Organization of Retina of the Mudpuppy, *Necturus maculosus*. I. Synaptic Structure

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THE VERTEBRATE RETINA is a portion of the central nervous system that is particularly advantageous for study of structural and functional relationships. It is readily accessible, highly ordered, and easily stimulated. It consists of five types of neurons whose perikarya are arranged in discrete layers, and the retinal synapses are all confined to two plexiform layers. Recent studies by electron microscopy have added significantly to our understanding of the synaptic organization of the vertebrate retina (13–15, 30, 35).

Retinal neurons are small, however, and recording from single cells in the retina has been difficult. Only from the ganglion cells or their axons have single-unit recordings been routinely made (1, 2, 21, 23, 27–29, 36, 39, 40). The few intracellular recordings obtained from more distal neurons in the retina suggest that many of the cells generate slow, graded potentials (3, 8, 9, 25, 36, 37) which make extracellular recording difficult to interpret (6).

A few years ago, Bortoff (3, 4) showed that it was possible to record intracellularly throughout the retina of the mudpuppy, *Necturus maculosus*. The nuclei of the cells in *Necturus* are extraordinarily large, which considerably increases the size of the cell perikarya. The present paper and the one following present an anatomical and physiological analysis of the *Necturus* retina. The principles of synaptic organization of the *Necturus* retina are similar to those of other vertebrates. Accordingly, discussion in this paper will emphasize features of the *Necturus* retina that are special to it and are particularly relevant to its physiology. For instance, in the outer plexiform layer of the *Necturus* retina the synaptic relations between receptor, bipolar, and horizontal cells are clearer than in any other retina so far described, and special attention will be paid to organization in the outer plexiform layer. Synaptic organization in the inner plexiform layer is quite similar to that previously described for the frog (13), and therefore this layer will be discussed in much less detail.

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METHODS

Live specimens of *Necturus maculosus* were decapitated, and the eyes dissected carefully from the head. The front of the eye, including cornea, iris, and lens was removed and the remaining eyecup immersed in 1.5% osmium tetroxide buffered with 0.1 M veronal acetate containing 0.8% calcium chloride and 30 mg/ml sucrose. Initial fixation was at 4 C for 20 min followed by 1 hr fixation at room temperature. The tissue was dehydrated in graded ethanol-water mixtures and embedded in Araldite 6005 epoxy resin. Sections were cut on a Porter-Blum MT-2 microtome, doubly stained with uranyl acetate and lead citrate, and studied in an RCA EMU-3G electron microscope.

For Golgi staining, retinas were dissected from the eyecup and processed according to the Colonnier modification of the Golgi-Kopsch method (5). This method employs an initial fixation with a glutaraldehyde dichromate mixture. After fixation and impregnation with silver nitrate, the retinas were embedded in a soft plastic mixture consisting of 25 parts Araldite resin, 20 parts Epon epoxy resin, 60 parts dodecenylsuccinic anhydride and 1–3% DMP-30 (2, 4, 6 tri, dimethylaminophenol). The plastic mixture was hardened for 6 hr in an oven at 60 C and was cut at 20 μ with glass knives.

RESULTS

Light microscopy

Figure 1 shows a thick section of a *Necturus* retina fixed for electron microscopy with osmium tetroxide, Araldite embedded, and stained for light microscopy by the Richardson method (33). Rod and cone receptors are easily distinguished by the shapes of their outer segments. There are a few double cones in the *Necturus* retina (7, 20), but none are illustrated in this section. The outer nuclear layer is about one and one-half cells thick and contains mainly receptor cell nuclei. However, displaced horizontal cell and bipolar cell perikarya are occasionally found in the outer nuclear layer (see below and 31).

The outer plexiform layer is very irregular in thickness, ranging from 2 to 10 μ. The inner nuclear layer contains only two to three layers of cells. The more distal cells are bipolars and horizontals, while the more proximal cells are mostly amacrine cells. The inner plexiform layer is about 20–25 μ in thickness, and proximal to it

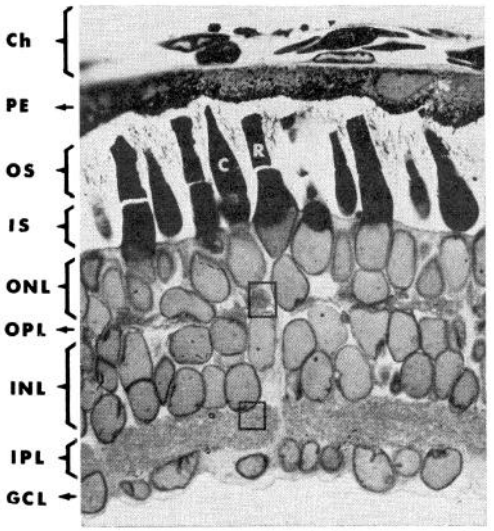


FIG. 1. Light micrograph of *Necturus* retina. Detailed description of the retina is given in the text. The two boxes enclose areas of the inner and outer plexiform layers that are roughly equivalent to the areas illustrated in the survey electron micrographs shown in Figs. 3 and 13. Ch, choroid; PE, pigment epithelium; OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; R, rod; C, cone. × 170.

are the perikarya of the ganglion cells. The cells throughout the *Necturus* retina are quite constant in size and measure 25–30 μ in diameter. (The seemingly smaller nuclei in the ganglion cell layer of this section are probably the cut edges of lobulated nuclei.)

The *Necturus* retina stains poorly with the Golgi method. Although the cell perikarya are large, the large size of the cells is caused by the very large nuclei, which do not usually stain with the Golgi method. The cytoplasm of the perikarya is often reduced to a thin, irregular sphere surrounding the nucleus (insert, Fig. 9). The processes of the cells are no larger than neural processes in other amphibian retinas, and many of them may be smaller. Thus, consistent and complete impregnation of any type of retinal cell is very rare in *Necturus*.

Figure 2 is a montage of photomicrographs of the best impregnated cells from 12 preparations. Figure 2*a* is a survey micrograph showing cells of the inner nuclear layer particularly well. At the top of the photomicrograph are stained receptor terminals, and fine processes can be seen extending from the terminals into the outer plexiform layer. One horizontal cell, two bipolars, and five amacrines are the other cells stained in this section. The remaining photomicrographs show examples of typical cells in the *Necturus* retina, or details of the cells.

Figure 2, *b* and *c*, illustrates examples of receptor terminals. The cone receptor terminals are usually situated directly subjacent to the nucleus of the cell (Fig. 2*b*), while rod terminals are often displaced laterally from the cell body (Fig. 2*c*). Fine processes extend from the terminals well into the outer plexiform layer. In Fig. 2*b* one such fine process ends in a knob (arrow) which is probably in contact with a cell perikaryon in the inner nuclear layer (see below).

Figure 2, *d* and *e*, shows typical bipolar and horizontal cells. The horizontal cell body is usually rounded with a flattened apical side. There are no processes on the horizontal cell that descend to the inner plexiform layer; but numerous processes extend from the apical side of the cell

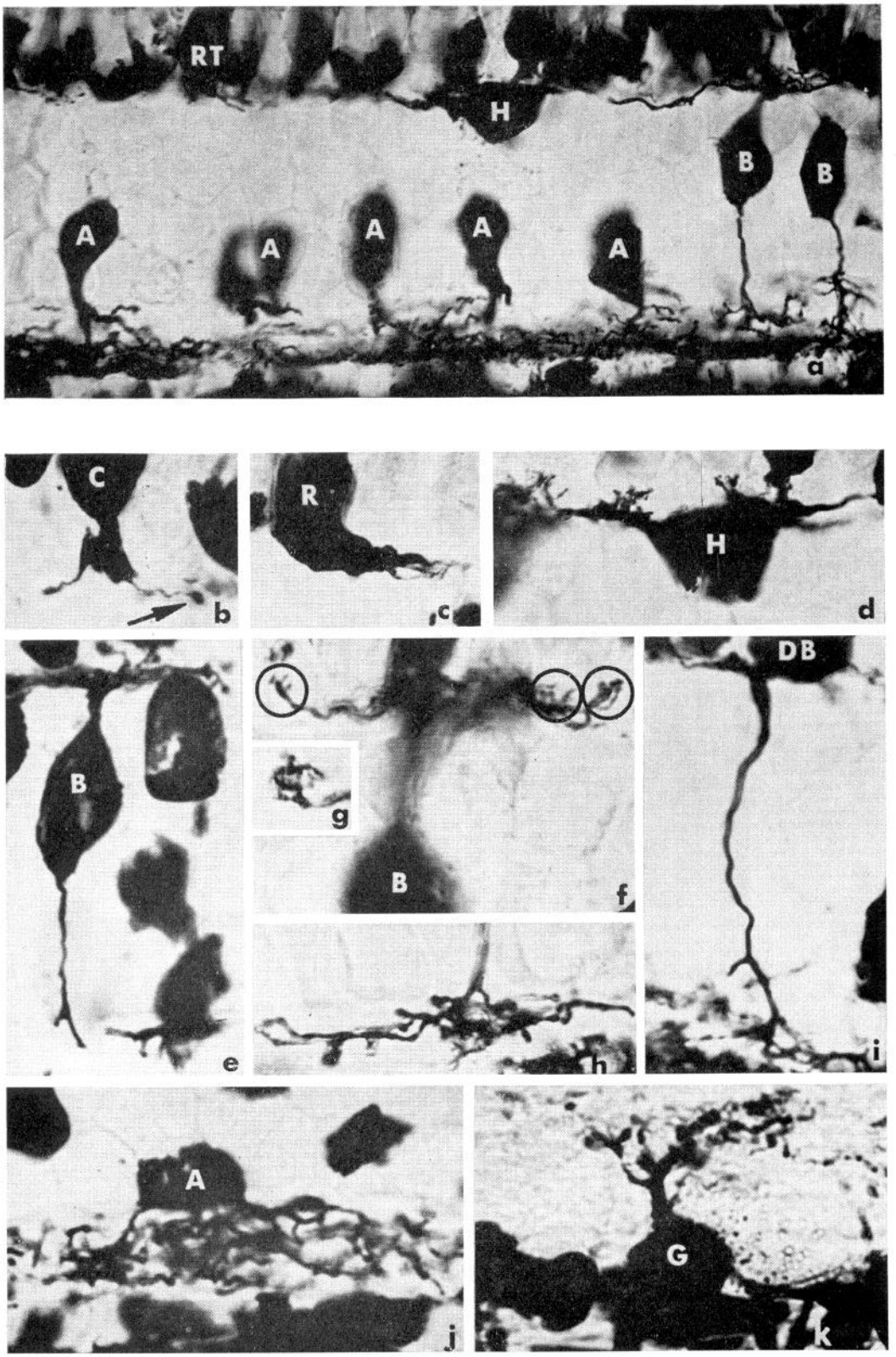


FIG. 2. Photomicrographs of cells in the *Necturus* retina stained by Golgi method. See text for detailed description of micrographs. RT, receptor terminal; H, horizontal cell; B, bipolar cell; A, amacrine cell; C, cone; R, rod; DB, displaced bipolar; G, ganglion cell.

perikaryon to run laterally in the outer plexiform layer. By contrast, the bipolar cell perikaryon is oval; the cell has a prominent, descending, axon-like process, and one large apical process that extends from the perikaryon to the outer plexiform layer where the process branches to run laterally. In addition, on many bipolar cells in *Necturus,* a Landolt club process extends from the large apical process and runs along the inner segments of the receptors to end at the external limiting membrane (10, 19). These criteria provide the most reliable means for distinguishing horizontal from bipolar cells by light microscopy.

