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RESEARCH ARTICLE

Opsin1-2, $G_q\alpha$ and arrestin levels at *Limulus* rhabdoms are controlled by diurnal light and a circadian clock

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SUMMARY

Dark and light adaptation in photoreceptors involve multiple processes including those that change protein concentrations at photosensitive membranes. Light- and dark-adaptive changes in protein levels at rhabdoms have been described in detail in white-eyed *Drosophila* maintained under artificial light. Here we tested whether protein levels at rhabdoms change significantly in the highly pigmented lateral eyes of wild-caught *Limulus polyphemus* maintained in natural diurnal illumination and whether these changes are under circadian control. We found that rhabdomeral levels of opsins (Ops1-2), the G protein activated by rhodopsin ($G_q\alpha$) and arrestin change significantly from day to night and that nighttime levels of each protein at rhabdoms are significantly influenced by signals from the animal's central circadian clock. Clock input at night increases Ops1-2 and $G_q\alpha$ and decreases arrestin levels at rhabdoms. Clock input is also required for a rapid decrease in rhabdomeral Ops1-2 beginning at sunrise. We found further that dark adaptation during the day and the night are not equivalent. During daytime dark adaptation, when clock input is silent, the increase of Ops1-2 at rhabdoms is small and $G_q\alpha$ levels do not increase. However, increases in Ops1-2 and $G_q\alpha$ at rhabdoms are enhanced during daytime dark adaptation by treatments that elevate cAMP in photoreceptors, suggesting that the clock influences dark-adaptive increases in Ops1-2 and $G_q\alpha$ at *Limulus* rhabdoms by activating cAMP-dependent processes. The circadian regulation of Ops1-2 and $G_q\alpha$ levels at rhabdoms probably has a dual role: to increase retinal sensitivity at night and to protect photoreceptors from light damage during the day.

Key words: *Limulus*, photoreceptors, dark adaptation, light adaptation, circadian rhythm, $G_q\alpha$ translocation, arrestin translocation, opsin expression.

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INTRODUCTION

Dark and light adaptation are processes that change the sensitivity of photoreceptors to light and permit them to function optimally over a broad range of illumination. Multiple mechanisms are thought to contribute. Rapid changes in sensitivity, those that occur in seconds to minutes, are largely driven by changes in intracellular free Ca^{++}. Slower processes, those with a time scale of many minutes to hours, include the translocation of proteins involved in the photoresponse into and out of the photosensitive compartment of photoreceptors. For example, in vertebrate rod photoreceptors and the rhabdomeral photoreceptors of *Drosophila*, light triggers an increase in the concentration of arrestin at photosensitive membranes – arrestin is the protein that quenches the photoresponse – and a decrease in the concentration of the G protein activated by rhodopsin. In the dark, the opposite occurs: the level of arrestin in the photosensitive compartment falls while the level of the G protein increases (reviewed in Frechter and Minke, 2006; Arshavsky and Burns, 2012). Protein translocations in rhabdomeral photoreceptors have been studied in detail in white-eyed *Drosophila* mutants maintained in artificial light. However, it is not known whether dark- and light-adaptive protein translocations are significant in wild-type animals exposed to natural illumination, and mechanisms underlying dark- and light-adaptive protein translocations are not fully understood in any system.

Photoreceptors also undergo more sustained, daily changes in structure and function that are predicted to impact their sensitivity. These are regulated by signals from circadian clocks. Specific effects of circadian signals on vertebrate rods and cones differ with the species but include outer segment membrane shedding (LaVail, 1976), cone retinomotor movements (Levinson and Burnside, 1981), the expression and modulation of photoreceptor ion channels (Ko et al., 2001; Ko et al., 2007) and opsin gene expression (Korenbrot and Fernald, 1989; Pierce et al., 1993; Li et al., 2005).

Effects of circadian signals on rhabdomeral photoreceptors are perhaps best understood from studies of the lateral compound eyes (LEs) of the horseshoe crab *Limulus polyphemus* (Linneaus 1758), a chelicerate arthropod. The sensitivity of *Limulus* LEs increases dramatically at night (Barlow et al., 1977; Barlow, 1983), and behavioral studies indicate that these animals see at night with their LEs nearly as well as during the day (Powers et al., 1991). Half of this increase in sensitivity is driven by signals from a central circadian clock (Pieprzyk et al., 2003). At night, the central clock activates a bilateral population of neurons that project from the chelicerial ganglia in the brain to all of the eyes – LEs, median ocelli and rudimentary eyes – through their optic nerves (Fahrenbach, 1973; Fahrenbach, 1981; Barlow et al., 1977; Barlow et al., 1980; Calman and Battelle, 1991; Kass and Barlow, 1992).

These clock-driven efferent neurons innervate all cell types in LEs (Fahrenbach, 1975; Fahrenbach, 1981), and release the biogenic amine octopamine (OA) from their axon terminals (Batelle et al., 1982; Battelle and Evans, 1984; Evans et al., 1983). OA stimulates a rise in cAMP in *Limulus* photoreceptors (Kaupp et al., 1982) and LE slices (Dalal and Battelle, 2010), and many of the known effects of clock input to LEs are mimicked by OA and other treatments that activate the cAMP cascade (reviewed in Battelle, 2002; Dalal and Battelle, 2010).

The known effects of clock input on LEs are diverse. Among the most dramatic are structural changes that increase photon catch. These changes are primarily responsible for large diurnal changes in the amplitude of electroretinograms recorded from LEs (Barlow et al., 1980; Chamberlain and Barlow, 1987; Kass et al., 1988). They are driven by the circadian clock and amplified by diurnal light (Chamberlain and Barlow, 1987).

The clock also influences the photoresponse. Intracellular electrical recordings from photoreceptors in LEs maintained in constant darkness showed that clock input decreases photoreceptor noise and increases the response per photon (gain) (Kaplan and Barlow, 1980; Barlow et al., 1987). Clock input also increases the duration of the elemental photoresponse or quantum bump (Kaplan et al., 1990) and regulates photoreceptor gene expression (Battelle et al., 2000b). Finally, clock input at night is required to prime a process called transient rhabdom shedding (TRS). TRS is triggered by the dim light of dawn and is characterized by a rapid, synchronous shedding of opsin-containing rhabdomeral membranes. TRS does not occur if clock input to the LE is blocked (Chamberlain and Barlow, 1979; Chamberlain and Barlow, 1984; Sacunas et al., 2002). A separate process of rhabdom shedding called light-driven shedding (LDS) is independent of clock input and becomes apparent as light intensity increases during the day (Sacunas et al., 2002).

The goals of the present study were to characterize changes in rhabdomeral levels of proteins critical for the photoresponse in wild-caught *Limulus* maintained under natural diurnal illumination and to test whether clock input influences these changes. In a previous study we showed that rhabdomeral levels of *Limulus* opsin 1-2 (RhOps1-2), the most abundant opsins in LE photoreceptors (retinular cells), changed dramatically from day to night and that the normal nighttime increase in RhOps1-2 required clock input (Katti et al., 2010). These results are confirmed and extended in the present study, in which we also examined diurnal and circadian changes in rhabdomeral levels of arrestin (RhArr) and $G_q\alpha$ ($RhG_q\alpha$), the alpha subunit of the G protein activated by rhabdomeral opsins. We show that RhOps1-2, $RhG_q\alpha$ and RhArr levels change significantly from day to night in natural illumination and that the levels of each change with a unique time course during dawn and dusk. We show further that RhOps1-2, $RhG_q\alpha$ and RhArr levels are influenced by the clock, and that the effects of the clock on RhOps1-2 and $RhG_q\alpha$ levels can be mimicked during the day with treatments that elevate cAMP in photoreceptors.

MATERIALS AND METHODS

Animals

Adult *Limulus* were collected from the Indian River near Melbourne, FL, USA, and maintained at The Whitney Laboratory in aquaria supplied with natural, continuously flowing seawater. Seawater depth was approximately 60 cm; its temperature was held between 18 and 20°C. Both males and females were used. Unless otherwise specified, animals were held in an aquarium room in which they were exposed to natural illumination provided through a skylight but not to direct sunlight. Light levels near the surface of the water in the holding tank were monitored continuously using a HOBO light data logger (Onset Computer Corporation, Pocasset, MA, USA). An example of these light levels during a typical day in August is shown in Fig. 1.

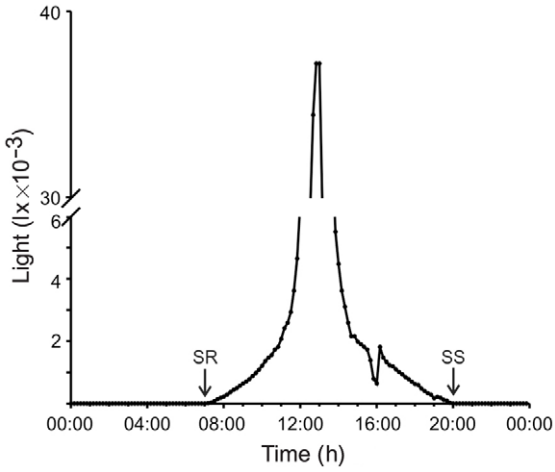

Fig. 1. Intensity of illumination over the *Limulus polyphemus* aquarium during a typical day in August (19 August 2011) measured with a HOBO light data logger and expressed in lux. Time is on a 24 h scale. Times of sunrise (SR) and sunset (SS) are indicated.

Reagents

Reagents were from Sigma-Aldrich (St Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA) unless otherwise specified.

