | 1.68 ± 0.09*** | 11.26 ± 1.16*** |
| **Arousal transition** | 3.96 ± 0.19*** | 1.34 ± 0.14*** | 14.35 ± 1.86*** |

Mean values ± standard errors are displayed. HR, Heart Rate; LF, Low Frequency; HF, High Frequency; HRV, Heart Rate Variability; REM, Rapid Eye Movement; NC, Narcolepsy with Cataplexy. *P < 0.05, **P < 0.01, ***P < 0.001.

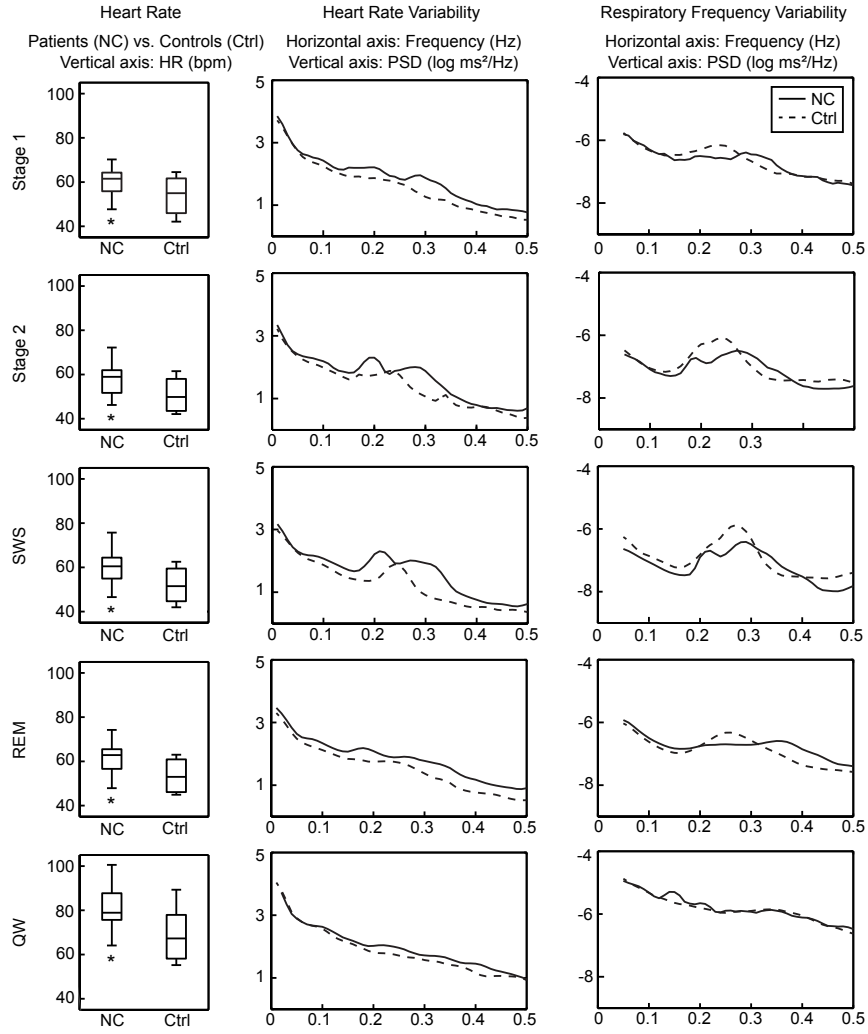


**Figure 1.** *The left column shows a box plot of the values for mean heart rate (HR) per participant in patients [narcolepsy with cataplexy (NC)] and controls (Ctrl) with HR (bpm) on the vertical axis. Asterisks (*) indicate differences between cases and controls (P < 0.05). The other two columns display the mean frequency spectrums of HR variability (center) and respiratory rate variability (right) with power spectral density (PSD; log ms²/Hz) plotted against frequency (Hz). Only epochs without apneas, leg movements, and arousal transitions were included in the analysis. Each row corresponds to a state, from top to bottom: sleep stage 1; sleep stage 2; slow-wave sleep (SWS); rapid eye movement sleep (REM); and quiet wakefulness (QW). HR in NC patients was increased in all states. Sleep-related respiratory frequency tended to be increased (P < 0.1) in NC cases.*

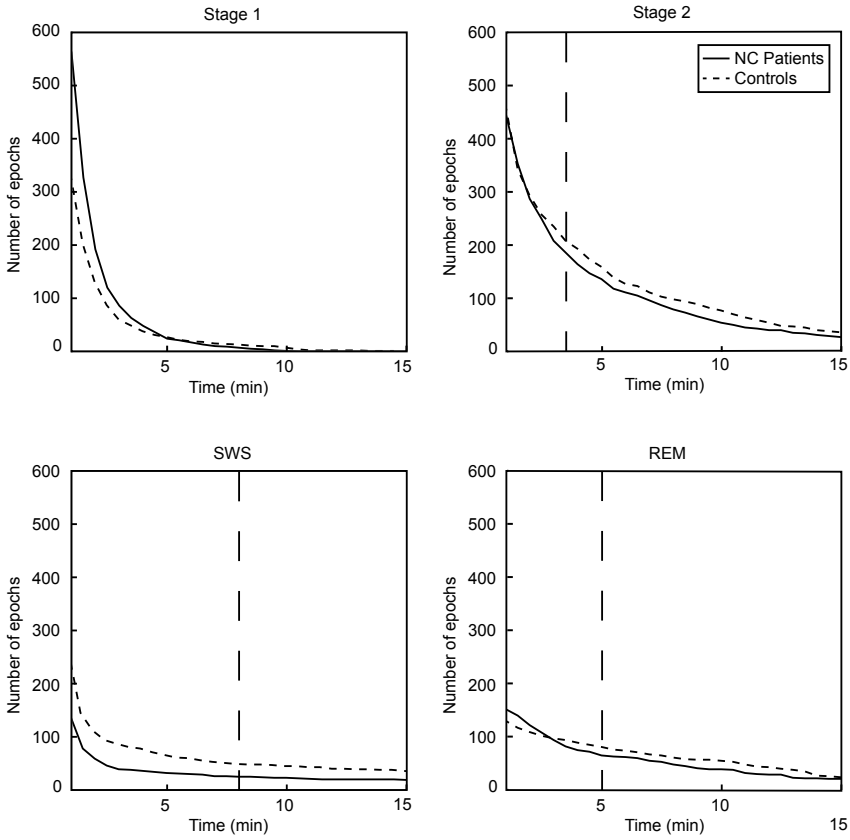


**Figure 2.** *The distribution of 30-s epochs per sleep stage. The x-axis represents the sleep state duration and the y-axis the total number of 30-s epochs per group. The dashed vertical line indicates the lowest median sleep state duration of both groups. This value was used to define the time window to assess the effects of sleep state duration.*

**Effects of sleep state duration on HR and HRV**

NC patients had the shortest median duration of sleep stage 2 (3.5 min vs. 4.5 min in controls) and REM sleep (5 min vs. 6.5 min), and the longest median duration of SWS (9 min vs. 8 min) (Figure 2). The changes in HR after sleep state onset in sleep stage 2, SWS, and REM sleep were similar for patients and controls (Figure 3). Changes in HRV over time were similar for patients and controls in REM and SWS, but a less marked decrease in LF/HF ratio was seen in patients during sleep state 2 ($P < 0.05$).

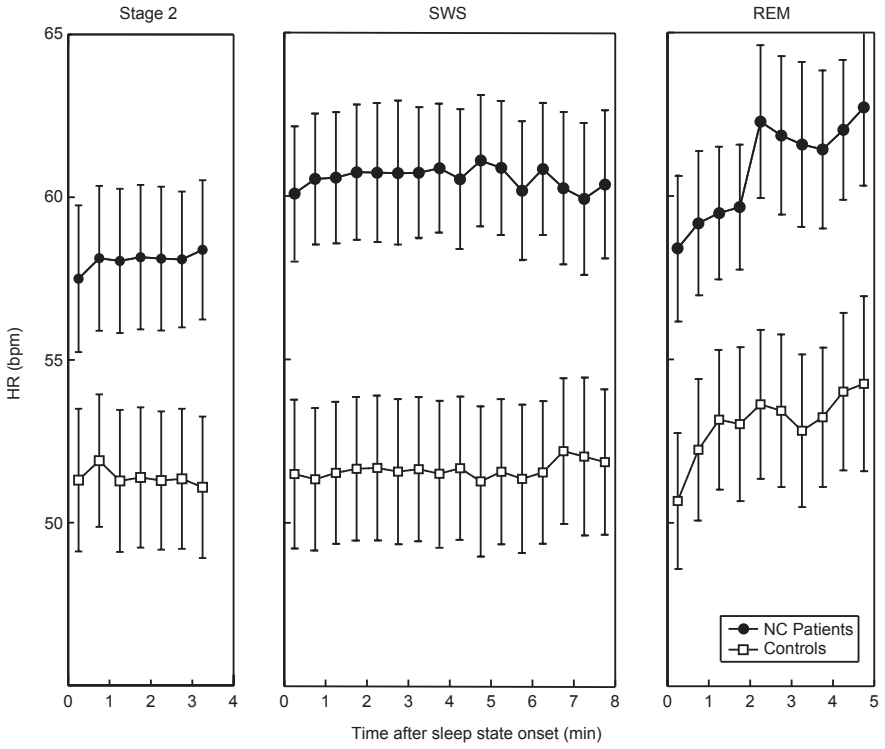


**Figure 3.** *Heart rate (HR) dynamics during sleep stage 2 (Stage 2), slow-wave sleep (SWS), and rapid eye movement sleep (REM). Mean group values indicate mean HR per participant per 30-s epoch. Only epochs without apneas, leg movements, and arousal transitions were included in the analysis. Error bars indicate standard errors of the mean. Overall HR was higher in patients in all sleep stages. The effects of sleep state duration on HR were similar between patients and controls.*

**HR and HRV during sleep state transitions**

HR increase during the transition from NREM to wake was significantly lower in the NC than in the control group (Figure 4). The change in the sympathovagal balance was similar for both groups. HR and HRV increase during the transition from NREM to REM sleep did not differ between the groups.

