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Ambient light detection is important for the synchronization of the circadian clock to the external solar cycle. Light signals are sent to the suprachiasmatic nuclei (SCN), the site of the major circadian pacemaker. It has been assumed that cone photoreceptors contribute minimally to synchronization. Here, however, we find that cone photoreceptors are sufficient for mediating entrainment and transmitting photic information to the SCN, as evaluated in mice that have only cones as functional photoreceptors. Using in vivo electrophysiological recordings in the SCN of freely moving cone-only mice, we observed light responses in SCN neuronal activity in response to 60-s pulses of both ultraviolet (UV) (λ_{max} 365 nm) and green (λ_{max} 505 nm) light. Higher irradiances of UV light led to irradiance-dependent enhancements in SCN neuronal activity, whereas higher irradiances of green light led to a reduction in the sustained response with only the transient response remaining. Responses in SCN neuronal activity decayed with a half-max time of ~9 min for UV light and less than a minute for green light, indicating differential input between short-wavelength–sensitive and mid-wavelength–sensitive cones for the SCN responsiveness. Furthermore, we show that UV light is more effective for photoentrainment than green light. Based on the lack of a full sustained response in cone-only mice, we confirmed that rapidly alternating light levels, rather than slowly alternating light, caused substantial phase shifts. Together, our data provide strong evidence that cone types contribute to photoentrainment and differentially affect the electrical activity levels of the SCN.

suprachiasmatic nucleus | cones | photoentrainment | in vivo electrophysiology | circadian

In addition to rods and cones, light is also sensed in the retina by a specialized subset of melanopsin-containing intrinsically photosensitive retinal ganglion cells (ipRGCs). ipRGCs incorporate input from rod and cone photoreceptors (1). Two types of cone photoreceptors are present in the murine retina: short-wavelength–sensitive cones (S-cones; λ_{max} 360 nm), which are maximally sensitive to ultraviolet (UV) light, and mid-wavelength–sensitive cones (M-cones; λ_{max} 508 nm), which are maximally sensitive to green light (2). The ipRGCs project to the suprachiasmatic nuclei (SCN) of the hypothalamus, hereby subserving photoentrainment of the major pacemaker for circadian rhythms in physiology and behavior (3). The ablation of ipRGCs results in the loss of photoentrainment of circadian rhythms to the environmental light–dark (LD) cycle (1). Although rod and cone photoreceptors are not essential for entrainment of the biological clock to the external LD cycle, both rod and cone photoreceptors influence the SCN, which is evidenced by the experimental finding that mice can entrain to an LD cycle in the absence of melanopsin (4, 5). In addition, recordings in SCN of melanopsin-deficient mice show preservation of sustained light responses in the SCN, the magnitude of which seems unaffected by the absence of melanopsin (6, 7).

Whereas rods are capable of driving photoentrainment at a wide range of light intensities (8), the majority of cone-only ($Opn4^{-/-}Gnat1^{-/-}$) mice, which lack melanopsin and functional rod signaling, show surprisingly large interindividual differences in their ability to entrain to LD cycles of white light, and some of them exhibit a positive phase angle of entrainment (9, 10). However, phase-shifting responses in mice lacking M-cones are attenuated (11, 12), which contradicts the reduced ability of $Opn4^{-/-}Gnat1^{-/-}$ mice to entrain to an LD cycle. The question is, therefore, to what extent cones contribute to photic entrainment and whether S- and M-cones contribute similarly to the entrainment of the circadian clock.

Photoentrainment is dependent on light-induced changes in SCN neuronal activity (13, 14). Typically, SCN neurons respond to light with a transient increase in SCN electrical activity followed by a sustained component throughout light exposure. Together, rod and cone photoreceptors can mediate light responses at the level of the SCN, including both the fast and the sustained components (6, 7, 15). These findings are consistent with rod- and cone-mediated responses recorded in ipRGCs (16–18). In this study, we determined the specific contribution of the S- and M-cone photoreceptors to circadian photoreception. We performed behavioral and in vivo electrophysiological recordings

Significance

Synchronization of our biological clocks to the environmental day–night cycle critically depends on daily exposure to light. Here, we show that cones transmit distinct photic information to the clock by performing recordings of clock neurons in freely moving mice with cones as their only photoreceptors. This is in contrast to the expectation that exclusively melanopsin and rods fulfil this role. Moreover, we show that especially short-wavelength–sensitive cones as compared to mid-wavelength–sensitive cones are important. The evidence for a role for cones implicates that clocks respond to a broad spectrum of colors rather than to blue light, which can be used to strengthen the clock in humans.

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in $Opn4^{-/-}Gnat1^{-/-}$ mice to determine the effects of λ_{max} 365 nm (UV) and λ_{max} 505 nm (green) light on photoentrainment and on light-induced responses in electrical activity of SCN neurons. Furthermore, we assessed the capability of $Opn4^{-/-}Gnat1^{-/-}$ mice to phase shift in response to intermittent monochromatic light pulses aimed to stimulate the UV- or green-sensitive cones.

Results

Behavioral Photoentrainment. Photoentrainment was tested in wild-type and $Opn4^{-/-}Gnat1^{-/-}$ mice under 12:12 LD cycles using either UV or green light during the light phase at relatively high irradiances (13 log photons/cm²/s, Fig. 1 *A* and *B* and *SI Appendix,* Fig. S2). Under green LD cycles, 13 out of 15 $Opn4^{-/-}Gnat1^{-/-}$ mice (87%) showed entrainment, while 2 out of 15 mice (13%) were not able to entrain to the LD cycle (Fig. 1*D*). A total of 6 out of 15 (40%) entrained $Opn4^{-/-}Gnat1^{-/-}$ mice showed a normal phase angle of entrainment (<1 h), whereas a total of 7 out of 15 mice (47%) entrained with a large positive phase angle of entrainment (>1 h). For UV light, all $Opn4^{-/-}Gnat1^{-/-}$ mice (13/13) entrained with only one mouse showing a positive phase angle of entrainment (>1 h; Fig. 1*D*). All wild-type mice entrained to UV and green light, with phase angles of entrainment of 6.9 ± 2.9 min and 0 ± 0 min, respectively (Fig. 1 *C* and *E*). Most of the

$Opn4^{-/-}Gnat1^{-/-}$ mice entrained to green light, albeit with a large phase angle of entrainment (164 ± 55 min). This was significantly larger than phase shifts in response to UV light (12 ± 5 min, Fig. 1*E*, Welch's *t* test *P* = 0.017). When exposed to constant darkness, animals started to free-run from the phase set by the preceding LD cycle (*SI Appendix,* Fig. S3). These results show that both S- and M-cones play a role in photic entrainment and that S-cones are sufficient to mediate normal entrainment.

