Subcellular Localization of Neutral Red Staining in *Limulus* Ventral Photoreceptors

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SUMMARY

Limulus ventral photoreceptors are vitally stained by neutral red. In other systems such staining has been correlated with the presence of monoamines or neuropeptides. The stained cellular components in ventral photoreceptors are clusters of small ovoids which have been identified as residual bodies. These structures are unlikely candidates for monoamine or neuropeptide synthesis or storage sites, but may be part of the cyclic synthesis and degradation of photosensitive membrane. While vital staining with neutral red is a particularly useful method for identifying certain classes of neurons *in vivo*, in the case of ventral photoreceptors, the association of the vital staining property with the presence of a particular class of neurotransmitter candidates has proven difficult. Neutral red is useful, however, for visualizing the segmentation of ventral photoreceptors *in vivo*.

INTRODUCTION

When invertebrate nervous system are incubated in dilute solutions of the dye neutral red, some neurons are selectively and specifically stained. This property is particularly useful for the identification of these specific neurons in dissections of living material. Such vital staining has been correlated with the presence of monoamines in some preparations. Originally some vitally staining neurons in the ventral nerve cord of leech were shown to contain serotonin (Stuart, Hudspeth, and Hall, 1974; Lent et al., 1979); later the dorsal unpaired median neuron (DUMETi) in locust was shown to vitally stain with neutral red and contain octopamine (Evans and O'Shea, 1978). Battelle, Kravitz, and Stieve (1979) showed that *Limulus* ventral photoreceptors are vitally stained with neutral red and that the somata-rich fraction of dissected nerve roots selectively synthesizes octopamine. Subsequently we localized the octopamine synthesis to efferent fibers intimately associated with the photoreceptor cell body (Battelle and Chamberlain,

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1980; Battelle, Evans, and Chamberlain, 1982; Evans, Chamberlain, and Battelle, 1983), but not to the photoreceptor cell itself.

In an effort to determine why ventral photoreceptors are stained by neutral red, we have localized the staining to a particular component of the cytoplasm. Staining is restricted to clusters of polymorphic residual bodies that appear to be part of an intracellular degradation pathway, possibly one involving the cycling of photosensitive membrane.

METHODS

Three types of experiments were conducted. Living tissue was vitally stained and examined in whole mounts, tissue was fixed and embedded for light and electron microscopy, and tissue was incubated in tritiated tyramine and processed for autoradiography.

To examine vitally-stained whole mounts, ventral optic nerves were dissected free from adult males and females. The nerve trunk, with photoreceptors, was maintained in artificial seawater while the vascular sheath enclosing the nerve was dissected away. Then it was immersed in either artificial seawater saturated with neutral red at pH 7.2 or in the sodium chloride equivalent of seawater (0.53M NaCl) at pH 7.2 with neutral red concentrations between .01 and 0.1 mg/ml. Some of these preparations were observed and photographed with a Nikon SMZ-10 stereomicroscope; others were mounted on microscope slides in staining solution, covered, observed, and photographed with a Nikon Biophot microscope.

For light and electron microscopy, animals were fixed by intracardial injection of the double aldehyde fixative used by Fahrenbach (1969) for *Limulus* (0.8% glutaraldehyde, 5% paraformaldehyde, 3% NaCl, 4.5% sucrose in 0.1M Sorensen's phosphate buffer at pH 7.2). Dissected ventral optic nerves were immersed in fixative overnight at 4°C, washed and postfixed in 1% OsO_4. Then they were dehydrated through alcohols and propylene oxide and embedded in Epon-Araldite. For light microscopy, 1 μm serial sections were cut on a Dupont Sorvall MT2B ultramicrotome using glass knives. Sections were mounted on glass slides and were stained with 1% toluidine blue whose pH was adjusted with saturated Li_2CO_3 in water. For electron microscopy, silver and gold sections were cut and mounted on copper grids, stained with uranyl acetate and lead citrate, and observed and photographed on a Siemens Elmiskop 101 electron microscope.

