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# Evidence that ganglion cells react to retinal detachment

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**Abstract**

Growth associated protein 43 (GAP 43) is involved in synapse formation and it is expressed in the retina in a very specific pattern. Although GAP 43 is downregulated at the time of synapse formation, it can be re-expressed following injury such as axotomy or ischemia. Because of this we sought to characterize the expression of GAP 43 after retinal detachment (RD). Immunoblot, immunocytochemical and quantitative polymerase chain reaction (QPCR) techniques were used to assess the level of GAP 43 expression after experimental RD. GAP 43 was localized to three sublaminae of the inner plexiform layer of the normal retina. GAP 43 became upregulated in a subset of retinal ganglion cells following at least 7 days of RD. By immunoblot GAP 43 could be detected by 3 days. QPCR shows the upregulation of GAP 43 message by 6 hr of detachment. To further characterize changes in ganglion cells, we used an antibody to neurofilament 70 and 200 kDa (NF) proteins. Anti-NF labels horizontal cells, ganglion cell dendrites in the inner plexiform layer, and ganglion cell axons (fasicles) in the normal retina. Following detachment it is upregulated in horizontal cells and ganglion cells. When detached retina was double labelled with anti-GAP 43 and anti-NF, some cells were labelled with both markers, while others labelled with only one. We have previously shown that second order neurons respond to detachment; here we show that third order neurons are responding as well. Cellular remodelling of this type in response to detachment may explain the slow recovery of vision that often occurs after reattachment, or those changes that are often assumed to be permanent.


*Keywords:* retinal detachment; growth associated protein 43; neurofilament; immunocytochemistry; immunoblot; quantitative PCR; ganglion cells; plasticity

## 1. Introduction

Evidence for the rearrangement of synaptic connections or the restructuring of dendritic arbors in the adult mammalian nervous system continues to increase, reflecting a change in the view that the nervous system is 'hard wired' during development. Growth associated protein 43 (GAP 43) is an intracellular membrane associated phosphoprotein that is expressed in neuronal growth cones during synaptogenesis (Skene, 1989; Benowitz and Perrone-Bizzozero, 1991a; Strittmatter et al., 1992). Following synapse formation, GAP 43 is downregulated (Benowitz and Perrone-Bizzozero, 1991b) but remains expressed in regions that are thought to retain some degree of plasticity such as pyramidal cells of the hippocampus and the molecular layer of the dentate gyrus (Benowitz et al., 1988; Skene, 1989; Gispen et al., 1991; Meberg and Routtenberg, 1991; Benowitz and Perrone-Bizzozero, 1991b; Kruger et al., 1993; Kapfhammer et al., 1994). Cells deficient for GAP 43 are capable of extending neurites; however, these processes are often abnormal (Baetge and Hammang, 1991; Aigner and Caroni, 1993; Aigner and Caroni, 1995). Non-neuronal cells expressing transgenic GAP 43 extend long fine filipodial processes (Zuber et al., 1989). GAP 43 knockout mice die early in the postnatal period and show a tangling of axons at the optic chiasm (Strittmatter et al., 1995) while transgenic mice overexpressing GAP 43 show enhanced sprouting of terminals in both the peripheral and central nervous system (Aigner et al., 1995). Therefore, GAP 43 is likely to be an important regulator of synaptogenesis during neurite outgrowth and development.

GAP 43 has a specific pattern of expression in the retina. Developmentally its expression begins in neurons shortly after they complete their migration at the time of optic axon elongation (De la Monte et al., 1989; Benowitz and

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Perrone-Bizzozero, 1991b). GAP 43 mRNA is expressed in retinal ganglion cells from E15 until P21 in the rat (Reh et al., 1993), and some inner nuclear layer (INL) cells also express GAP 43 mRNA (Reh et al., 1993). GAP 43 protein is first detected in the optic nerve and optic fiber layer (i.e. ganglion cell axons) but later disappears between P8 and P16 in the rat (Kapfhammer et al., 1994). GAP 43 protein also appears in the inner plexiform layer (IPL) at P5 in the rat retina where it remains in the adult (Kapfhammer et al., 1994). Indeed, in several species of adult mammals (rat, rabbit, and human), GAP 43 immunoreactivity has been reported in sublaminae of both plexiform layers (McIntosh and Blazynski, 1991).

Although GAP 43 is downregulated following the time of synaptogenesis in the CNS, it can be re-expressed after various types of injury such as axotomy and ischemia. GAP 43 immunoreactivity increases following lesion of the rat hippocampus (Masliah et al., 1991), and in the affected areas of dorsal lateral geniculate nucleus after binocular central retinal lesions (Baekelandt et al., 1994). Placing the adult mouse retina in culture under serum free conditions can induce the expression of GAP 43 in retinal ganglion cells (Meyer et al., 1994), while optic nerve crush prior to culture induced an earlier increase in GAP 43 immunoreactivity (Meyer et al., 1994). GAP 43 immunoreactivity increases in the rat retina after optic nerve axotomy if the injury is within 3 mm of the eye (Doster et al., 1991), and acute increase of GAP 43 expression also occurs after cortical ischemia and reperfusion injury (Stroemer et al., 1993; Goto et al., 1994; Stroemer et al., 1995; Li et al., 1998).

Retinal detachment (RD) occurs when the neural retina is separated from the retinal pigmented epithelium (RPE) and in humans can cause significantly reduced visual capacity or even blindness if the fovea is involved. From examining model systems of RD several specific morphological and biochemical changes termed the 'retinopathy of detachment' have been identified (Mervin et al., 1999. These changes include neurite sprouting from rod bipolar and horizontal cells (Lewis et al., 1998) indicating a certain degree of plasticity in the adult mammalian retina. Based on this study we can add changes in GAP 43 and neurofilament protein expression to the list of molecular changes that occur after RD. In this case it seems reasonably likely that the changes are mechanistically related to structural changes in ganglion cells as well as horizontal cells.

