Organization of the Retina of the Mudpuppy, *Necturus maculosus*. II. Intracellular Recording

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FROM ELECTRICAL RECORDINGS at the level of the optic nerve, it has been possible to specify many of the functions performed by the vertebrate retina. These include brightness detection (16, 18, 27), center-surround contrast detection (1, 8, 20, 23), and motion detection (3, 4, 26–28). It has not been possible, however, to determine how the retina organizes the visual message recorded at the optic nerve, primarily because intracellular recording from single cells distal to the ganglion cells has been difficult.

Detailed structural studies of the vertebrate retina, such as the one in the preceding paper (14), provide a framework within which the functional organization of the retina can be described. The anatomical studies show a limited number of clearly defined synaptic structures at which interaction between specific neurons can take place. In this paper we shall describe the intracellularly recorded response characteristics of each type of neuron in a vertebrate retina, and then relate the response of each neuron to the responses of those neurons to which it is synaptically coupled. By following the responses through the synaptic pathways, we can begin to describe how information from the visual field is abstracted and encoded in the retina.

Intracellular recording throughout most retinas has been difficult because even the finest available micropipettes fail to penetrate the small retinal neurons consistently without damage. Bortoff (5–7) showed that this difficulty could be overcome by recording in an animal with larger retinal neurons: the mudpuppy, *Necturus maculosus*. As described in the preceding paper (14), the retinal neurons in *Necturus* are about 30 μ in diameter, as compared to diameters of less than 10 μ for many cells in the retinas of frogs or mammals. This represents a difference in cell volume of 3^3, more than an order of magnitude. Bortoff was able to record intracellularly from cells throughout the retina of *Necturus* and to stain these cells for later identification. He described several response types in the retina, primarily those of the more distal neurons, which give slow, graded potentials. Bortoff used diffuse illumination in his experiments, and did not study the spatial organization of the receptive fields for each neuron. It will be shown here that study of spatial organization is crucial for interpreting the functional organization of the retina.

In other vertebrate retinas, intracellular recording and staining have been possible in only a few cell types. Fish, for example, have relatively large horizontal cells that can be penetrated easily, and these cells have been extensively studied. The potentials recorded from within these cells—the luminosity- or L-type S potentials—are sustained and graded with illumination over a limited range of intensity and are always hyperpolarizing (15, 24, 25, 27, 29, 30, 32–35, 37–39, 42). Recently, Tomita (40) and Kaneko and Hashimoto (21) obtained intracellular responses from fish cones and have shown that these receptor cells also respond with sustained, hyperpolarizing potentials that are graded over a limited range of intensity.

The finding of graded, hyperpolarizing potentials in both receptors and horizontal cells has been somewhat surprising. Svaetichin and his co-workers (24, 30, 37) have

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suggested that the horizontal cells are not neurons but glia-like cells. They have shown that horizontal cells are more easily affected by metabolic poisons than classical neurons and under these adverse conditions tend to polarize in the direction opposite to that of classical neurons. They have offered the hypothesis that such "glia" cells control transmission of information through the retina. It has been shown anatomically, however, that horizontal cells make synaptic connections similar to those of classical neurons, which indicates that horizontal cells should be considered as neurons (see 13, 14). Grundfest (17) has pointed out that since such cells as horizontal cells or receptors need not communicate over long distances, no axonal (conductile) process is necessary. Thus, it is not necessary that the cell be depolarized for initiation of impulse activity, and so the direction of polarization during activation may not be significant. Thus, excitation could be signaled with either hyperpolarizing or depolarizing potentials.

Intracellular recording from neurons in the inner nuclear layer other than horizontal cells has seldom been achieved. The few studies that have been made indicate that some units show impulse activity while others do not (5, 9, 22, 39, 41). These findings suggest that it is within the inner nuclear layer that the transition from slow-potential generators to spike generators takes place.

METHODS

In all experiments adult *Necturus*, about 12 inches long, were used. The animals were trapped in the rivers of Wisconsin, flown to Baltimore, and kept in a tank of cold spring water in a dark room until the time of the experiment. The animal was decapitated, and the anterior part of one eye dissected away. The head was placed in a contoured holder so that the eye could be properly positioned with respect to the stimulus and the electrode. During the dissection no attempt was made to remove the vitreous humor. Under these conditions the retina remained functional for as long as 6 hr. Drying seemed to be the primary cause of functional deterioration.

The micropipette electrodes were made with a Livingston-type spring puller, modified so that the pull was initiated only after a fixed heating interval. Corning, type 7740 capillary tubing with 0.8-mm outside diameter and 0.4-mm inside diameter was used. All electrodes were filled by heating to about 70 C and then boiling under reduced pressure for about 10 min. The electrodes, when filled with 2 M potassium chloride, had tip resistances of 100–150 megohms measured in Ringer solution. Similar electrodes filled with the staining solution of 4% Niagara blue (21), had resistances of about 700 megohms. Electrodes were used only on the day they were filled. Those that were stored for longer periods were less effective in penetrating cells.

To eject Niagara blue from the electrodes for staining, a negative potential of 400 v was applied across the electrode tip through a current-limiting resistance of 100 megohms (Fig. 1). Within a few seconds after the voltage was applied, the first few microns of the electrode tip were destroyed and stain began to enter the cell through the widened tip. The current flowing through the electrode tip was monitored via a loudspeaker. With practice it was possible to deliver the proper quantity of stain by "listening" to the flow of stain through the tip.

After the stain had been ejected, the eyecup was fixed for about 15 min in 6% glutaraldehyde and 2.5% potassium dichromate. This solution had a pH of 4, which kept the stain insoluble. The retina was then firm enough to be removed from the eyecup without tearing or folding. It was transilluminated, and the blue spot located with the aid of a dissecting microscope. A 2 x 2 mm square of tissue containing the spot was cut out, dehydrated, and embedded in soft plastic. Sections were cut at about 10 μ on a glass knife until the stained cell was located.

The first stage of the d-c recording amplifier consisted of a field effect transistor with an input impedance of 10^{13} ohms and an input capacitance of about 10^{-12} farads. Although negative capacitance was used to increase the bandwidth, the system was always band-limited by the high distributed capacitance and resistance at the pipette tip. With the very high impedance electrodes used in these experiments, action potentials were recorded only when the pipette tip was within the cytoplasm of a cell. A probable explanation for this is that the tip capacitance acts in parallel with the tip resistance when the electrode is in the cytoplasm, thus increasing the bandwidth; but the tip capacitance acts as a shunt when the tip of the electrode is outside the cell membrane, thus decreasing the bandwidth. Even the intracellularly recorded action potentials were

