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SYMPOSIUM

What the Clock Tells the Eye: Lessons from an Ancient Arthropod

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Synopsis Circadian changes in visual sensitivity have been observed in a wide range of species, vertebrates, and invertebrates, but the processes impacted and the underlying mechanisms largely are unexplored. Among arthropods, effects of circadian signals on vision have been examined in most detail in the lateral compound eye (LE) of the American horseshoe crab, *Limulus polyphemus*, a chelicerate arthropod. As a consequence of processes influenced by a central circadian clock, *Limulus* can see at night nearly as well as they do during the day. The effects of the clock on horseshoe crab LE retinas are diverse and include changes in structure, gene expression, and rhabdom biochemistry. An examination of the known effects of circadian rhythms on LEs shows that the effects have three important outcomes: an increase in visual sensitivity at night, a rapid decrease in visual sensitivity at dawn, and maintenance of eyes in a relatively low state of sensitivity during the day, even in the dark. All three outcomes may be critically important for species' survival. Specific effects of circadian rhythms on vision will certainly vary with species and according to life styles. Studies of the circadian regulation of *Limulus* vision have revealed that these effects can be extremely diverse and profound and suggest that circadian clocks can play a critical role in the ability of animals to adapt to the dramatic daily changes in ambient illumination.

Introduction

Sustained, daily changes in retinal structure and function are typically observed in animals that live in diurnal environments. These changes underlie the ability of visual systems to function optimally over large daily fluctuations in ambient illumination; many of these changes are regulated by signals from circadian clocks. Studies of the circadian regulation of vision are relatively limited. However, among invertebrates, effects of circadian signals on vision have been examined in most detail in the lateral compound eye (LE) of the American horseshoe crab *Limulus polyphemus*, a chelicerate arthropod. As a consequence of processes influenced by a central circadian clock, this animal sees at night nearly as well as it does during the day (Powers et al. 1991), and in *Limulus* LEs, the clock impacts almost every aspect of retinal function. It drives coordinated changes in retinal structure and in the physiology and biochemistry of photoreceptors in ways that are predicted to increase visual sensitivity at night. It also primes light-dependent processes predicted to produce a rapid down-regulation of visual sensitivity at first light.

This contribution describes the circadian organization of the *Limulus* visual system and summarizes known effects of clock input on the LE, with an emphasis on the clock's effects on concentrations of two proteins at photosensitive membranes (rhabdoms) that are critical for the photoresponse: opsin, an integral membrane protein that is the protein component of visual pigment, and the alpha subunit of the G-protein activated by visual pigment ($G_q\alpha$), a soluble protein. Data summarized here show that signals from the circadian clock drive increase in the concentrations of both proteins at the rhabdom at night, that the clock primes a rapid loss of opsin from the rhabdom at first light, and that these effects of the clock are mediated by cAMP. Also described are results that indicate the clock may produce a

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day-to-night change in the spectral sensitivity of photoreceptors.

Circadian organization of the *Limulus* visual system

Limulus has three different types of eyes. A pair of LEs and a pair of median ocelli are evident on the dorsal carapace. In addition, it has three pairs of what are referred to in the literature as ''rudimentary eyes'' (lateral, median, and ventral). These are larval eyes that develop before the compound eyes and median ocelli (Harzsch et al. 2006), and they persist in the adult (Fig. 1A). Each type of eye shows circadian changes in sensitivity to light (Barlow 1983; Kass and Renninger 1988).

The circadian clock that influences the sensitivity of *Limulus* eyes is located in the animal's brain (protocerebrum), and circadian signals reach the eyes via well-characterized clock-driven efferent neurons that project from the brain to the eyes (reviewed by Battelle 2006). The nature and location of the central circadian clock is not known, and some evidence suggests that there are two interconnected central clocks, one on each side of the brain (Kass and Barlow 1992). The central clock(s) can be entrained by illuminating any of the eyes mentioned above, as well as by illuminating extraocular photoreceptors in the tail (Hanna et al. 1988; Horne and Renninger 1988).

Although little is known about the clock itself, the clock-driven efferent neurons innervating the eyes are well characterized. Their cell bodies are located in bilateral clusters within the cheliceral ganglia deep in the protocerebrum, and anatomical and physiological evidence (Calman and Battelle 1991; Kass and Barlow 1992) suggests that each efferent neuron projects to all of the eyes (Fig. 1B). A critical feature of the clock-driven efferent input to the eyes is that it is active only at night and is silent during the day (Barlow 1983). In natural illumination, the efferents begin firing action potentials about 45 min before sunset and stop firing at about sunrise (Pieprzyk et al. 2003; Liu and Passaglia 2011). All cell types in the LE are innervated by the clock-driven efferents; thus, all cell types are potential targets for regulation by the circadian clock.

