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Permalink
https://escholarship.org/uc/item/7466c4zj

Journal
Nature, 545(7654)

ISSN
0028-0836

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Publication Date
2017-05-01

DOI
10.1038/nature22325

Peer reviewed
A rhodopsin in the brain functions in circadian photoentrainment in Drosophila

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Animals partition their daily activity rhythms through their internal circadian clocks, which are synchronized by oscillating day–night cycles of light. The fruitfly Drosophila melanogaster senses day–night cycles in part through rhodopsin-dependent light reception in the compound eye and photoreceptor cells in the Hofbauer–Buchner eyeteil. A more noteworthy light entrainment pathway is mediated by central pacemaker neurons in the brain. The Drosophila circadian clock is extremely sensitive to light. However, the only known light sensor in pacemaker neurons, the flavoprotein cryptochrome (Cry)2,3, responds only to high levels of light in vitro4. These observations indicate that there is an additional light-sensing pathway in fly pacemaker neurons5. Here we describe a previously uncharacterized rhodopsin, Rh7, which contributes to circadian light entrainment by circadian pacemaker neurons in the brain. The pacemaker neurons respond to violet light, and this response depends on Rh7. Loss of either cry or rh7 caused minor defects in photoentrainment, whereas both loss of caused profound impairment. The circadian photoreceptor to constant light was impaired in rh7 mutant flies, especially under dim light. The demonstration that Rh7 functions in circadian pacemaker neurons represents, to our knowledge, the first role for an opsin in the central brain.

Cry is a light detector in central pacemaker neurons that contributes to photoentrainment5,6. However, cry mutant flies still entrain to light–dark (L–D) cycles2,6. Therefore, we screened for an additional light sensor that functions in circadian photoentrainment using Drosophila activity monitors. We entrained flies under 12 h light–12 h dark (L–D) cycles for 4 days, and switched them to constant dark (D–D) conditions. Control flies (w1118) displayed two daily activity peaks during dawn and dusk, termed the morning and evening peaks (Fig. 1a; note these are double plots). Activity increased before the light and dark transitions, indicating anticipation of changes in light, which is a hallmark of the circadian clock. Another such hallmark is the ability to maintain activity patterns established under L–D cycles after being transferred to constant darkness (Fig. 1a and Extended Data Fig. 1a, h).

Mutation of cry causes only subtle effects on circadian behaviour3 (Extended Data Fig. 1b, h). Flies also show rhythmic behaviour after photoentrainment if they are missing the phospholipase C (PLC) NORPA2,5, which is required for phototransduction in the compound eye, or if they are doubly mutant for norpA and cry2,3 (Extended Data Fig. 1c, d, h). Phototransduction in Hofbauer–Buchner eyeteil photoreceptors couples to Rh6 and the TRPL channel7–9, but is independent of NORPA2. Flies triply mutant for rh5, rh6 and cry3,5 for norpA, cry and trp, or for norpA, trpl and cry are entrained by L–D cycles (Extended Data Fig. 1e–h). Thus, as proposed3, there is likely to be an additional light input pathway that influences the circadian clock preceding exposure to D–D.

The Drosophila genome encodes an uncharacterized opsin, Rh7 (Extended Data Fig. 2a), which shares 27–30% amino acid identity with other opsins in Drosophila melanogaster. Rh7 is conserved in other Drosophila species (79–99% identity) and in Aedes aegypti and Anopheles gambiae (49–52% identity)10. Photoreceptor cells in the compound eye and ocelli express six opsins (Rh1–Rh6; Extended Data Fig. 2b, c). However, a mutation (q560) that eliminates ocular photoreceptor cells and reduces levels of rh1 (also known as ninaE)–rh6 RNA did not reduce rh7 RNA levels (Fig. 1b). We performed RNA sequencing (RNA-seq) using RNA from flies expressing a cell death gene (GMR-hid) in ocular photoreceptor cells. The numbers of rh1–rh6 transcripts were reduced markedly, whereas the number of rh7 transcripts was unchanged (Fig. 1c). We did not detect Rh7 in the compound eye with Rh7 antibodies (see below; Extended Data Fig. 2b–e). We generated an rh7 null allele, rh71 (Extended Data Fig. 2f, g), and tested the light responses of rh71 mutant flies by performing electroretinogram (ERG) recordings. The ERGs of control and rh71 flies were indistinguishable (Extended Data Fig. 2h–j). Thus, Rh7 neither was expressed nor functioned in known photoreceptor cells.

To address whether Rh7 is a light receptor, we tested whether it could substitute for Rh1 in R1–6 photoreceptor cells. Expression of Rh7 rescued a wild-type-like ERG in the rh1 mutant (ninaE17; Extended Data Fig. 2k–m). To assess the light response due to Rh7 only, we eliminated the light responses from the remaining two photoreceptor cells (R7 and R8), which express other rhodopsins. Phototransduction was abolished in norpA24 flies (Fig. 1d, e). We restored a photoresponse in R1–6 cells of norpA24 flies by expressing a norpA1 transgene using the rh1 promoter (rh1>norpA; Fig. 1f). When we eliminated rh1 (ninaE17) from the norpA24,rh1>norpA flies, the flies were unresponsive to light (Fig. 1g). We restored a light response by expressing rh7 in the R1–6 cells (rh1>rh7; Fig. 1h). Thus, Rh7 is a light sensor and is capable of coupling to a Gq–PLC signalling pathway. We expressed Rh7 in HEK293T cells and found that it responded to light with a peak at 397 nm (Fig. 1i, j).

