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Neuroprotection and Regeneration of Adult Lesioned Retinal Ganglion Cells by the Modulation of Fibroblast Growth Factor-2 and Receptor Tyrosine Phosphatase-Sigma

par

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présentée par:

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SOMMAIRE

La capacité du système nerveux central (SNC) à survivre et à se régénérer par suite de lésions traumatiques ou lors des maladies dégénératives est fortement compromise chez les vertébrés supérieurs adultes. Le manque de survie découle de la perte du support trophique provenant des cellules environnantes et des cellules cibles (Burek and Oppenheim, 1996; Jacobson et al., 1997), ainsi que d'une perte générale de l'habilité à répondre aux signaux trophiques (Shen et al., 1999). De son coté, la régénération limitée est attribuable à un environnement hautement inhibiteur et une perte intrinsèque de la capacité de croître. Les deux scénarios aboutissent finalement à la mort neuronal par apoptose.

Les stratégies décrites dans cette thèse tentent de stimuler la régénération et la survie des cellules ganglionnaires de la rétine (CGR) adulte en réactivant leurs programmes intrinsèques de croissance et de survie. Plus spécifiquement, **nous avons testé l'hypothèse selon laquelle certains gènes impliqués dans le développement ordonné du système nerveux peuvent être modulés chez l'adulte dans le but de stimuler la croissance axonale et de provoquer la survie.** À cette fin, nous avons premièrement augmenté le niveau endogène du Facteur de Croissance des Fibroblastes-2 (FGF-2). Cette molécule s'avère être un puissant stimulateur de croissance axonale chez les CGR lors du développement (Dingwell et al., 2000). En utilisant des adéno-virus associés recombinants (AAV.FGF-2), nous avons introduit le gène codant pour FGF-2 dans les CGR matures, leurs fournissant ainsi des niveaux élevés de ce facteur. Nous démontrons ici que, par suite d'une blessure du nerf optique, cette approche augmente de

façon significative la repousse axonale des CGR et engendre une neuroprotection robuste mais transitoire.

Ultérieurement, nous avons cherché à identifier les mécanismes moléculaires responsables de la croissance axonale induite par FGF-2. Nous avons d'abord déterminé le niveau d'activation de certaines voies de signalisation susceptibles d'être stimulées par le récepteur de FGF (FGFR). Nos résultats ont démontré que l'activation de la kinase «extracellular-signal regulated kinases 1/2 » (Erk1/2) est amplifiée dans les rétines traitées avec l'AAV.FGF-2. Au contraire, des voies de signalisations comme celles de la phospholipase Cγ, de la «phosphoinositide-3-kinase » et de la protéine kinase C n'ont pas été stimulées. De plus, l'inhibition pharmacologique de Erk1/2 amène une réduction d'environ 80% du nombre d'axones de CGR pouvant se régénérer. Ceci établit un rôle important pour Erk1/2 dans la croissance axonale provoquée par FGF-2.

En raison de l'importance de la phosphorylation dans la survie et la régénération neuronale, nous avons étudié le rôle du *récepteur à protéine tyrosine phosphatase Sigma* (RPTPσ): un régulateur de la phosphorylation intracellulaire. L'implication de RPTPσ dans le développement du SNC est illustrée par son haut niveau d'expression dans le tissu nerveux embryonnaire et par le fait que son absence chez des souris transgéniques entraîne une réduction globale de la taille de leur cerveau (Elchebly et al., 1999; Wallace et al., 1999). En démontrant une hausse significative de la régénération des CGR lésées chez des souris RPTPσ (-/-), nous proposons que cette phosphatase inhibe la croissance axonale chez l'adulte. Nous avons également détecté une forte activation endogène de Erk1/2 et de Akt à chez les souris RPTPσ (-/-), suggérant que ces voies peuvent être impliquées dans l'augmentation de la repousse axonale observée chez ces animaux.

Les données présentées dans cette thèse suggèrent que la modulation des gènes impliqués dans le développement du SNC offre une stratégie prometteuse pour favoriser la croissance du SNC adulte lésé.

Mots clefs: Système Nerveux Central, Facteur de Croissance de Fibroblastes-2, Récepteur à Protéine Tyrosine Phosphatase-Sigma, Régénération Axonale, Survie Neuronale, Thérapie Génique, Cellule Ganglionnaire de la Rétine, Erk1/2.

SUMMARY

The ability of the central nervous system (CNS) to regenerate and survive following traumatic injury or disease is severely compromised in adult higher vertebrates. Current data ascribes this lack of CNS regrowth to both a highly growth-prohibitive environment and to the neuron`s own loss of intrinsic growth capacity. The lack of neuronal survival following CNS injury is mostly attributed to inadequate trophic support from neighboring and target cells (Burek and Oppenheim, 1996; Jacobson et al., 1997) as well as the neuron's overall loss of trophic responsiveness (Shen et al., 1999); both scenarios ultimately resulting in neuronal apoptosis.

The strategies described in this thesis attempt to promote the regeneration and the survival of adult lesioned retinal ganglion cells (RGCs) by reactivating their intrinsic growth and anti-apoptotic programs. More specifically, **we tested the hypothesis that genes involved in the orderly development of the nervous system can be modulated in the adult to ensure adequate neuroprotection and stimulate axonal regrowth.** In this aim, we first chose to increase levels of fibroblast growth factor-2 (FGF-2), a potent stimulator of RGC axon growth during development (Dingwell et al., 2000). Using recombinant adeno-associated virus (AAV), we introduced the gene coding for FGF-2 into mature RGCs, thus ensuring a sustained upregulation of this neurotrophic factor. We provide evidence that this approach significantly enhances RGC axonal regrowth following acute optic nerve injury and promotes robust but transient neuroprotection.

We subsequently sought to elucidate the molecular signaling mechanisms that regulate FGF-2-induced RGC axon growth. To address this question, we first proceeded to screen candidate pathways known to be recruited upon stimulation of FGF receptor (FGFR). Our data demonstrate that the extracellular-signal regulated kinases 1/2 (Erk 1/2) were stimulated in AAV.FGF-2 treated retinas, but not other pathways such as phosphoinositide 3-kinase, phospholipase Cγ or protein kinase C. Inhibition of Erk 1/2 with the pharmacological inhibitor PD98059 led to a ~80 % reduction in the number of regenerating RGC fibers and thus established Erk 1/2 as an important signaling pathway in FGF-2 mediated axonal re-growth.

As both neuronal survival and regenerative signals rely heavily on phosphorylation events, we investigated the role of Receptor Protein Tyrosine Phosphatase-Sigma (RPTP-σ), a regulator of intracellular phosphorylation. RPTP-σ's involvement in CNS development is illustrated both by its high levels of expression in the embryonic nervous system and by an overall reduction in brain size in transgenic animals lacking this protein (Elchebly et al., 1999; Wallace et al., 1999). By demonstrating that RPTP-σ (-/-) mice show a significant increase in RGC axon regrowth following acute injury when compared to wild type littermate controls, we provide evidence that RPTP-σ hinders CNS regeneration in the adult. This increased growth cannot be attributed to developmental defects as we found that knockout animals have normal retinal and optic nerve histologies and show identical time-courses of RGC death following lesion. Lastly, we show high levels of Erk 1/2 and Akt activation in RPTP-σ (-/-) mice, suggesting that these pathways may be implicated in the increased axonal growth observed in these animals.

The data presented in this thesis suggest that the modulation of genes involved in the sound development of the CNS is an adequate strategy to promote regenerative growth of adult injured CNS neurons.

Key words: Central Nervous System, Fibroblast Growth Factor-2, Receptor Tyrosine Phosphatase-Sigma, Axonal Regeneration, Neuronal Survival, Gene Transfer, Retinal Ganglion Cells, MAPK

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LIST OF ABBREVIATIONS

AAV	adeno associated virus
ANT	adenosine nucleotide translocator
Ad	Adenovirus
bHLH	basic helix-loop-helix
BRB	blood-retinal barrier
BDNF	brain derived neurotrophic factor
CaMKII	Ca^{2+}/calmodulin kinase II
CREB	cAMP-response element binding protein
CAM	cell adhesion molecule
CNS	central nervous system
CSPG	chondroitin-sulfate proteoglycans
CNTF	ciliary neurotrophic factor
cAMP	cyclic adenosine monophosphate
cyt C	cytochrome C
DCC	deleted in colorectal cancer
DNA	deoxyribonucleic acid
DSP	dual specificity phosphatase
E13	embryonic day 13
ECM	extracellular matrix
Erk	extracellular regulated-signal kinase
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor

FAK	focal adhesion kinase
GCL	ganglion cell layer
GDNF	glial cell line-derived neurotrophic factor
GAPs	GTPase activating proteins
GEF	guanine nucleotide exchange factor
HSV	herpes simplex virus
Ig	immunoglobulin
IP3	inositol triphosphate
INL	inner nuclear layer
IGF-1	insulin-like growth factor-1
ITR	inverted terminal repeats
LN	laminin
mm	millimeters
MAPK	mitogen activated protein kinase
MEK	MAP kinase/ extracellular signal-regulated kinase (ERK) kinase
MAG	myelin-associated glycoprotein
NGF	nerve growth factor
NT-3	neurotrophin-3
NT-4	neurotrophin-4
NgR	Nogo receptor
NLS	nuclear localization signal
Omgp	oligodendrocyte-myelin glycoprotein
ONH	optic nerve head

ONL	outer nuclear layer
PDK	3'-phosphoinositide-dependent protein kinase
PLC	phospholipase C
PKC	protein kinase C
PNS	peripheral nervous system
PTK	protein tyrosine kinase
RPTP	receptor protein tyrosine phosphatase
RTK	receptor tyrosine kinase
RGM	repulsive guidance molecule
RGC	retinal ganglion cell
RPE	retinal pigment epithelium
RNA	ribonucleic acid
RSK	ribosomal S6 kinase
Robo	roundabout
Shh	Sonic Hedgehog
TGF-β	transforming growth factor-Beta
VDAC	voltage dependant anion channel

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PREFACE

The principal focus of this thesis was to study the involvement of FGF-2 and RPTP-σ in the regeneration and survival of retinal ganglion cells (RGCs), a prototypic central nervous system (CNS) neuronal population. In order to familiarize the reader with the subject at hand, this thesis includes a thorough introduction on axonal growth by RGCs, the consequences of acute axonal injury (both on survival and regeneration) and a review of current strategies that may be employed to modulate gene expression to stimulate neuronal survival and axon regrowth. The remainder of the thesis is manuscript-based; it comprises three original research articles that have been published in (or submitted to) peer-reviewed journals, followed by a general discussion covering key topics pertaining to the articles.

"Consider that at the core of the mind is willpower. Whether there is success or failure, if one entrusts himself to the straightness of the path at the core of the mind, he will attain right-mindedness in either case. Severing oneself from desire and being like a rock or a tree, nothing will ever be achieved. Not departing from desire, but realizing a desireless right-mindedness- this is the Way"

- TAKUAN SOHO, *THE CLEAR SOUND OF JEWELS*

To my parents Jola and Slawek,

for their wisdom, guidance and support

and to Cindy, for sharing the highs and lows.

<u>Chapter I</u>

1. GENERAL INTRODUCTION

1.1 CENTRAL NERVOUS SYSTEM REGENERATION: A HISTORICAL PERSPECTIVE

The regeneration of the injured CNS has been a daunting task since the dawn of modern neurobiology. CNS neurons severed from their targets are incapable of surviving, regrowing axons and restoring function to affected structures. For these reasons, traumatic injuries and diseases of the CNS are of an irreversible nature. Although the intrinsic inability of lesioned CNS neurons to re-establish function was documented as far back as Ancient Egypt (Jackowski, 1995), it was only in the early 1900s, with the advent of histological and electrophysiological tools, that CNS regeneration began to be thoroughly explored.

In the late 1800s, neuroscientists first described that rather than being completely incapable of regeneration, transected CNS axons undergo a phase of abortive sprouting. It was however Santiago Ramón y Cajal, the father of modern neurobiology, using the novel reduced silver nitrate staining method, who confirmed without a doubt the existence of a phase of regenerative sprouting in the lesioned optic nerve, spinal cord and cerebral cortex (Ramon y Cajal, 1928). These attempts at regrowth were characterized by club-like terminals, hypertrophic varicosities and irregular branched growth; all suggesting that the sprouting events were not to yield full blown axonal growth. Nevertheless, these "frustrated regenerative acts" as they were named, gave the first indication that the CNS was capable of mounting a response to counter injury.

In the peripheral nervous system (PNS), Cajal noticed that regenerating fibers were oriented towards and associated with Schwann cells. This lead Cajal to postulate that these glial cells were a source of both trophic and tropic factors for the growing

axons. He went on to hypothesize that regenerative failure in the CNS was due to the absence of Schwann cells and the growth-promoting factors they secrete. These conclusions prompted Cajal and his student Tello to examine the outcome of conferring a PNS environment to transected CNS axons. To this effect, Tello transplanted peripheral nerve segments to the lesioned cortex of a rabbit (Tello, 1911). Thirty days later, he was able to observe growth of central cortical axons through these grafts. Tello pursued this work by grafting sciatic nerves to transected optic nerve stumps and noted a similar regenerative phenomenon. The main conclusion of these studies was that CNS neurons were intrinsically capable of regenerating their axons when exposed to a permissive PNS environment and that this response was supported by growth promoting factors secreted by Schwann cells in the degenerating grafts (Ramon y Cajal, 1928). Nevertheless, there was skepticism as to the exact origin of the neurons growing into the PN grafts. Were they CNS axons or axons originating from peripheral bundles associated with the nerve or surrounding muscles?

It was not until the early 1980's that Albert Aguayo and colleagues managed to unequivocally trace and confirm the origins of the regenerating axons. Using the techniques of retrograde tracing with horseradish peroxidase and fluorescent dyes, they were able to label cell bodies and axons of regenerating CNS neurons from spinal cord (Richardson et al., 1980; David and Aguayo, 1981; Richardson et al., 1982), brain (Benfey and Aguayo, 1982), cerebellum (Dooley and Aguayo, 1982) and retina (So and Aguayo, 1985).

These studies were seminal to our understanding of the regenerative potential of CNS neurons and paved the way to designing strategies for CNS repair. This work also

established a proof of principle for future investigations into the molecular basis of CNS survival and regeneration.

1.2 AXON GROWTH IN THE CNS

In order to better understand the dynamics required for axonal growth during adult CNS regeneration, I will first describe this process in the developing nervous system.

1.2.1 A Complex Neural Network

The nervous system is an intricate structure that receives sensory input from the environment, integrates the significance of the stimulus and consequently produces an adequate physiological response. It is made up of two structurally distinct yet interconnected components, the PNS and the CNS. The CNS (the brain and spinal cord in vertebrates) acts as the main processing unit of the nervous system. It receives motor and sensory information from a subset of PNS neurons and then uses other PNS neural components to generate a behavioral motor response.

The extreme complexity of these systems in humans is illustrated by the networks of hundreds of billions of neurons, each of which communicates with thousands of other neurons. It is thought that a single cubic centimeter of human brain contains over 50 million nerve cells (Kandel and Siegelbaum, 2000). To establish appropriate functional synaptic connections, a neuron must extend its axon over long distances into the maze of chemical signals and physical barriers and ensure that this axon arrives in proximity of the adequate target and generates a working contact. This

growth process relies essentially on the axon's ability to read and interpret molecular growth and guidance cues.

1.2.2 Growth Cone Dynamics

« I had the good fortune to behold for the first time that fantastic ending of the growing axon. In my sections of the spinal cord of three day chick embryo, this ending appeared as a concentration of protoplasm of conical form, endowed with amoeboid movements. It could be compared with a living battering ram, soft and flexible, which advances, pushing aside mechanically the obstacles which it finds in its path, until it reaches the region of its peripheral termination. This curious terminal club, I christened the growth cone. »

Santiago Ramon y Cajal, 1890,

Recollections of My Life

The oracular Cajal divulged this first accurate description of the neuronal growth cone over a century ago. Roughly two years later, while studying retino-tectal projections, Cajal was inspired by the leukocyte chemotaxis phenomenon and proposed that the growth cone is guided by "chemical flows" which allow it to find the secretory target (Ramon y Cajal, 1893). Another century would pass before these "chemicals flows" were identified and the mechanisms by which they act were elucidated.

As put forth by Cajal, the growth cone is a locomotory and sensory structure that transduces environmental cues and assures sound axonal growth and accurate connections. It comprises three principal regions: filopodia which are long finger-like

extensions of bundled fibrillary (F)-actin, the fan-like lamellipodia rich in cross-linked F-actin and a central core that contains mitochondria, microtubules and other organelles (Figure 1. A. p.9) (Dent and Gertler, 2003; Huber et al., 2003).

The filopodia are the major sensory elements of the growth cone. These highly motile protrusions probe the environment and transduce extracellular signals via a network of receptors on their outer membrane. The guidance cues encountered by the growth cone along its trajectory can be either attractive or repulsive, acting over long distances (chemoattraction and chemorepulsion) or short distances (contact-dependent attraction and repulsion) (Huber et al., 2003). Once the receptors on the growth cone come in contact with a given cue, it can respond by either advancing, stalling, turning or retracting. Axonal pathfinding is therefore dictated by the growth cone`s response to a series of attractive and repulsive cues which draw out the advancing axon`s trajectory.

The progression or the retraction of the growth cone is controlled by a balance of actin polymerization and depolymerization at the leading edge of both the filopodia and lamellipodia (Mallavarapu and Mitchison, 1999). The bundled F-actin in the periphery of the filopodia is connected to the microtubule network in the central core (Figure 1, p.9). Upon contact of growth cone receptors to an adhesive substrate, an attractive molecule will stimulate the filopodia to contract and pull the growth cone forward. Actin then polymerizes at the leading edge (+) of the filopodia and lamellipodia and disassembles at the trailing edge (-). The force generated by the addition of actin to the (+) end drives the filopodium forward. New membrane and receptors are then added at the leading edge to ensure that the growth/guidance process is adequately maintained. Microtubules subsequently assemble and extend from the axon shaft in the central core

towards the growth cone and thus leave behind a new segment of axon (Dent and Gertler, 2003). When subjected to repulsive cues, actin at the leading edge (+) depolymerizes resulting in the retrograde flow of F-actin and thereby causing the growth cone to stop, turn or retract.

1.2.3 Growth Cone Remodeling

Appropriate axonal navigation necessitates that the motor components of the growth cone function in response to the sensory apparatus of the filopodia. The coordinated function of these two elements implies that the receptors on the growth cone membrane not only mediate adhesion, but are also responsible for transducing an intracellular response via a network of secondary messengers capable of modifying the axonal cytoskeleton (Huber et al., 2003).

A possible mechanism linking adhesion molecule and growth factor signaling to the growth cone actin cytoskeleton involves Rho signaling pathways (Hall, 1998). The Rho family of small GTP-binding proteins influences actin filament assembly and disassembly as well as other growth cone remodeling events such as myosin-dependent retrograde flow of actin (Dickson, 2001). Rho GTPases are controlled via the state of their bound guanine nucleotide. When bound to GTP, they are able to recruit effector proteins and perform their tasks. Once the GTP is hydrolyzed to GDP by an intrinsic GTPase, the effectors are released and they are rendered inactive.

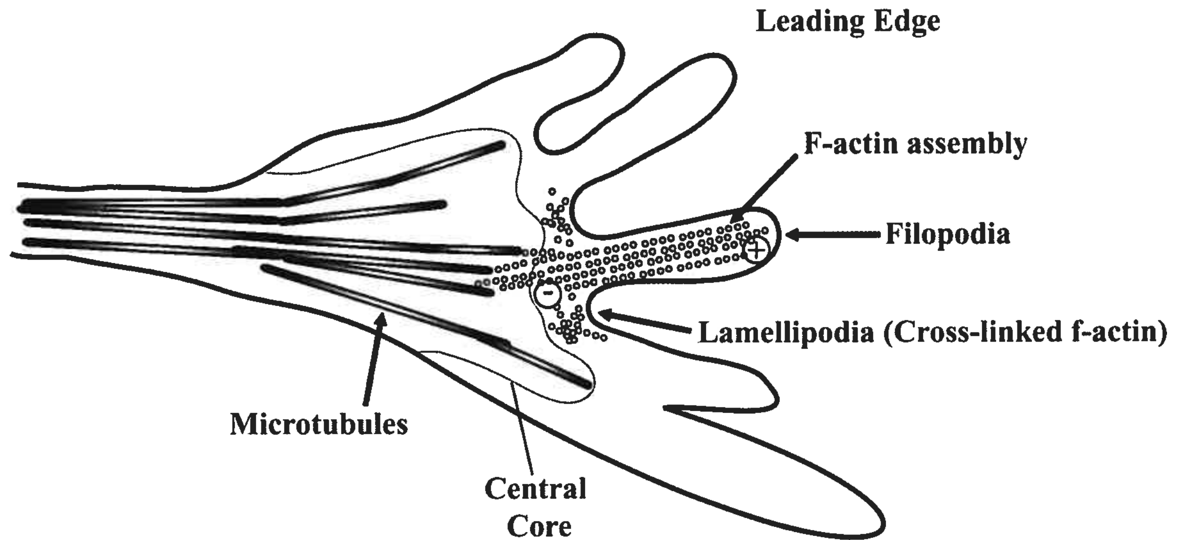
The best characterized members of the Rho family are Rho A, Cdc42 and Rac. Activation of Cdc42 or Rac in neuronal cells induces formation of filopodia and lamellipodia, respectively, and promotes neurite extension (Hall, 1998). Conversely,

activation of Rho A in neuronal cells provokes growth cone collapse and neurite retraction.

The equilibrium between activated GTP bound and inactivated GDP bound Rho is directed by the adverse activities of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). These effectors can be activated directly or laterally by growth cone receptors governing the growth/guidance process. This mechanism therefore allows the receptors to control the cytosekeletal rearrangements necessary to ensure proper and directed axonal growth. Based on these findings, a simplified model of Rho GTPase dependent growth cone dynamics involves attractive guidance cues that activate Cdc42 and Rac while growth inhibitory and repulsive signals activate Rho A (Figure 1. B, p.9).

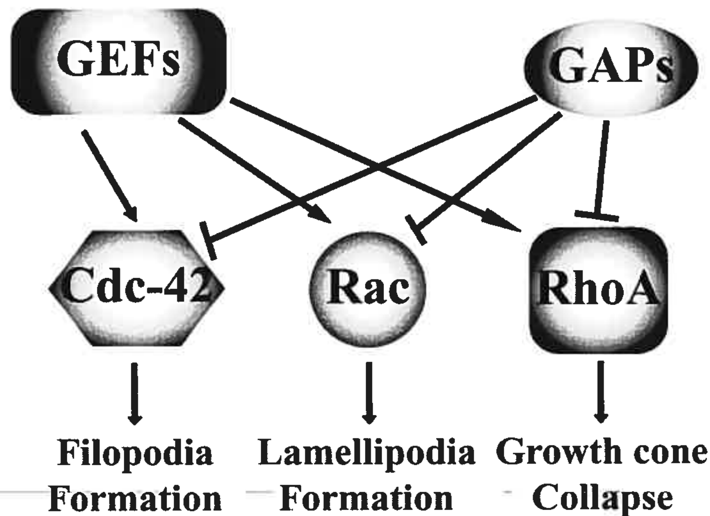
FIGURE 1.

A)



The organization of the neuronal growth cone.
The growth cone is composed of fan-like structures called lamellipodia and finger-like protrusions named filopodia. Lamellipodia are rich in cross-linked F-actin filaments while the filopodia are composed of bundled F-actin. The actin network in the periphery is linked to microtubules found in the central core of the growth cone and axon shaft. Once the growth cone advances, the actin cytoskeleton supports the translocation and polymerization of microtubules into more peripheral regions of the growth cone. Actin polymerization at the plus (+) end (leading edge of the growth cone) and depolymerization at the minus (-) end (the central growth cone) govern growth cone progression and retraction.

B)



Rho GTPases and growth cone dynamics.
Rho-like GTPases (e.g. Cdc 42, Rac 1 and Rho A) are controlled by the opposing activities of Rho GTPase-activating proteins (GAPs) and Rho GTPase nucleotide exchange factors (GEFs). GAPs turn Rho GTPases off by activating endogenous GTPases while GEFs turn them on by facilitating the exchange of GDP to GTP. Once in an activated state, Cdc 42 provokes the formation of filopodia, Rac stimulates the formation of lamellipodia and Rho A causes growth cone collapse.

1.2.4 The Retino-Collicular Pathway as a Model to Study CNS Repair

The visual system has a rich history as the model pathway for the CNS. From the early pioneering work of Santiago Ramon y Cajal to the modern experiments of Albert Aguayo, the retino-collicular pathway's experimental accessibility (outside the brain proper) and compartmentalized layout has made it a system of choice for the study of the CNS. Of all the cellular populations within the retina, retinal ganglion cells (RGCs) lend themselves particularly well to study neuroprotective and regenerative strategies. Their somata lie within the eye, while their axons project into the optic nerve and synapse with sub-cortical regions of the brain. This anatomical design permits for easy, minimally intrusive access as well as for selective labeling and manipulation of these cells.

Following transection of the optic nerve, RGCs die by apoptosis (Semkova and Krieglstein, 1999; Sofroniew et al., 2001) (described in section 1.4). The pattern of cell death following optic nerve transection is well documented and can be easily evaluated, thus facilitating the assessment of survival enhancing strategies (Berkelaar et al., 1994). The optic nerve itself is a convenient model for the study of CNS regeneration (Vidal-Sanz et al., 1987; Chierzi et al., 1999; Lehmann et al., 1999; Leon et al., 2000; Sapieha et al., 2003). Lesion paradigms such as the micro-crush lesion, offer a neatly defined injury site with minimal cavitation (Selles-Navarro et al., 2001); a phenomenon seen in conventional crush and cutting lesions (Giftochristos and David, 1988). These advantages allow for an accurate measurement of axonal regrowth into the CNS environment and thus make the optic nerve a choice system for investigation.

In this thesis, the rodent retino-collicular system was employed as model of the CNS. Using the optic nerve micro-crush lesion model, we studied the role of FGF-2 and RPTPσ in the regeneration of acutely injured RGC axons. Alternatively, with the optic nerve axotomy injury paradigm, we investigated the role of these two proteins in the neuroprotection of RGCs. The following sections describe the events leading to RGC generation during development and their axonal growth within the optic nerve.

1.3 THE GENESIS OF RGCs AND AXONAL GROWTH FROM THE RETINA TO THE MIDBRAIN

One of the most studied and best understood examples of axonal growth and guidance in the CNS is that of RGCs. The orderly wiring of RGCs to their midbrain targets is a truly elegant example of the awesome complexity of axonal growth and navigation. RGC axons are the only anatomical and functional pathway between the neural retina and the brain; appropriate axonal targeting to the mesencephalon, diencephalon and telencephalon is essential for vision. To ensure accurate targeting, RGC axons must travel within the eye, reach the optic disk, extend through the optic nerve, grow across the optic chiasm into the optic tract and connect with appropriate targets in the midbrain. This whole process is made up of several distinct steps: RGC generation, axon initiation, axon outgrowth and navigation and finally target recognition and innervation. These developmental stages of RGC axon growth are discussed bellow.

1.3.1 Origins of the Eye and Cellular Determination in the Developing Retina

The eye and its neural retina originate from evaginations of the embryonic diencephalic wall and remain connected to the brain by the optic stalk. This transitory bridge serves as the initial substrate for RGC axonal growth to the brain (Isenmann et al., 2003). A series of invagination events in the optic vesicle and optic stalk result in the formation of an opening termed the choroid fissure. In addition to being the site of formation of the retinal artery, it will be through this canal that the first RGC axons travel.

The retina is derived from a sheet of neuroepithelial cells that robustly express Pax-6; a critical gene for eye development in species as distantly related as humans and flies (Ziman et al., 2001). Although the exact mechanism is not clearly understood, the generation of the various retinal cell types occurs in a polar fashion across the thickness of the neuroepithelium. It is speculated that asymmetric segregation of mRNAs coding for cell fate determination genes as well as the segregation of proteins such as Numb, which inhibit Notch signaling and control neural cell fate, may be involved (Cayouette and Raff, 2002). The positional information may appear during gastrulation and neurulation when positional patterning genes are activated. Differentiation of retinal precursor cells starts in the center of the inner layer of the optic cup and radiates outwards towards the periphery (Prada et al., 1991; Holt and Harris, 1993). The positional information of precursor cells dictates cellular specificity and consequently influences vision acuity, sensitivity and movement analysis. RGCs are the first retinal cell types to differentiate, followed by horizontal cells, cone photoreceptors, amacrine cells, rod photoreceptors, bipolar cells and lastly Müller glia (Cepko et al., 1996).

1.3.2 Determinants of RGC Differentiation

The development of RGCs is driven by several protein factors including Sonic Hedgehog (Shh) and fibroblast growth factor-2 (FGF-2). These molecules and their roles in RGC generation are described in the following sections.

1.3.2.1 Sonic Hedge Hog and RGC Generation

Shh is a member of the hedgehog family of signaling proteins known to play multiple roles in CNS development. For example, Shh influences the proliferation of neuronal precursors, controls axon growth and induces oligodendrocyte formation (Ingham and McMahon, 2001). It is synthesized as a 45 kDa protein that is later proteolytically cleaved into a 19 kDa N-terminal secreted protein and a 25 kDa C-terminal secreted protein. It is the 19 kDa fragment that exerts all known biological function. Shh exerts its biological activity by binding to Patched, a 12-pass transmembrane protein found on the cell surface. This in turn relieves Smoothened, a 7-pass transmembrane G-protein-coupled receptor, of its normal inhibited state. Smoothened, considered as signaling arm of Shh, consequently either activates or represses gene expression (Ingham and McMahon, 2001).

In the developing vertebrate retina, Shh acts as a mitogen (Jensen and Wallace, 1997). It exerts its function early in the process of determining the RGC phenotype by activating Pax genes such as Pax-6. The latter stimulates basic helix-loop-helix (bHLH) transcription factors such as Math5 in mice (*Xath 5* in *Xenopus, atonal in drosophila).* These bHLH genes are expressed in the early stages of retinal development (E11-E15 in mice). They activate the transcription factor Brn3b, necessary for RGC differentiation

after the initial stage of commitment to this cellular fate (Gan et al., 1999). Shh is then expressed and secreted by already differentiated RGCs and stimulates its own production, thereby ensuring the concentric differentiation of retinal precursor cells towards a RGC phenotype (Zhang and Yang, 2001).

1.3.2.2 Fibroblast Growth Factor-2 and RGC Generation

The FGF family of neurotrophic factors plays multiple roles in the development and maintenance of the CNS and retina (reviewed in Eckenstein, 1994 and Hicks, 1998). Of the 23 currently identified members of this family, FGF-2 (basic FGF; bFGF) is the best characterized. FGF-2 was discovered in 1984 and, as its name suggests, was described as a mitogenic factor for fibroblasts (Thomas et al., 1984). It exerts its biological function by binding its high affinity FGF receptors (FGFR) and activating an array of signaling pathways such as mitogen activated protein kinase (MAPK) Erk 1/2 , PI-3K, PKC or PLCγ (Boilly et al., 2000) (described in section 1.7.2).

FGF-2 has been isolated in large quantities from the CNS (Eckenstein et al., 1991a; Eckenstein et al., 1991b). In the retina, FGF-2 immunoreactivity is present in the outer and inner nuclear layers throughout life and associated with RGCs between E14 and E18, a period during early development when RGCs are forming (de Iongh and McAvoy, 1993). At this time, FGF receptors (FGFR) are expressed throughout the whole of the neuroepithelium (Wanaka et al., 1991). Moreover, FGF-2 is expressed in the embryonic retinal pigment epithelium (RPE) and has been shown to enhance retinal growth and differentiation (Hicks, 1998). When FGF-2 is added to naive retinal explants from rats, an increased rate of RGC differentiation is observed. Conversely, treatment

with antibodies against FGF-2 slows their appearance (Zhao and Barnstable, 1996). Furthermore, overexpression of FGF-2 in *Xenopus laevis* retinal precursors, favours RGC production while reducing the number of Müller glial cells (Patel and McFarlane, 2000). These data suggest that FGF-2 plays an important role in stimulating the differentiation of RGCs in the neural retina.

1.3.3 Initiation of Axon Growth

The first RGCs originate in proximity to the optic fissure. The initial navigational assignment of RGC axons is to extend to the optic nerve head; thus, the first RGCs only travel short distances before entering the optic nerve. The process of axonogenesis is morphologically characterized by a thickening of the cytoplasm on the vitreal side of RGCs (Holt, 1989). Initial RGC axon growth within the retina occurs in contact with endfeet neuroepithelial cells which are simultaneously differentiating into Müller cells. The first axons to grow, termed the pioneering axons, extend directly to the fissure (Silver and Sidman, 1980). In rats, the first axons exit the retina at E13. These initial RGC axons must rely entirely on growth cone guidance cues in the neuroepithelium. More peripheral and therefore younger RGCs, arise in an outwardly concentric manner and elaborate their axons inward. To reach the optic nerve head, these newly formed RGC axons (the majority of retinal axons) use the established pioneer axons as guides in addition to the growth promoting Müller endfeet (Silver and Sidman, 1980; Holt, 1989; Williams et al., 1991). They come in contact and fasciculate with the existing axons by interacting with growth promoting molecules on the axons themselves. For example, L1, a member of the immunoglobulin superfamily, is present at points of contact between

new and elaborated axons and is thought to encourage axonal fasciculation on the way to the optic nerve head (Brittis and Silver, 1995). Inhibition of L1 causes retinal axons to stray and grow within the retina instead of heading directly for the optic nerve head (Brittis et al., 1995).

In addition to the cues on the axons themselves, the molecular environment of RGCs exerts a great influence on axonal outgrowth and pathfinding. For example, RGC axons grow along the vitreal surface of the retinal neuroepithelium while dendrites arborize on the opposite side. When RGC axons contact Müller cell endfeet, they turn 90° and head towards the fissure (Holt, 1989). Interestingly, in explant cultures, the axonal growth promoting properties of Müller cells remain confined to the endfeet on which robust axonal growth can be supported. Upon mechanical removal of the glial endfeet, however, RGC growth is suppressed (Stier and Schlosshauer, 1995). Furthermore, RGCs cultured on a bed of Müller cell somata elaborate dendritic processes (Bauch et al., 1998). RGCs plated next to Müller cells were able to grow axons, although growth cones collapse when they come in contact with the glia. The exact repulsive signal that mediates the Müller-somata induced repulsion of RGC axons has yet to be identified; however, these observations suggest that Müller cell bodies are inhibitory towards RGC axons while their endfeet constitute a permissive substrate.

Another inhibitory mechanism that prevents axonal extension into the retinal periphery involves a ring of chondroitin-sulfate proteoglycans (CSPGs), found in the extracellular matrix (ECM) adjacent to nascent RGCs (Brittis et al., 1992). When this ring is experimentally degraded during the period of RGC development, axons project abnormally into the periphery. The molecular environment ensures that nascent RGC

axons remain confined within the central retina to a zone delimited by RGC cell bodies on one side and Müller endfeet and vitreal basal lamina on the other. The mechanism described here illustrate a well coordinated molecular environment that ensures proper growth of RGC axons and safeguards against disordered axon growth within the retina.

1.3.4 Molecular Determinants of Intraretinal Axonal Growth

Müller cell endfeet are distributed in a continuous layer across the vitreal side of the retina (Holt, 1989), interspersed with basal lamina (Easter et al., 1984; Ledig et al., 1999b). This disposition permits receptors on RGC growth cones to come in contact with several ECM molecules such as laminin, fibronectin and nidogin, heparan sulfate proteoglycans such as agrin and perlecan as well as other CSPGs (Halfter, 1998). Proper RGC navigation is dependent on an adequate balance of attractive and repulsive directional cues present along its pathway. In the process of axonal elongation, growth cone adhesion to its substrate is an obligatory step to generate the necessary forces needed for axon outgrowth. In addition, the activation of intracellular signaling cascades is essential to link the extracellular cues to the axonal cytoskeleton. The following sections describe the receptors and ligands known to influence RGC growth. A general summary of these molecules found along the retino-collicular pathway is outlined in Figure 2 (p.24).

1.3.4.1 Laminins and Integrins

Laminin (LN) is an abundant growth promoting ECM molecule. LN receptors, termed integrins, are heterodimers of α and β subunits which link the axonal plasma

membrane to the ECM. Integrins are important constituents of focal adhesion sites: macromolecular signaling complexes that transmit extracellular signals to the cytoskeleton (Hynes, 1992; Aota and Yamada, 1997). At these sites, the cytoplasmic tyrosine kinases pp60^{c-src} and pp125 FAK (focal adhesion kinase) transduce an array of signals from integrins and growth factor receptors. FAKs are normally expressed in RGC filopodium; however, when tyrosine kinase activity is inhibited, axonal growth is blocked and the FAKs translocate to central regions of the growth cone (Worley and Holt, 1996). pp60^{c-src} is also expressed in RGC growth cones and plays a role in neurite outgrowth (Maness et al., 1988; Bixby and Jhabvala, 1993). The interplay between these two kinases is illustrated by the requirement of FAK activity in order for pp60^{c-src} to phosphorylate its substrates (Schaller et al., 1999). Therefore, following integrin binding, FAK is activated and recruits pp60^{c-src} to focal adhesion sites, allowing the latter to phosphorylate the cytoskeletal proteins required to ensure axonal growth.

1.3.4.2 Cellular Adhesion Molecules

Developing RGC axons as well as the substrates on which they grow abound with cell adhesion molecules (CAMs). The immunoglobulin (Ig) super family is a subfamily of adhesion molecules containing one to seventeen Ig-like domains (Huber et al., 2003). Members of this family are widely expressed on the surface of Müller cell endfeet, basal lamina and on previously elongated pioneer axons, where they act as growth cone guiding cues. The most studied Ig-superfamily CAMs are the neural CAMs (NCAMs) and the closely related L1. They are expressed on elongating axons of vertebrates as distantly related as fish and mammals. When NCAM function is inhibited

in chick embryos, RGC axons extend to the contra-lateral side and fail to exit the retina (Pollerberg and Beck-Sickinger, 1993). Similarly, blockage of L1 in rats promotes axons to grow at right angles to the pre-laid fascicles (Brittis et al., 1995).

Another large family of CAMs is the cadherins (calcium-dependent adherent proteins). The Neural (N)-cadherins, for example, are important in early axon fasciculation and growth and help stabilize retinal axons at their synaptic targets (Ranscht, 2000). Cadherins are linked to the actin cytoskeleton by their cytoplasmic domain via catenins. This interaction with the cytoskeleton, however, is not the driving force behind cadherin-induced axonal growth. Instead, a signaling mechanism, thought to be mediated by the cytosolic protein $p120^{ctn}$, must be triggered in addition to cell-cell adhesion for cadherins to stimulate axonal outgrowth (Shapiro and Colman, 1998; Provost and Rimm, 1999). $p120^{ctn}$, a member of the catenin family and originally described as a substrate of $pp60^{c\text{-}src}$, binds to the juxtamembrane region of N-cadherin. Overexpression of $p120^{ctn}$ results in long filopodial-like structures in fibroblasts (Reynolds et al., 1996), thereby making it an interesting effector of N-cadherin-induced axonal elongation.

The interest in CAMs as modulators of axonal growth stems from their capacity to act both as adhesion molecules and intracellular signal transducers (Walsh and Doherty, 1997). Although the exact mechanisms governing CAM-induced growth remain ambiguous, one possibility lies in that stimulation of either N-CAM, N-cadherin or L1 can lead to the phosphorylation of FGFR. Once activated, FGFR will recruit its downstream signaling cascades including MAPK and PLCγ, both shown to play a role in axonal growth (Doherty and Walsh, 1996). This coupling of FGFR and CAMs during

axonal growth was demonstrated when the expression of a dominant-negative FGFR blocked NCAM mediated FGFR activation and consequently impaired axonal outgrowth (Saffell et al., 1997). Thus, FGFR can be a signaling partner of CAMs and an important mediator of axonal initiation and elongation.

1.3.4.3 Receptor Protein Tyrosine Phosphatases

Axonal growth and guidance require the presence of many receptor types at the growth cone (e.g. integrins, CAMs, growth factor receptors) to sense, interpret and transmit extracellular signals to intracellular effectors. The mechanisms underlying the integration of extracellular cues is not fully understood; however, a large number of these signals are relayed via tyrosine phosphorylation. Protein tyrosine kinases (PTKs) play important roles in signal transduction and it is now clear that protein tyrosine phosphatases (PTPs) are equally crucial. These two groups of enzymes exert opposite activities, yet act in concert to ensure adequate cellular functioning (Ostman and Bohmer, 2001).

PTPs are largely classified in two categories: the transmembrane and non-transmembrane. The former are termed receptor PTPs (RPTPs) because they contain a ligand-binding site on their extracellular domain. Of the five major classes of RPTPs, there is increasing evidence for the role of type IIa RPTPs in axon guidance and growth (Stoker, 2001). Type IIa RPTPs are CAM-like proteins that have large extracellular domains made of Ig and fibronectin type III (FNIII) repeats, a structure akin to NCAMs and thus placing them in the Ig superfamily. On the cytosolic side, type IIa RPTPs are

composed of two phosphatase domains giving them the ability to modulate intracellular phosphorylation events.

One member of this family that is of particular interest in RGC axonal growth is RPTP-σ. The avian orthologue of RPTPσ, CRYPα, is strongly expressed in RGC axons during development (Stoker et al., 1995; Ledig et al., 1999b) and has been shown to influence axonal growth in developing Xenopus (Johnson et al., 2001) and chick retinas (Ledig et al., 1999a). Expression of dominant negative CRYPα increases the length of RGC neurites by 50% in cultured Xenopus retinal explants (Johnson et al., 2001). Moreover, the ligands for RPTPσ have recently been identified as the heparan sulfate proteoglycans (HSPG) agrin and collagen XVIII (Aricescu et al., 2002). These molecules are expressed along the basal lamina and glial endfeet of the retina, two areas where RGC axon grow during development. These findings make RPTPσ an interesting target for modulation to enhance RGC axon growth.

1.3.4.4 Fibroblast Growth Factors and RGC Growth

RGC development and axonal growth require stimulation by growth factors. One of the key families of neurotrophic factors involved in the development and modeling of the retina are the FGFs. As mentioned above, there is ample evidence that FGFs are influential in the differentiation of RGC progenitors (Park and Hollenberg, 1989; Pittack et al., 1991; Guillemot and Cepko, 1992). A role of FGFs in axonal growth is suggested by the observation that these growth factors are abundantly expressed in developing RGCs and throughout the entire optic pathway (de Iongh and McAvoy, 1993; McFarlane et al., 1995), while their receptors are expressed in the growth cones of extending RGC

axons (Cirillo et al., 1990). Moreover, FGF-2 has been shown to be a potent stimulator of RGC axonal outgrowth in *Xenopus* (McFarlane et al., 1995) and expression of a dominant negative, kinase defective FGFR results in a 40% reduction in the rate of RGC outgrowth.

1.3.5 Entering the Optic Nerve

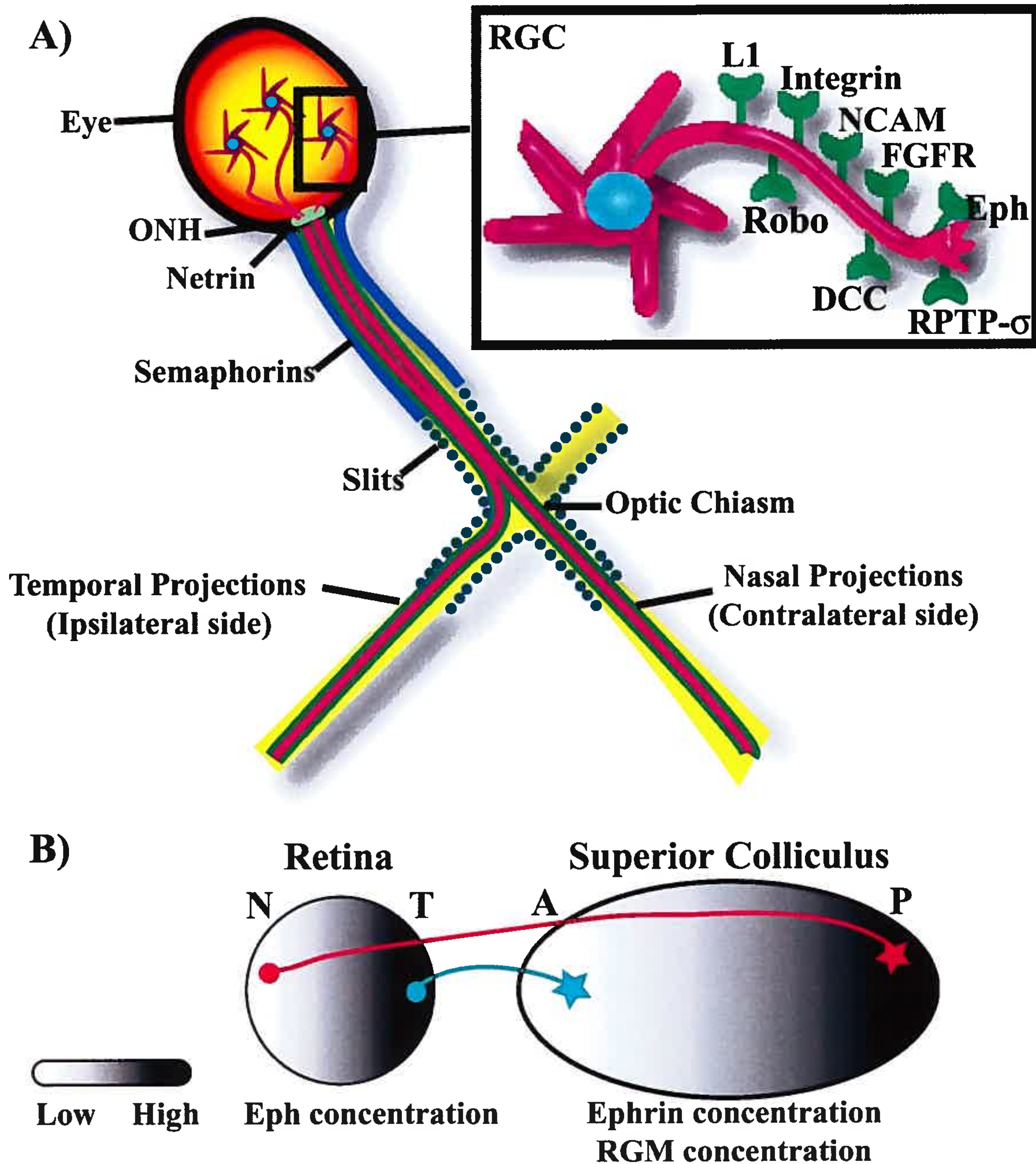
Growth cones undergo a variety of morphological changes along their trajectory. Their shape varies from a simple club-like structure to a highly elaborated cone with multiple filopodia (Holt, 1989). Interestingly, these structural changes depend on the growth cone`s position along its path; the more complex the steering decisions, the more complex the growth cone. For example, cones tend to be simple throughout the retinal surface and in the optic nerve where limited navigational decisions need to be taken. In contrast, they become much more structured in two zones along the pathway where crucial steering choices are made. These growth crossroads are the optic nerve head and the optic chiasm.

The exit of RGC axons from the retina is regulated by netrin-1 (Deiner et al., 1997), an axon guidance cue expressed by neuroepithelial cells at the optic nerve head (Figure 2, p.24). *In vitro*, RGC axons are attracted to artificially formed gradients of netrin-1. This turning is accompanied by a profound transformation in growth cone morphology similar to that seen *in vivo* at the optic nerve head (Holt, 1989; de la Torre et al., 1997). The attraction of RGC growth cones seems to be governed by the Deleted in Colorectal Cancer (DCC) receptor for netrin-1, which is expressed in RGC axons and when stimulated can increase cAMP levels (de la Torre et al., 1997). *In vivo*, the

attractive nature of netrin-1 is most likely contact-dependent, as homozygote knockout mice for netrin-1 are able to reach the optic nerve head, but then instead of entering, continue to grow errantly within the retina (Deiner et al., 1997).

The attraction of RGCs by netrin-1 can be transformed into a repulsive signal by altering intracellular cAMP levels or inhibiting protein kinase A (PKA) (Ming et al., 1997b). Elevated levels of cAMP provoke actin polymerization while reduced levels lead to depolymerization. This conversion is primordial for the orderly exit of axons from the optic nerve head. For example, laminin, which has been shown to reduce the netrin-1-DCC-mediated increase in cAMP levels and therefore transform growth cone attraction to repulsion (Hopker et al., 1999), is abundant on the vitreal surface of the retina. Therefore, when axons reach the optic nerve head, the presence of netrin-1 will establish lower cAMP levels on the vitreal side of the growth cone. Conversely, the high levels of netrin-1 in the optic nerve head will increase cAMP concentrations in that region of the growth cone. This gradient will favor growth cone actin polymerization on the optic nerve head side and consequently leads to axonal prolongation into the optic nerve, whereas depolymerization is likely to occur on the vitreal side (Dingwell et al., 2000). Once out of the eye, a sheath of semaphorines wrapped around the optic nerve prevents defasciculation through chemorepulsive forces (Figure 2, p.24).

FIGURE 2.



Guidance cues in the developing retino-collicular system.

A) A vast number of guidance cues are expressed along the developing retino-collicular system. For example, a ring of netrins found at the optic nerve head (ONH) helps RGC axons leave the eye and enter the optic nerve. Once axons exit the retina, proper axonal fasciculation is maintained through chemorepulsive forces exerted by a sheet of semaphorins around the optic nerve. At the optic chiasm, slits prevent the premature midline crossing of RGC axons. To properly interpret their environment, RGC axons express numerous receptors and adhesion molecules (inset) including the receptor for FGFs, fibroblast growth factor receptor (FGFR), the slit receptor Roundabout (Robo), the netrin receptor deleted in colorectal cancer (DCC), the ephrin receptor (Eph), receptor protein tyrosine phosphatase-Sigma (RPTP), neuronal cell adhesion molecule (NCAM), L1, and integrins. (B) The topographical innervation of the superior colliculus is dictated by concentrations of ephrins (and repulsive guidance molecule (RGM)) expressed along an anterior (A) - posterior (P) gradient and the corresponding nasal (N) - temporal (T) gradient of Eph receptors.

Adapted from Koeberle et al.

1.3.6 Decisions at the Optic Chiasm

In primates, RGC axons from the temporal half of the retina project onto ipsilateral subcortical regions of the brain, while axons originating from the nasal half cross-over at the optic chiasm to the contralateral side of the brain (Figure 2, p.24). In albino rats, ~ 99% of RGC axons cross-over at the chiasm and only ~1 % project to the ipsilateral side (Isenmann et al., 1999b).

The navigational decisions made at the chiasm are important for the proper topographical wiring of RGC axons yet the molecular factors mediating these events are poorly understood. Although local guidance cues contribute to the final path taken by the axon, it is also possible that the destiny of RGC axons, is determined prior to arriving at the chiasm. For example, axons originating in the ventro-temporal retina persistently form ipsilateral projections. Interestingly, it was found that the Zic2 zinc finger transcription factor is expressed exclusively in the ventro-temporal retina at a time when ipsilateral projections are being formed (Herrera et al., 2003). Moreover, both gain of function and loss of function experiments demonstrate that Zic2 by itself can redirect contralateral projections to ipsilateral ones. Another example of a change in neuronal biochemistry that can affect RGC decisions at the chiasm comes from mice deficient in GAP-43. Here, the knockouts showed randomized RGC crossing and abnormal ipsilateral turning (Sretavan and Kruger, 1998). Furthermore, as mentioned above, albino animals have a greatly reduced number of ipsilateral projections (Isenmann et al., 1999b). This has been attributed to a defect in the *tyrosinase* gene; a finding that was confirmed in animals with eye-specific albinism (Oa-1 knockout mice) (Incerti et al.,

2000). These examples provide evidence that the fate of axonal projections at the chiasm, may be governed by the biochemical make-up of RGCs themselves.

As RGC axons segregate at the optic chiasm it is important that nerve integrity be maintained. This is ensured by a family of inhibitory proteins known as the Slits. These proteins are structurally similar to other ECM molecules that contain EGF repeats, leucine-rich repeats and a laminin G domain. When slit proteins bind to their receptor Roundabout (Robo), they inactivate Cdc-42 and provoke growth cone collapse (Huber et al., 2003). Slits are expressed in the anterior and posterior regions of the developing optic chiasm where they prevent premature midline crossing of RGC axons. The spatial expression pattern of Slits 1 and 2 in the visual system indicates that they form an inhibitory wall, funneling growing axons along appropriate trajectories at the chiasm. They help maintain adequate fasciculation through chemorepulsive forces and their layout contributes to the formation of the optic chiasm (Plump et al., 2002) (Figure 2, p.26). In transgenic mice deficient in Slits 1 and 2, certain RGC axons cross prior and form a secondary chiasm. This phenotype does not involve all RGC axons (Plump et al., 2002), most likely due to variable expression of the Robo receptor, and therefore indicates that other directional cues are required.

1.3.7 Innervation of the Superior Colliculus

Retinal innervation of the superior colliculus (one of the major subcortical targets of RGC axons) occurs along a specific anterior/posterior and dorsal/ventral arrangement. Axons projecting from the temporal retina innervate the anterior superior colliculus, while axons from the nasal side target posterior regions. In addition, axons originating

from the dorsal retina reach the ventral superior colliculus, while those from the ventral side project to dorsal regions. Retinal projections are highly organized in that adjacent RGCs in the retina project to adjacent midbrain targets while maintaining their spatial organization. In order to accurately preserve this orderly distribution, guidance cues are distributed in a gradient like pattern. A gradient of guidance molecules requires far fewer proteins than the colossal task of assigning a distinct receptor-ligand to each axon-target combination and is therefore a considerably more efficient targeting mechanism (Sperry 1963).

Evidence for a chemical gradient that directs axonal growth came from experiments where RGC axons were collected from the posterior (temporal) or anterior (nasal) retina of a chick and plated on the membranes collected from the posterior or anterior tectum (avian superior colliculus) (Bonhoeffer and Huf, 1985). It was noticed that RGCs harvested from the posterior retina would only grow on the anterior tectal substrate whereas RGCs from the anterior retina would grow on both anterior and posterior tectal membranes. These observations indicated that a repulsive molecule was present in the posterior tectum and led to the identification of a vast family of receptor tyrosine kinases, the Eph kinases, and their membrane bound ligands: the ephrins (Bonhoeffer and Huf, 1985). It was later found, in both chicks and mice, that specific ephrins are expressed down an antero–posterior gradient in the superior colliculus, whereas a corresponding naso-temporal gradient of Eph receptors is present in the retina. Thus, axons traveling from the temporal retina, where Eph receptors abound, tend to avoid the inhibitory signals from ephrins expressed in the posterior colliculus. In the

nasal retina, axons express fewer Eph receptors and are consequently capable of innervating more posterior regions of the superior colliculus (Figure 2 B, p.24).

An additional guidance molecule that has been recently identified in the chick tectum is the Repulsive Guidance Molecule (RGM) (Monnier et al., 2002). As with ephrins, RGM is expressed along a concentration gradient from the anterior to the posterior pole of the tectum. RGM is capable of provoking the collapse of temporal (but not nasal) growth cones at concentrations significantly lower than ephrins. It will be interesting to further study the role and mechanism of action of this molecule in higher vertebrates.

1.4 <u>OPTIC NERVE AXOTOMY</u>

Following traumatic injury to the mammalian CNS, neurons die by apoptosis, necrosis or a medley of both. Neurons that have their axons sheared open will undergo fundamental cellular and biochemical changes. Notably, the physical separation of the cell body from its target results in the absence of retrograde transport of neurotrophic factors thought to be required for survival (Oppenheim, 1991). Furthermore, calcium influx destabilizes the cytoskeleton and contributes to the activation of apoptotic pathways and the ensuing cellular degradation. Neurons that survive are severely impaired and rendered inactive, at least transiently, by the dramatic variations in their ionic and metabolic environment. In brief, adult mammalian CNS neurons are unable to regenerate an axon and their soma dies or atrophies after lesion (Ramon y Cajal, 1928).

1.4.1 Acute Optic Nerve Injury and RGC Death

The optic nerve is formed by RGC axons extending from the retina to the brain and is therefore the transmission cable of visual input to the image processing centers of the brain. Any damage to this structure will compromise vision. Optic nerve afflictions include: i) glaucoma, in which an increase in intraocular pressure ravages the optic nerve at the optic nerve head; ii) multiple sclerosis, an autoimmune disease in which optic nerve myelin is degraded; iii) cancerous tumors which compress the nerve, and iv) acute trauma such as an orbital fracture. Although full transection (axotomy) of the optic nerve is unlikely to occur and traumatic injury of the optic nerve is very rare (Steinsapir and Goldberg, 1994), the optic nerve axotomy produces a well characterized time course of RGC death, permitting the accurate evaluation of neuroprotective and regenerative strategies. In addition, RGCs die by apoptosis after optic nerve axotomy as they do in glaucoma (Garcia-Valenzuela et al., 1995) and other CNS diseases. Thus, strategies to promote cell survival or regeneration in this system may be extrapolated to other neurodegenerative diseases affecting other neuronal populations, as well as for the treatment of traumatic CNS injuries such as spinal cord lesion.

1.4.2 Pattern of RGC Cell Death in the Axotomy Model

Transection of the adult rat optic nerve leads to a bi-phasic pattern of cell death. In the first and protracted phase, within 4 to 5 days after lesion, only a minute number of RGCs die. In the second and rapid stage, however, there is massive cell death. Seven days after axotomy, only 50 % of RGCs survive and, of these, less than 10 % remain 14

days after injury. Interestingly, approximately 5 % of RGCs remain viable up to 20 months after transection (Villegas-Perez et al., 1993).

Following optic nerve axotomy, RGC death has been described as apoptotic (Berkelaar et al., 1994b; Garcia-Valenzuela et al., 1994). Apoptosis is an energy consuming process that requires *de novo* protein synthesis. Apoptosis is characterized by cell shrinkage, condensation of nuclear chromatin, intranucleosomal DNA fragmentation, breakdown of the nuclear membrane and formation of small membrane bound vesicles termed apoptotic bodies (Garcia-Valenzuela et al., 1994; Cellerino et al., 2000; Nijhawan et al., 2000). The latter are then phagocytosed by microglial cells, thus avoiding an inflammatory response.

Although apoptosis is the active process by which RGCs die after axonal transection, a certain number of cells die by necrosis due to mechanical or inflammatory damage inflicted by the injury (Thanos et al., 1993; Bien et al., 1999). Necrosis typically occurs after toxic insult, hypoxia, energy depletion or other exogenous insults. It results in a swelling of the cell body and mitochondria, followed by perforation of the cell membrane resulting in the leakage of cellular contents and consequent inflammatory response. It is likely that there is a continuum between axotomy-induced apoptotic and necrotic cell death as both modes share common characteristics (Nicotera et al., 1999).

1.5 APOPTOSIS

The apoptotic machinery is present in all cells and is therefore considered to be an intrinsic suicide program. Neuronal survival relies on a detailed interaction between the cell and its environment. The neuron is thought to be continuously on the verge of

apoptosis, requiring survival signals to prevent its death (Raff. 1992; Raff et al., 1993). Neurotrophins, cytokines, growth factors, and other peptide ligands stimulate transmembrane receptors. which activate intracellular pathways and ultimately inhibit this internal death program (reviewed in Jacobson et al., 1997).

