Cone Photoreceptor Recovery after Experimental Detachment and Reattachment: An Immunocytochemical, Morphological, and Electrophysiological Study

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Purpose. To compare the morphologic and functional recovery of the retina after detachment and reattachment in an animal with a cone-dominant retina, the ground squirrel.

Methods. Ground squirrel (*Spermophilus beecheyi*) retinas were detached for 1 day and reattached for 7, 35, or 96 days ($n = 2$, each time point). Flicker ERGs were recorded 1 day after the detachment and at various times after reattachment. Contrast-response functions were measured for isochromatic modulation and for selective modulation of short-wavelength–sensitive (S) and middle-wavelength–sensitive (M) cones. At the end of the experiment, retinas were prepared for light microscopy or immunocytochemical staining with antibodies to rod opsin, S and M cone opsins, cytochrome oxidase, synaptophysin, glial fibrillary acidic protein (GFAP), cellular retinaldehyde–binding protein (CRALBP), interphotoreceptor–binding protein (IRBP), and peanut agglutinin lectin (PNA). Photoreceptor density maps were created from wholemount preparations labeled with biotinylated PNA and anti–S cone opsin. Cell counts of photoreceptor nuclei and cone outer segments (OS) were compared with flicker ERG data. Cell death was examined by the TUNEL method.

Results. Reattachment stopped photoreceptor cell death and reversed the disruption of interphotoreceptor matrix as well as the redistribution of Müller cell proteins. It also activated some astrocytes based on anti-GFAP staining. S- and M-cone OS showed a gradual recovery in length after reattachment, and this recovery continued to the longest time points examined. ERG contrast gains also recovered after reattachment, but these reached asymptotic levels by approximately a week after reattachment. There were significant correlations between outer nuclear layer (ONL) cell counts and ERG contrast gains. No differences were noted in the indices of recovery of M and S cones.

Conclusions. The ERG can be used to follow specifically the changes in the retina that occur after retinal detachment and reattachment. (*Invest Ophthalmol Vis Sci.* 2003;44:416–425) DOI:10.1167/iovs.02-0633

It has been suggested that retinal detachment has a greater morphologic impact on cones than on rods,[1] and it has been demonstrated in human patients that macular detachment has more severe consequences for visual outcome than does peripheral detachment.[2–6] These studies suggest that visual dysfunction and specific color vision defects may persist when the macula is involved, even though anatomic reattachment is successful. Tritan color vision defects occur frequently, suggesting that there is an enhanced susceptibility of S cones and/or of the S-cone signaling pathways.[7–9] Recent clinical studies have demonstrated that cone photopigments show a slow recovery after reattachment and that visual acuity supported by the reattached retina may continue to improve over the long term.[10,11] The recovery processes of cones and cone-based vision after retinal reattachment in humans remains poorly understood. Examining the detailed properties of cones after reattachment requires the study of animal models in which detachment and reattachment times can be determined precisely and in which detailed cellular studies can be performed in concert with electroretinogram (ERG) recordings. Studies of cats and rabbits have been valuable, but their disadvantage is that these species have only a modest number of cones widely scattered across the retina.[1,12–14] In this study, we used a detachment–reattachment model developed by using the cone-dominant ground squirrel retina. Many studies have shown that this species provides an excellent model for studying cones and cone vision.[15–22]

Recent animal studies examining the rescue of photoreceptor cells have mainly focused on the rescue of rods.[23–27] This is due, at least in part, to the interest in retinitis pigmentosa (RP), in which cell death occurs in rod photoreceptors early in the disease. Although cone cell death appears to be a secondary event in RP, rescuing cones is critical for visual function in retinal disease, including RP, age-related macular degeneration (AMD), and retinal detachment. In previous studies, histopathology and electrophysiology have both been used as means to assess rescue of photoreceptors in animal models.[25,26] Therefore, in the present study, we used both assays to assess the recovery of cones after damage caused by retinal detachment.

Material and Methods

All experimental procedures were designed to conform to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and to protocols approved by the Institutional Animal Care and Use Committee of the University of California Santa Barbara.

Retinal Detachments and Reattachments

Retinal detachments were created in the right eyes of adult California ground squirrels (*Spermophilus beecheyi*) of both sexes by infusing a

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balanced salt solution (BSS; Alcon, Ft. Worth, TX) slowly between the neural retina and the retinal pigment epithelium through a glass micropipette. The position and the size of each detachment were recorded at the end of each surgery. One day after the detachment, reattachments were performed by the injection of 20% sulfur hexafluoride (SF6) in filtered room air into the vitreous cavity. Intraocular pressure, before and after the gas injection, was controlled by tapping the anterior chamber with a 30-gauge needle. Successful reattachment was confirmed at the time of death by observation of the retina through a dissecting microscope. Control eyes and eyes injected with 20% SF6 in room air were used as the control ($n = 2$ for each).

Tissue Preparation

Seven, 35, and 96 days ($n = 2$, each time point) after reattachment surgery, the animals were killed with an overdose of pentobarbital sodium (120 mg/kg), and the eyes were enucleated and immersion fixed for 10 minutes in 4% paraformaldehyde in sodium cacodylate buffer (0.1 N; pH 7.4). The cornea and lens were then removed, and the eyecup was cut in half. One half of the eye was stored in the fixative solution. From this sample, small areas of retina were excised and embedded in low-melting-point agarose for immunocytochemical analysis by confocal microscopy. The other half was immersion fixed in 1% glutaraldehyde and 1% paraformaldehyde in sodium phosphate buffer (0.086 M; pH 7.3) overnight at 4°C for high-resolution transmitted light or ultrastructural analysis. The tissue was then fixed in phosphate-buffered osmium tetroxide (2%) for 1 hour and embedded in Spurr resin.

Immunocytochemistry

For confocal analysis, 100-μm-thick agarose-embedded sections were cut on a microtome (Vibratome; Technical Products International, Polysciences, Warrington, PA) and blocked overnight at 4°C in normal donkey serum (1:20) containing 0.1 M phosphate-buffered saline (PBS), 0.5% bovine serum albumin (BSA; Fisher Scientific, Pittsburgh, PA), 0.1% Triton X-100 (Roche Molecular Biochemicals, Indianapolis, IN), and 0.1% sodium azide (Sigma, St. Louis, MO), together referred to as PBTA. The next day, the sections were incubated in primary antibodies overnight at 4°C on a rotator. The sections were then rinsed in PBTA and incubated in donkey anti-mouse or anti-rabbit IgG conjugated to the fluorochrome Cy2, Cy3, or Cy5 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) overnight at 4°C on a rotator. Finally, the sections were mounted in 5% *n*-propyl gallate in glycerol and viewed on a laser scanning confocal microscope (model 1024; Bio-Rad, Hercules, CA). All antibody solutions were made in PBTA.

The primary antibodies used in this study were a mouse monoclonal antibody to rod opsin (Rho4D2, 1:50; provided by Robert Molday, University of British Columbia, Vancouver, British Columbia, Canada), two rabbit polyclonal antisera to short wavelength–sensitive (S) and medium/long wavelength–sensitive (M/L) cone opsins (JH455 and JH492, both 1:1,000; provided by Jeremy Nathans, The Johns Hopkins Medical School, Baltimore, MD), two mouse monoclonal antibodies to S and M/L cone opsins (OS-2 and COS-1, 1:10,000 and 1:1,000, respectively; provided by Ágoston Szél, Budapest, Hungary), a mouse monoclonal antibody to cytochrome oxidase (1 μg/mL; Molecular Probes, Eugene, OR), a rabbit polyclonal antibody to synaptophysin (1:100; Dako, Carpinteria, CA), a rabbit polyclonal antibody to glial fibrillary acidic protein (GFAP, 1:400; Dako), and a rabbit polyclonal antiserum to cellular retinaldehyde binding protein (CRALBP, 1:400) and a mouse monoclonal antibody to interphotoreceptor retinoid-binding protein (IRBP, 1:400; both provided by John Saari, University of Washington, Seattle, WA). In addition, some sections were stained with biotinylated peanut agglutinin (PNA) lectin (400 μg/mL; Vector Laboratories, Burlingame, CA). Outer segment lengths were measured from confocal images of sections stained with opsin antibodies with image-analysis software (ImageTool; http://ddsdx.uthscsa.edu/dig/itdesc.html, provided in the public domain by University of Texas Health Science Center, San Antonio, TX). Sampling areas were determined on the basis of the detachment map. The region of retina containing the highest detachment was used for comparison between different time points.