Fine processes from both the horizontal and bipolar cells extend to the receptors, and often these processes are observed to end just under the receptor cell nuclei. The dendritic spreads of bipolar cells have lateral extents of 80–100 μ. Measurements of horizontal cell spread are less reliable, but on several occasions horizontal cell processes were followed in one direction 150–200 μ from the cell perikaryon, suggesting that the total lateral spread of the processes of the cells is in excess of 300–400 μ. All the processes of the horizontal cells appear morphologically similar; no axon-like processes have been observed on horizontal cells in *Necturus* (10).

Figure 2, *f* and *g*, shows details of apical processes of bipolar cells, and two types of terminations are distinguishable. In Fig. 2*f*, the processes branch into fine twigs (circles) as they terminate; whereas in Fig. 2*g* the process ends with complex convolutions rather than branching into fine twigs. Figure 2*h* illustrates a large well-stained bipolar axon terminal with numerous processes. Figure 2*i* shows a displaced bipolar cell in the outer nuclear layer. Its perikaryon, oval like that of other bipolar cells, lies horizontally in the outer nuclear layer just above the outer plexiform layer. Displaced bipolars, such as this one, have been observed in a variety of vertebrate retinas (10).

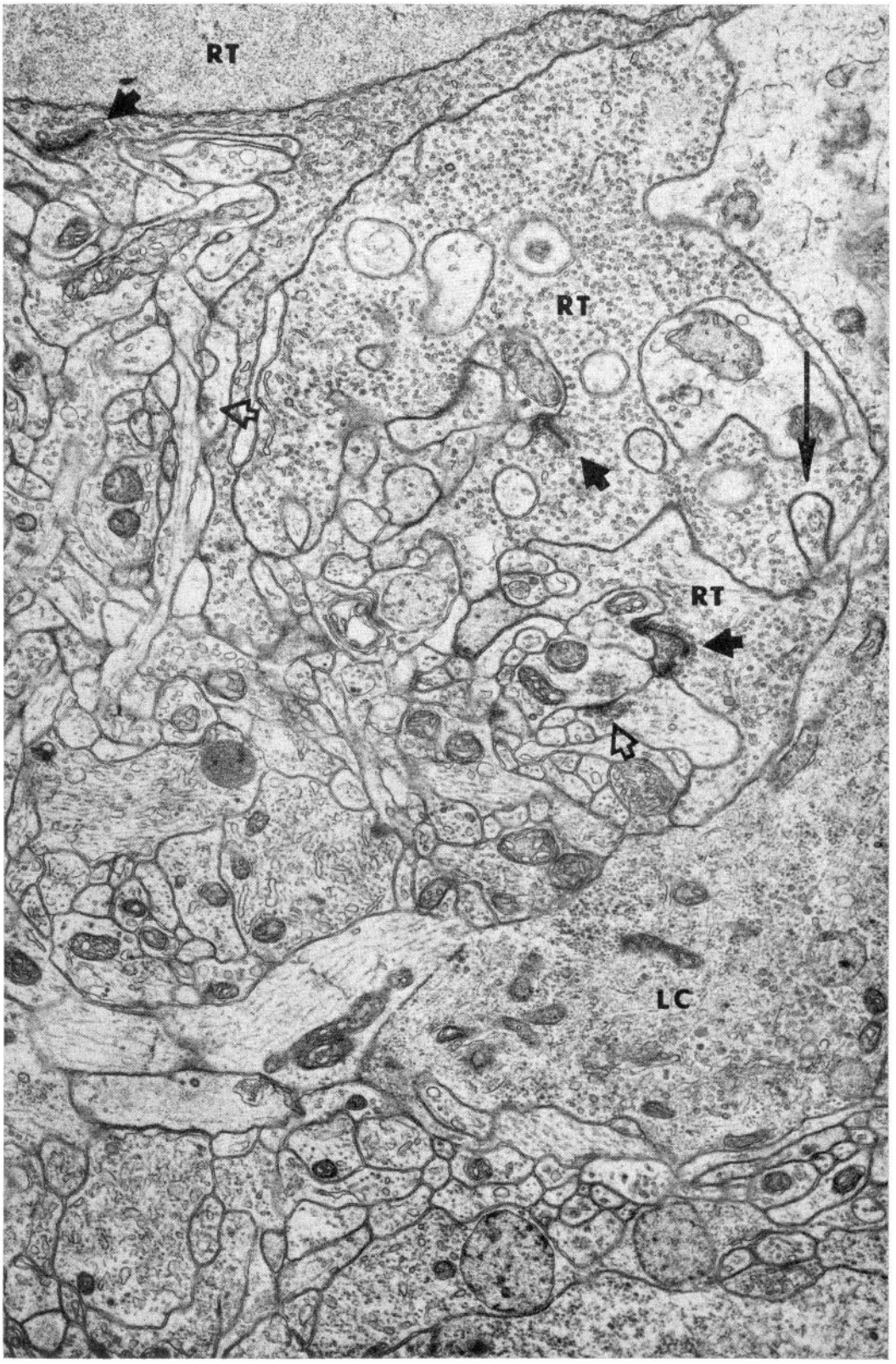
Figure 2*j* shows a large amacrine cell with numerous processes running laterally in the inner plexiform layer. Most amacrine cells have a single thick process which descends into the inner plexiform layer and then branches and spreads laterally for some distance (Fig. 2*a*). The cell in Fig. 2*j* has four smaller processes which extend from the cell perikaryon into the inner plexiform layer and run laterally. Figure 2*k* shows a ganglion cell whose dendrites are probably only partially stained. As here, most ganglion cells demonstrate a single thick process which extends from the perikaryon into the inner plexiform layer and then branches. No reliable measurements of the lateral extents of amacrine cell processes or ganglion cell dendrites have been obtained as yet from Golgi-stained material, but fragmentary observations suggest that processes from both cell types may have field diameters of up to several hundred microns.

Electron microscopy

OUTER PLEXIFORM LAYER. *Receptor terminals.* As noted above, the outer plexiform layer in *Necturus* is quite irregular in thickness. The receptor terminals are clustered in groups in the thicker portions of the outer plexiform layer (Fig. 1), and in such groupings the receptor terminals are layered two or three deep (Fig. 3). The groupings of synaptic terminals usually occur subjacent to receptor cells that can be identified as cones; such a grouping has yet to be observed subjacent to a clearly identified rod cell.

The cone terminals are positioned more distally in the groupings of receptor terminals in the outer plexiform layer as compared to the rod terminals, which tend to lie laterally and more proximally (Fig. 3). Adjacent terminals of both the rods and

FIG. 3. Low-power electron micrograph of outer plexiform layer. Portions of three receptor terminals (RT) are visible. The more distal terminals are probably cone terminals, while the small proximal terminal is probably a rod terminal. Note extensive areas of membrane apposition between terminals, and the finger of cytoplasm from one terminal that extends into the adjacent terminal (thin arrow). Probable synaptic contacts are marked by thick arrows. Filled arrows point to ribbon contacts, while the open arrows indicate conventional contacts. A large piece of a Landolt club process (LC) from a bipolar cell is seen at lower right of the micrograph. Landolt club processes extend through the outer plexiform layer, along the inner segments, and end at the external limiting membrane (19). × 13,000.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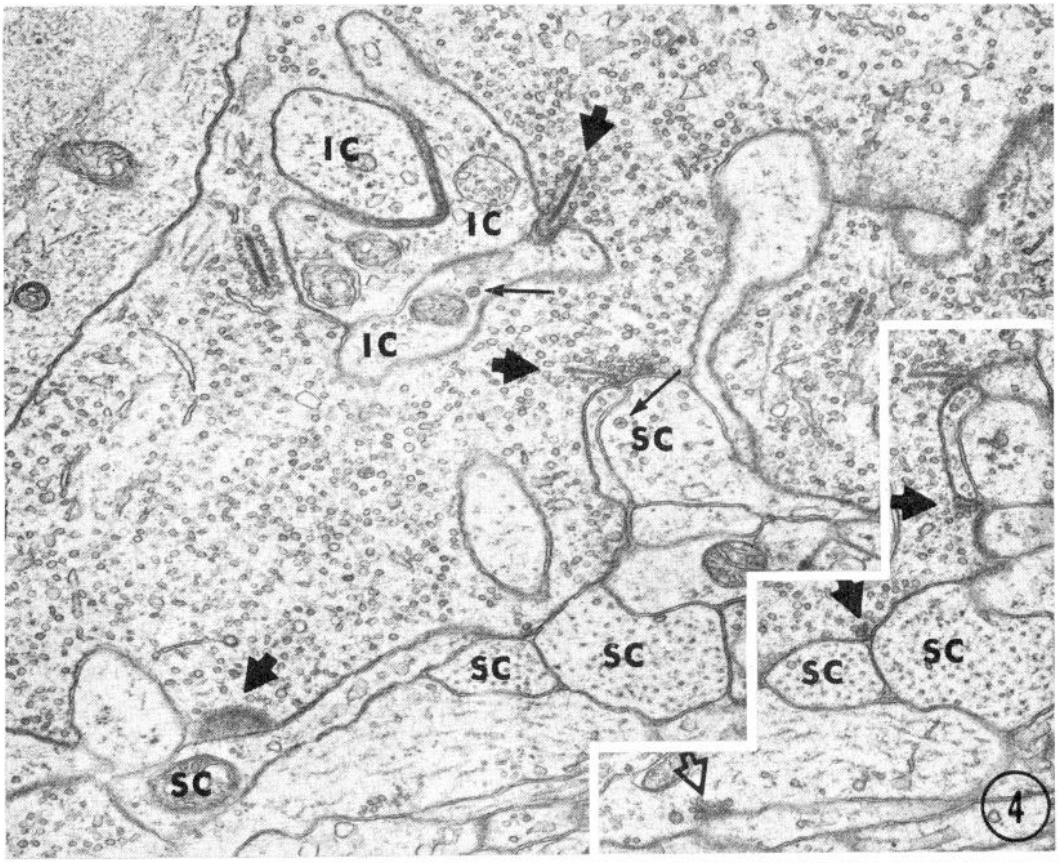
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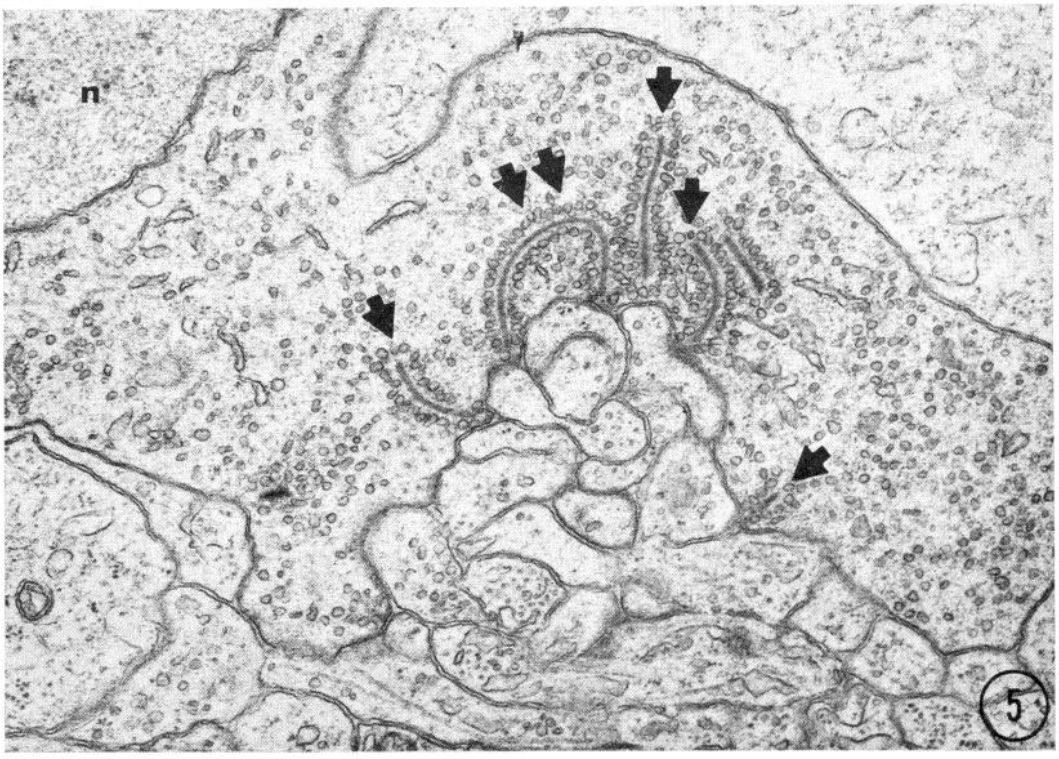
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cones show long extents (up to 6 μ) of apposed membrane without any intervening processes between the terminals; and occasionally a finger of cytoplasm from one receptor terminal extends into the cytoplasm of the adjacent receptor terminal (arrow, Fig. 3). No membrane or other specialization has ever been seen between the receptor terminals along these contact zones, although such specializations have been carefully looked for. Thus, there is no evidence for any type of specialized interreceptor contacts in *Necturus* (11, 12, 14, 34).

