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Organizational motifs for ground squirrel cone bipolar cells

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Abstract

In daylight vision, parallel processing starts at the cone synapse. Cone signals flow to On and Off bipolar cells, which are further divided into types according to morphology, immunocytochemistry, and function. The axons of the bipolar cell types stratify at different levels in the inner plexiform layer (IPL), and can interact with costratifying amacrine and ganglion cells. These interactions endow the ganglion cell types with unique functional properties. The wiring that underlies the interactions between bipolar, amacrine, and ganglion cells is poorly understood. It may be easier to elucidate this wiring if organizational rules can be established. We identify 13 types of cone bipolar cells in the ground squirrel, 11 of which contact contiguous cones with the possible exception of short-wavelength sensitive cones. Cells were identified by antibody labeling, tracer filling, and Golgi-like filling following transduction with an adeno-associated virus encoding for GFP. The 11 bipolar cell types displayed two organizational patterns. In the first pattern, 8-10 of the 11 types came in pairs with partially overlapping axonal stratification. Pairs shared morphological, immunocytochemical, and functional properties. The existence of similar pairs is a new motif that may have implications for how signals first diverge from a cone to bipolar cells, and then re-converge onto a costratifying ganglion cell. The second pattern is a mirror symmetric organization about the middle of the IPL involving at least 7 bipolar cell types. This anatomical symmetry may be associated with a functional symmetry in On and Off ganglion cell responses.

Keywords

ground squirrel; adeno-associated virus; inner plexiform layer; retina

Introduction

Parallel processing in the visual system begins when the signals in an individual cone photoreceptor are transferred to two functional bipolar cell classes, 'On' and 'Off', members of which depolarize and hyperpolarize, respectively, during a light step. Cones use glutamate as a transmitter, and the different response polarities in mammalian On and Off

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bipolar cells result from the expression of metabotropic (mGluR6; Masu et al., 1995) or ionotropic (AMPA/kainate; Slaughter and Miller, 1983; Attwell et al., 1987) glutamate receptors. On and Off functional classes can each be further divided into approximately 5-7 anatomical types based primarily on the level(s) of axonal ramification in the IPL (Wässle, 2004). Studies of response kinetics and receptor pharmacology suggest that some of these anatomical types receive distinct signals at the cone synapse (Awatramani and Slaughter, 2000; DeVries, 2000). Since the bipolar cells in a type are defined by their tiling of the retinal surface, the 10 or more types can thus carry multiple representations of the visual scene to the inner retina. In the inner retina, bipolar cells make layer specific connections with more than 30 types of amacrine and 15-20 types of ganglion cells (Masland, 2001; Wässle, 2004). The connections between most bipolar, amacrine, and ganglion cell types are poorly understood, but are likely to play an important role in endowing the ganglion cell types with their distinctive response properties.

One way to understand retinal parallel processing is to start by identifying the cone bipolar cell types, and then to characterize their functional properties and postsynaptic partners. Classification schemes based on immunomarkers, Golgi impregnation, genetic labels, and tiling criteria exist for the primate (Boycott and Wässle, 1991; Haverkamp et al., 2003b; Joo et al., 2011), rat (Euler and Wässle, 1995), mouse (Badea and Nathans, 2004; Ghosh et al., 2004; Pignatelli and Strettoi, 2004; Wässle et al., 2009), and rabbit retinas (Mills and Massey, 1992; McGillem and Dacheux, 2001; MacNeil et al., 2004), among other species (Chan et al., 2001). However, an understanding of the functional differences between the bipolar cell types has been slow to emerge due to the difficulty of targeting specific cells for electrophysiological recordings in most mammalian retinas. In contrast, cone bipolar cell types can be targeted for recording based on soma location and shape in the cone-dominant retina of the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) (West and Dowling, 1975; Kryger et al., 1998), and thus there is information on type specific function in this species (DeVries, 2000; DeVries et al., 2006; Li and DeVries, 2006; Li et al., 2010). However, the study of ground squirrel bipolar cell function is impeded by an incomplete classification: recent reports each describe 7-8 cone bipolar cell types (Fig. 1; Cuenca et al., 2002; Puller et al., 2011), whereas mouse and rabbit retinas contain at least 11 and 12 types, respectively (MacNeil et al., 2004; Wässle et al., 2009).

It may be easier to understand the functional roles and output circuits of the bipolar cell types if organizational rules can be established. A combination of anatomical and physiological studies has established at least one pattern of bipolar and ganglion cell organization that has predictive power: Off bipolar cell axons terminate in the outer half of the IPL (sublamina *a*), whereas On bipolar cell axons terminate in the inner half of the IPL (sublamina *b*) (Famiglietti et al., 1977; Kolb, 1979; Saito, 1987; Euler et al., 1996; Pang et al., 2004). Ganglion and amacrine cells also receive On or Off input based on the level at which their dendrites stratify in the IPL (Famiglietti and Kolb, 1976; Roska and Werblin, 2001) with minor exceptions (Dumitrescu et al., 2009; Hoshi et al., 2009; Anderson et al., 2011). This pattern can be used, for example, to predict the response polarity of newly described anatomical types of bipolar cells (Joo et al., 2011).

The evidence for additional organizational patterns is more equivocal. One idea is that the IPL has a mirror symmetric organization centered about the sublamina *a/b* border. Functionally, many types of On and Off ganglion cells come in pairs that respond to changes in light intensity of opposite polarity in nearly equivalent ways. Examples include the primate On and Off midget and parasol cells (Chichilnisky and Kalmar, 2002), cat On and Off X- and Y-cells (Enroth-Cugell and Robson, 1966), and the component On and Off responses of On-Off directionally selective (DS) cells (Barlow and Levick, 1965). Anatomically, the members of these functional pairs tend to have dendrites that stratify at

similar distances above and below the sublamina *a/b* border (Amthor et al., 1989; Watanabe and Rodieck, 1989; Wässle and Boycott, 1991). Against the idea of mirror symmetry, certain ganglion cell types, for example, the direction-selective JAM-B cell in the mouse (Kim et al., 2008), do not have obvious mirror partners. Ganglion cell temporal responses may also be ordered with respect to IPL stratification, with more sustained responses occurring at the distal and proximal borders and more transient responses occurring in the middle (Wu et al., 2000; Roska and Werblin, 2001). The evidence for either a mirror symmetry or temporal organization among bipolar cell types is more limited than for ganglion cells. Candidate mirror symmetric pairs include the On and Off midget cells in the primate (Boycott and Wässle, 1991), and also the On and/or Off stratifying calbindin or CaBP5 positive cells in the ground squirrel (Cuenca et al., 2002; Puller et al., 2011), mouse (Ghosh et al., 2004; Wässle et al., 2009), and primate (Jacoby et al., 2000; Haverkamp et al., 2003b) retinas. Based on our current understanding, it is not clear whether a bipolar cell mirror symmetry exists and has predictive power.

As a first step towards understanding the distinct functions and output circuits of bipolar cells in the ground squirrel retina, we determined the bipolar cell types and the laminar organization of their axon terminals. Using panels of antibody makers, cell injection, and adeno-associated virus (AAV) mediated transduction, we show that the ground squirrel retina contains 13 types of cone bipolar cells. Two types, including the blue bipolar cell, contact non-contiguous photoreceptors, whereas the remaining 11 types appear to contact contiguous cones in a patch with the possible exception of short-wavelength sensitive (S) cones (Li and DeVries, 2006). We identify a new and unexpected organizational motif, which is that of these 11 types, 8 to 10 come in pairs that share immunohistochemical, anatomical, and functional characteristics, including partial co-stratification. A second organizational motif is that On and Off bipolar cells comprise partially symmetric populations reflected across the sublamina *a/b* border.

Materials and Methods

Experimental animal

Adult thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) (Helgen et al., 2009) of either sex were obtained from TLS Research (Bloomington, IL). The use of animals was in accordance with the guidelines established by the National Institutes of Health and approved by the Animal Care and Use Committee of Northwestern University. The squirrels were kept in cages in the animal facility at Northwestern University on a cycle of 12 hours light and 12 hours dark.

Antibody characterization

The primary antibody sources, immunogens, hosts, and dilutions used for immunofluorescence are listed in Table 1. An affinity purified rabbit polyclonal antibody raised against recombinant human CaBP5 detects CaBP5 and not other members of the Ca²⁺-binding protein family in retinal extracts. Pre-incubation with CaBP5 eliminates labeling in sections from mouse retina (Haeseleer et al., 2000). A monoclonal antibody raised against calbindin D-28k purified from chicken gut labels a single 28 kDa band on immunoblots of brain homogenates of various mammalian species. The antibody labels hippocampal neurons in control mice but not in calbindin D-28k knock-out mice according to the manufacturer's product description. Antibodies raised against the carbohydrate epitope CD15 recognize a cone bipolar cell type with shared morphological features in the macaque, marmoset (DB6 bipolar cells; Chan et al., 2001; Lee et al., 2004), rabbit (Brown and Masland, 1999) and ground squirrel (Puller et al., 2011). A polyclonal antibody raised against human placental choline acetyltransferase recognizes a single 68 kDa band in

