Immunocytochemical Identification of Cone Bipolar Cells in the Rat Retina

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ABSTRACT
We studied the morphology of bipolar cells in fixed vertical tissue sections of the rat retina by injecting the cells with Lucifer Yellow and neurobiotin. In addition to the rod bipolar cell, nine different putative cone bipolar cell types were distinguished according to the position of their somata in the inner nuclear layer and the branching pattern and stratification level of their axon terminals in the inner plexiform layer. Some of these bipolar cell populations were labeled immunocytochemically in vertical and horizontal sections using antibodies against the calcium-binding protein recoverin, the glutamate transporter GLT-1, the alpha isoform of the protein kinase C, and the Purkinje cell marker L7. These immunocytochemically labeled cell types were characterized in terms of cell density and distribution.

We found that rod bipolar cells and GLT-1-positive cone bipolar cells occur at higher densities in a small region located in the upper central retina. This area probably corresponds to the central area, which is the region of highest ganglion cell density. A second peak of rod bipolar cell density in the lower temporal periphery matches the retinal area of binocular overlap. The population densities of the immunocytochemically characterized bipolar cells indicate that at least 50% of all bipolar cells are cone bipolar cells. The variety and total number of cone bipolar cells is surprising because the retina of the rat contains 99% rods. Our findings suggest that cone bipolar cells may play a more important role in the visual system of the rat than previously thought.

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synapses onto CB terminals (Kolb and Famiglietti, 1974, Famiglietti and Kolb, 1975). The ultrastructural details of the rod pathway have been analyzed in many different mammalian retinas, and it seems likely that all mammals have the same rod bipolar cell circuitry (Wässle et al., 1991).

Various antibodies have been described to label bipolar cells in the mammalian retina. Barnstable (1980; Barnstable et al., 1983) stained nearly all bipolar cells in the rat retina by using antibodies raised against crude retinal membranes (RET-B1). In addition, an antibody raised against rabbit olfactory bulb (ROB; MAb 115A10; Onoda and Fujita, 1987) stained almost all bipolar cells in the rat retina (Onoda, 1988). Antibodies raised against calbindin (CaBP D-28K) were shown to label bipolar cells along with other cell types in the monkey retina (Röhrenbeck et al., 1989; Martin and Grünert, 1992; Grünert et al., 1994). However, in the rat retina, only a few bipolar cells are weakly stained among other cell types (Feigenspan et al., 1993b; J.H. Brandstätter, personal communication).

Some recently developed antibodies are known to label bipolar cell populations—in some cases together with other retinal neurons—in the mammalian retina. An antibody raised against protein kinase C (PKC) has been found to label rod bipolar cells in various mammalian species (Nigishi et al., 1988; Greferath et al., 1990; Grünert and Martin, 1991; Müller and Peichl, 1991; Young and Vaney, 1991; Zhang and Yeh, 1991). An antiserum raised against the Purkinje cell marker L7 was also found to label rod bipolar cells (Berrebi et al., 1991; Grünert and Martin, 1991). Antibodies against the calcium-binding protein recoverin were found to label distinct populations of cone bipolar cells in different mammalian retinas and revealed two types of cone bipolar cells in the rat retina (Milam et al., 1993). An antibody raised against a glutamate transporter (GLT-1) purified from the rat brain (Danbolt et al., 1992) was recently found to label cone photoreceptors and two populations of putative cone bipolar cells in the rat retina (Rauen and Kanner, 1994).

Although these antibodies are available as markers of bipolar cell populations in the rat retina, a systematic examination of the bipolar cell morphologies and their spatial distributions is still missing. A brief report by Muller et al. (1993) describing a variety of bipolar types in the rat retina has not yet been followed up by a full-length paper. The rat retina is becoming a model system for in vitro studies of the mammalian retina (Karschin and Wässle, 1990; Suzuki et al., 1990; Yeh et al., 1990; Boos et al., 1993; Feigenspan et al., 1993a,b), and for this reason it is also important to know more details of rat bipolar cells. The cone-dominated retina of the macaque monkey has been shown to have 10 different types of cone bipolar cells (Boycott and Wässle, 1991; Grünert et al., 1994). Here we have investigated whether the rat retina, which is so clearly rod dominated, also has several different types of cone bipolar cells.

In this paper, we present evidence for 10 different types of bipolar cells in the rat retina that we distinguish morphologically after injecting the cells with Lucifer Yellow and neurobiotin. Five of these bipolar cell types could be labeled immunocytochemically in vertical and horizontal sections. Antibodies against the calcium-binding protein recoverin, the glutamate transporter GLT-1, an isoform of PKC, and the Purkinje cell marker L7 labeled distinct populations of these bipolar cells. The labeled cells were characterized in terms of their densities and spatial distri-butions. Our data indicate that at least 50% of all bipolar cells in the rat retina might be cone bipolar cells.

METHODS

Tissue preparation

We used retinas from adult albino rats (lab strain). The animals were deeply anesthetized with halothane and then decapitated. The eyes were removed and opened by an encircling cut along the ora serrata, and the vitreous was dissected out in HEPES buffered physiological saline containing (in mM): 137 NaCl, 5 KCl, 2 CaCl₂, 1 MgSO₄, 1 Na₂HPO₄, 15 glucose, and 10 HEPES (pH 7.4).

For tissue slices, which were used for the injections of Lucifer Yellow and neurobiotin, the retina was removed immediately from the sclera, transferred to a plastic Petri dish, and cut into quarters. One of the quarters was held with a pair of forceps, and, by "rolling" a scalpel blade over the edge, thin slices of 100–200 µm were cut (Boos et al., 1993). The slices were fixed for 4% paraformaldehyde (PA) in 0.1 M phosphate buffer, pH 7.4 (PB), for 20–30 minutes at room temperature and then rinsed several times (3 × 10 minutes) in 0.1 M phosphate buffer, pH 7.4.

For immunocytochemistry, the eyecup was fixed for 30 minutes in 4% PA, then the retina was carefully dissected and fixed again for 2 hours at room temperature. A sketch of the retina was made to determine its shape and the position of the optic disk. After rinsing in PB, the retinas were immersed in 30% sucrose in PB overnight before sectioning. Vertical frozen sections of 12-µm thickness were taken on a cryostat, collected onto gelatinized slides, air dried, and stored at −20°C. Horizontal frozen sections of 50- or 70-µm thickness were taken with a sliding microtome and processed free floating.

