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NEUROPEPTIDE MODULATION OF PHOTOSENSITIVITY

I. Presence, Distribution, and Characterization of a Substance P-like Peptide in the Lateral Eye of *Limulus*[1]

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Abstract

The lateral eye of *Limulus* is innervated by an efferent system of fibers containing a substance P-like peptide. They were detected and their distribution was studied using indirect immunocytochemical techniques and monoclonal and serum antibodies. These thin, efferent fibers travel up the optic nerve, cross the lateral plexus, and branch out profusely when reaching the ommatidial layer. Innervation is extended to more than one component of the ommatidia, including the pigment, retinular, and eccentric cells. Immunoreactive staining could be abolished by absorbing the antisera with as little as 1 μM synthetic substance P. The efferent character of the fibers was established by means of ligation experiments, a technique also used to determine their origin in the circumesophageal connectives. Radioimmunoassay with two different C-terminal serum antibodies confirmed the presence of substance P-like material in the eye in the amounts of 61.44 pg/μg of protein, or up to 18 ng/eye. Gel filtration chromatography of crude extracts of the lateral eye, followed by radioimmunoassay, revealed an elution pattern extremely similar but not identical to that of synthetic substance P. These results show that a system of efferent fibers containing a substance P-like peptide originates in cells in the circumesophageal ring and innervates the ommatidia of the lateral eye. Their distribution and origin suggest an involvement in the modulation of photosensitivity, as part of a larger, generalized, level-setting regulator that is driven by a circadian clock but can also be activated by other systems.

Substance P is one of several neuropeptides that can act synaptically or hormonally to regulate a variety of cell and tissue functions (Von Euler and Pernow, 1977; Skrabanek and Powell, 1980; Snyder, 1980). Much of the research on neuropeptides focuses on their molecular mechanism of action (Tixier-Vidal and Gourdji, 1981), in particular their "modulatory" effects on target cells (Kupferman, 1979; Barker and Smith, 1980; Zieglgansberger, 1980). Invertebrate nervous systems, which have served as useful models for the elucidation of general principles of neuronal function, can also be exploited for the study of peptidergic mechanisms (Haynes, 1980; O'Shea, 1982; Strumwasser, 1982).

An exploratory survey for the presence of interesting neuropeptides in identified neural networks of several arthropods (Mancillas et al., 1980, 1981a) led us to detect the presence of substance P-like immunoreactivity in the lateral eye of *Limulus* (Macillas et al., 1981b; Mancillas and Selverston, 1982). The compound eye of this ancient chelicerate offers many attractive features as a model system for cellular and molecular studies. The accessibility, large size, regularity, and small number of the neural components of the lateral eye have, in fact, facilitated the accumulation of detailed information about their anatomy (Fahrenbach, 1975; Chamberlain and Barlow, 1982) and physiology (Wolbarsht and Yeandle, 1967; Smith and Bauman, 1969; Ratliff and Hartline, 1974) and provided insight into basic principles of intercellular communication (Hartline, 1968). Interestingly, the pho-

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toreceptors' responsiveness is not static but is modulated by both extrinsic and intrinsic factors (Behrens, 1974; Barlow and Chamberlain, 1980).

Here, we report that a system of substance P-like immunoreactive efferent fibers, originating in the circumesophageal connectives, innervates several neural components of the lateral eye. Partial chemical and immunological characterization indicates that the immunoreactive material is a substance P-like peptide. In the accompanying article (Mancillas and Selverston, 1984), we describe the physiological and anatomical effects of substance P on the photosensitive apparatus of the lateral eye, in an attempt to elucidate the function of this efferent system. These studies open the way for detailed analysis of the membrane and molecular mechanisms of action of an important neuropeptide in a highly favorable simple system, where results can be readily translated into their consequences at the cellular, systems, and behavioral levels.

Materials and Methods

Specimens of *Limulus polyphemus* were obtained from Gulf Specimens (Panacea, FL) and kept in holding tanks supplied with running sea water. Illumination was cyclic, with equal periods of light and darkness. Both male and female adults, of sizes varying between 15 and 22 cm in prosomal width, were used for our experiments.

Immunocytochemistry. Lateral eyes, complete with the cornea and a segment of the optic nerve, were quickly dissected under normal room illumination and fixed in 4% paraformaldehyde/0.5% PBS at 4°C. After 10 to 20 hr the tissue was immersed in 30% sucrose/PBS at 40°C until it sank to the bottom. Serial sections (12 μm) were cut at −14°C in a Slee cryostat in transverse, oblique, and longitudinal planes and were collected on gelatin-coated slides. The slides were air dried for 30 min and were then subjected to immunocytochemical staining procedures.

A monoclonal anti-substance P antibody from rat (Cuello et al., 1979) (Accurate Chemicals) was used as the primary antibody for all of the experiments at a dilution of 1:100. Sections were also stained with an anti-substance P antiserum (SP_2, described below) of high specificity, used at a dilution of 1:1000 with 1 mg/ml of bovine serum albumin added. Both antibodies were diluted in 0.1 M PBS/0.3% Triton X-100. Substance P-like immunoreactivity (hereafter referred to as substance P-li) was localized using a rabbit anti-rat IgG-fluorescein isothiocyanate (FITC) in the case of the monoclonal antibody, and a goat anti-rabbit IgG-FITC in the case of the serum antibody. Both were diluted 1:25 in 0.1 M PBS/0.3% Triton X-100.

Sections were rinsed three times for 10 min with 0.1 M PBS and were incubated at 4°C for 15 to 24 hr in the primary antibody solution. After the incubation, the tissue was rinsed again three times and was incubated for 30 min in the secondary antibody solution. After three final rinses in PBS, the slides were air dried and a cover slip was added in paraffin oil. The sections were examined using a Zeiss Universal fluorescence photomicroscope with a Mercury epi-illumination light source (HBO-100) and equipped with a Zeiss filter pack (46-62-49-9904). The sections were photographed on Kodak Ektachrome (400 ASA or Tungsten 160 ASA) or Tri-X (400 ASA) pan film.

To test for specificity of the immunoreactive staining, anti-substance P antibody (1:100) was absorbed overnight with synthetic substance P (Sigma) at concentrations of 0.1, 1, 10, and 100 μM at 4°C. Control sections were added absorbed antiserum, antiserum which had been diluted and stored overnight at 4°C, nonimmune serum, or plain PBS, instead of freshly diluted primary antibody.

The same procedures were used for whole mount preparations, except that sectioning was obviously omitted and the tissue was run through an alcohol series and cleared with methyl salicylate (Sigma) before examination.

The location of the cell bodies of origin of efferent fibers innervating the lateral eye was investigated by using HRP and the fluorescent dye true blue. One thousand or 2000 units of HRP (Sigma) were injected subcorneally into lateral eyes. After survival times of 3 to 7 days, the complete circumesophageal ring (CER), lateral optic nerves (LON), hepatic artery (HA), and several dorsal nerves were dissected, fixed, treated following published procedures (Warr et al., 1981), and examined. Injections of 10 and 20 μl of a 5% aqueous suspension of true blue (kindly donated by P. Sawchenko) were applied subcorneally. Survival times were extended to 7, 12, and 14 days to allow transport through the long (up to 10 cm) optic nerve. Conventional techniques were used to process and examine the tissue (Sawchenko and Swanson, 1981). The CER was examined before and after double staining with anti-substance P antibodies.

Ligation experiments. Ligation experiments were performed to establish the direction of transport of the immunoreactive material and, thus, the direction of travel of the stained fibers.

Small portions of the dorsal carapace were removed directly over the segment of the nerve to be ligated. After identification of the desired nerve(s) and site(s), a piece of black surgical thread was tied snugly around the nerve and its ensheathing artery. Extreme care was exercised to make sure that the nerve had not been severed by the tight ligature. The removed piece of carapace was then replaced and sealed with wax. The subject was allowed to recover in complete darkness. The minimum survival time required for a clearly discernible accumulation of immunoreactivity in one side of the ligature, with the accompanying disappearance of staining on the other, was 3 days for most subjects. Survival times were extended up to 1 week or more when necessary. After that period, the entire CER, LON, lateral eye (LE), HA, and other relevant nerves were dissected and immediately fixed. The identity of the nerves and the precise location of the ligation site were corroborated during the dissection. The tissue was then treated and examined for the presence of substance P-li as described above.

Radioimmunoassay of extracts. Acetic acid extracts of LEs were prepared for radioimmunoassay as follows. Batches of four eyes were boiled for 10 min in 5 ml of 2

N acetic acid. The tissue was sonicated and then ground in a tissue homogenizer. This was followed by centrifugation for 15 to 20 min at 3000 rpm. The supernatant (crude extract) was then lyophilized and used immediately or stored at −70°C.

Radioimmunoassays were carried out following the procedure described by Buck et al. (1983). Two anti-substance P C-terminal serum antibodies (SP_2 and $SP_{228\text{-}8}$) were used. They were obtained after immunization of rabbits with synthetic peptide coupled to bovine thyroglobulin (SP_2) and human alphaglobulin ($SP_{228\text{-}8}$).

