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✉ For correspondence:

pingkalai.senthilan@uni-wuerzburg.de

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RHODOPSIN 7: An ancestral non-canonical photoreceptor shaping light-responsive behavior

Valentina Kirsch¹, Nils Reinhard^{1,2}, Heiko Hartlieb¹, Annika Mohr¹, Dirk Rieger¹, Peter Soba³,

Charlotte Helfrich-Förster¹, Pingkalai R Senthilan¹ ✉

¹Neurobiology and Genetics, Theodor-Boveri Institute, Biocenter, Julius-Maximilians-University Würzburg, Würzburg, Germany • ²University of Münster, Institute of Neuro- and Behavioral Biology, Münster, Germany • ³Institute of Physiology and Pathophysiology, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany

eLife Assessment

The study investigates, from multiple angles, the still-debated function of insect rhodopsin-7 (Rh7). The authors present **compelling** results for its ancient phylogenetic origin across pan-arthropods, a non-visual role based on expression analyses in the fly brain, an unusual G-protein signalling pathway, and - using behavioural genetics - that Rh7 affects how *Drosophila melanogaster* interprets and responds to light-dark transitions. Through this, the work provides **fundamental** new insights into the evolution and function of non-visual opsins.

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Abstract

Animals rely on light not only for vision but also to adapt behavior to their environment through non-image-forming pathways. In *Drosophila*, most rhodopsins mediate retinal image formation, but RHODOPSIN 7 (RH7) is widely expressed in the brain and optic lobe, acting as a non-canonical light sensor reminiscent of mammalian melanopsin. We combined expression mapping, behavioral assays, and phylogenetic comparisons to investigate its function. RH7 detects temporal changes in luminance, guiding rapid behavioral responses, and regulating daily activity patterns. Flies lacking RH7 are less active during the dark phase, while strains with constitutively active RH7 show increased activity. Phylogenetic analyses suggest that RH7 is an ancient rhodopsin present across panarthropods, representing an intermediate between G protein-coupled receptors and specialized rhodopsins, highlighting a key step in the evolution of non-visual light perception. These findings show that RH7 functions as a melanopsin-like sensor, shaping behavior, and illuminating the origins of light detection.

2 Introduction

The ability to detect and respond to light is fundamental for many organisms and can influence behavior through both visual and non-visual pathways. Vision relies on specialized photoreceptor cells that convert light into neural signals through a process called phototransduction. This allows the brain to interpret visual information and guide behavior. Rhodopsin chromoproteins, which are composed of the seven-helix membrane apoprotein opsin and the chromophore retinal, play a central role in this process. When a photon is absorbed, the chromophore undergoes a conformational change, transforming from 11-cis-3-hydroxyretinal to all-trans-3-hydroxyretinal. Then, structural changes occur in the protein, initiating signal transduction. As G protein-coupled receptors (GPCRs), rhodopsins first activate a trimeric G protein, which then initiates a cascade of downstream signaling events in phototransduction (Xiong and Bellen, 2013 [DOI](#)).

While the primary function of rhodopsins is image formation in the visual system, some are also known to participate in non-image-forming functions. Based on their specific roles, rhodopsins are classified as visual or non-visual (Kingston and Cronin, 2016 [↗](#); Leung and Montell, 2017 [↗](#); Terakita, 2005 [↗](#)). Several studies have highlighted the diverse functions of rhodopsins, including their involvement in thermosensation (Shen et al., 2011 [↗](#)), hearing (Senthilan et al., 2012 [↗](#)), proprioception (Zanini et al., 2018 [↗](#)), and taste (Leung et al., 2020 [↗](#)).

Opsins are classified into different groups, including ciliary opsins (c-opsins) and rhabdomeric opsins (r-opsins). While c-opsins trigger a $G_{\alpha\text{t}}$ transducin ($G_{\alpha\text{t}}$)-mediated signaling pathway leading to Na^+ channel closure and consequent hyperpolarization, r-opsins trigger a $G_{\alpha\text{q}}$ -mediated depolarizing signaling cascade. In humans, c-opsins in rods and cones are essential for visual perception, while melanopsin (OPN4), an r-opsin expressed in intrinsically photosensitive retinal ganglion cells (ipRGCs), is involved in the synchronization of circadian rhythms and enhances contrast sensitivity (Sonoda et al., 2018 [↗](#)). *Drosophila melanogaster* (*Dmel*) has seven rhodopsins (RH1-RH7), all belonging to the r-opsin group. While RH1-RH6 are expressed in well-characterized photoreceptors, the expression pattern and function of RH7 remain less clear and are still debated.

RH7 displays the canonical rhodopsin features, including seven transmembrane helices, a chromophore-binding lysine, and the LRTPXN motif located in the first intracellular loop (ICL1). However, two features mostly distinguish RH7 from other opsin groups: its extended N- and C-terminal tails, which are twice as long as in RH1-RH6, and the absence of the highly conserved QAKKMNV motif in the third intracellular loop (ICL3), important for G protein interaction (Figure 1A [↗](#)) (Senthilan and Helfrich-Förster, 2016 [↗](#)). Additionally, RH7 contains a fully palindromic RCSI motif within its second intron, in contrast to the non-palindromic RCSI found upstream of other rhodopsins (Datta and Rister, 2022 [↗](#); Rister et al., 2015 [↗](#); Senthilan and Helfrich-Förster, 2016 [↗](#)). The palindromic RCSI and its intronic location are likely key factors shaping the broader expression pattern of RH7. Tandem affinity purification of INTACT nuclei (TAPIN)-seq data indicate that *Rh7* is absent in retinal photoreceptors but present in visual processing neurons in the lamina and medulla (Figure 1B [↗](#)) (Davis et al., 2020 [↗](#)).

Previous studies on RH7 have yielded conflicting results regarding its function and expression, including discrepancies in the identified neuronal populations and associated behavioral phenotypes (Grebler et al., 2017 [↗](#); Kistenpennig et al., 2017 [↗](#); Leung et al., 2020 [↗](#); Leung and Montell, 2017 [↗](#); Ni et al., 2017 [↗](#); Senthilan et al., 2019 [↗](#)). Two factors may explain these discrepancies. First, RH7 is relatively large but likely adopts a compact, self-contained structure (Figure 1A [↗](#)). Its highly phosphorylatable N- and C-termini may interact with other proteins and further obscure relevant epitopes, making antibody detection nearly impossible. Second, the genetic background of the tested flies, particularly the presence of the *white* and *yellow* mutations as markers, appears to influence the *Rh7* phenotype, possibly due to overlapping expression sites. Both genes are expressed in the optic system (Davis et al., 2020 [↗](#)) (Figure 3-figure supplement 2D [↗](#)) and are known to affect neuronal function and behavior (Bond et al., 2024 [↗](#); Drapeau et al., 2003 [↗](#); Rickle et al., 2025 [↗](#)).

However, all previous studies, despite their differences, agree that *Rh7* is an atypical rhodopsin with non-visual functions, a hypothesis that is further supported by this study. We show that RH7 is distinct from other *Drosophila* rhodopsins. RH7 is predominantly expressed in optic lobe neurons and likely acts via a distinct signaling pathway, contributing to darkness detection and nocturnal activity. As an ancient rhodopsin, RH7 appears to contribute to basic visual functions, such as contrast processing as well as to non-visual functions like circadian synchronization, similar to melanopsins in mammals.

3 Material & Methods

3.1 Phylogenetics tree

Protein sequences were obtained from the NCBI RefSeq database (January 2026 annotation release, RS_2026_01). From these data, a dataset was compiled including all available invertebrate proteomes as well as selected vertebrate model organisms. Homologs of the opsins RH1, RH3, and

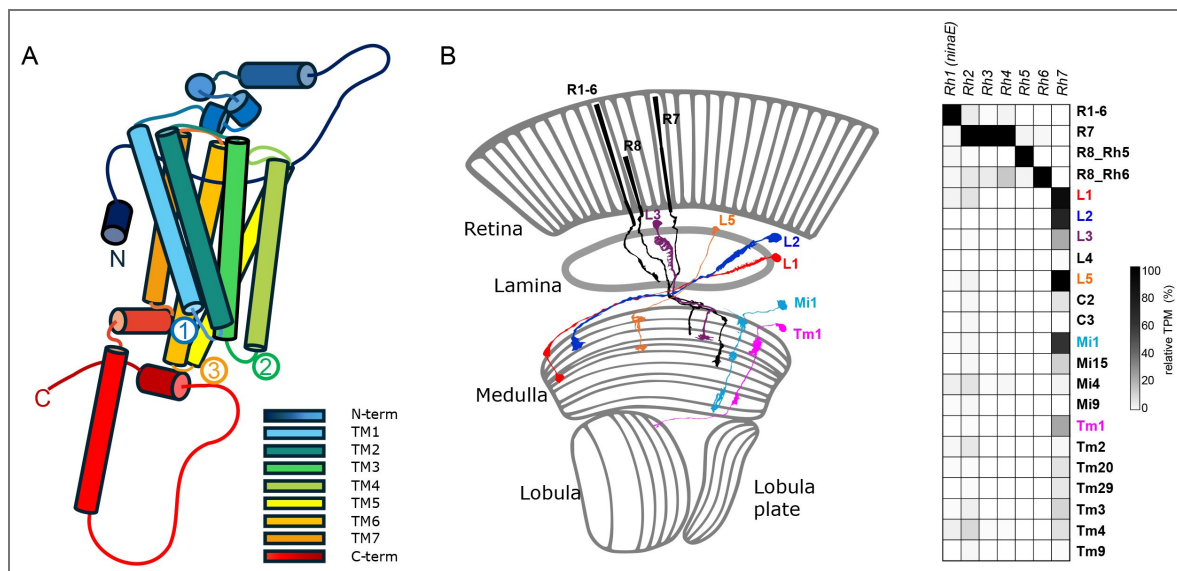


Figure 1. Predicted RH7 structure and expression pattern in the *Drosophila* optic system.

(A) Schematic representation of the predicted 3D structure of RH7 (GH14208p) based on AlphaFold (Jumper et al., 2021). The structure is color-coded using a continuous spectral gradient ranging from blue (N-terminus) to red (C-terminus). The three intracellular loops are highlighted and labeled in numbered circles: (1) ICL1 with the LRTPXN motif, (2) ICL2 with the highly conserved DRY motif, and (3) ICL3, which lacks the typical QAKMNV motif. **(B)** Left: Schematic representation of the *Drosophila melanogaster* optic system. The presumed *Rh7*-expressing cells, according to Davis et al., (2020) are shown (in color) in addition to retinal receptor cells (in black). Right: Heatmap of relative gene expression across *Drosophila* optic lobe cell types. TPM values were obtained by TAPIN sequencing (Davis et al., 2020) and normalized per gene (100% = maximum TPM per gene). Gray scale: from white (0%), to black (100%). Genes are shown on the x-axis, and cell types on the y-axis.

RH7 were identified using BLASTP searches with an E-value cutoff of $\leq 1e-40$. This procedure yielded sequences from 444 species. As *Paramacrobiotus metropolitani* is currently the only non-arthropod panarthropod represented in RefSeq, *Ramazzottius varieornatus* and *Hypsibius exemplaris* were manually added to improve representation of Tardigrada, resulting in a final dataset of 446 species. All sequences in FASTA format are provided in [Supplementary file 1](#).

To ensure balanced lineage representation in the phylogenetic tree, only one representative species per superfamily within each order was retained. Whenever possible, experimentally characterized species were preferentially selected as representatives. This resulted in 142 species with 558 protein sequences. Initial phylogenetic trees were examined to identify and remove obvious sequence duplicates, producing a final alignment of 470 protein sequences. Phylogenetic trees were constructed using Geneious Prime® (2026.0.2) with the Geneious Tree Builder. Protein sequences were globally aligned with free end gaps using the BLOSUM62 substitution matrix and gap costs of 10/0.1. Trees were used to assess evolutionary relationships among RH1, RH3, and RH7 opsins. Protein names, sequences, and accession numbers are provided in [Supplementary file 1](#). To confirm phylogenetic assignments, reciprocal BLAST searches were performed against the *D. melanogaster* proteome, ensuring clade consistency and homology validation.

3.2 Co-occurrence analysis of RH7 and DCRY

Drosophila-type cryptochrome (DCRY) and RH7 homologs were identified from the RefSeq dataset. DCRY sequences were validated based on motif searches and cluster properties following the approach described in [Deppisch et al. \(2022\)](#), and RH7 homologs were validated based on the phylogenetic analysis performed in this study. All species containing homologs of RH1, RH3, or RH7 (E-value $\leq 1e-40$) were included in the co-occurrence analysis. For species in which one of the proteins was detected but the other was not identified in RefSeq, additional searches were performed against clustered non-redundant (NR) protein datasets to detect potential DCRY or RH7 homologs missing from current RefSeq annotations.

3.3 GsX assay

To test the functionality of RH7, we used a GsX assay designed to determine which G_q proteins can bind to a given GPCR, in this case RH7 ([Ballister et al., 2018](#)). G protein coupling of GPCR constructs was examined using HEK293T or HEK293-AG7 cells, which lack the seven G_q subunits: G_{qS} -type (GNAS, GNAL), G_{qQ} -type (GNAQ, GNA11), $G_{q12/13}$ -type (GNA12, GNA13), and G_{qZ} (GNAZ). All cells were obtained from A. Inoue at Tohoku University ([Bowin et al., 2019](#)). HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Pan Biotech, Germany), 100 U/mL penicillin and 100 mg/mL streptomycin at 37 °C in 5% CO₂. The GsX assay was modified from Ballister et al. ([Ballister et al., 2018](#)). White 96-well plates (Greiner Bio-One) were coated with 0.1 mg/mL poly-L-lysine (Sigma Aldrich, USA) at 37 °C for 1 h prior to cell seeding. Receptor plasmids, G protein chimeras and the cAMP-sensitive luciferase reporter Glo22F (Promega) were co-transfected using Lipofectamine 2000 (Thermo Fisher, USA) and incubated at 37 °C in 5% CO₂ for 24 hours. The medium was replaced with L-15 medium (phenol red-free, 1% FBS) containing 2 mM beetle luciferin (in 10 mM HEPES, pH 6.9) and 10 mM 9-cis-retinal or all-trans retinal and incubated for 1 hour at room temperature in the dark. cAMP-dependent luminescence was measured using a Berthold Mithras multimode plate reader (Berthold Tech., Germany). Cells were activated with a 1-second light pulse (470 nm) using an LED light plate (Phlox Corp., France) or CoolLED pE-4000 (CoolLED, UK). Two technical duplicates were performed, and data were normalized to pre-activation baselines.

3.4 Locomotor Activity

The locomotor activity of individual male flies was monitored at 1-minute intervals using the *Drosophila* Activity Monitoring (DAM) system (Trikinetics, Inc., Waltham, MA), which detects infrared (IR) beam interruptions. Male flies (3 to 7 days old) were individually placed in recording glass tubes (~5 mm diameter) containing agar/sugar medium (2% agar; 4% sucrose) filled to one-third capacity and sealed with an air-permeable plug. Activity was quantified by counting the

number of infrared (IR) beam crossings per minute in the center of each tube. All recordings were performed under constant conditions of 60% relative humidity and 25 °C. A standard day was defined as 12 h light (30 $\mu\text{W}/\text{cm}^2 \sim 100$ lux, the spectrum of the light is shown in [Figure 3-figure supplement 1A](#)) and 12 h darkness (LD 12:12). In addition, experiments using individual wavelengths (469 nm, 500 nm, and 521 nm) were performed; the corresponding absorption spectra are shown in [Figure 3-figure supplement 1B](#). To assess light-off startle responses, which are brief clock-independent increases in activity after sudden light-off during the day, flies were repeatedly exposed to 30-minute dark pulses following 1.5 h illumination. Dark pulses were applied six times during each 12 h light phase. The experiments were conducted under “standard day” conditions consisting of 12 h light (30 $\mu\text{W}/\text{cm}^2$) and 12 h darkness.

To account for differences in baseline activity between genotypes, the activity data were normalized to the mean activity level by dividing the activity values of each time point by the mean activity of the respective fly across the entire 24 h recording period. For all experimental conditions, data from four consecutive days were averaged, omitting the first day to reduce effects of acclimatization.

In a second startle response assay (4-hour assay), we specifically aimed to assess the acute startle response independent of circadian influences. To minimize contributions of the circadian clock, flies were first rendered arrhythmic by exposure to constant light (120 $\mu\text{W}/\text{cm}^2$, ~ 500 lux) for two days. This led to sustained activation of the blue light receptor DCRY and continuous degradation of TIMELESS, resulting in a standstill of the molecular clock ([Deppisch et al., 2022](#); [Dolezelova et al., 2007](#); [Emery et al., 2000](#)). Subsequently, flies were subjected to three consecutive days of 4-hour light-dark cycles (6 cycles per day). Each cycle consisted of 3.5 hours of light (5 $\mu\text{W}/\text{cm}^2$ (~ 10 lux), 30 $\mu\text{W}/\text{cm}^2$ (~ 100 lux) or 120 $\mu\text{W}/\text{cm}^2$ (~ 500 lux)) followed by a 30-minute dark phase (no light or reduced light intensity of 5 $\mu\text{W}/\text{cm}^2$ or 30 $\mu\text{W}/\text{cm}^2$). This short-cycle paradigm prevents re-entrainment of circadian rhythms and allows isolation of the startle response itself (see schematic representation in [Figure 3-figure supplement 1C](#)). Startle activity was recorded during each cycle. The first 4 h cycle was excluded from analysis to avoid carryover effects from the prior light regime. Values from all remaining 17 cycles were averaged for analysis. Data were plotted with the 30-minute dark pulse centered on the time axis, resulting in two light periods of 105 minutes each, before and after the dark pulse. For normalization, the mean activity during the 105 minutes preceding light-off was set to one, and activity after light-off was expressed relative to this baseline.

3.5 Locomotor activity analysis

Activity data were analyzed in R using custom scripts. Raw activity traces were smoothed using cubic smoothing splines (`smooth.spline`, default settings). Morning and evening activity peaks were identified as the maximal smoothed activity within predefined windows (morning: 330–390 min; evening: 1050–1110 min). Peak amplitude was calculated as the mean activity across the peak maximum ± 2 min.

Activity onset and offset were detected from the smoothed signal using a threshold defined as 80% of the mean daily activity. An onset was defined when activity levels were first for at least 10 min below the threshold followed by at least 10 min of activity above the threshold together with a positive signal derivative; offsets were defined analogously using inverse criteria. Search windows were morning onset (180–360 min), morning offset (420–720 min), evening onset (750–1080 min), and evening offset (1080–1200 min). Daytime activity (avg activity day) was quantified as the summed smoothed activity from the light phase (361–1080 min). Siesta beginning time was defined as the morning offset. Siesta length was calculated as the time interval between morning offset and evening onset. Activity after lights-off (LOFF activity (min)) was calculated as the interval between the evening offset and lights-off (evening offset – 1080). Maximum activity after lights-off (LOFF max activity) was defined as the highest activity value recorded after lights-off, expressed as the maximum number of infrared beam crossings per minute.

3.6 Fly lines, Primers, and Antibodies

The lines listed below were used for the experiments in this study. To minimize background effects, all lines were previously brought on the same X chromosomal background derived from the control fly CantonS (CS), carrying wild-type alleles of the *yellow* (*y*⁺) and *white* (*w*⁺) genes, meaning any mutations in these genes are absent.

3.7 Generating the *Rh7-Gal4* line

We generated a *Rh7-Gal4* line to investigate *Rh7* expression. To this end, the *Rh7* promoter was cloned upstream of a *Gal4* coding sequence, driving *Gal4* expression in *Rh7*-expressing cells. Bioinformatic analyses suggested that the *Rh7* promoter is unusual and relatively large (Senthilan and Helfrich-Förster, 2016 [\[3\]](#)), hence we used a region including 1,080 bp upstream of exon 1 and extending into exon 2, which contains the translation start site. Due to the overall length of the target region (>8,000 bp), the construct was assembled in four fragments, which were sequentially cloned into the *pPTGAL* vector (Sharma et al., 2002 [\[3\]](#)). The four inserts were amplified from *D. melanogaster* genomic DNA using specific *Gal4* primer pairs (see Table 2 [\[3\]](#)). Insert 1 and insert 4 included artificial restriction sites (Eco52 and KpnI, respectively), while all other restriction sites were naturally occurring. Cloning began with insert 4 (see Figure 4-figure supplement 1 [\[3\]](#)) and proceeded stepwise with the remaining fragments. Final plasmids were amplified in *E. coli* NEB 10-beta and the construct integrity was confirmed by sequencing. The plasmid was injected into *w*¹¹¹⁸ flies by TheBestGene Inc., and the resulting transgenic line was crossed into the same *y*⁺*w*⁺ background as the controls (Table 1 [\[3\]](#)).