In Vivo Electrophysiology. To determine the underlying mechanisms of why S-cones entrain animals with a smaller phase angle than M-cones, we carried out in vivo electrophysiological recordings from the SCN in wild-type (*n* = 4) and $Opn4^{-/-}Gnat1^{-/-}$ mice (*n* = 4). We investigated the effects of light on the response properties of a subset of SCN neurons by exposing wild-type and $Opn4^{-/-}Gnat1^{-/-}$ mice to 60 s of varying intensities of UV (λ_{max} 365 nm; 12.7 to 14.5 log photons/cm²/s) and green (λ_{max} 505 nm; 13.3 to 14.9 log photons/cm²/s) light. Exposure to 60 s of monochromatic light of both wavelengths led to a robust increase in SCN neuronal activity with a typical transient overshoot (transient "on-excitation") in electrical discharge rate followed by a sustained component for the duration of lights on in both wild-type and $Opn4^{-/-}Gnat1^{-/-}$ mice. These response kinetics are characteristic
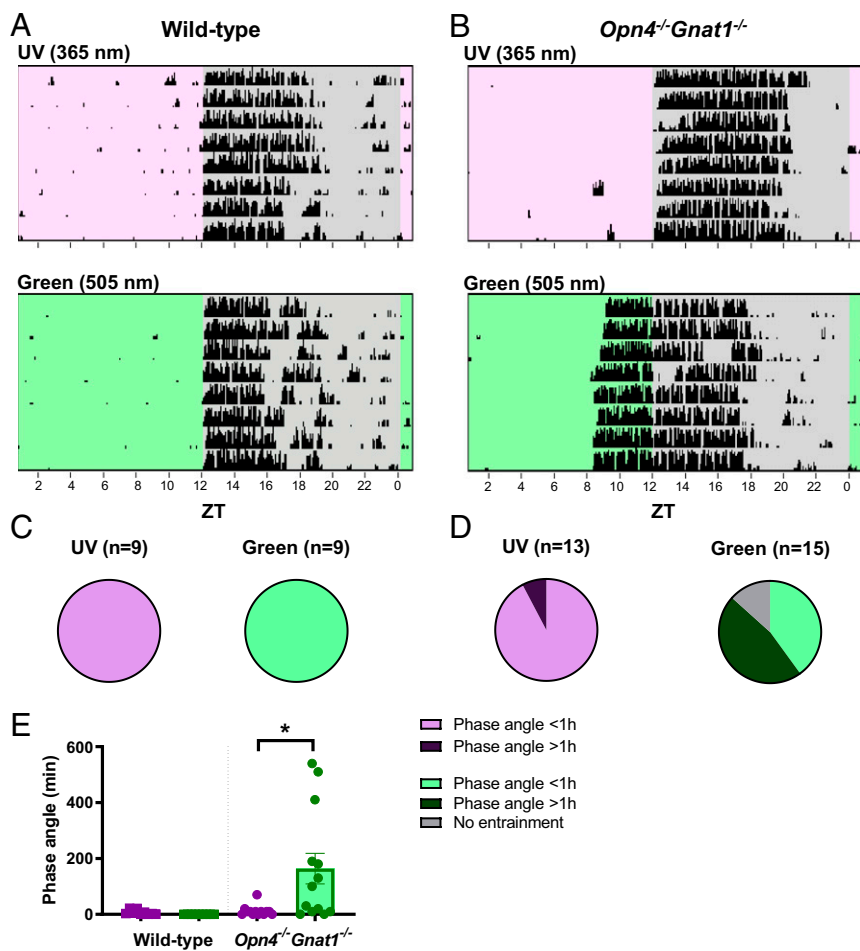
Fig. 1. Behavioral activity recordings of entrainment to LD cycles. (*A*) A representative example of actograms of a wild-type and (*B*) an $Opn4^{-/-}Gnat1^{-/-}$ mouse kept on a 12:12 LD cycle. The gray shading indicates the dark phase, and the purple- and green-colored backgrounds indicate the light phase with UV (λ_{max} 365 nm) and green light (λ_{max} 505 nm), respectively. The examples shown were from the same animals for UV and green light. Behavioral activity was measured using running-wheel activity; the black bars indicate when the mouse runs in a wheel, and the bin size is 5 min. (*C*) Pie charts of entrainment of wild-type animals and (*D*) $Opn4^{-/-}Gnat1^{-/-}$ animals. (*E*) Phase angle differences during 12:12 LD cycles compared to lights off in $Opn4^{-/-}Gnat1^{-/-}$ mice during UV (λ_{max} 365 nm) or green (λ_{max} 505 nm) light exposure (*P* = 0.017). **P* < 0.05.

for the SCN (19), and in the remaining analysis, we distinguish between the sustained response to light and transient responses.

In agreement with the photoentrainment experiments, UV light exposure had a more robust activation of the SCN neurons compared to green light. Specifically, exposure to UV light pulses resulted in sustained increments in SCN electrical discharge rate in wild-type (Fig. 2A) and $Opn4^{-/-}Gnat1^{-/-}$ mice (Fig. 2B) that were not different (Fig. 2C). Both wild-type mice and $Opn4^{-/-}Gnat1^{-/-}$ mice displayed transient on-excitations (Fig. 2D).

In contrast, for green light, the sustained response caused by high irradiance of light (14.9 log photons/cm^2/s) was significantly larger in wild-type mice (Fig. 3A) compared to $Opn4^{-/-}Gnat1^{-/-}$ mice ($P = 0.011$; Fig. 3 B and C; representative examples shown are from the same animals as for the UV light examples in Fig. 2). No differences were observed in the transient on-excitation of wild-type and $Opn4^{-/-}Gnat1^{-/-}$ mice (Fig. 3D). A linear mixed-effects model showed a significant color effect ($P = 0.002$) for the magnitudes of the sustained responses. For $Opn4^{-/-}Gnat1^{-/-}$ mice, the sustained response difference between UV and green light at the highest intensities was significant (245.9 $\pm$ 72.6 Hz; $P = 0.010$).

The remarkable difference between UV and green light in both photoentrainment and the 1-min light pulses prompted us to investigate the SCN firing for a prolonged light stimulation of 15 min (Fig. 4 A and B). For green light, the magnitude of the light response and the duration of the sustained response were significantly larger in wild-type mice compared to $Opn4^{-/-}Gnat1^{-/-}$ mice ($P = 0.041$ and $P < 0.001$, respectively; Fig. 4 C and D). However, the sustained responses were much higher in the UV compared to the green lights in $Opn4^{-/-}Gnat1^{-/-}$ mice. Specifically, 15-min pulses of UV light caused sustained light-induced increases in SCN neuronal activity with half-max times of 524.6 $\pm$ 200.4 s and 356.0 $\pm$ 216.8 s, respectively, in $Opn4^{-/-}Gnat1^{-/-}$ and wild-type mice, whereas 15-min pulses of green light led to light-induced responses that decayed to baseline levels quickly with half-max times of 24.6 $\pm$ 13 s compared to 747.0 $\pm$ 102.4 s in wild types. These results show that S-cones can maintain light responses in the SCN for prolonged periods of time, whereas M-cones fail at maintaining such responses, probably due to adaptation.

Phase Shifts. To investigate whether light adaptation plays a role in $Opn4^{-/-}Gnat1^{-/-}$ mice, we exposed mice to either high- or low-frequency pulses of white light for 15 min at circadian time (CT) 15.0 $\pm$ 0.14 h to assess the phase-shifting capacity (Fig. 5 A and B). In response to 60-s pulse trains, $Opn4^{-/-}Gnat1^{-/-}$ mice did not show significant shifts (0.15 $\pm$ 0.08 h; one-sample t test; $P = 0.11$), but when exposed to high-frequency 5-s pulse trains, significantly larger shifts were observed (0.77 $\pm$ 0.10 h; $P = 0.001$; Fig. 5C). In wild-type mice, 60-s pulse trains caused phase shifts of 1.53 $\pm$ 0.10 h and 5-s pulse trains of 1.57 $\pm$ 0.12 h. These shifts were not significantly different (paired t test; $P = 0.83$; Fig. 5C) but were significantly larger than phase shifts in $Opn4^{-/-}Gnat1^{-/-}$ mice (both groups $P < 0.001$).

