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Molecular Characterization of the Regenerating Retinal Ganglion Cells using Quantitative PCR and Immunohistochemistry

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This dissertation is dedicated to my family.

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Contents

1. Introduction

1.1. Research Background

The nervous system is extremely complex. It is widely accepted that the adult mammalian central nervous system (CNS) is unable to regenerate axons. Traumatic injury to the adult mammalian CNS permanently interrupts neural pathways, resulting in irreversible functional deficits [164]. Damage to the optic nerve leads to visual impairment and to irreversible blindness of the affected eye in severe cases. During the last two decades, research experimental strategies have been developed to facilitate axonal regeneration and restore interrupted neural connections between the eye and the brain with special emphasis being placed on understanding the mechanism involved in neuronal diseases.

Under normal circumstances, mature retinal ganglion cells (RGCs) fail to regrow their axons distal to the site of optic nerve (ON) injury. Moreover, lesions often result in death of the severed neurons. The failure of axons to regenerate in differentiated CNS tissue has been attributed to several factors, such as interaction with neurite growth inhibitors [188, 201] or the absence of growth-promoting substrates [195, 105] . Moreover, lesions often result in death of the severed neurons. Recently, it has been reported that optic nerve transection showed typical apoptotic cell death pattern. In rats, for example, about 72% [191] up to 90% [14] of the RGCs die within 2 weeks after transection of the optic nerve near its bulbar exit, and up to 95% of the RGCs undergo apoptosis [130] .

However, regenerative failure is not inevitable. Adult mammalian optic nerve axons are able to regenerate when provided with a suitable environment. There have been numerous studies on the regeneration of the injured optic nerves by applying neuroprotective reagents or by nerve transplantation [85, 228]. Ramon y Cajal [34] described Tello's observation that mature RGCs can regenerate axons through a peripheral nerve (PN) graft sutured to the cut end of the optic nerve and concluded that "the regenerative failure of the central paths is an accidental condition, due to the neuroglial environment." Expanding on this observation, Aguayo and colleagues [171, 32, 1] showed conclusively that mature RGCs and other CNS neurons retain an intrinsic capacity for axon growth in an altered environment, sparking renewed interest in the factors that support or inhibit nerve regeneration. All these studies constitute "proof of principle" that, provided that the axonal environment is modified, axonal regeneration can occur in the CNS of mammals, as it does spontaneously in the peripheral nervous system.

Injury to the adult optic nerve and treatment of the corresponding retina with microglia inhibitors [211, 212] or with neurotrophins results in a higher incidence of survival of the retinal ganglion cells (RGCs). Neurotrophic effects can be mimicked by the presence of a peripheral nerve piece used to replace the non-permissive environment of the optic nerve [221, 222]. Concomitant microglia directed neuroprotection and replacement of the inhibitory [190, 215] distal portion of the optic nerve with a peripheral nerve graft in vivo [221, 211, 212], or culturing the retina in vitro [211] showed a vigorous axonal regeneration.

Optic nerve transection causes irreversible degeneration of the RGC [223, 162]. The optic nerve provides the retina with various neurotrophic factors that are essential for the RGCs to survive. After axotomy, the ganglion cells ultimately die due to the deprivation of neurotrophins [193], altered gene expression [174, 110, 223], and various reactive oxygen species [99, 100].

The intracellular mechanisms of the nerve cell response to injury remain unknown. Regeneration of retinofugal axons has become one of the most prominent experimental models in restorative neurobiology. Understanding the mechanisms of regeneration and functional restoration in the central nervous system could be the first indicative steps towards therapeutic intervention.

1.2. The Role of Astrocytes in Axonal Regeneration

Astrocytes are the companions of neurons from the early stage of CNS formation, and are the most numerous cellular components of the CNS. Astrocytes have been involved in many functions including control of brain development and homeostasis [210], CNS immunity, and in regulation of development, function and efficiency of the synapse [156]. In the CNS of higher vertebrates, following injury, either as a result of trauma, genetic disorders, or chemical insult, astrocytes become reactive and respond in a typical manner, termed astrogliosis. Reactive astrogliosis is a prominent feature of astrocytes adjacent to injury.

Moreover, astrocytes play an important role in neural plasticity. It has been reported that neural reorganization can take place rapidly after nervous system injury even in the adult brain [18], and astrocytes are thought to support synaptic sprouting, formation of new polysynaptic connections and the maintenance of existing neuronal circuits [63, 57].

Many studies have demonstrated that reactive astrocytes synthesize a variety of growth

factors, cytokines and components of the extracellular matrix, many of which are known to rescuer damage neurons in various injury models [39, 129, 141].

1.2.1. Gliosis and Repair of Lesion

Neuron-glial and glial-glial interactions are thought to play a critical role during development [56, 58, 65, 66, 75], in neuronal survival and death after diverse insults to central nervous system (CNS) [63, 76].Within the CNS, the glial cells are responsible for maintaining homoeostasis. When the structural integrity of the nervous system is compromised glial cells, it become activated to restore homeostasis [143].

In the injured CNS, however, astrocytes appear as a key component of reactive gliosis [76, 172], which has been considered as one of the major impediments to neurone outgrowth and axonal regeneration [164, 168]. It has been suggested that gliosis can induce neuron degeneration, either physically or through the release of inhibitory molecules [19]. As radial glial cells, they constitute a major substratum for the migration of neurons [163]. Later, they subserve major functions in neuronal homeostasis, such as ionic balance [165] and energy supply [38]. The reactive gliosis, which occurs after an injury to the CNS and leads to the glial scar, consists mainly of reactive hypertrophic astrocytes. These reactive cells acquire new properties, leading to a non-permissive support for neurons. The glial scar constitutes a physical and chemical barrier that isolates the intact tissue, but also contributes to the failure for recovery post-injury [22]. In addition to physical or molecular barriers presented by glial scarring at the lesion site, it has been suggested that the normal myelinated CNS environment contains potent growth inhibitors or lacks growth-promoting molecules. And the blockage of astrocytes reactivity yielded some axonal regeneration [64].

1.2.2. Glial Fibrillary Acidic Protein (GFAP)

Astrogial reactivity is mainly characterized by a high overexpression of the major component of the gliofilaments GFAP. This GFAP over expression is related to the astroglial morphological response to injury. One conspicuous phenotypic change occurring in astrocytes during maturation and activation is the increase in the synthesis of GFAP [48]. Since astrocytic hypertrophy and an attendant increase in GFAP commonly occur in response to all types of nervous system injury, this major alteration in activating astrocytes directly associated with morphological changes may be related to the functional shift from

neurite- promoting to neurite-inhibiting elements [143].

It is now well established that GFAP is the principal 8.9 nm intermediate filaments in mature astrocytes of the CNS [49]. As a member of the cytoskeletal protein family, GFAP is thought to be important in modulating astrocyte motility and shape by providing structural stability to astrocytic processes. Increase of GFAP mRNA has been associated with Scrapies, Alzheimer's brains and Creutzfeldt-Jacob disease; was observed in a mechanical lesion to rat cerebral cortex, and cerebral freeze-injury [47].

Astrogliosis is characterized by rapid synthesis of GFAP intermediate filaments. The involvement of GFAP in the formation of astrocytic processes was demonstrated by using antisense inhibition of GFAP expression [225], thus opening up the possibility of modulating astrogliosis induced by injury [231, 232, 61]. Since GFAP accumulation is a prominent feature of astrocytic gliosis, inhibit GFAP synthesis using antisense oligonucleotides with the intention that this might delay scar formation resulting from a CNS injury. The delay in the formation of a physical barrier might allow the neurons and oligodendrocytes to re-establish a functional environment. In vitro studies using neuron-astrocyte co-cultures by Lefrancois et al [109] have showed that inhibition in GFAP synthesis leads to a reduction of astroglial hypertrophy and relieves the blockade of neurotic outgrowth that normally is observed after a lesion.

1.3. Neuronal Growth-Associated Protein GAP-43 and Neuron Regeneration

1.3.1. The Essential Role of GAP-43 during Initial Developmental Axon Growth

It has been postulated that GAP-43 plays an essential role in axon growth. This protein is a phosphoprotein found in highest concentrations in axonal growth cones [199, 13] and is expressed in various anatomical areas of the rat brain [153]. GAP-43 is involved in regulation of the actin cytoskeleton, mediating a link between membrane rafts and actin [55, 107, 31, 90, 170] and then expression falls with maturation.

The precise function of GAP-43 is not yet know, it has been observed to accumulate in the growth cone [68, 127, 198] where it is thought to play a role in signal transduction [13]. GAP-43 is expressed at high levels during initial developmental axon outgrowth.

However, whether GAP-43 plays a predominant role in elongation or guidance of axons has been a matter of debate for many years. The overexpression of GAP-43 in PC12 cells [29, 91, 140, 230] or in nonneuronal cells [235] in vitro induced the formation of neurite-

like filopodia while depletion of GAP-43 in primary sensory neurons on culture [3, 4], by antisense oligonucleotides resulted in the formation of thin neurites and very small growth cones. The overexpresssion of GAP-43 in transgenic mice also induced nerve sprouting [3]. On the other hand, the specific immunodetection of GAP-43 in axonal but not in dendritic growth cones [68, 69] supported the view that GAP-43 was mainly required for guidance rather than neurite elongation.

1.3.2. Overexpression of GAP-43 is Associated with Neuron Regeneration

Intrinsic factors associated with neuronal growth during embryonic development may be important for successful axon growth in mature neurons. Some of these factors are axonally transportable proteins associated with regenerative growth [196, 12]. One of the most abundant factors is GAP-43 which is highly expressed in developing and regenerating axon growth cones and in synaptic terminals of adult brain regions that exhibit plasticity [13, 200].

Evidence for the involvement of GAP-43 in axonal guidance of the visual system first came from the observation that its expression correlated with neurite outgrowth during development and regeneration following nerve injury [37, 199, 196, 204]. One of the most prominent changes in gene expression after peripheral nervous system (PNS) axotomy is the increase in GAP-43 [199]. Increased expression of GAP-43 may be one component of a metabolic response to axotomy, which is important for regenerative growth.

GAP-43 expression in the cell body is closely linked to both axonal growth and regeneration in normal animals [13, 196, 216]. In cultured neurons, it promotes neurite outgrowth branching and growth cone adhesion [4, 13]. An indication that the expression and presence of this protein in neurons may be damaging was provided by results on cerebella Purkinje cells of mice overexpression GAP-43 [21]. Although in the absence of axon injury these cells appear to survive, following axotomy, a considerable number of Purkinje cells died. Unlike in wild-type animals, in the transgenic animals extensive sprouts were seen at the site of injury [21]. GAP-43 is also playing a critical role in the topographic organization of the visual system [234]. However, the exact role of GAP-43 during axon growth and synaptic function remains to be determined.

1.4. Thymus-Specific Antigen (Thy-1) as RGCs marker

Thy-1 is a cell-surface signalling molecule of the immunoglobulin (Ig) superfamily

implicated in the regulation of neurite outgrowth, synaptic function and plasticity [132]. Although Thy-1 is a surface component of most neurons in the adult nervous system, it is present only on certain neurons during early development. Moreover, detectable antigen can be lost from cells which at earlier development were Thy-1 positive, whereas formerly Thy-1 negative cells can acquire high levels of the antigen during later stages of development. Thy-1 appears to be critically involved in neuron development [185].

In the eye, Thy-1 is found predominantly in the ganglion cell layer (GCL), with some staining also seen in the nerve fibre and inner plexiform layers (IPLs) [9]. Thy-1 can be detected in the inner retina of rats from the date of birth. However, levels were very low for the first few days of postnatal development. There was a sharp increase in Thy-1 levels at postnatal day 5, which reached a maximum towards the end of the second postnatal week. Thy-1 declined minimally from this point on, and remained at relatively constant level in adulthood [115].

The Thy1 gene codes for a 25 kDa cell surface glycoprotein [226] of unknown function. It is predominantly expressed by ganglion cells in the retina [9, 154], making it a useful marker for evaluating gene expression in these cells. Previous studies have shown that Thy1 message and/or protein were depleted after damage to the optic nerve and retina [154, 147, 106, 136], an observation that has been attributed to the loss of ganglion cells in affected retinas. In other experiments, however, the loss of Thy1 expression appeared to precede cell death [111]. Compared to the number of cells expressing Thy1 and the rate of cell loss in retinas exposed to two acute damaging stimuli in mice and chronic elevated IOP in a rat model of experimental glaucoma, Thy1 mRNA levels decreased prior to significant cell loss [184]. In addition, Thy1 expression also decreased in ganglion cells resistant to cell death, suggesting that this downregulation precedes a committed step in the apoptotic program of these cells.

1.5. ET-1 and ETB

Endothelin (ET) has been implicated in such a role as it can induce significant changes in the physiology of cultured astrocytes. ET promotes mitogenesis and increases intracellular Ca^{2+} levels [207, 117], increases nerve growth factor expression [103]. This peptide, first isolated from vascular endothelial cells [229], is localized in the CNS, and of its known isoforms (ET-1, ET-2, and ET-3) only ET-1 and ET-3 are found in neurons [62] and astrocytes [117]. ET agonists can stimulate astrocyte ET-1 production in vitro [40, 41]. The

biological actions of ET-1 are primarily mediated by two receptor subtypes, termed endothelin A receptor (ET_A) and endothelin B receptor (ET_B), cloned and sequenced in mammals [5, 178]. The ET_A receptors have selective affinity for ET-1 whereas the ET_B receptors have equal affinity for all isoforms.

1.5.1. Modulation Astrocyte Function in CNS

ET-1 is known to induce vasoconstriction of cerebral vessels resulting in decreased cerebral blood flow and neuronal damage [119]. The increased risk of late vasospasm of the basilar artery after head injury may be related to the loss of ET_B -receptor expression and ET_B -mediated vasodilation after neurotrauma [67]. Apart from a direct effect on the cerebral vasculature, lack of ET_B receptors may also result in an impairment of other cellular functions. ET_A and ET_B receptors are responsible for transducing the peptide's effects in the CNS; however, the ET_B receptor has been shown to be the primary receptor expressed on astrocytes in vitro [42]. Astrocytic endothelin receptors have autoregulatory [43] and eliminatory [74] tasks and also modulate astroglial activation [101], proliferation [207] as well as the permeability of astrocytic gap-junctions [17].Functional ET_B receptors have been found to protect this vulnerable cell population from apoptosis [44]. ET_B receptor which expressed by the majority of astrocytes were immunoreactive for GFAP in both the normal and crushed rabbit optic nerve. Optic nerve crush induced a marked increase in ET_B receptors and GFAP immunoreactivity without inducing a significant increase in the number of GFAP immunoreactivity astrocytes, suggesting that the crush-induced astrogliosis is due primarily to astrocyte hypertrophy. Pharmacological blockade of astrocyte ET_B receptors following CNS injury modulates glial scar formation and may provide a more permissive substrate for neuronal survival and regeneration [175].

Astrocytic endothelin receptors, e.g., mediate a reduction of gap-junctional permeability [17], thereby profoundly influencing astrocytic communication and also the propagation of apoptotic signals [114]. Autoregulatory and eliminatory functions of astrocytes rely on functional ET_B receptors [43, 74]. Interestingly, the expression of ET_B -receptor immunoreactivity was increased in the reactive astrocytes of lesioned brain indicating perhaps an attempt of the injured neural tissue to restrict the propagation of damage. Finally, microglial ET_B signaling might also be involved in the modulation of brain damage [227].

1.5.2. The Actions of ET-1 in Retina Neurons and Glia

The presence of the ET system in the eye is well established. ET-1 mRNA expression has been detected in the whole rat retina [24]. ET_A and ET_B are present in neural retinal rat membranes [33]. Autoradiographic studies have shown that ET_B receptors are present in neurones and glial cells of the neural retina [118]. The existence of both ET-1 and GFAP immunoreactivities within astrocytes in human and porcine retinas indicate that ET-1 may also be formed and stored within retinal glial cells thus playing a role in glial function [173]. The physiological significance of ET-1 expression in retinal glial elements is for the moment unknown. Astrocytic proliferation together with an excessive secretion of ET-1 have been reported in vivo, such as in cerebral focal ischemia, and subarachnoid haemorrhage [139, 227] Moreover, Sasaki et al. [180] demonstrated that ET-1 specifically stimulated the efflux of glutamate via ET_B receptors from cultured of rat astrocytes suggesting that ET-1 may exacerbate neuron degeneration. On the other hand, ET-1 acts as a growth factor for astrocytes, inducing DNA synthesis and proliferation [117].

The expression of ET-1 in neural, glial as well as vascular elements of the normal adult retina suggest that ET-1 may have a role in the maintenance of both neural and vascular integrity in the mature retina.

