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Untersuchungen zu Matrix Metalloproteasen und Metalloproteaseinhibitoren im verletzten und regenerierenden Sehnerv der Ratte.

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ZUSAMMENFASSUNG

Untersuchungen zu Matrix metalloproteasen und Metalloproteaseinhibitoren im verletzten und regenerierenden Sehnerv der Ratte.

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Ziele der Arbeit: Die Neuropathien des N. opticus, insbesondere jene, die in Folge axonaler Verletzungen auftreten (z.B. Glaukom oder Verletzung) sind häufige Ursachen der Erblindung. Das Ziel dieser Arbeit war die immunhistochemische Analyse der Rolle der Hauptregulatoren der extrazellulären Matrix, d.h. der Matrix Metalloproteasen (MMP) und der Tissue-Metalloproteaseninhibitoren (TIMP), bei der posttraumatischen Degeneration und bei induzierter Regeneration des Sehnervs. Zu diesem Zweck würde ihre unterschiedliche Expression im Bezug auf den zellulären Veränderungen in der Retina während der postnatalen Reifung, der axotomie-induzierten Degeneration und der axonalen Regeneration von Sehnervfasern in peripheren Transplantaten untersucht. Aus den Ergebnisse könnten Rückschlüsse zu den Mechanismen des posttraumatischen Überlebens und der Regeneration gezogen werden.

Methoden: Augen von erwachsenen Ratten wurden als Kontrolle benutzt**.** Die Degenerations- bzw. die Regenerationsfähigkeit der retinalen Ganglienzellen (RGZ) wurden anhand des Transections- bzw. Transplantationsmodels untersucht. Augen von Ratten im Alter der postnatalen Tage wurden für vergleichende Untersuchungen während der Entwicklung verwendet worden. Sowohl die Expression von MMP-1,-2,-3,-14 und TIMP-1,-3, als auch die zellulären Veränderungen sind immunohistochemisch untersucht. Zusätzlich wurden bekannte gliale und neuronale Marker hinsichtlich ihrer differentiellen Expression untersucht worden

Ergebnisse: MMP-1,-2,-3 werden in den regenerierenden Retinas hochreguliert und ihre Expression ähnelt der Expression der sich entwickelnden Retinas. Im degenerierenden Gewebe ist weder ein signifikanter Unterschied noch eine Herunterregulation nachzuweisen. MMP-14 ist gleich hochreguliert in beiden experimentellen Gruppen. TIMP-3 ist hochreguliert in den degenerierenden und herunterreguliert in den regenerierenden Retinas. Starke Expression von TIMP-1 ist nur in dem regenerierenden Gewebe nachzuweisen.

Schlußfolgerung: Es scheint, dass MMPs unterschiedlich zwischen De- und Regeneration reguliert werden. TIMP-1 könnte mit dem Überleben von RGZ assoziiert sein, während TIMP-3 mit Apoptosis assoziiert ist. Die Ergebnisse passen zur Vorstellung, dass Metalloproteasen und ihre Inhibitoren an De- und Regenerationsphänomenen beteiligt sind.

Tag der mündlichen Prüfung: 27.10.2010

This dissertation is dedicated to my friends and family
for their love and support.

Abbreviations

ADAM	a disintegrin and metalloprotease
AP-1	activator protein -1
BBB	blood-brain barrier
BDNF	brain derived neurotrophic factor
cAMP	adenosine 3',5'-cyclic monophosphate
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CREB	cAMP responsive element binding protein
CSPG	chondroitin sulfate proteoglycan
ECM	extracellular matrix
E	embryonic day
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FGF(r)	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GCL	ganglion cell layer
GDNF	glial cell derived neurotrophic factor
GFAP	glial fibrillary acidic protein
IGF	insulin like growth factor
IL-8	interleukin-8
ILM	inner limiting membrane

INL	inner nuclear layer
IOP	intraocular pressure
IPL	inner plexiform layer
MBP	myelin binding protein
MMP	matrix metalloprotease
NCAM	neural cell adhesion molecule
NFκB	nuclear transcription factor kappa-B
NGF	nerve growth factor
IF	intermediate filament
NFL	nerve fiber layer
NMDA	N-methyl-D-aspartic acid
NPC	neuronal progenitor cells
NADPHo	nicotinamide-adenine-dinucleotide-phosphate-oxidase
NT-3	neurotrophic factor 3
NT-4	neurotrophic factor 4
ONL	outer nuclear layer
ON	optic nerve
ONH	optic nerve head
OPL	outer plexiform layer
PKC	protein kinase C
PN	peripheral nerve
P	postnatal day
RGC(s)	retinal ganglion cell(s)

RNA	ribonucleic acid
RPE	retinal pigment epithelium
SC	superior colliculus
SD	standard deviation
TGF-b	transforming growth factor-b
TIMP	tissue inhibitor of matrix metalloprotease
TNF	tumor necrosis factor
TNF-a	tumour necrosis factor-a
Trk	tyrosine kinase

1. Introduction

1.1 Organization of the optic nerve

The optic nerve (ON) is responsible for visual sensory input to the brain. It is formed by afferent axons originating in retinal ganglion cells (RGCs) and oriented towards the vitreous body, where they make a sharp 90-degree turn in the direction of the optic disc. The axons then form fascicles and exit the eye-ball through the lamina cribrosa. At this point they get myelinated by oligodendrocytes and covered with three layers of meninges, the outermost of which – the dural sheath – is a direct extension of the sclera. Finally, RGC axons synapse in specific nuclei in the midbrain (Fujita et al; 2000).

1.2 Pathology of the optic nerve

Pathology of the optic nerve is a major cause of visual loss. Various pathological conditions of the optic nerve have been described, which can be classified into three major categories according to their localization and pathophysiology:

a) mitochondrial injuries;

b) retinal ganglion cell body injury, such as retinal ischemia and excitotoxicity; and

c) axonal injuries, such as optic nerve compression and transection, papilledema, inflammation, demyelination and glaucomatous optic neuropathy (Levin and Gordon, 2002). The latter is the most common chronic optic neuropathy and one of the leading causes of blindness in the western world, affecting almost 70 million individuals worldwide (Quigley HA, 1996; Coleman, 1999).

The optic nerve transection model is the main experimental method used to study RGC degeneration after optic nerve injury. Although uncommon in humans, the traumatic optic nerve lesion in experimental animals, such as rodents, offers several advantages: accessibility of the optic nerve to

microsurgery, a specific procedure protocol, a reliable qualification and quantification of RGC degeneration, and the possibility of assessing and establishing therapeutic approaches towards neuroprotection and regeneration (Morrison, 2005).

1.3 Anatomy and physiology of the retina and the optic nerve

1.3.1 Retinal anatomy

The retina is a highly organized laminated structure of the central nervous system (CNS). The basic multi-layered structure of the retina is the same in all vertebrates and consists of six main neuron types organized in the following layers, seen from the sclera towards the vitreous body:

1) Outer nuclear layer (ONL) where the bodies of photoreceptors (rods and cones) are located.
2) Outer plexiform layer (OPL) where the photoreceptor cells synapse with the bipolar and horizontal cells.
3) Inner nuclear layer (INL) which contains the horizontal cells, the bipolar cells, the amacrine cells, and the Müller cells' somata.
4) Inner plexiform layer (IPL) where the bipolar and amacrine cells synapse with the ganglion cell.
5) Ganglion cell layer (GCL) consisting of RGCs, the axons of which comprise the optic nerve.
6) Nerve fiber layer (NFL) which consists of the RGC axons converging from all parts of the retina towards the optic disc. Within the NFL there is already a retinotopic arrangement of axons, with those originating from peripheral ganglion cells positioned superficially (vitreally) within the retina (Ogden, 1983a) and then centrally within the nerve head (Minckler, 1989), and those originating centrally having the reverse arrangement. Those axons are often surrounded by glial cell processes which belong to the Müller cells or astrocytes that reside in that layer.

1.3.2 Retinal cell properties

The **glial cells** include the Müller cells, the astrocytes, the oligodendrocytes and the microglia. They play an important role in the physiology of the RGCs and the optic nerve.

The **Müller cells** constitute the main glial cells of the retina. They are radial, extend through the whole thickness of the retina, and offer structural and biochemical support to the RGCs and their axons. They also stabilize the complex retinal architecture, provide structural support to blood vessels, and prevent aberrant photoreceptor migration into the subretinal space.

The vitreal ends of Müller cell processes form expansions, the *endfeet*, which overlay the inner retina. Their basal lamina forms the inner limiting membrane (ILM). The Müller cell processes in the NFL, as well as the astrocytes that reside there, cover most of the RGC axons and the blood vessels (Ogden, 1983b). Their physiological role is very important in the maintenance of the extracellular matrix (ECM) that surrounds the RGCs because they buffer the K^+ concentration and remove glutamate from the extracellular space (Schwartz, 1993; Li and Puro, 2002). They also take part in the glucose homeostasis (Niemeyer, 1997) and respond to various growth factors and cytokines originating from themselves, the retinal pigmented epithelium (RPE) cells and the retinal neurons. Moreover, they secrete ECM molecules and take part in tissue remodeling (Agapova et al., 2001). In pathological conditions and under the effect of those factors Müller cells may proliferate and express the glial fibrillary acidic protein (GFAP), an intermediate-filament (IF) protein that is highly specific to cells of astroglial lineage.

The **retinal astrocytes** are glial cells of the retina found in its inner layers, especially the NFL, as well as in the optic nerve head. They share many common features with Müller cells concerning their physiology and the structural support they give to RGCs and their axons (Bussow, 1980). They

contribute to the maintenance of ionic homeostasis, the supply of energy to the RGCs in pathological conditions (e.g. ischemia) (Wender et al., 2000) and tissue remodeling (Hernandez, 2000). They also play a role in the integrity of the blood retinal barrier (BRB) (Janzer and Raff, 1987; Schnitzer, 1988).

The **optic nerve astrocytes** have an additional major role in supporting the axons in the optic nerve. They constitute the barrier between neurons and connective tissue, namely the central vein, the central artery and the laminar plates of the lamina cribrosa. This barrier is the glial limiting membrane, a dense and resilient membrane that forms the capsule of the ON. The optic nerve astrocytes seem to be important for optic nerve development, for they may serve as a substrate in which RGC axons grow (Lucius et al., 1996). After an injury followed by neuronal degeneration, astrocytes are the substrate for the procedure of gliosis, and form the so-called “glial scar” (Miller et al., 1986; Quigley et al., 1983). An upregulation of several ECM molecules, such as collagens and proteoglycans, takes place during the formation of this scar (Hermanns et al., 2001; Rhodes and Fawcett, 2004). Proteoglycans have been shown to inhibit the axonal outgrowth.

The number of astrocytes is regulated by the RGCs through several mechanisms (Burne and Raff, 1997). Glial cells appear to communicate with each other in various ways (e.g. through gap junctions) (Waxman and Black, 1984). All astrocytes express GFAP and can be immunohistochemically identified through this intermediate filament protein. Under pathological conditions, such as ON transection, they proliferate and are activated. The activation of the astrocytes is immunohistochemically detected by an increase in the expression of GFAP (Hernandez, 2000).

The **microglia** are a group of glial cells that act as immunocytes of the central nervous system, and are also found in the retina and the optic nerve. The activated microglia act as macrophages (Oehmichen, 1982) and are responsible for phagocytosizing degenerating axons and RGCs during

development (Sturrock, 1988) and in pathological conditions such as transection of the optic nerve (Garcia-Valenzuela et al., 2005). Microglia-macrophages can be identified with the ED-1 marker which recognizes a glycosylated protein expressed on their cell membrane (McLaurin et al., 1995).

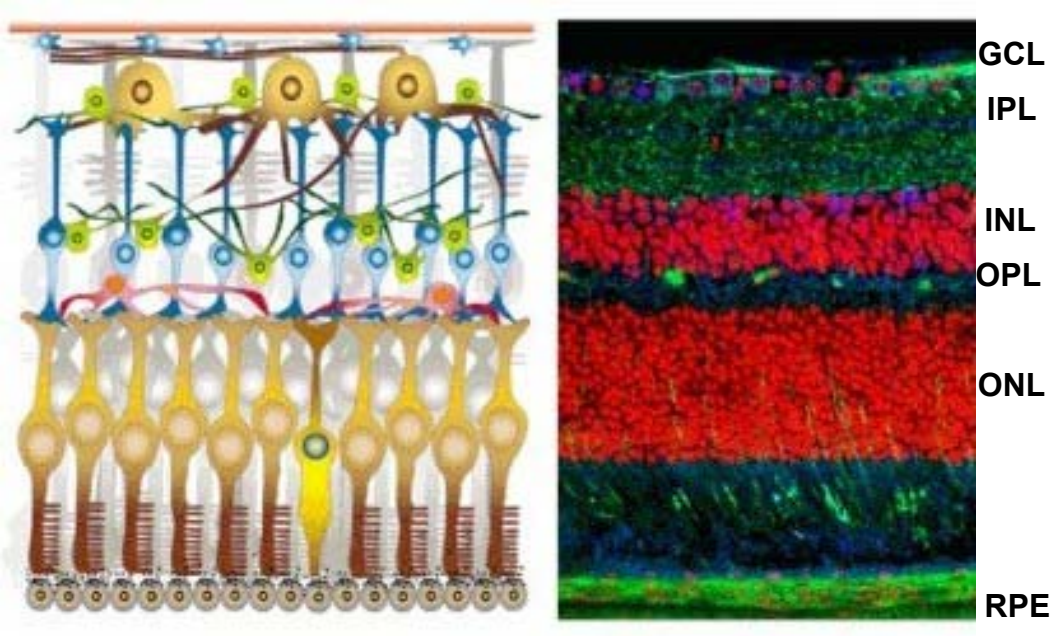


Fig.1 Left panel shows a schematic representation of the retina. Right panel shows the layers labeled with fluorescent probes. Retinal ganglion cells are found within the ganglion cell layer (GCL). Intermediate neurons are found within the inner nuclear layer (INL), and rod and cone photoreceptor cells are found within the outer nuclear layer (ONL). IPL and OPL are inner plexiform and outer plexiform layers where axons are found. RPE is pigmented epithelium. (Department of Biochemistry and Molecular Biology of the University of Texas - MD Anderson Cancer Center)

1.3.3 Development of the retina and the optic nerve

The neural tissue of the eye originates from the neural ectoderm and develops from two bilateral outgrowths of the neural tube, which evaginate to form the optic vesicle. The outer wall of each vesicle invaginates and the two-layered optic cup is created (Jacobson, 1966). The outer layer becomes the

monolayered RPE, and the inner layer of the optic cup develops into the future nervous retina (Hilfer, 1983).

The retinal maturation follows a centrifugal pattern beginning at the posterior pole and proceeding to the periphery. The RGCs are the first cell type to be produced and differentiated during retinogenesis (embryonic day (E) 13-19 in the rat) (Young, 1985; Dallimore et al., 2002), developing from the innermost part of the inner neuroblastic layer and migrating into the marginal zone of the nervous retina. They then send out fibers which grow towards the optic stalk and towards the brain targets (Dowling, 1970; Turner and Cepko, 1987; Wetts and Fraser, 1988; Holt, 1989). The RGCs that develop axons express the growth associated protein GAP-43. In the rat, more than 90% of the RGCs project to the contralateral superior colliculus (SC) (Dreher et al., 1985), which they innervate between E16 and postnatal day (P) 5 (Dallimore et al., 2002).

During the process of cell migration, differentiation and axon growth, a great proportion of the retinal ganglion cells die. In rat retinas, more than 90% of the newborn RGCs die in the period between birth and P6. This indicates that only the RGCs that manage to innervate their central targets receive the appropriate signals for their survival (Galli-Resta and Ensini, 1996). The death procedure is apoptotic (Young, 1984). In apoptosis cells drive themselves into a programmed “suicidal” death characterized by the condensation of nuclear chromatin, shrinkage of the cell soma, blebbing of the membrane, fragmentation of the cell into apoptotic bodies, and alteration of the cell surface properties. The latter leads the cell remnants to be phagocytosized by neighboring cells and macrophages, in this way avoiding the leakage of inflammatory content (Kerr et al., 1972). It is estimated that the programmed death of the RGCs that don’t manage to reach their targets takes approximately five days from their genesis (Farah and Easter, 2005).

1.3.4 Pathophysiology of the optic nerve cut

The failure of CNS neurons to regenerate after injury has been known about for more than 80 years (Ramon and Cajal, 1928). After optic nerve injury axons die back, the RGC somata undergo apoptotic cell death and the glial cells respond with proliferation, hypertrophy and the formation of a glial scar at the site of injury (Hall and Berry, 1989; Powell et al., 1997). Over the past two decades numerous ways of simulating RGC injury have been described, concerning the means of damaging the optic nerve (crush, transection), as well as where to locate the site of injury (proximal or distal to the ONH, intracranially or intraorbitally, etc.), (Cho and So, 1993; Zeng et al., 1995; Zhi et al.; 2005).

In the case of intraorbital axotomy close to the ONH the physiological consequences are well studied and documented. In adult rats this method of RGC injury leads to an initial attempt by the axons to regenerate. This fails and gives way to a wave of apoptotic death that begins four to five days after transection, reaching its highest point after approximately seven to eight days (Berkelaar et al., 1994; Garcia-Valenzuela et al., 1994; Bähr, 2000). Within 15 days almost 90% of the RGCs have been lost.

The reasons for this degenerative response have been thoroughly studied. At first it seems that the injury impairs the process of retrograde neurotrophic support that maintains mature RGCs (Ure and Campenot, 1997) or alters RGC responsiveness to factors in their close environment (e.g. via the downregulation of Trk receptors on their surface) (Cheng et al., 2002). Moreover, the intrinsic activation of apoptotic procedures involves the activation of cell death-receptors, differential expression of apoptotic and anti-apoptotic genes and proteins (Bax, Bcl, Caspases, Hsp27 etc.) and modulation of several transcription factors such as activator protein -1 (AP-1), p53, c-jun, c-Fos, CREB and NFκB (Isenmann et al., 2003). The inability to regenerate axons has also been attributed to changes in the ON environment, such as the myelin derived inhibitory molecules exhibited by the oligodendrocytes in the optic nerve

(Cadelli et al., 1992), and the formation of the glial scar at the site of injury by activated astrocytes. The latter secrete ECM molecules such as chondroitin sulfate proteoglycan (CSPG) which blocks axonal outgrowth (Powell et al., 1997). The reduced ability of macrophages to invade the injured optic nerve and enhance regeneration of axons to the distal stump has also been implicated (Perry et al., 1987). On the other hand, there are diverging opinions as to whether microglia activation promotes degeneration through the secretion of cytotoxic substances (Banati et al., 1993) or whether it has a beneficial effect on regeneration through the secretion of neurotrophic factors (Moore and Thanos, 1996; Batchelor et al., 1999).

1.3.5 RGC death in the developing and the injured retina

The cellular and nuclear features of the programmed cell death (PCD) that most newborn RGCs undergo during developmental apoptosis are to some extent similar to the ones observed during the ON axotomy-induced death of adult RGCs (Isenmann and Bähr, 1997; Isenmann et al., 1997; Cellerino et al., 2000). Inflammatory-necrotic procedures have also been implicated in the case of the latter (Thanos et al., 1993; Bien et al., 1999).

The survival or apoptosis signals seem to be neurotrophic factors:
a) transferred retrogradely to the RGCs from their central targets (Isenmann et al., 1999);
b) released from local resources in the retina (Herzog and von Bartheld, 1998);
c) secreted in an autocrine fashion, particularly after injury (Cheng et al., 2002).
Among those neurotrophines are the nerve growth factor (NGF), the brain derived neurotrophic factor (BDNF) and the neurotrophin 3 (NT-3). These react with specific receptors on the cell surface which belong to the tyrosine kinase (Trk) family or the p75-low affinity receptor (Frade et al., 1997; Frade and Barde, 1997; Bovolenta et al., 1996).

The intracellular mediators of retinal ganglion cell death are cysteine aspartyl-specific proteases called caspases. When caspases are activated, they initiate an intracellular cascade leading to the proteolysis of structural nuclear components and cell death (Earnshaw et al., 1999). The major protease involved is caspase-3, and it can be activated by two different pathways: an extrinsic one, involving death promoting receptors and ligands such as tumor necrosis factor (TNF) receptor (Ashkenazi and Dixit, 1998), and an intrinsic one involving the release of death-promoting molecules like cytochrome–c from the mitochondrium to the cytosol (Li et al., 1997).

1.3.6 Methods of enhancing RGC survival and regeneration

Research during the last 20 years has revealed that RGCs are capable of surviving and regenerating their axons when the properties of their surrounding environment are artificially altered. Aguayo and colleagues first demonstrated that transplantation of a peripheral nerve (PN) segment into the transected optic nerve prolongs the survival of RGCs, prevents axon degeneration and promotes their regeneration inside the graft (So and Aguayo, 1985). It has also been demonstrated that after connecting the peripheral nerve graft to the central targets in the brain, up to 10% of the total RGC population extend their axons along the former and form synapses within the latter (Vidal-Sanz et al., 1987; Thanos and Mey, 1995). In recent years, alternative ways of enhancing RGC survival and axon regeneration have been described, such as by the intraocular supply of neurotrophic factors (Mey and Thanos, 1993; Peinado-Ramon et al., 1996; Yan et al., 1999), anti-apoptotic and pharmacological substances (Heiduschka and Thanos, 2000; Huang et al., 2003; Kretz et al., 2006), intravitreal implantation of a PN segment or Schwann cells (Berry et al., 1996; Li et al., 2004), lens injury (Leon et al., 2000; Fischer et al., 2001), intraocular activation of macrophages (Yin et al., 2003), increasing the cAMP levels (Monsul et al., 2004), and by intraocular injection of the calcium-binding protein oncomodulin (Yin et al., 2006). All these ways - combined or not - have managed a rate of regeneration of up to 30% in some cases and indicate that

RGCs have an intrinsic capacity to survive injury and regenerate axons when they receive the appropriate direct or indirect ignition.

1.3.7 Survival and regeneration of RGCs after the transplantation of a peripheral nerve graft

As mentioned previously, the RGCs have the capacity to survive after axotomy for some time, and up to 10% of them have the ability to regenerate their axons inside a peripheral nerve graft over long distances within weeks, and to form functional synapses in the superior colliculus (Vidal-Sanz et al., 1987; Villegas-Perez et al., 1988). Peripheral nerves themselves have the ability to regenerate. After injury the distal nerve stump undergoes a process called *Wallerian Degeneration* in which axons degenerate and myelin debris is phagocytosized by invading macrophages, while Schwann cells remain intact and proliferate. Then growth cones coming from the axons of the proximal stump enter into tube formations created by Schwann cells (Bands of Brügner) (Bruck, 1997; Fawcett and Keynes, 1990). This regeneration promoting ability in peripheral nerves, as well as in grafts transplanted into the central nervous system, is attributed to the intrinsic features of Schwann cells (Negishi et al., 2001; Dezawa and Adachi-Usami, 2000).

