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Ultrastructure of photoreceptors and circadian pacemaker neurons in the eye of a gastropod, *Bulla*

JON W. JACKLET and WILLIAM COLQUHOUN

Department of Biology and Neurobiology Research Center, State University of New York at Albany, Albany, New York 12222, USA

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Summary

Each of the paired cephalic eyes of the marine gastropod, *Bulla*, is about 0.5 mm in diameter and contains about 1000 large photoreceptors, small photoreceptors, numerous pigmented support cells and about 130 neurons. The photoreceptors are of three types: large (90 μm × 20–30 μm) dense ones (PRLD) with elaborate narrow microvilli and aggregates of 650 Å clear vesicles in the cytoplasm; large clear ones (PRLC) with elaborate wide microvilli and aggregates of 650Å clear vesicles; small slender receptors (PRS) with a tuft of microvilli and lacking vesicle aggregates. Neurons (15–25 μm) containing dense-core 1000 Å vesicles are in the periphery of the retina or grouped in a collar around the neuropil below the photoreceptor layer. The 4–5 largest neurons are in the collar area. Correlation of neuron morphology with electrical activity was done with intracellular recording and Lucifer yellow injection of some of the larger neurons in the collar area whose action potentials contribute to the optic nerve impulses. Each one has an axon in the optic nerve and processes that go to the neuropil. They are the pacemaker neurons of the circadian rhythm in impulse frequency that is recorded from the optic nerve, since only their action potentials are correlated 1 : 1 with the optic nerve impulses. Gap junctions (with pentalaminar structure) are common between photoreceptors, glial cells, photoreceptors and glial cells, and neuronal processes in the neuropil, providing a basis for electrotonic coupling among retinal cells. There are about 2000 axons (diameter <3 μm) in the optic nerve, possibly one from each retinal photoreceptor and neuron plus efferent fibres from the brain. Accessory nerves, containing a few large axons, are seen in the optic nerve sheath.

Introduction

The eyes of *Bulla*, like the eyes of some other marine gastropods, contain circadian pacemakers that are precise biological timekeepers (Block & Friesen, 1981). A robust rhythm of compound action potential (CAP) frequency may be recorded from the optic nerve of the isolated eye *in vitro* or from the optic nerve of an intact animal. The frequency of CAPs changes rhythmically (with a period of about 24 h) from less than

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$1\ h^{-1}$ to more than $100\ h^{-1}$ in constant darkness and temperature, and is therefore a true circadian rhythm. It is similar to the rhythm in a related gastropod, *Aplysia* (Jacklet, 1969), whose study has resulted in considerable progress toward understanding the cellular mechanisms of circadian rhythms (reviewed, Jacklet, 1981). These eyes are among the best preparations known for studying the membrane and cellular bases of circadian rhythms.

The general features of the paired cephalic eyes of *Bulla* are similar to those of some other gastropod eyes including the marine gastropod *Aplysia* (Jacklet *et al.*, 1972) and the garden snail, *Helix* (Brandenburger, 1975; Eakin & Brandenburger, 1975). Each of these small (<1 mm diameter) eyes has a retina composed of an inner layer of photoreceptors and pigmented support cells next to the central lens, and an outer layer of fibre tracts neuropil and neurons at the base of the eye where the optic nerve originates and extends to the brain.

Bulla eyes are an important preparation for cellular studies of circadian rhythms because preliminary observations (Block & Friesen, 1981; Jacklet & Colquhoun, 1982) reveal that the photoreceptors are large and fewer in number than those in *Aplysia*. Also the neurons are in a compact cluster, accessible to study with microelectrodes. The action potentials of these neurons contribute directly to the CAPs in the optic nerve (Jacklet & Colquhoun, 1982), which exhibit circadian rhythm. Also, since surgically reduced eyes that still express a circadian rhythm contain the neuron cluster (Block & Wallace, 1982), the neurons appear to be responsible for the circadian rhythm. Accordingly, we examined the eye with light and electron microscopy to provide a structural basis for interpreting the results of electrophysiological investigations aimed at determining the function of the ocular photoreceptors and neurons in the generation and entrainment of the circadian rhythm.

Methods

Bulla gouldiana were obtained from Pacific Biomarine, Venice, CA, and kept in Instant Ocean aquaria at 16–17° C under LD (13 : 11). Animals weighing about 20 g with shell lengths of 40 mm were injected in the haemocoel with 4 ml isosmotic $MgCl_2$ to block synaptic activity and relax them just prior to dissection, which was performed several hours after lights-on. Eyes were washed in artificial sea water and immediately fixed in a solution of 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 containing 0.15, 0.25 or 0.35 M NaCl, at 5° C for 4 h. They were washed overnight in 0.1 M phosphate buffer containing 2.5% sucrose and post-fixed in 1% OsO_4 in 0.1 M phosphate buffer with 2.5% sucrose for 2 h at 4° C. Best preservation and least distortion of tissue were obtained with the fixative containing 0.15 M NaCl. Tissue was embedded in Mollenhauer's epoxy mixture II (Mollenhauer, 1964). Thick sections (5 μm) were cut and examined to find areas of interest using phase microscopy, and then thin sections (600–900 Å) were cut from the thick sections (Schabtach & Parkening, 1974). Thin sections were stained with 2% uranyl acetate in methanol for 15 min and Reynold's lead citrate for 3 min. Some eyes were fixed in formalin, dehydrated, embedded in paraffin and cut into 12 μm sections for viewing with light microscopy. Sections were stained with azure–eosin, which stained the large photoreceptor nuclei pale blue, while neuronal nuclei stained a distinctive deep blue. The

nuclei of each type in the sections were counted to estimate the total number of each cell type in the eye. Care was taken to avoid counting the same nucleus twice if it appeared in successive sections. Other eyes were prepared for intracellular recording and dye injection. After recordings were made to characterize the neurons, they were injected with the fluorescent dye Lucifer yellow (Stewart, 1978) contained in the electrode, to identify them subsequently with fluorescence microscopy as described previously for *Aplysia* eyes (Jacklet *et al.*, 1982).

Results

Bulla have paired, stalkless, cephalic eyes. In a 20 g animal, each eye is about 600 μm long and 450 μm in diameter and connected to the C.N.S. by an optic nerve about 3 mm long. The eye is a closed vesicle containing a clear prolate spheroidal lens *ca* 225 μm in minor diameter, a poorly defined cornea, and a complex retina composed of several cell types as shown in Fig. 1. The lens appears concentrically layered in electron micrographs with greatest electron density in the centre. It fills the anterior-middle chamber of the eye and adjoins the vitreous body enveloping the microvillous segments of the photoreceptors.

Eyes were extensively examined at the three levels shown in Fig. 1: A, B and C. Level A contains three types of photoreceptors (PR): a large electron-dense receptor (PRLD) up to 90 μm long and 20–30 μm wide with dense cytoplasm and microvilli; a large clear receptor (PRLC) of the same size with light cytoplasm and microvilli; and a small, slender, spindle-shaped receptor (PRS). Support cells (SC) with large dense pigment granules are abundant and interspersed with the distal segments of the photoreceptors. Level B contains a complex neuropil (NP) made up of the axons and branching processes of the neurons and photoreceptors of the eye, and perhaps terminals of efferent fibres. There is a population of neurons, lacking photoreceptive segments, clustered around the neuropil. The axons from the photoreceptors and neurons collect into the optic nerve, which was examined at level C. A complex capsule tissue, which contains vascular spaces from the arterial system, encloses the eye.

Morphology of retinal photoreceptors

At the photoreceptor level (A in Fig. 1) several layers may be distinguished (Fig. 2): a villous layer next to the lens containing the photoreceptor microvilli, a pigmented layer containing the pigmented support cells and the distal segments of the photoreceptors, and a nuclear layer containing the photoreceptor nuclei and bag-like cytoplasmic extensions of the photoreceptors. Axons and fibre processes of the photoreceptors appear in or at the base of the nuclear layer and project to the neuropil below it.

Two types of large photoreceptor occur in the retina (Fig. 2). Each is about 90 μm long and roughly pear-shaped being up to 30 μm wide at the level of the nucleus, although none of them appear so wide in Fig. 2. Each type has a large (*ca* 10 μm) nucleus surrounded by a voluminous vesicle-filled cytoplasm. The extensive cytoplasm often appears bag-like (a and b in Fig. 2) and encloses bundles of cell processes. The distal extensions of the photoreceptors pass through the pigmented layer and are capped with microvilli, which extend toward the lens. Conspicuous differences in electron density of

large photoreceptors are seen in Fig. 2, prompting their designation as photoreceptor large clear (PRLC) and photoreceptor large dense (PRLD). Other features also distinguish them including the number of cytoplasmic vesicles, the size of the microvilli and the morphology of the microvillous distal segment. The PRLC have relatively fewer cytoplasmic vesicles (Fig. 4) than PRLD (Fig. 5), although the diameter of the vesicles is about 650 Å in each case. Dark glycogen granules are commonly found in clusters with the vesicles in each type (Figs. 4, 5). The distal segment of a PRLC (arrowheads, Fig. 2) contains many large dense (0.5 μm) granules, confined to the pigmented layer and expands into a large bulb in the microvillous layer from which microvilli extend radially. In contrast the PRLD have a distal segment (arrows, Fig. 2) containing large dense granules, which are not confined to the pigmented layer but extend into the microvillous layer. The distal extension of the PRLD is dense and less voluminous than that of the PRLC and the microvilli are smaller in diameter (*ca* 1000–1300 Å) than those of PRLC (*ca* 1400–2000 Å) (Fig. 6). The villi of the PRLC expand at the tips to 3000 Å or more.

