Impaired Circadian Photoentrainment in *Opn5*-Null Mice

**HIGHLIGHTS**

- *Opn5*-null mice show impaired photoentrainment to UVA-light/dark (LD) cycles.
- Mice lacking melanopsin and functional rods/cones fail to entrain to UVA-LD cycles.
- *Opn5* alone is not sufficient for circadian photoentrainment.
- Retinal circadian clocks play a possible role in circadian photoentrainment.

Ota et al., *iScience* 6, 299–305 August 31, 2018 © 2018 The Authors.
https://doi.org/10.1016/j.isci.2018.08.010
Impaired Circadian Photoentrainment in Opn5-Null Mice

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SUMMARY
The master circadian pacemaker in mammals resides in the hypothalamic suprachiasmatic nuclei (SCN) and is synchronized to ambient light/dark cycles (i.e., photoentrainment). Melanopsin (Opn4) and classical rod-cone photoreceptors are believed to provide all the photic input necessary for circadian photoentrainment. Although the UVA-sensitive photopigment Opn5 is known to be expressed in retinal ganglion cells, its physiological role remains unclear and a potential role for Opn5 in the photoentrainment of the master clock has not been addressed. Here we report impaired photoentrainment and phase shifting to UVA light in Opn5-null mice. However, triple-knockout mice lacking all known functional circadian photoreceptors (i.e., rods, cones, and melanopsin) failed to entrain to UVA-light/dark cycles, despite the presence of Opn5, demonstrating that Opn5 alone is not sufficient for photoentrainment of the SCN clock. Since Opn5 is involved in the regulation of the retinal circadian clock, disrupted retinal function may cause impaired circadian photoentrainment in Opn5-null mice.

INTRODUCTION
Circadian rhythms are approximately 24-hr, cell-autonomous biological rhythms observed in virtually all living organisms. The mammalian circadian pacemaker resides in the hypothalamic suprachiasmatic nuclei (SCN). Daily physiological and behavioral activity patterns are regulated by the SCN, which is synchronized to ambient light/dark (LD) cycles via direct connections from retinal ganglion cells in the retina. Thus, circadian photoentrainment enables organisms to enhance their fitness by anticipating daily changes in food availability and predator activity. Melanopsin (Opn4) and classical rod-cone photoreceptive systems are widely believed to provide all photic input required for circadian photoentrainment (Hattar et al., 2003; Panda et al., 2003), and a potential role for other photopigments has not been addressed. Opn5 is a deep brain photopigment that regulates seasonal reproduction in birds (Nakane et al., 2010, 2014; Yamashita et al., 2010). In mammals, Opn5 is expressed in selected retinal ganglion cells and exhibits an absorption maximum in the UVA range (λmax = 380 nm) (Kojima et al., 2011; Yamashita et al., 2014). Although mammalian Opn5 has been demonstrated to be involved in the photoentrainment of local circadian clocks in the retina and the cornea, its involvement in photoentrainment of the SCN remains unclear (Buhr et al., 2015).

RESULTS AND DISCUSSION
Generation of Mice Deficient in Opn5 and Classical Rod-Cone Photoreceptors
To assess the physiological function of mammalian Opn5 in vivo, we generated Opn5-null (Opn5−/−) mice (Figures 1A–1D). Since mice also possess a UVA-sensitive cone opsin (Opn1sw; λmax = 360 nm), we generated mice deficient in Opn5 and classical rod-cone photoreceptors (Opn5−/−; rd1/rd1) by breeding Opn5-null mice with CBA/J mice harboring the retinal degeneration (rd1) mutation. Because the genetic background of Opn5-null mice was C57BL/6J, F2 hybrids of the parental strains were used to minimize the effect of genetic background, as suggested by the Jackson Laboratory. To examine the effects of Opn5 deficiency and the rd1 mutation on retinal morphology, we performed immunohistochemistry and analyzed the expression of different opsins. Information about the antibodies used is given in Table S1. The retina of Opn5-null mice was indistinguishable from that of wild-type (WT) mice, and immunopositive signals for rod (Rho) and cone (Opn1sw; Opn1mw) opsins and melanopsin (Opn4) were observed (Figure 1E). By contrast, although the rd1 mutation caused severe retinal degeneration and abolished the rod-cone photoreceptor layer, the retinal ganglion cell layer was intact and melanopsin expression was clearly observed in both rd1/rd1 and Opn5−/−; rd1/rd1 mutants (Figure 1E). Note that we have generated several antibodies against Opn5, but none of them were specific; therefore, we were unable to examine the expression of Opn5 in the different mutant mice.

