Modulations in irradiance directed at melanopsin, but not cone photoreceptors, reliably alter electrophysiological activity in the suprachiasmatic nucleus and circadian behaviour in mice

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Abstract

Intrinsically photosensitive retinal ganglion cells convey intrinsic, melanopsin-based, photoreceptive signals alongside those produced by rods and cones to the suprachiasmatic nucleus (SCN) circadian clock. To date, experimental data suggest that melanopsin plays a more significant role in measuring ambient light intensity than cone photoreception. Such studies have overwhelmingly used diffuse light stimuli, whereas light intensity in the world around us varies across space and time. Here, we investigated the extent to which melanopsin or cone signals support circadian irradiance measurements in the presence of naturalistic spatiotemporal variations in light intensity. To address this, we first presented high- and low-contrast movies to anaesthetised mice whilst recording extracellular electrophysiological activity from the SCN. Using a mouse line with altered cone sensitivity (Opn1mwR mice) and multispectral light sources we then selectively varied irradiance of the movies for specific photoreceptor classes. We found that steps in melanopic irradiance largely account for the light induced-changes in SCN activity over a range of starting light intensities and in the presence of spatiotemporal modulation. By contrast, cone-directed changes in irradiance only influenced SCN activity when spatiotemporal contrast was low. Consistent with these findings, under housing conditions where we could independently adjust irradiance for melanopsin versus cones, the period lengthening effects of constant light on circadian rhythms in behaviour were reliably determined by melanopic irradiance, regardless of irradiance for cones. These data add to the growing evidence that modulating effective irradiance for melanopsin is an effective strategy for controlling the circadian impact of light.

Keywords

behaviour, circadian, cone, constant light, electrophysiology, irradiance, melanopic lux, Melanopsin, radiance, suprachiasmatic nucleus
INTRODUCTION

Modern living and working practices result in daily patterns of light exposure, which differ substantially from those that the circadian system evolved under.1-3 Daytime light exposure is often low due to reduced exposure to natural daylight, whilst evening and night-time exposure is substantially increased due to the use of modern lighting and visual display technology.4,5 Collectively, such effects can disrupt circadian timing and impair sleep and are potentially associated with adverse health consequences.6-7 As such, there is now substantial interest in identifying ways of adjusting light exposure to support appropriate circadian entrainment and minimise the disruptive effects of light at night.

The simplest approach to adjust the circadian effects of light is simply to regulate overall light intensity (eg decreasing intensity over evening/night to minimise the phase-delaying effects or increasing it in the morning to maximise phase advances). Since, however, this is not always practical an alternate attractive possibility is to modulate spectral quality. The potential utility of such an approach stems from partial separability of the photoreceptor systems regulating the circadian response versus those required for visual perception. The brain region housing the master circadian clock, the suprachiasmatic nucleus (SCN), receives retinal projections from a specific subset of retinal ganglion cells (RGCs) that express the photopigment melanopsin, making them intrinsically photoreceptive (ipRGCs).8-11 Melanopsin is especially sensitive over a range of wavelengths (~480 nm) that are distinct from the cone photoreceptors responsible for much of our visual experience of the world.9,12-14 It follows that controlling energy over those wavelengths to which melanopsin is most sensitive should provide an attractive method for altering the “circadian efficiency” of light. Accordingly, there is substantial literature reporting that light containing more energy over shorter wavelengths is more effective for circadian responses15-20 (for review see refs 21,22). However, as the range of wavelengths over which cone photoreceptors are sensitive partly overlaps that of melanopsin, such modulations also typically alter effective intensity for one or more cone types and, therefore, other associated visual properties (eg “colour,” “brightness”). Since ipRGCs can also relay cone signals to the SCN (as reviewed23), Determining the extent to which effective light intensity reported by melanopsin and/or cones is an important determinant of the circadian impacts of light, therefore, has significant consequences for lighting design.

An international standard for quantifying effective intensity for melanopsin24 has recently been published, providing an opportunity to compare this parameter between light environments and to tailor the spectral content of light sources to maximise/minimise melanopic efficiency. It is thus important to know whether controlling “melanopic” exposure is an optimal strategy for regulating circadian responses and whether approaches changing some aspect of cone activity would likely be equally/more effective.

