

NEUROPROTECTIVE PROPERTIES OF CILIARY NEUROTROPHIC FACTOR  
ON THE SURVIVAL OF AXOTOMIZED RETINAL GANGLION CELLS *IN VIVO*

By

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**TITLE:** Neuroprotective properties of ciliary neurotrophic factor on  
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## ABSTRACT

Over 90% of retinal ganglion cells (RGCs) die after intraorbital optic nerve transection close to the eye. Several factors contribute to RGC death including: loss of neurotrophic support, overproduction of free radicals, glutamate-mediated excitotoxicity, activation of pro-apoptotic caspases (3 and 9), and reactive glia. In the present study, the model of optic nerve transection was used to study the neuroprotective mechanisms and effects of ciliary neurotrophic factor on the survival of axotomized retinal ganglion cells. It was demonstrated that axotomized RGCs die by apoptosis since overexpression of Neuronal Inhibitory Apoptosis Protein (NAIP), a potent and selective inhibitor of caspase 3, protected axotomized RGCs that would have otherwise died if untreated. Protection of axotomized RGCs was further enhanced by overexpression of Ciliary Neurotrophic Factor (CNTF) using adenoviral and lentiviral vectors. It was demonstrated that intraocular administration of adenoviral vectors selectively transduced retinal Müller cells, and injection of this vector into the optic nerve stump at the time of optic nerve transection selectively transduced a small percentage of RGCs. Regardless of the route of administration, adenoviral-mediated overexpression of CNTF protected axotomized RGCs. In comparison to adenoviral-mediated delivery, the delivery of CNTF using lentiviral vectors protected greater numbers of axotomized RGCs and for an extended period of time not typically seen using the optic nerve transection model.

It was assumed that viral-mediated transfer of CNTF rescued axotomized RGCs by directly activating the high affinity CNTF receptor alpha (CNTFR $\alpha$ ) expressed on RGCs. However, CNTF can also protect axotomized RGCs indirectly, by activating the low affinity leukemia inhibitory receptor beta (LIFR $\beta$ ) expressed on retinal astrocytes and Müller cells. It was demonstrated that viral-mediated overexpression of CNTF resulted in phenotypic changes in retinal glial cells (astrocytes and Müller cells) that may have increased their neuroprotective function. Overexpression of CNTF increased retinal levels of the gap junction protein, connexin 43 (Cx43), the intermediate filament glial fibrillary acidic protein (GFAP), the astrocyte-specific glutamate/aspartate transporter-1, GLAST-1, and the astrocyte specific enzyme, glutamine synthetase (GS). Taken together, these results suggest that CNTF is capable of protecting axotomized RGCs by directly binding to injured neurons or by modulating surrounding glia. Together, these results suggest that pharmacological interventions that maintain or upregulate CNTF expression may confer a clinically beneficial neuroprotection.



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## LIST OF ABBREVIATIONS

AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic
cAMP	adenosine 3',5',-cyclic monophosphate
ATP	Adenosine triphosphate
$\beta$ -gal	$\beta$ -Galactosidase
CAR	Coxsackie and adenoviral receptor
CNTF	Ciliary neurotrophic factor
CNTR $\alpha$	CNTF receptor alpha
CNS	central nervous system
CRE	cAMP response element
D-FITC	Dextran-FITC
EEA	excitatory amino acids
EEAC1	excitatory amino acid transporter
FG	FluoroGold
FITC	fluorescein isothiocyanate
GABA	$\gamma$ -gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GAS	gamma activated sequence
GCL	ganglion cell layer
GFAP	glial fibrillary acid protein
GS	glutamine synthetase
GLAST	glutamate astrocyte specific transporter
GLT-1	glutamate transporter
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
INL	inner nuclear layer
IPL	inner plexiform layer
ISRE	interferon-stimulated response element
JABs	JAK binding proteins

JAK(s)	Janus tyrosine kinase(s)
LIF	Leukemia inhibitory factor
LIFR $\beta$	LIF receptor beta
MAPK	mitogen activated protein kinase
NFL	nerve fiber layer
NMDA	<i>N</i> -methyl-D-aspartate
NO	nitric oxide
NOS	NO synthase
INOS	inducible NOS
nNOS	neuronal NOS
O <sub>2</sub> <sup>-</sup>	superoxide radical
ON	optic nerve
ONOO	peroxynitrate
ONL	outer nuclear layer
PCD	programmed cell death
PI3K	phosphatidylinositol-3 kinase PI-3K
PIAS	protein inhibitors of activated STATs
PLC	phospholipase C
PNS	peripheral nervous system
RGC(s)	retinal ganglion cell(s)
SC	superior colliculus
SH2	src homology 2
SHP-2	SH2 containing phosphatases
SOCS	suppressor of cytokine signaling
SSIs	STAT induced STAT inhibitors
STAT	signal transducers and activators of transcription

## GLOSSARY

Apoptosis	active cell death or cellular suicide
Caspase	proteins that are involved in the execution of apoptosis
Excitotoxicity	cell death by overactivation of excitatory amino acid receptors (NMDA and non-NMDA) by glutamate and its analogs
Gap junction	a direct cytoplasmic connection of adjacent cells as a result of pairing of two connexons
Gene therapy	involves the insertion of a functional gene or another molecule that contains an information sequence into a cell to achieve a therapeutic effect
GFAP	an intermediate filament protein expressed in astrocytes of the CNS
Glaucoma	rise in the pressure of the fluids within the eye sufficient enough to cause retinal damage and affect vision
IAPs	members of a protein family with the ability to inhibit cellular apoptosis
IL-6-type cytokines	a family of cytokine proteins that use the receptor component, gp130 for signaling
<i>In vitro</i>	occurring outside of the body
<i>In vivo</i>	occurring in within the body
Lentiviral vectors	Lentiviruses are a type of retrovirus that can infect both dividing and non-dividing cells because their preintegration complex (virus "shell") can get through the intact membrane of the nucleus of the target cell.

Neurodegeneration	a passive or active process whereby nervous tissue progressively degenerates resulting in loss of cognitive, sensory, and/or motor function of the nervous system.
Neuroprotection	the ability to promote neuronal survival.
Neurotrophic factor	a protein that promotes the survival of CNS and PNS nerve cells.
Retrograde tracer	molecules with varied chemical structures with the ability to be taken up by nerve terminals and retrogradely transported to the soma.
Reverse transcription	the process of converting RNA to DNA.
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis.
Tract tracing	the process whereby one uses either a retrograde or anterograde tracer for the purpose of determining efferent and afferent neuronal projections.
Western Blot	the SDS-page of proteins that are then transferred to membrane and probed with antibodies to detect specific proteins.

**CHAPTER 1**  
**GENERAL INTRODUCTION**

## **Axotomy as an animal model of neurodegeneration**

Axonal transection provides very useful paradigms to study cellular responses to injury, mechanisms of regeneration and plasticity, and processes that lead to nerve cell degeneration and ultimately their death. Moreover, models of axotomy are valuable for testing experimental therapeutic approaches (Price et al., 1992). Lesions can be made with great precision, and, depending on the neural system, location of the lesion, and age of the animal, these models provide the opportunity to examine a range of neuronal responses to injury. Many parameters influence the character, evolution, and outcomes of axotomy-related processes. The axons of the peripheral nervous system (PNS) have a high capacity for regeneration after injury, whereas injury to axons in the adult central nervous system (CNS) of higher species (i.e. mammals) does not generally result in regeneration. The most severe outcome of axotomy is cell death, which is very common in lesions of CNS neurons. Neurons of the PNS may also die if the transection is sufficiently close to the neuronal cell body or if lesions are performed in young animals (Tatagiba et al., 1997). Neuronal death is also a feature of pathologies of human neurodegenerative diseases, neurological and neuro-ophthalmic disorders including, amyotrophic lateral sclerosis (ALS), stroke, Parkinson's, Huntington's and Alzheimer's disease, spinal muscular atrophy (SMA), optic neuropathies, and retinitis pigmentosa (Osborne et al., 1999; Nicotera et al., 2000; Chader, 2002). For example, it has been shown that transected axons are common in the lesions of patients with

multiple sclerosis (MS), which could be one of the factors leading to the irreversible neurological impairment in this disease (Trapp et al., 1998). Likewise, optic neuropathies such as glaucoma irrevocably result in the loss of retinal ganglion cells (RGCs) in the inner retina, and continuous loss of these cells over time results in permanent loss of vision (Osborne et al., 1999, Levin, 2000). To date, studies of axotomy models have provided clues into the cellular/molecular events associated with neuronal death and the ways in which interventions can delay or prevent processes that lead to irreversible axon injury and neuronal loss.

### **Optic nerve transection: an *in vivo* model of CNS neurodegeneration**

Neuronal loss in several neurodegenerative diseases, such as glaucoma, Parkinson's and Alzheimer's disease involves a final common pathway (Nicotera et al., 2000a, 2000b). This suggests that mechanisms that lead to neuronal death are similar regardless of the initial stressor(s), and underscores the need for reliable and cost effective animal models of neurodegeneration. Currently there are several animal models that are used to investigate neuronal degeneration and regeneration after nerve injury in the CNS. Transection of the optic nerve in the rat is a valuable model for studying cellular responses to injury in the CNS. Moreover, the optic nerve transection model has been extensively used to develop neuroprotective strategies for neurodegenerative diseases, such as glaucoma (Osborne, 1999; Levin, 2001). Unlike some CNS lesion models,

optic nerve (ON) lesions in adult rats can be made with great precision that results in RGC death that occurs in a highly selective and time-dependent fashion.

The success of this model is afforded mainly due to the structure of the eye, the anatomical organization of the retina (Fig. 1), the accessibility to the optic nerve and the retinocollicular projection in the adult rat. First, optic nerve transection selectively injures only RGCs because these are the only retinal neurons that project an afferent axon from the retina to visual centers in the brain. In the adult rat the vast majority of RGCs transmit visual information to the contralateral superior colliculus (SC) via the optic nerve (Linden and Perry, 1983). Second, the optic nerve is an easily accessible CNS fiber tract that can be transected within the orbit of the eye in a consistently reproducible manner (Fig. 2A). After leaving the eye, RGC axons contained within the optic nerve become myelinated by oligodendrocytes and thus, have the same characteristics as white matter tracts in the brain. Two other CNS glia, astrocytes and microglia, are also found within the optic nerve tract (Dezawa and Adachi-Usami, 2000) as well as the retina, and after ON transection these cells respond with a variety of structural and molecular modifications typical for intrinsic CNS glia (reviewed in: Ridet et al., 1997; Aldskogius and Kozlova, 1998). Moreover, the ability to unequivocally quantify RGCs with minimal histological processing makes this model attractive to study neuronal degeneration (Fig. 2B, C, and D). Indeed, the layered structure of the retina simplifies the model, because RGCs are found as



a monolayer of neurons within the ganglion cell layer (GCL) of the retina (Fig. 3). However, as shown in Figure 3, RGCs are not the only cell type found in the GCL, which also contains cholinergic (ChAT) and GABAergic displaced amacrine cells (dACs), astrocytes and microglia. As Figure 4 illustrates, RGCs represent approximately 55% of the total cellular population of the GCL (Perry, 1981). Although a number of approaches have been used to quantify RGCs, including histological stains (Rabacchi et al., 1994; Manni et al., 1996) and immunohistochemical markers (Barnstable and Dräger, 1984; Manni et al., 1996), retrograde tract tracing techniques have proved the most useful and reliable method to separate functionally distinct classes of cells within the GCL (see Fig. 3 and 4) (Vidal-Sanz et al., 1988). Retrograde labeling techniques have also been useful for obtaining primary cultures of pure RGCs (Otori et al., 1998; Tehrani et al., 2000). Indeed, retrograde tract tracing using horseradish peroxidase (Linden and Perry, 1983), FluoroGold (Berkelaar et al., 1994; Di Polo et al., 1998) fast blue (Kügler et al., 1999), Dil (Vidal-Sanz et al., 1988) or fluorescent dextran conjugates (Koeberle and Ball, 1998) has proven to be the most selective method to identify retinal ganglion cells. The majority of studies pre-label RGCs before injury by stereotaxically injecting retrograde tracers into the SC where RGC axons terminate (Linden and Perry, 1983). However, it has recently been shown that the same quantity and quality of RGC labeling can be effectively achieved by injecting retrograde tracers directly into the proximal ending of the optic nerve at the time of transection (Koeberle and Ball, 1998;

Weise et al., 2000). Moreover, post-labeling of RGCs seems more favourable since this approach eliminates the need for multiple survival surgeries. Quantification studies of the survival of axotomized RGCs can be accurately determined from confocal photomicrographs taken from the mid-periphery of the ganglion cell layer in flatmounted retinas (see Fig. 5). Furthermore, RGC survival can be measured at different post-surgical time points to study the kinetics of RGC death (Vidal-Sanz et al., 1988; Berkelaar et al., 1994) or to compare the number of surviving RGCs between experimental (pharmacological or gene therapy) and control treatments (Koeberle and Ball, 1998, Di Polo et al., 1998; van Adel et al., 2002). Finally, retrograde tracing has been employed in this model to study the interactions between degenerating neurons and phagocytic microglia (Thanos, 1991, 1993, Thanos et al., 1992; Bodeutsch and Thanos, 2000). Microglia phagocytose cellular debris from dead retrogradely labeled RGCs, and become transcellularly labeled in the process (Kacza and Seeger, 1997). Taken together, these features make optic nerve transection an attractive model to study mechanisms that lead to, as well as those that prevent neuronal degeneration.

### **Response of retinal ganglion cells to optic nerve injury**

The primary outcome of optic nerve injury is RGC death. Complete transection of the optic nerve is a severe injury that results in the loss of the vast majority of retinal ganglion cells within two weeks (Berkelaar et al., 1994; Garcia-

Valenzuela et al., 1993; Garcia-Valenzuela and Sharma, 1998; Koeberle and Ball, 1998; Villegas-Perez et al., 1988). Cell death after ON transection has been shown to occur in a well-defined time course (Fig 6) by the process of apoptosis (reviewed in: Nickells, 1999; Bähr, 2000; Cellerino et al., 2000; Farkas and GrossKreuz, 2001). Four days after axotomy, RGC densities are unchanged, based on counting the number of retrogradely labeled neurons in the mid-periphery of the retina. With increasing survival times, the number of surviving RGCs quickly decreases to approximately 70% of the normal population by day seven, 30% by day ten, and 15-10% in animals surviving for 14 days post-axotomy. In comparison, both intraorbital crush lesions and intracranial transection of the optic nerve delay the onset and amount of RGC death (Villegas-Perez et al., 1993; Berkelaar et al., 1994; Yip and So, 2000). In addition to the distance from the site of injury, RGC survival is influenced by the size of the soma and by the age and species of the animal. For example, in the goldfish and frog, many RGCs survive axotomy and over a short period of time (1-2 months) can regenerate their axons, which remarkably leads to recovery of visual function (Murray and Grafstein, 1969; Bernhardt, 1989; Humphrey and Beazley, 1985; Waldeck and Grunberg, 1995). Although there is an initial response that increases transcriptional factors, survival factors, and growth associated factors, mammalian RGCs do not regenerate their axons after injury. Autologous peripheral nerve grafts have been used to increase RGC survival and promote the regeneration of a small population of RGCs that are resistant to

injury (Vidal-Sanz et al., 1987; Villegas-Perez et al., 1988; Bahr et al., 1992; Lau et al., 1994; Cho et al., 1999). Indeed, about 5-10% of RGCs appear to survive axotomy, and provided with the right conditions, have the ability to partially regenerate an axon. However, it is not exactly clear which endogenous characteristics influence some RGCs to survive optic nerve injury. Optic nerve transection in cats has revealed that vulnerability to axotomy differs among retinal ganglion cell types (Watanbe et al., 1997). In the mammalian retina, 11 different types of retinal ganglion cells have been identified based on their receptive field properties and morphology (O'Brien et al., 2002; Rockhill et al., 2002). In the cat retina, beta cells appear to be more vulnerable to axotomy (Watanbe et al., 1997, 2001), suggesting that RGC death is not a direct consequence of axotomy and that multiple pathways and regulatory mechanisms determine RGC survival after axonal injury.

### **Axotomized RGCs die by Apoptosis**

Despite the complexity of the pathways involved, it is clear that a variety of different insults can induce apoptotic cell death by converging on a similar final common pathway. Recent morphological, biochemical, and pharmacological evidence (summarized in Table 1) have indicated that RGCs die by an apoptotic mechanism after intraorbital or intracranial optic nerve injury, (Berkelaar et al., 1994; Bien et al., 1999; Chaudhary et al., 1999; Chierzi et al., 1998; Garcia-Valenzuela et al., 1993; Garcia-Valenzuela and Sharma, 1998; Isenmann et al.,

1997; and 1999; Kermer et al., 1998; and 2000; Kikuchi et al., 2000; Koeberle and Ball, 1998; Quigley et al. 1995). Moreover, RGC apoptosis has been described in humans with anterior ischemic optic neuropathy (Kerrigan et al., 1997) as well as primary open angle glaucoma (Levin et al., 1996). The fact that RGC apoptosis has been detected in these human disease processes further emphasizes the need to understand the mechanisms that cause experimentally induced RGC apoptosis, after optic nerve transection (Berkelaar et al., 1994), after intravitreal injection of excitatory amino acids (Li et al., 1999; Honjo et al., 2000), and during experimental glaucoma (Garcia-Valenzuela et al., 1995; Quigley et al., 1995). Before discussing in detail the mechanisms that lead to axotomy-induced RGC death, a brief description of neuronal apoptosis will be reviewed.

### **Neurodegeneration and cell death by apoptosis**

Apoptosis, or programmed cell death, occurs mainly in embryonic stages of development, and functions to control cell numbers and facilitate morphogenesis. Apoptosis can also be induced by diverse stimuli such as radiation, growth factor withdrawal, overproduction of reactive oxygen species (ROS), or mechanical injury to the cell such as nerve or axon transection. In the developing brain a high rate of apoptosis, or programmed cell death, is tolerated and even required to model the nervous system (reviewed in: Cellerino et al., 2000). However, an increased rate of apoptosis in the adult nervous system

underlies many chronic neurodegenerative disorders, including glaucoma, Parkinson's and Alzheimer's disease (reviewed in Leist et al., 1997; Levin, 1999, 2001). Cell death by apoptosis is classically defined by morphological changes at the ultrastructural level, including compaction and segregation of chromatin into narrow masses, internucleosomal DNA fragmentation, condensation of the cytoplasm, and convolution of the cell membrane (Wyllie et al., 1980). This is followed by disassembly of the cell into small membrane-enclosed apoptotic bodies that are phagocytosed by neighboring cells without inciting inflammation. The morphological changes of apoptosis are often contrasted with to cell death by necrosis. Necrotic cell death can be induced at any age by severe injurious changes in normal physiological conditions such as hypoxia, excessive inhibition or excitation, substrate deprivation, hyperthermia, and exposure to high concentrations of cytotoxins (Wyllie et al., 1980). The initial stages of necrosis are reversible and are marked by disaggregated polysomes, focal chromatin margination, and a slight swelling of mitochondria. Unrepaired cells enter an irreversible stage marked by cellular swelling, random DNA fragmentation, disruption of organelle and plasma membranes followed by uncontrolled release of cellular contents into the extracellular space causing an inflammatory response (Wyllie et al., 1980). In the past decade there has been an exponential increase in the number of reports of descriptions of cell death in the developing, adult and injured CNS. It has become increasingly clear that these early morphological descriptions of cell death represent the extreme ends of a

continuum of possible cell deaths (reviewed in: Nicotera, 2000; Nicotera et al., 2000).

Apoptosis has been shown to proceed via several biochemical cascades that are gene-directed, and since this is an active process it requires specific gene expression and energy production (Nicotera et al., 2000). Indeed, apoptosis can be blocked or slowed by inhibitors of RNA and protein synthesis (Miura et al., 1993). Recent work has demonstrated a decisive role for a class of aspartate-specific proteases of the interleukin-1 $\beta$ -converting enzyme (ICE) family known as caspases, which play a central role in the induction and execution of apoptosis (reviewed in Marks and Berg 1999, Nicotera et al., 2000a, 2000b). Diverse stimuli and insults causing apoptosis result in caspase activation, and caspase 3 and 9 have been shown to play a key role in neuronal degeneration (Marks and Berg, 1999). Evidence for the involvement of these caspases in axotomy-induced RGC apoptosis has been provided by studies applying peptide protease inhibitors, which preferentially inhibit caspase 3 (DEVD-CHO, DEVD-FMK, ZVAD-fmk and ZDVED-cmk), or caspase 9 (LEHD-CHO, and z-LEHD-fmk). Application of these inhibitors directly into the vitreous chamber at the time of axotomy has been shown to increase the survival of 30-35% of axotomized RGCs that would have otherwise died by apoptosis (Kermer et al., 1998, 2000; Chaudhary et al., 1999).

### **Pro-apoptotic and anti-apoptotic proteins determine the fate of a cell**

Cell survival and apoptosis are regulated by several key regulatory proteins that either promote or inhibit cell death by apoptosis (Hockenbery et al., 1993; Miller, 1999). For example, the *bcl-2* family of proteins has members that can inhibit (i.e. Bcl-2, Bcl-X, and Bcl-X<sub>L</sub>) or promote (i.e. Bax, Bad, and Bid) apoptosis (Korsmeyer, 1999). Bcl-2 can block apoptosis by preventing the efflux of cytochrome c from the inner membrane of mitochondria, thereby blocking caspase activation and the initiation of the apoptotic program (Kluck et al., 1997; Yang et al. 1997). It has been shown that *bcl-2* and *bcl-x* decrease, and *bax* mRNA increases after ON transection (Isenmann et al., 1997; Chaudhary et al., 1999; McKinnon et al., 2002). Such a shift in the intracellular ratio of anti-apoptotic to pro-apoptotic factors may lead to the activation of caspase 9 and subsequently caspase 3, ultimately leading to the death of the neuron (Kermer et al., 1998; 1999, 2000; Chaudhary et al., 1999; McKinnon et al., 2002). Consistent with these findings, axotomy-induced RGC death can be partially prevented in transgenic mice overexpressing *bcl-2* (Chierzi et al., 1998) or by intravitreal administration of *bax* antisense oligonucleotides (Isenmann et al., 1999) suggesting that induction of RGC apoptosis is via the mitochondrial pathway. These findings indicate that strategies aimed at increasing anti-apoptotic protein expression, or mimicking their function (i.e. caspase inhibition), could attenuate neuronal apoptosis and thus counteract neuronal degeneration in the CNS.



In addition to the *bcl-2* family, the Inhibitors of Apoptosis Proteins (IAP) are a family of anti-apoptotic proteins that are highly conserved throughout evolution (Liston et al., 1996). The baculovirus IAPs, Cp-IAP and Op-IAP, were the first members of the IAP family to be identified based on their ability to functionally complement the cell death inhibitor p35, a baculovirus protein that inhibits caspases (Crook et al., 1993; Clem and Miller, 1994). The first human IAP identified was the neuronal apoptosis inhibitory protein (NAIP). This gene was isolated based on its contribution to the neurodegenerative disorder, spinal muscular atrophy (SMA) (Roy et al. 1995). Subsequently, five human (c-IAP-1/HIAP-2/hMIHB, c-IAP-2/HIAP-1/hMIHC, XIAP/hILP, BRUCE, and Survivin) and two homologs from *Drosophila* (DIAP-1, DIAP-2/dILP) have been isolated (reviewed in Liston et al., 1997, Miller, 1999). The human IAPs contain domains of a highly conserved amino acid sequence in the N-terminal domains with strong homology to the baculoviral IAPs, Cp-IAP and Op-IAP, called BIR domains (Baculovirus IAP Repeats) defined by a  $Cx_2Cx_{16}HX_{6-8}C$  consensus sequence (Roy et al. 1995; Uren et al., 1998). The six human IAPs typically express one to three BIR domains critical for conserving their anti-apoptotic properties, specifically for inhibiting caspases (Roy et al., 1997; Tamm et al., 1998; Deveraux et al., 1999; Marks and Berg, 1999; Maier et al., 2002; Sanna et al., 2002). Recently, it has been demonstrated that the human IAPs are capable of suppressing apoptosis via direct caspase inhibition (Deveraux et al., 1997; Roy et al., 1997; Tamm et al., 1998, Maier et al., 2002). More specifically, NAIP,

XIAP, survivin, c-IAP-1, and c-IAP-2 inhibit cell death proteases, caspases 3 and 7. Indeed, IAPs can attenuate caspases-dependent cell death both *in vitro* and *in vivo* (Liston et al., 1996; Xu et al., 1997; Deveraux et al., 1997; Roy et al., 1998; Tamm et al., 1998; Perrelet et al., 2000). Furthermore, it has recently been demonstrated that viral-mediated overexpression of XIAP can protect retinal ganglion cells from axotomy-induced cell death (Kügler et al., 1999).

Taken together, these studies suggest that axotomy-induced RGC death is caspase-3-dependent, which is consistent with neuronal apoptosis observed after injury to other areas of the CNS (Gorman et al., 1998; Marks and Berg, 1999; Nicotera et al., 2000).

### **Multiple mechanisms contribute to retinal ganglion cell apoptosis after axotomy**

Although there is convincing evidence that RGCs ultimately die by apoptosis, the exact mechanisms that lead to the induction of apoptosis is not as clear. It has been hypothesized that the demise of injured RGCs in glaucoma and in animal models, such as optic nerve transection, is mediated by several mechanisms, including excitotoxicity caused by over stimulation of *N*-methyl-D-aspartate (NMDA) receptors (Kikuchi et al., 2000; Russelakis-Carneiro et al., 1996; Schettauf et al., 2000), reactive oxygen species (Koeberle and Ball, 1999; Levin, 1999; Levkovitch-Verbin et al., 2000), activation of glia (Kacza and Seeger, 1997; Morgan, 2000; Tezel and Wax, 2000; Thanos et al., 1994) as well

as neurotrophic factor deprivation (Pease et al., 2000; Yip and So, 2000). Consistent with this idea, it has been shown that RGC death can be partially prevented by intravitreal administration of glutamate receptor antagonists (Kikuchi et al., 2000), factors that inhibit microglia activation (Thanos et al., 1993), and several classes of growth factors (reviewed in: Weise et al., 2000; Yip and So, 2000) (summarized in Table 2).

### **Glutamate Excitotoxicity and RGC Death**

Excitatory amino acids, particularly glutamate, have considerable excitotoxic and oxidative potential (reviewed in Choi, 1992). As such, glutamate-mediated excitotoxicity has been implicated in neuronal death in glaucoma and after optic nerve transection (Levin, 1999; Kikuchi et al., 2000) as well as a number of other neurodegenerative diseases such as Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS) (Leist et al., 1997). The 'excitotoxicity hypothesis' posits that excessive synthesis and release of glutamate, faulty glutamate uptake, decreased glutamate degradation or decreased inhibition of excitatory neurons may lead to neuronal death (Choi, 1992; Leist et al., 1997, Hansson et al., 2000).

Under normal physiological conditions, extracellular glutamate is transported into glial and neuronal cells by several classes of excitatory amino acid transporters (EAAT). Although five EAATs (EAAT1-5) have been identified to date, the astrocyte specific L-glutamate/L-aspartate transporter GLAST-1

(EAAT1), plays a significant role in maintaining normal extracellular glutamate concentrations in the CNS (reviewed in: Anderson and Swanson, 2000). Excessive glutamate is actively transported into astrocytes, via GLAST-1, and is then metabolized to glutamine by the astrocyte-specific enzyme glutamine synthetase (GS) (Hertz *et al.* 1978; Martinez-Hernandez *et al.* 1977; Torgner and Kwamme, 1990). Recent evidence suggests that the intracellular glutamate can be buffered within the astroglial gap-junction coupled network (Hansson *et al.*, 2000). Inhibition of the synthesis of glial glutamate transporters, GLAST and GLT-1, using antisense oligonucleotide administration results in elevated extracellular glutamate concentrations, and neurodegeneration characteristic of excitotoxicity (Rothstein *et al.*, 1996).

In the mammalian retina, Müller glial cells and astrocytes express GLAST-1 (Derouiche and Rauen, 1995; Rauen *et al.*, 1996; Lehre *et al.*, 1997), although, Müller cells contribute to the majority of glutamate uptake in the retina (Rauen *et al.*, 1998). Antisense knockdown or pharmacological inhibition of GLAST-1 has been shown to cause significant changes in the retinal electroretinogram (Barnett and Pow, 2000), increase glutamate levels in the vitreous chamber (Vorwerk *et al.*, 2000), and cause RGC death (Vorwerk *et al.*, 2000). Furthermore, GLAST-1 deficient mice have been shown to have altered electroretinograms, and demonstrate more severe retinal degeneration after ischemia (Harada *et al.*, 1998). Conversely, mice deficient in the neuronal glutamate transporter EAAT2, exhibit normal electroretinograms and a similar response to retinal ischemia as

compared to control littermates. Together, these findings demonstrate an important role for GLAST-1 in preventing glutamate-mediated excitotoxicity in the retina, and in maintaining normal retinal excitatory neurotransmission. Furthermore, it has been reported that GLAST-1 levels are decreased in retinas from human eyes with glaucoma (Naskar et al., 2000). Thus, disruption of glutamate transport may contribute to excitotoxic RGC degeneration in the glaucomatous retina.

Under ischemic/hypoxic conditions due to vascular insufficiency, energy depletion causes a failure of glutamatergic cells to maintain membrane polarity. The cells enter a state of depolarization resulting in the release of glutamate that in turn activates *N*-methyl-D-aspartate (NMDA) receptors allowing calcium influx into the cell (Choi, 1992). The lack of available energy impairs glutamate reuptake, increasing the frequency and duration of NMDA channel activation. This state of hyperexcitability eventually results in excessive  $\text{Ca}^{2+}$  movement through NMDA channels, which generates a burst of free radicals (e.g., superoxide, nitric oxide, hydroxyl radical and peroxynitrite), mitochondrial damage, protease and lipase activation, and eventually cell death via apoptosis (Choi, 1992; Leist et al., 1998). Intraocular injections of NMDA, a glutamate analog, have been shown to cause degeneration of cells in the inner retina with RGCs being the most sensitive to glutamate-mediated degeneration (Lam et al., 1999). Similarly, cultured RGCs are extremely sensitive to high levels of extracellular glutamate (Levy and Lipton, 1990). Administration of the NMDA

receptor antagonist MK-801, has been shown to attenuate the loss of RGCs by NMDA-mediated excitotoxicity (Levy and Lipton, 1990; Lam et al., 1999). It has previously been demonstrated that there are elevated levels of glutamate in the eye following injury to the optic nerve (Yoles and Schwartz, 1998) and high levels of glutamate have been reported in human and primate eyes with glaucoma (Dryer et al., 1996). Furthermore, MK-801 has been shown to protect RGCs from cell death after partial crush or partial transection of the optic nerve (Russelakis-Carneiro et al., 1996; Yoles et al., 1997), and after complete optic nerve transection (Kikuchi et al., 2000).

Taken together, these findings indicate that glutamate-mediated excitotoxicity plays a significant role in the degeneration of RGCs after optic nerve injury. Furthermore, the most likely target for treatment would be to find factors that increase the kinetics of glutamate transport into glial cells (i.e. GLAST-1) and increase the amount of spatial buffering (i.e. Connexin 43 mediated coupling) of glutamate within the astroglial gap-junction coupled network (Hansson et al., 2000).

### **Glial Activation and RGC Death**

Activated glia can engage in a wide range of secretory activities. For example, activated microglia and astrocytes have been shown to secrete neurotoxins such as: nitric oxide (NO); glutamate; arachidonic acid; platelet activating factor (PAF); and the proinflammatory cytokines tumor necrosis factor-

$\alpha$  (TNF- $\alpha$ ), interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and interleukin-6 (IL-6) (reviewed in Cotter et al., 1999). Each of these factors is capable of leading to neuronal dysfunction and ultimately neuronal death. For example, IL-1 $\beta$  can activate the transcription of nuclear factor-kappa B (NF- $\kappa$ B), which can in turn, increase inducible nitric oxide synthase (iNOS) transcription in astrocytes (Cotter et al., 1999). Increases in iNOS activity lead to elevated levels of the free radical, nitric oxide (NO $^{\cdot}$ ), which can interact with superoxide anions (O $_2^{\cdot-}$ ) to form peroxynitrite (ONOO $^{\cdot}$ ). Peroxynitrite is a potent free radical that can impair energy metabolism, damage protein and DNA, and induce neuronal apoptosis (Torreilles et al., 1999). Consistent with this, it has been shown that iNOS positive microglia are detected in axotomized retinas, and intraocular injection of pharmacological inhibitors of iNOS protects axotomized RGCs (Koeberle and Ball, 1999). Arachidonic acid released from activated microglia can inhibit the uptake of glutamate via astrocyte transporters, and lead to elevated and potentially excitotoxic levels of glutamate (Breukel et al., 1997). Similarly, TNF- $\alpha$  has been reported to inhibit glutamate uptake by human astrocytes, and to potentiate glutamate neurotoxicity (Hu et al., 2000). A recent study has demonstrated immunohistochemical evidence of increased levels of TNF- $\alpha$  and its receptor, TNFR, in human glaucomatous retina (Tezel et al., 2001). In addition, the same group showed that glial cells secreted TNF- $\alpha$  as well as other noxious agents such as nitric oxide into the culture media, and facilitated the

apoptotic death of cocultured RGCs as assessed by morphology, TUNEL labeling, and caspase activation (Tezel et al., 2000). Based on these findings it seems evident that neurotoxic factors secreted from activated glial cells, particularly microglia, can trigger neuronal injury by eliciting an apoptotic cascade in injured neurons.

### **Neurotrophic Factors and RGC survival and regeneration**

Several lines of evidence suggest that the loss of target-derived growth factors may account for the loss of RGCs in glaucoma and in animal models of optic nerve injury (Pearson et al., 1992; Pease et al., 2000). Several different classes of neurotrophic factors have been demonstrated to provide trophic support for RGCs during development and in the adult retina (reviewed in: So and Yip, 1998; Weise et al., 2000b). Intraocular injection or viral mediated transfer of neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), neurotrophin 4 (NT-4), and ciliary neurotrophic factor (CNTF) have been shown to have survival promoting effects on axotomized RGCs (Mey and Thanos, 1993; Mansour-Robaey et al., 1994; Peinado-Ramon et al., 1996; Klocker et al., 1996; Di Polo et al., 1998; Koeberle and Ball, 1998; Cui et al. 1999, Weise et al., 2000). Although many of these trophic factors can promote RGC survival, only a few have been shown to promote axon regeneration (summarized in Table 3). For example, it was demonstrated that CNTF, but not BDNF exerted dramatic effects on axon



outgrowth in cultured RGCs (Jo et al., 1999). Moreover, these effects were not a simple consequence of enhanced cell survival, since other growth factors (i.e. BDNF) that also enhance cell viability did not affect outgrowth. Furthermore, of all the neurotrophic factors examined, only CNTF has been shown to promote the survival of RGCs and regeneration of their axons, *in vitro* and *in vivo* (Cho et al., 1999; Cui et al., 1999; Jo et al., 1999) (see Table 3). In addition to protecting axotomized RGCs, a single injection of recombinant CNTF has been shown to protect retinal neurons from the acute effects of ischemia-induced injury (Honjo et al., 2000), and from the damaging effects of constant light (LaVail et al., 1992).

### **CNTF as an endogenous neurotrophic factor for the retina**

Ciliary neurotrophic factor belongs to the interleukin-6 (IL-6) family of cytokines and was originally isolated from avian ocular tissue and named because of its survival promoting effects on ciliary ganglion neurons (Adler et al. 1979; Manthorpe et al., 1980). CNTF is a 23 kDa protein with low sequence similarity to any known protein (Inoue et al., 1996). CNTF lacks a consensus secretory signal sequence and is normally localized to the cytoplasm of glial cells in the central and peripheral nervous systems (Sendtner et al., 1994). However, under conditions of cellular stress CNTF can be secreted from glia by a yet unknown mechanism; and thus CNTF has been postulated to function as an injury-activated factor (Adler, 1993; Peterson et al., 2000). Both CNTF and the  $\alpha$ -subunit of the CNTF receptor complex are expressed during different phases

of retinal neurogenesis and differentiation (Ip et al., 1993; Kirsch and Hoffman, 1994, Heller et al., 1995; Kirsch et al., 1997). In the normal adult rat retina, CNTF is predominately localized in Müller glial cells, whereas retinal neurons, including RGCs, horizontal and amacrine cells, express the CNTFR $\alpha$  (Kirsch et al., 1997). Recently, it has been shown that CNTF and CNTFR $\alpha$  are upregulated in the rat retina after optic nerve transection (Chun et al., 2000; Ju et al., 2000b; Kirsch et al., 1998; Weise et al., 2000; Wen et al., 1995), after ischemic insult (Ju et al., 1999, and 2000a) and in light-stressed rats (Walsh, 2001). Indeed, elevation of endogenous or exogenous administration of CNTF is neuroprotective for retinal neurons, however the exact mechanisms remain largely unknown (Lavail et al., 1992; Mey and Thanos; 1993; Honjo et al., 2000, Peterson et al., 2000; Weise et al., 2000). Several possibilities have been suggested including, inhibition of glutamate-mediated excitotoxicity (Semkova et al., 1999; Petersen and Brundin, 1999; Petersen et al., 1999; Honjo et al., 2000), modulation of glial cells conferring an additional protective capacity (Lisovoski et al., 1997; Monville et al., 2001; Albrecht et al., 2002; Liang et al., 2001a), and activation of neuroprotective signal transduction pathways including the JAK (Janus tyrosine kinase) –STAT (signal transducers and activators of transcription) pathway (Peterson et al., 2000).

## **CNTF and signal transduction**

The CNTF receptor complex is composed of its own unique component, CNTFR $\alpha$  (72 kDa), and two shared signal transducing transmembrane subunits, gp130 and leukemia inhibitory factor (LIF) receptor-beta (LIFR $\beta$ , which is the LIF binding protein) (Davis and Yancopoulos, 1993) (Fig. 7). The CNTFR $\alpha$  is anchored to the cell membrane via a glycosyl-phosphatidylinositol (GPI) linkage, and therefore can exist as a membrane bound or soluble form (sCNTFR $\alpha$ ) following detachment by phospholipase C (PLC) (Sleeman et al., 2000). The membrane bound form mediates the mostly neuronal actions of CNTF, whereas the soluble form confers CNTF responsiveness to non-neuronal cells, such as glial cells that express the signal transduction components, gp130 and LIFR $\beta$  (Fig. 8) (März et al., 1999). Regardless, both function strictly as a ligand binding chain for CNTF and have no signal transduction role other than to increase the formation of the CNTF receptor complex (Inoue et al., 1996). This in turn induces intracellular signal transduction cascades via the JAK-STAT pathway (Fig. 7). gp130 (135 kDa) is widely distributed throughout all cell types and constitutes half of the high affinity binding sites for CNTF. It has two CNTF binding domains and six tyrosine residues that are contained within motifs for STAT3, STAT1 and SHP-2 (which can dephosphorylate the receptor) binding (Fukada et al., 1999). The LIFR $\beta$  (190 kDa) provides the other half of the high affinity binding site for CNTF, with one CNTF binding domain and tyrosine

binding residues for both STAT3 and STAT1 (Davis et al., 1993). The order of CNTF receptor activation begins with a 1:1 ratio of CNTF to CNTFR $\alpha$ . Two CNTF-CNTFR $\alpha$  dimers then attach to gp130 which causes LIFR $\beta$  to be recruited and form the full tripartite receptor complex (Stahl and Yancopoulos, 1994, Sleeman et al., 2000). The heterodimerization of gp130 and LIFR $\beta$ , which are initially unassociated, activates the intracellular signal transduction cascade (Schindler & Darnell, 1995). CNTF makes direct contact with all three receptor components (Davis et al., 1993). CNTF can bind with a lower affinity to the LIFR $\beta$ , and initiate the same signal transduction cascade as, gp130 and LIFR $\beta$  without CNTFR $\alpha$ , provided that the extracellular concentration of CNTF is significantly elevated (Monville et al., 2000).

CNTF is able to transduce a signal from its receptor on the cell membrane directly to genes in the nucleus via the JAK-STAT pathway (Cattaneo et al., 1999). In resting cells, JAKs are catalytically inactive protein kinases located directly on the cytoplasmic face of the receptor chains (Yeh & Pellegrini, 1999). STATs are ubiquitous cytoplasmic proteins that initially act as signal transducers by functioning as a substrate for JAKs. Interaction with JAKs results in the phosphorylation of STATs, which then translocate to the nucleus where they can function as a transcription factor (Schindler & Darnell, 1995, Imada & Leonard, 2000). All STATs have a single tyrosine close to residue 700 that becomes phosphorylated during cytoplasmic activation (Fukada et al., 1999). Both gp130

and LIFR $\beta$ , have constitutively associated JAKs (JAK1 and JAK2) that are activated by receptor dimerization. Binding of CNTF to its receptor mediates a conformational change that allows the tyrosine trans-phosphorylation of JAK1 (135KD) and JAK2 (130KD) (Stahl and Yancopoulos, 1994; Sleeman et al., 2000). The activated JAKs then phosphorylate specific tyrosine sites on gp130 and LIFR $\beta$  and these phosphotyrosine sites are then recognized by the src homology 2 (SH2) domains of STAT3 and, to a lesser extent, STAT1 (Heim, 1999). SH2 domains bind to the receptor phosphotyrosine sites and determine the selectivity of which receptor will bind to which STAT protein. STAT3 and STAT1 are then bound to the receptor complex where they become activated by tyrosine phosphorylation and are able to form homodimers and heterodimers through mutual phosphotyrosine interactions prior to nuclear translocation (Schindler & Darnell, 1995; Peterson et al., 2000). The method by which STATs enter the nucleus is unclear, however, once in the nucleus they bind to specific response elements in the promoters of target genes and transcriptionally activate these genes by RNA polymerase II (Imada & Leonard, 2000).

The major intracellular effect of CNTF is the initiation of STAT3 signal transduction (Sleeman et al., 2000). STAT3 (88KD) is phosphorylated at Tyr705 into pSTAT<sub>3</sub>, which can homodimerize or heterodimerize with STAT1 ( $\alpha$  91KD and  $\beta$  84KD), which is phosphorylated at Tyr701 (Fukada et al., 1999). The exact method of STAT regulated gene transcription is still not clear, but it seems

likely that gene promoters have STAT consensus motifs (Weber-Nordt et al., 1998). There are two identified conserved regions in STAT inducible genes. The gamma activated sequence (GAS) will recruit homodimers of STAT1 and interferon-stimulated response element (ISRE) will recruit STAT1 homodimers and heterodimers (Schindler, 1999; Schindler and Strenghlow, 2000). STAT3 may have the most flexibility in regulating gene transcription through its interaction with CRE (cAMP response element) binding proteins (Kojima et al., 1996). Recently, it has been shown that STAT3 inhibits transcription of the inducible nitric oxide synthase gene by interacting with NF-kB (Yu et al., 2002). Thus, one action of CNTF may be to inhibit the activation of iNOS in microglia and prevent the formation of ONOO<sup>-</sup>, which has been shown to contribute to the death of axotomized RGCs (Koeberle et al., 1999).

Gene transcription initiated by CNTF is varied and occurs within minutes of exposure (Yeh & Pellegrini, 1999). Application of exogenous CNTF leads to the increased transcription of choline acetyl transferase (ChAT), vasoactive intestinal polypeptide (VIP), GFAP, *bcl-2*, *junB*, and *c-fos*, (Rajan et al., 1996; Symes et al., 1997a; Weber-Nordt et al., 1998; Cattaneo et al., 1999). CNTF has been localized to the nucleus, which raises the possibility that CNTF could act directly on transcription and not only through the activation of STAT3 and STAT1 (Bajetto et al., 1999). For example, CNTF can induce activation of the gene for GFAP indirectly by STAT1, but there is also evidence suggesting the presence of CNTF-responsive elements near the GFAP promoter (Kahn et al., 1997). GFAP

is an astrocytic protein that is upregulated following any CNS trauma, which likely shares a neuroprotective role with CNTF.