Antibodies

All primary antibodies used in this study have been characterized for their specificity in *Limulus*. Anti-*Limulus* Ops1-2, a rabbit polyclonal antibody, was generated against the C terminus of *Limulus* Ops1 (Battelle et al., 2001). It cross-reacts with *Limulus* Ops2 and thus the immunoreactivity (ir) observed with this antibody is referred to here as Ops1-2-ir. Anti-*Limulus* Ops5, a mouse monoclonal antibody (2-449), was generated against the C terminus of *Limulus* Ops5 and is specific for this opsin (Katti et al., 2010). Anti-*Limulus* Arr, a mouse monoclonal antibody (4D10), was generated against the N terminus of Arr (Battelle et al., 2000a). The specificity of rabbit polyclonal anti-$G_{q/11}\alpha$ (C-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for *Limulus* $G_q\alpha$ was confirmed by Munger et al. (Munger et al., 1996). Anti-actin, a monoclonal antibody (AC-15), was purchased from Sigma-Aldrich. This antibody labels a single protein band on western blots of LE homogenates with an apparent molecular mass of 45 kDa, which is close to the molecular mass of actin (data not shown). Fluorescently labeled (Alexa Fluor) secondary antibodies were from Invitrogen (Carlsbad, CA, USA).

Tissue fixation and immunostaining

Tissues were fixed in ice-cold methanolic/formaldehyde, rehydrated in a graded series of methanol in water, and then incubated overnight at 4°C in 30% sucrose in 0.1 mol l^{-1} sodium phosphate buffer, pH 7.2. Light-adapted eyes were dissected and fixed in the light; dark-adapted eyes were dissected under infrared or dim red light and fixed in the dark. Frozen sections of LEs were prepared and immunostained as described previously (Battelle et al., 2001).

Image collection and analysis

These procedures were described in detail elsewhere (Katti et al., 2010). Briefly, ommatidia in immunostained cross-sections of LEs (Fig. 2) were viewed with a confocal microscope (Leica SP2 or SP5; Leica Microsystems, Mannheim, Germany). Typically, sections were double-labeled for Ops1-2 and Ops5 and separately for Arr and $G_q\alpha$. Double-labeled fluorescent images were acquired with sequential scans. Samples to be compared were immunostained at the same time using identical conditions and analyzed during a single confocal session using identical confocal settings.

The average intensity of RhOps1-2-, RhOps5-, RhArr- or Rh$G_q\alpha$-ir per μm^2 of rhabdom was determined using either ImageJ (National Institutes of Health, Bethesda, MD, USA) or Leica software. Rhabdom perimeters were outlined with a polygon tool to create one region of interest (ROI1; Fig. 2C). The area in the center of the rhabdom occupied by the dendrite of the eccentric cell was also outlined to create a second region of interest (ROI2). To determine mean intensities of immunoreactivity per μm^2 of rhabdom, the total intensity in ROI1 minus the total intensity in ROI2 was divided by the total area of ROI1 minus ROI2. By determining immunoreactivity per unit area of rhabdom, we control for differences in rhabdom area observed among ommatidia, including changes in rhabdom shape that occur with time of day. Rhabdoms in eight separate ommatidia were analyzed in each eye to determine the mean intensity per μm^2 at rhabdoms for that LE. For each experimental condition, LEs from a number of different animals were assayed. This number is presented in the descriptions of individual experiments.

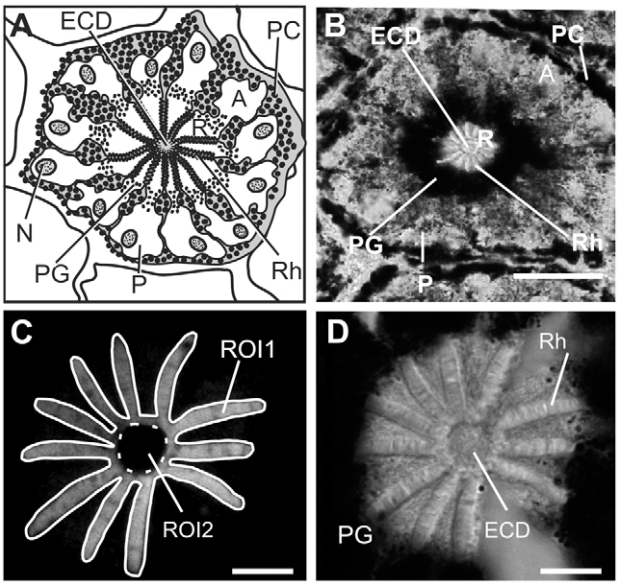

Fig. 2. Structure of *Limulus* lateral compound eye (LE) ommatidia seen in cross-section. (A) Schematic of a cross-section through one LE ommatidium with 12 photoreceptor cell bodies (P). The following are labeled: A, arhabdomeral segment; ECD, eccentric cell dendrite; N, nucleus; PC, pigment cell; PG, pigment granules in photoreceptors; R, rhabdomeral segment; Rh, rhabdom. (B) Low-power brightfield image of a cross-section of an ommatidium from a LE fixed during the day. Pigment granules are distributed throughout the cytoplasm of the A segment and concentrated at the junction between the R and A segments. Scale bar, 50 µm. (C) Confocal image of $G_q\alpha$-immunoreactivity in the R segment and proximal A segment of an ommatidium for a LE fixed during the day. Shown are the regions of interest (ROIs) drawn to quantify average immunoreactive intensities over rhabdoms. Total intensity of ROI1 minus ROI2 was divided by total area of ROI1 minus ROI2 to calculate the average intensity over rhabdoms per μm^2. Scale bar, 10 µm. (D) Brightfield image of the section shown in C showing the rays of the rhabdom in the R segment surrounded by a dense accumulation of PGs at the junction between the R and A segments of photoreceptors. Scale bar, 10 µm.

Additional approaches used in non-pigmented photoreceptors to measure changes in the distributions of the soluble proteins $G_q\alpha$ and arrestin between membrane and cytoplasmic compartments – by comparing cytoplasmic *versus* rhabdomeral levels of the proteins using immunocytochemistry and quantifying membrane-bound *versus* soluble protein levels on western blots – proved unreliable in studies of LE retinular cells. Pigment granules in retinular cells outside of the rhabdomeral segment (Fig. 2B,D) change their distribution from day to night (Kier and Chamberlain, 1990), and vary in density among animals. Because pigment granules absorb immunofluorescence, measurements of cytoplasmic immunofluorescence in these cells are unreliable. Quantification of soluble *versus* membrane-bound $G_q\alpha$ and arrestin in LEs at different times of the day were also confounded by the presence of $G_q\alpha$ and arrestin in photoreceptor axons (Munger et al., 1996; Battelle et al., 2001), which make up a significant but variable fraction of LE tissue volume. Furthermore, much extra-rhabdomeral arrestin remains associated with multivesicular body membranes that are prominent in the cytoplasm throughout the day (Fig. 3) (Sacunas et al., 2002), and it has not been possible to purify LE rhabdomeral membranes from extra-rhabdomeral membranes using biochemical approaches.

Effects of diurnal light and a circadian clock on protein levels at LE rhabdoms

1 – Time of day

LEs were dissected from animals at the time of day indicated in each experiment. Midday LEs were dissected 30 min after peak illumination in the aquarium room, typically about 13:30 h. Nighttime LEs were dissected under infrared or dim red light between 4 and 6 h after sunset (SS+4–6 h). Other times examined are expressed relative to sunrise (SR) or sunset (SS). At SR and SS it was light enough in our aquarium room to work without artificial light, but at 1 h before SR and 30 min after SS the aquarium room was dark. Results of three separate series of studies are presented, each involving a separate group of animals: (1) an initial series, which compared LEs fixed during the day, during deep night (SS+4–6 h) and at SR; (2) a dusk series; and (3) a dawn series.

2 – Neuronal clock input

Because signals from a central circadian clock reach *Limulus* eyes *via* an efferent neuronal projection through the optic nerves, clock input to LEs can be eliminated by cutting the optic nerve. Therefore, to examine effects of neuronal clock input on protein levels in LE rhabdoms, one lateral optic nerve of a group of animals was cut as described previously (Battelle et al., 2000b) while the other was left intact. All surgery was performed in the morning at least 3 h after SR. Animals were allowed to recover for 1 week after surgery before LEs were dissected and fixed. In one set of experiments, LEs were exposed to natural illumination during the week after surgery. In a second set, both LEs were patched with blackened beeswax (Battelle et al., 2000b) immediately after the optic nerve to one LE was cut, and were thereby maintained in the dark during the week following surgery. In both sets of experiments, the two LEs of an animal were dissected at the same time of day. Light-adapted eyes were dissected and fixed in the light; dark-adapted eyes were dissected under infrared or dim red light and fixed in the dark.

3 – Dark adaptation during the day

One LE of a group of animals was removed at midday in the light and fixed immediately in the light. The wound was covered with gauze and bandaged to reduce bleeding. The other LE was quickly patched with black electrical tape and duct tape to block light input, and the animal was returned to a tank in the naturally illuminated aquarium room. Four hours later, when it was still light in the aquarium room, animals were taken to an experimental darkroom where the patched eye was dissected under infrared or dim red light and fixed in the dark.

For the experiments described in 2 and 3 above, it is important to note that *Limulus* maintain their normal daily rhythms in the absence of light input to LEs. This is because the central circadian clock that influences the eyes can be entrained by light input to median and ventral eyes and to photosensitive cells in the tail (Hanna et al., 1988; Horne and Renninger, 1988). In the experiments described in 2 and 3 above, median, ventral and tail photoreceptors were exposed to natural, diurnal illumination.

