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Differential expression of cone opsin mRNA levels following experimental retinal detachment and reattachment

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Purpose: To identify changes in S- and M-sensitive cone opsin gene expression following retinal detachment (RD) and reattachment.

Methods: Cat retinas were detached for 1, 3, 7, or 28 days, or reattached after 1 h, 1 day, or 3 days of RD and fixed in 4% paraformaldehyde. Pieces of mid-peripheral retina were removed from the same region of each detached, normal (attached), and reattached retina and embedded in paraffin. Paraffin sections (8 μm) were processed for in situ hybridization using S- or M-cone opsins, rod opsin, or phosducin riboprobes in vitro transcribed from cat partial cDNAs. Labeled cells were counted to obtain the number of labeled cells/mm retina.

Results: The number of cells labeled with the anti-sense cone opsin riboprobes, and the intensity of this label, decreased after RD. The number of cones labeled with the anti-sense S-opsin riboprobe decreased to 42% of normal at 3 days of RD. The number of M-opsin mRNA-positive cones decreased to 4% of normal at 3 days of RD. The number of cells positive for M-opsin or S-opsin mRNA recovered to near normal levels after reattachment. Phosducin and rod opsin mRNA labeling was near normal in surviving rod photoreceptors after RD.

Conclusions: Cones and rods behave differently after detachment. There are significant obstacles to overcome in order to study the responses of cones after RD because surviving cells no longer label with antibodies used as cone markers in normal retina. The results of this study show that: (1) After RD, surviving cones decrease their expression of opsin mRNA while rods do not; (2) Upon reattachment of the retina, the cones once again begin to express their opsins; (3) Most cones survive short-term detachments; and (4) Defects in cone-based vision after reattachment may not be based mainly on the loss of cones but due to other changes in these cells, for example, reduced phototransduction and/or changes in synaptic connectivity to second order neurons.

Loss of sight in diseases such as retinitis pigmentosa is caused by the degeneration and inevitable death of photoreceptors [1]. Currently, there are no therapies that reverse the course of these diseases. Retinal detachment also induces degenerative changes in photoreceptors, including the loss of outer segments and cell death, but visual recovery to near pre-detachment acuity can occur upon retinal reattachment [2-9]. While there are other changes in the retina that probably complicate the return of normal vision after reattachment [10,11], outer segment recovery clearly plays a pivotal role. In a recent series of experiments we used a panel of 19 different molecular probes to study the response of photoreceptor proteins to different periods of detachment [12]. In particular, we found significant differences for the pattern of expression of opsin in rods and cones, which may reflect different mechanisms for survival in these two types of photoreceptors. Within a day of detachment, antibodies to rod opsin and to the short-wavelength-sensitive (S) and middle-/long-wavelength-sensitive (M/L) cone opsins began to intensely label the plasma membrane surrounding the whole photoreceptor cell. In the 1-3 day experimental detachments, the labeling with the anti-cone opsins began to disappear from the surviving cells. Finding a cone labeled with the anti-opsin antibodies was a rare event after 7 days of detachment [12,13]. Rod opsin immunolabeling remained robust even in detachments of 60 days duration [14] and, using Northern blot and in situ hybridization analysis [15], it was demonstrated that rod opsin mRNA continues to be expressed in long-term detached retinas.

In the experiments described here we used in situ hybridization to determine the pattern of mRNA expression for rod and cone opsins after experimental retinal detachment and reattachment. For comparison we also studied the expression of phosducin mRNA as an example of a protein whose expression increases in both rods and cones after detachment. We used detachment times of 1, 3, 7, and 28 days, the same detachment times used in our previous study of protein expression after detachment [12].

METHODS

Experimental Retinal Detachments and Reattachments: Retinal detachments were produced in the right eyes of cats using a refined version of methods published previously [10]. Briefly, the lens and vitreous were removed and a solution of 0.25% Healon (sodium hyaluronate; Pharmacia, Piscataway, NJ) in a balanced salt solution (BSS; Alcon, Fort Worth, TX) was infused between the neural retina and the RPE using a glass

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micropipette. The contralateral retinas were saved for use as normal controls. Animals were euthanized at 1, 3, 7, and 28 days after the detachment (n=2 for each time point) and the eyes were placed in 4% paraformaldehyde in 0.1 M Na Cacodylate buffer, pH 7.4.

