Functional histology of the retina

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Visual perception is a sensory process initiated at the retina, and completed in the cerebral cortex. Two main functions are currently performed by the retina: 1) the initial conversion of light energy into electric signals, phototransduction, which is carried out by photoreceptors; 2) a series of physiological processes performed by retinal interneurons (bipolar, horizontal and amacrine cells), in order to encode the different attributes of the visual stimuli (shape, movement and color) in electrical signals. The most direct pathway: photoreceptors - bipolar cells - ganglion cells is influence by two additional cell types: in the outer plexiform layer by horizontal cells that act on surrounding bipolar cells; and in the inner plexiform layer by amacrine cells, that modulate surrounding ganglion cells. Central nervous system, like retina, may be studied under several types of techniques. This chapter will deal with different microscopical techniques: iontophoretical intracellular injection, scanning and transmission electronic microscopy, and confocal immunofluorescence microscopy, that help us to understand the physiological mechanisms performed by retinal neurons.

Keywords: scanning; transmission electronic microscopy; confocal immunofluorescence microscopy; isolated cells; immunohistochemistry; iontophoretical intracellular injection.

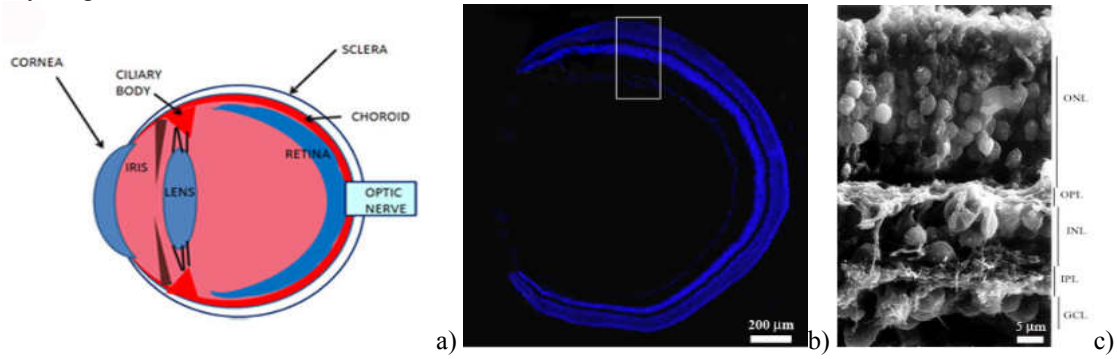
1. Anatomical structure of the eye

The eye is an organ specialized for the detection and analysis of light. The eye is a fluid chamber enclosed by three layers of tissue. The outermost of the three coasts of the eye consists of cornea, limbus and sclera. The transparent cornea makes up about 18% outer coat of the eye; the white opaque sclera accounts for most of the rest. Both tissues consist mainly of collagen fibers. The cornea is the principal refractive element of the eye. The sclera is rigid and resistant to penetration; it is able to protect the more delicate inner layers. The limbus is the region of transition from cornea to sclera.

The middle layer, the uveal tract, includes the iris, ciliary body and choroid. The iris is a layer disposed in the anterior pole of the eye, behind the cornea, that limits the quantity of light entering into the eye through the pupil. The ciliary body is adjacent to the iris and it consists in a ring of muscle cells encircling the anterior portion of the eye. The aqueous humor is formed at this site. The ciliary body musculature is part of the system for altering the refractive power of the lens, accommodation. The choroid is composed of blood vessels and pigmented epithelium. This epithelium is composed of dense melanin pigment to absorb and eliminate scattered light that might otherwise degrade the image.

The innermost eyeball layer is the retina that woks as computer that receives inputs from 100 million photodetectors, that are sampling the pattern of light and dark in the image formed by the optical system of the eye. The retina processes the light information that it receives from the surrounding world. This portion of the nervous system processes the light information and transmits it to the brain via the optic nerve which exits the eyeball from its posterior pole. Retina works detecting light in the retinal image and sending it to the brain, but not as a simple point-by-point representation of the image. In the retina, complex processes are carried out before sending the information to superior centers.

In general, good vision requires an in-focus retinal image, an appropriate pressure, integrity of vascular systems and anything that affects the milieu of the retina.



a) b) c)

Fig 1. **Structure of the eye**. **A** shows a schematic draw of the eye. **B** shows a cross section of the whole retina stained with DAPI to stain the nuclei and viewed under epifluorescence. **C** shows a cross retinal section viewed under scanning.

2. Retinal structure: layers and cells

Vertebrate retina is organized in superimposed layers, formed by the different cells. The retina contains five mayor types of cells: photoreceptors (rods and cones), bipolar cells, horizontal cells, amacrine cells and ganglion cells (RGC). In general, cell somas are grouped in three distinct nuclear layers, separated by two connecting layers plexiform layers, where synapses between cells are formed. The innermost layer is the ganglion cell layer, which contains the cell bodies of the ganglion cells and displaced amacrine cells. The next cell layer is the inner nuclear layer, which contains the cell bodies of the amacrine cells, the bipolar cells, and the horizontal cells; it may also contain some displaced ganglion cells. The next cell layer is the outer nuclear layer, which contains the cell bodies of the photoreceptors. Outside of these layers, the layer of photoreceptor outer segments contains the light-sensitive elements of the retina. Light must pass through vitreous humor and the different layers of the retina before reaching the outer segments of the photoreceptors. Interspersed between the ganglion cell layer and the inner nuclear layer is the inner plexiform layer, which contains the axons of bipolar cells, dendrites of ganglion cells and cell processes of amacrine cells (axons and/or dendrites). Between the outer and inner nuclear layers is the outer plexiform layer, which contains the axon terminals of photoreceptors, the dendrites of bipolar and cell processes of horizontal cells (axons and/or dendrites).

The basic system of retinal information processing consists on a direct pathway of visual information that flows from photoreceptors to bipolar cells to ganglion cells. The ganglion cells fire action potentials in response to light, and these impulses propagate down the optic nerve to the projection nuclei in the brain. This direct pathway is influenced by two transverse fluxes of modulatory signals coming from horizontal in outer plexiform layer and amacrine cells in inner plexiform layer. Horizontal cells receive input from the photoreceptors and project their processes laterally to influence surrounding bipolar cells. Amacrine cells receive input from bipolar cells and project their processes laterally to influence surrounding bipolar and ganglion cells. Both, horizontal and amacrine cells usually make electrical and chemical synapses with neighbor cells of the same type.

3. Design of histological techniques

Central nervous system may be studied under several types of techniques. Appropriate choosing of tissue is an important part of the study. Retina is a privileged part of the central nervous system for its accessibility and the possibility of study isolated from the rest of the eye.

For studying a tissue many histological techniques may be applied from a simple staining with hematoxiline-eosin to label nuclei and neuronal processes, respectively, to a Golgi staining that labels different cells depending on the cellular pH. Many works of Ramon y Cajal were made with this technique, since gives precious information of single cells, rather than the whole population of a tissue. The problem is we cannot decide which cell is stained.

