

# Visual Efference Neuromodulates Retinal Timing: In Vivo Roles of Octopamine, Substance P, Circadian Phase, and Efferent Activation in *Limulus*

Amanda R. Bolbecker, Corrinne C. M. Lim-Kessler, Jia Li, Alicia Swan, Adrienne Lewis, Jennifer Fleets and Gerald S. Wasserman

*J Neurophysiol* 102:1132-1138, 2009. First published 17 June 2009;  
doi: 10.1152/jn.91167.2008

## You might find this additional info useful...

---

Supplementary material for this article can be found at:

<http://jn.physiology.org/http://jn.physiology.org/content/suppl/2009/06/15/91167.2008.DC1.html>

This article cites 28 articles, 5 of which you can access for free at:

<http://jn.physiology.org/content/102/2/1132.full#ref-list-1>

Updated information and services including high resolution figures, can be found at:

<http://jn.physiology.org/content/102/2/1132.full>

Additional material and information about *Journal of Neurophysiology* can be found at:

<http://www.the-aps.org/publications/jn>

---

This information is current as of April 25, 2013.

# Visual Efference Neuromodulates Retinal Timing: In Vivo Roles of Octopamine, Substance P, Circadian Phase, and Efferent Activation in *Limulus*

Amanda R. Bolbecker,<sup>2</sup> Corrinne C. M. Lim-Kessler,<sup>3</sup> Jia Li,<sup>4</sup> Alicia Swan,<sup>1</sup> Adrienne Lewis,<sup>5</sup> Jennifer Fleets,<sup>1</sup> and Gerald S. Wasserman<sup>1</sup>

<sup>1</sup>Sensory Coding Laboratory, Department of Psychological Sciences, Purdue University, West Lafayette; <sup>2</sup>Department of Psychological and Brain Science, Indiana University, <sup>3</sup>Department of Psychology, Monmouth College, Indiana, <sup>4</sup>Division of Neuromuscular Disease, Department of Neurology, Harvard Medical School, and <sup>5</sup>Department of Family Medicine, Akron General Medical Center, Ohio State University

Submitted 21 October 2008; accepted in final form 13 June 2009

**Bolbecker AR, Lim-Kessler CC, Li J, Swan A, Lewis A, Fleets J, Wasserman GS.** Visual efference neuromodulates retinal timing: in vivo roles of octopamine, substance P, circadian phase, and efferent activation in *Limulus*. *J Neurophysiol* 102: 1132–1138, 2009. First published June 17, 2009; doi:10.1152/jn.91167.2008. Efferent nerves coursing from the brain to the lateral eye of the horseshoe crab, *Limulus polyphemus*, increase its nighttime sensitivity to light. They release octopamine, which produces a categorical increase of photoreceptor response duration in vitro. Analogous in vivo timing effects on the electroretinogram (ERG) were demonstrated when octopamine was infiltrated into the eye of an otherwise intact animal; nighttime ERGs were longer than daytime ERGs. Related effects on the ERG were produced by daytime electrical stimulation of efferent fibers. Surprisingly, in a departure from effects predicted solely from in vitro octopamine data, nighttime ERG onsets were also accelerated relative to daytime ERG onsets. Drawing on earlier reports, these remarkable accelerations led to an examination of substance P as another candidate neuromodulator. It demonstrated that infiltrations of either modulator into the lateral eyes of otherwise intact crabs increased the amplitude of ERG responses but that each candidate modulator induced daytime responses that specifically mimicked one of the two particular aspects of the timing differences between day- and nighttime ERGs: octopamine increased the duration of daytime ERGs and substance P infiltrated during the day accelerated response onset. These results indicate that, in addition to octopamine's known role as an efferent neuromodulator that increases nighttime ERG amplitudes, octopamine clearly also affects the timing of photoreceptor responses. But these infiltration data go further and strongly suggest that substance P may also be released into the lateral eye at night, thereby accelerating the ERG's onset in addition to increasing its amplitude.

## INTRODUCTION

Lim and Wasserman (2001) found that perfusion by octopamine, a neuromodulator, increased the duration of intracellular receptor potentials (RPs) evoked in vitro by brief (20 ms) light flashes in *Limulus* photoreceptors residing in excised eye slices. They called these responses categorical and prolonged potentials (CPPs) because such effects are categorical (i.e., either clearly present or totally absent) and because they prolong RPs by altering their characteristic shape.

These CPPs radically differ from more modest temporal scaling effects of octopamine (O'Day and Lisman 1985; Ren-

ninger et al. 1989) in that they involve a clear second response component that often begins after the transient RP waveform has begun to decline, they are only evoked by brief flashes, and they are primarily evoked by intermediate flash intensities.

Because such CPPs had only been observed in reduced in vitro preparations, they might be artifactual. One aim of the present experiments, therefore, was to determine whether CPPs exist in vivo when efferents from *Limulus*' brain release neuromodulators into its eye (Barlow et al. 1980; Battelle 2002; Battelle and Evans 1982; Calman and Battelle 1991; Kass and Barlow 1984).

Another aim was to re-examine Mancillas and Selverston's (1984) finding that subcorneal infiltration of substance P increased electroretinographic (ERG) amplitude and decreased its latency (i.e., accelerated its onset). Although somewhat controversial (cf. Chamberlain and Engbretson 1982), these findings, along with other evidence for the role of substance P (Lim et al. 2008a; Mancillas and Brown 1984; Mancillas and Selverston 1985), made substance P a candidate for investigation in intact or almost intact living animals. The present paper therefore examined the in vivo temporal effects of both candidate neuromodulators on the *Limulus* lateral eye ERG to see if they implied the presence of CPPs. This ERG is almost entirely a mass recording of RPs (Chapman and Lall 1967) although small optic nerve action potentials sometimes ride on its peaks (cf. Wasserman and Cheng 1996) (see Fig. 3).