## 2. Materials and methods

### 2.1. Retinal detachments

Cat retinas (right eye) were detached from the RPE for 1, 3, 7, or 28 days as described previously (Lewis et al., 1999). Briefly, the lens was removed by fragmentation and aspiration. Following removal of the vitreous a solution of 0·25% Healon (sodium hyaluronate; Pharmacia, Piscataway, NJ, USA) in balanced salt solution (Alcon; Fort Worth, TX, USA) was infused between the retina and RPE using a micropipette. Three animals were used at each time-point for immunohistochemical staining on tissue sections. One animal was used at the 7 day time-point for immunohistochemical staining in a wholemount preparation. Two animals at each time-point were used for Western blotting and quantitative PCR. The contralateral retinas were used as normal controls. All procedures adhered to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### 2.2. Immunoblot

Immunoblots were performed to determine the expression level of GAP 43 and to test the specificity of mouse monoclonal anti-GAP 43 to feline retina (Chemicon; Temecula, CA, USA). Retinas for immunoblot analysis were homogenized at 4°C in buffer containing 65 mM NaCl, 2 mM $MgCl_2$, 5 mM Tris–acetate, 0·1 mM phenylmethylsulfonylfluoride, 10 mM EDTA, 0·5% Triton X-100, and 2·5% SDS. Protein concentrations were determined by the amido schwartz assay (Schaffner and Weissmann, 1973). Twenty micrograms of protein were loaded per lane on a 7·5% SDS-polyacrylamide gel. Gels were run at 130 V for approximately one and a half hour. Proteins were transferred to nitrocellulose overnight at 4°C. Immunoblots were blocked in 3% nonfat dry milk in Tris buffered saline (20 mM Tris, 150 mM NaCl) containing 0·05% Tween 20 (pH 8) for 2 hr at room temperature. Blots were probed with 2 $\mu g\ ml^{-1}$ (1:250) mouse monoclonal anti-GAP 43 for 2 hr at room temperature. Following incubation, blots were washed and then incubated with HRP conjugated sheep anti-mouse immunoglobulins (Amersham Life Sciences; Piscataway, NJ, USA) for 2 hr at room temperature. Primary and secondary antibodies were diluted in blocking solution. Following washes with tris buffered saline, antigens were visualized using SuperSignal West Femto (Pierce; Rockford, IL, USA) and imaged with a CCD camera (Ultra Violet Products; Upton, CA, USA).

### 2.3. Immunocytochemistry

Retinal samples were prepared for study by confocal microscopy as described previously (Matsumoto and Hale, 1993). Briefly, retinas were fixed in 4% paraformaldehyde in sodium cacodylate buffer (0·1N, pH 7·4). Tissue samples, approximately 2 mm square, were excised and embedded in 5% agarose in phosphate buffered saline (PBS). 100 μm thick sections were cut on a vibratome (Technical Products International; Polysciences, Warrington, PA, USA) and blocked overnight in normal donkey serum (NDS, 1:20) in PBS at 4°C with continuous mixing. On the following day, the NDS was removed and sections were incubated overnight at 4°C with the monoclonal anti-GAP 43 diluted to 2 $\mu g\ ml^{-1}$ (1:250) in PBS containing 0·5% bovine serum

albumin, 0·1% Triton X-100, and 0·1% sodium azide (PBTA). On the next day sections were rinsed and incubated with Cy 3 conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories; West Grove, PA, USA) diluted to 7·5 μg $ml^{-1}$ (1:200) in PBTA. The following day sections were rinsed in PBTA, mounted in 5% *n*-propylgallate in glycerol and imaged with a laser scanning confocal microscope (Bio-Rad 1024; Hercules, CA, USA). Addition of secondary antibody alone was used as a control.

Mouse monoclonal anti-neurofilament 70 and 200 kDa subunits (NF, 1:200; Biomedia Corporation; Foster City CA, USA) and rabbit polyclonal anti-glial fibrillary acidic protein (GFAP, 10·25 μg $ml^{-1}$; 1:400; DAKO; Carpinteria, CA, USA) was also used on retinal sections in a series of double-label experiments. The procedure is the same as described above, except that after the blocking serum, the retinal sections were incubated in a mixture of both primary antibodies. The following day, the primary antibodies were removed; rinses performed and then secondary antibodies were added (7·5 μg $ml^{-1}$ donkey anti-mouse conjugated to Cy2 and 7·5 μg $ml^{-1}$ donkey anti-rabbit conjugated to Cy3; Jackson ImmunoResearch Laboratories). Sections were mounted and imaged as above.

To label retinal wholemounts, pieces of retina approximately the size of one retinal quadrant were treated for immunocytochemistry in the same manner as the sectioned tissue. Double labelling of wholemounts was performed using both anti-GAP 43 and anti-NF. Since both of these antibodies are monoclonals, the NF antibody was biotinylated by Vector Laboratories (Burlingame, CA, USA) and sequential labelling was performed. The wholemount retina was blocked in NDS (1:20) for 24 hr at 4°C. Blocking solution was removed and replaced with 2 μg $ml^{-1}$ anti-GAP 43 in PBTA. The following day the wholemount was rinsed and then incubated in 7·5 μg $ml^{-1}$ donkey anti-mouse conjugated Cy2 overnight. After rinsing, the whole mount was incubated with biotin conjugated anti-neurofilament (1:100). Following rinses, the wholemount was incubated overnight with avidin conjugated Cy3 (7·5 μg $ml^{-1}$, 1:100; Jackson ImmunoResearch Laboratories). The wholemount was then rinsed, mounted in n-propylgallate, and imaged as above.