Gel filtration chromatography. The crude extract was passed through a column (1.5 cm × 95 cm) of Bio-Rad P-4 and eluted with 10% formic acid. Fractions (5 ml) were collected at a rate of 4 ml/hr. Samples (1 ml) of each fraction were concentrated in a Savant rotary evaporator and subjected to radioimmunoassay using the SP_2 antibody. A second sample was subjected to radioimmunoassay with the $SP_{228\text{-}8}$ antibody. In both cases, the radioimmunoassay of each fraction was performed in duplicate. Another sample was subjected to the Bio-Rad dye reagent protein microassay, described elsewhere (Bradford, 1976).

Results

The LEs of *Limulus* are located in the prosoma, immediately below the ophthalmic ridge, which separates the subophthalmic and extracardiac region of the dorsal carapace (Fig. 1*A*). Their anatomy has been thoroughly described previously (Fahrenbach, 1975), but to enhance the clarity of this report their most salient features will be summarized. LON carries information from the eye to the protocerebrum or brain, which makes up the anterior portion of the CER. Each eye contains a layer of several hundred ommatidia, covered by a cornea (Fig. 1*B*). Below the ommatidial layer is a lateral plexus, which contains fibers involved in lateral interactions, as well as the axons of eccentric cells that form the bulk of the optic nerve. Each ommatidium is a complete functional receptor unit (Fig. 1*C*) served by its own private lens. Light is focused by the lens, passes through an aperture bordered by the distal pigment cells, and reaches the

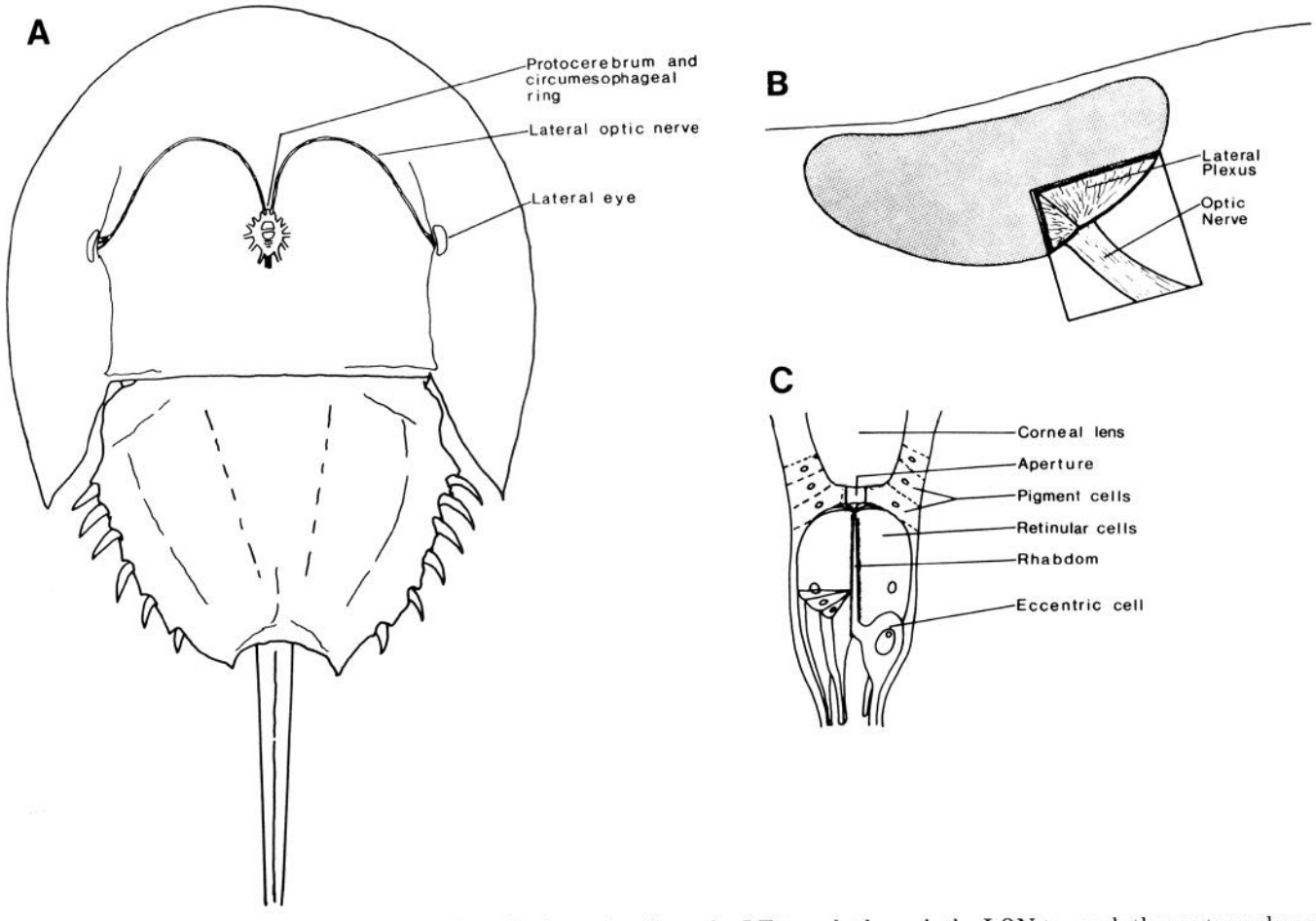

Figure 1. The LE system of *Limulus*. *A*, Visual information from the LE travels through the LON to reach the protocerebrum, located anterior to the esophagus in the ventral side of the animal. *B*, The LE is a complex eye, consisting of a layer of several hundred individual photoreceptor units, the ommatidia, covered by a cornea. The lateral plexus contains fibers involved in lateral interactions, as well as the axons of eccentric cells, that form the bulk of the optic nerve. *C*, Each ommatidium is served by a corneal lens, which focuses light that passes through an aperture bordered by the distal pigment cells, finally to reach the rhabdom. The photosensitive pigment is contained in the rhabdom, formed by invaginations of the membrane of the 10 to 13 photoreceptors or retinular cells that surround it, and the dendrite of an eccentric cell.

rhabdom, where the photosensitive pigment (rhodopsin) is located. The rhabdom is formed by the dendrite of an eccentric cell and membrane invaginations (rhabdomeres) of the 10 to 15 retinular cells that cluster symmetrically around it.

Distribution of substance P-like immunoreactivity in the LE system. An efferent system of substance P-li fibers innervates the lateral eye. This system of around two to three dozen, very thin (<0.2 μm) fibers can be detected as it travels up the distal portions of the LON (Fig. 2*C*), enters the eye, crosses the lateral plexus as distinct bundles (Fig. 2, *D* and *E*), and branches out profusely upon reaching the ommatidial layer. Innervation is extended to more than one component of the ommatidia, including the retinular cells (Fig. 3, *A*, *B*, and *E*), the distal pigment cells (Fig. 3, *C*, *D*, and *E*) and eccentric cells (Fig. 3, *A* and *F*). Fibers with large varicosities could sometimes be observed in the area of the rhabdom. At the light microscopic level, of course, we cannot establish the precise regions of actual synaptic contracts.

Serial reconstruction showed that individual fibers innervate more than one ommatidium (Figs. 2*A* and 3*F*) and terminate on more than one type of target cell but did not reveal any obvious geometrical or quantitative pattern in their distribution. Branches of single fibers follow more than one path to reach their targets. Some approach the ommatidia at their base (occasionally innervating eccentric cells) and ascend either on the lateral surface of retinular cells (Fig. 3, *E*, *left*, and *A*) or within the pigment cell partitions between the retinular cells, displaying multiple synaptoid enlargements throughout their length. However, most commonly they ascend in the spaces between ommatidia (Fig. 2*A*). Some branches penetrate the ommatidia midway along their length (Fig. 3*B*), making contact with retinular cells, and continue up to innervate distal pigment cells (Fig. 3*E*, *center*), whereas others reach the distal end of the ommatidia, where they either divide to innervate the distal pigment cells (Fig. 3, *C* and *D*) or descend to terminate on several retinular cells (Fig. 3*A*).

Specificity controls showed that substance P immunostaining can be totally abolished (Fig. 2*B*) by absorption of the anti-substance P antibodies with the synthetic undecapeptide at concentrations of 1, 10, and 100 μM