3.8 Generating UAS-constructs

For *Rh7*-rescue experiments, UAS constructs were generated to assess the ability of different *Rh7* variants to rescue the phenotype. *UAS-Rh7* carries the wild-type *Rh7* sequence, *UAS-Rh7^{Darkfly}* carries the Darkfly variant, and *UAS-empty* (*UAS-()*) serves as a negative control (no-rescue). The *Rh7* construct was prepared from the *D. melanogaster* cDNA using the primers UAS-Rh7-5' and UAS-Rh7-3' (Table 2 [\[3\]](#)), digested with EcoRI and BglII enzymes and ligated into the *pUASTattB* vector (Bischof et al., 2007 [\[3\]](#)). For the *UAS-empty* construct, the *pUASTattB* vector was used unmodified. The vectors were then injected into flies with CytoSite 2R, 55C4 (BDSC Stock #8621, containing attP1) by the company TheBestGene Inc. All lines were brought into a *+,+;Rh7⁰* background and finally crossed with flies carrying respective *Gal4*-constructs in *+,+;Rh7⁰* background and assayed. The *UAS-Rh7^{Darkfly}* construct was prepared with the primers UAS-Rh7-5' and UAS-Darkfly-3' (Table 2 [\[3\]](#)) with the same steps described above. All transgenic lines were crossed into the same *y*⁺*w*⁺ background as the controls (Table 1 [\[3\]](#)).

3.9 Optomotor response

To investigate the contribution of RH7 in the lamina and medulla to visual motion detection, male flies were individually tested for their optomotor walking behavior. Flies were raised under standard LD 12:12 conditions at 25 °C. Subsequently, they were maintained under constant light (LL, 120 $\mu\text{W}/\text{cm}^2$ (~500 lux)) for at least 3 days to induce arrhythmicity and minimize the influence of the circadian clock on the optomotor response. The optomotor response setup comprises: (1) an upright cylinder ($\varnothing$ 8 cm; H 4.5 cm) with vertical black and white stripes (2.2 cm width and 4 cm height) inside for visual stimulation and (2) a transparent Plexiglas arena ($\varnothing$ 3 cm; H 1.5 cm) with a tube for inserting the flies. The open space available for movement was only about 2 mm, limiting the flies to a single horizontal walking plane (Figure 5-figure supplement 1C [\[3\]](#)). The LED-illuminated cylinder with 6 white and 6 black stripes rotated clockwise (CW) and counterclockwise (CCW) at 16 rpm, equivalent to a frequency of 1.6 Hz. The flies were acclimatized for 2 minutes without light, followed by 2 minutes under CW rotation with light, a 1-minute rest without light, and then 2 minutes under CCW rotation with light. For the optomotor response (OR), the number of complete laps around the arena and complete body turns in the direction of the pattern movement within both 2-minute runs were added, while movements in the opposite

Table 1. Fly lines

Name	Genotype	Comment
WT control (wild-type control)	+,+,+	Strain CantonS (CS) (Horn et al., 2019)
<i>Rh7⁰</i>	+,+; <i>Rh7⁰</i>	From Kistenpfennig et al. (2017) crossed into the <i>y⁺w⁺</i> (CS) background.
<i>Rh7^{MIMIC}</i>	+,+; <i>Rh7^{MIMIC}</i>	From Kistenpfennig et al. (2017) crossed into the <i>y⁺w⁺</i> (CS) background.
<i>per⁰¹</i>	<i>per⁰¹</i> ;+,+	From (Konopka and Benzer, 1971)
<i>per⁰¹</i> ;; <i>Rh7⁰</i>	<i>per⁰¹</i> ;+; <i>Rh7⁰</i>	Double mutants carrying the mutations <i>period⁰¹</i> and <i>Rh7⁰</i>
<i>Rh7-Gal4</i>	+, <i>Rh7-Gal4</i>	Extended <i>Rh7</i> promoter (8,2 kb) construct, spanning ~1kb upstream of transcription start, extending to exon 2 incl. ATG, cloned into <i>pPTGAL</i> (Sharma et al., 2002) vector; injected by TheBestGene.
<i>Rh7-Gal4</i> ; <i>Rh7⁰</i>	+, <i>Rh7-Gal4</i> ; <i>Rh7⁰</i>	<i>Rh7-Gal4</i> line crossed into <i>Rh7⁰</i> background.
<i>UAS-empty</i> / <i>UAS-()</i>	+, <i>UAS-empty</i> ; <i>Rh7⁰</i>	An empty <i>pUASTattB</i> (Bischof et al., 2007) construct, with the UAS-backbone and mini-white, CytoSite 2R, 55C4, injected by TheBestGene, crossed into <i>Rh7⁰</i> background.
<i>UAS-Rh7</i>	+, <i>UAS-Rh7</i> ; <i>Rh7⁰</i>	<i>Rh7</i> under UAS control, generated with the <i>pUASTattB</i> (Bischof et al., 2007) vector, CytoSite 2R, 55C4, injected by TheBestGene, crossed into <i>Rh7⁰</i> background.
<i>UAS-Rh7^{Darkfly}</i>	+, <i>UAS-Rh7^{Darkfly}</i> ; <i>Rh7⁰</i>	<i>Rh7</i> lacking the last 21 amino acids, under UAS control, generated with the <i>pUASTattB</i> (Bischof et al., 2007) vector, CytoSite 2R, 55C4, injected by TheBestGene, crossed into <i>Rh7⁰</i> background.
<i>UAS-GFP-JFRC81</i>	+, <i>UAS-GFP</i> ;+	<i>JFRC81-10xUAS-IVS-Syn21-GFP-p10</i> (Pfeiffer et al., 2012)
<i>UAS-MCFO</i>	see Nern et al. (2015)	Bloomington <i>Drosophila</i> Stock Center #64091 (Nern et al., 2015)
Darkfly	see Izutsu et al. (2012)	This line has been maintained for decades in constant darkness and carries numerous mutations, including a truncated <i>Rh7</i> lacking the last 21 amino acids. Kindly provided from Naoyuki Fuse (Izutsu et al., 2012)
<i>w¹¹¹⁸</i>	<i>w¹¹¹⁸</i> ;+,+	Used for injection by the company TheBestGene
BDSC Stock #8621	<i>y1 w67c23</i> ; <i>P{CaryP}attP1</i>	Used for injection by the company TheBestGene

Table 2. Primers

Application	Name	Sequence (5'-3')
Gal4 driver construct	Rh7 Gal4 Eco52 (1)-5'	CGGCCGTAGTTCATGACGATGCCAGACCAG
	Rh7 Gal4 EcoRI (1)-3'	CAATCTGGCCTGAATAAGCCACGG
	Rh7 Gal4 EcoRI (2)-5'	CGGCCGTGGTCACAATGGGAATTCGCA
	Rh7 Gal4 SpeI (2)-3'	AGGCCGTTCACTAGTGAGCACTAC
	Rh7 Gal4 SpeI (3)-5'	GCGGCCGACGAGTCTAAGTGATGGGTCCG
	Rh7 Gal4 BglII (3)-3'	CGGGTTTGGCAGAATTTGAAGATCTG
	Rh7 Gal4 BglII (4)-5'	AGTTTCGCAGTGGAAAAGCACTTGC
	Rh7 Gal4 KpnI (4)-3'	GGTACCGATGGCCTCCATGACTGACTTG
UAS construct	UAS-Rh7-5'	CAAGGAATTCATGGAGGCCATCATCATGACGAC
	UAS-Rh7-3'	GTCAGATCTCAGAACTTACTCTGTTCCATGACG
	UAS-Darkfly-3'	CAAAGATCTACTCGTTCTCCTCCGCTCCTCG
qPCR	Rh7 qPCR-5'	AATCATCCGCTGTGAATGTAGGCAAG
	Rh7 qPCR-3'	AATCAGGAAGGTGCTCGACTTAGGTG
	alphaTub-5'	TCTGCGATTGATGGTGCCCTTAAC
	alphaTub-3'	GGATCGCACTTGACCATCTGGTTGGC

direction were subtracted. The result was then divided by the total number of cylinder rotations (4×16). A representative video of the optomotor response assay is provided as [Supplementary Video 1](#) [↗](#).

3.10 qPCR

For quantitative PCR (qPCR), total RNA was extracted from either five heads, five brains, 10 retinæ or 10 laminae of control flies. Flies were raised under standard LD 12:12 conditions at 25 °C. Tissues were carefully dissected from adult flies without prior fixation to avoid interference with RNA integrity and downstream extraction procedures. Dissections were performed during the light phase and were not restricted to specific time points. Total RNA was extracted using the Quick-RNA™ Microprep Kit (Zymo), and cDNA was synthesized with the QuantiTect Reverse Transcription Kit (Qiagen). For qPCR, three biological replicates were used for each tissue, with two technical replicates. qPCRs were performed using the SensiFAST™ SYBR® No-ROX Kit (Bioline) in the Qiagen Rotor-Gene Q qPCR machine, with raw data fit by dynamic tube and slope correction calculation using the Rotor-Gene Q Series software. Relative mRNA levels were calculated using the Δ -CT equation, with *α-tubulin* used as a reference gene (Δ CT = CT_{*α-tubulin*} - CT_{*Rh7*}). qPCR was performed with *Rh7* (*Rh7* qPCR-5', *Rh7* qPCR-3') and control (*αTub*-5', *αTub*-3') primers, each at 0.1 mM ([Table 2](#) [↗](#)).

3.11 Immunohistochemistry

Fluorescent immunohistochemistry was performed on 3 to 5 days old male flies. Whole flies were fixed for 3 h in 4% paraformaldehyde in 0.5% PBST (1xPBS with 0.5% Triton X-100) followed by three 10-minute rinses with PBS. The brains were dissected in 0.5% PBST and incubated overnight at 4 °C with 5% normal goat serum (NGS) or normal donkey serum (NDS) and 0.02% NaN₃ in 0.5% PBST, depending on the host species of the secondary antibodies. Antibody solutions contained the antibody in the concentration specified in [Table 3](#) [↗](#) and 5% NGS or NDS with 0.02% NaN₃ in 0.5% PBST. The brains were incubated for two days at 4 °C in primary antibody solution followed by five 10-minute washes with 0.5% PBST. Subsequently, the brains were incubated for at least 4 hours at room temperature or overnight at 4 °C with the secondary antibody solution containing the corresponding secondary antibodies ([Table 3](#) [↗](#)). The samples were washed five times for 10 minutes with 0.5% PBST before embedding in VectaShield 1000 mounting medium (Vector Laboratories, Burlingame, CA, USA) using object slides with spacers (~170 μm). The high-precision coverslips (170 ± 5 μm, No.1.5H) were sealed with FixoGum. Brains with retinæ attached were embedded with retinæ facing the object slide to ensure easy imaging from the posterior. Object slides were stored in the dark at 4 °C until scanning. In addition, individual neurons were visualized using the MultiColor FlpOut (MCFO) technique ([Nern et al., 2015](#) [↗](#)), which stochastically labels single cells with distinct membrane-targeted fluorescent tags. Flippase-mediated excision of FRT-flanked stop cassettes enables sparse and color-resolved labeling, allowing detailed analysis of neuronal shape and projections. Labeled brains were processed and mounted as described above.

3.12 *In situ* hybridization

For RNA *in situ* hybridization, we used the hybridization chain reaction v3 (HCR) method developed by Molecular Instruments ([Choi et al., 2018](#) [↗](#)). The flies were fixed and dissected as described for the fluorescent immunohistochemistry. The tissue was either used directly for *in situ* hybridization or dehydrated and stored in 99% methanol at -20 °C for up to 14 days. Dehydration was performed by sequential incubation in 30%, 50%, 70%, and 90% methanol in PBS (2 minutes each). Rehydration was performed in the opposite order. *In situ* HCR was performed using a modified protocol from [Bruce et al. \(2021\)](#) [↗](#). In short, *Rh7* probe mixes containing 20 probe pairs were designed by Molecular Instruments. Instead of using Proteinase K or detergent-based denaturation solutions, tissues were incubated in 0.5% PBST for 30 minutes. This was followed by a 30-minute incubation at 37 °C in preheated probe hybridization buffer (Molecular Instruments), and then an overnight incubation (≥ 20 h) at 37 °C in preheated probe hybridization buffer.

Application	Antibody	Dilution	Source	Reference
<i>Rh7-Gal4>UAS-GFP</i> MultiColor FlpOut	chicken anti-GFP	1:1000	Rockland, Limerick, PA, USA	AB_13970
	goat anti-chicken Alexa Flour® 488	1:400	Thermo Fischer Scientific	AB_2534096
	chicken Anti-V5	1:100	Fortis Life Sciences	A190-118A
	rat anti-HA (3F10)	1:100	Roche	AB_2687407
	goat anti- FLAG	1:500	Bethyl Laboratories	Thermofischer A190-101P
	donkey anti-chicken Alexa Flour® 488	1:400	Thermo Fischer Scientific	AB_2921070
	donkey anti-rat Alexa Flour® 555	1:400	Thermo Fischer Scientific	AB_2910652
	donkey anti-goat Alexa Flour® 594	1:400	Thermo Fischer Scientific	AB_2762828

Table 3. Antibodies used for immunohistochemistry

containing the *Rh7* probe mix (12 nM). After hybridization, the tissue was washed four times for 15 minutes each with preheated probe washing buffer (Molecular instruments) at 37 °C. This was followed by two washes at room temperature (5 minutes each) in 5xSSCT (5xSSC buffer with 1% Tween20). Next, the tissues were incubated for 30 minutes at room temperature in amplification buffer (Molecular Instruments) followed by incubation for at least 20 h at room temperature in amplification buffer containing the hairpins required for signal amplification. Hairpins were prepared by heating 3 μ M h1 hairpins and 3 μ M h2 hairpins separately in amplification buffer to 95 °C for 90 seconds. They were then cooled to room temperature for 30 minutes, protected from light, before being mixed and applied to the tissue. When hairpins were reused, the already mixed hairpins were reheated together. After five washes with 5xSSCT (2 x 5 minutes, 3 x 30 minutes) the tissues were either directly embedded in VectaShield as described above, or the normal fluorescent immunohistochemistry protocol was used for additional antibody labeling starting with blocking in 5% NGS or NDS.

3.13 Image acquisition and analysis

Images were acquired with a Leica TCS SP8 confocal microscope equipped with a photon multiplier tube and hybrid detector. A white light laser (Leica Microsystems, Wetzlar, Germany) was used for excitation. We used a 20-fold glycerol immersion objective (0.73 NA, HC PL APO, Leica Microsystems, Wetzlar Germany) for wholemount scans. We obtained 8-bit confocal stacks with 2048 $\times$ 1024 pixels with a maximal voxel size of 0.3 $\times$ 0.3 $\times$ 2 μ m and an optical section thickness of 3.12 μ m. Weak signals were amplified using line accumulation. Images were analyzed using Fiji. No changes in pixel intensity were applied other than linear changes in brightness and contrast.

4 Results

4.1 Evolutionary position and distribution of RH7

To determine the taxonomic distribution of RH7 among r-opsins, a phylogenetic tree was constructed. The analysis clearly identifies the three main clades of *Drosophila* rhodopsins: clade 1 (RH1, RH2, and RH6), clade 2 (RH3, RH4, and RH5), and clade 3, which corresponds to RH7. Unlike the first two clades, which include only rhodopsin homologs from other arthropods, the RH7 clade also contains homologs from tardigrades, forming a distinct branch within the clade (Figure 2A [↗](#)).

Adjacent branches contain more ancestral r-opsins from a broad range of metazoans, including mollusks, annelids, arthropods, and echinoderms. One early-diverging branch, highlighted in orange, corresponds to the vertebrate melanopsin clade, which itself appears to have split into two subgroups early in its evolution. While mammals have lost one of these lineages, all other vertebrates retain both. The placement of RH7 near the mammalian melanopsin clade and other early-diverging r-opsins suggests that it occupies an ancestral position within the r-opsin family. To assess the distribution of DCRY and RH7, we examined all species available in the RefSeq dataset, totaling 446 species. DCRY is widely distributed across Bilateria, whereas RH7 arose later within Panarthropoda. Among 360 panarthropod species, 58% possess a DCRY homolog and 68% an RH7 homolog. Overall, 57% of species contain both genes, 1% only DCRY, 11% only RH7, and 31% neither (Figure 2B [↗](#)). Nearly all species with DCRY (~98%) also retain RH7. Conversely, 17% of RH7-containing species lack DCRY. This pattern is observed across several insect orders, including basal hymenopterans of Tenthredinoidea, coleopterans, hemipterans, and dipterans. It is also seen in other arthropod lineages such as chelicerates, tardigrades, and some crustaceans. Together, these observations highlight the strong co-occurrence of RH7 and DCRY, as well as the existence of lineages where RH7 persists without DCRY, suggesting potential functional and evolutionary relationships between these photoreceptors.

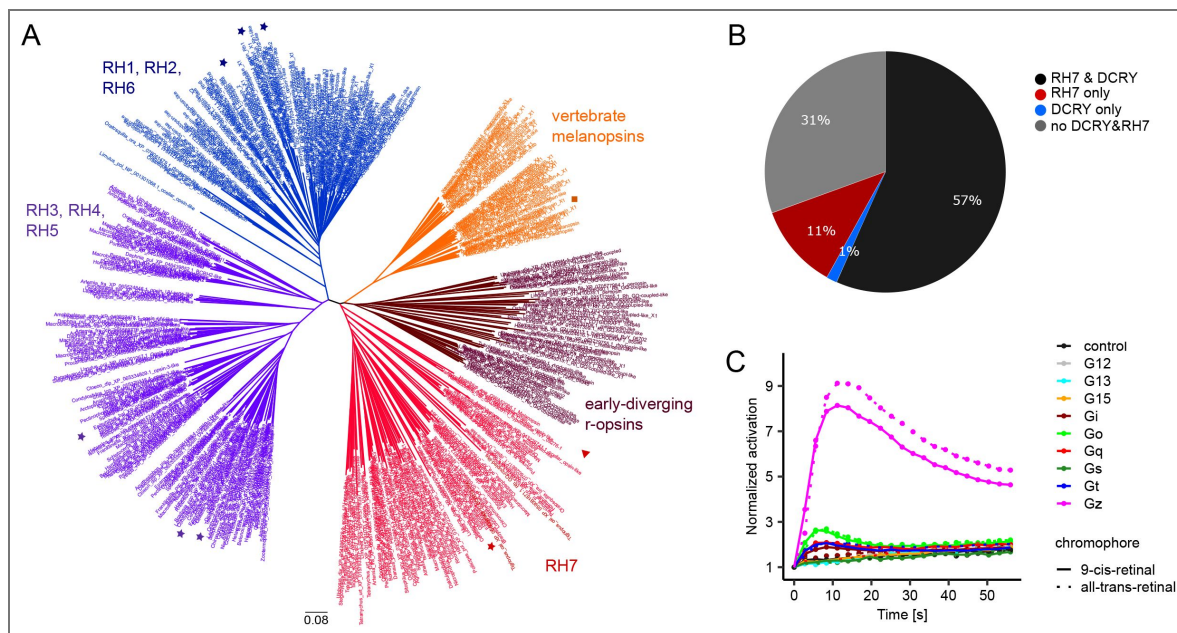


Figure 2. Evolutionary conservation, distribution, and signaling properties of RH7.

(A) Phylogenetic tree of 470 r-opsin protein sequences from 142 selected species, highlighting the clades containing the *Drosophila* rhodopsins: RH1, RH2, and RH6 (dark blue); RH3, RH4, and RH5 (purple); and RH7 (red). Early-diverging r-opsins are shown in brown, and vertebrate melanopsins in orange. *Drosophila* opsins are marked with stars in the corresponding colors. The four tardigrade RH7-like opsins are represented with a red triangle, and the human melanopsin is indicated by an orange square. **(B)** Distribution of RH7 and DCRY across 360 analyzed panarthropod species: 57% possess both genes (black), 12% possess only RH7 (red), 1% possess only DCRY (blue), and 32% lack both proteins (grey). **(C)** GsX assay showing that RH7 robustly activates the $G_{\alpha z}$ chimera in a light-dependent manner, with minimal differences between 9-cis-retinal and all-trans-retinal. No activation was detected with the $G_{\alpha q}$ chimera.