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Impaired Re-Entrainment to UVA-LD Cycles in Opn5-Null Mice

We then compared photoentrainment to UVA-LD cycles in WT mice, Opn5+/−; rd1/rd1 single mutant mice, and Opn5−/−; rd1/rd1 double mutant mice. Absorption spectra of the retinal photopigments present in these mice are illustrated in Figure S1A. Mice were initially kept under white-LD cycles and then transferred to UVA-LD cycles (λp = 365 nm, Figure S1B); wheel-running activity was recorded to measure entrainment (Figure 2A). The intensity of the UVA light was decreased every 2–10 weeks, concomitant with a 2-hr advance of the LD cycle, as described previously (Butler and Silver, 2011). Entrainment to the advanced UVA-LD cycles was determined by a transient interval of a free-running rhythm with a period shorter than 24 hr, which was then followed by a 24-hr rhythm with a stable phase angle of entrainment.

Double mutant mice (Opn5−/−; rd1/rd1) required more days for re-entrainment to new UVA-LD cycles than WT mice (Figure 2B), whereas the single mutants (Opn5−/− and rd1/rd1) exhibited intermediate phenotypes (Figure 2B) (e.g., WT: ~12 days, rd1/rd1: ~20 days, Opn5−/−: ~23 days, Opn5−/−; rd1/rd1: ~30 days at a log photon flux of 12.7). Half-maximal intensities (I50) for entrainment were higher for single and double mutant mice than for WT mice (Figures 2C and 2D). Although differences in the free-running period can affect the duration and light intensity required for re-entrainment, the free-running period of...
all 4 genotypes did not differ significantly (Figure S2). Together, these results demonstrated that Opn5−/−, rd1/rd1, and Opn5−/−; rd1/rd1 mice exhibit significantly slower rates of re-entrainment to UVA-LD cycles than WT mice. Although Opn5−/−; rd1/rd1 mice exhibited severe impairment in circadian photoentrainment, they were able to entrain to the higher intensity UVA-LD cycles, suggesting the involvement of additional photopigments. In the rd1/rd1 retina, most rods degenerate rapidly (within the first week after birth) and all rods disappear completely by about 7 weeks of age. By contrast, cones degenerate much more slowly, with some surviving for at least 18 months (Carter-Dawson et al., 1978). The involvement of UVA-sensitive cones in circadian photoentrainment is well established (van Oosterhout et al., 2012; Yoshimura and Ebihara, 1998). Therefore, it is possible that the remaining UVA-sensitive cone opsins and/or melanopsin may mediate UVA-dependent circadian photoentrainment in Opn5−/−; rd1/rd1 mice.

Impaired Phase Shifting and Fos Induction to UVA Light Pulses in Opn5-Null Mice

To further evaluate the ability of Opn5-null mice to respond to light for circadian functions, we examined the effect of phase-shifting light pulses under constant darkness (Figure 3). A single 15-min light pulse was given 4 hr after activity onset (i.e., circadian time 16). Retinal degenerate (rd1/rd1) mice showed decreased phase shifts to both UVA and orange light pulses (λp = 602 nm, Figure S1B) compared with WT mice (Figures 3A–3D). This is most likely due to the loss of classical photoreceptors, whose contribution to irradiance encoding and regulation of circadian rhythms is well known (Altinum et al., 2010; Lall et al., 2010; van Diepen et al., 2013; van Oosterhout et al., 2012; Yoshimura and Ebihara, 1996, 1998). Although Opn5−/− mice showed impaired phase shifting to UVA light pulses (Figures 3A and 3C), they showed normal phase shifting to orange light pulses (Figures 3B and 3D), indicating that impaired photoresponses observed in Opn5−/− mice are UVA light specific.
We also examined the induction of neuronal activation marker, Fos mRNA, in the SCN by UVA light (single 15-min pulse at a log photon flux of 12.3 cm$^{-2}$ s$^{-1}$). These results showed that UVA-dependent Fos induction was significantly decreased in Opn5$^{-/-}$ and rd1/rd1 single mutant mice, as well as in Opn5$^{-/-}$; rd1/rd1 double mutants (Figure 3E). However, the phenotype was much more severe in double mutant mice (Opn5$^{-/-}$; rd1/rd1) than in the single mutants (Opn5$^{-/-}$ and rd1/rd1) and correlates well with our phase shifting results in Figure 3C. All together, these results demonstrated that Opn5$^{-/-}$ mice have decreased photosensitivity to UVA light.

