iERM: neurofilament (red), GFAP (bl), sema 3a (gr)
Chapter 1
General Introduction
Epiretinal membranes (ERMs) are scar like sheets of cells and extracellular matrix that occur on the vitreal surface of the retina. The most common are idiopathic epiretinal membranes (iERM). However, ERMs also occur as a result of disease or trauma of the eye such as in proliferative vitreoretinopathy (PVR) in retinal detachment, proliferative diabetic retinopathy (PDR), and post-successful retinal detachment repair (ERMpRD). These membranes can become a serious problem when they become contractile and therefore visually threatening due to traction on the retina or by causing a traction retinal detachment. In all cases the membranes form from cells within the retina that begin to proliferate and migrate to the surface of the retina. It has been postulated that the formation of membranes in the eye is an aberrant healing response, with an initial proliferation phase after which the contraction phase occurs (Hiscott et al., 1985, Gilbert et al., 1988).

iERMs are composed of non-vascularised tissue growth along the inner limiting membrane (ILM) on the retinal surface. The prevalence of iERMs in the general population over 70 years of age is around 11.6% (McCarty et al., 2005). There is no underlying or preceding pathology in the ocular history associated with these membranes. However, the development of an idiopathic ERM is usually preceded by a posterior vitreous detachment (Foos, 1977). The iERMs can be asymptomatic, but may cause metamorphopsia, micro or macropsia of varying degrees. If they are symptomatic, surgical removal of the membrane by vitrectomy and peeling is an option.

PVR is a serious complication of retinal detachment (RD). It can occur either before surgery at presentation, or after surgical repair of the RD. It is a cellular proliferation on the retinal surface, causing contractile ERMs to form. PVR at presentation occurs in 5%-50% of RDs (Tseng et al., 2004). PVR is the most common cause of failure of RD surgery. The risk of occurrence is influenced by various factors such as duration of the RD and various intraocular factors (Kon et al., 2000).

Epiretinal membranes that occur after successful RD surgery are a recognised separate clinical entity. The ERMs that form post RD surgery (ERMpRD) occur in up to 6% of patients and behave differently to PVR membranes and more like iERMs (Uemura et al., 1992).

Proliferative diabetic retinopathy is potentially the most serious of the ocular complications due to diabetes. PDR can disrupt an already compromised macular function through traction or can cause a tractional retinal detachment. It often occurs late in the disease and is due to neovascular growth, development of neovascular epiretinal membranes, bleeding from the new vessels and vitreous contraction.

The most obvious cells involved in all types of epiretinal membrane formation are glia,
macrophages, RPE cells, and fibroblasts (Vinores et al., 1990; Heidenkummer and Kampik, 1992; Jerdan et al., 1989). Using light or electron microscopy, it has been shown that many cells seem to change morphological characteristics as the epiretinal membrane develops making it difficult to identify their origin (Kampik et al., 1981; Morino et al., 1990; Vinores et al., 1990). However, with the addition of immunocytochemistry, it has become easier to identify the different cell types.

Retinal pigment epithelial cells have not been found to be part of the cellular population in idiopathic ERMs, but glia, fibroblasts and immune cells are seen (Hiscott et al., 1984). Besides the cellular components ERMs have extracellular matrix components that have been identified to include actin, tenascin, fibronectin, laminin and collagen type I, III and IV in variable quantities (Sramek et al., 1989; Hiscott et al., 1990; Ioachim et al., 2005).

In this thesis the first 2 studies describe how ERM and ILM removal can be facilitated by the use of intraocular dyes.

Removal of ILM and ERMs requires surgical skill and experience. Non closure of macular holes and re-proliferation of macular puckers have both been related to inadequate membrane removal. To facilitate surgery and improve the surgical outcome, adjuncts to enhance membrane visibility have been sought. These have included the use of a slit beam illumination, triamcinolone (Furino et al., 2003), and stains such as indocyanine and infracyanine green (ICG), brilliant blue (Enaida et al., 2006a, 2006b), and trypan blue (Feron et al., 2002; Perrier and Sebag, 2003; Teba et al., 2003; Haritoglou et al., 2004).

