Targeting the Phosphatidylinositol-3 Kinase Pathway and the Mitogen-Activated-Protein Kinase Pathway through Thymosin-β4, Exercise, and Negative Regulators to Promote Retinal Ganglion Cell Survival or Regeneration

by

Mark Magharious

A thesis submitted in conformity with the requirements for the degree of Master of Science

Rehabilitation Sciences Institute
University of Toronto

© Copyright by Mark Magharious 2015
Targeting the Phosphatidylinositide-3 Kinase Pathway and the Mitogen-Activated-Protein Kinase Pathway through Thymosin-β4, Exercise, and Negative Regulators to Promote Retinal Ganglion Cell Survival or Regeneration

Mark Magharious

Master of Science

Rehabilitation Sciences Institute
University of Toronto

2015

Abstract

The phosphatidylinositide-3 kinase (PI3K) and mitogen-activated-protein kinase (MAPK) pathways mediate cellular survival in the presence of apoptotic stimuli. These pathways are known to promote the survival of injured retinal ganglion cells (RGCs), central nervous system neurons that project visual information from the retina to the brain. Injury to the optic nerve triggers apoptosis of RGCs. This work demonstrates that Thymosin-β4, a peptide involved in actin sequestration, both enhances RGC survival after injury and increases axonal regeneration. Moreover, Thymosin-β4 modulates the PI3K and MAPK pathways. In addition, this study demonstrates that exercise reduces apoptosis of injured RGCs, and explores the function of the PI3K and MAPK pathways in this process. Finally, small peptides are used to interfere with the functions of PTEN, a negative regulator of the PI3K pathway, as well as Erbin and BCR, negative regulators in the MAPK pathway. These peptides enhance RGC survival and axonal regeneration after injury.
Acknowledgments

I would like to take this opportunity to recognize all those who helped me through the process of researching and writing this thesis. Without their generous time and effort, I could not have completed this project.

First, I thank God for the many blessings in my life. Few people are ever given the opportunity to make a contribution to the scientific literature. To find myself among them, even with such a small contribution, is humbling and reminds me that I am truly blessed.

I would also like to thank my supervisor, Dr. Paulo Koeberle. Some years ago, Dr. Koeberle accepted me into his lab, even though I was inexperienced and had very minimal lab exposure. Not only did he welcome me into his lab, but he also took the time to personally teach me how to perform the techniques and procedures I would need for my very first research project, which I completed under his supervision. I am truly grateful for Dr. Koeberle’s encouragement and patience in all my research, especially throughout the process of this thesis. In the time I spent under Dr. Koeberle’s guidance, I have been able to mature both as a student and as a person. I will always cherish the time I spent in his lab.

I am also very grateful to my co-supervisor, Dr. Cindi Morshead. Her guidance and advice throughout my thesis have been invaluable. I sincerely appreciate all her wise counsel in this project, particularly at times when this study had not gone in the direction I had expected. Dr. Morshead helped focus my work, and set my project back on the right path. Moreover, Dr. Morshead has been very supportive throughout, and always willing to promptly answer my questions or provide a solution for my problems. I am unbelievably fortunate to have had her on my committee.

I am also thankful to Dr. Philippe Monnier. As a member of my thesis committee, Dr. Monnier has been very generous with his time and contributions to my project. His many questions and advice have continuously helped me ameliorate my study, and render my work more credible. I am privileged to have been able to work with him.

I would also like to express my gratitude to the Graduate Department of Rehabilitation Science (Rehabilitation Sciences Institute). All the members of the faculty and staff with whom
I have interacted have been very kind and welcoming. I sincerely appreciate their efforts to fit a square peg into a round hole. They were always very accommodating despite my research falling outside the “usual” rehabilitation field.

I would like to thank all my lab mates, and in particular Philippe D’Onofrio. It has been a great pleasure to learn, discuss, and travel with all of them. I will always relish the memories of our time together.

Finally, I want to recognize my parents, Michael and Lucy Magharious, as well as my sister, Parthenia. They have been a constant source of support in my life that has extended beyond my academic endeavours. Their love, patience, and encouragement have never faltered, and have helped me through the toughest times. I cannot fully express the full extent of my gratitude, and I am certain that I would not be where I am today without their help.
Table of Contents

Abstract ......................................................................................................................... ii

List of Tables .................................................................................................................. viii

List of Figures ................................................................................................................ ix

List of Abbreviations ...................................................................................................... x

Chapter 1: Introduction ................................................................................................. 1
  1.1. Research Hypothesis ............................................................................................. 2
  1.2. Research Objectives ............................................................................................. 2

Chapter 2: Literature Review ....................................................................................... 3
  2.1. Central Nervous System Neuron Survival After Injury ........................................ 3
  2.2. Retinal Ganglion Cells and the Optic Nerve as a Model for CNS Injury and Disease .................................................................................................................. 4
  2.3. Phosphatidylinositol-3-kinase (PI3K) Pathway in Cell Survival and Apoptosis ...... 8
  2.4. Mitogen-activated-protein kinase (MAPK) Pathway in Cell Survival and Apoptosis .................................................................................................................. 13
  2.5. Thymosin-β4 ....................................................................................................... 18
  2.6. Physical Exercise .................................................................................................. 22
  2.7. PDZ Domains and PDZ Binding Motifs ................................................................ 24
  2.8. Phosphatase And Tensin Homolog (PTEN) ......................................................... 25
  2.9. Erbin .................................................................................................................... 28
  2.10. Breakpoint Cluster Region (BCR) ...................................................................... 31

Chapter 3: Methods and Materials ............................................................................ 33
  3.1. Optic Nerve Transection and Optic Nerve Crush ................................................. 33
  3.2. Intraocular Injections .......................................................................................... 34
  3.3. Quantification of RGC Survival .......................................................................... 35
  3.4. Quantification of RGC Axon Regeneration ......................................................... 36
3.5. Western Blot ................................................................. 37

3.6. Treadmill Running of Animals ........................................... 37

Chapter 4: Results .................................................................. 39

4.1. Thymosin-β4 .................................................................. 39

  4.1.1. Exogenous Thymosin-β4 Enhances RGC Survival Following Optic Nerve Transection .......................................................... 39

  4.1.2. Exogenous Thymosin-β4 Promotes RGC Regeneration Following Optic Nerve Crush .......................................................... 42

  4.1.3. Exogenous Thymosin-β4 Increases the Activation of the PI3K and MAPK Pathways ......................................................... 45

4.2. Exercise Training ............................................................... 48

  4.2.1. Forced Light Treadmill Running Decreases the Activation of the PI3K Pathway But Not the MAPK Pathway .......................... 48

  4.2.2. Forced Heavy Treadmill Running Does Not Affect the Activation of the PI3K or MAPK Pathways ......................................... 50

  4.2.3. Forced Treadmill Running Partially Improves the Survival of RGCs at 7 Days Following Optic Nerve Transection ....................... 52

  4.2.4. Forced Treadmill Running Enhances the Survival of RGCs at 14 Days Following Optic Nerve Transection ............................ 55

4.3. PDZ Peptides .................................................................. 58

  4.3.1. Intraocular Delivery of PDZ Peptides Enhances RGC Survival Following Optic Nerve Transection .............................................. 58

  4.3.2. Gelfoam Application of PDZ Peptides at the Cut End of the Optic Nerve Increases the Number of Surviving RGCs Following Optic Nerve Transection ......................................................... 61

  4.3.3. RGC Axon Regeneration Following Optic Nerve Crush Can Be Enhanced Using a PDZ Peptide Directed Against PTEN ................. 64

Chapter 5: Discussion ............................................................. 67

5.1. Thymosin-β4 ................................................................. 67

5.2. Exercise, the PI3K and MAPK Pathways, and Adult Neuronal Survival ................. 68
5.3. PDZ Interactions in the PI3K and MAPK Pathways .............................................. 70

5.3.1. PTEN and the Phosphatidylinositol-3 Kinase Pathway .......................... 71

5.3.2. Erbin and the Mitogen-Activated-Protein Kinase Pathway ...................... 73

5.3.3. BCR and the Mitogen-Activated-Protein Kinase Pathway ....................... 74

Chapter 6: Conclusion .................................................................................................. 75

Chapter 7: Future Directions ....................................................................................... 76

Chapter 8: References.................................................................................................. 78
List of Tables

Table 1 – PDZ Peptides

................................................................. 35
List of Figures

Figure 2.2. Optic nerve transection and optic nerve crush ..................................................7
Figure 2.3. The phosphatidylinositol-3-kinase (PI3K) pathway ...........................................12
Figure 2.4. The mitogen-activated-protein kinase (MAPK) pathway ......................................17
Figure 4.1.1. Intraocular injection of exogenous Thymosin-β4 enhances RGC survival ..........40
Figure 4.1.2. Intraocular injections of exogenous Thymosin-β4 enhance axonal regeneration...43
Figure 4.1.3. Thymosin-β4 induces activation of the PI3K and MAPK pathways .....................46
Figure 4.2.1. Forced light exercise decreases the activation of Akt but not MAPK ...............49
Figure 4.2.2. Forced heavy exercise does not change the activation of either Akt or MAPK .....51
Figure 4.2.3. Forced treadmill running partially increases survival of RGCs on day 7 ..........53
Figure 4.2.4. Forced treadmill running enhances RGC survival at 14 days .........................56
Figure 4.3.1. Intraocular Delivery of PDZ peptides has different effects on RGC survival .......60
Figure 4.3.2. RGC survival following Gelfoam application of PDZ peptides .......................63
Figure 4.3.3. PDZ peptides that target PTEN enhance axonal regeneration .......................65
List of Abbreviations

ABL  Abelson murine leukemia viral oncogene homolog 1
AcSDKP N-acetyl-seryl-aspartyl-lysyl-proline
AF-6 Afadin
AFX FoxO4; Forkhead box protein O4
ANOVA Analysis of variance
APC Adenomatous polyposis coli
APTeX Aminopropyltriethoxysilane
ART Adhesive removal test
ARVCF Armadillo repeat protein deleted in velo-cardio-facial syndrome
BAD Bcl-2-antagonist of death
Bax Bcl-2-like protein 4
BBB score Basso, Beattie, and Bresnahan score
Bcl-2 B-cell lymphoma 2 protein; Apoptosis regulator Bcl-2
Bcl-X Bcl-2-like protein 1
Bcl-XL B-cell lymphoma-extra large; BCL2-like 1 isoform 1
BCR Breakpoint Cluster Region
BDNF Brain-Derived Neurotrophic Factor
Bim Bcl-2 interacting mediator of cell death; Bcl-2-like protein 11
CAD Caspase-Activated Deoxyribonuclease
CNS Central nervous system
cPLA2 Cytosolic phospholipase A2
CRE Cyclic AMP response elements
CREB Cyclic AMP response element-binding protein
CSPG Chondroitin sulphate proteoglycan
CXCL1 Corneal keratinocyte chemokine
Cy3 Cyanine 3
Dlg1 Disks large 1 tumor suppressor protein
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBP50</td>
<td>Ezrin-radixin-moesin-binding phosphoprotein 50</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>Elk-1</td>
<td>ETS domain-containing protein Elk-1</td>
</tr>
<tr>
<td>ErbB2</td>
<td>Receptor tyrosine-protein kinase erbB-2; HER2</td>
</tr>
<tr>
<td>Erbin</td>
<td>ErbB2-interacting protein</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulated kinase 1 and 2</td>
</tr>
<tr>
<td>FKHR</td>
<td>FoxO1; Forkhead box protein O1</td>
</tr>
<tr>
<td>FKHRL1</td>
<td>FoxO3; Forkhead box protein O3</td>
</tr>
<tr>
<td>FPI</td>
<td>Fluid percussion injury</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GAP-43</td>
<td>Growth associated protein-43</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GSK-3α/β</td>
<td>Glycogen synthase kinase-3 alpha/beta</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>hDLG</td>
<td>Human discs large tumor suppressor protein</td>
</tr>
<tr>
<td>HEK293 cells</td>
<td>Human Embryonic Kidney 293 cells</td>
</tr>
<tr>
<td>HER2</td>
<td>Receptor tyrosine-protein kinase erbB-2; ERBB2</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus 1</td>
</tr>
<tr>
<td>HTLV-I</td>
<td>Human T-lymphotropic virus type-1</td>
</tr>
<tr>
<td>ICAD</td>
<td>Inhibitor of CAD</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Insulin-like growth factor-I</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL-18</td>
<td>Interleukin 18</td>
</tr>
<tr>
<td>IL-1α</td>
<td>Interleukin-1α</td>
</tr>
<tr>
<td>Biological Term</td>
<td>Expanded Name</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin-linked kinase</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>Isobaric tags for relative and absolute quantification</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional adhesion molecule</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich repeat</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin-associated glycoprotein</td>
</tr>
<tr>
<td>MAGI-2</td>
<td>Membrane-associated guanylate kinase inverted 2</td>
</tr>
<tr>
<td>MAP2</td>
<td>Microtubule-associated protein 2</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated-protein kinase</td>
</tr>
<tr>
<td>MAST2</td>
<td>Microtubule associated serine/threonine kinase 2</td>
</tr>
<tr>
<td>MAST205</td>
<td>Microtubule-associated serine/threonine kinase 205 kDa</td>
</tr>
<tr>
<td>MCF-7 cells</td>
<td>Michigan Cancer Foundation-7 cells</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen activated ERK kinase</td>
</tr>
<tr>
<td>MEK1/2</td>
<td>Mitogen activated ERK kinase 1 and 2</td>
</tr>
<tr>
<td>Mint3</td>
<td>Munc18-1-interacting protein 3</td>
</tr>
<tr>
<td>MIP-2</td>
<td>Macrophage inflammatory protein 2</td>
</tr>
<tr>
<td>MMAC1</td>
<td>Mutated in multiple advanced cancers-1</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Matrix metalloproteinase-2</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix metalloproteinase-9</td>
</tr>
<tr>
<td>mNSS test</td>
<td>Modified Neurological Severity test</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MT6-MMP</td>
<td>Membrane type matrix metalloproteinase 6</td>
</tr>
<tr>
<td>mTORC2</td>
<td>Mammalian target of rapamycin complex-2</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
</tr>
<tr>
<td>NIH 3T3 cells</td>
<td>3-day transfer, inoculum 3 x 10^5 cells fibroblast cell line</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartic acid</td>
</tr>
<tr>
<td>Nod2</td>
<td>Nucleotide-binding oligomerization domain-containing protein 2</td>
</tr>
<tr>
<td>Omgp</td>
<td>Oligodendrocyte Myelin glycoprotein</td>
</tr>
<tr>
<td>p0071</td>
<td>Plakophilin-4</td>
</tr>
<tr>
<td>Pak1</td>
<td>p21 Activated protein kinase</td>
</tr>
<tr>
<td>PBD</td>
<td>Phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2)-binding domain</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC12 cells</td>
<td>Pheochromocytoma 12 cell line</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDK1</td>
<td>Akt phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD-95/Dlg1/ZO-1 homology</td>
</tr>
<tr>
<td>PDZK1</td>
<td>PDZ domain-containing protein 1; Na^+\text{}/H^+ exchange regulatory cofactor NHE</td>
</tr>
<tr>
<td>PEST motif</td>
<td>Proline, glutamic acid, serine, and threonine-rich sequence</td>
</tr>
<tr>
<td>PHF</td>
<td>Paired helical filaments</td>
</tr>
<tr>
<td>PHLPP</td>
<td>PH domain leucine-rich repeat protein phosphatase</td>
</tr>
<tr>
<td>PHTS</td>
<td>PTEN Hamartoma-Tumor Syndrome</td>
</tr>
<tr>
<td>PI-3,4-P_2</td>
<td>Phosphatidylinositol-3,4-bisphosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PI-4,5-P_2</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PI-4-P</td>
<td>Phosphatidylinositol-4-phosphate</td>
</tr>
<tr>
<td>PINCH</td>
<td>Particularly Interesting New Cys-His Protein</td>
</tr>
<tr>
<td>PIP_3</td>
<td>Phosphatidylinositol-3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>PRR2</td>
<td>Poliovirus receptor-related protein 2; nectin-2</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Postsynaptic density protein 95; Disks large homolog 4</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phophatase and Tensin Homolog</td>
</tr>
<tr>
<td>R9</td>
<td>Nonaarginine; 9 arginine residues</td>
</tr>
<tr>
<td>RGC</td>
<td>Retinal ganglion cell</td>
</tr>
<tr>
<td>RSK</td>
<td>90 kDa ribosomal S6 kinase</td>
</tr>
<tr>
<td>S473</td>
<td>Serine 473 of Akt</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal cord contusion injury</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Sema3A</td>
<td>Semaphorin 3A</td>
</tr>
<tr>
<td>Sema4D</td>
<td>Semaphorin 4D</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2 domain-containing inositol polyphosphate 5-phosphatase</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of Sevenless</td>
</tr>
<tr>
<td>SRE</td>
<td>Serum response element</td>
</tr>
<tr>
<td>Sur-8</td>
<td>Leucine-rich repeat protein SOC-2</td>
</tr>
<tr>
<td>T308</td>
<td>Threonine 308 of Akt</td>
</tr>
<tr>
<td>TAT</td>
<td>Transactivating regulatory protein</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline, containing 0.1% Tween 20</td>
</tr>
<tr>
<td>TEP1</td>
<td>Tensin-like phosphatase-1</td>
</tr>
<tr>
<td>Trk</td>
<td>Tyrosine kinase receptor</td>
</tr>
<tr>
<td>TrkA</td>
<td>Tropomyosin-related kinase A; Tyrosine kinase receptor A</td>
</tr>
<tr>
<td>TrkB</td>
<td>Tropomyosin-related kinase B; Tyrosine kinase receptor B</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labelling</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
Chapter 1: 
Introduction

When adult central nervous system (CNS) neurons are injured, they are unable to regenerate and are susceptible to apoptosis (programmed cell death) [1-3]. Permanent cellular and functional loss ensue. This process occurs after traumatic CNS injury, stroke (ischemia), and over the course of many CNS diseases. For this reason, it is necessary to understand the apoptotic pathways and molecular elements involved therein so that potential therapeutic treatments for CNS insults may be uncovered. The optic nerve contains the axons of the entire population of Retinal Ganglion Cells (RGCs), the projection neurons of the eye, which transmit visual information from the retina to the lateral geniculate nucleus and the superior colliculus of the brain. RGCs are CNS neurons, and thus, injury to the optic nerve leads to the failure of axonal regeneration and results in RGC apoptosis, leading to the permanent loss of visual function [4-13]. Herein, RGCs and the optic nerve are used as a model to explore molecular approaches for protecting CNS neurons following injury.