Our SCN recordings in $Opn4^{-/-}Gnat1^{-/-}$ mice revealed that UV light triggered sustained responses, whereas green light triggered transient responses. Therefore, we tested whether exposure to fast and slow light-pulse trains of UV and green light resulted in significant phase shifts. Wild-type animals showed phase shifts to both UV (fast 0.74 $\pm$ 0.11 h; $P = 0.0001$; one-sample t test, slow 0.68 $\pm$ 0.13 h; $P = 0.0009$) and green light (fast 1.41 $\pm$ 0.08 h; $P < 0.0001$, slow 1.54 $\pm$ 0.15 h; $P < 0.0001$, Fig. 6 A and C). $Opn4^{-/-}Gnat1^{-/-}$ mice showed phase shifts to UV for both fast (0.52 $\pm$ 0.12 h; $P = 0.005$; one-sample t test) and slow light-pulse trains (0.48 $\pm$ 0.15 h; $P = 0.021$), whereas green light showed significant phase shifts to fast (0.59 $\pm$ 0.12 h; $P = 0.001$) but not slow light-pulse trains (0.28 $\pm$ 0.17 h; $P = 0.153$; Fig. 6 B and C). Opn4$^{-/-}$Gnat1$^{-/-}$ mice, phase shifts to green 5-s light trains were not significantly larger than for green 60-s light trains ($P = 0.1415$; t test).
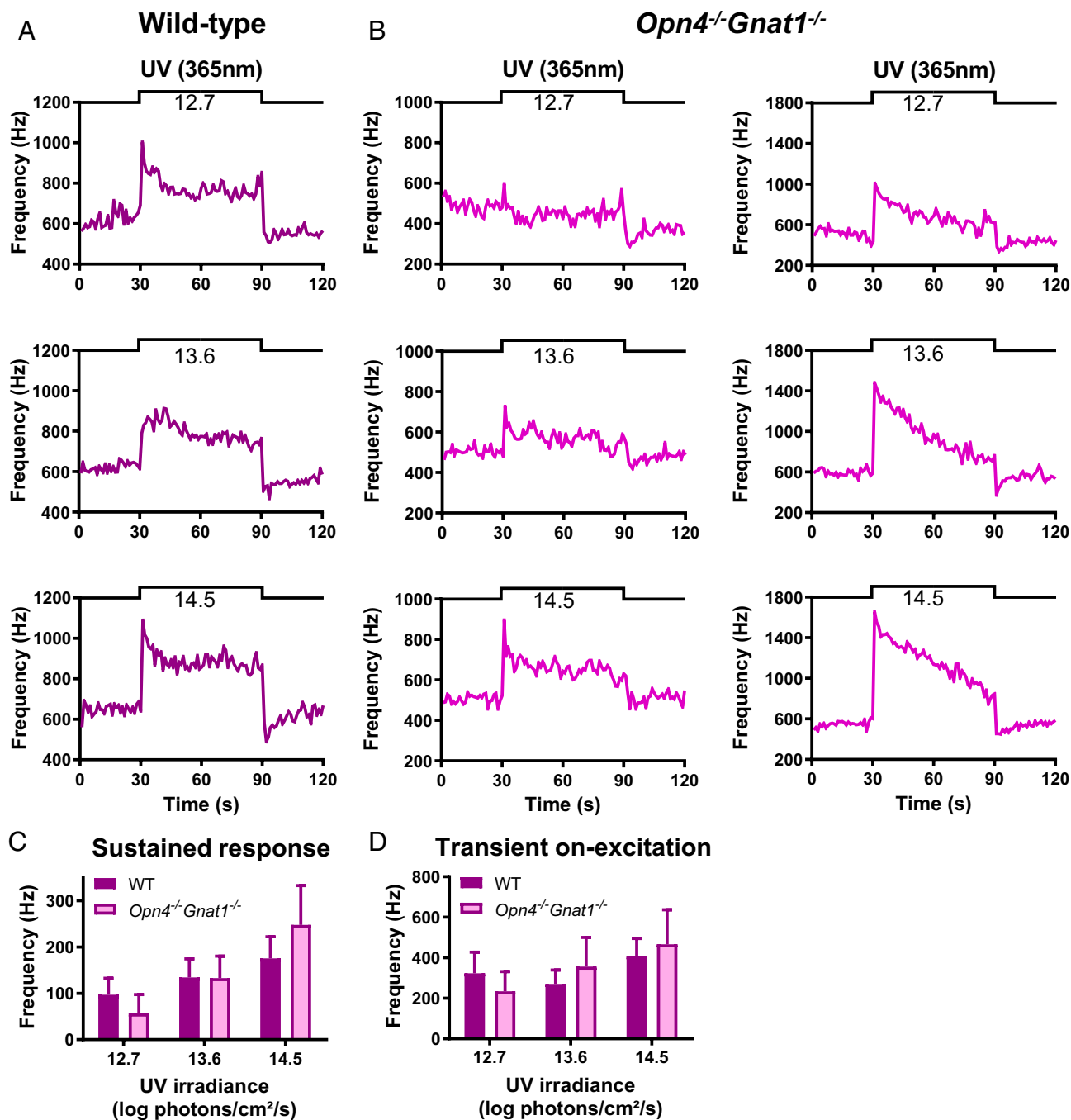
Discussion

In behavioral experiments, we observed that $Opn4^{-/-}Gnat1^{-/-}$ mice entrained to green light of 505 nm stimulating primarily M-cones. However, the phase angle of entrainment was larger than in wild-type mice. Under UV light of 365 nm stimulating primarily S-cones, the vast majority of animals showed normal entrainment in a way that was indistinguishable from entrainment in wild-type mice. Electrophysiological recordings of SCN neurons in freely moving animals showed transient responses to green light with half-max times of 24.6 $\pm$ 13.0 s (Fig. 4D). UV light appeared fully capable of mediating sustained increases in electrical discharge. Exposure to light-pulse trains that should prevent cone adaptation enhances the ability of phase shifting to light.

All $Opn4^{-/-}Gnat1^{-/-}$ mice entrained to LD cycles of UV light, from which only one entrained with an advanced phase angle. When maintained on LD cycles of green light, 13% of $Opn4^{-/-}Gnat1^{-/-}$ mice did not entrain. The other mice entrained normally (40%) or entrained with a large phase angle (47%; Fig. 1D). On average, phase angles of entrainment were significantly larger for green light than for UV light (Fig. 1E). The advanced phase angle of entrainment is a theoretical necessity to entrain to the environment when the sensitivity to external light is low and a larger delaying part of the phase response curve needs to be stimulated by light in order to achieve the required amount of correction (20). Therefore, the advanced phase angle of entrainment observed under green light could indicate a lower effectiveness of green versus UV light in photic entrainment, comparable to dim light versus bright light (21, 22). This interpretation is consistent with our electrophysiological data. Another potential explanation for the large phase angle could be a change in the shape of the phase response curve. Exposure to light pulses of a longer duration affects both the amplitude and shape of the phase response curve (23). As retinal mutant mice show occasionally more activity during the day as compared to wild-type mice, the daytime activity could alternatively reflect impaired masking responses (9).

