Bipolar Cells of the Mouse Retina: A Gene Gun, Morphological Study

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ABSTRACT

One of the key elements concerning our understanding of the organization of the mouse retina is the complete classification of the various types of bipolar cells. With the present study, we tried to contribute to this important issue. Unfortunately, most of the antibodies that stain specifically bipolar cells in the retina of other mammals hardly work for the retina of the mouse. We succeeded in overcoming this limitation by using a relatively novel technique based on the gene gun transfer of fluorescent dyes to cells. Hence, we were able to stain a considerable number of bipolar cells that could be characterized according to morphological and comparative criteria. We also performed a complete morphometric analysis of a subset of bipolar cells stained by anti–neurokinin-3 receptor antibodies. We found nine types of cone bipolar cells and one type of rod bipolar cell; these data are consistent with the findings of previous studies on the retinas of other mammals, such as rabbits, rats, and monkeys and with a recent study based on the mouse retina (Ghosh et al. [2004] J Comp Neurol 469:70–82). Our results also confirm the existence of a common structural similarity among mammalian retinas. It remains to be elucidated what is exactly the functional role of the various types of cone bipolar cells and what is the specific contribution they provide to the perception of a given visual stimulus. Most probably, each bipolar cell type constitutes a specialized channel for the computation of a selected component of the visual stimulus. More complex signal coding, involving the coordinated activity of various types of bipolar cells, could also be postulated, as it has been shown for ganglion cells (Meister [1996] Proc Natl Acad Sci USA 93:609–614). J. Comp. Neurol. 476:254–266, 2004.

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The central nervous system operates through both serial and parallel processing; the latter implicates that multiple, anatomically distinct areas or neural circuits execute various tasks at the same time, upon receiving a similar input signal (Grunert et al., 1994; Prut et al., 2001; Zaborszky, 2002; Chiry et al., 2003; Scott and Johnsrude, 2003). The retina makes no exception to this working strategy: many types of anatomically and functionally distinct ganglion cells, the last neurons in the retinal visual pathway, operate in parallel to provide the brain, through multiple sets of channels, information about the position, chromatic features, contrast, duration, and so on, of a given visual stimulus (Rodieck, 1998). The complexity of ganglion cell physiology originates partly from complex synaptic interactions taking place in the outer and inner plexiform layers of the retina; in turn, the complexity of retinal networks is reflected in the high variety of cellular types that compose each of the various classes of retinal neurons (Masland and Raviola, 2000).

In the past few years, the full catalog of neurons has been provided for the retina of various mammals and the puzzle composition is quickly progressing toward the identification of all the retinal cells for an increasing number of species. We know that the rabbit retina (and thus presumably, the retina of most mammals) contains 2 types of photoreceptors, 2 types of horizontal cells, approximately a dozen types of bipolar cells (McGillem and Dacheux, 2001), 23 types of amacrine cells, and, finally, 12 varieties of ganglion cells (Masland, 2001b). Shape, size, pattern of dendritic and axonal stratification, connectivity, staining properties, and so on, contribute to the definition of a cell...
type and to its assignment to a unique position within a classification scheme. Usually, the anatomical identification precedes in time the study of the physiological properties of a given cell; thus, we can expect that physiological studies will soon provide functional information about the vast majority of the above-mentioned cell types.

Of interest, retinal bipolar cells are among the most difficult neurons to classify: with the noticeable exception of rod bipolar cells (the second-order neurons that collect information from rod photoreceptors), the intrinsic features of cone bipolar cells, such as small size, large number of types, and similar molecular phenotypes, make their identification extremely difficult. With various techniques and through progressive steps of identification, a classification has been provided for the cone bipolar cells of the monkey (Boycott and Wassle, 1991; Grunert et al., 1994), rat (Euler and Wassle, 1995), and rabbit retinas (Cohen and Sterling, 1990; Mills and Massey, 1992; Grunert et al., 1994; Strettoi et al., 1994; Jeon and Masland, 1995; Merighi et al., 1996; McGillem and Dacheux, 2001).

Previous data based on quantitative analysis (Jeon et al., 1998), electron microscopy of serial sections (Tsukamoto et al., 2001), and immunocytochemistry (Haverkamp and Wassle, 2000) have provided a fundamental base of reference to study various neurons in the retina of the mouse; a complete description of bipolar cells for this important species was provided recently, while this work was in progress (Ghosh et al., 2004). The significance of completing the catalog of cell types of the mouse retina is obvious: the mouse is extremely relevant because of the high number of spontaneous mutations affecting retinal cells in this species. Many of these mutations, together with laboratory-based genetic manipulations, produce phenotypes that are exceptionally useful tools for studying human retinal diseases, including retinitis pigmentosa and glaucoma (Chang et al., 2002). Remarkably, recent literature has described extensive remodeling of bipolar cells in mouse retinas in which photoreceptors are degenerating because of a genetic disease (Strettoi and Pignatelli, 2000; Jones et al., 2003; Marc et al., 2003; Strettoi et al., 2003). Thus, the precise knowledge of the different types of bipolar cells normally present in the retina of the mouse is a crucial step for a better interpretation of data concerning several retinal dystrophies.