The clock-driven retinal efferents are octopaminergic, meaning they synthesize, store, and release

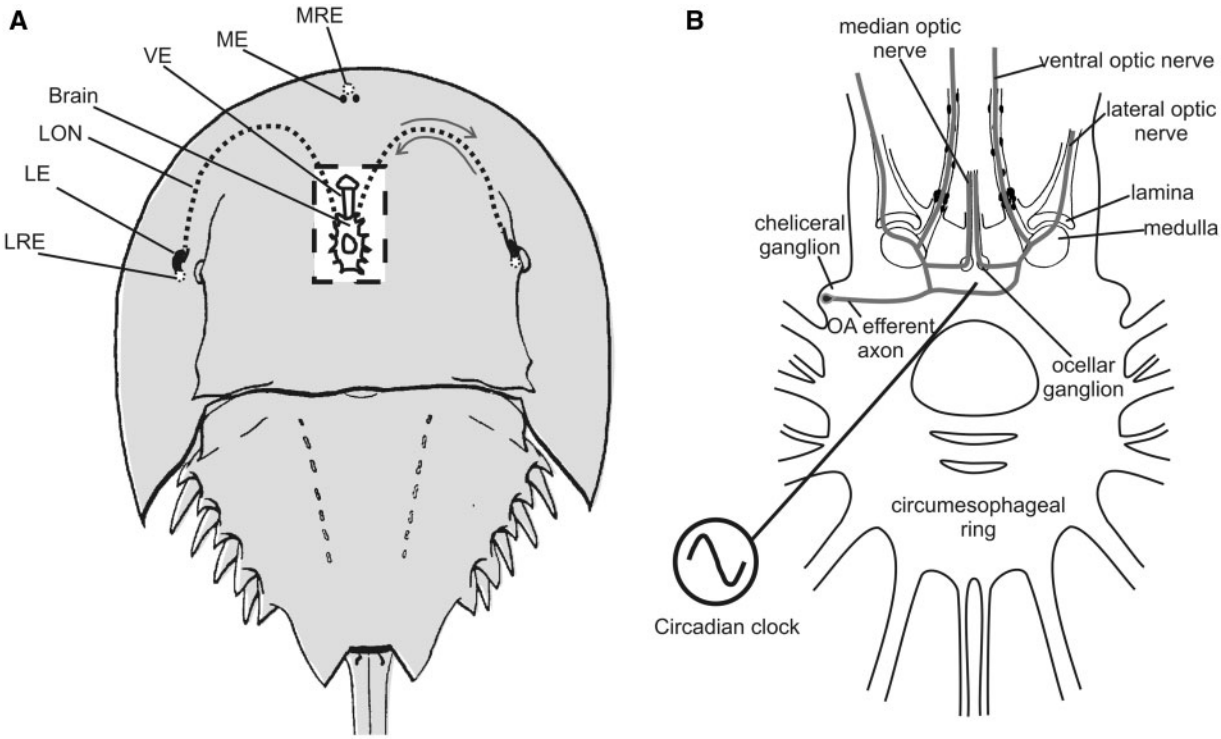

Fig. 1 (**A**) Dorsal view of *Limulus* showing the locations of its eyes. Rectangle in center: cut-away to show the locations of the brain and the ventral eyes lying just under the ventral cuticle. The dashed lines show the projections of the lateral optic nerves. The arrows indicate that information in the optic nerve travels in two directions, from the eye to the brain and from the brain to the eye. LE, lateral eye; LON, lateral optic nerve; LRE, lateral rudimentary eye; ME, median eye; MRE, median rudimentary eye; VE, ventral eye. (**B**) The brain and circumesophageal ring of *Limulus*. The circadian clock(s) regulating the eyes is(are) in the brain. The location of one clock-driven efferent neuron is diagramed along with its proposed projections. Bilateral clusters of these efferent neurons are in the cheliceral ganglia, and each is thought to project to all of the eyes through the optic nerves. Based on Calman and Battelle (1991).

the biogenic amine octopamine (Battelle et al. 1982). *Limulus* photoreceptors, and very likely other cell types in the LE, have receptors for octopamine that are coupled to adenylyl cyclase; thus, when octopamine is applied to *Limulus* photoreceptors, there is an increase in intracellular cAMP (Kaupp et al. 1982; Dalal and Battelle 2010). This leads to the prediction that the effects of clock input to *Limulus* eyes are mediated by an increase in cAMP levels in target cells, and, as is summarized in Table 1, for many known effects of clock input to *Limulus* eyes, this has been shown to be the case.

However, our understanding of the transmitter chemistry of the clock-driven efferent neurons is incomplete. Postsynaptic targets of clock input probably express more than one type of octopamine receptor, and some receptors may produce effects other than an increase in cAMP. Furthermore, in addition to octopamine, these efferents probably release one or more neuroactive peptides that may influence retinal functions. Terminals of the clock-driven efferents contain dense granules that are characteristic of peptidergic neurons in arthropods (Fahrenbach 1981), but the peptides have not yet been identified.

The clock-driven, octopaminergic efferents are not the only efferent inputs to LEs. There is an entirely separate Substance P/FMRFamide-containing efferent projection to LEs, that also has been implicated in the regulation of LE function (Chamberlain and Engbretson 1982; Mancillas and Brown 1984; Mancillas and Selverston 1984, 1985; Lewandowski et al. 1989; Bolbecker et al. 2009). However, since this projection has not yet been shown to be regulated by a circadian clock, a discussion of this projection is outside the scope of the current contribution.

