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Experimental Retinal Reattachment

A New Perspective

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Abstract

In the feline model, retinal detachment initiates a cascade of changes that include photoreceptor-cell "deconstruction," apoptotic death of some photoreceptors, neurite outgrowth from second- and third-order neurons, remodeling of photoreceptor synaptic terminals, and Müller-cell gliosis. We have previously shown that reattachment within 24 h halts or reverses many of these presumed detrimental changes. However, in patients with retinal detachments, reattachment cannot always be performed within this 24-h window. Moreover, recovery of vision following successful reattachment surgery in the macula is often imperfect. Here, we examine the ability of relatively long-term reattachment (28 d) to stop or reverse several cellular events that occur at 3 d of detachment. In contrast to earlier studies of reattachment, which focused on the regeneration of outer segments, we focus our attention here on other cellular events such as neuronal remodeling and gliosis. Some of these changes are reversed by reattachment, but reattachment itself appears to stimulate other changes that are not associated with detachment. The implications of these events for the return of vision are unknown, but they do indicate that simply reattaching the retina does not return the retina to its pre-detachment state within 28 d.

Index Entries: Retinal detachment; retinal reattachment; Müller cell; photoreceptor; rod; cone; neurite outgrowth; epiretinal membrane; gliosis.

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Introduction

Recovery of visual function after successful retinal reattachment surgery is often variable and less consistent than might be expected, considering surgical success. Various factors that are known to contribute to the outcome include detachment duration and height, age of the patient, extent of myopia *(1),* and the condition of the retina just prior to reattachment. We have recently shown that detachment of the neural retina from the retinal pigment epithelium (RPE) initiates extensive remodeling of both neurons and glia *(2).* Indeed, both molecular and morphological changes can be identified in all major classes of retinal cells within the first few days of detachment. One of the first observable changes is the degeneration of rod and cone outer segments *(3,4),* followed by apoptosis of some photoreceptors *(5).* Soon after, cones—but not rods—begin to lose the expression of many proteins, including their specific opsins *(6–8).* By d 2 after detachment, rod synaptic terminals, but not cone terminals, begin to withdraw toward their cell body, leaving the outer plexiform layer (OPL) highly disorganized *(4,9).* In concert with this withdrawal, there is an outgrowth of rod bipolar and horizontal-cell neurites, with some extending deep into the outer retina. Although bipolar-cell neurites appear to elongate toward their withdrawn rod terminals, horizontal-cell neurites usually elongate adjacent to reactive Müller cells with no particular reference to their presynaptic target *(2).* Within 7 d of detachment, many ganglion cells are immunopositive for neurofilament protein and growth-associated protein 43 (GAP 43), and these cells occasionally sprout neurites *(10).* Finally, Müller cells, which become activated as rapidly as 15 min after detachment *(11),* proliferate and hypertrophy extensively both within the retina and extraretinally *(12,13).* The proliferation peaks 3–4 d after detachment, but the growth of their processes, often extending into the subretinal space, continues as long as the retina is detached *(14).*

These changes are not restricted to animal models of detachment; similar events have been identified in tissue specimens obtained from human patients with complex detachments *(15).* Considering these extensive cellular changes, it is perhaps not surprising that successful reattachment surgery does not always return the patient's vision to pre-detachment levels, especially if the macula is involved. Indeed, improvement in visual acuity has been observed to continue for several years following surgery *(16),* which may be an indicator that reversal of some of these changes is a slow process. We have previously shown that reattaching the retina within 1 d could essentially halt the formation and progression of many of the remodeling events listed here *(17).* The effects of reattachment after a longer period of detachment are reviewed here. The events described occurred during experiments in which the retina was detached for 3 d—a time when many cellular events are well underway—and then reattached for 28 d. The methodology used (including surgical, histological, and immunocytochemical techniques) is standard for our studies of the feline model of detachment and reattachment *(17).*

Photoreceptor Cells

Histologically, retinas detached for 3 d and reattached for 28 d usually have an outer retina that is almost normal in appearance (compare the outer retina in Fig. 1A to that in Fig. 1B). However, in every retina examined, there are scattered patches of apparent poor recovery (Fig. 1C–F). These usually measure less than 1 mm long in a single section. In these regions, the outer segments appear to be shorter than normal, and are often disrupted. The inner segments often contain large vacuoles, and the outer nuclear layer (ONL) appears to be much more loosely packed with nuclei than normal. These affected zones often occur with an abrupt transition from the normal retina, although the entire region has been detached and reattached (Fig. 1C).

Outer-segment lengths decrease progressively after retinal detachment (Fig. 2). Reattachment of 28 d after 3 d of detachment is