The activated JAK-STAT pathway can be suppressed by receptor endocytosis, the actions of SHP-2 (SH2 containing phosphatases) on receptor phosphorylation, and by several newly discovered proteins (Hilton, 1999; Yeh & Pellegrini, 1999). These new proteins have been divided into several categories; SOCS (suppressor of cytokine signaling), JABs (JAK binding proteins), PIAS (protein inhibitors of activated STATs) and SSIs (STAT induced STAT inhibitors), all of which have been implicated in controlling CNTF signaling (Heim, 1999; Schindler, 1999).

The downstream signaling of STATs correlates better with the receptor activated components (gp130 and LIFR $\beta$ ) than with the JAKs that were initially activated by the ligand (Heim, 1999). It is via the SH2 domains of the STATs that shared receptors gain their ability to produce specificity in signal transduction by interacting with only a specific type and quantity of STATs (Schindler & Darnell, 1995). For example, CNTF and LIF share a high level of tertiary structure similarity but almost no amino acid homology (Inoue, 1996). Therefore, CNTF and LIF which share the ability to activate gp130 and LIFR $\beta$  may activate STAT3 and STAT1, but not at the same proportion of homo and heterodimer, or for the same period of time. Furthermore, the phosphotyrosine phosphatases that cease STAT3 and STAT1 activation also have unique kinetics. This differentiates CNTF and LIF based on the genes regulated by their respective

STATs. It is also known that CNTF binds with greater affinity to gp130, and LIF binds with greater affinity to LIFR $\beta$  (Sleeman et al., 2000). This would further delineate their individual signal transduction effects because phospholipase C (PLC) is primarily associated with gp130 and phosphatidylinositol-3 kinase (PI-3K) is primarily associated with LIFR $\beta$  (Boulton et al., 1994). Therefore, CNTF versus LIF would favour activation of PLC over PI-3K. CNTF is also implicated in the activation of the ras-MAPK (mitogen activated protein kinase) pathway. The tyrosine phosphorylation of gp130 would attract not only STATs but also SHP-2 (src-homology 2 phosphatase). Ras is a membrane-associated protein, often in close proximity to gp130, and the recruitment of SHP-2 by an activated gp130 leads to ras-MAPK signalling through the engagement of Raf1 (Peterson et al., 2000). Activation of the MAPK and PI-3K can result in cell survival via intracellular cascades including, the activation of numerous enzymes, interference with the induction and execution of apoptosis, and the transcriptional control of survival promoting genes (Weise et al., 2000; Kaplan and Miller, 2000; Kermer et al., 2001). Taken together, it appears as though there are multiple pathways that can be activated by CNTF and that these pathways may work in concert to mediate the neuroprotective effects of CNTF in the retina and brain (Peterson et al., 2000)



## Research Objectives

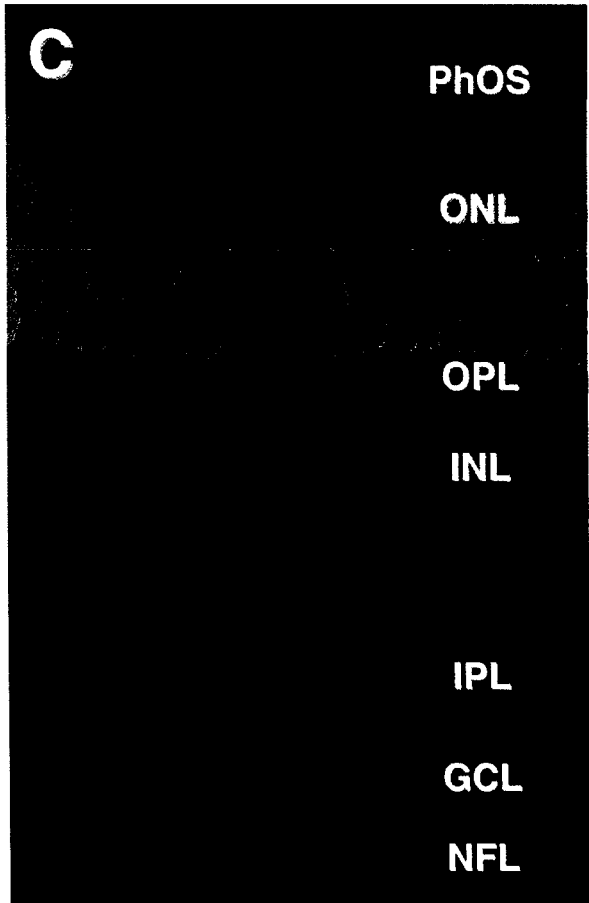
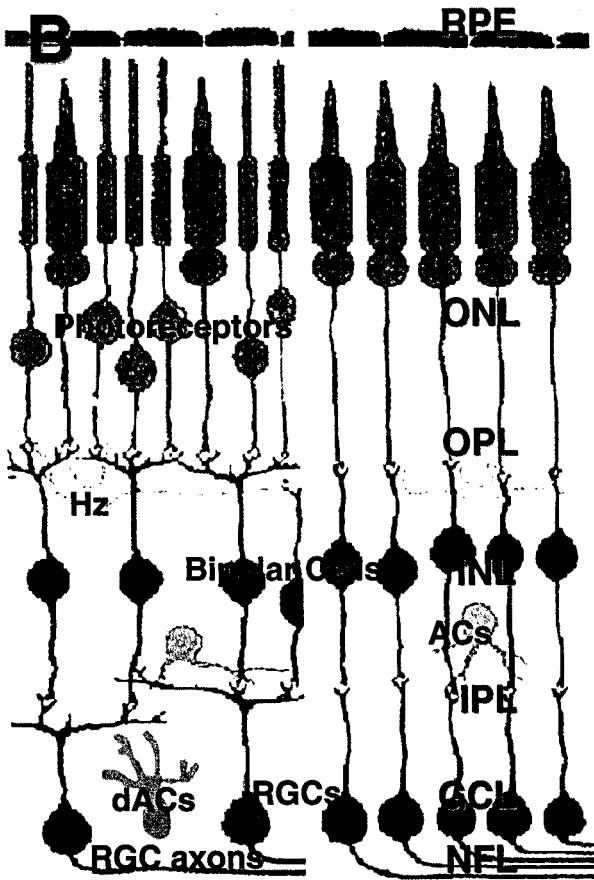
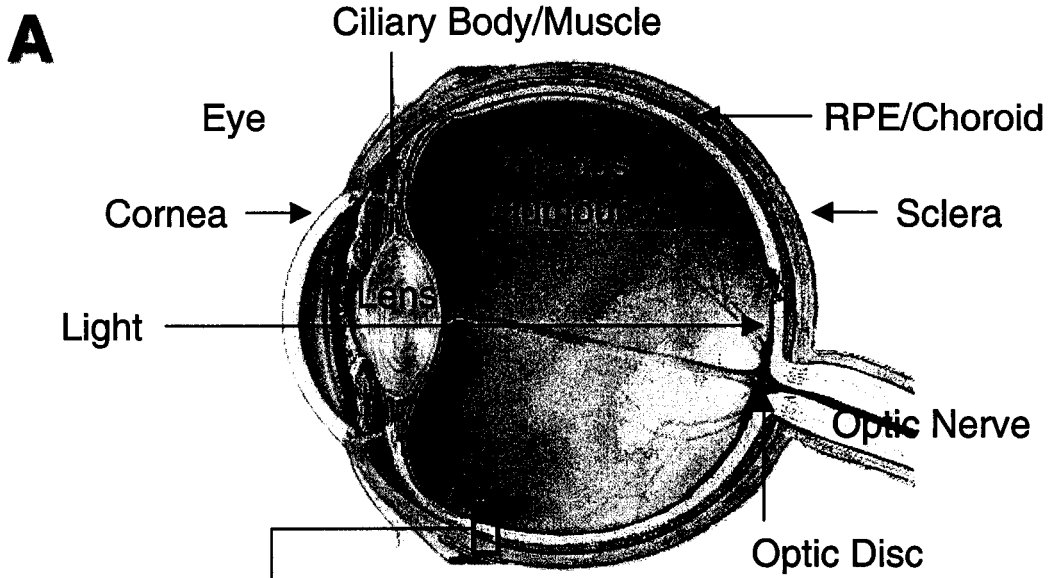
The role of apoptosis in neurodegenerative disorders has led to suggestions that treatments that offer neuroprotection by rendering specific neuronal populations apoptosis resistant may represent a potentially valuable therapy for such diseases. While this approach is still in its infancy, encouraging reports of *in vivo* apoptotic attenuation have been published using animal models such as optic nerve transections (Bonfanti et al., 1996; Cenni et al., 1996; Chierzi et al., 1998; Kermer et al. 1998; Porciatti et al., 1996).

Survival of injured neurons is a fundamental requirement for any regenerative therapy, thus it is of great clinical relevance to be able to interfere or inhibit the process of cell death by apoptosis. To this end, a well established *in vivo* model system of axonal injury and apoptosis was used in the present series of experiments: transection of the rat optic nerve. Indeed, intraorbital transection of the rat optic nerve results in the apoptotic cell death of more than 85% of RGCs 14 days after the lesion. The overall aim of this research, was to develop strategies to suppress neuronal apoptosis and promote neuronal survival. Since, CNTF has proven to be a promising candidate for promoting both the survival and regeneration of axotomized RGCs, this thesis examined the potential use of this cytokine for the treatment of retinal injuries involving RGCs (So and Yip, 1998; Weise et al., 2000a, 2000b). More specifically, the investigation examined the neuroprotective mechanisms of CNTF and related compounds to inhibit axotomy-induced apoptosis of RGCs in the adult rat. The information gained

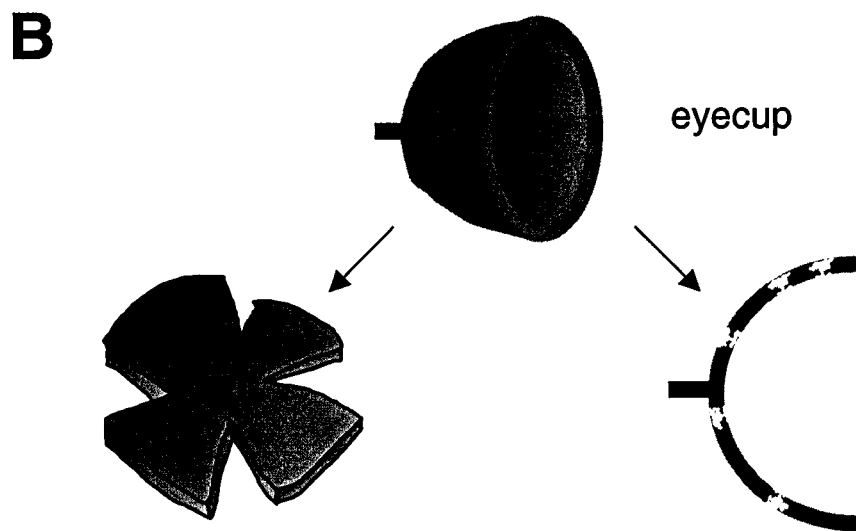
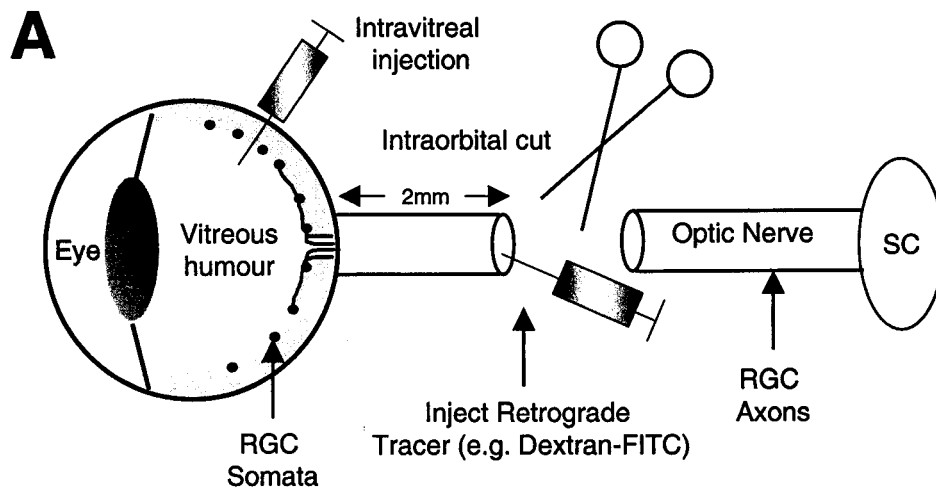
from this line of investigation will lead to a better understanding of neuronal apoptosis and its role in neurodegenerative disorders of the nervous system. Furthermore, the results will inform possible avenues for treatment of neurodegenerative diseases.

**Figure 1.** Schematic illustration of the mammalian eye and the anatomical organization of the retina. **(A)** Light energy travels through the anterior chamber of the eye where it is focused and projected through the posterior chamber (vitreous body) of the eye to the neural retina. The retina is made up of neurons that transduce light energy, encode visual information, and convey a neural representation of the visual world to the brain via the optic nerve. **(B and C)** A cross-sectional view of the retina showing its layered structure and the five main classes of retinal neurons within three distinct nuclear layers, separated by two synaptic (plexiform) layers. The retinal pigment epithelium (RPE) is the outermost layer of the retina, and is located at the back of the eye and associated with the photoreceptor outer segments (PhOS). The photoreceptor nuclei are located in the outer nuclear layer (ONL). Photoreceptors synapse with bipolar cell dendrites and horizontal cell processes in the outer plexiform layer (OPL). The somas of bipolar, horizontal and amacrine cells are found in the inner nuclear layer (INL). The synaptic networks of amacrine and bipolar cells are found in the inner plexiform layer (IPL) where their processes converge with the dendritic fields of retinal ganglion cells (RGCs) and displaced amacrine cells found in the ganglion cell layer (GCL). At the level of the GCL, RGC axons project through the nerve fiber layer (NFL) towards the optic nerve disc where they leave the eye and project to visual centers in the brain via the optic nerve (ON). Note: retinal glia (microglia, astrocytes and Müller cells) are not shown here. Astrocytes are restricted to the NFL, whereas Müller cells have their cell

bodies in the INL. The outer limiting membrane marks the distal border of Müller cells and the basal endfeet contribute to the inner limiting membrane of the retina. Microglia are found scattered throughout the retina.

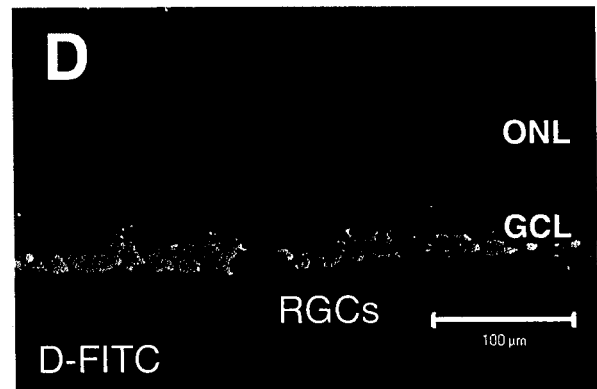
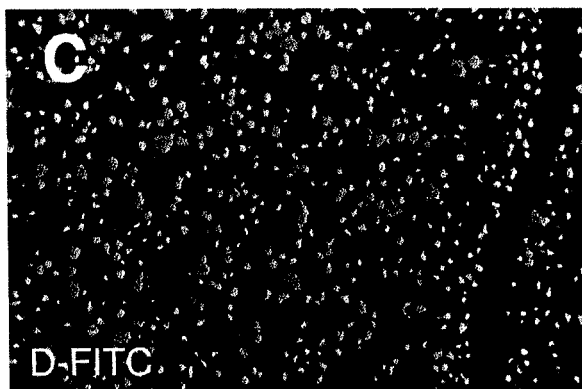


**Figure 2.** Illustration of the surgical procedures for optic nerve transection, intraocular injection, and retrograde labeling from the nerve stump and histological procedures used to isolate the retina for RGC quantification. **(A)** the optic nerve is transected within the orbit at a distance of 2mm from the optic nerve head. Pharmacological agents, viral vectors, or modified cell lines can be injected directly into the vitreous by making intraocular injections through the sclera. Retinal ganglion cells can be pre-labeled prior to ON transection by stereotaxic injection of a retrograde tracer into the contralateral superior colliculus (SC). Alternatively, RGCs can be retrogradely labeled immediately following optic nerve transection by injecting a retrograde tracer directly into the proximal nerve ending. **(B)** Eyecups are collected at different post-surgical times, immediately fixed and processed either as a flatmount or as a transverse section. **(C)** A representative confocal micrograph of a flatmounted retina showing the pattern of retrograde labeling 2 days after nerve injection of Dextran-FITC (D-FITC). **(D)** A transverse section of the retina showing the selective labeling of RGCs in the GCL 2 days after nerve injection of D-FITC. **Note:** transverse sections permit visualization of all retinal layers whereas photomicrographs of flatmounted retinas represent a single layer of the retina showing the distribution/density of labeled cells. The laser scanning confocal microscope can be used to optically section a flatmounted retina and permit the selective visualization of fluorescently labeled cells in different retinal layers.



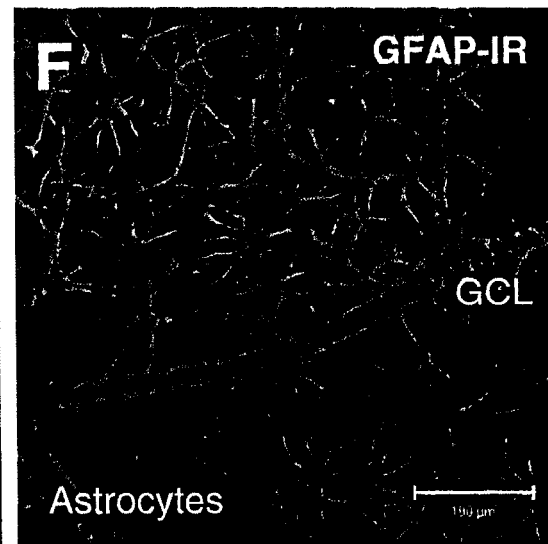
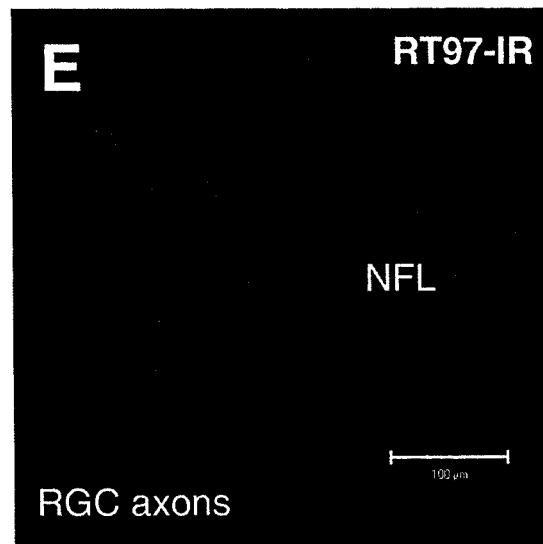
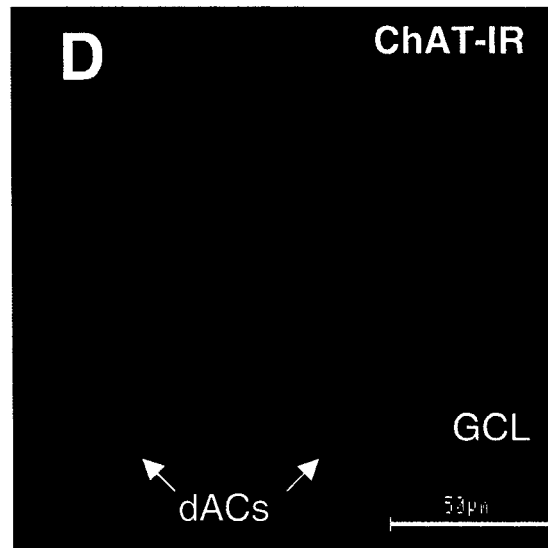
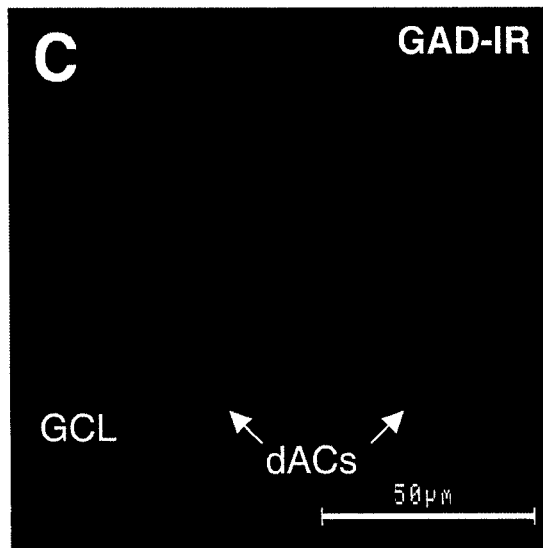
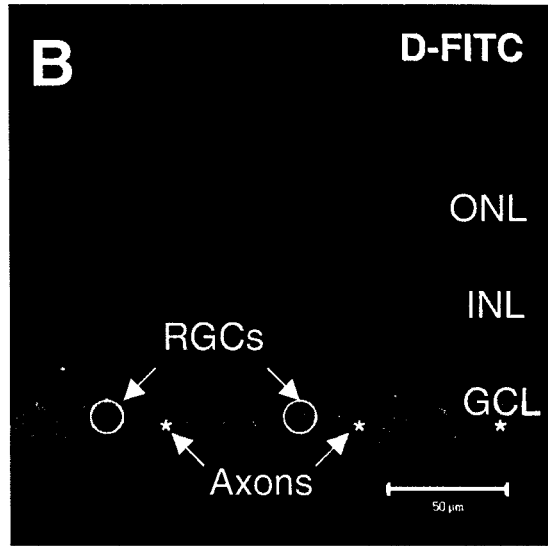
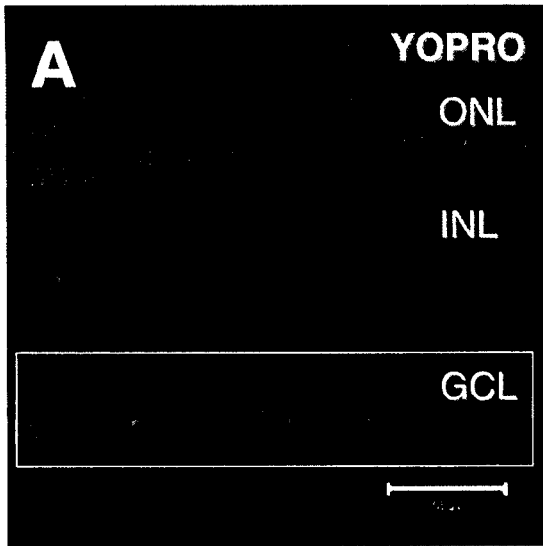
Retinal Flatmount

Transverse sections

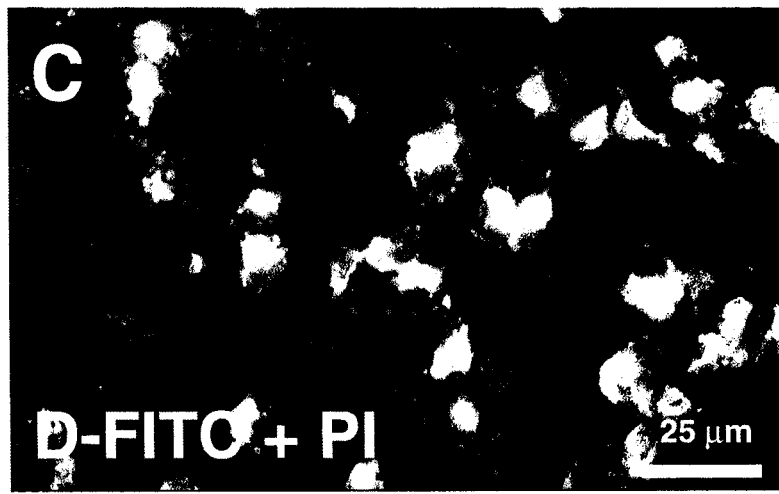


**Figure 3.** Photomicrographs of representative cell types in the retinal ganglion cell layer (GCL) of the adult rat retina. (A) a transverse section of a normal retina demonstrating the dense packing of yopro stained nuclei in the GCL (white box). (B) a transverse section of the retina showing that retrograde tracers can be used to selectively label only RGCs in the ganglion cell layer. (C) a small percentage of cells in the GCL are GABAergic displaced amacrine cells (dACs). (D) a transverse section demonstrating the presence of cholinergic dACs. (E) axons of retinal ganglion cells (stained with an antibody to RT97) stratify the GCL. (F) GFAP positive astrocytes are located just below the GCL and are located in the NFL together with RGC axons. This confocal micrograph of a flatmounted retina clearly demonstrates the contribution of astrocytes to the formation of the blood-retinal barrier.



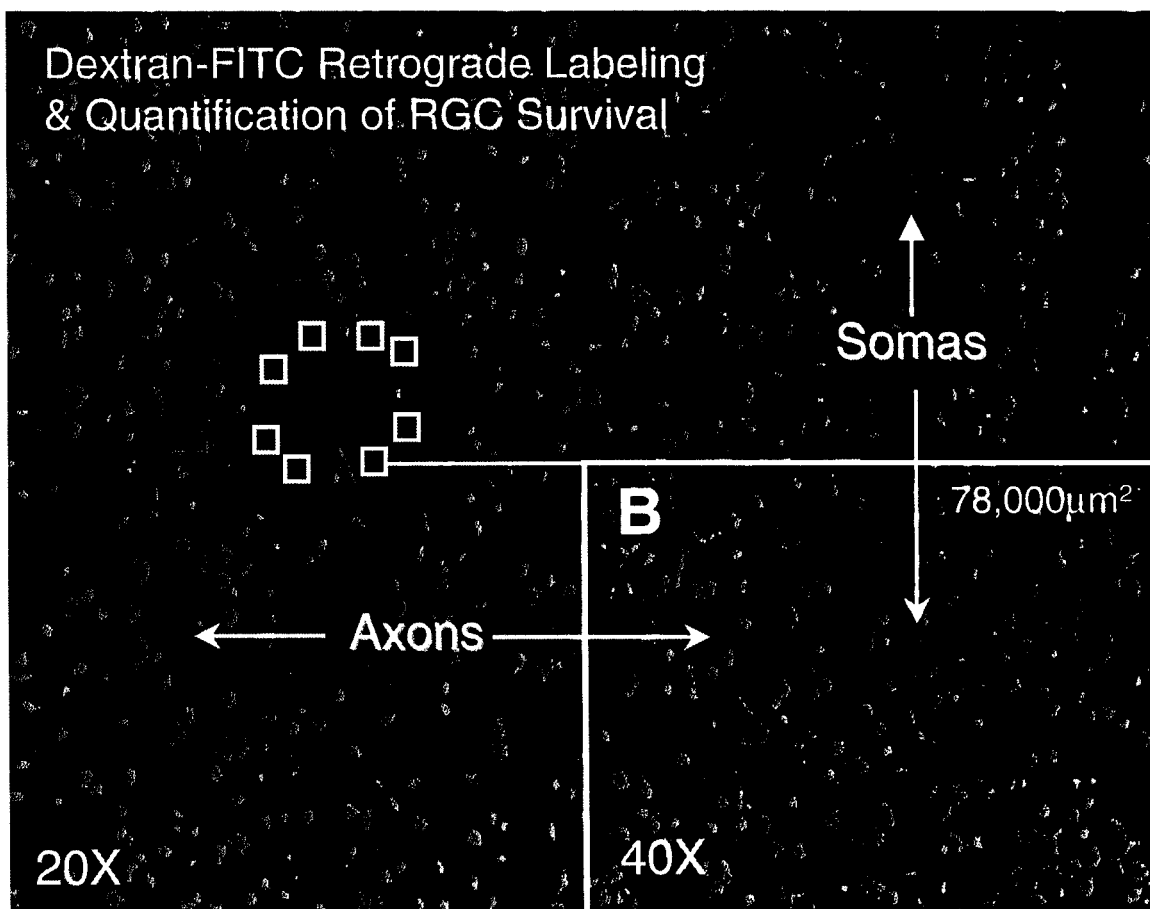


**Figure 4.** Estimate of the percentage of retinal ganglion cells in the GCL of the adult rat retina. (A) High magnification confocal micrograph of a flatmounted retina retrogradely labeled with Dextran-FITC. (B) The same retina counterstained with propidium iodide (PI) which labels all nuclei of cells in the GCL. (C) Double-labeled (D-FITC + PI) cells revealed that approximately 55% of the cells in the GCL are RGCs. Scale bar = 25  $\mu$ m.



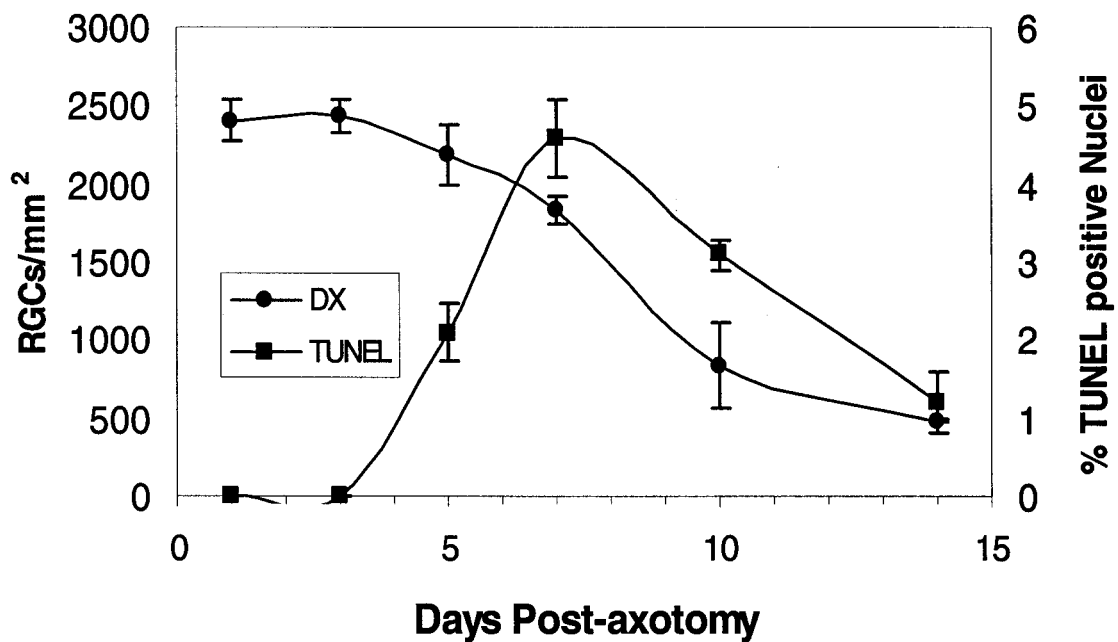
**Figure 5.** Quantification of retinal ganglion cell survival using retrograde tract tracing. (A) Confocal micrograph of a flatmounted retina 2 days after injection of the retrograde tracer, Dextran-FITC, into the nerve ending. Cartoon overlay of the retina showing the sampling regions taken from the mid-periphery or 3/6 of the retinal eccentricity. (B) The density of retinal ganglion cells is determined from each sampling area, which represents an area of 78,000  $\mu\text{m}^2$ .

# Dextran-FITC Retrograde Labeling & Quantification of RGC Survival



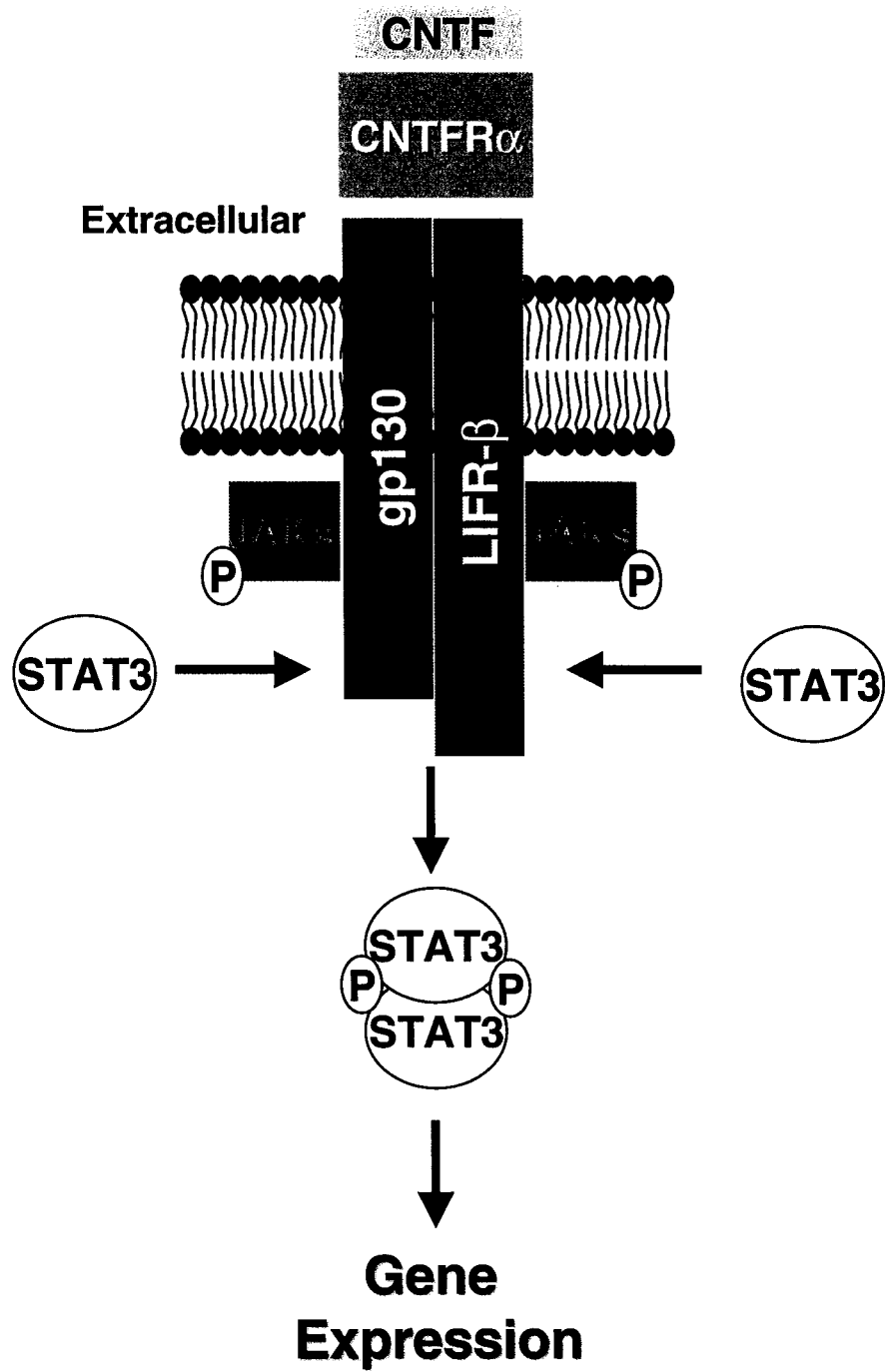
**Figure 6.** The loss of retinal ganglion cells after optic nerve transection and the appearance of TUNEL positive nuclei in the GCL. This graph shows that axotomy of all RGCs by ON transection results in the delayed death of ~80-90% of RGCs within 14 days. The results are based on plotting the mean densities (RGCs/mm<sup>2</sup>) of Dextran-FITC retrogradely labeled RGCs determined in flatmounted retinas isolated at 1, 3, 5, 7, 10, and 14 days post-axotomy (dpa). Also plotted are the mean percentages of TUNEL stained nuclei ( $\pm$  SD) counted in transverse sections at 5, 7, 10, and 14 dpa. **Note:** the peak rate of RGC death (7-10 dpa) coincides with the greatest number of TUNEL positive nuclei. Adapted with permission from Koeberle and Ball, 1998.

### Survival of Axotomized RGCs

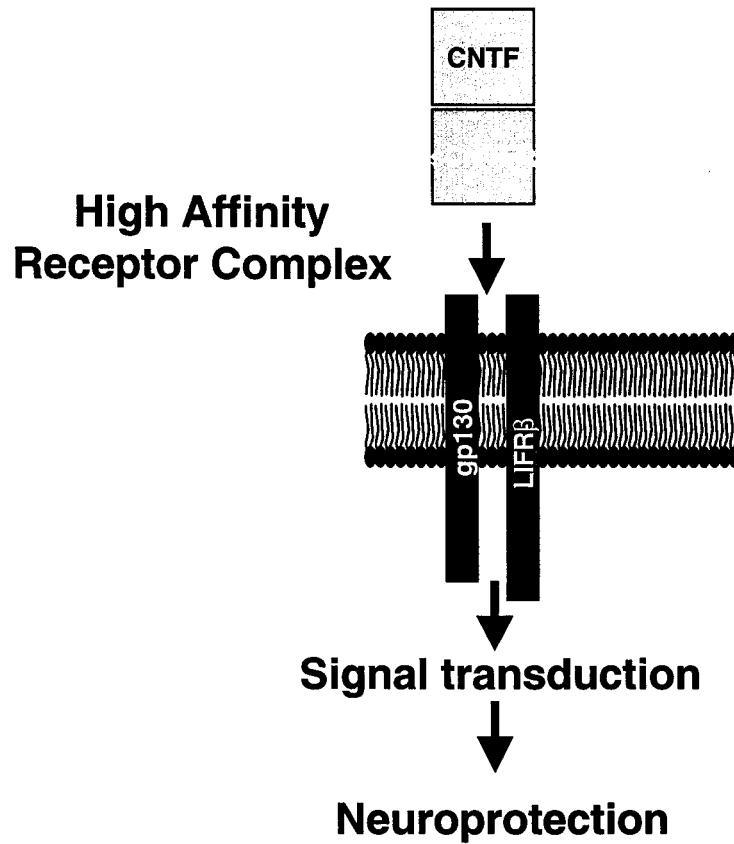
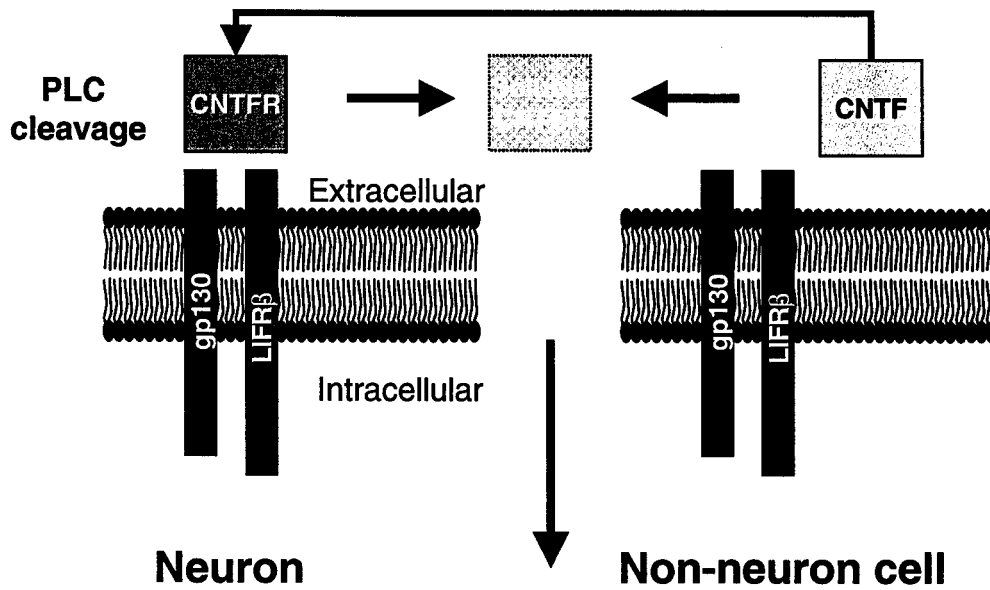


**Figure 7.** The CNTF receptor complex and activation of the JAK-STAT pathway in CNS neurons. The interaction of CNTF (light blue) with its  $\alpha$ -receptor (grey) induces dimerization of gp130 (dark blue) and LIFR $\beta$  (pink). This in turn brings members of the JAK (red) cytoplasmic kinases into juxtaposition, allowing them to transphosphorylate each other and to phosphorylate specific STAT (pale yellow) proteins. STATs are normally found in the cytoplasm in monomeric form and, following tyrosine-residue phosphorylation by the activated JAKs, they then migrate to the cytokine receptor (arrows), homo- or heterodimerize, and translocate to the nucleus where they bind to specific DNA elements found upstream of the genes that are induced by CNTF.





**Figure 8.** Possible neuroprotective actions of CNTF in the injured central nervous system. CNTF is secreted from non-neuronal cells such as astrocytes and acts as an injury-activating factor directly on injured neurons. Alternatively, CNTFR $\alpha$  is released (via PLC) from a neuron and the soluble form of the receptor (sCNTFR $\alpha$ , shaded grey) binds extracellular CNTF (light blue) and induces dimerization of gp130 (dark blue) and LIFR $\beta$  (pink) on a target cell that does not normally respond to CNTF. There is also evidence that CNTF can bind directly to the LIFR $\beta$  (Monville et al., 2001).



**Table 1.** Morphological, biochemical and pharmacological evidence for apoptosis of retinal ganglion cells after optic nerve injury.