We raised Rh7 antibodies, which stained two groups of cells in the brain, consistent with a subset of central pacemaker neurons (Fig. 2a and Extended Data Fig. 3a, c). The approximately 150 pacemaker neurons express Period (Per), a core component of the circadian clock11–13, and are classified as dorsal and lateral neurons (Extended Data Fig. 3a). The 15–16 lateral neurons include 5–6 dorsal lateral neurons (LNds), 4–5 large ventrolateral neurons (L-LNvs), and 4–5 small ventrolateral neurons (s-LNvs)14. Rh7 and Per were co-expressed in the LNvs (Fig. 2a–c), which express the neuropeptide pigment dispersing factor (PDF)15 (Fig. 2d–f). We also detected Rh7-positive neurons in the vicinity of dorsal neurons 1 (DN1s) (Extended Data Fig. 3a, c), half of which expressed cry16 (Extended Data Fig. 3d, e). However, these neurons did not co-stain with the cry reporter (cry-Gal4.E13 (refs 2, 17); Extended Data Fig. 3e). We did not detect anti-Rh7 staining in other central pacemaker neurons or in rh71 flies (Fig. 2g–i and Extended Data Fig. 3b).

Cry mediates rapid increases in action potentials evoked by blue light (450 nm peak) in 1-LNvs16–20. We compared the electrophysiological
Figure 1 | Rh7 is a light receptor. a, Actogram obtained with control flies entrained under L–D cycles and released to constant darkness (D–D). Red and blue arrows indicate morning and evening anticipation, respectively. n = 62. b, Quantitative real-time PCR analysis of opsin genes using RNA from heads. Error bars indicate s.e.m. n = 3 per genotype. c, OpsiRNA-seq mRNA levels were quantified as reads per kilobase of transcript per million mapped reads (RPKM). Error bars indicate s.e.m. d–h, ERG responses using 2-s light. d, Control flies. e, norpA024 mutants. f, Expression of norpA using the rh1 promoter (rh1>norpA) in a norpA024 background. g, Expression of rh1>norpA in a norpA024;ninaEi17 background. h, Expression of rh1>norpA and UAS-rh7 under the control of the rh1–Gal4 driver (rh1>norpA and rh1–rh7, respectively) in a norpA024;ninaEi17 background. i, HEK293T cells expressing Rh7 and stained with anti-Rh7. The DAPI stain indicates nuclei. Scale bar, 10 μm. j, Absorbance spectrum of Rh7 from HEK293T cells expressing Rh7.

responsiveness to white (400–1,000 nm) and violet (405 nm) light in control and rh71 flies. The L-NV responses to white and violet light were greatly diminished in rh71 flies (Fig. 2j, k, m–o). Control, norpA024 and rh1–norpa flies showed minimal or no response to orange light (550–1,000 nm; Fig. 2i).

To address the importance of Rh7 to entrainment, we first investigated its contribution to circadian phase changes in response to a nighttime light pulse, which shifts the phase of the clock23. The direction of the shift depends on when the light is presented23–25. Lights were turned on and off at Zeitgeber time (ZT)0 and ZT12, respectively. An early night light pulse (ZT14–18) produced a phase delay in controls, whereas a late night light pulse (ZT20–22) caused a phase advance23 (Fig. 3a). As previously reported23–25, cry1 flies displayed severely impaired phase shifting to early or late night pulses (Fig. 3a and Extended Data Fig. 4).

We exposed rh71 flies to nighttime light pulses. The phase delay was normal if the stimulus was presented early (ZT14; Fig. 3a and Extended Data Fig. 4). However if the light pulse occurred later, the degree of phase shift was significantly reduced (ZT16, P < 0.01; ZT18, P < 0.01; ZT20, P < 0.01; ZT22, P < 0.01; Fig. 3a and Extended Data Fig. 4). We knocked down rh7 RNA in wild-type flies using an L-NV-specific driver (pdf–Gal4) (Extended Data Fig. 3f–i) and then exposed the flies to light at ZT22. These flies exhibited the same phase advance defect as rh71 flies (Fig. 3b), indicating that Rh7 is required in PDF-positive neurons for normal photoentrainment.

Phase advances due to light pulses late at night (ZT22) correlate with light-induced degradation of the core clock protein Timeless (Tim)24. Tim degradation depends on binding to Cry23, or results from neuronal activation25. As light-induced neuronal firing is reduced in rh71 flies, light-induced degradation of Tim might be impaired. To test this hypothesis, we applied a light pulse at ZT22 and assayed anti-Tim signals in PDF neurons (L-NVs) 55 min later. In controls, light caused a 4.8-fold decline in Tim (Fig. 3c, f). However, in rh71 L-NVs, light caused only a slight reduction in anti-Tim staining, which was not statistically significant (Fig. 3d, f). We rescued this defect with a genomic transgene (Fig. 3e, f).

Another test of photoentrainment is to assess the number of days required to adjust to a delay in the light-to-dark transition. If control flies are entrained under L–D cycles, and then the transition from light to darkness is delayed by 8 h, they quickly re-entrain to the new L–D cycle (Fig. 3g, h). The evening peak shifts by 7.6 ± 0.1 h on day 1 and 7.9 ± 0.1 h on day 2 (Fig. 3g). Consistent with a previous study24, cry1 flies shifted by only 4.6 ± 0.2 h on day 1 and 6.5 ± 0.1 h on day 2, and required 3 days to establish stable peak activity (Fig. 3g, i). We
found that rh7+ flies displayed significant delays in phase shift (day 1, 6.5 ± 0.2 h; P < 0.01; day 2, 7.1 ± 0.1 h, P < 0.01; Fig. 3g, j) consistent with a contribution of Rh7 to photoentrainment. We rescued the defect with an rh7+ genomic transgene (Fig. 3g, k). The impairment in the phase-shift exhibited by rh7+ cry+ double mutant flies (day 1, 3.1 ± 0.2 h; day 2, 5.3 ± 0.2 h) was more severe than those seen in the single mutants (Fig. 3g, l).