This problem may solved by other techniques. For example, a cell may be selectively stained by iontophoretical intracellular injection of fluorescent tracers like Lucifer Yellow, or substances that may be processed like neurobiotin, making stable and durable preparations. Previously to cell staining is necessary to be able of visualize and identify it. A way of labeling RGCs is injecting into the targets (*superior colliculi*) or into the axonal pathways (optic nerve) a neuronal tracer. In addition, these techniques allow us to estimate neuronal populations in a selective way.

With the development of immunohistochemistry the microscopy gained a great advance. The possibility of selectively labeling a protein contained only in a type of cell allows studying specifically its structure and function. Initially immunohistochemistry were developed with H_2O_2 and diaminobencidine to obtain a brown stable labeling. These preparations could be observed along the time under light microscopy and without loss of staining. Later, immunofluorescence by adding a fluorocrome to the secondary antibody showed some advantages. It was takes less time and steps to complete the process. However, the fluorescence fades with time and exposition to the light. A new advance came with the application of different fluorocromes to the secondary antibody. It was possible labeling simultaneously, and differentiates, two or more proteins in the same preparation and to study their relationships. With the development of confocal immunofluorescence microscopy it was possible to improve focuses and images quality, to make co-localizations studies of two or more proteins in the same tissue, 3D reconstructions, and decreases fading of fluorescence.

Cellular ultrastructure could be studied in a more fine way by transmission electronic microscopy to analyze intracellular organelles. At this level, when the researcher is interested in the events on the surface (outer surface of the membrane) may be preferable to use scanning electronic microscopy. Also it is possible to study isolated cells after dissociating of their tissues. By this process cells should maintain their vitality and many times their morphology. At the present work are showed several samples of tissues and isolated cells studied by scanning.

This chapter will deal with different microscopical techniques (scanning and transmission electronic microscopy, and light and confocal immunofluorescence microscopy) to study the physiological mechanisms performed by retinal neurons. Below are described some of the experimental procedures applied to this study.

4. Experimental procedures

4.1 Animals and surgical procedures

Adult New Zealand rabbits weighing 1000-1500 g, adult C57 BL/6j mice and adult Sprague-Dawley rats were used in this study. All experimental procedures were carried out in accordance with the European Communities Council Directive (86/609/ECC) for the use of laboratory animals. The animals were anesthetized by intramuscular injection of 5% ketamine chlorhydrate solution (Ketalar, Parke-Davis) 0.85 ml/kg. The animals had also previously received 2% tiazine chlorhydrate solution (Rompun, Bayer) 0.35 ml/kg. All surgical procedures were done under anesthesia. To pre-label RGCs, optic nerve was exposed by lateral approach and 10 μl of 0.1% fast blue (Sigma) was injected into the optic nerve, 2 mm from the eyeball with a Hamilton microsyringe. Care was taken not to damage the blood vessels, especially the ophthalmic artery that enters the sclera from the ventral margin of the optic nerve. Preservation of the structure of the central retinal artery was confirmed by ophtalmoscopy. Prelabeling of RGCs by injection of neuronal tracer (fast blue, fluorogold, diI, and others) into the optic nerve allow us to count selectively RGCs to calculate populations in normal or abnormal situations.

4.2 Retinal ganglion cell labeling by intracellular injection of Lucifer Yellow and neurobiotin

Pre-labeled soma ganglion cells by injection of fast blue were easily identified under fluorescent microscopy, allowing the staining of RGCs by intracellular iontophoretic injection of Lucifer Yellow and neurobiotin. Labeling procedures have been described in detail previously [1]. Several days after labeling, the rabbits were sacrificed by an overdose of sodium pentobarbital. The retina was removed from the eyecup and the vitreous humor teased away from its surface with a paintbrush in oxygenated (95% oxygen, 5% carbon dioxide) Ames medium [2]. The retina was flattened and fixed with the ganglion cell layer uppermost to a plate of Sylgard (Dow Corning Corporation Mich., USA) in a chamber of oxygenated Ames medium. Fast blue-labelled ganglion cells were filled intracellularly with Lucifer Yellow following a procedure similar to that described by Tauchi and Masland [3]. Micropipette electrodes filled with 1% Lucifer Yellow (Sigma) and 3% Neurobiotin (Vector) in 0.1 M Tris buffer (pH 7.4) were used. The tip resistance was 20-100 MΩ. Lucifer Yellow was injected by negative pulse (1-3 nA, 1 Hertz, duration 500 msc) for about 1 min. Once the cell was impaled, Neurobiotin was ejected by passage of direct positive current (1-3 nA, 1 Hertz, duration 500 msc) for about 5 min. After this, the microelectrode was taken out the cell and the procedure repeated in other cells until the electrode tip becomes clogged. After filling several cells, the retinas were equilibrated in Ames medium for 30 min [4, 5], and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) pH 7.4, at 22ºC for 2 hours. The retinas were then washed in PB, cryoprotected in 30% sucrose in PB, and stored at -18ºC for further processing.

4.2.1 Processing of the retinal tissue

After defrosting at room temperature and washing in phosphate buffer, the retinas were incubated in 0.2% albumin and 0.1% Triton X-100 in 0.1 M PB for 10 min to avoid unspecific labeling and to make the retina permeable to antibodies, respectively. Then they were incubated with 1% methanol and 0.15 % H_2O_2 in PB for 15 min. The next step was an incubation of the retinas in Avidin-Biotin complex solution (ABC-kit, Vectastatin, Vector) for 2 h. Following incubation, retinas were washed in phosphate buffer and incubated in the dark; with 0.05% diaminobenzidine for 20 min. Neurobiotin was visualized after adding 0.03% H_2O_2 to the solution. The retinas were then washed and dehydrated through an ascending ethanol series and mounted in DEPEX.

4.2.2 Retinal shrinkage

All RGCs were drawn using *camera lucida* attached to a microscope (Leitz Laborlux 12) under a 25x objective. The retinal area was measured and the shrinkage due to the histological procedures was estimated. The distance between the optic disc and the cells were measured after injecting Lucifer Yellow (before any possible retraction), and compared with the distances after processing the retinas with diaminobenzidine and dehydrating. Some retinas had an area of shrinkage ranging from 0% to 10%, others had an area of shrinkage between 11% and 20%. Retinas with a larger shrinkage percentage were not used in the quantitative analysis.

