Antibody to Calretinin Stains AII Amacrine Cells in the Rabbit Retina: Double-Label and Confocal Analyses

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ABSTRACT

The AII or rod amacrine cell is a critical interneuron in the rod pathway of mammalian retinae. In this report, it is shown that commercially available antibodies to the calcium binding protein calretinin may be used to label the population of AII amacrine cells selectively. Calretinin-positive amacrine cells had the morphological attributes of AII amacrine cells. Double-labeling procedures showed that calretinin-positive somata were surrounded by dopaminergic varicosities and that calretinin-positive dendrites enclosed rod bipolar terminals, both as previously described for AII amacrine cells. By analyzing the surrounding kernel for each labeled pixel in the rod bipolar image, it is shown here that AII processes are adjacent to rod bipolar terminals at a level that far exceeds the random overlap present in images in which one label was rotated out of phase. Such a spatial relationship is indicative of synaptic connections, as well described for rod bipolar input to AII amacrine cells. AII amacrine cells also were double-labeled for calretinin and parvalbumin; however, a scattergram analysis of red versus green intensity showed that the parvalbumin antibody stained additional unidentified amacrine cells. In conclusion, at the appropriate dilution, calretinin antibodies are a useful marker for AII amacrine cells in the rabbit retina. J. Comp. Neurol. 411:3–18, 1999. © 1999 Wiley-Liss, Inc.

Indexing terms: retinal circuitry; rod bipolar cell; protein kinase C; tyrosine hydroxylase; parvalbumin

Rod and cone pathways are segregated in the first stages of the mammalian retina (Sterling, 1994). Cones contact multiple ON and OFF cone bipolar cells which, in turn, synapse with ON and OFF ganglion cells respectively, in the inner retina (Boycott and Wässle, 1991). In contrast, there is only one type of rod bipolar cell, which descends to sublamina 5 of the inner plexiform layer but rarely contacts ganglion cells directly. In the rabbit retina, more than 90% of rod bipolar cell synapses are made onto two amacrine cell types. One of these, identified by indoleamine uptake, contains γ-aminobutyric acid (GABA) and makes frequent reciprocal synapses with rod bipolar terminals (Sandell et al., 1989; Massey et al., 1992). The other cell that receives direct input from rod bipolar cells is a glycnergic amacrine cell known as the AII or rod amacrine cell (Raviola and Dacheux, 1987; Strettoi et al., 1990, 1992, 1994). The morphology of the AII amacrine cell was first described by Famiglietti and Kolb (1975). This cell has a characteristic bistratified morphology with a spray of lobules in sublamina a and many fine dendrites, with considerable overlap, in sublamina b (Mills and Massey, 1991; Vaney et al., 1991; Bloomfield et al., 1997). The matrix of AII processes at the bottom of the inner plexiform layer is the site of input from rod bipolar cell terminals (Strettoi et al., 1990, 1992, 1994).

The output of the AII amacrine cell funnels rod signals into the cone pathways by two distinct pathways that serve OFF and ON channels. The lobules of AII amacrine cells in sublamina a contact OFF cone bipolar cells through inhibitory glycnergic synapses, and the dendrites of AII amacrine cells in sublaminae 3 and 4 make gap junctions with ON cone bipolar cells (Strettoi et al., 1992, 1994). These gap junctions are presumed to be sign conserving, so that signals of opposite polarity are produced in ON and OFF cone bipolar cells. Thus, in the mammalian retina,
the AII amacrine cell is a critical interneuron in the rod pathway by which rod signals are merged into the cone pathways. Further studies of this cell type will be facilitated by use of an antibody to calretinin that can be used to label AII amacrine cells selectively.

Calretinin is a member of a group of calcium-binding proteins that are found commonly in the central nervous system. Calcium is a critical cellular messenger, and there are several related families of calcium-binding proteins, which include calretinin, calbindin, parvalbumin, guanylate cyclase activating proteins (GCAPs), recoverin, and calmodulin (Baimbridge et al., 1992; Polans et al., 1996). GCAPs 1 and 2 recoverin play roles in the regulation of the phototransduction cascade (Polans et al., 1996). The other calcium-binding proteins are thought to regulate the intracellular concentration of free calcium; however, exact details of their neuronal function are incomplete. Nevertheless, these calcium-binding proteins have proven to be extremely useful as morphologic markers for a variety of retinal neurons, particularly horizontal cells and bipolar cells (Röhrenbeck et al., 1987, 1989; Massey and Mills, 1996). However, it should be noted that there is considerable species variability in retinal staining patterns.

In the rabbit retina, antibodies to different calcium-binding proteins may be used for labeling different populations of retinal neurons. A calbindin antibody labels horizontal cells, a few wide-field amacrine cells, and one type of ON cone bipolar cell (Massey and Mills, 1996). Amacrine cells, including but not limited to AII amacrine cells, were labeled with a parvalbumin antibody (Casini et al., 1995; Strettoi and Masland, 1996). It has been reported previously that an antibody to calretinin labels many amacrine cells, including AII amacrine cells, and cells in the ganglion cell layer (Pasterk et al., 1990; Sandel and Jacobowitz, 1992). Strettoi and Masland (1996) labeled AII amacrine cells with calretinin but noted that another amacrine cell type also was labeled. Most recently, calretinin was colocalized with parvalbumin in AII amacrine cells, but these accounted for only 36% of the total calretinin-positive cells amacrine cells (Vo¨lgyi et al., 1997). In other words, there were nearly three times as many calretinin-labeled amacrine cells as AII amacrine cells, probably due to the high concentration of calretinin antibody. In addition, large ganglion cells also were labeled for calretinin (Vo¨lgyi et al., 1997).