**Opn4$^{-/-}$; Gnat1$^{-/-}$; Gnat2$^{-/-}$ Mice Lack the Ability to Photoentrain to UVA-LD Cycles**

Previous studies have shown that mice deficient in melanopsin and rod-cone photoreceptors (e.g., Opn4$^{-/-}$; rd1/rd1 double mutant mice and Opn4$^{-/-}$; Gnat1$^{-/-}$; Cnga3$^{-/-}$ [melanopsin; guanine nucleotide-binding protein z transducin 1; cyclic nucleotide-gated channel z3, respectively] triple-knockout [TKO] mice) failed to entrain to white-LD cycles; therefore, melanopsin and classical rod-cone photoreceptive systems are widely believed to account for all photic input required for circadian photoentrainment (Hattar et al., 2003; Panda et al., 2003). However, these studies used relatively dim white light (100–800 lx) (Hattar et al., 2003;
Panda et al., 2003). The illuminance under direct sunlight is approximately 100,000 lx, whereas that of sunrise or sunset on a clear day is approximately 400 lx. Thus, it is possible that the illuminance (i.e., 100–800 lx) and/or wavelengths (i.e., white light but not UVA light) used in these previous studies were not sufficient to stimulate Opn5. We therefore examined circadian photoentrainment to UVA-LD cycles in Opn4\(^{-/-}\); Gnat1\(^{-/-}\); Gnat2\(^{-/-}\) TKO mice (Gnat1/2: guanine nucleotide-binding protein \(\alpha\) transducin 1/2), which have mutations in all known circadian photoreceptor pathways (Figure S1A). WT and TKO mice were initially kept under white-LD cycles and then transferred to UVA-LD cycles. The intensity of UVA light was adjusted every 3–7 weeks. WT mice could entrain to white- and UVA-LD cycles, except at very low intensities of UVA light (log photon flux of 8.5–9.5 cm\(^{-2}\) s\(^{-1}\)); however, TKO mice failed to entrain to LD cycles of white light and all intensities of UVA light (Figure 4). Although 2 of 6 TKO mice (#1 and #2) showed free-running periods close to 24 hr, they failed to entrain to advanced UVA-LD cycles (Figure 4). These results demonstrated that the UVA-sensitive photopigment Opn5 alone is not sufficient for photoentrainment of the circadian pacemaker.

Figure 4. Photoentrainment Analysis of Opn4\(^{-/-}\); Gnat1\(^{-/-}\); Gnat2\(^{-/-}\) Mice
Representative double-plotted actograms of WT and Opn4\(^{-/-}\); Gnat1\(^{-/-}\); Gnat2\(^{-/-}\) TKO mice. The vertical and horizontal axes indicate the number of recorded days and the time of day over 2 days, respectively. Yellow, purple, and black bars on the right indicate different lighting conditions: white-LD cycles, UVA-LD cycles, and constant darkness (DD), respectively. The log photon flux of UVA light (log photons cm\(^{-2}\) s\(^{-1}\)) is also marked on the right. The timing of different LD cycles is illustrated at the top, and the assigned letter (a–d) for each LD cycle corresponds to the letter shown on the right. The red lines indicate when lighting conditions were changed. See also Figure S1.
Conclusions