Light Microscopic Analysis

For light microscopy, two different eyes for each time point were embedded in resin, divided into four regions extending from superior to inferior retina, sectioned at 1 μm, and stained with toluidine blue. Outer nuclear layer (ONL) cell counts from the entire retinal section were summed and the numbers normalized to cells per millimeter.

Retinal Wholemount Immunocytochemistry

Four eyes (one normal eye and three eyes with 1-day detached/35-day reattached retinas) were used to prepare retinal wholemounts. After the eyes were fixed, the cornea and lens were removed, and the retinas were carefully dissected from the eyecup and divided into quadrants by using the optic nerve head as a landmark. In fixed ground squirrel retinas, the retinal pigment epithelium (RPE) adheres firmly to the photoreceptor outer segments (OS) and cannot be removed without causing damage to the photoreceptors. To visualize the retina more clearly, the pigment granules in the RPE were bleached by incubating the retina for 1.5 hours into a solution of 10% H_2O_2 in PBS with 1 drop of a 29% ammonia solution per 10 mL.[28] After bleaching, the tissue was thoroughly washed in PBS. Retinal wholemount immunocytochemistry was then performed by first incubating the retinas with blocking solution for 6 hours and then staining with PNA (which stains all cones) and anti-S opsin (both 1:100,000) for 3 days. After the incubation, specimens were washed in PBTA and then incubated in a mixture of streptavidin-Cy3 and anti-rabbit IgG conjugated to the fluorochrome Cy2. The flatmounts were rinsed in PBTA and mounted in 5% *n*-propyl gallate in glycerol with the photoreceptor side up.

The wholemounted retinas were sampled along the vertical meridian at 1- or 2-mm intervals. At each of the selected retinal locations, confocal micrographs were taken with a 60× oil-immersion objective lens. The number of anti–S cone opsin and PNA-labeled cones were counted in fields 181.2 × 181.2-μm from confocal images either manually or automatically, using the image-analysis software (Image Tool). The number of M cones was obtained by subtraction of anti-S opsin–stained cells from those stained with PNA. These local densities were converted to cones per square millimeter at their respective positions on the wholemounted quadrant. Isodensity contours were fitted to the data and coded by a gray scale so that regions of highest density were black, with areas of decreasing density appearing in increasingly lighter shades of gray. The total number of cells was calculated by multiplying the mean density of each data point on the retina.

ERG Measurements

ERG measurements were made by using procedures that have been fully described.[22] In brief, ground squirrels were anesthetized with an injection of a mixture of ketamine (70 mg/kg) and xylazine (9 mg/kg), and the pupil of the test eye was dilated by topical application of atropine sulfate (0.04%) and phenylephrine hydrochloride (10%). The squirrel was positioned on a heating pad in a head holder. ERGs were differentially recorded with bipolar contact lens electrodes. A ground electrode was placed in the mouth. All recordings were made in an illuminated room (illuminance at the test eye was 150 lux). The recordings were performed in each animal at least 1 week before the detachment, 1 day after the detachment (just before the reattachment), and at various time points after the reattachment.

The stimulus was a spatially uniform field (116° × 101°), square-wave modulated at a rate of 37.5 Hz. Based on earlier measurements of the absorption properties of the two cone pigments in this species, the stimuli were designed to modulate the contrast seen by either the S or M cones or both of them together (isochromatic).[22] Two advantages of this stimulus arrangement are that the subject is held in a constant state of chromatic adaptation because the time-averaged luminance and chromaticity do not vary, and the use of cone-isolating stimuli allows discrimination of signals from single cone classes, an end that cannot be reliably accomplished with monochromatic or any other filter-produced spectral stimuli. Stimuli were generated on a computer-controlled color monitor (average luminance, 50 cd/m^2; Intellicolor 20-in. monitor, model 0461; Radius, San Jose, CA). The refresh rate of

the monitor was 75 Hz. The experimental control software was written in a commercially available program (MatLab; The Mathworks, Natick, MA; using the extensions provided by high-level Psychophysics Toolbox and low-level Video Toolbox.[29,30]) Analog hardware was used to window the amplified ERG signal with a sinusoid set to the fundamental frequency of the stimulus (37.5 Hz). This response was averaged over 50 stimulus presentations at each stimulus contrast, and five such averages were compiled at any given stimulus contrast. For each direction of modulation (i.e., S, M, isochromatic) we varied stimulus contrast from the highest obtainable (60% to 70%, depending on the direction of modulation) down to contrasts that led to minimal ERG responses (1% and less).

Results

Cone Recovery after Reattachment

After retinal detachment, the onset of degenerative changes in the ground squirrel retina is quite rapid. In this retina the JH492 antiserum to M/L cone opsin labels both S and M cones.[31] Therefore, we used the mouse monoclonal OS-2 antibody to S opsin, in a series of double-labeling experiments with the JH492 antibody, to compare simultaneously the OS regenerative capacity of the individual cone classes after reattachment (Figs. 1A, 1B). The results revealed similar trends in OS regeneration in the two cone classes (Figs. 1C, 1D).

Moreover, M- and S-cone OS length measurements show that the OS, which were shortened by detachment, recovered progressively after reattachment (Fig. 2C). At 96 days after reattachment, both M- and S-cone OS had reached approximately 70% of their normal lengths.

In the ground squirrel, the amplitude of the flicker ERG increases linearly with increasing contrast. Figure 2A shows the average M-cone contrast–response function obtained from a large sample ($n = 40$) of normal ground squirrels. The slope of the fitted line defines contrast gain for this condition. The variation across subjects was relatively small. Responses obtained from S-cone isolation exhibited a similar relationship but with a much lower contrast gain, presumably reflecting the great disparity in the relative numbers of M and S cones in the ground squirrel retina.[31] Retinal detachment reduces the amplitude of the ERG and lowers contrast gain. Figure 2A illustrates this fact with results obtained from six animals 24 hours after retinal detachment.

Figure 2B plots the contrast gains as a percentage of their predetachment levels at 24 hours after detachment and then at three time points after reattachment. As explained, detachment reduces contrast gain: In these six animals the M-cone contrast gain was approximately 32% of the normal, whereas the same detachment yielded an S-cone contrast gain that was approximately 53% of normal. Seven days after reattachment contrast gain had increased for both S and M cones (Fig. 2B). Beyond that point and up to more than 3 months, there were no clearly consistent changes in contrast gain for either cone class in this sample. For these animals the asymptotic values averaged approximately 59% of normal for the M cone and approximately 79% for the S cones. The two important outcomes of this experiment are that recovery as indexed by the ERG seemed nearly complete at approximately 1 week after reattachment and that there was no indication of a difference in the recovery of ERGs from isolated S and M cones.