These are:
a) the secretion of trophic factors and neurotrophins, such as NGF, BDNF, NT-4, ciliary neurotrophic factor (CNTF) and glial cell line-derived neurotrophic factor (GDNF) (Meyer et al., 1992; Springer et al., 1994; Friedman et al., 1992);
b) the secretion of extracellular matrix molecules such as fibronectin, laminin, merosin (laminin-2) and type IV collagen; and
c) the expression of a variety of cell adhesion molecules on their surface such as NCAM, L1 and N-cadherin.

The features mentioned in (b) and (c) not only offer a substrate for the attachment and extension of RGC processes, but also mediate an RGC-ECM

and RGC-Schwann cell interaction through membrane receptors (e.g. integrins) and initiate intracellular signals (Martini, 1994; McKerracher et al., 1996; Jung et al., 1997; Tomaselli et al., 1993). It has also been demonstrated that Schwann cells form gap junctions with RGC axons, implicating a direct transfer of several molecular signals or factors from one cell type to another (Dezawa and Adachi-Usami, 2000).

It is worth mentioning that the process of optic nerve regeneration to some extent resembles that of optic nerve development. The RGCs of the developing retina have the intrinsic ability to generate axons and reach their CNS targets, an ability which they later seem to lose (Goldberg et al., 2002). Moreover, the partial survival of the RGCs in the developing retina and the developmental cell death of the majority of them (Galli-Resta and Ensini, 1996) could be compared to the RGC survival and cell death witnessed in the regenerating retina. It has also been found that these two conditions have similarities, as well as differences, in their levels of gene expression (Zhou and Snider., 2006; Filbin, 2006), a fact that could help decrypt the mechanisms of survival and growth of RGCs and lead to possible interventions towards regeneration and neuroprotection.

All of the above leads to the conclusion that the peripheral nerve graft not only offers a “mechanically” beneficial environment for the regeneration and survival of the RGCs and their axons, but actually triggers the intrinsic capacity of the RGCs for this. It accomplishes this by sending signals which reach the RGC somata. These cause the RGCs to alter their intrinsic as well as their extrinsic properties, and to interact differently with their close environment, or even to reform it so that it promotes their survival and regeneration.

The molecules that are mainly responsible for the reforming of the extracellular environment and the regulation of the cell-matrix composition are the matrix metalloproteases and their inhibitors.

1.4 The matrix metalloproteinases family and their inhibitors

1.4.1 MMP structure and basic biology

MMPs comprise a family of 24 zinc- and calcium-dependent endopeptidases that have the ability to hydrolyze almost all components of the extracellular matrix and basement membranes, as well as non-matrix macromolecules. They have a decisive role in tissue remodeling, development, physiological procedures and disease (Matrisian, 1990; Shapiro, 1998; Puente et al., 2003).

They are characterized by a basic structural organization consisting of:
a) a signal peptide domain that targets them for secretion;
b) a propeptide domain containing a cystein-switch motif; and
c) an N-terminal catalytic domain containing a Zn-binding motif.
Most of the MMPs also have a hinge-region, and a C-terminal hemopexin-like domain, which is involved in the ECM-substrate binding and the interaction with tissue inhibitors of metalloproteases (Fig. 2).

Fig.2 Schematic representation of MMP structure. SP-signal peptide, PRO-propeptide domain, F-furin cleavage site, Catalytic domain containing a Zn-binding motif, C-terminal hemopexin-like domain.

Based on their structure, and whether they are bound to the cell membrane or secreted, MMPs are classified into six major groups (Table 1). MMPs are produced as inactive zymogens with the cystein motif of the propeptide binding to the Zn^{2+} catalytic site thereby preventing their activity. For the activation of the enzyme, a disruption of this endomolecular interaction is required and

accomplished through cleavage of the propeptide. The membrane-bound MMPs (MT-MMPs) are membrane associated, either through a transmembrane domain (MT-MMP-1, 2, 3 or 5) or through a GPI-anchor (MT-MMP-4 or 6) (Nagase et al., 2006). New roles for MMPs are currently being discovered, in addition to their “mechanical” reformation of the ECM. For example, the degradation of ECM components generates matricryptic sites, which allow new interactions between those sites and cell surface molecules, enhancing biological activities like cell migration and neurite outgrowth (Gilles et al., 2001; Giannelli et al., 1997; Koshikawa et al., 2000). MMPs are also found to liberate growth factors like IGFs and FGFs bound to the ECM (Rundhaug, 2005), and to generate chemokines and cytokines such as IL-8, TNF-a and TGF-b (Parks et al., 2004).

Table 1 MMP group classification according to substrates and structure.

Collagenases	MMP-1, 8, 13, 18
Gelatinases	MMP-2, 9
Stromelysins	MMP-3, 10
Matrilysins	MMP-7, 26
Membrane-bound MMPs	MMP-14, 15, 16, 17, 24, 25
Other MMPs	MMP-12, 19, 20, 22, 23, 28

1.4.2 TIMP structure and basic biology

In vertebrates there are four tissue inhibitors of metalloproteinases (TIMPs). TIMPs-1, 2 and 4 are diffusible secreted proteins, while TIMP-3 is bound to the ECM. Their structure can be divided into two domains: the N-terminal domain responsible for MMP-inhibitor activity, and the C-terminal domain responsible for the distinct properties of the four TIMPs. All MMPs can be inhibited by all TIMPs by forming an inhibitory 1:1 complex with them, with the exception of the transmembrane MMPs which are poorly sensitive towards TIMP-1 inhibition. Nevertheless, they differ in other properties, such as tissue distribution, transcriptional regulation, latent-MMP activation in some cases (TIMP-2 and MMP-2), as well as other biological activities, (endothelial cell proliferation, pro-apoptotic–anti-apoptotic effects, cell growth promotion etc.) (Brew et al., 2000).

The TIMPs also have various non-MMP-inhibitory properties that have been described in many studies. For example, TIMP-1 has the ability to promote growth in various cell types through a growth promoting domain of the molecule

independent of the MMP-inhibitory one (Jiang et al., 2002). TIMP-3, when overexpressed by adenoviral delivery, induces apoptosis in vascular smooth muscle cells, an effect not reproducible by synthetic MMP inhibitors (Baker et al., 1998). The pro-apoptotic effects of TIMP-3 are probably related to its ability to inhibit members of another family of metalloproteinases, the adamalysins (ADAMs), and particularly ADAM-17 which liberates the active ligands of TNF-a and TGF-b from their pro-forms (Itai et al., 2001; Wei et al., 2005).

1.4.3 Regulation of MMP activity

MMP activity can be regulated on many levels, such as gene transcription, mRNA stability, translational control, cell compartmentalization, zymogen activation and inhibition by endogenous inhibitors (Chakraborti et al., 2003). MMP zymogen activation is often the result of complex cascades, in which other proteinases also take part. Namely, pro-MMP-1 and 3 can be cleaved in the propeptide domain by serine proteases (Cuzner and Opdenakker, 1999); MMP-3 can activate pro-MMP-1 and 9 (Nagase et al., 1992, Ogata et al., 1992); and MT1-MMP (MMP-14) can activate MMP-9 through the activation of MMP-13 (Knauper et al., 2002; Cowell et al., 1998) and is the main activator of MMP-2 through an extensively studied mechanism, in which TIMP-2 also participates (Fig. 3) (Butler et al., 1998). By way of the latter procedure, the levels of TIMP-2 can modulate the activation of MMP-2 as well as MMP-14 autocatalysis (Hernandez-Barrantes et al., 2000).

Recently great importance has also been given to the factors regulating metalloproteinases at a transcriptional level. Especially in the nervous system, factors like NGF (Khan et al., 2002), EGF (Rooprai et al., 2000), TGF-b (Wick et al., 2001), integrins (Giraudon et al., 2000), cytokines like TNF-a and interleukin-1a (Hebert et al., 2003), chemokines (Cross and Woodroofe, 1999), reactive oxygen species (Kim G.W. et al., 2003), prostaglandins (Kyrkanides et al., 2002), cAMP (Muir, 1995), protein kinase C (Arai et al., 2003), glucocorticoids (Leco et al., 1994), inflammatory agents (Misse et al., 2001),

antibiotics (Sadowski and Steinmeyer, 2001), extracellular levels of other MMPs (Oh et al., 2004), neurotransmitters like serotonin (Shum et al., 2002) , and cell-cell or cell-matrix interaction (Sternlicht and Werb, 2001) can modulate the transcription of MMPs and TIMPs through various intracellular signal cascades.

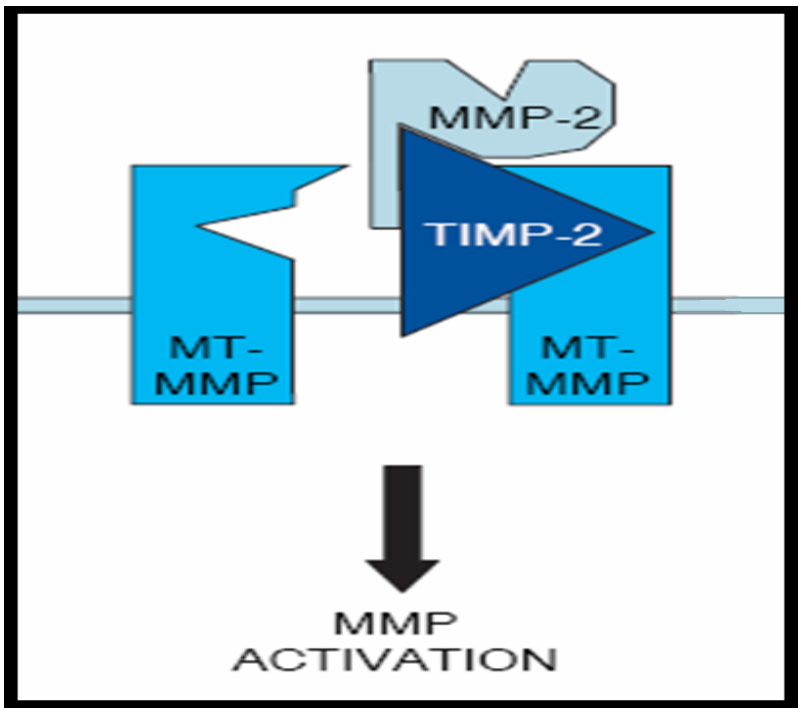


Fig.3 Activation of pro-MMP-2 by an MT-MMP with participation of a binary MT-MMP – TIMP-2 complex which exposes the zymogen to its activator. (Stetler-Stevenson and Seo, 2005; modified)

1.4.4 MMPs and TIMPs in the retina

In recent years much experimental work has been conducted in the field of matrix metalloproteinase expression in the retina in pathological conditions (Sivak and Fini, 2002). Some of it has focused on the pathophysiology concerning RGCs e.g. the association of RGC death with MMP-9 upregulation in a retinal ischemia model (Chintala, 2006). Few studies have focused on RGC death after ON injury. In one study, Hernandez and colleagues compared the

MMP-TIMP expression between a model of experimentally elevated intraocular pressure and one of ON injury after transection, focusing primarily on the ONH (Agapova et al., 2003). In another study, the expression of MMPs-TIMPs in a rat model of ON injury was compared between RGC degeneration and regeneration after intravitreal implantation of an SC, focusing mainly on the glial scar degrading activity of the proteases rather than on their retinal expression (Ahmed et al., 2005). In these cases it was also demonstrated that in normal control retinas there was a constitutive expression of MMP-2 and TIMP-1 and 2 by RGC axons. In cases of injury the additional expression of MMP-1, MMP-14, MMP-3 and MMP-9 was observed by reactive astrocytes and inflammatory cells like microglia (Agapova et al., 2003; Agapova et al., 2001).

As mentioned above, after optic nerve injury the retinal cells have been shown to exhibit different intrinsic and extrinsic properties which also influence the extracellular matrix. The extracellular matrix itself has a much more important physiological role than just being a structural scaffold. It can interact directly with the cells in various ways and regulate their behaviour (Schnaper and Kleinman, 1993). MMPs can modify the ECM and liberate growth factors and chemokines. TIMPs not only inhibit MMP activity, but also have a direct influence on cell survival. All these facts make it provoking to shed light on the expression of those proteins which are within the close extracellular environment of the RGCs, and to investigate their differential interactions with the ECM and their neighboring cells. The choice of the proteins examined was made based on the recent literature concerning MMP-expression in retinal injury (Table 2).

Table 2 **Display of the MMPs to be examined with their main substrates and activators.** (Chakraborti et al., 2003; modified)

Enzyme	MMP	ECM substrate	Non-ECM substrate	Activator of
Collagenase-1	MMP-1	Collagens (I, II, III, VII, VIII and X), gelatin, proteoglycan link protein, aggrecan, veriscan, tenacin, entactin	α1-PI, ILb-1, IGFBP-3, pro-TNF, MMP-2, MMP-9	MMP-2
Gelatinase A	MMP-2	Collagens (I, IV, V, VII, X, XI and XIV), gelatin, elastin, fibronectin, laminin-1, laminin-5, galectin-3, aggrecan, decorin, hyaluronidase-treated versican, proteoglycan link protein, osteonectin	IL-1b, α1-PI, prolysyl oxidase fusion protein, MMP-1, MMP-9, MMP-13	MMP-9, 13
Stromelysin-1	MMP-3	Collagens (III, IV, V and IX), gelatin, aggrecan, versican, hyaluronidase-treated versican, perlecan, decorin, proteoglycan link protein, large tenascin-C, fibronectin, laminin, entactin, osteonectin	α1-PI, antithrombin-III, ovosstatin, substance P, IL-1β, serum amyloid A, IGFBP-3, fibrinogen and cross-linked fibrin, plasminogen, MMP-1 'superactivation', MMP-2/TIMP-2 complex, MMP-7, 8, 9, 13	MMP-1, 7, 8, 9, 13
MT1-MMP	MMP-14	Collagens (I, II and III), casein, elastin, fibronectin, gelatin, laminin, vitronectin, large tenascin-C, entactin, proteoglycans	α1-PI, MMP-2, 13	MMP-2, 13

1.4.5 Purpose of the present experiments

The purpose of the present experiments is to explore whether matrix metalloproteases and their inhibitors are linked to RGC death and survival after optic nerve injury. An assessment of the differential expression of these molecules with the cellular changes observed in the degenerating, regenerating and developing optic nerve may help us to understand whether the extracellular matrix is involved in the neurodegenerative and neuroregenerative processes by replicating patterns of expression seen in the optic nerve development. The focus of this study will be set primarily in the direct environment of the RGC somata, in the retina. To stimulate optic nerve injury, the model of ON transection will be used. The MMP and TIMP expression will also be assessed in a time-based manner, since research has indicated that there are critical time points concerning the behaviour of RGCs after injury.

The possible qualitative and quantitative differences in MMP and TIMP expression that may arise between the different groups and at different time points will be recorded.

2. Materials and Methods

In this section, the chemical substances, instruments and materials used in the operations and experiments, as well as the electronic devices and appliances utilized, will be mentioned. In addition, the operative and laboratory methods employed will be described.

2.1 Substances, instruments and appliances

2.1.1 Buffers

PBS: "Phosphate buffered saline" is an isotonic to the normal serum saline solution containing sodium phosphate, potassium phosphate and sodium phosphate with neutral pH. To prepare a 10 liter stock of 10 x PBS:
800 g NaCl, 144 g Na_2HPO_4 x $2H_2O$ and 24 g KH_2PO_4 were dissolved in 8 l of distilled water (Ampuwa), and then topped up to 10 l. For 1 x PBS the former saline was diluted (1:10) with "Ampuwa".

2.1.2 Laboratory items

Petri dishes, diameter 35 mm and 60 mm

Nitrocellulose filter, black (Sartorious)

Micropipettes, 0.5-10 µl / 10-100 µl / 100-1000 µl and pipette tips (Eppendorf)

Gloves "Nitra-Tec" (Ansell)

Object glass-slides 76x26 mm / 3x1 inch (Mattrand) and cover-glasses 24x60 mm

Microtomer “HM 550 Cryostat” (MICROM) with compatible metal chucks

2.1.3 Operations material

Anesthetics:

Ketamin 10% (Ketaminhydrochlorid) 0.1 ml/100 gr bodyweight

Xylazin 2% (Xylazinhydrochlorid) 0.05 ml/100 gr bodyweight

Sutures :

4-0 used for scalp suture

5-0 used for skin-incision closure

6-0 used to stabilize the bulb

10-0 used in the nervus opticus-sciatic nerve anastomosis and the dural sheath resuture

Forceps and Scissors:

INOX Dumont “Biologie”, No 5 and No 3

Forceps “McPherson” No 7275 (HERMLE)

Tissue Forceps 0.6 and 1.2 mm (HERMLE)

“Castroviejo” and “Vanass” (Aesculap)

Others :

Antibiotic ointment “Gentamytrex” (Gentamicinsulfat, Bausch & Lomp)

Hydration-cream “Visidic” (Carbomer, Bausch & Lomp)

Drill Maxicraft 20.000 (PLUTON)

Gelfoam (Pharmacia & Upjohn) - Absorbable Gelatin

Fluorescence dye:
“4-Di-10ASP”: 4-(4-didecylaminostyryl)-N methylpyridiniumjodid (Molecular Probes), dissolved in Freund’s adjuvant

2.1.4 Microscopes and accessories

Carl ZEISS OPMI 19-FC

Carl ZEISS OPMI 9-FC

Fluorescence microscope:

“Axiophot” (ZEISS) with fluorescence filter 395-440 nm, 450-490 nm, 510-560 nm
Objectives neofluar 5x, 10x, 20x, 40x, 63x (oil)

Axiocam HRc camera (Zeiss)

Oil “Carl Zeiss” (immersion oil)

2.1.5 Hardware and software

Pentium 4 Processor (INTEL) equipped with an image acquisition board (FUTJITSU SIEMENS)

AxioVision Rel. 4.5 software (Zeiss)

Photoshop 8.0 software (Adobe Systems, San Jose, CA)

Celeron (INTEL-centrino) processor

2.1.6 Antibodies

Immunohistochemistry was applied to assess the MMP and TIMP expression as well as the cellular changes. Retinal-cell markers, antibodies against MMPs-1, 2, 3 and 14 and TIMPs-1 and 3 were utilized (Tables 3 and 4). NF-200 (TRITC) was double-stained with GAP-43 (Cy2). The metalloproteases and their inhibitors (Cy2 or FITC) were double stained with GFAP (TRITC) to examine possible colocalization with retinal astrocytes and activated Müller cells.

Table 3 List of Primary Antibodies. The supplier, code number, host species and dilution used are displayed.

Against	*Supplier*	*Code Number*	*Species*	*Dilution*
Glial Fibrillary Acidic Protein (GFAP)	Sigma	G 9269	Rabbit	1 : 100
GAP-43	Sigma	G 9264	Mouse	1 : 500
NF-200	Sigma	N 4142	Rabbit	1 : 200
MMP-1	GenWay	15-288-21115	Chicken	1 : 1000
MMP-2	Chemicon	MAB3308	Mouse	1 : 4000
MMP-3	Chemicon	MAB3306	Mouse	1 : 400
MMP-14	Chemicon	MAB3317	Mouse	1 : 400
TIMP-1	Chemicon	MAB3300	Mouse	1 : 200
TIMP-3	Chemicon	AB802	Rabbit	1 : 1000

Table 4 List of Secondary Antibodies. The supplier, code number, host species, dilution used, and the fluorescence substance in which the antibody was conjugated are displayed.

Against	*Supplier*	*Code Number*	*Species*	*Dilution*	*Conjugation*
Rabbit	**Jackson Immuno research**	**111-225 -045**	**Goat**	**1 : 200**	**Cy2**
Mouse	**Sigma**	**T7782**	**Goat**	**1 : 300**	**TRITC**
Rabbit	**Sigma**	**T6778**	**Goat**	**1 : 400**	**TRITC**
Chicken	**GenWay**	**GAY-FITC**	**Goat**	**1 : 200**	**FITC**
Mouse	**Jackson Immuno research**	**115-226-062**	**Goat**	**1 : 200**	**Cy2**

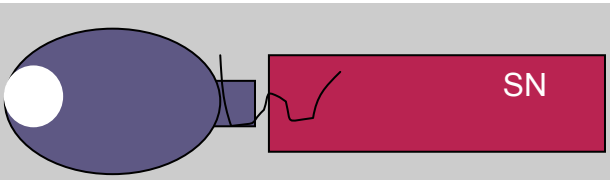
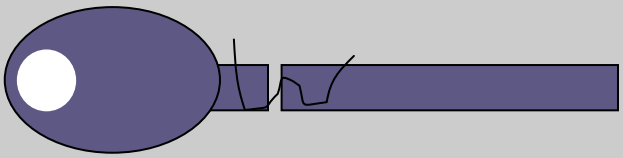
2.2 Laboratory animals

Sprague-Dawleys - an outbred strain of albino brown rats - aged about three (3) months and weighing about 250-350 gr were used. The project comprised nine groups of rats: two main operative groups with three subgroups each. In one group rats had their left optic nerve transected and resutured, to evoke RGC degeneration. The enucleation-timepoint of the operated eye was 4, 15 and 30 days postoperatively. According to literature the degenerative process starts on day 4, involves 90% of the RGCs by day 15, and concludes with a plateau leveling total degeneration later (Berkelaar et al., 1994; Garcia-Valenzuela et al., 1994; Bähr, 2000).

The rats in the second group received a peripheral (sciatic) nerve graft on the proximal part of the transected optic nerve in order to stimulate the partial regeneration of the RGCs and their axons. In this way the regeneration model

has a close resemblance to the operating procedure used for the degeneration model. As such, systematic errors originating from dissimilar operative manipulation are diminished. Moreover, the close environment of the RGC somata, the main focus area, is not directly influenced, as it would be with intravitreal PN transplantation for example. The postoperative time-subgroups were chosen to match the ones simulating ON degeneration in order to accomplish direct association between the two groups.

Table 5 The groups and number of rats used in the study.

SUBJECTS	4 days Post-operationally	15 days Post-operationally	30 days Post-operationally
Transplantation	**5 rats**	**5 rats**	**5 rats**
			
Transection	**5 rats**	**5 rats**	**5 rats**
			
Normal controls & developing	**Postnatal day 0**	**Postnatal day 15**	**Postnatal day 30**
	**3 rats**	**3 rats**	**3 rats**

In each of the six subgroups five rats were used. Additionally, three rats from the “transection” group and five rats from the “transplantation” group were used to draw a comparison with the normal control group in order to verify quantitatively

the degenerative and regenerative processes respectively. Three normal rats 3 months old were used as a control. Moreover, three P0 (Postnatal day 0) and three P15 (Postnatal day 15) rats were used to examine the expression of the metalloproteinases and their inhibitors during development. In a P0 rat, as mentioned above, the axon growth of many RGCs is still in progress, whereas other RGCs are in the process of programmed cell death. Fifteen days after birth this procedure has already been completed.

2.3 Operation techniques

2.3.1 Preoperational narcosis

The rats were anesthetized via intraperitoneal injection of a mixture of 0.1 ml Ketaminhydrochlorid and 0.05 ml Xylazin per 100 gr of weight. Animals were handled in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Institutional Animal Care and Use Committee. To confirm that the animals were in the stage of narcosis, the corneal reflex and the withdrawal reflex were controlled by mechanical irritation of the cornea and the footpad respectively. After confirming that the animals wouldn't suffer any pain, they were prepared to be set on the operation disc.

