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Circadian regulation of *Limulus* visual functions: A role for octopamine and cAMP

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Abstract The purpose of this contribution is to review our current understanding of the source and biochemistry of the circadian efferent input to the eyes of the American horseshoe crab *Limulus polyphemus* and the impact of this input on the structure, physiology and biochemistry of *Limulus* eyes. Special emphasis is given to the role of the biogenic amine octopamine and biochemical cascades it activates in the eyes. In addition to reviewing published data, we present new data showing that octopamine elevates cAMP levels in *Limulus* lateral eyes, and we partially characterize the pharmacology of the receptors involved in this response. We also present new data showing that octopamine regulates gene expression in *Limulus* lateral eyes by activating a cAMP cascade [*Current Zoology* 56 (5): 518–536, 2010].

Key words Horseshoe crab, Circadian rhythms, Octopamine, Photoreceptors, Substance P, Arrestin

1 Introduction

This contribution is in two parts. In Sections 2– 4, we review published evidence for the importance of central circadian clock(s) as regulators of vision in *Limulus*, describe the organization of the visual system and review what is currently known about the impact of circadian clocks on the structure and function of *Limulus* eyes. In Section 5, we focus on the role of the biogenic amine octopamine (OA) in mediating effects of the central circadian clock(s) on vision. We present new data showing that OA stimulates a rise in cAMP in *Limulus* lateral eyes (LEs), and that OA mimics the effect of the clock on visual arrestin (Varr) mRNA levels in LEs by activating the cAMP cascade. These new finding are discussed in the context of studies of OA receptors in other systems and other published studies which tested whether effects of the clock on *Limulus* vision are mediated by OA and through activation of the cAMP cascade.

2 Importance of Central Circadian Clocks for *Limulus* Vision

When American horseshoe crabs *Limulus polyphemus* are maintained in natural diurnal illumination, their compound LEs are about a million fold more sensitive to light at night then they are during the day (Barlow, 2001). This dramatic day/night change in sensitivity nearly compensates for the day/night change in ambient light levels in the animal's environment (Brown et al., 2004); thus *Limulus* are thought to see equally well during the day and the night. This idea is supported by behavioral studies which show that *Limulus* visual performance is similar during the day and night (Powers et al., 1991; Herzog et al., 1996).

Limulus use their LEs to find mates (Barlow et al., 1982; Duffy et al., 2006; Schwab and Brockmann, 2007; Saunders and Brockmann, 2010), and since spawning can occur during the day and during the night (Barlow et al., 1986), increased sensitivity of their LEs at night is presumably adaptive. Two things are required for LEs to achieve the full range of their normal, day/night sensitivity change, diurnal light and signals from an internal circadian clock.

The importance of a circadian clock for regulating LE sensitivity was first described by Barlow and his collaborators. They maintained animals in constant darkness and measured amplitudes of electroretinograms (ERGs) recorded from LEs in response to dim flashes of light. They showed that LEs become dra-

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matically more sensitive to light during the subjective night compared to the subjective day (Barlow et al., 1977; Barlow, 1983). They showed further that the responsiveness (amplitude of the photoresponse at a given light level) of LE photoreceptors increases during the subjective night (Barlow et al., 1987), and that increases in sensitivity and responsiveness correlate with the activity of efferent axons that project from the brain to the eyes through the optic nerves. These efferent axons fire action potentials during the subjective night and are silent during the day (Barlow et al., 1977; Barlow, 1983). Phase shifting studies confirmed the circadian nature of changes in LE functions and in the activity of efferent axons (Barlow, 1983). Direct evidence that efferent nerve activity was responsible for increased LE sensitivity and responsiveness came from a series of studies which demonstrated that these nighttime increases were abolished when efferent synaptic input to the eye was blocked by severing the lateral optic nerve (LON), and that LE sensitivity and responsiveness increased during the subjective day when efferent axons in the severed LON leading to the eye were electrically stimulated to activate their synaptic inputs to the eye (Barlow et al., 1977; Barlow, 1983).

Circadian, nighttime increases in ERG amplitudes were also recorded from ventral and median eyes (Kass and Renninger, 1988; Barlow, 1983). Circadian changes in median eyes depend on an intact optic nerve, and they are synchronous with those in LE (Barlow, 1983). Furthermore, bursts of action potentials recorded from efferent axons in all of the optic nerves—lateral, median and ventral—are synchronous (Kass and Barlow, 1992). This suggests the efferent projections in all optic nerves originate from the same cells or from groups of cells with tightly coupled activity.

Most of the *in vivo* studies described in the preceding paragraphs utilized animals maintained in constant darkness. More recently, Pieprzyk et al. (2003) showed that clock-driven efferent nerve activity contributes significantly to functional changes in LEs of animals maintained under natural diurnal illumination. Specifically, they found that in LEs with cut optic nerves, and thus deprived of synaptic input from the clock-driven efferent neurons (called here "clock input"), the nighttime increase in ERG amplitude was only half that observed in LEs that received normal clock input. It is also clear from a number of different studies that both clock input at night and diurnal light are required for LEs to achieve their normal daytime, light-adaptive state (Barlow et al., 1980; Chamberlain and Barlow, 1987; Kier and Chamberlain, 1990; Pieprzyk et al., 2003).

The role of circadian clocks in producing the dramatic diurnal changes in *Limulus* visual functions cannot be fully appreciated without first understanding the organization of the *Limulus* visual system.

3 Organization of the *Limulus* Visual System

3.1 General organization

Limulus has three major types of eyes, lateral compound eyes, median ocelli and rudimentary eyes. Its lateral compound eyes and median ocelli are apparent on the dorsal carapace of adult animals (Fig. 1A). Diagrams of a longitudinal and cross section of a LE ommatidium are shown in Fig. 1C. Its three types of rudimentary eyes—lateral, median and ventral—consist of clusters of large photoreceptors (Calman and Chamberlain, 1982; Fig. 1D) which differentiate in the embryo before the compound LEs and median ocelli. They presumably provide photic information to the embryo and larvae (Harzsch et al., 2006). The early development of the visual system and the visual performance of larvae and juvenile *Limulus* are discussed in more detail by Medina and Tankersley (2010).

In adult animals, the lateral rudimentary eyes are located under the carapace at the posterior edge of each LE and a fused pair of median rudimentary eyes is located under the carapace between the two median ocelli (Fig. 1A). The ventral rudimentary eyes consist of a pair of optic nerves that extend anteriorly from the brain and terminate in an end organ located beneath a wart-like structure visible on the ventral cuticle in front of the mouth (Fig. 1A and B). Ventral photoreceptors are scattered along the length of the ventral optic nerves, and clustered in the end organ and clustered close to where the ventral optic nerves exit the brain.

3.2 *Limulus* eyes are innervated by octopaminergic, clock-driven efferent neurons

All *Limulus* eyes are innervated by structurally identical efferent axons that project to the eyes through the optic nerves (Fahrenbach 1969; 1971; 1973; 1975; 1981; 1985; Fahrenbach and Griffin, 1975). Their axons are small, between 0. 5 and 2 μm in diameter, and their terminals have an ultrastructure that is typical for neurosecretory terminals in arthropods. They contain clear vesicles and large, dense granules, and they have a presynaptic, but no postsynaptic, density. These efferents innervate all cell types in LE ommatidia (Fig. 2A): cone cells that occupy the aperture at the base of the lens, guanophores, proximal and distal pigment cells, photoreceptors and eccentric cells. The latter are electrically