In this study, we attempt a complete classification of mouse bipolar cells by means of the recently developed technique of gene gun labeling (Gan et al., 2000; Kettunen et al., 2002) that we applied in a novel style to stain cells in vertical retinal sections. The technique allows the random introduction into cells of microparticles coated with lipophilic fluorescent dyes that then move freely along the membranes outlining cells in a Golgi-like manner. The efficiency of the procedure and the possibility to perform it in a very short time compensate for the impossibility to select for a desired cell type. This procedure can be used as an alternative to single-cell injections or photofilling. We found the method of gene gun staining particularly helpful to study the morphology of retinal cells in the mouse: several commonly used antibodies are cell-type specific for various mammals but provide little information when used for the retina of the mouse, in which they label too many cells or produce a very different pattern of staining (Jakobs et al., 2003).

By means of gene gun staining and immunocytochemistry with a neurokinin-3 receptor (NK3R) antibody (labeling a subset of bipolar cells), we were able to identify one type of rod bipolar cell and nine types of cone bipolar cells in the retina of the mouse. These findings are consistent with the present literature, as 10 types of bipolar cells have been described in the rat (Euler and Wassle, 1995) and 11 in the rabbit (Mills and Massey, 1992; McGillem and Dacheux, 2001) and monkey retina (Boycott and Wassle, 1991), and with the recent findings by Ghosh et al. (2004) for the retina of the mouse.

**MATERIALS AND METHODS**

**Animals**

C57BL6J and C3HPe (rd/rd) mice between 2 and 4 months of age were used for all the experiments. Animals were raised in a local animal house and kept in 12-hour artificial light/dark cycle. All mice were anesthetized with an intraperitoneal injection of Avertin (0.1 ml/5 g body weight). Animal procedures were conducted in accordance with the national regulations and the Association for Vision in Research and Ophthalmology (ARVO) statement. Anesthetized mice undergoing eye enucleation for gene gun labeling of the retina were immediately killed with an overdose of anesthetic after eye removal.

**Gene gun experiments**

**Retinal preparation.** A total of 24 retinas were used. Eyes (obtained from deeply anesthetized mice) were gently perforated with a thin needle and placed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF) solution (NaCl 119 mM, KCl 2.5 mM, MgCl2·6H2O 1.3 mM, CaCl2·2H2O 2.5 mM, NaH2PO4 1 mM, NaH2PO4·H2O 1 mM, glucose 11 mM). Eye cups were obtained by removing the cornea and lens; the retinas were isolated into the oxygenated medium under a dissecting microscope. The retinas were then flattened on the plastic stage of an automatic Millipore tissue chopper and vertically sectioned into 100-μm-thick slices. The slices were then transferred onto a round Millipore nitrocellulose filter (pore size, 0.4 micrometers) for subsequent labeling with the gene gun.

**Bullet preparation**

**DiI, DiO bullets.** A Bio-Rad 165-2431 gene gun device was used for the present study, together with its accessories (Tubing Prep Station and tubing cutter). The inside wall of 1 meter of Bio-Rad Tefzel tube was coated with a 0.1 mg/ml polyvinyl pyrrolidone (PVP) solution in 100% ethanol and then completely dried in the Bio-Rad Tubing Prep Station for 8 minutes. PVP residues were purged from the tubing with nitrogen gas. A total of 2–4 mg of lipophilic dyes (DiI and DiO; Molecular Probes #D-288 and D-275, respectively) were suspended in 200 μl of methylene chloride and mixed with 200-μg tungsten particles (size, 1.3 μm, Bio-Rad) on a clean glass slide. The mixture was spread on the slice surface with a thin plastic tip. After drying, coated particles were gently scraped from the slide with a clean razor blade onto a filter paper and transferred into a 15-ml Falcon tube filled with 3–5 ml of MilliQ water. The suspension was then transferred into the PVP-precoated tubing by means of a syringe fitted with a small piece of silicon tubing. Water was then pulled out gently from the PVP tubing with the syringe, and the tubing was dried with a low flow of nitrogen gas. Finally, the dried tubing was cut into 1.2-cm segments with a
Bio-Rad tubing cutter, and the bullets gently were shaken to improve particle dispersion. The bullets were stored in a dry cabinet away from light.

**Dextran bullets.** A total of 1.5–2 mg of fluorescent dextran (lysine fixable, Oregon Green 488 conjugated, molecular weight 10,000; Molecular Probes #D-7171) were suspended in 80–100 µl of MilliQ water and mixed with 25–40 mg of tungsten particles as described above. After drying, coated particles were transferred directly in the PVP-precoated tubing with a small plastic tip used as a funnel. Fine particle dispersion was achieved by holding the tubing into an ultrasonic bath for several minutes.