4 – *In vitro* incubations with OA and forskolin

As was described in the Introduction, many effects of the clock on LEs are mimicked by OA and treatments that activate the cAMP cascade. We therefore tested whether effects of the clock on dark-adaptive changes in rhabdomeral protein levels could be mimicked by incubating LE slices *in vitro* with OA or forskolin, a drug that directly activates adenylyl cyclase (Seamon et al., 1981). LEs from animals maintained under natural illumination were dissected at midday, and each eye was cut in half to yield four slices from each animal. One slice was fixed immediately in the light. The other three were preincubated in organ culture medium (OCM) (Katti et al., 2010) for 10 min in the light, and then incubated for 4 h in the dark at room temperature under one of three conditions: (1) OCM; (2) OCM plus OA (40 μm) and the phosphodiesterase inhibitor isobutylmethyl xanthine (IBMX) (1 mmol l^{-1}) (Beavo et al., 1970); and (3) OCM plus forskolin (10 μm) and IBMX (1 mmol l^{-1}). All incubation media contained 0.08% DMSO, the solvent for IBMX and forskolin. At the end of the 4 h incubation period, LE slices were fixed in the dark. RhOps1-2, RhOps5, $RhG_q\alpha$ and RhArr levels in all slices incubated in the dark were compared with those in the light-adapted control slice from the same animal.

RESULTS

RhOps1-2, $RhG_q\alpha$ and RhArr levels in retinular cells change during natural dusk and dawn

Figs 3 and 4 show that $RhG_q\alpha$ levels increase and RhArr levels decrease significantly from day to night. For completeness, these findings are plotted together with previous results (Katti et al., 2010) showing that RhOps1-2 levels roughly double from day to night while RhOps5 levels do not change significantly. The rays of the rhabdom clearly change shape from day to night. When viewed in cross-section, they are typically broader at night than during the day. But because total intensities over rhabdoms are normalized to rhabdom area (see Materials and methods) (Fig. 2), structural differences among rhabdoms are controlled for, and the data reflect changes in the concentration of each protein at the rhabdoms. Figs 3 and 4 reveal that in heavily pigmented compound eyes of a wild population of animals exposed to natural diurnal light, rhabdomeral concentrations of Ops1-2, $G_q\alpha$ and Arr, three proteins critical to the photoresponse, change significantly with a diurnal rhythm.

A major goal of this study was to determine whether diurnal changes in RhOps1-2, $RhG_q\alpha$ or RhArr levels are influenced by the circadian clock. Therefore, we first needed to characterize the dynamics of normal diurnal changes in rhabdomeral levels of these proteins and estimate when in our animals the clock begins to influence retinular cells during dusk and when light begins to exert its influence during dawn. All previous investigations of the dynamics of the effects of clock input and light on retinular cells used animals captured off the coast of New England and maintained under natural illumination at Woods Hole, MA, or Syracuse, NY. We used animals from a different breeding population (King et al., 2005), and the dynamics of light in Florida are much different from those in Massachusetts and New York. To estimate when in our

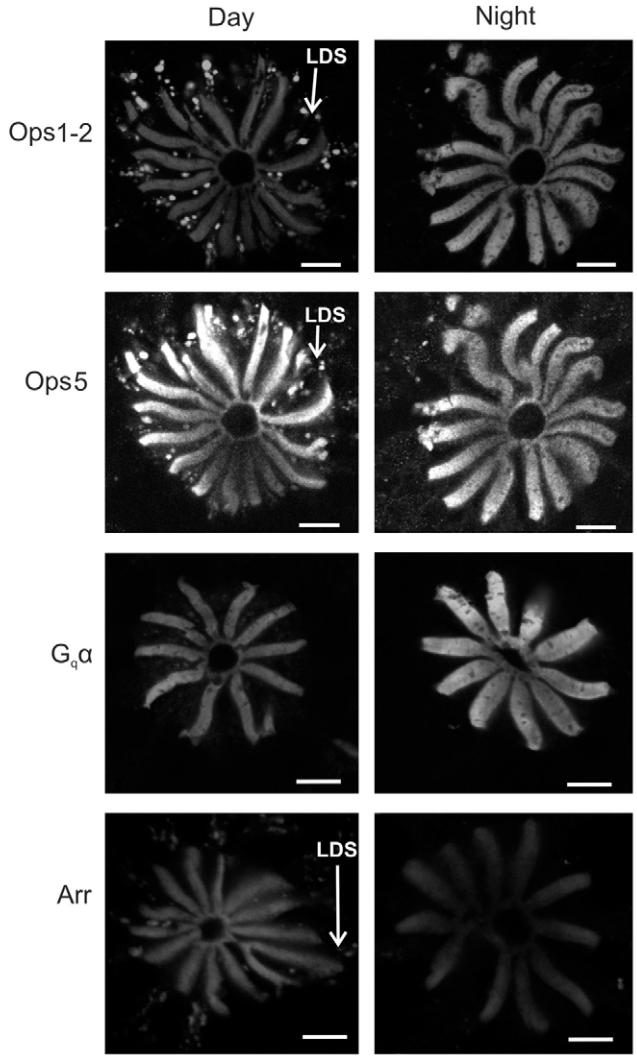

Fig. 3. Immunoreactive intensities of Ops1-2, $G_q\alpha$ and Arr over rhabdomeres changed significantly from day to night while that of Ops5 did not. Representative images of single confocal optical sections showing Ops1-2-, Ops5-, $G_q\alpha$- and Arr-ir in the R segment and proximal A segment of LEs (Fig. 2) fixed midday, ~30 min after peak illumination, and during the night, between 4 and 6 h after sunset. Images show the extremes of day-to-night structural changes in rhabdoms as viewed in cross-section. Daytime and nighttime images of each antigen were taken from frozen sections that were immunostained at the same time and imaged during a single confocal session with identical confocal settings. Day and night images of each antigen were intensified in Photoshop to exactly the same extent and then assembled in CorelDraw. LDS, immunoreactive membranes shed from rhabdomeres during the day by light-driven shedding, a clathrin-mediated endocytosis involving Arr as an adaptor protein (Sacunas et al., 2002). Scale bars, 10 μm.

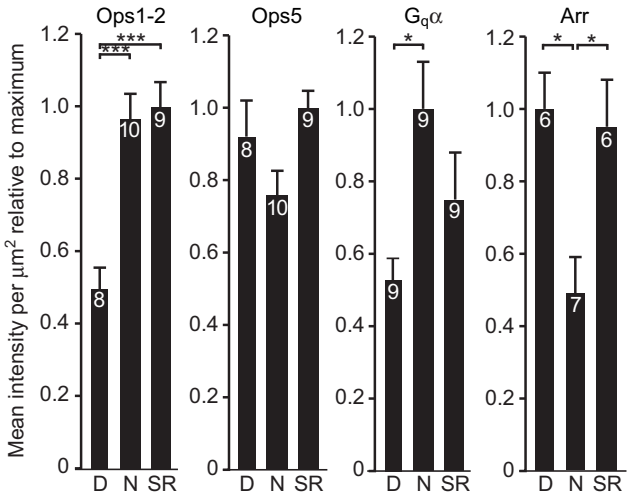

Fig. 4. RhOps1-2, RhG$_q\alpha$ and RhArr levels change significantly day to night while levels of RhOps5 levels do not. Quantification of average Ops1-2-, Ops5-, G$_q\alpha$- and Arr-ir per µm^2 of rhabdoms in LEs from animals maintained in natural illumination. Eyes were fixed during the day (D) between 7 and 10 h after sunrise, during the night (N) between 4 and 6 h after sunset, or at sunrise (SR). The mean immunoreactivity per µm^2 of rhabdom in individual ommatidia was determined as described in the Materials and methods and in the legend to Fig. 2. Data from eight different ommatidia in an eye were averaged to determine the mean intensity for that eye. Data for each antigen were pooled from three to four confocal sessions each involving eyes from two to four different animals at each time point; the numbers in each bar show the total number of animals assayed. Data are expressed as mean (±s.e.m.) immunoreactive intensities per µm^2 of rhabdom relative to the mean maximum immunoreactive intensity per µm^2. The significance of differences among time points was tested with a one-way ANOVA followed by the Student–Newman–Keuls test (***P<0.001, *P<0.05). Data for Ops1-2 and Ops5 are adapted from Katti et al. (Katti et al., 2010).

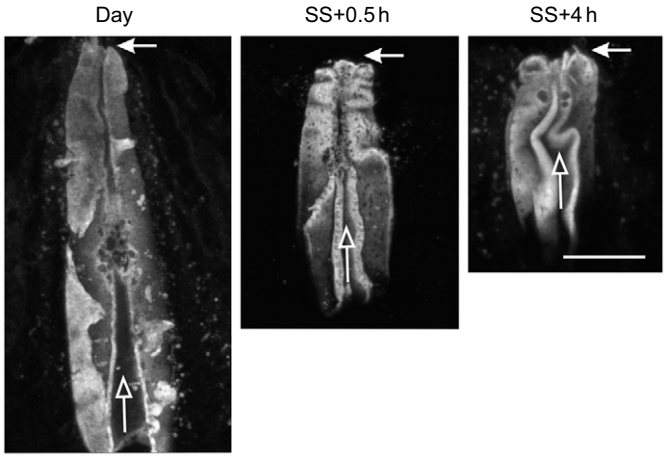

Fig. 5. Structural changes in rhabdoms begin before dark. Confocal images of longitudinal sections of LE rhabdoms immunostained for Ops5. LEs were fixed approximately 30 min after peak illumination during the day (Day), 30 min after sunset (SS+0.5 h) and during deep night (SS+4 h). Clock-driven changes in rhabdom structure are already well underway at the onset of darkness and thus must begin before dark. Images were collected in 0.5 µm steps. Maximum projections of between 10 and 15 sections are shown. Images were intensified in Photoshop and assembled in CorelDraw. Solid arrows, location of the aperture at the base of the lens; open arrows, location of the eccentric cell dendrite. Scale bar, 25 µm.

animals the clock exerts its influence on retinular cells during dusk, we examined structural changes in rhabdoms during dusk that are largely driven by the circadian clock (Chamberlain and Barlow, 1987). During dawn, we looked for structural changes associated with TRS, which is primed by the clock and triggered by dawn.