Two particularly important pathways involved in neuronal survival are the phosphatidylinositol-3 kinase (PI3K) pathway and mitogen-activated-protein kinase (MAPK) pathway [14-23]. These pathways function in the activation of intracellular enzymes, the transcription of survival promoting genes, and the inhibition of apoptotic pathways [24-26]. In this study, I investigate different mechanisms to augment the activity of these pathways, in an attempt to rescue RGCs after CNS injury.
1.1. Research Hypothesis

I hypothesize that increasing the activity of the PI3K or MAPK pathways, through exogenous Thymosin-β4 administration, physical exercise, or by means of interfering with negative regulators in the PI3K and MAPK pathways, will increase the survival or regeneration of RGCs following CNS injury.

1.2. Research Objectives

In order to test my hypothesis, I performed the following experiments:

i. Quantification of the survival and regeneration of injured RGCs following the delivery of exogenous Thymosin-β4, to determine if RGC survival is enhanced.

ii. Investigation of the effects of exogenous Thymosin-β4 on the activation of the PI3K and MAPK pathways in the adult retina, to determine if Thymosin-β4 augments the activity of these survival promoting pathways.

iii. Evaluation of the activation of the PI3K and MAPK pathways in the retinas of adult rats that have undergone a forced-treadmill running exercise program, to test whether exercise can bolster protective PI3K or MAPK activity.

iv. Quantification of the survival of injured RGCs in rats that have undergone a forced-treadmill running exercise program, to test for biological benefits of exercise on injured RGC survival.

v. Test to determine if PDZ peptides that interfere with negative regulators of the PI3K and MAPK pathways can promote RGC survival or regeneration after optic nerve injury.
Chapter 2:
Literature Review

2.1. Central Nervous System Neuron Survival After Injury

The inability of a central nervous system neuron to regenerate is in part due to the CNS microenvironment, which does not allow for axonal regeneration in the mammalian adult after injury [27, 28]. There are multiple contributing factors that contribute to this. First, unlike the peripheral nervous system, there are no Schwann cells to create a growth track for an extending axon in the CNS [1]. Schwann cells ordinarily synthesize the growth factors required for axonal growth in the peripheral nervous system [1]. Second, when a lesion occurs in the CNS, several growth-inhibiting compounds accumulate at the site of injury, including: Nogo, Myelin-Associated Glycoprotein (MAG), Oligodendrocyte Myelin glycoprotein (OMgp), Ephrin B3, Sema 4D, and Sema 3A [29-31]. If these compounds are inhibited, axonal regeneration can be restored. For instance, in the rat spinal cord, it was shown that when growing axons are treated with Nogo, the growth cones become retracted; however, when antibodies are used to block Nogo, spontaneous regeneration resumes [30, 32]. Additionally, MAG has been shown to inhibit axonal regeneration in vitro, and this effect can be reversed with antibodies directed against MAG [31]. Another factor that prevents axonal growth is the glial scar that forms at the site of injury and acts as a physical barrier [28]. It contains further growth-inhibiting compounds, such as Semaphorin 3A, Slit-1, Tenascin-R, and chondroitin sulphate proteoglycans (CSPGs) [33-41]. Axonal growth in the CNS is also hindered by the slow clearing of myelin and axonal debris. This differs from the peripheral nervous system, where debris is cleared more rapidly [42-45]. Finally, the immune response may halt regeneration in the CNS via neuroinflammation, which occurs after CNS injury and involves immune cells such as microglia [46]. Although immune cells have some beneficial effects in the CNS, such as the synthesis of growth factors and the removal of debris, they also release neurotoxic compounds that include free radicals, nitric oxide, and glutamate [47-51].

With so many factors hindering regeneration, injured CNS neurons succumb to apoptosis (programmed cell death). Apoptosis, which is different from necrosis, is a form of cell death that
preserves cell membrane integrity, showing condensation of the cytoplasm and the nucleus, a
decrease in cell volume, plasma-membrane bleb formation, and the morphological preservation
of organelle structure [52]. It involves neither the loss of cellular content, nor the initiation of an
inflammatory response [52], and it is genetically controlled [53]. It is now understood that
apoptosis plays a role in neuronal loss following stroke, spinal cord injury, and traumatic brain
injury [54-57], as well as in chronic degenerative diseases such as Alzheimer’s disease [58],
Huntington’s disease [59], Parkinson’s disease [60, 61], and Amyotrophic Lateral Sclerosis [62].
Therefore, understanding the molecular cascades that govern apoptosis in CNS neurons is
crucial.

2.2. Retinal Ganglion Cells and the Optic Nerve as a Model for
CNS Injury and Disease

Using the mammalian visual system, apoptosis in CNS neurons can be easily explored.
Light that enters the eye is detected by the photoreceptors in the retina. Visual information is
transmitted to the brain by a subset of neurons called Retinal Ganglion Cells (RGCs) that
synapse in the lateral geniculate nucleus and the superior colliculus, from which, the visual
signal is passed on to the primary visual cortex and other brain centres. Together, the axons of
RGCs in the eye form the optic nerve. During development, RGCs differentiate from
diencephalic precursors [63-65], and possess similar properties as other neural tube-derived CNS
neurons. Hence, when the optic nerve is injured, RGCs are unable to regenerate, and the majority
undergo apoptosis, leading to a permanent loss of visual function [4-13].

Together, the retina and optic nerve form a system that is well-suited for experimentation
in the CNS, for several reasons. First, the optic nerve is itself a simple and well-defined CNS
neuronal tract, which contains the sensory axons of an entire neuronal population. It possesses a
well-established microanatomy and function [66], and is easily accessed within the orbit of the
eye. More importantly, the optic nerve can be reproducibly transected or injured (Figure 2.2.) to
disrupt the axons of all the RGCs within the retina [7, 26]. The retina is housed in the vitreous
chamber of the eye. This fluid compartment can be used as liquid-sink for drug delivery to
RGCs, by way of intraocular injections (Figure 2.2.). The diffusion of chemicals through the
vitreous fluid ensures that any chemicals applied will act on all cells in the retina. This
overcomes the difficulties of effective drug diffusion that are encountered in solid brain tissue. Furthermore, RGCs can be selectively transfected by applying short interfering RNAs (siRNAs), plasmids, or viral vectors to the cut or injured end of the optic nerve [67-71] (Figure 2.2), permitting selective therapeutic targeting of RGCs without the confounding effects of bystander neurons or surrounding glia.

An additional advantage of the visual system is the well-characterized and reproducible time-course of RGC apoptosis. After axotomy or crush, RGC apoptosis is delayed 3-4 days. This brief time period provides an opportunity for experimental manipulations. Moreover, axotomizing (transecting) the optic nerve (Figure 2.2.A) causes the apoptotic death of ~90% of the RGC population within 14 days [8-11], while only ~70% of RGCs will die after crushing the optic nerve at this juncture [3] (Figure 2.2.B). After the rate of cell death declines, a large portion of the remaining cells survive long-term. Since the retina is a flat tissue and RGCs form its innermost layer, RGCs can be very accurately quantified. The survival of RGCs can be easily evaluated by applying a fluorescent tracer to the cut end of the optic nerve; by injecting the tracer into the superior colliculus one week prior to injury; or by injecting the tracer directly into the vitreous chamber. A tracer applied by the former two methods is retrogradely transported back to the retinal ganglion cell bodies. RGCs are then assessed by fixing the tissue, flat-mounting the retina, and quantifying the healthy cells under an epifluorescence microscope. More recently, protocols have used immunostaining for RBPMS or Brn isoforms to identify RGCs. Similarly, RGC axonal regeneration can be quantified through immunohistochemistry in fixed transverse sections of optic nerves labelled with antisera directed against growth associated protein-43 (GAP-43), a marker of regenerating retinal ganglion cell axons in both neonatal and adult RGCs [72-77].

Transected RGCs demonstrate condensed nuclei, DNA fragmentation, and form apoptotic bodies (i.e. fragmented and shrunken nuclear chromatin) [8, 78], all of which are hallmarks of apoptosis. Apoptosis is generally understood to be mediated by controllers, such as Bcl-2 and Bax, and executors, such as caspases [7]. In experimental studies, expression of the pro-apoptotic protein Bax has been shown to increase after optic nerve injury, while the expression of the anti-apoptotic proteins Bcl-2 and Bcl-X was decreased [79]. Furthermore, mice that overexpress Bcl-2 displayed decreased RGC death following an optic nerve lesion [80, 81]. As for the executors, caspases are a family of aspartate-specific cysteine proteinases that regulate
apoptosis [82]. In RGC apoptosis following axotomy (optic nerve transection), caspase-3, -6, -8 and -9 play pivotal roles, as all show increased activity [83-89]. Furthermore, when these caspases were inhibited by selective and broad spectrum caspase inhibitors, RGC survival following axotomy was enhanced [84-89]. In addition, inhibition of Caspase-6 and -8 showed increased axonal regeneration following optic nerve crush, suggesting that these caspases also play a role in the regenerative failure of RGCs following injury [89]. Increased levels of caspase-3, -8, and -9 were also detected in RGCs following ocular hypertension [90, 91], and ischemia [92, 93]. In traumatic spinal cord injury, apoptotic activation of caspase-3 has been shown to cause DNA fragmentation [94], possibly through Caspase-Activated Deoxyribonuclease (CAD) [95] or by cleaving and inhibiting the CAD inhibitor, ICAD [96]. Furthermore, in vitro studies in mammalian cells have identified another substrate of caspase-3, gelsolin, which has been associated with causing cell detachment, nuclear fragmentation, blebbing and DNA fragmentation following the induction of apoptosis [97].

When specific caspases were individually knocked out in experimental animals, different results were obtained. For instance, caspase-3 deficiency had profound effects on the brain development of knockout mice. These animals showed reduced CNS apoptosis, resulting in a variety of hyperplasias, ectopic cell masses, disorganized cellular structures and excessive neurons in the cortex, cerebellum, striatum, hippocampus and retina [98]. Caspase-6-deficient mice showed normal brain architecture at 3 months of age, but demonstrated an age-dependent increase in cortical and striatal volume [99]. Furthermore, caspase-6-deficient neurons were protected against excitotoxicity, nerve growth factor deprivation, and myelin-induced axonal degeneration in vitro [99]. Caspase-8 knockout mice died in utero, and showed impaired heart muscle development and congested accumulation of erythrocytes [100]. Furthermore, fibroblast strains derived from these embryos were resistant to death-receptor-mediated apoptosis, but were sensitive to drug-induced apoptosis [100]. Similarly, caspase-9 knockout mice showed embryonic lethality and defective brain development, involving markedly enlarged and malformed cerebrums [101, 102]. Caspase-9-deficient embryonic stem cells and fibroblasts were resistant to several apoptotic stimuli, including UV and gamma radiation [101], and caspase-9 deletion prevented the activation of caspase-3 in embryonic mice brains in vivo [102]. All together these results demonstrate the execution role that caspases play in CNS apoptosis, both in normal development and following lesion or disorder.
Figure 2.2. Optic nerve transection and optic nerve crush. (A) Optic nerve transection involves cutting the optic nerve a few millimeters away from the back of the eyeball. This induces the apoptotic demise of ~90% of the RGCs present in the retina within 14 days. (B) Crushing the optic nerve results in ~70% of RGCs in the retina dying by apoptosis in the subsequent 14 days. Intraocular injections into the vitreous chamber (V.C.) allow for the delivery of tracers, siRNAs, plasmids, viral vectors, or peptides to be applied to the entire population of cells in the retina. To selectively transfect RGCs, chemicals or peptides can be applied at the lesion site in the optic nerve.
2.3. Phosphatidylinositol-3-kinase (PI3K) Pathway in Cell Survival and Apoptosis

Phosphatidylinositol-3-kinases are a family of enzymes capable of catalyzing the phosphorylation of several phosphoinositide substrates. They are divided into three major classes (I, II, and III) based on their structural and functional homologies [103]. Class I PI3Ks are composed of four catalytic subunits and two distinct families of regulatory subunits, and hence are further subdivided into class IA and class IB. Classes IA, IB, and II are primarily located downstream of cell surface receptors [104], where class IA PI3Ks are responsive to growth factors, hormones, neurotransmitters, antigens, and inflammatory stimuli [103, 105]. Class III enzymes form part of the pathway that controls the passage of proteins, vesicles, and membranes through the endosomal and lysosomal compartments [106, 107].

Ligands, such as insulin, NGF, BDNF, and other growth factors, induce receptor tyrosine kinases to activate class IA PI3K through autophosphorylation [14, 16, 108-114]. In the plasma membrane, activated PI3K phosphorylates the phosphoinositides, phosphatidylinositol-4-phosphate (PI-4-P) and phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2), at the 3°-OH of the inositol ring to generate the second messengers, phosphatidylinositol-3,4-bisphosphate (PI-3,4-P2) and phosphatidylinositol-3,4,5-trisphosphate (PIP3), respectively [115-117] (Figure 2.3.). These second messengers activate protein kinase B (PKB), also called Akt [118-121]. It is believed that PIP3 binds PKB/Akt and recruits it to the membrane to bring it in close proximity to another PIP3-bound kinase, phosphoinositide-dependent kinase 1 (PDK1). PDK1 has a higher affinity for PIP3 than PKB/Akt [122], and in unstimulated cells, it resides in the plasma membrane as a constitutively active enzyme [123, 124]. Only when there are increased levels of D3-phosphoinositides present in the membrane, such as following stimulation of PI3K, PKB/Akt will shuttle to the membrane [125]. Co-localization of PKB and PDK1 was shown to lead to the phosphorylation of the catalytic domain of PKB by PDK1 at the threonine-308 site [125, 126]. This leads to the partial activation of PKB. To stimulate full kinase activity, PKB must be phosphorylated at both threonine-308 and serine-473 [127]. Phosphorylation at S473 is required to facilitate structural transition of the Akt kinase domain and allow substrates to align properly within its active site [128, 129]. While several kinases have been proposed as responsible for this additional phosphorylation [130], it is now believed that mammalian target of rapamycin
complex-2 (mTORC2) directly phosphorylates S473 (Figure 2.3.) and enhances the subsequent phosphorylation of PKB/Akt by PDK1 [131-133].

Protein Kinase B/Akt is expressed in mammalian cells as three closely related isoforms: PKBα (Akt1), PKBβ (Akt2), and PKBγ (Akt3). While each isoform is encoded by a separate gene, they share more than 80% of their amino acid sequence, and all possess a similar structural organization: an N-terminal pleckstrin homology domain, a central catalytic domain, and a C-terminal regulatory domain [134, 135]. PKBα (Akt1) is ubiquitously expressed at high levels [136-138]; PKBβ (Akt2) is highly expressed in insulin-sensitive tissues, such as the liver, skeletal muscle, and adipose tissue [139, 140]; and PKBγ (Akt3) is expressed most highly in the brain, testis, lung, mammary glands, and adipose tissue [141, 142].