For autoradiographic localization of synthesized octopamine, tissues were incubated overnight in saline (Battelle, 1980) containing 6×10^{-8} M ^{3}H-tyramine (NEN, specific activity 7.7 to 9.14 Ci/mM), rinsed in cold saline, and fixed with 2.5% glutaraldehyde in cacodylate buffer at pH 7.4 (Evans, Talamo, and Kravitz, 1975). Postfixation was done in 1% OsO_4. After dehydration in alcohols, tissues were embedded in Araldite 502. Serial 1 or 2 μm sections were cut and mounted on glass slides. These were coated with Kodak NT2B emulsion, exposed for 1–2 weeks, developed in Dektol, counterstained with toluidine blue, and observed and photographed with the light microscope. Biochemical characterization of the resulting radioactive products has been reported in detail elsewhere (Battelle, 1980; Evans et al., 1983). Of the radioactivity closely associated with photoreceptor cell bodies approximately 80% could be attributed to newly synthesized octopamine (58%) or octopamine metabolite (20%) which could be acid hydrolized back to octopamine.

RESULTS

Observations of the whole mount

When desheathed ventral optic nerves are placed in even dilute solutions of neutral red (0.01 mg/ml), within minutes the ventral photoreceptor somata become stained. The staining is visible while the nerve trunk is still in the neutral red solution, and is darker than the coloring of the staining solution itself. The photoreceptor axons which make up the majority of the volume of the nerve trunk do not stain. Figure 1 shows a micrograph and drawing of a stained nerve trunk. This preparation was photographed through the staining medium and demonstrates the apparent concentration, in terms of