### 2.4. Cell counts and montage

Metamorph software (Universal Imaging Corporation; West Chester, PA, USA) was used to calculate soma size using images collected from an anti-GAP 43 labelled retinal wholemount on the confocal microscope. Images were thresholded based on fluorescence intensity. The cells of interest were highlighted by integrated morphometry analysis and subsequently visually confirmed from those identified by the Metamorph software. The number of anti-GAP 43 labelling cells was plotted against their soma size.

### 2.5. Quantitative PCR

#### 2.5.1. Isolation of RNA

Retinas for RNA isolation were homogenized in 4·0 M guanidinium thiocyanate containing 0·1 M Tris–Cl and 1% β-mercaptoethanol. The resulting homogenate was layered on a 5·7 M CsCl/10 mM EDTA (pH 7·5) cushion and centrifuged at 32 000 rpm for 24 hr. The pellets were rinsed with 80% ethanol, followed by a second rinse with 100% ethanol and then air-dried. The pellets were then resuspended in $dH_2O$ and absorbance readings at 260 nm were used to quantify the amount of RNA. The RNA was then DNAsed by taking 100 μg aliquots of RNA and adding 8 μl to DNAse Buffer (40 mM Tris–HCl, 10 mM NaCl, 6 mM MgCl2, and 2 mM DTT) containing 0·5 μl RNAsin (Promega; Madison, WI, USA) and 0·5 μl RNAse free DNAse (Promega; Madison, WI, USA). The DNAse reaction was incubated at 37°C for 1 hr and then chilled on ice. Once cool, the DNAsed RNA was isolated using a RNEasy QIAgen kit (QIAgen; Santa Clarita, CA, USA) following the RNA clean up protocol. Integrity of the RNA was assessed using EtBr staining of an RNA gel.

#### 2.5.2. First strand synthesis

Twenty-five nanograms of total retinal RNA per PCR reaction was used to make cDNA. Three microlitres containing 750 ng of RNA was added to 17 μl of a mixture containing: 1X First Strand Buffer (Gibco BRL; Rockville, MD, USA), 1 mM dithiothreitol, 0·5 mM each dATP, dGTP, dCTP, dUTP (Promega; Madison, WI, USA), and 5·8 μM random hexamers (Promega; Madison, WI, USA). This mixture was heated to 90°C for 1 min, chilled on ice and then 0·5 μl of each Superscript Reverse Transcriptase (Gibco BRL; Rockville, MD, USA) and RNAsin (Promega; Madison, WI, USA) were added. After 10 min at room temperature, the reactions were placed at 50°C for 50 min. After this time, the resulting cDNA was either used in PCR reactions or frozen at −20°C. Reverse transcriptase was omitted from some first strand synthesis reactions for no reverse transcriptase controls. RNA was omitted from first strand synthesis reactions for no template controls.

#### 2.5.3. Sequencing of feline GAP 43

The mRNA sequence for feline GAP 43 was obtained by designing primers to a pileup of the sequences for *Homo sapiens*, *Mus musculus*, *Serinus canaria*, and *Xenopus laevis* (accession numbers M16228, M25667, U75453, and X87582, respectively). The sequences of the primers used are listed in Table 1 and the GAP2F and GAP3R were used to amplify a portion of the feline GAP 43 sequence. cDNA prepared as above was used to set up 25 μl endpoint PCR reactions consisting of the following reagents: 1X PCR Buffer (Promega; Madison, WI, USA), 2·5 mM $MgCl_2$, 200 μM each dATP, dGTP, dCTP, dUTP (Promega; Madison, WI, USA), 0·5 units Platinum Taq DNA Polymerase (Gibco BRL; Rockville, MD, USA), and 20 nM

Table 1
Sequences of primers used in this study

| Primer | Sequence | Location of sequence in human |
|---|---|---|
| GAP2F | 5′ AGATGGTATCAAACCAGAAGATA AAGC 3′ | 157 |
| GAP2R | 5′ CTTGTTATGTGTCCACGGAAGC 3′ | 234 |
| GAP3R | 5′ TCGGCTTGTTTAGGCTCCTC 3′ | 597 |
| 18S F1 | 5′ GCCGCTAGAGGTGAAATTCTTG 3′ | 949 |
| 18S R1 | 5′ CATTCTTGGCAAATGCTTTCG 3′ | 1014 |

primers. PCR amplification was carried out using the following temperature profile: 2 min at 95°C, 30 cycles of 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C. Products were purified for sequencing using a QIAquick PCR Purification Kit (QIAgen; Santa Clarita, CA, USA). The size and quantity of PCR product was assessed using a 2% agarose gel with Lambda BstE II (New England Biolabs; Beverley, MA, USA) and 100 bp ladder (Gensura; San Diego, CA, USA) markers. The resulting purified cDNA was sequenced at the University of California Santa Barbara sequencing facility.

### 2.5. QPCR reactions

The cDNA, no template controls, and no reverse transcriptase controls were used to set up 25 μl real-time quantitative PCR (QPCR) reactions. QPCR reactions contained the same reagents as endpoint PCR reactions with the exception of also containing 1X SYBR® Green I nucleic acid gel stain (S-7563, Molecular Probes; Eugene, OR, USA). Primers used for QPCR were GAPF2, GAPR2, 18SF1, and 18SR1. QPCR amplification was carried out on the ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Biosystems; (Heid et al., 1996)) using the following temperature profile: 3 min at 95°C, 45 cycles of 15 sec at 95°C, 90 sec at 60°C, 45 sec at 77°C, and 45 sec at 81°C.

QPCR products were evaluated on a 3·5% MetaPhor® agarose (Biowhittaker Molecular Applications; Rockland, ME, USA) gel using a 25 bp MW ladder (Promega; Madison, WI, USA). Melting temperature analysis was performed on the QPCR products to choose a temperature for data collection.