Effects of the clock on *Limulus* eyes

The known effects of the clock on *Limulus* LE retinas are diverse, ranging from changes in structure to gene expression (Table 1). Some effects result from processes directly driven by clock input (points 1–12 in Table 1). Each of these is predicted to increase sensitivity and responsiveness of LEs to light at night. Other effects involve processes triggered or driven by light but which must be ''primed'' by clock input. This means that if clock input is eliminated, these light-dependent processes do not occur. Among processes primed by clock input are light-dependent movements of screening pigment granules within photoreceptors and light-triggered transient rhabdom shedding (13 and 14 in Table 1). These ''clock-primed'' processes are predicted to decrease the sensitivity of LEs at first light.

Clock-dependent changes in retinal structure

Structural changes are among the most dramatic effects of clock input to LEs (Fig. 2). During the day in the light, the aperture at the base of the lens is long and narrow, the rhabdom is extended below the narrow aperture and photoreceptor screening pigment is clustered near the rhabdom. At night, pigment cells move away from the base of the lens, thereby widening and shortening the aperture. Photoreceptors move closer to the base of the lens, and the distal half of the rhabdom close to the base of the lens is thrown into extensive folds, increasing the probability that entering photons strike the rhabdom. Screening pigment also disperses away from the rhabdom, decreasing the probability of absorbing incoming photons. Structural changes continue in constant darkness, indicating they are driven by the clock, but their amplitude is reduced. Thus, the effects of the clock on structure are amplified by diurnal light. However, in the absence of clock input, no structural changes are observed, even in diurnal light, and the structure of the ommatidia becomes frozen in a configuration not seen in a normal eye (Chamberlain and Barlow 1987).

Another clock-dependent structural change is transient rhabdom shedding, a process triggered by the dim light of dawn and which must be primed by clock input the night before. During transient shedding, microvilli in the rhabdomeral rays rapidly and transiently become disorganized and membranes containing opsin are shed (Chamberlain and Barlow 1984). This process is distinct from a second membrane-shedding process called light-driven shedding that is not dependent on clock input, continues throughout the day in the light, and is a clathrin-mediated endocytosis of membranes containing opsin (Sacunas et al. 2002) (Fig. 3).

Clock-dependent changes in rhabdom biochemistry

As described above, clock-driven structural changes in ommatidia that begin at dusk are predicted to increase the capture of photons by rhabdoms. Recent studies, summarized below and described in detail by Battelle et al. (2013), indicate that as the clock drives changes in rhabdom structure, it also drives changes in rhabdom biochemistry that are predicted to increase the probability that an incoming photon initiates phototransduction. In these studies, we examined diurnal changes in the concentrations at rhabdoms of proteins that drive phototransduction, opsins, and $G_q\alpha$. There is good

Table 1 LE responses to clock input mimicked by OA and cAMP

Retinal response to circadian efferent input	References: describing retinal responses	Mimicked by OA or cAMP	References describing effects of OA or cAMP
1. Increase in sensitivity (ERG amplitude)	Barlow et al. (1977), Barlow (1983)	OA	Kass and Barlow (1984)
2. Decrease in latency of ERG	Bolbecker et al. (2009)	No	Bolbecker et al. (2009)
3. Change in structure (Fig. 2)	Barlow et al. (1980), Chamberlain and Barlow (1987)	OA inferred	Kass and Barlow (1984)
4. Photoreceptor screening pigment disperses (Fig. 2)	Chamberlain and Barlow (1987), Kier and Chamberlain (1990)	Not determined	
5. Increase in gain	Barlow et al. (1977), Kaplan and Barlow (1980), Barlow et al. (1987)	OA or cAMP	Kass and Renninger (1988), Renninger et al. (1989)
6. Decrease in noise	Barlow et al. (1977), Kaplan and Barlow (1980), Barlow et al. (1987)	OA or cAMP	Kass and Renninger (1988)
7. Quantum bumps longer	Kaplan and Barlow (1980), Barlow et al. (1987)	OA or cAMP	Renninger et al. (1989)
8. Increase in phosphorylation of MyoIII at PKA sites	Edwards et al. (1990), Cardasis et al. (2007)	OA or cAMP	Edwards and Battelle (1987), Battelle et al. (1998), Kempler et al. (2007)
9. Visual arrestin transcript decreases	Battelle et al. (2000)	OA or cAMP	Dalal and Battelle (2010)
10. Increase in opsin 1-2 protein levels in rhabdoms (Fig. 5)	Katti et al. (2010), Battelle et al. (2013)	OA or cAMP	Battelle et al. (2013)
11. Increase in $G_q\alpha$ protein levels in rhabdoms (Fig. 5)	Battelle et al. (2013)	OA or cAMP	Battelle et al. (2013)
12. Changes in ratio of co-expressed opsin proteins in rhabdoms (Fig. 7)	Katti et al. (2010)	OA and cAMP	Battelle et al. (2013)
13. Transient rhabdom-shedding primed (Fig. 3)	Chamberlain and Barlow (1979), Chamberlain and Barlow (1984)	OA or cAMP	Khadilkar et al. (2002), Runyon et al. (2004)
14. Photomechanical movements of screening pigment primed (Fig. 2)	Chamberlain and Barlow (1987), Kier and Chamberlain (1990)	Not determined	

evidence that the concentrations of these proteins in rhabdoms influence the sensitivity of photoreceptors to light (Stephenson et al. 1983; Frechter et al. 2007). The concentrations of these proteins at *Limulus* rhabdoms were quantified by immunocytochemistry and confocal microscopy using procedures described in detail elsewhere (Katti et al. 2010; Battelle et al. 2013). Briefly, the total immunoreactive intensity present over rhabdoms was divided by the total area of rhabdoms to produce a measure of protein concentration: average immunoreactive intensity per μm^2 of rhabdom.