A more general aim was to explore possible temporal effects of efferent neuromodulation by employing four different experimental designs. In the first, ERGs were collected for days from completely intact animals to evaluate the differences between waveforms collected during the day, when little efferent activity occurs, versus during the night, when maximal efferent activity causes octopamine to be released into the lateral eye (O'Day and Lisman 1985; Renninger et al. 1989). Animals in the next three designs were almost intact, save for the insertion of thin electrodes or mini-tubes into or near their lateral eyes. In a second design, efferent activity was induced during the day by shocking the lateral optic nerve; this should have released intrinsic octopamine (as well as the neuropeptide, substance P, another candidate efferent neuromodulator). In a third design, extrinsic octopamine was infiltrated into the lateral eyes of otherwise intact animals. Finally, a fourth design

Address for reprint requests and other correspondence: G. S. Wasserman, Sensory Coding Laboratory, Dept. of Psychological Sciences, Purdue University, West Lafayette IN 47907-2081 (E-mail: codelab@purdue.edu).

infiltrated extrinsic substance P. A preliminary report of this work has been given (Bolbecker et al. 2005).

## METHODS

Progress in this area has been retarded by insufficient descriptions of critical aspects of the methods employed. As a result, it has only recently become evident that our group has consistently perfused excised eyes with isotonic seawater while others had long ago switched to the use of severely hypotonic organ cultures, perhaps without realizing that such a change had been made (cf. Lim et al. 2008b for a full review). To ensure that our present ERG methods are fully disclosed, we have therefore provided a very extensive description of them in the supplementary material.<sup>1</sup> Only a very brief condensation of these methods has been provided in the following text.

The questions we posed concerned variations in the ERG waveform that may possibly occur over very long time periods. To investigate these questions reliably, we developed a compact gel/bellows salt-bridge electrode (cf. Bolbecker et al. 2007) and used it to collect long-term data from 13 experiments in which continuous long-term recordings (CLTRs) were taken during the day as well as during the night. All stimulation and data recording parameters were controlled by a Macintosh computer via an ADI (AD Instruments) MacLab/400 interface. ERGs were digitized at 400 sample/s and stored.

A dual flash paradigm was generally used in which 2.5-ms flashes were separated by 75 ms, although a single 5-ms flash was sometimes used. The ERGs evoked by this regimen were collected continuously for 2–5 days, and those taken under comparable conditions were averaged within conditions.

The quantitative temporal comparisons presented in the following text first involved scaling ERGs so that their peak amplitudes were equated. Then the times needed for an ERG to rise (or fall) to potential levels that were equal to half of these peak levels were determined. Such measurements involved interpolating between the two digital samples that straddled a half-peak potential. It will be seen in the following text that these ERGs change rapidly between the 2.5 ms that separate such half-peak straddle points, and so the resulting temporal interpolations were rather precise.

In those experiments in which time of day was the variable of interest, data were collected round the clock at a rate of six times per hour for several days. In these experiments, the conventional astronomical day was divided into four roughly equal organismically defined quadrants respectively representing the organismic day, its transition into the organismic night, the organismic night itself, and its transition back into the organismic day. These periods were thus each a bit longer than 6 h. Data were averaged within quadrants so that each of the response traces presented in the following text represents the average of many ERGs. More details on this method are given in the supplementary material.

However, the efferent stimulation and recording experiments as well as the extrinsic modulator infusion experiments collected data between 1,000 and 1,400 h because this would be during the circadian interval when natural efferent effects on lateral eye sensitivity have been shown to be at their lowest daytime level. (cf. Pieprzyck et al. 2003).

To stimulate the lateral optic nerve electrically, two straight insect pins were inserted through the carapace on either side of the nerve, near the anterior margin of the lateral eye. These pins were connected to a Grass SD9 Stimulator that generated electrical pulses. Once a useful stimulation amplitude had been set (see supplementary material), the same 75-ms interval that had been used between paired light flashes was used between paired electrical stimuli. Because excitability to external electrical stimulation recovers much more rapidly than light adaptation does, the interval between these electrical stimulus

pairs was 90 s (instead of the 10 min used for light pairs). This permitted the time course of efferent stimulation effects to be more fully captured. The efference data displays given below will thus always compare the average ERG produced during the hour before efferent stimulation commenced versus the average produced during the period following such stimulation. This latter period covered the time after stimulation during which the effects of efference were apparent and it extended to a maximum of  $\leq 2$  h after stimulation ended.

Prior to the onset of efferent stimulation, control ERGs were obtained for 1 h during the daytime. As before, these control recordings were averaged responses. Then efferent stimulation commenced and was maintained for 45–60 min after which efferent stimulation was terminated. ERG recording was suspended during the efferent stimulation period, and then it resumed for several hrs until the ERG had returned to its prestimulation baseline. Such experiments usually lasted for a total of 4–5 h, and they were repeated a day later if all seemed still in order.

Neuromodulator experiments involved the infiltration of octopamine and substance P into the lateral eye. Initial guidance for the octopamine aspect of this work came from dose-response characterizations of octopamine's effects on *Limulus* photoreceptors that had been provided by Renninger et al. (1989). Furthermore, two concentrations near the middle of the concentration range were chosen on the basis of the *in vivo* substance P work done by Mancillas and Selverston (1984) and the work done *in vitro* by Lim et al. (2008a). The basis for choice was the likelihood that these concentrations would be effective.

Using the approach described in the preceding text, substance P infiltration clearly increased the amplitude of the ERGs in 14 experiments of a total of 25 that had used 2.5-mM infiltrations. The other 11 fell into two categories: the infiltration produced no amplitude increase in eight cases while ERG amplitude declined substantially below the preinfiltration amplitude in the other three, indicating that the health of the eye or the crab was waning. This aspect of the present results then replicated results given in Mancillas and Selverston (1984). Another 19 experiments involved 250  $\mu$ M infiltrations of substance P; none of them increased ERG amplitude.