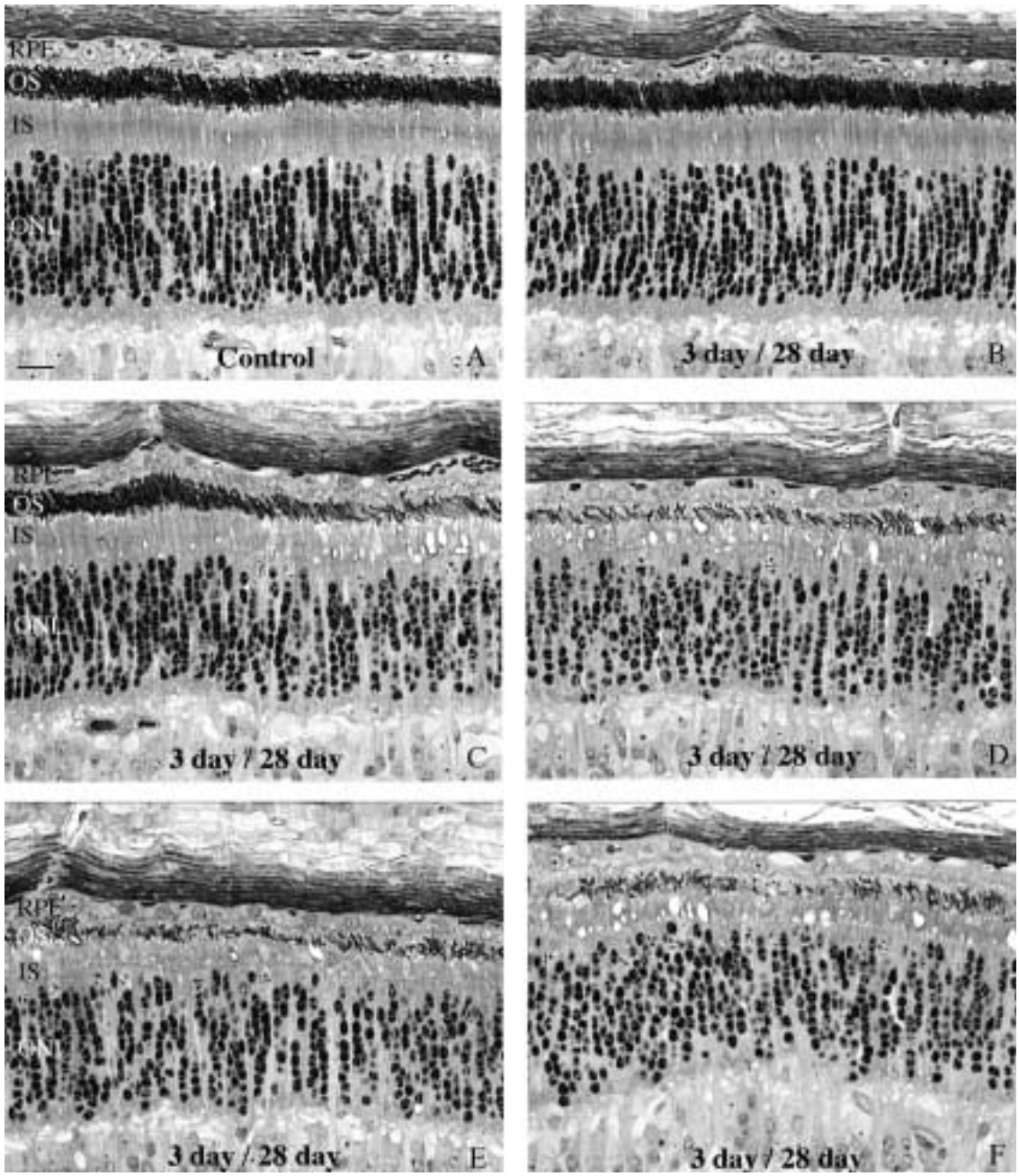


Fig. 1. Light micrographs of the outer retina from control eyes **(A)** and those with retinas reattached for 28 d following a 3-d detachment **(B–F)**. Most reattached regions appear almost normal; the outer segments (OS) are well-organized, and the nuclei in the outer nuclear layer (ONL), are tightly packed **(B)**. Scattered areas of poor recovery with shorter OS, vacuolized inner segments (IS), and fewer nuclei in the ONL are occasionally observed **(C–F)**. Transitions from a "normal" appearing retina to regions with poor morphological recovery are often abrupt **(C)**. RPE, retinal pigment epithelium. Scale bar = 20 μm.

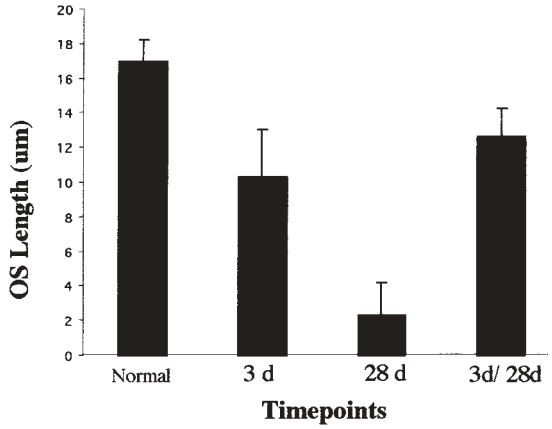


Fig. 2. The average width of the outer-segment layer in control retina and retinas detached for 3 d or 28 d, or detached for 3 d and reattached for 28 d (3 d/28 d).

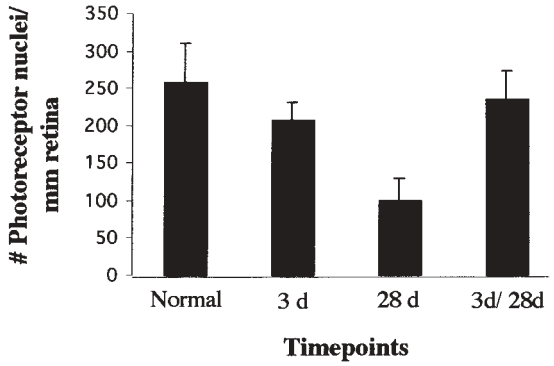


Fig. 3. The average number of photoreceptor nuclei per mm of retina in control eyes and those with 3-d or 28-d detachments, or those detached for 3 d and reattached for 28 d (3 d/28 d).

enough time for outer segments to recover some, but not all, of the length seen in the control retina. The width of the outer-segment layer in normal feline eyes averages about 17 µm in the region of retina used for these studies (Fig. 2). By d 3 of detachment, that value decreases to about 10.3 µm (with a further decrease to 2.3 µm at 28 d). In the reattached retinas, the width of this layer averaged 12.6 µm. Although this demonstrates that there has been some recovery from the 3-d detachment time-point ($p = 0.004$, using a two-sample t-test and assuming equal variance), this number is still significantly different from values in control eyes ($p < 0.00004$). Based on estimates of the rod turnover rate, these cells have the potential to recover their length within 1 wk of reattachment, but clearly, this does not occur. This issue is discussed in ref. *18*.

The control retinas have, on average, 257 photoreceptor nuclei/mm of retinal length (Fig. 3). After 3 d of detachment, this figure is reduced to an average of 207 nuclei/mm ($p = 0.03$). This number declines even further to an average of 100 nuclei/mm in the 28-d detachments ($p < 0.0005$). In the 3-d detached, 28-d reattached retinas, the number of nuclei/mm retina in the ONL is 235, or about midway between the control and 3-d detachment values. Since this value would not be considered statistically different from those in control animals ($p = 0.07$), it is clear that reattachment at 3 d greatly slowed the death of photoreceptor cells.

Photoreceptor-cell death after retinal detachment occurs via the apoptotic pathway *(5)*. Apoptosis begins within 1 d after detachment, peaks at d 3, and continues at low levels for as long as the retina is detached. At 3 d after detachment, there are, on average, 54.2 TdT-mediated dUTP nick-end labeling (TUNEL)-labeled photoreceptor cells per mm of retina (Fig. 4), and this number decreases to 6.0 cells/mm in 28-d detachments. TUNEL-labeled cells in the ONL can still be found after 28 d of reattachment (0.61 cells/mm retina), although this number is significantly less than in the 28-d detachments without reattachment ($p < 0.0002$). It is important to note that no TUNEL cells occur in a normal feline retina of this age.

As reflected by the data on outer segment-layer width—and shown here immunocytochemically—rod photoreceptors usually recover from the effects of being detached for 3 d. In the normal retina, labeling with the antibody to rod opsin is restricted to the outer segments (Fig. 5A), yet at 3 d of detachment, it becomes redistributed to the plasma membrane of the inner segments and cell bodies (Fig. 5B).

