

Intracellular Responses of the Müller (Glial) Cells of Mudpuppy Retina: Their Relation to b-Wave of the Electroretinogram

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THE ELECTRORETINOGRAM (ERG) was first recorded by Holmgren in 1865 (20), and it has proved to be a useful tool for studying retinal function for almost a century. Despite extensive studies, however, the cellular origins of the ERG components proximal to the receptors remain to be established. Quite recently it has become possible to record intracellularly from the retinal cells with fine micropipettes (2, 22, 34, 39), and thus the precise origins of the ERG components can now be investigated more directly.

Our views on the components of the ERG are based mainly on the classic studies of Granit (19, 20). He analyzed the waveforms of the ERG into three components: an initial negative wave (PIII), which lasts the duration of the stimulus; an early transient positive wave (PII); and a late transient positive wave (PI). Granit (19) named these components in order of their sensitivity to ether narcosis. These components interact to produce the a, b, and c waves, respectively, of the intact ERG (16).

PI (the c-wave) appears to originate in the pigment epithelium. Noell has shown that sodium iodate selectively abolishes the c-wave as it destroys the pigment epithelial cells (27, 28). Also, Brown and Wiesel (10) have reported intracellular records obtained from pigment epithelial cells and these records show a c-wave of reversed (negative) polarity with respect to the extracellular c-wave. Although the available evidence indicates the pigment epithelium as the probable c-wave generator, the functional sig-

nificance of this potential is not known. Many vertebrates, including the mudpuppy, lack a c-wave. The c-wave will not be considered in this paper.

Many workers, most notably Brown and his co-workers (5–8), have provided evidence that PIII (whose leading edge represents the a-wave of the intact ERG) is generated by the receptors. In mammals, Brown et al. have measured the PIII voltage-depth profile using micropipettes, and have localized the maximum PIII amplitude near the outer segments of the receptors (7). The polarity of PIII is positive at that point, suggesting that the receptors hyperpolarize to light. Support for this finding has come from intracellular records obtained from photoreceptors of carp, mudpuppy, and gecko (2, 36–39). These records show intracellular hyperpolarization of the receptor in response to light and are consistent with the findings of Brown et al. (5–8). Murakami and Kaneko in the frog (26) and Pautler et al. in the cat and rabbit (30) have demonstrated the presence of subcomponents that contribute to PIII. These workers have suggested that these subcomponents, termed the distal and proximal PIII, may arise from separate retinal structures. They agree, however, that the distal PIII probably originates in receptors. In the mudpuppy, we have found that the latency of the a-wave and the intracellularly recorded receptor response match closely, adding further evidence that the leading edge of PIII, the a-wave, originates in the receptors. The mechanism of the generation of the distal PIII has now been suggested by the experiments of Penn and Hagins (32).

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The cellular origin of PII (b-wave) has not been proven, but most workers have suggested that the b-wave generator lies in the inner nuclear layer. This idea was first suggested by Granit (20) and has since been supported by workers using micropipettes to record the intraretinal ERG (1, 4, 5, 13, 35). Such studies of the intraretinal ERG have shown that the b-wave results from a radially oriented dipole, since the b-wave reverses polarity as the electrode advances from the vitreous to the deeper retinal layers.¹

While there is no good evidence regarding the specific cellular origin of the b-wave, results indicate that the b-wave is initiated by membrane depolarization (as opposed to hyperpolarization). Granit first concluded this, based on the sensitivity of the b-wave to KCl and the general association of the b-wave with "excitation" (21). Some support for this idea has come from micro-electrode studies (4, 13, 20, 35) that show the b-wave to be negative at the level where the radially oriented nerve elements receive their primary synaptic input. In a recent study of the mudpuppy retina, Werblin and Dowling (39) have described the intracellular responses of the neuron types in that retina. Only three types of neurons were found to depolarize in response to a flash of light and thus can be considered as possible b-wave generators. These include the ganglion cells, the amacrine cells, and one class of bipolar neurons.

As a primary generator of the b-wave the ganglion cells can probably be dismissed since Noell has shown that a normal ERG is obtained following ganglion cell degeneration (27). Also, stimulation of the optic nerve does not affect the ERG or result in a detectable ERG (21). Furthermore the anatomical position of the ganglion cells

does not agree with the "localization" results of the b-wave obtained with micro-electrodes.

The amacrine cells, which lie in the proximal margin of the inner nuclear layer, produce a transient depolarization at "on" and "off" in response to a flash of light (39). With flashes of light one frequently observes one or two "spikes" riding on the transient depolarizations of the amacrine response (Fig. 1). Using extracellular microelectrodes in the frog, Burkhardt has recently separated the extracellular amacrine response from the intraretinal b-wave (11, 12). The extracellularly recorded amacrine response closely resembles in form and intensity-response relations the intracellularly recorded amacrine response. The absolute latency of the amacrine response, recorded either intra- or extracellularly, is similar to b-wave latency, but the peak latency of the amacrine response is considerably earlier than the peak time of the intraretinal b-wave (Table 1). Also, the extracellular amacrine response behaves quite differently to various stimulus spot sizes, position, and intensity, as compared with the intraretinal b-wave (11, 12). These results make it doubtful that the amacrine contribute directly to the b-wave (11, 12).

The bipolars in mudpuppy are of two types: one hyperpolarizing and the other depolarizing (39). Both types reveal a center-surround organization. Light falling on the center produces a sustained response, whereas light falling on the surround inhibits the center, driving the membrane potential closer to the dark-adapted resting level. To see the effects of the surround it is necessary that the center be illuminated since the surround never drives the membrane "beyond" the dark-adapted resting

TABLE 1. Latencies of retinal responses ($\log I = -1$)

	Absolute Latency, msec	Peak Latency, msec
Horizontal cell	30-40	
Bipolar cell	30-40	135-150
Amacrine cell	100-110	110-115
Müller cell	90-100	280-300
b-Wave	90-100	280-300

¹ Recently, PII in mammals has been divided into "subcomponents." Brown and Wiesel (9) have shown with vitreous recording in cats that light pulses of decreasing intensity result in the gradual loss of the b-wave and the appearance of a steady positive potential which they have termed the d-c component; according to their model, the off-effect of the mammalian ERG is a result of interaction between PIII and the d-c component. The d-c component has not been observed in amphibians; it will not be considered further here.

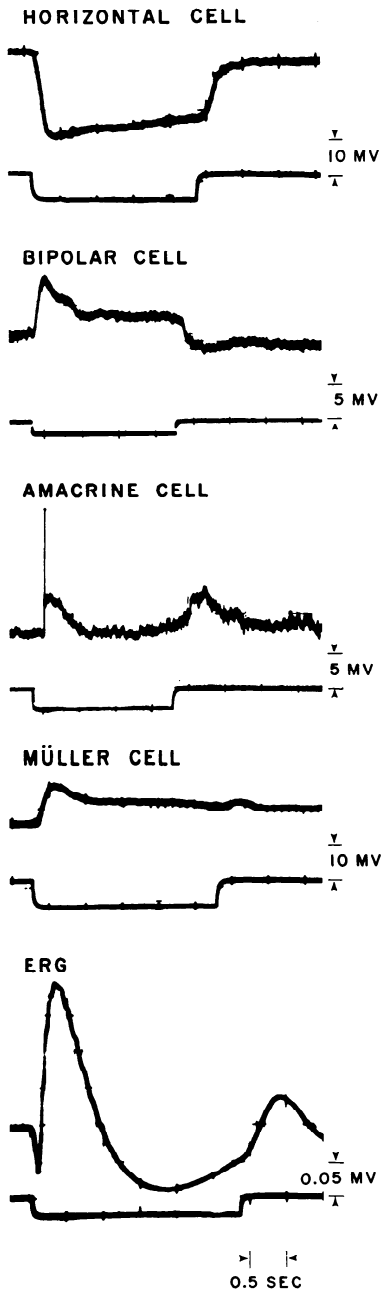


FIG. 1. Intracellular responses recorded from cells of the inner nuclear layer of the mudpuppy compared with the ERG. All responses were evoked by light flashes of constant intensity that uniformly illuminated the entire retina. All recordings are dc, except for the ERG (capacitance coupled, time constant ca. 1 sec). Horizontal and bipolar cell responses show a similar latency that is much earlier than the latency of the amacrine cell, Müller cell, or b-wave response. Although the amacrine cell has a latency similar to the b-wave, the peak delay time

potential. The effects of the surround are mediated through the horizontal cells and a delay exists between the more direct center response and the somewhat later surround effect. Thus light falling simultaneously on the center and surround (as is the case when eliciting the gross ERG) results in a peak appearing in the bipolar response caused by the delay from the inhibitory surround (Fig. 1). Although the appearance of this peak is somewhat similar to the b-wave, both the absolute and peak latency of the bipolar response occur considerably earlier in time as compared with the b-wave peak and absolute latency elicited with the same stimulus intensity (Fig. 1). For example, with flash intensities about 5 log units above b-wave threshold, the bipolar response latency is 30–35 msec, whereas the b-wave has a latency of 90–100 msec (Table 1). In addition, intensity-response curves for the bipolar response are considerably steeper than are the intensity-response curves for the b-wave of the ERG (39). The bipolar intensity-response curve saturates within 2 log units of threshold, whereas the b-wave increases in amplitude over some 4–5 log units of intensity. Thus, the bipolar response also does not parallel the b-wave in several crucial respects.