<b>Morphological Evidence</b>	<b>Reference</b>
1) Ultrastructural detection of apoptosis after ON axotomy	Barron et al., 1986
1) Ultrastructural features and TUNEL labeling after axotomy	Quigley et al., 1995
2) Ultrastructural features after optic nerve transection	Joo et al., 1999
3) Ultrastructural features and TUNEL labeling crush injury	Bien et al., 1999
4) TUNEL positive RGCs and DNA laddering after axotomy	Garcia-Valenzuela et al., 1994
5) Nuclear morphology and TUNEL labeling	Berkelaar et al., 1994
6) TUNEL positive RGCs after optic nerve crush	Isenmann et al., 1997
7) TUNEL positive RGCs after optic nerve transection	Koeberle & Ball, 1998
<b>Biochemical Evidence</b>	<b>Reference</b>
1) Bcl-2 and Bcl-x downregulated after optic nerve injury	Isenmann et al., 1997
	Chaudhary et al., 1999
2) Bax upregulated in axotomized RGCs	Isenmann et al., 1997
	Chaudhary et al., 1999
3) c-Jun activation after axotomy	Isenmann et al., 1997
4) NF-KappaB upregulated in axotomized RGCs	Choi et al., 1998
5) Caspase 9 activation after optic nerve transection	Kermer et al., 2000
6) p38 upregulated in axotomized RGCs	Kikuchi et al., 2000
7) Axotomized RGCs protected in bcl-2 transgenic mice	Chierzi et al., 1998
<b>Pharmacological Evidence</b>	<b>Reference</b>
1) Caspase-3 inhibitors rescue axotomized RGCs	Kermer et al., 1998
	Chaudhary et al., 1999
2) Bax antisense oligonucleotides rescues axotomized RGCs	Isenmann et al., 1999
3) Caspase-9 inhibitors protect axotomized RGCs	Kermer et al., 2000
4) p38 inhibitors rescue axotomized RGCs	Kikuchi et al., 2000
5) XIAP protects axotomized RGCs	Kügler et al., 2000
6) Aurintricarboxylic acid protects axotomized RGCs	Heiduschka & Thanos, 2000

**Table 2.** Neuroprotective strategies to rescue retinal ganglion cells after optic nerve injury.

**Glutamate Neurotoxicity**

Factor	Species	Protection	Reference
1) NMDA antagonist (MK801)	Cat	+++	Russelakis et al., 1996
2) NMDA antagonist (MK801)	Rat	+++	Kikuchi et al., 2000
3) NMDA and Non-NMDA antagonists	Rat	+++	Schuettauf et al., 2000
4) NMDA antagonist (ON delivery)	Rat	++	Vorwerk et al., 2001

**Microglia Activation**

Factor	Species	Protection	Reference
1) Microglia-suppressing factors	Rat	++	Thanos et al., 1993
2) iNOS inhibitors	Rat	++	Koeberle & Ball, 1999

**Oxidative Stress (e.g. Nitric Oxide)**

Factor	Species	Protection	Reference
1) (-)-Deprenyl	Rat	++	Buys et al., 1995
2) neuronal NOS inhibitors	Rat	++	Koeberle & Ball, 1999
3) Salicylic acid	Chick	++	Castagne et al., 1999

**Neurotrophic Factors**

Factor	Species	Protection	Reference
1) Brain derived neurotrophic factor	Rat	++++	Di Polo et al., 1998
2) Brain derived neurotrophic factor	Cat	++++	Chen & Weber, 2001
3) Nerve Growth Factor	Rat	++	Carmignoto et al., 1989
4) Neurotrophin-4/5	Rat	++	Clarke et al., 1998
5) Glial-derived neurotrophic factor	Rat	++	Koeberle & Ball, 1998
6) Neurturin	Rat	++	Koeberle & Ball, 2002

**Cytokines and other Growth Factors**

Factor	Species	Protection	Reference
1) Ciliary Neurotrophic Factor	Rat	++++	Mey & Thanos, 1993
2) Tumour necrosis factor- $\alpha$	Rat	+++	Diem et al., 2001
3) Insulin-like growth factor	Rat	++	Kermer et al., 2000

**Miscellaneous and Novel Compounds**

Factor	Species	Protection	Reference
1) Ca <sup>2+</sup> channel blocker (Flunarizine)	Rat	++	Eschweiler & Bahr, 1993
2) $\alpha$ 2-Adrenoreceptor Agonists	Rat	++	Yoles et al., 1999
3) Peptide vaccination	Mouse	+++	Fisher et al., 2001
4) Anti-myelin basic protein T cells	Rat	+++	Moalem et al., 2000
5) Heat Shock protein 72	Rat	+++	Park et al., 2001

Note: + = fair; ++ = moderate; +++ = high; ++++ = exceptional.

**Table 3.** The effects of neurotrophic factors on the survival and regeneration of retinal ganglion cells *in vitro* and *in vivo*.

<b>Neurotrophic Factor</b>	Survival <i>in vitro</i>	Neurite outgrowth <i>in vitro</i>	Survival <i>in vivo</i>	Nerve regeneration <i>in vivo</i>
Brain-derived neurotrophic factor (BDNF)	++	-	+++	-
Insulin-like growth factor-1 (IGF-1)			+	
Nerve growth factor (NGF)	+	+	+	-
Neurotrophin-3 (NT-3)	+	-	-	-
Neurotrophin-4 (NT-4)	+	+	+	-
Ciliary neurotrophic factor (CNTF)	+++	+++	+++	++
Fibroblast growth factor (FGF)	+	+	++	-
Glial derived neurotrophic factor (GDNF)	-	-	++	-
Neurturin	-	-	+	-

+ = fair, ++ = moderate, +++= high, - = no effect. (Adopted from: So and Yip, 1998; Weise et al., 2000)

## **CHAPTER 2**

### **Experiment I:**

Neuronal apoptosis inhibitory protein enhances the survival of axotomized retinal ganglion cells *in vivo*

#### **Research contributions:**

B.A. van Adel: optic nerve transection, nerve injections, retrograde labeling, immunohistochemistry, histochemistry, data analysis, and preparation of the manuscript for publication (Neuroscience – IBRO journal)

Nathalie Gendron & April Doyle: RT-PCR for NAIP Expression

**Neuronal apoptosis inhibitory protein enhances the survival of axotomized  
retinal ganglion cells *in vivo***

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**Keywords:** retinal ganglion cell, axotomy, retina, IAPs, neuronal apoptosis inhibitory protein, adenovirus, gene therapy, optic nerve transection, cell death, apoptosis

**Running Head:**

NAIP rescues axotomized RGCs



**Abstract:**

Neuronal apoptosis inhibitory protein (NAIP) is a member of a large family of anti-apoptotic proteins called Inhibitors of Apoptosis (IAPs). NAIP has recently been shown to prevent apoptosis by inhibiting the effector caspases 3 and 7. Over 90% of retinal ganglion cells (RGCs) die within 2 weeks after optic nerve transection by a caspases-dependent apoptotic mechanism. In this study the optic nerve (ON) was transected intraorbitally 2mm from the eye. RGCs were simultaneously transduced and retrogradely labeled by injecting a solution of adenoviral vector encoding the gene for human NAIP (Ad.NAIP) or bacterial  $\beta$ -galactosidase (Ad.lacZ) and the fluorescent tracer, Dextran-FITC into the proximal ON ending. Retinas treated with Ad.NAIP showed a 20% increase in surviving RGCs compared with control retinas treated with Ad.lacZ at 14 days post-axotomy. Real-time (quantitative) PCR using TaqMan chemistry did not detect a change in endogenous NAIP expression in axotomized retinas, which may have made axotomized RGCs more susceptible to death by apoptosis. These results suggest that elevation of endogenous IAPs, such as NAIP could serve as a potential treatment for retinal disorders involving RGC injury.

## Abbreviations

Ad: adenovirus  
Ad.CNTF: adenovirus encoding human CNTF  
Ad.lacZ: adenovirus encoding beta-galactosidase (RSV promoter)  
Ad.lacZ<sub>2</sub>: adenovirus encoding beta-galactosidase (CMV promoter)  
Ad.NAIP: adenovirus encoding NAIP  
Ad.XIAP: adenovirus encoding XIAP  
 $\beta$ -gal: beta-galactosidase  
BIR: Baculovirus IAP Repeats  
CAR: Coxsackie and adenoviral receptor  
CMV: cytomegalovirus  
CNTF: ciliary neurotrophic factor  
CNTFR $\alpha$ : CNTF receptor alpha  
dpa: days post-axotomy  
D-FITC: Dextran-FITC  
GCL: ganglion cell layer  
IAP(s): inhibitor(s) of apoptosis  
INL: inner nuclear layer  
IO: intraocular  
lacZ: cDNA coding for beta-galactosidase  
LIF: leukemia inhibitory factor  
LIFR $\beta$ : LIF receptor beta  
NAIP: neuronal apoptosis inhibitory protein  
NFL: nerve fiber layer  
NMDA: *N*-methyl-D-aspartate  
pfu: plaque forming units  
ON: Optic nerve  
RGC(s): retinal ganglion cell(s)  
RSV: rous sarcoma virus  
SC: superior colliculus  
x-gal: 5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside  
XIAP: X-linked IAP

## Introduction

Neuronal apoptosis inhibitory protein (NAIP) was the first human Inhibitor of Apoptosis (IAP) Protein to be identified as a candidate gene for the inherited childhood neurodegenerative disorder Spinal Muscular Atrophy (SMA) (Roy et al. 1995). Subsequently, five human homologues have been identified: X-linked IAP (XIAP), HIAP-1, HIAP-2, BIR-repeat-containing ubiquitin-conjugating enzyme (BRUCE) and Survivin (reviewed in Liston et al., 1997; LaCasse et al., 1998; Deveraux and Reed, 1999). The human IAPs contain domains of a highly conserved amino acid sequence in the N-terminal domains with strong homology to the baculoviral IAPs, Cp-IAP and Op-IAP, called BIR domains (Baculovirus IAP Repeats) defined by a  $Cx_2Cx_{16}HX_{6-8}C$  consensus sequence (Roy et al. 1995; Uren et al., 1998). The six human IAPs typically express one to three BIR domains critical for conserving their anti-apoptotic properties, specifically for inhibiting a class of aspartate-specific proteases of the interleukin-1 $\beta$ -converting enzyme (ICE) family known as caspases (Roy et al., 1997; Tamm et al., 1998; Deveraux et al., 1999; Marks and Berg, 1999; Maier et al., 2001; Sanna et al., 2002). The BIR2 domain of NAIP is critical for preserving the inhibitor properties on the effector caspases, 3 and 7 (Maier et al., 2001), which are activated by a variety of triggers. Indeed, NAIP can attenuate caspases-dependent cell death both *in vitro* and *in vivo* (Liston et al., 1996; Xu et al., 1996; Deveraux et al., 1997; Roy et al., 1998; Tamm et al., 1998; Perrelet et al., 2000). Overexpression of NAIP, using either plasmid or recombinant adenoviral vectors, was shown to

prevent different cell lines (CHO, Rat-1 and HeLa) from undergoing apoptosis after serum withdrawal, and after menadione or TNF $\alpha$  exposure (Liston et al., 1996). The administration of NAIP using adenoviral vectors prevents ischemic damage in the rat hippocampus (Xu et al., 1996), ameliorates the loss of nigrostriatal function in a rat model of Parkinson's disease (Crocker et al., 2001), and protects motor neurons from axotomy-induced cell death (Perrelet et al., 2000). Endogenous NAIP has been shown to be upregulated in a number of CNS structures, including the rat hippocampus following global ischemia (Xu et al., 1997). Conversely, the loss of endogenous NAIP after motoneuron axotomy in rats (Perrelet et al., 2002), and in a mouse model of traumatic brain injury (Hutchison et al., 2001) may increase the vulnerability of CNS neurons to injury-induced apoptosis. Consistent with this, it has been shown that hippocampal neurons are more vulnerable to excitotoxic damage in Naip1 knockout mice (Holcik et al., 2000). Together, these studies demonstrate that NAIP plays an important role in the regulation of neuronal apoptosis in the mammalian CNS.

The optic nerve transection model has been used extensively to develop neuroprotective strategies for neurodegenerative diseases such as glaucoma and to study the mechanisms of neuronal degeneration and regeneration. Transection of the adult rat optic nerve (ON) results in delayed neuronal death of approximately 85-90% of retinal ganglion cells (RGCs) 14 days after injury (Berkelaar et al., 1994; Peinado-Ramon et al., 1996; Klöcker et al., 1997; Koeberle and Ball, 1998; Kikuchi et al., 2000). There is accumulating evidence

from both animal models of glaucoma and optic nerve injury paradigms (i.e. transection and crush) that RGC death occurs via a caspase-dependent apoptotic pathway (Berkelaar et al., 1994; Lucius and Sievers, 1997; Bien et al., 1999; Chierzi et al., 1998; Garcia-Valenzuela et al., 1994; Kermer et al., 1998; 1999; Koeberle and Ball, 1998; Kügler et al., 1999; Nickells, 1999; Quigley et al., 1995; McKinnon et al., 2002). Injury-induced RGC apoptosis may be triggered by several events, including neurotrophic factor deprivation (Pease et al., 2000; Weise et al., 2000a; Yip and So, 2000), excitotoxicity caused by over stimulation of *N*-methyl-D-aspartate (NMDA) receptors (Kikuchi et al., 2000; Russelakis-Carneiro et al., 1996; Schettauf et al., 2000), increased production of reactive oxygen species (Huxlin and Bennett, 1995; Koeberle and Ball, 1999; Levin, 1999; Levkovitch-Verbin et al., 2000), and activation of microglia (Thanos et al., 1994; Kacza and Seeger, 1997; Kortuem et al., 2000; Morgan, 2000; Tezel and Wax, 2000;). Furthermore, it has been shown that the levels of anti-apoptotic factors, Bcl-2 and Bcl-x decrease and the pro-apoptotic factor, Bax increases after ON transection (Isenmann et al., 1997; Chaudhary et al., 1999; McKinnon et al., 2002). Such a shift in the intracellular ratio of anti-apoptotic to pro-apoptotic factors may lead to the activation of caspase 9 and subsequently caspase 3, and ultimately the death of the neuron (Kermer et al., 1998; 1999, 2000; Chaudhary et al., 1999; McKinnon et al., 2002). Consistent with these findings, axotomy-induced RGC death can be partially prevented in transgenic mice overexpressing *bcl-2* (Chierzi et al., 1998) or by intravitreal administration of *bax* antisense

oligonucleotides (Isenmann et al., 1999) suggesting that induction of RGC apoptosis is via the mitochondrial pathway. Evidence for the involvement of caspases in axotomy-induced RGC apoptosis has been provided by studies applying peptide protease inhibitors, which preferentially inhibit caspase 3 (DEVD-CHO, DEVD-FMK, ZVAD-fmk and ZDVED-cmk), or caspase 9, (LEHD-CHO, and z-LEHD-fmk) (Kermer et al., 1998, 2000; Chaudhary et al., 1999;). Application of these inhibitors directly into the vitreous chamber at the time of axotomy has been shown to increase the survival of 30-35% of axotomized RGCs that would have otherwise died by apoptosis. Furthermore, overexpression of the baculovirus pan-caspase inhibitor p35, or XIAP using viral vectors (Kugler et al., 1999; 2000; McKinnon et al., 2002) protects axotomized RGCs, whereas overexpression of the cowpox caspase inhibitor crmA (which is more specific for caspases 1 and 8) using adenoviral vectors, exerted no survival promoting effects on axotomized RGCs (Kugler et al., 1999). Taken together, these studies suggest that axotomy-induced RGC death is caspase-3-dependent, which is consistent with neuronal apoptosis observed after injury to other areas of the CNS (Gorman et al., 1998; Marks and Berg, 1999; Nicotera et al., 2000). Thus, overexpression of an anti-apoptotic protein that inhibits caspase 3 directly, such as NAIP, may serve as a potential treatment to reduce RGC death in optic neuropathies such as glaucoma.

To test this hypothesis we first determined the effect of optic nerve transection on retinal NAIP expression in order to further elucidate mechanisms

of RGC death. We then examined the effects of adenoviral-mediated gene transfer of NAIP on the survival of axotomized RGCs. The survival promoting properties of NAIP on axotomized RGCs were compared with the IAP family member XIAP, and the neurotrophic factor, CNTF, previously shown to be protective in this model (Weiss et al., 2000; van Adel et al., 2000), as well as with a control vector carrying the *lacZ* gene. Our results show that endogenous levels of NAIP do not change after axotomy, however overexpression of NAIP extended the survival period of axotomized RGCs *in vivo*.

## **Materials and Methods:**

### ***Animals***

Naïve, adult, female Sprague-Dawley rats were purchased from Charles River Canada (St. Constant, Quebec) at six weeks of age (200-250 g). Animals were acclimatized to the McMaster University Central Animal Facility (CAF) in a level B pathogen controlled environment for approximately 2 weeks prior to surgical procedures. The rats were housed individually in shoebox cages and maintained in good health under the care of a veterinarian and animal care technicians for the duration of the experiment. The animals were maintained on a 12 hr light-dark cycle (lights on at 8:00 a.m.) and both food and water were available *ad libitum*.

### ***Adenoviral vectors***

The construction, production, purification and titration of the adenoviral vectors used in this study and their *in vitro* and *in vivo* bioactivity have been previously described (Xu et al., 1997, Gravel et al., 1997). Briefly, a replication-deficient type 5, adenovirus encoding human NAIP or the bacterial enzyme *Escherichia coli*  $\beta$ -galactosidase under the transcriptional control of rous sarcoma virus (RSV) were generated by insertion of human NAIP or bacterial *E. coli*  $\beta$ -galactosidase expression cassette cDNA into the adenoviral vector backbone as previously described (Liston, et al., 1996; Xu et al., 1997). As a positive control we compared the neuroprotective effects of Ad.NAIP with Ad.XIAP since this is another IAP with similar caspase inhibitory properties (Maier et al., 2001) that



has been shown to be protective in this model (Kügler et al., 2000) and others (Eberhardt et al., 2000; Xu et al., 1999). Furthermore, we compared the neuroprotective effects of the IAP vectors (Ad.NAIP and Ad.XIAP) to a vector coding for a secreted form of the neurotrophic factor, CNTF (Ad.CNTF). The CNTF vector was driven by a cytomegalovirus (CMV) promoter, thus, we employed a second control vector encoding bacterial *E. coli*  $\beta$ -galactosidase driven by CMV (Ad.lacZ<sub>2</sub>). The adenoviral vectors used in the present study (Ad.NAIP, Ad.XIAP, Ad.CNTF, Ad.lacZ and Ad.lacZ<sub>2</sub>) are schematically illustrated in Figure 1A. The vectors were matched for particle content and used at  $1.2 \times 10^{10}$  pfu/ml.

***Optic nerve transection, retrograde labeling and application of adenoviral vectors to the nerve stump***

Using a surgical microscope (Zeiss, Germany), the optic nerve was approached within the orbit by making a skin incision along the superior rim of the orbital bone. The superior orbital contents were carefully dissected and the rectus muscles were reflected laterally. To gain visual access to the optic nerve and the surrounding meningeal sheath the eye was rotated in a temporal direction by transecting and then retracting segments of the extra-ocular muscles. Following rotation of the eye, the meningeal sheath was excised longitudinally and the optic nerve was cut approximately 2 mm behind the eye (see Fig. 1B). In untreated animals (n=18) retinal ganglion cells were retrogradely labeled by injecting 10  $\mu$ l of a 3% solution of Dextran-FITC (3000

MW, Molecular Probes, Eugene, OR) into the proximal nerve stump as previously described (Koeberle and Ball, 1998). A second group of animals was used to determine the number of RGCs in normal retinas by stereotaxically injecting a 3% solution of D-FITC into the superior colliculus (SC) as previously described (van Adel et al., 2001). For experimental animals, treated with adenoviral vectors, RGCs were simultaneously retrogradely labeled and transduced by injecting a 10  $\mu$ l cocktail consisting of a 3% solution of Dextran-FITC (3000 MW, Molecular Probes, Eugene, OR) and adenoviral vector (Ad.NAIP, Ad.XIAP, Ad.CNTF, Ad.lacZ or Ad.lacZ<sub>2</sub>) into the proximal nerve stump. Experimental nerve injections consisted of Ad.NAIP (n=5), Ad.XIAP (n=8), Ad.CNTF (n=6), and control injections consisted of Ad.lacZ (n=4) and Ad.lacZ<sub>2</sub> (n=4) (see Fig.1B and 1C). A separate group of animals received axotomy without treatment or tracer injection in order to determine the expression of NAIP after optic nerve transection. Animals in this group were sacrificed after: 12 hours, 1, 2, 4, 7, or 14 days post-axotomy (minimum of 4 retinas/group). During all surgical procedures, care was taken to avoid damage or disruption to the retinal blood supply. We performed postoperative ophthalmoscopy to verify the integrity of the retinal vasculature so that only those rats without a compromised retinal vasculature were included as experimental subjects.

### ***Isolation and preparation of retinas for histology***

At the endpoint of each experiment, animals were induced for 30 seconds in a 0.5 L dessicator jar containing a cotton swab soaked in 0.5ml isoflurane,

ethanized by an overdose of chloral hydrate (4 ml 7% chloral hydrate; intraperitoneal) and eyes were enucleated. The cornea and lens were dissected away and the remaining eyecups were fixed for 1.5 hours in a solution consisting of 4% paraformaldehyde and 2% sucrose, in 0.1 M Sorensen's phosphate buffer (0.1 M, pH 7.3). Following fixation, eyes were rinsed in PBS for 10 minutes and flatmounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) for visualization and quantification of RGC densities.

***RNA preparation and real-time (quantitative) PCR using The ABI Prism 7700***

Retinas were isolated at 1 and 12 hours, and at 1, 2, 4, 7, and 14 days post-axotomy and immediately stored at  $-80^{\circ}\text{C}$  until processed. Retinal RNA was extracted using Trizol or the RNeasy kit (Qiagen) and detection of NAIP expression was performed by real-time quantitative PCR (RT-PCR) simultaneously with GAPDH expression. RT-PCR reactions were performed in the ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Biosystems) with the TaqMan EZ RT-PCR kit (Perkin Elmer Applied Biosystems) and TaqMan Rodent GAPDH Control Reagent (Perkin Elmer Applied Biosystems). Briefly, 25  $\mu\text{l}$  RT-PCR reactions were set in triplicate for each sample and contained: 100 ng of total RNA, 1X TaqMan EZ Buffer, 3mM Mn(OAc), 300uM deoxy-ATP, -CTP, -GTP, 600  $\mu\text{M}$  deoxy-UTP, 100 nM rodent GAPDH primers and probe, 600 nM rat NAIP primers, 200 nM rat NAIP probe, 0.25 U AmpErase UNG and 2.5 U rTth DNA Polymerase. RT-PCR conditions

were: 2 min at 50°C, 30 min at 60°C, 5 min at 95°C, followed by 45 cycles of 15 sec at 94°C and 1 min at 60°C.

### ***X-gal histochemistry, $\beta$ -galactosidase, NAIP, XIAP and RT97***

#### ***Immunohistochemistry***

Several retinas were processed to determine the cellular localization of adenoviral transgene expression of  $\beta$ -galactosidase in retinas isolated 4 days after nerve injection of Ad.lacZ and Ad.lacZ<sub>2</sub> vectors. RT97 immunohistochemistry was performed on whole-mount normal and axotomized retinal preparations to examine the integrity of RGC axons in the nerve fiber layer. Transverse cryostat sections (14  $\mu$ m) or flatmounted retinas were processed using X-gal (5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside, Boehringer-Mannheim, Mannheim, Germany) histochemistry that in the presence of functional  $\beta$ -galactosidase, is converted to a blue reaction product in transduced cells. Retinal sections were washed three times in 100 mM PBS (pH 7.4) and then incubated in 2mM MgCl<sub>2</sub>, 25 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 25 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, and 1 mg/ml X-gal in PBS for 4-6 hours at room temperature. Upon completion of the staining, sections were washed three times in 100 mM PBS and coverslipped. Alternatively, immunofluorescent detection of *lacZ* transgene expression was carried out using standard immunohistochemistry procedures. Briefly, sections were incubated with a polyclonal antibody raised in rabbit

against  $\beta$ -galactosidase (3prime-5prime Inc, 1:2000). For axon labeling whole retinas were incubated in antisera directed against the 200-kDa neurofilament protein, RT97 (mouse anti-RT97, 1:500; Anderton et al., 1982, generously donated by Dr. L.C. Doering). We used our own mouse monoclonal antibody against NAIP (1:100) and a polyclonal antibody against XIAP (1:100) to determine the localization of these IAPs in the adult retina. Following primary antibody incubation, retinas were rinsed 3 x 10 minutes in PBS. Beta-galactosidase immunoreactivity was detected using a goat-anti-rabbit secondary antibody (1:500; Texas Red conjugated, pre-absorbed against rat serum proteins, Jackson ImmunoResearch, West Grove, PA), and RT97 immunoreactivity was detected with a goat anti-mouse secondary antibody (1:500, Jackson ImmunoResearch, West Grove, PA). Whole retinas were incubated in secondary antisera for 4 hours, rinsed 3 x 15 minutes in PBS and then coverslipped in Vectashield for visualization.

### ***Microscopy and Cell Counting***

Dextran-FITC fluorescence and  $\beta$ -galactosidase or RT97 immunofluorescence were visualized using a Zeiss Laser Scanning Confocal Microscope (LSM-510) with a 488 nm Ar laser excitation for FITC and 585 nm HeNe laser excitation for Texas red. X-gal histochemistry was visualized using transmitted light microscopy. Labeled RGCs were counted from confocal micrographs covering an area of 78,000  $\mu\text{m}^2$  as previously described (Koeberle and Ball, 1998). Each retina was divided into 4 radial quadrants, and confocal

micrographs of 12 areas (3 from each quadrant) from the mid-periphery (1/2 the retinal eccentricity) of each flatmounted retina were quantified. Microglial cells that had been transcellularly labeled with Dextran-FITC, due to phagocytosis of dead RGCs, were distinguished from intact RGCs on the basis of their size and morphology, and excluded from the cell counts. The results for separate retinas were pooled for statistical analysis and the mean RGC densities/mm<sup>2</sup> ± SD were calculated. The percentage of cells that were transduced following Ad.lacZ injection was calculated by comparing the number of X-gal positive or  $\beta$ -galactosidase immunoreactive cells to previously calculated densities of RGCs (approximately 2500 cells/mm<sup>2</sup>; Koeberle and Ball, 1998, 1999; van Adel et al., 2000).

### ***Statistical analysis***

Statistical analysis of the results was performed using SPSS 8.0. for Windows98. Statistical significance was determined using one-way ANOVA followed by Tukey's and Dunnet's post hoc comparisons for significance between groups at  $p < 0.05$ .

## Results

### *Effect of Optic Nerve transection on RGC Densities*

In normal rats, RGCs retrogradely labeled from the superior colliculus (SC) using D-FITC revealed a mean RGC density in the mid-periphery of the retina to be  $2474 \pm 61$  RGCs/mm<sup>2</sup> (mean  $\pm$  SD) (Fig.2A). Similar results were obtained when RGCs were retrogradely labeled at the time of axotomy by injecting D-FITC into the proximal nerve ending. As figure 2B shows, injection of D-FITC into the optic nerve labeled RGCs and axon fascicles as early as 2 days post-axotomy (dpa). Since no appreciable RGC death occurs within the first 4 days after optic nerve injury (Villegas-Perez et al., 1988; 1993; Peinado-Ramon et al., 1996; Koeberle and Ball, 1998), we observed no significant difference in the mean number of D-FITC labeled RGCs ( $2381 \pm 102$  RGCs/mm<sup>2</sup>: mean $\pm$ SD) at 2 dpa compared to normal retinas retrogradely labeled from the SC. However, at 7 and 14dpa there was a significant reduction in the number of D-FITC retrogradely labeled RGCs (Fig. 2C and D) and a concomitant increase in the number of phagocytic microglia. Microglia were easily distinguishable from D-FITC labeled RGCs since they are morphologically different from RGCs and have a significantly smaller diameter than RGCs (Thanos et al., 1992). These microglia become transcellularly labeled with D-FITC by active phagocytosis of RGC apoptotic bodies (Thanos, 1991, 1993). There was a moderate decline in the mean RGC density at 7 dpa ( $1709 \pm 166$  RGCs/mm<sup>2</sup>) compared to retinas from normal animals and animals surviving 2 dpa (Fig. 2E). Retinas isolated at

14 dpa revealed a drastic reduction in the mean RGC density ( $369 \pm 85$  RGCs/mm<sup>2</sup>), which had declined to 14.9% of the RGC density observed in normal retinas (Fig.2E). The greatest rate of RGC death occurred between 7 and 14 dpa and is consistent with previous reports in the literature (Berkelaar et al., 1994; Peinado-Ramon et al., 1996; Koeberle and Ball, 1998).

### ***Expression of NAIP in the normal and axotomized retina***

As shown above, approximately 15% of axotomized RGCs are resistant to axotomy-induced apoptosis without treatment. It is possible that this population of RGCs is able to survive axotomy due to high levels of endogenous NAIP expression. To investigate this possibility, we used immunohistochemistry and RT-PCR to identify NAIP expression in the normal and axotomized retina. Our previous studies demonstrated strong expression of a NAIP message in the retina of developing mouse embryos at E16.5 (Ingram-Crooks, 2002). Although the distribution of NAIP in the rat CNS has been reported (Xu et al., 1997), there have been no specific studies to date on the expression of NAIP in the normal or injured rat retina. Using a monoclonal antibody directed against NAIP, we detected moderate immunoreactivity of neuronal perikarya in all layers of the normal rat retina with the strongest labeling of the photoreceptor outer segments (Fig. 3A) and relatively weak labeling of RGCs, amacrine, horizontal and bipolar cells. A similar pattern of labeling was observed when we stained transverse sections with an antibody raised against XIAP (Fig. 3B). There was no staining observed in retinas when the primary antisera was replaced with pre-immune



serum, or when the primary antibody was excluded from the procedure. These results demonstrate that NAIP and XIAP are expressed in the normal rat retina, albeit at relatively moderate levels. Consistent with the immunohistochemistry analysis, we detected a message for NAIP in the normal adult rat retina using quantitative RT-PCR. We then examined the expression of NAIP at several different time points following complete optic nerve transection (Fig. 4). Quantitative RT-PCR of RNA isolated and purified from axotomized retinas at 1 and 12 hours, and at 1, 2, 4, 7, and 14 days post-axotomy, was performed using specific primers and probe for rat NAIP and GAPDH as an internal standard. There was no significant change in retinal NAIP levels from 1 hour to 14 days post-axotomy. Since RGCs represent less than 5% of the total neuronal population of the rat retina, it is possible that even with the loss of 85% of RGCs 14 days post-axotomy that injury-induced changes in NAIP messenger RNA levels may have been too small to be detected. It is unlikely that any significant changes in NAIP expression were missed due to the sensitivity of the procedure used in this study. Moreover, we analyzed several time points before RGC death occurs (4 days post-axotomy and beyond) as well as during the maximal rate of RGC death (7-10 days post-axotomy). Thus, these findings indicate that NAIP levels in the retina are not altered by optic nerve transection in adult rats.

#### ***Transduction of RGCs after nerve stump application of adenovirus***

Administration of Ad.lacZ directly into the proximal nerve stump immediately following optic nerve transection resulted in the selective

transduction of RGCs (Fig. 5). We found X-gal reactive or  $\beta$ -gal immunoreactive neurons and axons in only the ganglion cell layer (GCL) and nerve fiber layer (NFL) of flatmounted retinas (Fig. 5A-D). Examination of transverse retinal sections revealed that stained cells were indeed restricted to the GCL and were not present in other retinal layers or in retinas not transduced. At 4 days after viral administration, we observed pronounced differences in the staining intensities of X-gal reactive or  $\beta$ -gal immunoreactive RGCs and axons. About half of the RGCs transduced showed complete cytoplasmic filling (Fig 5B; large arrows), while the remainder appeared incompletely stained, and showed punctate cytoplasmic staining (Fig 5B; small arrows) suggesting large differences in the uptake and transport of viral vectors by individual axons. In addition, we did not observe a uniform distribution of stained RGCs in the GCL or axons in the NFL of flatmounted retinas. Some regions had higher densities of transduced RGCs (Fig. 5A and 5C), and some areas appeared totally void of stained somata or axons. We have observed a similar “patchy” pattern of labeling after transduction of RGCs in flatmounted retinas 7 days after stereotaxic injection of Ad.lacZ into the superior colliculus (SC) (van Adel et al., 2000). We observed no significant difference in the number or intensity of stained RGC somata and axons using either Ad.lacZ vector under the control of the RSV promoter (Ad.lacZ) or the CMV promoter (Ad.lacZ<sub>2</sub>). This result was consistent with a previous study that compared  $\beta$ -galactosidase activity in retinas after nerve

injection of plasmid vectors containing either the CMV or RSV promoter linked to the lacZ gene (Garcia-Valenzuela et al., 1997). Regardless of the promoter, nerve injection of Ad.lacZ resulted in the transduction of 5% of the normal population of RGCs. It is possible that we have underestimated the total number of transduced cells since we examined retinas 4 days post-axotomy/post-injection. This time point was selected in order to allow for histological examination of retinas prior to any RGC death (Koeberle and Ball, 1998). In addition, there is a delay in retrograde transport of the virus and transgene expression (Cayouette and Gravel, 1996; Kügler et al., 1999). Therefore, the large number of faintly stained cells suggests that it is possible that we did not detect the total number of transduced RGCs.

#### ***Administration of vectors encoding NAIP at the optic nerve stump rescues axotomized RGCs***

Adenoviral vectors encoding NAIP were injected into the optic nerve stump immediately following nerve transection and RGC survival was measured at 14 days post-axotomy and compared to retinas treated with Ad.XIAP, Ad.CNTF, Ad.lacZ, or Ad.lacZ<sub>2</sub>. Firstly, there was no significant difference in the number of surviving RGCs using either Ad.lacZ, or Ad.lacZ<sub>2</sub> as a control vector. We therefore pooled the data from both control groups and are subsequently presented as the Ad.lacZ group. Secondly, nerve stump injection of the control vector, Ad.lacZ (Fig. 6A) did not increase the number of surviving RGCs compared to untreated retinas at 14 dpa (Fig. 2D). The density of D-FITC

labeled cells in the Ad.lacZ group decreased to  $389 \pm 55$  RGCs/mm<sup>2</sup> (mean±S.D.) from the initial population density of  $2381 \pm 102$  RGCs/mm<sup>2</sup>. In contrast, injection of Ad.NAIP (Fig. 6B) into the optic nerve stump significantly enhanced RGC survival ( $744 \pm 127$  RGCs/mm<sup>2</sup>) compared to control injections at 14 days following optic nerve transection (Fig 6B). This difference represents a 15% increase in neuronal survival in Ad.NAIP treated retinas compared with Ad.lacZ. Similarly, nerve stump injection of Ad.XIAP (Fig. 6C) and Ad.CNTF (Fig. 6D) resulted in an increase in RGC survival at 14 dpa of 16% and 22% respectively. However, there was no significant difference between Ad.NAIP, Ad.XIAP or Ad.CNTF treatments (Fig. 6E). In addition to an increase in neuronal survival in retinas treated with nerve injections of Ad.NAIP, Ad.XIAP, or Ad.CNTF there was a corresponding decrease in the number of transcellular stained microglia compared to the control retinas.

***Effects of overexpression of NAIP on nerve fiber layer integrity after axotomy***

The integrity of axotomized RGC axons in the nerve fiber layer of the retina was examined using immunohistochemistry directed against the 200-kDa neurofilament protein, RT97 (Anderton et al., 1982). In normal retinas, RT97 stained small and large bundles of RGC axons in the nerve fiber layer (Fig. 7A). In contrast, axotomized retinas stained at 14 dpa with RT97 showed a drastic reduction in axons, and staining of RGC somata (Fig. 7B). The appearance of the remaining axons in axotomized retinas was characterized by varicosities

along their length giving the axons a beaded appearance that is characteristic of degenerating axons. A similar pattern of axonal loss and degeneration was observed in Ad.lacZ treated retinas at 14 days post-axotomy. In comparison, retinas with nerve injection of Ad.NAIP (Fig. 7D), Ad.XIAP (Fig. 7E), or Ad.CNTF (Fig. 7F) at the time of optic nerve transection had less axonal beading and thicker axon fascicles than control retinas at 14 days post-axotomy. Axonal integrity appeared highest in the Ad.CNTF treated retinas, presumably because CNTF has been previously demonstrated to be a survival and axogenesis factor for injured RGCs in vitro (Jo et al., 1999) and in vivo (Cho et al., 1999; Mey and Thanos, 1993; van Adel et al., 2000).

Despite the increased preservation of RGC axons in Ad.NAIP and Ad.XIAP, beaded axons were observed in these retinas. Regardless, axotomized retinas treated with Ad.NAIP, Ad.XIAP or Ad.CNTF had the appearance of thicker and more numerous axon fascicles in the nerve fibre layer, which correlated with increased survival of RGC somata.

**Discussion:**

The purpose of this study was to evaluate the effect of adenoviral-mediated transfer of NAIP on the survival of axotomized RGCs. It was apparent that endogenous responses within the retina are not sufficient to protect the vast majority of severely injured RGCs, as we observed no significant increase in retinal NAIP levels and a loss of 90% of RGCs 14 days after optic nerve transection. In contrast, overexpression of NAIP in axotomized RGCs by application of adenoviral vectors to the optic nerve stump immediately after transection rescued approximately 20% of axotomized RGCs that would have otherwise died. The expression of NAIP in the normal and axotomized retina, as well as the role of apoptosis in RGC death will be discussed below.

***Optic nerve transection is an in vivo model of neuronal apoptosis***

Apoptosis is an active, self-directed process of cell death, whereby an individual cell responds to internal and/or external cues and proceeds to die with characteristic biochemical and cytological features. In the developing brain and retina a high rate of apoptosis is tolerated and even required to model the central nervous system (CNS). However, an increased rate of apoptosis in the adult CNS underlies many neurodegenerative disorders, such as glaucoma, Parkinson's disease and stroke (reviewed in Leist and Nicotera, 1998). In the present study we used optic nerve transection in the adult rat since it has proved to be an excellent *in vivo* model to study mechanisms leading to neuronal apoptosis (Berkelaar et al., 1994, Koeberle and Ball, 1998; Quigley et al., 1995).

More importantly, we are concerned with RGC degeneration/death since this occurs in patients with glaucomatous optic neuropathy, which is one of the world's leading causes of blindness (Levin, 2001; Osborne et al., 1999). In glaucoma elevated intraocular pressure and ischemia are common stress factors thought to facilitate the demise of retinal ganglion cells. The molecular mechanisms of RGC death remain incompletely defined; as a result, there are currently no effective therapies to prevent visual loss in patients with glaucoma (Osborne et al., 1999). Loss of target derived neurotrophic factors, excitotoxicity, overproduction of free-radicals, mitochondrial inhibition, a shift in the ratio of anti-/pro-apoptotic proteins, as well as caspase 3 and 9 activation have recently been implicated in RGC death, as well as several other chronic neurodegenerative disorders (Leist and Nicotera, 1998; Osborne et al., 1999; McKinnon et al., 2002). In addition to its direct relevance for glaucoma (Levin 2001), transection of the optic nerve is a valuable model for studying degenerative and regenerative events in the mammalian CNS (Vidal-Sanz et al., 1991). Neuronal loss in several neurodegenerative diseases, including spinal muscular atrophy, Parkinson's Disease, and glaucoma involves a final common pathway. Recent studies examining neuronal cell death in various animal models have demonstrated a key role for caspase 3 in the apoptotic process (Gorman et al., 1998; Marks and Berg, 1999; Nicotera, 2000; Nicotera et al., 2000; Robertson et al., 2000). Indeed, caspase-3 activity has been demonstrated to play a decisive role in numerous apoptotic events including neuronal death following axon injury

(Kermer et al., 1998, 2000; Chaudhary et al., 1999; Perrelet et al., 2000, 2002) ischemia (Xu et al., 1997, 1999; Namura et al., 1998), and during CNS development (Urase et al., 1998; Woo et al., 1998). In this study we employed the optic nerve transection model and used retrograde labeling techniques to selectively label RGCs by injecting the fluorescent tracer, dextran-FITC, into the proximal nerve ending at the time of axotomy. We observed no RGC death over the first 4 days post-axotomy, whereas approximately 90% of RGCs died over the ensuing 10 days with the maximum rate of RGC death occurring between 7 and 10 days after optic nerve transection. This time course is consistent with previous reports from other laboratories (Berkelaar et al., 1994; Peinado-Ramon et al., 1996; Klöcker et al., 1997; Kikuchi et al., 2000), as well as our previous study that demonstrated that the temporal pattern, and specifically the delay in RGC death, coincides with the temporal pattern of TUNEL stained nuclei in sections from axotomized retinas (Koeberle and Ball, 1998).

### ***IAPs as endogenous neuroprotectants in the Retina: Implications for glaucoma***

The fate of a cell is controlled by several pro- and anti-apoptotic families of genes, including the inhibitor of apoptosis (IAP) family of proteins, that have been conserved in diverse species ranging from nematodes through mammals and viruses (Miller, 1999). Several lines of evidence suggest that in comparison with other anti-apoptotic proteins, such as p35 and CrmA, IAPs protect against the broadest spectrum of apoptosis signals (Miller, 1999; Marks and Berg, 2000).



Members of the IAP family, including NAIP, possess neuroprotective properties capable of rescuing neurons from various insults *in vitro* and *in vivo*. Recent *in vitro* data indicate that BIR2 domain of NAIP is a direct inhibitor of caspase 3 and 7 (Maier et al., 2002), which provides a mechanism for NAIP's neuroprotective properties using *in vivo* animal models where caspases-3 dependent apoptosis has been established (Xu et al., 1997a; Crocker et al., 2001; Perrelet et al., 2000, 2002). Indeed, overexpression of NAIP has been shown to protect neurons from ischemic and axotomy-induced apoptosis (Xu et al., 1997; Perrelet et al., 2000). Likewise, elevated levels of NAIP may protect neurons from damaging free radicals and may prevent cell death in Parkinson's disease (Crocker et al., 2000). Moreover, the presence of endogenous IAPs, such as NAIP, in the mammalian CNS may increase neuronal tolerance to severe injurious insults like ischemia and excitotoxicity (Holcik et al., 2000), which have been associated with several neurodegenerative disorders, including glaucoma. Conversely, a decrease in NAIP expression may have negative effects on motoneuron survival, such as in SMA (Roy et al., 1995).

High levels of NAIP have been demonstrated in the retinas of developing mouse embryos (Ingram-Crooks et al., 2002) and in normal adult rats (Xu et al. 1997b). During retinal development NAIP most likely functions as an important regulator protein during natural occurring programmed cell death, however, the exact role of NAIP in the adult retina remains unclear. In this study, we used immunohistochemistry and quantitative RT-PCR to examine the expression of

NAIP in the normal and axotomized retina. We first compared the expression of NAIP in the normal adult retina with XIAP, which has previously been characterized in this tissue (Kügler et al., 1999). The expression of NAIP was found in all layers of the retina with the most intense labeling found in the photoreceptor outer segments. We observed a very similar pattern of labeling using an antibody against XIAP, suggesting that these two IAPs possibly work in concert to suppress against the sporadic activation of caspases and thus prevent apoptosis in adult retinal neurons. We then used real-time (quantitative) PCR and examined NAIP expression at several different time points ranging from as early as 1 hour to 14 days after optic nerve transection. We observed no significant change in NAIP expression in axotomized retinas at any time interval examined in this study. This result was surprising since previous studies have reported injury-induced alterations in NAIP expression (Xu et al., 1997a; Hutchison et al., 2001, Perrelet et al., 2002). Although we used a sensitive and quantitative method (Taqman) to measure changes in mRNA levels, it is possible that we did not detect any changes in NAIP levels after axotomy since RGCs represent less than 5% of the total population of retinal neurons. It is tempting to propose that the lack of NAIP induction in this paradigm may partly explain the increased vulnerability of RGCs to apoptosis between 4-14 days post-axotomy. On the other hand, the delay in RGC death may result from endogenous NAIP levels in the retina being sufficient to protect axotomized RGCs within the first few days following ON transection. Consistent with this hypothesis, it has been

demonstrated that the activation of caspases in this model are delayed for 3 days and peak at 7 days post-axotomy (Kermer et al., 1999, 2000; Chaudhary et al., 1999). Moreover, it has also been shown that Bcl-2 and bcl-xl levels decrease and Bax levels begin to increase as early as one day after axotomy (Isenmann et al., 1997; Chaudhary et al., 1999). The lack of NAIP induction after axotomy may contribute to a mismatch in the ratio of intracellular anti-/pro-apoptotic factors, and thus may have lead to the induction and execution of apoptosis in axotomized RGCs at survival times of 4 days and beyond. It has previously been suggested that a reduction in NAIP levels after sciatic nerve axotomy in rats (Perrelet et a., 2002), or traumatic brain injury in mice (Hutchison et al., 2001), renders these CNS neuronal populations more likely to degenerate and die by apoptosis. Taken together, these results suggest that a shift in the concentration of anti-apoptotic and pro-apoptotic factors after optic nerve transection may decrease the chance for cell survival. The critical time of 4 days post-axotomy may represent a point where axotomized RGCs are committed to die by apoptosis. Further experiments are required to determine the precise role of endogenous NAIP expression in the normal and axotomized retina.