1.6. Nuclear Factor kappa B (NF-κB)

Nuclear factor kappa B (NF-κB), as a transcription factor, is one of the molecules involved in cell death. NF-κB exists in the cytoplasm as heterodimers or homodimers of Rel related proteins. The amino terminal region of the Rel homology domain contains DNA binding, dimerization, and nuclear localization domains. The predominant form of NF-κB is composed of NF-κB1 (p50) and RelA (p65), and is associated with inhibitor-κB (IκB) as an inactive form. Upon stimulation, I-κB is phosphorylated by IκB kinases, ubiquitinated, and subsequently processed to proteolytic degradation. The freed NF-κB translocates from the cytoplasm to the nucleus by exposing the nuclear localization signal and then binds to the target genes to activate transcription [128, 146]. NF-κB is activated by various stimuli, including stress or injury. So far, the known inducers for NF-κB activation are interlukin (IL)-1, tumor necrosis factor alpha (TNF-α), bacterial lipopolysaccharides, sphingomyelinase, oxygen free radicals, ultraviolet light and γ-irradiation [84, 155, 203]. The mechanism for anti-apoptotic role of NF-κB is suggested as the elevation of Bcl-2 expression by NF-κB. Furthermore, p50 knockout mice showed increased apoptosis and

the survival pathway by growth factors or cytokines also activate NF-κB through PI-3 kinase pathway [108, 131, 176, 208, 224, 233].

1.6.1. NF-κB in the Nervous Cells

NF-κB is widely known for its ubiquitous roles in inflammation and immune responses, as well as in control of cell division and apoptosis. These roles are apparent in the nervous system, but neurons and their neighbouring cells employ the NF-κB pathway for distinctive functions as well, ranging from development to the coordination of cellular responses to injury of the nervous system and to brain-specific processes such as the synaptic signalling that underlies learning and memory [124].

In the nervous system, NF-κB is modulated under physiological and pathological conditions, including developmental cell death and acute or chronic neurodegenerative disorders [8, 123]. The activation of NF-κB as a pro-apoptotic factor has been shown in neuronal cell death induced by TNF-α, glutamate, and reactive oxygen species in vitro [71, 70, 122, 159], while the anti-degenerative function also has been suggested. The activated NF-κB plays a protective role from the cell death in the injured ganglion cells [27]. NF-κB was observed in the ganglion cell layer after axotomy with immunohistochemistry [26]. It has been associated with synaptic plasticity since NF-κB is present in synaptic terminals and can be activated locally in such synapses [93, 126]. Moreover, physiological signals such as glutamate receptor binding and membrane depolarization induce NF-κB activation in hippocampal pyramidal neurons and cerebellar granule neurons in cell culture [70, 72, 94]. NF-κB activity is also greatly increased in brain cells following excitotoxic and apoptotic insults. In addition, activation of NF- κB before experimental insults such as exposure to glutamate, glucose deprivation, β-amyloid peptide, or oxidative molecules has been shown to protect neurons against apoptosis [6, 25, 92]. In contrast, treatment of neurons with κB decoy DNA that selectively blocks NF-κB activity abolished the protective effect of small doses of TNF-α [122]. In vivo, administration of κB decoy DNA to mice via intraventricular infusion before administration of the glutamate agonist kainite resulted in a significant increase of neuronal death in the CA1 and CA3 regions of the hippocampus, and mice lacking p50 exhibited increased damage to hippocampal pyramidal neurons [233]. Finally, studies of postmortem brain tissues from patients with neurodegenerative diseases such as Alzheimer's or Parkinson's diseases revealed increased NF-κB activity closely associated with the neurodegenerative process [86, 92]. These

results strongly suggest that NF-κB regulates apoptosis in response to stress in the nervous system, in addition to regulating apoptosis in a large variety of cells and tissues.

1.7. Heat Shock Proteins

Heat shock proteins (HSPs) represent a family of stress –activated proteins that participate in protein folding, repair, and degeneration [202]. HSPs range in molecular weight from 10 to 170 kDa. Although these proteins were initially identified in Drosophila in response to heat stress, they are found in all cells, and their expression is upregulated in response to different forms of cellular stress. The primary physiological function of HSPs is to act as molecular chaperones that assist in protein folding. Other functions include participation in activation of glucocorticoid receptors, polymerisation of actin, and transfer of proteins to lysosomes [202]. In addition, overexpression of specific HSPs has been shown to prevent apoptotic cell death [95, 11, 59]. The induction of HSPs involves both transcriptional activation and translational changes. Studies have provided evidence that depletion of adenosine triphosphate (ATP) and the induction of the mitogen-activated protein (MAP) kinase pathway play significant roles in the activation of specific HSPs [160].

HSPs are induced in the cells of many types of tissues by stressful stimuli and play important roles in cellular repair and protective mechanisms. Increasing attention is being focused on the neuroprotective effects of relatively low-molecular-weight HSPs, including HSP27 and HSP32, in the event of heat shock or other injuries [10, 23]. HSP27 has been shown to interact with the actin cytoskeleton, to modulate intracellular reactive oxygen species content, and to prevent apoptotic cell death triggered by a variety of stimuli, including TNF-α [60, 83]. Several mechanisms have been proposed to account for the HSP27-mediated negative regulation of programmed cell death. This small HSP specifically interacts with cytochrome *c* when released from the mitochondria to the cytosol, thus preventing the formation of the apoptosome. A premitochondrial effect at a higher HSP27 expression level has also been described [149, 179]. In vitro, this protein behaves as an ATP-independent chaperone that helps in the refolding of denatured proteins [138], a property that could be of key importance for understanding its in vivo functions.

In vivo studies demonstrated that transection of the optic nerve results in the expression of HSP27 in the visual system. HSP27 may be associated with enhanced survival of a subset of retinal ganglion cells, providing evidence of a protective role for HSP27 in CNS after neuronal injury [102]. And an increase in expression of HSP27 appears to be one

component of the neuroprotective events induced by ischemic preconditioning in the retina [112].

When overexpressed in response to various stimuli, HSP27 facilitates phosphorylated I-κBα proteasome-mediated proteolysis, which could account for the protective effect of this small stress protein [148].

1.8. The Aim of this Project

The ability of the adult mammalian central nervous system (CNS) to regenerate following injury is influenced by a balance between factors that promote and those that inhibit neuronal survival, regeneration, and sprouting. While the properties of the injured neurons affect the extent of neuronal survival and regeneration, glial cells at the site of injury are also critically involved in determining the regenerative outcome [53, 161]. The molecular mechanisms underlying neuronal degeneration, repair and regeneration after axon injury have yet to be identified.

It is therefore of crucial importance to characterize the different populations of cells, which may codetermine whether regeneration will take place or not. In this study the optic nerve of adult rats was transect (axotomy-group) or the optic nerve was transect and replaced with an autologous graft (grafting-group) to facilitate axonal regeneration.

To gain further insights into the molecular mechanisms responsible for regeneration of retina, the semi-quantitative RT-PCR, real time PCR and immunohistochemistry method were performed for differentials genes and protein expression in the retina. The purpose of the present study was to search for changes in gene expression in the regenerating retina following optic nerve grafting. We conducted a comparison of mRNAs in those retinas receiving PN grafted with those axotomized and used untreated retinas as controls. At various times elapsed after injury, those glial cell markers, neuronal markers and capillary endothelin were analysed.

1.9. Injury Model and Methods Used

1.9.1. Animal Model of Optic Nerve Axotomy and PN Grafting

The optic nerve has served as an important model for the study of the neuro/nonneuronal response to injury and to study axon regeneration in the CNS [54, 221, 222, 15]. Retinal ganglion cells and their associated projections to the brain have recently become an

attractive experimental model to study the issue of axonal regeneration in the CNS [35, 80, 88]. A number of experimental manipulations have been found to enhance the axonal regeneration of rodent RGCs after injury of the ON apposition of a peripheral nerve segment to the cut ON. Analysis of axon sprouting and regeneration after injury by anatomical tracing are aided by lesion models that produce a well-defined injury site.

Retinal ganglion cells have historically been a model cell-type for study both in vitro and in vivo, since they represent an accessible population of neurons of the central nervous system and have been examined in numerous experimental paradigms. Ganglion cells are also the primary retinal cell-type affected by several blinding diseases including glaucoma, tumor-associated optic nerve compression, and ischemic optic neuropathy.

1.9.2. Real Time Quantitative PCR

Real-time quantitative RT-PCR is the method of choice used to quantify mRNA expression of cytokines, which are often expressed at very low levels. It quantitates the initial amount of the template most specifically, sensitively and reproducibly, and is a preferable alternative to other forms of quantitative RT-PCR, which detects the amount of final amplified product. Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e., in real time) as opposed to the endpoint detection by conventional quantitative PCR methods. Real-time PCR quantitation eliminates post-PCR processing of PCR products (which is necessary in competitive RT-PCR). This helps to increase throughput, reduce the chances of carryover contamination and remove post-PCR processing as a potential source of error. In comparison to conventional RT-PCR, real-time PCR also offers a much wider dynamic range of up to 10^7-fold (compared to 1000-fold in conventional RT-PCR). This means that a wide range of ratios of target and normalizer can be assayed with equal sensitivity and specificity. It follows that the broader the dynamic range, the more accurate the quantitation.

The real-time PCR system is based on the detection and quantitation of a fluorescent reporter. This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The TaqMan probes [79] use the fluorogenic 5' exonuclease activity of Taq polymerase to measure the

amount of target sequences in cDNA samples. TaqMan probes are oligonucleotides longer than the primers (20-30 bases long with a Tm value of 10 ^{o}C higher) that contain a fluorescent dye usually on the 5' base, and a quenching dye (usually TAMRA) typically on the 3' base. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing (this is called FRET = Förster or fluorescence resonance energy transfer). Thus, the close proximity of the reporter and quencher prevents emission of any fluorescence while the probe is intact. TaqMan probes are designed to anneal to an internal region of a PCR product. When the polymerase replicates a template on which a TaqMan probe is bound, its 5' exonuclease activity cleaves the probe. This ends the activity of quencher (no FRET) and the reporter dye starts to emit fluorescence which increases in each cycle proportional to the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye.

TaqMan assay uses universal thermal cycling parameters and PCR reaction conditions. Because the cleavage occurs only if the probe hybridizes to the target, the fluorescence detected originates from specific amplification. The process of hybridization and cleavage does not interfere with the exponential accumulation of the product. One specific requirement for fluorogenic probes is that there is no G at the 5' end. A 'G' adjacent to the reporter dye quenches reporter fluorescence even after cleavage.

The threshold cycle (C_T) value is the cycle at which a significant increase in ΔRn is first detected. The threshold cycle is when the system begins to detect the increase in the signal associated with an exponential growth of PCR product during the log-linear phase. This phase provides the most useful information about the reaction (certainly more important than the end point). The important parameter for quantitation is the C_T. The higher the initial amount of cDNA, the sooner accumulated product is detected in the PCR process, and the lower the C_T value.

Relative gene expression comparisons work best when the gene expression of the chosen endogenous control is more abundant and remains constant, in proportion to total RNA, among the samples. By using an endogenous control as an active reference, quantitation of an mRNA target can be normalized for differences in the amount of total RNA added to each reaction. The use of this endogenous reference also normalizes for variation in reverse transcriptase efficiency among the different cDNA reactions. For this purpose, the most common choices are 18S RNA, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and

β-actin.

The real-time quantitative PCR method measures PCR product accumulation through a dual-labeled fluorogenic probe. This method provides very accurate and reproducible quantitation of gene copies. Unlike other quantitative PCR methods, real-time PCR does not require post-PCR sample handling, preventing potential PCR product carry-over contamination and resulting in much faster and higher throughput assays. The real-time PCR method has a very large dynamic range of starting target molecule determination. Real-time quantitative PCR is extremely accurate and less labour-intensive than current quantitative PCR methods.

2. Materials and Methods

2.1. Surgical Techniques

2.1.1. Material

CO2 Gas	Autolab PGStat 12, Nederland
10/0 suture (10-0 silk)	Ethicon, Hamburg, Germany
Operating microscope	OPMI 19, Zeiss, Jena, Germany)

2.1.2. Animals and Anaesthesia

Lister-Hooded rats, female

10% ketamine	Sanofi-Synthelabo, Paris, France
2% Xylazine	Sanofi-Synthelabo, Paris, France

The studies were performed at University of Münster. Totally 40 Adult female Lister hooded rats (Charles River Laboratories, Germany), weighting 200-230g and aged between 8 to 10 weeks were used for the experiments.

2.1.3. Surgical Procedures

Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the authorities Institutional Animal Care and Use Committee (University of Münster). Lister-Hooded rats were given free access to food and water under a normal 12 hour light-dark cycle. The rats were anesthetized by intraperitoneal injection of 10% ketamine (60–80 mg/kg) and 2%xylazine (10–15 mg/kg). Surgery was performed under visual control using an operating microscope. The rats were killed by CO_2 inhalation at various points, and the eyes were enucleated.

2.1.3.1. Optic Nerve Transection

Degeneration of RGCs was achieved by complete transection of the ON. The head of the anesthetized animal was positioned and fixed in a head-holder. A 1.5-2 cm incision was made in the skin above the left orbit. The ON was surgically exposed in its intraorbital segment under a surgical microscope. After partial resection of the lachrymal glands and extraocular muscles, the meninge of the optic nerve was opened longitudinally. Avoiding

injury to the ophthalmic artery, the ON was transected with microscissors at a distance of 0.5 mm behind the eye. The blood flow of retina was monitored by using a direct ophthalmoscope during the surgery.

2.1.3.2. Grafting of Peripheral Nerve Segments

The meninge of the left ON was opened and the nerve was completely transected intraorbitally 0.5 mm behind the optic nerve head. A 1.5 cm segment of autologous peripheral nerve (PN) was obtained from the peroneal branch of the right sciatic nerve (SN) and transplanted onto the ON stump using 10/0 suture (10-0 silk), as previously described in detail [221].

2.1.4. Animal Experimental Design

The RGCs regeneration mediated characterization study comprised three groups of animals: one control group were normal animals without any surgical treatment (N=6); another group of animals served with ON axotomy (n=15); the third group of animals were those with transected ON and grafting of a segment of PN to the cut end of the optic nerve (n=18). Axotomized rats were divided into two groups (n=3 per group) surviving for either 5 or14 days after optic nerve cut for immunostaining; and three groups (n=3 per group) for 5, 14 and 21 days for RNA isolation. The grafted rats were divided into three groups (n=6 per group), surviving for 5, 14, and 21 days after optic nerve cut and simultaneous PN grafting. All animals were sacrificed on each time schedule; left eyes were enucleated for immunohistochemistry and molecular analysis.

2.2. Immunohistochemistry

2.2.1. Preparation of Tissue Sections

Tissue-Tek O.C.T. compound	Sakura Company, Torrance, CA, USA
Gelatin-coated glass slides	Superfrost Plus; Fisher Scientific

Immediately following surgical eye removal, the whole eye was embedded in Tissue-Tek O.C.T. compound and then snap-frozen in liquid nitrogen. Frozen sections (10-12 μm) were cut longitudinally on a cryostat, and were thaw-mounted on gelatin-coated glass,

maintained at -20 °C until use.

2.2.2. Immunohistochemistry

The sections were incubated with antibodies to visualize the microenvironment within the retina.

2.2.2.1. Material

DAPI	Hoechst
10% fetal calf serum (FCS)	Carlsbad, CA, USA
Mowiol	Hoechst, Germany)
Microscope	Axiophot, Zeiss; Germany

2.2.2.2. Antibodies

2.2.2.2.1. First Antibody

Rabbit Anti-Hsp25 polyclonal Antibody (SPA-801)	Stressgene
Rabbit Anti-NF-κB p65 polyclonal Antibody (sc-109)	Santa Cruz
Rabbit Anti-I-κB-α polyclonal Antibody (sc-371)	Santa Cruz
Rabbit Anti-Endothelin polyclonal Antibody (E-1645)	Sigma
Mouse Anti-Rhodopsin monoclonal antibody (MAB5316)	Chemicon International
Mouse Anti-GAP-43 monoclonal antibody (G9264)	Sigma
Mouse Anti-GFAP monoclonal antibody (G3893)	Sigma

2.2.2.2.2. Second Antibody

CyTM2-conjugated*AffiniPure F (ab´) 2 Fragment Goat Anti-Mouse IgG (H+L)	Jackson ImmunoResearch, Westgrove, PA, USA

CyTM2-conjugated*AffiniPure Goat Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch, Westgrove, PA, USA

2.2.2.3. Immunohistochemistry Procedure

1) Frozen sections were fixed in cold methanol (store at -20^{o}C) for 10 mins.
2) Washed 3 x 5 mins in phosphate buffer saline (PBS).
3) Blocked with 10% fetal calf serum (FCS) in a humid chamber for 30 mins at room temperature.
4) The first antibody was diluted in FCS with different dilution (GAP-43 1:100; GFAP 1:400; the others 1:200), and the sections were incubated overnight at 4 ^{o}C.
5) After rinsing the slides for 3 x 5 mins in PBS.
6) The sections were incubated with an appropriate fluorescein-conjugated second antibody (1:200) for 30 mins at room temperature.
7) Wash 3 x 5 mins in PBS.
8) Finally the slides were cover-slipped using Mowiol with DAPI to stain nuclear, and viewed with the appropriate filter under a fluorescence microscope at a final magnification of x 200 or x 400.
9) Control samples were treated without the primary antibodies.