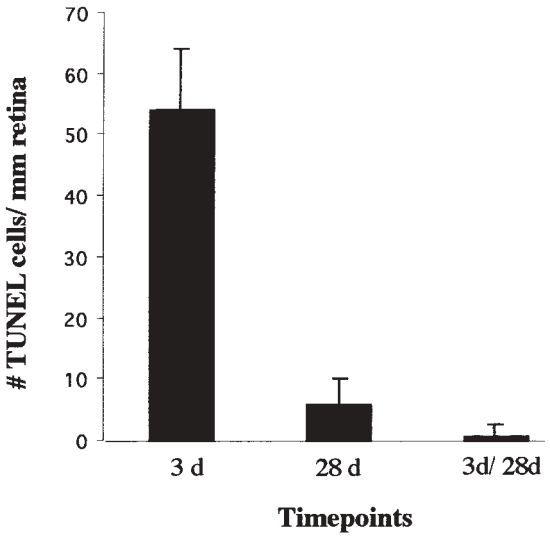
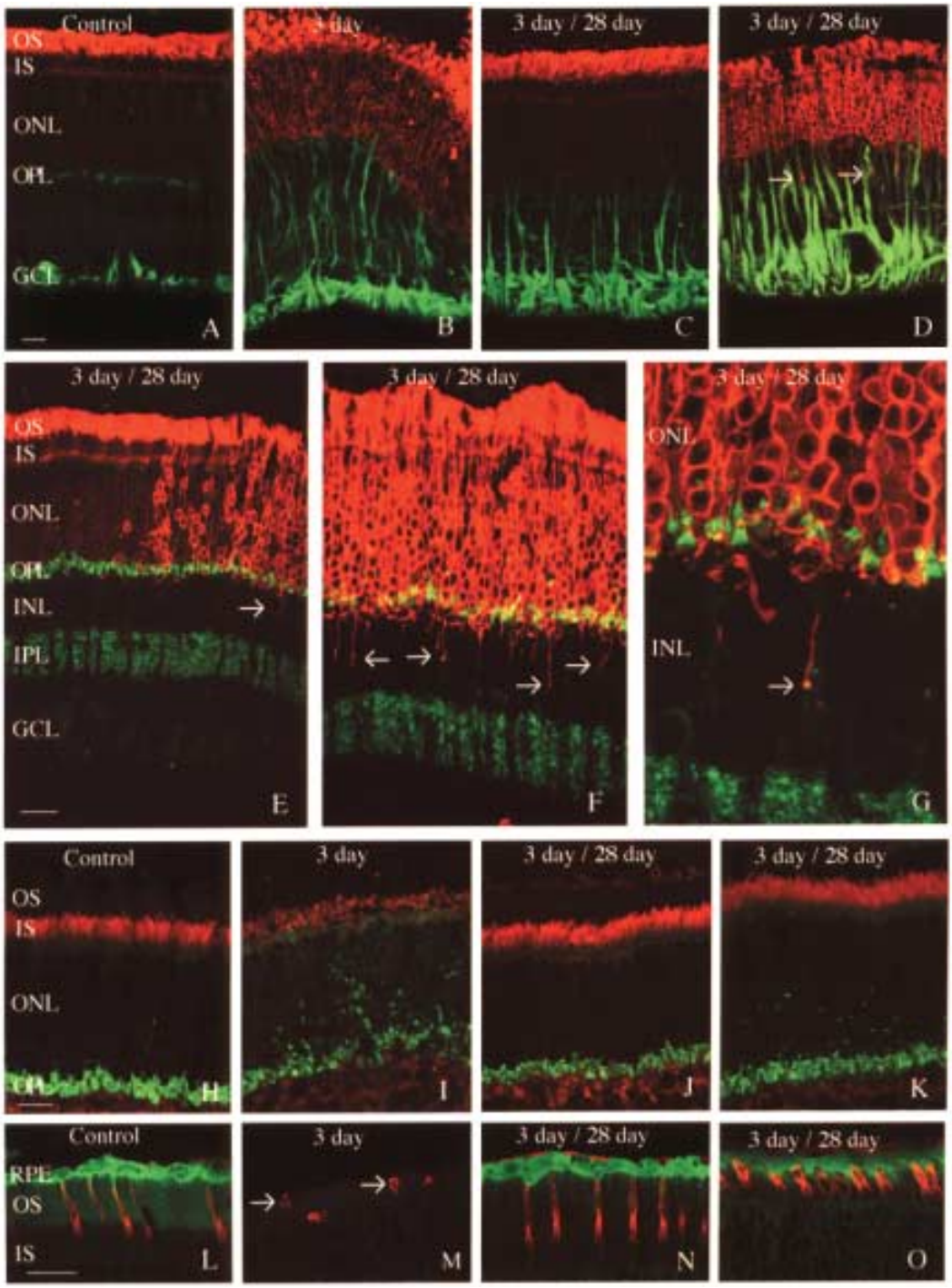


Fig. 4. The average number of TUNEL-positive cells per mm in retinas detached for 3 d or 28 d, or detached for 3 d and reattached for 28 (3d/28d). Low numbers of TUNEL-positive cells occur in both the 28-d detached and reattached retinas.

In the reattached retinas, this labeling is once again restricted to the outer segments over the majority of the reattached area (Fig. 5C). However, in some areas, it remains delocalized, and it is in these regions that we observe rod axons extending past the OPL into the inner retina (Fig. 5D–G). These rod axon outgrowths can be numerous along a length of the retina that demonstrates opsin delocalization (Fig. 5F). The presence of elevated anti-GFAP labeling in Müller cells confirms that the regions showing rod axon growth were both detached and reattached (Fig. 5C,D). Anti-glial fibrillary acidic protein (GFAP) labeling in the reattached retinas is equivalent to that observed in 3-d detached retinas, and does not appear to reflect the presence of delocalized opsin or rod axon outgrowth (Fig. 5B–D). (The Müller-cell response is presented in detail below.) Thus, it is unlikely that either is generated by a failure of reattachment. The transition between an area of good and poor outer-segment regeneration and the presence or absence of opsin redistribution can be quite abrupt (Fig. 1C,5E). However, there does not appear to be a complete correlation between the two events, as demonstrated in Fig. 5E, where there is an abrupt transition within a region of normal appearing rod outer segments to a region of intense opsin redistribution. Therefore, only slight disruption of the outer segments may be required to trigger opsin redistribution. Even in the areas of heavy opsin redistribution showing outer segment disorganization, overall outer segment disruption is not as severe as in the 3-d control detachments (compare Fig. 5B with Fig. 5F). This finding suggests that these cells are not in the process of degenerating, but are probably still undergoing regeneration. Indeed, the fact that they elongate axons with terminals that are immunopositive for the synaptic vesicle protein, synaptophysin, also suggests that they are regenerating and attempting to make functional synapses (Fig. 5G, arrow). In regions with no opsin delocalization, we have no means of observing these elongated rod axons. However, the lack of synaptophysin labeling in the inner nuclear layer (INL) suggests that they do not occur.

Elongation of rod axons occurs only after reattachment. When the retina is detached, some rod synaptic terminals retract into the ONL toward their respective cell bodies. At 3 d after detachment, many anti-synaptophysin-labeled rod terminals are observed in the ONL, leaving the layer of terminals in the OPL disrupted (Fig. 5I; compare to normal, Fig. 5H). After 1 mo of reattachment, most retinal regions appear to have reorganized their previously disrupted OPL, and few or no terminals remain in the ONL (Fig. 5J). In some areas, synaptophysin labeling is still fairly prominent within the ONL, suggesting that not all rod terminals have regrown (Fig. 5K). Overall, the process of rod photoreceptor regeneration seems to be a fairly remarkable one, in which many cells in the population appear to recapitulate at least part of their developmental program—that is, they develop an outer segment while extending their axon and synaptic terminal. Rod axon extension beyond the OPL is a phenomenon that has also been described during normal develop-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ment *(19).* These ectopic processes are eventually removed (i.e., they do not occur in adult retina); whether or not they undergo a similar fate after prolonged reattachment is unknown. Thus, adult rod photoreceptors remain remarkably dynamic in their ability to respond to changes in the retinal environment.