Structural changes

Dusk

Differences in rhabdom structure from day to night can be seen in ommatidia viewed in cross-section (Fig. 3). However, the timing of structural changes in rhabdoms during dusk is best observed in longitudinal sections. Fig. 5 shows semi-longitudinal sections of LE ommatidia fixed at midday, at the onset of darkness (SS+0.5 h) and during deep night (SS+4 h). Ops5-ir was selected as a marker of rhabdoms for this series because its intensity at rhabdoms does not change significantly with time of day. At midday, rhabdomeres were typically long and straight and their distal ends were narrow. During deep night, rhabdomeres were greatly shortened, and their distal ends were highly folded and wider than during the day. This day-to-night change is identical to that described previously for animals from Woods Hole, MA (Chamberlain and Barlow, 1987). In LEs of Florida animals fixed at the onset of darkness (SS+0.5 h), rhabdomeres had already changed substantially toward the nighttime state – their distal ends were clearly more folded and wider than during the day – but the transition to the nighttime state was incomplete.

Dawn

During TRS, microvilli in the rhabdomeral rays rapidly and transiently become disorganized and opsin-containing membrane is shed (Chamberlain and Barlow, 1979; Chamberlain and Barlow, 1984; Sacunas et al., 2002). To determine when TRS occurs in our animals, we double-labeled cross-sections of LEs fixed 1 h before SR (SR–1 h), at SR and 1 h after SR (SR+1 h) for Ops1-2-ir and actin-ir (Fig. 6). At SR–1 h, rhabdom structure was indistinguishable from that in LEs fixed during the night. The rays of the rhabdom were broad, RhOps1-2-ir was bright, no Ops1-2 immunoreactive debris was detected between rays and actin-ir was concentrated at the base of microvilli. At SR, the rays looked dramatically different. They were long and narrow and stained uniformly for actin. Actin-ir over the rays was also less intense at SR. This suggests that actin protein in rhabdomeral microvilli is partially degraded during TRS. However, actin-ir over the central ring of microvilli remained bright. This is consistent with electron microscopic observations showing that during TRS, microvilli of the central collar are largely spared from the degree of disorganization observed in the rays (Chamberlain and Barlow, 1984). Some Ops1-2-ir debris was detected between the rays at SR, indicating opsin-containing membrane was being shed. At SR+1 h, the structure of the rays and the distribution of actin-ir were similar to that seen at SR; however, Ops1-2-ir over rhabdoms was dramatically lower and a large amount of Ops1-2-ir was concentrated in debris between the rays.

From Figs 5 and 6 we conclude that the timing of structural changes in the retinular cells of Florida and Woods Hole animals during natural dusk and dawn are nearly identical when both sets of data are expressed relative to SS and SR. In both populations, structural changes must be underway before dark, indicating clock input to LEs of both groups begins before dark. In Woods Hole animals, clock input is estimated to begin approximately 45 min before SS (Pieprzyk et al., 2003). During dawn, TRS in both populations begins at approximately SR.

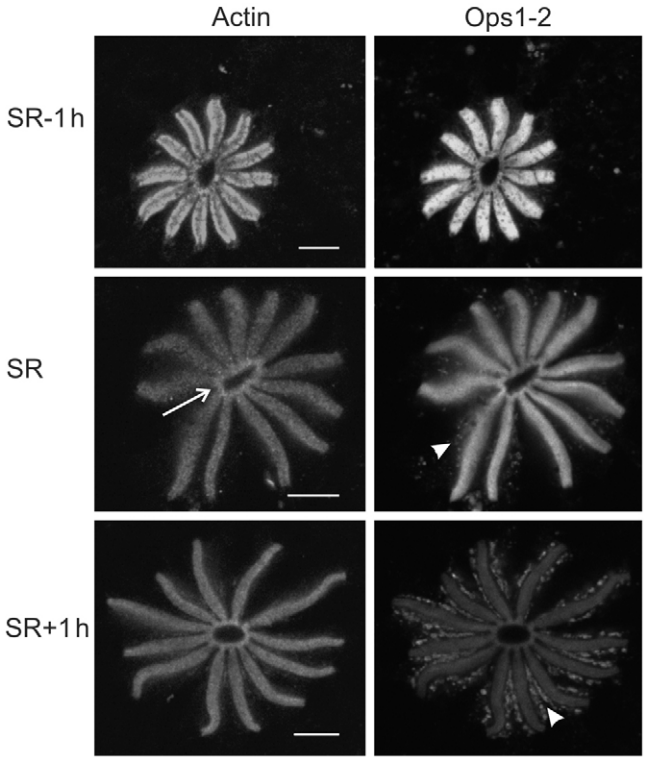

Fig. 6. Rhabdom structure and actin- and Ops1-2-ir at rhabdoms change during dawn. Confocal images of cross-sections of LE rhabdoms double-labeled for actin-ir and Ops1-2-ir. LEs were fixed at SR-1 h, SR and SR+1 h. Sections were immunostained at the same time and images were collected in a single confocal session using identical settings. Single optical sections are shown. Images were intensified in Photoshop and assembled in CorelDraw. Images of actin-ir were intensified together and images of Ops1-2-ir were intensified together. Arrow indicates the central collar of the rhabdom. Arrowheads indicate Ops-1-2-containing debris shed by transient rhabdom shedding. Scale bars, 10 μm.

Protein changes

Dusk

RhOps1-2, $RhG_q\alpha$ and RhArr levels were measured at several different times: midday, when clock input is silent; SS–1 h, just before clock input is estimated to begin; SS+0.5 h, the onset of darkness when structural changes in rhabdoms are clearly underway (Fig. 5); SS+1.5 h, early night; and SS+4 h, deep night (Fig. 7, Dusk).

RhOps1-2 levels varied considerably among animals during the afternoon and dusk. Nevertheless, average RhOps1-2 levels increased before SS as light levels fell and continued to increase gradually after dark. There was no sharp change in RhOps1-2 levels following the estimated onset of clock input. But by SS+1.5 h, levels were significantly higher than at midday and by SS+4 h they were at their maximum. RhOps5 levels, which were quantified in the same rhabdoms as Ops1-2, did not change significantly during dusk; however, $RhG_q\alpha$ levels increased with a time course similar to that of RhOps1-2. $RhG_q\alpha$ levels began to increase before SS, by SS+1.5 h they were significantly higher than at midday, and they continued to increase significantly to a maximum measured at SS+4 h. As with RhOps1-2, no sharp change in $RhG_q\alpha$ was observed following the estimated onset of clock input. By contrast, RhArr levels changed rapidly during dusk. At SS–1 h, when light levels in our aquarium room were already quite low (Fig. 1), RhArr was only slightly below its midday level. However, RhArr levels fell sharply between SS–1 h

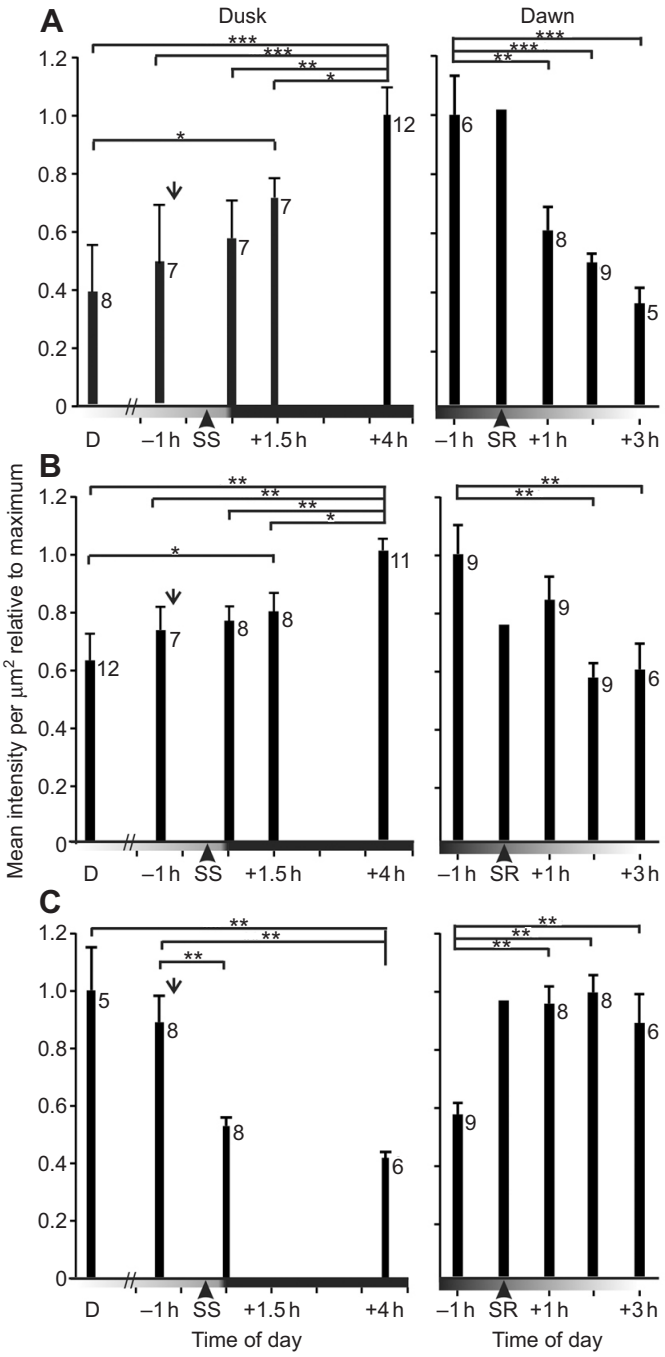

Fig. 7. RhOps1-2, $RhG_q\alpha$ and RhArr levels change with different time courses during dawn and dusk. The dusk and dawn series were assayed separately. Each contains data pooled from three separate confocal sessions; each confocal session included immunostained sections of LEs from two to four animals fixed at each of the different time points. Sections were imaged as described in the Materials and methods and in the legend to Fig. 2. Data are plotted as described in the legend to Fig. 4. On the abscissa, time is expressed as hours before or after sunset (SS) or sunrise (SR). The bar provides a rough indication of the change in light intensity during dusk and dawn. The dusk series includes a midday (D) time point. The downward arrows indicate the estimated time when circadian clock input to LEs begins (Fig. 5). The number of animals assayed at each time point is indicated next to each bar. Asterisks indicate significant differences (one-way ANOVA followed by the Student–Newman–Keuls test; ***P<0.001, **P<0.01, *P<0.05). The dawn series includes SR data from Fig. 4. (A) RhOps1-2. (B) $RhG_q\alpha$. (C) RhArr.