Western blots from tree shrew (Knabe et al., 2007) and rat (Anadon et al., 2000) brain. The labeling pattern obtained in this study is comparable to that in previous studies in the ground squirrel obtained with the same antibody (Cuenca et al., 2002; Cuenca et al., 2003; Puller et al., 2011). A mouse monoclonal antibody raised against a portion (aa 1-418) of the RII β regulatory subunit of human protein kinase A detects a single band of 53 kDa in Western blots from human endothelial cell lysates (manufacturer's technical data sheet) and mouse retina (Mataruga et al., 2007). The labeling pattern that we obtain is similar to that previously obtained from the ground squirrel retina with the same antibody (Puller et al., 2011). The antibody to PKC α is a rabbit polyclonal that was raised against the C-terminus of the human enzyme (Besalduch et al., 2010). It labels a single 80 kDa in Western blots using material from NIH/3T3 cell lysates, but cross-reacts to a small extent with the β I and β II isoforms (manufacturer's data sheet). Antibodies against PKC α label rod bipolar cells in a variety of mammals (Greferath et al., 1990). In the primate (Mills and Massey, 1999; Chan et al., 2001) and ground squirrel (Cuenca et al., 2002; Li et al., 2010; Puller et al., 2011), antibodies to PKC α also label cone bipolar cells. A rabbit polyclonal antibody raised against human recombinant recoverin recognizes a single 26 kDa band on Western blots (Wiechmann, 1996) and labels photoreceptors and bipolar cells in a variety of mammals including the macaque (Jacoby et al., 2000) and mouse (Haverkamp et al., 2003a). The labeling pattern observed here is similar to that previously reported in the ground squirrel (Cuenca et al., 2002; Puller et al., 2011). A rabbit polyclonal antibody raised against purified recombinant human secretagogin (amino acids 135-273; UniProtKB/Swiss-Prot Accession # O76038) labels a single 32 kDa band on Western blots of brain lysates and subpopulations of neurons in the mouse (Mulder et al., 2009b) and rat (Mulder et al., 2009a). A goat polyclonal antibody against purified recombinant human secretagogin (amino acids 164-276; UniProtKB/Swiss-Prot Accession # O76038) recognizes a single 32 kDa band on Western blots using lysates from the mouse pancreatic insulinoma β -TC cell line (manufacturer's data sheet). A different rabbit antibody raised against human recombinant secretagogin also recognizes a 32 kDa band in material from retinal and cerebellar lysates. This rabbit antibody recognizes cone bipolar cells in mouse, rat (Puthussery et al., 2010), and primate (Puthussery et al., 2011) retinas.

Tissue preparation

Animals were euthanized by intracardiac injection of 200 mg/kg pentobarbital. The procedures for making 100 μ m thick retinal slices and for intracellular tracer labeling have previously been described (DeVries et al., 2006). In brief, the eyes are enucleated and both the anterior pole and vitreous are removed. The retina and pigment epithelium are dissected free from the sclera, and a piece of the superior retina is placed vitreous side down onto filter paper (Millipore, Billerica, MA). The pigment epithelium is then removed. The retina and attached filter paper are cut into slices using a vertically mounted razor in a HEPES-buffered (10 mM HEPES, 15 mM NaCl, pH 7.35) AMES medium-based solution. Slices are either transferred to a chamber and mounted on a microscope (Zeiss Axioskop2) for whole cell patch clamp recording or directly fixed (see below). Whole cell patch pipettes contained a standard intracellular solution (DeVries et al., 2006) to which was added 10 mM Neurobiotin (Vector Laboratories, Burlingame, CA). Slices were removed from the recording chamber after cell filling and immediately fixed.

Immunocytochemistry

Tissue was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 2-3 hours at room temperature or overnight at 4 °C. The tissue was washed 6 times for 30 minutes with modified PB containing 0.5% Triton X-100 and 0.1% Na₃N (pH 7.4) and blocked overnight in modified PB containing 3% donkey serum. Slices were then incubated with primary antibody diluted in modified PB plus 3% donkey serum overnight at 4 °C.

After six washes each for 30 minutes, tissue was incubated in donkey secondary antibody for 2 hours at room temperature. For double or triple labeling, tissue was incubated in a mixture of primary antibodies raised from different species including mouse, rabbit, and goat at the dilutions indicated in Table 1. Tissue was then incubated in a mixture of the corresponding species-specific donkey secondary antibodies conjugated with different fluorophores. The Alexa Fluor® 488 conjugated secondary antibodies were obtained from Invitrogen (Carlsbad, Ca). Cy3- and Cy5- conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). All were used at a dilution of 1:200. Neurobiotin was visualized with streptavidin-Alexa Fluor® 488 conjugate (1:200, Invitrogen) or Cy3-conjugated streptavidin (1:200; Jackson ImmunoResearch). A similar procedure was used for whole-mounted retinas except that 2×2 mm pieces of retina were placed vitreal side down onto filter paper, peeled from the filter paper after fixation, and left to float in solution in a 24 well culture plate. Primary antibody incubation lasted 5–7 days followed by a secondary antibody incubation that lasted 2 days. Tissue was mounted in VECTASHIELD® Mounting medium (Vector Laboratories, Burlingame, CA). Control slices, in which primary antibodies were omitted, were processed in parallel. No specific staining was detected in these controls. Images were acquired with a Zeiss LSM-510 META confocal microscope and processed with LSM image software and Photoshop 10.0.1 (Adobe Systems, San Jose, CA). During image processing, the input-output functions for the color primaries were adjusted to enhance visibility. Adjustments were applied to the entire image. All images are from collapsed z-stacks.

Adeno-associated virus serotype 2 vector and intravitreal injection

The capsid of AAV2 was modified to contain phenylalanine (F) instead of tyrosine (Y) at six sites (Y252, 272, 444, 500, 704, 730F) (Petr-Silva et al., 2011). The virus contained single stranded DNA which coded for green fluorescent protein (GFP) under the control of a chicken β -actin promoter. Anesthesia was produced by injecting animals with ketamine (100 mg/kg) and xylazine (10 mg/kg) IP. The target eye received one drop of dilute betadine solution which was immediately followed by one drop of proparacaine (1%). A 27 gauge syringe needle was used to make a hole at a location 1–2 mm below the limbal margin near the medial epicanthus. Virus ($10 - 20 \mu\text{l}$; titer equal to $10^{12} - 10^{13}/\text{ml}$) was injected by inserting a blunt 30 gauge syringe-mounted needle through the access hole into the vitreal chamber. After injection, the syringe needle was slowly withdrawn. Antibiotic drops were then applied to the treated eye, and the animal was injected with meloxicam (1.0 mg/kg, SC), yohimbine (0.5 – 1.0 mg/kg, IM), and 0.9% NaCl solution (5 – 10 ml, SC). The typical incubation period was 6–10 weeks.

Bipolar cell reconstructions

Amira 5.3 (Visage Imaging GmbH, Berlin, Germany) was used to reconstruct the morphology of labeled bipolar cells from a series of confocal sections. Images in each optical section were segmented into cells using a threshold for guidance. A smoothed surface was then constructed from the segmented sections. Bipolar cell axon terminals were ‘skeletonized’ using Adobe Photoshop. A series of confocal images were imported into Photoshop from a QuickTime (Apple, Cupertino, CA) movie file. The Animation dialog was used to leaf through movie frames. Skeletons were diagrammed in a transparent overlay. A circle was placed at the position of each axon trunk in a section immediately prior to one in which branching occurred. Lines were then used to trace axons through sections from this initial branch point. Lines were drawn by eye to extend between the center of neighboring varicosities. Varicosities were attributed to a cell if they were connected by a continuous axon branch. In some cases, near the periphery of an axon’s field, the branch connecting a varicosity was too dim to resolve. In these cases, the varicosity was assigned to a nearby tree using its long axis as a guide. Errors in assignment at the receptive field periphery did not

appreciably change the estimates of axon tree area for the sample. Axon tree area was obtained by connecting the axon tips of a skeletonized bipolar cell to form a convex polygon. Cells with axon trees that intersected the edge of a section were excluded from the area analysis. However, all identified axon trunks were used to calculate the number of each cell type in a given area. Coverage was determined by multiplying the average axonal area for a bipolar cell in a class by the number of axons in the image and then dividing by the area of the image. Nearest neighbor distances were determined according to the method of Wässle and Riemann (1978). The reference points for this analysis were the locations of the axon terminals just prior to branching in the IPL. The tips of bipolar cell dendrites were located and counted by obtaining a stack of confocal images that spanned from a GFP-labeled bipolar cell soma to the top of the outer plexiform layer (OPL). The images were imported into Adobe Photoshop and viewed as an animation. Dendritic branches were followed by eye to determine their final end points.

Nomenclature

Cone bipolar (cb) cell types in our classification are numbered according to their level of axonal stratification from the top to the bottom of the IPL. When the terminals of two cell types overlap, the type designated 'b' is either bistratified or more broadly stratified when compared to the 'a' type. The classification omits a cb4 cell so as to preserve a partial mapping between the old (West, 1976; Linberg et al., 1996; DeVries, 2000; Cuenca et al., 2002; DeVries et al., 2006; Li and DeVries, 2006; Li et al., 2010) and new nomenclatures. West's b4 cell is a rod bipolar cell (West, 1976; Li et al., 2010). In the ground squirrel, the sublamina a/b border occurs in the middle of the IPL where the upper stratification of cb6b cells abuts the lower stratification of cb3b cells. Physiologically, cb3b and cb6b are Off and On bipolar cells, respectively (DeVries, 2000, and unpublished results).

Results

We identified 13 types of cone bipolar cells in the ground squirrel retina (Fig. 1). Eleven types contact contiguous cones with the possible exception of the minority S-cones, one type contacts non-contiguous photoreceptors, and one type exclusively contacts S-cones. Taken in combination, recent classification schemes (Cuenca et al., 2002; Puller et al., 2011) contain 11 of the 13 cone bipolar cell types that we describe. Previously identified cell types and nomenclatures are indicated in Figure 1.

Bipolar cells were grouped into types based on their immunolabeling patterns, morphology, and tiling. Rarely, an antibody labeled a single morphologically uniform type. This was the case, for example, for antibodies against the calcium binding protein recoverin (RCV). More commonly, bipolar cell types were recognized based on their labeling patterns when two or more antibodies were simultaneously applied. In some cases, we could not find a labeling pattern that distinguished between two closely co-stratifying types. In these cases, we individually labeled adjacent cells by either injection or by virally delivered GFP so as to better observe morphological differences and to determine the extent of axonal arbor overlap. Axonal arbor overlap should be limited for bipolar cells of the same type (Wässle et al., 2009).

