



# Cellular remodeling in mammalian retina: results from studies of experimental retinal detachment

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## Abstract

Retinal detachment, the separation of the neural retina from the retinal pigmented epithelium, starts a cascade of events that results in cellular changes throughout the retina. While the degeneration of the light sensitive photoreceptor outer segments is clearly an important event, there are many other cellular changes that have the potential to significantly effect the return of vision after successful reattachment. Using animal models of detachment and reattachment we have identified many cellular changes that result in significant remodeling of the retinal tissue. These changes range from the retraction of axons by rod photoreceptors to the growth of neurites into the subretinal space and vitreous by horizontal and ganglion cells. Some neurite outgrowths, as in the case of rod bipolar cells, appear to be directed towards their normal presynaptic target. Horizontal cells may produce some directed neurites as well as extensive outgrowths that have no apparent target. A subset of reactive ganglion cells all fall into the latter category. Müller cells, the radial glia of the retina, undergo numerous changes ranging from proliferation to a wholesale structural reorganization as they grow into the subretinal space (after detachment) or vitreous after reattachment. In a few cases have we been able to identify molecular changes that correlate with the structural remodeling. Similar changes to those observed in the animal models have now been observed in human tissue samples, leading us to conclude that this research may help us understand the imperfect return of vision occurring after successful reattachment surgery. The mammalian retina clearly has a vast repertoire of cellular responses to injury, understanding these may help us improve upon current therapies or devise new therapies for blinding conditions.

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## 1. Introduction

### 1.1. Cellular remodeling in the central nervous system

Two decades of studying experimental retinal detachment (and reattachment) have led us into new avenues of thinking about the responsiveness of mammalian retina to injury, especially with respect to the degree of cellular remodeling that occurs among the retinal neurons and glia. In general, the neural circuitry of the adult vertebrate retina has not been considered as a highly “plastic” or malleable component of the central nervous system. Its task, transducing light energy into electrical impulses and then encoding parts of a visual image for reconstruction by the visual centers of the brain, may seem best served by a relatively hard-wired system. For example, the fact that it is possible to identify specific neural circuits in the retina (Dowling, 1970; Kolb and Famigletti, 1976; Linberg et al., 2001b; Kolb et al., 2001) and that many of these circuits are

preserved between species could be interpreted to support this concept. Indeed, there are references dating back to the late 1800s describing the reactivity of neurons in the brain and spinal cord to injury. However, only recently have similar changes in the retina received much attention, especially from those who study photoreceptor degenerative diseases.

#### 1.1.1. Specific examples of retinal remodeling

In the last few years descriptions of cellular remodeling in the vertebrate retina have begun to appear with some regularity in the published literature. Prior to this, the few descriptions of structural remodeling came from studies of fish retinas where Wagner and colleagues (Wagner, 1975, 1980; Wagner and Ali, 1977) described the remodeling of cone photoreceptor synapses and horizontal cell processes in response to transitions of the light/dark cycle. Peichl and Bolz (1984) described structural remodeling of retinal neurons in response to severe retinal degeneration induced by kainic acid. It

was nearly a decade later that reports of cellular remodeling in mammalian retina began to appear with some regularity (Chu et al., 1993; Li et al., 1995; Lewis et al., 1998; Fariss et al., 2000). Thus, the published literature reinforced the impression of the retina as relatively static structurally. Even total photoreceptor cell loss was not regarded as causing significant changes to the neuronal components of the inner retina, probably due in part to the difficulties of visualizing the often subtle changes in organization that accompany such alterations. Now it seems likely that the retina shares with the rest of the CNS a significant capacity for remodeling of its cellular architecture. The functional consequences of such remodeling largely remain unknown, although various informal and anecdotal clinical reports along with a few systematic studies of human vision after retinal reattachment surgery suggest that there are functional consequences because visual recovery occurs over a much longer time-period than would be expected based on outer segment regeneration alone.

### 1.1.2. Photoreceptor cell death differs among models

Many of the recent descriptions of structural remodeling in mammalian retina are from observations in humans or rodent species in which massive photoreceptor cell death is induced by light damage or genetic mutations. The literature on this topic has recently been reviewed extensively by Marc et al. (2003), and data from the detachment models compliment data from those systems because in most species there is not massive photoreceptor cell death after detachment. Another important distinction is that the earliest, and most obvious damage induced by detachment, outer segment degeneration, is reversible by reattaching the retina. The reversibility of inner retinal changes has, for the most part not been studied in detail.

### 1.2. Goals

Here we will discuss remodeling events in the retinal cell types illustrated in Fig. 1, including: Retinal pigmented epithelium, Müller cells, photoreceptors, rod bipolar cells, horizontal cells, ganglion cells, and astrocytes.

### 1.3. Retinal detachment and reattachment as systems for studying remodeling

Animal models of retinal detachment have been available since the late 1960s (Kroll and Machemer, 1968). In early light and EM studies (Erickson et al., 1983; Anderson et al., 1983) outer segment degeneration, photoreceptor cell loss, Müller cell hypertrophy, and changes in the retinal pigmented epithelium (RPE) apical surface were all recognized as significant cellular

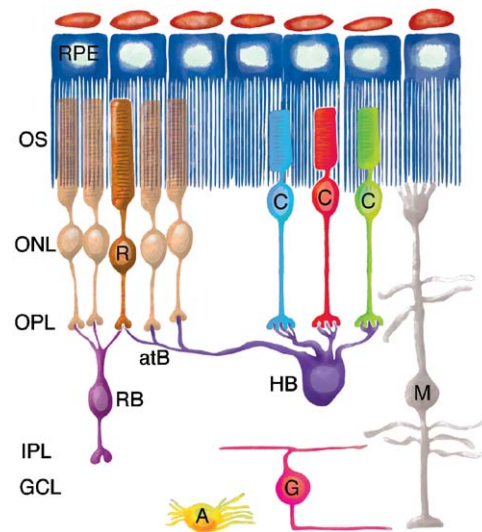


Fig. 1. A drawing representing the retinal cell types discussed in this paper. The outer segments (OS) of rod (R) and cone (C) photoreceptors interdigitate with specialized processes on the apical surface of the retinal pigmented epithelium (RPE). Their cell bodies comprise the outer nuclear layer (ONL), and their synaptic terminals end in the outer plexiform layer (OPL). Although the discussion in this paper is largely based on data from the feline retina, which has only two spectral types of cones (M- and S-sensitive), the 3 spectral classes of cones found in human retina are illustrated here. The dendrites of rod bipolar cells (RB) and the axon terminal (atB) of the B-type horizontal cell (HB) are postsynaptic to rod photoreceptors. Axons of rod bipolar cells terminate deep in the inner plexiform layer (IPL). The dendrites of the HB cell are postsynaptic to cones. The dendrites of ganglion cells (G) terminate in the IPL and their axons form the optic nerve. They are the third-order neurons of the retina, and their cell bodies form the ganglion cell layer (GCL). Müller cells (M) are the radial glia of the vertebrate retina. Their microvilli extend into the interphotoreceptor space adjacent to the ONL; their cell bodies lie in the INL, and they branch extensively in all retinal layers. The Müller cells terminate with specialized “endfeet” on the vitreal border of the retina. A population of astrocytes (A) lies among the GC axons and in the GCL. Blood vessels of the choriocapillaris lie just sclerad to the RPE monolayer and are the source of blood flow for both the RPE and photoreceptors. The retina proximal to the OPL is nourished by intraretinal vessels (not shown). The retina also has a population of microglial cells (not shown) that normally lie in the IPL and OPL. Artwork by Black Pixel, <http://www.blackpixel.net>.

events induced by detachment. More recently as we have gained the technological advantages of immunocytochemistry and confocal imaging (along with other methods of advanced imaging), many studies have shown the events after detachment to be far more complex than previously expected, and to include a significant amount of neural and glial remodeling. Presumably each cellular change induced by detachment indicates a change that is ideally reversed by reattachment in order for the retina (and vision) to return to its pre-detachment condition. Clearly, changes in the retina created by cell death are not going to be reversed by any current therapy. Other events may be reversed by reattachment, often incompletely and usually slowly—over a time-course that can vary from days to years.

Because experimental studies have, out of practical necessity, been limited in time we do not know what effects may continue for years after reattachment. Retinal circuitry may have a great deal of built in redundancy, in which case it may not be necessary to reverse all of the damage caused by detachment in order to have relatively normal vision. Likewise, photoreceptor outer segments may need only some fraction of their normal length in order to function with reasonable efficiency. The link between molecular expression, cellular architecture and recovery of normal vision remains relatively unexplored territory. The literature now clearly demonstrates that reactions to detachment occur across the retina, from photoreceptors to ganglion cells, and this research is still in an early stage. As a model system, retinal detachment has some advantages: (1) The ability to study events associated with both degeneration (after detachment) and recovery (after reattachment). (2) Control over the starting times for these two events, and the duration of each. (3) The effect of detachment height (the distance the neural retina and RPE are separated) can be controlled. (4) The extent (size) of a detachment can be controlled which also provides the opportunity to study precise transitions between detached and attached retinas.

#### 1.4. Defining remodeling

The difficulties of describing cellular events in the CNS are well documented from historical arguments that occurred well into the mid-20th century questioning the application of cell theory to neural tissue. The vast organizational complexity of CNS tissue, the huge numbers of neurons and glial cells involved, the vast range of intricacy in neuronal architecture, the small size of neuronal cell bodies relative to other cells, and the small size of the neuronal processes that intertwine to make up the traditional synaptic neuropil (or plexiform layers in the case of the retina) all combine to make convincing and reliable observations of changes in cellular morphology difficult-to-nearly impossible by the methods of traditional light and electron microscopy. Historically, discovery of the Golgi impregnation method was the breakthrough that allowed for a detailed description of individual neurons and their morphologic diversity (Ramón y Cajal, 1892).

##### 1.4.1. Making use of new technology to describe remodeling events

Similarly, immunocytochemistry and other current techniques that label individual cells or populations of cells have permitted the discovery of remodeling events in the retina in recent years. In many ways the Golgi method would be ideal for uncovering changes in neuronal morphology occurring as a result of injury or

insult to the nervous system, except that such discovery requires a reliable method that will give reproducible results in a large population of cells (to account for individual variation among cells). This is not a strength of the Golgi technique; well-known for its capriciousness and apparent randomness in producing stained cells. Recent advances in biological imaging (i.e., confocal microscopy), image processing, and cytochemical labeling (usually but not always by antibodies) have allowed for consistent, reliable means of documenting changes in cell morphology and the expression pattern of specific molecules. Thus data that have lead us to view the retina as a highly plastic tissue, one capable of undergoing significant cellular remodeling in response to injury. With this technology whole populations of cells can be reliably studied in different tissue samples. Antibody labeling of tissue sections can provide precise quantitative information under the right conditions (see Marc et al., 2003), or at least semi-quantitative comparisons of molecular expression levels if one is careful to keep control of variables in specimen preparation (fixation, hydration, dehydration, embedding medium, etc.), section thickness, and image collection parameters.

##### 1.4.2. Two studies that changed our views of retinal remodeling

The power of the newer technology can be illustrated by an example from data published from one of our earlier studies of retinal detachment. Erickson et al. (1983) published ultrastructural evidence suggesting that some rod photoreceptors retract their synaptic terminals after detachment. This conclusion was based on the observation that synaptic ribbons and vesicles came to lie in the cell body, adjacent to the nucleus instead of at the end of a rod axon (or synaptic spherule) in the outer plexiform layer (OPL). The limited sampling provided by electron microscopy made visualizing the extent of this event cumbersome and thus a number of questions were left unanswered by the study: was this a rare event, or did it occur widely throughout the photoreceptor population? Did it only occur in photoreceptor cells on the path to cell death? If it is a widespread phenomenon, then what are the effects on cells connected to the retracted rod terminals (i.e., rod bipolar dendrites and horizontal cell axon terminals)? These questions were answered only as antibodies became available to label the specific players: photoreceptor synaptic terminals, rod bipolar, and horizontal cells (Lewis et al., 1998). However, the ability to correlate changes in antibody labeling patterns with the ultrastructural images was critical, because protein expression profiles (e.g., amount, labeling pattern) can change dramatically in a cell responding to injury (as occurs in cone photoreceptors). Specimen preparation techniques can also lead to different results. Detached feline retinal tissue

embedded in wax using traditional dehydration techniques, provided an image of rod bipolar cells without dendrites, i.e. victims of extreme dendritic “pruning”, while the current technology (Matsumoto and Hale, 1993) in which specimens are not dehydrated, demonstrated prominent neurite outgrowth (with some significant pruning as well) from this same cell type (Lewis et al., 1998). Thus, advances in bioimaging technology have lead to some unexpected insight into the range and variety of the retina’s responses to injury. Speculation based on EM data in 1983 resulted in the following conclusion: “In addition to the effects of retinal detachment in the outer retina, we strongly suspect that the inner nuclear layer, inner plexiform layer, ganglion cell layer and, perhaps, more central areas of the visual system may be affected as well” (Erickson et al., 1983). Remodeling has now been firmly established in the inner retina, and leads one to believe more strongly that central changes, for example ganglion cell axonal arborizations and synaptic contacts, will be eventually identified as well. It is clear that the adult retina is not a static structure. Like the rest of the CNS, it has a wide repertoire of inherent responses to injury and many of these result in significant cellular remodeling.

#### 1.4.3. Levels of remodeling

In general, we are using the term “remodeling” to refer to structural changes in retinal cells, although these structural events will always be built on a foundation of molecular changes, and ultimately it is these that we will need to identify, understand and manipulate the mechanisms involved. Some of the changes that we describe can occur generally across a whole population of cells within the affected retinal area, although there is always inter-cell variation. For example, photoreceptor cell death changes the photoreceptor population and alters overall thickness of the outer nuclear layer (ONL). Outer segments are greatly shortened in all photoreceptors in a region of detachment, but the exact amount of outer segment loss may vary slightly from cell to cell. All inner segments appear to lose mitochondria and to show a “mixing” of organelles segregated normally to the ellipsoid (mitochondria) or myoid (ER, RER, Golgi). The apical surface of the RPE in a region of detachment is remodeled as the cells become “mounded” and their distinctive and highly specialized apical processes are replaced by a uniform fringe of microvilli (Figs. 2A–C, asterisk). Indeed, the latter may be the most invariant cellular remodeling that occurs in response to detachment. It appears to be constant from cell-to-cell and species-to-species. This surface must remodel again in response to reattachment as the apical processes are reformed, and here the remodeling is not so homogeneous (Figs. 2D and E); a good indicator that regeneration is not a perfect recapitulation of developmental events. In many cases, it is difficult to determine

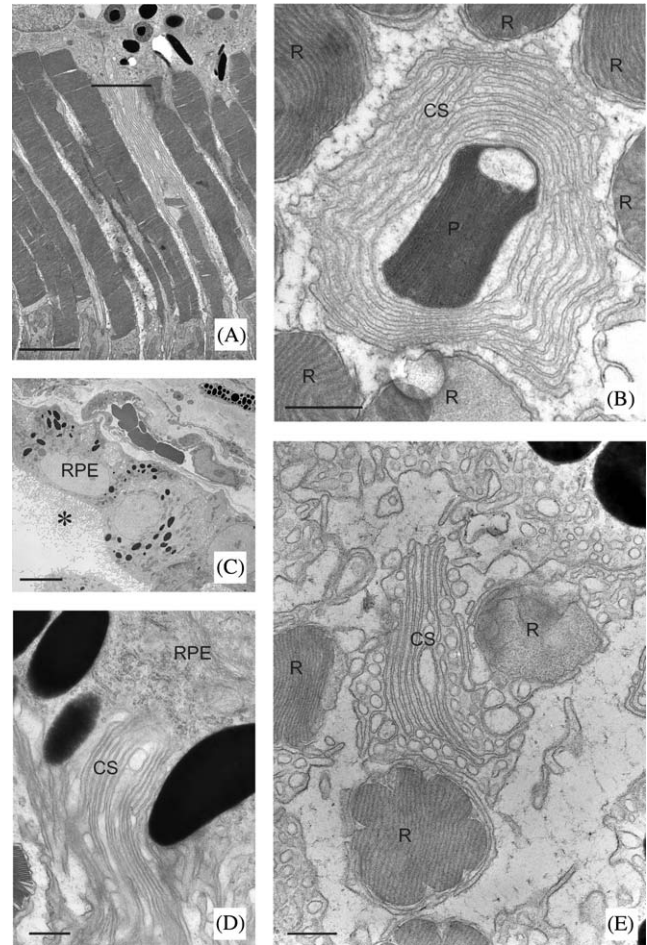


Fig. 2. A series of electron micrographs illustrating relationships between the RPE and photoreceptors. (A) A radial section through the normal feline retina. Rod (R) outer segments terminate close to the RPE monolayer (RPE). About 1/3 of their length is surrounded by microvillar and sheet-like apical processes from the RPE. Cone outer segments (C) are less than half the length of the rod outer segments and their outer segments are surrounded entirely by elaborate sheets of apical surface membrane referred to as the cone sheath (Reproduced with permission from Fisher and Anderson, 2001.). (B) A cone sheath in cross-section near the apical surface of the RPE (approximately in the location of the straight line in (A) showing the elaborate sheet-like apical processes that comprise the cone sheath (CS). The structure labeled “P” is a phagosome being transported within one of the apical processes from the tip of the cone outer segment to the RPE for digestion. The CS is surrounded by rod outer segments (R) in a regular array. (C) When the retina is detached, the specialized processes on the RPE apical surface are replaced by a fringe of unspecialized-appearing, homogeneous microvilli (\*). (D) Radial section near the RPE/photoreceptor interface in a retina detached for 3 days and reattached for 28 days. When the retina is reattached, the specialized RPE apical processes are regenerated. At this time of reattachment, CS are always recognizably “different” than in the normal retina. Their processes seem stouter and they are rarely observed in good alignment as shown in A. (E) A cross-section near the apical surface of the RPE in a retina detached for 3 days and reattached for 28 days. The processes that make up the CS are recognizable; though sheetlike they appear shorter and less concentrically organized than those in the normal retina (compare to (B)). In general the interface is not as regular as in normal retina, making it difficult to appreciate the array of rod outer segments around the cone sheaths. Scale bars = 2  $\mu$ m figures (A, C); 0.5  $\mu$ m figures (B, D, and E).

the number of cells within a population that are responding to detachment, and quantifying the responses may be critical to understanding the reaction to detachment and the recovery process. Cellular proliferation is stimulated in all non-neuronal cell types (Müller cells, RPE, astrocytes, vascular endothelium, pericytes) by detachment (Fisher et al., 1991), but the proportion of the different cell populations that proliferate is unknown. While all Müller cells appear to upregulate intermediate filament proteins, only some extend processes into the subretinal space or onto the vitreal surface, and both of these events result in serious ophthalmic diseases. Only a subpopulation of ganglion cells appear to remodel in response to detachment (Coblentz et al., 2003), and identifying which types may lead to a better understanding of some visual disturbances that occur after successful reattachment surgery.

#### 1.4.4. Retinal deafferentation revisited

Marc et al. (2003) discuss the remodeling of the inner retina as occurring largely in response to “deafferentation”, that is, the massive loss (usually approaching 100% but at varying rates) of photoreceptors in the retinas of animals and humans with inherited degenerations. They conclude that it is this deafferentation that leads to dramatic changes in the organization of the remaining retinal tissue; reorganization that is so extensive during the latter stages as to make it conceptually difficult to think of “recovery”, even if a new population of photoreceptor cells could be introduced into the tissue. Many of the remodeling events that we will describe here are similar to those occurring in the retinal degenerations, but they are often more subtle, may appear more quickly, and do not usually lead to gross rearrangements of the tissue. In the model system we have used for most of our studies (the feline retina), they are not occurring in response to a massive loss of photoreceptors. Indeed, more recent evidence (Claes et al., 2004) indicates that in mice in which both rod and cone degeneration is caused by knock-out mutations, early neuronal remodeling is very similar to what occurs in detachment, but with more dramatic, and drastic structural changes occurring in late stages when massive photoreceptor loss has occurred. Total photoreceptor cell death does occur after detachment in some species such as rabbit and ground squirrel. Interestingly, despite loss of virtually all of its photoreceptors after detachment, the ground squirrel retina shows little remodeling of inner retinal neurons and essentially none of the typical reactivity associated with Müller cells (Linberg et al., 2002a). Available evidence suggests that photoreceptor loss in human detachment is similar to that in the feline model (Chang et al., 1995). The changes that we have focused on are those that occur within hours, or days of detachment, because it is these changes that may be successfully manipulated by

therapeutic intervention and thus lead to better visual recovery. Except for the loss of photoreceptors, the hypertrophy of Müller cells, and the remodeling of the RPE, the neuronal remodeling that is associated with detachment cannot be appreciated by ordinary histological observation. While photoreceptor cell death may play a role in inducing these events, it is probably not the sole cause. Indeed molecular events that may lead to remodeling are induced very rapidly in Müller cells (Geller et al., 2001) before TUNEL positive photoreceptors (a measure of apoptotic cell death) appear in the ONL. Furthermore, remodeling is not just associated with the detachment and the subsequent degeneration of photoreceptors, but with the recovery phase after retinal reattachment. Until some method for replacing lost photoreceptors is found, it is not possible to study recovery in the models that lead to deafferentation of the inner retina.