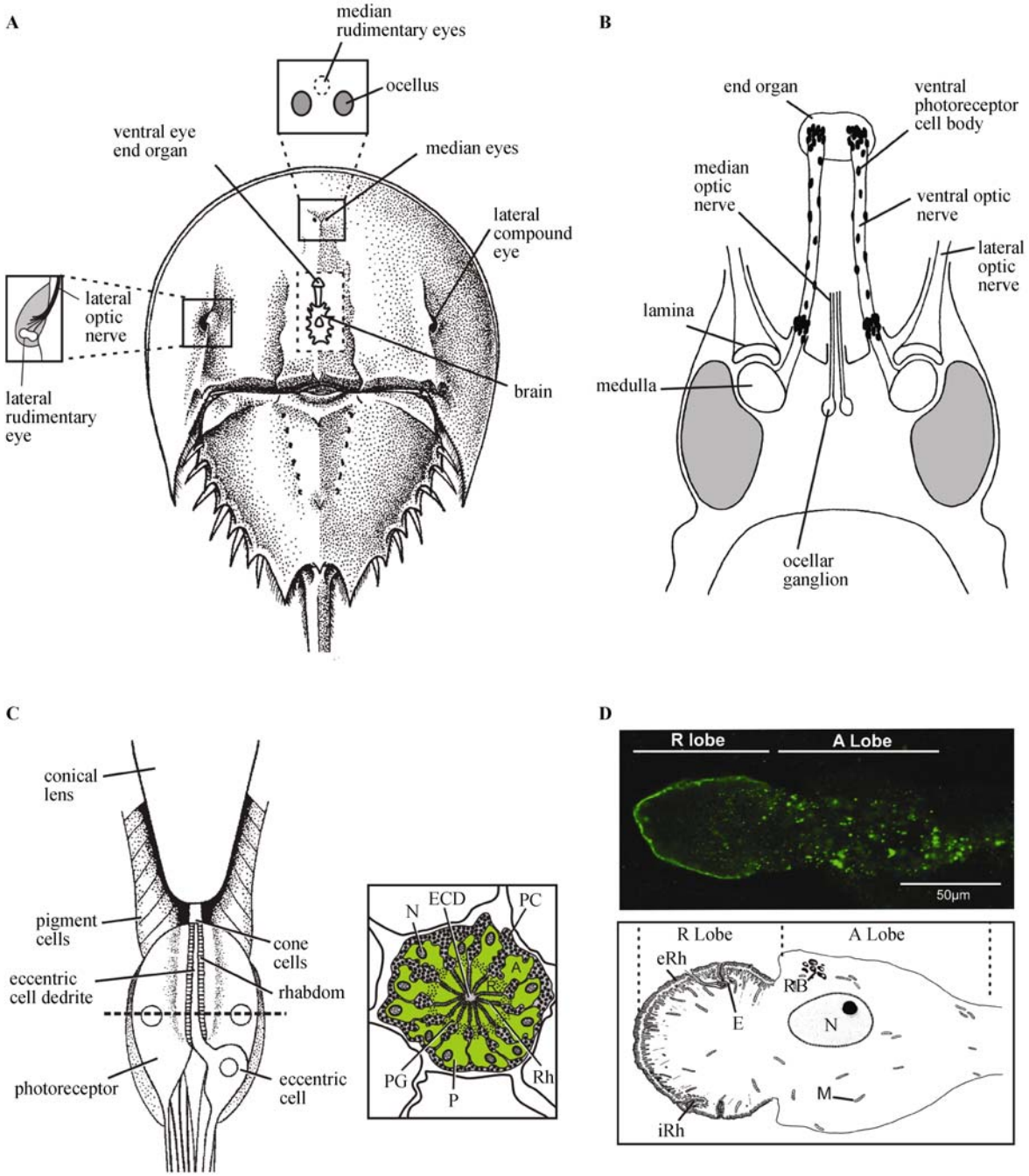

Fig. 1 Schematic of the *Limulus* visual system

A. Schematic of the dorsal view of *Limulus* showing the locations of its eyes. Box on the left: Enlargement of LE to show the location of the lateral rudimentary eye. Box above: Location of the median rudimentary eyes under the carapace between the median ocelli. Dotted rectangle in center: Cut-away to show the locations of the brain and ventral eyes which lie near the ventral surface. **B**. Schematic of a dorsal view of the protocerebrum (brain) and ventral optic nerves at the anterior end of the circumesophogeal ring. The locations of the optic ganglia (lamina and medulla) and ventral photoreceptor cell bodies (dark ovals) are indicated. **C**. On the left is a schematic of a longitudinal section through a lateral eye ommatidium showing the major cell types. The dashed line shows the approximate level of the cross section diagramed on the right. **D**. Upper panel: Section through a ventral photoreceptor immunostained for opsin1-2. The opsin immunoreactivity outside of the rhabdom is within shed rhabdomeral debris. Lower panel: A schematic of a ventral photoreceptor cell body (based on Calman and Chanberlain, 1982).

A: arhabdomeral segment; A lobe, arhabdomeral lobe; E, efferent axon; ECD, eccentric cell dendrite; eRh, external rhabdom; iRh, internal rhabdom; M, mitochondria; N, nucleus; P, photoreceptor; PC, pigment cell; PG, pigment granules in photoreceptors; R, rhabdomeral segment; R lobe, rhabdomeral lobe; RB, ribosomes; Rh, rhabdom.

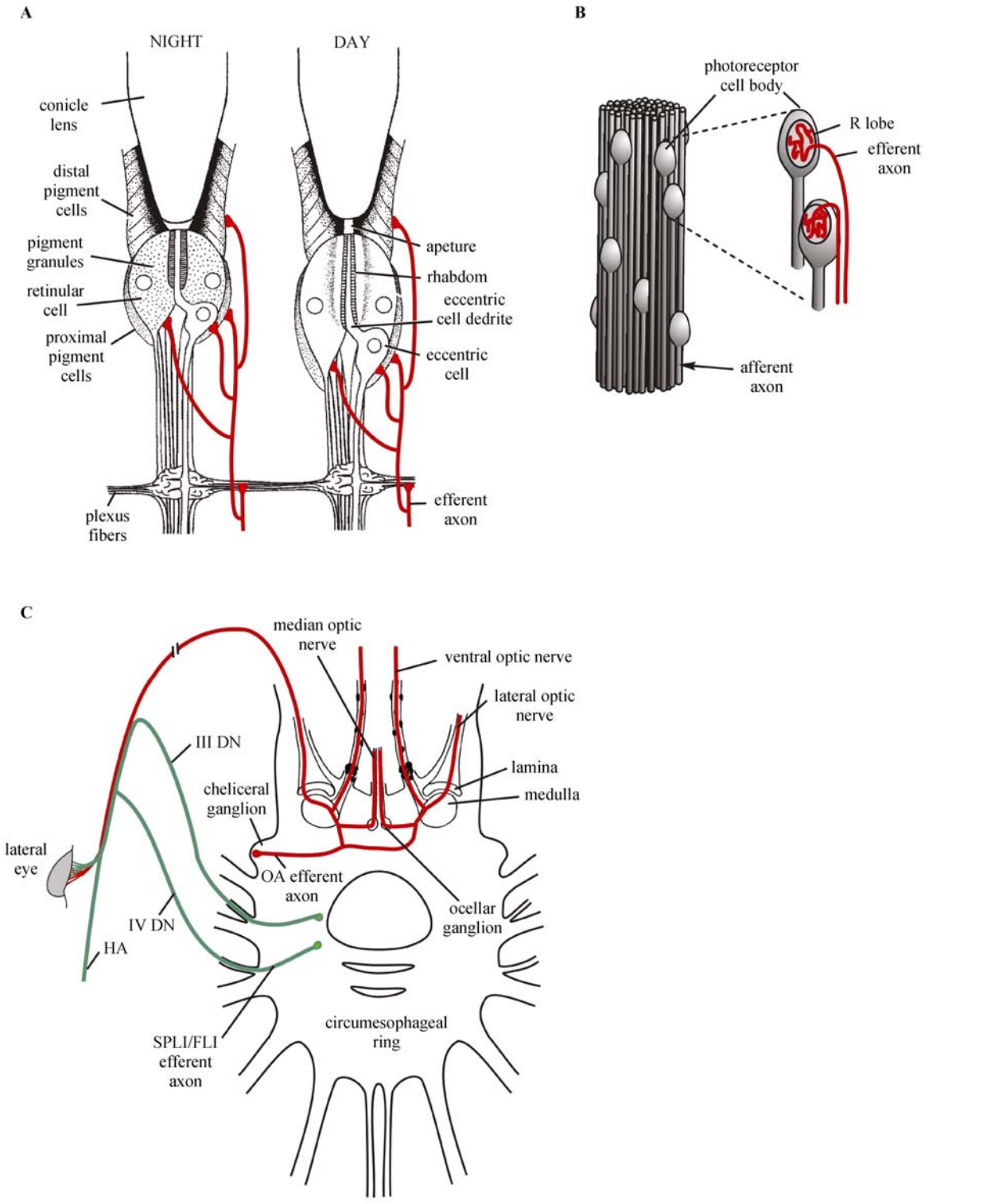

Fig. 2 Schematic of efferent projections to *Limulus* eyes

A. Schematic of longitudinal sections through LE ommatidia in their nighttime and daytime states (based on Barlow et al., 1980; Chamberlain and Barlow, 1987). Also modeled are projections of the OA-containing efferent neurons (red) showing the cell types they are known to innervate (based on Fahrenbach, 1981; 1985). **B**. Left: Schematic of a piece of a ventral optic nerve showing somata of ventral photoreceptors. Right: Models of two ventral photoreceptor somata with projections of OA-containing efferent projections (red) specifically targeting the rhabdomeral lobe (R lobe) (Battelle et al., 1982; Evans et al., 1983). **C**. Schematic of the brain and circumesophogeal ring of *Limulus* showing the locations of the cell bodies and projections of OA-containing efferent axons (red) and SPLI/FLI-containing efferent axons (green).
III DN, third dorsal nerve; IV DN, fourth dorsal nerve; HA, hepatic artery. (based on Calman and Battelle, 1991 and Mancillas and Brown, 1984).

coupled to photoreceptors and generate action potentials in response to light-stimulated photoreceptor depolarizations. Terminals of the same class of efferent axons innervate photoreceptors in the median eye and terminate specifically at the rhabdom of ventral eye photoreceptors (Fig. 2B) (Clark et al., 1969; Calman and Chamberlain, 1982; Battelle et al., 1982; Evans et al., 1983).

The large, dense granules —100 by 400 nm cylindrical structures that have a crystalline substructure and a depression at one end (Lee and Wyse, 1991)—observed in these efferents are characteristic of octopamine (OA)-containing neurons in *Limulus,* and there is good evidence these efferents use OA as a neurotransmitter. Radiolabelled tyramine, the immediate precursor to OA, is specifically taken up by these dense granule-containing axons and terminals, they synthesize and store OA from tyramine and tyrosine, and they release OA upon depolarization (Battelle et al., 1982; Evans et al., 1983; Battelle and Evans, 1984). The presence of dense granules as well as clear vesicles in these efferents suggests they contain and release more then one neuroactive molecule. Since dense granules are characteristic of neurons that release neuropeptides (For example see chapter 20 of Chapman, 1998), it is speculated that the OA-containing efferents also release one or more neuropeptides.