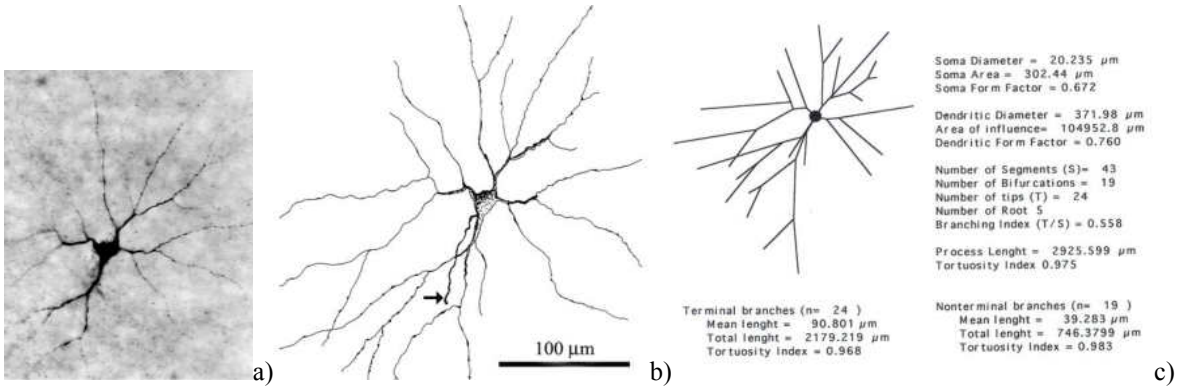


Fig 2. Morphometrical analysis of retinal ganglion cell. **A,** a RGC labeled by ionthophoretical injection of neurobiotin and developed with avidin-biotin and diaminobenzidine is shown. **B**, drawing of the same cell by *camera lucida.* **C**, computerized representation of the cell after morphometrical analysis

4.2.3 Morphometrical analysis

To study morphometrical features of RGCs (soma and dendritic field size, and number and length of dendritic branches, etc.) stained rabbit retinal ganglion cells were drawn using *camera lucida* projection as seen in whole-mount preparations and digitized using an HP-scanner with a resolution of 300 dpi. Gross image editing, such as removing large blocks of noise were performed with standard commercial software (Adobe Photoshop 5.0) on a 6100 Power Macintosh. See figure 2C for morphometrical analysis.

To obtain quantitative information about the true shape and morphology of neurons, the NIH Image program was used. This program has been developed at the US National Institutes of Health and is available on the Internet at http://rsb.info.nih.gov/nih-image [6]. A custom set of macros (modified version of the Neurite Macros v1.2, developed by Charles Thomas at the University of Wisconsin) allowed us to calculate different morphological parameters for each cell. Briefly, the first step was the acquisition of scale information followed by the acquisition of the soma and dendritic contours (convex hull around the dendritic area). The tracing of the dendritic tree were drawn by the operator via the mouse or a digitizing table (Kurta IS/ONE) connected to a 6100 Apple Power Macintosh. Once tracing has begun, the procedure permits the tracking of continuation points, branch points and end points to take place. From the traced tree, it is possible to automatically calculate non metric parameters such as the order of the neurite (e.g. primary, secondary, tertiary, etc.), number of branches, number of roots, number of bifurcations, number of terminal tips (topological degree) and sequences of branch order [7, 8, 9]. Using the scale information, metric variables such as the total dendritic length, dendritic length by branch order and so forth, were directly calculated. Fig. 2 summarizes the procedure for staining, drawing and morphometrical analyses performed on RGCs.

4.2.4 Data analysis

Morphometrical data of the cells was compared using a two-tailed *Student t* test. This was calculated using GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA.

4.3 Retinal cell dissociation

Dissociated retinal cells were prepared from adult rabbit retinas following a procedure described elsewhere [10]. Animals were deeply anaesthetized with urethane (loading dose, 1.5 kg^{-1}, i.p.) prior to enucleation. Both globes were hemisected from each animal, the vitreous removed and the retinas isolated and kept in Ames medium [2]. Since retinal eccentricity influences on the RGC size, same areas of both retinas were used for dissociation; a piece of *ca*. 0.5 cm^2 from the inferior-nasal quadrant, *ca*. 10 mm away from the optic nerve head was used for the dissociation. The retinal pieces were incubated separately for 30 min in standard Ringer solutions containing 40 U ml^{-1} papain (Worthington) and 0.1 mg ml-1 cysteine (Sigma, St. Louis, MO, U.S.A.) at 30ºC. Retinal pieces were rinsed with a standard Ringer solution containing 0.05% bovine serum albumin. The retinal fragments were mechanically dispersed on glass coverslip coated with concanavalin A (Sigma) (1 mg ml-1). This dissociation yielded a mixture of cells. Different retinal cells were identified by their characteristic morphology. The number of different neurons obtained per Petri dish was variable from day to day, from none to more than 10 cells. Cells were stored at 5ºC and the experiments were performed at room temperature within 1-4 hr after dissociation.

4.4 Scanning electron microscopy

Flat-mounted retinas, retinal cross-sections preparations, and isolated retinal cells were used for scanning electron microscopy (SEM). In the flat retina, the inner limiting membrane (ILM), constituted by the feet of the Müller cells [11] need to be picked off in order to clearly observe the RGCs and their membrane surface. To remove the ILM, we used a technique described in detail previously [12]. Briefly, a 2.5% poly-L-Lysine Hydrobromide solution was applied in a Petri dish. After an hour, the retina was extended with the ganglion cell layer down on the plate and glued to glass by poly-L-Lysine in dish perfused with Ames medium [2]. Five minutes later, the retina was carefully separated and taken to another plate with a Sylgard floor and fixed (ganglion cells layer up) with micro-pins. Then, the retinas were incubated 10 minutes at 30ºC in a Ringer solution containing 15 U/ml Papain (Worthington, Lakewood NJ, USA) and 1 mg/ml Cystein (Sigma, St.Louis, MO, USA). Retinas were washed with a continuous flow of Ringer to eliminate cellular debris (procedure modified from Edwards and cols.) [13]. Then, a retina patch of 1.7 x 2.24 mm was cropped and fixed in 1.5 % paraformaldehyde and 0.25% glutaraldehyde in phosphate buffered saline (PBS) for 2 hours at room temperature, rinsed and store at 4ºC.

For retinal cross-section SEM, patches of retinas were isolated from the eyes and strongly adhered to a nitrocellulose paper (Membrane Filters; Advance; Tokyo) ganglion cell layer down. Then, the retinas were vertically cut in 200 to 500 μm thick slices. The slices were lied down on a plate and stuck with poly-L-Lysine to expose the retinal layers. In the next step, retinas were cleaned and fixed following the protocol previously described, and then stored at 4ºC until fixation with osmium.

Retinal cross-sections and flat mount retinas without ILM were then immersed in 1% O_4Os in Milloning (1.87% monosodium phosphate and 0.42% sodium hydroxide in distilled water) for 1.5 hour at room temperature in absolute darkness. Retinas were washed with distilled water and dehydrated in ethanol series and pure acetone. In next step, acetone was mixed with liquid CO_2 (at room temperature and a pressure of 50 bar). When acetone was completely displaced by CO_2, the chamber was heated to 37ºC (at 80 bar) and liquid CO_2 became gas (g). In the critical point, chamber was slowly uncompressed to evacuate CO_2 (g) (Polaron E 300 and Polaron E 5000/5100). These procedures ensured the plasma membrane preservation and observations by scanning (DSM-950, Zeiss, Germany). Retinal shrinkage due to SEM procedures was taken into account and preparations with a shrinkage percentage larger than 20% were not used for analysis.

