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Experimental retinal detachment: a paradigm for understanding the effects of induced photoreceptor degeneration

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Introduction

In 1961 Dowling and Gibbons reported that vitamin A deficiency caused rod outer segments to degenerate, and that restoring vitamin A to the diet would allow the outer segments to regenerate. This was a remarkable observation. It was the first real demonstration of vitamin A's ability to regulate the presence or absence of the outer segment, and showed unequivocally the cellular mechanism underlying blindness caused by vitamin A deficiency. It was also the first true experimental demonstration that photoreceptors, a sensory neuron, retain the ability to regenerate their outer segment in adult mammals. Interestingly, in a subsequent study they (Dowling and Gibbons, 1962) also reported the presence of lamellar inclusions in the RPE cells, paving the way to describing a mechanism for outer segment regeneration. As shown later by Young (1967) and others (Young and Bok, 1969), these bodies (phagosomes) are the end result of the disc shedding part of the renewal cycle for vertebrate photoreceptor outer segments (Fig. 1). Thus, these cells have evolved an elegant and highly efficient cellular machinery to assure the structural integrity of their outer segments throughout the lifetime of the animal, and one that allows for outer segment regeneration. New discs and plasma membrane are constantly produced at the base of the cell, and under normal conditions, these are assembled into an outer segment (Fig. 1; Steinberg et al., 1980; see http://insight.med.utah.edu/Webvision/). In 1968, Kroll and Machemer demonstrated that outer segments would also degenerate if the retina is detached from the retinal pigmented epithelium (RPE), and that the phagosomes disappear from the RPE in detached retina (Kroll and Machemer, 1968). Subsequently they also demonstrated that outer segments would regenerate when the retina was reattached, and the phagosomes would reappear, thus demonstrating that the renewal cycle was re-established (Kroll and Machemer, 1969). Retinal detachment is providing us with a method for studying the dynamics and mechanisms of induced outer segment degeneration and regeneration, as well as a model system for studying many other aspects of retinal degeneration. At the same time, it provides direct information on an important condition that causes significant visual impairment, and even loss of sight in humans.

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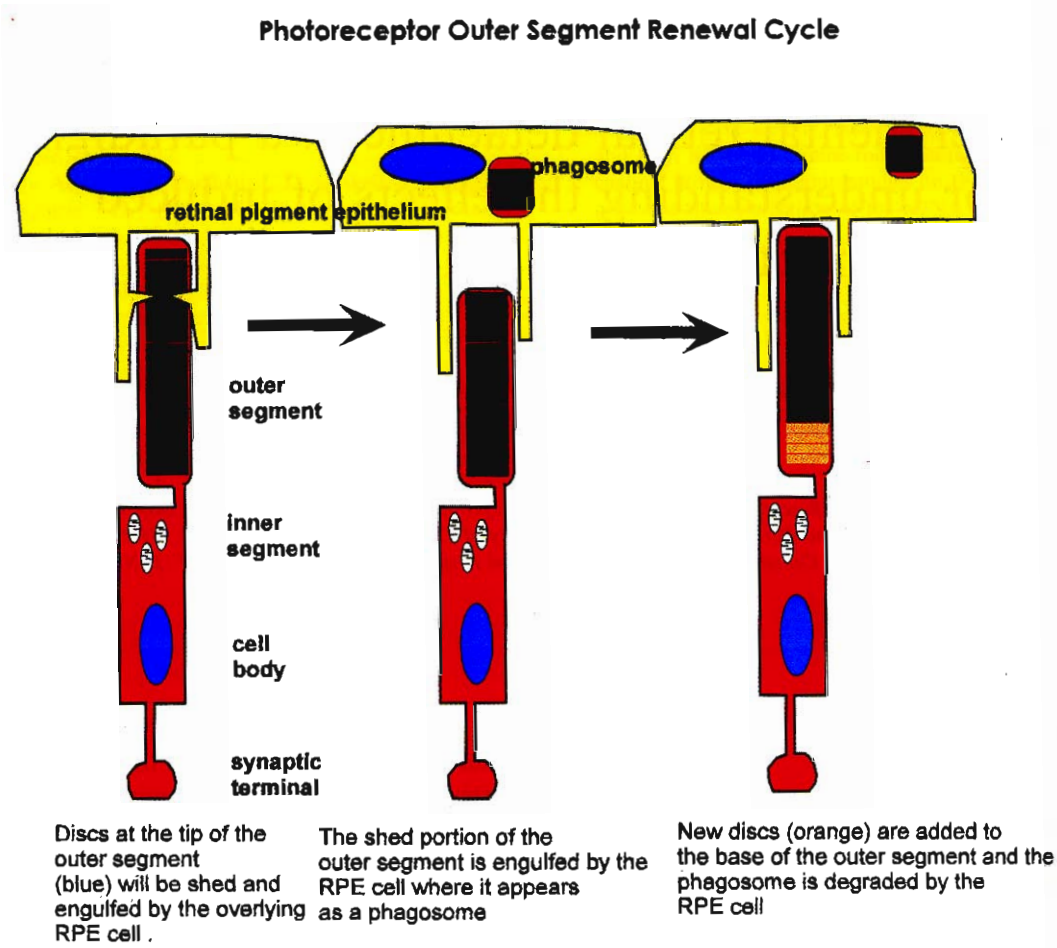

Fig. 1. A diagrammatic representation of the outer segment renewal cycle of vertebrate photoreceptor cells.

What is retinal detachment?

Under normal conditions the photoreceptor outer segments are in close apposition to the apical surface of the RPE (Fig. 2A). Indeed, this surface of the RPE extends long, and often quite elaborate processes that envelope the outer segments, often times reaching all the way to the inner segment (Steinberg and Wood, 1974; Steinberg et al., 1977; Anderson and Fisher, 1979; Fisher and Steinberg, 1982). It is these processes that participate actively in the phagocytosis of disc packets periodically shed from the outer segment. When this apposition is physically disrupted, and the sensory retina becomes separated from the apical surface, creating a new "subretinal" space from the interphotoreceptor space, a retinal detachment occurs (Fig. 2B). This separation can occur due to various reasons (trauma and tearing of the retina, severe myopia, traction by foreign cells on the vitreal surface of the retina, etc.) but always results in the accumulation of fluid between the neural retina and RPE and a subsequent enlargement of the space between the two. Clinically there are different types of detachments (Hay and Landers, 1994), and these have different etiologies, different effects on vision and visual recovery, and are produced by different underlying mechanisms. In our studies we have modeled a rhegmatogenous detachment, or a condition in which there is a small hole across the retinal layers, therefore exposing the