### ***Nerve Stump application of Ad.NAIP protects axotomized RGCs***

Since endogenous levels of NAIP are unchanged after ON transection, we hypothesized that overexpression of NAIP may protect axotomized RGCs. This seems likely since NAIP is a potent inhibitor of caspase 3 (Maier et al., 2002) and axotomy-induced RGC apoptosis has been shown to be caspase 3 dependent

(Kermer et al., 1998; Chaudhary et al., 1999). To test this hypothesis, we investigated the neuroprotective effects of adenoviral-mediated overexpression of NAIP on axotomized RGCs. NAIP was overexpressed in axotomized RGCs by directly injecting an adenoviral vector encoding the gene for NAIP into the nerve stump at the time of injury. Furthermore, we simultaneously applied the retrograde fluorescent tracer (Dextran-FITC) to the viral vector suspension so that we could quantify the total number of surviving RGCs at 14 days post-axotomy. We first showed that nerve injection of a control vector carrying the *lacZ* gene resulted in the selectively transduction of RGCs. Nerve injection of adenoviral vectors transduced less than 5% of the normal population of RGCs, a result that was consistent with similar studies employing nerve stump delivery of adenoviral vectors in this model (Kugler et al., 1999, 2000; Weise et al., 2000b; Koeberle et al., 2002). In addition to the low rate of RGC transduction after nerve injection of adenoviral vectors, we found that expression of the reporter transgene, *lacZ* was not long-lasting which was not surprising for type 5 adenoviral vectors containing *lacZ* as a control transgene (Easton et al., 1998; Kugler et al., 1999; Isenmann et al., 2001). We have reported similar short-term transgene expression using the same Ad.*lacZ* vector after intraocular injection (van Adel et al., 2000). Moreover, raising the viral titre does not increase the number of transduced cells or the duration of transgene expression, but merely increases the intensity of  $\beta$ -gal activity in transduced cells (Perrelet et al., 2000). Normally, adenoviral vectors gain access and integration into a cell via binding to

the cell surface coxsackie and adenoviral receptor, CAR (Barnett et al., 2002). It is possible that viral entry after nerve injection is a result of mechanical force or occurs via gross endocytosis since intraocular injection of the same adenoviral vector does not transfect RGCs, but rather is selective for retinal glia, with the greatest affinity for Müller cells (Di polo et al., 1998; van Adel et al., 2000; Weise et al., 2000). Application of Ad.NAIP to the nerve stump resulted in a 2 to-3 fold increase in RGC survival 14 days after ON transection. Similar results were obtained when using an adenovirus encoding XIAP, which has previously been shown to protect equivalent numbers of axotomized RGCs (Kügler et al., 2000). We did not expect to see differences in the survival promoting effects of NAIP and XIAP, since these two IAP family members have similar inhibitor kinetics for caspase 3 (Maier et al., 2001). Thus, these results further support a role for NAIP in regulating caspase 3-dependent neuronal apoptosis *in vivo*.

We also applied an adenoviral vector coding for the neurotrophic factor, CNTF, to the cut nerve stump. This was done to determine whether this neurotrophic factor might be more effective in RGC rescue than intracellular factors, such as NAIP and XIAP. Since CNTF lacks a conventional signal sequence, the vector used in this study contained an NGF leader sequence, permitting the CNTF protein to be secreted from transduced RGCs (Gravel et al., 1997). We previously demonstrated that intraocular injection of the Ad.CNTF vector selectively transduced retinal Müller cells and secretion of CNTF from transduced glia rescued approximately 50% of axotomized RGCs (van Adel et

al., 2000). In this study, nerve stump application of the Ad.CNTF vector was less effective than intraocular treatment, and no more efficient at protecting axotomized RGCs than treatment with either the Ad.NAIP or Ad.XIAP vectors, both of which code for intracellular proteins. Although the mechanisms of neuroprotection and anti-apoptotic properties afforded by CNTF (Inoue et al., 1996) are not as well-defined as NAIP or XIAP (Deveraux et al., 1999; Maier et al., 2001, Perrelet et al., 2002), the result was surprising since CNTF has recently been shown to protect neurons via directly acting on injured retinal neurons as well as indirectly through modulation of retinal glia (Jo et al., 1999; Peterson et al., 1999; Li et al., 2001; van Adel et al., 2001). In a similar study, nerve stump application of adenoviral vectors encoding NAIP, HIAP 1 and HIAP2 were shown to be as effective in motoneuron rescue as application of adenoviral vectors encoding the secreted forms of the neurotrophic factors BDNF and CNTF (Perrelet et al., 2000). Since very few RGCs were transduced after nerve stump application of Ad.CNTF, it is possible that not enough CNTF was present at any one time to rescue the same proportion of cells as seen following intraocular administration of Ad.CNTF (van Adel et al., 2000; Weise et al., 2000). This is likely, considering that nerve injection of Ad.CNTF protected about the same number of axotomized RGCs as a single intraocular injection of 4  $\mu$ g of recombinant CNTF (van Adel et al., 2002). Indeed, single or multiple intravitreal treatment(s) of CNTF below 1  $\mu$ g does not protect axotomized RGCs (Cho et al., 1999; Cui et al., 1999). Moreover, the CNTF protein has a half-life of only 1.5

minutes (Sendtner et al., 1997) and a single injection of relatively small amounts of rhCNTF or CNTF production from relatively few adenovirally-transduced RGCs may not have been sufficient to protect a large percentage of axotomized RGCs.

Interestingly, elevation of retinal NAIP, using adenoviral vectors also attenuated the loss of RGC axons in the nerve fiber layer of the retina. We expected to see differences in axon preservation with CNTF compared to NAIP or XIAP, since CNTF has previously been demonstrated to be a potent axogenesis factor for RGCs both in vitro (Jo et al., 1999) and in vivo (Cui et al., 1999). In support of the findings of this study, it was recently shown that adenoviral-mediated expression of NAIP decreased the loss of dopamine fibers in the rat striatum in a 6-OHDA model of Parkinson's Disease (Crocker et al., 2001). Our findings suggest that overexpression of NAIP may postpone the execution of apoptosis in axotomized RGCs by direct inhibition of caspase 3, and increased RGC responsiveness to endogenous trophic factors, such as CNTF, which has been shown to be up-regulated after optic nerve transection (Chun et al., 2000). Likewise, the delay in apoptosis afforded by NAIP treatment may allow additional interventions to further suppress cell death and promote regeneration of injured axons, and ultimately functional recovery. Further studies are required to determine the therapeutic window for and the amounts and the duration of NAIP treatment required to sufficiently inhibit apoptosis in axotomized RGCs. Moreover, the use of different viral vectors encoding NAIP, such as Adeno-associated and lentivirus, that have been shown to selectively transduce

the vast majority of RGCs after intraocular treatment, may increase the survival of RGCs (Dreyer et al., 1999; van Adel et al., 2002).

**Conclusion:**

The results of the present study showed that NAIP is expressed in the normal retina and its expression is unchanged after optic nerve transection. We have also shown for the first time that adenoviral mediated gene transfer of NAIP to the retina enhances the survival of axotomized RGCs *in vivo*. Adenoviral injection into the transected optic nerve stump increased RGC survival at 14 days post-axotomy rescuing approximately 2-3 fold more RGCs than control treatments. It is well known that the final common pathway in glaucoma is RGC death, thus a strategy to protect RGCs by inducing/increasing NAIP using pharmacological interventions may widen the field of glaucoma treatment.



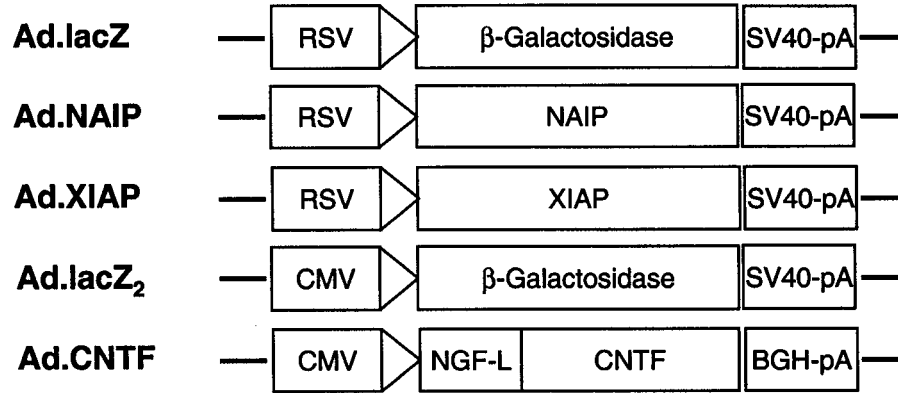
## **Acknowledgements**

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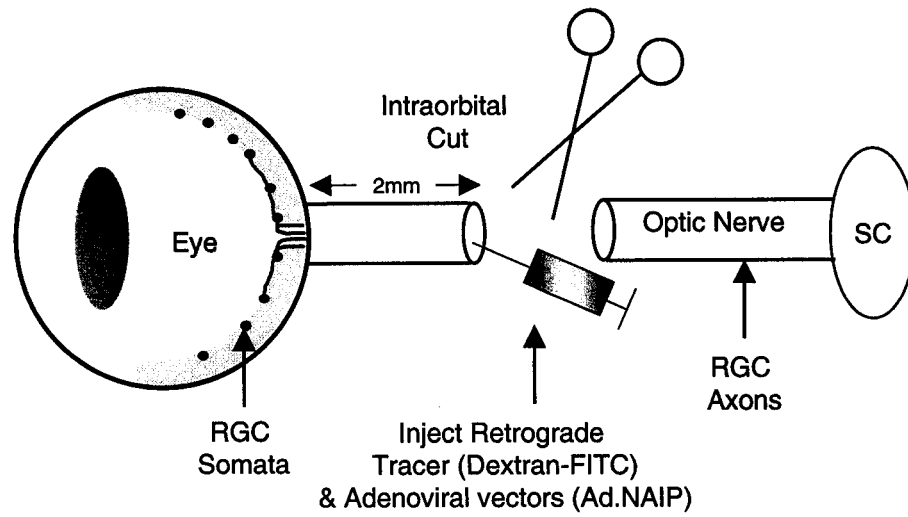
### Figure Legends:

**Figure 1. A:** Schematic representation of the replication-deficient, adenoviral vectors used in this study. The genome of the vectors were based on the *d/309* adenovirus type 5 mutant and functional elements are shown in detail: coding regions for human NAIP, XIAP or the bacterial enzyme *Escherichia coli*  $\beta$ -galactosidase fitted with a SV40 polyadenylation signal (SV40-pA) under the transcriptional control of rous sarcoma virus (RSV) promoter. The CNTF vector contains a nerve growth factor signal sequence-*cntf* chimeric gene (NGF-L CNTF) fitted with a bovine growth hormone polyadenylation signal (BGH-pA) and is driven by a cytomegalovirus (CMV) promoter. The Ad.lacZ<sub>2</sub> vector, is a control for the Ad.CNTF vector and was construct essentially the same with the deletion of the NGF-L sequence and the BGH-pA was replaced with an SV40 polyadenylation signal. **B:** Illustration of the surgical procedures used for optic nerve transection and co-application of viral vectors and retrograde tracer (D-FITC) to the proximal ending of the transected optic nerve. **C:** Experimental design used to test the effects of adenoviral-mediated transfer of NAIP on the survival of axotomized RGCs *in vivo*.

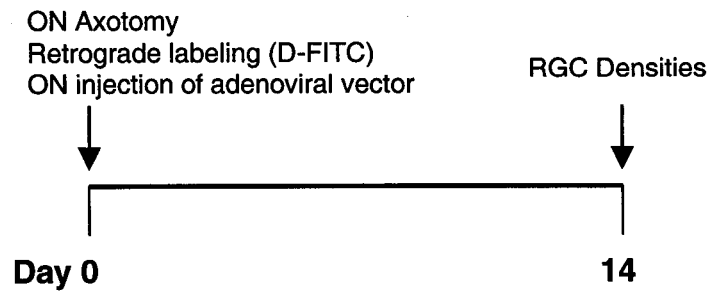
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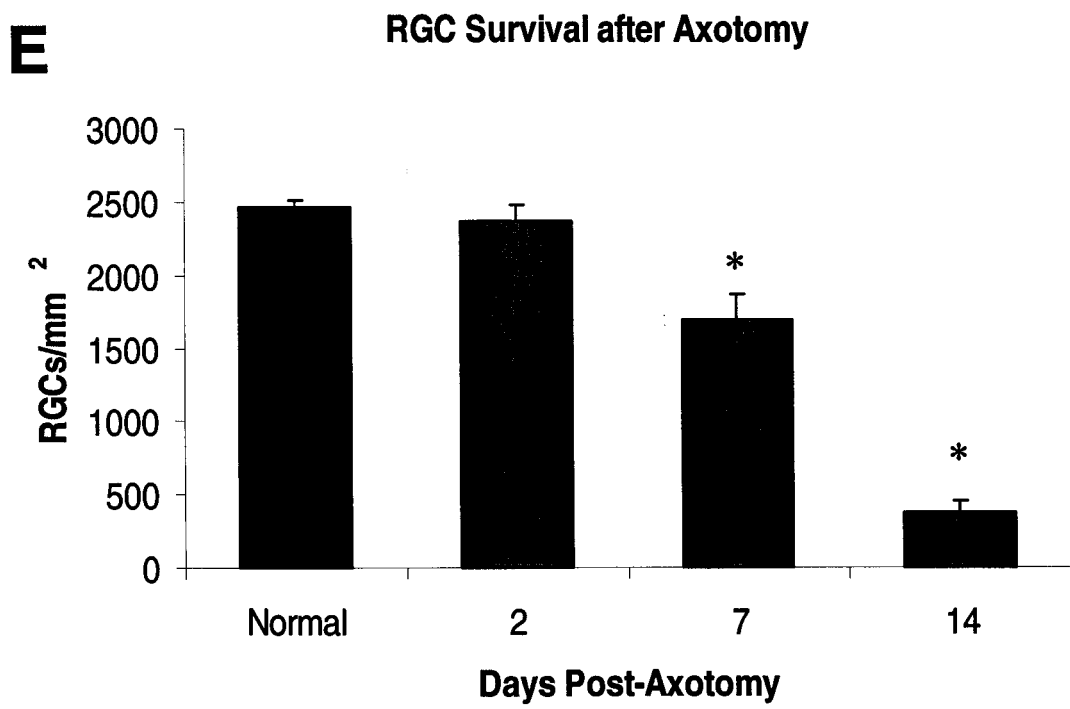
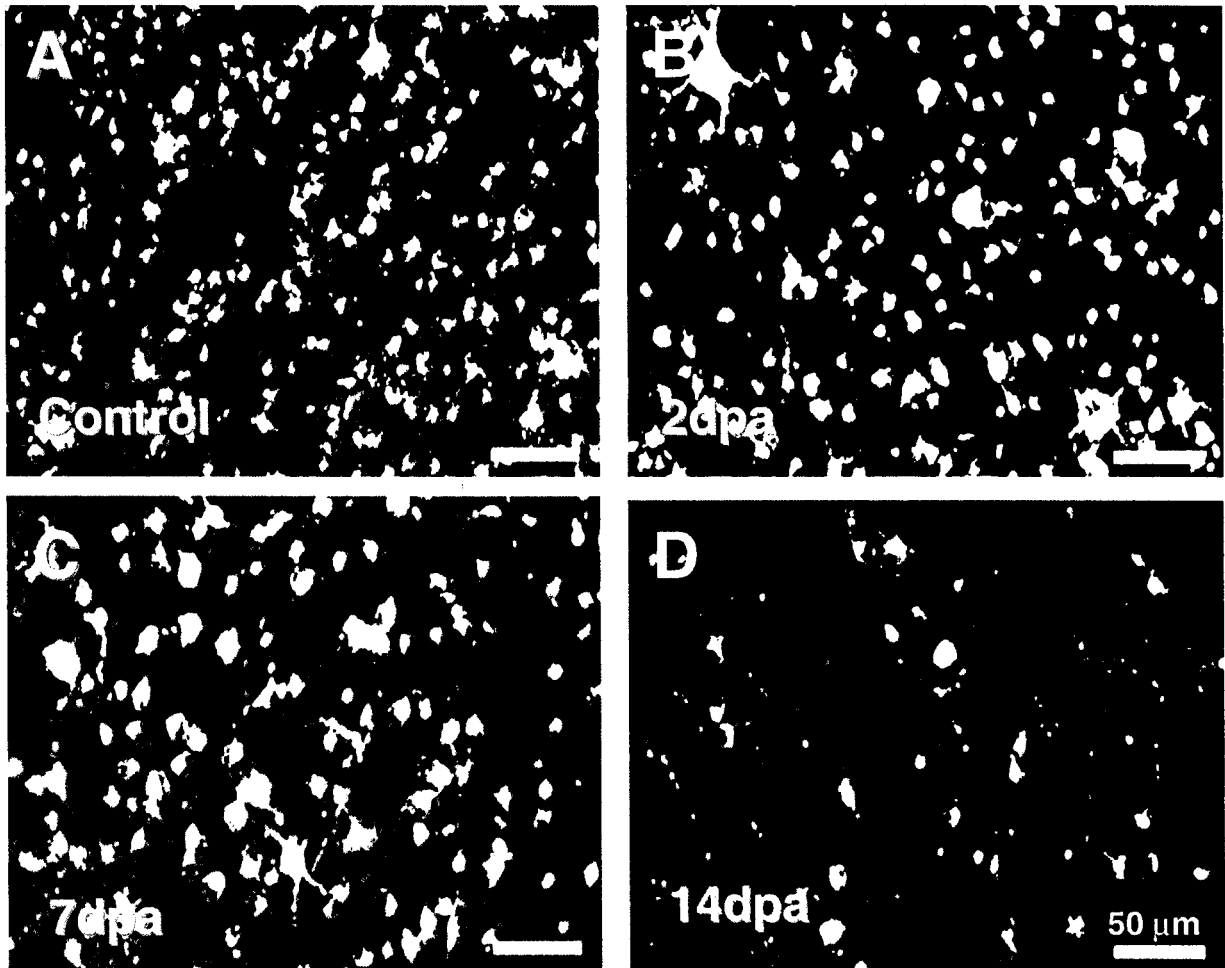
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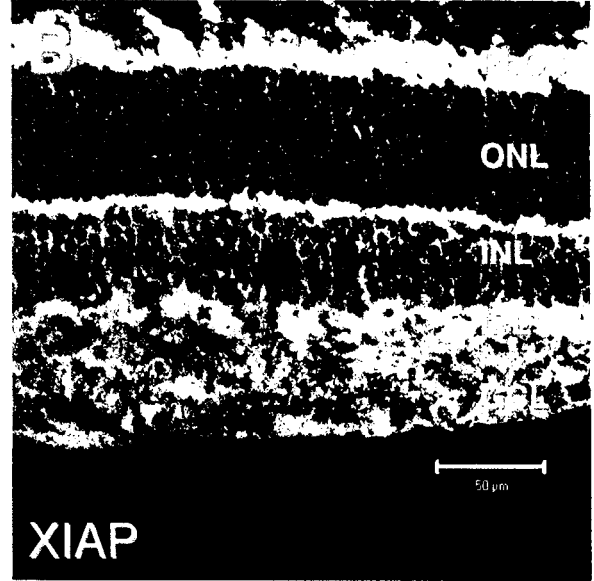
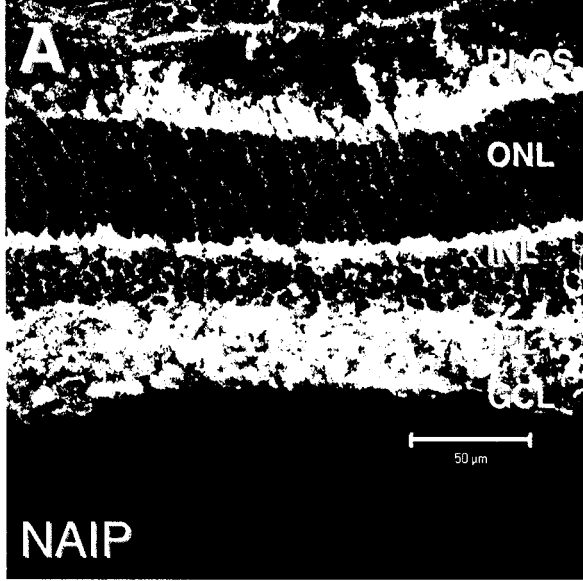
**C**



**Figure 2.** Time-course of retinal ganglion cell death after complete intraorbital optic nerve transection 2 mm from the eye. Confocal micrographs showing Dextran-FITC retrogradely labeled retinal ganglion cells in flatmounted retinas from a normal animal and at 2, 7, and 14 days after optic nerve transection. **A:** normal population of RGCs labeled in the rat retina after injection of D-FITC into the contralateral superior colliculus. **B:** no significant loss of RGCs at 2 dpa after injection of D-FITC into the proximal ending of the optic nerve. **C:** A noticeable loss of RGCs at 7 days post-axotomy and the appearance of transcellularly labeled microglia that have small cell bodies and ramified processes. **D:** only a few viable RGCs at 14 dpa and numerous transcellularly labeled microglia. **E:** Graph showing a significant decrease ( $p < 0.001$ ) in RGC densities (mean  $\pm$  standard deviation) in flatmounted retinas at 7, and 14 dpa. Bar = 50  $\mu\text{m}$ .



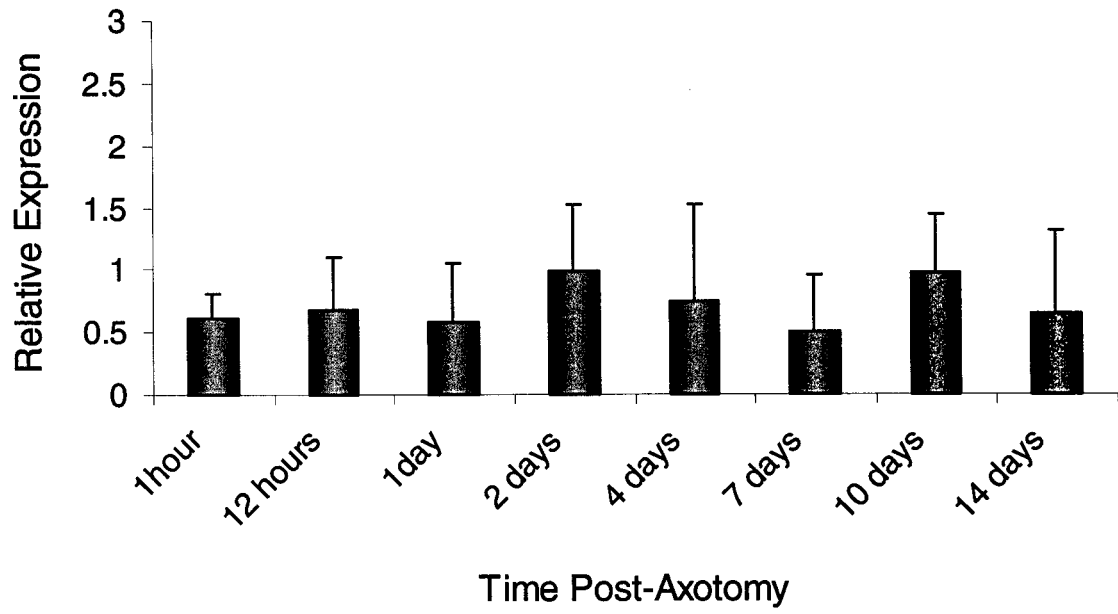
**Figure 3.** Confocal micrographs of NAIP (A) and XIAP (B) immunohistochemistry in transverse retinal sections. (A) Neuronal perikarya and processes were moderately labeled in all retina layers including, the ganglion cell layer (GCL), the inner nuclear layer (INL), the outer nuclear layer (ONL) and relatively intense staining of the photoreceptor outer segments (PhOS). (B) a similar pattern of labeling was observed in retinal sections analyzed for XIAP immunoreactivity. Bar = 50  $\mu$ m.



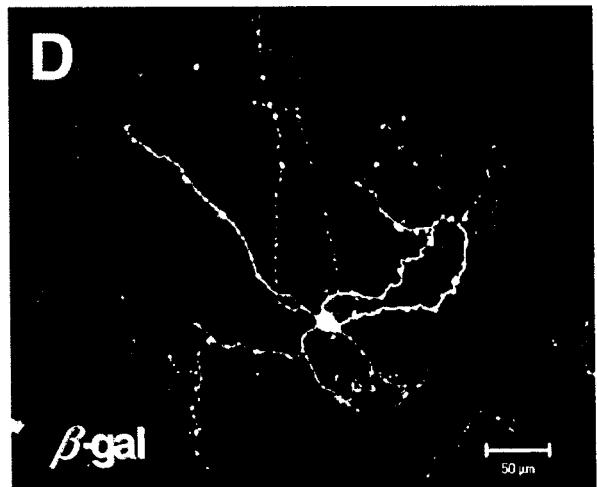
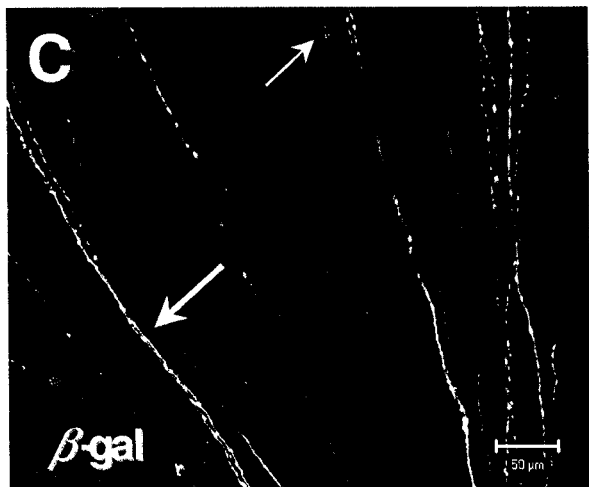
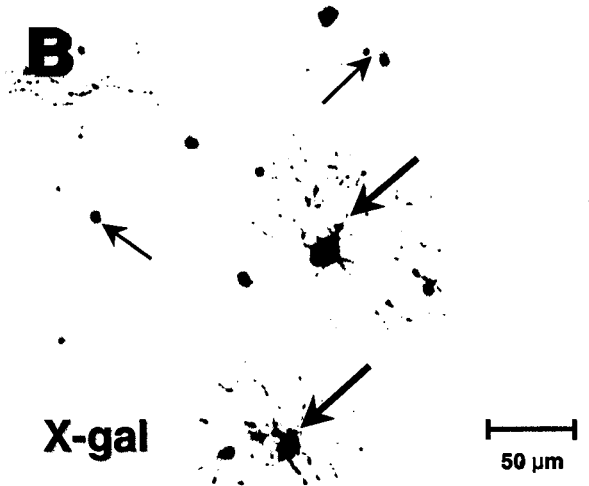
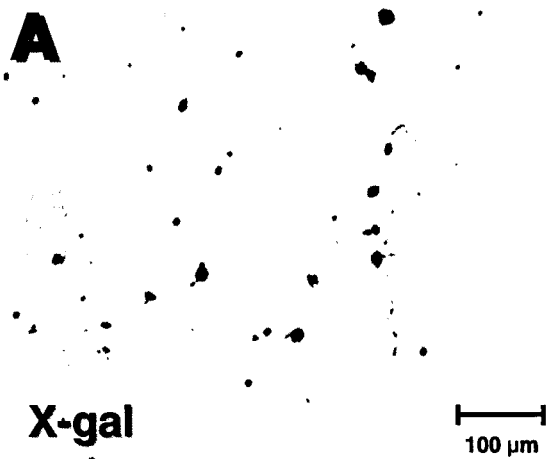
**Figure 4.** NAIP expression was not significantly changed in axotomized retinas examined at 1, and 12 hours, and 1, 2, 4, 7, 10 and 14 days post-axotomy. NAIP and GAPDH mRNA levels were assessed by quantitative multiplex RT-PCR using the ABI 7700 system (Applied Biosystems Inc.). NAIP expression in each sample is normalized to GAPDH mRNA levels. Average of normalized NAIP levels in retinas 1 hour post-axotomy is arbitrarily fixed at 1. Results are presented as relative expression of normalized NAIP levels. Each column is the average of a minimum of three separate samples. (hpa = hours postaxotomy, dpa = day(s) postaxotomy).



### NAIP Expression after ON Transection



**Figure 5.** Confocal and transmitted light micrographs showing the selective transduction of retinal ganglion cells by nerve stump application of an adenoviral vector encoding the bacterial enzyme *Escherichia coli*  $\beta$ -galactosidase. **A:** Retinal flatmount showing x-gal positive RGCs, 4 days after nerve injection of Ad.lacZ. **B:** high power photomicrograph of an X-gal stained retina showed a mixture of labeled cells; larger RGCs (large arrows) showed complete cytoplasmic and dendritic filling, and smaller RGCs had punctate cytoplasmic staining (small arrows). **C:** Beta-galactosidase immunoreactivity in a flatmounted retina 4 days after nerve injection of Ad.lacZ.



**Figure 6.** Neuroprotective effect of adenoviral-mediated transfer of NAIP, XIAP, CNTF or  $\beta$ -galactosidase ( $1.4 \times 10^{10}$  pfu/ml) on RGC survival at 14 days post-axotomy (dpa) in adult rats. The recombinant adenoviral vectors were co-administrated with D-FITC to the proximal nerve ending immediately following optic nerve transection. The number of surviving RGCs in each group was compared with the control treated with D-FITC alone. **A:** very few RGCs survived in Ad.lacZ treated retinas. **B:** Numerous RGCs were survived axotomy in retinas treated with Ad.NAIP. **C:** Similar protective effects were seen in Ad.XIAP treated retinas. **D:** Interestingly, similar numbers of axotomized RGCs were protected with Ad.CNTF treatment. **E:** Graph showing a significant increase ( $p < 0.001$ ) in the number of surviving RGCs (mean  $\pm$  standard deviation) in flatmounted retinas at 14 dpa in Ad.NAIP, Ad.XIAP and Ad.CNTF treated eyes compared to controls. Note: there was no significant difference between RGC survival in Ad.NAIP, Ad.XIAP or Ad.CNTF treated retinas.

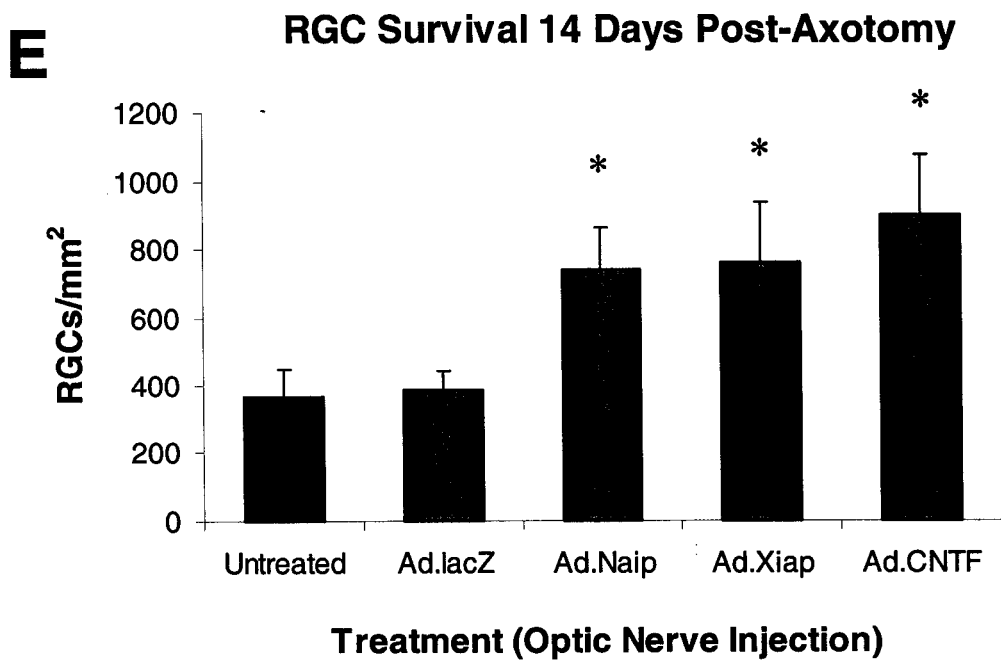
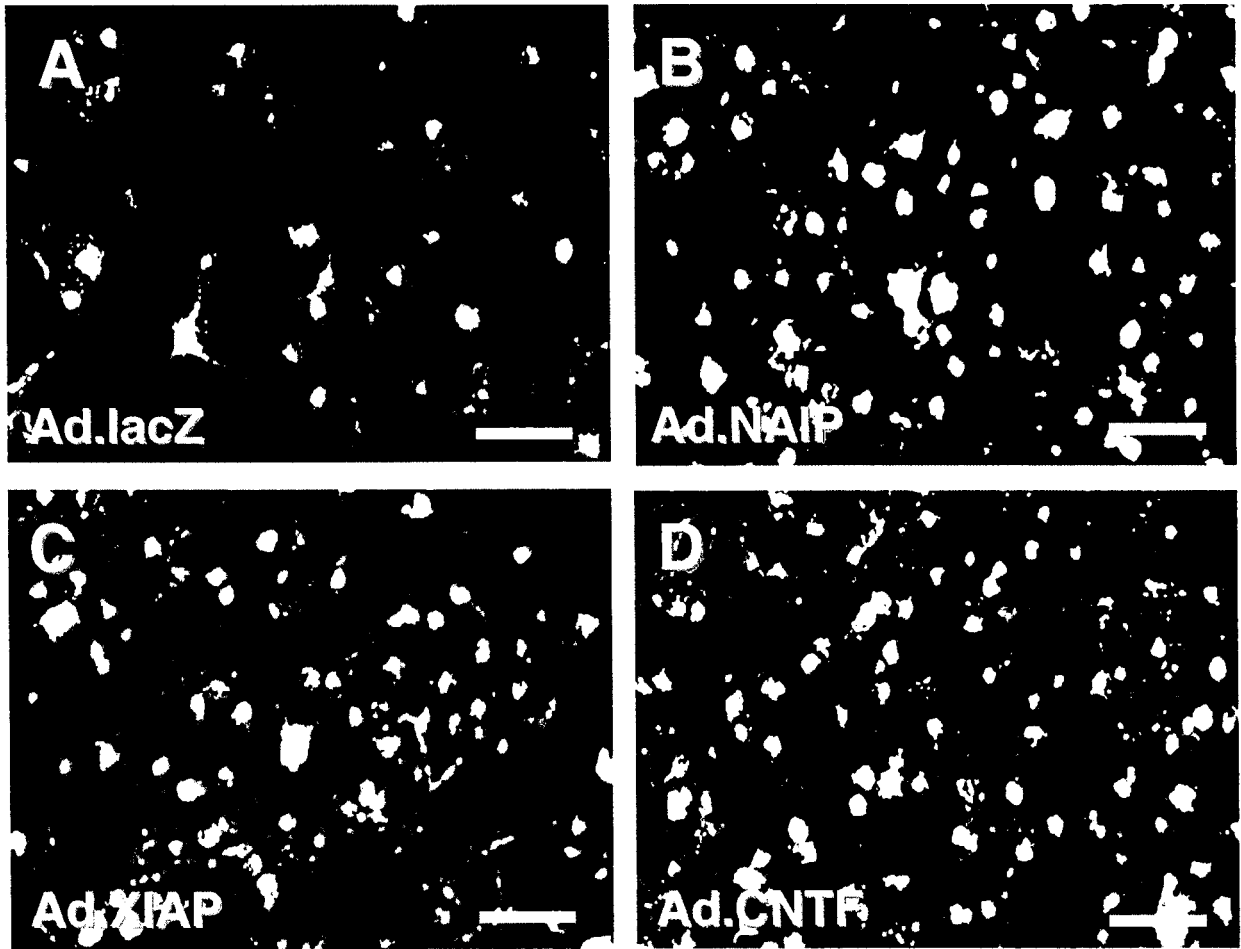
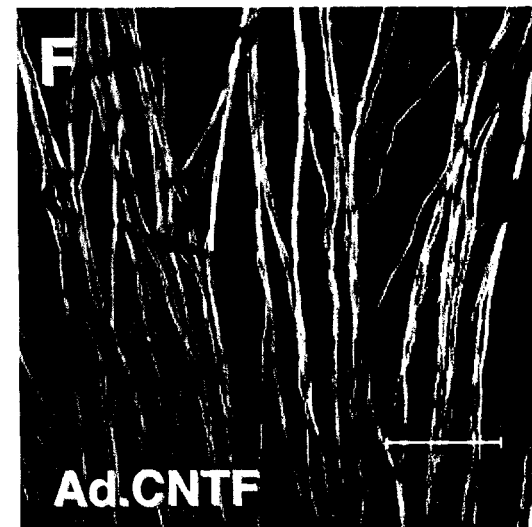
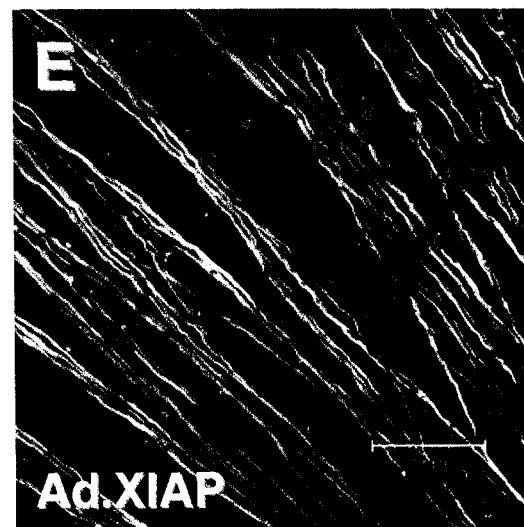
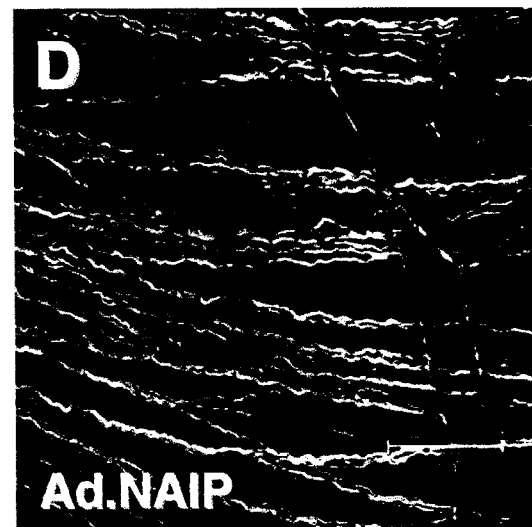
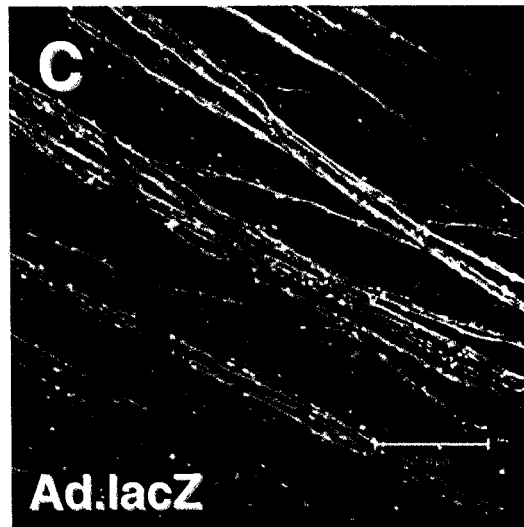
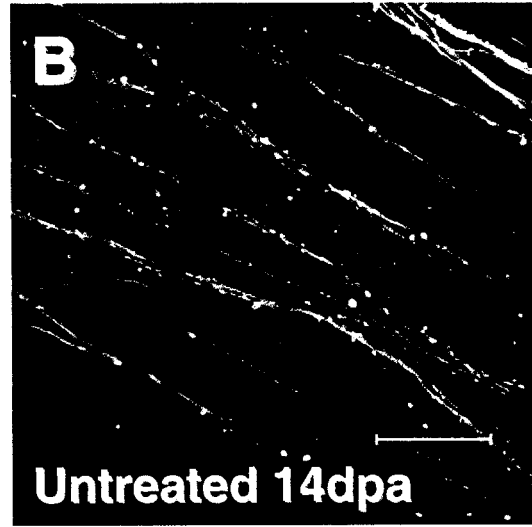
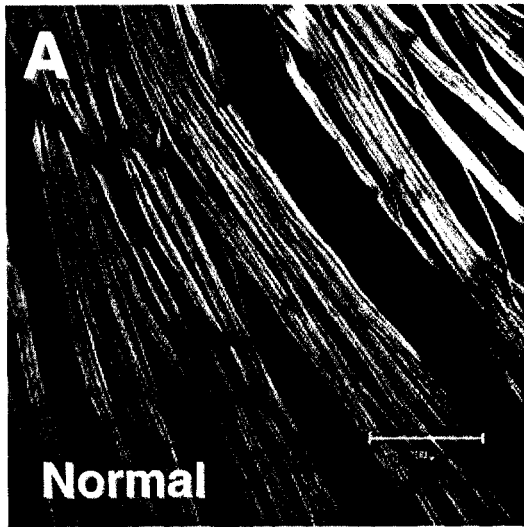
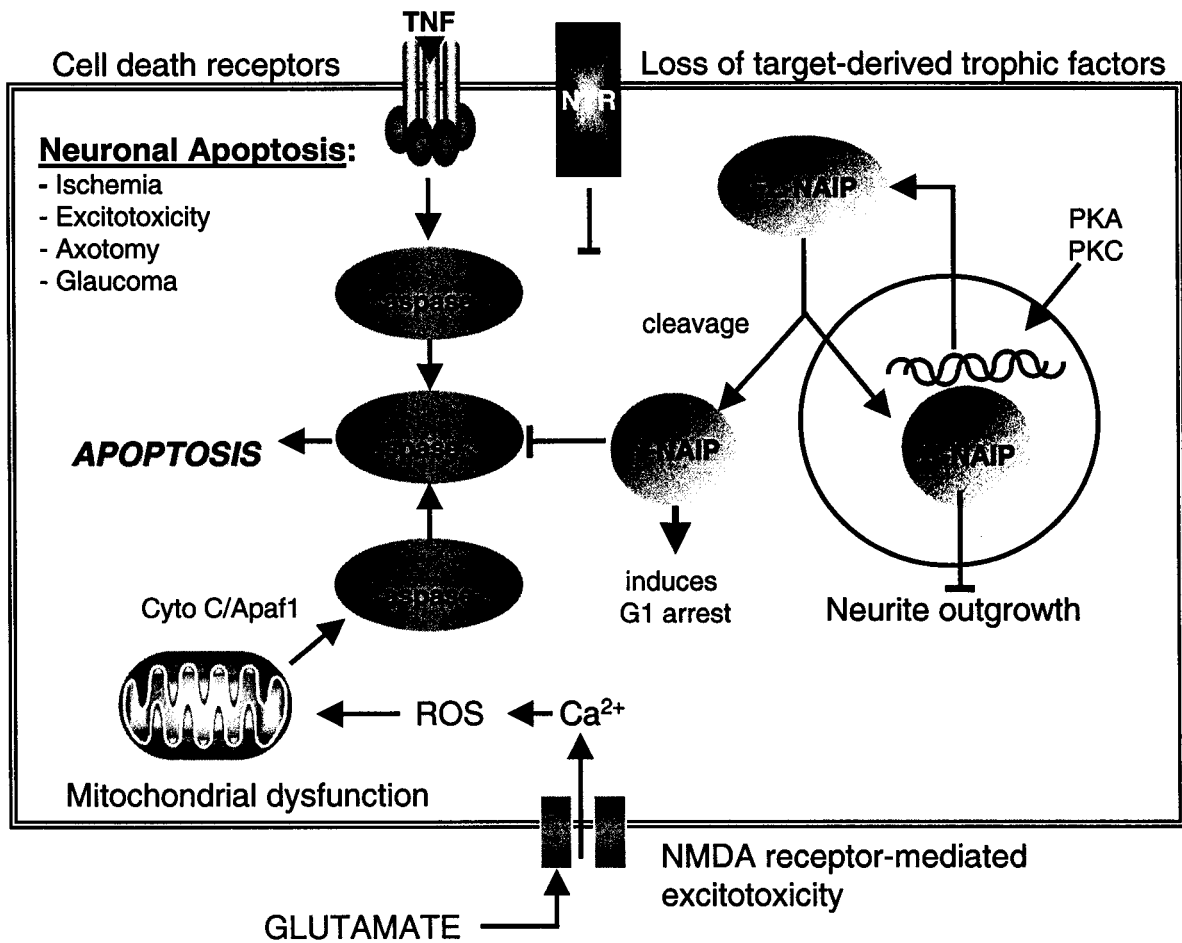


Figure 7. Confocal micrographs of RT97 immunohistochemistry in flatmounted retinas. **A:** RT97 immunoreactive RGC axons in a normal retina. **B:** drastic reduction in RGC axons at 14 days post-axotomy. Note the significant axonal beading (arrows), characteristic of axonal degeneration. **C:** Nerve injection of Ad.lacZ did not preserve RGC axons. **D, E and F:** Significantly more axons were observed 14 days postaxotomy in retinas with nerve injection of Ad.NAIP (D), Ad.XIAP (E) and Ad.CNTF (F).