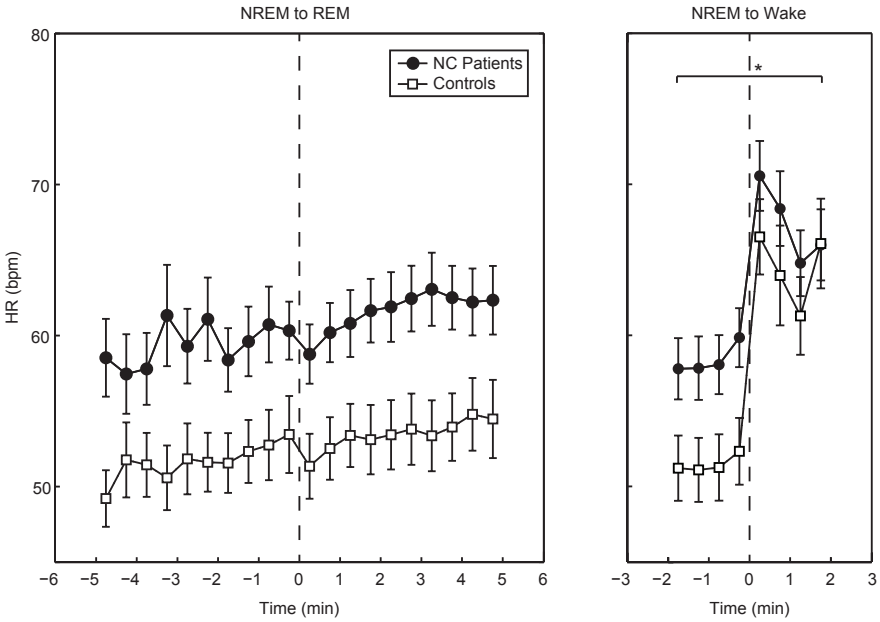


**Figure 4.** *Heart rate (HR) during transitions from non-rapid eye movement (NREM) sleep to rapid eye movement sleep (REM; left panel), and from NREM to Wake (right panel). Time (min) indicates the time to transition (at time = 0, dashed line). Mean group values indicate mean HR per participant per 30-s epoch. Only epochs without apneas, leg movements, and arousal transitions were included in the analysis. Error bars indicate standard errors of the mean. Asterisks (*) indicate differences in HR changes during transitions between cases and controls (P < 0.05). HR increase in response to the transition from NREM to REM was not different between cases and controls, but patients had a blunted HR augmentation in response to the transition from NREM to REM sleep.*

## Discussion

We found that narcolepsy is associated with a continuously elevated HR with unchanged HRV during sleep. Neither shorter sleep state duration nor frequent stage transitions could explain the higher HR in narcolepsy: (1) the effects of sleep state duration on HR were intact; (2) the increases in HR in patients following transitions were similar to controls (NREM to REM) or even less marked (NREM to wake). Taken together, these results suggest that the continuously elevated HR during sleep is not due to sleep fragmentation, but more probably reflects a direct effect of hypocretin deficiency.

**Autonomic control during sleep**

Physiological NREM sleep is characterized by a progressive increase in parasympathetic tone and a decrease in sympathetic activity, resulting in a decrease of HR and blood pressure[190,193,194]. Conversely, autonomic activity becomes unstable during REM sleep with irregular activation and deactivation of the central autonomic network, resulting in a relative increase of HR and blood pressure[190,193,194]. In line with previous work[178-180] we found that nocturnal heart rate was elevated in all sleep states without clear HRV changes[179]. In two studies HR during sleep was not higher in NC patients: in one older study[181] NC was diagnosed without proof of hypocretin deficiency and may therefore have introduced diagnostic bias. In the second recent paper indeed similar HR during sleep states was reported, but at the same time a less marked HR decline from wake to sleep was seen in NC patients vs. controls[182]. Taken together we believe that there is sufficient evidence for a higher nocturnal HR in NC. In contrast to previous work, we have corrected for effects of apnea, changes in respiratory variability and sleep fragmentation in NC, allowing us to improve our understanding of its etiology. Since no differences were found even when these differences were taken into account, we feel that there are no substantial shifts in sympathovagal balance during undisturbed sleep in NC. Not only HR, but also blood pressure was found to be increased during sleep in NC[178,183]. Both findings could theoretically be explained by sleep fragmentation[178], but we found no effect of sleep state duration on HR, so this explanation is not likely. Previous analysis of coupling of HR and blood pressure responses provided evidence that central feed-forward mechanisms are the likely underlying cause of the sleep-related autonomic response in NC[179]. The central autonomic network during NREM sleep may involve the hypothalamic ventrolateral preoptic area, central thermoregulatory and central baroreflex pathways, and command neurons in the pons and midbrain and is a major determinant of autonomic control during sleep[195]. The absence of congruous findings in sympathovagal balance may be explained by the fact that hypocretin neurons affect both sympathetic and parasympathetic outflow[196].

Interestingly, we found that NC patients tended to have a higher and more variable sleep-related respiratory frequency. These findings warrant further research. It might be postulated that this unstable respiratory frequency during sleep is associated with a decreased chemo reflex sensitivity and hereby explains the increased prevalence of apneas in NC patients[185]. To our knowledge, the association between chemoreflex sensitivity and apneas has only been demonstrated in hypocretin knock-out mice [197,198] and not in humans so far.

**Autonomic control during wakefulness**

Interestingly, two studies in NC reported an increased sympathovagal balance during wake[181,199], whereas this balance was unchanged in two others[179,200]. Obviously, these discrepancies could result from a type I error. Alternatively, it could be speculated that these differences are explained by differences in vigilance. Disturbed vigilance is one of the key symptoms in NC and typically fluctuates during the day. Impaired vigilance, if accompanied

by 'fighting against sleep', is likely to be accompanied by increased sympathetic outflow[199]. More definite conclusions could be drawn if HR changes during wake in NC are related to quantitative assessments of vigilance levels including Sustained Attention to Response Test[201]. The same holds for total power of HRV, which was found to be increased in one daytime study[200] but normal in another [199].

**Autonomic response to arousing events during sleep**

We found a blunted HR increase in response to awakening from NREM sleep in NC. Similarly, a diminished HR increase after arousal[180] and leg movements[180,184] has been reported in NC. HR increases during arousing events are accompanied by an increase in sympathetic tone[190]. Accordingly, blunted HR responses to arousal have been reported not only in peripheral autonomic disorders (pure autonomic failure[202], Parkinson's disease[203]), but also in central ones (multiple system atrophy[202], REM sleep behavior disorder[203]. In view of the absence of signs of peripheral vagal denervation[204,205] and the coupling of HR and blood pressure changes[179], autonomic alterations in NC probably reflect a central and not a peripheral site of action[185].



**Clinical implications**

Recent work suggested an increased prevalence of heart diseases and hypertension in NC[206]. The autonomic changes in NC (i.e. higher HR and non-dipping nocturnal blood pressure profile) may contribute to the increased cardiovascular risk. Alternatively, this higher risk may result from drug treatment. Further prospective studies are needed to determine whether the autonomic profiles in drug-naive patients may explain the increased propensity to cardiovascular disease.

