

# A Survey of Molecular Expression by Photoreceptors after Experimental Retinal Detachment

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**PURPOSE.** To describe changes in the localization patterns and levels of rod and cone photoreceptor proteins after experimental retinal detachment (RD).

**METHODS.** Cat retinas were detached for 1, 3, 7, or 28 days, at which time the eyecups were placed in fixative for immunocytochemical analysis or homogenized for biochemistry. Immunocytochemistry was performed using 19 probes for molecules known to be associated with photoreceptors. Protein concentrations were determined using enzyme-linked immunosorbent assay or Western blot analysis. Cone cell death was analyzed by double labeling with TdT-dUTP terminal nick-end labeling and cone-specific antibodies.

**RESULTS.** Although some cones died, many survived long-term RD. Although their profiles may have changed, rod photoreceptors continued to express most of the molecules studied as long as they were alive. In contrast, the cones failed to label with almost all probes specific to them after 3 to 7 days of detachment. The exception was phosducin, which localized to both rods and cones and, in 28-day detachments, increased to 180% of the amount in normal retina.

**CONCLUSIONS.** Rods and cones respond differently to RD. This difference may account for a faster return of rod vision and for the lingering changes in color vision and acuity that are often reported after successful reattachment surgeries. (*Invest Ophthalmol Vis Sci.* 2002;43:1234-1247)

There are approximately 15,000 new cases of nontraumatic retinal detachment every year in the United States.<sup>1</sup> Although reattachment surgery is remarkably successful, patients often describe permanent defects in color vision and a decline in visual acuity.<sup>2,3</sup> Furthermore, it has been shown that if the macula has been detached for even short periods, successful reattachment leads to a final visual acuity of 20/50 or better in only 39% of cases.<sup>4,5</sup> There are four main factors that affect the recovery of vision after reattachment surgery: preoperative vision, age, degree of myopia, and duration of the retinal detachment—the return of vision declining exponentially with the length of detachment.<sup>6</sup> Currently, there is no effective treatment other than surgery. In fact, recent treatment for

age-related macular degeneration involves translocation of the retina<sup>7,8</sup> after the production of a detachment in a procedure that is remarkably similar to that used in our model. Thus, information about detachment has become relevant to cases in which the retina is purposefully detached as part of a therapeutic process.

Retinal detachment induces many cellular changes in photoreceptors, including rapid degeneration of much of the outer segment (OS), disruption of the cytoskeleton, disruption of organelles in the inner segment (IS), loss of mitochondria, and withdrawal of synaptic terminals (ST) of the rods.<sup>9-13</sup> The effect on photoreceptors is only part of a more generalized response,<sup>14,15</sup> but it must be important in the recovery of vision after retinal reattachment. In this study, we sought to learn more about the responses of these cells by studying their expression of various proteins.

Most photoreceptor cell death occurs within the first few days of detachment, but cells continue to be lost from this layer as long as the retina remains detached.<sup>11,13,16,17</sup> The surviving rods continue to label with antibodies to OS proteins, such as rod opsin and peripherin/rds, and continue to synthesize RNA and protein.<sup>18-20</sup> There are, however, major differences in the antibody labeling patterns of these two proteins.<sup>20</sup> There are few comparable data on the responses of cones, except indications that short-wavelength-sensitive (S) cones may be more susceptible to damage than the middle- to long-wavelength-sensitive (M/L) cones.<sup>2</sup> In an earlier attempt to quantify the survival of cones after detachment we discovered that labeling with antibodies to the cone opsins and calbindin D, both reliable markers of cones in normal retina, was rapidly lost.<sup>21,22</sup>

In this study, we used a variety of probes to define more completely the expression of different molecules in rods and cones in an animal model of retinal detachment. We expected that their expression would decline as the cells underwent deconstruction and degeneration. Only partially matching that expectation, the results described herein contribute new information about what is known as “photoreceptor deconstruction,” a term used to describe degenerative changes in these cells that do not necessarily lead to cell death.<sup>13</sup> Perhaps this information can be exploited to improve the recovery of vision after reattachment or after other surgeries involving the photoreceptor-RPE interface. Portions of this work have been presented and published previously in abstract form.<sup>21,23</sup>

## MATERIALS AND METHODS

### Experimental Detachments

Cat retinas were detached by injecting a solution of 0.25% sodium hyaluronate (Healon; Pharmacia, Piscataway, NJ) in balanced salt solution (Alcon, Fort Worth, TX) between the neural retina and RPE with a glass micropipette (see details in Ref. 24). Detachments were created only in the right eyes. The left eyes were used as the control for all the experiments. At least two animals were used for each detachment time point at 1, 3, 7, and 28 days. All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of California Santa Barbara (UCSB) Institutional Animal Care and Use Committee.

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TABLE 1. Summary Information for Molecular Probes

Cell Specificity	Protein Recognized	Antibody Type	Concentration		Source†	Reference
			Immunocytochemistry	ELISA/Western Blot		
Cones	S-cone opsin	Polyclonal antibody	1:2000		J. Nathans <sup>a</sup>	Wang et al. <sup>66</sup>
	M/L-cone opsin	Polyclonal antibody	1:2000		J. Nathans <sup>a</sup>	Wang et al. <sup>66</sup>
	GCAP-1	Polyclonal antibody	1:2500		K. Palczewski <sup>b</sup>	Gorczyca et al. <sup>67</sup>
	Terminal disaccharides	Lectin/peanut agglutinin	400 µg/mL		Vector Labs <sup>c</sup>	Johnson et al. <sup>33</sup>
	Calbindin-D	Monoclonal antibody	1:1000		Sigma Chemical Co. <sup>d</sup>	Celio et al. <sup>68</sup>
Rods	Rhodopsin	Polyclonal antibody		1:2000	T. Shuster <sup>c</sup>	Shuster et al., <sup>69</sup> Walter et al. <sup>70</sup>
	Rhodopsin	Monoclonal antibody	1:50	1:50	R. Molday <sup>f</sup>	Hicks and Molday <sup>71</sup>
	GCAP-2*	Polyclonal antibody	1:1000	1:1500	K. Palczewski <sup>b</sup>	Otto-Bruc et al. <sup>30</sup>
	Rds/peripherin	Monoclonal antibody	1:20	1:50	R. Molday <sup>f</sup>	Molday et al. <sup>72</sup>
	Transducin-α	Monoclonal antibody	1:50	1:300	B. Fung <sup>g</sup>	Navon and Fung <sup>73</sup>
Mixed rod-cone	γ-PDE	Polyclonal antibody	1:1000	1:1000	B. Fung <sup>g</sup>	Fung and Griswold-Penner <sup>74</sup>
	Phosducin	Polyclonal antibody	1:450	1:1000	T. Shinohara <sup>h</sup>	Sunayashiki-Kusuzaki <sup>75</sup>
	Arrestin	Polyclonal antibody	1:2000	1:1500	B. Fung <sup>g</sup>	Fung, written communication (July 1991)
Matrix	Recoverin	Polyclonal antibody	1:500	1:1000	A. Dizhoor <sup>i</sup>	Dizhoor et al. <sup>76</sup>
	NSE	Polyclonal antibody	1:500		Polysciences <sup>j</sup>	Parma et al. <sup>48</sup>
	IRBP	Polyclonal antibody	1:200		J. Saari <sup>k</sup>	Eisenfeld et al. <sup>77</sup>

\* Cell specificity may be species dependent; see text for results.

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## Immunocytochemistry

**Confocal Microscopy.** Epifluorescence observation of labeled tissue sections was performed using a laser scanning confocal microscope (model 1024; BioRad, Hercules, CA). The tissue fixation, embedding, and sectioning were all performed according to the techniques of Hale and Matsumoto.<sup>25</sup> The 100-µm-thick sections were rinsed and incubated overnight at 4°C in PBS containing 0.5% bovine serum albumin (BSA), 0.1% Triton X-100, and 0.1% sodium azide (PBTA) plus normal donkey serum. The next day, this blocking serum was replaced with one of several primary probes. These consisted of the various polyclonal and monoclonal antibodies and the biotinylated lectin, peanut agglutinin (PNA), with various specificities as shown in Table 1. The next day the sections labeled with primary antibodies were rinsed in PBTA and incubated in either cy2- or cy3-conjugated secondary antibodies (1:200 in PBTA). The sections labeled with biotinylated PNA were incubated overnight with streptavidin-cy3 (1:250 in PBTA). Finally, the sections were rinsed in PBTA and mounted in 0.5% *n*-propyl gallate. Immunohistochemical experiments were performed on at least four sections from different retinal regions within two experimental eyes (at each detachment time point) and two contralateral normal eyes. Although detachment produced a "patchiness" of labeling with some antibodies (i.e., small areas of retina that showed no labeling), in general, the patterns for each of the antibodies studied were highly consistent among animals and between sections.