The colour science technique of receptor silent substitution represents an opportunity to determine melanopsin contributions to circadian light responses. In this approach, balancing changes in intensity across a number of wavelength bands are used to generate pairs of stimuli differing in effective intensity for only a subset (one or more) of the available photoreceptor class(es)25 (for review see ref. 26). This silent substitution strategy has been applied in a small number of human studies to show that selective increases in melanopic intensity can enhance light-dependent suppression of melatonin and increase alertness.27,28 Turning to more direct assays of the circadian light response, such melanopsin isolating stimuli drive increases in the firing rate of neurons in the suprachiasmatic nucleus (SCN, site of the master circadian oscillator) in mice.29,30 However, stimuli targeting only cone photoreceptors can also modulate SCN activity.29,30

While one function of cone input to the clock is to allow the daily variation in spectral composition (colour)
of ambient light to be used as a time-of-day cue, changes in effective intensity for cones (without a concomitant alteration in colour) also drive responses in the SCN. Circadian light responses are substantially disrupted in mice in which cones provide the only photoreception, while mice lacking cone function retain irradiance-dependent circadian responses. Conversely, a substantial contribution by cones to the circadian phase resetting effect of light in visually intact mice can be revealed using (trains of) short light pulses, consistent with electrophysiological evidence that cones primarily define the initial (500 ms⁻¹) component of the SCN response to abrupt changes in light intensity.

In summary, there is evidence for both melanopsin and cone contributions to the circadian response to changes in irradiance, with the degree of temporal contrast being a key determinant in their relative importance. One feature of natural light exposure that has not been explicitly investigated in those studies, is the presence of spatial patterns. Combined with changes in direction of gaze and object movement, these produce continuous spatiotemporal modulations in light falling on individual photoreceptors. Previous work revealing
cone-dependent circadian responses have used temporal contrast much greater than could be expected in nature.\textsuperscript{15,33,34} However, the use of stimuli devoid of spatial or temporal patterns in other circadian studies is also unnatural. This raises the question of whether melanopsin activity can account for a substantial fraction of the circadian-light response under more naturalistic conditions in which spatiotemporal contrast exists, but is substantially smaller than that previously used to reveal cone-dependent circadian responses. To this end, we set out to use the principles of receptor silent substitution to compare melanopsin and cone-driven circadian responses (SCN firing rate and changes in clock period) in the presence of realistic patterns of spatiotemporal contrast.

2 | MATERIALS AND METHODS

2.1 | Animals

All animal care and experimentation received institutional ethics committee and UK Home Office approval and were in accordance with regulations laid out in the UK Animals (Scientific Procedures) Act 1986, and the European Directive 2010/63/EU on the protection of animals used for scientific purposes. Male \textit{Opn1mw}~\textsuperscript{R} mice (RRID:MGI:2678771) were used for all experiments. All \textit{Opn1mw}\textsuperscript{R} mice were from a C57BL/6 background and had the murine middle wavelength cone opsin gene replaced by the human long-wavelength cone opsin gene, thereby shifting their spectral sensitivity from 508 to 556 nm.\textsuperscript{15,30,35} Food and water was available ad libitum. For electrophysiology male \textit{Opn1mw}\textsuperscript{R} mice aged >4 weeks were group housed on a reverse 12:12 light:dark cycles. Data were collected from mice aged between 6 and 31 weeks during their early subjective night when the SCNs electrical response to light is greatest.\textsuperscript{16} For the behavioural studies, male \textit{Opn1mw}\textsuperscript{R} mice aged 3-5 months were singly housed in wire top cages (32 × 16 × 14 cm) and locomotor activity was recorded using a passive infrared (PIR) system as previously described.\textsuperscript{36} The area directly under the food hopper and water bottle (10 × 16 × 14 cm) was blocked off, using a custom-made plastic barrier, so that the mouse was unable to enter these PIR dead spots.

2.2 | Light calibration

Light intensity was measured in 1 nm intervals between 380 and 780 nm using a calibrated spectroradiometer (DMC150; Bentham Instruments Ltd for the behavioural study and SpectroCal, Cambridge Research Systems for the electrophysiology studies). Each light channel was measured at a range of intensities to establish linear input-output relationships. These data were used to calculate the photon capture for each class of photoreceptor (Figures 1A, 2A and 3B) and the corresponding spectral compositions (Figures 1B, 2B and 3A) required to produce photoreceptor-specific light steps as previously described.\textsuperscript{31} In essence, we summed across each wavelength the number of photons produced by the stimuli, normalised to the Govardovskii nonogram\textsuperscript{37} for each photoreceptor (\(\lambda_{\text{max}}\): S cone opsin 365 nm, L cone opsin 556 nm, rhodopsin 498 nm, melanopsin 480 nm), and corrected for lens transmission.\textsuperscript{38} Melanopic equivalent daylight luminance (EDI) was calculated in accordance with CIE standard.\textsuperscript{24} In brief, spectral irradiance was weighted according to the sensitivity of melanopsin (\(\lambda_{\text{max}}\): 490 nm after adjusting for lens transmittance of the standard human observer) and corrected to obtain the lux value of natural (D65) daylight spectra that would provide an equivalent melanopic irradiance (0.754 lm mW\textsuperscript{-1}). Murine melanopic EDI was calculated using an identical method but replacing metrics for the human lens transmission with that of the murine lens transmission.\textsuperscript{38}