Injection of cells

Between 6 and 8 of the fixed slices were transferred to a filling chamber and placed with the cut side up. The slices were held in place by a grid of parallel nylon strings (Kornreich et al., 1987; Edwards et al., 1989). The filling chamber was placed on the fixed stage of a microscope (Zeiss ACM). The microscope was equipped with epifluorescence illumination and differential interference contrast (Nomarski) optics. A water immersion objective was used (Zeiss, 40/0.75 W; for further details, see Boos et al., 1993).

Sharp filling electrodes were pulled from borosilicate glass (outer diameter of 1.5 mm, wall thickness of 0.3 mm; Clark, Redding, UK) on a horizontal electrode puller (DMZ-Universal Puller, Zeitz Instrumente, Augsburg, Germany). The filling electrode contained 4% neurobiotin (NB; Vector; Vaney, 1991) and 1% Lucifer Yellow (LY, dilithium salt; Sigma) in 0.1 M Tris buffer, pH 7.6. The LY was used to control the filling procedure and to visualize the filled cells during the experiment, and NB was the intracellular marker for the subsequent permanent staining (Vaney 1991). For every experiment, the filling solution was freshly prepared from frozen stock solutions. The filling electrode was controlled by a Leitz micromanipulator for coarse movement and a piezoelectric manipulator (Physik Instrumente, Waldbronn, Germany) for fine adjustment. A positive holding current of less than 1 nA was applied to the electrode (Micro-iontophoresis programmer, Model 160, W-P Instruments) to prevent LY from diffusing out of the electrode tip. After the filling electrode had penetrated the cell membrane, a negatively charged ejection current (−2 to −5 nA)
was applied for 2 to 4 minutes to inject LY into the cell. Under fluorescent light, the morphology of the filled cell became visible and photomicrographs could be taken. The direction of the current was then reversed to inject the positively charged neurobiotin molecules for about 5 minutes.

We tried to fill bipolar cells in a systematic manner over large portions of each slice. To avoid an overlap of filled processes, only a few bipolar cells in a given area were injected, but the distance between these injection sites varied in an attempt to fill as many different types as possible. In addition, we avoided filling rod bipolar cells, which could be recognized by their soma size and position. Therefore, our data do not represent a truly random sample, and cell density cannot be determined solely by the number of each type of cell injected.

Staining neurobiotin-filled cells

Following the injection of LY and NB into cells, the slices were fixed once more in 4% paraformaldehyde for 1 hour and then rinsed in phosphate buffered saline (PBS). Then the slices were incubated in Extravidin-Peroxidase (Sigma, diluted 1:200 in PBS) for 1 hour. The NB-filled cells were visualized with 0.5% 3,3-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H$_2$O$_2$ in PB (Hsu et al., 1981). The slices were mounted between two coverslips in glycerol.

Antibodies

A polyclonal antibody against the 23-kD calcium-binding protein recoverin (Lambrecht and Koch, 1991) was used. The antibody was kindly provided by Dr. K.-W. Koch (Institut für Biologische Informationsverarbeitung, Jülich, Germany). It was raised in rabbit against purified recoverin isolated from bovine retinal photoreceptors (Lambrecht and Koch, 1992) and was used at dilutions of 1:2,000 or 1:4,000.

A polyclonal antibody raised in rabbit against the glutamate transporter GLT-1 from rat brain (Danbolt et al., 1992) was a kind gift from Dr. B.J. Kanner (The Hebrew University, Jerusalem, Israel). It was used at a dilution of 1:1,000.

A mouse monoclonal antibody against PKC was purchased from Amersham (clone MC5, RPN.536). The information supplied with the antibody states that it recognizes both type II (β) and type III (α) of PKC, although Walker et al. (1990) showed that the MC5 antibody cross reacts only with the α isozyme. For brevity we will use the terms “PKC immunoreactivity” or “PKC-positive cells” instead of type-II-PKC or α-PKC-like immunoreactivity. The PKC antibody was used at a dilution of 1:100.

The polyclonal antisem against the Purkinje cell differentiation marker L7 was raised in rabbit against a synthetic peptide conjugate predicted from the L7 amino acid sequence (Oberdick at al., 1988). The antibody was kindly provided by Dr. J.I. Morgan (Roche Institute, Nutley, NJ, USA) and was used at a dilution of 1:1,000.

Immunocytochemistry

In vertical cryostat sections and horizontal sliding microtome sections, an ABC method (Hsu et al., 1981) was used to demonstrate immunoreactivity. For some vertical sections, an indirect immunofluorescence method was used. Regardless of which procedure was followed, all steps, except for the incubation of the horizontal sections, were carried out at room temperature. All sections were preincu-
were found, and therefore reconstruction was necessary. To prevent double counting, areas containing labeled cells in two adjacent horizontal sections were omitted and the cell types were the position of the cell body in the INL and, usually all profiles could be recognized within a single section. To prevent double counting, areas containing labeled cells in two adjacent horizontal sections were omitted and the stained cells in the remaining area of each section were counted. Then the two horizontal sections were brought into register by matching the edges of the sections, the position of the optic disk, and the areas with labeled cells in more than one section.

For the density maps, at least 70 fields were counted. We tried to distribute these fields uniformly over the whole retina by using a regular grid (steps of 50 μm). At positions with limited staining quality, we tried to count the cells in a neighboring field. In areas with steeper gradients, the cells in a field between two grid positions were also counted.

The horizontal sections were fixed to gelatinized slides, where shrinkage has been found negligible (Wassle et al., 1975); therefore, the density data are not corrected for shrinkage. However, some shrinkage (5–10%) may occur as a result of the initial fixation and immunocytochemical processing prior to mounting on the slides.

## RESULTS

### Bipolar cell types in the rat retina

More than 100 bipolar cells were injected with LY and NB in vertical tissue sections (Fig. 1A–G). They differed in the position of their cell bodies in the INL and in the shape and stratification levels of their axons in the IPL. Camera lucida diagrams of the cells were made, and characteristic examples are shown in Figure 2. We observed one type of bipolar cell with the typical rod bipolar cell morphology (RB in Fig. 2) and nine additional bipolar cell types. In all other mammals examined to date, only one morphological type of RB has been found (Wassle et al., 1991). Assuming that this is also the case for the rat retina, the nine morphologically distinct bipolar cell types are probably cone bipolar cells. Confirmation of this identification with electron microscopy is currently lacking. For brevity, we will use the term ‘cone bipolar cell’ (CB) to describe those bipolar cells that differ from the classical morphology of the rod bipolar cell and that display similarity to cone bipolar cells of other mammals. However, this designation does not preclude the possibility of some input from rods to these bipolar cells.

