Evidence that neurites in human epiretinal membranes express melanopsin, calretinin, rod opsin and neurofilament protein

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ABSTRACT

Aims We have previously identified neurofilament-protein-containing neurites in human epiretinal membranes (ERMs). The aim of this study was to further characterise these neurites by examining the expression of additional specific proteins in human ERMs and to correlate this expression with various retinal disease conditions.

Methods Epiretinal membranes originating from 43 patients with proliferative vitreoretinopathy (PVR), proliferative diabetic retinopathy (PDR) or with no known pathology (idiopathic epiretinal membrane; iERM) were removed during vitrectomy at varying durations after diagnosis and immediately placed in fixative. The membranes were labelled immunohistochemically with different combinations of antibodies to the proteins melanopsin, calretinin and neurofilament (to identify subclasses of ganglion cells), rod opsin (to identify rod photoreceptors), synaptophysin and synaptic vesicle glycoprotein 2A (SV2) (identifies synaptic vesicles) and vimentin (identifies glial cells).

Results Anti-melanopsin-, anti-calretinin-, anti-neurofilament- and anti-rod-opsin-labelled neurites were routinely observed in the epiretinal membranes. Their presence did not appear to correlate with a specific disease condition or duration of the membrane. Generally neurites were observed in regions of glial cells.

Conclusions Based on the expression of selected markers for neurites, we show neurite processes in human ERMs of various aetiologies originating from rod photoreceptors and different populations of retinal ganglion cells, although there was no obvious correlation with specific disease condition. In addition, synaptophysin and SV2 labelling was observed associated with all types of neurites, indicating the presence of at least one component necessary for synaptic transmission. Our data suggest that the adult human retina retains a significant capacity for neuronal remodelling under various disease conditions.

INTRODUCTION

The adult retina has long been considered a relatively static neuronal system with little potential for plasticity. However, it is becoming progressively clear that it can undergo remodelling in response to various injuries and diseases. The classical response includes glial cell proliferation, hypertrophy and migration, as well as neuronal changes including neurite pruning and, surprisingly, neurite sprouting. These events have been shown to occur in retinal degenerations and dystrophies,[1–6] diabetes,[7 8] retinal detachment (RD),[9–13] macular pucker,[14] macular degeneration,[15] retinitis pigmentosa (RP)[16–18] and in the aged retina.[19 20] The newly generated neurites appear to originate from every class of retinal neuron including rod and cone photoreceptors, and bipolar, amacrine and ganglion cells, and these neurites often grow throughout the retina (for reviews see Marc *et al* and Fisher *et al*[21 22]).

Interestingly, neurite growth does not always stop at the boundaries of the retina. Once subretinal or epiretinal membranes (ERMs) form, regardless of the disease condition, neurites are invariably observed.[10 12 14 22] ERMs have been shown to contain various cell types including glial cells (Müller cells and astrocytes), retinal pigment epithelial cells, fibroblasts and immune cells[23–25] However, neurites are always found associated with Müller cells, suggesting that these cells are permissive to the growth of neurite processes.[12 14]

Neurites have been found in ERMs removed from patients with the retinal conditions proliferative vitreoretinopathy (PVR), proliferative diabetic retinopathy (PDR) and RD[12] in addition to idiopathic epiretinal membrane (iERM) removed from eyes with no known retinal injury or disease.[14] To date, only anti-neurofilament-labelled neurites have been identified in human ERMs.[12 14] Since neurofilament protein can be expressed by various cell types the goal of this study was to determine more precisely the cells of origin of neurites present in ERMs and correlate their presence with disease condition, using markers for proteins in cell types that have been shown to sprout neurites within the retina. We also used markers for synaptic vesicle proteins to determine if the neurites contained the machinery for synaptic transmission.

We show here that there are distinct neurite populations in the membranes as evidenced by staining with antibodies to the photopigments melanopsin and rod opsin, as well as the calcium-binding protein calretinin. In addition, these neurites grow on a bed of glial cells and many contain the synaptic vesicle proteins synaptophysin or synaptic vesicle protein 2A (SV2).