2.3.2 Preparation of the operation field

The anesthetized rats were shaved across the middle line of the scalp between the eyes and ears. The rats of the group planned to receive the graft were additionally shaved across the right femur. Each animal was fixated under a dissecting microscope on the operation dish by fixing its limbs with adhesive tabs. The head was stabilized in the desired position with a mouthclip. The tongue of the animal was pulled gently out to make sure that the air-intake remained unobstructed. A median incision was performed along the skull and the two parts of the scalp were drawn and held apart with two threads (4.0) on

each side, in order to keep the operation field accessible. An incision in the connective tissue was made with a scalpel across the edge of the frontal bone over the orbita, in order to achieve dorsal access to the orbital cavity. The revealed lacrymal glands were removed and the underlying *Musculus rectus superior* was slipped across. After removing the remaining gland-tissue, the *Musculus retractor bulbi*, which surrounds the optic nerve, was carefully incised and the optic nerve was revealed.

2.3.3 Transection and resuture of the optic nerve

The optic nerve was exposed in the same fashion as previously described. An incision was made across the upper half of the optic nerve sheath, approximately 3 mm intraorbitally from its root, with Vanass scissors. Two sutures 10.0 (Ethicon, Resorba, Nürnberg) were placed across the incision, in order to resuture the opposite sides of the neural sheath later. The optic nerve was carefully transected inside its sheath and the latter was resutured. Caution was taken to prevent traumatizing the retinal artery and cutting the prefixed sutures (Fig.4B). To compensate for the lost volume of the removed lacrymal glands, a pad of Gelfoam was placed posterior to the bulb. After applying an antibiotic cream, the scalp was resutured with a continuous “Donati-Blair” suture (4.0).

2.3.4 Transplantation of sciatic nerve graft onto the transected optic nerve

The anesthetized rats were stabilized on the operation dish and an incision was performed along the right femur. After cutting the femoral sheath, the femoral muscles were dissociated and the sciatic nerve was exposed (Fig.5D). The muscles, as well as the two edges of skin along the incision, were fixed wide open with threads. The exposed sciatic nerve was carefully dissociated from the surrounding tissue and a segment of it of approximately 3 cm was obtained and placed in a Petri dish filled with PBS, to be used for the autologous grafting.

After application of gentamicin sulfate ointment, the muscles were placed back in their original position. The skin was resutured with a continuous “Donati-Blair” suture (4.0).

The posterior pole of the left orbit and the origin of the optic nerve were exposed as previously described (preparation of the operation field). The bulb was stabilized with a 6-0 nylon suture through the *Musculus rectus superior* and the optic nerve was axotomized close to the eyeball, taking care not to damage the ophthalmic artery. The perineurium of one end of the sciatic nerve graft was sutured to the dural sheath of the part of the axotomized optic nerve proximal to the bulb by using three different sutures (Ethicon 10.0; Resorba, Nürnberg), so that a stable optic-sciatic nerve anastomosis was made (Fig.4C and 5). The distal part of the sciatic nerve graft was then placed subcutaneously under the scalp along a groove in the skull. The latter was drilled with a dentist-driller along the left orbit towards the occipital bone. In order to stabilize the anastomosis and compensate for the volume of the removed lacrymal glands, a pad of Gelfoam was placed posterior to the bulb. The scalp was resutured after application of an antibiotic cream on the wound across the midline.

2.3.5 Assessment of retinal artery integrity

During the operation, an eye gel (Vidisic) was applied to both eyes to prevent the corneas from getting dry. After the operation, the integrity of the retinal artery was assessed. An extra amount of eye gel was applied to the cornea, and the fundus was examined through a glass slide with the aid of a dissecting microscope to verify the preservation of the bloodflow in the retinal arteries.

2.3.6 Determination of the success of regeneration and degeneration

To verify the regenerative procedure in the group receiving the peripheral nerve graft, in contrast with the group receiving only the optic nerve transection, the method of retrograde labeling with 4Di-10Asp-crystals was utilized. The

surviving RGCs with their regenerating axons were labeled retrogradely 20 days postoperatively in five rats from the “transplantation” group and three rats from the “transection” one. The results were qualitatively and quantitatively compared with the retrogradely labeled retinas of the rats in the control group.

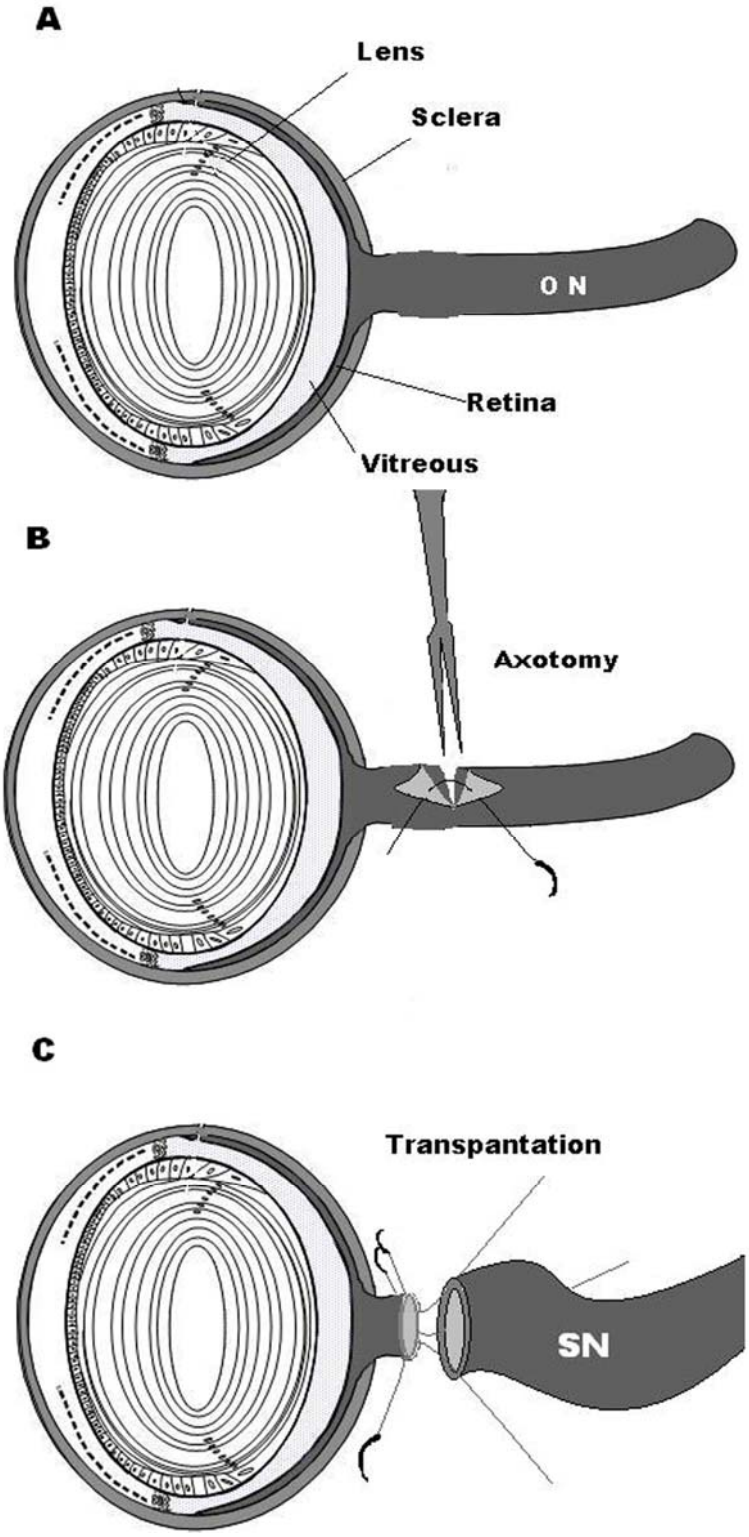


Fig.4 Graphic representation of the rat eye and the operational procedures.(A) Control, (B) Transection and resuture of Nervus Opticus, (C) Transplantation of SN.

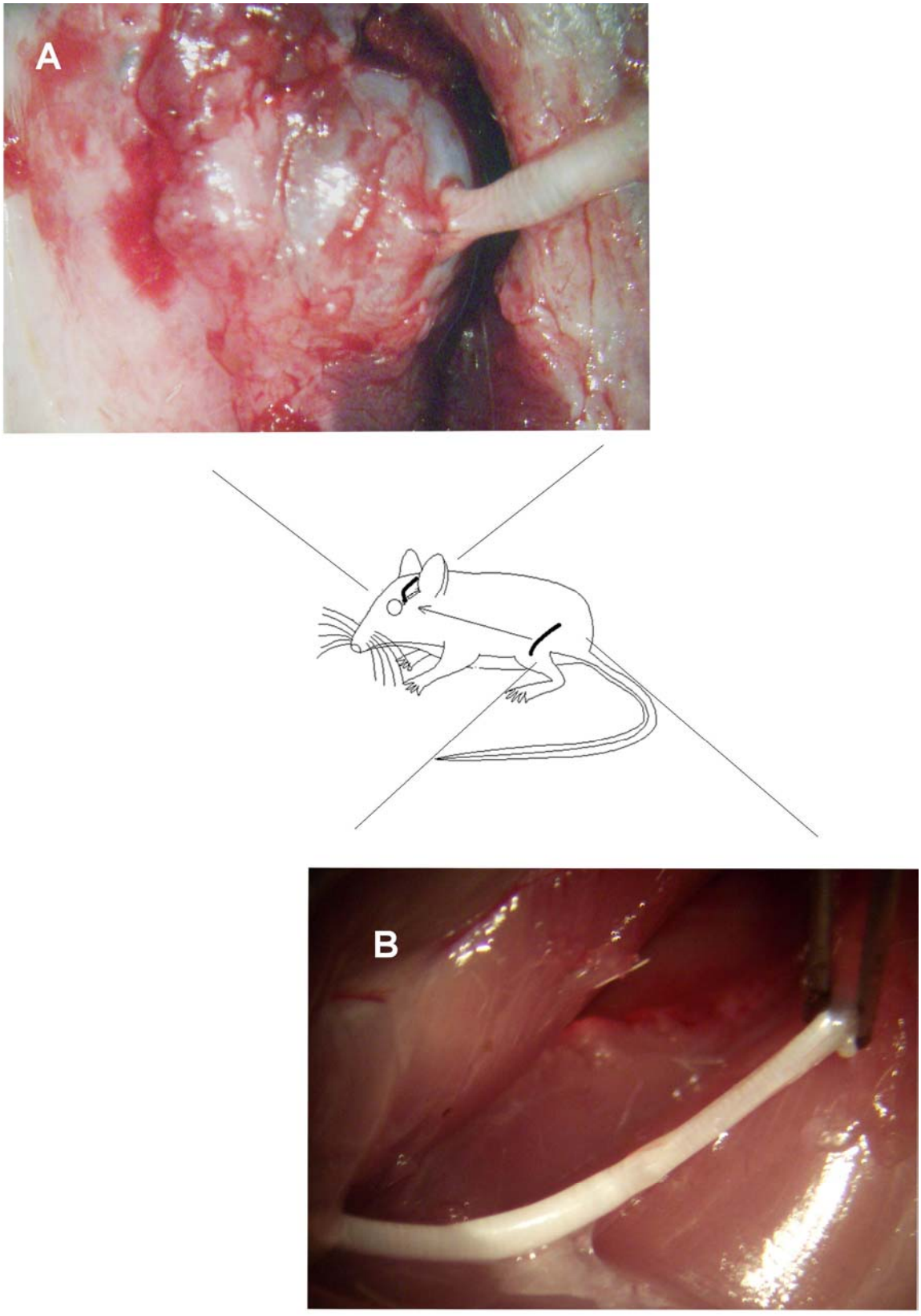


Fig. 5 Transplantation of the SN onto the proximal part of the ON (A). Exposure of sciatic nerve between the femoral muscles (B).

2.3.7 Retrograde labeling of the surviving RGCs

The technique of retrograde labeling is based on the ability of the living RGCs to receive specific molecular dyes and transport them via axonal transportation to the somata of the cells. In this case the dye-substance used was 4-(4-didecylaminostyryl)-N methylpyridiniumjodid (4Di-10Asp).

Group receiving the graft: 20 days after receiving the graft, five rats were once more anesthetized, and a small incision was performed on the scalp next to the region of the blind end of the sciatic nerve. The sciatic nerve was exposed, a small incision was made approximately 1 cm from its blind end, and the 4Di-Asp crystals were fixed inside the endoneurium. The incised perineurium was occluded with absorptive Gelfoam to avoid deposition of the crystals. The scalp was then resutured. After seven days the dye-substance was transported to the somata of the regenerated axons of the RGCs. The animals were then euthanized. The operated eyes were enucleated, and the retinas were excised, flat-mounted and qualitatively examined under a fluorescence microscope.

Group receiving the transection and normal control group: 20 days after having one of their ONs transected as mentioned, three rats, together with three normal controls, had their retinas retrograde labeled in order to verify the RGC degeneration in the first group.

An incision was made along the skull-midline and the two sides of the scalp were drawn and held apart with two threads (4.0). From the contralateral to the operated eye side of the skull-cap the periosteum was removed with a scalpel. A square flake (ca 5x5 mm) of the parietal bone proximal to the lambda (node of the sagittal and the occipital suture) was removed contralaterally to the operated eye. The meninges were dissected and the underlying cortex was aspirated with a Pasteur-pipette attached to a water-pump by a rubber tube. As soon as the underlying superior colliculus (SC) was exposed, its pial septum was perforated with a pair of fine forceps. The fluorescent dye crystals,

dissolved in Freund's adjuvant, were placed over the SC. The cortical vacancy was then filled with Gelfoam embedded in antibiotic eyedrops (Gentamytrex) and the scalp was resutured. Seven days later, the eyes were enucleated. The retinas were excised, flat-mounted on an object glass-slide, and examined with a fluorescence microscope.

2.3.8 Eye enucleation and quantitative examination of the retina

After the desired postoperative period, the rats were euthanized in a CO2 chamber. The eyes were removed with scissors and immediately set in PBS. The eyes of the rats receiving the fluorescent dye were placed under a dissecting microscope. An incision was made through the sclera posterior to the ciliary body, and the anterior segment was removed. Four radial incisions were then performed with Vanass scissors on the remaining eyecup, creating four quarters. The vitreous body was removed and the retina was carefully separated from the sclera with forceps. The optic nerve head was cut off the sclera with a pair of Vanass scissors. The detached retina was then transferred flat-mounted on a nitrocellulose filter (Sartorious), with its inner part looking upwards. The whole preparation was then placed in 4% Paraformaldehyde (PFA), on an objective disc and examined with epifluorescence microscopy. For the quantitative examination, the RGCs were counted in 20 different square areas of 0.096 mm^2 each in each retina. The RGC density was calculated by dividing the RGC number in all the square areas by 20 x 0.096.

2.4 Laboratory techniques

2.4.1 Preparation of the enucleated eyes for cryotomy

The eyes were enucleated as described above, and immediately transferred onto frozen cryo-gel (Tissue-Tec® O.C.T. compound) placed on metal holders. The eyes, along with a piece of the transected nerve, were placed on the cryo-gel parallel to the horizontal axis of the metal dish. Additional cryo-gel was

placed to cover the tissue completely. Then a plastic safe-for-freezing container was filled with liquid nitrogen. The metal dishes containing the tissue were gripped with a pair of tweezers at one edge and held into the gaseous phase of the liquid nitrogen until the embedding medium looked homogenously white. This technique leads to slow freezing and therefore reduced the formation of ice crystals in the tissue. The frozen eyes were then placed with the metal microtome-dishes in the microtome. Sagittal sections of the eyeball and the attached nerve 12 µm thick were acquired on coated (gelatinized) glass slides (Eppendorf). The sections were made at the level of the optic nerve head. With every coated glass slide four to five sections were thaw mounted and stored at -20 °C.

2.4.2 Immunohistochemical preparation of the cryosections

Double-staining immunohistochemistry was performed with Cy2 or FITC-conjugated and TRITC-conjugated secondary antibodies. MMPs and TIMPs were double-stained with GFAP, whereas NF-200 was double-stained with GAP-43. The frozen sections on the coated glass slides were drawn around with a solvent resistant pen (Dako Pen), creating a water repelling circle. This circle provides a barrier to liquids, such as antibody solutions or chromogenic substrates applied to the sections, making it feasible to obtain uniform immunohistochemical staining results, and to reduce the amount of reagents. The tissues were then fixed in methanol for 10 minutes at -20°C. After that they were rinsed in PBS three times for five minutes each time. To block non-specific binding they were incubated in blocking buffer 10% FCS (foetal calf serum) for 30 minutes at room temperature. The primary antibodies were diluted in FCS in a test tube and applied to the tissue. The dilution is patterned by the antibody-supplier. One tissue on each glass slide was covered with FCS deprived of primary antibodies, to be used as a negative control (Fig. 6). The tissues were then incubated overnight at 4°C. Next day the slides were rinsed in PBS three times for five minutes each time, after which the secondary antibodies, diluted as previously described, were applied to every tissue (negative controls as

well). After incubating for one hour in a dark chamber at room temperature, they were rinsed again in PBS three times for five minutes each time. At the end, the PBS was drained from the slides and a mixture of Dapi/Mowiol (Hoechst 33258) was applied to the tissues to stain the DNA. The tissues were then covered with a cover-glass slide. After letting the mowiol dry stable for five hours the preparations were ready to be studied under the fluorescence microscope.

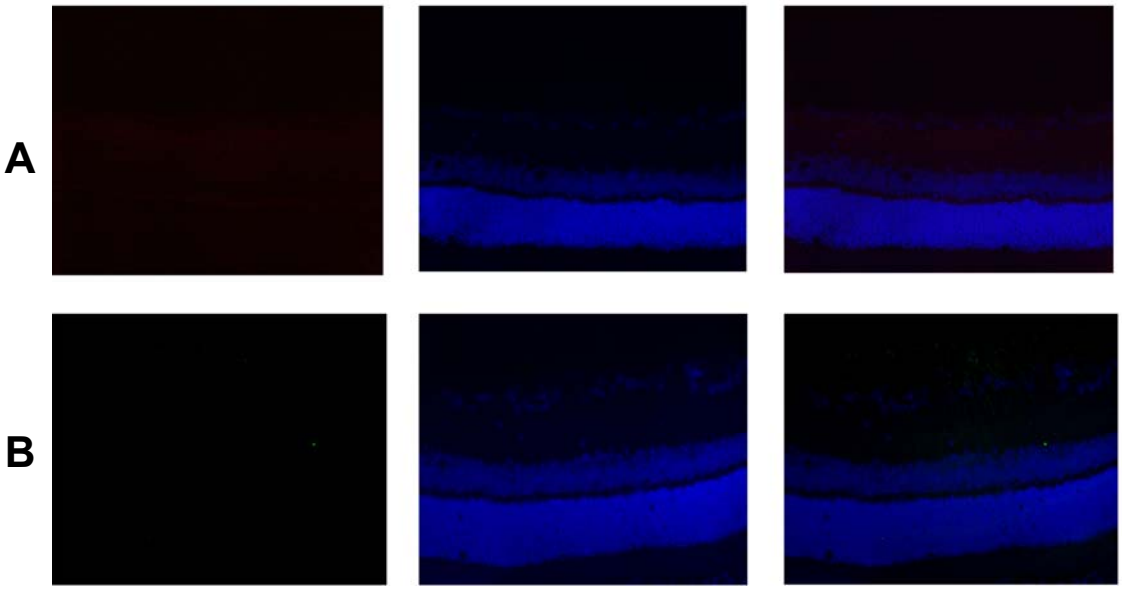


Fig.6 Negative-control stained sections incubated only with the secondary antibody did not exhibit any unspecific staining. (A) TRITC – Dapi – Merge. (B) FITC – Dapi – Merge.

2.4.3 Fluorescence Microscopy

The stained sections were examined under the fluorescence microscope "Axiophot" (Zeiss) equipped with reflected fluorescence illumination and digital imaging. Filter bandpasses were as follows:

1) UV excited, (395-440 nm) Blue fluorescence filter for DAPI detection;

2) Blue excited, (450-490 nm) Green fluorescence filter for Cy2 or FITC detection;

3) Green excited, (510-560 nm) Red fluorescence filter for TRITC detection.

Color digital images were collected with an Axiocam HRc camera (Zeiss) and a Pentium 4 Processor (INTEL) equipped with an image acquisition board (FUTJITSU SIEMENS) using AxioVision Rel. 4.5 software (Zeiss).

2.5 Result evaluation

Digital images were processed with Photoshop 8.0 software (Adobe Systems, San Jose, CA) using a Celeron (INTEL-centrino) processor. The images were then qualitatively studied. The fact that the immunostaining and the fluorescence microscopy were conducted under the same protocol for each tissue and at the same time made grading of protein expression, based on the intensity of the immunoreaction, reliable. The antibody expression in all the retinas belonging to the same group was qualitatively and quantitatively similar.

For the presentation of the results abbreviations of each experimental group will be used as follows:

(a) P0, P15, P90: for the subjects of postnatal days 0, 15 and 90 respectively.

(b) TS4, TS15, TS30: for the subjects that received the axotomy and resuture of the optic nerve and were euthanized on postoperative days 4, 15 and 30 respectively.

(c) TP4, TP15, TP30: for the subjects that received the peripheral nerve graft after axotomy of the optic nerve and were euthanized on postoperative days 4, 15 and 30 respectively.

3. Results

The qualitative and quantitative differences in MMP and TIMP expression between the groups, as well as the cellular changes, are going to be presented in this section. In the following figures of the retinal sagittal sections the inner layers are presented upside. In this experiment the same staining of every protein studied was consistently found in all animals of each group.

3.1 Quantitative control of regeneration and degeneration

For the quantitative examination, after being retrogradely labeled the RGCs were counted in 20 different square areas of 0.096 mm^2 each in each retina. The RGC density was calculated by dividing the RGC number in all the square areas by 20 x 0.096 (Fig.7 and 8).