Constant light (L–L) leads to arrhythmic circadian behaviour in wild-type flies6 (Fig. 4a, d). However, cry mutant flies remain rhythmic in constant light8 (93.1%; Fig. 4d and Extended Data Fig. 5a). Under L–L conditions, 19.0% of rh7+ flies are also rhythmic, and this phenotype is rescued by a wild-type rh7+ transgene (Fig. 4b–d and Extended Data Fig. 5b).

Because phototransduction promotes signal amplification and sensitivity to low light, we tested the effects of lack of Rh7 on rhythmicity by photoentraining rh7+ flies and then exposing them to constant dim light (10 lx). Few control flies maintained rhythmicity even under dim light, whereas all cry+ flies were rhythmic (Fig. 4e, h and Extended Data Fig. 5c). Notably, the majority of rh7+ flies maintained rhythmicity under constant dim light (66.7%; Fig. 4f–h). We restored wild-type responses with an rh7+ genomic transgene (Extended Data Fig. 5d). These data suggest that Rh7 is required for sensitizing the Cry-dependent circadian photopresponse under dim light conditions.

PDF-expressing LNVs promote light-dependent arousal26,27. To test whether Rh7 functions in arousal, we stimulated the flies at night (ZT22) with a 5-min white light pulse. The rh7+ flies exhibited a decreased light-incident arousal (Fig. 4i and Extended Data Fig. 5e) and a longer arousal delay than control flies (Extended Data Fig. 5f), and these defects were at least as great as those exhibited by cry+ flies30 (Fig. 4i and Extended Data Fig. 5e, f). Responses to red light were not impaired significantly in the mutant flies (Extended Data Fig. 5g). The rh7+ cry+ double mutant, but not the single mutants, exhibited a deficit in violet light arousal (405 nm; Extended Data Fig. 5h), indicating that the two light sensors compensate for each other during the arousal response.

We tested for a potential role of Rh7 in maintaining rhythmic behaviour during constant darkness (D–D) following L–D entrainment. The mutant flies showed rhythmic diurnal and circadian behaviour similar to that of wild-type flies (Fig. 4j, k, p). As previously shown1, cry+ flies also photoentrained and exhibited rhythmic diurnal and circadian behaviour (Fig. 4l, p). However, rh7+ cry+ double mutant flies showed profound deficits. Over one-third (37.2%) of the animals were arrhythmic (Fig. 4m, p), possibly owing to insufficient synchronization of the molecular clock between different groups of central pacemaker neurons in the absence of both light sensors intrinsic to these cells. The remaining rhythmic flies displayed much longer periodicity during D–D than did controls (control, 23.8 ± 0.08 h; rh7+ cry+, 27.3 ± 0.07 h, P < 0.01; Fig. 4j, n). rh7+ cry+ double mutants displayed similar impairments (Extended Data Fig. 6a–c). Thus, at least one of the two light receptors is required in pacemaker neurons for normal rhythmic behaviour. We rescued these defects with an rh7+ genomic transgene (Fig. 4o, p).

Although flies with single g800, rh7+ or cry mutations display photoentrainment impairments1, they are capable of circadian rhythmicity. Thus, any two of these three photoreceptors (Cry, Rh7 and gl-dependent) are sufficient for rhythmic circadian behaviour. However, Rh7 is insufficient on its own because g800 cry+ flies are circadian blind1. To test whether cry alone is sufficient for rhythmic behaviour, we subjected the rh7+ g800 double mutant flies to a L–D regime followed by D–D, and found that 25% were arrhythmic (Extended Data Fig. 6d–h). Thus, although Cry alone cannot preserve fully wild-type circadian behaviour, it enables flies to maintain greater rhythmicity than just Rh7 or gl-dependent photoreceptor cells. We exposed rh7+ g800 flies to a 5-min light pulse at ZT22 to test their circadian phase-response. Although rh7+ and g800 single mutants showed a decreased phase-advance, rh7+ g800 double mutant flies displayed a greater impairment (Extended

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**Figure 3 | Rh7 is a circadian light receptor.** a. Phase response of flies to 5-min white light at the indicated ZT. Negative and positive phase changes indicate phase delays and phase advances, respectively. Three independent assays per genotype (n = 8–32 per experiment). Total flies tested: ZT14, control, n = 56; cry+, n = 46; rh7+, n = 55. ZT16, control, n = 61; cry+, n = 52; rh7+, n = 57. ZT18, control, n = 61; cry+, n = 51; rh7+, n = 77. ZT20, control, n = 45; cry+, n = 39; rh7+, n = 48. ZT22, control, n = 54; cry+, n = 60; rh7+, n = 48. NS, not significant. Error bars indicate s.e.m. *P < 0.01. One-way ANOVA (Kruskal–Wallis test) followed by Dunn’s test. b. Phase responses to 5-min white light stimulation at ZT22. Three independent assays per genotype (n = 8–24 per experiment). + > rh7RNAi (UAS-rh7RNAi), n = 40; pdf–rh7RNAi (UAS–rh7RNAi and pdf–Gal4), n = 52. *P < 0.01, unpaired Student’s t-test. c-e. Flies were exposed to 5-min light stimuli at ZT22, and the LNVs were stained with anti-Tim and anti-PDF at day 5, the day cycle was extended by 8 h. Red dots indicate evening peaks.

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with their rhythmic behaviour under L–D cycles. Per oscillations in LNVs are altered in cry2 mutants.1,3 The trough and peak are delayed in s-LNVs, and there is a large reduction in oscillation amplitude in l-LNVs (Extended Data Fig. 9g–i). In rh7+/cry2 flies, Per levels remained high from ZT2 to ZT14 in s-LNVs and peaked shortly after dusk (ZT14) in l-LNVs (Extended Data Fig. 9j–l). PDF does not oscillate in control29 or rh7+ LNVs (Extended Data Fig. 9a, d).

