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Recoverin immunoreactivity in mammalian cone bipolar cells

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Abstract

Human, macaque monkey, and rat retinas were immunostained with a polyclonal antibody preparation against purified recoverin, a 23-kD calcium-binding protein isolated from bovine retina that localizes to rods and cones (Dizhoor et al., 1991). In addition to immunoreactive photoreceptors, we have identified subpopulations of recoverin-positive bipolar cells in all three species. Results from immunostaining with progressive dilutions of anti-recoverin and preadsorption of the antibody with a dilution series of purified recoverin showed that photoreceptors and bipolar cells had similar affinities for the antibody and suggested that the molecule recognized by the antibody in both cell types is recoverin. Immunoreactivity for recoverin and protein kinase C, a selective marker for all rod bipolar cells, was found in separate bipolar cell populations. Recoverin immunoreactivity is therefore a characteristic of certain cone bipolar cell types.

In rat retina, anti-recoverin labeled two morphologically distinct subpopulations of cone bipolar cells whose axonal arbors stratified at different depths in the inner plexiform layer (IPL). The bipolar cells labeled with anti-recoverin did not correspond to those that were reactive for calbindin, another cone bipolar cell marker.

Human and monkey retinas also had two populations of cone bipolar cells that were recoverin-positive. One population showed a distinct pattern of narrow bistratification at the outer border of the IPL and a regular mosaic arrangement of its axonal arbors, suggesting that the entire population of a single cone bipolar type was labeled. Cell density, dendritic morphology, and axonal-field size and stratification indicate that anti-recoverin selectively stains the flat midget (presumed OFF-center) cone bipolar cell type observed previously in Golgi preparations. By contrast the second bipolar cell population had axonal stratification in the inner half of the IPL and showed an unusual but consistent morphology and spatial distribution. Individual cells were intensely stained but were present at an extremely low density (~2-5 cells/mm^2). These cells had multibranched dendritic trees characteristic of the diffuse bipolar cell class, but very small axonal fields in the size range of the midget bipolar class. Neither of the two recoverin-positive bipolar cell types in monkey was labeled with anti-calbindin or anti-cholecystokinin. An antibody preparation against bovine pineal hydroxyindole-O-methyltransferase (HIOMT) labeled photoreceptors and bipolar cells that closely resembled the recoverin-positive bipolar cells in human and rat retinas. Preadsorption of this antibody preparation with purified recoverin abolished immunostaining of the bipolar cells, suggesting that the anti-HIOMT preparation contains antibodies against recoverin, which is known to be present in the bovine pineal gland.

Keywords: Retina, Cone bipolar cell, Recoverin, Calcium-binding protein, Macaque monkey

Introduction

In the mammalian retina, bipolar cells selectively contact either rods or cones and thereby establish the first synaptic step in parallel rod and cone signal pathways (for a recent review see Wässle & Boycott, 1991). Bipolar cells that contact rods constitute a single, depolarizing or ON-cell type that can be selectively stained with an antibody against protein kinase C (PKC) (Wood et al., 1988; Negishi et al., 1988; Graferath et al., 1990; Grünert & Martin, 1991; Young & Vaney, 1991; Zhang & Yeh, 1991; for review, see Wässle et al., 1991), or an antibody against L7, a putative growth factor (Berrebi et al., 1991; Grunert & Martin, 1991). By contrast, bipolar cells that selectively contact cones can be divided physiologically into both ON- and OFF-cell classes (e.g. Nelson & Kolb, 1983). An additional complexity beyond the ON-OFF dichotomy is that a large number of morphologically distinct cone bipolar types have been recog-

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nized in Golgi preparations (Polyak, 1941; Boycott & Dowling, 1969; Famiglietti, 1981; Kolb et al., 1981; Mariani, 1983, 1984; Boycott & Wässle, 1991; Boycott & Hopkins, 1991), or by electron-microscopic reconstruction (McGuire et al., 1984; Cohen & Sterling, 1990). It has been relatively difficult to characterize the morphological or physiological properties of the individual cone bipolar types and little is known about the functional significance of multiple cone pathways.

Immunostaining for neurotransmitters or transmitter-related molecules reinforces the suggestion of anatomical and functional diversity in cone bipolar cells. In macaque monkey, bipolar cells that selectively contact blue cones are labeled with anti-cholecystokinin precursor (CCK) (Kouyama & Marshak, 1992; Marshak et al., 1990). Some cone bipolar cells may also contain glycine (Cohen & Sterling, 1986; Wässle et al., 1986; Pourcho & Goebel, 1987; Hendrickson et al., 1988), γ-amino butyric acid (Wässle & Chun, 1989; Grünert & Wässle, 1990), glutamate (Ehinger et al., 1988), and hydroxyindole-O-methyltransferase (HIOMT), an enzyme involved in melatonin synthesis (Wiechmann & Hollyfield, 1987, 1989; Wiechmann & O'Steen, 1990). Antibodies against certain calcium-binding proteins (Pasteels et al., 1990; Verstappen et al., 1986; Hamano et al., 1990; Berrebi et al., 1991) and a monoclonal antibody made against rat olfactory bulb also label subpopulations of cone bipolar cells (Onoda & Fujita, 1987; Onada, 1988; Martin & Grünert, 1991). However, with the exception of the blue cone bipolar cell, in none of these studies has it been possible to relate the observed labeling patterns to single cone bipolar cell types that have been proposed on previous morphological or physiological grounds.

This report shows that two populations of cone bipolar cells, which can be distinguished by the depth of stratification of their axonal processes, are stained selectively in human, macaque monkey, and rat retinas with an antibody preparation against recoverin, a calcium-binding protein that was localized previously to rods and cones of bovine retina (Dizhoor et al., 1991). In the monkey retina, we show that one of the recoverin-immunoreactive bipolar cell types corresponds to the flat midget (presumed OFF-center) bipolar cell that has been observed previously in Golgi studies and is believed to be a major interneuron in a cone signal pathway that limits visual acuity in the primate.

Materials and methods

Tissue preparation

A total of 20 retinas were studied from the following species: human (2), monkey (*Macaca nemestrina*; 10), and albino rat (Sprague Dawley; 8). Normal human eyes were slit at the pars plana and fixed at 2 h or less *postmortem*. The monkey eyes were opened immediately *postmortem*, maintained in oxygenated Hank's balanced salt solution or Ames medium (Sigma Chemical Co., St. Louis, MO), and fixed 1 h or less *postmortem*. The rat eyes were removed, slit, and immersion fixed from animals that had been deeply anesthetized with sodium barbiturate; the rats were killed immediately by an anesthetic overdose. Experiments involving animal subjects conformed to the principles regarding the care and use of animals adopted by the American Physiological Society and the Society for Neuroscience. Human donor retinas were obtained from the Lions Eye Bank, University of Washington Medical Center, Seattle, WA (UW Human Subjects Approval No. 21-51-E).

Immunocytochemistry

The following primary antibodies were used in 0.3% Triton X-100 in phosphate buffered saline (PBS): anti-recoverin (1:500 to 1:5000, made in rabbit; Dizhoor et al., 1991), anti-PKC (1:50, made in mouse; Seikagaku Kogyo Co., Ltd., Chuo-ku, Tokyo, Japan), anti-CCK (1:100, R8B5, made in rabbit against a peptide fragment of the gastrin/CCK precursor; a gift from Dr. J. Del Valle), anti-calbindin (1:1000, made in mouse; a gift from Drs. M. Celio and A. Hendrickson), and anti-HIOMT (1:200, made in rabbit; a gift from Dr. A. Wiechmann). The specificity of the anti-recoverin was previously published (Dizhoor et al., 1991). Radial cryostat sections of retinas were processed for immunofluorescence or by the avidin-biotin peroxidase complex (ABC) method (Vector Elite Kit, Vector Labs., Burlingame, CA), using previously published methods (Milam et al., 1990). All eyes were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 2–6 h at room temperature. Portions of the retinas were dissected and placed in 30% sucrose in phosphate buffer at 4°C overnight. Cryostat sections of 12-μm thickness were mounted onto subbed slides, air dried, and stored at 20°C. For immunofluorescence, the sections were surrounded with plastic rings attached with fingernail polish, and incubated for 1 h at room temperature in blocking solution containing 1% horse serum, 1% bovine serum albumin, and 0.05% Triton X-100 in PBS, blotted gently, incubated overnight at 4°C in primary antibody diluted in PBS with 0.3% Triton X-100, rinsed in PBS, incubated for 30 min at room temperature in secondary antibody (goat anti-rabbit or anti-mouse immunoglobulin [IgG]) tagged with fluorescein isothiocyanate (FITC) or rhodamine, rinsed, and coverslipped in 90% glycerol in PBS containing 2% 1,4-diazabicyclo(2,2,2)octane. The retina sections that had been processed by the ABC method were dehydrated and coverslipped in Permount.