**Bright-field Microscopy.** The antibody to guanylate cyclase-activating protein-2 (GCAP-2; Table 1) was sensitive to tissue preparation procedures used for confocal microscopy, so an HRP method was used on tissue fixed for 2 hours and embedded in wax. After 2 hours in the paraformaldehyde solution (see above), the tissue was dehydrated in ethanol and embedded in wax (Paraplast Extra; Fisher Scientific, Tustin, CA). Sections of 4-µm thickness were placed on glass

slides that are designed to produce a 75-µm gap between paired slides (ChemMate; Ventana Medical Systems, Tucson, AZ). Slides with dewaxed, rehydrated sections were immersed in heat-induced epitope-retrieval buffer (Ventana Medical Systems) and steamed for 20 minutes. The slides were then processed in pairs using an automated tissue processor (TechMate 1000; Ventana Medical Systems). The sections were incubated for 2 hours in primary antibody diluted 1:1000 in antibody dilution buffer (ChemMate; Ventana Medical Systems) and then for 25 minutes in avidin-biotin-horseradish peroxidase complex (ChemMate, Ventana Medical Systems), followed by three 6-minute changes of diaminobenzidine (ChemMate; Ventana Medical Systems). The sections were then dehydrated and coverslipped for examination by bright-field microscopy (model BX60; Olympus, Lake Success, NY).

## TdT-dUTP Terminal Nick-End Labeling

As a measure of apoptotic cell death, we slightly modified the procedure of Gavrieli et al.,<sup>26</sup> as published by Alon et al.,<sup>27</sup> to accommodate the confocal microscopy antibody-labeling procedures. The 100-µm-thick agarose-embedded sections were rinsed in Tris-buffered saline (TBS; 20 mM Tris, 150 mM NaCl, and 0.2% Triton X-100 [pH 7.3]), incubated for 30 minutes in 10 µg/mL proteinase K, rinsed in TBS, and placed in terminal transferase buffer (TdT buffer: 30 mM Tris-HCl, 150 mM Na cacodylate, 1 mM cobalt chloride, and 0.2% Triton X-100 [pH 7.2]). To create a positive control, 0.01 U/µL DNase was added to the TdT buffer. After 30 minutes, the TdT buffer was removed, and a TdT reaction solution (4 µM biotin dUTP, 0.05 U/µL TdT) was added to the retinal sections. The reaction was allowed to continue for 2 hours at 37°C in a humidified chamber and then was quenched by replacing the reaction solution with 2× SSC (30 mM citric acid trisodium, 300 mM NaCl, and 0.2% Triton X-100 [pH 7.3]). The quenching solution was rinsed off with TBS, at which time the sections were blocked with 1%

BSA and incubated overnight in streptavidin-cy3 (1:200) in TBS at 4°C. The sections were mounted as for bright-field microscopy, for viewing by confocal microscopy.

Some TUNEL-labeled sections were also labeled with the anti-calbindin D antibody as a marker for cone photoreceptors. In this case, the sections were treated with the TUNEL procedure, rinsed, and labeled with the primary and secondary antibodies, as for the other sections. The proteinase K treatment was omitted to improve the quality of the antibody labeling for the TUNEL antibody double-labeling studies. This was shown not to affect the number of TUNEL-positive cells.<sup>27</sup> To estimate the amount of cone cell death, the number of calbindin D-positive, TUNEL-positive, and double-labeled cells per millimeter of retina were counted in confocal images. For this purpose, sections were selected from the midperipheral superior-nasal retina of control eyes or retinas with 1-, 3-, or 7-day detachments.

### Sandwich ELISA

Detergent-solubilized retinal homogenates were assayed for the presence of rod opsin by a sandwich ELISA detection method. Briefly, a rabbit polyclonal antibody produced against rod opsin (Table 1) was diluted in bicarbonate buffer (Pierce, Rockford, IL) and used to coat wells of a 96-well enzyme immunoassay (EIA) microtiter plate (ImmunoWare; Pierce, Rockford, IL). All incubations were done at room temperature. Unbound antibody was removed from the plate after 1 hour by repeated washing with cold Dulbecco's PBS solution containing 0.05% Tween 20 (Pierce). Nonspecific binding was blocked by incubating the wells for 2 hours in PBS containing 1% BSA. Homogenates from normal (control) eyes and those with detached retinas were assayed at the same dilution (1:200). After 1 hour, incubation was terminated by repeatedly washing the plates with wash buffer. The antibody RHO 4D2, an anti-rod opsin (see Table 1) was diluted 1:50 with wash buffer and added to the plates. After 2 hours, the antibody solution was rinsed off with wash buffer, and HRP-conjugated rabbit anti-mouse IgG (1:1000, Pierce) was added to all wells and allowed to incubate for 2 hours. A one-step 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) solution (Pierce) was used as a substrate for the HRP-catalyzed reaction. This solution was added to wells and allowed to react for 30 minutes in the dark. Absorbance values were measured at 405 nm with an EIA plate reader (model 2550; BioRad). The absorbance values obtained from samples of control retina were compared with those from detached retina. In each case, the control retina came from the completely normal, contralateral eye of the same animal. In addition, samples of homogenate from control eyes were serially diluted and assayed by ELISA to generate standard curves used to estimate the relative change in rod opsin content after detachment.

### Enzyme-Linked Immunosorbent Assay

An ELISA procedure was also used to quantify phosphodiesterase (PDE)- $\gamma$ , phosducin, transducin  $\alpha$ , peripherin/rds, arrestin, and recoverin levels after detachment. Except as described in this section, all conditions were the same as for the sandwich ELISA technique, including production of a standard curve for each antibody. The surfaces of the EIA plates were coated directly with retinal homogenates for 1 hour. The dilution used for each antibody is shown in Table 1. The retinal homogenate was diluted 1:300 for PDE $\gamma$ , 1:5000 for phosducin, 1:300 for transducin  $\alpha$ , 1:200 for peripherin/rds, 1:400 for arrestin, and 1:400 for recoverin. Each sample and ELISA were run in triplicate and plotted on a graph as the percentage of normal retina, which was defined as 100%. The data are presented as the mean  $\pm$  SEM.

### Western Blot Analysis

**Phosducin.** Retinas for SDS-PAGE and Western blot analysis were homogenized at 4°C in 0.02 M PBS containing 0.1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 0.5% Triton X-100, and 2.5% SDS. Protein concentrations were determined using a bicinchoninic acid protein assay (BCA; Pierce), and 50  $\mu$ g protein was loaded per lane of

an 11% to 23% gradient gel (Phorcast; Amersham, Arlington Heights, IL). The separated retinal proteins were transferred to nitrocellulose paper, blocked in 3% BSA in TBS, and incubated overnight in anti-phosducin (1:1500) diluted in 1% BSA in TBS. The blots were washed in TBS containing 0.5% Tween (TTBS) and incubated with goat anti-rabbit IgG conjugated to HRP (1:2000; Dako, Carpinteria, CA) for 1 hour. After washing in TTBS and TBS, the blots were placed in HRP color-development reagent (BioRad) for 15 minutes, rinsed in distilled water, and air dried.

**Guanylate Cyclase Activating Protein-2.** For the analysis of GCAP-2 levels, 20  $\mu$ g whole retinal homogenate was loaded onto a 5% to 12% gradient gel. The filter was blocked for 2 hours in 5% nonfat dry milk in TBS, and then the anti-GCAP-2 was added at a dilution of 1:5000 in TBS and BSA, and the blot was incubated overnight. Finally, the blot was washed, placed in enhanced chemiluminescence (ECL) reagents (Pierce) for 5 minutes, and exposed to film specific for the ECL system. Changes in the density of the bands on the blot were measured using a phosphorimager (Molecular Dynamics, Upland, CA).

## RESULTS

### TdT-dUTP Terminal Nick-End Labeling

It is already known that, after detachment, cell bodies in the outer nuclear layer (ONL) label by the TUNEL technique.<sup>16</sup> We determined that some TUNEL-positive photoreceptors (Figs. 1A, 1C) also labeled with the antibody to either calbindin D (Fig. 1B) or cone opsin (Fig. 1D), identifying them as cones. Although there were some TUNEL-labeled cells present at all detachment times examined, both the number present in the ONL and the percentage of these cells double labeled with the antibody to calbindin D were highest at 3 days of detachment (Table 2). These data also show that the number of anti-calbindin D-labeled cells decreased dramatically after 1 day of detachment.