## 2. A description of the model system

The retina as a developmental outgrowth of the brain is highly specialized for the transduction and primary processing of visual information. It is well-known that neuronal regeneration is extremely limited in the brain and spinal cord, but at the same time, these tissues react vigorously to injury in many ways: astrocytes migrate, proliferate, hypertrophy and form glial scars (Pekny et al., 1999), and oligodendrocytes migrate and express molecules on their surface that appear to inhibit axonal growth. Intriguingly, however, abortive axonal sprouts are common. There are fewer studies of similar reactions in the retina, but available evidence suggest that while some are shared, others are distinctively different than those in the brain and spinal cord. In part, the differences may reflect different cellular architectonics of the brain, spinal cord and retina. While photoreceptors have the highest metabolic rate of any cells in the body, there are no blood vessels among them—they are nourished almost solely by the capillaries of the choroid which lie on the opposite side of the RPE (see Fig. 1; Linsenmeier and Padnick-Silver, 2000). Traditional astrocytes are not scattered throughout the retina as they are in the brain and spinal cord, but reside only among the ganglion cells and their axons. And the retina has a large population of highly differentiated, polarized radial glia, Müller cells that may assume many of the functions of astrocytes in the brain and spinal cord, but are at the same time distinctively different from them. Ganglion cell axons in most species are not myelinated until after they enter the optic nerve, and thus, the retina does not have a population of oligodendrocytes, the myelin-producing glial cells. The retina does have a resident population of microglial cells that appear to be restricted to the inner retinal layers of the healthy eye.

## 2.1. Animal models of detachment

Several different species have been used in studies of detachment and reattachment. The choice of which species to use has been driven by many issues including among others: (1) how closely the retina resembles that of humans, (2) existing knowledge on the species (physiological studies, photopigment types, knowledge of retinal circuitry, genetics, etc.), (3) how reliably controlled detachments and reattachments can be created, and (4) the specific goals of the study. Different species do react differently to detachment and not all of these accurately reflect what little we do know about the reaction of the human retina. The rabbit retina (with about the same rod/cone ratio as the feline retina) is unusual in that it exhibits rapid degeneration of the photoreceptor layer, and eventual degeneration of much of the neural retina (Berglin et al., 1997; Faude et al., 2002). It provides a valuable model for rapid events occurring within minutes to a few days after detachment, but is probably not a good model for longer-term events. The California ground squirrel has a retina dominated by cones (Long and Fisher, 1983; Kryger et al., 1998), and thus is a potential model for the reactivity of the human macula (except that ground squirrel cones do not structurally resemble macular cones). The ground squirrel, however, shows a rapid and eventually complete degeneration of the photoreceptor layer, but almost no RPE or glial reactivity or neuronal remodeling (Linberg et al., 2002a; Sakai et al., 2001).

### 2.1.1. Developing new model systems

Developing a reliable method for producing large controlled detachments in the mouse eye has become a high priority in recent years in several labs (Nour et al., 2003; Yang et al., 2004) including our own. Current data from that model show many of the same reactions found in the feline model, although there may be less Müller cell reactivity. The height of a detachment, that is the distance separating the detached neural retina from the RPE, is probably an important parameter in human detachments and high detachments are harder to produce in mice because of the small size of the eye, and the fact that the lens fills much of the vitreous cavity. Nonetheless, the mouse model is an invaluable resource because it provides the opportunity to take advantage of genetic information and the techniques of molecular genetics.

## 2.2. The feline model

The experimental results that will form the basis for this report are largely derived from our own studies of feline detachment and reattachment because that is the system on which we have accumulated the largest database, and a system that provides many favorable

comparisons to known events in the human retina. Remodeling, the focus of this report, has not been the focus of many studies in other species, except in the broadest of terms (i.e. loss of cell populations such as photoreceptors, or Müller cell reactivity). The feline retina, like the peripheral human retina, is rod-dominated. It is a species with a robust intraretinal circulation (as in all species, excluded from the ONL). The feline eye is large, allowing for both easy surgical access, and for the production of defined detachments and simple reattachments using the same procedures as in human patients.

Our experimental procedure for producing detachments involves removing the lens and vitreous and the infusion of fluid into the interface between the RPE and neural retina through a glass micropipette with a tip diameter of about 100  $\mu\text{m}$  (Lewis et al., 1999). It has been argued (Aaberg, 1999) that this does not model a rhegmatogenous detachment because there is no large tear (referred to as a “retinal break”) through the retinal tissue. However, our observations of human detachment and reattachment tissue validate the feline model as producing generally the same cellular responses (Sethi et al., 2004). Our method for producing a reattachment is to perform a fluid–gas exchange, which drains the fluid from under the retina. Once the retina becomes flat the eye is flushed with 20% sulfur hexafluoride in filtered room air (Lewis et al., 2002).

## 3. Cell types remodeled by detachment (and reattachment)

### 3.1. Introduction

Fig. 1 shows the cell types that we have identified to date as structurally remodeled by detachment and reattachment, although it seems reasonable that other cells will be added to this list. We have only preliminary evidence for the structural remodeling of amacrine cells, although this has been described in late-stage human retinitis pigmentosa (Fariss et al., 2000). We have also observed some changes in retinal astrocytes as they proliferate and participate in the formation of epiretinal membranes but little else is known about the reactivity of these cells. Retinal microglia also respond robustly to detachment by assuming more macrophage-like morphological characteristics (Thanos et al., 1996).

### 3.2. Retinal pigmented epithelium

The morphology of the retinal pigment epithelium changes stereotypically after detachment in all species studied, although the morphological complexity of the apical surface varies significantly from species to species. In some, the processes are thick and filled with pigment

granules, in others they are fine, and sheetlike, or they can be combinations of sheet-like and tubular shaped processes (Long and Fisher, 1983; Anderson and Fisher, 1979; Steinberg et al., 1977; Steinberg and Wood, 1974; Fisher and Steinberg, 1982). In the cone-dominated ground squirrel retina there is virtually no distinguishing morphological features between the apical processes that drape the cones and those that drape the far less numerous rods. In species like cat, dog, rabbit, and human there are vast differences between those that interact with rods and cones (Anderson and Fisher, 1979; Steinberg et al., 1977; Steinberg and Wood, 1974; Fisher and Steinberg, 1982), with the “cone sheath” forming a unique structure consisting of a highly complex, multi-layered array of sheets of apical processes (Figs. 2A and B). Regardless of their structure in the normal eye, these apical processes all disappear after detachment, and are replaced very quickly, probably within hours, by a fringe of simple microvillus-like processes (Immel et al., 1986; Fig. 2C, asterisk). These remain virtually unchanged as long as the retina remains detached. In some species (cat, rabbit, human) the RPE proliferates with daughter cells migrating from the monolayer to form complex “cellular membranes” in the subretinal space, and sometimes migrating through retinal tears and into cellular membranes in the vitreous. In other species such as the California ground squirrel, the RPE remains mitotically quiet (Linberg et al., 2002a). Perhaps the most remarkable ability of the RPE is to re-form its complex apical processes after reattachment. In the case of the feline retina, this also means regenerating the highly complex cone sheaths as the cone outer segments regenerate. Indeed, cone sheaths are recognizable opposite regenerating cone outer segments with only a few basal discs (Fisher and Steinberg, 1982), thus its regeneration does not require the presence of a full-length, nor normally shaped outer segment. Moreover, the appearance of slightly truncated, often thickened or misaligned processes making up the cone sheaths are almost always a clear indicator that a portion of retina was detached at some earlier time. Although regeneration of this structure may never be a perfect recapitulation of its developmental complexity (compare the structures labeled “CS” in Figs. 2A, B, D and E), there must be some signaling mechanism between cones and the RPE that induces the regeneration of these intricately organized cone sheaths. Assuming that they serve some important physiological function, finding ways to improve their regenerative capacity, particularly in the macula, may be one of the subtle ways to improve vision after retinal reattachment surgery or any other disease or procedure that disrupts the RPE/photoreceptor interface. The surface of this monolayer is further perturbed as cells begin to proliferate within a day or two after detachment (Anderson et al., 1981). Newly proliferated cells can

expand in size or migrate out of the monolayer into the subretinal space where they can assume many complex geometric arrangements: single cells, long strands, or multiple layers that appear to replicate the original monolayer, or multiple layers with reversed apical–basal polarity. If the basal surface faces the neural retina then photoreceptor outer segment regeneration does not occur after reattachment (Anderson et al., 1986). It is not known if photoreceptor function is compromised when outer segments do regenerate in the presence of multiple layers of RPE. In diseases such as proliferative vitreoretinopathy, RPE cells can migrate into the retina and onto the vitreal surface through retinal tears where they can participate in the formation of cell assemblies in the vitreous known as “epiretinal membranes”. While their presence in the epiretinal membranes is convincing, their functional role in establishing the membranes and in the ensuing events that result in retinal detachments due to contraction of the cellular membranes, are still controversial. We will further address the formation of these membranes in the context of Müller cell remodeling.

### 3.3. Photoreceptor cells

#### 3.3.1. Photoreceptor deconstruction, outer segment degeneration and cell death

In the feline model, and probably in the human retina, detachment sends all of the photoreceptors, both rods and cones within the zone of detachment, along a pathway of structural changes that we have termed “deconstruction” (Mervin et al., 1999). The outer and inner segment structural response appears to be the same in both photoreceptor types, but the synaptic terminal responses differ. The term “deconstruction” was chosen as more accurately reflecting the overall changes in photoreceptors than the commonly used, and more general, “degeneration” because these cells enter a phase of very specific changes which always results in structural reorganization but only sometimes leads to cell death. In both feline and human retina it is clear that many cells survive detachment for very long periods of time; thus, not all cells are sent on a pathway that leads to cell death. Indeed, in these species, deconstructive changes may well occur as a mechanism to assure the cells’ survival under adverse environmental conditions. It is not clear why in some species (ground squirrel, rabbit) detachment leads to complete photoreceptor degeneration and cell death, more like the responses to light damage or to various gene-induced degenerations. Outer segment degeneration is a common theme in all of the photoreceptor degenerative conditions whether genetic, disease-, or injury-induced (Marc et al., 2003). In many such conditions, outer segment disc membranes can appear vesiculated or distorted prior to deterioration of the outer segment. In retinal detachment, the



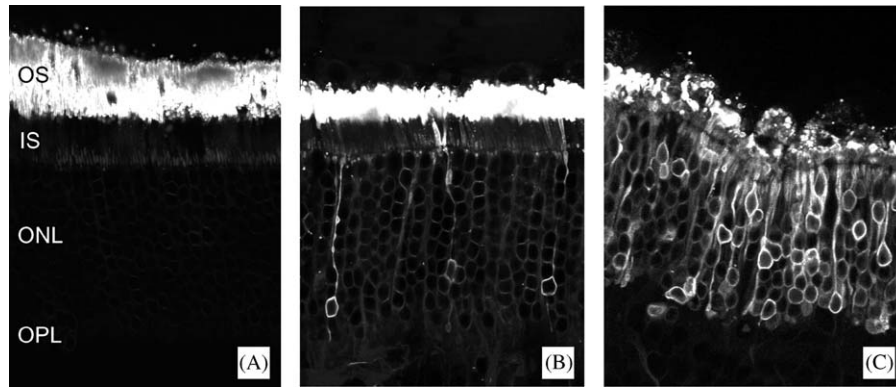


Fig. 3. A series of confocal images of normal and detached retina labeled with an antibody to rod opsin (provided courtesy of Dr. Robert Molday). (A) Normal retina. Only the layer of rod outer segments (OS) labels heavily with this antibody. (B) Retina detached for 3 days. When the retina is detached, the OS layer becomes thinner as the rod (and cone, see Fig. 4) OS degenerate. As the OS degenerate, the rod opsin antibody begins to prominently label the plasma membrane of rod cells in the outer nuclear layer (ONL). This plasma membrane “redistribution” of opsin can outline the entire cell to its synaptic terminal in the outer plexiform layer (OPL). (C) Retina detached for 28 days. The rod outer segments are greatly shortened and highly disrupted. However, even the smallest fragments of remaining OS continue to label with the antibody to rod opsin. Note the extensive redistribution of labeling in the ONL.

most common observation is that it appears as a simple process of outer segment shortening over a period of a few days. However, the mechanism by which outer segment material is discarded is unknown. It may occur by the controlled, sequential loss of disk packets from their tips as occurs during the outer segment renewal process (Young, 1967). It is difficult to understand how a closed cellular compartment such as a rod outer segment could lose part of its length by some simple “deterioration”. Whatever the mechanism, outer segment material is lost until only a few disks remain along with the connecting cilium (Anderson et al., 1983). Because it is unusual to see large collections of outer segment membranes or outer segment fragments in the subretinal space after detachment, these must be removed quickly, probably by a combination of phagocytosis by the RPE and macrophages that invade that space. Feline photoreceptors in detachments of several months duration frequently have a few discs (often distorted in shape) associated with the connecting cilium, and always show the presence of a cilium. Clearly the complex mechanism of disc morphogenesis is not lost in these cells (Steinberg et al., 1980) because once reattached, they retain their ability to once again reconstruct an outer segment through the production of evaginations of the connecting cilium, even though that reconstruction may not be structurally perfect. When examined in detail, some photoreceptor outer segments may retain a few more discs than others, but in general the loss is uniform across the detachment resulting in the complete remodeling of the outer segment layer (Figs. 3A–C). Remaining photoreceptor outer segments, even if composed of only a few discs are still positive for their respective opsin protein (Fig. 3; Lewis et al., 1991; Fariss et al., 1997) as well as other proteins.

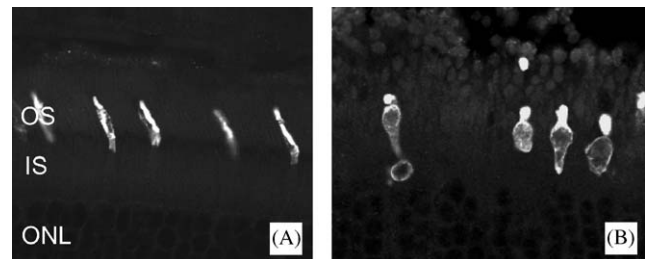


Fig. 4. A series of confocal images of normal and detached retina labeled with an antibody (provided by Dr. J. Nathans) to the medium (M)- and long (L)-wavelength-sensitive cone opsins. Of the two, the feline retina has only M cones. (A) Normal retina. Only the M-cone outer segments are labeled within the outer segment layer (OS). (B) Retina detached for 3 days. Cone OS are greatly shortened and distorted in the detached retina, and the antibody now labels the plasma membrane of the cone cells extending to the outer nuclear layer (ONL). After 3–7 days of detachment, it is rare to find labeled cones in detached retina. IS = inner segments.

As will be discussed below, cone photoreceptors demonstrate outer segment degeneration (Figs. 4A and B) but otherwise behave very differently from rods. Rod photoreceptors also continue to transport radiolabeled proteins into these shortened outer segments (Lewis et al., 1991) indicating that the general process of outer segment renewal (Young, 1967) continues even though a normal outer segment cannot be constructed in the absence of attachment to the RPE. While outer segment loss is also a characteristic of genetic based photoreceptor diseases, the end-product is always cell death (Marc et al., 2003). This is not necessarily the case after detachment. Although there may be islands in which the immunocytochemical data suggests complete photoreceptor loss in feline detachments of a month or more duration, many cells survive.

Most photoreceptor cell death after detachment is probably by apoptosis, although necrotic cell death may occur, especially among photoreceptor cells extruded into the subretinal space. Based on our results in the feline retina, there is an early period, around the first 3 days after detachment when about 20% of the photoreceptors die by apoptosis (Cook et al., 1995; Lewis et al., 2002), but even retinas detached for 450 days can retain at least 50% of their photoreceptors (Erickson et al., 1983). Although the greatest number of TUNEL positive cells occurs around day 3 of a feline detachment, and the number falls dramatically after that, there is always some apoptosis occurring in the detached retina. Observations of human detachments suggest that cell death by apoptosis is a part of the response to detachment, but as in the feline model, large portions of the photoreceptor population also survive (Chang et al., 1995). There is no information about the survivability of foveal photoreceptors after detachment. In other species, such as rabbit and ground squirrel, essentially all of the photoreceptors eventually die, leaving the inner retina in the “deafferented” state. In rabbits, but not ground squirrels, the inner retina shows signs of severe cell loss as well. Thus, outer segment degeneration is common to all photoreceptors after detachment, but other cellular responses differ between rods and cones, and between species.

### 3.3.2. Internal reorganization of photoreceptor inner segments

Photoreceptor inner segments undergo internal reorganization after detachment. In normal photoreceptors the most distal part of the inner segment is packed with mitochondria (the ellipsoid), reflecting the high metabolic rate of these cells, while the more proximal part (the myoid) contains the organelles associated with protein synthesis and trafficking. After detachment, electron microscopy as well as immunocytochemical labeling for mitochondrial components show a decrease in the number of mitochondria in the cells as well as a much less distinctive compartmentalization of all organelles (Anderson et al., 1983; Erickson et al., 1983; Mervin et al., 1999). Although losing mitochondria might be expected to have a profound effect on cells' survivability during a time of stress, there have been no definitive studies of this response, nor of the effect of detachment on photoreceptor energy metabolism in general. Deconstructing the highly differentiated state of photoreceptors after detachment may in fact provide a survival mechanism for these cells. Maintaining the highly compartmentalized, and polarized structure of a photoreceptor must be metabolically costly. Certainly, the overall high metabolic rate of photoreceptor cells is well-established (Winkler, 1983). Having the cells assume a much simpler organization may assure their survival under environmentally challenging condi-

tions. In the case of detachment that challenge would include hypoxia and probably hypoglycemia created by physically moving the retina farther away from its choroidal blood supply (Mervin et al., 1999; Lewis et al., 1999; Linsenmeier and Padnick-Silver, 2000) and perhaps the removal of crucial biological factors normally secreted from the RPE. This hypothesis is supported by studies in which providing excess environmental oxygen lessens photoreceptor deconstruction and cell death after detachment (Mervin et al., 1999; Sakai et al., 2001; Lewis et al., 2004).