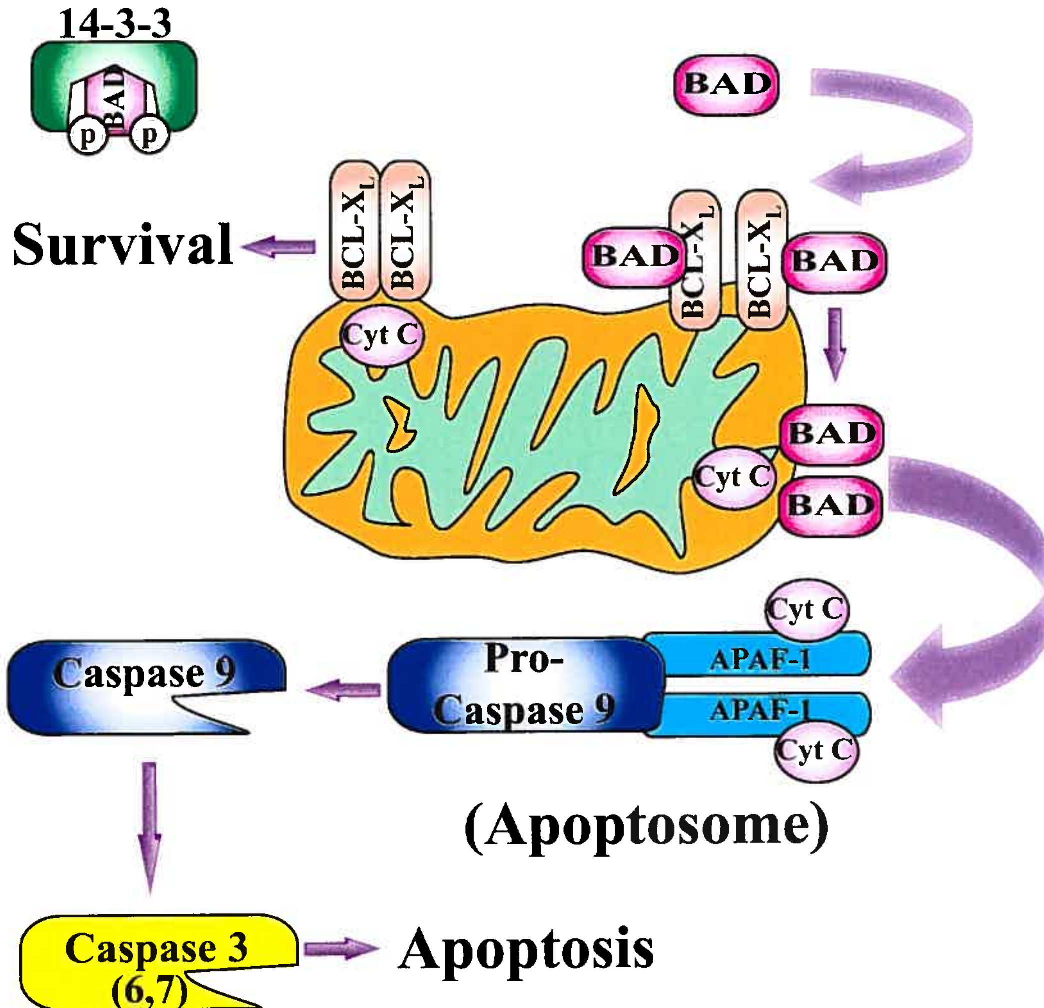
The apoptotic pathway can be induced either by stimulation of cell surface death receptors (Ashkenazi and Dixit, 1998) or through a mitochondrial pathway (Green and Reed, 1998). Both modes of activation result in the regulation of members of the Bcl-2 and caspase families (Merry and Korsmeyer, 1997; Green and Reed, 1998; Yuan and Yankner, 2000).

1.5.1 The Bcl-2 Family

The activation of the apoptotic program is regulated by members of the Bcl-2 family (reviewed in Merry and Korsmeyer, 1997). A subset of this family is said to be pro-apoptotic (Bad, Bax, Bak, Bok, Bid and Bik) and another anti-apoptotic (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and A-1) (Kearns et al., 1996). Bcl-2 proteins are situated on the cytoplasmic side of the outer mitochondrial membrane, the nuclear envelope and the endoplasmic reticulum.

Although there appears to exist several possible mechanisms by which members of the Bcl-2 family control apoptosis, one of the key functions is to control the release of pro-apoptotic factors, such as cytochrome C (cyt C), from the mitochondria.

FIGURE 3.



Bcl-XL prevents the loss of mitochondrial cytochrome C.
The balance between pro-apoptotic and anti-apoptotic members of the Bcl-2 family is paramount to the survival of CNS neurons. When anti-apoptotic members such as Bad are phosphorylated by growth factor-induced signaling cascades, they are sequestered in the cytoplasm by the protein 14-3-3. Conversely, dephosphorylated Bad translocates to the mitochondria and shifts the balance between anti- and pro-apoptotic Bcl-2 family members. Consequently, cytochrome C is released from the mitochondrial inter-membrane space, binds to Apaf-1 (Apoptosis protease activated factor) and together recruit and activate the pro-caspase-9. This complex known as the apoptosome, then releases active caspase-9 which in turn activates downstream effector caspases such as caspase-3, caspase-6 and caspase-7 and ultimately provokes neuronal apoptosis.

Growth factor-induced signaling cascades phosphorylate anti-apoptotic members such as Bad thereby making them targets for he cytoplasm by the protein 14-3-3. In contrats, when in a non-phosphorylated state, Bad translocates to the mitochondria and shifts the balance between anti- and pro-apoptotic Bcl-2 family members.

Consequently, there is leakage of cyt C which brakes the electron transfer chain and impairs energy production, increases the generation of reactive oxygen species and, most importantly for apoptosis, cyt C activates Apoptosis Protease Activated Factor-1 (Apaf-1) and pro-caspase-9 (Reed, 1997) (Figure 3, p.32). During apoptosis, cyt C leaks from the mitochondria and binds Apaf-1. This complex then recruits pro-caspase-9 and becomes the apoptosome, which can go on to activate the caspases-3, 6 and 7, also known as the executioner caspases.

A possible mode of action for anti-apoptotic members of Bcl-2 is that Bcl-2 and Bcl-X_L inhibit cyt C release by binding and forming heterodimers with pro-apoptotic Bcl-2 members such as Bax. Furthermore, Bcl-X_L can directly sequester Apaf-1 via its (Bcl-2 homology) BH4 domain. These events block Bax homodimerization and thus avert the formation of cyt C permeable channels. Other postulated pro-survival mechanisms mediated by members of the Bcl-2 family involve preventing the release of pro-apoptotic elements from the mitochondria (e.g. Smac, Diablo, cyt C and HtrA2/OMI) (Saelens et al., 2004). This may be achieved through maintaining mitochondrial membrane integrity by ensuring proper ATP for ADP exchange by the adenosine nucleotide translocator (ANT), or by acting directly on the voltage dependent anion channel (VDAC). These mechanisms which involve the interplay of pro-survival

Bcl-2 members with various mitochondrial exchange pore proteins safeguard against mitochondrial swelling and rupture (Bortner and Cidlowski, 2002).

Bcl-2 is expressed at high levels during development and is downregulated in the adult CNS and retina (Merry et al., 1994; Levin et al., 1997; Merry and Korsmeyer, 1997). Conversely, Bcl-X_L is expressed throughout adulthood in the CNS and in RGCs (Isenmann et al., 1997; Levin et al., 1997; Krajewska et al., 2002), suggesting that it can promote the survival of adult neurons. Interestingly, optic nerve axotomy leads to an increase in Bax expression in transected RGCs while levels of Bcl-X_L decline (Isenmann et al., 1997; Levin et al., 1997). These newly skewed proportions of anti- to pro-apoptotic members can contribute to axotomy-induced RGC cell death (Isenmann et al., 1997). Additional evidence stems from experiments where neuronal death was delayed when axotomized RGCs were transduced with Bax antisense oligonucleotides (Isenmann et al., 1999a) or injected with a permeable form of Bcl-X_L (Isenmann et al., 1999a). Furthermore, adult transgenic mice that overexpress Bcl-2 show considerably higher numbers of RGCs after axotomy than wild-type littermates (Cenni et al., 1996; Porciatti et al., 1996). Together, these results indicate that an increase in Bax leads to RGC apoptosis, while an increase in Bcl-2/Bcl-X_L protects RGCs from axotomy-induced death.

1.5.2 Caspases

During apoptosis, a vast array of intracellular proteins are cleaved and degraded. The family of cysteine-activated aspartate-specific proteases, known as caspases, was identified as the principal effectors of this protein breakdown. Caspases are catalytically

inactive zymogens (pro-enzymes) that require proteolytic cleavage to become activated (Thornberry and Lazebnik, 1998). They are classified with respect to the sequence of their amino-terminal pro-domain. Caspases that have death effector-domains, such as caspase-8 and 10, are activated when recruited to the intracellular death domains of receptors like CD95 (Apo-1/Fas) or tumor necrosis factor (TNF) receptor. Alternatively, caspases which have caspase-activating recruitment domains (CARDs), such as caspases 1, 2, 4, 5, 9, 11 and 12, are activated by multimeric complexes such as the Apaf-1/cytochrome C/caspase-9. Caspase bearing short pro-domains, such as caspase-3, can be activated by a majority of the aforementioned caspases. Additionally, caspases can be classified either as regulatory caspases that activate other caspases (e.g. 8, 9, 10) or as executioner (effector) caspases that actively dismantle the cell by acting on various protein substrates (e.g. 3, 6, 7).

In neurons, there are two well characterized caspase pathways involved in apoptotic cell death. The first requires binding of Fas Ligand and/or TNF to their cell surface receptors (Fas and TNF Receptor). This binding induces the receptors trimerization (Nagata, 1997) and once activated, the receptors subsequently recruit adaptor proteins such as Fas Associating Protein with Death Domain (FADD/MORT1), which summon pro-caspase-8 to the receptor where it becomes activated (Boldin et al., 1995; Muzio et al., 1996; Srinivasula et al., 1996). Once in an active state, casapase 8 will turn on other effector caspases such as caspase-3, 6 and 7 (Boldin et al., 1995; Muzio et al., 1996; Srinivasula et al., 1996). In addition, caspase-8 can cleave and activate pro-apoptotic Bcl-2 family member Bid, thereby provoking its translocation to the mitochondria and promoting cyt C release and consequently amplifying the caspase

response (Li et al., 1998; Luo et al., 1998). Of interest, Fas and TNF monomers have also been shown to self assemble independent of ligand binding, yet in this scenario, cells do not show recruitement of Caspase-8 (Seigel et al. 2000) .

The other major caspase pathway involves the apoptosome complex described above, where Apaf-1 associates with caspase-9 after cyt C leakage from the mitochondria. The formation of the complex leads to caspase-9 activation, which is then free to activate other effectors such as caspase-3, 6 and 7.

In RGCs, caspase-3 activation has been associated with RGC death after optic nerve axotomy (Kermer et al., 1999; Kermer et al., 2000b) and was found to be activated in an ocular hypertension rat glaucoma model (McKinnon et al., 2002). Moreover, inhibition of caspase-3 by protein inhibitors was shown to decrease the extent of cell death ensuing from optic nerve injury (Kermer et al., 1998; Chaudhary et al., 1999). The signals upstream of caspase activation in retinal degeneration remain a matter for debate. Nevertheless, caspase activity is far down the chain of events and occurs in the late stages of RGC degeneration. In order to achieve an efficient and sustainable approach for treating RGC apoptosis, it is imperative to address the causes and early initial events that lead to cell death rather then tend to the final consequences of the degenerative process.

1.5.3 Signaling and Apoptosis

Neuronal survival relies on the constant inhibition of the cell's intrinsic death program by neurotrophic factor signaling (Raff et al., 1993; Burek and Oppenheim, 1996). The exact nature of the stimulus required to tilt the balance in favor of survival is

likely quite complex and has yet to be fully defined. In the PNS, for instance, prevention of apoptosis can be achieved with single peptide trophic factors (Hamburger, 1993; Huang and Reichardt, 2001) such as nerve growth factor (NGF) or ciliary neurotrophic factor (CNTF). Survival of PNS neurons can also be maintained by either increasing intracellular levels of cAMP (Rydell and Greene, 1988) or by depolarizing the neurons (Franklin et al., 1995).

Conversely, CNS neurons appear to necessitate multi-factorial stimulation to stay alive (Snider, 1994; Meyer-Franke et al., 1995). Meyer-Franke et al. (1995) postulated that the survival of RGCs requires sustained stimulation by the various factors secreted from different cell types distributed along the optic pathway. For example, FGF-2 secreted from retinal cells, insulin-like growth factor-1 (IGF-1) and leukemia inhibitory factor (LIF) from astrocytes, a yet to be identified factor from oligodendrocytes and brain derived neurotrophic factor (BDNF) from the target cells in the superior colliculus (Meyer-Franke et al., 1995). In addition, *in vitro* paradigms indicate that the response of CNS neurons to trophic peptides can be greatly enhanced by either depolarization or an increase in cAMP levels (Hack et al., 1993; Hanson et al., 1998; Meyer-Franke et al., 1998). The multiple and varied stimulations required to maintain CNS neurons alive illustrate the intricacies that must be addressed when devising neuroprotective strategies for lesioned RGCs.

Neurotrophic factors, such as the neurotrophins (NGF, BDNF, NT-3, NT-4), CNTF and the FGFs, bind to and activate cell surface receptor tyrosine kinases (RTK). Ligand binding stimulates the autophosphorylation of tyrosine residues on the cytoplasmic moieties of the receptors. These phosphotryrosines then activate distinct

signaling cascades as they become docking sites for signaling molecules such as phosphoinositide-3 kinase (PI-3K), phospholipase Cγ and adaptor proteins such as Shc, Grb2 and Sos which trigger other transduction pathways (Figure 4, p.41). Here, we will focus on two pathways of particular importance for RGC survival; the PI-3K/Akt pathway and the Ras/Raf/MAPK pathway (Datta et al., 1999; Grewal et al., 1999).

1.5.3.1 The PI-3K/ Akt Pathway

PI-3K is present in the cytosol of neurons including RGCs and is activated by either the stimulation of RTKs, receptors indirectly coupled to tyrosine kinase (e.g. integrins), or receptors coupled to seven transmembrane G-protein receptors (Datta et al., 1999). Activation of these receptors results in the direct recruitment of PI-3K to intracellular tails of the phosphorylated receptor, or indirectly via the Ras pathway. Once stimulated, PI-3K promotes the formation of 3'-posphorylated phosphoinositides. These lipids in turn bind Akt and provoke its translocation from the cytoplasm to the plasma membrane (Franke et al., 1995; Franke et al., 1997), where it comes in close proximity with 3'-phosphoinositide-dependent protein kinases (PDKs). These kinases (which are themselves regulated by phospholipids) phosphorylate and activate the serine/threonine kinase Akt (Alessi et al., 1997).

Akt inhibits apoptosis by acting on several substrates (Figure 4, p.41) :

1) It phosphorylates pro-apoptotic Bad. When pro-apoptotic Bcl-2 family members, such as Bad, are in an unphosphorylated state, they tend to form heterodimers with anti-apoptotic proteins such as Bcl-X_L. consequently shifting the pro- to anti apoptotic equilibrium. The pro-apoptotic proteins subsequently form homodimers and

integrate into the mitochondrial membrane, ultimately resulting in apoptosis (Gross et al., 1998). Conversely, if Bad is phosphorylated, it will become a target for protein 14-3-3, which will sequester it in the cytosol thereby blocking apoptosis (Zha et al., 1996).

2) It inactivates IκB kinase (IKK), thereby releasing IκB (the repressor element of NFκB) and permitting NFκB-dependent transcription of survival genes.

3) It phosphorylates the pro-apoptotic transcription factors Forkheads, which favors their association with 14-3-3 protein and thus prevents their entry into the nucleus (Brunet et al., 1999). Akt has also been shown to phosphorylate and therefore activate the transcriptional activator cAMP-response element binding protein (CREB) and thus enhancing CREB-dependent transcription (Du et al., 1998). CREB is thought to mediate the transcription of pro-survival genes (Riccio et al., 1999).

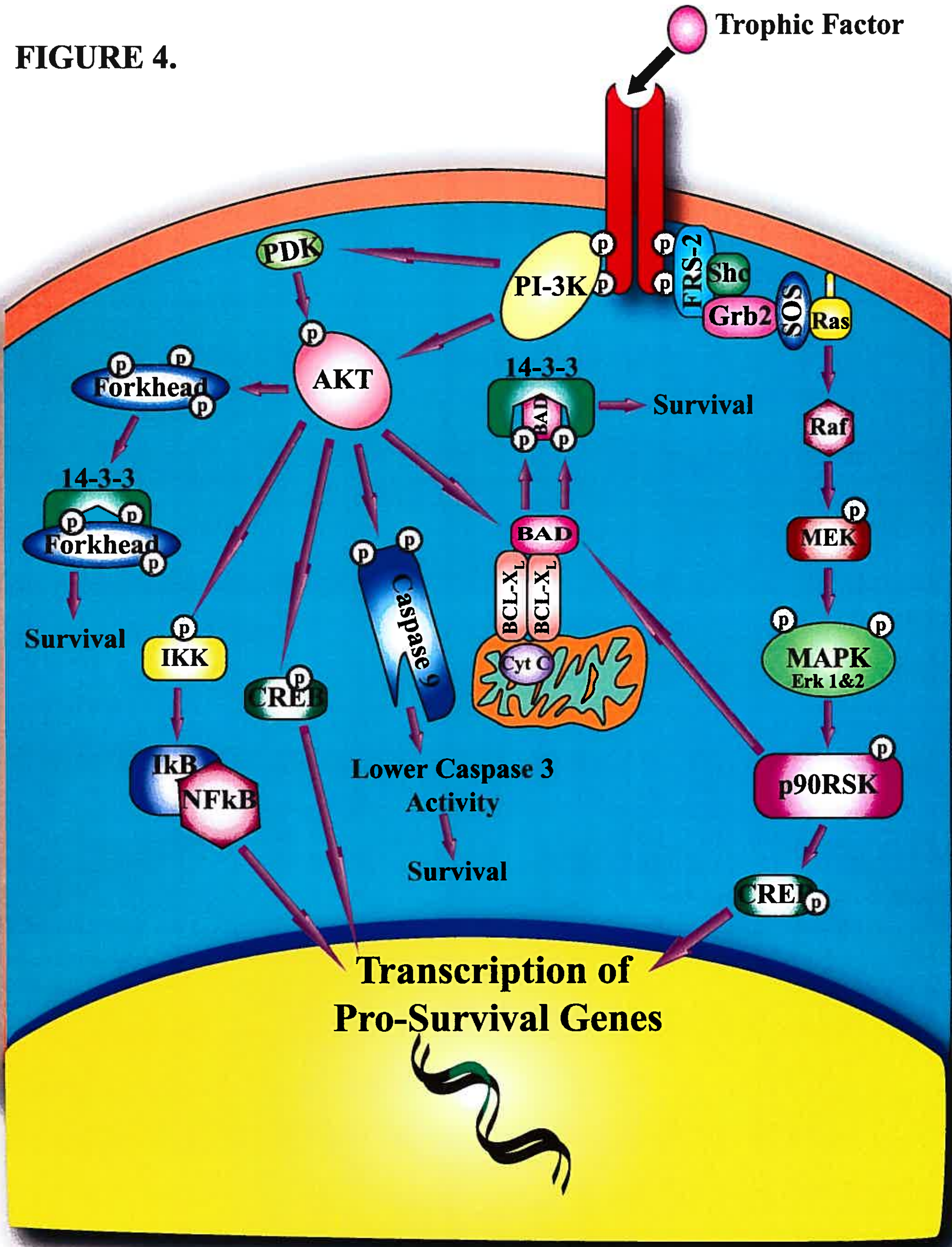
4) It phosphorylates and therefore directly inactivates caspase-9. This mechanism does not seem to be conserved throughout different species as the phosphorylation site for Akt found in human caspase-9 is not present in mice (Fuchs et al., 1998).

Although the PI-3K/Akt pathway is important for the prevention of apoptotic cell death by certain stimuli in a variety of neurons, other signaling cascades are often required. For example, IGF is neuroprotective for axotomized RGCs via a PI-3K dependent pathway (Kermer et al., 2000a), whereas BDNF induced neuroprotection is not compromised when the PI-3K is pharmacologically blocked (Klocker et al., 2000). Furthermore, RGC survival mediated by TrkB (the BDNF receptor) is achieved solely via the MEK/MAPK pathway (Cheng et al., 2002a).

Figure 4. Neuronal survival induced by growth factors.

The binding of neurotrophic factors such as NGF, BDNF, NT-3, NT-4, CNTF and FGFs provokes the dimerization and auto-phosphorylation of receptor tyrosine kinases. The phosphorylated tyrosine residues on the intracellular tails of the receptor provide docking sites for a variety of signaling and adaptor proteins. For example, following ligand binding, phosphoinositide 3-kinase (PI-3K) as well as the docking proteins growth factor receptor-bound protein 2 (Grb2), FGFR substrate-2 (FRS-2) and Shc are recruited to the activated receptor. PI-3K subsequently activates 3'-posphorylated phosphoinositides which translocate Akt to the inner surface of the plasma membrane where it is phosphorylated and activated by 3'-phosphoinositide-dependent protein kinase (PDK) (also activated by PI-3K). Akt then phosphorylates Bad, Forkhead, and caspase-9 consequently blocking the apoptotic pathway. Alternatively, Akt phosphorylates the cAMP-response element binding protein (CREB), resulting in the transcription of pro-survival genes or Akt phosphorylates IκB kinase (IKK) thus releasing IκB (the repressor element of NFκB) and permitting NFκB-dependent transcription of survival genes. Concurrently, the recruitment of FRS-2, Grb2 and Shc, attracts son of sevenless (SOS) nucleotide exchange factor which, stimulates the Ras-Raf-MEK-Erk pathway and activates pp90 ribosomal S6 kinase (RSK). As with Akt, RSK also phosphorylates Bad and CREB and the two may act synergistically to promote survival.

FIGURE 4.



Adapted fromYuan et al. 2000, Nature

1.5.3.2 The MEK/MAPK Pathway

The mitogen-activated protein kinase (MAPK) pathway is involved in a vast array of cellular functions from growth to apoptosis (Grewal et al., 1999). As mentioned above, upon neurotrophic stimulation, Shc, Grb2, SOS and other adaptor proteins are recruited to the activated RTK's cytosolic domain (Figure 4, p.41). This cascade of events leads to the activation of the small GTP-binding protein Ras and the subsequent phosphorylation of Raf, MAP kinase/ extracellular signal-regulated kinase (ERK) kinase (MEK) and ERK (Bonni et al., 1999). MAPK's modulation of apoptosis is achieved by the recruitment of members of the pp90 ribosomal S6 kinase (RSK). The latter, as did Akt, phosphorylates Bad, thereby preventing its death promoting functions. Of interest, Akt and RSK each phosphorylate Bad on a different serine residue, indicating that the MAPK and PI-3K pathways could act synergistically to prevent apoptosis (Datta et al., 1999). Moreover, RSKs also phosphorylate CREB and therefore stimulate the transcription of survival genes such as Bcl-2 (Riccio et al., 1999).

1.5.4 RGC Axotomy and Trophic Deprivation

RGCs, as other CNS neurons, receive trophic support from their midbrain targets (Bhattacharyya et al., 1997; Grimes et al., 1997; Riccio et al., 1997; Senger and Campenot, 1997). During development, RGCs undergo a period of cell death. This cell death can be exacerbated by partial or total removal of the superior colliculus and/or the lateral geniculate nucleus (their targets in the brain) (Hughes and Lavelle, 1975; Hughes and McLoon, 1979). In contrast, increasing the size of the target diminishes the extent of normally occurring death (Sengelaub and Finlay, 1981). These observations are in

agreement with the neurotrophic hypothesis suggesting that developing neurons are in competition for a limited supply of trophic factors secreted by their targets. Moreover, in the adult CNS, fully transected neurons tend to die, whereas neurons that have branched projections remain alive after axotomy given that one of the collateral branches remains intact (Bernstein-Goral and Bregman, 1997; Bernstein-Goral et al., 1997). This indicates that the intact collateral branches remain channels for retrograde trophic support. Of interest, RGCs transected closer to the optic nerve head degenerate at a faster rate than those axotomized closer to their targets with longer segments of intact axon in place (Berkelaar et al., 1994). Together, these data suggest that CNS neurons require a target-derived retrograde signal to survive.

The role of retrograde trophic support in the maintenance of adult neurons is however somewhat controversial. For example, when both the adult superior colliculus and lateral geniculate nucleus are chemically destroyed with kainate, a paradigm where axonal terminals remain intact, there is no effect on the survival of mature RGCs although their primary source of retrograde trophic support is removed (Perry and Cowey, 1979). Likewise, pharmacological inhibition of retrograde transport with lidocaine in neonatal rats, results only in a minor increase in cell death (Fagiolini et al., 1997). These results could be attributed to the high levels of trophic factors, such as BDNF and FGF-2, that are produced in the retina proper (Gao et al., 1997; Herzog and von Bartheld, 1998). For example, Müller cells are an important source of trophic factors in the inner retina. These glial cells envelope RGCs and can therefore influence their microenvironment. It has been demonstrated that conditioned medium from Müller cells is more effective at promoting survival during the period of naturally occurring

RGC death than extracts from the superior colliculus (Armson et al., 1987). Furthermore, this conditioned medium from Müller cells has been shown to promote neurite outgrowth from RGCs. The Müller cell medium, however, loses its potency as RGCs mature while the collicular extract retains its neuroprotective quality. Remarkably, after optic nerve injury, reactive Müller cells regain the ability to promote survival of mature RGCs thus indicating that these cells may be essential in the intrinsic response to retinal injury. There are a number of trophic factor sources within the visual system. For example, the superior colliculus produces BDNF (Sievers et al., 1989), the astrocytes in the optic nerve and superior colliculus produce CNTF and LIF (Stockli et al., 1991; Yamamori, 1991), cells of the outer nuclear layer and inner nuclear layer produce FGF-2 (Sapieha et al., 2003). Therefore, it is unlikely that retrograde trophic factor deprivation is the only factor leading to the death of adult, axotomized RGCs. For example, if this hypothesis were true, treatment with neurotrophic factors should block RGC death. Although trophic supplementation for axotomized RGCs has been intensively explored and is currently the most effective strategy to promote RGC survival, the effects are of a rather modest magnitude and a transient nature (Mansour-Robaey et al., 1994; Aguayo et al., 1996; Clarke et al., 1998; Di Polo et al., 1998; Shen et al., 1999). These observations suggest that other factors should also be considered during axotomy-induced degeneration.

For example, following axonal transection, a large percentage of neurotrophic receptors such as TrkB are downregulated from the cell surface (Cheng et al., 2002a). Furthermore, these neurons have reduced levels of cAMP after injury as a result of impaired synaptic connections or lower activity (Northmore, 1987). This loss of cAMP

has been shown to prevent the ability of MAPK to translocate following trophic stimulation (Shen et al., 1999). In fact, treatment with cAMP, or artificial induction of depolarization with K^+ and Ca^{2+} channel agonists, was required to reverse the observed lack of trophic responsiveness by recruiting TrkB (the high affinity receptor for BDNF) from intra-cellular stores to the cell membrane (Meyer-Franke et al., 1998). Alternatively, upregulation of TrkB within RGCs using AAVs has also proven to be an effective approach to enhance responsiveness to BDNF (Cheng et al., 2002a). These observations indicate that axotomized RGCs not only lack trophic stimulation, but also lose their ability to respond to trophic signals.

1.6 AXON REGENERATION IN THE CNS

In contrast to their PNS counterparts, lesioned CNS neurons are not only unable to survive but are also incapable of regenerating their axons. There exists a number of factors that can hinder mature CNS axons regrowth. For example, reduced trophic support, lower cAMP levels, inhibitory myelin proteins and a fortified repulsive glial scar. Importantly, it has been demonstrated that, when adult RGCs are given a permissive PNS environment, a certain percentage gains the ability to survive and regenerate their axon (Ramon y Cajal, 1928; David and Aguayo, 1981) (Richardson et al., 1980; Villegas-Perez et al., 1988). These findings demonstrate that given the right conditions (e.g. a permissive environment), axon regrowth can be achieved in the adult injured CNS. Finding the optimal determinants and stimulatory conditions to regenerate mature CNS neurons remains one of the great challenges in modern neurobiology. In the

following section I will examine the role of the inhibitory CNS environment versus the intrinsic capabilities of CNS neurons to regenerate.

1.6.1 The Inhibitory CNS Environment

The success of peripheral nerve grafts as bridges for CNS axonal growth suggested that components of the injured CNS environment such as myelin and the glial scar are important factors contributing to regeneration failure.

1.6.1.1 CNS Myelin

The inhibitory properties of CNS myelin were examined in the pioneering work of Martin Schwab and colleagues back in the late 80s. Their investigations showed that the growth inhibitory properties of a certain fraction of myelin preparation could be partially reversed by using a function blocking antibody raised against this fraction that they named IN-1. These observations eventually led to the discovery of a 250 kDa glycoprotein with axon growth inhibitory properties that was given the name Nogo (Caroni and Schwab, 1988).

Subsequently, another component of CNS myelin, myelin-associated glycoprotein (MAG), was found to play a growth inhibitory role (McKerracher et al., 1994; Mukhopadhyay et al., 1994). Originally described in 1973 (Everly et al., 1973), this member of the immunoglobulin superfamily, has both adhesive and growth inhibitory properties. Interestingly, MAG promotes neurite growth of E18 RGCs yet inhibits growth of postnatal RGCs (DeBellard et al., 1996), suggesting that either its receptor or one of its co-receptors could be developmentally expressed.

The most recent myelin growth inhibitor to be identified was oligodendrocyte-myelin glycoprotein (OMgp) (Kottis et al., 2002; Wang et al., 2002b). OMgp is a glycosyl-phosphatidylinositol (GPI)-linked glycoprotein with high levels of expression in mature oligodendrocytes (Quarles, 1997).

Remarkably, it was recently demonstrated that all three inhibitory molecules bind to the Nogo receptor (NgR) (Fournier et al., 2001; Liu et al., 2002; Wang et al., 2002b). Because NgR is a GPI linked protein lacking a transmembrane domain, it requires a binding molecule to transduce its inhibitory signal. One binding/transduction partner that was recently identified is the receptor p75 (Wang et al., 2002a; Wong et al., 2002). In fact, neurites cultured from p75 deficient mice were not inhibited by myelin (Wang et al., 2002a), suggesting a direct role of p75 in mediating the growth inhibitory effects of myelin. These novel findings indicate that it could be feasible to override the growth inhibitory effects of myelin by blocking a single target, such as the Nogo receptor, on which all of the myelin induced signaling converge. If accurate, this would greatly facilitate the design of therapeutic approaches to counter the growth inhibitory activity of CNS myelin.

1.6.1.2 The Glial Scar

Axonal injury in the CNS produces a scar that is inhibitory towards axon regrowth (Berry et al., 1983). The glial scar was first described in the 50s, as a physical barrier consisting of collagen aggregates, dense conjunctive tissue, neovascularization, macrophage infiltration and a glial limitans (Clemente and Van Breemen, 1955; Scott and Clemente, 1955). It is now well established that the glial scar is also rich in growth

inhibitory molecules. The lack of scar formation in the injured CNS of neonatal rats sparked further interest for its potential role in regenerative failure in the adult nervous system (Fawcett and Asher, 1999).

In the days ensuing a lesion, monocytes and macrophages enter the injured zone via the ruptured circulatory system. At this time point, the transected axons are undergoing what Cajal termed "short distance frustrated regenerative acts" (Ramon y Cajal, 1928). This phenomenon could potentially be attributed to the macrophages' degradation of inhibitory myelin or their secretion of growth promoting molecules such as FGFs (Nathan, 1987; Fawcett and Asher, 1999). Next, meningeal cells, oligodendrocyte precursors as well as fibroblasts enter the lesion site and intertwine with the capillaries, forming a dense conjunctive meshwork. Astrocytic processes later cross through the neuropile and interact with meningeal cells to create a tight barrier with the mesenchyme known as the glial limitans (Maxwell et al., 1990). The growth inhibitory molecules in the scar abound on the various cell types present in scar. For example, oligodendrocyte precursors express the chondroitin sulfate proteoglycan (CSPG) NG2 (Levine et al., 2001), while meningeal cells produce semaphorine III, tenascins and different proteoglycans (Ajemian et al., 1994; Ness and David, 1997; Pasterkamp et al., 1999). Of these, the most prominent inhibitors of axon growth in the glial scar are the CSPGs NG2, phosphacan, veriscan and neurocan. Although little is known about their mechanism of action, it seems that the glycosaminoglycan (GAG) chains are important for the transduction of the inhibitory signal, as enzymatic removal of these chains improves regeneration and functional recovery (Bradbury et al., 2002). Thus, paradoxically although CSPGs ensure proper axon path finding during development,

they become key molecules responsible for regenerative failure following injury in the adult CNS.

1.6.2 Intrinsic Regenerative Ability of RGCs

CNS neurons lose their intrinsic ability to grow axons shortly after postnatal development. Studies comparing RGC growth at different developmental periods have revealed that cells in cultures from E20 RGCs (1 day prior to birth) extend their axons ~10 times as fast as those from postnatal P8 rats (Goldberg et al., 2002a). Moreover, when transplanted into the developing brain, embryonic RGCs regenerate their axons significantly further. Interestingly, the loss of intrinsic axon growth capability after birth is accompanied by an increased ability to elaborate dendritic arbors. These data demonstrate a developmental switch in growth programs. Further investigations have attributed this change to retinal amacrine cells which signal to RGCs and irreversibly alter their ability to grow axons (Goldberg et al., 2002a).

There are several developmentally regulated changes that occur while the neuron is maturing. For example, there are pronounced differences between immature and mature cytoskqueletal proteins. This can include differences in post-translational modifications such as acetylation, phosphorylation, tyrosination/detyrosination as well as the binding of several forms of stabilizing proteins such as microtubule associated proteins (MAPs), all of which either enhance of hinder the axons ability to extend (reviewed in Dent and Gertler; 2003).

One of the inherent differences that may contribute to the mature neuron's inability to regenerate when compared to its embryonic counterpart is the changes in

endogenous cAMP levels. Adult neurons have lower levels of cAMP and this decrease is thought to reduce their ability to regrow an axon after transection (Cai et al., 2001). Varying levels of this cyclic nucleotide can determine the growth cone's response to guidance cues and neurotrophic factors (Ming et al., 1997a; Song et al., 1997; Song and Poo, 2001). Of relevance to axonal regeneration, increasing cAMP levels was shown to override the inhibitory effects of MAG and myelin (Cai et al., 1999a) and convert MAG mediated repulsion to attraction (Song et al., 1998). Moreover, injection of permeable cAMP analogues into the dorsal root ganglion (DRG) increased regeneration in the CNS dorsal column (Neumann et al., 2002; Qiu et al., 2002). This approach also yielded increased growth when DRGs were subsequently cultured on either permissive or inhibitory substrate (Neumann et al., 2002).

Modification of cAMP levels alters the growth cone response to guidance cues (Song et al., 1997); therefore, although axons are stimulated to regenerate, they may grow astray by their newly skewed internal chemistry. One plausible approach to overcome this issue is to examine the downstream molecules induced by elevated cAMP levels that allow a neuron to grow an axon on myelin. This question was addressed by Cai et al. (2002) in a study where they identified the enzyme Arginase 1 as one of the key downstream components in the cAMP pathway. Arginase 1 is involved in the synthesis of polyamines, and overexpression of either Arginase 1 or the addition of polyamines stimulated axonal growth on myelin (Cai et al., 2002). This approach allows to reap the benefits of elevated cAMP levels without actually increasing cAMP levels and consequently modifying growth cone turning.

1.6.3 Survival versus Regeneration of Lesioned CNS Neurons

The previous section focused on why adult CNS neurons are less prone to regenerate; however, there is evidence that adult mammalian RGCs are capable of regrowing axons within the injured optic nerve. Early evidence came from a study where Berry and colleagues introduced pre-degenerated segments of sciatic nerve into the vitreous chamber (Berry et al., 1996). Twenty days later, between 0.6% and 10% of RGCs grew axons 3 to 4 mm into the optic nerve. The conclusion from this study was that trophic factors liberated from the implant stimulated axonal growth.

The obvious questions then arise: what signals are required to stimulate the regeneration of transected CNS neurons and do these differ from the signals necessary for their survival? It seems intuitive that a neuron needs to survive in order to regenerate; however, is survival in itself sufficient? For instance, in the example above, Schwann cells in the degenerated nerve implant may have provided neuroprotective signals to the axotomized RGCs (Maffei et al., 1990); yet, it is impossible to determine if these survival signals are themselves contributing to axonal growth. In order to fully isolate the survival variable from the growth variable, investigators have used transgenic animals mutated for apoptotic genes. For example, transgenic mice that overexpress Bcl-2 retain 65% of their RGCs at 6 months after axotomy (Bonfanti et al., 1996; Cenni et al., 1996). In these mice, 100% of RGCs survived 1 month after intracranial optic nerve crush as compared to 44% in wild type mice (Chierzi et al., 1999). Nevertheless, the level of axon regeneration in these mice remained identical to controls unless they were

treated with the IN-1 antibody. This held true even when RGCs were presented with the permissive environment of a peripheral nerve graft (Inoue et al., 2002).

In experiments where RGCs were transfected with Bcl-2, postnatal RGCs survived *in vitro* without any trophic stimulation, yet were unable to extend axons on their own (Goldberg et al., 2002a). Similarly, Bax -/- DRG sensory neurons can be cultured without neurotrophic factors, but neurotrophins greatly enhance their axonal outgrowth (Lentz et al., 1999). The separation of survival and regeneration may explain why a small population of RGCs remains viable for up to 20 months after axotomy (Villegas-Perez et al., 1993) without regrowing their axons. This phenomenon also occurs in other CNS populations such as Purkinje cells (Dusart and Sotelo, 1994) and cortico-spinal motor neurons (Schwab and Bartholdi, 1996). Taken together, these studies indicate that CNS neurons that survive after lesion require extrinsic signals to regenerate their axons. The question remains as to which stimulus or combination of stimuli is required.

1.7 NEUROTROPHIC SUPPLEMENTATION FOR LESIONED NEURONS

Treatment of transected RGCs with trophic factors in order to prevent apoptosis and stimulate regrowth has been an avenue of research for more than twenty years (reviewed in Yip and So, 2000 and Chaum, 2003). As described above, axon transection not only reduces the availability of target-derived trophic factors but also compromises the intrinsic ability of neurons to respond to these stimuli. An attractive neuroprotective approach consists in boosting the levels of available neurotrophic factors. This is exemplified in experiments where Schwann cells or peripheral nerve segments

introduced into the vitreous chamber of rats promoted both RGC survival and regeneration (Maffei et al., 1990; Berry et al., 1996). It is presumed that Schwann cells or non-neuronal components of the graft (Schwann cells and fibroblasts) released a cocktail of trophic factors. Schwann cells, for example, secrete neurotrophins (e.g. NGF, BDNF, NT-4/5) and other trophic factors, such as FGFs (e.g. FGF-2), ciliary neurotrophic factor (CNTF), glial cell line derived neurotrophic factor (GDNF) and transforming growth factor-Betas (TGF-βs) (Dezawa and Adachi-Usami, 2000). Of these factors, FGF-2 has been associated with both survival and growth of RGCs and therefore its role in retinal injury repair as well as its mechanisms of action will be covered in greater detail in the following section.

1.7.1 FGF-2 and Retinal Injury

FGF-2 is expressed in the outer nuclear and inner nuclear layers of the adult retina (Sapieha et al., 2003). Following injury to the retina, endogenous levels of FGF-2 are upregulated (Wen et al., 1995; Cao et al., 1997; Wen et al., 1998) and following optic nerve injury, FGF-2 levels increase in photoreceptor cells and glial cells of the optic track (Kostyk et al., 1994). These observations suggest that FGF-2 may be involved in injury repair. This claim has been substantiated by a number of studies that demonstrate a neuroprotective role for this factor. In the adult retina, application of FGF-2 protein to the distally transected optic nerve stump of adult rats enhances survival of RGCs three-fold, 30 days after axotomy (Sievers et al., 1987). FGF-2 has been effective in delaying inherited retinal dystrophy in rats (Faktorovich et al., 1990, 1992), slowing age related photoreceptor loss (Lin et al., 1997) and preventing photoreceptor loss after

light damage (LaVail et al., 1992). Furthermore, it has been shown to exhibit transient protective effects in adult rat models of pressure-induced retinal ischemia models (Unoki and LaVail, 1994; Zhang et al., 1994). *In vitro*, FGF-2 stimulates neurite extension of adult rat RGCs (Bahr et al., 1989) and axon branching of cortical neurons (Szebenyi et al., 2001). Finally, our studies, presented in chapters 2 and 3 of this thesis, have demonstrated a role for FGF-2 in RGC axon regeneration (Sapieha et al. 2003).

1.7.2 FGF Signaling

FGFs provoke a vast range of biological effects when bound to their high affinity extracellular tyrosine kinase receptors, the FGFRs. There are currently four members of this family, FGFR1-4. FGFRs are transmembrane glycoproteins with three extracellular Ig-loops (I, II and III), a hydrophobic transmembrane domain and an intracellular tyrosine kinase domain (Johnson et al., 1991). The Ig-loop III determines FGF binding affinity to a given receptor and alternative splicing of this loop gives rise to various isoform for FGFR1-3, while FGFR4 is generated as a single splice product. Of importance, all four FGFRs have been localized in the retina (Cornish et al., 2004).

I*n vitro* studies indicate that each receptor and its diverse isoforms show distinct affinities for given FGFs (Ornitz et al., 1996). The ensuing signaling cascades are however likely to be very similar as there is a great degree of structural homology between the various receptors. In fact, a study in which chimeric FGFRs were made from the cytoplasmic domains of FGFR1, FGFR2 and FGFR4 and the extracellular domain of the PDGF receptor demonstrated that only the strength of tyrosine kinase activity and not the recruitment target of proteins was affected (Raffioni et al., 1999).

This suggests that the signaling cascades are conserved throughout the vast FGF family and numerous FGFRs and that only the intensity of the signals vary.

FGF activation of FGFRs is stabilized and enhanced by interaction with heparan sulfate proteoglycans (HSPGs). The latter are expressed either on the cell surface or in the embryonic ECM. HSPGs enhance the signaling of FGFs by preventing their diffusion from the target cells, by storing FGFs in the ECM, and by protecting them against thermal and proteolytic denaturing (Moscatelli, 1987; Flaumenhaft et al., 1990). In addition, HSPGs are thought to confer a steric advantage that facilitates and stabilizes the interaction between FGFs and FGFRs (Ornitz et al., 1992; Aviezer et al., 1994).

Similar to the majority of RTKs, FGFRs transmit their extracellular signal to the cell via a network of phosphorylation dependent events. FGFRs are activated in a stepwise fashion. First, a single FGF molecule binds to a single FGFR molecule, thus forming a FGF/FGFR complex. Second, two independent FGF/FGFR complexes are assembled with the assistance of HSPGs (Venkataraman et al., 1999; Stauber et al., 2000), and lastly, intracellular phosphorylation cascades are stimulated. Upon activation, FGFR1 has 7 tyrosine residues in its cytoplasmic domain that can serve as substrates for phosphorylation and the consequent recruitment of signaling effectors. In the following sections, two of the most prominent pathways ensuing from FGFR stimulation are described.

1.7.2.1 The SNT-1/FRS2 Pathway

A novel bridging/ adaptor protein was identified that is recruited via a mechanism which does not involve the phosphorylation of proteins containing SH2.

This protein was simultaneously reported by two groups and named SNT-1 (Wang et al., 1996) and FRS2 (Kouhara et al., 1997). Once activated, FRS2 recruits the adaptor proteins Grb-2/Sos which recruit the small GTPase Ras to the FGFR complex and consequently activate the MEK/MAPK pathway (Kouhara et al., 1997). Moreover, tyrosine phosphorylation of FRS2 after FGF stimulation can result in the activation of the PI-3K/Akt pathway via the recruitment of Grb2 (Ong et al., 2001). Of note, FRS2 can be constitutively associated with FGFR1, regardless of its state of activation (Ong et al., 2000). Interestingly, Trk receptors also recruit FRS2. However, this occurs only when Trks are activated (Ong et al., 2000; Yan et al., 2002). It is therefore possible that FGFR1 may influence Trk signaling by sequestering FRS2 from activated Trks.

1.7.2.2 The PLCγ Pathway

Upon phosphorylation of Tyr766 in the intracellular domain of FGFR1, PLCγ is recruited and associates via its SH2 domain (Mohammadi et al., 1991). PLCγ is a cytoplasmic protein that when activated cleaves phosphatidyl-inositol-4, 5-biphosphate to yield inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 provokes the release of intracellular Ca^{2+} stores from the endoplasmic reticulum; DAG and Ca^{2+} activate protein kinase C (PKC). Thereafter, DAG lipase converts DAG to arachidonic acid (AA). The later activates Ca^{2+} channels causing Ca^{2+} influx that stimulates the Ca^{2+}/calmodulin kinase II (CaMKII) and ultimately provokes neurite outgrowth (Williams et al., 1995). The PLCγ pathway seems to be involved in FGFR stimulated axonal growth (Doherty and Walsh, 1996; Lom et al., 1998). In culture, the inhibition of PLCγ by small molecules considerably impairs RGC axonal growth (Saffell et al.,

1997), whereas inhibition of components of the PLCγ pathway in the developing *Xenopus* significantly reduces neurite outgrowth (Lom et al., 1998).

1.8. GENE MODULATION FOR THE INJURED CNS, PART I: ADDING A GENE

A shortcoming of trophic factor supplementation strategies is the modest half-life of these proteins. Overcoming this issue to achieve sustained levels of trophic factors in or around the injured neuronal population is essential to better assess the true potential of these regenerative and neuroprotective strategies. An attractive method to achieve a perpetual upregulation of trophic factors is to use gene transfer technology. This approach is also commonly termed gene therapy. The following sections give an overview of current developments in this domain.

1.8.1 Gene Transfer Technology

Gene therapy is the transfer of a gene into a cell with the goal of preventing or treating a disease. In contrast to most conventional medicines that alleviate symptoms or non-specifically eradicate the cause, gene therapy alters a cell's program in order to help it or its neighboring cells function soundly. For example, the newly introduced gene (transgene) can increase the levels of neurotrophic factors or their receptors which are depleted as a result of injury (Cheng et al., 2002a). Furthermore, gene therapy can be used to upregulate genes whose expression is confined to defined periods of development (Sapieha et al., 2003). This can help the cells in their response to injury by activating developmentally relevant programs such as those used for axonal growth.

To date, most clinical studies involving gene therapy have been for cancer, where the primary objective is to eliminate cancerous cells. Here, the strategies are geared to enhancing certain inherent cellular traits such as immunogenicity (Stopeck et al., 1997) or to provoke apoptosis (Boussif et al., 1996; Swisher et al., 1999). In other approaches, the transgene can yield a toxic end product such as a pro-drug or an enzyme (Ram et al., 1997) or a functional copy of a mutant or absent gene is delivered in cases such as cystic fibrosis or mucopolysaccharidosis type VII, (Ohashi et al., 1997; Wagner et al., 1998). Gene transfer can be performed *in vivo,* where the host is treated directly with the vector, or *ex vivo,* where cells are treated *in vitro* and later transplanted into the tissue of choice.

Gene transfer offers a multilateral approach for treating a condition or illness; different strategies to deal with different genetic malfunctions can be implemented. For example, neurotrophin or growth factor therapy offers neuroprotection for a wide scope of neurodegenerative diseases. Gene augmentation therapy compensates for defective gene products or null mutations. Ribozyme therapy introduces a catalytic RNA which degrades mutated gene products. Pro-apoptotic therapy induces the cell's entry down the apoptotic pathway; of interest for anti-tumor therapies. Conversely, anti-apoptotic therapies sway the cell fate in favour of survival (Hauswirth and Beaufrere, 2000). In order for gene therapy to be considered a suitable option, certain prerequisites must be met; most evidently, knowledge of the genetic causes of the disease. Nevertheless, it is important to note that although it was first conceived as a therapeutic approach aimed at revolutionizing modern medicine, gene transfer technology has thus far proven more effective as an investigational tool.

1.8.2 Properties of Gene Transfer Vectors

Gene therapy requires an efficient means of introducing a transgene of choice into a target cell. Over the past fifteen years, advances in molecular biology and associated fields have permitted the conception of proficient vehicles for gene delivery. In order for a vector to be considered suitable for therapeutic use, it must exhibit certain desired properties:

1) Firstly, it must efficiently transfer the gene of choice. The vector must infect a sufficient population of target cells and lead to expression of physiological levels of the transgene. This must be achieved with the titers available and a restricted volume of injection due to the sensitivity of the neural environment.

2) In order to reap the benefit(s) of gene transfer, it is important to selectively infect the target cells. This may entail infection of the afflicted cells or neighboring cells when the gene's end-product is secreted. The site of administration, the vector and the promoter used, all play crucial roles for the specificity of infection.

3) Stable and persistent expression of the gene at adequate levels is required to treat a condition. The level and duration of expression will dictate the necessity for and the intervals of re-administration and ultimately the clinical feasibility of the therapy.

4) The vectors must provoke minimal toxic and immunogenic responses. Cytotoxicity can manifest itself as pharmacological side-effects (Bennett et al., 1994; Jomary, 1994; Li et al., 1994) or as an immune response against viral DNA or viral proteins, and may result in the loss of the host cells (Yang, 1994).

5) The vector's pathogenicity must be eliminated. At present, most of the viral vectors employed in gene transfers are attenuated forms of pathogenic human viruses.

6) Another issue, especially for vectors with clinical potential, is the ease of manufacturing. If the technology is to be taken from the bench to the clinic, copious amounts of a vector will need to be produced.

Three main classes of vectors have been explored; the physical, the non-viral and the viral. Physical transfer methods consist of electroporation or micro-injection techniques (needle free-injectors or Gene Gun). The rates of DNA introduction are limited; however, this approach is promising for certain specific applications such as *in vitro* or *ex-vivo* strategies. Non-viral protocols include introduction of naked DNA or DNA complexed with cationic molecules such as liposomes (Wolff, 1998; Weir, 1999). They are considered to be less efficient transducers with mostly transient expression (Hangai et al., 1996). Their advantage lies in the possibility of introducing large DNA inserts. In terms of rates of transfection, viral vectors such as adenovirus, adeno-associated virus and retroviruses have been the most efficient.

1.8.3 Viral Vectors for Retinal Gene Transfer

The major advantage of gene therapy for the retina is that, with a single intraocular injection, sustained expression of a given gene can be achieved. This reduces the need to repeatedly breach the blood-retinal barrier (BRB), thus reducing the damage inflicted by every injection. The vectors that are the most widely used in retinal gene therapy are described below. For a detailed account on how to produce viral vectors, please refer to Appendix B.

1.8.3.1 Adenovirus (Ad)

Ad vectors are an efficient tool to study the effect of *in vitro* and *in vivo* gene expression on neuroprotection. The major advantages of this system are that Ad can efficiently infect post-mitotic cells and that it can be easily grown to high titers. Ad contains a linear double-stranded DNA genome of approximately 36 kilobases (kb) encapsidated in an icosahedral protein shell. Immediate early genes (E1, E2, E3 and E4) orchestrate viral gene transcription and suppression of the host immune response, while late genes are necessary for viral assembly (Shenk, 1995). Most recombinant Ad belong to the group C Ad type 2 or 5. Ad vectors were initially generated with deletions of the early region 1 (ΔE1) that contains genes required for virus replication (Bett et al., 1994); this renders vectors replication defective and more suitable for gene transfer into mammalian cells. A major disadvantage of these early Ad vectors is the strong cytotoxic and immune response elicited upon infection of the host cells (Kovesdi et al., 1997). Recent versions of Ad vectors have been produced in which the entire viral genome, except for the terminal repeat regions required for viral assembly, has been replaced by exogenous gene sequences. These so-called "gutless" vectors exhibit considerably reduced immune response (Morsy et al., 1998; Schiedner et al., 1998b), but can only be produced in the presence of a helper virus that provides all the proteins required for viral replication (Parks et al., 1996).

1.8.3.1.1 Ad Tropism in the Retina

Although Ad vectors show efficient transduction in a wide variety of cell types *in vitro*, the cellular tropism for viral infection *in vivo* appears to be more complex. Our

studies using Ad vectors injected into the vitreous chamber of adult rat eyes demonstrated that Müller cells, the predominant glial cell in the retina, is the main target for Ad infection *in vivo* (Di Polo et al., 1998). This approach has proven to be useful for the delivery of genes encoding diffusible neurotrophins in order to promote neuroprotection of axotomized retinal ganglion cells. Ad vectors have also been shown to efficiently transduce the retinal pigment epithelium (RPE) following subretinal injections (Bennett et al., 1994; Li et al., 1994). Together, these studies indicate that non-neuronal cells in the adult retina are the preferred cellular targets for recombinant Ad. Nevertheless, some experimental conditions support limited Ad transduction of retinal neurons. For example, intraocular administration of Ad in animals at early developmental stages may result in modest infection of photoreceptors (Bennet et al., 1996). In addition, introduction of Ad to the brain (e.g. superior colliculi) or to the transected optic nerve stump results in retrograde transport of viral particles that mediate gene expression in retinal ganglion cells (Cayouette and Gravel, 1996; Kugler et al., 2000). Because all these studies involved transgenes directed by the ubiquitous cytomegalovirus (CMV) promoter, it is likely that the presence of cellular receptors for Ad in non-neuronal retinal cell types mediates the observed viral tropism *in vivo*. The coxsackievirus and adenovirus receptor (CAR) protein involved in Ad attachment and infection has been identified (Bergelson et al., 1997). In addition, integrins αvß3 and αbß5 participate in Ad internalization (Wickman et al., 1993). The specific cellular localization of these receptors in the retina remains to be defined.

1.8.3.2 Adeno-associated Virus (AAV)

AAV is a member of the *parvoviridae* family initially identified as a contaminant of Ad stocks. AAV requires a helper virus (e.g. Ad or herpes simplex virus) for replication. The wild-type virus houses a single stranded genome of 4680 base pairs (bp) containing two genes, *rep* and *cap*, that encode proteins involved in replication and encapsidation, respectively. The AAV genome is flanked by two identical 145-bp inverted terminal repeats (ITRs) which are essential for packaging, replication or integration. Recombinant AAV vectors derived from human parvovirus AAV-2 have been produced by substituting all viral sequences, except for the ITRs, for a transgene of interest (McLaughlin et al., 1988; Samulski et al., 1989). However, packaging of functional AAV particles requires the presence of the *rep* and *cap* proteins typically provided in *trans*. The recombinant AAV system has several advantages for *in vivo* gene transfer research: i) it is not pathogenic and has not been implicated in the etiology of any known human disease (Berns and Bohensky, 1987); ii) it mediates long-term transgene expression that persists for several months *in vivo* (Dudus et al., 1999; Guy et al., 1999b); iii) the absence of viral sequences results in minimal immune response or cytotoxicity in the target tissues (Xiao et al., 1996, 1998); and iv) it can efficiently infect post-mitotic cells *in vivo* (Podsakoff et al., 1994).

In the absence of helper virus, wild-type AAV can integrate at a specific site on the *q* arm of chromosome 19 to establish latent infection (Kotin et al., 1990). However, the lack of *rep* proteins has been shown to compromise integration specificity leading to random insertion of recombinant AAV (Flotte et al., 1994; Ponnazhagan et al., 1997). Although viral integration into the genome may contribute to the stability of AAV-

mediated transgene expression, a careful evaluation of the risks associated with insertional mutagenesis is required before implementing AAV-based therapies. A disadvantage of AAV vectors has been the size constraint for packaging genes larger than 4.7 kb. Although methods have been developed to increase the size of delivered transgenes by trans-splicing of two independent vectors co-administered to the same tissue (Yan et al., 2000), this remains a limitation of the AAV system. The laborious work needed to produce AAV vector stocks has often been regarded as a disadvantage of this vector system; however, the recent availability of reagents and improvements in the protocols, described below, have greatly facilitated the preparation of high-titer and pure AAV stocks.

1.8.3.2.1 AAV Tropism in the Retina

AAV-mediated gene expression can be restricted to photoreceptor cells when under the control of a well characterized murine rhodopsin promoter sequence (Flannery et al., 1997). More recently, retinal ganglion cells have been identified as the primary targets for AAV infection in the inner retina following intravitreal injection of viral vectors (Cheng et al., 2002a) Thus, subretinal injection of AAV vectors results mainly in gene transfer to photoreceptors and RPE cells, while intravitreal injection allows infection of cells in the ganglion cell layer. Unlike Ad, AAV appears to have a preferential tropism for retinal neurons rather than glial cells. This is consistent with studies in the brain showing AAV transduction of subsets of neurons rather than astrocytes, oligodendrocytes or microglia (Kaplitt et al., 1994; Bartlett et al., 1998).

Interestingly, genetic modification of capsid proteins has been shown to allow AAV targeting of cells normally resistant to infection (Girod et al., 1999).

1.8.3.3 Lentivirus (LV)

LV, a genus of retroviruses, consists of two identical single-stranded RNA molecules and enzymes required for replication within a viral protein core. Following virus internalization, the viral RNA is reverse transcribed into double-stranded DNA and transported to the cell nucleus (Panganiban, 1990). Viral DNA is then permanently integrated into the host genome to become a provirus. The retrovirus genome contains *gag*, *pol* and *env* genes flanked by long-terminal repeats (LTRs). These genes encode proteins essential for replication, encapsidation, internalization and reverse transcription. Replication-deficient recombinant retroviral vectors have been generated by substituting all viral genes for a transgene of interest with the exception of the *cis*-acting sequences required for vector propagation, such as the reverse transcription initiation site and the packaging site (Coffin, 1996). Functional recombinant retrovirus particles can be generated in culture when the *gag*, *pol* and *env* gene products are provided in *trans*.

Most retroviral vectors can only transfer genes into cells that are actively proliferating (Roe et al., 1993). Thus, their use in neuroprotective strategies which typically involve gene transfer into fully differentiated cells is rather limited. An exception to this rule are LVs, such as the human immunodeficiency virus (HIV), which can efficiently infect non-mitotic cells (Lewis and Emerman, 1994). This ability relies on nuclear localization signals in the preintegration complex that allow entry into the nucleus without the need for nuclear membrane fragmentation (Roe et al., 1993). Other

advantages of the LV system are its relatively large cloning capacity, close to 10 kb, its ability to mediate high levels of transgene expression *in vivo* and the lack of immune response in the target tissues (Naldini et al., 1996; Kafri et al., 1997). Recently, recombinant LV was shown to efficiently infect hematopoietic stem cells, extending its potential use as a therapeutic vector (Miyoshi et al., 1999).

The main concern with LV vector systems is the risk of generating replication competent recombinant (RCR) virus during the production of viral stocks. Because HIV is a human pathogen, considerable work has been done to increase biosafety of LV production systems. Other concerns include low vector titers and the risks associated with insertional mutagenesis as the vector integrates into the host genome. The issue of transgene silencing still requires further investigation. The lack of immune response associated with LV recombinant infection and its ability to stably integrate into host DNA are promising features for persistent gene expression. A systematic study of the time-course of expression mediated by LV vectors in the retina should resolve this issue.

1.8.3.3.1 Cellular Tropism of LV Vectors

LV-mediated gene transfer and expression in the retina *in vivo* was first characterized by subretinal injection of a vector carrying the green fluorescent protein (GFP) gene (Miyoshi et al., 1997). Using the ubiquitous CMV promoter, the main cellular targets of LV vectors were shown to be photoreceptors and RPE cells with some bipolar and Müller cells. When a rhodopsin-specific promoter was used, transgene expression was restricted to the photoreceptor layer (Miyoshi et al., 1999; Takahashi et

al., 1999). The infection pattern of LV vectors following intravitreal injection remains to be characterized.

An attractive feature of HIV-based vectors is their ability to efficiently infect cells *in vitro*. For example, RPE primary cultures have been transduced with LV to express GFP. Transgene expression persisted in the infected RPE cells following their transplantation into the subretinal space of a host (Lai et al., 1999). HIV-based vectors have been shown to efficiently transduce human $CD34^+$ hematopoietic stem cells that were capable of long-term engraftment in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (Miyoshi et al., 1999). Recently, neural stem cells have been isolated from the RPE in the ciliary margin of the adult eye (Tropepe et al., 2000). These cells can express retinal specific markers when they are differentiated *in vitro*. Thus, *ex vivo* transduction of stem cells using HIV-based vectors followed by retinal transplantation may be useful in the design of neuroprotective strategies.

1.9 GENE MODULATION FOR THE INJURED CNS, PART II: REMOVING A GENE

In contrast to the gene supplementation approach described above, where a given gene is added to obtain a beneficial effect, certain gene products are detrimental to the recovery of the lesioned CNS. For example, MAG promotes growth of immature neurons (rat E18 RGCs and DRGs younger then P4) but inhibits the growth of postnatal RGCs and older DRGs (DeBellard et al., 1996; Cai et al., 2001). In this regard, there are strategies designed to counter these proteins in order to obtain a desired effect (e.g.

neuronal survival or regeneration). In the following sections, I describe approaches that are currently available to silence gene expression *in vivo*.