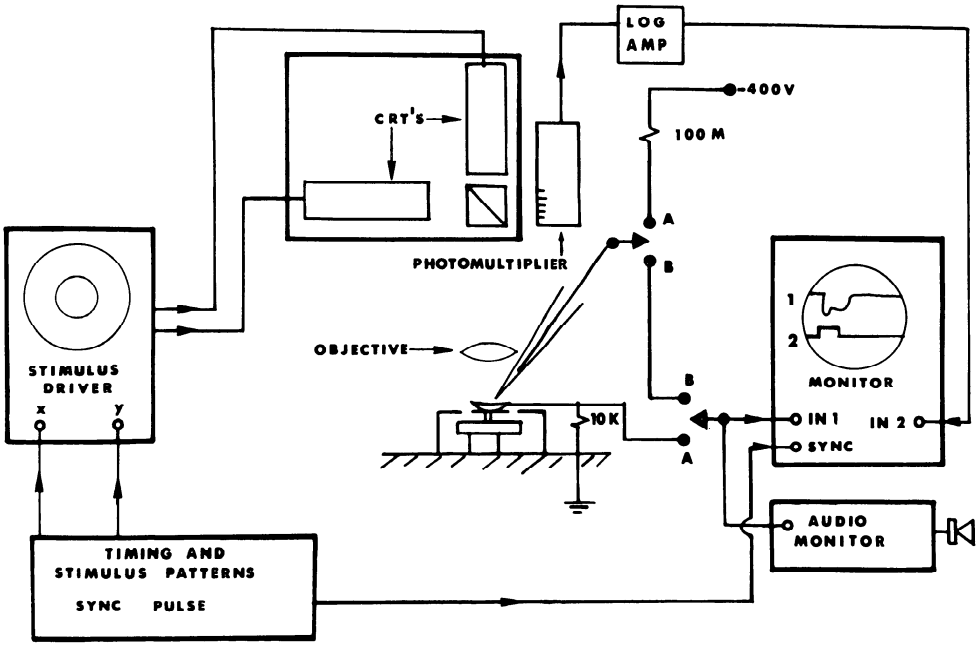


FIG. 1. Apparatus for stimulation, recording, and staining. Patterns generated on the faces of the two cathode-ray tubes (CRTs) are combined in the beam-splitting prism and focused on the retina. The other output from the prism illuminates the face of a photomultiplier. Output from the photomultiplier is linear with intensity throughout the range used here. This linear signal is logarithmically compressed and appears as the lower trace, 2, in all recordings presented in the paper. One log unit of intensity is represented by one vertical division. The electrode is coupled, either to the d-c recording amplifier and then to the oscilloscope (B) or to the negative 400-v staining potential (A), by the relay system. When the stain is ejected, current flows through the electrode, the preparation, and the 10-kilohm resistor to ground. The potential produced by the staining current across the 10-kilohm resistor is monitored by the audio amplifier. Timing is controlled so that the oscilloscope sweep begins in all recordings 200 msec before the retina is stimulated. In this way, timing for all recordings can be estimated from the initial segment of the trace.

attenuated by the limited bandwidth of the recording electrode. The recorded signal was amplified, displayed on an oscilloscope, and recorded on tape.

The retina was stimulated by patterns of illumination produced on the faces of two 1-inch cathode-ray tubes as shown in Fig. 1. The patterns were combined through a beam-splitting prism and focused on the retina. Each cathode-ray tube was driven by a master oscilloscope on which the stimulus pattern also appeared. A 1-cm pattern on the master oscilloscope corresponded to a 0.5-mm replica focused on the surface of the retina. The stimulus patterns could be monitored as they were moved over the surface of the retina and the receptive fields for units were plotted on the face of the master oscilloscope. The patterns in these experiments were simple, consisting of spots and annuli (Lissajous patterns) of continuously variable diameter and intensity. The width of the spot or line width of the annulus was 100 μ when focused on the retina. At high intensities the width of the spot or line width of the annulus increased somewhat, but receptive fields were plotted at lower intensities. The patterns were focused on the retina by viewing through a dissecting microscope; they were not noticeably distorted in passing through the vitreous or retinal tissue. The stimulus intensity was limited to a maximum of about 3.5 log units above threshold for units in the *Necturus* retina. The cathode-ray tube stimulators (RCA type 1EP1) are available with numerous phosphors having narrow emission spectra over the visible range. For most of these experiments phosphors which had emission peaks at 525 nm were used. When color-coded responses were looked for, tubes with phosphors with peak spectral emission at 425 nm or 600 nm were employed.

The light from the beam splitter at right angles to that focused on the retina fell on a photomultiplier tube (RCA type 1P21). The output from the photomultiplier was logarithmically compressed and appeared with each

recording as an indication of the total illumination energy falling on the retina. The size of the annulus could be varied continuously from a spot to a ring of many millimeters while maintaining a constant total output energy, although the intensity continuously decreased. This energy was recorded as being constant by the photomultiplier, independent of annular size.

In a typical experiment an annulus about 2 mm in diameter was flashed in the vicinity of the electrode tip as it penetrated the retina. The annulus was flashed for 1 sec every 5 sec, with a total energy corresponding to that of a spot 2 log units above threshold for a ganglion cell in the retina. A sequence of intracellular responses was often obtained—first a ganglion cell, then one or more distal neurons—during a single penetration. Cell penetration was signaled by the sudden appearance of a negative resting potential of about 30 mv. The resting potentials were quite variable in magnitude and not clearly related to the size of light-evoked responses. The receptive field for a penetrated unit was then determined, either in the dark or with the center of the field or the entire retina illuminated. Cells could often be recorded from for more than 15 min.

The term "receptive field" is used here to indicate the extent of the retinal surface over which a unit could be influenced by illumination, whether this illumination evoked or antagonized a response. In units with impulses, "threshold" is defined as the intensity of illumination at which impulses occurred. For the slow-potential generators, "threshold" refers to the intensity at which a response could be seen above the electrical noise.