Cell bodies of neurons that give rise to this class of efferent axons are located within the cheliceral ganglia at the anterior end of the circumesophogeal ring very near the protocerebrum. They were found by backfilling proximal segments of optic nerves with Neurobiotin (Calman and Battelle, 1991). Interestingly, backfilling any single optic nerve—lateral, median or ventral—consistently labeled cheliceral ganglion cells on both sides of the brain. This suggests each eye is innervated by efferent neurons from both sides of the brain. Backfilling a single optic nerve of any type also filled axons projecting out each of the other optic nerves. This suggests each efferent neuron branches in the brain and innervates more than one eye and more than one type of eye. The branching pattern of an idealized efferent neuron shown in Fig. 2C is based on reconstructions of fills from all three optic nerves (Calman and Battelle, 1991) and is consistent with observations that efferent axons in proximal regions of all optic nerves fire synchronously (Kass and Barlow, 1992).

Considerable evidence indicates that OA-containing efferent projections in the optic nerves are the clock-driven efferent axons which regulate circadian changes in the functions of *Limulus* eyes. As was described above, all three types of eyes are innervated by circadian efferent axons and all three show circadian changes in function. Circadian efferent nerve activity has been recorded from the proximal regions of each type of optic nerve (Kass and Barlow, 1992) where the OA-containing efferents are the only type of efferent axons present. In addition, the number of cell bodies that became labeled in an individual cheliceral ganglion following the most complete Neurobiotin backfills of optic nerves was 21, which correlates well with the 12 to 24 OA-immunoreactive cell bodies present in each cheliceral ganglion (Lee and Wyse, 1991). Finally, as will be discussed below, most known effects of circadian clock input to LEs can be mimicked by applying OA.

It must be emphasized here that the OA-containing cell clusters within the cheliceral ganglia which innervate the eyes are not the only OA-containing cell clusters within the *Limulus* central nervous system. Clusters of OA-containing cells are present in each segment of the circumesophogeal ring (Lee and Wyse, 1991), and as is described by Wyse (2010), OA is a major modulator of *Limulus* central pattern generators.

3.3 LEs are innervated by a second class of efferent neurons that may influence LE function

A second, entirely separate type of efferent projection has been implicated in the regulation of LE function. This projection contains Substance P (SP)-like and FMRFamide-like immunoreactivity (SPLI/FLI) (Chamberlain and Engbertson, 1982; Mancillas and Brown, 1984; Mancillas and Selverston, 1984; Lewandowski et al., 1989; Bolbecker et al., 2009). They arise from cell clusters located posterior to the cheliceral ganglia in the circumesophogeal ring, project out the third and fourth dorsal segmental nerves and join the LON near the LE (Mancillas and Brown, 1984; Mancillas and Selverston, 1985; Lewandowski et al., 1989) (Fig. 2C). Since SPLI/FLI axons are not present in proximal regions of any optic nerves, they cannot be the clock-driven efferent axons detected in optic nerves near the brain (Kass and Barlow, 1992). Another difference between octopaminergic and SPLI/FLI projections is that while octopaminergic projections from cells in the cheliceral ganglion appear to specifically innervate eyes, the SPLI/FLI projection to LEs appears to be part of a more generalized epidermal innervation (Chamberlain and Engbretson, 1982; Mancillas and Brown, 1984; Lewandowski et al., 1989).

Physiological studies show that injections of SP into LEs increase LE sensitivity to light (Mancillas and

Selverston, 1984; Bolbecker et al., 2009) and decrease the latency of the ERG (Bolbecker et al., 2009). Based on these findings, it has been proposed that SP participates in the circadian regulation of LE function. This possibility clearly deserves further investigation. For example, it would be important to determine whether the activity of the SPLI/FLI input to LEs is circadian. Furthermore, the distribution of the SPLI/FLI projections in LEs remains controversial with some (Marcillas and Brown, 1984) suggesting these axons innervate pigment cells, photoreceptors and eccentric cells while others (Chamberlain and Engbretson, 1982; Lewandowski et al., 1989) maintain they do not penetrate ommatidia. Finally, it must be pointed out that the SPLI/FLI projection cannot be required for all observed circadian changes in visual function because circadian changes are observed in ventral eyes (Kass and Renninger, 1988) where SPLI/FLI axons are absent (Chamberlain and Engbretson, 1982; Mancillas and Brown, 1984).

3.4 The central circuitry which drives and modulates circadian efferent nerve activity is complex and not fully understood

Bilaterally synchronous circadian efferent nerve activity can be recorded from all of the optic nerves *in vitro* in a preparation that includes only the protocerebrum (Kass and Barlow, 1992). This is strong evidence that the circadian oscillator(s) which drive the activity of octopaminergic efferent axons is located within the protocerebrum. When the protocerebral bridge in this preparation is cut, efferent axons continue to fire but their bilateral firing pattern becomes asynchronous. This suggests circadian oscillators are located on each side of the protocerebrum, and that these oscillators are synchronized by processes that pass through the protocerebral bridge. The cells in the cheliceral ganglion that give rise to the octopaminergic efferent projections may themselves be circadian oscillators; alternatively, and more probably, these retinal efferents may be driven by central oscillators located elsewhere. In scorpions, a close relative of *Limulus* in which circadian retinal efferent axons also originate from cells within cheliceral ganglia, the timing input from central oscillators is thought to be transmitted to retinal efferent axons via efferent axon collaterals in the optic ganglia (Fleissner and Heinrichs, 1982; Fleissner, 1983; Heinrichs and Fleissner, 1987).

The visual circuitry that sets the phase of central oscillators driving clock input to the eyes is complex and not yet understood. These oscillators can be phase shifted by illuminating any of the eyes individually or in combination (Horne and Renninger, 1988), indicating that each eye contains photoreceptors which can entrain the clock. However, each type of eye impacts the clock differently. Only small phase shifts are observed when each type of eye is illuminated individually and when ventral and median eyes are illuminated together. Larger phase shifts occur when LEs are illuminated in combination with ventral or median eyes, and the largest phase shifts occur when all three types of eyes are illuminated simultaneously. This suggests information from individual eyes is integrated centrally to phase shift the clock.

Surprisingly, whole animal illumination produces a greater phase shift than illuminating all of the anterior eyes simultaneously, suggesting extraocular photoreceptors also phase shift the clock (Horne and Renninger, 1988). Indeed, illuminating only the tail (telson) with white light produces large and consistent phase shifts, and occluding the tail consistently reduces the magnitude of the phase shift produced by general illumination (Hanna et al., 1988). These and other studies (Renninger et al., 1997) provide convincing evidence that photosensitive cells in the tail of *Limulus* can, by themselves, effectively phase shift the circadian oscillator(s) which drive efferent nerves innervating the anterior eyes. The tail photoreceptors have not yet been identified and their projections remain unknown.

Circadian efferent nerve input to the LE can be modulated acutely by illuminating the tail with white light or by illuminating the median eyes with UV light. Efferent nerve activity and LE sensitivity at night is transiently depressed by illuminating the tail (Hanna et al., 1985; 1988; Renninger et al., 1997) while LE sensitivity at night is enhanced by illuminating the ME with UV light (Herzog and Barlow, 1991). Stimulating median eye afferents *in vitro* in the isolated brain preparation also increases efferent nerve activity in both lateral and median optic nerves (Kass and Barlow, 1992).

4 Impact of Circadian Efferent Nerve Activity on *Limulus* Eyes

4.1 Structure and function

The overall effect of endogenous circadian efferent nerve activity is to increase the sensitivity and responsiveness of LEs to light at night and the nighttime responsiveness of median and ventral eyes. Structural and functional changes in the eyes that correlate with clock-driven increases in sensitivity and responsiveness have been studied most in LEs. These have been reviewed extensively elsewhere (Barlow et al., 1989) and are summarized here (Table 1).

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

Retinal response to circadian efferent input	References	Mimicked by OA or elevating cAMP	References
Sensitivity (ERG amplitude) increases	a, b	OA	c
ERG latency decreases	d	No	d
Structure changes (Fig. 1)	e, f	OA inferred	c
Responsiveness increases	a, g, h	OA or cAMP	i, j,
Noise decreases	a, g, h	OA or cAMP	i
Quantum bumps longer	g, h	OA or cAMP	j
Transient shedding primed	k, l	OA or cAMP	m, n
Photomechanical movements of pigment granules primed	f, o	Not determined	
Myo21 phosphorylation increases at PKA sites	p, q	OA or cAMP	r, s, t
Varr transcript levels decrease	u	OA or cAMP	This study

a. Barlow et al., 1977; b. Barlow, 1983; c. Kass and Barlow, 1984; d. Bolbecker et al., 2009**;** e. Barlow et al., 1980; f. Chamberlain and Barlow, 1987;g. Kaplan and Barlow 1980;h. Barlow et al., 1987; i. Kass et al., 1988; j. Renninger et al., 1989; k. Chamberlain and Barlow, 1979; l. Chamberlain and Barlow, 1984; m. Khadilkar et al., 2002; n. Runyon et al., 2004; o. Kier and Chamberlain 1990; p. Edwards et al., 1990; q. Cardasis et al., 2007; r Edwards and Battelle, 1987; s. Battelle et al., 1998; t. Kempler et al., 2007;u. Battelle et al., 2000.

LE ommatidia normally undergo diurnal changes in structure (Fig. 2A). Towards dusk, when efferents become active, the aperture at the base of the lens widens and shortens permitting more photons to reach underlying rhabdomeres. The rhabdom shortens and widens, adjusting to the larger aperture, and photoreceptor pigment granules disperse. These structural changes persist with lower amplitudes in constant darkness, but they are abolished, even in diurnal light, if clock input to the eye is eliminated by cutting the LON. Thus, clock input is required for structural changes to occur, and diurnal light increases their amplitudes.