1.9.1 RNA Interference

RNA interference (RNAi), or RNA silencing, is a naturally occurring gene silencing process first identified as a mechanism to fight viral infections. It was initially discovered in plants but is now known to occur in a wide scale of eukaryotes (Tijsterman et al., 2002). RNAi is the cell's endogenous response to double stranded (ds) RNAs. Once the dsRNA enters the cell, it is converted by the enzyme Dicer into short interfering (si) RNAs of ~22 nucleotides in length. The siRNAs are subsequently incorporated into a complex named RNA-induced silencing complex (RISC) which then catalyzes the cleavage and degradation of mRNAs with sequence homology to the initial dsRNA (Fire et al., 1998). As an experimental tool and potential clinical strategy, siRNAs are introduced into the cell by using mainstream transfection methods. In fact, delivery of siRNAs by viral vectors such as AAVs is currently being developed and is likely to yield a powerful tool for gene silencing in adult cells (Tomar et al., 2003).

With the completion of the sequencing of the human, rat and mouse genomes, a wealth of gene targets is currently available. Entire siRNA libraries can be designed and tested to identify the most effective inhibitors for a given gene. For example, a library of 148 siRNAs was recently constructed and screened for genes that may influence Akt phosphorylation (Hsieh et al., 2004).

Although a promising investigative and therapeutic tool, RNAi, as all novel approaches, has its current share of limitations. For example, it is complicated to design

highly specific RNAi triggers that have minimal homology with all other sequences of the genome. Consequently, non-specific gene silencing must be carefully screened for. Also, introduction of foreign dsRNAs may trigger the host's immune response. For example, production of siRNAs using bacteriophage polymerases can readily stimulate the cell's antiviral interferon pathways (Kim et al., 2004). Moreover, as with all therapeutic approaches, effective strategies must be devised to ensure efficient introduction of siRNAs into target cells.

Nevertheless, with a certain amount of fine tuning, RNAi based therapies are likely to become a potent tool for the treatment of a vast scale of diseases from HIV to amyotrophic lateral sclerosis (ALS) (Jana et al., 2004). Already, the virtues of RNAi for the treatment of retinal pathologies are starting to be seen, as it was successfully used to reduce retinal neovascularization by reducing the expression of the VEGF gene (Reich et al., 2003).

1.9.2 Antisense Oligonucleotides

Antisense oligonucleotides are single stranded DNA molecules that encode the complementary sequence of a specific target mRNA. Once antisense oligos bind their target mRNA, they inhibit its transcription and provoke its enzymatic degradation by endogenous nucleases, ultimately blocking protein synthesis. In the retina, Bax-antisense has been used to delay RGC degeneration after axotomy (Isenmann et al., 1999a). A disadvantage of this approach is that it yields, at best, rather limited transfection efficacy. Other disadvantages include severe limitations in terms of relative instability *in situ,* ability to cross the blood brain barrier or the blood retinal barrier and restricted

ability to enter into cells (Godfray and Estibeiro, 2003). For these reasons, this strategy has seen only limited use in the retina.

1.9.3 Ribozymes

Ribozymes are catalytic RNAs which can be designed to cleave other RNAs in a sequence-specific manner. They can be packaged into viral vectors and delivered to a target cell population. They have been successfully used to reduce levels of pathological mRNA species that encode deleterious proteins, such as mutant rhodopsin in diseases such as retinitis pigmentosa (Hauswirth and Lewin, 2000; Shaw et al., 2001).

1.9.4 Small Molecule Inhibitors

Small molecule inhibitors are the mainstay of the pharmacological industry. These molecules act by binding to a given protein (gene product) and thus rendering it inactive. As with any exogenous molecule, their stability, cellular penetration and general pharmacokinetics can be optimized in the lab and, as a result, they can become highly effective inhibitors. The major limitation of this approach is the complexity of designing and synthesizing these compounds. Because the proteins targeted by small molecules may have radically different structures from one another, the synthesis of each compound may require drastically different approaches. Consequently, there are massive costs associated with the development of small molecules. Furthermore, the specificity of these compounds may be limited by structural homology between the target protein and other proteins, thereby causing undesirable side effects.

1.9.5 Transgenic Knockout Animals

Although of limited therapeutic value, transgenic knockout (KO) animals offer the most penetrant form of gene silencing. They have become central to investigating mammalian gene function. Because the gene is eliminated in all cells, it mimics a scenario where a given compound or gene silencing strategy attains perfect elimination of its target gene.

Studies using KO mouse models have exposed numerous roles for genes that were already thoroughly studied (Lee and Threadgill, 2004). For a non-essential gene, viable homozygous KO animals can be generated. These animals provide useful models to assess the *in vivo* function of a gene or protein during development or post-natal life.

However, in conventional gene KO models, global gene deletions removes the gene from all cell in the organism. This may lead to developmental defects that may later interfere with functional studies. Therefore, it is often difficult to exclude that the observed phenotype in the adult is not a direct result of a developmental defect. Also, because all cells are affected, it may be complicated to attribute a given phenotype to a particular cell population in multi-cellular structures. Moreover, if the gene is vital for embryonic development, the KO will be lethal and the knowledge gained on the gene's function will be limited to restricted periods of embryonic development. To counter this, novel technologies have been developed that permit for the spatial and temporal KO of candidate genes.

For example, one method to ensure tissue and cell specific KO calls for the use of the *Cre/loxP* system. The *Cre recombinase* is an integrase of the E. coli bacteriophage P1. While in its lytic state, the P1 bacteriophage has linear double stranded DNA with

two 34-bp *lox P* sites at the end of each strand. The principal function of the *Cre recombinase* in the virus is to catalyze the site-specific recombination between the two *loxP* sites in order to circularize the DNA (Sauer and Henderson, 1988). To generate transgenic mice, the *loxP* sites can be inserted by homologous recombination into embryonic stem cells such that they flank the desired gene (*floxing* the gene). Subsequently, a homozygous mouse for the floxed gene is crossed with a mouse that has the *Cre* transgene under the control of a cell or tissue specific promoter. Consequently, in the homozygous progeny, the floxed gene will be eliminated by *Cre/loxP* recombination, specifically in the cells/tissue where the *Cre* gene associated promoter is active(Branda and Dymecki, 2004).

The temporal restriction of gene expression is a valuable tool as it permits to study the role of a given gene at a determined time point, while excluding the possibility that any abnormalities may be the result of developmental defects. These time-specific inducible KOs can be achieved by introducing a gene that can be turned on or off with a drug. First, the desired gene (e.g. a mutant construct) is introduced under the control of a tet-O inducible promoter. Tet-O, originally found in bacteria, requires the transcriptional regulator, *tetracycline transactivator* (tTA) to turn on transcription of its gene. Therefore, another mouse line is generated with tTA placed under the control of a tissue/cell specific promoter. In the homozygous progeny of these two lines, tTA binds to the tet-O promoter and expression of the transgene (e.g. a mutant construct) occurs. When the mouse is given the antibiotic doxycycline, the drug binds to tTA causing it to fall of the promoter and transcription is stopped. Thus administering the drug turns the gene off. Conversely, mice that express reverse tTA, (rtTA; a mutant form of tTA), can

be generated. In this scenario, the mouse requires doxycycline for the rtTA transactivator to activate transcription. Therefore, the transgene is only expressed when the animal is given the drug.

Transgenic knockout animals are invaluable tools in elucidating the roles of genes in mammals. With the application of new molecular technologies to the design of transgenic animal models, new functions will be attributed to proteins and consequently new therapeutic targets will arise.

1.10 OBJECTIVES OF THE THESIS

In the past 100 years, since the days of Cajal, our vision of the CNS and its propensity to survive and regenerate following injury has greatly evolved. With the advent of modern biochemistry and molecular biology, we are now better able to understand the causes and cellular mechanisms responsible for regenerative failure and neuronal death within the lesioned CNS. This knowledge can be used to design novel approaches geared at reversing the devastating consequences of CNS disease and injury.

In this thesis, we explore the hypothesis that genes involved in the orderly development of the nervous system can be modulated in the adult to achieve successful survival and regeneration of mature lesioned CNS neurons. More specifically, we seek to determine if either FGF-2 or RPTP-σ, which are both abundantly expressed during retinal development, participate in the regenerative process or the neuroprotection of axotomized adult RGCs. Additionally, we investigate the signaling mechanisms involved in these events.

Chapter II

FIBROBLAST GROWTH FACTOR-2 GENE DELIVERY STIMULATES AXON REGENERATION BY ADULT RETINAL GANGLION CELLS AFTER ACUTE OPTIC NERVE INJURY

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FIBROBLAST GROWTH FACTOR-2 GENE DELIVERY STIMULATES AXON REGENERATION BY ADULT RETINAL GANGLION CELLS AFTER ACUTE OPTIC NERVE INJURY

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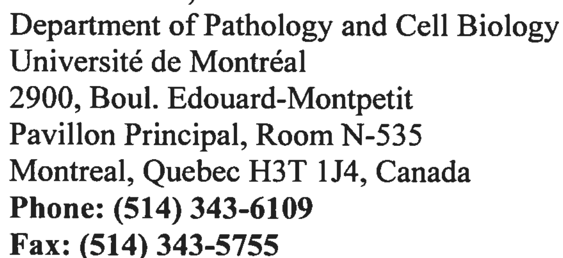

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2.1 ABSTRACT

Basic fibroblast growth factor (or FGF-2) has been shown to be a potent stimulator of retinal ganglion cell (RGC) axonal growth during development. Here we investigated if FGF-2 upregulation in adult RGCs promoted axon regeneration in vivo after acute optic nerve injury. Recombinant adeno-associated virus (AAV) was used to deliver the FGF-2 gene to adult RGCs providing a sustained source of this neurotrophic factor. FGF-2 gene transfer led to an increase in the number and length of axons that regenerated into the distal optic nerve. Detection of AAV-mediated FGF-2 protein in injured RGC axons correlated with growth distal to the lesion. The regenerative response to FGF-2 upregulation was supported by our finding that FGF receptor-1 (FGFR-1) and heparan sulfate (HS), known to be essential for FGF-2 signaling, were expressed by adult rat RGCs. FGF-2 transgene expression led to only transient protection of injured RGCs, thus the effect of this neurotrophic factor on axon extension could not be solely attributed to an increase in neuronal survival. Our data indicate that selective upregulation of FGF-2 in adult RGCs stimulates axon regrowth within the optic nerve, an environment that is highly inhibitory for regeneration. These results support the hypothesis that key factors involved in axon outgrowth during neural development may promote regeneration of adult injured neurons.

2.2 INTRODUCTION

Adult retinal ganglion cells (RGCs), like other central nervous system (CNS) neurons, fail to regenerate their axons after traumatic injury or disease. An appealing hypothesis is that the molecular cues that direct axonal extension during development may be used to stimulate regeneration in the adult injured CNS. Diffusible neurotrophic factors have a profound influence on neurite and axon growth during the maturation of the nervous system (Barde, 1989; Davies, 2000; Huang and Reichardt, 2001; Sofroniew et al., 2001). The fibroblast growth factor (FGF) family of neurotrophic factors play multiple roles in the development of the brain (Eckenstein, 1994; Abe and Saito, 2001) and the retina (Barnstable, 1991; Hicks, 1998). The biological activity of FGFs is primarily mediated through receptor tyrosine kinases (FGFRs), of which four distinct families have been identified (FGFR-1-4) (Klint and Claesson-Welsh, 1999a; Powers et al., 2000). Members of the FGF and FGFR families are expressed in the developing and adult retina of many species, including human (Gao and Hollyfield, 1992; Bugra et al., 1993; Song and Slack, 1994; Kinkl et al., 2002).

FGF-2, a well-characterized member of the large FGF family, has been identified as a potent stimulator of axon growth for developing RGCs (Dingwell et al., 2000). FGF stimulates neurite outgrowth of embryonic and early post-natal RGCs in culture (Lipton et al., 1988; Bahr et al., 1989; McFarlane et al., 1995). Importantly, *in vivo* expression of a dominant negative FGFR in *Xenopus* RGC axons significantly impaired their ability to grow along the developing optic tract (McFarlane et al., 1996). In the developing mammalian retina, FGFR function has been shown to be required for the orderly projection of RGC axons (Brittis et al., 1996). Astrocytes in the ganglion cell layer as

well as cells in the inner and outer nuclear layers express endogenous FGF-2 (Gao and Hollyfield, 1992; Kostyk et al., 1994; Ohsato et al., 1997). Interestingly, FGF-2 is upregulated after injury in cells of the inner nuclear layer and ganglion cell layer of the adult retina (Wen et al., 1995; Cao et al., 1997; Wen et al., 1998), as well as in the optic nerve (Eckenstein et al., 1991b) and in the optic tract (Kostyk et al., 1994) suggesting that this neurotrophic factor may be involved in neural repair.

FGFs have a high affinity for cell surface heparan sulfate proteoglycans (HSPGs) and require heparan sulfate (HS) to activate FGFRs *in vitro* and *in vivo* (Rapraeger et al., 1991; Yayon et al., 1991; Ornitz et al., 1992a; Lin et al., 1999). For example, in cells that lack endogenous heparan sulfate, FGF fails to activate its high affinity receptor, while FGF responsiveness is restored by addition of heparin or heparan sulfate (Yayon et al., 1991). Previous studies showed expression of FGFR-1 and HSPGs in the rodent inner retina including the ganglion cell layer (Blanquet and Jonet, 1996; Ozaki et al., 2000; Inatani and Tanihara, 2002; Kinkl et al., 2002), but given that RGCs, displaced amacrine cells and astrocytes populate this retinal layer, the specific cellular localization of these proteins was not established. Here we demonstrate that FGFR-1 and HS are expressed by adult rat RGCs, a finding that supports the ability of these neurons to respond to FGF-2.

A role for FGF-2 in the regeneration of adult injured RGCs in vivo has not been established. A previous study using intraocular administration of FGF-2 protein failed to show RGC regeneration into a peripheral nerve graft attached to the optic nerve after intracranial axotomy (Cui et al., 1999). Lack of RGC axon regrowth in this study may have been due to limited persistence of FGF-2 protein in the eye since FGFs have a short

half-life in vivo (Culajay et al., 2000; Unger et al., 2000). Here we used recombinant adeno-associated virus (AAV) serotype 2 to evaluate the effect of sustained FGF-2 transgene expression on axon growth. We recently demonstrated that AAV effectively infects adult RGCs upon injection of viral vectors into the vitreous chamber of the eye (Cheng et al., 2002). AAV offers several advantages for in vivo gene delivery such as minimal immune response in the host (Xiao et al., 1996; Xiao et al., 1997) and long-term transgene expression that persists for over 1 year after vector administration in the adult retina (Dudus et al., 1999; Guy et al., 1999a). The injury model used in our study was micro-crush lesion of the optic nerve (Selles-Navarro et al., 2001), which has clear advantages over other procedures such as conventional optic nerve crush or peripheral nerve grafts. Micro-crush lesion results in a well-defined lesion with a focal glial scar and absence of optic nerve cavitation, facilitating accurate identification of the injury site and quantification of axon growth. Because all RGC axons are completely transected after micro-crush lesion, there is no risk of confusing spared fibers, or axons undergoing late secondary degeneration as in partially transected nerves, for regenerating axons. Moreover, the proximal and distal portions of the optic nerve remain attached allowing us to test the effect of FGF-2 upregulation on axon regeneration within the growth inhibitory optic nerve environment.

2.3 EXPERIMENTAL METHODS

2.3.1 Preparation of Recombinant AAV Serotype 2 Vectors

A cDNA sequence encoding human FGF-2 was inserted downstream of the cytomegalovirus immediate early promoter/enhancer in the plasmid D10 (Wang et al.,

1997). This plasmid also contained a bovine growth hormone polyadenylation site and AAV type 2 terminal repeat sequences. An AAV control vector carrying the green fluorescent protein (GFP) gene, but lacking the FGF-2 gene, was generated in identical fashion. Packaging of AAV particles was performed by triple transfection of low passage human embryonic kidney 293 cells. Cells were grown in a 10-layer cell factory (Nalge Nunc, Naperville, IL) and cotransfected with the FGF-2 D10 vector, and helper plasmids pKSrep/cap and pBHG10. The virus was extracted by freezing and thawing the cells and the resulting suspension was then clarified by low speed centrifugation. Packaged AAV was purified by heparin affinity chromatography and concentrated by precipitation with polyethylene glycol. Viral titers were determined by DNA dot blot analysis (Srivastava et al., 1990) and by infectious center assay (Clark et al., 1995a) and were as follows: AAV.FGF-2 = 4×10^{12} infectious particles (i.p.)/ml; and AAV.GFP = 5×10^{12} i.p./ml. The AAV stocks contained less than 1 wild-type genome per 10^{10} recombinant AAV genomes. Bacterial endotoxin levels were assayed with *Limulus* amebocyte lysate (Associates of Cape Cod, Inc., Woods Hole, MA) and were less than 70 U/ml.

2.3.2 Surgical Procedures

i) Intravitreal injections

All surgical procedures were performed in adult female Sprague-Dawley rats (180-200 g) under general anesthesia (2% Isoflurane/oxygen mixture, 0.8 liters/min) and in accordance with the guidelines for the use of experimental animals (Olfert et al., 1993). Five µl of viral vectors, AAV.FGF-2 or AAV.GFP, or phosphate buffered saline

(PBS) were injected into the vitreous chamber of the left eye using a 10-μl Hamilton syringe with a 32-gauge needle. The contralateral, unoperated eyes or eyes from normal unoperated animals served as controls. The sclera was exposed and the tip of the needle was inserted at a 45° angle through the sclera and retina into the vitreous space. Following slow withdrawal of the needle, the injection site was sealed with surgical glue (Indermill, Tyco Health Care, Mansfield, MA). This route of administration avoided injury to structures of the eye, such as the iris or lens, shown to promote RGC survival and regeneration (Mansour-Robaey et al., 1994; Leon et al., 2000).

ii) Axonal growth

The effect of AAV.FGF-2 on RGC axon growth *in vivo* was tested using the experimental protocol shown in Figure 1A. We, and others, have observed that AAV-mediated transgene expression reaches a plateau between 3 and 4 weeks after administration of the vector into the rodent eye (Bennett et al., 1997; Ali et al., 1998b; Bennet et al., 2000; Cheng et al., 2002b) and persists thereafter (Guy et al., 1999a). This delay in the onset of gene product expression *in vivo* may arise from the need to convert single-stranded viral DNA to a double-stranded form prior to active transcription (Ferrari et al., 1996). Because of the slow onset in transgene expression directed by AAV, microcrush lesion of the optic nerve was performed 4 weeks after virus injection. The optic nerve was exposed and a 10-0 suture was used to tie a knot to completely constrict the nerve for 60 sec, after which the suture was carefully released. Thirteen days after microcrush lesion, 5 μl of 1% cholera toxin β subunit (CTβ, List Biological Laboratories, Campbell, CA) was injected into the vitreous chamber. Twenty-four hrs

later (2 weeks post-microcrush lesion), animals were sacrificed and the eyes were processed for optic nerve immunostaining as described below. Groups included rats injected with AAV.FGF-2 (n=4), controls injected with AAV.GFP (n=3) or saline (n=4), and non-injected animals with optic nerve lesion only (n=4).

iii) Neuronal survival

RGC survival experiments were performed as follows (Fig. 1B): 4 weeks after intraocular injection of viral vectors, the left optic nerve was transected at 1 mm from the optic nerve head avoiding injury to the ophthalmic artery. One week prior to axotomy, RGCs were labeled with 2% FluoroGold (Fluorochrome, Englewood, CO) in 0.9% NaCl containing 10% dimethyl sulfoxide (DMSO) by application of the tracer to both superior colliculi. Retinas were examined histologically at 7 and 14 days after optic nerve transection to determine the density of surviving RGCs. Following micro-crush lesion or axotomy, the vasculature of the retina was always monitored by fundus examination and animals showing signs of compromised blood supply were eliminated from this study. Groups included: i) animals sacrificed at 1 week after optic nerve lesion that received AAV.FGF-2 (n=7), AAV.GFP (n=3) or no injection (axotomy only, n=4, or micro-crush lesion only, n=4); and ii) animals sacrificed at 2 weeks after injury that received AAV.FGF-2 (n=2), AAV.GFP (n=4) or no injection (axotomy only, n=4, or micro-crush lesion only, n=4)

iv) Cell proliferation

The effect of FGF-2 gene delivery and optic nerve injury on retinal cell proliferation was assessed following the protocol illustrated in Figure 1C. Briefly, 3 weeks after intraocular injection of viral vectors, RGCs were labeled and subsequently axotomized. Rats received a daily intraperitoneal injection of BrdU (2.5 mg/100 g, Sigma, Oakville, ON) for 1 week following transection of the optic nerve. Eyes were then processed for immunocytochemistry as described below. Groups included animals injected with AAV.FGF-2 (n=3) and controls injected with AAV.GFP (n=3).

2.3.3 Retinal immunocytochemistry

For co-localization studies, RGCs were retrogradely labeled with FluoroGold as described above. One week later, animals (n=3-4/group) were perfused intracardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4) and the eyes were immediately enucleated. The anterior part of the eye and the lens were removed and the remaining eye cup was immersed in the same fixative for 2 hr at 4°C. Eye cups were equilibrated in graded sucrose solutions (10-30% in PB) for several hours at 4°C, embedded in O.C.T. compound (Tissue-Tek, Miles Laboratories, Elkhart, IN) and frozen in a 2-methylbutane/liquid nitrogen bath. Radial cryosections (16 µm) were collected onto gelatin-coated slides and processed as follows. Non-specific binding was blocked by incubating sections for 1 hr in 3% bovine serum albumin (BSA, Sigma), 5% normal goat serum (NGS) and 0.2% Triton X-100 (Sigma). Each primary antibody was added in 2% NGS, 0.2% Triton X-100 and incubated overnight at 4°C. Sections were then incubated with the appropriate secondary antibody, washed in PBS and mounted using

an anti-fade reagent (SlowFade, Molecular Probes, Eugene, OR). Alternatively, sections were processed with a biotinylated secondary antibody followed by avidin-biotin-peroxidase reagent (ABC Elite Vector Labs, Burlingame, CA) and incubation in a solution containing 0.05% diaminobenzidine tetrahydrochloride (DAB) and 0.06% hydrogen peroxide in PB (pH 7.4) for 5 min. Antibodies were: monoclonal anti-human FGF-2 (Type I, clone bFM-1, 2 μg/ml, Upstate Biotechnology, Lake Placid, NY), monoclonal anti-human FGF-2 (Type II, clone bFM-2, 10 μg/ml, Upstate Biotechnology), polyclonal anti-FGFR-1 that recognizes an epitope in the carboxy terminus of FGFR-1 (Flg C-15, 0.4 μg/ml, Santa Cruz Biotechnologies, Santa Cruz, CA), polyclonal FGFR-1 against an epitope corresponding to amino acids 22-97 within the extracellular domain of FGFR-1 (Flg H-76, 2 μg/ml, Santa Cruz Biotechnologies), monoclonal anti-heparan sulfate (HepSS-1, 10 μg/ml, Seikagaku Corporation, Tokyo, Japan), monoclonal anti-BrdU (6 μg/ml, Roche Diagnostics Canada, Laval, QC), Alexa 594-conjugated goat anti-mouse IgG (14 μg/ml, Molecular Probes), Cy3-conjugated goat anti-rabbit (3 μg/ml, Jackson Immunoresearch Laboratories, West Grove, PA) and anti-mouse Fab fragment (Jackson Immunoresearch Labs). Fluorescence was visualized with a Zeiss Axioskop 2 Plus microscope (Carl Zeiss Canada, Kirkland, QC), images were captured with a CCD camera (Retiga, Qimaging) and processed with Northern Eclipse image analysis software (Empix Imaging, Mississauga, ON).

2.3.4 Optic nerve immunostaining and quantification of axonal growth

Animals were perfused intracardially with 4% PFA and the optic nerves processed for immunocytochemistry as described above. Longitudinal optic nerve

cryosections (14 µm) were taken along the nasal-temporal plane and collected onto gelatin-coated slides. Non-specific binding was blocked with 3% BSA, 5% normal rabbit serum (Vector Laboratories, Burlingame CA) and 0.2% Triton X-100. Sections were then incubated overnight with goat CTβ antibody (1:4,000 dilution, List Biological Labs), followed by incubation with biotinylated rabbit anti-goat IgG (6 µg/ml, Vector Laboratories) and lastly, with DTAF-conjugated streptavidin (3.6 µg/ml, Jackson Immunoresearch Labs). GAP-43 expression was detected using anti-rat GAP-43 antibody (1:4,000 dilution, kind gift of Dr. L. Benowitz, Harvard Medical School) followed by biotinylated anti-rat IgG (6 µg/ml, Vector Laboratories) and by streptavidin-coupled Alexa Fluor 594 (2 µg/ml, Molecular Probes). Alternatively, a monoclonal anti-rat GAP-43 (2 µg/ml, Roche Diagnostics Canada) was used followed by incubation in Alexa 594-conjugated anti-IgG (14 µg/ml, Molecular Probes).

The extent of axonal regeneration was evaluated by counting the number of CTß-positive axonal profiles that extended beyond the site of injury. Axons that crossed a virtual line parallel to the lesion site at 50 µm, 100 µm, 250 µm, 500 µm and 1 mm were counted in three to four sections of optic nerve per animal. Sections were selected across the entire width of the nerve to analyze axon growth in both central and peripheral regions. The quantification of axons was carried out using two complementary methods: i) axons were counted directly on each section using a 100X objective (Zeiss), and ii) composite images of each nerve were generated at 60X magnification to verify axon counts with respect to the lesion site and for documentation. Both methods yielded identical results. During quantification, the injury site was clearly identified in the same optic nerve section by dark field microscopy. In addition, the location of the lesion site

was routinely confirmed in the same section or in an immediately adjacent section using hematoxylin/eosin staining. The thickness of the optic nerve was measured at each point where axons were counted and this value was used to calculate the number of axons per mm of nerve width. An estimate of the total number of axons (Σa) extending over a distance (*d*) was calculated by adding the number of axons in all 14-µm thick (*t*) sections of an entire nerve having a radius of *r*, according to the formula: $\Sigma a_d = \pi r^2$ X [average axons/mm]/*t* (Leon et al., 2000a). The length of individual regenerating axons was measured from the lesion site to the tip of the axon using Northern Eclipse image analysis software (Empix Imaging Inc., Mississauga, ON). Data analysis was performed using the GraphPad Instat program (GraphPad Software Inc., San Diego, CA) by a one-way analysis of variance (ANOVA) or an unpaired *t* test.

2.3.5 Quantification of Neuronal Survival

Rats were sacrificed by intracardial perfusion with 4% paraformaldehyde and both the left (optic nerve lesion) and right (intact control) retinas were dissected, fixed for an additional 30 min and flat-mounted vitreal side up on a glass slide. In addition, backlabeled retinas from normal, unoperated animals were used as controls. The ganglion cell layer was examined in whole-mounted retinas with fluorescence microscopy and FluoroGold-labeled neurons were counted in 12 standard retinal areas as described (Villegas-Perez et al., 1993). Data analysis and statistics were performed using the GraphPad Instat program by a one-way ANOVA test.

2.4 RESULTS

2.4.1 AAV-mediated FGF-2 protein is expressed by adult RGCs

For gene transfer experiments, a recombinant AAV vector containing a human FGF-2 cDNA was injected into the vitreous chamber of adult rat eyes (Fig. 1). Endogenous and virally-mediated expression of FGF-2 protein was examined using FGF-2 antibodies. Intact retinas or retinas infected with the control virus (Fig. 2A) showed positive FGF-2 labeling in few cells in the ganglion cell layer, and widespread staining of cells in the inner and outer nuclear layers. These results are consistent with previous reports describing the endogenous localization of FGF-2 in the adult retina (Gao and Hollyfield, 1992; Kostyk et al., 1994b; Ohsato et al., 1997). Only astrocytes in the ganglion cell layer, but not adult rat RGCs, have been shown to express endogenous FGF-2 (Kostyk et al., 1994b). Four weeks after single intravitreal injection of AAV.FGF-2, we observed positive FGF-2 immunolabeling in RGCs (Fig. 2B), in contrast to control retinas where FGF-2 staining was restricted to astrocytes. Consistent with our previous study (Cheng et al., 2002b), ~70% of the total RGC population was effectively infected with recombinant AAV. This is in agreement with a recent report showing that intraocular injection of AAV.GFP led to transduction of 60-70% of adult RGCs (Harvey et al., 2002). Positive FGF-2 labeling was also observed in some RGC dendritic processes (Fig. 2C) and processes in the nerve fiber layer (Fig. 2D). Identical results were obtained with the two FGF-2 antibodies used in this study.

The ganglion cell layer of the adult rat retina contains displaced amacrine cells, which account for ~40% of the total number of neurons in this layer (Perry, 1981), as well as astrocytes. We sought to confirm that AAV directed FGF-2 protein expression in

RGCs. For this purpose, we performed co-localization studies in retinas from eyes that received an intravitreal injection of AAV.FGF-2 followed by retrograde labeling of these neurons with FluoroGold. Our results confirmed that RGCs produced AAV-mediated FGF-2 (Fig. 2E-G). We did not detect expression of AAV.FGF-2 in other glial cells, such as Müller cells, in any of the infected retinas, a finding in agreement with our previous data showing that RGCs are the primary cellular target for AAV infection in the inner retina (Cheng et al., 2002b).

2.4.2 RGCs express the high affinity FGF-2 receptor, FGFR-1, and heparan sulfate

To assess the capacity of RGCs to respond to FGF-2, we examined the endogenous expression of FGFR-1, a member of the FGF receptor family for which FGF-2 shows high binding affinity (Ornitz et al., 1996). Incubation of retinal sections from normal, unoperated eyes with an antibody against the C-terminus of FGFR-1 (Flg C-15) demonstrated positive immunostaining in the ganglion cell layer and the outer nuclear layer, with faint labeling in the inner nuclear layer (Fig. 3A). An identical labeling pattern was observed using a different antibody against an epitope in the extracellular domain of FGFR-1 (Flg H-76). Co-labeling experiments with anti-FGFR-1 (Flg H-76) and FluoroGold confirmed FGFR-1 localization in RGC somata and processes in the nerve fiber layer (Fig. 3B-D) as well as in some displaced amacrine cells. Our results are consistent with a previous study showing FGFR-1 immunoreactivity in RGCs in the ferret retina (Quan et al., 1999).

We also investigated the localization of heparan sulfate (HS), typically associated with cell surface HSPGs, in the adult uninjured rat retina. Using an HS antibody, we

observed robust positive immunolabeling in the ganglion cell layer (Fig. 3E). Strong, positive HS labeling could be clearly visualized in many neurons. In contrast, diffuse HS staining was observed in the outer and inner nuclear layers suggesting the association of HS with extracellular matrix components. HS immunostaining of retinas labeled with FluoroGold showed co-localization of HS on RGC bodies and in processes in the nerve fiber layer (Fig. 3F-H). No staining was observed in control retinal sections in which the primary antibodies were omitted (not shown). These data indicate that key molecules involved in FGF-2 binding and activity, FGFR-1 and HS, are expressed by adult RGCs.

2.4.3 AAV.FGF-2 promotes regeneration of injured RGC axons

The effect of AAV.FGF-2 on axonal growth was tested after micro-crush lesion of the adult rat optic nerve (Fig. 1A). While all RGC axons are completely transected in this injury paradigm, the lesion site remains well-defined and the extent of the glial scar is minimized (Lehmann et al., 1999; Selles-Navarro et al., 2001a). Application of FluoroGold to the superior colliculi after the lesion resulted in absence of fluorescence in the retina, confirming that the optic nerve was completely transected and validating our technique (Figs. 4A and 4B). The lesion site was accurately identified during quantification of RGC axons by visualization of the optic nerve in dark field (Fig. 4C). In addition, we routinely confirmed the localization of the injury zone in the same section or in an immediately adjacent section using hematoxylin/eosin staining (Fig. 4D).

Extensive growth of RGC axons, visualized with CTß, was observed in all the eyes treated with AAV.FGF-2 (Figs. 5A-C). In contrast, AAV.GFP-treated eyes showed

RGC axons that retracted from the lesion site and few crossed over the glial scar (Fig. 5D and 5E). Quantitative analysis of axon growth demonstrated that FGF-2 gene transfer increased the number of regenerating axons at all distances examined from the injury site (Fig. 6A), particularly between 50 µm and 500 µm from the lesion. For example, the number of growing axons per mm of optic nerve at 50 µm, 250 µm and 500 µm was 76.8 ± 11 (mean ± SEM), 25.4 ± 12 and 7.3 ± 1.6, respectively. In addition, we estimated that the total number of axons per nerve that extended past 50 µm, 250 µm and 500 µm were: 668 ±35, 254 ± 30, and 61.5 ± 7, respectively. There was a 8.5-fold and 10-fold increase in the number of axons growing at 250 µm and 500 µm from the lesion, respectively, after AAV.FGF-2 injection compared to optic nerves from eyes treated with AAV.GFP. While individual axons could be visualized using CTß staining in optic nerve longitudinal sections, we cannot exclude the possibility that some fine axon fascicles, rather than single axons, were counted. This quantification error may result in an underestimation of the total number of growing axons in all experimental groups, but the overall fold-increase between AAV.FGF-2-treated and control groups would remain unchanged.

To evaluate if FGF-2 gene transfer had an effect on the length of the growing axons, we identified the longest axons in the experimental and control groups and measured their mean distance of growth from the lesion site. Our analysis showed that the average length of the longest axons in AAV.FGF-2-treated eyes at 2 weeks post-axotomy was 909 ± 55 µm (mean ± S.E.M.), whereas in eyes treated with the control virus it was 387 ± 20 µm (Fig. 6B). The short distance regeneration in AAV.GFP- and saline-treated eyes compared to injured control eyes that did not receive any intraocular

injection (Fig. 6A), could be attributed to retinal injury during injection which has been shown to increase FGF-2, FGFR-1 and ciliary neurotrophic factor (CNTF) mRNA levels in the retina (Wen et al., 1995; Cao et al., 1997; Cao et al., 2001). During all surgical procedures, we avoided injury to the lens and anterior structures of the eye shown to promote RGC regeneration and survival (Mansour-Robaey et al., 1994; Leon et al., 2000a), but injury to the retina itself is unavoidable upon insertion of the tip of the needle into the vitreous space. We observed a small, but not statistically significant ($p > 0.05$), increase in axon growth between AAV.GFP- and saline-injected eyes (Fig. 6A). Under these conditions, however, we always found enhanced axon growth induced by AAV.FGF-2 with respect to control eyes.

The growth-associated protein 43 (GAP-43) has been shown to be expressed in RGCs during axonal growth (Doster et al., 1991; Schaden et al., 1994; Berry et al., 1996). We used two different GAP-43 antibodies to confirm that AAV.FGF-2 induced axon growth after micro-crush lesion of the optic nerve. Robust GAP-43 staining was observed in axons growing past the injury site in eyes treated with AAV.FGF-2 (Fig. 6C). The pattern of GAP-43 immunostaining closely resembled that of CTß labeling (Fig. 6D). Double-labeling experiments demonstrated that all regenerating axons were immunopositive for both CTß and GAP-43 (Fig. 6E). Identical results were obtained with two different GAP-43 antibodies used in this study. Taken together, our data indicate that AAV-mediated gene transfer of FGF-2 increases the number and length of RGC axons that grow past the lesion the site in the injured rat optic nerve.

2.4.4 Selective upregulation of FGF-2 in RGCs, but not in optic nerve glia, is required for axonal growth

In intact, non-lesioned optic nerves, endogenous FGF-2 expression was detected in glial cell bodies organized in linear arrays along the anterior-posterior axis of the nerve (Fig. 7A). Following micro-crush lesion alone, in the absence of viral vector injection, we detected an increase in the number of FGF-2 immunoreactive glial cells (Fig. 7B). In addition, the linear organization of FGF-2 positive glial cells was disrupted with accumulation of cells around the lesion site. Importantly, endogenous FGF-2 was never detected in RGC axons in intact or injured, non-treated optic nerves. We then examined the correlation between axonal growth and FGF-2 transgene expression. At 2 weeks after micro-crush lesion, positive FGF-2 staining was clearly visualized in all CTß-labeled RGC axons growing into the distal optic nerve (Figs. 7C-E). Figs. 7F-H illustrate an individual RGC axon between 200 μm and 350 μm from the lesion site, co-labeled with CTß and FGF-2 antibodies. In contrast, FGF-2 expression was never detected in RGC axons of injured eyes injected with the control vector AAV.GFP (Fig. 7I-K). As expected, both AAV.FGF-2-treated and AAV.GFP-treated injured optic nerves showed endogenous positive FGF-2 immunoreactivity in glial cells. These results indicate that although micro-crush lesion alters the pattern of endogenous FGF-2 expression in the optic nerve, endogenous FGF-2 expression is never detected in RGC axons. More importantly, our data indicate that selective upregulation of FGF-2 in RGCs, but not in optic nerve glia, correlates with axon extension up to 1 mm from the lesion site.

2.4.5 FGF-2 gene delivery confers transient protection to axotomized RGCs.

To determine whether axonal growth produced by AAV.FGF-2 was due to an increase in the number of surviving RGCs, we investigated the neuroprotective effect of FGF-2 gene delivery after optic nerve axotomy (Fig. 1B). We first compared the time-course of RGC death induced by either axotomy or micro-crush lesion of the optic nerve. Our quantitative analysis demonstrated that both injury modalities led to a similar loss of RGCs within the first 2 weeks of injury (Fig. 8A), thus allowing us to compare AAV.FGF-2-mediated survival and regeneration. Flat-mounted retinas were examined at 7 and 14 days after optic nerve transection to determine the density of surviving RGCs identified by their FluoroGold label (Fig. 8A and Table 1). Macrophages and microglia that may have incorporated FluoroGold after phagocytosis of dying RGCs were excluded from our quantitative analysis based on their morphology and the presence of microglia specific markers as described (Cheng et al., 2002). A single intraocular injection of AAV.FGF-2 resulted in increased RGC survival at 1 week after axotomy: 74% of RGCs remained alive compared to only 49% neuronal survival induced by AAV.GFP. At 2 weeks after axotomy, however, treatment with AAV.FGF-2 protected only 13% of all RGCs, a survival rate that was not significantly different from that produced by the control virus (Fig. 8A).

FGF-2 is a potent mitogenic factor for retinal neurons during early development and for neural progenitor cells in the retinal ciliary margin (Hicks, 1998; Ahmad et al., 2000; Patel and McFarlane, 2000; Tropepe et al., 2000). To rule out that the increase in RGC density observed with AAV.FGF-2 at 1 week post-axotomy was due to cell proliferation, we first evaluated the number of FluoroGold-labeled neurons after

AAV.FGF-2 or AAV.GFP injection in the normal, intact retina. Our analysis demonstrated that the total number of RGCs after infection with either AAV.FGF-2 (2,056 ± 107, n=4, mean ± S.E.M.) or AAV.GFP (2,169 ± 153, n=3) was not statistically different (unpaired t test, P=0.628) (Fig. 8A). In addition, we evaluated retinal cell proliferation visualized by BrdU incorporation after AAV.FGF-2 or AAV.GFP injection and axotomy of the optic nerve (Fig. 1C). The pattern of BrdU-positive cells was similar in both AAV.FGF-2- and AAV.GFP-treated eyes at 1 week after nerve injury: while few BrdU-labeled cells were detected in the retinal fiber layer (Fig. 8B), a larger number of proliferating cells was observed at the optic nerve head (Fig. 8C). Double labeling experiments using a BrdU antibody in combination with FluoroGold demonstrated that the BrdU-positive cells were not RGCs (Figs. 8B and 8C). The location of BrdU-positive cells suggests that they may be retinal astrocytes, known to proliferate after retinal injury (MacLaren, 1996). These data indicate that AAV.FGF-2 promotes only transient survival, but not proliferation, of axotomized RGCs.

2.5 DISCUSSION

The idea has been put forward that factors that promote axon growth during development may facilitate axon regeneration by adult neurons in the injured CNS. In the adult visual system, injured RGCs re-express markers typically found during development, such as cytoskeletal proteins (McKerracher et al., 1993; Dieterich et al., 2002), growth associated proteins (Meiri et al., 1986; Moya et al., 1988; Doster et al., 1991), neurotrophic factors (Eckenstein et al., 1991b; Gao et al., 1997), adhesion and guidance molecules (Petrausch et al., 2000; Ellezam et al., 2001; Knoll et al., 2001;

Lang et al., 2001), metabolic substrates (Allen et al., 2001) and transcription factors (Ziman et al., 2001), suggesting that these neurons revert to a developmental growth mode when attempting to regenerate. In this study we tested the hypothesis that FGF-2, a potent stimulator of RGC axon growth in the developing visual system (McFarlane et al., 1995; Brittis et al., 1996; McFarlane et al., 1996; Dingwell et al., 2000), promotes regeneration of adult rat RGCs.

Using a gene transfer approach, we demonstrate that selective upregulation of FGF-2 in RGCs stimulated axonal extension distal to the lesion after acute optic nerve injury. Other neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin (NT)-3 or NT-4, failed to promote RGC axon growth into a peripheral nerve graft attached to the severed optic nerve (Cui et al., 1999). Intraocular injection of CNTF was shown to stimulate some RGC regeneration into a graft (Cui et al., 1999), but failed to enhance RGC axon growth into the optic nerve after crush injury (Leon et al., 2000). Thus, our study provides the first evidence that upregulation of a single trophic factor, FGF-2, can stimulate RGC axon extension within the growth inhibitory environment of the adult optic nerve perhaps by recapitulating the expression of some developmental genes associated with axonal growth. This idea is supported by our observation that GAP-43 was localized in all growing axons of AAV.FGF-2-treated eyes. GAP-43 is typically expressed by developing RGCs during axon growth and synaptogenesis, but it is downregulated in mature RGCs (Skene and Willard, 1981; Meiri et al., 1986). The level of GAP-43 is transiently upregulated after optic nerve injury (Doster et al., 1991; Fournier et al., 1997), but remains expressed at high levels only during active regeneration (Skene and

Willard, 1981; Benowitz and Lewis, 1983; Doster et al., 1991; Schaden et al., 1994; Berry et al., 1996).

Importantly, we found that all regenerating axons also expressed virally-mediated FGF-2 protein. Our data showed that endogenous FGF-2 was not present in mature RGC bodies or axons but only in glial cells of the ganglion cell layer and optic nerve. Although injury to the optic nerve increased the number of FGF-2-positive glial cells, particularly around the injury site, endogenous expression of this neurotrophic factor remained restricted to nerve glia. Optic nerve crush has been shown to increase endogenous FGF-2 expression within the retina in the outer nuclear layer, but not in RGCs (Kostyk et al., 1994). Our data indicate that injury-induced upregulation of endogenous FGF-2 was not sufficient to promote axon growth. In contrast, selective expression of FGF-2 in retinal ganglion neurons stimulated axonal extension distal to the lesion. Of interest, FGFR-1 mRNA and protein are known to increase in photoreceptor cells of the adult retina after focal mechanical injury (Ozaki et al., 2000; Cao et al., 2001). We can not rule out the possibility that endogenous FGFR-1 upregulation restricts RGC axon growth and survival by increasing the ability of neighboring cells to bind FGF-2, thus reducing the amount of neurotrophic factor available to RGCs. Our results suggest, however, that within the first two weeks of injury a sufficient quantity of virally-delivered FGF-2 is available to elicit RGC axon growth, perhaps overcoming the potential effect of increased retinal FGFR-1 as an FGF-2 sink.

FGF-2 lacks a classical signal sequence for secretion (Abraham et al., 1986), yet this neurotrophic factor is secreted into the extracellular milieu presumably via a non-conventional secretory pathway (Powers et al., 2000). Although the mechanism of action

of AAV-mediated FGF-2 is still undefined, it is possible that infected RGCs secrete FGF-2 into the extracellular space where it binds cell surface FGFRs and HS, in an autocrine manner, stimulating axon growth. The observation that AAV-mediated FGF-2 is found in growing axons suggests that this neurotrophic factor is anterogradely transported, thus it is also possible that local FGF-2 release from axon terminals can influence growth. Our observation that both FGFR-1 and HS, key molecules involved in FGF-2 binding and signaling, are localized in adult rat RGC bodies and axons support these possible mechanisms of FGF-2 action. Other FGFRs have been shown to be present in the rat inner retina (Blanquet and Jonet, 1996; Kinkl et al., 2002) and although unequivocal localization of these FGFRs on RGCs has yet to be established, we can not exclude their potential role in FGF-2-induced RGC regeneration. There is substantial evidence supporting roles for FGFR signaling in RGC axon extension, guidance and targeting during development (McFarlane et al., 1995; Brittis et al., 1996; McFarlane et al., 1996; Lom et al., 1998). Similarly, HSs are important regulators of axon growth and targeting in the developing visual system (Walz et al., 1997; Irie et al., 2002). For example, heparitinase removal of native HSs during formation of the *Xenopus* optic tract retarded retinal axon elongation (Walz et al., 1997). Thus, it is likely that FGFR-1 and HS play a role during RGC axon regrowth in the adult injured visual system.

Although FGF-2 gene delivery stimulated significant axon growth, this effect was restricted to within 1 mm from the lesion site. Other strategies using angiotensin II (Lucius et al., 1998), the Rho-A inhibitor C3 (Lehmann et al., 1999), antibodies against myelin (Papadopoulos et al., 2002), or lens injury (Leon et al., 2000) have also resulted in short distance RGC axon regrowth. The limited ability of RGC axons to regenerate

has been partly attributed to the growth inhibitory environment of the optic nerve, rich in proteins such as Nogo-A (Caroni and Schwab, 1988a; Caroni and Schwab, 1988b), myelin-associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994) and oligodendrocyte-myelin glycoprotein (OMgp) (Kottis et al., 2002) that restrict the extension of axons on myelin by interacting with the Nogo receptor (Fournier et al., 2001a; Liu et al., 2002; Wang et al., 2002b). When the optic nerve was replaced for the permissive environment of a peripheral nerve graft, RGC axons regenerated long distances and re-established functional synaptic connections with their targets (Vidal-Sanz et al., 1987; Keirstead et al., 1989). Nevertheless, only a small number of all RGCs, an average of 3% (Vidal-Sanz et al., 1987), were able to regenerate into the favorable graft environment. We estimated that after AAV.FGF-2 injection, ~2% of surviving RGCs were able to regrow an axon 250 μm past the lesion site within the growth inhibitory environment of the optic nerve. The limited regenerative capacity displayed by adult RGCs is further illustrated by the observation that strategies that promote long distance regeneration in the injured spinal cord produce only short distance axon growth in the optic nerve. For example, a myelin-based vaccine stimulated regeneration for distances up to 11 mm past the lesion site in the adult mouse spinal cord (Huang et al., 1999), while the same treatment produced a few hundred microns of regenerative axon growth in the optic nerve (Ellezam et al., 2003). Several studies indicate that a given treatment can lead to axon regeneration in spinal cord that is almost an order of magnitude larger than that produced in optic nerve (Bartsch et al., 1995; Lehmann et al., 1999; Dergham et al., 2002). It is apparent that other factors participate in the control of the regenerative capacity of RGCs, including inhibitory components of

the glial scar and/or changes in the neuronal expression of key molecules involved in axon growth.

Regeneration in the optic nerve is also likely to be limited by the death of large numbers of RGCs soon after injury: >90% of these neurons are lost at 2 weeks after axotomy. Our data indicate that FGF-2 upregulation produced transient survival, but not proliferation, of injured RGCs. The absence of FGF-2-induced survival at 2 weeks after lesion could not be attributed to loss of FGF-2 gene expression since here, and in previous studies (Dudus et al., 1999; Guy et al., 1999; Cheng et al., 2002), sustained AAV-mediated transgene expression was observed for several months after vector administration. Strategies involving other neurotrophic factors have proved to be more effective at promoting long-term survival of injured rat RGCs. For example, we previously demonstrated that TrkB gene delivery, in combination with exogenous BDNF supported the survival of 76% of RGCs at 2 weeks after axotomy (Cheng et al., 2002). The present study indicates that in spite of low RGC survival, FGF-2 upregulation enhanced RGC axon growth. The early increase in neuronal survival that we observed at 1 week after injury may have translated into more RGCs being capable of responding to FGF-2 with subsequent activation of their growth signaling pathways. Recent studies clearly show that RGC survival alone is not sufficient to promote axon regeneration. For example, upregulation of Bcl-2 in RGCs was sufficient to keep neurons alive, but failed to promote axon extension in culture (Goldberg et al., 2002), within the optic nerve (Lodovichi et al., 2002) or into a peripheral nerve graft (Inoue et al., 2002). These results suggest that signals that induce RGC survival or axon growth utilize different intracellular pathways. Future studies are required to establish if axon regeneration can

be enhanced when strategies that result in long-term RGC survival are combined with FGF-2 upregulation.

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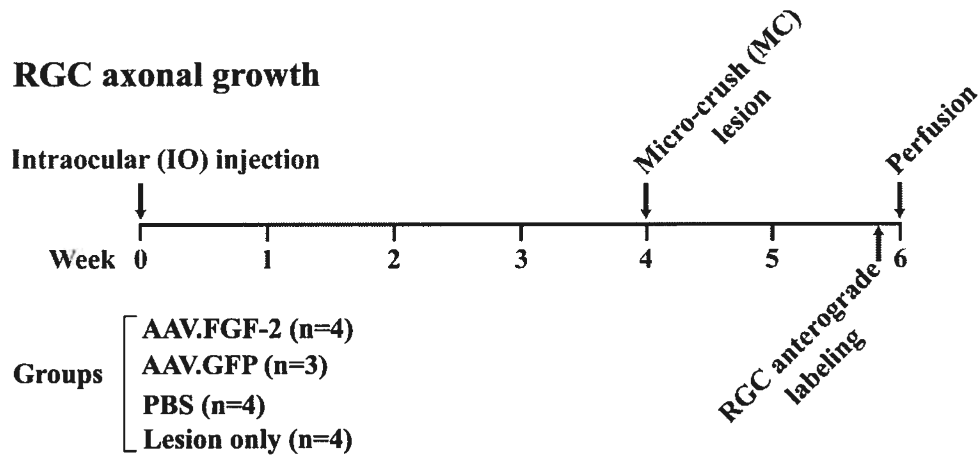
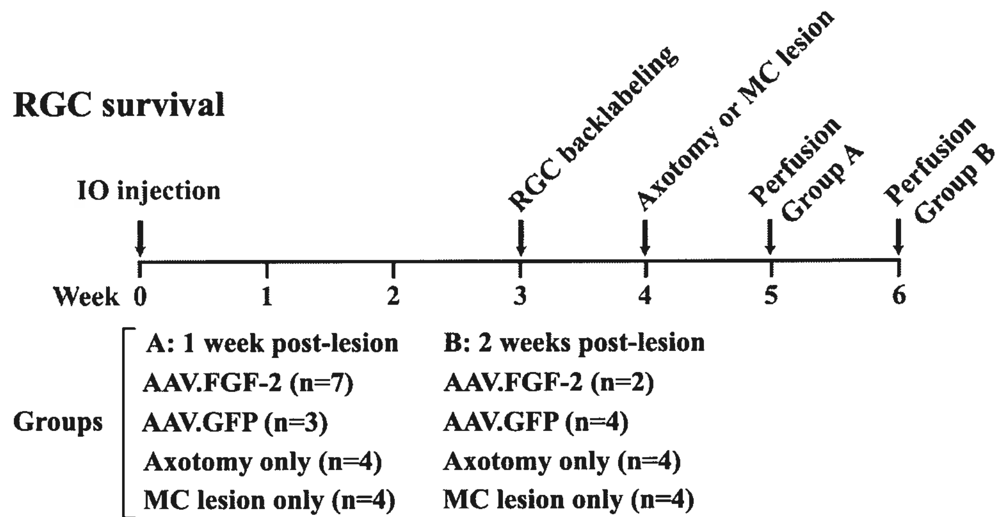
2.7 FIGURES

Figure 1. Experimental protocols used to test the effect of AAV.FGF-2 on RGC axon growth, survival or proliferation.

(A) Micro-crush lesion (MC) of the optic nerve was performed 4 weeks after intraocular (IO) injection of AAV.FGF-2, the time required for AAV-mediated transgene expression to reach a plateau in the adult rat retina. Thirteen days after injury, RGC axons were anterogradely labeled by IO injection of CT□ and 24 hrs later (2 weeks post-microcrush lesion), animals were sacrificed and the optic nerves were processed for analysis of axon growth. Control groups included animals that received a single intravitreal injection of AAV.GFP, saline or no injection. (B) For neuronal survival assays, RGCs were retrogradely labeled with FluoroGold and subsequently axotomized at 4 weeks after IO injection of AAV vectors. RGC survival was assessed by quantification of fluorescent neurons in whole-mounted retinas at 1 week (Group A) or 2 weeks (Group B) after optic nerve lesion. Control groups included animals that received a single injection of AAV.GFP or no injection. (C) For in vivo cell proliferation assays, AAV.FGF-2 or AAVGFP (control) were injected intravitreally and, 4 weeks later, the optic nerve was transected. RGCs were retrogradely labeled prior to axotomy. Following optic nerve lesion, rats received daily intraperitoneal injections of BrdU for 1 week after which animals were sacrificed and their retinas were processed for BrdU immunocytochemistry.

FIGURE 1.





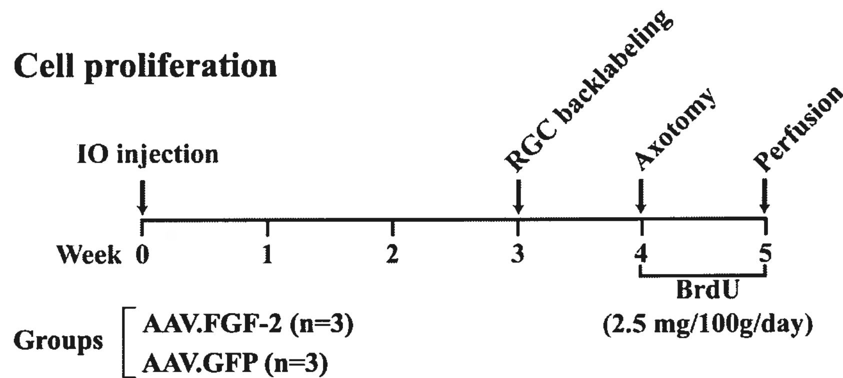


Figure 2. AAV mediates FGF-2 transgene expression in adult RGCs.

(A) FGF-2 protein expression in retinal sections from eyes injected with control AAV.GFP (n=3) was visualized by fluorescent microscopy using FGF-2 antibodies. FGF-2 immunostaining was detected in some astrocytes in the ganglion cell layer (GCL), and cells in the inner and outer nuclear layers (INL, ONL), but not in RGCs. An identical labeling pattern was found in normal, unoperated retinas (not shown). (B) Four weeks after single intraocular injection of AAV.FGF-2 (n=4), FGF-2 labeling was observed in ~70% of all RGCs. (C-D) Expression of AAV-mediated FGF-2 was also visualized in dendrites (C) and processes in the nerve fiber layer (D) of some of the virally transduced RGCs. In contrast, endogenous levels of FGF-2 in the INL and ONL remained unchanged after intraocular injection of AAV.FGF-2. (E-G) Expression of AAV-mediated FGF-2 in RGCs was confirmed by double-labeling using FluoroGold (E), and a FGF-2 antibody (F). Superimposition of the images shown in (E) and (F) demonstrated that RGCs expressed AAV-mediated FGF-2 (G). Identical results were obtained using two different FGF-2 antibodies. ONL: Outer Nuclear Layer, OPL: Outer Plexiform Layer, INL: Inner Nuclear Layer, IPL: Inner Plexiform Layer, GCL: Ganglion Cell Layer. Scale bars: 100 µm (A, B), 10 µm (C-D), 25 µm (E-G).

FIGURE 2.

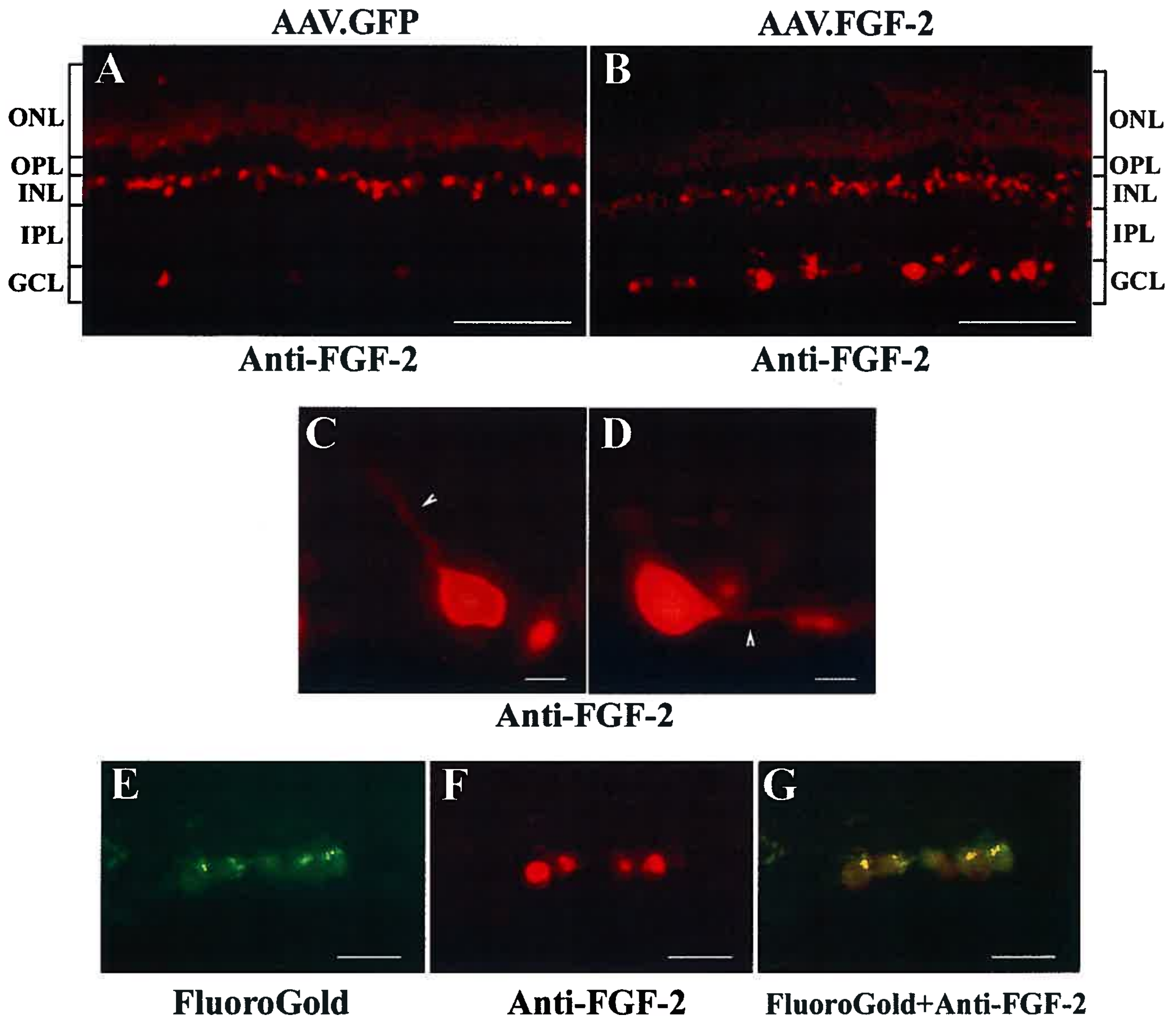


Figure 3. FGFR-1 and heparan sulphate (HS) are expressed by adult RGCs. Expression of endogenous FGFR-1 and HS by RGCs was investigated by immunohistochemistry in adult retinas from normal, unoperated eyes. (A) Incubation of retinal sections (n=3) with an antibody against the C-terminus of FGFR-1 (Flg C-15) demonstrated strong positive immunostaining in the ganglion cell layer (GCL) and outer nuclear layer (ONL), with somewhat fainter labeling in the inner nuclear layer (INL). (B-D) Expression of FGFR-1 protein in the GCL was confirmed using a different antibody against an epitope within the extracellular domain of FGFR-1 (Flg H-76). Co-labeling experiments with FGFR-1 antibody and FluoroGold detected FGFR1 in RGC cell bodies. (E) HS, typically associated with HSPGs, was examined using an HS antibody (HepSS-1). Positive HS labeling (n=3) was found associated with neurons in the GCL. In contrast, diffuse HS staining was observed in the ONL and INL. (F-H) HS immunostaining of retinas labeled with FluoroGold detected HS immunoreactivity in RGC somata and in axon bundles of the nerve fiber layer. IPL: Inner Plexiform Layer, OPL: Outer Plexiform Layer, PS: Photoreceptor Segments, RPE: Retinal Pigment Epithelium. Scale bars: 100 µm (A, E), 25 µm (B-D and F-H).