Correlations between Photoreceptor Histology and Electrophysiology

In earlier experiments on the ground squirrel, we found that the magnitude of the loss of ERG signal engendered by detachment was proportional to the total number of photoreceptors in the region of the detachment.[22] To examine this relationship in the reattached retina we compared the ERGs recorded from five squirrels (four taken from the previous experiment and an additional animal) to cell counts in the ONL. Because there were regional differences in the extent of photoreceptor degeneration after detachment and reattachment ONL cell counts were summed along a strip of tissue running vertically across the retina, so as to bisect the detachment zone (as illustrated in

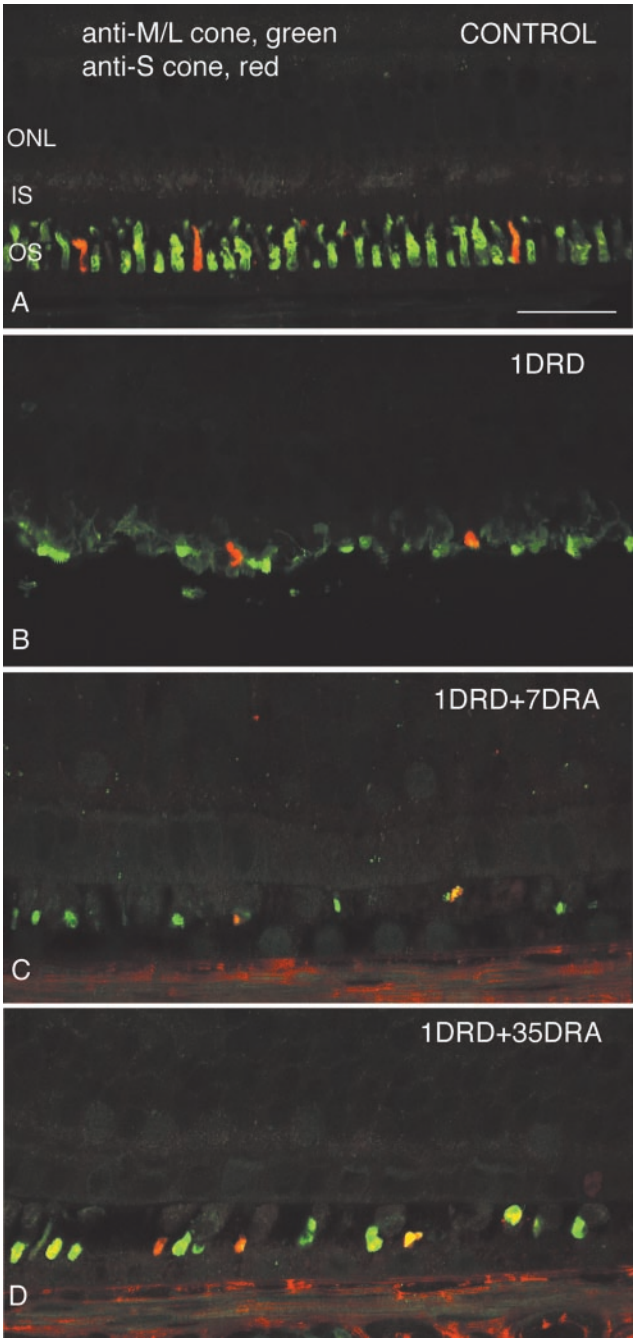

Figure 1. Laser scanning confocal micrographs showing the immunolabeling pattern for the antibody to M/L-cone opsin (anti-JH492; *green*) and S-cone opsin (anti-OS-2; *red*) in (**A**) control, (**B**) 1-day detached (1DRD, (**C**) 1-day detached/7-day reattached (1DRD+7DRA), (**D**) and 1-day detached/35-day reattached (1DRD+35DRA) retinal sections. This M/L-opsin antibody labels all cone OS in ground squirrels making the S-cone OS appear *yellow-red*. Cone OS were extensively disrupted at 1 day of detachment (**B**), were still short in 1-day detached/7-day reattached retinas (**C**), and became longer, but did not return to normal length, in 1-day detached/35-day reattached retinas (**D**). Note that there is a similar trend in the recovery of the length of M- and S-cone OS after reattachment. Bar, 20 μm.

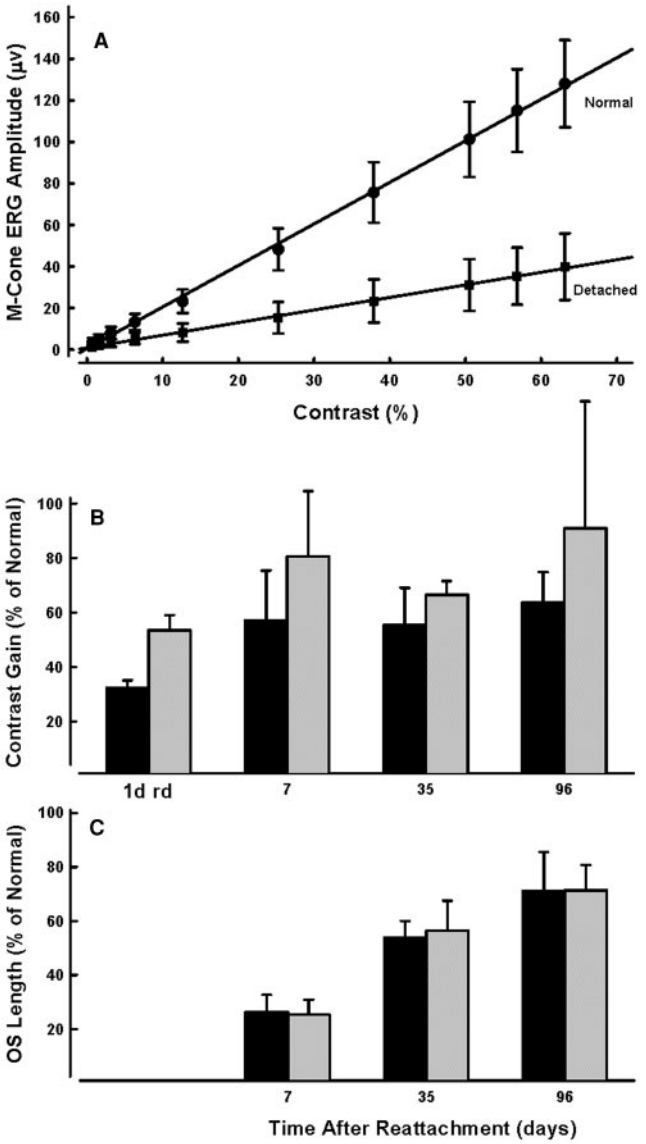

FIGURE 2. (**A**) Average M-cone contrast response functions obtained from ERG recordings in 40 normal ground squirrels and 6 squirrels in which the retina had been experimentally detached 24 hours before the recording. Data are the mean ± SD). In each case, the data have been best fit with linear regressions. The slope of the line defines contrast gain. (**B**) Contrast gain measurements in six squirrels at 1 day after retinal detachment and at three intervals after reattachment with results from two animals at each of the latter points. Results are plotted relative to control animals similarly tested and are shown separately for (▨) S- and (■) M-cone–isolating stimuli. (**C**) Measurements of outer segment lengths obtained from surviving S- and M-cones at three periods after retinal reattachment. Cone types are coded as in (**B**).

Fig. 3A). These counts were obtained from a control animal and from the five experimental animals. So estimated, ONL cell counts covered a range from approximately 50% of the normal to a value actually slightly greater than that obtained from the control (Fig. 3B). Because the identity of the cone types could not be ascertained in these counts, they are most appropriately compared with contrast gain values obtained for isochromatic modulation. As can be seen in Fig. 3B, these two measures were linearly related—that is, the greater the number of ONL cells in these reattached retinas the higher the ERG contrast gain.

In the sectioned material it was difficult to obtain an overview of the effect of detachment–reattachment on the photoreceptor cells. Therefore, we prepared wholemounts of samples from a control animal and four animals with 1-day detached/35-day reattached retinas. In this group of animals, an attempt was made to produce detachments of similar sizes and locations. All cone OS were labeled with the lectin PNA and the S cones with the antibody to S cone opsin. The number of M cones was determined by subtraction of anti-S cone– from PNA-stained cells. Figure 4 demonstrates the density maps of M- and S-cone populations from control (Fig. 4A) and reattached retinas (Figs. 4B–D). The control retina contained a total of approximately 6.7 million cones, a value similar to that previously reported for this species.[31] The map of M cones from this retina shows clearly an area of highest density that defines a horizontally oriented visual streak. In contrast, S-cone density in the control retina was flat across most of the retina, also as shown previously.[31] The M cone maps of the reattached retinas from three different eyes displayed similar patterns. In each case they had a continued disruption of the normal pattern with "holes" in the inferior retina. Although some variation occurred, there was remarkable consistency in the appearance of the gradients around these holes in cone density in the inferior retina. S-cone maps of reattached retina showed the lowest-density areas to be located in the inferior retina, consistent with the holes observed on M-cone maps.