MATERIALS AND METHODS

Human ERMs were collected during three-port vitrectomy surgery from 43 patients with different types of retinal pathologies including PVR (n=14), PDR (n=9) and iERM (n=17). Three ERMs were also from non-insulin-dependent diabetic patients without neovascular proliferation (NIDDM). One of the PDR membranes was large enough to be divided into four pieces and was used for four antibody combinations, and one was divided into two pieces for two antibody combinations. The

membranes were removed from the eyes at varying durations after diagnosis. The average durations were 5 weeks for PVR, 12 months for PDR, 8 months for iERM and 6 months for NIDDM. After removal, the membranes were immediately placed in fixative (4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4; Electron Microscopy Sciences, Fort Washington, Pennsylvania, USA) and stored at 4°C until used. The surgery was conducted at the Academic Medical Center, Amsterdam, The Netherlands.

The ERMs were processed whole, without embedding or sectioning. Following fixation the tissue samples were rinsed in phosphate buffer (PBS) and incubated in normal donkey serum (1:20) in PBS, 0.5% BSA, 0.1% Triton X-100 and 0.1% azide (PBTA) overnight at 4°C on a rotator. The following day various combinations of primary antibodies, in PBTA, were added overnight at 4°C on a rotator. The antibodies, and their concentrations and sources, are listed in table 1. Anti-SV2 was chosen in cases where the combination of primary antibodies did not allow the use of anti-synaptophysin, although both antibodies label synaptic vesicles in the neurite. Following rinsing of the primary antibodies in PBTA, the secondary antibodies conjugated to CY2, CY3 or CY5, were added together overnight at 4°C on a rotator (1:200 in PBTA). In two membranes the primary antibodies were omitted and only the secondary antibodies were added as negative controls. On the final day, the samples were rinsed in PBTA and a Hoechst nuclear stain was added at 1:5000 for 10 min. Without rinsing, the samples were then mounted on glass slides using 5% *n*-propyl galate in glycerol and viewed on an Olympus FluoView 500 laser scanning confocal microscope (New York, USA). Final images were collected as projections of five to 10 images taken at depths of 0.5 μm intervals.

RESULTS

All membranes showed the presence of neurites as identified by various combinations of antibodies to neurofilament protein, melanopsin, calretinin and rod opsin (table 2). Many of these proteins were double-labelled with antibodies to synaptophysin or SV2, both of which are normally present in synaptic vesicles.

No obvious correlation was observed with the specific antibody labelling of neurites and the disease condition or duration of the ERM before removal.

Anti-neurofilament staining was observed in all ERMs and in all disease conditions as reported previously (table 2).[12 14] The neurofilament labelling pattern appeared as long, thin processes, probably single fibres and thicker structures that most likely represented clusters of fibres (figures 1A,C–F), or as bulbous structures (figure 1A,C). Anti-synaptophysin labelling of vesicles was seen both in association with these neurofilament-labelled processes, as well as in areas without neurofilament labelling (figures 1A–D). Anti-neurofilament labelling was only observed in regions that contained anti-vimentin labelled glia cells (figures 1E) (see also Lewis *et al*[12])Anti-microtubule associated protein 2 (MAP2), a marker for dendrites,[26] labelled some neurites in all six ERMs where it was used, including PDR, PVR and iERMs. Many of the MAP2-labelled processes also labelled with anti-neurofilament (figures 1E–G).

Anti-calretinin labelling, typically observed in retinal ganglion cells, amacrine cells and horizontal cells,[27–29] was not as common as anti-neurofilament labelling, nor did it co-localise with neurofilament in the ERMs (data not shown). It was only observed in three of 11 ERMs including one iERM, one NIDDM and one PDR membrane (table 2; figure 2, green). The calretinin-labelled processes were always observed on a bed of anti-vimentin-labelled glia (figure 2, blue). Anti-SV2 labelling (figure 2, red) often co-localised with the anti-calretinin-labelled processes. In some cases, the vesicle labelling was only observed in specific regions of the calretinin-labelled processes (figure 2A, B). In other cases, it was observed throughout the processes appearing much more discrete than in the neurofilament-labelled neurites and often assuming a pattern that appeared vesicular or in association with 'beads' along the neurites (figures 2C–F).