ICG has been used for both epiretinal membrane and macular hole surgery. It stains the ILM intensely thereby facilitating surgery (Gandorfer et al., 2001a; Bainbridge et al., 2008). However, its use is not without risk. Several case reports and case series document adverse effects from a reduced visual recovery following surgery to the development of pigment alterations (Engelbrecht et al., 2002), and post operative vision loss (Haritoglou et al., 2001; Weinberger et al., 2001), particularly when retinal integrity is compromised (Gandorfer et al., 2001b). In vitro as well as in vivo toxicity has been demonstrated against the retinal pigment epithelium and retinal glia (Gandorfer et al., 2001a, 2001b; Weinberger et al., 2001; Engelbrecht et al., 2002; Gale et al., 2004; Haritoglou et al., 2004; Jackson et al., 2004; Kwok et al., 2004; Hirasawa et al., 2007). Due to the risks associated with the use of ICG, alternatives dyes have been sought to achieve the same goal, but with less potential toxicity.

Due to a superior safety profile as compared to ICG, membrane blue has been used by a number of European surgeons despite its lesser staining characteristics for the ILM (Teba et al., 2003; Michalewska et al., 2008; Hasler and Prunte, 2008).
The main issue with commercially available trypan blue is that it requires a fluid-air exchange to achieve adequate staining. However, this means a potentially difficult extra step in the surgery, with increased risk of retinal tears. We assessed the possibility of making trypan blue “heavy”, and being able to omit the fluid air exchange, yet to still have adequate staining of the ERM to peel safely. The studies in Chapter 2 and 3 describe an alternative method of staining the ILM and ERMs and the results of 2 clinical studies performed for ERM peeling and ILM peeling for macular holes.

With the aid of heavy trypan blue it was easier to visualise and collect ERMs and to further examine both the proliferation characteristics and ultrastructure immunohistochemically. We used the ERMs collected by this method for the further studies of ERMs in this thesis.

In order to better understand the origins of the proliferating cell types and their relationship to the 4 different disease conditions, PVR, PDR, iERM and ERMpRD, we used antibodies on whole mounted membranes, to the Ki-67 protein, which stains dividing cells, in combination with antibodies to specific proteins identified within glial, RPE and immune cells (which as described above are felt to be the most important cellular components in membrane formation (Vinores et al., 1990; Heidenkummer and Kampik, 1992; Jerdan et al., 1989)). We are able to quantify the dividing cell types and relate their numbers to the total number of cells present, as well as to the type of ERM and the disease duration.

In Chapter 4 we examined how proliferating glial, RPE and immune cells can be identified in the 4 main types of ERMs. The relative numbers of dividing cells varied between disease conditions and depended on the estimated duration of the ERM in the eye. Implications for disease progression, prognosis and treatment strategies are discussed.

The continued proliferation in ERMs, even after a significant period of time, is a sign of the dynamic character of the cell population in ERMs, especially as this “scar” tissue is often perceived to be inert. The retina is often compared to the central nervous system, but the scar tissue in the retina seems to be more active than (CNS) scar tissue, as the next chapters will demonstrate.

The adult CNS and retina have long been considered relatively static neuronal systems with little potential for plasticity. However, progressively it is becoming clear that they can undergo dramatic remodelling in response to various forms of injuries and diseases. The stereotypical response of the CNS to injury includes the activation of glial cells, resulting in their proliferation and hypertrophy throughout the damaged region (Sofroniew, 2005; Pekny and Nilsson, 2005). One consequence of this gliotic response can be impaired regeneration of neurons, as their processes are unable to migrate through the damaged area to re-establish synaptic connections. The newly formed glial “scar” acts as a physical barrier to regeneration.
and contains inhibitory molecules for neuronal growth (Schwab, 2004). Similar glial reactions can be seen in the retina following trauma. For example, glial activation occurs in retinal detachment, where there is a separation of the neural retina from the retinal pigment epithelium (RPE) (Fisher and Lewis, 2006). Within 3 days after detachment, Müller cells undergo cell division and hypertrophy within the detached area of the retina (Fisher et al., 1991; Geller et al., 1995). These activated cells can grow out of the retina into the newly created “subretinal space” resulting in subretinal fibrosis, or can grow onto the retina on the vitreal side and form PVR membranes (Lewis et al., 2003).

Of interest to this thesis is, in contrast to what occurs in other regions of the CNS, the reactive Müller cells in the “scar tissue” of the ERM appear to act as a permissive substrate for the growth of neurites. Within 3 days of detachment in the feline retina, neurites from horizontal and ganglion cells are invariably found growing adjacent to reactive Müller cells (Lewis et al., 1998; Coblentz et al., 2003; Fisher and Lewis, 2003). These newly formed neurites occur both within the retina as well as on the sub- and epiretinal surfaces, where they often extend for significant distances along glial scars (Fisher et al., 2005).