To identify ganglion cells in the sections processed for immunofluorescence, rats were anesthetized with Equithesin (University Hospital Drug Services, Seattle, WA) and each optic tract was injected with 1 μl of rhodamine dextran (Molecular Probes, Eugene, OR; average molecular weight, 10 kD, 2 mg in 20 μl lactated Ringer's solution). After 48-h survival to allow retrograde transport of the tracer, the rats were killed by an overdose of sodium barbiturate and the eyes were enucleated and fixed as above. Cryostat sections of retinas containing ganglion cells marked by rhodamine were processed for indirect immunofluorescence using anti-recoverin, followed by goat anti-rabbit IgG tagged with FITC. The same retinal region was photographed as a double exposure with alternate filters for FITC and rhodamine (Milam et al., 1990).

We used double-labeling immunofluorescence to determine if recoverin colocalized with PKC or calbindin. Cryostat sections were incubated with a mixture of the primary antibodies prepared in different species (anti-recoverin in rabbit and anti-PKC or anti-calbindin in mouse). After a rinse in PBS, the sections were treated with a mixture of secondary antibodies (goat anti-rabbit and -mouse IgG) tagged with rhodamine and FITC, respectively. The same retinal region was photographed as a double exposure with alternate filters for FITC and rhodamine. As primary antibodies against CCK and HIOMT were not available prepared in non-rabbit species, serial sections pro-

cessed with the antibody against recoverin and the second antibody were photographed as projection slides, and the images were superimposed using two projectors to determine if the recoverin-positive bipolar cells corresponded to those labeled with the second marker.

For wholemount preparations, free-floating pieces of macaque retina were incubated in blocking solution containing 1.5% goat serum in PBS and 0.3% Triton X-100 for 1 h at room temperature and transferred to the primary antiserum diluted in PBS with 0.3% Triton X-100 at 4°C for 2–7 days. After rinsing in PBS, the retinas were immunostained by the Vectastain ABC method according to the manufacturer's directions. In general, we found that penetration of the reagents into the intact retina was slow and that standard incubation times produced incomplete penetration into the middle third of the retina. Staining was enhanced when the retinas were incubated in the secondary antibody at 4°C for 2–3 days and in the ABC reagent at 4°C for 1–2 days.

Camera lucida tracings and measurements of axonal-field size and cell density were made on retinal pieces that were mounted flat on slides in a water-based solution of glycerol and polyvinyl alcohol. Measurements of the retinal pieces before immunostaining and after mounting suggested that there was no significant tissue shrinkage and no attempt was made to precisely quantify or correct for this factor.

Controls

Control sections from each species were treated with nonimmune rabbit serum and processed as above. Sections of human retina were also processed for immunofluorescence using a dilution series of anti-recoverin (1:500 to 1:100,000), or with anti-recoverin (1:5000), or anti-HIOMT (1:200) that had been preadsorbed for 1 h at room temperature with purified recoverin (0.01–50 μg/ml).

Results

Both rods and cones were immunoreactive with the antibody preparation against recoverin in human, macaque monkey, and rat retinas, as previously shown in bovine retina (Dizhoor et al., 1991). Additionally, we found intense labeling of certain bipolar cells in the three mammalian species. The bipolar cell labeling appeared to be the only significant immunoreactive cell population beyond the photoreceptors, although extremely rare immunoreactive cells were observed in the ganglion cell layer in the rat retina. We will first describe the bipolar cell staining in vertical sections of rat and human retinas. This is followed by a more detailed analysis of the bipolar staining in wholemounted macaque retinas with the goal of identifying the specific types of bipolar cells that are immunoreactive for recoverin.

Recoverin in the rat bipolar cells

In vertical sections of rat retina, intensely immunostained bipolar cells had somata in the middle of the inner nuclear layer and axons that branched at two distinct depths in the IPL (Fig. 1A). Some rat bipolar cell axons gave rise to short processes that arborized in the outer portion of the IPL and in others the axon extended close to the inner border of the IPL before terminating (Fig. 1A, asterisks). The axonal arbors within each band were morphologically distinct. The outer band consisted of relatively fine processes and small terminal varicosities and the inner band showed thicker branches and larger varicosities.

Rod bipolar cells stratify at the inner border of the inner nuclear layer so it was possible that the inner stratifying recoverin-positive cells corresponded to rod bipolars. The dendrites of the recoverin-positive bipolar cells were not clearly visible because of the intense staining of the photoreceptors, such that a distinction between rod and cone bipolar cells could not be made on the basis of morphology of the terminal dendrites. Anti-PKC selectively stains the rod bipolar cells and we used this antibody to determine if recoverin and PKC staining were localized in the same bipolar cells. As shown by double labeling of single vertical sections, anti-PKC did not colocalize with anti-recoverin (Fig. 1B). The PKC-immunoreactive rod bipolar somata were much more numerous than the recoverin-stained cells. The depth of stratification in the IPL of axons reactive for recoverin and PKC was also distinct. The inner band of recoverin staining was situated slightly more sclerad than the PKC staining, although there was a slight overlap between the two bands (Fig. 1B). We conclude that anti-recoverin selectively labels a subpopulation of cone bipolar cells in the rat retina. The two morphologically distinct bands in the IPL suggest strongly that at least two distinct cone bipolar cell types are recoverin immunoreactive, although the identity of these types remains unknown.

Recoverin in primate bipolar cells

In human and monkey retinas, recoverin immunoreactivity was also restricted to cone bipolar cells (Fig. 2), but the pattern of labeling in the inner plexiform layer was distinct from that found in rat retina. Instead of two distinct bands of immunoreactivity in the inner and outer portions of the IPL, two bands of label were found in the outer IPL (Figs. 2A–2C). The outermost of these two bands was directly adjacent to the inner nuclear layer and was separated from the inner band by a narrow, 2–3 μm gap. The inner band was thin and immunostained varicosities appeared to be somewhat sparsely distributed by comparison to the outer band, which was broader and more densely filled with processes and varicosities. The same basic pattern of labeling was present in sections from both the central and peripheral retina (Figs. 2A–2C). In addition, there was very rare staining of single bipolar cells with axonal processes that extended to the inner portion of the IPL (Figs. 3 and 4). These cells were intensely immunoreactive for recoverin and had thick axonal processes and large varicosities relative to the immunoreactive processes in the outer IPL. Their somata also appeared to be slightly larger than the commonly stained bipolar cell type.

In the primate retina, two general classes of cone bipolar cells have been previously distinguished by the nature of their dendritic contacts with cone pedicles. The midget bipolar cell generally has a single dendrite that contacts one cone pedicle (Boycott & Wässle, 1991), although cells located in the retinal periphery sometimes give rise to a bifurcating dendrite that contacts two pedicles (Boycott & Hopkins, 1991). By contrast, the diffuse bipolar cell shows a multibranched dendritic tree that contacts several cone pedicles. We therefore examined the branching patterns of the dendrites of the recoverin-immunoreactive cells in the macaque retina to determine if they fell into the midget or diffuse class. In vertical cryostat sections, the bipolar cells that stratified in the outer IPL gave rise to a single dendrite that extended to the level of the cone pedicles without