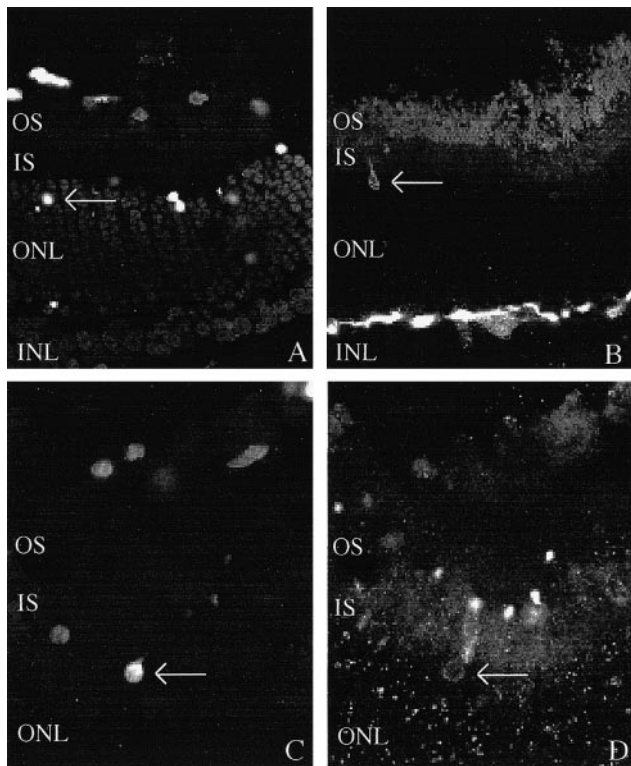
### Opsins

In the normal cat retina, the M/L-cone opsin, S-cone opsin, and rod opsin antibodies labeled the OS of their respective photoreceptor types (Figs. 2A, 2D, 2G). After detachment, the responses of the S- and M- cones (cats do not have L-cones) were qualitatively the same, although the S-cones appeared to lose their opsin protein more quickly. In both cases, at 3 days the antibodies continued to label truncated OS, but heavily labeled the plasma membrane of the entire cell as well (Figs. 2B, 2E). At this time, many fewer labeled cells were detected, although the pattern of cells that labeled was the same as at 1 day (data not shown). By 7 days, labeled cells were rare (Figs. 2C, 2F), but occasionally, labeled cell bodies without OS (Fig. 2C) or with just fragments of labeled OS (Fig. 2F, arrow) were observed.

Rod photoreceptors showed robust labeling at all times with the Rho-4D2 antibody. The redistribution of opsin into the proximal plasma membrane after detachment (Figs. 2G-I) was much more dramatic than in the cones, because bright labeling outlined the whole cell all the way to the synaptic-terminal (ST). As long as OS were present, they labeled as in normal retina, but as they shortened, more opsin labeling appeared in the cell body.<sup>20</sup> The antibody to rod opsin also gave a strong signal from the Golgi/rough endoplasmic reticulum (RER) region of the IS in normal retina, whereas we observed no labeling of this region with the antibodies to the cone opsins (Figs. 2A, 2D, 2G). This labeling tended to become more diffuse after detachment, as the organelles of the IS became dispersed (Figs. 2H, 2I).<sup>11</sup>

The quantitative ELISA for rod opsin showed a decrease to approximately 50% of control levels at 3 days and to 10% of control levels at 7 and 28 days (Fig. 2J). We were not able to





**FIGURE 1.** (A–D) Confocal images of 1-day retinal detachments. Separated images from TUNEL double-labeling experiments (A, C) with either anti-calbindin D (B) or anti-M/L cone opsin (D). At 1 day after detachment, there were several TUNEL-positive cells in the ONL, some of which could be identified as cones. (A, B, arrows) Cell bodies labeled with TUNEL and calbindin D, respectively. Note in (B) that calbindin D also labeled horizontal cells in the inner nuclear layer (INL). (C, D, arrows) Cells labeled with TUNEL and anti-M/L cone opsin, respectively. In (D) most of the labeling occurred around the IS, with only faint labeling of the cell body.

obtain a reliable signal with cone opsin antibodies, using either the ELISA or Western blot analysis, presumably because of the relatively sparse cone population in the cat's retina.<sup>22,28</sup>

### Phosducin

In normal retina, the antibody to phosducin most heavily labeled the IS and ST and lightly labeled the OS and cell bodies of both rods and cones (Fig. 3A). At one day of detachment, the ST labeling had almost completely disappeared, whereas the IS and cell body labeling increased slightly in intensity (Fig. 3B). As detachment time increased, this pattern became even more exaggerated, so that by 28 days the whole photoreceptor layer was intensely labeled (Fig. 3C). It appeared that rods and cones reacted similarly (i.e., labeling of the IS layer and ONL was homogeneous). Both the ELISA and Western blot data support

the immunocytochemical observations (Figs. 3D, 3E). The Western blot also showed one band at 28 kDa that is similar to molecular weights for phosducin in other species.<sup>29</sup> By 28 days ELISA shows a protein level that was 180% of that found in the control retina.

### Peripherin/Rds

A detailed analysis of changes in the distribution of this protein has appeared elsewhere.<sup>20</sup> Briefly, labeling was confined to the OS (to the disc rims) in normal retina, with occasional signal from small vesicles in the IS (Fig. 4A). The OS continued to label as long as they were present (Figs. 4B, 4C), but as they degenerated the pattern of labeling shifted to an accumulation of vesicular structures throughout the cytoplasm of the cell (Fig. 4C). The ELISA results showed a small decrease (~20%) in the amount of peripherin/rds in the retina at 3 days of detachment with a further decline to approximately 25% of control levels at 7 and 28 days of detachment (Fig. 4D).

### Transducin- $\alpha$

In the normal retina, antibody TF-15 to transducin- $\alpha$  labeled only the rod photoreceptors and labeled the IS most heavily, with lighter labeling of the OS and ONL (Fig. 4E). After 3 days of detachment, there was a slight decrease in the IS labeling and a slight increase in the labeling of the rod cell bodies (Fig. 4F). There was variability in labeling in the longer-term detachments where it sometimes showed a significant decline (Fig. 4G), whereas in other cases it remained relatively unchanged by comparison to the 7-day detachments. This variability was reflected in the ELISA (Fig. 4H) data in which there was a decline to 67% of normal at 3 days and then the amount remained relatively constant at approximately 70% at 7 and 28 days.

### Arrestin

The antibody to arrestin labeled primarily the OS and ST region of rods in normal retina (Fig. 4I). The OS in detachments of 1 day and longer continued to label, but the labeling intensity decreased in a heterogeneous manner (Figs. 4J, 4K). Terminal labeling was no longer detected at 3 days (Fig. 4J). The ELISA data showed a decrease in arrestin levels to approximately 70% of normal at 3 days and to 40% to 50% of normal at 7 and 28 days (Fig. 4L).

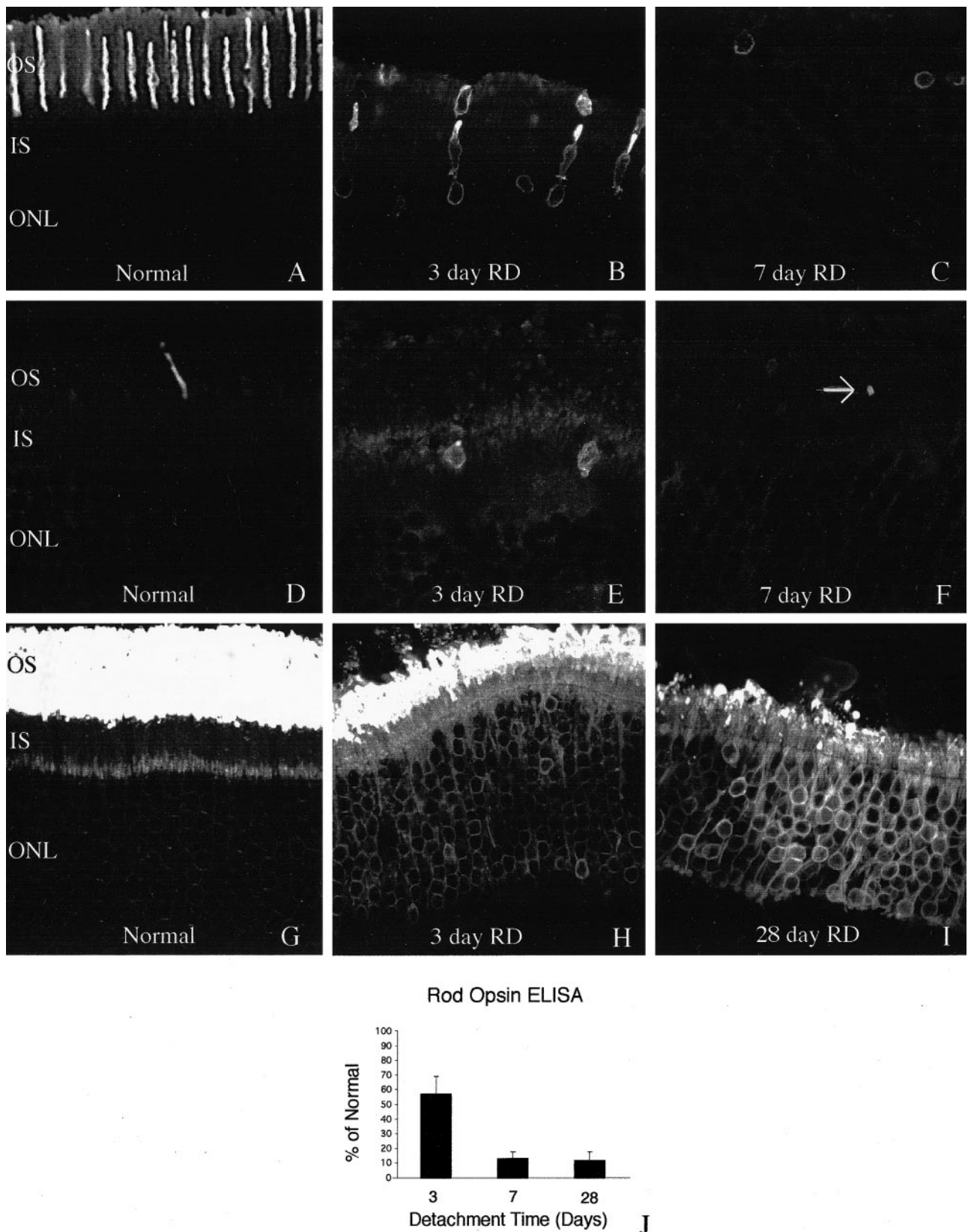
### Guanylate Cyclase Activating Protein-2