In the eye, rhodopsin signal through the PLC encoded by norpA. However, norpA and cry double mutant flies exhibit normal rhythmicity during constant darkness following L–D entrainment2,5,6 (Extended Data Fig. 1d, h), indicating that Rh7 signalling in LNVs is NORPA-independent. To investigate whether the other PLC3 (PLC21C) functions in PDF neurons, we combined two UAS–plc21C RNAi lines with the pdf–Gal4 driver (Extended Data Fig. 10a), and then exposed the flies to a white-light pulse for 5 min at ZT22. Knockdown of plc21C caused reductions in phase-advance at ZT22 (Extended Data Fig. 10b–g), similar to the effects of the rh7+ mutation (Fig. 3a).

In summary, our work establishes a role for a rhodopsin in the central brain. Rh7 is strategically expressed in PDF-positive cells, which appear to be master light input clock neurons that also receive input from the optical lobes26. The fact that PDF-positive neurons express two distinct light sensors (Rh7 and Cry) highlights their key role in circadian photoentrainment. In the mammalian retina, around 1% of retinal ganglion cells are intrinsically photosensitive (ipRGCs), and these cells function in circadian photoentrainment. ipRGGs are directly light sensitive owing to the expression of melanopsin and also receive light information from rods and cones. The striking similarities between Drosophila PDF-positive neurons and ipRGCs indicate a common strategy for circadian photoentrainment. Opsins are also expressed in the mammalian brain30, although their functions are unknown. Because light penetrates the mammalian skull, our findings raise the possibility that neurons in the mammalian brain also sense light and contribute to photoentrainment of circadian rhythms.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 24 June 2016; accepted 30 March 2017. Published online 10 May 2017.

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Supplementary Information is available in the online version of the paper.

Acknowledgements We thank A. Sehgal, M. Rosbash, M. Wu and P. Emery for fly stocks; A. Sehgal and S. Britt for antibodies; and E. Guzman, H. Zhou and the Next Generation Sequencing Core at the UCSB Biological Nanostuctures Laboratory for help with the RNA-seq. This work was supported by grants to C.M. from the National Eye Institute (EY008117) and the National Institute on Deafness and other Communication Disorders (DC007864), and to T.C.H. from National Institute of General Medical Sciences (GM102965 and GM107405).

Author Contributions J.D.N., T.C.H. and C.M. designed the study. J.D.N. and T.C.H. performed experiments, and all authors analysed the data. J.D.N. and T.C.H. wrote the manuscript with input from T.C.H. and L.S.B.

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Reviewer Information Nature thanks C. Desplan, R. Stanewsky and the other anonymous reviewer(s) for their contribution to the peer review of this work.
METHODS
No statistical methods were used to predetermine sample sizes. All behavioural data were collected in a random order. No blinding method was used in assessing experimental outcomes.

Fly stocks. The following flies were obtained from Bloomington Stock Center: isogenized w^{111}(BL5905), norpA^{224} (BL9048), ninaE-norpA (rh1-norpA); this is a direct fusion of the ninaE promoter to the norpA coding region; BL52276, ninaE–Gal4 (rh1–Gal4; BL9691), trp^{38} (BL23636), trp^{38} (BL23914), UAS–mcherry–NLS (Bloomington Stock Center); p{UAST}Gal4 (BL6900), and two UAS–p2Cl RNAi lines (01210, BL 31269 and 01211, BL31270). GMR–hsp{68} was obtained from the Drosophila Genetic Resource Center, Kyoto (108419). We used w^{111} as the control strain. The UAS–rh7 RNAi line (v478) was from VDRC Stock Center. The tim–Gal4 transgene was provided by A. Sehgal. The cry–Gal4 E13 transgene was from M. Roshbash. The cry^{+} and cry^{-} flies were provided by W. M. and the rh7^{0}, rh8^{0}, UAS-rh3, UAS-rh4 and UAS-rh5 lines were provided by C. Desplan. We also used ninaE^{117} flies.

Cloning of the rh7 cDNA and generation of transgenic flies. To clone the rh7 coding region, we prepared mRNA from w^{111} heads, performed reverse transcription (RT)-PCR using the following primers, and cloned the cDNA into the TOPO vector (pCR2.1-TOPO, Invitrogen). Primers: rh7 forward, GCGGGCGGACATGGAGCCGACATCATGAGC; rh7 reverse, GCGGCCGCCCTCAAGATTTCTCCTCGATAGC. To generate the UAS–rh7 transgene, we subcloned the rh7 open reading frame into the NotI site of the pUAST vector. To construct the plasmid for expression of rh7 in HEK293T cells, we subcloned the rh7 open reading frame between the BamHI and XbaI sites of the pCS2+ MT vector using the following primers: rh7 forward, ATCGAGTCTCAGATGAGCCGACATCATGAGC; rh7 reverse, ATTCGAGTCTCAGATGAGCCGACATCATGAGC. To generate transgenic flies expressing an Rh7–FLAG fusion protein, we first constructed the pUAST–FLAG vector using the following two oligonucleotides, which we annealed and cloned into the XhoI and XbaI sites of the pUAST vector: FLAG 5′-XbaI, TCGAGGGATTACAAGATGAGCAGACGAGAAT; FLAG 3′-XhoI, CTAGATGATTTGCTGATTGATTTAATACCCC. We amplified the rh7 coding region using the same forward primer as above, in conjunction with the following reverse primer to eliminate the stop codon: rh7 reverse, 5′-GGCGGCCGCGACTTCATGAGCGCCGACATCATGAGC. Both the UAS–rh7 and UAS–rh7–FLAG transgenic flies were obtained by germline transformation using w^{111} embryos (Bestgene Inc.).

To generate flies expressing an rh7^{+} genomic transgene (P{rh7^{+}}), a BAC genomic DNA clone (CH2218019G) was obtained from the P{acman} collection (GenBank). The germline transformation took advantage of site-specific integration using the F{3′}-attB/attP system (Bestgene Inc.).

