NEUROPEPTIDE MODULATION OF PHOTOSENSITIVITY

II. Physiological and Anatomical Effects of Substance P on the Lateral Eye of *Limulus*[1]

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Abstract

A system of efferent substance P-like immunoreactive fibers innervates the ommatidia of the *Limulus* lateral eye. Thus, we tested the physiological effects of substance P on the lateral eye by measuring the electroretinogram, a population potential reflecting the photoreceptors' response to light, under different experimental conditions. Substance P had no direct effect on the photoreceptors, but it induced an increase in their responsiveness to test flashes of light. The latency, magnitude, and duration of this reversible modulatory effect was dose-dependent. The lateral eye displays an endogenous circadian rhythm in its responsiveness to light. Application of exogenous substance P in the daytime causes an immediate rise as well as an increase in the nocturnal peak, while injection of one of its antagonists (D-Pro^2, D-Phe^7, D-Trp^9 substance P) in the afternoon retards the normal rise in sensitivity and reduces the nighttime levels. Passive incubation with substance P antibodies at midnight caused a drop to diurnal levels of photosensitivity. Short-term changes in photosensitivity, similar in their nature to the substance P-induced ones, were caused by arousing the subjects. Arousal had an effect on the ongoing circadian rhythm similar to that of substance P application. Thus, the substance P efferent system may regulate neural responsiveness in both a short-term, environmentally induced manner, as well as for level setting in a circadian fashion. The mechanism for substance P-induced increases in photosensitivity involves changes in ommatidial structure: contraction of distal pigment cells, resulting in an increased aperture, and contraction of the retinular cells and rhabdom, resulting in a wider diameter of the latter. These structural modifications result in a greater angle of acceptance and increased light quantum catch.

Neuropeptides, such as the undecapeptide substance P, which were first isolated from mammalian nervous systems, have now been found in several invertebrates that may serve as useful model systems (see Haynes, 1980; O'Shea, 1982; Strumwasser, 1982; for reviews).

We have found a substance P-like peptide in a system of efferent fibers that innervates the photoreceptive units of the lateral eye of *Limulus polyphemus* and originates in two cell clusters located in the circumesophageal connectives (Mancillas et al., 1981; Mancillas and Selverston, 1982; Mancillas and Brown, 1984). They appear to be part of a larger system of six pairs of substance P-li containing clusters in the posterior circumesophageal ring. This larger system may constitute an effector "neurosecretory" system involved in the regulation of neural responsiveness and activity by both a circadian clock and arousal or alert systems (J. R. Mancillas and A. I. Selverston, submitted for publication).

The lateral eyes of *Limulus* undergo circadian changes in their sensitivity to light (Barlow et al., 1977; Barlow and Chamberlain, 1980; Barlow, 1983) believed to be mediated by efferent fibers. Efferent fiber activity seems to bring about those increases in photosensitivity by a mechanism that involves: (*1*) changes in ommatidial structure and a resulting increase in angle of acceptance (Barlow et al., 1980) and (*2*) a decrease in photoreceptor noise or spontaneous quantum bumps (Kaplan and Barlow, 1980).

Given the presence and distribution of the efferent

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system containing a substance P-like peptide, we have explored the possible involvement of substance P or a related peptide in the circadian modulation of photosensitivity in the lateral eye and its underlying structural changes. Our results show that substance P can modify ommatidial structure, increase the photoreceptors' responsiveness to light, and alter ongoing endogenous circadian rhythms of photosensitivity. Substance P antagonists and antibodies, on the other hand, can depress the spontaneous nocturnal rises in photosensitivity. This suggests that the substance P-like peptide-containing efferent system is at least partially involved in circadian regulation of photosensitivity in the lateral eye. The apparent ability of the undecapeptide substance P and its pharmacological agents to interact with the postsynaptic structures indicates that this highly favorable model system (Mancillas and Brown, 1984) can be exploited to elucidate general questions about the cellular and molecular mechanisms of action of substance P.

Materials and Methods

Specimens of *L. polyphemus* were obtained from Gulf Specimens (Panacea, FL) and kept in running sea water tanks. Illumination was cyclic, with equal periods of light and darkness. Large size adults, mostly female, 20 to 24 cm in prosomal width, were used for our experiments.

Electroretinogram (ERG) measurements. Individuals were placed in a light-sealed box and clamped to the bottom of a shallow Plexiglas container with constantly aerated sea water. They were allowed to adapt to the experimental setting and display their endogenous circadian rhythm of photosensitivity by being left undisturbed in complete darkness, except for the application of test flashes of light as described below, for at least 2 days before any experiment was performed.

Light stimulation was provided by a Tiyoda 7012/MR-38A illuminator system and delivered to selected areas of the lateral eye via light guides. The population response, or ERG, was recorded with a wick electrode placed over the cornea (Fig. 1). A reference electrode was placed into the prosoma through a fine hole drilled on the carapace at the posterior-most end of the ophthalmic ridge. The signal obtained was passed through a Tektronix FM-122 AC-coupled amplifier, displayed in an RM 565 dual beam cathode ray oscilloscope (Tektronix), and filmed with a Grass C4N Kymograph camera. Continuous records were also kept on a Gould brush 260 recorder. All of the equipment was regulated by a Chrontrol timer, programmed to turn it on for appropriate brief periods of time at 10- or 30-min intervals, depending on the experiment.

A 1-sec test flash of light was delivered every 10 min during experiments where short-term effects were to be observed and measured. This was the shortest interval which would safely and reliably circumvent light adaptation with the intensities of illumination used. For chronic, continuous recordings that extended for several days, light stimulation was presented every 30 min. The intensity of the light pulse was chosen by calculating the dose response relationships (Fig. 2) and using intensities in the middle range of the curve.

Pharmacological experiments. Drugs were delivered through a fine needle implanted subcorneally at the posterior edge of the lateral eye (Fig. 1) and connected via a very fine polyethylene tubing to a Gilson microsyringe. This arrangement allowed injections at a steady, controlled rate without disturbing the subject, the electrodes, or the light guides. In most experiments, especially those where effects on spontaneous circadian rhythms were tested, implantations were performed at least 24 hr before any experimental manipulation. They were quickly performed under a dim red safety light during the lower sensitivity, diurnal phase of the circadian cycle. Experiments were continued only after verifying that the cycle established during the 2 previous days was unaltered by the implantation and/or control saline injections; otherwise they were discontinued.

All drugs were dissolved immediately prior to injection in room temperature saline solution with 0.1% bovine serum albumin (Reheis Co.) added to prevent adherence of substance P to surfaces. Every single experimental injection was preceded by a control injection of the carrier solution, delivered 15 to 30 min earlier. The total volume applied was 100 μl at a rate of 20 to 30 μl/min.

Testing the effects of arousal on photosensitivity was difficult, as we have not as yet found a reliable, quantitatively definable unconditioned stimulus that will consistently produce an unmistakable, quantifiable "arousal" response. Equally difficult is the quantification of arousal under our experimental conditions that require total darkness. Because of this and the unreliability of the stimuli used, showing a blockage of arousal-induced changes in photosensitivity in an unequivocal way was virtually impossible. Stimuli that can produce "arousal" (intense locomotor activity) include manipulation of the tail spine or cutting its tip and nocioceptive stimulation of the arthrodial membrane.

Effects on ommatidial structure. The effects of drugs on lateral eye structure were tested by *in vitro* incubation in solutions of different drugs. Subjects were kept in complete darkness or a controlled, cyclic light/dark environment for 2 days. Both lateral eyes were then rapidly excised from each subject at noon and cut in half. One half was used as control and incubated in saline, whereas the second one was incubated in a saline solution containing varying concentrations of substance P (Sigma and Peninsula), octopamine (Sigma), or a combination of substance P and D-Pro^2, D-Phe^7, D-Trp^9 substance P (Peninsula). Solutions were prepared at room temperature immediately before incubation. Incubations extended for periods of 30, 45, or 60 min, after which all half eyes were placed in a boiling water bath for 5 min, fixed, embedded, and sectioned.