FIGURE 3.

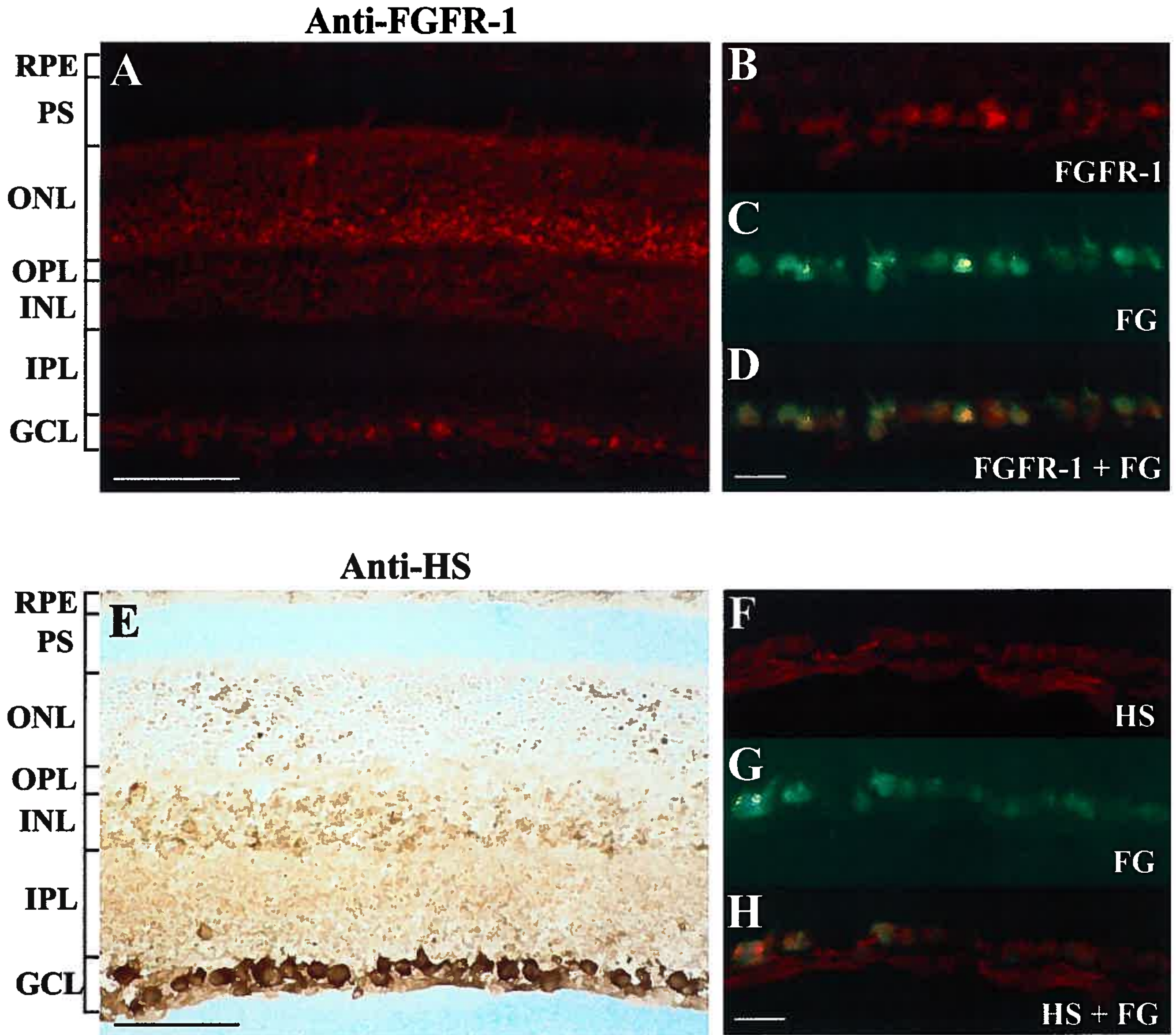


Figure 4. Micro-crush lesion results in complete transection of the optic nerve and a well-defined injury site.

(A) Whole-mounted retina visualized after application of the tracer FluoroGold to the superior colliculus that labeled the entire RGC population. (B) Micro-crush lesion of the optic nerve preceded application of FluoroGold, thus absence of fluorescence in the retina confirmed that the optic nerve was completely transected. (C-D) Micro-crush lesion typically resulted in a well-defined injury site in which the extent of the glial scar was minimized. The lesion site (white arrowheads) was accurately identified at 2 weeks post-injury by visualization of the optic nerve section in dark field during RGC axon quantification (C). The location of the injury site (black arrowheads) was routinely confirmed in the same section or in an immediately adjacent section by staining with hematoxylin/eosin (D). Scale bars: 200 µm (A, B), 150 µm (C, D).

FIGURE 4.

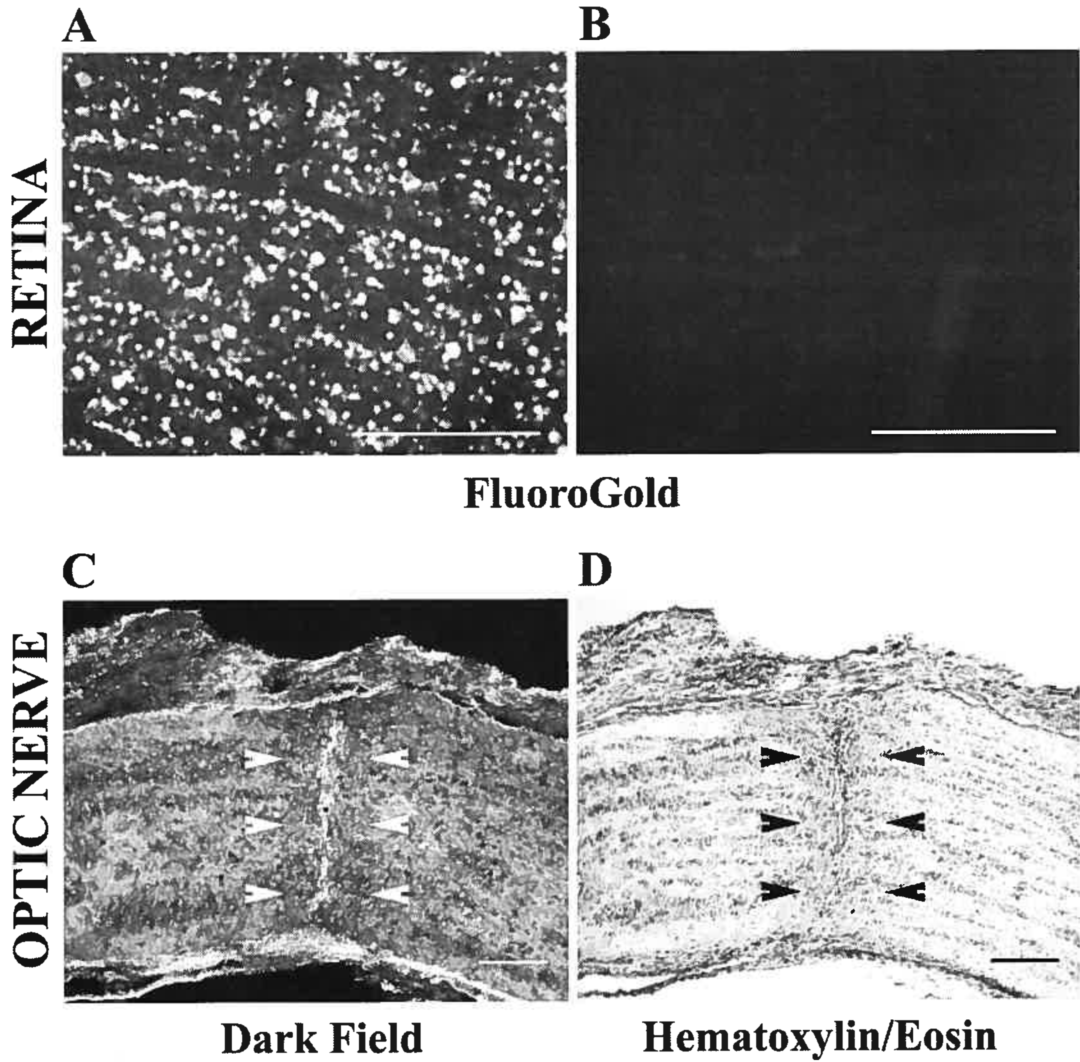


Figure 5. FGF-2 gene transfer promotes RGC axon growth after micro-crush lesion of the optic nerve.

(A) Optic nerve section immunostained with a CTß antibody showed extensive RGC axon growth following intravitreal injection of AAV.FGF-2. A large number of CTß-positive axons regenerated into the distal optic nerve past the lesion site (asterisks). (B) The same section shown in (A) was visualized with dark field, which allowed the clear identification of the injury site (asterisks). (C) Higher magnification image of an AAV.FGF-2-treated optic nerve showing numerous RGC axons growing between 100 μm and 350 μm from the lesion site. (D) Intravitreal injection of the control vector AAV.GFP resulted in lack of RGC axon growth. Many axons retracted from the lesion site (asterisks). (E) Same section shown in (D) visualized in dark field to locate the injury site (asterisks). Scale bars: 100 μm (A, B), 25 μm (C), 50 μm (D, E).

FIGURE 5.

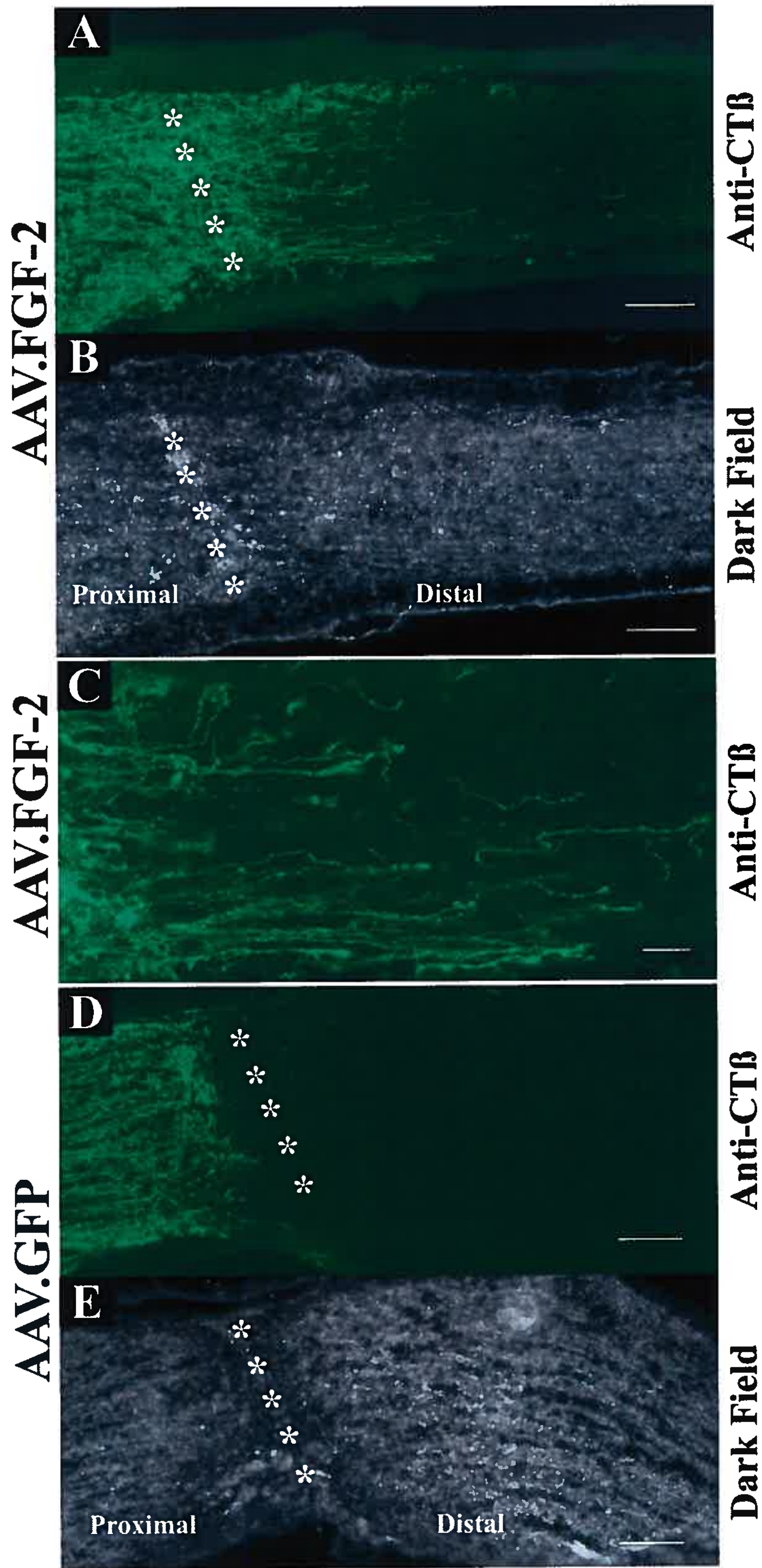
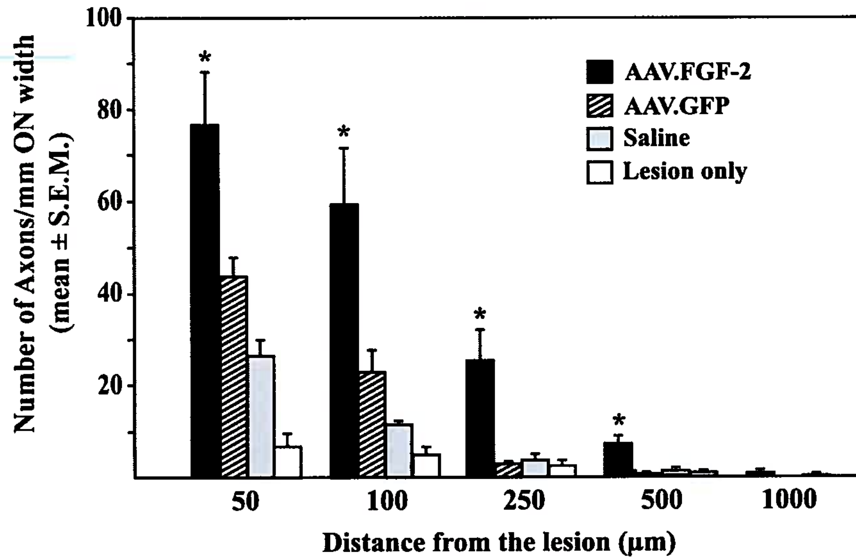
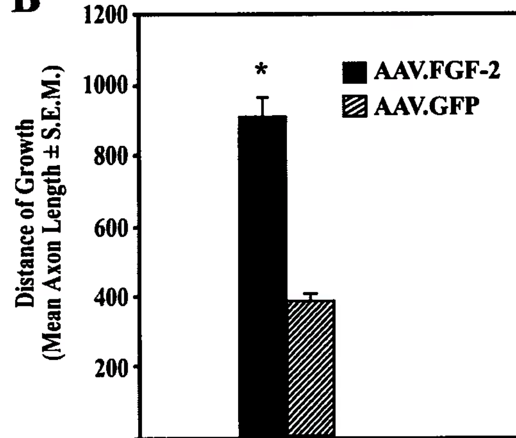


Figure 6. AAV.FGF-2-induced RGC axon regeneration within the injured optic nerve: quantitative and immunocytochemical analysis of growing axons.

(A) Number of RGC axons (mean ± S.E.M.) regenerating after a single intravitreal injection of AAV.FGF-2 (black bars, n=4), AAV.GFP (hatched bars, n=3) or saline (gray bars, n=4) at 2 weeks after optic nerve injury. Lesion only controls are shown with white bars (n=4). RGC axon growth in the AAV.FGF-2-treated group was significantly larger (asterisk) than in the AAV.GFP control group (ANOVA, $p < 0.05$), saline-treated group ($p < 0.01$) or lesion only group ($p < 0.001$). The difference in axon growth between AAV.GFP and saline control groups at 50 µm and 100 µm from the lesion site was not significant ($p > 0.05$). (B) The length of the longest regenerating axons in all optic nerve sections analyzed was measured and averaged. AAV.FGF-2 treatment led to a significant increase in the distance of growth (n=4) compared to AAV.GFP- treated eyes (n=3) (student t test, $p < 0.001$). (C-E) Fluorescent microscopy images of optic nerve sections following AAV.FGF-2 administration showed that all regenerating RGC axons, identified by their CTß-positive staining (D), also expressed the growth-associated protein GAP-43 (n=4) (C). (E) Superimposition of (C) and (D). Scale bars: 50 µm.

FIGURE 6.





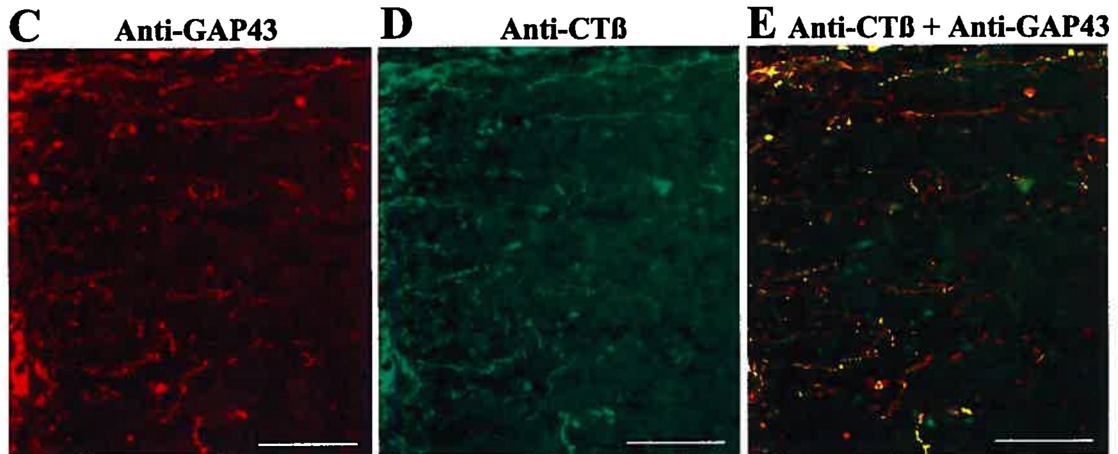


Figure 7. Selective upregulation of FGF-2 in RGCs, but not in optic nerve glia, is required for axonal regeneration.

(A) FGF-2 immunostaining of longitudinal sections of intact, non-injured adult rat optic nerve (n=3). Linear arrays of optic nerve glia expressed endogenous FGF-2. (B) Soon after micro-crush (MC) lesion, in the absence of viral infection, increased numbers of FGF-2 positive glial cells were detected in optic nerves (n=3). This pattern of staining persisted for at least 2 weeks after injury. Endogenous FGF-2 staining was never detected in RGC axons. (C-E) Immunofluorescence of optic nerve sections (n=3) following AAV.FGF-2 injection showed co-localization of FGF-2 and CTß staining in all axons distal to the lesion at 2 weeks after injury. Axons shown are between 100 µm and 250 µm from the lesion site. (F-H) Micrograph of a single RGC axon, between 200 µm and 350 µm from the lesion site, shows clear CTß and FGF-2 co-labeling. (I-K) Control eyes injected with AAV.GFP showed only endogenous FGF-2 expression in optic nerve glia but not in RGC axons. Scale bars: 200 µm (A, B); 40 µm (C-E), 20 µm (F-H), 50 µm (I-K).

FIGURE7

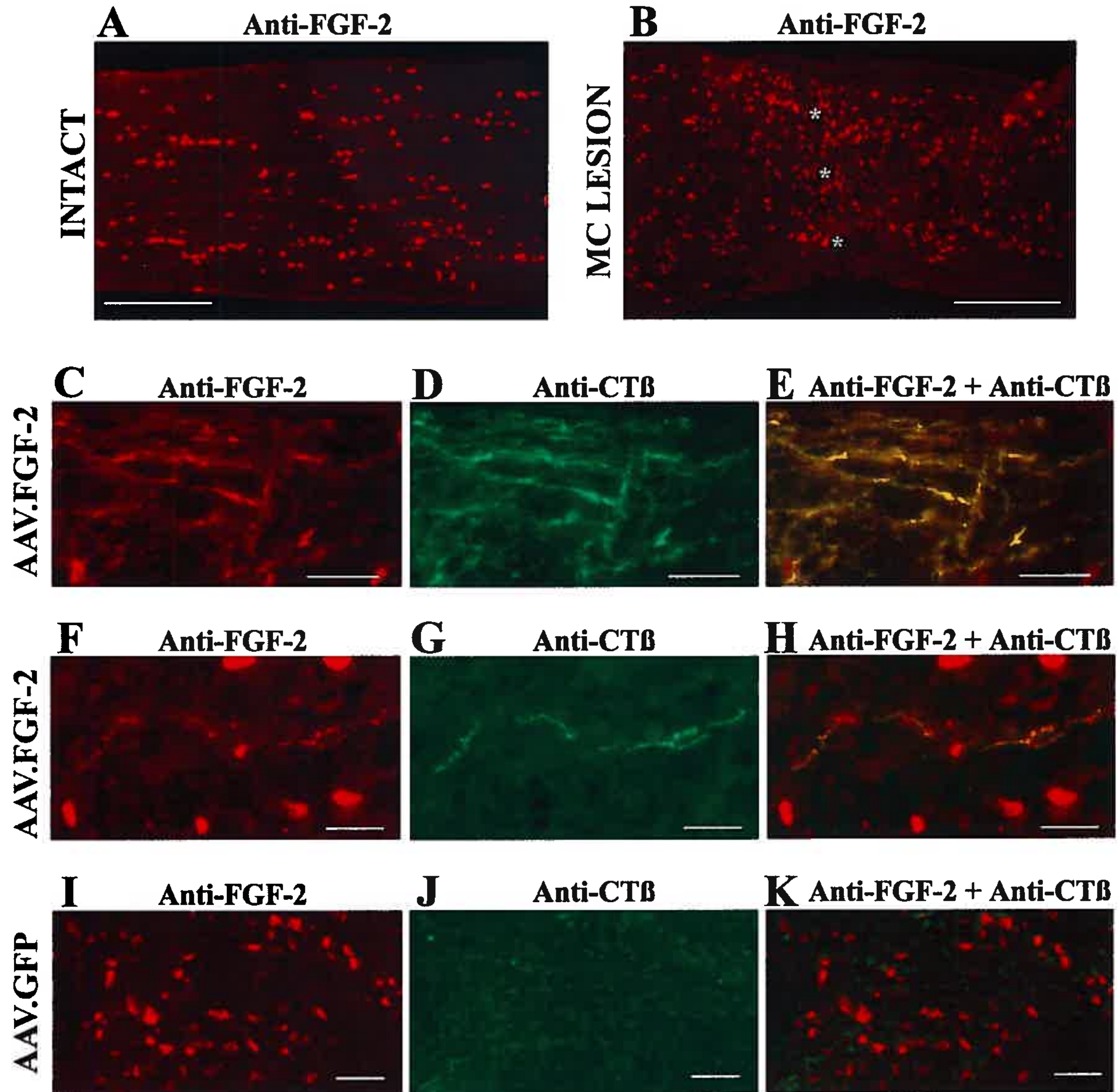
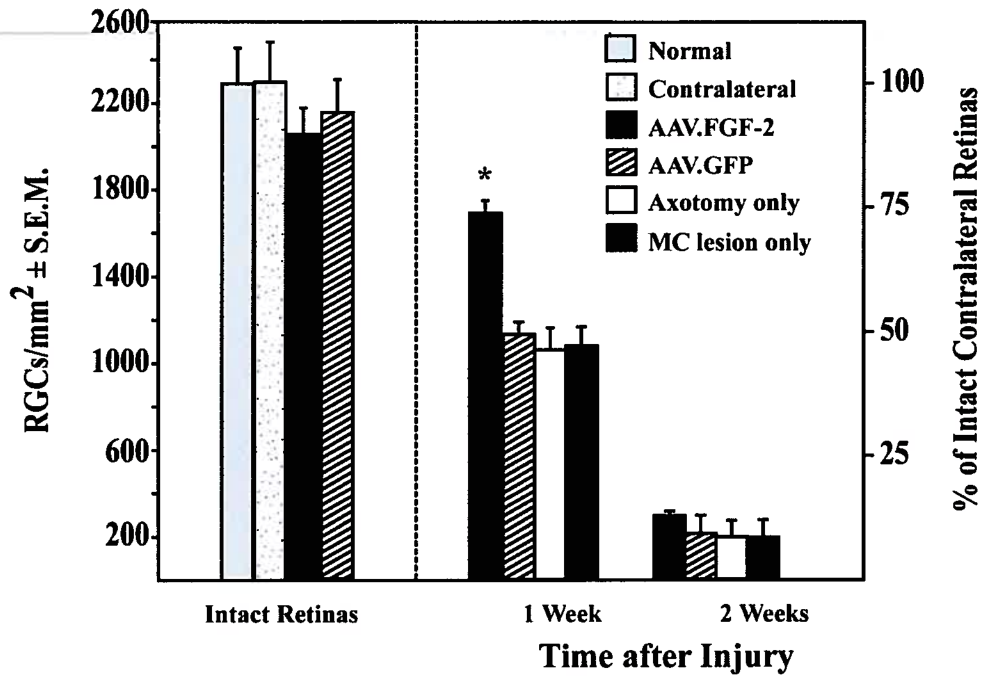
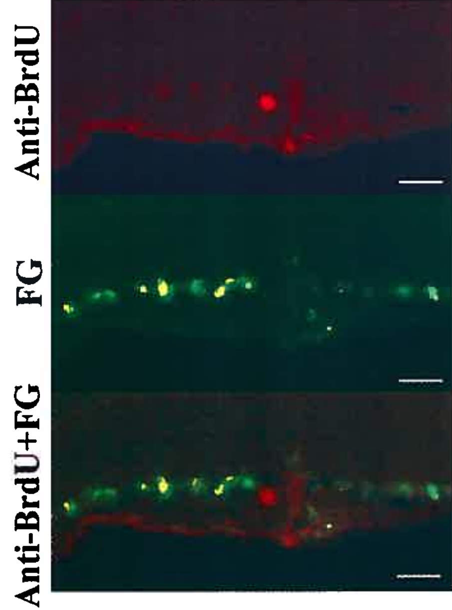


Figure 8. FGF-2 gene transfer promotes transient survival of axotomized RGCs.

(A) The density of RGCs in retinas from normal, unoperated animals (light gray bar, n=8) was similar to that found in retinas from eyes contralateral to axotomized eyes (stippled bar, n=15), indicating that optic nerve injury does not alter the total number of RGCs in the contralateral, unoperated retinas. The RGC density in normal retinas remained unchanged after injection of AAV.FGF-2 (solid bar, n=4) or AAV.GFP (hatched bar, n=4), which indicates that FGF-2 expression or viral infection by itself do not change the total number of ganglion neurons in intact retinas. Quantitative analysis of RGC survival following optic nerve axotomy showed that the neuroprotective effect of AAV.FGF-2 (solid bars, n=7) was significantly higher than that of AAV.GFP (hatched bars, n=3) or injury alone (white bars, n=3) at 1 week after axotomy (ANOVA, $p < 0.001$), but was not significantly different from that found in control retinas at 2 weeks post-injury. The time-course of RGC death was identical following either axotomy (white bars, n=4) or micro-crush lesion (dark gray bars, n=4) of the optic nerve. (B-C) To rule out that the increase in RGC density observed with FGF-2 gene transfer at 1 week after axotomy was due to cell proliferation, we examined BrdU incorporation after AAV.FGF-2 injection (n=3) and axotomy of the optic nerve. While few BrdU-labeled cells were detected in the retinal fiber layer (B), a larger number of proliferating cells was observed at the optic nerve head (ONH) (C). Double labeling experiments using a BrdU antibody in combination with FluoroGold demonstrated that the BrdU-positive cells in the retina (B) and ONH (C) were not RGCs. Scale bars: 25 μm (B), 50 μm (C).

FIGURE 8.





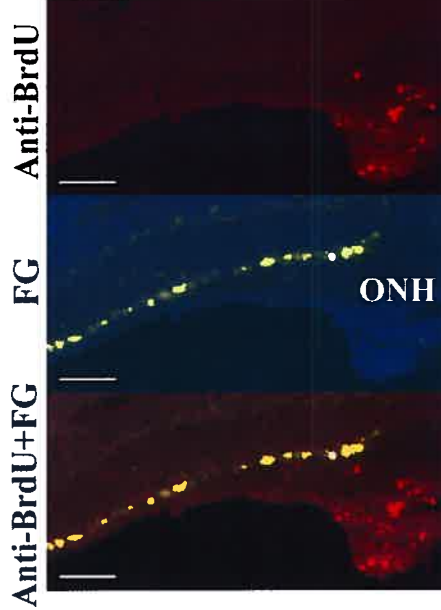


Table 1. Survival of Axotomized RGCs Following FGF-2 In Vivo Gene Transfer

RGCs/mm2 ± S.E.M.

Treatment	Intact Retina	Time After Axotomy	
Viral Vector		1 week	2 weeks
AAV.FGF-2	2,056 ± 107 n = 4	1,695 ± 154 (74%), n = 7	293 ± 24 (13%), n = 2
AAV.GFP	2,169 ± 153 n = 4	1,131 ± 53 (49%), n = 3	220 ± 77 (10%), n = 4
None	Contralateral: 2,291 ± 185 n = 15 Normal: 2,285 ± 162 n = 8	1,062 ± 100 (46%), n = 5	193 ± 77 (8.4%), n = 4

* Numbers in parentheses represent % of intact, contralateral retinas (100%).

Chapter III

EXTRACELLULAR SIGNAL-REGULATED KINASES 1/2 MEDIATE RETINAL GANGLION CELL AXON REGENERATION INDUCED BY FIBROBLAST-GROWTH FACTOR-2

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Extracellular Signal-Regulated Kinases 1/2 Mediate Retinal Ganglion Cell Axon Regeneration Induced by Fibroblast-Growth Factor-2

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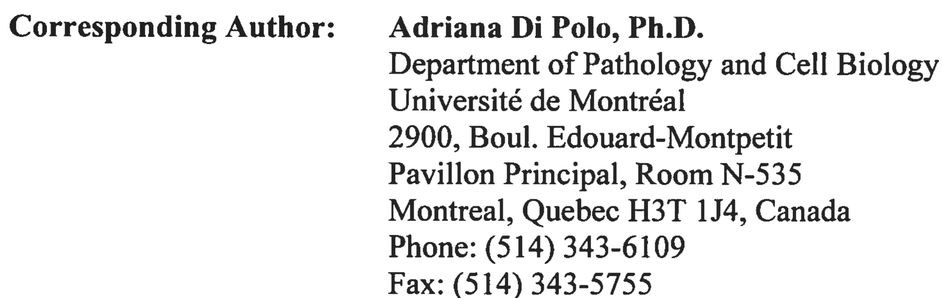

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3.1 ABSTRACT

The intracellular signaling mechanisms used by neurotrophic factors to promote axon growth in the mature, injured CNS are not well understood. We recently demonstrated that basic fibroblast growth factor (or FGF-2) stimulates adult retinal ganglion cell (RGC) axon regeneration within the injured optic nerve. Here we investigated the signaling cascades that control FGF-2-mediated RGC axon extension *in vivo*. For this purpose, recombinant adeno-associated virus (AAV) was used to deliver the FGF-2 gene to RGCs providing a sustained source of this neurotrophic factor. FGF-2 gene transfer led to a ~10-fold increase in the number of axons that extended past the lesion site compared to control nerves. Axon growth correlated with FGF-2-induced activation of the extracellular signal-regulated kinases 1/2 (Erk1/2), but not other signaling pathways including phosphoinositide 3-kinase, phospholipase Cγ or protein kinase C. Pharmacological inhibition of Erk1/2 activation resulted in ~80% decrease in the number of axons that regenerated at 0.5 and 1.0 mm from the site of injury. Our data demonstrate that the Erk1/2 pathway is a key signaling component in FGF-2-mediated axon regeneration in the mature, injured visual system.

3.2 INTRODUCTION

The fibroblast growth factor (FGF) family of neurotrophic factors play critical roles in the development of the central nervous system (CNS) (Barnstable, 1991; Eckenstein, 1994; Hicks, 1998; Abe and Saito, 2001). FGF-2 is the best characterized member of this large family, with well-documented roles in neurogenesis, survival and synaptic plasticity (Reuss and von Bohlen und Halbach, 2003). In the visual system, FGF-2 has been identified as a potent stimulator of axon growth for developing retinal ganglion cells (RGCs) (Brittis et al., 1996; McFarlane et al., 1996; Dingwell et al., 2000). Importantly, we recently demonstrated that FGF-2 gene transfer promotes regrowth of adult rat RGC axons in the injured optic nerve *in vivo* (Sapieha et al., 2003). The regenerative response to FGF-2 upregulation was supported by our finding that FGF receptor-1 (FGFR-1) and heparan sulfate, known to be essential for FGF-2 signaling, are expressed by adult RGCs (Sapieha et al., 2003).

Understanding the signal transduction events responsible for switching a CNS neuron to an axonal growth mode is a fundamental question in neurobiology. Much of our knowledge of the pathways that mediate FGF signaling is derived from biochemical studies using FGFR-1 as the prototypical FGF receptor (FGFR). The signaling pathways elicited by different FGFRs, however, are quite similar owing to the high degree of homology between the various receptor types (Johnson and Williams, 1993; Raffioni et al., 1999). Ligand binding leads to FGFR dimerization, tyrosine autophosphorylation (Ullrich and Schlessinger, 1990) and subsequent activation of FGFR substrate 2 (FRS2) (Klint et al., 1995), which recruits growth factor receptor-bound protein 2 (Grb2), son of

sevenless nucleotide exchange factor (SOS), and Src homology 2 phosphatase 2 (SHP2) (Hadari et al., 1998). These initial events promote the stimulation of the extracellular signal-regulated kinases 1/2 (Erk1/2) pathway (Schlessinger, 2004). In addition, FGFs stimulate phosphoinositide 3-kinase (PI3K) and the protein Akt by an indirect mechanism that involves phosphorylation of the docking protein Gab1 (Lamothe et al., 2004). FGFR-1 has also been shown to activate phospholipase Cγ (PLCγ) (Mohammadi et al., 1991; Peters et al., 1992), which catalyzes the conversion of phosphatidylinositol 4,5 biphosphate (PIP_2) to inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases Ca^{+2} from internal stores acting in concert with DAG to translocate protein kinase C (PKC) to the cell membrane and stimulate its activity (Schlessinger, 2004).

Here we used recombinant adeno-associated virus (AAV) serotype 2 carrying a human FGF-2 gene to investigate the signaling pathways that promote RGC axon growth. The injury model used in our study was micro-crush lesion of the optic nerve, which has clear advantages over other procedures such as conventional optic nerve crush or peripheral nerve grafts. Micro-crush lesion results in a well-defined injury site with a focal glial scar and absence of optic nerve cavitation, facilitating accurate identification of the injury site and quantification of axon growth (Sapieha et al., 2003). We demonstrate that *in vivo* FGF-2 upregulation leads to strong activation of retinal Erk1/2, but not PI3K, PLCγ or PKC. Furthermore, pharmacological inhibition of MEK, the obligate upstream activator of Erk1/2, markedly inhibited FGF-2 mediated RGC axon growth. This study identifies Erk1/2 as a key intermediary in the signaling pathway responsible for FGF-2-induced axon regeneration in the adult CNS.

3.3 EXPERIMENTAL PROCEDURES

3.3.1 Preparation of Recombinant AAV Serotype 2 Vectors

The human FGF-2 cDNA (provided by K. Rendahl, Chiron Corporation, Emeryville, CA) was inserted downstream of the hybrid cytomegalovirus (CMV) enhancer/ chicken ß-actin promoter in the plasmid pXX-UF12, a derivative of pTR-UF5 (Zolotukhin et al., 1996), containing the AAV terminal repeat sequences and a simian virus 40 polyadenylation sequence. The helper plasmid pDG (Grimm et al., 1998) that contains both the AAV genes (*rep* and *cap*) and helper genes required for AAV propagation was used to generate recombinant AAV. Vectors were packaged, concentrated and titered as previously described (Hauswirth et al., 2000). The number of infectious particles/ml (ip/ml) was determined by infectious center assay as described (McLaughlin et al., 1988) and was: 1.4×10^{12} ip/ml for AAV.FGF-2 and 2.9×10^{11} ip/ml for the control AAV containing green fluorescent protein, AAV.GFP. No helper adenovirus or wild-type AAV contamination was detected in these preparations.

3.3.2 Intravitreal injection of viral vectors

All surgical procedures were performed in adult female Sprague-Dawley rats (180-200 g) under general anesthesia (2% Isoflurane/oxygen mixture, 0.8 liters/min) and in accordance with the guidelines of the Canadian Council on Animal Care (www.ccac.ca). Injections into the vitreous chamber were performed on the left eye using a 10-µl Hamilton syringe adapted with a glass-pulled needle (~40 µm external diameter). The contralateral, unoperated eyes served as controls. The sclera was exposed and the tip of the needle inserted at a 45° angle through the sclera and retina into the

vitreous space. Following slow withdrawal of the needle, the injection site was sealed with surgical glue (Indermill, Tyco Health Care, Mansfield, MA). This route of administration avoided injury to structures of the eye, such as the iris or lens, shown to promote RGC survival and regeneration (Mansour-Robaey et al., 1994; Leon et al., 2000). AAV-mediated transgene expression reaches a plateau between 3-4 weeks after intravitreal administration of the vector (Bennett et al., 1997; Ali et al., 1998; Bennet et al., 2000; Cheng et al., 2002), thus subsequent surgical procedures were performed 3-4 weeks after AAV administration.

3.3.3 Retrograde RGC labeling and retinal immunohistochemistry

For co-localization studies of AAV-mediated FGF-2 in the inner retina, RGCs were retrogradely labeled 3 weeks after intravitreal injection of viral vectors. For this purpose, the fluorescent tracer FluoroGold (2%, Fluorochrome, Englewood, CO) was applied to both superior colliculi. One week later, animals were perfused intracardially with 4% paraformaldehyde (PFA) and the eyes and optic nerves were immediately enucleated and the anterior structures and the lens removed. Eye cups and nerves were incubated in 4% PFA for 2 hr at 4°C, followed by immersion in a 20% sucrose solution for 6 hrs at 4°C. Tissue was embedded in optimal cutting temperature (O.C.T.) compound (Tissue-Tek, Miles Laboratories, Elkhart, IN, USA) and frozen in a 2-methylbutane/liquid nitrogen bath. Radial retinal cryosections (16 µm) were collected onto gelatin-coated slides. Non-specific binding was blocked by incubating sections for 1 hr in 3% bovine serum albumin (BSA, Sigma, Oakville, Ontario), 5% normal goat serum (NGS) and 0.2% Triton X-100 (Sigma). Human FGF-2 monoclonal antibody (10

μg/ml, Upstate Biotechnology, Lake Placid, NY, USA) was added in 2% NGS, 0.2% Triton X-100 and incubated overnight at 4°C. Sections were then incubated with Alexa 594-conjugated goat anti-mouse (14 μg/ml, Molecular Probes, Eugene, OR), washed in PBS and mounted using an anti-fade reagent (SlowFade, Molecular Probes). Fluorescent staining was examined using a Zeiss Axioskop 2 Plus microscope (Carl Zeiss Canada, Kirkland, QC), pictures captured with a CCD video camera (Retiga, Qimaging, Burnaby, British Columbia) and analysed with Northern Eclipse software (Empix Imaging, Mississauga, ON).

3.3.4 Micro-crush lesion of the optic nerve and anterograde axon labeling

Axonal regrowth was investigated following optic nerve microcrush lesion. In this paradigm, all RGC axons are completely transected while leaving the proximal and distal nerve segments attached (Selles-Navarro et al., 2001; Sapieha et al., 2003). Briefly, the left optic nerve was exposed and a 10-0 suture used to tie a knot to completely constrict the nerve for 10 sec, after which the suture was carefully released. During this procedure, care was taken to avoid injury of the ophthalmic artery. The vasculature of the retina was always monitored by fundus examination and animals showing signs of compromised blood supply were eliminated from the study. Thirteen days after microcrush lesion, 5 μl of 1% cholera toxin β subunit (CTβ, List Biological Laboratories, Campbell, CA) was administered by intravitreal injection. Eighteen hrs after CTβ injection, two weeks post-microcrush lesion, animals were sacrificed and the eyes were processed for optic nerve immunostaining and axon quantification as described below. Animals received a single injection of AAV.FGF-2 (n=8), AAV.GFP

(n=3) or control PBS (n=4); or were left uninjected (n=4). At the time of lesion, a group of rats treated with AAV.FGF-2 received a single injection of the MEK inhibitor PD98059 (200 μM, Cell Signaling, Beverly, MA; n=3) or vehicle control (n=5).

3.3.5 Optic nerve immunohistochemistry and quantification of axon growth

Longitudinal optic nerve cryosections (14 μm) were taken along the nasal-temporal plane and collected onto gelatin-coated slides. Non-specific binding was blocked with 3% BSA, 5% normal rabbit serum (Vector Laboratories, Burlingame CA) and 0.2% Triton X-100. Sections were then incubated overnight with goat CTβ antibody (1:4,000 dilution, List Biological Labs), followed by incubation with biotinylated rabbit anti-goat IgG (6 μg/ml, Vector Laboratories) and lastly, with DTAF-conjugated streptavidin (3.6 μg/ml, Jackson Immunoresearch Labs, West Grove, PA). For co-labeling experiments, sections were simultaneously incubated overnight with a monoclonal anti-βIII tubulin antibody (21 μg/ml, Sigma) and anti-CTβ antibody followed by incubation with secondary antibodies.

The extent of axonal growth was evaluated by counting the number of CTß-positive axons that extended beyond the site of injury. Axons that crossed a virtual line parallel to the lesion site at 500 μm and 1 mm were counted in four sections of optic nerve per animal. Sections were selected across the entire width of the nerve to analyze axon growth in both central and peripheral regions. The quantification of axons was carried out using two complementary methods: i) axons were counted directly on each section using a 100X objective (Zeiss), and ii) composite images of each nerve were generated at 60X magnification to verify axon counts with respect to the lesion site and

for documentation. During quantification, the injury site was identified in the same optic nerve section by dark field microscopy. In addition, the location of the lesion site was routinely confirmed in the same section or in an immediately adjacent section using hematoxylin/eosin staining. The thickness of the optic nerve was measured at each point where axons were counted and this value was used to calculate the number of axons per mm of nerve width. Data analysis and statistics were performed using GraphPad Instat software (GraphPad Software Inc., San Diego, CA) by a one-way analysis of variance (ANOVA) test.

3.3.6 Western Blot Analysis

Freshly disected retinas were homogenized with an electric pestle (Kontes, Vineland, NJ) in ice-cold lysis buffer (20 mM Tris, pH 8.0, 135 mM NaCl, 1% NP-40, 0.1% SDS and 10% glycerol supplemented with protease inhibitors). Following a 15-min incubation on ice, samples were centrifuged at 13,000 rpm for 5 min at 4°C and the supernatants containing solubilized protein extracts were collected. Retinal samples (50-150 μg) were resolved on 10% SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad Life Science, Mississauga, ON). Non-specific binding was eliminated by incubating blots for 1 hr at room temperature in a solution of 3% bovine serum albumin in TBST (10 mM Tris, 150 mM NaCl, 0.2% Tween-20). Blots were then incubated overnight at 4°C with either of the following primary antibodies: polyclonal phospho-Erk1/2 that specifically recognizes Erk1/2 phosphorylated on Thr202/185 and Tyr204/187 residues (0.2 μg/ml, BioSource, International, Camarillo, CA), polyclonal pan Erk1/2 (2.3 μg/ml, BioSource

International), polyclonal phospho-Akt that recognizes Akt phosphorylated on Thr308 (0.6 μg/ml, Cell Signaling), polyclonal Akt (0.1 μg/ml, Cell Signaling), polyclonal phospho-PKC-α that recognizes PKCα phosphorylated on Ser657 (1.3 μg/ml, Upstate Biotech, NY) polyclonal phospho-PLC-γ1 that recognizes PLC-γ1 phosphorylated on Tyr783 (1:750, Cell Signaling), or monoclonal β-actin (0.5 μg/ml Sigma). Membranes were subsequently washed in TBST and incubated with anti-mouse or anti-rabbit peroxidase-linked secondary antibody (0.5 μg/ml, Amersham Pharmacia, Baie d'Urfé, QC) in 5% non-fat milk in TBST for 1 hr at room temperature. Blots were treated with a chemiluminescent reagent (ECL, Amersham Pharmacia) and exposed to X-OMAT (Kodak) imaging film. Densitometric analysis was performed using BioDoc Analyze 1.0 software (Biometra, Göttingen, Germany) on scanned autoradiographic films obtained from a series of 3 independent western blots each carried out using retinal samples from distinct experimental groups: i) AAV.FGF-2 (n=5), ii) AAV.GFP (n=5) and iii) intact, non-injected (n=5). The densitometric values obtained for the phosphorylated (active) proteins were normalized with respect to their loading controls (non-phosphorylated proteins) in the same blot to obtain the final phosphorylated/total protein ratios. Data analysis and statistics were performed using GraphPad Instat software (GraphPad Software Inc.) by a Student's t-test.

3.4 RESULTS

3.4.1 AAV serotype 2 directs FGF-2 transgene expression in adult RGCs

We prepared recombinant AAV serotype 2 containing a human FGF-2 cDNA under the control of the hybrid CMV immediate early enhancer/chicken beta actin

(CβA) promoter (AAV.FGF-2). This enhancer/promoter combination has been shown to direct more efficient transgene expression than with CMV alone (Acland et al., 2001). AAV.FGF-2 virus was injected into the vitreous chamber and retinas were examined at four weeks following vector administration, the time required for transgene expression to reach a plateau in the rodent retina (Bennett et al., 2000; Cheng et al., 2002). Adult RGCs do not express endogenous FGF-2 (Kostyk et al., 1994), thus AAV-mediated expression of this neurotrophic factor in the inner retina was examined by immunohistochemistry using an antibody against FGF-2. Four weeks after a single intravitreal injection of AAV.FGF-2, we observed positive FGF-2 immunolabeling in most cells of the ganglion cell layer (GCL) (Fig. 1A), whereas control eyes that received an injection of AAV.GFP lacked FGF-2 positive immunoreactivity (Fig. 1B).

We, and others, have shown that >70% of RGCs can be effectively infected when recombinant AAVs are administered into the vitreous chamber (Cheng et al., 2002; Harvey et al., 2002; Martin et al., 2002; Fischer et al., 2004). To confirm that AAV.FGF-2 mediated FGF-2 expression in RGCs, we performed co-localization studies in retinas from eyes that received a single intravitreal injection of the viral vector followed by retrograde labeling of RGCs using FluoroGold applied to the superior colliculus. Double-labeling experiments demonstrated that the vast majority of RGCs, visualized with FluoroGold (Fig. 1C), produced virally-mediated FGF-2 protein (Figs. 1E). This finding is in agreement with our previous data showing that RGCs are the primary cellular target for AAV infection in the inner retina (Cheng et al., 2002; Sapieha et al., 2003; Pernet et al., 2005). No signs of inflammation, cytotoxicity, abnormal

growth or immune reaction were detected in any of the eyes following administration of AAVs.

3.4.2 FGF-2 gene transfer promotes regrowth of RGC axons after acute optic nerve injury

The effect of AAV.FGF-2 on RGC axon growth *in vivo* was tested using the experimental protocol outlined in Figure 2. We, and others, have observed that AAV-mediated transgene expression reaches a plateau between 3 and 4 weeks after administration of the vector into the rodent eye (Bennett et al., 1997; Ali et al., 1998; Bennet et al., 2000; Cheng et al., 2002) and persists thereafter (Guy et al., 1999). This delay in the onset of gene product expression *in vivo* may arise from the need to convert single-stranded viral DNA to a double-stranded form prior to active transcription (Ferrari et al., 1996). Because of the slow onset in transgene expression directed by AAV, microcrush lesion of the optic nerve was performed 4 weeks after virus injection (Fig. 2). The anterograde tracer cholera toxin beta-subunit (CTß), which has been shown to be highly sensitive for revealing fine axonal morphology in the visual system (Mikkelsen, 1992; Angelucci et al., 1996; Matteau et al., 2003), was used to examine regenerating axons at 2 weeks post-injury.

Following a single injection of AAV.FGF-2, growing axons, visualized with CTβ, were observed up to several millimeters past the glial scar (Fig. 3A, B). Figure 3A shows a representative example of axonal growth up to ~2.5 mm from the site of injury. In contrast, eyes treated with a control virus containing the reporter gene GFP showed absence of RGC axons that successfully crossed over the glial scar (Fig. 3C, D). AAV

predominantly targets adult RGCs (Cheng et al., 2002; Martin et al., 2003; Fischer et al., 2004), thus no other neurons or glial cells are likely to contribute significantly to this regenerative response. Growing RGC axons were immunopositive for βIII tubulin (Fig. 3E), an isoform that is selectively expressed by RGCs (Cui et al., 2003; Yin et al., 2003) and upregulated during regeneration (Fournier and McKerracher, 1997). Co-localization of βIII tubulin with the anterograde tracer CTβ (Fig. 3F) confirmed that these axons were from regenerating RGCs (Fig. 3G). Moreover, growth cone-like structures were present at the tips of the axons further suggesting that these neurons were in an active growth state (Fig 3E-G, arrows).

3.4.3 AAV.FGF-2 stimulates retinal Erk1/2 activation

To elucidate the pathway(s) involved in AAV.FGF-2-mediated axonal outgrowth, we investigated the activation of known FGF-2 signaling effectors. For this purpose, we performed Western blot analyses of whole-retina homogenates using antibodies that specifically recognize the phosphorylated (active) forms of Erk1/2, Akt, PLCγ1 or PKCα. Retinal samples were obtained from three independent experimental conditions: i) eyes that received a single, intravitreal AAV.FGF-2 injection (n=5); ii) eyes that received a single, control AAV.GFP injection (n=5); or iii) intact, unoperated control eyes (n=5). Retinal tissue was rapidly dissected out; all samples were processed identically and analyzed in triplicate. Because mostly RGCs are infected by AAV (Cheng et al., 2002; Martin et al., 2003; Fischer et al., 2004), changes in protein phosphorylation reflect changes that occur in these neurons.

Treatment with AAV.FGF-2 induced a marked increase in phosphorylated Erk1/2, detectable at 4 weeks after vector administration, compared with control retinas that displayed low, basal levels of phospho-Erk1/2 (Fig. 4A). Densitometric analysis confirmed that a single AAV.FGF-2 injection led to a ~2-fold increase in phospho-Erk1 (Student's t-test, $p < 0.05$) and a 2.3-fold increase in phospho-Erk2 (Student's t-test, $p < 0.05$) with respect to control retinas (Fig. 4B). In contrast, we did not detect AAV.FGF-2-induced phosphorylation of Akt, PKCα or PLCγ1 (Figs. 4C-G). Quantitative analysis of densitometric signals confirmed that there were no significant differences in the levels of phospho-Akt (Fig. 4D, Student's t-test, $p = 0.3$) or phospho-PKCα (Fig. 4E, Student's t-test, $p = 0.3$) between AAV.FGF-2-treated and control retinas. We were not able to detect phospho-PLCγ1 in any of the retinal samples (Fig. 4G). A protein sample from human carcinoma (A431) cells stimulated with epidermal growth factor (EGF) was used as positive control for PLCγ1 activation (Fig. 4G). Taken together, these data indicate that AAV-mediated FGF-2 leads to selective activation of Erk1/2 in adult RGCs *in vivo*.

3.4.4 Erk1/2 mediate RGC axon growth induced by FGF-2

To determine whether Erk1/2 signaling was involved in FGF-2-mediated growth of transected RGC axons, we used the pharmacological inhibitor PD98059 that has been shown to selectively inhibit MEK1 (Dudley et al., 1995), the obligate upstream activator of Erk1/2. Eyes were injected with AAV.FGF-2 and, 4 weeks later, received a single intraocular injection of PD98059 or vehicle control at the time of micro-crush lesion of the optic nerve. First, the extent of Erk1/2 inhibition was investigated by Western blot analysis of whole retinal homogenates collected at 48 hrs or 14 days following PD98059

treatment. We previously established that a single intravitreal injection of 200 μM PD98059 (final intravitreal concentration: ~17 μM) effectively inhibited retinal Erk1/2 activation *in vivo* (Cheng et al., 2002), therefore this concentration was also used here. Our results show that a single intravitreal injection of PD98059 markedly blocked FGF-2-induced Erk1/2 phosphorylation at 48 hrs after administration of the inhibitor (Fig. 5A). As expected, the levels of phospho-Erk1/2 increased again at 14 days after administration of PD98059. The transient inhibitory effect of PD98059 is consistent with the short half-life of this drug that results in only temporary inhibition of Erk1/2 activation promoted by sustained FGF-2 upregulation. To verify the specificity of PD98059, we examined the phosphorylation levels of Akt following administration of this inhibitor. We found that the levels of phospho-Akt (Fig. 5B) were not altered in the presence of PD98059. These results confirmed the specificity of the inhibitory effect of this compound *in vivo*. Examination of histological sections and whole-mount preparations of the treated retinas did not show any inherent cytotoxic effect of PD98059 (not shown).

To investigate if Erk1/2 activation was responsible for FGF-2 induced RGC axon growth, we performed *in vivo* axon growth assays in the presence of PD98059 injected at the time of optic nerve lesion (Fig. 6). Administration of PD98059 (200 μM) resulted in striking inhibition of the regenerative effect produced by FGF-2 gene transfer. Quantitative analysis revealed that a single injection of PD98059 caused a ~80% reduction in the number of axons growing at 500 μm (ANOVA, $p < 0.001$) and 1 mm (ANOVA, $p < 0.0001$) from the lesion site. These data indicate that Erk1/2 are key signaling components that regulate AAV.FGF-2-mediated RGC axon growth.

3.5 DISCUSSION

Much research has focused on the glial inhibitory proteins that block regeneration in the CNS (He and Koprivica, 2004; McKerracher and David, 2004; Schwab, 2004), however, considerably less attention has been paid to identifying intracellular signaling pathways that promote axon regeneration. Although some data is beginning to emerge from *in vitro* studies (Goldberg, 2003), virtually nothing is known about the intracellular events that direct axonal growth *in vivo*. A study using early post-natal RGC cultures convincingly demonstrated that these neurons do not extend axons by default unless signaled to do so by growth factors (Goldberg et al., 2002b). This finding necessarily implicates components of the neurotrophic factor-signaling cascade in the ability of RGCs to regenerate axons. We recently demonstrated that gene transfer of FGF-2, a potent stimulator of axon outgrowth for developing RGCs (McFarlane et al., 1995; Brittis et al., 1996; McFarlane et al., 1996; Dingwell et al., 2000), promotes robust growth of adult RGC axons within the lesioned optic nerve (Sapieha et al., 2003). In the present study, we identified the Erk1/2 signaling cascade as an essential molecular component of FGF-2-mediated RGC axon growth *in vivo*.

Genetic studies in mice, frogs, flies and nematodes, along with studies *in vitro* have demonstrated that receptor-mediated induction of the Erk1/2 pathway is a central, evolutionarily conserved mechanism used by FGFs to elicit a broad spectrum of biological activities including cell growth, differentiation, and morphogenesis (Tang et al., 1995; Umbhauer et al., 1995; Sundaram et al., 1996; Saxton et al., 1997; Szebenyi et al., 2001). Of interest, *in vitro* studies have shown that Erk1/2 activation mediates neurite outgrowth in PC12 cells (Pang et al., 1995), retinal neurons (Perron and Bixby,

1999; Bonnet et al., 2004), sympathetic neurons (Atwal et al., 2000) and dorsal root ganglion neurons (Sjogreen et al., 2000; Wiklund et al., 2002). Our data is consistent with these reports and provides the first *in vivo* evidence for the involvement of Erk1/2 in neurotrophic factor-induced regeneration of adult, injured RGCs. We show that even transient inhibition of Erk1/2 activation using PD980590 was sufficient to markedly block regenerative growth induced by FGF-2. This suggests that Erk1/2 activation is essential soon after injury to successfully stimulate axon regeneration. It is well documented that injured CNS neurons undergo short-distance abortive sprouting shortly after injury (Ramon y Cajal, 1928; Schwab, 2002). It is likely that RGCs in this early growth-mode are stimulated to regenerate axons by increased levels of active Erk1/2.

Our finding that administration of PD98059 led to substantial (80%) but incomplete blockade of axon growth may be a consequence of its transient inhibitory effect on Erk1/2 activation. In addition, other, yet undefined, signaling molecules may participate in the RGC response to FGF-2. In support of the model that Erk1/2 does not act alone to promote RGC regeneration, recent data from our laboratory demonstrated that gene transfer of a constitutively active form of the obligate Erk1/2 activator, MEK1, by itself was not sufficient to promote axon outgrowth (Pernet et al., 2005). It is likely that FGF-2, signaling through its receptor, activates other intracellular cues that switch RGCs to an axon growth mode. Perhaps the clearest example of a rapid change in the ability of RGCs to extend axons is the dramatic switch that occurs at birth. RGCs cultured one day before birth can extend their axons up to 10-times faster than RGCs cultured one day after birth (Goldberg et al., 2002). The molecular mechanisms leading to this change of growth ability remain largely unknown. Of interest, it has been recently

demonstrated that exposure to neurotrophins primes cerebellar neurons for growth on inhibitory myelin substrates by elevating internal cAMP (Cai et al., 1999). Moreover, endogenous cAMP levels drop sharply after birth, which coincides temporally with loss of regenerative growth (Cai et al., 2001). These experiments demonstrate that neurotrophic factors can stimulation the intrinsic growth state of CNS neurons. Thus, we propose a model in which FGF-2 activates an intrinsic growth program in adult RGCs in which Erk1/2 act as key regulators of axon regeneration by modulation of downstream effectors.

The downstream targets of Erk1/2 involved in axon outgrowth are currently unknown. Molecules that stimulate FGFR-dependent axon growth such as laminin and N-cadherin (Doherty and Walsh, 1996; Lom et al., 1998) have been shown to cause the redistribution of active Erk1/2 to the plasma membrane in embryonic retinal neurons (Perron and Bixby, 1999). This suggests that non-nuclear targets, such as cytoskeletal proteins, are candidate substrates used by Erk1/2 to promote growth. For example, microtubule-associated proteins, which influence the stability of the microtubule network and are involved in axon outgrowth, are known to be phosphorylated by Erk1/2 (Roder H. M. et al., 1993; Garcia Rocha and Avila, 1995). Furthermore, Erk1/2 phosphorylate neurofilament proteins and inhibition of this phosphorylation leads to a reduction of neurite length in hippocampal cultures (Veeranna et al., 1998). It is also likely that successful axon regrowth depends on the ability of Erk1/2 to modulate gene expression by activating transcription factors and consequent protein synthesis (Sonenberg and Gingras, 1998; Grewal et al., 1999). A recent study demonstrated that the cAMP-response element binding (CREB) protein, a downstream target of Erk1/2,

contributes to CNS axon regeneration by overcoming myelin inhibition (Gao et al., 2004). Future studies will elucidate the downstream molecular mechanisms by which Erk1/2 regulates RGC axon regrowth in the mature, injured visual system.