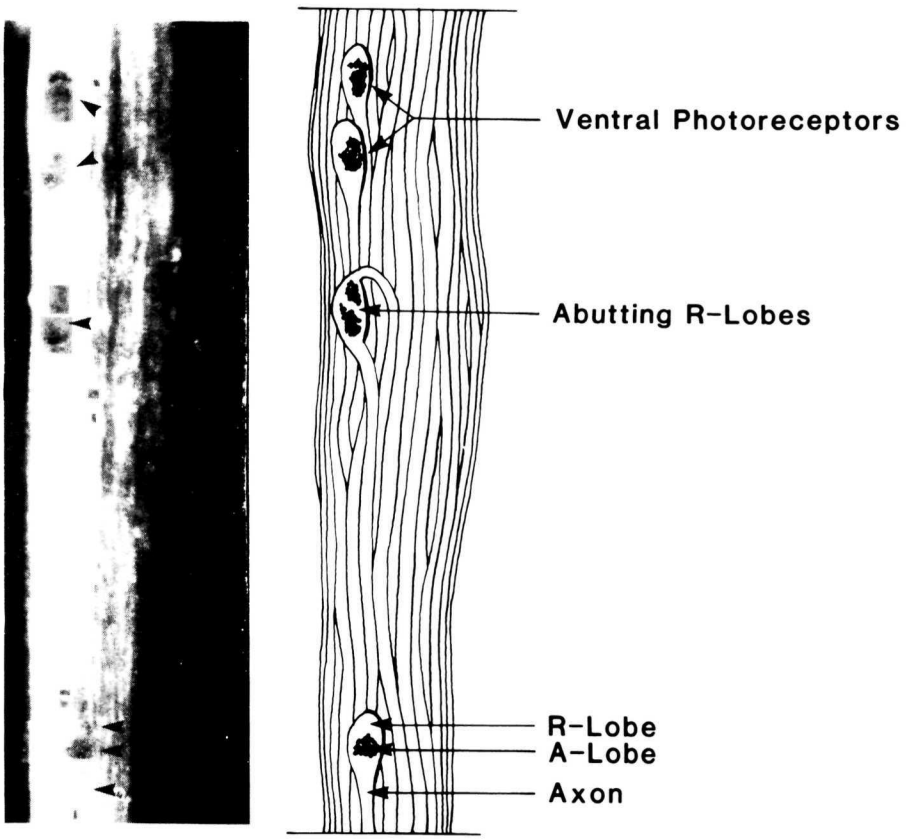

Fig. 1. *Limulus* ventral optic nerve bearing photoreceptor somata vitally stained with neutral red. The nerve (left) was photographed after 10 min immersion in 0.01 mg/ml neutral red in 0.52M NaCl at pH 7.2. The staining is blotchy with some parts of the soma devoid of stain. The photograph was taken through the staining medium. No post-staining wash was necessary. The sketch on the right is somewhat schematic. The ventral optic nerve connects the endorgan along the midline on the ventral surface of the animal with the ipsilateral optic medulla. Photoreceptor cells are sparsely scattered along the nerve trunk and tend to be concentrated in the endorgan and near the brain. The figure shows a portion of the nerve trunk from the middle of its length. See Clark, Millecchia, and Mauro, 1969; Stern et al., 1982; and Calman and Chamberlain, 1982 for more information on the anatomy.

color contrast, of the stain by the photoreceptor cell bodies. The stained somata appear blotchy and while all cells in a preparation are stained, large portions of individual cells may be unstained.

When stained cells are mounted under a coverslip and observed with a transmission light microscope, clusters of stained bodies are found around the cell nucleus (Fig. 2). In most cells two regions can be distinguished, corresponding to the rhabdomeral lobe and the arhabdomeral lobe (Stern et al., 1982; Calman and Chamberlain, 1982). The neutral red staining is restricted to the arhabdomeral lobe. Under higher magnification, clusters of small round forms about 1 μm in diameter can be resolved [Fig. 3(A)]. When a photoreceptor somata is squashed, the stained bodies flow out through the ruptured membrane with the rest of the cytoplasm, maintaining their size and shape, but reaggregating into larger clusters.