### 3.3.3. An overall comparison of rod and cone photoreceptor responses

The outer and inner segments of rods and cones undergo similar structural changes in response to detachment, but show many differences otherwise. A prominent response of rods is the withdrawal of their axon (Figs. 5A and B) and a reconfiguration of the single synaptic invagination with its 3–5 postsynaptic processes (Boycott and Kolb, 1973; Kolb, 1974). This withdrawal is manifested as a disruption of the layer of rod synaptic terminals at the distal border of the OPL. While this disruption is apparent in electron microscopic images, it is best appreciated when the terminals are labeled with any one of several antibodies to presynaptic proteins, such as synaptophysin (Fig. 5) or vesicle associated membrane protein (VAMP or, synaptobrevin). In the normal retina, the photoreceptor synaptic terminals form a compact layer on the distal border of the OPL. The large cone pedicles form a single,

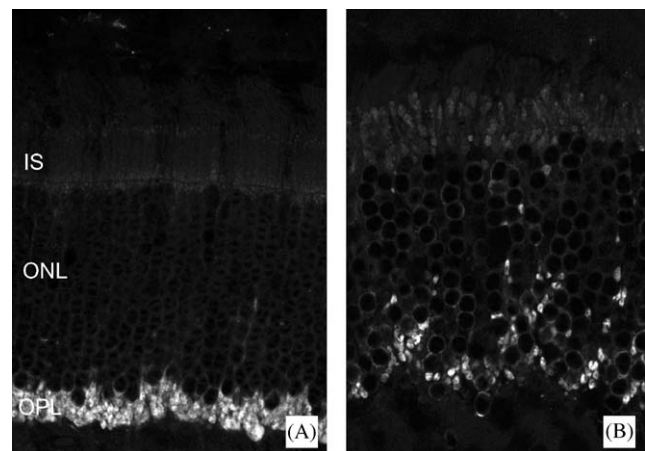


Fig. 5. Confocal images of normal and detached retina labeled with an antibody (Dako) to the synaptic vesicle protein, synaptophysin. (A) Normal retina. Antibody labeling is highly specific to the layer of photoreceptor synaptic terminals in the outer plexiform layer (OPL) illustrating the tightly packed ranks of photoreceptor terminals typical of normal retina. B. Retina detached for 3 days. The compact layer of anti-synaptophysin labeling is now dispersed with labeling prominent in the outer nuclear layer (ONL) revealing many retracted rod spherules remodeled by detachment. IS = inner segments.

interrupted row adjacent to the neuropil, while the more distally located, numerous, and smaller rod spherules occur 3–5 deep. After detachment, disruption of the layer of rod terminals is obvious because some labeled terminals now appear near the rod nuclei scattered throughout the ONL (Fig. 5B). This terminal withdrawal and reorganization begins quickly, with a few terminals appearing in the ONL within a day, and many recognizable there within 3 days of a detachment. The relatively low-power electron micrograph in Fig. 6 shows the highly compact organization of the rod terminals in the OPL in a normal feline retina. After detachment, ultrastructural observations show that the deep synaptic invagination become shallower, (arrows, Figs. 7A and B), and are eventually lost altogether (Figs. 7C and D). Also based on their ultrastructural appearance there are still membrane specializations associated with the “postsynaptic” processes terminating adjacent to the base of the retracted rod terminals (Figs. 7C and D). Based on electron microscopic observations, there may be a sequence of events in which the synaptic complexes of the rod spherules first become shallower than normal and the post-synaptic processes more “loosely” organized (Fig. 7B). Those terminals that do not retract may retain this type of organization, that is, they do not lose their synaptic invaginations but they do change their organization. All of these changes must be on a foundation of complex changes in protein expression, ranging from changes in adhesion molecules to those associated with transmitter release, vesicle recycling, and transmitter receptors. This is unexplored territory for these highly altered synapses. The cytoplasm of the withdrawn rod terminals continues to show a sparse population of synaptic vesicles and synaptic ribbons that vary more in size, configuration and location than normal. Most ribbons appear anchored to the membrane, even in the absence of a synaptic invagination, but some appear to be free-floating in the cytoplasm. While there are one or two long, arc-shaped synaptic ribbons in normal feline rod spherules (Boycott and Kolb, 1973; Migdale et al., 2003), the presence of 1–3 very short ribbons is common in the retracted terminals (arrowheads Fig. 7A, C and D). On the other hand we have also observed very long ribbons with sharp curves unlike the smooth arc of those in normal retina. The data from synaptic ribbon-specific labeling (see below) also suggests that these ribbons change dramatically after detachment.

Cone synaptic terminals respond differently from those of rods. The impression from both electron microscopy and immunocytochemical labeling studies is that the cone pedicles remain in their location within the OPL, that is, their axon does not withdraw like that of many rods, but that the pedicle itself undergoes unpredictable changes in shape. Some appear to flatten along the border of the neuropil while others appear

more club-shaped and smaller than normal. The axons of some become tortuous instead of streaming straight across the ONL from their cell body (Figs. 8A–C). The latter may be a response to the fact that cone cell bodies, which form a single row on the outer border of the ONL in the normal retina, appear to migrate deeper within that layer in response to detachment (Anderson et al., 1983); the axon, instead of shortening as the rod axons do, simply becomes folded among the rod cell bodies. In all cases however, these terminals appear to lose the 9–14 synaptic invaginations (Boycott and Kolb, 1973) that occur in normal feline cone pedicles (Figs. 9A and B), giving their base a flattened appearance (Fig. 8C) when observed by immunocytochemical labeling or electron microscopy (also see Erickson et al., 1983). As the invaginations become shallower the associated presynaptic ribbons appear to grow shorter, with many apparently disappearing (Figs. 9A and B). This response is dramatic when observed by immunocytochemical labeling when an antibody to CtBP2 is used to label presynaptic ribbons. This antibody recognizes a CtBP2 domain of a transcriptional repressor and part of the ribbon-specific protein known as “RIBEYE” (Schmitz et al., 2000). Labeling with the antibody shows that recognizable ribbons remain in rod terminals although most now appear as “clumps” of label rather than the characteristic “arcs” seen in the normal retina (compare the red colored synaptic ribbons in Figs. 10B and C to those in Fig. 10D). More dramatic is the change in labeling within the cone pedicles, because the characteristic array of ribbons (arrowheads, Fig. 10C) associated with each pedicle is no longer detected in the OPL (Fig. 10D). Ultrastructurally, some short ribbons remain within the affected cone terminals, and some post-synaptic processes are still recognizable (Fig. 9). Documenting changes in these complex cone synapses after detachment of more than 3 days is complicated by the fact that the expression of proteins in these cells rapidly decreases to levels that are below detection (Rex et al., 2002a; Linberg et al., 2001a). This response is shown clearly by labeling of normal and detached cones with an antibody to the  $\gamma$  subunit of the photoreceptor phosphodiesterase (PDE $\gamma$ ). The series of confocal images shown in Fig. 8 demonstrate the robust labeling observed in cones of the normal retina (Figs. 8A and B) and the decrease in labeling intensity in cones of 7-day detached retina (Figs. 8C and D). However, finding even a few faintly labeled cones in 7-day detached retina is challenging, the more common labeling pattern is shown in Fig. 11. In a normal retina there would be many green colored cones extending across the outer retina (with a morphological appearance of the labeled cones in Fig. 8A). In Fig. 11 there are long expanses of the retina in which only the rod outer segments (green) are labeled by the anti-PDE $\gamma$  and there are no labeled cones. The lack of cone labeling shown here is indicative of the

general down-regulation of proteins expressed in these cells. The observation of this same tissue by transmitted light or electron microscopy would clearly show the presence of recognizable cone nuclei and cone pedicles. Thus, the only observations of structural remodeling in cones in long-term detachments comes from electron microscopy with its inherent sampling issues. Nonetheless, the impression over many years of study is that one never observes structures within the ONL of detached retinas that can be identified as retracted cone terminals. The rod synapses made by rod terminals deep in the ONL of the detached retina have ultrastructural characteristics reminiscent of early developing photoreceptor synapses where synaptic ribbons and invaginations appear in perinuclear cytoplasm (Linberg and Fisher, 1990). Cone cell bodies are easily recognized by the distinctive pattern of chromatin in their nucleus, but we do not observe synaptic structures in a perinuclear location in these cells. What the two photoreceptor types do share is the loss of deep synaptic invaginations and changes in the number and organization of synaptic ribbons.

#### 3.3.4. Cone survival

Cell death occurs among both rods and cones, altering the overall population of each cell type. A quantitative understanding of cone survival, considering their critical role as the receptors for high acuity vision, is a vital issue in understanding the recovery of human vision after reattachment, but not one easily resolved. Most species used for detachment/reattachment studies do not have a fovea although they may have regions of increased cone density (the *area centralis* of cats or visual streak of rabbits or ground squirrels). Although feline cone nuclei can be recognized by positional and morphological criteria, their position can change dramatically after detachment making their recognition less reliable. Thus, the ideal means of sampling the surviving cells is to use some specific marker for the cones. But as stated above, this is extremely problematic because cone photoreceptors, as opposed to rods, down-regulate the expression of many proteins to the point where they are below the threshold of immunocytochemical detection (Rex et al., 2002a, b; Linberg et al., 2001a; John et al., 2000).

#### 3.3.5. The population of cones is heterogeneous

The lectin, peanut agglutinin (PNA), labels the extracellular matrix domain (matrix sheath) around cone photoreceptors (Johnson et al., 1986) and can be used to define the total population of cone cells. Fig. 12, left is a density map of PNA labeled cone sheaths in the superior temporal quadrant of a control feline retina. The density of cones closely matches that based on morphology (Steinberg et al., 1973), and demonstrates the elevated population in the *area centralis*. There are

specific antibodies that recognize the different opsins associated with different spectral classes of photoreceptors (Wang et al., 1992; Szél et al., 1985, 1988). The feline retina contains mid-wavelength sensitive (M) and short-wavelength sensitive (S) cones, and the latter are not distributed evenly over the retinal topography (Fig. 13, upper left). Whereas the total number of cones peaks in the *area centralis*, the density of S-cones is highest in the inferior retina but even there they comprise only about 20% of the cone population (Linberg et al., 2001a). The expression of specific photopigment genes defines distinct populations of photoreceptors, and photopigment proteins are expressed in all normal photoreceptors. The expression of other proteins by these cells is less consistent. An antibody to the calcium binding protein calbindin D labels entire cone photoreceptors in many species, including humans and felines. The density of calbindin D positive cells matches very closely the density of PNA labeled cone matrix sheaths in the peripheral retina, but there is significant disparity in the *area centralis* where the antibody fails to label a majority of cones (Fig. 14, left). This phenomenon also occurs in monkey and human retina where foveal cones do not label with calbindin D antibodies (Röhrenbeck et al., 1989; Pasteels et al., 1990; Haley et al., 1995). Heterogeneity in the expression of cone opsin genes is well-known, but it appears likely that there are other, less well-understood differences in the expression of proteins among the cone population which adds to the complexity of analyzing the responses of cones to injury.

#### 3.3.6. Protein expression in cone photoreceptors after detachment: analyzing the surviving cone photoreceptor array

As photoreceptor deconstruction proceeds, rods remain labeled by antibodies used to label them in the normal retina, although the intracellular *pattern* of labeling changes dramatically in some cases. As rod outer segments degenerate, rod opsin labeling begins to increase in the plasma membrane of the cells until the whole cell is outlined (see Fig. 3; Lewis et al., 1991; Fariss et al., 1997; Rex et al. 2002a; Linberg et al., 2002a). Thus antibodies to rod opsin remain a reliable marker for the presence of these cells and the redistribution of labeling is also a remarkable indicator of stress or injury to them. Antibodies to the cone opsins similarly begin to label the plasma membrane as the outer segment degenerates (Fig. 4). However after only 24 h of detachment many cones fail to label with these antibodies, or any of the other markers we have used (see Rex et al., 2002a). A similar phenomenon occurs in retinas of humans with late stage genetic degeneration (John et al., 2000), with the difference being that cone opsin expression appears to remain while the expression of other marker molecules does not. Thus, reliable

markers for cones in the normal retina are not that reliable for estimates of the cone population that survive an interval of detachment (Linberg et al., 2001a). Indeed, as shown in the quantitative study by Linberg et al. (2001a), if the lack of labeled cones after detachment was an accurate reflection of cone survival, then the effects of detachment on the cone population would be devastating. Further confounding the use of markers for cones is the fact that the response is not consistent from marker to marker nor even from one retinal region to another. In the central region of a control retina, anti-calbindin D labels about 19,700 photoreceptors/mm<sup>2</sup>, and the antibody to S-cone opsin about 1100/mm<sup>2</sup>. After 24 h of detachment these numbers drop to 9000/mm<sup>2</sup> (46% of control values) and 700/mm<sup>2</sup> (63% of control values), respectively. By 28 days of detachment there were no cells labeled with the anti-calbindin D, but 200/mm<sup>2</sup> (18% of control values) with the anti-S-opsin (Linberg et al., 2001a and see Figs. 13, lower left and 14A–C). When examining the detached retinas with these markers there are large areas in which no labeling appears at all (see the example for PDE $\gamma$  in Figs. 10 and 11), as if all of the cones in the region were gone. These dramatic changes are reflected in the isodensity maps for both S-opsin and anti-calbindin D labeling (Figs. 13 and 14). In the latter, the central area is nearly undefined by 1 day of detachment, and completely undefined after 3-days. Similarly, the number of S-cones drops from between 700 and 1100/mm<sup>2</sup> in the control retina to less than 300 in a 3-day detachment. Because of the relatively large bins used to create these maps, they do not show the substantial islands in which there were no labeled cones. The wide variations in labeled cones gives the retinal whole-mounts a “patchiness” that is not observed in control retinas where the transitions in cone density are smoothly graded.

Because no reliable marker has been found that continues to label cones after detachment, we attempted to approach the question of cone survival in another way: by examining the recovery of cone markers after reattachment (Linberg et al., 2002b). Based on observations in tissue sections, cones regain the expression of the various markers as they recover. Thus, we detached retinas for 3 days, a time that creates a significant drop in the cone population based on studies with PNA, anti-cone-opsin, or anti-calbindin D labeling. The superior retina in the right eye of three cats was detached for 3 days and then reattached for 28. The retinas were harvested and labeled with PNA (Fig. 12, PNA = all cones, only the superior temporal quadrant is illustrated) and the antibody to S-opsin (Fig. 13). Comparisons can be made to the maps for total cones (calbindin D labeling, control and 3 days, Figs. 14 left, and B; PNA labeling, control retina, Fig. 12) and S-cones (S-cone opsin labeling, control and 3 days, Figs. 13 left). There is

clearly recovery of both the PNA and S-cone population after reattachment; indeed a central-to-peripheral gradient is apparent in the PNA labeling pattern, although the density remains depressed; none of the animals recovered densities greater than 17,000–20,000 cones/mm<sup>2</sup> (Figs. 12A–C). Similarly, the S-cone population recovers, but recovery is not complete (Figs. 13A–C). The high density area of S-cones in the far periphery of the superior-temporal quadrant is not recovered at 28 days of reattachment, and in all three animals, recovery in the central retina was in the range of 300–700 cells/mm<sup>2</sup>, compared to the 700–1100 cells/mm<sup>2</sup> observed in the control retina (the detachment did not extend into the yellow/red colored area in the central area of Fig. 13C). Because these numbers are generated as counts from small sampling areas, and the contour lines on the maps drawn by eye, it is difficult to choose an average value for cone density within the reattached retina, but using estimates of cone density sampled over a fairly broad region, results from two animals with reattachments show a recovery of 40–60% of PNA labeling in the area centralis, and approaching 100% recovery in the periphery.

S-cone recovery seems more variable, ranging between zero and 40% with no pattern readily discernable across the retina. Whereas PNA labeled cone matrix sheaths were remarkably evenly distributed across the retina and showed a central to peripheral decline, S-opsin labeling showed large swatches within the reattached hemiretinal map with no visible labeling. These data demonstrates quantitatively that the absence of marker molecules in the detached retinas does not indicate the absence of cone photoreceptors because cells recovered across the entire retina, in some cases to numbers comparable to those in the control eyes. It is unknown of course, if there would be more recovery over a longer reattachment time.

Structurally, the S-cone outer segments in the 28-day reattached retina are not equivalent to those in the normal eye. They are often only punctuate “dots”, a fraction of their size in control retina. The S-cone population has been reported to be more sensitive to damage in human retinas with detachments (Nork et al., 1995), with all S-cones either lost or showing signs of “irreversible damage” within a few days of detachment. Our data from feline retina may support the concept that S cones are more fragile, more susceptible to cell death, and slower to recover than the M-cones, however it does not support the conclusion that all of the S-cones are irreversibly damaged.

Combined electrophysiological (ERG) and immunocytochemical studies in the cone-dominant ground squirrel retina did not show any particular difference in recovery of signals from S- and M/L-cones in that species (Jacobs et al., 2003). What may be the most remarkable conclusion from this and other studies, is the

ability of cones to stop expressing specific proteins when the cells are under some type of physiological stress, and then, at least in the case of reattachment, to begin re-expressing these proteins as part of a recovery process. In their studies of human retinal tissue, Nork et al. (1995) reported that carbonic anhydrase reactivity became an unreliable marker for M-cones after detachment, indicating that it may join the list of severely down-regulated proteins, and its loss may not be an indicator of irreversible damage.

It is worth re-emphasizing the differences in the labeling data for rods where the cells continue expressing all markers that have been studied to date, albeit with changes in the pattern of expression, even in retinas detached for as long as a month. Do primate cones show similar responses to detachment? The data of John et al. (2000) suggest that there may be differences, at least in the expression of visual pigments. Whether or not foveal cones show similar responses is an unanswered question. Are the structurally exceptional cones in the fovea (Anderson and Fisher, 1979; Hogan et al., 1971; Rodieck, 1998) with their very long outer segments and long axons more susceptible to damage than the peripheral cones? Even the peripheral cones are structurally different in the feline and human retinas (Anderson et al., 1978; Anderson and Fisher, 1979) so their reaction to injury and their capacity for recovery may differ as well.

A differential loss of M/L- and S-cones, or a differential recovery of these cells in humans may explain some of the color vision deficiencies reported in reattachment patients, and could underlie losses in visual acuity when the fovea is involved. One important component of the cones' response repertoire, the large down-regulation in the expression of key proteins, may contribute to their survival under stress, but may also account for the prolonged recovery period often experienced by patients with foveal detachments.

### 3.3.7. *Remodeling of photoreceptors after reattachment*

The fact that photoreceptor outer segments re-grow after reattachment has been recognized since 1968 (Machemer, 1968; Kroll and Machemer, 1969a, b). This remarkable ability of photoreceptors to recover their outer segment is mechanistically tied to the fact that these cells have in place machinery for the constant addition of new outer segment material (outer segment renewal; see Young, 1967). Thus they are poised to rebuild their outer segment as soon as favorable conditions allow. Studies of detached retina (Lewis et al., 1991) in fact show that radiolabeled proteins continue to be transported into the truncated outer segments of rods that remain after detachment. No comparable studies of protein incorporation have been done for cones after detachment. Both rods and cones undergo similar cycles of membrane renewal but our data indicate that the two

respond differently with respect to the production of proteins after detachment. Since cones downregulate the production of many outer segment proteins, they may also alter their membrane renewal cycle after detachment.

Immunocytochemical data suggest that molecules such as opsin, peripherin/rds, PDE $\gamma$ , arrestin and ROM-1 continue to be incorporated into rod outer segment membranes in the detached photoreceptors (Fariss et al., 1997; Lewis and Fisher, 2000; Lewis and Fisher, unpublished data), although the prominent labeling of the plasma membrane with rod-opsin antibodies and the labeling of intracellular vesicles with peripherin/rds (Fariss et al., 1997) and ROM-1 (Lewis, G.P. and Fisher, S.K., unpublished observations) antibodies suggest that protein targeting and trafficking is altered when the outer segment degenerates in the rod cells.

There is evidence from experimental data that the presence of opsin in the plasma membrane may make photoreceptors more vulnerable to apoptotic cell death (Zhang and Townes-Anderson, 2002). If this hypothesis is born-out, then the fact that cones quickly down-regulate opsin expression so that plasma membrane labeling occurs for a relatively short time may make them less susceptible to cell death. However, the continued production of critical outer segment structural proteins such as opsin and peripherin/rds by rods may assure that these cells are poised to enter a recovery phase rapidly upon return to the reattached state. There may be sufficient redundancy in the rod pathways that sacrificing even significant numbers of these cells is balanced by the advantage of rapid recovery of scotopic vision. Cones, which appear to cease their production of many outer segment proteins during an episode of detachment, may recover more slowly, but more of the initial population may survive. This hypothesis has not been explored systematically in experimental studies although there are suggestions of support if results from different species are compared.

The width of the outer segment layer in rod dominated feline retinas detached for 3 days and reattached for 28 days is about 13  $\mu\text{m}$  compared to 16.5  $\mu\text{m}$  in control eyes, or an average recovery of 78%. In the retinas of cone-dominated ground squirrels recovery of the outer segment layer is much slower. In animals reattached for 35 days after only 1 day of detachment, the outer segments have recovered less than 60% of their length and only about 70% of their length at 96 days of reattachment. The immunocytochemical observation of recovering rod and cone outer segments also suggests that recovered rods appear structurally more like those in a normal retina than do cones which are often distorted in shape. Recovering primate cones also show altered outer segment structure when observed by electron microscopy (Guérin et al., 1989). It is

now recognized that vision may continue to recover over years in reattachment patients (Liem et al., 1994; Ross, 2002) and this could be at least partly attributed to slow recovery of cone outer segments in the fovea. Further complicating recovery of cones is the fact that there is optical significance to the size ratio between the inner and outer segments (see discussion in Rodieck, 1998), the alignment of outer segments with respect to the pupil of the eye (the Stiles-Crawford effect, Stiles and Crawford, 1933; reviewed by Enoch, 1963 and in Rodieck, 1998), and they have a structurally specialized interface with the apical RPE as described earlier.