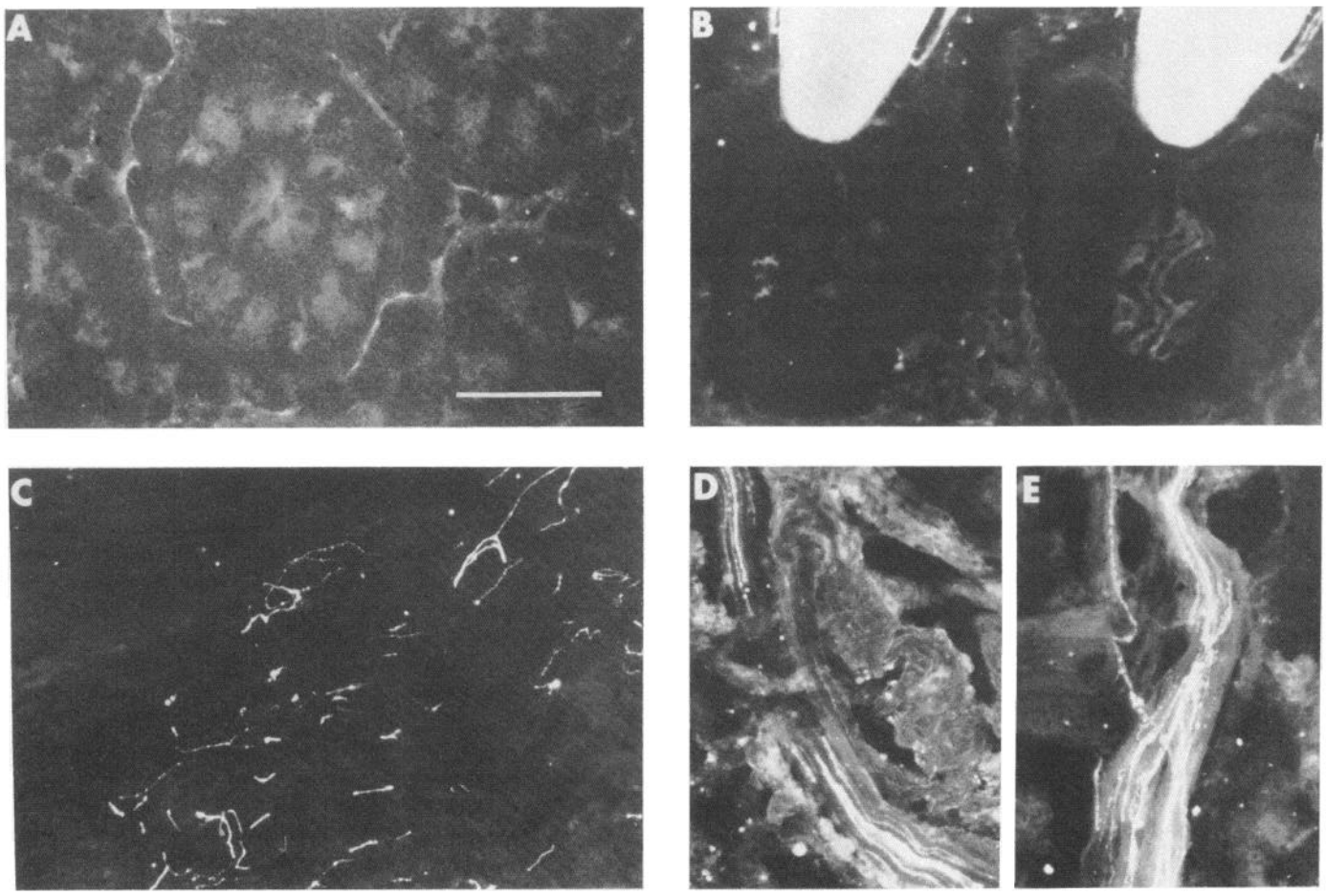

Figure 2. Substance P-like immunoreactive fibers in the LE. *A*, Cross-section of an ommatidium, with the rhabdom in the *center*, surrounded by 12 retinular cells. Portions of other ommatidia are visible in the *corners* of the picture. Substance P-li fibers can be seen in the spaces between ommatidia. *B*, Block control: longitudinal section through two adjacent ommatidia, showing lack of staining when the antibody was preabsorbed with 1 μM synthetic substance P. *C*, Substance P-li fibers in the distal portion of the optic nerve, close to the point of entrance to the LE. *D* and *E*, Substance P-li fiber bundles in the lateral plexus. The cornea is up in *B* to *E*. *Calibration bar:* 100 μm.

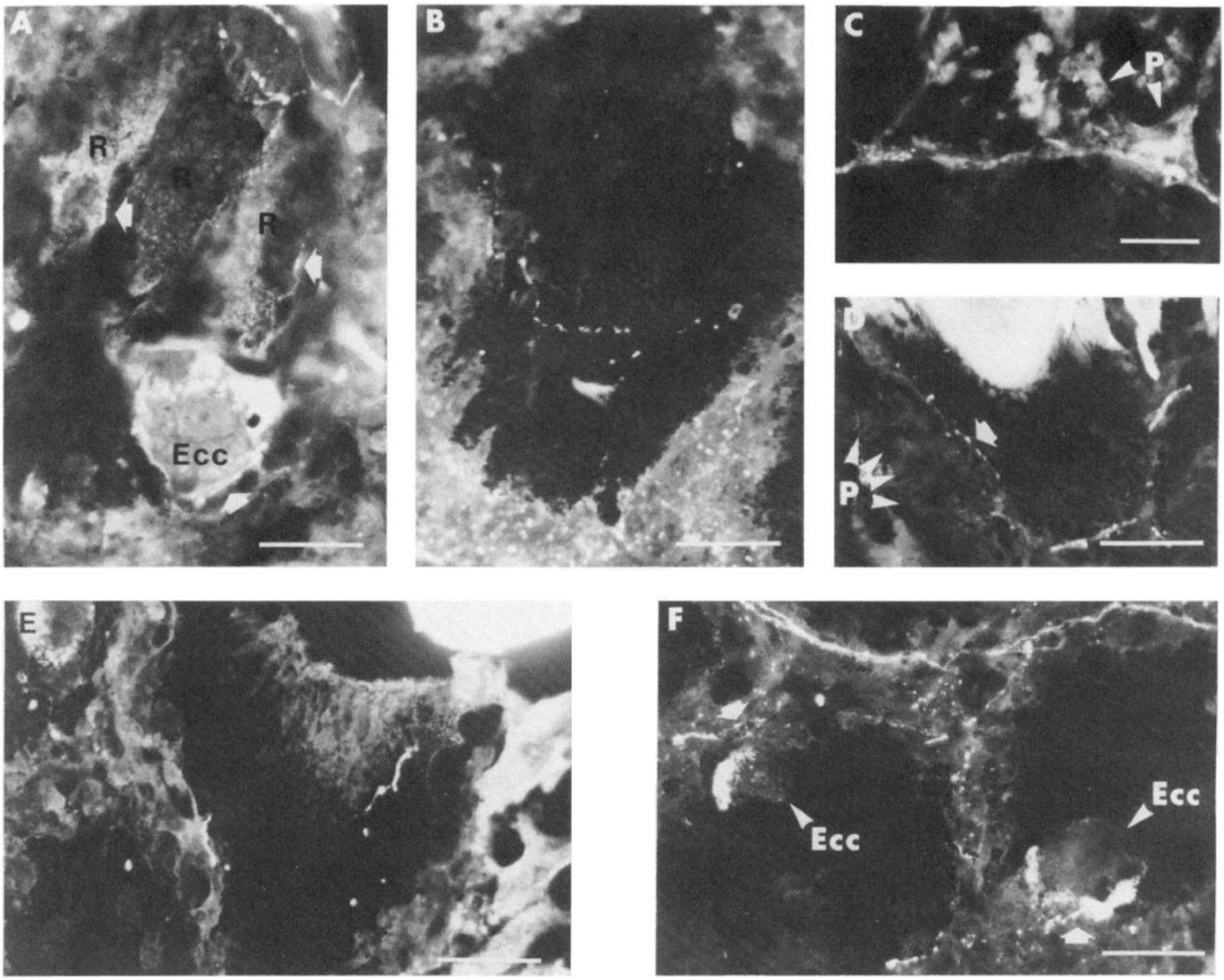

Figure 3. Substance P-li fibers innervate several components of the ommatidia. *A,* Three heavily pigmented retinular cells (*R*) can be seen surrounding the dendrite of an eccentric cell (*Ecc*), whose nucleus and nucleolus are discernible. Substance P-li fibers descend at the *top right*, branch out, and terminate on the retinular cells. Additional substance P-li fibers innervate the retinular cells on the *left* and *right* (*upper two broad arrows*). Apparent staining inside the cell bodies of eccentric cells in *A* and *F* reflects a bright orange autofluorescence not related to the immunocytochemically induced FITC green fluorescence. *B,* Longitudinal section through an ommatidium, showing a single substance P-li fiber innervating several retinular cells. *C,* Cross-section through two pigment cells (*P*), and *D,* longitudinal section through four pigment cells innervated by substance P-li fibers (*broad arrow* in *D*). At high magnification (*C*), the pigment inside the cells appears a bright brown-orange color. *E,* Substance P-li fibers innervating retinular (*left*) and pigment (*center*) cells. *F,* Substance P-li fibers (*top*) descend to innervate (*broad arrows*) the eccentric cells of two neighboring ommatidia shown in cross-section. *Calibration bar:* 25 μm in *C*; 50 μm in all others.

and partially blocked by 0.1 μM substance P. Omission of the primary antibody also resulted in the disappearance of the staining. Both the monoclonal (Cuello et al., 1979) and SP_2 antibodies have been characterized for their cross-reactivity with other known and related peptides and have been shown to be highly specific for substance P.

Ligation experiments. To establish the efferent character of the substance P-li-containing system, we ligated the LON at a site close to the point where it enters the LE. The contralateral LON of the same subject was used as control. After survival times of at least 3 days, accumulation of substance P-li in fibers on the proximal (brain) side of the ligation site can be observed (Fig. 4*A*), while very few, if any, stained fibers appear on the distal (LE) side. When both eyes were examined, those whose nerve had been ligated showed a gradual disappearance of stained fibers (Fig. 4*B*), whereas the contralateral eyes displayed a normal pattern of staining (Fig. 4*C*).