### 2.7. Data analysis

Quantification of the PCR data was based upon determining the cycle number at which a threshold signal ($C_t$) was achieved during the exponential phase of the reaction. Because amplification was equal and near 100% for all amplicons, a difference in 1 cycle is approximately equal to a 2-fold difference in RNA concentration (Heid et al., 1996). An average $C_t$ value was determined for the quadruplicates of each QPCR reaction. The $C_t$ values were normalized to 18S by taking the average GAP 43 $C_t$ value and subtracting the average 18S $C_t$ value to generate a delta $C_t$ ($\Delta C_t$). To assess the difference between normal and detached GAP 43 cDNA, the average $\Delta C_t$ for the normal cDNA was subtracted from the average $\Delta C_t$ for the detached cDNA giving a delta delta $C_t$ value ($\Delta\Delta C_t$). A ratio of detached over normal GAP 43 cDNA was generated by raising 2 to the exponent $\Delta\Delta C_t$ or $2^{\Delta\Delta C_t}$. Data from three independent experiments were averaged to generate the graph in Fig. 8 Error bars represent one standard deviation from the mean.

## 3. Results

By immunoblot analysis the antibody to GAP 43 recognizes a single band at 65 kDa, which is absent from the negative control lane (Fig. 1). GAP 43 in some species generally has a molecular weight of about 43 kDa, but this has been shown to vary significantly with the percentage of gel used (Benowitz and Routtenberg, 1987). Also GAP 43 is subject to phosphorylation that alters its apparent molecular weight (Benowitz and Routtenberg, 1987). A very light band is present in the normal retina. The intensity of this band increases at 1 day of detachment and continues to increase with longer time periods of detachment. Equal loading of lanes on the immunoblot was confirmed by Ponceau S staining (data not shown).

On retinal sections, anti-GAP 43 labelling three sublaminae of the IPL in the normal feline retina (Fig. 2(A)). Following 7 or 28 days of detachment, anti-GAP 43 labelling increased in a subpopulation of cell bodies in the ganglion cell layer (Fig. 2(B)–(D)). These labelling cells

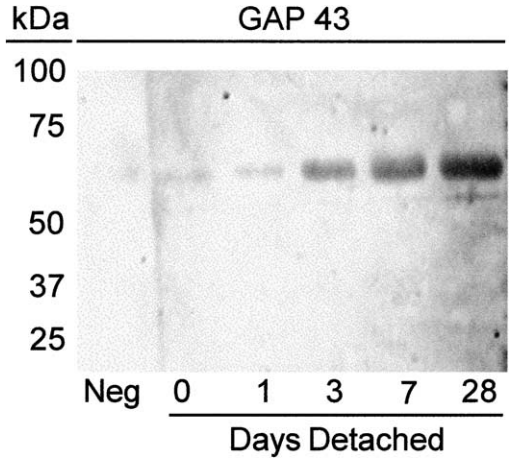


Fig. 1. An immunoblot of retinal homogenates at 1, 3, 7, or 28 days after detachment probed with antibody to growth associated protein 43 (GAP 43). Neg: negative control; 0: normal control retina homogenate. Anti-GAP 43 shows recognizes a species with an apparent molecular weight of 65 kDa whose abundance increases following detachment.

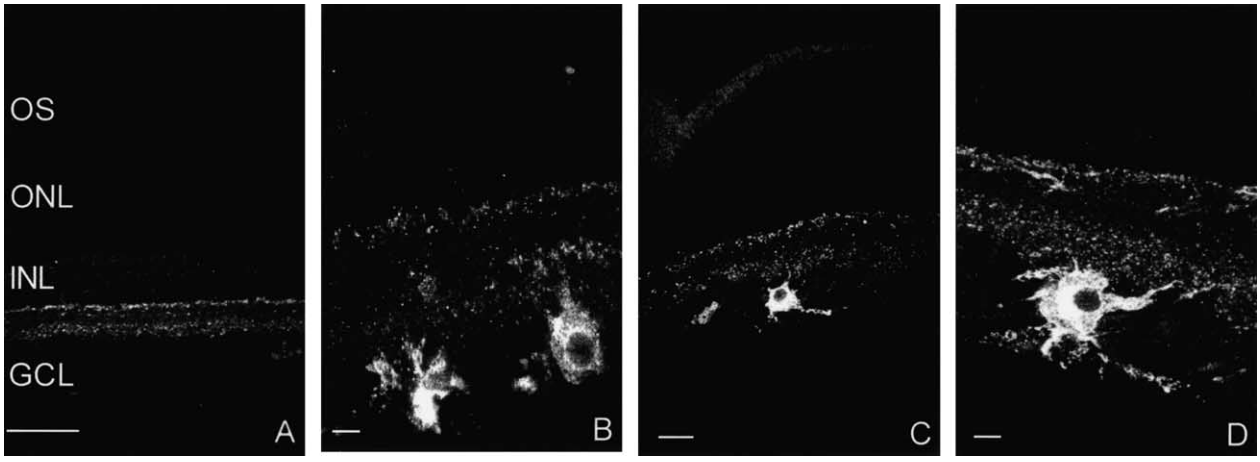


Fig. 2. Laser scanning confocal images of normal (A), 7 day detached (B), and 28 day detached ((C) and (D)) retinas labelling with anti-growth associated protein 43 (GAP 43). Anti-GAP 43 labelling three sublaminae of the inner plexiform layer in the normal retina. In the 7 and 28 day detached retinas it labelling some ganglion cells. (OS: outer segments; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer.) Bar = 25 μm.

were relatively infrequent in retinal sections therefore we used retinal wholemounts to determine if GAP 43 was expressed regionally in the retina or perhaps associated with the area around the small retinal hole created during the detachment surgery (i.e. an area in which optic axons may have been damaged). In retinal wholemounts the distribution of GAP 43 positive ganglion cells appeared to be random. That is, anti-GAP 43 labelling ganglion cells showed neither an association with the retinal hole made to create the detachment nor with any particular retinal region (Fig. 3(A)–(F)). In wholemount preparations ganglion cell axons appeared to have abnormally appearing short processes emerging from them (Fig. 3(A), (C), and (D)). Based on their appearance and cell body size anti-GAP 43 probably labelling one population of cells with a somal area of about 800 $\mu m^2$, although there is possibly a smaller group with a larger somal size around 1900 $\mu m^2$ (Fig. 4). These may be of the large or giant class of ganglion cells as defined by Kolb et al., 1981).