Much of the increased sensitivity observed in LEs at night can be attributed to the structural changes described above which increase photon capture (Barlow et al., 1980; Chamberlain and Barlow, 1987), but clock input also directly impacts the photoresponse. When clock input is active, the duration of the elemental photoresponse (quantum bump) increases, and the frequency of spontaneous depolarizations recorded in the dark (noise) decreases (Kaplan and Barlow 1980; Kaplan et al., 1990). Clock input also primes light driven processes in photoreceptors including synchronous transient rhabdom shedding, which occurs at first light (Chamberlain and Barlow 1979, 1984) and light-adaptive photoreceptor pigment migration (Chamberlain and Barlow 1987; Kier and Chamberlain, 1990). These processes are initiated by light but occur only in eyes that receive prior clock input.

4.2 Posttranslational modification of proteins

Clock input enhances the phosphorylation of the *Limulus* homologue of the *Drosophila* NINAC protein (Montell and Rubin, 1988; Edwards et al., 1990; Battelle, 1998; Cardasis et al., 2007). *Drosophila* NINAC and its *Limulus* homologue are unconventional myosins characterized by an N-terminal kinase domain, a myosin motor-like domain, one or more IQ calmodulin binding motifs and a C-terminal tail of varying lengths (Fig. 3A). Both are active kinases, bind calmodulin and bind actin (Porter and Montell, 1993; Porter et al., 1993; 1995; Ng et al., 1996; Hicks et al., 1996; Battelle et al., 1998; Kempler et al., 2007). Originally called class III myosins, NINAC and its homologues have recently been reclassified as class XXI myosins or Myosin21 (Myo21) based on the sequences of their myosin domains (Odronitz et al., 2009). In keeping with this most recent classification, here we refer to NINAC homologues as class XXI myosins and to the *Limulus* homologue as *Limulus polyphemus* Myo21 (LpMyo21).

LpMyo21 expression is photoreceptor specific, and its concentration in photoreceptors is high, roughly 2.4% and 5.8% of the total soluble protein in LE and LON, respectively (Cardasis et al., 2007). It is present throughout *Limulus* photoreceptors from their cell bodies to their terminals (Harzsch et al., 2006), and in daytime, light-adapted LE photoreceptors, it concentrates over the rays of the rhabdom (Fig. 3B). In *Drosophila melanogaster* (Dm) photoreceptors, where Myo21 is also highly expressed, the protein has been implicated in a variety of processes impacting photoreceptor function including the termination (Li et al., 1998; Liu et al., 2008) and modulation of the photoresponse (Chyb et al., 1999), calmodulin localization (Porter et al., 1993), pigment migration (Hofstee et al., 1996) and the translocations of Varr to, and $G_q\alpha$, from rhabdomeres (Cronin et al., 2004; Lee and Montell; 2004, but also see Satoh

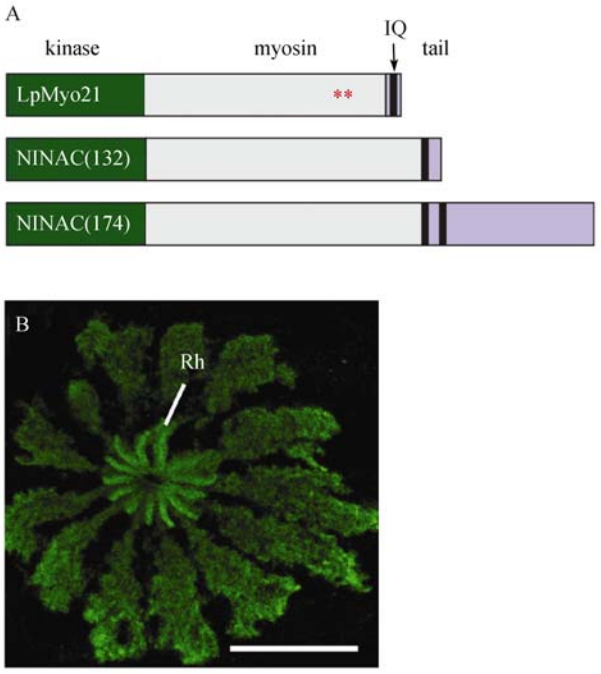

Fig. 3 LpMyo21, a photoreceptor protein targeted for modulation by the clock

A. Schematic of the domain structures of LpMyo21 and the *nina*C gene products of *Drosophila* which are splice variants. LpMyo21 is a target for clock-stimulated phosphorylation within its myosin-like domain (red asterisks). **B**. Fixed frozen section of a *Limulus* LE ommatidium immunostained for LpMyo21 immunoreactivity and imaged with a confocal microscope. A single optical section is shown. Rh: rhabdom. Scale bar = 50 μm. (Based on Battelle et al., 1998; Kempler et al., 2007; Cardasis et al., 2007).

and Ready 2005). Varr is the protein responsible for quenching phototransduction and $G_q\alpha$ is the G protein activated by rhabdomeral opsins. Furthermore, photoreceptors lacking DmMyo21 undergo a light-dependent retinal degeneration (Montell and Rubin, 1998). We speculate the modulation of LpMyo21 by the circadian clock mediates some of the circadian changes in *Limulus* photoreceptor function.

LpMyo21 is unusual in that its actin binding affinity is independent of ATP concentration, and it lacks motor activity (Kempler et al., 2007). Furthermore, it becomes phosphorylated in response to clock input at two canonical PKA sites (Pearson and Kemp, 1991) within and near an important actin binding region of the myosin domain. DmMyo21 binds to actin within the photosensitive microvilli, and when DmMyo21 is lacking, the actin within these microvilli is fragmented or missing even before eclosion (Hicks et al., 1996). Taken together, these observations lead us to propose that phosphorylation of LpMyo21 influences the stability of actin in the rhabdom. A clock-driven change in LpMyo21's affinity for actin in the microvillar cores of rhabdomeres could also change the concentration of LpMyo21 at the rhabdom and lead to changes in functions that depend on the kinase activity of LpMyo21. This idea is consistent with observations showing that the photoresponse is abnormal in *Drosophila* expressing a Myo21 lacking the kinase domain even though the photoreceptors are structurally normal (Porter et al., 1993). To understand how LpMyo21 influences photoreceptor function, it will be important to indentify its endogenous substrates.

4.3 Gene expression

Clock input to LEs controls an early step in the expression of visual arrestin (Varr) (Battelle et al., 2000). Varr is the protein responsible for terminating phototransduction in rhabdomeral photoreceptors (Dolph et al., 1993; Ranganathan and Stevens, 1995). In LE photoreceptors, the level of mRNA encoding Varr fluctuates with a diurnal rhythm (Fig. 4A) and is higher during the day than during the night. This day-night fluctuation persists undiminished in LEs deprived of diurnal light if they have intact optic nerves (Fig. 4B). However, the nighttime fall in Varr mRNA levels is eliminated if the LON is cut, depriving the LE of clock input, even when the eye is exposed to diurnal light (Fig. 4C).

Changes in Varr mRNA levels may influence the concentration of Varr protein at the rhabdom where the ratio between Varr and visual pigment is critical for determining the time course of the photoresponse (Dolph et al., 1993). Recall that Varr is responsible for quenching the photoresponse. Thus, a clock-regulated change in the concentration of Varr at the rhabdom could explain some of the circadian changes in the photoresponse described above such as the change in quantum bump duration. Experiments are in progress to test whether clock input does, in fact, reduce Varr at the rhabdom in nighttime eyes.

Clock input does not regulate mRNA levels for all proteins important for the photoresponse. For example, the levels of mRNAs encoding two very similar opsins, opsins1 and 2 (Ops1-2), the most abundant visual pigments expressed in LE photoreceptors (Katti et al., 2010), are regulated by light not by clock input (Dalal et al., 2003). However, a later stage of Ops1-2 expression is influenced by the clock.

In LEs exposed to normal clock input and natural diurnal illumination, Ops1-2 protein in the rhabdom is shed during the day under natural illumination and falls to about half its normal nighttime level (Katti et al., 2010). In order for rhabdomeral Ops1-2 to be restored to its higher nighttime level, new or recycled Ops1-2