There are about 1000 large photoreceptors in the eye. This number was estimated by two methods: counting the nuclei in serial sections of the eye, and calculating the number based on the density of receptors in the spherical retinal area surrounding the lens. Nuclei larger than 8 μm were counted in each 12 μm paraffin section of an eye stained with azure–eosin. Pale blue photoreceptor nuclei were easily distinguished from the deep blue neuronal nuclei. The total number of large pale blue nuclei was 1131. The calculated number of large photoreceptors depends on the assumptions made about the size and shape of the eye structures. Assuming that the lens is a sphere 224 μm in diameter it has a surface area of 157 550 μm^2. Each large photoreceptor will occupy 144 μm^2 of that surface if it is 12 μm from the centre of its distal segment to the centre of each near neighbour distal segment in a square array. Using these assumptions the calculated number is 1094. Obviously the calculated number is only a rough approximation of the actual number because of irregularities in the eye structure. For example, the lens in Fig. 1 is a prolate spheroid 224 μm in minor diameter but 300 μm in major diameter. This would make the calculation an underestimate due to the increased area. However, the cornea is free of photoreceptors and estimating the photoreceptor area as a sphere will compensate for the cornea. The distance from one distal segment centre to a neighbouring distal segment centre is quite variable. In Fig. 2 the distance is 10 to 15 μm, so 12 μm was used in the calculation. For comparisons, if 10 μm is used for

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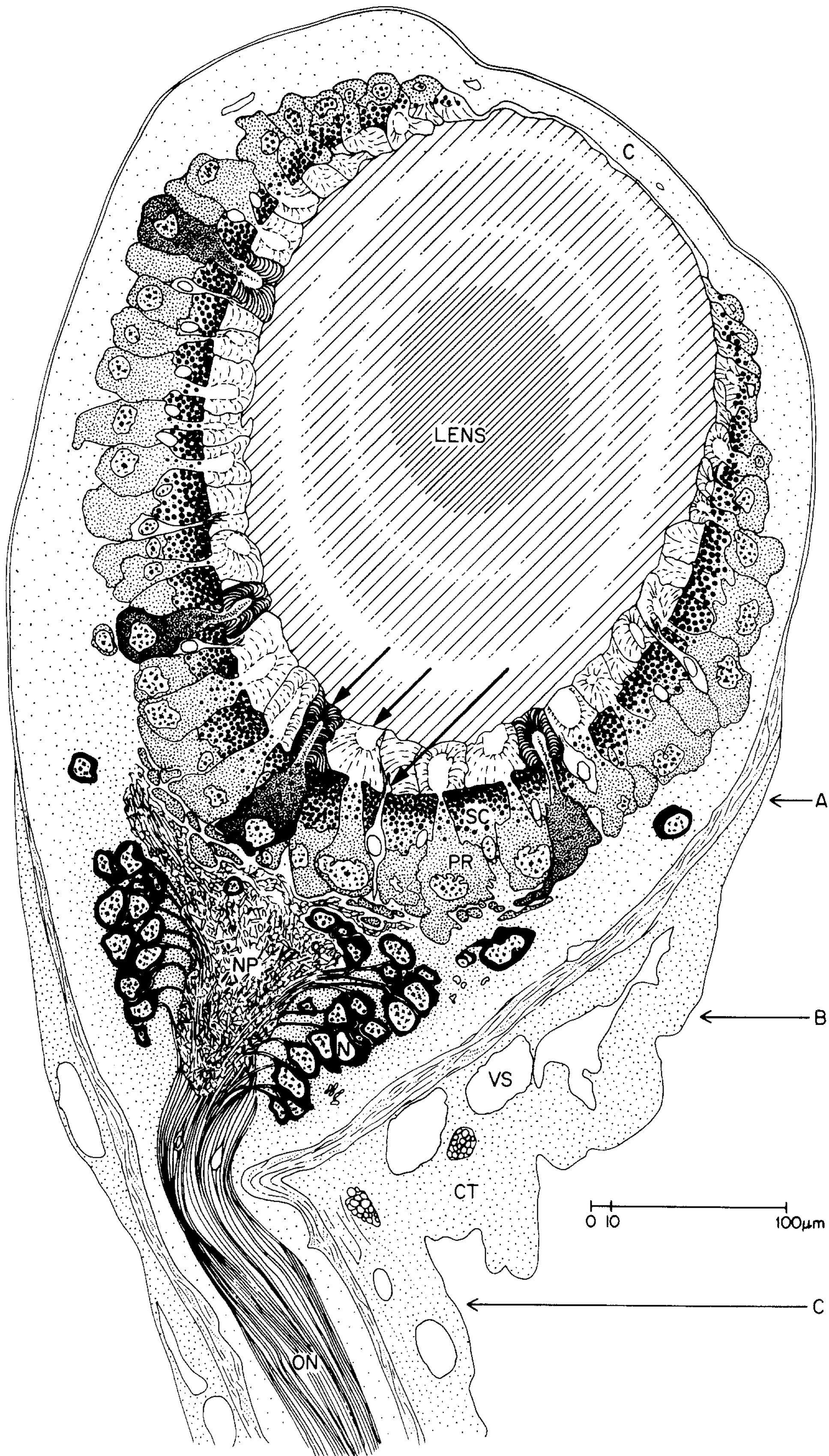
Fig. 1. Schematic diagram of the eye of *Bulla* drawn from a thin section of the entire eye. Major features are: the large concentrically layered lens, a pigment-less corneal area (c), a retina, surrounding the lens, composed of primary photoreceptors (PR) and pigmented support cells (SC) at level A; a cluster of neurons (N) and neuropil (NP) at level B, and the optic nerve (ON) at level C enclosed in a musculo-connective tissue capsule (CT) that also encloses vascular spaces (VS) from the arterial system. At level A representatives of the three photoreceptor types are identified with arrows. From left to right they are photoreceptor, large dense (PRLD); photoreceptor, large clear (PRLC); and photoreceptor small (PRS).



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the centre-to-centre distance then the number is 1575, if 15 μm is used the number is 700. Considering all the difficulties in the calculation, the actual number of nuclei counted in the stained sections is the best estimate. Assuming a 10% error in the counting of nuclei, there are approximately 1000 large photoreceptors, which agrees reasonably well with the calculated number.

The relative numbers of the two types of large photoreceptor cannot be determined by light microscopy because they cannot be distinguished unambiguously one from the other. In representative electron micrographs of large areas, such as Fig. 2 and other micrographs, each type appears to be represented in all areas of the retina and approximately in the ratio 2 PRLD to 3 PRLC.

The distal segment of a PRLC is shown in Fig. 7. It contains large (*ca* 5000 Å diameter) dense granules, microtubules, and a few vesicles and mitochondria. Microvilli originate from the distal bulb of cytoplasm. The photoreceptor has adhering zonules at junctions with the neighbouring pigmented support cell, which has short narrow microvilli bordering on the microvillous layer (Fig. 7). In some cases the cytoplasm of support cells extends 10 μm or so, into the microvillous layer before ending in short microvilli.

A type of small photoreceptor, PRS, is commonly found in the retina in addition to the conspicuous larger ones. It is not rare and in some cases two or three may occur within a few micrometres of each other (Fig. 3). Accurate estimates of the number of small photoreceptors are difficult to make, but it appears that there are several hundred of them in the retina. A PRS has a narrow distal segment that extends toward the lens and ends in a tuft of microvilli (Figs. 8, 9). It is flanked by pigmented support cells whose nuclei are often found near the PRS nucleus (Figs. 8, 10). The nucleus of a PRS is electron lucent and spherical (3–6 μm in diameter). It is found in the upper nuclear layer, above the nuclei of most of the large photoreceptors, just below the pigmented layer

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Fig. 2. Section of the retina at level A (Fig. 1) showing large photoreceptor types: photoreceptor large clear (PRLC) (arrowheads at distal segment) and photoreceptor large dense (PRLD) (arrows at distal segment). Photoreceptor processes are often enclosed by photoreceptor cytoplasm (above a, right of b). The two types of photoreceptors differ in their microvilli (area c and elsewhere), distal segments (arrows and arrowheads) and electron densities. Scale bar: 10 μm.

Fig. 3. Section of retina adjacent to the area shown in Fig. 1 illustrating small cells and nuclei. The clear nucleus on the left (large arrowhead) belongs to a small photoreceptor with its axon extending downward. The two clear nuclei to the right (arrowheads) are also from small photoreceptors. The two dense nuclei on the right (small arrowheads) belong to pigmented support cells. The nuclei are much smaller than the nucleus (n) of a large photoreceptor. The bag-like process (p) of a large photoreceptor appears below the small nuclei. Scale bar: 10 μm.

Fig. 4. Enlargement of area above (a) in Fig. 2 showing sparsely packed clear vesicles (*ca* 650 Å diameter) and clumps of dark glycogen granules in cytoplasm of PRLC. Scale bar: 0. 25 μm.

Fig. 5. Enlargement of area above (b) in Fig. 2 showing densely packed clear vesicles (*ca* 650 Å diameter) and clumps of dark glycogen granules in cytoplasm of PRLD. Scale bar: 0.25 μm.