To further identify which class(es) of ganglion cells were responding to detachment by increasing their expression of GAP 43, sections of normal retina were stained with anti-NF (Figs. 5 and 6). In the normal retina, anti-NF labelling horizontal cells, processes in the IPL, fasicles of ganglion cell axons, and lightly labelling an occasional ganglion cell body (Figs. 5(A) and 6(A)). Following 3, 7, or 28 days of detachment, NF was upregulated in a subpopulation of cell bodies in the ganglion cell layer (Figs. 5(B) and (C) and 6(B)–(D); data not shown for 3 day detached retinas). Occasionally short wispy processes labelling with anti-NF could be seen extending from labelling cell bodies in the ganglion cell layer after RD (Fig. 6(C)–(D)). These did not appear on the lightly labelling cells of the ganglion cell layer observed prior to detachment (Fig. 6(A)).

Newly formed processes from horizontal cells could be seen occurring in outer retina (Fig. 5 (C) and (D)). Often, these anti-NF labelling horizontal cell processes associated with Müller cell processes labelling with anti-GFAP and could be seen to weave around in GFAP scars on the photoreceptor surface of the retina in longer periods of detachment (Fig. 5(D) insert).

When the retinal wholemounts were double labelling with anti-GAP 43 and anti-NF, it appeared that the majority of the ganglion cells positive for GAP 43 also labelling for NF, although there were definite examples of cells that labelling with only one of the antibodies (Fig. 7).

To corroborate the data for GAP 43 protein, we analyzed the abundance of GAP 43 mRNA by QPCR. Since the sequence of the feline GAP 43 was not known, we designed primers to a consensus sequence of GAP 43 using the mouse, canary, human, and xenopus sequences available. Using the primers GAP3R and GAP2F we then generated and sequenced a 452 bp fragment. This fragment (accession number: AF481882) shows an 88% identity to the human sequence. The GAP2F and GAP2R primers were used for QPCR to generate a product of 78 bp. This 78 bp product was not present in the no reverse transcriptase controls and showed a melting temperature of 84°C. Using this primer set and normalizing a detached/normal value to 18S products, a trend of increased synthesis of GAP 43 mRNA after RD was observed. After the initial elevation of GAP 43 message, detached/normal values returned to normal between 7 and 28 days of detachment (Fig. 8).

## 4. Discussion

We have previously reported significant changes in first and second order neurons in response to RD. Here we report changes in third order neurons extending the effects of detachment far beyond the original assumption that its effects were largely on photoreceptors. GAP 43, a developmentally regulated protein, is expressed at low levels in the normal retina, is upregulated noticeably by 3 days of