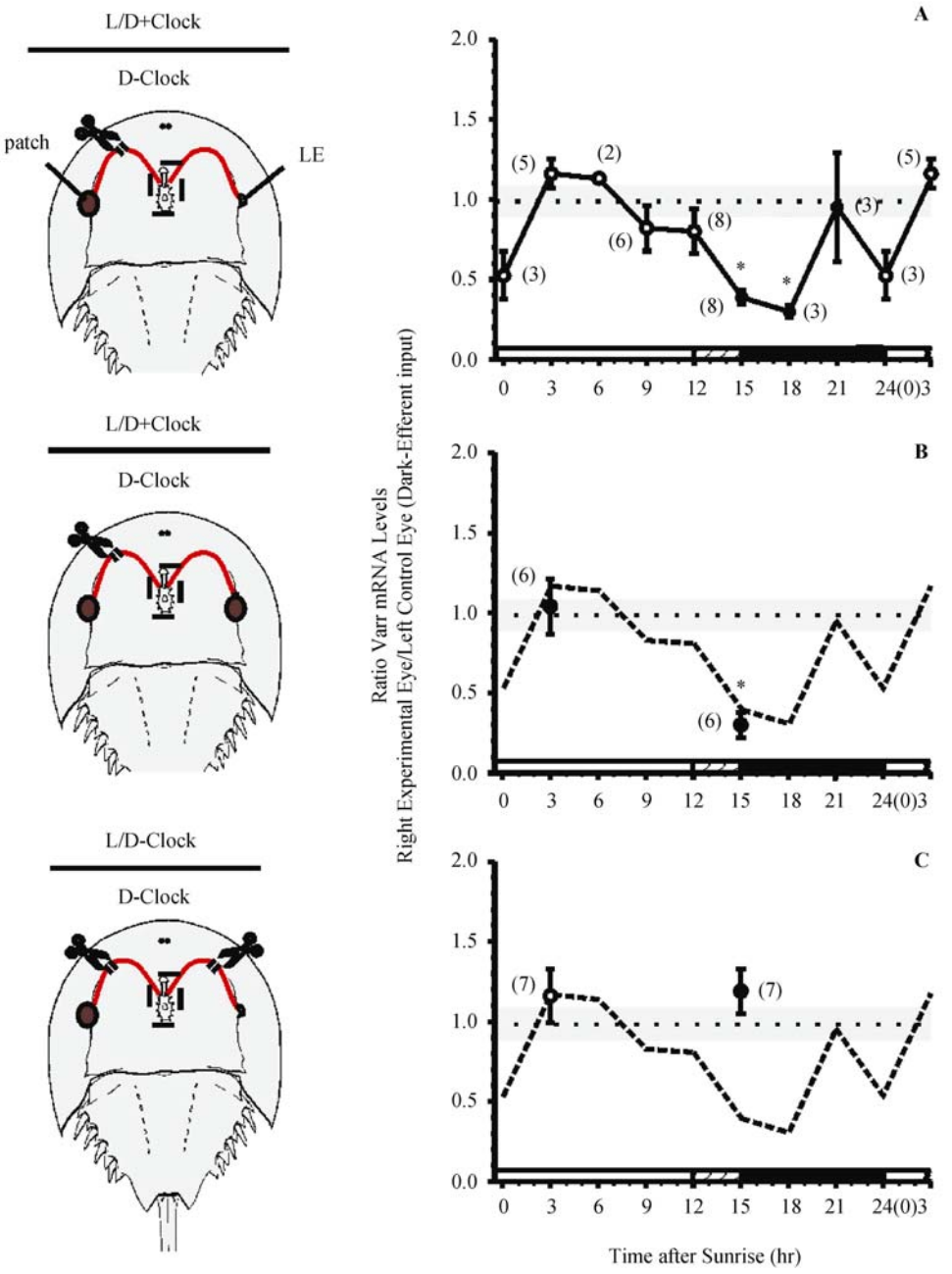

Fig. 4 Clock input regulates Varr mRNA levels

Varr mRNA levels in left control eyes that were patched (dark circle over the LE) to eliminate light input and had cut LONs (scissors and break in the LON) to eliminate clock input were compared to that in right experimental eyes that were: **A**. exposed to normal diurnal light and received clock input. **B**. maintained in the dark but received clock input. **C**. exposed to diurnal light and were deprived of clock input (had a cut LON). The cartoons on the left illustrate the comparisons made. Varr mRNA levels in each eye were measured relative to 18s ribosomal RNA. Comparisons of mRNA levels were always made between control and experimental eyes of the same animal, and the mean ratio ± the standard error of the mean was plotted against the time after sunrise the eyes were dissected from the animals. The 0 hr and 3 hr points at the left of the X axis are replotted as 24 hr and 3 hr on the right. All animals were maintained in natural light; the bar on the X axis shows when the animals experienced light (open bar), darkness (solid bar) and twilight (hatched bar). Experimental eyes were collected in the light (open circles) or in the dark (closed circles). Control eyes were always collected in the dark (infrared illumination). The numbers in parentheses indicate the number of animals assayed; asterisks indicate ratios that are significantly different from that obtained by comparing two untreated eyes from the same animal. The dotted line and shaded area show the mean ratio ±standard error of the mean obtained from comparisons of two untreated eyes of the same animal. The mean results obtained in A are replotted as dashed lines in B and C. The results show that the diurnal rhythm in Varr mRNA levels observed in panel A persists in eyes maintained in constant darkness (patched) if the eye receives clock input, i. e. has an intact LON (panel B). But the rhythm is eliminated, even in diurnal light, if clock input to the eye is eliminated (cut LON) as in panel C. (reprinted from Battelle et al., 2000 with permission from Cambridge University Press).

protein must be inserted into the rhabdom at the end of the day. In LEs with cut optic nerves, and thus deprived of clock input, the nighttime level of rhabdomeral Ops1-2 is about 36% lower than in nighttime LEs with intact optic nerves (Katti et al., 2010; Fig. 5A and B). Thus, clock input is required for the higher nighttime protein levels of Ops1-2 in the rhabdom to be fully re-established. Since eliminating clock input does not influence Ops1-2 mRNA levels (Dalal et al., 2003), one or more processes down stream of transcription must be clock-regulated, such as translation, or the transport or insertion of opsin into the rhabdom.

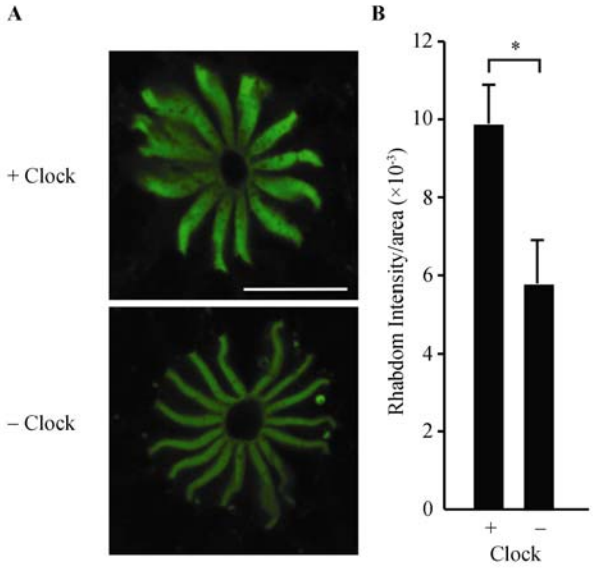

Fig. 5 Clock input regulates Ops1 levels at photosensitive rhabdomeres

A. Laser confocal images of single optical sections through LE ommatidia immunostained for Ops1. LEs were fixed at night in the dark and are from the same animal. One LE received normal clock input (+ Clock); the LON to the other eye had been cut depriving it of clock input (-Clock). The intensity of the Ops1 immunoreactivity at rhabdomeres appears lower in the ommatidium of the eye that lacked clock input (scale bar=50 μm). **B**. Shows the average intensities of Ops1 immunoreactivity ± the standard errors of the means over the rhabdomeres of LEs with and without clock input. The average intensity of Ops1 over the rhabdomeres in each eye was determined by averaging the intensity in 8 separate ommatidia and eyes from 12 different animals were analyzed * indicates P<0. 05 (Student's *t* test) (Based on Katti et al., 2010)

5 What Is the Role of Octopamine?

5.1 Introduction

The effects of clock input to *Limulus* eyes are clearly profound and diverse. A major goal and challenge is to characterize the underlying biochemistry. Some progress has been made. Fig. 6 shows a biochemical cascade thought responsible for many known effects of the clock. As was described above, there is substantial evidence that the clock-driven efferent axons which project out all the optic nerves synthesize, store and release the biogenic amine octopamine (OA), and that OA stimulates a rise in cAMP in ventral photoreceptors (Kaupp et al., 1982). Here we show that OA stimulates rise in cAMP in LEs and partially characterize the pharmacology of adenylyl cyclase-linked OA receptor(s) in lateral and ventral eyes. We also show that elevated cAMP and activation of cyclic-AMP dependent protein kinase (PKA) mimic the clock-driven fall of Varr mRNA levels in LE. Our results provide additional evidence that some effects of the clock on *Limulus* eyes are mediated by an OA-stimulated rise in cAMP and activation of PKA. They also suggest that OA-driven structural changes in LEs are mediated via a cAMP-independent cascade.

5.2 Materials and Methods

Animals Adult *Limulus* were either collected from the Indian River, near Cape Canaveral, FL, or purchased from the Marine Biological Laboratory, Woods Hole, MA. They were maintained in natural running seawater and in natural diurnal light provided by a skylight in the aquarium room. The temperature of the seawater was held at approximately 15°C.

Reagents The reagents were purchased from Fischer Scientific (www. fishersci. com) or Sigma-Aldrich (www. sigmaaldrich. com) unless otherwise specified.

cAMP Assays Tissues were dissected early afternoon into *Limulus* Ringer plus dextrose (LR) (Warren and Pierce, 1982). LEs were cut from the animals with a sharp razor blade, extraneous tissue was cut from the back of the eyes, and retinas were stripped from the cornea. Each LE was cut to produce four slices of roughly equal size, and each slice was incubated in 1 mL LR for at least 1hr in the dark at 4°C before they were assayed. Ventral optic nerves were desheathed, and photoreceptor cell body-enriched portions were dissected from the bulk of the optic nerve. Somata from each optic nerve were placed into a separate 1.5 mL conical test tube containing 50 μL organ culture medium (OCM) (Kass and Renninger, 1988) and incubated overnight in the dark at 4°C. The next morning the cells were adapted to ambient room light for at least 1hr while they were rinsed three times with LR.