Fig. 6. Enlargement of area above (c) in Fig. 3 showing microvilli of a PRLD (left) and a PRLC (right) with conspicuous differences in density and size. Scale bar: 0.7 μm.

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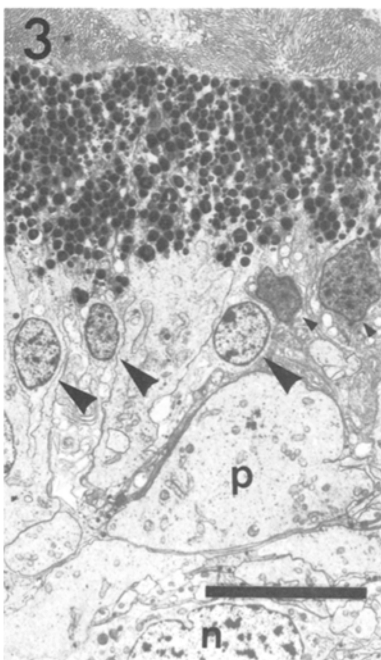
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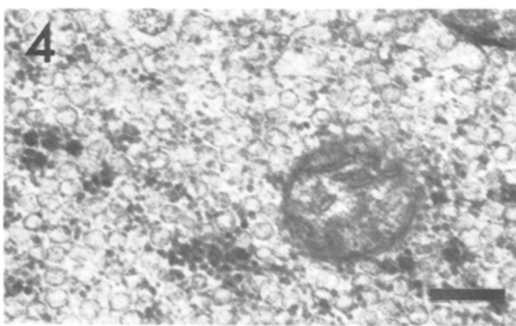
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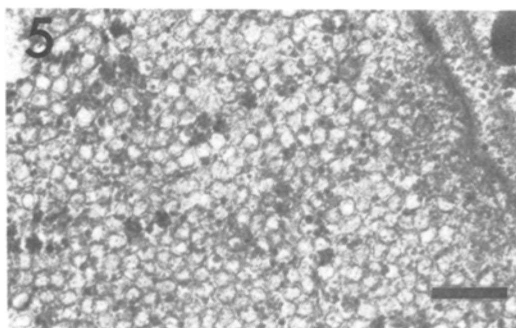
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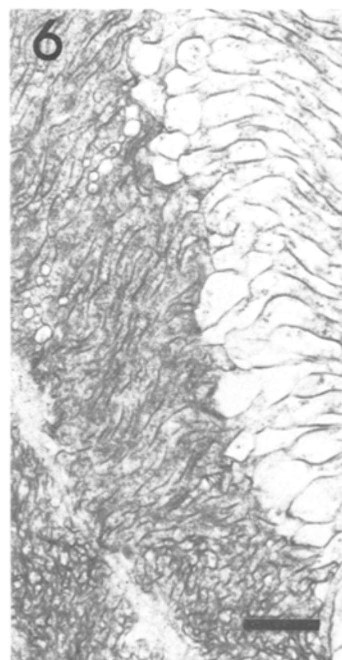
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(Figs. 1, 3). There is only a small amount of cytoplasm in a PRS and it lacks the aggregates of cytoplasmic vesicles seen in large photoreceptors but it does have Golgi elements and associated vesicles (Fig. 10). A prominent axon extends from the base of the PRS (Fig. 8) and the hillock is full of glycogen granules and mitochondria. Vesicle-filled cytoplasmic processes of large photoreceptors are commonly found near the soma and axon of a PRS (Figs. 8, 10), showing their intimate relationship and allowing the direct contrast of differences in their cytoplasmic characteristics.

Pigmented support cells contain numerous dense granules (*ca* 5000 Å). Their cytoplasm is rich with rough endoplasmic reticulum and mitochondria, suggesting that they are active in synthesis (Fig. 10). The nucleus is electron dense (in sharp contrast to the PRS nucleus, Fig. 10) and has a prominent nucleolus (Fig. 8). Pigmented support cells flank the distal segments of all the photoreceptors and their dense granules are largely responsible for the pigmented layer of the retina. Their cytoplasm rarely extends below the nuclear layer of the retina.

Neuropil and synaptic junctions

An extensive neuropil encircled by a collar of neurons lies below the photoreceptor layer at level B of the eye (Fig. 1). The neuropil, which is a site of presumed synaptic interaction between neural and photoreceptor elements, is shown in cross-section in Fig. 11. Many of the processes in the neuropil are broad and electron-lucent. They contain conspicuous microtubules (Fig. 11) and vesicles that are usually clustered along the edges of the processes (Fig. 11). As shown in Fig. 12 processes in the neuropil may contain clear vesicles of various sizes (*ca* 650–1000 Å) or a mixture of clear and dense vesicles or large dense vesicles (*ca* 1000–1500 Å). Occasionally vesicles may be seen clustered next to the membrane (Fig. 12) in a fashion reminiscent of a putative chemical synapse, but the classical morphology associated with chemical synapses in vertebrate

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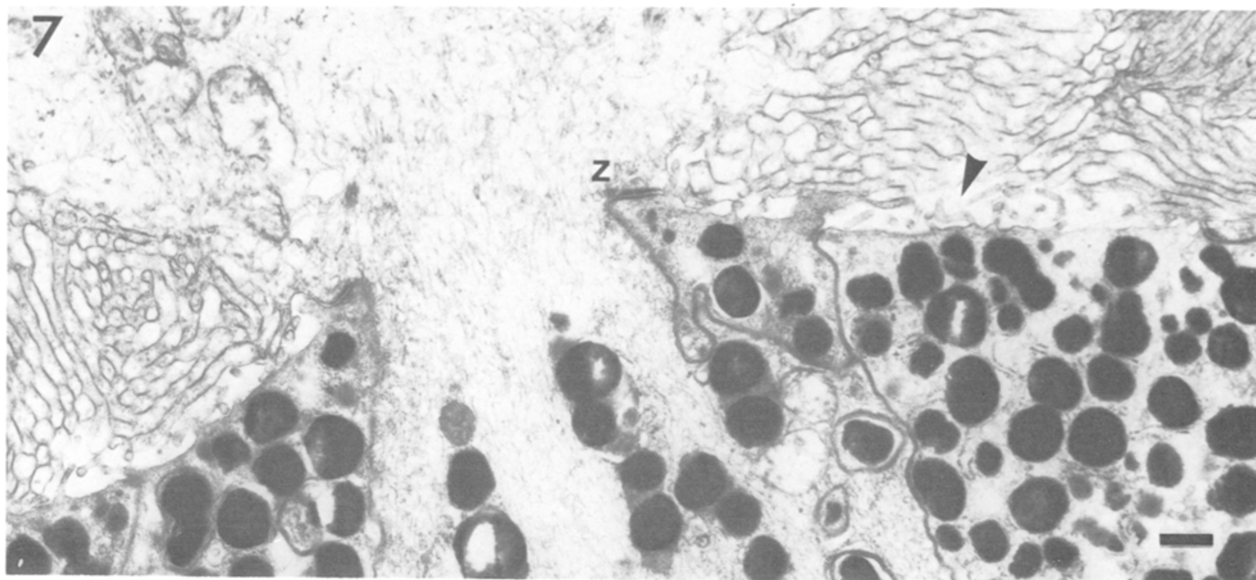
Fig. 7. A PRLC distal segment flanked by pigmented support cells. Zonules (Z) of adherence between photoreceptors and support cells are common. Support cells have short thin microvilli (arrowhead) and large dense pigment granules. Scale bar: 0.5 μm.

Fig. 8. A small photoreceptor, outlined with ink for clarity. The long thin distal segment (d) terminates in a tuft of microvilli and a prominent axon (a) extends from the soma. The distal segment is flanked by a pigmented support cell with a typical dense nucleus (ns) which contrasts with the clear oval nucleus (n) of the small photoreceptor. Vesicle filled cytoplasm of a large photoreceptor (p) borders the base of the small photoreceptor. Scale bar: 3 μm.

Fig. 9. Microvillous distal segment (d) of a small photoreceptor, flanked by support cells, ending in a tuft of microvilli. Scale bar: 1 μm.