Figure 5 compares the cell counts and ERG data obtained from these three animals with those of the control. There were no significant differences between the contrast gain values and photoreceptor cell counts for either the S-cone (t = 0.928;

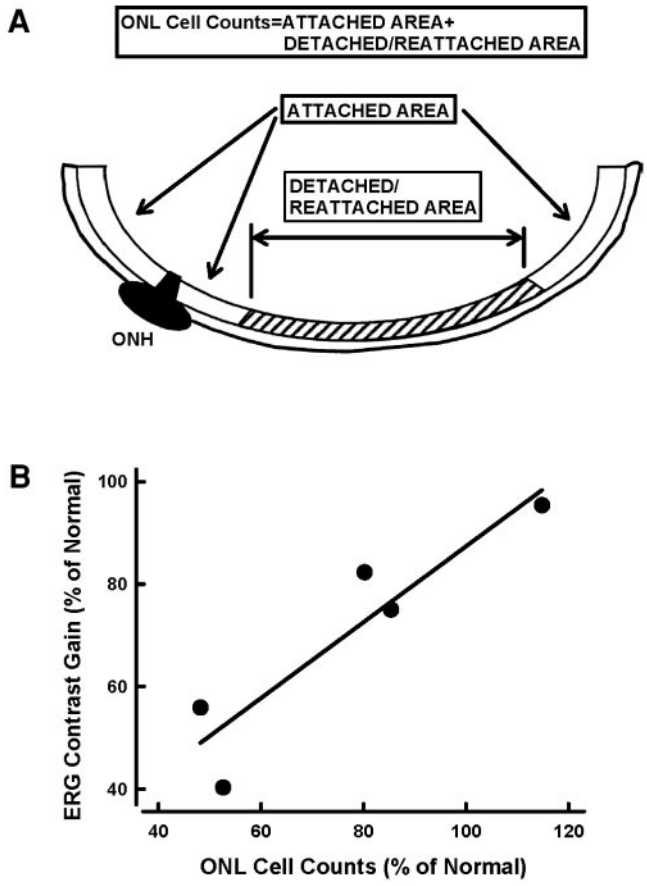

FIGURE 3. Relationship between ERG and receptor cell counts in reattached retinas (1DRD+35DRA) obtained from five ground squirrels. (**A**) An estimate of the number of receptors (specified as a percentage of normal retina) was obtained from counts made in sectioned material along a strip of tissue running vertically across the retina so as to bisect the region of detachment. (**B**) For isochromatic modulation, the ERG contrast gain is linearly related to the estimated number of cells (slope = 0.74; r^2 = 0.846).

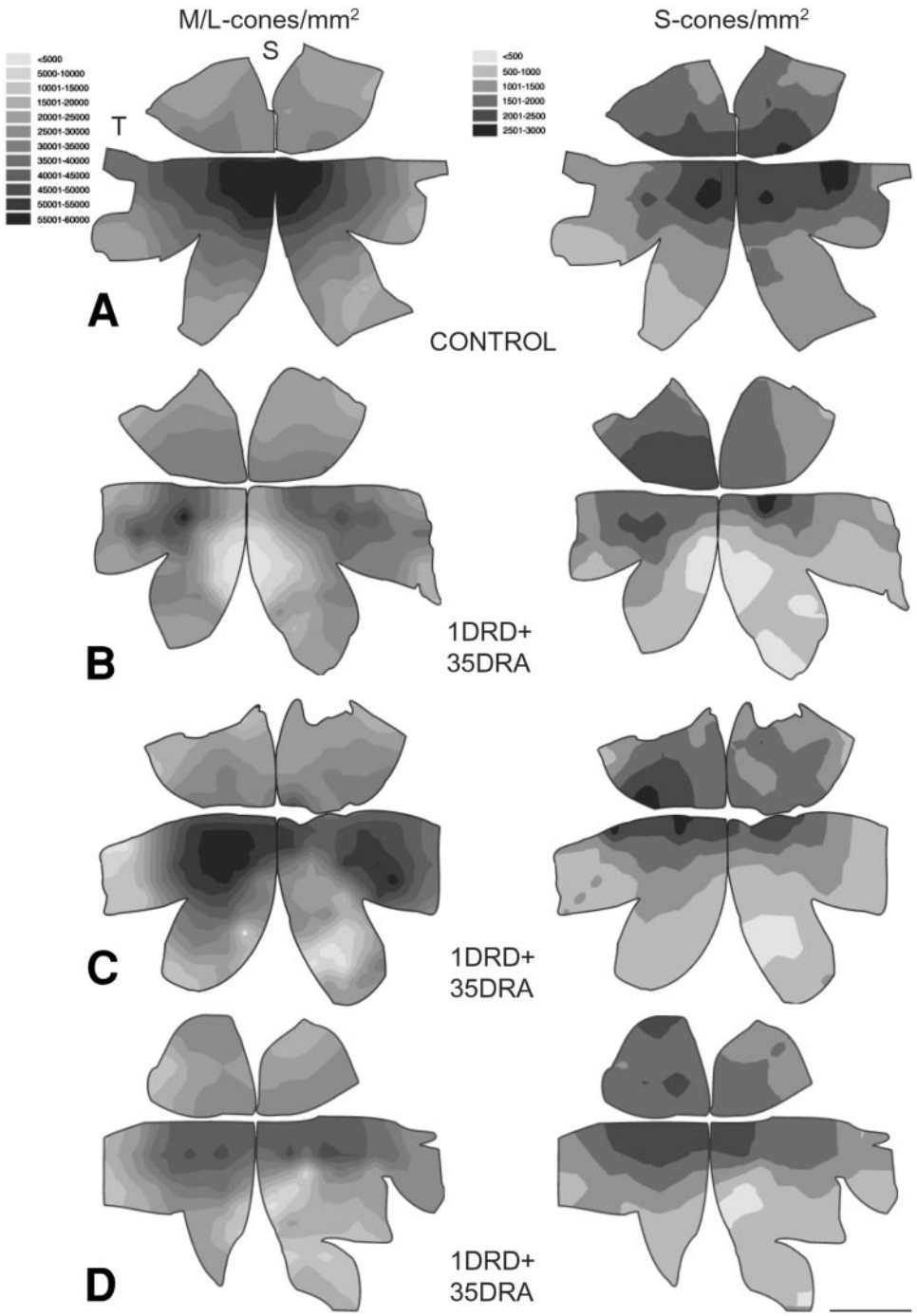

FIGURE 4. Density maps for the distribution of M and S cones in retinal wholemounts from (**A**) control and (**B–D**) three different eyes with 1-day detached/35-day reattached retinas (1DRD+35DRA). The density map for M-cone photoreceptors was identified by subtraction of cells stained by the JH455 antiserum from those labeled with PNA (**A–D**, *left*). In control retina, the peak density area was below the optic nerve head. It was in this area that the detachments generally occurred. The *numbers* indicate that cone outer segment recovery was still incomplete after 35 days of reattachment. The density map for S-cone photoreceptors was identified with JH455 antiserum (**A–D**, *right*). The lowest density area of S cones in reattached retinas was consistent with that of M cones. T, temporal; S, superior. Bar, 5 mm.

$df = 2$; NS) or the M-cone ($t = 0.781$; $df = 2$; NS) comparisons. In short, both this experiment and the previous one show that in the reattached retina there was a predictive relationship between the total number of viable photoreceptors and cone ERG contrast gains. Significantly, this relationship did not appear to differ for S and M cones ($F = 0.174$; $df = 1, 2$; NS).

Cone Cell Death

To examine cone cell death in detached and reattached retinas, double-labeling experiments with TUNEL and the anti-M/L cone opsin were performed (Fig. 6). In the 1-day detachments, TUNEL-positive cells were observed in the ONL and cones showed disruption of inner segment (IS) and OS and redistribution of opsin to the inner segment and cell body—all characteristic signs of photoreceptor degeneration (Fig. 6B). One striking observation was that most of the TUNEL-positive cells were double labeled with the antibodies to anti-M/L cone opsin. These data are consistent with our previous data in this species showing that cones appear to be more vulnerable to the effect of the detachment than rods.[32] In the 7- and 35-day reattachments, TUNEL labeling in the ONL was increasingly rare as the cone OS regenerated (Figs. 6C, 6D).