**Limitations**

One obvious limitation of our study is the small number of participants. This is the inevitable result of selecting a homogeneous group of recently diagnosed, hypocretin-deficient and drug-naive NC patients, and we feel that this outweighs the disadvantages of limited group size. The study did therefore not have enough power to detect small differences and we were not able to correct for multiple testing. However, we believe that our conclusions are valid since the results were similar in all sleep states and in line with previous work. PSG was performed only during one night. Participants may not have had ample opportunity to get used to the set-up, which may have interfered with their sleeping behavior. Due to the retrospective set-up we could not ensure complete blinding of technicians. As a consequence, certain features in NC including sleep fragmentation may have been overreported. As we accounted for the effects of transitions and sleep state durations, we believe that this did not affect our results. A potential limitation is that we did not record for each epoch whether it was associated with arousal or not. Arousals may cause minor transient effects sympathetic tone[190,202]. Nevertheless, we believe that this effect will be negligible as we included all epochs

during sleep (i.e., on average 860 per subject); and epochs with arousals will only constitute a minority. Moreover, those arousals with major effects on autonomic control will relate to apnea, leg movements, and transitions, factors that we accounted for. Another limitation is the lack of blood pressure measurements, thus preventing us to detail changes in baroreflex sensitivity and central autonomic control[179].

**Conclusions**

HR in narcolepsy is increased during the entire nocturnal sleep period and the increase is not due to shorter sleep state duration or frequent transitions. These findings are best explained by an important contribution of hypocretin deficiency per se.

## Supplemental material

**Table S1.** *The number of 'periods' of a sleep stage per participant for NC patients and controls.*

| | Number of periods | Median duration (min) |
|---|---|---|
| ***NC patients*** | | |
| **Stage 2** | 40.2 ± 2.9 | 3.5 |
| **SWS** | 12.8 ± 1.5 | 9 |
| **REM** | 13.3 ± 1.7 | 5 |
| ***Healthy controls*** | | |
| **Stage 2** | 37.0 ± 2.6 | 4.5 |
| **SWS** | 22.8 ± 3.0 | 8 |
| **REM** | 10.8 ± 1.7 | 6.5 |

Mean values ± standard errors are displayed for the number of 'periods' (i.e. series of consecutive epochs of a particular sleep stage) in sleep stage 2 (Stage 2), slow-wave sleep (SWS), and rapid eye movement (REM) sleep in narcolepsy with cataplexy (NC) patients and healthy controls. For each sleep state, a cut-off was calculated using the shortest median sleep state duration of both groups.

# CHAPTER 7

## Discussion

## General discussion

Environmental light exerts potent effects on human physiology and behavior that can last long after discontinuation of the exposure. These non-image forming effects of light are orchestrated by intrinsically photosensitive retinal ganglion cells (ipRGCs), which serve as relay stations for information on environmental light[1] and directly transduce this information from the retina to non-image forming areas in the brain[5]. These ipRGC-driven downstream structures include olivary pretectal nucleus (OPN) in the midbrain and the suprachiasmatic nucleus (SCN) and ventrolateral preoptic area (VLPO) in the hypothalamus, which have been characterized as the center for pupil size control[8], the biological clock[21], and the major sleep-promoting area[146], respectively. Accordingly, previous studies showed that ipRGCs were involved in the pupillary light reflex[7,207], photoentrainment of circadian rhythms[2,4], and photoinduced modulations of sleepiness[12,44].

Interestingly, ipRGCs not only receive extrinsic input from rods and cones, like regular image-forming retinal ganglion cells, but additionally possess an intrinsic phototransduction circuitry driven by melanopsin, which is a photopigment maximally sensitive to short-wavelength light[4]. Previous work showed considerable individual differences in the ipRGC-driven effects of light on sleep-related parameters including the timing of sleep, which are thought to arise, in part, from individual differences in the intrinsic melanopsin-signaling phototransduction circuitry[14]. Accordingly, human genetics studies demonstrated a relationship between sleep timing and melanopsin polymorphisms[25,26]. It however remains to be evaluated whether this interindividual variation involves actual functional differences in the intrinsic melanopsin-based phototransduction circuitry.

The present thesis therefore assessed functional variation in ipRGC-driven phototransduction by investigating post-illumination changes in sleep-related physiology and behavior using light with different wavelengths. With regard to short-wavelength illumination, we proposed and validated a method for quantifying the post-illumination pupil response (PIPR) after blue light in order to estimate the functionality of the intrinsic melanopsin-driven phototransduction circuitry. We subsequently assessed whether the intraindividual variation in the PIPR after blue light related to individual differences in sleep timing. Regarding long-wavelength illumination, we investigated the acute post-illumination effects of daytime red light on pupil diameter, response speed, heart rate, and sleep propensity, and compared these effects with the functional post-illumination effects of blue and green light exposure. In addition, we amplified on the association between heart rate and sleep propensity by examining cardiovascular alterations in individuals with excessive daytime sleep propensity (i.e., narcolepsy with cataplexy patients).

## Overview of findings

In the **second chapter**, we proposed and validated a method to estimate the OPN-mediated PIPR after blue light. This characteristic feature of the pupillary light reflex provides a specific readout for the functionality of an individual's intrinsic melanopsin-dependent phototransduction circuitry. The pupil diameter was continuously monitored in the left eye, while the right eye underwent a light exposure paradigm. This pupillometry procedure was designed with the aim to elicit a persistent steady-state PIPR after blue light. Hence, the pupil of the illuminated eye was pharmacologically dilated[62] and the light exposure paradigm included 5 minutes of continuous intense blue illumination[11], which was preceded by 5 minutes of bright red light[58]. The PIPR outcome measures showed very high test-retest reliability, and were moreover robust across office hours and preceding light levels. Secondary to a larger baseline pupil diameter, the PIPR was increased in a supine position, indicating that body position should be taken into account when interpreting the results from functional magnetic resonance imaging studies[15]. We concluded that our PIPR assessment is very reliable and robust, providing a stable trait-like biomarker for functionality of the intrinsic melanopsin-driven phototransduction circuitry.



We further developed the PIPR assessment procedures in the **third chapter**, where we presented an alternative method without pharmacological pupil dilation with the aim to relieve the participant's burden and to allow for application outside the ophthalmological clinic. In order to elicit a maximum PIPR, similar to the aforementioned method with mydriatics, the absence of pharmacological pupil dilation was compensated by an increase of illumination intensity. The test-retest reliability of this method was very high, both between consecutive days as well as between winter and summer trials. We concluded that individual differences in the PIPR can be reliably and feasibly assessed without mydriatics by instead increasing the light intensities. The robustness across seasons adds to the assumption that our approach for PIPR measurements yields trait-like estimates for the functionality of an individual's intrinsic melanopsin-signaling phototransduction circuitry.

In the **fourth chapter**, we investigated whether the functionality of the intrinsic melanopsin-driven phototransduction circuitry may contribute to an individual's sleep timing. We assessed readouts of the brain structures OPN and the SCN, which are both critically innervated by ipRGCs[120]: we correlated the OPN-mediated PIPR after blue light with the SCN-driven sleep timing. In an age group with known vulnerability to a delayed sleep phase (i.e., healthy adolescents and young adults), sleep timing measures from a chronotype questionnaire, a sleep diary, and actigraphy consistently showed that individuals with a stronger PIPR had later sleep timing. This indicates that young individuals with a delayed sleep have a stronger melanopsin-based responsiveness to blue light.

Intriguingly, an increasing number of mostly recent studies suggest that the melanopsin-signaling phototransduction circuitry is not only driven by short-wavelength light in the blue part of the spectrum, but also modulated by exposure to light with longer wavelengths. The **fifth chapter** therefore focuses on post-illumination effects of red light. Whereas isolated ipRGCs[30] and non-retinal cells with heterologous melanopsin expression[31] showed a persistent increase in output firing frequency after blue illumination, their intrinsic firing rate was also acutely suppressed following longer-wavelength light exposure. Given that ipRGCs target numerous downstream brain structures involved in autonomic control and sleep regulation[5,6], we considered the likely possibility that suppressed ipRGC firing rate following long-wavelength light has physiological and behavioral consequences[27-29]. Following red illumination, we here showed sustained pupil dilation, slower response speed along with an increased heart rate suggestive of increased effort fighting sleep, and augmented sleep propensity. On the other hand, after blue light exposure we found the established sustained pupil constriction but no alterations in the other parameters. Concertedly, the physiological and behavioral changes occurring during darkness after prior exposure to intense red light are compatible with widespread downstream effects of a suppressed ipRGC firing rate. The less consistent functional alterations after blue illumination suggest that the strength of the ipRGC photoresponse to blue light exposure may vary more between functional circuitries.

Two of the parameters assessed in the fifth chapter were further examined in the **sixth chapter**, where we evaluate the relationship between sleep propensity and heart rate under conditions of extreme sleepiness. We assessed heart rate in narcolepsy with cataplexy, which is a chronic disabling disorder with excessive daytime sleep propensity as one of its core symptoms[40]. Prior to bedtime, narcolepsy patients had a higher heart rate than sex- and age-matched healthy controls, indicating an increased sympathetic drive during wakefulness. Given that narcolepsy patients suffer from excessive daytime sleep propensity, the suggestion of increased sympathetic activation in narcolepsy patients outside nighttime hours is probably related to an amplified effort fighting sleep to remain awake when desired in daily life.

