All AII amacrine cells limit scotopic acuity in central macaque retina: A confocal analysis of calretinin labeling

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Abstract
We have used calretinin antibodies to label selectively the mosaic of AII amacrine cells in the macaque retina. Confocal analysis of double-labeled material indicated that AII dendrites spiral down around descending rod bipolar axons before enveloping the synaptic terminals. Processes from a previously observed dopaminergic plexus in the inner nuclear layer were observed to contact the somata of calretinin-positive AII somata. Intracellular neurobiotin injection revealed that AII amacrine cells are tracer coupled to other AII amacrine cells and to some unidentified cone bipolar cells. An analysis of the retinal distribution of macaque AII amacrine cells, including an area in and around the fovea, showed a peak density of approximately 5,000 cells/mm² at an eccentricity of 1.5 mm. Staining of AII amacrine cells in central retina with antibodies to calretinin was confirmed by confocal microscopy. These results indicate that calretinin antibodies can be used to label the AII amacrine cell population selectively and that primate AII amacrine cells share many of the features of previously described mammalian AII amacrine cells. The peak AII cell density closely matched the peak sampling rate of scotopic visual acuity. Calculations suggest that, in central macaque retina, where midget ganglion cells are more numerous, AII amacrine cells form the limit of scotopic visual acuity (Wässle et al. [1995] J. Comp. Neurol. 361:537–551). As the ganglion cell density falls rapidly away from the fovea, there is a cross-over point at around 15° eccentricity that matches the inflection point in a psychophysically derived plot of scotopic visual acuity versus eccentricity (Lennie and Fairchild [1994] Vision Res. 34:477–482). The correspondence between the anatomic and psychophysical data supports our interpretation that the anatomic sampling rate of AII amacrine cells limits central scotopic acuity. J. Comp. Neurol. 411:19–34, 1999. © 1999 Wiley-Liss, Inc.

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AII amacrine cells are the most numerous amacrine cell type in the mammalian retina. They comprise around 11% of total amacrine cells or one-fourth to one-third of the glycinergic amacrine cells (Vaney et al., 1991; Strettoi and Masland, 1996), and it is clear that they play a major role in retinal circuitry. AII amacrine cells receive a dominant excitatory input from rod bipolar cells and merge rod signals into the cone pathways through contacts with cone bipolar cells (Famiglietti and Kolb, 1975; Dacheux and Raviola, 1986; Raviola and Dacheux, 1987; Strettoi et al., 1992). The contacts with OFF cone bipolar cells occur by conventional inhibitory glycinergic synapses, whereas the transmission to ON cone bipolar cells is through gap junctions (Hampson et al., 1992; Bloomfield et al., 1997). Thus, the AII amacrine cell is an integral part of the most sensitive rod circuit of the retina (Smith et al., 1986; Sterling, 1994), suberving detection at the dimmest light levels.

Much of our understanding about rod pathways and the AII amacrine cell has been derived from the studies of cat, rabbit, or rat retina outlined above and in the accompanying paper (Massey and Mills, 1999). More recently, interest has turned to the analogous circuits of the primate retina, and, thus far, the general rules about rod pathways that...
we have learned about the mammalian retina are fully applicable. Several Golgi studies have produced examples of AII amacrine cells in the primate retina showing they are narrow-field, bistratified cells with distinctive varicosities in sublamina a of the inner plexiform layer (IPL; Boycott and Dowling, 1969; Mariani, 1990; Wässle et al., 1995). In an independent study, Wässle et al. (1995) used calretinin antibodies to stain the AII population and reported a peak density of just over 10,000 cells/mm². Analysis of this material in the electron microscope revealed that primate AII amacrine cells receive input from rod bipolar cells in sublamina b and input from cone bipolar cells in sublamina a. Calretinin-labeled AII amacrine cells made synaptic outputs to cone bipolar terminals in sublamina a and gap junctions between AII processes and from AII amacrine cells to cone bipolar cells. Some of the cone bipolar cells recently were identified as flat midget bipolar cells, indicating input to the parvocellular pathway (Grüner, 1997).

However, there has been some controversy over the use of calretinin antibodies to stain the AII amacrine cell population. Calretinin is one of a family of calcium-binding proteins that are expressed commonly by neurons, reflecting the importance and ubiquity of calcium as an intracellular messenger (Baimbridge et al., 1992). Unfortunately, there is substantial species variability in the staining patterns derived for antibodies to calcium-binding proteins. For example, calretinin antibodies stain AII amacrine cells in the rabbit and macaque retina but not in the rat retina. In addition, Zhang et al. (1996) have suggested that a second cell type is stained for calretinin based on the presence of calretinin-positive cells in the fovea, an area devoid of rods and neurons comprising the rod pathway. Electron microscopic analysis confirmed that these calretinin-positive foveal amacrine cells make synaptic connections atypical of AII amacrine cells. The possibility of a second calretinin-stained amacrine cell suggests that the peak density of AII amacrine cells may have been overestimated.