In this paper, we have confirmed and extended previous work with calretinin in the rabbit retina. Specifically, we will show that calretinin antibodies can be used to label AII amacrine cells selectively. We also have conducted a quantitative double-labeling study with parvalbumin and calretinin antibodies, showing that parvalbumin labels certain amacrine cells in addition to AII amacrine cells. Finally, in double-labeled material, we have used confocal microscopy to view the connections between rod bipolar cells and AII amacrine cells. An abstract of this work was presented previously (Massey and Mills, 1995).

**MATERIALS AND METHODS**

Under a protocol approved by the institutional Animal Welfare Committee, adult New Zealand White rabbits of either gender weighing 2–3 kg were deeply anaesthetized with urethane (loading dose, 1.5 gkg, i.p.), and the orbit was infused with 2% lidocaine prior to enucleation. The globe was hemisected, the vitreous was removed, and the retina was isolated from the inverted eye cup while it was submerged in fresh Ames and Nesbett medium (Ames and Nesbett, 1981). The isolated retina was floated onto filter paper photoreceptor side down and fixed in fresh 4% formaldehyde for 30–60 minutes. For Vibratome sections, retinal pieces were removed by flattening the sclera, laying a Millipore filter on the ganglion cell side, inverting this sandwich, and peeling away the sclera. This preparation, with the retina on filter paper ganglion cell side down, was fixed in 4% formaldehyde for 30 minutes, then the retina was removed from the filter paper and embedded in 5% agar. Vibratome sections were cut at 30 µm.

For intracellular dye injections, retinal cells were prelabeled with 4,6-diamino-2-phenylindole (DAPI) or Nuclear Yellow (Mills and Massey, 1991; Vaney, 1991). Rabbits were anesthetized with a ketamine/acepromazine/xylazine cocktail (3:1:1; 0.25 ml/kg, i.p.), and the orbit was irrigated with 2% lidocaine. Intavitreal injections were made with 15 µg Nuclear Yellow in 10–50 µl isotonic saline. One or two days later, the retina was isolated and mounted ganglion cell side up on filter paper, as described above. Alternatively, pieces of isolated retina were incubated in fresh Ames and Nesbett medium containing 5 µM DAPI, for 30–50 minutes to prelabel AII amacrine cells (Mills and Massey, 1991).

**Immunocytochemistry**

Immunocytochemical methods followed established protocols. After fixation, pieces of retina or free-floating Vibratome sections were washed six times for 30 minutes each in phosphate buffered saline, pH 7.4 (PBS), with 0.5% Triton X-100 and 0.1% sodium azide. The tissue was blocked in 3% goat serum in PBS/0.5% Triton X-100/0.1% sodium azide for between 3 hours and overnight. Then, 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 six 30-minute washes in PBS/0.5% Triton X-100/0.1% sodium azide, the tissue was incubated in the appropriate biotinylated secondary antibody overnight and then in streptavidin/ horseradish peroxidase (HRP) overnight and was developed with hydrogen peroxide and diaminobenzidine (DAB). Material processed with HRP/DAB was mounted in 50% glycerol. Sometimes, the mounting medium contained 0.1% nitroblue tetrazolium, and the HRP/DAB reaction product could be intensified photochromically by illuminating it with green light (530–560 nm; Vaney, 1992).

For double-labeled material, we used immunofluorescent goat secondary antibodies conjugated to fluorescein isothiocyanate (FITC) or indocarbocyanine (CY-3). Occasionally, if goat-derived primary antibodies were used for a double-labeling study, the tissue was blocked in 3% donkey serum, and the appropriate donkey secondary antibodies were used. Fluorescent preparations were mounted in 50% glycerol with 0.1% phenylenediamine to reduce fading. Specimens were viewed on an Olympus Vanox photomicroscope (Olympus, Tokyo, Japan) by using either blue or green epifluorescence. For double-label immunofluorescence, the green excitation was trimmed with a 530-nm long-pass filter, and the FITC emission was trimmed with a 520-nm interference filter to prevent cross-talk between the fluorochromes. For the double-labeling experiment with antibodies against calretinin and tyrosine hydroxylase (TOH), both primary antibodies were derived from...
rabbit. This material was processed sequentially with anticalretinin/CY-3 first, followed by anti-TOH/FITC. Processing in this order substantially reduced crossover labeling from the secondary antibodies, probably due to the higher dilution of the calretinin antibody.