Photoreceptor Mitochondria and Synaptic Terminals

In control retina, immunocytochemical labeling of the outer retina with the antibody to cytochrome oxidase occurred mainly in the IS of photoreceptor cells (Fig. 6E). At 1 day after detachment, the IS was disrupted and shortened and the cyto-

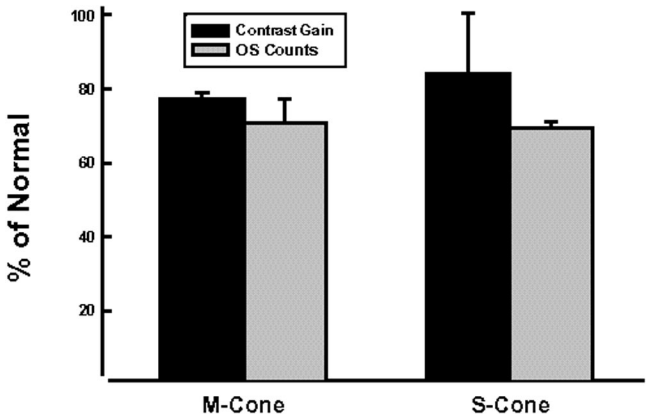

FIGURE 5. The relationship between the number of S and M cones obtained from flatmount counts in retinas of three squirrels after a 1-day detachment and a 35-day reattachment and the ERG contrast gains measured at the end of the reattachment period. There was no significant difference between the contrast gains and cell counts of either S- or M-cone subtypes.

chrome oxidase staining was reduced (Fig. 6F). After 7-days of reattachment, the labeling pattern did not substantially change, but the IS appeared to elongate (Fig. 6G). A more normal pattern of labeling was present in the 35-day reattached retinas, although the intensity of labeling with individual cells always appeared less than normal (Fig. 6H). The Müller cell labeling appeared to increase in intensity and then decrease again in the long-term reattachments.

Anti-synaptophysin labeled the synaptic terminals of rods and cones in the outer plexiform layer (OPL; Fig. 6E). In detached retinas there was a decrease in both the labeling intensity and the number of labeled terminals within the OPL (Fig. 6F). Labeling also occurred outside the synaptic layer within the photoreceptor cells. In 7- and 35-day reattached retinas, anti-synaptophysin labeling in the OPL recovered its intensity, and the number of labeled terminals in the OPL increased over those in the 1-day detachment, although significant gaps in the row of terminals continued to be present (Figs. 6G, 6H).

Glial, Rod, and Matrix Responses

Astrocytes were labeled with anti-GFAP in both control and detached retinas, but there was no labeling in Müller cells (Figs. 7A, 7B, 7F, 7G). Little change was observed at 1 day of detachment; however, after reattachment, the labeling was greatly increased in astrocytes. This is shown in sections taken en face (Figs. 7C–E) as well as radially (Figs. 7H–M). In some radial sections, labeled processes were observed originating from astrocyte cell bodies extending through the ONL (Figs. 7K, 7 L), indicating that most of the upregulation in GFAP was within astrocytes rather than Müller cells.

Labeling with rod opsin in control retina was restricted to the rod OS. At 1 day of detachment, rod opsin labeling was present in the truncated OS and became redistributed to the inner segment and cell body (Fig. 7G). This pattern gradually returned to a more normal appearance after reattachment, although some opsin labeling could still be observed in the cell bodies at 35 and 90 days after reattachment (Figs. 7I, 7J). Occasionally, we also observed rod axon outgrowth into the inner retina after reattachment (Fig. 7I).

Immunolabeling with the antibody to CRALBP occurred throughout the Müller cell and RPE cytoplasm in the control retinas (Fig 7N). After detachment for 1 day, the signal increased in Müller cell processes, especially in the ONL (Fig. 7O). After reattachment, the distribution of the CRALBP labeling became more similar to that in control retinas (Figs. 7P, 7Q, 7R).

Biotinylated-PNA bound to the cone sheaths in normal retina (Fig. 7N) and became severely disrupted at 1 day after detachment (Fig. 7O). After reattachment the labeling returned as OS grew in length (Figs. 7P–R), although it still did not appear normal at 90 days of reattachment.

To further examine the relationship between Müller cells, cones, and the RPE in reattached retinas, we performed double-labeling experiments with biotinylated PNA and anti-CRALBP on sections that contained regions of normal, nondetached retina (i.e., attached; Fig. 7S, left), a transition zone, and a 1-day detached and 35-day reattached zone (Fig. 7S; right). A

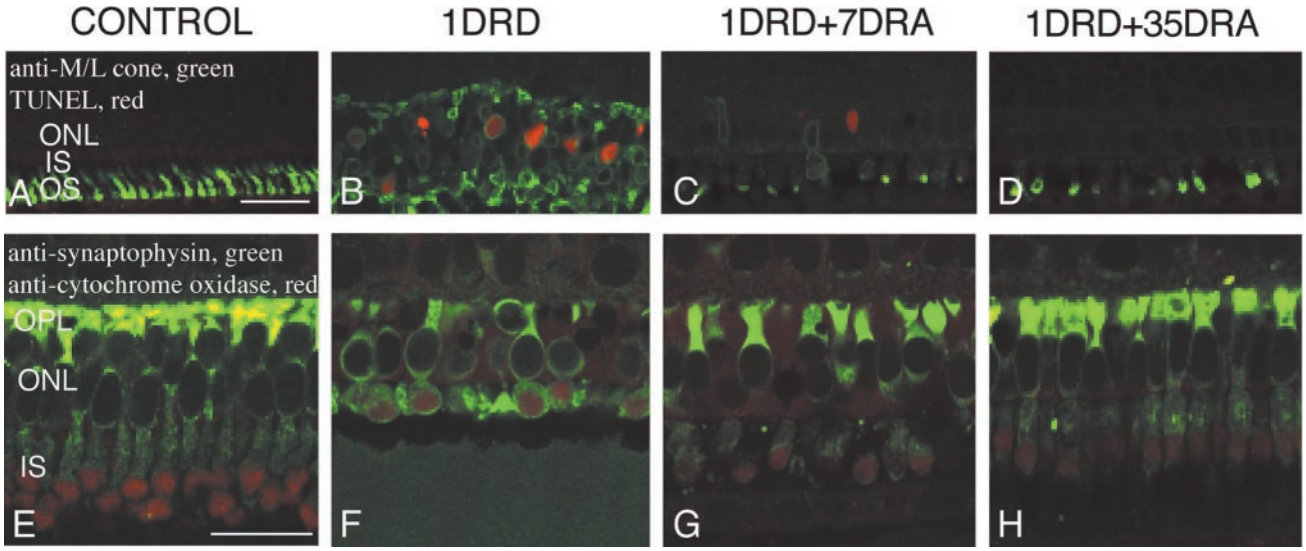

FIGURE 6. Laser scanning confocal micrographs showing the labeling patterns of the immunostaining agents listed in (**A**) and (**E**) in (**A**, **E**) control, (**B**, **F**) 1-day detached (1DRD), (**C**, **G**) 1-day detached/7-day reattached (1DRD+7DRA), and (**D**, **H**) 1-day detached/35-day reattached (1DRD+35DRA) retinal sections. Numerous photoreceptors were TUNEL positive at 1 day of detachment (**B**). After reattachment, TUNEL labeling decreased and the cone OS showed a gradual recovery (**C**, **D**). The reduction of anti-cytochrome oxidase in photoreceptor IS and anti-synaptophysin labeling in the synaptic terminals induced by the detachment (**F**) returned to normal levels after reattachment (**G**, **H**). OPL, outer plexiform layer. Bars, 20 μm.