Fig. 10. Dense nucleus (ns) of a pigmented support cell contrasts with the nucleus (n) of a small photoreceptor (lower centre). Support cells have extensive rough endoplasmic reticulum (er). A small photoreceptor has a prominent distal segment (d) and scant cytoplasm, and contains Golgi elements (g) and associated vesicles. Cells are adjacent to the process (p) of a large photoreceptor. Scale bar: 0.5 μm.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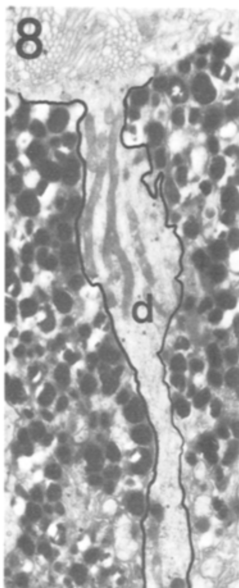
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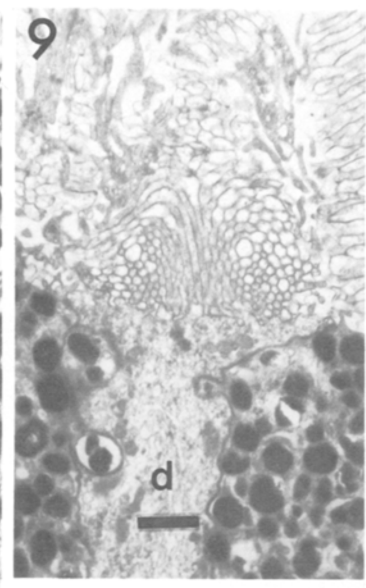
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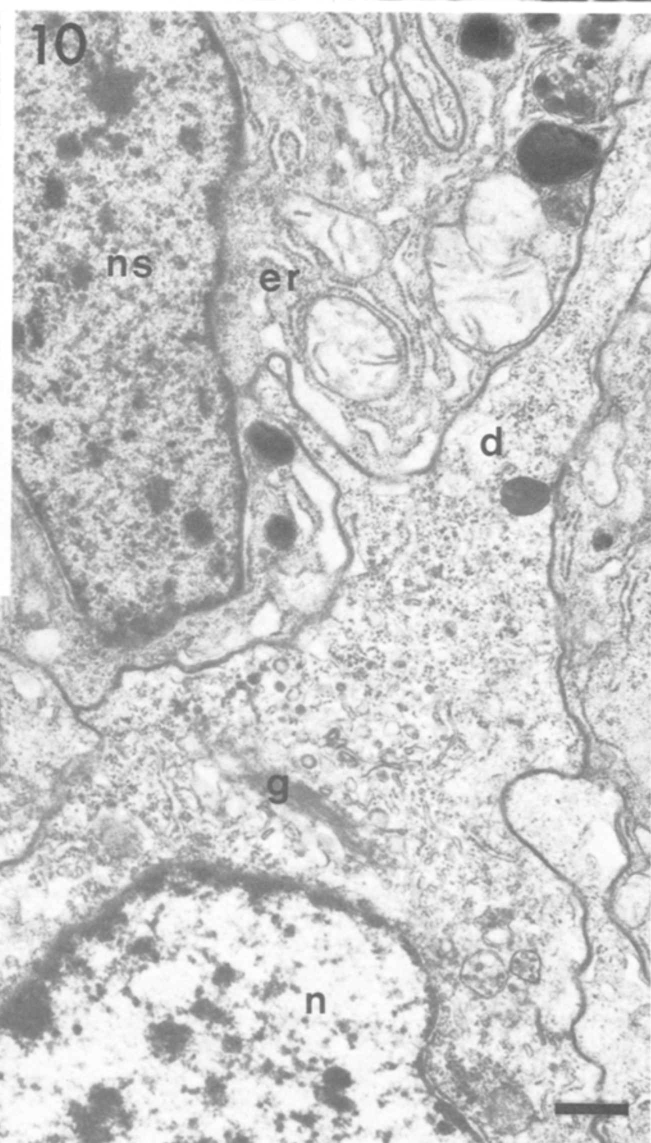
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tissue, such as widening of the cleft between processes, and presynaptic and postsynaptic membrane specializations are not seen. However, gap junctions, sites of possible electrical communication between processes, are plentiful in the neuropil. Usually the membranes of adjacent neural processes are distinctly separated over much of their area of apposition (Fig. 14) but the membranes do come together at specific sites to form a pentalaminar gap junction (Fig. 14) where dense bands alternate with light bands. The centre dark band, which represents the fused outer leaflets of the membranes, is more dense than the lateral dark bands, which represent the inner membrane leaflets of each of the membranes. The junctions in Fig. 14 are between processes found among many others in the neuropil (see Fig. 13). Each process contains clear vesicles of various sizes (*ca* 600–1000 Å) but the cells of origin of the processes cannot be determined. Outside the neuropil the processes of large photoreceptors can be identified unmistakably by tracing the processes from their cell bodies, which are full of clear *ca* 650 Å vesicles. Two identified PRLC processes containing 650 Å vesicles were identified with two glial processes interposed between them (Fig. 15). Gap junctions are evident between one PRLC and the adjacent glial cell and between the glial processes themselves (Fig. 16). The junctions extend a considerable distance along the membranes, providing large sites of probable intercellular interaction. A gap junction between two processes in the neuropil, each of which contained large dense-core vesicles, is shown in Fig. 17. The pentalaminar structure is seen in each example of a gap junction shown in Figs. 13, 16 and 17.

Fig. 11. Neuropil of the eye showing neural processes cut in various orientations. Microtubules (t) are seen in longitudinal section or cross-section (tx) and dense-core vesicles are seen along the edges of the processes (arrowhead). Scale bar: 1 μm.

Fig. 12. Variety of vesicles found in the neuropil. Some terminals contain clear vesicles, others a mixture of larger dense-core vesicles and small clear vesicles, others have large very dense vesicles with a few small clear ones. The arrowhead points to a cluster of vesicles adjacent to the membrane. Scale bar: 0.3 μm.

Fig. 13. Processes in the neuropil containing clear vesicles of various sizes, mitochondria and cross-sections of microtubules (tx). Enlargement of area in the rectangle is shown in Fig. 14. Scale bar: 0.5 μm.

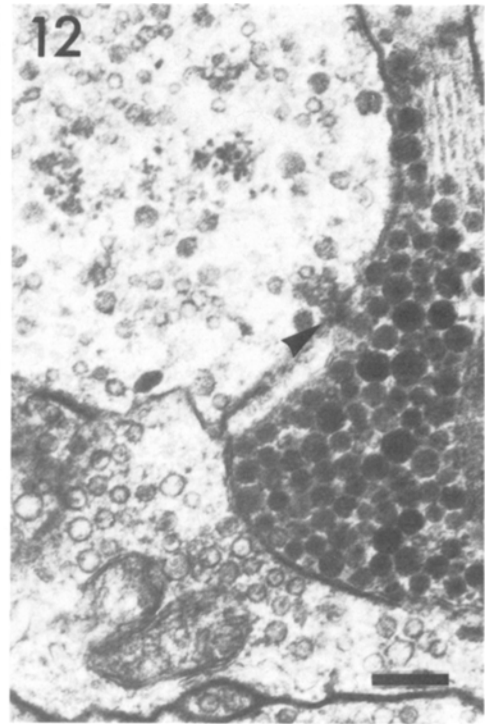
Fig. 14. Enlargement of area within the rectangle in Fig. 13. The membrane of each process is clearly separated from other processes at many points but membranes come together to form a gap junction (arrow) at one point and possibly at other points (arrowheads). Scale bar: 0.1 μm.

Fig. 15. Processes (p) of two PRLCs containing small clear vesicles, separated by two glial cell processes. Scale bar: 0.1 μm.

Fig. 16. Higher contrast reproduction of Fig. 15 showing membranes forming gap junctions (downward arrowhead) between a PRLC and a glial process, and between glial processes (upward arrowhead). Scale bar: 0.1 μm.

Fig. 17. Close membrane appositions forming a gap junction (arrowhead) between processes that contained both large dense-core and clear vesicles. Scale bar: 0.1 μm.

11
t
tx
t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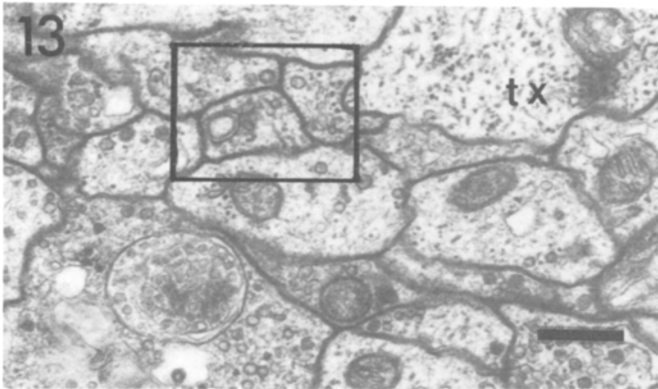
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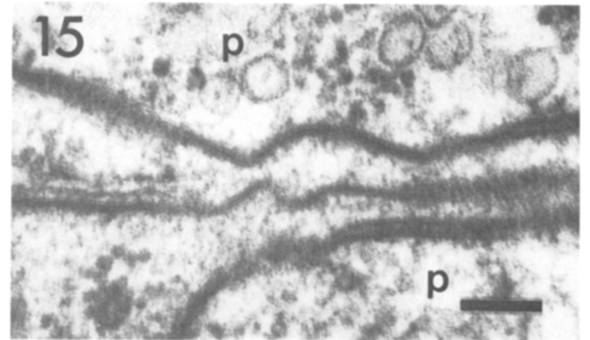
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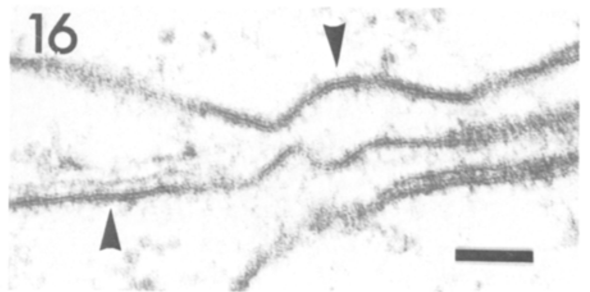
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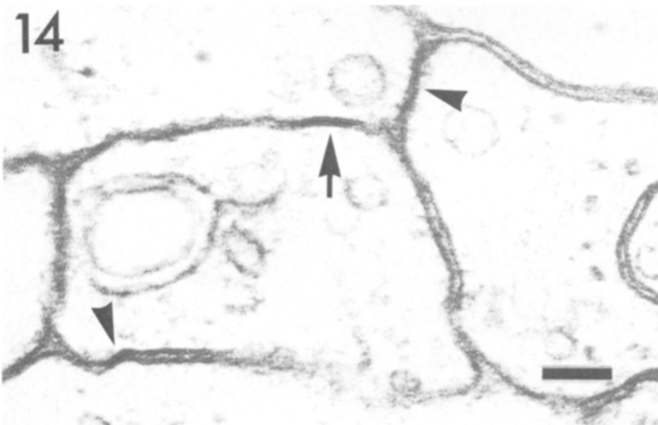
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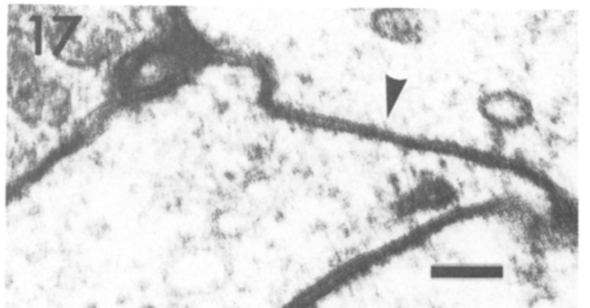
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Morphology of neurons