To examine effects of amines and potential OA receptor agonists in LEs, slices of LE from a single animal were incubated for 10 min at room temperature in 1 mL of LR containing 10^{-3} mol/L of the phosphodiesterase inhibitor IBMX (isobutyl methylxanthine) and

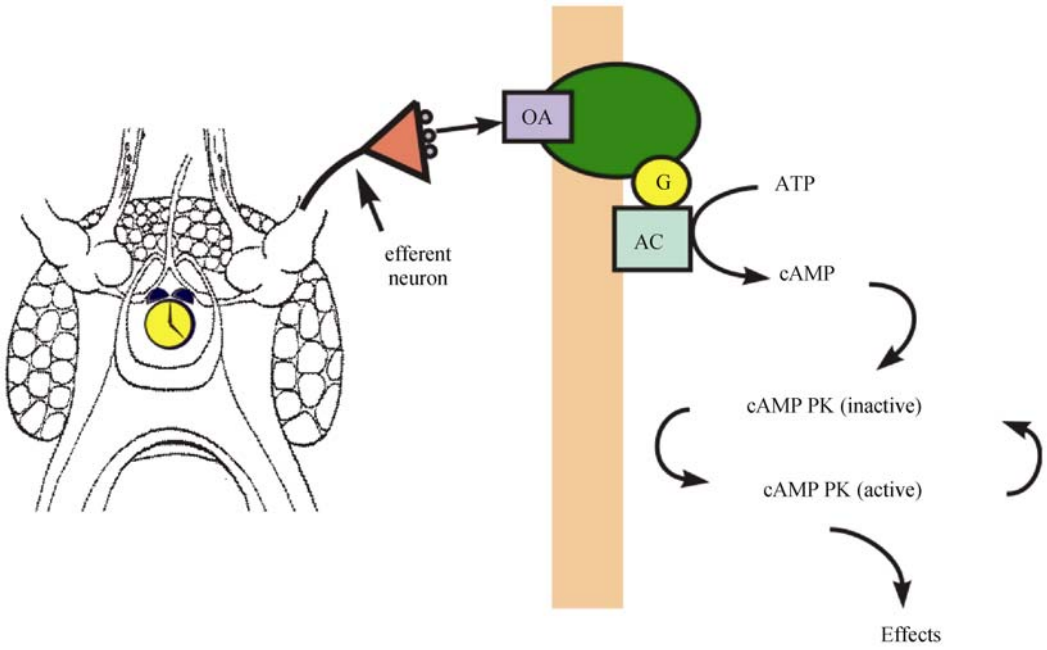

Fig. 6 Schematic of a biochemical cascade in photoreceptors known to be activated by clock input

Initial steps in the cascade are well established. Octopamine (OA) is released when the clock-driven efferent input to the eyes is activated, binds to a receptor coupled to a G protein that activates adenylyl cyclase (AC), stimulates a rise in cAMP and activates cAMP-dependent protein kinase (PK).

the concentration of amines or potential agonists as indicated in the figures. The concentration of intracellular cAMP in these slices was always compared to that in a LE slice from the same animal that had been incubated for 10 min in 10^{-3} mol/L IBMX only.

To examine effects of potential antagonists of the OA-stimulated rise in cAMP, LE slices were preincubated for 10 min in 1 mL LR plus 10^{-3} mol/L IBMX and the concentration of antagonist as indicated in the figures. Then the slice was transferred to another 1 mL of LR containing IBMX, the antagonist, and 2×10^{-5} mol/L OA. A separate LE slice from the same animal was preincubated for 10 min in LR plus IBMX only and incubated for an additional 10 min in IBMX plus 2×10^{-5} mol/L OA; another slice was incubated for 20 min in IBMX only.

At the end of the incubations, LE slices were blotted on filter paper, placed into 500 µL of 10% (vol/vol) perchloric acid (PCA), frozen on dry ice, and stored frozen at -20°C until they were processed further as follows. Tissues in PCA were thawed, disrupted completely with sonication (Heat Systems, W-225) and centrifuged for 10 min in a microfuge. A 450 µL volume of supernatant was removed to a separate tube, and remaining supernatant was drained from the pellet. The pellet was solublized in 500 µL 1 mol/L NaOH and used to determine protein concentration (Lowry et al., 1951) with bovine serum albumin as the standard. The supernatant was neutralized with 1 mol/L KOH and passed over a 1 mL cation exchange column (AG1 X8 Formate form, 100-200 mesh, BioRad, www.bio-rad.com) that had been washed extensively with H_2O. Neutralized aliquots (450 µL) of 10% PCA only or 10% PCA containing 10^{-12} moles cAMP were processed through separate columns along with each group of samples to monitor background and recovery. After samples were applied to columns, columns were rinsed with 10 mL of H_2O. cAMP was then eluted with 10 mL of 2 mol/L formic acid (Frandsen and Krishna, 1976). Aliquots (10 µL to 50 µL) of this eluate were dried under vacuum and assayed for cAMP using a radioimmunoassay (RIA kit from Biomedical Technologies, www. btiinc. com). Recoveries from control columns were usually greater than 90%. If recovery was lower, the experiment was discarded.

cAMP in ventral photoreceptors was assayed with a protocol similar to that described by Kaupp et al. (1982). Light adapted photoreceptors were incubated for 10 min at room temperature in 20 µL of 10^{-3} mol/L IBMX in LR without or with the drugs indicated in the figures. The concentration of cAMP in cells incubated with IBMX plus a drug were always compared to the concentration in cells from the same animals incubated with IBMX alone. The incubation was stopped by addition of 50 µL

ice cold 0.1 mol/L HCl. Samples were sonicated, heated in boiling water for 5 min, and centrifuged at 15,000 g for 15 min. The supernatant (65 μL) was collected, dried under vacuum and assayed for cAMP as described above. The pellet was solublized in 1 mol/L NaOH and assayed for protein using a microlowry procedure (Hess et al., 1978) with bovine serum albumin as the standard.

Varr mRNA assays LEs were dissected from daytime, light-adapted animals. Extraneous tissue was removed from the back of the eye and retinas were stripped from the cornea. Each retina was then incubated over night in the dark at 4°C in 1 ml OCM. The next day, LEs were incubated at room temperature in the light in fresh OCM containing 10^{-3} mol/L IBMX without or with drugs known to elevate or inhibit the rise of cAMP levels. Incubations were stopped by putting tissues into RNAlater (Ambion, Inc., www. ambion. com), and tissues were stored in RNAlater at 4°C until total RNA was extracted in TRIZOL reagent (Invitrogen, http://products. invitrogen. com).

Varr mRNA levels were assayed using quantitative Northern blots as described previously (Dalal et al., 2003) using a radiolabelled ribroprobe specific for Varr mRNA. A 215 bp region of Varr cDNA (nucleotide 870–1085) was amplified by PCR and subcloned into pGEM-T with SP6 and T7 promoters flanking the cloning sites. After the sequence was verified and the direction of the inset determined, a high specific activity (1–2 × 10^8 cpm/μg) α^{32}P-CTP-labeled 215 bp riboprobe was prepared by run-off transcription of the plasmid vector that had been linerarized with *AccI* restriction endonuclease (New England Biolabs, www. neb. com). The amount of radiolabelled Varr riboprobe that bound to Northern blots was normalized to the 18S rRNA band, as determined by ethidium bromide staining intensity of the gel before blotting. The radioactive labeling and ethidium bromide staining were both quantified using ImageQuant (GE Healthcare LifeSciences, www. gelifesciences. com).

To control for between-animal variations in Varr mRNA levels, we compared control and experimental eyes from the same animal. We used unpaired t-tests to determine whether the mean ratios of Varr transcript levels observed comparing control and experimental eyes of the same animal were significantly different from the ratios observed comparing two untreated eyes of an animal.

Both control and experimental eyes were incubated in 10^{-3} mol/L IBMX, and incubations were for 4h, about the time it takes for clock input to prime the eyes for clock-dependent, light-triggered responses such as transient rhabdom shedding (Chamberlain and Barlow, 1984), and roughly the time it takes for Varr mRNA levels to fall in LEs after dusk *in vivo* (Battelle et al., 2000, and Fig. 4). When inhibitors of adenylyl cyclase (MDL-12330A, Merck Chemicals, www. merck-chemicals. com) and cAMP-dependent protein kinase (H-89) were tested, they were added 10 min before OA or 8 Bromo cAMP.

5.3 Results

OA stimulates a rise in cAMP in LE and ventral photoreceptors. In the presence of 10^{-3} mol/L IBMX, (±)OA at 2 × 10^{-5} mol/L stimulates a time dependent rise in the concentration of cAMP in LE slices (Fig, 7A). This rise is linear for at least 10 min. IBMX alone produced a rise in cAMP that consistently plateaued after 2 min; no significant OA-stimulated rise was detected in the absence of IBMX. The magnitude of the IBMX-stimulated rise varied greatly among animals—between 2 and 7 times the concentration measured in untreated tissues—and the basal concentration of cAMP also varied widely among animals—between 3 to 20×10^{-12} mol/mg protein. To control for these animal variations, cAMP levels observed following incubations with drug plus IBMX were always normalized to the concentration of cAMP in a slice of LE from the same animal incubated with IBMX alone. Preliminary experiments verified that different slices of LE from an individual animal contained the same concentration of cAMP after incubations in 10^{-3} mol/L IBMX, and that the OA-stimulated rise in cAMP was the same in different slices of LE from an individual animal.