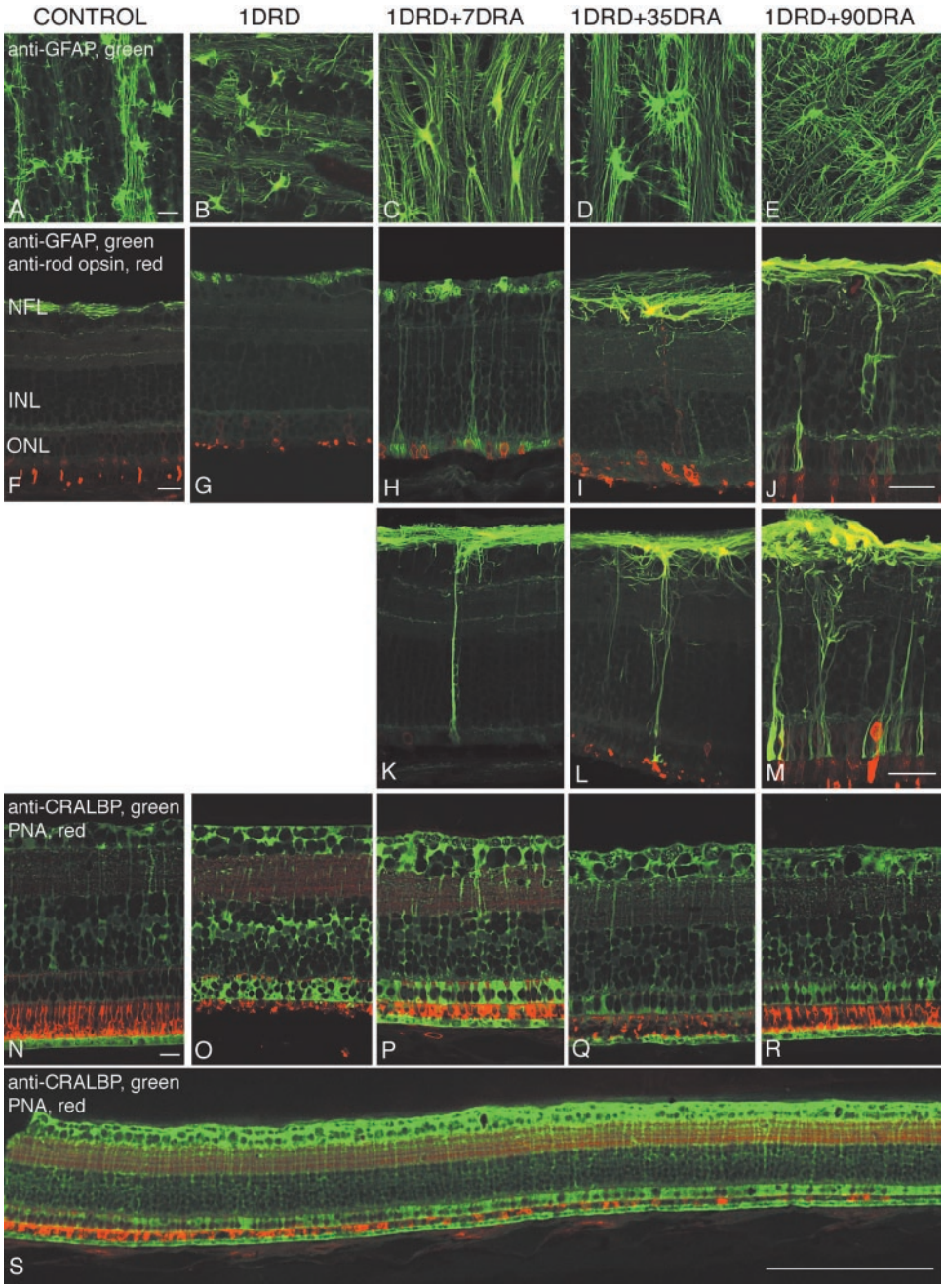

FIGURE 7. Laser scanning confocal micrographs showing the labeling pattern of the antibodies listed in (**A**), (**F**), (**N**), and (**S**) in sections of (**A**, **F**, **N**) control, (**B**, **G**, **O**) 1-day detached (1DRD), (**C**, **H**, **K**, **P**) 1-day detached/7-day reattached (1DRD+7DRA), (**D**, **I**, **L**, **Q**, **S**) 1-day detached/35-day reattached (1DRD+35DRA), and (**E**, **J**, **M**, **R**) 1-day detached/90-day reattached (1DRD+90DRA) retinas. There was a dramatic increase in the intensity of astrocyte labeling with anti-GFAP in sections of reattached retinas viewed en face (**C**–**E**) and in radial sections, in which label originated from astrocyte cell bodies and extended through the ONL (**H**–**M**). As reattachment time increased, more rod opsin labeling was observed (**H**–**M**). The intensity of anti-CRALBP labeling in Müller cells increased in detached retina (**O**), but decreased after reattachment (**P**–**R**), returning to levels more similar to those found in control retinas. PNA labeling of cone sheaths (**N**) was reduced after detachment (**O**) but returned after reattachment (**P**–**R**). A gradient of anti-CRALBP labeling in the Müller cells (but not RPE) and PNA labeling of cone sheaths is shown in a section (**S**) that includes normal (i.e., never detached) retina (*left*), a transition zone between normal and reattached retina (*middle*), and reattached retina (*right*). The more distant from the normal end of the section, the less PNA labeling and the more anti-CRALBP labeling were observed, particularly in the end foot and ONL. NFL, nerve fiber layer. Bars: (**A**–**E**) 50 μm; (**F**–**R**) 20 μm; (**S**) 200 μm.

gradient of CRALBP immunoreactivity in the Müller cells and PNA labeling of the cone matrix sheaths can be observed from normal to reattached retina, with CRALBP increasing and PNA decreasing. In contrast, CRALBP immunoreactivity in the RPE showed very little change across the entire region.

In agreement with previous results reported in this species[33,34] IRBP immunoreactivity was observed in the matrix surrounding the photoreceptor cells (Fig. 8A). In the 1-day detachments, the labeling had markedly dissipated (Fig. 8B). In the reatttachments, there was an increase in the labeling (Fig. 8C), but not to the extent found in the control retina.

DISCUSSION

Correlation between Histology and the ERG

Up to this point, there have been few studies specifically examining the relationship between physiological function and morphology of cone photoreceptors in retinal disease.[35–37] One goal of our study was to establish the relationship between morphologic and functional recovery of cones after retinal reattachment. In the ground squirrel model we found a significant correlation between the number of photoreceptor cells and contrast gains determined from ERG recordings. That correlation was established in two ways: first, by summing the photoreceptor number in the ONL from attached and detached–reattached regions. So measured, the correlation between contrast gain and photoreceptor number was high (Fig. 3B) even though the reattached retinas showed a patchiness in the number of cells in the ONL. Retinal wholemounts have many advantages for quantification of photoreceptor cells,[38] and so we also used them in conjunction with specific cytochemical staining to determine density maps for the remaining OS of individual classes of cones after reattachment. That

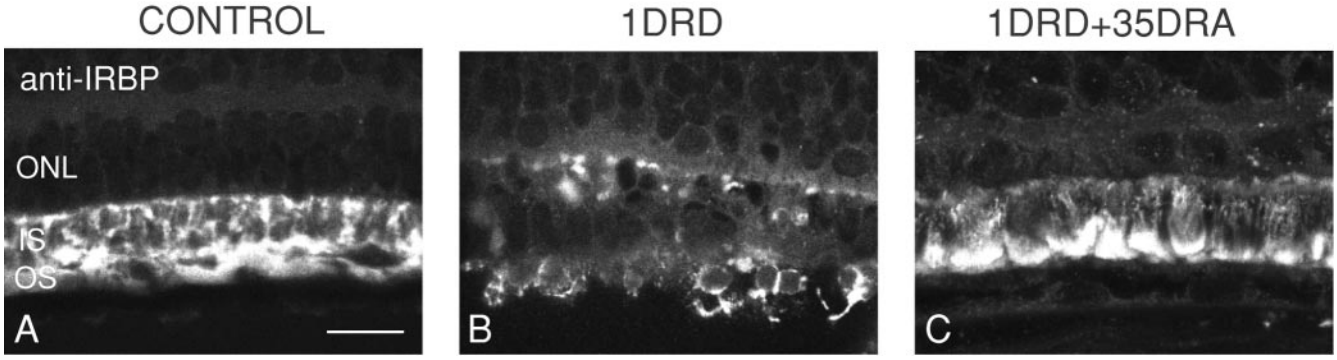

FIGURE 8. Laser scanning confocal micrographs showing the labeling pattern of anti-IRBP. In control retina (**A**) labeling was observed in the matrix between the IS and OS (**A**). This labeling was disrupted and reduced in (**B**) 1-day detached retinas (1DRD), and gradually returned (**C**) after reattachment (1DRD+35DRA) although the pattern was not completely normal. Scale bar, 20 μm.

index also correlated highly with the ERG contrast gain measurements.