The antibody to GCAP-2 labeled only the OS of the rod photoreceptors (Fig. 4M), and continued to label whatever portion of the OS remained after detachment (Figs. 4N, 4O). We never detected this protein outside the OS in normal or detached photoreceptors. Although we found this antibody specific to rod OS in the feline retina, Otto-Bruc et al.<sup>30</sup> observed that it labeled both rods and cones in the monkey retina.

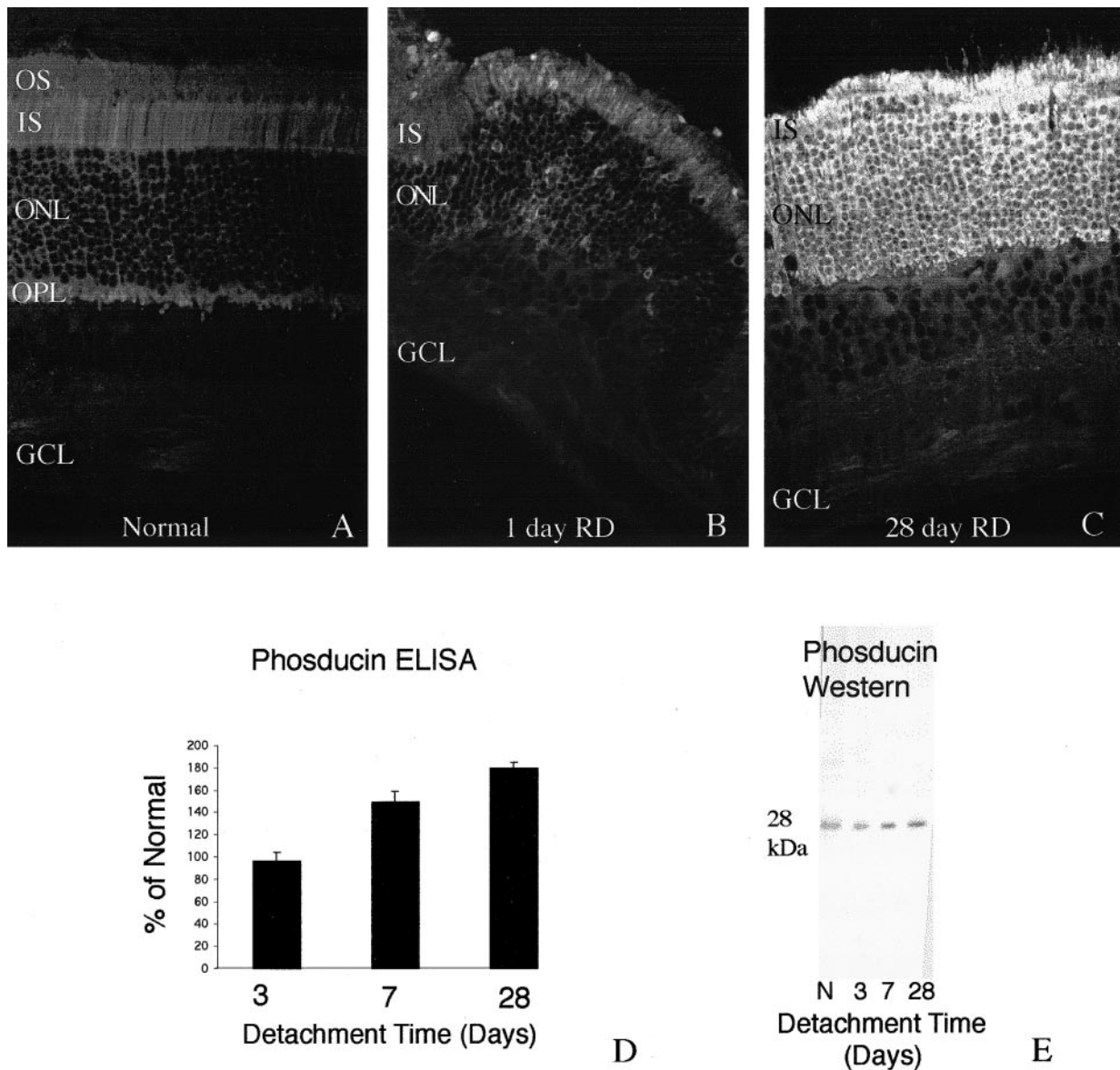
**TABLE 2.** Counts of Calbindin-D- and TUNEL-Labeled Photoreceptors in Normal and Detached Retina

	Normal			1-Day RD			3-Day RD			7-Day RD		
	Cat 1	Cat 2	Avg.	Cat 1	Cat 2	Avg.	Cat 1	Cat 2	Avg.	Cat 1	Cat 2	Avg.
Calbindin D	47	47	47	34	51	44	11	3.3	7	5.8	7.6	6.7
TUNEL	0	0	0	26	40	34	54	67	61	2.4	3	2.7
Calbindin D and TUNEL	0	0	0	1.6	0.4	1	1.6	0.2	0.9	0.3	0.2	0.25
% TUNEL cones			0			2.2			13			3.9

Data are cells per millimeter of retinal tissue. RD, retinal detachment.



**FIGURE 2.** Confocal images of antibody labeling at different detachment times. Labeling with the antibody to M/L cone opsin (A-C), the antibody to S-cone opsin (D-F), and the antibody to rod opsin (G-I). The M-cones were plentiful in the normal retina, and labeling was limited to the OS (A). The S-cones were relatively rare by comparison, but the labeling pattern was the same as that for M-cones in normal retina (D). At 3 days of retinal detachment, the OS of both cone types became shorter, and labeling appeared in the plasma membrane (B, E). By 7 days, almost no OS remained, and the plasma membrane labeling became fainter (C, F). Cells labeled with the antibody to S-cone opsin were rarely observed by 7 days (F, *arrow*). Labeling with the rod opsin antibody was distributed to the plasma membrane quickly after detachment (H); however, in contrast to cones, rods continued to label intensely with this antibody in long-term detachments (I). (J) Results from the quantitative ELISA experiments for rod opsin with results shown as a percentage of the protein in normal retina (i.e., 100%). Error bars, 1 SEM.



**FIGURE 3.** (A–C) Confocal images of anti-phosducin labeling at different detachment times. In normal retina, the antibody labeled OS and photoreceptor STs in the outer plexiform layer (OPL) and, to a lesser extent, in the OS and ONL (A). When the retina was detached, cells in the ONL began to show increased labeling, but the ST labeling disappeared (B). As detachment time increased, there was a steady increase in labeling intensity across the whole ONL (C). (D) Results from the quantitative ELISA experiments for phosducin shown as a percentage of the protein in normal retina (i.e., 100%). (E) Results from a Western blot analysis using the anti-phosducin antibody showed that it labeled only a single band at 28 kDa. The increased intensity of labeling on the blot as detachment duration increased agrees with the results of the immunocytochemical and ELISA experiments. Error bars, 1 SEM.

The antibody to GCAP-2 labeled a single band on a Western blot at the same molecular weight (25 kDa) as the band labeled when purified GCAP-2 was included as a positive control (Fig. 4P). Changes in the density of the bands on the blot from normal to longer-term detachments indicated a decrease to approximately 61% of normal levels at 3 days of detachment with a further decrease to approximately 20% of normal in the long-term detachment (Fig. 4Q), consistent with the immunocytochemical images.

**Guanylate Cyclase Activating Protein-1**

The GCAP-1 antibody labeled cones from OS to ST, whereas rods showed only relatively faint labeling of OS and IS (Fig. 5A). After 1 day of detachment, only very faint labeling was ob-

served (Figs. 5B, 5C). We did not detect a signal with this antibody in the ELISA or Western blot assays.