The opsins on which we focused are *Limulus* opsins 1 and 2. These are encoded by two closely related genes (Smith et al. 1993; Dalal et al. 2003) and quantitatively are the major opsins expressed in LE rhabdoms (Katti et al. 2010). The antibody used in this study was generated against the C-terminus of opsin 1. Since opsins 1 and 2 differ from one another by only one amino acid in this region, the two opsins cannot be distinguished immunocytochemically (Battelle et al. 2001). Therefore, the opsin immunoreactivity described here is the combined immunoreactivity of opsins 1 and 2 (Ops1-2).

The intensities of Ops1-2- and $G_q\alpha$-immunoreactivity (ir) at rhabdoms (RhOps1-2 and $RhG_q\alpha$) change significantly with a diurnal rhythm; the immunoreactive intensities of both proteins at rhabdoms was greater during the night compared with daytime (Fig. 4). The dynamics of these diurnal changes in natural illumination were quantified, and the circadian clock's role in producing these changes was tested by comparing RhOps1-2 and $RhG_q\alpha$ concentrations in LEs with and without clock input. Clock input to LEs was eliminated by cutting the lateral optic nerve (Fig. 1) (Battelle et al. 2013).

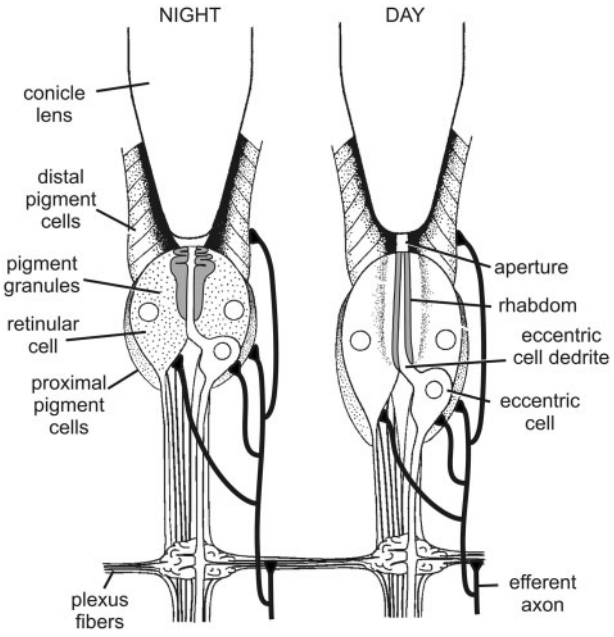

Fig. 2 Longitudinal sections through LE ommatidia in their daytime and nighttime states. Based on Barlow et al. (1980) and Chamberlain and Barlow (1987). See text for details. The schematic also shows projections of the clock-driven efferent neurons innervating all cell-types in the LE. Based on Fahrenbach (1981).

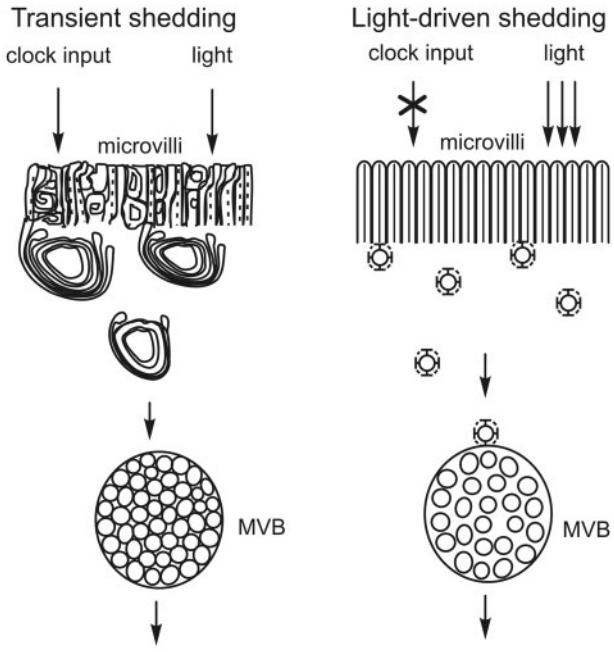

Fig. 3 Schematic differentiating clock-primed, light-triggered transient rhabdom-shedding from light-driven shedding. Transient shedding is primed by clock input during the night and triggered by the dim light of dawn. It is characterized by a rapid, transient disorganization of microvilli in the rays of the rhabdom and a breakdown of the actin cytoskeleton, accompanied by the formation of large whorls of opsin-containing membranes that accumulate between the rays of the rhabdom as densely packed multivesicular bodies (MVBs). Light-driven shedding is a progressive process driven by brighter light that does not require clock input. It is characterized by the clathrin-mediated endocytosis of opsin-containing membranes from the base of the microvilli that then accumulates in loosely packed MVBs. Transient shedding begins at about sunrise and is largely complete by 1 h after sunrise; light-driven shedding continues throughout the remaining daylight hours. Based on Chamberlain and Barlow (1979), Sacunas et al. (2002), and Battelle (2013).