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DISCLOSURE

A.D.P., W.W.H., the Université de Montréal, and the University of Florida could be entitled to patent royalties for inventions related to this work and W.W.H. owns equity in a company that may commercialize some of the technology described in this work.

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3.7 FIGURES

Figure 1. AAV serotype 2 directs FGF-2 transgene expression in adult RGCs.

FGF-2 protein expression was examined in retinal sections at 4 weeks after intraocular injection of AAV.FGF-2 (A) or control AAV.GFP (B). Adult RGCs do not express endogenous FGF-2, thus AAV-mediated FGF-2 was visualized by fluorescent microscopy following immunostaining with an FGF-2 antibody. Expression of AAV-mediated FGF-2 in RGCs was confirmed using the retrograde tracer FluoroGold (C). Superimposition of the images shown in (A) and (C) demonstrated that RGCs expressed AAV-mediated FGF-2 (E). Scale bars: 50 μm (A-E).

FIGURE 1.

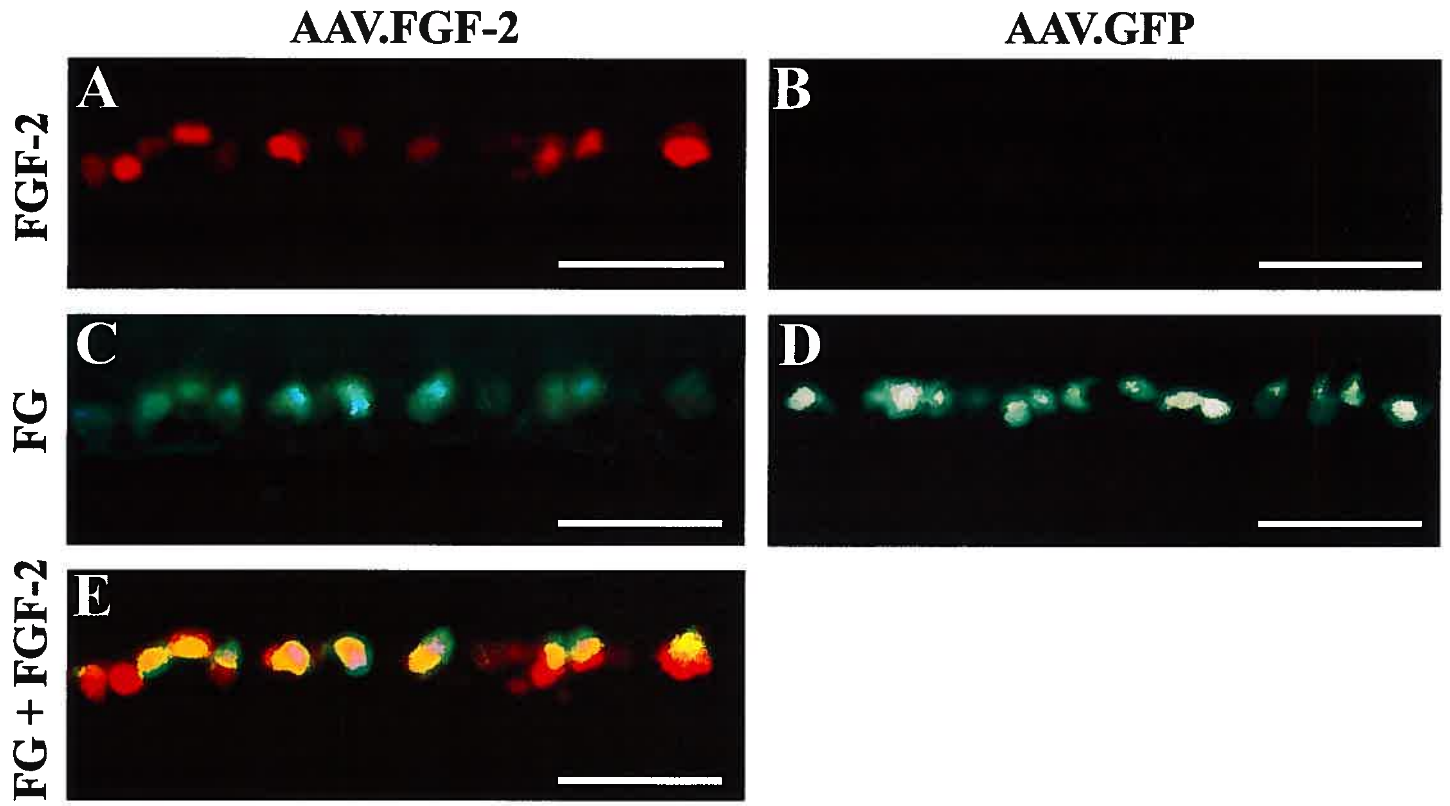


Figure 2. Experimental protocol used to investigate the effect of AAV.FGF-2 on RGC axon growth.

Micro-crush lesion (MC) of the optic nerve was performed 4 weeks after intraocular (IO) injection of AAV.FGF-2, the time required for AAV-mediated transgene expression to reach a plateau in the adult rat retina. Thirteen days after nerve injury, RGC axons were anterogradely labeled by IO injection of CTβ and, 24 hrs later (2 weeks post-microcrush lesion), animals were sacrificed and the optic nerves were processed for analysis of axon growth.

FIGURE 2.

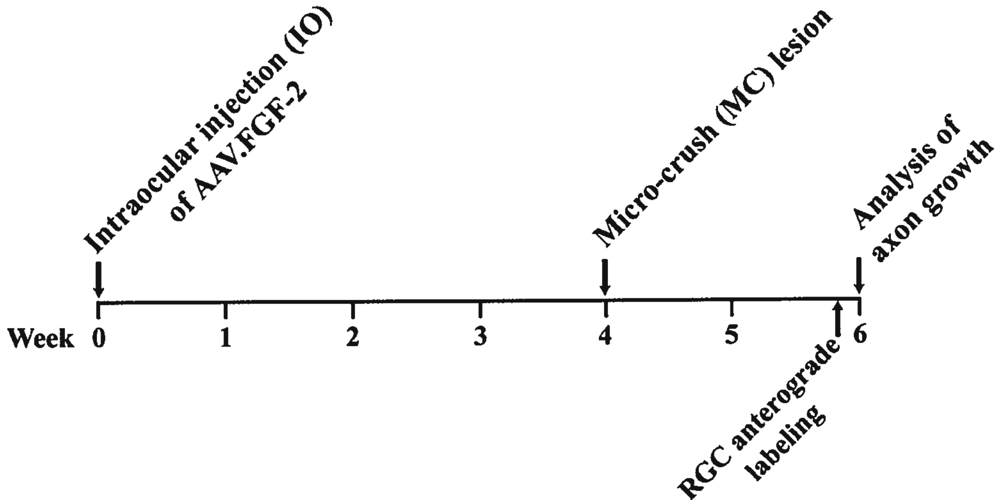


Figure 3. FGF-2 gene transfer promotes regrowth of RGC axons after acute optic nerve injury.

A) Representative optic nerve section immunostained with an antibody against the anterograde tracer cholera toxin ß (CTß) shows RGC axon growth following intravitreal injection of AAV.FGF-2. Two weeks after injury, a large number of CTß-positive axons (arrows) extended into the distal optic nerve past the lesion site (asterisks). (B) The same section shown in (A) was visualized with dark field, which allowed the unequivocal identification of the injury site (asterisks). (C) Intravitreal injection of control AAV.GFP did not provoke axonal regrowth past the site of lesion (D). (E-G) Higher magnification images of an AAV.FGF-2-treated injured optic nerve taken at ~ 1 mm from the site of lesion show RGC axons co-stained with β-III tubulin and CTß. β-III tubulin is an RGC-specific isoform that is upregulated during regenerative axon growth. The white arrows indicate structures at the tip of axons that appear to be growth cones. Scale bars: 250 μm (A-D), 25 μm (E-G).

FIGURE 3.

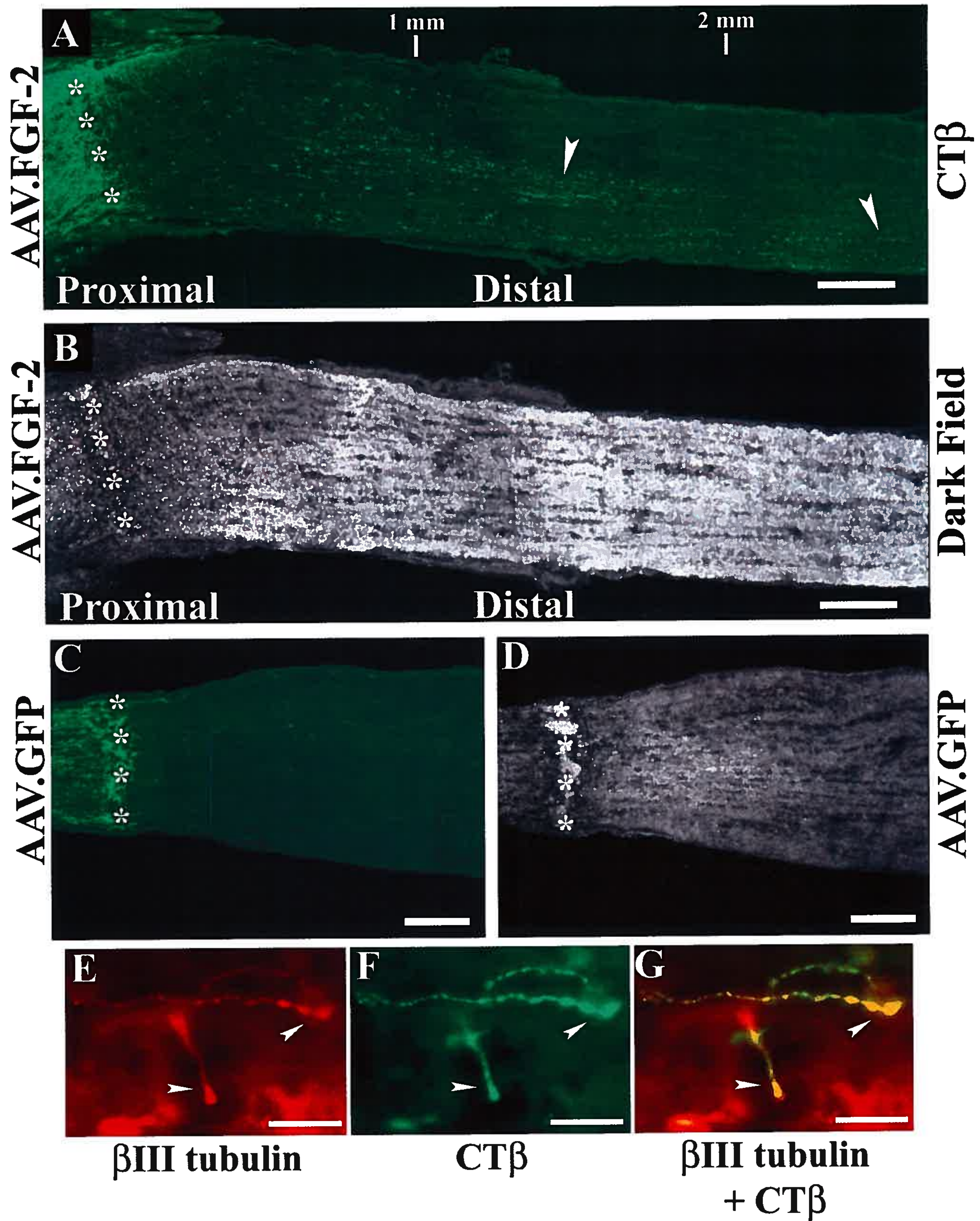


Figure 4. AAV.FGF-2 stimulates retinal Erk1/2 activation

Activation of FGF-2 candidate signaling pathways was investigated using Western blots of total retinal extracts (75-150 μg) probed with antibodies that selectively recognized phosphorylated (active) Erk1/2, Akt, PLCγ1 or PKCα. Protein samples were collected at 4 weeks following intraocular injection of AAV.FGF-2 (n=5) or control AAV.GFP (n=5). In addition, retinas from uninjected, intact eyes served as controls (n=5). (A) AAV.FGF-2 markedly increased phosphorylation of Erk1/2 compared with control retinas that displayed low, basal levels of phospho-Erk1/2. (B) Densitometric analysis confirmed that a single AAV.FGF-2 injection led to a ~2-fold increase in phospho-Erk1 (Student's t-test, $p < 0.05$) and a 2.3-fold increase in phospho-Erk2 (Student's t-test, $p < 0.05$) with respect to control retinas. In contrast, we did not detect changes in the levels of phospho-Akt (C and D, Student's t-test, $p = 0.3$) or phospho- PKCα (E and F, Student's t-test, $p = 0.3$) with respect to control retinas. (G) Phospho-PLCγ1 remained at low basal levels in all samples. Protein extracts from human carcinoma A431 cells stimulated with epidermal growth factor (EGF) was used as positive control for PLCγ1 activation. In all cases, lower panels represent the same blot as in the upper panels but probed with an antibody that recognizes total protein or β-actin used to confirm equal protein loading in each lane.

FIGURE 4.

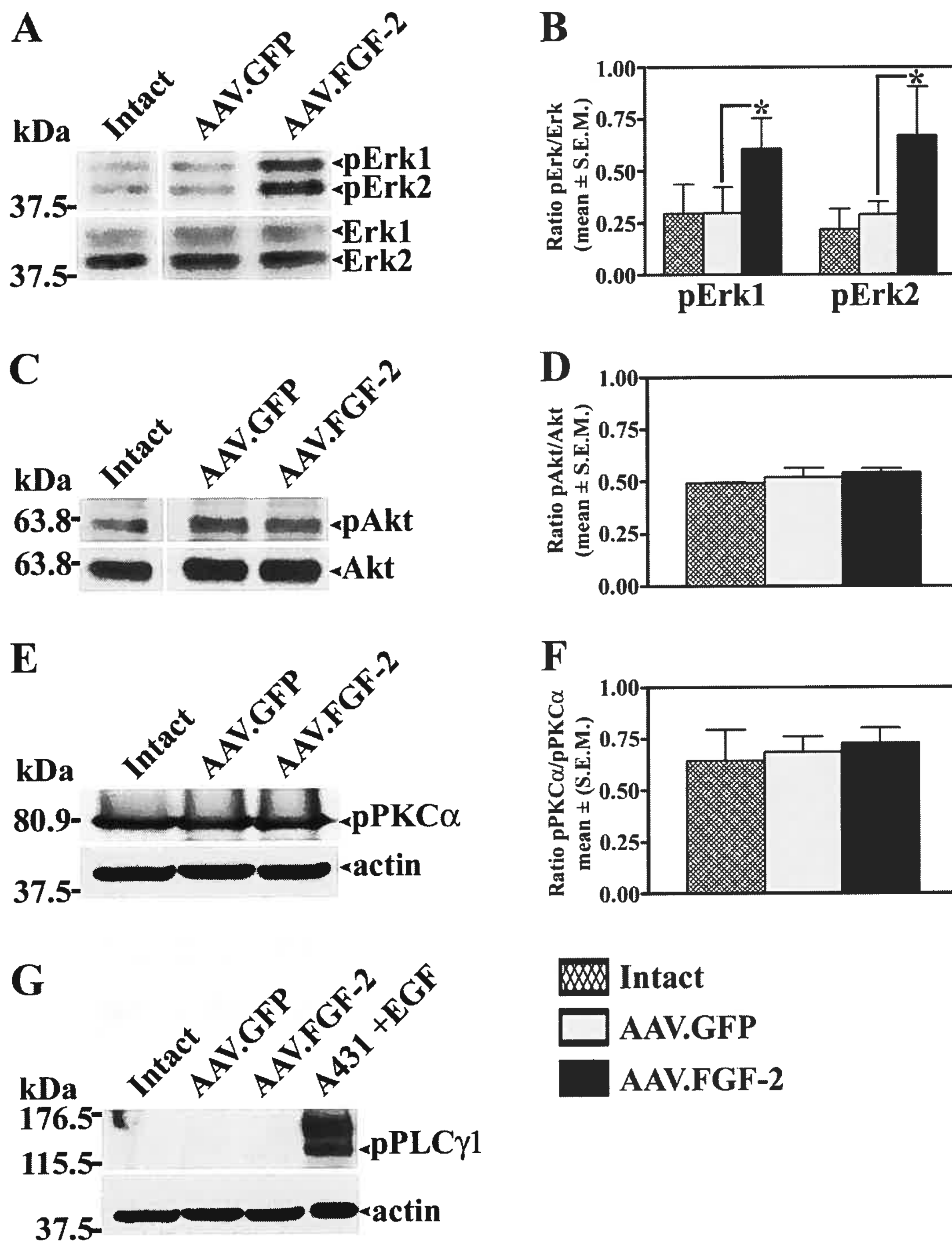
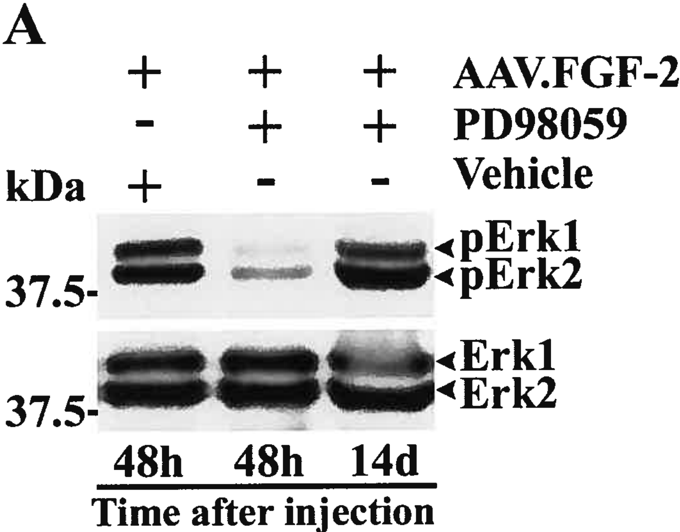


Figure 5. Selective *in vivo* inhibition of AAV.FGF-2-induced activation of retinal Erk1/2

Inhibition of Erk1/2 phosphorylation was examined at 48 hrs and 14 days following intraocular injection of 200 μM of PD98059. The intravitreal concentration of this compound was ~16.7 μM based on the estimated volume of the vitreous chamber in the adult rat eye (~60 μl). (A) Phosphorylated Erk1 and Erk2 were visualized with an antibody against phospho-Erk1/2 (Thr202/Tyr204 residues, top panel) and total Erk protein was visualized in the same blot reprobed with p44/42 MAP kinase antibody (bottom panel). In vivo Erk1/2 activation was effectively inhibited at 48 hrs after PD98059 administration. (B) Intraocular injection of 200 μM of PD98059, which effectively inhibited Erk1/2 activation, did not block Akt phosphorylation. This result confirms the biochemical specificity of the pharmacological inhibitor PD98059

FIGURE 5



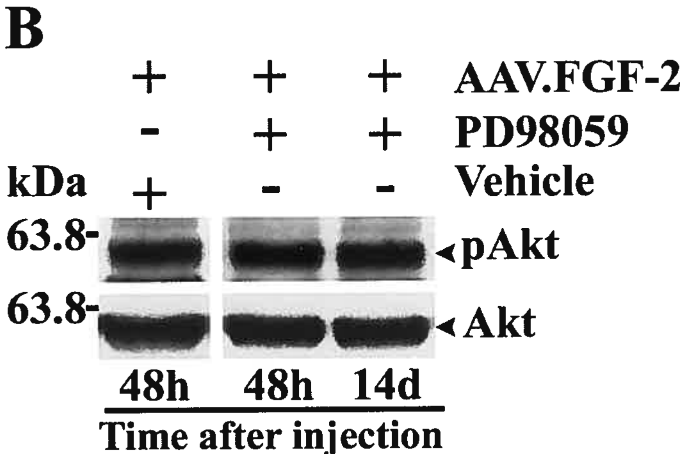
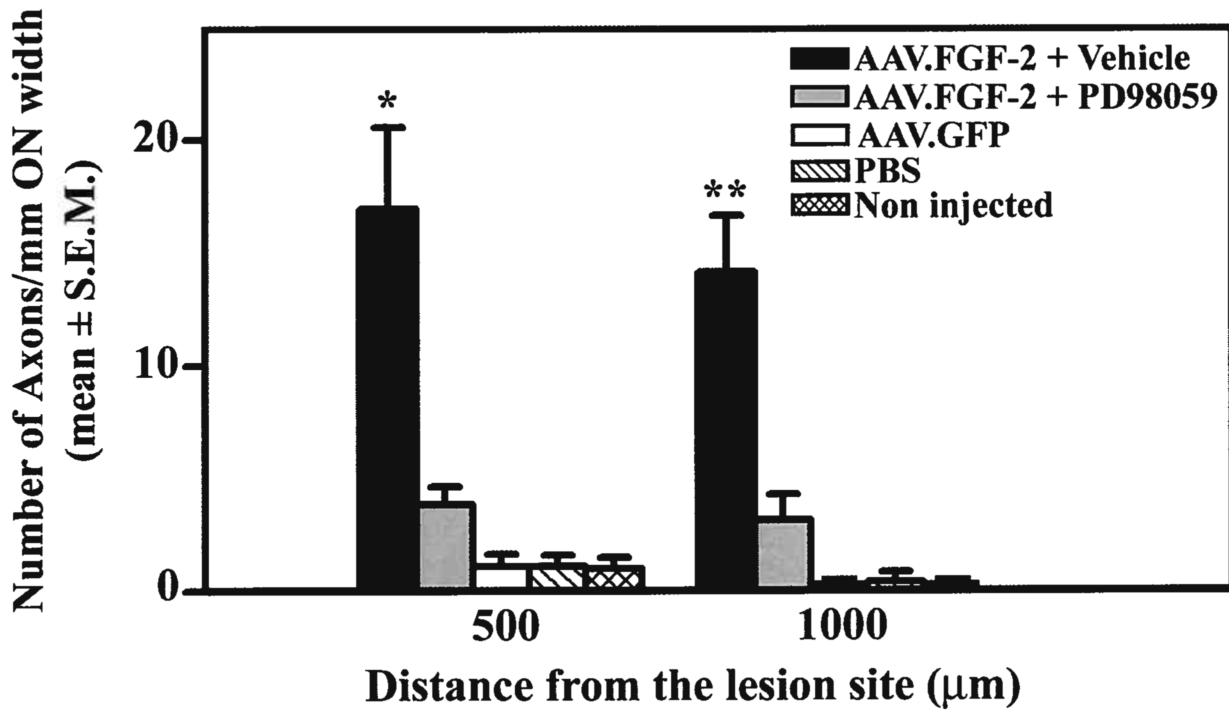


Figure 6. Erk1/2 mediate RGC axon growth induced by FGF-2

The density of RGC axons that regenerated following FGF-2 gene transfer was significantly reduced by specific inhibition of the Erk1/2 pathway with a single intraocular injection of PD98059 (200 μM) administered at the time of nerve lesion. Quantitative analysis of axon growth at 2 weeks post-injury demonstrated that PD98059 caused ~80% reduction in the number of axons extending at 500 μm (ANOVA, *: $p < 0.001$) and 1.0 mm (ANOVA, **: $p < 0.0001$) in the presence of AAV.FGF-2 (gray bars, n=3). Control groups included: i) animals treated with AAV.FGF-2 that received a single injection of vehicle at the time of nerve injury (solid bars, n=4), ii) animals treated with control AAV.GFP (open bars, n=3) or PBS (hatched bars, n=4), and iii) animals without any injection but that received micro-crush lesion of the nerve (grid bars, n=3).

FIGURE 6



Chapter IV

RECEPTOR PROTEIN TYROSINE PHOSPHATASE SIGMA INHIBITS AXON REGROWTH IN THE ADULT INJURED CNS

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RECEPTOR PROTEIN TYROSINE PHOSPHATASE SIGMA INHIBITS AXON REGROWTH IN THE ADULT INJURED CNS

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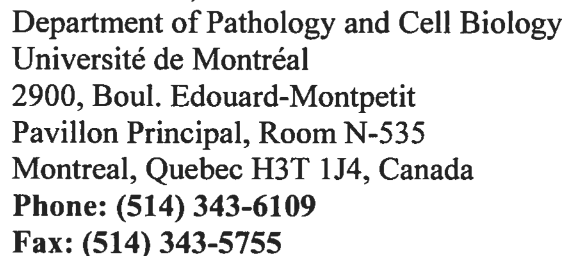

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4.1 ABSTRACT

Recently, receptor protein tyrosine phosphatase-σ (RPTPσ) has been shown to inhibit axon regeneration in injured peripheral nerves. Unlike the peripheral nervous system (PNS), central nervous system (CNS) neurons fail to regenerate their axons after injury or in disease. In order to assess the role of RPTPσ in CNS regeneration, we used the retinocollicular system of adult mice lacking RPTPσ to evaluate retinal ganglion cell (RGC) axon regrowth after optic nerve lesion. Quantitative analysis demonstrated a significant increase in the number of RGC axons that crossed the glial scar and extended distally in optic nerves from RPTPσ (-/-) mice compared to wild-type littermate controls. Although we found that RPTPσ is expressed by adult RGCs in wild-type mice, the retinas and optic nerves of adult RPTPσ (-/-) mice showed no histological defects. Furthermore, the time-course of RGC death after nerve lesion was not different between knockout and wild-type animals. Thus, enhanced axon regrowth in the absence of RPTPσ could not be attributed to developmental defects or increased neuronal survival. Finally, we show constitutively elevated activity of mitogen activated protein kinase (MAPK) and Akt kinase in adult RPTPσ (-/-) mice retinas, suggesting that these signaling pathways may contribute to promoting RGC axon regrowth following traumatic nerve injury. Our results support a model in which RPTPσ inhibits axon regeneration in the adult injured CNS.

4.2 INTRODUCTION

During development and regeneration of the nervous system, the growth cone responds to multiple guidance cues to direct axon extension and establish synaptic connections with appropriate targets. The ability of the growth cone to react to extracellular signals is largely regulated by cell surface receptors linked to a complex transduction machinery (Huber et al., 2003). Although protein tyrosine kinases are key components of these signaling pathways, it is now clear that receptor protein tyrosine phosphatases (RPTPs) are equally essential for growth cone function (Hunter, 1989; Stoker, 2001; Johnson and Van Vactor, 2003).

Type IIa RPTPs, a well-characterized group of the large RPTP family, have been shown to influence axon outgrowth and guidance during neural development (Johnson and Van Vactor, 2003). Members of this subfamily of RPTPs: RPTPσ, RPTPδ and LAR, are composed of two cytoplasmic phosphatase domains and extracellular immunoglobulin domains followed by fibronectin type III (FNIII) repeats, similar to the NCAM family of cell adhesion molecules. Mutations in *Drosophila* type IIa RPTPs DLAR and DPTP69D cause errors in axon guidance of motoneurons (Desai et al., 1996; Krueger et al., 1996), photoreceptors (Garrity et al., 1999; Newsome et al., 2000) and commissural neurons (Sun et al., 2000). HmLAR, a DLAR homolog in leech, is required for appropriate axon guidance and extension (Gershon et al., 1998; Baker and Macagno, 2000). In the developing *Xenopus* visual system, RPTPσ and RPTPδ control the rate of axon extension of retinal ganglion cell (RGC) axons (Johnson et al., 2001) while in the developing chick retina, RPTPσ regulates intraretinal RGC axon growth (Ledig et al., 1999a).

The participation of type IIa RPTPs in axon growth during development suggested that these proteins might also influence axon regeneration following traumatic injury in adult mice. It has been shown that RPTPσ, which is highly expressed in the developing and adult nervous system (Yan et al., 1993; Wang et al., 1995; Schaapveld et al., 1998), inhibits axon regeneration after peripheral nerve injury (McLean et al., 2002; Thompson et al., 2003). For example, enhanced axon regeneration in the sciatic nerve was observed in the absence of RPTPσ (McLean et al., 2002). More recently, Thompson et al. (2003) showed that mice deficient in RPTPσ displayed accelerated regeneration of axotomized facial nerve motoneurons, and that absence of RPTPσ increased the rate of axon outgrowth.

In contrast to their PNS counterparts, CNS neurons have a limited capacity to regenerate following injury or disease. A role for RPTPσ in post-traumatic axon growth in the adult CNS has not been established. Here we used the retinocollicular system of adult transgenic mice lacking RPTPσ to investigate the regenerative capacity of RGCs after microcrush of the optic nerve. This injury paradigm was chosen because it results in a focal glial scar and absence of optic nerve cavitation, facilitating accurate identification of the injury site and quantification of axon growth (Selles-Navarro et al., 2001; Sapieha et al., 2003). Furthermore, because all RGC axons are completely transected after microcrush lesion, there is no risk of confusing spared fibers with regenerating axons.

Little is known about the molecular components that mediate signaling triggered by RPTP family members. Nevertheless, a few candidate intracellular effectors of RPTP

signaling pathways have been identified, such as ableson tyrosine kinase (Abl), enabled (Ena), liprin-α and guanine-nucleotide exchange factor Trio (Johnson and Van Vactor, 2003). Interestingly, the type II RPTP Clr-1 in *C. elegans* has been shown to be an antagonist of the fibroblast growth factor receptor (FGFR) ortholog egl-15 (Kokel et al., 1998). In this study, the mutant Clr-1 phenotype was suppressed by loss of egl-15 and could be mimicked by overexpression of egl-15, suggesting that Clr-1 likely acts as a negative regulator of FGFR signaling pathways. Stimulation of receptor tyrosine kinases, such as FGFR, results in the activation of downstream signaling components, including the mitogen activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and Akt kinase, and phospholipase Cγ (PLCγ) (Huang and Reichardt, 2003; Segal, 2003). Thus, it is likely that RPTPs influence cellular function by regulating the activity of receptor tyrosine kinases (Jallal et al., 1992; Sorby and Ostman, 1996) or downstream signaling components.

Here we report that injured optic nerves from adult RPTPσ (-/-) mice showed increased numbers of RGC axons growing past the lesion site into the distal nerve. Lack of RPTPσ did not alter the time-course of RGC death, thus its effect on axon growth could not be attributed to increased neuronal survival. Moreover, histological analysis of retinas and optic nerves revealed no major structural differences between RPTPσ (-/-) and RPTPσ (+/+) mice. Finally, we show that the MAP kinases Erk1/2 and Akt kinase are activated in adult RPTPσ (-/-) mice retinas, suggesting that stimulation of these signaling pathways may increase axon growth by injured RGCs *in vivo*.

4.3 EXPERIMENTAL METHODS

4.3.1 Animals

RPTPσ knockout mice used in this study were generated and bred on a BALB/c background as previously described (Elchebly et al., 1999). All procedures were carried out using 4- to 6-month old female RPTPσ (-/-) or control wild-type littermates in accordance with the Canadian Council on Animal Care guidelines for the use of experimental animals in research (Olfert et al., 1993).

4.3.2 *In situ* hybridization

Sense and antisense cRNA probes corresponding to 478 bases (nucleotides 3461-3939) of the mouse *Ptprs* gene were generated. Probes were synthesized from cDNA templates with T3 (sense) (Roche, Laval, QC, Canada) or T7 (anti-sense) (Gibco BRL, Burlington, ON, Canada) RNA polymerases and digoxygenin (DIG) RNA labeling mix (Roche, Laval, QC, Canada). Mice were perfused intracardially with ice cold 0.1 M phosphate buffered saline (PBS, pH 7.4) containing 0.1% Diethyl Pyrocarbonate (DEPC) (Sigma, Oakville, ON, Canada). Eyes were immediately enucleated and the anterior structures and the lens were removed. The remaining eye cups were incubated in 4% paraformaldehyde (PFA) containing 0.1% DEPC for 2 hr at 4°C, followed by immersion in a sucrose solution (20% in PB) for 6 hrs at 4°C. Eye cups were embedded in optimal cutting temperature (O.C.T.) compound (Tissue-Tek, Miles Laboratories, Elkhart, IN, USA) and frozen in a 2-methylbutane/liquid nitrogen bath. Retinal cryosections (7 μm) were collected onto slides (Superfrost Plus; Fisher Scientific, Whitby, ON, Canada) and processed as follows. Endogenous peroxidase activity was quenched with 0.3%

hydrogen peroxide (Fisher Scientific) in methanol. Sections were pre-hybridized for 20 min at room temperature in 5X saline sodium citrate (SCC), 50% dimethyl-formamide, 5X Denhardt's solution, 1% SDS and 40 μg/ml single stranded salmon sperm. Hybridization was performed overnight at 56°C with 50 ng/ml of probe in 5X SSC, 50% dimethyl-formamide and 40 μg/ml single stranded salmon sperm. Sections were washed and incubated with a peroxidase conjugated anti-DIG antibody (Roche), followed by Tyramide Signal Amplification (TSA, Perkin Elmer Life Sciences, MA, USA) and 3,3N-Diaminobenzidine Tetrahydrochloride (DAB) Liquid Substrate (Sigma). Retinas were visualized with a Zeiss Axioskop 2 Plus microscope (Carl Zeiss Canada, Kirkland, QC, Canada). Images were captured with a CCD camera (Retiga, Qimaging, ON, Canada) and processed with Northern Eclipse image analysis software (Empix Imaging, Mississauga, ON, Canada)

4.3.3 Retinal and optic nerve histology

Mice were perfused intracardially with 3.6% glutaraldehyde in Sorensen's Buffer and eyes with optic nerves were immediately dissected out. The anterior structures of the eye as well as the lens were removed and the remaining tissue was incubated overnight in the same fixative. Samples were then incubated in 2% osmium tetroxide, dehydrated in graded alcohol baths and embedded in Epon resin (Marivac, Montreal, Canada). Sections of the retina (1-μm thick) were prepared along the dorsal-ventral plane of the eye and serial sections that passed through the optic nerve head, used as reference, were analyzed. The thickness of the outer nuclear layer (ONL), the inner nuclear layer (INL) and inner plexiform layer (IPL) was measured in the superior central retina in wild type

(n=5) and RPTPσ knockout (n=5) mice using Northern Eclipse image analysis software. In addition, RGC axons were counted in 1-μm thick transverse sections in wild-type (n=3) and RPTPσ knockout (n=3) optic nerves. Axons in five areas of each optic nerve section, encompassing a total area of 2,700 μm^2 per nerve, were quantified. The five optic nerve areas analyzed included one in the center of the nerve, two peripheral dorsal and two peripheral ventral regions. The total surface area per optic nerve cross section was measured using the Northern Eclipse image analysis software, and this value was used to estimate the total number of axons in each optic nerve.

4.3.4 Surgical procedures

i) Axonal growth

The microcrush lesion injury paradigm previously used in the rat optic nerve (Sapieha et al., 2003) was adapted for the smaller mouse eye. Briefly, the left optic nerve was exposed and a 10-0 suture was used to tie a knot to completely constrict the nerve for 10 sec after which the suture was carefully released. During this procedure, care was taken to avoid injury to the ophthalmic artery. The vasculature of the retina was always monitored by fundus examination and animals showing signs of compromised blood supply were eliminated from the study. Thirteen days after microcrush lesion, 2 μl of 1% cholera toxin β subunit (CTβ, List Biological Laboratories, Campbell, CA) was injected into the vitreous chamber using a 10-μl Hamilton syringe with a glass-pulled needle (~40 μm external diameter). Anterograde axonal tracing with CTß has been shown to be highly sensitive for revealing fine axonal morphology in the visual system (Mikkelsen, 1992; Angelucci et al., 1996; Matteau et al., 2003).

For injection of the CTß tracer, the sclera was exposed and the tip of the needle inserted at a 45° angle through the sclera and retina into the vitreous space. Following slow withdrawal of the needle, the injection site was sealed with surgical glue (Indermill, Tyco Health Care, Mansfield, MA). This route of administration avoided injury to the iris or the lens which has been shown to promote RGC survival and regeneration (Mansour-Robaey et al., 1994; Leon et al., 2000). Eighteen hrs after CTβ injection, two weeks post-microcrush lesion, animals were sacrificed and the eyes were processed for optic nerve immunostaining and axon quantification as described below.

ii) Neuronal survival

One week prior to optic nerve axotomy, RGCs were labeled with 2% FluoroGold (Fluorochrome, Englewood, CO) in 0.9% NaCl containing 10% dimethyl sulfoxide (DMSO) by application of the tracer to both superior colliculi. Retinas were examined histologically at 7 and 14 days after microcrush lesion to determine the density of surviving RGCs. Groups included: i) RPTPσ knockout mice (n=4) and wild type mice (n=4) sacrificed at 1 week after optic nerve lesion, and ii) RPTPσ knockout mice (n=3) and wild type mice (n=2) sacrificed at 2 weeks after injury.

4.3.5 Quantification of axonal growth and neuronal survival

For quantification of axonal growth, animals were perfused intracardially with 4% PFA in 0.1 M phosphate buffer (PB, pH 7.4) and the eyes with optic nerves were immediately disected and processed as described for *in situ* hybridization. Longitudinal nerve cryosections (14 μm) were taken along the nasal-temporal plane and collected

onto gelatin-coated slides. Non-specific binding was blocked with 3% BSA, 5% normal rabbit serum (Vector Laboratories, Burlingame CA) and 0.2% Triton X-100. Sections were then incubated overnight with goat CTβ antibody (1:4,000 dilution, List Biological Labs), followed by incubation with biotinylated rabbit anti-goat IgG (6 μg/ml, Vector Laboratories) and lastly, with DTAF-conjugated streptavidin (3.6 μg/ml, Jackson Immunoresearch Labs). Fluorescence was visualized using a Zeiss Axioskop 2 Plus microscope (Carl Zeiss). The extent of axonal growth was evaluated by counting the number of CTß-positive axons that extended beyond the site of injury. Axons that crossed a virtual line parallel to the lesion site at 50 μm, 75 μm, 100 μm and 200 μm were counted in four sections of optic nerve per animal. Sections were selected across the entire width of the nerve to analyze axon growth in both central and peripheral regions. The quantification of axons was carried out using two complementary methods: i) axons were counted directly on each section using a 100X objective (Zeiss), and ii) composite images of each nerve were generated at 60X magnification to verify axon counts with respect to the lesion site and for documentation. Both methods yielded identical results. The location of the lesion site was routinely confirmed in the same section or in an immediately adjacent section using an antibody against chondroitin sulfate proteoglycan (CSPG) (1:200, Sigma) followed by Alexa 594-conjugated goat anti-mouse IgG (14 μg/ml, Molecular Probes) or hematoxylin/eosin staining. The thickness of the optic nerve was measured at each point where axons were counted and this value was used to calculate the number of axons per mm of nerve width as described (Sapieha et al., 2003). Data analysis was performed using the GraphPad Instat program (GraphPad Software Inc., San Diego, CA) by an unpaired *t* test.

For quantification of neuronal survival, mice were sacrificed by intracardial perfusion with 4% PFA and both the left (optic nerve lesioned) and right (intact control) retinas were dissected, fixed for an additional 30 min and flat-mounted vitreal side up on a glass slide. The ganglion cell layer was examined by fluorescence microscopy and FluoroGold-labeled neurons were counted in a total of 12 retinal zones corresponding to a total area of 0.5 mm^2. The retinal zones analyzed included three areas in each of the eye quadrants at 0.5 mm, 1.0 mm and 1.5 mm from the optic nerve head. Data analysis and statistics were performed using the GraphPad Instat program by unpaired *t* test.

4.3.6 Western blot analysis and immunocytochemistry

For protein analysis, fresh retinas were rapidly dissected and homogenized with an electric pestle (Kontes, Vineland, NJ) in ice-cold lysis buffer: 20 mM Tris, 135 mM NaCl, 1% NP-40, 0.1% SDS, 10% glycerol containing Complete-Mini Protease Inhibitor Cocktail (Roche). Following a 15-min incubation on ice, samples were centrifuged at 13,000 rpm for 5 min at 4°C and the supernatants containing solubilized protein extracts were collected. Retinal extracts (150 µg) were resolved on 10% SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad Life Science, Mississauga, ON). Non-specific binding was eliminated by incubating blots for 1 hr at room temperature in a solution of 5% non-fat milk in TBST (10 mM Tris, 150 mM NaCl, 0.2% Tween-20). Blots were then incubated overnight at 4°C with either of the following primary antibodies: RPTPσ (17G7.2, 1:200) (Thompson et al., 2003), monoclonal phospho-Erk1/2 that specifically recognizes Erk 1/2 phosphorylated on Thr202/Tyr204 residues (0.8 µg/ml, Cell Signaling, Beverly, MA), polyclonal pan

Erk1/2 (2.3 μg/ml, BioSource International, Camarillo, CA), polyclonal phospho-Akt that recognizes Akt phosphorylated on Thr308 (0.6 μg/ml, Cell Signaling), polyclonal Akt (0.1μg/ml, Cell Signaling), polyclonal phospho-PLCγ1 that recognizes PLCγ1 phosphorylated on Tyr783 (1:750, Cell Signaling), or monoclonal β-actin (0.5 μg/ml Sigma). Membranes were subsequently washed in TBST and incubated with anti-mouse or anti-rabbit peroxidase-linked secondary antibody (0.5 μg/ml, Amersham Pharmacia, Baie d'Urfé, QC) for 1 hr at room temperature. Blots were treated with a chemiluminescent reagent (ECL, Amersham Pharmacia) and exposed to X-OMAT (Kodak) imaging film.

For retinal immunocytochemistry, animals were perfused intracardially with 4% PFA and the eyes dissected and processed as described for *in situ* hybridization. Radial cryosections (16 μm) were collected onto gelatin-coated slides. Non-specific binding was blocked by incubating sections for 1 hr in 3% bovine serum albumin (BSA, Sigma), 5% normal goat serum (NGS) and 0.2% Triton X-100 (Sigma). Primary antibody, phospho-Erk1/2 (2 μg/ml, BioSource) or phospho-Akt (1:20, Cell Signaling) was added in 2% NGS, 0.2% Triton X-100 and incubated overnight at 4°C. Sections were then incubated with Cy3-conjugated goat anti-rabbit (3 μg/ml, Jackson Immunoresearch Laboratories, West Grove, PA), washed in PBS and mounted using an anti-fade reagent (SlowFade, Molecular Probes, Eugene, OR). Fluorescence was visualized with a Zeiss Axioskop 2 Plus microscope (Carl Zeiss).

4.4 RESULTS

4.4.1 RPTPσ mRNA is expressed by adult mouse RGCs

Endogenous expression of RPTPσ mRNA in the adult mouse retina and optic nerve was investigated by *in situ* hybridization using a digoxigenin (DIG)-labeled RNA antisense probe specific for murine RPTPσ. Robust DIG labeling was detected in most cells in the ganglion cell layer (GCL) of RPTPσ (+/+) mice (Fig. 1A) indicating that RGCs, and possibly displaced amacrine cells found in the GCL (Perry, 1981), express this phosphatase. Many cells in the inner nuclear layer (INL), which contains cell bodies of bipolar, horizontal, amacrine neurons and Müller glia, were also DIG-positive. In contrast, we did not detect staining in retinal sections from RPTPσ (-/-) mice (Fig. 1B) or wild-type mice incubated with control sense probe (Fig. 1C). These findings are consistent with other studies showing that RGCs express the RPTPσ homologue, CRYP-α, in the developing chick and *Xenopus* retina (Ledig et al., 1999b; Johnson and Holt, 2000). RPTPσ mRNA was also detected in the optic nerves of adult RPTPσ (+/+) mice (Fig. 1D) but not in RPTPσ (-/-) mice (Fig. 1E) or in sections from RPTPσ (+/+) mice treated with sense probe (1F). DIG labeling was confined to glial cells organized in linear arrays along the antero-posterior axis of the nerve (Fig. 1G), a pattern typical of oligondendrocytes. These results demonstrate that RPTPσ is constitutively expressed in the retina and optic nerve throughout adult life.

4.4.2 Lack of RPTPσ does not alter retinal or optic nerve histology

To determine if lack of RPTPσ disrupted cellular migration or organization of the rodent visual system, we performed histological analysis of retinas and optic nerves from RPTPσ (-/-) mice and wild-type littermates at post-natal day (P) 6 and in adulthood. The outer plexiform layer (OPL) in the mouse retina begins to form at ~P4-P5. Photoreceptor precursors migrate through the OPL and reach their final destination in the outer nuclear layer (ONL), a process that is completed at ~P10 in the central retina (Rohrer et al., 1999). The position of the OPL during these stages of retinal development serves as an indication of precursor cell migration within the retina. At P6, both RPTPσ (+/+) and RPTPσ (-/-) mice retinas showed typical layered structures with similar cellular densities (Figs. 2A and 2B). Absence of RPTPσ did not alter the formation or location of the OPL, visualized as a gap between the photoreceptor nuclei, suggesting that the rate of photoreceptor migration was preserved.

Analysis of adult RPTPσ (-/-) mouse retinas revealed that the layered organization was identical to that found in wild-type control retinas (Figs. 2C and 2D). All retinal cells reached their final destination and photoreceptor inner and outer segments were fully elongated. The cellular density of each layer appeared normal in RPTPσ (-/-) retinas. To confirm this, the thickness of the inner plexiform layer (IPL), inner nuclear layer (INL) and ONL were measured and no significant differences between knockout (n=5) and wild-type (n=5) littermates were found (Fig. 2E). In addition, RGCs were retrogradely labeled by application of the fluorescent tracer FluoroGold to the superior colliculus and quantified on retinal whole-mounts (Fig. 2F). The number of neurons in wild-type retinas (4,447 ± 99 RGCs/mm^2, mean ± S.E.M,

n=4) was not significantly different from that found in RPTPσ (-/-) retinas (4,472 ± 151 RGCs/mm^2, n=4).

The histology of knockout and wild-type optic nerves was analyzed using light microscopy (Fig. 3) and no morphological difference was detected between the two groups (Figs. 3A and 3B). Furthermore, quantification of axons in five standard areas of knockout and wild-type optic nerves, corresponding to a total area of 2,700 μm^2 per nerve, revealed an equal number of axons (Fig. 3C). The total number of axons per nerve was calculated based on the total surface area per optic nerve cross section measured using image analysis software. We estimated a total of 55,757 ± 1,454 axons (mean ± S.E.M.; n=3) and 59,467 ± 1,421 axons (n=3) in RPTPσ (-/-) and RPTPσ (+/+) optic nerves, respectively. These values, which are not significantly different from each other (P>0.05), are consistent with a previous report quantifying the total number of RGC axons in the BALB/c mouse retina (Williams et al., 1996). To determine if the lack of RPTPσ favored the development of axons of a particular diameter, we analyzed the distribution of axonal size in optic nerve cross-sections. No significant difference was found in the distribution of axonal size between RPTPσ (-/-) and RPTPσ (+/+) nerves (Fig. 3D). Together, these data indicate that absence of RPTPσ does not alter retinal or optic nerve structures.

4.4.3 Absence of RPTPσ does not influence RGC survival after optic nerve transection

First, we investigated if axotomy induced a change in the expression of RPTPσ in the wild-type retina. Western blot analysis was performed using protein samples from

retinas collected at 3, 5 and 7 days after microcrush lesion of the optic nerve. RPTPσ was detected using a monoclonal antibody specific for the intracellular subunit of this phosphatase (Thompson et al., 2003). Our results indicate that optic nerve lesion did not alter RPTPσ protein levels in the retina (Figure 4A). Consistent with this, the pattern of retinal RPTPσ mRNA expression examined by *in situ* hybridization with a DIG-labeled RPTPσ probe remained unchanged at 3 and 5 days after nerve lesion (data not shown).

To determine if RPTPσ played a functional role in RGC survival, we examined the time-course of cell death in RPTPσ (-/-) and wild-type mice following optic nerve transection. FluoroGold-labeled RGCs were counted on retinal flat-mounts at 7 and 14 days after microcrush lesion to assess the density of surviving RGCs (Fig. 4A). Both knockout and wild-type mice showed a similar pattern of axotomy-induced RGC death. At 7 days after optic nerve transection, 2,033 ± 358 RGCs/mm^2 (mean ± S.E.M.) or 2,020 ± 232 RGCs/mm^2 remained alive in RPTPσ (-/-) (n=4) or RPTPσ (+/+) (n=4) mice, respectively. While at 14 days, only 1,174 ± 81 RGCs/mm^2 or 984 ± 86 RGCs/mm^2 survived in knockout (n=3) or wild-type (n=2) retinas, respectively. No significant difference was found in the number of RGCs between RPTPσ (-/-) and RPTPσ (+/+) retinas at one week (*t* test, P=0.97,) or two weeks (*t* test, P=0.22) after injury. Macrophages and microglia that may have incorporated FluoroGold after phagocytosis of dying RGCs were excluded from our quantitative analysis based on their morphology and the presence of microglia specific markers as described (Cheng et al., 2002).

4.4.4 RPTPσ (-/-) mice display enhanced RGC axon regrowth after acute optic nerve lesion

The functional influence of RPTPσ on the ability of RGCs to regrow an axon was tested two weeks after microcrush lesion of the adult mouse optic nerve. To validate the microcrush lesion model in the mouse visual system, FluoroGold was applied to the superior colliculi after transection of the left optic nerve and retinas from both the operated and the contralateral, intact, eyes were examined. Lack of fluorescent labeling in the injured retina confirmed that RGC axons in the optic nerve were fully transected (Fig. 5A and 5B). While all RGC axons are completely severed in this injury paradigm, the lesion site remains well-defined and the extent of the glial scar is minimized (Lehmann et al., 1999; Selles-Navarro et al., 2001). The lesion site was identified by immunostaining with an antibody against chondroitin sulfate proteoglycan (CSPG), an extracellular matrix protein that accumulates following lesion (Selles-Navarro et al., 2001) (Fig. 5C). In addition, we routinely confirmed the localization of the injury zone in the same section or in an immediately adjacent section using hematoxylin/eosin staining (not shown).

Increased axonal extension past the lesion site, visualized with the anterograde tracer cholera toxin ß subunit (CTβ), was observed in all RPTPσ (-/-) eyes (Fig. 6A and 6B), while considerably fewer axons crossed the glial scar in wild-type nerves (Fig. 6C). Quantitative analysis of axon growth demonstrated that absence of RPTPσ significantly increased the number of axons that extended into the distal optic nerve (Fig. 6D). This effect was apparent at all distances examined from the injury site, producing an increase in the number of growing axons between 46% and 73% with respect to wild-type optic

nerves. These data suggest that RPTPσ inhibits adult RGC axon extension in the injured mammalian visual system.

4.4.5 Retinal MAPK and Akt are activated in adult RPTPσ (-/-) mice

To investigate putative signaling mechanisms involved in enhanced RGC axon growth detected in the absence of RPTPσ, we performed western blot analysis of retinal proteins using antibodies that specifically recognize the phosphorylated forms of MAPK, Akt and PLCγ1. Robust activation of the MAP kinases, Erk1 and Erk2, was observed in intact adult RPTPσ (-/-) retinas compared to basal levels of Erk1/2 phosphorylation found in wild-type retinas (Fig. 7A). A moderate increase in phosphorylated Akt was also detected in the absence of RPTPσ (Fig. 7B). In contrast, low levels of phosphorylated PLCγ1 remained unchanged in both knockout and wild-type retinas (Fig. 7C). To identify the cells within the retina that upregulated expression of active MAPK and Akt in the absence of RPTPσ, we examined the expression of these molecules by immunocytochemistry using phospho-specific antibodies. For this purpose, RPTPσ (-/-) and RPTPσ (+/+) retinal sections were mounted on the same slide, processed simultaneously for immunofluorescence and microscope images were captured using identical conditions. Our data showed an increase in phospho-Erk1/2 staining in RPTPσ (-/-) retinal cells, particularly in cells of the GCL and INL, compared to the basal levels found in RPTPσ (+/+) retinas (Figs. 7D, 7E). Interestingly, staining with a phospho-Akt antibody revealed staining only in cells in the GCL and fiber layer of RPTPσ knockout retinas compared to basal levels found in wild-type retinas (Figs.

7F, 7G). These results indicate that lack of RPTPσ results in activation of the MAPK and Akt kinase signaling pathways in adult RGCs.

4.5 DISCUSSION

The identification of RPTPσ as a key regulator of axon extension and guidance during development has sparked interest in its potential role in the control of axon growth following injury (Haworth et al., 1998; Xie et al., 2001). Recent studies demonstrated that absence of RPTPσ increased axonal regeneration in peripheral nerves following crush injury (McLean et al., 2002; Thompson et al., 2003). It is well established that injured PNS neurons have the ability to extend an axon toward peripheral targets with subsequent restoration of sensory and motor function. In contrast, adult mammalian CNS neurons fail to regenerate an axon after injury. The inability of CNS neurons to regenerate an axon has been generally attributed to an inhibitory glial environment (Yiu and He, 2003; Raisman, 2004; Silver and Miller, 2004) however, emerging data indicate that signals intrinsic to neurons play a significant role in the control of axon growth (Cai et al., 1999; Lehmann et al., 1999; Cai et al., 2002; Goldberg et al., 2002).

Here we used the retinocollicular system of RPTPσ knockout mice to investigate the role of this protein phosphatase in the regulation of axon growth by adult injured RGCs, a prototypical CNS neuronal population. Our *in situ* hybridization analysis demonstrated that RPTPσ mRNA is expressed by adult mouse RGCs. This finding agrees with previous reports that RPTPσ homologs are expressed by RGCs in developing chick (Ledig et al., 1999b) and *Xenopus* (Johnson and Holt, 2000). In

addition, we found that glial cells within the optic nerve express RPTPσ, consistent with the observation that Schwann cells in the adult mouse sciatic nerve produce RPTPσ (McLean et al., 2002). Nerve injury often leads to marked changes in neuronal gene expression (Plunet et al., 2002). Thus, we examined whether optic nerve transection affected RPTPσ expression by RGCs. We demonstrate that expression of RPTP-σ mRNA and protein by RGCs remained unaltered after optic nerve transection suggesting that this protein is present in lesioned RGCs and therefore able to play an active role in inhibiting axon regrowth. Similarly, altered RPTPσ gene and protein expression was not detected in facial motoneurons after facial nerve crush (Thompson et al., 2003). In contrast, RPTPσ mRNA increased in adult dorsal root ganglia and in injured sciatic nerve during regeneration (Haworth et al., 1998; McLean et al., 2002). Taken together, these results suggest that injury-induced changes in RPTPσ gene expression may be differentially regulated among cell populations.

Transgenic mice lacking RPTPσ exhibit growth retardation, delayed peripheral nerve development and altered development of the olfactory bulb, pituitary gland and hypothalamus (Elchebly et al., 1999; Wallace et al., 1999). Our morphological analysis identified no phenotypic difference between RPTPσ (-/-) and RPTPσ (+/+) retinas during development and in adult mice. In addition, the structure of the optic nerve was unaltered in RPTPσ (-/-) mice. These results are consistent with a study in which expression of dominant negative CRYP-α, an RPTPσ homolog, did not affect cell fate determination or population size in *Xenopus* retina, yet it enhanced RGC neurite outgrowth *in vitro* (Johnson et al., 2001). Of interest, mice expressing a mutant RPTPδ,

another type IIa RPTP, have a CNS that appears histologically normal but exhibit impaired learning and enhanced long-term potentiation (Uetani et al., 2000). The preservation of retinal and optic nerve structure in RPTPσ knockout mice could be attributed to functional redundancy as other type IIa RPTPs are expressed in vertebrate RGCs (Fuchs et al., 1998b; Ledig et al., 1999b; Johnson and Holt, 2000).

Here we demonstrate that absence of RPTPσ stimulates RGC axon outgrowth after acute optic nerve injury. Quantitative analysis showed a significant increase in the number of injured axons that successfully extended past the lesion site in RPTPσ knockouts with respect to wild-type littermate controls. This effect could not be attributed to a larger number of RGCs, and therefore axons, in RPTPσ (-/-) mice because the time-course of axotomy-induced RGC death was similar in knockout and wild-type animals. Our data is supported by previous studies in the developing visual system. For example, expression of dominant negative RPTPσ increased the length of RGC neurites in developing *Xenopus* retinal explants (Johnson et al., 2001). Together, these findings suggest that RPTPσ activity is detrimental for RGC axon growth. Importantly, RPTPσ has been postulated to slow the rate of axon extension during regeneration of injured PNS neurons. For example, absence of RPTPσ stimulated axon regeneration and recovery after crush injury in adult sciatic (McLean et al., 2002) and facial (Thompson et al., 2003) nerves. Our study, however, provides the first demonstration that RPTPσ negatively regulates axon growth in the adult CNS following traumatic injury.

The mechanism by which RPTPσ regulates growth cone motility and axon extension remains largely unknown. Genetic analysis of DLAR function in *D. Melanogaster* suggests that type IIa RPTPs regulate cytoskeletal organization through

proteins that include the guanine-nucleotide exchange factor Trio (Debant et al., 1996; Awasaki et al., 2000; Bateman et al., 2000), the tyrosine kinase Abl (Wills et al., 1999a; Wills et al., 1999b) and the Abl substrate Enabled (Wills et al., 1999a). However, at present, there is no evidence that these proteins act as downstream effectors for any type IIa RPTP family member in mammals. An alternative approach to identify mediators of RPTPσ action is to investigate pathways already known to be involved in axon growth. Although little is known about the signaling pathways that direct axon growth by adult neurons *in vivo*, work using primary neuronal cultures have identified components involved in neurotrophic factor-induced axon extension (Brann et al., 1999; Perron and Bixby, 1999; Atwal et al., 2000; Liu and Snider, 2001; Goldberg et al., 2002; Markus et al., 2002; Edström and Ekström, 2003). For example, the neurotrophin receptor TrkB has been shown to modulate both MAPK and PI3K/Akt pathways to promote axon growth by cultured neonatal sympathetic neurons (Atwal et al., 2000), Another study demonstrated that inhibition of the MAPK pathway, blocked FGF-induced neurite outgrowth in embryonic chick retinal neurons (Perron and Bixby, 1999). More recently, the PI3K/Akt kinase pathway was implicated in axonal outgrowth from explants of adult mouse dorsal root ganglia (Edström and Ekström, 2003). Thus, RPTPσ may regulate axon growth by interacting with signaling components downstream of receptor tyrosine kinases. This idea is supported by our results indicating that both MAPK and Akt kinase are constitutively active in adult RPTPσ (-/-) retinas, notably in RGCs. Thus, MAPK and PI3K/Akt kinase are candidate signaling mechanisms by which RPTPσ may promote RGC axon outgrowth.

RGC axon growth in RPTPσ (-/-) mice was restricted to short distances from the lesion compared to other recent studies that report axon growth as far as 1 mm from the injury site after intravitreal injection of cAMP (Monsul et al., 2004) or lens injury (Yin et al., 2003; Fischer et al., 2004). A number of factors may contribute to these differences, including the use of rats in these latter studies, compared to the use of mice by us. Furthermore, these other studies each induced some form of retinal injury shown to upregulate expression of endogenous growth factors (Wen et al., 1995; Cao et al., 1997; Cao et al., 2001), which may contribute to promoting axon regeneration in the optic nerve.

The ability of RGC axons to regenerate is limited in part by the growth inhibitory environment of the optic nerve, rich in proteins such as Nogo-A (Caroni and Schwab, 1988a; Caroni and Schwab, 1988b), myelin-associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994) and oligodendrocyte-myelin glycoprotein (OMgp) (Kottis et al., 2002) that restrict the extension of axons on myelin by interacting with the Nogo receptor (Fournier et al., 2001; Liu et al., 2002; Wang et al., 2002b). This limited regenerative capacity by adult RGCs is further illustrated by the observation that strategies that promote long distance regeneration in the injured spinal cord produce only short distance axon growth in the optic nerve (Bartsch et al. 1995; Lehmann et al. 1999; Huang et al. 1999; Dergham et al. 2002; Ellezam et al. 2003). Importantly, emerging data suggest that overcoming inhibitory signals is not sufficient to promote axon growth but that activation of an intrinsic growth program in neurons is also required for axon regeneration in the CNS. For example, during retinal maturation, a signal from amacrine cells triggered an irreversible loss of the intrinsic axon growth

ability of RGCs (Goldberg et al., 2002). Moreover, suppression of Nogo receptor activity was not sufficient to promote extensive RGC regeneration unless these neurons were stimulated with macrophage-derived factors (Fischer et al., 2004). Future studies will establish if RPTPσ inhibition combined with strategies that overcome growth inhibitory signals or stimulate active growth will promote long-distance axon regeneration.

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4.7 FIGURES

Figure 1. RPTPσ is expressed by adult RGCs and optic nerve glia.