We observed light-induced increases in SCN electrical activity in response to all light intensities of UV light investigated. These responses were of the sustained type. No significant differences were found between $Opn4^{-/-}Gnat1^{-/-}$ and wild-type mice in the SCN responses to UV light (Fig. 2). Additionally, we observed transient on-excitations in response to all intensities of UV light in both wild-type and $Opn4^{-/-}Gnat1^{-/-}$ mice with no significant difference between the groups. Previous studies using retinal transgenic mice showed that rod signaling contributes to and is sufficient for photoentrainment (8). A potential role for cones has been implicated but not unambiguously proven (6, 7). Here, we show that photoentrainment and SCN light responsiveness is preserved in mice that lack both melanopsin and rod signaling and have cones as their only photoreceptors. Hence, all photoreceptors are, by themselves, sufficient for photoentrainment.

In contrast to UV light, the highest intensity of green light investigated failed to elicit sustained responses in SCN firing rate in $Opn4^{-/-}Gnat1^{-/-}$ mice (Fig. 3 B and C). The absence of this sustained response in $Opn4^{-/-}Gnat1^{-/-}$ mice may be explained by desensitization of M-cones at high irradiances (10). In wild-type mice, light pulses of high irradiance of green light did lead to large-magnitude sustained responses in SCN neuronal activity, which are probably driven by melanopsin-containing ipRGCs (λ_{max} 471 nm) and/or rods (6, 10, 24).
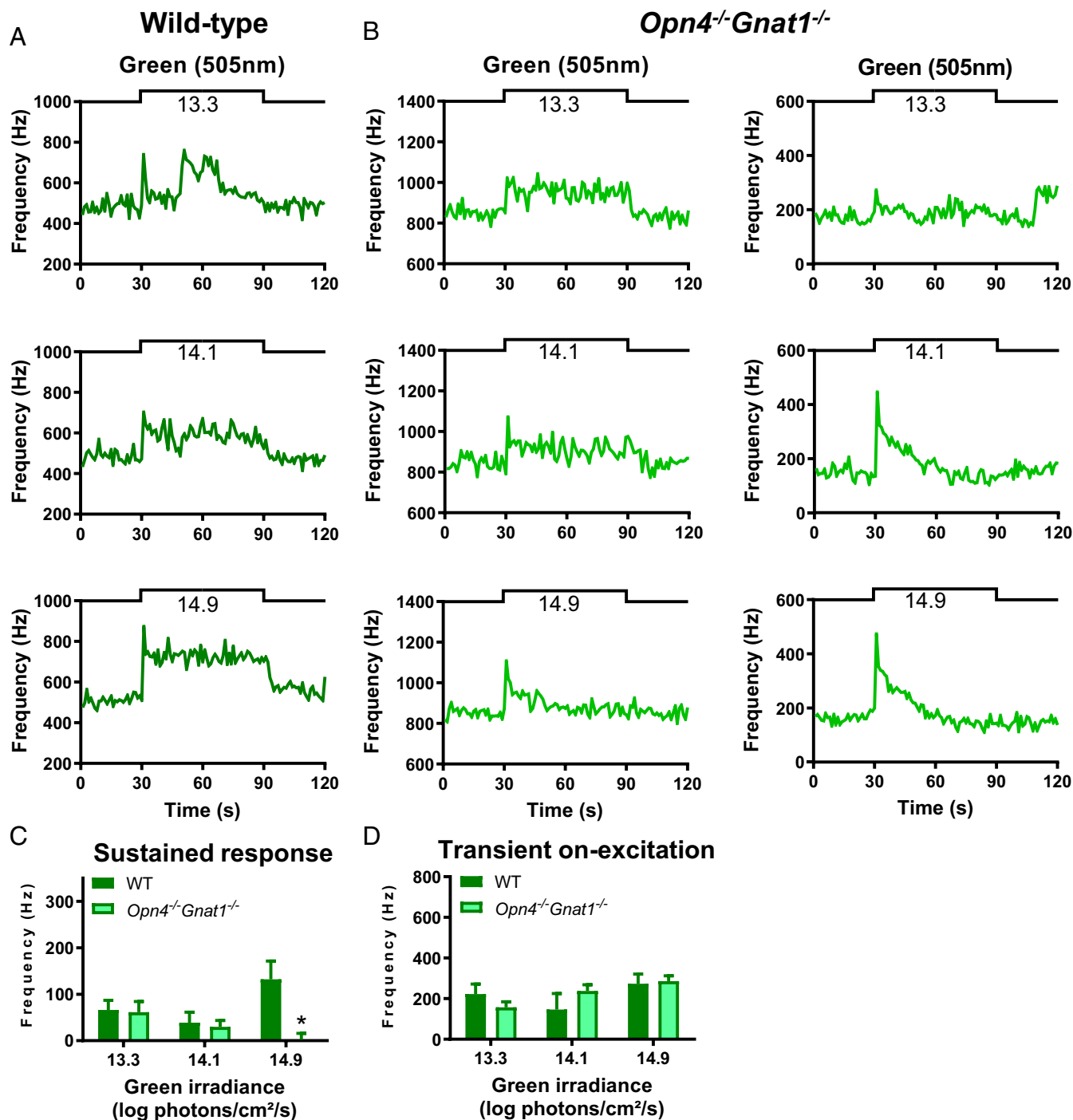
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Fig. 2. SCN in vivo electrophysiological responses to 1-min UV (λ_{max} 365 nm) light exposure. (*A*) Wild-type and (*B*) $Opn4^{-/-}Gnat1^{-/-}$ mice were exposed to 1-min UV light pulses indicated in a step diagram above each graph. For wild-type mice, one representative trace of SCN electrical activity is depicted per irradiance, with the photon flux shown on top of each graph as log photons/cm²/s. Representative traces are shown for two $Opn4^{-/-}Gnat1^{-/-}$ mice. Bin size is 1 s. (*C*) Bar graphs showing mean ($\pm$ SEM) sustained responses in SCN electrical activity as a function of irradiance of UV light in wild type (filled bars, $n = 4$) and in $Opn4^{-/-}Gnat1^{-/-}$ ($n = 4$). (*D*) No significant differences were found in transient on-excitations.

The impaired photoentrainment to green light correlates with the absence of sustained SCN responses in the electrophysiological data. The intensity of green light was likely inadequate to fully elicit sustained SCN responses, as our electrophysiological recordings showed that low but not high intensities of green light elicit small, sustained levels of elevated electrical activity (Fig. 3*C*). These findings provide evidence that M-cones are able to contribute to photoentrainment but with a reduced capacity compared to wild-type mice. In previous studies, $Opn4^{-/-}Gnat1^{-/-}$ mice

showed impaired photoentrainment or photoentrainment with advanced phase angles, similar to results from green light in this study (9, 10). These studies made use of LD cycles with white incandescent light tubes, which generally lack UV light. Our data suggests that the photoentrainment of $Opn4^{-/-}Gnat1^{-/-}$ mice in white light will significantly improve when UV is present in the spectrum, as it would be in an outdoor setting.