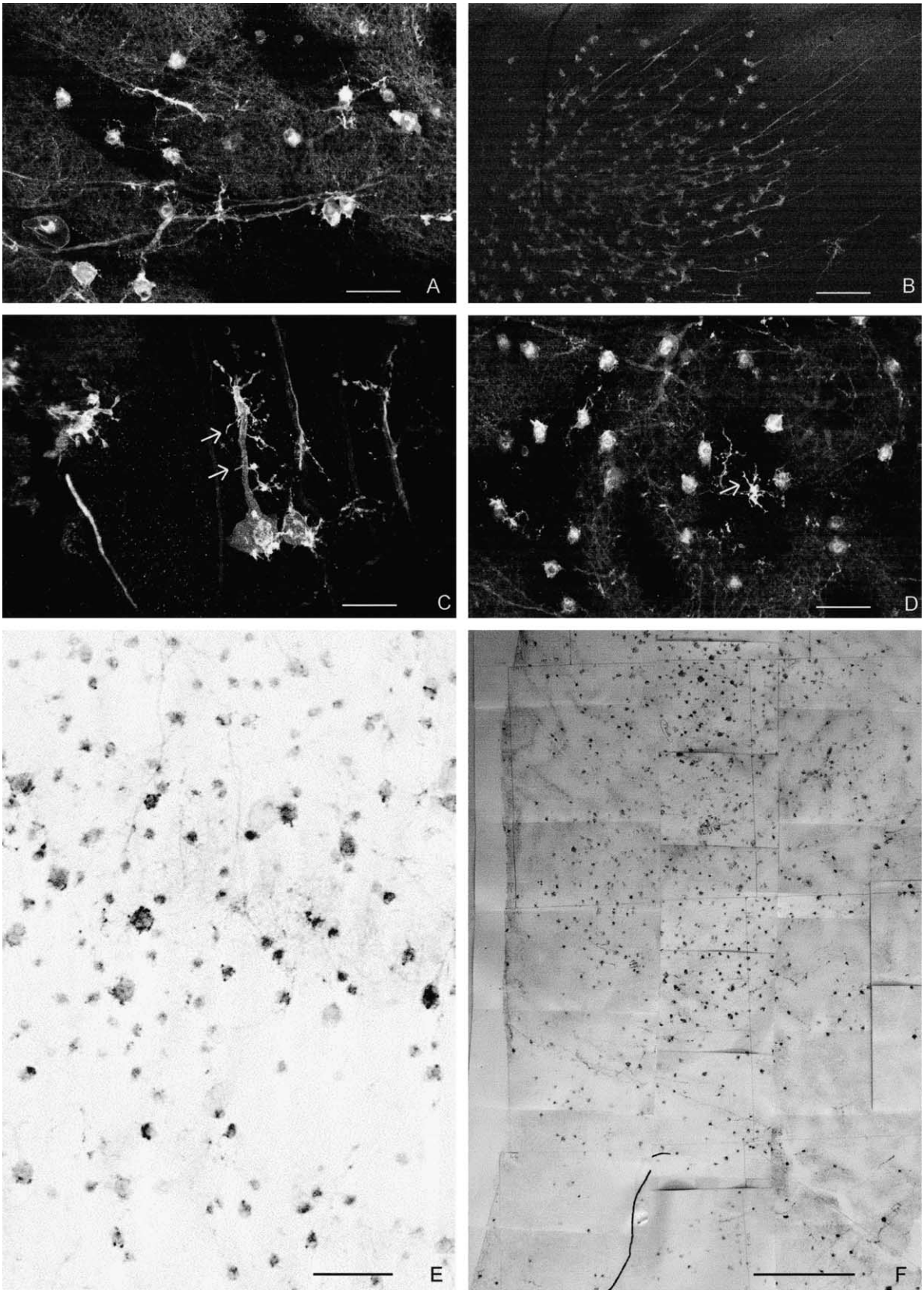


Fig. 3. Laser scanning confocal images from a 7 day detached retina labelling with anti-growth associated protein 43 (GAP 43). Images in (A)–(D) are examples from regions illustrating that these cells are distributed randomly. The images in (E) and (F) are inverted. (F) shows a larger area of the wholemount distant from the small hole made by the detachment surgery where labelling cells are still plentiful. Arrows show potential neurites growing from labelling axons. Bar = 25 μm, (A)–(E), 25 000 μm, (F).

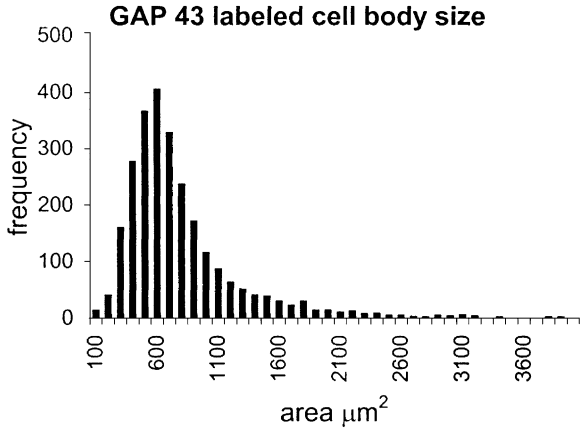


Fig. 4. Frequency histogram for somal areas of anti-GAP 43 labelling ganglion cells from the retinal wholemount made from a 7 day detachment. The GAP 43 labelling ganglion cells appear to be from a single population with an average somal area of 800 $\mu m^2$. There is a possibility of a second class of ganglion cells with a somal area of 1900 $\mu m^2$.

detachment, and is apparently still increasing at 28 days. With the GAP 43 antibody we get a staining pattern in normal retina that has been reported previously in other species (McIntosh and Blazynski, 1991; Kapfhammer et al., 1994). Data from the QPCR experiments indicate that the message for GAP 43 rises within 6 hr of detachment and returns to normal around 7 days (Fig. 8). Both the immunoblot and the immunocytochemistry show continued higher levels of GAP 43 protein in the 28 day detachments (Figs. 1 and 2). In fact, the large variation observed in the 28 day QPCR samples makes it possible that message levels actually do remain elevated in the latter time points (Fig. 8).

The upregulation of GAP 43 in ganglion cells is most likely a result of the detachment and not due to the other aspects of the intraocular surgery because controls in which only the lens and vitreous are removed showed no upregulation (data not shown). Furthermore, there did not appear to be any association of the increased immunostaining with the retinal hole made by the detachment pipet when it was examined in wholemount preparation, thus making it unlikely that the upregulation is due to injury of the ganglion cell axons.

Functionally GAP 43 is associated with axon growth and synaptogenesis as well as synaptic remodeling (Skene, 1989; Oestreicher et al., 1997). Thus, our results seem to have three major implications. First, they indicate that the ganglion cells, third order neurons in the retina, are responding to a pathological event associated with the RPE/photoreceptor interface. Second, they show that the changes in ganglion cells involve a molecule associated with plastic changes elsewhere in the CNS (Oestreicher et al., 1997). And third the change appears to be limited to a subset of ganglion cells, for the most part the same cells showing an upregulation of NF. We have previously shown significant structural remodeling of rod terminals, rod bipolar dendrites, horizontal cell processes, and Müller cells after detachment (Lewis et al., 1995; Lewis et al., 1998). Although we can't rule out trans-synaptic signaling of some type as the mechanism underlying the neuronal changes, it seems more likely that they are in response to either the release of some diffusible factor that stimulates these cells or repression of some factor that holds them in check. Indeed, the injection of bFGF (FGF2) into the vitreous induces many of the same plastic changes in Müller cells as detachment (Lewis et al., 1992). Although bFGF does not appear to effect neuronal structure, neither has this been extensively studied nor its effect on the expression of molecules such as GAP 43.