The ommatidial structural parameters compared included aperture diameter, distance between the lens and the rhabdom's distalmost edge, and rhabdom length and diameter. Rhabdom elongation was selected as the more reliable and convenient parameter for quantitative comparison between eyes subjected to different treatments. To allow comparison between eyes of different subjects, the data were normalized by dividing the rhabdom's length by the diameter, yielding the rhabdom elongation ratio. The rhabdom elongation ratio of the ommatidia from each half eye was obtained and averaged. The
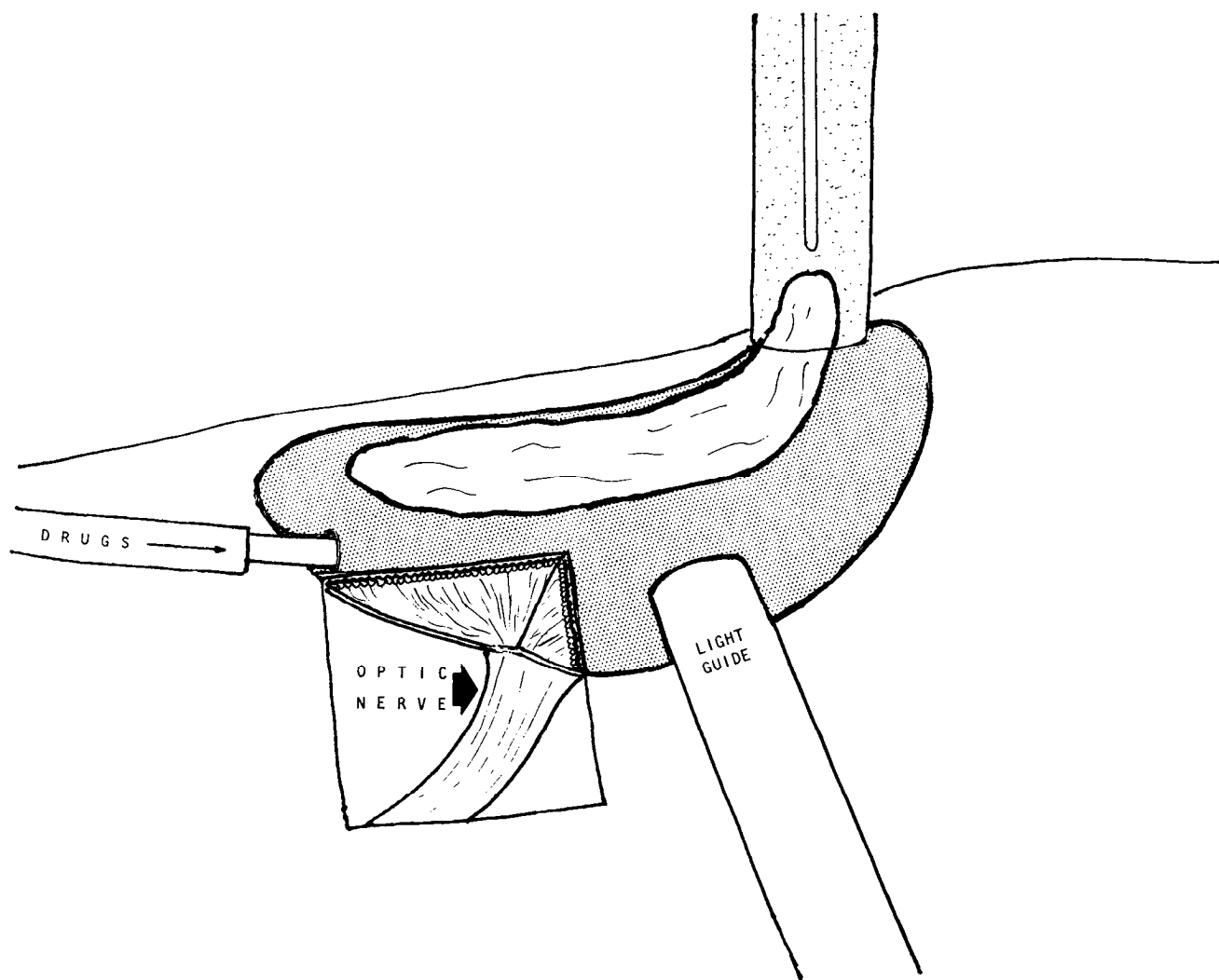
Figure 1. Schematic diagram of the experimental set-up. Light pulses were delivered to a population of photoreceptors through a light guide positioned in front of the cornea. The response was recorded with a wick electrode placed over the cornea. A very fine needle, connected to a micrometer syringe by thin plastic tubing, was inserted subcorneally and used to inject drugs.

Student's t test was used to determine the statistical significance of the differences in average rhabdom elongation ratio observed between any eye and its control half eye.

Other putative neurotransmitter substances used for these experiments were obtained from Sigma.

Results

The ERG. Photoreceptor responsiveness was monitored by measuring the amplitude of the ERG induced by a 1-sec test flash of light. The main component of the ERG is a transient negative wave, which is believed to represent the depolarization of a population of photoreceptors (Svaetichin, 1956; Chapman and Lall, 1967). To select the most appropriate intensity for the test light pulse, we determined the dose response characteristics of the ERG by measuring its amplitude in response to a 1-sec light pulse of varying intensities, presented every 10 min in a random order. These measurements were performed well within the lower sensitivity/diurnal

phase of the circadian cycle demonstrated by the subjects during the previous 2 to 4 days. Figure 2 (*left*) shows the relationship observed. When, instead of its absolute value, the log of the intensity is plotted in the abscissa, the relationship becomes linear (Fig. 2, *right*).

Substance P effects on photosensitivity. Subcorneal injection of substance P (10^{-9} to 10^{-7} mol), induced a clear increase in the photoreceptors' responsiveness to light. Figure 3 shows the results from a subject whose lateral eyes were presented with a test flash of light every 10 min. Injection of 10^{-7} mol of substance P into the right eye (Fig. 3, *bottom traces*) at noon, causes a 2-fold increase in ERG amplitude that peaks by 10 min (Fig. 3, *bottom center*) and slowly recovers (Fig. 3, *bottom right*), while simultaneous injection of saline in the contralateral eye of the same subject (Fig. 3, *top traces*) has no effect. The effect of substance P is dose-dependent (Fig. 4), with higher dosages reducing its latency, increasing its intensity, and prolonging its duration.

As can be seen, the time course of this modulatory

effect is very slow, with a latency that ranges from seconds to several minutes, and a duration that can extend over an hour. Notice that an eye receiving a control injection of the carrier saline solution under identical conditions (Fig. 4, *dashed line and open circles*)

shows no alteration in its response to light. Additionally, all experimental injections were preceded (15 to 30 min) by control saline injections which had no observable effect. Other putative peptide neurotransmitters, including enkephalin, β-endorphin, and physalemin (10^{-3} M),
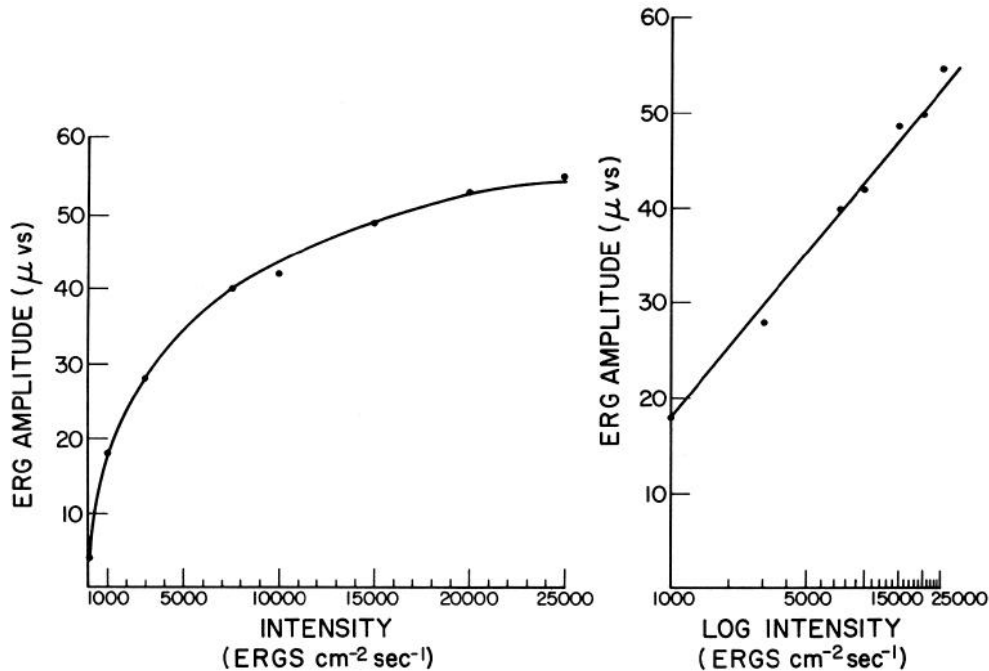