Off bipolar cells

We divided Off bipolar cells into six types. All six had axon terminals that ramified in the upper or distal half of the IPL (sublamina *a*). We started our classification by labeling with an antibody to the calcium-myristoyl switch protein RCV (Ames et al., 1996), since, in a previous report (Puller et al., 2011), the RCV antibody labels a single, uniform population of ground squirrel bipolar cells. RCV positive bipolar cells (Fig. 2A) had spherical somata that

were located in the middle of the inner nuclear layer (INL), and wispy primary dendrites and axons. RCV positive cell axon terminals ramified in two strata. An upper stratum, near the middle of sublamina *a*, “rests” on top of and partially overlaps with the upper choline acetyltransferase (ChAT) labeled band (Fig. 2D). A lower RCV positive stratum occurs at the sublamina *a/b* border. A small number of varicosities were located on axon branches between the two strata (Fig. 2F). Cuenca et al. (2002) first identified a RCV positive bistratified bipolar cell in the ground squirrel, which corresponds to the b3 cell in West’s classification (West, 1976; see also DeVries, 2000). The same cell type was identified by Puller et al. (2011). We classify this RCV positive cell as type cb3b.

Antibodies against the calcium binding protein secretagoin (SCG), which labels multiple bipolar cell types in the rat and mouse retina (Puthussery et al., 2010), also labeled several bipolar cell types in the ground squirrel (Fig. 2B). One or more strongly labeled bipolar cells had axons that terminated in sublamina *b*, and will be considered later. More weakly labeled somata gave rise to two layers of processes in sublamina *a*. Double labeling with an antibody to RCV showed that the weakly labeled somata and axon terminals belonged to two bipolar cell types (Fig. 2C): A RCV positive cell with a bistratified terminal distribution, the aforementioned cb3b, and a RCV negative cell that is more monostратified (Fig. 2C, *inset*), the cb3a. The terminal arborizations of cb3a cells overlap with the lower cb3b cell stratum. The morphological similarities and differences between cb3a and cb3b cells can be more easily appreciated when the cells are individually labeled in the same section, either by tracer injection or by AAV2-mediated transduction (Fig. 2E,F). In Figs. 2E,F, the cell on the left is a cb3b cell since it is bistratified and RCV positive (Fig. 2E), whereas the cell on the right, a cb3a, is mostly monostратified and lacks RCV labeling (Fig. 2E). cb3a cell axons start to divide at the level of the cb3b cell upper stratum, where they frequently display a cluster of varicosities or small branches (Fig. 2E-G). While the lower terminals of cb3b cells and the terminals of cb3a cells overlap, the results in Fig. 2E,F suggest that cb3a cells terminate slightly more distally in the IPL. The RCV negative/SCG positive cb3a bipolar cell corresponds anatomically to the RCV and calretinin positive b9 cell of Cuenca et al. (2002), although, in our hands, cb3a cells were not labeled by antibodies to RCV or calretinin.

An antibody to calbindin (calb) labeled at least two types of bipolar cells with axons that stratified in sublamina *a* and *b*, respectively (Fig. 3) (DeVries, 2000). The bipolar cell type that terminated in sublamina *a* had a large goblet-shaped soma with a stout axon (Fig. 3A-C). The terminal contained relatively large, monostратified varicosities. Triple-labeling with antibodies to RCV and ChAT showed the following sequence from the top to the bottom of sublamina *a*: an upper RCV positive stratum, an upper ChAT band, a layer containing calb positive terminals, and a lower RCV positive stratum. Fig. 3C shows the morphology of an injected calb positive Off bipolar cell (DeVries, 2000). Cells with a morphology similar to this calb positive cell were also labeled by antibodies to the Ca²⁺ binding protein CaBP5 (Fig. 3D) (Puller et al., 2011), and with antibodies to a hyperpolarization and cyclic-nucleotide gated channel (HCN1; Light and DeVries, unpublished result). The calb positive cb2 cell corresponds to the b2 in DeVries (2000) and Cuenca et al. (2002), and the cb2 in Puller et al. (2011), but may not correspond to West’s b2, which terminates above the upper cb3b stratum (West, 1976).

An antibody to the Ca²⁺ binding protein CaBP5 lightly labeled a band of terminals near the top of the IPL (Fig. 4A). This light band of CaBP5 labeling was first identified by Puller et al. (2011). In favorable sections, we could trace axons back to lightly labeled somata that were located in the middle of the INL. These somata could further be distinguished by their labeling with an antibody against the II β regulatory subunit of protein kinase A (PKA; Fig 4B,C). Careful inspection (Fig 4C) suggested that there might be two types of CaBP5 and

PKA positive Off bipolar cells. In one type (arrows), PKA-labeled cytoplasm formed a crescent around a central CaBP5 positive nucleus. This type had a thin axon. In the other type, a spindle-shaped soma (arrowheads) gave rise to a sturdy axon in a region in which CaBP5 and PKA labeling overlapped. Even in the most favorable images, these differences were subtle, so we sought to distinguish between the two cell types by injecting neighboring bipolar cells and then selecting those with axons that terminated above the layer formed by the terminals of cb2 cells for further analysis. An example is shown in Fig. 4D. We injected Neurobiotin into three bipolar cells and then labeled the slice with an antibody to RCV. The leftmost injected bipolar cell was a cb3a. The two bipolar cells on the right had terminals that overlapped in a region between the top of sublamina *a* and the upper stratum of RCV positive cb3b cells. Since both injected cells were labeled by Neurobiotin, their processes were disentangled by tracing in individual sections followed by reconstruction (Fig. 4E,F). The reconstruction showed that the cell on the right had an axon that branched upon entering the IPL, and then expanded to form a narrow cluster of terminals that ended just above the upper ramification of cb3b cells (Fig. 4D). The terminals of the cell on the left extended laterally immediately upon entering the IPL, and branched in a loosely-organized fashion throughout the upper portion of sublamina *a* (Fig. 4F). The idea that these cells are different types is supported both by the differences in morphology and the apparent violation of tiling implicit in the extensive overlap of axonal arborizations (Wässle et al., 2009). The rightmost cell in Fig. 4D-F will be called cb1a and its immediate neighbor, cb1b. cb1a and cb1b cells were routinely encountered in GFP labeled material (Fig. 4G). GFP labeled cb1a cells were strongly CaBP5 positive (9 out of 9 GFP-labeled cells with Cb1a-like morphology; Fig 4H), whereas CaBP5 labeling was weaker in cells with cb1b-like morphology (strong labeling in 8 and weak labeling in 9 of 17 GFP positive cells). We have previously classified the cb1a cell as a b7 (DeVries, 2000), but it most closely corresponds to West's b2.

Less frequently, we encountered a sixth type of cell with processes that ramified in sublamina *a*. This cell type had a large spherical soma that was located low in the INL either above or among the amacrine cell somata (Fig. 5). The cell was not labeled by our panel of antibodies and, because of the low location of its soma (Fig. 5A-C), was not routinely targeted for labeling via whole cell recording. Characteristically, a short, stout axon gave rise to an umbrella-shaped arborization (Fig. 5A,B). Axon processes penetrated both the upper cb3b stratum and ChAT band to terminate among the terminal varicosities of cb2 cells (Fig. 5A). In contrast, neither the terminals of cb1a or cb1b cells penetrated the upper cb3b layer. A total of 5 similar cells were filled. Fig. 5D shows a GFP-labeled cell, as well as nearby cb3a and cb1b cells. The patterns of axonal arborization clearly differ between the cb1b and new cell, with the horizontal span of the new cell's axon terminals being about twice that of the cb1b cell's terminals. A fine process emerged from the top of the cell and ascended to make a few sparse branches in the OPL (Fig. 5B,C). The small caliber of the ascending process made it difficult to image the contacts at the photoreceptor terminals, particularly in GFP-labeled material (Fig 5D). Visual inspection of the few cells in this sample suggested that this type contacted between 2-5 non-neighboring photoreceptors, at least some of which were middle wavelength sensitive (M) cones. Cone type in these experiments was determined by the arrangement of postsynaptic AMPA and kainate receptors (Li and DeVries, 2006; Li et al., 2010). Due to the fine caliber of the cell's ascending process, its lack of varicosities, and the abrupt ending of each branch at a photoreceptor terminal, we consider this new type to be a bipolar cell. For comparison, in the ground squirrel, the processes of dopaminergic interplexiform cells ascend through the inner nuclear layer and then travel horizontally in the OPL without branching. The horizontally oriented processes contact cone terminals at bouton-like thickenings (Cuenca et al., 2002; Saszik and DeVries, unpublished observation). Since the pattern of photoreceptor connectivity is, at present, unclear, we designate this cell as type 'X', and group it with the blue bipolar cell (see below). West's b7 cell may correspond to either the cb1b or 'X'.

On cell types

We distinguished seven cone bipolar cell types with axon terminals that ramified in the lower half of the IPL (sublamina *b*) including a blue cone bipolar cell. Antibodies against calb labeled two bipolar cell types with axons that ramified near the middle of sublamina *b* (Fig. 6A,B and Fig. 3). Similar to the calb positive Off bipolar cell, the cb2, these On bipolar cells had large goblet-shaped somata, stout axons, and prominent terminal varicosities. Labeling with an antibody to protein kinase C α (PKC α) subdivided the calb-labeled cells into two types, one PKC α positive (cb5b) and the other nearly devoid of PKC α labeling except for a small rim beneath the membrane (cb5a). The different labeling patterns are illustrated in Fig. 6A,B, which show, in cross-sectional view, a stratum just above the lower ChAT band that contains the terminals of calb/PKC α positive cb5b cells (yellow) and calb positive cb5a cells (red). Processes that are solely PKC α positive come from either rod bipolar cells (rb) or a type of multistratified bipolar cells that is further described below. Although difficult to appreciate in Fig. 6A,B, the stratification of cb5b cells was broader than that of cb5a cells. Both cell types were also labeled by antibodies to CaBP5 (Fig. 3). Evidence that cb5a and cb5b cells comprise distinct anatomical classes comes from viewing the overlap of their axonal arborizations in a thin confocal layer in the flat mount orientation (Fig. 6C). The processes of the two cell types appear to form two networks. An analysis of axon tree spacing (see below) confirms this impression. A b5 cell that is similar to the cb5 cell types was first described in Golgi stained material by West (1976). Cuenca et al. (2002) identified a labeling pattern consistent with two types of b5 cells, and found that one of the b5 cell types was bistratified in sublaminae *a* and *b*, an observation that we have been unable to confirm.