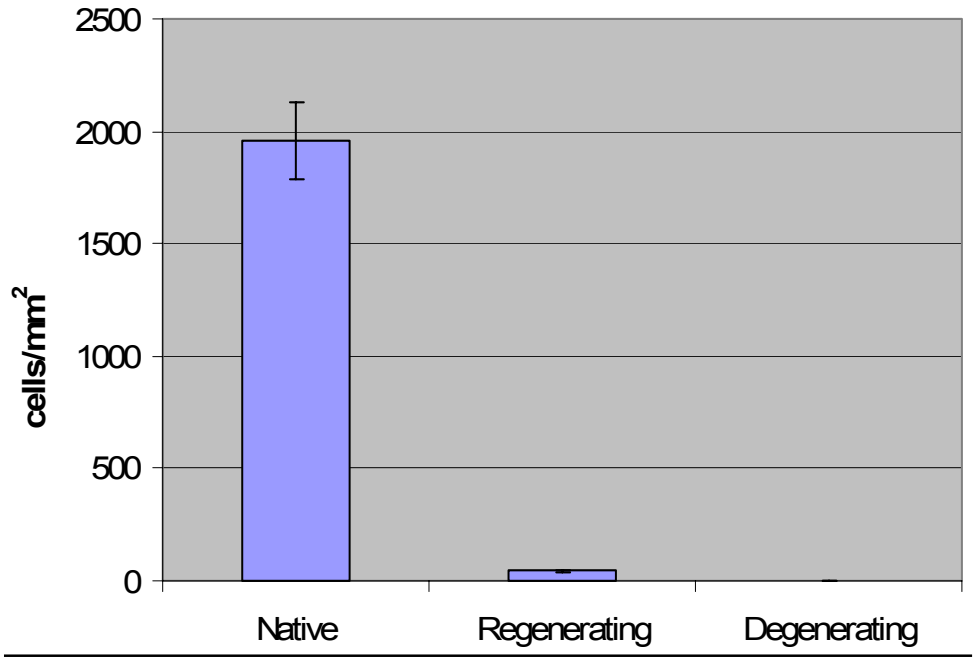


Fig.7 Histogram presenting the average number of RGCs per square milimeter of retina in the three different groups and standard deviation (SD). The normal control retinas have an average of 1970 RGCs per square milimeter. The average number of RGCs that regenerate after grafting of the PN to the axotomized ON is 47 cells/mm^2. In contrast, RGCs have degenerated in the retinas of the eyes receiving axotomy.

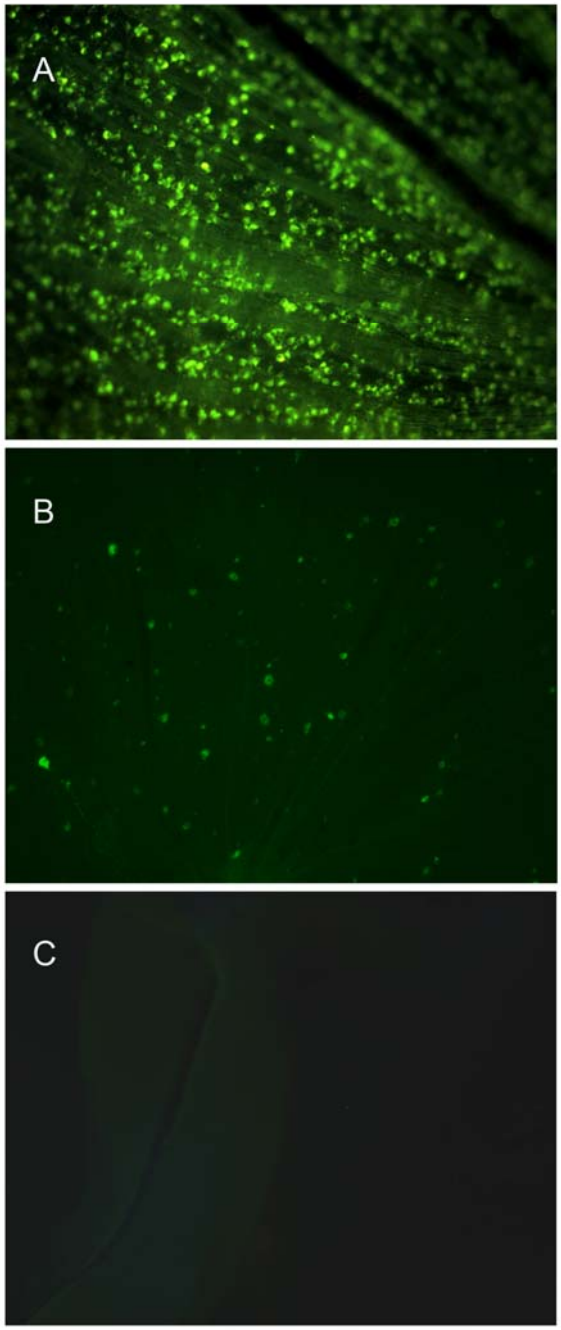


Fig.8 Flat mounted retinas seven days after being retrogradely labeled with 4Di-10ASP (Green). (A) Normal controls (native). (B) Transplantation of PN (regenerating). (C) Transection and resuturation of ON (degenerating). Qualitative estimation proves the regenerative properties of the second in comparison to the latter.

3.2 Qualitative control of cellular changes in the retina and the optic nerve

3.2.1 RGCs

The neuronal growth-associated protein GAP-43 and the mature neuronal marker NF-200 were used for the marking of axon-growing and quiescent RGCs respectively. In the first postnatal days during development (P0 – P10) there is vivid expression of GAP-43, indicating axonal sprouting (Dreher et al., 1985).On P90 the NF-200 is normally expressed, especially in the NFL (Fig.9, 10 and 11).

In the retina of the eyes that received a graft, there is immunoexpression of both antigens, which colocalize to a great extent. As indicated by the immunoreactivity of GAP-43, the regenerative procedure is moderately obvious in TP4, becomes more vivid in TP15 and evens out in TP30 (Fig.9). The neuronal marker NF-200 is expressed in a similar fashion, indicating the preservation to some extent of the neuronal tissue in the former group (Fig.10). GAP-43 is also expressed in the IPL and OPL, indicating that ganglion cells may remodel their neuronal network together with the other cell types in the damaged retina (Dijk et al., 2007).

In the retinas with the transected optic nerve, an attempt at regeneration during the first post-traumatic days is detected. There is good immunoexpression of GAP-43 in the retinas of TS4. The RGCs, however, degenerate during the following days. The expression of both GAP-43 and the neural axon detector NF-200 gradually decreases, so that in the TS-30 retina there is no expression of either antigen, indicating total RGC degeneration (Fig. 9, 10 and 11).

GAP-43

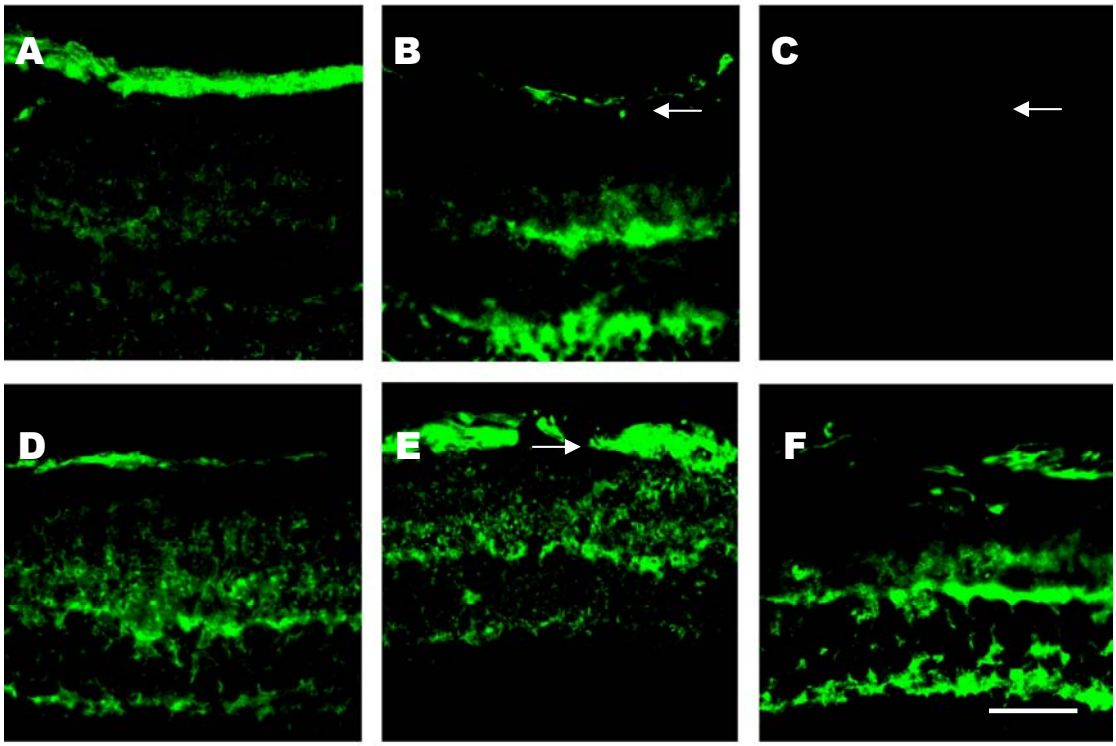


Fig.9 Retinal expression of GAP-43 seen at 20x magnification. (A) TS4. (B) TS15. (C) TS30. (D) TP4. (E) TP15. (F) TP30. The regenerative procedure is moderately obvious in TP4, becomes more vivid in TP15, and evens out in TP30. GAP-43 is also expressed in the IPL and OPL, indicating that ganglion cells may remodel their neuronal network together with the other cell types in the damaged retina (Dijk et al., 2007). The arrows indicate the GCL. The contrast between the regenerative procedures in the retina of the ON receiving the graft and the degenerative procedure in the transected one is also indicated by the arrows. (Bar = 20 µm).

NF-200

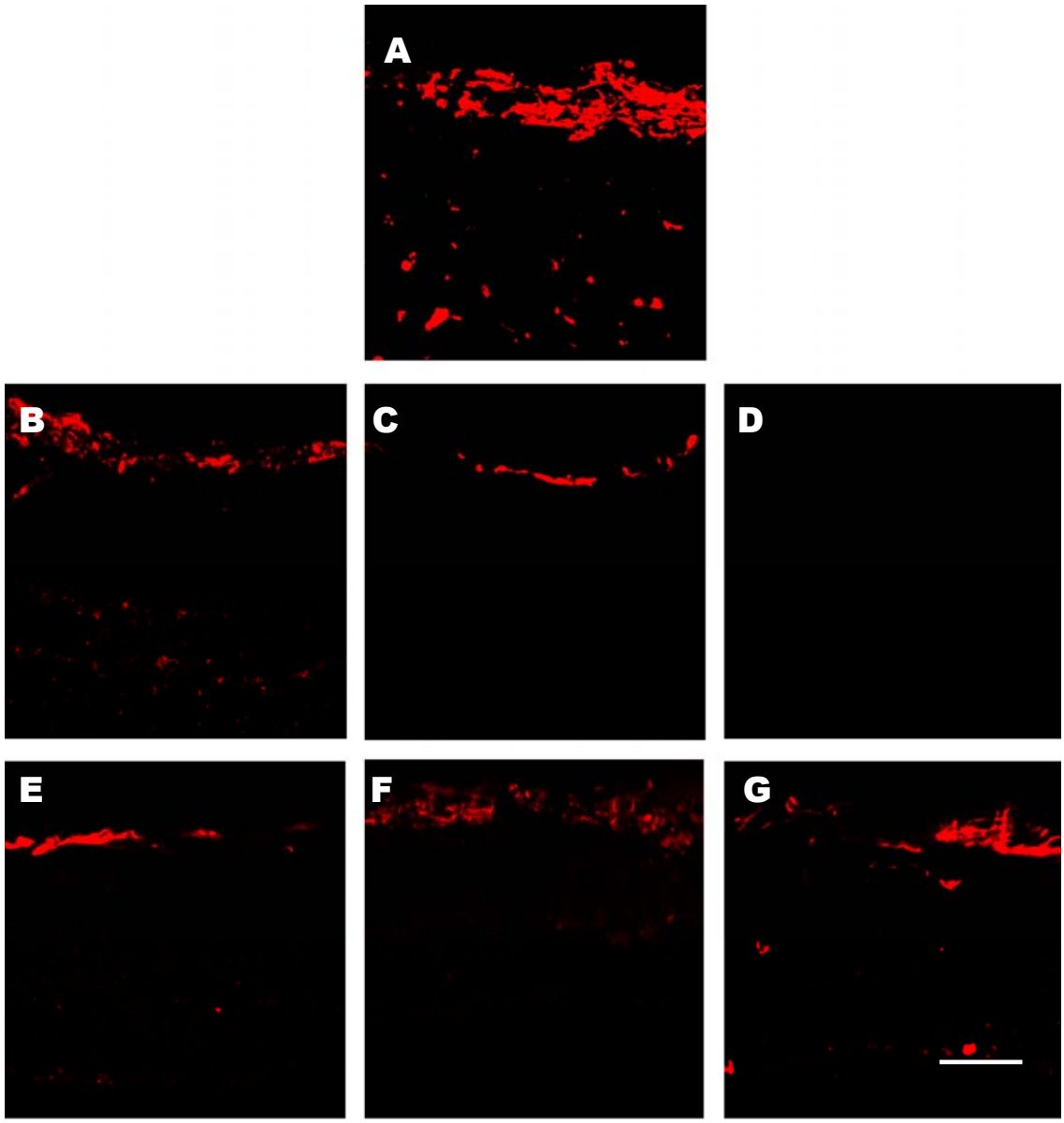


Fig.10 Retinal expression of NF-200 seen at 20x magnification. (A) P90. (B) TS4. (C) TS15. (D) TS30. (E) TP4. (F) TP15. (G) TP30. The progressive decrease of the neuronal marker in the degenerating group leads to its total absence in TS30 (D), whereas in the regenerating group it is preserved (E, F and G). (Bar = 20 µm).

MERGED (GAP-43 – DAPI – NF-200)

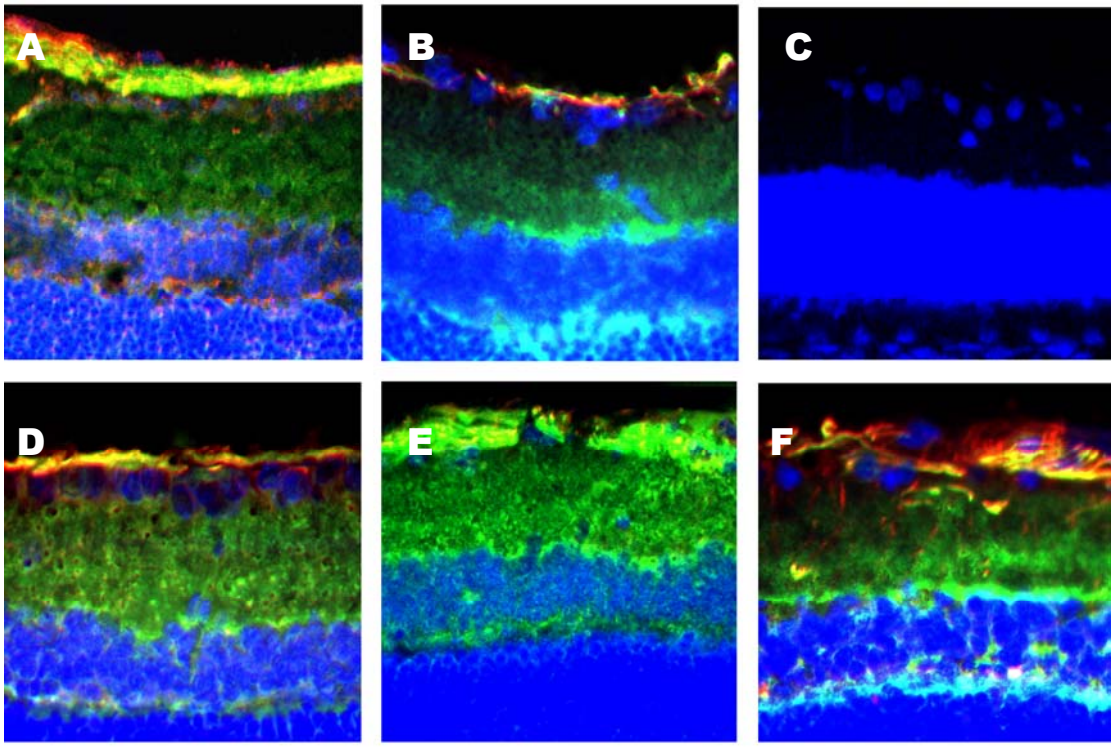


Fig.11 Retinal expression of GAP-43 and NF-200 seen at 20x magnification. (A) TS4. (B) TS15. (C) TS30. (D) TP4. (E) TP15. (F) TP30. The fainter expression of GAP-43 in TP30 in comparison to NF-200 indicates the moderation of the regenerative process (F). In TS30 all neural markers have disappeared (C). Green=GAP-43, Red=NF-200, Blue=DAPI

3.2.2 Glial cells

As would be expected, in the developing and mature retina, the astrocyte marker glial fibrillary acidic protein (GFAP) is expressed by astrocytes. These reside in the inner layers of the retina, as well as in the lamina cribrosa and optic nerve. After injury of the optic nerve caused by transection, astrocytes and Müller cells respond by accumulating GFAP, as the higher fluorescence of this protein indicates (Lewis and Fisher, 2003) (Fig.12).

GFAP

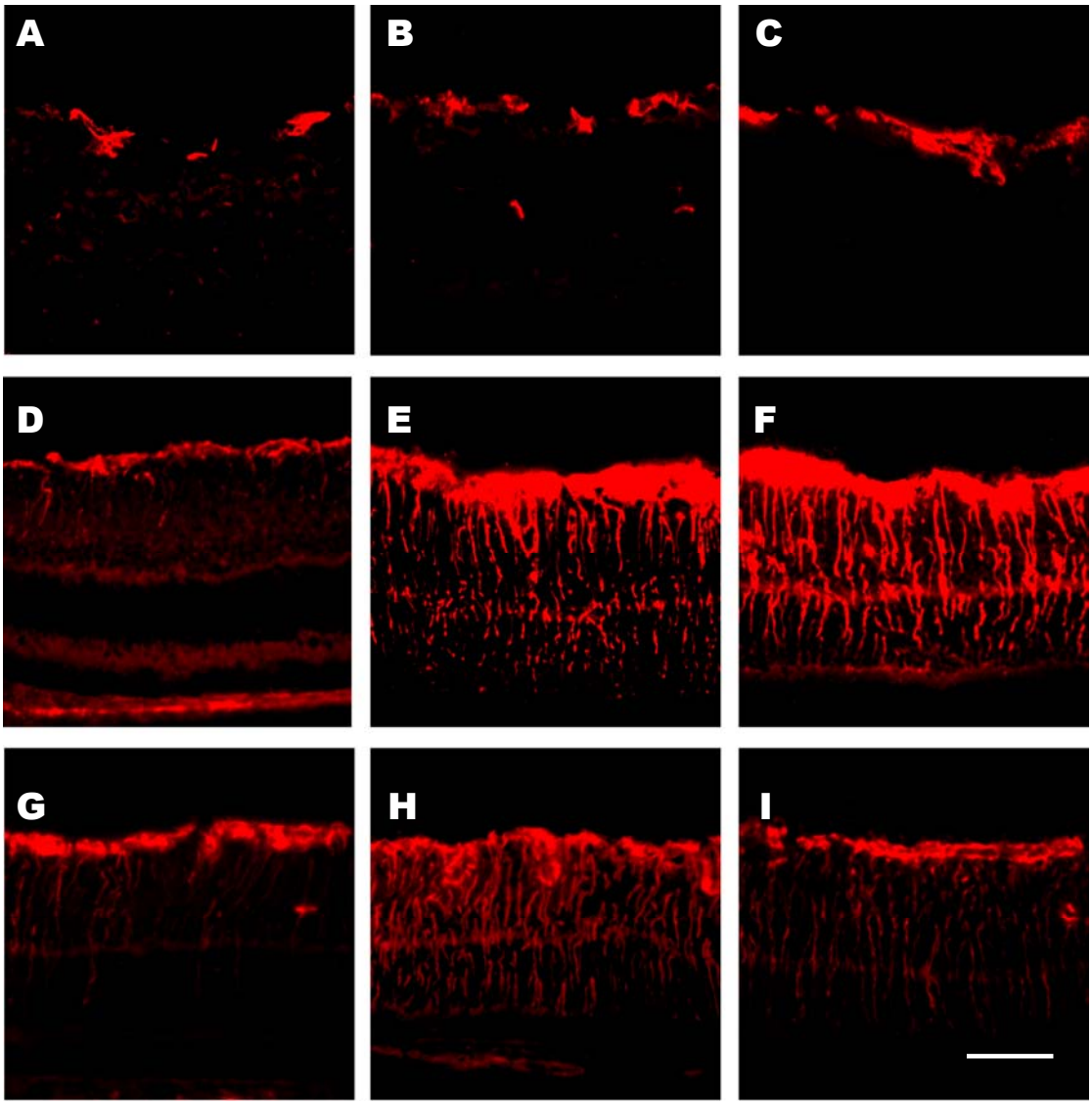


Fig.12 Retinal expression of GFAP seen at 20x magnification. (A) P0. (B) P15. (C) P90. (D) TS4. (E) TS15. (F) TS30. (G) TP4. (H) TP15. (I) TP30. After optic nerve transection, the expression of GFAP in astrocytes and Müller cells is activated. In TS4 and TP4 GFAP expression is initiated (D and G), whereas there is an obvious enhancement of GFAP fluorescence in later postoperative days (E, F, H and I). (Bar = 20 µm)

The chronological pattern of GFAP immunoreactivity following injury in the degenerating group points to an increase in the protein expression over time. In TS4 and TP4 the Müller cell and astrocyte activation is initiated, whereas there

is an obvious enhancement of GFAP expression in TS15 and TP15 which remains in TS30 and TP30 (Fig.12D, E, F, G, H and I).

3.3 Differential expression of the matrix metalloproteases and their inhibitors

3.3.1 MMP-1

During development, the immunoexpression of MMP-1 in the GCL is vivid and follows a diffuse pattern (Fig.13A, Fig.14A). Later, in the mature normal retina, the protein expression is vivid in the GCL and the INL, and seems to have an accumulative intracellular pattern rather than a diffuse extracellular one, possibly inside vesicles (Baba et al., 2004), (Fig.15F arrow). Double-staining with GFAP indicates that there is no immunocolocalization with the astrocyte somata (Fig.13B, Fig.14B).

In the first post-injury days (TS4, TP4), the immunoexpression of MMP-1 in the ECM of the outer retinal layers is not significantly altered and has a similar pattern to that found in mature normal tissue (Fig.13B, C and E).

Nevertheless, in later post-traumatic days the patterns in the groups of degenerative and regenerative tissue are different. During regeneration, the protease is diffusely expressed in the inner layers of the retina (IPL, GCL), rather than contained in intracellular vesicles. In the degenerating retina there seems to be some secretion of the protein in the ECM, but not as much as in the regenerating retina, and the protein expression pattern is minimally altered in comparison to the first post-injury days (Fig.13D, C and E). Double-staining and pictures of higher-magnification of the retina do not prove any immunocolocalization between MMP-1 and GFAP (Fig.14A, B, C, D, E and F; and Fig.15A, B, C, D, E and F).