In view of the problems of associating the b-wave with one of the known retinal responses, we decided to reinvestigate the mudpuppy retina, hoping to find intracellular responses that produced a more suitable match for the b-wave. We have been successful in recording intracellularly from units we believe to be Müller (glial) cells of the retina. These cells produce a depolarizing response to light, which in the best examples appear remarkably similar to the vitreally recorded b-wave (see Fig. 10). This paper will describe the structure of the Müller cells in the mudpuppy retina and the intracellular responses obtained from

is much earlier than the peak delay time of the b-wave. The Müller cell response is the intracellular slow potential that best matches the b-wave in latency, peak delay, and waveform. The range of latencies and peak delay times measured for neurons, Müller cells, and the b-wave are listed in Table 1.

these cells. Comparisons of the Müller cell responses and the b-wave of the ERG will be provided.²

METHODS

Necturus maculosus varying in size from 8–14 inches were flown to Baltimore from the Midwest. The animals were kept in aerated water-filled tanks maintained at about 6 C until their use in experiments. In general the larger animals (12 inches or more) tended to have an unusually viscous vitreous humor; this proved difficult to remove and sometimes interfered with our ability to penetrate the retina with microelectrodes. Often the vitreous was viscous enough to cause bending of the microelectrode tip. This problem was less noticeable with the smaller animals, and they were generally preferred.

Animals selected for experimentation were decapitated and following removal of the eye, the cornea, iris, and lens were excised to expose the entire retina. Dissection was carried out under a dissecting microscope and dim illumination. Most of the vitreous was removed by absorption onto a small piece of tissue paper. The eyecup was placed on a small piece of Ringer-soaked cotton and mounted on a chlorided silver plate. The silver plate had a spherical impression that provided a snug fitting for the cotton surrounding the eyecup.

Microelectrodes were made from borosilicate glass tubing (1.0 mm od, 0.5 mm id) pulled on a modified Livingston puller (39). In the present series of experiments all but a few records were obtained using dye-filled electrodes. The electrodes were filled in either of two ways. In one method, the electrodes were immersed in

dye and boiled at 80 C under reduced pressure. The second method used was to pull electrodes using glass tubing which had a small bundle of fiber glass fibers threaded through the lumen. Electrodes pulled in this manner were easily filled by injecting the dye into the nontapered end; the dye diffused to the tip within 5 min. Electrodes filled by either method had resistances of 100 to 200 megohms measured in Ringer.

Electrodes selected for use were mounted in an electrode holder filled with the dye solution. A chlorided silver wire contacted the solution and was led to a switch box. The switch box was mechanically isolated from the preparation; it allowed the introduction of two different current sources to the microelectrode. In the normal position the microelectrode was connected to a cathode follower, the output of which was fed to one beam of a Tektronix 502A oscilloscope. A second switch position shunted the microelectrode by a bridge that provided current pulses of 5–20 na. A third switch position disconnected the microelectrode from the cathode follower and connected it to a larger current source, a 400-v supply passed through a 100-megohm current-limiting resistor. This method of current passing has been described previously (39).

To record the gross ERG we used a Ringer-soaked cotton wick sealed in a small tapered glass tube lowered into the eyecup until it contacted the vitreous. A chlorided silver wire contacted the cotton wick, and the light-induced potentials were recorded between the vitreous and the grounded silver plate. The signals were amplified and displayed on one beam of a 502A Tektronix oscilloscope. All recordings shown are d-c recordings, except when noted.

Usually, ERG recordings and Müller cell recordings were not obtained simultaneously. The eyecup of mudpuppy is small (less than 4 mm in diam) and it proved desirable to explore the entire retinal surface with micropipettes without interference from the larger wick electrode. Furthermore, the ERG of mudpuppy is comparatively uniform among animals, if they are in good condition and provided the experiment does not last longer than about 2 hr. In some preparations we measured the gross ERG first and then removed the gross electrode to begin retinal penetrations with microelectrodes. In many instances, however, microelectrode penetrations were initiated immediately after dissection and gross ERG records were not obtained.

Light stimulation

Background and stimulus light were obtained from a 45-w quartz-iodide lamp. Aperture images

² For the past 15 years, slow hyperpolarizing potentials have been recorded intracellularly from various retinas, particularly the fish retina (34, 36). These potentials have been called S potentials, and recent evidence shows they arise from the horizontal cells (39) (see Fig. 1). The S potentials behave differently to metabolic inhibitors, temperature change, and NH_3 , as compared with classical neuronal potentials, and thus it has been suggested, particularly by Svætichin and his colleagues (34), that the S potentials are glial potentials. Most investigators presently classify the horizontal cells as neurons, and thus it seems that S potentials should be considered as neuronal potentials. It is universally agreed that Müller cells of the retina are true glial cells; no reports on intracellular responses of Müller cells have appeared heretofore in the literature. It should be pointed out, however, that before the cellular origin of the S potentials was shown to be the horizontal cells it was postulated that some of the S potentials (C type) might arise in the Müller cell (34). This view is no longer tenable.

from stimulus and background beams were focused on the retina by adjusting an objective while observing the preparation through a dissecting microscope. The background and stimulus spots could be moved independently and provided variable spot diameters of 160 μ to 6 mm.

Intensity of the light beams was controlled by neutral-density filters. Stimulus duration and frequency were controlled by electromagnetic shutters. Light intensity was calibrated with a Macbeth illuminometer and maximum intensity was 490 mW. The entire light system was mounted on a bench separated from the preparation. Thus, light intensity and spot position could be altered without causing significant mechanical vibration to the preparation. Recordings from the oscilloscope were photographed on 35-mm film with a Grass camera.

Intracellular staining

To stain cells intracellularly, a number of different dyes and combinations of dyes were tried. In early experiments electrodes were filled with a saturated (ca. 6%) solution of Niagara sky blue, and the dye was ejected by passing relatively large amounts of current, usually sufficient to break the pipette tip. This method has proved suitable for staining the cell bodies of mudpuppy neurons (39). However, this technique proved disappointing in staining the Müller cell. The stain remained localized in the cytoplasm at the point of dye ejection, and the large currents used to eject the Niagara sky blue tended to disrupt the Müller cell membrane and deposit traces of dye into surrounding tissue.

More successful staining was obtained in later experiments by filling electrodes with a saturated solution containing a combination of Niagara sky blue, Procion yellow, and methyl blue (ca. 6% each). Dye could be ejected from such microelectrodes by passing intermittent current pulses of 5–20 na (electrode negative) while still recording the response, and it was found that dye spread throughout the cell. Usually the cell was lost after about 1 min of current passing. At that point the electrode was withdrawn and moved to one side of the recording site 0.5–1 mm away. The electrode was then lowered into the retina and connected to the large current source. Enough dye was ejected until a small dark spot was visualized at the electrode tip (a dissecting microscope was necessary to see this); this usually required about 20 sec of current application. The eyecup was allowed to remain for about 30 min and was then placed in a solution of 1% osmium tetroxide dissolved in Ringer and adjusted to pH 4 with acidic buffer. After fixation of about 15 min the eyecup was placed under a dissecting microscope; the sclera and choroid were removed and a 2-mm section of retina that contained the large stain spot was cut out so as to include the original recording site (it was not possible to detect the original recording site at this point). The tissue was placed in the osmium tetroxide for another 45 min, dehydrated, and embedded in plastic according to the protocol outlined by Werblin and Dowling (39). Sections were cut on a Porter-Blum microtome at 10 μ . Sections were examined both under the light microscope and a fluorescence microscope.