Figure 2. The electroretinogram: dose response relationships. The amplitude of the electroretinogram was plotted as a function of the intensity of a 1-sec light pulse. Measurements were performed from noon to 2 P.M. on the eyes of animals kept in complete darkness for over 24 hr and during the experiment. The relationship becomes linear when the log of the intensity is plotted in the abscissa (*right*).
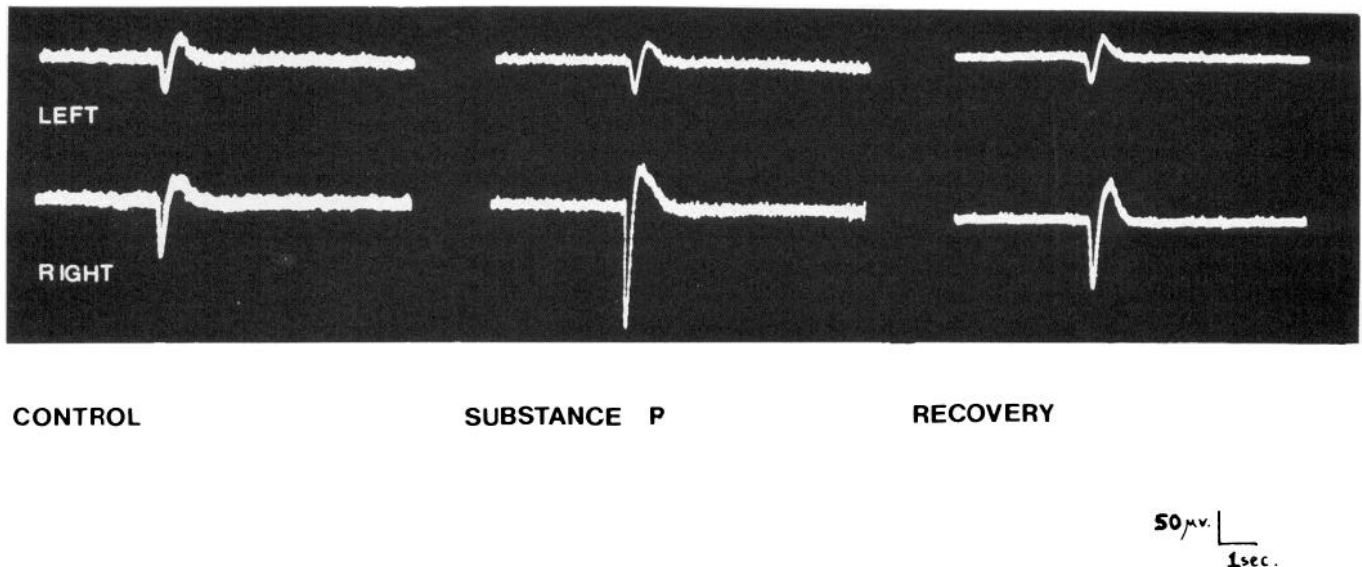

Figure 3. Substance P-induced increases in photosensitivity. The electroretinogram was recorded simultaneously from both lateral eyes, which were presented with light pulses every 10 min. The left eye (*top traces*) was unaffected by an injection of saline (*top center*). The right eye (*bottom traces*) received a simultaneous injection of substance P-containing saline. Ten minutes after the injection, the ERG displayed a larger than 2-fold increase (*bottom center*) over the control response (*bottom left*). An hour later, the ERG had almost completely recovered to its pre-injection level of responsiveness (*bottom right*). *Calibration:* 50 μV, 1 sec.
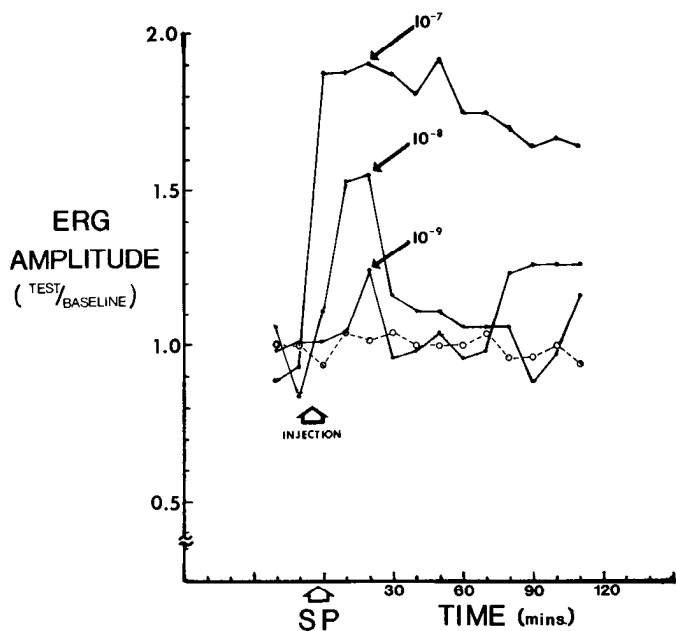
Figure 4. Substance P modulation of photosensitivity: dose response relationships. The amplitude of the ERG elicited by 1-sec light pulses delivered every 10 min is plotted as a function of time after the injection of different amounts of substance P. The *dashed line* shows the lack of effect of control saline injection. Application of larger doses of substance P shortened the latency of the induced rises in photosensitivity and increased their intensity and duration. The values of the ERG amplitude were normalized by dividing them by an average of the three responses preceding the beginning of the experiments, which were performed between noon and 4 and 5 P.M. The rises seen in the three curves at the end of the experiment reflect phenomena explained in Figure 6.

had no effect. Finally, the effects of substance P can be blocked by D-Pro^2, D-Phe^7, D-Trp^9 substance P (see Fig. 13 for related data).

Substance P effects on circadian rhythms of photosensitivity. The lateral eye displays a spontaneous circadian rhythm in photosensitivity (Fig. 5). One-second test flashes of light presented every 30 min elicit an ERG whose amplitude varies depending on time of day, with higher amplitude responses observed at night and lower ones during the day. The length of the free-running circadian cycle, under continuous darkness and in the absence of any other external temporal cues (Aschoff, 1960), deviates slightly from the 24-hr period of the solar day and varies from subject to subject.

Slight individual differences were also observed in the waveform of the circadian oscillations in photosensitivity, which in most subjects resembled a quasisinusoidal curve, with a slight negative slope in the nocturnal plateau (as seen most clearly in Figs. 6 and 8). Subjects, for instance, varied in the slope of the rise and fall in photosensitivity. The eye recorded from in Figure 5 shows a rise period of 3 to 3½ hr and a fall period of 3 to 4½ hr, whereas those in Figures 6 and 8 display a rise period of 2 and 3½ hr respectively, and a fall period of 6 hr and 3 to 3½ hr during the "control" days preceding experimental manipulation.

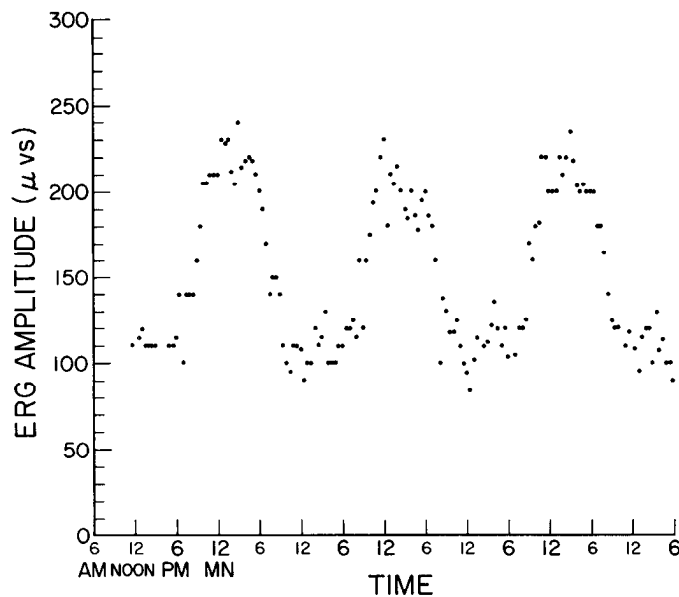Differences in the amplitude of the circadian oscillations are clearly observable (compare Figs. 5 to 8, and

Figure 5. The lateral eye displays a spontaneous circadian rhythm in photosensitivity. The eyes were presented with a test flash of light every 30 min for several days, and the ERG was recorded. The amplitude varied depending on the time of day, with a plateau of higher values at night and lower values during the day. In this time scale, the rise and fall in values appears relatively sharp in most cases. However, in another time frame they are very slow. The shortest rise period observed, for example, was 1½ hr. The length of the free-running circadian cycle varied with each subject.