MMP-1

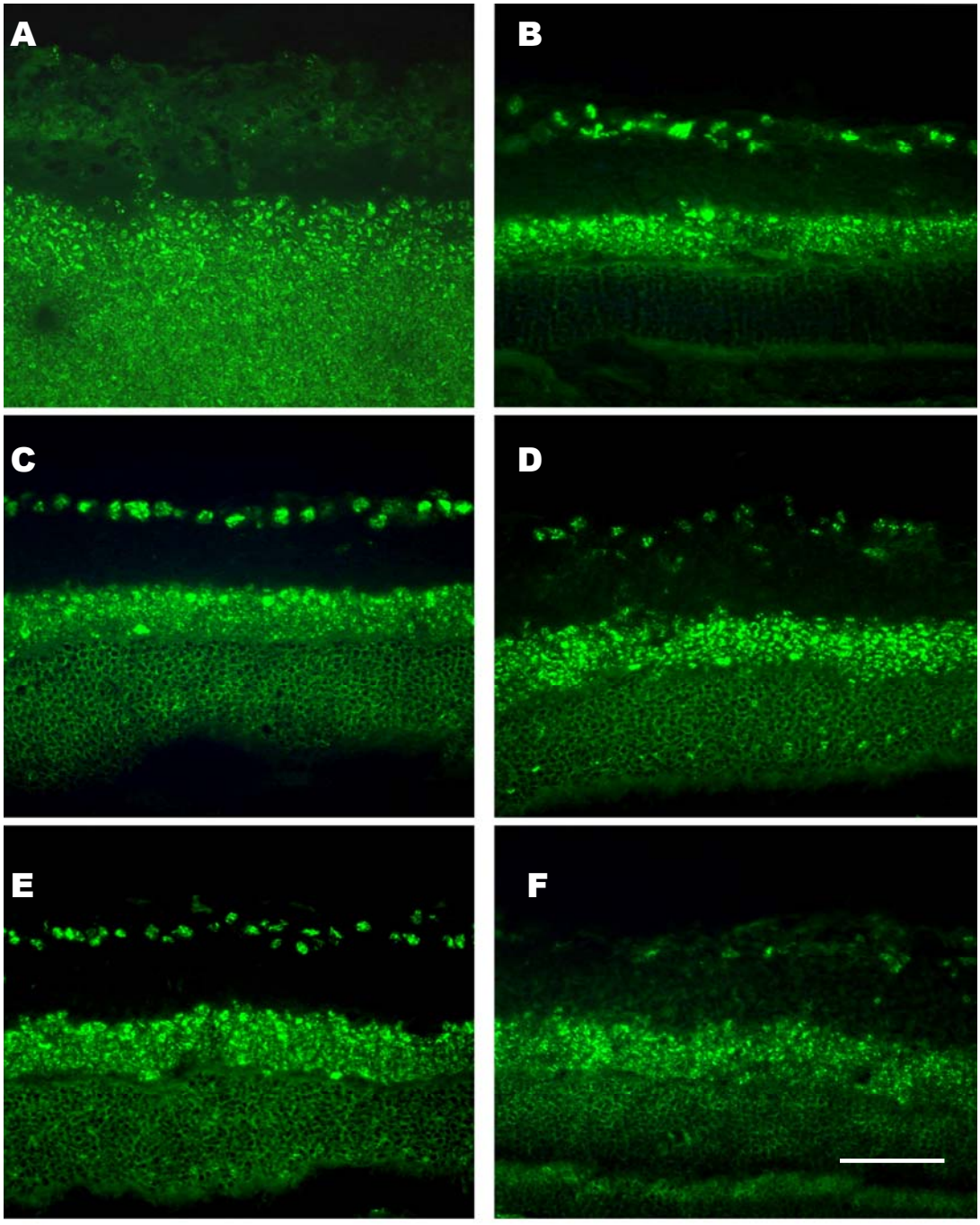


Fig.13 Retinal expression of MMP-1 seen at 20x magnification. (A) P0. (B) P90. (C) TS4. (D) TS30. (E) TP4. (F) TP30. (Bar = 20 µm). MMP-1 seems to be located mostly intracellularly in the normal and early post-traumatic tissue (B, C and E). In later post-traumatic days there seems to be some extracellular expression in the degenerating tissue (D), whereas in regenerating tissue there is vivid extracellular expression of the enzyme (F).

MERGED (MMP-1 – DAPI – GFAP)

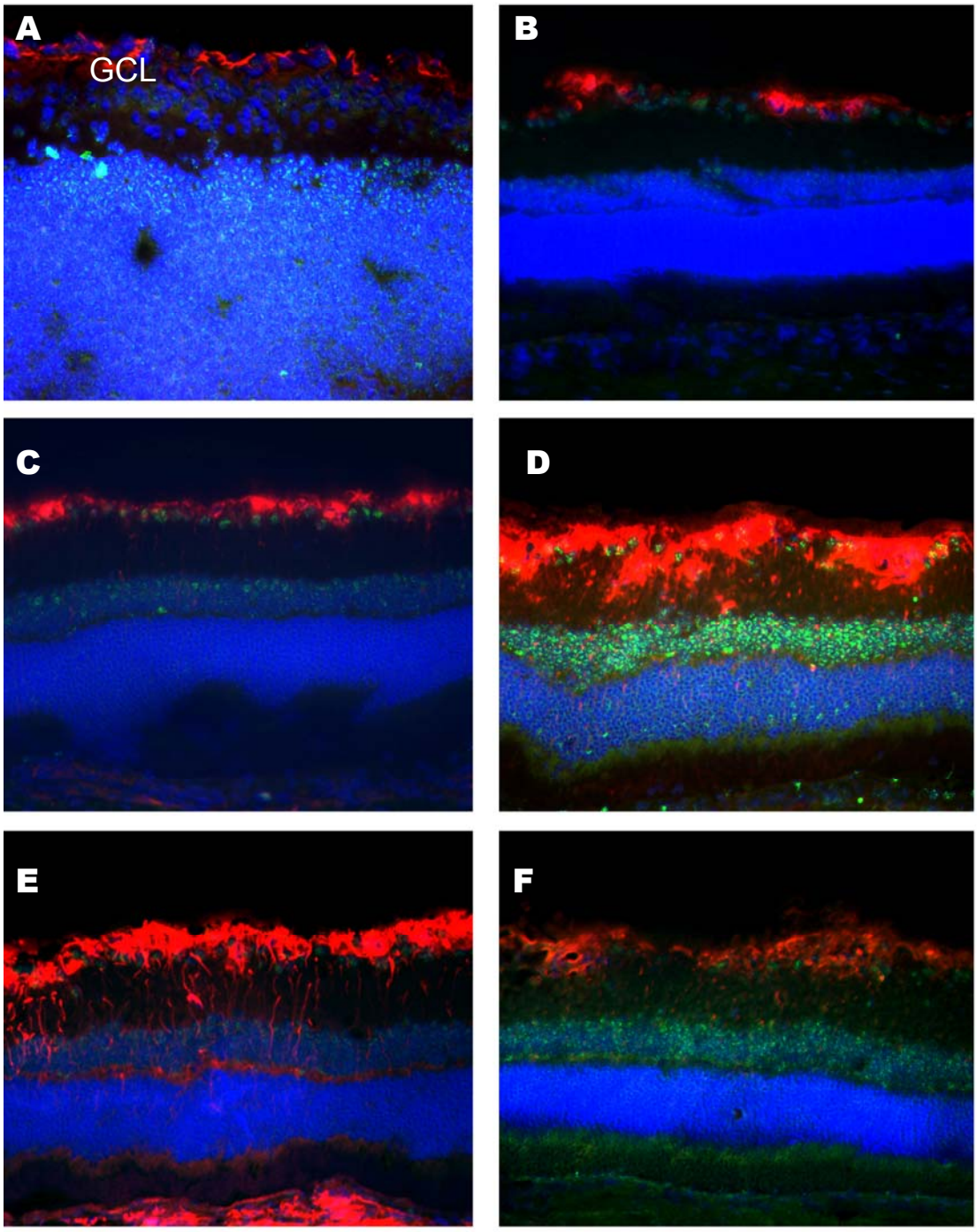


Fig.14 **Retinal expression of MMP-1 and GFAP merged with DAPI seen at 20x magnification. The enzyme appears to be expressed mainly in the INL and GCL. There is no colocalization with the retinal astrocytes. (A) P0. (B) P90. (C) TS4. (D) TS30. (E) TP4. (F) TP30. Green=MMP-1, Red=GFAP, Blue=DAPI.**

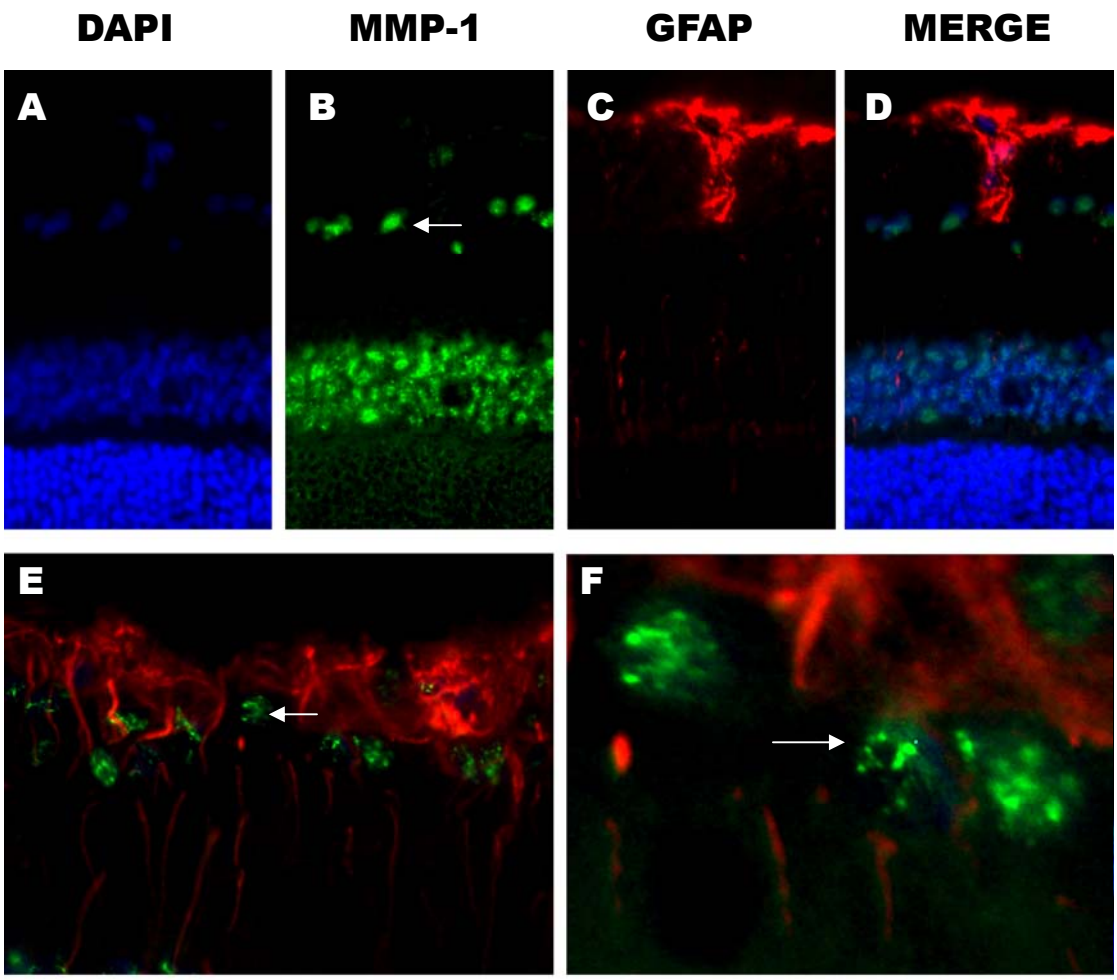


Fig.15 (A) TS4 40x retinal cell nuclei colored with DAPI, (B) MMP-1 in TS4 40x retina, (C) GFAP in TS4 40x retina, (D) merged, (E, F) TP4 63x retina merged. MMP-1 appears to be localized in intracellular vesicles (arrows). The vesicles containing the protease show no colocalization with GFAP (red).

3.3.2 MMP-2

In the case of MMP-2 there is strong immunoreactivity of the protein during development, indicated by its vivid expression in P0 and P15, while in P90 there is only residual expression (Fig.16 A, B and C).

From the first post-surgery days there is an obvious divergency in the immunoexpression of MMP-2 between the regenerating and degenerating

retinas. In TP4 the expression of MMP-2 is more vivid in the INL and the GCL compared to P90, while in TS4 it remains unaltered (Fig.16C, D and G).

Later, 15 and 30 days postoperatively, there is a stronger expression of the antigen in the inner layers, especially the GCL and the INL, of the retinas receiving the graft. On the other hand there is no significant change in the retinas of the degenerating group when they are compared to the control group (Fig.16E, F, H and I).

Gelatinase A is a secreted protease, therefore it has a diffuse pattern of immunoexpression. However, MMP-2 seems to colocalize marginally with GFAP to a great extent (Fig.17 and Fig.18). This could be explained by the fact that MMP-2 activation takes place on the cell membrane by the membrane-bound MMP-14 (Butler et al., 1998).

The former results demonstrate that MMP-2 is expressed in the retina during regeneration. The marginal colocalization of MMP-2 with GFAP suggests that the astrocytes and the activated Müller cells play a role in the activation of the protease.

MMP-2

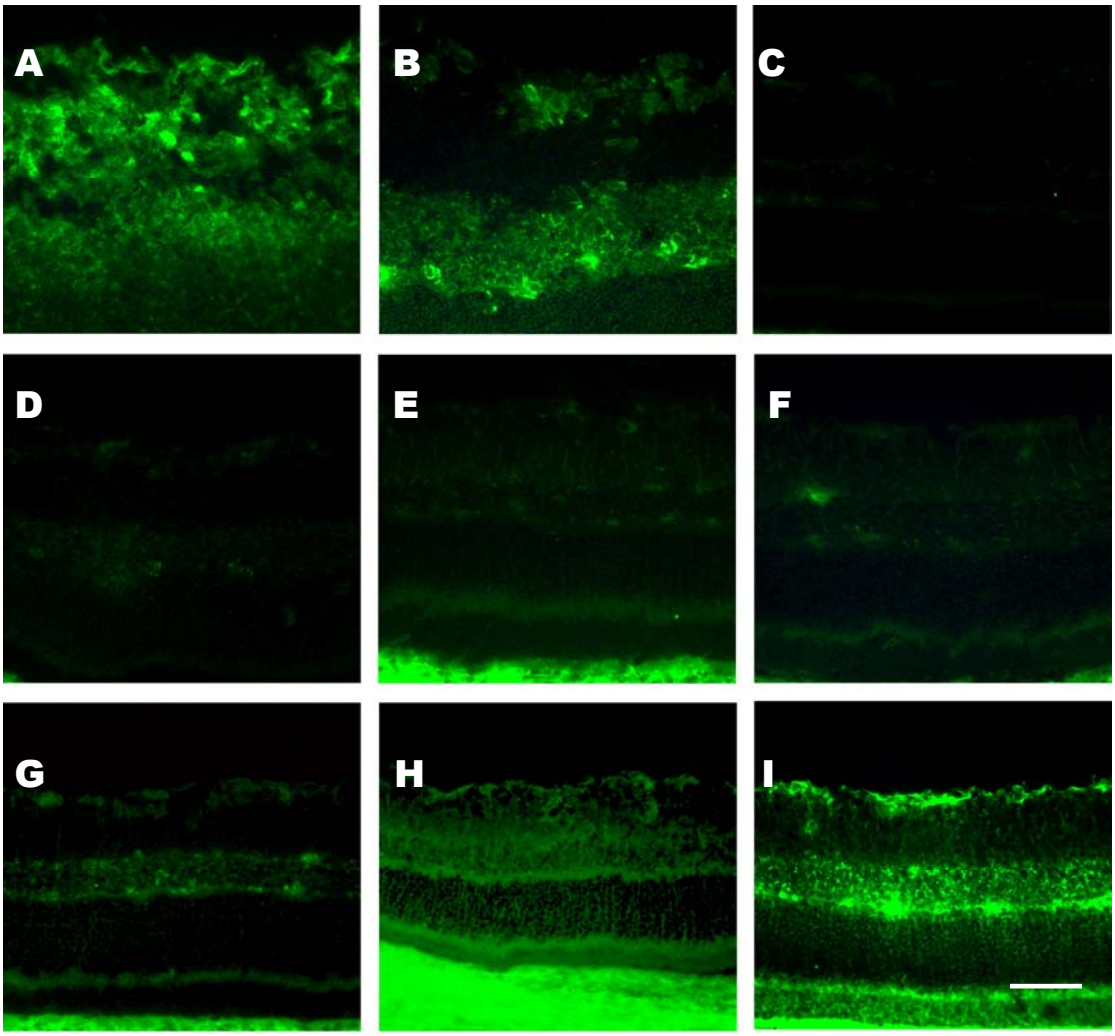


Fig.16 **Retinal expression of MMP-2 seen at 20x magnification. (A) P0. (B) P15. (C) P90. (D) TS4. (E) TS15. (F) TS30. (G) TP4. (H) TP15. (I) TP30. (Bar=20 µm). There is vivid expression of the protease in the retina during development (A and B). In the normal and degenerating retinas there is no significant expression of MMP-2 (C, D, E and F). In the regenerating tissue there is expression of the antibody against MMP-2, especially in the INL and the GCL, which intensifies with time (G, H and I).**

Table 6 **Grading of MMP-2 immunoreactivity.**

P0	**+ + + +**	**P15**	**+ + +**	**P90**	**+**
TS4	**+**	**TS15**	**+**	**TS30**	**+**
TP4	**+ + +**	**TP15**	**+ + + +**	**TP30**	**+ + + + +**

MERGED (MMP-2 – DAPI – GFAP)

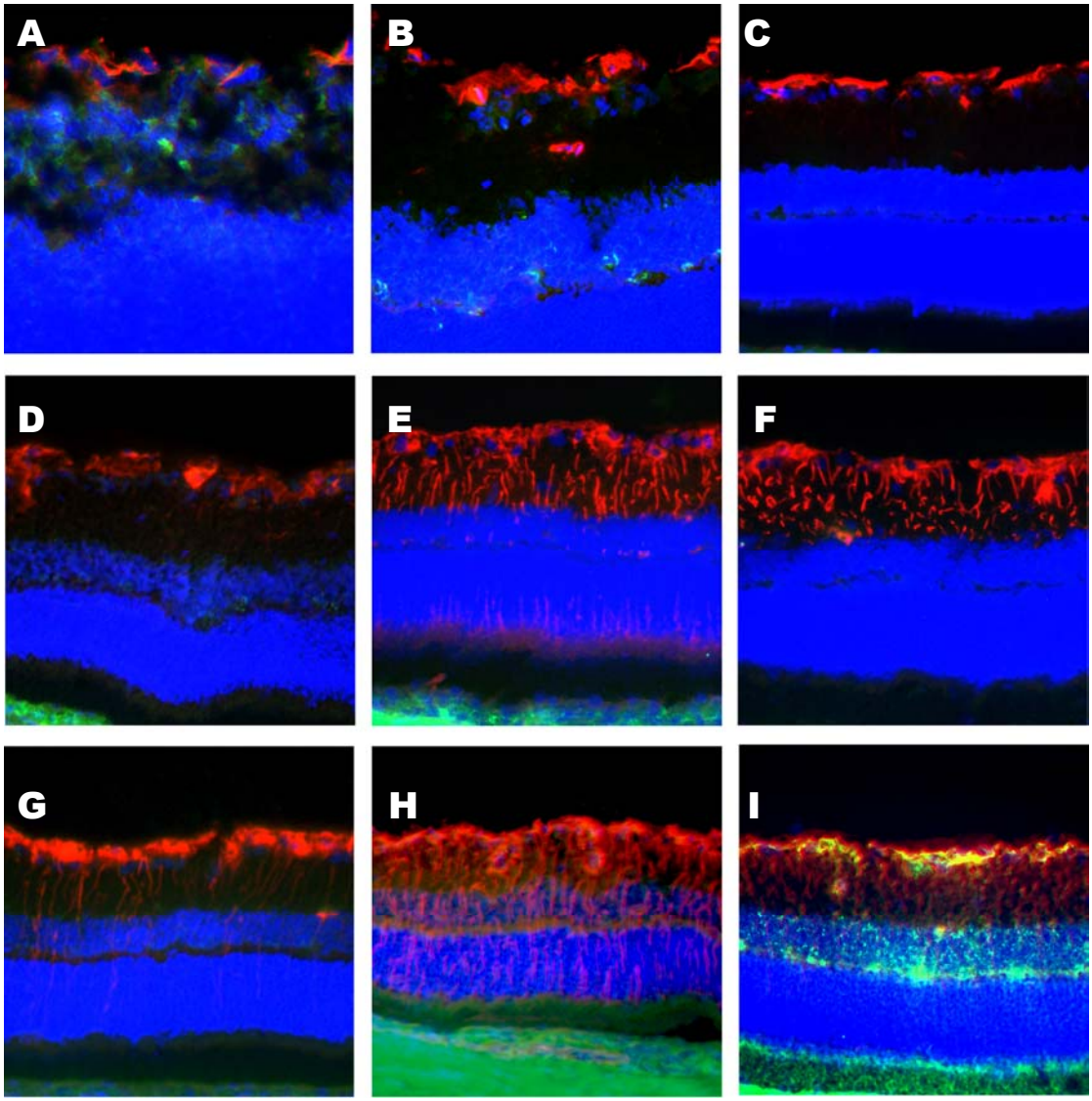


Fig.17 **Retinal expression of MMP-2 and GFAP seen at 20x magnification. (A) P0. (B) P15. (C) P90. (D) TS4. (E) TS15. (F) TS30. (G) TP4. (H) TP15. (I) TP30. There is partial immunocolocalization of the protease with the activated glial cells in the INL and the GCL (I). Green=MMP-2, Red=GFAP, Blue=DAPI**

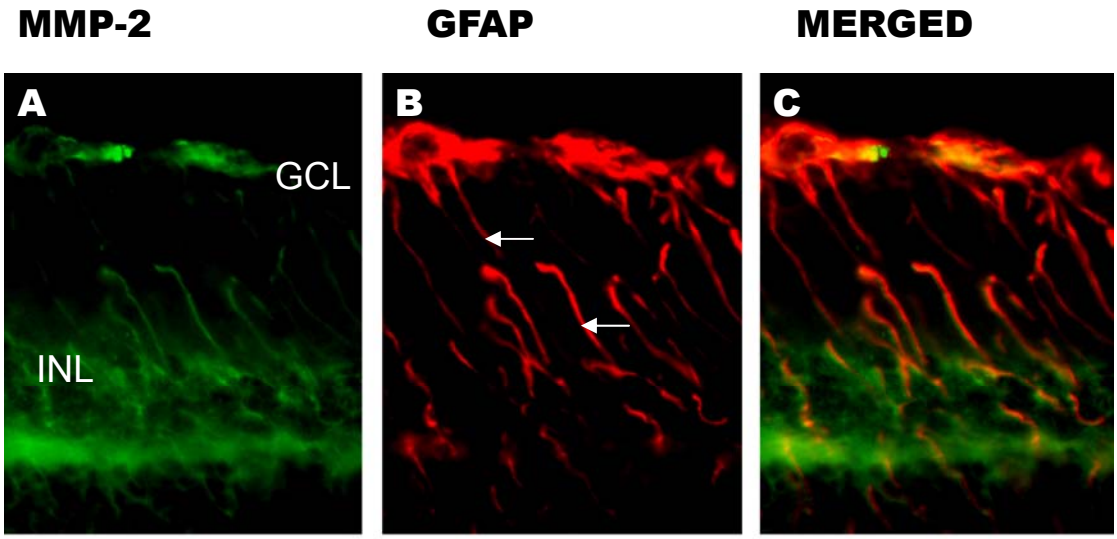


Fig.18 Localization of MMP-2 in close proximity to GFAP in T15 retina seen at 63x magnification. The processes of the activated Müller cells are indicated by the arrows. Green =MMP-2, Red =GFAP.