We presented trains with 60 or 5 s of UV or green light to $Opn4^{-/-}Gnat1^{-/-}$ mice in order to trigger the transient responses

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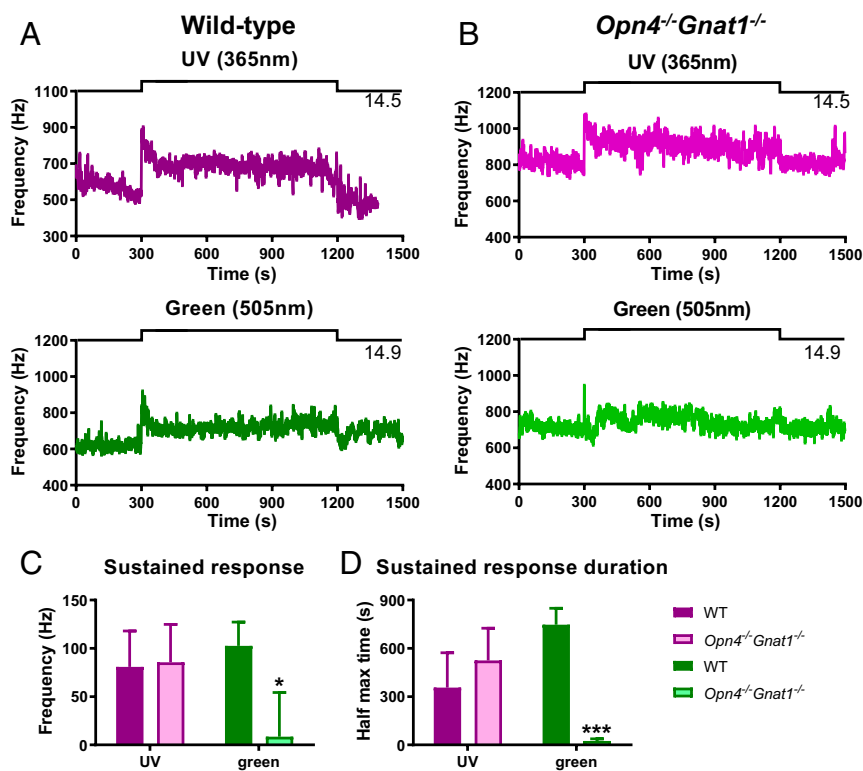Distinct contribution of cone photoreceptor subtypes to the mammalian biological clock

Fig. 3. SCN in vivo electrophysiological responses to 1-min green (λ_{max} 505 nm) light exposure. (A) Wild-type and (B) $Opn4^{-/-}Gnat1^{-/-}$ mice were exposed to 1-min green light pulses indicated in a step diagram above each graph. For wild-type mice, one representative trace of SCN electrical activity is depicted per irradiance, with the photon flux shown on top of each graph as log photons/cm²/s. Representative traces are shown for two $Opn4^{-/-}Gnat1^{-/-}$ mice. The examples shown here for green light were from the same animals as the examples for UV light in Fig. 2. Bin size is 1 s. (C) Bar graphs showing mean (± SEM) sustained responses in SCN electrical activity as a function of irradiance of green light in wild type (filled bars, $n = 4$) and in $Opn4^{-/-}Gnat1^{-/-}$ ($n = 4$). At 14.9 log photons/cm²/s of green light, the light response is significantly different between wild-type and $Opn4^{-/-}Gnat1^{-/-}$ mice (independent t test, $P = 0.011$). (D) No significant differences were found in transient on-excitations.

repeatedly. In all conditions, we observed significant phase shifts with the exception of green 60-s pulse trains. We attribute this to the fact that green light pulses elicit only short elevations in firing rate. Previous studies showed that mice lacking the M-cones exhibit reduced phase-shifting capacity in response to long-wavelength light, while the response to short-wavelength light

was not altered (11, 12). The differences in phase-shifting responses were only present in response to short light pulses (1 and 5 min), a fact which, in correspondence with our study, indicates a role for M-cones in the initial part of light detection. More evidence that cones contribute significantly during exposure to short discontinuous light stimuli was observed in studies with

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Fig. 4. SCN in vivo electrophysiological responses to UV (λ_{max} 365 nm) or green (λ_{max} 505 nm) light exposure of 15 min. (*A*) Wild-type (upper traces) and (*B*) *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ (lower traces) mice were exposed to 15-min light pulses indicated in a step diagram above each graph. One representative trace of SCN electrical activity is shown per group and per wavelength, with the photon flux shown next to each graph as log photons/cm²/s. Bin size is 1 s. (*C*) Bar graphs showing mean ($\pm$ SEM) light response in SCN firing frequency caused by 15-min light pulses of UV or green light in wild-type (*n* = 4) and *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ (*n* = 5) mice. Sustained light responses induced by 15 min of green light are significantly smaller in *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ compared to wild-type mice (independent *t* test, *P* = 0.041). (*D*) Bar graphs showing mean ($\pm$ SEM) half-max time of the light response in SCN-firing frequency caused by 15-min light pulses of UV or green light in wild-type (*n* = 4) and *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ (*n* = 5) mice. The duration of the sustained response is significantly shorter in *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ than in wild-type mice (independent *t* test, *P* < 0.0001). **P* < 0.05; ***P < 0.0001.
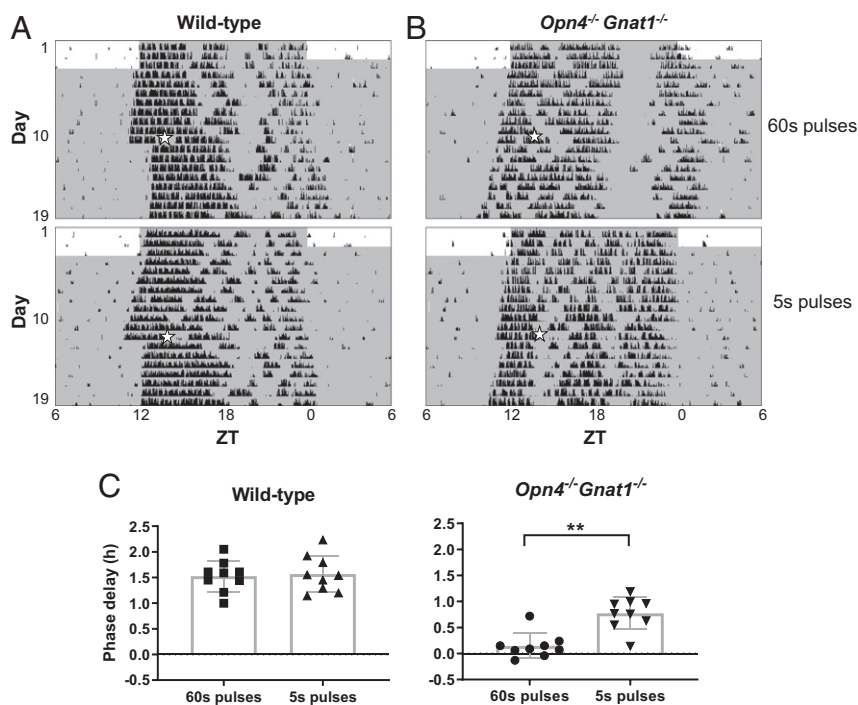
long-wavelength–sensitive cone (L-cone) knockin mice (*Opn1mw*^{R}), in which the mouse M-cones are replaced by the human L-cones. Long-wavelength red light as series of 1-min pulses led to significantly larger phase shifts in behavioral activity compared to a continuous light stimulus of 15 min (10). In both studies, 1-min light pulses were sufficient to phase shift the behavioral rhythm, while in this study, *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice did not show phase shifts in response to 1-min light pulses (Fig. 5 *B* and *C*). A possible explanation is that the irradiance in this study (15.2 log photons/cm²/s) was higher than in the previous studies (both less than 15 log photons/cm²/s) (10, 11). Our SCN recordings revealed that, surprisingly, the response magnitude to green light diminished for higher light intensities.