Labeling with an antibody to PKC α also revealed a layer of fine processes that started above and ended below the layer containing cb5 cells (Fig. 6A,B). According to Cuenca et al. (2002) and Puller et al. (2011), processes in this region are likely to come from bistratified b6 cells (West, 1976). To study the morphology of broadly stratifying cells in sublamina *b*, we labeled bipolar cells with GFP following transduction with AAV2. By comparing neighboring cells, we provisionally identified two types with similar but distinct axonal ramifications (Fig. 7). One type was bistratified, with an upper band of processes that ramified at the sublamina *a/b* border just below the terminals of cb3b cells. The other type was more narrowly multi-stratified, with an upper termination just below that of the first type. We classify the more bistratified cell as a cb6b and the narrower, multistratified cell as a cb6a. It was difficult to appreciate the differences in stratification pattern between the cb6a and cb6b cells without lamination landmarks. In the ground squirrel, in addition to the typical upper and lower bands, ChAT lightly labels a third band of putative cholinergic processes that ramify in the middle of the IPL (Cuenca et al., 2002; Cuenca et al., 2003). GFP positive cells were visualized in retinal cross-sections that were also labeled for ChAT (Fig. 8A-C). The two labeled cb6b cells in Fig. 8A and the left-most labeled cb6b cell in Fig. 8B have an upper set of processes that ramify at the same level as the middle ChAT band and a lower ramification that overlaps with the lower ChAT band. In contrast, the right-most labeled cb6a cell in Fig. 8B and the two cb6a cells in Fig. 8C overlap slightly with the middle ChAT band and terminate at the upper border of the lower ChAT band with a small amount of overlap.

We next examined whether one or both types of cb6 cell were PKC α positive. Figure 8D shows a cb6 type bipolar cell that was filled with tracer during intracellular recording. This bi-/multi-stratified cell had a lower set of terminals that ramified at the top of the lower ChAT band, consistent with its identification as a cb6a cell. The cell was PKC α positive. We obtained one additional filled cell that had cb6a-type morphology and PKC α labeling. Cb6a cells were also labeled with GFP following transduction by AAV2. Out of 11 GFP positive cb6a cells, all were strongly PKC α positive. In contrast, Fig. 8E,F shows a

bistratified GFP-labeled cb6b cell with processes that do not overlap with strong PKC α labeling, although some weak labeling at the soma cannot be excluded. Of 11 GFP positive cells that had cb6b type morphology, six lacked PKC α labeling altogether, whereas five had weak PKC α labeling. The upper ramification of the cb6b cell abutted the sublamina a/b border, and partially overlapped with processes descending from the lower stratum of RCV positive cb3b cells (Fig. 8G). These descending processes were first described by Puller et al. (2011). West (1976) described a multistratified cell that he called a b6 which is morphologically similar to the cb6a. Using West's classification, DeVries (2000) identified a bistratified b6 cell that shares morphological features with the cb6b.

Two types of cone bipolar cells had axon terminals that ramified beneath the lower ChAT band. These two types could be distinguished based on their labeling patterns for antibodies to CaBP5, SCG, and CD15. The first type was strongly labeled by antibodies to both CaBP5 and SCG (Fig. 9A-B), and had axon terminals that stratified just beneath the lower ChAT band. This cell may not have a counterpart in West's classification, and will be called cb7b. cb7b cells had large somata that were located near the top of the INL, and stout axons (Fig. 9C,D). A similar CaBP5 positive cell type was identified by Puller et al. (2011). The second type of cell, the cb7a, was labeled strongly by an antibody to CD15, and ramified at the bottom of the IPL (Fig. 9E-G). Antibodies against SCG labeled cb7a cells at the soma, axon (Fig. 9E,F), and axon terminals (Fig. 9C; *e.g.*, the layer beneath the GFP labeled cell). CD15 positive cells were weakly labeled by the antibody to CaBP5, which was more consistently evident in the soma (Fig. 9G) than in the axon terminals (Fig. 9B,C). cb7a CD15 positive cells were remarkable for their even spacing. This cell may also not have a counterpart in West's classification, but was recently described by Puller et al. (2011).

Two types of bipolar cells, the blue cone and rod bipolar cell, make selective contacts with photoreceptors. Their morphology has previously been described (Li and DeVries, 2006; Li et al., 2010; Puller et al., 2011). Both cell types have processes that ramify at the bottom of the IPL.

Cb2 cells: one type or two?

cb2 and cb5a/b cells share many properties. All three types have large goblet-shaped somata, label with antibodies to calb and CaBP5, and have axon terminals with large boutons that ramify in the middle of sublamina *a* and *b*, respectively. The similarities between cb2 and cb5 cell types prompted us to more critically examine whether cb2 cells might also be comprised of two separate classes. We first investigated whether the calb positive cb2 cells could be further subdivided based on CaBP5 labeling, but found, instead, that both antibodies labeled identical populations ($n = 4$ slices). We then searched for cells with cb2-like morphology that did not label with either calb or CaBP5. GFP labeled bipolar cells were first-identified as cb2 cells based on anatomical criteria in slices. These criteria included the shape and location of the soma, the approximate level of termination of the axon, and the size of the varicosities. Of 24 GFP positive cb2 cells identified using morphological criteria, all were calb positive. Similarly, a different group of 9 identified cb2 cells were all CaBP5 positive (*e.g.*, Fig. 2D). Thus, by the available immunohistochemical criteria, anatomically identified cb2 cells comprise a uniform class.

If cb2, cb5a, and cb5b cells each comprise single classes, then their axon terminal arborizations should tile the retina with an even spacing (Wässle et al., 2009). We characterized tiling by marking the locations of cb2, cb5a, and cb5b axons at a point just prior to the start of arborization. The results are illustrated in Fig. 10 for a retinal flat mount in which all three cell types were particularly well labeled. Within each of the three classes, nearest neighbor plots of axon spacing showed a minimum distance of 10 μ m (Fig. 10C). A clear minimum spacing is the expected behavior when bipolar cells belong to a single class.

When two classes were combined and treated as one (*e.g.*, cb5a and cb5b), there was no minimum spacing. Similar results were obtained in a total of 4 retinas. The results are consistent with the idea that cb2, cb5a, and cb5b cells comprise three distinct classes.

We next considered whether the retinal coverage of cb2 cells was increased so as to 'balance' the combined coverage of cb5a and cb5b cells. By counting either PKC α - or calb-labeled somata or axon trunks in 7 images from 5 different retinas, we found the following percentages for each cell type: cb2, $43 \pm 1\%$; cb5a, $24 \pm 3\%$; and, cb5b, $33 \pm 2\%$; (mean $\pm$ S.D.; the sample contained a total of 850 cb2, cb5a, and cb5b cells in roughly 220,000 μm^2 ; 100% in this sample equals the sum of the percentages of the cb2, cb5a, and cb5b cells). To obtain a coverage factor for each cell type, we used well-labeled tissue (Fig. 10A) to reconstruct the axon arbors for a number of cells of each type, and to fit the arbors with a convex polygon. For the piece of retina containing the region shown in Fig. 10A, cb2 cells had a mean polygon area of $550 \pm 120 \mu\text{m}^2$ ($n = 22$) and a coverage factor of 0.92 (26 axon terminals in an area of 15,600 μm^2 as shown in Fig. 10A,B); cb5a cells had an area of $640 \pm 120 \mu\text{m}^2$ ($n = 14$) and a coverage factor of 0.45 (11 axon terminals); and, cb5b cells had a mean polygon area of $480 \pm 130 \mu\text{m}^2$ ($n = 17$) and a coverage of 0.65 (21 axon terminals). The greater coverage of cb2 cells can also be appreciated by inspection in Fig 10B. The axonal branches of neighboring cb2 cells often touched and occasionally crossed, and gaps between cells were minimal. Branches that touched or overlapped were more rare among cb5a and cb5b cells, and gaps between the cells were more visible. The axonal arborization areas for the bipolar cell types are likely to depend on retinal eccentricity, which was not systematically controlled in these experiments. However, we made the reasonable assumption that the axon areas retain the same ratio irrespective of retinal eccentricity. In Fig. 10B, the areas have ratios of 1.0 : 1.2 : 0.9 (cb2 : cb5a : cb5b). In a total of three retinas, the area ratios were $1.0 : 1.2 \pm 0.2 : 0.8 \pm 0.2$. Given the percentage of each cell type per area listed above, we calculated a coverage factor ratio of 1.0 : 0.7 : 0.6 or 1 : 1.3 (cb2 : cb5a + cb5b). The results suggest that the cb2 cell could provide an anatomical input in sublamina *a* that could partially balance the combined inputs of cb5a and cb5b cells in sublamina *b*.

Quantitative comparison of bipolar cell morphologies

Within an antibody-labeled type, it was difficult to analyze the morphology of individual cells due to numerous process crossings between neighbors, both in the OPL and IPL. In contrast, tracer injection clearly delineated the morphology of individual cells, but the small number of labeled cells in any one slice made it difficult to make comparisons across types. To circumvent these problems, we virally transduced bipolar cells to express GFP, and then analyzed the morphology of individual cells in flat-mounted retinas. At a density of 50-100 labeled bipolar cells/mm 2 , well-labeled tissue typically contained several members of each type in close proximity.