## RESULTS

### *Response waveform template*

Under octopamine perfusion *in vitro* (cf. Lim et al. 2008a), intracellularly recorded CPP waveforms are dramatically different from those of ordinary RPs. An approximate reconstruction of how these differences would be expressed in an ERG recording can be seen in Fig. 1.

### *CLTR day versus night experiments*

As noted in the preceding text, it might once have been predicted that nighttime traces would have slower onset latencies and longer durations (i.e., later response onset and prolonged offset) because efferent nerves release octopamine at night and because octopamine alone has been demonstrated to have delaying effects *in vitro* (Lim and Wasserman 2001; Lim et al. 2008a; Renninger et al. 1989).

To provide a more objective and agnostic account of the results of the present work, comparisons between the timing of day- and nighttime lateral eye responses were made in two ways: graphically by overlaying ERGs scaled to the same peak amplitude and numerically by calculating the half-peak onset and offset times as well as their half-peak durations. Figure 2 therefore shows ERGs from a CLTR day versus night experi-

<sup>1</sup> The online version of this article contains supplemental data.

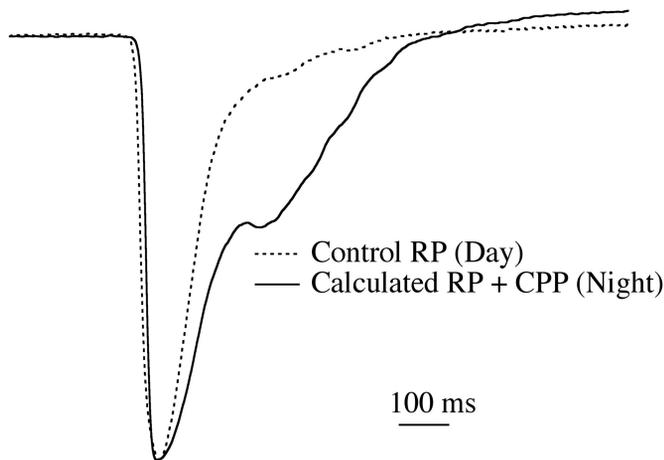


FIG. 1. Electoretinographic (ERG) traces constructed from in vitro intracellular data. —, the ERG waveforms calculated from in vitro responses evoked during octopamine perfusion. Appropriate ratios (see supplementary material for details) of categorical and prolonged potential (CPP) responses and receptor potentials (RPs) without CPPs (cf. Lim and Wasserman 2001) were integrated in the composite waveform. The dotted waveform was generated from in vitro Control experiments perfused with seawater. These predictions show that octopamine produces both temporal rescaling and prolongs the duration.

ment the results of which were quite characteristic in that 12 of the 13 experiments of this type also produced this same pattern. It will be recalled that these curves are averages of data that were collected six times per hour for  $\sim 6$  h/day and for several days.

As predicted, these data exhibit a clearly prolonged ERG at night: its overall duration (taken between the half-peak onset and half-peak offset points) was 204.8 ms at night while the half-peak duration during the day was only 147.4 ms, giving a prolongation of 57.4 ms. This prolongation is composed of a 12.5-ms nighttime half-peak onset latency increase plus a 44.9-ms increase in the half-peak offset latency. Moreover, the overall shape of the decay phase is much slower at night. These data also suggest that temporal summation may be greater during the night because the ERG has a slight double peak that appears during the day and is absent at night. However, this

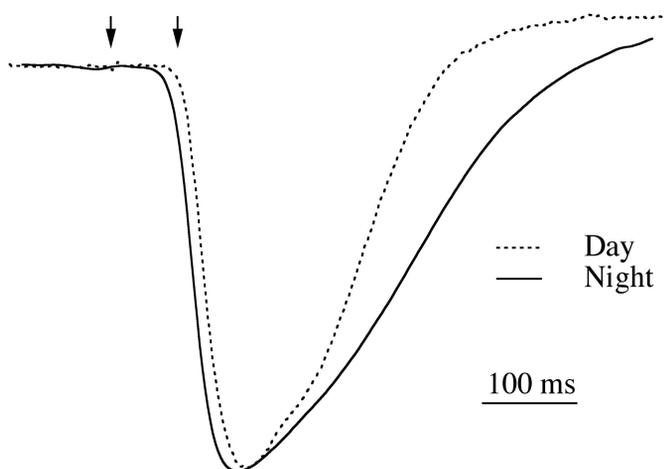


FIG. 2. Nighttime ERGs are faster (i.e., response onsets occur earlier) and longer (i.e., response offsets occur later) than daytime ERGs. The composite nighttime ERG (—) has a faster onset and a longer duration than the daytime composite trace (---). Arrows represent the dual light flashes.

increase is clearly at the limit of the resolution of our method and should be treated as a suggestion.

Interestingly, Fig. 2 also clearly shows a feature that is quite unlike the pattern that would have been expected from the effect of octopamine alone: the latency of the average nighttime ERG (77.7 ms) was clearly faster (i.e., it had an earlier onset) than that of the daytime ERGs (90.2 ms). Ten of the 13 experiments produced results with this kind of difference.