Outer segments degenerate on all photoreceptors after detachment, and the available information suggests that they recover on all surviving photoreceptors after reattachment, although with more structural diversity than occurs during development. The opposite pole of rod cells must also recover in those cells in which the axon withdraws. We can draw this conclusion based on the re-organization of the layer of synaptic terminals that occurs in the reattached retina when they are observed by immunolabeling with antibodies to synaptic proteins. After a month of reattachment, the outer border of the OPL is again composed of a relatively compact layer of rod synaptic terminals, in definite contrast with the highly disrupted layer occurring in the detached retina (compare Figs. 5B–15A). There have been no detailed structural, molecular, or physiological analyses of these regenerated synapses. Because some rods continue to express opsin in their plasma membrane after reattachment, we were able to identify rod axons that extend well beyond the OPL and into the inner retina, terminating at different levels in the INL and inner plexiform layer (IPL) (arrowheads, Figs. 15B–D). The endings of these “overgrown” axons also label with antibodies to the proteins associated generally with synaptic vesicles, synaptophysin and VAMP (not shown), but it has not been determined if they form actual synapses. While they appear to be relatively common in immunolabeled tissue, they are sufficiently rare to make their study by electron microscopy a daunting task.

A population of rods also “overshoot” their synaptic target layer during early retinal development providing much the same picture as we observed in the reattached retina (Johnson et al., 1999). A similar phenomenon of rod axons growing into the inner retina has been observed extensively in human retinas with advanced inherited retinal degeneration (Fariss et al., 2000) and in those with a history of complex detachments and reattachments (Sethi et al., 2004). It is curious that they do not seem to occur in the detached feline retinas, but only appear after reattachment. The mechanisms underlying these outgrowths are unknown, but it seems reasonable to hypothesize that similar factors are acting in the reattached and developing retina. Some signal must initiate or allow the regrowth of retracted rod

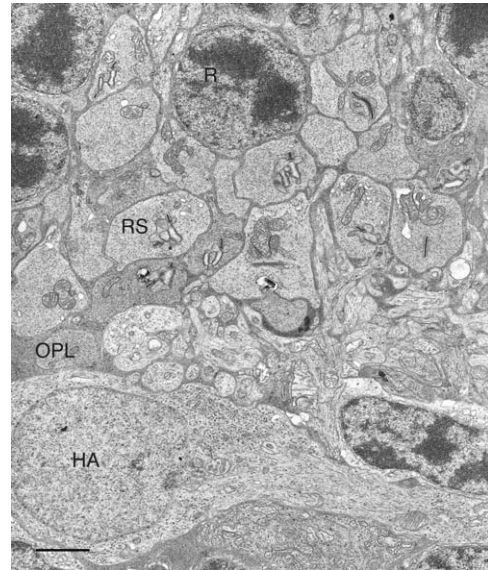


Fig. 6. An electron micrograph through the outer plexiform layer (OPL) of normal retina. The layer of rod spherules (RS) is tightly organized distal to the neuropil of the OPL. HA = A-type horizontal cell; R = rod nucleus in the outer nuclear layer. Scale bar = 2  $\mu$ m.

axons into the OPL after reattachment. And, in turn, there must be a mechanism for signaling the axon to stop growing at its proper location in the OPL. Rod axon overgrowth may result from a failure of this “stop” mechanism. Not all rod terminals retract, some remain in the OPL, so it is also conceivable that sprouts or branches from these terminals account for growth into the inner retina, instead of these being the result of continued growth by lengthening of retracted axons. These axon outgrowths are usually beaded, and often make abrupt turns in the inner retina, unlike the simple, straight rod axons found in normal retina. Their ectopic terminals have fine filopodia-like extensions (Figs. 15C and D, arrowheads). Whether or not the overgrown axons remain in place, or are eventually retracted, or even if the cells bearing them eventually die is unknown for both development and reattachment. To date, there is little data on the “redifferentiation” or remodeling of the extraordinarily complex synapses of the cone terminals after reattachment. Cone pedicles with synaptic invaginations do re-appear in retinas detached for 7 days and reattached for 28. In general, the redifferentiation of photoreceptor synapses, along with profiles for the expression of specific pre- and post-synaptic molecules after reattachment is unexplored territory, but one that seems critical to fully understand the events related to visual recovery.

### 3.4. Remodeling of second- and third-order neurons

#### 3.4.1. Rod bipolar cells

Rod bipolar cells in the feline retina have apical dendrites that innervate the rod spherules. Each rod

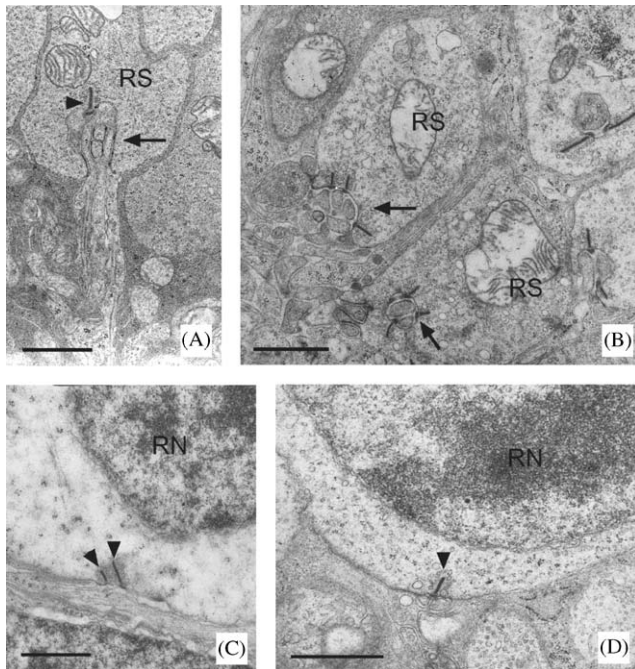


Fig. 7. A series of electron micrographs illustrating morphological changes in rod synapses after detachment. (A) Normal retina. Notice the tight organization of the postsynaptic processes that enter the synaptic invagination (arrow) of the rod spherule (RS). A pre-synaptic ribbon (arrowhead) lies in the RS cytoplasm just facing the synaptic invagination. Dendrites from rod bipolar cells and the outermost branches of B-type horizontal cell axon terminals form the processes post-synaptic to a rod spherule. (B) Rod spherules in a detached retina. The synaptic invaginations (arrows) are much shallower, and the postsynaptic processes more loosely organized than in the normal retina. Also notice that there are 3 synaptic ribbons at each invagination. (C, D) Two examples of rod synapses formed by rods whose axons have retracted so that the perinuclear synapses lie in the mid outer nuclear layer. Short synaptic ribbons (arrowheads) front on the rod plasma membrane at sites lacking any deep synaptic invaginations. Some specialization of the presumed pre- and post-synaptic membranes is still evident. RN = rod nucleus. Scale bar = 1  $\mu$ m.

spherule is usually presynaptic to two different rod bipolar cells, and each rod bipolar cell contacts between 16 and 20 rod spherules (Freed et al., 1987). The rod bipolar dendrites penetrate deeply into the invagination of the rod spherule to terminate opposite one of the two (on average) synaptic ribbons (Figs. 6 and 7A; Boycott and Kolb, 1973). The general relationship between rod bipolar cells and rod spherules can be observed by confocal imaging using antibodies (Negishi et al., 1988; Wässle et al., 1991) to label each cell type (Fig. 16A, green = anti-synaptophysin, synaptic terminals, red = anti-PKC, rod bipolar cells). In the normal feline retina the compact layer of rod spherules stands out (Figs. 5A and 16A, green). Rod bipolar cells have multiple branched apical dendrites that terminate in the invaginations of the spherules (Fig. 16A, red), and do not extend into the ONL. As the layer of rod terminals becomes increasingly disrupted with detachment time,

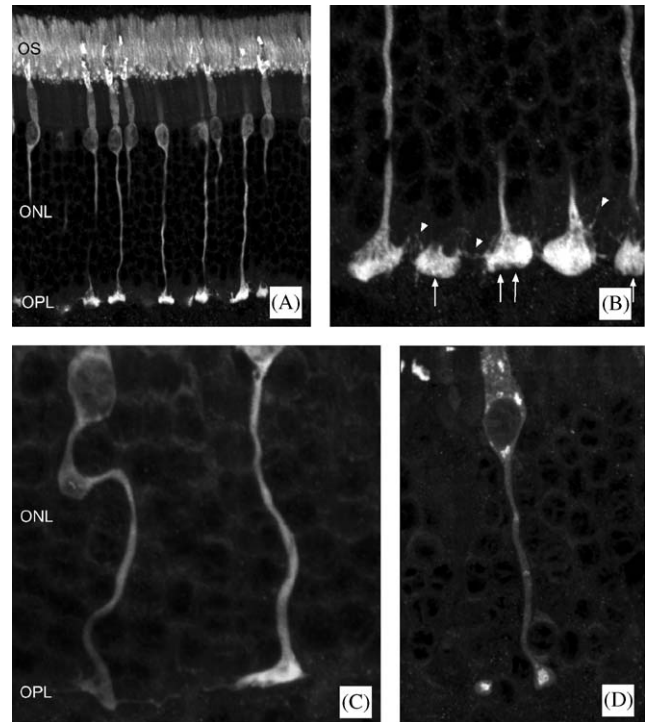


Fig. 8. A series of confocal images of normal and detached retina labeled with an antibody to the  $\gamma$  subunit of the photoreceptor-specific phosphodiesterase (provided by Dr. B. Fung). (A) Normal retina. The antibody labels both rod and cone outer segments (OS), but labels cones in their entirety from their OS to their synaptic terminals in the outer plexiform layer (OPL). Note that the cone cell bodies lie in a row along the outer border of the outer nuclear layer (ONL). (B) A higher magnification image of the labeled cone pedicles in the normal retina. Fine telodendria (arrowheads) can often be observed extending from the pedicles. The small “indentations” visible on the base of a pedicle (arrows) represent the multiple synaptic invaginations that occur in these complex terminals. (C–D) Cone pedicles in retina detached for 7 days. In general, labeled cones are difficult to find at this time when labeling is far fainter even than at 3 days. Cone axons often assume tortuous paths across the outer nuclear layer. The shape of their pedicle is highly variant when compared to those in normal retina.

there is a concomitant emergence of fine, tapered dendritic processes that reach from the rod bipolar cells into the ONL, usually ending adjacent to withdrawn rod synaptic terminals (arrowheads, Fig. 16B). Such remodeled dendritic branches are readily apparent within 3 days of a detachment and they remain as long as the retina is detached. The growth of these dendrites appears specific, that is, they grow towards the retracted terminals, their presynaptic target in the normal retina (arrowheads, Figs. 16B and C). This apparent specificity contrasts with the responses of some horizontal cell processes and these ganglion cell processes that remodel after detachment.

The remodeled rod bipolar dendrites are obvious because they extend into the ONL, an area in which there are normally no anti-PKC labeled processes. The prominence of the long outgrowths may tend to obscure the fact that there probably is also “pruning” of



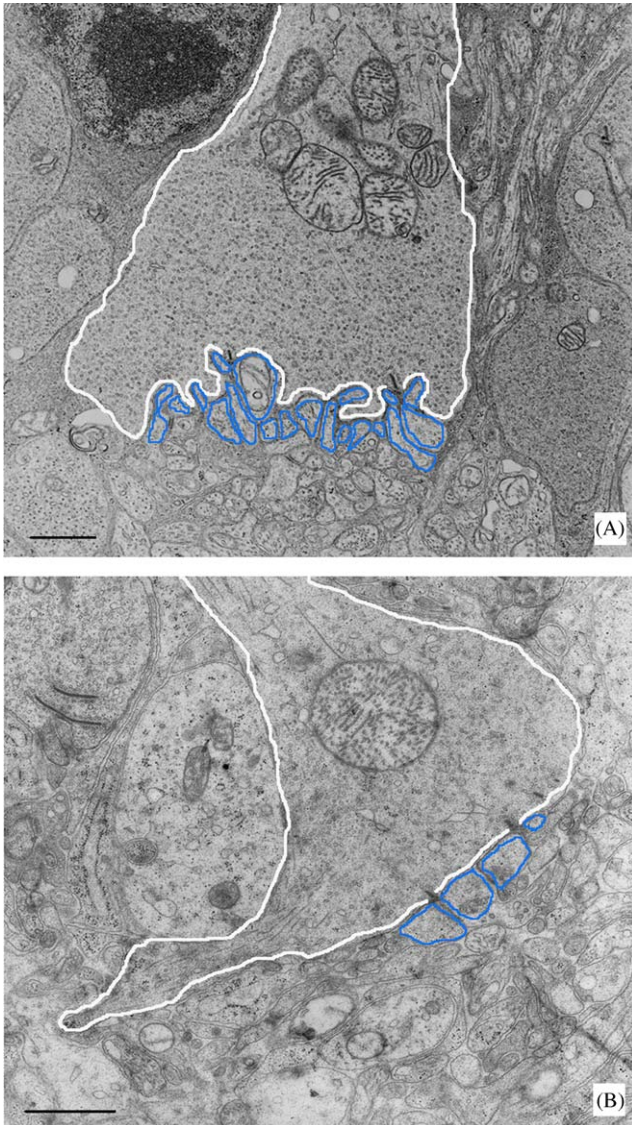


Fig. 9. Electron micrographs of cone synaptic terminals in normal (A) and detached (B) retina. In both, pedicles are outlined in white while identifiable post-synaptic processes are outlined in blue. Notice the lack of deep invaginations and the greatly reduced number of post-synaptic processes in the pedicle from the detached retina. Also notice that the cytoplasm of the normal terminal is filled with synaptic vesicles but that this population is greatly reduced in the detached retina. The synaptic ribbons in the pedicles from detached retina appear shortened and greatly reduced in number. Although difficult to determine by electron microscopy, this conclusion is supported by data from labeling with the antibody to CtBP2 shown in Fig. 10. Scale bar = 1  $\mu$ m.

dendritic branches on these cells. In control retinas the dendrites appear as fine, “wispy” outgrowths extending from the cell body into the OPL (Fig. 16D). In detached retina these are not nearly as prominent, leading to the impression that some dendrites have withdrawn while others have grown in length (Fig. 16E). Pruning of dendrites appears to be the major response of bipolar cells in other forms of retinal degeneration (Marc et al., 2003), but in those cases the response appears late in the

disease, when “deafferentation” due to loss of photoreceptor cells is well-advanced. Some of these dendritic outgrowths can be long, reaching across most of the ONL. Indeed, in long-term detachments (28 days), where a large number of rod terminals have withdrawn, these dendritic outgrowths can be quite stout (Fig. 16F). Although never quantified, in some long-term detachments the rod bipolar outgrowths appear too few in number to innervate the large number of retracted rod terminals, perhaps an indicator that dendritic pruning becomes more extensive as detachment time increases. The extent of remodeling of these dendritic trees is difficult to estimate from sectioned material and would be better appreciated in wholemounts where the dendritic branching pattern is more obvious and easier to describe. However, because the antibody to PKC labels all of the rod bipolar cells it would be difficult to sort out single cells. Here data from the classic Golgi technique or from single cells filled by dye injection could be invaluable.

#### 3.4.2. Horizontal cells

The feline retina has two morphologically distinct horizontal cells (Fig. 17); one with stout tapering dendrites and no axon (A-type) and the other with somewhat finer, highly branched dendrites and a long thin axon that forms an elaborate axon terminal (B-type; Dowling et al., 1966; Fisher and Boycott, 1974). Painstaking observations of Golgi impregnated cells by electron microscopy demonstrated that the dendrites of both the A- and B-type cells innervate the cone terminals, while only the branches of the axon terminal innervate rod spherules (Kolb, 1974). The axon of the B-type cell apparently provides only a metabolic link between the two compartments because it does not generate action potentials, and its passive electrical properties assure no communication via non-regenerating electrical signals (Nelson et al., 1975).

Observations by electron microscopy also show that the A-type cell is particularly rich in neurofilaments (Fisher and Boycott, 1974) which correlates with the fact that the A-type cell stains with the classic, silver-based “neurofibrillar” silver stains (Gallego, 1971). Neurofilaments are not completely absent from the B-type cell but EM data suggests that they are relatively scarce by comparison to their high density in the A-type cell. We and others have used antibodies to the calcium binding proteins, calbindin D and calretinin, to study horizontal cells. In addition, we have added a third antibody, that to the 70 and 200 Kd subunits of the complex neurofilament protein, in order to differentiate the subtypes in feline retina. Our original studies of remodeling of these cells came from observations of calbindin D labeled tissue, where both types of horizontal cell labeled (red cells, Fig. 18A). What we observed after detachment were calbindin D-positive,

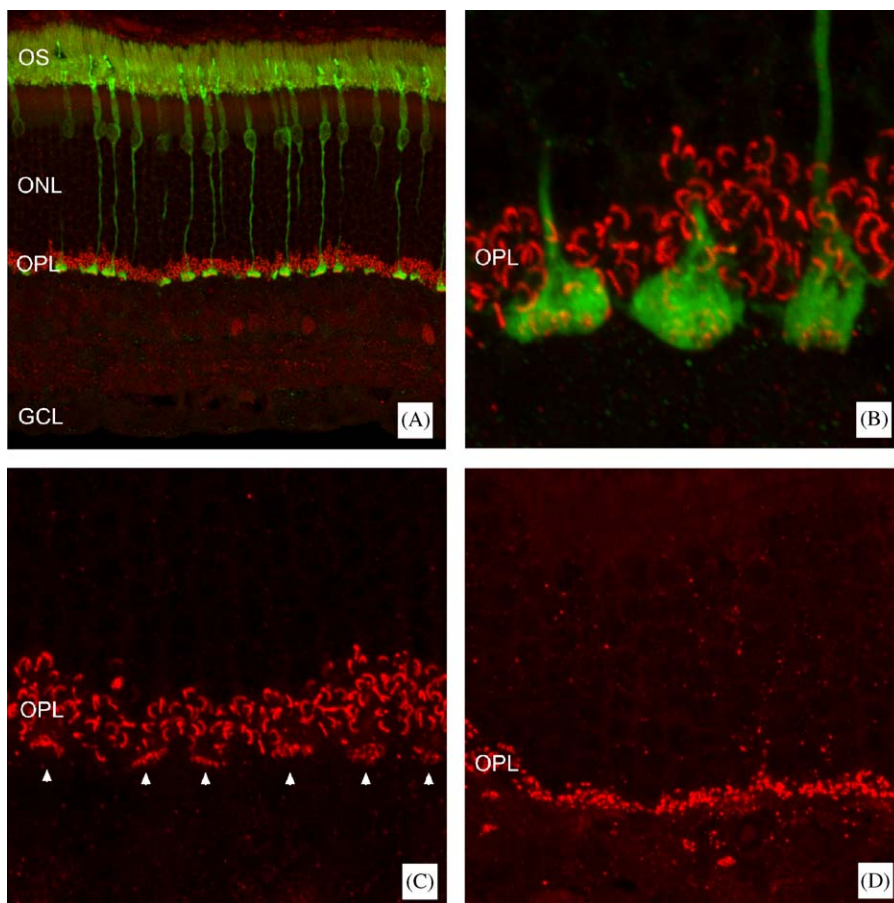


Fig. 10. A series of confocal images from normal and detached retina in which the tissue was labeled with the antibody to PDE $\gamma$  (green) and with anti-CtBP2 (red; B.D. Transduction Labs), which labels synaptic ribbons. (A) Normal retina. The antibody to PDE $\gamma$  labels rod and cone outer segments (OS) and cone cells in their entirety. The antibody to CtBP2 labels specifically the synaptic ribbons in rod spherules and cone pedicles (green) in the outer plexiform layer (OPL). (B) Normal retina. A higher magnification image of the layer of photoreceptor synaptic terminals. Note the large number of synaptic ribbons that appear labeled in the cone pedicles (their yellow color coming from the superposition of the green and red labeling). Note the arcuate structure of the individual rod ribbons. (C) Normal retina. In this image the green (PDE $\gamma$  labeling) channel is turned off to better show the organized arrays of presynaptic ribbons associated with the individual cone pedicles in the OPL (arrowheads). (D) Retina detached for 3 days, CtBP2 labeling only. Note the lack of organized arrays of cone synaptic ribbons in the OPL, and the much smaller “clumped” appearance of ribbons in rod terminals. Labeling in the outer nuclear layer (ONL) represents ribbons in retracted rod terminals. GCL = ganglion cell layer.

prominent, often fairly thick, beaded processes extending into the ONL from the OPL (red process, left in Fig. 18A, green processes Fig. 18C). These same outgrowths label with the antibody to neurofilament protein. Indeed, there is a large increase in the intensity of horizontal cell labeling with this antibody after detachment (red, OPL, Figs. 19A and B), most likely indicating an increase in the complexity of the neurofilament cytoskeleton in these cells, although the A-type cell always labels more heavily than the B-type cell (Linberg et al., 2004).