Anti-melanopsin-labelled neurites were observed in six out of eight ERMs including iERM, PDR and PVR membranes (table 2; figure 3, green). Generally these processes appeared distinct from the anti-neurofilament-labelled processes (figure 3A). In a few cases, however, there was some faint labelling of melanopsin-positive neurites with anti-neurofilament (figure 3B,C; arrows). While anti-SV2 labelling was often observed in the vicinity of melanopsin-labelled processes, the two typically did not co-localise (figure 3D).

Anti-rod-opsin-labelled neurites were observed in three out of seven ERMs, including iERM, PVR and PDR membranes (table 2; figure 4). These rod neurites were distinct from those neurites labelled with anti-neurofilament (figure 4A,B).The rod-opsin-positive neurites generally co-localised with anti-synaptophysin labelling, which often appeared as small uniform vesicles or in densely labelled areas that could be resolved as sites of heavy accumulation of smaller vesicles (figure 4C-E). The anti-rod-opsin-labelled neurites were often, but not always, associated with anti-vimentin-labelled glia (eg, figure 4B).

Several of the membranes were double-labelled with both anti-vimentin and anti-glial fibrillary acidic protein (GFAP) and we observed co-localisation of the two proteins, indicating that the vimentin labelling represents glial cell labelling and not

Table 1 Antibodies used for the study along with their source and concentrations

Antibody	Species	Concentration	Source
Neurofilament protein	Mouse	1:500	Biomeda, Hayward, California, USA
Melanopsin	Rabbit	1:400	Affinity BioReagents, Golden, Colorado, USA
Calretinin	Rabbit	1:500	Chemicon, Temecula, California, USA
Rod opsin (rho 4D2)	Mouse	1:100	Robert Molday, University of British Columbia, Canada
Synaptophysin	Rabbit	1:100	Dako, Carpinteria, California, USA
Synaptic vesicle glycoprotein 2A (SV2)	Mouse	1:250	DSHB, Iowa City, Iowa, USA
Vimentin	Chicken	1:2000	Chemicon, Temecula, California, USA
Microtubule associated protein 2 (MAP2)	Mouse	1:100	Sigma, St Louis, Missouri, USA
Hoechst		1:5000	Invitrogen, Carlsbad, California, USA
Secondaries: donkey anti-CY-2, -3, -5		1:200	Jackson ImmunoResearch, West Grove, Pennsylvania, USA

Table 2 The antibody combinations used on different membrane types and whether the labelling was present (+) or absent (−)

Study	ERM type	NF	Calretinin	Melanopsin	Rod	Synapto physin or SV2	VIM	MAP2
Synaptophysin+NF	PVR5	+				+		
	PVR16	+				+		
	PDR1	+				+		
	PDR6	+				+		
	iERM2	+				+		
	iERM4	+				+		
Calretinin+NF	iERM16	+	−					
	iERM15	+	−					
	PVR17a	+	−					
	PVR12b	+	−					
	PVR9	+	−					
	PVR11	+	−					
Rod opsin+synaptophysin+VIM	iERM1				−	+	+	
	iERM8				−	+	+	
	PVR1				+	+	+	
	PDR1				+	+	+	
	PDR2				−	+	+	
	NIDDM1				−	+	+	
Calretinin+SV2+VIM	iERM2		−			−	+	
	iERM5		+			+	+	
	PVR2		−			−	+	
	PDR1		+			+	+	
	NIDDM2		+			+	+	
Melanopsin+NF+VIM	iERM3	+/−		+			+	
	iERM6	+/−		+			+	
	PVR3	+/−		+			+	
	Niddm3	+/−		−			+	
	PDR1	+/−		+			+	
Melanopsin+SV2+VIM	iERM7			+		+	+	
	PVR4			−		−	+	
	PDR1			+		−	+	
NF+synaptophysin	PDR2a	+				+		
	PDR5a	+				+		
	PDR7a	+				+		
	PVR2a	+				+		
	PVR4a	+				+		
	iERM8a	+				+		
	iERM9a	+				+		
	iERM17	+				+		
MAPII+NF+VIM	PVR12	+					+	+
	PVR21	+					+	+
	PDR2	+					+	+
	PDR7c	+					+	+
	iERM11	+					+	+
	iERM13	+					+	+
Rod opsin+VIM	iERM5				+		+	