#### Efferent stimulation

Three experiments that had provided clear whole nerve recordings also clearly demonstrated an increase in ERG amplitude after the stimulation period. Figure 3 shows the results from one such efferent stimulation experiment. Its *top panel* shows a time series of ERG peak amplitudes over a period that lasted  $\sim 6$  h. The investigation naturally stopped collecting ERGs during stimulation. Consequently, gaps appear in the presented time series that were derived from such experiments. The  $\uparrow$  particularly indicate the start of two 30-min periods of efferent stimulation; in both cases, efference caused ERG

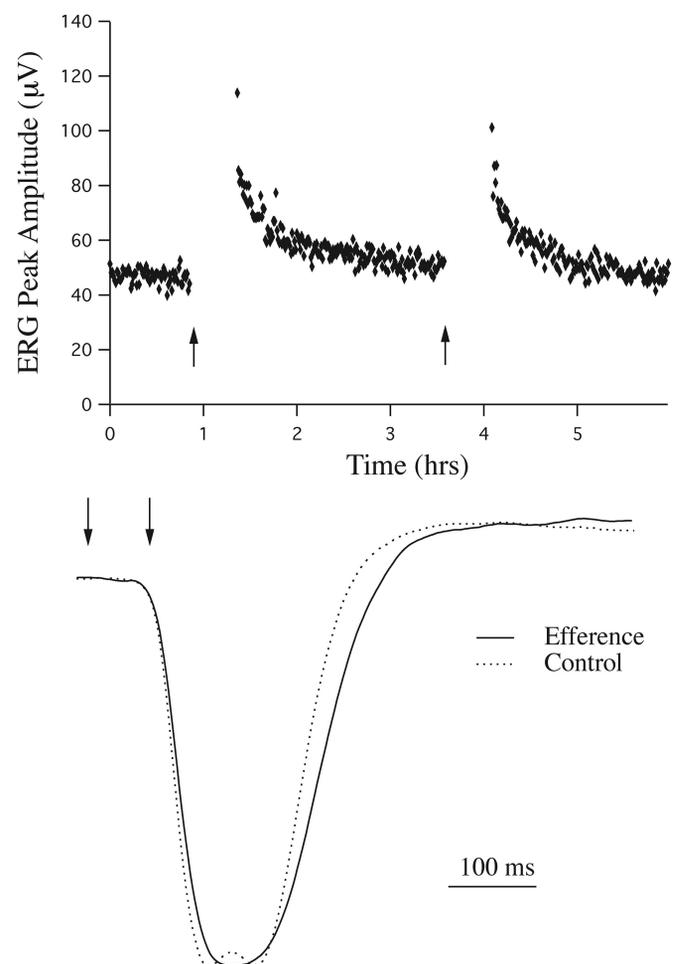


FIG. 3. Efferent stimulation increases ERG amplitude and changes its timing. *Top panel*: a portion of a multi-day time series of ERG peak amplitudes.  $\uparrow$ , the onset of 2 30-min periods of optic nerve stimulation. ERG amplitude doubled after each stimulation period. *Bottom panel*: efference produced longer half-peak latencies as well as longer half-peak response durations relative to control; it also decreased temporal resolution as indicated by the abolition of the double peak seen in the control ERG.

amplitude at least to double relative to that of control ERGs recorded prior to stimulation.

On a much faster time scale, the *bottom panel* of this figure presents an overlay of averaged, scaled ERGs from both the prestimulation control and the postafferent stimulation conditions. The overlay clearly shows that efferent stimulation had slowed ERG timing in several ways: after efferent stimulation, ERGs had longer half-peak onset latencies (106.9 vs. 96.9 ms) as well as longer half-peak offset latencies (266.8 vs. 244.4 ms). The combined effect of these changes was a 12.4-ms increase in half-peak response duration (159.8 vs. 147.4 ms).

The impression created by this graphical display was at first sight incompatible with the numerical characterization given above in that the onset difference displayed by the figure seems to be less than the calculated 10 ms numerical difference. That this impression is illusory was confirmed by direct measurements; we speculate that it was produced by the fact that the space between the traces is small relative to their thickness.

This experiment provided an even clearer indication of temporal summation than the previous one. Specifically, the ERGs evoked by these dual flash stimuli delivered after efferent stimulation produced one melded response peak compared with the prestimulation average response to the same dual flashes that doubtless exhibited two discrete peaks. This rather clear demonstration that efferent activation decreases this indicator of temporal resolution was representative of all successful stimulation experiments. Finally, it may be noted that the overall temporal pattern of these ERGs is consistent with the predicted *in vivo* effects of octopamine.

#### Octopamine infiltration

Results from an octopamine experiment that lasted almost 2 days and which produced results typical of all six such experiments that met our data quality criteria are shown in Fig. 4. Its *top panel* shows a time series the symbols of which portray the peak amplitudes of all of the ERGs recorded over this entire time. Octopamine was infiltrated in the middle of the daytime waning of the ERG cycle at the time marked by the second arrow. That caused a rapid and dramatic increase in ERG amplitude that lasted for hours. Then the ERG amplitude gradually returned toward the daytime preinfiltration baseline that had prevailed prior to the nighttime increase in sensitivity (cf. Khadilkar et al. 2002).

This same time series provides four arrows that mark the groups of ERGs that contributed to the averaged traces overlain in the *bottom* of Fig. 4. As has been the practice in this work, the ERGs in that overlay were scaled to have the same peak amplitude. They reflect ERGs taken in sequence during the first night, as a control during the next day just prior to infiltration, during that same next day at the peak of the octopamine-induced increase in ERG amplitude, and during the second night.

The first night exhibited a half-peak onset latency of 95.2 ms and that was clearly delayed during the following day to a value of 100.2 ms; the exact same 100.2-ms latency was recorded after octopamine had been infiltrated a little later during the same day, and that coincidence made the graphical traces of their onsets overlay and the character generator produced an odd melding of their two interrupted lines. By contrast, the arrival of the second