Synaptic terminals can be observed in the ONL by electron microscopy (Fig. 6A–D). Thus, the anti-synaptophysin labeling in the ONL is indeed an indicator of retracted rod synaptic terminals, and not just indicative of a redistribution of the protein. Figure 6 shows the ultrastructure of the OPL and ONL in the reattached retina. Both rod and cone terminals are recognized in the OPL (Fig. 6A), and the structure of this layer usually appears to be relatively normal, although the layering of rod spherules is broader. The rod terminals observed in the ONL contain synaptic ribbons, vesicles, and postsynaptic processes (Fig. 6B–D). These ectopic synaptic terminals can stratify at all levels of the ONL; we show one at about its midpoint (Fig. 6B) and a more distal example lying next to a cone-cell body near the outer limiting membrane (Fig. 6D). Whether the postsynaptic processes originate from the same cell types as in the normal retina (e.g., rod bipolar and B-type horizontal-cell axons) or occur in a normal triadic configuration has not yet been determined. We also do not know whether these postsynaptic processes reattached to the terminals during the reattachment period, or if they actually remained "attached" to the rod terminals as they retracted.

Anti-cytochrome oxidase labels mitochondria in both rod and cone inner segments, and is believed to reflect information about the metabolic state of a cell (Fig. 5H–K). After 3 d of detachment, there is an obvious decrease in the labeling intensity of this region (Fig. 5I) compared to normal (Fig. 5H). After reattachment, the labeling pattern usually returns close to that observed in the normal retina (Fig. 5J).

Fig. 5. Double-label laser scanning confocal images showing changes in the distribution of several proteins in detached and reattached retinas. *Rod opsin* (**A–G**, red). In control retina, anti-rod opsin labels only the outer segments (OS) **(A).** At 3 d of detachment, shortened and disrupted OS continue to label, as does opsin delocalized to the plasma membrane of the cell body in the ONL **(B).** At 28 d of reattachment after a 3-d detachment, labeling, in most cases, is present only in the OS **(C)** but in some regions, rod OS recovery is less, and labeling persists in the ONL. In regions where opsin is delocalized, axon growth from rods is observed extending into the inner retina **(D–G, arrows).** *Glial fibrillary acidic protein, GFAP* (**A–D**, green). In control retinas, anti-GFAP labels intermediate filaments in Müller-cell endfeet and astrocyte processes in the ganglion-cell layer (GCL) **(A).** At 3 d of detachment, anti-GFAP labeling increases in Muller cells extending into the ONL **(B).** The reattached retinas show a similar pattern of anti-GFAP labeling **(C,D).** *SynaptophysinM* (**E–K**, green). In control retina, anti-synaptophysin labels rod and cone synaptic terminals in the outer plexiform layer (OPL) **(H).** At 3 d of detachment, some anti-synaptophysin labeling is observed in the ONL, leaving the OPL disorganized **(I).** Following reattachment, the synaptophysin labeling again appears to be localized to the OPL **(E–G,J)** except for scattered regions in which labeling still occurs in the ONL **(K).** Anti-synaptophysin labeling is also observed in rod axon terminals that have extended into the inner nuclear layer (INL) (**G**, arrow). *Cytochrome oxidase* (**H–K**, red). In control retina, anti-cytochrome oxidase labels mitochondria in rod and cone inner segments (IS) **(H).** At 3 d of detachment, the intensity of labeling decreases **(I).** Following reattachment **(J,K),** the labeling intensity is greater than in the detached retina **(I),** but often not as intense as in the normal controls **(H).** *Biotinylated peanut agglutinin (PNA)* (**L–O**, red). In control retina, PNA labels the extracellular matrix (ECM) associated with cone photoreceptors **(L).** At 3 d of detachment, labeling is greatly diminished and present only around the cone IS (**M**, arrows). Following reattachment, the cone matrix sheath labeling appears to be close to normal length in most retinal regions **(N),** but remains greatly diminished in some **(O).** *Cellular retinaldehyde-binding protein, CRALBP* (**L–O**, green). In control retina, anti-CRALBP labeling is present in the RPE monolayer and the fine RPE apical processes that extend to cone OS **(L).** Following reattachment, most regions appear to be close to normal, having reformed the RPE apical processes **(N),** although in regions where the cone matrix sheath remains degenerate, the RPE apical processes are also absent **(O).** In such regions, the cone matrix sheath and the RPE appear to merely abut one another. (**Note:** No RPE is present in the detached sections shown in **M.**) Scale bars = 20 μm.