In addition to their ability to sense fast changes in light intensity, cones seem to provide information on slow changes in spectral composition as well (25). In vivo electrophysiological measurement in SCN of *Opn1mw*^{R} mice revealed distinct responses in SCN neuronal activity induced by the activation of S- or L-cones. Moreover, the circadian entrainment of the *Opn1mw*^{R} mice improved in LD cycles with natural spectrum twilight as opposed to LD cycles with twilight, which lacked changes in color, or to rectangular LD cycles, indicating a biological relevance of "color vision" by the SCN (26). Our findings are difficult to reconcile with findings from a study using a silent substitution paradigm. In this study, higher L-cone stimulation, rather than S-cone stimulation, led to larger circadian responses (27). Yet, like our study, there is a consistency in the finding that cones contribute to photic entrainment.

It has been questioned whether rod signaling is completely absent in the *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mouse model (28, 29). Any residual rod responses in this mouse model are likely to be negligible, as in our study, the mice showed largest sensitivity to UV light compared to green light, while rods are most sensitive to green light. If residual rod activity would have been present, the difference between the UV and green response in *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice would have been opposite to what we observed, and responses to green light would have been larger than to UV light.

The majority of murine cone photoreceptors coexpress S-opsin and M-opsin (30) in reverse dorsal–ventral gradients, with the highest S-opsin expression in the ventral retina and highest M-opsin expression in the dorsal retina (31). A small population of murine cone photoreceptors in the retina expresses S-opsin exclusively (32). Relative expression of S-opsin and M-opsin determines light sensitivity of the cone photoreceptors. Cone photoreceptors can be classified as S-cones if their phototransduction is mainly driven by S-opsins, whereas phototransduction in M-cones is predominantly activated by M-opsins (2).

The melanopsin photopigment plays a crucial role in regulation of the sustained response to light. More recently, it became evident that rod and cone photoreceptors also contribute to sustained light signaling to the SCN (6, 7, 15, 19). Our data show that, in the absence of both melanopsin and rod photoreceptor signaling, cone subtypes have distinct contributions to photoentrainment and SCN electrical activity. The implication is that the clock of species with relatively more cones, such as humans,

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Fig. 5. Behavioral phase shifts to intermittent white-light exposure. (*A*) Representative single-plotted actograms of wild-type and (*B*) *Opn4**Gnat1* mice exposed to 60- or 5-s light pulses for 15 min. The time of the light-pulse train is indicated with a white star. The examples shown were from the same animals for 60- and 5-s pulses. (*C*) Mean (± SEM) phase shifts of wild-type mice (*n* = 9) and *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice are plotted. In *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice, 5-s pulses cause significantly larger phase shifts than 60-s pulses ($P = 0.001$), whereas the different pulse durations do not result in significantly altered phase delays in wild-type mice ($P = 0.83$). ▲, ▼, ■, and ● indicate individual data points; **$P = 0.001$.

shows a broad spectral sensitivity range. This can potentially be used to strengthen their circadian system.

In laboratory studies, cages are often designed to ensure uniform illumination levels to obtain properly defined experimental conditions. In nature, animals are expected to be exposed to large fluctuations in illumination levels due to the movement of the animal in and out of the shade or burrow. Also, in human daily life, abrupt alterations in light intensity are normally perceived (33). Therefore, cone photoreceptors may have a larger contribution to photoentrainment in nature than what is expected from laboratory studies.
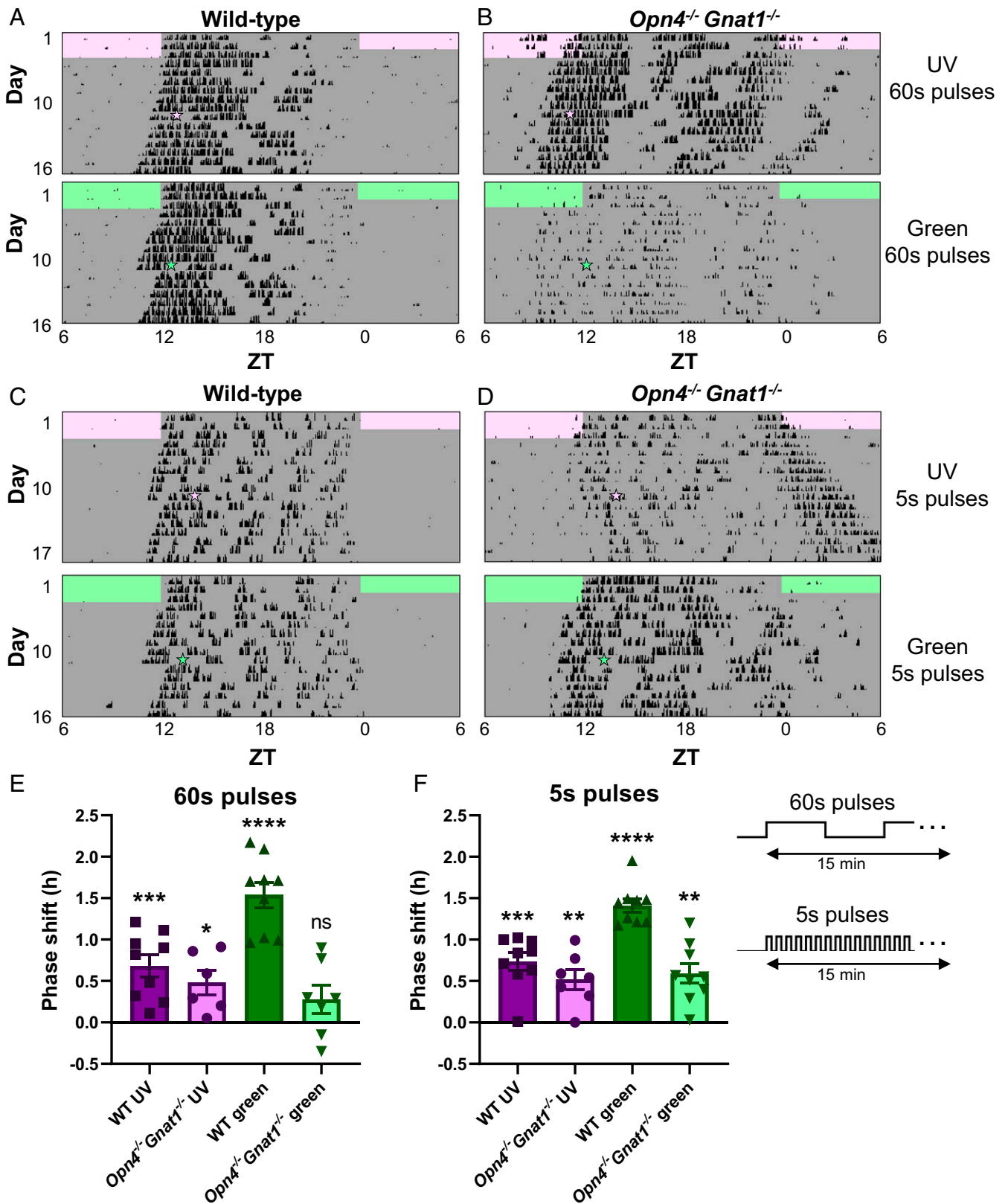
Materials and Methods

Animals. Experiments were approved by the ethical committee of the Leiden University Medical Center and were carried out according to their guidelines. *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice originated from the laboratory of Samer Hattar (Johns Hopkins University, Baltimore) and were backcrossed on a C57/Bl6 background at the Leiden University Medical Center. Experiments were carried out with male mice (ages 3 to 10 mo) with homozygous knockouts of the *Opn4* and *Gnat1* genes. Wild-type animals (ages 3 to 10 mo) from the same background were obtained from Charles River Laboratories to minimize differences apart from potential developmental alterations.