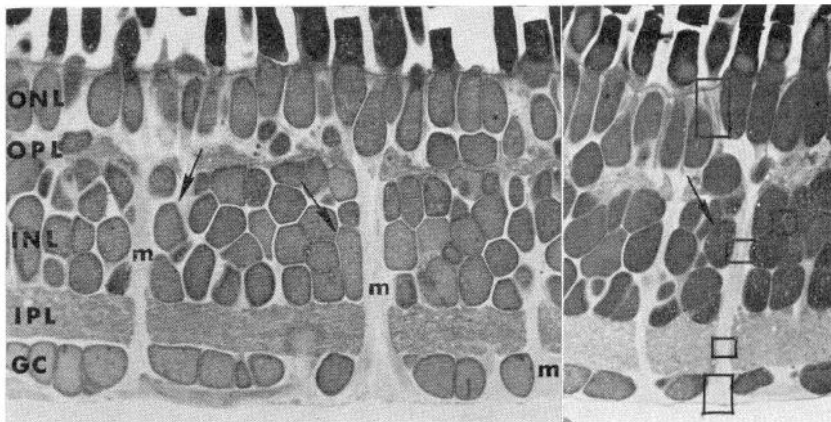


FIG. 2. Light micrographs of the mudpuppy retina showing the prominent Müller cells (m). Cells extend from the outer limiting membrane (top arrow) to the inner limiting membrane (bottom arrow). Note that the Müller cells extend laterally along the inner limiting membrane to form a widened end foot. Arrows indicate probable Müller cell nuclei. Enclosed areas are similar to the areas illustrated in the electron micrographs. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GC, ganglion cells. $\times 240$.

Light and electron microscopy

Live specimens 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.

For light microscopy, 2- μ -thick sections were cut on a Porter-Blum MT-2 microtome and stained by the Richardson method (33). For electron microscopy, thin sections were cut on the same microtome, doubly stained with uranyl acetate and lead citrate, and studied in an RCA EMU-3G electron microscope.

RESULTS

Light microscopy

Figure 2 shows light micrographs of the mudpuppy retina that illustrates well the prominent Müller cells found in this retina. These cells extend from the outer limiting membrane at the base of the inner segments through the retina to the inner limiting membrane. Throughout most of the retina the Müller cells display a fairly constant column of cytoplasm of some 15–20 μ width. At the inner surface of the retina the Müller cell columns widen, forming substantial end feet, often 30–40 μ across. In addition, fine processes of the Müller cells, which cannot be resolved in the light microscope, extend laterally from the columns so that all of the retinal elements are at least partially enveloped by glial cell cytoplasm.

The nuclei of the Müller cells lie usually in the inner nuclear layer and often are quite elongated. Probable Müller cell nuclei are indicated by arrows. The boxes on the right side of the figure enclose areas similar to those illustrated in the electron micrographs.

Electron microscopy

Figure 3 is a low-power electron micrograph illustrating the distal portion of a Müller cell, from the external limiting membrane to the outer plexiform layer. As can be seen from this micrograph, the external limiting membrane is not a true

membrane but a series of junctional complexes. Extending beyond the level of the external limiting membrane are fine villous processes that run distally between the inner segments of the receptors. Along the outer margin of the Müller cells of the mudpuppy retina, there are numerous deep infoldings of the cell membrane that extend almost to the outer plexiform layer.

Such infoldings have been described in the toad retina; they have not been seen in the Müller cells of the mammalian retina (25). Their possible function has recently been discussed (25). These infoldings appear platelike and are parallel so that in any one section the membranes observed all have approximately the same orientation. The insert of Fig. 3 shows a section at right angles to the plane of the infoldings; the orientation of the infoldings in the main portion of Fig. 3 is about parallel with the section.

The junctional complexes at the level of the external limiting membrane are seen clearly in the inset of Fig. 3 and at high magnification in Fig. 4. Two types of junctional complexes are observed. Between Müller cell processes, the complex consists of an area of close membrane apposition surrounded by an "adhering zonule" (18, 25) (Fig. 4, *a* and *b*). In the area of close membrane approximation the distance between the cytoplasm of the adjacent processes is narrowed to 140 Å. Assuming a membrane thickness of 55–60 Å, this indicates an extracellular space of only 20–30 Å. Thus, these close membrane appositions are probably "gap" junctions (3).

No other close membrane junctions have been observed in the mudpuppy retina, although similar close junctions are seen between the pigment epithelial cells at their apical borders (14) (Fig. 4*d*). The dimensions of these pigment epithelial cell junctions in mudpuppy are similar to those observed between the Müller cell processes, suggesting that these too are gap junctions (3).

The second type of junctional complex occurs between Müller cell processes and receptor cell inner segments. These junctions consist mainly of an extensive adhering zonule (Fig. 4*c*); no extensive close membrane junction has been observed along these complexes. Typically, however,

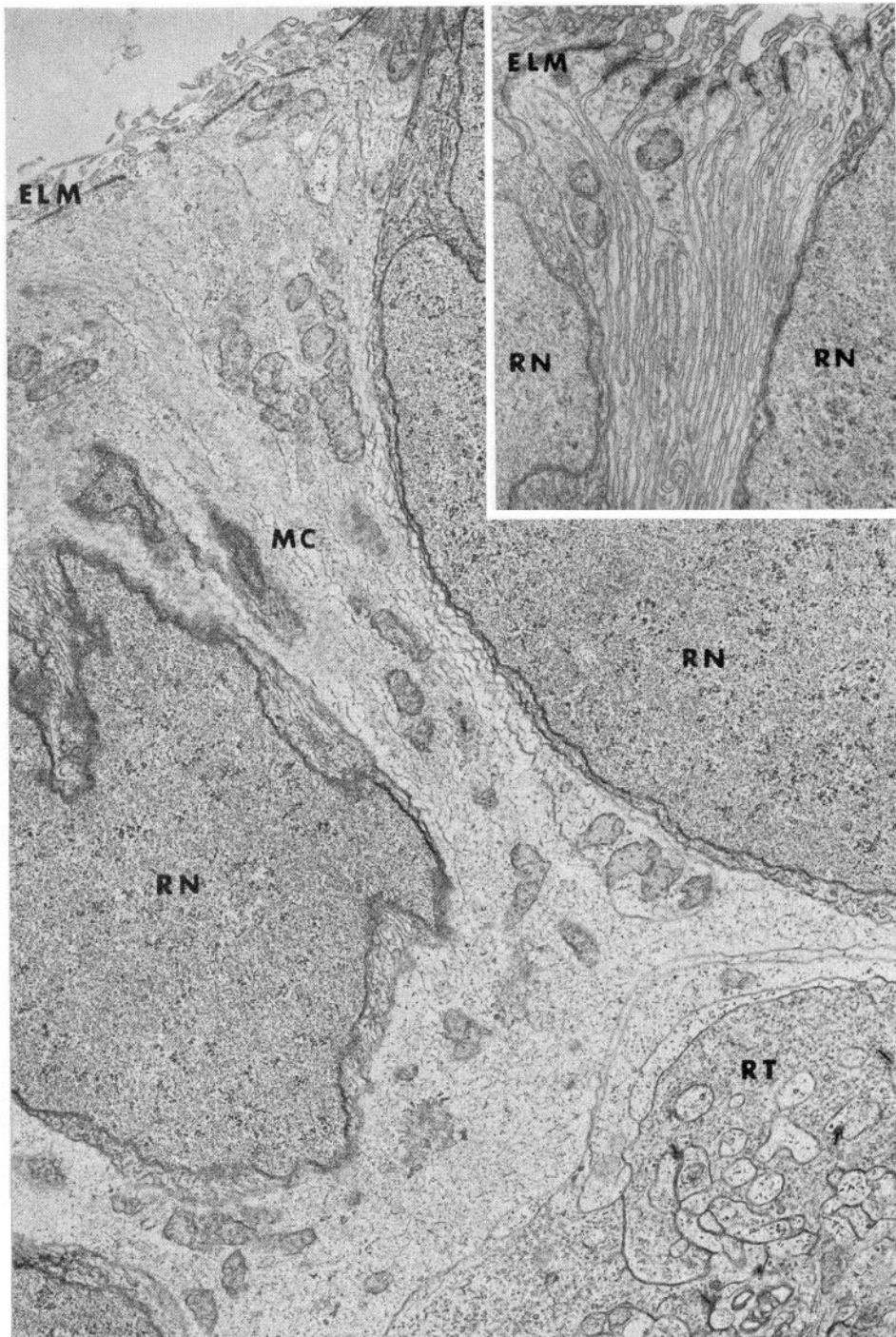


FIG. 3. Electron micrographs of the distal portion of the Müller cell. The external limiting membrane (ELM), consisting of a series of junctional complexes, is shown at the top of the micrographs. Numerous deep infoldings into the Müller cell extend from the ELM almost to the level of the receptor terminals (RT). Infoldings are all parallel so that in any one section the membranes are seen in approximately the same orientation. Infoldings are parallel with the plane of the large micrograph; in the inset the infoldings are cut at right angles to the plane of the section. RN, receptor cells. $\times 7,800$; inset, $\times 15,300$.