Cone terminals can often be positively identified by tracing along the cell to the outer segment. The terminal portion of the cone cell is usually just below or adjacent to the nucleus, making such tracing easy. Neural processes contact these cone terminals in two ways. They either penetrate deep into the receptor terminal or make superficial contacts on the basal portion of the terminal. Synaptic ribbons are associated with both types of contact (see below and Fig. 4).

The rod terminals are more difficult to identify positively, because they are usually displaced laterally from the rest of the cell body and the outer segment (Fig. 2*c*). Occasionally, one can trace a rod receptor terminal to the cell body or find the terminal near the cell body. Figure 5 shows an example. In such rod terminals no invaginated processes have been observed; all the contacts with these terminals are superficial. Synaptic ribbons are associated with these contacts, and the ribbons in such terminals are often quite long and frequently are arcuate. Thus, one ribbon may be associated with more than one contact point along the terminal (Fig. 5).

Invaginated contacts. Rather large processes are often observed invaginated deeply into the cone receptor terminals. Some of these processes contain a few synaptic vesicles (including an occasional granulated vesicle) and are identified as horizontal cell processes (Fig. 4, and see below). Others contain a variety of cytoplasmic organelles and are identified as bipolar dendrites. In many cases, however, the invaginating processes show so little cytoplasmic structure that identification of the processes cannot be made (Fig. 7*a*, for instance). The invaginating processes are often opposed to synaptic ribbons that are located in the adjacent receptor cytoplasm. In single sections, only two processes appear to be associated with a synaptic ribbon. With serial sections, however, it is possible to show that more than two processes may be related to one synaptic ribbon. Figure 6 is a partial reconstruction by serial section of the invaginated processes related to one synaptic ribbon. On the right are drawings of two single sections, which show in each case only two processes in relation to the ribbon. On the left is a drawing based on 12 sections, which shows that at least three processes are associated with the ribbon.

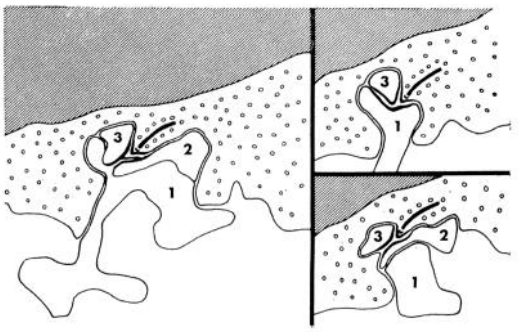
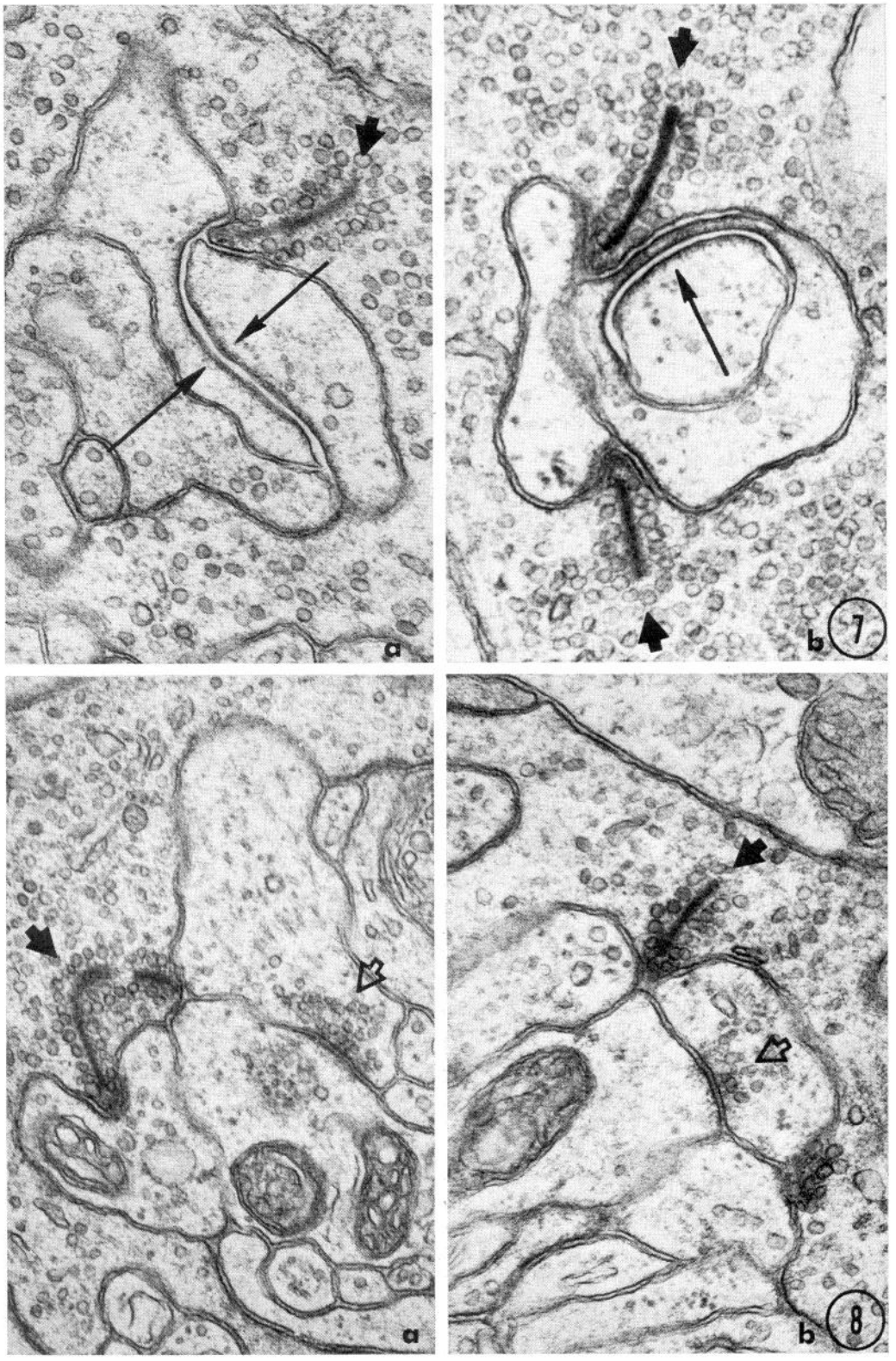


FIG. 6. Drawing of the relations of three invaginating processes with one synaptic ribbon, based on a partial serial section reconstruction. In single sections (right), only two processes appeared associated with the ribbon. Serial section reconstruction (left) shows that at least three processes are associated with the ribbon.

FIG. 4. Portion of a cone receptor terminal showing both invaginated (IC) and superficial contacts (SC). Synaptic ribbons (filled arrows) are related to both types of contact. The insert is from an adjacent section, showing additional synaptic ribbons (in the terminal) that are associated with processes making superficial contacts. Also in the insert, a conventional synaptic contact (open arrow) is suggested in a nearby, neurotubule-filled process. Thin arrows point to granulated synaptic vesicles in presumed horizontal cell processes. Note that a third process is adjacent to the unmarked ribbon in the insert. Only two processes appeared related to this ribbon in the other section. × 22,000.

FIG. 5. Portion of a rod receptor terminal. All contacts with the terminal are superficial contacts. Synaptic ribbons (filled arrows) are long, and some are arcuate. One arcuate ribbon (double filled arrow) appears associated with two contact points. n, nucleus of rod cell. × 27,000.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Since this was not a complete reconstruction, it is possible that even more processes are related to this synaptic ribbon.

Such serial reconstructions show that single processes that are invaginated into the receptor terminals have a twisting and turning configuration in the terminals, and come into relation to several ribbons. This finding is strengthened by Golgi-stained material which shows that small processes in the outer plexiform layer often have a twisting form as they terminate (Fig. 2*g*). In several retinas a precise anatomical arrangement of processes in the invaginations has been described (14, 30, 34, 35). Horizontal cell processes terminate more distally and laterally in the invaginations, whereas the bipolar dendrites end more proximally and centrally. In *Necturus* it has not been possible as yet to decide whether such a precise spatial arrangement of horizontal and bipolar cell processes exists in the invaginations.

Near the location where the invaginating processes are adjacent to the synaptic ribbon, some synaptic specialization is usually seen (Fig. 7*a*). In these areas the plasma membranes of both the receptor terminal and the invaginating processes often appear denser, and fine filaments extend from the plasma membrane into the cytoplasm of the invaginating process along the contact zone. This filamentous specialization, the so-called subsynaptic web, is similar to that observed along the postsynaptic membrane at a number of synapses in the nervous system (18, 38). At the invaginated ribbon synapses of the receptors, the synaptic cleft is widened to about 300–400 A.

A further consistent observation is that the membrane specializations described above are not always confined to the region immediately adjacent to the synaptic ribbon, but rather that a considerable zone of membrane specialization (up to 1 μ in length) is often observed between the invaginating processes themselves (arrow, Fig. 7, *a* and *b*). In such cases the intercellular space is widened along the entire length of the contact zone; the apposed membranes appear denser, and subsynaptic webs are seen in the cytoplasm of the processes all along the contact. In Fig. 7*b* it is especially convincing that two of the invaginating processes have a specialized contact zone with each other that is quite similar to the specialization between the invaginating processes and receptor terminal. The zones of specialized contact between invaginating processes suggest that interactions may occur between the invaginating processes. No conventional type of synaptic contact has been observed between invaginating processes in the receptor terminals; therefore, if interaction between invaginating processes does occur, these zones are likely sites for such interaction. This will be discussed further below.

Superficial contacts. On the basal portion of both the rod and cone receptor terminals, numerous groups of processes make superficial contacts on the terminals. These processes do not penetrate into the terminal to any great extent, but usually just dent the surface of the terminal (Figs. 4 and 5). Synaptic ribbons are often observed in the receptor terminal cytoplasm, pointing between two processes that contact the terminal. In single sections only two processes appear to be associated with one ribbon, but fortuitous single sections or serial sections show that three or more processes

FIG. 7. *a:* Membrane specializations observed at the invaginated synaptic contacts of the receptor terminals. Specializations, consisting of membrane densification, subsynaptic webs along the plasma membrane of the invaginating processes, and a widened synaptic cleft, are not confined to the area just adjacent to the ribbon (filled arrows), but extended areas of specialization are observed between the invaginating processes themselves (thin arrows). *b:* One invaginating process appears enclosed in a second invaginating process. This is probably due to the angle of section through the two processes as they pass by one another. Micrograph shows clearly that specialization between the invaginating processes is quite similar to the specialization observed between the receptor terminal and the invaginating process. × 61,200.