In eyes with intact optic nerves that receive natural clock input, the concentration of RhOps1-2 begins to increase gradually before dark and continues to increase gradually after dark until it reaches a maximum concentration at about 4 h after sunset (Fig. 5A, + Clock). The concentration of RhOps1-2 remains high throughout the night and into dawn, then falls sharply between sunrise and 1 h after sunrise (SR + 1). This sharp decline correlates with clock-primed, transient rhabdom shedding. From previous studies of transient rhabdom shedding, it was not clear whether it was a mechanism for opsin turnover or a mechanism that reduces the opsin concentration at rhabdoms. Figure 5 shows clearly that transient shedding rapidly decreases the concentration of Ops1-2 in rhabdoms. After SR + 1, RhOps1-2 falls more slowly until at 3 h after sunrise it is at the same level observed at midday. The more gradual decline is attributed to light-driven shedding because it also occurs in LEs without clock input and thus is driven solely by light.

In eyes with severed optic nerves (− Clock), the RhOps1-2 concentration also increases gradually before dark, until it is about 20% above the midday level, but then the increase stalls, and throughout the night, in LEs without clock input, RhOps1-2 remains about 40% below the concentration in eyes with clock input. However, by 1 h after sunrise, after transient rhabdom shedding in LEs with clock input has occurred, RhOps1-2 concentrations in LEs with and without clock input are the same, and they remain the same throughout the day.

Similar results were obtained when $RhG_q\alpha$ concentrations were examined (Fig. 5B). In eyes with intact optic nerves and natural clock input (+ Clock), the $RhG_q\alpha$ concentration increases gradually through dusk and into the night, but at night, in eyes with severed optic nerves and no clock input, the $RhG_q\alpha$ concentration remains significantly below that observed in eyes with clock input.

These results indicate that clock input is required for maximum dark-adaptive increases in Ops1-2 and in $G_q\alpha$ concentrations at rhabdoms. This was tested further by examining changes in RhOps1-2 and in $RhG_q\alpha$ concentrations after 4 h of daytime

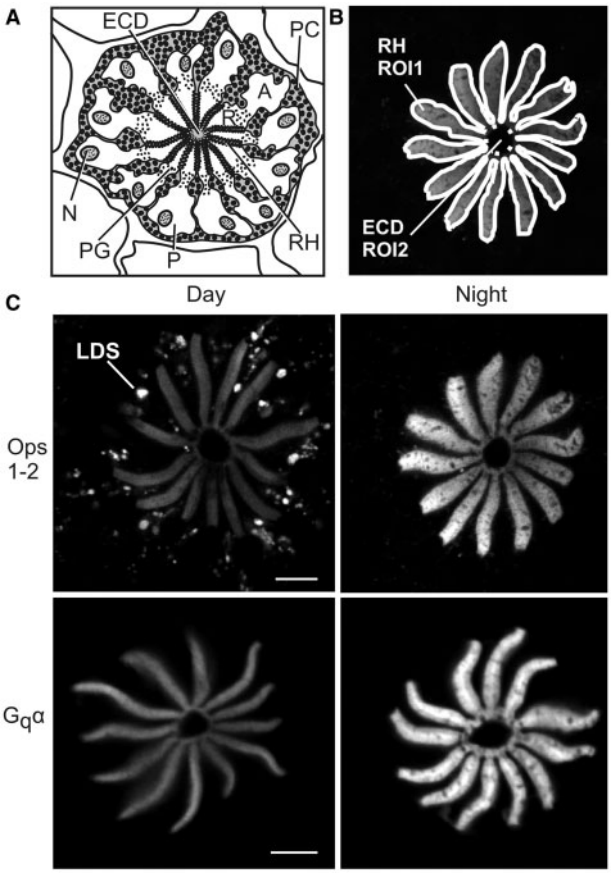

Fig. 4 Immunoreactive intensities of Ops1-2 and $G_q\alpha$ over rhabdoms changes significantly from day to night. (**A**) Cross-section of a LE ommatidium. The following are labeled: A, arhabdomeral segment; ECD, eccentric cell dendrite; N, nucleus; PC pigment cells; PG, pigment granules in photoreceptors; R, rhabdomeral segment; and RH, rhabdom. (**B**) Confocal image of Ops1-2-ir in the R-segment and proximal A-segment of an ommatidium from a LE fixed during the night. 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/μm^2. (**C**) Representative images of single confocal optical sections showing Ops1-2- and $G_q\alpha$-ir in the R-segment and proximal A-segment of LEs fixed at midday (Day) and during the night (Night), between 4 and 6 h after sunset. Day-images and night-images of each antigen were immunostained and imaged together during the same confocal session, using identical confocal settings. Images were intensified in Photoshop to exactly the same extent and then assembled in CorelDraw. LDS, opsin-containing membranes shed during the day by light-driven shedding. Scale bar = 10 μm.

dark-adaptation *in vivo*. Because clock input to LEs is naturally silent during the day, we predicted that during daytime dark-adaptation, changes in RhOps1-2 and in $RhG_q\alpha$ concentrations would be similar to those observed during nighttime dark-adaptation in LEs without clock input. This is what we found. With 4 h of daytime dark-adaptation, RhOps1-2 concentrations increase significantly (approximately 20%) but also remain significantly below the concentration in normal nighttime dark-adapted eyes. The $RhG_q\alpha$ concentration does not increase at all during 4 h of daytime dark-adaptation and remains significantly below that in normal eyes dark-adapted at night.