In this paper, we have confirmed and extended previous work using calretinin antibodies to label primate AII amacrine cells (Paskett et al., 1996; Sandell and Jacobowitz, 1992), most recently by Wässle et al. (1995). Specifically, we have used double-labeling procedures and confocal microscopy to show that AII amacrine cells can be labeled selectively by calretinin antibodies. In addition, we have mapped the distribution of calretinin-positive cells in the fovea and revised the peak density for primate AII amacrine cells downward. Finally, we have made intracellular dye injections to reveal the morphology of single AII amacrine cells and their coupling patterns. Our results indicate that primate AII amacrine cells follow the pattern established in other mammalian retinas. Macaque AII amacrine cells reach a peak density of almost 5,000 cells/mm² at an eccentricity of 1.5 mm, which matches the peak of scotopic visual acuity (Lennie and Fairchild, 1994).

MATERIALS AND METHODS

Tissue preparation

Primate eyes were obtained from animals that had been used for other experimental purposes not related to the eye, commonly, although not exclusively, recording from the visual cortex. All procedures followed protocols approved by the institutional animal welfare committee. Enucleation was performed shortly after death, and the eye was hemisected at once. After removing the vitreous, the retina was isolated and immediately placed into freshly oxygenated Ames and Nesbett (1981) solution. Before intracellular dye injections, AII amacrine cells were labeled by incubation in 18 μM 4.6-diamidino-2-phenylindole (DAPI) for 1 hour, as described elsewhere for the rabbit retina. Primate retinæ intended for immunocytochemistry were fixed in freshly prepared 4% paraformaldehyde for 60 minutes.

Immunocytochemistry

Immunocytochemical methods followed established protocols and are described in detail in the accompanying paper (Massey and Mills, 1999). Briefly, after fixation, pieces of retina or free-floating Vibratome sections were washed in phosphate-buffered saline, pH 7.4 (PBS), with 0.5% Triton X-100 and 0.1% sodium azide and blocked overnight in 3% goat serum in PBS/0.5% Triton X-100/0.1% sodium azide. The tissue was incubated in primary antibody with 1% goat serum/0.5% Triton X-100/0.1% sodium azide for 5–10 days. Controls lacked primary antibody and were blank. After washing, single-labeled preparations were visualized with immunofluorescent secondary antibodies conjugated to indocarbocyanine (CY-3) and mounted in 50% glycerol with 0.1% phenylenediamine to reduce fading. Some preparations were incubated with streptavidin-horseradish peroxidase (HRP) and developed with hydrogen peroxide and diaminobenzidine (DAB), as described in the accompanying paper (Massey and Mills, 1999). Double-labeled preparations were visualized with CY-3 and fluorescein isothiocyanate or indodicarbocyanine (CY-5). Confocal microscopy was performed on a Zeiss LSM-410 (krypton-argon laser, 488, 568, and 647 lines; Zeiss, Thornwood, NY), and there was no crossover between channels. Images were processed in Photoshop software (Adobe Systems, Mountain View, CA0 and printed on a Cordonic 1600 color printer (Cordonic, Inc., Cleveland, OH). Manipulation of the images was restricted to threshold and brightness adjustments to the whole image. No image sections were enhanced selectively and digital filtering was not used.

Antibodies

Rabbit or goat polyclonal antibodies to calretinin were obtained from Chemicon (Temecula, CA). Preliminary dilution studies showed reduced background and increased selectivity for AII amacrine cells at a dilution of 1:5,000 or 1:10,000. For Vibratome sections, dilutions of 1:50,000 or 100,000 were used. A mouse antibody to the α isoform of protein kinase C (PKC; 1:1,000) was obtained from Transduction Laboratories (Lexington, KY). A rabbit polyclonal antibody to PKC (1:5,000 or 10,000) was obtained from Chemicon. Mouse antityrosine hydroxylase (anti-TOH) was a generous gift from Dr. J.W. Haycock (1:6,000). Goat or donkey secondary antibodies, conjugated with fluorochromes, were obtained from Jackson Immunochemicals (West Grove, PA) and were used at a dilution of 1:200.

Intracellular dye injection

Intracellular dye injections followed previously published methods (Mills and Massey, 1991; Massey and
Isolated pieces of live retina were mounted ganglion cell side up, and cells were impaled under visual control by using pipettes tip filled with 4% neurobiotin (Vector Laboratories, Burlingame, CA) and 0.5% Lucifer Yellow-CH in 50 mM Tris back filled with 3 M lithium chloride. After penetration, Lucifer Yellow was ejected with negative current to confirm cellular morphology, and then neurobiotin was ejected with positive current (1 nA, 3 Hz) for 4 minutes. Then, the retina was fixed in 4% formaldehyde for 30 minutes, washed in PBS/0.5% Triton X-100/0.1% sodium azide, and reacted overnight with 1:200 streptavidin conjugated to CY-3. Cells injected with only Lucifer Yellow were photooxidized with epifluorescent illumination in the presence of DAB to provide an opaque reaction product (Maranto, 1982; Sandell and Masland, 1988; Mills and Massey, 1991).