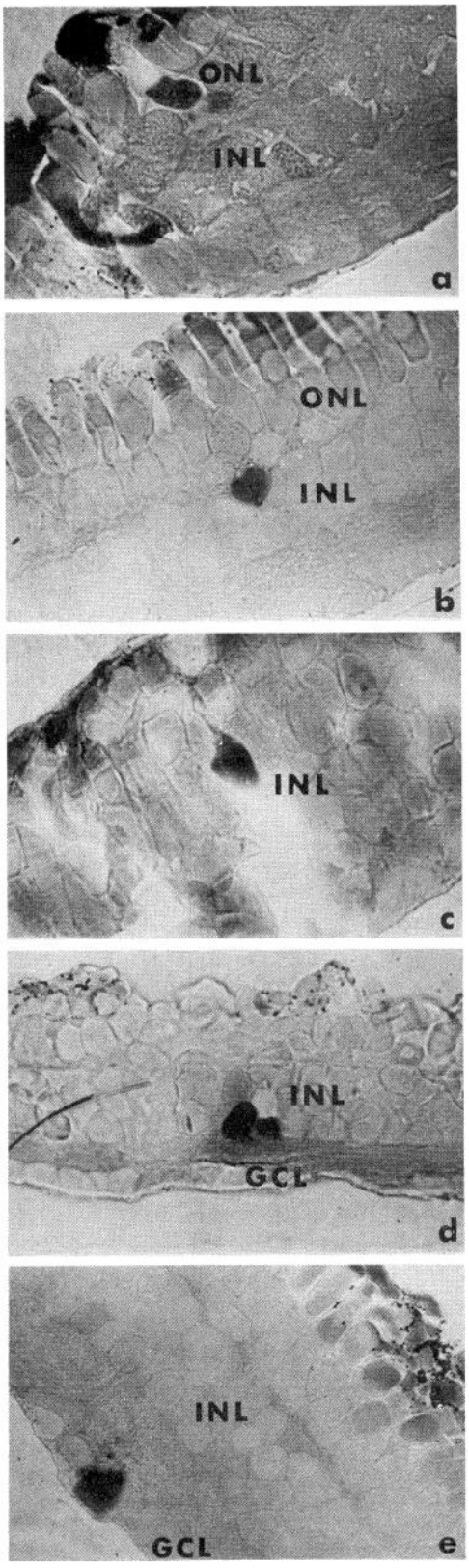
RESULTS

Intracellular staining

To identify the type of cell generating a particular response, it was necessary to stain many cells intracellularly after recording from them. Stained cells, corresponding to different response types, tended to group at characteristically different depths within the retina, and thus it was possible in most cases to identify with confidence the intracellularly stained cells by their shape and depth (see 14). For example, bipolar cells almost always lie within the outer half of the inner nuclear layer and have radially elongated cell bodies. The amacrine cells almost always lie along the inner margin of the inner nuclear layer. The position of horizontal cells is more variable, lying both distal and proximal to, as well as within, the outer plexiform layer. However, they usually show a wide, flattened surface on one side, where processes leave the cell to enter the outer plexiform layer. Examples of intracellularly stained cells of each type are shown in Fig. 2.

Because the cells are large, it is easy to distinguish a fully stained cell (ca. 40% of those stained) characteristic of a good intracellular staining, from a region of partially stained cell membranes (ca. 60% of the cases) characteristic of extracellular staining. Only the former result was used as a criterion for cell type. Receptors and ganglion cells were the easiest to distinguish—both by depth measurements made during penetration and by staining—because these cells are separated from the other cell types by a plexiform layer (Fig. 2 *a, e*). About 5 of each of these 2 types of cell were stained, but at least 50 of each type were recorded from during these experiments.

Cells that stained consistently along the inner margin of the inner nuclear layer were identified as amacrine cells (Fig. 2*d*). Twenty stained amacrine cells were recovered, and more than 100 were recorded from. Cells identified as bipolar cells were usually found within the outer half of the inner nuclear layer. Fourteen such cells were stained and more than 100 recorded from. The stained bipolar cell shown in Fig. 2*c* is typically oval, and shows staining of its Landolt club process. The horizontal cell shown in Fig. 2*b* is located at the outer margin of the inner nuclear layer, and shows a flattened apical surface, typical of many horizontal cells. Twenty-five horizontal cells were stained and recovered successfully, but many more were recorded from during the course of these experiments. As noted above, it was often possible to obtain a sequence of intracellular recordings while penetrating the retina with a single pipette. The sequence of responses was always consistent with that predictable from the position of the stained cells. This added further support to the identification of cell types.

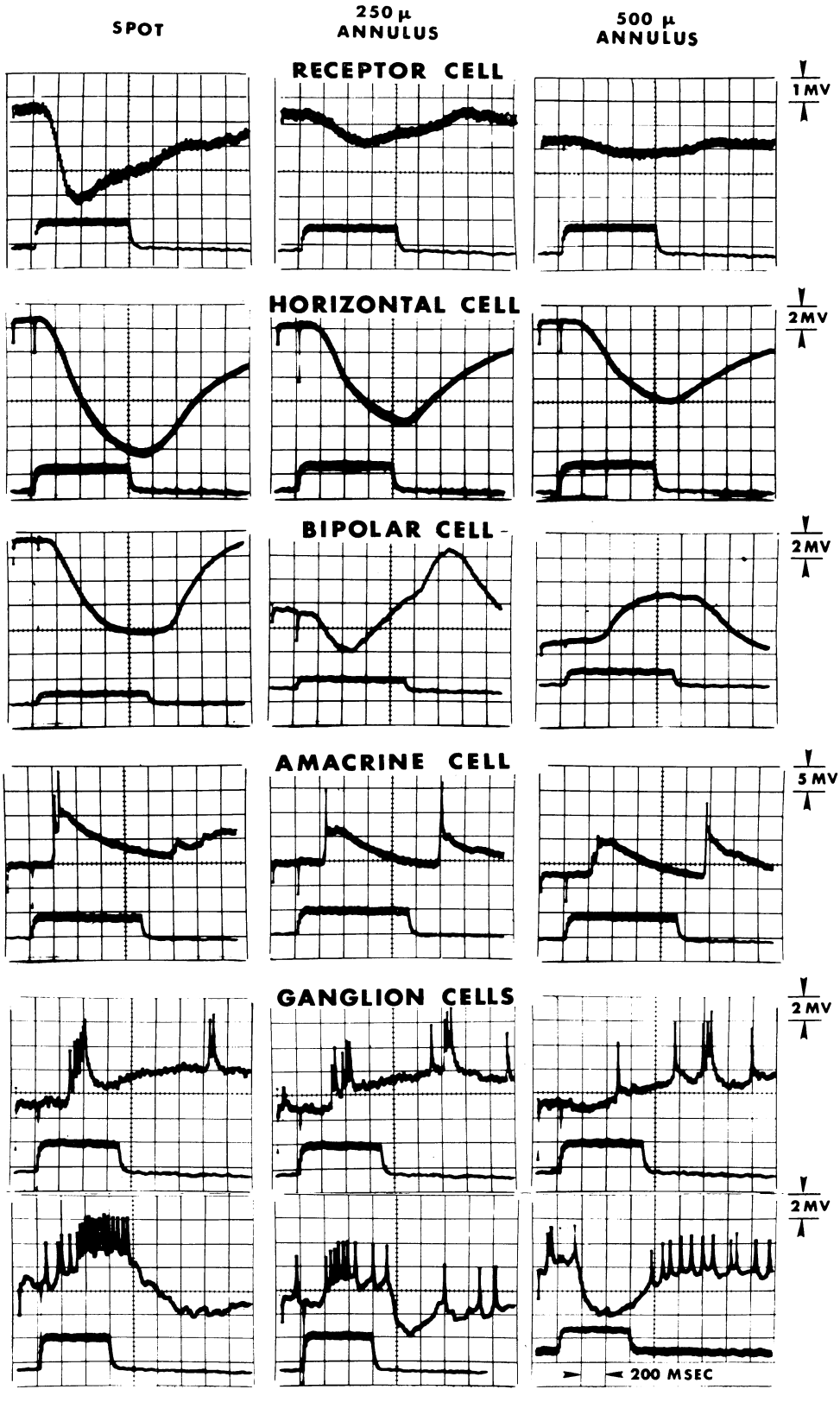


Intracellular recording

Intracellularly recorded responses from each of the types of retinal neurons in *Necturus* are shown in Fig. 3. Neurons in the distal retina respond with slow, graded, mostly hyperpolarizing potentials, as shown in the upper half of the figure. In the proximal retina (lower half of the figure) most neurons respond with transient, depolarizing potentials on which impulses are superimposed.