**Phosphodiesterase-γ**

In the normal retina, the antibody to PDEγ labeled both rod and cone OS (Fig. 5D). Cone photoreceptors labeled intensely, from OS to ST, whereas the rods showed faint labeling for these parts of the cell (Fig. 5D). This labeling pattern was maintained at 3 days, but the rod ST labeling had disappeared, and cone ST labeling was much fainter (Fig. 5E). By 7 days, labeling became sporadic in the cones, decreasing in both its frequency and intensity (Fig. 5F). The labeled ST appeared to have undergone significant changes in shape at these times. The shortened rod OS appeared to label as intensely as in



control retina, but the remainder of the cell was unlabeled. The pattern was similar at 28 days (data not shown). The ELISA data showed a decrease in the amount of PDE $\gamma$  in a 3-day detached retina to approximately 86% of that in control retina (Fig. 5G). By 7 days the amount had decreased to 36% of normal, but at 28 days it had increased slightly to 50%, probably reflecting the variation that occurs between detachments.

### Recoverin

In normal retina the antibody to recoverin labeled both types of photoreceptors in their entirety, but labeled cones more heavily than rods (Fig. 5H). After detachment the labeling became fainter, and cones were no longer distinguishable (Figs. 5I, 5J). The ELISA data for this antibody showed a decrease in recoverin levels after 3 days to approximately 20% of that in normal retina, and this level remained relatively stable with detachment time (Fig. 5K).

### Calbindin D

Outside the area centralis, the antibody to calbindin D labeled the OS, IS, cell bodies, axons, and ST of all cones (Fig. 6A). Within the area centralis, however, approximately 25% of the cones failed to label with this antibody, a phenomenon first identified in primate fovea.<sup>31,32</sup> Although some anti-calbindin D labeling persisted after detachment, the staining with this antibody became unpredictable (Fig. 6B). In general the labeling decreased with detachment time, so that by 28 days, only an occasional stained cone was observed. At shorter times, however, cones in one portion of a section sometimes stained intensely, whereas the remainder of the section showed no staining. Based on the observation of the tissue by bright-field microscopy, the absence of calbindin D labeling did not correlate with the absence of cones.<sup>22</sup> In cones that labeled at day 7 after detachment, a shift in the location of the cell body was observed in some cells (Fig. 6C). Calbindin D also labeled horizontal cells (see Figs. 6B, 6C) and here the labeling intensity remained relatively unchanged after detachment.

### Neuron-Specific Enolase

The whole neural retina labeled with the anti-neuron-specific enolase (NSE; Fig. 6D). Cone IS and ST labeled more intensely than those of the rods (Fig. 6D). At 1 day, labeling was patchy in the ONL and greatly diminished in intensity across the whole retina (Fig. 6E). After 3 days, the only labeling of the photoreceptor layer was attributable to background, whereas some faint labeling sometimes occurred in the inner retina (Fig. 6F).

### Peanut Agglutinin

The lectin PNA recognizes terminal disaccharides of proteoglycans and binds to the extracellular domains around cone OS and IS, where it defines the cone matrix sheath (Fig. 7A).<sup>33</sup> In

our experiments, PNA binding outlined the entire cone from the OS to ST (Fig. 7A). At 1 day of detachment, labeling was the same as in control retina, except that there was an obvious shortening of the cone-matrix sheath (not shown). By 3 days, the PNA labeling in the interphotoreceptor space appeared disorganized (Fig. 7B). The cone-matrix sheath disappeared as the OS degenerated, and by 7 days it appeared as if the matrix labeling had collapsed over the IS (Fig. 7C). Labeling of the axon and ST of the cones persisted to some extent after the labeling in the interphotoreceptor matrix had disappeared (the most intense labeling we observed at 3 and 7 days is illustrated in Figs. 7B, 7C). However, by 28 days of detachment only very faint labeling remained in the outer retina, and it could be observed only by significantly increasing the gain on the photomultiplier of the confocal microscope (data not shown).

### Interphotoreceptor Retinoid-Binding Protein

As in other species,<sup>34-42</sup> interphotoreceptor retinoid binding protein IRBP in normal cat retina localized to the interphotoreceptor matrix (IPM) between the outer limiting membrane and the apical surface of the RPE (Fig. 7D). There was no detectable IRBP immunoreactivity at any of the postdetachment times examined (Fig. 7E, 1 day detached).

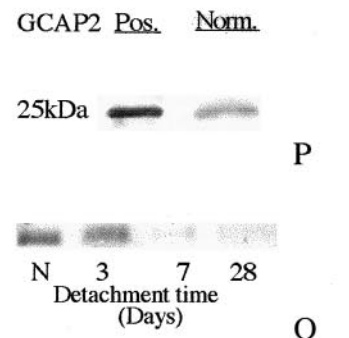
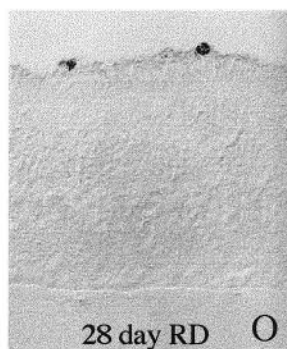
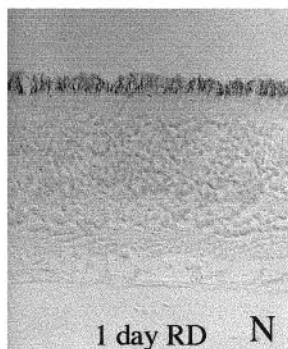
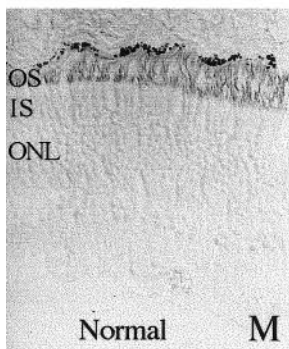
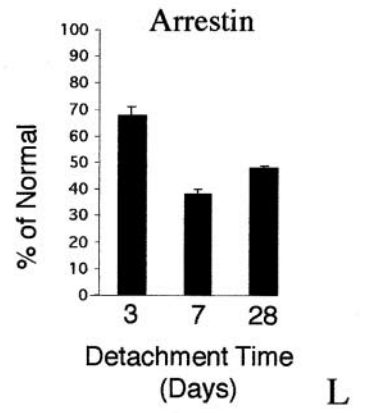
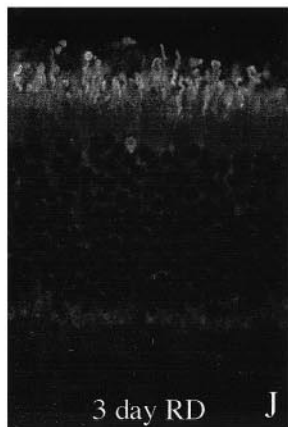
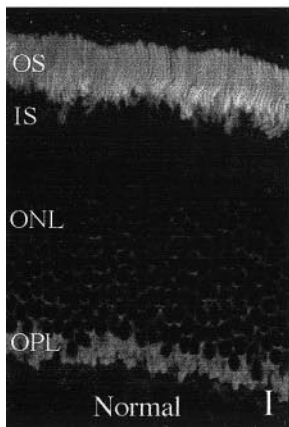
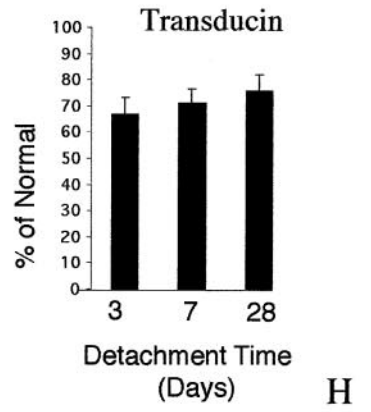
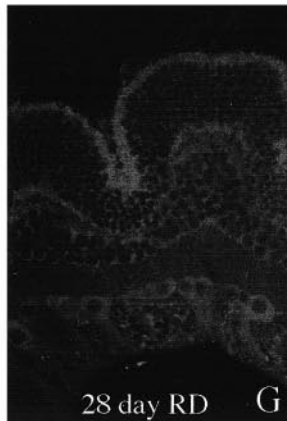
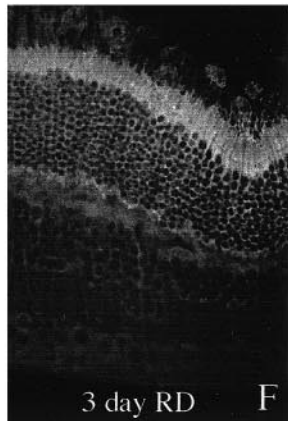
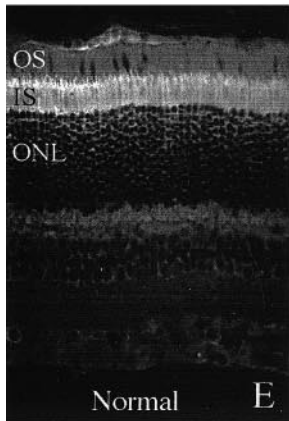
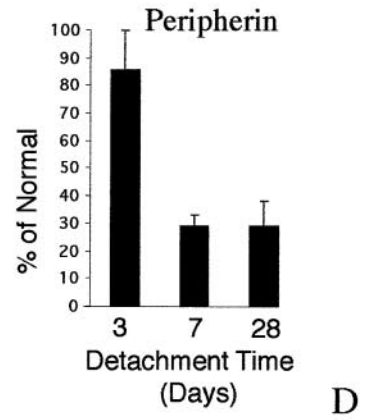
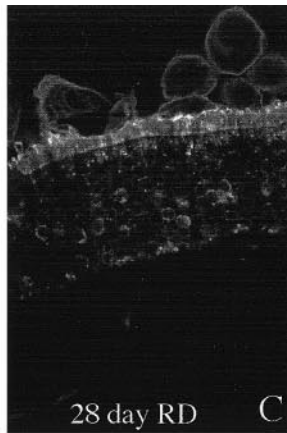
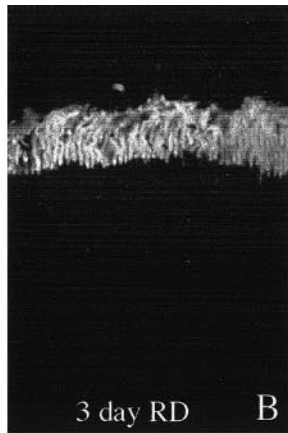
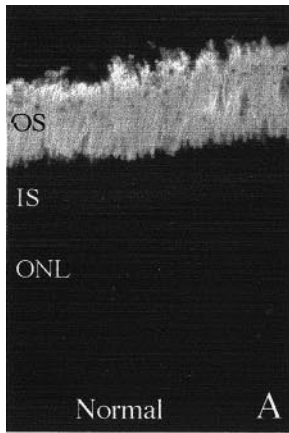
### DISCUSSION