In the present study, we demonstrated impaired photoentrainment to UVA light in Opn5-null mice. However, Opn4<sup>−/−</sup>; Gnat1<sup>−/−</sup>; Gnat2<sup>−/−</sup> TKO mice that lack melanopsin and functional rod-cone photoreceptors, but still possess Opn5, failed to photoentrain to LD cycles of both white and UVA light. This means that Opn5 is required for maximum UVA sensitivity, but it alone is not sufficient for entrainment to UVA-LD cycles. Although it is possible that Gnat1 and/or Gnat2 are involved in the phototransduction of Opn5, this seems unlikely, since they are thought to be expressed in different cell types, with Opn5 being expressed in retinal ganglion cells and Gnat1/2 in rod and cone photoreceptors, respectively (Buhr et al., 2015). The mammalian retina itself has a local circadian clock (Buhr and Van Gelder, 2014; Tosini and Menaker, 1996), which is photoentrainable in vivo and ex vivo and does not require the SCN. Photoentrainment of the retinal clock is mediated by Opn5 and not by melanopsin or rod-cone photoreceptors (Buhr et al., 2015). Thus, the retinal circadian clock is likely to be disrupted in Opn5<sup>−/−</sup> mice. Many retinal functions are known to be under the control of the circadian clock. For example, photoreceptors undergo daily cycles of renewal and shedding of outer segment disc membranes, which are fundamental for the maintenance of photoreceptor health (La Vail, 1976). Rod and cones are anatomically coupled by gap junctions, and the retinal circadian clock controls the extent and strength of rod-cone electrical coupling, leading to changes in retinal sensitivity between the daytime and nighttime (Ribelayga et al., 2008). Furthermore, disruption of the retinal circadian clock has been demonstrated to result in abnormal retinal transcriptionsal responses to light and defective inner retinal electrical responses to light (Storch et al., 2007). It is therefore plausible that the impaired photoentrainment we observed in Opn5-null mice is caused by disruption of the retinal circadian clock.

Although Opn5 is expressed in retinal ganglion cells, it alone is not sufficient for photoentrainment of the circadian pacemaker in the SCN. However, remarkably, Opn5 still contributes to circadian light responses even in the presence of functional rods, cones, and melanopsin light responses.

Limitations of the Study

Our study focused primarily on behavioral analysis of circadian locomotor activity of various mutant mice (except Figures 1 and 3E). Therefore, we do not fully understand the underlying mechanism of impaired photoentrainment and phase shifting in Opn5-null mice. Further molecular analysis of the retina of Opn5-null mice might be required in future studies to characterize this mechanism.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods, two figures, and one table and can be found with this article online at https://doi.org/10.1016/j.isci.2018.08.010.

ACKNOWLEDGMENTS

We thank Dr. Kathy Tamai for comments on the manuscript, Mr. Kousuke Okimura for technical support, Dr. Willem J. DeGrip for the Rho antibody, and Dr. Keisuke Ikegami for helpful discussions. We also thank the Nagoya University Radioisotope Center for use of their facilities. This work was supported by the Funding Program for Next Generation World Leading Researchers (NEXT Program) initiated by the Council for Science and Technology Policy (CSTP) (LS055), JSPS KAKENHI “Grant-in-Aid for Specially Promoted Research” (Grant Number 26000013), Human Frontier Science Program (RGP0030/2015), Grant-in-Aid for JSPS Fellows (14J03915), and Program for Leading Graduate Schools “Integrative Graduate Education and Research in Green Natural Sciences (IGER),” MEXT, Japan. WPI-ITbM is supported by World Premier International Research Center Initiative (WPI), MEXT, Japan.

AUTHOR CONTRIBUTIONS

T.Y. conceived and directed the work; W.O. and Y.N. performed research; S.H. provided new materials; W.O. and T.Y. analyzed data; all authors discussed the results and commented on the manuscript; W.O. and T.Y. wrote the paper.
DECLARATION OF INTERESTS
The authors declare no competing interests.

Published: August 31, 2018
Accepted: August 10, 2018
Revised: July 25, 2018
Received: December 21, 2015

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Supplemental Information

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Figure S1. Absorption spectra of retinal photopigments for each genotype, and spectral profiles of light sources used in this study. Related to Figures 1E, 2, 3 and 4.