4.2 Functional distinction of RH7 from canonical *Drosophila* opsins

We investigated RH7 signaling by examining its interaction with different G_{α} proteins using a GsX assay (Ballister et al., 2018). This assay employs chimeric G_{α} proteins that share a core derived from $G_{\alpha}s$, which triggers cAMP production upon activation. The C terminal 14 amino acids of each chimera, critical for GPCR binding, are replaced with sequences from other G_{α} subtypes, such as $G_{\alpha}q$ or $G_{\alpha}z$. This allows each chimera to mimic the coupling specificity of a distinct G protein while ensuring that any productive GPCR interaction results in a measurable increase in cAMP levels. For this analysis, we co-expressed RH7 with various GsX chimeras in HEK cells and stimulated RH7 with brief light pulses. We observed robust activation with the $G_{\alpha}z$ chimera, with both 9-cis-retinal and all-trans-retinal (Figure 2C). Although there is no $G_{\alpha}z$ homolog in *Drosophila*, related G_{α} subunits such as $G_{\alpha}o$ and $G_{\alpha}i$ are expressed in the fly. Nevertheless, these results revealed two key findings: (1) RH7 can be activated by light, and (2) RH7 does not appear to signal via the $G_{\alpha}q$ -mediated pathway, unlike other *Drosophila* rhodopsins. These findings indicate that RH7 exhibits a signaling profile distinct from other insect rhodopsins.

4.3 *Rh7⁰* mutants show altered daily activity pattern & reduced light-off startle responses

To study the influence of RH7 on the daily locomotor activity of flies, wild-type (WT) control flies and *Rh7⁰* mutants were examined under standard LD 12:12 conditions (Figure 3A). During the day, mutants and wild-type controls showed the morning and evening activity typical of *Drosophila melanogaster* with a siesta in between. However, *Rh7⁰* mutants showed a significant increase in daytime activity compared to WT controls. In addition, mutants displayed a significantly shortened siesta, which also started later than in controls. Another prominent difference was observed in the startle response after light-off, characterized by a transient increase in locomotor activity lasting approximately 5–10 minutes. While WT control flies showed a pronounced startle response after lights-off, this response was strongly reduced in *Rh7⁰* mutants and terminated more rapidly after lights-off. To determine whether these behavioral differences depended on the spectral composition of the light, locomotor activity was additionally analyzed using distinct wavelengths (Figure 3-figure supplement 1B). Overall, locomotor activity patterns were comparable across the different wavelength conditions, and the differences between WT and *Rh7⁰* mutants remained evident.

4.4 Startle response to lights-off persists without a functional clock

The startle response, triggered by the sudden light-off, differs from circadian-regulated evening activity and can be triggered during the day as well. To test the startle response, we performed 30-minute dark pulses every 1.5 hours during the light phase on a standard LD 12:12 day and recorded the fly's activity. We observed that the startle responses of both WT controls and *Rh7⁰* mutants were generally lower in the morning than in the afternoon (Figure 3B). The circadian clock and thus the internal state of flies appear to modulate the startle response, as can be seen in clockless *per⁰¹* mutants. *per⁰¹* mutants exhibited a startle response that remains constant throughout the day. This startle response is significantly and consistently reduced in the *per⁰¹;+;Rh7⁰* double mutants (Figure 3B). These results show that the startle response is modulated by the circadian clock but is not dependent on it, and that RH7 plays an important role in this response.

In a second assay (4-hour assay), flies were rendered arrhythmic by continuous exposure to bright light to isolate the startle response from circadian influences. We then tested how different light intensities affected this response using repeated 4-h cycles over 3 days, consisting of a 3.5 h light phase at different intensities (5, 30, or 120 $\mu\text{W}/\text{cm}^2$) and a 30-minute dark phase. Smaller intensity changes were assessed by replacing the dark phase with dim light (5 or 30 $\mu\text{W}/\text{cm}^2$), while the light phase remained brighter (30 or 120 $\mu\text{W}/\text{cm}^2$, respectively). Under all tested conditions, *Rh7⁰* mutants exhibited a significantly lower startle response than WT controls. Both genotypes increased the startle response with increasing light intensity, but this reached only significance for

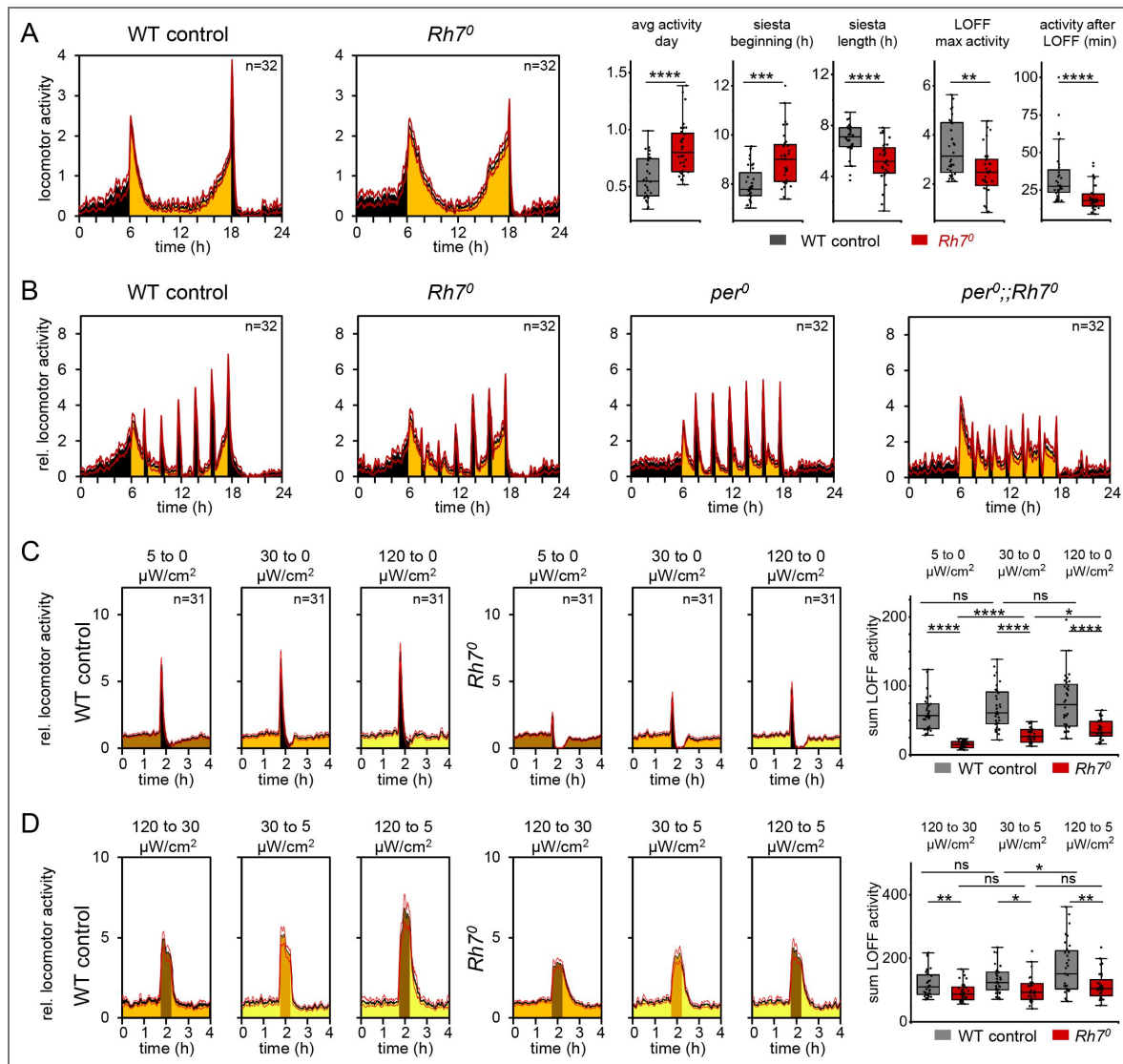


Figure 3. Altered locomotor activity and light-off responses in $Rh7^0$ mutant flies.

(A) Left, average locomotor activity profiles of wild-type (WT) and $Rh7^0$ mutant flies under standard conditions (12 h light (30 $\mu W/cm^2$):12 h dark, 25 °C). Right, box plots showing daytime average activity, siesta onset, siesta duration, maximum activity after lights-off (LOFF), and LOFF response duration. **(B)** Average activity profiles of WT, $Rh7^0$, per^0 , and $per^0;Rh7^0$ flies during a 12 h light (30 $\mu W/cm^2$):12 h dark cycle at 25 °C. During the light phase, the light was switched off for 30 min every 1.5 h to assess the light-off startle response. Activity was normalized to the mean activity of each fly across the entire 24 h recording period. Non-normalized activity profiles are shown in Figure 3-figure supplement 2A. **(C)** Light-off startle responses after 3.5 h of light exposure at 5, 30, or 120 $\mu W/cm^2$, followed by a 30 min dark period. **(D)** 4h assay with dimmed light. After 3.5 h of light exposure, illumination was reduced for 30 min to either 5 or 30 $\mu W/cm^2$ instead of being switched off completely (**** p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05, Mann-Whitney U-Test, BH correction, R-studio).

Rh7⁰ mutants (Figure 3C [↗](#)). *Rh7^{MiMIC}* mutants, an independent *Rh7* mutant line retaining reduced but detectable *Rh7* mRNA levels, displayed a similar phenotype to *Rh7⁰* mutants (Figure 3–figure supplement 2B, 2C [↗](#)). The phenotype persisted in trans-heterozygous *Rh7⁰/Rh7^{MiMIC}* flies but was absent in heterozygous controls, further supporting a role of RH7 in mediating the startle response (Figure 3–figure supplement 2E, 2F [↗](#)).

To investigate whether the startle response is also induced by reductions in light intensity rather than transitions to complete darkness, we performed the same assay while maintaining residual light levels of 5 or 30 $\mu\text{W}/\text{cm}^2$ during the “dark phase”. Transitions to complete darkness induced a pronounced, short-lasting startle response, whereas transitions to residual-light conditions resulted in sustained elevated activity throughout the 30-minute stimulus without a sharp transient peak (Figure 3D [↗](#)). Across all conditions, WT flies showed the strongest responses. *Rh7⁰* mutants displayed similar qualitative response patterns, but with a markedly reduced magnitude compared to WT controls (Figure 3D [↗](#)). In WT flies, larger differences in light intensity tended to evoke stronger responses. In contrast, *Rh7⁰* mutants showed largely unchanged response levels across the different light conditions.

4.5 *Rh7* is expressed in the optic lobe and clock neurons

To determine the tissue-specific expression of *Rh7*, we performed qPCR analyses using *α -tubulin* as a reference gene. The expression of *Rh7* was highest in the lamina, followed by the brain, while only minimal levels were detected in the retina (Figure 4A [↗](#)). Additionally, we performed *Rh7* RNA *in situ* hybridization chain reaction (HCR), which verified the strongest *Rh7* expression in the lamina and medulla (Figure 4B [↗](#)). We further observed considerably weaker expressions in neurons of the central brain (Figure 4B [↗](#)). No *in situ* hybridization was detected in *Rh7⁰* mutants (Figure 4C [↗](#)).

To further investigate *Rh7* expression, we generated a *Rh7* promoter-driven *Gal4* line (*Rh7-Gal4*). Since the *Rh7* gene expression regulatory elements are not only located within the promoter but also extend into the transcriptional region, we cloned a large part of this region, including exon 1, intron 1 and part of exon 2, together with the start codon (Figure 4–figure supplement 1 [↗](#)). When comparing GFP expression driven by the newly generated *Rh7-Gal4* with the *Rh7* RNA *in situ* hybridization and qPCR data, we again observed the strongest expression in the lamina and medulla. Interestingly, also the *Rh7-Gal4* line showed additional expression in neurons of the central brain (Figure 4D [↗](#)). To further characterize *Rh7*-expressing neurons, we used MultiColor FlpOut (MCFO) (Nern et al., 2015 [↗](#)), which stochastically labels neurons when crossed to a *Gal4*-driver line. Using MCFO, we identified at least two cell types of the optic system based on their characteristic arborization pattern in the medulla: lamina neuron 5 (L5) and medulla intrinsic neuron 1 Mi1 (Figure 4E [↗](#)). Importantly, both neurons were also present in the TAPIN RNA-seq analysis, with L5 showing the strongest *Rh7* expression (Davis et al., 2020 [↗](#)). Next, we used an antibody against the clock protein PERIOD to test for colocalization, given that previous studies showed *Rh7* expression in subsets of clock neurons (Kistenpennig et al., 2017 [↗](#); Ma et al., 2021 [↗](#); Ni et al., 2017 [↗](#)). We observed colocalization of GFP and PER in some DN_{1p}, DN_{1a}, and DN₂ neurons (Figure 4F [↗](#)) but not in the LN_vs contrary to Ni et al. (2017) [↗](#). To test whether *Rh7* expressing clock neurons also express *dcry*, we performed simultaneous RNA *in situ* hybridization for *dcry* and *Rh7*. As suggested by single cell RNA sequencing data, the clock neurons expressing *Rh7* are CRY-negative with the DN_{1a} forming the only exception (Figure 4–figure supplement 1B [↗](#)).

To validate the expression pattern of the *Rh7-Gal4* line, we performed *in situ* hybridization with *Rh7-Gal4>GFP* flies. All GFP-labeled cells were also detected by RNA *in situ* hybridization, although the signal was less pronounced in cells of the central brain. Some cells, particularly in the optic system, were detected only by *in situ* hybridization and not by the *Gal4* line. This suggests an even broader expression of *Rh7* than shown by the *Gal4* line (Figure 4G [↗](#), 4H [↗](#)).

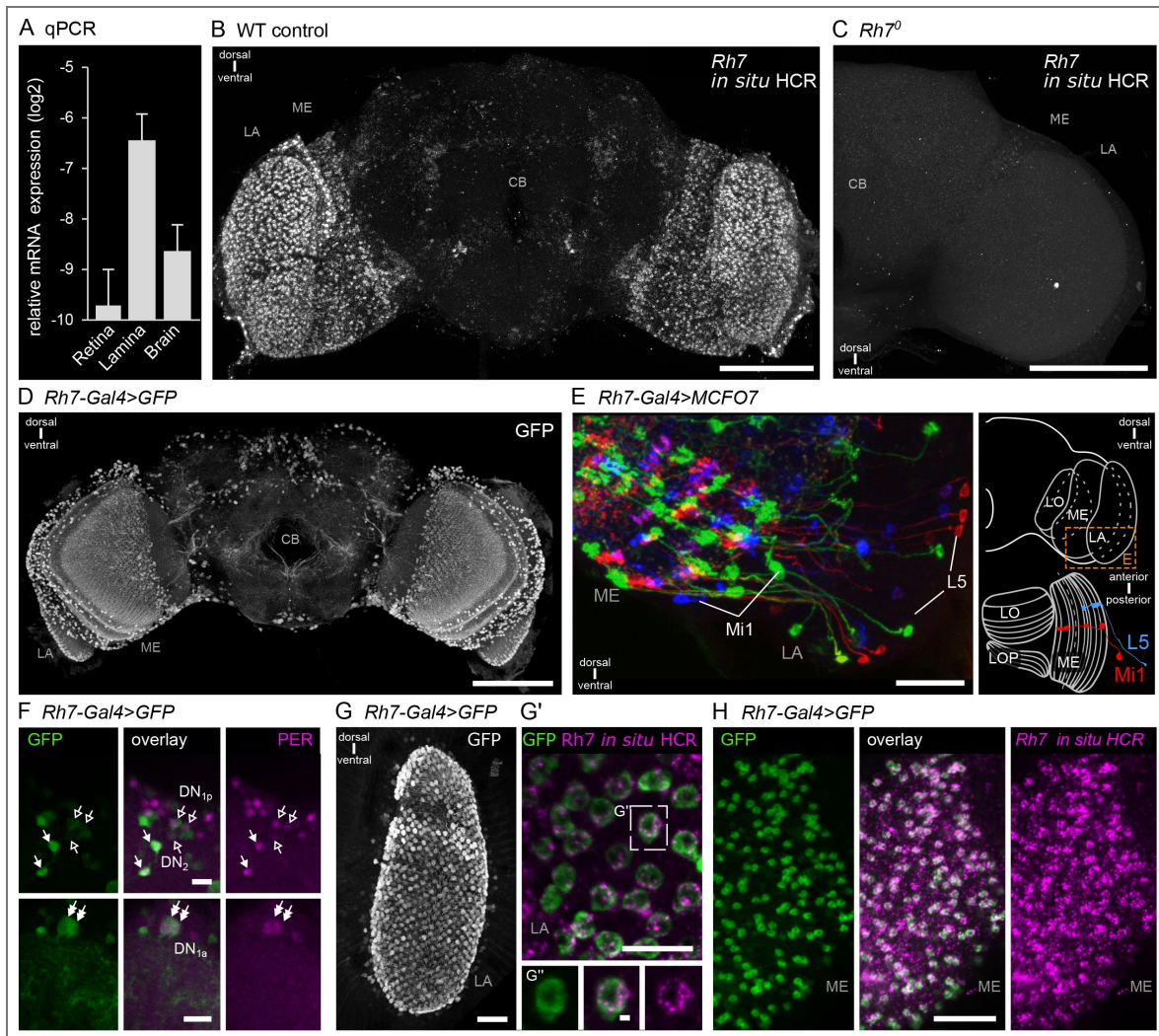


Figure 4. Expression pattern and cellular localization of *Rh7* in the optic lobe and circadian neurons.

(A) Relative qPCR analysis revealed strong *Rh7* expression in the lamina, followed by the brain, with minimal background expression in the retina. (B) *Rh7 in situ* HCR in wild-type (WT) controls revealed strong *Rh7* signals in the lamina (LA) and medulla (ME), and weaker signals in the central brain (CB). (C) *Rh7⁰* mutants showed no detectable *Rh7 in situ* HCR signal, confirming the specificity of the probe and the loss of *Rh7* expression in the mutant. (D) *Rh7-Gal4*-driven *UAS-GFP* expression revealed robust labeling in the optic system, particularly in the lamina and medulla, as well as in various brain cells. (E) *Rh7-Gal4*-driven MCFO labeling identified lamina neurons L5 and the medulla neuron Mi1. Right: schematic illustration of the detected neurons in the optic lobe. (F) Immunostaining of *Rh7-Gal4>UAS-GFP* flies with anti-GFP and anti-PER antibodies revealed co-labeling in clock neurons, suggesting that a subset of circadian neurons expresses *Rh7*. Open arrows mark DN_{1p} and arrows mark DN₂; double arrows mark DN_{1a}. (G) & (H) *in situ* HCR in *Rh7-Gal4>UAS-GFP* flies revealed that all GFP-labeled cells in the lamina (G) and medulla (H) also showed *Rh7* transcript signal, confirming *Rh7* expression in these neurons. Scale bar in B, C, D represents 100 μ m, in E, G, H 25 μ m, in F, G' 10 μ m and in G'' 1 μ m.

4.6 C-terminal truncation of RH7 affects dark-phase behavior

The so-called Darkfly was kept in complete darkness for 57 years (1400 generations) accumulating mutations that may have enhanced its adaptation to perpetual darkness (Izutsu et al., 2012 [↗](#)). Notably, the mutant carries a nonsense mutation in the *Rh7* gene, resulting in the loss of the final 21 amino acids at the C-terminus. qPCR and immunohistochemistry analyses indicate preserved *Rh7* transcript levels and expression patterns, suggesting that the truncated RH7 protein is indeed expressed (Figure 3-figure supplement 2C [↗](#), Figure 5-figure supplement 1B [↗](#)). When we exposed the Darkfly to dark pulses in a 12:12 light-dark cycle, they exhibited the opposite phenotype to *Rh7⁰* mutants, showing a stronger startle response (Figure 5A [↗](#)), possibly reflecting RHODOPSIN 7 adopting a constitutively active form in the Darkfly strain, driving the enhanced behavioral response (Figure 5B [↗](#)).

To validate the role of RH7 in the startle response, we conducted a rescue experiment by expressing *Rh7* in *Rh7⁰* mutants using the *UAS-Rh7* construct driven by *Rh7-Gal4*. As a control for cloning and insertion artifacts, we used a *UAS-empty* line with the same insertion site as *UAS-Rh7*, driven by *Rh7-Gal4* in the *Rh7⁰* background. In the dark pulse experiment, *Rh7-Gal4>UAS-empty;Rh7⁰* (“no-rescue”) flies behaved like mutants, while *Rh7-Gal4>UAS-Rh7;Rh7⁰* flies (“*Rh7* rescue”) resembled WT controls (Figure 5B [↗](#)). To express a truncated RH7 protein lacking the last 21 amino acids, as found in the Darkfly, we crossed the *UAS-Rh7^{Darkfly}; Rh7⁰* line with the *Rh7-Gal4; Rh7⁰* line (“*Rh7^{Darkfly}* rescue”). We observed an increased startle response in the offspring, most prominently during the second half of the light phase (Figure 5B [↗](#)). Under standard LD 12:12 conditions, both *Rh7* rescue and *Rh7^{Darkfly}* rescue flies showed reduced average daytime activity compared to no-rescue flies. Similar effects were observed for siesta length, with *Rh7^{Darkfly}* rescue flies additionally differing significantly from *Rh7* rescue flies. Differences in siesta beginning were also detected, with significant changes observed between no-rescue flies and *Rh7^{Darkfly}* rescue flies, as well as between *Rh7* rescue and *Rh7^{Darkfly}* rescue flies (Figure 5C [↗](#)). In the 4-hour assay, in which the flies were made arrhythmic we found a clear rescue of the startle response in *Rh7-Gal4>UAS-Rh7;Rh7⁰* flies compared to the *Rh7-Gal4>UAS-empty;Rh7⁰* controls. Furthermore, *Rh7-Gal4>UAS-Rh7^{Darkfly};Rh7⁰* flies showed a significantly increased startle response (Figure 5C [↗](#)).