Retinal detachment is a condition that almost certainly produces hypoxia and probably hypoglycemia of the outer retina.<sup>13,43</sup> Part of the response to this condition involves the death of some photoreceptors, and finding ways to prevent these cells from dying would be beneficial. Of equal interest, however, are the changes in photoreceptors that survive a period of detachment, because they retain at least some capacity for recovery. Eventually, we may be able to preserve their overall status or find some way to improve their regenerative capacity. In the current study, we examined the responses of a wide variety of molecules that are associated with photoreceptors. The data continue to emphasize that photoreceptors do not simply degenerate after detachment, but undergo very specific changes, some of which lead to structural degeneration. The data also show clearly that rods and cones react differently.

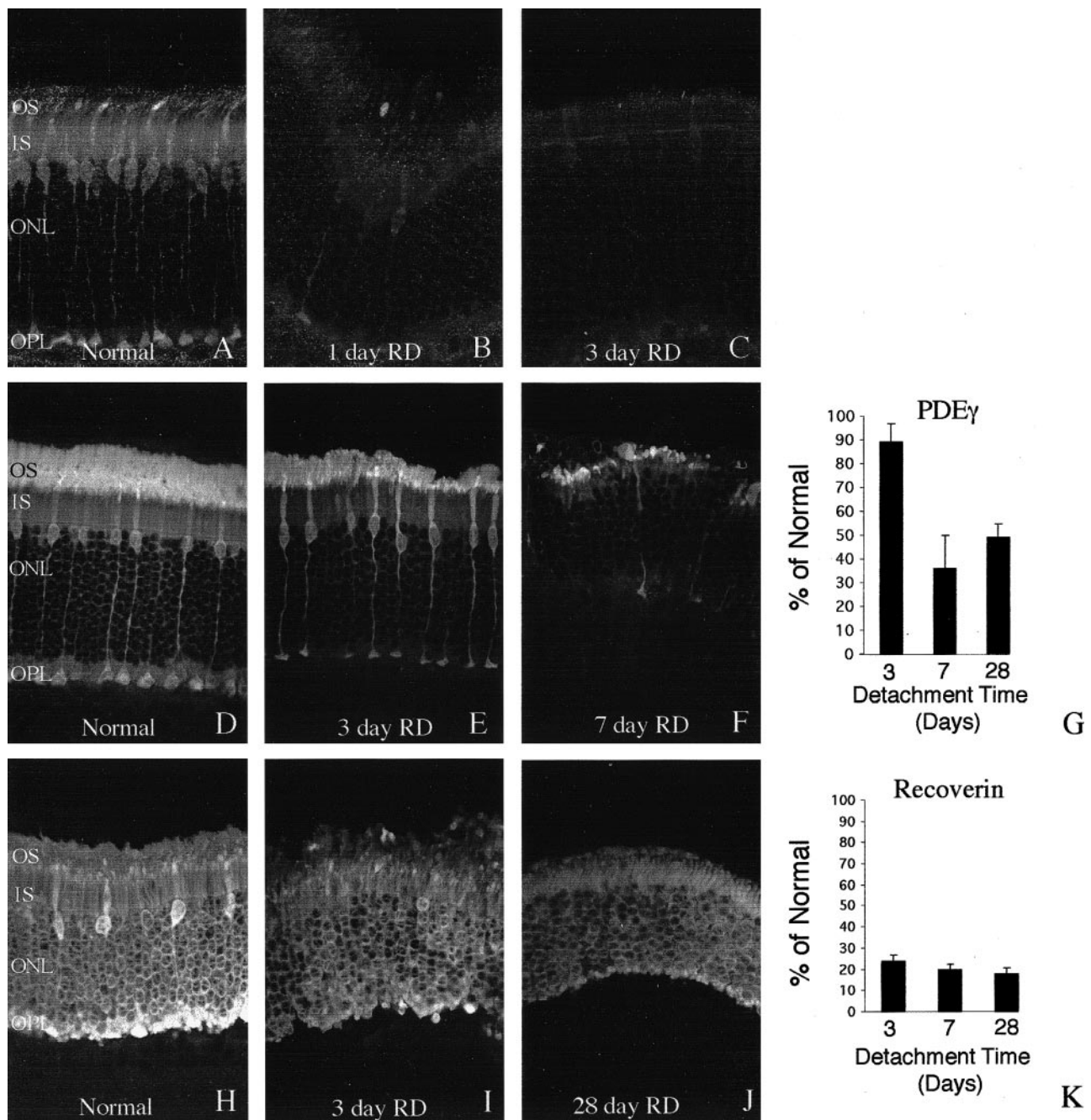
### Photoreceptor Death and Survival

Many photoreceptor cells die after detachment,<sup>11,17</sup> and this cell death is most likely through apoptosis.<sup>13,16,44,45</sup> How many cones die and by what mechanism, seemingly simple questions, have not been easily answered in the species commonly studied, because their eyes are heavily rod dominated. Even with cone-specific probes such a study in rod-dominated retinas is difficult because of the rapid decline in the expres-

**FIGURE 4.** (A-C) Confocal images of anti-peripherin/rds labeling at different detachment times. The antibody labeled rod OS in normal retina (A) and continued to label OS as long as they were present (B). As OS degenerated, label accumulated over structures in the cytoplasm of the rods (C). (D) Results from the quantitative ELISA assay for peripherin/rds shown as a percent of the protein in normal retina (i.e., 100%). (E-G) Confocal images of anti-transducin- $\alpha$  labeling at different detachment times. In normal retina, the OS and IS of the photoreceptors were labeled most intensely (E). In detached retina, this labeling increased slightly in the ONL (F) and then became highly variable, with some regions showing almost no labeling (G). (H) Quantitative ELISA data for transducin- $\alpha$  as a percentage of the amount in normal retina shows that, overall, there was a reduction in the amount of protein by 3 days, but then a relatively steady state level was maintained. (I-K) Confocal images of anti-arrestin labeling at different detachment times. In normal retina the antibody intensely labeled the OS layer as well as ST in the outer plexiform layer (I; OPL). The OS labeling persisted and correlated with the amount of OS material remaining, but the ST labeling disappeared after 3 days of detachment (J, K). (L) Quantitative ELISA data for arrestin as a percentage of the amount in normal retina. (M-O) Bright-field images of anti-GCAP-2 labeling at different detachment times. Labeling was restricted to rod OS in normal retina (M). In detached retina, labeling remained associated only with the OS remnants (N, O). (P) Western blot of purified GCAP-2, run as a positive control (Pos.), and of normal retinal homogenate (Norm.), demonstrating that the antibody labeled bands at similar molecular weights. (Q) Western blot results with the GCAP-2 antibody showing the decline in the amount of protein that occurs with detachment time. Error bars, 1 SEM.