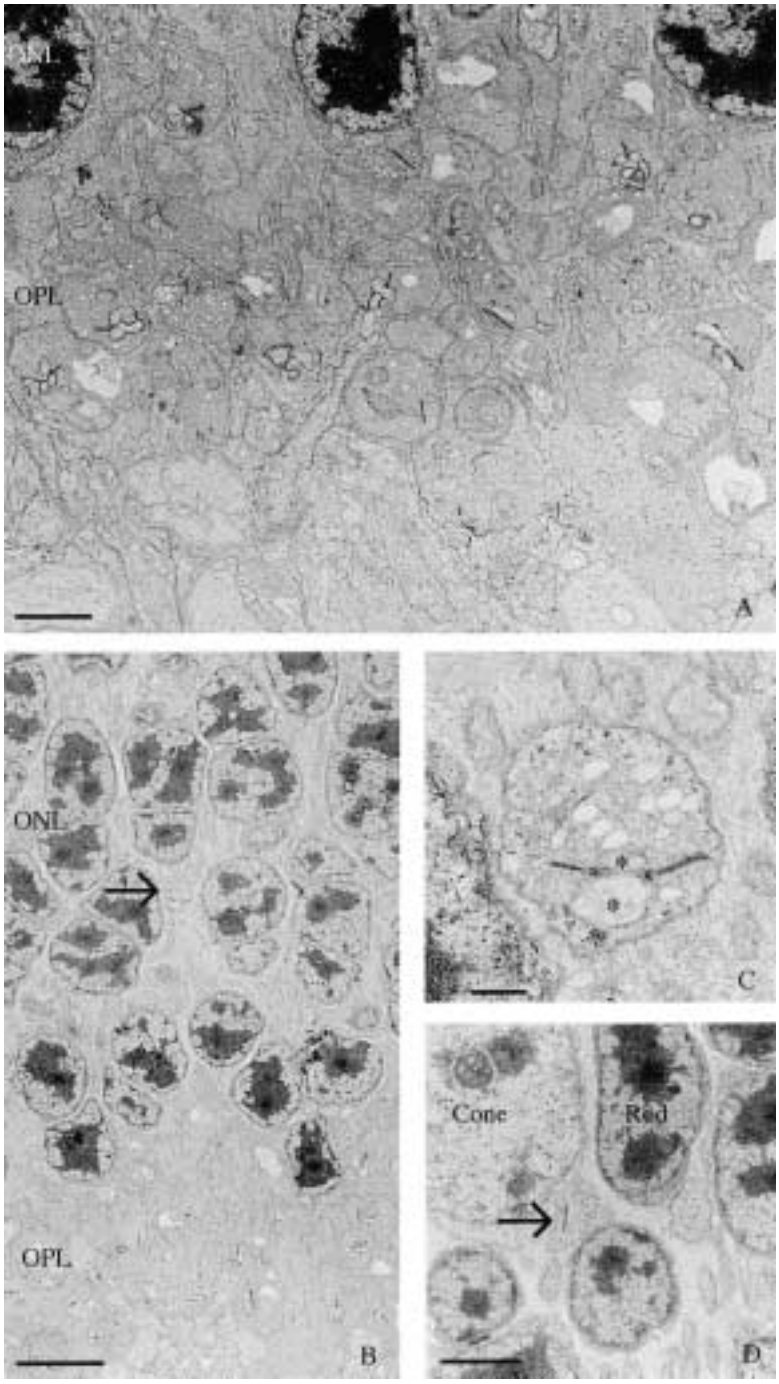


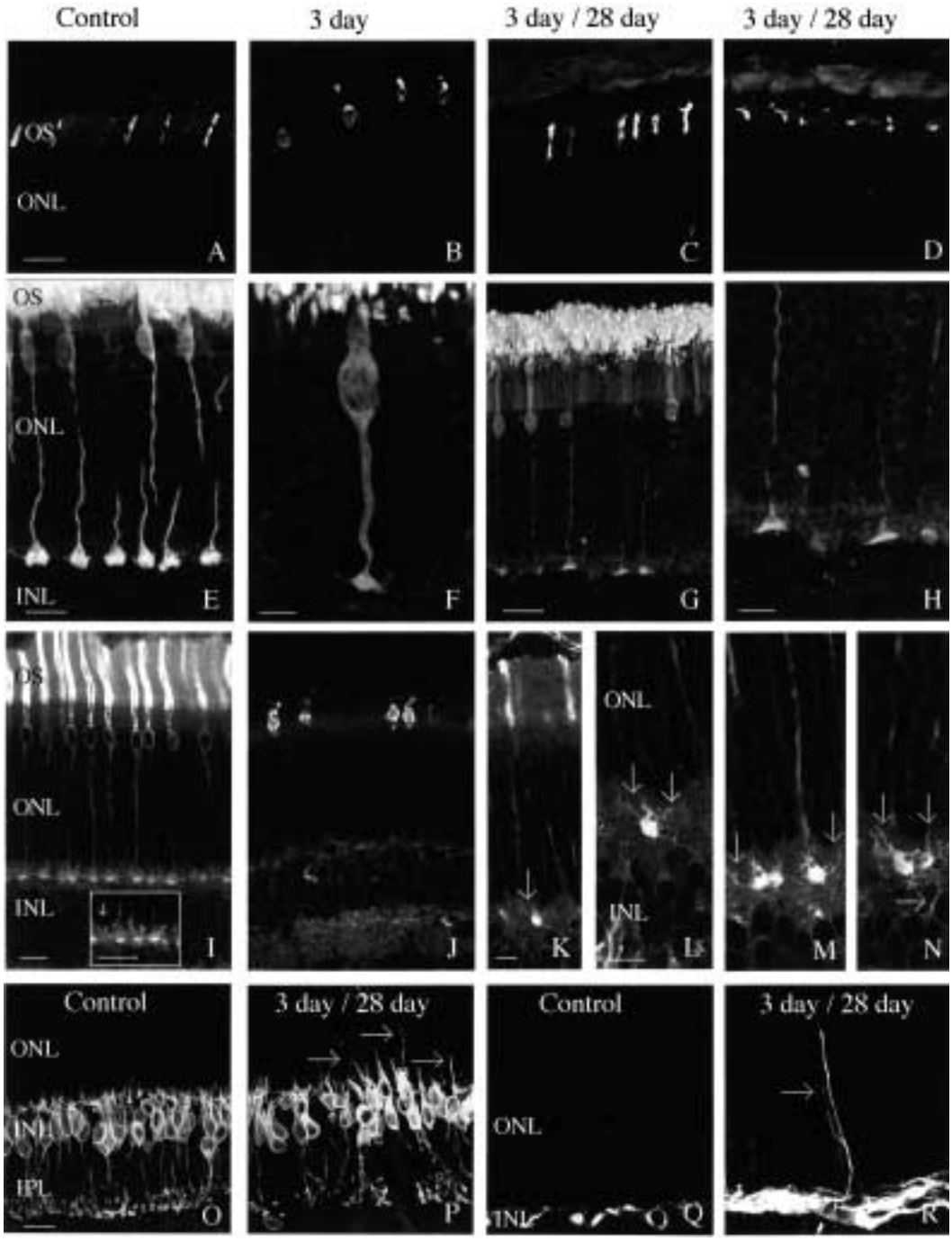
Fig. 6. Electron micrographs of the OPL and ONL in retinas reattached for 28 d following a 3-d detachment. Generally, the OPL is well-organized following reattachment **(A),** although in some regions, rod terminals can still be observed in the ONL **(B–D**, arrows). These terminals can exist at all levels of the ONL. The example in **B** (and at higher magnification in **C**) is nearer the OPL, and that in **D** is just beneath the outer limiting membrane. The terminals are recognized by the presence of synaptic ribbons **(B–D)** and associated synaptic vesicles **(C).** In the appropriate plane of section, postsynaptic processes appear within a synaptic invagination (**C**, asterisk). Scale bar = **A,** 2 μm; **B,** 5 μm; **C,** 0.5 μm; **D,** 2 μm.

Areas in which a lower intensity was maintained in the inner segments (Fig. 5K) appear to be associated with regions in which rod regeneration may not be complete, as evidenced by the synaptic terminals that are present in the ONL.

Cone photoreceptor outer segments respond similarly to those of rods. Indeed, "patchy" areas of poor recovery were observed both in the appearance of cone outer segments (Fig. 7A–D) and in the relationship of the cone matrix sheath to the RPE apical processes (Fig. 5L–O). In a normal cat retina, the cone outer segments are less than one-half the length of rod outer segments, and thus end about halfway across the interphotoreceptor space. Fine sheet-like anti-cellular retinaldehyde-binding protein (CRALBP)-labeled RPE processes reach each cone and envelop it in a complex sheath *(20,21),* and these extensions are enveloped by the cone matrix sheath (Fig. 5L). Following detachment, the complex RPE apical processes revert to a homogeneous fringe of microvilli, and of course, since the RPE and retina are physically separate, there is no further CRALBP labeling associated with cones. Patterns of peanut agglutinin (PNA) binding indicate that the matrix sheath is highly degenerate (Fig. 5M, arrows). Following reattachment, as cone outer segments regenerate, the matrix sheath once again elongates to form a relatively normal interaction with the anti-CRALBP-labeled RPE apical processes (Fig. 5N). In some areas of reattachment, however, the matrix sheath appears to abut the apical surface of the RPE with the fine, elongate processes missing (Fig. 5O). Since there is virtually no chance that any given cone will be reapposed to its original location on the RPE surface, the presence of an outer cone segment is likely to stimulate the RPE to regenerate the specialized apical processes that enwrap individual cones, although at 28 d, many of these appear to be less than perfect.