Demonstrating clear structural changes in the GAP 43 expressing ganglion cells has proven difficult because

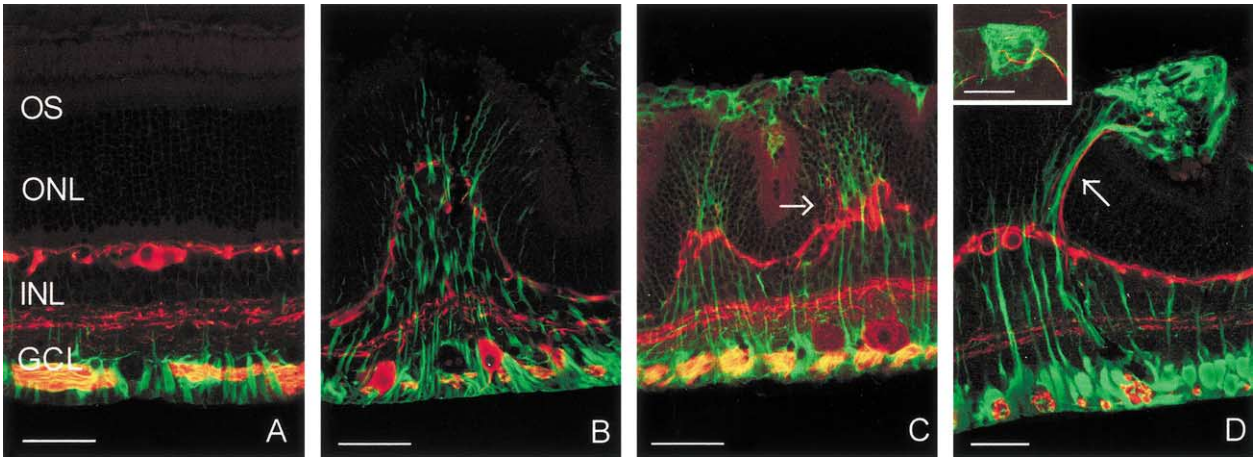


Fig. 5. Laser scanning confocal images showing labelling of anti-neurofilament (NF; red) and anti-glial fibrillary acidic protein (GFAP; green) from normal (A), 7 day detached (B) and (C), and 28 day (D) detached retinas. In the normal retina, anti-NF labelling filaments in horizontal cells, processes in the IPL and in ganglion cell axons; anti-GFAP labelling intermediate filaments in Müller cell end feet. In detached retina, anti-GFAP labelling increased in Müller cells and anti-NF labelling increased in some ganglion cell bodies and horizontal cell processes. These horizontal cells processes many times appeared to associate with Müller cell scars (arrows, (C) and (D)). (OS: outer segments; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer.) Bar = 25 μm.

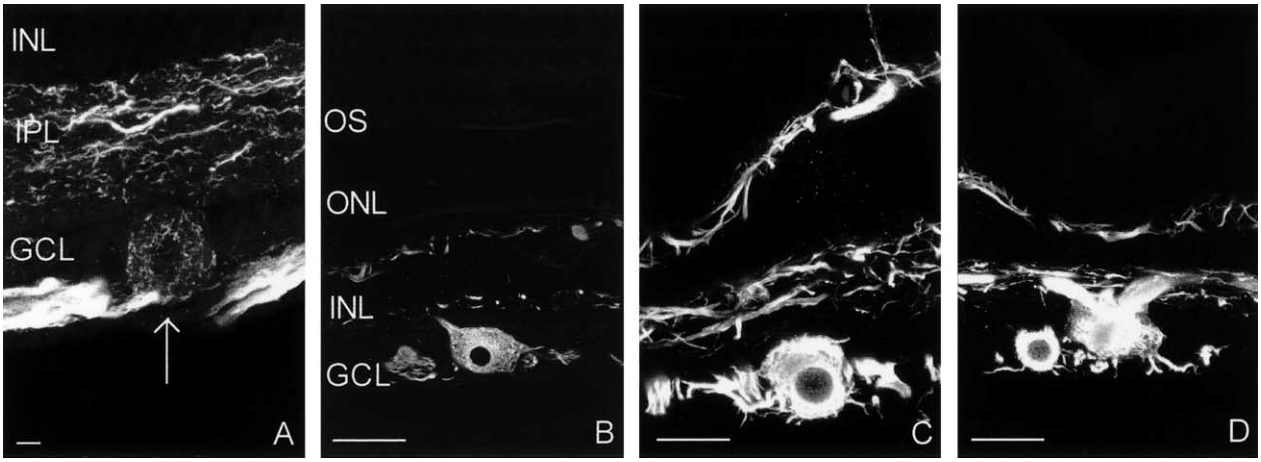


Fig. 6. Laser scanning confocal images of labelling with anti-neurofilament (NF) from normal (A), and 7 day detached (B)–(D) retinas. Labelling ganglion cells in the normal retina were rare and labelling was weak. The gain had to be significantly increased in order to acquire the image in (A). In the detached retinas anti-NF labelling was much more intense in ganglion cells (B–D) and sometimes these cells showed the presence of unusual processes that appeared to be growing from their cell bodies (C) and (D). (OS: outer segments; ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer.) Bar = 25 μm.