For each type of neuron the response both to focal (left side of figure) and to annular (right side of figure) illumination centered on the receptive field was recorded. These two measurements provided sufficient information to characterize each response type. For example, the receptor has a narrow receptive field such that spot illumination evokes a much larger response than annular illumination. The horizontal cell has a broad, uniform receptive field, so that both spot and annular illumination evoke a sizable hyperpolarizing response. The bipolar cell responds with a sustained polarization when the center of its receptive field is illuminated. The sustained response is reduced when illumination is added at the periphery of the receptive field (right column). The units proximal to the bipolar cell reflect the antagonism between center and periphery established at the level of the bipolar cell, but these units are depolarizing and spike-generating neurons.

FIG. 2. Intracellularly stained cells such as these were used to establish the identity of recorded responses. The receptor cells (*a*) always show stain in the inner segment or nucleus. The horizontal cell (*b*) shown here is located along the outer margin of the inner nuclear layer and has a flattened distal surface where processes extend into the plexiform layer. Bipolar cells (*c*) are located in the outer half of the inner nuclear layer. The bipolar cell morphology of an elongated cell body is represented in this section, although the overall preservation of this retina is poor. Amacrine cells (*d*) stain typically along the inner margin of the inner nuclear layer. In the example shown here there is a faint blue staining of an adjacent cell, probably due to leakage of the stain. The deeply stained cell is almost certainly the cell recorded from, but in any case the layering of the stain along the inner margin of the inner nuclear layer is unequivocal. Ganglion cells (*e*) are easily identified in the single row of cells proximal to the inner plexiform layer. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.


SPOT
250 μ ANNULUS
500 μ ANNULUS
RECEPTOR CELL
1 MV
HORIZONTAL CELL
2 MV
BIPOLAR CELL
2 MV
AMACRINE CELL
5 MV
GANGLION CELLS
2 MV
2 MV
200 MSEC

The characteristics of each type of response will be discussed in detail in the following sections. The recordings in Fig. 3 are typical examples of about 80% of the recordings made in *Necturus*. The other 20% (not discussed here) represent either rare response types, damaged units, or those that, for unknown reasons, are not easily or consistently penetrated.

RECEPTOR RESPONSE. The receptor response is a hyperpolarization, consisting of an initial transient that decays to a steady level and is graded with stimulus intensity. When illumination is removed the potential returns very slowly to the base line, with a small "off" transient. Receptor responses are illustrated in Figs. 3, 4, and 5.

The response occurs after a latency of about 50 msec and has a time to peak of 50 msec when elicited with a stimulus about 1 log unit above threshold. Resting potentials are quite variable in the receptors, but are typically 30 mv. The response is only about 5 mv in magnitude. It is graded over a relatively narrow range of intensities —less than 2 log units from threshold. At higher intensities the latency and time to peak decrease, but the magnitude of the response remains unchanged.

Figure 5 shows an experiment in which a receptor was recorded from while a 100-μ spot, centered on the receptor, was flashed. This was done first with the retina in the dark and again while steady annular illumination (250 μ in radius, centered on the receptor, and of equal total energy as the stimulating spot) illuminated the retina. The two superimposed traces indicate that the receptor response was not altered by the annular illumination, so that under these conditions there is no evidence of a surround effect. In the more proximal retina it will be shown that under the same conditions a dramatic surround effect is observed.

The response from cones in fish has been recorded intracellularly by Tomita (40). He also reported a hyperpolarizing response that is sustained and graded with intensity. He has seen no appreciable difference in response when stimulating with a spot or disk of similar illumination centered on the receptor, indicating that the receptor is not susceptible to a "surround effect." The lack of a surround effect is a characteristic feature of the receptor and is illustrated also in Fig. 3. The significance of this will be discussed below with respect to the more proximal neurons.

Recordings from the receptors were probably always obtained from the inner segments or nuclei of the cells, since this is where the stain was located. The outer segments were often lost during the preparation of the tissue for histologic study following staining, so that it has not been possible to distinguish the responses of rods and cones. The inner segments of all re-

FIG. 3. Recordings show the major response types in the *Necturus* retina and the difference in response of a given cell type to a spot and to annuli of 250- and 500-μ radius. Receptors have relatively narrow receptive fields, so that annular stimulation evokes very little response. Small potentials recorded upon annular stimulation were probably due to scattered light. The horizontal cell responds over a broader region of the retina, so that annular illumination with the same total energy as the spot (left column) does not reduce the response significantly (right columns). The bipolar cell responds by hyperpolarization when the center of its receptive field is illuminated (left column). With central illumination maintained (right trace; note lowered base line of the recording and the elevated base line of the stimulus trace in the records) annular illumination antagonizes the sustained polarization elicited by central illumination, and a response of opposite polarity is observed. In the middle column the annulus was so small that it stimulated the center and periphery of the field simultaneously. The amacrine cell was stimulated under the same conditions as the bipolar cell, and gave transient responses at both the onset and cessation of illumination. Its receptive field was somewhat concentrically organized, giving a larger "on" response to spot illumination, and a larger "off" response to annular illumination of 500-μ radius. With an annulus of 250-μ radius, the cell responded with large responses at both on and off. The ganglion cell shown in the upper row was of the transient type and gave bursts of impulses at both on and off. Its receptive-field organization was similar to the amacrine cell illustrated above. The ganglion cell shown in the lower row was of the sustained type. It gave a maintained discharge of impulses with spot illumination. With central illumination maintained, large annular illumination (right column) inhibited impulse firing for the duration of the stimulus. The smaller annulus (middle column) elicited a brief depolarization and discharge of impulses at on, and a brief hyperpolarization and inhibition of impluses at off.

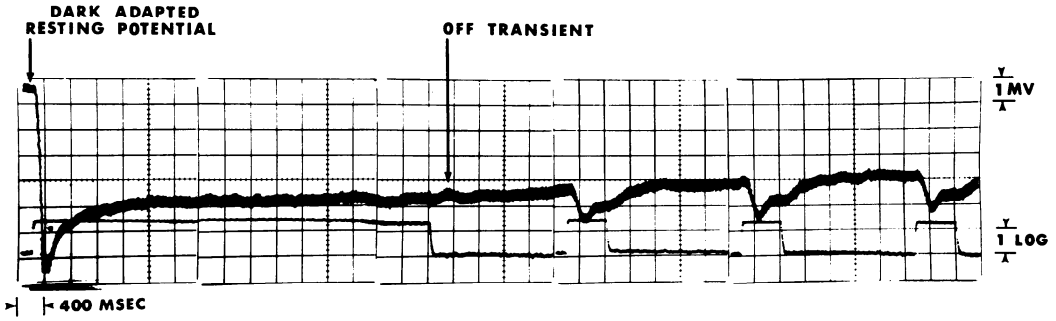


FIG. 4. Time course of response for a receptor. Response of the dark-adapted receptor begins with a large initial transient which decays to a steady hyperpolarization that is graded with the intensity of stimulus. When the stimulus is terminated, the response begins to decay to the original resting potential, with a surprisingly small off-transient. Additional stimuli of the same intensity, presented before the cell fully dark adapts, evoke responses with small initial transients that decay to the same steady hyperpolarized level that is independent of the state of adaptation.

ceptors in *Necturus* are of comparable size, so there is no apparent reason to suppose that the electrode should have penetrated either rods or cones preferentially.