PKB/Akt is involved in various cellular and physiological processes, such as metabolic functions, including glucose transport, glycogen synthesis, glycolysis, and protein synthesis, as well as other functions involving cell cycle progression, cell growth, cell differentiation, cell survival and anti-apoptotic functions, angiogenesis, and motility [121, 143]. It has been shown that the activation of PKB/Akt is essential in the growth factor-mediated survival of cultured neurons [144]. Upon phosphorylation, PKB has been observed to translocate to the nucleus [145, 146]. Furthermore, activated PKB/Akt phosphorylates various targets (Figure 2.3.). For instance, active PKB/Akt phosphorylates and inhibits caspase-9, thereby preventing the cell from undergoing apoptosis [147]. PKB/Akt also phosphorylates the pro-apoptotic protein, BAD (Bcl-2-antagonist of death) [148-150]. Phosphorylated BAD associates with protein 14-3-3, thereby preventing BAD heterodimerization with Bcl-2 and Bcl-XL and the subsequent apoptotic progression [121, 151, 152]. In the BAF/3 lymphoid cell line, it was shown that the expression of catalytically active Akt mutants promoted the expression of Bcl-2 and c-Myc, and thereby inhibited apoptosis and stimulated cell cycle progression [153]. It has also been shown that the survival factor, platelet-derived growth factor (PDGF), activates NF-κB through PI3K and PKB/Akt [154]. NF-κB activation is required for the expression of c-Myc, a central regulator of cell growth, death and differentiation, and for cell proliferation. Activated PKB/Akt also phosphorylates and inhibits FKHR (FoxO1), FKHRL1 (FoxO3), and AFX (FoxO4), members of the Forkhead family of transcription factors [155-157]. This family of transcription factors is involved in regulating the genes for cell growth, proliferation, and differentiation. Upon phosphorylation, FKHRL1 associates with 14-3-3 proteins, and is retained in the cytoplasm [155]. Thus, it is unable to translocate to the nucleus, where it normally induces the expression of
pro-apoptotic genes. Another target of activated PKB/Akt is Bim, the Bcl-2 interacting mediator of cell death. Upon phosphorylation by Akt, Bim binds to protein 14-3-3, and is unable to execute its pro-apoptotic activities [158]. PKB/Akt can also maintain cell survival by inhibiting the release of cytochrome c from the mitochondria, which is a potent activator of the apoptotic caspase cascade [159, 160]. Furthermore, in axotomized RGCs, it has been shown that the protective effects of insulin-like growth factor-I (IGF-I) are mediated through the PI3K-dependant phosphorylation of Akt and the inhibition of caspase-3 [161]. Through these various methods, PI3K and PKB/Akt prevent apoptosis and mediate cell survival (Figure 2.3.).

Conversely, the action of PKB/Akt can be opposed through dephosphorylation of threonine-308 and serine-473. Several phosphatases negatively regulate PKB/Akt activity in this manner. Protein phosphatase 2A (PP2A), a ubiquitously expressed cytoplasmic serine/threonine phosphatase, dephosphorylates Akt at both T308 and S473 [162, 163]. Another phosphatase, PHLPP, named for PH domain leucine-rich repeat protein phosphatase, has two isoforms, PHLPP1 and PHLPP2, which specifically dephosphorylate S473 of Akt [164, 165]. Other phosphatases indirectly inhibit PKB/Akt activity by interfering with PIP₃. Phosphatase and tensin homolog (PTEN), a tumor suppressor protein, has been shown to negatively regulate the intracellular levels of PIP₃ in cells and dephosphorylate PIP₃ in vitro [166-168] (Figure 2.3.). Similarly, SH2 domain-containing inositol polyphosphate 5-phosphatase (SHIP) causes the breakdown of PIP₃ to PI-3,4-P₂ [169, 170].

The importance of the PI3K-PKB/Akt pathway is highlighted when disruption of the pathway occurs. When different isoforms of PKB/Akt are knocked out in animal models, different phenotypes ensue. PKBα (Akt1)-knockout mice are viable, but have reduced body weight and a retardation of growth compared to the wild-type animals. They also demonstrate spontaneous apoptosis in the testes and thymus, as well increased susceptibility to apoptosis at the cellular level [171, 172]. Mice that are deficient of PKBβ (Akt2) are hyperinsulemic and hyperglycemic due to impaired action of the hormone on liver and skeletal muscle [173]. In PKBγ (Akt3)-knockout mice, there was a reduction in brain size and brain weight [174, 175]. Since all these knockout animals were viable and only showed subtle differences in phenotype, it has been suggested that the three Akt isoforms, at least in some part, are able to compensate for each other [143]. Hence, double-knockout animals show much more severe phenotypes. For instance, Akt1/Akt2 double-null mice exhibited severe dwarfism, atrophy of multiple organ systems, failed adipogenesis, and early neonatal lethality [176]. Likewise, mice that were
deficient of both Akt2 and Akt3 were viable but were glucose and insulin intolerant, and exhibited a reduction in body weight, as well as a reduction in brain and testicular size [177].

On the other hand, overexpression of the molecules involved in the PI3K-PKB/Akt pathway is often associated with tumor formation. Generally, Akt hyperactivation is found more frequently in poorly differentiated tumors that are more invasive, grow faster, and are less responsive to treatment [143]. For instance, an E17K mutation in PKBα (Akt1) results in increased plasma membrane recruitment. This mutation has been identified in human breast, colorectal, ovarian, lung, and melanoma clinical cancer specimens [178-182]. Mutations in the other Akt isoforms, Akt2 and Akt3, have also been identified in various human cancers, such as ovarian, breast, prostatic, and pancreatic cancer [183-186]. Mutations may also occur upstream of Akt in the PI3K pathway. Mutations in the PI3K subunit p110α (PIK3CA) have been identified in human breast, ovarian, gastric, esophageal, and lung cancers [187-191]. Furthermore, mutations, as well as deletions or promoter methylation silencing, in the tumor suppressor, PTEN, lead to an increased activation of PKB/Akt, and are featured in many human cancers [192]. Alterations may also occur in the growth factor receptor or in Ras, and these result in PKB/Akt hyperactivity [193].

In the brain, the importance of the PI3K-PKB/Akt has been demonstrated in numerous experiments. Several studies have shown that Akt signalling is necessary in neuronal protection following trophic factor deprivation, oxidative stress, and ischemic injury [144, 194, 195]. Furthermore, the dysregulation of PKB/Akt activity occurs in several neurodegenerative diseases, including Alzheimer’s disease [196, 197], Parkinson’s disease [198], and Huntington’s disease [199], as well as in the pathobiological processes underlying spinocerebellar ataxia [200]. Moreover, Akt likely plays a role in higher brain functions as there have been several reports of impaired Akt signalling in patients suffering from schizophrenia or mood disorders [201-207].
Figure 2.3. The phosphatidylinositol-3-kinase (PI3K) pathway. PI3K is activated upon ligand binding to the appropriate receptor tyrosine kinase. PI3K phosphorylates PI-4,5-P$_2$ to PIP$_3$. PIP$_3$ recruits Akt to the cell membrane, where Akt is phosphorylated at T308 and S473, and activated. Activated Akt is involved in various cellular and physiological processes, including the inhibition of apoptosis and the induction of cell survival. PTEN negatively regulates the PI3K pathway by reducing the number of PIP$_3$ second messengers.
2.4. Mitogen-activated-protein kinase (MAPK) Pathway in Cell Survival and Apoptosis

The mitogen-activated-protein kinase (MAPK) pathway is evolutionarily conserved in all eukaryotes, and has been shown to participate in diverse cellular processes, such as cell division, differentiation, migration, and apoptosis [208]. MAPKs are a large superfamily of serine/threonine kinases, for which several major subfamilies have been identified in mammals. These include the extracellular signal-regulated kinase 1 and 2 (ERK1/2), which are primarily activated by mitogenic stimuli and are preferentially involved in regulating cell growth and differentiation [208]. Other members encompass the stress-activated MAP kinases, which are the c-Jun N-terminal kinases (JNK) and the p38 kinases. These respond to such stressful stimuli as inflammation, cytokines, UV irradiation, growth factor deprivation, heat or cold shock, osmotic stress, and inhibitors of DNA and protein synthesis [209]. Furthermore, these stress-activated kinases have been shown to play roles in axonal outgrowth [210, 211], insulin signaling regulation [212], and immune cell function [213]. Each MAPK subfamily is activated through a protein kinase cascade, where each downstream kinase serves as a substrate for the upstream activator. Thus, a cascade typically consists of the MAPK, a MAPK activator (MAPK kinase or MEK), and a MEK activator (MEK kinase or MAPK kinase) [208].

In the extracellular signal-regulated kinase (ERK) subfamily (Figure 2.4.), when the appropriate ligand, such as a growth factor or cytokine, binds to its receptor tyrosine kinase or following mitogenic stimulation, activation of the receptor occurs through autophosphorylation [214]. The receptor then binds to an adaptor protein, Grb2, via its Src homology 2 (SH2) domain. Grb2 (growth factor receptor-bound protein 2) subsequently binds Son of Sevenless (SOS), a guanine nucleotide exchange factor [214-216]. Thus, activation of the receptor tyrosine kinase results in the recruitment of SOS to the plasma membrane, where the small GTPase, Ras is localized. SOS is able to release GDP from Ras, and allow it to bind GTP and become activated [217]. Ras can be activated through various other means, and functions as a molecular switch in the control of the intracellular signalling pathways involved in many fundamental cellular processes [218]. Ras is frequently mutated in human tumors [219]. GTP-bound Ras recruits Raf (MAPK kinase kinase) to the membrane (Figure 2.4.), where Raf becomes activated [220-225]. Raf is responsible for the phosphorylation and activation of the mitogen activated ERK kinase 1 and 2 (MEK1 and MEK2) (MAPK kinases) [218, 226-228]. MEK1 and MEK2 phosphorylate
ERK1 and ERK2 (MAPKs) [214, 229, 230] (Figure 2.4.). Additionally, ERK1/2 can be activated by various neurotransmitters and hormones through increased intracellular calcium levels via stimulation of either G protein-coupled receptors or ligand-gated ion channel-coupled receptors [231-234]. Activated ERK1 and ERK2 phosphorylate a variety of substrates in both the nucleus and the cytoplasm.

ERK1 and ERK2 regulate numerous important cellular functions, such as proliferation, differentiation, apoptosis, and synaptic plasticity, as well as many aspects in neural development. ERK1 and ERK2 have a 95% common amino acid sequence among humans, mice, and rats [235], and are 84% identical to each other [236]. Furthermore, they are both activated by the same kinases, MEK1 and MEK2, and both appear to have identical substrate specificities [237, 238]. However, some differences are revealed in transgenic animals. ERK1 knockout mice were found to have no gross morphological abnormalities and an enhancement in striatum-dependent long-term memory, as measured by passive avoidance tests [239]. On the other hand, ERK2 knockout mice died in utero, while heterozygous ERK2 knockouts showed anatomical impairments [240]. When ERK2 was deleted in neural precursor cells only, the mice developed a normal brain structure and no initial behavioural abnormalities [241]. However, it was soon uncovered that these animals have a decrease in neural progenitor cell proliferation, resulting in a thinning of cortical layers II/III [242]. In adult rats, ERK1 and ERK2 mRNA is most highly expressed in the nervous system, where ERK1 shows a uniform expression and ERK2 shows a higher expression in the forebrain [236]. Using in situ hybridization histochemistry, ERK1 mRNA was identified in the olfactory bulb, cortex, regions of the hippocampus, amygdala, nucleus basalis of Maynert, substantia nigra, some hypothalamic and brainstem nuclei, the cerebellum, and in neurons of the spinal cord [243]. ERK2 mRNA was observed in the cerebral cortex, olfactory bulb, hippocampus, amygdala, basal ganglia (except the globus pallidus and endopeduncular nucleus), basal nucleus, thalamus, hypothalamus, brain stem nuclei, cerebellum and neurons in the spinal cord [243].

In the CNS, there have been several means identified by which ERK1/2 can be activated. In experimental animals, MAPK can be activated following electroconvulsive shock [244, 245], general seizure activity [246], or transient brain ischemia [247, 248]. Thus, both depolarization and stimulation of glutamate receptors are capable of activating ERK1/2. This has been supported by findings in cultured cortical neurons, where spontaneous synaptic activity led to the activation of MAPK [249]; and in cultured hippocampal neurons, where stimulation of NMDA
receptors [232] or glutamate receptors [250] led to the phosphorylation of MAPK. Furthermore, the involvement of the MAPK pathway following neurotrophic factor signalling has also been demonstrated. For instance, MAPK activation following NGF stimulation is critical in the differentiation of PC12 cells and sympathetic neurons [251-254]. Since NGF induced a sustained MAPK activation, it has been suggested that prolonged MAPK activation is required for its translocation to the nucleus and neuronal differentiation of PC12 cells [255]. MAPK activation has also been reported following exposure to BDNF in cultured hippocampal neurons [256, 257] and in cultured cortical astrocytes [258]. Moreover, MAPK can be activated through G-protein coupled receptors. In rat 1a fibroblasts, MEK (MAPK kinase) was stimulated through the acetylcholine muscarinic M2 receptor, coupled to G\textsubscript{i}, and this effect was insensitive to a tyrosine-kinase inhibitor [259]. Similarly, in PC12 cells, cAMP is able to stimulate the MAP kinase cascade through the activation of the small G-protein, Rap1 [260].

Once activated, MAPK phosphorylates a large number of targets (Figure 2.4.), which some have estimated as greater than 600 [214]. Many of these are involved in cell survival and proliferation. For instance, among the various cytoskeletal components which MAPK phosphorylates is microtubule-associated protein 2 (MAP2) [261]. Since highly phosphorylated MAP2 has reduced ability to stabilize microtubules and actin filament bundles, it has been suggested that the action of MAPK may contribute to synaptic remodeling or dendritic arborisation in neonatal development [261]. Furthermore, MAPK translocates to the nucleus and directly phosphorylates the transcription activator, Elk-1 [262]. Elk-1 is one of the ternary complex factors, which form part of the multicomponent complex that binds with the c-fos promoter, serum response element (SRE), for the activation of transcription of the proto-oncogene c-fos [263]. Phosphorylation of Elk-1 by MAPK is critical for transcription to take place [264, 265]. Elsewhere, it has been found that activated MAPK promotes the survival of postnatal RGCs of rats both in vitro and in vivo [266, 267].

Other targets of activated MAPK are the 90 kDa ribosomal S6 kinases (RSKs) [268-270]. Different RSKs have been identified, and their functions, following MAPK phosphorylation, have been partially determined. Downstream of the MAPK cascade, Rsk1, once activated, directly phosphorylates and inhibits the pro-apoptotic protein, BAD [271], thereby contributing to the survival functions associated with MAPK. Rsk2 phosphorylates the transcription factor CREB (cAMP response element-binding protein) following MAPK activation, and thereby
induces transcription of the genes associated with the cAMP response elements (CRE) gene [272].

A role for MAPK in several disease processes has also been implicated. It has been observed that during brain ischemia, phosphorylation of the cytosolic phospholipase A2 (cPLA2) occurs, and may play a role in the associated free fatty acid-mediated neurotoxicity [273-275]. Phosphorylation of cPLA2 is mainly catalyzed by ERK1 and ERK2, and results in the translocation of cPLA2 to the membrane and in increased intrinsic activity of the enzyme [276-279]. This is further supported by observations that MAPK is activated following brain ischemia [247, 248]. Another disease where MAPK involvement has been suggested is Alzheimer’s disease. Experimentations in the brains of Alzheimer’s patients typically uncover abnormal paired helical filaments (PHFs), in which tau, a microtubule-associated protein, is a major component. Unlike normal tau, pathological tau is hyper-phosphorylated and this may compromise its ability to bind to and stabilize microtubules [280]. Several studies have supported the involvement of MAPK in the phosphorylation of tau to produce PHFs [281, 282].

Other less-well understood associations between MAPK and disease have been made. For instance, it was found that in 1% of autism cases, there is a 16p11.2 microdeletion [283, 284]. This genetic loci contains the gene of the p44 MAPK (Erk1) [285]. Another condition, termed Noonan syndrome, results from mutations in the genes encoding some of the elements upstream to the Erk cascade, such as Shp, SOS, Ras, and Raf [286, 287]. Patients with Noonan syndrome show diverse phenotypes that may include developmental delays, behavioural and learning disabilities, pulmonic stenosis, short stature, and facial dysmorphia, and either normal or abnormal cognitive development [288-290]. Other syndromes that involve mutations in the components of the MAPK cascade include neurofibromatosis type I [291], LEOPARD syndrome [292], cardio-facio-cutaneous syndrome [293], and Costello syndrome [293, 294].