For the reattachment procedure, the detachment was produced with BSS in place of Healon to allow for a better reapposition of the retina to the RPE. At 1 h, 1 day, or 3 days the retina was reattached (n=3 for each time point). The animals with reattachments at 1 h or 1 day were allowed to survive until 3 days after the detachment surgery, while those with reattachments after 3 days were allowed to survive for a total of 28 days after the detachment. To produce a reattachment, first a fluid-gas exchange was performed, being careful to drain the fluid from under the retina. After the retina was flat, 20% sulfur hexafluoride in filtered room air was flushed through the eye. The contralateral eyes were used as normal controls. All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Paraffin Embedding: Pieces of mid-peripheral retina, approximately 4 mm square, were excised from the eye and rinsed in phosphate buffer. The tissue was dehydrated in a graded ethanol/H_2O series/ (30, 70, and 100%), submersed in 100% toluene at room temperature, and incubated at 60 °C in toluene and paraplast. Finally, the tissue was incubated in multiple solutions of 100% paraplast and then submerged in ice water.

cDNA Cloning: RNA was extracted from normal and detached retinas using the technique of Chomczynski and Sacchi [16]. cDNA was made from the purified RNA using an in vitro reverse transcriptase reaction [17]. The phosducin sequence was obtained through the genbank database. The phosducin PCR primers used were as follows: 5'-ACA GGA CCC AAA GGA GTA ATA AAT G-3' (forward); and 5'-GGT AGT AAT CCA TAT TCA TTT AGG-3' (reverse). The PCR product of the expected molecular weight was gel purified using a QIAquick gel extraction kit (Qiagen, Germany), subcloned into pBKS-, and sequenced to confirm its identity. The cat rod opsin cDNA was obtained as a gift from D. Farber (UCLA). The cat cone opsin sequences and cDNA in pBKS+ were obtained as gifts from S. Yokoyama (Syracuse University).

In Situ Hybridization (ISH): The purified cDNA was used to make riboprobe for in situ hybridization. To synthesize riboprobes, linearized cDNAs were in vitro transcribed and labeled with digoxygenin according to the manufacturers protocol (Boeringer Mannheim, Indianapolis, IN). A slightly revised version of previously published methods [18,19] was used for ISH. Wax embedded retinal sections (6 µm thick) from normal and experimental retinas were placed on the same slide. The sections were melted on a hot plate, de-waxed in xylene, and rehydrated in a graded EtOH/H_2O series (100, 70, and 30%). The sections were submitted to proteinase K (5µg/ml; Boeringer Mannheim, Indianapolis, IN) digestion for 10 min, fixed in 4% paraformaldehyde in PBS, and acetylated for 10 min followed by an incubation in 2 mg/ml glycine in PBS. Pre-hybridization solution (deionized formamide, 20X NaCl, $NaPO_4$, EDTA (SSPE), Poly A, herring sperm DNA, yeast tRNA, and Denhardt's solution.), was added to the sections and they were incubated at 65 °C for 1 h in a hydration chamber. Following pre-hybridization, digoxygenin-labeled riboprobe (0.1µg/ml rhodopsin, 0.8µg/ml S-cone opsin, 0.8µg/ml M-cone opsin, or 0.4µg/ml phosducin) in hybridization solution (deionized formamide, 20X SSPE, dextran sulfate, poly A, herring sperm DNA, and tRNA) was applied to the tissue samples. Digoxygenin-labeled sense riboprobes (at the same concentrations as the anti-sense probes) were added to normal retinal sections as negative controls. All sections were incubated with the specified riboprobe at 65 °C in mineral oil overnight. After hybridization, the coverslips were removed and the sections were washed in decreasing concentrations of sodium chloride and sodium citrate solution, pH 7.0 (SSC; 2X, 0.5X, 0.1X) for 10 min at room temperature. The sections were then submittted to a high stringency wash in 0.1X SSC at 68 °C for at least 2 h, blocked in TNTB (Tris, NaCl, Triton-X-100, 0.1% BSA) plus 2% BSA for 30 min, and incubated in anti-digoxygenin-alkaline phosphatase (1:1000 in TNTB) for 1 h. Sections were rinsed in solutions of TNTB, TN (Tris and NaCl, pH 9.3), and TNMZ (Tris, NaCl, $MgCl_2$ and $ZnCl_2$, pH 9.3). The chromagen mixture [NBT and BCIP (Fisher, Tustin, CA), and 10% PVA in TNMZ] was then added to the sections and incubated at 37 °C in the dark for up to 24 h. After sufficient development the color reaction was quenched with H_2O. Finally, retinal sections were dehydrated (in 30, 70, and 100% EtOH), covered with a glass coverslip, and visualized using standard light microscopy. Sense and anti-sense retinal sections were developed for the same amount of time.