Using any of these three antibodies, we detect two sub-types of outgrowths from these cells; those that terminate adjacent to retracted rod spherules (arrowheads, Figs. 18A and B), i.e. “directed”, and those that do not, i.e. “undirected” (examples in Figs. 18A, 19B, 20D and E). Our initial studies focused almost entirely on the wild, seemingly undirected horizontal cell

neurites because of their prominence and their unusual nature. Whether these strikingly dramatic outgrowths serve some functional or survival role for the horizontal cells or are merely vestiges of some injury response is a complete unknown. In detachments of 3 days or longer, the latter category of outgrowths were often observed extending beyond the outer limiting membrane and into the subretinal space. This always occurs in conjunction with Müller cell processes (green, Fig. 19B and C) that had expanded into the subretinal space to form glial scars. Also, within the neural retina the “undirected” horizontal cell processes (those that did not terminate adjacent to a retracted rod terminal), were invariably spatially associated with Müller cell processes showing a large upregulation of intermediate filament proteins, i.e., those that are presumably highly reactive. Once in the subretinal space the horizontal cell outgrowths (red, Fig. 19D) were never observed growing on the

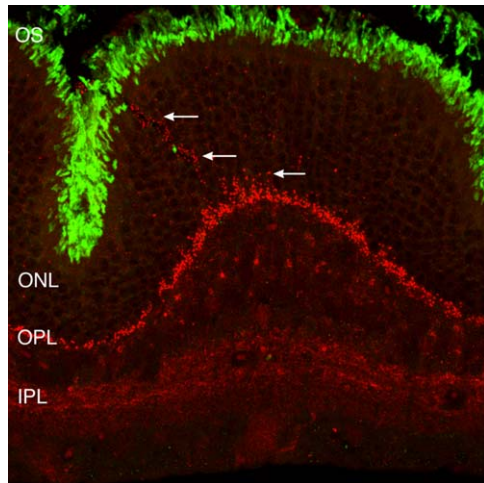


Fig. 11. A low-power confocal image of region around a retinal fold in a 7-day detached retina to demonstrate the prominent reduction in expression of specific protein molecules by cones after detachment. The section was labeled with the antibodies to PDE $\gamma$  (green) and CtBP2 (red). Note that while rod outer segments (OS) still label with the antibody to PDE $\gamma$  there is a complete lack of cone labeling proximal to the outer segment layer (compare to Fig. 10A). Also notice the large amount of CtBP2 labeling found in the outer nuclear layer (ONL) arising from ribbons in retracted rod spherules (arrows). IPL = inner plexiform layer; OPL = outer plexiform layer.

exposed photoreceptor cells themselves, but always embedded within a meshwork of Müller cell processes that covered the exposed photoreceptors (green, Fig. 19D), where they could run for long distances.

Based on their structural appearance, the labeled horizontal cell outgrowths also fall into different categories. Some are fairly thick processes descending from the horizontal cells into the inner retina, although these were observed with much less regularity than those ascending into the ONL. The descending processes were also observed by Marc et al. (1998, 2003) in the detached feline retina and in other forms of retinal degeneration. Indeed, they seem to be the most commonly encountered form of horizontal cell remodeling in cases of extreme photoreceptor loss (Marc et al., 2003). It should be noted that descending horizontal cell processes are sometimes observed in normal retina (Silviera et al., 1989; Kolb et al., 1994), and may become more numerous in retinas with reactive photoreceptors. The “undirected” ascending processes in both sectioned material and whole mounts appear in two structural types; one composed of thin, often beaded cylindrical processes and the other as flattened broad processes. These can both occur singly and in clusters (Fig. 18C). Within the subretinal space the long extended processes, all appear thin and cylindrical (Fig. 19D). But, thicker, fleshier processes also occur there, and these often branch profusely, giving rise to complex assemblies reminiscent of sparse versions of axon terminals of the B-type cell within the OPL (Fig. 20F).

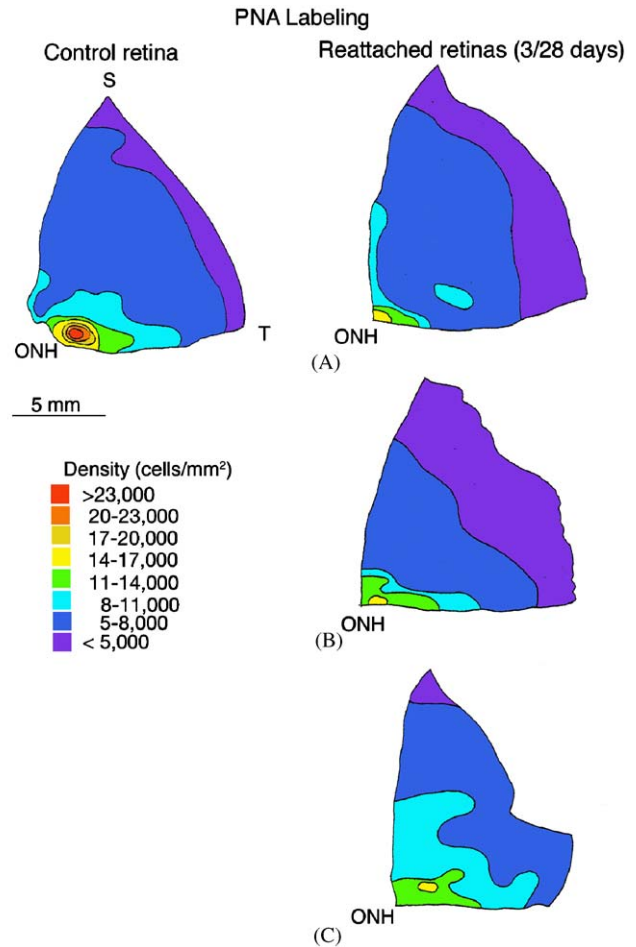


Fig. 12. Density profiles for PNA labeling in normal (left) and reattached retinas (A–C). The lectin PNA binds specifically to extracellular matrix domains around cone outer segments (cone matrix sheath). In these experiments, biotinylated PNA was bound to retinal wholemounts and visualized by fluorescence imaging after incubation of the tissue in streptavidin conjugated to the fluorochrome Cy3. The wholemounts were systematically photographed and the number of labeled cone sheaths was counted. Isodensity contours were drawn by eye on the maps of the wholemounts. A retinal detachment was created that covered most of the superior (S)–temporal (T) quadrant. After 3 days the retina was surgically reattached and the tissue harvested 28 days later. While the general central to peripheral gradient of cone density is restored in the reattached retina, the area of highest cone density, or *area centralis* (the red/orange area in the control retina) has not. In the 3 experimental eyes (A, B, C), the density of cones sheaths in central retina was only about 50% of that in normal retina. ONH = optic nerve head.

#### 3.4.2.1. The origin of the horizontal cell outgrowths.

The dendrites of the A- and B-type horizontal cells both connect to cone terminals and only the fine arborizations of the B-type axon terminal system connect to rods. Synaptic terminals of both photoreceptor types remodel, albeit differently, in response to detachment. Determining the origin of the horizontal cell outgrowths may provide clues to mechanisms that initiate neuronal remodeling. The horizontal cells

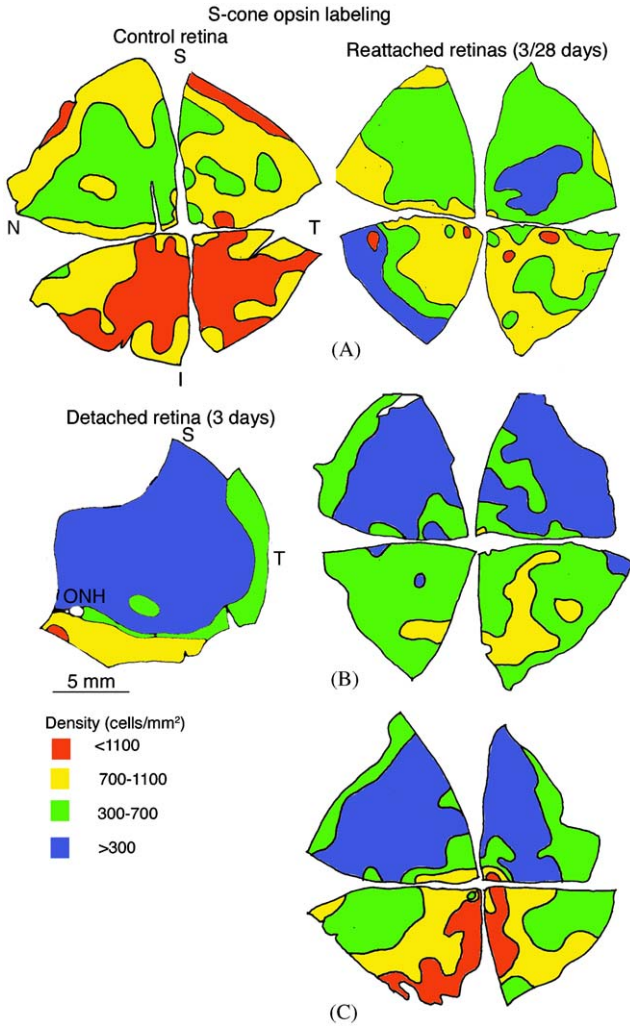


Fig. 13. Isodensity contours for S-cones in normal (top left), detached (bottom left) and reattached feline retinas (A–C). The S-cones were labeled in retinal wholemounts with an antibody to S-cone opsin (provided by Dr. J. Nathans), which was then visualized by using a secondary antibody bound to the fluorochrome Cy2. Isodensity profiles were constructed as described for Fig. 12. The density profiles for S-cones is very different than that for total cones (PNA labeling, Fig. 12 control). The greatest density of S-cones occurs in the inferior retina (Control retina). When the retina is detached for 3 days, the number of labeled S-cones drops dramatically (the yellow and orange areas inferior to the optic nerve head (ONH) were not detached). In the three reattached retinas (A, B, C), most of the superior retina was detached for 3 days and then reattached for 28. S-cone labeling in the reattached retinas was patchy and of low density by comparison to the normal retina. Many of the sampled areas showed no S-cone labeling at all in the reattachments.

may be responding in general to deconstruction of photoreceptors, photoreceptor cell death, changes in the release of transmitter (or other factors) from photoreceptor synaptic terminals, or even to the reactive Müller cells or microglia. Understanding more specifically the origin of the outgrowths may help narrow the search for what stimulates them. To accomplish this, we used a variety of antibodies to determine immunocyto-

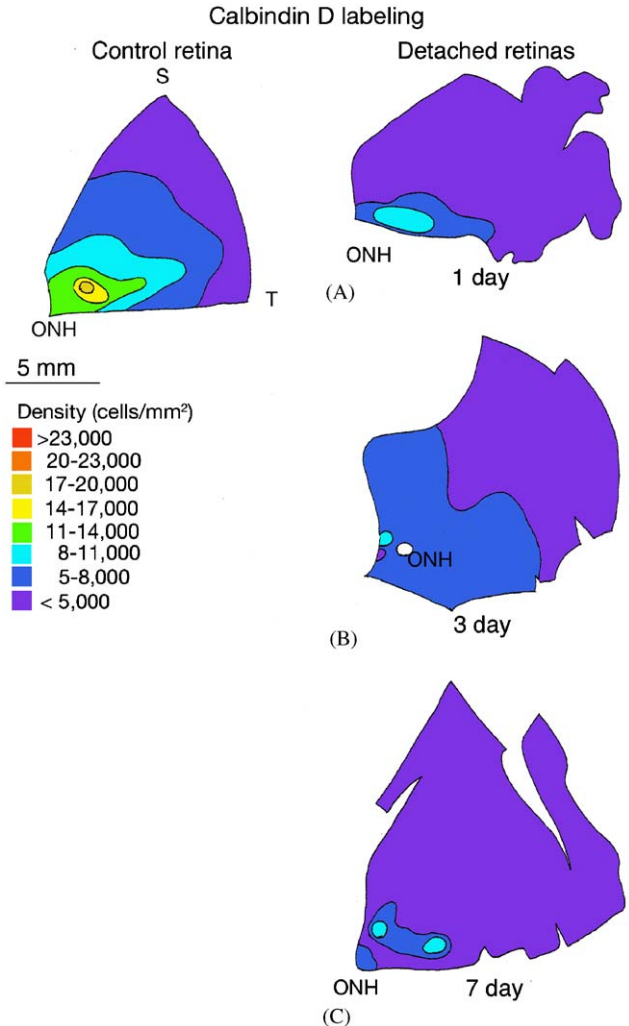


Fig. 14. Isodensity contours for cones labeled with an antibody to the calcium binding protein calbindin D in normal (left) and detached (right) feline retinas. The labeling protocol and production of the isodensity maps was as in Figs. 12 and 13. In the periphery, this antibody binds to all cone outer segments. However in the area centralis many cones fail to label, hence the difference between the density profiles in the normal central retina here and in Fig. 12 control. After detachment (A, B, C) there is a rapid drop in the number of labeled cones with the central to peripheral gradient essentially disappearing.

chemical labeling signatures specific to each horizontal cell type. By labeling with a combination of antibodies to neurofilament protein, calbindin D, and calretinin and assigning the output colors as green, blue, and red, respectively we determined that the characteristically shaped A-type cells are blue/white in color (i.e. heavily labeled with all 3 antibodies; HA in Figs. 20A–C), while cells with the appropriate shape and location to be the B-type are red or near red in color (due to the near lack of labeling with the anti-neurofilament antibody; HB in Figs. 20B and C). The outgrowths into the ONL, including the long processes that reach the subretinal space and those that end adjacent to retracted rod

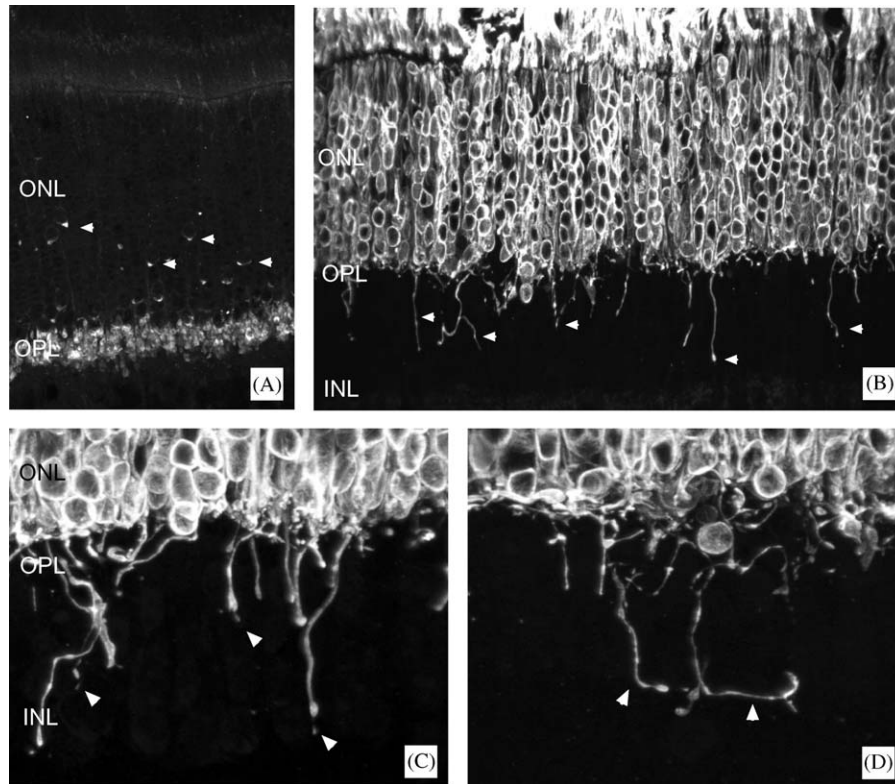


Fig. 15. A series of confocal images showing the labeling of synaptic terminals (A) and rod photoreceptors (B–D) in retinas detached for 3 days and reattached for 28. (A) Labeling with an antibody to the synaptic vesicle protein synaptophysin shows the relative return of organization to the layer of photoreceptor terminals in the outer plexiform layer (OPL) after a month of reattachment (compare to the labeling pattern in Fig. 5B for detached retina). Although some labeled terminals (arrowheads) still exist in the outer nuclear layer (ONL), their number is dramatically reduced. (B, C, D). Labeling with the antibody to rod opsin demonstrates the growth of rod axons (arrowheads) into the inner retina after reattachment. These overgrown axons appear to terminate with a typical rod spherule appearance and have very fine telodendria-like structures associated with them. INL = inner nuclear layer.

terminals (not shown) all bear an immunochemical labeling signature closer to that of the B-type cell than that of the A-type (arrows Figs. 20D, E and F). When an outgrowth that gives rise to a branched process in the subretinal space is traced back through the ONL, it is found to arise from a complex plexus in the OPL with a structure characteristic of the axon terminals bearing the B-type cell immunochemical signature (arrows, Fig. 20D and E). Many undirected processes often course for long distances with a uniform diameter (Fig. 19C), but others terminate by elaborating a branched structure within the subretinal space (Fig. 20F). The directed processes end adjacent to retracted rod spherules. Thus, the evidence points most directly to all of these processes as arising from the axon terminal of the B-type horizontal cell. The specificity of this sprouting response is still another indicator of the independence of the axon terminal from the cell body of these complex interneurons. Although the rod bipolar cells are exposed to the same changing environment as the horizontal cells, the former do not seem to produce “undirected” processes; all of the neurites seem to end near a retracted rod terminal. Perhaps this represents some fundamental difference

between the responses of dendrites and axons. Although growth in response to the release (or lack) of some factor from the rods is one possibility, another mechanism is suggested from studies of cultured neurons in which mechanical tension can elicit neurite outgrowth (Lamoureux et al., 2002). Thus, if the rod bipolar cell dendrites and the horizontal cell axon terminal endings remain mechanically connected to retracting rod spherules, tension generated by the retracting rod spherules may initiate a growth response from the post-synaptic neurons. Whether or not the same mechanism accounts for one or both of the directed and undirected outgrowths is an unanswered question.