**Figure 8.** Schematic representation of an axotomized RGC to illustrate the neuroprotective properties of NAIP on injured CNS neurons. Axotomy-induced stress factors, such as glutamate excitotoxicity initiate a cascade of events that lead to the activation of caspase-9 and caspase-3 that ultimately leads to the demise of the cell. NAIP is able to inhibit caspase 3 activation and thus block the apoptotic cascade and in concert with several factors and possible reverse the process and promote cell survival.





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## **CHAPTER 3**

### **Experiment II**

**Delivery of Ciliary Neurotrophic Factor via Lentiviral-Mediated Transfer Protects Axotomized Retinal Ganglion Cells for an Extended Period of Time.**

#### **Contributions:**

B. A. van Adel: optic nerve transection, intraocular injections, retrograde labeling, immunohistochemistry, histochemistry, data analysis, and preparation of the manuscript for publication (Human Gene Therapy).

Dr. Corinne Kostic: viral vector production and purification

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Delivery of Ciliary Neurotrophic Factor via Lentiviral-Mediated Transfer Protects

Axotomized Retinal Ganglion Cells for an Extended Period of Time.

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**Keywords:** retinal ganglion cell, axotomy, retina, ciliary neurotrophic factor, lentiviral vector, gene therapy, optic nerve transection, cell death, apoptosis

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**Running Head:** Protection of axotomized RGCs by Lentiviral-delivery of CNTF

## **Abstract**

Ciliary neurotrophic factor (CNTF) has recently been demonstrated to be one of the most promising neurotrophic factors to improve both the survival and regeneration of injured retinal ganglion cells (RGCs) (Weise et al., 2000). In the present study, we used optic nerve transection as an *in vivo* model to evaluate the effectiveness of a self-inactivating, replication deficient, lentiviral-mediated transfer of human ciliary neurotrophic factor (Lv-CNTF) on the survival of axotomized adult rat RGCs. Counts of Dextran-FITC retrogradely labeled RGCs revealed that the percentage of RGCs was drastically reduced (<90% cell death) 21 days after optic nerve transection. Retinal sections stained with X-gal revealed that intravitreal injection of the control LacZ-expressing lentiviral vector (LV-LacZ) resulted in the transduction of RGCs and retinal pigment epithelium (RPE) cells. A single intravitreal injection of LV-CNTF at the time of axotomy significantly enhanced RGC survival at 14 and 21 days post-axotomy compared to controls. These results demonstrate for the first time that rapid and prolonged delivery of CNTF using lentiviral-mediated gene transfer to the retina is an effective treatment for rescuing axotomized RGCs for an extended period of time. These results suggest that early and continuous administration of CNTF could serve as a potential treatment for retinal disorders involving optic neuropathy and RGC injury such as in glaucoma.

## Overview Summary

Glaucoma is a progressive optic neuropathy that leads to the degeneration of retinal ganglion cells (RGCs) and thus permanent visual field loss. Several lines of evidence suggest that RGC death may be prevented by the continuous supply of neurotrophic factors. However, repeated injections into the eye can cause severe damage. In this study we took advantage of the rapid and prolonged transgene expression afforded by lentivirus to delivery the gene for human ciliary neurotrophic factor (CNTF) to the injured retina. We demonstrated that a single intraocular injection of a lentivirus encoding CNTF immediately after optic nerve injury significantly increased RGC survival at 14 and 21 days post-axotomy compared to a control vector encoding  $\beta$ -galactosidase. To our knowledge this is the first report of extended RGC survival with a single treatment strategy at the time of injury, and the first study to evaluate the neuroprotective effects of lentiviral vectors using the model of optic nerve transection.

## Abbreviations

$\beta$ -Gal:	beta-galactosidase
CNTF:	ciliary neurotrophic factor
CNTR $\alpha$ :	CNTF receptor alpha
dpa:	days post-axotomy
D-FITC:	Dextran-FITC
GCL:	ganglion cell layer
INL:	inner nuclear layer
IO:	intraocular
LacZ:	cDNA coding for beta-galactosidase
LIF:	leukemia inhibitory factor
LIFR $\beta$ :	LIF receptor beta
LV:	lentivirus
NFL:	nerve fiber layer
pfu:	plaque forming units
PGK:	phosphoglycerate kinase 1
PGK-CNTF:	lentivirus encoding human CNTF
PGK-LacZ:	lentivirus encoding beta-galactosidase
ON:	Optic nerve
RGC(s):	retinal ganglion cell(s)
SC:	superior colliculus
SIN:	self-inactivating
x-gal:	5-bromo-4-chloro-3-indoyl- $\beta$ -D-galctopyranoside
WPRE:	woodchuck post-transcriptional regulatory element



## **Introduction**

The retina detects light, processes spatial and temporal aspects of vision, and relays this information to the visual centers via the optic nerve (ON). Glaucoma is one of the leading causes of blindness and is characterized by a progressive optic nerve damage and neuronal cell loss (Levin, 2001). Because the optic nerve is a central nervous system (CNS) white matter tract, injury to RGC axons ultimately leads to the selective death of retinal ganglion cells (RGCs) in the inner retina and failure to regenerate the injured axon. In glaucoma, elevated intraocular pressure and ischemia are two stress factors that are believed to contribute to the demise of RGCs. Since the exact molecular mechanisms of RGC death remain poorly defined there are currently no effective therapies to prevent visual loss in patients with severe glaucoma or acute trauma to the optic nerve (Osborne et al., 1999).

Transection of the adult rat optic ON has proven to be a valuable in vivo animal model to investigate mechanisms leading to RGC death and for screening the effects of neuroprotective compounds on the survival of axotomized RGCs (Levin, 2001). When the rat optic nerve is completely transected 2mm from the eye, over 85% of RGCs die within 14 days after injury (Berkelaar et al., 1994; Peinado-Ramon et al., 1996; Klöcker et al., 1997; Koeberle and Ball, 1998; Kikuchi et al., 2000). There is accumulating evidence from both animal models of glaucoma and optic nerve injury paradigms (i.e. transection and crush) that RGC cell death occurs via a caspase-dependent apoptotic cascade (Berkelaar et

al., 1994; Bien et al., 1999; Chierzi et al., 1998; Garcia-Valenzuela et al., 1994; Isenmann et al., 1999; Kermer et al., 1999, 2000; Koeberle and Ball, 1998; Kügler et al., 1999; Levin, 1999; Li et al., 1999; Quigley et al., 1995). It has been hypothesized that RGC death in glaucoma and ON transection is mediated by several mechanisms, including neurotrophic factor deprivation (Weise et al., 2000; So and Yip, 2000), excitotoxicity caused by over stimulation of N-methyl-D-aspartate (NMDA) receptors (Kikuchi et al., 2000; Russelakis-Carneiro et al., 1996), increased production of reactive oxygen species (Koeberle and Ball, 1999; Levin, 1999; Levkovitch-Verbin et al., 2000), as well as cytokine-mediated activation of retinal glial cells (Kacza and Seeger, 1997; Koeberle and Ball, 1999; Morgan, 2000; Tezel and Wax, 2000; Thanos et al., 1992). Consistent with this idea, it has been shown that RGC death can be partially prevented by intravitreal administration of glutamate receptor antagonists (Kikuchi et al., 2000), factors that inhibit microglia activation (Thanos et al., 1993, Koeberle and Ball, 1999, 2002), and several classes of growth factors (Yip and So, 2000). Single or repeated intraocular injection(s) of neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), neurotrophin 4 (NT-4), and ciliary neurotrophic factor (CNTF) have been shown to have survival promoting effects on axotomized RGCs (Mey and Thanos, 1993; Koeberle and Ball, 1998; Peinado-Ramon et al., 1996; Cui et al., 1999). Furthermore, only CNTF has been shown to promote the survival of RGCs and

regeneration of their axons, in vitro and in vivo (Cho et al., 1999; Cui et al., 1999; Jo et al., 1999).

In addition to protecting axotomized RGCs, a single injection of recombinant CNTF has been shown to protect retinal neurons from the acute effects of ischemia-induced injury (Honjo et al., 2000), or the damaging effects of constant light (LaVail et al., 1992). However, chronic retinal degenerative diseases such as glaucoma will likely require continuous pharmacological intervention. Although neurotrophic factors show great promise for treating retinal disease in animal models, their use in humans where continuous delivery is necessary maybe limited by several factors, including the damage caused by repeated injections into the eye. Recently, several classes of viral vectors, including adenovirus (Ad), adeno-associated virus (AAV) and lentivirus (LV), have been employed in animal models of photoreceptor degeneration to overcome the necessity of multiple and potentially harmful injections (Cayouette and Gravel, 1997; Liang et al., 2001a, 2001b). There have been several reports using adenoviral (Di Polo et al., 1998; Isenmann et al., 1998; Weise et al., 2000), and AAV vectors (Dryer et al., 1999) in animal models of RGC injury. However, to date there have been no studies to investigate the use of lentiviral vectors for the protection of injured RGCs.

The use of genetically modified lentiviral vectors has been shown to be an efficient method for rapidly transferring genes to the retina and the rest of the CNS (de Almeida et al., 2001; Déglon et al., 2000, 2002; Hottinger et al., 2000;

Kordower et al., 1999, 2000; Miyoshi et al., 1997; Zufferey et al., 1997; Zuffery, 2002). Moreover, the vector has been shown to transduce both dividing and non-dividing cells such as neurons and to integrate transgenes into the chromosomes of their targets. This results in rapid and prolonged expression of neuroprotective proteins without invoking a severe immune response (Miyoshi et al., 1997, Déglon et al., 2000). For these reasons alone, lentiviral vectors may represent an efficient and effective gene delivery system for testing and evaluating neuroprotective proteins in animal models of rapid neuronal degeneration as seen after optic nerve transection in rodents. Miyoshi et al., (1997) demonstrated that subretinal injection of a lentiviral vector encoding the Green Fluorescent Protein into newborn and adult rats resulted in stable transgene expression in the retina for up to 12 weeks.

The aim of the present study was to examine the effects of a single intravitreal injection of a lentivirus encoding the gene for human CNTF (LV-CNTF) or bacterial  $\beta$ -galactosidase (LV-LacZ, control) on the survival of axotomized rat RGCs. We took advantage of the fact that lentivirus transgene expression is rapid and prolonged, and thus, injected the vector immediately after ON transection. We subsequently measured RGC survival up to 21 days post-axotomy (dpa), which is considered to be an extended period of time for this model. We found, that prolonged administration of CNTF using lentiviral vectors enhanced the survival of axotomized RGCs at 14 and 21 dpa compared to control treated retinas. Moreover, prolonged administration of CNTF using

lentiviral vectors enhanced the survival of axotomized RGCs greater than a single injection of recombinant human CNTF at the time of optic nerve transection. Therefore, it would appear that the events leading to RGC death are initiated soon after axotomy and that intervention at this time by CNTF, whether it is by gene delivery or protein injection, is protective.

## **Materials and Methods**

### **Animals**

Naïve, adult, female Sprague-Dawley rats were obtained from Charles River Canada (St. Constant, Quebec) at six weeks of age (200-250 g). Animals were acclimatized to the McMaster University Central Animal Facility (CAF) in a level B pathogen controlled environment for approximately 2 weeks prior to surgical procedures. The rats were housed individually in shoebox cages and maintained in good health under the care of a veterinarian for the duration of the experiment. All rats were maintained on a 12 hr light-dark cycle (lights on at 7:00 a.m.) and both food and water were available *ad libitum*.

### ***Optic Nerve Transection and Retrograde Labeling***

Animals were deeply anesthetized with 7% Chloral Hydrate (420 mg/kg; ip) and placed in a Kopf stereotaxic head holder. With the aid of a surgical microscope (Zeiss, Germany), the optic nerve was approached within the orbit by making a skin incision along the superior rim of the orbital bone and the superior orbital contents were carefully dissected and the rectus muscles were reflected laterally. To gain visual access to the optic nerve and surrounding menigeal sheath the eye was rotated in a temporal direction by transecting and then retracting segments of the extra-ocular muscles. Following rotation of the eye, the menigeal sheath was excised longitudinally and the optic nerve cut 1.5-2 mm behind the eyeball (see Fig. 1B). Retinal ganglion cells were retrogradely labeled

by injecting 10  $\mu$ l of a 3% solution of Dextran-FITC (3000 MW, Molecular Probes, Eugene, OR) into the proximal nerve stump as previously described (Koeberle and Ball, 1998). Care was taken to avoid damage or disruption to the retinal blood supply. Postoperative ophthalmoscopy was performed to confirm the integrity of the retinal vasculature and rats with a compromised retinal vasculature were not included as experimental subjects. Another group of animals was used to determine the number of RGCs in normal retinas by stereotaxically injecting a 2% solution of FluoroGold (Fluorochrome Inc. Englewood, CO) into the superior colliculus (SC) as previously described (Koeberle and Ball, 2002).

### ***Lentiviral Vectors***

The construction of the lentiviral vectors used in this study and their in vitro and in vivo bioactivity have been previously described (Dégion et al., 2000; de Almeida et al., 2001). Briefly, A replication-deficient, self-inactivating lentivirus encoding human CNTF (LV-CNTF) or the bacterial enzyme *Escherichia coli*  $\beta$ -galactosidase (LV-LacZ) under the control of mouse phosphoglycerate kinase 1 (PGK) were generated by insertion of a human CNTF containing a sequence for the murine immunoglobulin signal peptide (since CNTF lacks a leader sequence) or bacterial *E. coli*  $\beta$ -galactosidase expression cassette cDNA (LacZ) into the SIN-W-PGK vector backbone as previously described (Dégion et al., 2000). The

lentiviral vectors used in the present study (LV-CNTF or LV-LacZ) are schematically illustrated in Figure 1A. The vectors were matched for particle content (de Almeida et al., 2001) and used at a level of 480,000 ng p24/ml.

### ***Administration of Viral vectors and recombinant CNTF***

Immediately following optic nerve transection, animals received intraocular injections of a viral vectors or recombinant protein into the posterior chamber of the eye as previously described (Koeberle and Ball, 1998). One group of animals received an intravitreal injection consisting of a 5  $\mu$ l suspension of LV-CNTF or LV-LacZ (minimum of n=3/per group). A second group of animals received an intravitreal injection of a 5  $\mu$ l of recombinant human CNTF (0.8  $\mu$ g/ $\mu$ l, Leinco Technologies, St. Louis, MO) dissolved in 5  $\mu$ l sterile PBS, or a control injection of PBS vehicle. Another group of animals that were not axotomized received an intraocular injection consisting of a 5  $\mu$ l suspension of PGK.LacZ to localize of transgene expression in the retina. For all injections, a 30 gauge needle was used to puncture the sclera within 3 mm of the optic nerve head. Intraocular injections were administered using a 5  $\mu$ l SGE microlitre syringe (Type A-RN, Austin, TX; 0.11 mm outer diameter needle). Care was taken to avoid damage to the lens, or the anterior structures of the eye, since this has been shown to cause the secretion of growth factors and increase RGC survival (Mansour-Robaey et al., 1994; Leon et al., 2000). Following intraocular



injections, the needle was held in place for 2 minutes, withdrawn slowly, and the puncture in the sclera was immediately sealed using Vetbond tissue cement (3M, St. Paul, MN). Upon completion of all surgical procedures animals were monitored during a recovery period and then returned to the vivarium and kept under the care of a veterinarian during the course of the experiment.

### ***Tissue Preparation***

Rats were euthanized by an overdose of anesthetic and their eyes enucleated at 14 or 21 days following optic nerve transection. The cornea and lens were dissected away and the remaining eyecups were fixed for 1.5 hours in a solution consisting of 4% paraformaldehyde, and 2% sucrose, in 0.1 M Sorensen's phosphate buffer (0.1 M, pH 7.3). Following fixation, eyes were rinsed in PBS for 10 minutes and flatmounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) to determine RGC densities, or sectioned on a cryostat and processed for localization of lentiviral-mediated gene expression of  $\beta$ -galactosidase.

### ***$\beta$ -Galactosidase Staining in the Retina***

Several retinas were processed to determine the cellular localization of viral transgene expression of  $\beta$ -galactosidase at 1, 7, and 21 days post-injection. Transverse cryostat sections (14  $\mu$ m) or flatmounted retinas were processed

using X-gal histochemistry (5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside, Boehringer-Mannheim, Mannheim, Germany) or  $\beta$ -galactosidase immunohistochemistry (rabbit polyclonal-anti- $\beta$ -galactosidase; 1:500, Five Prime Three Prime, Boulder, CO). In the presence of functional  $\beta$ -gal, X-gal is converted to a blue reaction product in transfected cells. Retinal sections were washed three times in 100 mM PBS (pH 7.4) and then incubated in 2mM MgCl<sub>2</sub>, 25 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 25 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, and 1 mg/ml X-gal in PBS for 4-6 hours at room temperature. Upon completion of the staining, sections were washed three times in 100 mM PBS and coverslipped. Alternatively, immunofluorescent detection of  $\beta$ -gal transgene expression was carried out using standard immunohistochemistry procedures. Briefly, sections were incubated with a polyclonal antibody raised in rabbit against  $\beta$ -gal (3prime-5prime Inc, 1:2000). Following primary antibody incubation, retinas were rinsed 3 x 10 minutes in PBS. Beta-galactosidase immunoreactivity was detected using a goat-anti-rabbit secondary antibody (1:500; Texas Red conjugated, pre-absorbed against rat serum proteins, Jackson Immunoresearch, West Grove, PA). Whole retinas were incubated in secondary antisera for 4 hours, rinsed 3 x 15 minutes in PBS and then coverslipped in Vectashield for visualization.

#### Microscopy and Cell Counting

Dextran-FITC fluorescence and  $\beta$ -galactosidase immunofluorescence were visualized using a Zeiss Laser Scanning Confocal Microscope (LSM-510). X-gal histochemistry was visualized using transmitted light microscopy. Labeled RGCs were counted from confocal micrographs covering an area of 78,000  $\mu\text{m}^2$  as previously described (Koeberle and Ball, 1998). Each retina was divided into 4 radial quadrants, and confocal micrographs of 12 areas (3 from each quadrant) from the mid-periphery (1/2 the retinal eccentricity) of each flatmounted retina were quantified. Microglial cells that had been transcellularly labeled with Dextran-FITC, due to phagocytosis of dead RGCs, were distinguished from intact RGCs on the basis of their size and morphology, and excluded from the cell counts. The results for separate retinas were pooled for statistical analysis and the mean RGC densities/ $\text{mm}^2 \pm \text{SD}$  were calculated.

### ***Statistical analysis***

Statistical analysis of the results was performed using SPSS 8.0. for Windows98. Statistical significance was determined using one-way Analysis of variance (ANOVA) followed by Tukey's and Dunnet's post hoc comparisons for significance between groups at  $p < 0.05$ .

## Results

### *Effect of Optic Nerve transection on RGC Densities*

In normal rats, RGCs retrogradely labeled from the superior colliculus (SC) using FluoroGold (FG) revealed a mean RGC density in the mid-periphery of the retina to be  $2461 \pm 50$  RGCs/mm<sup>2</sup> (mean  $\pm$  SD) (Fig.2E). Similar results were obtained when RGCs were retrogradely labeled at the time of axotomy by injecting Dextran-FITC (D-FITC) into the proximal nerve ending. As figure 2A shows, injection of D-FITC into the optic nerve labeled RGCs and axon fascicles as early as 2 days post-axotomy (dpa). Since no appreciable RGC death occurs within the first 4 days after optic nerve injury (Villegas-Perez et al., 1988; 1993; Peinado-Ramon et al., 1996; Koeberle and Ball, 1998), we observed no significant difference in the mean number of D-FITC labeled RGCs ( $2381 \pm 102$  RGCs/mm<sup>2</sup>: mean $\pm$ SD) at 2 dpa compared to normal retinas retrogradely labeled from the SC using FG. In addition, there was no sign of any transcellularly labeled microglia, which become labeled with D-FITC by active phagocytosis of RGC apoptotic bodies (Thanos, 1991). However, at 7, 14 and 21 dpa there was a significant reduction in the number of D-FITC retrogradely labeled RGCs (Fig. 2B, C, and D) and a concomitant increase in the number of phagocytic microglia. These cells were easily distinguishable from D-FITC labeled RGCs since they are morphologically different from RGCs and have a significantly smaller diameter than RGCs. There was a moderate decline in the mean RGC density at 7 dpa ( $1709 \pm 166$  RGCs/mm<sup>2</sup>) compared to retinas from normal animals and

animals surviving 2 dpa (Fig. 2E). In contrast, retinas isolated at 14 dpa, revealed a drastic reduction in the mean RGC density ( $369 \pm 85$  RGCs/mm<sup>2</sup>), which had declined to 14.9% of the density of RGCs observed in normal retinae (Fig.2E). A small additional decline was observed at 21 dpa ( $193 \pm 26$  RGCs/mm<sup>2</sup>). The greatest rate of RGC death occurred between 7 and 14 dpa.

### ***Transduction of retinal cells after intravitreal delivery of LV-LacZ***

Twenty-one days after intravitreal administration of LV-LacZ,  $\beta$ -galactosidase was expressed primarily in retinal neurons of the ganglion cell layer (GCL) and a small population of retinal pigment epithelial (RPE) cells (Fig. 3). Approximately 25% of retinal neurons in the GCL were transduced, whereas less than 1% of the total population of RPE cells was transduced 21 days after intraocular injection of LV-LacZ in normal eyes. In the adult rat retina, approximately 55% of the GCL is composed of RGCs, with the remaining cells being displaced amacrine cells. Since the CNTF transgene in the current study was engineered to be secreted we were not concerned with selective RGC transduction using lentiviral vectors because we have previously shown that adenoviral mediated transfer of a secreted form of CNTF from infected Müller cells increased the survival of non-transduced axotomized RGCs at 7 and 14 dpa (unpublished results). The densities of transduced RPE cells varied between <1% to approximately 5%. The highest transduction of RGCs and RPE cells was

observed nearest to the injection site. This gradient most likely resulted from diffusion of the vector from the injection site.

### ***Effect of LV-CNTF on the Survival of Axotomized RGCs***

We tested the effects of lentiviral vectors encoding human CNTF on the survival of axotomized RGCs at 14 and 21 after optic nerve transection. These time points were selected for this experiment because previous studies have shown that approximately 90% of axotomized RGCs die within 14 days after optic nerve transection (Villegas-Perez et al., 1988; 1993; Peinado-Ramon et al., 1996). Without treatment few RGCs survive axotomy, however there is a small population of RGCs (5-10%) that survive several months after optic nerve transection (Villegas-Perez et al., 1993). In this experiment, we evaluated RGC rescue in axotomized retinas by determining the number of Dextran-FITC retrogradely labeled RGCs treated with LV-CNTF or LV-LacZ. At 14 and 21 days after optic nerve axotomy, retinas that had received control intraocular injections of LV-LacZ (Fig. 4A & C) had a significantly amount of RGC death. The few RGCs that remained were interspersed between transcellularly labeled microglia. RGC densities in LV-LacZ treated retinas at 14 ( $373 \pm 102$  RGCs/mm<sup>2</sup>) and 21dpa ( $166 \pm 14$  RGCs/mm<sup>2</sup>) represented approximately 16% and 7% of the original RGC population ( $2381 \pm 102$  RGCs/mm<sup>2</sup>) respectively, and were not statistically different from the mean number of RGCs observed in untreated retinas. In contrast, intraocular administration of LV-CNTF significantly

enhanced RGC survival compared to control treatment (Fig. 4B & D). RGC densities in LV-CNTF treated animals at 14 ( $1673 \pm 128$  RGCs/mm<sup>2</sup>) and 21 dpa ( $755 \pm 178$  RGCs/mm<sup>2</sup>), represented 70% and 32% of the normal RGC density respectively (Fig.4 E). We also compared the survival of axotomized RGCs at 14 dpa in retinas treated with either a single intraocular administration of 4  $\mu$ g of rhCNTF or injection of LV-CNTF at the time of axotomy. The mean RGC density of rhCNTF treated retinas ( $688 \pm 41$  RGCs/mm<sup>2</sup>) was significantly higher than control retinas treated with PBS ( $425 \pm 81$  RGCs/mm<sup>2</sup>). Treatment of axotomized retinas with LV-CNTF rescued far more injured RGCs than a single treatment of recombinant CNTF (Fig. 4F). These findings demonstrate that CNTF delivery after optic nerve transection is capable of extending the survival of severely injured RGCs *in vivo*. In addition, our results suggest that prolonged delivery of CNTF may serve as potential treatment for patients with optic nerve neuropathies, such as glaucoma.

## Discussion

The purpose of this study was to evaluate the effect of lentiviral-mediated transfer of human CNTF on the survival of axotomized RGCs. Moreover, we wanted to determine if the effects of LV-CNTF were capable of extending the survival of RGCs to an endpoint of 21 days, an improvement that would be considered unusual in this model. In untreated retinas, it was apparent that endogenous responses within the retina are not sufficient to protect severely injured RGCs, as we observed that fewer than 10% of RGCs survive 21 days after optic nerve transection. In contrast, exogenous delivery of CNTF, whether by direct injection of recombinant CNTF protein or via lentiviral-mediated gene transfer of CNTF rescued a large population of axotomized RGCs that would have otherwise died. Moreover, we observed a dramatic and extended increase in RGC survival following optic nerve injury when we injected a LV-CNTF immediately after ON transection.

In this study, we used optic nerve transection in the adult rat to elucidate lentivirus mediated gene transfer because it has proved to be a useful model to study the time course of neuronal death after injury (Berkelaar et al., 1994; Di Polo et al., 1998; Koeberle and Ball, 1998, 1999, 2002). Since the vast majority of RGCs die between 7-14 days after ON axotomy, few studies have investigated the neuroprotective effects of gene delivery or pharmacological agents on RGC survival beyond 14 days. Indeed, it has proved very difficult to protect



axotomized RGCs beyond 14 days using pharmacological agents or adenoviral vectors (Di Polo et al., 1998). In the present study we wanted to determine if axotomized RGCs could be protected beyond 14 dpa because of the demonstrated rapid and prolonged transgene expression of neurotrophic factors using lentivirus (Déglon et al., 2000, 2002; Hottinger et al., 2000; de Almeida et al., 2001). We demonstrated that prolonged administration of CNTF by lentiviral-mediated gene transfer to the retina considerably enhanced RGC survival at 14 and 21 days post-axotomy. This survival effect was not observed with the control vector encoding bacterial *E. coli*  $\beta$ -galactosidase. To our knowledge, this is the first report of extended RGC survival using a single treatment at the time of axotomy, as well as the first report of the application of a lentivirus gene delivery system using optic nerve transection as an *in vivo* animal model. We showed an increase in RGC survival at 14 dpa with a single large bolus injection of 4  $\mu$ g of rhCNTF at the time of axotomy. Indeed, single or multiple intravitreal treatment(s) of CNTF below 1  $\mu$ g does not have survival-promoting effects on axotomized RGCs (Cho et al., 1999; Cui et al., 1999). In addition to the increase in RGC survival at 21 days, RGC survival at 14 dpa was substantially higher after a single injection of LV-CNTF compared to a single injection of rhCNTF. This difference most likely resulted because the CNTF protein has a short half-life and a single injection of rhCNTF would not remain in the axotomized retina long after treatment (Sendtner et al., 1997). Thus, it is not surprising that the rapid and

prolonged neurotrophic support provided by the lentiviral vector is more efficient for RGCs protection even when nanogram level of viral-mediated production of CNTF is delayed by approximately 24 hrs (de Almeida et al., 2001).

The demonstration of prolonged RGC survival is in agreement with our previous studies that demonstrated long-term neuroprotective properties of lentiviral-mediated gene transfer of neurotrophic factors on injured CNS neurons (Dégion et al., 2000; de Almeida et al., 2001). Using the same lentiviral vector, we recently showed that pretreatment of the rat striatum with LV-CNTF 3 weeks before quinolinic acid lesions significantly reduced the extent of neuronal loss in the striatum and prevented the loss of motor function (de Almeida et al., 2001). In another model, we showed that pretreatment of the substantia nigra with LV-GDNF was able to protect nigral dopaminergic neurons after medial forebrain bundle axotomy (Dégion et al., 2000). The most significant difference between these results and the findings of the present study is that the increase in RGC survival was obtained by injecting LV-CNTF immediately after optic nerve transection. This suggests that lentivirus mediated gene transfer is effective as a delivery system in both chronic and acute injury paradigms, probably due to the rapid onset and prolonged transgene expression not afforded by other vector systems. Although, adenoviral vectors also allow rapid transgene expression, we observed that the expression of the CNTF or LacZ transgene was not long lasting (5-7 days) based on western blotting data (unpublished results). Indeed, intraocular injection of an adenoviral vector encoding human CNTF immediately

following axotomy protected fewer axotomized RGCs for the first 14 days, but afforded very little protection at 21 days post-axotomy (unpublished data). Together these findings suggest that it is both the rapid onset and prolonged expression of lentiviral-mediated CNTF that leads to the significant improvement in RGCs survival in the optic nerve transection model.

An additional factor that might affect a particular vector's effectiveness in delivering cell survival genes is the potential for activating extrinsic and intrinsic reactions to the vector itself. Moreover, the injured neuron may become less responsive to neurotrophic factors over time. For example, the expression of BDNF encoded by an adenoviral vector was shown to be limited to approximately 10 days in normal animals, but could be extended to at least 30 days by concurrent administration of the immunosuppressant, FK506 (Di Polo et al., 1998). Despite the fact that the immunosuppressed rats showed prolonged expression of BDNF there was no further enhancement on the survival of axotomized RGCs. The authors concluded that the survival promoting effects of BDNF on axotomized RGCs is not limited to its availability or reactions to the vector, but rather to injury-induced changes in the retina that limit the survival promoting properties of BDNF (Di Polo et al., 1998). The demonstration that a single treatment of LV-CNTF enhanced both the number of surviving RGCs and the time-course of survival over a single treatment of Ad.CNTF mediated gene transfer or rhCNTF suggests that intrinsic properties of injured RGCs do not limit their responses to survival factors in general.

There are several constraints that may limit the practical use of adenovirus for the treatment of chronic retinal degenerative diseases such as glaucoma. Repeated injections of adenoviral vectors would have to be made to ensure long-term expression, that may damage the eye, particularly to the lens. In addition, adenoviral vectors have been shown to cause severe immune response within the eye and brain using moderate titers (Easton et al., 1998). Moreover the immune response elicited by adenoviral vectors may decrease the period of transgene expression (Easton et al., 1998; Koeberle and Ball, 2002; Thomas et al., 2001).

In comparison to adenoviral and lentiviral vectors, Adeno-associated (AAV) vectors display much slower kinetics, but similar to lentivirus, display long-term transgene expression in the retina (Liang et al., 2001, 2001). Subretinal or intravitreal injection of AAV.CNTF has been demonstrated to protect photoreceptors in animal models of retinitis pigmentosa (RP), even though expression of the transgene was not detected until 4 weeks after the injection (Liang et al., 2001, 2001). Other studies have reported similar delays in transgene expression after injection of AAV vectors into CNS tissue (Dryer et al., 1999; Mandel et al., 1999). Based on these reports it seems unlikely that adenoviral or AAV vectors will be suitable gene delivery systems for human retinal degenerative diseases requiring immediate clinical intervention. However, they will remain useful to further advance our understanding of the pathways leading to neuron death in the CNS (Di Polo et al., 1998; Gravel et al., 1997, Koeberle et al.,

2002; Kügler et al., 1999, 2000; Mandel et al., 1999; Perrelet et al., 2000; Weise et al., 2000; Xu et al., 1997).

Despite the widely reported neuroprotective properties of CNTF on CNS structures like the retina, the exact mechanisms of action remain unknown. Several possibilities have been suggested, including: inhibition of glutamate-mediated excitotoxicity, a phenotypic shift in glial cells that confers an additional protective capacity, activation of neuroprotective signal transduction pathways including the JAK/STAT pathway, and blockade of multiple stages of the apoptotic pathway (Honjo et al., 2000; Monville et al., 2000; Peterson et al., 2000; Yan et al., 2000). In the adult rat retina, CNTF is predominately localized to Müller glial cells, whereas retinal neurons, including RGCs, horizontal and amacrine cells express the CNTF receptor alpha (CNTFR $\alpha$ ) (Kirsch et al., 1997). The CNTF receptor complex is composed of three basic components: CNTFR $\alpha$ , gp130, and gp190 (LIF $\beta$ ). The CNTFR $\alpha$  is a GPI linked receptor, and therefore functions strictly as the ligand binding chain for CNTF having no signal transduction role other than to increase the formation of the CNTF receptor complex (Inoue et al., 1996). This in turn induces intracellular signal transduction cascades via the JAK (Janus kinase) - STAT (signal transducer and activator of transcription) pathway. Consistent with this mechanism, we observed increases in Stat3 phosphorylation after adenoviral-mediated CNTF delivery in this model (unpublished results) suggesting that this pathway is activated in lentiviral-

mediated CNTF delivery. Endogenous CNTF lacks a consensus secretory signal sequence, and is only secreted from glia under conditions of stress, leading to the hypothesis that CNTF acts as an “injury factor” (Sendtner et al., 1991, 1997). Indeed, the expression of CNTF and CNTFR $\alpha$  are elevated in the rat retina after optic nerve transection (Chun et al., 2000; Ju et al., 2000; Kirsch et al., 1998; Weise et al., 2000; Wen et al., 1995), after ischemic insult (Ju et al., 1999, and 2000) and in light-stressed rats (Walsh, 2001). Elevation of endogenous CNTF levels may serve to protect injured neurons in the inner retina, presumably by activating the high affinity CNTFR $\alpha$  receptor (Weise et al., 2000). The neuroprotective mechanisms of CNTF on photoreceptor degeneration are intriguing, since CNTFR $\alpha$  is not localized in the outer retina (Kirsch et al., 1997; Liang et al., 2001, 2001). Indeed, CNTF treatment has demonstrated excellent histological protection of photoreceptors, however, such treatments have not resulted in physiological preservation of retinal function in animal models (Liang et al., 2000, 2001). Recently, it was demonstrated that CNTF binds with low affinity to the leukemia inhibitory receptor beta (LIFR $\beta$ ), which is expressed predominately on mature astrocytes (Monville et al., 2001). Indeed, there is experimental evidence to suggest that CNTF may activate retinal astrocytes and Müller cells, and increase their endogenous neuroprotective capacity (Peterson et al., 2000). The elevation of pSTAT<sub>3</sub> observed in Müller cells after CNTF treatment or after retinal injury may increase the expression of neuroprotective

factors and thereby protect photoreceptors from light-induced death (Peterson et al., 2000). At the same time, these glial modulations may be masking the positive physiological impact of photoreceptor protection by altering the electroretinogram b-wave (Liang et al., 2001). The indirect action of CNTF on retinal glia may protect neurons in the inner retina after axotomy or NMDA-induced cell death (Honjo et al., 2000; Weise et al., 2000). Together, these studies suggest that CNTF and its receptor components are important factors involved in protecting the injured retina and the use of the lentiviral vector LV-CNTF may be a useful tool to elucidate those mechanisms and to address the discordance of functional and structural results reported in the literature (Liang et al., 2000, 2001).

Even if the downstream mechanisms of CNTF have not been clearly identified, the potential therapeutic properties of CNTF have been well established in preclinical studies using rodents and non-human primate models of CNS degenerative disease such as Huntington disease (Anderson et al., 1996; Emerich et al., 1996, 1997; Emerich 1999; Mittoux et al., 2000). Those promising results lead to a phase I clinical study using encapsulated cells secreting CNTF as a potential therapy for Huntington disease (Bachoud-Levi et al., 2000). The same strategy was evaluated in a study involving patients with amyotrophic lateral sclerosis and without adverse effects, making CNTF a good candidate as the basis for the development of a new therapy (Aebischer et al., 1996).

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We have shown that lentiviral-mediated gene transfer of CNTF to the retina enhances and prolonged the survival of axotomized RGCs in vivo when injected at the time of injury. Furthermore, our results suggest that there is a potential window for therapeutic intervention for RGC rescue by lentiviral-mediated CNTF delivery immediately after injury. Although these results are promising, further studies are required to evaluate the effects of long-term exposure of the retina to exogenous CNTF, to identify the therapeutic window for efficient RGCs rescue, and to identify the neuroprotective mechanisms of CNTF. Rapid and prolonged lentiviral-mediated gene transfer will allow us to approach these questions.

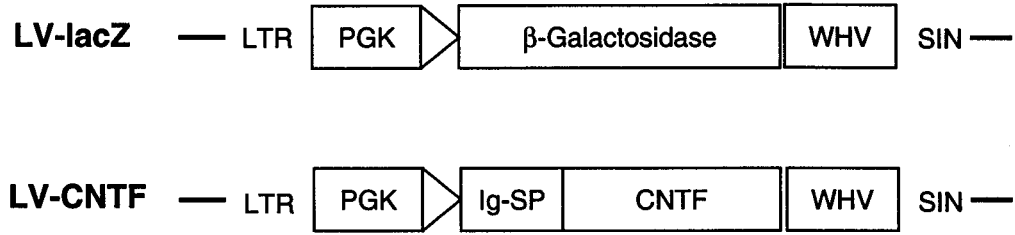
## **Acknowledgements**

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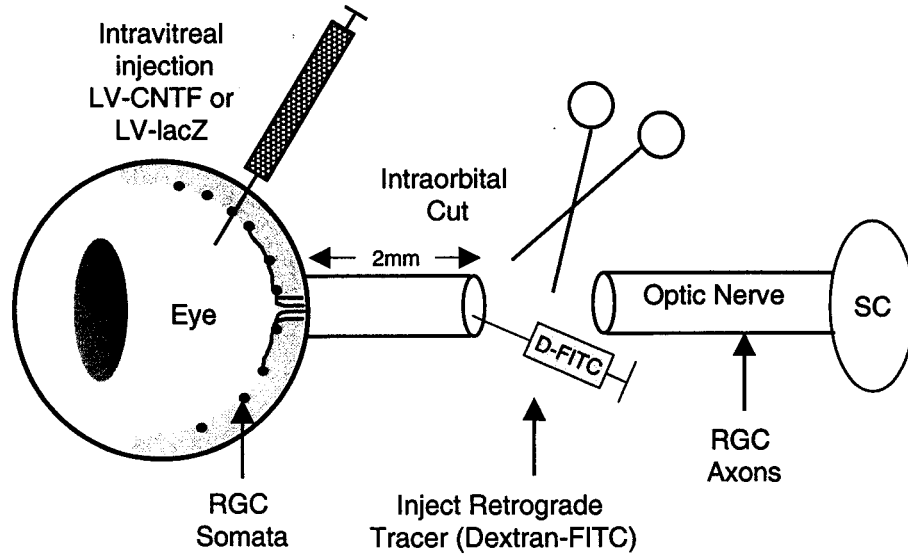
## Figure Legends

**Figure 1. A:** Schematic representation of the replication-deficient, self-inactivating lentiviral vectors used in this study (adopted from: de Almeida et al., 2001). Functional elements are shown: coding regions for human CNTF or the bacterial enzyme *Escherichia coli*  $\beta$ -galactosidase under the control of mouse phosphoglycerate kinase 1 (PGK) promoter and the post-transcriptional regulatory element of the woodchuck hepatitis virus (WHS). A self-inactivating vector (SIN) was produced by deleting the 400-bp *EcoRV-PvuII* fragment of the U3 region at 3' LTR. **B:** Illustration of the surgical procedures used for optic nerve transection, retrograde labeling at the nerve stump and intravitreal injection of viral vectors into the posterior chamber of the eye. **C:** Experimental design to test the long-term effects of lentiviral-mediated transfer of CNTF on axotomized RGCs *in vivo*.