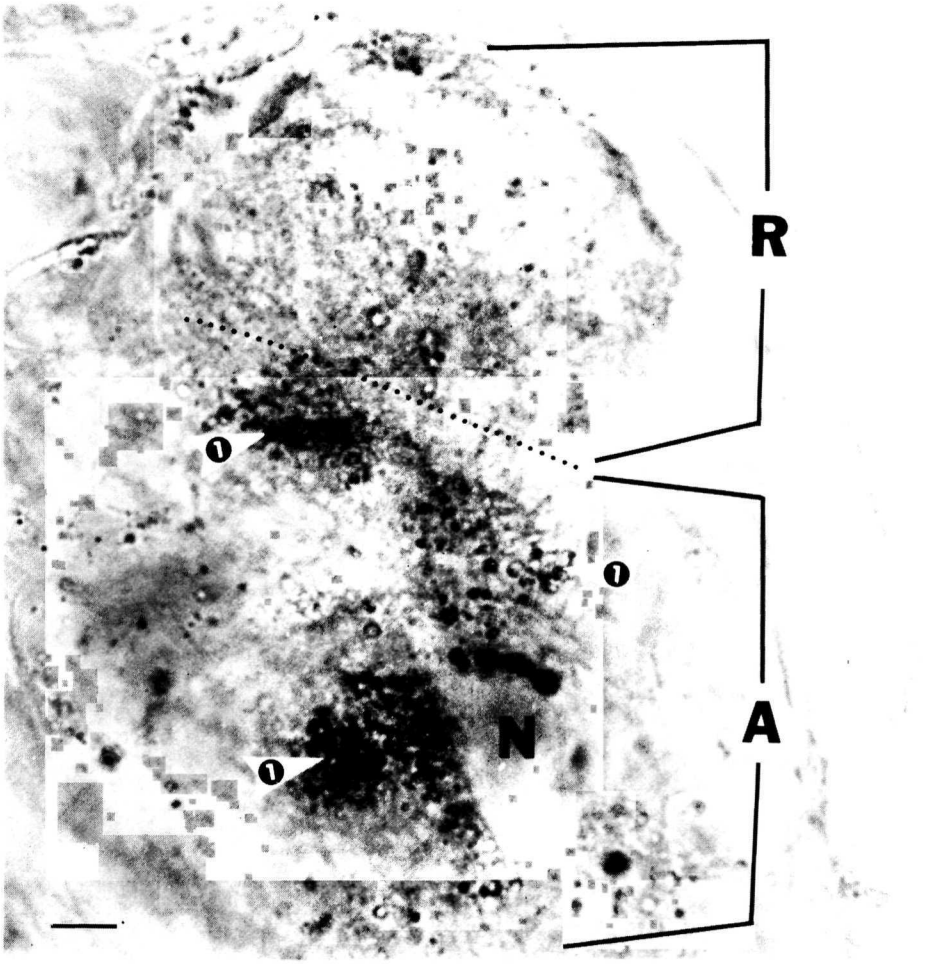

Fig. 2. Neutral red stained whole mount. Sometimes the segmentation of ventral photoreceptors is revealed by neutral red straining. The residual bodies which vitally stain are restricted to the arhabdomeral lobe (A). The rhabdomeral lobe (R) is devoid of stained structures. Residual bodies (#1) occur in clusters some of which surround the nucleus (N). The dotted line represents the approximate division between the two lobes. The distribution of organelles in the cytoplasm changes gradually in this region. The bar represents 7.7 μm.

Observations of sectioned somata with light microscopy

In sections stained with toluidine blue, numerous clusters of darkly stained bodies are present [Fig. 3(B)]. These clusters were also observed in the lateral rudimentary photoreceptors by Fahrenbach (1970) who called them residual bodies. The residual bodies observed in sections have exactly the appearance and distribution of vitally-stained bodies in whole mounts. In ventral photoreceptors residual bodies are found only in those parts of the cell that do not contain rhabdom (arhabdomeral lobe); the rhabdomeral lobe is free of these and other inclusions such as lipid droplets. This explains why photoreceptor somata in vitally-stained whole mounts have large unstained regions.