The criteria used to group CBs into different morphological types were the position of the cell body in the INL and, most importantly, the branching pattern and the stratification level of the axon terminal system within the IPL. Because the dendrites are much finer than the axon terminals and because background staining in the OPL was relatively high, criteria such as shape and width of the dendritic trees could not normally be used for classification (Fig. 1). For the same reasons, the drawings in Figure 2 probably show only parts of the dendritic trees. However, unquestionably the dendritic trees of different types of bipolar cells differ substantially in size as reflected in Figure 2. In the classification scheme shown in Figure 2, the nine CB types are arranged according to the stratification level of their axon terminal systems in the IPL. We have subdivided the IPL into five strata of equal width, starting in the outermost part of the IPL. There is a striking relationship between soma position and stratification level in that the cell bodies that are closer to the OPL have axons that descend and stratify more deeply in the IPL.

Type 1 CB (n = 6, Fig. 1B) is an outer cone bipolar cell with a flat stratification in stratum 1 of the IPL and only one ascending primary dendrite that ramifies sparsely. Type 2 (n = 12, Fig. 1C) and type 3 (n = 15, Fig. 1D) CBs have similar dendritic tree shapes but show diffuse stratification at different levels of the outer half of the IPL, that is, in strata 1–2 and 2, respectively. Whereas the axon terminal system of type 2 looks a bit disordered, type 3 shows a "well-arranged" treelike branching pattern. Type 4 (n = 4, Fig. 1E) is a diffuse CB stratifying in both strata 1 and 2. We could inject only a few cells of this type; therefore, we believe it to be very rare. Type 5 (n = 10, Fig. 1F, left) and type 6 (n = 6, Fig. 1F, right) both have a very narrow stratification. The branching pattern of their axon terminal systems is very similar; their axons descend into the middle of the IPL where they terminate in flat side branches in two distinct thin bands in stratum 3 (type 5) and at the border of strata 3 and 4 (type 6). By using Nomarski optics, the two cell types can be distinguished by their stratification level within the IPL relative to a band of higher optical density (Fig. 1A, arrow), which is localized at the border of strata 3 and 4 and represents the inner cholinergic band. In some of the injected cells, the lateral extent of the axon terminal system was found to be larger than shown in the drawing. Type 7 (n = 16, Fig. 1A) and type 8 (n = 10, Fig. 1A) are diffuse CBs with their axon terminal systems in the inner part of the IPL. Type 7 stratifies in strata 3 and 4, whereas type 8 stratifies in stratum 5 near the border of the GCL.

![Fig. 1. Photomicrographs show bipolar cells injected with Lucifer Yellow (LY) and neurobiotin (NB). Ten types of bipolar cells as diagrammed in Figure 2 are shown here following 3',3-diaminobenzidine tetrahydrochloride (DAB) processing and viewed with Nomarski optics. Throughout the figure, the large arrows point to cell bodies and arrowheads point to axons. A: Layers of the retina, outer nuclear layer (ONL), etc., are distinguishable in this retinal slice that displays three injected bipolar cells. The bipolar cell on the left is a type 7 cell and stratifies in the inner inner plexiform layer (IPL) well above the ganglion cell layer (GCL). The bipolar cell in the middle is a type 5 cell, displaying a flat stratification directly above the optical dense band (small arrow), which represents the inner cholinergic band. The bipolar cell on the right is type 8 and shows a diffuse stratification in the inner "well-arranged" treelike branching pattern. Type 4 (n = 4, Fig. 1E) is a diffuse CB stratifying in both strata 1 and 2. We could inject only a few cells of this type; therefore, we believe it to be very rare. Type 5 (n = 10, Fig. 1F, left) and type 6 (n = 6, Fig. 1F, right) both have a very narrow stratification. The branching pattern of their axon terminal systems is very similar; their axons descend into the middle of the IPL where they terminate in flat side branches in two distinct thin bands in stratum 3 (type 5) and at the border of strata 3 and 4 (type 6). By using Nomarski optics, the two cell types can be distinguished by their stratification level within the IPL relative to a band of higher optical density (Fig. 1A, arrow), which is localized at the border of strata 3 and 4 and represents the inner cholinergic band. In some of the injected cells, the lateral extent of the axon terminal system was found to be larger than shown in the drawing. Type 7 (n = 16, Fig. 1A) and type 8 (n = 10, Fig. 1A) are diffuse CBs with their axon terminal systems in the inner part of the IPL. Type 7 stratifies in strata 3 and 4, whereas type 8 stratifies in stratum 5 near the border of the GCL.](image-url)
Bipolar cells immunoreactive for recoverin

In the primate retina, flat midget bipolar cells have been found to be immunoreactive for recoverin and their spatial mosaic has been analyzed (Milam et al., 1993, Grünert et al., 1994; Wässle et al., 1994). The labeling of the rat retina with this antibody is shown in Figures 3A and 4. In Figure 3A, the ONL and the OPL are heavily stained, whereas only some bipolar cells are labeled in the INL. The axons of these stained bipolar cells run into the IPL where their axon terminal systems form two diffuse bands: a denser band in the outer IPL and a sparser band in the inner IPL close to the GCL (Milam et al., 1993; Sassoe-Pognetto et al., 1994). The cell bodies of the recoverin-immunoreactive bipolar cells differ in size and staining intensity. The open arrowheads in Figure 3A point to weakly stained, more slender cell bodies with axon terminal systems that form the inner IPL band within sublamina b. These cells are most likely to be ON cone bipolar cells.

Comparing the morphologies of the injected bipolar cell types (Fig. 2) with the recoverin immunostaining, the labeled cells in the INL could be identified as types 2 and 8 (Fig. 3B). A small gap (small arrow in Fig. 3A) in the outer band formed by the axon terminals raises the possibility that two types have been labeled. However, camera lucida drawings made from immunostained sections (not shown), it was found that every labeled bipolar cell with an axon ending in the outer IPL sends processes into both substrata. Therefore these neurons belong to one type of OFF bipolar cell, whose axon subdivides into two narrow strata.