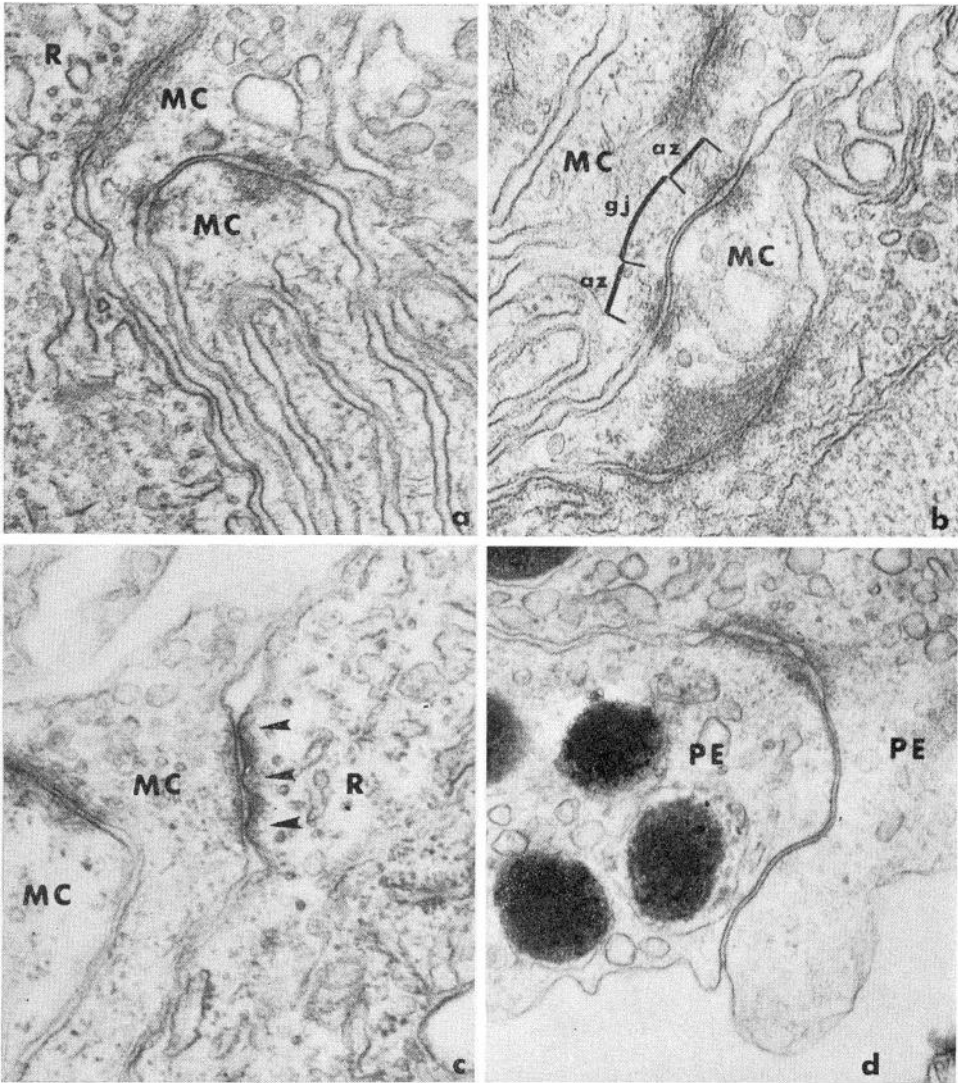


FIG. 4. High magnification micrographs of junctional complexes seen at the level of the external limiting membrane (*a-c*) and between the pigment epithelial cells (*d*). The complex between Müller cell processes (MC) (*a* and *b*) consists of a prominent close membrane apposition (gj) surrounded by an adhering zonule (az). Dimensions suggest that these close membrane appositions are "gap" junctions. Between Müller cell processes and receptor cell (R) (*c*), the complex consists of a large adhering zonule interrupted by a few closely apposed adhering spots (arrows). Between pigment epithelial cells (PE) at their apical border (*d*) is an extended gap junction and smaller adhering zonule. *a-c*, $\times 58,200$; *d*, $\times 54,600$.

there are tiny areas along the adhering zonule where the extracellular space is significantly narrowed. These spots are usually not as closely spaced along the zonule as in Fig. 4*c*, which illustrates these spots well. Similar zones are seen in the published micrographs of the toad retina (25).

In the mudpuppy retina, Landolt club processes extend from many of the bipolar cells to the external limiting membrane

(15). Junctional complexes are observed between Müller cell processes and Landolt club processes and these are similar to the complexes between Müller cell processes and receptor cell inner segments (Fig. 4*c*).

Figure 5 shows Müller cells and their relation to adjoining elements in the inner nuclear layer and inner plexiform layer. In the inner nuclear layer the neurons are mostly separated by Müller cell cytoplasm