2.3 | In-vivo electrophysiology

2.3.1 | Surgery

Mice were anaesthetised with urethane (intraperitoneal injection 1.55 g kg\textsuperscript{-1}, topped up subcutaneously if required). Once fully under anaesthesia, the mouse was placed in a custom-made stereotaxic frame (Narishige) and the skull was fixed in position using bite and ear bars. The skull was exposed with a sagittal incision and a hole was drilled, 0.98 mm lateral to bregma. The dura was removed to expose the brain. The ipsilateral eye was covered.

A Buzsaki32L electrode (NeuroNexus Technologies) was coated in fluorescent dye (CM-DiI; Invitrogen) and inserted at an angle of 9° from the midline about the sagittal axis. The electrode was lowered using a micromanipulator (M0-10; Narishige) until slight flection was observed and then raised 50-100 \(\mu\)m. This led to a depth between 5.5 and 6 mm, which corresponds to the location of the SCN according to a stereotaxic mouse atlas.\textsuperscript{39} A test stimulus was presented to check for light responses. This test stimulus consisted of a 5 second light pulse (\(6 \times 10^{13}\) photons cm\textsuperscript{-2} s\textsuperscript{-1}; 10 second inter-stimulus interval) for 5–10 minutes. If no light responses were detected the electrode was repositioned. After detecting light responses mice were left for 30 minutes to allow brain activity to settle from possible trauma due to electrode placement. The core body temperature of the mouse was monitored and regulated using a homeothermic heat mat (Harvard Apparatus), and fluid was replenished with a 0.1 mL subcutaneous injection of Hartmann’s solution approximately every 2 hours. Data were recorded using Recorder64 (Plexon). Signals were amplified by a 20x gain AC-coupled headstage (Plexon) followed
by preamplifier conditioning providing a total gain of $3500\times$. Data were filtered using a high-pass Butterworth filter set at 300 Hz. Signals passing a 35 µV threshold were timestamped and their waveform digitised at a rate of 40 KHz. All data were stored on a hard drive for offline analysis.

2.3.2 | Light source

The movie was played in greyscale under various multispectral backlights through a custom-made projector as previously described$^{40}$ consisting of 4 primary LED light sources (Phat-light PT-120 Series (Luminus Devices), $\lambda_{\text{max}}$ and full width at half max (FWHM): 405 nm (FWHM = 15 nm), 455 nm (FWHM = 10 nm), 525 nm (FWHM = 25 nm), 630 nm (FWHM = 15 nm)). The intensities of the 4 LEDs were controlled by a ChipKit Uno32 microcontroller to produce several multispectral backlights (Figures 1B and 2B). Light intensity was reduced by attaching a combination of glass neutral density filters to the projector.

2.3.3 | Stimuli

A 5-second test pulse with 10-second inter-stimulus interval was used to identify electrode channels recording from light
responsive neurons. The test pulse was a spatially uniform presentation of the energy step that was used in the corresponding experiment. Multiunit signals detected at each channel were considered light-responsive if the average firing rate during the 5-second stimuli was statistically different from the preceding 5 seconds (Paired-T test, $P < .01$). To assess melanopsin and cone influences on SCN firing rate in the presence of spatiotemporal contrast we used two movie clips: (a) a low spatiotemporal contrast movie consisting of a web-browsing epoch captured using Bandicam (Bandisoft, Korea), (b) a high-spatiotemporal contrast movie recorded whilst traversing an urban environment. Both clips were rendered into monochrome and presented at 30 frames per second on a loop using video editing software (VSDC: Free video editor, Flash-Integro LLC). In the initial experiments (Figure 1), movie clip 1 only was run continuously, with the spectrum presented to the mouse changing upon every cycle of the movie (5 minutes duration). In subsequent experiments (Figure 2), which use both movie clips, a 30 seconds segment of each movie was looped with the spectral composition of the movie changing every 3 minutes (6 repeats of the movie clip).

To estimate the impact of the two movies on the variation in light input to the SCN over time, we modelled frame by frame variation in effective photon flux falling within the receptive field of typical SCN neurons (modelled as a 10° Gaussian, Figures 1D-E and 2E) at each point across the screen. Frame by frame variation in effective photon flux ranged from 0% to 88.6% and 0% to 53.7% for high- and low-contrast movies, with the probability of a >5% change occurring being 35× higher for the former (0.175 vs. 0.005, for high- and low-contrast movies, respectively, Figure 2F). In both cases, the overall irradiance of the movies varied little between frames (Figure 2D, 1.1 and 0.1% for high- and low-contrast movies, respectively).