Figure 4 shows horizontal sections immunostained with the recoverin antibody. A section through the outer INL (Fig. 4A) reveals two regular patterns of somata which differ in size and staining intensity: the larger, strongly stained cells (arrowheads) are the putative OFF CBs that send their axons to the outer IPL (Fig. 4B), and the smaller, weakly stained cells (arrows) are the putative ON CBs with axons stratifying in the inner IPL (Fig. 4C).

We counted the immunolabeled cell bodies at different retinal locations to calculate the cell densities for both populations. The total density of the recoverin-positive bipolar cells ranged from 3,600 to 7,200 cells/mm². The proportion of the weakly stained ON CBs was approximately 44% (2,900/mm²) in the high density area and about 30% (1,000/mm²) in the peripheral retina. The density distribution of recoverin-immunoreactive CBs is shown in Figure 5. A large area of high density forms a slight diagonal, streaklike band along the nasal-temporal axis, with a shallow maximum in the nasal periphery. The density decreases gradually toward the periphery.

Bipolar cells immunoreactive for the glutamate transporter (GLT-1)

Immunostaining of bipolar cells with antibodies against the glutamate transporter (GLT-1) has been described elsewhere (Rauen and Kanner, 1994; Grünert et al., 1994; Brandstätter et al., 1995); however, an analysis of the cell types involved has not been done in rat. The labeling of the rat retina with this antibody is shown in Figure 6A. Cones and bipolar cells are strongly immunoreactive, whereas...
both the OPL and IPL show a diffuse labeling similar to that shown by Rauen and Kanner (1994) in their study. In accord with the putative membrane localization of the GLT-1 antigen, the DAB reaction product is confined to the cell membranes. The bipolar cell axons can be followed to the middle of the IPL, where they terminate in a narrow band in either stratum 3 or at the border of strata 3 and 4. While at the microscope, we sometimes could resolve single axon terminal systems that stratify either in the outer or the inner narrow immunolabeled band. This suggests that two different bipolar cell types might be stained. In addition, some less intensely labeled cell bodies can be found. The large arrow in Figure 6A points to the outermost cell layer of the INL, where these more weakly stained somata are located. In the IPL, a few weakly labeled axons can be followed through both GLT-1 bands. The axon terminals cannot be resolved because of the relatively strong background staining. The soma position and the axon terminal shape of these weakly labeled cells suggest that they are rod bipolar cells (Rauen and Kanner, 1994). To test this hypothesis we performed a double-labeling experiment for GLT-1 and PKC, which selectively stains rod bipolar cells in the rat retina (Greferath et al., 1990). We compared the staining patterns in vertical sections (not shown) and found that 13% (n = 426) of all stained cells in the INL showed immunoreactivity for both antibodies. Almost all of the more weakly stained GLT-1-immunoreactive cell bodies in the outermost INL were also immunoreactive for PKC. This suggests that the weakly GLT-1-immunoreactive bipolar cells are in fact rod bipolar cells. Although only about 40% of the RBs were labeled for GLT-1, we presume that the GLT-1 antibody generally cross reacts with RBs. The reason for the "incomplete" labeling might be a low level of GLT-1 expression by RBs. Nevertheless, we cannot exclude the possibility that there is a distinct subpopulation of rod bipolar cells that is GLT-1 positive. The ratio of PKC-labeled RBs to GLT-1 CBs is estimated to be 10:9, based on the double-labeling experiments.

The GLT-1 staining pattern (Fig. 6A) suggests the existence of two similar types of cone bipolar cells with flat stratification and therefore confirms our proposed classification scheme with the differentiation of type 5 and type 6 CBs (Fig. 6B). Horizontal sections immunolabeled by the GLT-1 antibody are shown in Figure 7. Figure 7A shows the cell mosaic in a section through the middle of the INL. The arrowheads point to GLT-1-immunoreactive cells that appear to be encircled by the DAB reaction product. In Figure 7B, the axons of the GLT-1-immunoreactive cells leaving the INL are visible. The axon profiles (arrowheads in Fig. 7B) are relatively constant in diameter and staining intensity. This suggests that only the strongly stained GLT-1 CB axons and not the weakly stained RB axons are visible. A section through the middle of the IPL (not shown), where the GLT-1 CB axons stratify, revealed granular labeling, but individual axon terminal systems could not be resolved.

For the cell density measurements, we counted GLT-1-immunoreactive axon profiles in horizontal sections at the level of the outer IPL and calculated a GLT-1 CB density ranging from 8,600 to 16,400 cells/mm². Figure 8 shows the corresponding density map. The cell distribution is quite uniform in the central and mid-peripheral regions, with a slight increase of cell density toward the central retina. A large density peak is located in the superior mid-peripheral...
Fig. 4. Recoverin-immunoreactive cone bipolar cells in a horizontal section of the rat retina. A: A horizontal section with the focal plane in the outer INL is shown. The arrowheads point to the more intensely stained somata of the putative OFF-type cone bipolar cells. The arrows point to the more weakly stained cell bodies of the putative ON-type cone bipolar cells. B: The same section with the focal plane in the outer IPL is shown. The outer recoverin-immunoreactive (putative OFF) dendritic plexus is visible. C: In the same section, with the focal plane in the inner IPL, the inner and sparser recoverin-immunoreactive (putative ON) dendritic plexus is visible. Scale bar = 50 μm.

Immunoreactivity of rod bipolar cells

Rod bipolar cells can be selectively labeled with antibodies against the α isozyme of PKC in many mammalian retinae. The PKC staining pattern has been described in detail (Greferath et al., 1990), but an analysis of the RB density and distribution in the rat retina is still missing. Because PKC immunoreactivity was also found in some amacrine cells (Negishi et al., 1988), it was desirable to find a more selective marker of RBs to count them. An antibody, raised against a peptide corresponding to the predicted amino acid sequence of the Purkinje cell differentiation marker L7 (Oberdick et al., 1988), provides such a marker for RBs in a variety of species (Berrebi et al., 1991; Grünert and Martin, 1991).

Double-labeling experiments with PKC and L7 antibodies in vertical sections revealed that in rat retina 95% of all labeled bipolar cells showed immunoreactivity for both antisera, whereas the remaining 5% were only stained by the L7 antibody (U. Grünert, personal communication). Therefore PKC, as well as L7, can be used as a marker for rod bipolar cells. However, the bipolar cells exclusively labeled by the L7 antibody might represent a subpopulation of a rare but distinct bipolar cell type.