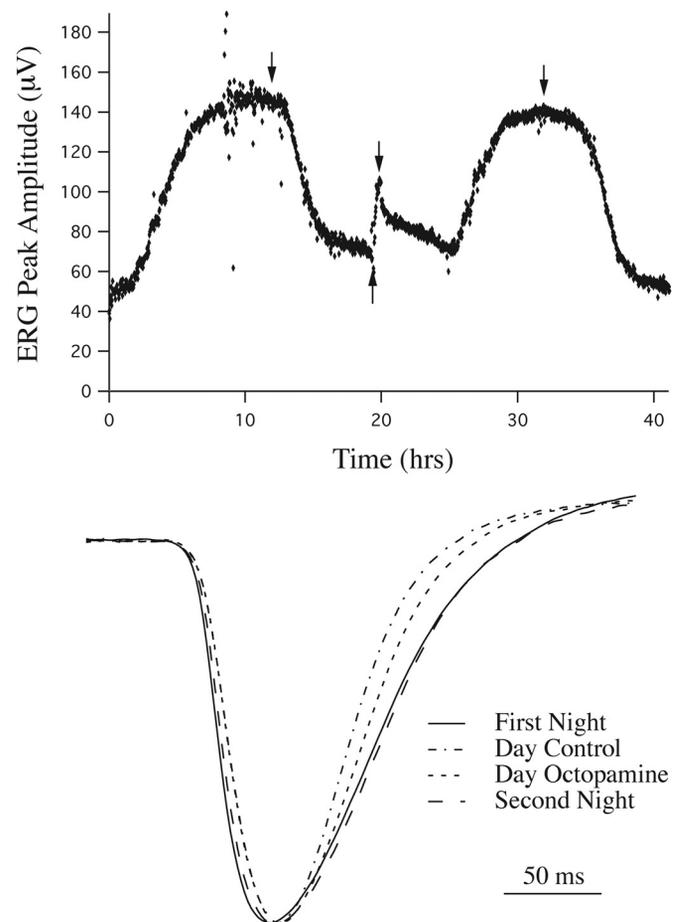


FIG. 4. Octopamine increases both the amplitude and duration of ERG responses. *Top panel*: a time series for the peak amplitudes of ERGs collected over 2 days. The middle 2 arrows indicate when octopamine was infiltrated; these data demonstrate that octopamine increases ERG amplitude. *Bottom panel*: overlays of ERGs collected at the times indicated by the 4 arrows given in the *top panel*. These data indicate that octopamine and nighttime ERG traces are both longer in duration than the daytime trace, but only the onset of the nighttime trace is faster (i.e., occurs earlier).

night clearly accelerated the ERG to a half-peak onset latency of 97.7 ms, close to its original value. It now appears that these nighttime onset accelerations are due to the endogenous release of substance P (see results below).

The offset latencies were similarly consistent with the notion that that parameter was influenced by both the endogenous and the experimental influence of octopamine with the former being stronger. This last difference is expressed in the fact that the first night's offset of 215.1 ms shortened to only 194.7 ms during the day, yet the infiltration of octopamine during the day prolonged the offset to only 204.6 ms, while the arrival of the second night's endogenous contribution further prolonged it to 220.1 ms.

These half-peak waveform effects demonstrate that extrinsic octopamine clearly prolonged ERG half-peak duration (104.4 ms) relative to the daytime control (94.5 ms), although this prolongation did not fully reach that found endogenously during either night (119.9 and 122.4 ms). It should be noted that, in two of the six experiments including this one, octopamine did not slow the onset latency of the ERG relative to the daytime trace. But in no case did it speed up the latency of the response, and it was always slower than the nighttime latency.

### Substance P infiltration

Although the daytime octopamine infiltration experiments and efferent stimulation results partially mimic some of the temporal characteristics of nighttime ERGs, they alone clearly cannot account for the acceleration in onset latency usually observed at night in such ERGs. But further information on this issue comes from substance P infiltrations done at similar moments in the day/night cycle. Of the 14 high-concentration (2.5 mM) infiltration experiments that met our data quality criteria, 7 produced an increase in amplitude that was followed within several hours by a gradual decrease to the preinfiltration daytime level. In the other seven experiments, the increase in amplitude after substance P infiltration actually persisted long enough to merge with the nighttime phase of the circadian rhythm; this merger appeared in those time series as an early onset of the nighttime sensitivity increase. Both because of the early increase in sensitivity (preceding that of a normal night by  $\geq 2$  h) and because the slope of the increase in ERG amplitude was steeper than that of the circadian-mediated nighttime increases, these experiments were interpreted as successful infiltrations. Thus all 14 high concentration substance P infiltrations increased the size of the ERG.

Figure 5 illustrates such work with results from an experiment that lasted 2 days. The composition of this figure parallels that of Fig. 4, and the arrows in the *top panel* again point to those data that contributed to the various averaged waveforms overlain in the *bottom panel*. Inspection of this *top panel* indicates that the infiltration of the higher (2.5 mM) dose of substance P increased ERG amplitude by  $\sim 40\%$  following infiltration, and then the ERG returned to its preinfiltration daytime amplitude, which was followed by the normal nighttime increase in ERG amplitude.

The *bottom panel* expands the time base and overlays averaged and scaled comparisons of preinfiltration night- and daytime control ERGs with daytime ERGs taken under substance P. As before, these data exhibited very consistent results. Although small differences can be seen, there was no remarkable difference in the shape or amplitude of the nighttime ERGs in night two of the time series (i.e., following substance P infiltration) although a slight decrease in nighttime ERG amplitude can be seen in the unscaled *top panel* by comparing *night 2* relative to *night 1*. In all, 10 of the 14 experiments lasted 2 days and, although differences in amplitude between *nights 1* and *2* were sometimes observed, no systematic differences existed. Sometimes *night 1* was larger, other times *night 2* was.

But strikingly, as this overlay also shows, substance P *decreased* the latency of the response relative to the daytime preinfiltration ERG. The 92.7-ms half-peak onset latency of the substance P ERG is faster (i.e., occurs earlier) than the onset of both the nighttime preinfiltration ERG and the daytime control ERG that was recorded prior to infiltration. This equivalence held even when the computation was extended out to a resolution of 0.001 ms; the first night and daytime onsets were still identical at 100.152 ms. Moreover, the onset of the ERG during the second night was virtually identical at 100.158 ms., although the graphics generator actually provided a slight indication of this last difference in the figure trace.