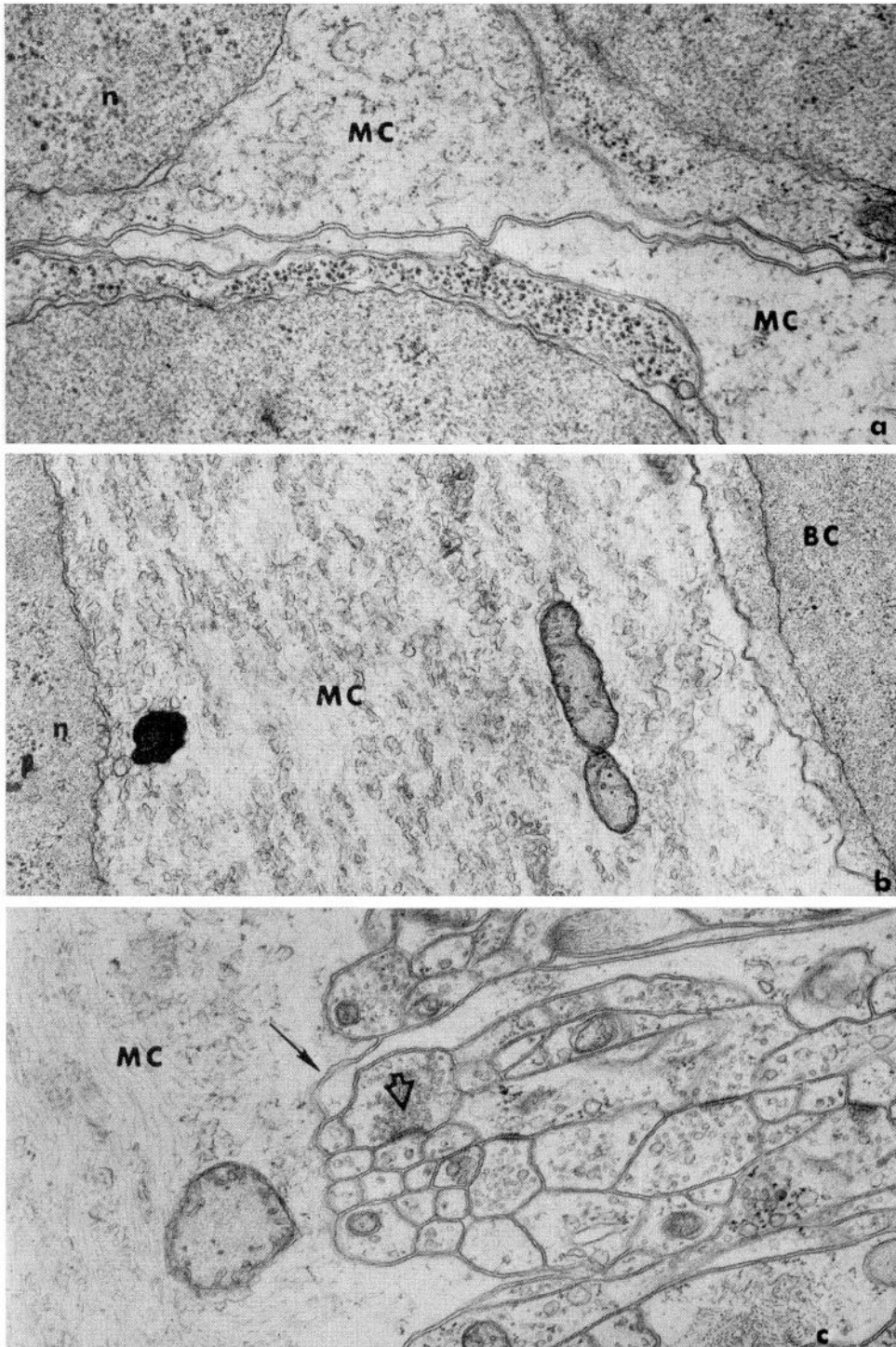


FIG. 5. Müller cell cytoplasm (MC) in the inner nuclear and inner plexiform layers. *a*: Shows portions of two Müller cells in the inner nuclear layer. n, Müller cell nucleus. $\times 25,600$. *b*: Shows a column of Müller cell cytoplasm in the inner nuclear layer. BC, bipolar cell. $\times 10,900$. *c*: Shows a column of Müller cell cytoplasm in the inner plexiform layer. Fine processes of Müller cells extend between the neuronal elements and may reach to adjacent Müller cells (small arrow). The large arrow marks a synapse. $\times 20,000$.

(Fig. 5a), although not infrequently two neurons are seen in apposition for some distance without any intervening glial cell. The extracellular space between glial cell processes and between glial and neuronal cell processes ranges between 150 Å and

200 Å throughout the retina, except at the junctional complexes along the outer margin of the retina.

The cytoplasm in a column of a Müller cell running through the inner nuclear layer is illustrated in Fig. 5b. It is charac-

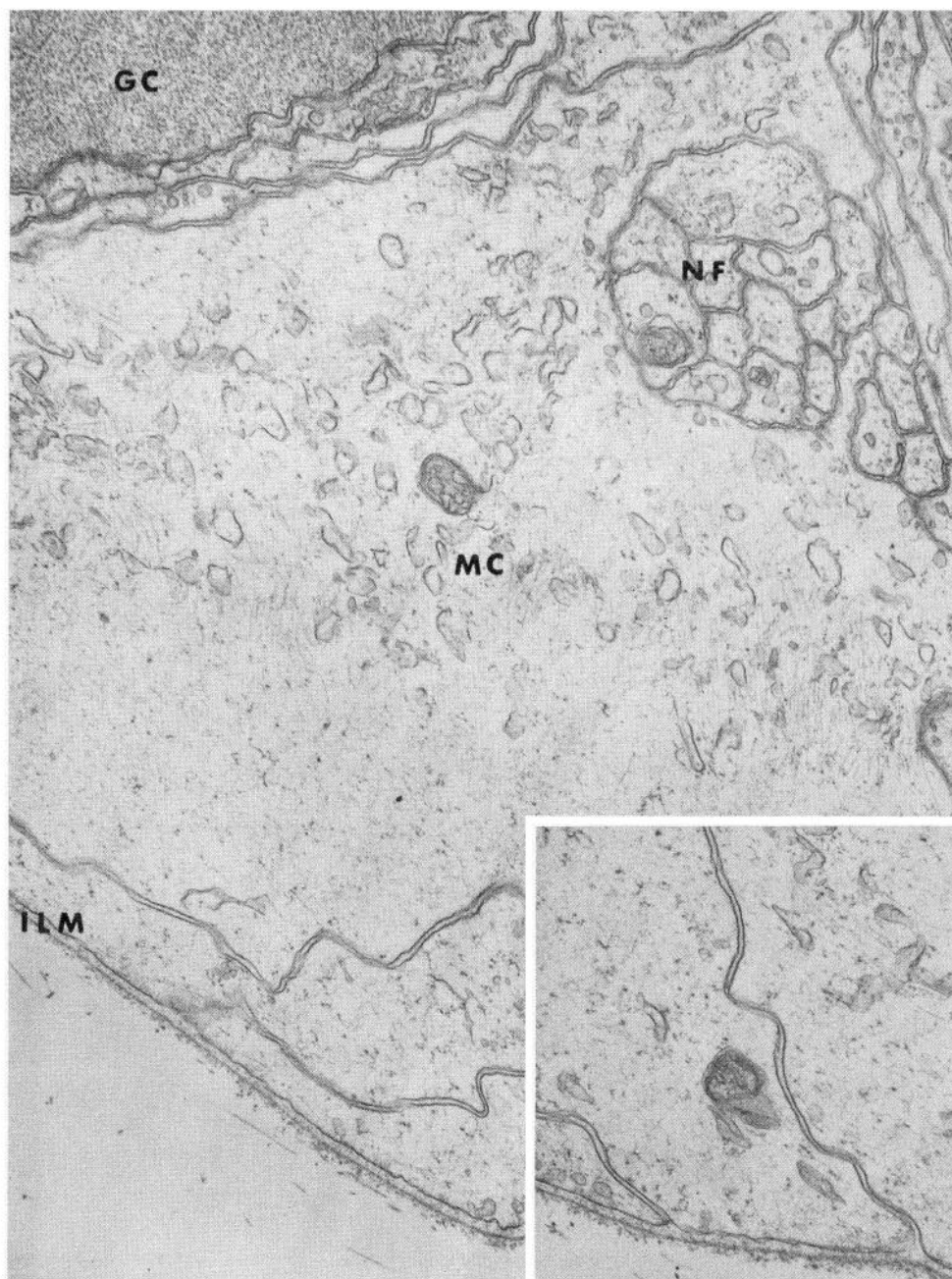


FIG. 6. The expanded basal (end foot) region of the Müller cell. A few infoldings into the Müller cell occur along the inner limiting membrane, but no junctional complexes are seen between Müller cell processes at this level. NF, nerve fibers; GC, ganglion cells. $\times 35,200$; inset, $\times 31,300$.

terized by small, irregular profiles of endoplasmic reticulum, occasional mitochondria, and a few electron-dense inclusion bodies. Fine fibrils, oriented vertically, are observed running throughout much of the Müller cell cytoplasm (Figs. 5 *b*, *c* and 6). Relatively low amounts of glycogen are seen in Müller cell cytoplasm in most preparations of mudpuppy retina, and often the adjoining neuronal cytoplasm shows comparatively more glycogen particles (Fig. 5*a*). Figure 5*c* shows a portion of a column of Müller cell cytoplasm in the inner plexiform layer. Processes from the column extend laterally to run between groups of neuronal elements. Note the long glial cell process, presumably from another Müller cell, that extends between the neuronal processes and slightly invaginates the Müller cell column (thin arrow).

The inner surface of the mudpuppy retina is illustrated in Fig. 6. The inner limiting membrane is seen to consist of a basement membrane and the plasma membrane of the Müller cells. The substantial pocket of Müller cell cytoplasm formed at this level by the expanding end foot of the Müller cell is illustrated well in this micrograph. Occasional infoldings of the plasma membrane of the Müller cell are seen on the inner surface of the retina but they are not as extensive as those described in the toad or reptile retinas (25, 31). No junctional complexes of Müller cell processes are observed along the inner surface of the retina (Fig. 6, insert).

Figure 7 is a summary diagram depicting the structural features of the Müller cells in the mudpuppy retina. The drawing is described in the figure legend.