HORIZONTAL CELL RESPONSE. The intracellularly recorded horizontal cell response, like that of the receptor, is hyperpolarizing and sustained with intensity. Figure 3 shows, however, that the horizontal cell response is slower, with a latency of about 100 msec and a time to peak of over 300 msec. At the cessation of illumination, the horizontal cell response decays with a similar slow time course, lasting about 300 msec.

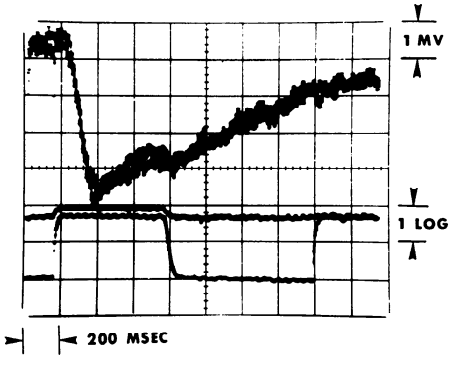


FIG. 5. The autonomy of the receptor response is illustrated by these two superimposed records. One record was obtained with spot illumination flashed on the receptor with the retina in the dark. For the second record, an annulus (of 250-μ radius) around the electrode was maintained on (elevated base line in stimulus recording), and then the receptor was illuminated with the same spot as in the first case. The two records are almost identical, indicating that under these conditions illumination of the surround does not affect the response of the receptor.

The magnitude of the response is graded with intensity over about 3 log units. However, the shape of the intensity-response curve depends on the configuration of the stimulus. Figure 6 shows that the response

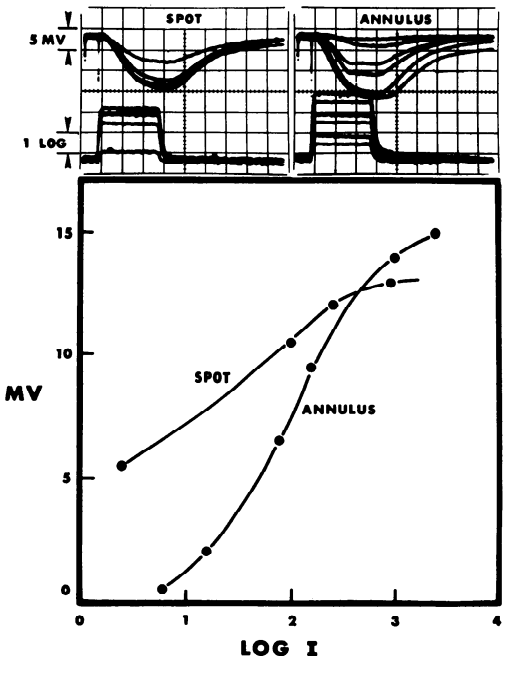


FIG. 6. Summation properties of the horizontal cell. The intensity-response relations of the horizontal cell were measured with stimuli of two different configurations. In one experiment all of the energy is confined to a spot of illumination. In the other the stimulus energy is distributed in an annulus with a radius of 0.5 mm. The response is greater, and saturates at a higher total energy level, when the stimulus is diffuse than when it is concentrated.

is greater and saturates at a higher absolute intensity when annular rather than spot stimulation is used. This suggests that the potential recorded in the horizontal cell is formed through the weighted summation of potentials from many sites, each of which can be saturated. Figure 3 shows that the horizontal cell summates over a wide area (compared to the receptor), since the response is not greatly reduced for stimulation with an annulus of 250- to 500-μ radius.

The potentials recorded from horizontal cells resemble the non-color-coded, or luminosity-type S potentials recorded in fish (15, 25, 34, 39, 42). The resting potential is typically 30 mv, with a response potential as large as 20 mv. The units summate over a wide area of the retina and are graded and sustained with intensity. No units corresponding to the chromaticity or C-type S potentials, which change their polarity of response with wavelength, have been observed in *Necturus* (25, 33).

BIPOLAR CELL RESPONSE. Like receptors and horizontal cells, bipolar cells generate only slow, graded potentials in response to illumination of the retina. However, unlike receptors and horizontal cells, the receptive field of the bipolar cell is concentrically organized into two antagonistic zones. The polarization of the cell in response to illumination of the center of the receptive field is antagonized by additional illumination falling on the periphery of the field (Fig. 3). About half of the units hyperpolarize to central illumination, while the other half depolarize. Representative recordings of each type of cell are shown in Fig. 7. In both cases additional illumination of the retina at the periphery of the receptive field antagonized the response to central illumination. Illumination of the periphery alone, however, did not polarize the cell in the opposite direction to that of the central illumination. Peripheral illumination appears only to turn off the bipolar responses to central illumination.

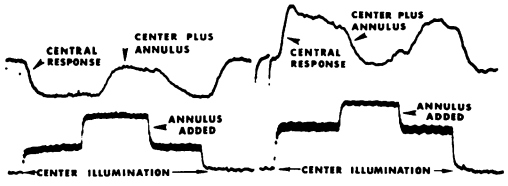


FIG. 7. The antagonistic effect of annular illumination on the bipolar cell response. In each experiment the center illumination of the receptive field for the bipolar cell was maintained on while an annulus of 250-μ radius was flashed. The bipolar cell polarization produced by central illumination was antagonized by the annular illumination. This was true for both the hyperpolarizing type (left) and the depolarizing type (right) of bipolar cell.

The bipolar responses bear a superficial similarity to the color-coded C-type S potentials (25, 33) in that it is possible to polarize the cell in opposite directions by the addition of appropriate stimuli. We have, however, been unable to find any evidence for color coding in the reversal of the bipolar response.

The magnitude of the bipolar cell response is typically 10 mv, starting from a resting potential of about 30 mv. The latency of the response to central illumination is about 100 msec at the intensity levels used in these experiments, which were within 3 log units of threshold. The center of the receptive field is about 100 μ in diameter, whereas an annulus with a radius of 250 μ is most effective in eliciting the antagonistic surround response. Smaller annuli scattered considerable light into the center of the field, whereas larger annuli fell outside the most efficient antagonistic peripheral zone.

Figure 8 shows intensity-response relations for a typical bipolar cell. These curves show how the response to central illumination increased with increasing intensity for three different levels of fixed illumination at the periphery of the receptive field. The curves demonstrate that the potential of the bipolar cell can be differentially controlled by varying the ratio of total flux energy falling at the center and periphery of its receptive field. To illustrate this more clearly, the points on the curves in Fig. 8 have been replotted in Fig. 9, with polarization potential rather than annular energy as the parameter. These curves show that under steady-state conditions the bipolar cell behaves as a center-surround contrast detector. The magnitude of the bipolar response can be held

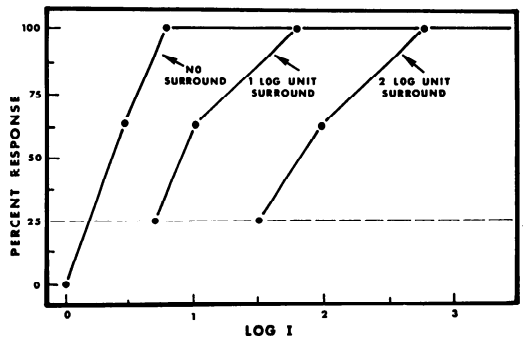


FIG. 8. Intensity-response relations for the bipolar cell. The bipolar response is determined by two parameters: energy falling in the center and energy falling in the periphery of the unit's receptive field. The three curves plotted here for a typical bipolar cell show how the polarization increases with increasing central illumination for three different values of peripheral illumination. The level of saturation is the same in all cases, but the dynamic range for the response is shifted along the intensity axis as the surround illumination is increased. Dotted line above the resting potential indicates that in this cell the surround could not turn off the polarization completely.