4.7 RH7 modulates optomotor responses

The *Drosophila* optomotor response is a reflex that stabilizes locomotion by counteracting perceived environmental motion with its own movement. Motion detection relies on the processing of spatiotemporal changes in luminance across the visual field. The optic system, particularly the lamina neurons, plays an important role in this response (Yang and Clandinin, 2018 [↗](#)). We found that *Rh7⁰* mutants show deficits in the optomotor response, while the Darkfly showed an increased optomotor response (Figure 5D [↗](#)). When the rescue flies were tested, the *Rh7-Gal4>UAS-empty;Rh7⁰* flies behaved like the *Rh7⁰* mutants, whereas the *Rh7-Gal4>UAS-Rh7;Rh7⁰* flies showed a significant rescue, with no significant difference from WT controls. In contrast, *Rh7-Gal4>UAS-Rh7^{Darkfly};Rh7⁰* flies showed a significantly higher optomotor response than the WT controls and the *Rh7-Gal4>UAS-Rh7;Rh7⁰* flies. This response was similar to the Darkfly (Figure 5E [↗](#)).

5 Discussion

5.1 RH7 is an ancestral and structurally distinct opsin

Although RH7 has been known for nearly 25 years, its function remains poorly understood. This is partly due to its atypical expression outside the retina and its divergence from canonical visual rhodopsins. Phylogenetic analyses suggest that RH7 is an early-diverging r-opsin within Panarthropoda. Homologs identified in arthropods and tardigrades (Figure 2A [↗](#)) indicate a deep evolutionary origin and place RH7 within an ancient opsin lineage. Neighboring branches include early-diverging r-opsins from lophotrochozoans and echinoderms, as well as melanopsins from vertebrates. This phylogenetic position supports the ancestral character of RH7 and suggests that it

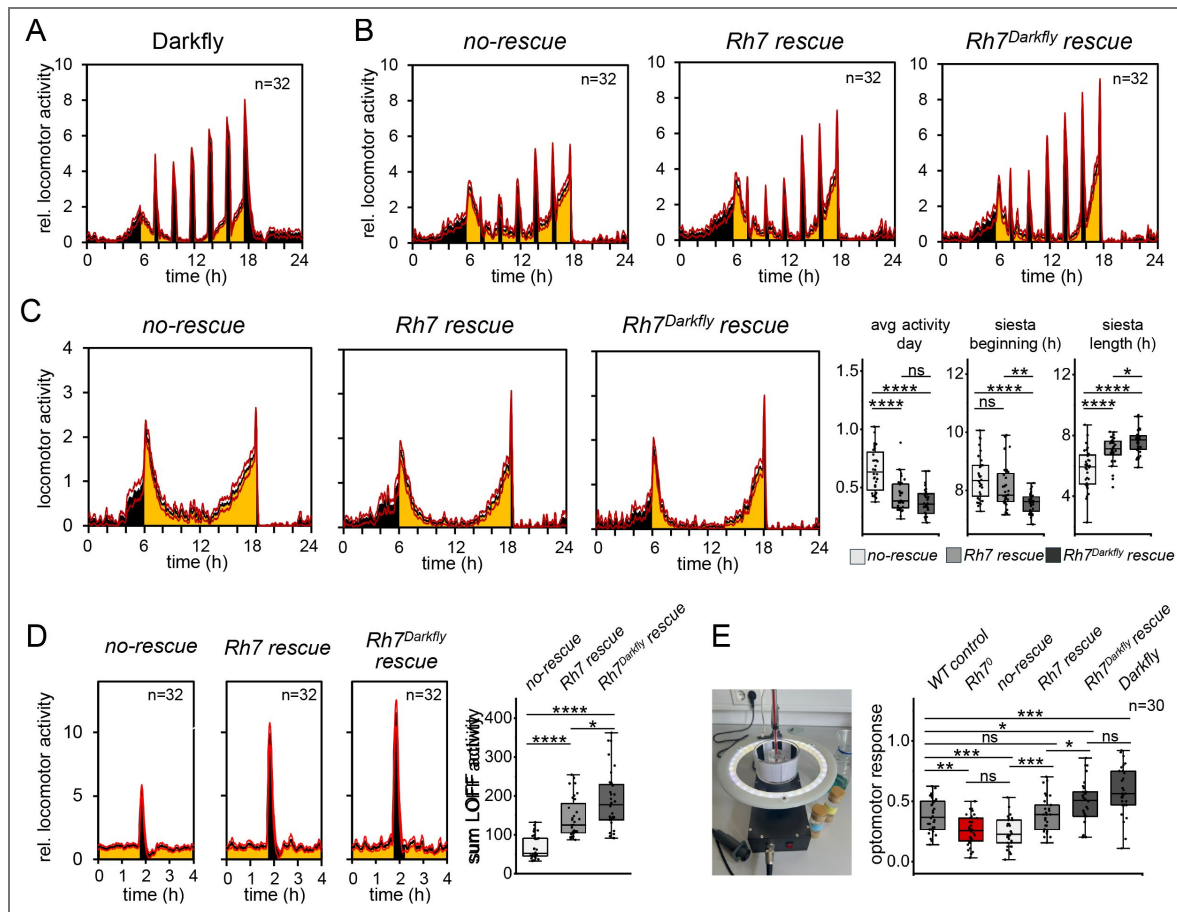


Figure 5. Functional characterization of *Rh7* rescue and *Darkfly* rescue lines.

(A) & (B) Normalized average daily activity of *Darkfly* and rescue fly lines recorded under standard conditions (12 h light (30 $\mu\text{W}/\text{cm}^2$); 12 h dark, 25 °C). During the light phase, lights were turned off for 30 min every 1.5 h. **(A)** *Darkfly* flies exhibit an enhanced startle response to light-off. **(B)** No-rescue (*Rh7-Gal4; Rh7⁰>UAS-empty; Rh7⁰*) display a phenotype similar to *Rh7* mutants, whereas the *Rh7* rescue line (*Rh7-Gal4; Rh7⁰>UAS-Rh7; Rh7⁰*) resembles WT controls. The *Rh7^{Darkfly}* (*Rh7-Gal4; Rh7⁰>UAS-Rh7^{Darkfly}; Rh7⁰*) rescue shows an enhanced startle response similar to *Darkfly*, particularly during the second half of the day. **(C)** *RH7* rescue alters daytime activity and siesta parameters under LD conditions. Both *Rh7* rescue and *Rh7^{Darkfly}* rescue flies showed reduced average daytime activity compared to no-rescue flies. Similar effects were observed for siesta length, including a significant difference between *Rh7* rescue and *Rh7^{Darkfly}* rescue flies. For siesta beginning (h), significant differences were observed between no-rescue flies and *Rh7^{Darkfly}* rescue flies, as well as between *Rh7* rescue and *Rh7^{Darkfly}* rescue flies. **(D)** To measure the startle response, the 4-hour assay was performed. The *Rh7* rescue line restored the enhanced startle response that was absent in the no-rescue mutants, which behave like *Rh7⁰* mutants ($p < 0.0001$). The *Rh7^{Darkfly}* rescue showed an even stronger startle response, significantly higher than the *Rh7* rescue line ($p < 0.001$). **(E)** Optomotor response assays show that no-rescue flies behave like *Rh7⁰* mutants, with no significant difference between them. *Rh7* rescue flies respond like WT controls. Both *Rh7⁰* mutants and no-rescue flies differ significantly from WT and *Rh7* rescue flies ($p < 0.01$). *Darkfly* and *Rh7^{Darkfly}* rescue flies do not differ from each other but show significant differences compared to WT and *Rh7* rescue flies ($***p < 0.001$, $**p < 0.01$, $*p < 0.05$, Mann-Whitney U-Test, BH correction, R-studio).

may retain features of early opsin function, as reflected by RH7-related opsins in tardigrades, which have been linked to monochromatic vision (Fleming et al., 2021 [↗](#); Hering and Mayer, 2014 [↗](#)). Many species that possess RH7 inhabit sun-exposed environments, suggesting that RH7 may contribute to light sensitivity under natural conditions. As our results with different light contrast and the optomotor tests suggest, Rh7 may enhance contrast sensitivity, which is advantageous for insects when they move rapidly through environments with highly variable light contrast, for example, under trees between shaded and well-lit areas.

The ancestral character of RH7 is further reflected in its unusual structural features with long N- and C-terminal regions carrying a high density of predicted phosphorylation sites. This suggests a high potential for post-translational regulation and possibly increased signaling flexibility. The extended termini distinguish RH7 from the more compact structure of typical rhodopsins and may represent an intermediate stage between canonical GPCRs and specialized visual rhodopsins (Figure 6A [↗](#)). In addition, RH7 lacks several conserved motifs found in other *Drosophila* rhodopsins, which likely emerged during the specialization of visual opsins. The absence of these features, together with the extended termini, supports the idea that RH7 occupies a structurally and evolutionarily distinct position. A more detailed comparison of these motifs and their functional implications is presented in section 5.5 [↗](#).

Besides its structural divergence, the developmental origin and central localization of RH7-expressing neurons support its ancestral photoreceptive role. RH7 is expressed in central visual neuropils such as the lamina and medulla that process input from peripheral photoreceptors. Unlike canonical r-opsins, which are primarily expressed in specialized retinal photoreceptor cells, RH7 expression in central brain regions suggests an ancient form of non-image-forming photoreception integrated directly within neural circuits. This idea is further supported by the presence of opsins expressed in brain regions of other early-diverging bilaterians, such as annelids and mollusks (Arendt et al., 2004 [↗](#); Vöcking et al., 2015 [↗](#); Wollesen et al., 2019 [↗](#)).

While our data show *Rh7* expression in the optic lobe and central brain, previous work also reports atypical expression in retinal support cells and multidendritic neurons (Charlton-Perkins et al., 2017 [↗](#); Lazopulo et al., 2019 [↗](#)). We did not detect signals in these cells, likely due to low expression levels and background fluorescence. However, the low *Rh7* mRNA levels detected by qPCR (Figure 4A [↗](#)), together with the faint staining reported by Kistenpfennig et al. (2017) [↗](#), may indicate *Rh7* expression in retinal support cells. Overall, these findings are consistent with our observations and further highlight the non-canonical expression of RH7. RH7 thus likely represents a central, evolutionarily conserved opsin for light sensing, predating the split between sensory input and processing centers.

5.2 Divergence from canonical signaling: first insights into RH7 phototransduction

The signaling properties of RH7 were explored using heterologous GsX assays, which clearly indicated that RH7 can be activated by light (Figure 2C [↗](#)) but does not interact with $G_{\alpha q}$. This lack of interaction appears plausible, as $G_{\alpha q}$ expression is restricted to retinal cells, whereas *Rh7* is predominantly expressed in lamina and medulla neurons (Davis et al., 2020 [↗](#)). Instead, RH7 appears to interact with mammalian $G_{\alpha z}$. Although *Drosophila* lacks a direct homolog of $G_{\alpha z}$, the related inhibitory subunits $G_{\alpha i}$ and $G_{\alpha o}$ from the same family may serve as functional analogs. Supporting this possibility, *in silico* protein–protein interaction predictions using FlyPredictome (Kim et al., 2024 [↗](#)), based on AlphaFold-Multimer modeling and interface confidence scoring, indicate a potential interaction between RH7 and $G_{\alpha i}$.

If RH7 indeed couples to a $G_{\alpha i}$ family member, this would represent a notable exception among *Drosophila* rhodopsins by engaging an inhibitory G protein pathway. This possible reversal of signaling direction recalls the mammalian system, where melanopsin in ipRGCs activates an excitatory $G_{\alpha q}$ pathway, while classical retinal rhodopsin signals through an inhibitory transducin ($G_{\alpha t}$) pathway belonging to the $G_{\alpha i}$ family (Contreras et al., 2021 [↗](#); Gao et al., 2019 [↗](#); Koyanagi

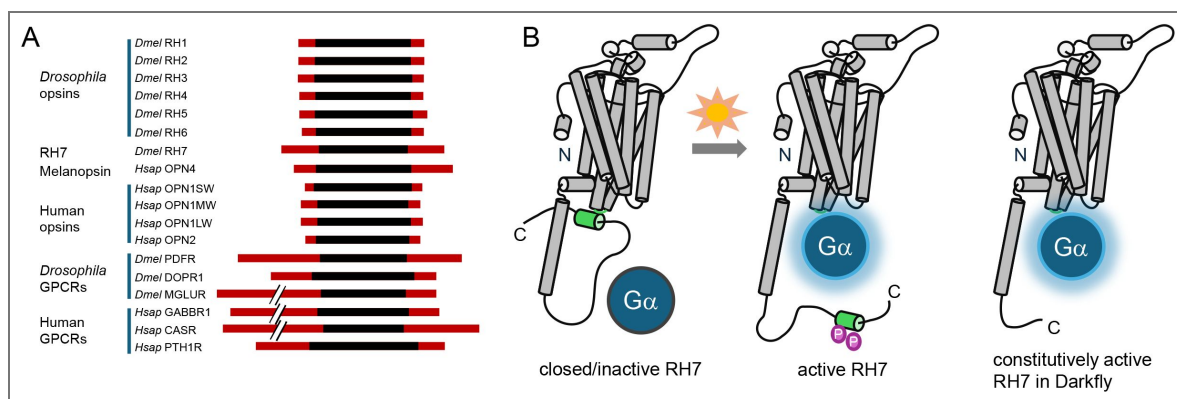


Figure 6. Structural features and hypothetical activation states of RH7.

(A) Protein lengths of *Drosophila* and human opsins are shown alongside randomly selected non-opsin GPCRs. General GPCRs are significantly longer, primarily due to extended N- and C-termini, whereas opsins are shorter and largely confined to the transmembrane core. RH7 and OPN4 (melanopsin) display intermediate protein lengths relative to both groups. The red bars represent the N- and C-termini of the proteins, and the black bars represent the core protein, spanning from the 1st to the 7th transmembrane domain. Additional information, including full protein names and UniProt IDs, is provided in [Supplementary table 1](#). **(B)** Left: Predicted 3D structure of RH7 based on AlphaFold, representing a closed conformation. Center: A putative open conformation that may result from light-induced conformational changes and subsequent C-terminal phosphorylation (magenta), potentially promoting G protein binding, as suggested by [Maggio et al. \(2023\)](#). Right: Hypothetical structure of RH7 in the Darkfly variant, possibly stabilized in an open, constitutively active state, independent of light.

and Terakita, 2008 [\[1\]](#)). The use of opposing G protein pathways in these systems, despite their evolutionary distance, may reflect a conserved principle that links photoreceptor polarity to downstream signaling.

5.3 RH7 in the optic lobe: A modulator of early visual processing

Rh7 is broadly expressed in the optic lobe, particularly in the lamina and medulla, which are key regions for early visual processing. These areas receive input from outer photoreceptors and are involved in contrast and motion detection. We found strong *Rh7* expression in L5 lamina neurons and Mi1 neurons of the medulla. L5 neurons pass signals from outer photoreceptors to the medulla and show strong contrast adaptation (Matulis et al., 2020 [\[2\]](#)). Mi1 neurons contribute to motion and contrast processing (Behnia et al., 2014 [\[3\]](#); Matulis et al., 2020 [\[2\]](#)). The expression of *Rh7* in Mi1 neurons may thus suggest a potential role in modulating contrast-related signals, although the functional connection remains to be directly tested. These computations rely on changes in light intensity over space and time, linking the functional properties of L5 and Mi1 neurons to general luminance-based visual processing.

Our behavioral data support a role for RH7 in modulating these visual functions. Flies lacking *Rh7* show reduced optomotor responses, suggesting they are less sensitive to moving visual stimuli. In contrast, the Darkfly strain, which expresses a truncated variant of RH7, shows an increased response in the same assay (Figure 5 [\[4\]](#)). Rescue experiments confirm that this effect depends on *Rh7*: expression of full-length RH7 in *Rh7⁰* mutants restores normal behavior, while the truncated form of the Darkfly enhances the optomotor response. These results indicate that RH7 influences visual processing in the optic lobe. The enhanced behavior seen with the Darkfly variant suggests that changes in RH7 structure can affect its function. This possibility is discussed further in section 5.6 [\[5\]](#).

5.4 RH7 in circadian regulation and behavioral flexibility

Fly activity is shaped by both the circadian clock and external light cues, which are detected by specialized photoreceptors (Helfrich-Förster, 2017 [\[6\]](#); Rieger et al., 2003 [\[7\]](#)). In natural environments, these cues can vary considerably. The observation that many RH7-containing species inhabit sun-exposed environments suggests that RH7 may contribute to light sensitivity and appropriate behavioral responses under such conditions. This possibility is discussed further in the next section. In *Drosophila*, this is reflected in circadian activity patterns, to which RH7 contributes. Under standard LD 12:12 conditions, *Drosophila melanogaster* displays two peaks of locomotor activity in the morning and evening separated by a midday siesta (Helfrich-Förster, 2000 [\[8\]](#)). This rhythm is controlled by the circadian clock and its associated neuronal network. Flies experience two rest phases: a pronounced one at night and a shorter one during the siesta, both primarily regulated by dorsal clock neurons (DNs) (Guo et al., 2018 [\[9\]](#)). The DN₁ subclass plays a key sleep-promoting role and includes CRY-positive and CRY-negative neurons that may contribute differently to the two rest phases (Guo et al., 2016 [\[10\]](#); Lamaze and Stanewsky, 2020 [\[11\]](#)).

RH7 is expressed in dorsal clock neurons, especially in CRY-negative DN₁, with the exception of the two DN_{1a} neurons (4F). While we could confirm RH7 expression in the DNs, we did not detect it in lateral clock neurons (Figure 4B, D [\[12\]](#)), as previously suggested by Ni et al. (2017) [\[13\]](#). Ni et al. (2017) [\[13\]](#) reported that RH7 plays a central role in circadian light adaptation and observed its expression in lateral neurons (LN_vs). However, Senthilan et al. (2019) [\[14\]](#) later found that the original antibody staining in LN_vs was likely artifactual, and Lazopulo et al. (2019) [\[15\]](#) further demonstrated that RH7's functional effects on light-dependent behavior occur outside the LN_vs. In addition to its role in DNs, RH7 may also influence circadian regulation via the optic lobe. Its expression in the lamina and medulla, along with possible synaptic connections between Mi1 neurons and PDF-expressing neurons (Reinhard et al., 2024 [\[16\]](#)), and the presence of PDF receptors in lamina neurons (Bastian et al., 2025 [\[17\]](#); Davis et al., 2020 [\[18\]](#)), suggest a functional link between early visual processing centers and the circadian network. Rather than acting as a dominant regulator, RH7 appears to exert subtle, context-dependent effects shaped by environmental conditions and genetic background. As a result, its contribution may be masked by other

photoreceptive pathways or background variation, leading to differing phenotypes across studies. Furthermore, the extent to which certain behaviors are restored may depend on how closely the *Rh7-Gal4* driver recapitulates the endogenous *Rh7* expression pattern, as some *Rh7*-expressing cells, especially in the visual system, were detected only by *in situ* hybridization (Figure 4G, 4H). Together, our findings contribute to a better understanding of RH7 function in light-dependent behavior and provide a basis for future studies to further define its potential role in circadian entrainment.

5.5 Evolutionary retention of RH7 in sun-exposed species

The repertoire of opsins and cryptochromes varies considerably among species, reflecting lineage-specific adaptations to different light environments. Yet, a consistent evolutionary link exists between RH7 and DCRY within the light-sensing network. While DCRY is evolutionarily older and widespread across Bilateria, RH7 arose later within Panarthropoda. Consequently, many non-Panarthropoda species possess DCRY, but not RH7. In contrast, nearly all arthropod species that retain DCRY also possess RH7 (approximately 98%), indicating a strong evolutionary link. However, RH7 can exist independently of DCRY. Lineages that have lost DCRY often retain RH7, particularly in species exposed to high sunlight, especially during early developmental stages (e.g., certain sawflies and some beetles).