Generation of rh7^{+} mutant flies by homologous recombination. We produced the plasmid for knocking out rh7 by ends-out homologous recombination as follows. We PCR amplified two homologous arms (left, 3.2 kb and right, 3.3 kb) using the following primers: left arm forward, AATTGCTGGGATGGAGCCGACATCATGAGC; right arm forward, ATCTCTAGATCAGAACTTACTCTGTTCCATGAC. Both the left arm forward, GCGGCCGCACCATGGAGGCCATCATCATGACG; and right, ATCAGATCTCACCATGGAGGCCATCATCATGACG were designed to delete 540 base pairs spanning this region in the control (w^{111}) sequence. This construct was designed to delete 540 base pairs (5′-TTGCTGGGATGGAGCCGACATCATGAGC–3′) and insert this sequence into the non-coding region of rh7 (4949059–4949047, Drosophila genome release r6.14). The germline transformation took advantage of site-specific integration using U6{+} (BL21), purified the recombinant protein using glutathione sepharose beads (GE Healthcare Life Science). We expressed the fusion protein in Escherichia coli (BL21), purified the recombinant protein using the pGEX6P-1 vector (GE Healthcare Life Science). We expressed the fusion protein in Escherichia coli (BL21), purified the recombinant protein using glutathione sepharose beads (GE Healthcare Life Science) and generated antisera in a rabbit (Covance). We affinity purified the antibodies by conjugating the antigen to Affi-Gel 10 (Bio-Rad).

Immunohistochemistry on whole mounts of Drosophila brains. We performed immunohistochemistry using whole-mounted fly brains as described previously. Briefly, we fixed dissected brains for 15–20 min at 4 °C in 4% paraformaldehyde in phosphate buffer (0.1 M Na_2P_4, pH 7.4) with 0.3% Triton-X-100 (Sigma), followed by primary
antibodies at 4°C for 24 h. After three washes in PBT, the brains were incubated overnight at 4°C with the following secondary antibodies from Life Technologies: anti-mouse Alexa Fluor 488 or 568 Dyese, anti-rabbit Alexa Fluor 488 or 568 Dyese or Alexa dyese. The brains were washed three times with PBT and mounted in VECTASHIELD mounting medium (Vector Labs) for imaging. For Rh7 and PDF co-staining (Fig. 2d–i), four brains were examined.

Immunohistochemistry for circadian clock proteins (Tim and Per). To analyse light-mediated degradation of Tim (Fig. 3c–f), we entrained the flies for 3 days under 12 h light–12 h dark cycles (~600 lx LED white light). The flies were then exposed to a 5-min LED light stimulation (~600 lx) at ZT22, kept in the dark for 55 min, fixed at ZT23 under a red photographic safety light (for 45 min), and dissected for whole-mount immunostaining. Flies that were not exposed to the nocturnal light treatment were fixed and stained at the same time.

To examine Per staining at different ZT points (Extended Data Fig. 9), flies were entrained for 4 days under 12 h light (~400 lx)–12 h dark cycles, and were collected at the indicated ZTs. For nighttime samples, we handled the flies under a red photographic safety light. We prefixed whole flies at 4°C with 4% paraformaldehyde in PBT for 45 min before dissecting out the brains. After the dissections, the brains were fixed again for 30–60 min at 4°C in 4% paraformaldehyde in PBT.

We used the following primary antibodies: anti-Rh7 (1:250, rabbit), anti-Per (1:1,000, guinea pig), anti-Tim (1:1,000, rat)\(^{40}\), anti-PDF (1:1,000, C7 mouse monoclonal antibody from the Developmental Studies Hybridoma Bank), anti-dSir (1:500, mouse, Clontech Catalog #632392). The Per and Tim antibodies were contributed by A. Sehgal. The secondary antibodies (Thermo Fisher Scientific) were anti-rat Alexa Fluor 568 Dyese and anti-guinea pig Alexa Fluor 555 Dyese. We acquired the images using a Zeiss LSM 700 confocal microscope.

Immunostaining of whole-mounts of the Drosophila retina. To perform whole-mount staining of the retina (within the eye cup) and fixed the tissue at 4°C in 4% paraformaldehyde in PBT for 20 min. After washing briefly in PBT, we blocked the retina for 1 h in PBT plus with 5% normal goat serum. We used the following primary antibodies: anti-Rh7 (1:250, rabbit), anti-Rh3 (1:200, mouse, gift from S. Britt, University of Colorado, Denver) and anti-Rh5 (1:200, mouse, gift from S. Britt, University of Colorado, Denver). The secondary antibodies were: anti-rabbit Alexa Fluor 568 Dyese (1:1,000) and anti-mouse Alexa Fluor 488 Dyese (1:1,000).

Circadian behaviour to assess rhythmicity and periodicity. Circadian experiments were performed at 25°C using the Drosophila Activity Monitoring (DAM) System (Trikinetics). Individual 3–7-day-old male flies were loaded into monitoring tubes, which contained 1% agarose (Invitrogen) and 5% sucrose (Sigma) as the food source. The flies were entrained to 12 h light–12 h dark cycles for 4 days and released to constant darkness or constant light (10 lx for dim light conditions and 400 lx for bright light conditions, unless indicated otherwise) for at least 6 days to measure periodicity.

Data collection and analyses were performed using Clocklab (Actimetrics). Activity data for each fly were binned every 30 min for the circadian analyses. To obtain the periodicities, data from constant darkness were subjected to \( \chi^2 \) periodicogram analyses and fast Fourier transfer analysis using Clocklab software. The rhythm strength of a fly was measured as the power minus the significance \( (p < 0.05) \). Flies were considered arrhythmic based on either a rhythm strength of a fly was measured as the power minus the significance \( (p < 0.05) \).