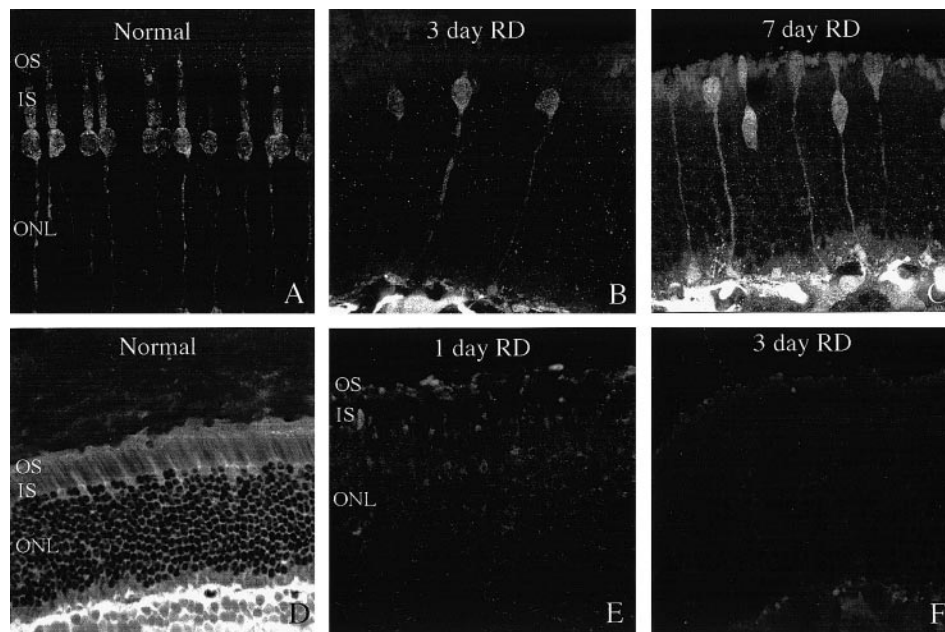




**FIGURE 5.** (A-C) Confocal images of labeling with the antibody to GCAP-1 at different detachment times. In normal retina the antibody labeled cone OS and IS and cone ST in the OPL (A). This labeling disappeared rapidly after detachment (B, C), although an occasional labeled cone (B) could still be found at 1 day. (D-F) Confocal images of labeling with the antibody to the  $\gamma$ -subunit of PDE at different detachment times. This antibody labeled all OS in normal retina but intensely labeled the remainder of the cone photoreceptors as well (D). At 3 days after detachment, labeling of the OS rudiments continued, although it appeared to decline in intensity in some regions. The labeling of the cones overall became fainter, and the cone ST began to show a distinctive change in shape (E). By 7 days, OS labeling was erratic, and labeling of the inner portions of the cones was weak and occurred only rarely in the ONL (F). (G) Quantitative ELISA data for the PDE- $\gamma$  antibody expressed as a percentage of the protein in normal retina (i.e., 100%). (H-J) Confocal images of labeling with the antibody to recoverin at different detachment times. In normal retina, this antibody labeled the entire photoreceptor layer, but labeled cones with more intensity than rods (H). This pattern persisted after detachment, but with decreasing overall intensity (I, J), and the organized labeling of the ST shown in (H) disappeared. (K) Quantitative ELISA data for the antibody to recoverin expressed as a percentage of the protein in normal retina. There was a rapid decline in the amount of this protein in the retina. Error bars, 1 SEM.

sion of cone markers (described later).<sup>21-23,46</sup> During the first few days of detachment however, the cones still reliably labeled with the antibody to calbindin D. Hence, cells that double label with this antibody and with TUNEL are certainly cones dying, presumably by apoptosis. Although the number

of cells acquired during our study were not large because of the paucity of cones, it appears that the same pattern of cell death occurs in cones as in rods. Cones began dying during the first day, probably with a maximum occurring around 3 days, and continued to some extent as long as the retina was de-



**FIGURE 6.** (A–C) Confocal images of labeling with the antibody to calbindin D at different detachment times. This antibody labeled cone cells in normal retina (A) as well as horizontal cells (B, C, *bottom* of images; the horizontal cell layer is not shown in A). After detachment, the labeling became much more faint and erratic, staining some cones in their entirety but not others (B, C). The results shown in (C) were unusual in the number of cones labeled within the field, but it demonstrates the shift in position of the cone cell bodies that can occur after detachment. (D–F) Confocal images of labeling with the antibody to NSE at different detachment times. In normal retina the antibody labeled the entire photoreceptor layer with cones showing slightly more intense labeling than rods (D). Labeling of the photoreceptor layer was significantly less intense than that occurring in the inner retina (D, *bottom*). After detachment, labeling rapidly disappeared from the photoreceptor layer and inner retina (E, F).

tached. Thus, there did not appear to be a large chronological difference in the pattern of rod and cone cell death. Humans recover cone-based vision after reattachment, and so significant numbers of cones must survive. This is supported by the fact that we did not find massive numbers of TUNEL-labeled cones at any time examined, and that at all times it was possible to find surviving cones by careful search of the ONL.

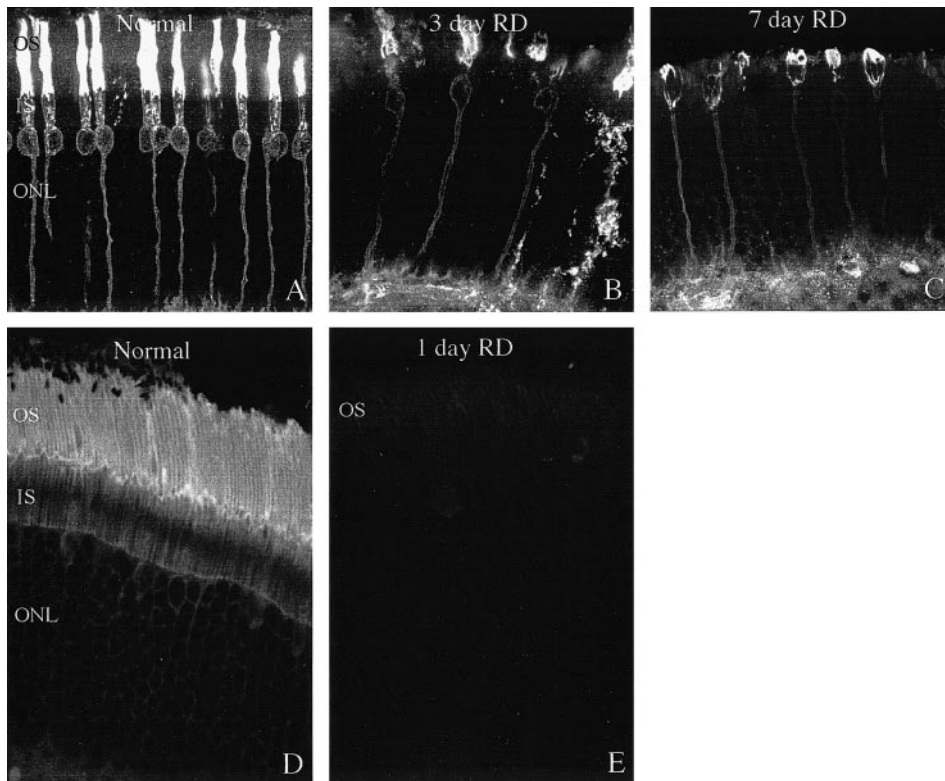
### Photoreceptor Deconstruction: Changes in Molecular Expression

Figure 8 summarizes the data for each probe used in this study. Immediately obvious is the great difference between rods and cones.

The loss of most of the OS is a response common to all photoreceptors after detachment. Cones appear to behave similarly to rods in this regard.<sup>13</sup> On average, the OS layer is reduced to approximately 10% of normal width after a week of detachment,<sup>13,14</sup> but there is no strict correlation between protein expression by the photoreceptors and OS length. The amount of rod opsin, peripherin/rds, recoverin, and GCAP-2 in long-term detachments correlated reasonably well with rod OS length, and yet the antibody to recoverin recognized the whole photoreceptor and did not even label the OS particularly heavily. By comparison, the antibody to arrestin was heavily localized to the OS, but its expression in the long-term detachments did not match OS length. The antibodies to recoverin, phosducin, transducin- $\gamma$ , and NSE labeled portions of the photoreceptors other than the OS, and their expression in the long-term detachments differed widely, with NSE disappearing completely, whereas phosducin increased to 180% of normal over the course of these experiments. In the case of cones, nearly all the markers became rapidly undetectable within days

of detachment. Their loss correlated with the loss of the OS, a clear difference from the rods. For example, labeling with the antibodies to GCAP-1, NSE, and the lectin PNA was not limited to the OS, and these all declined to undetectable levels as the OS degenerated. Labeling with antibodies to PDE- $\gamma$  and calbindin D, both of which label the whole cone cell in normal retina,<sup>22</sup> similarly disappeared from most of the cone cells. The difference between rods and cones was underscored by the data with the transducin- $\alpha$  antibody. The ELISA data showed that close to 70% of this protein remained in a retina detached for 28 days. This correlates well with the fairly intense immunofluorescent label that remained in the rods but not in adjacent cones, which are devoid of label. The loss or the inconsistent nature of labeling with the cone markers makes quantitating cone survival after detachment difficult.<sup>22</sup>

In addition to the quantitative differences in the expression of the various molecular markers, there were also differences in their patterns of expression after detachment. The increased labeling of the plasma membrane of rods with antibodies to rod opsin is an absolute hallmark of detachment and probably all photoreceptor degenerative diseases.<sup>19,20</sup> Both types of cone exhibited a variation in which they show a rapid redistribution, but then a rapid loss of labeling. Our impression was that the S-cones underwent this sequence slightly faster than the M-cones, but we have no unequivocal data to show this to be the case. In the rare cone in which a small amount of OS survived in a long-term detachment, only the OS labeled with an anti-opsin antibody. The data suggest that rods may continue producing their opsin but that cones do not. This is supported by data showing the continued detection of opsin message in rods but not in cones after detachments.<sup>47</sup> Although rhodopsin and peripherin/rds are both targeted to the disc membranes of the



**FIGURE 7.** (A–C) Confocal images of labeling with the lectin PNA at different detachment times. In the feline retina, the lectin intensely labeled the matrix around the cone OS as well as the entire cell (A). When the retina was detached, this labeling became weaker and fragmented, often only partially outlining the surviving cones (B, C). (D, E) Confocal images of labeling with the antibody to IRBP in normal and 1-day detached retina. In normal retina labeling with this antibody was restricted to the matrix surrounding the photoreceptor OS and IS (D). We detected no labeling in retinas detached for 1 day (E) or more.