4.5 Immunohistochemistry, immunofluorescence, confocal mycroscopy

Retinas were dissected and fixed by immersion for 2 hours at room temperature in a solution containing 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Rinsed in 0.1 M PBS (pH 7.4) and incubated with 1% sodium borohydride for 30 min in 0.1 M phosphate buffer (pH 7.4). This step was carried out for decreasing to minimal the auto fluorescence of paraformaldehyde. The retinas were incubated in 0.2% albumin and 0.5% Triton X-100 in 0.1 M PB for 1 hour at 4ºC in stirring to avoid unspecific labeling and to make the retina permeable to antibodies, respectively.

Then, the tissues were incubated with the monoclonal antibody. For example, to study axons and somas of RGC Anti-β Tubulin Isotype III was used at a working dilution of 1:500 (Sigma, clone SDL.3D10, Product No. T8660) in 1 % albumin 0.1 M PB (pH 7.4) for 4 days at 4ºC in stirring. After several rinsed, the retinas were immersed in fluorescent secondary antibodies (1:200) for 2 days at 4ºC in stirring. They were sheep anti-mouse IgG species-specific whole antibody (Amersham, Arlington Heights IL, USA). After rinsing, retinas were washed in 0.1 M PB (pH 7.4), mounted in fluoromount Vectashield (Vector Laboratories), and cover slipped for viewing by laser confocal microscopy (Leica TCS SP2 Leica Microsystems).

Using other primary antibodies we can label different retinal cells, part of them, or a combination. Some examples are:

Rhodopsin labels rod photoreceptors; calbindin that labels preferentially horizontal cells, but also cone bipolar cells; α-PKC labels rod bipolar cells; Sodium-potasium-cloro cotransporter (NKCC1) labels horizontal cells; K-Cl cotransporter (KCC2) labels ON- cone bipolar cells; Bassoon antibody labels presynaptic cytomatrix protein Bassoon, a major component of the photoreceptor ribbon; melanopsin labels intrinsically photosensitive RGC (a RGC subtype discovered recently); Tiroxine hidroxilase labels dopaminergic amacrine cells; α1-GABA subunit antibody labels gabaergic amacrine cells; and many more. Also is possible a selective staining based on a selective protein affinity like Peanut agglutinin, a lectin with high binding affinity for galactose-galactosamine disaccharide. Peanut agglutinin binds preferentially to cones, not to rods.

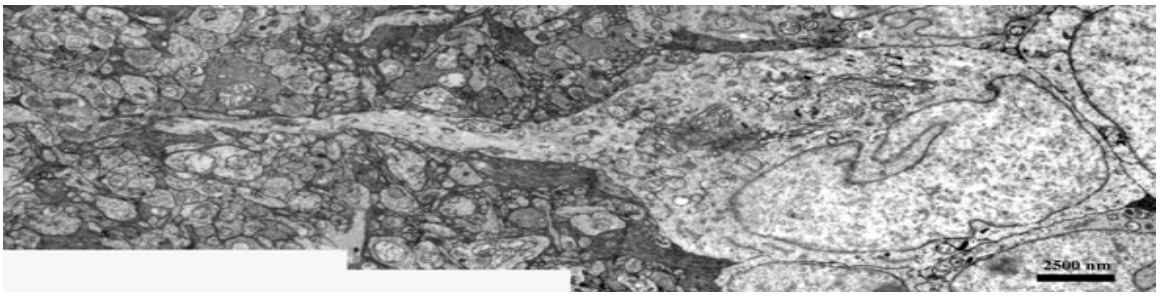


Fig. 3. Micropicture of a RGC. Notice the initio of a neural process, nucleus, mitochondrias, lisosomal apparatus and other cellular organelles.

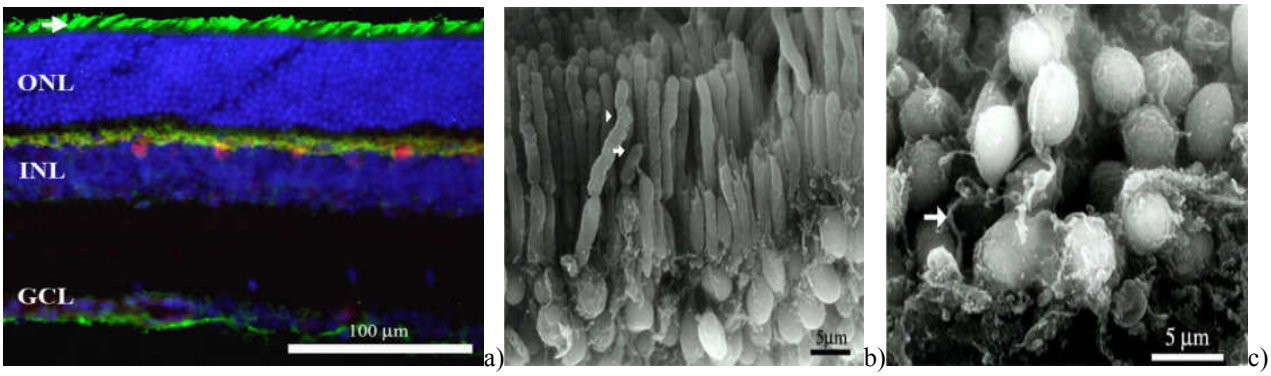
4.6 Immunohistochemistry, transmission electron microscopy

Retinas were dissected and fixed by immersion for 2 hours at room temperature in a solution containing 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M PBS (pH 7.4) with the addition of 0.15 mM CaCl2, 4% sucrose. Retinas were then fixed in 4% paraformaldehyde, 0.15 mM CaCl2, 4% sucrose and bicarbonate phosphate buffer (pH 10.4) at 4ºC overnight. To avoid tissue damage after freeze, retinas were exposed to a gradient of sucrose (15% thirty minutes, 20% one hour and 30% overnight) at 4ºC and quickly freeze and unfreeze. This step was carried out to make retinas permeable to antibodies; otherwise the use of triton-X would destroy membranes. To avoid non specifically labeling, retinas were incubated in 10% normal serum in 0.1 M PBS (pH 7.4) at 4ºC in stirring. Then, the tissues were incubated with the monoclonal antibody Anti-β Tubulin Isotype III at a working dilution of 1:500 (Sigma, clone SDL.3D10, Product No. T8660) in 1 % albumin in phosphate buffer (pH 7.4) for 4 days at 4ºC in stirring. After several rinsed, the retinas were immersed in secondary antibodies (1:200) for 2 days at 4ºC in stirring. They were sheep anti-mouse IgG biotinylated species-specific whole antibody (Amersham, Arlington Heights IL, USA). After rinsing, retinas were incubated in Vectastatin, ABC-kit (Vector, Burlingame, California, USA) in a 1:250 dilution in 0.1 M PBS for one day at 4ºC in stirring. Finally, retinas were washed in phosphate buffer and incubated with 0.05% diaminobenzidine and 0.03% H_2O_2 in 0.1 M PBS until the labeled cells were visualized by the light microscope.