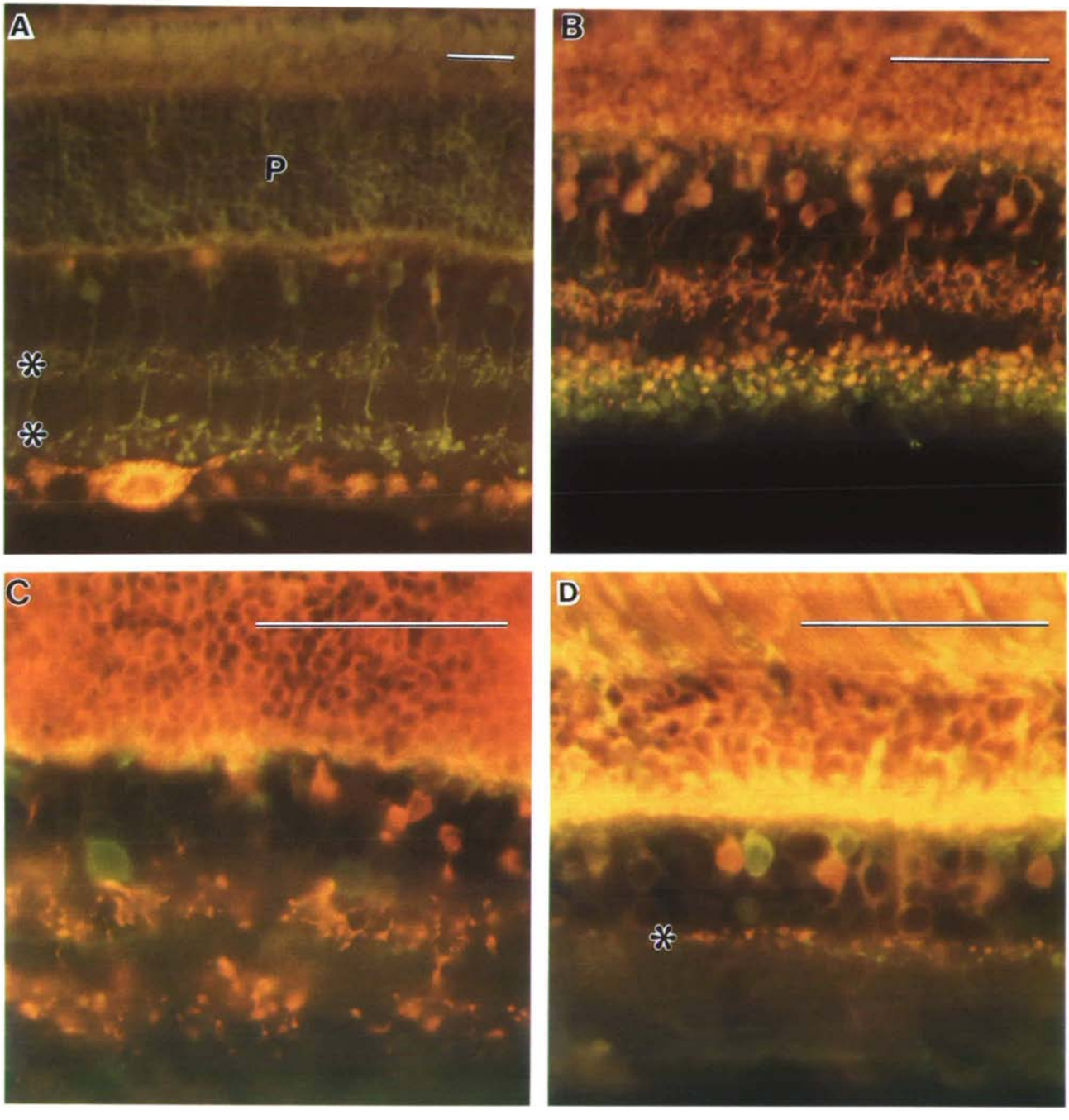


Fig. 1. Recoverin-immunoreactive neurons in rat and human retinas. A: Rat retina. In addition to reactive (yellow green) photoreceptor somata (P), bipolar cells with somata in the middle of the inner nuclear layer are also recoverin-positive. The axonal arbors of these bipolar cells form two distinct strata (*) in the outer and inner portions of the inner plexiform layer (IPL). The outer stratum contains relatively fine processes with small varicosities and the inner stratum contains thicker processes and larger varicosities. The ganglion cells are labeled with retrogradely transported rhodamine dextrin granules (red) injected into the optic tract 48 h prior to sacrifice. B: Rat retina. Double labeling with anti-recoverin (red) and anti-PKC (green) shows that the two markers for bipolar cells do not colocalize. The PKC-reactive axonal band (green) lies in the innermost IPL and overlaps slightly with the inner band of recoverin-reactive axon terminals. The PKC-reactive axon terminals are somewhat coarser than those in the recoverin-reactive bands. The PKC-positive somata are faintly stained. C: Rat retina. Double labeling with anti-recoverin (red) and anti-calbindin (green) shows that separate bipolar somata are labeled with these two markers. The calbindin-positive axons lie outside the plane of focus. D: Human retina. Labeled as in part C. Anti-recoverin (red) and anti-calbindin (green) labeled axonal bands overlap in the outer IPL (*) but the somata labeled by the two antibodies are clearly separate. Scale bars = 100 μm.

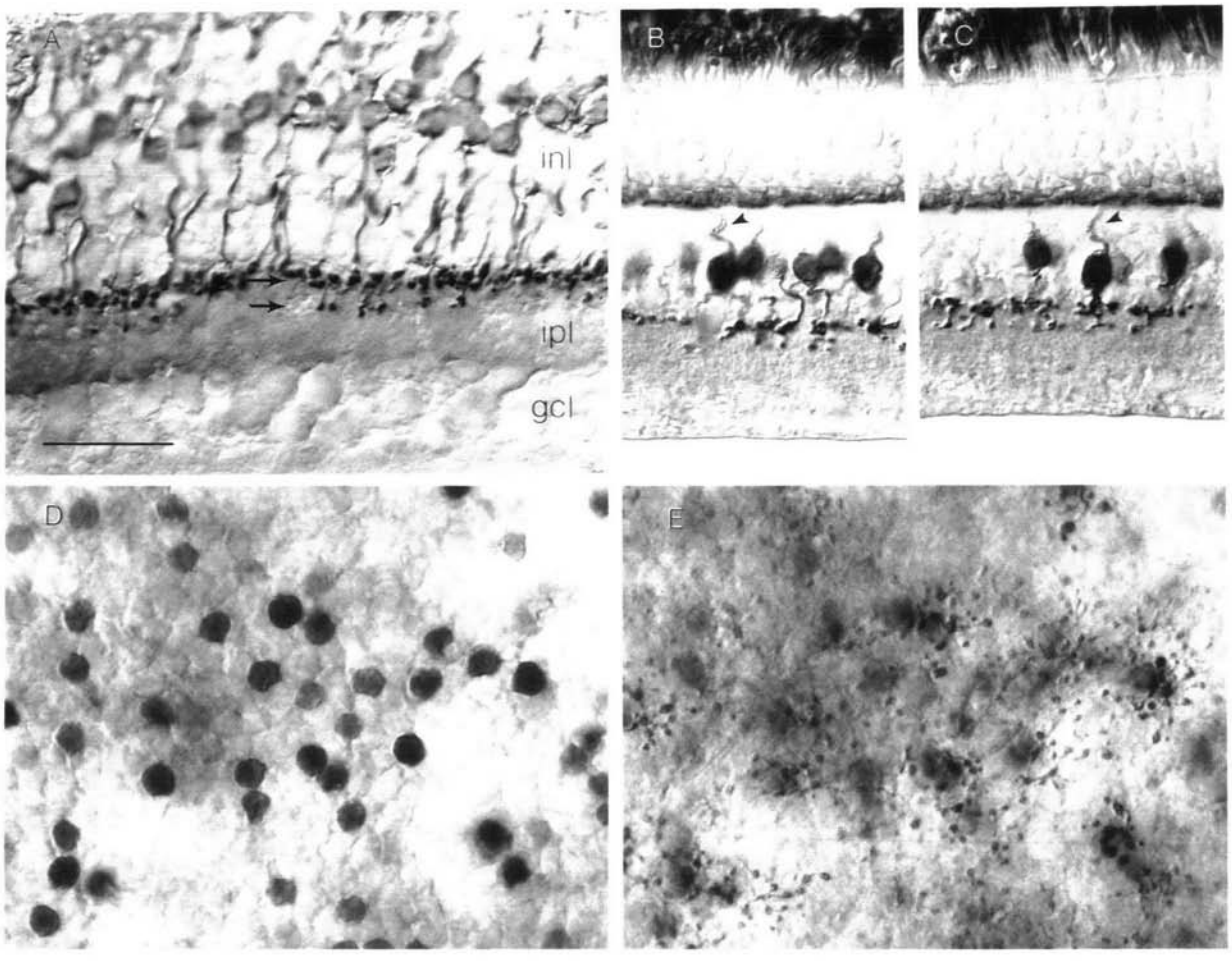
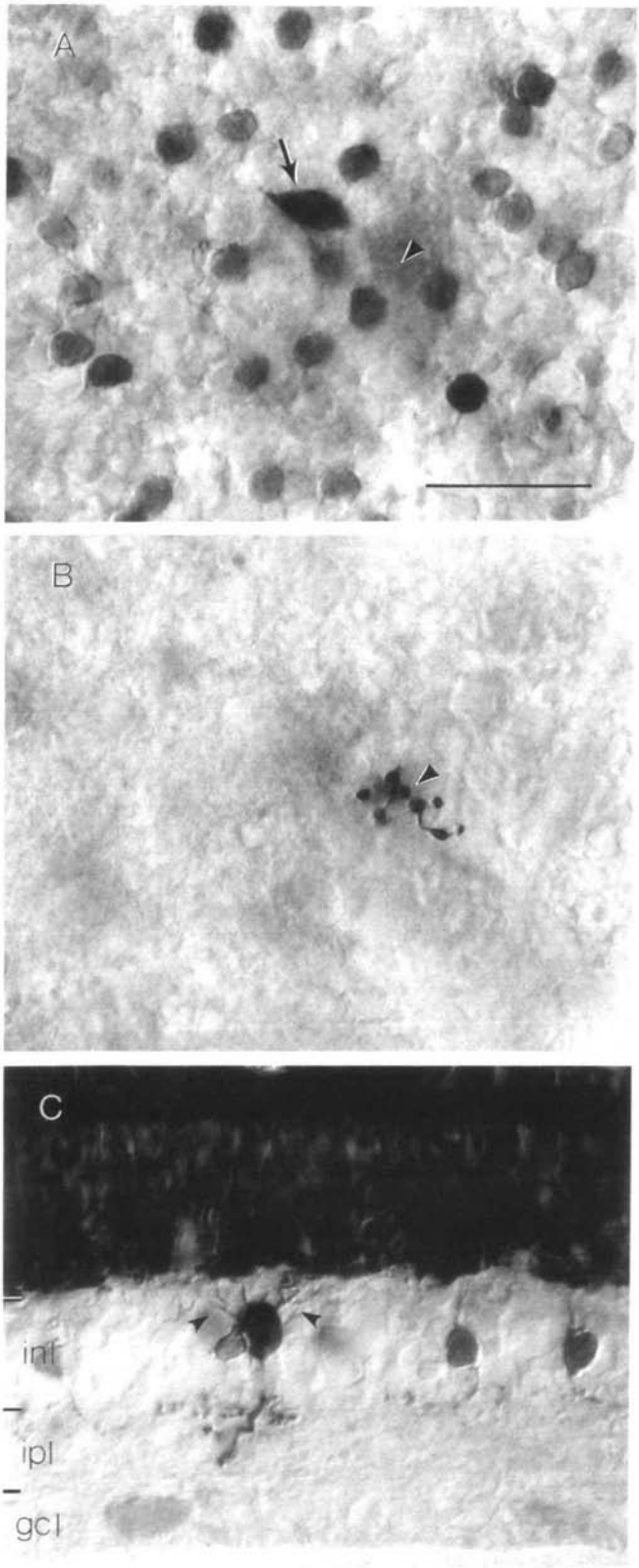