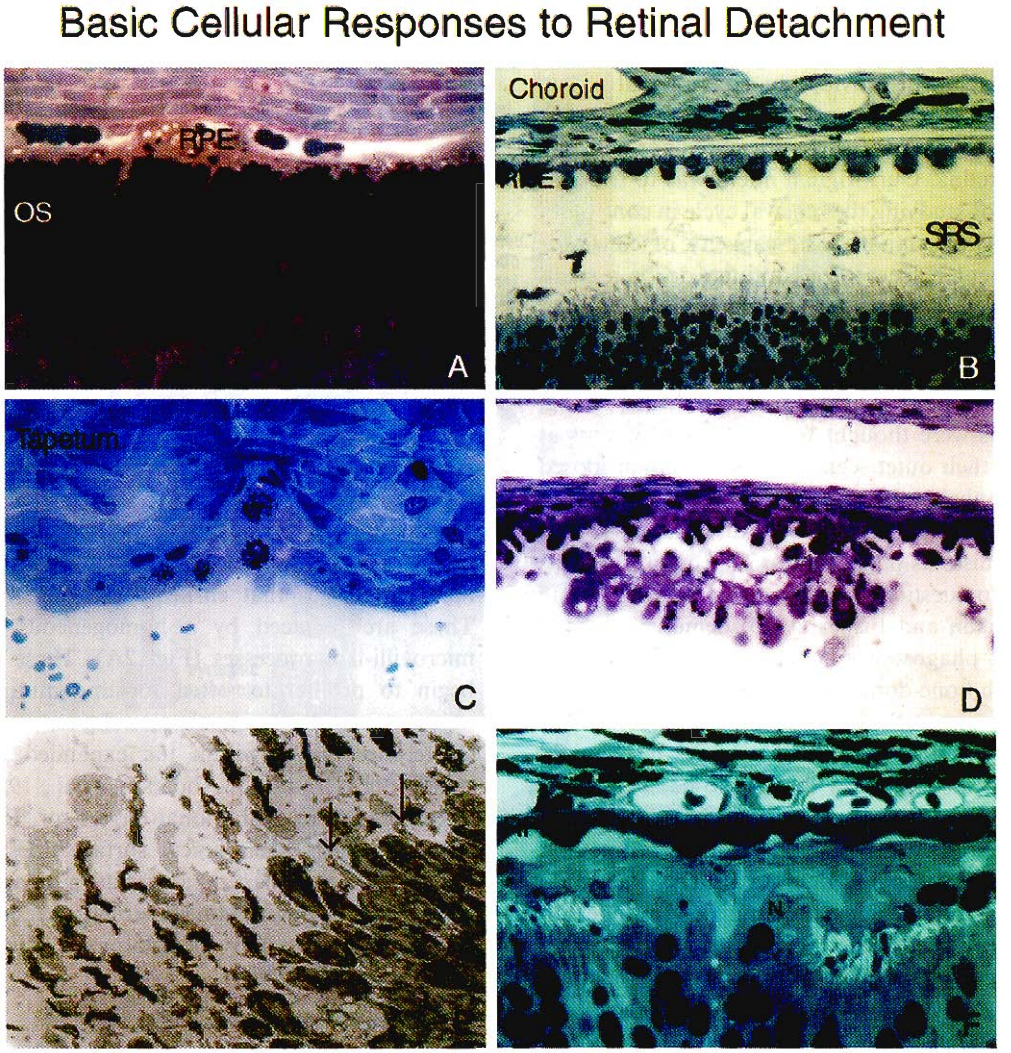

Fig. 2. Micrographs illustrating the basic cellular responses to retinal detachments: outer segment degeneration, RPE cell de-differentiation, proliferation, and gliosis. **A**. A light micrograph of the outer segment (OS)/RPE interface in normal cat retina. The "gaps" between the darkly stained rod outer segments mark the location of cone outer segments. The small, round inclusions in the RPE are phagosomes. A single phagosome appears just above the cone on the left. **B**. The interface shown in "**A**" after retinal detachment. There is now a subretinal space (SRS) measuring about 50 μm across, and this is filled with debris from degenerating outer segments. Note the scalloped appearance of the RPE and loss of apical processes. **C**. An autoradiogram showing tritiated-thymidine labeling of RPE cell nuclei 3 days after detachment. **D**. An assembly of RPE cells in the expanded subretinal space, a result of RPE proliferation. **E**. An electron micrograph of the interface shown in **B**, and showing that the debris in the space is composed of outer segment fragments. The 2 arrows mark photoreceptor cilia. Reprinted, with permission, from Lewis et al., 1999a. **F**. A light micrograph showing a large Müller cell scar in the subretinal space of a reattached cat retina. There is little outer segment regeneration on surviving photoreceptors beneath the scar. N, a Müller cell nucleus in the subretinal space.

subretinal space to components of vitreous and vice-versa. In some cases detachments occur when cells on the vitreal surface contract pulling the retina away from RPE, often times also leaving a tear across the retina. Such traction detachments often occur secondarily to successful surgical repair of a rhegmatogenous detachment, due to the proliferation of cells on the vitreal surface. In almost all cases this is a serious, nearly untreatable, ultimately blinding disease if the fovea is involved.

Why study retinal detachment?

Retinal detachment provides an experimental system in which to study many of the transformations that can occur between the biology of normal and abnormal retina. Our original intent was to use it as a method for studying the renewal cycle in cone outer segments. Understanding the capacity of cones for outer segment regeneration continues to be a significant problem. Young's original hypothesis of outer segment renewal excluded cones from the renewal cycle as described by rods and shown in Fig. 1, and postulated a different mechanism for them (Young, 1971). Rods were thought to construct new discs at the base of their outer segment. Cones were proposed to replace only "molecular components," of the outer segment and to not construct new discs nor dispose of packets of discs as phagosomes. This hypothesis was brought into question when Hogan et al. (1974) along with Anderson and Fisher (1975) demonstrated the presence of phagosomes in the human fovea and in species with cone-dominated retinas. Disc shedding from cones meant that they must also undergo disc morphogenesis in some form. The basic "mechanics" of these processes are now thought to be the same in rods and cones (Steinberg et al., 1980). Unlike the situation in rods, there was (and still is) no method available for quantifying the rate at which the cones renew their outer segment (Young, 1971; Anderson et al., 1978; Fisher et al., 1983). Thus, we reasoned, after failing to make cone-dominant species vitamin A deficient, that an animal model of retinal detachment and reattachment should provide the opportunity for measuring the rate at which cone outer segments re-grow, and thus, a measure of the rate at which these cells produce new membranes. In the course of the initial studies, it became obvious that the responses to retinal detachment are much more rich and interesting than we had anticipated. Indeed, the availability of antibody and molecular probes is now allowing us to continue identifying a host of cellular responses in addition to those initially identified in the early anatomic studies. These include: complex photoreceptor "deconstruction," proliferation of non-neuronal cells, changes in protein expression and amino acid profiles of most if not all retinal cell types, and plastic changes in second order neurons. Methods for altering these responses may ultimately lead to ways of improving vision after reattachment surgery.

The retinopathy of detachment

Detachment initiates a "cascade" of events leading to numerous cellular changes that have recently been termed the "retinopathy of detachment" (Mervin et al., 1999), an apt term considering that every retinal layer is involved in the etiology of the response. Each cell-type studied so far has its own characteristic response signature.

RPE cell "de-differentiation"

The complex apical surface of the RPE cells assume a much "simpler" appearance as it loses the elaborate apical processes that ensheath the outer segments. These are replaced by a homogeneous fringe of microvilli-like processes (Fig. 2A). These cells also begin to proliferate, often forming mutiple layers adjacent to the original monolayer, or separate clusters or layers within the expanded subretinal space (Fig. 2C and D; Anderson et al., 1981, 1983). The presence of additional layers of RPE cells, especially when their apical surface does not face the photoreceptor layer, can result in areas in which photoreceptor outer segments do not regenerate even though the retina may be anatomically re-apposed (Anderson et al., 1986).

Photoreceptor cell deconstruction: the basic rod response

Photoreceptors ultimately respond to detachment in one of two ways: they die by apoptosis (Cook et al., 1995; Chang et al., 1996; Mervin et al., 1999) or they undergo a series of "deconstructive" changes (Fig. 3). These have been best characterized in rods and can be divided into several events involving different compartments of the cell.

Outer segment degeneration

Degeneration of the outer segment of the rods is the most dramatic and obviously rapid manifestation of

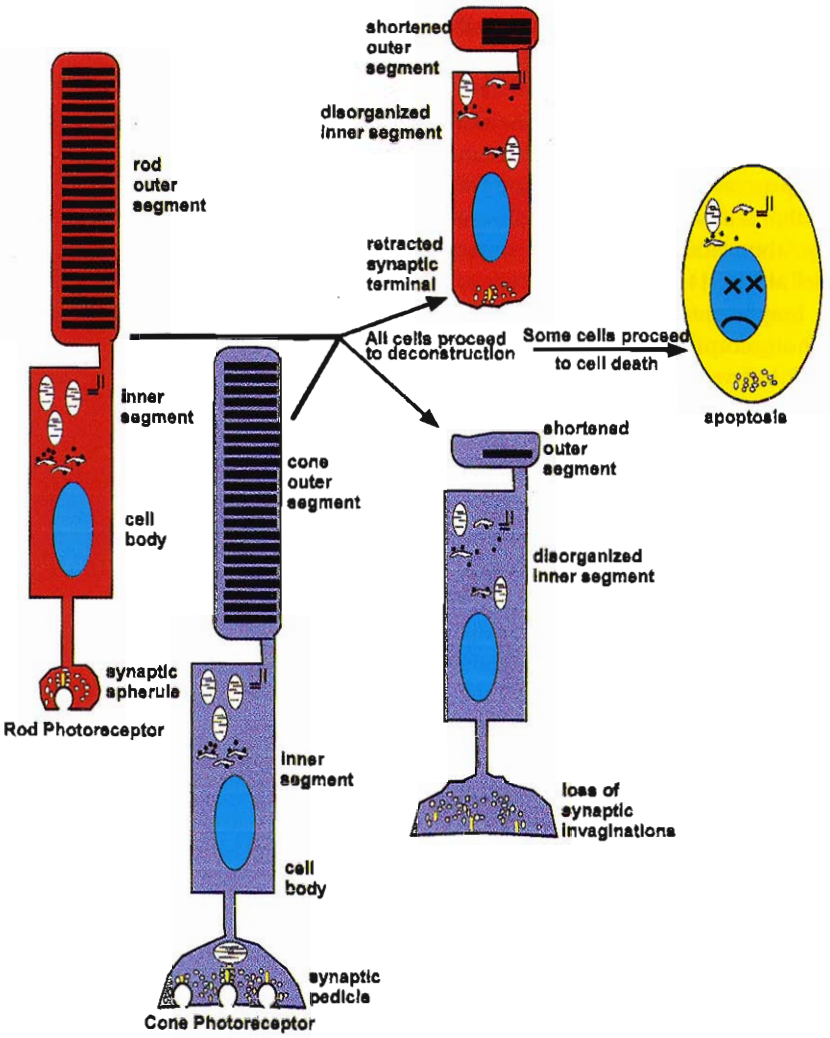

Fig. 3. A diagrammatic representation of the changes photoreceptor cells undergo after detachment. Almost immediately after separation from the RPE photoreceptors begin to undergo the process of "deconstruction." Some cells go on to survive in a highly modified state (cells in the middle) while other die by apoptosis. Cone outer segments appear to degenerate more quickly and completely than rods but their synaptic terminals do not retract.