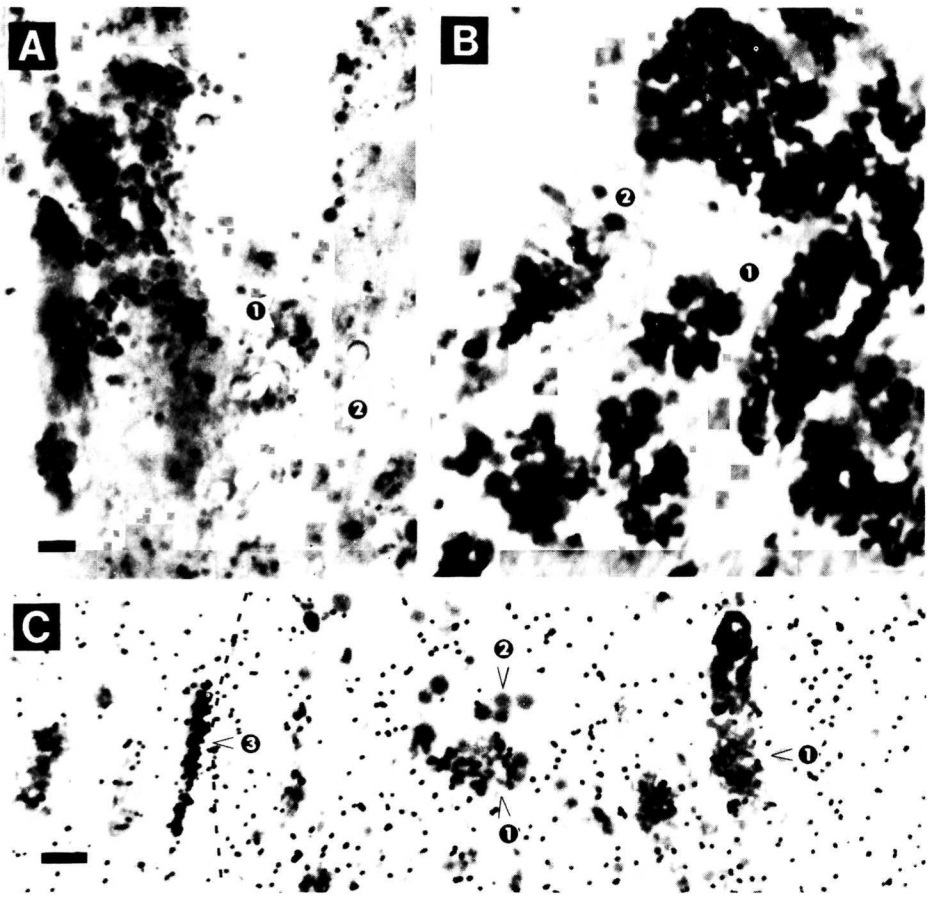

Fig. 3. Residual bodies in ventral photoreceptors which vitally stain with neutral red. *A* is a light micrograph of a vitally-stained whole mount. Residual bodies (#1) appear as clusters of round particles about 1 μm in diameter. Lipid droplets (#2) are visible as circular, unstained profiles. The staining conditions were the same as for Figure 1. *B* shows a light micrograph of tissue fixed, embedded in plastic, sectioned at 1 μm, and stained with toluidine blue. The dark blue residual bodies (#1) correspond to those stained with neutral red in *A*. Lipid droplets appear as grey circles (#2). *C* shows an autoradiograph of tissue incubated in tritiated tyramine. Eighty percent of the silver grains indicate newly synthesized octopamine or octopamine metabolites. There is no selective activity associated with the residual bodies (#1) nor lipid droplets (#2). Label appears over the efferent fibers (#3) running within the glial sheath covering the periphery of the cell body (see Evans et al., 1983). The dashed line indicates the boundary between the photoreceptor cytoplasm on the right and the glial sheath on the left. The bar in *A* represents 5 μm in *A* and *B*. The bar in *C* represents 5 μm.

Autoradiographic Localization of Octopamine Synthesis

Battelle, Kravitz, and Stieve (1979) first reported a selective association of octopamine synthetic activity with tissues rich in ventral photoreceptor cell bodies. Because vital staining with neutral red has been correlated with the presence of octopamine in some neurons (Evans and O'Shea, 1978; Wallace et al., 1974), we examined specifically the possibility that the stained residual bodies were in some way involved with octopamine synthesis or the storage of synthesized octopamine, precursor, or metabolite. Figure 3(C) shows an autoradiograph of a section of a ventral photoreceptor which was incubated in tritiated tyramine. No silver grains were selectively associated with clusters

of residual bodies. No concentrations of silver grains above background were observed over any structures in the cytoplasm of ventral photoreceptors. Within the cell body, only the efferent processes which invaginate the soma along glial fingers were labelled (Battelle and Chamberlain, 1980: Battelle, Evans, and Chamberlain, 1982; Evans, Chamberlain, and Battelle, 1983). These are only found in the rhabdomeral lobe associated with rhabdom, well separated from the portions of the cytoplasm which contain the stained residual bodies. We conclude that these cells do not synthesize octopamine, and specifically that the residual bodies which stain with neutral red are not involved with the synthesis or storage of octopamine.