Fig. 2. Recoverin immunoreactivity in the retina of *M. nemestrina*. A: In central retina (1-mm eccentricity), stained bipolar cell bodies occupy 2–3 tiers of cells in the middle of the inner nuclear layer. Axonal fields form a narrow, dense band at the outer border of the IPL (upper arrow); a minor, slightly more vitread band, is also present (lower arrow). Vertical 10-μm cryostat section. B: In midperipheral retina (5-mm eccentricity), immunoreactive bipolar cells are at a lower density but the two immunoreactive bands in the outer IPL remain intensely labeled. In addition, at this density single dendrites can be traced from each cell to the level of the cone pedicles (arrowhead). Section as in (A). C: In peripheral retina (8-mm eccentricity), most recoverin-immunoreactive bipolar cells show a single dendrite that extends to the level of the cone pedicle (arrowhead). This dendritic morphology is characteristic of the midget class of cone bipolar cells. Section as in (A). D: Spatial distribution of immunoreactive bipolar cell bodies in retinal wholemount preparation at an eccentricity of 5–6 mm. E: Plane of focus is shifted to the IPL in the same field shown in the previous panel. Mosaic of neighboring axonal arbors in the outer IPL of the immunostained cone bipolars are shown. Scale bar in (A) = 50 μm and also applies to (B–E).

branching (Figs. 2B and 2C). This was the case in both the central and peripheral retina, although occasional bifurcated dendrites were found in the retinal periphery. These cells thus appeared to correspond to the midget class of cone bipolar cells. However, it is important to note that we did not observe the actual points of contact with the cone pedicle because of intense immunoreactivity in the pedicle itself. In addition, some diffuse bipolar types have dendrites that branch very close to the pedicle (Boycott & Wässle, 1991). By contrast, the rarely stained bipolar cells clearly showed the multibranched dendritic tree characteristic of the diffuse bipolar cell class (Figs. 3C and 4).

The midget bipolar cells have been divided further into two distinct types: invaginating and flat midget bipolar cells. Invaginating midget bipolars (presumed ON-type) form the central element within the cone triad and their axon stratifies in the inner portion of the IPL (Kolb, 1970). The flat midget bipolars (presumed OFF-type) make basal contacts with the cone pedicles and stratify in the outer portion of the IPL, adjacent to the inner nuclear layer (Kolb, 1970). Thus, the finding of recoverin immunoreactivity in bipolar cells that have unbranched dendrites and axonal processes that are stratified at the outer border of the IPL suggests strongly that recoverin is a selective marker for the flat midget, presumed hyperpolarizing bipolar cell type in the primate retina.

Further evidence that anti-recoverin selectively labels the OFF-midget bipolar cell type comes from a preliminary analysis of the mosaic organization of these cells observed in wholemount preparations where the morphology of single bipolar cell axonal arbors was revealed in detail (Figs. 2D–2E and 5–7). The axonal arbors of each cell were narrowly bistratified (Fig. 5). The axons branched into an outer arbor composed of relatively large varicosities. From this part of the arbor, one or two thin



processes extended further vitread and arborized sparsely to create a second arbor that was smaller in size, more sparsely branched and tended to have smaller varicosities. We observed this bistratified pattern in every well-stained cell. The morphology of individual cells therefore accounted for the bistratified pattern of staining in the outer IPL that was observed in vertical sections (Figs. 2A-2C).

In addition to a characteristic morphology, the recoverin-immunoreactive cone bipolars also showed the mosaic organization characteristic of a single cell type (Wässle & Riemann, 1978). Axonal-field area increased from ~100 μm^2 at 5-mm eccentricity to ~250 μm^2 at 10-mm from the fovea. Conversely, the cell density decreased from 5500 cells/mm^2 to ~2000 cells/mm^2 over the same eccentricity range. The result was a constant mean coverage of 0.58. Although the coverage is very low and neighboring cells did not overlap, the regular, space-filling arrangement of the axon arbors suggested strongly that all members of the population were revealed by the recoverin immunostaining (Fig. 6).

The rarely stained diffuse bipolar cells were also observed in the retinal wholemounts. These cells were intensely stained but appeared at an extremely low density of only 2-5 cells/mm^2. By contrast with the commonly stained cells, the axonal arbors were monostratified and had much thicker processes and larger varicosities as seen in the vertical sections of the retina (Figs. 3C and 4B). The arbor size was small, about 15 μm in diameter, and did not appear to show any change in size over the eccentricity range in which they were observed (Fig. 7). Because of this uniformity in axon arbor size, at 5-mm eccentricity the rare cells had slightly larger arbors than the commonly stained cells, but in the retinal periphery the reverse was true, and the common cells achieved a larger size than the rarely stained cells (Fig. 7). A summary of the differences that were found between the two populations of cone bipolar cells in the macaque retina are given in Table 1.

Colocalization with other cone bipolar cell markers

Primate blue cone bipolar cells can be labeled selectively with anti-CCK (Kouyama & Marshak, 1992; Marshak et al., 1990). The dendritic trees of these cells are large and sparsely branching as they extend to selectively contact the small population of blue cone pedicles. The axonal fields of these cells are also large and stratify close to the inner border of the IPL. Therefore, it was unlikely that either of the recoverin-immunoreactive cell types in the primate could correspond to blue cone bipolar cells. As expected no colocalization was found when adjacent sections were reacted for anti-recoverin and anti-CCK (data not shown).

Fig. 3. Recoverin immunoreactivity in rarely stained cells in the macaque retina. A: Immunoreactive bipolar cell bodies in retinal wholemount preparation. Arrow indicates a single, intensely stained cell with a slightly larger soma size than the rest of the immunoreactive population. B: Plane of focus is shifted to the inner half of the IPL revealing the axonal arbor of this cell. The same point in both (A) and (B) is indicated by the arrowheads. All of the other bipolar cells arborize in the outer IPL as shown in Fig. 2E. C: Vertical 10-μm cryostat section through one of the rarely stained cells reveals the multibranched dendritic tree characteristic of the diffuse class of cone bipolar cells (arrowhead). Part of the axonal arbor can also be seen extending into the inner IPL. Scale bar in (A) = 50 μm and applies also to (B) and (C).

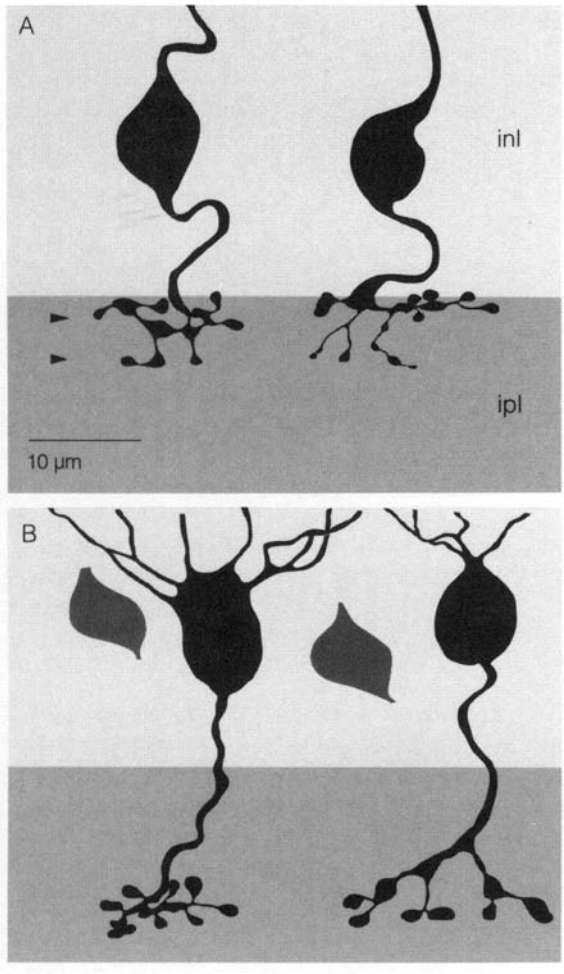


Fig. 4. Camera-lucida tracings of four examples of the recoverin-immunoreactive cone bipolar cells in macaque taken from the retinal periphery summarizes the morphological differences between the two cell types. A: Cone bipolar cell with single dendrite that extends to the level of the cone pedicles and a narrowly bistratified arbor in the outer IPL. The two levels of stratification are indicated by the arrowheads. B: Rarely stained cone bipolar with a multibranched dendritic arbor and a monostratified axonal arbor in the inner half of the IPL. The rarely stained cells also appear to have a larger cell body and thicker axonal processes than the commonly stained cells. Scale bar in (A) applies also to (B).