Behavioral Activity Recordings. Behavioral activity rhythms were assessed using running wheels and passive infrared sensors. Wild-type (*n* = 9) and *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice (*n* = 16) were housed individually in plastic cages, which were equipped with running wheels to stimulate active behavior of the mice in a measurable way. Cases of insufficient running-wheel activity to detect behavioral activity rhythms were excluded from the analysis. The number of rotations of the wheel was recorded using a ClockLab data acquisition system (Actimetrics) and stored on a computer in 1-min bins. Food and water were available ad libitum during the experiment. Mice were housed in a 12:12 LD schedule. All animals were exposed to LD cycles with UV light (365 nm, NCSU033B, Nichia) and LD cycles with green light (505 nm, LXML-PE01-0070, RS Company) during the light phase at an irradiance of 13 log photons/cm²/s.

To assess phase-shifting capacity, naive wild-type and *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice (both *n* = 9) on 12:12 LD cycles for 10 d were released in continuous darkness for 7 to 8 d. At CT15.0 ± 0.14 h, corresponding to the delaying part of the phase response curve (7), mice were exposed to a train of white-light pulses (15.2 log photons/cm²/s; 40 to 800 nm; Truelight 18W5500K) in a time window of 15 min. These trains consisted of either 60- or 5-s pulses, with 60- or 5-s intervals in between the pulses, respectively. Phase shifting in response to UV (365 nm) and green light (505 nm) was assessed using the same light-pulse parameters as described above for white light. Naive wild-type and *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice (both *n* = 18) were entrained to 12:12 LD cycles of UV and then released in constant darkness for 7 d. Mice were exposed to either fast (*n* = 9 per group) or slow light pulses of UV light (*n* = 9 per group) followed by 7 d of constant darkness. The animals exposed to fast UV light pulses repeated the same protocol with fast green light pulses, and the animals exposed to slow UV pulses were exposed to slow green pulses. Data were excluded if mice showed insufficient wheel running to detect a behavioral activity rhythm or if the timing of the light pulse was more than 3 h from CT15. Similar irradiances were used as in the photoentrainment experiments (13 log photons/cm²/s). The resulting behavioral phase shifts were quantified by fitting regression lines through the activity onsets before and after the light pulses and calculating the difference between the lines at the day of the light pulse. The first day after the light pulse was excluded for analysis to account for transients.

In Vivo Electrophysiological Experiments. Extracellular activity of SCN neurons was recorded in freely moving wild-type and *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice (both *n* = 4). Tripolar stainless steel electrodes (Plastics One) were implanted in an anesthetized mouse. The mouse was fixed in a stereotactic frame (Stoelting), and the electrodes were implanted in the brain, targeting the SCN. Two polyimide-insulated twisted electrodes were aimed at the SCN under a 5° angle 0.61 mm lateral from the bregma and 5.38 mm ventral to the dura and used for differential recording. The third uncoated electrode was placed in the cortex as a reference. The electrodes were fixed to the skull of the mouse with the use of additional screws and dental cement. After a week of recovery, mice were connected to a custom-designed recording chamber to measure extracellular activity of SCN neurons while the animal was able to move freely. The electrical signal was amplified, and the bandwidth was

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Fig. 6. Behavioral phase shifts to intermittent UV and green light exposure. (*A*) Representative single-plotted actograms of wild-type and (*B*) *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice exposed to 60- (*C* and *D*) or 5-s UV and green light pulses for 15 min. The time of the light-pulse train is indicated with a purple or green star. (*E*) Bar graphs of the phase-shift magnitude in response to 60-s and (*F*) 5-s light pulses. All groups showed significant phase shifts to trains of 5-s UV or green light and to trains of 60-s of UV light. Trains of 60-s of green light did not evoke a significant shift in *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice ($P = 0.153$). ns = nonsignificant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. ■, ●, ▲, and ▼ indicate individual data points.

filtered between 0.5 and 5 kHz. The differential amplifier was based on the design of Yamazaki et al. (34). The electrical SCN signal was digitized at 25 kHz using Spike2 hardware and software (Cambridge Electronic Design) and stored for offline analysis.

A custom-designed sphere was introduced in the recording setup to expose the animal to specific monochromatic wavelengths of light. The diameter of the sphere was 30 cm, and it was coated with high-reflectance paint (barium sulfate; WRC-680; Labsphere Inc.) to ensure uniform illumination levels. At the top of the sphere, high-power UV (365 nm, NCSU033B, Nichia) or green (505 nm, LXML-PE01-0070, RS Company) LEDs were positioned at the top of the sphere with a baffle to prevent the animal directly looking into the light source. The wavelength and intensity of light were measured with a calibrated spectrometer (AvaSpec2048, Avantes). Animals were exposed to near-monochromatic light of λ_{max} 365 ± 4.5 nm (UV) and λ_{max} 505 ± 25 nm (green) aimed at CT15, which corresponds to the light-sensitive zone of the phase response curve (13). For both colors, mice were first exposed to a 1-min pulse of each irradiance, starting with the lowest intensity pulse and ending with the highest followed by a 15-min light pulse. The order of different colors was randomized, and between all light pulses were darkness intervals of 9 min. Animals were exposed to all light pulses on the same day.

For detailed analysis of the response characteristics to light, the digitized recordings were imported in MATLAB as waveform data using parts of the sigTOOL SON Library, including data from light sensors. Imported waveform data were triggered at fixed voltage amplitude settings. Time and amplitude of the action potentials were used for analysis. Digitized action potentials were counted in 1-s bins. For a detailed analysis of population activity, a baseline recording was used to create spike amplitude histograms. On the basis of this amplitude histogram, thresholds were set in such a way that the average number of counts within each threshold window were equal. Threshold windows were nonoverlapping and started above noise level. Action potentials were counted within each step of a set threshold for the remainder of the recording.

For the 60-s light pulses, the magnitude of the sustained response was defined as the difference between the multiunit activity (MUA) average of the complete pulse excluding the initial 15 s and the MUA average of the 30 s of baseline preceding the light pulse. The transient on-excitation was calculated as the difference between the MUA average in 30 s of baseline before the light pulse and the first second of the light pulse.

For the 15-min pulses, the sustained response was defined as the difference between the average MUA during the light pulse with the first 15 s excluded and the average MUA during 100 s of baseline preceding the light pulse. Sustained response duration was quantified as the last time point at which the MUA did not decline below the half-maximum value for more than 30 s uninterrupted, with the half-maximum value calculated as the average between the mean MUA in the first 3 s of the light pulse and 100 s of baseline.

Histology. At the end of each recording mice were killed in a CO_2 chamber. A small electrolytic current was passed through the electrodes to mark the recording site. Brains were taken out and kept in 4% paraformaldehyde solution containing potassium ferrocyanide. The brains were sectioned coronally and checked with a microscope for reconstruction of the recording site (*SI Appendix*, Fig. S1).