and SS+30 min, and by SS+30 min they were not significantly different from their lowest level measured during deep night.

Dawn

The times assayed were selected to examine changes RhOps1-2, $RhG_q\alpha$ and RhArr levels around the time of TRS. Preliminary studies showed that protein levels at SS+4 h and SR–1 h were not significantly different; therefore, SR data from Fig. 4 are included in the Dawn series of Fig. 7.

During dawn, rhabdomeral levels of each protein changed with a different time course. At SR, RhOps1-2 levels were similar to those measured at night (Fig. 4) and at SR–1 h, even though TRS had clearly begun (Fig. 6, SR). However, RhOps1-2 levels dropped sharply between SR and SR+1 h as Ops1-2-ir debris accumulated between rhabdomeres (Fig. 6, SR+1 h). During the next 2 h, between SR+1 h and SR+3 h, RhOps1-2 levels fell more gradually until at SR+3 h the level of RhOps1-2 was similar to that measured at midday. This suggests that TRS, a process that is primed by the circadian clock, causes a rapid drop in RhOps1-2 during the hour after SR. This is followed by a slower drop in RhOps1-2 levels, which is probably due to LDS (see Discussion).

RhOps5 was quantified in the same rhabdoms as RhOps1-2. It also fell during the morning although less precipitously. Between SR–1 h and SR+3 h RhOps5 dropped an average of 35%; however, because RhOps5 levels varied considerably among animals, this decrease did not reach statistical significance. Also, by midday, the average level of RhOps5 was at least as high as that measured during the night (Fig. 4); therefore, unlike RhOps1-2, RhOps5 levels must increase during the day.

Changes in $RhG_q\alpha$ levels during dawn and early morning are more difficult to interpret because of large variations among animals, particularly at SR (Fig. 4), but $RhG_q\alpha$ levels appeared to fall gradually during the morning until they reached midday levels at about SR+2 h. By contrast, RhArr levels changed rapidly at dawn. At SR–1 h, RhArr was still at its low, deep night level, but by SR, when the light intensity in our aquarium room was still dim (Fig. 1), RhArr had already increased to the same high level observed at midday.

Effect of eliminating circadian clock input on diurnal changes in rhabdomeral protein levels

To test whether clock input influences diurnal changes in rhabdomeral protein levels, we cut the optic nerve to one LE of a group of animals to eliminate clock input to that eye and left the other optic nerve intact. Animals were maintained in natural illumination for at least a week after the optic nerve was cut; then both LEs of an individual animal were dissected and fixed at the same time at one of a number of different times of day: midday, SS–1 h, SS+30 min, SS+2.5 h, SS+4–6 h, SR–1 h, SR+1 h, SR+2 h or SR+3 h. To eliminate between-animal variations, we compared rhabdomeral protein levels in LEs from the same animal, one with clock input (+Clock) and the other without (–Clock). The average –Clock/+Clock ratio for each protein was then compared with the mean ratio obtained from assays of LEs from the same animal when both LEs had intact optic nerves (+Clock/+Clock; Table 1).

In LEs fixed from SR+1 h to SS+30 min, −Clock/+Clock and +Clock/+Clock ratios of RhOps1-2, $RhG_q\alpha$ and RhArr were not significantly different. Therefore, clock input had no detectable influence on rhabdomeral levels of the three proteins during the day through dusk. However, the clock significantly influenced rhabdomeral levels of all three proteins during the night (Table 1). We reported previously that at SS+4–6 h, RhOps1-2 was significantly lower in –Clock compared with +Clock LEs (Katti et al., 2010). For completeness, these data are included in Table 1. New assays revealed a similar difference at SR–1 h; thus the difference is maintained throughout the night. $RhG_q\alpha$ levels were also significantly lower during the night in –Clock compared with + Clock LEs (Table 1). This difference was detected at SS+2.5 h and was sustained throughout the night. RhArr levels were significantly higher in –Clock compared with +Clock LEs at SS+4 h, but this difference disappeared by SR–1 h. Taken together, these results suggest that clock input to LEs exposed to natural diurnal light is necessary for normal nighttime increases in RhOps1-2 and $RhG_q\alpha$ levels and the normal nighttime decrease in RhArr levels.

Effect of dark adaptation during the day on rhabdomeral protein levels

To examine further the effect of clock input on rhabdomeral protein levels, we tested whether they changed in LEs dark adapted during the day. In these experiments, the optic nerves to all LEs were intact. However, because clock input to LEs is silent during the day, we predicted that dark adaptation during the day would produce changes in rhabdomeral protein levels similar to those observed at night in –Clock LEs.

Table 1. Effect of light and clock on mean ratios of immunoreactive intensities over rhabdoms per μm^2 for each protein

	Relative intensity over rhabdoms per μm^2		
Time of day	Ops1-2	$G_q\alpha$	Arr
Right–Clock/Left+Clock[a]			
SS+2.5 h	n.d.	0.74±0.09 (8)*	n.d.
SS+4–6 h	0.61±0.06 (12)***,[c]	0.83±0.05 (8)*	1.33±0.08 (5)*
SR–1 h	0.66±0.04 (6)**	0.70±0.06 (8)**	0.95±0.13 (7)
SR+1 h	0.90±0.09 (6)	1.07±0.06 (7)	0.93±0.12 (7)
Right+Clock/Left+Clock[b]			
Midday	1.12±0.1 (6)	1.07±0.1 (5)	0.99±0.11 (5)

Immunoreactive intensities were determined as described in Fig. 2. Time of day is expressed relative to sunset (SS) and sunrise (SR). Midday is 30 min after peak illumination in the afternoon. Asterisks indicate that the mean ratio is significantly different from that obtained by comparing two LEs from the same animal both with intact optic nerves (+Clock/+Clock). ***P<0.001, **P<0.01, *P<0.05 (Student's t-test). n.d., not determined.

[a]Data are mean ± s.e.m. ratios of immunoreactive intensities in lateral compound eyes (LEs) of the same animal. LEs were exposed to natural diurnal light. The optic nerve to the right eye was cut to eliminate neural clock input 7 days before the experiment (−Clock); the optic nerve to the left LE remained intact so the left LE received normal clock input (+Clock). The number of animals assayed is in parentheses.

[b]Data are mean ± s.e.m. ratios of immunoreactive intensities in two LEs of the same animal. These LEs had intact optic nerves and were exposed to natural diurnal light.

[c]Data are from Katti et al. (Katti et al., 2010).

One LE from a group of animals was dissected 30 min after peak illumination during midday and fixed in the light. The other LE was immediately patched to exclude light, and animals were returned to a shallow tank and exposed to natural illumination for an additional 4 h. Animals were then taken to an experimental dark room where patched eyes were dissected and fixed in the dark. Four hours was selected as the length for daytime dark adaptation because all three proteins studied were at their maximum nighttime dark-adapted levels by SS+4 h. Frozen sections of daytime light- and dark-adapted LEs were immunostained and imaged together with sections from +Clock LE from a separate group of animals that had been fixed at night (SS+4 h) in the dark. Relative levels of RhOps1-2, $RhG_q\alpha$ and RhArr in the three sets of eyes were quantified and compared.

During 4 h of daytime dark adaptation, RhOps1-2 levels increased significantly (23%) but remained significantly below levels in +Clock LEs fixed at night in the dark (35%) (Fig. 8). Interestingly, the increase in RhOps1-2 during 4 h of daytime dark adaptation is nearly identical to that observed in –Clock LEs dark adapted during the night (Table 1). Surprisingly, $RhG_q\alpha$ did not increase in LEs dark adapted during the day. These results further support the idea that clock input is required for RhOps1-2 and $RhG_q\alpha$ to reach maximum levels in the dark. They also show that, with respect to rhabdomeral levels of these two proteins, dark adaptation during the day is not equivalent to dark adaptation during the night. However, RhArr decreased during daytime dark adaptation to the same level observed during the night in +Clock LEs (Fig. 8). Thus, during the day, RhArr can fall to normal nighttime dark-adapted levels without clock input.