RESULTS

Lucifer Yellow injection

After the fluorescent labeling procedures devised for the rabbit retina (Mills and Massey, 1991), pieces of primate retina were incubated in DAPI, and labeled somata were dye injected with Lucifer Yellow or neurobiotin. A dye-injected AII amacrine cell from the midperiphery is shown in Figure 1. This cell was filled with Lucifer Yellow and subsequently photooxidized. It is immediately obvious that the primate AII amacrine cell shares many characteristics with the well-described counterparts of the cat and rabbit retina. There are many fine dendrites bearing lobules in sublamina a and numerous smooth dendrites that branch profusely in sublamina b. Similar to other mammalian species, the dendritic field in sublamina b is larger than the corresponding field of lobules in sublamina a, as described previously by Mariani (1990) and Kolb et al. (1992).

Calretinin immunocytochemistry

Light microscopy. Examples of calretinin immunocytochemistry in the wholemount primate retina are shown in Figure 2. The focal series in Figure 2A–C, which was photographed with a ×63 oil-immersion objective, shows calretinin-positive amacrine cells from the midperipheral retina. The AII amacrine cell somata in the inner nuclear layer (INL), adjacent to the IPL, form a nonrandom mosaic (Fig. 2A). There are no adjoining somata in this area of low density. Figure 2B, from sublamina a of the IPL, shows a field of many varicosities up to 3 µm in diameter. Some of the varicosities, depending on the exact level of focus, can be traced back to the calretinin-positive somata. Finally, shifting the focus to deep in sublamina b of the IPL, a matrix of fine processes is revealed (Fig. 2C). Some components of the matrix can be followed through larger processes back to the labeled somata in Figure 2A. The bistratified dendritic tree, with varicosities in sublamina a, may be compared with the dye-injected AII amacrine cell shown in Figure 1. The morphologic appearance of the amacrine cells stained by the calretinin antibody indicates that they are AII amacrine cells. In contrast to the rabbit retina, very few primate ganglion cells were stained by either of the calretinin antibodies.

Similar to rabbit retina, a higher dilution of the calretinin antibody (1:50,000 or 1:100,000) was required to maintain specificity in sectioned material. We believe that this resulted from the lack of penetration barriers, such as the inner limiting membrane, which consists of densely packed Müller cell end-feet that form a substantial diffusion barrier. In vertical sections, the calretinin antibody

Fig. 1. An AII amacrine cell, from wholemount primate retina filled with Lucifer Yellow and subsequently photooxidized (DIC optics). A: Focus in sublamina a of the inner plexiform layer. The soma is just out of focus, but a spray of lobules connected by fine dendrites is visible. Large background structures are retinal blood vessels. B: The same field with the focus in sublamina b showing smoother dendrites with a much larger dendritic field.
Fig. 2. Calretinin immunocytochemistry in the primate retina. A–C: Focal series, visualized with horseradish peroxidase (HRP)/diaminobenzidine (DIC optics). A: Focus in the inner nuclear layer (INL) shows a mosaic of dark, calretinin-positive somata. B: Focus in sublamina a shows many the lobules of many AII amacrine cells, some of which can be traced back to the calretinin-labeled somata. C: Focus deep in sublamina b shows the matrix of AII amacrine cell dendrites.

D,E: Immunofluorescence visualized with indocarbocyanine (CY-3). D: AII amacrine cells in the peripheral retina, 9 mm from the fovea. The plane of focus is slightly oblique, so that lobules in sublamina a can be seen in the lower part of the field. E: AII amacrine cells in the region of peak density, 1.5 mm from the fovea. Some somata are out of focus, because they are displaced to the second row of amacrine cells in the INL.
brightly and selectively stained numerous amacrine cells, which, in peripheral retina, were immediately adjacent to the IPL. Figure 3 is a stereo pair of high-resolution confocal images showing amacrine cells stained for calretinin. All three cells show the clear characteristics of AII amacrine cells. The central cell has a stout, descending stalk that may be followed down to the bottom of the IPL, where there is a continuous matrix of fine processes. This is consistent with the overlap expected from the relatively large size of the dendritic field in sublamina b (Fig. 1B). Sublamina a is filled with numerous lobules that can be traced back to the parent somata. This positively identifies each of the cells as AII amacrine cells.

Figure 2D,E shows two areas from the same retina in the far periphery (9 mm from the fovea) and in the region of peak density (1.5 mm from the fovea). These figures were prepared from immunofluorescent material, because this method results in better penetration, particularly for thicker parts of the retina close to the fovea. The peripheral field is slightly oblique, so that the lobules of AII amacrine cells can be seen in the lower part of the image (Fig. 2D). At the periphery, the somata form a regular mosaic with only one pair of adjoining cells (Fig. 2D). Both of these cells were confirmed as AII amacrine cells by focusing under high power. In contrast, at an eccentricity of 1.5 mm, the AII amacrine cell density is high enough so that many cells are adjacent. In fact, the density is so high that some of the calretinin-labeled somata are pushed higher into the INL, into the second row of cells. These cells are out of focus in Figure 2E.