**Bullet delivery**

**DiO, DiI bullet delivery.** The gene gun was loaded with the bullets and the helium pressure set at 100 psi. Each disk of Millipore filter paper, supporting the retinal slices, was placed on top of a plastic Petri dish covered with white filter paper; the gun was positioned perpendicularly to it and 2 cm above the retinal surface. Just before shooting, a Falcon cell culture insert with pores of 3 µm was interposed between the gene gun and the specimen. After one or two shots, retinal specimens were rinsed in ice-cold oxygenated medium for 10 minutes and fixed for 30 minutes in 4% paraformaldehyde and phosphate buffer 0.1 M, pH 7.4. Retinal slices were counterstained with 1 µM propidium iodide for 10 minutes. After rinsing, the specimens were mounted onto a glass slide, cover-slipped in buffer, and examined with a Zeiss Axioplan microscope equipped with fluorescence and with a color AxioCam camera interfaced with the Zeiss Axiovision software for image analysis. Selected cells were also extensively studied with a Leica TCS-NT confocal microscope equipped with a krypton–argon laser. Files containing images of the cells were analyzed with AxioVision.

**Dextran bullet delivery.** After the shots, retinal specimens were rinsed with an oxygenated ACSF solution in a 36°C thermostatic bath for 1 hour, then fixed as above for 1 hour. Retinal slices were then processed according to the immunocytochemistry protocol for a retinal whole-mount preparation by using NK3R antibodies (see below) and protein kinase C (PKC) rabbit polyclonal antibodies (1:200; from Sigma), to label cone and rod bipolar cells, respectively.

**Identification and characterization of cells**

More than 200 cells were stained with the gene gun; cells with the best orientation and staining features (n = 117) were used for the classification. The numbers of stained exemplars for each cell type are: RB, n = 30; CB1, n = 14; CB1.2, n = 15; CB2, n = 11; CB3, n = 17; CB3.4, n = 5; CB4.5, n = 5; CB4a, n = 7; CB4b, n = 8; CB5, n = 5.

After choosing a bipolar cell properly labeled, an image was obtained with the confocal microscope and processed with AxiosVision. The inner plexiform layer (ipl) borders were traced at the inner limit of the inner nuclear and at the outer limit of the ganglion cell layer. A grid composed of five lines equally spaced according to the thickness of the ipl was overlaid to the image of each cell. Cells were divided into groups according to the position of their axonal arborization with respect to the five strata of the ipl. We found that often adjacent cells were also labeled, together with the bipolar cell of interest. At times, costained cells had a well-established pattern of stratification, such as AII amacrine or rod bipolar cells. This favorable coincidence helped considerably in the characterization of new cell types, providing an internal control (see Fig. 1). After grouping the cells according to the level of stratification of their axonal arbors, we took into account (1) the shape and size of their axonal arborization, and (2) the shape and size of their dendritic tree. All measures are expressed as mean values and standard errors.

**Immunocytochemistry**

Immunocytochemistry (ICC) was performed on retinal sections to visualize the pattern of stratification of cone bipolar cells labeled by anti-NK3R antibodies (NK3R, from Chemicon, and also a gift from Dr. R. Shigemoto), with respect to the labeling of cholinergic amacrine (stained with a goat polyclonal antibody against choline acetyltransferase, ChAT, from Chemicon), and of AII amacrines (stained with disabled-1 rabbit antiserum, kindly donated by Dr. B. Howell).
BIPOLAR CELLS OF THE MOUSE RETINA

Five anesthetized mice were perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer 0.1 M, pH 7.4, enucleated; and their eyes cut open. Eye cups were rinsed, infiltrated with 25% sucrose, embedded in Tissue Tek, and frozen at −22°C. Vertical sections, 14-μm-thick, were cut on a cryostat and collected on coated slides. Retinal sections were incubated for 2 hours in a solution containing 5% bovine albumin (BSA), and 0.3% Triton X-100 in phosphate-buffered saline (PBS). Primary and secondary antibodies were diluted in a solution containing 1% BSA and 0.1% Triton X-100 in PBS; primary antibodies were applied overnight at 4°C; secondary antibodies were applied for 2 hours at room temperature. In multiple-labeling experiments, antibodies were applied in sequence. Secondary antibodies were conjugated to Alexa-568 and Alexa-488 (Molecular Probes). For double labeling with two rabbit polyclonal antibodies, sections were incubated first with the primary antibody; then, an anti-rabbit Fab, conjugated with Alexa-488, was used as a secondary antibody. The sections were then incubated with a solution containing a very high concentration of unconjugated anti-rabbit Fab antibody at room temperature for 3 hours. The sections were then processed with the other primary antibody, followed by an anti-rabbit secondary antibody conjugated with Alexa-568.