Vertical sections of the rat retina stained for L7 (Fig. 9A) and PKC (Fig. 9B) are shown side by side for comparison. Both antibodies label bipolar cell somata that are located in the outermost part of the INL, although occasionally bipolar cells in the middle of the INL are labeled. Whereas L7 immunoreactivity fills the cell body, the PKC staining is confined to the cell membrane. In both sections, the RB axons can be followed to the inner border of the IPL, where they form varicose terminals. Fewer axon terminal systems seem to be labeled in the L7-immunostained section than in the PKC-stained section. It has been reported that L7 immunoreactivity is reduced in the axons of RBs (Grünert and Martin, 1991; Martin and Grünert, 1992). A PKC-immunoreactive amacrine cell (arrow) is shown in Figure 9B. Figure 9C shows a camera lucida drawing of a LY-injected rod bipolar cell.
A horizontal section labeled with the L7 antibody reveals the dense packing of rod bipolar cell somata in the outer INL (Fig. 10A). The arrows in Figure 10A point to rare but more strongly stained cell bodies that seem to have a regular distribution across the retina and might correspond to the bipolar cells exclusively stained by the L7 antibody. Figure 10B–C shows two different horizontal sections at the level of the inner INL. Figure 10B shows a PKC-immunostained section and Figure 10C shows an L7-labeled section. In both micrographs, the cell bodies are interspersed with strongly immunolabeled RB axons. Amacrine cell somata (Fig. 10B, arrowheads), which seem to be arranged in clusters, show PKC immunoreactivity.

For the cell density measurements, we counted labeled axon profiles in horizontal sections through the inner INL or the outer IPL at different retinal eccentricities. For both PKC- and L7-immunostained sections, the calculated rod bipolar cell density was in the same range, from 13,000 to 29,000 cells/mm² (mean = 20,000) for PKC and from 12,100 to 27,900 cells/mm² (mean = 20,300) for L7. The 5% exclusively L7-immunoreactive bipolar cells have a density ranging from 600 to 1,000 cells/mm², although it is not clear whether these cells are a distinct population of bipolar cells.

Figure 11 shows a cell density map of a retina immunostained by the L7 antibody. The cell density is relatively high in the central and mid-peripheral retina, and only in the far periphery is a significant decrease in cell density found. In the inferior temporal retina, a large region of high cell density can be observed, and a smaller peak of cell density is located in the upper mid-peripheral retina, approximately 1 mm from the optic disc. The general features of the RB distribution were similar in both L7- and PKC-immunostained retinas.

DISCUSSION
Bipolar cell types in the rat retina
In this study, we presented morphological evidence for nine putative cone bipolar cell types in the rat retina. The density and distribution of four of these cone bipolar cell types was assessed using two different antibodies: PKC and L7. The density of rod bipolar cells, which were stained exclusively by the L7 antibody, was calculated in horizontal sections through the inner INL or the outer IPL at different retinal eccentricities. The rod bipolar cell density ranged from 13,000 to 29,000 cells/mm² (mean = 20,000) for PKC and from 12,100 to 27,900 cells/mm² (mean = 20,300) for L7. The 5% exclusively L7-immunoreactive bipolar cells have a density ranging from 600 to 1,000 cells/mm², although it is not clear whether these cells are a distinct population of bipolar cells.

Figure 11 shows a cell density map of a retina immunostained by the L7 antibody. The cell density is relatively high in the central and mid-peripheral retina, and only in the far periphery is a significant decrease in cell density found. In the inferior temporal retina, a large region of high cell density can be observed, and a smaller peak of cell density is located in the upper mid-peripheral retina, approximately 1 mm from the optic disc. The general features of the RB distribution were similar in both L7- and PKC-immunostained retinas.
Fig. 6. GLT-1-immunoreactive cone bipolar cells in a vertical section of the rat retina. A: A vertical cryostat section immunolabeled with the GLT-1 antibody is shown. Cone bipolar cell somata in the middle of the INL are labeled. The arrowheads point to immunostained axons that run into the IPL where the axon terminal systems form two dense bands (small arrows). The large arrow points to the outermost cell layer of the INL, where the more weakly stained somata of rod bipolar cells are located. B: A camera lucida drawing of the two corresponding injected bipolar cell types 5 and 6 (Fig. 2) is shown. Scale bar = 50 μm.

populations, as well as the population of RBs, were characterized using immunocytochemical markers.

By injecting bipolar cells with LY, we found nine putative cone bipolar cell types and one rod bipolar cell. They were distinguished by the position of their somata in the INL and the branching pattern and stratification level of their axon terminal systems within the IPL. Because detailed electron microscopical studies concerning the bipolar-photoreceptor synapses in the rat retina are lacking, it is unclear whether the nine putative cone bipolar cell types are exclusively cone connecting bipolar cells or whether they are “mixed” bipolar cells with contacts to both rods and cones. In the retinae of lower vertebrates, such mixed inputs to bipolar cells are common (Ishida et al., 1980; Saito et al., 1985). Among mammals, a “mixed connecting” bipolar cell type has been suggested for squirrels (West, 1978). Muller et al. (1993), using electron microscopy, presented preliminary evidence for the existence of at least one mixed rod-cone bipolar cell type in the rat retina. They found a bipolar cell type ramifying in strata 1–2 that received wide-cleft basal contacts from cone pedicles and narrow-cleft nonribbon contacts from rod spherules, which led them to propose the existence of additional pathways for rod information in the rat retina. Muller et al. (1993) also reported, in addition to the rod bipolar cell, seven other bipolar cell types. As of yet, they have not published a full-length report; thus, a comparison to the bipolar types found in our study is not possible.

In the cat retina, the border between the OFF and the ON sublamina divides the IPL into the outer one-third (sublamina a) and the inner two-thirds (sublamina b; Nelson et al., 1978; Peichl and Wässle, 1981; Cohen and Sterling, 1990a,b). Although electrophysiological evidence for functional sublaminae within the IPL of the rat retina is still wanting, the IPL stratification levels of ganglion cells suggest that the subdivision of the IPL into an OFF and ON sublamina might exist and, similar to the cat retina, that the border might be shifted toward the outer retina (Peichl, 1989). The reason for this shift may be the broad band of rod bipolar cell terminals, which occupies a large portion of the inner part of the INL (Voigt, 1986; Greferath et al., 1990). The IPL stratification levels of the nine cone bipolar cell types suggest that types 1–4, with stratification in the outer IPL, could be physiological OFF-type bipolar cells, whereas types 6–9, with stratification in the inner IPL, could be ON-type bipolar cells. For the type 5 bipolar cell, which stratifies in the middle of the IPL, it cannot be clearly predicted whether the cell is of the physiological ON or OFF type.