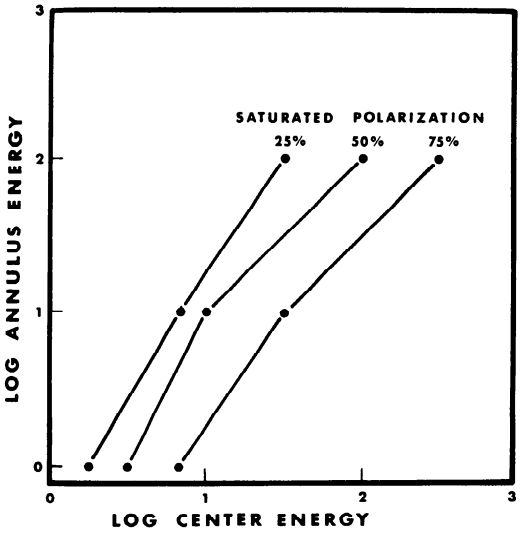


FIG. 9. Contrast detection properties of the bipolar cell. The abscissa and ordinate in this figure represent the total energies of stimulation falling in the center and periphery of receptive field for the bipolar cell. Each curve represents the relative quantities of each energy required to keep the polarization in the bipolar cell fixed. These curves indicate that the polarization is maintained by a fixed ratio of energy falling at the center versus the surround of the field. The curves suggest that the difference function is computed before saturation of either component. If this were not the case it would be impossible to increase the polarization of the unit after it had once been saturated.

constant for a fixed ratio of center-to-surround illumination over a wide range of absolute intensities.

The response to central illumination always precedes the antagonism evoked by peripheral illumination, regardless of the relative intensities of central and peripheral illumination. This is illustrated in Fig. 10. For these recordings, an annulus with radius of 250 μ was used throughout. The annulus was sufficiently bright so that scatter into the center of the receptive field generated an initial center response (here a hyperpolarization). The effect of the scatter was reduced in each successive (lower) trace by increasing sustained illumination at the center of the receptive field. In the lowermost record of Fig. 10 the central illumination was so great that the unit was saturated and no center response was evoked by the annulus. In all cases except the last the center hyperpolarizing response preceded the antagonism by more than 100 msec. Because of this latency difference there is in the bipolar cell a transient center response to any change in illumination even if there is not a change in contrast. The response is maintained only if the center-surround contrast is altered.

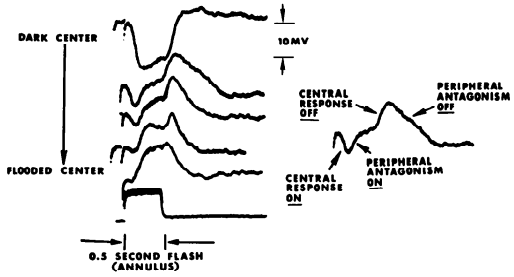


FIG. 10. Latency difference between the center and surround response of the bipolar cell. The series of responses shown here was elicited by annular stimulation sufficiently bright to yield considerable scatter into the center of the field. Maintained intensity at the center of the field was gradually increased to reduce the effect of scatter from the flashing annulus. The effective ratio of stimulation of center and surround is thereby gradually shifted to favor the surround. However, for any ratio of central and surround stimulation, the central response always occurs first. This is shown by the transient hyperpolarization in all traces except the lowermost trace. No center response is observed in the lowermost trace because the maintained central illumination was saturating.

AMACRINE CELL RESPONSE. At the level of the inner plexiform layer in the retina, a transition occurs from units that generate slow, sustained, graded, mostly hyperpolarizing potentials in response to steady illumination, to units that generate transient, depolarizing, regenerative potentials in response to changes in illumination. The transition from the bipolar cell response to the amacrine cell response is the first example of this.

Intracellularly recorded amacrine cell responses are shown in Fig. 3. The resting potential of these cells is typically 30–40 mv, whereas light-evoked responses are up to 30 mv in magnitude. The light-evoked responses consist typically of one or two spikes superimposed on a large, transient, depolarizing slow potential that lasts for about 250 msec. Such transient responses are seen at both the onset and cessation of illumination. Although the magnitude of the regenerative portion of the response is fixed, the latency of the spike varies dramatically with intensity of illumination, as illustrated in Fig. 11. Latencies of over 600 msec at threshold, and less than 200 msec at intensities 3 log units above threshold, were seen in these units.

The threshold for the amacrine response is easy to measure because of the nature of the all-or-none regenerative potentials. Thresholds are at least as low as the measured thresholds for receptors and bipolar cells. This suggests that we do not see the true threshold for the slow-potential units, which is probably buried in the noise of the recording system. This suggests that visual information can be carried by the slow-potential generators in the distal retina with little sign of electrical activity recorded intracellularly in these units.

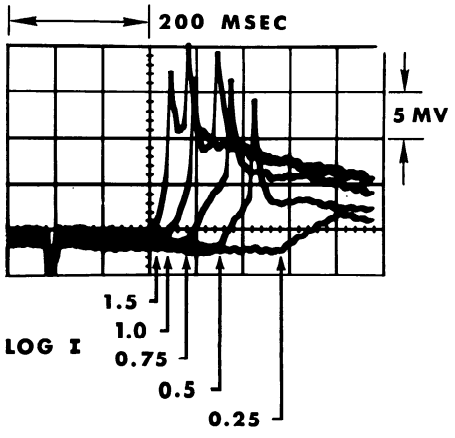


FIG. 11. Latencies of the amacrine response as a function of stimulus intensity. Five responses obtained with different intensities of stimulation were superimposed. Recordings show how the latency decreases with increasing stimulus intensity although the form of the response—one or two spikes superimposed on a transient slow potential —remains relatively unchanged.

Threshold measurements for amacrine cells were made to square pulses of light stimulation, because amacrine cells respond best to change in illumination. If the stimulus intensity was varied slowly enough, it was possible to increase the intensity by many orders of magnitude above threshold without evoking a regenerative response in an amacrine cell.

The dimensions of the receptive fields for the amacrine cells have been difficult to determine accurately. Some units had very broad, uniformly sensitive fields and responded at both "on" and "off" to illumination of any area of the receptive field. Others had narrow centers (measuring 100–200 μ) and larger surrounds like the receptive fields of the bipolar cells (Fig. 3). These units responded at on to central illumination, and at off to peripheral illumination. With diffuse illumination they responded at both on and off.

GANGLION CELL RESPONSES. The response of most of the ganglion cells in the retina of *Necturus* was transient, consisting of a brief burst of impulses superimposed on a small membrane depolarization. Such responses are illustrated in Fig. 3. The rate of impulse firing was roughly proportional to the membrane polarization, thus distinguishing this response from that of the amacrine cell, which consists of only one or two spikes superimposed on a large depolarization (Fig. 3 and Fig. 11). Resting potentials were quite variable and low in the ganglion cells, never exceeding 40 mv. Many of the units recorded from appeared to be spontaneously active, but this may be because they were often damaged upon penetration. The micropipette had to puncture through the internal limiting

membrane just before it penetrated a ganglion cell.