## Clinical implications and future perspectives

Chapter 2 and 3 of the present thesis were devoted to the design and development of PIPR assessment procedures allowing for an accessible and reliable estimate of an individual's functionality of the melanopsin-based phototransduction circuitry. Our method showed almost perfect within-subject test-retest reliability and was robust across preceding light levels, daytime hours, and even seasons, suggestive of biomarker properties. We showed that the test cannot only be reliably performed with mydriatics but also without pharmacological pupil dilation. Accordingly, the method can be applied both in the ophthalmological clinic as

well as in the numerous non-ophthalmic research fields for which PIPR assessments are highly relevant, such as sleep[208], cognition[17], alertness[16], and mood[94]. The high reliability indicates that our PIPR assessment method is sensitive to detect differences within and between individuals, allowing the method to be used in case-control studies on individual differences and in intervention studies that expect effects on the non-image forming visual system.

A specific population of interest for the application of PIPR assessments in case-control studies is formed by the narcolepsy with cataplexy patients that we addressed in chapter 6. Narcolepsy with cataplexy is caused by loss of hypocretin producing neurons in the lateral hypothalamus[171]. Hypocretin neurons show activity during wakefulness and are part of the sleep regulatory circuitry that also involves the SCN and VLPO[209]. Interestingly, recent work suggested that hypocretin is also involved in the functionality of ipRGCs[210]. The hypocretin deficiency of narcolepsy with cataplexy patients may therefore impair ipRGC functionality, likely resulting in disrupted photoentrainment of the sleep-wake cycle[116], which may contribute to the typical narcolepsy with cataplexy complaints of disturbed night sleep and excessive daytime sleep propensity[40]. This possible impaired ipRGC-functionality in narcolepsy with cataplexy may be revealed by our currently ongoing PIPR assessments.



Another disorder in which it would be interesting to apply our PIPR method is delayed sleep phase syndrome. In chapter 4 we demonstrated, in a population with an age range with a high prevalence of delayed sleep phase syndrome[33], that individuals with a later sleep timing have a stronger PIPR after blue light. Given that the sleep-wake cycle is the most explicit behavioral marker of circadian rhythmicity[19,20], our findings indicate that hyperresponsiveness to blue light may alter circadian photoentrainment resulting in a phase delay. Our suggestion was supported by others[211] and marks blue-light hyperresponsiveness of the intrinsic melanopsin-driven phototransduction circuitry as one of the possible biological underpinnings of delayed sleep phase syndrome.

Although chapters 2 and 3 demonstrated that our PIPR estimates are robust across daytime hours, suggesting that the presumed amplified photoresponse in patients with delayed sleep phase syndrome is continuous across daytime, the hazard of their blue-light hyperresponsiveness is probably largest around light exposure during late hours. In circadian photoentrainment, indeed, bright light in the evening and early night results in a sleep phase delay[113], whereas morning bright light advances the sleep phase[112]. The relationship between blue-light hyperresponsiveness and delayed sleep timing may not solely result from circadian phase shifting, however, given that bright light in the morning is more critical for circadian photoentrainment than light exposure in the evening[212]. It may therefore also involve an acute effect, possibly through the photoinduced suppression of melatonin, which is a sleep-promoting hormone that generally starts accumulating in the hours prior to bedtime[213]. Evening blue illumination activates the intrinsic melanopsin-driven phototransduction

circuitry, resulting in an amplified downstream ipRGC output and a consequential acute suppression of melatonin[2,4]. This sleep-impeding effect may even persist beyond light offset and into the sleep period[214], much like the PIPR phenomenon that we described in chapter 2 and 3. Others added to our pupillary findings by demonstrating that bright light in the early night induced an larger melatonin suppression in delayed sleep phase syndrome[23]. It may therefore prove fruitful, especially for patients suffering from delayed sleep phase syndrome, to avoid evening exposure to blue light, not only to preserve circadian photoentrainment but also to prevent possible adverse acute effects.

Conversely, our findings in chapter 5 suggested that if individuals are exposed to light with the appropriate intensity and spectral properties it may actually serve as a nightcap. We showed in daytime experiments that subjects left in the dark after having been exposed to bright red light become sleepier, as indicated by a faster transition to sleep. This post-illumination sleep facilitation likely involves the VLPO[145], which plays a pivotal role in the transitions between wakefulness and sleep [146]. The functionality of the VLPO depends on the direct[215] and particularly the indirect[216] input from the SCN and it therefore seems probable that the post-illumination effects on sleep propensity may vary across the day. For practical applications, future studies could evaluate whether pre-dark red light exposure elicit similar effects at bedtime, and whether such an intervention is effective in individuals that experience difficulties initiating sleep. Such difficulties are highly prevalent in delayed sleep phase disorder[33] and insomnia disorder[165]. If effective, these populations may benefit from this potential novel form of light therapy.

If subjects left in the dark after intense red light exposure had to perform a simple reaction time task, their response times slowed in spite of a likely increased effort as indicated by a higher heart rate. An increased heart rate has been suggested to be the most reliable electrocardiographic marker of mental effort during task performance[151]. This is in line with chapter 6, showing an increased heart rate during wakefulness in narcolepsy patients, which generally struggle against excessive daytime sleep propensity. Others demonstrated a higher heart rate during wakefulness in narcolepsy as well and likewise proposed that it may involve a large effort fighting against sleep[217]. Accordingly, in chapter 2 we characterized the larger baseline pupil diameter in a sleep-permissive body position as a reflection of increased sympathetic activity[35] resulting from an increased effort to keep the eyes open and remain awake in spite of the temptation to relax or give in to sleep. In addition, electroencephalography studies involving sleep deprivation showed that the decline in task performance[218] and the rise of subjective sleepiness[219] were associated with an increase in beta-power, which is considered as a marker for sympathetic activation[39,220]. Our findings further support the importance of evaluating task demands and mental effort when interpreting changes in central and autonomic nervous system readouts[221].

## Conclusions

The present thesis contributed to our assessment tools and knowledge with respect to the non-image forming effects of light on autonomic control, alertness, and sleep. In chapter 2 and 3 we developed a robust and feasible PIPR assessment method allowing for a widespread quantification of the functionality of the intrinsic melanopsin-based phototransduction circuitry. The method has now been adopted by several researchers and clinicians utilizing it to better understand sleep-related complaints in different disorders. In chapter 4 we applied this method to identify individual differences in functionality of the intrinsic melanopsin-signaling phototransduction circuitry and showed that individuals with a later sleep timing have a stronger PIPR after blue light. Our physiological and behavioral findings in chapter 5 indicated that a bright red light stimulus could even be used to facilitate sleep onset. Chapter 2, 5, and 6 highlighted that task demands and mental effort should be taken into account in order to allow for a correct interpretation of alterations in the functional estimates of the central and autonomic nervous system. It appears timely to consider large-scale follow-up studies to add to the multivariate fingerprint of the physiological and behavioral effects of light and to further evaluate the clinical application of a novel angle on light therapy in the treatment of sleep disorders including delayed sleep phase syndrome, sleep-onset insomnia, and narcolepsy with cataplexy.

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# APPENDICES

## Summary

The non-image forming effects of environmental light are critically mediated by intrinsically photosensitive retinal ganglion cells (ipRGCs) that are characterized by their expression of melanopsin, which is a photopigment that is maximally responsive to light in the blue part of the spectrum. The ipRGC-mediated effects of light can typically endure far beyond light offset, with widespread functional consequences including changes in various aspects of human sleep. The present thesis investigated such sleep-related post-illumination effects in the context of physiology and behavior.

Interestingly, it has been demonstrated that the photomodulation of sleep-related dimensions varies considerably between individuals, which may involve functional variation in the melanopsin-signaling phototransduction circuitry. We therefore first designed a method for estimating an individual's functionality of the intrinsic melanopsin-driven phototransduction circuitry from the characteristic sustained pupil constriction after a blue light stimulus, which is a feature of the pupillary light reflex that is almost entirely dependent on the prolonged ipRGC response following light exposure. We subsequently evaluated whether the individual differences in this so-called post-illumination pupil response (PIPR) after blue light were associated with the intraindividual variation in sleep timing. The scope this thesis however not only included blue light exposure but also light with other wavelengths, in particular red illumination. We investigated the post-illumination effects of daytime red light on pupil diameter, response speed, heart rate, and sleep propensity, and compared these effects with the functional changes after blue and green light exposure. Following up on these functional changes, we investigated the specific association between heart rate and sleep propensity in more detail.