Finally, many mutations along the MAPK cascade have been detected in human cancers [214]. Mutations in MAPK, as well as in components such as Ras, Raf, MEK1/2 can vary in type and occur in different locations, resulting in different human cancers, such as leukemia, melanoma, and many others.
Figure 2.4. The mitogen-activated-protein kinase (MAPK) pathway. Induction of the receptor tyrosine kinase leads to the activation of SOS, which subsequently removes GDP from Ras, and allows Ras to bind GTP. GTP-bound Ras is activated and binds Raf, leading to the activation of Raf. Raf activates the MAPK cascade, leading to the activation of ERK1/2 and the induction of cell survival and proliferation. Erbin and BCR negatively regulate the MAPK pathway by binding Ras and disrupting the interaction of Ras with Raf.
2.5. Thymosin-β4

After completing a large scale iTRAQ proteomics study of the retinal tissue of adult rats whose optic nerves had been injured (crushed) (see [295]), our lab uncovered several hundred differentially expressed proteins. Among them, we found that the levels of Thymosin-β4 were increased on day 3 post-injury, the time point that immediately precedes the active phase of RGC apoptosis. Then, on day 4, when RGCs begin to die at a substantial rate, Thymosin-β4 levels took a sharp dip.

Thymosin-β4 is a small 43 amino acid intracellular polypeptide, first isolated from bovine thymus tissue [296]. After its initial discovery, Thymosin-β4 was found to play a role in T-lymphocyte differentiation and in the inhibition of macrophage migration [297]. It also exerts effects on the hypothalamus and pituitary gland by stimulating the secretion of luteinizing hormone releasing factor [298]. Thymosin-β4 was soon found present in a number of other tissues, including the brain, spleen, lung, liver, and heart muscle [299], and it is synthesized by several cell lines [300]. Not long after, Safer et al. discovered that Thymosin-β4 functions as a G-actin sequestering peptide in human platelets. They found that Thymosin-β4 forms a 1:1 complex with G-actin, and inhibits G-actin polymerization at approximately equimolar concentrations [301, 302]. By maintaining the pool of actin monomers, Thymosin-β4 controls the assembly and disassembly of actin filaments, thereby regulating the dynamics of the actin cytoskeleton. It is now understood that G-actin sequestering is a property that all β-Thymosins possess [303-306], and requires amino acids 17 to 22 (L-K-K-T-E-T) [306-308].

Thymosin-β4 has been shown to play several roles throughout the body, among which is its role in promoting angiogenesis [309-312]. It was found that Thymosin-β4 transfection increases the rate of attachment and spreading of endothelial cells on matrix components, and that Thymosin-β4 is one of the major early genes induced in cultured endothelial cells. [309]. Moreover, Thymosin-β4 has a chemoattractant effect on endothelial cells in vitro; it stimulates the migration of human umbilical vein endothelial cells in vivo; and increases the production of matrix metalloproteinases by endothelial cells, which are required to degrade the basement membrane in angiogenesis [311, 313, 314]. In addition, Thymosin-β4 facilitates endothelial cell migration by reducing its interaction with G-actin at the leading edge, and allowing actin to be available for filament assembly [315]. Thus, Thymosin-β4 coordinates actin polymerization with
metalloproteinase synthesis to promote endothelial cell motility [315]. Similarly, in tumor cells, Thymosin-β4 expression promotes the formation of pseudopodia-like extensions [316]. By activating cell migration and angiogenesis, Thymosin-β4 is believed to stimulate tumor metastasis [317]. Furthermore, Thymosin-β4 regulates other factors that promote angiogenesis and tumor metastasis. *In vitro* studies have shown that Thymosin-β4 increases the production of vascular endothelial growth factor (VEGF) [317]; vascular endothelial-cadherin, which is important for normal vascular development, vascular stabilization, and endothelial survival; and angiopoietin-2, which is important for angiogenic outgrowth, vessel remodeling, and vessel maturation [318]. Thymosin-β4 also down-regulates Von Willebrand factor, which is involved in various cardiovascular events that are associated with endothelial dysfunction [318].

Another well-established feature of Thymosin-β4 is its role in wound healing. Thymosin-β4 has been shown to promote wound healing in several different experimental models. When Thymosin-β4 was applied topically or intraperitoneally in the rat full-thickness dermal wound model, it increased the re-formation of the epithelium, the contraction of the wound, collagen deposition, and angiogenesis [319]. Moreover, Thymosin-β4 was able to significantly improve wound healing in diabetic and aged-mice, both of which ordinarily show delayed wound healing [320]. Interestingly, polymorphonuclear leukocytes (neutrophils) and platelets, which are among the first cells that enter the wound site, both contain the highest concentrations of Thymosin-β4 [321]. Not surprisingly then, Thymosin-β4 demonstrates anti-inflammatory properties (described below), which may contribute to its wound healing effects.

Thymosin-β4 also possesses important functions related to heart development and repair. In isolated epicardial explants of mice, Thymosin-β4 treatment stimulated the extensive outgrowth of cells, restored the pluripotency and triggered the differentiation of fibroblasts, smooth muscle cells, and endothelial cells for the formation of new vessels [322]. It promoted myocardial and endothelial cell migration in the embryonic heart, and allowed post-natal cardiomyocytes to retain this property [323, 324]. Furthermore, it stimulated capillary-like tube formation of adult coronary endothelial cells [324]. It was shown that following coronary artery ligation in mice, Thymosin-β4 increased the activation of the survival kinase, Akt/Protein Kinase B, *in vivo*, enhanced early myocyte survival, and improved cardiac function [323]. When Thymosin-β4 was knocked-down in the heart, there was a significant reduction of N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP), a pro-angiogenic cleavage product [322]. Furthermore, knockdown impaired heart vessel development, resulting in a thin myocardium, reduced smooth
muscle recruitment and differentiation, failed ventricular contraction, and an abnormal aorta and pulmonary arteries [322].

Thymosin-β4 also promotes corneal repair in several models of eye injury. A single 5 μg dose of Thymosin-β4 enhanced and accelerated the re-epithelialization of the corneas of adult rats in vivo, which had been experimentally wounded [325]. Similar results were obtained when the corneas were injured by an alkali burn, which causes a more severe wound to the eye [326]. This property of Thymosin-β4 is attributed to its ability to increase cell-cell and cell-matrix attachments, as well as its ability to increase conjunctival epithelial cell migration [327], and decrease the expression of three major matrix metalloproteinases: MMP-2, MMP-9, and MT6-MMP (membrane type matrix metalloproteinase 6) [328]. While this effect on matrix metalloproteinases contradicts what Thymosin-β4 does during angiogenesis (described above), it is likely tissue-specific to the cornea [329]. Furthermore, Thymosin-β4 has been shown to inhibit injury-induced apoptosis in the eye. A single application of Thymosin-β4 demonstrated decreased deleterious mitochondrial alterations by maintaining mitochondrial membrane integrity, decreased cytochrome C release from the mitochondria, and increased Bcl-2 expression in ethanol-exposed human corneal epithelial cells in vitro [330]. It also inhibited the activity of caspase-2, -3, -8, and -9 in the same corneal injury model [330]. Other in vitro studies showed that Thymosin-β4 inhibits apoptosis initiated by benzalkonium chloride in human conjunctival epithelial cells and human corneal epithelial cells [331].

Following an eye injury, the host inflammatory response is one of the major factors that determine the outcome of recovery. Numerous ocular complications result from the infiltration of polymorphonuclear leukocytes into the stroma of the cornea. When Thymosin-β4 was applied topically after an alkali injury, there was a reduction in the infiltration of polymorphonuclear leukocytes, as well as a decrease in corneal keratinocyte chemokine (CXCL1) and macrophage inflammatory protein-2 (MIP-2) chemokine expression [328]. These molecules are functionally homologous to human interleukin-8 (IL-8), which is chemotactic for neutrophils (polymorphonuclear leukocytes). Furthermore, a single 25 μg dose of Thymosin-β4 was able to modulate the production of corneal cytokines in vivo, 6 and 24 hours after epithelial debridement with heptanol [325]. At 6 hours post injury, mRNA transcript levels of IL-1α, IL-1β, IL-6, and IL-18 were increased, while those of IL-1β and IL-6 were further increased at 24 hours post-injury [325]. In human corneal epithelial cells, Thymosin-β4 treatment up-regulated the expression of anti-oxidative enzymes, reduced intracellular reactive oxygen species and
increased cell viability following the induction of apoptosis by hydrogen peroxide [332]. Moreover, in a dose-dependent manner Thymosin-β4 enhanced Natural Killer cell cytotoxicity in vitro, as demonstrated by a greater degranulation of cytolytic granules [333].

The ability of Thymosin-β4 to affect cell migration has implications in the brain as well. Thymosin-β4 is differentially expressed in the developing rat cerebellum, particularly in the premigratory granule cells, in growing neuronal processes, in Golgi epithelial cells, and in Bergmann processes [334]. Furthermore, a two-week nerve growth factor (NGF) treatment on PC12 rat pheochromocytoma cells induced a 10-fold increase in Thymosin-β4 levels [335]. These results suggest a role of Thymosin-β4 in neurite outgrowth during development. Following focal ischemia, Thymosin-β4 is dramatically up-regulated in the infarcted brain area [336]. Moreover, an application of Thymosin-β4 attenuated glutamate-induced toxicity in both primary cortical neuron cultures and in rat hippocampal slices, while an intracerebroventricular administration of Thymosin-β4 reduced in vivo hippocampal neuronal loss following kainic acid excitotoxicity [337]. It is believed that Thymosin-β4 promotes neural survival in part by a dose-dependent up-regulation of the protein L1, which is important for cell survival and outgrowth of cultured spinal cord neurons [338]. Thymosin-β4 has also been tested in other models of neurological injury. In the rat embolic stroke model, a regular intraperitoneal administration of Thymosin-β4 improved scores in the animal adhesive removal test (ART) and the modified Neurological Severity (mNSS) test. It also promoted axonal remodeling [339]. Similarly, in the experimental autoimmune encephalomyelitis mouse model of multiple sclerosis, Thymosin-β4 treatment improved neurological and functional outcome by nearly 50% when compared to controls, and showed reduced inflammatory infiltrates and increased levels of oligodendrogenesis [340]. Finally, in the rat model of traumatic brain injury, Thymosin-β4-treated animals showed improved results in the Morris water maze test, the foot fault test, and the modified Neurological Severity Score (mNSS) test [341]. These animals also showed reduced hippocampal cell loss, enhanced angiogenesis and neurogenesis in the injured cortex and hippocampus, and increased oligodendrogenesis in the CA3 region [341]. Together, these results indicate that Thymosin-β4 possesses a valuable therapeutic potential for injured nerves and brain tissue.
2.6. Physical Exercise

It is well established that physical exercise has beneficial effects throughout the body. Several studies have indicated that exercise produces positive effects in the regeneration of peripheral nerves. Following peripheral nerve injury in animal models, exercise was shown to improve axon regeneration and elongation [342-344], muscle re-innervation [345], and sensory and motor function [346, 347]. Further studies have revealed that exercise can benefit human patients with peripheral neuropathy as demonstrated by improved nerve conduction velocities, improved muscle function and strength, and by preventing the onset or progression of neuropathy [348-350].

However, as previously explained, the peripheral nervous system is very different from the CNS. Nevertheless, there are numerous indications that the benefits of exercise extend into the CNS. Two studies carried out in normal adult mice showed that voluntary exercise in a running wheel can increase neuronal proliferation, survival, and net neurogenesis in the dentate gyrus of the hippocampus [351, 352]. Furthermore, voluntary running in these animals also enhanced long-term potentiation (LTP) in the dentate gyrus, and improved spatial learning, as tested in the Morris water maze [351]. Similarly, voluntary running was shown to improve locomotor recovery following spinal cord contusion injury (SCI) in mature rats, as tested by the BBB locomotor rating scale, the BBB sub scale, the thoracolumbar height test, the Gridwalk test, and the CatWalk test [353].

Several studies have offered clues to identify how the benefits of exercise are conveyed at the molecular level. Among other things, exercise was shown to increase brain-derived neurotrophic factor (BDNF) in the hippocampus and caudal cerebral cortex [354, 355], as well as the spinal cord of adult rats [356-358]. Other molecules that were shown to increase after exercise in the spinal cord include the BDNF receptor, TrkB; synapsin I, the synaptic mediator of the action of BDNF in neurotransmitter release; growth-associated protein 43 (GAP-43); and cyclic AMP response element-binding protein (CREB) [357]. Another study found that one week of voluntary running was able to reverse the increases in the growth inhibiting molecules, myelin-associated glycoprotein (MAG) and Nogo-A, in the hippocampus, following fluid percussion injury (FPI), a model of traumatic brain injury (TBI) in adult rats [359]. The FPI model of TBI was also shown to reduce the levels of GAP-43 and synaptophysin, which are
markers of axonal growth and synaptic growth, respectively [359]. Not only did voluntary exercise reverse these effects, but it also increased the levels of protein kinase A [359], which is involved in the BDNF signalling pathway for overcoming the inhibitory effects of myelin [360]. Interestingly, all these effects were abolished when the researchers used a chimera TrkB-IgG antibody to block the signalling of BDNF, suggesting that the effects of exercise are carried out through BDNF [359].

BDNF, a neurotrophin that supports the function and survival of neurons, was shown to activate both the MAPK and PI3K/ERK pathways in the adult rat retina, following optic nerve axotomy [20]. Likewise, NGF, another neurotrophin, is also able to activate these pathways [361-363]. Thus, as expected, several studies have demonstrated that the PI3K and MAPK pathways can be activated following different exercise regiments. For instance, a forced moderate-intensity treadmill exercise program (8 weeks, 5 days/week), in D-Galactose-induced aged rats, increased the levels of several molecules in the PI3K pathway [364]. The aged animals that underwent the exercise program showed increased levels of NGF, TrkA, phosphorylated PI3K, and phosphorylated Akt compared to the control aged animals [364]. They also showed decreased levels of caspase-3, and a reduction in the number of apoptotic cells as measured by a TUNEL assay [364].

Similarly, a study was performed in a transgenic mouse model of Alzheimer’s disease, the Tg-NSE/PS2m model, in which the human mutant of the PS2 gene is expressed in the brain [365]. After completing a 12 week exercise program (12 m/min, 60 min/day, 5 days/week), exercised transgenic animals performed significantly better in a water maze test than transgenic animals that were sedentary [365]. Furthermore, both normal and transgenic animals that underwent the exercise program showed decreased levels of β-amyloid 42, caspase-3, cytochrome c, and Bax in the hippocampus, compared to their inactive counterparts [365]. Exercise also decreased the levels of phosphorylated tau, phosphorylated JNK, and phosphorylated p38 MAPK in the hippocampus of transgenic animals, while increasing the levels of phosphorylated ERK1/2 [365]. Likewise, exercise increased the levels Bcl-2, NGF, BDNF, phosphorylated CREB, phosphorylated PI3K, phosphorylated Akt, and phosphorylated GSK-3α/β in the hippocampi of both normal and transgenic mice, compared to their sedentary counterparts [365]. The hippocampi of transgenic mice that underwent the exercise program also showed significantly less apoptotic cells, as measured by TUNEL staining, compared to the control transgenic mice [365].
Therefore, it is evident that exercise exerts positive effects on the survival of CNS neurons. This evidently involves the action of neurotrophins and is likely carried out through the PI3K and MAPK pathways.

2.7. PDZ Domains and PDZ Binding Motifs

PDZ (PSD95/Dlg1/ZO-1 homology) protein interacting domains are the most common type of protein-protein interaction [366]. They function as modules and scaffolds for protein-protein interactions. PDZ domains were first discovered in three proteins, from which they were named: PSD95, a postsynaptic density protein expressed in neurons [367]; its Drosophila homologue, a disc large tumor suppressor septate junction protein, Dlg1 [368]; and zonula occludens-1 (ZO-1), a tight-junction protein [369]. PDZ domains are approximately 90 amino acid residues in length [370-373], and are typically made up of six β-strands, and two α-helices [374]. Most PDZ containing proteins contain multiple PDZ domains [370-373]. PDZ domains act as specific interaction ‘pockets’ for PDZ binding motifs [366, 370], which are often found at the C-termini of target proteins [375]. PDZ motifs are usually about four residues long, where amino acid 0 (the C-terminal residue) and residue -2 are the most important for recognition by the PDZ domain [370]. There are four types of PDZ motifs based on their amino acid composition. Type I is comprised of [S/T]-X-Φ-COOH; type II of Φ-X-Φ-COOH; type III of Ψ-X-Φ-COOH; and type IV of D-X-V-COOH (where X is any amino acid, Φ is any hydrophobic residue, and Ψ is any basic and hydrophilic residue) [370, 375]. PDZ domains can also bind to other PDZ domains, internal PDZ motifs, other internal peptide sequences, and even lipids [370-373]. In this way, PDZ domains form complex protein interaction webs, which govern many molecular processes, such as targeting, clustering, cycling, and membrane expression of receptors, transporters and ion channels [371-373]. Thus, PDZ-containing proteins are essential in linking various intracellular signalling pathways and networks [371-373].