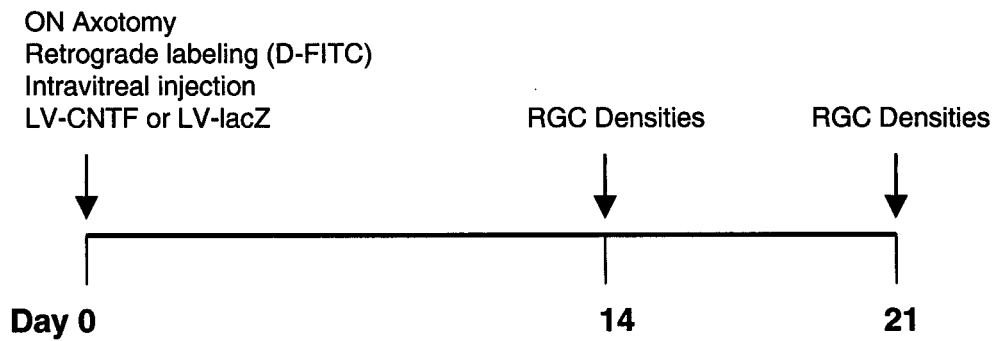
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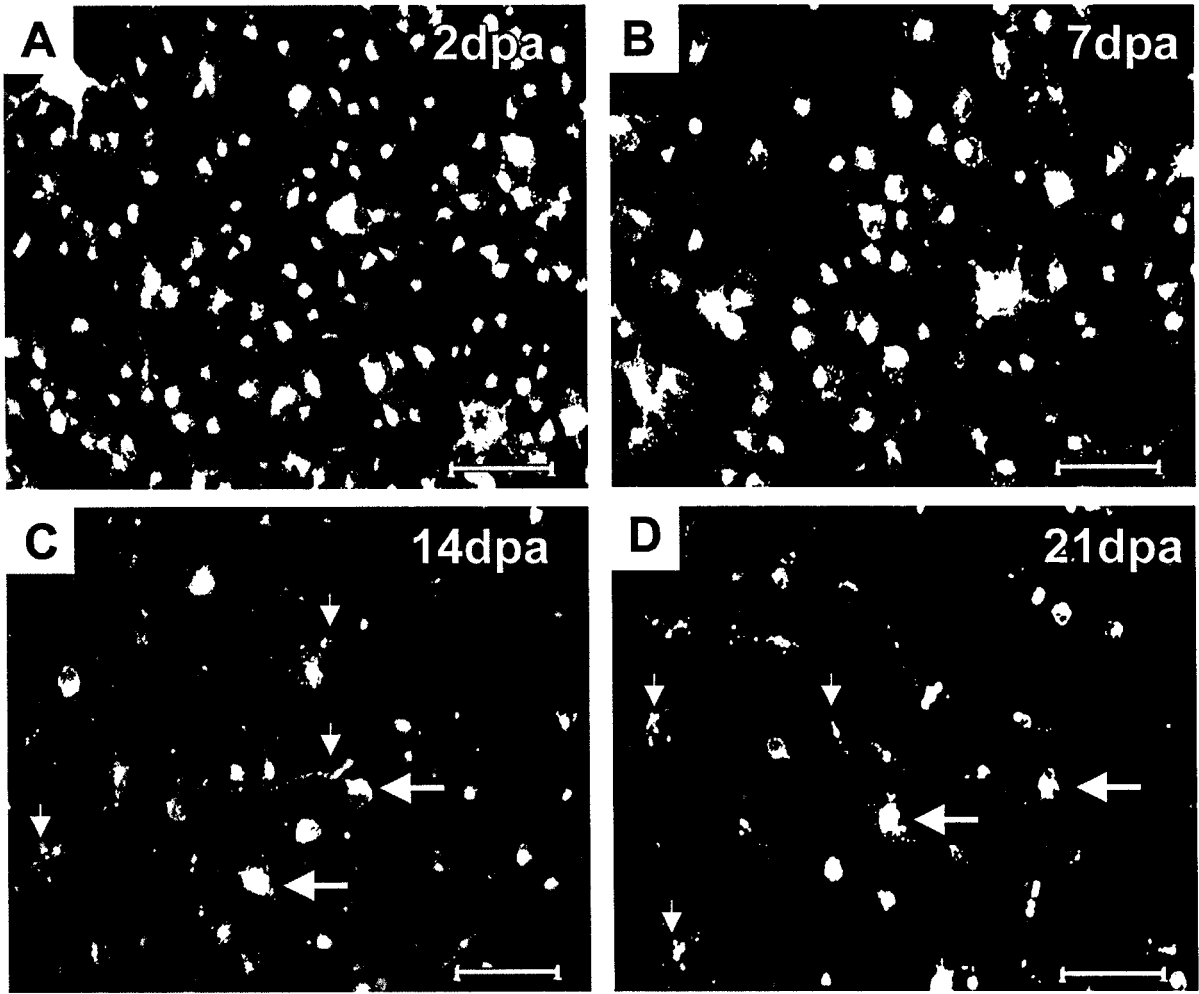
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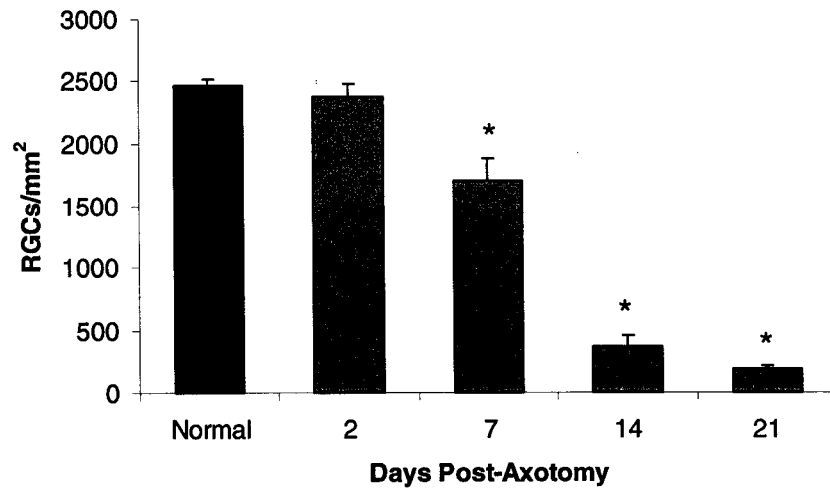


**Figure 2.** Few RGCs survive complete intraorbital optic nerve transection 2 mm from the eye. Confocal micrographs showing Dextran-FITC retrogradely labeled retinal ganglion cells in flatmounted retinas at 2, 7, 14, and 21 days after optic nerve transection. **A:** RGC labeling with dextran-FITC 2 dpa. **B:** A noticeable loss of RGCs at 7 days post-axotomy and the appearance of transcellularly labeled microglia that have small cell bodies and ramified processes. **C:** few viable RGCs at 14 dpa and numerous transcellularly labeled microglia. **D:** a small population of RGCs remained at 21 dpa. **E:** Graph showing a significant decrease ( $p < 0.001$ ) in RGC densities (mean  $\pm$  standard deviation) in flatmounted retinas at 7, 14 and 21 dpa. Note: normal counts were obtained from retrogradely labeling RGCs from the SC in normal animals. Bar = 50  $\mu$ m.



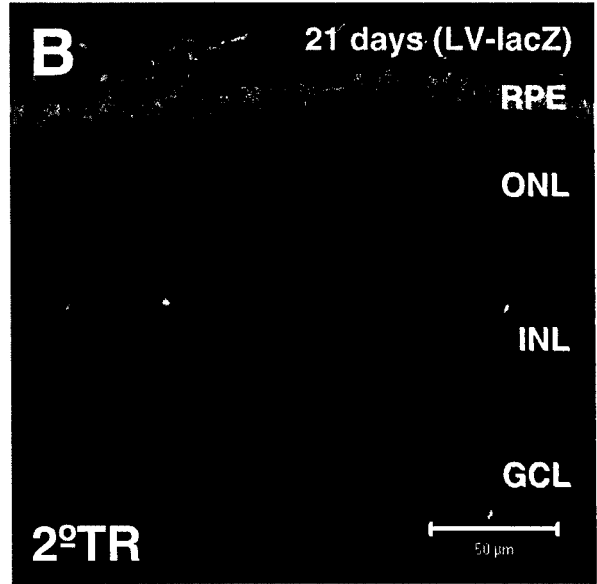
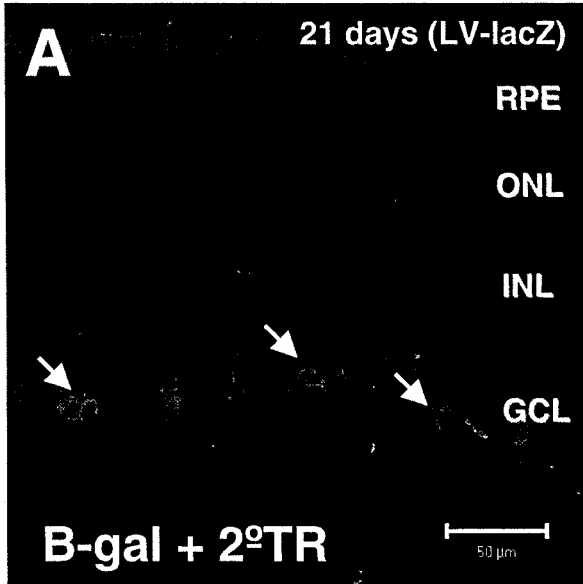
**E**

**RGC Survival after Axotomy**

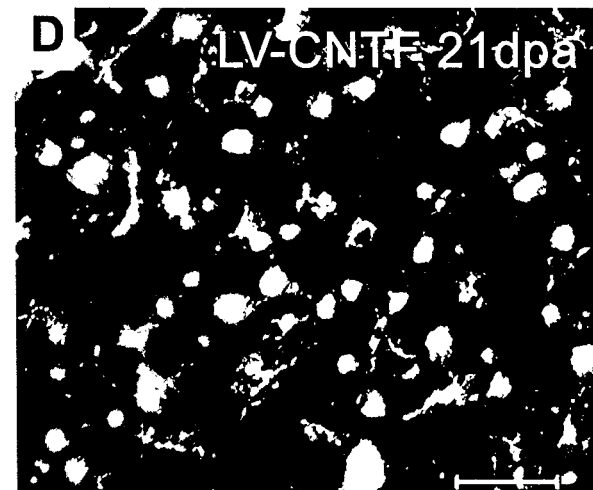
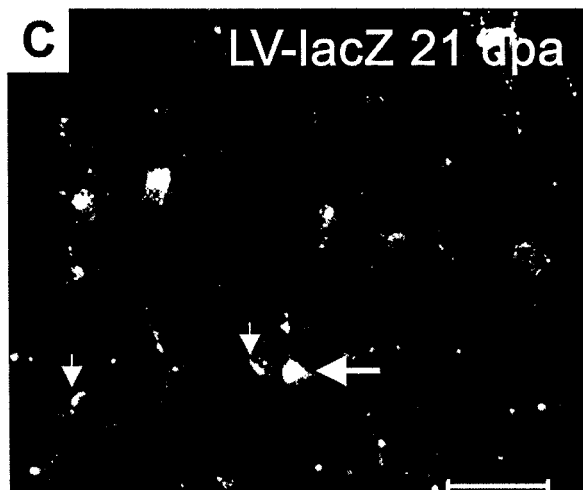
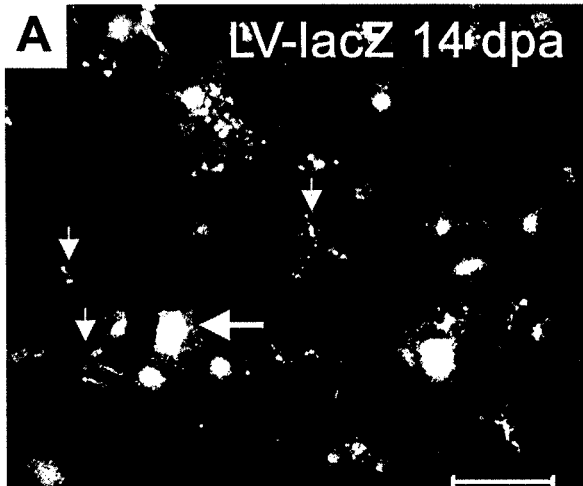


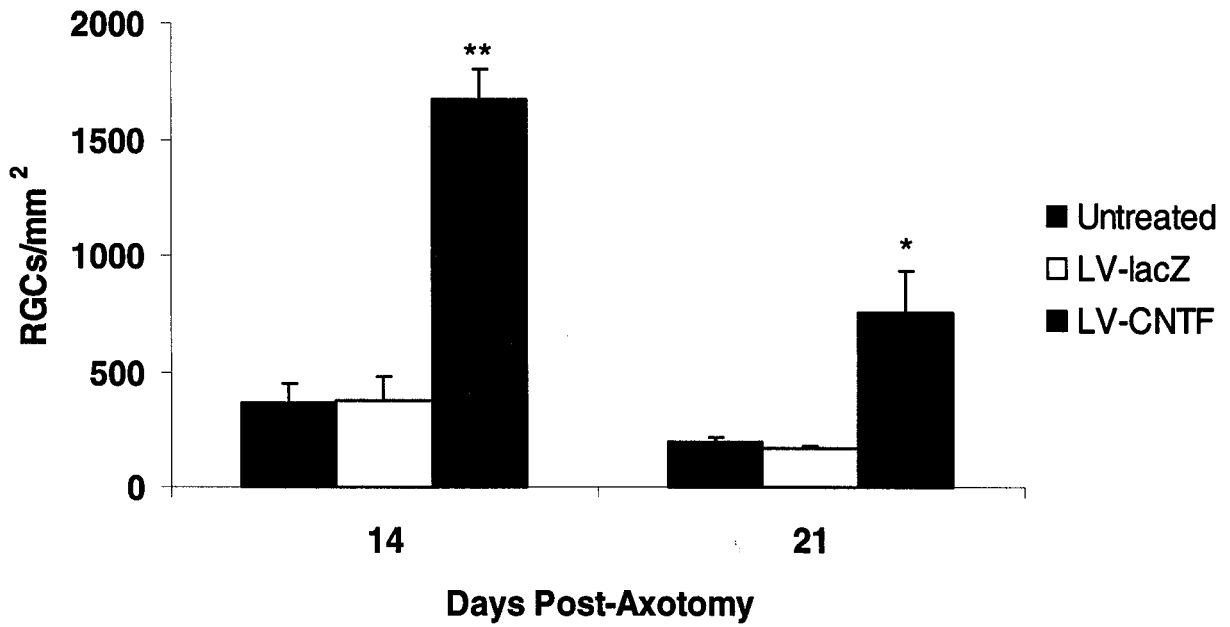
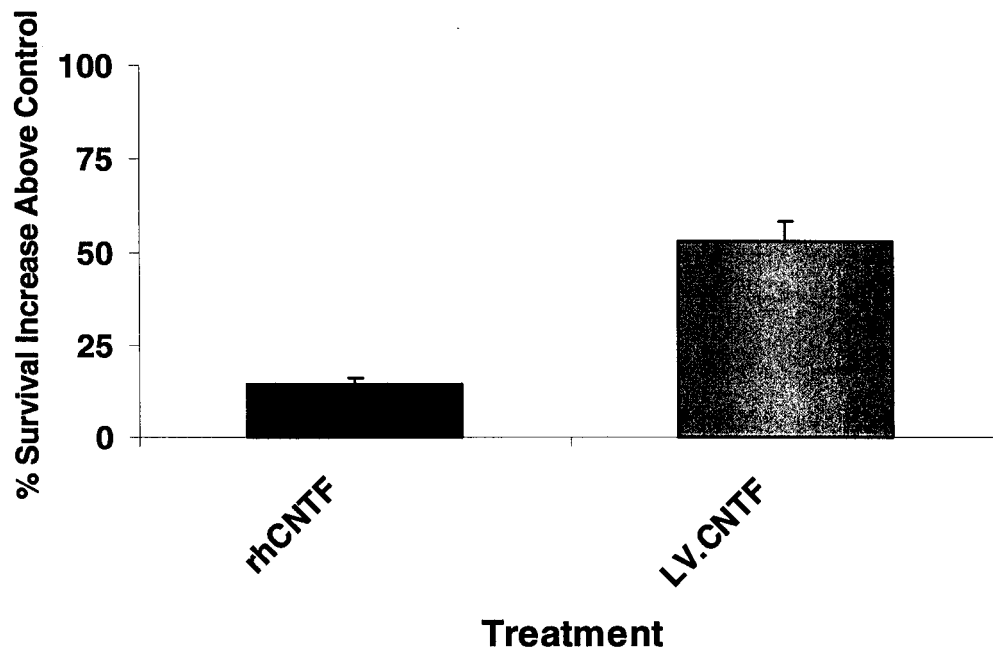
**Figure 3.** Confocal and transmitted light micrographs of retinal cells transfected by intraocular administration of LV-lacZ: Transverse retinal section showing  $\beta$ -galactosidase immunoreactivity in RGCs and RPE cells, 21 days after intraocular viral injection. Labeled RGCs (arrows) showed complete nuclear and cytoplasmic filling. Bar = 50  $\mu$ m.





**Figure 4.** Survival of axotomized RGCs after intravitreal administration of LV-CNTF or LV-lacZ vector. Confocal micrographs showing Dextran-FITC retrogradely labeled RGCs in flatmounted retinas at 14, and 21 dpa treated with either LV-CNTF or LV-lacZ. **A:** RGC survival at 14dpa with LV-lacZ treatment. **B:** increased RGC survival at 14dpa with intravitreal injection of LV-CNTF immediately following ON axotomy. **C:** very few RGCs at 21dpa with LV-lacZ treatment. **D:** RGCs survival at 21 dpa with LV-CNTF. **E:** Graph showing a significant increase ( $p < 0.001$ ) in RGC densities (mean  $\pm$  standard deviation) in flatmounted retinas at 14 and 21 dpa in LV-CNTF treated eyes compared to controls. **F:** percentage of RGCs rescued relative to control treatment at 14 dpa ( $P < 0.05$ ). Bar = 50  $\mu$ m.



**E****LV-CNTF & RGC Survival****F****RGC Survival 14 Days Post-Axotomy**

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## **CHAPTER 4**

### **Experiment III**

#### **Ciliary Neurotrophic Factor Protects Retinal Ganglion Cells from Axotomy- Induced Apoptosis via Modulation of Retinal Glia**

##### **Research contributions:**

B. A. van Adel: optic nerve surgeries, intraocular injections, western blotting, data analysis, immunohistochemistry, histochemistry, and preparation of the manuscript for the European Journal of Neuroscience.

Dr. Jennifer Arnold (NRC): protein purification, Western blotting and data analysis (densitometry)

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Ciliary Neurotrophic Factor Protects Retinal Ganglion Cells from Axotomy-Induced Apoptosis via Modulation of Retinal Glia.

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**Keywords:** retinal ganglion cell, Müller cell, astrocytes, axotomy, retina, ciliary neurotrophic factor, optic nerve transection, cell death, apoptosis

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**Running Head:**

Neuroprotective actions of CNTF on axotomized RGCs



## **Abstract**

Adenoviral-mediated transfer of CNTF to the retina rescued retinal ganglion cells (RGCs) from axotomy-induced apoptosis, presumably via activation of the high affinity CNTF receptor alpha (CNTFR $\alpha$ ) expressed on RGCs. CNTF can also activate astrocytes, via its low affinity leukemia inhibitory receptor beta (LIFR $\beta$ ) expressed on mature astrocytes (Monville et al., 2001, *Mol. & Cell. Neurosci.*, 17: 373-384) suggesting that CNTF may also protect injured neurons indirectly by modulating glia. We tested if adenoviral-mediated overexpression of CNTF in normal and axotomized retinas can increase the expression of several glial markers previously demonstrated to have neuroprotective function in the injured brain and retina. Using western blotting, the expression of glial fibrillary acid protein (GFAP), glutamate/aspartate transporter-1 (GLAST-1) and glutamine synthetase (GS) was examined 7 days after intravitreal injections of Ad.CNTF or control Ad.LacZ. Compared to controls (PBS or Ad.LacZ), intravitreal injection of Ad.CNTF there were significant changes in the expression of CNTFR $\alpha$ , pSTAT3, GFAP, GLAST, GS, and Cx43 in normal and axotomized retinas. Taken together, these results suggest that the neuroprotective effects CNTF may result from a shift of retinal glia cells to a more neuroprotective phenotype. Moreover, the modulation of astrocytes may buffer high concentrations of glutamate that has been shown to contribute to the death of RGCs after optic nerve injury.

## Abbreviations

Ad:	adenovirus
Ad.CNTF:	adenovirus encoding human CNTF
Ad.lacZ:	adenovirus encoding beta-galactosidase
$\beta$ -gal:	beta-galactosidase
CAR:	Coxsackie and adenoviral receptor
CMV:	cytolomegalovirus
CNTF:	ciliary neurotrophic factor
CNTFR $\alpha$ :	CNTF receptor alpha
Cx43:	connexin 43
dpa:	days post-axotomy
dpi:	days post-injection
D-FITC:	Dextran-FITC
GCL:	ganglion cell layer
GFAP:	glial fibrillary acidic protein
GLAST-1:	glutamate/aspartate transporter-1
GS:	glutamine synthetase
ILM:	inner limiting membrane
INL:	inner nuclear layer
IPL:	inner plexiform layer
IO:	intraocular
JAK:	janus kinase
lacZ:	cDNA coding for beta-galactosidase
LIF:	leukemia inhibitory factor
LIFR $\beta$ :	LIF receptor beta
NFL:	nerve fiber layer
pfu:	plaque forming units
PhOS:	photoreceptor outer segment
OLM:	outer limiting membrane
ON:	optic nerve
ONH:	optic nerve head
ONL:	outer nuclear layer
RGC(s):	retinal ganglion cell(s)
SC:	superior colliculus
STAT:	signal transducer and activator of transcription
X-gal:	5-bromo-4-chloro-3-indoyl- $\beta$ -D-galctopyranoside

## Introduction

Ciliary neurotrophic factor (CNTF) belongs to the interleukin-6 (IL-6) family of cytokines and was originally isolated from avian ocular tissue and named because of its survival promoting effects on ciliary ganglion neurons (Adler et al. 1979; Manthorpe et al., 1980). Since then there have been numerous reports on the neuroprotective actions of CNTF on CNS neurons including retinal ganglion cells (RGCs) and photoreceptors, however, the exact mechanisms remain largely unknown (Lavail et al., 1992; Clatterbuck et al., 1993; Mey and Thanos, Gravel et al., 1997; Cayouette and Gravel, 1997; 1993; Honjo et al., 2000). Several possibilities have been suggested including, inhibition of glutamate-mediated excitotoxicity (Semkova et al., 1999; Petersen and Brundin, 1999; Petersen et al., 1999; Honjo et al., 2000), modulation of glial cells conferring an additional protective capacity (Lisovoski et al., 1997; Monville et al., 2001; Albrecht et al., 2002; Liang et al., 2001a), activation of neuroprotective signal transduction pathways including the JAK (Janus tyrosine kinase) –STAT (signal transducers and activators of transcription) pathway (Peterson et al., 2000), as well as modulating neuroinflammation (Linker et al., 2002).

In the normal adult rat retina, CNTF is predominately localized in Müller glial cells and astrocytes, whereas retinal neurons, including RGCs, horizontal and amacrine cells, express CNTF receptor alpha (CNTFR $\alpha$ ) (Kirsch et al., 1997). The CNTF receptor complex is composed of its own unique component, CNTFR $\alpha$ , and two shared signal transducing transmembrane subunits, gp130

and leukemia inhibitory factor (LIF) receptor-beta (LIFR $\beta$ ) (Davis and Yancopoulos, 1993). The CNTFR $\alpha$  is anchored to the cell membrane via a glycosyl-phosphatidylinositol (GPI) linkage, and therefore can exist as a membrane bound or soluble form (sCNTFR $\alpha$ ). Regardless, both function strictly as a ligand binding chain for CNTF and have no signal transduction role other than to increase the formation of the CNTF receptor complex (Inoue et al., 1996). This in turn induces intracellular signal transduction cascades via the JAK-STAT pathway (Cattaneo et al., 1999; Sleeman et al., 2000). CNTF-mediated activation of this pathway is intriguing since CNTF lacks a consensus secretory signal sequence and is normally localized to the cytoplasm (Sendtner et al., 1994). However, under conditions of cellular stress CNTF can be secreted from glia by a yet unknown mechanism, and thus has been postulated to function as an injury-activated factor (Adler, 1993; Peterson et al., 2000). In agreement with this hypothesis, it has been shown that the expression of CNTF and CNTFR $\alpha$  are elevated in the rat retina following optic nerve (ON) transection, mechanical injury, ischemic insult, and light-induced damage (Wen et al. 1995, 1998; Kirsch et al., 1998; Ju et al., 1999, 2000; Chun et al., 2000; Weise et al., 2000; Walsh, 2001). Elevation of endogenous CNTF levels may serve to protect injured neurons in the inner retina, presumably via activation of CNTFR $\alpha$  (Chun et al., 2000). Alternatively, CNTF may protect neurons indirectly through an autocrine loop involving astrocytes and LIFR $\beta$ , which is expressed predominately on

mature astrocytes (Monville et al., 2001). The elevation of pSTAT<sub>3</sub> observed in Müller glia cells and nerve fiber layer (NFL) astrocytes after exogenous CNTF or Axokine (a CNTF analog) treatment provides experimental evidence that CNTF can activate retinal glia, and indirectly protect retinal neurons, such as photoreceptors, which do not express CNTFR $\alpha$  (Peterson et al., 2000). Taken together, these studies suggest that CNTF and its receptor components are important factors involved in protecting the injured retina.

Although the neuroprotective activity of CNTF against axotomy-induced apoptosis of RGCs has been well established (Mey and Thanos, 1993; van Adel et al., 2000; 2002; Weise et al., 2000), little is known about the mechanisms underpinning this neuroprotection. Several factors contribute to the demise of RGCs after optic nerve transection, including, glutamate excitotoxicity, reactive oxygen species, growth factor deprivation, and caspase activation (Kermer et al., 1998, Koeberle and Ball, 1999; So and Yip, 1998; Kikucki et al., 2000). Since exogenous CNTF treatment has been shown to increase the intermediate filament, glial fibrillary acidic protein (GFAP) in cultured astrocytes (Kahn et al., 1997; Levison et al., 1998; Monville et al., 2001) and after administration to the intact brain or eye (Lisovoski et al., 1997; Hudgins and Levinson, 1998; Peterson et al., 2000) we explored the possibility that CNTF may protect axotomized RGCs by increasing the expression of GFAP and several other glial markers, including the gap junction protein, connexin 43 (Cx43), the astrocyte-specific glutamate/aspartate transporter-1, GLAST-1, and the astrocyte specific enzyme,

glutamine synthetase (GS), previously demonstrated to have a neuroprotective function for the injured retinal or brain (Rothstein et al., 1996; Gorovits et al., 1997, Heidinger et. al., 1999; Barnett and Pow, 2000, Hansson et al., 2000; Vorwerk et al., 2000; Siushansian et al., 2001). In the present study we showed that overexpression of CNTF via adenoviral-mediated transfer increased the levels of CNTFR $\alpha$ , pSTAT<sub>3</sub>, GFAP, GLAST, and Cx43 in axotomized retinas compared to control treatments. Thus, it appears that in addition to the direct actions, CNTF may also protect axotomized RGCs via indirect actions by modulating retinal glia, which may in turn prevent the excitotoxic cascade known to contribute to the death of axotomized RGCs (Kikuchi et al., 2000).

## **Materials and Methods**

### ***Animals***

Naïve, adult, female Sprague-Dawley rats were obtained from Charles River Canada (St. Constant, Quebec) at six weeks of age (200-250 g). Animals were acclimatized to the McMaster University Central Animal Facility (CAF) in a level B pathogen controlled environment for approximately 2 weeks prior to surgical procedures. The rats were housed individually in shoebox cages and maintained in good health under the care of a veterinarian for the duration of the experiment. All rats were maintained on a 12 hr light-dark cycle (lights on at 7:00 a.m.) and both food and water were available *ad libitum*. Upon completion of all surgical procedures animals were monitored during a recovery period and then returned to the vivarium and kept under the care of a veterinarian during the course of the experiment.

### ***Adenoviral vectors***

The construction, production, purification and titration of the adenoviral vectors, and their *in vitro* and *in vivo* bioactivity, have been previously described (Braciak et al., 1993; Gravel et al., 1997). A control vector was designed with the E3 region of the adenovirus genome deleted, and a cassette containing *E. coli lacZ* gene, encoding  $\beta$ -Galactosidase driven by a cytomegalovirus (CMV) promoter, and the nuclear localization signal of the simian virus 40 (SV40) large T antigen, and SV40 polyadenylation signal were, inserted into the E1 region of the adenovirus genome by homologous recombination. The adenoviral vector

encoding CNTF was obtained from the laboratory of Dr. Claude Gravel (Laval University, Quebec) and was constructed in a similar way, except that the *lacZ* gene was replaced with a prepro-NGF leader sequences-CNTF chimeric gene fitted with a bovine growth hormone polyadenylation signal. As a positive control we compared the neuroprotective effects of Ad.CNTF with Ad.IL-6 since both cytokines belong to the IL-6-type cytokine family and use gp130 for receptor mediated signaling (reviewed in: Stahl and Yancopoulos, 1994, Richardson, 1994). The adenoviral vectors, designated as Ad.lacZ, Ad.CNTF, and Ad.IL-6 (schematically illustrated in Figure 1A) were propagated in 293 cells, purified and titers diluted to  $1.2 \times 10^{10}$  plaque forming units [pfu]/ml using vector vehicle consisting of 10 mM Tris-HCl, pH 7.4, 1 mM MgCl<sub>2</sub>, and 10% glycerol.

### ***Optic nerve transection***

Animals were deeply anesthetized with 7% Chloral Hydrate (420 mg/kg; ip) and placed in a Kopf stereotaxic head holder (David Kopf Instruments, Tujunga, CA). With the aid of a surgical microscope (Zeiss, Germany), the optic nerve was approached within the orbit by making a skin incision along the superior rim of the orbital bone. The superior orbital contents were carefully dissected and the rectus muscles were reflected laterally. To gain visual access to the optic nerve and surrounding meningeal sheath the eye was rotated in a temporal direction by transecting and then retracting segments of the extra-ocular muscles. Following rotation of the eye, the meningeal sheath was excised longitudinally and the exposed optic nerve was cut ~2 mm behind the eyeball



(see Fig. 1B). Care was taken to avoid damage or disruption to the retinal blood supply. Postoperative ophthalmoscopy was performed to verify the integrity of the retinal vasculature and any rat with a compromised retinal vasculature was excluded from the study.

### ***Retrograde labeling***

To quantify retinal ganglion cell survival after axotomy in animals with and without treatment, RGCs were retrogradely labeled by injecting 10  $\mu$ l of a 3% solution of Dextran-FITC (3000 MW, Molecular Probes, Eugene, OR) into the proximal nerve stump at the time of axotomy as previously described (Koeberle and Ball 1998). (see Fig. 1B). In several normal animals RGCs were retrogradely labeled by stereotaxically injecting D-FITC or a 2% solution of FluoroGold (Fluorochrome Inc. Englewood, CO) into the superior colliculus (SC) as previously described (van Adel et al., 2000).

### ***Intraocular injections***

Intraocular injections were administered using a 5  $\mu$ l SGE microlitre syringe (Type A-RN, Austin, TX; 0.11 mm outer diameter needle). Care was taken to avoid damage to the lens and the anterior structures of the eye, since this has been shown to cause the release of growth factors and increase RGC survival (Mansour-Robaey et al., 1994; Leon et al., 2000). After injecting the solution, the needle was held in place for 2 minutes and withdrawn slowly and the puncture in the sclera was immediately sealed using Vetbond tissue cement (3M, St. Paul, MN). To determine the neuroprotective properties of CNTF on

axotomized RGCs, animals received intraocular injections of adenoviral vectors or recombinant protein. One group of animals received intravitreal injections of a 5  $\mu$ l suspension ( $1.2 \times 10^{10}$  pfu/ml) of Ad.CNTF, or Ad.lacZ (minimum of n=3/per group) and survived 7, 14, and 21 days after axotomy (dpa). A second group of animals received an intravitreal injection of one of the following: 5  $\mu$ l of recombinant human CNTF (0.8  $\mu$ g/ $\mu$ l, Leinco Technologies, St. Louis, MO), or recombinant mouse LIF (0.8  $\mu$ g/ $\mu$ l, R&D Biochemicals, Canada) dissolved in 5  $\mu$ l of sterile PBS; a 5  $\mu$ l suspension of Ad.CNTF vector diluted tenfold (1/10) ( $1.2 \times 10^9$  pfu/ml), or Ad.IL-6 ( $1.2 \times 10^{10}$  pfu/ml); or control injections of PBS vehicle or Ad.lacZ ( $1.2 \times 10^{10}$  pfu/ml). Another group of animals received intraocular injections of 5  $\mu$ l suspension of Ad.lacZ or Ad.CNTF without axotomy in order to determine the type and quantity of cells transduced in the retina as well as the timecourse of transgene expression. In addition, several animals received an intraocular injection of 5  $\mu$ l of undiluted Ad.lacZ ( $3.4 \times 10^{10}$  pfu/ml) to assess whether recombinant adenovirus is toxic to retinal neurons in non-axotomized animals. To evaluate viral toxicity, RGCs were pre-labeled by injecting FG into the SC one week before intravitreal administration of adenovirus. The number of FG pre-labeled RGCs from retinas treated with adenoviral vector was compared to normal and control (saline injected) retinas 21 days following injection of Ad.lacZ into the vitreal chamber of the eye.

### ***Isolation and preparation of retinas for histology***

Rats were euthanized by an overdose of anesthetic and their eyes enucleated. The cornea and lens were dissected away and the remaining eyecups were fixed for 1.5 hours in a solution consisting of 4% paraformaldehyde and 2% sucrose in 0.1 M Sorensen's phosphate buffer (0.1 M, pH 7.3). Following fixation, eyes were rinsed in PBS for 10 minutes and flatmounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) to determine RGC densities, or sectioned on a cryostat and processed histochemically. One group of animals was used to determine the timecourse of RGC death at 1, 7, 14, 21, and 28 days post-axotomy. A second group of animals was used to determine the pattern of GFAP, GLAST, GS, and Cx43 labeling in the normal and axotomized retina. A third group of animals was used to determine the cells transduced in the retina after intravitreal injection of adenoviral vectors. A fourth group of animals was used to determine the neuroprotective properties of adenoviral-mediated overexpression of CNTF at 7, 14, and 21 dpa relative to control treatments (Fig. 1C). The survival promoting effects of Ad.CNTF were compared to Ad.IL-6 and Ad.lacZ as well as recombinant CNTF or LIF at 14 dpa.

### ***Antibodies***

Immunohistochemistry and Western blotting were performed using the following list of antibodies obtained commercially: FG (rabbit polyclonal, Chemicon, 1:1000), GFAP (mouse monoclonal, Sigma G3893, 1:200, or goat

polyclonal, Santa Cruz, 1:200), Vimentin (mouse monoclonal, Boehringer-Mannheim F814-318, 1:100),  $\beta$ -galactosidase (rabbit polyclonal, Five Prime Three Prime, Boulder, CO, 1:500), CNTF (goat polyclonal, Santa Cruz, 1:200), CNTFR $\alpha$  (goat polyclonal, Santa Cruz, 1:200), pSTAT<sub>3</sub> (goat polyclonal, Santa Cruz, 1:150), Cx43 (rabbit polyclonal, Zymed, 1:1000), GS (rabbit polyclonal, Santa Cruz, 1:200), GLAST-1 (guinea pig polyclonal, Chemicon, 1:500), THY-1 (mouse monoclonal, Chemicon, 1:400).

### ***Immunohistochemistry***

Immunoreactivity was performed using the indirect immunofluorescence method. The details of the staining procedures have been previously described (Phokeo and Ball, 2000; Koeberle and Ball, 2002; Zhang et al., 2002). Primary antibodies were detected using FITC or Texas Red conjugated secondary antibodies (BioCan, 1:200). Immunofluorescence was visualized using a Zeiss LSM 510 confocal microscope with a 488 nm Ar laser excitation for FITC and 585 nm HeNe laser excitation for Texas Red.

### ***X-gal histochemistry***

Several retinas that had received intravitreal injections of Ad.lacZ were processed to determine the cellular localization of  $\beta$ -galactosidase using X-gal histochemistry (5-bromo-4-chloro-3-indoyl- $\beta$ -D-galctopyranoside, Boehringer-Mannheim, Mannheim, Germany). Retinal sections were washed three times in 100 mM PBS (pH 7.4) and then incubated in 2mM MgCl<sub>2</sub>, 25 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 25

mM  $K_4Fe(CN)_6$ , and 1 mg/ml X-gal in PBS for 4-6 hours at room temperature. Upon completion of the staining, sections were washed three times in 100 mM PBS, coverslipped and visualized and photographed using transmitted light microscopy.

### ***Cell counting***

D-FITC fluorescence and FG immunofluorescence were visualized using a Zeiss Laser Scanning Confocal Microscope (LSM-510). Labeled RGCs were counted from confocal micrographs covering an area of  $78,000 \mu m^2$  as previously described (Koeberle and Ball, 1998). Each retina was divided into 4 radial quadrants, and confocal micrographs of 12 areas (3 from each quadrant) from the mid-periphery (1/2 the retinal eccentricity) of each flatmounted retina were quantified. Microglial cells that had been transcellularly labeled with D-FITC due to phagocytosis of dead RGCs were distinguished from intact RGCs on the basis of their size and morphology, and excluded from the cell counts. The results for separate retinas from the same treatment group were pooled for statistical analysis and mean ( $\pm$  SD) RGC densities (/mm<sup>2</sup>) are presented.

### ***Isolation and preparation of tissues for Western blotting***

Animals were euthanized by an overdose of anesthetic and their eyes enucleated and used for quantitative protein analysis. Two groups of animals were used to determine the time-course of transgene expression (CNTF and  $\beta$ -galactosidase) after intravitreal injection of Ad.CNTF or Ad.lacZ into normal non-axotomized eyes. Retinas from this group of animals were isolated at 1, 7, 14,

and 28 days post-injection. A third group of animals was used to study the expression of CNTF, CNTFR $\alpha$ , Cx43, GFAP, GLAST, GS, and pSTAT<sub>3</sub> in normal or axotomized retinas 7 days after intraocular injection of Ad:CNTF and compared to control Ad.lacZ or PBS injections.

### ***Western blotting***

Rat retinas from control and all surgical groups were homogenized and the protein was extracted using Trizol. Protein pellets were solubilized in 2% SDS and protein determination was done following the Bradford method. Equal amounts (20 $\mu$ g) of protein were diluted in 6x sample buffer, boiled for 5 minutes, and then separated by 10-15% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were probed with commercially available antibodies for CNTF, CNTFR $\alpha$ , Cx43, GFAP, GLAST, GS and pSTAT<sub>3</sub> diluted in 1% BSA Fraction V in TBST at 4°C overnight. Membranes were then washed three times in TBST and probed with alkaline phosphatase conjugated goat anti-rabbit IgG, donkey anti-goat IgG, or goat anti-guinea pig IgG (Jackson Laboratories, 1:4000) for 1hr. Membranes were then washed three times in TBST and colourimetric detection was by BCIP/NBT. Membranes were then scanned and optical densities of labeled bands were measured using imaging software (ImagePro for Windows). Densitometric data are presented as the relative optical density based on a percentage increase or decrease from normal control retinas arbitrarily set at 100%.

### ***Statistical analysis***

Statistical analysis of the results was performed using SPSS 8.0. Statistical significance was determined using one-way Analysis of variance (ANOVA) followed by post hoc comparisons for significance between groups at  $p < 0.05$ .

## Results

### ***Effect of optic nerve transection on RGC densities and THY-1 expression***

In normal rats, RGCs retrogradely labeled from the SC using D-FITC revealed a mean ( $\pm$  SD) RGC density in the mid-periphery of the retina to be  $2461 \pm 50$  RGCs/mm<sup>2</sup> (Fig. 2A). Similar results were obtained when RGCs were retrogradely labeled at the time of axotomy by injecting D-FITC into the proximal nerve ending (Fig. 2B.). We observed no significant difference in the mean number of D-FITC labeled RGCs ( $2381 \pm 102$  RGCs/mm<sup>2</sup>) at 2 dpa compared to normal retinas retrogradely labeled from the SC using either D-FITC or FG ( $p=.506$ ). This result is consistent with previous studies that have shown that no appreciable RGC death occurs until 4 days after optic nerve transection (Villegas-Perez et al., 1988; Villegas-Perez et al., 1993; Peinado-Ramon et al., 1996; Koeberle and Ball, 1998). By contrast, at 7, 14, 21, and 28 dpa there was a significant reduction in the number of D-FITC retrogradely labeled RGCs ( $F_{(5,57)} = 1283.9, p<.001$ , ANOVA) (Fig. 2C, D, and E) and a concomitant increase in the number of phagocytic microglia. These cells were easily distinguishable from D-FITC labeled RGCs since they are morphologically different from RGCs and have a significantly smaller diameter than RGCs (Thanos, 1991; 1993). As Fig.2G shows there was a relatively moderate decline in the mean RGC density at 7 dpa ( $1709 \pm 166$  RGCs/mm<sup>2</sup>). Retinas isolated at 14 dpa revealed a drastic reduction in the mean RGC density ( $369 \pm 85$  RGCs/mm<sup>2</sup>), which had declined to 14.9% of the density of RGCs observed in normal retinas (Fig. 2E). Although, a



further decline was observed at 21 ( $193 \pm 26$  RGCs/mm<sup>2</sup>) and 28 dpa ( $174 \pm 30$  RGCs/mm<sup>2</sup>), the greatest rate of RGC death occurred between 7 and 14 dpa and is consistent with previous reports (Villegas-Perez et al., 1988; Villegas-Perez et al., 1993). We also examined the change in the expression of the retinal ganglion cell marker, THY-1 following optic nerve transection (Barnstable and Dräger, 1984) as a possible alternative method to using retrograde labeling to measure RGC survival. Using double labeling immunohistochemistry, it was demonstrated that THY-1 labeling in the ganglion cell layer of the normal rat retina was colocalized with RGCs retrogradely labeled with FG from the SC (Fig. 3A and 3B). Using western blotting, it was shown that there was a drastic reduction in the expression of THY-1 ( $F_{(3,8)} = 34.7$ ,  $p < .001$ , ANOVA) at 1 (~47% reduction over non-axotomized controls,  $p < .005$ ), 7 (~65% reduction,  $p < .001$ ), and 14 (~80% reduction,  $p < .001$ ) dpa compared to normal retinas (Fig 3C). This result is in agreement with a recent study that demonstrated the loss of *thy-1* mRNA occurs well in advance of any detectable RGC death (Schlamp et al., 2001). Thus, the measurement of total retinal THY-1 expression does not appear to be a practical method for following RGC death.

### ***Transfection of retinal glia after intravitreal delivery of Ad.lacZ***

Intraocular administration of Ad.lacZ resulted in the transduction of primarily retinal Müller cells (Fig. 4). Müller cell somata and radial processes extending from the inner limiting membrane (ILM) to the outer limiting membrane (OLM) were labeled with markers for  $\beta$ -galactosidase ( $\beta$ -gal) (Fig. 4A, and B).

The end feet of transduced Müller cells were easily visualized in flatmounted retinas (Fig. 4C). An average of less than 1% of the total population of retinal Müller cells were transduced following intraocular injection of Ad.lacZ, although the densities of transduced cells varied between <1% to approximately 5% in different areas of the retina. In addition, a very small number of astrocytes in the nerve fiber layer were transduced by intraocular injections of Ad.lacZ. We also observed small immune-like cells in the nerve fiber layer (NFL) adjacent to the vitreous (Fig. 4D). It has recently been reported that intravitreal injection of adenoviral vectors encoding the gene for bacterial  $\beta$ -galactosidase evokes a host immune response in the retina that was not detected with viral vectors encoding neurotrophic factors (Isenmann et al., 2001). We were concerned that Ad.lacZ-induced immune responses in the retina may contribute to the death of RGCs in non-axotomized retinas since previous reports have demonstrated a delayed cytotoxicity after adenovirus administration into the CNS (Easton et al., 1998). To test this hypothesis, we pre-labeled RGCs by stereotaxically injecting FG into the SC and one week later made intraocular injections of Ad.lacZ (using a higher titre than used for neuroprotection studies:  $3.4 \times 10^{10}$  pfu/ml) and RGC survival was quantified 21 days post-injection (dpi) in flatmounted retinas (Fig. 4E and F). There was no significant difference ( $p=.204$ ) in the numbers of FG retrogradely labeled RGCs in eyes with intraocular injection of Ad.lacZ or PBS (Fig. 4G). This result suggests that although the Ad.lacZ vector evoked an immune response in

the retina, it was not cytotoxic to RGCs over the time period used to study RGC survival in this study.

***Adenoviral-mediated transgene expression in retina glia is not long-lasting***

To provide further evidence of the successful transduction of retinal cells after intraocular injections of adenoviral vectors, a group of non-axotomized animals received intraocular injections of Ad.lacZ or Ad.CNTF, and homogenized rat retinas were probed for transgene expression using Western blotting analysis. As shown in Fig. 5A, a  $\beta$ -gal-immunoreactive band of approximately 66 kDa was present in extracts from normal and PBS or Ad.lacZ treated retinas. Densitometric analysis of the  $\beta$ -gal-like immunoreactive proteins revealed that intraocular injection of the control Ad.lacZ vector at 1 and 7 days post-injection showed a significant increase in  $\beta$ -gal expression (Fig. 5A). Western blot analysis of retinal lysates using a polyclonal antibody raised against CNTF demonstrated the presence of two prominent immunoreactive bands with the molecular sizes of 23 (monomer) and 46 kDa (dimer). The same two bands were also detected in blots with control rat recombinant CNTF and have been observed in several previous reports (Kirsch et al., 1997; Liang et al., 2001). Densitometric analysis of the 23 kDa band demonstrated a significant increase in CNTF expression at 7 days after intravitreal Ad.CNTF treatment (Fig. 5B). Intravitreal control injections of PBS or Ad.lacZ did not significantly increase retinal CNTF levels (see Fig. 12A, and Table 1). Based on these results it was

apparent that adenoviral vectors do not result in long-term transgene expression as there was no significant increase in  $\beta$ -galactosidase or CNTF expression in retinas examined at 14 or 28 days after intravitreal treatment. Nevertheless, the Ad.CNTF vector used in this study was able to elevate retinal CNTF levels beyond four days, a critical period at which axotomized RGCs begin to die (Berkelaar et al., 1994).