2.3. Molecular Biology Work

2.3.1. Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. RNases are difficult to inactivate and even minute amounts are sufficient to degrade RNA. When handling RNA, proper microbiological, aseptic techniques should always be used to avoid RNase contamination. Creating and maintaining an RNase-free environment, always wear gloves while handling regents and RNA samples. Use sterile, disposable plastic ware and filtered pipette tips. All solutions should be made with DEPC treated water. Glassware should be baked at 180 ^{o}C for 5 hours. To minimize degradation of RNA by limiting the activity of endogenous RNases, store RNA at -80 ^{o}C.

2.3.2. DEPC H_2O

0.1% DEPC H_2O	1000ml	
	1000 ml	bidistilled H_2O
	1 ml	DEPC (Diethylpyrocarbonate)

Stirred for 6-8 hours at room temperature; then let incubate overnight in a fume hood. The next day residual DEPC is removed by autoclaving. Solution was stored at room temperature.

2.3.3. RNA Extraction

2.3.3.1. Buffer, Enzyme and Chemicals

RNeasy Mini Kit (74104):	QIAGEN GmbH, Germany
Buffer RLT	
Buffer RW1	
Buffer RPE	
RNase-free water	
RNase-Free DNase Set (79254):	QIAGEN GmbH, Germany
DNase I (1500 Kunitz units)	
Buffer RDD	
RNase-free water	
100% Ethanol	Merck
70% Ethanol	Merck
β-Mercaptoethanol (β -ME)	Merck

2.3.3.2. Expendable Material and Devices

RNeasy mini spin columns	QIAGEN
Collection tubes (1.5, 2 ml)	QIAGEN
20-gauge needle RNase- free syringe	
Microcentrifuge	Heraeus
Spectrophotometer	Eppendorf
Vortex-Genie 2	Scientific Industries

2.3.3.3. RNA Isolation Procedure

Total RNA from retina was extracted using the RNeasy Mini Kit according the protocol of the manufacture. Contaminating DNA was removed with RNase-free DNase.

1) Pooled the retina (not more than 30mg) in 350µl buffer RLT with fresh added β –ME (1:100).
2) Homogenized retina tissue by passing legate at least 5 times through a 20-gauge needle fitted to an RNase- free syringe.
3) Centrifuge the tissue lysate for 3 min at 13,000rpm speed in a microcentrifuge. Carefully transferred the supernatant to a new microcentrifuge tube by pipetting.
4) Added 1 volume (350µl) of 70%ethanol to the cleared lysate, and mixed immediately by pipetting.
5) Transferred all solution including any precipitate to an RNeasy mini column placed in a 2 ml collection tube, centrifuge for 1 min at >10,000 rpm. Discarded the flowthrough.
6) Pipetted 350µl Buffer RW1 into the spin column, and centrifuge 15 sec at >10,000 rpm. Discarded the flowthrough.
7) Added 10µl DNase stock solution to 70µl Buffer RDD, mixed gently. Pipetted whole 80µl incubation mix onto the spin column membrane, and placed on the bench top for 15 min in room temperature.
8) Pipetted 350µl Buffer RW1 into the spin column, and centrifuge 15 sec at >10,000 rpm. Discarded the flowthrough.
9) Transferred the RNase column into a new collection tube. Pipetted 500µl Buffer RPE onto the column, and centrifuge 15 sec at >10,000 rpm. Discarded the flowthrough.
10) Added 500µl Buffer RPE onto the column, and centrifuge 2 min at >10,000 rpm.
11) To elute, transferred the RNase column into a new collection tube, add 30µl RNase-free water onto the membrane, centrifuge 1 min at >10,000 rpm. Added 30µl RNase-free water; centrifuge 1 min to get a total volume of 60µl RNA.

The concentrations of RNA were determined by measuring absorbance at 260 nm (A_{260}) in a spectrophotometer. The ratios (A_{260}/A_{280}) of all samples were between 1.75 and 2.00.

2.3.4. Reverse Transcription (RT)

2.3.4.1. Material

M-MLV Reverse Transcriptase	Promega
5X Reaction Buffer	Promega
DTT (100mM)	Promega
dNTP's	Amersham
oligo-d(T)-Primer (5´-pd(T)12-18-3´)	Promega
Different size of filter tips	Greiner
Eppendorp (0.5, 0.2 ml), RNase- free	Biozym
Filter tips, RNase- free	Greiner

2.3.4.2. Devices

Blockthermostat BT 200	Kleinfeld Labortechnic
Trio-Thermoblock	Biometra
-20^{o}C Freezer	AEG

2.3.4.3. Procedure

The RNA-dependent DNA-polymerase activity (reverse transcription, RT) transcribes complementary DNA (cDNA) from an RNA template. This activity allows synthesis of cDNA for cloning, PCR, RNA sequencing, and primer extension. Total RNA (1ng) was used to generate first strand cDNA by random priming with reagents and protocols used as recommended by manufacturers. RNA was reverse-transcribed using M-MLV reverse Transcriptase.

Component	Volume/reaction	Final concentration
Master Mix		
5x Buffer RT	5.0µl	1x
DTT (100mM)	2.0µl	10nM
dNTP's (10mM)	1.0µl	5mM
Oligo-d(T) primer (10µM)	1.0µl	1µM
RNase inhibitor	1.0µl	20µ
M-MLV	1.0µl	200µ
RNase- free water	Variable	
Template RNA	Variable	1ng
Total volume	20.0µl	-

1) Thawed template RNA solution on ice.
2) For each template RNA sample, pipeted variable volume with containing 1ng RNA, add RNase-free water to bring a total volume of 9.0µl.
3) For denaturation, incubated for 10 min at 70 ^{o}C.
4) Immediately put on ice for 10 min.
5) Prepared a fresh Master Mix on ice according to the table above.
6) Added 11µl Master mix to the individual tubes containing the denatured of template RNA, mix by pipeting.
7) Incubated for 60 min at 37 ^{o}C.
8) To inactivate M-MLV Reverse Transcriptase by heat the reaction mixture to 93 for 5 min followed by rapid cooling on ice.
9) cDNA were keeping in -20 ^{o}C for further use.

2.3.5. Polymerese Chain Reaction (PCR)

2.3.5.1. Devices

GeneAmp PCR-System 9700 Perkin Elmer

2.3.5.2. Material

TITANIUMTM Taq DNA Polymerase Kit (8434-1) Clontech

Taq DNA Polymerase

10X Taq PCR Buffer

2.3.5.3. Primers

Primer pairs were carefully chosen to avoid amplification of genomic DNA. Primers were picked up from the sequence by using program "Primer3". As the stably expressed reference gene (housekeeping gene) β-actin was used.

β-actin primers

forward, 5'-CGCAGTTGGTTGGAGCAAAC-3'

reverse, 5'-AAGCAATGCTGTCACCTTCCC-3'

products 225 bp

GFAP primers

forward, 5’-GCTTACTACCAACAGTGCC-3’
reverse, 5’-CCACCGTCTTTACCACGAT-3’
products 853 bp

Hsp27 primers

forward, 5’-CCTCTTCGATCAAGCTTTCG-3’
reverse, 5’-CTTTCTTCGTGCTTGCCAGT-3’
products 318 bp

Endothelin-1 primers

forward, 5’-ACTTCTGCCACCTGGACATC-3’
reverse, 5’-GGCTCGGAGTTCTTTGTCTG-3’
products 200 bp

EAAC-1 primers

forward, 5’-AGAAATTCTGATGCGGATGC-3’
reverse, 5’-GGCAGGCTTCACTTCTTCAC-3’
products 349 bp

Stat 6 primers

forward, 5’-GGGGACTGCTACCAGAACACT-3’
reverse, 5’-ATCTGTGAGGAGCCATCCTG-3’
products 358 bp

Stat 3 primers

forward, 5’-GACCCGCCAACAAATTAAGA -3’
reverse, 5’-CGACATCCCCAGAGTCCTTA -3’
products 299 bp

Rhodopsin primers

forward, 5’-GGATTCACCACCACCCTCTA -3’
reverse, 5’-CAATCCCACATGAACACTGC -3’
products 301 bp

Calmodulin primers

forward, 5’-ACTGGGTCAGAACCCAACAG -3’
reverse, 5’-TTAGCTTTTCCCCGAGGTTT -3’
products 236 bp

GM-CSF primers

forward, 5’-GATGCCATCAAAGAAGCTCTG -3’

	reverse, 5'-AGTGGCTGGCTATCATGGTC -3'
	products 202 bp
Thy-1 primers	
	forward, 5'-GGTGAACCAGAACCTTCGAC -3'
	reverse, 5'-CAGCAGCCAGGAAGTGTTTT -3'
	products 346 bp
Parvalbumin primers	
	forward, 5'-GGCCTGAAGAAAAAGAGTGC -3'
	reverse, 5'-TTCTTCAACCCCAATCTTGC -3'
	products 204 bp
GAP-43 primers	
	forward, 5'-GGCTCTGCTACTACCGATGC -3'
	reverse, 5'-GGCTTGTTTAGGCTCCTCCT -3'
	products 225 bp
HO-1 (Hsp32) primers	
	forward, 5'-AAGGCTTTAAGCTGGTGATGG -3'
	reverse, 5'-AGCGGTGTCTGGGATGAACTA -3'
	products 669 bp

Component	Volume/reaction	Final concentration
Master mix		
10x Buffer	2.5µl	1x
dNTP's (25mM)	1.0µl	0.2mM
forward primer (100pmol/µl)	0.125µl	0.5pmol/µl
reverse primer (100pmol/µl)	0.125µl	0.5pmol/µl
A-Taq DNA Polymerase (20 µ/µl)	0.125µl	0.05µ/µl
RNase- free water	19.48µl	
Template RNA	2.5µl	
Total volume	25.0µl	

2.5 µl of RT reaction, representing 1ng input RNA was used as template for PCR amplification in a 25µl reaction volume. cDNA was amplified by PCR using Taq polymerase. Specific 20 base oligonucleotide primer pairs were added to the PCR reaction

vials and 35 cycles were performed at 30 sec 94 °C, 30 sec 60 °C, and 30 sec 72 °C. PCR were carried out in a Perkin-Elmer 9700 thermocycler. Each primer pair amplified a single band of the expected sized. No template control served without cDNA, instead of RNase-free water.

2.3.6. Gel Electrophoresis PCR Products

Buffers and Chemicals:

Phosphate buffer saline (PBS), pH 7.4

NaCl	9g/l	Sigma
KH_2PO_4	0.27g/l	Sigma
Na_2HPO_4 x 2 H_2O	1.42g/l	Sigma

ad H_2O to total volume 1,000 ml

5X TBE running buffer (Tris-Borat-EDTA-Buffer), pH 8.0

Tris (Tris-(Hydroxymethyl -aminomethan))	54.0g/l	Merck
Boric acid	27.5g/l	Sigma
Na_2EDTA x 2 H_2O	3.72g/l	Merck

ad H_2O to total volume 1,000 ml

5X TE buffer (Tris-EDTA), pH 8.0

10mM Tris/HCl	Merck	
1mM EDTA	Merck	

ad DEPC-water to total volume 1,000ml

6X Ficoll-buffer, 0.4 ml

60% (w/v) Ficoll	100µl	Sigma
1% Bromophenol blue	100µl	Sigma
RNase-free water	200µl	Sigma
Seakem-LE-Agarose		FMC Bioproducts
Ethidium Bromide		Sigma
Digital gel Camera		Zeiss

5.0µl PCR-amplified DNA fragments were resolved on 1.5% agarose gel containing 0.4 µg/ml ethidium bromide, running with 1X TBE buffer at 120 V, 40 mins. Bands were visualized with 302 nm UV light and photographed using a digital gel camera.

2.3.7. Quantitative Real-Time PCR

2.3.7.1. Material

2x TaqMan Universal PCR Master Mix (P/N 4324018)	Applied Biosystems
96-well optical reaction plates	Applied Biosystems
TaqMan probes	MWG, The genomic Company, Germany
Primers	MWG, The genomic Company, Germany

2.3.7.2. Device

ABI 5700 Sequence Detector	Perkin Elmer, Foster City, CA, USA
Primer Express version 1.0 software	Perkin Elmer, Foster City, CA, USA

Changes in mRNA levels of specific genes were quantified by Real time quantitative PCR. To validate the amount and quality of source of RNA, transcript levels of a housekeeping gene β -actin were measured.

Primer and TaqMan probe sequences were chosen with assistance of software Primer Express version 1.0. Accession Numbers for sequences used were M16228 for GAP-43, NM_017009 for GFAP, NM_012673 for Thy-1, NM_012548 for ET-1, X57764 for ETB-1, and NM_031144 for β -actin.

2.3.7.3. TaqMan Primer and Probe Design

1) The Primer Express software designs primers with a melting temperature (Tm) of 58-60 ^{o}C, and probes with a Tm value of 10 ^{o}C higher. The Tm of both primers should be equal.
2) Primers should be 15-30 bases in length.
3) The G+C content should ideally be 30-80%.
4) The run of an identical nucleotide should be avoided. This is especially true for G, where runs of four or more Gs are not allowed.
5) The total number of Gs and Cs in the last five nucleotides at the 3' end of the

primer should not exceed two. This helps to introduce relative instability to the 3' end of primers to reduce non-specific priming.

6) Maximum amplicon size should not exceed 400 bp (ideally 50-150 bases). Smaller amplicons give more consistent results because PCR is more efficient and more tolerant of reaction conditions.
7) The probes should not have runs of identical nucleotides (especially four or more consecutive Gs), G+C content should be 30-80%, there should be more Cs than Gs, and not a G at the 5' end.

2.3.7.4. Primers

GAP-43 primers

forward, 5'-CCGAGGCTGACCAAGAACA-3',
used at 300nmol;
reverse, 5'-TGAGAAAGGGCAGGAGAGACA-3',
used at 300nmol.
Product 88bp.

GFAP primers

forward, 5'-TCAGTGCGGAGTCTCATGGA-3',
used at 300nmol;
reverse, 5'-GTCCTCCCCTCCTGATTGC-3',
used at 300nmol.
product 86bp.

Thy-1 primers

forward, 5'-TGACAGCCTGCCTGGTGAA-3',
used at 300nmol;
reverse, 5'-TCATGCTGGATGGGCAAGT-3',
used at 300nmol.
product 78bp.

β-actin primers

forward, 5'-GGAAATCGTGCGTGACATTAAAG-3',
used at 300nmol;
reverse, 5'-CGGCAGTGGCCATCTCTT-3',
used at 300nmol.

product 73bp.

ET-1 Primers

forward, 5'-AAGAGAGGTTGAGGTGTTCCCTAA-3',
used at 300nmol;
reverse, 5'-CATCCCCAGACAGCAAGAAGA-3',
used at 300nmol.

ETB-1 Primers

forward, 5'- GCACTTCAGCACGGCTCTTAA-3',
used at 300nmol;
reverse, 5'- ACCCCTGAATAGAGTTTGAGTTCTTG-3',
used at 300nmol.

2.3.7.5. TaqMan Probes

TaqMan probes were labeled at the 5'-end with the reporter dye 6- FAM (6-carboxyfluorescein; emission λmax=518 nm) and at the 3'-end with the quencher dye TAMRA (6-carboxytetramethylrhodamine; emission λmax=582 nm).

The following sequences were used as probes:

GAP-43 5' (FAM)-TTTCCACGTTGCCCCCACCTGAA-(TAMRA)-3',
used as 200nmol/l.

GFAP 5' (FAM)- CTGCATCTCCAACTAACAGGATACCACC-(TAMRA)-3',
used as 200nmol/l.

Thy-1 5' (FAM)- ACCTTCGACTGGACTGCCGTCATGA-(TAMRA)-3',
used as 200nmol/l.