11). We cannot, however, determine to what extent they represent real differences between subjects or if they are due solely to differences in the relative intensity and average angle of incidence of the stimuli reaching each eye, the size of the photoreceptor population recorded from, and other variables that clearly influence the ERG's amplitude but are impossible to replicate exactly when recording the ERG from different eyes. In fact, while both eyes in any one subject displayed identical waveform and length in their circadian oscillations, the amplitude of their ERG (see Fig. 3) and the magnitude of the circadian changes in photosensitivity were frequently different. The eye shown in Figure 8, for example, displayed a 2.5-fold nocturnal increase, while the response of the contralateral eye (not shown) increased 3.5-fold.

The spontaneous circadian rhythm of photosensitivity recorded from any one eye, however, is relatively constant in all of its parameters over a period of several days and is not altered by control saline injections through a subcorneally implanted needle (see "Materials and Methods"). This permits the evaluation of the effects of applying different drugs and other experimental variables. Comparisons can be made between subjects by normalizing the data and comparing the relative magnitude of any changes.

Injection of 5×10^{-7} mol of substance P (Fig. 6, *arrow*) caused a rapid 2.3-fold increase in the amplitude of the photoreceptors' responses (Fig. 6, *solid line*), which peaked within ½ hr and began to decline. Complete recovery from the modulatory effects of substance P was interrupted 1½ hr after injection by the normal endoge-
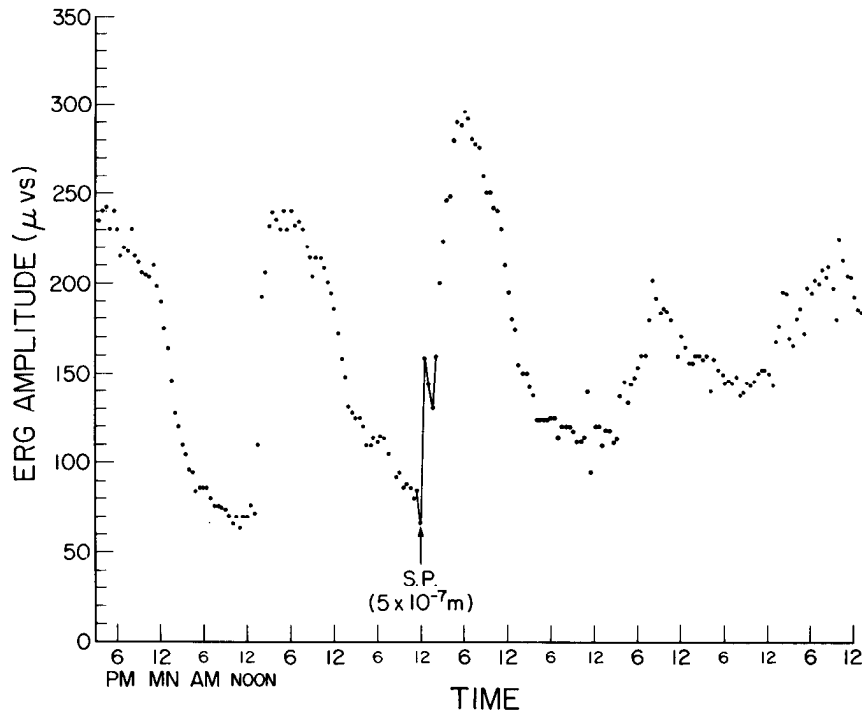
Figure 6. Effects of substance P on the circadian rhythm. The endogenously controlled fluctuations in photosensitivity were monitored by presenting a test 1-sec light flash every 30 min. Injection of substance P (*arrow*) produced an immediate rise in photosensitivity (*solid line*) which peaked within ½ hr and began to decline. Complete recovery from the effects of substance P was interrupted 1½ hr after injection by the normal endogenous rise in responsiveness. (This can also be observed at the end of the curves shown in Fig. 4.) The nocturnal values were considerably higher than on the preceding nights. The converse is true on the following night, perhaps due to some compensatory mechanism. The rhythm then begins to normalize slowly.
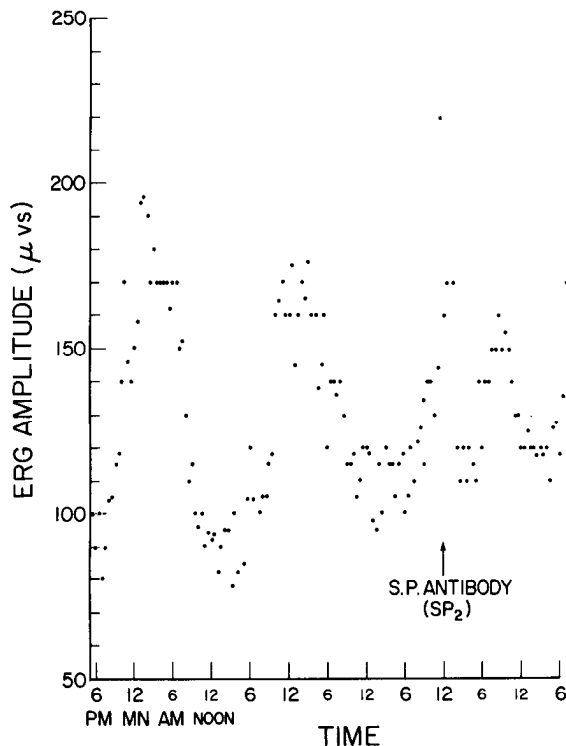

Figure 7. Passive incubation in substance P antibodies. The lateral eyes were monitored as in the preceding figures. Injection of a substance P serum antibody at midnight (*arrow*) caused a drop in the photoreceptors' responsiveness to their diurnal level. This drop occurred with a latency of 1½ to 2 hr and lasted for 4 hr, after which photosensitivity returned to nocturnal levels for the remainder of that period.

nous rise in photosensitivity. In addition to the short-term modulatory effect of substance P, a 25% increase in the amplitude of the responses during the nocturnal plateau can be observed as a result of the injection. As will be seen later, the opposite effect is observed after application of a substance P antagonist. Notice that during the following night the nocturnal values are much lower, perhaps due to a compensatory mechanism, and the rhythm begins to normalize only slowly.

The nocturnal rises in photosensitivity of the lateral eye are induced by the activity of efferent fibers (Barlow et al., 1977). Since (*1*) substance P can induce similar increases and alter the endogenous rhythm, and (*2*) efferent fibers that contain a substance P-like peptide innervate the ommatidia of the lateral eye, we explored the possibility that the endogenous, efferent-induced nocturnal rises in photosensitivity may be accomplished by the release of a substance P-like peptide.

To this end, we tested the effects of application of D-Pro[7], D-Phe[7], D-Trp[9] substance P, a peptide analogue of substance P which acts as an antagonist (Folkers et al., 1981), and passive incubation in SP$_2$, an anti-substance P serum antibody (Mancillas and Brown, 1984) on the spontaneous circadian rhythms of photosensitivity. Both were found to have the same effect of antagonizing efferent-induced increases in responsiveness, although the antibody was much more effective.