A unifying concept in anatomical studies of the retina suggests that cell types with similar morphology form functional ON/OFF pairs (Famiglietti and Kolb, 1976; Peichl, 1989). In the rat retina, Peichl (1989) showed that different types of ganglion cells form ON/OFF pairs in such a way that outer parts of the OFF sublamina correspond with outer parts of the ON sublamina. It is very likely that at least some types of cone bipolar cells follow the stratification pattern of ganglion cells (Famiglietti and Kolb, 1976), but exceptions from this scheme have also been reported (Nelson and Kolb, 1983; Saito, 1987). In the rat retina, the following ON/OFF pairs can be defined according to morphological similarities: types 1/5 or 6, types 2/7, and types
For the type 4 CBs and either type 5 or type 6 CBs, no morphological counterparts could be assigned.

**Immunocytochemical labeling of bipolar cells in the rat retina**

The morphologies of single LY-/NB-injected bipolar cells were compared with immunolabeled bipolar cells. We were able to correlate types 2, 5, 6, and 8 of Figure 2 to different immunolabeled bipolar cell populations. Types 2 and 8 probably correspond to the two bipolar cell populations labeled by the recoverin antibody. The cell bodies of type 2 bipolar cells are large and intensely stained by recoverin. The position of their axon terminals, which form a diffuse band within the outer part of the IPL, suggests that these cells may be OFF bipolar cells. Type 8 bipolar cells have more weakly stained, slender somata and their axon terminals form the inner recoverin-immunoreactive IPL band. Thus, these cells are probably ON bipolar cells.

The morphologies of types 5 and 6 suggest that these cells correspond with the bipolar cell populations labeled by the GLT-1 antibody. Their axon terminal systems stratify in strata 3 and 4/5, respectively, forming two thin, but dense, bands in close proximity to the dendritic plexus of displaced cholinergic amacrine cells (Voigt, 1986; Brandstätter et al., 1995). Because cholinergic amacrine cells are involved in direction selectivity (Masland and Ames, 1976; Ariel and Daw, 1982), Brandstätter et al. (1995) suggested that GLT-1-positive bipolar cells contribute to the circuitry underlying direction selectivity.

With the antibodies used in the present study, bipolar cell populations corresponding to LY-injected cell types 1, 3, 4, 7, and 9 were not immunostained. Type 1 bipolar cells show a flat stratification in stratum 1 of the IPL close to the border to the INL. Although we found no recoverin-immunoreactive bipolar cells stratifying exclusively in the outer part of the outer IPL band, it cannot be excluded that type 1 cells might contribute to this band.

Relatively few of the filled cells were of types 4 and 9, thus we propose that these cells each comprise a sparse population of bipolar cells. Type 4 shows a diffuse stratification in both strata 1 and 2. It was shown in other mammalian retinas (Boycott and Wässle, 1991; Young and Vaney, 1991; Mills and Massey, 1992) that many types of bipolar cells show territorial behavior. Assuming that this is the case for the type 4 CB suggests that the narrow axon terminal system has a higher cell density than predicted from the filling frequency. The dendritic tree of type 9 is relatively broad, and the sparse axon terminal system ramifies in
In other mammals, a bipolar cell type with a comparable morphology has been described: the blue cone bipolar cell (Famiglietti, 1981; Mariani, 1984; Kouyama and Marshak, 1992). The rat retina has very few blue cones, comprising only 0.05% of all photoreceptors (Szél and Röhlich, 1992); thus, the type of bipolar cells connecting to blue cones might also be very rare. When considering the morphological similarities and the presumed low density, type 9 cells are reasonable candidates to be the blue cone bipolar cell of the rat retina.

Only one type of interplexiform cell has been described in the rat retina (Perry and Walker, 1980). Because the somata of these interplexiform cells are positioned in the inner INL close to the border to the IPL, it is unlikely that these cells have been mistakenly identified as bipolar cells.

**Bipolar cell densities**

Rod bipolar cells were labeled with antibodies against PKC and L7. The density for both immunostained populations agreed fairly well and ranged from about 12,000 to 28,000 cells/mm² (Table 1). Double-labeling experiments with the two antibodies suggested the possibility that an additional bipolar cell subpopulation was stained exclusively by the L7 antibody. If this is the case, then the cell density for this presumed subpopulation would range from 600 to 1,000 cells/mm², corresponding to 5% of all L7-immunoreactive bipolar cells. In the horizontal sections, some L7-positive bipolar cells display a more intensely stained soma and seem to be distributed in a regular pattern in horizontal sections. These cells could be the 5% PKC-negative bipolar cells found in the double-labeling experiments. In vertical sections, however, these cells were not detected; neither more strongly stained somata nor different branching pattern of axons could be observed.

The GLT-1-immunoreactive CBs with a density ranging from 8,600 to 16,400 cells/mm² comprise the largest CB population in the rat retina found in this study (Table 1). The density of the recoverin-immunoreactive CBs ranged from 3,600 to 7,200 cells/mm² (Table 1). Putative ON-type and OFF-type recoverin CBs could be distinguished by the staining intensity of their cell bodies and the stratification level of their axons. Putative ON-type CBs made up approxi-
Fig. 9. Rod bipolar cells in vertical sections of the rat retina. A, B: Two different vertical cryostat sections with immunolabeled rod bipolar cells (RBs) are shown. Their axons run through the IPL and terminate close to the border of the GCL. A: RBs immunostained by the L7 antibody are visible. B: RBs immunolabeled with the protein kinase C (PKC) antibody are shown. The arrowhead points to a PKC-immunoreactive amacrine cell. C: A camera lucida drawing of the corresponding injected bipolar cell type RB (Fig. 2) is shown. Scale bars = 50 μm.

approximately 37% of all recoverin-immunoreactive bipolar cells, equivalent to 1,000 to 2,900 cells/mm².