β -actin 5' (FAM)- AGCTGTGCTATGTTGCCCTAGACTTCGAGC-(TAMRA)-3',
used as 200nmol/l.

ET-1 5' (FAM)- CTTCGTTTGCATCCGCTGGTAGCAA-(TAMRA)-3',
used as 200nmol/l.

ETB-1 5' (FAM)- CCTCACTGCACTCTCAGCCCACTTACATTTAA-(TAMRA)-3',
used as 200nmol/l.

2.3.7.6. PCR Amplification

Component	Volume/Reaction	Final Concentration
Master Mix (2X)	12.5µl	1X
Forward primer (100pmol/µl)	1.5µl	300nM
Reverse primer (100pmol/µl)	1.5µl	300nM
TaqMan Probe (100pmol/µl)	0.5µl	200nM
RNase- free water	8µl	
Template RNA	1µl	
Total volume	25.0µl	

Real-Time Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR)

Oligonucleotide sequences corresponding to the selected gene transcripts examined by RT-PCR were designed using Primer Express software and are available on request from the author. One µg of total cellular RNA from the retinal tissue specimens was subjected to reverse transcription using M-MLV reverse Transcriptase and oligo-dT, following the manufacturer's protocol (as described above). After first strand synthesis, an equivalent of 50 ng of starting total cellular RNA (1/20th of the cDNA reaction) was added to three triplicate PCR reactions containing 1x TaqMan Universal PCR Master Mix, 300nmol/L forward primer, and 300nmol/L reverse primer in a final volume of 25 µl. The Universal master mix contains ROX (6-carboxy-X-rhodamine), the passive reference fluorochrome that normalizes for pipetting volume errors. Each reaction was performed in triplicate in 96-well Optical reaction plates that cycled at 2 min at 50 ^{o}C, 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, and 60°C for 1 minute on a GeneAmp 5700 sequence detection system. Fluorescent data were converted into cycle threshold (Cт) measurements using the 5700-system software and exported to Microsoft Excel. Before this analysis, the performance of each primer pair was tested by making a reference RNA cocktail containing RNA from all samples analyzed. One µg of this cocktail was converted to cDNA and a series of twofold serial dilutions were subjected to quantitative PCR analysis as described above. Plots of log (input cDNA) versus CT were examined to verify linearity.

Each assay included ``no template´´ control and standard curve.

2.3.7.7. Quantify the Amount of Template

2.3.7.7.1. Methods

There are three methods to quantify the amount of template:

1) Absolute standard method: In this method, a known amount of standard such as in vitro translated RNA (cRNA) is used.
2) Relative standard: Known amounts of the target nucleic acid are included in the assay design in each run.
3) Comparative C_T method: This method uses no known amount of standard but compares the relative amount of the target sequence to any of the reference values chosen and the result is given as relative to the reference value (such as the expression level of resting lymphocytes or a standard cell line).

2.3.7.7.2. The Comparative C_T Method ($\Delta\Delta C_T$)

The comparative C_T method ($\Delta\Delta C_T$) for relative quantitation of gene expression:

This method enables relative quantitation of template and increases sample throughput by eliminating the need for standard curves when looking at expression levels relative to an active reference control (normalizer). For this method to be successful, the dynamic range of both the target and reference should be similar. A sensitive method to control this is to look at how ΔC_T (the difference between the two C_T values of two PCRs for the same initial template amount) varies with template dilution. If the efficiencies of the two amplicons are approximately equal, the plot of log input amount versus ΔC_T will have a nearly horizontal line (a slope of <0.10). This means that both PCRs perform equally efficiently across the range of initial template amounts. If the plot shows unequal efficiency, the standard curve method should be used for quantitation of gene expression. The dynamic range should be determined for both (1) minimum and maximum concentrations of the targets for which the results are accurate and (2) minimum and maximum ratios of two gene quantities for which the results are accurate. In conventional competitive RT-PCR, the dynamic range is limited to a target-to-competitor ratio of about 10:1 to 1:10 (the best accuracy is obtained for 1:1 ratio). The real-time PCR is able to achieve a much wider dynamic range.

As long as the target and normalizer have similar dynamic ranges, the comparative C_T method ($\Delta\Delta C_T$ method) is the most practical method. It is expected that the normalizer will have a higher expression level than the target (thus, a smaller C_T value). The calculations for the quantitation start with getting the difference (ΔC_T) between the C_T

values of the target and the normalizer:

$\Delta C_T = C_T$ (target) - C_T (normalizer)

This value is calculated for each sample to be quantitated (unless, the target is expressed at a higher level than the normalizer, this should be a positive value. It is no harm if it is negative). One of these samples should be chosen as the reference (baseline) for each comparison to be made. The comparative $\Delta\Delta$ C_T calculation involves finding the difference between each sample's Δ C_T and the baseline's Δ C_T. If the baseline value is representing the minimum level of expression, the $\Delta\Delta$ C_T values are expected to be negative (because the Δ C_T for the baseline sample will be the largest as it will have the greatest C_T value). If the expression is increased in some samples and decreased in others, the $\Delta\Delta$ C_T values will be a mixture of negative and positive ones. The last step in quantitation is to transform these values to absolute values. The formula for this is:

Comparative expression level = $2^{-\Delta\Delta C_T}$

This study designed an untreated control group animal to modify the gene change of the experimental groups. Relative quantification methods were used to describe the change in expression of the target gene relative to an untreated control group at time zero in a time-course study. The $2^{-\Delta\Delta C_T}$ methods were used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments. The $\Delta\Delta$ C_T method was used to calculate fold expression levels relative to the average value of normal control RNA specimens (the calibrator). Expression of each gene in each sample was normalized to the signal obtained for a β -actin control performed on the same plate. Fold expression change for each sample was defined as $2^{-\Delta\Delta C_T}$ where $\Delta\Delta$ C_T was defined as the difference between the sample's Δ C_T ($C_{T_{gene}}$-$C_{T_{\beta\text{-actin}}}$) and the average normal control reference's Δ C_T ($C_{T_{gene}}$-$C_{T_{\beta\text{-actin}}}$).

2.3.7.8. Standard Curve

Performance of primer pairs and probes was tested using a series of twofold serial dilutions of a cocktail containing cDNA from all samples analyzed (equivalent of 1 µg total RNA). Plots of log (input cDNA) *versus* C_T were examined to verify amplification linearity. Slopes of the standard curves were also determined to assess amplification efficiency. The C_T values were plotted *versus* the log of the initial amount of cDNA to give the standard curve shown in Figure 1. Each sample and standard is done in triplicate.

2.3.7.9. Amplification Plot

An amplification plot is the plot of fluorescence signal *versus* cycle number. In the initial of PCR, there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The parameter C_T is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Figure 2 shows amplification plots of β –actin, GFAP, GAP-43, Thy-1, ET-1 and ETB genes.

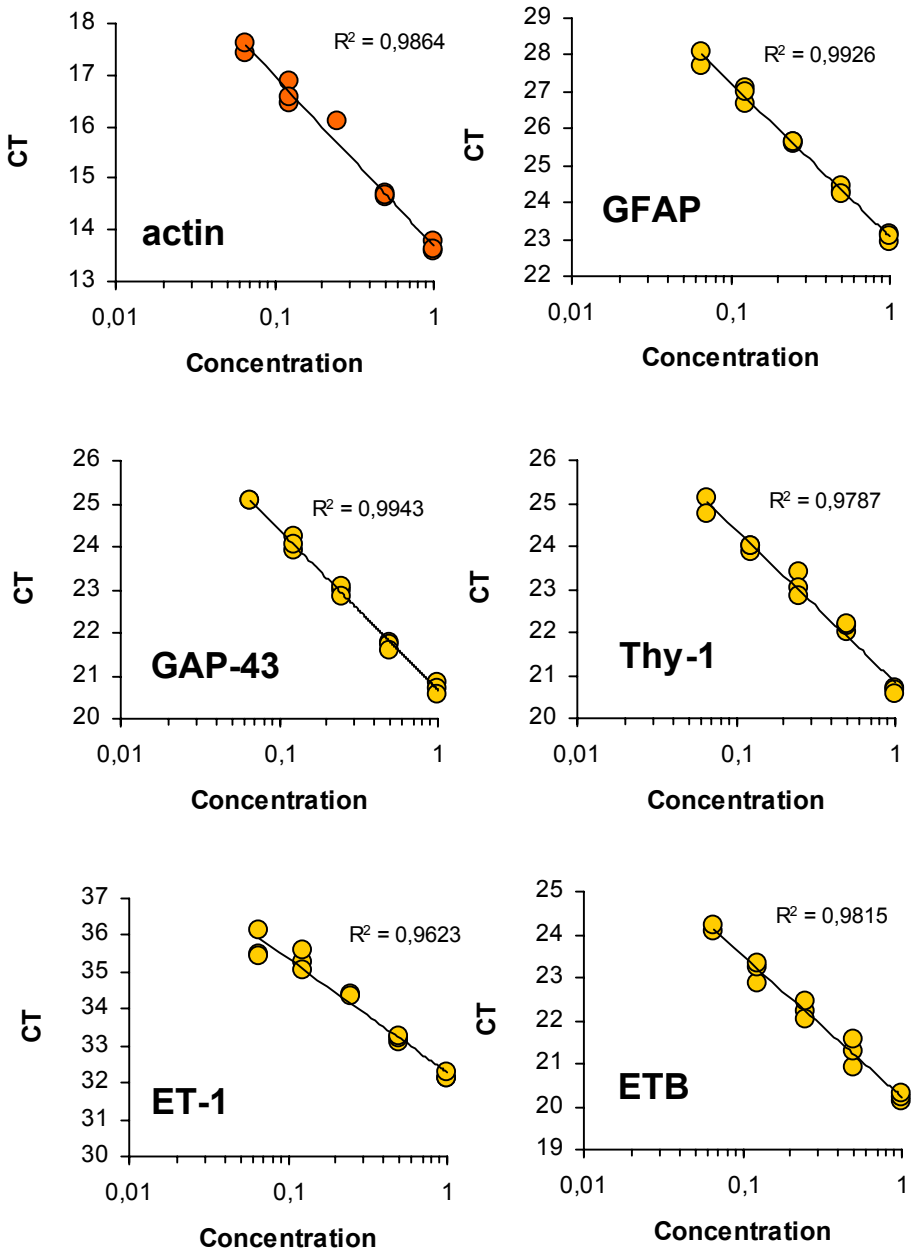

Figure 1. Standard curves. Concentrations of cDNA are plotted against treshold cycles (C_T).

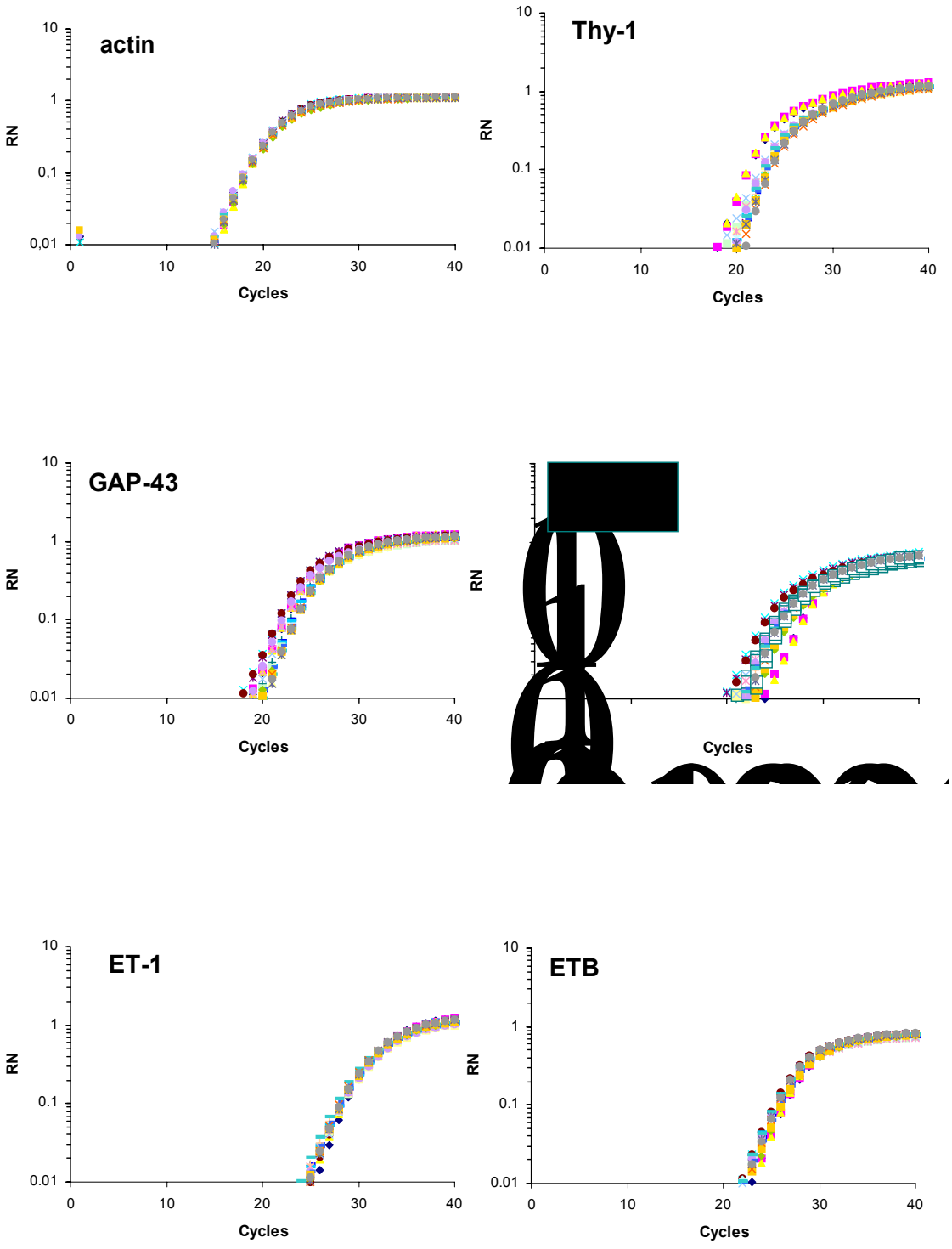

Figure 2. Representative amplification plots for β – actin, GFAP, GAP-43, Thy-1, ET-1 and ETB from retinal cDNA showing the increase in fluorescence signal (RN, y axis) with increasing cycle number (x-axis). The threshold (C_T) for signal detection was set at a RN of 0.02.

3. Results

3.1. Immunohistochemistry

Cryostat sections were examined from representative specimens to detect changes of protein expression.

3.1.1. GFAP

As expected, GFAP was confined to the astrocytes within the ganglion cell layer (GCL) and the nerve fibre layer (NFL) (Fig. 2F). Axotomized retinas showed a clear upregulation of GFAP at 5 and 14 days after axotomy (Fig. 2 A, B). GFAP-immunoreactive astrocytic cells and their processes were mainly observed in the NFL, GCL and inner plexiform layer (IPL) (Fig. 2 A, B). The immunostaining in the inner nuclear layer (INL), outer plexiform layer (OPL) and outer nuclear layer (ONL) (Fig. 2 A, B) was confined to Müller cells and their processes (arrowheads). Some GFAP-immunoreactive cells and processes could be observed to span through all retinal layers, including the outer segment of the ONL, and some processes extended even up to the photoreceptor layer (PRL). Compared to the injured retinas, grafted and regenerating retinas expressed lower levels of GFAP that was virtually restricted to the astrocytes with the Müller cells to show no upregulation at 5, 14 and 21 days after grafting (Fig. 2 C-E). The data consistently show that degeneration of RGCs imposes higher expression of GFAP compared to partially degenerating and regenerating RGCs as occurred in the grafting paradigm.

3.1.2. GAP-43

The phosphoprotein GAP-43 has been shown to play a critical role during development of the CNS. In the retina of rats, GAP-43 is expressed in RGCs during development and postnatal. Then the protein is downregulated to disappear in adulthood. Indeed, staining of control and axotomized sections revealed virtually no positive immunoreactivity shown in GCL (Fig. 4 A, B and F). On the other hand, grafting of SN piece resulted in clear upregulation of GAP-43 in GCL at 5 days (Fig. 4 C, arrowhead). And GAP-43 immunoreactivity was strongly upregulated in 14 days post grafting (Fig. 4 D, arrowhead). After 21 days, only few GAP-43 were expressed on GCL (Fig. 4 E). These were indicating that regenerating RGCs express GAP-43.