Many effects of clock input on LEs are mediated by an increase in cAMP. We therefore tested whether the same might be true for the clock-driven increases in RhOps1-2 and $RhG_q\alpha$ concentrations. This was tested with *in vitro* experiments in which, during daytime dark-adaptation, slices of LEs were incubated without or with treatments known to increase intracellular cAMP: octopamine, the neurotransmitter released from the clock-driven efferent neurons that activates postsynaptic receptors coupled to adenylyl cyclase (Kaupp et al. 1982; Dalal and Battelle 2010) or forskolin, a direct activator of adenylyl cyclase (Beavo et al. 1970) (Fig. 6). These treatments significantly increased the dark-adaptive rise of RhOps 1-2 and $RhG_q\alpha$ concentrations by an average of 35% ($P<0.05$, $N=13$) and 65% ($P<0.01$, $N=10$), respectively (Battelle et al. 2013). These results provide evidence that, as is the case for many other effects of clock input to *Limulus* eyes (Table 1), the clock-dependent rise in RhOps1-2 and $RhG_q\alpha$ is mediated by a rise in cAMP.

The clock's effects on concentrations of RhOps1-2 may change the spectral sensitivity of LE photoreceptors

It is clear from the experiments described above that in LEs with intact optic nerves and endogenous clock input, the concentration of RhOps1-2 roughly doubles from day to night. Ops1-2, however, is not the only opsin present in rhabdoms of LE photoreceptors. The receptors contain at least one additional opsin, opsin 5 (Ops5) that belongs to a clade of opsins predicted to have a spectral sensitivity that is blue-shifted compared with Ops1-2 (Katti et al. 2010). Unlike RhOps1-2, RhOps5 concentrations do not change significantly from day to night and are not influenced by the clock (Katti et al. 2010). As a result, the relative levels of Ops1-2 and 5 in rhabdoms change significantly from day to night (Fig. 7). Quantification of relative concentrations of Ops1-2 and 5 in rhabdoms indicates that at night the mean molar ratio of Ops5 in rhabdoms is 20% ($N=6$) that of Ops1-2. This ratio is estimated to increase to 40% during the day because of the dramatic decrease in RhOps1-2 accompanied by no decrease in Ops5 (Fig. 6). Thus, although Ops1-2 remains the dominant opsins in rhabdoms both by day and by night, Ops5 should contribute more to the photoreceptor's response during the day. If the spectral