To relate the movie to the visual experience of mice, we used a previously described method to calculate the spatial contrast, a mouse retinal ganglion cell with centre surround antagonism would experience when centred at a given point on each movie frame. To keep consistent with the temporal contrast analysis, we choose a 10-degree centre and a 15-degree surround receptive fields. Spatial contrast was calculated as follows:

$$\text{Contrast}_{ON-Off}(x, y) = \frac{I_{\text{Centre}}(x, y) - I_{\text{Surround}}(x, y)}{I_{\text{Centre}}(x, y) + I_{\text{Surround}}(x, y)}.$$
Where \( I_{\text{centre/surround}}(x,y) \) is the average pixel intensity at a point after convolving with either the centre (Figure S1B,G) or surround (Figure S1C,H) Gaussian filters. Across the entire movie, the magnitude of the spatial contrasts were greater in the high-contrast movie (Figure S1E,J, 95% of contrasts were below 4.5% and 10.5% in magnitude for low- and high-contrast movies, respectively). These values are consistent with “natural” images for mice which typically have a range up to 20%, with the majority falling below 10% contrast.42

One identified feature that distinguishes “natural” images from artificial images with equivalent spatial frequency composition is that luminance and contrast are largely independent in the former.43 To confirm the extent to which this property holds true for our naturalistic movies, we performed similar analysis to this previous study,\(^4\) to extract the relationship between spatial contrasts (\( \text{Contrast}_{\text{ON-OFF}} \)) and brightness (\( I_{\text{centre}} \), with data from each frame of the movie normalised according to the median contrast/brightness of that image (Figure S1K,N). From this distribution, the conditional probability was calculated for contrast at each given brightness (Figure S1L,O) and for brightness at each given contrast (Figure S1M,P). Consistent with previous findings,\(^4\) the distribution of luminance and contrast in the higher contrast movie was largely independent for contrasts and brightness values within ±0.75 log units of the median (~0.2 to 5 x median; indicated by near horizontal and vertical bands in Figure S1O,P, respectively). Across the whole range of observed values, the overall correlation between brightness and contrast in the high-contrast movie exhibited a weak negative correlation (average within frame correlation: Spearman's \( r = −0.27 \), Interquartile range \(-0.42 \text{ to } −0.17 \)), again consistent with previous findings (\( r = −0.2 \))\(^4\) and reflecting portions of the images taken up by sky where brightness was high but local contrast low. Equivalent properties were also true of the low-contrast movie (Figure S1K-M, average within frame correlation: \( r = −0.24 \), interquartile range \(-0.59 \text{ to } −0.22 \)), although the overall range of contrasts and, especially, brightness were compressed such that the extremes of the relationships seen previously in natural images (and in our high-contrast movies) were less represented.

\[ \text{Contrast} = \frac{I_{\text{ON}} - I_{\text{OFF}}}{I_{\text{centre}}} \]

\[ \text{Brightness} = I_{\text{centre}} \]

\[ r = \text{Spearman's rank correlation coefficient} \]

\[ \text{Interquartile range} = \text{The middle 50% of the data range} \]

**2.4 | Behaviour**

**2.4.1 | Light source**

The light-tight cabinet was illuminated from above using 4 custom-made lightboxes as previously described \(^3\). Each lightbox consisted of two smart RGBW LED bulbs (LIFX A60; LIFX) and six violet LEDs (peak emission 405 nm, Led Engin LZ1-00UA00-00U7; RS Components). The LIFX bulbs were connected wirelessly over a local network, UV bulbs were connected to LED drivers (T-Cube; Thorlabs) via a multichannel analogue output module (NI 9264; National Instruments). LED intensities were then controlled on a second by second basis using a PC running Python (2.7.10). Each box was fitted with a PTFE diffuser and light intensity could be reduced with the addition of neutral density gels (Lee Filters; Andover) which reduced intensity by a factor of 10-fold. The interior of the cabinet was painted white to provide uniform illumination.

**2.4.2 | Stimuli**

Starting with a background stimulus (Figure 3A,B: “M-C”), we designed additional stimuli by modulating the spectra such that light intensity was increased 6-fold for just rods and melanopsin (Figure 3A,B; “M+C”), L and S cones (Figure 3A,B; “M-C+”) or for all opsins (Figure 3A,B; “M+C+”).