detachment (Fig. 2B; Kroll and Machemer, 1968; Anderson et al., 1983). The outer segments of both rods and cones quickly shorten after detachment. In the cat retina, rod outer segments average 15–20 μm in length depending on retinal location, and within 7 days of detachment the average length will decrease to 4–5 μm (Lewis et al., 1999a). Even at 7 days, many photoreceptors will have no outer segment recognizable by light microscopy. In terms of outer segment regenerative capacity it is significant that the connecting cilium never disappears from these cells (Fig. 2E). Autoradiographic studies show that newly synthesized proteins are still transported to the connecting cilium and surviving portion of the outer segment, which may consist of only a few disorganized discs (Lewis et al., 1991). Thus, the remaining discs do not appear to be remnants of the original outer segment, but are most likely new discs, representing an attempt by the rod to continue outer segment production. The rods may be able to add some new

discs in the absence of RPE attachment, but they never construct a fully-formed outer segment. Why is this? Detached frog photoreceptors, which are probably more resistant to degeneration than their mammalian counterparts, in culture continue adding new discs in an apparently normal process and at a normal rate for several hours, but within 2 days, 75% of the cells are forming structurally abnormal discs at the outer segment base (Hale et al., 1991). Thus, the process of disc morphogenesis may eventually fail to produce normal discs in a photoreceptor detached from the RPE. There is a small but very specific aggregate of filmentous actin in the connecting cilium, in the region of disc morphogenesis (Vaughan and Fisher, 1987). The drug cytochalasin D disrupts this filamentous actin cytoskeleton, and treatment of photoreceptors with this drug produces abnormal discs (Williams and Fisher, 1987; Vaughan and Fisher, 1987). Hale et al (1996) showed that disrupting the filamentous actin cytoskeleton in the connecting cilium essentially stopped the cells from being able to construct new discs. Detachment also disrupts the f-actin cytoskeleton of the rod photoreceptor cell, and it may be this disruption that accounts for the inability of the cells to construct a normal outer segment (Lewis et al., 1995). In the presence of excess brain-derived neurotrophic factor (BDNF), the rods can construct surprisingly long and normal-appearing outer segments (Lewis et al., 1999a). It is possible that BDNF's action is to maintain the cytoskeleton of these cells, allowing normal outer segment formation to occur.

By what mechanism does the actual degeneration of the outer segment occur? Fragments of outer segments, and even individual discs can be recognized by TEM in the expanded subretinal space (Fig. 2E). Phagosomes are often observed within macrophages lying over the shortened outer segments (Anderson et al., 1983; Lewis et al., 1991), but are absent from the RPE. Although the dynamics of outer segment loss have not been studied, it seems unlikely that there is a simple "disintegration" of the outer segment, but rather a progressive shortening. Does the cell "release" packets of the outer segment in an organized/regulated manner such as occurs during the normal disc shedding cycle? The major evidence against this method of deconstruction is a lack of observed, regular packets of discs in the subretinal space, and the experimental evidence that normal rod disc shedding cannot occur in the absence of attachment to the RPE (Williams and Fisher, 1987). Does the outer segment degeneration proceed until the metabolic load on the cell is reduced to a level that compensates for the reduced availability of oxygen and other metabolites across the expanded subretinal space (see Linsenmeier and Padnick-Silver, 2000). When detached, does the rod cell actually regulate the number of new discs produced to match the availability of oxygen, or has the detachment simply disrupted the cytoskeleton (as discussed above), so that the cell can no longer form discs at its normal rate? These questions are yet to be answered experimentally. Experiments in which animals are kept in a hyperoxic (70% oxygen) environment however, do suggest a relationship between these events, i.e. the availability of oxygen may prevent the deconstruction of the cytoskeleton and thus allow outer segment formation (Mervin et al., 1999). On the other hand, the two may be independent of one another.

Inner segment de-compartmentalization

Inner segments are the home of an immense population of mitochondria as well as most of the machinery for protein synthesis and processing, with organelles being organized into compartments (the ellipsoid and myoid). Upon detachment this organization is disrupted, and the organelles intermingle (Erickson et al., 1983). Labeling with an antibody to the protein cytochrome oxidase confirms the ultrastructural observation that mitochondria are rapidly lost from these cells (Fig. 4D, E; Mervin et al., 1999). This is an important issue since these cells have a metabolic rate much higher than that of other neurons (Winkler, 1983). When animals are kept in an increased oxygen environment after detachment the mitochondria are preserved (Fig. 4D–F, J–L) suggesting that their loss is regulated by the lack of available oxygen from the choroidal circulation (Mervin et al., 1999). Disruption of the tubulin cytoskeleton, and its maintenance in hyperoxia is also easily observed in the inner segment and connecting cilium, although the tubulin cytoskeleton is actually lost throughout the rod cell (Fig. 4G–I).

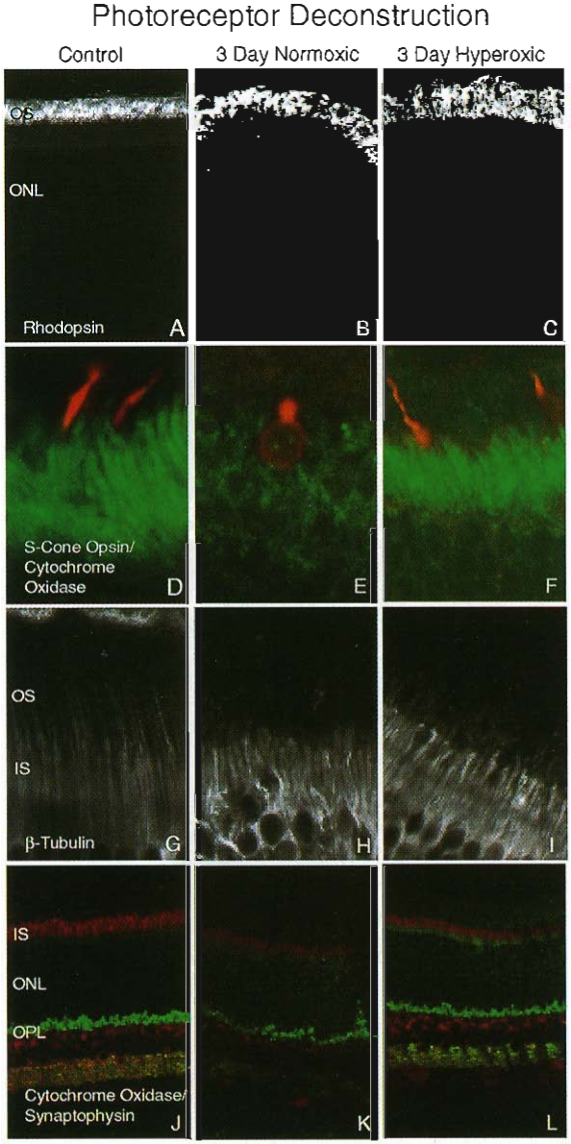

Fig. 4. Antibody labeling shows various aspects of photoreceptor deconstruction after detachment in normoxia (2 left columns) and their mitigation by oxygen therapy (right column). ONL, outer nuclear layer; OS, outer segment layer. **A,B,C.** Labeling with a rod-opsin antibody (kindly provided by Dr. R. Molday). In the control eyes (**A**) labeling is limited to the rod outer segments (and the Golgi/RER regions of the inner segment) while after detachment in a normoxic animal, labeling spreads to the plasma membrane surrounding the whole cell (**B**). In hyperoxia the label is once again confined to the outer segment, and the outer segments approach normal length and appearance. **D,E,F.** Double-label images after labeling with an antibody to the short-wavelength sensitive cone-opsin (red), and an antibody to cytochrome oxidase (green) which labels the mitochondria. (The antibody to S-cone opsin was kindly provided by Dr. J. Nathans). Cone outer segments degenerate quickly, and labeling of the plasma membrane is not as dramatic as for rods. Hyperoxia maintains the cone outer segments and mitochondria. **G,H,I.** The tubulin cytoskeleton is labeled with an antibody to beta-tubulin. The labeling in the region marked OS represents labeling of the connecting cilium, and that labeling is virtually lost in the normoxic detached retina, while that in the inner segment region (IS) assumes a more diffuse and amorphous appearance. The inner segment labeling appears more organized in hyperoxia, and the connecting cilium labeling is maintained, although not as well oriented as in a normal retina. **J,K,L.** Double labeling with the antibody to cytochrome oxidase (red) and an antibody to synaptophysin (green). The latter shows the order and organization of the photoreceptor synaptic terminals in normal retina (**J**), the loss of this organization in detached retina (**K**), and its maintenance by oxygen therapy (**L**). Also note the loss of mitochondrial labeling (red) in detached retina and compare this to normal and oxygen-treated detached retina. Reprinted, with permission, from Mervin et al. (1999).