### ***Effect of Ad.CNTF on the survival of axotomized RGCs***

We tested the effects of an adenoviral vector encoding human CNTF on the survival of axotomized RGCs at 7, 14 and 21 days after ON transection. RGC survival was determined by counting the number of D-FITC retrogradely labeled RGCs in flatmounted retinas treated with Ad.CNTF or Ad.lacZ. Control intraocular injections of Ad.lacZ (Fig. 6A, 6C and 6E) did not demonstrate any significant increase in RGC survival compared to untreated axotomized retinas at 7, 14 and 21 dpa. RGC densities in Ad.lacZ treated retinas at 7, (1636  $\pm$  160 RGCs/mm<sup>2</sup>), 14 (328  $\pm$  46 RGCs/mm<sup>2</sup>) and 21dpa (193  $\pm$  26 RGCs/mm<sup>2</sup>) represented ~66%, 13% and 8% of the original RGC population (2461  $\pm$  50 RGCs/mm<sup>2</sup>). In contrast, intraocular administration of Ad.CNTF significantly enhanced RGC survival compared to control treatment (Fig. 6B, 6D and 6F). The densities of surviving RGCs in Ad.CNTF treated animals at 7 (2383  $\pm$  94 RGCs/mm<sup>2</sup>), 14 (1353  $\pm$  225 RGCs/mm<sup>2</sup>) and 21 dpa (366  $\pm$  91 RGCs/mm<sup>2</sup>), represented 96%, 55%, and 15% of the normal RGC density, and a significant increase in RGC survival of 30% at 7 ( $p < 0.01$ ), 42% at 14 ( $p < 0.001$ ), and 7% at

21 ( $p < 0.05$ ) dpa respectively (Fig.4 G). By contrast, a ten-fold dilution of the Ad.CNTF protected significantly less axotomized RGCs (10.5%) than injections of undiluted Ad.CNTF at 14 dpa. No neuroprotection was seen at dilutions of 100, or 1000 fold of the stock Ad.CNTF vector suggesting that a critical number of viral particles are needed to obtain a significant increase in cell survival.

#### ***Effects of recombinant CNTF and LIF on the survival of axotomized RGCs***

The survival of axotomized RGCs at 14 dpa in untreated and PBS control injected retinas was compared to a single intraocular injection of 4  $\mu$ g of rhCNTF, or rmLIF, as well as an adenoviral vector encoding the gene for rodent interleukin-6 (Ad.IL-6). The mean RGC density of rhCNTF treated retinas ( $688 \pm 41$  RGCs/mm<sup>2</sup>) or rmLIF treated retinas ( $547 \pm 39$  RGCs/mm<sup>2</sup>) was significantly higher ( $p < 0.05$ ) than control retinas treated with PBS ( $325 \pm 91$  RGCs/mm<sup>2</sup>) (Fig.7A). This result was not surprising since both LIF and CNTF signal through LIFR $\beta$  (Stahl and Yancopoulos, 1994). We also tested the neuroprotective properties of Ad.IL-6 vector, since this cytokine has previously been shown to be neuroprotective (Carlson et al., 1999) and shares gp130 as a common receptor component with CNTF and LIF (Gadiant and Otten, 1997; Fukada et al., 1999). In comparison to the control Ad.lacZ we consistently observed less surviving RGCs in Ad.IL-6 treated retinas at 14 dpa, demonstrating that in this model IL-6 is not neuroprotective (Fig. 7A). Taken together, these results suggest that activation of the LIFR $\beta$  may mediate the survival-promoting properties of IL-6-

type cytokines, such as CNTF and LIF on the survival of axotomized RGCs *in vivo* (Fig. 7B).

#### ***Effects of CNTF on nerve fiber layer integrity after axotomy***

The integrity of RGC axons in the nerve fiber layer of retinas isolated at 14 dpa was examined using immunohistochemistry directed against the 200-kDa neurofilament protein, RT97 (Fig. 8) (Anderton et al., 1982). In normal retinas, RT97 stained small and large bundles of RGC axons in the nerve fiber layer (Fig. 8A). By contrast, untreated or axotomized retinas treated with a control injection of PBS (Fig 8B) or Ad.lacZ (Fig. 8E), showed a drastic reduction in the amount of RT97-immunoreactive RGC axons. The appearance of the remaining axons in these axotomized retinas was characterized by varicosities along their length giving the axons a beaded appearance that is characteristic of degenerating axons. In comparison, retinas treated with rmLIF (Fig. 8C), rhCNTF (Fig. 8D), or Ad.CNTF (Fig. 8F) at the time of optic nerve transection had less axonal beading and thicker axon fascicles than control retinas at 14 dpa. Axonal integrity appeared highest in the Ad.CNTF treated retinas, presumably because of the increased production of CNTF in these retinas.

#### ***Expression of GFAP, GLAST, GS, and Cx43 in the normal retina***

The expression of the glial markers GFAP, GLAST-1, GS and Cx43 were examined in the normal rat retina using double label immunohistochemistry and confocal microscopy. It was possible to differentiate retinal glia since nerve fiber layer astrocytes express GFAP (Fig. 9A-C) and Müller cells express vimentin

(Fig. 9D-F) as their primary intermediate filament. We observed no colocalization of vimentin and GFAP immunoreactivity in the normal rat retina. Vimentin immunoreactive Müller cells, and GFAP immunoreactive astrocytes were double labeled with antisera directed against the glutamate transporter, GLAST-1 (Fig. 10A-F). Similarly, both Cx43 (Fig. 10G-J) and GS (Fig. 10O) were colocalized to vimentin and GFAP immunoreactivity glia in transverse retinal sections from normal eyes. The most intense Cx43 immunoreactivity was observed in the retinal pigment epithelial (RPE) (Fig. 10J). In flatmounted retinas, Cx43 (Fig. 10K) and GFAP (Fig. 10L) staining clearly delineated the blood retinal barrier. The vitreal surface of retinal blood vessels was covered by a meshwork of Cx43 and GFAP immunoreactive astrocyte processes. There was also intense Cx43 and GFAP immunoreactive astrocyte staining in the optic nerve head (Fig. 10M and N). In summary, these results show that GLAST-1, GS, and Cx43 are expressed in nerve fibre layer astrocytes and Müller glial cells in the normal rat retina and are consistent with previous reports in the literature (Riepe and Norenburg, 1977; Rauen et al., 1996; Ball and McReynolds, 1998; Johnson et al., 2000).

Immunohistochemical analysis of GFAP, GLAST-1, GS and Cx43 staining was examined in transverse sections of retinas isolated at 7 days post-axotomy (Fig. 11). There was a robust increase in GFAP (Fig. 11A and B) and Cx43 (Fig. 11G and H) expression in retinal Müller cells after optic nerve transection. There was an increase in the intensity of GLAST-1 (Fig. 11C and D) and GS (Fig. 11E

and F) immunoreactivity in astrocytes and Müller cells at 7 dpa compared to control retinas. These results were confirmed using Western blotting analysis (Fig. 11B and Table 2).

***Effects of virally-mediated overexpression of CNTF on the expression of CNTF, CNTFR $\alpha$ , Cx43, GFAP, GLAST, GS, pSTAT<sub>3</sub> in the normal and axotomized retina***

Western blotting analysis was used to examine the effects of CNTF treatment on the expression of CNTFR $\alpha$ , pSTAT<sub>3</sub>, GFAP, GLAST, GS, and Cx43 at 7 days after intravitreal injection of Ad.CNTF or Ad.lacZ in normal and axotomized retinas. Retinas were isolated at 7 days after treatment since it was demonstrated that retinal levels of CNTF peak 7 days after intravitreal injection of Ad.CNTF. We first determined that intraocular injection of Ad.CNTF increased CNTF levels by approximately 2.25 fold in normal retinas compared to control treatment with PBS or Ad.lacZ. This in turn resulted in the elevation CNTFR $\alpha$ , pSTAT<sub>3</sub>, GFAP, GLAST, and Cx43 and a decrease in GS levels in non-axotomized normal retinas (Fig. 12A and Table 1). Similar increases of CNTFR $\alpha$ , pSTAT<sub>3</sub>, GFAP, GLAST, and Cx43 were seen 7 days after stereotaxic injection of the same number of Ad.CNTF particles into the SC. We hypothesized that a similar modulation of retinal glia may take place in axotomized retinas treated with high levels of exogenous CNTF. Indeed, at 7 days following optic nerve transection, Ad.CNTF administration significantly



increased retinal CNTF levels (5.75 fold), which resulted in increased levels of CNTFR $\alpha$ , pSTAT<sub>3</sub>, GFAP, GLAST, GS, and Cx43 expression compared to non-treated axotomized retinas as well as axotomized retinas that received control injections of PBS or Ad.lacZ (Fig. 12B, and Table 2). Furthermore, in comparison to controls, non-treated axotomized retinas showed elevated levels of GFAP, GLAST, GS, and Cx43 at 7 dpa, which is in agreement with the immunohistochemistry analysis of these factors in transverse retinal sections (Fig. 11A-H and Table 2).

Taken together, these results demonstrate that overexpression of CNTF via adenoviral vectors had significant effects on the survival of axotomized RGCs as well as the expression of several neuroprotective glial markers, and provides evidence that CNTF treatment may have indirect neuroprotective effects on axotomized RGCs.

## Discussion

### ***CNTF is a survival factor for axotomized RGCs***

Intravitreal injection of recombinant CNTF or overexpression of CNTF using viral vectors, such as adenovirus, adeno-associated virus, or lentivirus has been shown to protect retinal ganglion cells (RGCs) from axotomy-induced apoptosis (Mey and Thanos, 1993; Weise et al., 2000; van Adel et al., 2002), neurons in the inner retina from NMDA-mediated cell death (Honjo et al., 1999) and photoreceptors from apoptosis following exposure to constant light (Lavail et al., 1992; Coyouette and Gravel, 1997; Peterson et al., 2000; Liang et al., 2001a, Bok et al., 2002). Consistent with these findings we demonstrated that CNTF delivery after optic nerve transection, whether by single intraocular treatment of recombinant protein or overexpression using adenoviral vectors, significantly increased the survival of axotomized RGCs. We also observed an increase in the integrity of RGC axons in the nerve fiber layer of axotomized retinas treated with Ad.CNTF, which is in agreement with previous studies that have demonstrated that CNTF is a potent axogenesis factor for RGCs both *in vitro* (Jo et al., 1999) and *in vivo* (Cui et al., 1999). We also compared the neuroprotective effects of a single intraocular injection of CNTF with LIF, since LIF has previously been shown to protect axotomized CNS neurons (Cheema et al., 1994; 1998) and shares the same two signalling receptors with CNTF, gp130 and LIFR $\beta$  (Stahl and Yancopoulos, 1994; Sleeman et al., 2000). Indeed, both recombinant CNTF and LIF protected approximately equal proportions of

axotomized RGCs, suggesting that activation of the CNTF receptor complex by CNTF, or direct dimerization of gp130 and LIFR $\beta$  by LIF, result in similar neuroprotective signal transduction cascades. We also compared the neuroprotective properties of adenoviral-mediated overexpression of CNTF with IL-6, since IL-6 can protect CNS neurons (Carlson et al., 1999) and as with CNTF, IL-6 signals via gp130 activation (Richardson, 1994; Fukada et al., 1999; März et al., 1999). In Ad.IL-6 treated retinas there were less surviving RGCs than control retinas or untreated retinas, which indicates that in this system IL-6 was not neuroprotective, but rather destructive. It is not known if RGCs express the receptor for IL-6R, nevertheless, overexpression of IL-6 in the retina resulted in the death of a small population of RGCs that had previously been shown to survive axotomy for an extended period of time (Villegas-Perez et al., 1993).

***CNTF may protect RGCs by directly binding to the CNTF receptor alpha expressed on RGCs***

It seems likely that CNTF is able to protect axotomized RGCs by binding directly to the CNTFR $\alpha$ , which has been localized to RGCs (Kirsch et al., 1997). In support of the direct activation of the CNTF receptor complex on RGCs, it was recently shown that intravitreal injection of CNTF or Axokine (a CNTF analog) resulted in an increase in the amount of intracellular phosphorylated STAT3 (pSTAT<sub>3</sub>) in retinal ganglion cells (Peterson et al., 2000). In the present study, western blotting analysis demonstrated that intraocular injection of Ad.CNTF into normal and axotomized retinas resulted in a dramatic increase in CNTFR $\alpha$  and

pSTAT<sub>3</sub>. The increase in CNTFR $\alpha$  may have enhanced the responsiveness of axotomized RGCs to exogenous levels of CNTF. Moreover, the CNTF encoded by the adenovirus used in this study was engineered so that it would be secreted from cells transduced by the vector (Gravel et al., 1996; Coyouette and Gravel, 1997). Although intraocular injection of adenoviral vectors was shown to transduce only a small percentage of retinal Müller cells, western blotting showed that seven days after injection of the Ad.CNTF the overall levels of CNTF in the retina had more than doubled. This alone may account for the increased protection of axotomized RGCs at 7, 14 and 21 days after optic nerve transection, as well as the increased protection of RGCs in Ad.CNTF treated retinas compared to a single intraocular treatment of 4  $\mu$ g of recombinant CNTF. Indeed, single or multiple intravitreal treatment(s) of CNTF below 1  $\mu$ g does not protect axotomized RGCs (Cho et al., 1999; Cui et al., 1999) suggesting that CNTF must be delivered in high concentrations. Moreover, the CNTF protein has a half-life of only 1.5 minutes (Sendtner et al., 1997) and a single injection of relatively small amounts of rhCNTF or CNTF production from relatively few adenovirally-transduced Müller cells, as in the cases with injections of a ten-fold dilution of the Ad.CNTF, may not have been sufficient to protect a large proportion of axotomized RGCs.

Taken together, these results as well as results from previous studies (Mey and Thanos, 1993; Peterson et al., 2000 Weise et al., 2000) indicate that

the neuroprotective effect of CNTF may occur through direct actions on RGCs. Activation of the CNTFR $\alpha$  expressed on axotomized RGCs may have resulted in the stimulation of the JAK-STAT signalling pathway as well as the ras-MAPK and PI-3K pathways, which have been shown to be neuroprotective for RGCs as well as other CNS neurons (Kermer et al., 2000; Klocker et al., 2000; Peterson et al., 2000; Weise et al., 2000; Alonzi et al., 2001). For example, CNTF has been shown to prevent glutamate-mediated excitotoxicity (Petersen and Brundin, 1999), thus CNTF –dependent activation of these signal transduction pathways may work in concert to modify the expression or function of glutamate receptors and thus reduce the duration of excitotoxic stimulation, which has been shown to contribute to the demise of axotomized RGCs (Kikuchi et al., 2000).

***CNTF may also protect RGCs indirectly by binding to the LIF receptor beta expressed on retinal glia.***

Several lines of evidence suggest that CNTF may also have indirect neuroprotective properties through modulation of astrocytes. First, CNTF has been shown to induce hypertrophy of astrocytes and it significantly affects astrocyte phenotype (Winter et al., 1995; Lisovoski et al., 1997; Albrecht et al., 2002). Second, CNTF can bind with a lower affinity to the LIFR $\beta$ , and initiate the same signal transduction cascade as gp130 and LIFR $\beta$ , without CNTFR $\alpha$ , provided that the extracellular concentration of CNTF is significantly elevated (Monville et al., 2000). The heterodimerization of gp130 and LIFR $\beta$ , which are

initially unassociated, activates the intracellular signal transduction cascade of the JAK-STAT and ras-MAPK pathways (Monville et al., 2000). Consistent with this finding, retinal Müller glial cells, which do not express CNTFR $\alpha$ , have been shown to increase their cytoplasmic levels of pSTAT<sub>3</sub> as early as 30 minutes after intraocular CNTF or Axokine treatment, as well as to increase their GFAP content (Peterson et al., 2000). The increase in pSTAT<sub>3</sub> may have resulted from CNTF binding directly to the LIFR $\beta$ , or the presence of the soluble CNTFR $\alpha$  (sCNTFR $\alpha$ ) may have conferred a complete receptor complex to mediate the high-affinity binding of CNTF to Müller cells *in vivo*. Similarly, exposure of cultured astrocytes to CNTF has been demonstrated to stimulate hypertrophy and activate the JAK-STAT pathway and induce transcriptional expression of GFAP (Kahn et al., 1997; Levison et al., 1998). Furthermore, astrocytes transduced with an adenovirus encoding a secreted form of CNTF show enhanced support of neuronal populations *in vitro* (Smith et al., 1996). Indeed, there is considerable evidence that CNTF can modulate the astrocyte phenotype. However, most analyses of the actions of CNTF on mature astrocytes have been based upon morphologic assessment and measuring increases in GFAP, while many other glial markers with known neuroprotective properties have not been examined. Thus, we explored the possibility that CNTF may protect axotomized RGCs by increasing the expression of GFAP as well as several other glial markers, including the gap junction protein, connexin 43 (Cx43), the astrocyte-

specific glutamate/aspartate transporter-1, GLAST-1, and the astrocyte specific enzyme, glutamine synthetase (GS). These markers were selected since their anatomical distribution in retina glia has been well documented (Rauen et al., 1996, 1998; Ball and McReynolds, 1998; Chen and Weber, 2002), and they have previously been demonstrated to have neuroprotective properties against high levels of excitotoxic glutamate (Rothstein et al., 1996; Gorovits et al., 1997, Heidinger et al., 1999; Barnett and Pow, 2000, Hansson et al., 2000; Vorwerk et al., 2000; Siushansian et al., 2001). Overexpression of CNTF via adenoviral-mediated transfer significantly increased the levels of GFAP, GLAST, and Cx43 in normal retina, and increased the expression of GFAP, GLAST, GS, and Cx43 in axotomized retinas compared to control treatments. Thus, it appears that in addition to the direct actions, CNTF may also protect axotomized RGCs via indirect actions by modulating retinal glia.

### ***Conclusions***

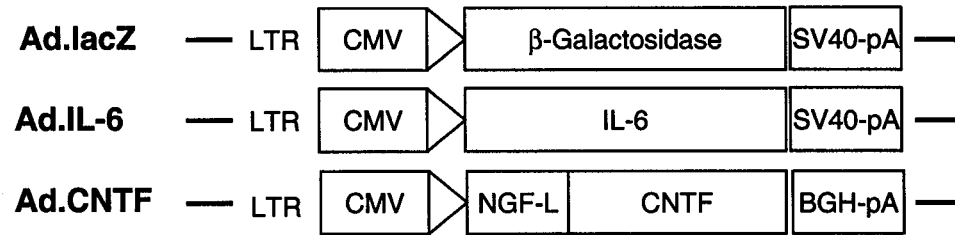
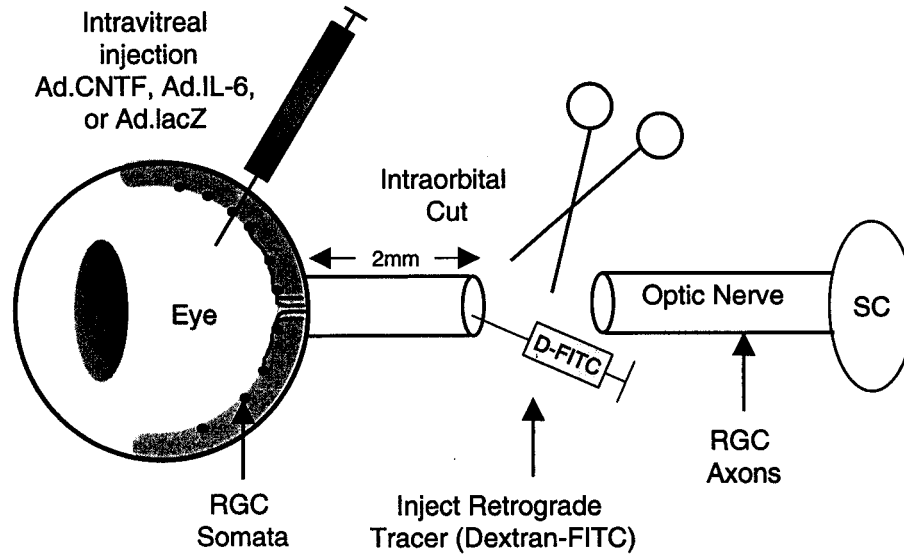
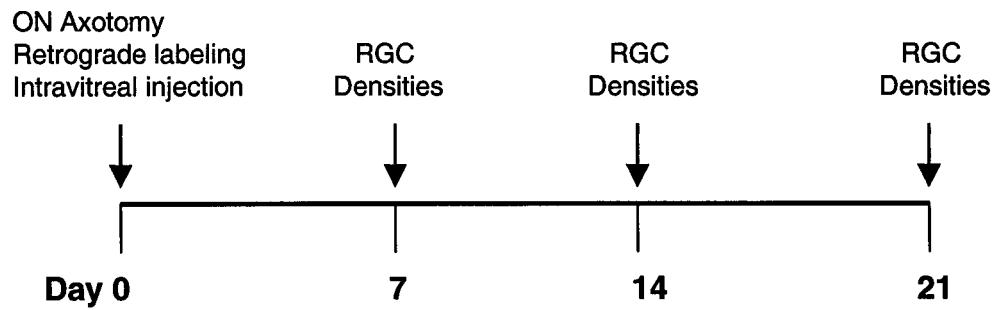
In summary, CNTF is a survival factor for severely injured retinal neurons. There is evidence to suggest that CNTF can act directly on injured RGCs. We have also raised the possibility that CNTF may protect RGCs indirectly by increasing the expression of several neuroprotective glial markers in axotomized retinas. The increased retinal expression of GFAP, GLAST-1, GS, and Cx43 in Müller glial cells may protect the injured retina from excitotoxic concentrations of glutamate believed to contribute to the demise of RGCs in glaucoma and in animal models of optic nerve injury.

## **Acknowledgements**

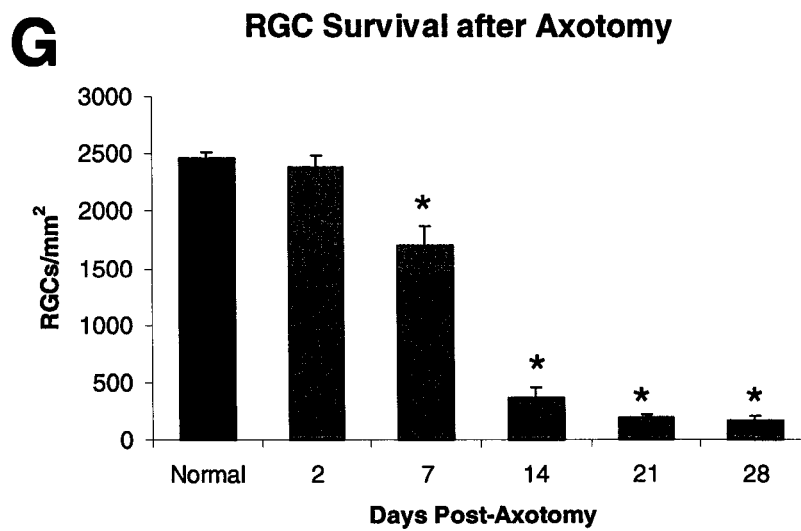
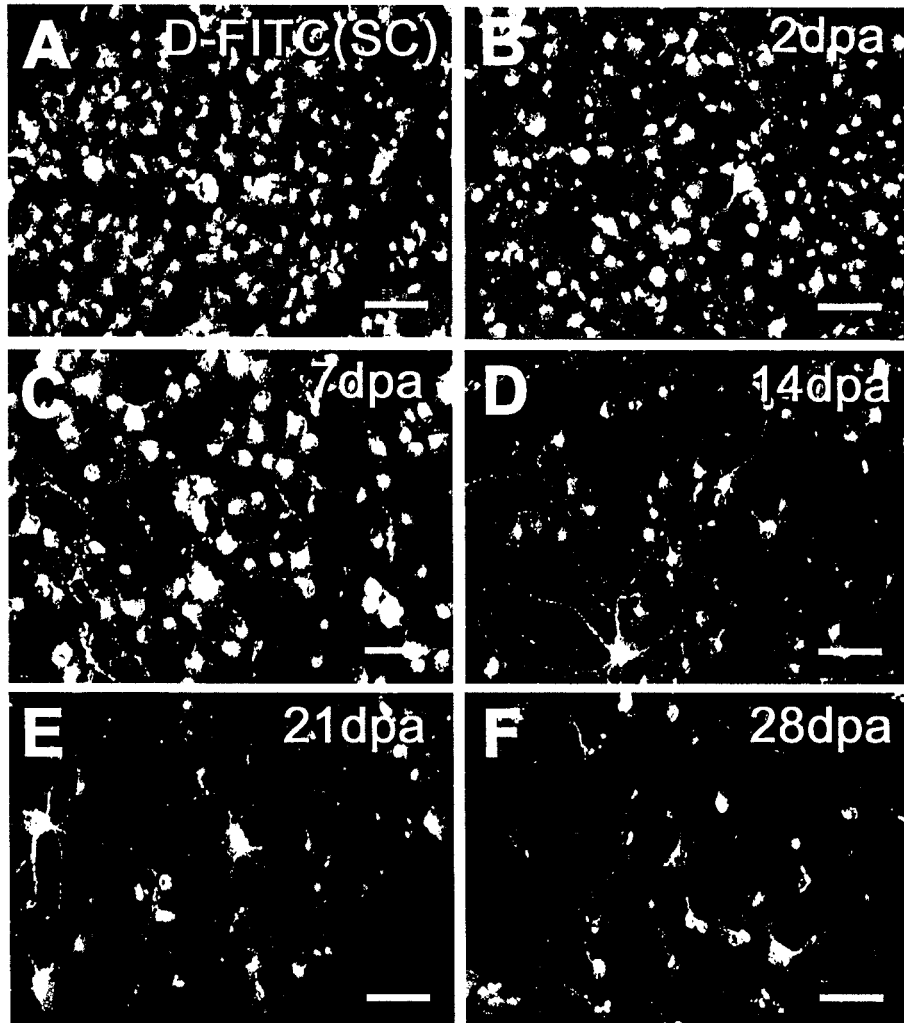
This research was supported by a grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) operating grant OGP0171190 to Dr. Ball. B. van Adel is supported by a studentship from CIHR and Dr. J.M. Arnold is supported by a fellowship from NSERC.



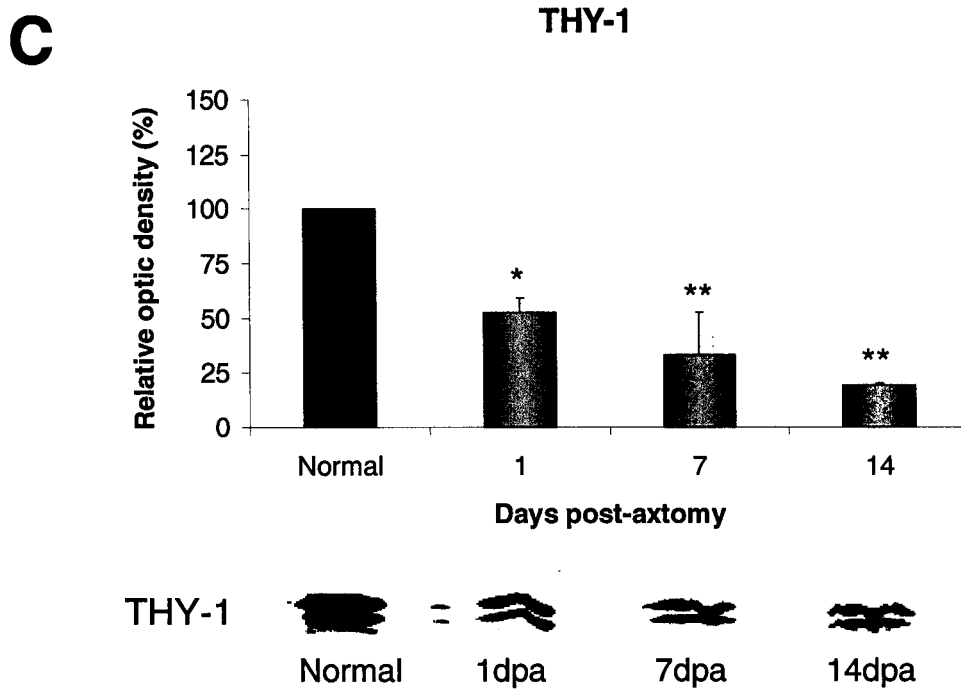
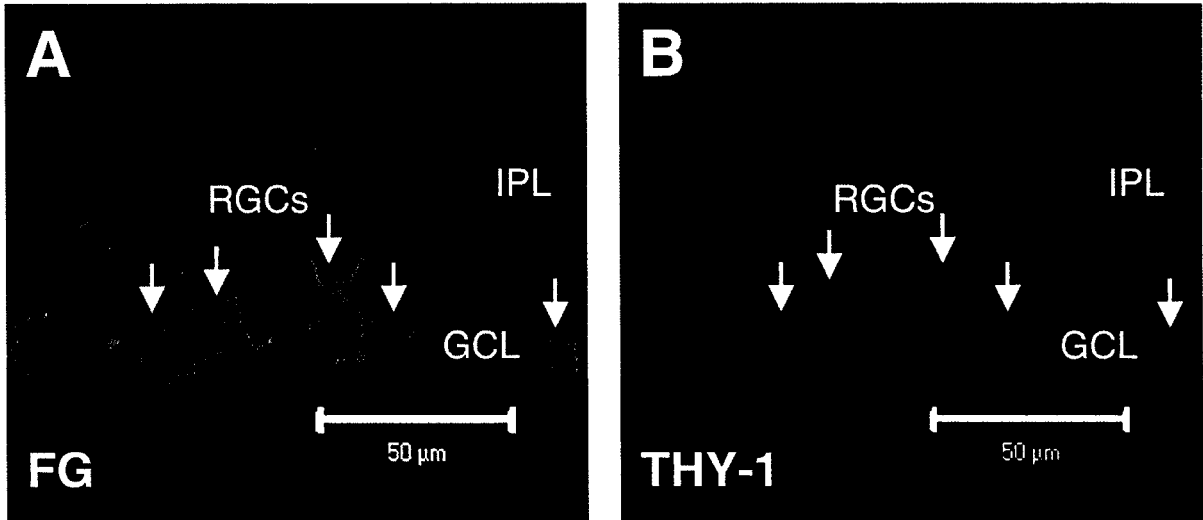
**Figure 1.** Summary of the adenoviral vectors, surgical procedures and experimental design. **(A)** Schematic representation of the replication-deficient adenoviral vectors used in this study. The genome of the vectors were based on the *d/309* adenovirus type 5 mutant and functional elements are shown in detail: coding regions for human CNTF, mouse IL-6 or the bacterial enzyme *Escherichia coli*  $\beta$ -galactosidase fitted with a SV40 polyadenylation signal (SV40-pA) under the transcriptional control of the cytomegalovirus (CMV) promoter. The CNTF vector contains a nerve growth factor signal sequence-*cntf* chimeric gene (NGF-L CNTF) fitted with a bovine growth hormone polyadenylation signal (BGH-pA). **(B)** Illustration of the surgical procedures used for optic nerve transection, retrograde labeling at the nerve stump and intravitreal injection of viral vectors and recombinant proteins into the vitreous chamber of the eye. **(C)** Experimental design to test the neuroprotective effects of overexpression of CNTF on the survival of axotomized RGCs *in vivo*. The neuroprotective effects of Ad.CNTF on the survival of axotomized RGCs at 14 dpa were compared with the following intraocular treatments: recombinant CNTF, recombinant LIF, a ten-fold dilution of the Ad.CNTF vector (Ad.CNTF 1/10) as well as an adenoviral vector encoding the gene for mouse interleukin-6 (Ad.IL-6).

**A****B****C**

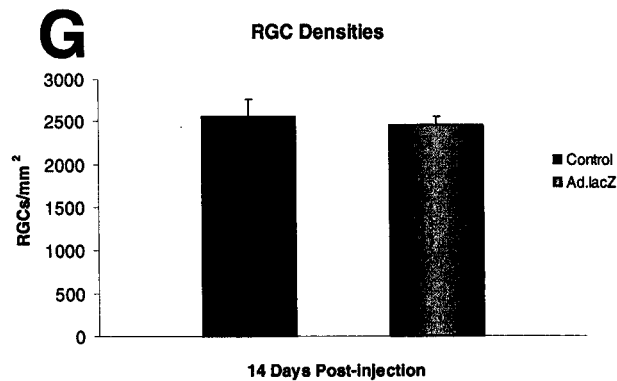
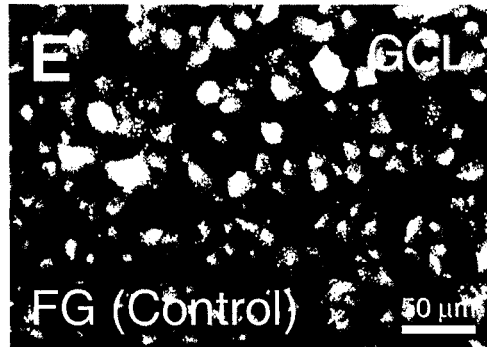
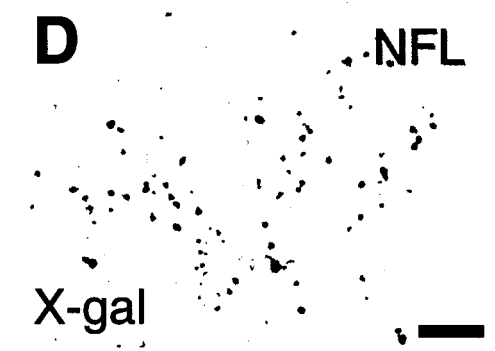
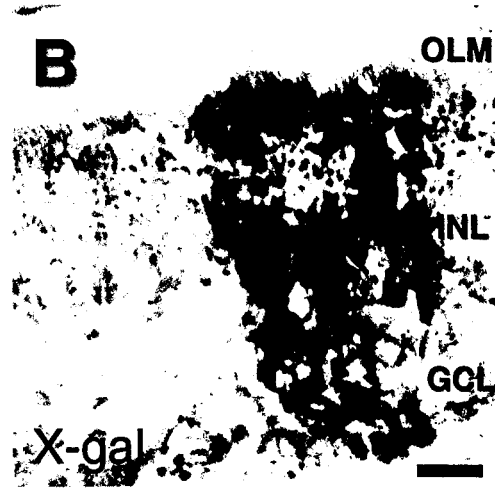
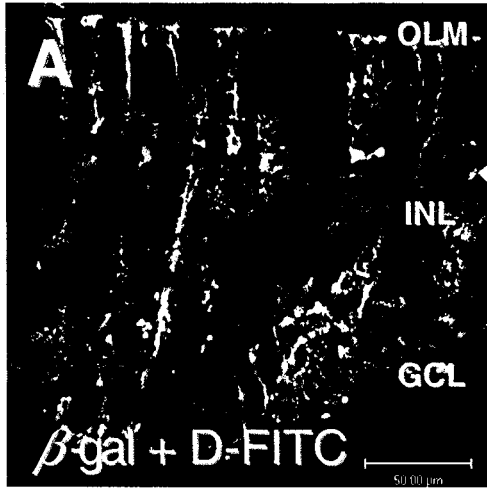
**Figure 2.** Confocal micrographs showing D-FITC retrogradely labeled RGCs in flatmounted retinas from a normal animal and at 2, 7, 14, 21, and 28 days after optic nerve transection 2 mm from the eye. **(A)** normal population of RGCs labeled in the rat retina after stereotaxic injection of D-FITC into the contralateral superior colliculus. **(B)** no significant loss of RGCs at 2 days post-axotomy (dpa). Note: RGCs were retrogradely after injection of D-FITC into the proximal ending of the optic nerve. **(C)** A noticeable loss of RGCs at 7 dpa and the appearance of transcellularly labeled microglia that have small cell bodies and ramified processes. **(D, E, and F)** a drastic reduction in RGCs at 14 (D), 21 (E) and 28 (F) dpa. **G:** Graph showing a significant decrease in RGC densities (mean  $\pm$  SD) in flatmounted retinas at 7, 14, 21, and 28 dpa ( $F_{(5,57)} = 1283.9, p < .001$ ). Post hoc analyses between groups showed no significant difference between the number of retrogradely labeled RGCs in normals and the 2 dpa group ( $p = .506$ ), however in comparison to normals or animals surviving 2 dpa, there were significant differences in the number of surviving RGCs at 7, 14, 21, and 28 dpa ( $*p < .001$ ). Bars = 50  $\mu$ m.



**Figure 3.** THY-1 is localized to RGCs in normal retinas and is dramatically reduced after optic nerve transection. **(A and B)** confocal micrographs showing the colocalization of FG retrogradely labeled RGCs (A) and THY-1 immunoreactivity in RGC somata (B). Bars = 50  $\mu$ m. **(C)** Immunoblot analysis of THY-1 expression in the adult rat retina after axotomy. Western blotting analysis showed a drastic reduction in the expression of THY-1 at 1, 7, and 14 days post axotomy (dpa). Protein extracts were prepared from dissected retinas and identical amounts of total protein (20  $\mu$ g/lane) were applied to the gel. The intensity of THY-1 immunoreactive bands was determined by densitometric analysis. Bars represent the mean ( $\pm$  SD) relative optical density as a percentage of normal retinas (100%) from N=3 replicates per treatment. Statistical analysis was performed using one-way ANOVA ( $F_{(3,8)} = 34.7$ ,  $p < .001$ ) followed by post-hoc (\* $p < .005$ , \*\*  $p < .001$ ).

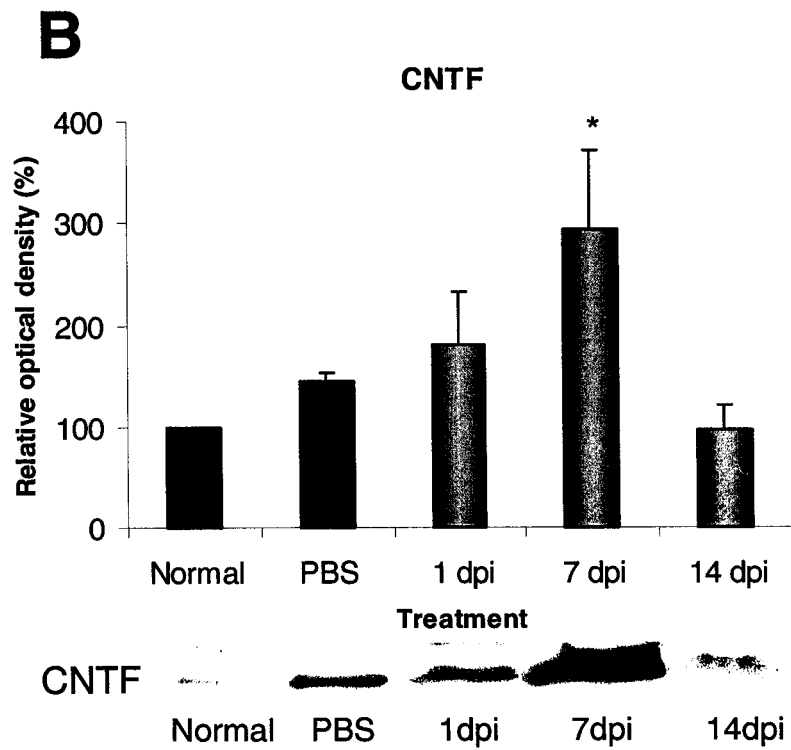
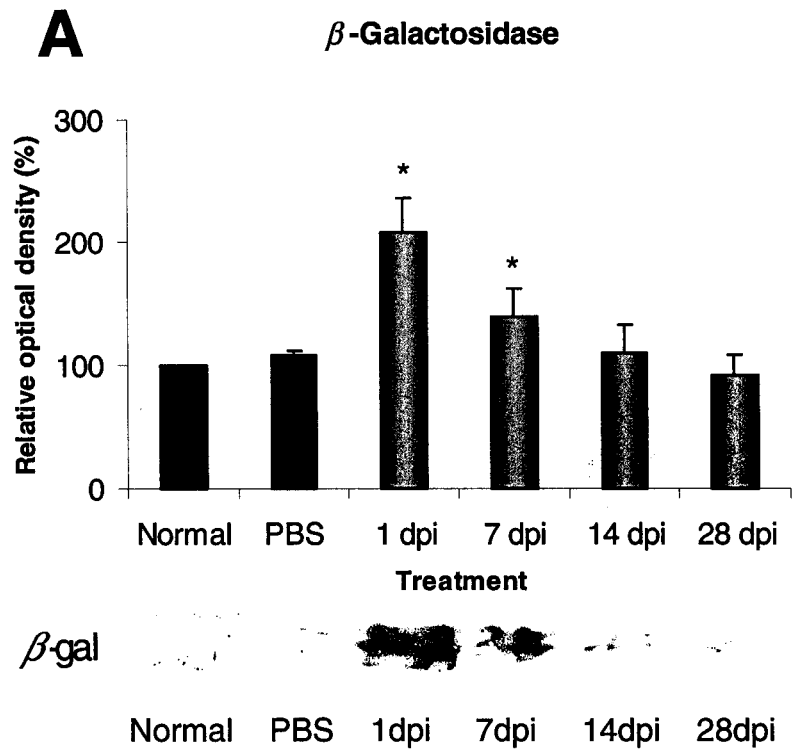


**Figure 4.** Confocal and transmitted light micrographs showing transgene expression at 2 and 7 days after intravitreal administration of Ad.lacZ. **(A)** a transverse retinal section showing  $\beta$ -galactosidase immunoreactivity in retinal Müller glial cells 2 days after intraocular viral injection. **(B)** A similar and specific staining pattern of retinal Müller cells with X-gal histochemistry. **(C)** Retinal flatmount showing Müller cell end feet in the ganglion cell layer, 7 days following intraocular injection of Ad.lacZ. **(D)** A photomicrograph taken of the same retinal flatmount as in C and focused in the nerve fiber layer (NFL), adjacent to the vitreous revealed small immune-like cells stained with X-gal. **(E and F)** Flatmounted retinas showing the numbers of FG retrogradely labeled RGCs in non-axotomized retinas 21 days after a control intraocular injection of PBS (E) or Ad.lacZ (F). **(G)** no significant loss in the number of FG retrogradely pre-labeled RGCs (mean  $\pm$  standard deviation) after intraocular Ad.lacZ treatment ( $p = .204$ ). Bars = 50  $\mu$ m.