In a previous experiment with this ground squirrel model we found that the reduction in contrast gain produced by retinal detachment is directly proportional to the number of cones in the detachment zone and that this relationship holds for both the S- and M-cone subtypes.[39] In the present experiment ERG contrast gain was shown to be directly proportional to the number of cones that survived after reattachment, and this held for both S and M cones, as well. In sum, in this model, ERG contrast gain can be used to sensitively index changes in the retina after experimental retinal detachment and reattachment. Note that in each case the recording index correlates with some aspect of the number of photoreceptors. Beyond the mere number of cells, those photoreceptors that survive after reattachment obviously undergo organizational changes including, prominently, a regrowth of the OS. Whereas OS growth in ground squirrel cones continued for at least 3 months after reattachment (Fig. 2B), the recovery of ERG contrast gain appeared to become asymptotic much sooner, so that ERG contrast gain seemed to show little change beyond the time point when the surviving photoreceptors had still achieved only approximately one third of normal OS length (compare Figs. 2B and 2C). Because contrast gain could be argued to provide a prediction of contrast sensitivity of the animal, this relationship implies that at least some aspects of functional vision may reach asymptotic levels before the complete regrowth of photoreceptor OS. We caution, however, that this implication is drawn from study of a relatively small number of animals. Further investigation of this relationship is clearly warranted.

S- and M-Cone Comparisons

Previous reports have suggested that S cones are more vulnerable to damage than the other cone types.[7] Nork et al.,[9] presented evidence obtained from human detached retinas that S cones are more susceptible to the degenerative effects of detachment than the other two cone types. Recently, Hayashi and Yamamoto[35] showed that after successful anatomic reattachment the M/L-cone ERG increases significantly, but that of the S cones does not improve. These results suggest that there is an enhanced susceptibility of S cones and/or S-cone signaling pathways, and this may explain the tritan color vision defects that are often observed after retinal reattachment. In the present study, however, there were no striking histologic or immunocytochemical differences in the recovery process of OS between M and S cones after reattachment. Neither were there any differences in the ERG measurements to suggest any difference in the viability of S and M cones. Finally, the density maps of S and M cones from the reattached retinal wholemounts showed similar patterns.

Several factors may account for the differences between our results and those in previous studies. First, our procedure for reattachment surgery was not conventional scleral buckling surgery but pneumatic retinopexy. As Hayashi and Yamamoto[35] pointed out, scleral buckling surgery may reduce ocular blood flow and lead to hypoxia in the outer retina, resulting in selective impairment of the S-cone system. Second, in human detachments there may be multiple factors involved, such as trauma, ischemia, and inflammation, and these may irreversibly damage the S cones. Third, it has been suggested that standard color vision tests to detect tritan defects can be prone to artifact and thus the association of retinal detachment with tritan color vision could be overstated.[40] Finally, of course, the regenerative capacity and response properties of S cones may vary in different species. Against this latter possibility, we note that the S-cone system in humans and ground squirrels share many properties,[22] and so the present finding that S cones are not more vulnerable to the effect of the detachment than the M-cones could be more than just a curiosity in this species. In support of that possibility, Rex et al.[41] have recently shown in the feline retina that after 28 days of reattachment the recovery of mRNA labeling is comparable for the M and S cones.

Cone Cell Death

In the present study, the TUNEL assay revealed that some cones are dying at 1 day after detachment and reattachment stops the progress of this event. Although the key factors for cessation of the cell death by reattachment remain unknown, we believe that alleviating the hypoxia of the photoreceptor layer plays an important role because of previous results showing that hyperoxia during a period of detachment, in both squirrel and feline models, reduces cone cell death.[32,42] Moreover, the preservation of OS, mitochondria and synaptic terminals that occurs with reattachment is also observed after hyperoxia treatment during detachment.[32,42] Finally, the death of photoreceptors after detachment is largely due to apoptosis[43] and this process is generally considered to be quite rapid. We do not know, however, whether reattachment allows photoreceptors to exit from the apoptotic program once it is initiated or whether it simply stops cells from entering the pathway.

One of the most striking features of the reattached retina in the present study was the gradual reduction in the number of cone photoreceptors and the lengths of cone OS toward those regions that had been highly detached (Fig. 7S). This appears not to depend on the condition of the RPE (at least with

respect to the overall appearance and anti-CRALBP labeling). We have reported that cone cell death is directly related to retinal elevation in ground squirrel retinal detachments.[32] Therefore, the difference we observed along the detached region is probably associated with the extent of cone degeneration that occurs during the period of detachment. Thus, preventing the degeneration during an episode of detachment may be significant in promoting visual recovery after reattachment, even if the duration of the detachment is very short.

Glial Response

Astrocytes and Müller cells have long been known to react to injury of the retina and are presumed to play a key role in the response to injury. In rod-dominated species, both cell types are stimulated to divide after detachment[44,45]; however, the Müller cells show a much more dramatic increase in labeling with antibodies to intermediate filaments. In the cone-dominated ground squirrel retina, the Müller cells alter levels of various proteins such as glutamine synthetase (GS) and CRALBP, indicating that they are responding to detachment, but they generally do not upregulate intermediate filament proteins. It appears that the astrocytes have taken over this response in the ground squirrel. Long GFAP-positive processes can be observed originating from astrocyte cell bodies and growing deep into the outer retina. These processes appear most often after reattachment, although they can occasionally occur as a result of detachment. Although the levels of GS and CRALBP in Müller cells appear to return to a more normal distribution after reattachment, GFAP-positive processes can still be observed for months after reattachment, extending the width of the retina. It will be interesting to see whether the differences in reactivity between Müller cells and astrocytes are a unique feature of the ground squirrel retina or whether they are a function of high cone density. The latter would have implications for glial changes in the fovea of humans.

Recovery of the Distribution of Proteins

Liem et al.[10] reported that foveal densitometry shows a slow recovery of the cone photopigments after reattachment. They concluded that the improvement may be attributed to regrowth and realignment of photoreceptor OS and metabolic recovery of the retinal pigment epithelium-photoreceptor complex inferred from an improved time constant of photopigment regeneration. Our results showing that IRBP immunoreactivity and PNA labeling of the cone matrix sheaths, which recovered from the disruption after reattachment, may be consistent with their conclusion by implying a continued long-term improvement of the molecular environment in which the OS function. Similarly, the gradual return to more normal distributions of cytochrome oxidase and synaptophysin in photoreceptors would have implications for the slow return of good vision after reattachment.