The control genotype for the electrophysiological recordings was w*;GAL4-dORK-NC1-GFP. The cry\(^{110}\) and rh7\(^{11} \) recordings were performed using w*;GAL4-dORK-NC1-GFP cry\(^{110}\) and w*;GAL4-dORK-NC1-GFP rh7\(^{11}\), respectively.

Statistical analyses. To analyse two sets of data, we used the unpaired Student's t-test. To compare multiple sets of behavioural data, we used a one-way ANOVA (Kruskal–Wallis test) followed by Dunn's test. Data are presented as mean \( \pm \) s.e.m. We used Fisher's exact test to analyse the percentages of rhythmic flies. For the patch-clamp recordings, the data are presented as mean \( \pm \) s.e.m. Values of \( n \) refer to the number of measurements for each condition. All data were analysed using SigmaPlot 11 (Systat Software Inc.) or Prism 6 (Graphpad Software).

Cell transfection, membrane extraction and spectral photometry. The HEK293T cells were obtained from the ATCC, which authenticates their lines. This line has not been tested for mycoplasma contamination. The HEK293T cells were cultured to 70% confluency and transfected with 2\( \mu \)g pCS2–MT–rh7 plasmid per 10-cm dish. We used the FuGENE HD Transfection Reagent (Cat.E2311) to perform the transfections. Cells were harvested 24–36 h after transfection and stored at ~80°C. For reconstitution of Rh7 with the chromophore, the HEK293T cells were resuspended in cold PBS (pH 7.4, Quality Biological Inc.) supplemented with a protease inhibitor cocktail (Sigma P8340) and incubated with 40 μM 11-cis-retinal in the dark for 4 h. We prepared membrane protein extracts by resuspending membrane pellets in 0.1% CHAPS in PBS, rotating for 2 h at 4°C, then centrifuging (14,000 g) for 20 min at 4°C. The supernatants were removed and analysed with a UV3600 UV-Nir-Nir Spectrometer (Shimadzu). To obtain the spectral absorption for Rh7, we used membrane extracts from untransfected cells as the blank.

ERG recordings. ERG recordings were performed by filling two glass electrodes with Drosophila Ringer's solution (3 mM CaCl\(_2\), 182 mM KC1, 46 mM NaCl, 10 mM Tris pH 7.2) and placing small droplets of electrode cream on the surface of the compound eye and the thorax to increase conductance. We inserted the recording electrode into the cream on the surface of the compound eye and the reference electrode into the cream on the thorax. Flies were dark adapted for 1 min before stimulating with a 2-s pulse using a halogen light (~30 mV/cm\(^2\) unless indicated otherwise). The ERG signals were amplified with a Warner electrometer and recorded with a Powerlab 4/30 analogue-to-digital converter (AD Instruments).

Patch-clamp recordings. Patch-clamp measurements were performed acutely dissected adult fly brains as described previously\(^{14,15}\). Briefly, all patch-clamp recordings were performed during the daytime to avoid clock-dependent variance in firing rate. All l-LNVs were recorded within a relatively narrow daytime window, and recordings for each genotype were normally distributed for the time of day and did not vary significantly among all three genotypes. l-LNV recordings were made in whole-cell current clamp mode. After allowing the membrane properties to stabilize after whole cell break-in, we recorded for 30–60 s in the current clamp configuration (unless otherwise stated) under near dark conditions (~0.05 mV/cm\(^2\)) before the lights were turned on. Lights-on data were collected for 5–20 s and this was followed by 60–120 s of darkness.

Multiple light sources were used for these studies. We used a standard halogen light source on an Olympus BX51 WI microscope (Olympus USA) for all experiments with white light (400–1,000 nm, 4 mW/cm\(^2\)). Orange light (550–1,000 nm; 4 mW/cm\(^2\)) for electrophysiological recordings was achieved by placing appropriate combinations of 25 mm long- and short-pass filters (Edmund Industrial Optics) over the halogen light source directly beneath the recording chamber. We changed the filters during the recordings to internally match the neuronal responses to different wavelengths of light. Recordings using 405 nm violet light (0.8 mW/cm\(^2\)) were obtained using LEDs obtained from Prizmatix 405 LED (UHP-Mic-Led-405), which provide >2 W collimated purple light (405 nm peak, 15 nm spectral half width). Light was measured for all sources using a Newport 818-UV sensor and the Newport Power/Energy Meter (842-PF, Newport Corporation) and expressed as mW/cm\(^2\).

The control genotype for the electrophysiological recordings was w*;GAL4-dORK-NC1-GFP. The cry\(^{110}\) and rh7\(^{11}\) recordings were performed using w*;GAL4-dORK-NC1-GFP cry\(^{110}\) and w*;GAL4-dORK-NC1-GFP rh7\(^{11}\), respectively.

Data availability. All data are available from the corresponding author upon reasonable request.

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Extended Data Figure 1 | Circadian photoentrainment in flies lacking cryptochrome and proteins required for phototransduction in the compound eye. 

**a**–**g.** Average actograms exhibited by flies of the indicated genotypes maintained under L–D for 4 days and then released to constant darkness. 