Certain cone bipolar cells are labeled with anti-calbindin in some species (Verstappen et al., 1986; Hamano et al., 1990; Pasteels et al., 1990). The calbindin- and recoverin-positive bipolar cells did not colocalize in sections of rat or human retina (Figs. 1C and 1D). In rat retina, the calbindin-positive axons in the IPL formed several thin laminae that did not coincide with the two recoverin-positive laminae, and separate bipolar somata were labeled with the two antibodies (Fig. 1C). In human retina, the band in the IPL that was immunoreactive for calbindin overlapped the pair of IPL laminae that were labeled with anti-recoverin, but the somata labeled with the two antibodies were clearly separate (Fig. 1D).

Wiechmann and co-workers showed that an antibody preparation against HIOMT labeled a population of cone bipolar cells in human and other vertebrate retinas (Wiechmann & Hollyfield, 1987). We reacted adjacent sections of human and rat retinas with anti-recoverin and anti-HIOMT and found that both antibodies appeared to label the same populations of bipolar cells. The cone bipolar cells labeled with both antibody preparations were very similar in number, location of somata, and axonal ramification patterns in the IPL (data not shown). To test for cross reactivity of the two antibody preparations, we adsorbed the anti-HIOMT (1:200) with purified recoverin (5–50 μg/ml) and found that adsorption of anti-HIOMT with each concentration of recoverin blocked all staining of cone bipolar cells in human and rat retinas. The same result was obtained independently by Dr. A. Wiechmann (personal communication), who also found that rat bipolar staining was blocked by preadsorption with recoverin.

Photoreceptors and bipolar cells show similar affinities for the recoverin antibody

Control sections from all species were treated with nonimmune rabbit serum (1:500–1:5000) and showed no specific labeling. To determine the relative recoverin immunoreactivity of cone bipolar cells and photoreceptors in human retinas, we preincubated the anti-recoverin with purified recoverin in a dilution series (0.01–5 μg/ml). This dilution series revealed that both photoreceptors and bipolar cells were strongly stained (0.01–0.1 μg/ml), weakly stained (0.5–1 μg/ml), or blocked (5 μg/ml) at the same dilutions of recoverin. The areas of human retina that remained reactive at the highest concentration of adsorbed recoverin (0.5–1 mg/ml) were the rod and cone outer segments, photoreceptor synapses, and the bipolar somata. Immunofluorescence using progressive dilutions of the antibody against recoverin revealed that staining of both photoreceptors and bipolar cells in human retinas was strong (1:500–1:5000), weak (1:50,000–1:75,000), or nondetectable (1:100,000) at the same dilutions. These observations suggest that bipolar and photoreceptor cells in fixed human retinas have similar affinities for anti-recoverin and that the molecule in bipolar cells that is recognized by this antibody is recoverin.

Discussion

Colocalization with other bipolar cell markers

We have identified two populations of recoverin-immunoreactive bipolar cells in the retinas of human, monkey, and rat. These bipolar cells are not immunoreactive for PKC, CCK, or calbindin. Have the recoverin-immunoreactive bipolars been observed with any other antibodies that stain bipolar cells? Antibodies against PEP19, a putative calcium-binding protein also found in cerebellar neurons, marks cone and rod bipolar cells in mouse and rabbit retinas (Berrebi et al., 1991). More cone bipolars are labeled with anti-PEP19 than with anti-recoverin so that it remains possible that a subset of the PEP19 immunoreactive cells also contain recoverin. Recoverin shows ~60% identity with visinin, a 23- or 24-kD calcium-binding protein first demonstrated in avian cones (Yamagata et al., 1990). However, visinin has been localized to horizontal cells and amacrine cells but not to bipolar cells in primate retina (Hatakenaka et al., 1985).

The present study has revealed that the same cone bipolar cell types are labeled with anti-recoverin and anti-HIOMT in rat and human retinas. However, adsorption studies performed by Dr. Alan Wiechmann (personal communication) and ourselves

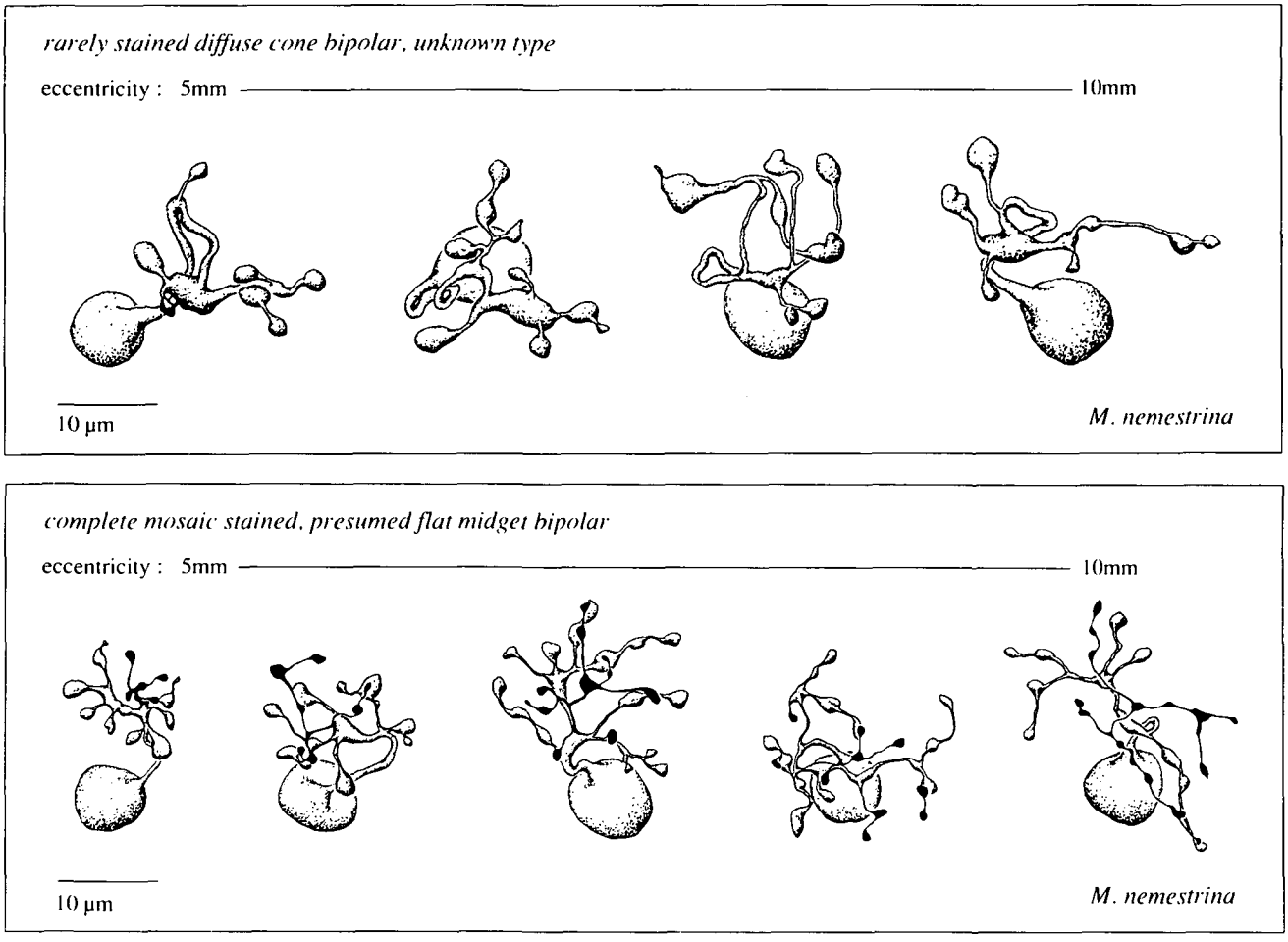


Fig. 5. Camera-lucida tracings of the axonal arbors of rarely stained (upper panel) and commonly stained (lower panel) recoverin-immunoreactive cone bipolar cells from a wholemount preparation of macaque retina. Over an eccentricity range of 5–10 mm the rarely stained cells show no obvious change in axonal-field size. By contrast, the commonly stained cells increase in size over this eccentricity range (see also Figs. 6 and 7). The outer and inner strata of these bistratified cells are indicated by the stippled and solid shading, respectively. The inner branches are smaller in size and are composed of finer processes and smaller varicosities.