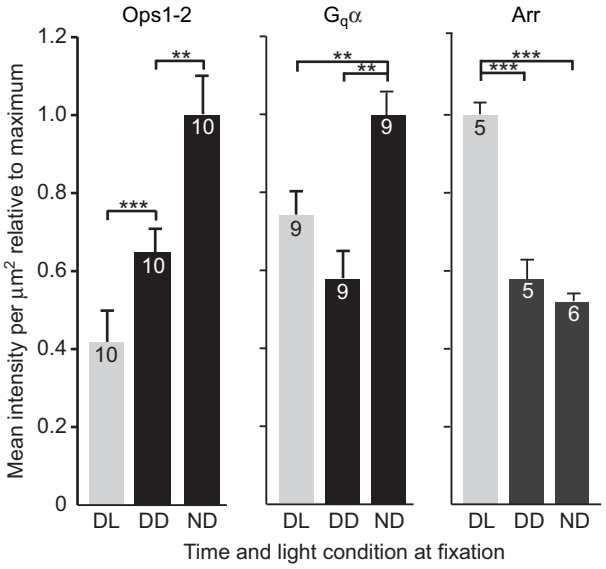

Fig. 8. Effect of dark adaptation during the day compared with during the night on levels of RhOps1-2, $RhG_q\alpha$ and RhArr. One LE of a group of animals was dissected at midday in the light and fixed immediately (day-light, DL; light gray bars). The other LE was patched and dissected in the dark 4 h later (day-dark, DD). RhOps1-2, $RhG_q\alpha$ and RhArr levels in these LEs were compared with those in LEs fixed during the night in the dark (night-dark, ND). Data are expressed as described in the legend to Fig. 4 relative to the maximum level of each protein. Asterisks indicate significant differences (one-way ANOVA followed by the Student–Newman–Keuls test; ***P<0.001, **P<0.01).

Effect of clock input on rhabdomeral protein levels in continuous darkness

We next asked whether clock input significantly influenced rhabdomeral protein levels in LEs maintained in constant darkness. For these experiments, the optic nerve to one LE of a group of animals was cut and both LEs were patched to eliminate light. These LEs are designated Dark–Clock and Dark+Clock, respectively.

The LEs of each animal were dissected and fixed at the same time in the dark either at midday or at SS+4–6 h, and we determined the Dark–Clock/Dark+Clock ratio for each protein in each pair of eyes. We then asked whether the mean daytime and nighttime Dark–Clock/Dark+Clock ratios were significantly different from one another. Recall that at night one LE receives clock input while the other does not, but during the day, neither eye receives clock input. Therefore, if the ratio changed significantly from day to night, this would mean that clock input at night had an effect in constant darkness. For example, if the nighttime ratio was significantly lower, this would be interpreted to mean that clock input at night significantly increased the rhabdomeral level of a protein. We observed no significant difference between nighttime and daytime ratios for RhOps1-2, $RhG_q\alpha$ or RhArr (Table 2). Therefore, the effects of the clock on the levels of these proteins at rhabdoms are detected only in diurnal light.

Effects of elevating cAMP on changes on rhabdomeral protein levels during daytime dark adaptation *in vitro*

Many effects of the clock are mimicked by OA and by elevating cAMP in photoreceptors (Battelle, 2002; Dalal and Battelle, 2010). Therefore, we tested *in vitro* whether OA, which activates adenylyl cyclase-coupled receptors, or forskolin, a direct activator of adenylyl cyclase, mimic the effects of clock input on RhOps1-2, RhOps5, $RhG_q\alpha$ or RhArr levels during daytime dark adaptation. Both LEs from an animal were dissected midday in the light and cut into four similarly sized slices. One slice was fixed immediately in the light. The other three were incubated in the dark for 4 h at room temperature in one of three conditions as detailed in the Materials and methods: (1) OCM, (2) OCM plus OA and IBMX, and (3) OCM plus forskolin and IBMX. In the presence of IBMX, OA and forskolin dramatically elevate cAMP in LEs (Edwards and Battelle, 1987; Dalal and Battelle, 2010). At the end of the incubation period, LE slices were fixed in the dark, and RhOps1-2, RhOps5, $RhG_q\alpha$ and RhArr levels were quantified in each slice. Levels in each dark-adapted slice were compared with those in the light-adapted slice from the same animal (Fig. 9). Dark adaption *in vitro* in OCM with no additions produced an approximate 20% increase in RhOp1-2 and RhOps5, $RhG_q\alpha$ did not increase significantly, and RhArr decreased by approximately 30%. Treatment of slices with OA and forskolin, each in the presence of IBMX, significantly enhanced the increase in RhOps1-2 and $RhG_q\alpha$ levels in the dark but had no effect on dark-adaptive changes in RhOps5 or RhArr levels.

DISCUSSION

We show here that in the heavily pigmented LEs of *Limulus* maintained in natural illumination, Ops1-2, $G_q\alpha$ and Arr, three proteins critical for the photoresponse, significantly change their concentrations at the rhabdom with a diurnal rhythm. We show further that these diurnal changes are produced by complex interactions between processes regulated by light and by a central circadian clock. Our results extend previous findings that showed that clock input to LEs is required for normal nighttime increases in RhOps1-2 levels (Katti et al., 2010), and that clock input regulates Arr transcript levels (Battelle et al., 2000b). In addition, we provide evidence that increases in $RhG_q\alpha$ levels in the dark are under circadian control in *Limulus*, and that effects of the clock on

Table 2. Effect of clock alone (Dark–Clock/Dark+Clock) on mean ratios of immunoreactive intensities over rhabdoms per μm^2 for each protein

	Relative intensity over rhabdoms per μm^2		
Time of day	Ops1-2	$G_q\alpha$	Arr
Midday	0.86±0.09 (8)	0.91±0.05 (8)	1.08±0.17 (8)
Night	1.05±0.07 (7)	0.77±0.11 (7)	1.02±0.13 (7)

Immunoreactive intensity was determined as described in the Materials and methods and in Fig. 2. Eyes were collected midday or at night, 4–6 h after sunset. Data are expressed as mean ± s.e.m. ratios of immunoreactive intensities in lateral compound eyes (LEs) of the same animal. One LE was patched and had its optic nerve severed at least 7 days before the experiment (Dark–Clock). The other eye was also patched but had an intact optic nerve (Dark+Clock).

increases in $RhG_q\alpha$ and RhOps1-2 levels are mediated by cAMP. By contrast, RhOps5 levels are not influenced by the clock or cAMP. The circadian regulation of RhOps1-2, $RhG_q\alpha$ and RhArr levels probably contribute to increased LE sensitivity during the night and protect photoreceptors by preventing maximum dark adaptation during the day. Our results add rhabdomeral biochemistry to the already substantial list of processes in *Limulus* LEs that are regulated by the circadian clock, and add Ops1-2 renewal and dark-adaptive $G_q\alpha$ translocation to the list of clock-regulated processes that are probably mediated by activation of the cAMP cascade.

RhOps1-2 levels are regulated by complex interactions between light- and clock-driven processes

Diurnal changes in RhOps1-2 levels in LEs with and without clock input are summarized in Fig. 10. The figure is a composite of means of data shown in Figs 4 and 7 together with values determined from the mean ratios of RhOps1-2 in LEs with and without clock input measured at different times of day. Ratios showing significant differences are presented in Table 1. From these data we draw a number of conclusions about how diurnal light and signals from the circadian clock interact to regulate RhOps1-2 levels.

During the day in the light, RhOps1-2 must renew continuously because even as RhOps1-2 is continuously shed (Sacunas et al., 2002), RhOps1-2 levels do not change significantly between SR+3 h, when light levels are low, and midday, when light levels are more than an order of magnitude brighter (Fig. 1). Clock input to LEs is silent during the day; therefore, RhOps1-2 renewal during the day in the light must be independent of the clock. Two additional observations indicate that RhOps1-2 is renewed in the absence of clock input. RhOps1-2 levels increase by approximately 20% when LEs are dark adapted during the day *in vivo* and *in vitro* (Figs 8, 9). RhOps1-2 levels also increase by approximately 20% when LEs without clock input are dark adapted during the night (Fig. 10, SS+0.5 h).

However, in the absence of clock input, RhOps1-2 levels remain significantly below that observed when LEs with clock input are dark adapted during the night (Table 1, Figs 8, 10). Therefore, clock input at night drives additional RhOps1-2 renewal. The rate of clock-driven renewal is slow, reaching a maximum several hours after the onset of clock input. And because the clock's effects on RhOps1-2 levels were detected only in LEs exposed to diurnal light, we conclude that the clock regulates processes specific for RhOps1-2 renewal following light-dependent shedding.

Mechanisms regulating RhOps1-2 renewal are not yet known, and clock-independent and -dependent mechanisms may be different. During both day and night, RhOps1-2 may be renewed from a pool of newly synthesized Ops1-2 and/or by reinsertion of Ops1-2 from a pool of shed membranes. New Ops1-2 synthesis may be important for clock-independent RhOps1-2 renewal during the day because Ops1-2 transcript levels become elevated during the day in the light (Dalal et al., 2003). However, RhOps1-2 transcript levels fall in the dark to the same extent in LEs with and without clock input; therefore, the clock-dependent increase in RhOps1-2 renewal during the night must involve processes downstream of transcription. Multiple processes may be involved, from translation to transport. Our finding that RhOps1-2 renewal is enhanced by treatments that elevate cAMP (Fig. 9) provides strong evidence that one or more of these processes is regulated by cAMP.