Synaptic terminal withdrawal and regression

The synaptic endings of surviving rods are also affected strikingly by detachment. Structurally, the synaptic terminals of the rods appear to withdraw from their normal location on the border of the outer plexiform layer so that the synaptic structures now appear within the cell body, instead of at the end of the "axon," (Figs. 4J,K; 5A,B,E,F; Erickson et al., 1983). Thus, the layer of rod spherules becomes disrupted, no longer forming a compact zone at the interface of the outer nuclear and outer plexiform layers (ONL, OPL) (Figs. 4J, K; 5A–C; Lewis et al., 1998, 1999a; Mervin et al., 1999). Since the rod nuclei form many rows within the ONL, their terminals begin to assume a random scattering throughout that layer as they withdraw towards the cell body (Fig. 5C). Ultrastructurally, the distinctive invaginations with their complex array of postsynaptic processes disappear from these terminals (Fig. 5G). They now assume a configuration reminiscent of developing photoreceptors (Linberg and Fisher, 1990) where the ribbons and vesicles are located in a small rim of basal cytoplasm, often closely juxtaposed to the nucleus, and are apposed directly to the flattened presynaptic membrane (Fig. 5G; Erickson et al., 1983). Postsynaptic processes can still be clearly identified by electron microscopy, even

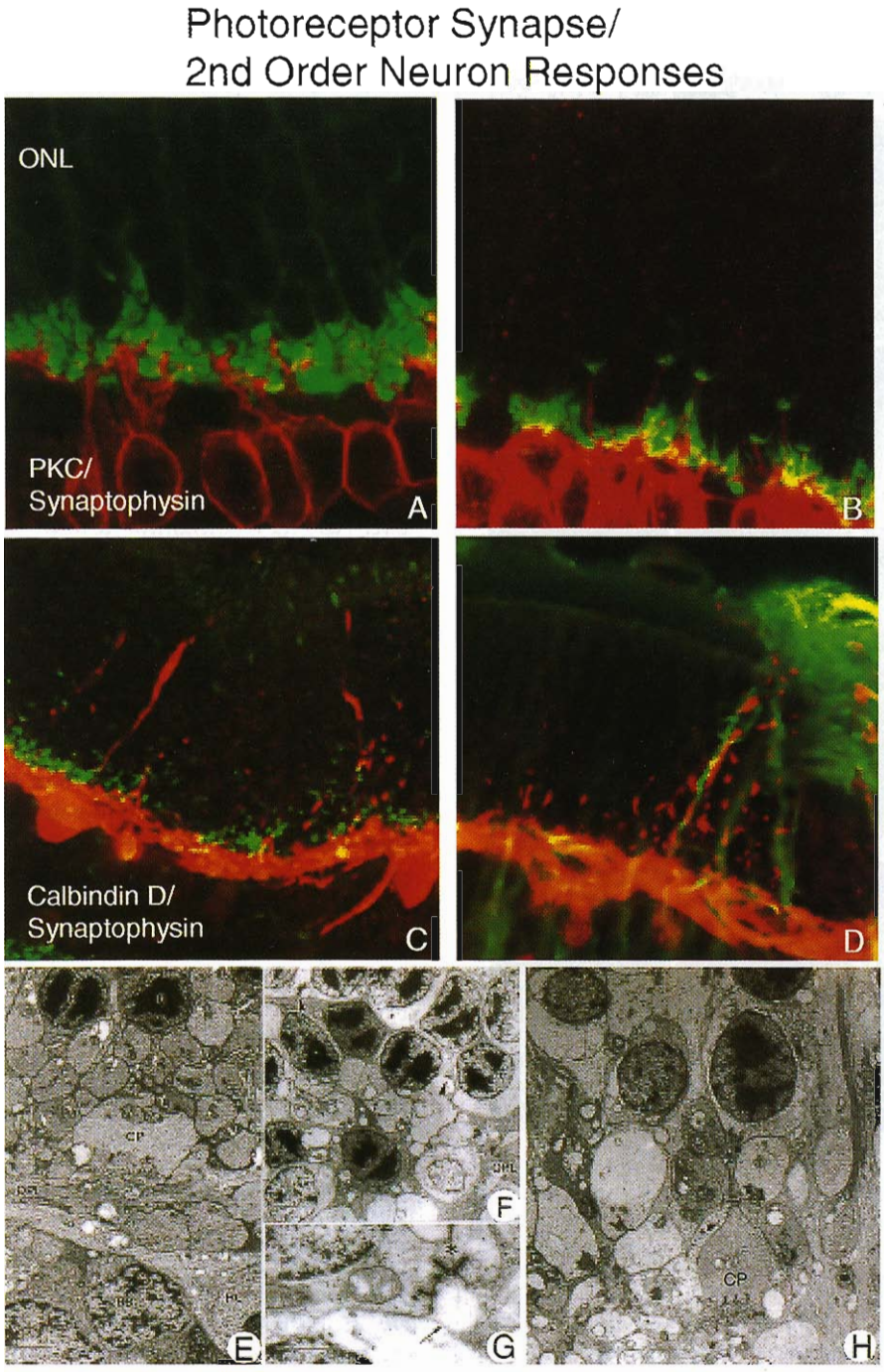

Fig. 5. The response of rod photoreceptor synaptic terminals and second-order neurons to detachment. **A**,**B**. Double-label images with antibodies to synaptophysin (green) that labels the photoreceptor terminals and protein kinase C (red) that labels the rod bipolar cells and their dendrites. Note the loss of synaptophysin labeling from the layer of terminals in the detached retina, the shift of this labeling into the ONL as rod terminals retract, and the extension of labeled neurites from the rod bipolar cells to the retracted rod terminals. **C**. Labeling of retracted rod terminals with an antibody to synaptophysin (green) and outgrowths from horizontal cells with an

when there is no invagination, and when the synaptic structures lie deep in the ONL, far from the original zone of synaptic terminals. The spatial relationship between these processes appears to be much different than that occurring within the invagination. Presumably the deep synaptic invagination into the rod terminal plays some role in the physiology of information processing at this, one of the most complex synapses in the whole central nervous system. What happens to the physiology of this synapse, and to information processing in the rod pathway when the postsynaptic processes are no longer within the confined space of an invagination?

Shifting the focus to cones

Rod degeneration has been studied in many animal models, although an emphasis on studying cones would seem highly compelling considering the devastating effects of foveal damage by trauma or diseases such as age-related macular degeneration. Presumably the reason for this is the ease with which rods are studied (being much more abundant than cones in most mammalian species), and the variety of naturally occurring mutations that affect rods, providing models for retinitis pigmentosa. There are, however, about 10 million people in the United States affected by age-related macular degeneration, compared with about 100,000 afflicted with retinitis pigmentosa. In both genetically induced retinal degenerations, and degeneration induced by constant light exposure, cone cells appear to survive until late in the degeneration. Data from the detachment model is providing a more detailed picture of the cones' responses. Many cones do, indeed, appear to survive the degeneration induced by detachment, but they may do so utilizing a different mechanism than the rods. Certainly these two differ greatly in how they express various proteins during an episode of detachment. Using that as a criterion, cones appear more susceptible to degeneration than rods. The cell bodies of the cones in cat retina are arrayed among the cell bodies of rods in the outermost row of the ONL, and thus, they have long axons that traverse the ONL to terminate in large, elaborate synaptic pedicles in the OPL (Fig. 6A). These axons do not retract as do those of the rods, but the synaptic pedicles change their overall shape, generally losing the large number of invaginations that populate the pedicle base (Figs. 5, 6B), and losing their large, distinctive mitochondria as well. The cone nuclei often shift their location to deeper into the outer nuclear layer. Whether this is an active relocation, or passive movement due to remodeling of the ONL as large numbers of rods die is not known. However, the most distinctive differences between rods and cones emerge when the retina is labeled with various antibodies. For example, rods continue to label intensely with antibodies to rhodopsin even in very long-term detachments (Fig. 7A–C). Outer segments consisting of only a few discs continue to label at the same relative intensity as an intact outer segment, but at the same time there is a distinctive shift in the labeling pattern. Within a day of detachment there is a recognizable increase in labeling of the plasma membrane around the inner segment and cell body. With time this labeling increases in intensity, and extends into the plasma membrane around the synaptic terminal so that eventually the whole cell is outlined (Fig. 7B; Lewis et al., 1991; Fariss et al., 1997). This pattern continues in surviving rods as long as the retina remains detached. It is not known if it is reversed when the retina is reattached and the

antibody to calbindin D (red). Note how some of these stout outgrowths run the width of the ONL. **D**. Labeling of horizontal cell outgrowths in detached retina with an antibody to calbindin D (red) and Müller cell processes with an antibody to GFAP (green). Note how the outgrowths seem to follow the hypertrophied Müller cell processes. **E**. An electron micrograph of the layer of photoreceptor synaptic terminals and the outer plexiform layer (OPL) in normal cat retina. CP, a cone pedicle. HC, horizontal cell; R, rod nucleus; RS, rod spherules. **F**. An electron micrograph showing disruption of the OPL and retraction of rod terminals in detached retina. The arrowheads mark rod synapses within the ONL. **G**. A higher magnification electron micrograph of a rod synapse in a cell that has retracted its synaptic terminal. The asterisk marks synaptic ribbons and the arrow a post-synaptic process. Fig. **E**, **F** and **G**, reprinted, with permission, from Lewis et al., 1998. **H**. An electron micrograph of the OPL after detachment showing that cone pedicles (CP) change their shape but do not retract from their normal location on the border of the outer plexiform layer. Note the large mitochondria in the CP in **E**, and the tiny remnants of mitochondria in the CP in **H**. Also the cone terminal in **H** has ribbons along its base, but no deep invaginations.