Retinas were placed overnight in cold 0.1M cacodylate-sucrose, pH 7.6 and post-fixed in cold 1% buffered OsO_4, pH 7.3 for one hour. Then, they were placed in aqueous 2% uranyl acetate and dehydrated and embedded in Epon 812.

Sectioning for electron microscopic examination followed standard procedures and was accomplished with an ultramicrotome (Vitracut E, Reichert-Jung, Austria). Segments of retinas were sectioned in the transverse plane (semithin, 1-2 μm) and stained with toluidine blue for light microscopy study. Blocks of retinas were then cut again and thin sections (60-80 nm thick) were stained in lead citrate and viewed under a transmission electron microscope (M-10, Zeiss, Germany).

5. Retinal cells



a) b) c)

Fig. 4. Photoreceptors. A, Retinal cross-section, immunohistochemically labeled with NKCC1 (Na-K-Cl-Cotransporter) antibody (green) and calbindin (red). NKCC1 stains outer segments of photoreceptors (arrow in the upper) and horizontal cells. Calbindin stains horizontal cells. GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outr nuclear layer. **B**, Outer and inner segments of photoreceptors viewed under scanning microscopy. A cone is pointed by an arrow and a rod by a arrowhead. **C**, Somas and terminals of photoreceptors. Notice how a photoreceptor terminal (arrow) contacts with the outer plexiform layer.

5.1 Photoreceptors, rods and cones

Almost all vertebrates have morphologically two types of photoreceptors, which can be classified as rod and cone cells [14]. Rods function in dim-light vision, whereas cones mediate bright-light and color vision. Rods and cones are differentiated on the basis of the shape of their outer segments (see Fig 4).

Rod outer segments contain a set of membranous disc, derive from the surface membrane of the cell but no longer continuous with the surface membrane or with each other. They resemble a stack of coins. Cone outer segments are composed mainly of infoldings of the surface membrane of the cell. Outside the foveal region, these outer segments taper slightly, and this gives the cones their name.

Other factors that may help in the identification are axonal terminal size and position, and soma location in the outer nuclear layer; it is smaller and closer to the outer plexiform layer in the case of rods. In most retinas there is a single type of rods, but amphibians that have two types of rods, red and green, sensible to these wave lengths [14]. Most species have multiple types of cones. Usually they are three cone types, like in primates, and are termed L, M, and S, which absorb maximally in low, medium and supra frequency, respectively. These frequencies correspond to the three basic colors: red, green and blue, respectively. Spectral sensitivity of L and M are relatively close in the low frequency end of visible spectrum, while S cone lies at the high frequency end. These different absorptions are due to the presence of different pigments in the outer segments of the photoreceptors.

Usually, the spatial distribution of S cones across the retina differs from that of L and M cones. They are only 10% of cone population and are absent of the fovea.

All photoreceptors have an outer segment (containing the visual pigments), an inner segment, a perikaryal region which contain the cell nucleus, and a terminal. Usually, rods have longer outer segments and smaller spherical terminals. Cones have a shorter outer segment, a fatter inner segment, and a larger terminal than rods. However, there are exceptions for both types.

Photoreceptor terminals are termed cone pedicles and rod spherules. Processes from cone pedicles contact with other cone pedicles as well as rod spherules. At each such contact there is a low-resistance junction termed gap junction that provides a partial electrical coupling between the two cells. In each pedicle or spherule there is an invagination and just above lies a specialized structure termed synaptic ribbon that plays an important role in the transmission of signals from a photoreceptor to the horizontal and bipolar cells.

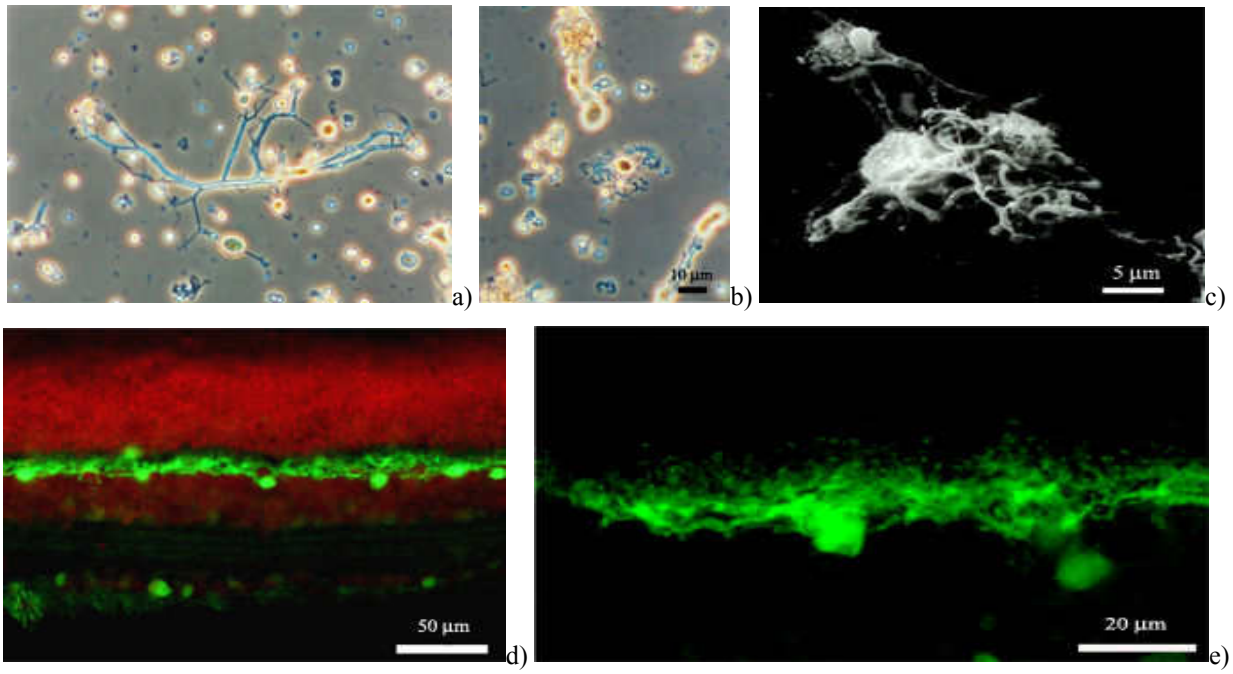


Fig. 5. Horizontal cells. **A**, isolated horizontal cell type HII (light microscopy). **B,** isolated horizontal cell type I (light microscopy). **C,** isolated horizontal cell type HII under scanning microscopy. **D,** Cross-retinal section immunohistochemically labeled with calbindin (labels horizontal cells and cone bipolar cells) and Propidium iodure to label retinal nucleus. **E,** horizontal cells in detail. Notice small dots in the upper that correspond to dendritic synapses.