3.1.3. NF-κb

The transcriptional factor NF-κb seems to be a universal heterodimer, which is utilized to control histones by translocation from the cytosol into the nucleus of cell. Within the nervous system NF-κb plays a pivotal role and may exert functions associated with axonal growth. Indeed, while in normal retinas NF-κb is located within the cytoplasm (Fig. 5 F) it migrates into the nucleus 5 days after axotomy (Fig. 5 A), and then disappear obviously due to the death of the RGCs. In the regenerating retinas NF-κb is migrating into nucleolus-like structures.

3.1.4. HSP27

Heat shock proteins, first identified in Drosophila and later in almost all organisms, are indicators of cellular stress. HSP27 has been characterized in injured nerve cells including neurons in the retina. Imminohistochemically, HSP27 is expressed in RGCs in normal retinas (Fig. 6 F). The protein seems redistributed within the RGCs 5 days after cut of the optic nerve (Fig. 6 A). By 14 days after cut of the optic nerve when the retina is almost depleted of RGCs, no HSP27 is detected in the ganglion cell layer (Fig. 6 B). In grafted retinas, HSP27 is continually expressed up to 21 days postsurgery indicating that regenerating RGCs continue to express the molecule over long periods of time (Fig. 6 C-E).

3.1.5. Rhodopsin

As expected, no changes in the rhodopsin expression were monitored after axotomy or grafting. This indicates that manipulations at the level of the optic nerve do not obviously influence the outer layers of the retina as seen in the content of Rhodopsin. Rhodopsin served therefore as the invariable parameter throughout the different groups and times postsurgery.

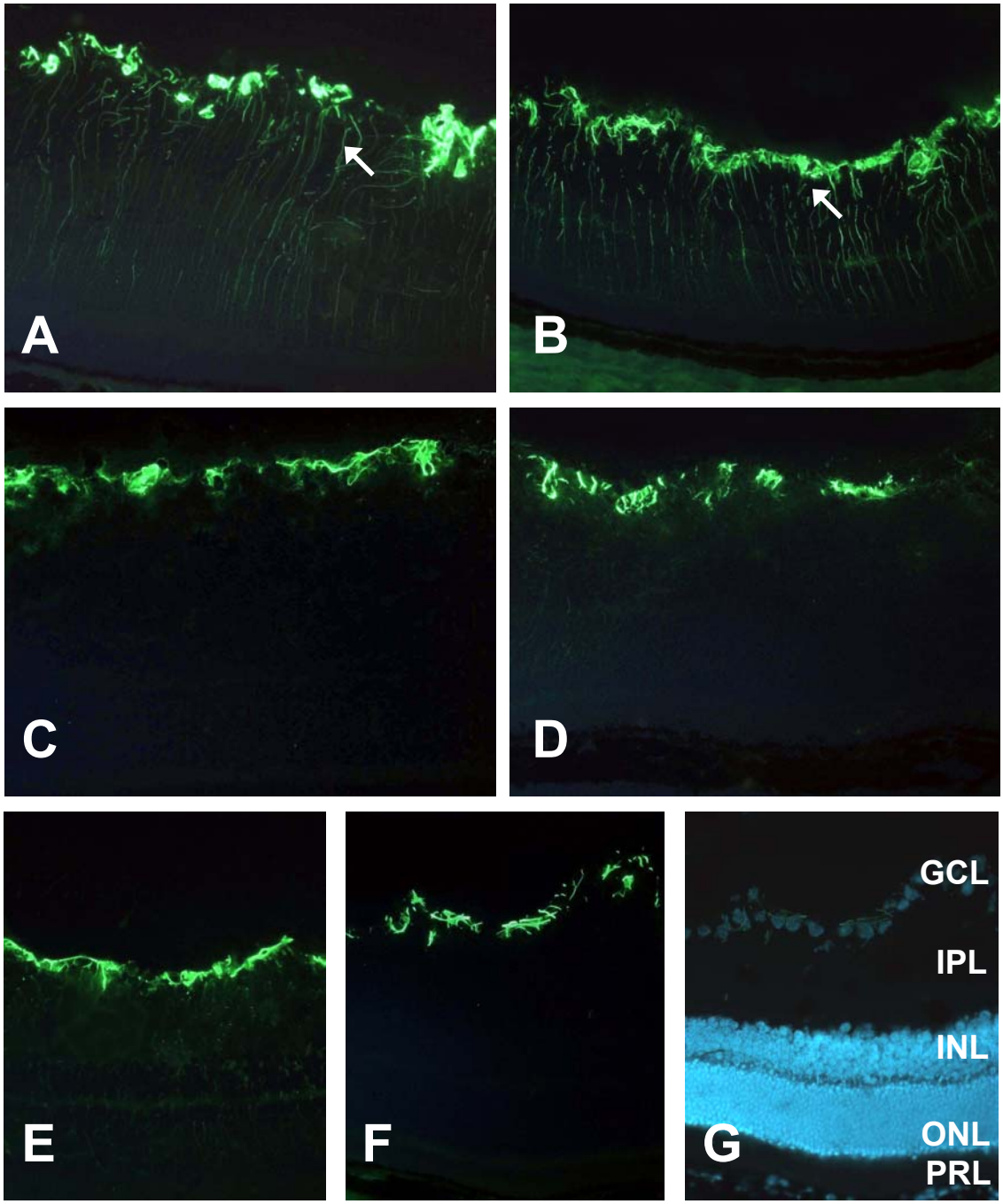

Figure 3. Expression of GFAP in axotomized (A, B), grafted (C-E), and control (F) retinas, (G) DAPI nuclear stain (blue signal).

Abbreviation: GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; ONL: outer nuclear layer; PRL: photoreceptor layer.

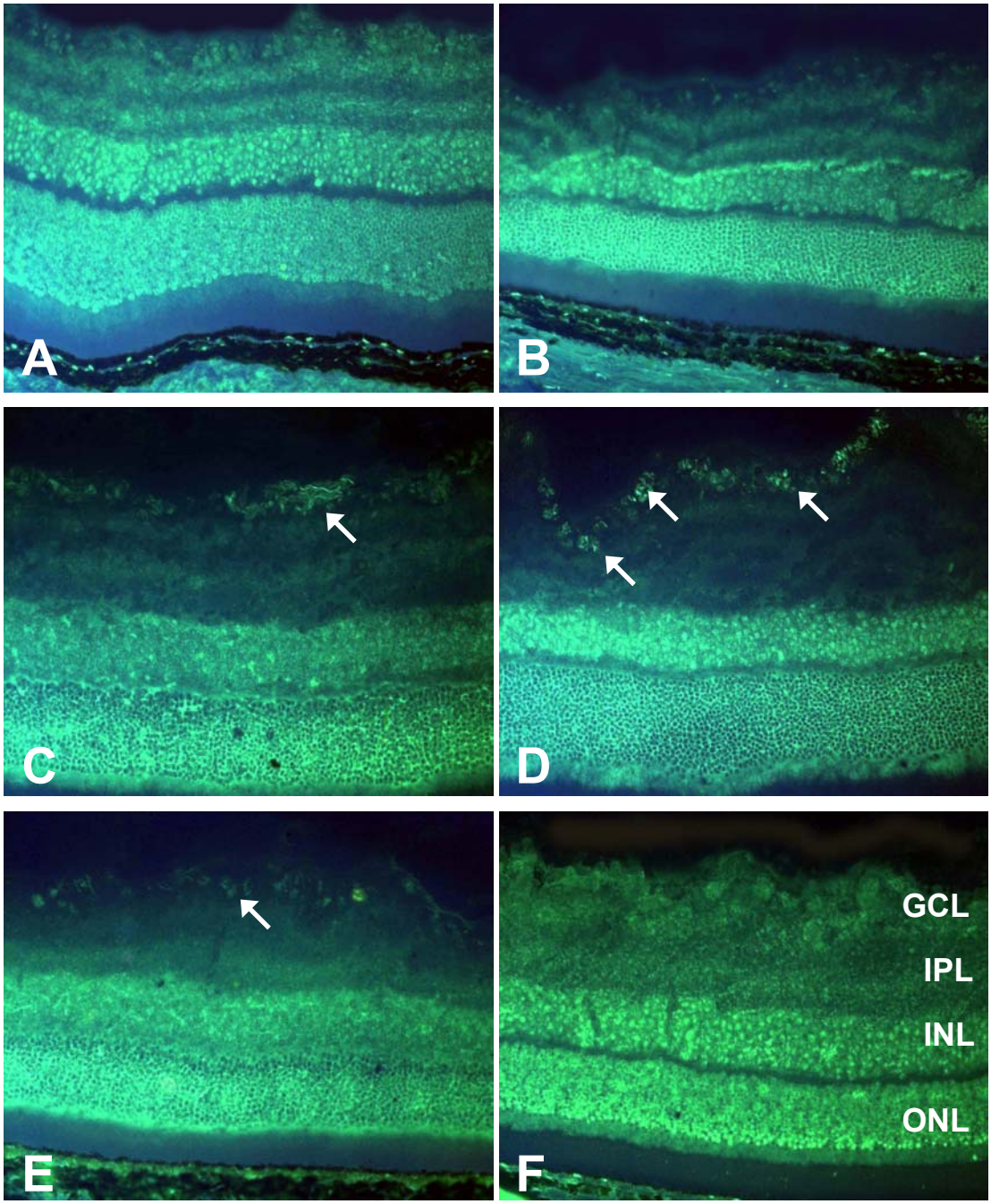

Figure 4. Photomicrographs of GAP-43 immunostaining in axotomized (A, B), grafted (C-E), and control (F) retinas of rats. GAP-43 immunoreactivity in the ganglion cell fibre layer (C and D arrowhead).

Abbreviation: GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; ONL: outer nuclear layer.

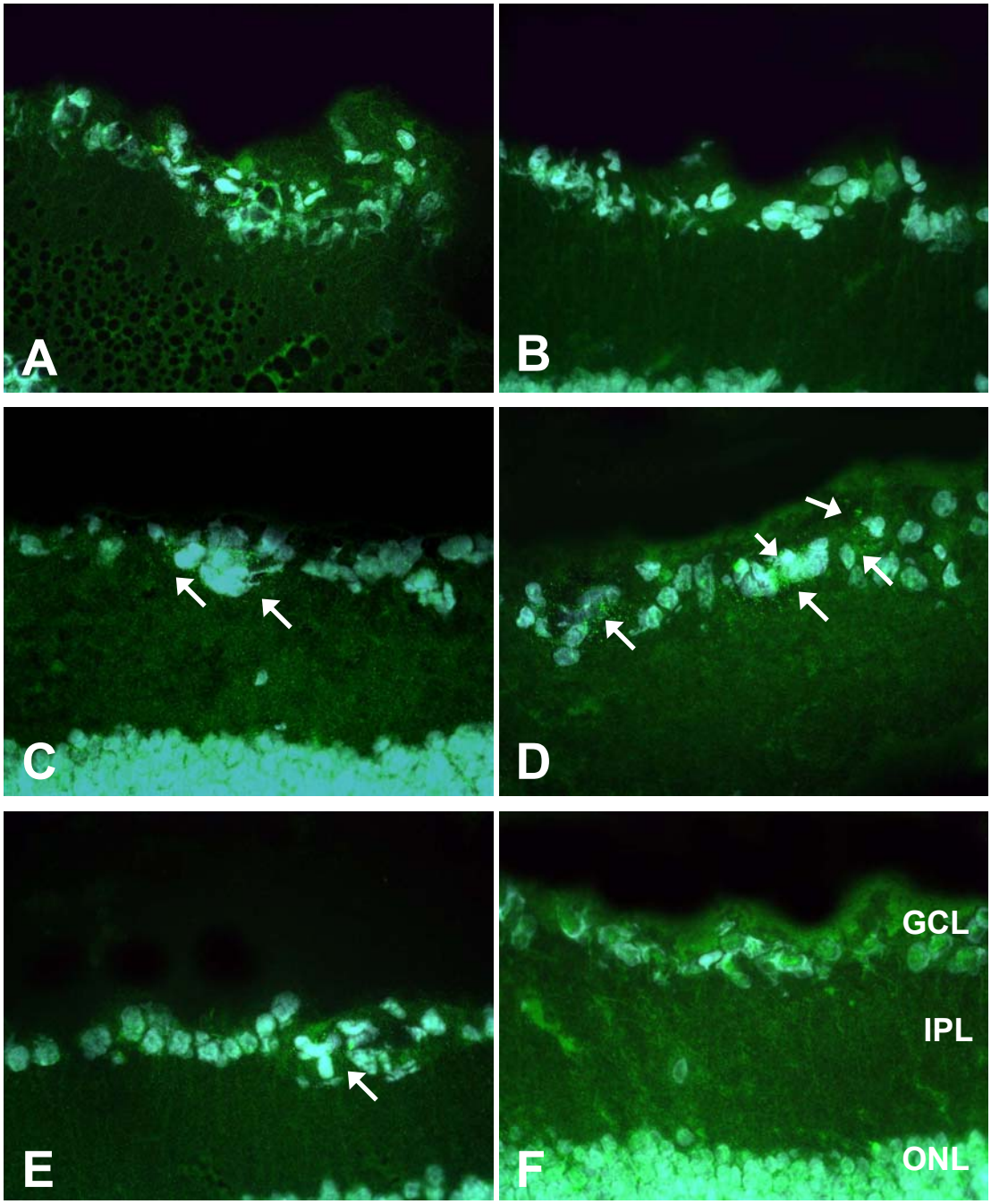

Figure 5. A, B) Expression of NF-κb within the GCL of axotomized retinas at 5 (A) and 14 (B) days after optic nerve cut. Arrows point to the localization of the staining. C, D, E) Regenerating RGCs at 5 (C), 14 (D) and 21 (E) days after grafting express the molecule which is translocated into the nucleus (arrowheads).

Abbreviation: GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; ONL: outer nuclear layer.

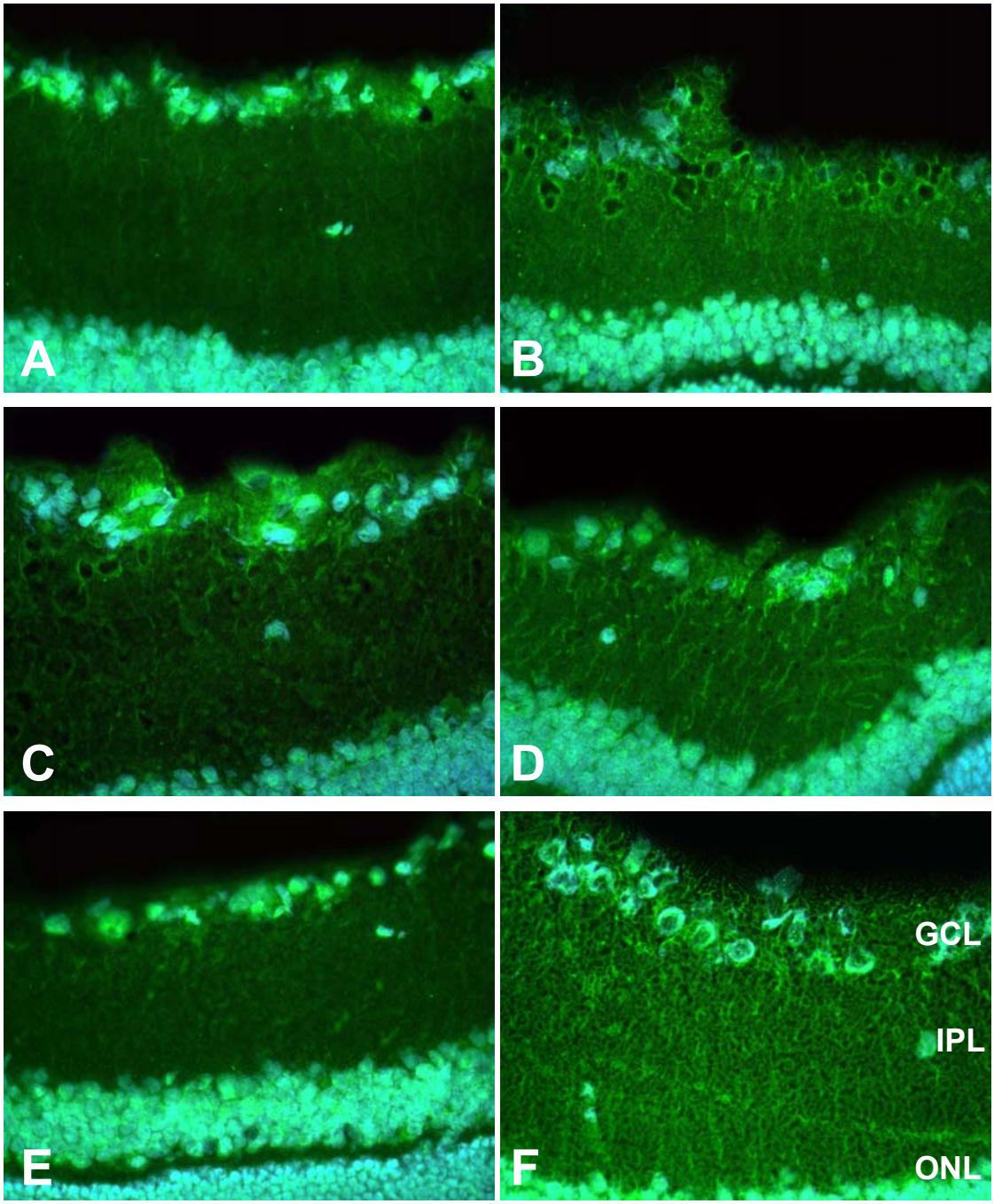

Figure 6. Expression of HSP27 in axotomized retinas 5 (A) and 14 days (B) after axotomized. The disappearence of HSP27 is (B) points to the degeneration of RGCs. In contrast, regenerating retinas at 5 (C), 14 (D) and 21 (E) days express HSP27, although the number of cells is lower, due to the degeneration of most RGCs despite of regeneration. F) Control retina. Abbreviation: GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; ONL: outer nuclear layer.