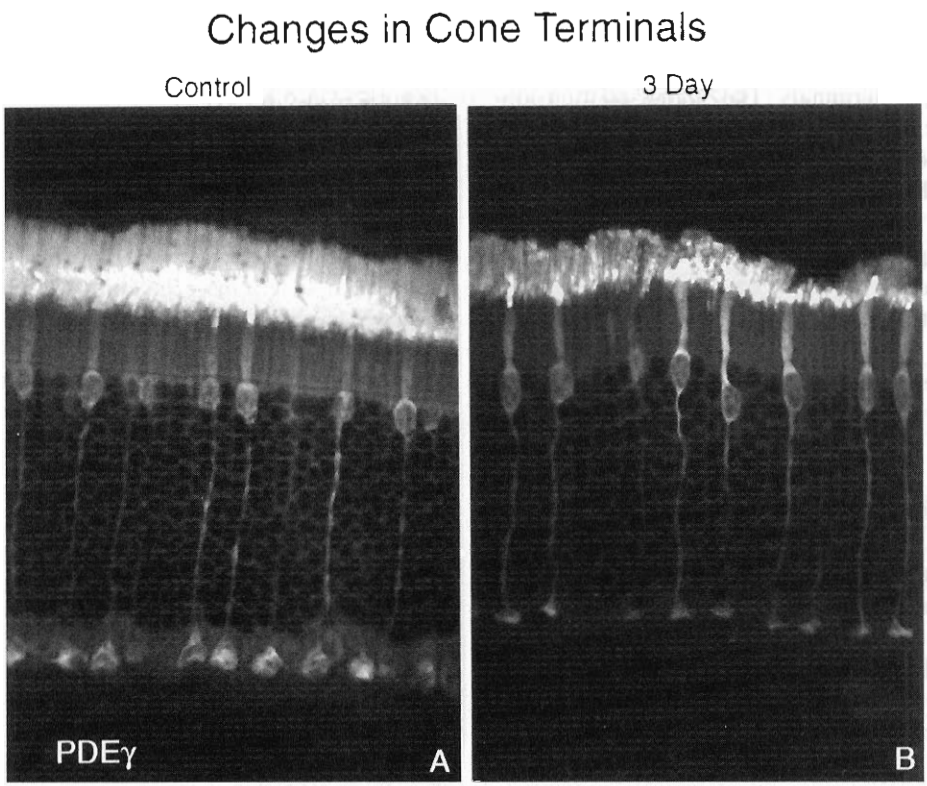

Fig. 6. An antibody to the γ subunit of phosphodiesterase (kindly provided by B. Fung) heavily labels the outer segments of the rods, but cone photoreceptors in their entirety. Note the significant change in the size and shape of the cone terminals when the retina has been detached for 3 days (**B**), and the fact that these terminals do not withdraw from their normal location along the OPL.

rods begin to regenerate an outer segment, or whether it represents some permanent change to the cell. It is still unknown how this opsin finds its way into the plasma membrane. Whether it is through some form of "reverse flow," from the degenerating outer segment, or whether it represents opsin that is inserted into the plasma membrane because it continues to be synthesized but cannot be used to construct an outer segment (Fariss et al., 1997) remains unresolved. There is evidence supporting the latter hypothesis, but there is also no obvious reason why both mechanisms could not be operating. Cones show a different pattern. There is a short time (about 3 days) during which the plasma membrane may show a slight increase in labeling intensity with a cone opsin antibody (Figs. 4E, K, 7E). This labeling rarely extends into the synaptic terminals, and disappears completely from the majority of surviving cones after about 1 week (Fig. 7F). Essentially the same trend occurs when other photoreceptor-specific antibodies are used. Although the pattern and intensity of label will vary, rods continue to express all of the proteins studied to date even in detachments of a month's duration. Indeed, in the case of phosducin, there is actually an increase in the amount detected in the rods, while others such as opsin and peripherin/rds shift their pattern of expression (Fisher et al., 1996; Fariss et al., 1997). As derived from a variety of studies using quantitative ELISAs, immunohistochemistry, and in situ hybridization, proteins produced by rods continue to be expressed, even though their pattern and location may be significantly different from those in normal retina (Fisher et al., 1996; Fariss et al., 1997). In the cones, however, the expression of each protein studied to date declines to very near the limits of detection by immunocytochemistry within a few days (Rex et al., 1997, 2000), or their presence becomes so sporadic that only an

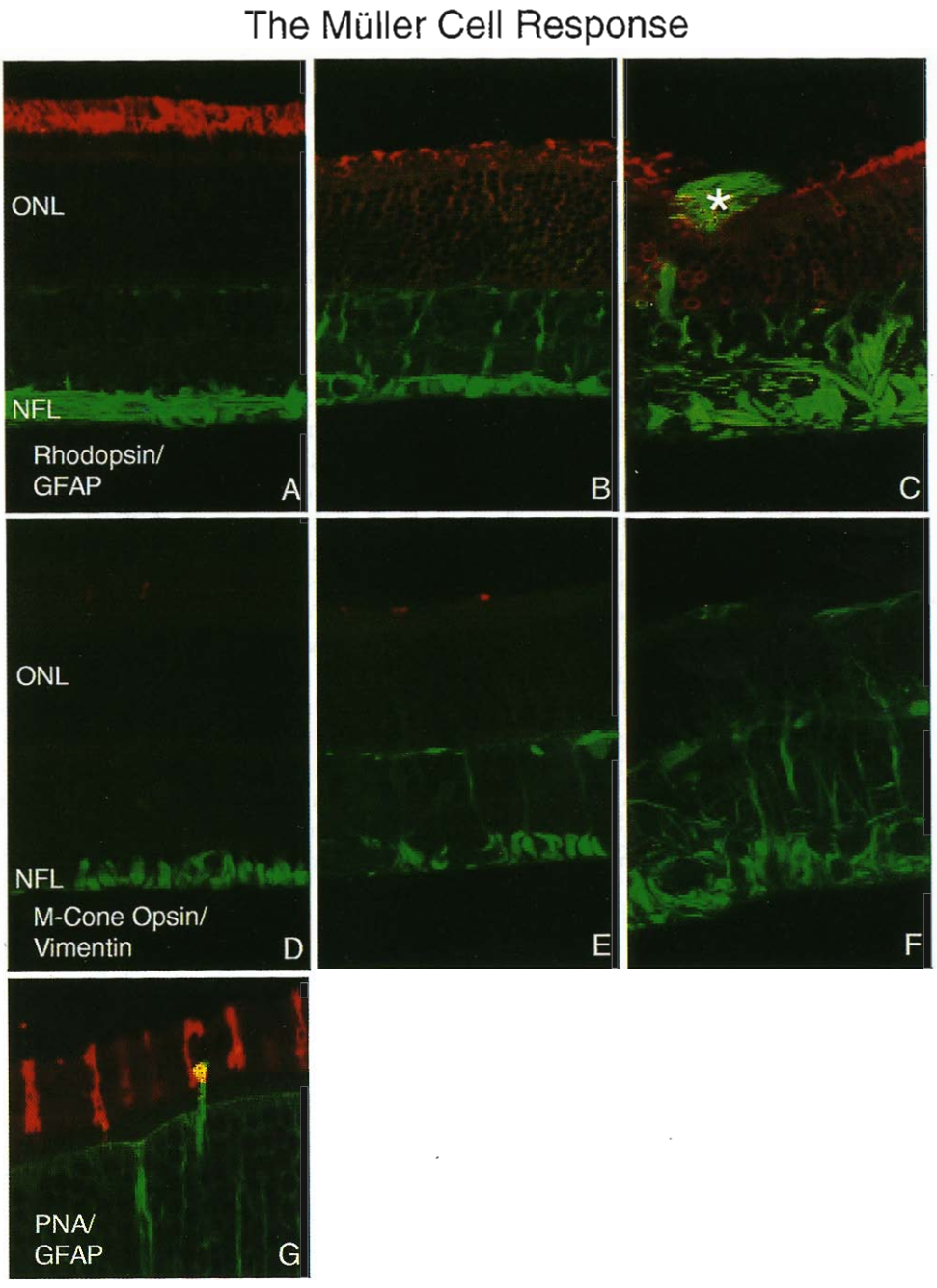

Fig. 7. The gliotic response of Müller cells to detachment demonstrated by immunolabeling with antibodies to intermediate filaments. ONL, outer nuclear layer; NFL, nerve fiber layer. **A**,**B**,**C**. Anti-GFAP (green) labels astrocytes in the optic nerve and faintly labels processes of Müller cells in normal retina. The rod outer segments are labeled with anti-rod opsin (red). Note the progressive labeling of the Müller cell processes with detachment time. In **C**, there is a large Müller cell scar (*) in the subretinal space. **D**,**E**,**F**. Labeling with anti-vimentin and an antibody to the M-cones. Anti-vimentin does not label astrocytes and shows the same Müller cell response as the anti-GFAP. **G**. Peanut agglutinin (PNA) is used to label all cones (red) in this section of retina detached for 3 days; the anti-GFAP (green) labeling shows how the leading edge of an expanding Müller cell process associates with a cone inner segment.