It was also clear that as a result of the ligation, not only was there an apparent accumulation of material in immunoreactive fibers, but we could see a larger number of stained fibers (at least three dozen). We had previously observed that the total number of fibers varied slightly from subject to subject (12 to 24 fibers). More significantly, some variability was also apparent along the

length of the nerve, with more fibers detectable in the proximity of the eye. It seems likely that as substance P-li is transported from the cell body to the terminals, the amounts contained in the middle segments of the very thin axons of some fibers are small enough to go undetected. Accumulation caused by placing a ligation would raise the amount of antigen to a detectable level, raising the number of stained fibers observable.

We attempted to locate the cell bodies of origin of the substance P-li efferent fibers by injecting eyes with HRP and the retrogradely transported fluorescent dye true blue. We failed to obtain staining of cell bodies or fibers with either method, in all tissues examined. These tissues included all the components of the CER, the LONs, HA, and 3rd and 4th dorsal nerve. No HRP-stained fibers were observed anywhere in the optic nerve. Examination of the injection sites showed the presence of the true blue and HRP in extracellular spaces but no uptake by neuronal structures, even after 3, 7, or 14 days. Thus, we resorted to tracing of the immunocytochemically stained fibers, complemented with ligation experiments that allowed us to determine their direction of travel.

The only previously described neural connection between the LE and the central nervous system is the LON, which originates in the dorsolateral surface of the brain or protocerebrum (see Fig. 1). This made the brain, where we found several substance P-li-staining cell types, the obvious candidate to contain the cell bodies of origin of the efferent system. Only a few (one to six), coarser, substance P-li fibers, however, could be observed leaving the brain through the LON (Fig. 5*B*) or in the one-half to one-third of the nerve proximal to the brain. (Curiously, those fibers seem to travel outside the nerve, in the wall of the artery that ensheaths it.) Suspecting reduced concentrations of antigen during transport through the long axons (up to 10 cm) to be responsible, we placed a ligation roughly halfway between the LE and the protocerebrum (Fig. 5*A*). Surprisingly, this resulted in accumulation of immunoreactivity in the distal (LE) side (Fig. 5*C*) and its disappearance from the proximal (brain) side (Fig. 5*D*). Ligations closer to the eye resulted in the reverse pattern of accumulation (Fig. 4*A*), suggesting that the substance P-li fibers enter the LON at some point in between.

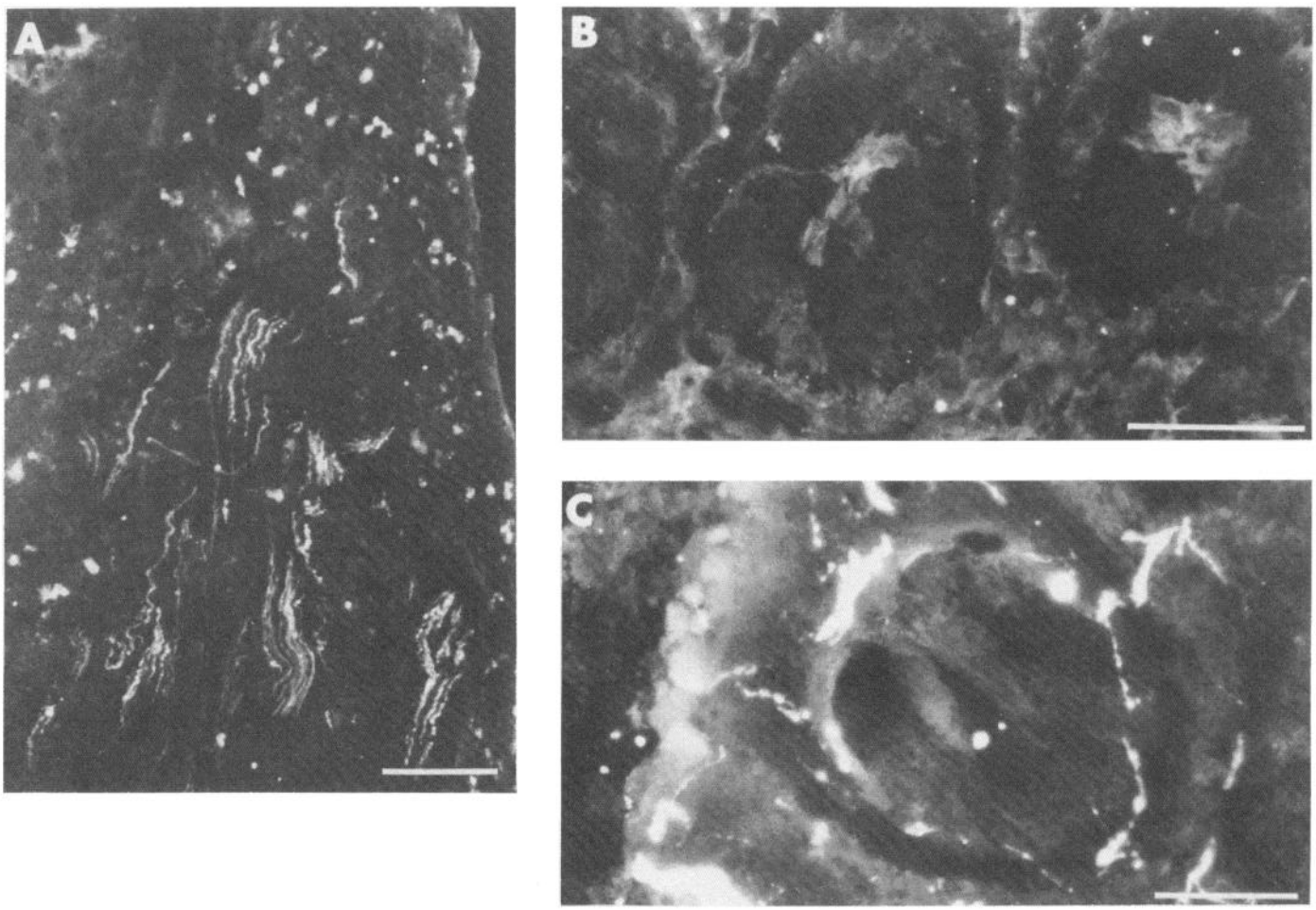

Figure 4. Efferent character of the substance P-li fibers. *A,* Accumulation of substance P-li fibers in the proximal side of an LON ligated close to its entrance into the eye. *B,* A cross-section through the ommatidia of LE whose optic nerve was ligated shows the disappearance of substance P-li fibers. *C,* Similar section through the contralateral eye of the same animal shows the normal pattern of substance P-like immunoreactive staining. *Calibration bar:* 100 μm in *A* and *B*; 50 μm in *C*.