FIG. 8. Typical arrangements of processes at the superficial contacts of the receptor terminals. Two or three processes are associated with a ribbon at one contact point (filled arrows), and often one of these processes makes a conventional synaptic contact on an adjacent process within 1–2 μ of the ribbon contact of the receptor (open arrows). Neurotubules are often seen in the processes making the conventional contacts (*a*), suggesting that these are horizontal cell processes. × 41,000.

may relate to one ribbon (Fig. 4). At these superficial contact points some membrane specialization between terminal and contacting process is seen, but the specialization is less clear than that observed at the invaginated contacts.

A striking and important finding in relation to the superficial contacts is the frequent observation of a classic conventional synapse between two processes just adjacent to their synaptic contact with the receptor terminal (Fig. 8). These more conventional synaptic contacts are characterized by a cluster of synaptic vesicles close to the presumed presynaptic membrane, and specialization on both pre- and postsynaptic membranes similar to that described at other synapses in the nervous system. Fine filaments are observed bridging the synaptic cleft and in the cytoplasm of the postsynaptic process, close to the presumed postsynaptic membrane. The presynaptic process often shows numerous neurotubules in addition to synaptic vesicles and is, therefore, identified as a horizontal cell process. The postsynaptic process usually contains no neurotubules or synaptic vesicles and is identified as a bipolar dendrite. Evidence for these identifications is presented below.

Bipolar and horizontal cell processes and synapses. Horizontal and bipolar cells can often be recognized along the margin of the inner nuclear layer, and the processes of these cells can be followed for some distance in the outer plexiform layer. These two cell types can be differentiated by the shape of the cell perikaryon (Fig. 2), by the presence of a descending axon process on bipolar cells, and by the morphology of processes. Large bipolar cell processes contain numerous cytoplasmic organelles similar to those seen in the cell body, and these processes are therefore easily identified. Fewer cytoplasmic organelles are observed in the smaller bipolar dendrites, and identification is less positive. In some cases the processes may appear quite empty.

On the other hand, horizontal cell processes (regardless of size) do not retain the characteristics of horizontal cell cytoplasm; rather, shortly after emerging from the soma, the processes lack most organelles and show only numerous neurotubules (Fig. 9). In such horizontal cell processes, scattered clusters of synaptic vesicles are seen, and often such processes are observed making conventional synaptic contacts on adjacent processes (Figs. 3, 4, 8, and 11). Occasionally, horizontal cell processes can be followed to the receptor terminals, where they make superficial contacts with the terminal (Fig. 10). Just adjacent to such contacts the horizontal cell process may make a synaptic contact on an adjacent process. The neurotubules do not appear to extend to the very tip of the horizontal cell processes (Fig. 10). This may explain why some processes making conventional synaptic contacts in the outer plexiform layer do not show neurotubules (Figs. 8*b* and 11*a*), and perhaps why processes deep in the invaginations do not often show neurotubules, although such processes are presumed to be horizontal cell processes because they contain synaptic vesicles.

Conventional synapses between horizontal cell processes and other processes are observed scattered throughout the outer plexiform layer (Fig. 11). Most of these contacts are on processes that contain numerous cytoplasmic organelles and are probably bipolar cell dendrites. In 12 randomly obtained micrographs from the outer plexiform layer showing conventional synapses on large processes, 11 of the postsynaptic processes were identified by their organelles as bipolar cell dendrites, and the other one as a horizontal cell process because it contained only neurotubules. In Fig. 11*b* a horizontal cell process makes synaptic contact with the perikaryon of a bipolar cell, identified as such because a descending axon-like process was observed to extend from the cell.

In summary, horizontal cell processes in the outer plexiform layer of *Necturus* are most easily identified by the numerous neurotubules they contain. Such horizontal cell processes make conventional synaptic contacts on adjacent processes, most of which are probably bipolar dendrites.

Additional observations. Golgi-stained retinas show that fine processes extend from the receptor terminals through the outer plexiform layer to the inner nuclear layer.

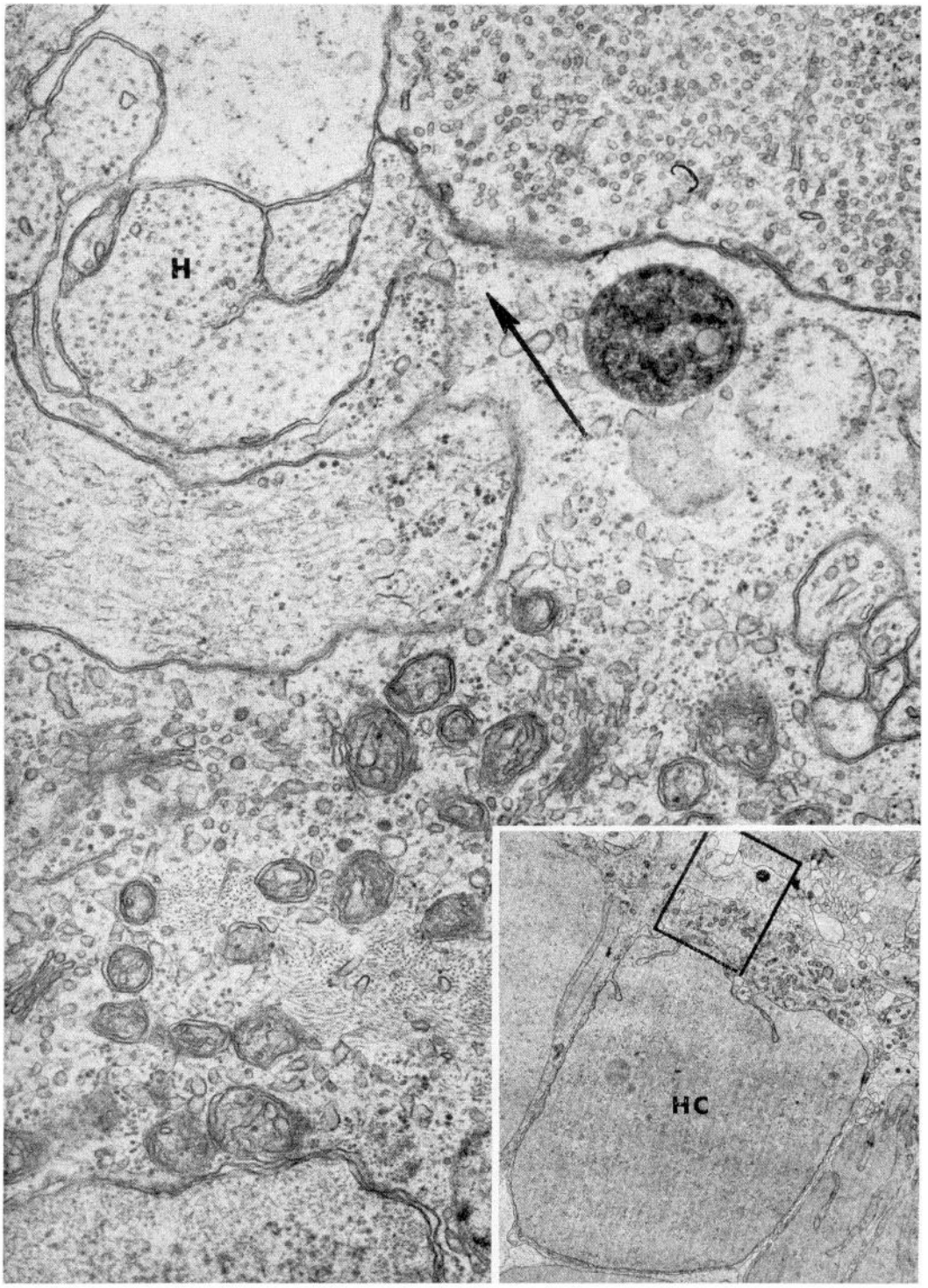
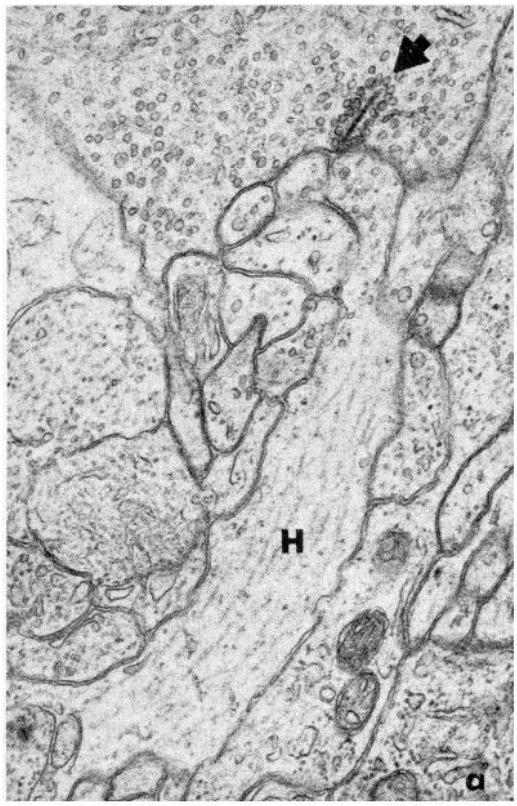