Ultrastructure of vitally-staining residual bodies

Figure 4 shows the ultrastructure of residual bodies in *Limulus* ventral photoreceptors. In a longitudinal section of a single photoreceptor [Fig. 4(A)], the residual bodies appear only in the arhabdomeral lobe from which the axon originates. The rhabdomeral lobe which contains the photosensitive rhabdom is devoid of them. The residual bodies themselves exhibit a variety of shapes and textures. Although generally round, some possess indentations and corners. All appear to be membrane bound. Features of the internal structure include zones with crystalline arrays of substructure, lamellated areas, and voids. These residual bodies closely resemble lysosomal structures associated with photosensitive membrane turnover in the retinular cells of the *Limulus* lateral eye (Chamberlain and Barlow, 1979), and in other invertebrate photoreceptors (e.g., White, Gifford, and Michaud, 1980), although this process has not yet been studied in detail in ventral photoreceptors.

DISCUSSION

Why are some neurons vitally stained with neutral red? Such neurons have been associated with serotonin or a catecholamine in the leech (Stuart, Hudspeth, and Hall, 1974; Lent et al., 1979; Lent, 1981; Glover and Kramer, 1982), proctolin in orthopterous insects (O'Shea and Adams, 1981—although the authors state that the vital staining with neutral red probably indicates the presence of a monoamine), and octopamine in cockroach (Dymond and Evans, 1979), grasshopper (Goodman et al., 1979), locust (Evans and O'Shea, 1978), and lobster (Wallace et al., 1974) to mention just a few examples. On the other hand, the data of Webb and Orchard (1980) suggest that in the leech there are octopamine-containing neurons which do not stain specifically with neutral red. Furthermore there is a large literature which documents that neutral red stains lysosomal organelles in other tissues (e.g., Dingle and Barrett, 1969; Bulychev, Trouet, and Tulkens, 1978; Nemes et al., 1979). Since the mechanisms by which neurons are vitally stained by neutral red are still unknown, the full significance of their vital staining is uncertain.

We cannot completely eliminate the possibility that such vital staining indicates the presence of a monoamine or neuropeptide in *Limulus* ventral photoreceptors. Battelle (1980) did not find synthesis of dopamine, serotonin, acetylcholine, or γ-aminobutyric acid. Likewise surveys with antibodies (Chamberlain and Engbretson, 1982) did not uncover any immunoreactivity indicating the presence of substance P, leucine enkephalin, vasoactive intestinal polypeptide, glucagon, bombesin, ranatensin, oxytocin, somatostatin, or neurotensin in ventral photoreceptors. The octopamine synthesized by