Table 1. *Morphology of recoverin-immunoreactive cone bipolar cell types in macaque retina*

	Commonly stained	Rarely stained
Dendritic morphology	Single dendrite, occasional dendritic bifurcation	Multibranched dendritic arbor
Axonal morphology	Narrowly bistratified, major and minor bands	Monostratified
Axonal stratification	Major band: ~0–20% Minor band: ~30–40%	~60–80%
Cell density (cells/mm^2) (5–10 mm eccentricity)	5500–2000	Rare, 2–5 cells/mm^2
Axonal field area (μm^2)	~110–300	~100–200
Axonal-field coverage	0.58	—
Bipolar cell type	Flat midget cone bipolar, presumed hyperpolarizing	Unknown diffuse bipolar type
Functional pathway	Single cone connecting, high acuity, color selective, OFF-center midget pathway	Unknown

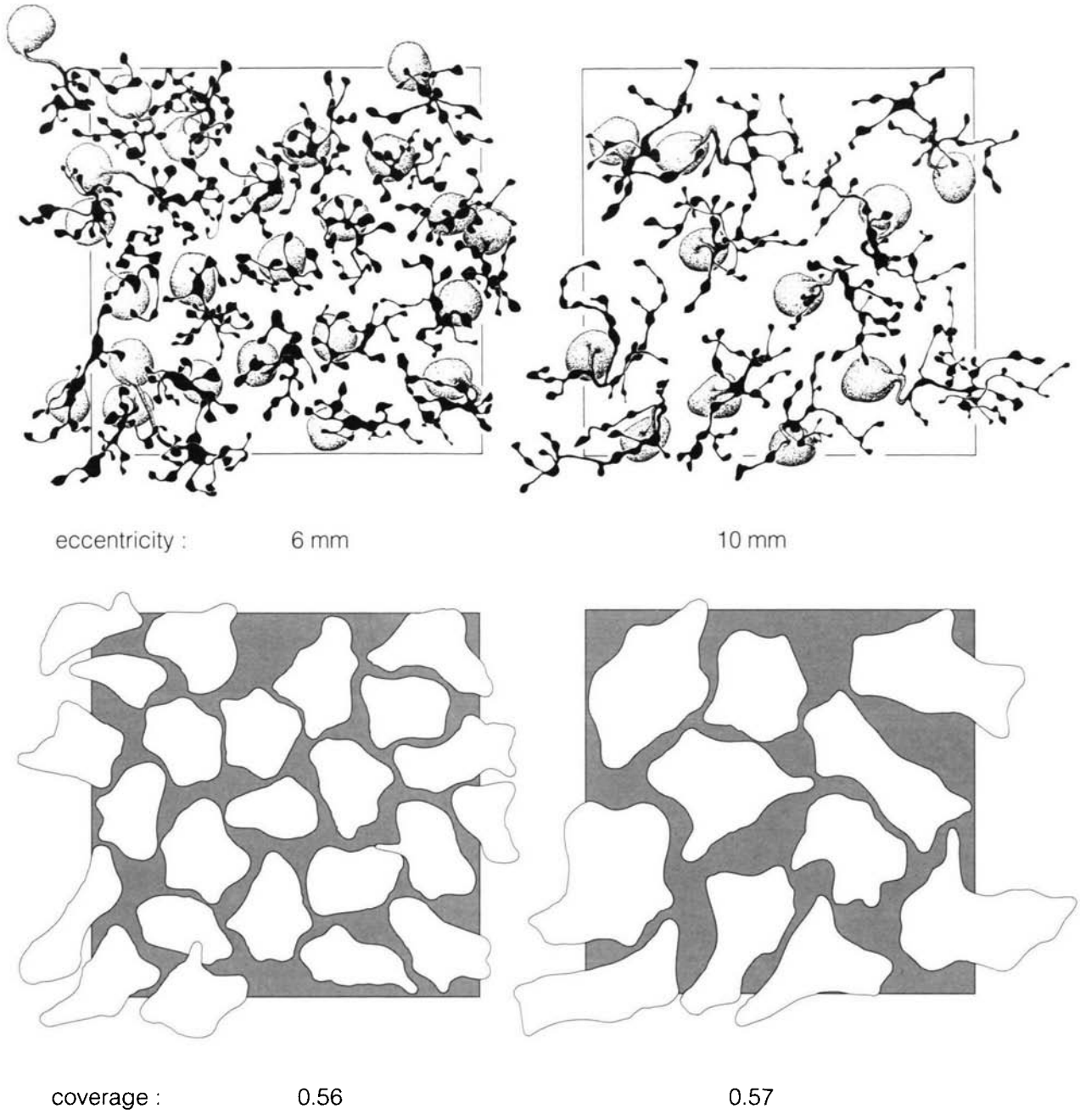


Fig. 6. Mosaic organization of recoverin-immunoreactive cone bipolar cells in macaque. In a wholemount preparation, the commonly stained bipolar cells appear as a single, morphologically homogeneous population of cells with a distinctive spatial distribution. The upper panels are camera-lucida tracings of the cell bodies (stippled) and axonal fields (solid) of all of the cells stained in a small patch of retina (the window is 60 μm on a side) at 6-mm and 10-mm eccentricity. The orderly tiling of the axonal trees are shown in the bottom panels by tracing around the perimeter of each arbor in the mosaic. The arbor size increases with increasing eccentricity but for both patches the coverage and regularity of the mosaic remain constant.

indicate that recoverin is probably the molecule in cone bipolar cells that is recognized by this antibody preparation against HIOMT. Sequence analysis of recoverin and a bovine HIOMT reveals low identity (10%) and overall similarity (40%) (Dr. John Crabb, personal communication), and the adsorption results suggest that the antibody preparation against bovine pineal HIOMT contains antibodies against recoverin, which is known to be present in this organ (Dizhoor et al., unpublished observations). Recoverin was identified independently by Polans et al. (1991) as the retinal antigen that is involved in the cancer-associated retinopathy (CAR) syndrome. Polans et al. (1991) localized the CAR antigen by immunocytochemistry to rods, cones, and some neurons of the inner nuclear layer of human retinas. It is very likely that at least some of the inner nuclear layer neurons labeled with anti-CAR antigen correspond to the cone bipolar cells that are labeled with anti-recoverin.

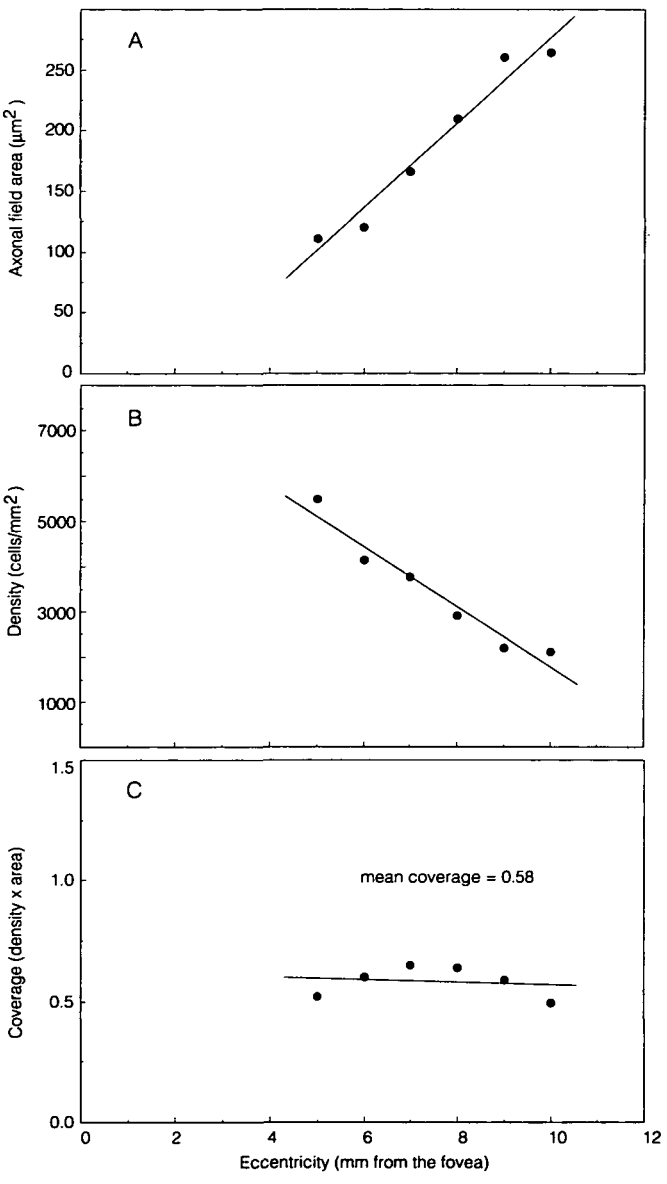


Fig. 7. Scatterplots of axonal-field diameter, cell density, and coverage for the commonly stained recoverin-immunoreactive bipolar cells in the macaque. Axonal-field size (A) is inversely correlated with cell density (B), over the sampled eccentricity range. C: This relationship gives rise to a relatively constant coverage (cell density × axonal field area). Straight lines fit to data points by linear regression.