Statistics. For comparing the phase angles of entrainment in behavioral data, Welch's *t* test was used. Electrophysiological data were analyzed using a linear mixed-effects model to test for differences between colors while correcting for genotype and light intensity. The magnitude of the MUA response was used as a dependent variable, and light intensity and color were specified as repeated variables. In phase-shifting assays, we used paired *t* tests to assess differences between phase shifts in response to fast or slow light-pulse trains. One-sample *t* tests were used to show whether phase shifts were significantly different from 0 min.

Data Availability. Data can be found in Dataset S1.

1. A. D. Güler et al., Melanopsin cells are the principal conduits for rod-cone input to non-image-forming vision. *Nature* **453**, 102–105 (2008).
2. S. S. Nikonov, R. Kholodenko, J. Lem, E. N. Pugh Jr, Physiological features of the S- and M-cone photoreceptors of wild-type mice from single-cell recordings. *J. Gen. Physiol.* **127**, 359–374 (2006).
3. R. Y. Moore, N. J. Lenn, A retinohypothalamic projection in the rat. *J. Comp. Neurol.* **146**, 1–14 (1972).
4. N. F. Ruby et al., Role of melanopsin in circadian responses to light. *Science* **298**, 2211–2213 (2002).
5. S. Panda et al., Melanopsin (Opn4) requirement for normal light-induced circadian phase shifting. *Science* **298**, 2213–2216 (2002).
6. H. C. van Diepen, A. Ramkisoensing, S. N. Peirson, R. G. Foster, J. H. Meijer, Irradiance encoding in the suprachiasmatic nuclei by rod and cone photoreceptors. *FASEB J.* **27**, 4204–4212 (2013).
7. F. van Oosterhout et al., Ultraviolet light provides a major input to non-image-forming light detection in mice. *Curr. Biol.* **22**, 1397–1402 (2012).
8. C. M. Altimus et al., Rod photoreceptors drive circadian photoentrainment across a wide range of light intensities. *Nat. Neurosci.* **13**, 1107–1112 (2010).
9. N. Mrosovsky, S. Hattar, Diurnal mice (Mus musculus) and other examples of temporal niche switching. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* **191**, 1011–1024 (2005).
10. G. S. Lall et al., Distinct contributions of rod, cone, and melanopsin photoreceptors to encoding irradiance. *Neuron* **66**, 417–428 (2010).
11. O. Dkhissi-Benyahya, C. Gronfier, W. De Vanssay, F. Flamant, H. M. Cooper, Modeling the role of mid-wavelength cones in circadian responses to light. *Neuron* **53**, 677–687 (2007).
12. A. Dollet, U. Albrecht, H. M. Cooper, O. Dkhissi-Benyahya, Cones are required for normal temporal responses to light of phase shifts and clock gene expression. *Chronobiol. Int.* **27**, 768–781 (2010).
13. J. H. Meijer, B. Rusak, G. Gänshirt, The relation between light-induced discharge in the suprachiasmatic nucleus and phase shifts of hamster circadian rhythms. *Brain Res.* **598**, 257–263 (1992).
14. T. M. Brown, J. Wynne, H. D. Piggins, R. J. Lucas, Multiple hypothalamic cell populations encoding distinct visual information. *J. Physiol.* **589**, 1173–1194 (2011).
15. N. C. Aggelopoulos, H. Meissl, Responses of neurones of the rat suprachiasmatic nucleus to retinal illumination under photopic and scotopic conditions. *J. Physiol.* **523**, 211–222 (2000).
16. K. Y. Wong, A retinal ganglion cell that can signal irradiance continuously for 10 hours. *J. Neurosci.* **32**, 11478–11485 (2012).
17. S. Weng, M. E. Estevez, D. M. Berson, Mouse ganglion-cell photoreceptors are driven by the most sensitive rod pathway and by both types of cones. *PLoS One* **8**, e66480 (2013).
18. T. M. Schmidt, P. Kofuji, Differential cone pathway influence on intrinsically photosensitive retinal ganglion cell subtypes. *J. Neurosci.* **30**, 16262–16271 (2010).
19. E. Drouyer, C. Rieux, R. A. Hut, H. M. Cooper, Responses of suprachiasmatic nucleus neurons to light and dark adaptation: Relative contributions of melanopsin and rod-cone inputs. *J. Neurosci.* **27**, 9623–9631 (2007).
20. D. E. Nelson, J. S. Takahashi, Sensitivity and integration in a visual pathway for circadian entrainment in the hamster (Mesocricetus auratus). *J. Physiol.* **439**, 115–145 (1991).
21. J. Aschoff, R. Wever, Uber Phasenbeziehungen zwischen biologischer Tagesperiodik und Zeitgeberperiodik. *Z. Vgl. Physiol.* **46**, 115–128 (1962).
22. J. Aschoff, "The phase-angle difference in circadian periodicity" in *Circadian Clocks*, J. Aschoff, Ed. (North-Holland, Amsterdam, 1965), pp. 262–276.
23. M. Comas, D. G. Beersma, K. Spoelstra, S. Daan, Phase and period responses of the circadian system of mice (Mus musculus) to light stimuli of different duration. *J. Biol. Rhythms* **21**, 362–372 (2006).
24. A. J. Emanuel, M. T. Do, Melanopsin tristability for sustained and broadband phototransduction. *Neuron* **85**, 1043–1055 (2015).
25. T. M. Brown, Using light to tell the time of day: Sensory coding in the mammalian circadian visual network. *J. Exp. Biol.* **219**, 1779–1792 (2016).
26. L. Walmsley et al., Colour as a signal for entraining the mammalian circadian clock. *PLoS Biol.* **13**, e1002127 (2015).
27. J. W. Mouland, F. Martial, A. Watson, R. J. Lucas, T. M. Brown, Cones support alignment to an inconsistent world by suppressing mouse circadian responses to the blue colors associated with twilight. *Curr. Biol.* **29**, 4260–4267.e4 (2019).
28. A. E. Allen, M. A. Cameron, T. M. Brown, A. A. Vugler, R. J. Lucas, Visual responses in mice lacking critical components of all known retinal phototransduction cascades. *PLoS One* **5**, e15063 (2010).
29. P. D. Calvert et al., Phototransduction in transgenic mice after targeted deletion of the rod transducin alpha -subunit. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 13913–13918 (2000).
30. M. L. Applebury et al., The murine cone photoreceptor: A single cone type expresses both S and M opsins with retinal spatial patterning. *Neuron* **27**, 513–523 (2000).
31. S. Hughes, T. S. Watson, R. G. Foster, S. N. Peirson, M. W. Hankins, Nonuniform distribution and spectral tuning of photosensitive retinal ganglion cells of the mouse retina. *Curr. Biol.* **23**, 1696–1701 (2013).
32. S. Haverkamp et al., The primordial, blue-cone color system of the mouse retina. *J. Neurosci.* **25**, 5438–5445 (2005).
33. R. Dobb et al., The impact of temporal modulations in irradiance under light adapted conditions on the mouse suprachiasmatic nuclei (SCN). *Sci. Rep.* **7**, 10582 (2017).
34. S. Yamazaki, M. C. Kerbeshian, C. G. Hocker, G. D. Block, M. Menaker, Rhythmic properties of the hamster suprachiasmatic nucleus in vivo. *J. Neurosci.* **18**, 10709–10723 (1998).

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