A preliminary assessment suggested that three morphological features might be useful for comparing the different cell types: dendritic tree area, axon arbor area, and dendrite contact density or its inverse, contact territory. Figure 11 shows the axon and dendrite morphologies for the 11 diffuse cell types obtained from 2 different flat-mount preparations in which cells of the same type fortuitously had similar areas (see Fig. 12A). The results are quantified in a scatterplot of dendritic tree area versus axon terminal area (Fig. 12A) and in a histogram of contact territory as a function of cell type (Fig. 12B) for a larger sample of cells from a total of four retinas. The most compact dendrite and axon arborizations occurred in Off cb3a/b and On cb6a/b cells. These cells also had a high density of cone contacts or, inversely, a small territory per contact. Off cb2 and On cb5a/b had larger dendrite and axon areas, and a relatively high density of cone contacts. Finally, cb1b, cb7a, and cb7b cells had the largest dendrite and axon areas. Along with the cb1a cell, these three types had a marked sparsity of cone contacts. A progression in dendrite and axon area (Fig. 12A) is evident for the Off cell

types beginning with a clustering of points for cb3a and cb3b cells near the origin and extending outward to cb1b cells. On cell types displayed a similar, but less well-organized progression: cb6a and cb6b cells generally had the smallest areas and cb7a and cb7b the largest. An analysis of territory per contact (Fig. 12B) emphasized the low contact density in cb1a/b and cb7a/b cells, which can be compared to a much higher density in cb3a/b and cb6a/b cells. The results support the idea that cells that closely co-stratify and share antibody markers also have similar dendrite and axonal morphologies when viewed in the whole mount orientation.

Discussion

Based on stratification pattern, morphology, immunocytochemistry, and function, we suggest two organizing principles for ground squirrel bipolar cells. The first principle is that bipolar cells tend to come in closely related pairs. The second principle is that the bipolar cell types are mirror symmetric with respect to the sublamina *a/b* border. For both organizational motifs, certain cell types follow the pattern more closely than others.

Evidence for bipolar cell pairs

Ground squirrel bipolar cell types tend to come in pairs with axonal arborizations that closely co-stratify. Eleven of the bipolar cell types appeared to contact all of the cones in a patch with the possible exception of the minority S-cones. Of the 11 types, between 8 and 10 could be grouped into pairs. Pairs shared immunohistochemical markers, morphological features, and, where studied, functional properties. However, paired cells were not identical with respect to all properties. Evidence for and against various pairings is detailed below.

cb3a/b—Evidence supporting similarity comes from immunocytochemistry, morphology, and function. cb3a and cb3b cells were both positive for SCG and negative for calb and CaBP5. Morphologically, both types had small spherical somata that were located near the middle of the INL, and axonal processes that partially overlapped just above the sublamina *a/b* border. cb3b cells had an exuberant distal process, yet cb3a cells also tended to have a small process or several boutons in the same layer. cb3a/b cells were distinguished from other Off bipolar cell types in having a combination of three features: small dendritic trees, small axonal arborizations, and dense cone contacts (Fig. 12). Functional information on cb3a/b cell synapses was obtained by reanalyzing data from DeVries (2000), and reclassifying the cells based on images obtained at the time. The responses of cb3a and cb3b cell glutamate receptors at the cone synapse are both ~80% blocked by a kainate receptor selective agonist (SYM2081; Table 2; Donevan et al., 1998; DeVries, 2000) and become unblocked at a distinctively slow rate after agonist removal (Table 2). In addition, cb3a and cb3b cells are unique in expressing a high level of GluK1 message (Ryan and DeVries, 2010). For both cell types, ~20% of the synaptic response is blocked by the AMPA receptor antagonist GYKI53655 (Lindstrom and DeVries, unpublished observation). cb3a and cb3b cells also had differences. Only cb3a cells were RCV-positive, and cb3a cells had a robust distal stratification.

cb1a/b—cb1a and cb1b cells have many similar features, but also differ in a key respect. cb1a/b cells are unique in being jointly labeled by antibodies to PKA and CaBP5. In cross section, the axon terminals of the two types partially co-stratified within the uppermost layer of the IPL (*i.e.*, distal to the ChAT band). In the flat-mount view, the two types were unique among Off bipolar cells in that they both made sparse cone contacts (Fig 11, 12B). Indeed, cb1a cells frequently made a single cone contact (Fig. 6H in DeVries et al., 2006). The synaptic responses of cb1a and cb1b cells were almost completely blocked by the kainate receptor agonist SYM2081 (Table 2; DeVries, 2000), and were unaffected by the AMPA

receptor antagonist GYKI53655 (DeVries, 2000). Although the sample is small, both types appear to rapidly recover sensitivity following a pulse of SYM2081 (Table 2). In contrast to the cb3 cell types, GluK1 message was beneath the level of detection in cb1 cells (Ryan and DeVries, 2010). One of the main differences between the cb1a and cb1b cells was the size of both the axonal and dendritic trees. Unlike cb1b cells, cb1a cells had small axon and dendrite spans, similar those found in cb2 and cb3 cells.

cb5a/b—cb5a and cb5b cells have many common features. Both cell types are strongly labeled by antibodies to calb and CaBP5. cb5b cells were PKC α positive, as were cb5a cells but to a lesser extent. Morphologically, both types have large somata and stout axons that contain large terminal varicosities. The terminals of both cell types costratify in the middle of sublamina *b*. The areas and contact densities of cb5a/b cell dendrites were difficult to evaluate, since few examples were found in GFP labeled flat-mounts. However, values for dendrite area and contact territory were roughly intermediate between those of cb6 and cb7 cells. Functionally, cb5a and cb5b cells can be distinguished from all other On and Off bipolar cell types by having a peak transient, voltage-dependent Na⁺ current in range of -100 to -1200 during a step from -70 to -30 mV. The Na⁺ current tended to be larger in cb5b cells than in cb5a cells, and cb5b cells could generate 30 – 40 mV action potentials in response to light (Saszik and DeVries, 2012). Thus cb5a/b cells costratify and have similar morphology, label with similar antibodies, and have a sizeable Na⁺ current in contrast to other bipolar cell types. Aside from the difference in PKC α labeling intensity, the main difference between the types is that the axon terminals of cb5a cells were about 30% larger, by area, than those of cb5b cells.

cb6a/b—cb6a and cb6b cells had small, ovoid somata that were located at the very top of the INL. Both types had wispy primary axons that were bi- or multistratified in partially overlapping IPL strata. Both cell types had small dendrite and axon areas when viewed in the flat-mount orientation, and high dendritic contact densities. cb6a and cb6b cells differed in that the former was more consistently PKC α positive. There is no functional data on cb6a/b cells, but similarities in dendrite and axon areas, contact densities, and broad stratification, argues that the two cell types comprise a pair.

cb7a/b—The case that these two cells comprise a pair is more equivocal than for the other cell types. Both types label with SCG and CaBP5, a property that is unique among the ground squirrel bipolar cells. Both neuron types also have large axonal and dendritic trees and make sparse cone contacts. The physiological properties of cb7 cells are not known. Cb7a cells differ from cb7b cells in three obvious ways: cb7a cells label strongly for CD15, have very large dendrite and axon areas, and have consistent gaps in dendrite tiling (Puller et al., 2011).

Bipolar cells with overlapping terminal strata are quite common, having been described in a number of species. For example, in the primate retina, the terminals of DB2 and DB3 cells are partially overlapping (Boycott and Wässle, 1991); and, in the mouse retina, Types 1 and 2 and Types 3 and 4 have overlapping terminals (Wässle et al., 2009). However, in our view, terminal overlap is not a diagnostic criterion for describing cell pairs. Rather, other properties such as immunohistochemical markers, morphological features, or synaptic function must also be shared, and this is evidently not the case for primate DB2 and DB3 cells (Haverkamp et al., 2003b). Bipolar cells in other species that fit the criterion for pairs are frequently described as hard to distinguish. Examples include the Type 3a and 3b and Type 5a and 5b bipolar cells in the mouse (Mataruga et al., 2007; Wässle et al., 2009). In the case of Type 5 cells, the existence of two subtypes was inferred from an anomalously high cell density in the absence of cell-specific markers. We speculate that pairs occur in many

different species, and result when two types diverge from a single precursor bipolar cell type during recent evolution.

The close stratification of 'paired' bipolar cell axon terminals may have implications for signaling to ganglion cells. A simple assumption is that when the axon terminals of a bipolar cell pair co-stratify with the dendrites of a ganglion cell, that ganglion cell will receive input from both bipolar cells. Three scenarios that might lead to different signals in each bipolar cell in a pair involve differences in the number of cones sampled (*e.g.*, cb1a/b), differences in amacrine cell inhibition (*e.g.*, cb3a/b), and differences in membrane currents (*e.g.*, cb5a/b). In the case of cb1a/b cells, we speculate that their temporal properties are similar due to having similar receptors at the cone synapse, but that they signal different spatial frequencies due to differences in the numbers of contacted cones. If both cb1 cells provide input to the same ganglion cell, and that input is non-linear (Demb et al., 2001), then the ganglion cell will be able to respond over a broader range of high spatial frequencies. In the case of cb3a/b cells, the density of cone contacts and number of cones contacted is similar in both types. Both cell types express a similar complement of kainate and AMPA receptors and thus receive nearly identical temporal cone inputs. However, one obvious difference between the types is that the cb3b cell ramifies near the top of the upper ChAT band, and has an opportunity to receive input from directional starburst amacrine cell neurites (Demb, 2007) that is lacking for the main cb3a cell stratum. The two cb3 cells would then relay different responses to a common postsynaptic ganglion, one shaped by motion related inhibitory input from the GABAergic starburst cells and the other not. Motion related signals might similarly shape the responses of cb6b and not cb6a cells. Finally, in the case of cb5a/b cells, while both have Na⁺ currents, the cb5b cells is more likely to generate an action potential at light onset (Saszik and DeVries, 2012). A common postsynaptic ganglion cell might then combine signals from the more graded responding cb5a cells and spiking cb5b cells. It must be emphasized that these scenarios for alternative processing in bipolar cell pairs are, at present, purely speculative.