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References

1. Anderson DH, Guerin CJ, Erickson PA, Stern WH, Fisher SK. Morphological recovery in the reattached retina. *Invest Ophthalmol Vis Sci.* 1986;27:168–83.
2. Gundry MF, Davies EW. Recovery of visual acuity after retinal detachment surgery. *Am J Ophthalmol.* 1974;77:310–314.
3. Chisholm A, McClure E, Foulds WS. Functional recovery of the retina after retinal detachment. *Eye.* 1975;95:167–172.
4. Tani P, Robertson DM, Langworthy A. Prognosis for central vision and anatomic reattachment in rhegmatogenous retinal detachment with macula detached. *Am J Ophthalmol.* 1981;92:611–620.
5. Burton TC. Recovery of visual acuity after retinal detachment involving the macula. *Trans Am Ophthalmol Soc.* 1982;80:475–497.
6. Sasoh M, Yoshida S, Kuze M, Uji Y. The multifocal electroretinogram in retinal detachment. *Doc Ophthalmol.* 1997;94:239–252.
7. Marré M. The investigation of acquired color vision deficiencies. In: Hiler A, ed. *Color 73.* New York: John Willey & Sons Inc; 1973:99–135.
8. Barca L, De Luka A, Passani F. Color discrimination (100-hue test) after successful surgical treatment of retinal detachment. *Doc Ophthalmol.* 1985;39:349–353.
9. Nork TM, Millecchia LL, Strickland BD, Linberg JV, Chao GM. Selective loss of blue cones and rods in human retinal detachment. *Arch Ophthalmol.* 1995;113:1066–1073.
10. Liem ATA, Keunen JEE, Meel GJ, Norren D. Serial foveal densitometry and visual function after retinal detachment surgery with macular involvement. *Ophthalmology.* 1994;101:1945–1952.
11. Kusaka S, Toshino A, Ohashi Y, Sakaue E. Long-term visual recovery after scleral buckling for macula-off retinal detachments. *Jpn J Ophthalmol.* 1998;42:218–222.
12. Fisher SK, Anderson DH. Cellular effects of detachment on the neural retina and the retinal pigment epithelium. In: Ryan SJ, ed. *Retina*: *Retinal Reattachments: Surgical Principles and Techniques*. St. Louis, MO: Mosby; 2001:1961–1986.
13. Mori T, Tsue TT, Marmor MF. Electrical responses from detached retina inside the intact rabbit eye. *Invest Ophthalmol Vis Sci.* 1986;27:1040–1043.
14. Kim SD, Naoi N, Maruiwa F, Sawada A. Electrical responses from locally detached retina and its recovery after reattachment. *Ophthalmologica.* 1996;210:195–199.
15. Jacobs GH, Blakeslee B, McCourt ME, Tootell RBH. Visual sensitivity of ground squirrels to spatial and temporal luminance variations. *J Comp Physiol.* 1980;136:291–299.
16. Jacobs GH. Blakeslee B, Tootell RBH. Color discrimination tests on fibers in the ground squirrel optic nerve. *J Neurophysiol.* 1981;45:903–914.
17. Jacobs GH, Neitz J, Crognale M. Spectral sensitivity of ground squirrel cones measured with ERG flicker photometry. *J Comp Physiol.* 1985;156:503–509.
18. Crognale M, Jacobs GH. Temporal properties of the short-wavelength cone mechanism: comparison of receptor and postreceptor signals in the ground squirrel. *Vision Res.* 1988;28:1077–1082.
19. Jacobs GH. Duplicity theory and ground squirrels: linkages between photoreceptors and visual function. *Vis Neurosci.* 1990;5:311–318.
20. Jacobs GH, Neitz J, Krogh K. Electroretinogram flicker photometry and its applications. *J Opt Soc Am A.* 1996;13:641–648.
21. Linberg KA, Lewis GP, Charteris DG, Fisher SK. Experimental detachment in a cone dominant retina. *Invest Ophthalmol Vis Sci.* 1999;40(4):**S**951. Abstract nr 5020.
22. Jacobs GH, Calderone JB, Sakai T, Lewis GP, Fisher SK. An animal model for studying cone function in retinal detachment. *Doc Ophthalmol.* 2002;104:119–132.
23. Faktorovich EG, Steinberg RH, Yasumura D, Matthes MT, La Vail MM. Photoreceptor degeneration in inherited retinal dystrophy delayed by basic fibroblast growth factor. *Nature.* 1990;347:83–86.
24. Valter K, Maslim J, Bowers F, Stone J. Photoreceptor dystrophy in the RCS rat: roles of oxygen, debris and bFGF. *Invest Ophthalmol Vis Sci.* 1998;39:2427–2442.
25. Machida S, Chaudhry P, Shinohara T, et al. Lens epithelium-derived growth factor promotes photoreceptor survival in light-damaged and RCS rats. *Invest Ophthalmol Vis Sci.* 2001;42:1087–1095.
26. Cao W, Tombran-Tink J, Elias R, Sezate S, Mrazek D, McGinnis JF. In vivo protection of photoreceptors from light damage by pigment epithelium-derived factor. *Invest Ophthalmol Vis Sci.* 2001;42:1646–1652.

27. Lewis GP, Linberg KA, Geller SF, Guerin CJ, Fisher SK. Effects of the neurotrophin BDNF in an experimental model of retinal detachment. *Invest Ophthalmol Vis Sci* 1999;40:1530–1544.
28. Petry HM, Erichsen JT, Szél A. Immunocytochemical identification of photoreceptor populations in the tree shrew retina. *Brain Res.* 1993;616:344–350.
29. Brainard DH, Calderone JB, Nugent AK, Jacobs GH. Flicker ERG responses to stimuli parametrically modulated in color space. *Invest Ophthalmol Vis Sci.* 1999;40:2840–2847.
30. Pelli DG. The Video Toolbox software for visual psychophysics: transforming numbers into movies. *Spat Vis.* 1997;10:437–442.
31. Kryger Z, Galli-Resta L, Jacobs GH, Reese BE. The topography of rod and cone photoreceptors in the retina of the ground squirrel. *Vis Neurosci.* 1998;15:685–691.
32. Sakai T, Lewis GP, Linberg KA, Fisher SK. The ability of hyperoxia to limit the effects of experimental detachment in cone-dominant retina. *Invest Ophthalmol Vis Sci.* 2001;42:3264–3273.
33. Anderson DH, Neitz J, Saari JC, et al. Retinoid-binding proteins in cone-dominant retinas. *Invest Ophthalmol Vis Sci.* 1986;27:1015–1026.
34. Porrello K, Bhat SP, Bok D. Detection of interphotoreceptor retinoid binding protein (IRBP) mRNA in human and cone-dominant squirrel retinas by in situ hybridization. *J Histochem Cytochem*. 1991;39:171–176.
35. Hayashi M, Yamamoto S. Changes of the cone electroretinograms to color flash stimuli after successful retinal detachment surgery. *Br J Ophthalmol.* 2001;85:410–413.
36. Falsini B, Iarossi G, Porciatti V, et al. Postreceptoral contribution to macular dysfunction in retinitis pigmentosa. *Invest Ophthalmol Vis Sci*. 1994;35:4282–4299.
37. Sugawara T, Sieving P, Bush R. Quantitative relationship of the scotopic and photopic ERG to photoreceptor cell loss in light damaged rats. *Exp Eye Res.* 2000;70:693–705.
38. Curcio CA, Sloan KR, Kalina RE, Hendrickson AE. Human photoreceptor topography. *J Comp Neurol.* 1990;292:497–523.
39. Jacobs GH, Calderone JB, Sakai T, Lewis GP, Fisher SK. Effects of retinal detachment on S and M cone function in an animal model. In: Mollon JD, Pokorny J, Knoblauch K, ed. *Normal and Defective Colour Vision*. Oxford, UK: Oxford University Press. In press.
40. Mollon JD. What is odd about the short-wavelength mechanism and why is it disproportionally vulnerable to acquired damage? In: Verriest G, ed. *Colour Vision Deficiencies.* Vol 6. The Hague: W. Junk; 1982;145–149.
41. Rex TS, Lewis GP, Geller, SF, Fisher SK. Differential expression of cone opsin mRNA levels following experimental retinal detachment and reattachment. *Mol Vis.* 2002;8:114–118.
42. Mervin K, Valter K, Maslim J, Lewis GP, Fisher SK, Stone J. Limiting photoreceptor death and deconstruction during experimental retinal detachment: the value of oxygen supplementation. *Am J Ophthalmol*. 1999;128:155–164.
43. Cook B, Lewis GP, Fisher SK, Adler R. Apoptotic photoreceptor degeneration in experiment retinal reattachment. *Invest Ophthalmol Vis Sci.* 1995;36:990–996.
44. Fisher SK, Erickson PA, Lewis GP, Anderson DH. Intraretinal proliferation induced by retinal detachment. *Invest Ophthalmol Vis Sci*. 1991;32:1739–1748.
45. Geller SF, Lewis GP, Anderson DH, Fisher SK. Use of the MIB-1 antibody for detecting proliferating cells in the retina. *Invest Ophthalmol Vis Sci.* 1995;36:737–744.