5.2 Horizontal cells

Most vertebrate retinas contains only two types of horizontal cells: a cell with a short axon that typically runs 400 µm or further, before ending in a prominent terminal expansion called telodendritic arbor, this cell is termed HI; and an

axonless cell, termed HII. Axonless cells have not been observed in primate retinas; and it may be that other retinas are lacking of these horizontal cell types [15].

Electron microscopy of Golgi-stained horizontal cells from the cat has shown that the processes of the axonless cell and the dendritic processes of the axon cell connect exclusively with cone terminals, whereas the axon terminal processes of the axon cell end exclusively in the rod terminals [16]. However, in other retinas such segregation of cone and rod inputs has not been observed.

Horizontal cells respond to illumination with sustained graded potentials. Usually, they do not generate action potentials, and the axon of the horizontal cell is sufficiently long that the graded electrical activity taking place in the telodendritic arbor is isolated from that of the dendritic arbor.

Each rod synaptic terminal contacts with two to five rod bipolar cells. Each rod bipolar cell contacts with 30-50 rods. Horizontal cells work by lateral inhibitions modulating the magnitude of rod bipolar cell activation by rods. In the first form of lateral inhibition are involved HI horizontal cells telodendritic arbor, rods and rod bipolar cells. A greater stimulation of the telodendritic arbors of the horizontal cells by rods decreases the effect on the rod bipolar cells. Thus, the effect of light intensity depends more on differences in intensity within the image, such as at a border, than on the overall level of light intensity.

A second example of lateral inhibition involves dendrites of HI, cones, and cone bipolar cells. The dendrites of adjacent HI are strongly coupled by gap junctions, which mediate spatial effects. The dendritic terminals of HI cells receive from both L and M cones, but not from S cones.

The third example of lateral inhibition involves HII cells, S cones and the cone bipolar cells that contact with them. HII horizontal cells do not have axon, but short dendritic branches and are also strongly coupled to one another via gap junction.

Although HII contact in equal measure with S cones and with a mixture of L and M cones, and since there are ten times more L and M cones than S cones, L and M do not appear to be strongly influenced by HII. This neural circuit seems to have a role on color vision.

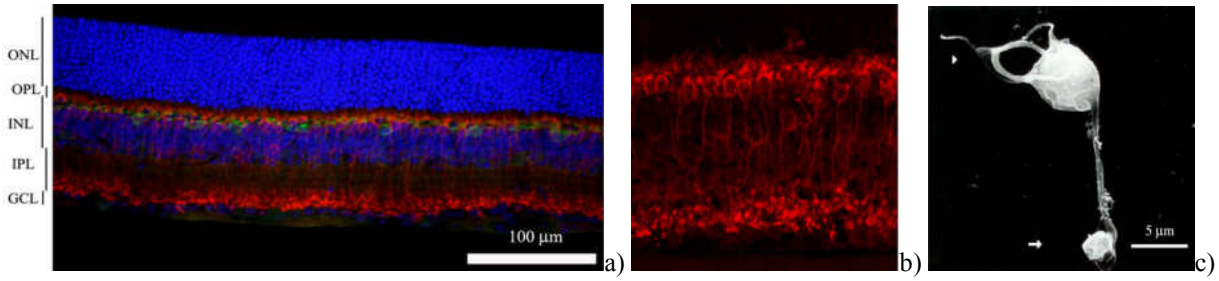


Fig. 6. Bipolar cells. A, retinal cross-section labeled with α-PKC antibody to stain rod bipolar cells, (red) and DAPI to label nuclei (blue). **B,** rod bipolar cells in detail, bipolar cell somas are placed in the upper position, above are the dendritic processes. From the inferior part of somas grow a unique axonal process that finish in the rod bipolar terminal. **C**, an isolated bipolar cell viewed under scanning microscopy. Notice dendritic processes (arrowhead) in the upper and axon terminal (arrow) in the bottom.

5.3 Bipolar cells

Bipolar cells connect outer and inner retina. Their dendritic processes make synapses with photoreceptors and horizontal cells in the outer plexiform layer and their axon terminals contact with amacrine and ganglion cells in the inner plexiform layer. Their somas are placed in the inner nuclear layer, between both synaptic layers.

The first classification of bipolar cells depends on the type of photoreceptor they receive input, rod bipolar cells and cone bipolar cells. These cone bipolar cells make two different kinds of synaptic connections with cones. One type of the cone bipolar cells extends dendrites into the center of the invaginations of the cone receptor terminal (they are called invaginating bipolar cells), and the axon terminals of these cells end deep in the inner plexiform layer. Attending to its function, these cells are termed ON-bipolar cells, because they are activated by an increase in light intensity. The other cell type makes contacts along the flattened base of the cone receptor terminals (they are called flat bipolar cells), and the axon terminals end in the distal part of the inner plexiform layer. This type responds to decreases of light intensity, it is also termed OFF-bipolar cells.

Especially interesting is the synaptic contact made into the cone pedicle, in the triad. The most distinctive of these are the invaginating, or central, processes directly below the synaptic ribbon. Usually, there are three processes (from two horizontal cells and one bipolar cell) in an invagination, which are aligned along the length of the ribbon. Bipolar cells that receive from photoreceptors via invaginating processes all appear to have axonal arbors that stratify in the ON-sublayer of the inner plexiform layer. Other bipolar cells are triad associated, or semi-invaginating processes, branch on either side of the invaginating processes, and constitute a heterogeneous group. Some are OFF bipolar cells and others

are ON bipolar cells. The remaining bipolar cell processes are non-triad associated or flat processes, and are OFF bipolar cells.

In the other extreme of the bipolar cell, there are two postsynaptic processes apposed to the bipolar terminal synaptic ribbons, and this postsynaptic arrangement has been called a dyad [17]. The two postsynaptic processes of a dyad are most often a ganglion cell dendrite and an amacrine cell process or two amacrine cell processes [18]. It has been reported gap junctions between bipolar cells in some species [19, 20].

Different types of bipolar cells may be distinguished: midget, diffuse, S cone, and giant. Midget bipolar cells are recognized by their small and compact dendritic arbors, which generally receive from only one cone, although in the peripheral retina a few receive from two cones [21]. Midget bipolar cells can receive from cones via invaginating contacts (ON) or via triad-associated contacts (ON or OFF). Diffuse mean bipolar cell with dendritic processes that spread out in the outer plexiform layer to receive from a number of photoreceptors. S cone bipolar cells have invaginating dendritic processes that receive exclusively from S cones. Their axonal arbors ramify in the inner most portion of the inner plexiform layer, and are presynaptic to a ganglion cell type termed small bistratified.