ERM, epiretinal membrane; iERM, idiopathic epiretinal membrane; MAP2, microtubule associated protein 2; NIDDM, non-insulin dependent diabetic patients without neovascular proliferation; NF, neurofilament; PDR, proliferative diabetic retinopathy; PVR, proliferative vitreoretinopathy; Rod, rod opsin; SV2, synaptic vesicle glycoprotein 2A; VIM, vimentin.
+/−, positive but light staining.

dedifferentiated RPE cells (data not shown). Due to the restrictions of antibody combinations only vimentin was used to label the membranes in combination with the neuronal markers. Anti-vimentin labelling of glia was observed in all membranes where the antibody was used. As retinal astrocytes in general do not label with vimentin antibodies, and Müller cells label with both vimentin and GFAP antibodies,[30 31] it is assumed that the glia labelled in the membranes are primarily Müller cells.

DISCUSSION

ERMs can form in eyes with no known pathology, as well as in eyes with severe retinal disruption such as RD or diabetes. Our results show that regardless of the disease condition or length of time that the membrane is present in the eye, neurites are invariably observed in all membranes. Indeed, the interval between diagnosis and removal ranged from 5 weeks (PVR) to 8–12 months (iERM and PDR), and there was no apparent difference in the type of neurites or the extent of their growth. This suggests that neurite growth begins almost immediately upon formation of any ERM, following Müller cell processes (the preferred substrate for neurite growth) as they grow onto the retinal surface. The early appearance of neurites in these ERMs agrees with our studies of RD in animal models and in human ERMs, where neurite sprouting occurs rapidly.[9 12–14 32] In addition to neurofilament-labelled neurites, we show neurites that are positive for melanopsin, calretinin and rod opsin. Many of these neurites also show labelling for the synaptic vesicle markers, synaptophysin or SV2.