For whole-mount ICC, retinas were separated from the pigment epithelium immediately after perfusion-fixation and subsequently fixed for additional 30 minutes. Samples were rinsed in PBS for 15 minutes and placed in blocking solution (5% BSA, 0.3% Triton X-100, PBS), overnight, at 4°C. NK3R antibodies were diluted 1:100 in a solution containing 1% BSA and 0.1% Triton X-100 and PBS, in which the retinas were incubated for 4–5 days (4°C). After extensive rinsing with PBS, the retinas were finally incubated in the secondary antibody (goat anti-rabbit Alexa 568, diluted 1:400 as above). The retinas were rinsed in PBS and flattened on glass slides with a thin brush. To avoid retinal deformation during covering, a thin layer of transparent paint was deposited beyond the retinal border with a PapPen. The retinas were then mounted in Vectashield (Vector) and covered-slipped. The specimens were analyzed at the confocal microscope. Three whole-mount retinas were used for data sampling. In each retina, 20 (250 × 250 μm) regularly spaced fields were sampled with the confocal microscope, along the four major retinal axes. The entire thickness of the inner nuclear layer was scanned, and the distribution of NK3R-positive cells was visualized in digital images representing collapsed views of the inner nuclear layer (extended focus images). Overlapping of cell bodies along the z axis was limited; thus, individual cell bodies could be clearly identified and counted by using high-resolution images displayed on a large-size monitor. Fine adjustments of digital images were made with Adobe Photoshop 7.0. An average number of 320 cells were counted in each field. The total number of NK3R-positive cells in each retina was obtained, multiplying the average density of cells in one retina by the area of that retina. To measure retinal areas, retinal profiles were drawn at the microscope using an optical drawing apparatus and the images were digitalized at 300 dpi. Areas were calculated by means of MetaMorph software for image analysis (version 4.6r8).

Statistical analysis

By using the xy coordinates of NK3R-positive cells, we analyzed the nearest neighbor distribution to obtain the regularity index (RI), defined as the ratio between the mean and the standard deviation of nearest neighbor distribution of distances. Cell coordinates were computed using a self-made software for analysis (Galli-Resta et al., 1999).

Cholinergic amacrine cells morphology

Two additional retinas were used to measure accurately the stratification level of the cholinergic amacrine dendrites within the ipl. After perfusion-fixation, retinas were isolated from the pigmented epithelium, infiltrated with 30% sucrose overnight, flattened on a sheet of Parafilem, and quickly frozen in Tissue Tek on a cryostat stage. Retinal blocks were then rotated 90 degrees and sectioned vertically in the cryostat at 12 μm. Retinal sections were stained with anti-ChAT antibodies as above, revealed with Alexa-488 secondary antibodies, and counterstained with propidium iodide. High-resolution images of a focal depth of 8 μm were obtained with the confocal microscope and analyzed with the AxioVision analysis software. From each image, a set of three measures was taken: (1) the distance between the inner border of the inl and the outer boundary of the outer cholinergic plexus, (2) the distance between the inner border of the inl and the outer boundary of the inner cholinergic plexus, and (3) the thickness of the inl.

RESULTS

We have identified a total number of 10 cell types, among which 9 are cone bipolar cells (CB) and 1 is a rod bipolar cell (RB). To produce a classification, we took into account the classic electrophysiological distinction of retinal bipolar cells into OFF and ON cells: the first hyperpolarize in response to a stimulus that falls in the center of their receptive field, while ON bipolar cells undergo a depolarization in response to a central light stimulus. The physiological classification has a well-known anatomical correlate: the axonal arborizations of OFF bipolar cells ramify in the outer portion of the ipl, the so-called sublamin a, which composes 40% of the ipl width. On the other hand, ON bipolar cells have axons that reach the innermost portions of the ipl, also known as sublamin b, which composes the remaining 60% of the ipl thickness. Sublamin a is additionally divided in two equally thick layers, layer 1 and 2, whereas sublamin b composes layers 3, 4, and 5.

We designated each bipolar cell as an RB or as a CB based on morphological criteria; we assigned to each bipolar cell a number from one to five, according to the layer of axonal stratification in the ipl. In addition, adjacent bipolar or amacrine cells having a known pattern of stratification were used as an internal control for the classification (see Fig. 1). We measured the diameters of the dendritic tree and axonal arborization (average measures reported with the standard error, SE).

Cone bipolar cells in sublamin a

We identified three distinct types of cone bipolar cells whose axons end in sublamin a of the ipl. The first one (CB1) has a very short axon and an axonal arborization...
confined within layer 1 (Fig. 2A). The diameter of the axonal arborization is approximately 27 ± 1.5 μm. Dendrites are scarcely ramified and flat, covering a mean region of 22 ± 1 μm.

The second cell (CB2) has a flat axonal arborization 25.5 ± 1.9 μm wide, stratifying in layer 2 of the ipl. The dendritic arbor is also flat and has a mean extension of 18.3 ± 1.6 μm. This type of cone bipolar is undoubtedly a rare one, as we did not find it frequently during the examination of the gene gun-treated retinas (Fig. 2B).

The last type of cone bipolar cell (CB1,2) has a large and complex axonal arborization that tends to occupy entirely layer 1 and a large fraction of layer 2 of the ipl (Fig. 2C), but it extends horizontally only 23 ± 3 μm. Dendrites are flat and cover a region of 14.4 ± 1.9 μm in diameter. This particular kind of cone bipolar cell is labeled very frequently with the gene gun, with a rate of recurrence that is second only to that of rod bipolar cells. This finding suggests that this type of cone bipolar cell could represent one of the largest populations among bipolar cell types.