Identification of cone bipolar cell types

Several pieces of morphological evidence suggest strongly that anti-recoverin selectively labels the population of flat midget (presumed OFF-center) bipolar cells in the macaque retina. First, the great majority of reactive bipolar cells have a single dendrite that extends unbranched to the cone pedicle, a characteristic of the single cone contacting midget bipolar cells (Boycott & Wässle, 1991). Second, the axonal fields stratify narrowly near the outer border of the IPL at about 0–20% depth, which corresponds to the depth of stratification for the flat midget bipolar cells that have been observed by Golgi impregnation (Boycott & Dowling, 1969; Boycott & Wässle, 1991). This depth of stratification suggests that the flat midget bipolars give hyperpolarizing light responses (Famiglietti & Kolb, 1976). Third, the axonal-field size falls into the size range previously suggested for the midget bipolars. The axonal fields of the recoverin-immunoreactive cells in the macaque flatmounts were about 10–15 μm in diameter at 5–7 mm eccentricity. This matches closely the measurements made for flat midget bipolar cells in a recent study of Golgi-impregnated cells (Boycott & Wässle, 1991). Fourth, the density of the recoverin-immunoreactive bipolars that we have measured is close to that found for cone photoreceptors in the macaque (Packer et al., 1989). This would be predicted for the midget system since there should be a single ON- and OFF-center type for each cone. Our preliminary density counts (Fig. 7) provide the first confirmation of that expectation and further support the previous finding (Boycott & Wässle, 1991) that, even in the retinal periphery, most midget bipolar cells contact only one cone pedicle. Final proof that recoverin is localized to the OFF-center midget bipolar cells will require electron-microscopic demonstration of immunoreactivity localized to the basal contacts at the cone pedicle (e.g. Hopkins & Boycott, 1992).

One consistent feature of the morphology of the recoverin-immunoreactive bipolars that has not been observed in previous studies is the presence of a second, minor stratification of these cells at ~40% depth in the IPL. A few midget bipolars with such a bistratified morphology were illustrated in the classic work of Polyak (1941; his Fig. 54), although he did not comment on this feature. The significance of this bistratification is not yet apparent but it suggests that the synaptic relationship between the midget bipolar and the midget ganglion cell type may be more complex than previously suggested (Kolb & Dekorver, 1991; Calkins et al., 1992).

Our results also provide the first picture of the mosaic organization of a single cone bipolar type in the macaque retina. The recoverin-immunoreactive cells showed a constant but very low mean coverage of ~0.6 over the eccentricity range of 5–10 mm (Figs. 6 and 7). A similar coverage has been found for the PKC-immunoreactive rod bipolar cells in the rabbit retina, suggesting that this type of mosaic organization may be characteristic of bipolar cells in general (Young & Vaney, 1991).

The rarely stained cone bipolar cells are presently an enigma, given the current understanding of bipolar cells in the macaque retina. These cells have the dendritic morphology of diffuse bipolar cells, which have been shown to nonselectively connect to several cone pedicles within their fields (Boycott & Wässle, 1991). However, they are clearly not identical to any known diffuse bipolar cell type because the axonal arbor size of this rarely stained cell is about the same as that for the midget bipolar cells and in the periphery may even be smaller. The very intense staining of these single, isolated cells is also an oddity. It is not clear whether this reflects partial staining of a larger population with a density and mosaic organization as we described for the midget bipolar cells or whether this density is an unprecedented but characteristic feature of this cell type. The former possibility seems more likely given the results in the rat. As in the macaque, the population of bipolars in the rat that stratified in the outer IPL showed finer processes and varicosities than the coarser inner stratifying population. It seems possible then that analogous bipolar populations are reactive in both the rats and monkeys but that in the monkey the inner branching type is only rarely stained.

Significance of recoverin in cone bipolar cells

Cyclic guanosine monophosphate (cGMP)-gated channels are well documented in photoreceptor outer segments, which contain several types of molecules (e.g. transducin, phosphodiesterase, arrestin, rhodopsin kinase, rhodopsin phosphatase, and phosducin) that function in the cyclic nucleotide cascade of phototransduction (reviewed in Hurley, 1992). Generation of the photoreceptor's electrical response to light involves the hydrolysis of cGMP and subsequent closure of the cGMP-gated channels. Recoverin is believed to play a key role in the visual process by mediating the activation of guanylate cyclase activity in conditions of low calcium concentrations, and thereby promoting restoration of the pre-illumination level of cGMP (Koch & Stryer, 1988; Dizhoor et al., 1991; Lambrecht & Koch, 1991). Recoverin-like protein is also reported to have a Ca^{2+}-dependent effect on cGMP phosphodiesterase activity in frog rod outer segments (Kawamura & Murakami, 1991).

The function of recoverin in cone bipolar cells is unknown. However, recent evidence suggests that depolarizing bipolar cells in certain submammalian retinas have glutamate receptors that are linked to a cGMP-gated channel (Nawy & Jahr, 1990, 1991; Shiells & Falk, 1992*a*,*b*). Glutamate release by photoreceptors in the dark is thought to close ion channels by increasing the rate of cGMP hydrolysis, mediated by a G-protein process analogous to the enzyme cascade that underlies the light response in outer segments (discussed by Yamashita & Wässle, 1991), although the channels in bipolars are different from those of rods (Shiells & Falk, 1992*b*; Wässle et al., 1992). Recoverin immunoreactivity in cone bipolar cells suggests that recoverin or a closely related protein might be involved in regulation of cGMP levels as occurs in photoreceptor outer segments during recovery from light stimulation (Dizhoor et al., 1991). However, the evidence that recoverin is localized essentially to the presumed *hyperpolarizing* midget bipolar cells in macaque is a surprise, given the evidence for cGMP-regulated channels (cited above) and a slower time course for the glutamate induced current found in *depolarizing* bipolar cells (e.g. Copenhagen et al., 1983). Therefore, one can also suggest that recoverin in bipolar cells may regulate a Ca^{2+}-dependent process different from that in photoreceptor cells.

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References

Berrebi, A.S., Oberdick, J., Sangameswaran, L., Christakos, S., Morgan, J.I. & Mugnaini, E. (1991). Cerebellar Purkinje cell markers are expressed in retinal bipolar neurons. *Journal of Comparative Neurology* **308**, 630–649.

Boycott, B.B. & Dowling, J.E. (1969). Organization of the primate retina: Light microscopy. *Philosophical Transactions of the Royal Society B* (London) **255**, 109–184.

Boycott, B.B. & Hopkins, J.M. (1991). Cone bipolar cells and cone synapses in the primate retina. *Visual Neuroscience* **7**, 49–60.

Boycott, B.B. & Wässle, H. (1991). Morphological classification of bipolar cells of the primate retina. *European Journal of Neuroscience* **3**, 1069–1088.

Calkins, D.J., Schein, S.J., Tsukamoto, Y., Masarachia, P. & Sterling, P. (1992). Parallel pathways to midget ganglion cells in macaque fovea. *Investigative Ophthalmology and Visual Science* **33**, 1173.

Cohen, E. & Sterling, P. (1986). Accumulation of [^{3}H]-glycine by cone bipolar neurons in the cat retina. *Journal of Comparative Neurology* **250**, 1–7.

Cohen, E. & Sterling, P. (1990). Demonstration of cell types among cone bipolar neurons of cat retina. *Philosophical Transactions of the Royal Society B* (London) **330**, 305–321.

Copenhagen, D.R., Ashmore, J.F. & Schnapf, J. (1983). Kinetics of synaptic transmission from photoreceptors to horizontal and bipolar cells in turtle retina. *Vision Research* **23**, 363–369.

Dizhoor, A.M., Ray, S., Kumar, S., Niemi, G., Spencer, M., Brolly, D., Walsh, K.A., Philipov, P.P., Hurley, J.B. & Stryer, L. (1991). Recoverin, a calcium sensitive activator of retinal rod guanylate cyclase. *Science* **251**, 915–918.

Ehinger, B., Ottersen, O.P., Storm-Mathisen, J. & Dowling, J.E. (1988). Bipolar cells in the turtle retina are strongly immunoreactive for glutamate. *Proceedings of the National Academy of Sciences of the U.S.A.* **85**, 8321–8325.

Famiglietti, E.V., Jr. (1981). Functional architecture of cone bipolar cells in mammalian retina. *Vision Research* **21**, 1559–1563.

Famiglietti, E.V., Jr. & Kolb, H. (1976). Structural basis for ON- and OFF-center responses in retinal ganglion cells. *Science* **194**, 193–195.

Graferath, U., Grünert, U., & Wässle, H. (1990). Rod bipolar cells in the mammalian retina show protein kinase C-like immunoreactivity. *Journal of Comparative Neurology* **301**, 433–442.

Grünert, U. & Martin, P.R. (1991). Rod bipolar cells in the macaque monkey retina: Immunoreactivity and connectivity. *Journal of Neuroscience* **11**, 2742–2758.

Grünert, U. & Wässle, H. (1990). GABA-like immunoreactivity in the macaque monkey retina: A light and electron microscopic study. *Journal of Comparative Neurology* **297**, 509–524.

Hamano, K., Kiyama, H., Emson, P.C., Manabe, R., Nakauchi, M. & Tohyama, M. (1990). Localization of two calcium-binding proteins, calbindin (28 kD) and parvalbumin (12 kD), in the vertebrate retina. *Journal of Comparative Neurology* **302**, 417–424.

Hatakenaka, S., Kiyama, H., Tohyama, M. & Miki, N. (1985). Immunohistochemical localization of chick retinal 24 kDalton protein (visinin) in various vertebrate retinae. *Brain Research* **311**, 209–215.