Evidence for mirror symmetry

Bipolar cells in the ground squirrel exhibit a remarkable core of mirror symmetry across the sublamina *a/b* border. This core extends from just above the upper ChAT band to just below the lower ChAT band. Mirror symmetric populations include the cb3b and cb6b cells, the cb3a and cb6a cells, and the cb2 and cb5 cells. In the latter case, there are two cb5 types, but the greater coverage of the single cb2 type might be viewed as creating a parity between the two layers. Whether symmetry exists outside of this central core is less clear. Two cell types, the cb1a and cb1b, terminate above the upper ChAT band and two, the cb7a and cb7b, terminate below the lower ChAT band. The argument that these cell types represent symmetric populations is based on their cone contact density and, with the exception of cb1a cells, on the large size of their dendrite and axon trees. Outside of the ground squirrel retina, well-characterized examples of mirror symmetric bipolar cell populations include the On and Off midget bipolar cells in the primate, and On and Off bipolar cells in several species that are variously calbindin or CaBP5 positive and which ramify near the middle of sublaminae *a* and *b*, respectively (Massey and Mills, 1996; Haverkamp et al., 2003b; Ghosh et al., 2004).

A symmetrical organization of bipolar cells could underlie the observed symmetries in the responses of ganglion cell types. Mirror symmetric responses are characteristic of primate On and Off midget and parasol cells (Chichilnisky and Kalmar, 2002), cat On and Off X- and Y-cells (Enroth-Cugell and Robson, 1966), On and Off brisk transient cells in the rabbit (DeVries and Baylor, 1997), and the component On and Off responses of On-Off DS cells (Barlow and Levick, 1965). Anatomically, the members of these functional pairs tend to have dendrites that stratify at similar distances above and below the sublamina *a/b* border.

For example, in the primate, parasol ganglion cell dendrites ramify close to the center of IPL and are sandwiched between the dendrites of midget ganglion cells (Watanabe and Rodieck, 1989). In the cat retina, α , β , and δ cells have a mirror symmetry around the sublamina *a/b* border (Wässle and Boycott, 1991). In addition, On-Off DS cells have two sets of dendrites that ramify in the middle of sublaminae *a* and *b*, respectively, and which co-stratify with the processes of two mirror symmetric classes of amacrine cells, the On and Off starburst cells (Vaney et al., 1981; Tauchi and Masland, 1985). Bipolar cell properties contribute to the characteristic responses of the ganglion cell types (Demb et al., 1999). Hence an anatomical and functional symmetry in bipolar cells, in combination with an anatomical symmetry in amacrine and ganglion cells, could contribute to the mirror symmetric properties of ganglion cells.

Nomenclature and cross-species comparisons

West (1976) originally classified seven bipolar cell types (b1-7, including a rod bipolar cell) in the ground squirrel retina without regard for stratification sequence. Rather than adding seven new types to the original scheme, we have established an alternative nomenclature that is based on the sequence of terminal stratification in the IPL. Ghosh et al. (2004) and Wässle et al. (2009) used a similar approach to classify mouse bipolar cells. A different approach to naming is based on dividing the IPL into 5 layers of equal thickness (MacNeil et al., 2004; Pignatelli and Strettoi, 2004; Puller et al., 2011). This approach is convenient in rod-dominant retinas because the boundary between layers 2 and 3 corresponds to the sublamina *a/b* border. However, we eschewed a classification based on 5 equal layers for three reasons: First, the middle layer in the rod poor ground squirrel retina would contain both On and Off bipolar cells; second, several bipolar cells would terminate in a layer necessitating arbitrary designations such as *a-c*; and third, several bipolar cell types would terminate in multiple strata, requiring designations such as cb3,4 (MacNeil et al., 2004; Pignatelli and Strettoi, 2004; Puller et al., 2011) or cb1-2n (MacNeil et al., 2004). These complicated designations may be effective in print, but do not lend themselves to everyday conversation in the laboratory. Based on the aforementioned similarities in stratification, morphology, and immunolabeling, we divided ground squirrel cone bipolar cells into six major types and subdivided each type, except for the cb2, according to whether the subtype was either bistratified or more broadly stratified (b) or monostратified or less broadly stratified (a). The mouse classification also contains an *a/b* distinction (Wässle et al., 2009). The new ground squirrel classification lacks a cb4 cell so as to preserve a correspondence with West's original scheme (*i.e.*, the b2, b3, b5, and b6 cells correspond to the cb2, cb3, cb5, and cb6 cells) and our own previous work (DeVries, 2000; DeVries et al., 2006; Li and DeVries, 2006; Li et al., 2010). We use the designation "cb" rather than either "b" or "db" to preclude, in the former case, confusion with West's original classification, and in the latter, to avoid the implication that there is a contrasting midget bipolar cell class. Finally, we leave two exceptional bipolar cell types outside of the classification, a blue cone-contacting bipolar cell and a unusually broadly stratified bipolar cell that contacts a small number of non-contiguous photoreceptors.

Our classification includes most of the previously identified ground squirrel bipolar cells, but also describes several new types. West (1976) recognized seven types of bipolar cells (Fig. 1). We now recognize his b1 and b4 cells as the blue bipolar (bb) and rod bipolar (rb) cells, respectively. Other corresponding cells include the b3 (cb3b), b5 (cb5a or cb5b), and b6 (cb6a). West's original b2 probably corresponds to the cb1a, and he most likely failed to label or recognize the calb and CaBP5 positive b2 cell (cb2) using the Golgi method. The single dendritic contact of West's b7 cell most closely corresponds to our X cell, although we cannot exclude that the corresponding cell is the cb1b. Linberg et al. (1996), also using Golgi-stained tissue, but in the whole mount orientation, confirmed West's classification and

added a new type, the b8, which is bistratified in sublaminae *a* and *b*. We did not find a similarly bistratified cell. Rather, immunolabeling (Cuenca et al., 2002) suggests that the b8 and our cb5a may be the same cell (Fig. 1). Alternatively, some differences in retinal anatomy between *I. tridecemlineatus* and *O. beecheyi* (formerly *S. beecheyi*; Helgen et al., 2009) may be expected, insofar as they are phylogenetically distant among the Marmotini (Helgen et al., 2009). Puller et al. (2011) recently extended West's classification scheme by labeling with a panel of antibodies including those against RCV, CaBP5, PKC α , and CD15. Our own classification is a superset of theirs: We recognize six Off bipolar cell types whereas they recognized three, and we found six On bipolar cell types (excluding the bb and rb) whereas they found four. The differences result from including two different antibodies, SCG and calb, in our panel, and from the use of single cell labeling techniques.

Ten to twelve bipolar cell types have been described in a number of mammalian retinas including rabbit, mouse, rat, and primate. However, comparisons across species can be tricky due to changes in protein sequence and consequent antigenicity, the pleomorphic distribution of widely used calcium binding protein markers such as RCV, CaBP5, calbindin, and SCG, and shifts in IPL lamination patterns due, for example, to variable percentages of rod bipolar cells. However, there are some cell types in which morphology, antibody labeling, and stratification are strikingly similar across species. For example, an antibody to CD15 labels a bipolar cell that stratifies at the bottom of the IPL in the ground squirrel (cb7a) (Puller et al., 2011), macaque (DB6) (Lee et al., 2004), and marmoset (DB6) retina (Chan et al., 2001). CD15 also labels an On-stratifying bipolar cell in the rabbit, but this cell ramifies at a different level from those in the aforementioned species (Brown and Masland, 1999); CD15 labels only an Off bipolar cell (Type 2) in the mouse (Wässle et al., 2009) and an additional Off bipolar cell, the Off midget, in the marmoset (Lee and Grünert, 2007). Antibodies to RCV label an Off bipolar cell type in a number of species. A broadly stratifying RCV positive cell ramifies near the middle of sublamina *a* in the rabbit (Massey and Mills, 1996), mouse (Pignatelli and Strettoi, 2004), rat (Euler and Wässle, 1995), ground squirrel (Cuenca et al., 2002), and primate (Wässle et al., 1994). However, RCV may not label an equivalent type in all of the species. For example, the ground squirrel RCV positive cb3b cell ramifies above and within the upper ChAT band and at the sublamina *a/b* border, whereas the mouse RCV positive bipolar cell (Type 2) ramifies within and above the upper ChAT band, but not below (Ghosh et al., 2004; Pignatelli and Strettoi, 2004). Antibodies to PKC α label rod bipolar cells in the ground squirrel (Li et al., 2010) as in other mammals. However, in the ground squirrel, PKC α also labels cone bipolar cells that ramify in the middle of sublamina *b* (*i.e.*, cb5b and cb6a cells). PKC α labeling is also described for DB4 cone bipolar cells in the primate (Grünert et al., 1994; Haverkamp et al., 2003b; Lee and Grünert, 2007). We have identified a putative Off bipolar cell type, the X, that has a large multistratified axon terminal and sparsely contacts overlying photoreceptors. Simultaneous labeling with antibodies to S-cone opsin suggest that the presynaptic cones are unlikely to be S-cones (Li and DeVries, unpublished observation). An Off cone bipolar cell with similarly sparse cone contacts has been described in the cat (CBa6; Pourcho and Goebel, 1987) and a presumed On bipolar cell with similar features occurs in a primate (Joo et al., 2011).