A collar of neurons that encircles the neuropil lies below the photoreceptor layer in level B of the eye (Fig. 1). This level is seen in cross-section in Fig. 18 where the neuron cell bodies form an arc around the central neuropil. The neurons are often in direct apposition to one another or only separated from one another by thin glial processes. They are oval-shaped with a prominent branching axon extending into the neuropil (see neuron outlined in Fig. 18). Each neuron cell body, like the large neuron shown in Fig. 19, is replete with mitochondria, Golgi elements, ribosomes, endoplasmic reticulum and dense-core vesicles. Dense granular aggregates occur in both the soma (Fig. 19) and the axon (Fig. 21). These aggregates contain dense-core vesicles and clusters of dense granules about the size of glycogen granules. In the soma dense-core vesicles *ca* 1000 Å (or less) in diameter appear to arise from the extensive Golgi apparatus (Fig. 20). Also prominent are large (*ca* 1 μm diameter) membrane-bound dense bodies, which contain vesicles or membraneous lamellar structures (Figs. 19, 20). Where cell bodies of neurons were directly juxtaposed as shown in Fig. 20, the membranes were examined carefully for the presence of gap junctions, but none were found. The axon of a neuron is broad and electron lucent compared to the soma, suggesting that many of the large electron-lucent processes observed in the neuropil (Figs. 11, 18) may be those of neurons. It extends into the neuropil where processes branch from it. Axons like the one in Fig. 22 were traced in sequential micrographs for 30 μm. A neuropilar branch of the axon giving rise to dendrite-like processes is confirmed by dye injection of the individual neuron shown in Fig. 27. Occasionally, neurons are observed with dendrite-like

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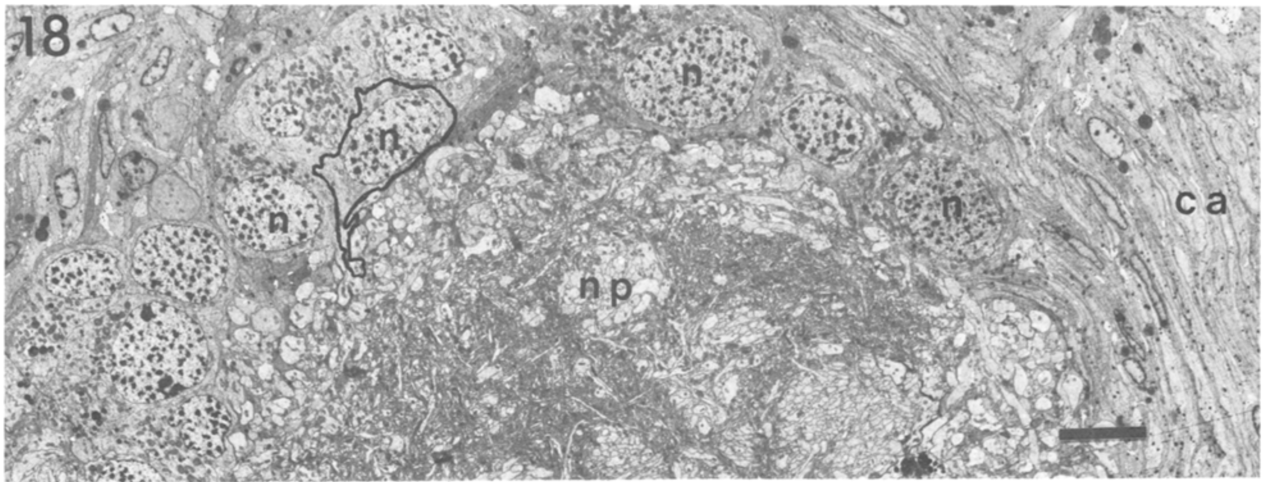
Fig. 18. Cross-section through the eye at the level of the neuropil and neurons (level B, Fig. 1). The neuronal nuclei (n) form an arc around the central neuropil (np) containing individual fibres and bundles of fibres cut in various orientations. One neuron is outlined in ink to show its shape, and its axon which enters the neuropil. An extensive cellular capsule (ca) encloses the collar of neurons. Scale bar: 10 μm.

Fig. 19. Nucleus (n) and cytoplasm of a large neuron. The neuron has a large axon (a), numerous Golgi elements (g), mitochondria, membraneous bodies (b), dense-core vesicles, and vesicular aggregates (arrowhead). Scale bar: 1 μm.

Fig. 20. Intersection of three neuronal cell bodies, similar to those shown in Fig. 18. The membranes (small arrowheads) of adjacent neurons are directly apposed but were never found to have gap junctions between them. Cytoplasm is replete with ribosomes, endoplasmic reticulum, Golgi elements (g), mitochondria (m), dense-core vesicles (arrowhead) and membraneous bodies (b). Scale bar: 0.3 μm.

Fig. 21. Axon (a) of the neuron shown in Fig. 19. It contains large mitochondria, microtubules, dense-core vesicles (arrowheads) and vesicular aggregates (large arrowhead). Another neuron, (nucleus at n), apposes the axon directly at some points. Scale bar: 1 μm.

Fig. 22. Extension of the axon (a), shown in Fig. 21, into the neuropil containing various other fibres. The axon is characteristically electron lucent and contains microtubules and dense-core vesicles (arrowheads). Scale bar: 1 μm.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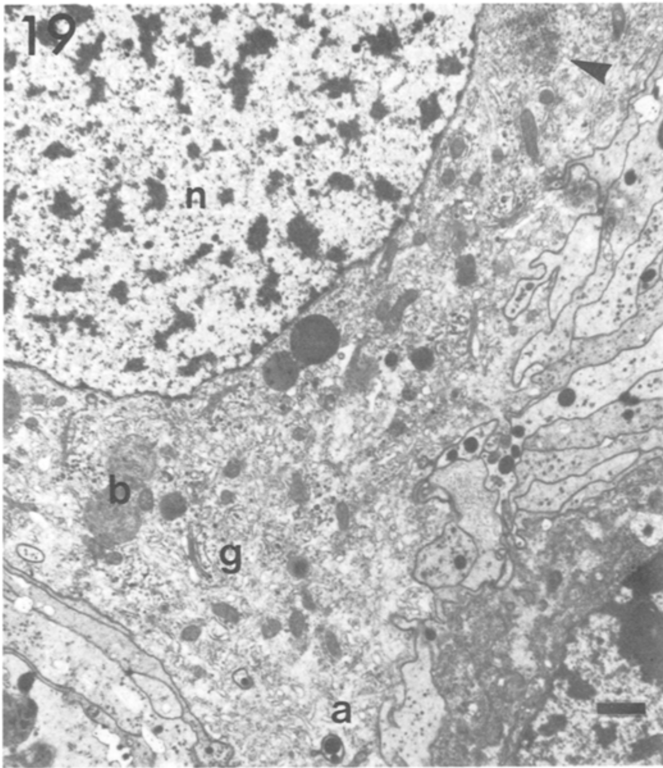
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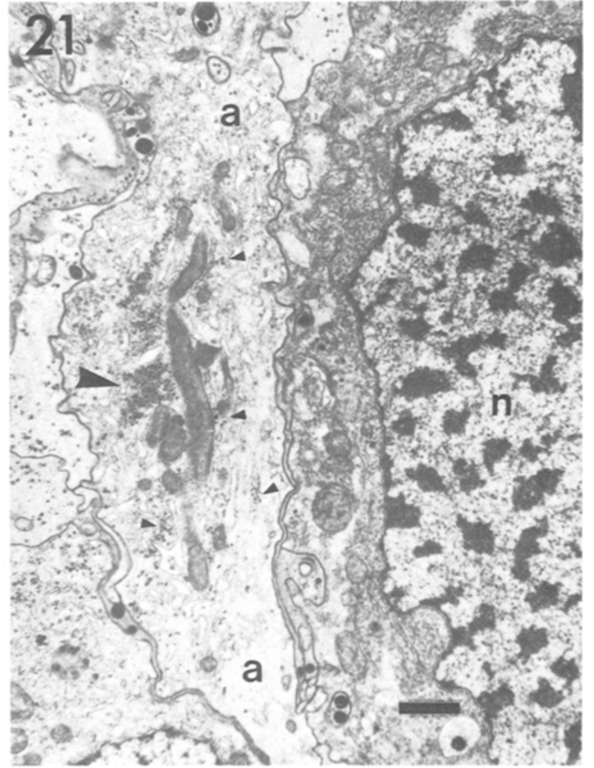
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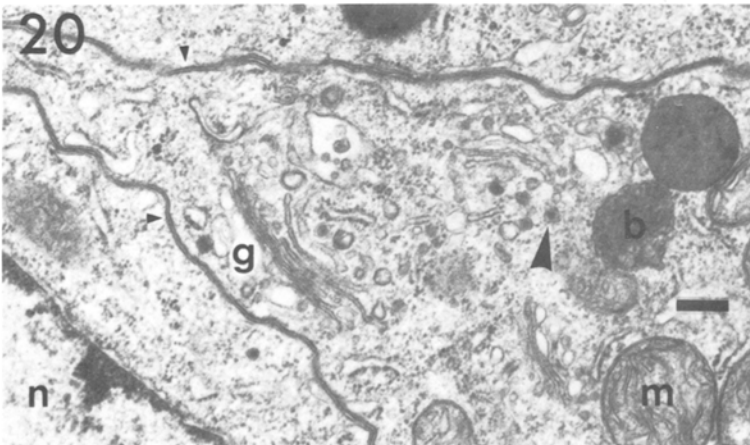
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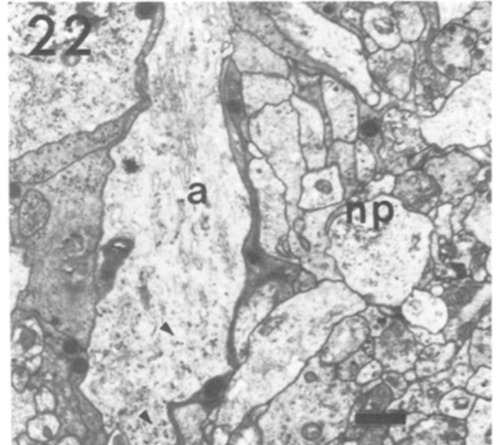
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processes extending from the soma for a short distance (Fig. 23). These processes are more electron dense than the axons and contain clear vesicles in the apparent terminals, as shown in Fig. 23. The neurons with dendrite-like processes have typical Golgi elements and dense-core vesicles in the soma as seen in other neurons. The area of the neuropil containing the presumed dendritic process (Fig. 23) was like the rest of the neuropil, rich in vesicle-laden processes and axonal processes containing microtubules and dense-core vesicles.