Hendrickson, A.E., Koontz, M., Pourcho, R.G., Sarthy, P.V. & Goebel, D.J. (1988). Localization of glycine-containing neurons in the *Macaca* monkey retina. *Journal of Comparative Neurology* **273**, 473–487.

Hopkins, J.M. & Boycott, B.B. (1992). Synaptic contacts of a two-cone flat bipolar cell in a primate retina. *Visual Neuroscience* **8**, 379–384.

Hurley, J.B. (1992). Signal transduction enzymes of vertebrate photoreceptors. *Journal of Bioenergetics and Biomembranes* **24**, 219–226.

Kawamura, S. & Murakami, M. (1991). Calcium-dependent regulation of cyclic GMP phosphodiesterase by a protein from frog retinal rods. *Nature* **349**, 420–423.

Koch, K-W. & Stryer, L. (1988). Highly cooperative feedback control of retinal rod guanylate cyclase by calcium ions. *Nature* **334**, 64–66.

Kolb, H. (1970). Organization of the outer plexiform layer of the primate retina. *Philosophical Transactions of the Royal Society B* (London) **258**, 261–283.

Kolb, H., Nelson, R. & Mariani, A. (1981). Amacrine cells, bipolar cells, and ganglion cells of the cat retina: A Golgi study. *Vision Research* **21**, 1081–1114.

Kolb, H. & Dekorver, L. (1991). Midget ganglion cells of the parafovea of the human retina: A study by electron microscopy of serial sections. *Journal of Comparative Neurology* **303**, 617–636.

KOUYAMA, N. & MARSHAK, D.W. (1992). Bipolar cells specific for blue cones in the macaque retina. *Journal of Neuroscience* **12**, 1233–1252.

LAMBRECHT, H.-G. & KOCH, K.-W. (1991). A 26 kD calcium binding protein from bovine rod outer segments as modulator of photoreceptor guanylate cyclase. *European Molecular Biology Organization Journal* **10**, 793–798.

MARIANI, A.P. (1983). Giant bistratified bipolar cells in monkey retina. *Anatomical Record* **206**, 215–220.

MARIANI, A.P. (1984). Bipolar cells in monkey retina selective for the cones likely to be blue-sensitive. *Nature* **308**, 184–186.

MARSHAK, D.W., ALDRICH, L.B., DEL VALLE, J. & YAMADA, T. (1990). Localization of immunoreactive cholecystokinin precursor to amacrine cells and bipolar cells of the macaque monkey retina. *Journal of Neuroscience* **10**, 3045–3055.

MARTIN, P.R. & GRÜNERT, U. (1991). Immunoreactivity of rod and cone bipolar cells in the macaque monkey retina. *Investigative Ophthalmology and Visual Science* **32**, 991 (Abstract #1587).

MCGUIRE, B.A., STEVENS, J.K. & STERLING, P. (1984). Microcircuitry of bipolar cells in cat retina. *Journal of Neuroscience* **4**, 2920–2938.

MILAM, A.H., DE LEEUW, A.M., GAUR, V.P. & SAARI, J.C. (1990). Immunolocalization of cellular retinoic acid binding protein to Müller cells and/or a subpopulation of GABA-positive amacrine cells in retinas of different species. *Journal of Comparative Neurology* **296**, 123–129.

NAWY, S. & JAHR, C.E. (1990). Suppression by glutamate of cGMP-activated conductance in retinal bipolar cells. *Nature* **346**, 269–271.

NAWY, S. & JAHR, C.E. (1991). cGMP-gated conductance in retinal bipolar cells is suppressed by the photoreceptor transmitter. *Neuron* **7**, 677–683.

NEGISHI, K., KATO, S. & TERANISHI, T. (1988). Dopamine cells and rod bipolar cells contain protein kinase C-like immunoreactivity in some vertebrate retinas. *Neuroscience Letters* **94**, 247–252.

NELSON, R. & KOLB, H. (1983). Synaptic patterns and response properties of bipolar and ganglion cells in the cat retina. *Vision Research* **23**, 1183–1195.

ONADA, N. (1988). A monoclonal antibody specific for a subpopulation of retinal bipolar cells in vertebrates. *Neuroscience Research* **8**, 113–125.

ONADA, N. & FUJITA, S.C. (1987). A monoclonal antibody specific for a subpopulation of retinal bipolar cells in the frog and other vertebrates. *Brain Research* **416**, 359–363.

PACKER, O., HENDRICKSON, A.E. & CURCIO, C.A. (1989). Photoreceptor topography of the retina in the adult pigtail Macaque (*Macaca nemestrina*). *Journal of Comparative Neurology* **288**, 165–183.

PASTEELS, B., ROGERS, J., BLACHIER, F. & POCHET, R. (1990). Calbindin and calretinin localization in retina from different species. *Visual Neuroscience* **5**, 1–16.

POLANS, A.S., BUCZYLKO, J., CRABB, J. & PALCZEWSKI, K. (1991). Photoreceptor calcium binding protein is recognized by autoantibodies obtained from patients with cancer-associated retinopathy. *Journal of Cell Biology* **112**, 981–989.

POLYAK, S.L. (1941). *The Retina.* Chicago, Illinois: University of Chicago Press.

POURCHO, R.G. & GOEBEL, D.J. (1987). A combined Golgi and autoradiographic study of ^{3}H-glycine-accumulating cone bipolar cells in the cat retina. *Journal of Neuroscience* **7**, 1178–1188.

SHIELLS, R.A. & FALK, G. (1992*a*). The glutamate-receptor linked cGMP cascade of retinal ON-bipolar cells is pertussis and cholera toxin-sensitive. *Proceedings of the Royal Society B* (London) **247**, 17–20.

SHIELLS, R.A. & FALK, G. (1992*b*). Properties of the cGMP-activated channel of retinal on-bipolar cells. *Proceedings of the Royal Society B* (London) **247**, 21–25.

VERSTAPPEN, A., PARMENTIER, M., CHIRNOAGA, M., LAWSON, D.E.M., PASTEELS, J.L. & POCHET, R. (1986). Vitamin D-dependent calcium binding protein immunoreactivity in human retina. *Ophthalmic Research* **18**, 209–214.

WÄSSLE, H. & BOYCOTT, B.B. (1991). Functional architecture of the mammalian retina. *Physiology Review* **71**, 447–480.

WÄSSLE, H. & CHUN, M.H. (1989). GABA-like immunoreactivity in the cat retina: Light microscopy. *Journal of Comparative Neurology* **279**, 43–54.

WÄSSLE, H., GRÜNERT, U., COOK, N.J. & MOLDAY, R.S. (1992). The cGMP-gated channel of rod outer segments is not localized in bipolar cells of the mammalian retina. *Neuroscience Letters* **134**, 199–202.

WÄSSLE, H. & RIEMANN, H.J. (1978). The mosaic of nerve cells in the mammalian retina. *Proceedings of the Royal Society B* (London) **200**, 441–461.

WÄSSLE, H., SCHÄFER-TRENKLER, I. & VOIGT, T. (1986). Analysis of a glycinergic inhibitory pathway in the cat retina. *Journal of Neuroscience* **6**, 594–604.

WÄSSLE, H., YAMASHITA, M., GREFERATH, U., GRÜNERT, U. & MÜLLER, F. (1991). The rod bipolar cell of the mammalian retina. *Visual Neuroscience* **7**, 99–112.

WIECHMANN, A.F. & HOLLYFIELD, J.G. (1987). Localization of hydroxyindole-O-methyltransferase-like immunoreactivity in photoreceptors and cone bipolar cells in the human retina: A light and electron microscope study. *Journal of Comparative Neurology* **258**, 253–266.

WIECHMANN, A.F. & HOLLYFIELD, J.G. (1989). HIOMT-like immunoreactivity in the vertebrate retina: A species comparison. *Experimental Eye Research* **49**, 1079–1095.

WIECHMANN, A.F. & O'STEEN, W.K. (1990). Hydroxyindole-O-methyltransferase in rat retinal bipolar cells: Persistence following photoreceptor destruction. *Brain Research* **506**, 14–18.

WOOD, J.G., HART, C.E., MAZZEI, G.J., GIRARD, P.R. & KUO, J.F. (1988). Distribution of protein kinase C immunoreactivity in rat retina. *Histochemistry Journal* **20**, 63–68.

YAMAGATA, K., GOTO, K., KUO, C.H., KONDO, H. & MIKI, N. (1990). Visinin: a novel calcium binding protein expressed in retinal cone cells. *Neuron* **2**, 469–476.

YAMASHITA, M. & WÄSSLE, H. (1991). Responses of rod bipolar cells isolated from the rat retina to the glutamate agonist 2-amino-4-phosphonobutyric acid (APB). *Journal of Neuroscience* **11**, 2372–2382.

YOUNG, H.M. & VANEY, D.I. (1991). Rod-signal interneurons in the rabbit retina: I. Rod bipolar cells. *Journal of Comparative Neurology* **310**, 139–153.

ZHANG, D. & YEH, H.H. (1991). Protein kinase C-like immunoreactivity in rod bipolar cells of the rat retina: A developmental study. *Visual Neuroscience* **6**, 429–437.