Expression of endogenous RPTPσ was investigated by *in situ* hybridization of adult wild-type retinas and optic nerves. Incubation of retinal sections with a digoxigenin (DIG)-labeled RNA anti-sense probe specific for mouse RPTPσ revealed strong positive staining in the ganglion cell layer (GCL) and inner nuclear layer (INL) (A), as well as in glial cells of the optic nerve (D). A higher magnification of an optic nerve section showed that DIG-positive glial cells formed linear arrays along the antero-posterior plane, a pattern typical of oligodendrocytes (G). Incubation of retinal (B) or optic nerve (E) sections from RPTPσ (-/-) mice with the anti-sense probe confirmed absence of RPTPσ expression. Sections from wild-type animals incubated with control sense probe did not show staining, thus confirming the specificity of our assay (C, F). IPL: Inner Plexiform Layer. Scale bars: 50 μm (A-C, G), 100 μm (D-F).

FIGURE 1.

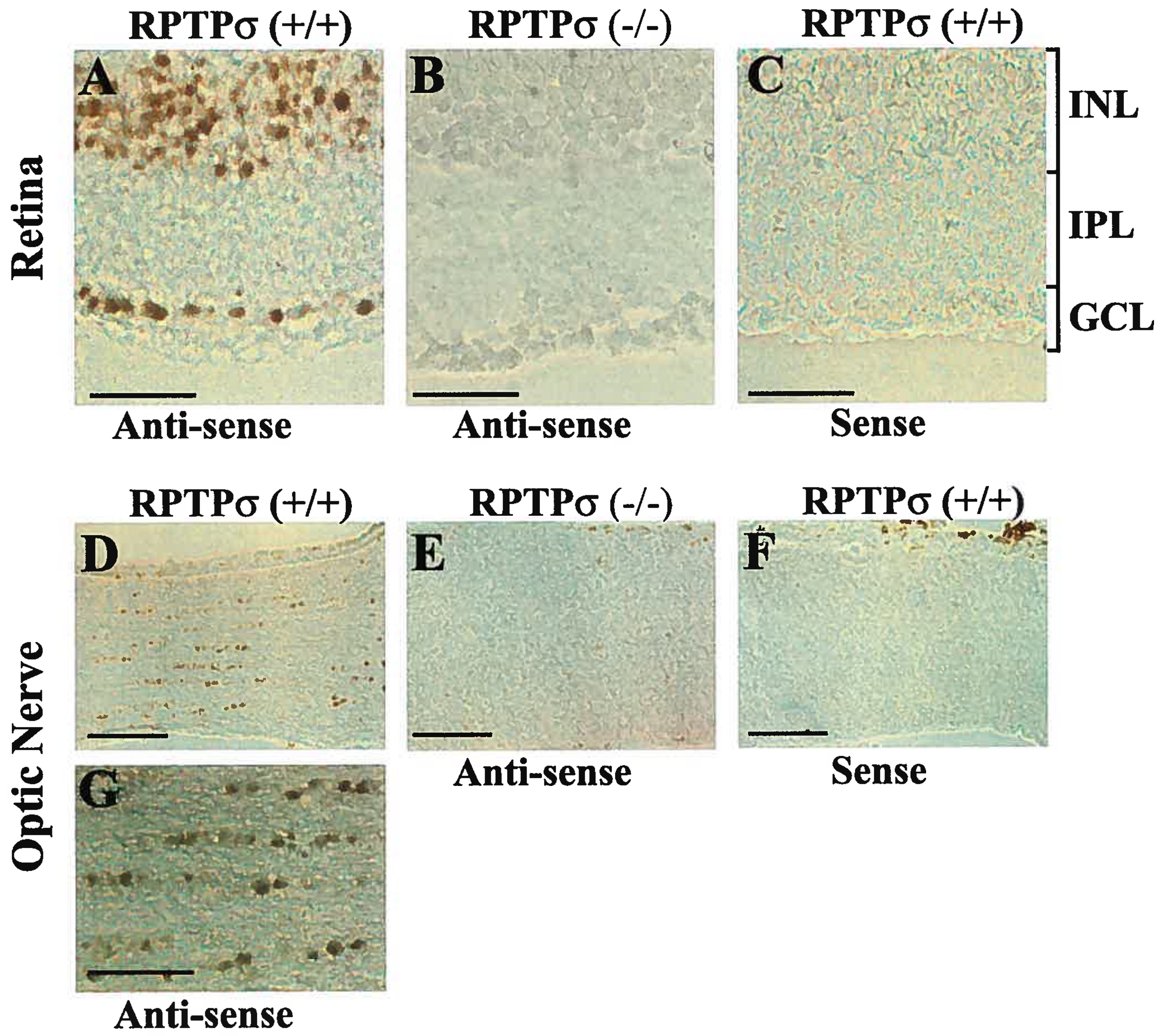
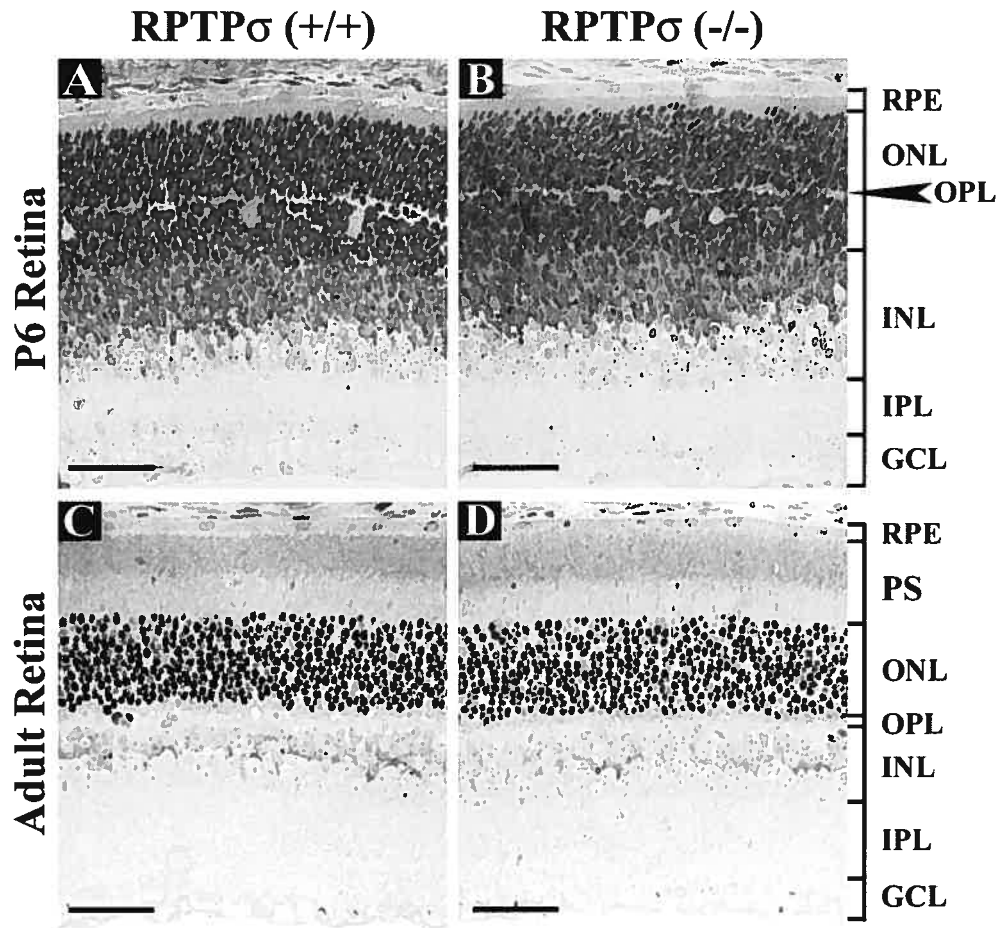
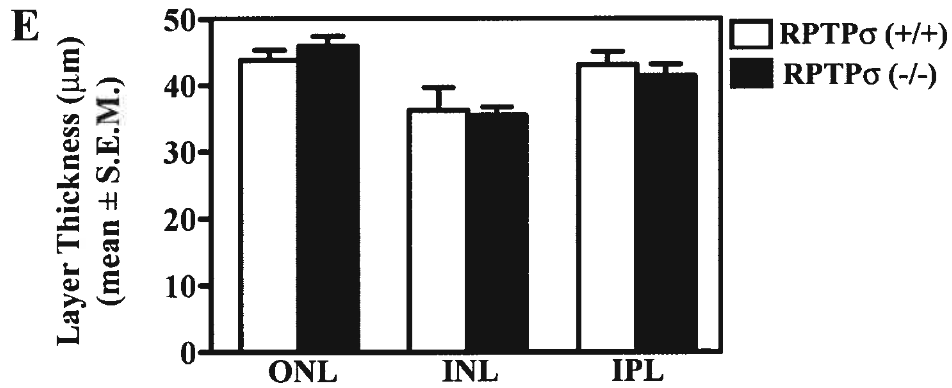


Figure. 2. RPTPσ deficiency does not alter retinal structure.

Epon embeded sections (1-μm-thick) stained with toluidine blue show normal retinal development in RPTPσ (-/-) mice as compared to wild-type littermate controls (A, B). Note the matching position of the outer plexiform layer (OPL) in both knockout and control retinas suggesting that cellular migration was not affected by RPTPσ removal. The retinal structure was identical in adult wild-type and RPTPσ knockout mice (C-D). Quantitative analysis of the thickness of retinal layers revealed no significant difference between RPTPσ (+/+) (n=5) and RPTPσ (-/-) (n=5) mice (unpaired t-test; confidence interval= 95%; ONL: p=0.3472; INL: p=0.8196; IPL: p=0.5633) (E). Moreover, the density of FluoroGold-labeled RGCs was similar in control (n=4) and RPTPσ (-/-) (n=4) animals suggesting that RPTPσ is not essential for normal RGC development (F). GCL: Ganglion Cell Layer, IPL: Inner Plexiform Layer, ONL: Outer Nuclear Layer, RPE: Retinal Pigment Epithelium. Scale bars: 50 μm (A-D).

FIGURE 2.





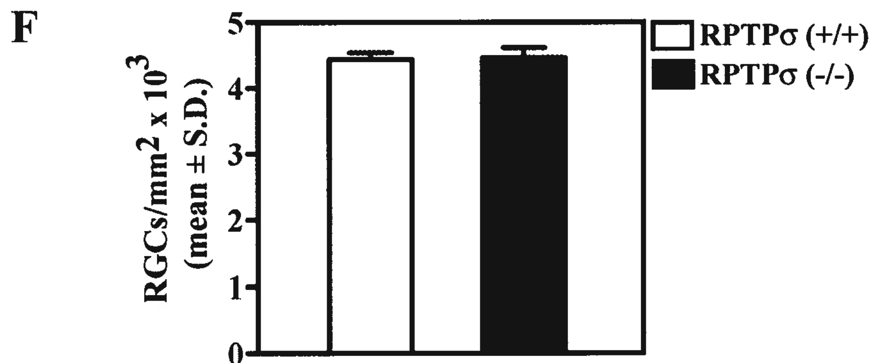


Figure 3. Lack of RPTPσ does not affect optic nerve structure.

Epon embeded sections of optic nerve stained with toluidine blue revealed a similar morphology between RPTPσ (-/-) and RPTPσ (+/+) mice (A, B). Quantification of the total number of axons in transverse optic nerve sections confirmed a comparable axon density in wild-type (n=3) and knockout (n=3) nerves (C). Furthermore, analysis of axonal calibre, measured as the area of the axon in an optic nerve cross section, demonstrated a similar distribution of axon size between RPTPσ (+/+) (n=3) and RPTPσ (-/-) (n=3) (D). Scale bars: 25 μm.

FIGURE 3.

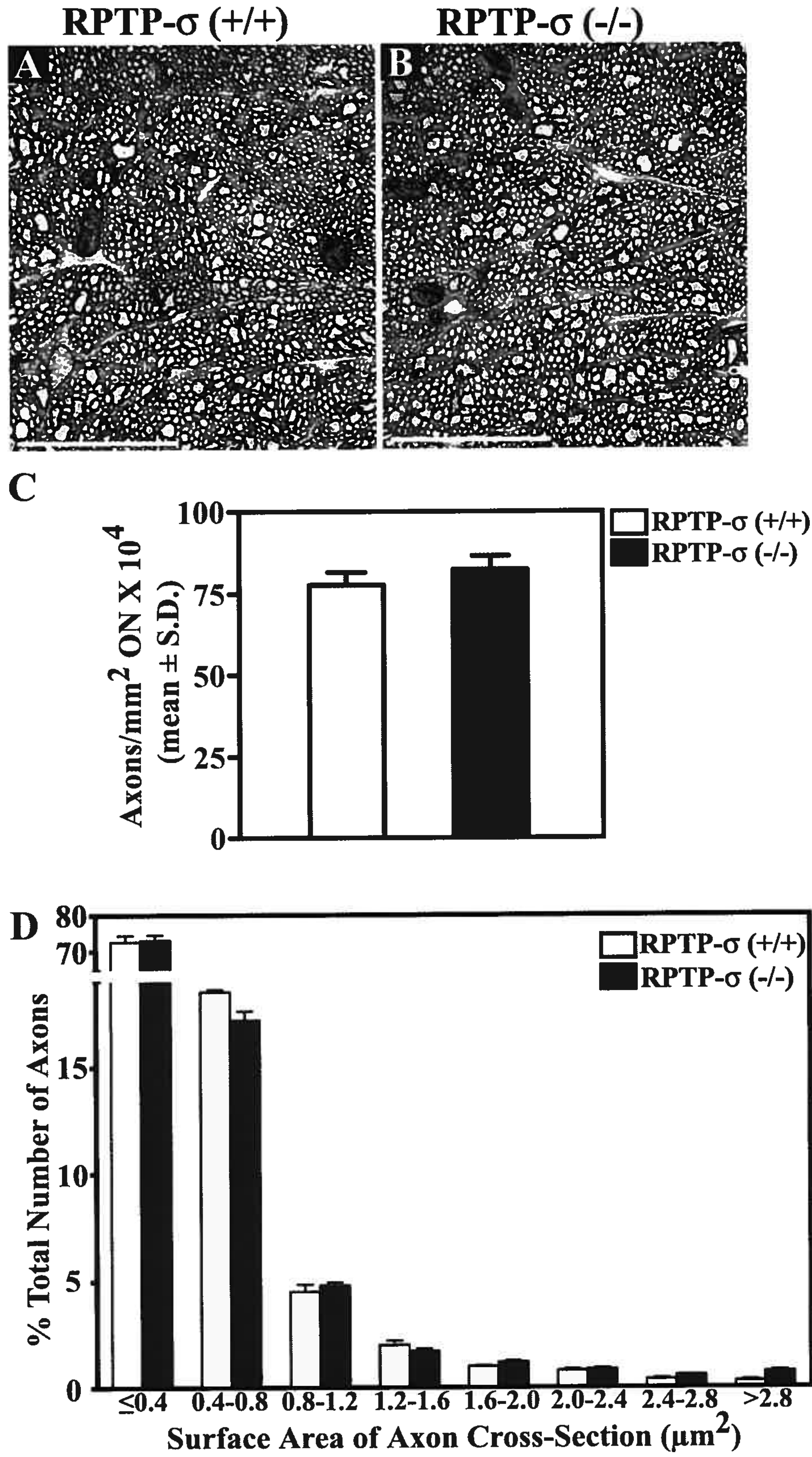


Figure 4. Retinal RPTPσ protein continues to be expressed after optic nerve injury but does not regulate RGC survival.

Whole retinal homogenates were obtained from wild-type intact or operated mice at 3, 5 or 7 days after nerve transection. The level of retinal RPTPσ was examined by immunoblot (150 μg per lane) using a monoclonal antibody raised against the intracellular domain of RPTPσ (A). The uniform intensity of the immunoreactive bands corresponding to RPTPσ suggests that the protein level of this phosphatase is not affected by injury. Bottom panel: same blot was re-probed with an antibody against ß-actin to visualize total protein. (B) RGC densities in retinas from intact wild-type mice (white bars, n=4) were similar to those found in intact RPTPσ knockouts (solid bars, n=4) suggesting that RPTPσ is not involved in the developmental regulation of the RGC population size. The time-course of RGC death after micro-crush lesion was virtually identical between wild-type (white bars) or knockout (black bars) mice. A similar number of RGCs survived at 1 week (wild-type: n=4, knockout: n=4) or two weeks (wild-type: n=2, knockout: n=3) after injury. These data indicate that RPTPσ does not influence the survival of axotomized RGCs.

FIGURE 4.

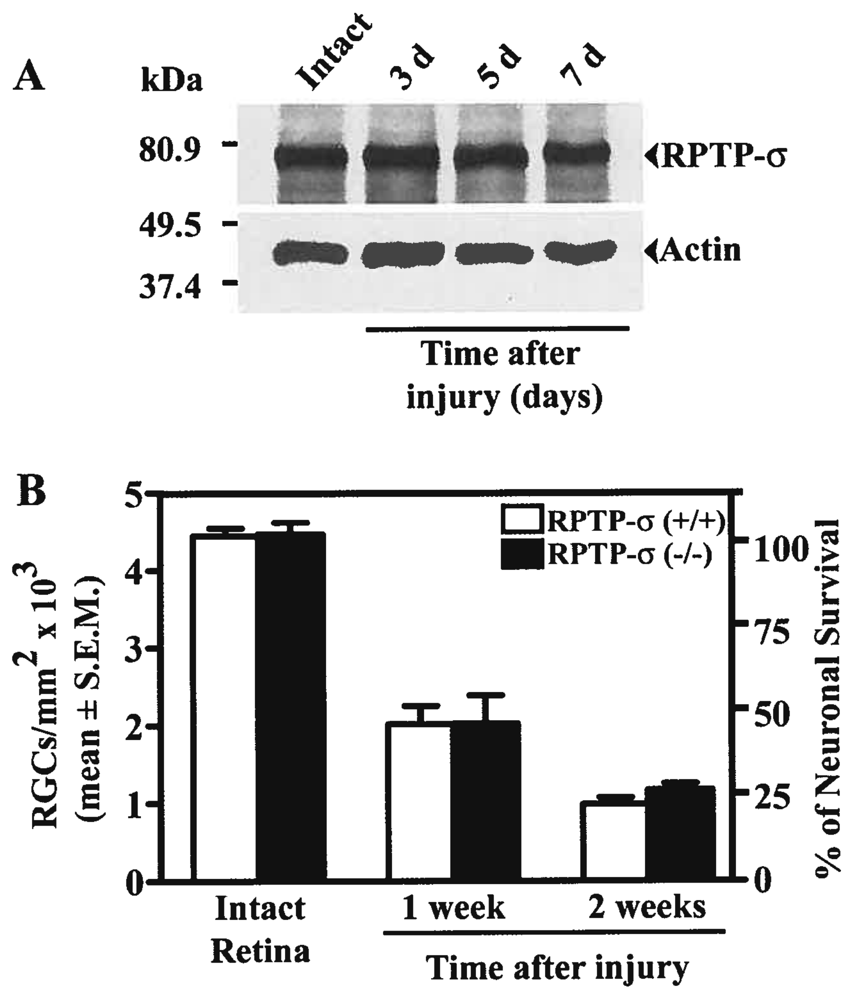


Figure 5. Micro-crush (MC) lesion of the mouse optic nerve results in complete transection of RGC axons.

(A) Whole-mounted mouse retina visualized after application of the tracer FluoroGold to the superior colliculus to label the entire RGC population. (B) MC lesion of the optic nerve preceded application of FluoroGold, thus absence of fluorescence in the retina confirmed that the optic nerve was completely transected. (C) Optic nerve MC typically resulted in a well-defined injury site in which the extent of the glial scar was minimized. The lesion site (asterisks) was accurately identified at 14 days post-crush by immunostaining against chondroitin sulfate proteoglycan (CSPG), an extracellular matrix protein that accumulates following injury. Scale bars: 250 μm (A, B) and 100 μm (C).

FIGURE 5.

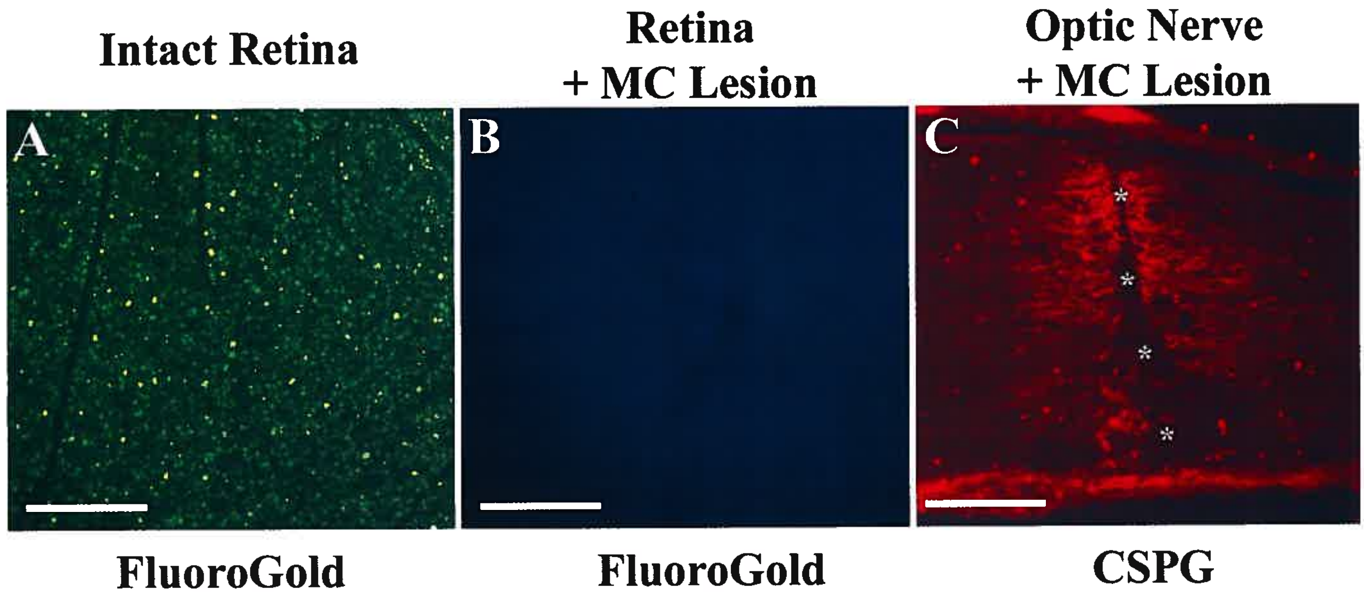


Figure 6. RPTPσ (-/-) mice show enhanced regrowth of transected RGC axons.

(A) Optic nerve longitudinal section from a RPTPσ (-/-) mouse immunostained with a CTß antibody showing regrowth of fully transected RGC axons. A significant number of CTß-positive axons extended into the distal optic nerve past the lesion site (asterisks). (B) Higher magnification image of a RPTPσ (-/-) optic nerve showing numerous RGC axons growing between 50 μm and 100 μm from the lesion site. (C) Minimal RGC axon outgrowth was observed in nerve sections from wild-type control mice. (D) Number of RGC axons (mean ± S.E.M.) extending past the lesion site in RPTPσ (+/+) (white bars: n=6) or RPTPσ (-/-) (solid bars: n=7) mice at two weeks after optic nerve transection. RGC axon growth in the RPTPσ (-/-) group was significantly larger than in the RPTPσ (+/+) control group (unpaired-*t*-test, $p < 0.05$). Scale bars: 100 μm (A, C) and 50 μm (B).

FIGURE 6.

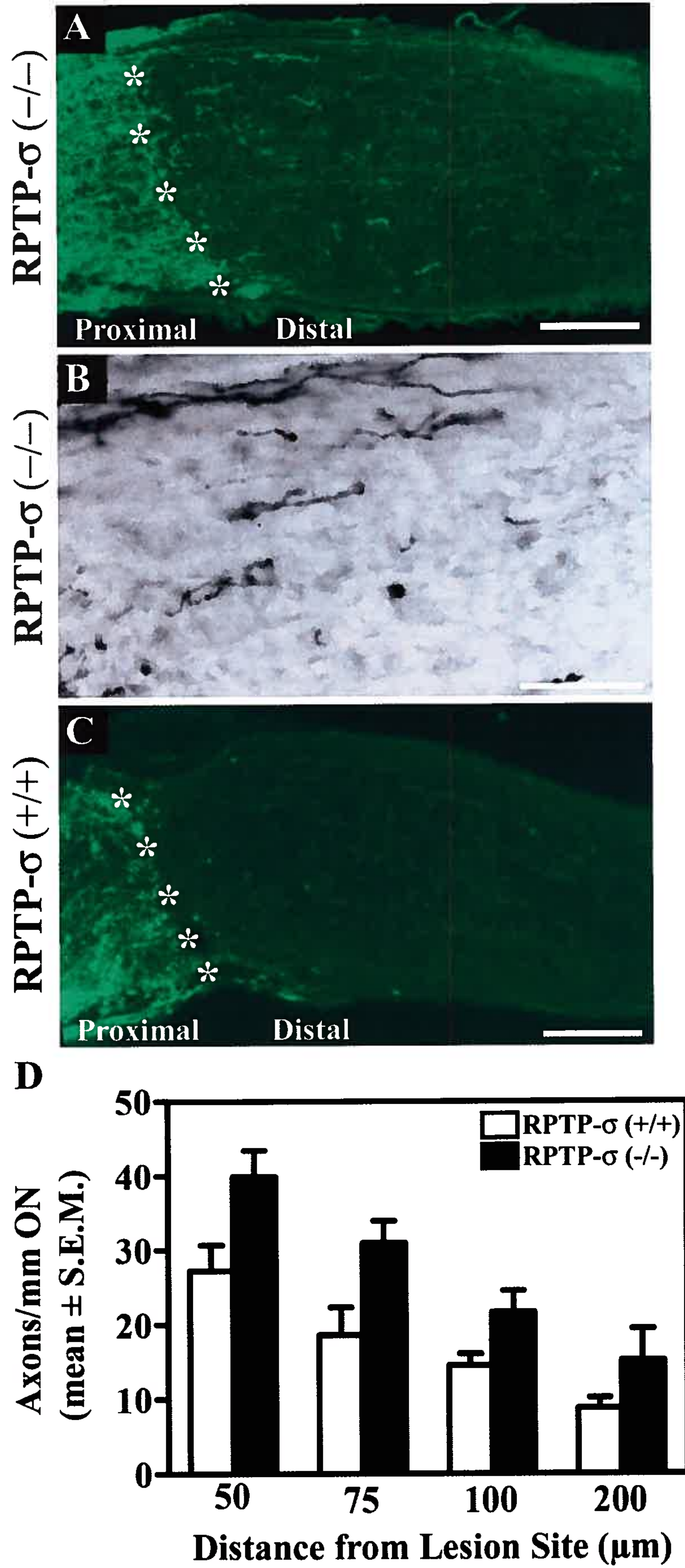
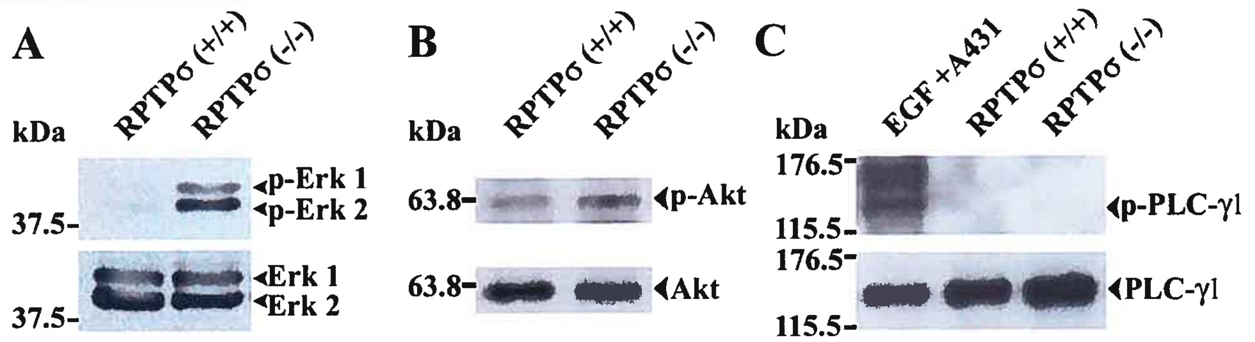
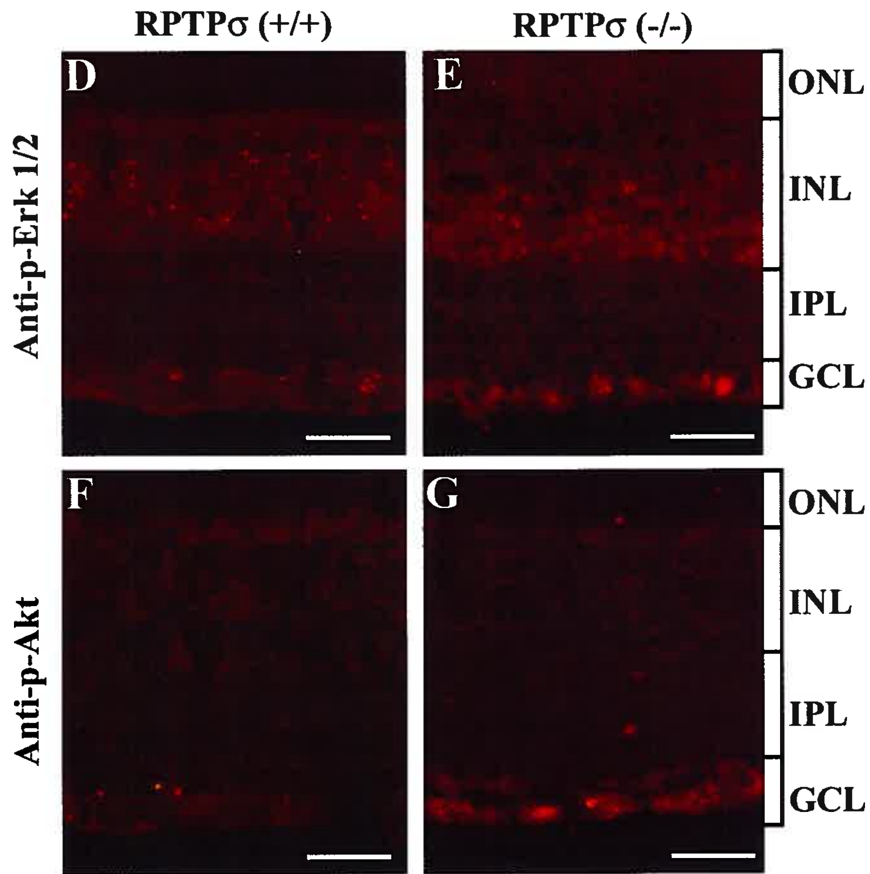


Figure 7. MAPK and Akt kinase pathways are constitutively active in RPTPσ (-/-) mice retinas.

(A)Robust activation of Erk1/2 kinases was detected in RPTPσ (-/-) mice retinal homogenates analyzed by western blots. (B) A modest stimulation of Akt kinase was detected in RPTPσ (-/-) retinal homogenates. (C) The levels of phosphorylated PLCγ1 were very low and remained unchanged between RPTPσ knockout and wild-type retinas. A protein sample from human carcinoma (A431) cells stimulated with epidermal growth factor (EGF) was used as positive control for PLCγ1 activation. Lower panels in (A), (B) and (C) show the same blot re-probed with antibodies that recognize non-phosphorylated Erk1/2, Akt or PLCγ1 to verify equal protein loading. The endogenous activity of Erk1/2 kinases and Akt kinase detected in RPTPσ (-/-) retinas was confirmed by immunofluorescence. Upregulation of phospho-Erk1/2 was observed in cells of the ganglion cell layer (GCL) and inner nuclear layer (INL) of RPTPσ (-/-) retinas (E) compared to RPTPσ (+/+) controls (D). Increased phospho-Akt staining in RPTPσ (-/-) mice (G) with respect to wild-type controls (F) was confined to the ganglion cell and fiber layer. Scale bars: 50 μm (C, D).

FIGURE 7.





Chapter V

GENERAL DISCUSSION

5. GENERAL DISCUSSION

It is well established that, as CNS neurons mature, they lose the ability to regenerate their axon after acute injury. This disparity between embryonic and adult neurons stems from intrinsic properties (Cai et al., 1999; Cai et al., 2001; Goldberg et al., 2002a) as well as from changes in their environment.

The studies presented in this thesis focused on the modulation of genes that are robustly expressed in the developing visual system, as a strategy to reactivate the neuron's intrinsic growth program and as a means to promote neuronal survival. In addition, we explored the possible signaling pathways involved in enhancing regenerative growth in the adult CNS.

5.1 SEARCHING FOR GENES INVOLVED IN REGENERATION

The identification of genes that can either stimulate or inhibit CNS regeneration is a challenging task. One approach calls for the comparative analysis of gene expression within the CNS at periods when axons are growing (immature neurons) versus when they become senescent (mature neurons). Using subtractive differential analysis or DNA micro-array technology, there are still far too many candidates from the ~30, 000 genes expressed in neurons (Hahn and Laird, 1971; Grouse et al., 1972) to test individually. Furthermore, the quest is complicated by the likelihood that more than one gene may need to be modulated to promote adequate axon regrowth. To make matters worse, a single cell in the CNS expresses ~20, 000 to 30, 000 distinct mRNAs and ~99% of these mRNA transcripts are rare. In fact, ~99% of the mRNA transcripts make up less than 30% of the total mass of mRNA (Hahn and Laird, 1971; Grouse et al., 1972; Hahn et al.,

1978). This signifies that the vast majority of genes are expressed at very low levels, making it considerably more difficult to detect them. It is therefore possible that regeneration associated genes (RAGs) could fall into this category of low abundance mRNAs. Moreover, because of the massive transcriptional activity observed in developing tissues, the hunt for RAGs is further convoluted by the abundance of genes that regulate processes other than axonal growth and regeneration. Although it would be simpler to assume that there are marked differences in RAG expression between developing and mature tissues, there is evidence that certain genes that influence axon growth do not show dynamic changes in their expression. For example, it is well documented that the myelin associated protein Nogo, is highly inhibitory towards axon growth (reviewed in David and Lacroix, 2003). Nevertheless, Nogo mRNA is highly expressed in both the developing and mature CNS (Josephson et al., 2001) and does not vary after spinal cord or cortical lesion (Huber and Schwab, 2000). The same pattern was found with RPTP-σ described in chapter 4 of this thesis.

Following the identification of a candidate gene, whether it be by differential screen or by analyzing the biochemical properties of the protein, it is necessary to conduct tests *in vivo* to ascertain its role. It is logical to predict that, certain genes, such as those coding for the transcription factor c-jun and the growth cone transduction proteins GAP-43 and CAP-23 (Herdegen et al., 1997; Frey et al., 2000; Bomze et al., 2001), should be upregulated to enhance regeneration, while other genes, such as myelin inhibitor NogoA/B and $p75^{NTR}$, should be downregulated (Hannila and Kawaja, 1999; Walsh et al., 1999; Kim et al., 2003). In this thesis, both strategies were described. First we investigated the ability of FGF-2 to promote survival and regeneration by

upregulating the FGF-2 gene in adult transected RGCs. Second, we investigated the effect of RPTP-σ gene removal from the mouse genome and studied its impact on RGC survival and regeneration.

5.2 SUSTAINED UPREGULATION OF FGF-2 IN INJURED ADULT RGCS

In the first two articles presented in this thesis (Chapters 2 & 3), we upregulated FGF-2 protein in mature RGCs using an AAV containing the FGF-2 transgene. FGF-2 is typically associated with developing RGCs during periods of axon extension and growth in the embryonic retina (de Iongh and McAvoy, 1993) and is absent altogether from adult RGCs (Kostyk et al., 1994; Sapieha et al., 2003). By increasing its levels in the adult, we were able to test if FGF-2 was capable of influencing the adult RGC injury response. Following AAV mediated gene transfer, elevated levels of FGF-2 persisted in mature RGCs for at least 2 months after injection, spanning the entire duration of the experiment. We found that this treatment promoted enhanced regeneration of injured RGCs, while having a robust yet transient effect on cell survival.

Due to the 4-week incubation period required to achieve optimal transgene expression with AAVs, the dosing protocol was designed so that the plateau of FGF-2 expression coincided with the initial protracted phase of RGC death (day 0 to day 5 after axotomy) (Berkelaar et al., 1994). During this period, untreated neurons unsuccessfully attempt to sprout new axons in what is known as “frustrated regenerative acts” (Ramon y Cajal, 1928). This suggests that during this phase of abortive growth, RGCs mount a response to counteract the effects of injury. Consequently, the neurons may be more responsive to survival and growth promoting treatments.

5.3 NEUROPROTECTION AND FGFS

5.3.1 FGF-2, FGF-5 and FGF-18 Promote Transient Survival of Axotomized RGCs

In addition to FGF-2, we also investigated the neuroprotective qualities of two other members of the FGF family: FGF-5 and FGF-18 (Appendix C). As with FGF-2, we employed AAVs to attain sustained, long term expression of these proteins in RGCs. These factors were of interest as both AAV.FGF-5 and AAV.FGF-18 had been shown to slow photoreceptor degeneration in transgenic models of light damage (Lau et al., 2000; Green et al., 2001). Using the optic nerve axotomy injury paradigm, we observed that one week after lesion, retinas treated with AAV.FGF-5 or AAV.FGF-18 showed 68% and 67% RGC survival respectively. This survival was of a comparable magnitude to the 74% obtained with AAV.FGF-2 and significantly higher than control AAV-GFP injected eyes, which showed 49% survival. Nevertheless, as with AAV.FGF-2, the neuroprotection was transient and declined to 12% survival two weeks after injury (control AAV.GFP injected retinas dropped to 10% survival).

5.3.2 FGFR1 Expression in the Injured Retina

We had previously demonstrated that the survival effect promoted by BDNF after TrkB gene transfer was considerably larger than that observed with AAV.FGF-2 (Cheng et al. 2002; Sapieha et al. 2003). One of the principal theories explaining the lack of long term survival following axotomy suggests that RGCs lose responsiveness to trophic factors after injury (Shen et al., 1999) (Goldberg and Barres, 2000). This can be explained by the fact that lesioned CNS neurons downregulate certain neurotrophic

receptors, such as Trks after injury (Venero et al., 1994; Kobayashi et al., 1997; Liebl et al., 2001; Cheng et al., 2002) and thereby become less apt to activate pro-survival signaling cascades.

In order to explain the short-lived survival effect of FGFs, we investigated the post-axotomy behavior of the FGF receptor, FGFR1, that binds FGF-2, FGF-5 and FGF-18. Interestingly, following RGC axotomy, Western blot analysis showed a persistent increase in FGFR1 protein expression within the retina (Appendix D). This data is consistent with a previous study demonstrating that retinal FGFR1 mRNA is upregulated after focal mechanical injury to the retina (Wen et al., 1995). Immunohistochemical analysis of axotomized retinas, revealed a pronounced increase in FGFR1 protein predominantly in photoreceptor cells, but not in RGCs (Figure 1. B, Appendix D). Similarly, FGF-2 protein was reported to be upregulated in photoreceptor cells following an optic nerve crush (Kostyk et al., 1994). In fact, photoreceptor cells are thought to have an α2 adrenergic receptor-coupled mechanism which induces FGF-2 upregulation and can consequently attenuate light damage (Wen et al., 1996). Taken together, these data may explain the peculiar finding by Bush and Williams (1991) that optic nerve transection protects photoreceptor neurons against light damage (Bush and Williams, 1991).

The observed increase in FGFR1, however, does not confer a survival advantage to axotomized RGCs. FGF-2 and FGF-18 are secreted, thus it is possible that FGFR1 upregulation in neighboring photoreceptors acts as an FGF sink, consequently depriving RGCs of these factors.

5.4 SOMATIC EXPRESSION OF FGF-2 IS REQUIRED TO STIMULATE REGENERATION

The location of a growth promoting stimulus is key to activating axonal growth. Should the treatment be delivered to the cell body or to the distal end of the transected axon? This question has been addressed in detail in studies on neurotrophin-induced axon growth using Campenot chambers (Campenot, 1994). In this culture system, neurons have their cell bodies and their distal axons in separate physical compartments. Early studies on PNS neuronal cultures revealed a dissociation between cell survival and axonal growth, depending on where the stimulus was applied. For example, NGF promoted survival when added to the soma. However, when NGF was added to the axonal terminal, it stimulated both axonal growth and survival (Campenot, 1994). It was later demonstrated that neurotrophin stimulation of the distal axon provoked phosphorylation of axonal Trks and the consequent activation of Ras/MAPK pathways (Riccio et al., 1997; Senger and Campenot, 1997). Moreover, when Erk1/2 was inhibited within axons, there was a significant decrease in axonal outgrowth (Atwal et al., 2000). In developing RGCs, it was shown that BDNF stimulation of axonal terminals enhanced dendritic arborization, while stimulation of dendrites reduced arborization (Lom and Cohen-Cory, 1999). These data are examples of the increasing evidence suggesting that the location of stimulation determines the neuron's response to a given molecule.

The retino-collicular system resembles an *in vivo* Campenot chamber in that the cell bodies are within the retina, in the eye, and the axon terminal in the brain. It is known that shortly after CNS injury, macrophages and other cells infiltrate the lesion site and secrete, amongst other factors, FGF-2 (Finklestein et al., 1988; Logan et al.,

1992). Increased FGF levels attract oligodendrocyte precursors and fibroblasts which enter the lesion site and form dense ramifications, contributing to the growth inhibitory glial scar (Fawcett and Asher, 1999). It is therefore established that FGF-2 levels are elevated at the site of a CNS lesion.

In our study, we demonstrated that, following optic nerve micro-crush, there is a reorganization and build-up of FGF-2 positive cells around the site of lesion. Because FGF-2 is secreted, it is likely that local concentrations of FGF-2 are elevated in the proximity of transected axons. Nevertheless, this presence of FGF-2 at the lesion site is not sufficient to promote either axonal regrowth or RGC survival. In fact, we showed that FGF-2 needs to be upregulated in RGC cell bodies to promote survival or regenerative axon growth. Although anterograde transport of FGF-2 was observed in regenerating RGCs, the protein itself is newly synthesized in the soma. Moreover, in Chapter 3, we showed a substantial reduction in axonal regeneration when MEK was inhibited upon application of a pharmacological blocker to the RGC body. This is in contrast with the MEK mediated growth of cultured developing sympathetic neurons, which is exclusively controlled locally at the axon (Atwal et al., 2000). Taken together, these data provide evidence that FGF-2 stimulation is required at the cell body and not at axon terminals in order to promote survival and regeneration and survival of adult lesioned RGCs *in vivo* (Figure 5-1, p231).

5.5 RPTP-σ HINDERS RGC REGENERATION

In Chapter 4 of this thesis, we provide the first *in vivo* evidence that expression of the type IIa phosphatase, RPTP-σ, impedes the regrowth of injured adult mammalian

CNS neurons. Although RPTP-σ has been identified as a crucial protein for nervous system development, its role in the adult CNS was unknown (Elchebly et al., 1999; Wallace et al., 1999). Using the optic nerve micro-crush lesion paradigm, we investigated RPTP-σ's involvement in the regeneration and survival of adult RGCs following acute injury. We report that axonal regrowth of transected RGCs was significantly enhanced in adult RPTP-σ (-/-) mice while the rate of survival remained unchanged. Furthermore, we showed that even if adult mouse RGCs express RPTP-σ, a lack of this protein does not affect their phenotype or population density. The exact mechanism of action of RPTP-σ remains to be elucidated, but our data indicate that it plays an important role in the regulation of axon outgrowth. The following sections speculate on the possible modes of actions of RPTPs in the context of axon regeneration.

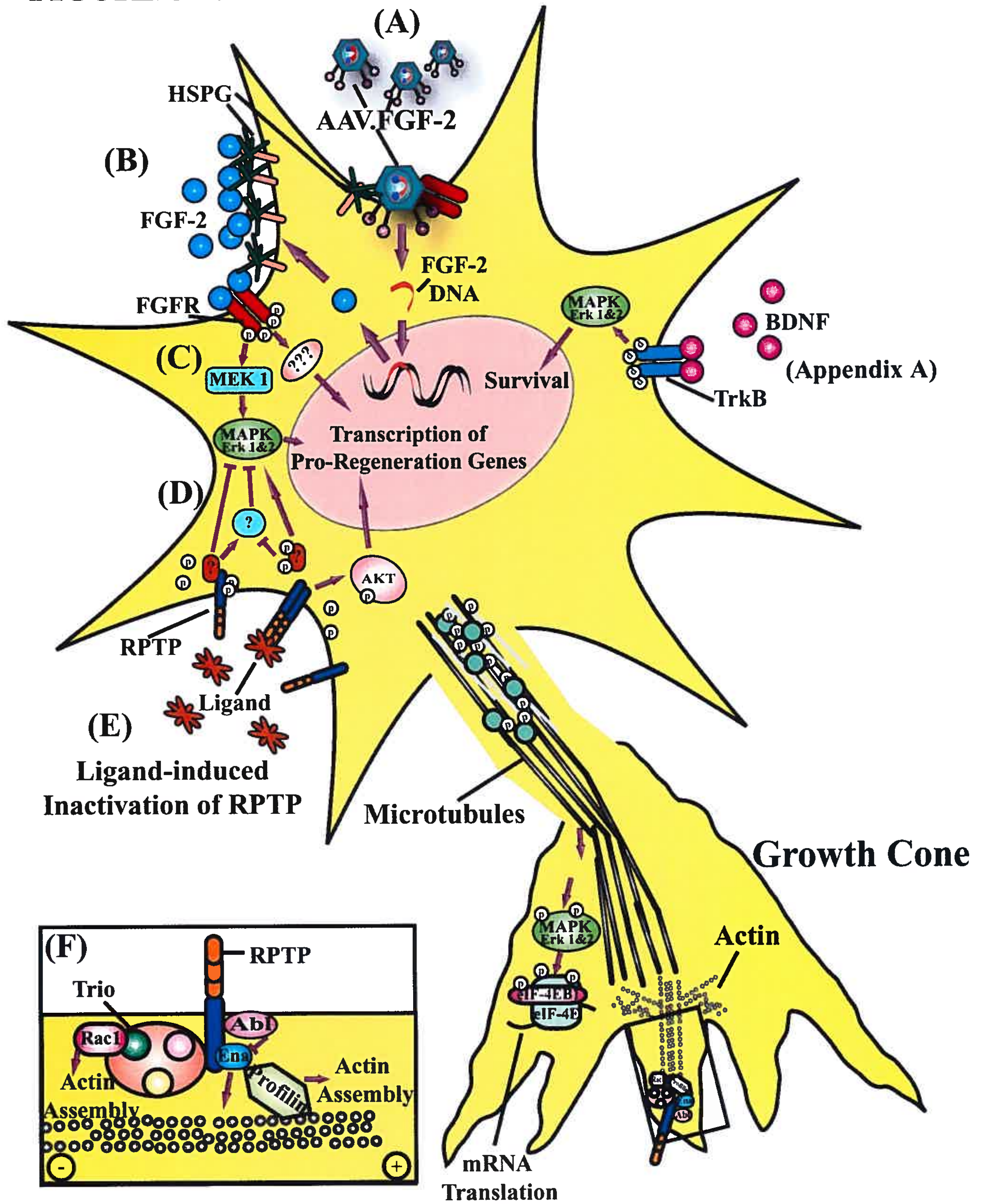
Figure 5-1. The working model and general summary.

This figure depicts the major conclusions presented in this thesis as well as data relevant to our findings. (A) AAV.FGF-2 readily infects and expresses its transgene in adult RGCs (Chapter 2). We identified on mature RGCs, FGFR and heparan sulfate proteoglycan (HSPG), two surface molecules required for AAV integration. The expression of these proteins likely contributes to the high rates of infection. (B) FGF-2 binds to HSPGs with high affinity and requires this surface glycoprotein for efficient signaling through FGFR. (C) Upon stimulation of FGFR by FGF-2, signaling cascade(s) that ultimately result in axonal regeneration are activated (Chapters 2 & 3). We have identified Erk1/2 as an important player in the signaling events leading to FGF-2-induced RGC re-growth (Chapter 3). Nevertheless, another, yet to be identified signaling effector is likely to be required to achieve growth, as activating Erk1/2 by itself is insufficient to promote RGC regeneration (Pernet et al.).

(D) We demonstrated that RPTP-σ impedes RGC regeneration following acute injury (Chapter 4). Moreover, removal of RPTP-σ results in both the activation of Erk1/2 and Akt and provokes axonal regrowth after acute injury (described in greater detail in Figure 5-2). (E) The ligand induced inactivation hypothesis stipulates that RPTP-σ is catalytically active until it binds its ligand. Once the ligand bound, RPTP-σ subunits dimerize and cause its inactivation. In this scenario, a RPTP-σ knockout animal would mimic continuous ligand binding.

(F) A theoretical model of RPTP action at the growth cone. The actin modulating proteins Ableson (Abl) and Enabled (Ena) can bind directly to RPTP. Ena, a common substrate for both Abl and RPTP, can promote axon elongation by binding directly to actin and interfering with actin-capping proteins or by recruiting the protein Profilin which can promote actin assembly. Alternatively, RPTP interacts with the Rho guanine exchange factor Trio. Through its D1 domain, Trio can activate Rac 1 and consequently promote actin assembly.

FIGURE 5-1.



5.6 RPTP-σ SIGNALING

5.6.1 How Does Ligand Binding Influence RPTP-σ Signaling?

The interactions between RPTP-σ and its ligands and effectors remain largely unknown. Putative ligands for cPTP-σ (chick RPTP-σ) have been identified along the developing avian retinotectal pathway (Haj et al., 1999) and, more precisely, the heparan sulfate proteoglycans agrin and collagen XVIII have been identified as ligands for RPTP-σ (Aricescu et al., 2002). Agrin is present in the optic nerve and the tectobulbar pathway (Halfter et al., 1997) of the developing chick, indicating that RPTP-σ may interact with this substrate during embryogenesis. Site-directed mutagenesis of the extracellular domain of RPTP-σ revealed that binding interactions are mediated by a group of amino acids in the first Ig domain. This motif is highly conserved and therefore suggests that other species are likely to show similar receptor-ligand interactions (Aricescu et al., 2002). It remains to be determined what ligand-receptor interplay occurs in adult lesioned nerves and whether this interaction (or lack of) could ultimately contribute to regeneration failure.

5.6.2 Ligand-induced Inactivation of RPTPs

As with RPTP's substrates, the mechanisms triggered after RPTP ligand binding have yet to be defined. Several examples exist of a ligand-induced inactivation of RPTP catalytic activity. For instance, structural studies of the type IV RPTP-α, a distant relative of RPTP-σ, show that following ligand binding, a dimerization event occurs where the phosphatase domain of one subunit inserts its amino-terminal helix-turn-helix into the catalytic domain of the other and renders it inactive (Bilwes et al., 1996). In this

scenario, ligand-mediated dimerization may block the access of the phosphatase to its substrate and prevent dephosphorylation. Further evidence from the type V RPTP-β and the type I CD45, support the idea that ligand-induced dimerization may reduce phosphatase catalytic activity (Desai et al., 1993; Majeti et al., 1998; Majeti et al., 2000). Moreover, in the chick, RPTP-σ ligand/receptor interaction reduces the rate of retinal axon outgrowth (Ledig et al. 1999a), while transfecting *Xenopus* RGCs with a kinase deficient RPTP-σ, increases the rate of axonal outgrowth (Johnson et al. 2001). These data provide evidence that ligand binding to RPTP-σ promotes outgrowth by inactivating its catalytic activity.

The previous examples propose that a signaling response is initiated once phosphatase activity is blocked. This most likely occurs by the modification in levels of key phospho-tyrosine residues (either negative or positive regulatory phospho-tyrosine residues) on adaptor and effector signaling proteins with specialized phospho-tyrosine binding domains (e.g. Src homology 2 (SH2) and phospho-tyrosine binding (PTB)). De-phosphorylation of these residues influences their ability to bind and to activate their substrates and therefore affects cellular signaling (Tonks and Neel, 2001).

In accordance with the ligand-induced inactivation model, knockout animals for the phosphatases would simulate perpetual ligand binding and therefore produce continuous catalytic inactivation. In this context, the following scenario may explain the axonal regeneration that we report. The ligands for RPTP-σ are present along the developing retino-collicular path, thus leading to catalytic inactivation during embryogenesis. These ligands may later be developmentally downregulated or completely absent from the adult. Therefore, after RGC transection, when the distal axon

is once again within the optic nerve, the original substrates could be absent and consequently, the catalytic activity of the phosphatase turned on; a scenario that is exactly the opposite of what occurs during development.

RPTP-σ would not be the first protein that is permissive during development and later becomes inhibitory in mature life. For example, another member of the Ig-superfamily, MAG, is permissive to axonal growth during embryonic development, yet is highly inhibitory in the adult. For example, growth of embryonic E18 RGCs or DRGs younger than P4 is stimulated when plated on MAG substrate, whereas older RGCs or DRGs are stunted when grown on this same substrate (DeBellard et al., 1996; Cai et al., 2001). This suggests that, as with RPTP-σ, there is a developmental switch that changes MAG from being growth promoting to growth inhibitory. It will therefore be interesting to compare the rates of axonal extension of RPTP-σ (-/-) and wild-type RGC cultures at various developmental stages (embryonic versus postnatal).

Nevertheless, not all RPTPs bare the same quaternary structures and therefore the mechanisms of action observed in the aforementioned RPTPs cannot be extrapolated to all RPTPs. For example, the crystal structure of the type II RPTP-μ and LAR reveal that in these RPTPs, the helix-turn-helix domain that blocks phosphatase activity in RPTP-α would not be able to interact with the opposing monomer because of steric hindrance (Hoffmann et al., 1997; Nam et al., 1999). Therefore, a model reconciling all RPTPs cannot yet be built.

5.6.3 Separate Functions for the Extracellular and Intracellular Domains

Another possibility is that ligand binding does not influence phosphatase activity. It is possible that the extracellular domains of RPTPs merely act as cell adhesion molecules or as part of a receptor complex, independent of their cytoplasmic activity. The extracellular domains might contribute to localizing the RPTPs to defined regions of the plasma membrane where their catalytic activity is required, such as at focal adhesion sites (FAs) (Serra-Pages et al., 1995), while the intracellular domains would be entirely controlled by cytoplasmic effector proteins.

Several studies in which RPTP loss of function mutants are rescued demonstrate that the catalytic activity of the phosphatase is essential for proper function. For example, the phenotype of mutant *Drosophila* phosphatase, DPTP69D, cannot be rescued with catalytically inactive DPTP69D (Garrity et al., 1999). Furthermore, removing the phosphatase activity of PTP-μ simulates its loss of function phenotype with regards to axonal outgrowth on N-cadherin (Burden-Gulley and Brady-Kalnay, 1999), while the rescue of the mutant phosphatase Clr-1 phenotype in C. *elegans* also requires PTP catalytic activity (Kokel et al., 1998). Although these studies demonstrate the need for phosphatase activity, they do not indicate that the catalytic function is modulated by ligand binding and therefore we do not rule out a mechanism in which the extracellular and intracellular constituents function independently.

5.7 RPTPS AND GROWTH DYNAMICS

5.7.1 Cytosolic Effectors of RPTP Signaling

Contrarily to RTKs which add phosphate groups to their substrates, RPTPs remove phosphate groups, thereby making the identification and detection of substrates more difficult. An efficient strategy to detect the downstream targets of RPTP activity has yet to be devised even if a few candidate molecules have been identified, using substrate-trapping mutants and yeast interaction assays. For example, Ableson (Abl), a non-receptor protein tyrosine kinase, was shown in *Drosophila* to be antagonistic towards the DLAR (a *Drosophila* type IIa phosphatase) signaling pathway (Wills et al., 1999a). Overexpression of Abl yields a DLAR (-/-) phenotype while co-expressing wild-type Abl and DLAR, rescues this effect. Moreover, suppressing the Abl gene reverses the DLAR mutant phenotype. Although a direct *in vivo* interaction has yet to be shown, *in vitro* studies have demonstrated that the cytoplasmic domain of DLAR binds to, recruits and dephosphorylates the Abl kinase. These results strongly suggest, that DLAR and Abl mediate opposite effects on the same substrates (Wills et al., 1999b) (Figure 5-1, F, p.231).

To further elucidate the downstream events in this pathway, genetic screens to determine potential substrates for Abl have identified Enable (Ena) as a likely candidate. This actin binding protein, binds the phosphatase domains of DLAR (and DPTP69D) *in vitro* and can itself be phosphorylated by Abl and dephosphorylated by DLAR (Wills et al., 1999a). In addition phosphorylation levels of Ena are severally reduced in Abl mutants, further suggesting that Abl is a substrate for Ena (Gertler et al., 1995).

The role of Ena on axonal elongation is likely to be mediated through its actin binding domain which associates with the barbed end of actin filaments and promotes growth by interfering with actin-capping proteins (Bear et al., 2002). Ena has also been shown to interact with *profilin* (Reinhard et al., 1995). Although the exact function of *profilin* in the Abl pathway remains unclear, it plays an important role in motor axon outgrowth by regulating the growth cone actin cytoskeleton (Wills et al., 1999b). The involvement of Abl and Ena in the signaling of a type IIa phosphatase offers a direct link between this phosphatase signaling and the actin cytoskeleton (Figure 5-1, F, p.231).

5.7.2 RPTPs and Rho GTPases

Although a clear picture of RPTP signaling remains to be elucidated, certain data suggest that RPTPs are regulators of the Rho family of GTPases. The Rho guanine exchange factor (GEF), Trio, was shown to physically associate with the cytoplasmic domain of mammalian LAR (Debant et al., 1996a) and chick-PTP-σ (Johnson et al., 2003) (Figure 5-1, F, p.231). Trio consists of three domains: two GEF domains (GEF-D1 and GEF-D2) and a protein kinase domain (Debant et al., 1996). *In vitro*, the GEF-D1 domain has Rac1 activity and GEF-D2 RhoA GEF activity. Interestingly, overexpression of dominant negative Rac1 yields a DLAR (-/-)-like axon guidance phenotype (Kaufmann et al., 1998), supporting that Trio is acting on Rac1, downstream of DLAR. Concurrently, *in vivo*, Trio was found to enhance Abl and interact with Ena (Liebl et al., 2000). Nevertheless, while Trio is clearly involved in a variety of cytoskeletal remodeling events (Seipel et al., 1999; Blangy et al., 2000), its function downstream of type IIa RPTPs remains to be defined.

5.8 ERK1/2 ACTIVATION AND RGC REGENERATION

In this thesis, we showed two *in vivo* examples in which an increase in axon regeneration correlated with an increase in Erk1/2 phosphorylation. In the first case, we demonstrated that FGF-2 upregulation increased the levels of activated Erk 1/2 and that this rise was essential for regenerative growth. In the second scenario, we showed that removal of the gene coding for RPTP-σ significantly enhanced axonal regrowth after injury and promoted constitutive activation of Erk1/2. While both studies indicated that Erk1/2 activation correlates with axonal growth, the downstream targets of Erk1/2 remain to be determined. Several prospective substrates for Erk1/2 phosphorylation can influence axon growth. For example, Erk1/2 can activate nuclear transcription factors such as CREB that participate in gene expression and somal protein synthesis (Grewal et al., 1999) an influence axonal growth. Other attractive substrates include microtubule-associated proteins (MAPs) such as Tau and MAP2 (Roder et al., 1993; Garcia Rocha and Avila, 1995) and neurofilament proteins (Veeranna et al., 1998) which are all involved in the process of axonal growth. Lastly, the translational repressor eIF-4EB1 and translational activator eIF-4E at axonal terminals (Campbell and Holt, 2001; Ming et al., 2002) have been shown to be stimulated by Erk 1/2 with implications on local protein synthesis and axonal guidance, but may be also be an interesting target to consider for axonal growth.

Interestingly, recent work from our laboratory has shown that robust activation of the Erk1/2 pathway, although beneficial for neuronal survival, is in itself insufficient to promote regrowth (Pernet et al. 2005). In this study, a constitutively activated MEK1 (MEK.CA) construct was delivered to RGCs by means of an AAV, but was completely

incapable of enhancing growth after injury. There are at least two possible explanations to reconcile these findings with my observations: either 1) a separate pathway must be co-activated in order to obtain growth (Figure 5-1, C, p.231) or, 2) over-activation of the Erk1/2 pathway may have adverse affects on axonal integrity.