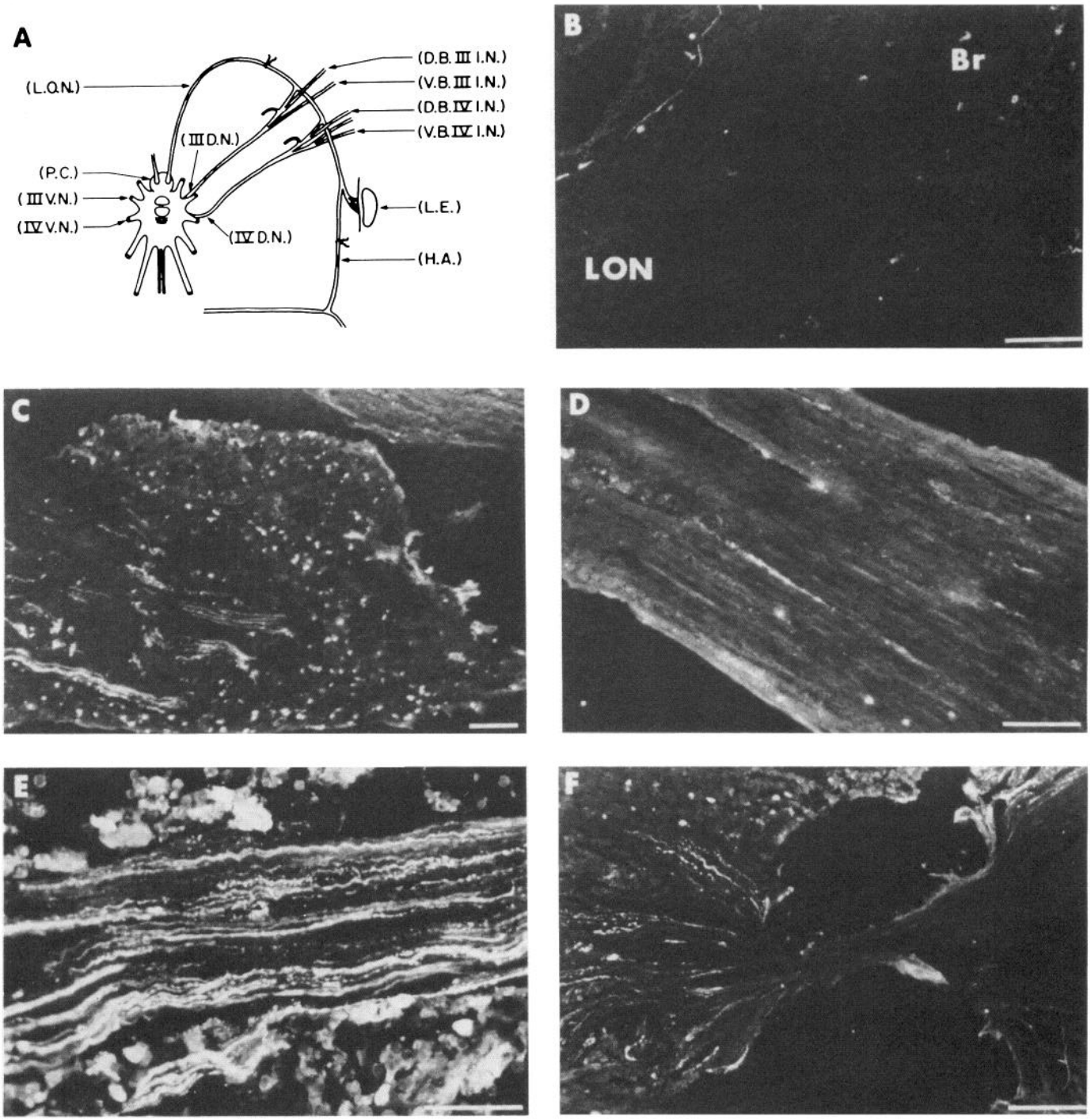

Figure 5. The substance P-li efferent fibers innervating the eye do not originate in the brain. *A,* Schematic diagram showing the protocerebrum (*P.C.*) connected via the lateral optic nerve (*L.O.N.*) to the lateral eye (*L.E.*). The LON is enclosed in an artery which anastomoses with the hepatic artery (*H.A.*). The latter, in turn, connects with an extension of the second lateral artery, which originates in the heart. The locations of two ligatures placed on the LON and HA are indicated. The diagram is further described in Figure 6*A*. *III V.N.* and *IV V.N.*, 3rd and 4th ventral nerves; *III D.N.* and *IV D.N.*, 3rd and 4th dorsal nerves; *D.B. III I.N.* and *D.B. IV I.N.*, dorsal branches of the 3rd and 4th integumentary nerves; *V.B. III I.N.* and *V.B. IV I.N.*, ventral branches of the 3rd and 4th integumentary nerves. *B,* Horizontal section through the dorsolateral edges of the brain (Br), at the point of exit of the LON. The substance P-li efferent system cannot be observed, although some fibers (i.e., *top left*) leave to innervate the wall of the artery which surrounds the LON. *C,* Section through an optic nerve, showing the accumulation of substance P-li fibers on the distal (LE) side of a ligation placed on the LON (see *A* for location). *D,* Similar section through the proximal (brain) side of the ligation, showing the absence of substance P-li fibers. *E,* Substance P-li fibers can be detected in the HA. This longitudinal section shows a segment of the HA between the LE and the ligation site in the artery marked in *A*. *F,* Accumulation of fibers in the LE side (*left*) of a ligation placed on the HA. Little staining appears in the heart side (*right*) of the ligation. *Calibration bar:* 50 μm in *E*; 100 μm in all others.

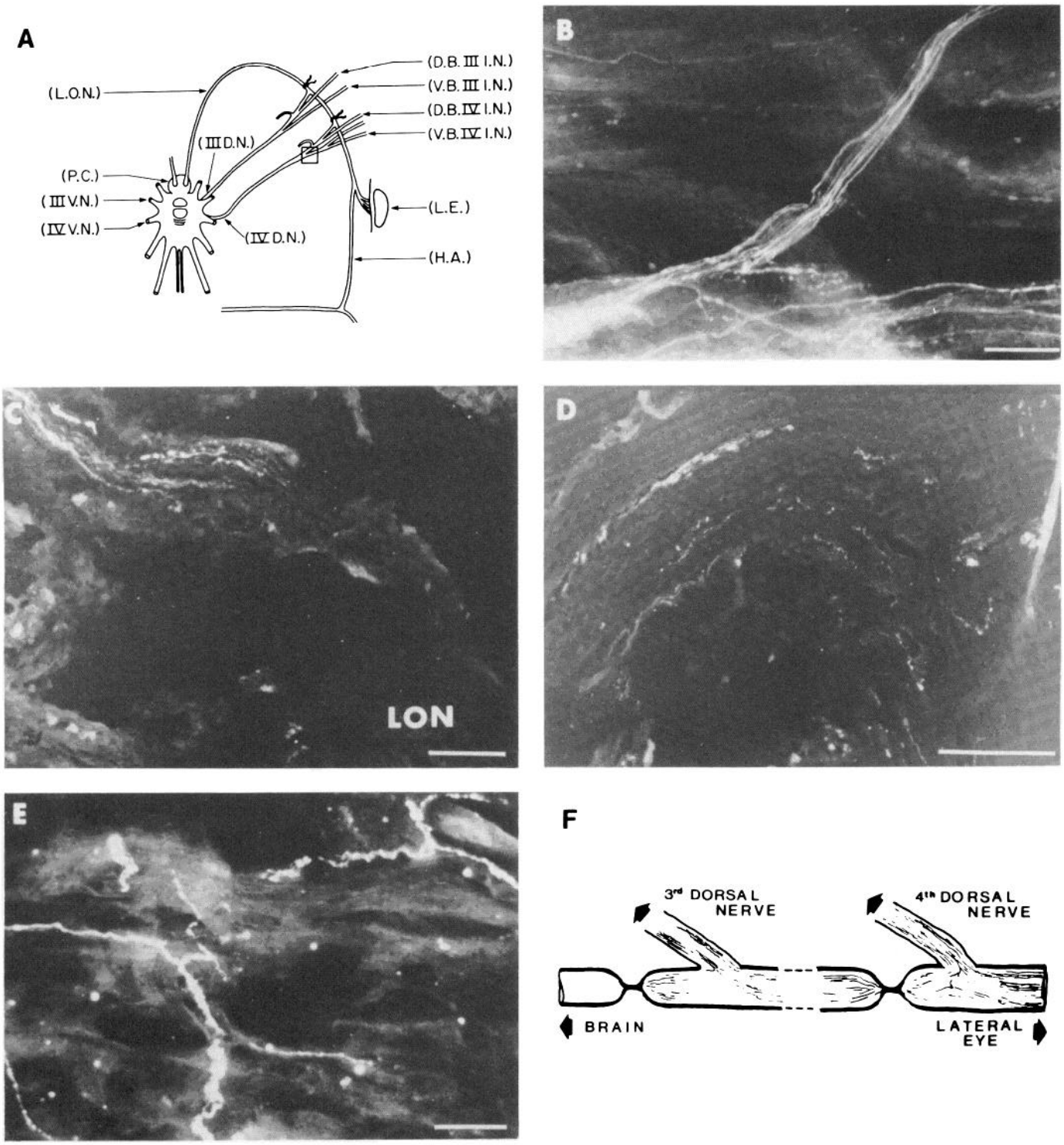

Figure 6. Origin of the substance P-li fibers. *A,* Schematic diagram showing the protocerebrum (*P.C.*), LON, and LE. The PC is the anterior portion of the CER. Eight ventral and dorsal nerves leave the CER sequentially. Branches of the 3rd and 4th dorsal nerves anastomose with the LON. Ligatures were placed on the LON close to the sites where they do. The *box* indicates the area shown in *B*. *B,* Substance P-li fibers in the 3rd dorsal nerve continue into the integumentary nerve and into its dorsal branch. Substance P-li fibers can be seen here leaving the dorsal branch of the 3rd integumentary nerve via a small branch which joins the LON. *C,* Substance P-li fibers described in *B*, as they approach the LON. *D,* Substance P-li fibers in a similar branch of the dorsal branch of the 4th integumentary nerve as it anastomoses with the LON (*right*). *E,* Branching of substance P-li fibers can be observed in the area where they enter the LON. *F,* Summary of the accumulation of substance P-li after ligations were placed on the LON as shown in *A*. Sections shown in *C* to *E* are from this experiment. These fibers, then, are efferents traveling mainly in the direction of the LE and HA. *Calibration bar:* 25 μm in *E*; 100 μm in *B* to *D*.

The largest structure that anastomoses with the LON in its distal half is the HA (Fig. 5*A*). Examination of the artery revealed the presence of substance P-li fiber bundles traveling inside the arterial wall of the periphery of the otherwise empty lumen (Fig. 5*E*). Ligation of the artery resulted in preferential accumulation in the LE side, with only a few fibers remaining in the heart side (Fig. 5*F*).

We then examined several small branches that seem to leave the LON as it courses from the brain to the LE. Small numbers of substance P-li fibers could be observed in several of them. Some travel toward the dorsal carapace, where they seem to terminate. However, two larger branches leave the optic nerve to travel in a ventromedial direction at a point slightly anterior to where the integumentary nerve extensions of the 3rd and 4th dorsal nerves pass the LON vertrally and dorsally, as they course from the CER to the carapace (Fig. 6*A*). (The 3rd and 4th dorsal nerves are unique in that they lack cardiac and intestinal branches, making the integumentary branch simply an extension of these nerves.) The two LON branches seem to be one and the same with thin offshoots of the dorsal branch of the 3rd and 4th integumentary nerves (Fig. 6*A*). Substance P-li fibers can be traced leaving the CER through the 3rd and 4th dorsal nerves, continuing into the integumentary nerves and into their dorsal branch. A small population of these fibers leaves the dorsal branch of the 3rd and 4th integumentary nerves (Fig. 6*B*), passes through the thin offshoots mentioned above, and enters the LON (Fig. 6, *C* and *D*). These fibers could be observed to ramify extensively in the area where they enter the LON (Fig. 6*E*), with ramifications going anteriorly and leaving the LON at different points through the small LON branches that seem to terminate in the carapace, while others travel posteriorly, toward the LE.