For confocal microscopy, FITC and CY-3 were used as fluorochromes, and specimens were viewed on a Noran Odyssey with argon ion laser (488 nm and 529 nm lines; Noran Instruments, Inc., Middleton WI) or a Zeiss LSM410 with krypton-argon laser (488 nm and 568 nm lines; Zeiss, Thornwood, NY). Images of anti-PKC versus anticalretinin were obtained sequentially with the argon laser by using a ×63 objective (N.A. 1.4; 512 × 480 pixels, 0.18 μm/pixel; 488 nm, long pass 515 pixels; 529 nm, long pass 590 pixels). A series of five to ten images in 0.5-μm steps in the Z-axis were combined into one plane. These images showed crossover between the two channels for FITC and CY-3. This was corrected for by calculating the mean overlap from eight to ten single-labeled structures and subtracting this fraction from each channel (Breij et al., 1993). The crossover from FITC to CY-3 was estimated as 29%, and the crossover from CY-3 to FITC was estimated as 46%. This correction reduces the dynamic range of the image, so that the intensity levels were expanded to fill the full range of 255, and background intensity <20 was removed. The antiparvalbumin versus anticalretinin material was analyzed with the krypton-argon laser, and there was no crossover between channels. A single image was obtained at low power (×20 objective; 512 × 512 pixels; 488 nm, band pass 515–540 nm; 568 long pass 590 nm). Zeiss LSM software was used for the cluster analysis and masking (Fig. 9): No background was subtracted from this material. Images were processed in Photoshop by Adobe Iversion 2.5 or 3.0; Adobe Systems, Mountain View, CA) and printed on a Codonics 1600 color printer (Cordonies, Inc., Cleveland, OH).

Antibodies

Rabbit and 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 1:10,000. For Vibratome sections, dilutions of 1:50,000 or 100,000 were used. Mouse antiparvalbumin was obtained from Sigma Immunochemicals (St. Louis, MO) and was used at a dilution of 1:1,000. A mouse antibody to protein kinase C (PKC) was obtained from Amersham (Buckinghamshire, United Kingdom) and was used at a dilution of 1:100. Rabbit anti-TOH was a generous gift from Dr. J.W. Haycock and was used at a dilution of 1:6,000. All primary antibodies were aliquoted and stored at –20°C. Secondary antibodies, goat (or donkey) anti-mouse and goat (or donkey) anti-rabbit, biotinylated or conjugated with fluorochromes, were obtained from Jackson Immunochemicals (West Grove, PA) and were used at a dilution of 1:200.
Intracellular dye injection

Isolated pieces of live retina were mounted ganglion cell side up on a fixed stage microscope equipped with epifluorescence and viewed with a long working distance objective (×40; SLWD; Nikon, Tokyo, Japan), as described previously (Mills and Massey, 1992). 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, then neurobiotin was ejected with positive current (1 nA, 3 Hz) for 4 minutes. Several dye injections were made at different locations, and the retina was perfused for 30 minutes more to allow for cellular diffusion. 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 with HRP, FITC, or 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).

Quantitative analysis of double-labeled material

The goal of quantitative analysis is to compare the original image with pseudorandom images derived from the same data to show that an objective relationship occurs in the original image that is lacking in the pseudorandom images. In other words, it should be possible to differentiate between the real, original image and the pseudorandom images derived from it. One way to generate pseudorandom images for comparison is by rotating one label out of phase by 90°, 180°, or 270°. This is achieved readily by separating the color channels in Photoshop, which also can be used to calculate the overlap between two colors. Pixels of a given color were selected and then forced to saturation with the "fill" command to give a single uniform shade. When this was done for both labels, quantitative data for each label could be obtained from the "histogram" command, and double-labeled structures were seen as a third color that also could be counted.

In addition, calculations were run on a Silicon Graphics Workstation (SGI, Mountain View, CA) to compare the surrounding 3 × 3 kernel for every red (rod bipolar) pixel in the image. For each red pixel, a score of 4 out of 9 green pixels in the kernel was counted as a hit (i.e., evidence for adjacency), and the number of hits were accumulated for the whole image. Then, the green (AII) channel of the image was stepped out of registration by a variable integer, up to ±100 pixels in the X and Y dimensions. The kernel analysis around each red pixel was repeated, and the numbers of hits at each location were plotted as contours in the Z-axis against X and Y displacement. This operation was run for the original image and for one image with the labels out of phase by 180°. The results show a central peak, indicating a high level of adjacent labeling for the two antibodies, only for the original image (see Fig. 7).

RESULTS

Dye injection

For comparative purposes, a focal series through a dye-injected AII amacrine cell from the rabbit retina is presented in Figure 1. This cell was injected with Lucifer Yellow, which subsequently was photooxidized in the presence of DAB to yield an opaque product. The cell body is in the inner nuclear layer, adjacent to the inner plexiform layer, and several fine processes connect the soma to a spray of lobules 2–3 μm in diameter in sublamina a. A thick primary dendrite descends into the inner plexiform layer before branching into many tapering dendrites deep in sublamina b of the inner plexiform layer. This is the classic description of the mammalian AII amacrine cell, whereby the bistratified tree with a spray of lobules in sublamina a is diagnostic for this cell type (Famiglietti and Kolb, 1975; Vaney, 1985; Mills and Massey, 1991; Vaney et al., 1991). Although AII amacrine cells form numerous gap junctions with cone bipolar cells and other AII amacrine cells, only a single cell is recovered after the injection of Lucifer Yellow. Because horizontal cells show extensive dye coupling after the injection of Lucifer Yellow, apparently, there is a charge or size barrier for gap junctions of the inner retina. In contrast, dye injection with neurobiotin shows extensive coupling between AII amacrine cells and certain cone bipolar cells (Vaney, 1991; Mills and Massey, 1995).