Injection of SP$_2$ at midnight (Fig. 7, *arrow*), 1½ hr after the nocturnal peak was reached, resulted in a drop in the photoreceptors' responsiveness to their diurnal level. This drop was not immediate, but had a latency of approximately 1 to 1½ hr and lasted for 4 hr, after which
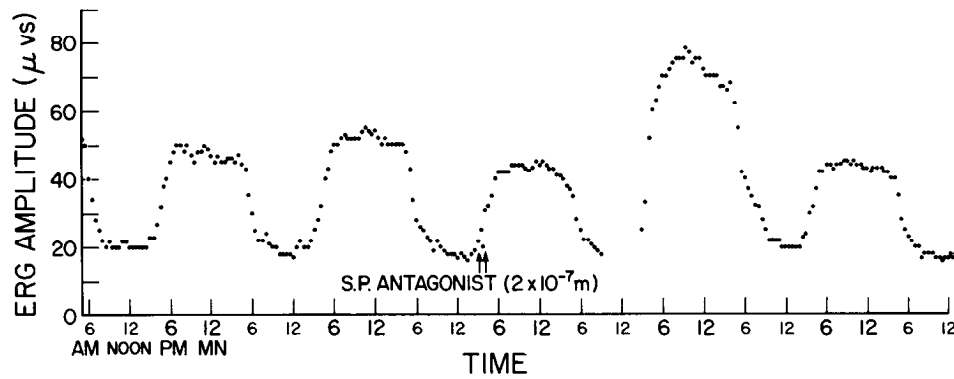
Figure 8. D-Pro^2, D-Phe^7, D-Trp^9 substance P blocks efferent activity. The substance P antagonist was injected into an eye being monitored as in the preceding figures. A first injection was delivered right at the onset of the nocturnal rise, followed by a second one an hour later. This produced a slight downward shift in the slope of the curve and the lowering of the values in the nocturnal plateau. Notice that the nocturnal peak on the following night is considerably higher than normal, again probably due to some compensatory mechanism. The rhythm finally stabilizes, although with a lower nocturnal level than before the experimental manipulation.

the eye returned to its nighttime level of photosensitivity for the remainder of the nocturnal phase. The long latency of the effects of passive incubation in SP$_2$ is not surprising, since at the time of injection the photoreceptors have already undergone the efferent-induced morphological changes (Barlow et al., 1980) that underlie increased photosensitivity. As shown earlier, the effects of exogenous substance P are long-lasting, with a duration that can be longer than an hour. Thus, the blockage by SP$_2$ of the effects of the efferent transmitter released after its injection would not be observed until the long-lasting effects of the transmitter released prior to injection wore off. More significantly, when nocturnal levels of sensitivity are induced by stimulation of efferent fibers *in situ* (Barlow et al., 1977; Barlow, 1983), the ERG amplitude does not drop immediately after stimulation ceases, but it decays with a time constant of about an hour. Cutting the optic nerve at night results in a permanent drop to daytime levels of photosensitivity, which, as the same authors show (Barlow et al., 1977; Barlow, 1983), occurs gradually over a period of 1 to 2 hr. Thus, application of agents that block the action of substance P seems to mimic the cessation of spontaneous or induced efferent fiber modulation of photosensitivity.

In addition to their short-term depression of the higher nocturnal levels of photosensitivity, agents that block substance P action can cause longer term alterations of the circadian rhythm similar to those shown in Figure 6 but of the opposite nature. This was observed by injecting D-Pro^2, D-Phe^7, D-Trp^9 substance P (*arrows*, Fig. 8), right at the onset and during the spontaneous "afternoon" rise in sensitivity, instead of within the nocturnal plateau. The two injections (2 × 10^{-7} M each) were given an hour apart. As seen in Figure 8, this caused a brief drop after the first injection (*point* between the tips of the *double arrows*) and a slight depression of the rising values of the ERG when compared to the corresponding values the previous 2 days. In addition, the nocturnal peak and all of the responses during the nocturnal plateau were consistently lower than on previous cycles. Notice that on the following day, the nocturnal values are considerably
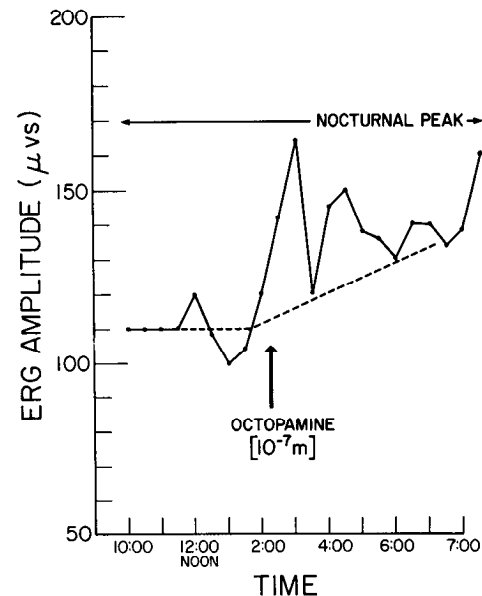

Figure 9. Octopamine-induced increases in photosensitivity. A 1-sec test flash of light was delivered every 10 min, and the ERG's amplitude was recorded. The *dashed line* represents the expected endogenously controlled rise in responsiveness, estimated from that observed on previous days on the same subject. Injection of octopamine caused a rise in photosensitivity similar in its characteristics to that induced by substance P. Notice the irregularity of the curve during recovery.

higher than normal, again probably due to a compensatory mechanism. The rhythm finally stabilized, although with a lower nighttime plateau than before the experimental manipulations. The effects, then, are the exact opposite of those seen in Figure 6 after substance P injection.

Finally, we were able to confirm the reports of Kass and Barlow (1980) that octopamine can also induce increases in the amplitude of the ERG (Fig. 9).

Effects of arousal on photosensitivity. While working out our experimental procedures, it became noticeable that disturbing the subjects or applying some nociocep-

tive stimuli could induce intense motor activity and other signs of apparent arousal. This frequently produced an increase in the photoreceptors' responsiveness to light pulses, even though no other variable had been introduced. This was later corroborated by observing and
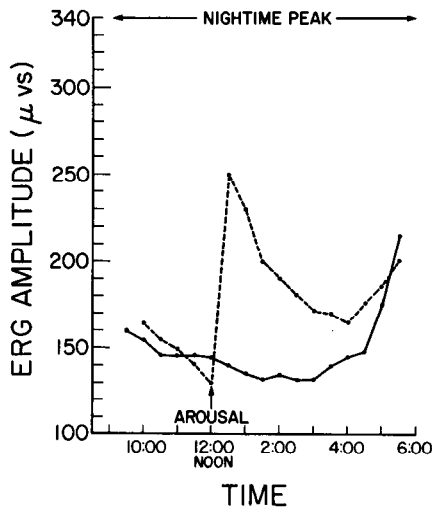

Figure 10. Arousal-induced increases in photosensitivity. Subjects were kept completely undisturbed in a dark box, except for the presentation of a 1-sec test light flash every 30 min. The *solid line* traces the responses to test flashes delivered with an increased frequency of one per 10 min during the period shown in the abscissa. The *dashed line* show a similar measurement performed the following day. Arousing the animal by delivering nocioceptive stimulation caused an increase in photosensitivity remarkably similar to the substance P-induced one in its temporal characteristics.

recording the effects on photosensitivity of "arousal" induced by nocioceptive stimulation.

As shown in Figure 10, arousing the subject during the day can result in a 60% increase in the amplitude of the ERG (*dashed line*). The *solid line* represents the values obtained from the same eye during the corresponding period the day before. Since we have not as yet devised a satisfactory way to quantify "arousal" reliably, we cannot establish a quantitative relation (dose response curve). As it is obvious, the curve shown in Figure 10 looks similar to those observed after injection of substance P or octopamine. Thus, it is possible that the apparent modulatory effects of arousal could be mediated by one of those substances. This possibility could be tested by blocking those effects with an antagonist of those neuroeffectors. Unfortunately, however, the lack of a reliable unconditioned stimulus that can unequivocally and consistently elicit arousal (see "Materials and Methods"), the current technical impossibility of quantifying such a state, and the lack of an established quantitative relationship between levels of arousal and increases in photosensitivity make any attempt to demonstrate unequivocally a blockage or depression of arousal-induced increases in photosensitivity virtually impossible. Since the causal factor is not constant or quantified, any alleged blockage or depression we could observe could always be ascribed to a different or insufficient level of arousal.