Ligations were placed on two LON sites, just anterior to where the substance P-li fibers coming from the 3rd and 4th dorsal nerves make their entrance (Fig. 6*A*). The results are summarized in Figure 6*F*. Staining virtually disappeared from the brain side of the ligation placed anterior to the point of entrance of the fibers from the 3rd dorsal nerve, while immunoreactivity accumulated on the LE side. Immunoreactive fibers accumulated on both sides of the second, more posterior site. Immunoreactive fibers accumulated on both sides of the second, more posterior site. More fibers accumulated on the brain side, reflecting the larger number of them coming into the LON from the 3rd dorsal nerve than from the 4th dorsal nerve.

The cell bodies of origin of these two populations of fibers appear to be contained in two of the six bilaterally symmetrical pairs of substance P-li-containing cell clusters located in the circumesophageal connective and subesophageal mass. The approximate location of these cell bodies and the trajectory of their projection fibers are shown schematically in Figure 7. These clusters are described in a separate report detailing the distribution of substance P-li cells and fibers in the central nervous system (J. R. Mancillas and A. I. Selverston, submitted for publication). The number of stained cell bodies in

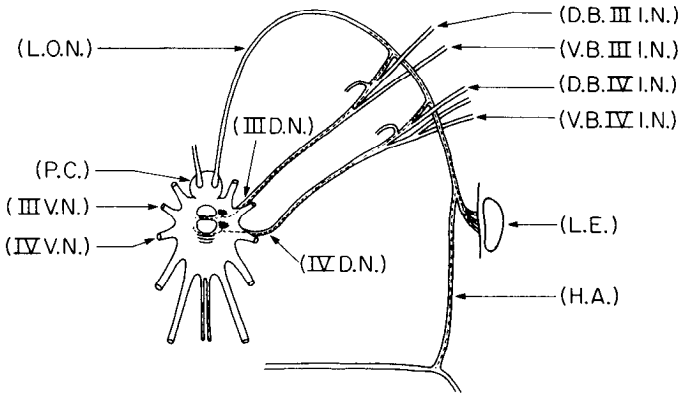

Figure 7. Location of the cell bodies of origin of the substance P-li efferent fibers. Schematic diagram showing the approximate location in the CER of the cell bodies that give rise to the efferent fibers innervating the LE. The trajectory followed by their projection fibers is indicated by *dashed lines*. Abbreviations are as in Figure 5*A*.

the cluster associated with the 3rd dorsal nerve, however, approximates the number of stained fibers from that nerve that enter the LON (around two dozen), while the same is true of the cluster associated with the 4th dorsal nerve (approximately a dozen cells and fibers). The combined number of the two cell and fiber systems is close to the total number of fibers entering the LE. It must be noted that these are approximate figures, based on counts of fibers in ligated nerves, while normal, stained nerves yield lower counts. Additionally, there is significant variability from subject to subject, due most likely to varying amounts of antigen in the axons than to actual numbers of fibers.

Biochemical and immunological characterization. The nature of the immunoreactive material was investigated by subjecting crude acidic extracts of LEs to substance P radioimmunoassay and gel filtration chromatography.

Radioimmunoassays revealed the presence of 61.44 pg of substance P-like immunoreactive material/μg of protein, with individual eyes containing up to 18 ng of immunoractivity. Figure 8 shows the elution profile of crude extracts subjected to gel filtration chromatography in a Bio-Rad P-4 column followed by radioimmunoassay. Substance P-like immunoreactivity from the LEs (Fig. 8, *shaded histogram*) displays an elution pattern that overlaps with and is extremely similar to that of the undecapeptide substance P (Fig. 8, *dashed line*), although not completely identical. (Iodinated Tyr^8 substance P, shown in Figure 8, and unlabeled synthetic substance P displayed the same elution pattern.) Notice the clear separation of the immunoreactivity from the protein (Fig. 8, *solid line curve*). As Table I shows, a single chromatographic run of a relatively small starting sample can yield a 26-fold purification (or almost 72-fold, if only the large peak, fractions 17 to 20, is considered).

The small disparity in chromatographic behavior between substance P-like material from the LE and synthetic substance P suggests that the former is a slightly larger molecule. The fact that both the monoclonal and the SP_2 serum antibody recognize the material during

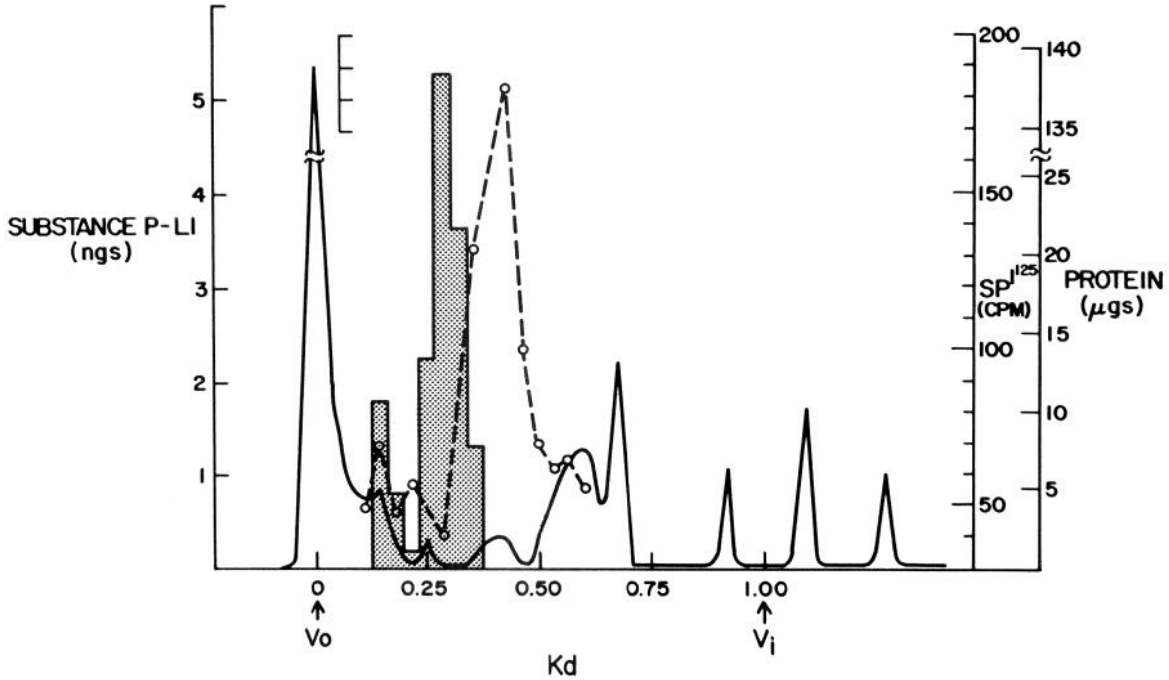

Figure 8. Elution profile of the substance P-li contained in the LEs. A crude acidic extract of the LE was subjected to gel filtration through a column (1.5 cm × 95 cm) of Bio-Rad P-4 and eluted with 10% formic acid. Fractions (5 ml) were collected at a rate of 4 ml/hr. Two different substance P antibodies were used for radioimmunoassay of 1-ml samples of each fraction. Another milliliter was subjected to the Bio-Rad dye reagent protein microassay. The solid curve (——) indicates the amount of protein in each fraction. The *shaded histogram* corresponds to the area containing substance P-li. The *open circles* and *dashed line* (O–O–O) show the elution pattern of a sample of [^{125}I]Tyr^{8} substance P applied to the column. Unlabeled synthetic substance P displayed the same elution pattern. Notice the separation of substance P-li from the protein and how the elution profile of substance P-li from the LE overlaps with and is extremely similar to that of substance P, but not completely identical. V_0, void volume, determined with blue dextran; V_i, included volume, determined with phenol red; K_d, distribution coefficient, used to normalize the elution volume (V_e) and calculated according to the formula:

$$K_d = \frac{V_e - V_0}{V_i} \text{ or } K_d = \frac{V_e - V_0}{V_t - V_0}$$

TABLE I

Partial isolation of substance P from the lateral eye of Limulus polyphemus

Protein content was determined with the Bio-Rad dye reagent protein assay. Starting sample = 1.36 eye equivalents; 1 eye equivalent = 125 mg wet weight.

Purification Step	Substance P-lir	Total Protein	Specific Activity	Recovery	Purification
	pg	*μg*	*pg/μg*	%	
Crude extract (1 eye equivalent)	18,125	295	61.44		
Crude extract (starting sample)	24,650	401.2	61.44		
Fractions 14 to 20 (Bio-Rad P-4)	15,250	9.375	1626.66	62	26.47
Fractions 17 to 20	12,425	2.812	4417.77	50	71.89

immunocytochemical staining, however, implies that they share a similar amino acid sequence.