3.3.3 MMP-3

MMP-3 is vividly expressed in the developing retina, especially in the GCL. Its expression disappears during development. In the mature normal retina, the immunostaining of this protease is not significant (Fig.19A and B).

Post-surgically, the expression of MMP-3 follows reverse patterns between the two groups.

In the group receiving the graft the expression of MMP-3 is enhanced during the post-surgical period. In the TP4 retina, the MMP-3 immunoreactivity is significant, while in TP30 there is vivid expression of the protein throughout the retina (Fig.19E and F).

In the group with the degenerating retinas, the expression of MMP-3 shows only a marginal difference from that in the normal tissue (Fig.19C and D).

Double-staining with GFAP shows no significant colocalization with the Müller cells or the astrocytes (Fig. 20).

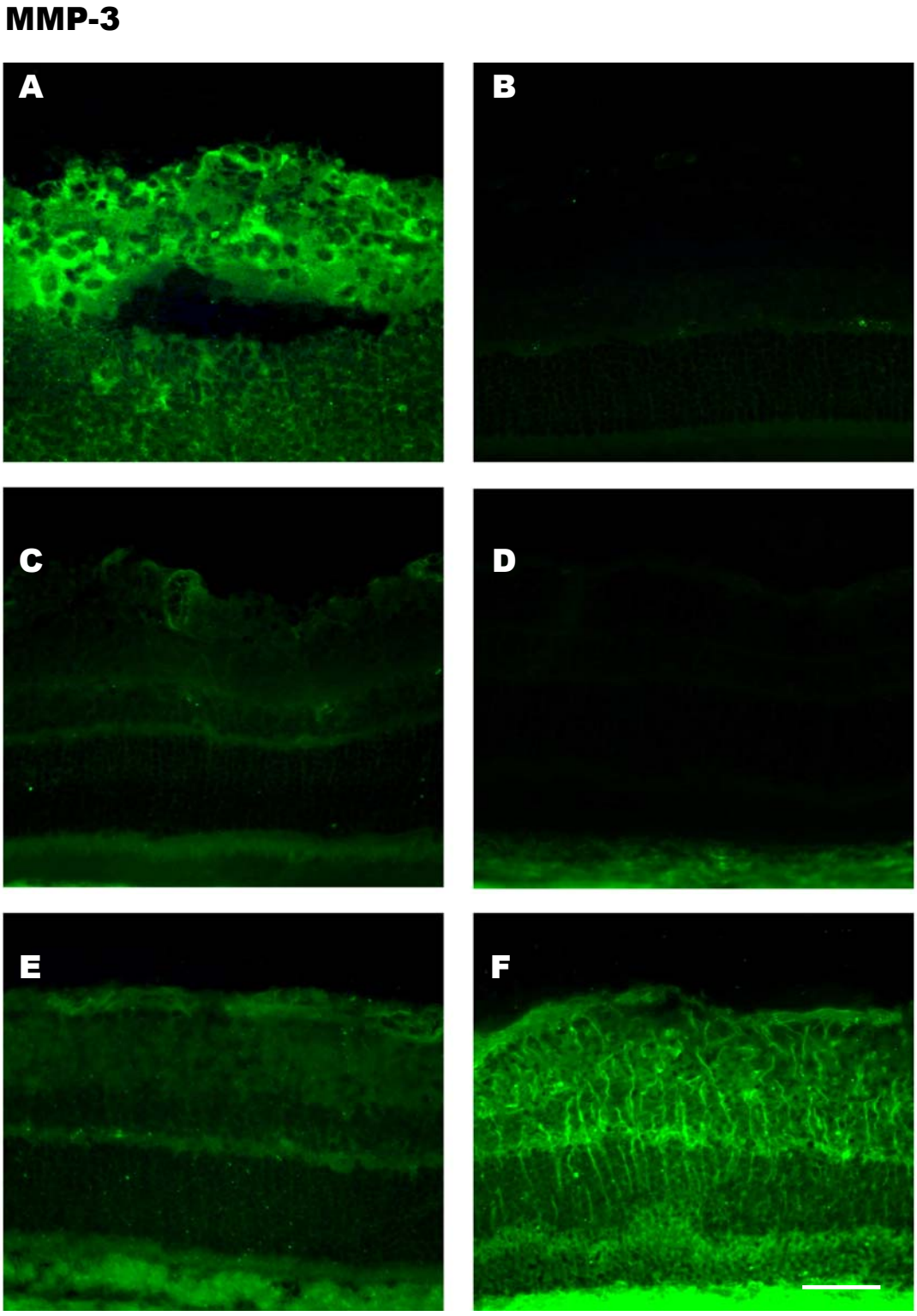


Fig.19 **Retinal expression of MMP-3 seen at 20x magnification (A) P0. (B) P90. (C) TS4. (D) TS30. (E) TP4. (F) TP30. In the developing as well as in the regenerating retina there is vivid expression of the protease (A, E and F). In the normal and degenerating tissue the immunoreactivity of the protein is insignificant (B, C and D).**

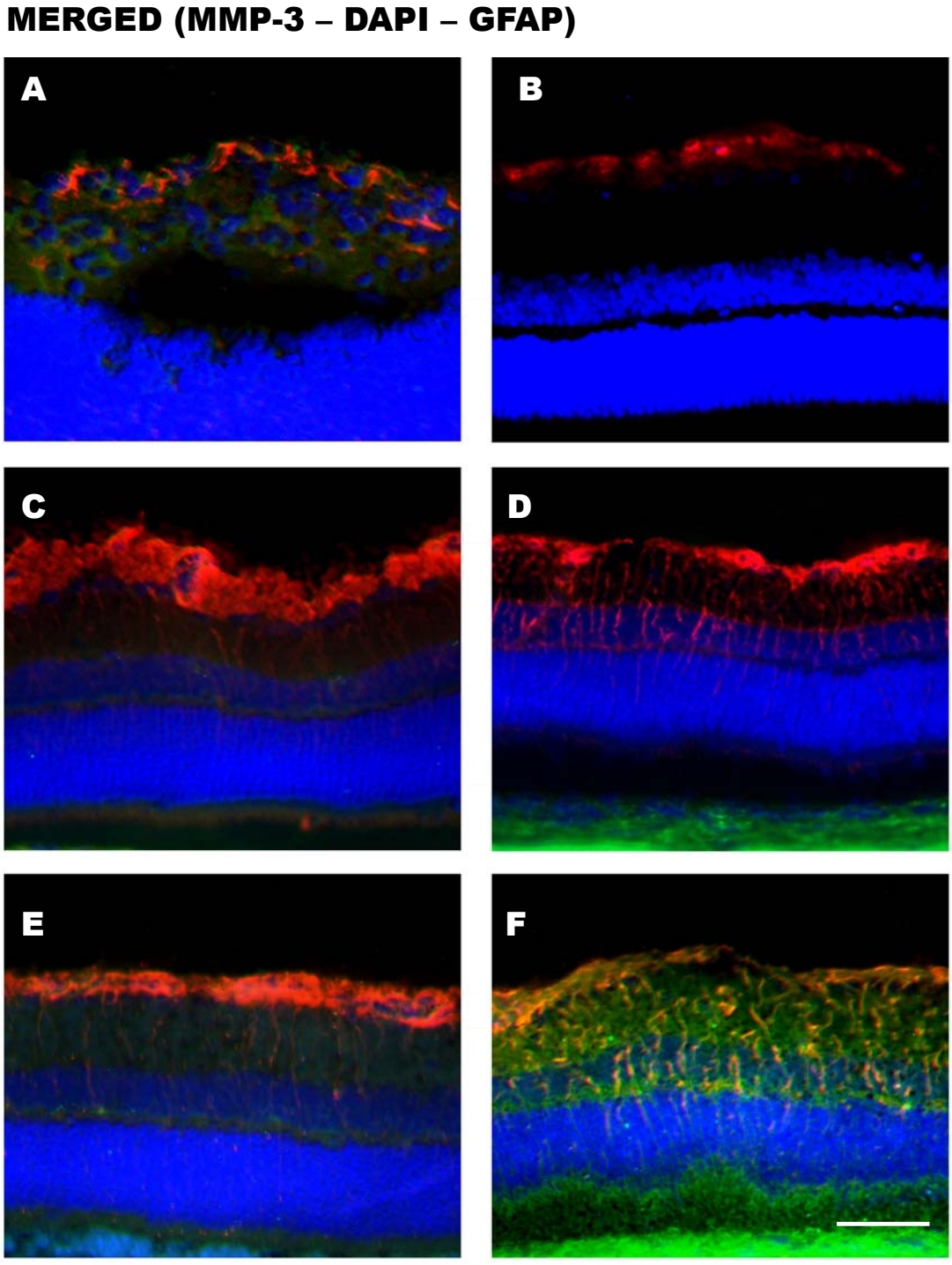


Fig.20 Partial colocalization of MMP-3 with GFAP seen at 20x magnification of the retina. (A) P0. (B) P90. (C) TS4. (D) TS30. (E) TP4. (F) TP30. Green=MMP-3, Red=GFAP, Blue=DAPI. (Bar=20 µm). There is no significant colocalization with the Müller cells or the astrocytes. However, the intensity of the immunoexpression of the protein seems to be increased in the ECM-glial cell membrane interface (F).

Table 7 Grading of MMP-3 immunoreactivity. The protease is expressed in the developing and regenerating retinas.

P0	+ + + +	P90	+
TS4	-	TS30	-
TP4	+	TP30	+ + + + +

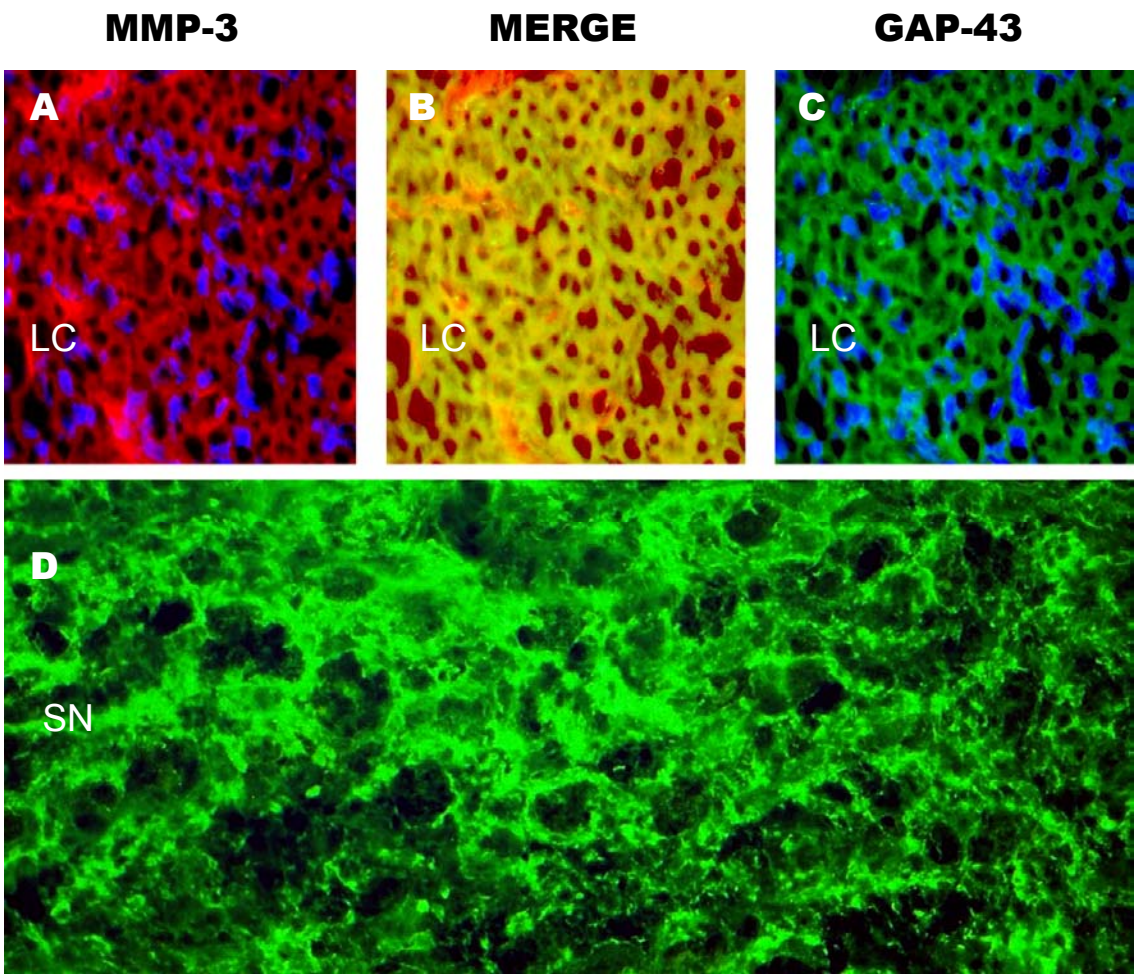


Fig.21 Colocalization of MMP-3 with GAP-43 in the lamina cribrosa seen at 40x magnification of TP15 (A, B and C). Red=MMP-3, Green=GAP-43, Blue=DAPI. In the lamina cribrosa there is almost absolute colocalization of MMP-3 with the regenerating axons of the ganglion cells. (D) Expression of MMP-3 (Green) in the sciatic nerve.

In the case of MMP-3 there is a pattern of diffuse expression of the protein. In TP30 there seems to be only marginal colocalization with the glial cells, where the intensity of the immunoexpression of the protein seems to be increased in the ECM-glial cell membrane interface (Fig.20F).

Moreover MMP-3 was strongly present in the sites of the regenerating axons inside the lamina cribrosa and the sciatic nerve, where it strongly colocalizes with the regenerating axons of the RGCs, as the immunocolocalization with GAP-43 indicates (Fig.21).

3.3.4 MMP-14

The membrane-bound metalloproteinase MMP-14 is immunoreactive in the inner layers of the retina during development, and becomes downregulated in mature tissue (Fig.22A, B and C).

Post-traumatically, it seems that the cells, especially in the IPL and the GCL of the retina, upregulate the expression of the membrane-bound protease in both groups, with a slight predominance in the group with the regenerating retinas (Fig.22D and G). However, in all the retinas of the TP15 group, there appears to be a decrease in the immunoreactivity of the protein, while it is re-enhanced in TP30 (Fig. 22H and I). This initially inconsequential pattern can be explained by considering the fact that MMP-14 plays a role in the activation of MMP-2, the immunoexpression of which is upregulated in TP15. The epitope recognized by the anti-MMP-14 antibody may be engaged in the course of MMP-2 activation during this period post-SN transplantation (Fig. 22 and Table 8).

Double-staining with GFAP and MMP-14 immunoexpression shows partial colocalization. This indicates that MMP-14 is expressed on the membrane of glial as well as other cells, and in their processes (Fig.23).

MMP-14

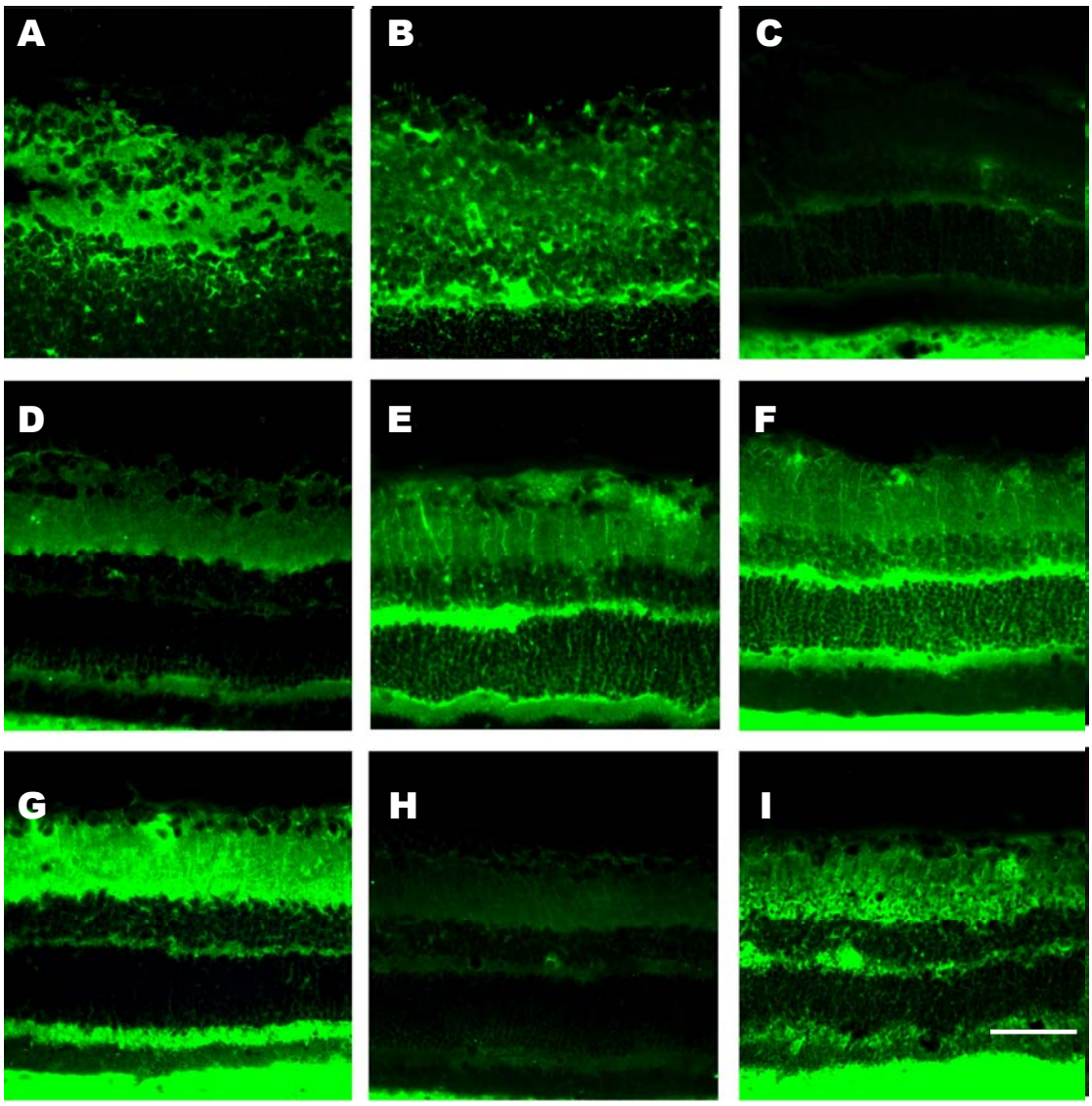


Fig.22 Retinal expression of MMP-14 seen at 20x magnification (A) P0. (B) P15. (C) P90. (D) TS4. (E) TS15. (F) TS30. (G) TP4. (H) TP15. (I) TP30. (Bar=20 µm). Post-traumatically there seems to be an upregulation of the membrane-bound protease (D and G). However, in all the retinas in the T15 group there appears to be a decrease in the immunoreactivity of the protein, and an enhancement in TP30 (H and I). This can be explained by the activation mechanism of MMP-2 by MMP-14.

Table 8 Grading of MMP-14 immunoreactivity in the retinas of the different groups.

P0	**+ + + +**	**P15**	**+ + +**	**P90**	**+**
TS4	**+ +**	**TS15**	**+ + + +**	**TS30**	**+ + + +**
TP4	**+ + +**	**TP15**	**+**	**TP30**	**+ + + +**

MERGED (MMP-14 – DAPI – GFAP)

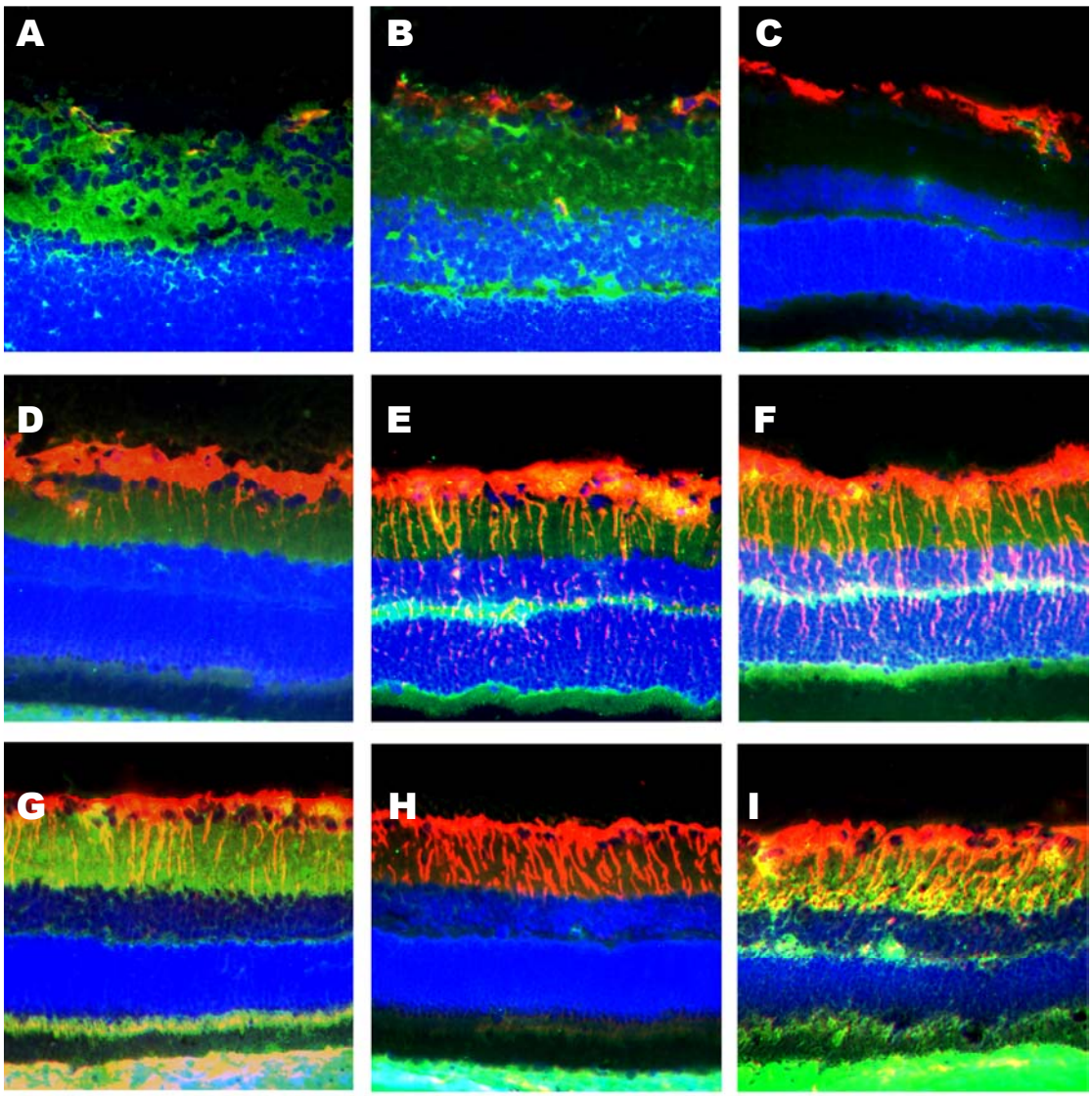


Fig.23 **Retinal expression of MMP-14 merged with GFAP seen at 20x magnification. (A) P0. (B) P15. (C) P90. (D) TS4. (E) TS15. (F) TS30. (G) TP4. (H) TP15. (I) TP30. Double-staining with GFAP and MMP-14 immunoexpression shows partial colocalization (D, E, F, G and I). Green=MMP-14, Red=GFAP, Blue=DAPI.**

3.3.5 TIMP-1

During development the expression of TIMP-1 is marginal throughout the retina. In the mature retina there seems to be some residual expression of this protease-inhibitor (Fig.24A and B).