A striking finding is that the same amount of Ops1-2 added to rhabdoms at night by clock-dependent renewal is rapidly removed from rhabdoms in the morning by TRS (Fig. 10), another clock-regulated process (Chamberlain and Barlow, 1979; Chamberlain and Barlow, 1984) that is mediated through activation of the cAMP cascade (Runyon et al., 2004). TRS is triggered by light but it must be primed by 3 to 4 h of clock input the previous night (Chamberlain and Barlow, 1984) or by treatments that elevate cAMP in LEs (Runyon et al., 2004). TRS is synchronous along the length of the rays of the rhabdom, does not involve clathrin or arrestin, as does light-driven shedding (LDS) (Sacunas et al., 2002), and is characterized by the rapid and transient breakdown of rhabdom microvillar structure as the membrane is internalized (Chamberlain and Barlow, 1979; Chamberlain and Barlow, 1984) (Fig. 6). A previous study showed that Ops1-2 was shed during TRS (Sacunas et al., 2002) but did not clarify whether TRS is a mechanism for rapid RhOps1-2 turnover or a processes resulting in net removal of Ops1-2 from the rhabdom. Our current findings show clearly that TRS results in rapid net removal of Ops1-2 from rhabdoms.

After TRS, RhOps1-2 levels continue to fall by another 20% but at a slower rate (Fig. 10). This slower net loss of RhOps1-2 occurs in LEs with and without clock input; therefore, the loss must be attributed to LDS. As was described above, LDS of Ops1-2 containing membranes continues throughout the day in the light, but because RhOps1-2 does not continue to fall after SR+3 h, during most of the day Ops1-2 shedding must be balanced by renewal.

An even more dramatic light-dependent downregulation of rhabdomeral opsin levels than reported here has been described in white-eyed mutants of the mosquito *Aedes aegypti* (AeOps1) (Hu et al., 2012). In the mosquito, opsin removal is probably similar to LDS described here; however, unlike in *Limulus*, rhabdomeral opsin levels in the mosquito appear to be regulated entirely by light and dark.

RhOps5 levels are not regulated by the clock

Limulus Ops5 is co-expressed with Ops1-2 in almost all retinular cells, and Ops5-containing membranes are shed during the day in the light (Fig. 3) (Katti et al., 2010). Yet RhOps5 levels do not change significantly from day to night in LEs exposed to diurnal light. This indicates that shedding must be balanced by renewal during the day and night. Furthermore, neither clock input (Katti et al., 2010) nor treatments that elevate cAMP (Fig. 9) influence RhOps5 levels. Clearly, RhOps1-2 and 5 levels are regulated differently. As a result

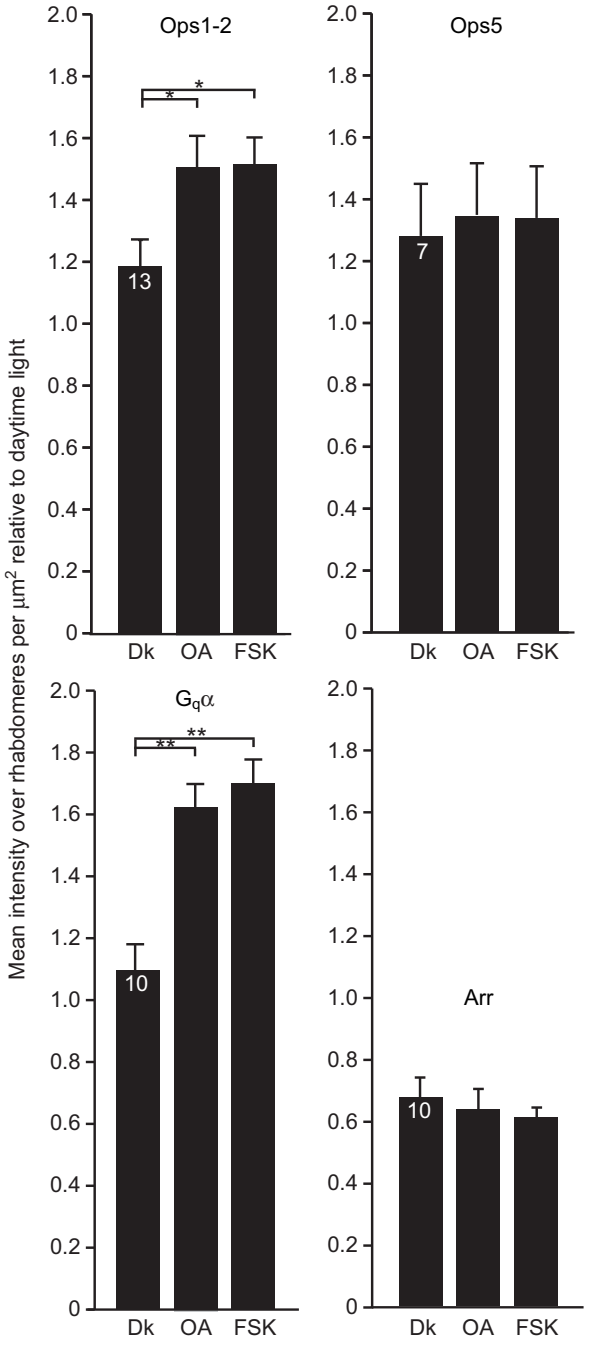

Fig. 9. Octopamine and forskolin increase levels of RhOps1-2 and $RhG_q\alpha$ during daytime dark adaptation *in vitro*. LEs were dissected from animals at midday. Each LE was cut in half to yield four slices from each animal. One slice from each animal was fixed immediately in the light. The other three slices were incubated for 4 h at room temperature in the dark in OCM + 0.08% DMSO and one of the following: Dk, no further additions; OA, octopamine + IBMX; or FSK, forskolin + IBMX. RhOps1-2, RhOps5, RhOps $G_q\alpha$ and RhArr were quantified in each slice as described in the Materials and methods and the legend to Fig. 2. Data are expressed as mean (±s.e.m.) immunoreactive intensities per μm^2 of rhabdom. Levels in each dark-adapted slice of an animal were normalized to those in the light-adapted slice of the same animal when the light adapted level is expressed as 1. Mean ± s.e.m. relative levels are presented for the number of animals shown in the first bar of each data set. Asterisks indicate significant differences among the dark-adapted treatments (one-way ANOVA followed by the Student–Newman–Keuls test; $^{**}P<0.01$, $^{*}P<0.05$).

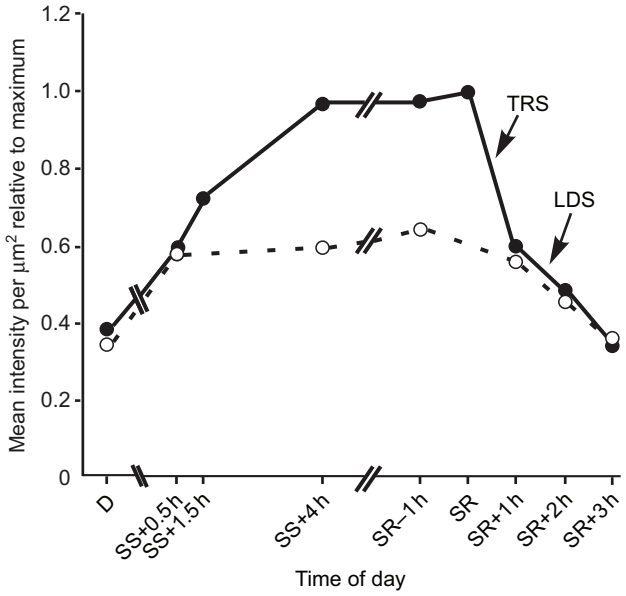

Fig. 10. Summary of diurnal changes in RhOps1-2 levels in LEs with (filled circles) and without (open circles) clock input. Data for levels in LEs with clock input are the means from Figs 4 and 7. Data for LEs without clock input are from Table 1 and other data described in the Results, which showed that RhOps1-2 levels were the same in LEs with and without clock input. Time of day is plotted as hours after SS and before and after SR. D, midday. The sharp drop in RhOps1-2 between SR and SR+1 is attributed to transient rhabdom shedding (TRS). It is observed only in LEs with clock input. The slower decline in RhOps1-2 observed between SR+1 and SR+3, which is seen in LEs with and without clock input, is attributed to light-driven shedding (LDS).

of this differential regulation, the ratio of Ops1-2 to Ops5 in rhabdoms changes from day to night (Katti et al., 2010).

The clock regulates the increase in $RhG_q\alpha$ levels in the dark

When LEs with normal clock input are exposed to diurnal light, $RhG_q\alpha$ levels are higher at night than during the day (Figs 3, 4, 7). This finding is consistent with results from other invertebrates showing that $RhG_q\alpha$ levels are higher in the dark than in the light (Narita et al., 1999; Terakita et al., 1996; Kosloff et al., 2003; Frechter et al., 2007). A surprising finding is that dark-adaptive increases in $RhG_q\alpha$ levels in *Limulus* require clock input. This conclusion is based on experiments showing that $RhG_q\alpha$ levels are consistently higher at night in LEs with clock input compared with LEs without clock input (Table 1), that $RhG_q\alpha$ levels do not increase in LEs dark adapted during the day *in vivo*, when clock input is silent (Fig. 8), or *in vitro* (Fig. 9), and that $RhG_q\alpha$ levels increased during dark adaptation *in vitro* when LEs were incubated under conditions known to mimic many effects of the clock (Fig. 9; and see below). Because effects of the clock on $RhG_q\alpha$ levels were only observed in eyes exposed to diurnal light (Table 2), we conclude that the clock targets processes that increase $RhG_q\alpha$ levels during dark adaptation.