To test this further, we used a third C-terminal antibody, $SP_{228\text{-}8}$, to perform a second radioimmunoassay of the Bio-Rad P-4 fractions and the crude extract. Table II compares the cross-reactivity of SP_2 and $SP_{228\text{-}8}$ to some selected tachykinins and substance P analogues. Similar data for the monoclonal antibody have been reported by Cuello et al. (1979). The three antibodies display a very different pattern of cross-reactivity. Yet, radioimmunoassay with $SP_{228\text{-}8}$ yielded the same results as radioimmunoassay with SP_2. Thus, we conclude that the LE contains a substance P-like molecule that is slightly larger but immunologically indistinguishable from the undecapeptide. Our data are consistent with it being either a higher molecular weight form of substance P or a new member of the substance P family of peptides, the tachykinins.

Discussion

Our results show that two cell clusters in the CER (J. R. Mancillas and A. I. Salverston, submitted for publication) give rise to a discrete efferent fiber system that innervates several components of the ommatidia of the LE, including the retinular, eccentric, and distal pigment cells.

The correlation between the number of stained fibers in this efferent system entering the LON and reaching the eye with the number of cell bodies that seem to give rise to them, as well as the observed branching in the area of the anastomoses, suggests that we are observing one discrete system with multiple targets. Indeed, the existence of six similar pairs of substance P-li-containing

TABLE II

Percentage cross-reactivity of selected tachykinins and substance P analogues to two substance P antisera

Percentage of cross-reactivity was determined by comparing the ED_{50} of the different peptides. ED_{50} of substance P = 100%.

Antiserum	Substance P	Physalemin	Eledoisin	Bombesin	Des-Arg[1] SP	Des-Met[11] SP	Tyr-Gly SP	*m*-iodo Tyr[6] SP
SP_2	100%	0.01%	0.4%	0	2%	2%	45%	100%
SP_{228}	100%	95%	7%	0.1%	100%	0.01%	90%	100%

cell clusters in the posterior CER and similar substance P-li fiber projections in most dorsal and ventral nerves (J. R. Mancillas and A. I. Selverston, submitted for publication) suggests that the substance P-li visual efferents may be part of a larger system that innervtes multiple organs in *Limulus*. This generalized system may regulate the daily activity rhythm of the animal as a whole (Rao and Habibulla, 1973; Fahrenbach, 1981; J. R. Mancillas and A. I. Selverston, submitted for publication), and perhaps also mediate modulation of sensory and motor activity during short-term arousal (Mancillas and Selverston, 1984) and other integrated organismic responses.

Efferent fiber systems in chelicerate eyes. While in some arthropods circadian regulation of sensory and motor systems is hormonally mediated (Kleinholz, 1966; Aréchiga and Huberman, 1980), in others it seems to be regulated by efferent innervation. Among chelicerates, the median eyes of the North African scorpion were found to display a circadian rhythm in photosensitivity (Fleissner, 1972, 1974). Neurosecretory efferent fibers that innervate the ommatidia (Fleissner and Schliwa, 1977) appear to mediate these changes in sensitivity (Fleissner and Fleissner, 1978) which are believed to be accomplished mainly by pigment migration in the retinular cells (Fleissner, 1974). Their cell bodies have been found in the lower lateral posterior margins of the supraesophageol ganglion by Fleissner and Heinrichs (1982), who postulate the "circadian clock" to reside in the scorpion's brain. Rao and Habibulla (1973) have found that neurosecretory clusters of cells in the posterior CER, with a distribution similar to those we found in *Limulus*, are associated with circadian rhythms of activity. Thus, the scorpion's circadian clock must either consist of several separate but tightly coupled oscillators or, if located in one place, have several strategically distributed, follower, effector neurosecretory cells.

Neurosecretory efferent fibers innervating the lateral eye of *Limulus* have been described by Fahrenbach (1969, 1973). Their main targets appear to be the pigment cells, eccentric cell dendrite, retinular cells, and cone cells (Fahrenbach, 1973, 1981). Subsequent physiological investigations established that circadian efferent activity is responsible for morphological cycles in the rhabdom (Chamberlain and Barlow, 1979; Barlow et al., 1980; Barlow and Chamberlain, 1980), in ommatidial acceptance angle (Levinson et al., 1979), and for decreases in photoreceptor noise and increases in visual sensitivity (Barlow et al., 1977a, 1980; Barlow and Chamberlain, 1980; Kaplan and Barlow, 1980; Barlow, 1983).

The evidence for circadian rhythms in visual physiology being mediated by efferent fibers (believed to be those described by Fahrenbach) comes mainly from experiments where: *(1)* cessation of their spontaneous occurrence was achieved by cutting the optic nerve; *(2)* their occurrence was induced by stimulation of the optic nerve; *(3)* their onset was correlated with spontaneous bursts of activity in the LON. It must be noted that the site of transection or recording, when either was performed in the physiological investigations cited, was close to the LE, a site which would not discriminate between fibers in the LON coming from the brain, from the circumesophageal connectives via the dorsal nerves, or entering the nerve through the HA. The complete, precise neural pathways mediating the efferent influences, including their cell bodies of origin, have not been previously determined. Recently, however, it was reported that efferent activity can be recorded from the LON in an excised brain preparation (Eisele et al., 1982).

Several features of the fibers stained in our study, including their diameter, their distribution in the eye, and the paths they follow to penetrate the ommatidia and reach their targets, match Fahrenbach's (1973, 1981) description of his neurosecretory fibers. Thus, it appears that the substance P-li fiber system we have described here and reported on previously (Mancillas et al., 1981b; Mancillas and Selverston, 1982) corresponds to the neurosecretory fibers described by Fahrenbach, which innervate the ommatidia. Ultrastructural immunocytochemistry will be necessary to reveal whether the similarities in innervation patterns hold up at the ultrastructural level. The larger number of efferent fibers observed in our studies, three dozen versus Fahrenbach's one dozen (Fahrenbach, 1981), can be explained by the much smaller size of the animals used in his observations: 3 cm (1981) and 5 to 12 cm (1973) in prosomal width, versus 15 to 22 cm in our studies. Additionally, small concentrations of material in some of the fibers may obscure the presence of a larger number of fibers. We could consistently at least double the number of visible fibers by ligation of the optic nerve and the subsequent accumulation of stained material.

Neurotransmitters in the LE of Limulus. Ours is not the first report of a putative neurotransmitter assigned to efferent fibers innervating the LE. Serotonin was proposed by Barlow et al. (1977b; Barlow and Chamberlain, 1980) as the transmitter mediating the circadian changes in photosensitivity that they had observed, but that hypothesis has apparently been abandoned (Barlow, 1983). Using immunocytochemical techniques and an anti-serotonin antibody, we have been unable to find serotonin immunoreactivity in the LE (J. R. Mancillas, unpublished observations).

More recently, Batelle et al. (1982) have localized octopamine synthesis *in vitro* in efferent fibers in the ventral eye and LE. Newly synthesized octopamine could

also be released by potassium depolarization. Kass and Barlow (1980) have also reported that octopamine injection in the LE increases the amplitude of the electroretinogram. Although it is tantalizing to speculate that a substance P-like peptide and octopamine coexist in neurosecretory efferents to the LE, the data at hand do not resolve between: *(1)* existence of one efferent fiber system containing two putative neurotransmitters or *(2)* two populations of efferent fibers, with only one of them corresponding to Fahrenbach's neurosecretory fibers. (Although the number of fibers observed by the latter would seem to preclude it, one could also imagine two subsets of Fahrenbach's neurosecretory efferents, one octopaminergic and one containing a substance P-like peptide.)

On the one hand, the presence of octopamine in the posterior CER, where the presumed cell bodies of origin of the substance P-li efferent system are located, has been suggested by the finding of large amounts of octopamine-induced adenylate cyclase activity (Atkinson et al., 1977). On the other hand, octopamine uptake, synthesis, and release have been demonstrated in fibers innervating the ventral eye (Batelle et al., 1982), whereas we could not observe substance P-li in that organ. However, it must be remembered that coexistence of putative neurotransmitters in one cell or neuronal population does not mean obligatory coexistence in every system which contains one of them. More significantly, however, a few octopamine-synthesizing fibers have been observed to leave the brain through the LON (B. A. Batelle, personal communication), whereas the only substance P-li fibers that we could see on that site were those that appeared to innervate the artery. Localization of the cell bodies of origin of the octopamine-synthesizing efferents in the LE with complete tracing of their projection pathways, or double staining experiments, will be necessary to resolve conclusively the question of possible coexistence.

Substance P-like immunoreactive fibers and circadian rhythms of photosensitivity. We have explored the possibility of involvement of substance P or a related peptide in one of the physiological changes undergone by the LE, which are believed to be mediated by efferent activity (Barlow and Chamberlain, 1980; Barlow, 1983). Some evidence suggests that substance P may indeed be at least partially involved in circadian changes in photosensitivity and morphology (Mancillas and Selverston, 1984). It has been postulated (Barlow and Chamberlain, 1980) that the "circadian clock" regulating time-locked morphological cycles of the rhabdom and circadian changes in photosensitivity is located in the brain and that all of the efferent fibers leave the brain through the LON. As we mentioned before, the nerve transection and efferent stimulation experiments on which that proposal was based were performed at a site in the LON that would not discriminate between fibers coming into the nerve from the brain or entering at other sites, as the substance P-li fibers do. However, it must be pointed out that ours is the first report on the connections between the LON and the dorsal nerves, as well as on the existence of nerve fibers in the HA. Thus, assuming a protocerebral origin of *all* efferent fibers was, at the time, the only logical conclusion of those experiments. Although some other efferent fibers may leave the brain through the LON, our data show that the substance P-immunoreactive ones that innervate the LE follow a different path and appear to originate in a nonprotocerebral site.