This pattern suggests that RH7 contributes to light sensitivity and the regulation of behavior under natural, sunlit conditions Meyerhof et al. (2024) reported altered light-dark activity in *Rh7* mutants, highlighting a modulatory role for RH7 in behavioral responses to ambient light. In our LD and wavelength assays, WT flies showed higher activity during dawn and dusk and a pronounced siesta, whereas *Rh7⁰* mutants displayed increased daytime activity and a reduced siesta. Avoiding activities at midday, when light intensity is at its highest under natural conditions, is consistent with a protective role of RH7, as suggested by Lazopulo et al. (2019) under high-intensity blue light. The moderate light intensities in our experiments were likely below the threshold for strong avoidance. Yet they still revealed behavioral differences between WT and *Rh7⁰* flies. These results support a role for RH7 in mediating protective behavioral responses to environmental light. While its precise functional contribution outside of *Drosophila* remains to be determined, the widespread retention of RH7 across diverse light-exposed species highlights its likely adaptive and conserved role in detecting and responding to environmental light.

5.6 RH7 is essential for locomotor activity during darkness

Although activity during darkness is regulated by the circadian clock, the startle response to light-off is independent of it. Dark-pulse experiments show that both WT controls and *per⁰* mutants, which lack a functional circadian clock, exhibit a strong startle response, consistent with the absence of diel modulation of the response in *per⁰* mutants (Figure 3B). While the circadian clock seems to modulate the magnitude of the response, it is not essential for its occurrence (Figure 3B). Moreover, this phenotype persists in flies rendered arrhythmic during the 4-hour assay. The critical role of RH7 is supported by trans-heterozygous combinations with *Rh7^{MIMIC}*, which show the same phenotype as *Rh7⁰* mutants, and by rescue experiments restoring normal behavior (Figure 3-figure supplement 2B).

The Darkfly strain, bred in complete darkness for many generations, provides an intriguing case for RH7 function in sustained darkness. It carries an RH7 variant with a 21-amino acid deletion in the C-terminal tail, a region rich in predicted phosphorylation sites. Structural modeling based on AlphaFold predicts an interaction between this tail and the receptor core, specifically the unique ICL3 loop of RH7 (Figure 1A), which lacks the QAKKMNV motif found in other *Drosophila* rhodopsins. Such intramolecular interactions are known to mediate autoinhibition in GPCRs, where the C-terminal tail stabilizes an inactive conformation that shifts to an active state upon receptor stimulation (Maggio et al., 2023).

We hypothesize that the deletion in the Darkfly variant weakens this autoinhibitory interaction and thus facilitates constitutive activation (Figure 6B). This is supported by the observation that deleting the final 21 amino acids increases startle response and optomotor response, approaching

levels seen in Darkfly flies (Figure 5 [↗](#)). However, the unnormalized locomotor activity data (Figure 5–figure supplement 1A [↗](#)) reveal generally elevated locomotor activity in the Darkfly strain, likely reflecting additional genetic changes accumulated during long-term maintenance in constant darkness (Izutsu et al., 2012 [↗](#)), which may also influence neural development or synaptic connectivity. For example, nonsense mutations in *HisCl1* and *Vha100-1* could alter visual processing and neural signaling (Alejevski et al., 2019 [↗](#); Williamson et al., 2010 [↗](#)), while missense mutations in *DopR*, *bgm*, and development-associated transcription factors *Sox21b* could modulate activity, sleep, and neural development (Jiang et al., 2016 [↗](#); McKimmie et al., 2005 [↗](#); Min and Benzer, 1999 [↗](#)). Taken together, these findings highlight the pivotal role of RH7 in modulating behavior in darkness and demonstrate that structural changes can fine-tune RH7 signaling to adapt to environmental conditions.

5.7 Parallels and divergence between *Drosophila* RH7 and other non-canonical Opsins

This study highlights significant similarities between RH7 and other noncanonical rhodopsins, particularly mammalian melanopsin (OPN4). Both RH7 and melanopsin are expressed in neurons downstream of classical photoreceptors, with RH7 in lamina and medulla neurons of the optic lobe and melanopsin in the ipRGCs, suggesting analogous roles in processing visual and circadian information. Functionally, both opsins contribute to circadian regulation as well as contrast and motion detection. Structurally, they share elongated N- and C-terminal regions that are intermediate in length relative to canonical rhodopsins and typical GPCRs. These extended regions harbor multiple phosphorylation sites that are likely crucial for their unique signaling mechanisms. In addition, we identified five conserved sequence motifs shared between RH7 and melanopsins, including two in the C-terminal region and others within the transmembrane core, one of which extends into the conserved DRY motif in the second intracellular loop (ICL2) (see Figure 6–figure supplement 1A, 1B [↗](#)).

Furthermore, RH7 and melanopsin activate phototransduction cascades distinct from those of classical visual opsins, potentially producing opposing effects. This reflects an evolutionary adaptation that supports diverse functions in both visual and nonvisual photoreception. However, while melanopsin expression in mammals is largely confined to the visual system, *Drosophila* RH7 appears to have acquired additional functions in the brain. This divergence may reflect evolutionary adaptations to differing anatomical constraints: the small, light-permeable brain of *Drosophila* may permit direct light sensing beyond the eyes, whereas in mammals, the evolution of a larger brain enclosed by a light-impermeable skull may have limited such extraocular photoreception to specialized retinal pathways.

Although melanopsin is predominantly expressed in retinal ganglion cells, it has also been detected in various brain regions (Aviles-Trigueros et al., 2012 [↗](#); Nissilä et al., 2017 [↗](#)). Additionally, other non-canonical opsins such as encephalopsin and neuropsin are present in the human brain, with their functional roles still under investigation (Buhr et al., 2015 [↗](#); Nissilä et al., 2012 [↗](#); Upton et al., 2022 [↗](#), 2021 [↗](#)). In this context, the expression of RH7 in the *Drosophila* brain, outside of clock neurons, may reflect similar non-visual roles. While its brain expression is relatively weak compared to its prominent presence in the optic lobes, it is consistently observed using both *Rh7-Gal4* reporters and *in situ* hybridization. The identity of the non-clock neuronal populations expressing *Rh7* remains to be determined, and further work, such as double labeling with cell-type-specific markers, will be necessary to clarify this. In these neurons, RH7 might contribute to higher-order visual processing, sensorimotor control, general arousal, or aspects of neuronal development.

These findings open new possibilities to study how noncanonical opsins like RH7 contribute to complex brain functions beyond just sensing light. Understanding the roles of RH7 can give important clues about how light-sensing systems evolved and how they work together with brain circuits to control behavior. Future research using genetics, anatomy, and physiology will be important to fully understand all the functions of RH7 in *Drosophila* and other animals.

6 Conclusion

Our study provides a key insight into the unresolved functional diversity of opsins. Unlike canonical rhodopsins involved in image formation, RH7 acts as a fundamental photoreceptor mediating simple light-off signals to diverse neuronal populations. As an evolutionarily ancient opsin, RH7 likely represents a primordial photoreceptive system predating the specialization of complex visual pathways. Structurally and functionally, RH7 exhibits characteristic features of G protein-coupled receptors (GPCRs), positioning it as an evolutionary intermediate between ancestral GPCRs and specialized visual opsins. This is reflected in its atypical sequence motifs, extended terminal domains, and distinct signaling properties, which collectively blur the distinction between classical visual and non-visual photoreception.

Functionally, RH7 modulates early visual processing in the lamina and might contribute to circadian regulation in dorsal clock neurons. Its phylogenetic and mechanistic parallels to mammalian melanopsin further support its role as a GPCR-like photoreceptor integrating environmental light cues with intracellular signaling cascades to regulate physiology and behavior. Notably, many RH7-retaining species, with or without DCRY, inhabit environments with high light exposure. This ecological context may indicate a potential adaptive role for RH7 in mediating light sensitivity in naturally sun-exposed environments. In summary, RH7 exemplifies how ancient opsins have diversified to couple photoreception with GPCR-mediated signaling beyond image formation. Its expression in brain regions highlights a conserved evolutionary strategy: the integration of light sensitivity into central neural circuits, underscoring the deep-rooted link between sensory and neurodevelopmental architectures.

Data availability

All data supporting this study are available within the main text or the Supplemental Information. Additional details can be obtained from the corresponding author upon request.

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Additional information

Author contributions

V.K. performed fly activity assays, optomotor experiments, generated UAS-construct lines, and carried out MCFO staining and qPCR analyses. N.R. performed immunohistochemistry and in situ hybridizations. H.H. generated the *Rh7-Gal4* line. A.M. contributed additional fly activity assays. D.R. established experimental setups and contributed to experimental design. P.S. carried out the GsX assay. C.H.-F. contributed to conceptual discussions, provided critical input throughout the project, and performed dissections for qPCR. P.R.S. designed the experiments, coordinated the study, analyzed experimental results, performed phylogenetic analyses, and wrote the manuscript. All authors reviewed and approved the final manuscript.

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Author ORCID iDs

Dirk Rieger:  <https://orcid.org/0000-0001-5597-5858>

Peter Soba:  <https://orcid.org/0000-0002-6163-4686>

Pingkalai R Senthilan:  <https://orcid.org/0000-0003-3921-8886>

Additional files

[Figure 3 - supplementary figure 1](#) 

[Figure 3 - supplementary figure 2](#) 

[Figure 4 - supplementary figure 1](#) 

[Figure 5 - supplementary figure 1](#) 

[Figure 6 - supplementary figure 1](#) 

[Supplementary file 1](#) 

[Supplementary table 1](#) 

[Supplementary video 1](#) 

References

Alejevski F, Saint-Charles A, Michard-Vanhée C, Martin B, Galant S, Vasiliauskas D, Rouyer F (2019) The HisCl1 histamine receptor acts in photoreceptors to synchronize *Drosophila* behavioral rhythms with light-dark cycles. *Nature Communications* **10**:252 <https://doi.org/10.1038/s41467-018-08116-7> | [PubMed](#)

Arendt D, Tessmar-Raible K, Snyman H, Dorresteyn AW, Wittbrodt J (2004) Ciliary Photoreceptors with a Vertebrate-Type Opsin in an Invertebrate Brain. *Science* **306**:869-871 <https://doi.org/10.1126/science.1099955> | [PubMed](#)

Aviles-Trigueros M, Matínez-Garrido F, Nadal-Nicolás F, Agudo-Barriuso M, Selles-Navarro I, Vidal-Sanz M (2012) Melanopsin-like Photopigment Expression In Adult Mammal Brain. *Investigative Ophthalmology & Visual Science* **53**:4336

Ballister ER, Rodgers J, Martial F, Lucas RJ (2018) A live cell assay of GPCR coupling allows identification of optogenetic tools for controlling Go and Gi signaling. *BMC Biology* **16**:10 <https://doi.org/10.1186/s12915-017-0475-2> | [PubMed](#)

Bastian FB, Cammarata AB, Carsanaro S, Detering H, Huang W-T, Joye S, Niknejad A, Nyamari M, Mendes de Farias T, Moretti S, *et al.* (2025) Bgee in 2024: focus on curated single-cell RNA-seq datasets, and query tools. *Nucleic Acids Research* **53**:D878-D885 <https://doi.org/10.1093/nar/gkae1118> | [PubMed](#)

Behnia R, Clark DA, Carter AG, Clandinin TR, Desplan C (2014) Processing properties of ON and OFF pathways for *Drosophila* motion detection. *Nature* **512**:427-430 <https://doi.org/10.1038/nature13427> | [PubMed](#)

Bischof J, Maeda RK, Hediger M, Karch F, Basler K (2007) An optimized transgenesis system for *Drosophila* using germ-line-specific ϕ C31 integrases. *Proceedings of the National Academy of Sciences* **104**:3312-3317 <https://doi.org/10.1073/pnas.0611511104> | [PubMed](#)

- Bond SM**, Peralta AJ, Sirtalan D, Skeelee DA, Huang H, Possidente DR, Vecsey CG (2024) Differential regulation of sleep by blue, green, and red light in *Drosophila melanogaster*. *Frontiers in Behavioral Neuroscience* **18** <https://doi.org/10.3389/fnbeh.2024.1476501> | PubMed
- Bowin C-F**, Inoue A, Schulte G (2019) WNT-3A-induced β -catenin signaling does not require signaling through heterotrimeric G proteins. *The Journal of Biological Chemistry* **294**:11677-11684 <https://doi.org/10.1074/jbc.AC119.009412> | PubMed
- Bruce HS**, Jerz G, Kelly S, McCarthy J, Ponerantz A, Senevirathne G, Sherrard A, Sun DA, Wolff C, Patel NH (2021) Hybridization Chain Reaction (HCR) In Situ Protocol. *protocols.io* <https://doi.org/10.17504/protocols.io.bunznvf6>
- Buhr ED**, Yue WWS, Ren X, Jiang Z, Liao H-WR, Mei X, Vemmaraju S, Nguyen M-T, Reed RR, Lang RA, *et al.* (2015) Neuropsin (OPN5)-mediated photoentrainment of local circadian oscillators in mammalian retina and cornea. *Proceedings of the National Academy of Sciences of the United States of America* **112**:13093-13098 <https://doi.org/10.1073/pnas.1516259112> | PubMed
- Charlton-Perkins MA**, Sandler ED, Buschbeck EK, Cook TA (2017) Multifunctional glial support by Semper cells in the *Drosophila* retina. *PLoS genetics* **13**:e1006782 <https://doi.org/10.1371/journal.pgen.1006782> | PubMed
- Choi HMT**, Schwarzkopf M, Fornace ME, Acharya A, Artavanis G, Stegmaier J, Cunha A, Pierce NA (2018) Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. *Development* **145**:dev165753 <https://doi.org/10.1242/dev.165753> | PubMed
- Contreras E**, Nobleman AP, Robinson PR, Schmidt TM (2021) Melanopsin phototransduction: beyond canonical cascades. *The Journal of Experimental Biology* **224**:jeb226522 <https://doi.org/10.1242/jeb.226522> | PubMed
- Datta RR**, Rister J (2022) The power of the (imperfect) palindrome: Sequence-specific roles of palindromic motifs in gene regulation. *BioEssays: News and Reviews in Molecular and Developmental Biology* **44**:e2100191 <https://doi.org/10.1002/bies.202100191> | PubMed
- Davis FP**, Nern A, Picard S, Reiser MB, Rubin GM, Eddy SR, Henry GL (2020) A genetic, genomic, and computational resource for exploring neural circuit function. *eLife* **9**:e50901 <https://doi.org/10.7554/eLife.50901> | PubMed
- Deppisch P**, Helfrich-Förster C, Senthilan PR (2022) The Gain and Loss of Cryptochrome/Photolyase Family Members during Evolution. *Genes* **13**:1613 <https://doi.org/10.3390/genes13091613> | PubMed
- Dolezelova E**, Dolezel D, Hall JC (2007) Rhythm Defects Caused by Newly Engineered Null Mutations in *Drosophila*'s cryptochrome Gene. *Genetics* **177**:329-345 <https://doi.org/10.1534/genetics.107.076513> | PubMed
- Drapeau MD**, Radovic A, Wittkopp PJ, Long AD (2003) A gene necessary for normal male courtship, yellow, acts downstream of fruitless in the *Drosophila melanogaster* larval brain. *Journal of Neurobiology* **55**:53-72 <https://doi.org/10.1002/neu.10196> | PubMed
- Emery P**, Stanewsky R, Hall JC, Rosbash M (2000) A unique circadian-rhythm photoreceptor. *Nature* **404**:456-457 <https://doi.org/10.1038/35006558> | PubMed
- Fleming JF**, Pisani D, Arakawa K (2021) New Tardigrade Opsins and Differential Expression Analyses Show Ontogenic Variation in Light Perception. *Genome Biology and Evolution* **13**:evab164 <https://doi.org/10.1093/gbe/evab164> | PubMed
- Gao Y**, Hu H, Ramachandran S, Erickson JW, Cerione RA, Skiniotis G (2019) Structures of the Rhodopsin-Transducin Complex: Insights into G Protein Activation. *Molecular cell* **75**:781-790.e3. <https://doi.org/10.1016/j.molcel.2019.06.007> | PubMed
- Grebler R**, Kistenpfennig C, Rieger D, Bentrop J, Schneuwly S, Senthilan PR, Helfrich-Förster C (2017) *Drosophila* Rhodopsin 7 can partially replace the structural role of Rhodopsin 1, but not its physiological function. *Journal of Comparative Physiology A* **203**:649-659 <https://doi.org/10.1007/s00359-017-1182-8> | PubMed

- Guo F, Holla M, Díaz MM, Rosbash M (2018) A Circadian Output Circuit Controls Sleep-Wake Arousal in *Drosophila*. *Neuron* **100**:624-635.e4. <https://doi.org/10.1016/j.neuron.2018.09.002> | PubMed
- Guo F, Yu J, Jung HJ, Abruzzi KC, Luo W, Griffith LC, Rosbash M (2016) Circadian neuron feedback controls the *Drosophila* sleep--activity profile. *Nature* **536**:292-297 <https://doi.org/10.1038/nature19097> | PubMed
- Helfrich-Förster C (2017) The *Drosophila* Clock System. In: Kumar V (Ed). *Biological Timekeeping: Clocks, Rhythms and Behaviour* Springer India. pp. 133-176 https://doi.org/10.1007/978-81-322-3688-7_6
- Helfrich-Förster C (2000) Differential control of morning and evening components in the activity rhythm of *Drosophila melanogaster*--sex-specific differences suggest a different quality of activity. *Journal of Biological Rhythms* **15**:135-154 <https://doi.org/10.1177/074873040001500208> | PubMed
- Hering L, Mayer G (2014) Analysis of the Opsin Repertoire in the Tardigrade *Hypsibius dujardini* Provides Insights into the Evolution of Opsin Genes in Panarthropoda. *Genome Biology and Evolution* **6**:2380-2391 <https://doi.org/10.1093/gbe/evu193> | PubMed
- Horn M, Mitesser O, Hovestadt T, Yoshii T, Rieger D, Helfrich-Förster C (2019) The Circadian Clock Improves Fitness in the Fruit Fly, *Drosophila melanogaster*. *Frontiers in Physiology* **10** <https://doi.org/10.3389/fphys.2019.01374> | PubMed
- Izutsu M, Zhou J, Sugiyama Y, Nishimura O, Aizu T, Toyoda A, Fujiyama A, Agata K, Fuse N (2012) Genome Features of "Dark-Fly", a *Drosophila* Line Reared Long-Term in a Dark Environment. *PLoS ONE* **7**:e33288 <https://doi.org/10.1371/journal.pone.0033288> | PubMed
- Jiang Y, Pitmon E, Berry J, Wolf FW, McKenzie Z, Lebestky TJ (2016) A Genetic Screen To Assess Dopamine Receptor (DopR1) Dependent Sleep Regulation in *Drosophila*. *G3 Genes | Genomes | Genetics* **6**:4217-4226 <https://doi.org/10.1534/g3.116.032136> | PubMed
- Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Žídek A, Potapenko A, *et al.* (2021) Highly accurate protein structure prediction with AlphaFold. *Nature* **596**:583-589 <https://doi.org/10.1038/s41586-021-03819-2> | PubMed
- Kim A-R, Hu Y, Comjean A, Rodiger J, Mohr SE, Perrimon N (2024) Enhanced Protein-Protein Interaction Discovery via AlphaFold-Multimer. *bioRxiv* <https://doi.org/10.1101/2024.02.19.580970> | PubMed
- Kingston ACN, Cronin TW (2016) Diverse Distributions of Extraocular Opsins in Crustaceans, Cephalopods, and Fish. *Integrative and Comparative Biology* **56**:820-833 <https://doi.org/10.1093/icb/icw022> | PubMed
- Kistenpfennig C, Grebler R, Ogueta M, Hermann-Luibl C, Schlichting M, Stanewsky R, Senthilan PR, Helfrich-Förster C (2017) A New Rhodopsin Influences Light-dependent Daily Activity Patterns of Fruit Flies. *Journal of Biological Rhythms* **32**:406-422 <https://doi.org/10.1177/0748730417721826> | PubMed
- Konopka RJ, Benzer S (1971) Clock Mutants of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America* **68**:2112-2116 <https://doi.org/10.1073/pnas.68.9.2112> | PubMed
- Koyanagi M, Terakita A (2008) Gq-coupled Rhodopsin Subfamily Composed of Invertebrate Visual Pigment and Melanopsin. *Photochemistry and Photobiology* **84**:1024-1030 <https://doi.org/10.1111/j.1751-1097.2008.00369.x> | PubMed
- Lamaze A, Stanewsky R (2020) DN1p or the "Fluffy" Cerberus of Clock Outputs. *Frontiers in Physiology* **10** <https://doi.org/10.3389/fphys.2019.01540> | PubMed
- Lazopulo S, Lazopulo A, Baker JD, Syed S (2019) Daytime colour preference in *Drosophila* depends on the circadian clock and TRP channels. *Nature* **574**:108-111 <https://doi.org/10.1038/s41586-019-1571-y> | PubMed
- Leung NY, Montell C (2017) Unconventional Roles of Opsins. *Annual Review of Cell and Developmental Biology* **33**:241-264 <https://doi.org/10.1146/annurev-cellbio-100616-060432> | PubMed