A new approach to selectively labeling bipolar cells

There are two general approaches to identifying bipolar cell types for light microscopy. The first approach targets cell types with antibody markers or, more recently, uses transgenic mice in which fluorescent proteins are expressed in bipolar cell types under promoter control (Wässle et al., 2009). The advantages of cell type selective markers include ease of use, the ability to study class features such as density and morphological uniformity, and the ability to compare multiple classes in the same preparation by using different antibodies. Disadvantages can include a lack of markers for certain classes, a lack of marker specificity,

difficulty with delineating the morphology of individual cells, and, in the case of transgenic labeling, a focus on mouse retina. The second approach uses techniques that label individual bipolar cells with varying degrees of cell-type bias. Individual labeling techniques include the Golgi method (Boycott and Wässle, 1991), cell injection (Ghosh et al., 2004), photofilling (MacNeil et al., 2004), biolistics (Pignatelli and Strettoi, 2004), and randomly activated genetic reporters (Badea and Nathans, 2004). The advantages of these approaches include the ability to label bipolar cell types in the absence of known markers, the ability to discern the morphology of individual cells, and, when unbiased techniques are used, the ability to estimate cell density and coverage (MacNeil et al., 2004). Disadvantages may include an inability to distinguish between two costratifying types based on morphological criteria alone (*e.g.*, the cb5a and cb5b), difficulty combining antibody labeling with the other labeling methods, and signal lability in the case of photofilling.

We have introduced a new method for individually labeling bipolar cells. This method uses a capsid mutant of AAV2 that is intravitreally injected (Petr-Silva et al., 2011). The capsid proteins contains tyrosine to phenylalanine changes at six surface locations that are expected to reduce proteasome digestion, thereby enhancing expression and apparent tissue penetration. Qualitatively, intravitreal injection of unmutated wild-type capsid AAV2 led to widespread GFP labeling in inner retinal neurons including ganglion, amacrine, and occasionally bipolar cells. A quadruply mutated AAV2 (Petr-Silva et al., 2011) produced dense ganglion cell labeling and minimal amacrine and bipolar cell labeling. The sextuple mutant shown here produced selective bipolar cell labeling. All of the bipolar cell classes that were identified by immunohistochemistry were also represented among the GFP labeled cells following viral transduction. It is beyond the scope of the present paper to determine whether the viral-mediated labeling is unbiased, but inspection does not rule out that possibility. One advantage to viral transduction with GFP is that labeling can be readily combined with other antibody markers. Disadvantages of AAV2-mediated transduction include a 4-8 week incubation period and a patchy distribution of labeling. Our data suggests that capsid mutations can affect cell tropism in addition to the intended effect on reducing intracellular virus particle degradation.

In summary, we have used several approaches to identify and classify bipolar cells in the ground squirrel retina. We identified two organizational motifs. In the first motif, there are 4-5 pairs of cells with similar stratifications, morphologies, immunolabeling patterns, and, where studied, function. The second motif consists of a core of mirror symmetry across the sublamina a/b border. This symmetry involves 7 cone bipolar types, but may extend to all of the 'diffuse' types that contact the majority M-cones. Bipolar cells initiate parallel processing by carrying signals between an individual cone and multiple types of amacrine and ganglion cells. Schemes for organizing bipolar cells into anatomical or functional groups have the potential to simplify the task of understanding early stages of parallel retinal processing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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types (cb)	1a	1b	2	3a	3b	5a	5b	6a	6b	7a	7b	X	bb
PKARIIβ	++	++		++	++								
CaBP5	++	+/-	+++			+++	+++			+	+++		
Calb			+++			+++	+++						
PKCα						+	+++	++	+/-				
RCV					+++								
SCG				++	++					+	+++		
CD15										+++			
West	b2				b3		b5	b6				b7	b1
Cuenca			b2	b9	b3	b8	b5	b6					b1
Puller	cb1	cb2		cb2,3		cb4	cb3,4	cb5b	cb5a				bb

Figure 1.

Cone bipolar cell types of the ground squirrel retina and antibody markers (see Table 1). Off and On bipolar cell types, which terminate in sublamina *a* and *b*, respectively, are separated by the thick horizontal bar between cb3b and cb5a cell types. Bipolar cell types that contact non-contiguous cones are separated from other bipolar cell types by the thick horizontal bar between cb7b and X cells. The gray bars show the approximate locations of the ChAT-labeled bands. The qualitative intensity of antibody labeling ranged from absent (blank) to weak (+/-) to very strong (+++). Previously identified cell types and classification schemes (West, 1976; Cuenca et al., 2002; Puller et al., 2011) are listed below.

Figure 2.

Classification of cb3a and cb3b Off bipolar cells. **A-C.** Section was labeled with antibodies to RCV (green) and SCG (red). **A.** RCV positive cells are bistratified. RCV also labels the cone terminals at the top of the section. **B.** SCG strongly labels cells with axons that ramify in sublamina *b* and more weakly labels the somata and axon terminals of cells that ramify in sublamina *a*. **C.** The SCG positive bipolar cells that ramify in sublamina *a* are either RCV positive (yellow) and thus cb3b cells or RCV negative and thus cb3a cells (24 sections $\times$ 0.37 μm thickness each). **C, inset.** An enlarged view of cb3a and cb3b somata and processes in the area delimited by the dashed box. **D.** The top cb3b cell stratum rests on top of a ChAT-positive band ($22 \times 1.0 \mu\text{m}$). **E.** cb3a (right) and cb3b (left) cells were injected (INJ) with Neurobiotin tracer via whole cell patch pipettes. The soma of the cb3b cell is RCV

positive ($20 \times 1.0 \mu\text{m}$). **F.** cb3a (right) and cb3b (left) cells labeled with GFP following transduction by AAV2 ($35 \times 0.76 \mu\text{m}$). **G.** A cb3a cell was labeled with Neurobiotin tracer following intracellular recording. The cell has a small lateral process (arrow) at the same level as the upper stratification of cb3b cells ($15 \times 0.4 \mu\text{m}$). Scale bars equal $10 \mu\text{m}$. The approximate distal and proximal borders of the IPL are marked at the side of panels with solid lines. The approximate location of the sublamina *a/b* border is shown by a dashed line. A magenta-green version of this figure is included in the supplementary results.

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Figure 3.

Classification of cb2 Off bipolar cells. **A-C.** Calb labels two bands of bipolar cell terminals in sublamina *a* and *b*, respectively (red). The calb positive terminals in sublamina *a* ramify, **A.**, between the processes of the bistratified RCV positive cb3b cell and, **B.**, beneath the upper ChAT band ($18 \times 1.1 \mu\text{m}$). **C.** A calb positive cb2 cell labeled by tracer injection ($20 \times 1.0 \mu\text{m}$). **D.** A cb2 bipolar cell expressing GFP is labeled by an antibody to CaBP5 ($7 \times 1.1 \mu\text{m}$). Scale bars equal $10 \mu\text{m}$.

Figure 4.

Classification of cb1a and cb1b cells. **A.** An antibody to CaBP5 faintly labeled a band of processes at the top of the IPL (asterisk). The axons that extend into this band originated in lightly labeled somata. **B.** An antibody to PKA-RII β (PKA) labels somata in the INL and processes in several layers of the IPL. **C.** The same section with PKA and CaBP5 labeling superimposed. Some lightly-labeled CaBP5 positive somata are PKA positive. Somata can be tentatively divided into two types based on their labeling patterns. One type (arrows) typically has a crescent of PKA positive cytoplasm around the nucleus, whereas in the other type (arrow heads), PKA and CaBP5 labeling overlap near the axonal pole ($18 \times 0.38 \mu\text{m}$). **D.** Three bipolar cells were injected with tracer and then the section was labeled with an antibody to RCV ($27 \times 1.0 \mu\text{m}$). **E.** The two bipolar cells on the right were reconstructed from individual confocal sections. Their axonal processes have different patterns of ramification and extensively overlap. **F.** A superimposed DIC image from the same set of confocal sections shows that the terminals of both cell types arborize at the top of sublamina *a*. **G.** An example of adjacent cb1a and cb1b cells from AAV2 labeled tissue ($54 \times 0.5 \mu\text{m}$). **H.** A GFP labeled cb1a cell was also labeled by an antibody to CaBP5 ($3 \times 1.1 \mu\text{m}$). Scale bars equal $10 \mu\text{m}$. A magenta-green version of this figure is included in the supplementary results.

Figure 5.

Evidence for a type of bipolar cell with processes that begin to ramify near the top of the IPL. **A.** A bipolar cell was injected with tracer (red) and then the section was labeled with an antibody to RCV (blue). The bipolar cell on the right (X) has terminal branches that cross the upper stratum of cb3b cells ($104 \times 0.38 \mu\text{m}$). The slice also contains a filled cb3a cell. **B.** Volume rendering of the labeled cell on the right in A. The arrow head indicates a putative site of cone contact. **C.** The soma of the rightmost cell is located near the bottom of the INL, as shown by a DIC image, and has axonal processes that begin to ramify near the top of the IPL. A dendritic branch ends at a cone terminal (corresponding arrowheads in B. and C.). Cell images were obtained by collapsing 6 contiguous sections from the larger z-stack shown in A. and superimposing the result on a DIC image from a section in the middle of the 6 sections. **D.** A type X cell is located between cb3a and cb1b cells in GFP labeled

material. The fine dendrite is difficult to resolve as it ascends toward the photoreceptor layer ($60 \times 1.0 \mu\text{m}$). Scale bars equal $10 \mu\text{m}$.

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Figure 6.

Classification of cb5a and cb5b cells. **A.** Vertical section from a retina labeled with antibodies to calb, PKC α , and ChAT ($19 \times 0.5 \mu\text{m}$). Calb positive axons ramify just above the lower ChAT band and come in two types: PKC α positive (yellow, cb5b) and negative (red, cb5a). **B.** An enlarged section from A., annotated according to cell type and with ChAT labeling removed (rb, rod bipolar cell). **C.** A collapsed stack of horizontal sections showing the ramifications of calb and PKC α positive cb5b and calb positive cb5a cells ($14 \times 0.7 \mu\text{m}$). Scale bars equal $10 \mu\text{m}$. A magenta-green version of this figure is included in the supplementary results.

Figure 7. Bistratified (cb6b) and more narrowly multistratified (cb6a) bipolar cells in sublamina *b*. GFP labeled material. The image is not a montage ($56 \times 1.0 \mu\text{m}$). Scale bar equals $10 \mu\text{m}$.

Figure 8.