**h.** Summary of circadian rhythmicity of flies in **a**–**g.** Rhythm strength of a fly was measured as $p - s$. 

| Genotype | n | Rhy (%) | p-s | Periodicity |
|----------|---|---------|-----|-------------|
| control  | 43| 97.7    | 88.3 (4.9) | 23.3 (0.07) |
| cry01    | 33| 97.0    | 92.1 (7.3) | 23.5 (0.04) |
| norpA406 | 27| 100.0   | 87.9 (6.5) | 23.3 (0.10) |
| norpA406,cry01 | 16| 96.6    | 63.1 (6.9) | 24.0 (0.21) |
| rh502;rh601,cry01 | 15| 100.0   | 83.0 (11.2) | 24.0 (0.11) |
| norpA406,cry01,-trpMc | 15| 93.3    | 112.8 (11.0) | 23.7 (0.17) |
| norpA406,rh502,rh601,cry01 | 23| 100.0   | 109.7 (6.9) | 23.6 (0.11) |
Extended Data Figure 2 | Rh7 is an extraretinal opsin. a, Phylogenetic tree constructed with protein sequences corresponding to the indicated opsins. The full name for A. gambiae OP10 is GPROP10 (VectorBase).

b, Cartoon showing a longitudinal view of the main structures in the Drosophila visual system, including the retina, lamina and medulla. The blue units represent ommatidia, which comprise eight photoreceptor cells (R1–8) and support cells. c, Cartoon showing the photoreceptor cells in a single ommatidium. The six outer photoreceptor cells (R1–R6) are represented in blue and express Rh1<sup>42,43</sup>. The central R7 photoreceptor cell (purple) expresses Rh3 or Rh4<sup>42,43</sup>, while the R8 photoreceptor cell (green) expresses Rh5 or Rh6<sup>44–46</sup>.

d, A wild-type retina stained with anti-Rh7 (red) and anti-Rh3 (green).
e, A wild-type retina stained with anti-Rh7 (red) and anti-Rh5 (green). Scale bars, 30 μm. No Rh7-positive staining was detected in the retina.
f, Generation of rh7<sup>1</sup> by homologous recombination. Shown are cartoons of the wild-type rh7 locus (top) and the genomic organization of the rh7<sup>1</sup> allele (bottom). The triangles (P1–P4) indicate the primers used to verify the rh7<sup>1</sup> mutation.
g, Confirmation of the rh7<sup>1</sup> mutation by PCR. We prepared genomic DNA from control (w<sup>1118</sup>) and rh7<sup>1</sup> flies and performed PCR using the P1–P2 and P3–P4 primer pairs. The positions of DNA markers (kb) are indicated to the right. See Supplementary Information for the raw images of the PCR gels.
h, ERG amplitudes of control and rh7<sup>1</sup> flies using 2-s white light pulses of the indicated intensities. Error bars indicate s.e.m., n = 4.
i–m, ERGs from flies of the indicated genotypes. The event markers below the ERGs indicate light pulses. i, Control flies. j, rh7<sup>1</sup> flies. k–m, Testing for rescue of the reduced ERG amplitude and loss of on- and off-transients in ninaE<sup>E17</sup> flies with an rh7<sup>1</sup> transgene. k, Control flies. l, ninaE<sup>E17</sup> (rh1 mutant). m, ninaE<sup>E17</sup> fly expressing UAS–rh7 in R1–R6 cells under control of rh1–Gal4 (ninaE<sup>E17</sup>, rh1>rh7).
Extended Data Figure 3 | Expression of Rh7 in non-Cry neurons in the dorsal brain. a, Cartoon of a fly brain showing different groups of clock neurons. The boxed areas indicate locations of two groups of Rh7-positive cells. b, rh7+ brain stained with anti-Rh7. c–e, Double labelling of the dorsal region of the brain with a Cry neuron reporter (cry-Gal4E13>UAS-mCherry-NLS) and anti-Rh7. c, Anti-Rh7. d, Anti-mCherry. e, Merge of c and d. f, Control brain stained with anti-Rh7. g, Control brain stained with anti-PDF. h, pdf-Gal4>rh7RNAi brain stained with anti-Rh7. i, pdf-Gal4>rh7RNAi brain stained with anti-PDF. Scale bars: b–e, 20 μm; f–i, 10 μm.
Extended Data Figure 4 | Actograms showing representative behaviour of control and mutant flies before and after a 5-min light pulse at the indicated ZT. Red dots connected by dashed red lines indicate evening peaks before and after the light pulse. Each yellow arrow indicates exposure to a 5-min ~600 lx LED light pulse.
Extended Data Figure 5 | Circadian responses to constant light and light-dependent arousal in rh7 flies. a, b, Flies of the indicated genotypes were entrained under L–D cycles and subsequently released to constant ~400 lx light (L–L). c, d, Flies of the indicated genotypes were entrained under L–D cycles and subsequently released to constant ~10 lx light (L–L). e, Quantification of the effect of a 5-min white light pulse on arousal. Arousal was quantified as increases in total bin crosses during the 5-min light stimulation compared to the total bin crosses during the 5 min before light stimulation. f, Quantification of the time required to reach maximum activity after white light stimulation. g, h, Quantification of the effects on arousal of a 5-min exposure to red (625 nm) (g) or violet (405 nm) LED lights (h). Error bars indicate s.e.m. One-way ANOVA (Kruskal–Wallis test) followed by Dunn’s test. *P < 0.05, **P < 0.01. Number of flies tested: norpA<sup>24</sup>, n = 16; other genotypes, n = 24.
Extended Data Figure 6 | Effects of multiple light input pathways on circadian behaviour. a, b, Actograms showing rhythmic and arrhythmic \textit{rh7} \textit{cry} \textsuperscript{d} double mutants. The flies were entrained under L–D cycles and subsequently released to constant darkness. c, Percentages of rhythmic and arrhythmic flies. Fisher's exact test, **\( P < 0.01 \). Number of flies tested: control, \( n = 16 \); other genotypes, \( n = 30 \). d–h, Circadian behaviour of \textit{g \textsuperscript{lo}} and \textit{rh7} \textit{g \textsuperscript{lo}} double mutant flies. The flies were entrained to L–D cycles for 4 days and subsequently released to constant darkness. d, Percentages of rhythmic and arrhythmic flies. e–h, Average actograms showing the activities of flies of the indicated genotypes. Number of flies tested: control, \( n = 46 \); \textit{rh7}, \( n = 34 \); \textit{g \textsuperscript{lo}}, \( n = 38 \); \textit{rh7} \textit{g \textsuperscript{lo}}, \( n = 40 \). i, Phase response of the indicated genotypes to 5-min white light stimulation at ZT 22. Error bars indicate s.e.m. One-way ANOVA (Kruskal–Wallis test) followed by Dunn's test. **\( P < 0.01 \). Flies tested: control, \( n = 54 \); \textit{rh7}, \( n = 49 \); \textit{g \textsuperscript{lo}}, \( n = 53 \); \textit{rh7} \textit{g \textsuperscript{lo}}, \( n = 57 \).
Extended Data Figure 7 | Rescue of the rh7<sup>1</sup> cry<sup>b</sup> photoentrainment defect by expression of rh7 in pacemaker neurons. 