- Leung NY, Thakur DP, Gurav AS, Kim SH, Di Pizio A, Niv MY, Montell C (2020) Functions of Opsins in *Drosophila* Taste. *Current biology: CB* **30**:1367-1379.e6. <https://doi.org/10.1016/j.cub.2020.01.068> | PubMed
- Ma D, Przybylski D, Abruzzi KC, Schlichting M, Li Q, Long X, Rosbash M (2021) A transcriptomic taxonomy of *Drosophila* circadian neurons around the clock. *eLife* **10**:e63056 <https://doi.org/10.7554/eLife.63056> | PubMed
- Maggio R, Fasciani I, Petraghano F, Coppolino MF, Scarselli M, Rossi M (2023) Unraveling the Functional Significance of Unstructured Regions in G Protein-Coupled Receptors. *Biomolecules* **13**:1431 <https://doi.org/10.3390/biom13101431> | PubMed
- Matulis CA, Chen J, Gonzalez-Suarez AD, Behnia R, Clark DA (2020) Heterogeneous Temporal Contrast Adaptation in *Drosophila* Direction-Selective Circuits. *Current Biology* **30**:222-236.e6. <https://doi.org/10.1016/j.cub.2019.11.077> | PubMed
- McKimmie C, Woerfel G, Russell S (2005) Conserved genomic organisation of Group B Sox genes in insects. *BMC Genetics* **6**:26 <https://doi.org/10.1186/1471-2156-6-26> | PubMed
- Meyerhof GT, Easwaran S, Bontempo AE, Montell C, Montell DJ (2024) Altered circadian rhythm, sleep, and rhodopsin 7-dependent shade preference during diapause in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences* **121**:e2400964121 <https://doi.org/10.1073/pnas.2400964121> | PubMed
- Min K-T, Benzer S (1999) Preventing Neurodegeneration in the *Drosophila* Mutant bubblegum. *Science* **284**:1985-1988 <https://doi.org/10.1126/science.284.5422.1985> | PubMed
- Nern A, Pfeiffer BD, Rubin GM (2015) Optimized tools for multicolor stochastic labeling reveal diverse stereotyped cell arrangements in the fly visual system. *Proceedings of the National Academy of Sciences of the United States of America* **112**:E2967-E2976 <https://doi.org/10.1073/pnas.1506763112> | PubMed
- Ni JD, Baik LS, Holmes TC, Montell C (2017) A rhodopsin in the brain functions in circadian photoentrainment in *Drosophila*. *Nature* **545**:340-344 <https://doi.org/10.1038/nature22325> | PubMed
- Nissilä J, Mänttari S, Särkioja T, Tuominen H, Takala T, Timonen M, Saarela S (2012) Encephalopsin (OPN3) protein abundance in the adult mouse brain. *Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology* **198**:833-839 <https://doi.org/10.1007/s00359-012-0754-x> | PubMed
- Nissilä JS, Mänttari SK, Särkioja TT, Tuominen HJ, Takala TE, Kiviniemi VJ, Sormunen RT, Saarela SYO, Timonen MJ (2017) The distribution of melanopsin (OPN4) protein in the human brain. *Chronobiology International* **34**:37-44 <https://doi.org/10.1080/07420528.2016.1232269> | PubMed
- Pfeiffer BD, Truman JW, Rubin GM (2012) Using translational enhancers to increase transgene expression in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America* **109**:6626-6631 <https://doi.org/10.1073/pnas.1204520109> | PubMed
- Reinhard N, Fukuda A, Manoli G, Derksen E, Saito A, Möller G, Sekiguchi M, Rieger D, Helfrich-Förster C, Yoshii T, et al. (2024) Synaptic connectome of the *Drosophila* circadian clock. *Nature Communications* **15**:10392 <https://doi.org/10.1038/s41467-024-54694-0> | PubMed
- Rickle A, Sudhakar K, Booms A, Stirtz E, Lempradl A (2025) More than meets the eye: mutation of the white gene in *Drosophila* has broad phenotypic and transcriptomic effects. *Genetics* **230**:iyaf097 <https://doi.org/10.1093/genetics/iyaf097> | PubMed
- Rieger D, Stanewsky R, Helfrich-Förster C (2003) Cryptochrome, compound eyes, Hofbauer-Buchner eyelets, and ocelli play different roles in the entrainment and masking pathway of the locomotor activity rhythm in the fruit fly *Drosophila melanogaster*. *Journal of Biological Rhythms* **18**:377-391 <https://doi.org/10.1177/0748730403256997> | PubMed
- Rister J, Razzaq A, Boodram P, Desai N, Tsanis C, Chen H, Jukam D, Desplan C (2015) Single base pair differences in a shared motif determine differential Rhodopsin expression. *Science* **350**:1258-1261 <https://doi.org/10.1126/science.aab3417> | PubMed

- Senthilan PR**, Grebler R, Reinhard N, Rieger D, Helfrich-Förster C (2019) Role of Rhodopsins as Circadian Photoreceptors in the *Drosophila melanogaster*. *Biology* **8**:6 <https://doi.org/10.3390/biology8010006> | PubMed
- Senthilan PR**, Helfrich-Förster C (2016) Rhodopsin 7–The unusual Rhodopsin in *Drosophila*. *PeerJ* **4**:e2427 <https://doi.org/10.7717/peerj.2427> | PubMed
- Senthilan PR**, Piepenbrock D, Ovezmyradov G, Nadrowski B, Bechstedt S, Pauls S, Winkler M, Möbius W, Howard J, Göpfert MC (2012) *Drosophila* auditory organ genes and genetic hearing defects. *Cell* **150**:1042-1054 <https://doi.org/10.1016/j.cell.2012.06.043> | PubMed
- Sharma Y**, Cheung U, Larsen EW, Eberl DF (2002) PPTGAL, a convenient Gal4 P-element vector for testing expression of enhancer fragments in *drosophila*. *genesis* **34**:115-118 <https://doi.org/10.1002/gene.10127> | PubMed
- Shen WL**, Kwon Y, Adegbola AA, Luo J, Chess A, Montell C (2011) Function of rhodopsin in temperature discrimination in *Drosophila*. *Science* **331**:1333-1336 <https://doi.org/10.1126/science.1198904> | PubMed
- Sonoda T**, Lee SK, Birnbaumer L, Schmidt TM (2018) Melanopsin Phototransduction Is Repurposed by ipRGC Subtypes to Shape the Function of Distinct Visual Circuits. *Neuron* **99**:754-767.e4. <https://doi.org/10.1016/j.neuron.2018.06.032> | PubMed
- Terakita A** (2005) The opsins. *Genome Biology* **6**:213 <https://doi.org/10.1186/gb-2005-6-3-213> | PubMed
- Upton BA**, Díaz NM, Gordon SA, Van Gelder RN, Buhr ED, Lang RA (2021) Evolutionary Constraint on Visual and Nonvisual Mammalian Opsins. *Journal of Biological Rhythms* **36**:109-126 <https://doi.org/10.1177/0748730421999870> | PubMed
- Upton BA**, Nayak G, Schweinzger I, D'Souza SP, Vorhees CV, Williams MT, Earl BR, Lang RA (2022) Comprehensive Behavioral Analysis of Opsin 3 (Encephalopsin)-Deficient Mice Identifies Role in Modulation of Acoustic Startle Reflex. *eNeuro* **9** <https://doi.org/10.1523/ENEURO.0202-22.2022> | PubMed
- Vöcking O**, Kourtesis I, Hausen H (2015) Posterior eyespots in larval chitons have a molecular identity similar to anterior cerebral eyes in other bilaterians. *EvoDevo* **6**:40 <https://doi.org/10.1186/s13227-015-0036-0> | PubMed
- Williamson WR**, Wang D, Haberman AS, Hiesinger PR (2010) A dual function of V0-ATPase a1 provides an endolysosomal degradation mechanism in *Drosophila melanogaster* photoreceptors. *Journal of Cell Biology* **189**:885-899 <https://doi.org/10.1083/jcb.201003062> | PubMed
- Wollesen T**, McDougall C, Arendt D (2019) Remnants of ancestral larval eyes in an eyeless mollusk? Molecular characterization of photoreceptors in the scaphopod *Antalis entalis*. *EvoDevo* **10**:25 <https://doi.org/10.1186/s13227-019-0140-7> | PubMed
- Xiong B**, Bellen HJ (2013) Rhodopsin homeostasis and retinal degeneration: lessons from the fly. *Trends in Neurosciences* **36**:652-660 <https://doi.org/10.1016/j.tins.2013.08.003> | PubMed
- Yang HH**, Clandinin TR (2018) Elementary Motion Detection in *Drosophila*: Algorithms and Mechanisms. *Annual Review of Vision Science* **4**:143-163 <https://doi.org/10.1146/annurev-vision-091517-034153> | PubMed
- Zanini D**, Giraldo D, Warren B, Katana R, Andrés M, Reddy S, Pauls S, Schwedhelm-Domeyer N, Geurten BRH, Göpfert MC (2018) Proprioceptive Opsin Functions in *Drosophila* Larval Locomotion. *Neuron* **98**:67-74.e4. <https://doi.org/10.1016/j.neuron.2018.02.028> | PubMed

Peer reviews

Reviewer #1 (Public review):

Summary:

The study investigates the *Drosophila* non-visual light receptor rhodopsin7 with regard to its role in light information processing and resulting consequences for behavioral patterns and circadian clock function. Using behavioral, in situ staining, and receptor activation assays together with different fly mutants, the authors show that rhodopsin7 is an important determinant of activity under and response to darkness, which likely signals via a pathway distinct from other, visual *Drosophila* rhodopsins. Based on phylogenetic analysis, the authors further discuss a potentially conserved functional role of non-visual photoreceptors like rhodopsin7 and the mammalian melanopsin light information processing and circadian clock modulation.

Strengths:

The manuscript follows a very clear structure with all investigations logically building onto each other. Background information and methodology are provided in appropriate detail so that readers can fully understand why and how experiments were conducted. It is further praiseworthy that the authors provide the details that allow also non-experts in the field to fully understand their approaches. Experimental work was conducted in a highly standardized manner, and also considered potential "side-aspects" like the consequences of temperature cycles and changed photoperiods. The detailed and clear description of the obtained results makes them very convincing, with (almost) all observable patterns being addressed.

By highlighting the evolutionary old phylogenetic position of rhodopsin7 and its conservation across numerous clades, the authors provide strong reasoning for the relevance of their work, also pointing out the similarities to the mammalian melanopsin. The postulated hypothesis regarding protein structure and functioning, as well as the role in light information processing and behavioral and circadian clock modulation are well based on the authors' observations, and speculative aspects are correctly pointed out.

Weaknesses:

Where the manuscript still has potential for improvement is the discussion, which in its current form does seem slightly self-contained and does not fully integrate the findings of previous studies on *Drosophila* rhodopsin7. As the introduction specifically points out that previous findings have been contradictory, this seems like a missed opportunity. Further details on this are provided in the recommendations below.

Similarly, the manuscript currently lacks a discussion of the possible relevance of rhodopsin7 (and other non-visual light receptors in other organisms) in the context of a species' environment and lifestyle, i.e., what is the relevance/benefit of having rhodopsin7 in the fly's everyday life? While this clearly involves speculation, when done carefully, it can elevate the paper's relevance from a primarily academic to a societal one.

An additional point concerns the title and abstract, which postulate rhodopsin7 roles in contrast vision as well as motion and brightness perception. Contrast remains poorly defined in the text, leaving it ambiguous whether it refers to bright/dark contrasts, e.g., along edges, or the temporal contrast that results from dark pulses (startle response). While the latter seems to apply here, the former is likely more intuitive. Thus, this aspect should be rephrased (also in the title) or properly clarified early on. Regarding motion detection, this is backed up by the optomotor response results, but the findings stand somewhat isolated from the other results, lacking a clear connection aside from general visual processing. Lastly, brightness perception is mentioned in the abstract, but never again, possibly due to inconsistent phrasing throughout the manuscript.

<https://doi.org/10.7554/eLife.109811.2.sa3>

Reviewer #2 (Public review):

Summary:

This is a very interesting paper bringing new and important information about the poorly understood rhodopsin 7 photoreceptive molecule. The very ancient origin of the gene is revealed in addition to data supporting a signaling pathway that is different from the one known for the canonical rhodopsins. Precise expression data, particularly in the optic lobe of the fly, as well as clear behavioral phenotypes in responses to light changes, make this study a strong contribution to the understanding of the still-debated function of rhodopsin 7.

Specific comments

(1) Title and abstract: Contribution of Rh7 to circadian clock regulation

a) It is not that clear to me what rhodopsin does in terms of circadian regulation (even though its function might be circadianly regulated). The clear role in the light/dark distribution of activity might not be circadian per se, but mostly light/dark- driven and there is no evidence here for a role in the entrainment of the clock.

b) The authors should cite Lazopulo, which nicely shows that Rh7 has an important role in peripheral neurons to allow flies to escape from blue light (see below).

(2) Figure 2 C

The finding showing that Galphaz but not Galphaq can trigger signaling from light-excited Rh7 is a very intriguing finding to better understand Rh7 function. Since Galphaz is related to Gi/o, it would be interesting to test those, for example, by expressing RNAi with Rh7-gal4 and testing the Light-dark or light-off response behavior.

(3) Figures 3-4

The change in the locomotor activity distribution between light and dark in LD conditions provides a nice assay for Rh7 function. Since Lazopulo et al. (2019) have shown that wild-type but not Rh7 mutants do escape from blue light, it would be important to compare and discuss these LD behavior data with the Lazopulo results. Precisely, is this nighttime preference linked to blue light?

The expression data are really nice and show that Rh7 is mostly a non-retinal photoreceptor. However, the paper would be strongly reinforced by correlating this with the LD behavior. The LD phenotype should be tested in flies with Rh7 expression rescued under Rh7gal4 control (as done for the startle response). This is important to show whether the expression pattern is likely responsible for the described Rh7 function in LD. If L5 and or M11 drivers are available, they should be used to rescue Rh7? Since expression in some clock neurons is shown, the rescue experiment should also be done with a clock neuron driver.

In the same line, can the LD phenotype (or startle response phenotype of Figure 4) be restored by expressing Rh7 under ppk control, as shown for the blue light avoidance phenotype by Lazopulo et al?

Finally, the Rh7 "darkfly" rescued flies should be tested in LD.

<https://doi.org/10.7554/eLife.109811.2.sa2>

Reviewer #3 (Public review):

Summary:

While our knowledge regarding visual opsins is largely very good, a lot more uncertainty exists around the role of non-visual opsins. Using the power of the *Drosophila melanogaster* model system, Kirsh et al. investigate the role of the non-visual opsin Rhodopsin7 (Rh7). Expression analysis, based on Rh7-Gal4>UAS-GFP and HRC in situ staining, reveals strong expression in the optic lobes and somewhat weaker, but nevertheless extensive expression in the brain. An investigation of motor activity reveals that loss of function leads to an altered day and night rhythm, specifically decreasing activity during the dark phase. These flies were also less sensitive, but still responsive to a light-induced startle response and showed deficiencies in the optomotor response. To further investigate how Rh7 may modulate these responses, inspired by the Dark line of flies (which were kept in the dark for ~1400 generations) and which has accumulated C-terminal related losses, the authors conducted rescues with an intact and a C-terminal-deficient Rh7 and were able to pinpoint that region as an important driver of related behavioral shifts. These findings are particularly intriguing as Rh7 represents an ancient opsin with phylogenetic and mechanistic parallels to mammalian melanopsin.

Strengths:

The paper is well-written and contains high-quality data with appropriate sample sizes, and the conclusions are well supported.

Weaknesses:

No weaknesses were identified by this reviewer, but the following recommendations are made:

(1) The authors should clarify exactly what tissues were taken for the comparative qPCR. This is particularly interesting in terms of the retina. Since Rh7 appears not to be expressed within the photoreceptor cells of the retina, this raises the important question as to which cells it is expressed in. To address this important question, it would also be helpful to include an expression analysis of the retina itself (by extending the RH7-GFP expression patterns and/or adding HCR in situ of the ommatidia array). The cell types of the retina are very well classified, and some evidence already exists for Rh7 expression in support cells (e.g., Charlton-Perkins et al., (2017); PMID: 28562601). This study has a unique opportunity to investigate this further by adding these critical data for a more complete picture of Rh7.

(2) Mammalian opsins should be included in the phylogenetic analysis illustrated in Figure 2A and indicate their position on the tree. This will allow readers to better put the authors' statements regarding the intermediate position of Rh7 into perspective. In addition, note that the distinction between red and deep red is easy to miss regarding the Rh7 cluster. Perhaps the authors could use a more distinct colour scheme, for example, orange and deep red.

(3) More details should be provided on the optomotor response experiments. Specifically, specifications of the frequencies used for the optomotor response are needed. Results show a relatively large level of variation, which may be due to different angular perspectives that flies may have had while viewing the stimulus. If possible, provide videos as examples, as they will make it clearer to viewers how much flies could move around in the setup (from the methods, it seems they could move within the 2.2 of the 3 cm diameter of the arena, which would lead to substantial differences in the visual angle of the viewed grating.

<https://doi.org/10.7554/eLife.109811.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public review):

(1) Where the manuscript still has potential for improvement is the discussion, which in its current form does seem slightly self-contained and does not fully integrate the findings of previous studies on Drosophila rhodopsin7. As the introduction specifically points out that previous findings have been contradictory, this seems like a missed opportunity.

We thank the reviewer for this helpful suggestion and agree that the previous version of the Discussion did not sufficiently integrate earlier studies on Drosophila RH7. In the revised manuscript, we have expanded and restructured the discussion to explicitly incorporate key studies, including those by Ni et al. (2017), Senthilan et al. (2019), Lazopulo et al. (2019), and Meyerhof et al. (2024). In Discussion 5.4, we directly address similarities and discrepancies, such as differences in reported expression patterns, behavioral phenotypes, and experimental conditions. We use these inconsistencies to better position our findings and propose that RH7 acts as a modulatory photoreceptor with context dependent effects, which may explain the variability observed across studies. These changes provide a more integrated and coherent view of RH7 function.

(2) Similarly, the manuscript currently lacks a discussion of the possible relevance of rhodopsin7 (and other non-visual light receptors in other organisms) in the context of a species' environment and lifestyle, i.e., what is the relevance/benefit of having rhodopsin7 in the fly's everyday life?

We agree that the ecological relevance of RH7 deserved a more detailed discussion. According to the suggestion, we have now expanded the discussion to address the ecological and functional relevance of RH7 in the context of environmental light conditions and species-specific lifestyles (see Section 5.5). In particular, we now highlight that RH7 is frequently retained in species inhabiting sun-exposed environments and that its presence correlates with conditions of high light exposure, especially during early developmental stages. Together with our functional data in Drosophila, showing a role of RH7 in light dependent behavioral flexibility and circadian regulation, this supports the idea that RH7 contributes to the detection and integration of environmental light cues under natural conditions.

(3) An additional point concerns the title and abstract, which postulate rhodopsin7 roles in contrast vision as well as motion and brightness perception. Contrast remains poorly defined in the text, leaving it ambiguous whether it refers to bright/dark contrasts, e.g., along edges, or the temporal contrast that results from dark pulses (startle response). While the latter seems to apply here, the former is likely more intuitive. Thus, this aspect should be rephrased (also in the title) or properly clarified early on.

Thank you for this comment. We agree that the term “contrast” was ambiguous, as it can refer to spatial contrast (e.g., edge detection) or temporal contrast (changes in light intensity). Our study focuses on temporal contrast, such as light-dark transitions. Accordingly, we have revised the manuscript, replacing “contrast” with the more precise term “temporal changes in light intensity” throughout the relevant sections. In addition, to clarify this further, we conducted additional experiments with graded changes in illumination. These showed that Rh7⁰ mutants have reduced responses compared to wild type in both abrupt (e.g. 30 → 0 $\mu\text{W}/\text{cm}^2$) and gradual (e.g. 30 → 5 $\mu\text{W}/\text{cm}^2$) transitions, indicating that RH7 contributes to detecting temporal changes in light intensity.

(4) Regarding motion detection, this is backed up by the optomotor response results, but the findings stand somewhat isolated from the other results, lacking a clear connection aside from general visual processing.

We have clarified the connection between motion detection and the other visual assays. In the Results section (4.7), motion detection is now described as “relying on the processing of spatiotemporal changes in luminance across the visual field,” explicitly linking it to general luminance-based visual processing. In the Discussion (5.3), we added the concise statement “These computations rely on changes in light intensity over space and time” to further emphasize this connection. We believe these changes make the relationship between the optomotor results and the other assays clearer and easier to follow.