occasional, surviving cell shows immunolabeling. The data showing a dramatic increase in phosducin in the photoreceptors, and the fact that there is such a wide range of values for the expression of other rod-specific proteins indicates that these changes are almost certainly highly regulated, and not simply a "metabolic shut-down" of the rods as might be expected.

A logical interpretation of this data would be that individual rods continue producing opsin at near normal levels, and that they place much of this opsin into the plasma membrane because they cannot use it to construct an outer segment. The sharp decline in overall opsin levels comes from the combined loss of outer segments and rod cell death, and is not an indicator of the capacity of individual cells to synthesize the protein. This may position these cells favorably to begin re-constructing their outer segment as soon as favorable environmental conditions return, e.g. when the retina is reattached. Cones on the other hand, appear to enter a state where they stop expressing protein molecules, including their specific opsins. Why would the rods and cones differ so dramatically in their responses? Perhaps each has independently evolved mechanisms of self-protection and survival under "hostile" conditions. Rods far out-number cones in the feline (and primate) retina, so there may be enough redundancy in their numbers that even a significant loss of cells can be tolerated with relatively little effect on rod-mediated vision. Thus, some rods may be "culled" from the population when there is a lack of oxygen and nutrients from the RPE/choroid, allowing those that survive to continue synthesizing molecules used in transduction, and thus, be poised to regenerate rapidly. Because there are far-fewer cones, and because they tend to be concentrated in central retina and are critical to high-acuity vision, their loss, even in relatively small numbers may be visually significant. Thus, having all of the cones reduce their metabolic activities to a minimum level may be advantageous, and allow for survival of more of these cells.

If the metabolic effect on cones is indeed to stop their expression of various proteins as a means of survival, then it would be predicted that a greater percentage of the cone than rod population would survive, and that their recovery might be slower than that of the rods. Because of the loss of cone-specific markers, quantitating the number of cones that *survive* in the rod-dominated cat retina has proven exceptionally difficult (Linberg et al., 2000). We have two sets of studies underway that will help answer some of the questions of cone survival, and recovery. The first is a study of reattachment in the cat. In a retina detached for 7 days cone markers already provide an unreliable estimate of surviving cells. For example, the number of cones in the mid-periphery that label with an antibody to S-cone opsin is reduced to about 2.7% of the number in normal retina, and yet using another marker (anti-calbindin D) we would predict that 50% of the cones survive in this portion of the retina (Linberg et al., 2001). We can, however, detach the retina for 7 days and then reattach it for varying periods of time and use various cone markers to compare the number of cones at various reattachment intervals to those in normal and detached retina. If they are present in greater numbers after reattachment, than after detachment, they must have survived since new photoreceptors are not added in adult mammalian retina. The other study entails experimental detachments and reattachments in a retina that is cone-dominated (Linberg et al., 1999, 2000). With cones in the majority, do we find the same responses as in the rod-dominated retinas? Understanding the intriguing differences between rods and cones may be a significant step in conquering the sight-impairing diseases that affect the macula.

Photoreceptor cell death

It has been known now for over 15 years that significant numbers of photoreceptor cells die after detachment in both animal models (Erickson et al., 1983) and in humans (Wilson and Green, 1987). Apoptosis is probably the predominant mechanism of cell death (Cook et al., 1995; Chang et al., 1996). It appears that about 80% of the outer nuclear layer survives during 3 days of a detachment (Mervin et al., 1999). Since the cat retina is heavily rod-dominated, this is probably a good measure of rod survival. Quantitating the number of cones that die or survive has so far proven difficult as discussed above. Superficially, our data point to cones being lost more quickly than the rods. When anti-calbindin D

labeling is used as a marker to count the number of cones there is a reduction from 7,000/mm^2 in the mid-peripheral retina of control eyes to 3,900/mm^2 in a comparable area of a 3-day detachment, a reduction to 56% of control values. When an antibody to the short-wavelength sensitive opsin is used to count the S-sensitive cones, they are reduced from 1,100 to 200/mm^2, a reduction to 18% of control values. Thus by either method it would *appear* that cones are lost much more rapidly than rods, and perhaps S-cones more quickly than the M-cones in this species (Linberg et al., 2001). But the fact is that we have no reliable marker for cones once they have been detached for as little as 3 days, and what we may well be describing is the loss of proteins, not the loss of cells. Thus, the important question of cone-survival remains a frustratingly unresolved one.

Effects on the inner retina

Plastic changes in second-order neurons

Initial ultrastructural observations showed the presence of post-synaptic processes at retracted rod photoreceptor terminals (Erickson et al., 1983), even those terminals that were deep in the ONL. Subsequent studies using antibodies that specifically label rod bipolar cells (anti-PKC), and horizontal cells (anti-calbindin D), showed that these observations were explained by the apparent growth of neurites from these cells (and presumably other second order neurons as well) (Lewis et al., 1998 and Fig. 5A–D). Indeed, these outgrowths appear to temporally accompany the retraction of the rod terminals, and by 3 days they are extensive across the zone of detachment. An interesting phenomenon occurs in longer-term detachments because the processes can often be identified growing past the layer of photoreceptors to the outer edge of the ONL where they appear to end "blindly." This latter effect was particularly prominent among the calbindin-D positive (horizontal cell) processes (Fig. 5C). Many of these growing processes also appear to run among or parallel to the processes of Müller cells. Whether they are in some way actively attracted to the Müller cells (Fig. 5D), which are undergoing many changes of their own, or whether this is a passive phenomenon is unknown. Do third and fourth order neurons (amacrine and ganglion cells) react to detachment? Although detailed evidence is not yet available, preliminary evidence suggests that they do.

Müller cells and gliosis

In the brain and spinal cord, injury results in a rapidly mounted, stereotypical response by astrocytes known as gliosis. This can involve proliferation of these cells as well as their extensive hypertrophy to form glial scars in the region of the injury. In the feline (also rabbit, and probably primate) retinas, there is extensive proliferation of astrocytes after detachment, but the cells seem to remain relatively stable in terms of their size and location (Fisher et al., 1991; Geller et al., 1995). Müller cells, however, mount a robust astrocytic-like response. Indeed, their response is rapid, dramatic and can result in a complete remodeling of the structural appearance of the retina. They also play, along with the RPE cells, a serious role in proliferative diseases that can occur secondarily to detachment and cause blindness. One of these, proliferative vitreoretinopathy, or the proliferation and eventual contraction of cells on the vitreal surface of the retina, occurs in about 7–10% of successful reattachment surgeries. Basic structural changes can be recognized in Müller cells within a day or two as they increase the number of intermediate (GFAP and vimentin-containing) filaments in their cytoplasm. Within 3 days their processes can be seen growing into the subretinal space, and becoming thickened within the retina (Fig. 7). As detachment time proceeds, these changes become more exaggerated as their cytoplasm fills with newly synthesized intermediate filaments, and the processes enlarge within the retina and expand on the retinal surfaces (Fig. 7). Utilizing a variety of methods one can develop an appreciation for the complex response mounted by the Müller cells as they undergo significant transformations after detachment:

1. They proliferate. Normally quiescent, like the RPE, Müller cells begin to proliferate within a day, with the response reaching a peak at 3–4 days (Fig. 8J,K), but continuing at some low level