**Cone bipolar cells in sublamina b**

We identified a total number of six types of cone bipolar cells that send their axons within layers 3, 4, and 5 of the ipl. One type (CB3) of cone bipolar cell has an axonal arbor that stratifies in the outermost part of the sublamina b (Fig. 2D); this cell has a bushy axonal arborization with a compact aspect and very short branches extending 24.2 ± 3 μm horizontally in the ipl. Dendrites are scarcely ramified and seem to cover a small region of the outer plexiform layer (opl). They extend horizontally 19.5 ± 3 μm.

The second bipolar cell type (CB3,4) has a broad axonal arbor (28.5 ± 2 μm) that spans throughout layer 3 and layer 4 of the ipl and also a wide dendritic arbor extending for 24 ± 0.5 μm in the opl (Fig. 2E).

Two varieties of cone bipolar cells (CB4a, CB4b) have axonal arborizations that end in layer 4 of the ipl; however, both the axonal arborizations and the dendrites of these cells have different morphologies. CB4a (Fig. 2F) has a flat axonal arbor extending for 23 ± 2 μm within layer 4 and a dendritic arbor composed of short and scarcely ramified dendrites that elongate 17.4 ± 2 μm in the opl. On the contrary, CB4b (Fig. 2G) has a bushy axonal arborization (20 ± 1 μm wide) that fills layer 4 almost completely. Dendrites are moderately flat and scarcely elongated in the opl (16 ± 1 μm).

The fifth cone bipolar (CB4,5) has a very wide axonal arborization, covering the entire thickness of layers 4 and 5 of the ipl and extending horizontally for 32 ± 9 μm. It has few and short dendrites, extending for 24 ± 2.6 μm in the opl (Fig. 2H).

The last cone bipolar cell (CB5) seems to be an uncommon type. It has a very large and flat axonal ending that covers a wide extension of the ipl (36 ± 1.3 μm). The dendritic tree is also sparsely branched and occupies a large portion (50 ± 4 μm) of the opl (Fig. 2I).

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**Fig. 2.** Panel showing bipolar cell types labeled with the gene gun technique; nuclei are lightly stained with propidium iodide. **A–C:** Cone bipolar cells with axonal arbors in sublamina a of the ipl. C is the cell type most likely stained by anti–neurokinin-3 receptor antibodies. **D–I:** Cone bipolar cells with axonal arbors in sublamina b of the ipl. H and I are two rarely encountered types. **L:** A rod bipolar cell. opl, outer plexiform layer; inl, inner nuclear layer; ipl, inner plexiform layer; gcl, granule cell layer; CB, cone bipolar cell; RB, rod bipolar cell. Scale bars = 20 μm in C (applies to A–C), L (applies to D–I).
Figure 2 (Continued)
Rod bipolar cells

There is one type of rod bipolar cell (RB5) in the mouse retina; this cell has a well-defined morphology and pattern of stratification and has been studied extensively by means of ICCH (Jeon et al., 1998; Strettoi and Pignatelli, 2000; Strettoi and Volpini, 2002) and electron microscopy (Tsukamoto et al., 2001). This cell has a typical club-shaped axonal ending of small diameter (11 ± 1 μm), composed of several varicosities mostly restricted to layer 5 of the ipl. The cell body resides in the outermost part of the onl and the dendritic arborization is rich. Dendritic processes cover a little region of 12.5 ± 1μm of diameter in the opl but they are considerably long as they reach the rod spherules, which occupy a more external position with respect to the cone pedicles (Fig. 2L). Rod bipolar cells are by far the easiest cells to stain with the gene gun method. This finding is not surprising, because these neurons alone account for approximately 50% of all the bipolar cells in the mouse retina (Strettoi and Volpini, 2002).

Immunocytochemistry of bipolar cells

Double-labeling experiments have been performed to study the spatial association between similar bipolar cells stained with different antibodies and to clearly define the stratification level of their axonal arborizations. Both anti-NK3R and anti-recoverin antibodies label cone bipolar cells with axonal arbors confined to the sublamina a of the ipl, comprising mostly layer 1 and the outer 40% of layer 2 (Fig. 3A,B). NK3R antibodies uniformly stain a thick plexus of axonal arborizations and a perceptible high
number of cell bodies in wild-type sections; on the contrary, anti-recoverin antibodies stain only few cells per section with the staining intensity variable among cells (Fig. 3A,B).

The axonal arbors of recoverin-positive bipolar cells overlap with the plexus stained by NK3R antibodies. Each cell body labeled by recoverin-antibodies is also labeled with NK3R antibodies; however, the reverse is not true (Fig. 3B). We applied the same anti-recoverin antibody on vertical sections from a retina of one retinal degeneration mutant mouse (Fig. 3A,B). We succeeded in finding single bipolar cells simultaneously stained with dextran and NK3R antibodies, unambiguously confirming the existence of a cone bipolar type, with the axonal arbor located in layers 1 and 2 of the ipl, that is positive for NK3R (Fig. 5A,B). To validate the method, dextran-labeled rod bipolar cells were successfully double stained with anti-PKC antibodies (Fig. 5C).