Blocking peptides, containing the amino acids that match the length and sequence of the PDZ binding motif, can be synthesized and easily modulate any PDZ interaction [366]. In blocking a specific PDZ interaction, a lot can be understood about a protein’s function. Furthermore, the small length of the PDZ binding motif provides an opportunity to create
blocking peptides of a minimal length. There have been many successful examples that used small peptides to block PDZ interactions [366].

2.8. Phosphatase And Tensin Homolog (PTEN)

Phosphatase and tensin homolog (PTEN) is a phosphatase that negatively regulates the PI3K pathway. Also called MMAC1 (mutated in multiple advanced cancers-1) and TEP1 (tensin-like phosphatase-1), PTEN was first reported in 1997 by three independent groups; two of which were trying to identify the potential tumor suppressor gene located at chromosome 10q23 that is often mutated or deleted in human cancers [376-378].

PTEN is a 403 amino acid peptide, containing a protein tyrosine phosphatase domain and extensive homology to tensin and auxilin [377]. Crystal structure analysis of PTEN reveals several important features. First, PTEN contains a phosphatase domain (aa 7-179), similar to other protein phosphatases, but with an enlarged active site to accommodate phosphoinositide substrates [379]. This N-terminal phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2)-binding domain (PBD) is important for the interaction of PTEN with the membrane and its catalytic activity [380, 381]. PTEN also contains a C2 domain (aa 186-351), in which mutations to the basic residues reduce its ability to bind to the membrane and suppress tumors [379]. Both the C2 domain and the PBD are essential for PTEN’s function.

The C-terminal tail (aa 353-403) contains several motifs that are important for the physiological regulation of PTEN, including several phosphorylation sites, two PEST motifs, and a PDZ binding motif [382, 383]. Although the tail was not thought to be crucial for the phosphatase activity of PTEN, it has been shown that it is important in maintaining PTEN’s stability, based on the phosphorylation of three important residues, S380, T382, and T383 [382]. Phosphorylation at these sites increases the breakdown of PTEN through polyubiquitination and proteasome-mediated degradation, and also decreases PTEN’s binding affinity to its interacting partner molecules [384, 385]. The PDZ binding motif of PTEN (aa 401-403) is important for binding to several molecules, including hDLG (human discs large tumor suppressor protein), MAST205 (microtubule-associated serine-threonine kinase 205 kDa), and MAGI-2 (membrane-associated guanylate kinase inverted 2) [386, 387]. It has been shown that the binding of PTEN to a PDZ domain of MAGI-2, a membrane-localized multi-domain scaffolding protein, recruits
PTEN to the plasma membrane and increases PTEN protein stability [387, 388]. In the human hepatocarcinoma 7721 cell line, it was shown that MAGI-2 was able to up-regulate PTEN activity, by increasing protein stability rather than increasing mRNA levels, and that MAGI-2 was able to inhibit cell migration and proliferation in a PTEN-dependant manner [389]. Furthermore, binding to MAST kinases facilitated the phosphorylation of PTEN by those kinases [388]. When a rabies virus, containing a viral G-protein with a C-terminal PDZ binding motif similar to that of PTEN, was used to infect neuroblastoma cells, there was a disruption of the interaction between PTEN and MAST2, as well as a cellular re-localization of PTEN and an induction of neuronal survival [390]. It has been suggested that by interacting through its PDZ binding motif, PTEN shifts into an open or active conformation, as its C-tail becomes sequestered away from interacting with the C2-domain and its membrane-binding regions become exposed [391]. Together, these findings propose that a disruption between PTEN and its PDZ-binding partners would ultimately lead to a decrease in the function of PTEN.

PTEN’s function involves the activity of both a protein phosphatase and a lipid phosphatase [166-168, 376]. Its primary cellular substrate is the phospholipid, phosphatidylinositol-3,4,5-trisphosphate (PIP₃), which PTEN hydrolyses to phosphatidylinositol-4,5-bisphosphate (PI-4,5-P₂) [166-168] (Figure 2.3.). By reducing the number of PIP₃ second-messengers, PTEN negatively regulates the PI3K pathway by decreasing PIP3-dependant membrane recruitment of kinases and the activation of PKB/Akt (Figure 2.3.). Therefore, PTEN inhibits cell survival, cell growth, and cell proliferation, which is critical in the prevention of tumor formation.

It is thought that PTEN’s protein phosphatase activity is primarily involved in inhibiting cellular migration, which is carried out through its C2 domain [392]. This has been demonstrated in mesodermal cells in the chick embryo [393], glioma cells [394], NIH 3T3 cells in the dermal wound healing model [395], and other cells [396]. The ability to inhibit cellular migration likely allows PTEN to suppress tumor progression. Hence, it was shown that in a human bladder cancer cell line, both the wild-type PTEN and a G129E mutant PTEN, which lacks lipid phosphatase activity, were able to inhibit cancer cell invasion; while, a G129R mutant, which lacks both protein and lipid phosphatase activity, was unable [397]. It should be noted that the G129E mutant was unable to suppress colony formation, suggesting that lipid phosphatase activity is essential in inhibiting cell growth. Elsewhere, it was shown that the G129E mutant was able to arrest cell cycle progression in MCF-7 cells, while both the G129R mutant and a similar mutant,
H123Y, were ineffective; indicating an important role of the protein phosphatase activity in cell cycle arrest [398]. PTEN is also believed to be involved in maintaining chromosomal integrity, since PTEN has been found localized at the centromeres, and disruption to PTEN leads to centromere breakage, chromosomal translocations, and double-stranded DNA breakage [399].

Thus, PTEN plays an important role in limiting cell survival and proliferation. Indeed, germ line mutations in PTEN result in the PTEN Hamartoma-Tumor Syndromes (PHTS), which include Cowden disease (tumors in breast, thyroid, or skin) [400], Bannayan-Riley-Ruvalcaba syndrome [401], Proteus syndrome, and Proteus-like syndrome [402]. Furthermore, certain types of cancers have a high frequency of PTEN mutations, including primary glioblastoma [403], endometrial carcinoma [404-406], and primary prostate cancer [407]. Even subtle changes in PTEN levels can cause profound effects on tumor susceptibility [408, 409]. Transgenic animals have further demonstrated the importance of PTEN. PTEN knockout mice showed early embryonic lethality, while heterozygous PTEN knockouts were viable, but developed lymphomas as well as neoplasms in multiple different organs, including the breast, prostate, adrenal medulla, endometrium, and intestines [410-412]. Furthermore, mice models with tissue specific deletions of PTEN resulted in cancer of the bladder [413], breast [414], endometrium [415], liver [416], lung [417], prostate [418], and in T-lymphocytes [419].

Finally, PTEN is also heavily involved in the central nervous system, in both development and in adulthood in such processes as neurogenesis, neuronal migration, neuronal size control, neurite outgrowth, synaptogenesis, and synaptic plasticity [420-424]. In the adult brain, PTEN was found to be preferentially expressed in neurons, particularly in Purkinje neurons, olfactory mitral neurons, and large pyramidal neurons [425]. At the subcellular level, PTEN is localized in both the nucleus and cytoplasm [426]. PTEN can also be found in the axon and in growth cones, where it rapidly accumulates on the growth cone membrane during the growth cone collapse response following semaphorin 3A (Sema3A) signalling [427]. It is believed that PTEN is necessary following the signalling of several neurite outgrowth inhibitors, such as Sema3A, Sema4D, and MAG (myelin-associated glycoprotein) in dorsal root ganglion, hippocampal, cortical, and spinal neurons [427-430]. Thus, unsurprisingly, deletion of PTEN in adult RGCs promoted robust axonal regeneration after optic nerve injury [431]. Similarly, reducing PTEN, either through genetic deletion or through short hairpin RNAs (shRNAs), increased corticospinal tract sprouting and axon regeneration, as well as enhanced synaptic formation in different models of spinal cord injury [432, 433].
PTEN mutations or deletions have been suggested as possible causes for several neurological disorders, such as macrocephaly, ataxia, seizures, mental retardation, and autism [425, 434-437]. Furthermore, in Alzheimer’s disease, a significant loss of PTEN, as well as an altered distribution of PTEN, were detected in hippocampal CA1 neurons in the subiculum, the entorhinal cortex, and in the angular gyrus of affected patients; and PTEN was found to accumulate in intracellular neurofibrillary tangles [438, 439]. Although the exact mechanism of PTEN’s effect in Alzheimer’s disease is poorly understood, there are several indications that PTEN affects the phosphorylation, binding to microtubules and aggregation of tau [440, 441]. Alterations of the levels of PTEN have also been reported in the rat model of transient cerebral ischemia, particularly in the damaged brain regions [442]. Moreover, several studies have shown that neuronal survival can be mediated or enhanced by the down-regulation of PTEN activity [443-445].

2.9. Erbin

Erbin, named ErbB2 interacting protein, was initially identified as a HER2 (ErbB2) binding protein in epithelial cells, and was shown to target HER2 to the basolateral membrane [446]. However, soon after, this function was challenged [447]. Nevertheless, it has been established that Erbin is a member of the family of LAP proteins, which are defined by a combination of leucine-rich repeats (LRRs) and PDZ domains [448]. LAP proteins are adaptor proteins which are bound to the membrane and contain a unique composition of 16 LRRs at the N-terminus, and up to 4 PDZ domains at the C-terminus [449]. The LRRs are typically 20-29 amino acid motifs containing a conserved 11 amino acid consensus unit (L-X-X-L-X-L-X-X-N/C-X-L, where X is any amino acid) [450]. LAP proteins have been shown to have important functions in numerous processes, such as worm and fly development, establishing and maintaining epithelial cell polarity, and epithelial cell morphogenesis, as well as acting as scaffolding molecules in various polarized cells, such as epithelial cells and neurons [449-453].

The structure of Erbin is composed of 16 LRR motifs (aa 23-391) located at the N-terminal end, and a single PDZ binding domain (aa 1280-1368) at the C-terminus [446]. In between, Erbin contains an LRR-like domain (aa 392-429) and an intermediary region containing proline-rich stretches [446]. The PDZ domain of Erbin, unlike most PDZ domains, is made up of
six β-strands and only a single α-helix [454], and numerous proteins have been identified as ligands for this PDZ domain. This list includes APC (adenomatous polyposis coli) [455], β-catenin [455], c-Rel [455], Tax of HTLV-I (Tax of the human T-lymphotropic virus type-1) [455], p0071 (plakophilin-4) [453, 456], δ-catenin [457], and ARVCF (armadillo repeat protein deleted in velo-cardio-facial syndrome) [457]. Other proteins with which Erbin has been shown to interact include PSD-95 [458], β4-integrin [459], bullous pemphigoid antigen 1 (dystonin) [459], Sur-8 [460], Nod2 [461], and EBP50 [462]. Proteins, such as p0071, δ-catenin, and ARVCF, are components of the adherens junctions; while others, such as β4-integrin, and bullous pemphigoid antigen 1, are involved in cellular attachment to substrates. Thus, unsurprisingly, Erbin has been localized to postsynaptic densities, where it interacts with postsynaptic density proteins, and therefore, is widely expressed in the brain [458].

Several studies have indicated that Erbin may play a negative role in the regulation of the MAPK pathway (Figure 2.4.). Tax1, a protein that is synthesized by the HTLV-I virus, is essential in transforming T cells in the process of immortalizing cells upon viral infection. Tax1 has been shown to interact with the PDZ binding domain of Erbin [463]. When Tax1 was overexpressed in Erbin-positive MCF-7 cells, there was an increase in the phosphorylation of Ras, Raf, MEK1/2, and ERK1/2, suggesting an important role for Erbin in the MAPK pathway [463]. Furthermore, the expression of Erbin in neuregulin-stimulated muscle cells decreased the transcription of an acetylcholine receptor subunit gene, a process which had been shown to require ERK activation [464]. Similarly, in COS-1 cells stimulated by neuregulin, Erbin expression decreased the amount of phosphorylated ERK and decreased ERK kinase activity; this effect required the LRR domain of Erbin [464]. This concept was further supported by observations that Erbin inhibited the differentiation of PC12 cells following NGF induction, a process in which the ERK pathway is known to play an important role; and this effect was similarly replicated using only the LRR domain of Erbin [464].

To further clarify the action of Erbin in the MAPK pathway, additional experiments were performed. These have revealed that Erbin expression did not affect ERK activation directly, instead it decreased the interaction between active Ras and Raf [464]. Erbin was found to associate with active Ras, but not inactive Ras or Raf. Thus, it was suggested that Erbin inhibits the ERK pathway by disrupting the interaction between active Ras and Raf (Figure 2.4.). Another study revealed that Erbin interacts with Sur-8 via its LRR domain [460]. Sur-8 is a scaffolding protein that is necessary for the Ras-Raf complex, and the expression of Erbin was
shown to attenuate the interaction of Sur-8 with active Ras and Raf [460]. This effect was reversed using shRNAs directed against Erbin [460]. Further proof that Erbin regulates the MAPK pathway was given in another study in which Erbin expression was reduced in primary cultures of Schwann cells, using siRNAs directed against Erbin [462]. These cells showed altered cell-cell interactions, disruption of E-cadherin junctions, increased cell proliferation, and elevated levels of phosphorylated ERK. The authors observed that these phenotypes can be rescued by using a pharmacological inhibitor of ERK kinase, suggesting that Erbin acts to negatively regulate the MAPK pathway [462].

As previously indicated, Erbin also binds HER2 (ErbB2), a receptor tyrosine kinase and a member of the epidermal growth factor receptor (EGFR) family. HER2 is a proto-oncogene, which functions in normal mammalian development, as well as cellular transformation and tumorigenesis, and is overexpressed in a large percentage of breast cancers [465]. Erbin was found to co-localize with HER2 on the lateral membrane of human intestinal epithelial cells [446]. A crystallography study showed that the PDZ domain of the human Erbin protein binds to a E-Y-L-G-L-D-V-P peptide sequence, which corresponds to the C-terminal residues of human ErbB2 (aa 1247-1255) [466]. Specifically, the Erbin PDZ domain directly interacts with the -7 tyrosine residue (tyrosine-1248) of HER2, which subsequently becomes stabilized through hydrophobic interactions inside the PDZ domain pocket [466]. However, phosphorylation of the tyrosine -7 residue abolishes the interaction of HER2 with the Erbin PDZ domain [466], and hence, it has been suggested that Erbin preferentially binds to unphosphorylated HER2 [467]. Furthermore, phosphorylation of the tyrosine-1248 residue following HER2 activation has been shown to be an essential step in the mitogenic signalling of this receptor tyrosine kinase [468]. By binding unphosphorylated HER2 and preventing the phosphorylation of the tyrosine-1248 residue, Erbin may be acting as a negative regulator of HER2 activity. Since HER2 has been shown to carry out its activity through the MAPK pathway [465, 469-473], binding to HER2 may be another means by which Erbin acts to negatively modulate the MAPK pathway. Therefore, interfering in the PDZ interaction between Erbin and HER2 (ErbB2) may positively regulate the MAPK pathway and promote its cell survival functions.
2.10. Breakpoint Cluster Region (BCR)

Breakpoint cluster region (BCR) is a multi-domain protein that is most commonly associated with the Philadelphia chromosome (t9:22). The Philadelphia chromosome is an abnormal chromosomal fusion that results from a reciprocal translocation between chromosome 9 and chromosome 22, in which the gene of BCR is fused with the gene of another kinase, ABL. The BCR-ABL fusion protein is a constitutively active and highly up-regulated tyrosine kinase, and commonly occurs in chronic myelogeneous leukemia and acute lymphocytic leukemia [474-476].

The normal gene for BCR, which is located on chromosome 22, encodes a 160 kilodalton phosphoprotein [477-479]. The human BCR protein has several enzymatic functions, as it contains a serine/threonine protein kinase domain, a GTPase-activating protein (GAP) domain, and has a guanine nucleotide exchange factor (GEF) function [480, 481]. BCR also contains a PDZ binding motif at its C-terminal end (aa 1268-1271), with which it binds to AF-6 (afadin) [482].