**2.4.3 | Protocol**

Four batches of 6 male Opn1mwR mice (aged 3-8 months) were singly housed under constant light conditions for a month, whilst their locomotor activity was recorded using a passive infrared system. Two cohorts experienced either the M+C+ followed by the M+C- stimuli (or vice versa) such that changes in melanopic/rhodopic irradiance experienced by these animals were accompanied by opposing changes in cone-opic irradiance. By contrast, the other two cohorts experienced M-C- followed by M+C+ stimuli (or vice versa), such that changes in melanopic/rhodopic irradiance were accompanied by changes in cone-opic irradiance.

\[ P < .05 \]

\[ \text{One sample Wilcoxon’s Test against 0} \]

\[ \text{Post hoc Sidak’s multiple comparisons were performed wherever the relevant main ANOVA terms identified a significant effect at} \]

\[ \text{Six additional stimuli were designed by modulating the spectra such that light intensity was increased 6-fold for just rods and melanopsin (Figure 3A,B; “M+C”), L and S cones (Figure 3A,B; “M-C+”) or for all opsins (Figure 3A,B; “M+C+”).} \]

\[ \text{ALFA} \]

\[ \text{SPEARMAN’S RANK CORRELATION COEFFICIENT} \]

\[ \text{INTERQUARTILE RANGE} \]

\[ \text{LOG UNITS} \]

\[ \text{MEDIAN} \]

\[ \text{UNIFORM ILLUMINATION} \]

\[ \text{LUMINANCE AND CONTRAST IN THE HIGH-CONTRAST MOVIE} \]

\[ \text{WIDER RANGE OF OBSERVED VALUES} \]

\[ \text{CONTRASTS AND BRIGHTNESS VALUES WITHIN ±0.75 LOG UNITS OF THE MEDIAN} \]

\[ \text{HIGHER CONTRAST MOVIE WAS LARGELY INDEPENDENT FOR CONTRASTS AND BRIGHTNESS VALUES WITHIN ±0.75 LOG UNITS OF THE MEDIAN} \]

\[ \text{LOW- AND HIGH-CONTRAST MOVIES, RESPECTIVELY}. \]

\[ \text{CONSISTENT WITH PREVIOUS FINDINGS} \]

\[ \text{THE DISTRIBUTION OF LUMINANCE AND CONTRAST IN THE HIGHER CONTRAST MOVIE WAS LARGELY INDEPENDENT FOR CONTRASTS AND BRIGHTNESS VALUES WITHIN ±0.75 LOG UNITS OF THE MEDIAN} \]

\[ \text{PARTICULARLY TO THE PHOTORECEPTOR SPECIFIC STEPS} \]

\[ \text{THE MICE WERE ACCOMMODATED TO THE PHOTORECEPTOR SPECIFIC STEPS} \]

\[ \text{CONTRASTS AND BRIGHTNESS VALUES WITHIN ±0.75 LOG UNITS OF THE MEDIAN} \]

\[ \text{THE CONDITIONAL PROBABILITY WAS CALCULATED FOR CONTRAST AT EACH GIVEN BRIGHTNESS (FIGURE S1L,O) AND FOR BRIGHTNESS AT EACH GIVEN CONTRAST (FIGURE S1M,P).} \]

\[ \text{ACROSS THE WHOLE RANGE OF OBSERVED VALUES, THE OVERALL CORRELATION BETWEEN BRIGHTNESS AND CONTRAST IN THE HIGH-CONTRAST MOVIE EXHIBITED A WEAK NEGATIVE CORRELATION (AVERAGE WITHIN FRAME CORRELATION: SPEARMAN’S R = −0.27, INTERQUARTILE RANGE −0.42 TO −0.17), AGAIN CONSISTENT WITH PREVIOUS FINDINGS (R = −0.2).} \]

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2.4.4 | Passive Infrared system

The PIR system was created to the design detailed by Brown et al. The PIR sensor was positioned 22 cm above the cage and recorded the percentage of time where movement was detected (10-second bins with a 10 ms resolution).

2.4.5 | Analysis

Activity is presented as actograms using a custom-made MATLAB script, where activity was smoothed across 30 minutes. Chi-Squared periodogram analysis was run on a 13-day window of activity for each condition (Figure 3E,F). The first day of activity following a change in the lighting condition was omitted. Analysis using a mixed-effects linear model that included the order of stimulus as a random effect and stimulus type as a fixed effect was performed using SPSS. Graphs were produced in GraphPad prism (8.1.2, GraphPad Software Inc) and MATLAB.