The pupillometry procedures for estimating the specific functionality of the intrinsic melanopsin-dependent phototransduction circuitry were presented in the **second chapter**. This novel methodology was designed such that we would measure a persistent steady-state PIPR in one eye after exposing the other eye to a 5-minute intense blue light stimulus. Hence, the blue illumination was preceded by 5 minutes of bright red light and the pupil of the illuminated eye was pharmacologically dilated using mydriatic eye drops. Our method showed almost perfect test-retest reliability, and was moreover robust across office hours and preceding light levels. We moreover found that the PIPR was increased in a lying body position, which was secondary to a larger baseline pupil diameter. This finding is particularly relevant to pupillometry routines that are applied in a supine position (e.g., functional magnetic resonance imaging studies and assessments in bed-ridden patients). We concluded that the presented method provides a robust PIPR estimate, allowing for a reliable quantification of the functionality of an individual's intrinsic melanopsin-driven phototransduction circuitry in various experimental designs including case-control and intervention studies.

Although the aforementioned PIPR assessment method was demonstrated to be widely applicable, the use of mydriatics means that ophthalmological expertise is required, thereby hampering the feasibility of performing the procedures in the many non-ophthalmological research communities to which ipRGC functionality is relevant. In the **third chapter,** we therefore designed an alternative method for eliciting a maximum PIPR after blue light without pharmacological pupil dilation by instead increasing the illumination intensity, thereby moreover avoiding the negative side effects of mydriatics for the participants. The day-to-day test-retest reliability of this more accessible method was very high, comparable to the previously established paradigm with mydriatics. In addition, the PIPR outcomes measures were stable across the winter and summer. Taken together, both the mydriatic as well as the non-mydriatic PIPR assessment method yielded trait-like individual biomarkers of the functionality of the intrinsic melanopsin-signaling phototransduction circuitry, allowing for a widespread application. At this moment, indeed, several research departments and clinical institutions from various disciplines have already adopted our methodology.

In the **fourth chapter,** we applied the PIPR assessments to evaluate whether intraindividual differences in sleep timing relate to the functionality of the intrinsic melanopsin-based phototransduction circuitry. We included a healthy group within an age range with a high prevalence of a delayed sleep phase (i.e., adolescents and young adults), in which we correlated the PIPR after blue light with sleep timing assessed in three ways: using a chronotype questionnaire, a sleep diary, and actigraphy. Consistent across all sleep timing measures, individuals with a later sleep timing had a more pronounced PIPR after blue light. In conclusion, hyperresponsiveness to blue light was identified as a functional underpinning of late sleep and should be considered in the clinical treatment of delayed sleep phase syndrome.



In contrast with the PIPR after blue light, recent evidence suggests that melanopsin-mediated functional changes beyond illumination offset are not only critically driven by short-wavelength light. Electrophysiology experiments showed that the output firing rate of melanopsin-containing cells can be promptly silenced following discontinuation of long-wavelength illumination, possibly resulting in an output firing frequency even below the characteristic spontaneous spike rate that ipRGCs possess in the long-term absence of light. The **fifth chapter** added to this previous work by evaluating the post-illumination effects of red light on human physiology and behavior. Given that ipRGCs target numerous downstream brain structures involved in autonomic control and sleep regulation, we assessed the wavelength-dependent post-illumination consequences for pupil diameter, task performance, and sleepiness levels. Indeed, following intense red illumination we demonstrated sustained pupil dilation, slower response speed during an alertness assessment task along with a higher heart rate indicative of increased effort fighting sleep, and amplified sleep propensity, all suggestive of an extinguished downstream ipRGC output. Conversely, after blue light exposure we found

no post-illumination changes, except for the established sustained pupil constriction. For practical applications, the sleep-permissive changes in darkness following red light exposure may provide a starting point for a novel form of bright light treatment for individuals that experience difficulties initiating sleep.

Following up on the aforementioned findings during alertness task performance, we examined the relationship between sleepiness and heart rate in more detail in the **sixth chapter**. We assessed heart rate in patients suffering from narcolepsy with cataplexy, which generally struggle against excessive daytime sleep propensity. During wakefulness, narcolepsy patients had a higher heart rate, indicating an increased sympathetic drive under conditions of extreme sleepiness, which may possibly involve an amplified effort of fighting against sleep when remaining awake is desired. This is in line with chapter 5 showing an increased heart rate along with a slower response speed on an alertness task in darkness after red illumination, indicating that more effort is required for performance when sleepiness levels are high.

Taken together, the findings in the present thesis added to our assessment tools and knowledge with respect to the non-image forming effects of light on the functional circuitries involved in human sleep regulation. In chapter 2 and 3, we presented a PIPR after blue light assessment method that yields a robust and feasible estimate of the functionality of an individual's intrinsic melanopsin-based phototransduction circuitry. In chapter 4, we showed that the PIPR after blue light was more pronounced in individuals with a later sleep timing. Following red light, sleep propensity is promoted, as indicated by our physiological and behavioral findings in chapter 5. Chapter 6 added to chapter 2 and 5 indicating that task demands and mental effort should be taken into account in order to correctly interpret changes in physiological and behavioral reflections of the central and autonomic nervous system. It appears timely to consider large-scale follow-up studies to contribute to the multivariate fingerprint of the non-image forming effects of light on human physiology and behavior and to further evaluate the clinical application of light therapy in the treatment of sleep disorders.

## Samenvatting

Intrinsiek lichtgevoelige retinale ganglioncellen (ipRGCs) in het netvlies zijn van groot belang voor de niet-beeldvormende effecten van omgevingslicht. Deze cellen worden gekenmerkt door de expressie van melanopsine, een fotopigment dat het sterkst reageert op licht in het blauwe deel van het spectrum. De functionele effecten van licht waarbij ipRGCs een rol spelen, waaronder veranderingen in diverse aspecten van de menselijke slaap, kunnen aanhouden tot lang na het stoppen van de blootstelling aan licht. In dit proefschrift onderzoeken we dergelijke slaapgerelateerde verlate effecten van lichtblootstelling in de context van fysiologie en gedrag.

Effecten van licht op slaapgerelateerde dimensies verschillen aanzienlijk tussen individuen. Mogelijk speelt functionele variatie in het melanopsinesysteem hierbij een rol. Om dit te kunnen onderzoeken hebben we eerst een methode ontworpen om de functionaliteit van het melanopsinesysteem van een individu te kwantificeren aan de hand van de karakteristieke langdurige pupilconstrictie die optreedt na stimulatie met blauw licht. Deze zogenaamde 'post-illumination pupil response' (PIPR) na blauw licht is bijna volledig toe te schrijven aan een aanhoudende activiteit van ganglioncellen die melanopsine tot expressie brengen. Vervolgens hebben we bepaald of individuele verschillen in de PIPR na blauw licht samenhangen met de individuele variatie in de gebruikelijke tijd om te gaan slapen en op te staan. In dit proefschrift richten we ons echter niet alleen blauw licht, maar ook op blootstelling aan licht met andere golflengtes en dan met name rood licht. We hebben onderzocht hoe pupildiameter, reactiesnelheid, hartslag en slaapneiging veranderen na blootstelling aan rood licht en hoe deze na-verlichtingseffecten zich verhouden tot functionele veranderingen na blauw en groen licht. Tenslotte hebben we het specifieke verband tussen hartslag en slaapneiging nader onderzocht.

In het **tweede hoofdstuk** beschrijven we een nieuwe methode voor pupillometrie om de functionaliteit van het melanopsinesysteem te bepalen. Bij deze methode wordt het ene oog 5 minuten blootgesteld aan intens blauwe licht, waarna de PIPR van het andere oog wordt gemeten. Om een langdurige, stabiele PIPR te genereren wordt de blauwe verlichting voorafgegaan door 5 minuten fel, rood licht en wordt de pupil van het belichte oog farmacologisch verwijd met behulp van mydriatische oogdruppels. Onze methode bleek gedurende kantooruren een bijna perfecte test-hertestbetrouwbaarheid te hebben. De uitkomsten van herhaalde metingen waren praktisch hetzelfde, ongeacht het tijdstip en de mate van voorafgaande verlichting. Daarnaast ontdekten we dat de PIPR sterker was als de metingen liggend werden ondergaan. Dit was het gevolg van een grotere pupildiameter voorafgaand aan de lichtblootstelling. Deze bevinding is met name relevant voor pupilmetingen die in een liggende positie worden uitgevoerd (bijv. functionele magnetische resonantie imagingexperimenten, 'constant routine' (slaap-) onderzoek en metingen bij

bedlegerige patiënten). Concluderend kunnen we stellen dat de beschreven methode een robuuste bepaling van de PIPR oplevert, waarmee het mogelijk is om de functionaliteit van het intrinsieke melanopsinesysteem van een individu op betrouwbare wijze te kwantificeren in verschillende soorten onderzoek, waaronder patiënt-controle- en interventie-experimenten.