Cone-specific antibodies show patterns that suggest varying degrees of recovery of cone outer segments (Fig. 7A–D). In addition, there is no apparent difference between the recovery of the M and S cones (data for S cones not shown). Studies now in progress *(22)* should reveal whether or not there is a quantitative difference in the recovery of the population of these two cone types. A study of the cone-specific electroretinograms (ERGs) in the ground squirrel retina suggests that, in this species, there is not *(23).*

The responses of cone synaptic terminals were examined using an antibody to phosphodiesterase gamma (PDEγ; Fig. 7E–H) and biotinylated peanut agglutinin (PNA; Fig. 7I–N). Anti-PDEγ labels entire cone cells as well as outer rod segments in the feline retina. In the control retina, the cone pedicles appear dome-shaped as they expand from their axon, and then flatten to form the presynaptic surface (Fig. 7E). Fine, lightly labeled telodendria extend laterally from them. At 3 d of detachment, many of the cones no longer label with the antibody *(6),* but in the cones that still do, the terminals appear to have a more flattened, compressed morphology (Fig. 7F). At 28 d of reattachment, the cone terminals continue to resemble those found in the detached retina more closely than in the normal retina, even in areas where the outer segments appear normal (Fig. 7G,H). Biotinlyated PNA labeling outlines the entire cell, including the matrix sheath, cell body, axon, and basal portion of the cone pedicle (Fig. 7I). Fine, faintly labeled telodendria can also be visualized with this technique (Fig. 7I, inset, arrow). Following a 3 d detachment, labeling is still observed in the matrix associated with the outer cone segment, although it is highly disrupted (also shown in Fig. 5M), but the remainder of the cell, including the synaptic terminal, is no longer labeled (Fig. 7J). The labeling returns at 28 d of reattachment, but the appearance of the brightly labeled cone terminals (Fig. 7K–N) is very different from that in control retina. Long, seemingly disorganized telodendria projecting from the terminal are now observed (Fig. 7K–N, arrows). The data from the PDEγ and biotinylated PNA indicate that cones do not retract their synaptic terminals as rods do, but they do alter their morphology dramatically following detachment, and reattachment does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not restore them to normal. Whether their physical connection with second-order neurons or functional synaptic transmission with them has been affected is unknown.

Rod Bipolar and Horizontal Cells

In general, the second-order neurons studied thus far appear to have a relatively normal morphology after 1 mo of reattachment. Antibodies to protein kinase C (PKC) and neurofilament protein (70- and 200-kDa subunits) were utilized to show the fine neurites extending from rod bipolar and horizontal cells into the ONL after detachment (Fig. 7O,Q, normal retina only) (detachment data not shown; *see* refs. *9,10*). Following reattachment, these are far less frequently observed than in a 3-d detachment, and are only rarely found in the ONL (Fig. 7P,R, arrows). Interestingly, rod bipolar neurites appear to extend toward synaptic terminals in the ONL, while horizontal cell neurites often extend into the outer retina adjacent to reactive Müller cells (data not shown) *(2).*

Müller Cells

Retinal reattachment appears to halt the well-characterized increase in GFAP and vimentin in Müller cells initiated by detachment, although it does not cause an obvious reduction of these intermediate filament proteins. In the normal retina, anti-GFAP (Fig. 5A) and anti-vimentin (not shown) label the endfoot portion of Müller cells. (GFAP also labels astrocytes and horizontal cells; Fig. 5A). Following detachment, these two intermediate filament proteins increase in both amount and extent in the Müller cells, extending well into the ONL by 3 d (Fig. 5B). Following reattachment, this labeling pattern continues in Müller cells in all retinal areas that were sampled. However, the fact that these two proteins did not increase over the levels found in the 3-d detached retinas is a good indication of some specificity in this response (Fig. 5C,D). Although increases in intermediate filaments in Müller cells are associated with many forms of retinal degeneration *(24,25),* the response is poorly understood and is often considered to be a "generic" response on the part of these cells. Our experiment suggests a fairly tight control over

Fig. 7. Laser scanning confocal microscrope images showing changes in the distribution of several proteins in the detached and reattached retina. *M-cone opsin* **(A–D).** Anti-M cone opsin labels only M outer cone segments (OS) in the control retina **(A).** At 3 d of detachment, anti-M cone opsin labels the degenerate cone OS, and the opsin that is delocalized to the inner segment (IS) and cell body **(B).** Following reattachment, labeling shows that most M cones are near normal length, and opsin is not delocalized **(C)** although some regions still have shorter, more poorly organized cone OS **(D).** *Phosphodiesterase gamma (PDEγ)* **(E–H).** In control retina, anti-PDEγ labels rod and cone OS as well as cone-cell bodies, axons, and synaptic pedicles that appear dome-shaped **(E).** At 3 d of detachment, the most obvious change occurs in the cone pedicles, which now appear more flattened **(F).** Following reattachment, the terminals often retain this flattened morphology (**G, H; H = higher magnification of G**). *Biotinlyated peanut agglutinin, PNA* **(I–N).** In control retina, PNA labels the matrix around the entire cone from the OS to the synaptic terminal, where it forms a distinct "bar" along the base of the terminal **(I).** Fine telodendria that extend from the pedicles are also labeled (**I**, inset). At 3 d of detachment, PNA labeling of cones is lost except for truncated cone matrix sheaths **(J).** Following reattachment, the labeling of matrix around the entire cone cell returns, but the cone pedicles now appear to be much more brightly labeled, and long, fine telodendria can be observed extending in apparent random directions from the terminal (**K–N**, arrows). *Protein kinase C, PKC* **(O–P).** In control retina, anti-PKC labels rod bipolar cells **(O).** Following reattachment, the anti-PKC labeling appears, for the most part, similar to that observed in control retina (not shown) but occasionally, fine neurite outgrowths can be seen extending into the outer retina (**P**, arrows) similar to what is observed in the detached retina (not shown). *Neurofilaments* **(Q,R).** In control retina, anti-neurofilament protein labels horizontal cells in the INL **(Q).** Following reattachment, the labeling generally appears similar to that observed in control retina (not shown), but rarely, long neurite outgrowths can be observed extending from horizontal cells into the outer retina (**R**, arrow). Scale bar = 20 μm, A–D; 10 μm, E–R.