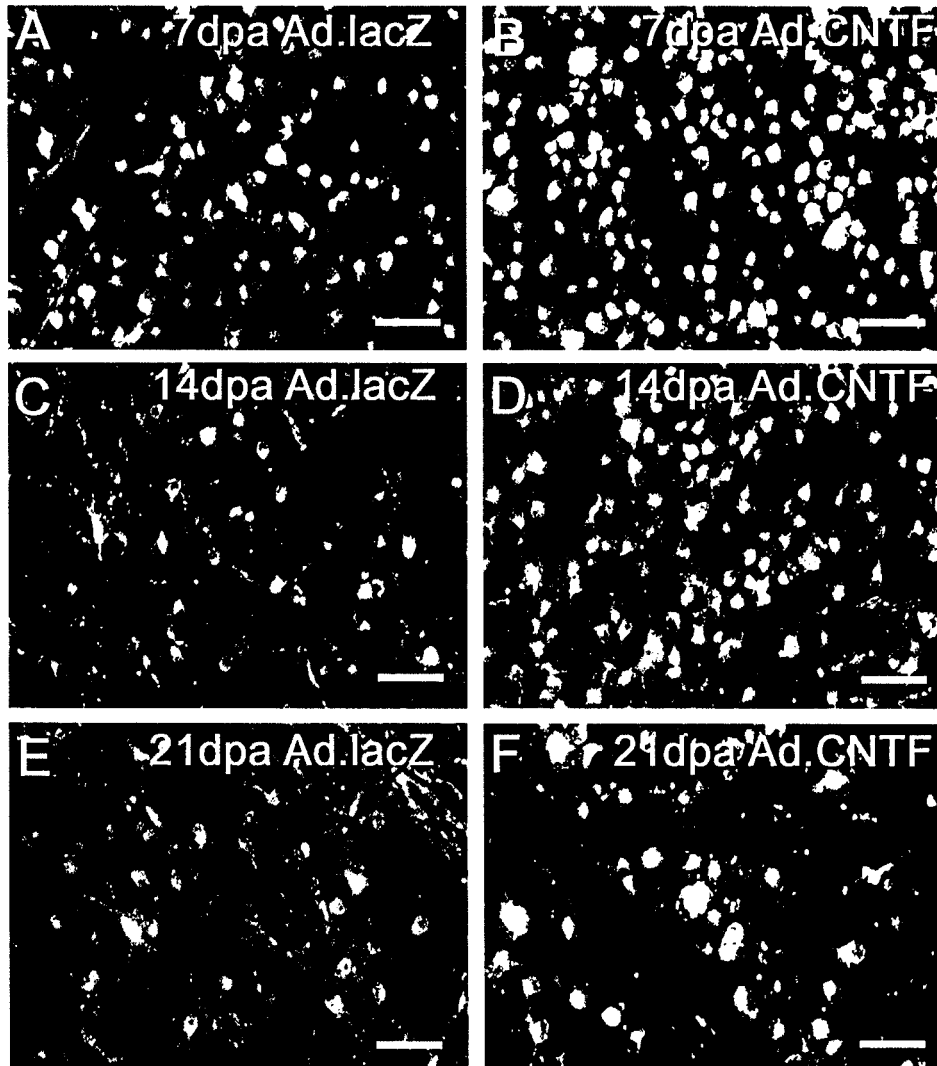




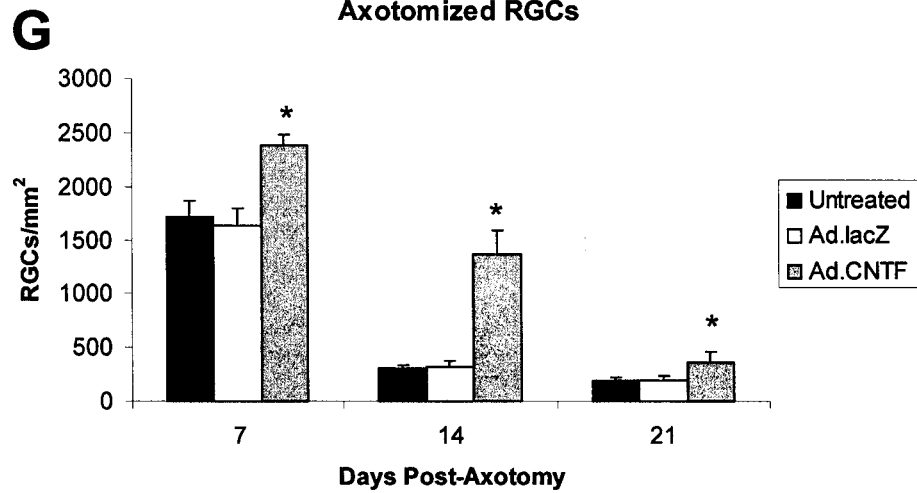
**Figure 5.** Timecourse of  $\beta$ -galactosidase and CNTF expression after intraocular injection of Ad.lacZ or Ad.CNTF in non-axotomized eyes. **(A)** A representative immunoblot demonstrated a defined band stained with anti- $\beta$ -galactosidase, at the approximate molecular weight of 66 kDa in normal, PBS injected, and at 1, 7, 14, and 28 days post-injection (dpi) of Ad.lacZ. Densitometric analysis of  $\beta$ -galactosidase protein levels (mean  $\pm$  standard deviation) demonstrated a significant increase in expression at 1 and 7 dpi ( $*p<0.05$ ). **(B)** Densitometric analysis of immunoblots stained for CNTF showed a significant increase in CNTF expression at 7 days after intraocular administration of the Ad.CNTF vector ( $*p<0.05$ ). As shown in this representative immunoblot, the CNTF antibody detected a band at 23 kDa in the normal (control) retina and for experimental retinas at specific days following Ad.CNTF or PBS treatment.



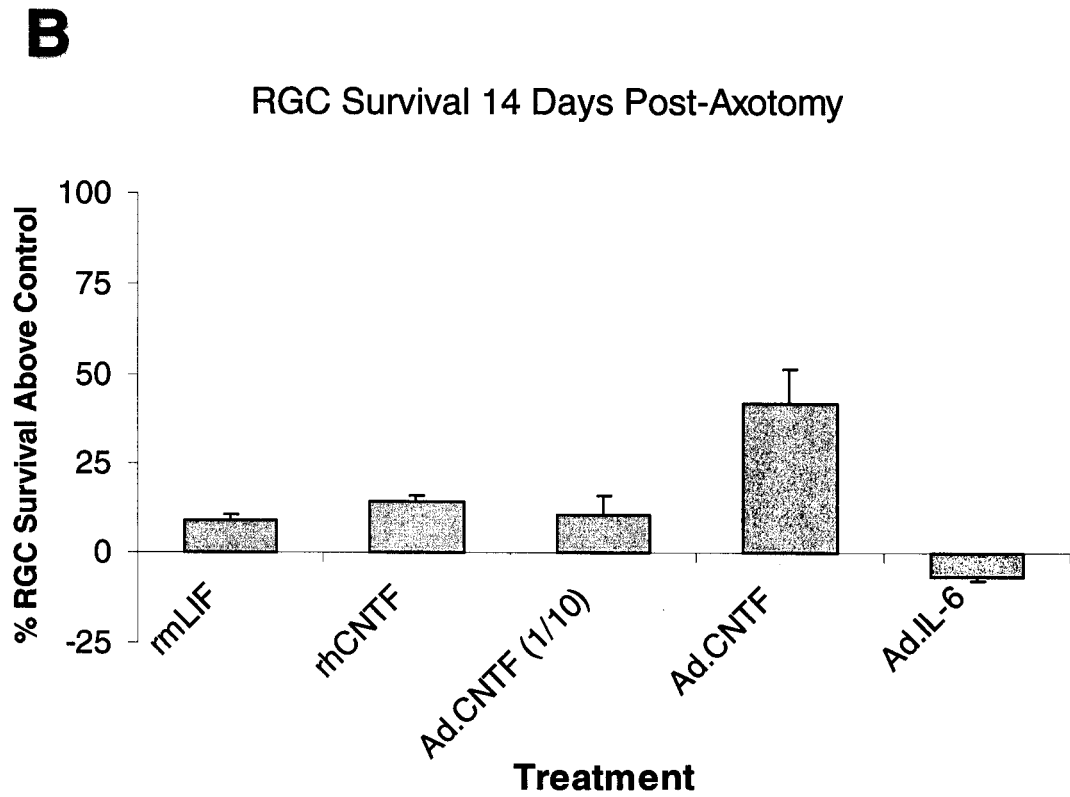
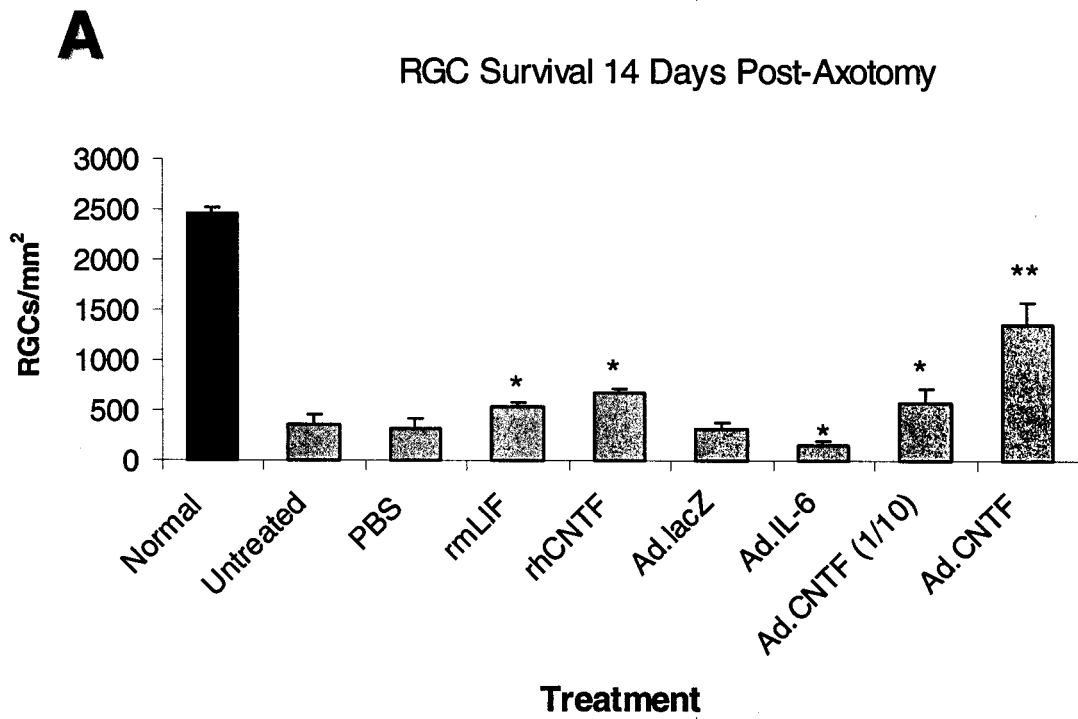
**Figure 6.** Survival of axotomized RGCs after intravitreal administration of Ad.CNTF or Ad.lacZ immediately following optic nerve transection. Confocal micrographs showing D-FITC retrogradely labeled RGCs in flatmounted retinas at 7, 14 and 21 dpa treated with the control Ad.lacZ (A,C, and E) or Ad.CNTF (B, D, and F) vector. **(A)** RGC survival at 7dpa with Ad.lacZ treatment. **(B)** increased RGC survival at 7dpa with intravitreal injection of Ad.CNTF. **(C and E)** Few RGCs at 14 (C) and 21dpa (E) with Ad.lacZ treatment. **(D and F)** RGC survival was significantly increased at 14 (D) and 21 dpa (F) in retinas treated intravitreally with Ad.CNTF. Bars in A-F = 50  $\mu$ m. **(E)** Graph showing a significant increase ( $*p \leq 0.002$ ) in RGC densities (mean  $\pm$  SD) at 7, 14, and 21 dpa in Ad.CNTF treated eyes compared to untreated or control Ad.lacZ treated retinas.



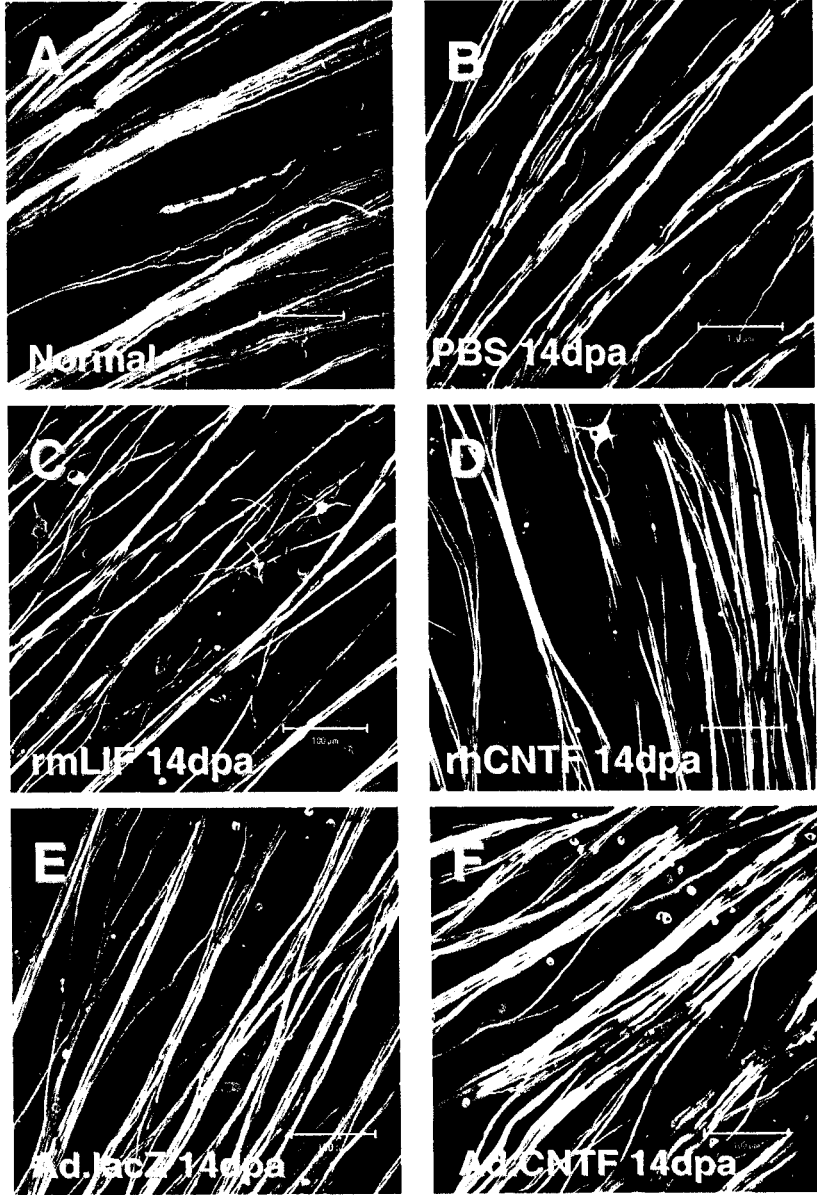
**Adenoviral-Mediated Transfer of CNTF Rescues Axotomized RGCs**



**Figure 7.** CNTF is a survival factor for axotomized RGCs. **(A)** Comparison of the survival (mean  $\pm$  SD) of RGCs at 14 dpa after control PBS or Ad.lacZ injection, or after injection of 4  $\mu$ g of rmLIF or rhCNTF, or following a 5  $\mu$ l injection of Ad.IL-6, Ad.CNTF diluted 10 fold (1/10) or Ad.CNTF. Note: the number of RGCs in normal (non-axotomized) retinas is shown (black bar). **(B)** In comparison to their respective controls, rmLIF, rhCNTF, Ad.CNTF (1/10) and Ad.CNTF significantly increased the survival of RGCs at 14 dpa. Treatment with Ad.IL-6 significantly decreased the survival of axotomized RGCs in comparison to controls. Statistically significant when compared with untreated, PBS, or Ad.lacZ (\* $p$ <0.005, \*\* $p$ <0.0001 ANOVA followed by Tukey's).

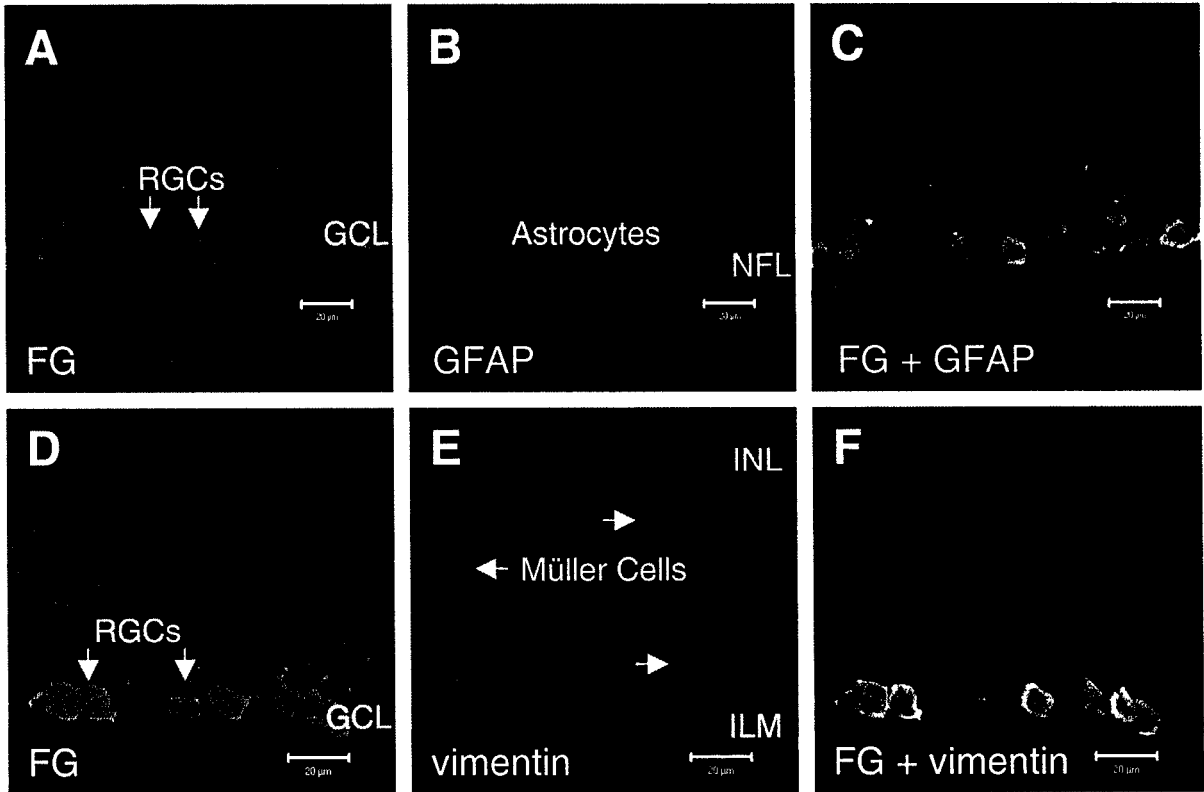


**Figure 8.** Confocal micrographs of RT97 immunohistochemistry in flatmounted retinas. (A) RT97 immunoreactive RGC axons in a normal retina. (B) Drastic reduction in RGC axons at 14 days post-axotomy in PBS treated retinas. (C and D) A single treatment of 4  $\mu$ g of rmLIF (C) or CNTF (D) had some effect on axon preservation in axotomized retinas. (E) Intraocular injection of Ad.lacZ did not preserve RGC axons. (F) Significantly more axons were observed 14 days postaxotomy in retinas with a single Ad.CNTF treatment. Bars = 100  $\mu$ m.

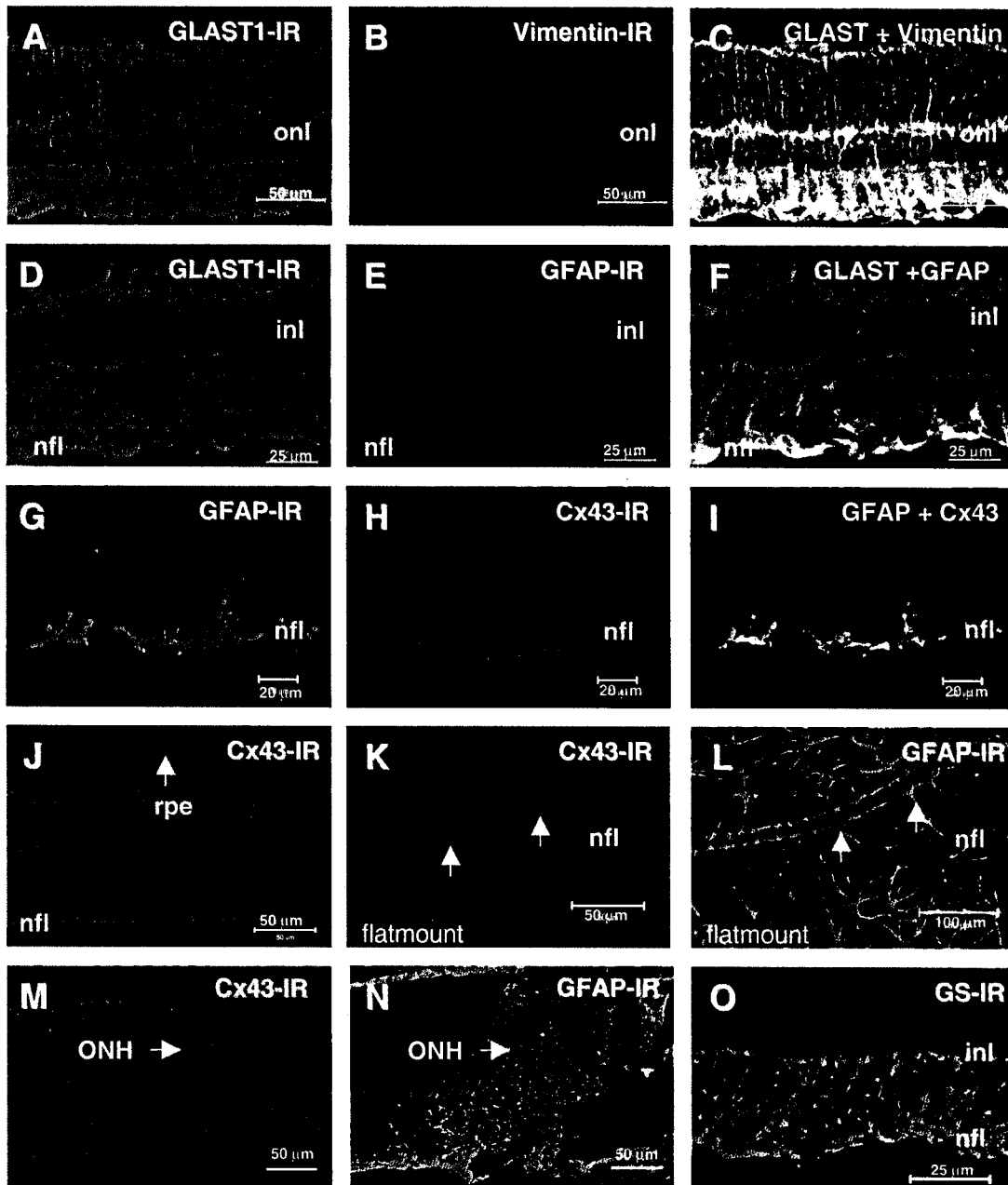




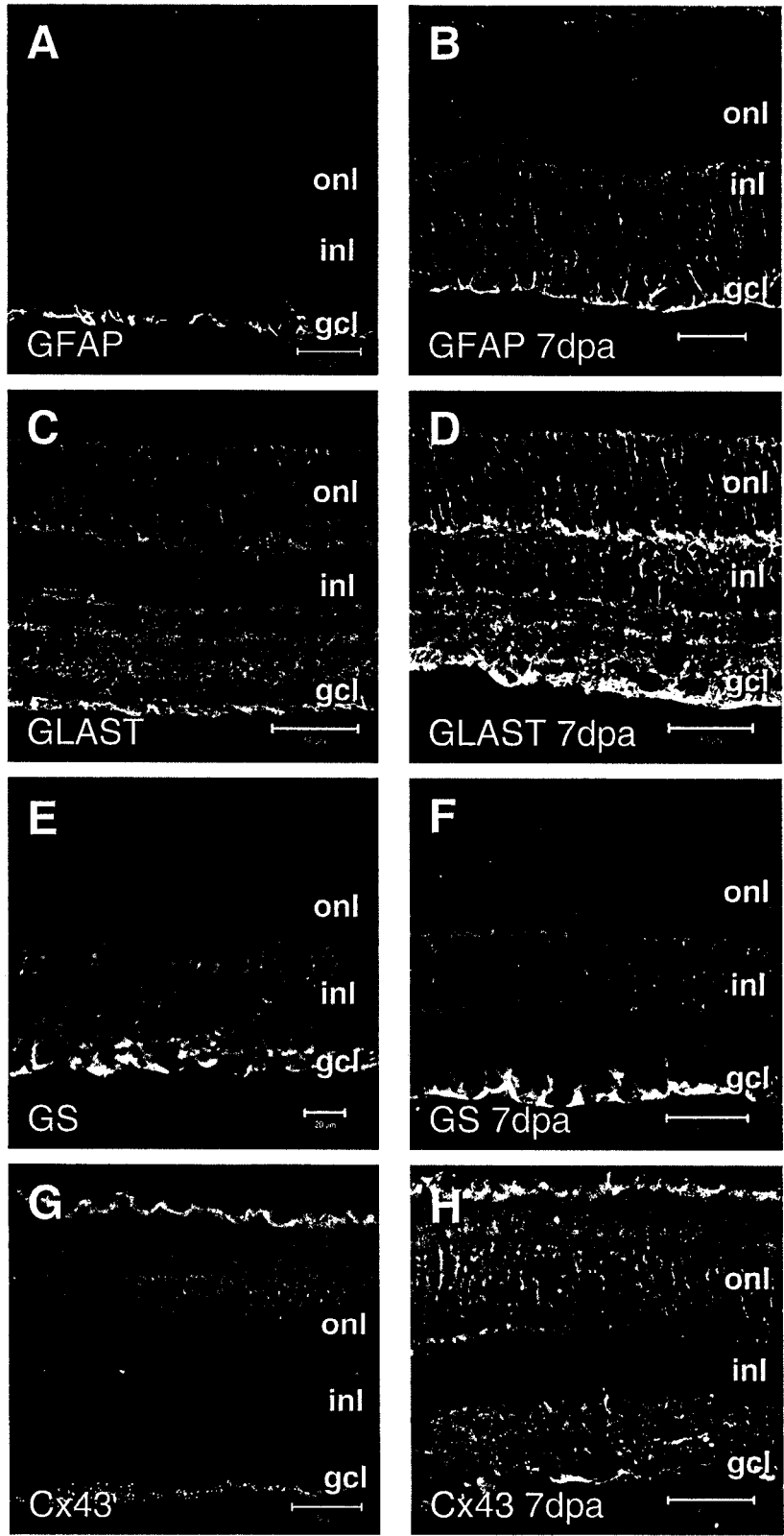
**Figure 9.** Double label immunohistochemistry directed against Fluorogold (FG) and GFAP or Vimentin in normal rat retina. RGCs were retrogradely labeled from the contralateral superior colliculus with 2% FG. (A and B) FG immunoreactivity RGCs (A) in the ganglion cell layer (GCL) and GFAP immunoreactivity astrocytes (B) in the nerve fiber layer (NFL). (C) Overlay of micrographs in (A) and (B). (D and E) FG immunoreactivity RGCs in the GCL, and Vimentin immunoreactivity Muller cells (E) with their cell bodies in the inner nuclear layer (INL) and their endfeet form the inner limiting membrane (ILM). (F) Overlay of micrographs in (D) and (E). Bars = 20  $\mu$ m.



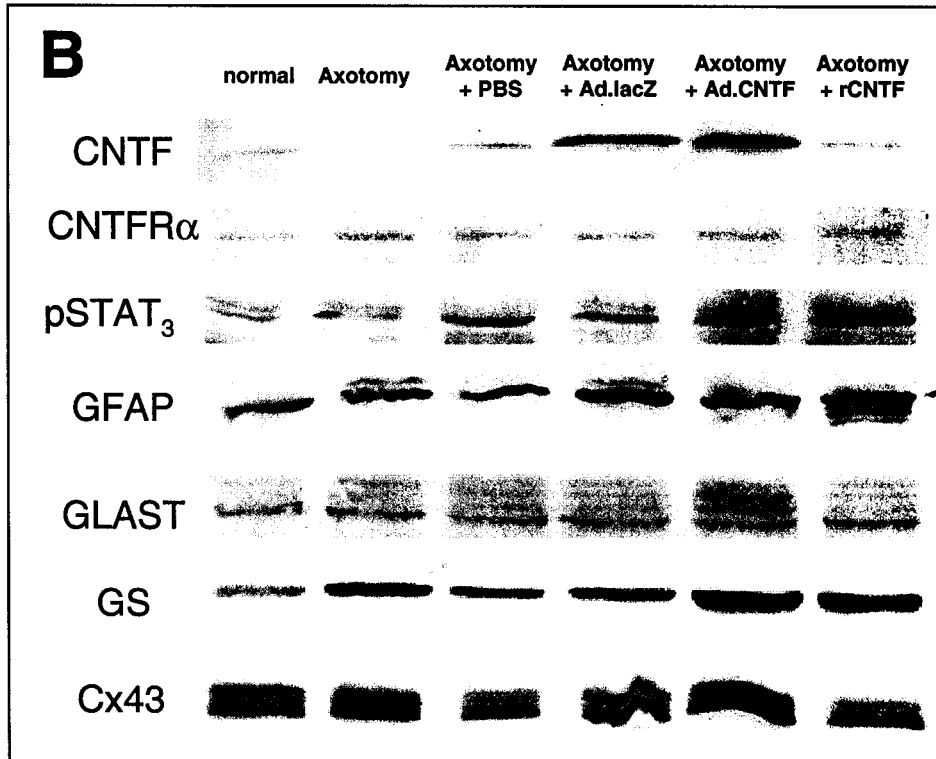
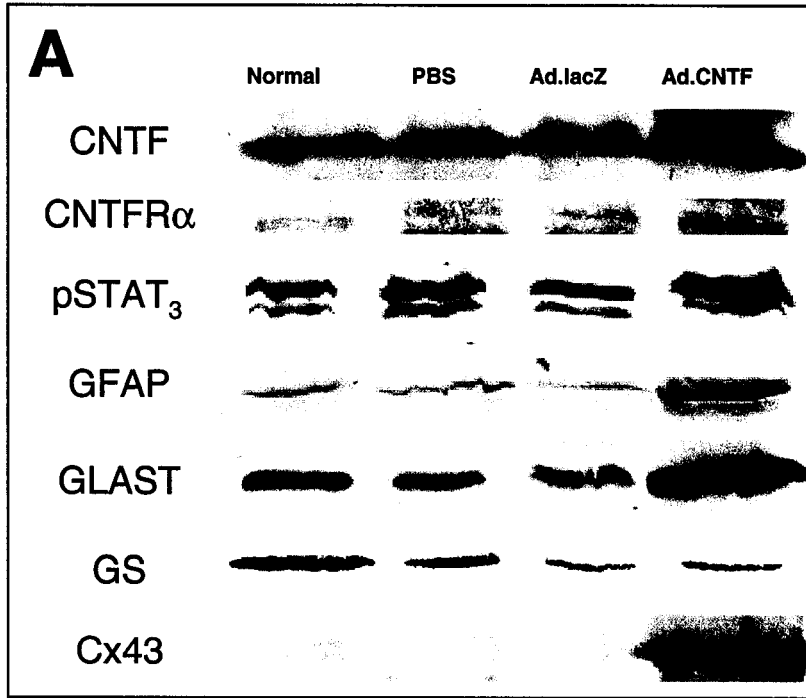
**Figure 10.** Confocal micrographs of GFAP, GLAST-1, GS, and Cx43 immunohistochemistry in transverse sections and flatmounts from normal retinas. (A) GLAST-1 immunoreactivity in Müller cells colocalized with (B) Vimentin immunoreactivity, as shown in (C) (yellow). (D) GLAST-1 immunoreactivity was also colocalized with (E) GFAP in retinal astrocytes, as demonstrated in (F). (G) GFAP immunoreactive astrocytes in the nerve fiber layer (nfl) colocalized with (H) Cx43 immunoreactivity, as shown in (I). (J) Cx43 immunoreactivity was also seen in the retinal pigment epithelial (rpe) (small arrows). In flatmount preparations (K) Cx43 immunoreactive, (L) and GFAP immunoreactive astrocytes formed a dense meshwork around retinal blood vessels (small arrows). (M) intense Cx43 immunoreactivity, (N) and GFAP immunoreactive astrocytes were also observed at the level of the optic nerve head (ONH). (O) GS immunoreactive astrocytes and Müller cells in the normal retina. inl= inner nuclear layer



**Figure 11.** Comparison of GFAP, GLAST-1, GS, and Cx43 immunohistochemistry in transverse retinal sections from normal and retinas isolated at 7 days post-axotomy (dpa). (A and B) GFAP labeling was restricted to nerve fiber layer (NFL) astrocytes in normal retinas (A) but was dramatically increased in Müller cell processes at 7 dpa (B). (C and D) Compared to normal retinas (C) GLAST-1 immunoreactivity appeared to be more intense in the axotomized retinas. (E and F) A similar trend was observed when comparing GS labeling in normal retinas (E) and retinas isolated 7 dpa (F). (G and H) In contrast to normal retinas (G) Cx43 labeling was more intense in axotomized retinas as noted by the intense staining of Müller cell processes at 7 dpa. gcl= ganglion cell layer, inl= inner nuclear layer, onl= outer nuclear layer.



**Figure 12.** Representative immunoblots of CNTF, CNTFR $\alpha$ , pSTAT<sub>3</sub>, GFAP, GLAST, GS, and Cx43 from normal (A) and axotomized (B) retinas treated with PBS, Ad.lacZ, or Ad.CNTF. (A) Compared to normal retinas or PBS, or Ad.lacZ treated retinas, intravitreal treatment of non-axotomized retinas with Ad.CNTF resulted in elevated levels of CNTF, CNTFR $\alpha$ , pSTAT<sub>3</sub>, GFAP, GLAST, and Cx43. (B) elevated levels of CNTF, CNTFR $\alpha$ , pSTAT<sub>3</sub>, GFAP, GLAST, GS and Cx43 after in axotomized retinas treated immediately following ON transection with Ad.CNTF. Note the increase in pSTAT<sub>3</sub>, GFAP, GLAST, GS, and Cx43 in non-treated axotomized retinas compared to non-axotomized normal retinas. Note: all retinas in B were isolated a 7 days post-axotomy (dpa).





**Table 1.** Summary of glial modulations in normal retinas following intravitreal treatment with PBS, Ad.lacZ, or Ad.CNTF.

	<b><u>Intraocular treatments in normal retinas</u></b>		
	<b>PBS</b>	<b>Ad.lacZ</b>	<b>Ad.CNTF</b>
<b>CNTF</b>	88 ± 33	104 ± 16	223 ± 17**
<b>CNTFR<math>\alpha</math></b>	98 ± 10	90 ± 24	330 ± 177*
<b>pSTAT<sub>3</sub></b>	115 ± 10	126 ± 34	336 ± 72*
<b>GFAP</b>	1120 ± 34	127 ± 39	368 ± 51**
<b>GLAST-1</b>	107 ± 17	96 ± 14	180 ± 13**
<b>GS</b>	103 ± 6	89 ± 14	80 ± 7
<b>Cx43</b>	25 ± 14	18 ± 13	471 ± 52**

Data are given as mean ± standard deviation and represent the relative optical density as a percentage of normal retinas (100%) from N=3 replicates per treatment. Statistical analysis was performed using one-way ANOVA followed by post-hoc testing.

\* $p < 0.05$ , \*\* $p < 0.001$ : when compared with controls (PBS and Ad.lacZ)

**Table 2.** Summary of the effects of optic nerve transection and overexpression of CNTF on the levels of several neuroprotective glial cell markers at 7 days post-axotomy.

	<u>Intraocular treatment at the time of ON transection</u>				
	Axotomy Non-treated	Axotomy + PBS	Axotomy + rhCNTF	Axotomy + Ad.lacZ	Axotomy + Ad.CNTF
<b>CNTF</b>	11 ± 4*	121 ± 49	160 ± 57	173 ± 84	572 ± 144*,§
<b>CNTFR<math>\alpha</math></b>	160 ± 2*	164 ± 2*	160 ± 32*	171 ± 6*	196 ± 17*
<b>pSTAT<sub>3</sub></b>	70 ± 40	91 ± 37	130 ± 35	116 ± 16	348 ± 57*,§
<b>GFAP</b>	183 ± 21*	200 ± 36*	259 ± 41*	220 ± 68*	356 ± 85*,§
<b>GLAST-1</b>	196 ± 19*	200 ± 12*	211 ± 10*	196 ± 26*	245 ± 12*,§
<b>GS</b>	496 ± 65*	470 ± 127*	524 ± 49*	488 ± 118*	736 ± 36*,§
<b>Cx43</b>	281 ± 29*	233 ± 40*	246 ± 108	221 ± 19*	465 ± 57*,§

Data are given as mean ± standard deviation and represent the relative optical density as a percentage of normal retinas (100%) from N=3 replicates per treatment. Statistical analysis was performed using one-way ANOVA followed by post-hoc testing.

Note: all experimental retinas were isolated at 7 dpa.

\* $p < 0.05$  when axotomized retinas are compared with normals

§ $p < 0.05$  when compared with control treatments (PBS and Ad.lacZ)

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**CHAPTER 5**

**THESIS SUMMARY**



## THESIS SUMMARY

The primary purpose of this series of experiments has been to determine the neuroprotective effects, and possible mechanisms of these effects, of ciliary neurotrophic factor on axotomized retinal ganglion cells. Three sets of experiments were conducted to determine the most effective method of delivering CNTF to the retina and to explore the neuroprotective mechanisms *in vivo*. The results and their implications are discussed below.

### **Experiment I: NAIP overexpression protects RGCs: comparison with CNTF**

Knowing how central nervous system (CNS) neurons, such as retinal ganglion cells, die is important for a complete understanding of mechanisms that lead to neurodegeneration. In the case of glaucoma and related optic neuropathies, the loss of RGCs occurs through a form of cell death known as apoptosis. As more information is gathered about the molecular pathways of apoptosis, it may be possible to prolong the life of RGCs and thus prevent visual loss.

Recent morphological, biochemical, and pharmacological evidence have demonstrated that axotomized RGCs die by apoptosis (Berkelaar et al., 1994; Bien et al., 1999; Garcia-Valenzuela et al., 1993; Quigley et al. 1995). Moreover, RGC apoptosis has been described in humans with optic neuropathy (Kerrigan et al., 1997) as well as glaucoma (Levin et al., 1996). The fact that RGC apoptosis

has been detected in the human disease process further emphasizes the need to understand the mechanisms that cause axotomy-induced apoptosis (Berkelaar et al., 1994).

This study demonstrated that the expression of Neuronal Inhibitory Apoptosis Protein (NAIP), a potent caspase-3 inhibitor, is not changed in the retina after optic nerve transection. Since activation of caspase-3 contributes to the apoptotic death of axotomized RGCs, we hypothesized that overexpression of NAIP would rescue RGCs after optic nerve transection. It was demonstrated for the first time that viral-mediated elevation of retinal NAIP had survival promoting effects on axotomized RGCs. These findings strengthen the literature indicating that optic nerve transection is a viable *in vivo* animal model of neuronal apoptosis. Moreover, CNTF was just as effective at inhibiting RGC apoptosis as NAIP, suggesting that CNTF can inhibit apoptosis by a yet unknown mechanism. Finally, it should be mentioned that inhibition of neuronal apoptosis in this model was assessed histologically, and functional measures could not be obtained since optic nerve transection is too severe an injury.

### **Experiment II:**

The second experiment of this thesis employed a lentiviral vector encoding the gene for CNTF to try and further enhance the survival of axotomized RGCs compared to retinas treated with adenoviral vectors encoding CNTF. The use of lentiviral vectors may be promising for retinal disorders since

it has been shown that these vectors produce rapid and prolonged expression of the encoded transgene without evoking an immune response. In this study, a lentiviral vector encoding human CNTF (LV-CNTF) was injected into the posterior chamber of the eye immediately following ON transection. Treatment with LV-CNTF resulted in an extended period of RGC protection not normally seen using the optic nerve transection as a model of RGC injury. Moreover, the lentivirus protected more RGCs than any other treatment employed in this series of experiments. Furthermore, lentivirus was effective in protecting axotomized RGCs when administered immediately after the injury. This further demonstrates that the properties of lentiviral vectors allow for rapid transgene expression and can protect CNS neurons within a relatively short window after traumatic injury. Thus, lentiviral vectors may be used for both acute and chronic disorders of the nervous system.

### **Experiment III: Direct and indirect neuroprotective mechanisms of CNTF**

The final experiment of this thesis attempted to elucidate some of the possible mechanisms of CNTF on axotomized RGCs. There is evidence to suggest that exogenous CNTF can modulate retinal glial. Thus, in addition to acting directly on axotomized RGCs, CNTF may also protect axotomized RGCs indirectly. To test this hypothesis, normal and axotomized retinas received intraocular injections of an adenoviral vector carrying the gene for CNTF (or control treatments) and the expression of several known glial markers including,

Cx43, GFAP, GLAST-1, and GS were measured. It was demonstrated that Cx43, GFAP, GLAST-1, and GS levels increased after optic nerve transection. Furthermore, viral-mediated overexpression of CNTF increased the expression of CNTFR $\alpha$ , pSTAT<sub>3</sub>, GFAP, GLAST-1, and Cx43, in normal and axotomized retinas. These results suggest that in addition to the direct neuroprotective mechanisms of CNTF on axotomized RGCs, CNTF treatment modulates retinal glia, and presumably increases their neuroprotective capacity, and protects RGCs from axotomy-induced excitotoxicity. Furthermore, these findings were also demonstrated in the brain after stereotaxic injection of Ad.CNTF into the superior colliculus, suggesting that astrocytes in different regions of the CNS respond to high levels of CNTF in a similar fashion. This was the first study to demonstrate that CNTF upregulates GLAST-1, GS, and Cx43 in the normal and injured CNS. The results of this study also suggest that therapeutic strategies can be developed to target CNS glia. In addition, these findings further demonstrate the supportive role CNS glia play in maintaining homeostasis and neuronal survival.

### ***Significance of Thesis***

Suppression of neuronal apoptosis, or programmed cell death, is the starting point to developing neuroprotective strategies for the treatment of neurodegenerative disorders such as glaucoma, amyotrophic lateral sclerosis (ALS), stroke, Parkinson's disease, and Huntington's disease. Moreover, the

molecular mechanism of neuronal apoptosis can be used as a target for the development of efficacious pharmacotherapies that might be useful in preventing or limiting irreversible axon injury and neuronal degeneration in these nervous system disorders.

The studies described within this thesis suggest that CNTF may have a potential for neuroprotection in glaucoma treatment as well as other neurodegenerative disorders. Even if the downstream mechanisms of CNTF have not yet been fully identified, the potential therapeutic properties of CNTF have been well established in preclinical studies using rodents and non-human primate models of CNS degenerative disease such as Huntington's disease (Anderson et al., 1996; Emerich et al., 1996, 1997; Emerich 1999; Mittoux et al., 2000). Those promising results lead to a phase I clinical study using encapsulated cells secreting CNTF as a potential therapy for Huntington's disease (Bachoud-Levi et al., 2000). The same strategy was evaluated in a study involving patients with amyotrophic lateral sclerosis without adverse effects, making CNTF a good candidate for the basis of the development of a new therapy (Aebischer et al., 1996).

Despite the fact that CNTF can protect retinal neurons there are several obstacles yet to overcome before this trophic factor can be considered for clinical trials. For example, CNTF treatment has demonstrated excellent histological protection of photoreceptors, however, such treatments have not resulted in physiological preservation of retinal function in animal models (Liang et al., 2000,

2001). Furthermore, in this study viral vectors were used to overexpress CNTF (possible at dosages that were not physiologically relevant) and we observed “histological protection” of only a portion of axotomized RGCs, suggesting that no single treatment can rescue the entire population of injured neurons. To date, no study has reported protection of the entire population of RGCs using the optic nerve transection model. This suggests that no single factor on it’s own can protect injured CNS neurons for extended periods of time. Thus, the quest for the magic “neuroprotective cocktail” continues.

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