In Table 1, the densities of RBs and CBs are compared. Despite the fact that only a fraction of the morphologically identified CBs could be counted from our immunocytochemical experiments, they comprise 48% of all immunocytochemically labeled bipolar cells. The density of the bipolar cell populations immunoreactive for recoverin, GLT-1, and L7/PKC is relatively high, and the space within the outer INL for the other cone bipolar cell types is limited. This suggests that the remaining cone bipolar cell populations may be much smaller and the total proportion of cone bipolar cells would not be larger than 60% of all bipolar cells.

Additional, but more indirect, evidence for the existence of a large proportion of cone bipolar cells in the rat retina comes from the immunocytochemical staining of dissociated retinal cells. It was shown, in a preparation of cells freshly dissociated from the rat retina, that all cells with typical bipolar cell morphology were labeled by the PKC antibody (Greferath et al., 1990; Karschin and Wässle, 1990). Because only RBs are immunoreactive for PKC, this suggests that only rod bipolar cells maintain their typical morphology during the dissociation process, whereas cone bipolar cells do not. Greferath et al. (1990) provided evidence for a loss of dendrites and axons of CBs during dissociation. They applied the ROB antibody (MAb 115A10), which was raised against a homogenate of rabbit olfactory bulb (Onoda and Fujita, 1987), to homogenates and dissociated retinal cells. In the retinal sections, both rod bipolar cells and ON cone bipolar cells were labeled. In the dissociates, rod bipolar cells were labeled, as were many round cell bodies, which obviously had lost their processes. These round cell bodies may well represent the populations of cone bipolar cells labeled by ROB in the retinal sections (Greferath et al., 1990; Grünert et al., 1994).

Spatial distribution of bipolar cell types

Here we compare the spatial distributions of rod bipolar cells, GLT-1-immunoreactive cone bipolar cells, and recoverin-positive cone bipolar cells with the distribution of other cell types in the rat retina.

In the nasal and inferior portions of the retina, the density of middle-to-long wavelength sensitive cones (M cones) is slightly increased. The other photoreceptor types, rods and short wavelength-sensitive cones (S cones), show a relatively even distribution over the whole rat retina (Copers, 1983; Szél and Röhlrich, 1992). The distribution of recoverin-immunoreactive CBs follows, to some extent, that of the M cones. Horizontal cells are distributed more uniformly across the rat retina (Peichl and González-Soriano, 1994), so a comparison with bipolar cell distributions is not useful.

A peak of ganglion cell density is located in the upper retina at a distance of about 20° (~1 mm in adult rats) from the optic disk, slightly shifted to the temporal retina. In this so-called central area, the ganglion cell density reaches 4,000 cells/mm² (Fukuda, 1977; Jeffery, 1985). The peak densities of both rod bipolar cells and GLT-1-positive cone bipolar cells are probably colocalized within the central area. Wässle et al. (1993) found that the density of AII amacrine cells also increases in the upper retina. The increase in density of RBs, AII cells, and ganglion cells within the central area suggests that spatial resolution is improved as a result of a lower convergence of rod signals onto ganglion cells. It should be emphasized, however, that the central area in the rat is not as pronounced as the central area of
Fig. 10. Rod bipolar cells in horizontal sections of the rat retina. Horizontal cryostat sections immunostained with either the L7 or the PKC antibody are shown. A: In the horizontal section, with the focal plane in the outer INL, the L7-immunoreactive rod bipolar cell (RB) somata are visible. The arrows point to more intensely stained cell bodies that seem to have a regular distribution across the retina and might correspond to a small subpopulation of cone bipolar cells. B,C: Two different horizontal sections with the focal plane at the level of the border of IPL and GCL are shown. Immunolabeled axon profiles of RBs are visible. B: The section was immunostained with the PKC antibody. PKC-labeled amacrine cell bodies (arrowheads), interspersed with axon profiles of RBs, are visible. C: In this section, the axon profiles of RBs immunostained with the L7 antibody are visible. Scale bar = 50 μm.

the cat, the visual streak of the rabbit, or the fovea of the primate (Hughes, 1977).

We found a second, larger peak of RB density located in the lower temporal and peripheral retina. Jeffery (1985) found, at the same retinal location, a crescent-shaped area where approximately 10% of the α ganglion cells project to the ipsilateral visual cortex. He concluded that in this area binocular vision might be possible. It is possible that the increased density of rod bipolar cells in the lower temporal retina coincides with this area of binocular vision.

It has been shown for the rabbit retina (Young and Vaney, 1991; Mills and Massey, 1992) and for the macaque monkey retina (Boycott and Wässle, 1991) that the axon terminals of many types of bipolar cells show territorial behavior and therefore their axonal field coverage approaches 1. By assuming that the axon terminals of the injected bipolar cells were completely labeled, the cell density can roughly be estimated from the diameter of their axon terminal systems. For both the recoverin-positive type 2 and type 8 cone bipolar cells, this estimation matches the results from the cell density measurements, indicating an axonal field coverage of approximately 1. For the GLT-1-immunoreactive cone bipolar cells, the ratio of type 5 to type 6 cells could not be evaluated, but the diameter of their axon terminal systems suggests that their axonal fields might overlap. Because specific immunocytochemical markers are not available for the other types of cone bipolar cells, an estimation of their cell density is of limited use. Further detailed analysis of single axon terminal systems in horizontal sections is needed to answer the question of axonal coverage in the rat retina.

Cone bipolar cells in mammalian retina

In the mammalian retina, many different types of cone bipolar cells have been recognized by using various methods. In the rabbit retina, 9 types have been described from Golgi-stained material (Famiglietti, 1981). Other studies have confirmed the existence of two of these types and recognized one additional type (Mills and Massey, 1992). In the cat retina, 8 to 11 types of cone bipolar cells have been
Fig. 11. Spatial density map for rod bipolar cells of the rat retina. The spatial density of rod bipolar cells (RBs; L7 immunolabeled) was counted from their descending axons. In the inferior temporal periphery, the RB density is increased in a large area. A smaller peak of cell density is located in the upper mid-peripheral retina about 1 mm from the optic disc. The general features of the RB distribution were similar in both L7- and PKC-immunostained retinas. Scale bar = 1 mm.