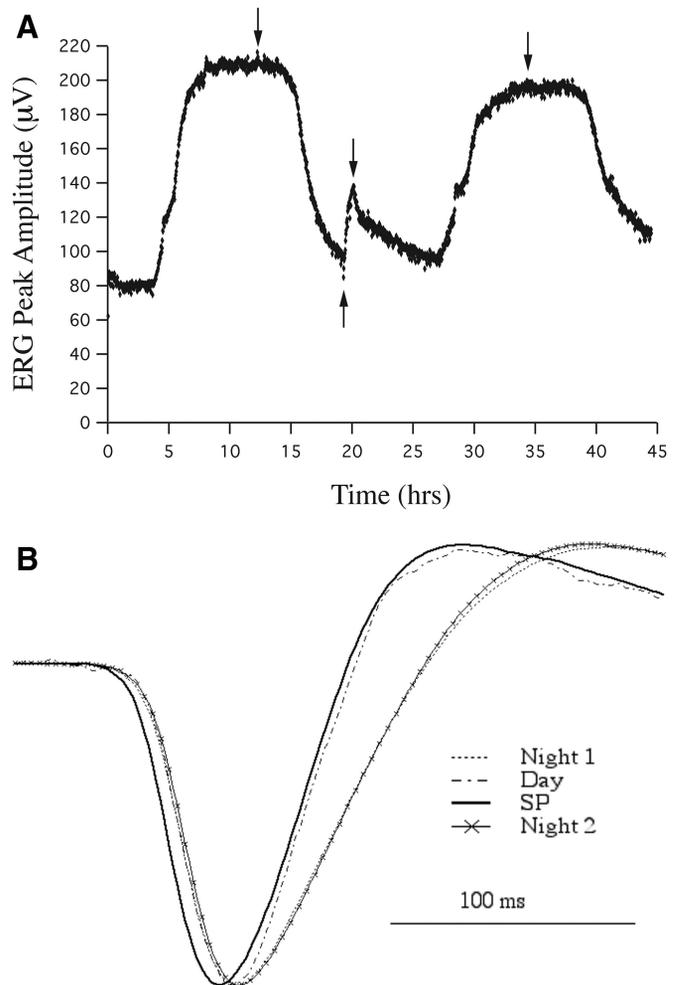


FIG. 5. Substance P increases the amplitude and decreases the latency of ERG responses. Each symbol in this 2-day time series represents the peak amplitude of an individual ERG. *Top panel*: substance P increased ERG amplitude. The 2nd arrow indicates when 2.5 mM of substance P was injected; the 3rd arrow shows the subsequent increase in ERG amplitude following substance P infiltration. The 4 arrows in the *top panel* point to the ERG waveforms that contributed to the averaged ERGs shown in the *bottom panel*. These waveforms show that ERGs after infiltration of substance P clearly had faster latencies (i.e., response onsets occurred earlier) than both night- and daytime ERGs.

Similarly invariant effects were found on the offset latencies wherein the offsets measured on both nights were identical at 200.1 ms while the day offset occurred at 177.5 ms and the substance P offset occurred at 175.0 ms. Such a trend appeared in every experiment. The conjoint effect of the latency changes produced at onset and offset was an *increase* in overall response duration from the 77.4 ms value measured during the day to 82.4 ms under substance P infiltration and finally to a value of 99.9 ms observed identically during both nights. In the particular case illustrated in Fig. 5, these duration changes are somewhat more difficult to discern because of the way the character generator melded the traces but the effects become clear when the numerical values of the half-peak points are considered.

### DISCUSSION

The present study was undertaken to determine whether evidence consistent with CPP occurrence existed in vivo and to

investigate the temporal effects of the candidate neuromodulator substance P on lateral eye ERGs. We investigated several specific hypotheses: 1) qualitative comparisons of temporal characteristics of daytime and nighttime ERGs would show nighttime ERGs to have accelerated onset latencies and to be prolonged compared with daytime ERGs, 2) daytime efferent nerve stimulation would replicate temporal features of nighttime ERGs, 3) daytime octopamine infiltration into the lateral eye would result in ERGs with increased onset latency and prolonged durations, and increased amplitude, and 4) daytime infiltration of substance P would accelerate onset latency and increase amplitude of ERGs.

The ERGs collected during the day and at night addressed the question of whether nighttime waveforms were prolonged; the present data showed that they were, indicating that CPPs do occur *in vivo*. In addition, ERG latency was also faster (i.e., response onsets occurred earlier) at night, contrary to what one would expect if effects were purely due to octopamine. Those results therefore indicate that another mechanism was affecting this change in ERG timing at night. The present data demonstrate that the likely candidate mechanism is substance P, which Mancillas and Brown (1984) and Mancillas and Selverston (1985) had suggested might also be released into this eye by nighttime efference.

Further support for the existence of endogenous CPPs came from efferent stimulation experiments, which produced a later onset and a prolongation of ERGs; this result is entirely consistent with an efferent-dependent release of octopamine into the eye and is phenomenologically similar to effects shown by the octopamine template generated from intracellular CPPs (see Fig. 1), although this result differed from the nighttime response because no acceleration of ERG onset latency was observed (see Fig. 2).

Because nerves of different diameters exist in the lateral optic nerve (Borsellino et al. 1965; Calman and Battelle 1991), differential activation of an octopaminergic subpopulation of fibers could cause prolongation without response acceleration. Alternatively, some populations may have been over-stimulated and were damaged or died. Both of these scenarios are possible, especially given that nerve responses tended to disappear after efferent nerve responses had been recorded after long periods of stimulation or after stimulation at high voltages. This was interpreted as a failure of the efferent nerves.