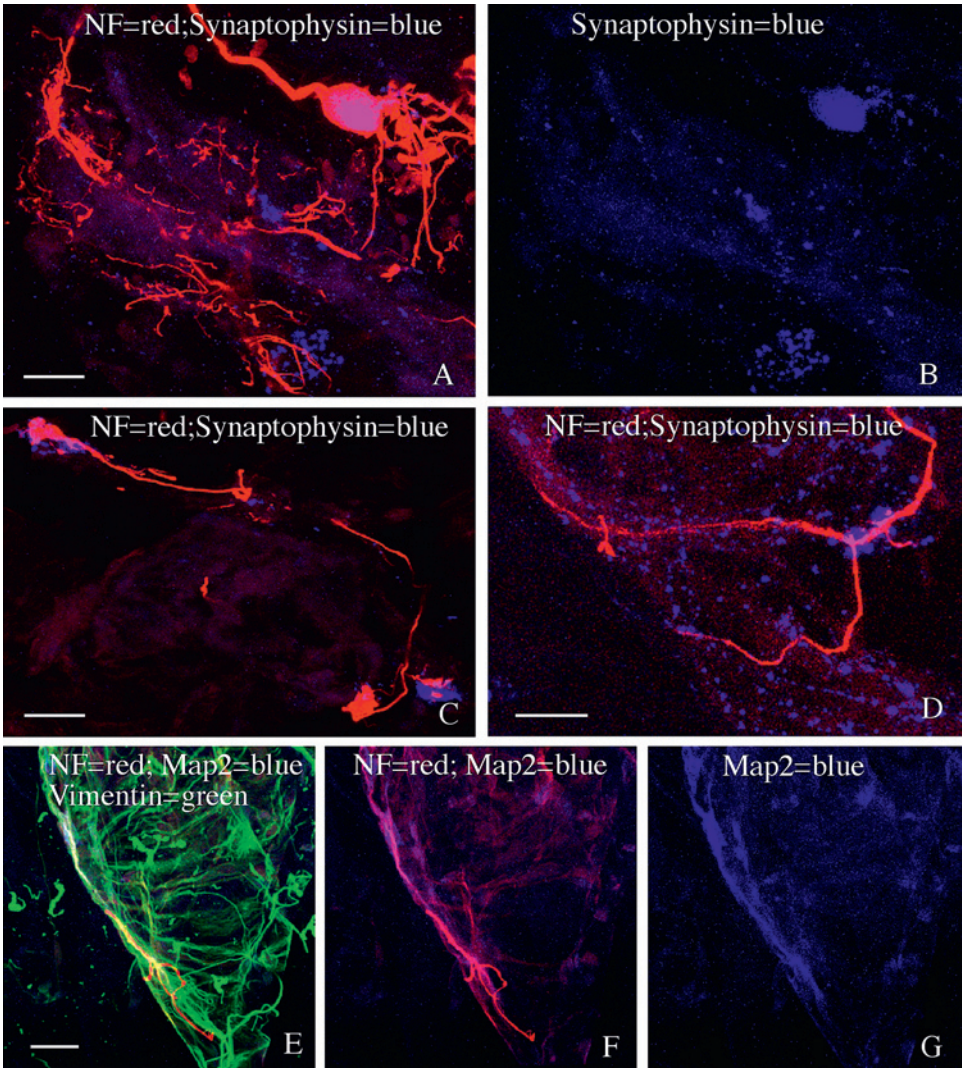

Figure 1 Confocal micrographs of epiretinal membranes labelled with anti-neurofilament protein (red; A—F), anti-synaptophysin (blue; A—D), anti-vimentin (green; E) and anti-microtubule associated protein 2 (MAP2) (blue; E—G). In some cases, anti-synaptophysin labelling appears to overlap with the neurofilament-labelled processes (A—C). The labelling that does not overlap with neurofilament presumably would be present in a different population of neurites. MAP2 labelling overlaps with some neurofilament-labelled processes (E—G). The close association between anti-neurofilament and anti-MAP2 labelled neurites with anti-vimentin-labelled glia produces the appearance of yellow (red + green) or white (red + green + blue) labelling of the long neurites in E (E and F are the same image with different colour combinations). B is the same as A with the red channel turned off. A—C and E—G are from idiopathic epiretinal membrane (iERMs); D is from proliferative diabetic retinopathy (PDR). Scale bars: A—C and E—G, 50 μm; D, 20 μm.

Neurofilament protein is a cytoskeletal protein that is expressed in the dendrites and axons of most ganglion cells in the human retina. The expression of this protein is upregulated rapidly in a subset of ganglion cells within a few days after experimental RD of the feline retina.[11 32] Within 1 week of RD in animal models, fine processes can be seen extending from the cell body and later growing out of the retina into ERMs. This appears to be a common phenomenon in human retina too, since neurofilament-labelled neurites were observed in 100% of the ERMs examined in both this study and in a previous one.[12] While it is not clear if these most closely resemble axons or dendrites, we did observe co-labelling with microtubule-associated protein 2 (MAP2) in some neurites, suggesting that these may form a heterogeneous mixture of processes that are dendritic and axon-like.

Calretinin is a calcium-binding protein found in the cytosol of amacrine and ganglion cells in the ganglion cell layer, and amacrine and horizontal cells in the inner nuclear layer in the human retina.[27—29] Importantly, the neurofilament protein antibody used in our study does not label horizontal or amacrine cells in the human retina.[10] In addition, calretinin did not label the same processes as the neurofilament antibody in the ERMs, again suggesting that the neurites arise from a heterogeneous collection of neurons within the retina. Calretinin-labelled processes were observed less frequently than neurofilament-labelled processes; since there is no evidence for neurites growing into ERMs from amacrine cells in other studies, these data, along with those detailed above, suggest that the origin of the anti-calretinin-labelled processes is most probably from a different sub-class of ganglion cell. Interestingly, the calretinin-labelled neurites were consistently co-labelled with SV2.

Melanopsin is a photopigment expressed by the intrinsically photosensitive retinal ganglion cell (ipRGC) as first described by Provencio *et al*[33 34] The ipRGCs form part of the retino—hypothalamic tract[35] and are involved in regulation of the circadian rhythm[36 37] and photoentrainment.[38] The ipRGC expresses melanopsin in both dendrites and axons.[39] In our study the frequency of ipRGC neurites in the ERMs was relatively high despite the fact that these ganglion cells form a small minority of all the ganglion cell population.[36 39] These neurites are clearly distinct from the neurofilament- and calretinin-labelled neurites. Although some of the melanopsin-labelled neurites did show faint co-localisation with neurofilament, these processes were obviously different from those labelled brightly with the neurofilament antibody. It is possible that these double-labelled neurites may be another subset of ipRGCs since two subpopulations of ipRGCs have been identified in mice.[40 41] Our study confirms that of Vugler *et al*,[6] using the Royal College of Surgeons (RCS) rat with retinal dystrophy, demonstrating that ipRGCs remodel during degeneration. Finally, while the ipRGCs have been shown to have synaptic vesicles at the junctions with amacrine and bipolar cells