Quantitation: Photoreceptor cells labeled with either the anti-sense probe for S-cone opsin or M-cone opsin were counted in 20 sections each of normal, detached, and reattached, mid-peripheral retina. The length of each of the retinal sections was measured and the number of labeled cells per mm of retina was calculated. The average total length of retina examined for each condition was 200 mm of normal retina and 40 mm of detached or reattached retina. Statistical analysis was performed using ANOVA. For the M-opsin studies: df=6,18; F=12.2; and p<0,001. For the S-opsin studies: df=6,17; F=4.43; and p<0.01.

RESULTS

Retinal Detachment: In normal retina, the anti-sense riboprobes for the S-, M-, and rod opsins all heavily labeled the inner segments and cell bodies of specific photoreceptors (Figure 1A,B,C). The probe for phosducin, on the other hand, lightly labeled all of the cell bodies in the outer nuclear layer (ONL; Figure 1D). Cells labeled with the S-opsin probe were much more infrequent than those labeled with the M-opsin probe (Table 1), as would be expected based on the distribution of the S- and M-cones in feline retina [13,20]. When the retina was detached, there was a rapid decline in the number of cells labeled with the cone opsin probes (Figure 1E,F,I,J). Our observations suggest that all of the rod cells present at any detachment time still label with the anti-sense probe to rod opsin at an intensity similar to that seen in control retina (Figure 1G,K). Likewise, the probe for phosducin continued

to recognize all cells in the ONL, with slightly more intense labeling than is observed in normal retina (Figure 1H,L). In the 28-day detachments, this probe labels more intensely a subset of cell bodies on the most distal edge of the ONL (Figure 1L). On average, we found about 37 cells/mm of retina labeled with the S- and M-opsin probes in the control eyes (Table 1). This number decreased to 7.6 cells/mm at 1 day of detachment, 2.6 cells/mm at 3 days of detachment, and 1.9 cells/mm at 28 days of detachment (Table 1). The greatest change occurred in the number of labeled M-cones, this number dropping to 15% of the value for control retina in the 1 day detachments, and about 4% and 2% in the 3 and 28 day detachments (Table 1). By comparison, about 73% of the S-cones found in control retina were labeled in the 1 day detachments, with a decline to 42% and 36% of control values in the 3 and 28 day detachments, respectively (Table 1). The S-cones