The protocerebrum does contain substance P-li cells (Chamberlain and Engbretson, 1982), but if they were the source of the LE substance P-li efferents, they would have to travel posteriorly to the circumesophageal connectives and leave through the dorsal nerves. Since we could not trace every single fiber completely and unequivocally, we cannot rule out this possibility completely. The more likely possibilities, however, are: *(1)* the circadian clock is located in the neurosecretory, substance P-li-containing clusters of the circumesophageal connectives; *(2)* there are several clocks, functioning independent of each other; *(3)* the circadian clock consists of several tightly coupled oscillators distributed in more than one site; *(4)* the circadian clock resides in the brain, but there are several strategically located, follower, effector neurosecretory cells. Given the evidence from studies in scorpions (Rao and Habibulla, 1973) and crickets (Tyshchenko, 1973), we favor the last hypothesis and believe the substance P-li efferent system to be one such effector, follower system. This will be discussed in another article (J. R. Mancillas and A. I. Selverston, submitted for publication).

It is important to keep in mind that there is no reason to think that circadian changes in sensitivity or activity are exclusive to the visual system, which in *Limulus* is secondary to chemo- and mechanoreception (Wyse, 1971). Furthermore, a pacemaker or circadian clock may not innervate all of its varied and multiple ultimate targets, but it may itself only innervate and regulate a small number of effector neural and endocrine organs, which, in turn, innervate multiple targets or release a variety of hormones on a particular schedule.

In a comprehensive description of the distribution of substance P-li in the protocerebrum of *Limulus*, Chamberlain and Engbretson (1982) have confirmed our report (Mancillas et al., 1981; Mancillas and Selverston, 1982) of the presence of substance P-li fibers in the LE. However, they concluded that they were part of "a generalized innervation of the corneal epidermis" and not related to the physiologically or anatomically defined efferent systems mentioned above. Their conclusion was based on the following four arguments.

First, they report not observing penetration of the ommatidia by the substance P-li fibers, but that, instead, they appeared to "run between the ommatidia up to the level of the corneal epidermis." Although their one figure of the LE shows many branches leaving the fibers and going to the ommatidia (Fig. 2*B*, Chamberlain and Engbretson, 1982), the brown reaction product of the peroxidase-antiperoxidase immunocytochemical technique that they used gets obscured by the dark pigment contained by the retinular and pigment cells, making it impossible to trace the stained fibers within the ommatidia. As we have shown in our results, we performed careful and thorough serial reconstructions using FITC-conjugated secondary antibodies that produce a green fluorescent staining. This allowed clear visualization of

the fibers and unequivocal determination of their actual distribution within the ommatidia.

Second, they point out that efferents to the lateral and ventral photoreceptors appear structurally identical and both synthesize octopamine, while no substance P-li fibers are present in the ventral eye. Despite their structural similarities, no evidence has been reported showing common cell bodies of origin for any efferents innervating the ventral eye and LE. On the other hand, while the LE photoreceptors show clear circadian rhythms of photosensitivity, all efforts to show similar changes in the ventral eye using electrophysiological (Kaplan and Barlow, 1980) or behavioral (Wasserman, 1973) techniques have failed. Thus, the fact that both the circadian oscillations in photoreceptor responsiveness and substance P-li fibers are present in the LE, while both are absent in the ventral eye, is consistent with an association between such fibers and modulation of photosensitivity and is not an argument against it.

Third, they cite the experiments by Barlow et al. (1977a) as evidence that "the lateral eye receives its efferents via the lateral optic nerve," while substance P-li fibers are present only in its distal end. As we have discussed above, these nerve transection and stimulation experiments could not discriminate between fibers originating in the brain or entering the LON at other points. Cyclic efferent activity can be recorded from the LON in excised brains (Eisele et al., 1982), but it was synchronized with activity in the ventral eye nerve, where, as mentioned above, no circadian rhythms of *photosensitivity* have been found.

Finally, they cite Fahrenbach's (1979) finding of "fibers structurally similar to visual efferents in the corpora pedunculata," while substance P "immunoreactive fibers in the corpora pedunculata do not correspond to this fiber type." However, no evidence exists that the type C "neurosecretory" afferents of the corpora pedunculata alluded to share a common origin with the LE efferents. In fact, unlike these fibers, the C afferents do not display synaptoid endings but form normal synapses, with both pre- and postsynaptic specializations (Fahrenbach, 1979). Fahrenbach (1979) notes that "it is not inconceivable to suppose" a common cell group of origin for both fiber systems, but he warns that this is based on circumstantial evidence and that "there is no a priori reason for excluding the more caudally located neurosecretory cells, of which more than 400 are found in the subesophageal mass" (Fahrenbach, 1973), as the origin of the LE efferents.

Although we do not believe it can be *conclusively* said that the substance P-li efferent system is a component in the expression of circadian rhythms by the LE (let alone the only mediator), we do think that its anatomical distribution and the pharmacological evidence suggest it (Mancillas and Selverston, 1984) and, at least, clearly indicate an involvement in the modulation of photosensitivity, whether it is exerted in a phasic or circadian fashion.

Distribution of the substance P-li projection and chemical nature of the material. As mentioned before, the connections between the dorsal nerves and the LON, and the presence of nerve fibers in the HA, were previously unknown. Although the pathway followed by substance P-li fibers from the CER to the LE may seem unusual, it does constitute a shorter route to the eye than through the brain and LON. Developmental studies may show how this unusual structure is arrived at. As discussed in another report (J. R. Mancillas and A. I. Selverston, submitted for publication), the position of the cell bodies in the posterior CER, close to the nerves that supply important motor structures, as well as chemosensory and mechanoreceptors that are more abundant than the LE photoreceptors (over 4 million versus 10,000) (Hayes, 1966; Wyse, 1971), is, in fact, a logical one and consistent with the "visual" efferents being part of a generalized system controlling circadian rhythms of activity.

The presence of nerve fibers in the HA is not unusual in the sense that most nerves in *Limulus* are ensheathed by arteries (Dumont et al., 1965). The presence of fibers in this artery had apparently been overlooked because of their small number and size. Their possible role in innervation and modulation of the heart will be the subject of another report, along with the presence of substance P-li fibers in the wall of most arteries (Fig. 5*B*), where they may be involved in regulation of blood flow (J. R. Mancillas, manuscript in preparation; Mancillas, 1983).

Our immunocytochemical, radioimmunoassay and chromatographic data indicate that the substance P-like immunoreactive material is extremely similar, but not identical, to the undecapeptide substance P. Our results are consistent with the existence of either a higher molecular weight form of substance P or a new member of the tachykinin family of peptides. If either of these possibilities is confirmed, it would not be unprecedented if this peptide would also be found in many other organisms, including mammals. Substance P, in fact, has been found to have a widespread phylogenetic distribution (Skrabanek and Powell, 1980).

The substance P-li efferent projection as a model system. Ours is one of a now long list of reports of the presence in invertebrates, including the simple and primitive *Hydra* (Grimmelikhuijzen et al., 1982), of neuropeptides first found in mammalian nervous systems (see Haynes, 1980; Gardner and Walker, 1982; O'Shea, 1982 for reviews). The *Limulus* LE efferent system preparation, however, offers some unique features as a model system. *(1)* The preparation is extraordinarily sturdy. Intracellular recordings and presynaptic stimulation can be performed *in situ* (Kaplan and Barlow, 1980), in the excised eye, or in the slice preparation (Adolph and Tuan, 1972), which allows precise control of the surrounding environment. *(2)* The postsynaptic cells are extremely large (eccentric cells: 50 μm in diameter, retinular cells; up to 200 μm in length; Fahrenbach, 1975). Since they are a large, virtually homogeneous population, several experiments can be performed in more than one cell of the same subject, and the data from several subjects can be pooled. *(3)* The physiology and structure of the postsynaptic targets have been thoroughly studied over the years. Their function and output are very well characterized. *(4)* Their output can be measured precisely and translated to its physiological implications. Their activity can be regulated by manipulating a clearly quantitatively definable stimulus—light. *(5)* There is a possibility, remote as it may be, that two putative neurotransmitters,

octopamine and a substance P-like peptide, coexist in the efferent fibers. At the very least, however, those two putative transmitters seem to interact with one another to modulate the activity of their target (Mancillas and Selverston, 1984), even it they are anatomically segregated. *(6)* The function of the presynaptic input, the efferent fibers, is not completely obscure. The accompanying article (Mancillas and Selverston, 1984), in fact, attempts to shed some light into this matter. *(7)* Results at the molecular level can be easily translated into possible consequences at the cellular, systems, and behavioral levels.

The efferent fiber system of the LE, then, offers an opportunity to elucidate at the cellular and molecular level important questions about peptidergic mechanisms of action, central "gating" of sensory inputs, interactions between neurotransmitters, and biological rhythms.

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