FIG. 9. Horizontal cell process (H) in continuity with the cell soma (thin arrow). The process contains few of the cytoplasmic organelles seen in the soma cytoplasm but shows, instead, numerous neurotubules. Insert shows a low-power micrograph of the whole horizontal cell (HC). Note its flattened apical margin. The box encloses the area illustrated at higher magnification. × 31,000; insert × 3,000.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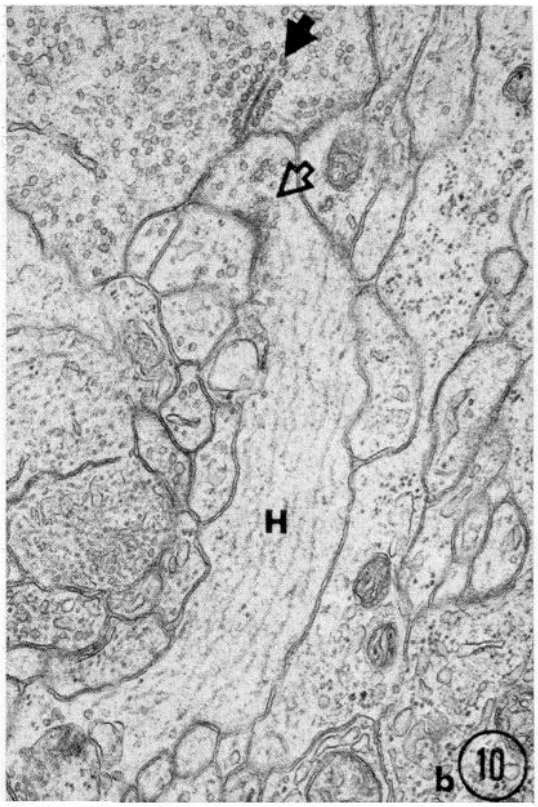
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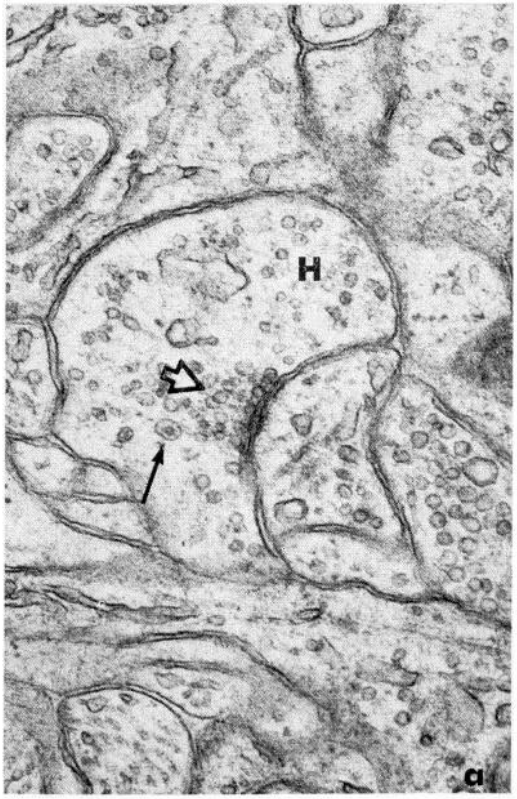
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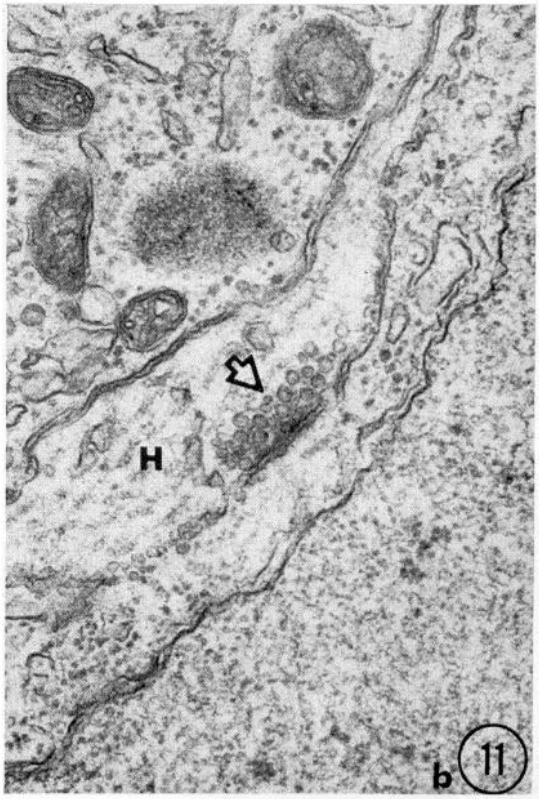
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On occasion, processes containing numerous synaptic vesicles and synaptic ribbons are observed in contact with the perikarya of both bipolar and horizontal cells (Fig. 12). The ribbons appear associated with such contact points, suggesting that axosomatic contacts between receptors and bipolar and horizontal cells are made in the *Necturus* retina. Some membrane specialization, especially on the receptor membrane, is seen at these contacts; usually the ribbon is positioned so that it points between the cell soma and one or two other processes (Fig. 12, *a, b,* and *c*). This is the first reported observation of receptor somatic contacts on second-order neurons in the visual system, and its significance is unclear. However, in Golgi-impregnated material, fine processes that extend from the receptor terminals into the outer plexiform layer have been observed in a variety of species (10, 13). Thus, it is possible that such contacts are present in other retinas.

INNER PLEXIFORM LAYER. The synaptic contacts observed in the inner plexiform layer of *Necturus* are morphologically similar to the synaptic contacts observed in the inner plexiform layer of other vertebrate retinas. As in other vertebrates, two types of synapses can be distinguished: ribbon synapses and conventional synapses (Fig. 13) (13, 14, 22, 30). Ribbon synapses are characterized by a synaptic ribbon in the presynaptic process, and the conventional synapses are characterized by a dense cluster of synaptic vesicles in the presynaptic process. Both types of contact show some synaptic membrane specializations (see below).

There are many more conventional contacts than ribbon contacts in the inner plexiform layer of *Necturus* (Fig. 13), which is the finding in most vertebrate retinas (13, 32, and see below). The ribbon synapses in the inner plexiform layer have been shown in several species to belong to the bipolar terminals (14, 17, 32) and this is believed to be the case for *Necturus* also. Conventional synapses, on the other hand, have been identified in amacrine cell processes in all vertebrate retinas so far studied (13, 14); in *Necturus* also, we have observed known amacrine processes to make conventional synaptic contacts. Thus it will be assumed that here in the inner plexiform layer of *Necturus,* as elsewhere, the conventional synapses are those of the amacrine processes, whereas ribbon contacts are those of the bipolar terminals.

In a number of processes in the inner plexiform layer of *Necturus,* believed to be amacrine processes, large granular synaptic vesicles are observed (Fig. 13). The majority of these processes are found in the more distal portion of the inner plexiform layer, just under the inner nuclear layer. Occasionally a process containing granular vesicles is observed to make a synaptic contact of the conventional (amacrine) type, but the granular vesicles do not appear associated with these contacts (Fig. 13). Evidence has been presented that granular vesicles probably contain catecholamines (33, 41); and the present observations that amacrine processes occasionally contain granular vesicles agree with recent light-microscopic studies showing that a small percentage of the amacrine cells in the vertebrate retina show catecholamine fluorescence (24, 26). Granular vesicles are never observed in ribbon-containing processes, and no catecholamine fluorescence has been observed in bipolar cells. These observations add further evidence that processes making conventional contacts are amacrine cell processes, whereas those making ribbon contacts are bipolar terminals.

Ribbon contacts. As in all other vertebrate retinas so far studied, the ribbons in the inner plexiform layer of *Necturus* are oriented in the presynaptic cytoplasm of the bipolar terminal so that they point between two adjacent postsynaptic processes. Synaptic specializations consisting of

FIG. 10. *a:* Neurotubule-filled (presumably horizontal cell) process making a superficial contact on a receptor terminal. × 22,000. *b:* In a nearby section, it is seen that the neurotubule-filled process makes a conventional synaptic contact on an adjacent process. × 22,000.

FIG. 11. *a:* Presumed horizontal cell process making a conventional contact (open arrow) deep in the outer plexiform layer. The thin arrow points to a large granular synaptic vesicle in the process. × 45,000. *b:* A neurotubule-filled process making a conventional contact onto the perikaryon of a bipolar cell, identified as such by its descending axon process. × 48,000.

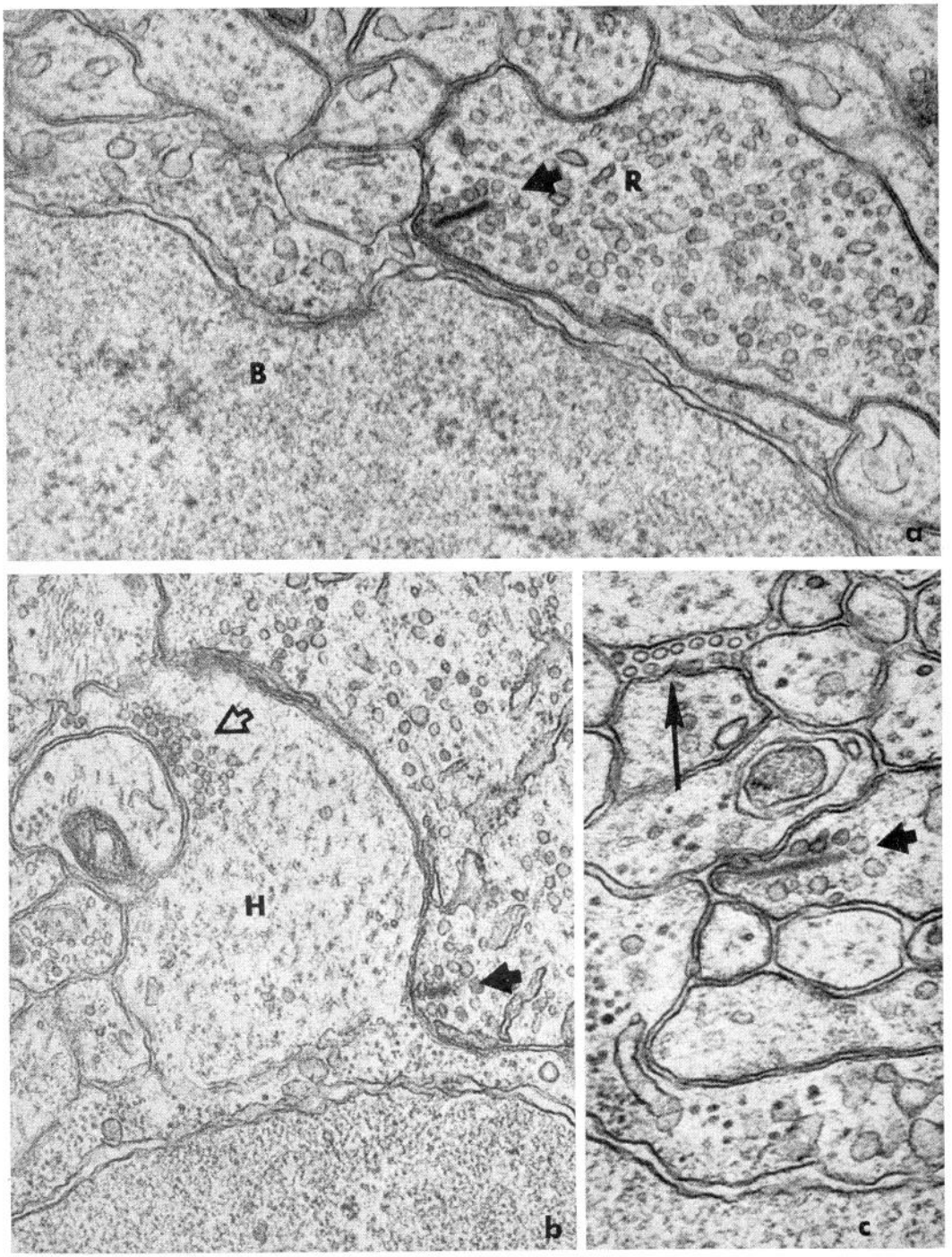


FIG. 12. *a:* Ribbon-containing process, probably from a receptor terminal, making an axosomatic contact (filled arrow) onto a bipolar cell perikaryon (B). × 33,000. *b:* A ribbon-containing process making an axosomatic contact (filled arrow) onto a horizontal process (H) as the process comes off the cell perikaryon. The horizontal cell process itself makes conventional contact onto a third process (open arrow). × 27,000. *c:* Another example of an axosomatic contact by a ribbon-containing process. Extracellular tubules (thin arrow) are occasionally observed in the outer plexiform layer of *Necturus*. These tubules appear to have no contact or relation with adjacent processes. × 70,000.