**Double labeling with antibodies and dextrans**

Lipophilic dye staining is inadequate for a successive antibody labeling because of the scarce persistence of these dyes in tissues treated with permeabilizing agents. To unambiguously characterize some of the bipolar cells, we labeled chopped retinal slices with the gene gun using tungsten particles coated with Oregon Green–conjugated, lysine-fixable dextrans. Dextran showed an intrinsic bias in the staining of retinal cells; the advantage is that they can be fixed with para- formaldehyde; hence, the tissue can be processed for ICCH. Dextran gun staining was consequently followed by labeling with NK3R antibodies.

We succeeded in finding single bipolar cells simultaneously stained with dextran and NK3R antibodies, unambiguously confirming the existence of a cone bipolar type, with the axonal arbor located in layers 1 and 2 of the ipl, that is positive for NK3R (Fig. 5A,B). To validate the method, dextran-labeled rod bipolar cells were successfully double stained with anti-PKC antibodies (Fig. 5C).

**Quantitative analysis of NK3R-positive cells**

We performed a systematic analysis of the population of NK3R-positive cells, measuring the average total number of these cells in the mouse retina and describing the cell distribution and the regularity of their mosaic. We found a mean number of \(93,152 \pm 3,670\) (SE) NK3R-positive...
cells in the mouse retina with a mean density of 5,290 ± 169 (SE) cells per square millimeter (Fig. 6).

The distribution of NK3R-positive cells along the major retinal axis (nasal to temporal and dorsal to ventral) follows a rule common to the majority of the cell types of the retina: The density of the cell bodies decreases approximately 50% from the center to the periphery along the vertical meridian, whereas there is a 25% decrease along the horizontal meridian.

We determined the RI of NK3R-positive cells to establish whether they follow a random distribution over the retinal plane. The regularity index is defined as the ratio

Fig. 5. A–C: Cone bipolar cell, corresponding to type CB1/2 of our classification, gene gun stained with Oregon Green–conjugated dextrans, and with antibodies against neurokinin-3 (red fluorescence). A: The digital overlay of two separate red and green confocal images, each spanning a very thin depth in the ipl. B,C: Arrowheads points to cell body and dendrites of the cell. D: Rod bipolar cell stained with green dextrans and protein kinase C antibodies (red signal). in E,F: Note the evident double labeling of the cell body (arrowheads). G,H: Axonal endings (arrowheads) and axon (arrows) of another rod bipolar cell double stained as above. opl, outer plexiform layer; ipl, inner plexiform layer. Scale bar = 20 μm in H (applies to A–H).
between the nearest neighbor mean value (nn), for a determined population of cells, and the associated standard deviation \((\sigma_{nn})\): \[ R_I = \frac{nn}{\sigma_{nn}}. \] The higher the \( R_I \) value is, the more regularly spaced the cells are.

The control for this experiment was set as follows. For each sampled retinal field, characterized by a certain number and distribution of cells, we generated a virtual field with the same features (size, number of cells) but with a random cell distribution, using self-made software (Galli-Resta et al., 1999). In generating the random distribution, the software takes into account the diameter of the cell bodies as a minimal distance between cell centers. Thus, we obtained a set of random distribution of cells matching the features of the experimental ones. We then calculated the RI value for the simulated random distribution and the RI value of measured samples. The RI for the random cell distribution was 3.0, whereas the calculated RI of the real cell population was 3.6. This finding suggest the existence of a small degree of regularity in the distribution of NK3R-positive cell, and it does not exclude the possibility that NK3R antibodies stain more than one population of cone bipolar cells.

**DISCUSSION**

Until very recently (Ghosh et al., 2004), there were no exhaustive studies aimed at a classification of bipolar cells for the retina of the mouse. However, a deeper knowledge of the cell types constituting the retina of this species is important: Although this animal's life is dominated by senses other than sight, it has been demonstrated clearly that (1) a fundamental retinal plan is maintained across different mammalian species (Masland, 2001a), therefore, the mouse retina is a good model for understanding retinal organization in other mammals including humans; and (2) the mouse is irreplaceable when it comes to studying several cell, retinal and ocular pathologic conditions also affecting humans (Chang et al., 2002).

In this study, we applied a recent technique, the gene gun labeling, to identify bipolar cell types in the retina of the mouse; we found nine types of morphologically distinct cone bipolar cells and one type of rod bipolar cell. We used the gene gun method to overcome the scarce availability of antibodies that stain bipolar cells as single types in the mouse retina.

The peculiar characteristic of the gene gun technique is that the dye-coated bullets spread over the retinal slices in a completely random way. This randomness should cause cells to be stained accordingly to their relative frequency; consequently, we should expect a correlation between the staining rate of a cell type and its specific abundance in the retina. This principle has not been exploited systematically in this work, and we did not perform any study to demonstrate this relationship; however, we considered big differences in the staining rate of two different cell types as indicative of differences in their relative abundance. In addition, by using anti-NK3R, an antibody that selectively stains cone bipolar cells terminating in sublamina a of the ipl, we performed a quantitative and mosaic analysis for this specific population of presumptive OFF cone bipolar cells. Finally, we succeeded in combining gene gun labeling with ICCH for at least some cell exemplars, providing unequivocal identification. For our taxonomy of bipolar cells, we referred mostly to the classic work performed in 1995 by Euler and Wassle for the retina of the rat, a closely related species.