3 | RESULTS

We first set out to investigate whether modulating melanopic irradiance represents a viable strategy to control SCN activity under naturalistic conditions, in which light is presented not as a diffuse stimulus as often is the case for circadian experiments, but including spatiotemporal patterns. Here, we introduced spatiotemporal contrast initially by superimposing light intensity, we used multispectral stimuli in conjunction with the sensitivity range of melanopsin than rods. This well-validated mouse line expresses the human long-wavelength sensitive opsin (556 nm) instead of the native murine middle wavelength sensitive cone opsin (508 nm). This shifts the peak wavelength sensitivity of the MWS cone away from that of rod opsin and melanopsin, allowing greater scope for modulating effective intensity for melanopsin independent of cones (and vice versa). We generated patterned visual stimuli using a bespoke DLP projector system, modified to accept light from 4 independently controllable, spectrally distinct, LEDs. In this way, we were able to adjust the spectrum of projected greyscale images in such a way as to produce an increase in effective intensity for melanopsin (~8-fold, 77% Michelson contrast) but not cones (“Mel” step Figure 1A,B), or a simple increase in brightness impacting all photoreceptors (“Energy step”). Note that although melanopsin-directed steps would also increase effective intensity for rods (Figure 1A), thanks to the similarity in spectral sensitivity of these photoreceptors, our stimuli are presented at or above the intensities expected to produce rod saturation. In this way, we could present intermittent “Mel” or matched “Energy” steps in light intensity across the scene, in the presence of ongoing variations in local radiance (Figure 1C-E). In addition, we could adjust baseline intensity by the inclusion of neural density (ND) filters in the projected light path to present these steps at three different starting irradiances.

Recording electrophysiological activity in the SCN of anaesthetised mice exposed to repeated “Mel” and “Energy” steps revealed responses to both stimuli (Figure 1F,G). In both cases, multiunit firing rates were higher during the step at the higher irradiances (One sample Wilcoxon’s Test, Melanopsin step: ND0 < .0001, ND1 < .0001, ND2 < .0001, Energy step: ND0 < .0001, ND1 < .0001, ND2 = .15). A mixed-effects linear model analysis was applied to determine whether there was a significant difference in the response to “Mel” versus “Energy” steps. This analyses returned a significant effect of irradiance (ND F = 7.52, P < .01), but not of stimulus type (Mel step vs. Energy Step F = 1.82, P = .31), nor of the interaction between irradiance and stimulus (ND x Stim F = 1.05, P = .37). As the “Mel” and “Energy” steps were matched for melanopsin contrast, it follows that the response to these irradiance steps could, to a first approximation, be accounted for by the action of melanopsin, with no detectable contribution from cones. The range of irradiances over which SCN responses became apparent is consistent with previous reports that the SCN is responsive across this irradiance range and more consistent with the sensitivity range of melanopsin than rods.

Given the evidence that cone influences on the circadian clock wane under extended light exposure, it was interesting that response magnitude decayed over the 5 minutes steps, especially at the highest background irradiance (Figure 1F,H). However, there was no suggestion that this reflected a change in the balance of cone versus melanopsin influences on firing, as responses to “Mel” steps declined across this period, as shown by comparing the first and last minute of the response to the step (Figure 1H, Mixed-effects linear model: ND F = 4.78, P = .03, 1st vs. 5th minute epoch F = 4.55, P = .08, ND x Epoch F = 6.60, P < .01, Sidaks Pairwise comparison: ND0: P < .01, ND1: P = .06, ND2: P = .79) and were equivalent to those elicited by “Energy” steps at even the earliest time points (Figure 1F insets).

The observation that the melanopsin and energy responses are comparable implies that cone-derived illuminance signals have little impact on SCN maintained firing rate in this paradigm. To directly address the effect of cones we designed a “cone” step, and associated contrast-matched “melanopsin” and “energy” steps (Figure 2A-C). In addition, to address the possibility that the level of spatiotemporal contrast in the
first experiment was too small to reveal a strong influence of cones, we set out to present these steps superimposed on movies with low versus high ongoing spatiotemporal contrast (movies of web browsing vs. walking around a suburban environment; Figure 2D-F). Based on the previous experiment, we decided to focus our efforts at the high background light intensities at which we had observed the largest responses.