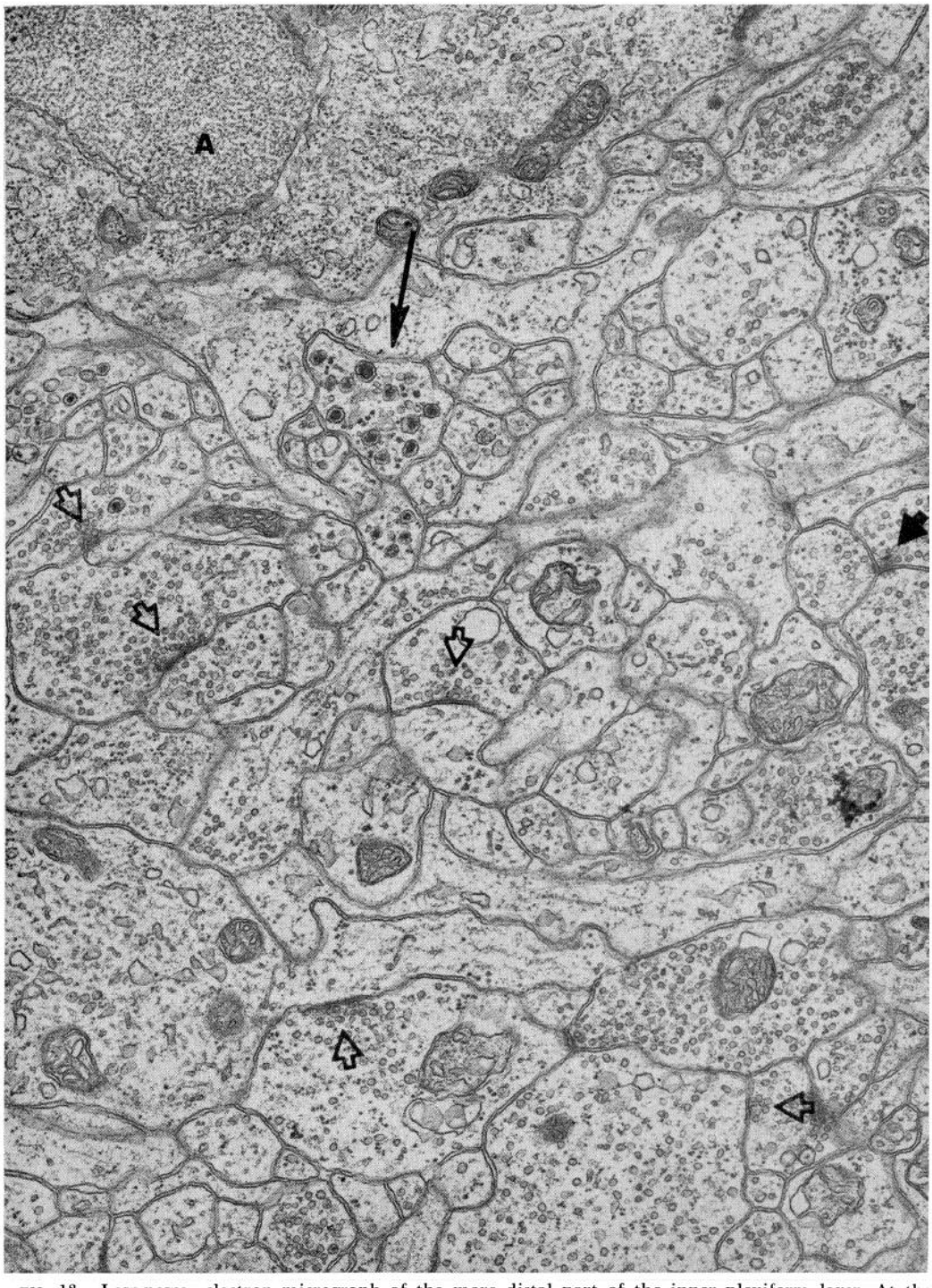
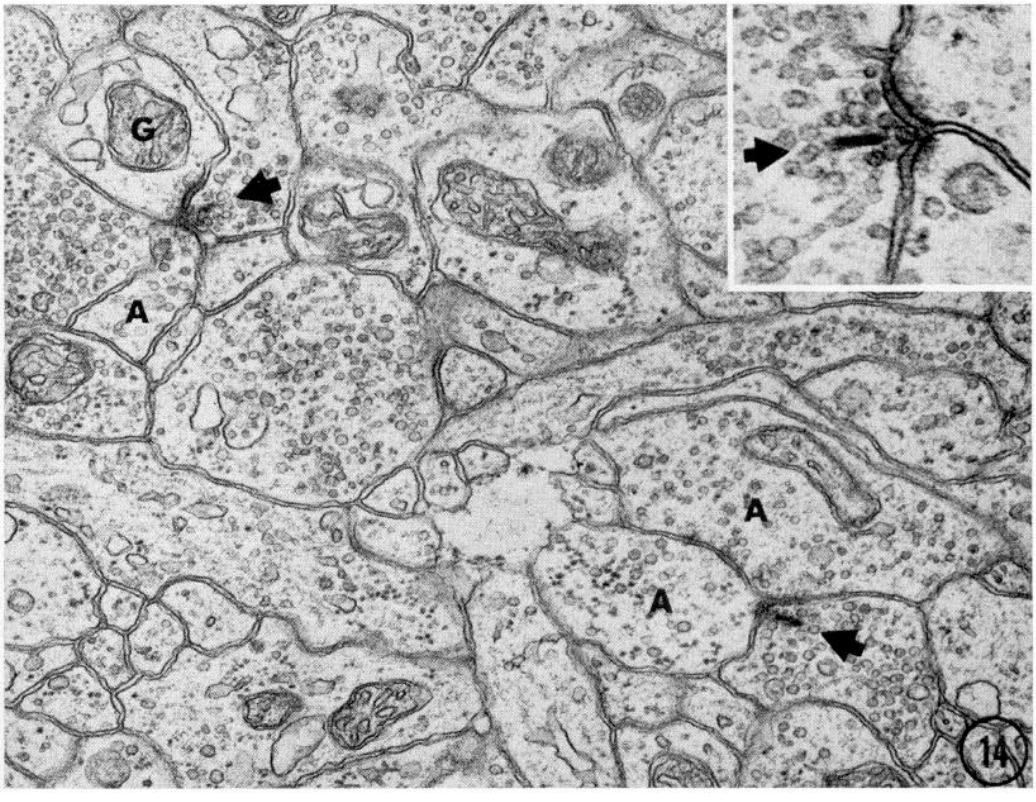
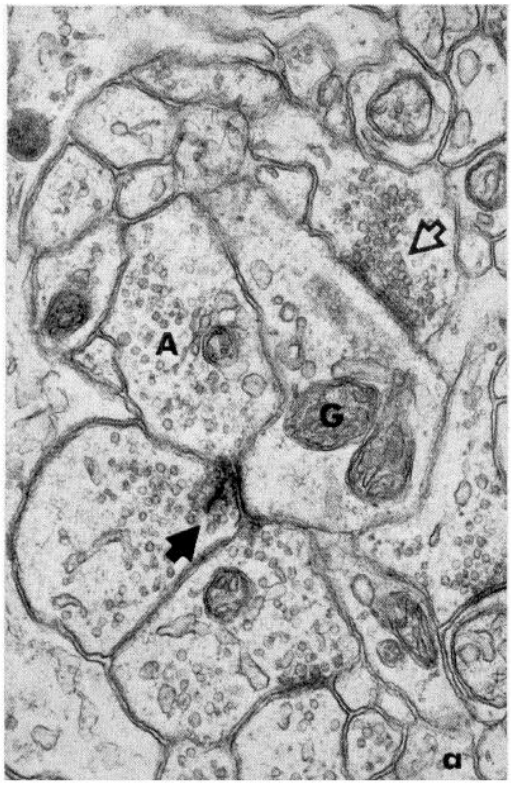


FIG. 13. Low-power electron micrograph of the more distal part of the inner plexiform layer. At the top of the micrograph is a portion of an amacrine cell perikaryon. Probable synaptic contacts are marked with thick arrows. Open arrows indicate conventional contacts; filled arrow, a ribbon contact. Several processes contain numerous large granular synaptic vesicles (thin arrow). × 20,000.


G
A
A
A
14


A
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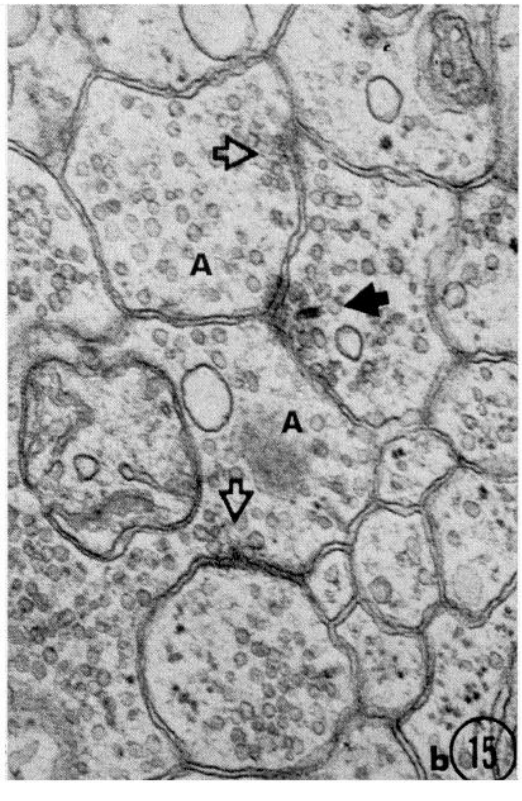

A
A
b
15

membrane densification on both sides of the contact, subsynaptic webs on the membranes of both postsynaptic processes, a widened synaptic cleft, and filaments in the synaptic cleft are seen at these ribbon contacts (Fig. 14, insert). Such specializations suggest that at ribbon synapses two postsynaptic processes are simultaneously contacted.

At the ribbon contacts of the bipolars in *Necturus*, the postsynaptic processes may be morphologically similar or they may be different in appearance (Fig. 14). If they are morphologically similar, both processes usually contain synaptic vesicles; if the processes are morphologically different, only one process usually contains synaptic vesicles, and the other contains none. In the frog retina a similar arrangement of postsynaptic processes was observed at ribbon contacts; and it was argued that when both postsynaptic processes contained synaptic vesicles, they were most likely to be both amacrine processes. On the other hand, when the two postsynaptic processes were different and one of the postsynaptic processes contained no synaptic vesicles, this suggested that one postsynaptic process was a ganglion cell dendrite and the other an amacrine process (13).

The evidence at hand favors the foregoing interpretations for the *Necturus* retina also. For example, in some instances in which both postsynaptic processes contain synaptic vesicles, the two processes themselves are observed to make synaptic contacts of the conventional (amacrine) type (Fig. 15*b*). In the other instances, in which one postsynaptic process contains no synaptic vesicles, this process closely resembles the ganglion cell dendrites, which in *Necturus* often appear less electron-dense than either amacrine processes or bipolar terminals (Figs. 14 and 15*a*).

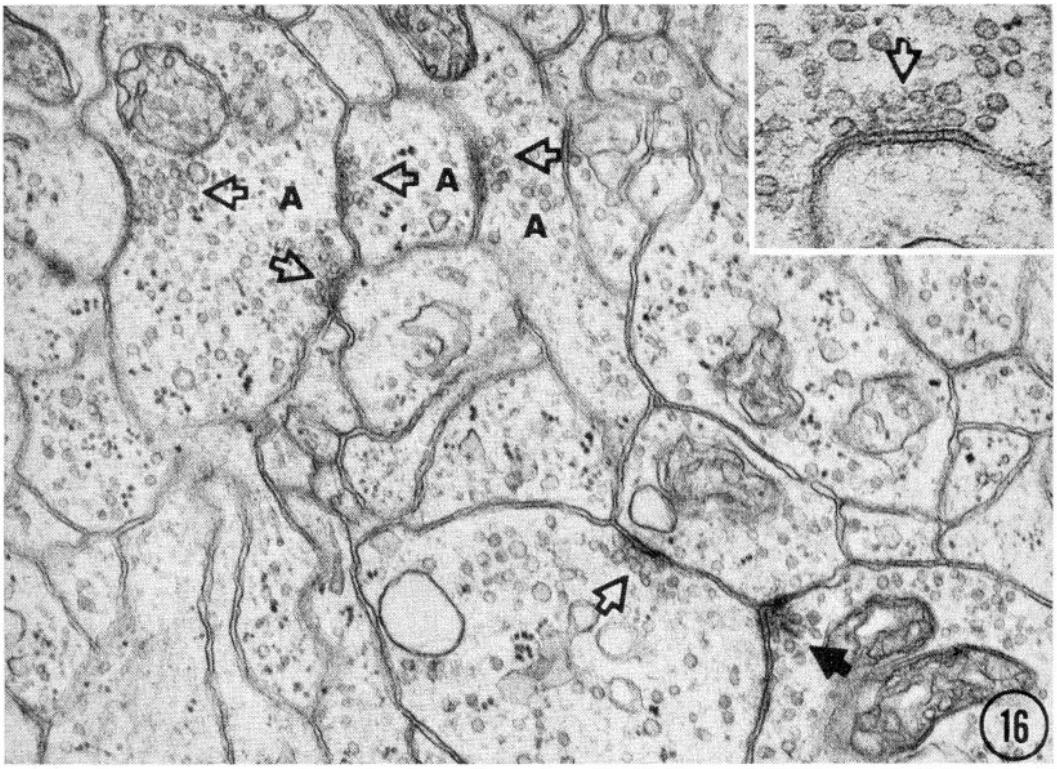
A survey of 54 ribbon contacts in *Necturus* showed that in 29 cases both postsynaptic elements contained synaptic vesicles, whereas in the other 25 instances one postsynaptic process contained synaptic vesicles and the other contained no vesicles. This suggests that at over half of the ribbon contacts in *Necturus* both postsynaptic processes are amacrine processes; at the other ribbon contacts, one postsynaptic process is a ganglion cell dendrite and the other an amacrine process.