In both ciliated and rhabdomeral photoreceptors, the increase in $RhG_q\alpha$ levels within the photosensitive compartment during dark adaptation is attributed to $G_q\alpha$ translocation from the cytoplasm to photosensitive membranes (Kosloff et al., 2003; Frechter et al., 2007; Arshavsky and Burns, 2012). We were unable to directly assay $G_q\alpha$ translocation in LE retinular cells (see Materials and methods); however, because the mechanism is broadly conserved in evolution,

we think it reasonable to speculate that the same mechanism underlies the changes in rhabdomeral $G_q\alpha$ levels observed in this study. The regulation of $G_q\alpha$ translocation is not understood in any system. Our study provides the first evidence that the dark-adaptive translocation of $G_q\alpha$ to rhabdoms can be regulated by a circadian clock (Table 1, Fig. 8) and is mediated by cAMP-dependent processes (Fig. 9). The clock- and cAMP-regulated increase in rhabdomeral $G_q\alpha$ we describe in *Limulus* is clearly different from what has been described in *Drosophila* photoreceptors, where light- and dark-adapted changes in $G_q\alpha$ at the rhabdoms are regulated entirely by light and darkness (Kosloff et al., 2003; Frechter et al., 2007).

In *Drosophila*, the unconventional class III myosin NINAC (neither inactivation nor afterpotential C) is thought to play a role in $G_q\alpha$ translocation from the cytoplasm to the rhabdom (Cronin et al., 2004; Lee and Montell, 2004). NINAC motor activity has not been demonstrated; therefore, it has been proposed that $G_q\alpha$ translocation involves reversible associations of $G_q\alpha$ with the cytoplasmic and rhabdomeral isoforms of NINAC (Montell, 2012). *Limulus* eyes express a single homolog of NINAC that is distributed throughout the photoreceptor, concentrated at the rhabdoms (Battelle et al., 2001), and is a major target for phosphorylation by the circadian clock *via* activation of cAMP-dependent protein kinase (Edwards and Battelle, 1987; Battelle et al., 1998; Kempler et al., 2007; Cardasis et al., 2007). *Limulus* myosin III is not a molecular motor (Kempler et al., 2007); however, based on the findings in *Drosophila* and the results presented here, it is interesting to speculate that the clock- and cAMP-dependent phosphorylation of *Limulus* myosin III plays a role in the clock-dependent translocation of $G_q\alpha$ to the rhabdom.

The circadian clock enhances the decrease in RhArr levels at night

In nighttime LEs, the decrease in RhArr levels is enhanced by the circadian clock; this is demonstrated in Table 1 (SS+4–6 h). However, in daytime LEs, which receive no signals from the central clock, RhArr levels decrease to the same level observed in nighttime LEs with clock input. Furthermore, treatments that increase cAMP levels in photoreceptors *in vitro* do not enhance the magnitude of the dark-adaptive decrease in RhArr during the day. Thus, there is a difference between nighttime and daytime LEs with respect to the influence of clock-driven processes on RhArr levels.

Some of our findings suggest a direct relationship between RhArr and Arr transcript levels. For example, our finding that clock input enhances the nighttime decrease in RhArr levels is consistent with results showing that clock input and elevated cAMP reduce Arr transcript levels (Battelle et al., 2000b; Dalal and Battelle, 2010). Our finding that RhArr levels in LEs with clock input increase before SR (Table 1) is also consistent with the observation that Arr transcript levels increase before dawn in LEs with clock input (Battelle et al., 2000b), as clock input slows before dawn (Barlow, 1983; Liu and Passaglia, 2011). However, other experiments reported here indicate that RhArr and Arr transcript levels are not directly related. In a previous study (Battelle et al., 2000b), the clock's effects on Arr transcript levels were observed in LEs maintained in constant darkness, but in the present study we saw no effect of the clock on RhArr levels in LEs maintained in constant darkness (Table 2). Furthermore, Arr transcript levels fall in daytime LEs incubated in the dark *in vitro* under conditions that elevate cAMP (Dalal and Battelle, 2010). Here we show that similar treatments of daytime LEs do not enhance the fall in RhArr levels. In view of these conflicting findings, mechanisms by which the clock influences nighttime RhArr levels at night are unclear.

Light- and dark-dependent arrestin translocations are responsible for the major changes in RhArr levels in the photoreceptors of *Drosophila* and mammals. We were unable to obtain direct evidence for Arr translocation in this study (see Materials and methods); however, because arrestin translocation, like $G_q\alpha$ translocation, is broadly conserved in evolution, we think it reasonable to speculate that the same mechanism functions in *Limulus*. Some evidence from *Drosophila* suggests that Arr translocation from the cytoplasm to the rhabdom, like the translocation of $G_q\alpha$, involves NINAC (Lee and Montell, 2004; but see Satoh and Ready, 2005). In *Limulus*, increases in $G_q\alpha$ and Arr at the rhabdoms have very different dynamics. $RhG_q\alpha$ increases slowly at dusk and the increase is clearly clock-dependent, whereas RhArr increases rapidly at dawn and is independent of clock input. However, these differences do not preclude involvement of the *Limulus* myosin III, the homolog of NINAC, because its functions are modulated both by Ca^{++} (Battelle et al., 1998), which rises rapidly and dramatically in *Limulus* photoreceptors in response to light (Lisman et al., 2002), and cAMP, which increases in photoreceptors in response to clock input at night.

Clock-dependent changes in RhOps1-2 and $RhG_q\alpha$ levels probably contribute to high LE sensitivity at night and low sensitivity during the day

Much of the nighttime increase in LE sensitivity to light has been attributed to clock-dependent structural changes in ommatidia, like those shown in Fig. 5 and described in detail elsewhere (Barlow et al., 1980; Chamberlain and Barlow, 1987; Kier and Chamberlain, 1990). These structural changes increase the probability that photons enter the retina and strike rhabdomeral membranes. The clock-dependent increase in RhOps1-2 and $RhG_q\alpha$ levels at night are also expected to enhance photoreceptor sensitivity by increasing the probability that photons entering the retina strike a rhodopsin molecule. Gain may be increased by increasing the number of $G_q\alpha$ molecules activated by the photoisomerization of rhodopsin. A study from *Drosophila* provides direct evidence that rhabdomeral levels of $G_q\alpha$ can contribute to the sensitivity of rhabdomeral photoreceptors (Frechter et al., 2007).

As was described in the Introduction, the clock directly influences photoreceptor physiology by decreasing noise, increasing gain and increasing the duration of quantum bumps. A clock-dependent increase in $RhG_q\alpha$ levels could enhance gain, and a clock-dependent decrease in RhArr levels could increase bump duration (Xu et al., 1997). But the relationship between clock-dependent changes in photoreceptor physiology and the rhabdom biochemistry described here is not clear. The physiological experiments were performed using animals maintained for more than 24 h in the dark. In our study, no significant clock-dependent changes in RhOps1-2, $RhG_q\alpha$ or RhArr levels were observed in LEs maintained for more than 24 h in the dark (Table 2). This argues that clock-dependent changes in photoreceptor physiology are unrelated to the changes in rhabdom biochemistry we describe. However, our immunocytochemical assays that, by necessity, pooled results from many animals with variable responses are considerably less sensitive than the physiological assays, which consisted of continuous electrical recordings from an individual photoreceptor. Our assays would not have detected small clock-driven changes in rhabdom biochemistry, which might significantly impact photoreceptor function. Nevertheless, in diurnal light, the clock clearly influences rhabdom biochemistry in ways expected to increase photoreceptor sensitivity and responsiveness to light at night.

Equally important may be the clock's role in achieving and maintaining low photoreceptor sensitivity and responsiveness during

the day. Light alone appears responsible for the daytime decrease in $RhG_q\alpha$ levels; however, clock input at night is required to prime the rapid shedding of RhOps1-2 at first light, and this probably contributes to a rapid decrease in visual sensitivity in the morning (Pieprzyk et al., 2003). Furthermore, because clock input is required for maximum increases in RhOps1-2 and $RhG_q\alpha$ levels in the dark, low photoreceptor sensitivity is maintained throughout the day, even in the dark.

Complex regulation of light and dark adaptation in *Limulus*

Why is the regulation of light and dark adaptation in *Limulus* so complex? Perhaps it is because *Limulus* use their LEs to find mates when they spawn (Barlow et al., 1982; Duffy et al., 2006; Schwab and Brockmann, 2007; Saunders et al., 2010), and they spawn during the day and night (Cohen and Brockmann, 1983). Thus, their LEs must function optimally under both very low and very bright light conditions. Optimal LE function probably depends on the day to night changes in retinal structure and photoreceptor physiology described previously and the rhabdom biochemistry described here. In addition, *Limulus* often bury in the mud for long periods. Therefore, it may be particularly important for *Limulus* to maintain their LEs in a low sensitivity state during the day, even in the dark, to prevent retinal damage should they emerge into the bright light of day. A complex regulation of light and dark adaptation like that observed in *Limulus* may be present in other species with lifestyles that place similar demands on their visual systems, and it may be typical among chelicerates. Like *Limulus*, scorpions and spiders show circadian changes in visual sensitivity, and their eyes are similarly innervated by octopaminergic efferent neurons driven by central circadian clocks (Fleissner and Fleissner, 1985; Yamashita, 2002).

LIST OF ABBREVIATIONS

IBMX	isobutylmethylxanthine
ir	immunoreactivity (also immunoreactive)
LDS	light-driven shedding
LE	lateral compound eye
NINAC	neither inactivation nor afterpotential C
OA	octopamine
OCM	organ culture medium
RhArr	arrestin at rhabdoms
$RhG_q\alpha$	alpha subunit of $G_{q/11}$ at rhabdoms
RhOps1-2	opsin1-2 at rhabdoms
ROI	region of interest
SR	sunrise
SS	sunset
TRS	transient rhabdom shedding

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AUTHOR CONTRIBUTIONS

Experiments were designed by B.-A.B. All authors participated in the execution of the study, in the interpretation of results, and in producing the final manuscript.

COMPETING INTERESTS

No competing interests declared.

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