Intracellular staining

Intracellular recordings from Müller cells were obtained from a wide range of retinal depths extending from the internal limiting membrane to the outer plexiform layer. This finding differentiates the Müller cell response from the neurons of the retina; different neuronal recordings were only obtained at specific retinal depths consistent with the anatomical distribution of the retinal neurons (15, 39). Support for this observation has come from intracellular staining with Niagara sky blue. Figure 8

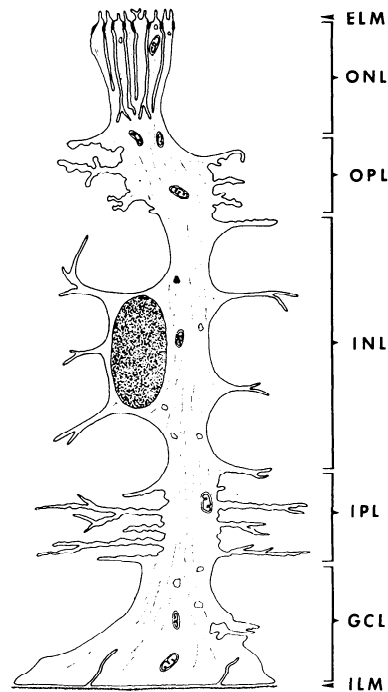


FIG. 7. Drawing of a section of a Müller cell in the mudpuppy retina based on light and electron microscopy. The principal features are the numerous, deep infoldings into the cell at its apical margin, junctional complexes at the level of the external limiting membrane, numerous processes that extend from the main column of cell cytoplasm to fit around the neuronal elements, and the expanded end-foot portion of the cell. These features are all illustrated in the accompanying electron micrographs.

a, *b*, and *c* shows the results of using large currents (μA) to eject dye from a micropipette filled with Niagara sky blue, after recording a typical light-induced slow potential. As noted above, once the Niagara sky blue is injected it does not diffuse through the cell cytoplasm but remains localized, presumably at the site of recording. In addition, passing such large currents frequently disrupts the Müller cell membrane and deposits dye in adjacent tissue. Figure 8*a* shows dye in a Müller cell end foot near the internal limiting membrane. The stain can be followed in the column of Müller cell cytoplasm well into the inner plexiform layer. Though part of an adjacent ganglion cell was also stained, it is primarily the Müller cell process that contains the dye.

Figure 8*b* shows dye deposited in cyto-

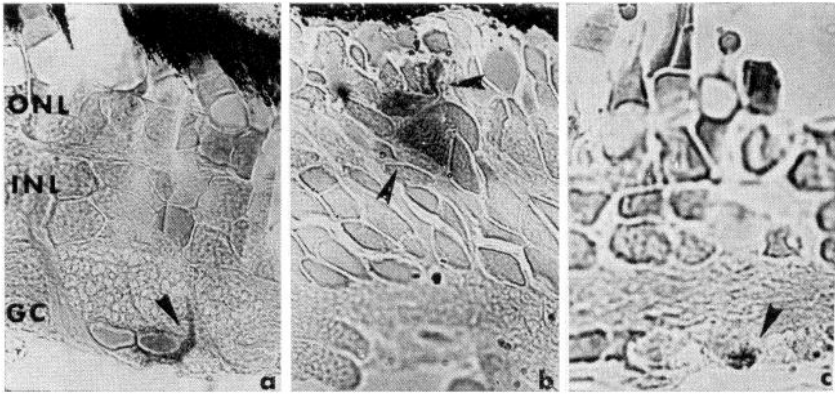


FIG. 8. Intracellular staining with Niagara sky blue ejected with relatively large current pulses. Slow potentials recorded from these sites were almost identical in waveform. *a*: Shows stain deposited near the internal limiting membrane; a column of dye can be seen in a Müller cell extending around a ganglion cell body up into the inner plexiform layer (arrow). *b*: Shows stain deposited in cytoplasm in the outer plexiform layer. A small filament of stained cytoplasm extends between the nuclei of the outer nuclear layer (arrow). Although it is difficult to judge from the micrograph, the dye is continuous with a Müller cell emerging from the inner nuclear layer. *c*: Shows a small amount of dye deposited in cytoplasm at the edge of the internal limiting membrane. The Müller cell is the only retinal cell that has cytoplasm approximating the internal limiting membrane.

plasm in the outer plexiform layer. Although it is difficult to decide from the photograph, observation of the tissue under the light microscope indicates the stain is in Müller cell cytoplasm. A filament of dye is seen extending between receptor nuclei toward the external limiting membrane. In this example no staining appears in any neuron. Figure 8c shows a small amount of dye, deposited just at the level of the internal limiting membrane. The stain must be in Müller cell cytoplasm since the Müller cell is the only retinal cell that approximates the internal limiting membrane.

Thus the Niagara sky blue technique was useful in demonstrating that similar intracellular potentials were recorded from a wide range of retinal depths and this confirmed our micrometer measurements of relative electrode position. However, the technique as employed did not stain the entire cell and furthermore sometimes caused cell disruption, forcing dye into adjacent structures.

The problems of using Niagara sky blue were eliminated with a second staining technique, which resulted in more complete staining of Müller cells (see METHODS). Fig-

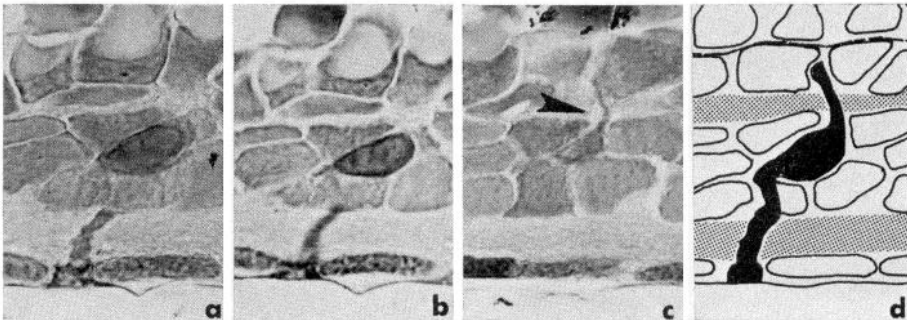


FIG. 9. An example of a Müller cell stained intracellularly with a combination of Niagara sky blue, methyl blue, and Procion yellow (see METHODS). Current pulses of 10–20 na were passed for about 45 sec, at which time the electrode slipped out of the cell. The three micrographs on the left are different focal planes of the same cell to show the extent of staining. *a*: Shows a column of dye in the cell beginning at the internal limiting membrane, extending to the inner nuclear layer. *b*: Shows that the Müller cell nucleus in the inner nuclear layer has also been stained. *c*: Shows a column of dye from the Müller cell nucleus extending to the outer nuclear layer. *d*: A reconstruction of the stained cell.

ure 9 shows one result when using a micropipette filled with Niagara sky blue, Procion yellow, and methyl blue.³ In this experiment small (10–20 na) current pulses were applied while monitoring the cell's response to light. Unfortunately, the cell was lost after about 45 sec of dye ejection and we were unable to stain the cell entirely. However, enough dye was passed to show considerable detail of the Müller cell; it has been stained from the internal limiting membrane to the outer nuclear layer. Three different focal planes of the cell are shown in Fig. 9, and also a reconstruction of the cell drawn by observing the tissue under high-power light microscopy.

Intracellular responses

About 100 eyes were used in the present series of experiments and about 30 stable intracellular records were obtained from Müller cells. Initially, intracellular records were obtained with very fine micropipettes (200-megohm resistance in Ringer) and staining with these pipettes showed that Müller cell responses were obtained from a wide range of retinal depths. However, the very fine pipettes also readily penetrated neurons, and the ratio of neuron to Müller cell recordings was very high. Better results were obtained by using somewhat larger micropipettes (ca. 100-megohm resistance); these reduced the number of neuronal recordings and also restricted the retinal depth at which Müller cell records were obtained. With the larger pipettes Müller cell responses were most commonly seen immediately after penetrating the internal limiting membrane. This is consistent with the anatomy of the Müller cells (see above), which broaden near the inner limiting membrane and present a face to a pipette that may be 30–40 μ in diameter. Penetration of a Müller cell was always accompanied by a rapid negative d-c shift of the recording trace. The extent of the d-c shift

was quite variable; in many instances it could not be measured, but in those we were able to measure it ranged from about 20 to 85 mv (negative). Equally as variable as the d-c shift were the amplitudes of the Müller cell responses, which varied from about 2 to 15 mv. Light-induced Müller cell responses were always positive-going (depolarizing) and no evidence of negativity (hyperpolarization) was ever observed regardless of stimulus intensity.