Calretinin immunocytochemistry

At high concentrations, the calretinin antibodies stained a large number of retinal cells, including most amacrine cells, as reported previously by Vögeli et al. (1997). However, in wholemount retina labeled with a 10,000:1 dilution of antibody, a regular mosaic of amacrine cells was stained, with somata predominantly in the innermost row of the inner nuclear layer (Fig. 2). Shifting the focus to sublamina a shows that many of the cell bodies protrude into the inner plexiform layer, and some are displaced there. Each soma is surrounded by stained lobules, some of which can be traced easily to the stained cell bodies through fine processes (Fig. 2B). Occasionally, a neighboring pair of cells are stained; however, their dendritic tree branch in opposite directions, as reported previously for close pairs of AII amacrine cells in dye-injected material (Vaney et al., 1991). Deeper still, in sublamina b of the inner plexiform layer, a relatively dense meshwork of overlapping processes is stained (Fig. 2C). These are the morphological characteristics of AII amacrine cells.

Occasionally, in the process of surveying a wholemount rabbit retina containing approximately 500,000 AII amacrine cells, calretinin-positive amacrine cells were observed with all of the attributes of AII amacrine cells, except that the fine dendrites normally found in sublamina 5 of the inner plexiform layer ramified in the outer plexiform layer. It was possible to trace back from this unusual structure in the outer plexiform layer to an...
otherwise normal AII soma connected to lobules in sub-

lamina a by fine dendrites. These upside-down AII ama-
crine cells occurred infrequently, on the order of 10–20 per 
retina, mostly in the far periphery. Thus, we suggest that 
they probably are a minor developmental error rather 
than interplexiform cells or a novel cell type.

Population counts of calretinin-stained amacrine cells 
from two wholemount preparations yielded a peak density 
of 2,465 cells/mm² at the visual streak. At 10 mm eccentric-
ity in the inferior retina, the cell density fell to 728 
cells/mm². At 10 mm superior, the cell density was slightly 
lower at 616 cells/mm². These numbers compare closely 
with previously reported estimates of AII distribution 
(Mills and Massey, 1991; Vaney et al., 1991; Casini et al., 
1995; Strettoi and Masland, 1996) and suggest further 
that calretinin may be used to stain the population of AII 
amacrine cells selectively. The appearance of the calretinin-
positive cells in a vertical section is shown in Figure 3. In 
Vibratome sections, penetration of the antibody was im-
proved greatly, and many cells were stained at the dilu-
tions used for retinal wholemounts. However, AII ama-
crine cells were the most prominent, and they could be 
identified easily. Selectivity for AII amacrine cells was 
followed back to labeled cell bodies. Most labeled cells had 
very thick primary dendrites that branched laterally deep 
in sublamina b of the inner plexiform layer. In these 
vertical cross sections, the cells stained with the calretinin 
antibody displayed the well-known characteristics of AII 
amacrine cells.

The calretinin antibody also labeled cells in the ganglion 
cell layer and the overlying ganglion cell axons (Fig. 4), as 
also reported by Völgyi et al. (1997). The cells were 
identified as ganglion cells because axons could be ob-
served leaving the soma or a primary dendrite to join the 
fiber layer. A mixed group of ganglion cells was labeled 
with this antibody. At the two eccentricities shown in 
Figure 4, labeled cell bodies cover a range of sizes; how-
ever, some ganglion cells, observed by DIC optics, appar-
ently were unlabeled. This is not an artifact, because 
labeled and unlabeled cells may be adjacent, and the 
underlying meshwork of AII amacrine cell processes is 
stained evenly. It is possible that the calretinin antibody is 
selective for certain classes of ganglion cell; however, we 
did not pursue this issue further.

Double labeling

We chose to confirm the identification of calretinin-
labeled AII amacrine cells by examining the relationship 
with other well-described retinal neurons in the rod path-
way. If the calretinin antibody truly stains AII amacrine 
cells, then this should be reflected in the pattern produced 
in combination with other retinal markers.
Dopaminergic amacrine cells are a sparse group of wide-field amacrine cells that form a dense meshwork of fine dendrites, laden with varicosities, in sublamina 1 of the inner plexiform layer, adjacent to the inner nuclear layer. A few processes descend to sublaminae 3 and 5 of the inner plexiform layer to form lacy networks, but their density is far less than that of the matrix in sublamina 1.

AII amacrine coupling is modulated by dopamine (Hampson et al., 1992), and AII amacrine cells, which often protrude into the inner plexiform layer, are well known to fill the prominent rings in the matrix of dopaminergic amacrine cell processes (Türk and Stone, 1979; Voigt and Wässle, 1987; Casini et al., 1995). Thus, a double-labeling experiment with antibodies to TOH provides another way to test the selectivity of the calretinin antibody for AII amacrine cells.