Neurite outgrowth has been shown to occur in the retina in retinal degenerations and dystrophies (Li et al., 1995; Milam et al., 1998; Fariss et al., 2000; Strettoi et al., 2000; Jones et al., 2003; Jones and Marc, 2005; Beltran et al., 2006; Fei, 2008; Vugler et al., 2008), diabetes (Meyer-Rusenberg et al., 2007; Gastinger et al., 2008), macular degeneration (Sullivan et al., 2007), retinitis pigmentosa (Li et al., 1995; Fariss et al., 2000) and in normal aging of the retina (Liets et al., 2006; Eliasieh et al., 2007). The newly generated neurites appear to originate from essentially every class of retinal neuron including rod and cone photoreceptors, bipolar, amacrine and ganglion cells and these neurites often grow throughout the retina. (Fisher et al., 2005; Marc et al., 2003). We have elaborated on these previous findings.

**Chapter 5** shows that ganglion cell neurites not only occur within the retina, where it would be possible to assume the neurite is looking for a synaptic connection, but can also be found in ERMs in human patients with reactive epiretinal and subretinal membranes after retinal detachment with PVR or PDR. It could be presumed that there are no synaptic goals within ERMs, which has important implications for the interpretation of the significance of the neurite sprouting. The neurites in ERMs are only observed in regions of glial growth suggesting that the neurites are using the glia as a scaffold for growth. In these cases neural growth seems to be a secondary reaction to the severe insult of retinal detachment or PVR and PDR.

However, in **Chapter 6**, we examined idiopathic epiretinal membranes for the presence of neurites and glial tissue. In contrast to what would be expected, in idiopathic ERMs, where
there is no significant injury to the retina, ganglion cell neurites also extend outside the neural retina in association with glial cells. This finding seems to indicate that neurites seem to be a universal finding in samples of sub- and epiretinal membranes removed from patients during vitrectomy surgery, adding to the mounting evidence that there is a significant capacity for structural remodelling among some classes of adult retinal neurons (Li et al., 1995; Fariss et al., 2000; Marc et al., 2003).

Having found neurites sprouting from presumed ganglion cells, using neurofilament staining, in ERMs as described in Chapter 5 and 6, we were interested in investigating neural plasticity or reactivity from other neuronal cell types in the retina growing into ERMs. Since neurofilament protein can be expressed by a number of cell types, the goal of this study was to determine more precisely the neuronal cell types present in epiretinal membranes of various aetiologies using different antibody stains. In addition to neuronal stains, we used markers for synaptic vesicle proteins to determine if the neurites contained the machinery for making synaptic connections. We observe in Chapter 7 that there are distinct neurite populations in the membranes, as evidenced by staining with melanopsin, calretinin, and rod opsins, and that many of the neurites grow on a bed of glial cells and they contain the synaptic vesicle proteins synaptophysin or SV2.

Neural plasticity within the mature adult retina has been described previously (Li et al., 1995; Fariss et al., 2000; Marc et al., 2003). However, the descriptions of this plasticity have always been for neurons within the retina, never outside of the retina. Also these studies have been in single diseases entities within the retina, not several diseases causing ERMs outside of the retinal layers, such as diabetes, retinal detachment or iERMs.

In this thesis, we have shown that ERMs are continually active in the eye. In Chapter 4 ERMs proliferate for a much longer time than expected, even if it is at a low rate. More unexpected is the finding that neurites from all types of retinal neurons can be found in ERMs of all types. This can be in PVR and PDR membranes, where there has been significant retinal trauma, as shown in Chapter 5 or with minimal retinal disruption in iERMs as described in Chapter 6. In Chapter 7 the origin of different neurites can from different neurons within the retina is described, showing that most retinal neurons are able to sprout neurites.

Why these neurites may occur, the factors that influence their growth, and their significance is discussed with the help of a literature review in Chapter 8.
References


Fisher SK, Lewis GP. Müller cell and neuronal remodeling in retinal detachment and reattachment and their potential consequences for visual recovery: a review and reconsideration of recent data. Vis Res 2003;43:887-897.