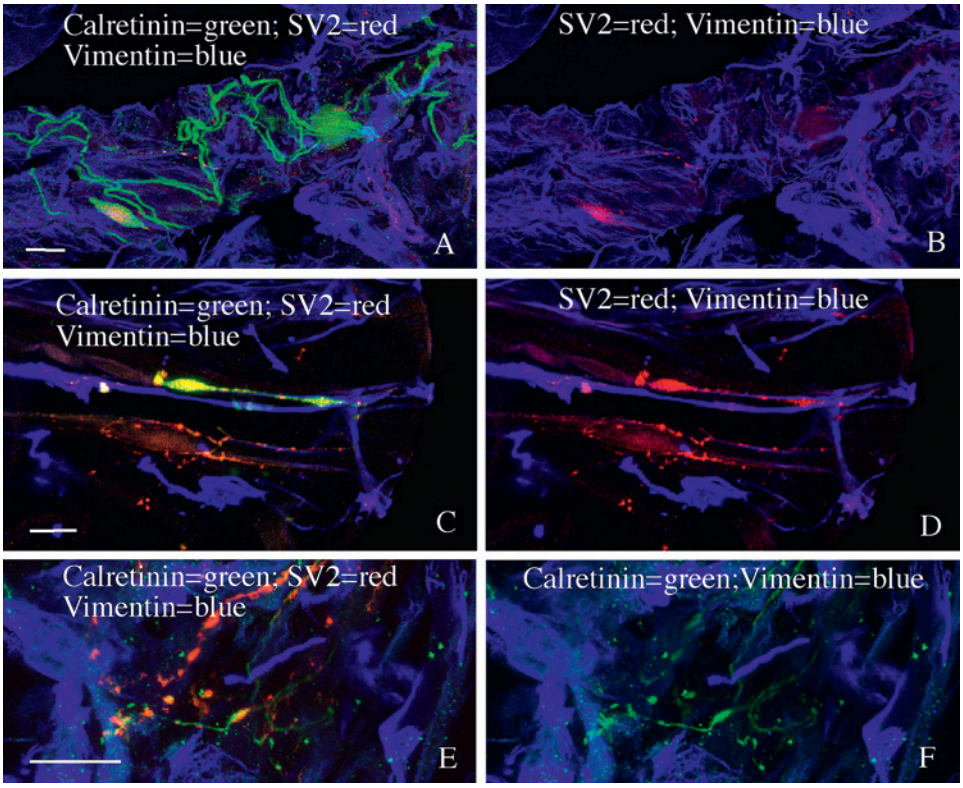

Figure 2 Confocal micrographs of epiretinal membranes labelled with anti-calretinin (green), anti- synaptic vesicle glycoprotein 2A (SV2) (red) and anti-vimentin (blue). Anti-calretinin-labelled processes can appear tortuous (A, B) or beaded (C—F), but invariably contain at least some anti-SV2 labelling. B and D are the same as A and C with the green channel turned off. F is the same as E with the red channel turned off. A and B are from proliferative diabetic retinopathy (PDR); C and D are from non-insulin-dependent diabetic patients without neovascular proliferation (NIDDM); E and F are from idiopathic epiretinal membranes (iERMs). Scale bars: A—D, 20 μm; E, F, 10 μm.

in the mouse retina,[42] they do not appear to label with anti-SV2 in the ERMs.