OS, they redistribute to completely different compartments after detachment. The implications of this have been discussed in detail previously.<sup>20</sup> We also found that antibodies to ROM-1, a protein that colocalizes with peripherin/rds at the disc rims, also colocalized with peripherin/rds antibody labeling in intracellular vesicles after detachment (data not shown). The localization of other molecules after detachment remained, for the most part, associated with the compartment in which they occurred in normal retina.

Both NSE and IRBP immunolabeling decreased to undetectable levels in the outer retina very rapidly. Because rods are thought to produce both of these, they clearly form an exception to the data for other proteins expressed in rods. These have in common their release by photoreceptor cells,<sup>56,48–50</sup> and there is evidence for a massive release of NSE in human retinal detachments.<sup>51</sup> NSE may in fact have a neuroprotective role.<sup>50</sup> We also observed a significant decline in labeling intensity in the inner retina with this antibody after detachment (not shown), but the decrease was less consistent than in the outer retina. IRBP is found at decreased levels in animals with progressive rod–cone dystrophy,<sup>52</sup> but in this case it correlates simply with the loss of photoreceptor cells, which was not true in this study. Experiments with skate retina show that infusing IRBP into the subretinal space significantly increased the rate of rhodopsin regeneration that was profoundly affected by even a momentary detachment in those experiments.<sup>53</sup> The presence or absence of IRBP in this region may have a significant effect on the surviving photoreceptors and perhaps on their ability to maintain or regenerate an OS. The analysis of subretinal fluid from human detachments shows the presence of small amounts of IRBP,<sup>54,55</sup> and so it is possible that this soluble protein does not completely disappear in the cat detachments but that it is present in small amounts that are lost during the tissue fixation and processing. It is also possible that contamination of subretinal fluid samples from surrounding attached retina occurred in the human studies, providing the small and variable amount of IRBP in the samples. It will be of

special interest to determine the patterns for the return of NSE and IRBP after reattachment and to see whether they correlate with photoreceptor recovery.

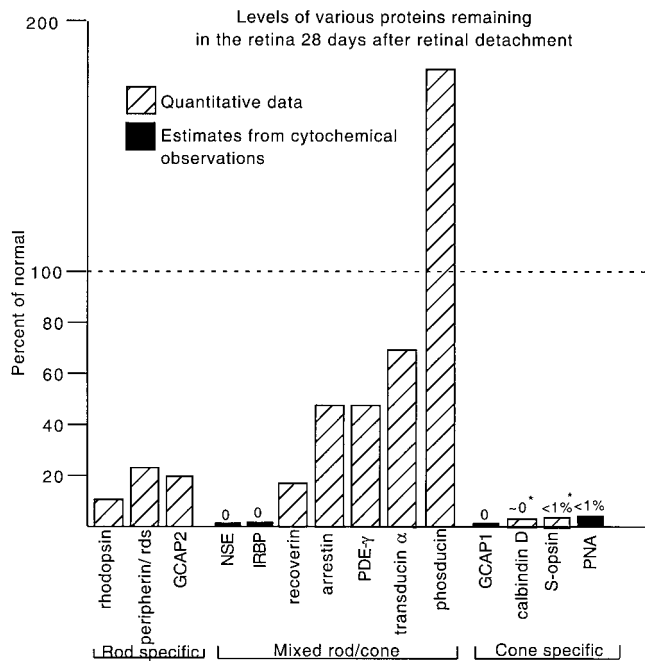
The lectin PNA presents an interesting case, because it did not label a specific protein but rather provided a robust marker for extracellular matrix components around cones (the cone-matrix sheath). Based on these results, the matrix sheath disappeared as the cone OS degenerated. Even the staining that surrounded the inner portions of the cones became unreliable.<sup>22</sup> Intense, fragmented labeling often remained around the inner portions of a few cones, but we do not know whether this indicates continued renewal of these components or simply residual material. Certainly, the PNA-binding domain around cones was disrupted in detached retina, probably an overall reflection of the altered metabolic status of the cones. Experiments are underway to determine whether reattachment results in regeneration of the cone matrix components in tandem with regeneration of the cone OS.

An increase in the amount of phosducin in photoreceptors also has been described during degeneration in progressive rod–cone degeneration (prcd) dogs<sup>52,56</sup> and in transgenic rhodopsin<sup>+/–</sup> mice.<sup>57</sup> Phosducin is the only marker we have studied that continues to be reliably expressed in cones. This conclusion comes from the fact that there were no “blank spaces” in the ONL indicative of unstained cells in the 28-day detachments. Whether this molecule plays some special role in damaged photoreceptors remains unknown. Based on data from the other species it may be hypothesized that its overexpression precedes or leads to cell death. That seems unlikely, given that we know that many photoreceptors can survive months of detachment.<sup>11</sup>

### Rods and Cones

John et al.<sup>58</sup> showed similar rod–cone differences in two specific human mutations that produce retinitis pigmentosa. There are, however, some differences in the results. In those





**FIGURE 8.** A semiquantitative summary of results found with the different probes used in this study. With the exceptions of NSE and IRBP, rods continued expressing, to some extent, all the molecular components studied. In some cases the level of expression remained quite high (arrestin, transducin- $\alpha$ , and PDE- $\gamma$ ) or even increased (phosducin) in the rods. In other cases the level of detection roughly matched the decline in the rod OS with detachment time (rod opsin, peripherin/rds, GCAP-2, recoverin). In the cones the markers rapidly became unreliable (calbindin D, PNA) or nearly undetectable (cone opsins, GCAP-1) after detachment. Labeling with calbindin D and PNA was restricted to only a few patches of cells in the region of detachment. In most areas they too were nearly undetectable. Data are based on counts of antibody-labeled cones in Table 1.<sup>15</sup>

two mutations, the staining disappeared only for three cytoplasmic proteins, 7G6, calbindin D, and X-arrestin, but remained for the cone opsins and peripherin-2 (the cones in their study still had OS). In other mutations examined at late stages when cones are degenerating, reactivity for all the markers remain. After detachment, the changes described herein were consistent—indeed, so consistent that they can be used to assess the overall status of the photoreceptor cells.<sup>13,14</sup> John et al.<sup>58</sup> interpret these changes in the cones to presage the death of specific cells. We do not believe this is the case in detachment. The events occur in all cones, yet we know that significant numbers of cones must survive, because humans recover cone vision after surgical reattachment. We are testing this difference in ongoing reattachment experiments. If the disappearance of the proteins is an indicator of cell death, then we should see very few cones in a retina reattached after being detached for 3 to 7 days, because at that time most of our molecular markers have disappeared from these cells.

Our current interpretation of these and other data<sup>22</sup> is that rods and cones have different mechanisms for surviving a prolonged period of environmental stress induced by detachment. Rods, which are present in large numbers and can probably be lost in fairly large numbers without significantly affecting photopic visual function, continue to express virtually their whole repertoire of protein molecules. Thus, fairly large numbers of rods may be killed, but the surviving rods are poised to begin functioning immediately after return of favorable conditions. The survival of a maximum number of cones is almost certainly critical to the return of functional high-

acuity vision. Cones have a metabolic rate estimated to be approximately 15 times that of rods, based on the number of mitochondria in each of these cell types,<sup>59,60</sup> and detachment with its accompanying hypoxia of the photoreceptor layer may have more profound effects on them. Thus, to survive, it may be imperative that the cones enter a state that conserves metabolic energy. They may accomplish this by halting the production of molecular components that are not essential. This may result in a longer recovery period for cones. Indeed, it is well known among retinal surgeons that both acuity and color vision continue to recover for months in patients with reattached retinas.<sup>61–65</sup> The mechanism proposed in this report may provide an explanation for this observation. Providing an environment that maximizes the ability of both rods and cones to maintain themselves in the face of adverse conditions may slow the loss or hasten the return of vision after retinal reattachment.<sup>13,14</sup>

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