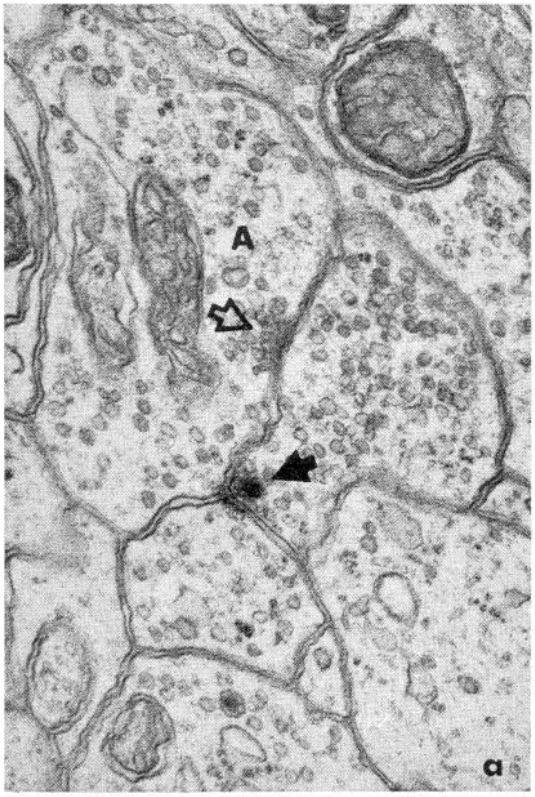
Conventional contacts. The conventional contacts in the inner plexiform layer are similar morphologically to most synapses described throughout the nervous system (18). A dense cluster of synaptic vesicles is positioned close to the presumed presynaptic membrane, and some membrane densification is observed on both the pre- and postsynaptic membranes. However, the synaptic cleft is only slightly widened at these conventional synapses in the inner plexiform layer, and a subsynaptic web is not well developed along the postsynaptic membrane at most of these contacts.

Conventional contacts in the inner plexiform layer in *Necturus* are observed on ribbon-containing (bipolar) terminals (Fig. 17*a*), ganglion cell dendrites (Fig. 17*b*), and other amacrine processes (Figs. 16 and 17*b*). Serial synaptic contacts between two or more presumed amacrine processes are occasionally seen (Figs. 16 and 17*b*), and the last process in the sequence may be a ganglion cell dendrite (Fig. 17*b*), or a bipolar terminal, or another amacrine cell process (Fig. 16). Reciprocal synapses between ribbon-containing processes and processes making conventional contacts are also observed

FIG. 14. Ribbon contacts in the inner plexiform layer. The ribbon in the presynaptic cytoplasm points between two postsynaptic processes. Both postsynaptic processes may contain synaptic vesicles, in which case both elements are probably amacrine processes (A); or only one postsynaptic process shows synaptic vesicles, in which case one postsynaptic process is probably an amacrine process (A) and the other is a ganglion cell dendrite (G). Insert shows a ribbon synapse at high magnification. × 25,000; insert × 61,000.

FIG. 15. *a:* Ribbon contact in the inner plexiform layer (filled arrow). One postsynaptic process contains synaptic vesicles and is probably an amacrine cell process (A). The other process contains no synaptic vesicles and is most likely a ganglion cell dendrite (G). The ganglion cell dendrite is also contacted by a process making a conventional synapse on it (open arrow). × 22,000. *b:* Ribbon contact on two synaptic vesicle-containing processes. Both processes appear to be making synaptic contacts of the conventional type (open arrow), indicating that both are amacrine processes (A). × 37,000.


A
A
A
16


A
a

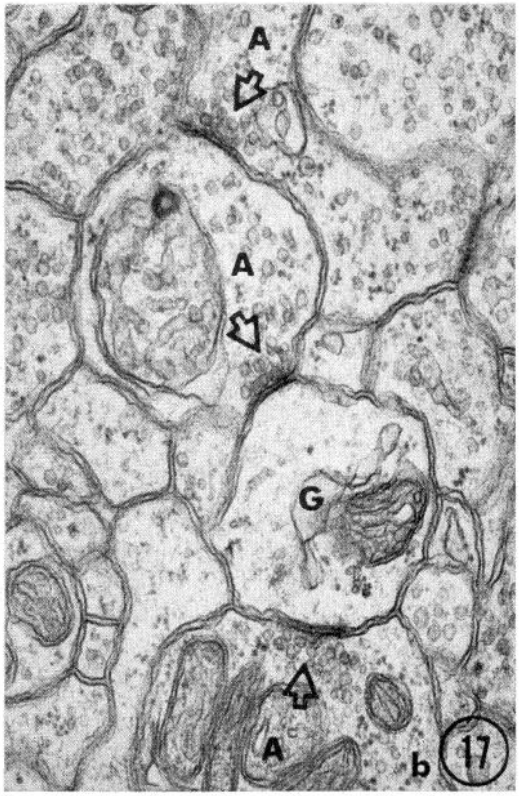

A
A
G
A
b
17

TABLE 1. *Incidence of synaptic contacts in inner plexiform layer of Necturus*

Type of Contact	No.		Percent		Incidence Per Unit Area, μ^2	
	a	b	a	b	a	b
Ribbon synapse	14	13	15	11	0.017	0.012
Conventional synapse	76	99	82	83	0.090	0.092
Conventional synapses in series	3	8	3	6	0.003	0.009

Montage a: 21 μ × 40 μ = 840 μ^2; montage b: 22 μ × 49 μ = 1,082 μ^2.

(Fig. 17*a*). All the above synaptic arrangements have been observed and described in some detail for the frog retina (13).

In a previous paper (13) an argument was presented that the relative numbers of types of synaptic contacts, and the absolute numbers of synaptic contacts per unit area, could be related to complexity of ganglion cell physiology in several vertebrates. It is of interest to compare relative and absolute numbers of types of synaptic contacts in *Necturus* with the retinas of some of these other species. Table 1 presents data from two montages of the inner plexiform layer prepared from different specimens of *Necturus* retina. The data are broken down into relative and absolute numbers of ribbon synapses, conventional synapses, and conventional synapses in series (serial synapses) per unit area. The proportion of conventional synapses versus ribbon synapses is approximately 7:1. This ratio is lower than in the frog retina, which has a 10:1 ratio of conventional synapses versus ribbon synapses. However, the ratio in *Necturus* is higher than that found in the primate retina, which has a ratio of about 2 conventional synapses to each ribbon contact. The incidence of serial synapses (5%) in *Necturus* is also intermediate between that of the frog and primates. In the frog, about 10% of the conventional synapses are arranged in series, whereas in primates only about 1% of the conventional synapses are in series. Finally, the absolute number of all synaptic contacts per unit retinal area in *Necturus* lies between those for the frog and primates. In *Necturus* the average is 0.112 contacts/μ^2; whereas in primates the number is 0.075 contacts/μ^2 and in frog 0.236 contacts/μ^2. Thus, the anatomy of the inner plexiform layer in *Necturus* suggests a complexity of synaptic interaction in the inner plexiform layer that is intermediate between that of primates and the frog (13). The significance of these observations will be discussed more fully below.

DISCUSSION

Outer plexiform layer

In the outer plexiform layer of *Necturus* synaptic relationships between receptor terminals, bipolar cell dendrites, and horizontal cell processes appear clearer than in any other retina so far described. In the primate outer plexiform layer, for example, no synaptic contacts of the horizontal cells have yet been described. In *Necturus,* however, it is possible to clearly identify horizontal cell synapses, and to show that horizontal cell processes synapse mainly on bipolar cell dendrites. Occasionally, horizontal cell processes synapse on other horizontal cell processes, but horizontal cell processes never synapse back onto the receptor terminals. Thus, there is no morphological evidence of feedback between hori-

FIG. 16. Complex synaptic arrangements in the inner plexiform layer. In the lower part of the micrograph, a ribbon-containing process contacts two postsynaptic processes (filled arrow), one of which makes a conventional contact on the other postsynaptic process (open arrow). At the top of the micrograph, a serial synaptic arrangement between processes making conventional synaptic contacts is seen. All of these processes are presumably amacrine processes (A). Insert shows a conventional synaptic contact in the inner plexiform layer at high magnification. × 27,500; insert × 71,000.

FIG. 17. *a:* Reciprocal or feedback synaptic contact (arrows) between a ribbon-containing process and a process making a conventional synaptic contact (A). × 37,500. *b:* A serial synaptic arrangement ending on a ganglion cell dendrite (G). Processes in the inner plexiform layer making conventional synaptic contacts are believed to be amacrine processes (A). × 27,500.

zontal cells and receptor terminals, and the great majority of synapses of the horizontal cells appear to be feed-forward synapses onto the bipolar cells.

Often a horizontal cell synapse is observed adjacent to a ribbon contact of the receptor terminal on the horizontal cell process. In such cases the horizontal cell process is both pre- and postsynaptic along a short portion of its length. Thus, horizontal cell processes are similar in this regard to amacrine cell processes in the inner plexiform layer which are both pre- and postsynaptic along their length (13–15).

Superficial and invaginated synaptic contacts of receptors have been described in all retinas so far studied (13, 14, 30). Synaptic specializations for the superficial contacts have not been observed in most of these retinas. In *Necturus,* synaptic ribbons, similar to the synaptic ribbons associated with the invaginated synaptic contacts, are invariably found in the presynaptic cytoplasm adjacent to the sites of superficial contacts on the receptor terminals. This provides firmer ground for suggesting that functional synaptic contact is made at both the superficial and invaginated contacts of receptor terminals. It is not clear why no ribbon or other presynaptic specialization is seen at superficial contacts in other retinas.

The reason for the invaginated synaptic contacts in receptor terminals has long been a puzzle. In primates no synaptic contacts of horizontal cells have been observed, and thus there has been speculation that perhaps the horizontal cells function by regulating receptor-bipolar transmission within the invaginations (14). This might explain the purpose of the invaginations and how horizontal cells operate in primates. In *Necturus,* horizontal cell processes clearly make classic synaptic contacts onto bipolar cell dendrites and perikarya, and thus there is now evidence for a conventional type of synaptic interaction between horizontal and bipolar cells, at least in the *Necturus* retina. These conventional synapses, however, appear to be associated mainly with the superficial contacts. No conventional synapses have been observed between two processes that are deeply invaginated into the receptor terminal. As already noted, long stretches of membrane specialization are observed between invaginated process and receptor terminal adjacent to the synaptic ribbon. This suggests that perhaps there is interaction between processes in the invaginations of receptor terminals, and this lends support to the notion that the invaginations allow for interaction between invaginating processes in some way not presently understood. Such interactions would not occur between processes making superficial contacts; for these latter processes to interact, a more classic type of synaptic contact is required, and this is observed. Whether processes from a single horizontal cell make both superficial and invaginated contacts is not known.