(A) Schematic diagram of the absorption spectra of the retinal photopigments in the mutant mice used in this study. Modified from Kojima et al. (Kojima et al., 2011). (B) Schematic diagram of the spectral profile of the UVA and orange light sources used in this study. The UVA light source is a fluorescent lamp (Philips), and the orange light source is a light-emitting diode (LED) (Beamtec). Values are shown as relative energy (%).
Figure S2. Free-running period of wild type and mutant mouse strains used in this study. Related to Figures 2 and 3.

Values represent the mean ± SEM. No significant differences were observed between genotypes ($p > 0.05$, one-way ANOVA followed by Scheffé’s post-hoc test; $n = 6–8$).

| Name   | Manufacturer                  | Product number | Dilution | Host    | Monoclonal/ Polyclonal |
|--------|-------------------------------|----------------|----------|---------|------------------------|
| Opn1sw | Santa Cruz                    | sc-14363       | 1:10000  | Goat    | Polyclonal             |
| Opn1mw | Chemicon                      | AB5405         | 1:8000   | Rabbit  | Polyclonal             |
| Rho    | Dr. Willem J. DeGrip          | CERN886        | 1:500    | Rabbit  | Polyclonal             |
|        | (Schalken and DeGrip, 1986)   |                |          |         |                        |
| Opn4   | Advanced Targeting Systems    | AB-N38         | 1:10000  | Rabbit  | Polyclonal             |

Table S1. Antibodies used in this study. Related to Figure 1E.
Transparent Methods

Animals
Male and female F2 progeny generated by intercrosses between Opn5-/- mice (C57BL/6J background) and rd1/rd1 mice (CBA/J strain) were used in this study. Opn4-/-; Gnat1-/-; Gnat2-/- mice (hybrids between C57BL/6N and 129S strain) were also used in this study. All animals were kept in a controlled environment (white-LD cycle [12/12-hr]; room temperature 22–24°C) until experiments were performed. Food and water were provided ad libitum. All animal procedures in this study were approved by the Animal Experiment Committee of Nagoya University.

Generation of Opn5-null mice
We manipulated the murine Opn5 locus by targeted replacement of the Opn5 gene with a cDNA encoding EGFP (Clontech) (Figure 1A). In the targeting construct, the EGFP gene replaces 127 bp of the first exon of Opn5 starting from the translational start site. Genomic fragments flanking the murine Opn5 gene were used to generate the Opn5 targeting vector. Following homologous recombination and isolation of ES cell clones that harbored the expected construct (replacement of Opn5 by EGFP), the Neo gene flanked by two loxP sites was then excised. The targeted ES cell clones were injected into blastocysts to generate chimeric mice. Germline transmission of the mutant allele yielded heterozygous mutant Opn5+/- mice, which were intercrossed to generate homozygous Opn5-/- mice. Because we used C57BL/6J-derived ES cells, the genetic background of Opn5-null mice was C57BL/6J. Primers used for genotyping were as follows (see also Figure 1A and 1C):

p1, 5’-gtgtaacatctctcactag-3’
p2, 5’-ggtagggcagttggttcaa-3’
p3, 5’-ctgaacttgtggccgtttac-3’

Deficiency of Opn5 mRNA was examined by RT-PCR. Primers for RT-PCR were designed at exons 1 and 7 as follows:

forward, 5’-atggccttggaaccacactgccccctcagc-3’
reverse, 5’-aggttgtcttttgccaccacgtc-3’