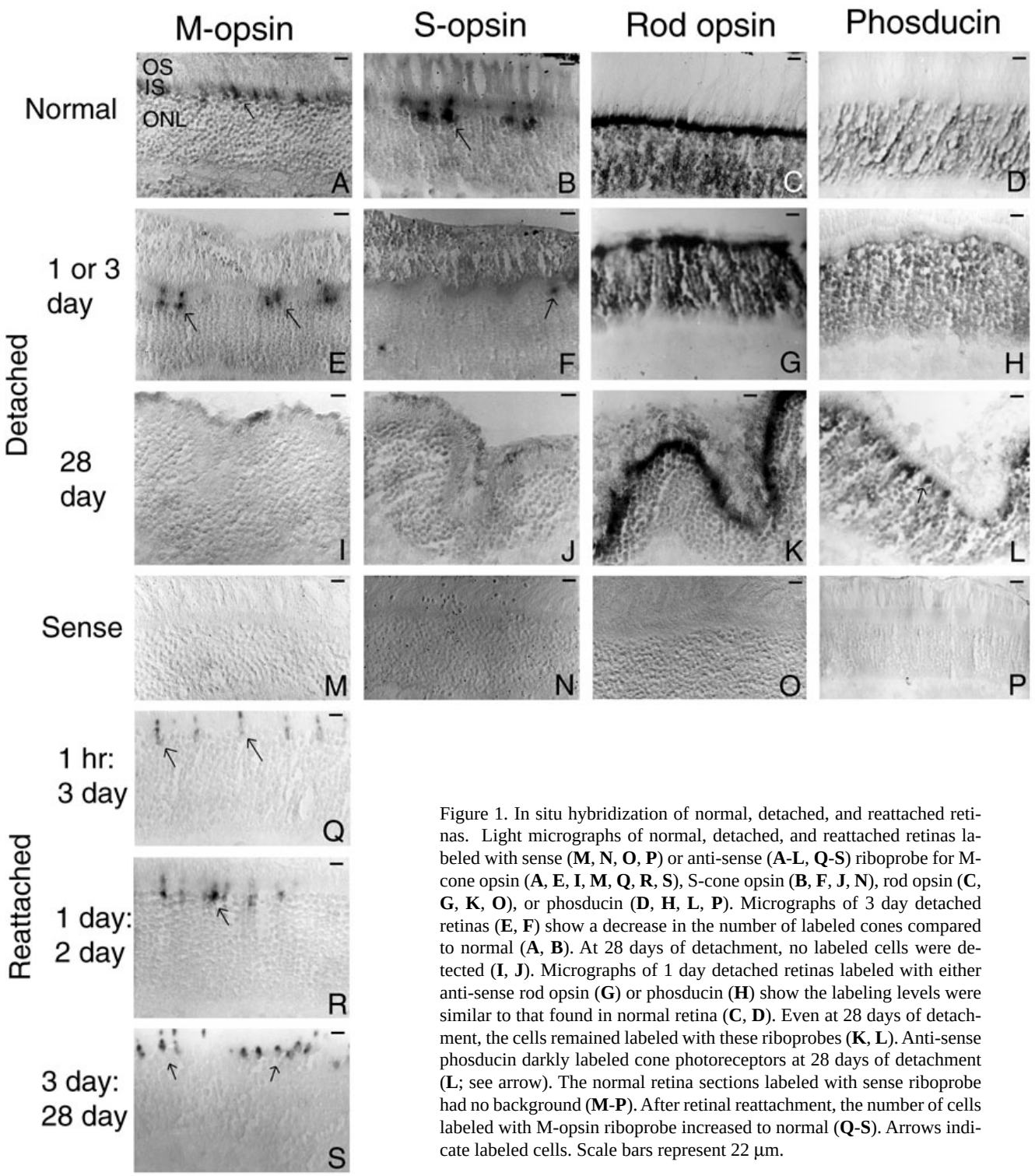

Figure 1. In situ hybridization of normal, detached, and reattached retinas. Light micrographs of normal, detached, and reattached retinas labeled with sense (**M**, **N**, **O**, **P**) or anti-sense (**A**-**L**, **Q**-**S**) riboprobe for M-cone opsin (**A**, **E**, **I**, **M**, **Q**, **R**, **S**), S-cone opsin (**B**, **F**, **J**, **N**), rod opsin (**C**, **G**, **K**, **O**), or phosducin (**D**, **H**, **L**, **P**). Micrographs of 3 day detached retinas (**E**, **F**) show a decrease in the number of labeled cones compared to normal (**A**, **B**). At 28 days of detachment, no labeled cells were detected (**I**, **J**). Micrographs of 1 day detached retinas labeled with either anti-sense rod opsin (**G**) or phosducin (**H**) show the labeling levels were similar to that found in normal retina (**C**, **D**). Even at 28 days of detachment, the cells remained labeled with these riboprobes (**K**, **L**). Anti-sense phosducin darkly labeled cone photoreceptors at 28 days of detachment (**L**; see arrow). The normal retina sections labeled with sense riboprobe had no background (**M**-**P**). After retinal reattachment, the number of cells labeled with M-opsin riboprobe increased to normal (**Q**-**S**). Arrows indicate labeled cells. Scale bars represent 22 μm.

are sparse in normal feline retina [13,19], and a reduction to less than half possibly creates sampling variability across a relatively small retinal area such as occurs when using histologic sections. Indeed, in the 7 and 28 day retinas, there are long expanses in the histological sections that showed no labeled cells with either probe (Figure 1I,J). Only the decline in cones labeled with the M-opsin probe in the 3 and 28 day detachments were statistically significant. Reacting retinal sections with the sense cRNA for each of the various probes produced no labeling (Figure 1M,N,O,P).