Most of the neurons are alike, being 15–20 μm in diameter with a nucleus 10–15 μm in diameter (Fig. 18) but there are four or five neurons in each eye which are somewhat larger. In the collar area where extensive observations were made, all the neurons seem to have Golgi elements and large dense-core vesicles like the neurons shown in Figs. 19, 20 and 23. The total number of neurons in an eye was estimated by counting the neuronal nuclei of eyes prepared for light microscopy and stained with azure–eosin.

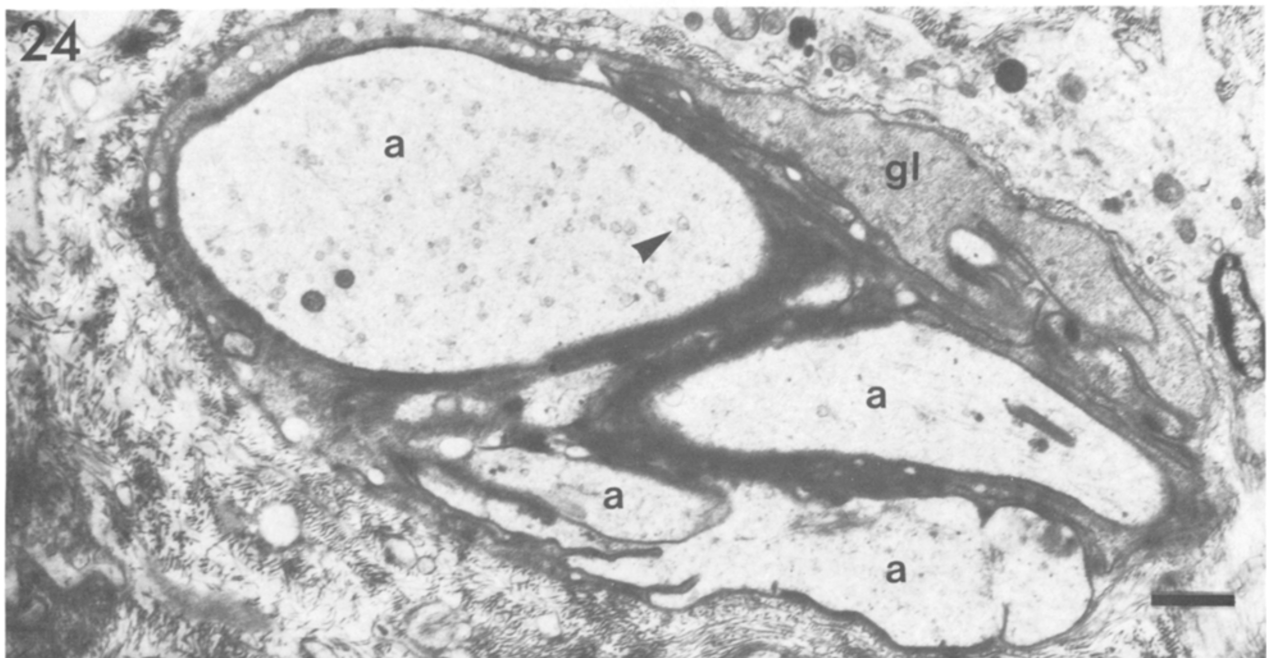
The nuclei in two eyes were counted, one contained 128 and the other 130. Each eye had one very large neuron (cell body *ca* 25 × 40 μm) and three or four large neurons (*ca* 20 × 35 μm) in the lower part of the collar of neurons at level B (Fig. 1). In both eyes the distribution of neurons was similar. About 70 neurons are within the collar of neurons at level B (Fig. 1); the rest are between levels A and B (Fig. 1) in the periphery of the retina, mixed with the basal portions of the photoreceptors.

Morphology of optic nerve

The optic nerve is a 50 μm diameter bundle of axons enclosed by a thick heterogeneous capsule (Fig. 1). In addition to the main nerve, thin accessory nerves are found in the optic nerve sheath (Fig. 24). They contain a few large (up to 4 μm) axons wrapped by a glial cell embedded in the nerve sheath. Some of the axons contain clear vesicles (*ca* 1500 Å in diameter) or clear vesicles with a small dense core (Fig. 24). The accessory nerves branch from the main nerve as shown in Fig. 25 and the axons may expand upon leaving the nerve because they are larger than the majority of axons in the main nerve where the accessory nerve branches (Fig. 25) and the largest (*ca* 4 μm) axons are in the accessory nerve. Points where the accessory nerve left the main nerve were difficult to find suggesting that only a few accessory nerves leave the main nerve at this level. The cell bodies that give rise to the axons in the accessory nerves are unknown.

Fig. 23. Neuronal process (p) extending into the neuropil. Nucleus (n) of the neuron is shown with an adjacent neuronal nucleus (n) on left. Neuronal cytoplasm contains Golgi elements (g), endoplasmic reticulum, dense-core vesicles and mitochondria. This electron-dense process contrasts with electron-lucent axons (a) and contains clear vesicles (v). Clear (arrow) and dense-core (arrowhead) vesicles are contained within other processes in the neuropil. Scale bar: 1 μm.

Fig. 24. Axons (a) of an accessory nerve. Axons are enclosed in a glial process (gl) and collagenous material of the optic nerve sheath. Clear vesicles are plentiful in the axoplasm and some are dense-cored (arrowhead). Scale bar: 1 μm.


24
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gl
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Axons of the optic nerve are clustered in subgroups by the radially arranged glial cells, which frequently have nuclei in the centre of the nerve (Fig. 25). Axons within the optic nerve may be individually enclosed by glial cells (Fig. 26) whether they are large or small, and often a group of axons (large and small) are enclosed by glial cells. Axons contain microtubules, mitochondria and vesicles. Some of the larger axons contain dense-core 1000 Å vesicles (Fig. 26). Smaller axons contain clear 650 Å vesicles, similar to those found in photoreceptors.

All the axons in two electron microscope sections of the optic nerve (like Fig. 25) were counted. One contained 1998 axons and the other 2005. Some axons appear elliptical in cross-section and therefore have a minor and a major diameter. The true diameter is assumed to be the minor diameter and the major diameter results from oblique sectioning of the axon. Seven per cent (140) of the axons are larger than 0.8 μm and the largest axon is about 3.0 μm. Seventy-eight per cent of the axons are less than 0.55 μm in diameter.