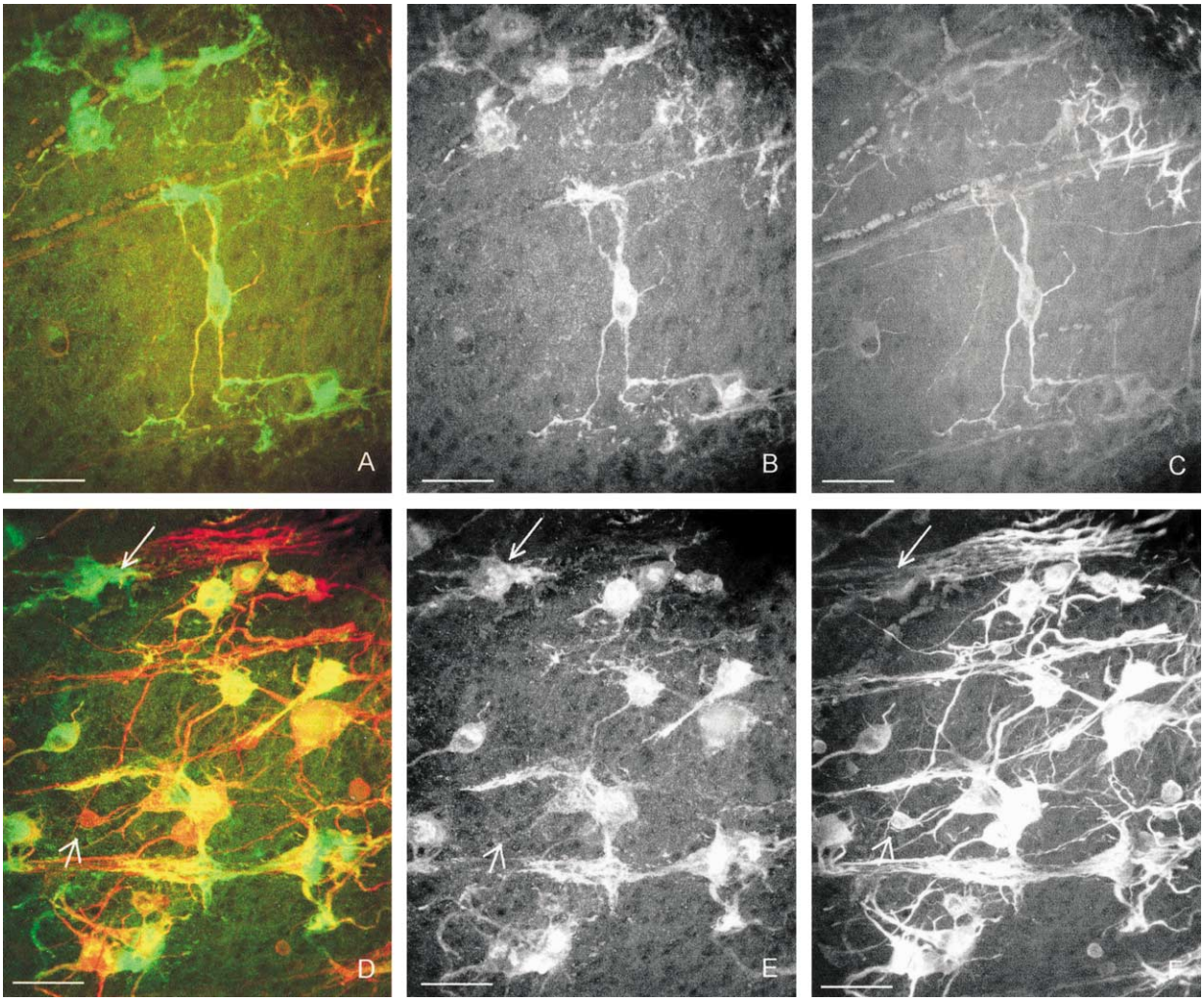


Fig. 7. Seven day detached retinal wholemount double labelling with anti-growth associated protein 43 (GAP 43; green) and anti-neurofilament (NF; red). While some cells labelling with both markers, others labelling with only one. Arrows indicate cells that only labelling with one of the two markers. Bar = 25 μm.

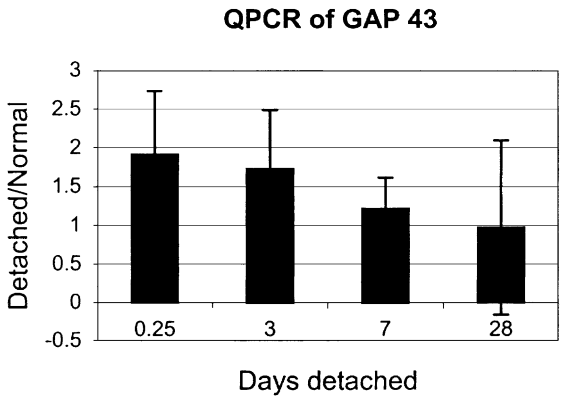


Fig. 8. Quantitative PCR results of the relative values for detached over normal. GAP 43 mRNA increased at 0·25 and 3 days and decreased to normal levels by 7 and 28 days. Error bars are 1 standard deviation.

the anti-GAP 43 does not label them in the normal retina. Therefore we have no good comparative data. However the morphology of cells labelling with the antibody to GAP 43 and that to NF is highly suggestive of structural changes. These cells have labelling processes coming off their basal surface, where dendrites do not normally arise. In addition, their labelling dendrites often appear 'wooly and tangled,' which is atypical of ganglion cell dendritic arbors (Fig. 6, Kolb et al., 1981). Also, the upregulation of NF in these cells is suggestive of structural remodeling since this cytoskeletal component is associated with structural stability. While the data on size distribution of the GAP 43 positive cells suggest they belong to either the large or giant class, we cannot really define them much more exactly (Kolb et al., 1981). Unfortunately our attempts to stain these cells in wholemount by the Golgi technique have so far failed.

Foveal detachments in humans, even those of short duration, can have lasting effects on visual function. Visual recovery can be slow, occurring over months and perhaps even years (Liem et al., 1994). While most effects of detachment have been explained by effects on photoreceptors, this and our earlier data suggests strongly that effects beyond the photoreceptors at the level of second and third order neurons, may play a role in some of the visual changes that occur. Indeed similar changes in second and third order neurons have been shown to occur in human retinas following detachment (Sethi et al., 2001). Plastic changes in neuronal connections that result in changes in the retinal circuitry, therefore, may explain the slow recovery time for certain visual functions after reattachment surgery.

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