In this material, shown in Figure 5, the somata of calretinin-positive amacrine cells can be seen occupying holes in the dopaminergic matrix. Furthermore, the calretinin-positive somata are ringed by closely apposed, TOH-positive varicosities, which Voigt and Wässle (1987) have shown to be sites of synaptic interaction, at least in the cat retina. Close examination of this material in the confocal microscope (not shown) shows that there are further TOH-positive varicosities associated with lobules in sublamina a that are stained for calretinin and connected to positively stained amacrine cell bodies by fine dendrites. The calretinin-positive amacrine cells bear stout, descending axons that also may be contacted by TOH-labeled varicosities deeper in the inner plexiform layer. These observations indicate that the calretinin antibody selectively labels AII amacrine cells and confirm that the major synaptic output of the dopaminergic amacrine cells is onto AII amacrine cells.

PKC

The dominant input to AII amacrine cells comes from rod bipolar cells. Therefore, we conducted a double-labeling experiment by using anticalretinin to label AII amacrine cells and an antibody to PKC to label rod bipolar cells. The confocal photomicrograph in Figure 6 is from a retinal wholemount with the focal plane at the level of the rod bipolar terminals in sublamina 5 of the inner plexiform layer. The large, round structures shown in red are the characteristic terminals of rod bipolar cells that descend to this level in the retina. The processes shown in green were labeled with anticalretinin, and they form the matrix of AII amacrine cells deep in sublamina b. The AII amacrine cell processes are associated intimately with the rod bipolar terminals. Every rod bipolar terminal is contacted by at least one green process, and the rod bipolar terminals often fill holes or branch points in the AII amacrine cell matrix. There are no rod bipolar terminals that are not contacted by a process from an AII amacrine cell. A quantitative analysis of this double-labeled material indicates that the association between rod bipolar terminals and the calretinin-positive processes far exceeds the level of coincidence (see below). This close association between rod bipolar cell terminals and the calretinin-positive processes was expected from previous work on the anatomy of the rod pathway, and it provides strong confirmation that the calretinin-labeled cells are indeed AII amacrine cells.

Quantitative analysis of rabbit retina double-labeled for PKC and calretinin

Given the number of rod bipolar terminals and the density of the AII amacrine cell meshwork in sublamina 5 of the inner plexiform layer, it is inevitable that there would be some coincidental overlap between the two...
Fig. 5. Double-label of wholemount rabbit retina with anti-tyrosine hydroxylase (TOH), visualized with fluorescein isothiocyanate (FITC), and anti-calretinin (CR), visualized with indocarbocyanine (CY-3). A: Focus in sublamina a adjacent to the inner nuclear layer, anti-TOH labels a single, large, dopaminergic amacrine cell and a matrix of fine dendrites in which rings of varicosities can be seen. Within each ring, faint crossover labeling of calretinin-positive amacrine cells may be observed due to the sequential use of two rabbit primary antibodies. B: In the same field shown in A, the mosaic of calretinin-positive amacrine cells that fill the rings in the dopaminergic matrix identifies these cells as AII amacrine cells. The arrowhead in each frame indicates an out-of-focus AII amacrine cell that is displaced to the inner plexiform layer.
immunocytochemical markers. Of course, the level of coincidental contacts will rise with the density of the two mosaics. Thus, the problem is to distinguish between random coincidence and the close targeted association, which indicates a true connection. Based on electron microscopic evidence, there is no doubt that AII amacrine cells receive input from rod bipolar cells, but how can this be extracted from the confocal photomicrograph in Figure 6? To simulate coincidental overlap, the calretinin image (originally shown in green) was rotated through 180° and merged with the PKC image for comparison with the original data. Labeled profiles were thresholded, and positive pixels were assigned to one of two gray levels to produce the black-and-white images shown in Figure 7. Rod bipolar terminals are shown as dark gray, and calretinin-labeled processes are shown as light gray outlined in black so that the overlap may be observed.

The resulting image in the original orientation is shown in Figure 7A; it is related closely to Figure 6, and there is a strong association between the rod bipolar terminals and calretinin-positive processes. The pseudoimage, with the calretinin-labeled AII matrix rotated 180°, is shown in Figure 7B. In this out-of-phase image, there still appear to be many contacts between rod bipolar terminals and AII amacrine cells processes, but this is at the level of coincidence. Compared with the real image in the original orientation (Fig. 7A), there is relatively little open space in the rotated image.

Close comparison of the real image and the pseudoimage does show some differences, even on a qualitative level. In the real image, there are much larger black areas with no label (Fig. 7A). This is because the green processes do not cover areas where there are no red terminals (or vice versa). In the pseudoimage, the open space is reduced by single-labeled processes that are not correlated with the other label. In the real image, one can find red terminals literally outlined in green. However, occasionally, similar things appear in the out-of-phase pseudoimage, presumably by chance. In the real image, the green processes sometimes appear swollen where they contact red bipolar terminals. This detailed level of intimacy is almost entirely missing in the pseudoimage.