We identified three types of cone bipolar cells whose axons end in sublamina a of the ipl and, thus, belong presumably to the physiological type of the OFF cone bipolar cells: we named them CB1, CB2 and CB1.2 (Fig. 7). There are good morphological indications that CB1.2 is a cell type that is also labeled by means of NK3R antibodies, because of the close resemblance of the axonal arborization and the identical spatial relationship that the latter establishes with the lobular appendages of AII amacrine cells and with the dendrites of cholinergic amacrine cells. This finding can be observed clearly in ICCH preparations, in which NK3R-positive bipolar cells have been immunostained together with cholinergic and AII amacrine cells.

Recently, Tsukamoto et al. (2001), have reconstructed various neurons of the mouse retina from continuous series of sections examined at the electron microscope. In particular, the authors describe two types of OFF bipolar cells, named B1 and B2. The characteristics of the cell designated as B1 by the authors make that very similar to our type CB1.2: B1 has the axonal arborization spreading through 35% of sublamina a, thus covering completely layer 1 and a half of layer 2. The axonal arbor overlaps perfectly the lobular appendages of one AII amacrine cell that also has been reconstructed in the same series and that establishes conventional synapses with the axonal ending of B1. Indeed, we found a precise spatial segregation of axonal endings of NK3R-positive cells and AII lobular appendages, revealed by immunostaining with DB1. The possibility that B1 could represent the cell type stained by NK3R antibodies has also been suggested by Haverkamp et al. (2003).

We also used the double staining with NK3R and ChAT antibodies to define the stratification level of NK3R cone bipolar axonal endings with respect to the position of the cholinergic bands, which we measured accurately. We found that the innermost portion of the axonal arbors of NK3R-positive cells overlaps with the outer cholinergic band, confirming that the axonal arborization of these cone bipolar cells encompasses layer 1 and partially occupies layer 2. These observations indicate that NK3R-positive cone bipolar cells, which comprise the CB1.2 type, have the possibility to establish contacts with both cholinergic and AII amacrine cells.

NK3R antibodies stain cone bipolar cells that are also stained by recoverin antibodies; however, the latter label only a subpopulation of NK3R-positive cells. There are two interpretations of this fact: a possibility is that NK3R antibodies label two or even more types of cone bipolar cells, which share very similar morphologies, and that are unmasked by recoverin labeling. This possibility is actually strengthened by the fact that (1) the RI for these cells is not particularly high, and (2) the number of cells stained with NK3R antibodies is quite large (over 90,000 cells per retina). It has been calculated that the total number of cone bipolar cells in the retina of the mouse is approximately 400,000 (Jeon et al., 1998; Strettoi and Pignatelli, 2000; Strettoi and Volpini, 2002); if each of the 10 cell types had to be represented equally, we should expect an average number of 40,000 cells per type, per retina. Yet, we do not know what is the relative frequency of the various types of cone bipolar cells or their specific

**Figure 7**
regularity index; thus, we can expect dense as well as rare types, as it happens for amacrine cells (MacNeil et al., 1999). On the other hand, the retinal histogram of NK3R shows a monotone trend, that could be produced both by the distribution of a single type as well as by two subpopulations with a very similar density profiles across the retina. For instance, it could be that the cell type that we designated as CB1 is also labeled by NK3R antibodies. In that case, the plexus stained by NK3R antibodies would be composed by the axonal arbors of CB1 and CB1.2. The other possibility is that NK3R-positive cells do represent a congruent type. Observations suggesting this possibility come from two sets of experiments. First, retinal sections do not stain homogenously with recoverin antibodies: some bipolar cells display a strong staining while others are weakly labeled; consequently, only few cells per section are clearly visible. It might be that the level of immunoreactivity for recoverin in bipolar cells depends upon a variety of conditions. In adult retina of rd/rd mice, in which photoreceptors had degenerated almost completely, recoverin antibodies label the same morphological types of bipolar cells but with a uniform and intense staining. Also, the number of recoverin-positive cells in the rd/rd retina seems much higher than normal, strongly recalling the staining pattern with NK3R antibodies of both rd/rd and normal retinas. It could be that recoverin antibodies detect a subpopulation of cone bipolar cells that are simply in a different functional or metabolic state, without actually discriminating a type. The same also holds for caldendrin staining: this antibody labels a subpopulation of cone bipolar cells sharing very similar features with NK3R-positive cells (Haverkamp and Wassle, 2000; Strettoi et al., 2002).

Tsukamoto et al. described in the retina of the mouse a cone bipolar cell with a flat axonal arbor stratifying in the innermost part of the sublamina a and denominated B2. We identified a cone bipolar cell (CB2) that perfectly matches the morphology and the stratification profile of B2. CB2 also seems to correspond to type 3 cone bipolar cell described in rat retina by Euler and Wassle.