AF-6 is a component of both tight junctions and adhesion junctions that is involved in connecting these junctional complexes with the cortical actin cytoskeleton [483]. It contains a PDZ binding domain through which it binds to various other ligands, including the junctional adhesion molecule, JAM [484]; the poliovirus receptor-related protein, PRR2 (nectin) [485]; and several members of the Eph receptor tyrosine kinases [486, 487]. AF-6 has also been shown to bind to other proteins, such as the small GTPases, Ras [488] and Rap1 [489]; fam, a deubiquitinating enzyme [490]; the tight junction protein, ZO-1 [488]; ponsin, a vinculin-binding protein [491]; and profilin, a modulator of actin polymerization [489]. AF-6 is critical in regulating cell-cell junctions, since AF-6 knockout mice died 10 days post coitum and demonstrated cell-cell junction defects, as well as reduced cellular polarity of neuroepithelial cells [492].

Radziwill et al. found that AF-6 and BCR were co-localized in epithelial cells at the plasma membrane [482] (Figure 2.4.). More specifically, they determined that these proteins interacted via the PDZ domain of AF-6 and the PDZ binding motif of BCR, and this interaction required an intact BCR kinase. Hence, they determined that BCR phosphorylates AF-6 at the threonine-893 residue, and this residue is important in the regulation of the interaction between
BCR and AF-6 [482]. Furthermore, AF-6 and BCR form a trimeric complex with Ras (Figure 2.4.), where AF-6 binds Ras via its Ras-binding domain [482]. When a PDZ mutant (V1271A) BCR protein was used, the interaction between AF-6 and Ras was reduced, suggesting that BCR increases the interaction between AF-6 and Ras. Once again, this required the kinase activity of BCR. Moreover, it was found that BCR negatively regulates Ras-mediated ERK signalling, and this requires that BCR contains both an intact PDZ binding motif and a functional kinase domain [482]. Although the exact mechanism was not precisely resolved, it is likely that AF-6-bound Ras is unable to become activated, and hence, unable to recruit Raf to the membrane, where Raf would become activated and trigger the ERK/MAPK pathway cascade. Nevertheless, it still quite evident that BCR binds to AF-6, which subsequently binds to Ras, and BCR negatively regulates Ras-mediated ERK signalling. Thus, it is suggested that disrupting the PDZ interaction between BCR and AF-6 would positively regulate the MAPK pathway.

There are several other proteins and functions that have been associated with BCR. It is believed that BCR is involved in the production of superoxides as a negative regulator of Rac, a small GTPase [493]. In BCR knockout mice, there was an increased septic shock response and increased tissue injury by neutrophils following gram-negative endotoxin exposure [493]. Furthermore, neutrophils from BCR knockout mice produced more reactive oxygen metabolites, were more sensitive to priming stimuli, and showed a 3-fold increase in p21 Rac2 membrane translocation, compared to neutrophils from control animals [493]. BCR has also been shown to reduce the activation of the protein kinase, Pak1 (p21 activated protein kinase), which has been reported as an activator of different MAPK pathways [494]. Another protein that has been associated with BCR is the protein 14-3-3, which has been shown to be phosphorylated by BCR, and has been shown to facilitate the binding of BCR to Raf [495, 496]. More recently, other proteins have been identified that interact with BCR via its PDZ binding motif. For instance, PDZK1, a protein that is localized at the apical membrane of epithelial cells and binds to several apical membrane proteins, has 4 PDZ domains [497]. It was found that BCR binds to the first PDZ domain of PDZK1 via its PDZ binding motif [497]. Similarly, BCR was found to bind to the first PDZ domain of Mint3, a protein that is localized to the Golgi apparatus and is involved in protein processing and vesicular trafficking [497]. Finally, BCR is also able to specifically bind to the PDZ domain of Erbin via its PDZ binding motif [498].
Chapter 3:  
Methods and Materials

3.1. Optic Nerve Transection and Optic Nerve Crush

All animals were cared for according to the Canadian Council on animal care. Adult Sprague-Dawley rats, free of common pathogens, were used in all experiments. These were kept at the University of Toronto animal facilities, in a pathogen-controlled environment and housed in standard cages.

Optic nerve transection (Figure 2.2.A) was performed as previously described [499]. For surgical procedures, animals were individually anesthetized with isoflurane (2%; 0.8% L/min oxygen flow rate) and placed in a stereotaxic frame with anesthesia delivered through a gas mask to maintain sedation. To anesthetize the cornea, Lidocaine eye drops (Alcon) were used, and ophthalmic eye ointment was applied to the cornea to prevent desiccation during surgery. To access the optic nerve, an incision was made in the tissue covering the superior border of the orbital bone, followed by dissection of the superior orbital contents and retraction of the overlying rectus muscles. The dural sheath surrounding the optic nerve was cut longitudinally to avoid damaging the blood vessels supplying the retina. For optic nerve transection, the nerve was carefully lifted from the meningeal sheath, and cut within 2 mm of the back of the eye. A small piece of Gelfoam, soaked in 2% FluoroGold (Sigma-Aldrich), was then placed over the transected end of the optic nerve in order to retrogradely label the RGCs (Figure 2.2.A). A subset of animals that underwent optic nerve transection received an application of Gelfoam soaked in a solution of PDZ peptide (10 mg/mL) in order to directly target RGCs, as opposed to global targeting of the retina via intraocular injection (Table 1). The RGCs of these animals were labelled by injecting FluoroGold into the vitreous chamber of the eye. A similar procedure was used for optic nerve crush (Figure 2.2.B), except that the optic nerve was kept in place while it was crushed using fine self-closing forceps (#N5, Dumont), held steadily for 6 seconds. Orbital contents were then returned to their original location, and the initial incision was closed. Following surgery, the animals were placed in a recovery cage under a heat lamp, and were given an intraperitoneal injection of ketoprofen (5 mg/mL, dosage for rats; 0.1 mL/100 g body
weight) to minimize postoperative discomfort. Ophthalmic eye ointment was again applied to the cornea to ensure that it remained hydrated during the recovery period.

3.2. Intraocular Injections

Optic nerve transection is a reproducible model of CNS apoptosis that causes the death of approximately 90% of RGCs at 14 days after injury [8-11]. Since RGC apoptosis commences 3-4 days after injury, there is a window for drug delivery to target the apoptotic machinery. Furthermore, by delivering a drug via intraocular injection (Figure 2.2.), the drug diffuses within the vitreous chamber that houses the retina, ensuring that all retinal cells are exposed. Intraocular injections, as previously described [500], were given in order to assess the effects of different treatments on RGC survival or regeneration, following optic nerve transection or crush, respectively. Thymosin-β4 injections (4 μl) were delivered at 3 and 10 days following surgery.

In order to inhibit negative regulators of the PI3K or MAPK pathways, PDZ-motif peptides were synthesized and delivered to the retina after RGC injury (Table 1). These peptides consisted of two components covalently bound together: an active component composed of an amino acid sequence containing the PDZ binding motif involved in the target PDZ interaction; and a protein transduction component to mediate the transportation of the peptide into the cell. Protein transduction domains that we used included a fragment of the HIV TAT-protein (Y-G-R-K-K-R-Q-R-R-R [501-503]); or, a sequence of nine arginine residues (R9-), which has also previously been shown to be effective for cell permeation [504-508]. Five peptides were used in this study: TAT-4A (control polyalanine peptide of similar length to PDZ motifs of 4 amino acids), TAT-PTEN, R9-PTEN, TAT-ERBIN, and R9-BCR (See Table 1). These PDZ peptides were dissolved in water and delivered (4 μL, 10 mg/mL) via intraocular injection at 3 days following optic nerve transection. TAT-PTEN and R9-PTEN (4 μL, 10 mg/mL) were delivered on day 3 and day 10 following optic nerve crush.
<table>
<thead>
<tr>
<th>PDZ Peptide</th>
<th>Transduction Component</th>
<th>Sequence of Active Component</th>
<th>Source of Active Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAT-4A</td>
<td>TAT</td>
<td>A-A-A-A</td>
<td>4 alanine residues to serve as a control</td>
</tr>
<tr>
<td>TAT-PTEN</td>
<td>TAT</td>
<td>D-Q-H-S-Q-I-T-K-V</td>
<td>C-terminal residues of PTEN protein</td>
</tr>
<tr>
<td>R9-PTEN</td>
<td>R9</td>
<td>D-Q-H-S-Q-I-T-K-V</td>
<td>C-terminal residues of PTEN protein</td>
</tr>
<tr>
<td>TAT-ERBIN</td>
<td>TAT</td>
<td>D-S-W-V</td>
<td>C-terminal residues of δ-catenin, ARVCF and p0071</td>
</tr>
<tr>
<td>R9-BCR</td>
<td>R9</td>
<td>Q-S-I-L-F-S-T-E-V</td>
<td>C-terminal residues of BCR protein</td>
</tr>
</tbody>
</table>

**Table 1 – PDZ Peptides**

To deliver the intraocular injections, animals were anesthetized with isoflurane and placed in a stereotaxic frame under continued anesthesia (2% isoflurane; 0.8% L/min oxygen flow rate) delivered through a gas mask. Alcaine eye drops (Alcon) were applied to the cornea in order to anesthetize the cornea. To deliver the solution, a pulled glass micropipette attached to a 10 μL Hamilton syringe via a hydraulic coupling was used. The injection was made into the vitreous chamber of the eye, posterior to the limbus, with care taken to prevent damage to the lens or the anterior structures of the eye, which have previously been shown to secrete confounding growth factors [74, 509]. After injecting the appropriate solution, the pipet was held in place for 5 seconds in order to prevent reflux, and then carefully withdrawn from the eye. Injections were carried out under a surgical microscope to visualize pipet entry into the appropriate location and to confirm delivery of the solution into the vitreous chamber.

### 3.3. Quantification of RGC Survival

Animals were sacrificed at 7 or 14 days after optic nerve transection. The eyes were enucleated and the eye cups, containing the retinas, were fixed in 4% paraformaldehyde for 1 hour. The retinas were then extracted, flat-mounted, and coverslipped in a solution of 50:50 glycerol/PBS. RGCs, stained with FluoroGold, were visualized using an Andor iXon 885+ EMCCD camera (Andor Technology, Belfast, Northern Ireland), attached to a Leica DM LFSA microscope (Leica Microsystems, Concord, Canada), with a Sutter Lambda XL light source (Quorum Technologies, Guelph, Canada). To measure RGC density, samples were taken at 3 different eccentricities of retinal quadrants: inner (1/6 of the retinal radius from center), middle (1/2 of the retinal radius from center), outer (5/6 of the retinal radius from center). Samples were
grouped according to eccentricity, and expressed as a mean ±SEM. Statistical significance between experimental and control samples was calculated using ANOVA followed by post hoc analysis using Tukey’s post hoc comparisons.

### 3.4. Quantification of RGC Axon Regeneration

To assess the regeneration of RGC axons, specimens were examined at 21 days after crushing the optic nerve. On day 21, animals were given an intracardial perfusion of 4% paraformaldehyde, and their optic nerves were carefully removed. Nerves were immediately fixed in 4% paraformaldehyde overnight at 4°C. They were then rinsed in PBS and cryoprotected in 30% sucrose in PBS for 7 days. The optic nerves were then sectioned using a Leica CM1950 cryostat microtome. Transverse sections, 14 μm in thickness, were collected onto slides coated with APTEX (Sigma), and stored at -20°C until immunostaining was performed.

Immunohistochemistry was performed with antisera directed against growth associated protein-43 (GAP-43), which is expressed in neuronal growth cones during development and axonal regeneration [72-77]. RGCs have been shown to express GAP-43 during axon outgrowth [510-513]. Transverse sections of the nerves were incubated overnight at 4°C with primary antisera (rabbit polyclonal, 1:250, Cell Signaling Technology/NEB), diluted in PBS containing 0.3% Triton X-100 and 3% normal goat serum. Following the incubation period with primary antibody, sections were rinsed three times in PBS for 15 minutes. This was followed by a 3 hour incubation period with Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) at room temperature, and three subsequent 15 minute rinses in PBS. The sections were then coverslipped with 50:50 glycerol/PBS. Four equally spaced sections across the width were taken from each optic nerve. These were examined and quantified using a Leica DM LFSA microscope (Leica Microsystems, Concord, Canada) at 20× objective, with an Andor iXon 885+ camera (Andor Technology, Belfast, Northern Ireland), and with EM gain applied. The total number of regenerating axon growth cones was quantified within bins of the optic nerve. Starting at the crush site and moving distally, the bins were 0-250 μm, 250-500 μm, and >500 μm. The total number of regenerating axons in each bin was averaged and expressed as a mean ±SEM. Statistical analysis was performed by ANOVA and Tukey’s post hoc test.
3.5. Western Blot

In order to assess the activation of the PI3K and MAPK pathways following different treatments, western blots on whole retinas were performed. Upon sacrificing the animals, eyes were enucleated and the retinas were immediately extracted in ice-cold PBS. Retinas were then placed individually in 400 μL solutions of ice-cold SDS lysis buffer (2% SDS, 0.3% DTT, 10% glycerol in 40 mm Tris-Cl, pH 6.8). Each retina was sonicated and then heated to 90°C for 8 minutes. This was followed by centrifugation (12,000 rpm, 10 min, 4°C), and separation of the protein samples from the pelleted debris. The protein samples were then stored at -80°C until further use.

To separate total protein fractions, SDS-PAGE was performed on Bio-Rad TGX Gels (10% acrylamide). This was followed by semidry electrotransfer to nitrocellulose membranes (0.2 μm pore size), and immunoblotting. Blots were initially blocked for 1 hour at room temperature with 5% milk in Tris-buffered saline, containing 0.1% Tween 20 (TBS-T). Blots were then incubated with the primary antibody (in 1% milk in TBS-T) solution overnight at 4°C on a platform rocker. The blots were washed three times (15 minutes in TBS-T), and then incubated with secondary antibody. This incubation was carried out for 1 hour at room temperature, on a platform rocker. The blots were subsequently washed with TBS-T for 15 minutes three times.

To image the blots, chemiluminescent immunoreactive complexes were visualized using a Bio-Rad Fluor-S Max imager (Bio-Rad, Mississauga, ON). Loading was verified by re-probing the blots with antisera directed against GAPDH (1:1000; rabbit polyclonal; Cell Signaling Technology). To quantify the bands, densitometry was performed. The density of each band was normalized against the density of the corresponding GAPDH band for each lane. Normalized densitometry values for each experimental group were averaged ±SEM, and statistical analysis was performed by ANOVA followed by post hoc analysis using Tukey’s post hoc comparisons.

3.6. Treadmill Running of Animals

Adult Sprague-Dawley rats were used to assess the effects of treadmill training on RGC survival and intracellular messenger activation after injury. A total of 18 animals were used. All
animals were run on an Exer 3-lane treadmill (Columbus Instruments, Columbus, OH). Six animals were used to evaluate the effects of exercise on the activation of the PI3K and MAPK pathways. These animals were randomly divided into light exercise and heavy exercise subgroups. Light exercise animals underwent a 3 week (5 days/week) exercise program, in which they were run at 15.0 m/min for varying durations, starting at 10 minutes/day and increasing to 40 minutes/day. Thus, these animals ran a total daily distance between 150-600 m. Heavy exercise animals underwent a 3 week (5 days/week) exercise program, running at 22.0-25.0 m/min for 60 minutes/day, accumulating a total daily distance of 1320-1500 m. On the final day of training, animals were sacrificed and the tissue was extracted for western blotting. Western blots were used to assess the levels of Akt, phosphorylated Akt, MAPK, and phosphorylated MAPK in exercised and normal adult rats.

The other 12 animals underwent a 2 week (5 days/week) exercise program, running at 20.0 m/min for 20-60 minutes/day. These animals ran a total daily distance of 400-1200 m. On the final day of the exercise program, the animals underwent an optic nerve transection, as described above. RGC survival was assessed, as described above, in six animals at 7 days after transection and at 14 days post-transection in the remaining six animals.
Chapter 4: Results

4.1. Thymosin-β4

4.1.1. Exogenous Thymosin-β4 Enhances RGC Survival Following Optic Nerve Transection

Our previous iTRAQ study [295], in which the rat retinal proteome was characterized following optic nerve crush, found that Thymosin-β4 was differentially expressed after injury. Protein levels of Thymosin-β4 increased at 3 days following optic nerve crush, which is the time point that precedes the rapid phase of RGC apoptosis. This was followed by a rapid decrease in Thymosin-β4 protein levels on day 4, when RGC death is noticeable. Since Thymosin-β4 is a small soluble polypeptide, which has been shown to play a role in tissue repair [301, 302, 311, 312, 319, 320], I sought to determine if exogenous application of Thymosin-β4 could suppress RGC apoptosis following optic nerve transection.