The OA-stimulated rise in cAMP levels was concentration dependent (Fig. 7B). The response threshold with (±) OA was between 10^{-8} and 10^{-7} mol/L, the response increased sharply between 2x10^{-6} and 2x10^{-4} mol/L, the half maximum response was achieved with 2×10^{-6} mol/L, and the maximum response with about 2×10^{-4} mol/L. When the enantiomers of OA (gift from J. M. Midgley) were assayed individually at 10^{-5} mol/L, each produced a significant rise in cAMP, but the stimulation observed with D(-) OA was much greater than that observed with L(+) OA (Fig. 7C). Other biogenic amines were substantially less effective than (±) OA at elevating LE cAMP levels. Tyramine, dopamine and norepinephrine produced only small increases in cAMP levels, and histamine produced none at all.

Among the potential OA receptor agonists tested on LE, XAMI (2,3-xylaminomethyl-2′-imidazoline) (gift

from N. Orr, Orr et al., 1991) was more potent than (±) OA with a half maximal response observed with 10^{-7} mol/L. NC5 (2-(2,6-diethylphenyl-imino)imidazoline) was also more potent than (±) OA with a half maximal response obtained with 10^{-7} mol/L. However the maximum response obtained with NC5 was only about 80% of that observed with $2x10^{-5}$ mol/L (±) OA. The rise in cAMP levels obtained with synephrine was similar to that obtained with NC5, but NC7 (2-methyl-4-chlorophenylimino)imidazoline), naphazoline (2-(1-naphthylmethyl)-2-imidazoline) and tolazoline (2-benzyl-4,5-dihydro-1*H*-imidazole) were considerably less effective (NC5 and NC7 were gifts from J. A. Nathanson; Nathanson, 1985).

The effects of agonists on LE and ventral photoreceptors were different (Fig. 7C compared to 7D). Specifically, when naphazoline at 10^{-5}mol/L was applied to ventral photoreceptors it stimulated a rise in cAMP as effectively as 2×10^{-5} mol/L ± OA (roughly a 5-fold increase over the IBMX control), whereas when applied to LE slices at the same concentration, it much less effective than OA.

A number of drugs reported to be OA receptor antagonists in other systems (for example see Evans and Maqueira, 2005; Marqueira et al., 2005) were tested for their ability to block the rise in cAMP in LE slices incubated with 2×10^{-5} mol/L (±) OA. Each antagonist was tested at 10^{-4} mol/L. Chlorpromizine (3-(2-chloro-10*H*-phenothiazin-10-yl)-*N*,*N*-dimethyl-propan-1-amine) (*n*=4), metoclopramide (4-amino-5-chloro-*N*-(2-(diethylamino) ethyl)-2-methoxybenzamide) (*n*=2), clozapine (8-chloro-11-(4-methylpiperazin-1-yl)-5*H*-dibenzo[*b*,*e*][1,4]diaze pine) (*n*=5) and yohimbine (17α-hydroxy-yohimban-16α-carboxylic acid methyl ester) (*n*=2) did not inhibit the OA-stimulated rise in cAMP. Phentolamine (3-[4,5-dihydro-1*H*-imidazol-2-ylmethyl-(4-methylphen yl)-amino]phenol) and mianserin ((±)-2-methyl-1,2,3,4, 10,14b-hexahydrodibenzo[*c*,*f*]pyrazino[1,2-*a*]azepine) were roughly equally effective and inhibited the OA-stimulated rise in cAMP by 53±6% (*n*=14) and 40± 6% (*n*=12), respectively.

OA and elevated cAMP mimic the clock's effect on Varr mRNA levels. Having established that OA stimulates a rise in cAMP in LEs *in vitro*, we asked whether OA and elevated cAMP mimics the clock-dependent fall in LE Varr mRNA levels (Fig. 4) and whether the activation of PKA is involved. The results of these studies are shown in Figure 8. We found that Varr mRNA levels were significantly lower in LEs incubated with 4×10^{-5} mol/L (±) OA plus 10^{-3} mol/L IBMX or 10^{-4} mol/L 8-Bromo cAMP plus 10^{-3} mol/L IBMX compared to control eyes incubated with IBMX alone. The OA-stimulated fall in Varr mRNA was blocked by the adenylyl cyclase inhibitor MDL-12330A, further evidence that OA influences Varr mRNA levels through generation of cAMP. PKA is implicated in this response because the 8-Bromo cAMP-stimulated fall in Varr mRNA levels was blocked by the PKA inhibitor H-89. By themselves MDL-12330A, and H-89 had no significant effect on Varr mRNA levels.

5.4 Discussion

The results presented here show that OA stimulates a rise in cAMP in *Limulus* LE. We also show that activation of OA receptors coupled to activation of adenylyl cyclase and PKA mimic the effects of the clock on Varr mRNA levels. These findings add to evidence that many effects of the clock are mediated through an OA-stimulated activation of the cascaded shown in Fig. 6. By contrast, an analysis of the pharmacology of receptors in LE responsible for the rise in cAMP suggest that other OA-driven biochemical cascades may be responsible for circadian structural changes in LE.

Some OA receptors expressed in LE are probably β-adrenergic like OA receptors. Data presented in Fig. 7 and previous studies (Kaupp et al., 1982) clearly show that cells in the LE and ventral photoreceptors express OA receptors that activate adenylyl cyclase. Since tyramine is relatively ineffective at elevating cAMP levels, these receptors are probably not octopamine/tyramine receptors, but rather β-adrenergic like OA receptors (OA β-receptors) (Evans and Maqueira , 2005). However, the effects of agonists and antagonists of the OA-stimulated rise in cAMP in LE described here do not fit the pharmacological profile of any OA β-receptors described in insects. Species differences could be a major reason. In addition, 5 different cell types in LEs are innervated by OA-containing efferent terminals and each cell type may express multiple types of OA β-receptors in different ratios. In insects, three different types of OA β-receptors have been identified, and each exhibits a unique pharmacology (Evans and Maqueira, 2005). So the response measured in a complex tissue like LE probably reflects a mixture of responses from different receptor types.

OA mediates many known effects of the clock on Limulus photoreceptors by activating the cAMP cascade and PKA. New data presented here and in previous studies provide strong evidence that OA mediates many clock-regulated changes observed in *Limulus* eyes by activating the biochemical cascaded modeled in Fig. 6.

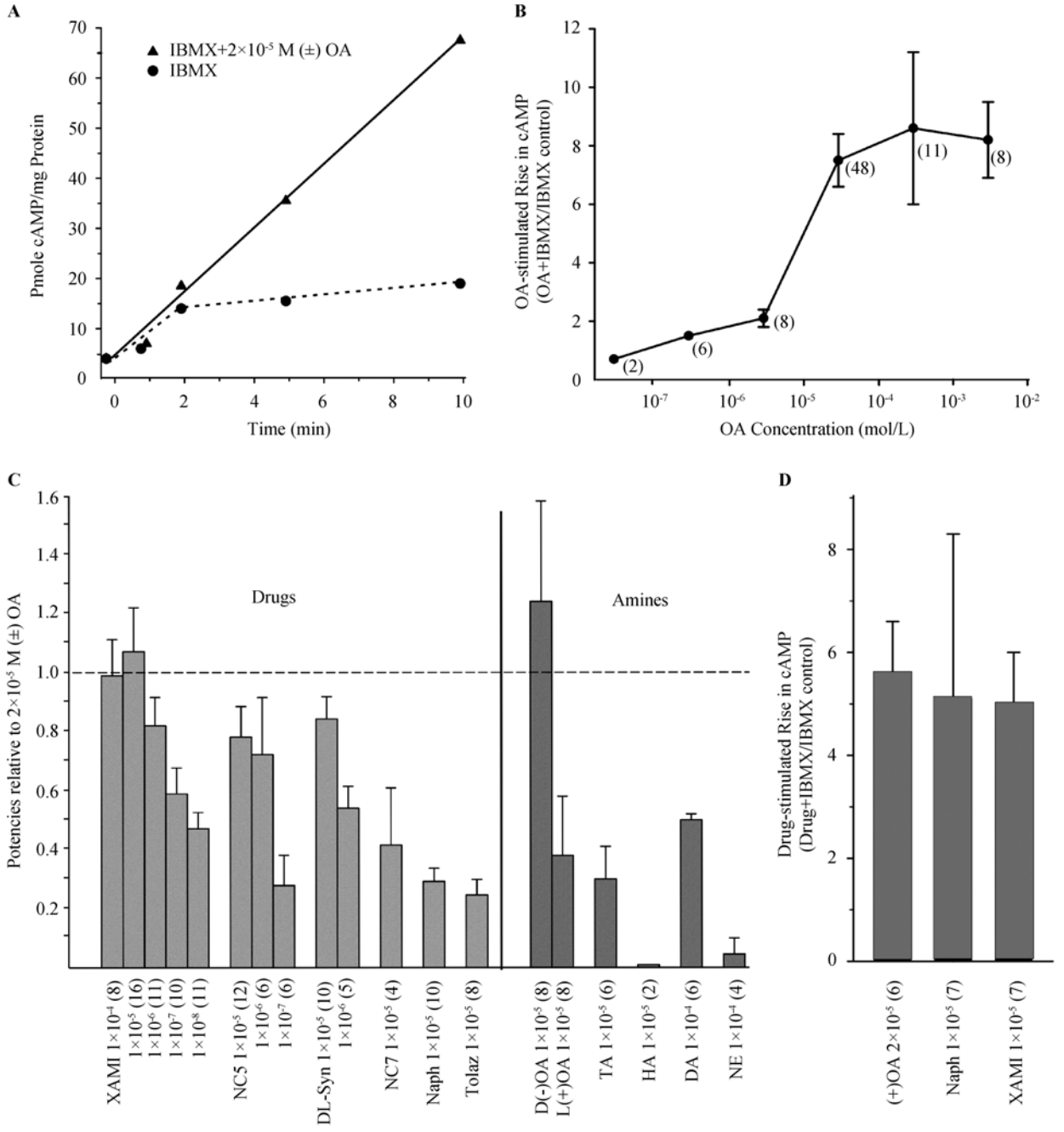

Fig. 7 OA stimulates a rise in cAMP in LE slices

A. Time course of the elevation of cAMP in LE slices incubated with 10^{-3} mol/L IBMX alone (M) or 10^{-3} mol/L IBMX plus 2×10^{-5} mol/L octopamine (OA) (▲). The results of a single experiment are shown in which all LE slices were from the same animal. Similar results were obtained in three separate experiments. All subsequent assays proceeded for 10 min. **B**. Rise in cAMP observed in LE slices incubated with different concentrations of OA plus 10^{-3} mol/L IBMX compared to that observed in slices incubated with IBMX alone. The means ± the standard errors of the means are plotted. The number of different animals assayed is shown in parentheses. **C**. Effects of potential OA receptor agonists and other biogenic amines on cAMP levels in LE slices. IBMX at 10^{-3} mol/L was included in all incubations. The rise in cAMP observed with potential agonists and other amines is plotted relative to that observed in a separate slice of LE from the same animal incubated with 2×10^{-5} mol/L (±) OA, which is expressed here as 1.0 (dashed line). **D**. OA and drug stimulated rise in cAMP in ventral photoreceptors.