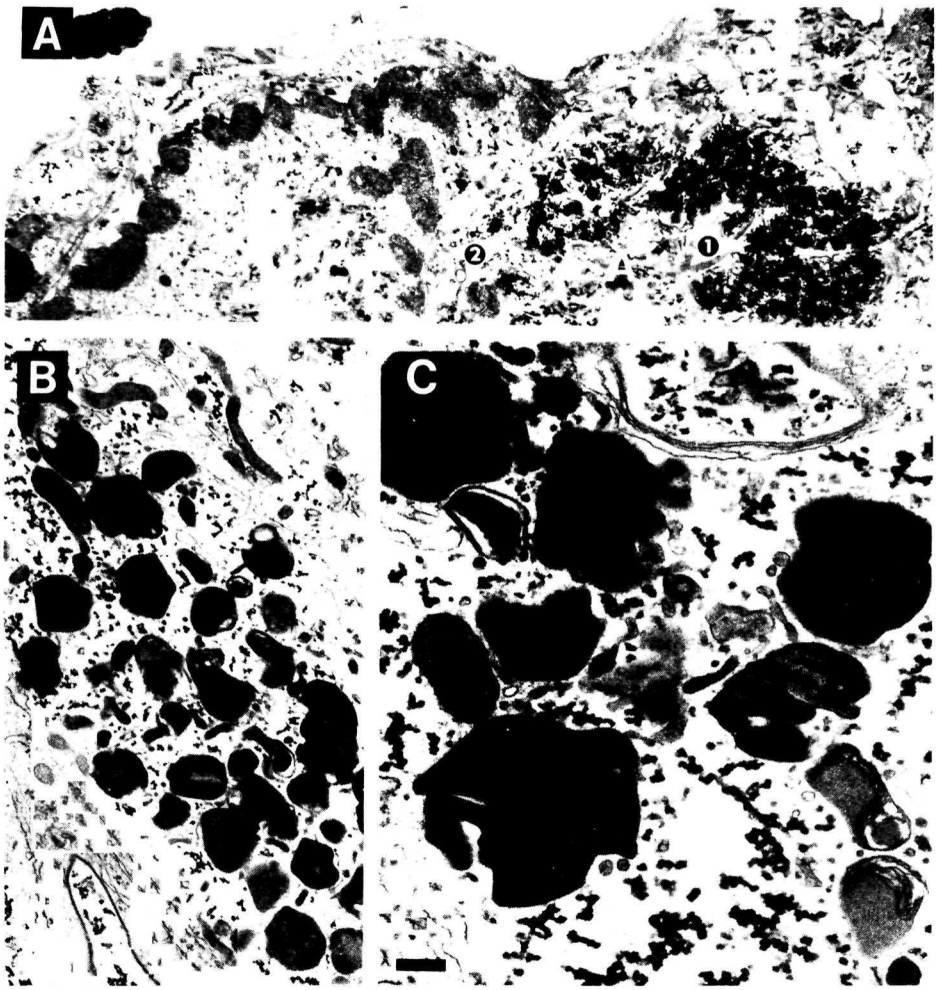

Fig. 4. Ultrastructure of vitally-staining residual bodies in *Limulus* ventral photoreceptors. *A* shows a low magnification micrograph of a longitudinal section of a single ventral photoreceptor. The rhabdomeral lobe (R) contains the rhabdom. The arhabdomeral lobe (A) contains the residual bodies (#1) and lipid droplets (#2). Compare *A* with Figure 2. *B* shows a clump of residual bodies and glycogen between two regions of cytoplasm containing mitochondria and endoplasmic reticulum. *C* shows residual bodies at higher magnification, indicating their diversity of internal structure. The bar represents 1.6 μm in *A*, 0.6 μm in *B*, and 0.23 μm in *C*.

somata-rich fractions of the ventral optic nerve is localized to the efferent fibers which innervate the rhabdomeral lobe (Evans, Chamberlain, and Battelle, 1983). Until we know that substances are synthesized and released by these photoreceptors, however, possible relationships between vital staining with neutral red and transmitter candidates in this case will remain unknown.

The residual bodies which stain with neutral red in ventral photoreceptors are excellent candidates for lysosomes involved in the cycling of photosensitive membrane or some other cellular component. Such residual bodies are a common feature of invertebrate photoreceptors (e.g., White, Gifford, and Michaud, 1980) and vertebrate pigment epithelium (e.g., Herman and Steinberg, 1982). On the other hand, they do not resemble any vesicles or granules that have been associated with the synthesis, storage, or transport of neurotransmitters or neurosecretory products of which we are aware. Our findings

suggest that the vital staining of ventral photoreceptors in *Limulus* may be more related to the vital staining of lysosomes than to the vital staining of monoaminergic or peptidergic neurons.

Limulus ventral photoreceptors are structurally segmented with one type of segment (rhabdomeral lobe) bearing all the photosensitive membrane, and the other type (arhabdomeral lobe) containing all the metabolic machinery of the neuron and giving rise to the axon (Stern et al., 1982; Calman and Chamberlain, 1982). Residual bodies are restricted to the arhabdomeral lobe of ventral photoreceptors (Calman and Chamberlain, 1982). Since these cells are widely used for physiological studies of phototransduction, and since they occur both singly and in clusters of 2–6 or more somata (Stern et al., 1982; Calman and Chamberlain, 1982) which are not easily distinguished in physiological preparations, the ability to vitally stain one lobe selectively may aid in the separation of single cells from cell clusters, and in the identification of the different segments of individual cells.

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