Figure 10 shows a series of Müller cell and ERG responses elicited by light flashes of comparable intensities. The Müller cell response and the b-wave of the ERG are nearly identical in latency at all intensities. The waveform of the two responses is also

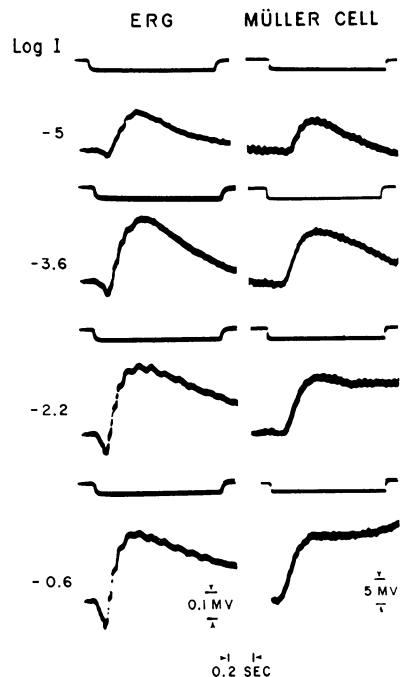


FIG. 10. An example of Müller cell responses (*right column*) compared to the ERG (*left column*) elicited by light flashes of 4 different intensities. At low light intensities (–5.0; –3.6) the b-wave and Müller cell responses show a very similar waveform. At higher intensities (–2.2; –0.6), the ERG appears more complex due to the increased a-wave amplitude and the appearance of prominent “oscillatory potentials” that ride on the envelope of the b-wave. No a-wave or oscillatory potentials are seen in the Müller cell recordings. Also, to bright light flashes (–2.2; –0.6), the Müller cell response tends to remain above the base line for the duration of the flash. The same tendency can be seen in the b-wave, but it is not as striking as in the Müller cell response.

³ Although the dye mixture contained Procion yellow, observation under the fluorescence microscope did not show good fluorescence. It is possible that the presence of the blue dyes “blocked” the ability of Procion yellow to fluoresce. Observation of the sections under the light microscope showed dark stain (probably mainly methyl blue) throughout much of the cell (Fig. 9).

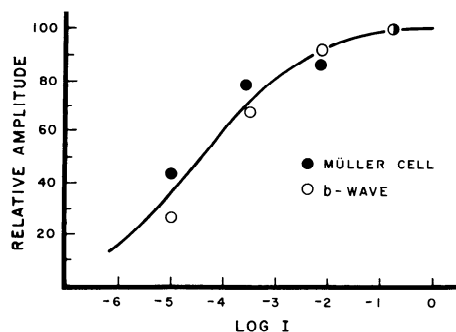


FIG. 11. Intensity-response curves for the Müller cell and b-wave shown in Fig. 10. The b-wave amplitude was measured from the a-wave peak to the b-wave peak. Both the b-wave and the Müller cell response amplitudes are similarly related to $\log I$ over the 5 log units of intensity tested.

similar, although there are some differences. The slopes of Müller cell responses evoked with the two lower intensities match very closely the respective b-waves, but the responses elicited with the brighter light flashes show that the b-wave has slightly greater slope. Note that oscillatory potentials are present on the b-wave but not on the Müller cell responses. In the lower traces the Müller cell response also does not show signs of returning to the base line while the light is on. This tendency to

"saturate," particularly at higher intensities, was a common observation although, as pointed out below, it is comparatively exaggerated in the lower trace of Fig. 10. However, comparing the ERGs of Fig. 10, it is also evident that the b-wave tends to remain above the base line, and this too becomes more exaggerated with light flashes of increasing intensity.

Figure 11 shows the amplitude-intensity curves for the traces of Fig. 10. Both the Müller cell and the b-wave show a dynamic range of about 5 log units; this range is much greater than the values reported for bipolars ($1\frac{1}{2}$ log units) or amacrine (2 log units) (12, 39). Thus the relative amplitude-intensity curves of the bipolars and the amacrine are much steeper than the b-wave and Müller cell curves, and show saturation to light intensities 3–4 log units lower than those required for b-wave and Müller cell saturation.

Recordings from Müller cells showed some variation in amplitude and waveform. Examples of some of these variations are shown in Fig. 12. The Müller cell responses are compared to the ERGs elicited by light flashes of the same intensity. At the termination of the light flash an off-effect was usually observed in most Müller

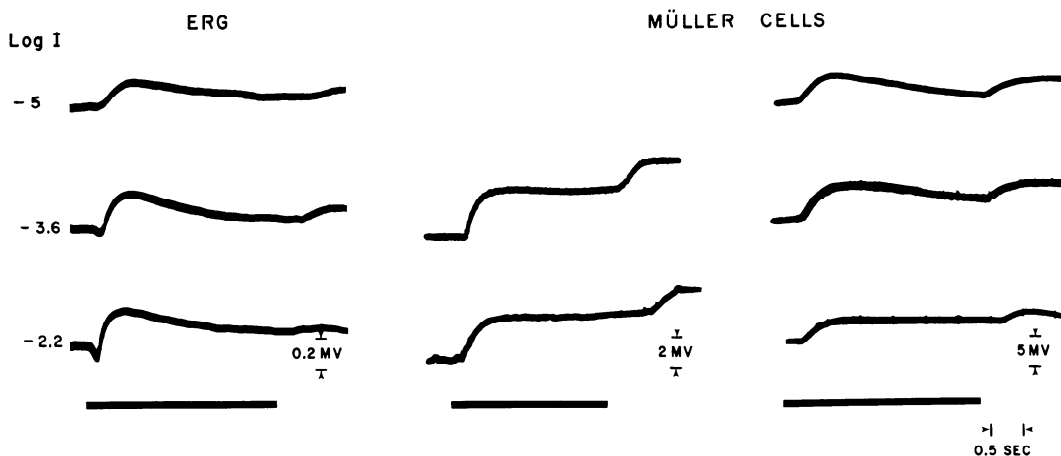


FIG. 12. Variations in the waveform of Müller cell recordings (*middle and right columns*), compared to the ERG (*left column*). Some Müller cell recordings (*right column*) displayed a waveform very similar to the b-wave, while others (*center column*) tended to remain above the base line during the light flash. Some Müller cell responses (*right column*) declined in amplitude to relatively bright light flashes. Müller cell recordings that had responses greater than 10 mv (Fig. 10) did not decline in amplitude to bright light flashes and saturated at about the same intensity as the b-wave. Note that a positive-going off-effect is observed in the ERG and Müller cell recordings. In all examples the off-effect declines in amplitude to relatively bright light flashes.

cell recordings provided that the flash duration was longer than about 1 sec. The off-effect was more prominent with light stimuli of intermediate intensities and was usually not observed with light flashes brighter than -1 log unit. The same relationship was apparent for the off-effect of the mudpuppy ERG.

The receptive fields of the Müller cell responses proved to be very large (ca. 1–2 mm). No center-surround organization was observed; rather, stimulation anywhere in the receptive field depolarized the cell.

As noted above, most responses from Müller cells were recorded just after penetration of the internal limiting membrane. When the cell was lost the electrode usually had withdrawn into the vitreous. However, on several occasions (particularly when the Müller cell was recorded from deeper retinal layers) the electrode dislodged from the cell and remained in the retina, presumably in the extracellular spaces. On these occasions an extracellular intraretinal ERG was

recorded, with a b-wave of opposite polarity as compared to the intracellular Müller cell response. An example of this is shown in Fig. 13. The upper trace shows an intracellular Müller cell response recorded at a sweep speed of 500 msec/cm. The middle pair of records show intra- and extracellular Müller cell responses recorded at 200 msec/cm to show the similarity of latency and rise time of the two responses. Note that the gain of the two records is 5 and 1 mv, respectively. The lowermost record is the extracellular response recorded at 1 sec/cm to show the small positive a-wave that can be seen in the extracellular recordings. An a-wave is never seen in the intracellular (Müller cell) records, regardless of stimulus intensity. It is interesting that the few times we were able to record an intraretinal ERG, the response appeared just after (and in one case just before) recording a Müller cell response. Ordinarily the amplitude of the ERG was too small to be resolved in the noise of the intracellular recording system.

One of the important features of the ERG is its ability to resolve flickering light. In the frog and bird, Granit (20) showed that it is mainly the PIII component of the ERG that responds to flicker. This appears to be the case also for the mudpuppy retina. Figure 14 shows the response to flicker of a Müller cell, a horizontal cell,

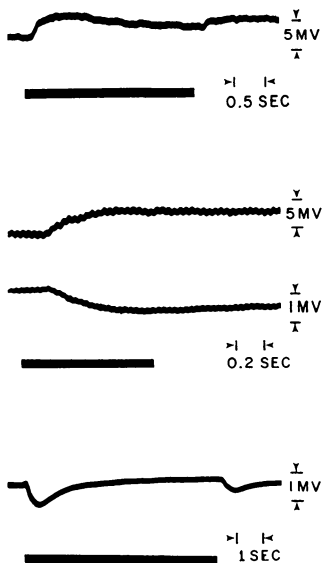


FIG. 13. Intra- and extracellular Müller cell responses. The top trace shows an intracellular Müller cell response recorded at 0.5 sec/cm. The middle two records show intra- and extracellular Müller cell responses recorded at 0.2 sec/cm. The latency and rise time of the two responses are very similar. The lower record shows the extracellular response recorded at 1 sec/cm to show the early, positive a-wave. An a-wave is never seen in the intracellular Müller cell recordings regardless of stimulus intensity.