Fig. 6. Confocal image from wholemount rabbit retina double-labeled with anti-calretinin (CR; green) and antiprotein kinase C (PKC; red). The focus is deep in the inner plexiform layer at the level of the rod bipolar terminals, which are well stained for PKC. The meshwork of calretinin-positive profiles is associated intimately with the rod bipolar terminals; every rod bipolar terminal contacts calretinin-labeled processes. This indicates that the calretinin antibody labels AII amacrine cells. See text and Figure 7, which establish that the association between the two antibody labels is much greater than random.
Quantitatively, all of the red terminals (110 of 110) appear to be contacted by green processes in the real image. In the pseudomage, only 79 of 110 rod bipolar terminals were contacted by green calretinin-positive processes, and it is easy to find red terminals on their own. However, there still is a great deal of overlap between the two labels. Because this image is 180° out of phase, this is coincidence. Of course, the level of coincidental hits will rise with the density of the two mosaics. Actually counting the label overlap (by counting pixels that are red and green) shows that the pseudomage has only half as much overlap as the real image.

We also determined the number of green pixels in a $3 \times 3$ kernel surrounding every red pixel, taking 4 as a hit, and then stepped the green image away from the red image by a variable number of pixels (maximum 100; 1 pixel = 0.2 µm). The spatial correlation between rod bipolar terminals and the AII amacrine cell matrix. Registration is defined as four green pixels within a $3 \times 3$ kernel around each red pixel. The total number of hits for the whole image is plotted against translation of one image against the other in X and Y. The plots were derived from the images in A and B. C: The two immunolabels show a large peak in the center of the plot that falls off rapidly to a random level as one image is translated relative to the other. This indicates a close spatial relation between the two labeled profiles, which is consistent with synaptic contact. D: The same operation performed when the calretinin image was rotated by 180°. Now, there is no peak in the center of the plot; the association between the two immunolabels is no greater than chance.
µm) in both the X and Y dimensions. The hits were plotted as contours over the X-Y translation, and the operation was repeated on the pseudoimage. The real image had a pronounced peak, approximately three times background, which fell off as the images were moved out of registration (Fig. 7C). The half-width at half-peak height was 6 µm, which is a measure of the small movement required to destroy image registration. This indicates a real association between the two sets of labeled profiles. The out-of-phase image had no such peak (Fig. 7D). In other words, the correlation between the two labels in the pseudoimage is no greater than random chance. This analysis shows that we are able to differentiate objectively between the random associations in a pseudoimage and the real contacts in the original image, which indicate synaptic connections between PKC-labeled rod bipolar terminals and calretinin-labeled AII amacrine cell dendrites.

**Parvalbumin**

Antibodies to parvalbumin have been reported to stain AII amacrine cells in both the rat and rabbit retina (Wässle et al., 1993; Casini et al., 1995). Therefore, we conducted double-labeling experiments with antibodies to parvalbumin and calretinin to examine the correlation between the two staining patterns. Figure 8 shows an example taken from midinferior rabbit retina stained with goat anticalretinin (1:5,000) and mouse antiparvalbumin (1:1,000). The anticalretinin image, visualized with CY-3, is shown in red, and the antiparvalbumin image, tagged with FITC, is shown in green. Yellow profiles indicate colocalization.
The most prominent cells were double labeled and appear yellow, which indicates staining for calretinin and parvalbumin. Of 102 calretinin-positive cells, 102 (100%) were double labeled for parvalbumin. These cells were easily identified as AII amacrine cells by their morphology. The double-labeled somata formed a regular mosaic, with only one pair of adjacent cells in this field, and each cell was connected to lobules in sublamina a of the inner plexiform layer. The dendrites in sublamina b also were double labeled, but the calretinin staining was notably brighter in this region. There are essentially no red somata in this frame, because all of the bright calretinin-labeled AII amacrine cells were double labeled for parvalbumin and, thus, appear yellow. The background staining for calretinin was dim; however, a few other somata were visible lightly at this antibody dilution. However, these unidentified amacrine cells were discriminated easily from the brightly labeled AII amacrine cells. In addition, only the right calretinin-positive cells were double labeled for parvalbumin.

The parvalbumin antibody stains other amacrine cells in addition to AII cells. Of 120 parvalbumin-positive cells, 108 (85%) were double labeled for calretinin and, thus, are colored yellow. These cells had the characteristics of AII amacrine cells, as reported previously by Casini et al. (1995). In this field, an additional 18 cells (15%) were labeled only for parvalbumin (shown in green), some of which are as bright as AII amacrine cells in the parvalbumin image. These non-AII cells often make adjacent pairs with AII amacrine cells.

This double-labeled material was analyzed further, as illustrated in Figure 9. The separate gray-scale images (which were combined and colored to make Fig. 8) are shown for anticalretinin (Fig. 9A) and antiparvalbumin (Fig. 9B). The scattergram of green versus red intensity for each pixel in Figure 9E clearly shows two limbs (green only along the Y-axis and double labeled at 45°). This indicates the presence of at least two separate populations of cells. There is an additional small cluster (low intensity, red only) along the X-axis close to the origin.