5.8.1 MAPK/Erk1/2 may Require a Co-signal to Promote Axon Growth

We demonstrated in Chapter 3 that Erk1/2 is required for RGC re-growth, yet Erk1/2 alone does not promote RGC regeneration (Pernet et al., 2005). These data suggest that Erk1/2 is permissive towards growth, but another independent signal is simultaneously or subsequently required to trigger growth. This model would reconcile why Erk 1/2 is needed to promote growth, but incapable of stimulating it when upregulated by itself. Evidence for this comes from *in vivo* experiments, where the MAPK cascade is required for the differentiation of naive PC12 cells to a neuronal like state (Pang et al., 1995) and yet, once they take on a neuron-like phenotype, the MAPK pathway is no longer required for neurite outgrowth (Sano and Kitajima, 1998). In this situation, MAPK is permissive but not instructive in the process leading to axonal growth. Similarly, in Chapter 3, we found that transiently blocking MAPK during the initial response to injury severely compromised the ability of RGCs to regenerate. This suggests that Erk 1/2 is an essential signal in the initial phase of growth. We also examined the involvement of PKCα, PLCγ and AKT; all downstream effectors of FGFR and all having documented roles in axonal growth (Huang and Reichardt, 2003; Segal, 2003; Doherty and Walsh, 1996; Boilly et al., 2000). These pathways, were not activated after AAV.FGF-2 injection, however. Thus it is likely that other yet to be identified

cascades must be activated concurrently with MAPK to achieve CNS axonal regeneration.

5.8.2 Adequate Levels of Erk1/2 are Required for Axonal Growth

Paradoxically, over activation of Erk1/2 may be detrimental to the neuron (Pei et al., 2002; Ferrer et al., 2003). Point mutations in the catalytic domain of MEK1 (as those in MEK1.CA) result in a four to five-hundred fold increase in activity. This level of activation may deviate from physiologically relevant levels and have detrimental effects on the stability of certain secondary substrates of MEK1, such as the microtubule and neurofilament networks. These could be consequently severely destabilized as a result of over-phosphorylation and hence be unable to adequately elongate (Roder et al., 1993; Garcia Rocha and Avila, 1995; Veeranna et al., 1998). For example, elevated levels of phospho-MEK1 and phospho-Erk1/2 have been associated with the progression of neurofibrillary degeneration in Alzheimer's disease (Pei et al., 2002). Similarly, elevated levels of phospho-Erk1/2 have been detected in neurofibrillary tangles and pre-tangles in familial tauopathies (Ferrer et al., 2003).

5.8.3 FGF-2 and Erk1/2 Activation

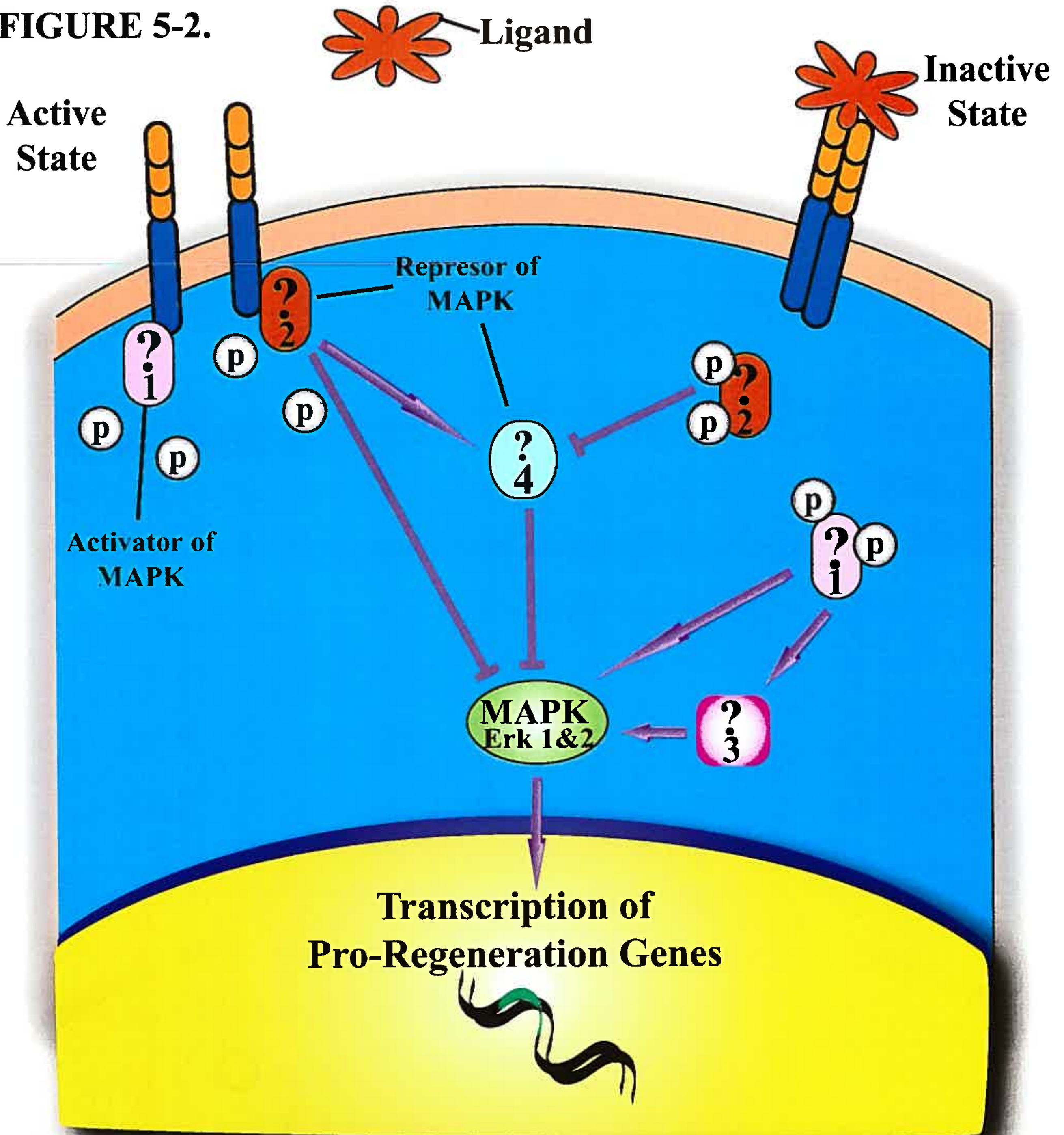
If Erk1/2 requires a specific window of activation intensity to promote growth, how would FGF-2 induce adequate stimulation of this pathway? Interestingly, FGF receptors have a built in "capacitor" that permits them to modulate the intensity of their cascades. Studies have shown that regardless of the stimulus, the signaling pathways activated by FGFRs are heavily conserved, varying primarily in intensity (Raffioni et al.,

1999). This intrinsic adjusting mechanism suggests that the intensity at which a given pathway is stimulated may in fact be a crucial variable for biological function. In addition, it ensures that the signaling cascades originating from FGFRs remain at physiological levels and consequently have physiologically relevant effects.

5.8.4 RPTP-σ and Erk1/2 Activation

How is Erk1/2 activated in RPTP-σ (-/-) mice and does its activation promote axonal growth? To explain the elevated levels of phospho-Erk1/2 observed in RPTP-σ (-/-) mice, the catalytic substrate of this phosphatase would have to be an upstream element in the MAPK pathway. For example, in a wild-type animal, the activated RPTP-σ would repress the given upstream signaling effectors that activate Erk 1/2. Conversely, in absence of RPTP-σ or when in an inactive state, the upstream activators in these pathways would remain phosphorylated and consequently, Erk 1/2 would be turned on (Figure 5-2).

The activation of MAPKs requires phosphorylation of both a tyrosyl and a threonyl residue. Therefore, either a dual specificity phosphatase (DSP) that can dephosphorylate both phospho-Tyr and phospho-Thr or two distinct single specificity phosphatases that can separately remove each phosphate group would be needed to silence MAPKs (Tonks and Neel, 2001). For this reason, it is unlikely that RPTP-σ (which can only catalyze the dephosphorylation of tyrosyl groups) acts directly on the MAPKs. It probably does so through intermediaries. Alternatively, RPTP-σ may modulate Erk 1/2 by directly influencing the signaling of RTKs. This can be accomplished either by limiting ligand independent signaling (Jallal et al., 1992) or by controlling the phosphorylation levels of activated RTKs (Sorby and Ostman, 1996).



Model for RPTPσ-mediated activation of Erk1/2.
RPTPσ (-/-) mice show endogenously elevated levels of activated Erk1/2, indicating that RPTPσ suppresses Erk1/2 in wild-type animals. Because Erk1/2 is activated by phosphorylation of both a threonyl and a tyrosyl residue, RPTPσ (being a tyrosine phosphatase) could not by itself turn off Erk1/2. Therefore, when in an active state, RPTPσ may either: 1) silence (de-phosphorylate) an activator of Erk1/2 (pink oval) or 2) activate a repressor of Erk1/2 (orange oval). Both mechanisms can either act alone or as part of a signaling pathway. The pink square (3) represents an intermediary effector in a stimulatory pathway while the blue oval (4) represents an effector in an inhibitory pathway.

5.10 GENERAL CONCLUSIONS

The research presented in this thesis focused on strategies to promote the survival and regeneration of RGCs after axonal transection. Our approach consisted in altering levels of two selected genes that are abundantly expressed in the developing nervous system: FGF-2 and RPTP-σ. In the first study, presented in Chapter 2 of this thesis, we made use of AAV vectors to re-express the FGF-2 gene within mature RGCs and studied its effects on survival and regeneration. This work provided the first evidence that a viral-based *in vivo* ocular gene therapy can be used to enhance axonal regrowth after acute injury of adult CNS neurons. Furthermore, it demonstrated that FGF-2, known to be an important modulator of axonal growth during development, may be harnessed to play a beneficial role in adult CNS regeneration.

These investigations were pursued in Chapter 3 by examining the signaling mechanisms underlying the observed regrowth. These experiments identified a central role for the MAPKs Erk1/2 in this process. Although our work strongly suggested the participation of Erk 1/2 in FGF-2-mediated axonal regrowth, it is likely that other, yet to be identified pathways, need to be co-activated to ensure adequate regrowth.

In Chapter 4 of this thesis, we investigated the role of RPTP-σ on RGC survival and regeneration. Although the retinas and optic nerves of RPTP-σ (-/-) mice did not show any morphological or cellular alterations when compared to wild-type animals, we observed a significant increase in adult RGC axonal regeneration. This enhanced growth was once again accompanied by increased Erk1/2 activation.

The studies described in this thesis helped identify two candidate molecules, FGF-2 and RPTP-σ, whose levels may be modulated to enhance CNS axonal regrowth

in the adult. The advent of novel and more penetrant strategies to regulate gene expression as well as the combination with approaches designed to overcome the myelin inhibitory environment are likely to yield even more promising results. Lastly, in this thesis we have outlined strategies that demonstrate the merits of gene modulation as an approach to enhance the regenerative capacities of adult lesioned CNS neurons.

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APPENDIX A

TrkB GENE TRANSFER PROTECTS RETINAL GANGLION CELLS FROM AXOTOMY-INDUCED DEATH IN VIVO

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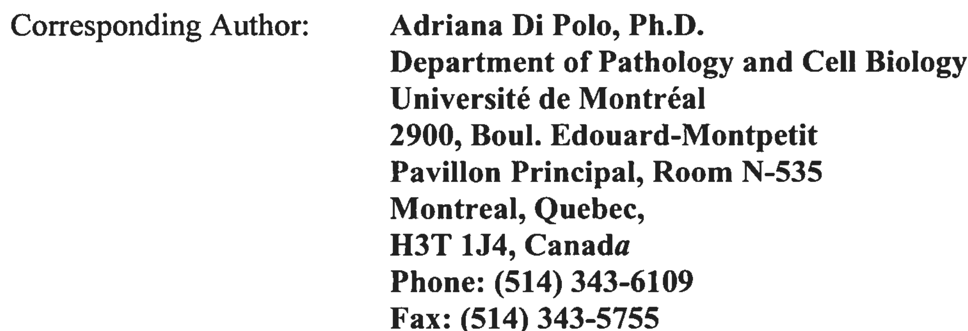
Running Title: Rescue of Axotomized RGCs by TrkB Gene Transfer



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ABSTRACT

Injury-induced downregulation of neurotrophin receptors may limit the response of neurons to trophic factors compromising their ability to survive. We tested this hypothesis in a model of CNS injury: retinal ganglion cell (RGC) death following transection of the adult rat optic nerve. TrkB mRNA rapidly decreased in axotomized RGCs to ~50% of the level in intact retinas. TrkB gene transfer into RGCs combined with exogenous BDNF administration markedly increased neuronal survival: 76% of RGCs remained alive at two weeks after axotomy, a time when >90% of these neurons are lost without treatment. Activation of MAP kinase, but not PI-3 kinase, was required for TrkB-induced survival. These data provide proof-of-principle that enhancing the capacity of injured neurons to respond to trophic factors can be an effective neuroprotective strategy in the adult CNS.

Key words: retinal ganglion cells, axotomy, gene transfer, TrkB, MAP kinase, cell survival

INTRODUCTION

Trauma or disease often leads to neuronal cell death and loss of functional connections. Strategies to promote the recovery of the injured central nervous system (CNS) have been limited by the death of large numbers of neurons soon after damage. Neurotrophins play important roles in the survival response of adult neurons after injury (Lewin and Barde, 1996; Sofroniew et al. 2001; Huang and Reichardt, 2001). In the visual system, brain-derived neurotrophic factor (BDNF) has been identified as a potent survival factor for axotomized retinal ganglion cells (RGCs) (Mey et al., 1993; Mansour-Robaey et al., 1994; Aguayo et al. 1996; Peinado-Ramon et al., 1996; Klöcker et al., 1998; Chen and Weber, 2001), known to express the BDNF receptor TrkB (Jelsma et al., 1993; Pérez et al., 1995; Rickman et al., 1995). For example, a single intravitreal injection of BDNF supports the survival of virtually all RGCs up to one week after axotomy (Mansour-Robaey et al., 1994). In contrast, nearly one-half of axotomized RGCs die in untreated retinas.

Long-term studies on the survival of RGCs have demonstrated that the effect of exogenous BDNF is temporary: it delays, but does not prevent, the onset of RGC death (Mansour-Robaey et al., 1994; Di Polo et al., 1998, Clarke et al., 1998). Administration of BDNF by repeated intravitreal injections or osmotic minipumps failed to extend the time-course of RGC protection (Mansour-Robaey et al., 1994, Clarke et al., 1998). Moreover, delivery of BDNF by adenovirus-infected retinal glial cells provided a sustained source of neurotrophin but resulted in transient RGC rescue (Di Polo et al., 1998). The mechanism underlying this loss of trophic support is poorly understood.

A possible explanation for the short-lived neuroprotective effect of BDNF is that the intrinsic capacity of RGCs to respond to this neurotrophin is compromised by injury (Di Polo et al. 1998). To test this hypothesis we addressed the following questions. First, are there axotomy-induced changes in TrkB gene expression in adult RGCs? Because TrkB mediates the response of RGCs to BDNF, we examined if axotomy leads to detectable changes in TrkB mRNA synthesis. Second, does *in vivo* upregulation of TrkB receptor expression extend RGC survival? To address this issue, we used recombinant adeno-associated viral (AAV) vectors to deliver the TrkB gene into adult RGCs. In contrast to adenovirus, AAV evokes minimal immune response in the host (Xiao et al., 1996; Xiao et al., 1997) and mediates long-term transgene expression that can persist in the retina for at least one year after vector administration (Dudus et al., 1999; Guy et al., 1999).

Finally, what signaling pathways mediate survival triggered by TrkB activation in adult RGCs *in vivo*? Upon binding to Trk receptors, neurotrophins stimulate multiple signaling pathways, including the MEK/mitogen activated protein (MAP) kinase and the phosphatidylinositol 3 (PI-3)/Akt kinase pathways (Segal et al., 1996; Kaplan and Miller, 2000). Studies on the participation of these pathways in neurotrophin-induced survival have been limited to neurons in culture (Xia et al., 1995; Bartlett et al., 1997; Dudek et al., 1997; Crowder and Freeman, 1998; Meyer-Franke et al., 1998; Skaper et al., 1998; Bonni et al., 1999; Dolcet et al., 1999; Klesse et al., 1999; Atwal et al., 2000; Orike et al., 2001) or at early developmental stages *in vivo* (Anderson and Tolkovsky, 1999; Hetman et al., 1999; Hee Han and Holtzman, 2000). Thus, the role of

neurotrophin-activated signaling pathways in the survival of adult CNS neurons *in vivo* remains undefined.

In this study, we demonstrate that TrkB mRNA levels are reduced in RGCs soon after transection of the optic nerve and before the onset of cell death. TrkB gene delivery to RGCs, in combination with exogenous BDNF, markedly extends the survival of these neurons following axonal injury. Our results indicate that activation of the MEK/MAPK pathway, but not the PI-3K/Akt pathway, mediates TrkB-induced RGC rescue.

MATERIALS AND METHODS

Quantitative *In Situ* Hybridization

Antisense oligonucleotide probes corresponding to base pairs (bp) 2324-2368 of the tyrosine kinase domain of full-length rat TrkB (44-mer) and bp 1899-1944 of the tyrosine kinase domain of full length rat TrkC (48-mer) were labeled with ^{35}S-dATP (NEN, Markham, ON) using terminal deoxynucleotidyl transferase (GIBCO Biotechnologies, Burlington, ON). These probes correspond to non-homologous regions of TrkB or TrkC cDNAs, respectively. *In situ* hybridization was performed on radial cryosections (7-10 µm) prepared from fresh retinal tissue. Hybridization was carried out essentially as described (Dangerlind et al., 1992). Briefly, sections were hybridized overnight at 42°C with 10^7 cpm of radiolabeled probe in 1 ml of hybridization solution in saline sodium citrate (SSC) containing formamide (50%), dextran sulfate (100 g/lt), sarcosyl (1%), phosphate buffer (20 mM, pH 7.0), salmon sperm DNA (500 g/lt), and dithiothreitol (200 mM). Following hybridization, slides were washed in SSC and then dehydrated in ascending concentrations of ethanol. Slides were dipped in Kodak NTB2

autoradiographic emulsion (Kodak, Rochester, NY), dried and stored in a light proof box at 4°C for 1-2 months. Sections were then developed for 5 min in Kodak D-19 at 20°C, rinsed and fixed for 5 min in Kodak fix. Tissue sections were counterstained with 0.25% thionin (Sigma, Oakville, ON) and mounted in Entellan (VWR, Mississauga, ON). Negative controls included sections hybridized with sense probes, sections treated with RNAse A (50 µg/ml, Boehringer-Mannheim, Laval, QC) at 37°C for 30 min and slides incubated with 100-fold excess of unlabeled TrkB or TrkC receptor probe. An image analysis system (Image1, Universal Imaging Corp., West Chester, PA) was used to determine the number of autoradiographic silver grains per neuron. The average number of grains per RGC was counted for experimental and control retinas, mounted on the same slide, and normalized to the levels found in the corresponding contralateral intact retina. Data and statistical analysis was performed using SigmaStat (Jandel, Corte Madera, CA).

Preparation of Recombinant AAV Vectors

A construct containing a c-myc epitope tagged form of full-length rat TrkB, provided by Dr. P. Barker (McGill University), was used to generate AAV vectors. The TrkB gene was inserted downstream of the CMV promoter in plasmid pTR-UF5 (Zolotukhin et al., 1996) containing the AAV terminal repeat sequences and a simian virus 40 polyadenylation sequence. An AAV control vector containing the GFP gene, but lacking the TrkB gene, was generated in identical fashion. Packaging of AAV vectors was performed as described previously (Hauswirth et al., 2000). Briefly, low passage human 293 cells were co-transfected with pTR-UF5-TrkB and the helper

plasmid pDG (Grimm et al., 1998) that contains both the AAV genes (*rep* and *cap*) and the adenovirus genes required for AAV propagation. After harvesting the cells, the virus was extracted by freezing and thawing the cells and the supernatant was then clarified by low speed centrifugation. Crude cell lysates containing AAV were loaded onto Iodixanol (Nycomed Pharma, Denmark) density step gradients for purification. The fraction containing AAV was further purified by heparin affinity chromatography (Sigma). Purified AAV was concentrated and desalted by centrifugation through Biomax 100K filters (Millipore, Mississauga, ON) according to the manufacturer's instructions. Viral titers, determined by quantitative-competitive polymerase chain reaction and by infectious center assay (Hauswirth et al., 2000), were in the order of 5 x 10^{12} physical particles/ml. No helper virus was used in this preparation to avoid contamination of AAV stocks with adenovirus. Wild-type AAV contamination was below the levels of detection (one part in 10^7).

Surgical Procedures

Animal procedures were performed in accordance with the guidelines for the use of experimental animals (Olfert et al., 1993). All surgeries were performed in female adult Sprague-Dawley rats (180-200 g) under general anesthesia (7% chloral hydrate; 0.42 mg per g of body weight, i.p.). Viral vectors (5 µl total volume) were injected into the vitreous chamber in the dorsal hemisphere of the left eye using a 10-µl Hamilton syringe fitted with a 32-gauge needle. The tip of the needle was inserted through the sclera and retina into the vitreous space using a posterior approach. This route of administration avoided injury to other structures of the eye, such as the iris or lens,

shown to promote RGC survival (Mansour-Robaey et al., 1994; Leon et al., 2000). The right eye was left untouched and served as internal contralateral control for each animal. For RGC survival experiments, cells were retrogradely labeled with 2% FluoroGold (Fluorochrome, Englewood, CO) in 0.9% NaCl containing 10% dimethyl sulfoxide (DMSO) by application of the tracer to both superior colliculi. Seven days later, the left optic nerve was transected 0.5-1 mm from the optic nerve head. In some experimental groups, the following agents were injected intravitreally in the superior hemisphere in a total volume of 5 μl at the time of axotomy: the MEK inhibitor PD98059, 100-200 μM in phosphate buffered saline (PBS) containing 20% DMSO (New England Biolabs [NEB], Mississauga, ON); the PI-3 kinase inhibitor LY294002, 100-800 μM in PBS with 20% DMSO (Sigma); BDNF protein, 1 μg/μl in PBS containing 1% bovine serum albumin (provided by Regeneron Pharmaceuticals, Tarrytown, NY) or vehicle (PBS or PBS containing 20% DMSO). Rats were sacrificed by intracardial perfusion with 4% paraformaldehyde and both the left (optic nerve lesion) and right (intact control) retinas were dissected, fixed for an additional 30 min and flat-mounted vitreal side up on a glass slide for examination of the ganglion cell layer.

Immunocytochemistry

Rats were perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) and the eyes were immediately enucleated. The anterior part of the eye and the lens were removed and the remaining eye cup was immersed in the same fixative for 2 hr at 4°C. Eye cups were equilibrated in graded sucrose solutions (10-30% in PB) for several hours at 4°C, embedded in O.C.T. compound (Tissue-Tek, Miles

Laboratories, Elkhart, IN) and frozen in a 2-methylbutane/liquid nitrogen bath. Radial cryosections (6-12 μm) were collected onto gelatin-coated slides and processed. Sections were incubated in 10% normal goat serum (NGS), 0.2% Triton X-100 (Sigma) in PBS for 30 min at room temperature to block non-specific binding. Each primary antibody was added in 2% NGS, 0.2% Triton X-100 and incubated overnight at 4°C. Sections were then incubated with the appropriate secondary antibody for 1 hr at room temperature, washed in PBS, mounted using an anti-fade reagent (SlowFade, Molecular Probes, Eugene, OR) and visualized with fluorescent microscopy (Zeiss Axioskop 2 Plus) Antibodies: Anti-c-myc antibody (5 μg/ml; Oncogene, Cedarlane Laboratories, Hornby, ON), Anti-GFAP (5 μg/ml; Chemicon International, Temecula, CA), Anti-Vimentin (5 μg/μl; Chemicon), FITC-conjugated Isolectin B4 (20 μg/ml, Sigma) and ED-1 (10 μg/ml, Chemicon), fluorophore-conjugated goat anti-mouse IgG (red, 4 μg/ml, Alexa™ 594, Molecular Probes).

Neuronal Quantification

For quantification of AAV-mediated TrkB expression in neurons in the ganglion cell layer, histological sections of the retina were produced along the dorsal-ventral plane of the eye and serial sections that passed through the optic nerve head, used as reference, were analyzed. The entire number of labeled cells per section was then counted using a fluorescent microscope. Six to eight serial sections per eye were typically counted per experimental animal. For neuronal survival studies, the ganglion cell layer was examined in whole-mount retinas with fluorescence microscopy and FluoroGold-labeled neurons were counted in 12 standard retinal areas as described

(Villegas-Perez et al., 1993). Data analysis and statistics were performed using the SigmaStat program (Jandel) by a one-way ANOVA or a Student's *t* test (paired groups).

Western Blot Analysis

Fresh retinas were rapidly dissected and homogenized with an electric pestle (Kontes, Vineland, NJ) in ice-cold lysis buffer: 20 mM Tris (pH 8.0), 135 mM NaCl, 1% NP-40, 0.1% SDS and 10% glycerol supplemented with protease inhibitors (1 mM phenylmethyl sulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.5 mM sodium orthovanadate). After incubation for 30 min on ice, homogenates were centrifuged at 10,000 rpm for 10 min, the supernatants were removed and resedimented for an additional 10 min to yield solubilized extracts. Alternatively, 200-300 μg of protein was immunoprecipitated with anti-pan Trk 203, provided by D. Kaplan, as described (Stephens et al., 1994). Retinal extracts (75-100 μg) or immunoprecipitated samples were resolved on 8% SDS polyacrylamide gels and transferred to nitrocellulose filters (Bio-Rad Life Science, Mississauga, ON). To block non-specific binding, filters were placed in 10 mM Tris (pH 8.0), 150 mM NaCl, 0.2% Tween-20 (TBST) and 5% dry skim milk for 1 hr at room temperature. Blots were then incubated for 16-18 hrs at 4°C with each of the following primary antibodies: anti-phospho-p44/42 MAP kinase (0.8 μg/ml, NEB), anti-p44/42 MAP kinase (20-80 ng/ml, NEB), anti-phospho-Akt (0.6 μg/ml, NEB), anti-Akt (0.1 μg/ml, NEB) or anti-phosphotyrosine (4G10, 1 μg/ml, Upstate Biotechnology, Waltham, MA). Membranes were washed in TBST and incubated in anti-rabbit or anti-mouse peroxidase-linked secondary antibodies (0.5 μg/ml, Amersham Pharmacia, Baie d'Urfé, QC) for 1 hr at room temperature. Blots

were developed with a chemiluminescence reagent (ECL, Amersham Pharmacia) and exposed to X-OMAT (Kodak) imaging film.

RESULTS

TrkB mRNA Levels are Downregulated in Axotomized RGCs

We investigated the changes in TrkB gene expression in axotomized RGCs by quantitative *in situ* hybridization. An oligonucleotide probe specific for the catalytic domain of full-length rat TrkB was used in these experiments. Probe hybridization to intact retinas produced a robust positive signal, visualized by dark field microscopy at low magnification, in cells of the ganglion cell layer and inner nuclear layer (Figure 1A). One week after axotomy, the hybridization signal was markedly reduced only in the ganglion cell layer (Figure 1B). No signal was detected in retinal sections hybridized with a sense probe or treated with RNAse A (not shown). The number of autoradiographic silver grains was quantified in single RGC cell bodies, visualized with thionin staining in intact and injured retinas using bright field microscopy (Figures 1C and 1D). Experimental and control retinas were mounted on the same slide and processed under identical conditions. As early as three days after axotomy, TrkB mRNA in RGCs was decreased by ~40% (11.9±4 grains/cell, mean ± standard deviation) relative to unoperated contralateral retinas (100%= 20.3±2 grains/cell) (Figure 1E). The decline in TrkB gene expression was detected prior to the onset of RGC death that typically starts at five days post-axotomy (Berkelaar et al., 1994). The average number of grains per RGC continued to decrease during the first week after injury and remained low, between 45% (8.9±4 grains/cell) and 60% (12±2 grains/cell) of the contralateral

control levels, thereafter. These results indicate that the reduction of grain density in RGCs can not be attributed to cell death caused by axotomy but to reduced TrkB mRNA levels in surviving RGCs. Amacrine cells, which are known to express TrkB (Cellerino and Kohler, 1997), populate the inner nuclear layer and the ganglion cell layer (Perry, 1981) but do not project axons to the optic nerve. Because amacrine cells are typically smaller than RGCs, cellular profiles of less than 70 μm^2 were identified as amacrine cells as described (McKerracher et al., 1993). In contrast to RGCs, TrkB mRNA levels in amacrine cells remained unchanged after axotomy (Figure 1 F) confirming our ability to distinguish between these two cellular populations.

AAV-mediated TrkB is Predominantly Expressed by Adult RGCs

For gene transfer experiments, a recombinant AAV vector containing a c-myc tagged full-length rat TrkB gene under control of the CMV promoter was produced. Intravitreal injection of AAV.TrkB resulted in TrkB protein expression exclusively in the ganglion cell layer, as detected by immunostaining with an antibody against c-myc (Figure 2A). No other retinal layer showed positive c-myc immunostaining. Robust c-myc labeling was localized on neuronal cell bodies and RGC axons in the fiber layer. Intact contralateral retinas or retinas infected with a control virus did not show positive c-myc staining. Because displaced amacrine cells account for ~40% of the total number of neurons in the ganglion cell layer of the rat retina (Perry, 1981), we sought to identify the cellular targets of AAV infection in this system. For this purpose, we performed co-localization studies in retinas from eyes that received a single intravitreal injection of AAV.TrkB followed by retrograde labeling of RGCs with the tracer FluoroGold applied

to both superior colliculi. Double-labeling experiments demonstrated that the majority of RGCs, visualized with FluoroGold (Figure 2B), also produced virally-mediated TrkB (Figure 2C). AAV.TrkB transgene expression reached a plateau at 3-4 weeks after administration of the vector and persisted for at least 10 weeks in the intact retina. The mechanism underlying the delay in the onset of gene product expression *in vivo*, characteristic of AAV vectors (Malik et al., 2000), remains undefined but may be related to the need to convert input viral single-stranded DNA to a double-stranded form prior to gene expression (Ferrari et al., 1996).

To determine the efficacy of adult RGC transduction by AAV, we quantified the number of c-myc and FluoroGold-labeled cells in retinal sections at 4 weeks after vector administration. An average of 68% of FluoroGold-labeled neurons were also c-myc positive, indicating that RGCs were successfully transduced by the AAV.TrkB vector (Figure 2D). A small population of c-myc positive cells (~8%) was not labeled with FluoroGold and may represent amacrine cells transduced by AAV or RGCs that did not incorporate the retrograde tracer. Immunostaining with an antibody against glial fibrillary acidic protein (GFAP) or vimentin, in combination with c-myc, did not show co-localization (not shown), indicating that glial cells in the retina were not infected by AAV. Taken together, these results indicate that RGCs are the primary target for AAV transduction in the adult inner retina.

TrkB Upregulation Protects RGCs from Axotomy-Induced Cell Death

The effect of AAV.TrkB on RGC survival *in vivo* was tested using the experimental protocol outlined in Figure 3A. Four weeks after intraocular injection of

viral vectors, RGCs were retrogradely labeled and subsequently axotomized. Retinas were examined histologically at 7, 14, 21 and 28 days after optic nerve transection to determine the density of surviving RGCs (Figure 3B and Table 1). A single intraocular injection of AAV.TrkB resulted in moderate, but significant, neuroprotective effect at one and two weeks after axotomy. For example, AAV.TrkB supported the survival of 27% of RGCs compared to 9.6% neuronal survival induced by the control virus at two weeks after injury. We then examined if AAV.TrkB treatment combined with a single intravitreal injection of BDNF protein at the time of axotomy could potentiate RGC survival. This approach yielded markedly higher RGC densities at all times examined (Figure 3B). At two weeks after axotomy, AAV.TrkB with BDNF protected 76% of RGCs while independent administration of BDNF or AAV.TrkB promoted 38% or 27% neuronal survival, respectively. This effect, although reduced, was still significant at three and four weeks after axotomy resulting in higher neuronal densities and better preservation of cellular integrity than with AAV.TrkB (Figure 4A and 4B) or BDNF alone.

To determine whether TrkB gene transfer was involved in this effect, we examined the correlation between neuronal survival and transgene product expression. At three weeks after axotomy, virtually all surviving RGCs (~96%) also expressed AAV-mediated TrkB (Figures 4C and 4D). Positive c-myc staining was clearly visualized on the surface of RGCs that remained alive, suggesting that TrkB delivered by AAV was effectively transported to the membrane. Together, these results strongly suggest that upregulation of receptor expression supports RGC survival in this injury model. Microglia and macrophages, that may have incorporated FluoroGold after

phagocytosis of dying RGCs, were excluded from our analysis of neuronal survival based on their morphology which can be easily identified in retinal whole mounts (Figure 4B). In addition, the identity of these cells was confirmed by immunostaining with antibodies against the microglia and macrophage markers Isolectin-B4 and ED-1 (not shown).

Retinal MAPK and Akt are Activated Following TrkB Gene Transfer

To identify the signaling pathways involved in AAV.TrkB-induced neuroprotection we examined whether TrkB gene transfer had an effect on receptor tyrosine phosphorylation, the initial step for transduction of survival signals. Western blot analysis of immunoprecipitated Trk proteins using an antibody against phosphotyrosine demonstrated TrkB activation at 5 weeks (Figure 5A) and 10 weeks (not shown) following intravitreal injection of AAV.TrkB. We then compared AAV-induced TrkB tyrosine phosphorylation with that produced by a single intravitreal injection of BDNF recombinant protein. Robust TrkB tyrosine phosphorylation was detected at 48 hrs after administration of BDNF (Figure 5A) but not at 10 days after injection of the neurotrophin (not shown). These data indicate that AAV.TrkB induced moderate but sustained levels of TrkB activation, while BDNF protein provoked robust but transient stimulation of this receptor. Injection of PBS or AAV.GFP did not have any effect on TrkB activation (Figure 5A).

We next examined the stimulation of known downstream components of the TrkB signaling cascade, the MAP kinases Erk1/2 and Akt, using antibodies that specifically recognized the phosphorylated forms of these kinases. Of interest, low but

detectable levels of Erk1/2 and Akt phosphorylation were observed in intact uninjected retinas indicating basal activation of these kinases (Figures 5B and 5C). In contrast, intraocular injection of AAV.TrkB, in the absence of exogenous BDNF, resulted in marked activation of both Erk1 and Erk2 compared to the low levels of phosphorylation observed in control retinas injected with PBS (Figure 5B) or AAV.GFP (not shown). Similarly, a marked increase in Akt phosphorylation was observed following injection of AAV.TrkB (Figure 5C). These results clearly demonstrate stimulation of both the MAPK and the Akt pathways in RGCs following AAV-mediated TrkB gene transfer. As in the case of TrkB phosphorylation, AAV.TrkB-induced activation of Erk1/2 and Akt was detected at 5 weeks and 10 weeks after intraocular administration of the viral vector (Figure 5B and 5C).

TrkB-Induced Survival of Axotomized RGCs *In Vivo* Occurs Via a MAPK-Dependent Pathway

To determine whether MAPK and/or Akt signaling were involved in TrkB-mediated survival of axotomized RGCs, pharmacological inhibitors of specific components of each of these pathways were used. PD98059 selectively inhibits MEK (Dudley et al., 1995), the upstream activator of MAPK, and LY294002 is a selective inhibitor of PI-3K (Weber et al., 1997), an upstream Akt activator. First, we determined the concentration of these compounds required to effectively block AAV.TrkB-induced activation of retinal Erk1/2 and Akt *in vivo*. For this purpose, eyes were injected with AAV.TrkB and four weeks later, the inhibitor PD98059 or LY294002 was delivered by a single injection into the vitreous chamber. Inhibition of Erk1/2 or Akt phosphorylation

was analyzed by western blots of whole retinal homogenates collected at 48 hrs or 14 days after administration of each compound (Figure 6). The inhibitory effect of PD98059 or LY294002 *in vivo* was dose dependent. Intravitreal injection of 200 μM of PD98059 effectively blocked Erk1/2 phosphorylation (Figure 6A), and injection of 800 μM of LY294002 inhibited Akt phosphorylation (Figure 6B). Because the volume of the vitreous chamber in the adult rat eye is approximately 60 μl, the final intravitreal concentration of these drugs was ~16.7 μM for PD98059 and ~66.7 μM for LY294002, well within the range shown to work effectively *in vitro* (Atwal et al., 2000). Of interest, effective inhibition of AAV.TrkB-induced retinal MAPK and Akt activation was sustained for at least 14 days following administration of these drugs (Figures 6A and 6B). There was no reduction in the phosphorylation of Erk1/2 in the presence of LY294002 (Figure 6B). Similarly, phosphorylation of Akt was not decreased with PD98059 (Figure 6A). These results confirmed the specificity of the inhibitory effect of each of these compounds *in vivo*. Examination of histological sections and whole mounts of the treated retinas found no inherent toxic or pro-survival effect of these chemicals.

We then carried out *in vivo* RGC survival assays after AAV.TrkB delivery in the presence of PD98059 or LY294002 injected intravitreally at the time of optic nerve transection (Figure 7 and Table 1). Administration of PD98059 (200 μM) resulted in complete inhibition of the survival effect produced by TrkB gene transfer. In this situation, RGC density was low and similar to that found in retinas treated with the control virus AAV.GFP. In contrast, blockade of PI-3K activation with LY294002 (800 μM) did not change the neuroprotective effect induced by AAV.TrkB. These results

demonstrate that while the MEK/MAPK pathway is essential for TrkB-mediated survival of injured adult RGCs *in vivo*, the PI-3K/Akt pathway has no apparent role in this survival effect.

DISCUSSION

Injury-induced changes in the expression of critical components of survival pathways may exacerbate the death of CNS neurons. Here we demonstrate that expression of TrkB, a key player in BDNF-induced survival, is downregulated in adult RGCs following axotomy close to the eye. The striking reduction in TrkB mRNA within two weeks of axotomy correlated with the inability of RGCs to survive in response to BDNF, despite sustained neurotrophin delivery (Di Polo et al., 1998). Downregulation of neuronal Trk receptors has been reported in the damaged rat spinal cord (Kobayashi et al., 1997; Liebl et al., 2001) and brain (Venero et al., 1994) suggesting that this is a common response to acute injury among some CNS neurons. In contrast to TrkB, BDNF mRNA levels have been shown to increase transiently in cells of the ganglion cell layer after optic nerve crush (Gao et al., 1997), but there is no evidence that this local upregulation delays the onset of RGC death. Taken together, these results suggest that reduced TrkB expression in injured RGCs contributes to their desensitization to exogenous, and possibly endogenous, BDNF.

This hypothesis was tested by increasing the capacity of axotomized RGCs to respond to BDNF following TrkB gene delivery. A key issue in the success of these experiments was to identify a vector system for efficient infection of adult RGCs *in vivo*. Previous studies showed that intravitreal injection of AAV resulted in transduction of

cells in the ganglion cell layer (Grant et al., 1997; Ali et al., 1998; Dudus et al., 1999), but given that both RGCs and amacrine neurons populate this retinal layer, the specific cellular tropism of this virus was not established. Our data indicate that RGCs are the main target for AAV infection in the inner retina. Furthermore, we show that an average of 68% of the total RGC population was effectively infected by AAV and, in some individual retinas, up to 75% of RGCs were transduced. Interestingly, heparan sulfate proteoglycan, the primary receptor responsible for AAV attachment to the host cell (Summerford and Samulski, 1998), is expressed on adult RGCs (A. Di Polo, unpublished observations) providing a likely mechanism behind the tropism of this virus in the inner retina.

Two main findings support our conclusion that AAV.TrkB promotes the survival of RGCs by augmenting their capacity to respond to BDNF. First, the neuroprotective effect of AAV.TrkB dramatically increased when it was combined with a single intraocular injection of BDNF. This survival effect was apparent at all times examined but was particularly striking at two and three weeks after axotomy. For example, an average of 76% of RGCs remained alive at two weeks post-axotomy, a time when typically less than 10% of these neurons survive in the absence of treatment. Although the total number of neurons in all experimental groups was lower at three weeks after injury, the combined effect of AAV.TrkB and BDNF yielded an ~6-fold higher number of surviving neurons than AAV.TrkB alone. Furthermore, AAV.TrkB and BDNF administration effectively extended RGC survival by at least 10 days, a substantial amount of time compared to the shorter effect of BDNF protein alone (Mansour-Robaey et al., 1994; Peinado-Ramon et al., 1996; Clarke et al., 1998) or BDNF gene transfer (Di

Polo et al., 1998) which delayed RGC death by only 2 days. To our knowledge, this level and extent of survival has not been previously achieved in a model of acute RGC death.

Secondly, we found a tight correlation between expression of AAV-mediated TrkB and neuronal survival. At three weeks after axotomy, ~96% of the surviving RGCs expressed c-myc tagged TrkB suggesting that upregulation of this receptor increased the capacity of these neurons to respond to BDNF. Interestingly, studies using highly purified post-natal day 8 RGC cultures have shown that TrkB internalization is likely to contribute to the loss of trophic responsiveness after axotomy (Meyer-Franke et al., 1998; Shen et al., 1999). Although we can not rule out the possibility that TrkB internalization restricts RGC survival in this model, our results suggest that within the first weeks of injury a sufficient quantity of virally-delivered TrkB is available to elicit cell survival following trophic stimulation, perhaps overwhelming any internalization process.

Our data demonstrate that although both MAPK and Akt pathways were activated following TrkB gene transfer, only selective inhibition of MEK blocked AAV.TrkB-induced survival while PI-3K inhibition had no effect. This finding strongly suggests that the MEK/MAPK pathway, but not the PI-3K/Akt pathway, is necessary for AAV.TrkB-induced survival of axotomized RGCs. MEK inhibition has also been shown to block the ability of early post-natal RGCs to survive after axonal injury (Shen et al., 1999). Therefore, the MEK/MAPK pathway appears to be a common survival signaling mechanism used by both developing and adult axotomized RGCs in response to TrkB stimulation. MEK/MAPK activity may regulate the survival of RGCs by activating the

transcriptional factor CREB, which is known to mediate BDNF-induced survival of cultured cerebellar neurons (Bonni et al., 1999). Of interest, CREB phosphorylation in RGCs has been observed following BDNF stimulation in mouse retinal organ cultures (Wahlin et al., 2000).

It is now well established that Trk activation leads to the stimulation of multiple signaling pathways in the same neuron (Segal and Greenberg, 1996; Kaplan and Miller, 1997; Klesse et al., 1999). Recent *in vitro* studies in NGF-dependent neonatal sympathetic neurons, have indicated that while TrkA uses PI-3K to stimulate cell survival, TrkB uses both PI-3K and MEK (Atwal et al., 2000). There is growing evidence, however, indicating that the specific role of each of these pathways on Trk-induced survival appears to be tightly dependent on neuronal type and injury modality. Although the PI-3K pathway is critical in growth factor-mediated survival upon trophic deprivation in a variety of PNS and CNS neurons *in vitro* (Kaplan and Miller, 2000), the MAPK pathway has been implicated in the survival induced by neurotrophins following physical or chemical injury. MAPK signaling is also responsible for the *in vitro* protection of cerebellar granule neurons from oxidative stress (Skaper et al., 1998), cortical neurons from camptothecin-induced apoptosis (Hetman et al., 1999), and sympathetic neurons from cytosine arabinoside toxicity (Anderson and Tolkovsky, 1999). *In vivo*, the MAPK pathway has been shown to protect the neonatal brain from hypoxic-ischemic injury (Hee Han and Holtzman, 2000). What then is the role of PI-3K/AKT activation in adult RGCs following TrkB stimulation? Although not apparently involved in BDNF/TrkB-induced survival, this pathway may mediate other, as yet undefined, functions closely related to the RGC response to trophic stimulation such as

axonal regrowth. Interestingly, the PI-3K pathway has been implicated in RGC protection by insulin growth factor and tumor necrosis factor alpha (Kermer et al., 2000; Diem et al., 2001) suggesting that different neurotrophic factors promote the survival of adult RGCs by distinct intracellular signaling mechanisms.

In summary, our findings indicate that downregulation of TrkB following RGC injury is a key mechanism underlying the short-lived survival effect produced by exogenous BDNF. We report a novel neuroprotective strategy based on TrkB gene delivery *in vivo* that significantly enhances the survival of RGCs following axotomy. This strategy may have therapeutic potential in the injured CNS, however, future studies are required to resolve important issues such as the efficacy of this approach in models of protracted neuronal death and to evaluate the functional state of the rescued neurons.

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FIGURES

Figure 1. TrkB mRNA in intact and injured adult rat retinas

Dark field micrographs after *in situ* hybridization using a probe that selectively recognizes the tyrosine kinase domain of full-length rat TrkB are shown for intact (A) and axotomized (B) retinas. TrkB mRNA is predominantly expressed in RGCs and displaced amacrine cells in the ganglion cell layer (GCL), as well as in amacrine cells in the inner nuclear layer (INL). After transection of the optic nerve, TrkB mRNA was reduced only in cells of the GCL (B, 1 week after axotomy). Bright field micrographs of the RGC layer of intact (C) and axotomized retinas (D, 1 week after axotomy) from sections counterstained with toluidine blue were used to quantify the number of grains corresponding to the TrkB probe on neuronal cell bodies. Size criteria were used to distinguish RGCs (>70 μm^2) from displaced amacrine cells (<70 μm^2). The non cross-reactivity of the TrkB probe with TrkC, another neurotrophin receptor found in neurons of the ganglion cell layer, was established by using a 100-molar excess of either unlabeled TrkC probe or TrkB probe in the hybridization solution. Unlike the excess of TrkB probe, the TrkC probe did not compete out the TrkB hybridization signal (not shown). (E) Time-course of TrkB mRNA downregulation in axotomized RGCs was assessed by quantitative *in situ* hybridization. The average number of grains per RGC was normalized to the level found in contralateral intact retinas (100%). TrkB mRNA was reduced in RGCs, but not in amacrine cells (F), to ~50-60% of the levels in intact control retinas (n=4-6 per time point, P<0.001). This decrease in TrkB gene expression was detected as early as 3 days after axotomy before the onset of RGC death. ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bars: 50 µm (A,B) and 25 µm (C,D).

FIGURE 1.

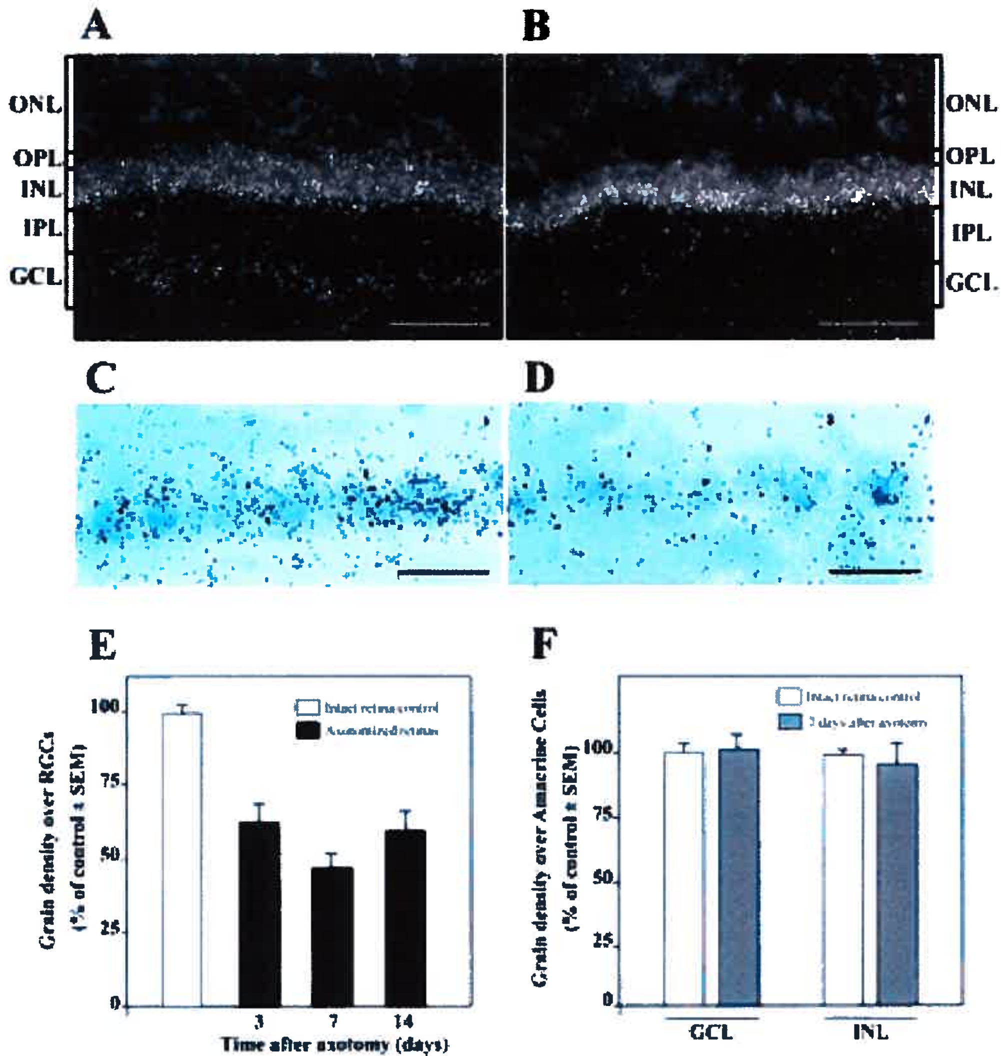
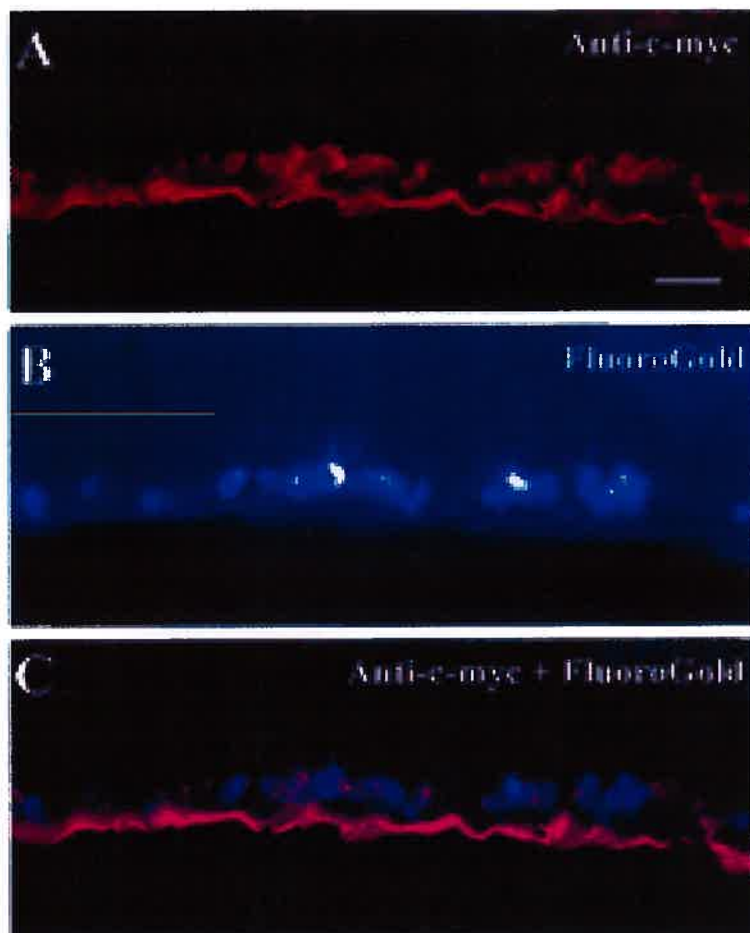


Figure 2. AAV-mediated TrkB gene product expression in adult RGCs

Fluorescent microscopy images of retinal sections following intraocular injection of AAV.TrkB. (A) Virally-mediated TrkB was visualized using an anti-c-myc antibody that stained neuronal cell bodies in the ganglion cell layer as well as RGC axons in the fiber layer. (B) RGCs were identified by retrograde labeling upon application of the tracer FluoroGold to the superior colliculus, the main target for these neurons in the rat brain. (C) Superimposition of the images in (A) and (B) demonstrates that the vast majority of RGCs expressed the AAV.TrkB gene product. Adjacent sections labeled with FluoroGold alone did not show bleed-through between filters (not shown). Scale bar: 10 μm. (D) Quantification of the number of cells in the ganglion cell layer expressing AAV-mediated TrkB protein at 4 weeks after intraocular injection of the vector. The total number of RGCs per retinal section was assessed by their FluoroGold label (FG+) and compared to the number of cells co-stained with an anti-c-myc antibody (c-myc+), that recognized AAV-mediated TrkB, and FluoroGold. An average of 68% of the total number of RGCs per retinal section expressed the AAV.TrkB transgene ($n=4$, $P<0.001$). Few c-myc positive cells (~8%) were not labeled with FluoroGold (FG-). These results indicate that RGCs are the primary cellular target for AAV infection in the inner retina.

FIGURE 2.



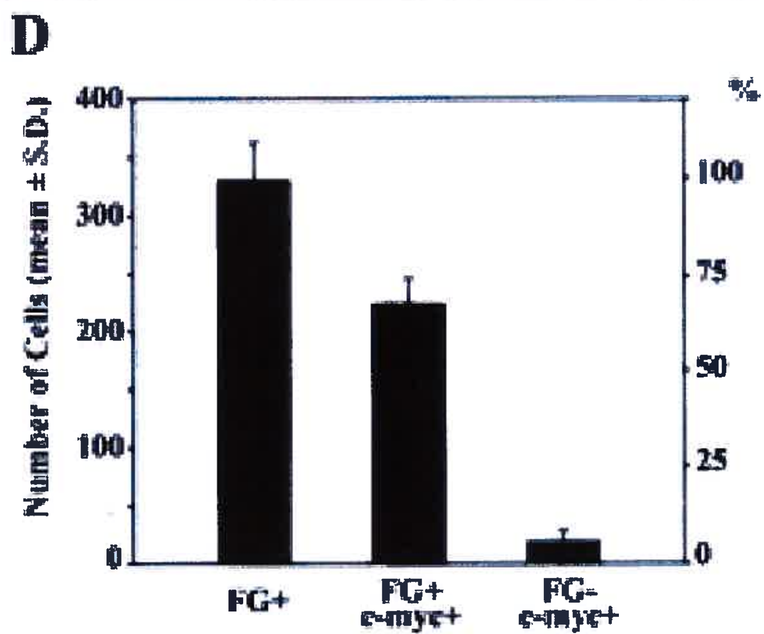


Figure 3. Effect of TrkB gene transfer on the protection of axotomized RGCs *in vivo*

(A) Outline of the experimental protocol used to test the effect of AAV.TrkB on RGC survival. Four weeks after intraocular injection of viral vectors, the time required for transgene expression to reach a plateau in the adult rat retina, RGCs were retrogradely labeled with FluoroGold and subsequently axotomized. RGC survival was assessed by quantification of fluorescent neurons in whole-mounted retinas. (B) Quantitative analysis of RGC survival following intravitreal injection of: AAV.TrkB (gray bars), BDNF recombinant protein (hatched bars), AAV.TrkB and BDNF protein (solid bars), and AAV.GFP (open bars) at 7, 14, 21 and 28 days after optic nerve transection (n= 3-15 rats per group, Table 1). The density of RGCs in intact unoperated retinas is shown as reference (stippled bar). The neuroprotective effect of AAV.TrkB was greatly enhanced when combined with a single intravitreal injection of BDNF at the time of axotomy. The synergistic effect of AAV.TrkB and BDNF was significantly larger than independent administration of AAV.TrkB or BDNF protein at all times examined ($P < 0.001$).

FIGURE 3.

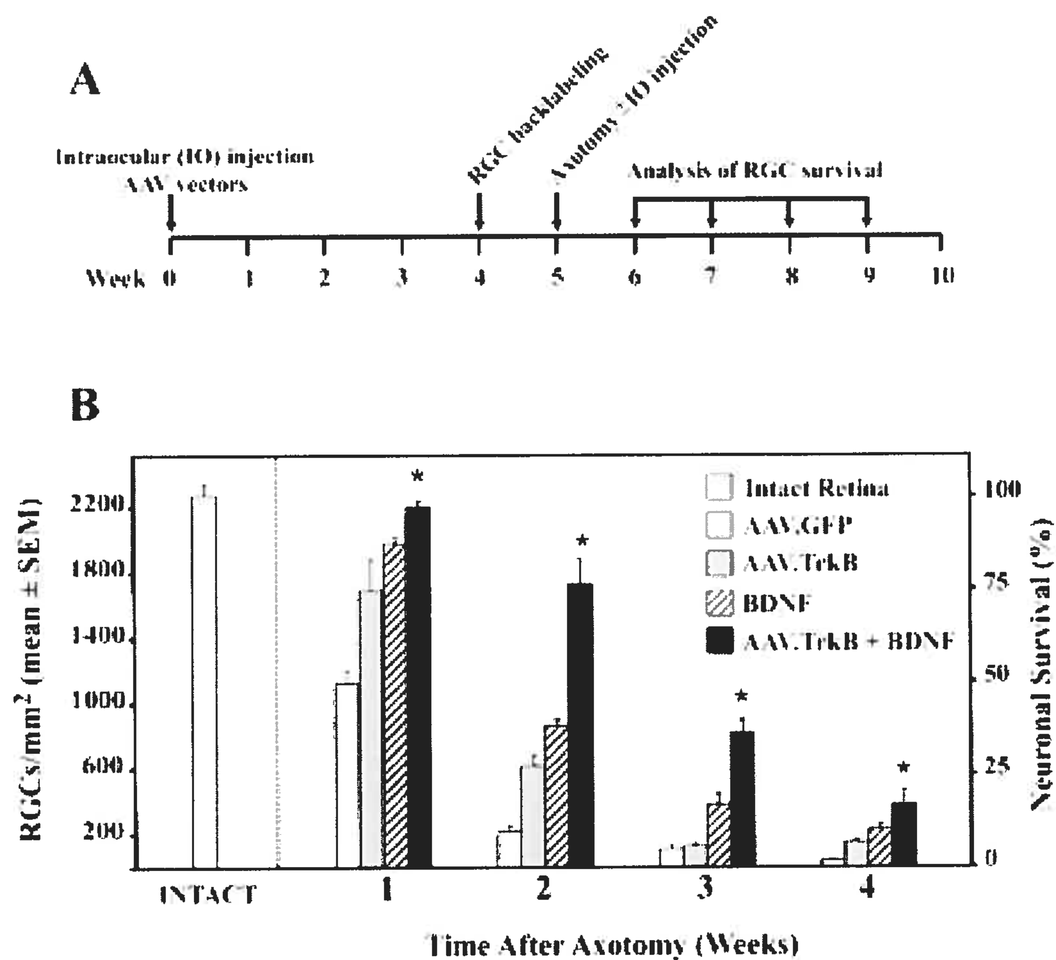


Figure 4. Correlation of RGC survival and AAV-mediated TrkB expression in injured retinas

Flat-mounted retinas show FluoroGold labeled RGCs after intravitreal administration of AAV.TrkB and BDNF protein (A) or only AAV.TrkB (B) at 3 weeks after axotomy. Microglia that may have incorporated FluoroGold after phagocytosis of dying RGCs were distinguished by their morphology (arrows) and excluded from our quantitative analyses. The identity of these cells was also confirmed by immunostaining using microglia and macrophage markers (not shown). Scale bar: 20 µm. (C) Fluorescent microscopy images of retinal sections following AAV.TrkB and BDNF administration show co-localization of anti-c-myc labeling, used to visualize AAV-mediated TrkB, and FluoroGold, used to identify surviving RGCs, at 3 weeks after axotomy. Scale bar: 10 µm. (D) Quantification of the number of cells in the ganglion cell layer co-stained with FluoroGold (FG+) and anti-c-myc (c-myc+) indicate that an average of ~96% of surviving RGCs express AAV-mediated TrkB protein. The population of RGCs labeled with both markers (FG+, c-myc+) is not significantly different from the total number of surviving RGCs (FG+) (n=4 per experimental group, p=0.769). These data suggest that TrkB upregulation increases RGC responsiveness to BDNF and enhances neuronal survival.