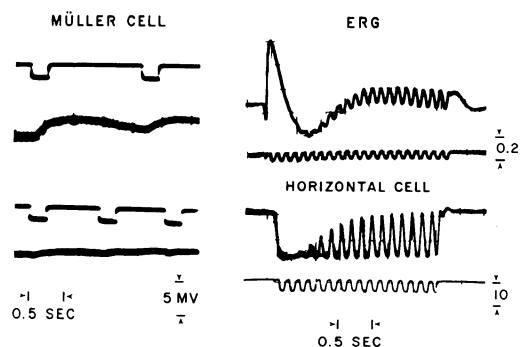


FIG. 14. Traces shown in the left column demonstrate the inability of the Müller cell to follow flickering light. The lower trace shows that flicker fusion frequency has almost been attained to flashes delivered at only 1/sec, and the Müller cell slowly summates. The upper trace of the right column shows the ERG (capacitatively coupled) that easily follows light flashes delivered at the rate of 3/sec. The lower trace shows a horizontal cell response that also follows flicker well, at rates comparable to those of the ERG.

and the ERG. The ERG and horizontal cell respond well to flickering light, up to 18 flashes/sec. In both cases the response to flickering light is a downward (negative) deflection. This is shown most clearly at the termination of the stimulus in both records where it can be seen that the base line is at the top of the potential following the flicker.

The Müller cell resolves flicker poorly, as might be expected from the above observations. The fastest flicker we have observed a Müller cell to follow is 2–3 flashes/sec; with higher flicker rates, the Müller cell potential slowly summates.

DISCUSSION

The present results show that the Müller cells of the mudpuppy respond after light stimulation of the retina with slow, depolarizing potentials. The features that distinguish the Müller cell responses are: 1) Müller cell responses are obtained from nearly all retinal depths, whereas recordings from specific neurons are obtained from discrete retinal layers consistent with their anatomical distribution. 2) Resting membrane potentials obtained from the Müller cells are considerably greater than the maximum values observed in retinal neurons, although a wide range of resting potentials is recorded. 3) Intracellular staining of such units is consistently found in Müller cells. 4) Müller cell responses are the only slow potentials observed in the retina whose latency and waveform appear similar to the b-wave of the ERG.

Intracellular recordings obtained from various retinal cells have now been reported for several species. It seems likely that other investigators may also have recorded from Müller cells, which in some retinas are larger than the neurons. Recently, Byzov has recorded slow, depolarizing intracellular responses from the frog and axolotl retina that match the b-wave of the ERG closely (13). He has termed these “slow bipolar” responses, but has not identified their origin by intracellular staining. Byzov’s records of the “slow bipolars” look identical to our records obtained from Müller cells. Also like the Müller cell response,

Byzov’s “slow bipolars” resolve flickering light poorly. In frogs especially, the bipolar cells are small and the possibility of successful and stable intracellular recordings from these neurons appears low. It seems likely to us that Byzov’s “slow bipolars” are Müller cell recordings.

Our results suggest that Müller cells are involved in one of the current paths associated with the b-wave; it remains to be shown how the Müller cell potentials are generated. In a few experiments it was possible to record responses of opposite polarity after the electrode slipped from the cell or just before the electrode penetrated the Müller cell membrane (see above). This suggests that the intracellular responses recorded from the Müller cells were generated across the Müller cell membrane. If the Müller cell membrane were simply a passive resistance to current flowing from other cells, one would expect to see, for example, an a-wave in the intracellular records, since the extracellular intraretinal ERG shows an a-wave as well as a b-wave; this was never observed.

An additional argument against the Müller cell behaving as a passive resistance to current flowing from other cells is that the largest responses were recorded intracellularly, whereas the extracellular ERG was always small and usually only recorded under special circumstances (i.e., immediately before penetrating the Müller cell membrane or just after leaving the Müller cell). A passive resistance theory could be supported only if the intracellular responses were smaller than the extracellular response, because of the voltage drop across the Müller cell membrane.⁴

Our observations on Müller cell responses are similar to the observations on glial cell

⁴ This argument assumes that no large discontinuities of retinal resistance are placed between the internal and external limiting membranes. To date there seems to be no morphological or physiological evidence that such barriers exist. If this were not true then it might be possible for the Müller cell to behave as a preferred pathway for current produced by other cells, and intracellular Müller potentials could thus be larger than extracellular potentials. If, however, such barriers did exist then the intracellular Müller response should be a derivative of the extracellular waveforms, but this does not appear to be the case.

responses made elsewhere in the nervous system. In the glial cells in the optic nerve of the mudpuppy, Orkand et al. (29) have shown that the glial cells are impassive to currents flowing down the optic nerve fibers. The glial cells behave as K^+ electrodes and produce potentials in response to changes in the K^+ concentration of the extracellular space; this was true whether the changes in K^+ concentration were produced artificially (by changing the bath medium) or "naturally" by light or electrical stimulation of the optic nerve (24, 29). The glial potentials produced in this manner tend to be relatively slow compared to the nerve action potential. Orkand and colleagues have shown that potentials produced by the glial cells are capable of initiating extracellular current flow (29). Although the glia are relatively short, they are connected by low resistance contacts; thus potentials generated at one locus of the cell are capable of spreading to neighboring cells, and inducing current flow just as in a passive nerve (23).

The possibility of a Müller cell potential initiating extracellular current flow is tenable only if a portion of the Müller cell initiates the response. If the entire Müller cell generated a potential which was everywhere equal, the extracellular current produced would be small. Thus, it seems necessary that the Müller cell initiate current flow from a "focal" point somewhere along the Müller fiber.

In recent years, studies of the intraretinal ERG have suggested that the b-wave origin is a very restricted space deep within the retina. The most thorough of these studies has been carried out very recently in the rabbit by Faber, who has done a second spatial derivative analysis of the intraretinal voltage profiles corrected for variation in retinal resistance (17). From these data Faber has been able to plot the "sources" and "sinks" of the extracellular b-wave current. The sink of the b-wave lies in the outer plexiform layer, and the sources lie distally and proximally from that site. The maximum b-wave positivity is recorded in the vitreous.⁵ From these data Faber has

argued that the Müller cells are the only retinal cells that can explain the distribution of extracellular current flow of the b-wave.

It would appear, therefore, that the cells whose activity results in the depolarization of the Müller cell lie in the distal portion of the inner nuclear layer. The responses of these cells (the horizontals and bipolars) have a considerably shorter latency and faster time course than the Müller cell response (Fig. 1, Table 1). This, however, is expected from the results of Orkand et al. (29) who have shown that depolarization of the glial cells in the optic nerve of the mudpuppy begins well after the neural activity initiating the depolarization begins and that the rise time of the depolarization is likewise slow. In conclusion, our findings support the notion that a local depolarizing potential occurs in Müller cells as a result of light-induced activity of distal retinal neurons. This depolarization causes a radial flow of current through the retina that is recorded as the b-wave of the ERG.

SUMMARY

Intracellular slow potentials were recorded from the Müller (glial) cells of the mudpuppy retina. Such responses were recorded from a wide range of retinal depths, a finding consistent with the anatomical extent of the Müller cells. Localization of the responses was confirmed by intracellular staining. Resting potentials of the Müller cells were in general larger than the resting potentials of the retinal neurons. Light-induced slow potentials recorded from Müller cells were slower than the neuronal responses. They were depolarizing and showed a latency and waveform similar to the b-wave of the ERG. The Müller cell is the only retinal cell thus far tested that shows an intensity-response curve similar to the b-wave: the neuronal responses reach maximum amplitude with light flashes of low or intermediate intensity, whereas the Müller cell response and b-wave saturate

small spots of light centered over the intraretinal electrode (1). Their results disagree with Faber's in some respects. However, Faber's technique has eliminated several variables not controlled in Arden and Brown's experiments; a full discussion of these discrepancies is presented in Faber's thesis (17).

⁵ Arden and Brown have determined the "voltage-depth" profiles of the cat intraretinal ERG, using

only to intense light flashes. The Müller cell response also shows a depolarizing off-response that is similar to the off-effect of the ERG. Suggestive evidence is presented that the Müller cell may generate the b-wave by a K^+ -regulated mechanism similar to glial cell potentials reported elsewhere in the nervous system.

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