Some ganglion cells responded at on, others responded at off, and some responded at both on and off. In addition to these ganglion cells that responded transiently with illumination, some cells responded with a sustained discharge of impulses when the center of the cell receptive field was illuminated. In such units the sustained discharge elicited by central illumination was inhibited by additional illumination at the periphery of the receptive field, as shown in Fig. 3. The receptive-field organization of these units was very much like that of the bipolar cells. The similarity between these sustained, spike-generating units and the bipolar response is illustrated in Fig. 3 where the membrane potentials for both cell types, although opposite in polarity, follow the same time course for all three conditions of stimulation. The dimensions of the receptive fields for the spike-generating cells were roughly similar to the dimensions for the bipolar cell receptive field, having centers no greater than 200 μ.

A small number of the sustained-type units have been stained, and they were located in the inner nuclear layer. We refer to these cells as ganglion cells because *a*) they show large spikes superimposed on a small membrane depolarization as in the normally situated ganglion cells, *b*) there are sustained-type units in the optic nerve of *Necturus* (19), and *c*) the discharge of these units resembles the responses of ganglion cells found in the frog (19), cat (23), rabbit (3), ground squirrel (28), and primates (20). This suggests that these cells may be the so-called displaced ganglion cells, or Dogiel cells, known to occur in many retinas (11). It will be most interesting if further experiments show that the displaced ganglion cells in the retina have different properties from those of cells located in the ganglion cell layer, as the results here suggest.

DISCUSSION

Outer plexiform layer

The mechanisms by which slow-potential generators in the retina communicate at their synaptic contacts are not understood. However, by comparing the dimensions of receptive fields and cell processes it is possible to infer the neural pathways in the outer plexiform layer and to suggest how the receptive field of the bipolar cell is formed.

The central part of the bipolar cell receptive field, measured physiologically, is roughly 100 μ in width. It is surrounded by an antagonistic region, which is best stimulated with an annulus of 250-μ radius. The dimensions of the central region of the bipolar cell receptive field correspond closely to the width of the dendritic fields of the bipolar cells, as measured in Golgi-stained material (14). No bipolar cell so far observed has a dendritic spread large enough to encompass both center and surround regions of the bipolar cell receptive field. However, horizontal cell processes extend laterally in the outer plexiform layer over distances of 200–400 μ, approximately the dimensions of the surround. Thus, the activity produced by an annulus of 250-μ radius could reach bipolar cell dendrites via the horizontal cell processes. This requires that the horizontal cell be driven by receptors in the periphery and modulate receptor-to-bipolar activity at the center of the bipolar cell receptive field. The synaptic organization observed in the outer plexiform layer of *Necturus* is consistent with this suggestion.

Horizontal cell processes contact receptor terminals together with the bipolar cell dendrites, and both appear to be driven by the receptors. Horizontal cell processes also make specialized contacts with adjacent bipolar cell dendrites, often close to the ribbon synapses of the receptors. The synaptic specializations seen at these contact points suggest that these contact points are probable sites of interaction between horizontal cell processes and bipolar cell dendrites (14).

Thus, anatomical evidence shows that the processes of horizontal cells extend far enough to mediate the receptive-field surround, and that the processes are both postsynaptic to receptors and presynaptic to bipolar cell dendrites. The physiology shows that the effect of the surround is to

antagonize the bipolar cell central response. This suggests that the bipolar cell is polarized by receptors directly at the center of its receptive field and this polarization is antagonized by surrounding receptors acting through horizontal cells, so that differences in levels of illumination in these two areas control bipolar cell polarization. Figures 8 and 9 show that it is the ratio of energy falling in each of these areas that determines that polarization.

There is no direct physiological evidence that the horizontal cell exerts a controlling effect on the bipolar cell, but there have been some suggestive experiments. Byzov (10) recorded the local ERG in the frog while passing current through a micropipette which was located inside an S-potential unit, probably a horizontal cell. He was able to control the magnitude of the local ERG in response to a flash of fixed intensity by passing current through the S-potential unit via the electrode. Since the horizontal cell is situated between the receptors and the presumed site of ERG generation, Byzov's experiment suggests that the horizontal cell can regulate the effect of transmission from receptors to the more proximal retina.

To explain his results, Byzov (10) has postulated that horizontal cells exert their effect presynaptically back onto the receptors. However, we have seen no suggestion of a surround antagonism in the receptors under the same conditions which evoke the surround effect in the bipolar cells, and there is no anatomical suggestion for synapses back onto receptors (14). Tomita (40) also has found no effect of surround illumination in the intracellularly recorded receptor response. Although lack of an apparent surround effect in receptors recorded intracellularly does not prove its absence, both the anatomical and physiological evidence suggest that the horizontal cell acts proximal to the receptors, on the bipolar cell dendrites. Thus it would seem most likely that horizontal cells mediate the surround of the bipolar cell receptive field by antagonizing the effect of direct receptor-to-bipolar transmission.

The results indicate that one of the functions of the outer plexiform layer, in which neurons showing sustained, graded responses interact, is to register sustained differences in intensity as graded polarizations in the bipolar cell. In this way, contrasting boundaries can be accentuated in the visual information brought to the inner plexiform layer by the bipolar cell.

Inner plexiform layer

The neurons proximal to the bipolar cell—the amacrine and ganglion cells—respond to changes in illumination by depolarizing and initiating impulse activity. Ganglion cells showing this behavior have been studied for over 30 years, but amacrine cell activity has not been previously identified although it has probably been recorded before (22, 31, 36, 39).

In the experiments reported in this paper the units we identify as amacrines, generating one or two spikes superimposed on a large membrane depolarization, were consistently found located along the inner margin of the inner nuclear layer. The histology in *Necturus* shows that this region is almost always populated by amacrine cells, and almost never by ganglion cells or bipolar cells (14). In the frog, similar spike-generating units which show a larger membrane depolarization associated with impulse activity than do ganglion cells were also found in the inner nuclear layer (31, 39). These units have not been identified but it was demonstrated that such units cannot be driven when the optic nerve is stimulated antidromically, indicating that they are not ganglion cells (22).

A comparison of the responses recorded under the same stimulus conditions in the bipolar and amacrine cells shows that the amacrine cell is active only during the initial part of the transient in the bipolar cell response, whether this transient is positive-going or negative-going, or at on or off. This raises the question of how the relatively sustained bipolar response is converted to a transient response across the bipolar-to-amacrine synapse. An anatomical observation may have bearing on the question. Adjacent to each ribbon synapse of the bipolar cell terminal, at which there is a bipolar-to-amacrine synapse, there is a return synapse from amacrine to bipolar terminal. If the return synapse were inhibi-

tory, the amacrine cell could turn off its own excitation in some way. However, the solution to this problem requires further investigation.