Syn=synephrine; Naph = naphazoline; Tolaz = tolazoline, TA = tyramine; HA = histamine; DA = dopamine; NE = norepinphrine.

Studies from a number of different laboratories show that applying OA, or other drugs that elevate cAMP, mimic known effects of the clock on *Limulus* photoreceptor physiology (Table 1). Furthermore, the concentration dependence of the effects of (±) OA and the relative potencies of the individual OA enatiomers on increasing the responsiveness of LE photoreceptors *in vitro* (Renninger et al., 1989; Renninger and Farrell, 1996) is similar to that reported here for the rise in cAMP stimulated by (±) OA and its individual enantiomers (Fig. 7B and C). These correlations strengthen the idea that OA's effects on photoreceptor physiology are mediated through cAMP. When OA and drugs that elevate cAMP are applied to ventral photoreceptors *in vitro*, quantum bump duration increases, noise decreases and responsiveness increases just as in LE photoreceptors (Kass and Renninger 1988). Since no pigment cells are present in ventral eyes, these must be direct effects on photoreceptors and independent structural changes. In addition, OA and drugs that elevate cAMP increase the rate of dark-adaptation in ventral photoreceptors (O'Day and Lisman, 1985), suggesting the rate of dark-adaptation is also under clock control.

An OA-stimulated activation of PKA has been implicated in a number of other clock-regulated processes. These include the reduction in Varr-mRNA levels shown here (Fig. 8), the phosphorylation of LpMyo21 at sites phosphorylated by the clock (Kempler et al., 2007; Cardasis et al., 2007) and the priming of transient rhabdom shedding (Runyon et al., 2004). The phosphorylation of LpMyo21 and OA-primed rhabdom shedding are each inhibited by phentolamine (Edwards and Battelle, 1987; Khadilkar et al., 2002), an effective inhibitor of the OA-stimulated rise in cAMP in LE (Fig. 7). Taken together, these findings suggest strongly that direct effects of the clock on photoreceptors are mediated by an OA-stimulated activation of the cAMP cascade.

Other biochemical cascades involved in the circadian regulation of vision. Although the OA-stimulated activation of the cAMP cascade clearly plays a major role in the circadian regulation of *Limulus* vision, other biochemical cascades may be involved as well. For example, although OA clearly increases the amplitude of the ERG recorded from LEs in response to dim flashed of light, which has been interpreted to reflect a change in structure (Barlow et al., 1980; Chamberlain and Barlow, 1987), the pharmacology of this response (Kass and Barlow, 1984) does not match that of the OA-stimulated rise in cAMP reported here. Subcorneal injections of naphazoline and tolazoline are more effective than OA

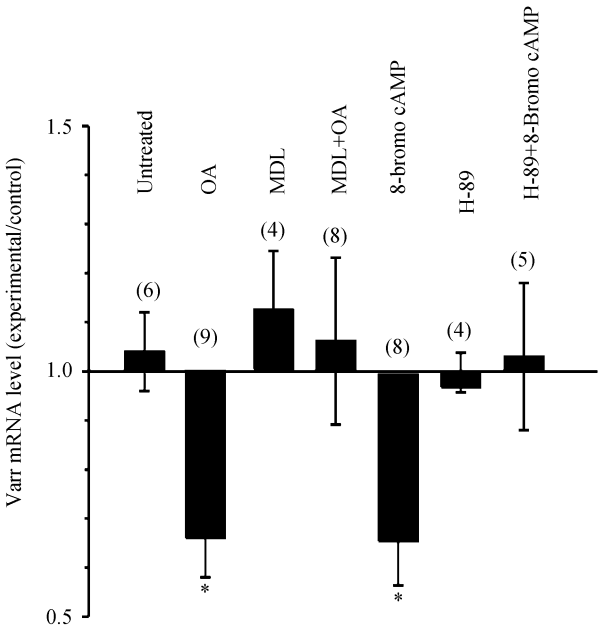

Fig. 8 Effects on Varr mRNA levels of OA and drugs that activate or inhibit the cAMP cascade

Results are expressed as the level of Varr mRNA in experimental eyes relative to that in a control eye from the same animal. Control eyes were incubated with 10^{-3} mol/L IBMX. Experimental eyes were incubated with 10^{-3} mol/L IBMX plus the reagents indicated above the bars. Concentrations are given in mol/L: OA=octopamine, $4x10^{-5}$; MDL= the adenylyl cyclase inhibitor MDL-12330A, 10^{-4}; 8-bromo cAMP,10^{-4}; PKA inhibitor H-89, $3x10^{-5}$ Unpaired *t*-tests were used to determine whether the mean ratios of Varr transcript levels observed comparing control and experimental eyes of the same animal were significantly different from the ratios observed comparing two untreated eyes of an animal (first bar). In parentheses is the number of separate pairs of eyes assayed. *: $P< 0.05$

at increasing LE ERG amplitude, but relatively ineffective at elevating cAMP levels in LE tissue (Fig. 7C). Furthermore, the OA-receptor antagonist clozipine effectively inhibits the OA and clock-stimulated rise in LE ERG amplitude, but not the OA-stimulated rise in cAMP in LE tissue (this study). This leads us to propose that the effects of OA on LE structure are not mediated by receptors which activate adenylyl cyclase.

In addition, OA may not be the only neuroactive molecule that influences circadian changes retinal structure and function. The amplitude of the LE ERG achieved following subcorneal injections of even high concentrations of OA during the day rarely reaches the level achieved with natural clock input at night or by electrically stimulating the LON during the day (Kass and Barlow, 1984). As was mentioned above, it has long been suspected that OA-containing efferents also contain and release peptides, but these peptides have not been identified. Peptides purified from *Limulus* central

nervous system (Gaus et al., 1993) modulate LE sensitivity in complex ways; however none of these is concentrated in LE (Gaus et al., 1997). They could influence the eyes as circulating hormones.

The Substance P and FMRFamide-like peptides present in the second efferent projection to LE could influence LE functions. Indeed, subcorneal injections of Substance P into LEs increase the amplitude of LE ERGs (Mancillas and Selverston, 1984), speed the time course of the ERG (Bolbecker et al., 2009) and alter LE structure (Mancillas and Selverston, 1984) in ways that are similar to the effects of clock input. It is also highly likely that electrical stimulations of the LON performed in many of the experiments described above stimulated both the OA-containing and the SPLI/FMLI -containing efferents. However, it is not yet known whether the activity of the SPLI/FMLI efferent projection is circadian, and, as was described above, the innervation of cells within ommatidia by SPLI/FMLI fibers remains controversial.

6 Summary and Unanswered Questions

Much has been learned about the anatomy, circuitry and biochemistry of clock-driven efferent input to *Limulus* eyes and the impact of the activation of this input on *Limulus* visual functions, but many unanswered questions remain. The cell bodies that give rise to the clock-driven OA-containing efferent axons have been located in the brain, but the central circuitry that drives and modulates these cells is largely unknown. The neurotransmitter chemistry of OA-containing, clock-driven efferent neurons is only partially known, and the relevance to LE circadian function of the efferent projection containing SubstanceP- and FMRFamide-like substances remains unclear. OA mimics most of the known effects of the clock on *Limulus* eyes, and many, but not all, involve the activation of OA receptors linked to adenylyl cyclase. A more complete understanding of the effects of OA on *Limulus* eyes would be enhanced by the molecular characterization of OA receptors in *Limulus* eyes and the down-stream cascades they activate. LpMyo21 has been identified as a major target for modulation by OA and the clock via phosphorylation, but the downstream functional consequences of this phosphorylation event are not yet known, and other substrates for clock-regulated phosphorylation and their functions await discovery. Clock input and an OA-stimulated activation of the cAMP cascade regulates an early step of Varr expression (transcription) and clock-input regulates a later step of Ops1-2 expression. The clock probably regulates the expression of a number of different photoreceptor proteins in a coordinated way and this action of the clock is just beginning to be investigated. The unique structure and organization of the *Limulus* visual system, the diverse and profound effects of the circadian clock on its visual processes, and the ability now to successfully combine anatomical, electrophysiological, biochemical and molecular approaches to study these eyes, make *Limulus* particularly useful for detailed analyses of the effects of the circadian clock on primary sensory cells.

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