(5) Lastly, brightness perception is mentioned in the abstract, but never again, possibly due to inconsistent phrasing throughout the manuscript.

We agree that the terminology was not used consistently throughout the manuscript. To improve clarity and consistency, we replaced the term “brightness perception” in the Abstract with “temporal changes in luminance”, which more accurately reflects the focus of our study.

Reviewer #2 (Public review):

(1) It is not that clear to me what rhodopsin does in terms of circadian regulation (even though its function might be circadianly regulated). The clear role in the light/dark distribution of activity might not be circadian per se, but mostly light/dark- driven and there is no evidence here for a role in the entrainment of the clock.

We thank the reviewer for this important point. We agree that our current data does not provide sufficient evidence for a direct role of RH7 in circadian regulation or clock entrainment. In this revision, we have examined LD (12:12) behavior in more detail and report changes in several activity parameters (Figure 3A). While these differences could in principle involve circadian mechanisms, our experiments do not allow us to distinguish between circadian effects and acute light-driven modulation of locomotor activity. We therefore present these results without interpreting them as evidence for a circadian function of RH7, and instead focus on light-driven behavioral effects under LD conditions.

(2) The authors should cite Lazopulo, which nicely shows that Rh7 has an important role in peripheral neurons to allow flies to escape from blue light (see below).

We thank the reviewer for pointing this out. We have now cited Lazopulo et al. (2019) at multiple relevant points in the manuscript, specifically regarding atypical RH7 expression in peripheral neurons, the functional effects of RH7 outside the LNvs, and its protective role in light-dependent behavior (discussion 5.1, 5.4, and 5.5). We believe these additions ensure that the manuscript accurately reflects the existing literature and appropriately acknowledges the contribution of Lazopulo et al. to understanding RH7 function.

(3) The finding showing that Galphaz but not Galphaq can trigger signaling from light-excited Rh7 is a very intriguing finding to better understand Rh7 function. Since Galphaz is related to Gi/o, it would be interesting to test those, for example, by expressing RNAi with Rh7gal4 and testing the Light-dark or light-off response behavior.

We agree that testing additional Gi/o-type Ga subunits would be highly informative for further dissecting RH7 signaling. However, in our preliminary experiments, RNAi-mediated knockdown of Rh7 itself did not result in a robust or statistically significant behavioral phenotype under our conditions. This is consistent with previous reports that RNAi in *Drosophila* can show variable efficiency and often results in incomplete knockdown, leading to weak or undetectable phenotypes (Dietzl et al., 2007; Green et al., 2014; Perkins et al., 2015).

Given these limitations, we did not further pursue RNAi-based approaches targeting additional Ga subunits in the current study. Instead, we complemented our experimental findings with additional comparative and *in silico* analyses using FlyPredictome, which support a potential association between RH7 and Gi/o-type signaling components. These results have now been incorporated into the revised manuscript (Discussion 5.2). We agree that further functional validation of Gi/o-type G protein involvement, for example using genetic mutants, interaction assays or alternative approaches, would be an important direction for future studies.

(4) The change in the locomotor activity distribution between light and dark in LD conditions provides a nice assay for Rh7 function. Since Lazopulo et al. (2019) have shown that wildtype but not Rh7 mutants do escape from blue light, it would be important to compare and discuss these LD behavior data with the Lazopulo results. Precisely, is this nighttime preference linked to blue light?

To address this point, we conducted additional experiments, which are now included in the supplementary materials. Instead of the usual white light–dark (LD) cycles, we tested blue light–dark and green light–dark conditions. However, we observed only minor differences and no significant shift in light preference, as reported by Lazopulo et al. (2019). A likely explanation for this discrepancy is the difference in experimental light intensity. In our assays, we used moderate light intensities, approximately 30 $\mu\text{W}/\text{cm}^2$ for white light and 5–6 $\mu\text{W}/\text{cm}^2$ for color lights. Lazopulo et al. used substantially higher light intensities (2480 $\mu\text{W}/\text{cm}^2$ for white light and approximately 600 $\mu\text{W}/\text{cm}^2$ for colored lights). Even the higher intensities used in our arrhythmia experiments ($\sim 120 \mu\text{W}/\text{cm}^2$) remained well below those applied in their study.

Importantly, the behavioral paradigms differ: Lazopulo et al. assessed acute light preference/avoidance, whereas our experiments quantify locomotor activity distribution across the day under LD conditions. The latter is strongly shaped by circadian regulation and integrates multiple internal and external factors. Therefore, wavelength-dependent avoidance behavior observed under acute conditions may not directly translate into changes in daily activity patterns under moderate LD conditions. Although we cannot directly replicate the strong avoidance of blue light or preference for green light described by Lazopulo et al., our results do not contradict those of Lazopulo. The reduced activity during the midday phase in the wild-type flies, which is less pronounced in Rh7⁰, may suggest a potential protective function or preventive behavior in the WT flies. Since the moderate light intensities used in our LD assays are much less aversive, no strong wavelength-dependent preference was observed.

(5) However, the paper would be strongly reinforced by correlating this with the LD behavior. The LD phenotype should be tested in flies with Rh7 expression rescued under Rh7gal4 control (as done for the startle response). This is important to show whether the expression pattern is likely responsible for the described Rh7 function in LD.

We agree that assessing LD behavior in flies with Rh7 expression rescued under Rh7-Gal4 control is important to link expression pattern to function. We have now performed these experiments. The Rh7-Gal4–driven rescue results in a substantial recovery of the LD locomotor phenotype, bringing it closer to control levels than the UAS-empty condition. While the rescue does not fully reach wild-type levels, the observed trend and significant differences across specific parameters support a functional contribution of Rh7-expressing cells. These data are now included in Figure 5C and described in section 4.6.

(6) If L5 and or M11 drivers are available, they should be used to rescue Rh7? Since expression in some clock neurons is shown, the rescue experiment should also be done with a clock neuron driver.

Thank you for this helpful suggestion. We agree that cell type-specific rescue using L5- and/or Mi1 drivers, as well as clock neuron drivers, would be highly informative.

However, implementing these experiments is currently not straightforward. Targeting these neuronal populations requires the use of split-Gal4 driver lines, which can differ in expression strength and are therefore not quantitatively comparable across combinations. In addition, the required genotype would be highly complex: the two split-Gal4 components will be located on separate chromosomes, the UAS-Rh7 transgene is on another chromosome, and the Rh7⁰ mutation resides on the third chromosome. Combining all necessary elements in a single line would therefore require extensive genetic recombination. Importantly, we aim to avoid introducing mutations or markers on the X chromosome, as these are known to influence behavioral readouts. However, many of the available lines are in a white⁻ background, and assembling the full rescue genotype while maintaining a controlled genetic background across all chromosomes is therefore particularly challenging. For these reasons, we have not pursued these rescue experiments in the current study but consider them an important direction for future work.

(7) In the same line, can the LD phenotype (or startle response phenotype of Figure 4) be restored by expressing Rh7 under ppk control, as shown for the blue light avoidance phenotype by Lazopulo et al?

We thank the reviewer for this suggestion. However, in our experiments, we did not observe a blue-light avoidance phenotype comparable to that described by Lazopulo et al. (2019). Since this behavioral effect did not occur in our assay, we did not perform rescue experiments with ppk-regulated Rh7 expression. As previously discussed, the absence of pronounced blue light avoidance in our experimental setup is likely due to the moderate light intensities we used, which are significantly lower than those applied in Lazopulo et al.

Therefore, a ppk-mediated rescue would not be meaningful in our context.

(8) Finally, the Rh7 "darkfly" rescued flies should be tested in LD.

We agree that testing Rh7Darkfly rescue flies under LD conditions provides important insight into Rh7 function. We have now performed these experiments. The Rh7Darkfly rescue leads to a marked shift of the LD locomotor phenotype toward the Darkfly phenotype, with a stronger effect than observed in the Rh7 rescue lines. While the rescue does not fully recapitulate either wild-type or Darkfly controls, the observed trend supports a contribution of RH7 to the modulation of LD behavior in this genetic background. These data are now included in Figure 5C.

Reviewer #3 (Public review):

(1) The authors should clarify exactly what tissues were taken for the comparative qPCR. This is particularly interesting in terms of the retina. Since Rh7 appears not to be expressed within the photoreceptor cells of the retina, this raises the important question as to which cells it is expressed in. To address this important question, it would also be helpful to include an expression analysis of the retina itself (by extending the RH7-GFP expression patterns and/or adding HCR in situ of the ommatidia array). The cell types of the retina are very well classified, and some evidence already exists for Rh7 expression in support cells (e.g., Charlton-Perkins et al., (2017); PMID: 28562601). This study has a unique opportunity to investigate this further by adding these critical data for a more complete picture of Rh7.

We thank the reviewer for this helpful comment. We have now clarified in the Materials and Methods exactly which tissues were collected and analyzed for the comparative qPCR experiments. Regarding Rh7 expression in the retina, in our hands, we did not detect signals

in retinal support cells, likely due to low expression and masking by autofluorescence. However, low Rh7 mRNA levels detected by qPCR in retinal tissue, together with the faint staining reported by Kistenpennig et al. (2017), suggest that expression in these cells is possible. We have added this clarification, along with the observation by Lazopulo et al. (2019), who reported Rh7 expression in multidendritic neurons, to the Discussion (section 5.1) to provide a more complete overview of RH7 localization.

(2) Mammalian opsins should be included in the phylogenetic analysis illustrated in Figure 2A and indicate their position on the tree. This will allow readers to better put the authors' statements regarding the intermediate position of Rh7 into perspective. In addition, note that the distinction between red and deep red is easy to miss regarding the Rh7 cluster. Perhaps the authors could use a more distinct colour scheme, for example, orange and deep red.

In the revised Figure 2A, we have included both mammalian melanopsins and lophotrochozoan opsins, which support our interpretation of RH7 as a primitive opsin. The color scheme has been updated to more clearly distinguish the different clades, and tardigrades are now represented with a red triangle rather than a separate color. These changes improve clarity and better highlight the intermediate phylogenetic position of RH7.

(3) More details should be provided on the optomotor response experiments. Specifically, specifications of the frequencies used for the optomotor response are needed. Results show a relatively large level of variation, which may be due to different angular perspectives that flies may have had while viewing the stimulus. If possible, provide videos as examples, as they will make it clearer to viewers how much flies could move around in the setup (from the methods, it seems they could move within the 2.2 of the 3 cm diameter of the arena, which would lead to substantial differences in the visual angle of the viewed grating).

We would like to thank the reviewer for this valid point. We agree that, due to the relatively simple design of our optomotor apparatus, the flies may have different viewing angles of the rotating grid. To illustrate this point, we have added representative video footage and further photographs of the experimental setup to the supplementary material. A more sophisticated optomotor apparatus that maintains a fixed viewing angle for the animals would certainly reduce experimental variability. Nevertheless, our current, simple setup yielded robust and statistically significant optomotor responses. To further strengthen the robustness of these results, we have increased the sample size from $n = 20$ to $n = 30$, thereby confirming the observed effects.

Recommendations for the authors:

Reviewing Editor Comments:

(1) Claimed impact of rh7 on circadian regulation.

As the data are presented at the moment, there is no evidence for a role in circadian regulation. At present, the data can be fully explained by the acute effects of light on behavior. Either remove the circadian from the text or consider specific entrainment and rescue experiments (see suggestions from Reviewer 2).

We thank the editor for this important comment. We agree that the original version of the manuscript overinterpreted the role of RH7 in circadian regulation, and that the observed phenotypes can be explained by acute effects of light on locomotor behavior under LD conditions. In the revised manuscript, we have substantially restructured and clarified the interpretation of our findings. We now explicitly frame our results as light-driven behavioral effects under LD (12:12) conditions and have removed claims suggesting a direct role of RH7 in circadian entrainment. Furthermore, we have also added new rescue experiments,

including Rh7-Gal4-driven Rh7 and Rh7Darkfly rescue, which provide further functional insight. These data show partial restoration of the locomotor phenotype and genotype-dependent shifts in activity patterns, supporting a modulatory role of RH7 in light-dependent behavior, while not directly addressing circadian clock function.

We have further revised the text to ensure that conclusions remain strictly aligned with the presented data. In addition, we now emphasize that our data provide a basis for future studies exploring the potential role of RH7 in circadian entrainment.

(2) Improved discussion section

*Both Reviewers 1 and 2 point out several aspects that should be better taken care of, especially embedding the new results into the context of the existing literature on rh7-
Lazopulo et al 2019, Ni et al 2017, Meyerhof et al 2024 were particularly mentioned.*

We have addressed the reviewers' suggestions by revising the Discussion section. We now refer to Meyerhof et al. (2024), Lazopulo et al. (2019), and Ni et al. (2017) at multiple points throughout the Discussion to better place our findings in the context of the existing literature on RH7 function. These references are now included in Sections 5.1, 5.4, and 5.5 of the Discussion.

(3) The addition of vertebrate melanopsins, as pointed out by Reviewer 3 (they suggest mammalian, but consider adding other vertebrate and also lophotrochozoan proteins), in the phylogenetic tree will help to put the evolutionary ancestry claim into a better perspective.

We performed new bioinformatic analyses using RefSeq proteomes and carefully selected representative species across relevant clades to construct a balanced phylogenetic tree. These additions support our interpretation of RH7 as a primitive opsin and help place its intermediate phylogenetic position into better perspective. The results are presented in the revised Figure 2A, which now also includes mammalian melanopsins and lophotrochozoan opsins.

(4) There were several questions raised on the qPCR and optomotor response experiments (Reviewer 3), as well as the definition of contrast (Reviewer 1).

We have addressed the reviewers' comments by substantially improving the clarity and presentation of the qPCR and optomotor response experiments, as well as the definition of "contrast." We now provide a more detailed description of the qPCR analysis in the Methods section. For the optomotor response, we have added representative images and videos of the experimental setup to the Supplementary Material to increase transparency. We further clarified that the term "contrast" was previously used ambiguously and may refer to different concepts. In the revised manuscript, we now consistently refer to "temporal changes in light intensity" to specify that our study focuses on light-dark transitions. In addition, we included additional experiments with graded changes in illumination, which show reduced responses in Rh7 mutants compared to wild type under both abrupt and gradual transitions.

(5) Supplementary Material needs sorting

Accession numbers are in Supplementary File 2, not Supplementary File 1. Supplementary Material S2B is in Supplementary File 3 - this is really confusing. Supplementary Materials S3 is probably Supplementary File 1, and the phylogenetic tree shown there actually looks identical to the tree shown in Figure 2A of the main manuscript.

We sincerely apologize for the confusion regarding the Supplementary Material numbering and file assignments. This issue likely arose during the upload process or transfer from the bioRxiv version of the manuscript. We have now carefully checked and corrected the

Supplementary Files to ensure that all accession numbers, figures, and files are correctly assigned and consistently labeled throughout the revised submission.

Reviewer #1 (Recommendations for the authors):

(1) Here, I will address specific aspects of the manuscript, elaborating on the points mentioned in the public review and also listing detailed suggestions.

As mentioned in the public review, the discussion could do a better job of relating the obtained findings to previous publications. For example, the paper by Ni et al. (2017) investigates the role of *Drosophila* rhodopsin7 in circadian light entrainment and its interplay with *dcry*, which seems relevant in the context of rhodopsin7 apparently being only present in CRY-negative clock neurons (except DN1a).

We thank the reviewer for this helpful suggestion. We have expanded the manuscript to further explore the potential relationship between RH7 and DCRY. Specifically, we performed additional bioinformatic analyses, which are now presented in Results Section 4.1, and in discussion sections 5.4 and 5.5 addressing this aspect. While previous observations by Ni et al. (2017) and Kistenpfennig et al. (2017) have already pointed to a possible relationship between RH7 and DCRY, our phylogenetic analyses provide additional support for such a connection from an evolutionary perspective. Although our data do not yet allow us to infer the precise functional nature of this relationship, they further strengthen the evidence that Rh7 and DCRY are evolutionarily and functionally linked.

(2) Another relevant paper by Meyerhof et al 2024 (doi:10.1073/pnas.2400964121) investigates the role of rhodopsin7 in shade preference and startle responses to short light pulses. The lack of shade preference and the altered startle response in rh7 mutants could be related to the authors' own findings on altered behavior in darkness. Further, the paper shows rh7 mutants to more rapidly return to baseline activity after a light pulse, which is somewhat reminiscent of the abrupt activity decrease in rh7 mutants at the start of the dark phase (18h). Generally, I recommend using the contradictory findings mentioned in the introduction as a starting point to describe where the new findings expand previous knowledge and possibly resolve contradictions.

We thank the reviewer for this suggestion. We have revised the Discussion to integrate our findings with the available literature, including the recent study by Meyerhof et al. (2024), and explicitly compare similarities and differences between the reported phenotypes and our own observations.

These aspects are now addressed in the newly added Section 5.5, where we discuss the evolutionary retention of RH7 in sun-exposed species and relate our phylogenetic findings to the emerging evidence that RH7 may play a conserved role in mediating responses to sun-exposed and shaded environments.

(3) One specific aspect that does not fully convince me is the interpretation of the behavioral patterns concerning the short photoperiods (Figure 3C). The authors state that rh7 mutants lose the flexibility to shift their activity into the dark phase. However, drawing a vertical line through panels A, B, and C, it rather seems that activity peak times in wildtypes are fixed to 6-7h and 17-18h, while rh7 mutants do actually shift their peak activities with the decreasing photoperiod. A way of quantifying this would be to determine the phase differences between M- and E-peaks. It may also help to align plots in a way so that lightson of long and short photoperiod plots align. There is clearly a difference between WTs and mutants, but I would be more careful in calling this a reduction in flexibility.

We thank the reviewer for this comment. We also noticed that the behavioral patterns under short photoperiods can be interpreted in different ways. Based on this comment, as well as

the comments from the other reviewers and the handling editor, we decided not to further discuss the potential circadian component in this manuscript. We agree that additional and more targeted experiments will be necessary to better separate circadian effects from light-induced behavioral responses. These experiments are beyond the scope of the present study but will be important to further investigate the potential role of RH7 in circadian regulation. Therefore, we revised the manuscript to focus on the light-induced behavioral effects of RH7 and avoided drawing conclusions about circadian flexibility that are not yet fully supported by our data. In this process, we removed the data showing activity under short days from the manuscript.

Below, I list individual points according to their line number in the manuscript:

(1) Line 1: Consider rephrasing the title based on the above comment regarding contrast.

We have revised the title accordingly.

The new title is: RHODOPSIN 7: An ancestral non-canonical photoreceptor shaping light-responsive behavior

(2) Line 10: Vision is also used to adapt to the environment, so maybe change to "...also to otherwise/indirectly adapt behavior..."

We have revised the sentence as suggested to clarify the non-image-forming role of light in behavioral adaptation.

"Animals rely on light not only for vision but also to adapt behavior to their environment through non-image-forming pathways."

(3) Line 15: Consider rephrasing the title based on the above comment regarding contrast, motion, and brightness perception.

We thank the reviewer for this suggestion. The title has been revised accordingly. The new title is: RHODOPSIN 7: An ancestral non-canonical photoreceptor shaping light-responsive behavior.

(4) Lines 23-25: This reads as if light perception and vision are the same. Please clearly distinguish to make clear that vision is only one way in which light can be perceived and influence behavior.

We have revised the text to clarify that vision represents only one modality of light perception and that light can influence behavior via both visual and non-visual pathways. "The ability to detect and respond to light is fundamental for many organisms and can influence behavior through both visual and non-visual pathways. Vision relies on..."

(5) Lines 26-27: Here and also later in the introduction, it is hard to follow how different groups of opsin light receptors relate to each other (e.g., opsins (in general,) rhodopsins, r-opsins, c-opsins, non-visual opsins), etc.) This is also related to the fact that opsins can be differentially grouped based on phylogeny/structure (e.g., r- vs. c-opsins) or role/localization (e.g., visual vs. non-visual). Please revisit the introduction with a non-expert reader in mind, and try to address the groups hierarchically. In fact, a small tree-like figure of the different groups may be a simple solution.

We thank the reviewer for this helpful suggestion. We have revised the Introduction to simplify the presentation of opsin classification. c-Opsins and r-opsins are now introduced only briefly to place RH7 within the rhabdomeric opsin family. Since *Drosophila* contains only rhabdomeric opsins and these are the focus of our study, we did not further expand the Introduction with a detailed discussion of c-opsins or add a classification figure.

(6) Line 33: Please expand to "...image formation in the eye, some..." to be more precise. We have revised the sentence to clarify that rhodopsin-mediated image formation refers to visual systems.

"While the primary function of rhodopsins is image formation in visual systems, some are also known to participate in non-image-forming functions."