The general patterns of the octopamine infiltration data are again in accordance with the expectation that the latencies of the octopamine-influenced ERG and the daytime control ERG are both slower than either of the nighttime ERG traces. Results from these octopamine infiltration experiments are also consistent with results from the efferent stimulation experiments.

The substance P infiltration results replicate the finding reported in Mancillas and Selverston (1984) in which substance P increased ERG amplitude during the day.

The present results have broad implications because there are two ways to affect the efficacy of neural signals. One is to alter their amplitude, and much research has specifically characterized the amplitudes of the photoreceptor potentials evoked by light stimuli. But another way is to alter their timing, although investigations that have specifically examined changes in the timing of such RPs tend to be less common. Examinations of circadian rhythms of the RPs present in the

*Limulus* lateral eye (cf. Barlow 1983) provide a striking example of the effect of this asymmetry of interest because such rhythm work necessarily extends over several days while RP timing effects are manifested on a millisecond time scale. Because of this large difference of scale, circadian amplitude variations have been quite well characterized, whereas the circadian rhythms expressed in their timing have been more or less neglected.

Our laboratory, by contrast, has a long history of investigating the timing of retinal signals (cf. Bolbecker et al. 2002, 2003). Unlike earlier work, the present data were collected with the degree of temporal precision necessary to evaluate the status of substance P as a second efferent neurotransmitter in the lateral eye of *Limulus*. Previous physiological research on *Limulus* ERGs, cited in the preceding text, had focused almost exclusively on the ability of similar manipulations to affect ERG amplitude. Indeed because data collection in many such experiments necessarily lasted for days, the automatic devices employed to record and store the data often stored only the maximum amplitudes of individual ERGs. Hence the actual waveforms of most or even all of the ERG responses might never even have been examined. But even when more complete waveforms were reported, the time scales employed were generally severely compressed, making the temporal properties of even those more complete ERGs rather obscure (cf. Barlow 1983; Fig. 2). However, the present results clearly show that substance P accelerates responses when infiltrated during the day, mimicking the reduction in response latency observed at night.

There are some limitations on the present approach because the cellular impetus for it perforce came from work done with intracellular microelectrodes recording from single receptor cells that were stimulated by a particular intensity of light; by contrast, ERGs reflect contributions from thousands of photoreceptor cells each exposed to a different light intensity and each located at a different electrical distance from the ERG electrode. Therefore a detailed quantitative prediction of the exact shape of the ERG waveform is not possible absent a complete network model of the electrotonic characteristics of the entire eye. However, as shown in the preceding text, qualitative predictions about the directions of changes in the ERG can be tested with such single-cell data.

These results strengthen the conclusion first tentatively drawn by Mancillas and Selverston (1984) that efferents release a substance P-like peptide into the lateral eye in a circadian fashion and that that peptide acts as a neuromodulator/neurotransmitter that mediates an increase in lateral eye sensitivity at night. Moreover the effects of daytime substance P infiltration clearly suggest a mechanism for the heretofore-unreported phenomenon of nighttime ERG onset acceleration, which is the opposite of what one would expect if octopamine were the only agent acting on this system.

Converging evidence for the role of substance P in the acceleration of visual responses comes from recent *in vitro* experiments by Lim et al. (2005) and Lim-Kessler' (2008a) in which substance P was perfused onto the lateral eye while RPs were recorded intracellularly. Those results support the present *in vivo* ERG findings because substance P both substantially increased RP sensitivity and accelerated RP responses. This acceleration was more evident in experiments done at colder temperatures but was also evident to varying degrees at ambi-

ent temperatures. This difference, coupled with the labile character of work done with substance P at ambient temperatures, may explain why the prior literature was inconclusive on this matter. These findings also suggest that the present findings with regard to substance P may be more pronounced at colder temperatures.

Interestingly, mass spectroscopic work currently in progress (J. Li, L. M. Stroup, E. A. Syverson, J. D. Brahmhatt, and G. S. Wasserman, unpublished data) has suggested that the substance P molecule itself is not unstable per se. Rather the expression of the effects of this modulator may be affected by other entities residing in this eye. If this suggestion is borne out, substance P in the *Limulus* eye may share properties displayed by peptides resident in other preparations, such as rat plasma (Couture and Regoli 1981) and guinea pig lung (Stimler-Gerard 1987). However, fuller understanding of this system awaits the results of chemical and electrophysiological studies of interactions between octopamine and substance P. Such studies are now in progress (J. Li, L. M. Stroup, E. A. Syverson, J. D. Brahmhatt, and G. S. Wasserman, unpublished data).

Despite these complexities, taken together, the present results lead to the following interim conclusion: substance P and octopamine efferent systems act in concert to increase ERG amplitude and in opposition to effect bidirectional changes in ERG timing observed at night.

#### ACKNOWLEDGMENTS

We are particularly indebted for the expert technical assistance received from Dr. Elwood K. Walls, who generously assisted in developing an in vivo infiltration technique. Several others also supported us in various aspects of this work: K. Beck, V. Traverso, A. Orchard, and C. Christadoss provided very useful assistance with data collection. H. Yin, as always, freely gave us expert technical help and prepared the complex graphical displays presented herein.