Obvious clusters of cells with similar red and green intensity were selected by eye. The double-labeled cells form a cluster of variable intensity at 45°. Selecting the pixels within the ellipse and masking the remaining cells produced the image in Figure 9C. These cells are all calretinin- and parvalbumin-positive; they are AII amacrine cells and correspond to the yellow double-labeled cells in Figure 8. This image closely resembles the calretinin-only image (Fig. 9A), which suggests that the calretinin antibody is virtually specific for AII amacrine cells. Selecting the cells within the ellipse by the green axis revealed a second population of cells (shown in Fig. 9D). These cells are parvalbumin-positive but not calretinin-positive. This image indicates the amacrine cells, in addition to AII cells, which are parvalbumin-positive and correspond to the green cells in Figure 8. Most of the adjacent pairs in the parvalbumin image (Fig. 9B) contain one of these non-AII amacrine cells. They may be subdivided further into two types on the basis of intensity. One type of cell was stained as brightly as AII amacrine cells. These cells are wide-field amacrine cells and are less numerous than AII amacrine cells, with primary dendrites deep in the inner plexiform layer, similar to a subset of parvalbumin-stained amacrine cells in the rat retina (Wässle et al., 1993). Another population was labeled more lightly, but these amacrine cells are unidentified at this time. Finally, selecting the small ellipse close to the origin on the red axis revealed a population of amacrine cells that was calretinin-positive but not parvalbumin-positive (Fig. 9F). These cells were few in number, poorly labeled, and difficult to differentiate from the background. They were discriminated easily from AII amacrine cells in the calretinin-only image. Again, this suggests that the calretinin antibody is relatively selective for AII amacrine cells in the rabbit retina.

**DISCUSSION**

**Identity of calretinin-positive amacrine cells in rabbit retina**

At an appropriate dilution, the calretinin antibodies used in this work labeled a distinct mosaic of amacrine cells. These cells were identified as AII amacrine cells by several complementary methods. First, the labeled cells have the well-known morphological features of AII amacrine cells, i.e., a bistratified amacrine cell with lobules in sublamina a and smooth dendrites in sublamina b that form an overlapping matrix. Second, the calretinin-labeled cells formed a regular mosaic with a density that matches previous reliable estimates of the AII amacrine cell density (Mills and Massey, 1991; Young and Vaney, 1991; Casini et al., 1995; Strettoi and Masland, 1996). Occasionally, adjacent pairs of calretinin-labeled cells were observed, but both cells could be identified as AII amacrine cells at high magnification. Usually, the primary dendrites of each pair member ran in opposite directions, as reported previously for dye-injected examples of adjacent AII amacrine cells (Young and Vaney, 1991). Third, calretinin-labeled cells filled the rings in the dopaminergic amacrine cell matrix, and they were surrounded by dopaminergic varicosities, as described previously for AII amacrine cells (Voigt and Wässle, 1987; Casini et al., 1995). Fourth, the calretinin-labeled cells formed a close association with rod bipolar terminals deep in sublamina b of the inner plexiform layer. This is the site of rod bipolar input to AII amacrine cells, and it is consistent with calretinin-labeling of the AII amacrine cell matrix. Fifth and finally, calretinin was
colocalized with parvalbumin, which has been described previously as labeling AII amacrine cells in the rabbit retina (Casini et al., 1995). Other non-AII cells labeled for parvalbumin were not labeled with the calretinin antibodies. In summary, the pattern of labeling obtained with calretinin antibodies is consistent with the distribution, morphology, and synaptic connections of AII amacrine cells in the rabbit retina.

In our hands, antibodies to calretinin may be used to label the AII amacrine cell population selectively; and, recently, we have used these antibodies in double-labeling procedures to show that AII amacrine cells express the glycine transporter (Koomen et al., 1996). However, the selectivity is partially dependent on the dilution of the primary antibody. At higher concentrations of primary antibody, many other unidentified amacrine cells were labeled in the rabbit retina, as reported previously by Völgyi et al. (1997). Those authors used the Chemicon rabbit anticalretinin antibody at a dilution of 1,000:1 on frozen sections and reported that calretinin-positive cells were three times as numerous as parvalbumin-stained amacrine cells. On the basis of morphology and colocalization with parvalbumin, some of the calretinin-labeled cells were identified as AII amacrine cells; however, clearly, many other amacrine cells also were stained (Völgyi et al., 1997). In the cat retina, the same calretinin antibody at a dilution of 500:1 stained photoreceptors, horizontal cells, and many amacrine cells, including AII cells (Goebel and Pourcho, 1997). We found that antibody concentration was critical for obtaining specificity for AII amacrine cells. This was especially true in vertical sections in which dilutions up to 100,000 were required to improve selectivity for AII amacrine cells. This finding may be explained partly by improved penetration in the sectioned material. In whole-mount material, a dilution of 10,000:1 selectively labeled AII amacrine cells (Figs. 2, 6). The calretinin antibodies also labeled certain ganglion cells (Fig. 4), but these were differentiated easily from AII amacrine cells, because the somata were located in the ganglion cell layer. Although there is considerable species variability in the staining patterns of calcium-binding proteins, calretinin antibodies also may be used to label AII amacrine cells in the primate retina (Wässle et al., 1995; Zhang et al., 1996; Mills and Massey, 1999).