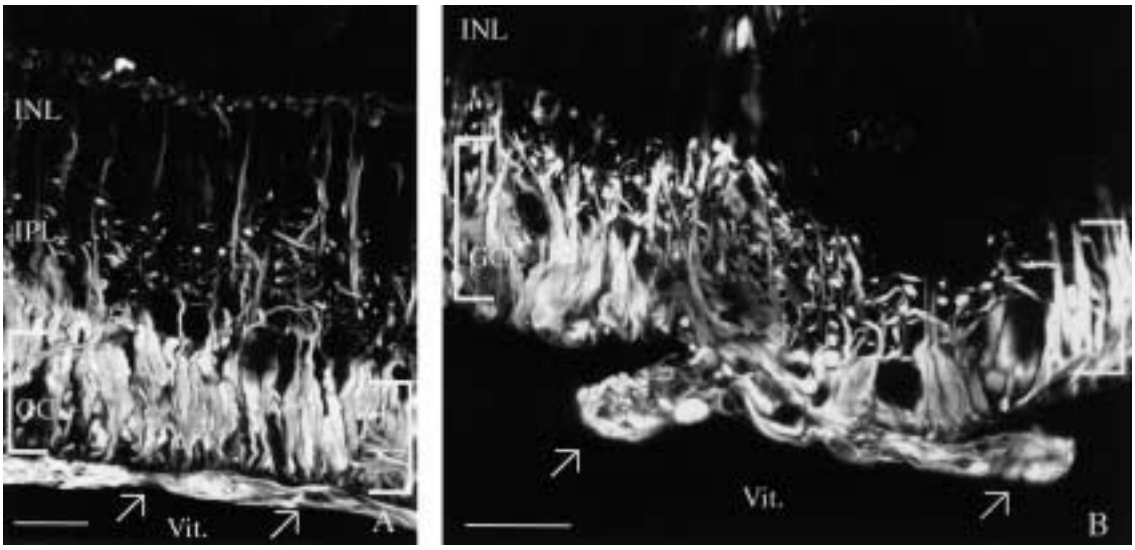

Fig. 8. Laser scanning confocal microscrope images of anti-GFAP labeling of epiretinal membranes that form in the vitreous cavity of retinas detached for 3 d and reattached for 28 d. Anti-GFAP labels intermediate filaments in Müller cell endfeet (**A,B**, brackets) and processes extending through the inner plexiform layer (IPL) and INL. Müller cell processes that extend along the vitreal surface of the retina—e.g. "epiretinal membranes"—are also labeled (**A,B**, arrows). These epiretinal membranes can be observed growing long distances on the surface of the retina **(A)**, or in the beginning stages of their formation, just extending from the ganglion-cell layer (GCL) through the inner limiting membrane into the vitreous cavity (Vit.) **(B)**. Scale bar = 20 μm.

the response. The fact that the intermediate filaments have not receded back to control levels at 28 d may reflect the stability of the proteins, but it may also suggest that they are functionally essential for reactive Müller cells. In detachments that are longer than 3 d, Müller cells routinely grow into the subretinal space, forming large glial scars (data not shown; *see* ref. *26*) and reattachment at d 3 effectively inhibits this process. However, reattachment appears to stimulate the growth of Müller cells onto the vitreal surface of the retina (Fig. 8A,B, arrows). In some cases, these "epiretinal membranes" can be quite expansive as they grow along the inner limiting membrane (Fig. 8A), and in other cases they are just beginning to form as Müller cell endfoot processes hypertrophy into the vitreous cavity (Fig. 8B). Vitreal epiretinal membranes have not been observed in simple feline detachments of 28 d in duration, but they have been observed in 20% of the reattached eyes.

Cytoplasmic proteins in Müller cells respond differently to detachment and reattachment than the structural proteins, GFAP, and vimentin. In the normal retina, antibodies to glutamine synthetase (GS), CRALBP, and carbonic anhydrase II (CA II) primarily label Müller cells, with the heaviest labeling occurring in the inner portion of the cell (Fig. 9A,D,G, respectively). As shown previously *(14),* Müller cells appear to downregulate the expression of these cytoplasmic proteins following detachment (Fig. 9B,E,H). Following reattachment, the intensity of the labeling does not return completely to control levels; all retinal regions show a consistent, light labeling of the Müller cells that appears to be closer to the intensity observed in detached retina than that observed in a normal retina (Fig. 9C,F,I). In the case of CA II, the Müller cell labeling was so light that it was not discernable from that associated with other cells in the inner retina (Fig.

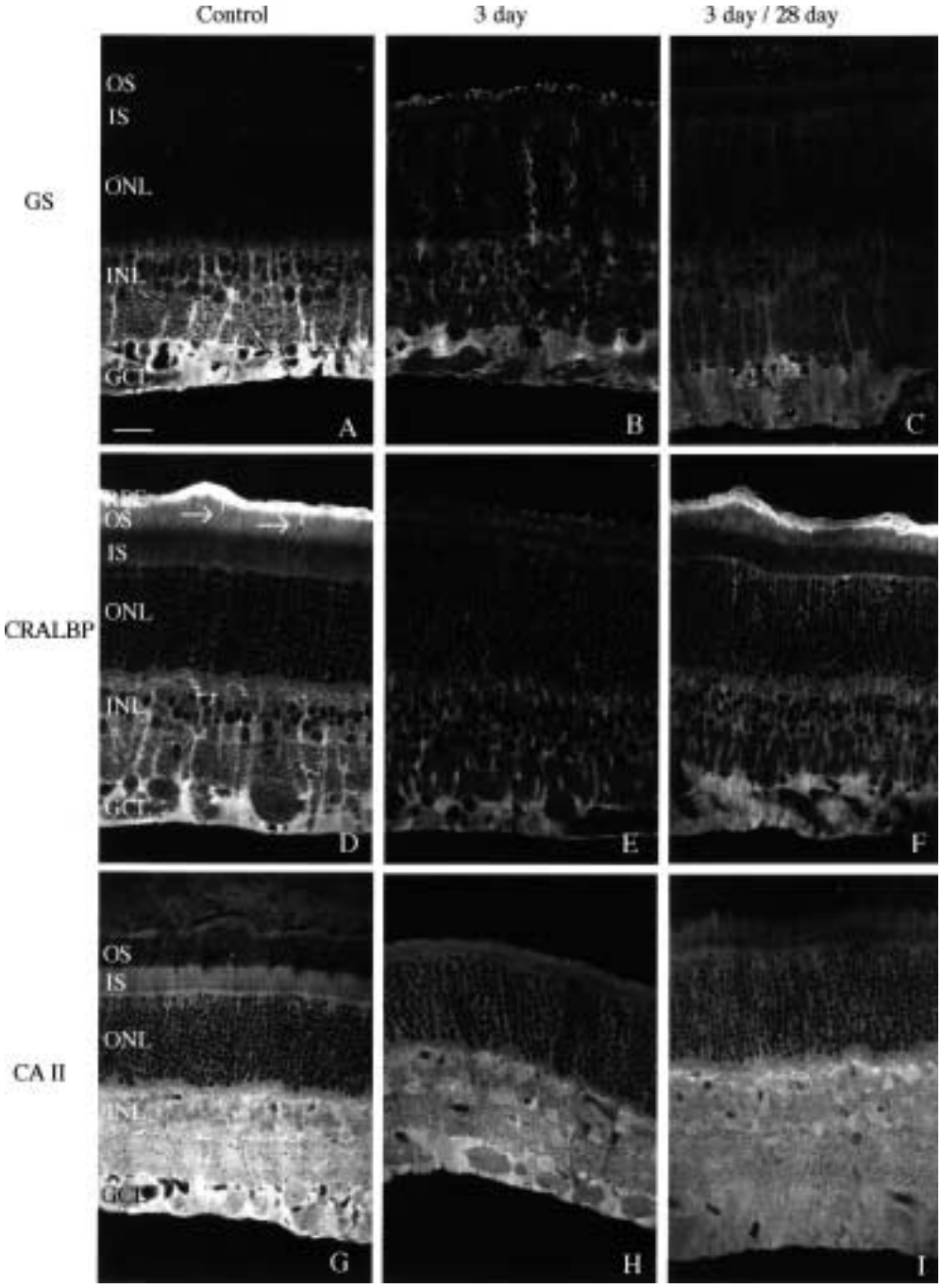

Fig. 9. Laser scanning confocal microscrope images showing the changes in cytoplasmic proteins in Müller cells following detachment and reattachment. Anti-glutamine synthetase (GS) **A–C**, anti-CRALBP **D–F**, anti-carbonic anhydrase II (CA II) **G–I**. Labeling in control retina with antibodies to all three proteins occurs primarily in the "inner" half of the Muller cells **(A,D,G)**. The overall labeling intensity decreases in retinas detached for 3 d **(B,E,H)**. In retinas detached for 3 d and reattached for 28, the labeling intensity and pattern has not returned to normal **(C,F,I)**. The intensity of anti-CRALBP labeling in the RPE following reattachment **(F)** is also less than observed in normal RPE **(D)**. OS, outer segments; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion-cell layer. Scale bar = 20 μm.