Retinal Reattachment: Whereas detaching the retina for 1 or 3 days resulted in a decreased number of cones labeled with the probes for both cone opsins, reattachment after 1 h, 1 day, or 3 days of detachment appeared to prevent or reverse this loss (Figure 1Q-S, Table 1). When the retina was detached for 1 h, then reattached for 3 days, we found a statistically indistinguishable number of labeled cones as in the control retina. If the retina was detached for 1 day and reattached for 2 days, there was about 75% of the control population of M-cones and about 90% of the population of S-cones labeled with their respective probes. If the retina was detached for 3 days and reattached for 28 days, the number of both cone types labeled approached 90% of the values in control retina. None of the differences were statistically significant when compared to control retina (Table 1). Labeling of reattached retina with the probe to phosducin produced the same pattern as in control retinas (not shown).

DISCUSSION

In studies of human retinal detachments using methods to detect the presence of the enzyme carbonic anhydrase, it was concluded that the majority of S-cones are either lost or irreparably damaged within a few days of detachment [6]. Indeed, data obtained using a variety of antibodies as markers for cones suggests that, in general, the majority of cones might be lost from the retina after detachment [12,13]. However, John et al. [21] observed in retinas, from patients with retinitis pigmentosa, the presence of cells that did not label with cone-specific markers but had the morphology of cones. During the course of our studies (this report and [12,13]), we have made similar observations in detached retina. As reported here, the cRNA probe that recognizes the mRNA for phosducin heavily labels a population of cells post-detachment that is most likely composed of cones, based on their location and appearance. Our data also suggest that reattachment after 1 or 3 days of detachment, times when there is a decrease in the number of cones labeled using either cone-specific antibodies or mRNA probes, results in essentially a return to the normal number of cones labeled with either the probe for S- or M-opsin. In addition, Jacobs et al. [22] recently used quantitative ERG measures to demonstrate a significant recovery of both S- and M-cone function in reattached ground squirrel retina. Finally, their study also showed no difference between the recovery of S- and M-cone [22]. It is true that some cones are lost after short-term detachment, but the true number that survive is probably much greater than suggested by antibody or mRNA labeling results. We qualify this conclusion only because the overall sparse population of cones in the feline retina, along with significant regional variation in their population, makes accurate sampling in sectioned material difficult. A more precise determination of the number of cones surviving in the reattached retinas will need to come from sampling in retinal wholemounts [14,20], a study that is in progress.

The data presented here extends previous observations of differences between protein expression in rods and cones after detachment to include alterations in mRNA expression [12-14]. They show a specific and significant decline in the number of cones labeled with the opsin probes, and thus presum-

Table 1. Quantitation of M- and S-cone opsin mRNA in normal, detached, and reattached retinas

Experimental Conditions	N	Average number of cells/mm of retina (±SEM) M-opsin	p value	mm retina	S-opsin	p value	mm retina
Control	8	33.8±5.02	-	230	3.29±0.45	-	230
Detached							
1 day	2	5.16±3.00	<0.005	48	2.41±0.84	1.00	32
3 day	2	1.25±1.02	<0.005	44	1.38±1.12	>0.05	25
7 day	2	1.21±1.18	<0.005	23	0.74±0.50	<0.005	37
28 day	2	0.69±0.56	<0.005	40	1.19±0.32	1.00	40
Detached/Reattached							
1 h/3 day	3	40.7±6.30	1.00	21	4.85±0.45	>0.05	35
1 day/2 day	3	25.4±2.33	1.00	25	2.95±0.29	1.00	37
3 day/28 day	3	28.8±2.40	1.00	46	2.96±0.96	1.00	67

Cells labeled with anti-sense riboprobes to either M- or S- cone opsin were counted in sections from control (normal, attached), detached, and reattached retinas. N = number of animals studied. The p values were determined using ANOVA and a post-hoc t-test. For the M-opsin studies: df=6,18 and F=12.2. For the S-opsin studies: df=6,17 and F=4.43.

ably a specific down-regulation of cone opsin mRNA shortly after detachment without a similar change in the rods. It might be supposed that the cones down-regulate message production in general, but this is probably not the case because the probe for phosducin continues to label cells presumed to be cones after detachment. The data presented here lend further support to the hypothesis that rods and cones have different mechanisms for surviving the stress (including hypoxia and probably hypoglycemia) induced by detachment [8,12,13,23,24]. Cones, the photoreceptors for high-acuity vision, may have developed a mechanism for survival that includes stopping the production of specific protein molecules. Rods, which continue to express almost all proteins that we have examined to date [12,13], may be more vulnerable to cell death after detachment, but may also recover more quickly or more completely after reattachment.

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