(7) Lines 46-55: This detailed information about the different *Drosophila* rhodopsins is not referred to anywhere else in the manuscript. I suggest shortening and focusing on *rh7*.

We thank the reviewer for the suggestion. We have shortened the section to focus on RH7, as the other rhodopsins are not discussed elsewhere in the manuscript. The revised text now highlights the debated expression and potential function of RH7.

"*Drosophila melanogaster* (Dmel) has seven rhodopsins (RH1-RH7), all belonging to the opsins group. While RH1–RH6 are expressed in well-characterized photoreceptors, the expression pattern and function of RH7 remain less clear and are still debated."

(8) Line 55: The statement that the expression of *Rh7* remains debated also occurs later in the introduction (line 88). If in line 55, it is about the expression sites specifically, references should be added. Otherwise, it may be sufficient to only address this aspect in line 88.

The statement in line 55 specifically refers to the expression sites of RH7. References to Ni et al., 2017 and Senthilan et al., 2019 have been added to support this point.

(9) Line 61: Which of these features distinguishes rhodopsins from other opsin groups?

We thank the reviewer for this comment. The RH7 section has been thoroughly revised to clarify both the features shared with other rhodopsins and those that distinguish RH7 from other opsin groups. Key distinguishing features, including the extended N- and C-terminal tails and the absence of the QAKKMN motif in ICL3, are now highlighted at the beginning of the paragraph, while additional *Drosophila*-specific features, such as the palindromic RCSI, are discussed subsequently.

"RH7 displays the canonical rhodopsin features, including seven transmembrane helices, a chromophore-binding lysine, and the LRTPXN motif located in the first intracellular loop (ICL1)...."

(10) Figure 1: For panel B (left), please increase label sizes. For panel B (right), the color scale makes it very hard to see low-level expression (e.g., in *Mi15* and below). Please try adjusting the color scale and/or consider only showing the cell types also indicated in the left part of the panel. For *ninaE*, please add "aka *Rh1*" or similar for the less informed reader.

We have revised Figure 1 accordingly. Specifically, we increased the label sizes in panel B (left) to improve readability. For panel B (right), we adjusted the color scale to better visualize low-level expression and improve overall clarity. In addition, we updated the labeling of *ninaE* to "Rh1 (*ninaE*)" to aid accessibility for a broader audience.

(11) Lines 88-90: What exactly were the contradictions that emerged from previous studies? Also see the comment in the public review regarding the discussion of the results.

Thank you for pointing this out. We have clarified this in the revised manuscript. "Previous studies on RH7 have yielded conflicting results regarding its function and expression,

including discrepancies in the identified neuronal populations and associated behavioral phenotypes”

(12) Line 91: Briefly explain the characteristics of white and yellow mutations so the reader can assess why these are relevant here.

We have clarified the role of the white and yellow genes in the genetic background. The revised text now reads:

“Both genes are expressed in the optic system (Davis et al., 2020; Supplementary Material) and are known to affect neuronal function and behavior (Bond et al., 2024; Drapeau et al., 2003; Rickle et al., 2025).”

In addition, we have visualized the expression data for yellow and white from Davis et al. in the Supplementary Material for more clarity.

(13) Lines 100-101: Circadian entrainment is not a typical visual function. Please rephrase.

The sentence has been revised for clarity and now reads:

“As an ancestral rhodopsin, RH7 appears to contribute to basic visual functions, such as contrast processing, as well as to non-visual functions like circadian synchronization, similar to melanopsins in mammals.”

(14) Line 105: The exact address is not necessary, mention BLASTP is enough.

Thank you. We have now removed the exact address and we now simply refer to BLASTP.

(15) Line 108-109: If default settings were used, the details are not needed.

We have omitted the details, as they are not necessary.

(16) Line 113: These settings seem to repeat line 108. Needed?

We thank the reviewer for pointing this out. In the original version, the settings in line 108 referred to the BLAST search, while those in line 113 pertained to the phylogenetic tree, where the parameters are essential, because phylogenetic inference depends on dataset- and method-specific choices. However, this entire section has now been revised, as the phylogenetic tree has been completely rebuilt with a much broader dataset, including mammalian melanopsins and lophotrochoan r-opsins.

(17) Line 122: Without further context, these abbreviations are non-informative and confusing to many readers. Provide details or rephrase. The same applies to DMEM, FBS, and Glo22F in the same paragraph. While many readers will understand instantly, keep the diverse audience of eLife in mind.

We have now added explanations for all abbreviations in the paragraph to improve clarity for a broad audience. Specifically, the Ga subunits (GNAS, GNAL, GNAQ, GNA11, GNA12, GNA13, GNAZ) are now clearly defined, and DMEM, FBS, and Glo22F are also explained as Dulbecco’s Modified Eagle Medium, fetal bovine serum, and a cAMP-sensitive luciferase reporter, respectively. These changes should make the methods clearer and more accessible to all readers.

(18) Line 144: "constant conditions" is not ideal as it could be misinterpreted as DD or LL. Please rephrase.

We agree with the reviewer and have removed the phrase “constant conditions” to avoid potential misinterpretation.

“All recordings were performed under 60% relative humidity and 25 °C...”

(19) Line 148: Stating Lux values for Drosophila is not informative as the unit is based on the spectral sensitivity of the human eye. Ideally, provide a spectrum of the used light source as a supplement.

We thank the reviewer for this suggestion. We agree that lux values alone are not informative for *Drosophila*, and we have now included the spectrum of the light source in Figure 3–figure supplement 1A.

“A standard day was defined as 12 h light (30 $\mu\text{W}/\text{cm}^2 \sim 100$ lux, the spectrum of the light is shown in Figure 3–figure supplement 1A) and 12 h darkness”. In addition, to ensure complete consistency of the experimental conditions, we repeated some earlier experiments using the same light source as in the rest of the study, since the exact light source used in the initial experiments could no longer be verified. This ensures that all behavioral experiments presented in the revised manuscript were performed using the same light source.

(20) Lines 149-153: The startle response protocol is hard to understand when only reading it. It could help to state when (relative to lights-on) the first dark pulse was given and how many dark pulses were given per light phase.

We have clarified the timing and frequency of the dark pulses in the Methods section. We now specify that the first dark pulse was applied 1.5 h after lights-on and that six dark pulses were delivered during each 12 h light phase.

(21) Lines 159ff: The 4-hour startle response assay is not clearly distinguished from the previously mentioned assay (30 min D + 1.5h L). I could help to start with e.g., "In a second assay..." and to clearly state why this additional assay was performed. That is, what information can the 4-hour assay provide that the previous assay could not?

We have revised the text to clearly introduce the 4-hour startle response assay as a second, distinct paradigm (“In a second startle response assay...”).

We also clarified its purpose: in contrast to the initial assay, this paradigm was designed to measure the acute startle response independently of circadian influences. To this end, flies were rendered arrhythmic and exposed to repeated 4-hour light–dark cycles, preventing re-entrainment and isolating the startle response.

In addition, we now refer to a schematic of the light regime in Figure 3–figure supplement 1C.

(22) Lines 161-163: As flies were kept in LL aside from the dark pulses, it could be more intuitive to talk about 72h or three 24h intervals rather than 3 days. Otherwise, one might assume that the initial constant light was followed by 3 days of LD plus dark pulses.

We have revised the text to emphasize the total duration (“72 h”) to avoid confusion with a 24-hour LD regime. In addition, we now provide a schematic of the light regime in the Supplementary Material (Figure 3–figure supplement 1C).

(23) Line 172: Please write out CantonS once, as otherwise it only occurs in Table 1. Also, clarify that y+ and w+ refer to the (absence of the) yellow and white mutation, respectively.

We have revised the text to write out Canton-S (CS) upon first mention and clarified that y^+ and w^+ denote wild-type alleles of the yellow and white genes, respectively, meaning any mutations in these genes are absent.

(24) Table 1: Consider adding "(truncated RHSOPSIN7)" or similar to the "Darkfly" cell.

We have updated Table 1 to indicate that the Darkfly line carries a truncated RH7. The description now reads: "This line has been maintained for decades in constant darkness and carries numerous mutations, including a truncated RH7 lacking the last 21 amino acids."

(25) Table 2: In the formatted paper, Table 2 should appear after section 3.5 so the reader can properly interpret the applications and names.

We have placed Table 2 immediately after section 3.5 as suggested.

(26) Table 3: Consider adding the product IDs of the used antibodies for convenience.

We have added the product IDs of all antibodies used to Table 3 for convenience.

(27) Lines 180ff: This section should initially state the purpose of establishing the rh7-Gal4 line, i.e., the expression of selected gene in rh7-expressing cells. Again, keep your (lee-informed) readers in mind.

We have revised the section to clarify the purpose and design of the Rh7-Gal4 line. It now states that the line was generated to identify Rh7-expressing cells and explains the use of a relatively large promoter region including exon 1 and 2.

(28) Line 190 w¹¹¹⁸ flies do not occur in Table 1. While used by a commercial company here, please still include them for completeness.

We have added the w¹¹¹⁸ line to Table 1 for completeness, as well as the BDSC Stock #8621 line, which was used as the background for the UAS-construct flies.

(29) Lines 192ff and 203ff: As for lines 180ff, start with a brief description of what the purpose of the respective works was.

We have revised the section to start with a brief description of the purpose of the rescue experiments. The text now clearly states that UAS constructs were generated to assess the ability of different Rh7 variants to rescue the phenotype, with UAS-Rh7 as the wild-type rescue, UAS-Rh7^{Darkfly} carrying the Darkfly variant, and UAS-empty serving as a control. We have added an introductory sentence to the optomotor section in Materials & Methods to clarify the purpose of the experiments: "To investigate the contribution of Rh7 in the lamina and medulla to visual motion detection, male flies were individually tested for their optomotor walking behavior." This makes clear why the assay was performed before describing the setup and measurements.

(30) Line 225: Just to be safe, make sure to not mix up Δ -CT and $2\Delta\Delta$ CT (see Livak & Schmittgen 2001, doi: 10.1006/meth.2001.1262).

We confirm that Δ CT was indeed used to calculate relative mRNA levels. To clarify this, we have now added the formula to the text: Δ CT = CT _{α -tubulin} – CT_{Rh7}.

(31) Line 229: Please state the light (cycle) conditions under which the flies were raised, as well as the sampling times at which they were fixed.

We have now specified the light cycle conditions under which the flies were raised (12 h light:12 h dark at 25 °C) in the Methods section. For qPCR experiments, tissues (retina, lamina, and brain) were carefully dissected from adult flies without prior fixation to preserve RNA integrity. Dissections were performed during the light phase and were not restricted to specific time points. These details have been added to the revised manuscript (section 3.10).

(33) Lines 296-297: This brief description could go at the start of section 3.2.

The sentence describing the GsX assay (“To test the functionality of RH7, we used a GsX assay designed to determine which Ga proteins can bind to a given GPCR, in this case RH7 (Ballister et al., 2018)”) has been moved to the Methods section (now 3.3) as a brief introduction to the approach.

| (34) Line 305: Clarify that 2 different retinals were tested.

We clarified that two different retinals were tested. The text now reads: “We observed robust activation with the Gaz chimera, with both 9-cis-retinal and all-trans-retinal (Figure 2C).”

| (35) Figure 2A, B: I suggest using a different color for the RH3,4,5, branch for better distinction from RH7.

We have generated a new tree and adjusted the branch colors to improve clarity between RH3,4,5, branch and RH7.

| (36) See comment above regarding the shifting/flexibility of WTs vs. rh7 mutants.

Following the reviewers and editor's recommendations, we revised the manuscript to focus on the light-dependent effects of RH7 and no longer interpret our data in terms of shifting ability or circadian flexibility. The corresponding statements have been removed or revised accordingly.

| (37) Line 348: Please change "occur" to "be triggered".

The wording has been updated as suggested, replacing “occur” with “be triggered” in the text.

| (38) Line 355: I would mention the double mutants only after the results from the rh7 single mutants were described.

We have adjusted the order of the text accordingly and now introduce the double mutants only after describing the Rh7 single mutants.

| (39) Line 362: See comment on line 159ff.

We have added a sentence at the beginning of the section to clearly introduce the 4-hour startle response assay as a second, distinct paradigm: “In a second startle response assay (4-hour assay), designed to measure the acute response independently of circadian influences...” This clarifies its purpose and distinguishes it from the initial assay.

| (40) Line 368: Write "rh7 mRNA" to be precise.

We have updated the text to use “Rh7 mRNA” for precision, as suggested.

| (41) Figure 5: If possible, please sort the panels in the order in which they are mentioned in the text. For panel E (right), the orientation of the lower schematic, relative to the upper one and the left panel, is unclear. Is it possible to add orientation indicators? Alternatively, could L5 and Mi1 neurons be indicated in the upper schematic instead?

We thank the reviewer's helpful suggestion. Since reorganizing the figure panels was challenging and would have affected the overall layout, we instead revised the corresponding text to ensure that the order of panel descriptions matches the figure arrangement. To address the concern regarding panel E, we have added orientation indicators to the schematic to clarify the relative positioning.

| (42) 419 Add "A)".

We have added an A).

(43) Line 432: Later on (line 598), it is mentioned that the other accumulated mutations in Darkfly may also have affected the observed patterns. Are there any specific mutations worth highlighting in this context aside from rh7?

We thank the reviewer for this comment. In response, we have expanded the Discussion to highlight additional mutations in the Darkfly genome that may contribute to the observed phenotype. Nonsense mutations in HisCl1 and Vha100-1 could affect visual processing and neuronal signaling, potentially altering sensory input and synaptic function. Missense mutations in DopR, bgm, and developmental regulators such as Sox21b may further modulate activity, sleep, and neural development. We have updated the Discussion accordingly to make these points clear.

(44) Lines 435-436: Unchanged mRNA levels do not necessarily equal the presence of the translated protein. Is there another way to properly confirm this? E.g., rh7 HCR in Darkfly?

We agree that unchanged mRNA levels alone do not confirm the presence of translated protein. While *in situ* HCR does not directly measure protein levels, it provides additional information on the cellular localization and presence of Rh7 transcripts beyond qPCR measurements. We have therefore performed Rh7 *in situ* in Darkfly and included this data in Figure 5—figure supplement 1B.

(45) Line 439: "activity" is too vague based on only the altered dark pulse response. I would rather suggest factors like increased protein concentrations/stability, increased sensitivity, constitutive activation, etc.

By "activity," we refer to the molecular activity of RHODOPSIN 7 as reflected in the behavioral response. The Results have been revised to:

"When we exposed the Darkfly to dark pulses in a 12:12 light-dark cycle, they exhibited the opposite phenotype to Rh7⁰ mutants, showing a stronger startle response (Figure 6A), possibly reflecting RHODOPSIN 7 adopting a constitutively active form in the Darkfly strain, driving the enhanced behavioral response."

(46) Lines 442-446: The no-rescue control does not fully resemble WTs as they lack the gradual activity decrease after lights-off. The abrupt decrease (also in the 2 rescues) is more reminiscent of the rh7 mutants. This could be discussed in the context of what was mentioned at the start of this section (Meyerhof et al 2024 paper).

We agree that the rescue lines do not fully restore all aspects of the WT control locomotor pattern. One possible explanation is that the Rh7-Gal4 driver does not fully represent the endogenous Rh7 expression pattern. As described in the Results section, our *in situ* hybridization analysis identified additional Rh7-expressing cells, particularly in the optic system, that were not detected by the Rh7-Gal4 line (results, section 4.5). We have added this point to the Discussion to address how the broader Rh7 expression pattern may contribute to the observed behavioral effects (discussion 5.4).

(47) Line 472: "restored the reduced startle response" reads a bit convoluted. Better write e.g., "restored the enhanced startle response that was absent in the no-rescue mutants".

We have revised the text as suggested to clarify the phrasing.

(48) Lines 533-535: Please provide a reference for the statements about L5 and Mi1 functional roles. Also, this is the only part where there is some connection made between rh7 (expressed in Mi1) and contrast processing (which involves Mi1). As this is a rather weak connection, please consider the last paragraph of the public review section.

We have added references to support the functional roles of L5 and Mi1 neurons. L5 neurons relay signals from outer photoreceptors to the medulla and show strong contrast adaptation (Matulis et al., 2020). Mi1 neurons contribute to motion and contrast processing (Behnia et al., 2014; Matulis et al., 2020). To address the connection between Rh7 expression and contrast processing, we have carefully phrased the text to indicate a potential role of Rh7 in modulating contrast-related signals in Mi1, while noting that the functional link remains to be directly tested. We believe this wording, together with the added references, provides proper support for the statements and maintains a cautious interpretation.

If contrast perception shall indeed be investigated, this may be done by performing the optomotor assay with gray stripes of different contrast, with stripes where the edges are not sharp but show a gradual change from black to white.

We agree that contrast perception can be assessed in more detail using stimuli with defined contrast levels and gradual luminance transitions. However, our aim was not to directly measure contrast sensitivity, but rather to investigate behavioral responses to changes in luminance. We have clarified this distinction in the revised manuscript and now specify that the optomotor response reflects processing of spatial and temporal changes in luminance, while the light-off startle response measures responses to abrupt changes in luminance.”

(49) Lines 546-547: Are there references on how activity or behavior in general can affect external light cues?

We have added appropriate references to support this statement. In particular, we now cite Rieger et al. (2003), which demonstrates direct effects of light on locomotor activity (masking), and Helfrich-Förster (2017), which provides a comprehensive overview of circadian regulation and light-dependent behavioral modulation. These additions clarify how external light cues can directly influence behavior in addition to circadian clock control.

(50) Lines 557-560: See comment above regarding the shifting/flexibility of WTs vs. rh7 mutants.

We thank the reviewer for this comment. We agree that assessing shifting and behavioral flexibility would require dedicated experiments, such as jet lag or entrainment assays. We have therefore revised the focus of the manuscript and now primarily discuss RH7 function in light-dependent behavioral responses rather than broader flexibility of the circadian system. Future studies will further explore the potential contribution of RH7 to behavioral adaptation and flexibility using dedicated experimental approaches.

(47) Lines 564-567: How many species possess dcry but not rh7? This is an important distinction, as otherwise one could assume that both must always co-occur. Alone, this provides only limited support for a potential functional interplay. The findings by Ni et al. (2017) regarding dcry and rh7 in entrainment could, however, help strengthen this point.

We have clarified the evolutionary relationship between DCRY and RH7 in the revised manuscript. We now specify that, although DCRY is widespread across Bilateral and many non-panarthropod species possess DCRY without RH7, RH7 and DCRY show a strong evolutionary association within arthropods, with approximately 98% of DCRY-retaining arthropod species also retaining RH7. At the same time, the presence of RH7 in some lineages lacking DCRY suggests that RH7 may retain functional relevance in the absence of DCRY. Together with previous findings on the functional interaction between RH7 and DCRY in light adaptation (Ni et al., 2017), these evolutionary patterns support a conserved connection between the two light-sensing systems, while the exact nature of this relationship remains to be further investigated. These points are now shown and discussed in section 4.1 and 5.5.

(48) Lines 579-580: Here, one could explicitly mention that this is supported by the lack of diel startle response modulation in the *per* mutants (Figure 4A).

We have modified the text to explicitly highlight this point. The sentence now reads: “Dark-pulse experiments show that both wild-type controls and *per*⁰ mutants, which lack a functional circadian clock, exhibit a strong startle response, consistent with the absence of diel modulation of the response in *per*⁰ mutants (Figure 4A).”

(49) Lines 596-597: The increased night activity in *Darkfly* seems largely limited to the first half of the night (compare e.g. Figure 6A and Figure 3A). Again, the loss/alteration of *rh7* seems to have its major effect after a transition from light to dark (see lines 442-446, start of the section).

We thank the reviewer for pointing out this issue. The wording in the previous version could have been misleading. By “dark phase,” we meant the 30-minute periods without lighting during the dark-stimulus experiments, and not the “night phase.” We have revised the text to clearly refer to the startle response.

We also clarify that the increased locomotor activity observed in the “*Darkfly*” strain outside of the startle response is likely caused by additional background mutations that accumulated during long-term rearing in constant darkness, and not exclusively by the *RH7* deletion.

(50) Line 622: See comments regarding contrast and motion perception. Do the N- and C-terminal regions of *RH7* and melanopsin share similarities in sequence or additional characteristics aside from phosphorylation sites? This could support a conserved role.

We thank the reviewer for this suggestion, which prompted us to perform a more detailed analysis of conserved features between *RH7* and melanopsins. In addition to the extended and potentially highly phosphorylatable N- and C-terminal regions, we identified several conserved sequence motifs, including two in the C-terminal region and others within the transmembrane core. One of these motifs extends into the conserved DRY motif in the second intracellular loop (ICL2). These findings are now described in the Discussion (section 5.7) to highlight potential structural similarities beyond phosphorylation sites. The corresponding sequence alignments are provided in the supplementary material.

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