Classification of cb6a and cb6b cells. **A-C.** Retinal slices containing cb6a and cb6b type cells labeled with GFP. **A.** GFP labels two similar cb6b bipolar cells which have a lower stratification that spans the lower ChAT band and an upper stratification that overlaps with a central ChAT band ($96 \times 0.36 \mu\text{m}$). **B.** Two cb6 cells, one (left side) with a bistratified cb6b-type morphology and the other (right side) with more narrowly multistratifying terminals (cb6a type). The top level of the cb6a cell ramification starts just below the central ChAT band and ends just within the upper border of the lower ChAT band ($43 \times 0.8 \mu\text{m}$). **C.** Two GFP labeled cb6a bipolar cells ($38 \times 0.8 \mu\text{m}$). **D.** A cb6a bipolar cell was filled with tracer during recording, fixed, and then labeled with an antibody to PKC α . The filled bipolar cell was PKC α positive ($23 \times 0.45 \mu\text{m}$). **E, F.** A GFP labeled cb6b type bipolar cell which has processes that ramify in two strata. PKC α co-labeling is minimal within the area

delimited by the cell border. Faint PKCa labeling near the top of the IPL is contributed by amacrine cells whose somata are visible just above the INL/IPL border ($14 \times 1.1 \mu\text{m}$). **G.** cb6b (INJ) and cb3b (RCV) positive cells are symmetrically organized relative to the sublamina *a/b* border ($34 \times 1.1 \mu\text{m}$). Scale bars equal $10 \mu\text{m}$. A magenta-green version of this figure is included in the supplementary results.

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Figure 9.

Classification of cb7 cells. **A.** An antibody to CaBP5 labels a broad band of axon ramifications in sublamina *b*. **B.** Co-labeling with an antibody against SCG divides the processes in sublamina *b* into at least two narrower bands, an upper CaBP5 positive band containing cb5 cell terminals and a lower CaBP5 and SCG positive band containing cb7 cell terminals (purple; $18 \times 0.4 \mu\text{m}$). The cb7 band can be further subdivided into an upper and lower portion based on stronger and weaker CaBP5 labeling, respectively. **C-D.** A GFP labeled cb7b type bipolar cell also labels with antibodies to CaBP5 and SCG ($24 \times 1.1 \mu\text{m}$). **E.-G.** cb7a bipolar cells are positive for both SCG and CD15 and ramify at the bottom of sublamina *b*. Inspection of the somata suggests that CD15 positive cells are also slightly positive for CaBP5 ($26 \times 0.8 \mu\text{m}$). Scale bars equal $10 \mu\text{m}$.

Figure 10.

Analysis of retinal tiling for the axon arborizations of cb2, cb5a, and cb5b cells. **A.** Collapsed confocal stack containing the axon terminals of cb2 (upper; calb positive; $18 \times 0.4 \mu\text{m}$), cb5a (middle; calb positive; $10 \times 0.4 \mu\text{m}$) and cb5b (lower; calb and PKC α positive) bipolar cells. The locations of the main axon trunks (overlay, circles) and branches (lines) were traced through individual confocal sections. **B.** Skeletonized axons arbitrarily colored. Scale bar equals $10 \mu\text{m}$. **C.** Histograms of the nearest neighbor distances for each axon trunk. The histograms for cb2, cb5a, and cb5b cells show a minimum distance of approximately $10 \mu\text{m}$. The lowermost histogram shows a similar analysis obtained by combining cb5a and cb5b cells without regard for type, and illustrates the absence of a minimum spacing when types are inadequately distinguished. The histograms were fitted with a Gaussian curve (red line) that provided a measure of mean distance ($\bar{x}$) and spread (σ equals the standard deviation of the Gaussian fit). The blue line plots the expected distribution of nearest neighbor distances for a random distribution of axons at the given density (Wässle and Riemann, 1978).

Figure 11.

Axons and dendrites of bipolar cells in 11 classes imaged in the flat-mount orientation and arranged according to process area from smallest to largest. The top two rows show the dendrites (upper row) and axon terminals (lower row) of representative Off bipolar cells. The bottom two rows similarly show the dendrites and axon terminals of On bipolar cells. Individual images were obtained from two retinas in which individual bipolar cells were labeled with GFP by viral transduction. The types of GFP-labeled bipolar cells were identified by referring to the simultaneous labeling patterns of SCG and either PKC α or RCV in the flat mount (labeling not shown). Types cb1a, cb1b, cb2, and cb3a were from one piece ($\sim 1 \text{ mm}^2$) of retina and the remaining cells, with the exception of the cb7a, were from the other piece of retina. cb7a cells were from a third piece of retina that was labeled with GFP and CD15 antibody. The processes shown are labeled with CD15. The terminals of neighboring cells, which did not overlap with the center cell, were removed from the image. The axon and dendrite diameters of corresponding cell types in the two GFP-alone labeled retinas were similar (see Fig. 12A). To facilitate comparison, the image of the cb7a cell was demagnified by 2.2-fold. A reduction by this factor brought the data points for the GFP labeled non-cb7a cells in this preparation into register with the data points for cells in the same classes from the other two preparations (see Fig. 12). The scale bar equals $5 \mu\text{m}$ and applies to all images except for the image of the cb7a cell where it equals $11 \mu\text{m}$. The circle shows the average diameter of a cone inner segment in these retinas (area = $29.4 \pm 8.1 \mu\text{m}^2$, $n = 15$). Inner segment areas were measured by searching for instances in which cones were

partially or fully enveloped by GFP-labeled Müller cells. Dendrites: 10-24 optical sections, 0.4 μm thickness. Axons: 12-29 optical sections, 0.6 – 0.75 μm thickness.

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Figure 12.

Quantitative analysis of bipolar cell axonal and dendritic ramifications. **A.** Plot of dendrite area versus axon terminal area. Areas were determined by fitting the tips of the distal processes in a collapsed image stack with a convex polygon. To facilitate comparison, the dendritic tree areas of Off bipolar cells are plotted upward from the origin as positive values, whereas those of On bipolar cells are plotted downward from the origin as positive values. The plot contains results from 4 ~1 mm² pieces of retina. Arborization areas from three of the retinas were plotted without scaling insofar as the data points from corresponding cell types overlapped. Results from a fourth piece of retina were scaled down by 2.2-fold so that the data points of 6 cells (1 cb1b, 1 cb2, 1 cb3a, 1 cb3b, 2 cb5a) overlapped with those of the corresponding types from the other 3 retinas (6 data points not plotted). Data points from 7 CD15 positive cb7a cells in the fourth retina are plotted (a data point for an eighth cb7a cell, which had the smallest dendrite area, was obtained from one of the first three retinas). **B.** Histogram of contact territory (number of apparent cone contacts/dendrite convex polygon area) for the cells in 4 flat-mounted retinas (mean $\pm$ standard error). Cone location was not taken into account in this calculation because cones form a dense semi-regular array in this species (Szmajda and DeVries, 2011). p values were determined using a Wilcoxon Rank Sum test and indicate the probability that the means of two samples are different. When a horizontal bar is present but lacks a p value, the hypothesis that the two means are different was rejected at the $p < .05$ level.

Table 1

Primary Antibodies

Antibody	Immunogen	Dilution/species	Source and catalog number
CaBP5	recombinant mouse calcium binding protein 5	1:400 rabbit, polyclonal	F. Haeseleer, University of Washington, Seattle, WA
Calb	calbindin D-28k purified from chicken gut	1:2,000 mouse, monoclonal	Swant, Bellinzona, Switzerland Code No.: 300, Lot No.: 07 (F)
CD15	U-937 histiocytic cell line, reacts with lacto-N-fucopentaose III	1:100 mouse, monoclonal	BD Pharmingen, 559045
ChAT	choline acetyltransferase from human placenta	1:100 goat, polyclonal	Chemicon, AB144P
PKARI β	protein kinase A, regulatory subunit II β , aa 1-418	1:500 mouse, monoclonal	BD Transduction Laboratories, 610625
PKC α	A synthetic peptide corresponding to aa 651-672 at the C-terminus of human PKC α	1:100 rabbit, polyclonal	Santa Cruz, sc-208
RCV	recombinant human recoverin	1:500 rabbit, polyclonal	Chemicon, AB5585
SCG	secretagogin recombinant human protein epitope signature tag	1:500 rabbit, polyclonal	Sigma-Aldrich, HPA006641
SCG	recombinant human secretagogin aa 164-276	1:500 goat, polyclonal	R&D Systems, AF4878
GFP	Green fluorescent protein derived from the jellyfish <i>Aequorea victoria</i>	1:500 goat, polyclonal	Abcam, ab6673

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Block of cone to Off bipolar cell synaptic transmission by a kainate receptor selective agonist

Table 2

SYM 2081 (20 μ M) ^a	cb1 ^b		cb3 ^b	
	cb1a	cb1b	cb3a	cb3b
synaptic response decrease (%)	91.5 $\pm$ 8.4 (4)		79.3 $\pm$ 12.5 (8)	
	82 (1) ^c	92 (2) ^d	78.8 $\pm$ 16.8 (4)	79.0 $\pm$ 11.1 (3)
recovery τ (s)	4.7 $\pm$ 2.4 (4)		41.7 $\pm$ 14.1 (8)	
	3.0 (1)	6.3 (2) ^e	35.8 $\pm$ 12.8 (4)	28.5 (2) ^f

^aThe membrane voltages of a cone and postsynaptic bipolar cell were controlled with whole cell voltage clamps. A train of pulse depolarizations applied to the cone produced a train of postsynaptic current responses. SYM 2081 was applied to the synapse from a nearby puffer pipette. The percent suppression of the synaptic response and exponential recovery time constants after the puff are shown (mean $\pm$ S.D. where applicable). The numbers in parentheses are cell pairs. The data is reanalyzed from DeVries (2000).

^bformerly b7 and b3 bipolar cells (DeVries, 2000).

^cA few cells in the sample could not be further subdivided based on the available anatomical data.

^drange = 87, 97.

^erange = 4.4, 8.1.

^frange = 10, 47.