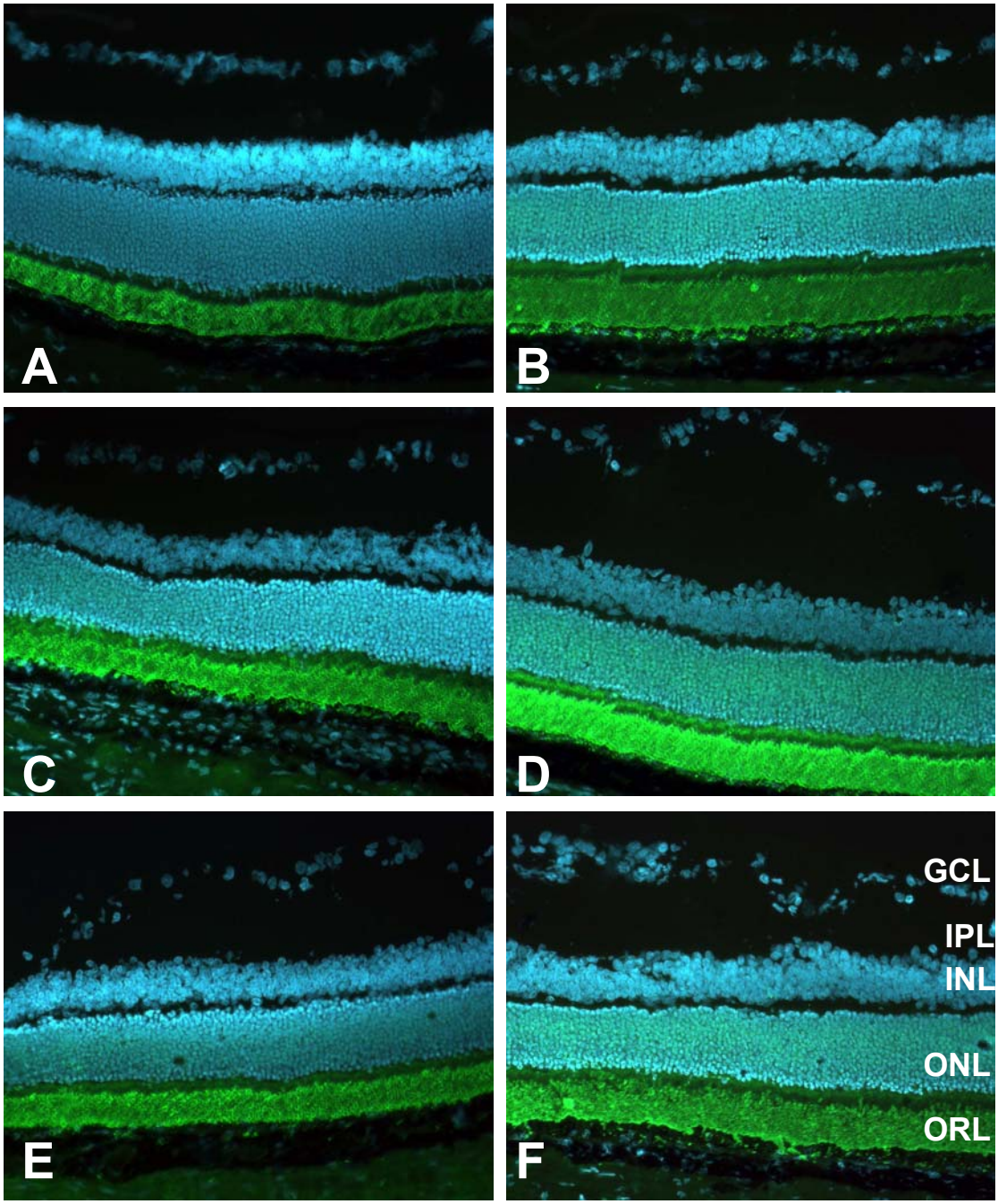

Figure 7. Immunohistochemical detection of Rhodopsin. The photoreceptor specific molecule is expressed in similar amounts throughout the period of axotomy (Fig. A, B) and regeneration (Fig. C-E). Control retina (Fig. F).

Abbreviation: GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; ONL: outer nuclear layer; PRL: photoreceptor layer.

3.2. RT-PCR

Changes in mRNA levels were first estimated by semi-quantitative RT-PCR. Rhodopsin and β-actin mRNA levels were used to normalize the amount of retinal cDNA synthesized. We have detected changes in mRNA levels of thirteen genes in rat retinas by RT-PCR after optic nerve transected and grafted. These include growth-associated protein GAP-43; neuron specific marker Thy-1; glial fibrillary acidic protein GFAP; endothelin; stress-response genes (HSP27 and HO-1); transcription factors (NF-κB, Stat3 and Stat6); glutamate transporters (EAAC-1); and calcium-binding protein (parvalbumin, Ca^{2+}/Calmodulin).

Since conventional RT-PCR detects the amount of final amplified product, from the results of those genes, no obvious changes (Fig. 8) were observed.

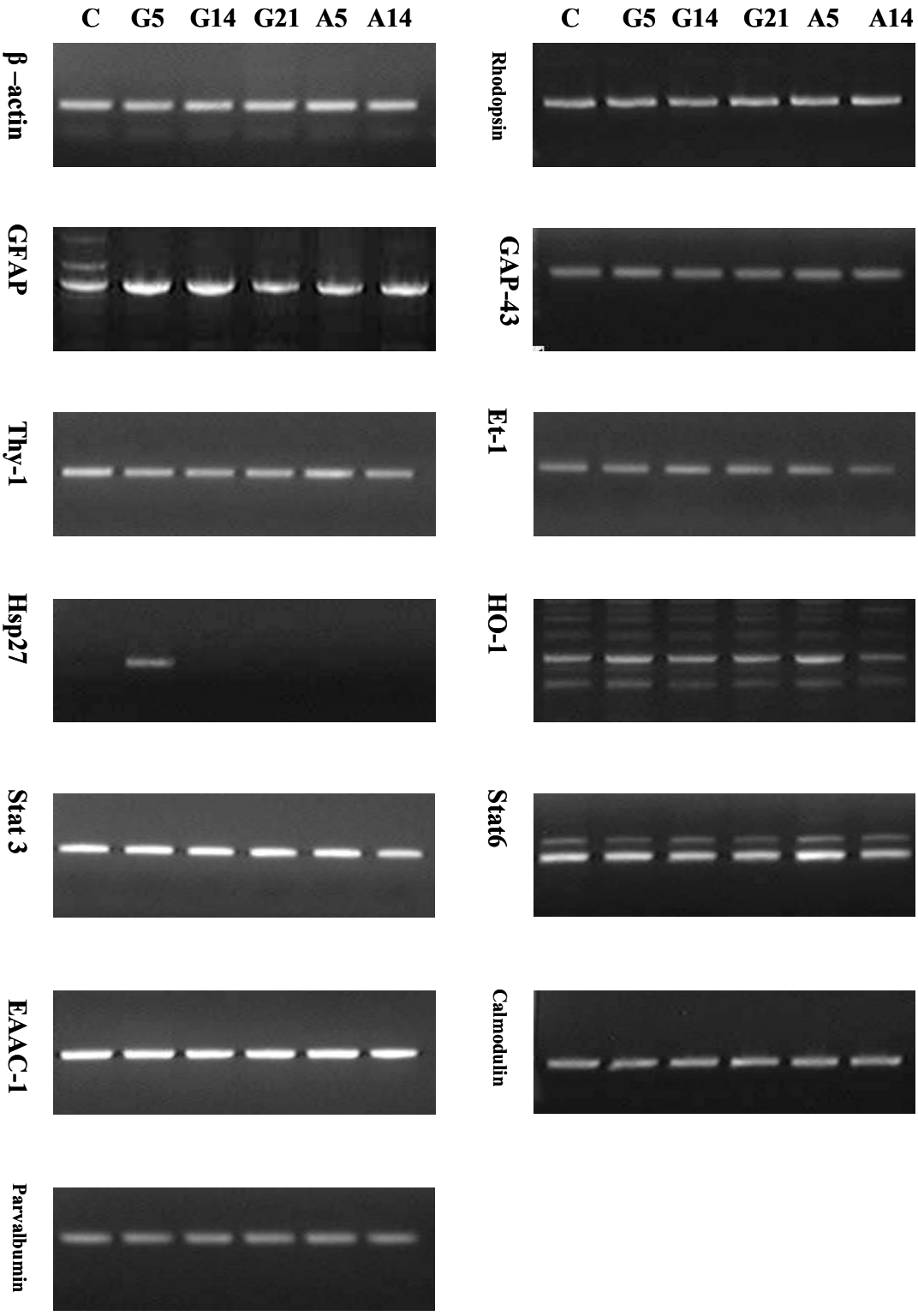

Figure 8. Estimation of mRNA level for several genes in total retina after grafted and axotomized using semi-quantitative RT-PCR. Expression of β-actin was used as an internal control of RNA amount in each sample. C as normal control; A5, A14 as axotomized 5 and 14 days; G5, G14 and G21 as grafted 5, 14, and 21 days.

3.3. Real time PCR

3.3.1. Statistical Comparisons

All in vivo experiments were repeated three times. And all values given in the text and figures are expressed as means ±SEM, with an indicating the number of observations. Statistical analysis was done by ANOVA followed by post-hoc testing using the LSD test (Statistica, Statsoft Inc., Tulsa OK). Significance was considered at $p<0.05$.

3.3.2. Results of the experiment

3.3.2.1. GFAP

After 5 days, retinal expression of GFAP was significantly increased as compared to controls in both axotomized and grafted animals ($p<0.01$ vs. controls). However, GFAP expression did not differ significantly between grafted and axotomized animals at any time-point (Fig. 9).

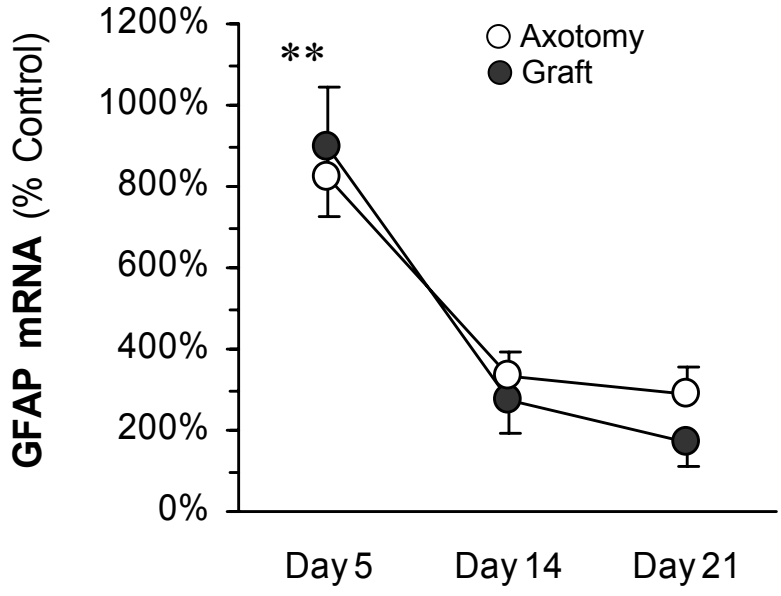

Figure 9. Retinal expression of GFAP in axotomized and grafted animals over time. Data are expressed as percentage of control. Mean±sem; n=3. ** $p<0.01$

3.3.2.2. GAP-43

After 5 days, retinal expression of GAP-43 was significantly increased as compared to controls in grafted animals (146±11%, $p<0.05$), whereas no such increase was observed in the axotomized group (108±31%, $p<0.05$ vs. grafted animals). At later time-points, GAP-43 expression was significantly decreased as compared to controls in both treatment groups ($p<0.05$). This decrease was less pronounced in grafted animals, but in contrast to day 5, differences did not reach statistical significance (day 14: 64±12% vs. 39±4%, $p=0.14$; day 21: 46±6% vs. 42±1%, $p=0.84$).

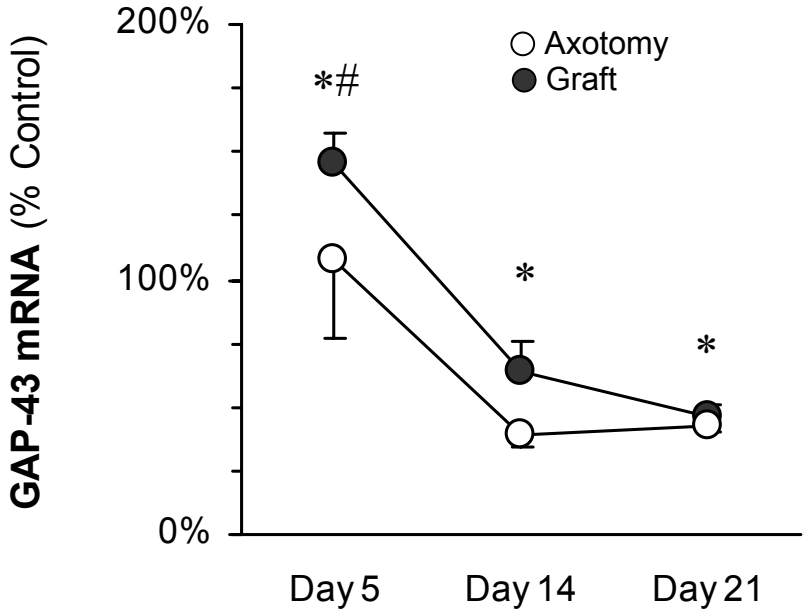

Figure 10. Retinal expression of GAP-43 in axotomized and grafted animals over time. Data are expressed as percentage of control. Mean±sem; n=3. * $p<0.05$

3.3.2.3. Thy-1

Retinal Thy-1 expression was found to be significantly decreased as compared to controls at all time-points, ($p<0.01$). Thy-1 expression did not differ significantly between grafted and axotomized animals (Fig.11).

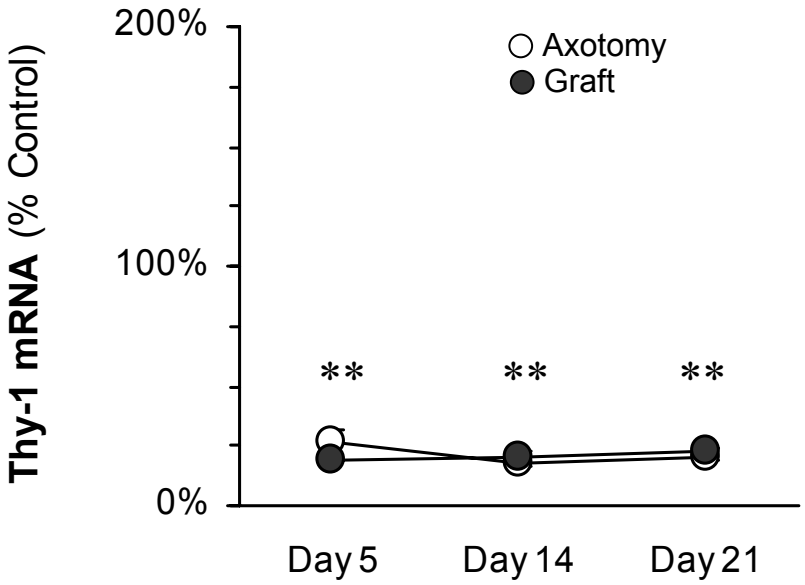

Figure 11. Retinal expression of Thy-1 in axotomized and grafted animals over time. Data are expressed as percentage of control. Mean±sem; n=3. ** $p<0.01$

3.3.2.4. ET-1

Increases in retinal ET-1 expression over time appeared to be less pronounced in grafted animals as compared to axotomized animals (Day 14: 108±24% vs. 148±21%). However, differences in retinal ET-1 expression over time or between groups failed to reach statistical significance (ANOVA: $p=0.20$) (Fig. 12).