FIGURE 4

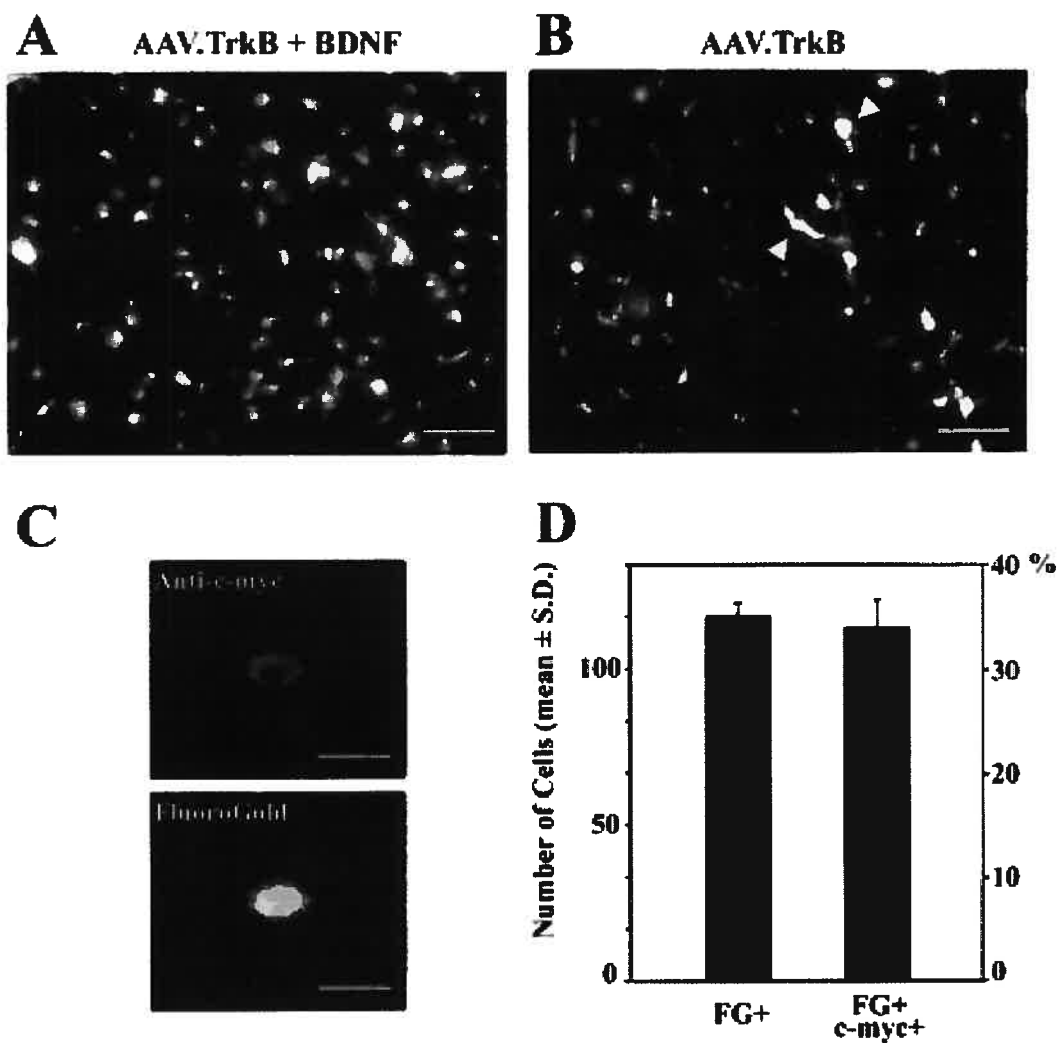


Figure 5. *In vivo* activation of retinal components of the BDNF/TrkB signaling pathway following TrkB gene transfer to RGCs

(A) Tyrosine phosphorylation of retinal TrkB at 5 weeks after a single intraocular injection of AAV.TrkB was examined by immunoprecipitation of ~300 µg of retinal protein with an anti-pan Trk (203) antibody followed by Western blot analysis using anti-phoshotyrosine (4G10) antibody. Controls included anti-pan Trk immunoprecipitated samples from retinas at 5 weeks of AAV.GFP injection and 48 hrs after BDNF or PBS injection. The bottom panel shows the same blot probed with an antibody that recognizes the intracellular domain of TrkB ($TrkB_{in}$) (B) Activation of the MAP kinases Erk1 and Erk2 was investigated using Western blots of total retinal extracts (75-100 µg) probed with an antibody that selectively recognizes both Erk1/2 phosphorylated on Thr202/Tyr204 residues. Stimulation of Erk1/2 was detected at 5 and 10 weeks after intravitreal injection of AAV.TrkB. Controls include retinal samples collected at 48 hrs or 10 weeks after injection of BDNF protein. Bottom panel shows the same blot re-probed with a p44/42 MAP kinase antibody to visualize total Erk protein. (C) Akt activation at 5 and 10 weeks after TrkB gene transfer was detected using a phospho-Akt specific antibody that recognizes Akt phosphorylated on Thr308. Controls are similar as for (B). Bottom panel shows the same blot re-probed with an anti-Akt antibody that allows visualization of total Akt protein.

FIGURE 5

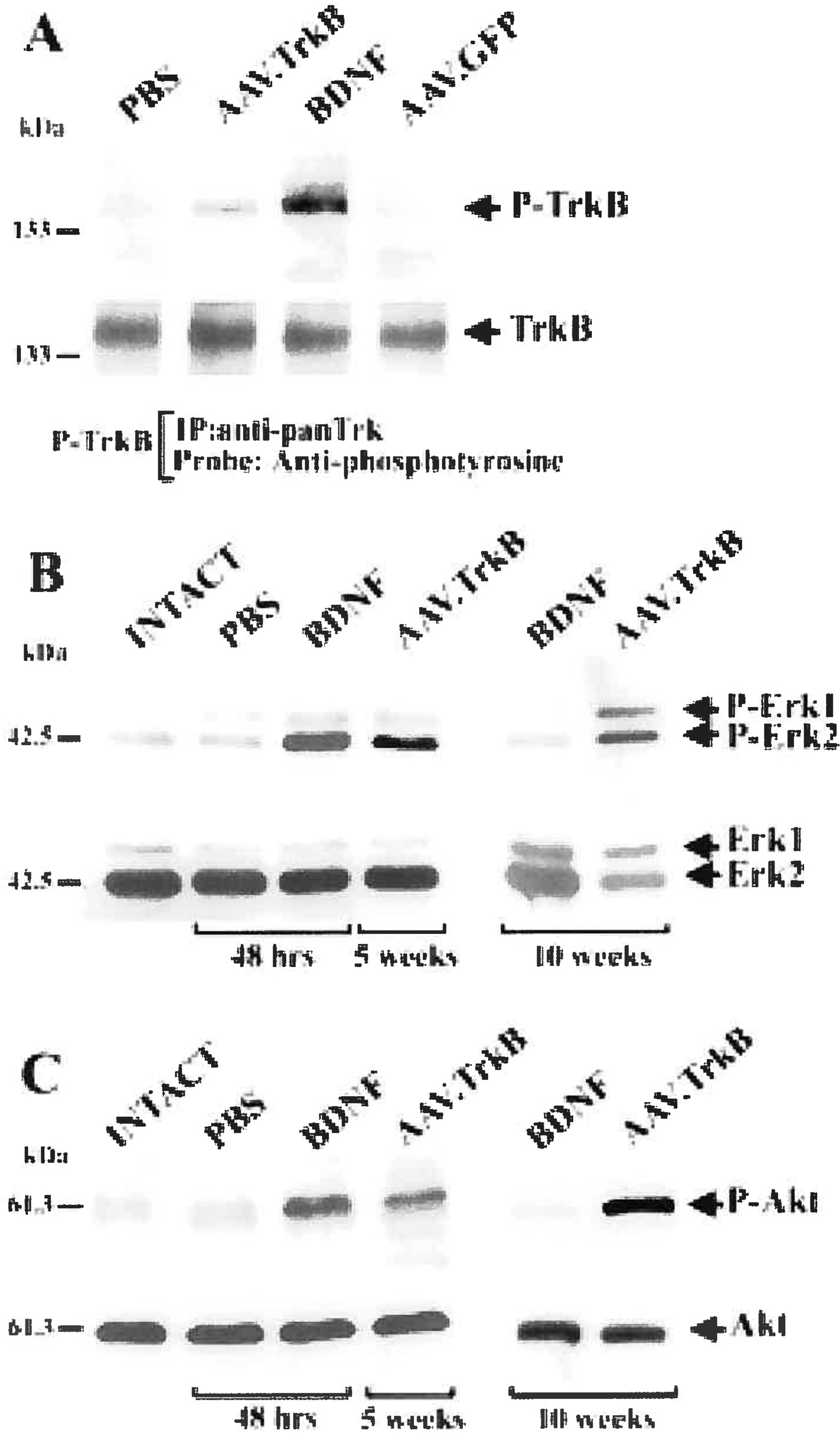


Figure 6. Selective inhibition of AAV.TrkB-induced activation of retinal MAPK and Akt

(A) Western blots of whole retinal homogenates show dose-dependent blockade of AAV.TrkB-induced activation of Erk1/2 with PD98059, a selective inhibitor of MEK. Inhibition of Erk1/2 phosphorylation was observed at 48 hrs and 14 days following intraocular injection of 200 μM of PD98059. The intravitreal concentration of this compound was ~16.7 μM based on the estimated volume of the vitreous chamber in the adult rat eye (~60 μl). Phosphorylated Erk1 and Erk2 were visualized with an antibody against phospho-MAPK (Thr202/Tyr204 residues, top panel) and total Erk protein was visualized in the same blot reprobed with p44/42 MAP kinase antibody (bottom panel). Intraocular injection of 200 μM of PD98059, which effectively inhibited Erk1/2 activation, did not block Akt phosphorylation (right panels). This result confirms the biochemical specificity of the pharmacological inhibitor PD98059 (B) Akt phosphorylation induced by TrkB gene transfer was blocked at 48 hrs and 14 days after single intravitreal injection of 800 μM LY294002 (intravitreal concentration: ~66.7 μM), a selective inhibitor of PI-3K. Activated Akt was visualized with an antibody against phospho-Akt (Thr308, top panel), total Akt protein was visualized in the same blot reprobed with an anti-Akt antibody (bottom panel). Administration of 800 μM of LY294002, which inhibited Akt stimulation, did not block Erk1/2 phosphorylation confirming the specificity of LY294002 (right panels).

FIGURE 6.

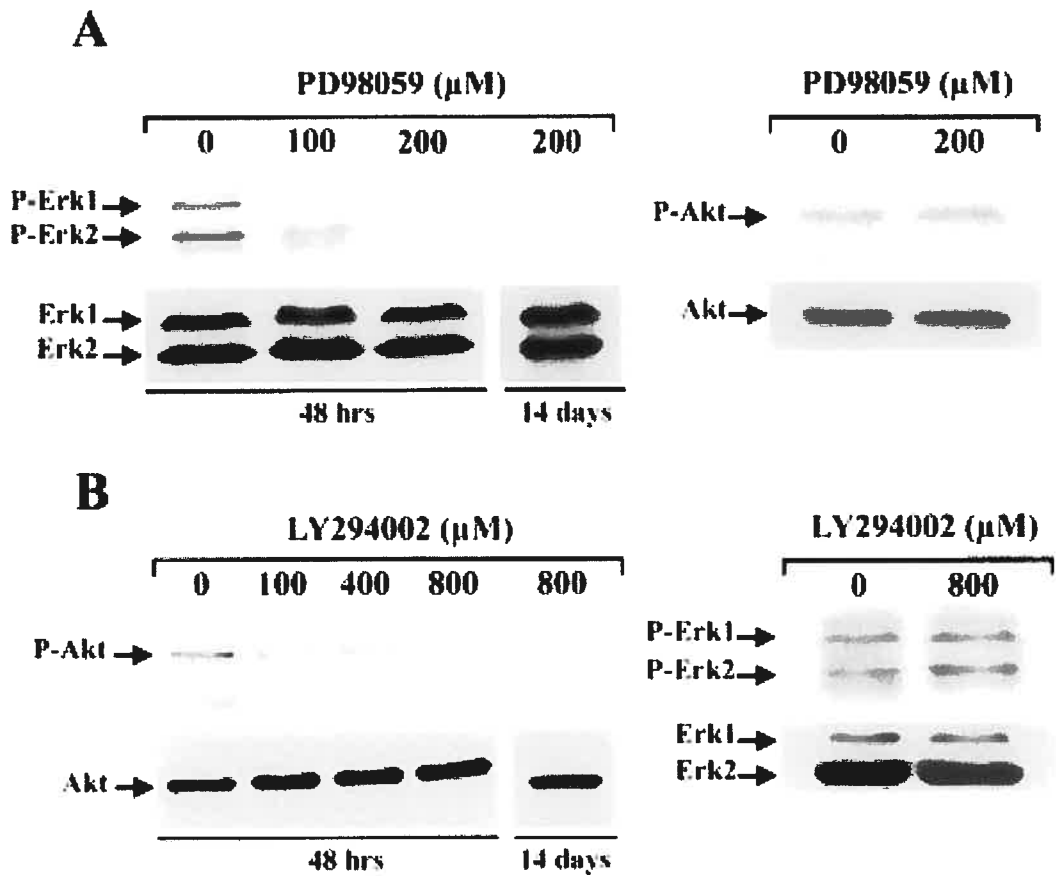


Figure 7. TrkB uses MAPK activation to promote the survival of adult RGCs *in vivo*

(A) Effect of the pharmacological inhibitors PD98059 and LY294002 on AAV.TrkB-induced RGC survival. The density of RGCs that survived following TrkB gene transfer with AAV.TrkB was significantly reduced by specific inhibition of the MEK/MAPK pathway with a single intraocular injection of PD98059 (200 μM) at the time of axotomy ($P<0.001$, t test). In contrast, AAV.TrkB-induced neuronal survival was not significantly changed by injection of LY294002 (800 μM), a specific inhibitor of PI-3K ($P= 0.798$, t test).

FIGURE 7.

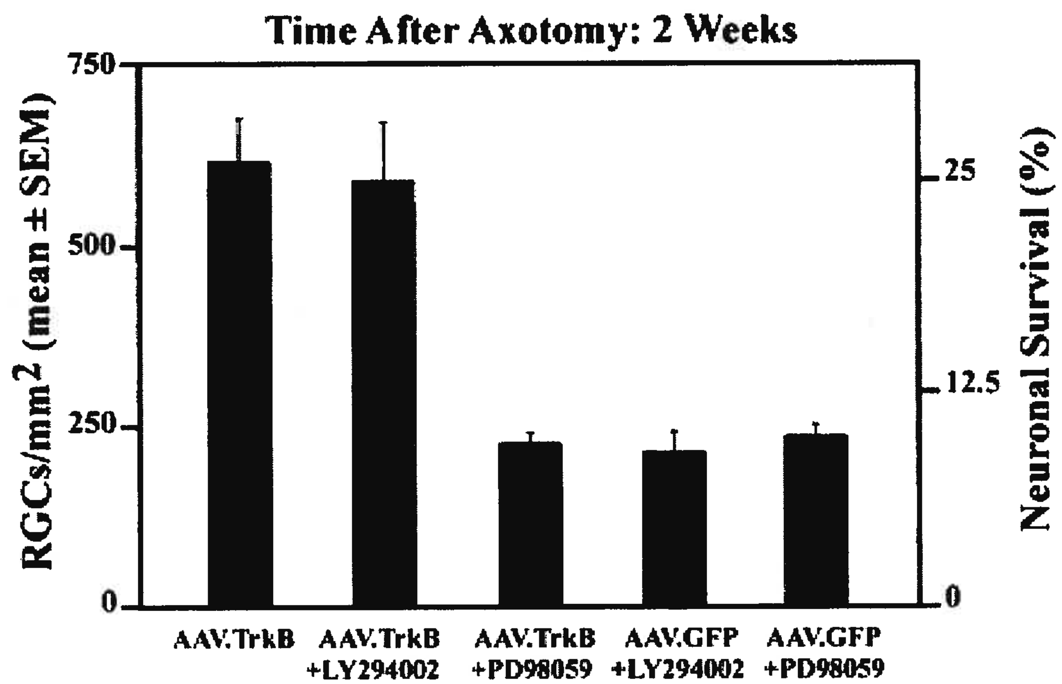


Table 1. Survival of Axotomized RGCs Following *In Vivo* Gene Transfer RGCs/mm^2 ± S.E.M. (% of intact contralateral retinas)

Treatment		Time After Axotomy			
Viral Vector	Agent Injected*	1 week	2 weeks	3 weeks	4 weeks
AAV.GFP	–	1131 ± 53 (50%), n=3	220 ± 32 (10%), n=5	104 ± 24 (5%), n=3	38 ± 2 (2%), n=3
AAV.TrkB	–	1692 ± 190 (75%), n=4	620 ± 60 (27%), n=7	124 ± 10 (6%), n=8	152 ± 10 (7%), n=3
AAV.TrkB	BDNF Protein	2203 ± 24 (97%), n=4	1732 ± 150 (76%), n=4	817 ± 79 (36%), n=5	378 ± 88 (17%), n=5
–	BDNF Protein	1981 ± 42 (87%), n=4	866 ± 32 (38%), n=5	381 ± 62 (17%), n=4	226 ± 26 (10%), n=4
AAV.TrkB	LY294002	–	594 ± 81 (26%), n=6	–	–
AAV.TrkB	PD98059	–	227 ± 15 (10%), n=5	–	–
AAV.GFP	LY294002	–	210 ± 17 (9%), n=3	–	–
AAV.GFP	PD98059	–	234 ± 13 (10%), n=3	–	–

* Intravitreal injection performed at the time of axotomy
Contralateral intact retinas: 2271 ± 64 RGCs/mm^2 (100%), n=15

APPENDIX B

HARNESSING THE GENE: STRATEGIES FOR RETINAL NEUROPROTECTION

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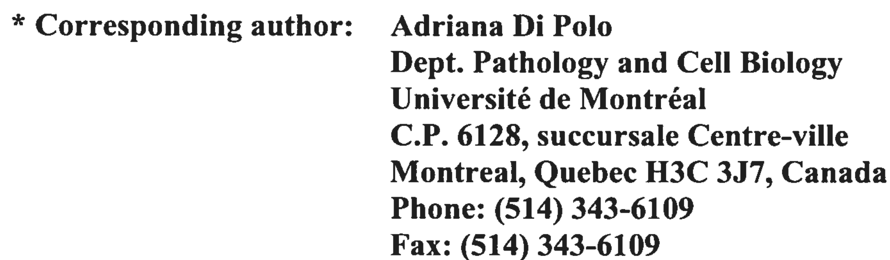

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1. Introduction

Gene transfer technology is gaining ground as a tool to investigate and promote neuroprotection in the retina. In the past few years, a number of studies have established proof-of-principle for the efficacy of gene delivery using viral vectors to enhance neuronal survival in animal models of retinal diseases. Several factors have contributed to the progress in this area such as the elucidation of the genetic basis of inherited retinal diseases, the availability of natural, experimental or transgenic animal models, and the development of recombinant viral vectors suitable for *in vivo* gene delivery.

The design of appropriate neuroprotective strategies is the first step in tackling the complex problem of neuroprotection in the retina. A sensible strategy should consider several factors including: i) the cell type affected, ii) the mechanism and cause of death, iii) the appropriate target cell for gene transfer, iv) the time-course of death, v) the developmental stage of the experimental animal at the moment of therapy, vi) the optimal viral vector for gene delivery, vii) the time-course of vector-mediated transgene expression, and viii) the available animal models. There may be as many neuroprotective schemes as there are retinal diseases or injury models. The goal of this chapter is to provide the reader with an updated account of current methods and tools for viral gene transfer that may serve as a guide to investigate neuroprotection in the retina.

2. Neuroprotective strategies

A variety of gene delivery strategies have been explored to promote neuronal survival in the retina following injury or disease. A gene transfer protocol that leads to the complete and permanent rescue of degenerating retinal neurons is yet to be

established. However, the following strategies successfully enhanced cell survival by delaying, in some cases considerably, the time-course of neurodegeneration.

2a) Gene supplementation: Many retinal disorders are due to genetic defects that result in a lack of one or more essential gene products. Delivery of a healthy copy of the mutated gene to the affected cell may restore loss-of-function deficits and lead to neuroprotection. This approach has been tested in the retinal degeneration (*rd*) mouse, a model of autosomal recessive human retinitis pigmentosa (RP). Defects in the rod-specific cGMP phosphodiesterase ß-subunit (ß-PDE) underlie irreversible and rapid photoreceptor loss in this model (Bowes et al., 1990; Pittler and Baehr, 1991). Transient rescue of the *rd* phenotype was achieved following delivery of the wild-type ß-PDE cDNA into the retina using several viral vectors: adenovirus (Ad) (Bennet et al., 1996), encapsidated Ad (gutted vector) (Kumar-Singh and Farber, 1998), adeno-associated virus (AAV) (Jomary et al., 1997a) and lentivirus (LV) (Takahashi et al., 1999). Furthermore, Ali et al. demonstrated that supplementation of the peripherin-2 gene using AAV vectors resulted in the preservation of outer segment ultrastructure and function for up to 10 months in the *rds* mouse (Ali et al., 2000), a model for autosomal dominant RP and macular dystrophy.

2b) Ribozyme therapy: Retinal diseases caused by dominantly inherited mutations may result in the production of abnormal gene products that affect cellular trafficking, metabolism and function. Cell death due to accumulation of these toxic products can be minimized by delivery of ribozymes, small RNA molecules that can be designed to

cleave mutant RNA transcripts while leaving the wild-type mRNAs intact. Lewin et al. demonstrated morphological and functional protection of photoreceptors following AAV-delivery of ribozymes in a mutant rhodopsin transgenic rat model of autosomal dominant RP (Lewin et al., 1998). Long-term studies on the effectiveness of this form of therapy indicated that the progression of photoreceptor loss was considerably delayed for up to 8 months of age (LaVail et al., 2000).

2c) Neurotrophic factor therapy: The main limitation of the gene correction therapies mentioned above is that the genetic basis of prevalent forms of retinal disorders, such as glaucoma or age-related macular degeneration, remains unknown. In addition, retinal diseases that arise from mutations in several genes (polygenic) or a combination of environmental and genetic factors represent a challenge in the design of potential gene therapies. Alternative therapies would involve the use of neurotrophic factors, with a broad spectrum of action, capable of providing a more generic form of neuroprotection. Neurotrophic factors bind to specific cell surface receptors triggering intracellular signaling pathways that lead to neuronal survival (Lewin and Barde, 1996; Kaplan and Miller, 2000; Huang and Reichardt, 2001a). Because most neurotrophic factors are secreted and diffuse well within the retina, this type of approach allows to select either the affected cell or its supporting cells as targets for gene transfer. For example, delivery of the brain-derived neurotrophic factor (BDNF) gene to Müller glial cells using an Ad vector resulted in temporary protection of retinal ganglion cells in an optic nerve axotomy rat model (Di Polo et al., 1998). Ad-mediated delivery of ciliary neurotrophic factor (CNTF) has been shown to slow photoreceptor loss in the *rd* and *rds* mouse

(Cayouette and Gravel, 1997; Cayouette et al., 1998). Basic fibroblast growth factor (bFGF) delivered by Ad vectors delayed retinal degeneration in the Royal College of Surgeons (RCS) rat (Akimoto et al., 1999). More recently, AAV-mediated delivery of bFGF delayed photoreceptor cell death in transgenic rats carrying a mutant rhodopsin (Lau et al., 2000a) and retinal ganglion cell loss after axotomy (Sapieha et al., 2001).

2d) Inhibition of apoptosis: Apoptosis or programmed cell death appears to be a common mechanism of neuronal loss in the injured or degenerating retina. Retinal ganglion cells have been shown to die by apoptosis after optic nerve axotomy and in experimental glaucoma (Berkelaar et al., 1994a; Garcia-Valenzuela et al., 1995a; Quigley et al., 1995). Photoreceptors also die by apoptosis in several inherited mouse models of RP (Chang et al., 1993; Portera-Cailliau et al., 1994), the RCS rat (Tso et al., 1994), light-induced retinal damage (Abler et al., 1996) and experimental retinal detachment in the rat (Cook et al., 1995). The identification of intracellular components of the cell death program (e.g. bcl-2 family members and caspases) has motivated experimental strategies involving transfer of anti-apoptotic genes. For example, gene delivery of bcl-2 using Ad resulted in moderate protection of photoreceptors in the *rd* mouse model (Bennett et al., 1998a). Recently, Ad-mediated gene transfer of the X-linked inhibitor of apoptosis protein (XIAP) resulted in partial protection of retinal ganglion cells following axotomy (Kugler et al., 2000) and high intraocular pressure (McKinnon et al., 2001).

3. Selection of a gene delivery system

Genes can be introduced into cells via non-viral and viral vectors. Non-viral methods include liposome-mediated DNA transfer, DNA carried on ballistic metal particles ("gene gun") and micro-injection techniques (Mahato et al., 1999). These gene transfer methods often yield low DNA transduction rates that result in limited transgene expression *in vivo*. In contrast, viral vectors have the ability to efficiently infect proliferating and post-mitotic cells and support adequate transgene expression levels. Improvements in the design of these vectors and the methods to increase viral titers and purity have contributed to their popularity. What makes a viral system suitable for therapeutic use? Let us imagine an ideal vector with the following characteristics: i) it can transduce a large number of target cells; ii) it mediates gene expression specifically in the target cells; iii) it allows stable expression of the delivered gene product at adequate levels; iv) it is safe, entailing no toxic or immunogenic response in the target tissue; v) it has no limitation in the size of DNA that it can accommodate; and vi) it is easy to produce in large volumes of high purity, high titer stocks. Although such perfect vector does not yet exist, there are a number of useful viral vectors available for gene transfer research. Here we will focus on Ad, AAV and LV vectors, the most widely used vectors in retinal neuroprotection research (Table 1). The following sections are not intended to endorse any vector in particular but to highlight their advantages and disadvantages for different gene transfer applications.

4. Adenovirus (Ad)

Ad vectors are an efficient tool to study the effect of *in vitro* and *in vivo* gene expression on neuroprotection. The major advantages of this system are that Ad can efficiently infect post-mitotic cells and that it can be easily grown to high titers. Ad contains a linear double-stranded DNA genome of approximately 36 kilobases (kb) encapsidated in an icosahedral protein shell. Immediate early genes (E1, E2, E3 and E4) orchestrate viral gene transcription and suppression of the host immune response, while late genes are necessary for viral assembly (Shenk, 1995). Most recombinant Ad belong to the group C Ad type 2 or 5. Ad vectors were initially generated with deletions of the early region 1 (ΔE1), that contains genes required for virus replication (Bett et al., 1994), rendering vectors replication defective and more suitable for gene transfer into mammalian cells. A major disadvantage of these early Ad vectors is the strong cytotoxic and immune response elicited upon infection of the host cells (Kovesdi et al., 1997). Recent versions of Ad vectors have been produced in which the entire viral genome, except for the terminal repeat regions required for viral assembly, has been replaced by exogenous gene sequences. These so-called "gutless" vectors exhibit considerably reduced immune response (Morsy et al., 1998; Schiedner et al., 1998b), but can only be produced in the presence of a helper virus that provides all the proteins required for viral replication (Parks et al., 1996). Because it is often difficult to fully separate the "gutted" vector from the helper virus, we will focus here on novel methods to produce recombinant Ad vectors without the need for helper virus (see below).

4a) Ad tropism in the retina

Although Ad vectors show efficient transduction of a wide variety of cell types *in vitro*, the cellular tropism for viral infection *in vivo* appears to be more complex. Our studies using Ad vectors injected into the vitreous chamber of adult rat eyes demonstrated that Müller cells, the predominant glial cell in the retina, is the main target for Ad infection *in vivo* (Di Polo et al., 1998). This approach has proved to be useful for delivery of genes encoding diffusible neurotrophins to promote neuroprotection of axotomized retinal ganglion cells. Ad vectors have also been shown to efficiently transduce the retinal pigment epithelium (RPE) following subretinal injections (Bennett et al., 1994; Li et al., 1994). Together, these studies indicate that non-neuronal cells in the adult retina are the preferred cellular targets for recombinant Ad. Nevertheless, some experimental conditions support limited Ad transduction of retinal neurons. For example, intraocular administration of Ad in animals at early developmental stages may result in modest infection of photoreceptors (Bennet et al., 1996). In addition, introduction of Ad to the brain (e.g. superior colliculi) or to the transected optic nerve stump results in retrograde transport of viral particles that mediate gene expression in retinal ganglion cells (Cayouette and Gravel, 1996; Kugler et al., 2000). Because all these studies involved transgenes directed by the ubiquitous cytomegalovirus (CMV) promoter, it is likely that the presence of cellular receptors for Ad in non-neuronal retinal cell types mediates the observed viral tropism *in vivo*. The coxsackievirus and adenovirus receptor (CAR) protein involved in Ad attachment and infection has been identified (Bergelson et al., 1997). In addition, integrins αvß3 and αbß5 participate in

Ad internalization (Wickman et al., 1993). The specific cellular localization of these receptors in the retina remains to be defined.

4b) Immunological response to Ad and transgene expression

Most studies have shown robust Ad-mediated transgene expression detectable soon after vector administration, but this expression is transient and decreases within a few weeks. This has been attributed mainly to a host immune reaction to viral and transgene products in which the infected cells are targeted for rapid T-cell mediated clearance (Hoffman et al., 1997; Reichel et al., 1998). This is supported by the observation that Ad-mediated gene expression is prolonged in immunodeficient animals (Hoffman et al., 1997). It is possible that other factors such as promoter silencing or loss of viral DNA, since the Ad genome remains episomal, contribute to this limited expression (Isenmann et al., 2001). As such, Ad represents an attractive system in gene therapy applications that require the expression of a gene product for a limited amount of time. For applications that require sustained transgene expression, several approaches have been explored. Repeated administration of Ad to enhance transgene expression has proved to be inefficient due to the production of neutralizing antiviral antibodies (Yang et al., 1994; Yang et al., 1995b). Alternative strategies have been implemented to by-pass the natural immune response. Daily administration of immune-suppressant drugs, delivered by subcutaneous injections (Di Polo et al., 1998) or osmotic minipumps, can effectively sustain transgene expression in the retina for several months. Similarly, co-administration of modulators of the immune response, such as CTLA4-Ig, prolong Ad-mediated gene expression (Ali et al., 1998a).

The ocular immune response elicited by Ad has been shown to depend on the route of administration of the vector. It is now clear that injection of Ad vectors into the vitreous space leads to a stronger immunological reaction than subretinal injection (Bennet et al., 1996; Hoffman et al., 1997). This finding highlights important differences in the ocular immune privilege response between the intravitreal and subretinal compartments following Ad injection. Interestingly, the immune response triggered by intravitreal administration of Ad vectors has been shown to promote a moderate degree of neuroprotection (Di Polo et al., 1998; Reichel et al., 1998). This has been partially attributed to the production of cytokines and neurotrophic factors by activated T cells (Noalem et al., 2000).

4c) Preparation of Ad vectors

E1 or E1/E3 deleted Ad vectors are widely used in most gene transfer applications. Most methods to generate such vectors rely on standard molecular biology and tissue culture techniques. Traditional protocols for the production of recombinant Ad involve homologous recombination of two pieces of DNA: i) a plasmid carrying the gene of interest, usually replacing the E1 genes, and the 5' end of the Ad genome, and ii) the 3' end of the Ad genome. Both DNA constructs are co-transfected into low passage 293 cells, a human embryonic kidney cell line engineered to express the E1 gene products in *trans* to allow replication and propagation of Ad (Graham et al., 1977). Recombination events result in the production of functional viral particles that have incorporated the gene of interest into their genome. Single viral clones are identified as plaques using standard overlay techniques and are further characterized by polymerase

chain reaction (PCR) or dot-blot analysis of viral DNA. Viral clones containing the gene of interest are then propagated in 293 cells. The purification of Ad preparations is routinely done by cesium chloride gradient ultracentrifugation and the viral titer is established using standard plaque titration assays. A detailed protocol of this method has been described elsewhere (Csaky, 2001). Commercial kits for the production of Ad vector using this technique are available (Qbiogene, Carlsbad, CA).

This approach has proved to be useful but time-consuming due to the low efficiency of homologous recombination in mammalian cells and the need for repeated rounds of plaque purification to eliminate contamination with wild-type virus. A modification of this approach has been developed where Ad is reconstituted in yeast (Ketner et al., 1994) or bacteria (Crouzet et al., 1997; He et al., 1998) which are endowed with a more efficient recombination machinery and are easier to manipulate than mammalian cells. In addition, the recombinant viral DNA is isolated from single clones allowing to generate homogeneous Ad preparations, free of contaminating wild-type virus, following transfection into 293 cells.

Recently, a novel method that does not require homologous recombination has been developed to generate E1/E3- or E1/E4-deleted recombinant Ad by *in vitro* ligation (Mizuguchi and Kay, 1998, 1999). First, the gene of interest is inserted into a shuttle plasmid to generate an expression cassette. Then, this cassette is excised and ligated into another vector containing the complete Ad genome with E1/E3 or E1/E4 deletions. The resulting recombinant Ad DNA is grown and purified following transformation into bacteria. This DNA is then used to transfect 293 cells where viral particles carrying the desired expression cassette are propagated. Because transfection is done with DNA from

a single clone, there is no need to screen plaques following transfection. Commercial kits to generate recombinant Ad by this method are available (Adeno-X^{TM}, Clontech Laboratories, Palo Alto, CA). One drawback of this technique is the need to transform bacteria and transfect 293 cells with large plasmids which may result in poor yields. Alternative methods using cosmid technology have been designed to select clones containing full-size genomes while excluding small and incomplete DNAs that are often produced following transformation of bacteria with large plasmids (Danthinne and Werth, 2000).

5. Adeno-associated virus (AAV)

AAV is a member of the *parvoviridae* family initially identified as a contaminant of Ad stocks. AAV requires a helper virus (e.g. Ad or herpes simplex virus) for replication. The wild-type virus houses a single stranded genome of 4680 base pairs (bp) containing two genes, *rep* and *cap*, that encode proteins involved in replication and encapsidation, respectively. The AAV genome is flanked by two identical 145-bp inverted terminal repeats (ITRs) which are essential for packaging, replication or integration. Recombinant AAV vectors derived from human parvovirus AAV-2 have been produced by substituting all viral sequences, except for the ITRs, for a transgene of interest (McLaughlin et al., 1988; Samulski et al., 1989). However, packaging of functional AAV particles requires the presence of the *rep* and *cap* proteins typically provided in *trans*. The recombinant AAV system has several advantages for *in vivo* gene transfer research: i) it is not pathogenic and has not been implicated in the etiology of any known human disease (Berns and Bohensky, 1987); ii) it mediates long-term

transgene expression that persists for several months *in vivo* (Dudus et al., 1999; Guy et al., 1999b); iii) the absence of viral sequences results in minimal immune response or cytotoxicity in the target tissues (Xiao et al., 1996, 1998); and iv) it can efficiently infect post-mitotic cells *in vivo* (Podsakoff et al., 1994).

In the absence of helper virus, wild-type AAV can integrate at a specific site on the q arm of chromosome 19 to establish latent infection (Kotin et al., 1990b). However, the lack of *rep* proteins has been shown to compromise integration specificity leading to random insertion of recombinant AAV (Flotte et al., 1994; Ponnazhagan et al., 1997b). Although viral integration into the genome may contribute to the stability of AAV-mediated transgene expression, a careful evaluation of the risks associated with insertional mutagenesis is required before implementing AAV-based therapies. A disadvantage of AAV vectors has been the size constraint for packaging genes larger than 4.7 kb. Although methods have been developed to increase the size of delivered transgenes by trans-splicing of two independent vectors co-administered to the same tissue (Yan et al., 2000), this remains a limitation of the AAV system. The laborious work needed to produce AAV vector stocks has often been regarded as a disadvantage of this vector system, however, recent availability of reagents and improvements in the protocols, described below, have greatly facilitated the preparation of high-titer and pure AAV stocks.

5a) AAV tropism in the retina

AAV-mediated gene expression can be restricted to photoreceptor cells when under the control of a well characterized murine rhodopsin promoter sequence (Flannery

et al., 1997). More recently, retinal ganglion cells have been identified as the primary targets for AAV infection in the inner retina following intravitreal injection of viral vectors (Cheng et al., 2002a) Thus, subretinal injection of AAV vectors results mainly in gene transfer to photoreceptors and RPE cells, while intravitreal injection allows infection of cells in the ganglion cell layer. Unlike Ad, AAV appears to have a preferential tropism for retinal neurons rather than glial cells. This is consistent with studies in the brain showing AAV transduction of subsets of neurons rather than astrocytes, oligodendrocytes or microglia (Kaplitt et al., 1994; Bartlett et al., 1998). Interestingly, genetic modification of capsid proteins has been shown to allow AAV targeting of cells normally resistant to infection (Girod et al., 1999).

5b) Time-course of AAV-mediated transgene expression

Recombinant AAV vectors have a slow onset of detectable transgene expression in the retina, which typically reaches a plateau between 1 and 8 weeks following administration of the vector depending on the animal species (Bennett et al., 2000). In rodents, peak expression is normally found 3 to 6 weeks post-injection of the vector (Bennett et al., 1997; Ali et al., 1998b; Guy et al., 1999b; Cheng et al., 2002a). Similar time-dependent increases in AAV-mediated expression have been observed in brain (Lo et al., 1999), muscle (Fisher et al., 1997) and liver (Snyder et al., 1997a). The mechanism for this *in vivo* delay to reach peak expression levels in the retina has not been determined, but may be related to the requirement of the single-stranded AAV genome to be converted to a double-stranded form for the transgene to be expressed (Ferrari et al., 1996; Malik et al., 2000). This feature of AAV-mediated gene expression

should be taken into consideration when designing neuroprotective strategies. Experimental paradigms with early onset and fast retinal neurodegeneration may be less amenable to AAV-based therapies.

5c) Preparation of AAV vectors

Because AAV depends on the presence of certain Ad helper genes to replicate and mount an effective infection, traditional methods have relied on the use of Ad for packaging recombinant AAV. These protocols involve co-transfection of two constructs, a vector plasmid carrying the transgene expression cassette and a helper plasmid that provides the *rep* and *cap* genes, into cells infected with helper Ad. Although separation of the resulting recombinant AAV from Ad has been routinely performed on cesium chloride (CsCl) gradients, column chromatography or by heat-inactivation, Ad remains a frequent contaminant of these preparations. The presence of helper virus can result in unwanted cellular host immune response (Monahan et al., 1998) confounding the interpretation of the results following *in vivo* gene transfer of contaminated AAV stocks. These concerns were addressed by the development of constructs that provide the essential helper genes in a plasmid, but lack the structural and replication Ad genes (Grimm et al., 1998; Matsushita et al., 1998; Salvetti et al., 1998; Xiao et al., 1998). For example, the AAV *rep* and *cap* genes and the Ad helper genes have been introduced in a single helper plasmid, pDG (Grimm et al., 1998), eliminating the need for Ad infection. In addition, the use of helper plasmids has been shown to improve the titer of recombinant AAV stocks possible due to strong *cap* gene expression that enhances virus encapsidation (Grimm and Kleinschmidt, 1999).

Current methods of AAV preparation in most laboratories are based on co-transfection of low passage 293 cells with a vector plasmid containing the transgene of interest flanked by the ITRs and a helper plasmid. Once the AAV particles are assembled within the host cells, the virus is extracted by freezing and thawing the cells and subsequently purified. Traditional AAV vector purification protocols that involve precipitation of the virus with ammonium sulfate followed by repeated rounds of CsCl density gradient centrifugation lead to poor viral recovery and low infectivity. A new purification strategy based on density gradient purification using iodixanol has been reported (Hauswirth et al., 2000). Iodixanol (Nycomed Pharma, Roskilde, Denmark) is a non-ionic iodinated density gradient media which, unlike CsCl gradients, does not promote aggregation of AAV and cell proteins that normally make the purification of AAV particles difficult. The fraction containing the AAV is further purified on a heparin/agarose column. This step relies on the high affinity of this virus for heparan sulfate proteoglycan (Summerford and Samulski, 1999), its main cellular receptor for attachment and internalization (Summerford and Samulski, 1998). The eluted fraction containing the virus is then concentrated and its titer is estimated.

In the absence of helper virus, AAV can not mount a productive (lytic) infection. Exposure of cells to AAV does not result in the formation of plaques as with Ad. Consequently, indirect methods are used to estimate how much virus is present in a given stock. Because not all virion particles are necessarily capable of successful infection, virus quality is often assessed as the ratio of physical particles to infectious particles in a given stock. A quantitative competitive (QC)-PCR method is currently used to estimate total viral particles or copies of recombinant AAV genomes (Conway et

al., 1997). DNA dot blot analysis can also be used to estimate total viral particles (Snyder et al., 1997b), but it is more time-consuming and unreliable than PCR-based methods. Infectious particles are determined by an infectious center assay (ICA) using the C12 cell line (Clark et al., 1995b) that contains integrated wild type AAV *rep* and *cap* genes. ICA involves infection of C12 cells with the AAV preparation followed by infection with Ad. The recombinant AAV genome is amplified in those cells successfully infected by AAV virions. Subsequently, the number of cells expressing detectable amounts of recombinant AAV DNA are quantified using radiolabeled probes (Hauswirth et al., 2000).

Methods for manufacturing high-titer AAV stocks based on transient transfection of 293 cells in the absence of infectious helper virus have proved particularly useful for pre-clinical studies which require rapid testing of therapeutic genes in a variety of animal models. While this method provides versatility, it is laborious and expensive to scale up due to the large amounts of DNA needed for transfection and the low efficiency of co-transfection protocols. As AAV vectors move toward the clinical arena, improved methods to produce large quantities of pure, high-titer stocks are required. Packaging cell lines engineered to contain the AAV *rep* and *cap* genes have been particularly useful (Clark et al., 1995b; Inoue and Russell, 1998). The transgene of interest can be delivered to packaging cells via a recombinant Ad to generate recombinant AAV stocks in the presence of helper Ad (Gao et al., 1998; Liu et al., 1999). Alternatively, the *rep* and *cap* genes have been delivered via a recombinant herpesvirus to cells that carry a stably integrated AAV provirus with the transgene of choice (Conway et al., 1999). These strategies are promising for scaling up AAV stocks, but they inevitably result in

contamination with helper viruses and require thorough purification prior to any clinical application.

6. Lentivirus (LV)

LV, a genus of retroviruses, consists of two identical single-stranded RNA molecules and enzymes required for replication within a viral protein core. Following virus internalization, the viral RNA is reverse transcribed into double-stranded DNA and transported to the cell nucleus (Panganiban, 1990). Viral DNA is then permanently integrated into the host genome to become a provirus. The retrovirus genome contains *gag*, *pol* and *env* genes flanked by long-terminal repeats (LTRs). These genes encode proteins essential for replication, encapsidation, internalization and reverse transcription. Replication-deficient recombinant retroviral vectors have been generated by substituting all viral genes for a transgene of interest with the exception of the *cis*-acting sequences required for vector propagation, such as the reverse transcription initiation site and the packaging site (Coffin, 1996). Functional recombinant retrovirus particles can be generated in culture when the *gag*, *pol* and *env* gene products are provided in *trans*.

Most retroviral vectors can only transfer genes into cells that are actively proliferating (Roe et al., 1993b). Thus, their use in neuroprotective strategies which typically involve gene transfer into fully differentiated cells is rather limited. An exception to this rule are LVs, such as the human immunodeficiency virus (HIV), which can efficiently infect non-mitotic cells (Lewis and Emerman, 1994b). This ability relies on nuclear localization signals in the preintegration complex that allow entry into the nucleus without the need for nuclear membrane fragmentation (Roe et al., 1993b). Other

advantages of the LV system are its relatively large cloning capacity, close to 10 kb; its ability to mediate high levels of transgene expression *in vivo* and the lack of immune response in the target tissues (Naldini et al., 1996b; Kafri et al., 1997). Recently, recombinant LV was shown to efficiently infect hematopoietic stem cells extending its potential use as a therapeutic vector (Miyoshi et al., 1999).

The main concern with LV vector systems is the risk of generating replication competent recombinant (RCR) virus during the production of viral stocks. Because HIV is a human pathogen, considerable work has been done to increase biosafety of LV production systems. The latest methods to generate safer HIV-based vector systems are described below. Other concerns include low vector titers and the risks associated with insertional mutagenesis as the vector integrates into the host genome. The issue of transgene silencing still requires further investigation. The lack of immune response associated with LV recombinant infection and its ability to stably integrate into host DNA are promising features for persistent gene expression. A systematic study of the time-course of expression mediated by LV vectors in the retina should resolve this issue.

6a) Cellular tropism of LV vectors

LV-mediated gene transfer and expression in the retina *in vivo* was first characterized by subretinal injection of a vector carrying the green fluorescent protein (GFP) gene (Miyoshi et al., 1997). Using the ubiquitous CMV promoter, the main cellular targets of LV vectors were shown to be photoreceptors and RPE cells with some bipolar and Müller cells. When a rhodopsin-specific promoter was used, transgene expression was restricted to the photoreceptor layer (Miyoshi et al., 1999; Takahashi et

al., 1999). The infection pattern of LV vectors following intravitreal injection remains to be characterized.

An attractive feature of HIV-based vectors is their ability to efficiently infect cells *in vitro*. For example, RPE primary cultures have been transduced with LV to express GFP. Transgene expression persisted in the infected RPE cells following their transplantation into the subretinal space of a host (Lai et al., 1999). HIV-based vectors have been shown to efficienly transduce human $CD34^+$ hematopoietic stem cells that were capable of long-term engraftment of non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (Miyoshi et al., 1999). Recently, neural stem cells have been isolated from the RPE in the ciliary margin of the adult eye (Tropepe et al., 2000). These cells can express retinal specific markers when they are differentiated *in vitro*. Thus, *ex vivo* transduction of stem cells using HIV-based vectors followed by retinal transplantation may be useful in the design of neuroprotective strategies.

6b) Preparation of LV vectors

LV has a more complex genome and, consequently, a more complicated replication cycle than other retroviruses. In addition to the three structural *gag*, *pol* and *env* genes, LV has additional regulatory genes: *vif*, *vpr*, *vpu*, *tat*, *rev* and *nef* genes (Trono, 1995). Traditional methods to generate recombinant LV vectors involved a vector plasmid with all viral genes deleted, containing only the transgene of interest flanked by the essential *cis*-acting elements. The viral proteins required for virus propagation were provided in *trans* by co-transfection of a helper plasmid into 293 cells. This procedure resulted in low titer preparations and in high risk for generating RCR virus. This is a major concern

because the considerable overlap that exists between *cis*-acting sequences in HIV vectors and helper constructs increases the chance of homologous recombination leading to the production of RCR viral particles.

A better understanding of the HIV replication cycle has led to new advances in the field of LV vector packaging. The latest generation of LV vectors have only three of the nine HIV-1 genes: *gag*, which encodes the main structural proteins; *pol*, responsible for the production of viral replication proteins and *rev*, encoding a regulator required for *gag* and *pol* gene expression. The HIV envelope has been substituted by other viral envelope proteins such as the vesicular stomatitis virus G-protein (VSV-G) due to its high stability and broad tropism (Yee et al., 1994). Methods to generate recombinant LV particles from three or four separate transcriptional units containing *gag/pol*, *rev*, VSV-G and the transgene of interest following transfection into 293 cells have been recently described (Naldini et al., 1996b; Dull et al., 1998b). By distributing the required sequences in multiple plasmids, the risk of creation of RCR virions was minimized. In addition, the overlap between vector and helper sequences was markedly reduced.

The safety level for production of LV has been further improved by the development of self-inactivating (SIN) HIV-1-derived vectors. Two independent research groups have reported HIV-based vectors in which the regulatory sequences (promoter and enhancer elements) contained in the 3' LTR have been deleted (Miyoshi et al., 1998b; Zufferey et al., 1998). By eliminating these *cis*-acting elements, the virus loses its capacity for gene expression directed by both LTRs resulting in inactivation of the integrated virus in the infected cells. In addition, the expression of the transgene of interest can only be controlled by an internal heterologous promoter. A stable, high-output packaging cell

line to produce this latest generation of LV vectors has been recently reported (Xu et al., 2001). These results represent the latest advancement in the efforts to render HIV-based vectors safe for clinical use. Although these novel vectors greatly minimize the risk of RCR virus infection to users during manipulation and preparation, the biosafety of LV vectors *in vivo* has yet to be demonstrated.

7. Summary

Several independent research laboratories have recently established proof-of-principle for the efficacy of gene therapy for neuroprotection in animal models of retinal injury or disease. Three viral systems, Ad, AAV and LV, have been used for most gene delivery applications to the retina both *in vitro* and *in vivo*. The pros and cons inherent to each of these vectors need to be carefully weighed when designing a gene transfer strategy. Recent progress in the methods to create these recombinant viruses has greatly increased the ability of researchers to generate high-titer and pure stocks while reducing production times. In addition, the likelihood of adverse reactions in the host tissues and the risk of RCR virus generation have been minimized. As these viral vectors progress toward pre-clinical and potential clinical human trials, highly stringent standards for their efficacy, biosafety, ease for scaling up production and purity will be expected. Future *in vivo* characterization of these viral vectors will be essential to fully assess if these requirements are met for clinical applications.

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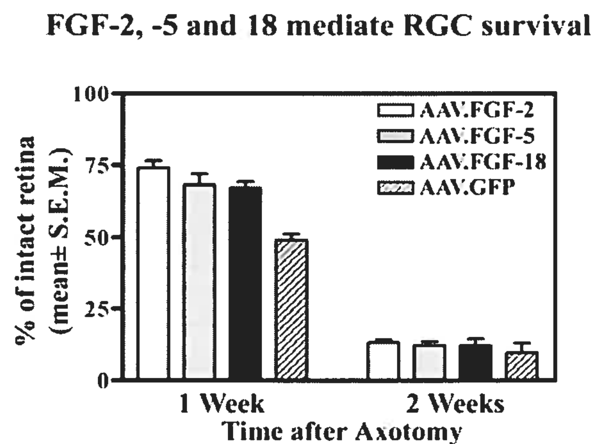
APPENDIX C

AAV.FGF-2, AAV.FGF-5 OR AAV.FGF-18 PROMOTES TRANSIENT SURVIVAL OF AXOTOMIZED RGCS

We investigated the neuroprotective effects of FGF-2, FGF-5 and FGF-18 on adult RGCs following acute optic nerve lesion. In order to achieve sustained levels of these factors, we employed AAV technology as described in chapter 2 of this thesis. One week after axotomy, FGF treated eyes displayed a marked increase in RGC survival compared to their GFP controls. AAV.FGF-2 injected eyes showed 74% survival, AAV.FGF-5 treated eyes, 68% survival and AAV.FGF-18 eyes bore 67% survival. Control AAV-GFP injected eyes yielded 49% survival. Two weeks following optic nerve transection, RGC numbers in treated eyes dropped to control levels. AAV.FGF-2 treated eyes declined to 13% survival, AAV.FGF-5 and AAV.FGF-18 injected eyes to 12% while control AAV.GFP injected yielded 10% survival. These experiments suggest that AAV mediated gene transfer of members of the FGF family (FGF-2, FGF-5 or FGF-18) transiently protects lesioned RGCs (Figure 1).

Appendix C
FIGURE 1

FGF-2, -5 and 18 mediate RGC survival

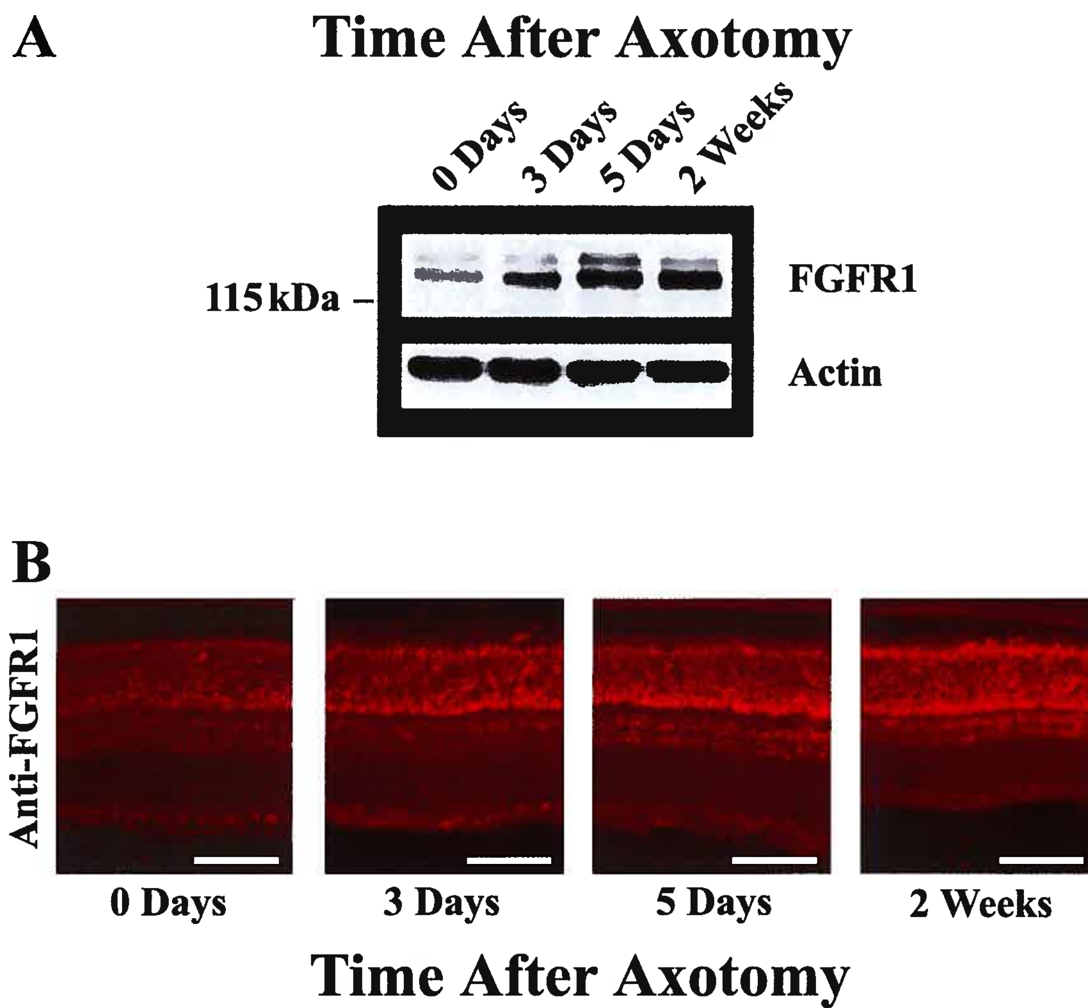


Appendix D

FGFR1 IS UPREGULATED IN PHOTORECEPTOR CELLS FOLLOWING OPTIC NERVE TRANSECTION

To determine whether optic nerve transection influenced FGFR1 levels in the retina, axotomized retinal samples were analyzed by western blot and immunohistochemistry on cryosections. Surprisingly, Western blot of total retinal homogenates, revealed a pronounced increase in FGFR1 protein after optic nerve transection (Figure 1. A). We then sought to identify the source of this increase by immunohistochemistry on retinal cryosections. Interestingly, we localized the increase in FGFR1 predominantly on photoreceptor cells while observing only a minimal increase in RGCs (Figure 1. B). Three days following lesion, immunohistochemical labelling significantly intensified in photoreceptor cell bodies of the outer nuclear layer (ONL) and to a considerably lesser extent in the ganglion cell layer (GCL). At 5 days post-axotomy, the enhanced signal persisted in the ONL but returned to basal levels in the GCL. This pattern was observed for at least 14 days following axotomy. These results demonstrate that FGFR1 protein is upregulated in photoreceptor cell bodies following optic nerve axotomy. Scale bars: 100 µm (B)

Appendix D
FIGURE 1.



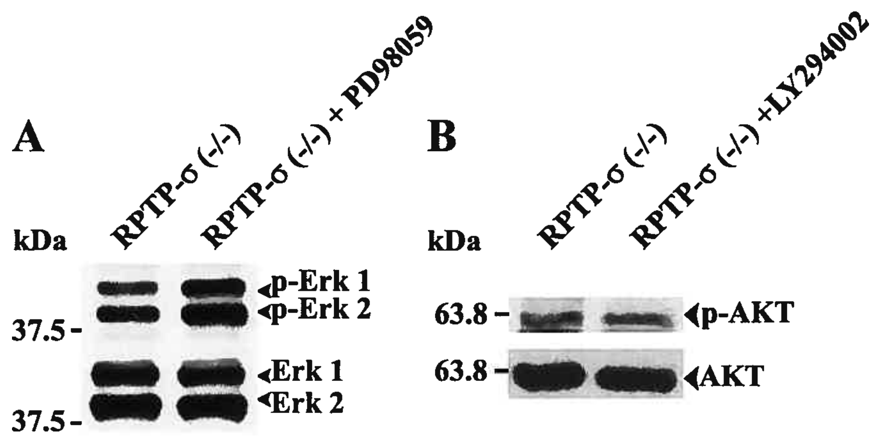
APPENDIX E

TECHNICAL LIMITATIONS WHEN INHIBITING ERK 1/2 AND AKT IN THE MOUSE RETINA

We previously reported that intraocular injection of PD98059 or LY294002 at a concentration of 200 μM or 800 μM, respectively, effectively inhibited Erk1/2 or Akt activation in the adult rat retina (Cheng et al. 2002). Injection of these compounds into RPTPσ (-/-) mice eyes, however, failed to block Erk1/2 or Akt activation (Figure 1. A,B). The use of higher concentrations of these inhibitors resulted in the formation of dense precipitates that had toxic effects *in vivo*.

In some cases, intraocular injection of these inhibitors even caused a slight increase in activation of retinal Erk1/2 or Akt (Figure 1A). These results could be partly attributed to retinal injury during injection which has been shown to increase FGF-2, FGFR-1 and CNTF levels in the retina (Wen et al., 1995; Cao et al., 1997; Cao et al., 2001), leading to further stimulation of retinal Erk1/2 or Akt. Given that the mouse eye is considerably smaller than the rat eye, it is expected that the injury effect produced by the proportionately larger needle will have a dramatic effect on the expression and release of endogenous factors in the mouse retina. Although these are extremely important experiments that are worth pursuing to investigate the role of both Erk1/2 and Akt in axonal growth in RPTPσ (-/-), the technical difficulties encountered preclude us from carrying out this work using this approach and further studies *in vitro* will be required to fully address this issue.

Appendix E
FIGURE 1



APPENDIX F

ARTICLE CONTRIBUTIONS

As first author in the article **"Fibroblast Growth Factor-2 Gene Delivery In Vivo Stimulates Axonal Regeneration of Adult Retinal Ganglion Cells After Acute Optic Nerve Injury" (Chapter 2)** I carried out all of the technical procedures; i.e. surgeries, immunohistochemical and biochemical analysis. Furthermore, I performed the majority of the data analysis, wrote the initial version of the manuscript and collaborated in the writing of the subsequent versions with Adriana Di Polo.

As first author in the article **"Extracellular Signal-Regulated Kinases 1/2 Mediate Retinal Ganglion Cell Axon Regeneration Induced by Fibroblast-Growth Factor-2" (Chapter 3)** I performed all of the technical procedures, wrote the initial version of the manuscript and collaborated in the writing of further versions with Adriana Di Polo.

As first author in the article **"Receptor Protein Tyrosine Phosphatase Sigma Inhibits Axon Regrowth in the Adult Injured CNS"(Chapter 4)**, I carried out all of the surgical procedures, immunohistochemistry and biochemistry and data analysis. Moreover, I wrote the first version of the manuscript and collaborated with Adriana Di Polo in the writing of subsequent versions.

In the article **"TrkB Gene Transfer Protects Retinal Ganglion Cells from Axotomy-Induced Death *In Vivo*" (Appendix A)** my contribution consisted in

performing certain surgeries and certain Western blot analysis as well as generating the graphs in figures 3 and 7 in the manuscript.