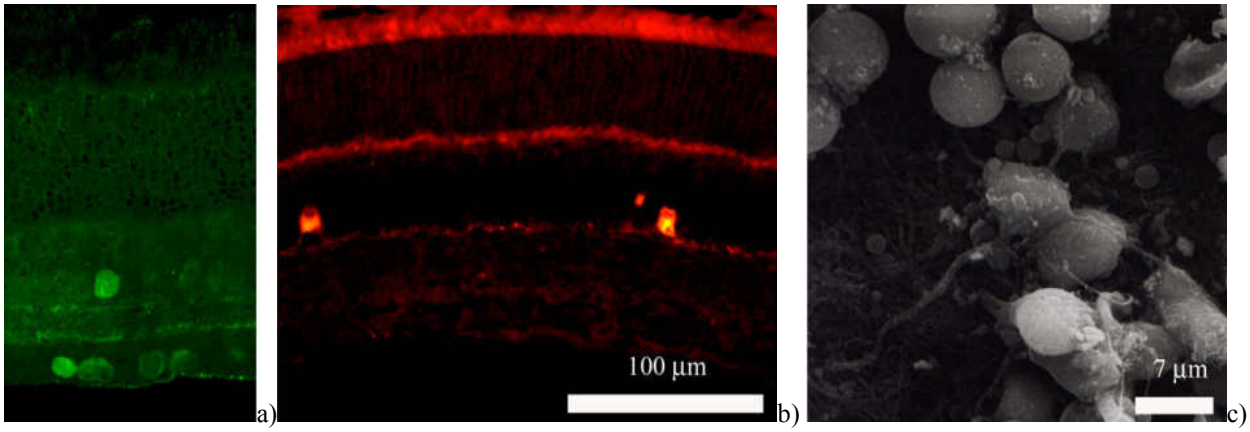


Fig. 7. Amacrine cells. **A,** retinal cross-section labeled with ChAT (Choline Acetyltransferase) that stains amacrine cells containing this enzyme. Somas of these amacrine cells are distributed in inner nuclear layer and ganglion cell layer. **B**, retinal cross-section labeled with tiroxine hidroxilase antibody. This antibody stain cells placed only in the inner nuclear layer**. C,** Amacrine cells viewed under scanning in a very closer relationship. Scale is the same in A and B.

5.4 Amacrine cells

Ramón y Cajal discovered a group of neurons in the retina that had no axon, and he called a-macr-ine (*a*: lacking, *macros*: long, *inos*: fibers) [22]. There are many different types of amacrine cells, which vary greatly in size, morphology, and function. Cajal classified amacrine cells into two major types: diffuse that extends their processes throughout the thickness of the inner plexiform layer; and stratified that extend their processes on one or few strata of the inner plexiform layer. Diffuse amacrine cells may be sub-classified in narrow and wide-field (on the basis of the dendritic spread); and mono, bi, multi-stratified (on the basis of the strafication levels in the inner plexiform layer). Amacrine cells constitute the most diverse group of cell types within the retina with respect to morphology, size, and retinal coverage. There appear to be 30 to 40 types, but the functions of most are unknown. However, we know that many ganglion cell properties, such as directional selectivity or local edge detection is responsibility of amacrine cells. One of the most complete and recent classifications is given by Masland [23, 24].

Cell bodies of amacrine cells are placed in the innermost layer of the inner nuclear layer, in the middle of the inner plexiform layer (A1 amacrine cell), and in the ganglion cell layer (displaced amacrine cells).

Amacrine cells make numerous synapses onto ganglion cell dendrites, bipolar cell terminals, interplexiform processes and other amacrine cell processes [17]. Electron microscopy has shown that amacrine cell processes have characteristics of both axons and dendrites. Thus, amacrine cell processes can be both pre- and postynaptic over a very short portion of their length [18]. There are, at least, two types of synaptic arrangements. First, a postsynaptic amacrine cell process makes a synapse back onto the bipolar terminal a short distance away, these are called reciprocal synapses. Second type of arrangement, one amacrine cell process makes a synapse onto an adjoining amacrine cell process, which makes a nearby synapse onto a third element (a ganglion cell dendrite, a bipolar cell terminal, or another amacrine cell processes). These are called serial synapses. Another kind of communication between amacrine cells processes are gap junctions that serve to extend the receptive field size of the cells.

Attending to the diversity amacrine cell properties, we can show four main cell types: ON-starburst, OFF-starburst, dopaminergic, and A1.

Starburst amacrine cell are present in every vertebrate class, and recognized by their distinctive morphology. Radiate processes extend from the cell body, branch, thicken, and branch again near their endings. Most of the synaptic contacts

occur away from the cell body, at the thickenings. This gives place to two subtypes, OFF- starburst amacrine cells, stratified in the off-sublayer of the inner plexiform layer; and ON- starburst amacrine cells stratified in the on-sublayer of the inner plexiform layer. They release acetylcholine (in excitatory synapses) and GABA (in inhibitory synapses).

Dopaminergic amacrine cells are also common to all vertebrates. Their somas lie in the most inner portion of the inner nuclear layer, and stratify in the outer portion of the inner synaptic layer to contact with AII amacrine cells. Its function is to control the degree of AII interconnection by gap junctions.

A1 amacrine cells are characterized by two components: a dense dendritic field, and a more sparsely branched tree of axon-like processes that extends for some millimeters beyond the dendritic arbor. A1 amacrine cells are coupled together showing a regular array across the retina. They are stratified at the border between the ON- and OFF sublayers of the inner plexiform layer; and thus can respond in both ways. Whatever the different types of amacrine cells do, their ultimate role is to influence the response properties of ganglion cells, to which we now turn.

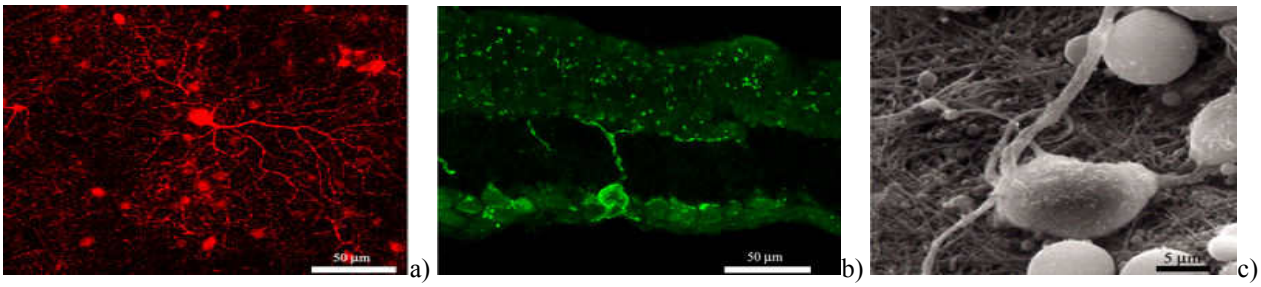


Fig. 8. Retinal ganglion cells. **A**, ganglion cells in a flattened retina. These cells were labeled by retrograde injection of DTMR (dextrotetramethyl rodamine) into the optic nerve. **B,** Cross-retinal section labeled with melanopsin antibody. **C**, a RGC soma on the inner plexiform layer viewed under scanning.