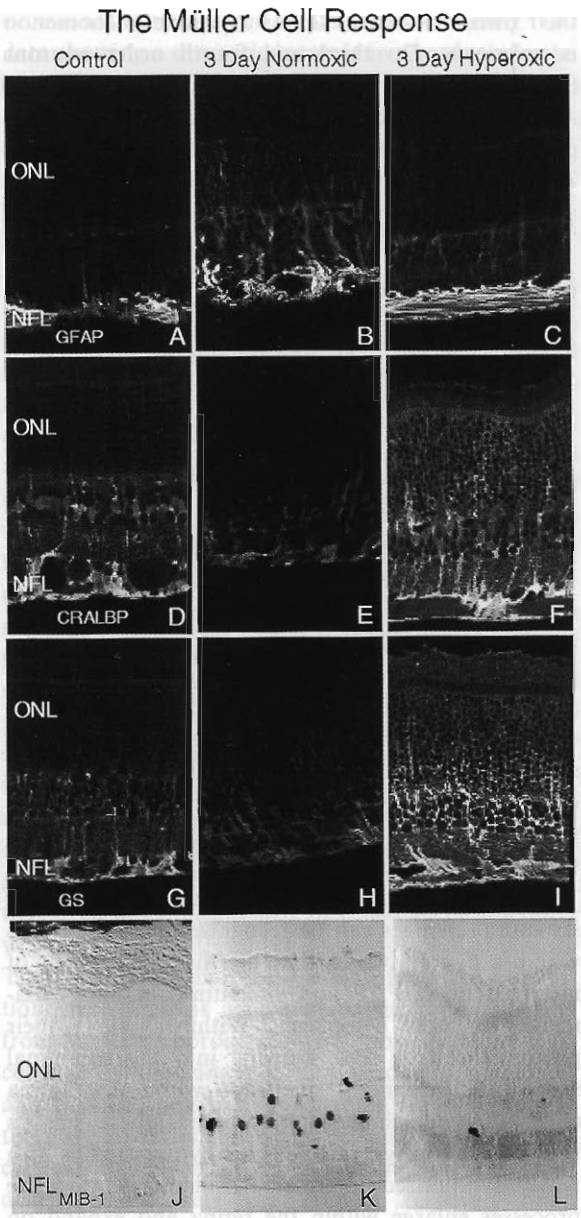

Fig. 8. Müller cell reactivity and responsiveness to hyperoxia. The left two columns are from animals (control or 3-day detached) kept in a normoxic environment, the right-hand column has data from 3-day detached retinas in which the animals were kept in an environment of 70% oxygen. **A,B,C.** Anti-GFAP labeling demonstrates the hypertrophy of Müller cells by 3-days in normoxia, and the significant lack of this response in animals kept in hyperoxia. **D,E,F.** Cellular retinaldehyde binding protein (CRALBP) is normally present throughout the Müller cell cytoplasm (**D**), and rapidly decreases when the retina is detached (**E**). Hyperoxia not only maintains but appears to increase the amount of CRALBP in the Müller cells after detachment (**F**). **G,H,I.** The pattern of labeling with anti-glutamine synthetase is remarkably similar to that of CRALBP. **J,K,L.** Labeling with the MIB-1 antibody to the Ki-67 antigen shows the presence of cells that have begun dividing. Routinely no labeled cells occur in normal retinae (**J**). The number of labeled cells is at its maximum at 3 days of detachment (**K**), and is greatly reduced by hyperoxia.

as long as the retina remains detached (Fisher et al., 1991; Geller et al., 1995).

2. They expand in size. Müller cells normally have a fairly thin, sometimes branched, "trunk" that runs from the vitreal border to the level of the outer limiting membrane where their apical microvilli extend into the space between neural retina and RPE. The processes within the neural retina begin to grow in diameter until they can be fairly massive in size, cutting a huge swath through the retina (Figs. 7B; 8B). It might be thought that these expand to fill space left behind by dying neurons, except that they expand across the entire retina and neurons are lost only from the ONL. As mentioned above, they extend processes out of the neural retina (Fig. 2F). Antibodies to intermediate filament proteins clearly show this response (Fig. 7C). The processes that grow into the subretinal space seem to have a special affinity for cones cell bodies (Fig. 7G; Lewis and Fisher, 2000), perhaps due to the expression of the growth factor bFGF by these cells (Mervin et al., 1999). Growth of these processes on the photoreceptor surface interferes absolutely with outer segment regeneration in reattached retina (Fig. 2F). Growth occurs on the inner retinal surface as well, but only in detachments of long duration (28 days or more) in the feline model. As part of this response, their nuclei often migrate into the outer retina or even the subretinal space (Fig. 2F), but interestingly, never towards the inner retina.
3. They change their profile of protein expression. Besides the dramatic increase in GFAP and vimentin expression (Figs. 7; 8A,B), the expression of many other proteins change as well. Their cytoplasm rapidly becomes nearly devoid of such proteins as carbonic anhydrase C, cellular retinaldehyde binding protein (Fig. 8D,E), and

glutamine synthetase (Fig. 8G,H; Lewis et al., 1989; Lewis et al., 1994).

4. They change their amino acid profile. The loss of the glutamine synthetase correlates with a very large accumulation of glutamate (and a commensurate decline in glutamine) by these cells (Marc et al., 1998). The significance of this change is not known but the release or accumulation of glutamate during an episode of detachment may contribute to the overall responses of the retina, since glutamate is a powerful neurotoxin (Marc et al., 1998).
5. They react rapidly, with their response seemingly mediated through immediate early-response signaling pathways. The extracellular signaling kinase, ERK, becomes phosphorylated in Müller cells (and RPE cells) within 15 min of detachment. There is apparent de novo production of *c-fos* mRNA, and increased c-Fos and c-Jun (members of the AP-1 transcription factor) protein expression in both cell types within 2 h of a detachment (Geller et al., 2001).

Non-neuronal cells play an important part in the response to detachment

Thus, at least 2 non-neural cell types, the RPE and Müller cells have been demonstrated to play major roles in the retina's responses to detachment. They react quickly, transform their morphology, proliferate, and extensively change their metabolism (although data on the latter changes in RPE are scant), and these changes can have significant effects on the recovery process. A third cell type, the astrocytes that reside in the inner retinal layers also clearly react because they proliferate vigorously, but the significance of their response remains enigmatic. Understanding the role of the non-neuronal cells in maintaining the health of the normal and damaged retina remains an important challenge.

Mechanisms

We now have a few clues as to mechanisms that may be operating to produce the numerous changes that result from detachment:

1. Basic fibroblast growth factor (bFGF), probably plays a major role. When injected into the vitreous of normal eyes it causes Müller cells to divide (Fig. 9A), and to increase the number of intermediate filaments in their cytoplasm (Lewis et al., 1992). It also binds to Müller cells in what is mostlikely a receptor mediated event (Fig. 9B; Lewis et al., 1996). Furthermore, there is an apparent release of bFGF from the inner retina after detachment (Fig. 9C,D), which is prevented by hyperoxia (Fig. 9E,F; Mervin et al., 1999). Hyperoxia also appears to increase the expression of bFGF by cones after detachment (Fig. 9E,F). The receptor for bFGF is phosphorylated rapidly after detachment (Geller et al., 2001).
2. Hypoxia of the outer nuclear layer may be responsible for many of the changes observed after detachment. Increasing the availability of environmental oxygen can both prevent photoreceptor cell deconstruction and the Müller cell gliotic response (see below; and Mervin et al., 1999; Lewis et al., 1999b). Modeling studies suggest that even relatively shallow detachments can produce significant hypoxia of the photoreceptors (Linsenmeier and Padnick-Silver, 2000).

Figure 10 shows proposed relationships between hypoxia, the inner retina, and bFGF that could result in the genesis of the changes that constitute the retinopathy resulting from a retinal detachment (Lewis et al., 1999b).

Preventing the retinopathy of detachment

Current experimental evidence suggests that the management of several different responses after detachment may be necessary to improve visual recovery above that obtained by the sophisticated procedures of modern retinal surgery: (1) preventing photoreceptor deconstruction and death, (2) improving the recovery capacity of photoreceptors after reattachment, and (3) preventing changes in non-neuronal (specifically the RPE and Müller cells) cells that may contribute to imperfect recovery or undesirable long-term side effects. There is now