Correlated structure and function of neurons

The retinal cells were injected with Lucifer yellow after recording their activity to correlate morphological cell type with electrical activity. The cells in level A (Fig. 1) are photoreceptive and respond to light with slow, graded potentials. In preliminary studies (Jacklet & Colquhoun, 1982) there appear to be several types of graded depolarizing responses, with or without action potentials, but none of the receptors produces action potentials correlated with the CAPs that occur in the optic nerve. The CAPs are the impulses that exhibit the circadian rhythm of impulse frequency. However, the neurons found in level B are spontaneously active in darkness and their action potentials are correlated 1 : 1 with the CAPs in the optic nerve. A large neuron marked with the dye Lucifer yellow is shown in Fig. 27 as well as the spontaneous dark and light evoked action potentials, synchronous with the optic nerve CAPs, which were recorded from the neuron prior to injection of the dye. This identifies the neuron as one of the output neurons of the circadian rhythm. The injected neuron appears to be one of the larger 4–5 neurons of the eye. The soma was brightly fluorescent so it is assumed that the neuron was completely filled with dye and the complete form of the neuron was observed including the dendritic branch that extended about 20 μm into the neuropil.

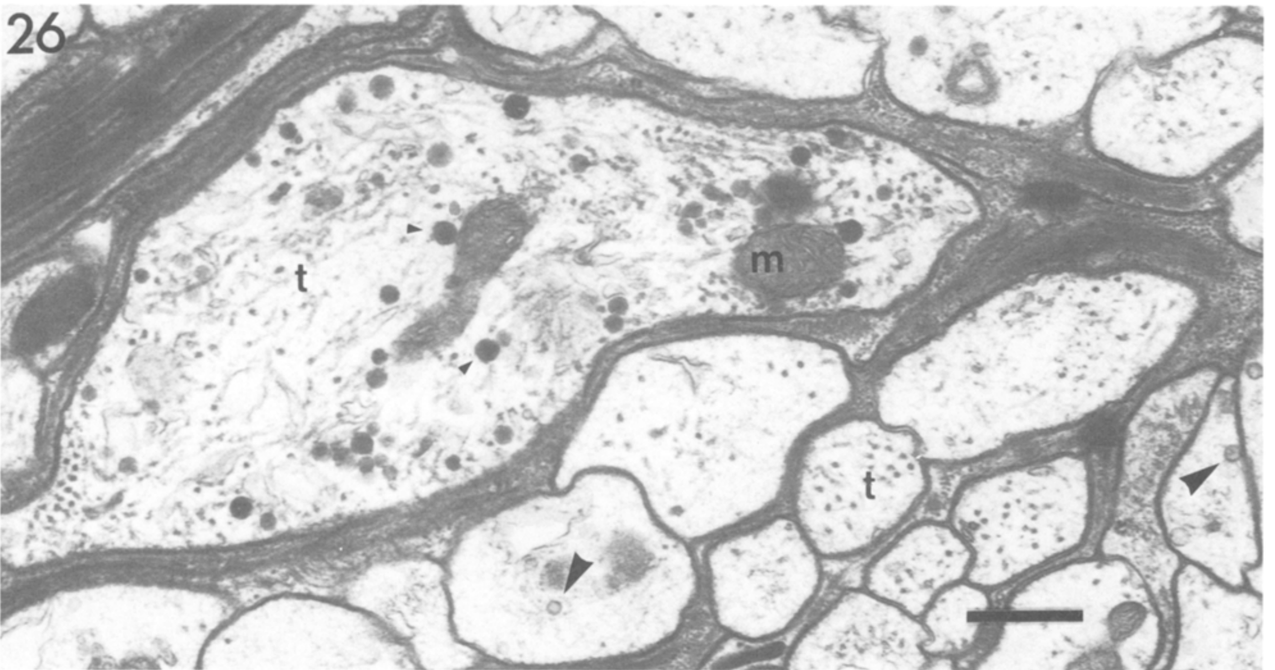
Fig. 25. Cross-section of the optic nerve taken at level C (Fig. 1). Axons are grouped into bundles wrapped by glial cells and several glial nuclei appear in the centre of the nerve. The nerve is enclosed in a complex capsule (ca) of cellular elements. An accessory nerve (ac) emerges from the nerve and contains several large axon-like processes. Scale bar: 4 μm.

Fig. 26. Enlargement of the axons within the rectangle of Fig. 25. Microtubules (t) cut in several orientations are evident in many axons and dense-core vesicles about 950–1050 Å in diameter (small arrowhead) appear in the larger axon. Smaller clear vesicles (large arrowheads) are seen in some smaller axons. Some axons are directly apposed to one another while others are wrapped by glia. Scale bar: 0.5 μm.

25

ac

ca



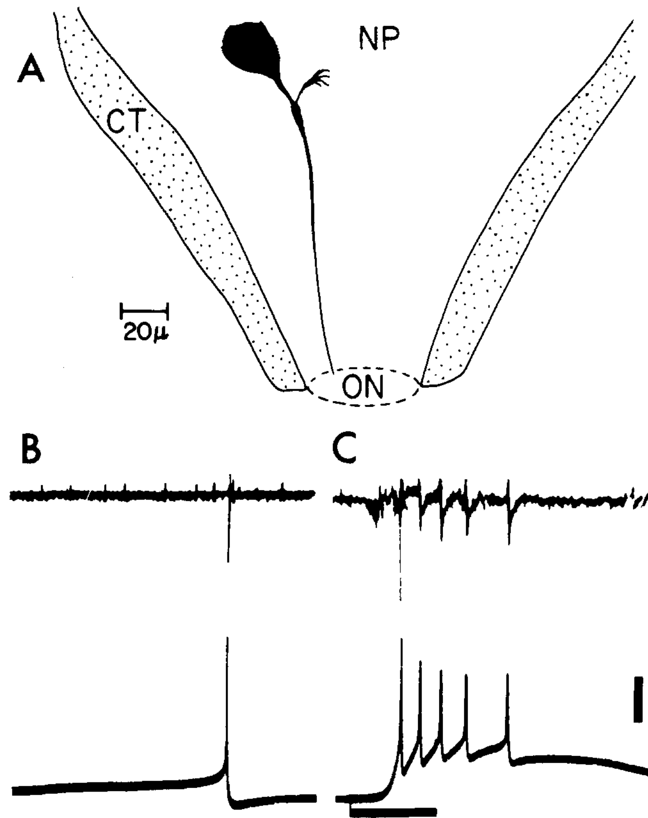


Fig. 27. (A) Diagram of a large neuron, injected with Lucifer yellow, in the cluster at the base of the eye (level B, Fig. 1). It has a dendritic process extending into the neuropil (NP) and an axon contributing to the optic nerve (ON). The spontaneous spikes in darkness (B) and spikes in response to light (C) were recorded before dye injection. The large CAPs in the optic nerve (top traces) are correlated 1 : 1 with spikes in the neuron (bottom traces). Scale bars: top traces 50 μV; bottom traces 20 mV; time, 1 s light pulse in C. The same bar is 4 s in B.

The soma was in the collar of neurons, as shown in Fig. 18. Five other neurons, whose action potentials were correlated 1 : 1 with the optic nerve CAPs were injected. None of them filled as completely as the one shown in Fig. 27, but four out of five were among the neurons clustered around the neuropil (Fig. 1), and each one had an axon in the optic nerve. One was in level A in the periphery of the retina just outside the collar of neurons.

Discussion

The eyes of *Aplysia* and *Bulla* both exhibit circadian rhythms in optic nerve CAP activity and the neurons of each eye are believed to be the output neurons of the rhythm (Block &

Wallace, 1982; Jacklet *et al.*, 1982) and perhaps the circadian pacemakers themselves. There are about 1000 neurons (called secondary neurons) in the eye of *Aplysia* (Jacklet, 1973) distributed in small clusters throughout the peripheral layer of the retina (Jacklet, 1973; Jacklet *et al.*, 1982). In *Bulla* some of the neurons are distributed in a similar way but about 70 of the 130 neurons are in a compact collar surrounding the neuropil. Eyes that were surgically reduced until only 30–50 neurons remained were able to express a circadian rhythm with normal periodicity (Block & Wallace, 1982) showing that the neurons are sufficient to produce the rhythm but leaving in doubt the contribution of the other neurons. Similar reduction of the *Aplysia* eye had shown that cells in the eye base including 150–200 neurons were sufficient to express the circadian rhythm (Jacklet & Geronimo, 1971), but since the neurons are not in a compact cluster they could not be isolated as precisely as they were in *Bulla*. Thus, probably in both eyes the neurons at the base of the eye are able to produce the rhythm and it is not known if the other neurons contribute to the rhythm in normal eyes. This study did not reveal any conspicuous differences in the structure of the neurons that would suggest differences in function.

In the surgically reduced eyes of *Bulla* the CAP frequency was reduced to about one-half (Block & Wallace, 1982), suggesting that other neurons might contribute to the firing rate in normal eyes. This raises questions about how many neurons contribute to the CAP and what interactions are needed to produce the CAP and regulate their frequency. These questions need to be addressed with simultaneous recordings from neuron pairs, to specify the interactions between neurons, and subsequent staining to identify the neurons. The techniques are now available to visualize Lucifer yellow-stained neurons by electron microscopy utilizing a photo-oxidation reaction involving diaminobenzidine (Maranto, 1982), allowing the correlation between structure and function to be carried to the ultrastructural level.

The neurons in *Bulla* eye are similar to the neurons in *Aplysia* eye (Luborsky-Moore & Jacklet, 1977; Jacklet *et al.*, 1982). Each is monopolar and about 15–25 μm in diameter containing 1000 Å dense-core vesicles. Short dendrite-like processes occur on the soma, and the axon is broad and electron lucent. Axons typically branch near the axon hillock in *Bulla* and *Aplysia* as shown by Lucifer yellow injection and the main axon continues into the optic nerve. Neurons similar to those in *Bulla* and *Aplysia* are seen in *Arthoracophorus* (Eakin *et al.*, 1980) and *Helix* but they are fewer in number (12 estimated for *Helix*) and are called ganglion cells (Brandenburger, 1975).