Hoewel we hebben laten zien dat de eerdergenoemde PIPR-bepalingsmethode breed toepasbaar is, zorgt het gebruik van mydriatica ervoor dat oogheelkundige expertise vereist is bij de uitvoering ervan. Dit vormt een belemmering voor het toepassen van de metingen in de vele niet-oogheelkundige onderzoeksgebieden waarvoor de functionaliteit van het melanopsinesysteem van belang is. Daarnaast kunnen deelnemers last krijgen van de negatieve bijwerkingen van het toedienen van mydriatica. Vandaar dat we in het **derde hoofdstuk** een alternatieve methode beschrijven, die gebruik maakt van een hogere lichtintensiteit om zo een maximale PIPR na blauw licht op te wekken zonder farmacologische pupilverwijding. De test-hertestbetrouwbaarheid van deze meer toegankelijke methode was zeer hoog, vergelijkbaar met het protocol dat we beschrijven in hoofdstuk 2. De PIPR-uitkomstmaten waren niet alleen overeenkomstig als de methode herhaald werd op opeenvolgende dagen, maar ook bij de vergelijking tussen metingen in de winter en de zomer. We kunnen dus concluderen dat zowel de mydriatische als de niet-mydriatische bepalingsmethode een karakteristieke PIPR na blauw licht oplevert, die kan worden beschouwd als een stabiele individuele eigenschap. Dit geeft aan dat onze PIPR-bepalingen breed toegepast kunnen worden als biomarker voor de functionaliteit van iemands melanopsinesysteem. Op dit moment is onze methodologie dan ook overgenomen door een aantal andere onderzoeksafdelingen en klinische instellingen uit verschillende disciplines.

Het **vierde hoofdstuk** beschrijft hoe we onze PIPR-bepalingsmethode hebben gebruikt om vast te stellen of individuele verschillen in slaaptijden verband houden met de functionaliteit van het melanopsinesysteem. We hebben de PIPR na blauw licht gemeten bij gezonde adolescenten en jongvolwassenen, omdat een groot aantal mensen in deze leeftijdsgroep een verlate slaapfase vertoont. Vervolgens hebben we de PIPR na blauw licht gecorreleerd met drie bepalingen van slaaptijden (vragenlijst, dagboek en actigrafie). Deze bepalingen lieten consistent een sterkere PIPR na blauw licht zien bij individuen met een latere slaaptijd. De conclusie van dit onderzoek luidt dat een overmatig sterke reactie op blauw licht mogelijk kan bijdragen aan verlate slaaptijden. Het meten van deze reactie zou van waarde kunnen zijn in de klinische evaluatie en behandeling van het vertraagde slaapfasesyndroom.

Recent onderzoek heeft aangetoond dat langdurige effecten niet alleen optreden na afloop van licht met een korte golflengte, zoals de PIPR na blauw licht, maar ook na verlichting met langere golflengtes. Elektrofysiologische experimenten lieten zien dat de elektrische activiteit van cellen die melanopsine bevatten direct uitdooft na afloop van oranje of groengeel licht en mogelijk is de vuurfrequentie van dergelijke cellen, waaronder ipRGCs, dan zelfs nog lager dan

tijdens een langdurige periode zonder licht. Naar aanleiding van deze eerdere bevindingen, hebben we in het **vijfde hoofdstuk** de fysiologische en gedragsmatige veranderingen na afloop van verschillende kleuren licht onderzocht. We laten zien dat er in duisternis, na blootstelling aan heldere rood licht, sprake is van een aanhoudende pupilverwijding. Ook was de reactiesnelheid tijdens een alertheidstaak vertraagd, terwijl de hartslag versnelde, wat tijdens het uitvoeren van een taak een teken is van verhoogde mentale inspanning. Tenslotte vielen deelnemers na rood licht ook sneller in slaap. Al deze veranderingen zijn in overeenstemming met onderdrukking van de vuurfrequentie van ipRGCs na licht met een lange golflengte. Wat betreft praktische toepassingen zouden de slaap-versterkende veranderingen in duisternis na roodlichtblootstelling een uitgangspunt kunnen vormen voor een nieuwe manier van lichttherapie voor mensen die problemen hebben met in slaap vallen.

Naar aanleiding van de eerdergenoemde bevindingen tijdens het uitvoeren van een alertheidstaak, hebben we in het **zesde hoofdstuk** de relatie tussen slaperigheid en hartslag nader onderzocht. We bestudeerden de hartslag bij patiënten die lijden aan narcolepsie met kataplexie. Deze patiënten hebben doorgaans last van extreme slaperigheid overdag, wanneer het wenselijk is om wakker te blijven. We ontdekten dat de narcolepsiepatiënten een hogere hartslag hadden tijdens de periode wakker zijn, wat wijst op een verhoogde sympathische activatie. Mogelijk duidt dit weer op een grotere inspanning, om te vechten tegen de slaap, in overeenstemming met onze bevindingen in hoofdstuk 5. Daarin beschreven we dat de vertraagde reactiesnelheid tijdens een alertheidstaak na rood licht samenging met een versnelde hartslag, wat erop kan duiden dat er een grotere mentale inspanning vereist is als er sprake is van toegenomen slaperigheid.

Concluderend kunnen we stellen dat de onderzoeken die in dit proefschrift beschreven staan een waardevolle aanvulling vormen op de bestaande kennis en toepassingen op het gebied van niet-beeldvormende effecten van licht op de functionele systemen die betrokken zijn bij de regulatie van slaap. We beschrijven een robuuste en toegankelijke methode om de functionaliteit van iemands melanopsinesysteem te bepalen aan de hand van de PIPR na blauw licht en laten zien dat deze PIPR na blauw licht sterker is bij late slapers. We beschrijven ook dat men sneller in slaap valt na blootstelling aan rood licht. Daarnaast tonen we aan dat er rekening moet worden gehouden met de mate van mentale inspanning als fysiologische en gedragsmatige parameters worden gebruikt voor de bepaling van de functionaliteit van het centrale en autonome zenuwstelsel. Onze methoden en bevindingen leggen het fundament voor grootschaligere, multidimensionale vervolgonderzoeken om de vele niet-beeldvormende effecten van licht op fysiologie en gedrag verder te onderzoeken en de klinische toepassingen van lichttherapie bij slaapstoornissen te verbeteren.

## Résumé

La lumière influence de manière importante de multiples aspects de notre physiologie et de nos comportements. L'information lumineuse non-visuelle est principalement médiée par un sous-type de cellules ganglionnaires de la rétine (ipRGCs) qui exprime un photopigment, la mélanopsine. La présence de ce photopigment, dont la sensibilité à la lumière est maximale dans le spectre de couleur bleue, rend ces cellules intrinsèquement photosensibles. La lumière exerce, via les ipRGCs, divers effets qui s'observent pendant la période d'exposition lumineuse, mais aussi après l'extinction de la lumière, affectant différents aspects du sommeil humain. Ce travail de thèse s'est intéressé aux effets de la lumière sur la physiologie et le comportement humain survenant après l'extinction de la lumière (post-illumination), en prenant en compte également la variabilité interindividuelle.

En effet, les effets de la lumière sur le sommeil varient considérablement d'un sujet à un autre, ce qui suggère que la sensibilité de la phototransduction mélanopsinergique varie selon les individus. Pour atteindre notre objectif nous avons donc conçu un appareil de mesure qui permet d'étudier spécifiquement la phototransduction médiée par les cellules à mélanopsine. Cet outil analyse la contraction pupillaire à la lumière (réflexe pupillaire) et plus particulièrement la réponse tardive (soutenue dans le temps) suivant l'application de lumière bleue, une caractéristique du réflexe pupillaire hautement spécifique du système mélanopsinergique. Par la suite, nous avons évalué si les différences interindividuelles de cette réponse pupillaire post-illumination (nommée PIPR) à la lumière bleue étaient associées aux horaires de sommeil des sujets, c'est-à-dire à leur chronotype. De plus, notre travail ne s'est pas limité à l'étude de la réponse à la lumière bleue, mais aussi à d'autres longueurs d'onde, en particulier la lumière rouge. Nous avons ainsi étudié les effets post-illumination de la lumière rouge administré de jour sur la réponse pupillaire (diamètre de la pupille, vitesse de réponse), le rythme cardiaque, et la propension au sommeil, en comparant les modifications induites par la lumière rouge à celles induites par la lumière bleue ou par la lumière verte. Nous avons également analysé plus en détails la relation entre l'évolution du rythme cardiaque et la propension au sommeil.

La procédure de réalisation et d'analyse de la pupillométrie permettant d'évaluer la phototransduction mélanopsinergique est présentée dans le **deuxième chapitre**. Cette méthodologie novatrice a été conçue de façon à nous permettre de mesurer une PIPR stable mesurée au niveau d'un œil après avoir exposé l'autre œil à une lumière bleue pendant 5 minutes. Afin d'optimiser la réponse pupillaire post-illumination, la pupille des sujets était dilatée avec l'administration d'un collyre mydriatique et l'exposition à la lumière bleue était précédée d'une exposition de 5 minutes à une lumière rouge intense. La fiabilité test-retest de notre méthode était quasiment parfaite et consistante quel que soit l'horaire de la journée auquel était effectué le test et le niveau d'exposition lumineuse auquel était exposé le sujet

avant la réalisation du test. La mesure de PIPR était influencée par la position, augmentée en position couchée, le diamètre pupillaire de base étant plus grand permettant ainsi d'obtenir une plus grande constriction. Ce résultat est particulièrement intéressant à prendre en compte pour l'interprétation des pupillométries réalisées en routine en position couchée (ex., études en imagerie par résonance magnétique fonctionnelle et évaluations chez les patients alités). En conclusion de ce chapitre, la méthode que nous avons développée permet d'obtenir une mesure robuste de PIPR et ainsi une évaluation fiable de la phototransduction mélanopsinergique qui pourra ainsi être appliquée à divers paradigmes expérimentaux, que ce soit des études cas-contrôles ou des études interventionnelles.