Confocal microscopy. We used confocal microscopy to obtain more detailed images of the calretinin-positive cells as an aid to identification. An example from peripheral retina is shown in Figure 4A. In this high-magnification field, eight 0.5-µm optical sections were combined. Each of six calretinin-positive somata adjacent to the INL had numerous fine branches connected to a dense field of lobules in sublamina a. Thus, for peripheral retina, like the light microscopy described above, the calretinin-positive cells could be identified individually as AII amacrine cells on the basis of their morphology. Each soma also was connected to a matrix of fine processes in sublamina b (Fig. 4B). Central retina is thicker, presenting a barrier to antibody penetration and imaging. Moreover, the dendritic arbor of AII amacrine cells is very small in central retina, only slightly larger than the soma, as also observed in Golgi stained examples from this region (Wässle et al., 1995; Fig. 1), so that many of the lobules in sublamina a are directly underneath the soma. Nevertheless, in an area near to peak density at 1.5 mm eccentricity, all 25 calretinin-positive cells could be followed to lobules in sublamina a (Fig. 4C). Every cell also had descending branches that contributed to a deeper matrix of processes in sublamina b that could be traced when a stack of confocal images was animated. In central retina, the sublamina b matrix was considerably denser than in peripheral regions (Fig. 4B,D). These characteristics positively identify all the calretinin-labeled cells in this field as AII amacrine cells and, in a simplistic fashion, puts $\frac{1}{26}(4\%)$ for the upper limit of calretinin-positive, non-AII amacrine cells. In fact, the incidence of calretinin-positive, non-AII cells is far less than 4%.

Foveal distribution

In one wholemount retina, we were able to map the foveal distribution of calretinin-positive cells. Within the foveal pit (Fig. 5), there were only a few scattered calretinin-positive cells, three within a radius of 200 µm from the
Within the first ring of blood vessels, at a radius of approximately 300 µm, there were 45 calretinin-stained somata. Outside the first ring of blood vessels, the density climbs sharply, and the level of focus changes steadily as it moves away from the central area up the foveal slope. The shape of the calretinin-positive cells within the central fovea is unusual. They are narrowly stratified due to the thin extent of the IPL at this eccentricity, and they are elongated. They do not have the normal narrow-field morphology of AII amacrine cells, but they still bear varicosities. By the first ring of blood vessels (~300 µm), the calretinin-labeled cells clearly are recognizable as AII amacrine cells (Fig. 6). A Z-axis projection of this material shows the bistratified appearance of the calretinin-positive cells that is typical of AII amacrine cells (Fig. 6B). It also can be observed that the cells in this area, just outside the first ring of blood vessels, lie on the foveal slope.

Presence of non-AII, calretinin-positive amacrine cells.

Because others have reported that calretinin antibodies stain several amacrine cell types, we searched diligently for calretinin-positive, non-AII amacrine cells. This was possible only in central retina using the confocal microscope. In the central region, a few displaced AII amacrine cells with somata in the IPL or the ganglion cell layer were observed, but they contributed to the AII matrix in an otherwise normal fashion. Rarely (n = 3), calretinin-positive, wide-field amacrine cells were observed. These cells had large, lightly stained somata that produced long dendrites, which ramified in the middle of the IPL. In peripheral retina, essentially all of the calretinin-positive cells were AII amacrine cells. There were a few faintly stained ganglion cells and other amacrine cells, but they

Fig. 4. Confocal views of calretinin-stained peripheral (A,B) and central (C,D) macaque retina. In each case, the identity of all cells as AII amacrine cells was established by tracing each soma to the characteristic lobular appendages in sublamina a (A,C) and to the arbor of finer processes in sublamina b (B,D). In peripheral retina, the identity of the calretinin-positive cells is readily apparent. In central retina, most cells could be traced to lobules, but the confirmation was achieved best by animating a stack of confocal images. The density of somata is much higher in central retina, as is the density of the matrix in sublamina b.
Fig. 5. A map of the calretinin-positive somata in the foveal region of macaque retina. Within the first ring of retinal blood vessels, there are very few stained cells. Outside the first ring of blood vessels, approximately 300 µm from the center of the fovea, the numbers rise rapidly.
Fig. 6. Confocal views of calretinin-positive cells in the foveal pit and on the foveal slope at an eccentricity of 300 µm. A: An en face view of the drop in AII amacrine cell density as the foveal area begins. B: A z-axis rotation of the view shown in A establishes the identity and shows the positioning of these cells. The lobular appendages of the calretinin-stained AII amacrine cells are evident in both A and B. The horizontal line in B shows the depth of the optical sections shown in A. Because of the slope in this area of the retina, somata to the right in A are in focus; however, to the left of this frame, the level of focus is in the inner plexiform layer.
were discriminated easily from the bright AII cells. We conclude that calretinin antibodies, at a 10,000:1 dilution, selectively stain AII amacrine cells with less than 2% contribution from other amacrine cells.

Distribution of calretinin-positive amacrine cells

A density plot of calretinin-positive somata was obtained from well-stained immunofluorescent material in one wholemount macaque retina (Fig. 7). The identity of calretinin-positive cells as AII amacrine cells was confirmed by confocal microscopy in the central region. The distribution showed a peak density for AII amacrine cells of almost 5000 cells/mm² at an eccentricity of 1.5 mm. It was necessary to take measurements at 0.5-mm intervals to obtain the peak density. The peak density of calretinin-positive cells was confirmed in another macaque retina, although, in this second preparation, we were unable to confirm the identity of individual cells as AII amacrine cells due to the staining quality. The AII density declined smoothly with further eccentricity to 800 cells/mm² at an eccentricity of 10–12 mm. This results in a ratio of 6.25 for peak:peripheral density. The nearest-neighbor distance in central from confocal images was measured as 8.8 μm.