#### REFERENCES

- Barlow RB Jr.** Circadian rhythms in the *Limulus* visual system. *J Neurosci* 3: 856–870, 1983.
- Barlow RB Jr, Chamberlain SC, Levinson JZ.** *Limulus* brain modulates the structure and function of the lateral eyes. *Science* 210: 1037–1039, 1980.
- Battelle B-A** Circadian efferent input to *Limulus* eyes: anatomy, circuitry and impact. *Microsc Res Tech* 58: 345–355, 2002.
- Battelle B-A, Evans JA.** Efferent fibers to *Limulus* eyes synthesize and release octopamine. *Science* 216: 1250–1252, 1982.
- Bolbecker AR, Cheng Z, Felsten G, Kong K-L, Lim CCM, Nisly-Nagele SJ, Wang-Bennett LT, Wasserman GS.** Two asymmetries governing neural and mental timing. *Conscious Cogn* 11: 265–272, 2002.
- Bolbecker AR, Cheng Z, Wasserman GS.** Time versus size: which characteristic of a neural response carries more information? *Biol Cybern* 88: 73–78, 2003.
- Bolbecker AR, Lewis AR, Swan AA, Carlson K, Fleet JR, Beck KE, Wasserman GS.** Stable bellows cup electrode demonstrates low-frequency properties of long-term electroretinographic recordings in the *Limulus* lateral eye. *J Neurosci Methods* 159: 252–260, 2007.
- Bolbecker AR, Lim CCM, Li J, Traverso V, Orchard A, Christadoss CS, Brahmhatt J, Beck KE, Lewis AR, Fleet J, Carlson KW, Hoyt C, Collins MD Jr, Swan A, Wasserman GS.** Are photoreceptors in the attention spotlight? Efferent neuromodulators accelerate and/or retard the time course of photoreceptor responses evoked by light. *J Vis* 5: 680a, 2005.
- Borsellino A, Fuortes MGF, Smith TG.** Visual responses in *Limulus*. *Cold Spring Harb Symp Quant Biol* 30: 429–443, 1965.
- Calman BG, Battelle BA.** Central origin of the efferent neurons projecting to the eyes of *Limulus polyphemus*. *Vis Neurosci* 6: 481–495, 1991.
- Chamberlain SC, Engbretson GA.** Neuropeptide immunoreactivity in *Limulus*. I. Substance P-like immunoreactivity in the lateral eye and protocerebrum. *J Comp Neurol* 208: 304–315, 1982.
- Chapman RM, Lall AB.** Electroretinogram characteristics and the spectral mechanisms of the median ocellus and the lateral eye in *Limulus polyphemus*. *J Gen Physiol* 50: 2267–2287, 1967.
- Couture R, Regoli D.** Inactivation of substance P and its C-terminal fragments in rat plasma and its inhibition by Captopril. *Can J Physiol Pharmacol* 59: 621–625, 1981.
- Kass L, Barlow RB, Jr.** Efferent neurotransmission of circadian rhythms in *Limulus* lateral eye. I. Octopamine-induced increases in retinal sensitivity. *J Neurosci* 4: 908–917, 1984.
- Khadilkar RV, Mytinger JR, Thomason LE, Runyon SL, Washicosky KJ, Jinks RB.** Central regulation of photosensitive membrane turnover in the lateral eye of *Limulus*. I. Octopamine primes the retina for daily transient rhabdom shedding. *Vis Neurosci* 19: 283–297, 2002.
- Li J.** *Comodulation of Limulus Lateral Eye Photoreceptors by Efferent Neuromodulators* (PhD dissertation). W. Lafayette, IN: Purdue University, 2009.
- Li J, Stroup LM, Syverson EA, Brahmhatt JD, Wasserman GS.** Physical, chemical, and biological factors in the comodulation of *Limulus* lateral eye photoreceptors by two efferent neuromodulators. Personal communication, 2009.
- Lim CCM, Wasserman GS.** Categorical and prolonged potentials are evoked when brief, intermediate-intensity flashes stimulate horseshoe crab lateral eye photoreceptors during octopamine neuromodulation. *Biol Signals Recept* 10: 399–415, 2001.
- Lim-Kessler' CCM, Bolbecker AR, Li J, Wasserman GS.** Osmotic properties of *Limulus* seawaters and organ cultures: an unrecognized issue. *Vis Neurosci* 25: 103–105, 2008a.
- Lim-Kessler' CCM, Bolbecker AR, Li J, Wasserman GS.** Visual efference in *Limulus*: In vitro temperature-dependent neuromodulation of photoreceptor potential timing by octopamine and substance P. *Vis Neurosci* 25: 83–94, 2008b.
- Mancillas JR, Brown MR.** Neuropeptide modulation of photosensitivity. I. Presence, distribution, and characterization of a substance P-like peptide in the lateral eye of *Limulus*. *J Neurosci* 4: 832–846, 1984.
- Mancillas JR, Selverston AL.** Neuropeptide modulation of photosensitivity. II. Physiological and anatomical effects of substance P on the lateral eye of *Limulus*. *J Neurosci* 4: 847–859, 1984.
- Mancillas JR, Selverston AI.** Substance P-like immunoreactivity is present in the central nervous system of *Limulus polyphemus*. *J Comp Neurol* 238: 38–52, 1985.
- Nisly-Nagele SJ, Wasserman GS.** Dissociating sensory and cognitive contributions to visual persistence. I. Photoreceptor response duration as a function of flash intensity, adaptation state, and candidate code. *Biol Cybern* 85: 167–183, 2001.
- O'Day PM, Lisman JE.** Octopamine enhances dark adaptation in *Limulus* ventral photoreceptors. *J Neurosci* 5: 1490–1496, 1985.
- Pieprzyk AR, Weiner WW, Chamberlain SC.** Mechanisms controlling the sensitivity of the *Limulus* lateral eye in natural lighting. *J Comp Physiol [A]* 189: 643–653, 2003.
- Renninger GH, Schimmel R, Farrell CA.** Octopamine modulates photoreceptor function in the *Limulus* lateral eye. *Vis Neurosci* 3: 83–94, 1989.
- Stimler-Gerard NP.** Neutral endopeptidase-like enzyme controls the contractile activity of substance P in guinea pig lung. *J Clin Invest* 79: 1819–1825, 1987.
- Wasserman GS.** *Limulus* psychophysics: dark adaptation in the ventral eye. *J Exp Psychol* 104: 68–76, 1973.
- Wasserman GS, Cheng Z.** Electroretinographic measures of vision in horseshoe crabs with uniform versus variegated carapaces. *Biol Signals* 5: 247–262, 1996.