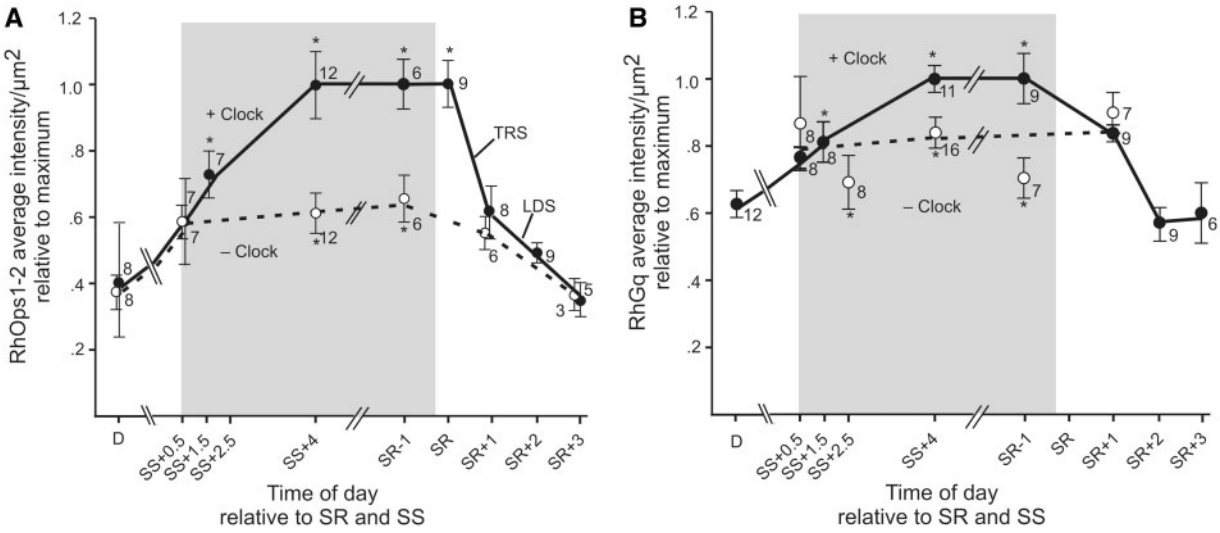

Fig. 5 Clock input drives increases in concentrations of (**A**) RhOps1-2 and of (**B**) Rh $G_q\alpha$. The average intensity of RhOps1-2- and $RhG_q\alpha$-ir per μm^2 of rhabdom is plotted versus time of day in hours relative to sunset (SS) and sunrise (SR). All animals were exposed to natural illumination. The shaded area indicates when it was dark in the room. The figure summarizes three series of studies: (1) A Dusk Series that included LEs fixed between midday (D) and SS + 4. (2) A Dawn Series that included LEs fixed from SR-1 to SR + 3. Concentrations of RhOps1-2 and $RhG_q\alpha$ did not change significantly between SS + 4 and SR-1; therefore, these series could be combined. The data are normalized relative to the maximum concentration. All eyes assayed in Series 1 and 2 had intact optic nerves and thus received normal clock input (+Clock) (filled circles and dotted line). The asterisk in the dusk series indicates when concentrations of rhabdomeral protein were significantly higher than at midday (D). In the dawn series, the asterisk indicates when RhOps1-2 concentrations were significantly higher than at SR + 1 and $RhG_q\alpha$ concentrations were significantly higher than at SR + 2 ($P < 0.05$). (3) In series 3, direct comparisons were made between RhOps1-2 or $RhG_q\alpha$ concentrations in LEs from the same animal, one with, and the other without, clock input. The open circles and dashed line shows the average concentrations of RhOps1-2 or $RhG_q\alpha$ in LEs lacking clock input (− Clock) relative to that in LEs with clock input (+ Clock). The asterisks (*) associated with these points indicate when there was a significant difference between RhOps1-2 or $RhG_q\alpha$ concentrations in LEs with, and without, clock input. Means are plotted ± SEM. The number at each time-point indicates the number of animals assayed. The fall in concentration of RhOps1-2 during the morning is attributed to transient rhabdom shedding (TRS), and light-driven shedding (LDS), as indicated. For details, see Battelle (2013).

sensitivity of Ops5 is significantly different from that of Ops1-2, this could produce a day-to-night shift in the LE's spectral tuning. Experiments designed to test this hypothesis are in progress.

Conclusions

Considering together all the known influences of the circadian clock on *Limulus* vision (Table 1), three important outcomes can be identified: an increase in visual sensitivity at night that is driven by the clock, a rapid decrease in visual sensitivity at dawn that is primed by the clock, and because clock input is silent during the day, maintenance of eyes in a relatively low state of sensitivity during the day, even when the eyes are in the dark. Increased visual sensitivity at night and a rapid decline in sensitivity at dawn may be critical for allowing the eyes to function optimally in very low light and in extremely bright light. Optimal visual function during the day and night may be critical for survival since *Limulus* use their eyes to find mates during spawning, and they spawn both during the day and at night (Barlow et al. 1982; Schwab and Brockmann 2007). Furthermore, *Limulus* often bury in the mud for long periods. Therefore, it may be particularly important for *Limulus* to maintain their eyes in a state of low sensitivity during the day, even in the dark, to prevent retinal damage should they emerge into the bright light of day.

Another potentially important consequence of the clock's influence on *Limulus* photoreceptors is a day-to-night shift in relative levels of co-expressed opsins in photosensitive membranes that may produce daily changes in the photoreceptor's spectral tuning. *Limulus* photoreceptors provide the first example of photoreceptors in which the levels of co-expressed opsin proteins in rhabdoms are regulated differently by light and a circadian clock, but evidence is accumulating that the photoreceptors of many invertebrates express multiple opsins. *Limulus* photoreceptors provide the first example for the differential regulation of opsin protein co-expression at