Interestingly, however, arousal can induce the longer term effects of substance P injection on the circadian rhythm depicted in Figure 6. As seen in Figure 11, arousing a subject at the onset of the spontaneous rise in photosensitivity (*arrows*) leads to a sharper than
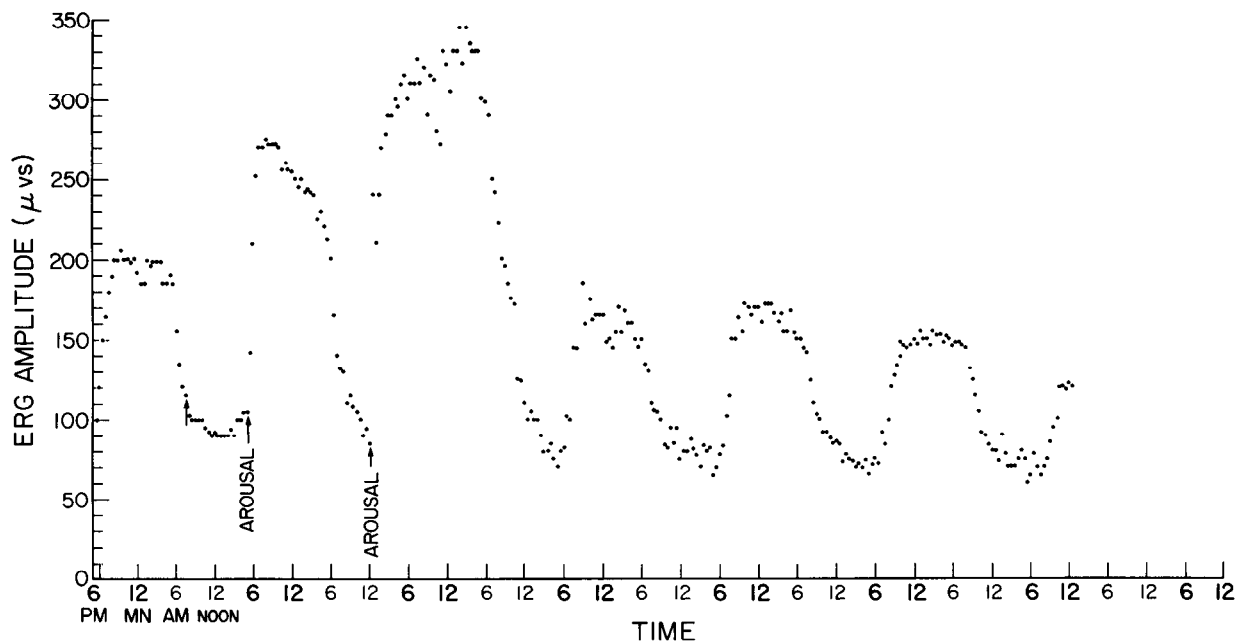
Figure 11. Effects of arousal on the circadian rhythm. Arousing the animal at the onset of the endogenously controlled rise in photosensitivity (*first arrow*) resulted in a sharper rise and a higher nocturnal level of responsiveness than on the preceding nights. Delivery of intense nocioceptive stimulation during the middle of the diurnal period (*second arrow*) produced an immediate rise to the nocturnal levels and a clearly higher nocturnal peak, as well as an extension of the period of higher photosensitivity (almost 24 hr instead of 12). Notice the reduced level of nocturnal responsiveness in the following days, perhaps due to a compensatory mechanism.

normal rise and considerably higher nocturnal responses than in preceding cycles. The amplitude of the circadian oscillations was greatly reduced on the days following the two cycles of experimentally induced higher nocturnal levels, a phenomenon similar to that observed in Figure 6. It appears, then, that either the systems mediating endogenous circadian and arousal-induced elevations in photosensitivity can interact with one another, or both phenomena are mediated by one anatomical system.

Effects of substance P on ommatidial structure. It is believed that one of the mechanisms by which efferent fibers cause circadian oscillations in photosensitivity in *Limulus* involves the inducement of morphological changes (Barlow et al., 1980). Thus, we tested the effects of substance P on ommatidial structure by incubating isolated lateral eyes *in vitro* in saline solutions containing different concentrations of the synthetic undecapeptide.

During the day, light focused by the lens passes through a narrow aperture (15 to 20 μm in diameter) to reach the elongated, photosensitive rhabdom. Portions of distal pigment cells provide a separation of about 30 μm between the lens and the photoreceptors or retinular cells. This "diurnal" structure is unchanged by 30- or 60-min control incubations in saline at noon (Fig. 12, *left*). Simultaneous incubations in substance P-containing saline (Fig. 12, *right*) induced: (*1*) an increased aperture diameter by radial retraction of the distal pigment cells processes, (*2*) a close apposition of the lens and the distal end of the rhabdom and retinular cells, and (*3*) an enlarged diameter and reduced length of the rhabdom, as well as an apparent contraction of the retinular cells.

Barlow et al. (1980) report that these morphological changes, associated with the nocturnal phase, follow the circadian rhythm of efferent optic nerve activity, no longer occur cyclically if the optic nerve is cut, and can be induced by stimulation of the nerve during the day. Thus, substance P can mimic one of the morphological changes associated with efferent optic nerve activity.

Finally, the effects of substance P on rhabdom structure were selected for quantitative comparisons (Fig. 13). These effects are dose-dependent. As can be seen by
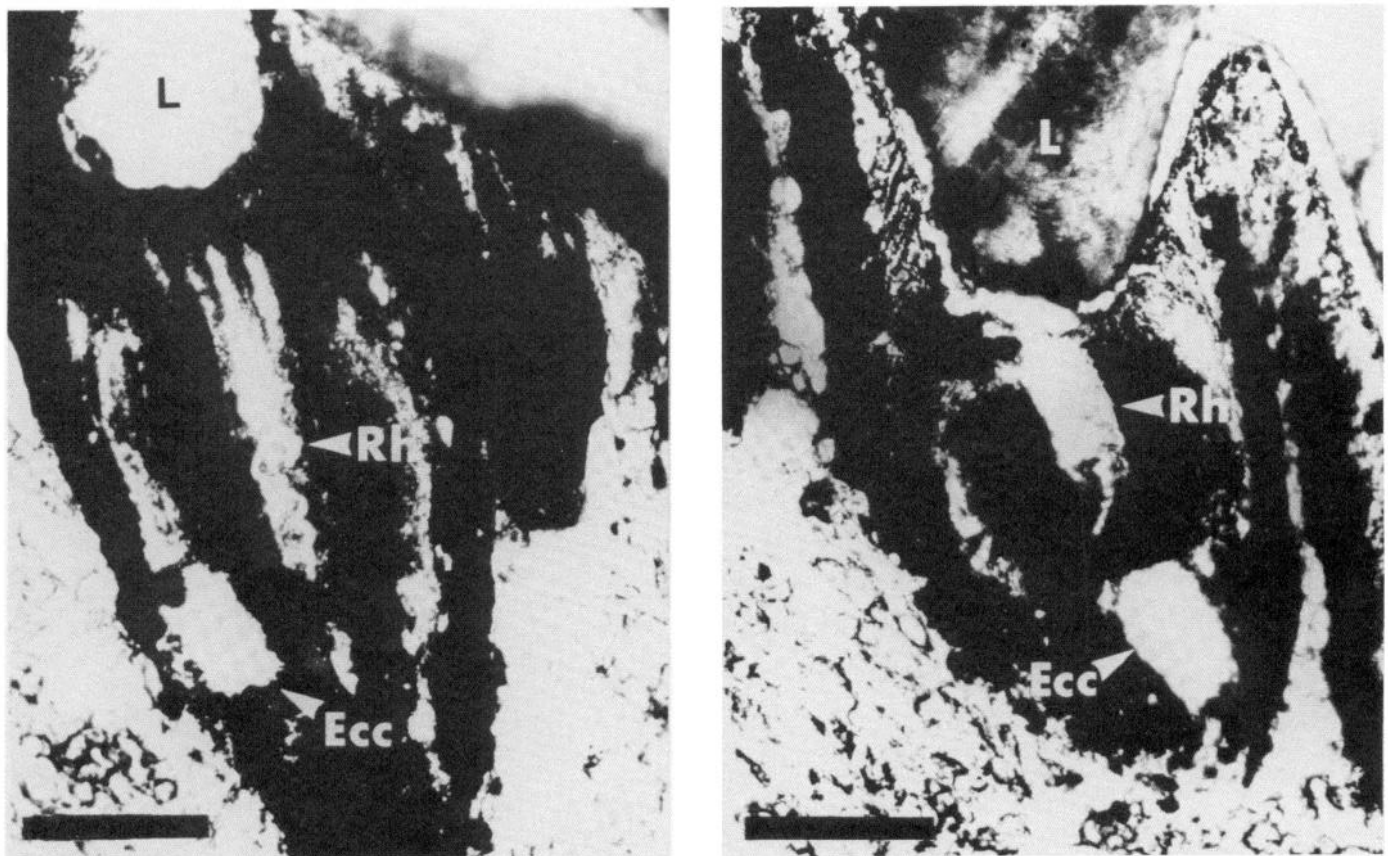