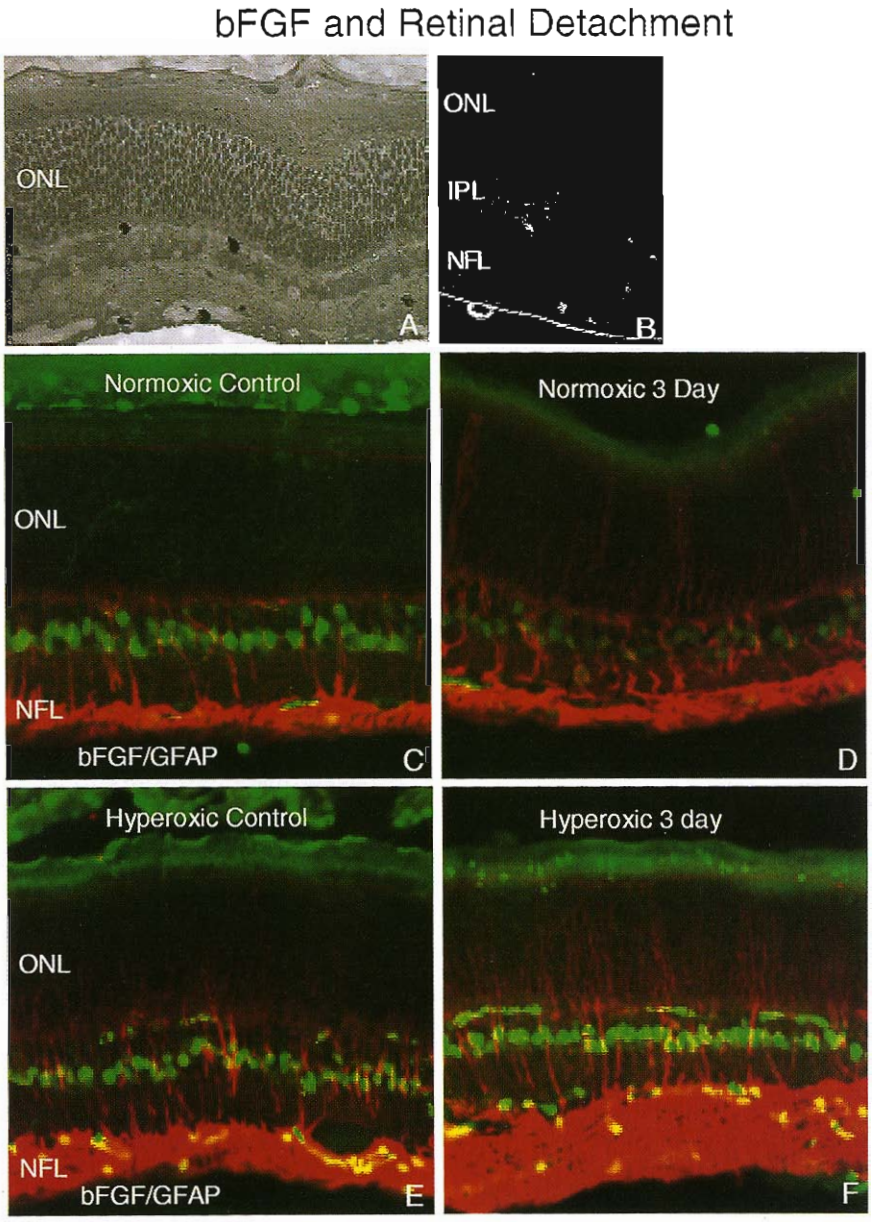

Fig. 9. Basic fibroblast growth factor bFGF and detachment. **A**. Labeling with the MIB-1 antibody 3 days after the injection of bFGF into the vitreous shows the presence of proliferating cells. **B**. When biotinylated bFGF is injected into the vitreous and detected with avidin-Cy3, it is first found associated mainly with the inner limiting membrane and within the extracellular space of the inner retina, 6 h later, it appears as punctate labeling associated with Müller cells, astrocytes, the RPE, and as a row in the OPL. At higher magnification the latter can be seen as within the synaptic invaginations of the photoreceptors. **C,D**. The localization of bFGF protein (green) and GFAP (red) in normal, normoxic retina (**C**), and normoxic 3-day detached retina (**D**). The protein appears to be rapidly lost from the inner retina after detachment. Note the faint labeling of cone outer segments in detached retina. **E,F**. The localization of bFGF protein (green) and GFAP (red) in normal hyperoxic retina (**E**), and hyperoxic 3-day detached retina (**F**). The hyperoxia may slightly increase the amount of bFGF in cells of the inner nuclear layer. In hyperoxic detached retina (**F**), the loss of bFGF from inner retina is prevented by the hyperoxia, and the labeling of cones is much more intense than in hyperoxic control retina.

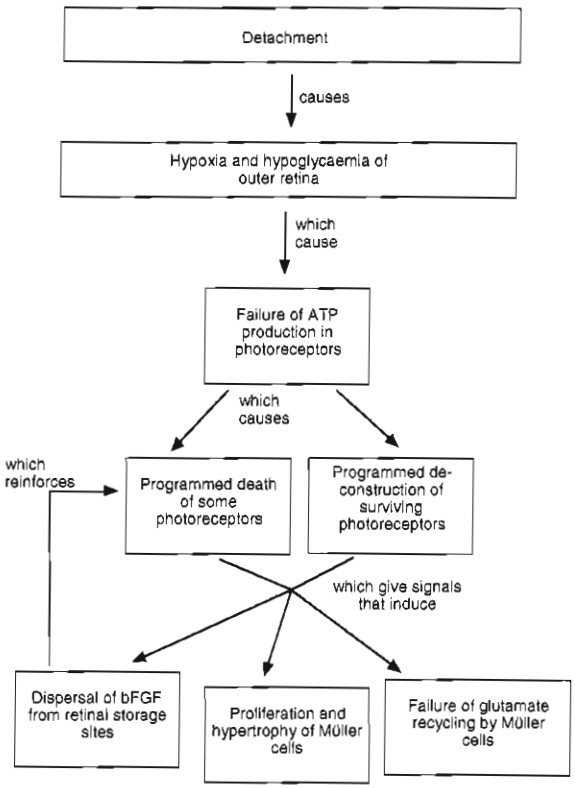

Fig. 10. An hypothesized scheme for the interactions of hypoxia and intracellular signaling to produce the retinopathy of detachment. Reprinted, with permission, from Lewis et al. (1999).

evidence that the neurotrophin BDNF (brain-derived neurotrophic factor) can slow the effects of detachment, and promote the production of remarkably well-formed outer segments in the absence of attachment to the RPE (Lewis et al., 1999a). BDNF treatment also reduces proliferation within the retina, and may prevent the long-term formation of glial scars. Keeping experimental animals in an atmosphere enriched with oxygen can have a similar effect including: preventing the loss of photoreceptor cells, preventing or slowing photoreceptor deconstruction (Fig. 4; Mervin et al., 1999), and mitigating the gliotic response of Müller cells (Fig. 8; Lewis et al., 1999b). Presently, the mechanisms through which the neurotrophin works is not well understood, especially since its effects on photoreceptors may be indirect, thus perhaps lessening its attractiveness as an adjunct to surgical therapy. Treatment of detachments with hyperoxia though has considerable rationale and attraction. Modeling shows that detachments as shallow as 100 μm can create serious hypoxia of the photoreceptor layer, and that increasing environmental oxygen can have significant effects on oxygen available to the photoreceptor cells (Linsenmeier and Padnick-Silver, 2000). Short-term oxygen therapy is in use worldwide on a daily basis with essentially no adverse effects (Mervin et al., 1999).

Conclusions and future perspectives

While this research has specifically focused on retinal detachment, it is hoped that its outcome will be to produce a better understanding of retinal degenerations in general, and, perhaps lead us to clues that will be useful in preventing photoreceptor degeneration not just in cases of detachment, but in such diseases as age related macular degeneration, where millions suffer from a loss of sight with little hope for treatment. Clearly we must find ways to prevent photoreceptor cells from dying, and to stimulate those that survive to maintain the molecular machinery necessary for structural integrity, and visual transduction. We must also understand how rods and cones differ and how they are alike. These two subtypes of cells may react differently to degeneration induced by injury than to degeneration induced by genetic mutation. We have lagged significantly in our understanding of cone degenerations by comparison to those of rods. We must also determine if the primate fovea presents a specialized environment with its own set of rules and regulations by comparison to the rod-dominated periphery. We must seek to understand the role of non-neuronal cells in retinal degenerations, not only in the obvious proliferative diseases but also the role of these cells in maintaining the healthy and injured retina. New technologies such as gene array analysis will undoubtedly speed-up our understanding of the retina's response to injury, or its response to biological factors such as trophic molecules that may prove useful in treating degenerative diseases.

We have spent well over a decade studying retinal detachment as an "undesirable" condition, but as we enter the 21st Century, we find that retinal surgeons are now creating large retinal detachments as part of

experimental therapy to treat specific events in the subretinal space, especially in the neovascular changes that occur in age-related macular degeneration (Lewis et al., 1999). Therapies also are being proposed for diseases like retinitis pigmentosa that involve the injection of factors or biological vectors into the subretinal space, creating a temporary detachment (Flannery et al., 1997; Lewin et al., 1998; Lau et al., 2000). Will these detachments be sufficient to initiate the cascade leading to proliferative diseases months or years later? Will photoreceptors, already rendered fragile by an underlying pathological condition die in response to these treatments simply by virtue of their separation from the RPE? Can we treat these patients with something as simple as oxygen therapy and prevent these undesirable events? Although these are questions with specific clinical ramifications, their answers will come from the continued study and appreciation of basic retinal biology, through the continued study of the retina with the most modern methods available to biologists. It is the inspiration and dedication of scientists like John Dowling and his continuing, untiring advocacy for taking advantage of the most modern technology available to understand the retina that will lead us into a new era for the treatment of blinding diseases.

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