After optic nerve transection, the immunoexpression of the protein follows different patterns in each group. During degeneration the protease inhibitor is downregulated with time until there is no significant expression of TIMP-1 in TS30 (Fig.24C and D).

On the other hand, the production of the protein is progressively upregulated during regeneration, resulting in strong immunoreactivity of the protein in TP30, localized especially in the GCL, the INL and the ONL, where the cell somata reside (Fig.24E and F).

Further coimmunostaining with GFAP reveals a partial immunocolocalization of TIMP-1 with the glial cells and their processes inside the retina (Fig.25).

Table 9 Grading of TIMP-1 immunoreactivity in the retinas of the different groups.

P0	**+**	**P90**	**+ +**
TS4	**+ +**	**TS30**	**+**
TP4	**+ +**	**TP30**	**+ + + + +**

TIMP-1

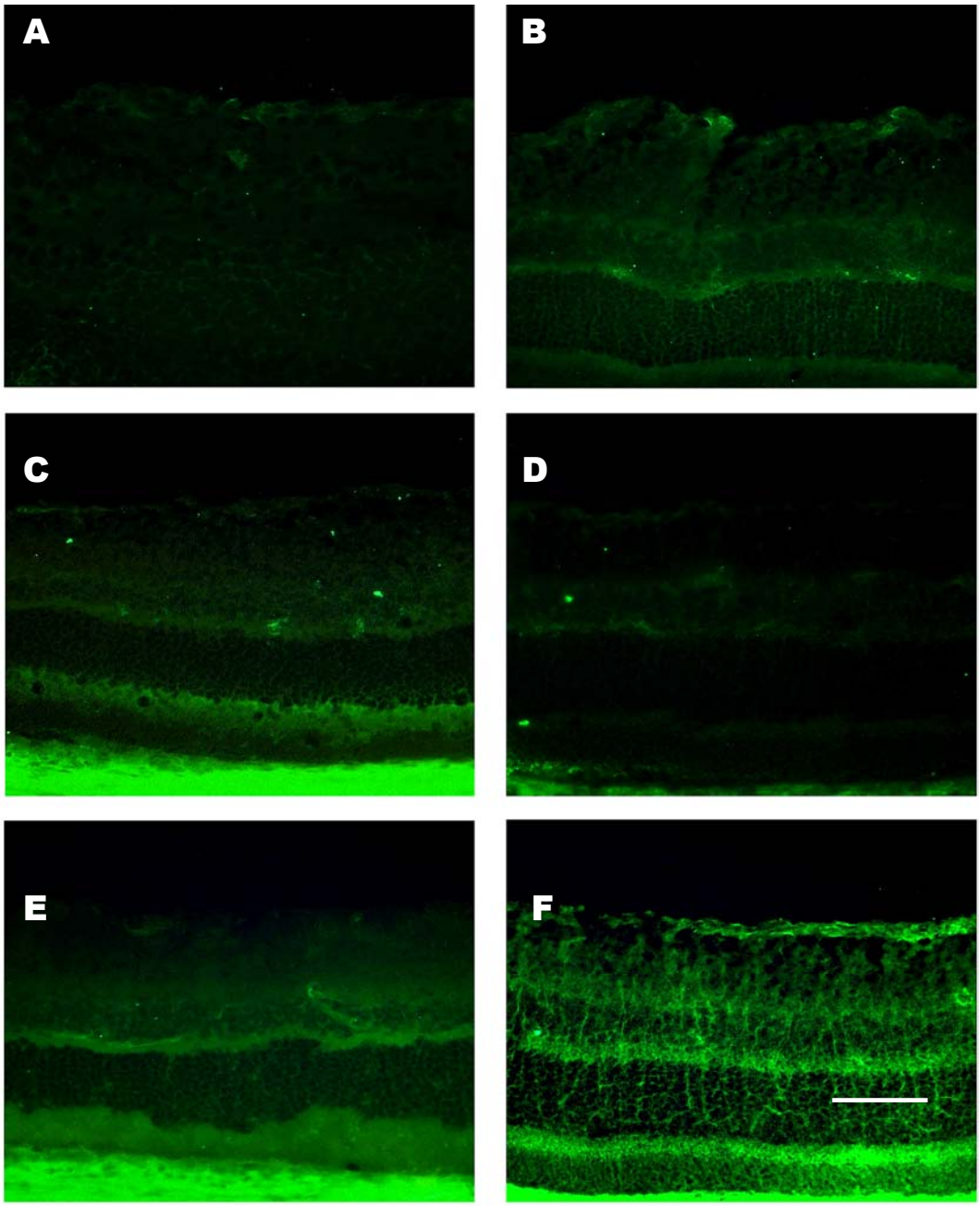


Fig.24 Retinal expression of TIMP-1 seen at 20x magnification. (A) P0. (B) P90. (C) TS4. (D) TS30. (E) TP4. (F) TP30. (Bar=20 µm). During development, as well as in the mature retina, the expression of TIMP-1 is marginal (A and B). During degeneration the protease inhibitor is downregulated with time until there is no significant expression of TIMP-1 in TS30 (C and D). In the regenerating retina, the production of the protein is progressively upregulated, resulting in strong immunoreactivity of the protein in TP30, localized especially in the GCL, the INL and the ONL (E and F).

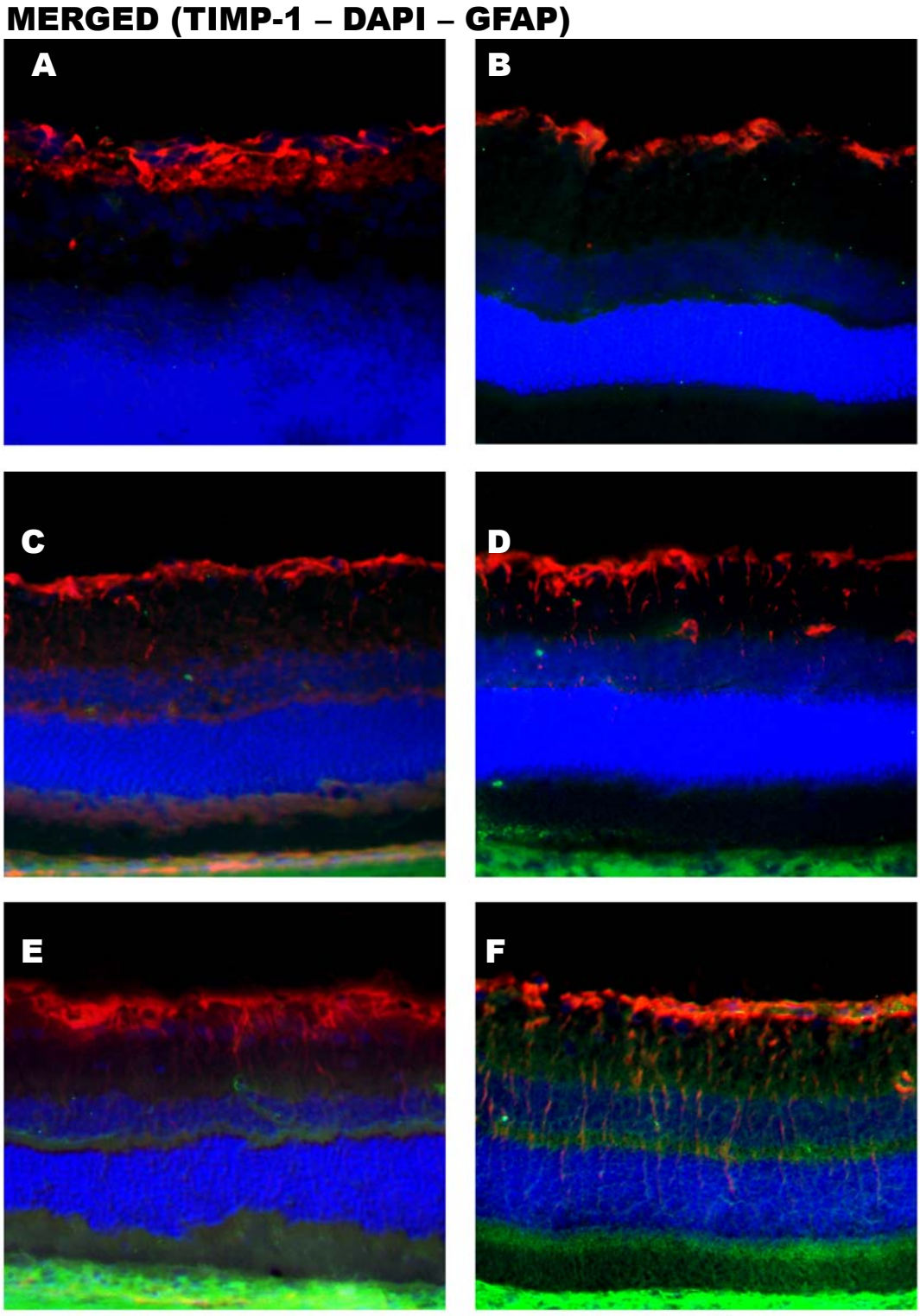


Fig.25 **Retinal expression of TIMP-1 along with GFAP seen at 20x magnification. (A) P0. (B) P90. (C) TS4. (D) TS30. (E) TP4. (F) TP30. Coimmunostaining with GFAP reveals a partial immunocolocalization of TIMP-1 with the glial cells and their processes inside the retina. Green=TIMP-1, Red=GFAP, Blue=DAPI.**

3.3.6 TIMP-3

During retinal development in P0, there is an absence of TIMP-3 immunoreactivity (Fig.26A).

After all the RGCs have reached their central targets in P15, the tissue inhibitor is vividly expressed throughout the GCL, INL and ONL of the retina (Fig.26B), and its expression is maintained slightly downregulated in the mature retina of the control group (Fig.26C).

In the degenerating group the immunoexpression pattern of the protein is maintained and remains unaltered in correlation with time, so that the pattern exhibited in TS30 resembles that of P90 (Fig.26D).

In contrast, in the regenerating retinas the protein immunoexpression is downregulated, until there is only a faint expression of TIMP-3 in TP30.

After coimmunostaining with GFAP, TIMP-3 is expressed throughout the extracellular matrix without any specific colocalization with the glia. This accords with the fact that TIMP-3 is a molecule that binds to the ECM and probably the cell membranes too (Fig.27).

Table 10 Grading of TIMP-3 immunoreactivity in the retinas of the different groups.

P0	**+**	**P15**	**+ + + + +**
P90	**+ + +**		
TS30	**+ + + +**	**TP30**	**+ +**

TIMP-3

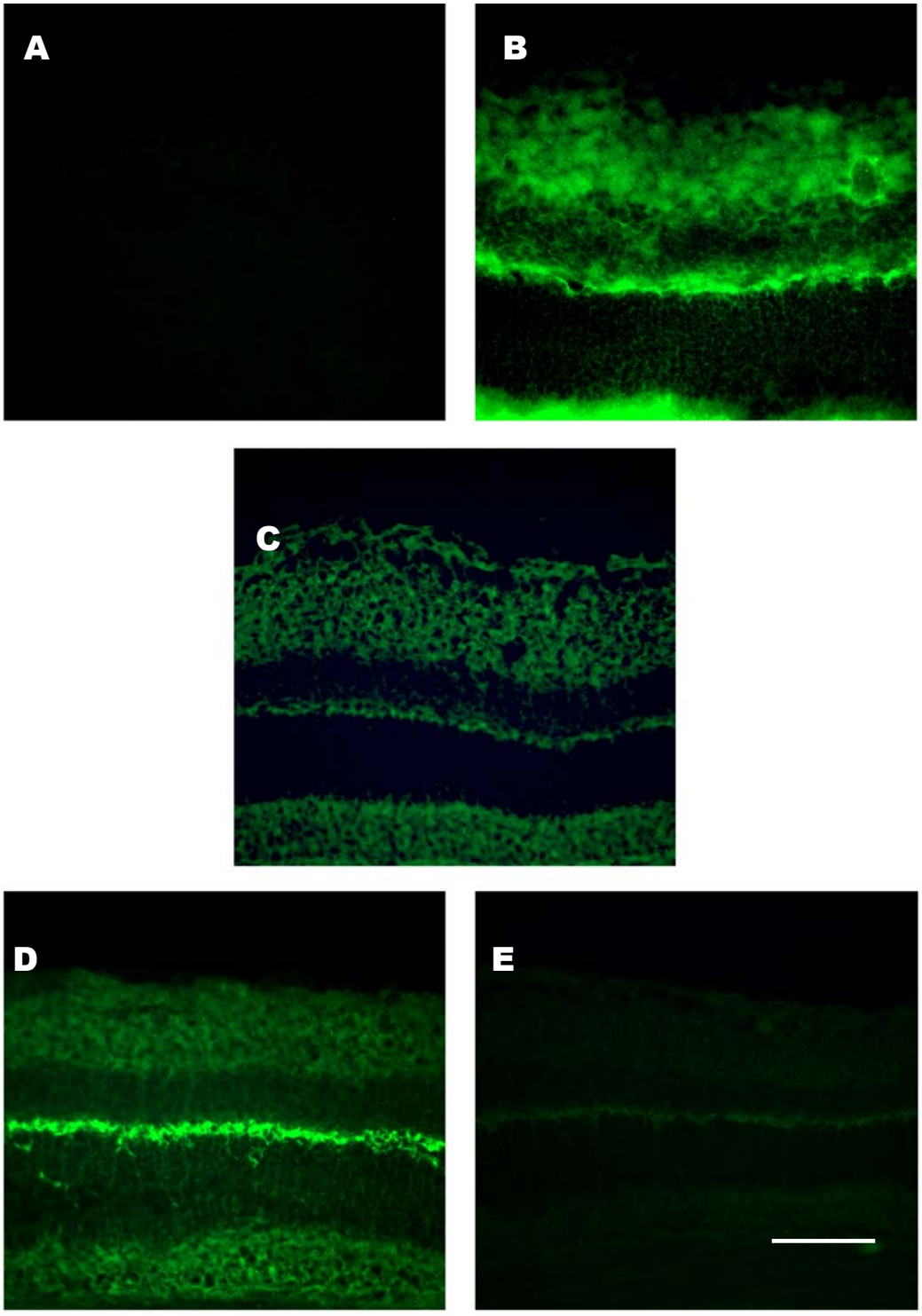


Fig.26 Retinal expression of TIMP-3 seen at 20x magnification (A) P0. (B) P15. (C) P90. (D) TS30. (E) TP30. (Bar=20 µm). During the first postnatal days, as well as during regeneration, there is no expression of the protein (A and E). During the later days of retinal development, as well as during degeneration, there is vivid expression of the protein in the GCL, INL and ONL (B and D).

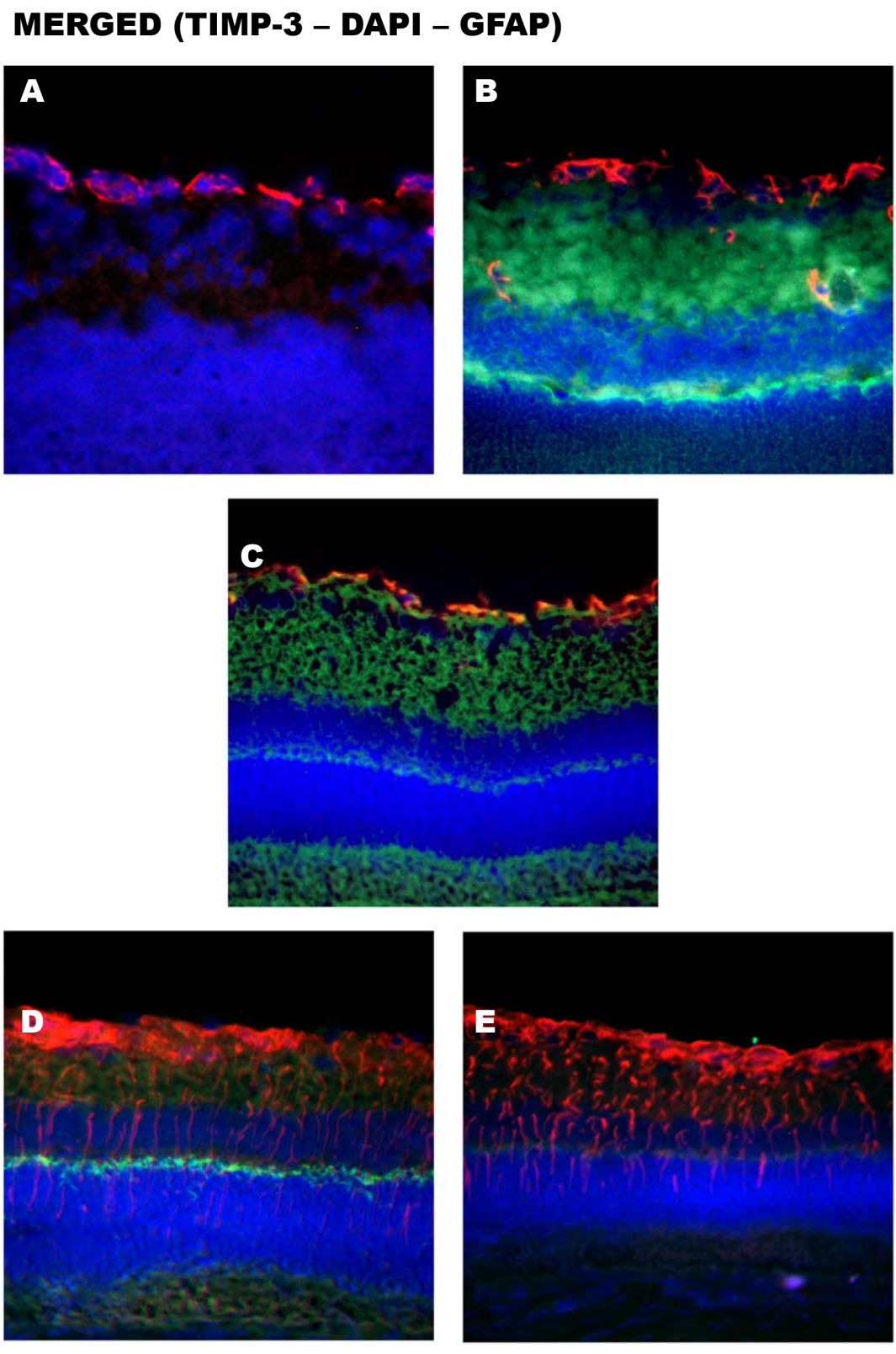


Fig.27 Retinal expression of TIMP-3 with GFAP seen at 20x magnification. The protein does not show any colocalization with the glia. (A) P0. (B) P15. (C) P90. (D) TS30. (E) TP30. Green=TIMP-3, Red=GFAP, Blue=DAPI

4. Discussion

4.1 MMPs and TIMPs in neuronal cell physiology

According to literature MMPs participate in many inflammatory responses. So far there have been described both primate and rodent models of nerve injury, mechanical or caused by excitotoxins and other neurotoxic agents, which exhibit many changes in the level and activity of many MMPs and TIMPs at the site of injury (Agapova et al., 2003; de Castro et al., 2000; Hebert et al., 2003).

Their activity has in some cases been considered undesirable, making them targets for therapeutic inhibition. For example, MMPs are speculated to impair the blood-brain barrier function by degrading components of the basal lamina, thus promoting inflammation and producing neurotoxicity (Rosenberg, 2002; Lo et al., 2002; Yong et al., 2001; Mandal et al., 2003). Additionally, MMPs may induce neuronal death either by direct action on the ECM (Gu et al., 2002) or by interfering with the proteolytic shedding of extracellular death receptors and death ligands on the cell surface (Wetzel et al., 2003).

On the other hand, it is also believed that MMPs may be involved in neuronal regeneration and injury repair in the adult nervous system, and are probably important in neurogenesis during development. Their neuroregenerative properties have been attributed to their ability to clear cellular debris and remodel the damaged extracellular matrix, as well as to facilitate axonal regrowth and remyelination by degrading chondroitin sulfate proteoglycans and other growth inhibiting molecules (Hayashita-Kinoh et al., 2001; Zuo et al., 1998; Ferguson and Muir, 2000; Duchossoy et al., 2001; Larsen et al., 2003). Moreover, MMPs are also responsible for the activation of growth factors and cytokines such as IGFs, FGFs, TGF-b, EGF and TNF-a, by releasing them from their ECM binding proteins (Sethi et al., 2000). Consequently they play a pivotal

role in controlling the signals elicited by matrix molecules, which regulate the growth and development of cells.

In development metalloproteinases are also indirectly implicated in the regulation of apoptosis and programmed cell death by modulating synapse formation between neuronal cells (Vaillant et al., 2003) or by interfering with the cell-ECM and cell-cell adhesion mechanisms, causing the cells to detach from their environment and leading to a form of PCD called anoikis (Boudreau et al., 1995; Lukashev and Werb, 1998). Especially in the retina, the inner limiting membrane (ILM) is believed to play a vital role in the survival, axon growth, and differentiation of RGCs (Halfter et al., 2005). Remodeling of the ILM may therefore lead to different cell-matrix interactions that may drive the cells into apoptotic pathways. On the other hand, application of MMP inhibitors during development results in decreased axon elongation and misguidance of the retinotectal tract (Hehr et al., 2005).

Taking all of this into consideration, MMPs and their regulators, TIMPs, seem to have an ambiguous role in neural tissue damage and healing. In order to decrypt this role, their expression has to be studied spatiotemporally under those conditions.

This study reports on the different patterns of MMP and TIMP expression in the retina of rat eyes in correlation with retinal cell changes during development, degeneration and regeneration. The focus of this study is the retina, and especially the GCL.