Other explanations for the meandering horizontal cell processes could include their adhesion to the surfaces of growing Müller cell processes, or to specific trophic molecules released by the growing Müller cells. In experiments with cultured neurons, the sprouting neurites were identified as axon-like. However, there is no evidence suggesting that dendrites could not be stimulated to grow by the same mechanism, especially *in vivo*. It is interesting that the outgrowths appear rapidly, essentially concurrently with the appearance of retracted

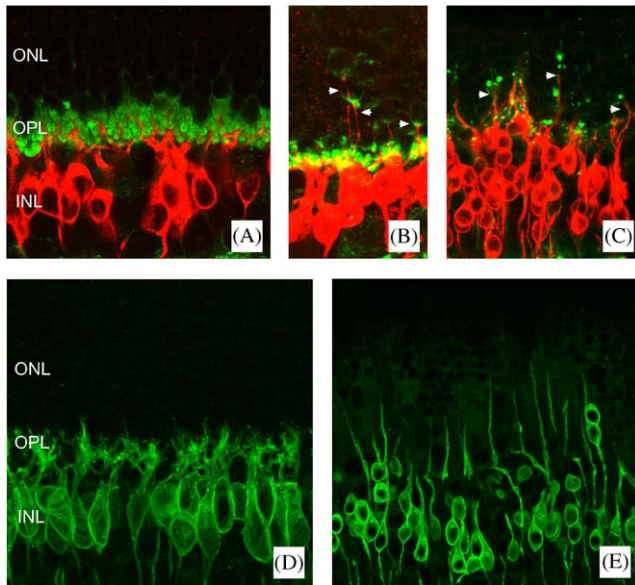


Fig. 16. A series of confocal images illustrating the remodeling that occurs in rod bipolar cells after detachment. In figures A–C, the rod bipolar cells are labeled with an antibody to protein kinase C (PKC, red; Biomed Res Labs, Inc.) and photoreceptor synaptic terminals in the outer plexiform layer (OPL) are labeled with an antibody to the synaptic vesicle protein, synaptophysin (green). In figures D and E only anti-PKC labeling (green) is shown. (A) Cell bodies of the rod bipolar cells reside in the inner nuclear layer (INL), while their dendrites specifically enter the synaptic invaginations of the rod spherules where they are post-synaptic. (B, C) After detachment of 3 days, many fine rod bipolar outgrowths (arrowheads) extend into the outer nuclear layer (ONL) where they terminate adjacent to retracted rod spherules. (D) In the normal retina the rod bipolar cell dendrites are very fine, giving a “thin and wispy” appearance to the dendritic tree of these cells. (E) After longer term detachment (28 days), the rod bipolar dendrites have a thicker appearance as they grow into the ONL, and many of the short, “whispy” processes appear to be missing. This may be indicative that both dendritic pruning and dendritic outgrowth play roles in the remodeling of these interneurons. INL = inner nuclear layer.

axon terminals, which could be interpreted as correlative evidence that they remain connected to the rod spherules and are being “towed” by the retracting terminal. There is no published evidence for changes in the axon terminals of the rod bipolar cells in the IPL. Observations with the anti-PKC antibody do not indicate any dramatic changes, however such changes may be subtle, or occurring at the ultrastructural level and not revealed in the immunostaining pattern. Rod bipolar cells transmit their information through a well-characterized neural circuit involving the AII amacrine cell which transmits information from the rod bipolar axon to a cone bipolar cell which in turn relays the information to both off- and on-center ganglion cells (there is no direct rod-bipolar-ganglion cell circuit) (Famiglietti and Kolb, 1975). Thus, there may be changes in this specific circuitry; changes that may be revealed only by physiological studies or ultrastructural reconstructions of the synaptic circuitry of these cells.

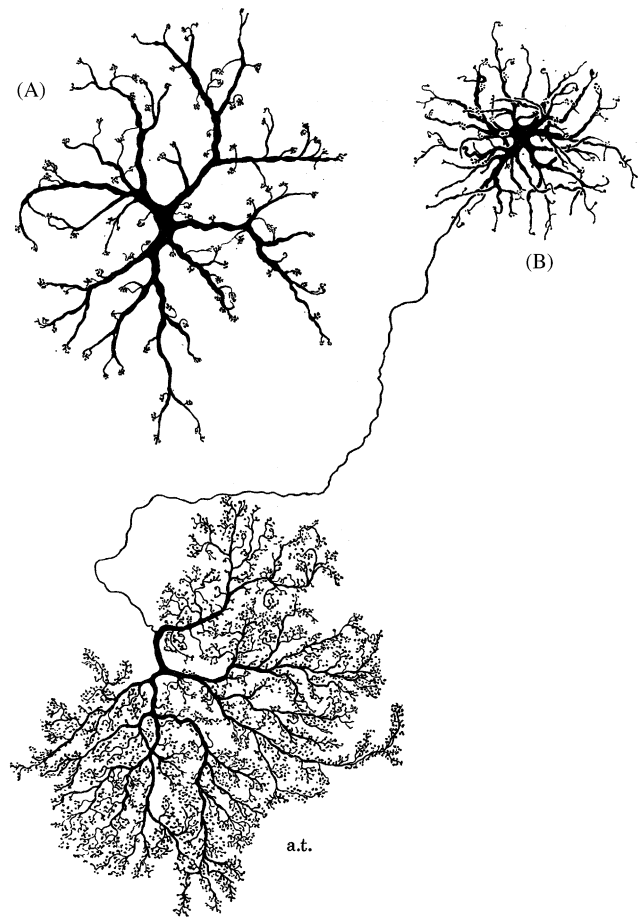


Fig. 17. Drawings of Golgi stained horizontal cells from the feline retina. (A) The A-type cell, with a dendritic spread of about  $250\mu\text{m} \times 250\mu\text{m}$  has no axon and its dendrites contact only cone photoreceptors. (B) The B-type cell has a smaller dendritic field than the A-type cell (about  $150\mu\text{m} \times 150\mu\text{m}$ ) but an elaborately branched axon terminal system (a.t.). The dendrites of the B-type cell contacts only cones, while the axon terminal contacts only rods. Reprinted with permission from Fisher and Boycott (1974).

### 3.4.3. Ganglion cells

A subset of ganglion cells remodels vigorously in response to detachment. This may seem somewhat surprising since they are the retinal neurons farthest removed from the site of the detachment. It is important, however, not to lose sight of the fact that ganglion cells sit among the endfeet of the Müller cells—cells in direct physical contact with the actual site of the injury (the subretinal space) via their apical microvilli. Müller cells also react to detachment very quickly (Geller et al., 2001), with many changes in gene expression and thus could easily influence the ganglion cells. Additionally, ganglion cell responsiveness could be dependent on the trans-neuronal changes originating with changes at the rod and cone synapses. Clearly the cellular remodeling of retinal neurons as described here will be eventually linked mechanistically to molecular changes in these cells. To date, changes in growth

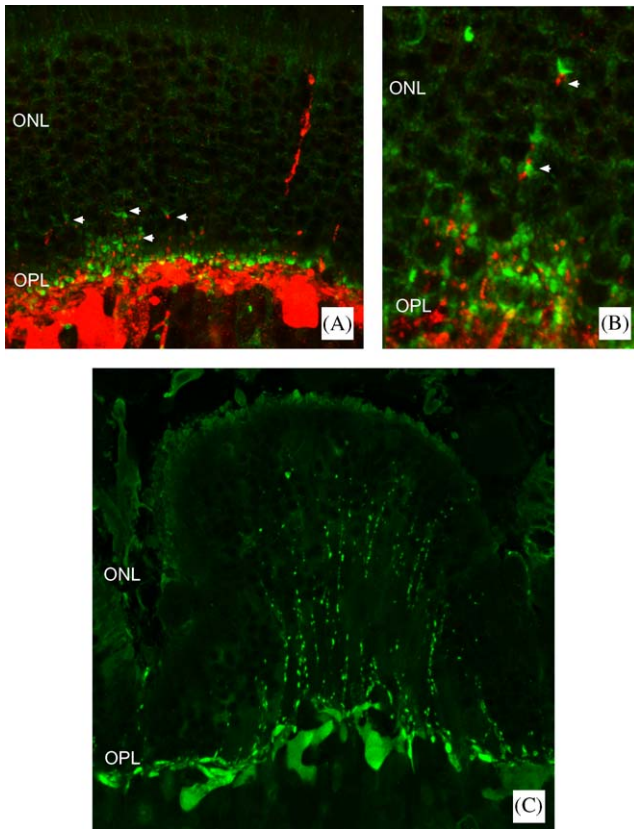


Fig. 18. A series of confocal micrographs showing the remodeling of horizontal cells after detachment. (A, B) Horizontal cells (red) are labeled with the antibody to calbindin D (Sigma), and the synaptic terminals of photoreceptors (green) with the antibody to synaptophysin. Notice the many fine red processes from the horizontal cells that extend above the layer of synaptic terminals into the outer nuclear layer (arrowheads, ONL). Many of these appear to terminate adjacent to withdrawn rod terminals, but some do not, such as the thick process in figure (A). (C) A cluster of fine, beaded horizontal cell outgrowths extending well into the outer nuclear layer after 7 days of detachment. OPL = outer plexiform layer.

associated protein 43 (GAP 43) expression in ganglion cells and changes in neurofilament protein expression in ganglion and horizontal cells are the only two such molecular linkages identified.

**3.4.3.1. GAP 43 expression, neurofilament protein expression, and ganglion cell remodeling.** Growth associated protein 43 expression is generally associated with axonal growth cones and synaptogenesis (Skene, 1989; Benowitz and Perrone-Bizzozero, 1991a, b; Strittmatter et al., 1992). Although expression is retained in some neurons (e.g., pyramidal cells of the hippocampus and cells in the molecular layer of the dentate gyrus: Benowitz et al., 1988; Skene, 1989; Gispén et al., 1991; Meberg and Routtenberg, 1991; Benowitz and Perrone-Bizzozero, 1991a, b; Kruger et al., 1993; Kapfhammer et al., 1994), it is generally down-regulated significantly after the developmental period associated with synaptogenesis (Benowitz and Perrone-Bizzozero, 1991b). Cells

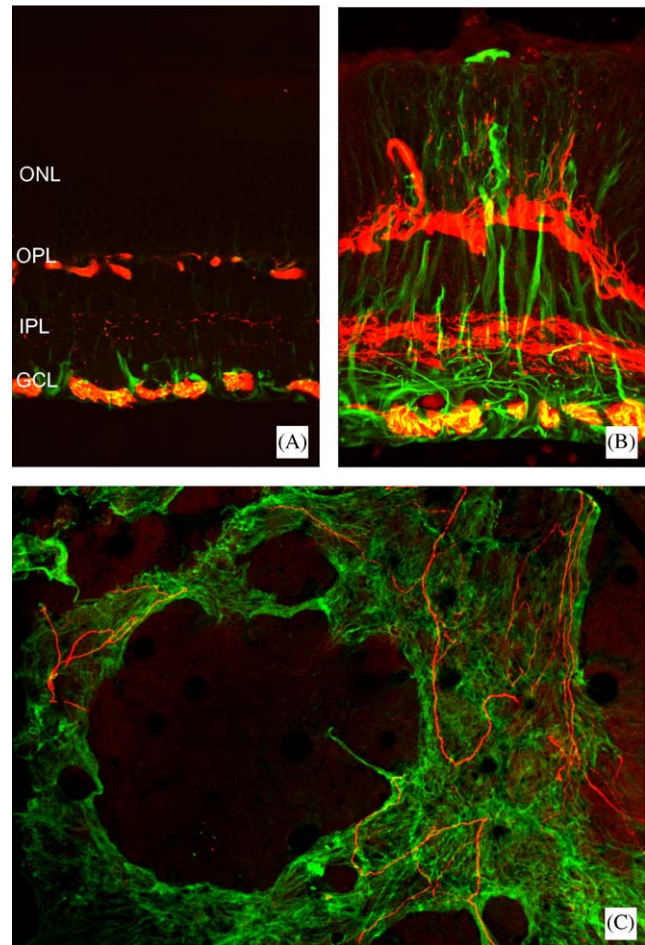


Fig. 19. A series of confocal images showing the remodeling of horizontal cells after detachment with anti-neurofilament labeling. In all cases the tissue was labeled with an antibody to the 70 and 200 kD subunits of neurofilament protein (red) and an antibody to glial fibrillary acidic protein (GFAP, green; Dako) to show the presence of Müller cells. (A) In the normal retina, there is prominent labeling with the anti-neurofilament antibody of thick horizontal cell processes in the outer plexiform layer (OPL), probably those of the A-type horizontal cell, and of ganglion cell axons in the ganglion cell layer (GCL) and some labeling of fine ganglion cell dendrites in the inner plexiform layer (IPL). (B) After 7 days of detachment there is a robust increase in labeling of the horizontal cells (and ganglion cells) with the anti-neurofilament antibody, and many horizontal cell processes of varying size extend well into the outer nuclear layer (ONL). Notice that the area showing the strong responsiveness of horizontal cells also shows a high degree of Müller cell reactivity by the increased presence of GFAP in the outer retina. (C) An image of the subretinal space from a detached retina taken from a retinal wholemount. Notice the long, fine anti-neurofilament labeled outgrowths of horizontal cells (red) in the subretinal space where they are always associated with Müller cell processes (green) that have grown from the neural retina and into that space.

in culture, including non-neuronal cells that are transfected to express GAP 43, extend long, fine filipodial processes (Zuber et al., 1989). Mice in which GAP 43 has been genetically removed show a tangling of axons in the optic nerve (Strittmatter et al., 1995) while mice over-expressing GAP 43 show an enhanced

sprouting of axon terminals by both central and peripheral neurons (Aigner et al., 1995). Thus, it appears that GAP 43 is not just important for synaptogenesis, but also normal neurite outgrowth, normal axon growth, and terminal branching.

GAP 43 expression in the retina appears as the ganglion cells migrate into their specific layer of the retina and begin the process of axon outgrowth. It remains in adults as stratified labeling within the IPL (De la Monte et al., 1989; Benowitz and Perrone-Bizzozero, 1991b; Reh et al., 1993; Kapfhammer et al., 1994; McIntosh and Blazynski, 1991). This is the pattern observed in the adult feline retina (Coblentz et al., 2003). Immunoblot analysis also reveals that there is some GAP 43 protein expressed in normal adult feline retina (Coblentz et al., 2003).

Immunoblot, immunocytochemical, and real-time PCR data all showed a rise in GAP 43 expression beginning a day after a retinal detachment and continuing to increase to the last experimental timepoint at 28 days. Quantitative PCR shows an increase in GAP 43 message levels to about twice control levels at 6 h of detachment. Immunohistochemistry data reveal a population of ganglion cell bodies that are heavily labeled

in the 7-day postdetachment animals (red processes, Fig. 21A; Coblentz et al., 2003). The GAP 43-positive ganglion cells also show a major change in labeling with the same anti-neurofilament protein antibody used to show changes in horizontal cells. In the normal retina, this antibody labels only a few processes in the IPL, and ganglion cell axons (red, IPL and GCL labeling, Fig. 19A; Coblentz et al., 2003). After detachment it heavily labels processes in the IPL, and cell bodies in the ganglion cell layer (red, IPL, GCL labeling Figs. 19B, 21B and C). This labeling co-localizes with that for GAP-43. After detachment the GAP 43/neurofilament-positive ganglion cells exhibit an unusual morphology, unlike any reported in the literature for feline (or other mammalian retinae) ganglion cells, with numerous small, spikey processes extending from their cell body toward the nerve fiber layer (Fig. 21C; Coblentz et al., 2003). As detachment time increases, there is an astonishing increase in GAP 43/neurofilament-positive neurites that extend from the labeled ganglion cells and course completely across the neural retina and into the subretinal space (Fig. 21B and C, arrows). These processes are generally of a uniform caliber without obvious branching.

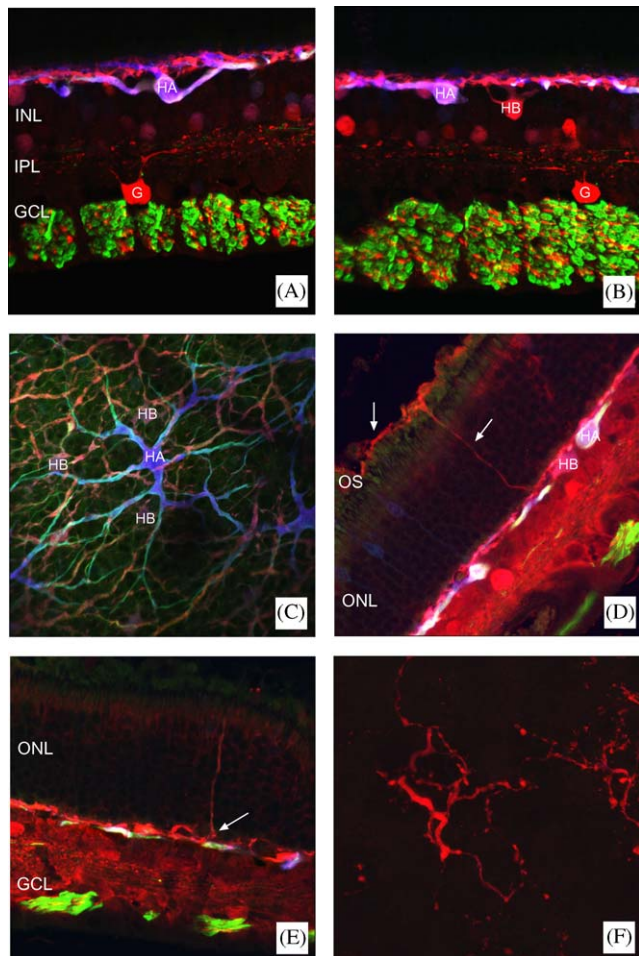


Fig. 20. A series of confocal microscope images showing data suggesting that the remodeled horizontal cell processes arise from the axon terminal system of the B-type cell. In all cases the tissue was labeled with 3 antibodies: anti-calretinin (red), anti-calbindin D (blue), and anti-neurofilament (green). (A, B) Radial sections through normal retina. There are two populations of horizontal cells based on the labeling signature, location and structure. The A-type (HA) cells demonstrate a “blue/white” color, stout processes and a cell body that lies on the outer border of the inner nuclear layer (INL). The B-type cells are red, have finer processes emerging from the cell body, and lie lower in the INL. G = calretinin-positive ganglion cells with their cell bodies in the ganglion cell layer (GCL) and dendrites extending into the inner plexiform layer (IPL). (C) An image taken at the plane of the outer border of the inner nuclear layer showing in a flat-mounted retina the two types of horizontal cell. The plane of focus is at the level of the A-type cells (HA) with the smaller, finer B-type cells (HB) lying slightly deeper in the tissue. (D) A radial section through a retina detached for 28 days showing one of the fine horizontal cell outgrowths (arrows) rising through the outer nuclear layer (ONL) to the subretinal space where it branches and grows along the surface of the degenerated outer segments (OS). The fine branch is red in color as are the cell bodies and processes of the B-type cell. HB = B-type cell body, HA = A-type cell body. (E) A radial section through a retina detached for 28 days showing one of the fine horizontal cell outgrowths (arrow) rising through the outer nuclear layer (ONL). The red color of the process gives it the labeling signature of the B-type cell. This process arises from a dense plexus on the border of the outer plexiform layer characteristic of the axon terminal system of these cells. GCL = ganglion cell layer. (F) A section through the subretinal space in a flat-mounted retina detached for 28 days. There are 3 sets of red branching processes in this figure, with each originating from single processes identical to those shown in Figs. 20D and E. These single processes were traced back through the outer nuclear layer to the outer plexiform layer where they arise from a fine plexus of processes with the structure of B-type cell axon terminals.



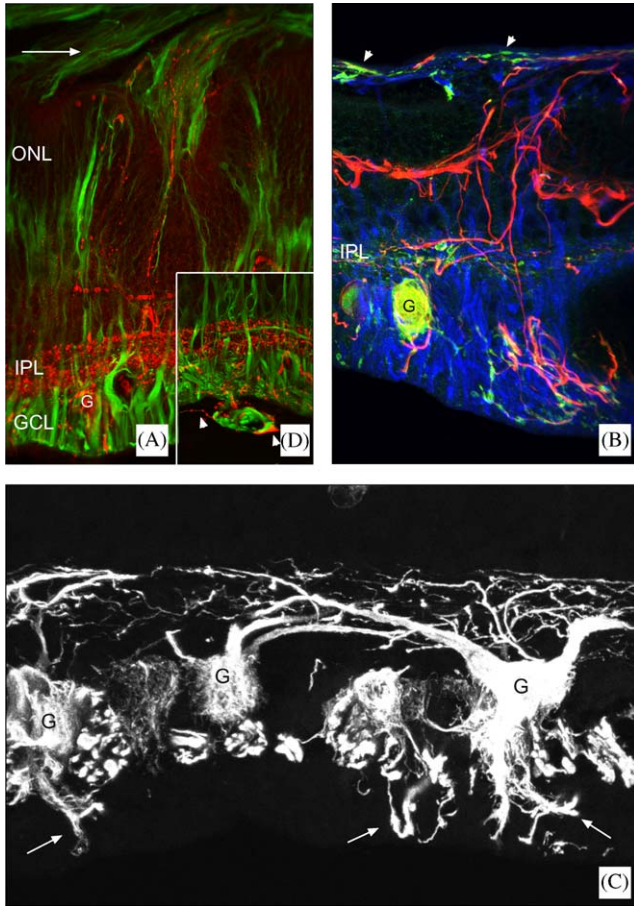


Fig. 21. A series of confocal images showing the reactivity of ganglion cells to retinal detachment. An antibody to growth associated protein 43 (GAP 43, red; Chiron), labels only fine processes in the inner plexiform layer (IPL) of normal adult feline retina, and no cell bodies (data not shown). (A) When the retina is detached for 28 days, anti-GAP 43 labeling increases dramatically, labeling a subpopulation of cell bodies in the ganglion cell layer (GCL), many processes in the IPL, and some processes that grow beyond the IPL into the outer nuclear layer (ONL) and often into the subretinal space (arrow). These GAP 43-positive processes in the outer retina are always closely associated with reactive Müller cell processes (anti-GFAP, green). (B) 28 day detached retina triple-labeled with antibodies to neurofilament protein (red), GAP 43 (green), and GFAP (blue). The anti-neurofilament labeling pattern for normal retina is shown in Fig. 19A. A subpopulation of ganglion cell bodies label heavily with both the neurofilament and GAP 43 antibodies giving the yellow color to the cell labeled “G”. Processes colored red represent remodeled process from the horizontal cells (both ascending into the ONL and descending into the inner retina). The fine yellow/green processes in the subretinal space (arrowheads) represent outgrowths from remodeled ganglion cells. As shown in Fig. 21A and previously, the outgrowths in the subretinal space, whether from ganglion cells or horizontal cells are always associated with outgrowths of Müller cells. (C) The inner retina after detachment labeled with the anti-neurofilament protein antibody. Recall (Fig. 19A), that labeling with this antibody in the normal retina is limited to fine processes in the IPL. Here, large ganglion cells label, along with their processes in the IPL. Notice the unusual appearance of these labeled ganglion cells with the many branched, “spikey” processes growing from their basal surface towards the vitreous (arrows). (D) After retinal *reattachment* neurites from ganglion cells (arrowheads) can grow into the vitreous in association with Müller cell endfeet (green) that form epiretinal membranes.