3.3.2.5. ET_B

No significant changes of retinal ET_B receptor expression were observed over time or between groups (ANOVA: $p=0.81$) (Fig. 13).

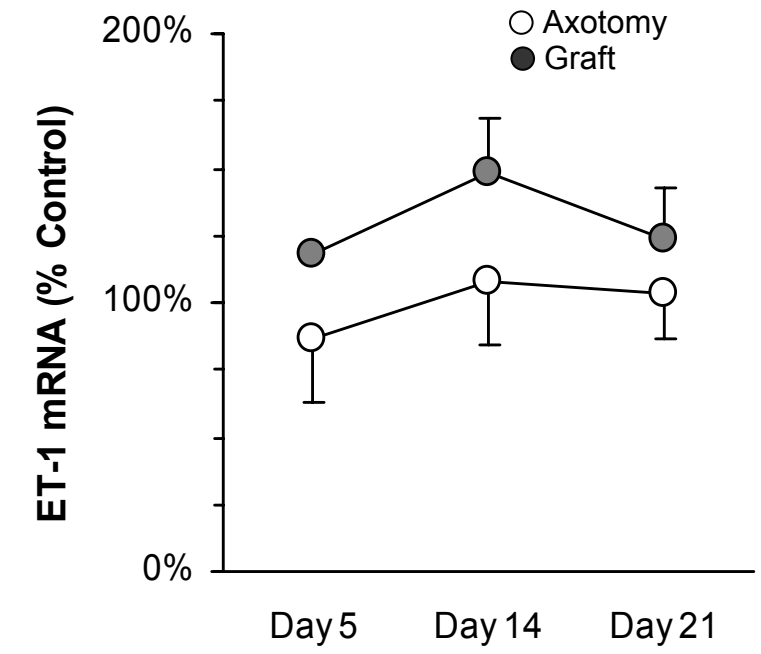

Figure 12. Retinal expression of ET-1 in axotomized and grafted animals over time. Data are expressed as percentage of control. Mean±sem; n=3.

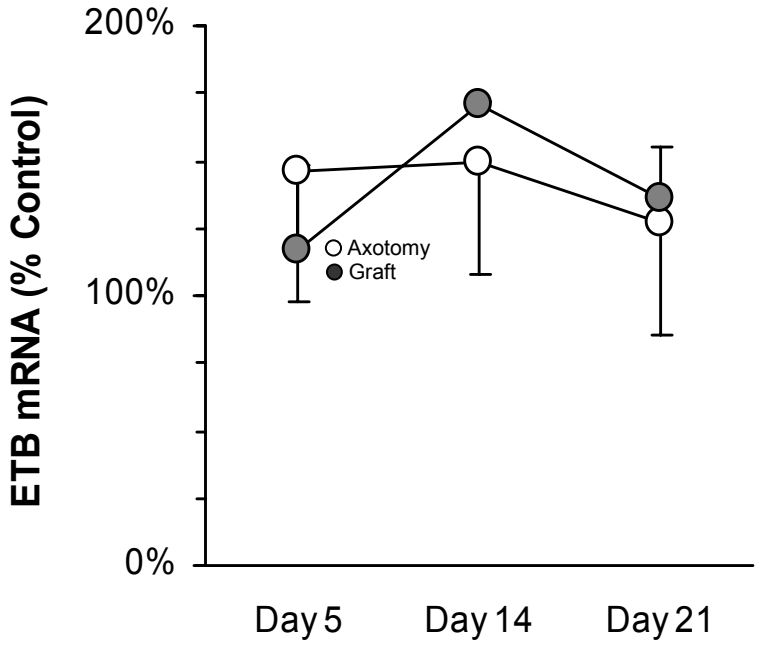

Figure 13. Retinal expression of ET_B in axotomized and grafted animals over time. Data are expressed as percentage of control. Mean±sem; n=3.

4. Discussion

4.1. Upregulation of GFAP Expression

4.1.1. Reactive Hypertrophy of Astrocytes

GFAP is a sensitive marker to monitor astrocytic responses to various injuries, including axotomy. As we can see from immunohistochemistry, axotomized retinas showed a clear upregulation of GFAP at 5 and 14 days post surgery. GFAP-immunoreactive astrocytes and their processes were mainly observed in the NFL and GCL. On the other hand, compared to the injured retinas, GFAP-immunoreactive astrocytes in grafted and regenerating retinas showed upregulated expression at 5, 14 days post surgery, and returned to control level 21 days after grafting. In addition, at mRNA level from quantitative real time PCR showed after 5 days, that retinal expression of GFAP was significantly increased as compared to controls in both axotomized and grafted animals ($p<0.01$ vs. controls). However, GFAP expression did not differ significantly between grafted and axotomized animals at any time-point.

In the normal retina, astrocytes are reported to perform many other functions such as wrapping around ganglion cell axons, contacting other glial cells (e.g. Müller cells) to form adherent junctions and also playing a role in potassium buffering and in neuronal signalling [104].One conspicuous phenotypic change occurring in astrocytes during maturation and activation is the major increase seen in the synthesis of intermediate filament proteins, especially GFAP [47, 46, 28]. This major alteration in maturing astrocytes, directly associated with morphological changes, raises the possibility of their role in the functional shift from neurite-promoting to neurite-inhibiting elements, something that is still unknown. The two paradigms reported here are consistent with the fact that astrocytes are actively involved in the posttraumatic retinal repair.

Recent findings indicate that astrocytes play a more expansive role in brain physiology than previously considered. In culture, astrocytes enhance synaptic efficacy, increase the number of functional neuronal synapses, and are necessary for the maintenance of neuronal synaptic stability [213]. Astrocytes are critical for neuronal survival [30] and contribute significantly to the maintenance of ion concentrations and clearance of glutamate from the synaptic cleft [166, 218]. Astrocytes express diverse neurotransmitter receptors and their activation increase intracellular free calcium [217].

The expanding base of knowledge regarding astrocyte function in normal brain physiology suggests that astrocytes may play an important role in retina pathophsiology associated with optic nerve injury.

Astrocytes play a significant role in normal neuron function, including active neuronal-glia signaling and maintenance of homeostasis in the extracellular microenvironment. Reactive astrocytosis represents hypertrophy and hyperplasia of astrocytes in response to CNS injuries. Early impairment of astrocyte function or early loss after optic nerve transection may compromise critical neuronal-glia interactions and thus may play a significant role in outcome after injury.

4.1.2. Active Müller Cells of Axotomized Retina

While astrocytes are located in the inner surface at the GCL, Müller cells have long processes spanning from the inner retina to the outer limiting membrane and support retinal structures lying in-between. Our results showed that immunostainined Müller cells and their processes in the INL, OPL and ONL. Some cells and processes could be observed to span throughout all retinal layers, including the outer segment of the ONL. After the optic nerve was transected, both types of glial cells become reactive and upregulated GFAP. Compared to the injured retinas, GFAP-immunoreactive Müller cells were not seen in grafted and regenerating retinas throughout all time point. This is an intriguing finding pointing to a sophisticated response of Müller cells, which seem to discern between degenerating and regenerating RGCs.

GFAP and vimentin form intermediate filaments, a part of the cytoskeleton, in astrocytes and Müller cells; the production of intermediate filaments increases in reactive astrocytes and Müller cells under pathological conditions and after transplantation in the brain and the retina [7]. Increased production of intermediate filaments is reportedly associated with the formation of glial scarring that obstructs axonal growth [151, 125]).

The Müller cells, which do not express significant amounts of GFAP in normal retina, have been shown to induce or upregulate their GFAP expression following a wide variety of pathological states in the retina including age-related retinal degeneration [36], retinal ischemia [106], inherited retinal dystrophy [177], retinal hyperoxia [152], induced retinal degeneration [45], glaucoma [209] and diabetes [113]. Thus, GFAP-positive staining has been demonstrated to be reliable indicator of most if not all acute and chronic neuroretinal pathology.

The results of the present experiments supported the proposal that Müller cells may play a role in the cell degeneration after axotomy. The increased glial cells may be activated to clear the neuron degeneration products [150]. Those were thought to recognize a variety of neuronal signals and actively control levels of K^+, H^+ and neurotransmitters such as glutamate in the extracellular space of the retina [137]. The immunoreactivities of Müller cells indicate that glial cells are activated by the changes in retinal environment linked to the neuronal degeneration in the axotomized retina.

4.1.3. The Role of Glial Scar in Degenerating and Regenerating Retina

It has been suggested that glial scarring after neural injury obstructs axonal regrowth by forming physical and diffusion barriers that separate the intact regions of the retina and CNS from the damaged area [51].Glial scarring by reactive astrocytes may also occur and is thought to be an attempt by the CNS to restore homeostasis after injury by isolating the damaged region [53]. Glial scarring may have other beneficial effects since astrocytes contribute to the removal of excitotoxins such as glutamate, restore and maintain the ionic environment, and provide neurotrophic support [134]. However, glial scarring has often been regarded as detrimental since it may interfere with subsequent neural repair or axonal regeneration [172], impede regeneration [167], and contribute to the development of seizures. The failure of axonal regeneration in the mammalian CNS is current attributed to the glial environment of the lesion site, which elaborates a multitude of inhibitory factors. The glial scars within the retina do not account for direct inhibition of axonal regowth because the optic nerve axons are cut behind the eye cup, and therefore, axonal stumps do not hit on potentially inhibiting astrocytic scars.

Recent studies suggest that astrocytes may be severely damaged and lost following CNS injury prior to the development of reactive gliosis [116].The role of astrocytes in uptake of glutamate may have particular importance to the traumatically injured brain where large fluxes of extracellular glutamate [50, 96] likely contribute to acute excitotoxic processes [145, 77]. Specifically, early impairment of astrocyte function after optic nerve injury may compromise maintenance of homeostasis in the extracellular microenvironment and disrupt critical neuronal-glia interactions. Damage to or loss of astrocytes has important implications for neuronal survival and brain function.

Astrocytes are the most numerous types of glia cells and provide many important functions to support neurons, including exchange of metabolic and nutritional material, maintenance

of ion concentrations, and clearance neurotransmitters in the vicinity of neurons [218]. Astrocytes have historically been viewed as nonexcitable structural support cells of the brain that maintain a healthy environment and provide trophic factors for neurons. The view of the role of astrocytes has expanded considerably due to recent observations of their participation in neuronal signalling [219, 220].

The majority of studies examining astrocytes after neuronal injury have focused on astrocytic activation characterized by enhanced expression of GFAP and glial scarring. Indeed, the CNS responds within days to injury by producing reactive astrogliosis or glial scarring [125]. Although not completely understood, glial scarring is thought to be a response by the CNS to restore homeostasis by isolating the damaged region [53]. However, the glial scar may interfere with any subsequent neural repair or axonal regeneration [172].

4.1.4. Grafting may Inhibit Astroglial Hypertrophy and Induce Functional Changes, Beneficial for Retina Regeneration

Our data revealed a significant increase in GFAP immunoreactive after axotomy, and a limited immunoreactivity in the transplant group. In addition, in the transplant group, the reaction was confined to an astrocytic reaction induced by lesion, and was not shown in Müller cells. This shows that although both types of cells may regulate their GFAP content under certain circumstances, they respond differentially to a certain lesion.

Recently several study have been undertaken to determine whether an imposed decrease in GFAP synthesis would affect neurite outgrowth. In a neuron-astrocyte coculture under experimental conditions in which astrogliosis has been elicited by a mechanical lesion [232], using antisense GFAP mRNA have been observed that phenotypic changes, consecutive to the decrease in GFAP synthesis, induced a functional shift to neurite-promoting elements [231]. Inhibition of GFAP synthesis leading to a reduction of astroglial hypertrophy relieves the blockade of neuritic outgrowth that normally is observed after a lesion [109].

These results show that inhibition in GFAP synthesis, leading to a reduction of astroglial hypertrophy, relieves the blockade of neuritic outgrowth that normally is observed after a lesion. The mechanisms may involve changes in the secretion of extracellular matrix molecules by astrocytes.

Our findings suggest that astrocytes are particularly vulnerable to nerve injury and that

damage to their integrity may compromise critical glia-neuron interactions. Such a disturbance would disrupt the maintenance of homeostasis in the extracellular environment and likely contribute to subsequent neuronal damage.

Briefly, the mechanisms, which involved in the retina regeneration after optic nerve grafting remain poorly understood. The reactive gliosis, which occurs after an injury to the CNS and leads to the glial scar, has been considered as one of the major impediments to neurite outgrowth and axonal regeneration. A glial scar consists mainly of reactive, hypertrophic astrocytes. These reactive cells acquire new properties, leading to a non-permissive support for neurons. Astrogial reactivity is mainly characterized by a high overexpression of the major component of the gliofilaments, GFAP. This GFAP overexpression is related to the astroglial morphological response to injury. Based our data from immunohistochemistry, less GFAP expression on grafted retina indicate that regenerating retina may modulate of GFAP synthesis, reversing the hypertrophic phenotype, might also reverse the blockage of neuritic outgrowth observed after a lesion. Although our quantitative real-time PCR did not show significantly mRNA level change, the mean of GFAP message level were lower in grafting group compare to those axotomized groups as could see. Especially at later time points of 14 and 21 days showed obviously different. Those changes fit protein level change nicely. The statistical analysis failed to differ both group may due to the data variable and the small amount of animals of this experiment. Or it may due to message RNA changed earlier then protein, our time course fail to catch it. For further experiment, Western blot may be used for measuring the protein level changes.

4.2. GAP-43 Upregulation in Regenerated Retina

In mature mammals, RGCs are unable to regrow injured axons and soon undergo apoptotic death. These well known events are a paradigm of regenerative failure in the CNS and may mimic pathophysiological sequelae that underlie degenerative diseases. The failure to regenerate spontaneously has been attributed to downregulation of growth associated proteins, and in particular of GAP-43. However, as shown before, RGCs can regenerate their axons *in vivo* through a peripheral nerve graft, and an average about 15-17% RGCs survive and regrow their axons [211].

The phosphoprotein GAP-43 has been shown to play a critical role during development of

the CNS. In the retina of rats, GAP-43 is expressed in RGCs during development and postnatal. Then the protein is downregulated to disappear in adulthood. Because RGCs only express GAP-43 during axon outgrowth, probes for this protein enable to visualize RGCs in a growth state [127, 133, 37, 182, 16]. Developmentally, RGCs express this protein during axon outgrowth and synaptic refinement, then downregulate it as their connections mature [196]. Normally, GAP-43 is transiently upregulated after axotomy and declines as RGCs undergo atrophic changes [37]. Levels remain high only if regeneration is sustained [197, 12, 37, 16].

As expected, intact normal retina showed no staining at all in our experiment. Axotomized sections revealed RGCs unstained. On the other hand, grafting of SN piece resulted in clear upregulation of GAP-43 in GCL at 5 days. A dramatic increase in the immunostaining of RGCs was seen with in the overlying fiber layer at 14 days. GAP-43 could no longer be detected at 21 days. These were indicating that regenerating RGCs expressed GAP-43.

Increase GAP-43 expression in grafted retina also be conformed in mRNA level by using quantitative real time PCR. As the data show, a transient upregulation of GAP-43 was seen in grafted animals, whereas no such increase was observed in the axotomized group. At later time-points (14 and 21 days), GAP-43 expression was significantly decreased as compared to controls in both treatment groups. This result is a line of evidence that GAP-43 upregulation is causally connected with regenerative growth, although the mechanism of GAP-43 involvement remains elusive.

4.2.1. Role in Neural Development and Structural Plasticity

Convergent evidence from many labs indicates that GAP-43 plays a key role in guiding the growth of axons and modulating the formation of new connection. One clear indication of this comes from a recent study in transgenic mice, overexpression of GAP-43 leads to the spontaneous formation of new synapses and enhanced sprouting after injury. Thus, even in the absence of additional trophic factors, GAP-43 enables neurons to sprout new terminals, and can therefore be considered an intrinsic determinant of the neuron's growth state [4]. Primary sensory neurons from embryonic chick, when grown on poly-L-lysine, failed to extend axons when treated with antisense oligonucleotides complementary to portions of GAP-43 mRNA [2].