5.5 Ganglion cells

Ganglion cells send a message to the brain in the form of action potentials, which are influenced by amacrine cells and bipolar cells that the ganglion cells receive from. In general, bipolar cells increase the ganglion cell activity and amacrine cells tend to decrease.

Most ganglion cell types respond in specialized ways. For example, some cells show directional selectivity (respond in one direction, but not in the opposite one). This selectivity is a property of some amacrine cells these ganglion cells receive from, rather than a property created by the ganglion cells from the signal received.

Although the number of different types of ganglion cells is uncertain, based upon differences in morphology, might be close to 30 types. Each ganglion cell type is specialized for coding some particular aspect of the visual world (contrast, color, movement).

On the basis of the morphology, Boycott and Wässle described in cat the basic RGCs types, [25]: α RGCs were characterized for a large soma and a monostratified dendritic arborization. This dendritic tree was composed of smooth and large processes that branched out at relatively acute angles to form a particular radial branching pattern, with virtually no spines or dendritic thickenings; β RGCs showed a small perykaria and dendritic tree; and more branches; and γ RGCs that form a more heterogeneous group that has been sub-classified in more groups. These researchers observed how the soma and dendritic size of the RGCs changed with the retina eccentricity, and established, in the cat retina, the morpho-physiological correlation of X- and Y- cells with β- and α-, respectively.

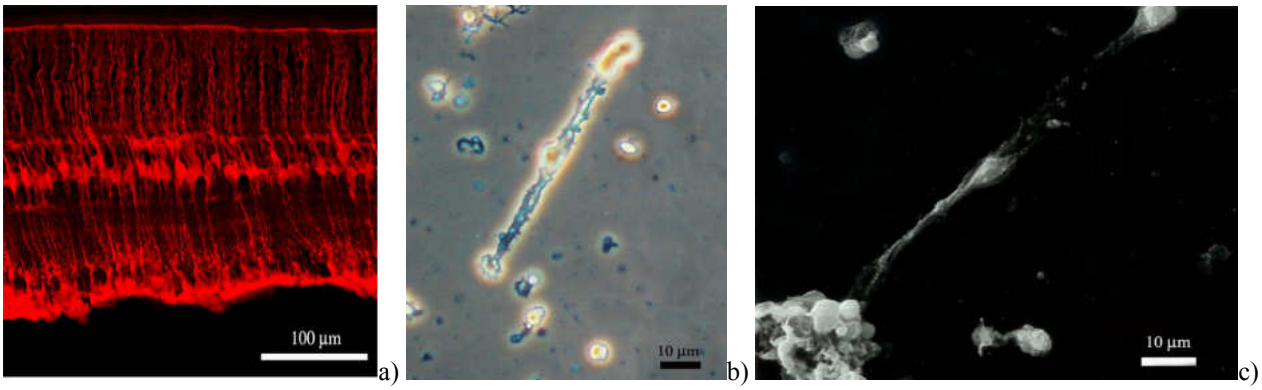
Classically, from a physiological point of view, mammalian RGCs have been divided, in concentric and no concentric receptive field. Concentric receptive field may have a central part that respond with depolarization to lightness (ON), or to darkness (OFF), or to changes in illumination (ON-OFF). Non concentric receptive field RGCs are responsible for specific stimulus features, such as movement. Kuffer discovered the concentric, center-surround organization of ganglion cells in the mammalian retina [26].

Different types of RGCs have different targets. In the primate retina, tonic ganglion cells with color-specific and small concentric receptive fields project to the parvocellular layers of the lateral geniculate nucleus (LGN), and phasic ganglion cells with non-color-specific large concentric receptive fields project to the magnocellular LGN layers.

In the primate retina, we distinguish three RGCs groups: midget, parasol, and small bistratified. Midget RGCs constitute about 70% of the total ganglion cell population, with a highest percentage in central eccentricities. Midget RGCs are termed ON- and OFF-. The group of ON-midget bipolar cells is composed of two cell types, L cone ON-midget bipolar cells and M cone ON-midget bipolar cells. The population of OFF-midget RGCs shows territorial domains. However, their density is about 70% higher, and their dendritic fields are smaller than those of ON-midget.

Parasol ganglion cells compose an 8-10% of the ganglion cell population. Parasol RGCs receive from diffuse bipolar cells, have larger dendritic fields, and collect from many more cones than midget RGCs. As with midget RGC, they can be sub-classified in ON-parasol and OFF-parasol ganglion cells. Each type also forms tight territorial domains.

Small bistratified ganglion cells compare S cones with M and L cones. Their dendrites arborize in both the ON- and OFF- sub-layers of the inner synaptic layer.



a) b) c)

Fig. 9. Müller cells. **A,** Cross-retinal section immunohistochemically labeled with glutamine synthetase. This antibody stains Müller cells from feet (lower position, inner limiting membrane) to apical microvilli (upper position, outer limiting membrane). **B,** isolated Müller cell viewed under light microscopy. **C** isolated Müller cell viewed under light microscopy.

5.6 Muller cells

The Müller (radial glial) cells are the predominant glia of the vertebrate retina. These cells extend vertically through the retina, from the distal margin of the outer nuclear layer to the inner margin of the retina. The distal border of Müller cells is marked by the outer limiting membrane, which consists of junctional complexes made between the processes of different Müller cells or between the processes of Müller cells and photoreceptors. The proximal border of the cells is marked by the inner limiting membrane, which is formed by the fusion of Müller cell feet [11]. The nuclei of Müller cells are usually in the middle of the inner nuclear layer.

Müller cells arise, together with rod photoreceptor cells, bipolar cells, and a subset of amacrine cells, from common precursor cells during a late proliferative phase. One Müller cell and a number of such neurons seem to form a columnar unit within the retinal tissue. Whereas 'extracolumnar neurons' (ganglion cells, cone photoreceptor cells, horizontal cells, and another subset of amacrine cells) are born and start differentiation before most Müller cells are generated. Such neurons need to develop metabolic capacities sufficient to support their own survival, whereas late-born ('columnar') neurons seem to depend on a nursing function of Müller cell [27].

The close relationship of Müller cells with neurons is reflected by a multitude of functional interactions between neurons and Müller cells, including a 'metabolic symbiosis' and the processing of visual information. Müller cells are also responsible for the maintenance of the homeostasis of the retinal extracellular milieu (ions, water, neurotransmitter molecules, and pH). Müller cells also seem to be involved in the control of angiogenesis, and the regulation of retinal blood flow. Every disease of the retina is associated with a reactive Müller cell gliosis which supports the survival of retinal neurons via a release of neurotrophic factors, the uptake and degradation of the excitotoxin, glutamate, and the secretion of the antioxidant, glutathione. However, these reactive Müller cell gliosis also may accelerate the progress of neuronal degeneration [28].

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