Double-labeling studies

Our initial goal was to test the specificity of the calretinin antibody for AII amacrine cells by using double-labeling procedures designed to visualize the known synaptic connections of AII amacrine cells. If the expected patterns were seen in the double-labeling experiments, then this would confirm the identity of the calretinin-positive cells. In conducting these experiments, we not only observed the predicted connections, but the calretinin antibody revealed new details of retinal circuitry related to the rod pathway and AII amacrine cells.

PKC

Antibodies against the α isoform of PKC are well known to stain rod bipolar cells in many species, including primates (GREFERATH ET AL., 1990). However, the primate retina is slightly unusual, in that certain cone bipolar cells also are stained. A low-power view of the macaque retina stained for PKC is shown in Figure 8A. Among the prominent rod bipolar cells, DB4 cone bipolar cells also are stained. This is seen most obviously by the two rows of dendritic terminals corresponding to the level of rod spherules and cone pedicles and the band in the IPL corresponding to the level of cone bipolar terminals (Fig. 8A, arrow). To confirm the presence of cone bipolar cells, a high-power view of the IPL is shown in the adjoining figure (Fig. 8B). At this magnification, the branch point of the DB4 cone bipolar cells may be observed above the level of rod bipolar terminals (arrows, Fig. 8B).

This material also was double-labeled with the calretinin antibody (Fig. 9). Against a background of red bipolar cells, the calretinin-positive somata are located predominantly next to the IPL. The top half of the IPL is filled with lobules, and, at the bottom of the IPL, there is a dense band of calretinin-positive dendrites enveloping the terminals of rod bipolar cells (Fig. 9A). In a high-magnification view of the IPL, it can be seen that the cone bipolar terminals are relatively free of calretinin-positive dendrites, and there is little evidence of any contact (Fig. 9B). The lower half of the IPL is almost an AII-free zone, although we would expect to find sparse contacts with several cone bipolar terminals that are the sites of gap-junction input. When the calretinin-labeled AII processes cross this zone, they can be seen to follow the route of rod bipolar axons. The AII processes traverse the lower half of the IPL and spiral down around the descending axons of rod bipolar terminals. The individual terminals of rod bipolar cells, which are sites of synaptic output, are surrounded extensively and intimately by calretinin-positive processes. In summary, these double-labeled confocal images reveal the connections expected of AII amacrine cells and suggest that the calretinin antibody may be used to label AII amacrine cells selectively.

TOH

TOH immunocytochemistry reveals the dendrites of dopaminergic amacrine cells that form a dense matrix in sublamina a of the IPL, adjacent to the INL. In whole-mount views of many species, including primate, there are prominent rings of varicosities in the dopaminergic matrix that encircle AII amacrine somata (TÖRK and STONE, 1979; VOIGT and WÄSSLER, 1987; WÄSSLER ET AL., 1993, 1995; CASINI ET AL., 1995; MASSEY and MILLS, 1999). There also is a sparse network of dopaminergic processes running in the INL. In vertical sections of retina double labeled for TOH and calretinin, it can be seen that the dopaminergic processes in the INL appear to contact calretinin-positive somata. Frequently, there is a varicosity adjacent to the top of each calretinin-positive soma, as shown in Figure 9C. This further suggests that calretinin selectively labels AII amacrine cells in the macaque retina.