Cependant, pour pouvoir l'appliquer à de multiples champs d'investigation, la nécessité de dilater la pupille des sujets constitue un facteur limitant. En effet, ceci nécessite d'avoir au préalable une évaluation ophtalmologique approfondie et ceci est de plus contraignant pour les sujets. Nous avons donc optimisé les conditions de réalisation du test afin de se passer de la dilatation pupillaire. Ceci a été fait en augmentant l'intensité lumineuse et ces nouvelles conditions de réalisation du test sont décrites dans le **troisième chapitre** de la thèse. Nous avons alors validé ces nouvelles conditions de réalisation de la pupillométrie en démontrant que la fiabilité test-retest inter-jour était très élevée et très proche de celle observée après dilatation de la pupille et non affectée par la saison puisque les valeurs de PIPR étaient comparables en hivers et en été. Finalement, les deux méthodes, avec ou sans dilatation de la pupille, nous ont permis de mettre au point un biomarqueur spécifique du fonctionnement de la phototransduction mélanopsinergique de chaque individu (« marqueur-trait »). Cette méthodologie peut être appliquée à de nombreux domaines d'investigation comme en témoigne l'intérêt actuel de nombreux départements cliniques ou de recherches, de disciplines variées, pour cette nouvelle méthodologie.

Nous avons ensuite utilisé la mesure de PIPR pour évaluer si les différences interindividuelles d'horaires de sommeil ou chronotype étaient liées à des différences de fonctionnement de phototransduction mélanopsinergique (**chapitre 4**). Pour ceci, nous avons inclus un groupe de sujets sains avec une surreprésentation de sujets avec retard de phase et donc une majorité d'adolescents et d'adultes jeunes. Nous avons corrélé la valeur de PIPR de chaque individu avec son chronotype, les horaires de sommeil étant évalués de trois manière différentes: questionnaire de chronotypage, agenda de sommeil et actimétrie. Les sujets avec des horaires de sommeil plus tardifs ou « sujets du soir » avaient une mesure de PIPR après administration de lumière bleue plus élevée, et la corrélation était observée avec les trois méthodes de mesure du chronotype. Cette méthode semble donc très pertinente pour avoir une évaluation fonctionnelle du fonctionnement mélanopsinergique chez des sujets avec retard de phase, et ceci pourrait avoir un intérêt majeur en clinique pour le suivi des patients ayant un syndrome de retard de phase du rythme circadien.

De récentes données de la littérature suggèrent que le fonctionnement mélanopsinergique post illumination c'est-à-dire après extinction de la lumière est affecté non seulement par une exposition initiale à la lumière bleue mais aussi à des longueurs d'ondes plus grande. Ainsi, en électrophysiologie, la fréquence de décharge des cellules à mélanopsine pendant la période de temps suivant une exposition à de grandes longueurs d'onde (rouge) est quasi-nulle ou très faible, à un niveau plus faible que la fréquence de décharge de repos de ces cellules en l'absence prolongée de lumière. Nous nous sommes donc ensuite intéressés chez l'homme à l'influence de la lumière rouge sur la physiologie et le comportement en analysant plus spécifiquement les effets post illumination c'est-à-dire survenant après extinction suivant l'application d'une lumière rouge (**cinquième chapitre**). Considérant que les ipRGCs projettent sur de nombreuses structures cérébrales en particulier impliquées dans le contrôle autonomique et la régulation du sommeil, nous avons évalué, en fonction du spectre de couleur de lumière administrée, les effets post-illumination sur différents paramètres, le diamètre pupillaire, la performance et le niveau de somnolence. Suite à une exposition à une lumière rouge intense, nous avons observé une dilatation soutenue de la pupille, des performances plus lentes lors de tâches de vigilance, une plus grande propension au sommeil et une fréquence cardiaque plus élevée. Ceci suggère que le sujet effectue un plus grand effort mental pour lutter contre la pression de sommeil. Ces résultats sont donc cohérents avec une extinction d'activité des ipRGCs. En revanche, après une exposition à la lumière bleue, nous n'avons pas observé de changements post-illumination, sauf en ce qui concerne la constriction soutenue de la pupille. Finalement, cette observation représente un point de départ intéressant pour le développement de nouvelles formes de thérapie lumineuse pour les personnes qui éprouvent des difficultés à s'endormir.

Dans la continuité des résultats précédents observés au cours de taches de vigilance, nous avons examiné plus en détails les relations entre la somnolence et la fréquence cardiaque (**sixième chapitre)**. Nous avons évalué la fréquence cardiaque chez des patients souffrant de narcolepsie avec cataplexie (un trouble du sommeil caractérisé entre autres par des accès de somnolence diurne la journée) qui, de manière générale, luttent contre une somnolence excessive durant la journée. Au cours de l'éveil, les patients narcoleptiques avaient un rythme cardiaque plus élevé, indiquant une augmentation de l'activité sympathique dans des conditions de somnolence extrême. Ceci suggère un effort plus important pour lutter contre le sommeil afin de pouvoir rester éveillé. Cela est cohérent avec les observations du chapitre 5, où il est démontré qu'une augmentation du rythme cardiaque et une diminution des temps de réaction lors de tâche de vigilance est observée lors de la période suivant une exposition à la lumière rouge, suggérant que des efforts supplémentaires sont requis pour maintenir un niveau de performance satisfaisant lorsque la pression de sommeil est élevée.

Pris dans leur ensemble, les résultats de ce travail de thèse ont permis d'améliorer notre compréhension des effets non-visuels de la lumière sur la physiologie et le comportement

humain. Nous avons développé et validé un premier outil permettant d'évaluer la phototransduction mélanopsinergique chez l'homme, ce qui ouvre des perspectives d'applications très larges. Nous avons ainsi montré que les mesures obtenues (PIPR) étaient propres à chaque individu et corrélaient par exemple avec le chronotype des sujets. Enfin, nous rapportons un résultat original et novateur en montrant qu'une exposition à une lumière rouge intense facilite dans un deuxième temps (effet post illumination) la propension au sommeil. Enfin, l'ensemble de ces résultats encourage la réalisation de travaux futurs, notamment sur de plus grands échantillons de sujets, afin de mieux comprendre l'influence non-visuelle de la lumière sur la physiologie et le comportement humain. Nos observations incitent également à poursuivre l'évaluation des applications cliniques de la luminothérapie dans le traitement des troubles du sommeil.

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Wisse Pier VAN DER MEIJDEN

# Lumière, physiologie et comportement

**Résumé**

La lumière influence de manière importante de multiples aspects de notre physiologie et de nos comportements. L'information lumineuse non-visuelle est principalement médiée par un sous-type de cellules ganglionnaires de la rétine (ipRGCs) qui exprime un photopigment, la mélanopsine. La présence de ce photopigment, dont la sensibilité à la lumière est maximale dans le spectre de couleur bleue, rend ces cellules intrinsèquement photosensibles. La lumière exerce, via les ipRGCs, divers effets qui s'observent pendant la période d'exposition lumineuse, mais aussi après l'extinction de la lumière, affectant différents aspects du sommeil humain. Ce travail de thèse s'est intéressé aux effets de la lumière sur la physiologie et le comportement humain survenant après l'extinction de la lumière (post-illumination), en prenant en compte également la variabilité interindividuelle. Nous avons développé et validé un premier outil permettant d'évaluer la phototransduction mélanopsinergique chez l'homme, ce qui ouvre des perspectives d'applications très larges. Nous avons ainsi montré que les mesures obtenues (PIPR) étaient propres à chaque individu et corrélaient par exemple avec le chronotype des sujets. Enfin, nous rapportons un résultat original et novateur en montrant qu'une exposition à une lumière rouge intense facilite dans un deuxième temps (effet post illumination) la propension au sommeil. Enfin, l'ensemble de ces résultats encourage la réalisation de travaux futurs, notamment sur de plus grands échantillons de sujets, afin de mieux comprendre l'influence non-visuelle de la lumière sur la physiologie et le comportement humain. Nos observations incitent également à poursuivre l'évaluation des applications cliniques de la luminothérapie dans le traitement des troubles du sommeil.