Exogenous Thymosin-β4 was delivered by intraocular injection at 3 days after axotomy, and RGC survival was assessed at 14 days. Control animals had few surviving RGCs at 14 days post-axotomy (Figure 4.1.1.A). In contrast, retinas of the animals that received an intraocular injection of Thymosin-β4 showed a marked improvement in RGC survival at the same time-point (Figure 4.1.1.B). When the density of surviving RGCs at 14 days was quantified, Thymosin-β4 treated retinas showed an approximate three-fold increase in RGC survival compared to control retinas (Figure 4.1.1.C). Retinas treated with Thymosin-β4 had a significantly higher density of RGCs (p<0.001) at all three retinal eccentricities (inner, middle, outer) when compared to control treatments. These findings demonstrate that Thymosin-β4 reduces adult RGC apoptosis induced by traumatic axonal injury.
Figure 4.1.1.
Figure 4.1.1. Intraocular injection of exogenous Thymosin-β4 enhances RGC survival after optic nerve transection. (A, B) Epifluorescence micrographs of flat-mounted retinas, showing FluoroGold-labelled RGCs fixed at 14 days following optic nerve transection. Intraocular injections were delivered at 3 days following injury. Arrows demarcate some of the surviving RGCs in each treatment. Bar = 50 μm. (A) Sample retinal tissue from control retinas that received vehicle injections. (B) Sample retinal tissue from treatment animals that received an intraocular delivery of Thymosin-β4. (C) Quantification of RGC density (mean ± SEM) at 14 days after optic nerve transection. Cell densities were quantified at three different retinal eccentricities (inner, middle, outer). Intraocular injection of exogenous Thymosin-β4 significantly increased RGC survival after optic nerve injury in all three retinal eccentricities (**p<0.001; n=6 for each).
4.1.2. Exogenous Thymosin-β4 Promotes RGC Regeneration Following Optic Nerve Crush

Having determined that Thymosin-β4 can improve RGC survival, I next sought to test whether Thymosin-β4 could promote axonal regeneration in RGCs. Animals underwent an optic nerve crush and were subsequently given intraocular injections of Thymosin-β4 at 3 and 10 days post-surgery. Axon regeneration was evaluated at 21 days after injury. Both control and Thymosin-β4-treated nerves showed axonal sprouting in the nerve stump on the proximal side of the crush site; however, few axons extended through the crush site in control animals, and only for relatively short distances (Figure 4.1.2.A). On the other hand, Thymosin-β4-treated animals showed enhanced axon regeneration into the distal myelinated optic nerve (Figure 4.1.2.B) and axon regeneration was significantly greater at all distances examined (p<0.001) (Figure 4.1.2.C). This data indicates that Thymosin-β4 enhances axon regeneration within the normally non-permissive environment of the adult CNS.
Figure 4.1.2.

A

Control

B

Thymosin-β4

C

Mean Axon Number/Section

---

0-250 μm

250-500 μm

>500 μm

*** p<0.001
Figure 4.1.2. Intraocular injections of exogenous Thymosin-β4 enhance axonal regeneration of RGCs following optic nerve crush. (A, B) Epifluorescence micrographs of longitudinal sections of optic nerves fixed at 21 days after optic nerve crush and immunolabelled for GAP-43. Intraocular injections were delivered at 3 and 10 days following injury. Crush site is indicated by arrowhead. Arrows demarcate some of the regenerating axons in each section. The retina (not visible) is towards the left-hand side of each image. Bar = 250 μm. (A) Optic nerve sample from a control animal that received vehicle injections showing minimal axon regeneration past the crush site. (B) Thymosin-β4 treated nerve showing enhanced axon regeneration following optic nerve crush. (C) Quantification of regenerating axons (mean ± SEM) in three different bins (0-250 μm, 250-500 μm, >500 μm) distal to the lesion site. Thymosin-β4 significantly increased axon regeneration after optic nerve crush compared to a control vehicle treatment (***p<0.001; n=4 for each).
4.1.3. Exogenous Thymosin-β4 Increases the Activation of the PI3K and MAPK Pathways

The mechanisms by which Thymosin-β4 exerts its effects on RGCs remain unknown, but they are suspected to involve two critical pathways for neuronal protection and survival: the phosphatidylinositol-3 kinase (PI3K) pathway and the mitogen-activated-protein kinase (MAPK) pathway [14-23]. I therefore evaluated the ability of Thymosin-β4 to activate these pathways at 4 and 24 hours after intraocular injection. Both pathways involve activation by phosphorylation at key residues. PI3K activation is evaluated by the PI3K-dependant phosphorylation of Akt (Protein Kinase B) at serine-473, which is both indicative of PI3K activity and required for Akt function. MAPK is activated by MEK phosphorylation of the threonine-202/tyrosine-204 residues.

At 4 and 24 hours following an intraocular injection of Thymosin-B4, phosphorylation of Akt and MAPK was assessed (Figure 4.1.3.A,B). A significant increase in Akt phosphorylation was found at both 4 (p<0.05) and 24 hours (p<0.01) (Figure 4.1.3.C), relative to controls (vehicle injection). Since there was no major difference in the levels of total Akt between experimental and control animals, the increases in Akt phosphorylation likely occurred within the existing intracellular pools.

Increases in MAPK phosphorylation were observed at 24 hours (p<0.01), but not at the 4 hour time point (Figure 4.1.3.C). Just as with Akt, levels of total MAPK remained unchanged. Together, these findings suggest that Thymosin-β4 induces an early and prolonged phosphorylation of Akt, but a delayed phosphorylation of MAPK.
Figure 4.1.3.

A

B

C

![Image of Western Blot with Protein Expression Analysis](image-url)

**Mean Normalized Blot Density**

- **pAkt 4h**
- **Akt 4h**
- **pMAPK 4h**
- **MAPK 4h**
- **pAkt 24h**
- **Akt 24h**
- **pMAPK 24h**
- **MAPK 24h**

*Vehicle*  
*Thymosin-β4*
Figure 4.1.3. Thymosin-β4 induces activation of the PI3K and MAPK pathways in the normal adult retina. (A, B) Western immunoblots of whole retinal lysates at 4 hours (A) or 24 hours (B) following an intraocular vehicle injection (Normal) or an intraocular injection of Thymosin-β4 (Tβ4 I.O.). Both active (phosphorylated; pAkt or pMAPK) and total Akt and MAPK were probed at each time-point. GAPDH loading controls are shown below each group of blots. (C) Normalized blot densitometry values (mean ± SEM) for each test of both experimental groups. Thymosin-β4 treated retinas showed a significant increase in Akt phosphorylation at 4 hours (*p<0.05) and 24 hours (**p<0.01) without increasing total Akt levels. MAPK phosphorylation was not affected at 4 hours, but was significantly increased at 24 hours (**p<0.01). Total MAPK levels did not change significantly. (n=4 for each treatment at each time point)
4.2. Exercise Training

4.2.1. Forced Light Treadmill Running Decreases the Activation of the PI3K Pathway But Not the MAPK Pathway

Several studies have demonstrated that exercise can increase the levels of neurotrophins such as BDNF and NGF in the CNS [354-358, 364, 365]. Furthermore, the PI3K and MAPK pathways have been found to be activated following exercise [364, 365]. Thus, I sought to assess the activation of these pathways in the retina following forced treadmill running. Normal adult rats completed a 3-week light exercise program, after which their retinal tissues were extracted for Western blots. Once again, activation of the PI3K and MAPK pathways was assessed using antibodies directed against specific residues in Akt (Ser 473) and MAPK (Thr 202/Tyr 204), respectively.

The levels of phosphorylated Akt in the retinal tissue of the animals that underwent the light exercise program were significantly lower (p<0.001) than those of the normal sedentary animals (Figure 4.2.1.A,C). However, there was no significant difference in the levels of total Akt between both groups, suggesting that exercise increased the dephosphorylation and not the breakdown of Akt in the retina. On the other hand, the levels of phosphorylated MAPK were not significantly different between normal sedentary and exercised retinas (Figure 4.2.1.B,C). No difference was found in the levels of total MAPK between both groups. This data suggests that in normal adult retinas, light exercise negatively regulates the PI3K pathway by promoting the dephosphorylation of Akt, but does not affect the MAPK pathway.
Figure 4.2.1. Forced light exercise decreases the activation of Akt, but not MAPK, in the normal adult retina. (A, B) Western immunoblots of whole retinal lysates of control sedentary rats (Normal) and rats that underwent a forced light exercise program (Exercise). Both groups were probed for active (phosphorylated; pAkt or pMAPK) and total Akt (A) and MAPK (B). GAPDH loading controls are shown below each group of blots. (C) Graph depicting the mean normalized densitometry values (± SEM) of each group of blots for both treatments. A forced light exercise program caused a significant decrease in activated Akt levels (**p<0.001), without significantly changing the levels of total Akt. There was no significant difference in the levels of activated and total MAPK between exercise and sedentary animals. (Normal n=3; Exercise n=6)
4.2.2. Forced Heavy Treadmill Running Does Not Affect the Activation of the PI3K or MAPK Pathways

Following the results obtained with a light exercise program, I sought to determine how a heavy exercise program could affect the activation of the PI3K and MAPK pathways in retinas of normal adults. To do so, adult rats were completed a 3-week treadmill running regimen, in which the rats were run at higher speeds for longer durations. The retinal tissues were then removed, prepared, and run through Western blots.

There were negligible differences found in the levels of phosphorylated Akt between the retinas of normal sedentary and the heavy exercised animals (Figure 4.2.2.A,C). Moreover, there was no significant difference in the level of total Akt between both groups. Similarly, animals that completed the heavy exercise program did not have a significant difference in the levels of phosphorylated MAPK compared to the normal sedentary animals (Figure 4.2.2.B,C). Furthermore, while levels of total MAPK were greater in the retinas of animals that underwent the heavy exercise program compared to sedentary animals, this difference was not significant. These findings indicate that in normal adults, heavy exercise neither promotes nor impairs the activation of the PI3K or MAPK pathways.
Forced heavy exercise does not change the activation of either Akt or MAPK in the normal adult retina.

(A, B) Western blot analysis of sedentary (Normal) and heavy exercise (Exercise) retinas, directed against activated (phosphorylated; pAkt or pMAPK) and total Akt (A) and MAPK (B). Corresponding GAPDH loading controls are shown below each group. (C) Quantification of normalized densities (mean ± SEM) of activated and total Akt and MAPK blots of each treatment. A forced heavy exercise regimen did not significantly change the levels of Akt or MAPK, nor alter their activation in the normal adult retina. (Normal n=3; Exercise n=6)
4.2.3. Forced Treadmill Running Partially Improves the Survival of RGCs at 7 Days Following Optic Nerve Transection

Having assessed the activation of the PI3K and MAPK pathways in normal adult retinas following forced treadmill running, I sought to determine if these effects correlate positively or negatively to the survival of RGCs following optic nerve transection. Since the experiment with a light exercise program suggested that forced treadmill running down-regulates the PI3K pathway, I assessed the survival of RGCs following optic nerve transection at 7 days following injury. At this time point, there are still enough RGCs alive to assess possible detrimental effects on cell survival. After completing a 2-week exercise program, adult rats underwent bilateral optic nerve transection, and their retinas were extracted 7 days later for quantification of RGC survival.

Animals that underwent forced treadmill running did not have fewer surviving RGCs compared to control animals (optic nerve transection only). In fact, exercised retinas showed improved RGC survival in the outer retinal eccentricity (Figure 4.2.3.A). However, the inner and middle eccentricities did not show any significant changes in RGC density between the exercised and control animals. In the outer eccentricity, exercised retinas had a significantly greater RGC density (p<0.05) than control retinas (Figure 4.2.3.B). Therefore, these results suggest that exercise may partially decrease the apoptosis of adult RGCs following optic nerve transection.
Figure 4.2.3.
Figure 4.2.3. Forced treadmill running partially increases the survival of RGCs following optic nerve transection at 7 days following injury. (A) Epifluorescence micrographs showing FluoroGold-labelled RGCs in three retinal eccentricities (inner, middle, outer) of sedentary control (left column) and exercise (right column) animals that underwent a bilateral optic nerve transection. Retinas were extracted and fixed at 7 days following injury. Arrows demarcate some of the surviving RGCs in each section. Bar = 50 μm. (B) Quantification of RGC densities (mean ± SEM) in the three retinal eccentricities at 7 days post-transection. At 7 days following optic nerve transection, forced treadmill running significantly enhanced the survival of RGCs in the outer retinal eccentricity only (*p<0.05; n=12 for each). No significant differences were found between control and exercise animals in the inner and middle retinal eccentricities.
4.2.4. Forced Treadmill Running Enhances the Survival of RGCs at 14 Days Following Optic Nerve Transection

In order to further characterize the effect of exercise on the survival of RGCs in the optic nerve transection model, I measured RGC densities at 14 days following injury. Adult rats underwent 2-weeks of forced treadmill running, followed by bilateral optic nerve transection. Animals were sacrificed 14 days later, and RGC densities were subsequently measured.

Animals that underwent the exercise regimen showed a greater number of surviving RGCs at 14 days following axotomy (Figure 4.2.4.A). Control animals, that did not undergo forced treadmill running, had few surviving RGCs at this time point. Exercised retinas had a significantly higher density of RGCs (\(p<0.001\)) at all three retinal eccentricities (inner, middle, outer) when compared to control retinas (Figure 4.2.4.B). These findings demonstrate that exercise protects adult RGCs from apoptosis caused by traumatic injury to the optic nerve.
Figure 4.2.4.
Figure 4.2.4. Forced treadmill running enhances RGC survival at 14 days following optic nerve transection. (A) Epifluorescence micrographs of flat-mounted retinas extracted and fixed 14 days after optic nerve transection. FluoroGold-labelled RGCs of control sedentary (left column) and exercise (right column) retinas are shown at three retinal eccentricities (inner, middle, outer). Arrows demarcate some of the surviving RGCs in each section. Bar = 50 μm. (B) Quantification of the density of surviving RGCs (mean ± SEM) at 14 days following optic nerve transection in all three retinal eccentricities. Forced treadmill running significantly improved the survival of RGCs at 14 days following optic nerve transection in all three retinal eccentricities (**p<0.001; n=12 for each).
4.3. PDZ Peptides

The PI3K and MAPK pathways are controlled by negative regulators that keep them in check. PTEN phosphatase hydrolyses phosphatidylinositol-3,4,5-trisphosphate (PIP₃) into phosphatidylinositol-4,5-bisphosphate (PI-4,5-P₂), and thus reduces the number of PIP₃ second messengers that are required for the activation of Akt (PKB) [166-168]. Erbin binds to active Ras, as well as Sur-8, and disrupts the interaction between Ras and Raf, which is required in the MAPK cascade [460, 464]. Similarly, BCR forms a trimeric complex with AF-6 and Ras, inhibiting Ras-mediated ERK signalling [482]. PTEN, Erbin, and BCR possess c-terminal PDZ binding motifs or PDZ binding domains that facilitate interactions with their target proteins. We synthesized peptides that individually mimic the PDZ binding motifs involved in the PDZ interactions of PTEN, Erbin, or BCR. By doing so, these peptides can act as competitive inhibitors of the normal PDZ interactions of PTEN, Erbin, or BCR, thereby potentially inhibiting their function. These peptides were delivered either though an intraocular injection or by application of a peptide-soaked piece of Gelfoam to the cut end of the optic nerve, and RGC survival and regeneration after optic nerve transection and crush respectively were assessed.