Again, we found that both melanopsin and energy steps reliably increased SCN firing (Figure 2G). The impact of the cone step appeared to differ between low- and high-contrast stimuli and, indeed, there was a significant impact of movie contrast (and interaction between step type and contrast) on the response (Figure 2G,H, Mixed-effects linear model: Contrast: $F_{1,8,2} = 38.32, P < .0001$, Step type: $F_{2,26.6} = 2.26, P = .12$, Interaction Contrast x Step type: $F_{2,16.9} = 8.41, P < .01$). The level of spatiotemporal contrast had no noticeable impact on response to melanopsin steps (Figure 2H, Sidak’s Pairwise Comparisons, $P = .57$), but was a significant determinant of responses to cone and energy steps (Figure 2H, Sidak’s Pairwise Comparisons: Cones: $P < .0001$; Energy: $P < .0001$). Thus, the impact of both the cone and energy steps on SCN maintained firing was greater for the low-contrast movie (Figure 2G). The response of the SCN to cone steps (at least for the low-contrast movie) suggests that cones can make a substantial contribution to the SCN light response under these conditions, and indeed, we found that the energy response could be adequately approximated by the linear sum of the responses elicited by melanopsin and cone-directed steps (Figure 2H, Mixed-effects linear model: Energy vs. Linear Sum: $F_{1,25.1} = 0.39, P = .54$; Contrast: $F_{1,17.8} = 8.08, P = .01$, Interaction: $F_{1,25.1} = 0.01, P = .92$). In summary, this second experiment revealed that a cone illumination signal impacted SCN firing under the low- but not a high-contrast movie, while the influence of melanopsin was consistent across conditions.

Our electrophysiological recordings from the SCN thus indicate that in the presence of ongoing spatiotemporal contrast, increases in melanopic irradiance consistently elevate SCN firing rates, whereas increases in “cone-opic” irradiance excite the SCN less consistently. To determine the relative importance of the irradiance signals from the two photoreceptors for circadian control of behaviour, we used the silent substitution approach to generate background lights that allowed us to test the impact of increasing “melanopic” irradiance alongside simultaneous increases or decreases in “cone-opic” irradiance (Figure 3A,B). The free-running period ($\tau$) of circadian behavioural rhythms in mice (including the OpnlmR$^R$ line$^{15,31}$) lengthens in a predictable manner as a function of light intensity.$^{52}$ Thus, we would expect that the stimuli that have a higher effective circadian intensity would produce a longer $\tau$. Initially, mice were housed under a light with relatively high “melanopic” and low “cone-opic” intensity (M+C-) before being switched to “cone-opic” high and “melanopic” low lighting (M-C+) or vice versa (Figure 3C,E). In almost every case ($n = 11/12$), $\tau$ was longer under the high melanopic, low cone-opic, stimulus (Figure 3G) implying that melanopsin is the dominant influence under these conditions. To put this in context, we then compared how $\tau$ lengthens when melanopic and cone-opic irradiance changed together (M-C- and M+C+, Figure 3D,F). A comparison across all conditions was consistent with the conclusion that the $\tau$ lengthening effect is driven by melanopic irradiance (Figure 3G Mixed-effects linear model: Melanopic Irradiance: $F_{1,43.0} = 19.24, P < .0001$), with cone-opic irradiance having negligible effect on $\tau$, either on its own (Conopic Irradiance: $F_{1,43.0} = 0.10, P = .76$), or by modulating the effect of melanopsin (Interaction Melanopic x Conopic Irradiance: $F_{1,43.0} = 0.17, P = .68$). Irrespective of the order of presentation, there was a clear effect of melanopic irradiance on $\tau$ (Sidak’s Pairwise Comparisons: M-C-> M+C+: $P < .001$, M+C- -> M-C+: $P < .001$, M-C- -> M+C+: $P < .01$, M+C+ -> M-C+: $P < .01$).

## DISCUSSION

Previous studies addressing the relative importance of cone versus melanopsin irradiance signals on circadian responses have primarily employed light exposure paradigms devoid of spatial patterns (eg ganzfeld domes or diffusing filters). Here, we address this question under more naturalistic conditions in which any change in background lighting is superimposed upon ongoing variations in local light intensity across the visual scene. Our most important finding is that modulations in spectral content targeting melanopic irradiance represent a reliable method for controlling circadian light responses under more naturalistic conditions. Thus, melanopsin steps increased SCN firing when superimposed upon movies depicting different degrees of ongoing spatiotemporal contrast and across a range of background light conditions. Furthermore, in our behavioural experiments, the circadian period lengthening effects of light were defined by its melanopic irradiance irrespective of concurrent changes in effective intensity for cones. These findings imply that the most reliable influences on the mouse circadian clock (across the light intensities studied here) come from melanopsin. Although, in principle, the present findings may not directly translate to humans, these data re-enforce the growing evidence that modulating melanopic irradiance is likely to be an effective strategy for adjusting the circadian impact of light exposure in humans.$^{21,27,28}$