9I). The RPE labeling by anti-CRALBP also appeared to be somewhat diminished in the reattached retina (Fig. 9F) in comparison to normal retina (Fig. 9D), although this change was not as obvious as that in the Müller cells. In addition, the presence of RPE microvilli, brightly labeled by this antibody in the normal retina (Fig. 9D, arrows), were often not apparent in the reattached retina (Fig. 9F).

Non-Neuronal-Cell Proliferation

As shown in previous studies, retinal detachment induces proliferation of all non-neuronal retinal-cell types, including astrocytes, Müller cells, endothelial cells, pericytes, and microglia, with the greatest number of proliferating cells occurring on d 3 and 4 *(12,13).* In a retina detached for 1, 3, 7 or 28 d, we counted, respectively, an average of 0.65, 49.94, 16.78, and 1.93 cells/mm of retina labeled with the MIB-1 antibody used for detecting proliferating cells (Fig. 10). These cells consisted of Müller cells, astrocytes, endothelial cells, pericytes, and microglia. Twenty-eight days after reattachment on d 3, we counted 2.07 MIB-1-labeled cells/mm retina—a significant reduction compared to the 3- and 7-d detachments, but not markedly different from the 1- ($p = 0.08$) or 28- ($p = 0.44$) d detachments. Thus, there is a low level of cellular proliferation that continues in the reattached retina, a fact that may be significant to secondary diseases such as proliferative vitreoretinopathy (PVR). On the other hand, we do not know the exact fate of these proliferating cells, and their identification provides a challenge for future research.

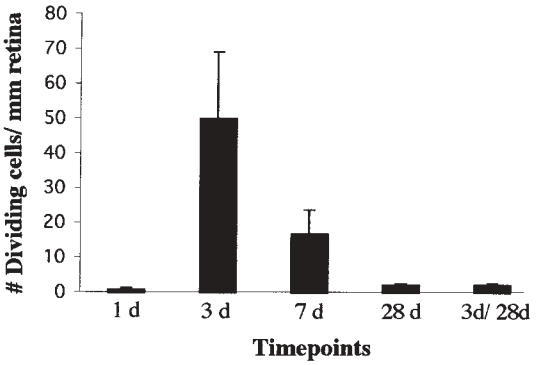

Fig. 10. The number of MIB-1-labeled cells (e.g., proliferating cells) per mm of retina at various periods of detachment (1, 3, 7, 28d) and reattachment (3 d detached, 28 d reattached) times. There is a low level of proliferation that continues in retinas detached for 3 d and reattached for 28 d (3d/28d).

Implications of the Changes That Occur After Reattachment

Three days of detachment are sufficient to induce significant changes in both neurons and glia in the retina, and 28 d of reattachment is sufficient for the retina to reverse many but not all of these changes. Because some of the changes induced by detachment are either permanent or seemingly very stable (photoreceptor-cell death, glial changes), and because some of the changes seem fairly profound in the context of CNS function (synaptic retraction) it may appear to be an over simplification to think of the retina as returning to its pre-detachment (e.g., "normal") status following reattachment. Instead, it appears that it must undergo a series of "re-remodeling" events, and that some of these lead to a new set of parameters such as rod axon extension and the growth of Müller cells into the vitreous cavity.

Most prior studies of reattachment have focused on outer segment regeneration *(18,27–29).* Over much of the reattached retinal area, the outer segments and their interface with the RPE were indistinguishable from a "normal" retina. However, there were abrupt transitions to distinct patches of retina in which the outer segments were short and the cone-ensheathing apical RPE processes were either short or absent. We currently have no explanation for these patches. Regeneration appears to lag behind in these areas but the cause remains unknown. We have not yet determined whether such areas eventually

will "catch up" to the rest of the retina. These patches are not associated with areas in which RPE proliferation or glial scars are inhibiting outer-segment regeneration *(30).* It is possible that reattachment is not uniform across a broad area, in effect creating micro-detachments. This suggests that there is some actual physical interaction between retinal cells and the RPE apical surface that is critical to initiating the full regenerative potential of each tissue. If this "patchiness" has any effect on visual recovery, presumably it would be most serious in the fovea, and would depend upon the size of the poorly regenerated areas. The effect that shorter than normal outer segments have on vision is also unknown. In the ground squirrel retina, M and S cone outer segments that have regenerated to only a small fraction of their normal length are able to generate an ERG that is nearly equivalent in size to control values (about 80% of pre-detachment amplitude) *(23).* This slight reduction in the cone-specific ERG correlates better with a loss of photoreceptor cells than outer segment length.

By immunocytochemistry, the shape of cone terminals did not appear normal 28 d after reattachment. Perhaps most importantly, the deep synaptic invaginations into cone pedicles, easily observed by confocal microscopy in the control retinas, were not observed in the reattached retinas. Whether this has an effect on the cone pathway is unknown, but it suggests that the cone photoreceptors have not returned completely to their pre-detachment state.

Perhaps equally important to the changes in photoreceptors are the sustained changes in the expression of proteins by Müller cells. Our impression is that the expression of all five proteins studied in Müller cells is "arrested" at the 3-d time-point. This is perhaps more surprising for the three soluble proteins than for the two intermediate filament proteins, considering their stability. Each of the soluble proteins presumably has some important role in maintaining the retinal environment, and the data may reflect the fact that this environment has not returned to the pre-detachment state. The arrest of GFAP and vimentin in the 3-d detachment pattern may be of functional significance. The expansion of Müller cell processes into the subretinal space occurs in processes filled with intermediate filaments. Studies using vimentin and/or GFAP-knockout mice suggest that intermediate filaments are essential for astrocyte hypertrophy and glial scar formation *(31).* Thus, preventing the rising expression of the intermediate filaments within these cells may effectively inhibit subretinal scar formation.

It has been reported that vision may take months or years to maximally recover following repair of macular detachments *(16),* suggesting that the morphological and biochemical changes such as those described here will continue for a long time. In addition, rod axon extensions into the inner retina and the formation of epiretinal membranes are two significant events we have observed only in reattached retinas. The significance of cellular "membranes" in the vitreous to diseases such as PVR is well-documented, yet the significance of the rod axon extension is unknown. Studies have not yet established whether there is a regression of these axons back to the OPL or if their cells of origin eventually die. Data from human retinas with diseases such as retinitis pigmentosa (RP), however, would suggest that the elongated rod axons persist for a long period of time *(32).* It seems unlikely that the epiretinal membranes will regress with longer reattachment times, and more likely that they will grow until severe PVR may cause the retina to again detach from the RPE. The remodeling of both neurons and glia described in the animal models will be most relevant if they also occur in humans. Observations of retinectomy tissue samples taken from "complex" human detachments suggest that most are indeed part of the repertoire of responses that occur in human retinas *(15).* Outer-segment regeneration is likely to be only one part of the equation for recovery after reattachment. Re-establishing the RPE/photoreceptor interface, synaptic circuitry, and the full expression of Müller cell proteins such as GS, CAII, and CRALBP may all be significant elements of the recovery process.

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