Rod neurite sprouting was first described in vivo in the disease RP in human donor retinas where long intra-retinal rod-opsin-positive neurites were found.[16] Since then, rod neurite sprouting has been described within the retinal layers in different forms of retinal dystrophy in animals models and humans.[2–4 13 18 21 22 43] These studies suggest that rod axons terminate within the inner retina as they do during development.[44] We show here that these neurites can continue their growth outside the retina into an

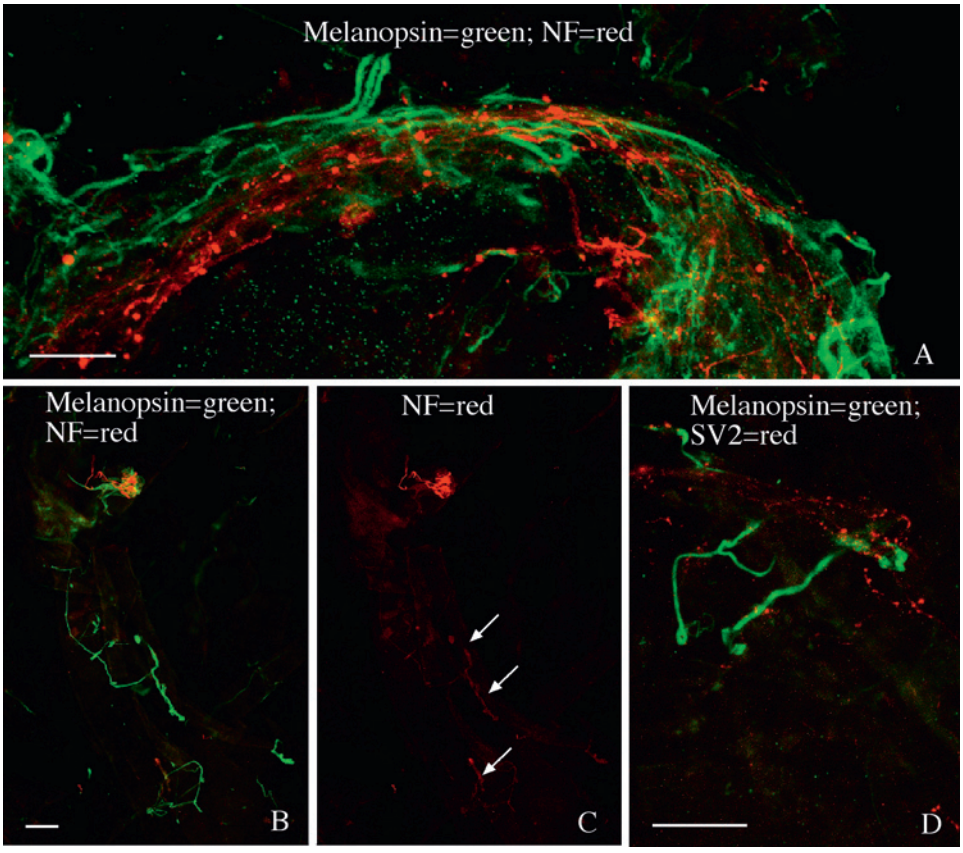

Figure 3 Confocal micrographs of epiretinal membranes labelled with anti-melanopsin (green; A—D), anti-neurofilament (red; A—C) and anti-synaptic vesicle glycoprotein 2A (SV2) (red; D). Melanopsin-positive processes appear to intermingle with neurofilament-positive processes (A—C), although faint neurofilament labelling can be observed in some melanopsin-labelled neurites when the green channel is removed (C; arrows). Anti-SV2 labelling appears in the vicinity of melanopsin-labelled processes although the two typically did not co-localise. A is from proliferative vitreoretinopathy (PVR); B—D are from idiopathic epiretinal membranes (iERMs). Scale bars: 50 μm.