Our data do not provide a clear resolution of the question of the extent to which cone measures of ambient light intensity are an important influence on the clock. Our previous work revealed that changes in colour detected by cones (ie,
the ratio of L- vs. S-cone activation) modulate the circadian response to light in mice but suggested that irradiance signals provided by cones were relatively unimportant for circadian measures of ambient light intensity.31 Consistent with those findings, of the 5 circumstances in which we presented light steps in our electrophysiological experiments, only one revealed a measurable cone influence on SCN firing. Thus, the magnitude of responses to melanopsin steps was equivalent to those of matched “energy” steps (that also impacted cones) across the three background light intensities in experiment 1 and for the high-contrast movie in experiment 2. Only for the low-contrast movie in experiment 2 were energy responses higher, and a response to cone steps apparent. The implication from experiment 2 that higher levels of ongoing spatiotemporal contrast actually inhibit the ability of cones to impact SCN firing is counterintuitive but may reflect contrast adaptation, which is a known feature of cone vision.53,54 Low spatiotemporal contrast though is not sufficient to reveal cone responses, as we did not detect an effect of cones under experiment 1 which also employed a low-contrast movie. A number of differences between the two experiments could explain the divergent outcome including the magnitude of the step (smaller in experiment 2) the background “colour” (invariant across all steps, but different between experiments 1 and 2) or the background irradiance. In the case of the latter, effective irradiance for cones in experiment 2 was largely contained within the range tested in the first experiment, although the effective irradiance for melanopsin was higher in experiment 2. Alongside the degree of spatiotemporal contrast, any of the factors listed above (alone or in combination) could, in principle, modulate the extent to which cone-opic irradiance might influence SCN activity.

The other photoreceptors that likely do play an important role in circadian measures of ambient light intensity are rods.15,55 In this study, all of the stimuli designed to present steps in melanopsin would also have presented steps in rods. We describe the responses here in terms of melanopsin photoreception because we are working at light intensities at which rods are expected to be at or near saturation (while acknowledging that rods may be active at surprisingly high light levels56). We have not attempted to discriminate rod from melanopsin responses partly because the similarity in their spectral sensitivity (λ_max melanopsin = 480 nm, rod opsin = 498 nm in mice) makes this difficult to achieve using silent substitution, but also because the distinction has little practical relevance for lighting design. As rod opsin and melanopsin are sensitive to similar parts of the spectrum, effective intensity for these receptors strongly covaries for any broad-spectrum real-world light exposure, and any practical lighting design targeting melanopic irradiance would have nearly equivalent impact on rods.

How do the stimuli presented here compare with real world levels of light exposure? In our electrophysiological experiments, the melanopic output of the projector system was comparable to that of a standard laptop (Toshiba, satellite pro, range: 12.7-13.8 log_10 melanopic photons cm^-2 s^-1 vs. the projector: 13.3 log_10 melanopic photons cm^-2 s^-1). Similarly, melanopic irradiance in our electrophysiological and behavioural experiments was within the physiological range. To facilitate comparisons with human exposure, we adapted the concept of melanopic equivalent daylight illumination (“melanopic EDI”) established in the CIE S026 standard24 to mice, by using the mouse melanopic efficiency function. Melanopic EDI describes the illumination, in photopic lux, of daylight required to achieve an equivalent melanopic irradiance. The melanopic EDI for our behavioural and electrophysiological experiments were 17.8 and 20.8 lux, respectively, which equates to ~30 lux of a 4000 k fluorescent light source and is in line with levels typically experienced in the home environment.5

It should be noted that whilst the constant light paradigm is useful for dissecting photoreceptor contributions due to the linear relationship between light intensity and period, it can be disruptive to both activity levels and rhythm strength, especially when animals are housed in constant light for extended periods of time. Here, we specifically designed the experiment to mitigate against this by keeping exposure comparatively brief and controlling for order effects. It would also be informative in the future studies to assess the extent to which cone vs. melanopic irradiance signals influence entrainment. In principle, the silent substitution approaches employed here could be extended to such studies, however, limitations in the degree in which day vs. night-time irradiances can be selectively modulated for specific photoreceptor classes may pose a practical impediment to how informative such studies will be.

In conclusion, modulating melanopic irradiance is an effective method for altering the impact of light on the circadian system in mice. By contrast, although we did find evidence for a cone contribution to irradiance responses, this was not reliable across experimental conditions. Further investigation is needed to determine whether the results shown here in mice are indeed translatable across Mammalia, including humans. However, alongside previous studies in humans,21,27,28 these data support the practice of modulating melanopic irradiance as a method for altering the circadian impact of artificial light.

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AUTHOR CONTRIBUTION
RJL, TMB and JWM designed the experiments. FM and JWM constructed and calibrated the experimental
apparatus. JWM performed the experiments. JWM, and TMB performed the analysis. TMB, RJL and JWM wrote the manuscript.

DATA AVAILABILITY STATEMENT
Raw data and analysis code will be provided upon request by the Lead Contact, Tim Brown (timothy.brown@manchester.ac.uk).

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