The activity of the ganglion cells in the *Necturus* retina can be interpreted in terms of the intracellular activity of the neurons that provide their synaptic input. The anatomy suggests two types of synaptic input. Ganglion cells can be driven directly by bipolar cells at the synaptic ribbon or by amacrine cells through conventional synapses, and it has been postulated that some ganglion cells may be driven primarily by bipolar cells while others may receive their major input from the amacrine cells (12, 14). The physiology shows that there are two distinct forms of ganglion cell response: some ganglion cells respond like bipolars, having concentrically organized receptive fields and showing sustained responses which can be antagonized. However, most ganglion cells in the *Necturus* retina behave more like amacrine cells, giving transient responses at on or off or both. Thus the suggested anatomical dichotomy seems to have a physiological expression.

The sustained type of ganglion cell having a concentrically organized, antagonistic receptive field has been described for many vertebrates (2, 3, 19, 23). Some of the special properties of these units, described previously, are consistent with the behavior of bipolar cells in *Necturus* as well as ganglion cells. For example, Barlow, Fitzhugh, and Kuffler (2) in the cat and Barlow and Levick (4) in the rabbit showed that the antagonistic surround is best elicited when the retina is light adapted. This behavior applies to bipolar cells in *Necturus* because the surround can only "turn off" the polarization resulting from central stimulation (Figs. 3 and 7). Barlow and Levick (4) also found in the rabbit that the central response always precedes the peripheral response, regardless of the relative intensities of the two. Figure 10 shows this phenomenon clearly for the bipolar cell in *Necturus,* and Fig. 3 for the ganglion cell.

The on-off units in *Necturus* seem to follow more closely the activity of amacrine cells (see Fig. 3) from which cells the anatomy suggests they receive their primary synaptic input (14). Anatomical studies in many vertebrates (12, 14, 27) suggest that the more complex processing in the retina is carried out in the inner plexiform layer, probably by amacrine cells. The electrophysiology of the neurons in *Necturus* shows that amacrine cells are primarily responsive to temporal changes in illumination, and that their activity is particularly suitable to accentuate dynamic properties of the visual world such as motion. Further work should serve to elucidate the mechanisms by which the complex retinal functions are performed in the inner plexiform layer.

In conclusion, the data presented in this paper show that two transformations are made in tandem in the retina at each of the plexiform layers. Figure 12 shows schematically how these transformations are accomplished. At the outer plexiform layer a contrast detection is effected so that the final bipolar cell polarization reflects the ratio of energy falling in the center of its receptive field to the energy falling over a wider peripheral area. This transformation appears to be accomplished primarily at the base of the receptor. Here processes from the bipolar and horizontal cells receive input from the receptors, whereas the horizontal cell processes feed across to adjacent bipolar dendrites. It is suggested that the effect of the horizontal cell contacts is to antagonize the effect of the receptor-to-bipolar response. Because horizontal cells have a much wider dendritic spread than do bipolars, a center-surround antagonism is formed. All elements involved in the synaptic interaction are slow-potential generators, mostly hyperpolarizing, and the responses are all graded and sustained with illumination. Some ganglion cells with response properties similar to those of the bipolars carry the transformation effected at the outer plexiform layer to higher centers.

The inner plexiform layer operates on the transformed information coming to it via the bipolar cells. Here the dynamic qualities of the visual image—temporal changes in either the intensity or configuration—are detected and amplified by the amacrine cell system. Amacrine cells impinge on ganglion cells and drive them

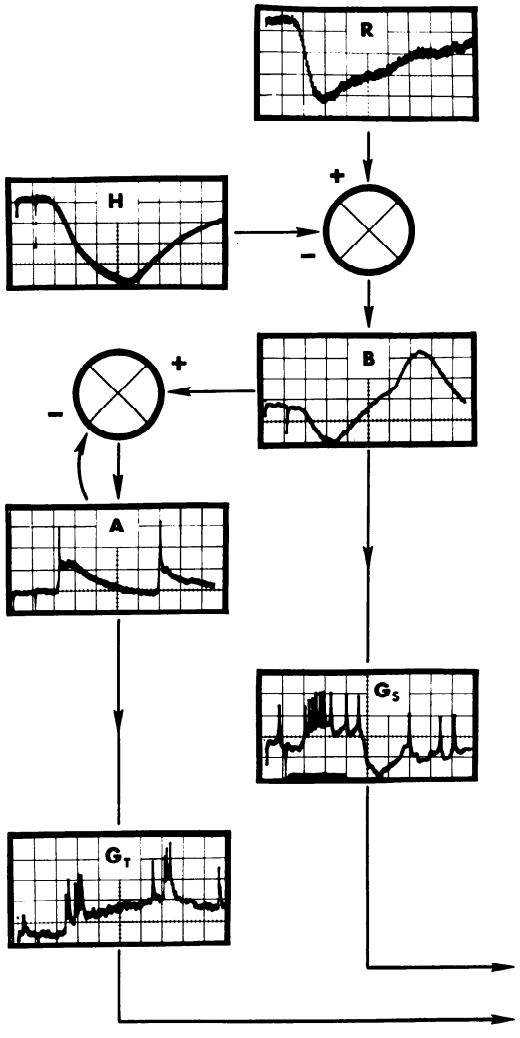


FIG. 12. Summary diagram of synaptic organization of the retina. Transformations taking place in each plexiform layer are represented here by summing junctions. At the outer plexiform layer the direct input to the bipolar cell from the receptor is modified by input from the horizontal cells. At the inner plexiform layer the bipolar cell drives some ganglion cells directly. These ganglion cells generate a sustained response to central illumination, which is inhibited by additional annular illumination; and thus these cells follow the slow, sustained changes in the bipolar cells' response. Bipolar cells also drive amacrine cells, and the diagram suggests that it is the amacrine-to-bipolar feedback synapse that converts the sustained bipolar response to a transient polarization in the amacrine cell. These amacrine cells then drive ganglion cells which, following the amacrine cell input, respond transiently.

transiently. The ganglion cells so driven carry information about the changes in the characteristics of the visual field. The optic nerve, which contains axons from both types of ganglion cells, thus carries information both about relative intensities in the visual field and temporal changes in these intensities.

SUMMARY

The responses of neurons throughout the retina of the mudpuppy have been studied by intracellular recording with micropipettes. These neurons were subsequently identified by intracellular staining. The results of the electrophysiological studies were correlated with the synaptic organization of the retina of *Necturus*, and through this combined study of structure and function it has been possible to begin a description of the functional organization in the inner and outer plexiform layers of the retina.

Neurons that form synapses at the outer plexiform layer—the receptors, horizontal cells, and bipolar cells—respond with slow, graded, sustained potentials to illumination. Receptors and horizontal cells always respond by hyperpolarizing, whereas bipolars may either hyperpolarize or depolarize in response to illumination. The polarization of a bipolar cell, produced by illumination at the center of its receptive field, is antagonized when the area surrounding the receptive field is illuminated.

In the inner plexiform layer, the neurons driven by the bipolars—the amacrine and ganglion cells—respond to illumination by depolarizing. Depolarization beyond a threshold level in these cells results in classical regenerative "spike" activity. Amacrine cells respond transiently to either increasing or decreasing intensity of illumination, provided the change is rapid enough. Most ganglion cells respond transiently to changes in illumination. A few ganglion cells respond tonically to steady illumination within their receptive fields.

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