GAP-43 in axon elongation in embryonic cells and its decline during maturation has been correlated with the loss of regenerative ability within the CNS. According to data from

various models, GAP-43 may work via modulation of signaling cascades rather than autonomously causing growth [52]. This result has generally been interpreted as showing that, although these neurons are intrinsically capable of regenerating their axons, this can only occur outside the inhibitory influences that normally prevail in the CNS.

4.2.2. Correlation of regenerative axonal growth with GAP-43

GAP-43 was expressed within the RGCs along regenerating retina. Former studies showed GAP-43 as a crucial role in regenerative growth. Identification of GAP-43 in growth cone membranes [198, 235]) and its involvement in optic tract formation [13, 234, 205, 206] revealed that this protein is associated with neurite growth in general, but also with synaptic plasticity.

It has been postulated that the neuronal growth associated protein GAP-43 plays an essential role in axon elongation. Although termination of developmental axon growth is generally accompanied by a decline in expression of GAP-43, a subpopulation of dorsal root ganglion (DRG) neurons retains constitutive expression of GAP-43 throughout adulthood. Peripheral nerve regeneration occurring subsequent to injury of the peripheral axon branches of adult DRG neurons is accompanied by renewed elevation of GAP-43 expression. Lesions of DRG central axon branches in the dorsal roots are also followed by some regenerative growth, but little or no increase in GAP-43 expression above the constitutive level is observed.

How does axonal growth correlate with GAP-43 upregulation? From previous it was shown that first axons enter a SN graft 5 days after surgery [221].This is in agreement with our finding that GAP-43 content increases at 5 days. Maximum numbers of axons were observed in regenerating retinas at around 14 days after grafting by addition of newly formed growth cones between day 5 and day 14 [212]. This is in line with the present finding that GAP-43 shows its peak expression at 14 days after surgery. The down regulation of GAP-43 observed at 21 days indicates that regenerative growth is declining at this stage. Indeed, in blind-ending SN grafts as used in this study, no additional axons are growing within the graft at this stage and some decline in the numbers of axons may be observed [212] depending on the length of the SN graft. This strong correlation between the different stages of regeneration and the GAP-43 expression is proving that the molecule plays a crucial role as assumed by the former studies [189].

4.3. NF-κB may play a Neuroprotective Role in the Early Response to Regenerated RGCs

NF-κB is a transcription factor, which is activated by various stimuli. Activated NF-κB plays a protective role from the cell death in the injured ganglion cells [27]. NF-κB plays a role in cell death as a pro- or anti-apoptotic transcription factor. It has been hypothesized that NF-κB is activated to transcribe the essential genes for cell death or survival [131]. Within the nervous system NF-κB plays a pivotal role and may exert functions associated with axonal growth.

Since the translocation of NF-κB from the cytoplasm to the nucleus is essential for NF-κB activation, we examined its activation with immunohistochemistary. Indeed, while in normal retinas NF-κB is located within the cytoplasm, it translocated into the nuclei of RGCs in regenerating retinas.

The immunohistochemical data clearly showed that a greater amount of NF-κB was observed in the nucleus than in the cytoplasm of the grafted retina. NF-κB expression was upregulated 5 days after both axotomized and grafted retina. A dramatically increase could be seen at 14 days after grafting with the immunostaining to disappear at 14 days after axotomy, perhaps due to the death of the RGCs. In the regenerating retinas, NF-κB migrated into the nucleus to aggregate within nucleolus-like structures. These results confirm that the positively stained NF-κB in immunohistochemistry is due to the activation and translocation from the cytoplasm to the nucleus. This specific pattern of molecular movement suggested that NF-κB may play a neuroprotective role in the early response to regenerated RGCs. The mechanism of how NF-kB functions in regenerating retina remains to be elucidated.

NF-κB influences the expression of a complex array of genes in the nervous system and, in general, the genes serve important functions in cellular responses to injury and in neuronal plasticity. Genes encoding several different injury-responsive cytokines are induced by NF-κB in glia and neurons. In the developing rat nervous system, levels of NF-κB activity change, with levels peaking in the cerebellum during the early postnatal period at a time when synaptogenesis is occurring [186]. A peptide inhibitor of NF-κB (SN50) blocks the ability of nerve growth factor (NGF) to prevent death of cultured sympathetic neurons [120], suggesting a role for NF-κB in the control of neuronal death during development of the nervous system. The presence of NF-κB in synaptic terminals located at considerable distances from the neuronal cell body, and its ability to be activated locally at those sites,

strongly suggest that this transcription factor modulates synaptic function [146].

Finally, NF-κB may play an important role in the apoptotic death of neurons that occurs during normal development of the nervous system. Such natural neuronal death is thought to be regulated by electrical activity in neurons and by competition of neurons for a limited supply of neurotrophic factors produced by target cells upon which synapses form.

Injury to the brain or spinal cord induces a cascade of signaling events that stimulate NF-κB activation in injured neurons and in injury responsive glial cells. Cellular and molecular analyses of brain and spinal cord tissues in experimental rodent models of stroke, epileptic seizures, and traumatic injury have begun to reveal the complex functions of NF-κB in modifying neuronal degeneration and recovery.

It has become clear that NF-κB influences the neurodegenerative process by directly affecting gene expression in neurons themselves and by indirectly regulating gene expression in glial cells. Data from studies of mice lacking the p50 subunit of NF-κB suggest that, overall, NF-κB activation enhances ischemic neuronal death [187], but its effects differ between cell types such that, whereas activation of NF-κB in microglia promotes ischemic neuronal degeneration, activation of NF-κB in neurons may increase their survival after a stroke. Cell culture studies have clearly shown that activation of NF-κB in neurons protects them against excitotoxic and metabolic insults relevant to the pathogenesis of stroke, including glucose deprivation and exposure to glutamate [25, 233].

Levels of NF-κB activity are increased in cerebral cortex within hours of traumatic brain injury in rats, after which they remain elevated for at least 24 hours. Within neurons in the injured cortex, levels of p65 immunoreactivity increase first in the axons and subsequently in neuronal cell bodies [142]. Neighboring microglia and astrocytes also show increased levels of p65. This increase in immunoreactivity persists for many months, particularly in the margins of the progressively enlarging ventricle, suggesting a role for NF-κB in a prolonged inflammatory process.

The emerging data described above suggest that NF-κB plays important roles in cellular responses to injury of the nervous system in both acute and chronic neurodegenerative conditions. Proteins involved in NF-κB signaling are therefore potentially important targets for therapeutic intervention in an array of neurological disorders. In general it appears that activation of NF-κB in neurons protects them against degeneration, whereas activation of NF-κB in microglia promotes neuronal degeneration.

The latter studies have shown that the neuroprotective effect of dietary restriction involves

induction of a mild stress response that results in increased production of heat-shock proteins.

4.4. HSP27

Heat shock proteins, first identified in Drosophila and later in almost all organisms, are indicators of cellular stress. HSP27 has been characterized in injured nerve cells including neurons in the retina. These data were confirmed in the present study by showing HSP27 in the ganglion cells layer of normal retinas. The protein seems redistributed 5 days after the optic nerve transection. By 14 days after transected nerve when the retina is almost deleted of RGCs, no HSP27 is detected in the ganglion cell layer. In grafted retinas, HSP27 is continually expressed up to 21 days postsurgery indicating that regenerating ganglion cell layer continues expressing the molecule over long periods of time. However, the immunohistochemical data show a less consistent staining, which needs to be further approved. In addition, it is not clear from the images whether RGCs or astrocytes within the ganglion cell layer account for the positive staining.

HSP27 were found to be overexpressed in some areas of the brain under simulated pathological conditions [97, 98, 157, 158, 87] as well as in the pathological conditions of serious brain diseases, such as Alexander's disease, Alzheimer's disease, and Parkinson's disease [89, 194, 169]. Of particular interest is the fact that HSPs are easily induced in astrocytes, which are relatively injury resistant (Soltesz, 1996) and therefore have often been used for in vitro experiments designed to investigate the mechanisms of their induction [135, 78, 121, 183]. A recent study [10] showed that this stress-induced protein was simultaneously induced in Bergmann glial cells, a specialized astrocyte, in rat brain and were then transported into the radial fibres, which project into the synaptic-enriched molecular layer of the cerebellum. Thus, the induction of HSP27 in the astrocytes of CNS appears to be common under certain stress-related conditions, and the induction mechanism remained to be determined.

Crush-injured rat sciatic nerves induction of HSP27 was examined using the combined technique of immunofluorescent labelling and confocal laser scanning microscopy (CLSM), followed by electron microscopy (EM) and immunoelectron microscopy (IEM). In this study demonstrated that the induction of HSP27 is specific to the Schwann cells (SCs) columns, which are known to guide axonal sprouts during the regeneration of nerves, and their associated axons. SCs were already known to show dramatic functional

and morphological alterations during the process of Wallerian degeneration and the ensuing regeneration after axotomy [73, 81].Thus, a strong induction of HSP27 can be detected in a certain phase of SCs that have been transformed by axotomy. Moreover, the induction of HSP27 was accompanied by the re-expression of the intermediate filament protein, one of the cytoskeletal components of SCs, so the HSP27 play a role in modulating the cytoskeletal dynamics of SCs during the regeneration of the peripheral nerves [82].

Experimental evidence suggests that the brain responds to injury in a regionally heterogeneous manner and that the stress response is differentially regulated among cell types in the CNS [181, 192]. Increased expression of heat shock proteins (HSPs) has been observed in CNS following ischemia, trauma, hyperthermia and exposure to neurotoxins [20, 144, 214]. The presence of HSPs in astrocytes in vitro and in vivo supports the idea reported by Sharp et al. [192] that glial cells have a functional role in brain injury.

4.5. Thy-1 and ET-1

In the CNS, Thy-1 is expressed predominantly on neurons and serves as a specific marker for neurons. In the eye, Thy-1 is found predominantly in the GCL. From our data of quantitative real time PCR, retinal Thy-1 mRNA expression was significantly decreased as compared to controls at all time-points ($p<0.01$). And Thy-1 expression did not differ significantly between grafted and axotomized animals. This may be explained by the low abundance of RGCs within the retina as they make up less than 1% of all cells. Quantitative changes in ganglion cell specific genes are therefore limited, because one starts with non-specific RNA prepared from the entire retina. In addition, even in the regenerating retina, only 15 to 20% of ganglion cells survive and regenerate, therefore further limiting the chance to detect differences between regenerating and degenerating retinas. Thy-1 may be an extremely sensitive marker for isolated RGCs, because,in spite of neuroprotection and accessibility of the attached PN graft, most of the ganglion cells (between 80-85%) die progressively after axotomy therefore further diluting the percentage of RGCs within retinal tissue [211].

Since ETB were expressed by astrocytes and regulate astrocytes hypertrophy in crush injured CNS [175], we detected both ET-1 and ET-B mRNA level. The results appeared less specific to detect differences between de- and regenerating retinas. ET-1 and ETB both molecules are expressed in capillary endothelial cells, which show less exit in both

degenerating and regenerating retina. So we consider both molecules as controls in addition to glial and neuronal markers. They may change their patterns of expression in long-term experiments utilising retinas, which survive months after surgery, in order to examine advanced gliosis connected with neovascularization.

5. Summary

Molecular Characterization of the Regenerating Retinal Ganglion Cells using Quantitative PCR and Immunohistochemistry

The aim of this study was to detect whether neuronal, glial and endothelial markers change their expression patterns after transection of the optic nerve in adult rats and after the transect axons gain the opportunity to regrow within an autologous graft of sciatic nerve. The ability of the adult mammalian central nervous system and the retina to regenerate following injury is influenced by a balanced activity between factors that promote, and of those that inhibit neuronal survival, regeneration, and sprouting. It was found by immunohistochemistry that the properties of the injured ganglion cells affect the extent of neuronal survival and regeneration by showing specific expression of neuron-specific markers like GAP-43 and Thy-1. On the other hand, glial cells at the site of injury are also critically involved in determining the regenerative outcome as shown by detecting specific regulation of glial markers like GFAP. To gain further insights into the molecular mechanisms responsible for regeneration of retina, the semi-quantitative RT-PCR and real time PCR was performed for differential gene expression in the retina. A comparison of mRNAs in those retinas receiving sciatic nerve grafts with those in axotomized retinas and untreated retinas was conducted. At various times elapsed after injury, glial cell markers, neuronal markers and endothelin were analysed. The data confirmed the immunohistochemical studies and revealed specific regulation of GAP-43 and GFAP, but no specific patterns for Thy-1, ET-1 and ETB, as expected from their cellular expression patterns. In spite of the high sensitivity of the model used in the present study and in the reliability of the methods, the molecular mechanisms underlying neuronal degeneration, repair and regeneration after axon injury have yet to be identified.

It is therefore of crucial importance to characterize the different populations of cells which may codetermine whether regeneration will take placing or not.

Tierversuchgenehmigung: Bezirksregierung Münster, 20.4.2001, Aktenz: 50.0835.1.0

6. Zusammenfassung

Molekulare Charakterisierung der regenerierenden retinalen Ganglienzellen mittels quantitativer PCR und Immunhistochemie

Das Ziel dieser Untersuchung war es, zu erfassen, ob neuronale, gliale und endotheliale Marker nach einer Durchtrennung des Sehnervs der adulten Ratte und nachdem die durchtrennten Axone die Möglichkeit erhalten, innerhalb eines autologen Transplantates aus dem Ischiasnerven zu wachsen, ihre Expressionsmuster verändern. Die Fähigkeit des adulten Säuger-ZNS und der Retina, nach einer Verletzung zu regenerieren, wird durch eine ausgewogene Aktivität zwischen wachstumsfördernden und hemmenden Faktoren beeinflusst. Mittels Immunhistochemie wurde gefunden, dass die Eigenschaften der verletzten Ganglienzellen das Ausmaß des neuronalen Überlebens und die Regeneration beeinflussen: Es konnte gezeigt werden, dass neuron-spezifische Marker wie GAP-43 und Thy-1 reguliert exprimiert wurden. Auf der anderen Seite, waren auch gliale Zellen an der Verletzungsseite am Ausgang der Regeneration involviert, was durch die Erfassung einer spezifischen Regulation glialer Marker wie GFAP gezeigt werden konnte. Um weitere Einblicke in die molekularen Mechanismen der retinalen Regeneration zu erhalten, wurde eine semi-quantitative RT-PCR und eine Echtzeit-PCR für die differentielle Genexpression durchgeführt. Es wurde ein Vergleich von mRNAs aus Retinae mit Nerventransplantat, solcher aus axotomierten Retinae und aus unbehandelten Kontrollen durchgeführt. Zu verschiedenen Zeiten nach der Verletzung wurden gliale, neuronale und endotheliale Marker analysiert. Die Daten bestätigten die immunhistochemischen Befunde und zeigten eine spezifische Regulation von GAP-43 und GFAP, nicht aber von Thy-1, ET-1 und ETB, was aus ihrer zellulären Expression erwartet wurde. Trotz der hohen Sensitivität des benutzten Modells und der Reliabilität der Methoden sind die molekularen Mechanismen der neuronalen Degeneration, der Reparatur und der Regeneration nach axonaler Verletzung noch zu analysieren. Es ist deshalb von großer Bedeutung, verschiedene Populationen von Zellen zu charakterisieren, die darüber mitbestimmen, ob Regeneration stattfindet oder nicht.

Tierversuchgenehmigung: Bezirksregierung Münster, 20.4.2001, Aktenz: 50.0835.1.0

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8. Abbreviations

ATP	adenosine triphosphate
CLSM	confocal laser scanning microscopy
CNS	cental nervous system
C_T	threshold
$\Delta\Delta C_T$	the comparative C_T method
DAPI	4', 6-diamidino-2-phenylindol
DRG	dorsal root ganglion
EM	electron microscopy
ET-1	endothelin-1
ETA	endothelin A receptor
ETB	endothelin B receptor
FCS	fetal calf serum
GAP-43	growth associated protein-43
GFAP	glial fibrillary acidic protein
GCL	ganglion cell layer
HSPs	heat shock proteins
Hsp27	heat shock protein 27
Hsp32	heat shock protein 32
IEM	immunoelectron microscopy
Ig	immunoglobulin
INL	inner nuclear layer
IPL	inner plexiform layer
IPLs	inner plexiform layers
MAP	mitogen-activated protein
MMLV-RT	moloney murine leukaemia virus reverse transcriptase
NF-κB	nuclear factor kappa B
NFL	nerve fibre layer
NGF	nerve growth factor
ON	optic nerve
ONL	outer nuclear layer
OPL	outer plexiform layer
PNS	peripheral nervous system

PRL	photoreceptor layer
RGC	retinal ganglion cells
SCs	Schwann cells
SN	sciatic nerve
Tm	melting temperature
TNF-α	tumor necrosis factor alpha

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