### TABLE 1. Ranges of Cell Density (cells/mm²) Across the Retina (Maxima and Minima) for Different Cell Types Directly Involved in Rod and Cone Pathway of the Rat Retina

<table>
<thead>
<tr>
<th>Photoreceptors</th>
<th>Bipolar cells</th>
<th>Cone bipolar cells (CBs)</th>
<th>GLT-1-positive CBs</th>
<th>Recoverin-positive CBs</th>
<th>Other CB types</th>
<th>All amacrine cells</th>
<th>Ganglion cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rod</td>
<td>206,000</td>
<td>12,000</td>
<td>8,600</td>
<td>3,000</td>
<td>&gt; 11,600</td>
<td>2,000</td>
<td>2,600</td>
</tr>
<tr>
<td>Cones</td>
<td>3,800</td>
<td>28,000</td>
<td>16,400</td>
<td>7,200</td>
<td>25,000</td>
<td>7,000</td>
<td>4,000</td>
</tr>
</tbody>
</table>

1From Coers (1983).
3From Wässle et al. (1995).

Although cat, rabbit, monkey, and rat retinas have some notable differences in retinal circuitry, similarities exist between many of the cell types from all classes of retinal cells. However, a comparison between cell types in different mammals is difficult, especially when it is based only on morphology and immunoreactivity. With some caution applied, both the morphology and immunoreactivity of a certain cell type can be useful to find counterparts in other mammalian retinas. This has been demonstrated for the rod bipolar cell, whose morphology and immunoreactivity seem to be well preserved in different mammals (Negishi et al., 1988; Greferath et al., 1990; Grünert et al., 1991; Müller and Peichl, 1991; Young and Vaney, 1991; Zhang and Yeh, 1991).
Bipolar cell types can be classified by the branching pattern and the stratification level of their axon terminal systems within the IPL. In the rat retina, we recognized cell types with a flat stratification in the IPL (types 1, 5, 6) and cell types whose axon terminals ramify more broadly (types 3, 8) or even in more than one stratum of the IPL (types 2, 4, 7, 9). A similar rough classification can be made with the cone bipolar cells that have been described for the macaque monkey (Boycott and Wässle, 1991), the cat, and the rabbit retina (Famiglietti, 1981).

Similarities of bipolar cells across species can be noted in vertical sections. For example, the type 1 CBs in the rat retina resemble the DB1 cell in the macaque monkey retina (Boycott and Wässle, 1991) and the CBa1 and the DAPI Ba1 cells in the rabbit retina (Cohen and Sterling, 1990a; Mills and Massey, 1992). There are also resemblances between the type 3 CBs (rat), DB2 (macaque monkey), DAPI Ba2/ CBa2 (rabbit), and the “varicose flat” bipolar cell in the cat retina (Famiglietti, 1981). Except for differences in the stratification level within the IPL, type 5 and type 6 CBs resemble DB3 and DB5 in the macaque monkey retina and the various flat stratifying bipolar cells in the cat and the rabbit retina (Famiglietti, 1981). Our study thus supports the view that bipolar cell architecture in the mammalian retina might be highly conserved (Famiglietti, 1981; Wässle and Boycott, 1991).

Using immunocytochemical results for a comparison between cone bipolar cell types in different mammals is rather questionable. Immunocytochemical similarities between cell types do not necessarily imply functional similarities, given the diversity of protein isoforms available for antibody binding. In addition, a cursory examination of immunocytochemical similarities between bipolar cells in this study and bipolar cells characterized in other mammals suggest that these comparisons are inappropriate in our case. For example, in the rat retina, two diffuse cone bipolar cells, an inner (type 2) and an outer (type 8) stratifying type, are recoverin immunoreactive, whereas, in the macaque monkey retina, only an outer stratifying midget bipolar cell (FMB in Wässle et al., 1994) is labeled by this antibody. The same midget bipolar cell is also immunoreactive to GLT-1, whereas the two CB types of the rat retina, which stratify narrowly in the middle of the IPL, are GLT-1 positive.

Thus, it is difficult to make morphological and immunocytochemical comparisons between the cone bipolar cells described in mammalian retina. A more reliable, and appropriate, basis for such comparisons will come from a combination of anatomical and functional studies. At this time, very little is known about functional properties of different cone bipolar cells. Anatomy offers some clues: the axon stratification level within the IPL possibly indicates whether or not the cell will show a physiological ON or OFF type of response, and the cell density may give an indication of the highest spatial frequency that the bipolar cell could transmit.

Physiological recordings from ganglion cells indicate that the light signal is separated into different "channels," which differ in their temporal, spatial, and chromatic transfer characteristics (Levick, 1975; Lee et al., 1989). It is likely that the bipolar cells that provide input to these ganglion cells play a role in determining these channels. Supporting this view are in situ hybridization studies and immunocytochemistry that have revealed differential expression patterns of glutamate receptor subtypes (Hughes et al., 1992; Hamassaki-Britto et al., 1993; Brandstätter et al., 1994) and GABA, and glycine receptor subunits (Brecha, 1992; Greferath et al., 1993, 1994) in the INL and IPL. These findings may reflect functional differences in bipolar cell populations that contribute to the separation of signal properties. Thus, different types of bipolar cells could represent the first element of parallel input channels with distinct functional properties.

**Photopic vision of the rat**

Rats are nocturnal animals and, accordingly, about 99% of their photoreceptors are rods. Early electrophysiological experiments suggested the existence of both scotopic and photopic mechanisms in the rat retina (Green, 1971, 1973). More recently, Jacobs et al. (1991) described two photopic sensitivities in the electroretinogram of rats, indicating a functional two-cone system, although behavioral tests failed to produce any evidence for the existence of color vision in rat (Neitz and Jacobs, 1986). In agreement with the electrophysiological data, two types of cones have been demonstrated in the rat retina by using cone-specific antibodies directed against visual pigments (Szél and Röhlich, 1992; Szél et al., 1993). Seven percent of all cones are presumed to be sensitive to short wavelengths, whereas the remaining cones probably contain pigments that are sensitive to middle-to-long wavelengths.

In the present study, we have given strong evidence for the existence of a variety of different putative cone bipolar cell types, which probably outnumber rod bipolar cells and may contribute to the photopic system of the rat. These results are consistent with the existence of a two-cone system in the rat retina and further supports the idea of photopic vision in the rat.

In addition, the diversity of cone bipolar cell types is consistent with the model proposed by Strettoi et al. (1990, 1994) that cone bipolar cells may serve as interneurons in the rod pathway. Although they receive no direct input from rods, cone bipolar cells would, in such a model, play an important role in scotopic vision.

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**LITERATURE CITED**


BIPOLAR CELLS IN THE RAT RETINA