Neurobiotin injection: Primate retina

When Lucifer Yellow was used as an intracellular dye (Fig. 1), there was no visible sign of dye coupling. However, when neurobiotin, an intracellular tracer with a lower molecular weight and slight positive charge, was injected...
Fig. 8. Vertical section of macaque retina stained with an antibody against protein kinase C (PKC). **A:** Low-power photomicrograph showing many brightly stained rod bipolar cells with prominent terminals deep in the inner plexiform layer (IPL) adjacent to the ganglion cell layer (GCL). A fainter band corresponding to DB4 cone bipolar terminals also is observed just above the rod bipolar terminals (arrow). The somata are a combination of rod and cone bipolar cells. Another clue about the mix of rod and cone bipolar cells can be seen in the outer plexiform layer (OPL), where there are two levels of dendritic terminals (arrowheads) corresponding to cone pedicles and the slightly higher rod spherules. **B:** At high magnification, the faint band seen in the IPL is made up from a distinct cell type, cone bipolar DB4 (axons labeled by arrows), which ramifies above the rod bipolar axon terminals. ONL, outer nuclear layer.
Fig. 9. Cells double-labeled with anticalretinin. **A:** Rod bipolar cells interspersed with DB4 cone bipolar cells stained with anti-PKC are shown in red. Two levels of dendritic terminals are seen in the OPL (arrowheads). Anticalretinin staining is shown in green. The rod bipolar cells descend deep in sublamina 5, where they associate intimately with AII amacrine cells. The arrow indicates the level of DB4 terminals in the IPL. **B:** High-power enlargement of the rectangle from A. The termination of the PKC-stained cone bipolar cell can be seen faintly (arrow). Anticalretinin-stained AII amacrine cells seem to surround the stalks of rod bipolar cells as they descend to sublamina 5, where the association is maintained. **C:** Anticalretinin-stained AII amacrine cells are shown in red. Dopaminergic amacrine cells (antityrosine hydroxylase (anti-TOH)-positive) are shown in green. Apart from the known rings that the TOH-positive processes make around the bottoms of AII amacrine cells in sublamina 1, there are processes that course through the INL, appearing to make contact with the tops of AII amacrine cells. Arrowheads indicate appositions between dopaminergic processes and AII cell bodies.
into primate AII amacrine cells, dye coupling to other AII amacrine cells became apparent (Fig. 10). The injected cell is brighter and is marked by a little dye leakage. Surrounding the injected cell is a regular mosaic of dye-coupled somata, each surrounded by a spray of lobules that serves to identify them as AII amacrine cells (Fig. 10A). Focusing down to sublamina b of the IPL shows a matrix of overlapping dendrites typical of AII amacrine cells (Fig. 10B). The labeled structures (out of focus in Fig. 10A) are retinal blood vessels. In another example, the dye-injected cell is particularly well-stained, and, again, there is a surrounding mosaic of labeled cells that are discernible as AII amacrine cells by focusing at the level of the lobules in sublamina a (Fig. 11A). The somata of these cells are brightly labeled and clearly focused in the first tier of the INL, adjacent to the IPL (Fig. 11B). Shifting the focus higher into the INL reveals four more lightly labeled somata (Fig. 11C, arrows). No dendrites could be observed; however, from their position in the INL, these cells were identified as bipolar cells. At this peripheral eccentricity and consequent low density, no AII amacrine cells were found above the first row of cells in the INL. These results indicate that AII amacrine cells in the primate retina are dye coupled, presumably through gap junctions, to other AII amacrine cells and to cone bipolar cells, as reported in other mammalian species.

**DISCUSSION**

Identity of calretinin-positive amacrine cells in primate retina.

Antibodies to calcium-binding proteins show considerable species variation in their staining patterns (Pasteels et al., 1990). For example, antiparvalbumin labels horizontal cells in the primate retina but not consistently in rat or rabbit retinae (Röhrenbeck et al., 1989; Wässle et al., 1993; Casini et al., 1995). In the rabbit retina, a calbindin antibody labels horizontal cells and an ON cone bipolar cell (Röhrenbeck et al., 1987; Massey and Mills, 1995); however, in the primate retina, anticalbindin labels an OFF cone bipolar cell, DB3, and possibly another cone bipolar cell, DB5 (Grünert et al., 1994). Therefore, there is no a priori reason, based on the rabbit data, that calretinin should label AII amacrine cells in the primate retina, although others have reported similar labeling patterns of an unidentified bistratified amacrine cell in both rabbit and primate retina (Pasteels et al., 1990; Sandell and Jacobowitz, 1992).

In the primate retina, the calretinin antibodies stain a population of amacrine cells with the morphologic appearance of AII amacrine cells, as revealed by Golgi staining or intracellular dye injection of single cells (Fig. 1; see Figs. 56, 58, and 60 in Boycott and Dowling, 1969; see also Mariani, 1990; Kolb et al., 1992). This confirms and extends a recent independent report by Wässle et al. (1995). The labeling of AII amacrine cells is clear in peripheral retina, in which the cells are sufficiently well stained that the features of individual cells can be determined at high magnification. Like in the rabbit retina, it is important to increase the selectivity of the calretinin antibody by using an appropriate dilution of the primary antibody. In this study, we used a dilution of 5,000 or 10,000. In peripheral retina, all of the calretinin-positive cells appear to be AII amacrine cells, and there are very few dimly stained non-AII cells. In central retina, the details are not so clear, and it was necessary to use the confocal microscope at high resolution to confirm that the vast majority of calretinin-positive cells are AII amacrine cells. In addition, vertical sections of central primate retina show that all of the calretinin-positive cells have the characteristic morphology of mammalian AII amacrine cells (Fig. 3; Wässle et al., 1995). The primate AII amacrine cell mosaic is not as regular as in the rabbit, but this may be due to the presence of blood vessels in the
A similar irregular mosaic also is seen by using dye injection into a single AII amacrine cell (Fig. 10).

**Double-labeling immunocytochemistry**

It is well known in many species that rod bipolar cells contact AII amacrine cells deep in sublamina 5 of the IPL (Famiglietti and Kolb, 1975; Strettoi et al., 1990, 1992; Wässle et al., 1995). This was confirmed easily in double-labeling studies, which showed that PKC-labeled rod bipolar terminals were surrounded by calretinin-labeled AII processes. In turn, this experiment supports our contention that calretinin antibodies may be used to label AII amacrine cells selectively. These results also are consistent with electron microscopy, which showed calretinin-positive AII processes postsynaptic to large, vesicle-packed terminals with ribbon synapses that were identified easily as rod bipolar terminals (Wässle et al., 1995). In addition, AII amacrine cell processes appear to cross the lower IPL by spiraling down around rod bipolar axons (Fig. 9). This suggests a developmental sequence by which AII processes envelop rod bipolar axon terminals.