In summary, the hypothesis may be made that horizontal-bipolar interaction occurs in the invaginations of the receptor cells. The morphological evidence for this interaction is the extensive zone of membrane specialization observed between invaginating processes. For processes making superficial contacts on receptor terminals, synaptic contacts are more conventional in nature; and classic synapses are observed between horizontal and bipolar cell processes that superficially contact the receptors. It is possible that the processes which invaginate into the receptor terminals may also interact by conventional synapses that are located deep in the outer plexiform layer. (As yet we have not traced invaginating processes sufficiently to test this possibility.) If this proves to be so, the function of the invaginated contacts in receptor terminals remains obscure. In any case, it is clear that receptors in *Necturus* contact both horizontal and bipolar cell processes and that horizontal cell processes contact and interact primarily with bipolar cell dendrites. Thus horizontal cells provide in the outer plexiform layer a pathway for lateral interaction such that receptors outside the dendritic spread of a bipolar cell can still communicate with the bipolar cell via the horizontal cell processes. In this way we might expect bipolar cells to respond to annular illumination that exceeds in diameter the dendritic spread of the bipolar cells. In the following paper, physiological evidence for such interaction will be presented.

Inner plexiform layer

In *Necturus,* as in all vertebrate retinas, the inner plexiform layer is much more extensive than is the outer plexiform layer. There are many more synaptic contacts in the inner plexiform layer, and a much greater variety of synaptic structure is observed. Thus, it is to be expected that amacrine and ganglion cell physiology is more complex than bipolar and horizontal cell physiology, and that more complex synaptic interactions are mediated in the inner plexiform layer.

In the inner plexiform layer, bipolar terminals synaptically contact both amacrine cell processes and ganglion cell dendrites. The amacrine processes, in turn, make feedback synapses onto the bipolar terminals, feed-forward synapses on ganglion cell dendrites, and feed-forward and lateral contacts on other amacrine cell processes. Amacrine synapses are also arranged in series, suggesting successive interactions between amacrine processes.

Since there are considerably more amacrine synapses than bipolar synapses in the inner plexiform layer, and since at more than one-half of the bipolar synapses both postsynaptic elements are amacrine processes, the probability is high that many ganglion cells in *Necturus* are driven primarily by amacrine cells, as was suggested for many ganglion cells in the frog retina (13). In such instances, the amacrine cell may be a true interneuron, interposed between the bipolar and ganglion cell. Other ganglion cells, however, do have direct contacts with bipolar terminals, and this suggests that two types of ganglion cells may exist in *Necturus:* one driven primarily by amacrines, the other driven primarily by direct bipolar contacts. Thus we might expect some ganglion cells to closely follow bipolar cell responses, and other ganglion cells to follow amacrine cell activity. In the following paper, physiological evidence for this is presented.

Comparative retinal organization

Figure 18 is a drawing illustrating the synaptic connections we have observed in the *Necturus* retina and how they may be arranged. The details of the connections are described in the figure legend.

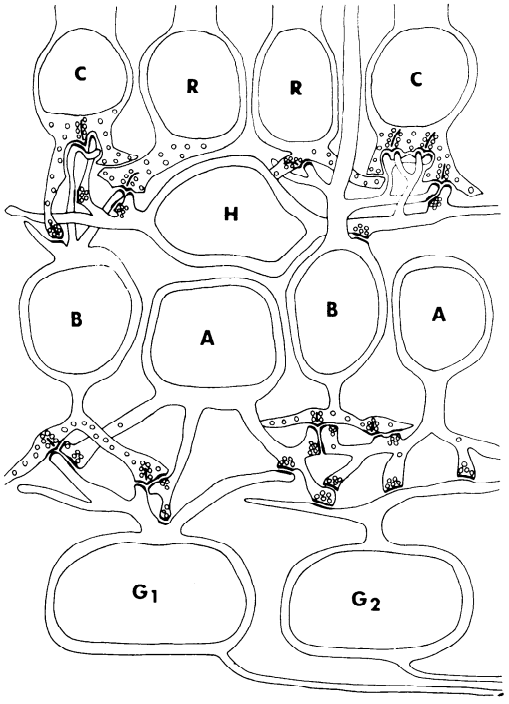


FIG. 18. Summary diagram of the *Necturus* retina. Terminals of the cones (C) lie directly under the cell, while the terminals of the rods (R) extend laterally and often make synaptic contacts under the cone terminals. Bipolar (B) and horizontal cell processes (H) invaginate deeply into the cone terminals and also make superficial contacts on the basal portion of both the rod and cone terminals. Ribbons are associated with both types of contact, suggesting that both are synaptic. At these ribbon contacts, two or more processes are observed. Horizontal cell processes make conventional synaptic contacts on bipolar dendrites and occasionally on bipolar cell perikarya. Often, a horizontal cell synapse is just adjacent to a superficial ribbon contact of the receptor terminal. Occasional horizontal cell to horizontal cell synapses are observed, but they are not illustrated here. In the inner plexiform layer, bipolar cell terminals make contacts at synaptic ribbons. Two postsynaptic processes are opposite the ribbons; they may both be amacrine processes (A) or one may be an amacrine process and the other a ganglion cell dendrite (G). Amacrine cell processes make conventional synaptic contacts: *1)* back onto the bipolar terminals, *2)* on adjacent amacrine processes, and *3)* on ganglion cell dendrites. Serial synaptic contacts between amacrine processes are frequently observed. In the diagram two types of ganglion cells are suggested, one driven primarily by bipolars (G_1), the other driven primarily by the amacrines (G_2). In the latter situation the amacrine cells involved may be considered as true interneurons, interposed between the bipolar and ganglion cells.

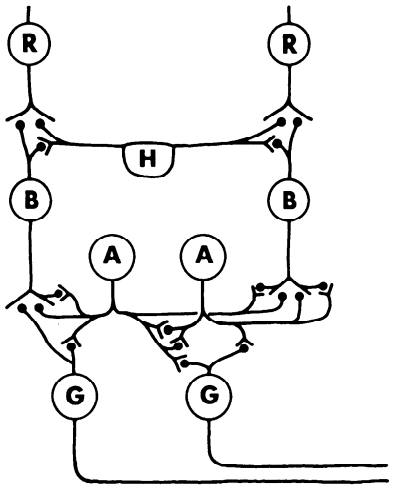


FIG. 19. Schematic wiring diagram of the *Necturus* retina. See text for details. R, receptors; H, horizontal cell; B, bipolar cells; A, amacrine cells; G, ganglion cells.

A schematic wiring diagram for the *Necturus* retina is shown in Fig. 19. The diagram shows that in the outer plexiform layer the receptors drive both the bipolar and horizontal cells, and the horizontal cells synapse laterally on adjacent bipolar cells. In the inner plexiform layer, bipolar terminals drive both ganglion and amacrine cells. The amacrine cells, in turn, make feedback synapses on bipolar terminals, feed-forward synapses on ganglion cells, and feed-forward and lateral, serial synapses on other amacrine cell processes. Two types of ganglion cells are postulated. One (left side of the diagram) receives mostly direct input from the bipolars, whereas the other (right side of diagram) receives its primary input from the amacrine cells.

This diagram (Fig. 19) could serve also to describe synaptic connections of other vertebrate retinas, since similar synaptic interconnections have been found in all vertebrate retinas so far studied (13, 14, 16). The striking way in which vertebrate retinas differ is in the relative proportions of types of synaptic contacts in the inner plexiform layer. So, for example, in the primate and cat retina there are numerous direct bipolar-ganglion cell contacts, relatively few amacrine-amacrine and amacrine-ganglion cell contacts, and almost no serial synaptic contacts between amacrine processes. The inner plexiform layer of the primate retina is better described, therefore, by the simpler (left-hand) side of Fig. 19. Its ganglion cells receive mostly direct input from bipolar neurons (13, 14, 16).

In the frog or pigeon, on the other hand, there are few direct bipolar-ganglion contacts observed, but numerous bipolar-amacrine, amacrine-amacrine, and amacrine-ganglion contacts, and many serial synaptic contacts between amacrine processes (13, 16). The inner plexiform layer of the frog retina is better described by the more complex (right-hand) side of Fig. 19. Its ganglion cells receive less direct input from bipolar cells, and appear to be driven primarily by the amacrine cells.

The physiology of the ganglion cells of these animals can be correlated with this differing retinal organization. Most of the primate and cat ganglion cells have a relatively simple, antagonistic center-surround receptive-field organization (21, 23). On the other hand, frog and pigeon ganglion cells have more varied and complex receptive-field organizations (27, 28). For example, many ganglion cells in these latter animals respond best to moving stimuli of a specified configuration.

In other retinas, such as those of rabbit or ground squirrel, some of the ganglion cells are functionally of the primate and cat type, while others are of the more complex type, as in frog and pigeon. Anatomical examination of these retinas shows relative proportions of types of synaptic contacts intermediate between those of primates and cats versus frogs and pigeons (2, 29). Rabbit and ground squirrel retinas, like the *Necturus* retina, are therefore described better by the overall proportions of types of synaptic contacts illustrated in Fig. 19.

In summary, with more anatomical and physiological complexity in a retina, more amacrine synapses are observed and there is more evidence for amacrine-amacrine and amacrine-ganglion cell interaction. In retinas with complex ganglion cell responses, amacrines are probably true interneurons interposed between bipolar terminals and ganglion cell dendrites. This suggests that the amacrine cell may be the cell type in the retina primarily responsible for mediat-

ing complex features of ganglion cell responses such as movement detection. In the following paper, physiological evidence is provided that this is likely to be the case.

SUMMARY

The synaptic organization of the *Necturus* retina has been studied by light and electron microscopy. The outer plexiform layer is irregular in thickness, and the receptor terminals cluster in the thicker portions of the layer. Bipolar and horizontal cell processes invaginate deeply into the cone terminals and also make superficial contacts on the basal portions of both the rod and cone terminals. Synaptic ribbons are associated with both types of contact, suggesting that both are synaptic. At these ribbon contacts, two or more postsynaptic processes are observed. Horizontal cell processes make conventional synaptic contacts primarily on bipolar cell dendrites and occasionally on bipolar cell perikarya or horizontal cell processes. Often the horizontal cell synapse is located just adjacent to a superficial ribbon contact of the receptor terminal. Horizontal cell processes extend further laterally than do bipolar cell dendrites, and thus these observations suggest that the horizontal cells mediate a lateral interaction in the outer plexiform layer such that distant receptors can affect the bipolar cell response through horizontal processes.

In the inner plexiform layer, bipolar terminals make contacts at synaptic ribbons. Two postsynaptic processes are opposite the ribbons; they may be amacrine processes or one may be an amacrine process and the other a ganglion cell dendrite. Amacrine cell processes make conventional synaptic contacts: *1)* back onto the bipolar terminals, *2)* on adjacent amacrine processes, and *3)* on ganglion cell dendrites. Serial synaptic contacts between amacrine processes are frequently observed. The observations suggest that there may be two functional types of ganglion cell in the *Necturus* retina, one driven primarily by bipolar terminals, the other driven primarily by the amacrines. In the latter case, the amacrine cells may be true interneurons, interposed between the bipolar and ganglion cells.

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