**a**–**c**, Actograms of control flies and rh7<sup>1</sup> cry<sup>b</sup> flies harbouring only the UAS–rh7 or pdf–Gal4 transgenes. Number of flies tested: control, n = 16; UAS–rh7<sup>1</sup>/+; rh7<sup>1</sup> cry<sup>b</sup>, n = 52; pdf–Gal4<sup>1</sup>/+; rh7<sup>1</sup> cry<sup>b</sup>, n = 21. 

**d**–**f**, Actograms of rh7<sup>1</sup> cry<sup>b</sup> flies expressing UAS–rh7 in pacemaker neurons under the control of tim–Gal4 or pdf–Gal4 as indicated. Number of flies tested: UAS–rh7/tim–Gal4; rh7<sup>1</sup> cry<sup>b</sup>, n = 37; UAS–rh7/pdf–Gal4; rh7<sup>1</sup> cry<sup>b</sup>, n = 23; UAS–rh7–flag/pdf–Gal4; rh7<sup>1</sup> cry<sup>b</sup>, n = 21. 

**g**, Percentages of rhythmic and arrhythmic flies of the indicated genotypes. Fisher’s exact test, *P < 0.05.
Extended Data Figure 8 | Rescue of rh7<sup>1</sup> cry<sup>b</sup> photoentrainment defect by expression of other fly rhodopsins. a–f, Controls showing actograms of rh7<sup>1</sup> cry<sup>b</sup> flies harbouring UAS–rhodopsin transgenes only, and of rh7<sup>1</sup> cry<sup>b</sup> flies expressing the indicated rhodopsin genes in pacemaker neurons under the control of pdf–Gal4. Number of flies tested:

UAS–rh3<sup>+/–</sup>; rh7<sup>1</sup> cry<sup>b</sup>, n = 56; UAS–rh4<sup>+/–</sup>; rh7<sup>1</sup> cry<sup>b</sup>, n = 46; UAS–rh5<sup>+/–</sup>; rh7<sup>1</sup> cry<sup>b</sup>, n = 24; UAS–rh3<sup>pdf</sup>–Gal4; rh7<sup>1</sup> cry<sup>b</sup>, n = 64; UAS–rh4<sup>pdf</sup>–Gal4; rh7<sup>1</sup> cry<sup>b</sup>, n = 25; UAS–rh5<sup>pdf</sup>–Gal4; rh7<sup>1</sup> cry<sup>b</sup>, n = 16.

g, Percentages of rhythmic and arrhythmic flies of the indicated genotypes. Fisher’s exact test, *P < 0.05, ***P < 0.001.
Extended Data Figure 9 | Per oscillates in control, *rh7*<sup>1</sup>, *cry*<sup>b</sup> and *rh7*<sup>1</sup> *cry*<sup>b</sup> flies. a, d, g, j. Flies of the indicated genotypes were entrained under L–D cycles for 4 days and the brains were dissected on the 5th day. The ZT times indicate when the brains were fixed and dissected for staining with anti-Per (Per, upper rows, red) and anti-PDF (PDF, lower rows, green) as indicated. At least one s-LNV (s) and one l-LNV (l) are labelled in the images obtained at each ZT to facilitate identification of LNVs. Scale bars, 10 μm. b, c, e, f, h, i, k, l. Quantification of relative Per levels in s-LNVs and l-LNVs of flies of the indicated genotypes. The image quantification was performed using ImageJ. The y axes indicate relative Per intensities. The Per intensities in ZT2 of the control flies were designated as 100. For control flies, ZT10, n = 6; ZT22, n = 8; n = 5 for all other time points. For *rh7*<sup>1</sup>, ZT2, n = 9; ZT6, n = 8; ZT10, n = 6; ZT14, n = 8; ZT18, n = 8; ZT22, n = 7. For *cry*<sup>b</sup>, ZT2, n = 8; ZT6, n = 9; ZT10, n = 8; ZT14, n = 7; ZT18, n = 10; ZT22, n = 8. For *rh7*<sup>1</sup> *cry*<sup>b</sup>, n = 5 for all time points. Error bars indicate s.e.m.
Extended Data Figure 10 | Knockdown of plc21C in PDF-positive neurons impaired circadian phase response. a, Quantitative real-time PCR analysis of plc21C mRNA using RNA prepared from whole adults. The plc21C expression levels in each sample were normalized using rp49 expression. The control was w1118. Centre values indicate the average and error bars indicate s.e.m. One-way ANOVA (Kruskal–Wallis test) followed by Dunn’s test. **P < 0.01. b, Phase response of the indicated genotypes to 5 min white light stimulation at ZT22. One-way ANOVA (Kruskal–Wallis test) followed by Dunn’s test. **P < 0.01. pdf–Gal4/+; plc21C–RNAi01211/+; plc21C–RNAi01211, n = 37; pdf–Gal4/+; plc21C–RNAi01210, n = 32. Error bars indicate s.e.m. c–g, Examples of behaviour before and after the 5-min light pulse. The yellow arrows indicate the times of the 5-min white light pulses (~600 lx). The red dots connected by dashed red lines indicate the evening peaks before and after the light pulse.