Figure 12. Substance P induces changes in morphology. The lateral eye is made up of several hundred ommatidia. The main components of an ommatidium can be seen in these two longitudinal sections. The lens (*L*) focuses light that passes through an aperture to reach the rhabdom (*Rh*). The photoreceptors or retinular cells (white profiles filled with dark pigment) surround the rhabdom. An eccentric cell (*Ecc*) conveys visual information to the brain. Incubating lateral eyes in saline (*left*) at noon had no effect on the morphology typically observed during the daytime, which includes a narrow aperture (not in the plane of section) and an elongated rhabdom and retinular cells. Simultaneous 30-min incubations in substance P induced changes in morphology, including a widening of the aperture (by contraction of the pigment cells), close apposition of the lens and rhadbom, and contraction of the rhabdom and retinular cells, resulting in increased rhabdom diameter. This is the structure typically observed at night and which permits a greater angle of acceptance to incident light and, consequently, a greater quantum catch. *Calibration bar*: 100 μm.
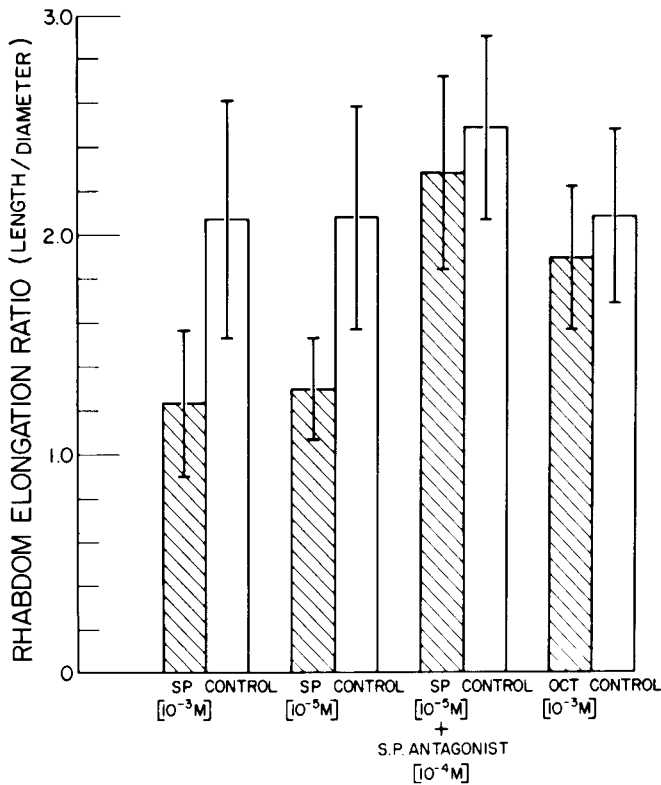
Figure 13. Effects of substance P on rhabdom structure. Lateral eyes were excised at noon and cut in half. One half was incubated in saline, while the second one was incubated in a saline solution containing either substance P at two concentrations (10^{-3} M or 10^{-5} M), substance P (10^{-5} M) plus D-Pro^2, D-Phe^7, D-Trp^9 substance P (10^{-4} M) or octopamine (10^{-3} M). The length and diameter of the rhabdom were measured in sections of all eyes. To allow comparison between eyes of different animals, the length was divided by the diameter and a ratio was obtained. The averages of this ratio for the half eyes incubated for 30 min under each experimental conditon (*shaded bars*) are compared with their saline controls (*open bars*). Notice that the average of the rhabdom elongation ratio is extremely similar for three of the four controls. Incubation in substance P (*first two histograms*) caused a statistically significant (p = <0.01 in both cases) difference in the elongation ratio. This change in rhabdom structure was blocked by co-incubation with D-Pro^2, D-Phe^7, D-Trp^9 substance P. Octopamine had no detectable effect on rhabdom structure.

comparing the *first two shaded histograms* on the *left*, a dose of 10^{-5} M is already close to the saturation plateau. The differences between the ommatidia incubated in substance P for 30 min (Fig. 13, *first two shaded histograms*) and their controls (Fig. 13, *first two open histograms*) are statistically significant (p = <0.01 in both cases). (The reason that both doses are presented in this figure is to allow comparison with a high dose of octopamine (10^{-3} M) and a more reasonable dose (10^{-5} M) being blocked by a substance P antagonist.) Co-incubation of substance P with D-Pro^2, D-Phe^7, D-Trp^9 substance P blocked the changes in rhabdom structure (Fig. 13, *third pair of histograms*). However, octopamine had no statistically significant effect on rhabdom structure during *in vitro* incubation (Fig. 13, *fourth pair of histograms*).

The rhabdom elongation ratio ranges between 1 (nocturnal minimum) and 4 (diurnal maximum) under normal conditions. However, notice that it does not exceed 3, and the controls' averages are between 2 and 2.4 in the data presented in Figure 13. The main reason is that for those particular experiments the subjects were kept in constant darkness for over 48 hr prior to incubation. Prolonged constant darkness has been observed to reduce significantly the elongation of the rhabdom (Barlow and Chamberlain, 1980).

Discussion

The sensitivity of the photoreceptors of the lateral eyes of *Limulus* can be modulated by several naturally occurring factors. The best documented influences are lateral interactions (Ratliff et al., 1969; Hartline and Ratliff, 1972), ambient illumination (Hartline and McDonald, 1947; Beherens, 1974), and modulatory influences by a circadian clock located in the central nervous system (Barlow et al., 1977; Barlow and Chamberlain, 1980; Barlow, 1983). There are several mechanical and electrophysiological mechanisms by which the photoreceptors' responses to light can be regulated, among them pigment migration (Miller and Cawthon, 1974; Beherens, 1974), morphological changes (Beherens, 1974; Barlow et al., 1980), and changes in signal to noise ratio (Dodge et al., 1968; Kaplan and Barlow, 1980). Changes in membrane properties or in intracellular concentration of selected ions are also likely to be involved (Brown and Lisman, 1975; Lisman and Strong, 1979).

Substance P as a transmitter or neuromodulator in the lateral eye. Our results show that substance P induces an increase in the receptors' photosensitivity. This modulatory effect has a long time course and is reversible and dose-dependent. The mechanism by which it is accomplished appears to involve changes in ommatidial structure: contraction of distal pigment cells, resulting in an increased aperture, and contraction of the rhabdom and retinular cells, resulting in a wider diameter of the former. These structural modifications result in a greater angle of acceptance to light and increased quantum catch (Barlow et al., 1980). The changes induced by substance P can be blocked by its antagonist, D-Pro^2, D-Phe^7, D-Trp^9 substance P.

Substance P also alters the endogenous circadian rhythm in photosensitivity by increasing the amplitude of the nocturnal plateau, whereas injection of its antagonist in the afternoon retards the rise in sensitivity and reduces the nighttime levels. If the injection is applied after the plateau has been reached, a short-term, reversible drop to diurnal levels is observed.

Our data are consistent with an involvement of a substance P-like peptide in the efferent-controlled circadian rhythms of photosensitivity.

It is now commonly accepted that certain criteria must be met for a substance to be accepted as the transmitter of a synaptic pathway or the mediator of a particular physiological function (Cooper et al., 1982). (*1*) The substance must be present in the presynaptic structures. (*2*) It must be released by those structures when selectively stimulated. (*3*) Application of the substance must mimic the action of the transmitter released by the

presynaptic fibers when stimulted. (4) Pharmacological agents must have identical effects in blocking or potentiating postsynaptic responses to both the neurally released and the injected exogenous substance.