Thus, the picture that emerges for remodeled ganglion cells is very much like that of the remodeled horizontal cell axon terminals, inasmuch as both produce a large number of “undirected” processes that can grow for long distances through the retina and into the subretinal space. The neurites from ganglion cells also tend to appear adjacent to the processes of reactive Müller cells either within the retina or in the subretinal space. The upregulation of both GAP 43 and neurofilament protein is specific to the region of detachment, and does not occur in control eyes in which the lens and vitreous were removed but no detachment created.

There is not yet evidence that the ganglion cell neurites are directed to any specific presynaptic targets. To date, the only evidence is for their “undirected” growth. We cannot conclude that only ganglion cells showing the molecular upregulation remodel because we only have data for cells that label with the markers, i.e. those showing responses to detachment. At present, we have no reliable way of imaging ganglion cells that do not show the upregulation of GAP 43 and neurofilament peptides. We also have essentially no information about the stimulus for ganglion cell remodeling. It does seem reasonable that GAP 43 upregulation is part of the initiating events for remodeling, while neurofilament protein upregulation may be a means of providing structural support for these newly grown processes.

**3.4.3.2. Which ganglion cell type(s) remodel?** The data for horizontal cells point toward remodeling of the axon terminal of the B-type cells. It is clear from the immunohistochemical labeling that only a subpopulation of ganglion cells shows the upregulation in GAP 43 and neurofilament protein expression after detachment. Feline ganglion cells exist in several structural and functional classes. Size measurements from retinal whole-mounts were used to produce a frequency distribution for the somal area of the GAP 43 labeled ganglion cells. The data show a large peak at  $600\ \mu\text{m}^2$ , but also a small population of cells clustering around  $3000\ \mu\text{m}^2$  (Coblentz et al., 2003), suggesting that it is the  $\alpha$ -ganglion cells (Kolb et al., 1981; Boycott and Wässle, 1974) that remodel. Certainly the wholemounts demonstrate clearly that only a subset of ganglion cell bodies become GAP 43- and neurofilament-positive after detachment. And it seems likely that remodeling is limited and does not occur across all size-classes of ganglion cell.

**3.4.3.3. Ganglion cell remodeling after reattachment.** In the detached retinas, the GAP 43 or neurofilament-positive ganglion cell outgrowths seem to occur as two types: short “spikey” processes that occur on the basal part of the soma, and long, thin processes that grow wildly across the neural retina and into the subretinal space. Retinal reattachment appears to elicit the

extensive growth of neurites into the vitreous where they associate with epiretinal membranes produced by Müller cells (arrows, Fig. 21C; arrowheads, Fig. 21D). Thus, the response in the vitreous is similar to the response in the subretinal space, neurite outgrowths associate with reactive Müller cell processes, but the vitreal growth is initiated only after reattachment, probably because reattachment also initiates the growth of Müller cells into the vitreous cavity (Lewis et al., 2003). This data is also concordant with data from pathology specimens of human vitreal membranes showing the presence of anti-neurofilament labeled processes (Sethi et al., 2004). Initial observations of the labeled processes in human pathology samples seemed to indicate that the surgical procedures resulted in severed ganglion cell axons, which presumably would lead to the death of these cells. Data from the animal model suggest that these may represent neurites that have grown from ganglion cells as a result of detachment and reattachment. Removing these neurites may not have any consequences for the survival of ganglion cells.

#### 3.4.4. Initiation and origin of remodeled neuronal processes: general discussion

During development or in culture, undefined processes extending from neuronal cells are usually referred to as “neurites”. Given their origin, structure, and termination next to rod terminals, it seems reasonable to identify the processes that extend from rod bipolar cells and specifically contact the rod spherules as “dendrites”. While we first referred to the processes from horizontal cells as “neurites”, because their origin was not clear, it appears more likely based on their immunochemical signature, structure (long, thin, and unbranched for most of their length), and termination, when they are “directed”, that the horizontal cell processes represent growth of the B-type horizontal cell axon terminal. When these do branch in the subretinal space, their structure is also reminiscent of the axon terminals in the OPL, i.e., thick, branching extensions (see Kolb, 1974). It is perhaps surprising that some of these B-type cell axon terminal outgrowths try to “recapitulate” an axon terminal in the subretinal space since their normal presynaptic targets (rod spherules) are missing.

Unlike horizontal cells, all types of ganglion cell have both dendrites and axons. Thus the case for identifying the newly remodeled processes is not so obvious. The heaviest labeling with the anti-neurofilament antibody in normal retina occurs in retinal ganglion cell axons, but there is some labeling associated with the IPL, presumably of ganglion cell dendrites, although this has not been determined with certainty. The basal outgrowths on the ganglion cells in detached retina at first appear “spikey” and tapered, a description more applicable to dendrites than axons. However, the

processes that presumably grow from them into the neural retina and eventually into the subretinal space and vitreous are long, cylindrical, and non-tapering, i.e., axon-like in their appearance. During development, GAP 43 expression is generally associated with axons and thus the combination of labeling with the anti-GAP 43 and neurofilament antibodies along with the structure of the processes suggests that they are best classified as “axons”. In the normal retina ganglion cells are not multi-axonal; however, the concept of multiple axons is not completely novel since there are documented cases of retinal neurons that do, at least in structural terms, have them (Mariani, 1985, 1990; Dacey, 1990). Moreover in experiments with cultured CNS neurons, multiple axons could be elicited by “towing” of processes from the cells (Lamoureux et al., 2002). Indeed, these experiments may provide clues to the stimulus for neuronal outgrowths observed in our studies.

Experiments with chick and rat brain neurons in culture have demonstrated a link between mechanical tension, neurite elongation and axonal specification (Zheng et al., 1991; Chada et al., 1997; Lamoureux et al., 1997, 1998, 2002). Mechanical tension was applied to cultured mammalian hippocampal neurons by fine glass needles coated with poly-lysine and concanavalin A. The glass needle adhered to the plasma membrane of the experimental cell and a calibrated pulling force was applied. The results showed that mechanical tension applied to a cultured neuron can initiate the outgrowth of neurites and cause the elongation of neurites to “considerable length” (Lamoureux et al., 2002). These elongated neurites were of uniform caliber, i.e. having the architecture of axons, not the tapered structure of dendrites. The results also showed that these “towed” neurites express the axonal marker molecules, dephospho-tau and L-1. Furthermore, additional axons can be stimulated to grow from the neurons by towing, even after a primary axon has already been established, thus showing the capacity of these cells to develop multiple axons. Up to 4% of early developing hippocampal neurons have 2 axons (Ruthel and Hollenbeck, 2000), and differentiated dendrites of hippocampal neurons in culture can become axons (Bradke and Dotti, 2000).

Tension on the bipolar cell dendrites and the horizontal cell axon terminals could be exerted by the retracting rod spherules. However, data from the horizontal cells imply that tension on the axon terminal endings innervating the retracting spherules stimulates the growth of adjacent processes (i.e., those not terminating at spherules but growing through the retina) and this was not the case in the manipulated hippocampal neurons, where untowed sibling processes did not demonstrate outgrowths (Lamoureux et al., 2002). Perhaps the undirected processes result when the target of an outgrowth is lost, i.e., through the death of rod photoreceptor cells. We also have no evidence for any

mechanical changes that would provide force to ganglion cell dendrites. It is not known whether the processes presynaptic to these ganglion cells undergo structural remodeling, but it seems likely given the emerging data on such events in the retina. Thus, sprouting may not be in response to loss of presynaptic targets, nor mechanical tension applied by retracting presynaptic terminals. However, one possibility that could apply equally to horizontal cells and ganglion cells is the application of mechanical tension by rapidly hypertrophying Müller cell processes. This would only require the presence of adhesion between the two cell types, not an unlikely possibility. As Müller cells expand throughout the retina and into the subretinal space or vitreous, the horizontal and ganglion cell outgrowths would then simply be “towed” with them. An interaction of this type would explain why ganglion cell neurites do not appear in the vitreous until after Müller cells invade that space following reattachment.

#### 3.4.5. Are the remodeled neurites functionally significant?

There is no real answer to this question at the present time. The retracted rod spherules continue expressing at least some synaptic proteins (synaptophysin, RIBEYE; Figs. 5, 7, 10 and 11; Lewis et al., 1998; bassoon; Claes et al., 2004), the fact that the actual synapse with the extended bipolar and horizontal cell processes is architecturally abnormal does not mean that some form of neurotransmission cannot occur. It may be at least sufficient to preserve the integrity of the pre- and post-synaptic sites until reattachment allows recovery. The “aberrant axons” grown by horizontal and ganglion cells pose another issue. It seems difficult to imagine them serving some functional capacity. Perhaps they are the by-product of a developmental program which is re-instituted as part of the response to detachment (e.g. the release of soluble factors or mechanical “tugging”). If they are indeed axons, and they never innervate a target they may be completely nonfunctional. Likewise, the overgrown rod axons may express pre-synaptic proteins but not be involved in any type of functional connection.

Although difficult to assign any specific functional meaning, it seems worth mentioning the similarities between the responsiveness of horizontal cells and ganglion cells and glial cells (astrocytes in the brain and spinal cord, and Müller cells in the retina). All of these show extensive structural remodeling that is accompanied by an upregulation of intermediate filament proteins (GFAP and vimentin in the case of glial cells; neurofilament protein in the case of neurons). Perhaps these neurons differ from the rod bipolar cells (they are all larger in size, for example), or perhaps all remodeled cells exhibit an upregulation of cytoskeletal proteins, but the specific changes simply have not yet been identified.

### 3.5. Glial cell remodeling

#### 3.5.1. Müller cells

Photoreceptors, retinal pigment epithelium, and Müller cells are the cells most reactive to detachment. Müller cells hypertrophy. They also show nuclear migration and cell division along and growth into the subretinal space. All of these are obvious by simple histological observation (Machemer, 1968; Erickson et al., 1983; Anderson et al., 1983, 1986).

##### 3.5.1.1. The intermediate filament cytoskeleton and remodeling.

The more recent availability of immunocytochemical technology made it possible to correlate the structural hypertrophy of these cells with increased expression of cytoskeletal proteins including tubulin, glial fibrillary acidic protein (GFAP) and vimentin (Lewis et al., 1989, 1995). Electron microscopy also shows a large increase in the number of intermediate filaments in the cytoplasm of these reactive cells (Fig. 22; Erickson et al., 1987). Astrocytes in the brain and spinal cord react similarly, especially with respect to an upregulation of GFAP (Kerns and Hinsman, 1973; Eng and DeArmond, 1981). What makes the Müller cell GFAP response so dramatic is the fact that these filamentous proteins are almost exclusively localized to the endfoot region in a normal retina (red/yellow labeling, Fig. 23A), or in some species such as rats and mice, not detectable by current immunocytochemical technology (Figs. 23C, Bignami and Dahl, 1979; Eisenfeld et al., 1984; Sarthy and Ripps, 2001). On injury there is a tremendous amount of cytoskeletal remodeling that occurs in these cells that correlates closely with changes in overall morphology. Indeed the correlation is so close that they are generally assumed to be functionally linked events. In support of this argument is the fact that in the ground squirrel retina Müller cells do not hypertrophy in response to detachment, and their intermediate filament cytoskeleton remains unchanged (Linberg et al., 2002a). Following brain injury astrocytic scar formation is impaired in *vim*<sup>-/-</sup>/*GFAP*<sup>-/-</sup> mice, but not in mice lacking only one of the two intermediate filament genes (Pekny et al., 1999). Whether a similar principle applies to Müller cells remains to be determined.

##### 3.5.1.2. The endfoot as the origin of the intermediate filament response.

Within a day of a detachment intermediate filament proteins within the endfoot become more dense in number, often forming whorl-like or wavy bundles (red/yellow labeling, Fig. 23B; Erickson et al., 1987). They appear to grow from this distal mass, extending both into branches of the endfoot, which increase in size and in number with detachment time, and apically towards the cell body and then into the outer retina (Fig. 23; Lewis et al., 1995,

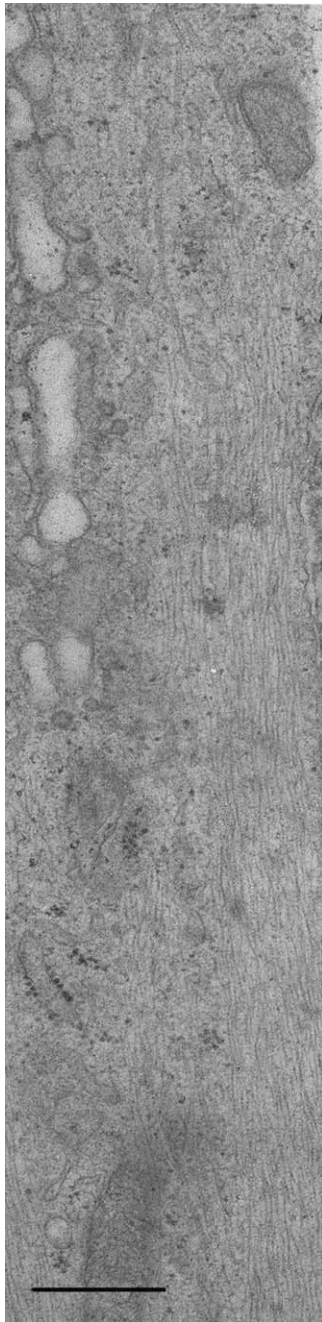


Fig. 22. An electron micrograph showing the presence of many 10 nm diameter filaments in the cytoplasm of a Müller cell in the outer retina after retinal detachment. These intermediate filaments label with antibodies to both GFAP and vimentin and in the normal retina are limited to the endfoot region of these cells. Scale bar = 0.5  $\mu$ m.

2003). The cell bodies and nuclei of Müller cells are located in the inner nuclear layer, more-or-less midway between the endfoot and the apical microvilli. Thus, the intermediate filament response of these cells is somewhat unusual inasmuch as the new proteins appear to be assembled at a site distant from the nucleus. Immunocytochemical studies indicate that there are apparently unpolymerized subunits for these proteins in the

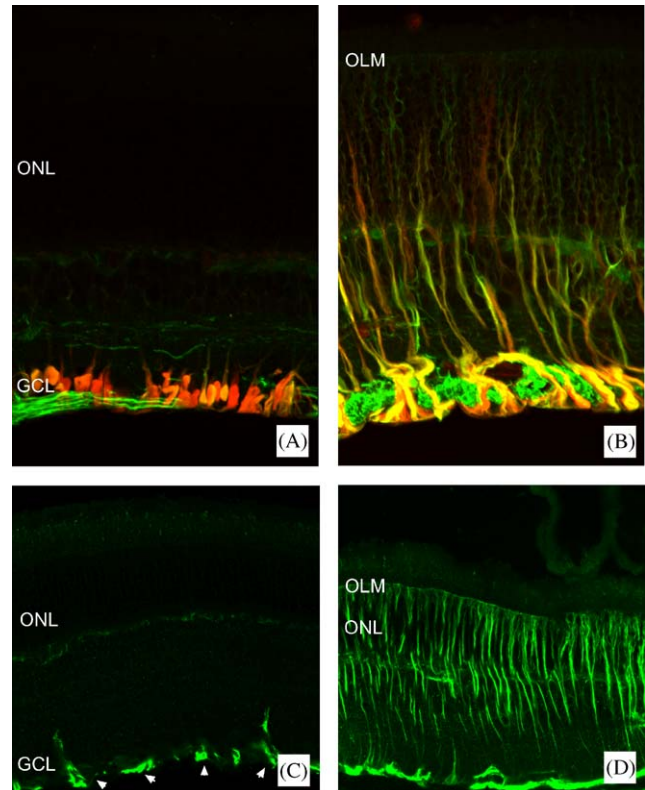


Fig. 23. A series of confocal microscope images from detached retinas labeled with antibodies to the intermediate filament proteins GFAP (green) and vimentin (red). (A) In the normal retina, intermediate filaments are concentrated in the endfoot region of the Müller cells with vimentin predominating over GFAP (astrocytes, however, show a great preponderance of GFAP in their cytoplasm). (B) After 3 days of detachment, the intermediate filament cytoskeleton is showing growth from the endfoot into the outer retina. There is also a shift towards the yellow and green colors indicating that GFAP labeling has increased more than the labeling for vimentin. (C) Mouse retina labeled with the antibody to GFAP. In some species such as the mouse, there is no detectable GFAP in Müller cell endfeet, the only labeling occurring in astrocytes (arrowheads). (D) When the mouse retina is detached for 7 days, the GFAP reactivity of Müller cells is striking, with labeling now extending across the entire neural retina to the outer limiting membrane (OLM). ONL = outer nuclear layer; GCL = ganglion cell layer.

cytoplasm and these may add to existing intermediate filaments in the endfoot thus resulting in their growth. However ultrastructural in situ hybridization data indicate that the mRNA for GFAP may be transported from the cell body to the endfoot where new GFAP may be synthesized (Erickson et al., 1992). Whether there is a similar transport of message apically as the intermediate filaments grow beyond the cell body, or even into Müller cell “scars” on the retinal surface has not been investigated. The transport of mRNA, with localized protein synthesis, into distant locations away from the nucleus was regarded as somewhat unusual, but is now recognized as an important functional mechanism in dendrites (see Job and Eberwine, 2001).

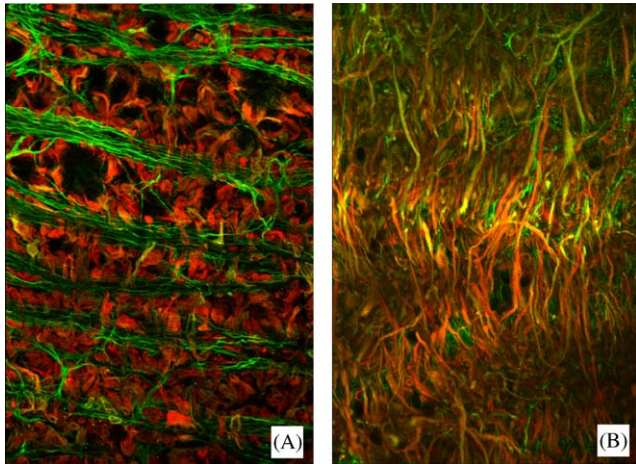


Fig. 24. Confocal images of retinal wholemounts labeled with the antibodies to GFAP (green) and vimentin (red) and viewed from the ganglion cell surface to show changes in the Müller cell endfeet after detachment. (A) Normal retina. The endfeet are “hoof-shaped” and their labeling is predominated by vimentin. The long thin green processes are processes of astrocytes following the bundles of ganglion cell axons across the surface of the retina. The “holes” represent ganglion cell bodies which are negative for both proteins. (B) In a retina detached for 7 days, the endfeet processes have grown extensively on the retinal surface. Astrocyte processes appear disorganized in these regions. Ganglion cell axon bundles are no longer as prominent, probably due to them being overlain by the thicker endfeet processes.