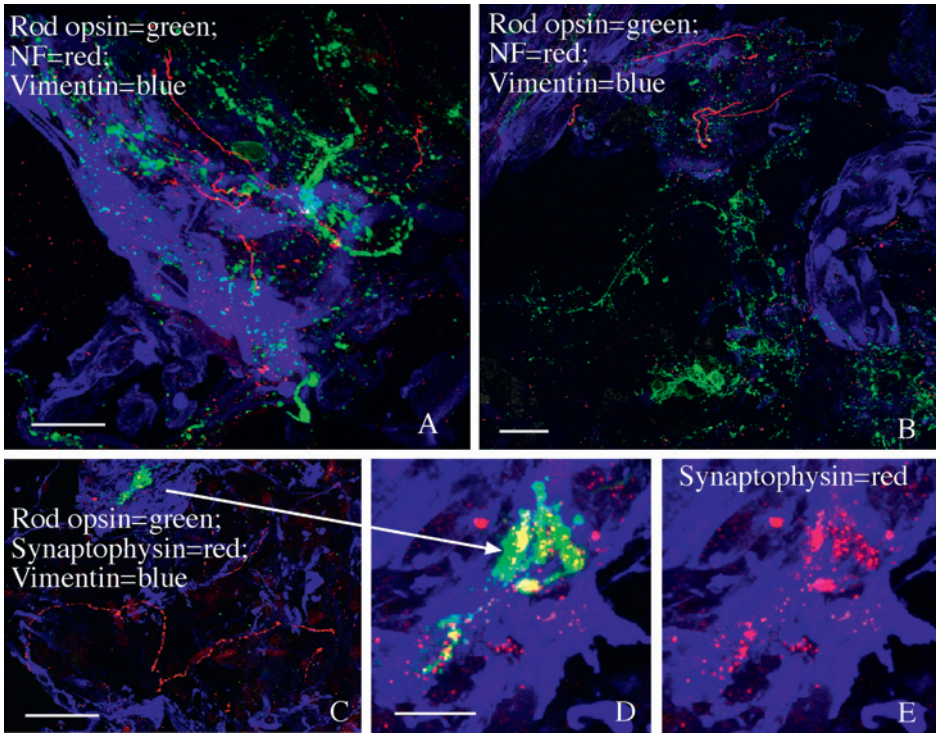

Figure 4 Confocal micrographs of epiretinal membranes labelled with anti-rod opsin (green; A—D), anti-neurofilament (red; A, B), anti-synaptophysin (red; C—E) and anti-vimentin (blue; A—E). Anti-rod opsin labelling does not overlap with neurofilament protein (A, B), but does overlap with anti-synaptophsyin labelling (C—E). Some synaptophysin labelling can be observed away from the rod opsin labelling indicating the presence of other neuronal cell types not labelled for in the present study. D is a higher magnification of C; E is the same as D with the green channel turned off. All images are from idiopathic epiretinal membranes (iERMs). Scale bars: A, B, 50 μm; C, 20 μm; D, E, 10 μm.

ERM. The fact that rod neurites grow the entire thickness of the retina and into iERMs suggests that there need not be significant damage to photoreceptors to stimulate growth. Moreover, since vision can often return to normal after iERM removal it would appear that the extraordinary growth of rod axons has minimal effect on vision. This may not be surprising since changes in small populations of rod photoreceptors probably would not affect typical scotopic visual function. Rod opsin labelling was not observed co-localised with neurofilament since these proteins do not overlap in the retina. However, we did observe synaptophysin to be co-localised to rod neurites, an observation congruent with the presence of synaptophysin labelling in elongated rod neurites within the retina in a feline model of RD,[43] the canine RP model and in human RP.[4 16–18] There is no evidence that these neurites form functional synaptic connections.

The presence of neurites is universal in ERMs, reinforcing the concept that significant disturbance of the retina is not necessary to induce the remodelling of both rod photoreceptors and ganglion cells. While it is not known what initiates neurite growth, it appears that molecules in the membranes, and in particular associated with Müller cells, may act as an attractant or stimulus for their outgrowth. Indeed, neurites in various pathologies have been shown to grow preferentially on a substrate of Müller cell glia, one of the main cellular components of ERMs.[11 12 14 45–49]

Although the specific protein(s) that instigates neural growth and creates the permissive environment is unknown, it has been shown that human idiopathic and diabetic ERMs express neural growth factors and receptors, such as nerve growth factor (NGF), basic fibroblast growth factor (bFGF) and glial cell line-derived growth factor (GDNF), and the receptors p75 neurotrophin receptor and tyrosine kinase receptors trkA, trkB and trkC,[50–53] which might be involved in neurite growth.

CONCLUSION

It is clear from this and other studies that adult retinal neurons retain the ability to remodel given the appropriate cues. Here we demonstrate the presence of neurites derived from rod photoreceptors as well as three sub-types of retinal ganglion cell in ERMs. This growth appears unrelated to the particular disease condition since neurites were observed in all conditions examined, irrespective of the disease being severely disruptive to the retina, as in PVR and PDR, or minimally disruptive as in iERM. Future studies are aimed at identifying factors that may be involved in initiating and guiding this neurite growth.

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Competing interests None to declare.

Patient consent Obtained.

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