The electrical events in *Bulla* neurons are similar to those in *Aplysia* (Jacklet *et al.*, 1982) but the cell bodies appear to be more active in generating pacemaker and spike activity and, therefore, the *Bulla* eye is a more favourable preparation for electrophysiological studies. Neuronal spikes in each eye are correlated 1 : 1 with the optic nerve CAPs. Since the CAP activity is produced by the synchronous firing of a group of neurons it requires neuronal interactions of some sort to produce it. Attempts to block the CAP activity in *Aplysia* with low Ca^{2+}–high Mg^{2+} solutions, known to block chemical synapses, have failed (Jacklet *et al.*, 1982) indicating that the interactions are electrotonic. There is indirect evidence of electrotonic coupling among *Aplysia* neurons (Jacklet *et al.*,

1982), including dye coupling between neurons in a cluster. Gap junctions are thought to mediate electrotonic coupling among neurons in other systems and in *Bulla* neuropil they are commonly seen between cell processes including presumed neuron–neuron contacts. No gap junctions were found between cell bodies of adjacent neurons, however, suggesting that electrotonic coupling should be expected between the processes of the neurons rather than directly between the cell bodies. The electrotonic coupling among neurons is expected to be effective, allowing electrical current flow among the neurons, because direct intracellular depolarization by current injection into a single neuron in the collar area is sufficient to drive the CAP activity (Jacklet & Colquhoun, 1982). The gap junctions appeared pentalaminar in the fixatives used here and conform to the structure of other gap junctions (for a review see Staehelin, 1974). Gap junctions also occur widely in the *Aplysia* retina, between photoreceptors (Strumwasser *et al.*, 1979) and between neurons (Luborsky-Moore & Jacklet, 1977; Strumwasser *et al.*, 1979).

Aggregates of clear vesicles (*ca* 650 Å) in the cytoplasm are a conspicuous feature of large *Bulla* photoreceptors. They are commonly found in large photoreceptors of other gastropods although the size and packing density in the cytoplasm varies among species. Vesicles are about 800 Å in diameter in *Helix* (Eakin & Brandenburger, 1975), 600–800 Å in *Hermissenda* (Eakin *et al.*, 1967) and 500–700 Å in *Aplysia* (Jacklet *et al.*, 1972). They have been studied in *Helix* and are believed to contain calcium (Eakin & Brandenburger, 1975, 1980) and vitamin A (Eakin & Brandenburger, 1968) and to be involved in phototransduction.

There are two distinct types of large photoreceptors in the *Bulla* retina but only one large type in other gastropods studied. One of these, the PRLD, is electron dense, being packed with 650 Å cytoplasmic vesicles, and it has microvilli 1000–1300 Å in diameter that form an elaborate dense brush-like distal segment. This photoreceptor is similar to the large type (called sense cell I or SCI), which may be specialized for night vision, found in the nocturnal arboreal slug, *Athoracophorus* (Eakin *et al.*, 1980). The other large photoreceptor in *Bulla* (PRLC) has a very conspicuous distal segment that expands into a clear cytoplasmic bulb about 10 μm in diameter from which microvilli (1400–3500 Å) radiate. It is tempting to speculate that the two large photoreceptor types are used for different light conditions because *Bulla* may be active during the day or at night. However, they are typically active during the night and bury themselves in the gravel of the aquarium during the day. Block (1982) has shown that *Bulla* locomotion is circadian and nocturnal in normal animals, but becomes diurnal and light driven in eyeless animals.

The PRS cells of the *Bulla* eye are similar to those found in other gastropod eyes. They are called sense cell type II (SCII) in *Helix* (Brandenburger, 1975) and *Athoracophorus* (Eakin *et al.*, 1980). Similar cells are seen in the *Aplysia* retina (Jacklet & Colquhoun, unpublished observation) and the retina of *Strombus* (Gillary & Gillary, 1979). In every case the cells are elongate and spindle-shaped, with one end of the spindle forming a long distal segment ending in a short tuft of microvilli and the other end of the spindle

forming a prominent axon extending from the small soma to the neuropil. Many glycogen granule aggregates and mitochondria appear in the cytoplasm of the axon hillock but no clear vesicle aggregates are seen like those in large photoreceptors. The PRS cells are possibly the same type as the *Aplysia* upper retinal cells noted (but not completely described) by Strumwasser *et al.* (1979).

Surprisingly, the reduced eyes studied by Block & Wallace (1982), devoid of the organized photoreceptive layer, still responded to light with increased CAP firing and the circadian rhythm could be phase-shifted by a light pulse. The neurons remaining in the eyes are apparently able to respond to light directly, although they were not observed in this study to have any specialized photoreceptive apparatus like the primary photoreceptors. However, many central neurons in gastropods respond to light (Arvanitaki & Chalazonitis, 1961) and they contain no specializations beyond cytoplasmic organelles containing pigment (Baur *et al.*, 1977). In the reduced eyes the small unitary activity normally recorded in the optic nerve along with the CAP activity was absent from the light responses suggesting that the photoreceptors produce the unitary activity in normal eyes and they have axons in the optic nerve. The appearance of stained axons in the optic nerve after injection of *Bulla* photoreceptors with Lucifer yellow (Jacklet & Colquhoun, 1982) supports the view that photoreceptors of *Bulla* have axons in the optic nerve just as they do in *Aplysia* (Jacklet & Rolerson, 1982).

Although there are many neuronal and photoreceptor processes containing vesicles of various sorts in the neuropil, chemical synapses with the classical features found in vertebrate tissue are not obvious. This seems to be the case with chemical synapses in marine gastropod nervous systems and eyes (Luborsky-Moore & Jacklet, 1977). In *Hermissenda* eyes where the individual photoreceptor cells were traced morphologically with horseradish peroxidase and where functional synapses were known to occur there was scant evidence of classical features (Crow *et al.*, 1979).

A wide range of dense-core vesicle sizes (480–2000 Å) was noted in the axons and cell bodies of (secondary) neurons of *Aplysia* (Luborsky-Moore & Jacklet, 1977). The average size was 1120 Å in the soma and 1060 Å in axons. A similar wide range was noted in *Bulla* neurons with a typical size of about 1000 Å. Processes in the neuropil containing dense-core vesicles often have smaller clear vesicles as well, a feature which characterized catecholaminergic neurons (Geffen & Jarrott, 1977). A larger type of (1500 Å) dense-core vesicle with granular contents was noted in the retinal neuropil of *Aplysia* (Luborsky-Moore & Jacklet, 1977), and seen infrequently in *Bulla*. It is similar in texture but smaller in size than the 1800 Å dense-core vesicles in the peptidergic bag cells of *Aplysia* (Haskins *et al.*, 1981). Some of the variation in the size and density of vesicles found in the neuropil may be explained by activity induced changes. Electrical stimulation of bag cell processes reduced the number of dense-core vesicles and increased the number of small clear vesicles in the end-feet release sites in the neural sheath, suggesting that the ratio of dense-core/clear vesicles changes with functional release of hormone (Haskins *et al.*, 1981), as has also been suggested for release of serotonin (Shkolnik & Schwartz, 1980).

The optic nerve of *Bulla* contains about 2000 fibres, enough for each receptor and neuron to have an axon in the optic nerve, since there are about 130 neurons, about 1000 large photoreceptors and an undetermined number of small photoreceptors. There are probably efferent fibres in the *Bulla* optic nerve also (Roberts & Block, 1982) just as there are in *Aplysia* (Luborsky-Moore & Jacklet, 1976), where they arise from neurons in the cerebral ganglion. Many, if not all, the photoreceptors and neurons are expected to have axons in the nerve because those photoreceptors (Jacklet & Colquhoun, 1982) and neurons (this study) that have been injected with dye have had axons in the optic nerve. Also, the *Bulla* eye is similar to the *Aplysia* eye, and dye injection studies have shown that each type of photoreceptor and neuron has an axon in the optic nerve (Jacklet & Rolerson, 1982; Jacklet *et al.*, 1982). The axons of neurons are among the largest (3 μm) in the neuropil so it is expected that many of the larger axons in the optic nerve may be from neurons, especially those that contain dense-core 1000 Å vesicles.

An unusual feature of the *Bulla* eye is the accessory nerve fibres found in the sheath of the optic nerve. They clearly branch from the main optic nerve and ramify in the sheath. The cells from which these fibres originate are presently unknown as well as the type of endings or contacts they might make in the sheath. It is also unknown whether the branches are restricted to the part of the optic nerve examined in this study or if they occur over the length of the optic nerve. A wider study utilizing selective staining of cells addressing these questions would surely be important for a clearer insight into the function of the eye and optic nerve. The release of substances at a neurohaemal organ perhaps similar to the bag cell release sites on the pleural–abdominal connective of *Aplysia* (Haskins *et al.*, 1981) is possible. Backfilling the *Aplysia* eye with Procion yellow dye (Jacklet, 1976) occasionally revealed fibres outside the optic nerve; perhaps they were similar to the accessory nerves seen in *Bulla* and these two eyes might have a similar organization of accessory fibres. Peptides are known to be released from the eye of *Aplysia* (Harf *et al.*, 1976) but the release sites are not known.

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