Within a day or two of detachment, branching of the endfeet and their growth along the vitreal surface of the retina is obvious using the antibodies to GFAP and vimentin (Figs. 23B and 24). Strikingly, these endfoot extensions, while often quite long, remain adjacent to the retina, within the basement membrane that lines the interface between the retina and vitreous. As the endfeet expand, the major trunk and side branches of the Müller cells grow thicker and more expansive within the neural retina (Erickson et al., 1983). Müller cell processes also fill in space left by dying photoreceptors. The extreme case is observed in the ground squirrel where virtually all photoreceptors die and the space that was defined as the ONL is now comprised solely of elaborately branched Müller cell processes (Linberg et al., 2002a). Müller cells are joined to each other and to adjoining photoreceptors at the outer limiting membrane by adhering junctions. Beyond this the apex of the Müller cell consists of microvilli that extend into the interphotoreceptor matrix and label heavily with an antibody to the protein CD-44 (Chaitin et al., 1994).

**3.5.1.3. Subretinal growth of Müller cells after detachment.** As intermediate filaments expand into the apical region of the activated Müller cell cytoplasm, some Müller cell microvilli begin to grow, expanding into the subretinal space where they elaborate into striking structures (Figs. 25A and B). GFAP and vimentin can

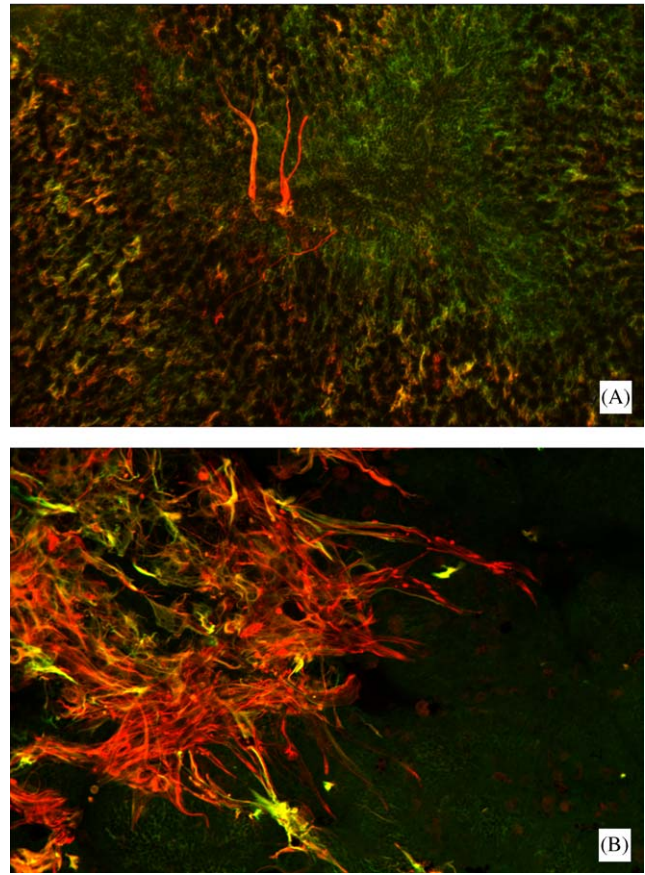


Fig. 25. Confocal microscope images of the subretinal space in retinal wholemounts prepared after detachment and labeled with antibodies to vimentin (red) and GFAP (green). (A) An image from a retina detached for 3 days showing the initial outgrowth of a Müller cell process (red) from the neural retina and into the subretinal space. Notice that the background is filled with reactive Müller cell processes that remain within the neural retina, and their green color indicates the great predominance of GFAP. Those processes that grow into the subretinal space show a predominance of vimentin. (B) An image from the subretinal space of a retinal wholemount prepared from a 28 day detachment. Now there is a large “membrane” growing in the subretinal space composed of overlapping Müller cell processes. Vimentin continues to predominate over GFAP in these subretinal membranes.

copolymerize to form intermediate filaments, but we have observed in double-labeling studies that there is a differential expression of the two in feline Müller cells. In the normal retinas, vimentin seems the predominate protein, so that the endfeet label most heavily with this antibody and labeling extends farther into the neural retina than does that for GFAP. In the main trunk of the *reactive* cells, GFAP seem to be the predominant intermediate filament protein. The exception seems to occur in cases where the apical microvilli begin to grow into the subretinal space, and then vimentin labeling predominates (Figs. 25A, red tapering, forked processes). This imbalance in expression continues as the processes expand into the subretinal space where they can form multiple layers of very long strands of

cytoplasm (Fig. 25B; Lewis and Fisher, 2003). The “leading edge” of the growing processes shows a predominance of anti-vimentin labeling, with GFAP expression becoming increasingly intense at the point of exit from the neural retina. These subretinal scars can have a significant physiological effect. Their presence does not appear to prevent physical reattachment of the retina, but does appear to inhibit the regeneration of outer segments (Anderson et al., 1986).

**3.5.1.4. Reattachment stimulates growth of Müller cells into vitreous.** While the endfoot region reacts by branching and growth, a new reaction occurs in the feline retina in response to reattachment. Now the branched endfoot processes that remained closely adherent to the vitreal surface of the retina begin growing into the vitreous. While it is the apical microvilli that appear to expand into the subretinal space forming the thick Müller cell processes in a glial scar, on the vitreal surface it appears to be the “branchlets” of the endfoot that expand into the vitreous. These appear as fine, flat “ribbons” of cytoplasm extending away from the neural retina into the vitreous cavity (Figs. 26A and B). These delicate, flattened processes have a very different morphology from the fine, tapered, and branched processes that

expand into the subretinal space. In these endfoot outgrowths, GFAP expression dominates over that of vimentin (Fig. 26A and B). While vimentin expression “leads the way” in the expanding Müller cell processes within the subretinal space, it is GFAP that holds this position in the endfoot processes that grow into the vitreous (Figs. 26C and D).

The growth of Müller cells into the vitreous in both feline and human reattachments is a clear indicator that they form an important component of “fibroproliferative” diseases such as proliferative vitreoretinopathy. The growth of these cellular processes into the vitreous can have significant consequences on vision because they can become contractile (Ryan, 1985) to cause wrinkling and eventual re-detachment of the neural retina.

Although it is our impression that the membranes originate from Müller cell processes that grow into the vitreous, other cell types, including retinal pigment epithelial cells and astrocytes (arrows, Fig. 26D) can also be components of these vitreal membranes. Understanding the molecular expression profiles for the reactive Müller cells as well as determining the growth factors, cytokines, or neurotrophins they respond to may eventually lead to preventative therapy for these diseases.

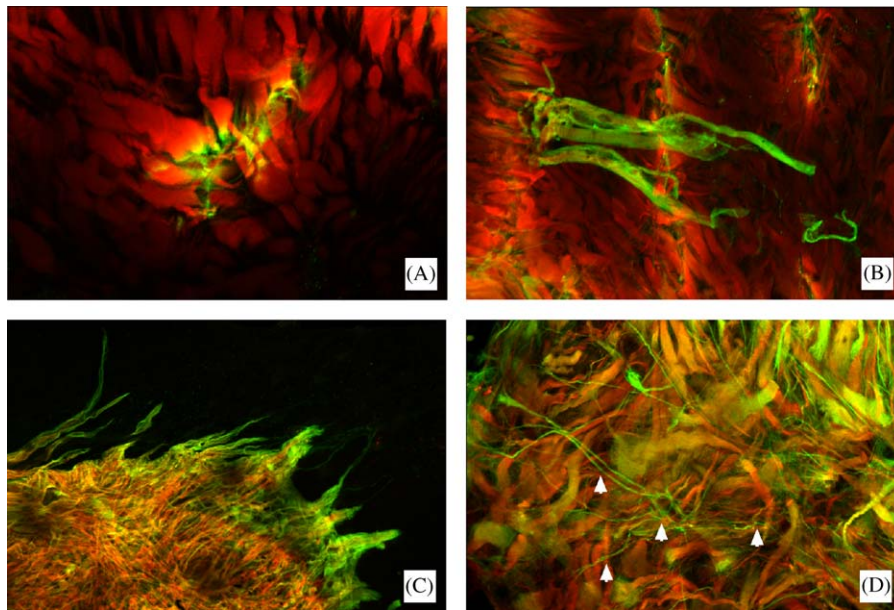


Fig. 26. Confocal microscope images of the vitreal surface of retinal wholemounts prepared after the retinas were detached for 3 days and reattached for 28 days. The wholemounts were labeled with antibodies to vimentin (red) and GFAP (green). (A) In this relatively high magnification image of the retinal surface, the Müller cell endfeet are still largely hoof shaped. The red color indicates a predominance of vimentin. However in the vitreal edge of some of these endfeet GFAP is beginning to show predominance (yellow/green color). (B) An image showing the outgrowth of ribbon-like extensions of the Müller cells into the vitreous. Notice that the within the retina the endfeet are red, indicating a predominance of vimentin labeling, while the endfeet outgrowths into the vitreous are green, indicating a shift in the balance of intermediate filament protein expression towards GFAP. (C) In some regions, complex epiretinal “membranes” are found growing on the vitreal surface of the wholemounts. These complex membranes consist mainly of Müller cell processes in which the “leading” edge of the membrane shows a predominance of GFAP (green). (D) A higher magnification image showing the complex structure of the epiretinal membranes with their mixture of Müller cell endfeet and branched cells presumed to be astrocytes based on their structure and labeling with the antibody to GFAP (arrowheads).

**3.5.1.5. General consequences of Müller cell remodeling.** We have already discussed the fact that the expanded Müller cell processes within the retina and on the retinal surfaces appear to provide some attraction for the neurites that grow from horizontal and ganglion cells after detachment. Thus, Müller cell remodeling cannot be viewed as a “benign” activity. It is almost a certainty that these cells are also functionally altered as they remodel and this may have profound effects on retinal physiology. Their expression of such enzymes as glutamine synthetase and carbonic anhydrase decreases significantly (Lewis et al., 1994) and this can have significant effects on both glutamate concentration within the retina (Marc et al., 1998) and the pH of the surrounding environment, in turn leading to decreased neuronal stability. It is likely that there are many more changes in the molecular expression profiles of activated Müller cells, but that these changes simply have not yet been identified.

### 3.5.2. Astrocytes

In the normal retina, astrocytes reside among the axons of the ganglion cells as they course over the surface of the retina towards the optic nerve head. Here they have the characteristic stellate appearance of astrocytes in the CNS, with their long arms appearing to reach out to the ganglion cell axons. Some astrocytes also appear around the ganglion cell bodies, and close to major dendritic trunks of these cells. There is little known about the reactive capacity of these cells in the retina. They proliferate in response to detachment (Fisher et al., 1991; Geller et al., 1995) and observations of optic nerve fiber layer in retinal wholemounts show that their regular array and stellate shape are lost as the endfeet of the Müller cells expand on the retinal surface (Lewis and Fisher, 2003). Whether this is in response to the presence of the growing endfeet or to some other stimulus is unknown. Nor is it known if their shape and organized array returns once the retina is reattached. Based on immunochemical labeling data it seems likely that astrocytes are one of the cell types that also occur in fibroproliferative scars within the vitreous (arrowheads, Fig. 26D). Astrocytes elsewhere in the CNS are known to secrete a variety of factors, thus, their reactivity may be an important, poorly understood response to detachment.

## 4. Retinal remodeling, detachment and reattachment

Studies of detached (and reattached) retina have shown us that the mammalian retina has remarkable remodeling capabilities. Fig. 27 shows in summary form the remodeling of the neural retina described here. The only change of this type for which we may have some underlying mechanistic explanation exists is outer

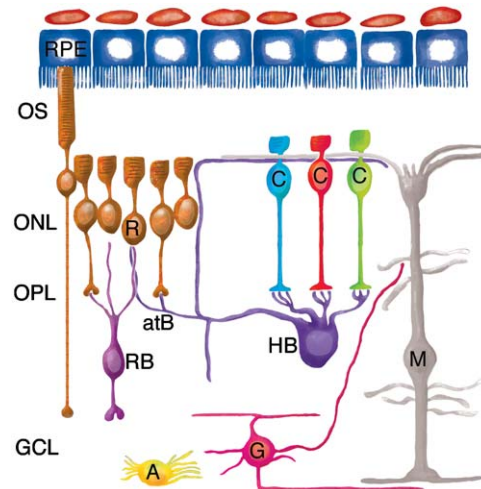


Fig. 27. A drawing showing the remodeling of feline retinal cells as discussed in this paper. The outer segments of rod (R) and cone (C) photoreceptors are greatly shortened by detachment and separated from the apical surface of the pigmented epithelium (RPE). The synaptic terminals of many rods are withdrawn from the outer plexiform layer (OPL) presumably by retraction of the rod axon. After reattachment some rod axons grow into the inner retina (shown by the cell on the left) where they form structurally specialized endings that express some presynaptic proteins. Cone terminals remain in place after detachment, although they undergo significant ultrastructural remodeling. The dendrites of rod bipolar cells (RB) grow into the outer nuclear layer (ONL) where they always appear to terminate adjacent to withdrawn rod terminals. There may also be some pruning of RBC dendrites in longer-term detachments. The axon terminals of the B-type horizontal cell (HB) remodel extensively, with some processes terminating, like those from the RB cells, next to withdrawn rod spherules, while others grow wildly into the outer retina, and often into the subretinal space adjacent to reactive Müller cells. Horizontal cell axon terminals can also grow into the inner retina, although this reaction appears less frequent than growth into the outer retina. A subpopulation of ganglion cells (G) that upregulate both GAP 43 and neurofilament expression extend short spiky processes from the base of their cell body after detachment, but can also grow thickened processes into the outer retina where they behave much like the outgrowths from horizontal cells and appear to associate with reactive Müller cells. In the reattached retinas, ganglion cell processes can also grow into epiretinal membranes formed by Müller cell growth into the vitreous. Müller cells (M) are highly reactive to detachment. They greatly upregulate the expression of intermediate filament proteins and after detachment their microvilli can grow out of the neural retina expanding in the subretinal space to form membranes or glial scars on the exposed photoreceptor outer segments. After detachment their endfeet expand but remain within the inner limiting membrane. After reattachment their specialized endfeet can grow into the vitreous where they form epiretinal membranes as part of the disease, proliferative vitreoretinopathy. Astrocytes (A) proliferate and often appear in epiretinal membranes but their responses to detachment have not been characterized in detail. Artwork by Black Pixel, <http://www.black-pixel.net>.

segment regeneration. One of the most important discoveries in retinal cell biology was that of the phenomenon of outer segment renewal (Young, 1967). Although the details of the molecular machinery at work in this process are still not completely understood over three decades after its discovery, we do know that it

provides a mechanism for vertebrate photoreceptors to regenerate their outer segment on a continuous basis. This occurs through the process of forming of sequential outgrowths or “evaginations”, of disc surface and rim membrane from the connecting cilium (Steinberg et al., 1980) which then mature into the stacks of discs characteristic of vertebrate photoreceptors. Reattachment seems to place the photoreceptors back into a permissive environment that allows the molecules being synthesized, to be assembled into a structurally organized, functional outer segment. This permissive environment could involve some molecular signaling between the RPE and photoreceptors, because supplementing detached retinas with the brain-derived neurotrophic factor (BDNF) maintains outer segments in the detached retina (Lewis et al., 1999), or it may be a function of something as basic as the oxygen concentration in the subretinal space. Increasing the oxygen concentration of the environment for animals with detachments has a significant effect on outer segment maintenance, and photoreceptor cell death in at least two species; cats and ground squirrels (Mervin et al., 1999; Sakai et al., 2001; Lewis et al., 2004). Neither BDNF nor hyperoxia maintains the differentiated RPE apical surface in the detached retina, suggesting that the neural retina provides some signal that keeps this complex surface in its differentiated state. Furthermore, this signaling function must be restored upon reattachment allowing the apical surface to re-differentiate.

Another unknown player in interactions between the neural retina and RPE is the complex interphotoreceptor matrix, with its biochemically and structurally distinct domains for rods and cones (Johnson et al., 1986). This matrix is lost after detachment, so its role in outer segment survival and in maintaining the architecture of the apical surface is unknown. Although neither the distinctive “cone matrix sheaths” nor cone sheaths in feline retina regain a completely normal organization after reattachment—at least not within a month—the fact is that both do regenerate to some degree and remain specifically associated with cone outer segments; an indication of some form of signaling between these photoreceptors and the RPE.

#### *4.1. Do Müller cells respond independently of photoreceptors?*

Hyperoxia, if administered immediately upon production of a detachment, can greatly reduce Müller cell reactivity (Lewis et al., 1999); if its administration is delayed by 24 h it is less effective, even though photoreceptor rescue is highly effective (Lewis et al., 2004). This, along with the very rapid reaction of Müller cells to detachment in which they demonstrate an

increase in ERK phosphorylation within 15 min (Geller et al., 2001), suggests that they are reacting independently to detachment, not just downstream to the damage or death of photoreceptors. The independence of Müller cell reactivity from photoreceptor cell death is dramatically supported by comparative data from detachments in the California ground squirrel. In this species photoreceptor cell death is rapid and nearly complete, while Müller cells remain largely unreactive (Linberg et al., 2002a). Müller cell apical microvilli extend into the interphotoreceptor matrix. They are highly invested with the protein CD-44 which has the capacity to interact with molecules like hyaluronic acid (Lesley et al., 1993), a prominent component of that space (Hageman and Johnson, 1990). The signaling capacity of the microvilli has not been studied in any detail, and may hold an important link in the chain leading to Müller cell reactivity.

The remodeling changes in second- and third-order neurons also may be thought of as “secondary” to photoreceptor reactivity. The vigorous reactivity of rod bipolar cells and B-type horizontal cell axon terminals suggests this is the case. We have not yet observed clear evidence for A- or B-type horizontal cell dendritic remodeling, although the reaction of cone-connected bipolar cells is still unexplored because we have not had available specific markers for these cells that work reliably in the feline retina. However, if the cone-connected horizontal or bipolar cells do not show the same extensive reactivity of the rod-connected cells, then this may be another indicator that it is the actual physical retraction of the rod synaptic terminals that provides the stimulus for remodeling.

It is more difficult to derive specific hypotheses to test for the initiation of structural remodeling in ganglion cells. Even if the rod bipolar cell axons remodel in response to detachment, there is no direct link between them and ganglion cells. Thus, if the changes are initiated through physiological changes in the rod pathway, it will have to be mediated by other interneurons. Other possibilities include some type of signaling from reactive Müller cells, or the release of diffusible factors that effect vulnerable cells. Glutamate could serve as such a general signal since immunocytochemical data suggests disruption of the glutamate recycling pathway in Müller cells (Lewis et al., 1994, 1999; Marc et al., 1998).

## **5. Future directions**

Although there are many unanswered questions, data from the detachment model, as well as data from a variety of other studies of retinal degeneration now suggests that retinal neurons remain capable of significant structural remodeling in adult mammals. This in



turn may provide increased optimism for a variety of therapies for blinding diseases in which photoreceptor degeneration is the primary cause of visual loss. Although preventing photoreceptor cell death is the optimum therapy in these diseases, alternatives to this daunting challenge include technology for replacing photoreceptors, whether it is by way of cellular transplantation (Aramant and Seiler, 2004) or the use of progenitor cells (Fischer and Reh, 2001, 2003; Tropepe et al., 2003). This success, however, would be hollow if the second-order neurons did not retain sufficient plasticity to form functional connections with the new photoreceptors. It may seem far-fetched at the present that such connections would form circuitry for functional vision. However, the degree of remodeling we have observed may be an indicator that the inner retina has more of a capacity for remodeling itself than previously imagined. Studies of patients with foveal reattachments indicating that visual recovery may occur over years, not weeks, could conceivably represent the re-formation of appropriately functional retinal circuits, or even a remodeling of the RPE/photoreceptor interface to properly align the foveal cones. In the case of detachment, preventing both photoreceptor and Müller cell reactivity may also be key to treating the injury and preventing threats to sight through diseases such as subretinal fibrosis, and proliferative vitreoretinopathy. Adjuncts to therapy do not seem so distant in these cases, since treatment with something as simple as elevated oxygen concentration appears to help attain these goals in animal models (Mervin et al., 1999; Lewis et al., 1999, 2004; Sakai et al., 2001).

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