In the primate retina, dopamine amacrine cells form a rich plexus in sublamina a of the IPL, adjacent to the INL. A double-labeling experiment with TOH antibody to label the dopaminergic cells showed extensive contacts around the base of AII amacrine cells. These have been described previously as the dopaminergic rings seen in wholemount preparations (Wässle et al., 1995). In addition, a fine network of processes was observed in the INL. These fibers have been reported previously. Hokoc and Mariani (1987) noted very fine, 0.5 µm-diameter, TOH-positive processes that either ended in the INL or coursed back to the IPL. Occasionally, these processes were observed to reach the outer plexiform layer, but they never arborized there. Holmgren (1982) observed dopaminergic processes in the INL, sometimes surrounding unlabeled cell bodies. Dopaminergic processes in the INL also have been observed in human retina (Kolb et al., 1992). We can now identify the targets of the dopaminergic processes in the INL as calretinin-positive AII amacrine cells (Fig. 9C). TOH-positive processes frequently terminated with a varicosity on the top of an AII amacrine cell. The significance of this finding is unknown. Most of the dopamine input to AII amacrine cells occurs at or near the soma or primary dendrite, perhaps because the effects of dopamine are mediated biochemically through intracellular adenylate cyclase (Hampson et al., 1992). However, the identification of the targets in the INL does end speculation that these outwardly directed dendrites might be interplexiform cells.

**Intracellular dye injection**

Dye injection with Lucifer Yellow reveals that primate AII amacrine cells share the common features of mammaalian AII amacrine cells. Compared with rabbit AII amacrine cells, there are more slender processes (Massey and Mills, 1999). Lucifer Yellow injections yield only single cells. However, as in other species, Lucifer Yellow is too
Population counts

The peripheral numbers for AII density are in close agreement with Wässle et al. (1995). This is a straightforward matter, because individual calretinin-positive cells can be observed well enough to confirm their identity as AII amacrine cells. In the central area, details were too obscure due to the thickness of the retina unless the confocal microscope was used. Then, the characteristics of AII amacrine cells could be observed by animating a stack of confocal images. Thus, we have a high degree of confidence in the cell counts even from central retina. We measured a peak density of almost 5,000 cells/mm² at an eccentricity of 1.5 mm. This estimate is substantially lower than the peak density of 10,626 cells/mm² at 1 mm eccentricity reported previously by Wässle et al. (1995). Those authors also were unable to observe details or even count calretinin-positive cells in central regions of retinal whole mounts. Instead, they cut vertical sections and corrected for section thickness and shrinkage. This difference in methodology may have produced a higher estimate of peak density, especially if the sections were cut through a small area of high AII density, because there is some local regional variation in cell density.

It is also possible that additional cells were labeled by the calretinin antibody, as suggested by Zhang et al. (1996), who reported a population of calretinin-positive, wide-field amacrine cells in addition to AII amacrine cells. In the present work, we occasionally have observed lightly stained, wide-field amacrine cells; however, at the antibody dilution used, they were rare and accounted for less than 1% of calretinin-positive AII amacrine cells. It is clear that antibody concentration and even the choice of visualization also may be variables here, because HRP methods have a nonlinear amplification step that tends to compress the range of staining by pushing all labeled cells to saturation. This can make all cells well labeled instead of producing a range of intensity so that lightly labeled cells can be discriminated. In the accompanying paper on AII amacrine cells in the rabbit retina, we also note that the selectivity of calretinin for AII amacrine cells was improved by the appropriate dilution: 10,000 for whole-mounts and as high as 100,000 for Vibratome sections (Massey and Mills, 1999).

Visual acuity and the distribution of AII amacrine cells in the primate retina

It is well known that peak cone density in the fovea matches the area of highest visual acuity and that the cone density slightly exceeds measures of photopic visual acuity. In contrast, the rod density has a peak at 18–20°, whereas the area of maximum scotopic acuity occurs at 5° eccentricity. Thus, rather than being limited by the photoreceptors, it has been suggested that other components of the rod pathway that are present at a far lower density, such as the AII amacrine cells, form a bottleneck to limit scotopic acuity (Wässle et al., 1995).

The rod pathway for maximum acuity is thought to be rods → rod bipolar cells → AII amacrine cells → midget bipolar cells → midget ganglion cells (or P cells). The M-cell (or parasol ganglion cell) array is too sparse to support maximum scotopic resolution (Curcio and Allen, 1990; Lennie and Fairchild, 1994), but there is good evidence for rod input to midget ganglion cells (Lee et al., 1997). In support of this pathway, it has been reported that AII amacrine cells contact both ON and OFF midget bipolar cells (Dacey, 1996; Grünert, 1997). Rod bipolar cells have two to three times the AII density and are therefore unlikely to present a limit to acuity (Grünert and Martin, 1991; Grünert et al., 1994). In addition, both flat and invaginating midget bipolar cells have a one-to-one correspondence with cones in central retina, and their densities far exceed those of the AII mosaic. Finally, estimates of midget ganglion cells in central retina also are far higher than the density of AII amacrine cells (Lennie and Fairchild, 1994; Wässle et al., 1995). Thus, there is strong evidence in central retina that AII amacrine cells...
have the lowest density in the rod pathway. Therefore, we compared our density values with psychophysically derived measures of scotopic visual acuity.