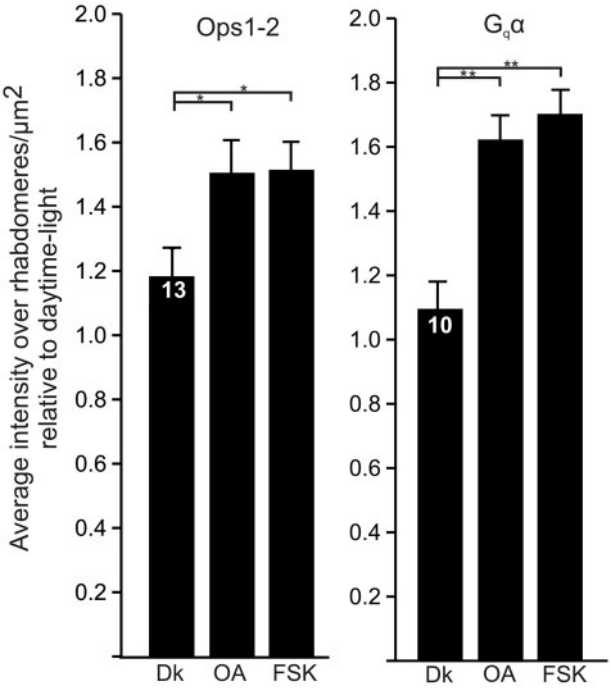

Fig. 6 Octopamine and forskolin increase concentrations of RhOps1-2 and Rh$G_q\alpha$ during daytime dark-adaptation *in vitro*. LEs were dissected from animals at midday. Each LE was cut in half to yield four slices from each animal. One slice from each animal was fixed immediately in the light. The other three slices were incubated 4 h at room temperature in the dark in an organ-culture medium (Katti et al. 2010) +0.08% dimethyl sulfoxide and one of the following: Dk, no further additions; OA, octopamine (40 μM)+ IBMX (1 mM); FSK, forskolin (10 μM) + IBMX (1 mM). RhOps1-2 and Rh$G_q\alpha$ were quantified in each slice as described in Fig. 5. Data are expressed as mean immunoreactive intensities/ μm^2 of rhabdom ± SEM. Rhabdomeral protein concentrations in each dark-adapted slice were normalized to those in the light-adapted slice of the same animal. The light-adapted concentrations are expressed as 1. The number of animals assayed is shown in the first bar of each dataset. The significances of differences among the dark-adapted treatments are as follows: $^*P < 0.05$; $^{**}P < 0.01$. From Battelle et al. (2013).

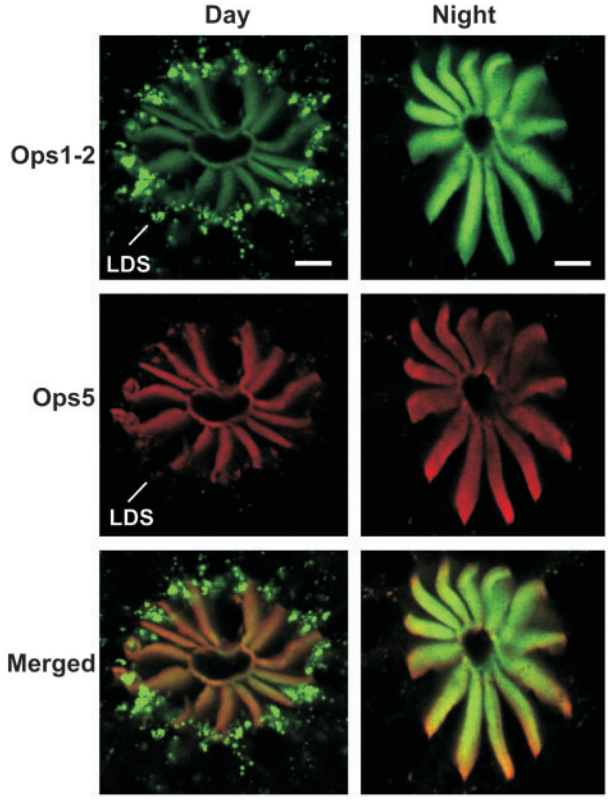

Fig. 7 The relative level of Ops1-2 and Ops5 in rhabdoms changes from day to night. Shown for each time-point are confocal images of sequential scans of single optical sections and their merged images (Ops1-2-ir, green; Ops5-ir, red). Sections were immunostained at the same time and imaged during a single confocal session using identical settings. All images were intensified in Photoshop to exactly the same extent. During the night, the intensity of RhOps1-2-ir is considerably higher than during the day, whereas the intensity of RhOps5-ir does not change significantly from day to night. Rhabdomeral membrane shed during the day by LDS contains both Ops1-2- and Ops5-ir, although Ops1-2-ir in debris is clearly more intense. Scale bar = 10 μm.

rhabdoms by light and a circadian clock, but as more species are investigated the phenomenon may be discovered to be wide-spread. Studies with *Limulus* also provide the first evidence that the clock's influence on levels of rhabdomeral opsin is exerted downstream of transcription (Dalal et al. 2003; Katti et al. 2010; Battelle et al. 2013) and involves processes regulated by cAMP.

Light-dependent and dark-dependent changes in the concentration of G-proteins activated by photopigments are observed in photosensitive membranes in both ciliated and rhabdomeral photoreceptors, and across species these changes are attributed to the translocation of the G-protein between the cytoplasmic and photosensitive compartments (Frechter et al. 2007; Arshavsky and Burns 2012). In no system are the mechanisms regulating these translocations understood. G-protein translocations in rhabdomeral photoreceptors have been examined in detail only in retinas of *Drosophila*, a preparation only marginally influenced by circadian rhythms. Studies of *Limulus* eyes provide the first evidence that $G_q\alpha$ translocation to photosensitive membranes in the dark can be regulated by a circadian clock and that this process involves regulation by cAMP.

Circadian changes in visual sensitivity have been observed in a wide range of arthropod species (reviewed by Fleissner and Fleissner 1988) but the processes impacted and the underlying mechanisms are largely unexplored. Mechanisms similar to those described in *Limulus* may be typical among

chelicerates. For example, the eyes of scorpions and spiders show circadian changes in sensitivity and, like *Limulus*, are innervated by clock-driven, octopaminergic efferent neurons that are active at night and silent during the day (Fleissner and Fleissner 1985; Yamashita 2002). Specific effects of circadian rhythms on vision will certainly vary with species and according to life styles. Studies of the circadian regulation of vision in *Limulus* have revealed that these effects can be extremely diverse and profound. Clearly, an understanding of the circadian clock's effects on vision is required for a full understanding of how animals adapt to the dramatic daily changes in ambient illumination.

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