**Mots-clefs:** Lumière, effets non-visuels, couleur de lumière, physiologie, comportement

**Abstract**

The non-image forming effects of environmental light are critically mediated by intrinsically photosensitive retinal ganglion cells (ipRGCs) that are characterized by their expression of melanopsin, which is a photopigment that is maximally responsive to light in the blue part of the spectrum. The ipRGC-mediated effects of light can typically endure far beyond light offset, with widespread functional consequences, including changes in various aspects of human sleep. The present thesis investigated such sleep-related post-illumination effects in the context of physiology and behavior. In chapter 2 and 3, we presented a post-illumination pupil response (PIPR) after blue light assessment method that yields a robust and feasible estimate of the functionality of an individual's intrinsic melanopsin-based phototransduction circuitry. In chapter 4, we showed that the PIPR after blue light was more pronounced in individuals with a later sleep timing. Following red light, sleep propensity is promoted, as indicated by our physiological and behavioral findings in chapter 5. Chapter 6 added to chapter 2 and 5 indicating that task demands and mental effort should be taken into account in order to correctly interpret changes in physiological and behavioral reflections of the central and autonomic nervous system. It appears timely to consider large-scale follow-up studies to contribute to the multivariate fingerprint of the non-image forming effects of light on human physiology and behavior and to further evaluate the clinical application of light therapy in the treatment of sleep disorders.

**Key words:** Light, non-image forming effects, wavelength-dependency, physiology, behavior

## PhD portfolio

**Wisse P. van der Meijden**

**PhD period:** April 2013 - September 2018

**PhD supervisors:** prof. dr. Eus J. W. van Someren, prof. dr. Patrice Bourgin, and prof. dr. Christian Cajochen

| **Courses** | **Year** | **ECTS** |
|---|---|---|
| Workshop Model-Based Cognitive Neurosciences (ABC) | 2014 | 0.4 |
| Multilevel Analysis (EpidM) | 2014 | 0.8 |
| Functional Neuroanatomy (ONWAR) | 2015 | 1.3 |
| Neuroimaging Tool-kit (Donders) | 2017 | 1.3 |

**Oral presentations**

| | | |
|---|---|---|
| NeuroTime Annual Meeting, Beuggen, Germany<br>*The effect of red and blue light exposure on pupil diameter and vigilance* | 2013 | 0.5 |
| NeuroSIPE Symposium, Doorn, The Netherlands<br>*Alertness promoting factors in health and disease* | 2013 | 0.5 |
| NeuroTime Annual Meeting, Amsterdam, The Netherlands<br>*The alerting effect of light: modification by behavioral state, circadian cycle and individual differences* | 2014 | 0.5 |
| NeuroTime Annual Meeting, Basel, Switzerland<br>*The alerting effect of light: modification by behavioral state, circadian cycle and individual differences* | 2015 | 0.5 |
| NeuroTime Annual Meeting, Strasbourg, France<br>*The alerting effect of light: modification by behavioral state, circadian cycle and individual differences* | 2016 | 0.5 |
| Chronotherapy Network Netherlands, Amsterdam, The Netherlands<br>*Individual differences in sleep timing relate to melanopsin-based phototransduction in healthy adolescents and young adults* | 2016 | 0.5 |
| NIN Neuroscience Symposium, Amsterdam, The Netherlands<br>*The alerting effect of light: modification by behavioral state, circadian cycle and individual differences* | 2017 | 0.5 |
| EBRS Congress, Amsterdam, The Netherlands<br>*Wavelength-dependent post-illumination effects on human physiology and behavior* | 2017 | 0.5 |

**Poster presentations**

| | | |
|---|---|---|
| World Congress on Sleep Medicine, Valencia, Spain | 2013 | 0.5 |
| *Time- and state-dependent analysis of autonomic control in narcolepsy: higher heart rate with normal heart rate variability* | | |
| ONWAR Annual Meeting, Zeist, The Netherlands | 2013 | 0.5 |
| *The effect of red and blue light exposure on pupil diameter and vigilance* | | |
| ONWAR Annual Meeting, Zeist, The Netherlands | 2014 | 0.5 |
| *The effect of red and blue light exposure on pupil diameter and alertness* | | |

**Supervised internships**

| | | |
|---|---|---|
| Brit Giesbertz, Master Biomedical Sciences | 2013 | 2 |
| *The effects of posture, light and skin temperature on vigilance* | | |
| Jessica Bruijel, Master Biomedical Sciences | 2014 | 2 |
| *The effects of blue and red light and antidepressants on ipRGC functioning* | | |
| Jesminne Castricum, Master Biomedical Sciences | 2015 | 2 |
| *The effect of different colors of light on sleep onset latency* | | |
| Liz Vink, Master Biomedical Sciences | 2015 | 2 |
| *The effects of red, green and blue light on pupil diameter and alertness* | | |
| Mana Fazel, Bachelor Psychobiology | 2016 | 1 |
| *Assessment of vigilance using pupillometry in type 1 and 2 narcolepsy* | | |
| Thomas Adrian, Bachelor Psychobiology | 2017 | 1 |
| *Post-illumination pupil response after blue light in type 1 narcolepsy: a case study* | | |
| Alexandra Heijdelberger, Bachelor Psychobiology | 2017 | 2 |
| *A beneficial effect of blue-light-blocking glasses on daytime performance in high school students* | | |
| Britt Bouter, Bachelor Psychobiology | 2017 | 2 |
| *Blue-light-blocking spectacles as a countermeasure for the effect of screen use on sleep quality in high school children: a combination of subjective and objective measures* | | |
| Laura Koster, Bachelor Psychobiology | 2018 | 2 |
| *The effects of blue light emitted from electronic devices on sleep quality and duration in high school students* | | |
| Yvonne Kuiper, Master Nutrition & Health | 2018 | 2 |
| *The relationship between sleep characteristics and the time of eating corrected for waking up times* | | |

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# Lumière, physiologie et comportement

**Résumé**

La lumière influence de manière importante de multiples aspects de notre physiologie et de nos comportements. L'information lumineuse non-visuelle est principalement médiée par un sous-type de cellules ganglionnaires de la rétine (ipRGCs) qui exprime un photopigment, la mélanopsine. La présence de ce photopigment, dont la sensibilité à la lumière est maximale dans le spectre de couleur bleue, rend ces cellules intrinsèquement photosensibles. La lumière exerce, via les ipRGCs, divers effets qui s'observent pendant la période d'exposition lumineuse, mais aussi après l'extinction de la lumière, affectant différents aspects du sommeil humain. Ce travail de thèse s'est intéressé aux effets de la lumière sur la physiologie et le comportement humain survenant après l'extinction de la lumière (post-illumination), en prenant en compte également la variabilité interindividuelle. Nous avons développé et validé un premier outil permettant d'évaluer la phototransduction mélanopsinergique chez l'homme, ce qui ouvre des perspectives d'applications très larges. Nous avons ainsi montré que les mesures obtenues (PIPR) étaient propres à chaque individu et corrélaient par exemple avec le chronotype des sujets. Enfin, nous rapportons un résultat original et novateur en montrant qu'une exposition à une lumière rouge intense facilite dans un deuxième temps (effet post illumination) la propension au sommeil. Enfin, l'ensemble de ces résultats encourage la réalisation de travaux futurs, notamment sur de plus grands échantillons de sujets, afin de mieux comprendre l'influence non-visuelle de la lumière sur la physiologie et le comportement humain. Nos observations incitent également à poursuivre l'évaluation des applications cliniques de la luminothérapie dans le traitement des troubles du sommeil.

**Mots-clefs:** Lumière, effets non-visuels, couleur de lumière, physiologie, comportement

**Abstract**

The non-image forming effects of environmental light are critically mediated by intrinsically photosensitive retinal ganglion cells (ipRGCs) that are characterized by their expression of melanopsin, which is a photopigment that is maximally responsive to light in the blue part of the spectrum. The ipRGC-mediated effects of light can typically endure far beyond light offset, with widespread functional consequences, including changes in various aspects of human sleep. The present thesis investigated such sleep-related post-illumination effects in the context of physiology and behavior. In chapter 2 and 3, we presented a post-illumination pupil response (PIPR) after blue light assessment method that yields a robust and feasible estimate of the functionality of an individual's intrinsic melanopsin-based phototransduction circuitry. In chapter 4, we showed that the PIPR after blue light was more pronounced in individuals with a later sleep timing. Following red light, sleep propensity is promoted, as indicated by our physiological and behavioral findings in chapter 5. Chapter 6 added to chapter 2 and 5 indicating that task demands and mental effort should be taken into account in order to correctly interpret changes in physiological and behavioral reflections of the central and autonomic nervous system. It appears timely to consider large-scale follow-up studies to contribute to the multivariate fingerprint of the non-image forming effects of light on human physiology and behavior and to further evaluate the clinical application of light therapy in the treatment of sleep disorders.

**Key words:** Light, non-image forming effects, wavelength-dependency, physiology, behavior