The results presented here and in the previous report (Mancillas and Brown, 1984) provide evidence that a substance P-like peptide partially fulfills those criteria in the lateral eye. First, a substance P-like peptide is present in efferent fibers that innervate the photoreceptors and the distal pigment cells (Mancillas and Brown, 1984). Examination at the ultrastructural level will be necessary to confirm its presence in synaptic or synaptoid structures. Those fibers seem to originate in substance P-li cell clusters (J. R. Mancillas and A. I. Selverston, submitted for publication) that have been associated with circadian rhythms of activity in another chelicerate (Rao and Habibulla, 1973).

Second, application of synthetic substance P mimics the efferent-mediated nocturnal rises in photosensitivity and alters the amplitude of ongoing circadian oscillations. It also induces the morphological changes that follow the circadian rhythm of efferent optic nerve activity and which are believed to underlie those increases in photosensitivity.

Third, anti-substance P antibodies and a substance P antagonist depress the efferent-mediated higher nocturnal levels of photosensitivity. As noted before, the SP_2 antibody was more effective than $D\text{-}Pro^2$, $D\text{-}Phe^7$, $D\text{-}Trp^9$ substance P in suppressing the effect of efferent activity on the photoreceptors. This may be because this substance P analogue is not a very potent antagonist (Hanley, 1982). Another possible explanation is that it may not compete as well with the substance P-like peptide contained by efferent fibers in the lateral eye, as it competes with substance P for binding of its postsynaptic receptor. The antibody, on the other hand, seems to make no distinction between the two peptides (Mancillas and Brown, 1984). Alternatively, the superior action of the antibody may simply reflect a higher relative dose being contained in the aliquots injected.

We have not as yet demonstrated the fourth criterion, release from presynaptic structures, but will attempt to do so in the near future.

In must be stressed that not only is the evidence listed not complete, but that even if it can be conclusively demonstrated that a substance P-like peptide or *any other substance* is used as a neurotransmitter in the lateral eye, caution must be exercised in attributing to it the mediation of a specific, well defined physiological role. Although, as mentioned before, the photoreceptors' responsiveness can be modified by several factors to achieve the same physiological objective—increased or decreased photosensitivity—this is brought about by different external and internal conditions and fits into the context of different integrated organismic responses. Some of the factors modulating photosensitivity probably utilize different mechanisms (pigment migration, structural modifications, electrophysiological changes) and are mediated by different structures (local interactions, efferent fibers, hormones). However, others may have access to the same structures and/or mechanisms. By the same logic, more than one neurotransmitter may

have access to the same molecular and cellular mechanism, even if released under different conditions or for different organismic purposes.

Under ideal laboratory conditions, those various mechanisms, with different presumed physiological functions, can be isolated. It is obvious, though, that they interact with one another in the normal life of the organism and may even do so in some of the most carefully controlled experiments. Thus, although one may be able to demonstrate the inducement of a particular effect (i.e., increases in photosensitivity) by application of a neuroeffector, establishing conclusively what specific physiological function this subserves requires additional evidence of a specific correlation between the neuroeffector and that function. In the case of substance P, that evidence, by no means conclusive, consists of the alteration of endogenous circadian rhythms of photosensitivity by the peptide and its antagonist, the depression by the latter and the antibody of the efferent-mediated higher nocturnal levels, and the apparent association of the cell bodies of the substance P-li system with circadian rhythms of activity.

Relationships between substance P and octopamine. Octopamine has been found to be synthesized and released by efferent fibers in the lateral eye (Batelle et al., 1982) that resemble those containing a substance P-like peptide (Mancillas and Brown, 1984), as well as the neurosecretory efferents described by Fahrenbach (1981). The possibility of the coexistence of a substance P-like peptide and octopamine in the same fibers has been discussed in the preceding article (Mancillas and Brown, 1984).

Kass and Barlow (1980) have reported that octopamine can increase the amplitude of the ERG, an effect that is dose-dependent, blocked by clozapine, and exhibits a time course similar to that caused by endogenous efferent activity. In addition, they report that clozapine decreases ERG amplitude at night. We have been able to confirm the effects of octopamine on ERG amplitude. Since they have been published only in the form of an abstract, we have included here the data in Figure 9 simply to allow comparison between the effects of substance P with those of octopamine. Recently, Kass and Barlow (1982), recording intracellularly from single photoreceptors, found that octopamine injection could reproduce the electrophysiological characteristics associated with the nocturnal phase. We could not observe any effects of octopamine on ommatidial structure, and the authors mentioned have not reported examining the anatomical effects of the amine.

Interestingly, they do note (Kass and Barlow, 1980) that octopamine increased the amplitude of the ERG to *half* the elevated nighttime level. Thus, it is possible that, whether contained and released by the same fibers or by anatomically segregated systems, octopamine and a substance P-like peptide act synergistically to bring about the full complement of endogenously controlled nocturnal increases in photosensitivity. One could imagine, for example, that binding of one of them (i.e., substance P) to its postsynaptic receptors may induce a cascade of events leading to the morphological changes observed, while improvement of the signal to noise ratio (Kaplan

and Barlow, 1980), increases in gain, and other electrophysiological changes contributing to the increases in photosensitivity may require the action of the second one (i.e., octopamine) on the postsynaptic membrane.

Alternatively, octopamine and the substance P-like peptide may be associated with separate organismic responses (i.e., arousal versus circadian rhythms) but have the photoreceptors as a common target and share the ability to cause increases in photosensitivity, either by a common or different mechanisms.

A complicating factor in elucidating the role of octopamine in the visual system is its presence in the ventral eye. While the lateral eye clearly exhibits regular circadian rhythms of photosensitivity, efforts to demonstrate similar rhythms in the ventral eye, using electrophysiological (Kaplan et al., 1980) and behavioral techniques (Wasserman, 1973), have been unsuccessful. This is not altogether surprising, since the ventral eye does not have the same structure of the compound lateral eye but consists of a small number of loosely arranged photoreceptor cells (Clark et al., 1969). Thus, the mechanism postulated to underlie the efferent-mediated circadian rhythms—changes in ommatidial structure (Barlow et al., 1980)—could not operate in this eye. Consistent with substance P's association with circadian rhythms in photosensitivity and its ability to induce changes in ommatidial structure, substance P-li efferent fibers are abundant in the lateral eye but are absent in the ventral eye (Chamberlain and Engbretson, 1982; Mancillas and Brown, 1984). However, octopamine has been found to be synthesized and released by structurally identical efferent fibers in both the lateral and ventral eye (Batelle et al., 1982).

The association of other efferent-mediated phenomena (Barlow and Chamberlain, 1980) with any putative neurotransmitter has not yet been demonstrated or even explored.

Substance P, arousal, and circadian rhythms. In addition to the factors listed at the beginning of this discussion which can influence photosensitivity, we found that "arousal" can induce increases in photoreceptor responsiveness and alter endogenous circadian rhythms. Although we could not quantitate those effects, they looked qualitatively similar to the effects of substance P, suggesting an association between the two. Our lack of a reliable method of producing a quantitatively measurable "arousal" response makes it difficult to get unequivocal answers to whether the influence of arousal on the lateral eye can be blocked by substance P antagonists.

An association between substance P and arousal, in addition to its mediation of circadian rhythms of photosensitivity, would open an interesting possibility. The cell bodies of the efferent system containing a substance P-like peptide are located in two of the ganglia of the circumesophageal connectives (Mancillas and Brown, 1984; J. R. Mancillas and A. I. Selverston, submitted for publication). Similar cells in the scorpion have been associated with circadian rhythms of activity (Rao and Habibulla, 1973). However, since the circadian clock is believed to reside in the brain (Tyshchenko, 1973; Barlow et al., 1977; Fleissner and Fleissner, 1978), we believe the substance P-li efferent system to be a follower, neurose-

cretory effector system (Mancillas and Brown, 1984; J. R. Mancillas and A. I. Selverston, submitted for publication). It is possible, then, that access to this system is not exclusive to the circadian clock. Other systems, such as those integrating general "alert," "arousal," or escape responses when the animal confronts threatening situations, may also be able to activate it.

Thus, the efferent system containing a substance P-like peptide may be a general neurosecretory effector system capable of modulating sensory, and perhaps also motor, activity which can be activated by different systems, under different conditions, and for different integrated organismic responses that require enhanced sensory sensitivity. The existence of such a system would be more economical and efficient, but this hypothesis must await experimental confirmation.

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