4.2 Evaluation of the results

According to literature, in normal retinas RGCs express MMP-2, TIMP-1 and TIMP-2 constitutively, while most other proteinases are secreted on a need-only basis from RGCs and other retinal cells into the ECM, where they become activated (Hernandez, 2000; Agapova et al., 2003; Ahmed et al., 2005).

In normal control retinas, most of the MMP-1 is detectable in intracellular vesicles, and is therefore in a zymogen form, mainly in the GCL and IPL. The vesicles containing the protein do not colocalize with the GFAP and are probably contained inside microglial cells which reside in those layers (Thanos et al., 1992).

In developing and regenerating retinas MMP-1 seems to have a similar pattern of immunoexpression. During development it is expressed vividly throughout the retina. This is to be expected since major changes concerning the ECM, such as synapse reformation and PCD, take place during this period (Vecino et al., 2004). During regeneration it seems that the enzyme inside the vesicles is secreted into the ECM. Cell death and survival through molecular signals is regulated by cell adhesion to the ECM via integrin attachment, direct or indirect connections to the actin cytoskeleton, growth factor receptors and intracellular signal transduction cascades. For example, it has been reported that the loss of β1 integrin engagement in detached cells suppresses EGFR expression, whose activation stimulates the Erk/MAPK cascade and suppresses anoikis (Reddig and Juliano, 2005). MMP-1 is not only responsible for the degradation of major molecules of the ECM, but has also been associated with neuronal cell death by enhancing caspase activity through activation of α2β1 integrin cell receptors - a mechanism that is irrelevant to its proteolytic properties. This was proved by the use of an MMP-1 inhibitor and a catalytic mutant of pro-MMP-1 where neuronal death was not avoided with the former and was stimulated with the latter (Conant et al., 2004b). MMP-1 was also postulated to activate the release of MMP-9 from both neural cells and monocyte/macrophages through a G protein

coupled receptor (Conant et al., 2002). The RGC–degenerative role of MMP-9 is well established: for example, activation of matrix metalloproteinase-9 via neuronal nitric oxide synthase contributes to NMDA-induced retinal ganglion cell death (Manabe et al., 2005), whereas an intravitreous injection of a membrane depolarization agent causes retinal degeneration via matrix metalloproteinase-9 (Mali et al., 2005).

The above could indicate pro-apoptotic as well as toxic effects of MMP-1. The results of this study suggest that during development and regeneration the microglia probably partially secrete the constitutively expressed intracellular zymogen. This probably happens to a limited extent in the case of degenerating tissue. This fact does not seem to correlate with the apoptotic properties of MMP-1 mentioned above.

Nevertheless, MMP-1 also seems to have other properties that enhance survival after injury in the tissue that expresses it. Namely, in some experiments it was postulated that for HIV dementia, the release and activation of MMP-1 via the Tat (HIV transactivating protein) was partly responsible for the activation and invasion of monocytes that released neurotoxins (Conant et al., 2004a). In later experiments it was found that MMP-1 actually attenuated the neurotoxicity of the Tat in fetal neuronal cultures by its enzymatic cleavage, revealing a self protecting neuronal mechanism rather than a toxic one (Rumbaugh et al., 2006). Those mechanisms are associated with the prevention of microglial activation. Indeed, whether MMPs exert pro- or anti-inflammatory effects via cleavage of inflammatory cytokines has not been clarified yet (Parks et al., 2004). It could be postulated that the secretion of MMP-1 is related to the apoptotic death of a portion of the RGCs, though it rescues some from the toxic death that the activated microglia could induce.

MMP-2 seems to have similar properties. MMP-2 (Gelatinase A) plays a critical role in BBB (blood-brain barrier) disruption, and astroglial and microglial cell activation, as well as in the pathogenesis of white matter lesions after chronic

cerebral hypoperfusion in rodents (Nakaji et al., 2006). MMP-2 is also an important metalloprotease, taking part in the reformation of the extracellular matrix and the retinal interneuronal synapses. According to literature, it is the only metalloprotease expressed constitutively from the RGCs. The results of this study show a minimal constitutive expression of MMP-2, followed by a minimal or no increase during the degeneration of RGCs. On the other hand, the expression of MMP-2 progressively increases during regeneration, and follows an expression-pattern the reverse of that witnessed during development. This actually indicates that MMP-2 expression enhances regeneration. Its intense expression during development is probably due to the structural reformation that takes place during this period, since MMP-2 has been shown to trigger the migration of retinal progenitor cells (Suzuki et al., 2006). The MMP-2 migration promoting properties are based, among other things, on its ability to facilitate cell motility by revealing an encrypted form of laminin-5 in the ECM (Giannelli et al., 1997). MMP-2, together with MMP-9, is implicated in axonal regrowth across the injury site, by cleavage of the CSPG by MMP-2, and the MBP (myelin binding protein) by MMP-9 (Ahmed et al., 2005).

However, it is possible that this “mechanical” regenerative property of MMP-2 is not the only factor involved. CSPGs are generally upregulated in the CNS after injury (Properzi et al., 2005). It has also been found that a disaccharidic degradation product of CSPG seems to have a neuroprotective influence on RGCs after an elevated intraocular pressure (IOP), possibly by modulating the immune responses of T-cells and microglia/macrophages to a neuroprotective level (Bakalash et al., 2007).

Taking into account all the above, MMP-2 could have an anti-inflammatory effect, something that accords with the results concerning microglia activation in this study.

Moreover, MMP-2 cleaves FGFR-1 and releases its soluble ectodomain, which can still bind FGFs but lacks the ability to promote its signal (Levi et al., 1996).

FGF-2 increases Bcl-2 and decreases Bax when applied to the optic nerve after axotomy (Rios-Munoz et al., 2005), and it promotes axonal growth and survival via upregulation of BDNF and TrkB in ganglion cells (Soto et al., 2006). However, FGF-2 also induces astroglial and microglial reactivity in vivo (Goddard et al., 2002). According to the above, it is possible that MMP-2 suppresses the inflammatory reaction through the cleavage of FGFR-1.

As far as the origin of MMP-2 is concerned, in the case of regenerative tissue glial cells seem to have a major, although not exclusive, role in its secretion. The difference between the strong expression of MMP-2 during development and that seen during regeneration is that in the former group the gelatinase A seems to be secreted primarily by retinal neurons, whereas in the latter group there is also colocalization with glial cells. Results in other studies indicate that astrocytes are a more important intrinsic cellular source of MMP-2 than neurons under normal and neuroinflammatory conditions (Liu et al., 2007). On the other hand, MMP-2 activity is reported to be induced in rat NPCs (neural progenitor cells) by pro-inflammatory cytokines, such as TNF-a and IFN-gamma, to mobilize them for promoting reparative processes (Ben-Hur et al., 2006).

Another factor that contributes to an increase in MMP-2 activity inside the retina is the augmentation of MMP-14 expression that appears to take place on RGC as well as glial cell membranes after injury. This activation seems most prominent in the regenerative tissue during the first post-injury days. Besides, membrane-type 1 MMP (MMP-14) is also reported to increase after injury in superior cervical ganglion (SCG) neurons with a time course that correlates with that of MMP-2 activation (Leone et al., 2005).

Paradoxically, the expression of MMP-14 seems to be transiently downregulated in TP15 and re-enhanced in late postoperative days in the regenerative retina. This inconsequential pattern can be explained by taking into consideration the activation mechanism of MMP-2. Activation of pro-MMP-2 by MMP-14 is one of the most extensively studied mechanisms, in which MMP-14

acts as a receptor for TIMP-2 which binds to the active site of the former. This binary complex acts as a receptor for progelatinase A, which binds via its C-terminal domain to the same domain of TIMP-2. Then another MMP-14 molecule positioned in close proximity interacts with the first MMP-14 molecule via their hemopexin domains and cleaves the propeptide of pro-MMP-2 (Butler et al., 1998). Interestingly, the antibody used in this study to mark MMP-14 (Chemicon, catalog number: MAB3317) is generated against the hemopexin domain of MMP-14 which is occupied during the MMP-2 activation procedure. The above suggests that MMP-2 activation takes place after the first post-surgery days in the retinas of the regenerating tissue - in contrast with the retinas of the degenerating tissue. This, combined with the fact that there is a repression of microglial activation in regenerating retinas, could support the assumption that MMP-2, like MMP-1, could exert anti-inflammatory effects. Worth mentioning is also the fact that MMP-2 is inhibited by the N-terminal domain of TIMP-3 (Bond et al., 2000), whereas knockdown of TIMP-3 with small interfering RNA (siRNA) was shown to significantly increase MMP-2 activation in astrocytes (Liu et al., 2007). TIMP-3, according to the results of this study, has an enhanced expression during degeneration and is downregulated during regeneration.

TIMP-3 is also related to MMP-3 activity. MMP-3 has so far been associated in many ways with the induction of apoptosis in neurons as well as in microglia activation. Pro-MMP-9 is activated by MMP-3 (Dreier et al., 2004). Active MMP-3 released from stressed dopamine (DA) neurons is responsible for microglial activation and the generation of NADPHo-derived superoxide, and it eventually enhances nigrostriatal DA neuronal degeneration (Kim et al., 2007). Also, recombinant MMP-3 (cMMP-3) is shown to extensively release TNF-alpha from microglia and to activate the nuclear factor-kappaB pathway, while these microglia responses were totally abolished by pre-incubation with an MMP-3 inhibitor. These results strongly suggest that the release of the active form of MMP-3 can be a distinctive signal of neuronal apoptosis by activating microglia and subsequently exacerbating neuronal degeneration. Therefore, the release

of MMP-3 from apoptotic neurons may play a major role in degenerative human brain disorders (Kim et al., 2005).

On the other hand, it has been shown that MMP-3 activation causes transmembrane agrin cleavage on neurons after an ischemia/reperfusion injury (Sole et al., 2004). In retinal neurons agrin increases the efficacy of FGF-2 stimulation of neurite outgrowth, mediated by the FGF receptor, by increasing the affinity between FGF-2 and the FGF receptor. In particular, basic fibroblast growth factor (FGF-2) binds to agrin with high affinity, agrin augments a transient early phosphorylation of ERK in the presence of FGF-2, and augments and sustains FGF-2 mediated increases in c-fos phosphorylation (Kim M.J. et al., 2003). Those facts could indicate an attenuation of the regeneration by MMP-3.

The data in this study indicate that MMP-3 is expressed in the developing and the regenerating retina, but not in the control and not in the degenerating retina. The colocalization with GFAP and GAP-43 shows that MMP-3 is primarily positioned on the surface of RGCs, but is also on glial cells. To interpret the results, the expression of TIMP-3 should also be taken into account.

TIMP-3 follows a pattern opposite to that of MMP-3. The apoptotic properties of TIMP-3 have been extensively studied and attributed to its MMP-inhibitory N-terminal domain. It has been shown to induce apoptosis in the nervous system (Wetzel et al., 2003), as well as to inhibit angiogenesis by functioning as an antagonist to the VEGF-2 receptor (Qi et al., 2003). TIMP-3 is also related to the death of the RPE cells in Sorsby's fundus dystrophy, a degenerative retinopathy connected to a mutation in the TIMP-3 molecule and attributed to resistance in the turnover of the mutant molecule (Majid et al., 2002, Langton et al., 2005). Moreover, TIMP-3 has been shown to promote apoptosis of smooth muscle cells. TIMP-3 is generally shown to sensitize cells to apoptosis induced by toxic stimuli via the stabilization of death receptors (Ahonen et al., 2003).

Death receptor-mediated apoptosis is a critical component in neuronal death in the developing and adult mammalian nervous system (Raoul et al., 2000). Fas and its ligand FasL are cell surface mediators of apoptosis. Binding of Fas ligand (FasL) to Fas initiates intracellular signaling cascades that ultimately terminate in caspase-dependent cellular demise (Wallach et al., 1998). Metalloproteinase activity on the cell surface strongly influences neuronal vulnerability to extracellular death signals. MMP-3 can modulate Fas activation by proteolytic shedding of FasL from the cell surface (Matsuno et al., 2001). Conversion of membrane-bound FasL to its soluble form by MMP-3 may attenuate or enhance its death-promoting activity depending on the target cell type (Ferguson and Griffith, 2007). In embryonic neurons the transmembrane form of FasL is more efficient at inducing apoptosis than its soluble form, suggesting that its shedding from MMP-3 in neurons could have neuroprotective results (Wetzel et al., 2003; Qiu et al., 2002). For example doxorubicin (Dox) treatment of cultured primary neurons derived from embryonic rat cerebral cortices induced Fas-dependent neuronal apoptosis. Dox-induced Fas-mediated neuronal apoptosis required metalloproteinase-inhibition by the tissue inhibitor of metalloproteinase-3 (TIMP-3), and was extenuated by matrix metalloproteinase-3 (MMP-3) activity (Wetzel et al., 2003). In addition, dox-induced neuronal apoptosis has been shown to be regulated by the balance of MMP-3 and TIMP-3 in rat cortical cultures, and strain dependent differences in doxorubicin sensitivity appear to correlate with differences in the pattern of expression of TIMP-3, Fas, FasL and MMP-3 mRNA levels (Wetzel et al., 2004). Therefore, it seems that the MMP-3/TIMP-3 balance, after the induction of apoptotic stimuli, is implicated in regulating RGC sensitivity to death receptor-mediated apoptosis. In the regenerating tissue this balance is shifted towards MMP-3 prevalence. In the quiescent tissue where no apoptotic stimulus is induced, the prevalence of TIMP-3 does not seem to induce apoptosis.

The survival enhancing properties of TIMP-1 in nerve tissue have also been recorded. The expression of TIMP-1 in neurons and astrocytes, possibly with the involvement of the transcriptional factor c-Fos, is induced as part of the

neural defence mechanism against injury (Jaworski et al., 1999). Firstly TIMP-1 is reported to have homeostatic and protective functions (Gardner and Ghorpade, 2003) by limiting inflammation (von Gertten et al., 2003) and by protecting the basement membrane from uncontrolled degradation (La Fleur et al., 1996). It has also been proposed that it exerts non-MMP-inhibiting neuroprotective effects by inhibiting glutamate-induced calcium entry stimulated by MMP-9 (Tan et al., 2003).

In another study, although no differences were found in CNS seizure injury between wild-type (WT) and TIMP-1 knock-out (KO) mice, the TIMP-1 KO mice were resistant to excitotoxicity and did not undergo the typical unorganized fiber sprouting observed in WT mice. In the same study it was also indicated that the lack of TIMP-1 paradoxically attenuated the increase in the activity of MMPs observed in the injured WT mice. In addition, it was demonstrated that TIMP-1 in a pathological situation is also implicated in the synaptic mechanisms which underlie learning and memory in physiological conditions (Jourquin et al., 2005). TIMP-1 could play a similar role in synapse preservation in the retina after an injury-signal. In a similar study concerning a model of experimental autoimmune encephalomyelitis (EAE), although CD4+ T-cell immune responses to myelin in wild-type (WT) and TIMP-1-/- mice were similar, analysis of CNS tissues from TIMP-1-/- mice revealed more severe myelin pathology than that of WT mice. This disruption of myelin was associated with both increased lymphocyte infiltration and microglia/macrophage accumulation in the brain parenchyma. It was also suggested that the induction of TIMP-1 by astrocytes during EAE in WT mice represents an inherent cytoprotective response that mitigates CNS myelin injury, through the regulation of both immune cell infiltration and microglia activation (Crocker et al., 2006). In another encephalomyelitis model an upregulation of TIMP-1 as well as MMP-3 mRNA was reported (Ulrich et al., 2006).

The link between the inflammatory response elicited by nerve injury and subsequent neurodegeneration has also been indicated in another study, in which TIMP-1 also appeared to have a significant role (Swanberg et al., 2006).

In an IOP-mediated retinal injury in a hereditary rodent model, a remarkable upregulation of TIMP-1 gene expression was found, possibly caused by a self-protective cell mechanism (Naskar and Thanos, 2006). In another related study, an elevated IOP correlated positively with a significant increase in MMP-9 activity and TIMP-1 expression (Guo et al., 2005). In the present study TIMP-1 was shown to upregulate in the regenerating tissue and to decrease in the degenerating one, something that accords with the studies mentioned above. This attributes a neuroprotective role to TIMP-1. In the developing tissue there is no expression of TIMP-1, possibly because of the inhibiting effect it would have on MMP-1 and MMP-2 activity, both of which are necessary during the developmental process. As mentioned before, these MMPs seem to have both advantages (anti-inflammatory properties) and disadvantages (direct mischievous effect on RGC environment) as far as survival and regeneration are concerned.

The expression of TIMP-1 also differs between the regenerating and the developing tissue, possibly because in the latter apoptosis seems to be a desired phenomenon to some extent.

Additionally, TIMP-1 seems to have anti-inflammatory effects that contribute to the survival of the neurons, since it is clear that microglia activation and inflammation are associated with progressive neuronal apoptosis in neurodegenerative disorders.

4.3 Conclusion and Future Perspectives

In this study, the correlation between the expression of MMPs and TIMPs in the retina in the three different groups indicates that MMPs-1, 2, and 3 are upregulated in the regenerating tissue, and their expression resembles that seen in the developing tissue. In the degenerating tissue the expression of those proteases is either not significantly altered or downregulated. The RGC survival in the regenerating retinas is probably due to the anti-inflammatory properties that MMPs-1 and 2 seem to exert, therefore “saving” the remaining RGCs from the toxic effects of microglial activation. MMP-14 expression is upregulated equally in both groups, probably due to signals produced after injury, but in the regenerating group it seems to play an important role in MMP-2 activation. MMP-3 expression is mostly related to the expression of TIMP-3, which is upregulated in the degenerating tissue and downregulated in the regenerating one. The ratio of MMP-3/TIMP-3 seems to be important in the induction of apoptosis after an apoptotic stimulus and is higher during regeneration. After not receiving the preserving trophic factors from the SC, the RGCs may be programmed to be eliminated as “useless”, whereas the ones receiving “maintenance signals” from Schwann cells do not induce the expression of TIMP-3. The elevated expression of TIMP-1 witnessed only in the regenerating tissue could be explained as an effort by the surviving RGCs to eliminate neurotoxic signals in their environment. Taking these results into consideration, together with the progress achieved in the intervention of MMP and TIMP regulation at all possible levels (transcription, translation, secretion, activation and inhibition), this study may provide valuable insights into how to reinforce the survival properties of RGCs exogenously. For example, the expression of TIMP-1 could be amplified with viral vectors, while TIMP-3 expression could be attenuated at the mRNA level by anti-sense mRNA. On the other hand, the regenerative properties of the MMPs that promote axon elongation could be limited to the site of axonal sprouting, thus avoiding their deleterious effects on the cell somata, while the administration of anti-inflammatory agents could substitute for their anti-inflammatory activities.

A body of experimental literature suggests that the principles of ON-axotomy-mediated RGC death may also apply to degenerative conditions following glaucoma injury (Quigley, 1999; Quigley et al., 1995; Quigley et al., 2000; Pease et al., 2000), as well as to other RGC axonal injuries (Levin and Gordon, 2002). This study could lead to a partial decoding of the properties of the regenerating RGC environment, and in this way open new avenues for pharmaceutical intervention in the case of optic nerve injury and neurodegenerative disorders of the RGCs, such as glaucoma.

5. Literature

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19. April 2004

Tierschutz;
Durchführung von Versuchen an Wirbeltieren

Ihr Antrag vom 13.02.2004

Genehmigungsbescheid

Sehr geehrter Herr Prof. Dr. Dr. Thanos,

gemäß § 8 Tierschutzgesetz (TierSchG) in der zur Zeit gültigen Fassung (BGBl. I S. 1105, 1818) wird Ihnen die Genehmigung zur Durchführung des nachstehenden Versuchsvorhabens erteilt:

"Proteomisch - genomische Analyse der regenerierenden Rattenretina"

Leiter des Versuchsvorhabens und sein Stellvertreter sind:

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Diese Genehmigung gilt bis zum **30.04.2006**.

Zur Durchführung des Versuchsvorhabens dürfen folgende Tiere verwendet werden:

200 Ratten

Mit dem Versuchsvorhaben darf erst nach Vorliegen dieser Genehmigung begonnen werden.

Sofern der Leiter des Versuchsvorhabens oder sein Stellvertreter wechselt, ist mir dieser Wechsel unverzüglich anzuzeigen.

Diese Genehmigung wird zurückgenommen, wenn bei der Erteilung die Voraussetzungen nach § 8 Abs. 3 TierSchG nicht gegeben waren.

Sie wird widerrufen, wenn die Voraussetzungen nach § 8 Abs. 3 TierSchG nicht mehr gegeben sind und dem Mangel nicht innerhalb einer von mir zu bestimmenden Frist abgeholfen wird; sie kann widerrufen werden, wenn ihre Bedingungen und Auflagen nicht erfüllt werden oder den Vorschriften des § 9 Abs. 2 TierSchG wiederholt oder grob zuwidergehandelt worden ist.

Ein Antrag auf Verlängerung der Genehmigung ist rechtzeitig vor Ablauf der Frist bei mir zu stellen.

Ich weise darauf hin, dass diese Genehmigung eine evtl. erforderliche Zustimmung nach dem Gentechnikgesetzt für Arbeiten mit transgenen Tieren nicht einschließt.

Da das Versuchsvorhaben überwiegend im öffentlichen Interesse erfolgt, wird von der Erhebung einer Verwaltungsgebühr Abstand genommen.

Rechtsbehelfsbelehrung

Gegen diesen Bescheid kann innerhalb eines Monats nach Bekanntgabe Widerspruch erhoben werden. Der Widerspruch ist bei mir unter der oben genannten Anschrift schriftlich oder zur Niederschrift einzureichen.

Sollte die Frist durch das Verschulden eines von Ihnen Bevollmächtigten versäumt werden, so würde dessen Verschulden Ihnen zugerechnet werden.

Ich bitte um umgehende Rückgabe des beiliegenden Empfangsbekenntnisses.

Mit freundlichen Grüssen
Im Auftrag

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Betr.: Tierschutz;
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Bezug: Ihr Antrag vom 22.11.2000, hier eingegangen am 10.01. 2001, und Ergänzungen vom 20.03.2001, hier eingegangen am 28.03.2001

G e n e h m i g u n g s b e s c h e i d:

Sehr geehrte Frau Dr. Naskar,

gemäß § 8 Tierschutzgesetz (TierSchG) in der Fassung der Bekanntmachung vom 25. Mai 1998 (BGBl. I. S. 1105) wird Ihnen die Genehmigung zur Durchführung nachstehenden Versuchsvorhabens erteilt:

Pathomechanismen der glaukomatösen Nervenschädigung: Entwicklung eines Tiermodells in der Ratte und Anwendung neuroprotektiver Substanzen.

Leiterin des Versuchsvorhabens und ihr Stellvertreter sind:

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Sollte die Frist durch das Verschulden eines von Ihnen Bevollmächtigten versäumt werden, so würde dessen Verschulden Ihnen zugerechnet werden.

Ich bitte um umgehende Rückgabe des beiliegenden Empfangsbekenntnisses.

Mit freundlichen Grüßen
Im Auftrag

Dr. Piontkowski