Recently, five bipolar cell types have been described in the mouse retina by means of immunocytochemical techniques (Haverkamp et al., 2003). The authors identified two new types of bipolar cells by using a single antibody against a calcium binding protein (CaB5): one OFF cone bipolar cell stratifying in layer 2 and one ON cone bipolar...
cell stratifying in layer 3 of the ipl. CB2 seems to match the first of these two cell types. We identified six types of bipolar cells stratifying in the sublamina b of the ipl (CB3, CB4a, CB4b, CB3,4, CB4,5, CB5; Fig. 7).

CB3 has a bushy axonal arbor confined within layer 3 of the ipl. Even though the morphological match is not perfect, CB3 could correspond to the type 5 or the type 6 bipolar cell of the rat retina (Euler and Wassle, 1995). CB4a has a narrow axonal arbor localized in layer of the ipl. The position of its axon corresponds to 75% of the ipl; it could correspond to the type 8 bipolar cell of the rat retina (Euler and Wassle, 1995). CB4b, with a bushy and not very wide axonal arborization localized in the layer 4, could be the counterpart of the type 6 cell of the rat retina (Euler and Wassle, 1995).

To our knowledge, there is no evident parallel, in other mammalian species, for CB3,4; it could be the first time that this particular type of cone bipolar cell has been observed in a rodent retina. We are aware of the fact that this kind of cell is quite rare, because we labeled it at a low frequency with the gene gun.

CB4,5 also does not seem to be very abundant; compared with the other bipolar cells, it has a huge axonal arborization, spanning throughout layer 4 and layer 5 of the ipl; it could correspond to the type 8 bipolar cell of the rat retina (Euler and Wassle, 1995).

The last cone bipolar cell we identified, CB5, is undoubtedly similar to the type 9 cone bipolar cell of the rat retina. Its peculiar morphology, exhibiting sparsely branching dendrites that cover a wide area in the opl and a narrow axonal arborization, are strongly reminiscent of the blue cone bipolar cell described in other species (Boycott and Wassle, 1991; Euler and Wassle, 1995). Anti-PMCA1 antibodies stain distinctly a narrow stratum corresponding to the plane of axonal ramification of this cell type (Krizaj et al., 2002).

While this work was in progress, a detailed analysis of bipolar cells in the mouse retina studied with a different method was published (Ghosh et al., 2004). Our classification is very similar to that proposed in that study, differing only for these aspects: (1) we identify only one CB in layer 1 (CB1), (2) we describe an additional CB in layers 3 and 4 of the ipl (CB3,4). We find it remarkable that two completely different methods (intracellular injections and gene gun labeling) produced very close classifications for cell types that are undoubtedly very heterogeneous and hard to visualize.

It remains to be elucidated what is exactly the functional role of the various types of cone bipolar cells and what is the specific contribution they provide to the perception of a given visual stimulus. Each visual stimulus is perceived by the visual cortex as an ensemble of differentiated information concerning the intensity, the frequency, and the direction of the stimulus itself (Kundel et al., 2000); nevertheless, it remains unclear in which part of the visual system this multi-channel processing begins.

The retina represents an exceptional candidate to initially split visual information in its fundamental components. This split could be achieved through functional distinct bipolar cells acting in parallel. It seems very likely that this could happen just at the first retinal synapse beyond photoreceptors. It is known that many cone bipolar cells contact a single cone pedicle, a phenomenon called divergence, but it has yet to be demonstrated whether the anatomical divergence corresponds to the physiological subdivision of the stimulus components. An evident functional subdivision is created at the outer retinal synapses through the dichotomy between ON and OFF channels. It is well known that this dichotomy is established by the presence of different types of glutamate receptors upon the dendrites of diverse types of cone bipolar cells. In addition, it has been observed that the different types of OFF and ON cone bipolar cells can provide separate channels for high-frequency and low-frequency information. This is made possible by the presence on the dendrites of the bipolar cells of different types of AMPA and kainate receptors (DeVries, 2000).

Recently, Roska and Werblin provided data supporting the idea that bipolar cells can discriminate between the sustained and the transient components of the light stimuli (Cohen and Sterling, 1992; Freed, 2000a,b; Roska and Werblin, 2001; Werblin et al., 2001). This possibility would arise from the ordered anatomical structure of the retina: each type of cone bipolar cell should be able to provide a characteristic stimulation onto a selected type of ganglion cell, stratifying at the same level of the ipl. In-
hibition, on the contrary, would take place more diffusely by means of amacrine cells (Masland, 1986, 2001a). In this complex circuitry, the presence of a variety of cone bipolar cell types would reflect a variety of parallel functions; the capability of computation of the cone pathway is then exploited by the rod pathway as well, by means of the connections between rod bipolar cells and AII amacrine cells. It is known that these amacrine cells gain access to ganglion cells mainly through contacts established with the axonal endings of cone bipolar cells (Strettoi et al., 1994; Hack et al., 1999). Thus, the complete knowledge of the bipolar cell morphology and pattern of stratification represents a fundamental tool to integrate and understand information deriving from retinal physiology.

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