Immunohistochemistry
Mice were deeply anesthetized with isoflurane and perfused with saline followed by neutral buffered formalin (NBF). Brains were removed, post-fixed for 24 hr in NBF and soaked in 20% sucrose in 0.1
M phosphate buffer (PB, pH 7.4) for 24 hr. The brains were then frozen using dry ice, and coronal frozen sections (20-µm thickness) were prepared using a cryostat. After air-drying, sections were placed in 0.1 M phosphate-buffered saline (PBS, pH 7.4). The sections were soaked in antigen retrieval solution (Histo VT One, Nacalai Tesque), heated in a microwave for 7 min, and quenched for 1 hr at room temperature (RT, ~25°C). Sections were washed in 0.1 M PBS, and endogenous peroxidase activity was blocked with 0.03% H2O2 in 0.1 M PBS at RT. The samples were then washed in 0.1 M PBS, followed by a wash in 0.1 M PBS supplemented with 0.3% Triton X-100 (PBST; pH 7.4), and incubated in blocking solution (1.5% normal goat or rabbit serum in 0.1 M PBST) at RT. Sections were then incubated with each antibody (listed in Table S1) overnight at RT. Samples were washed with 0.1 M PBST, incubated with N-Histofine Simple Stain Mouse MAX PO (R) or (G) (Nichirei Biosciences) for 1 hr at 30°C, and then washed with 0.1 M PBS. Signal detection was carried out by incubating with 0.25 mg/ml 3,3’-diaminobenzidine and 0.006% H2O2 in 0.1 M PBS. Finally, samples were washed with 0.1 M PBS, then washed with distilled water, dehydrated with increasing concentrations of ethanol, cleared in xylene, and mounted on slides with Entellan new solution (Merck).

**Monitoring circadian activity rhythms**

Twelve-week-old male and female mice were housed in individual cages equipped with running wheels, and the cages were placed in a light-tight box (136.7 × 42.5 × 42.5 cm). Light in the box was provided by fluorescent lamps (white light: Panasonic FHF32EX-N-H, 4,150 lux at the top of the cage; UVA light: Philips TL-D 36W/08 low-pressure mercury vapour fluorescent lamp, peak wavelength 365 nm with half-bandwidth 13.9 nm) or a light-emitting diode (LED) (orange light: Beamtec LT40OS, straight tube LED, peak wavelength 602 nm with half-bandwidth 21.6 nm). The spectral profile of the UVA- and orange-light source is illustrated in Figure S1B. Light intensity was controlled using a neutral density filter (Fujifilm ND 0.9) and measured at the top of the cage (Monotech QTM-101). The UVA-light intensity was less than that observed under direct sunlight in Nagoya, Japan (~16 log photons cm−2 s−1). Wheel-running activities were recorded using the Chronobiology Kit (Stanford Software Systems).

**Circadian phase-shifting light pulses**

Mice were kept under a white-light LD cycle (12/12-hr) for 2 weeks and transferred to constant darkness (DD). After 3 weeks in DD, mice were placed in a wire mesh cages (10 ×10 ×10 cm) and exposed to a 15-min UVA- or orange-light pulse at CT16, with CT12 defined as activity onset. Four
intensities of UVA- and two intensities of orange-light pulses were examined as shown in Figures 3C and 3D. Night-vision goggles (Ninox, Armasight) were used for all operations in the dark.

**In situ hybridization of Fos mRNA**

Since Fos expression peaks 30 min after stimulation (Radzioch et al., 1987), brains were collected 30 min after the beginning of the light pulse (log photon flux of 12.3) and rapidly frozen on dry ice. Fos mRNA was examined by in situ hybridization. Non-perfused, frozen sections (20-µm in thickness) were prepared using a cryostat (CM3050S, Leica Microsystems) and probed with $^{33}$P-labeled oligonucleotides as previously described (Yoshimura et al., 2000). Four 45-mer oligonucleotide probes were designed for the mouse Fos gene (GenBank: NM_010234) and used as a mixture to increase the sensitivity. Hybridization of the probes was carried out overnight at 42°C. Two high-stringency washes were performed at 55°C. Samples were air-dried and exposed to BioMax MR Film (Eastman Kodak) for 4 weeks with $^{14}$C-Standard slide (American Radiolabeled Chemicals). Densitometric quantification of the signal was performed using the Multi Gauge software (Fujifilm). The probe sequences were as follows:

5’- tcactgtctgttcggaaccgcctacgtctcttcag - 3’
5’- tccaggagccacagacatctctctggaagccaggtcag - 3’
5’- atctggcacagagcgggaggtctctgagccactgggcctag - 3’
5’- ctggaggccagatgtgatgatgatgatgatgactgaggtgcag - 3’

**Statistical Analysis**

All data are shown as the mean ± SEM. Statistical analyses were performed using one-way ANOVA, followed by Scheffè’s post-hoc test.
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