The peak density of AII amacrine cells was approximately 5,000 cells/mm² at an eccentricity of 1.5 mm. This is similar to the peak density for AII amacrine cells in the cat retina (Vaney, 1985; Sterling et al., 1988). Taking 1° as 200 µm for the macaque retina (de Monasterio et al., 1985; Wässle et al., 1995), 1.5 mm corresponds to an eccentricity of 7.5° (but recall that data were collected in 0.5-mm or 2.5° increments). Now, psychophysical measurements show that the maximum scotopic acuity for humans reaches six or seven cycles/degree at approximately 5° (Lennie and Fairchild, 1994). Earlier data taken from lower eccentricities actually show a peak at 5° (Mandelbaum and Sloan, 1947), which is close to the peak in AII density reported here. From sampling theory, we can predict the spacing of a detector array that would be able to discriminate seven cycles/degree, as follows: converting to mm gives 35 cycles/mm, and, simply from the Nyquist limit, the detector density must be twice the stimulus frequency to give 70 detectors per millimeter. Squaring to calculate the detectors per unit area gives 4,900/mm² for a square array. The center-to-center distance (R) of cells arranged in a hexagonal array is best calculated from the density (D) as R = (\sqrt{3}/2D)\times10^{-3} (Lamb and Simon, 1976; Lennie and Fairchild, 1994). This yields a corrected density of 4,243 cells/mm², which is slightly lower than the peak density of AII amacrine cells reported here. These calculations also predict that the detector spacing for a hexagonal array should be 15.3 µm or 4.5 minutes of arc. The nearest-neighbor distance in the area of peak density was measured as 8.8 µm. However, the density of AII amacrine cells is not isotropic, and the nearest-neighbor distance yields a low estimate of spacing (de Monasterio et al., 1985). By contrast, the average between-cell distance calculated by using Delaunay triangulation (de Monasterio et al., 1985) was 15.2 µm, which agrees quite well with the calculated detector spacing of 15.3 µm. Thus, there is a reasonable match between the peak density of AII amacrine cells reported in this study and maximum scotopic acuity. The correspondence between the anatomic peak density and the peak scotopic acuity supports the interpretation that AII amacrine cells limit scotopic acuity in central retina (Wässle et al., 1995).

Finally, the revised number for the peak AII density shifts the crossover point between the limit imposed by AII density and that imposed by ganglion cell density as it falls rapidly away from the fovea. The crossover is taken as the point where the AII density equals 40% of the ganglion cell density, because, in central retina, midget ganglion cells comprise 80% of the ganglion cell population, and they are present in ON and OFF pairs (Wässle et al., 1995). The point at which the AII:ganglion cell ratio reaches 0.4, when ganglion cell density becomes the limit of scotopic acuity, is at 3.25 mm or 16° eccentricity. Inside this area, AII amacrine cell density is the lowest in the rod pathway through the retina and, thus, imposes the limit to scotopic visual acuity. Beyond 16°, the paucity of midget ganglion cells accounts for the limit of scotopic acuity. This compares favorably with the data of Lennie and Fairchild (1994). Those authors measured scotopic visual acuity with sinusoidal gratings and found an inflection in the curve at 15° (Fig. 12, arrow). Outside 15°, the resolution limit was close to that predicted by sampling from the midget ganglion cell mosaic, assuming that a sampling element is formed by an ON center and OFF center pair of cells (Fig. 12; see Fig. 4 in Lennie and Fairchild, 1994).

We have combined the data on scotopic acuity and cell density in Figure 12. This shows the calculated acuity from the P ganglion cell mosaic taken from Lennie and Fairchild (1994), as calculated by them from the data of Curcio and Allen (1990). It also shows the calculated scotopic acuity if the AII amacrine cell mosaic determined the limiting spatial density and the measured scotopic acuity data of Lennie and Fairchild (1994). It illustrates that there is a shift in which mosaic determines scotopic acuity, with a crossover at about 15°, confirming the suggestion of Wässle et al. (1995). Outside 15°, the acuity curve (Fig. 12, open circles) follows the ganglion cell sampling limit (Fig. 12, gray line). Centrally, the acuity curve falls below the ganglion cell curve to follow the AII sampling limit (Fig. 12, black line). The correspondence between these calculations is very satisfying and lends further support to the revised peak density for AII amacrine cells of 5,000 cells/mm² in the macaque retina.

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**LITERATURE CITED**


![Fig. 12. The calculated sampling limits from the AII amacrine cell mosaic (black line), using the densities from this study (Fig. 7), crosses the calculated sampling limit from P cells (gray line) at about 15°. The scotopic acuity curve measured by Lennie and Fairchild (1994) is plotted by the circles. It appears that AII amacrine cell densities limit scotopic acuity below about 15°, whereas decline in the density of P cells limits scotopic acuity beyond this eccentricity. P-cell sampling limits were taken from Lennie and Fairchild (1994). The arrow indicates the inflection point in the scotopic acuity curve at 15°.](image-url)