Comparison between calretinin and parvalbumin labeling

From double-labeling experiments, the calretinin antibodies appeared to be more selective for AII amacrine cells than the parvalbumin antibody, which also labels a population of non-AII cells in rabbit and rat retinæ (Figs. 7, 8; Wässle et al., 1993; Casini et al., 1995; Strettoi and Masland, 1996). Casini et al. (1995) also noted the presence of a small population (4%) of non-AII amacrine cells that were stained for parvalbumin. In the analysis presented in Figures 7 and 8 from midperipheral inferior retina, we estimated that 15% of the parvalbumin-labeled amacrine cells were non-AII cells. This minor difference could be accounted for by regional variation in the distribution of parvalbumin-labeled cells. Strettoi and Masland (1996) preferred to use parvalbumin rather than calretinin to label AII amacrine cells, but they also noted that other unidentified amacrine cells were stained lightly for parvalbumin and suggested that this was a source of variability in counting the AII population. Theoretically, the calretinin antibody should be preferred for labeling AII amacrine cells selectively; however, in practice, the parvalbumin antibody shows better penetration in central retina and, because it is a mouse monoclonal, will be useful for double-labeling experiments. The availability of calretinin primary antibodies raised in both rabbit and goat also will facilitate double-labeling strategies.

Use of confocal microscopy to visualize putative connections

It is often impossible to look at a single image and be sure that there is a relation between two labels. For example, most observers who were shown the AII/rod bipolar terminal images (Figs. 6, 7) were content with the apparent level of association in the out-of-phase image alone, although they were less so compared with the real image. Much of this problem comes from the high density of the two labels, which leads to frequent coincidental overlap. The difficulty in extracting a spatial relationship may seem counterintuitive. In fact, our visual systems function as pattern detectors to such an extent that a false relationship may be extracted from random signals, where no real pattern exists. This inherent bias can be overcome by a statistical analysis of the image. If there is a true difference between a given real image and a random superposition, then we should be able to demonstrate the difference objectively. One way to do this is to compare the contacts in the original image with random images generated by rotating one label out of phase by 90°, 180°, or 270°. This strategy has been employed successfully in at least three examples from retinal circuitry: 1) to demonstrate the input from cholinergic amacrine cells to a ganglion cell in the cat retina (Vardi et al., 1989); 2) to indicate inputs to ON parasol ganglion cells in primate retina (Jacoby et al., 1996); and 3) as evidence for input from calbindin cone bipolar cells to ON ganglion cells in the rabbit retina (Massey et al., 1996). In addition, we have analyzed the rod bipolar/AII material to give a measure of adjacency or a spatial relationship.

The use of selective markers for both rod bipolar cells and AII amacrine cells enabled us to label an entire field of potential contacts. In the case of rod bipolar input to the fine dendrites of AII amacrine cells, for which there is already ample and definitive electron microscopic evidence (Dacheux and Raviola, 1986; Raviola and Dacheux, 1987; Strettoi et al., 1990), our analysis showed that the level of association between the labeled profiles was approximately three times higher than when the independent labels were moved out of registration. The central peak indicating registration in the real image was missing when one label pattern was rotated by 180° (Fig. 8). There was no association greater than random chance for any displacement value in the images rotated out of phase. Thus, we believe that this confocal analysis indicates a connection between rod bipolar cells and the calretinin-labeled dendrites of AII amacrine cells. It follows from these data that calretinin may be used to label the matrix of AII dendrites.

Confocal analysis of putative connections in retinal circuitry undoubtedly will prove useful in further studies of this type. Significant time savings can be realized by minimizing the need to embed and section material. At the very least, confocal microscopy can be used to identify promising potential candidates for further analysis with electron microscopy. Failure to demonstrate contacts between two independent labels would seem to preclude
synaptic input. However, it is also dear that confocal microscopy has its limitations. This type of quantitative assessment may fail in a case in which there is a low density of synaptic input and a high density of nonsynaptic dendrites or for a diffuse neuron with multiple postsynaptic targets. In both cases, a small signal would be lost against a high background of random noise. Definitive evidence for synaptic input can be gained only from electron microscopy. Synapses cannot be observed in confocal images, and the direction of synaptic transmission is unknown. Nevertheless, it is possible to use confocal microscopy to uncover a quantitative spatial relationship between labeled profiles. Objective quantitative analysis greatly strengthens the casual observation of processes that appear to touch. Invagination or the swelling of apposed terminals between two independently labeled cell types also strongly suggests synaptic contact. However, the possibility of serial synapses or common sites of input/output should be borne in mind. We also are aware of heterologous cell types that are tracer coupled, apparently through gap junctions, e.g., AII amacrine cells and cone bipolar cells. When electron microscopy is used to demonstrate the nature of the usual contact, this type of confocal analysis also can be useful for producing boundary estimates of synaptic convergence or divergence.

ACKNOWLEDGMENTS

We thank W.S. Sunny Liu and Andrew Zych for their contributions to this project and Ulrike Grünert for helpful comments. Andrew Zych developed the computer algorithm to compare alignment in double-labeled images. This work was supported by grants from the National Institutes of Health (EY65015 to S.C.M., EY10121 to M.S.L.), and from Research to Prevent Blindness (an unrestricted award to the Department of Ophthalmology and Visual Science and the Dolly Green Special Scholar Award to S.L.M.).

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