4.3.1. Intraocular Delivery of PDZ Peptides Enhances RGC Survival Following Optic Nerve Transection

PDZ peptides were administered 3 days following optic nerve transection, and RGC survival was quantified at 14 days. Animals that received intraocular injections of the control peptide (TAT-4A) showed RGC survival comparable to those that received vehicle injections (Figure 4.3.1A,B). RGC survival was significantly greater in the animals that received intraocular injections of TAT-PTEN or TAT-ERBIN (Figure 4.3.1C,D). RGC densities following TAT-PTEN injections were significantly greater at all three eccentricities (inner, middle, and outer) compared to vehicle (**p<0.001) and TAT-4A (###p<0.001) injections (Figure 4.3.1F). Similarly, RGC densities in the retinas that received an injection of TAT-ERBIN were significantly greater compared to vehicle (**p<0.01 at the inner eccentricity; ***p<0.001 at the middle and outer eccentricities) and TAT-4A retinas (##p<0.01 at the inner eccentricity; ###p<0.001 at the middle and outer eccentricities) (Figure 4.3.1F). Conversely, only
RGC survival in the outer eccentricity was significantly greater (#p<0.01) in R9-BCR retinas when compared to TAT-4A retinas at the same eccentricity (Figure 4.3.1.F). This data indicates that the TAT-PTEN and TAT-ERBIN PDZ peptides, when delivered by intraocular injections, are capable of reducing RGC apoptosis following optic nerve transection, whereas the R9-BCR peptide provides only a marginal benefit.
Figure 4.3.1. Intraocular Delivery of PDZ peptides has different effects on RGC survival following optic nerve transection. (A-E) Epifluorescence micrographs of flat-mounted retinas, showing FluoroGold-labelled RGCs fixed at 14 days following optic nerve transection. Intraocular injections were delivered at 3 days following transection. Arrows demarcate some of the surviving RGCs in each treatment. Bar = 50 μm. (A) Control retina given a vehicle intraocular injection had minimal RGC survival following transection. (B) Control retina that received an intraocular injection of TAT-4A showed few surviving RGCs, comparable to vehicle control retinas. (C) Injured retina injected with the TAT-PTEN peptide following optic nerve transection showed enhanced RGC survival. (D) Increased RGC survival was also observed in injured retinas that received an intraocular injection of the TAT-ERBIN peptide. (E) An injured retina that received the R9-BCR peptide showed some improvements in RGC survival as compared to control retinas. (F) Quantification of the density of surviving RGCs (mean ± SEM) at 14 days after optic nerve transection. RGC densities were quantified at three different retinal eccentricities (inner, middle, outer). Intraocular injections of TAT-PTEN and TAT-ERBIN significantly increased RGC survival in all three retinal eccentricities compared to either control treatment. R9-BCR only significantly increased RGC survival in the outer eccentricity as compared to the TAT-4A control. (** = p<0.01 as compared to vehicle control; *** = p<0.001 as compared to vehicle control; ### = p<0.001 as compared to TAT-4A control; # = p<0.01 as compared to TAT-4A control; Vehicle n=8; TAT-4A n=4; TAT-PTEN n=4; TAT-ERBIN n=10; R9-BCR n=6)
4.3.2. Gelfoam Application of PDZ Peptides at the Cut End of the Optic Nerve Increases the Number of Surviving RGCs Following Optic Nerve Transection

Since my primary interest was the survival of RGCs following optic nerve transection, I assessed the direct effects of peptides when transduced into injured RGCs. To selectively target RGCs, drugs, siRNAs, plasmids, or viral vectors, can be applied to the cut end of the optic nerve [67-71]. These are taken up by the axons of the RGCs, and retrogradely transported back to the cell bodies, thereby eliminating any confounding effects of bystander neurons or the surrounding glia. Following adult rat optic nerve transection, I immediately applied a small piece of Gelfoam soaked in a peptide solution to the cut end of the nerve.

Retinas that received either the vehicle or the TAT-4A treatments showed few surviving RGCs at 14 days post-axotomy (Figure 4.3.2.). Much greater RGC survival was seen in the retinas that received TAT-PTEN or TAT-ERBIN in gelfoam. When compared to vehicle treatments, TAT-PTEN-treated retinas demonstrated significantly greater levels of RGC survival at all three eccentricities (**p<0.001 at the inner and middle eccentricities; *p<0.05 at the outer eccentricity) (Figure 4.3.2.). However, when compared to the TAT-4A retinas, only the inner eccentricity of TAT-PTEN retinas showed significantly greater RGC densities (###p<0.001; Figure 4.3.2.). Among all PDZ peptide treatments tested, the greatest RGC survival was seen in the retinas that received a gelfoam application of TAT-ERBIN. These showed RGC densities that were approximately three times greater than vehicle or control (TAT-4A)-treated retinas. RGC densities were significantly greater at all three eccentricities (inner, middle, and outer) when compared to either vehicle (***p<0.001) or TAT-4A (###p<0.001) gelfoam treatments (Figure 4.3.2.). When the R9-BCR peptide was applied to the transected end of the optic nerve, RGC survival at 14 days post-axotomy was only slightly increased compared to vehicle and TAT-4A treatments at the same time point. RGC densities of R9-BCR retinas were significantly greater (*p<0.05) only in the outer eccentricity when compared to the vehicle-treated retinas (Figure 4.3.2.). No significant differences were observed in the inner and middle eccentricities compared to vehicle treatments, or when any eccentricity was compared to TAT-4A treatments (Figure 4.3.2.). These results demonstrate that both the TAT-PTEN and TAT-ERBIN peptides decrease RGC apoptosis following optic nerve transection, when the peptides are applied at the
cut end of the optic nerve or selectively transduced into RGCs. In contrast, the R9-BCR peptide, applied in this manner, is not as effective at decreasing RGC apoptosis.
Figure 4.3.2. RGC survival after optic nerve transection has varying outcomes following Gelfoam application of PDZ peptides. Graph depicting mean quantification of RGC density (± SEM) at 14 days following optic nerve transection. RGC densities were quantified at three different retinal eccentricities (inner, middle, outer). Treatments were delivered by a soaked piece of Gelfoam applied directly to the transected nerve end, immediately after the nerve was cut. When compared to vehicle control treatment, Gelfoam application of TAT-PTEN or TAT-ERBIN peptides significantly increased RGC survival in all three retinal eccentricities. Gelfoam application of the R9-BCR peptide only increased RGC survival in the outer retinal eccentricity. Compared to the TAT-4A controls, TAT-ERBIN enhanced RGC survival in all three retinal eccentricities, while TAT-PTEN significantly improved survival in the inner eccentricity only. (** = p<0.001 as compared to vehicle controls; * = p<0.05 as compared to vehicle controls; ### = p<0.001 as compared to TAT-4A controls; Vehicle n=6; TAT-4A n=4; TAT-PTEN n=4; TAT-ERBIN n=4; R9-BCR n=8)
4.3.3. RGC Axon Regeneration Following Optic Nerve Crush Can Be Enhanced Using a PDZ Peptide Directed Against PTEN

I found that the TAT-PTEN peptide was effective in decreasing RGC apoptosis following optic nerve transection. Moreover, it had previously been reported that PTEN is localized to growth cones of budding axons [427], and may be involved in the signalling of several neurite outgrowth inhibitors, such as Sema3A, Sema4D, and MAG [427-430]. Furthermore, upon reduction of PTEN levels, either through genetic deletion or through shRNAs, previous investigators observed increased axonal regeneration in the damaged corticospinal tract [431-433]. Thus, I sought to determine if the TAT-PTEN peptide is able to promote axonal regeneration in the optic nerve crush model. Following crush injury, I applied the TAT-PTEN peptide to the lesion site via gelfoam or through targeting the retina globally via intraocular injection. I also tested an R9-PTEN peptide via intraocular injection for comparison. RGC axon regeneration was assessed at 21 days after injury.

Optic nerves of the animals that received vehicle treatments showed minimal axonal regeneration past the crush site (Figure 4.3.3.A). Most regenerating growth cones in this treatment were observed in the 0-250 μm bin. Conversely, nerves that were treated with either TAT-PTEN or R9-PTEN showed much greater axonal regeneration in all three bins (0-250 μm, 250-500 μm, and >500 μm) (Figure 4.3.3.B, C, D). Interestingly, these treatments showed markedly greater axonal regeneration in the furthest bin from the crush site (>500 μm). Compared to control (vehicle) treatment, animals that were treated with TAT-PTEN intraocular injections showed a significantly greater number of regenerating growth cones in all three bins (**p<0.001 in the 0-250 μm and >500 μm bins; **p<0.01 in the 250-500 μm bin) (Figure 4.3.3.E). Similarly, nerves that received intraocular injections of R9-PTEN showed a significantly greater number of regenerating growth cones in all three bins (**p<0.001 in the 0-250 μm and >500 μm bins; *p<0.05 in the 250-500 μm bin) (Figure 4.3.3.E). The greatest axonal regeneration was seen in the nerves that received a TAT-PTEN Gelfoam application, wrapped directly around the nerve (Figure 4.3.3.C). These nerves showed a significantly greater number of regenerating growth cones (**p<0.001) in all three bins (Figure 4.3.3.E). Therefore, these results clearly demonstrate that the TAT-PTEN and R9-PTEN peptides are capable of enhancing RGC axon regeneration following injury in the optic nerve.
Figure 4.3.3.
Figure 4.3.3. Continued

Figure 4.3.3. PDZ peptides that target PTEN enhance axonal regeneration in RGCs following optic nerve crush. (A-D) Epifluorescence micrographs of GAP-43-immunolabelled sections of optic nerves fixed at 21 days after optic nerve crush. Intraocular injections (I.O.) were delivered at 3 and 10 days following injury; TAT-PTEN-soaked Gelfoam (C) was applied immediately to the lesion site after crush. Crush site is indicated by arrowhead. Arrows demarcate some of the regenerating axons in each section. The retina (not visible) is towards the left-hand side of each image. Bar = 250 μm. (A) Nerve that received vehicle intraocular treatment showed minimal axonal regeneration beyond the crush site. (B, C) Nerves treated with the TAT-PTEN peptide either by intraocular delivery (B) or via Gelfoam application (C) showed enhanced axonal regeneration into the distal optic nerve. (D) Intraocular R9-PTEN-treated nerve showed similar enhancement of axonal regeneration. (E) Quantification of the mean number of regenerating axons (± SEM) at 21 days following optic nerve crush. The number of axons was quantified in three bins distal to the crush site (0-250 μm, 250-500 μm, >500 μm). Intraocular injections (I.O.) of TAT-PTEN or R9-PTEN significantly increased RGC axon regeneration compared to vehicle controls in the corresponding bin. The greatest number of regenerating axons was observed in the nerves that received the TAT-PTEN peptide applied directly at the lesion site (Nerve). (** = p<0.001; *** = p<0.001; * = p<0.05; vehicle I.O. n=8; TAT-PTEN I.O. n =6; TAT-PTEN gelfoam n =6; R9-PTEN I.O. n=4)
Chapter 5: Discussion

5.1. Thymosin-β4

Thymosin-β4, a small intracellular peptide, is found in several body tissues, including the brain, spleen, lung, liver and heart muscle [299]. In a previous iTRAQ study of the retinal proteome following optic nerve injury, Thymosin-β4 was also localized to the retina [295]. Following traumatic injury of the optic nerve, Thymosin-β4 protein levels increase before the onset of RGC apoptosis, but decrease drastically at the onset of apoptosis [295]. In this study, I demonstrated that exogenous Thymosin-β4 increases RGC survival in vivo, when delivered by intraocular injection after optic nerve transection. Furthermore, I found that Thymosin-β4 is capable of enhancing RGC axon regeneration. Following optic nerve crush, animals that received intraocular injections of Thymosin-β4 had a greater number of extending growth cones beyond the site of injury, compared to control animals. These results indicate that Thymosin-β4 is a potent neuroprotective agent for injured adult CNS neurons, and fall in line with previous findings that demonstrate the protective role that Thymosin-β4 plays throughout the body.

In a rat full-thickness dermal wound model, topical or intraperitoneal application of Thymosin-β4 increases the re-formation of the epithelium, and contraction of the wound [319]. In the heart, Thymosin-β4 is involved in the formation of new vessels, promotes myocardial and endothelial cell migration, and is able to enhance myocyte survival following coronary artery ligation [322-324]. Furthermore, Thymosin-β4 enhances and accelerates the repair of the corneal epithelium following wound induction [325, 326]. In human corneal epithelial cells, Thymosin-β4 protects against oxidative stress and increases cell viability following hydrogen peroxide-induced toxicity [332]. Following focal ischemia, Thymosin-β4 is up-regulated in the infarcted brain area [336]. Moreover, Thymosin-β4 protects primary cortical and hippocampal neurons from glutamate-induced toxicity in vitro, and hippocampal neurons following kainic acid excitotoxicity in vivo [337]. Reduced hippocampal cell loss, as well as enhanced angiogenesis, enhanced neurogenesis, and increased oligodendrogenesis in the CA3 region are also seen in Thymosin-β4-treated animals in the rat model of traumatic brain injury [341]. These findings,
taken together with the results obtained in this study, suggest a potential therapeutic function for Thymosin-β4. Thymosin-β4 may be useful in treating visual diseases, such as glaucoma, where RGCs die by apoptosis.

On the other hand, the specific mechanisms by which Thymosin-β4 promotes cell survival are not well understood. Several studies have offered clues: in corneal epithelial cells, Thymosin-β4 treatment following ethanol exposure increases the expression of Bcl-2, an anti-apoptotic protein [330]. Thymosin-β4 also inhibits the activity of caspases-2, -3, -8, and -9, which are involved in executing apoptosis [330]. In primary cultured neurons, Thymosin-β4 treatment enhances the expression of the protein L1, a transmembrane cell adhesion molecule, and the effects of Thymosin-β4 on neurite outgrowth and neuronal survival are attenuated by antibodies directed against L1 [338]. In the heart, Thymosin-β4 forms a functional complex with PINCH (Particularly Interesting New Cys-His Protein) and integrin-linked kinase (ILK), resulting in the activation of protein kinase B (Akt) [323]. In mice, both ILK and Akt are up-regulated following Thymosin-β4 treatment after coronary artery ligation [323]. Similarly, I found that the level of activated Akt, a key member of the PI3K pathway, increased following Thymosin-β4 treatment. I also observed an increase in the level of phosphorylated MAPK, without an increase in the total MAPK levels. In axotomized RGCs, the PI3K and MAPK pathways are critical for cell survival following axotomy [19-23]. My results demonstrate that Thymosin-β4 activates both the PI3K and MAPK pathways, and these represent additional mechanisms by which Thymosin-β4 may prevent the apoptosis of adult CNS neurons. To further establish the role of these pathways in the survival and regenerative effects of exogenous Thymosin-β4, additional studies can be carried out in which exogenous Thymosin-β4 can be applied in combination with a PI3K inhibitor, such as LY294002, or a MEK inhibitor, such as PD98059. If these inhibitors are able to abolish or significantly decrease the survival or regenerative effects of Thymosin-β4, this would provide a more definitive link between Thymosin-β4 and the PI3K and MAPK pathways.

5.2. Exercise, the PI3K and MAPK Pathways, and Adult Neuronal Survival

The extent to which the effects of exercise carry through into the CNS is not as clear as in the peripheral nervous system. In the PNS, exercise improves axon regeneration and elongation
following injury [342-344], as well as muscle re-innervation [345], and sensory and motor functions [346, 347]. There are several indications that exercise can provide a similar benefit to neuron survival and regeneration in the CNS. For instance, in the fluid percussion injury (FPI) model of traumatic brain injury (TBI), one week of voluntary running is able to reverse the associated increases in the growth inhibiting molecules, MAG and Nogo-A, in the hippocampus of adult rats [359]. In the CNS, MAG and Nogo are known to accumulate at the lesion site following an injury, where they inhibit axonal regeneration [30-32]. If these molecules are inhibited, axonal sprouting can resume [31, 32]. Hence, not surprisingly, voluntary exercise increases neuronal proliferation, survival, and net neurogenesis in the dentate gyrus of the hippocampus [351, 352]. Furthermore, exercise enhances long-term potentiation (LTP), spatial learning, and locomotor recovery following spinal cord contusion injury (SCI) [351, 353].

In this study, animals that underwent a 2-week exercise program showed decreased RGC apoptosis at 7 and 14 days following optic nerve transection compared to control sedentary animals. These results agree with findings in D-Galactose-induced aged rats, where animals that underwent an 8-week forced exercise program showed a reduced number of apoptotic cells in the hippocampal dentate gyrus [364]. Similarly, exercise reduced the number of apoptotic cells in the hippocampi of transgenic mice of the Tg-NSA/PS2m model of Alzheimer’s disease [365]. Taken with my results, it is evident that exercise has a significant effect in the CNS, particularly, in reducing CNS neuronal apoptosis.

The mechanism by which exercise contributed to reducing RGC apoptosis was also examined in the present study. Previous findings indicated that neurotrophins in the CNS were increased in response to exercise: BDNF surges were seen in the hippocampus, cerebral cortex, and spinal cord of exercised adult rats [354-358], and TrkB (the BDNF receptor), synapsin I, GAP-43, and CREB were all found to be present at increased levels following exercise [357]. BDNF supports the function and survival of neurons, and activates both the PI3K and MAPK pathways in the retina [20]. Similarly, NGF, another neurotrophin, activates the PI3K and MAPK pathways [361-363]. In this study, I measured the levels of activated (phospho) Akt and MAPK in normal exercised animals to evaluate the potential effects exercise may exert on these pathways. My results yielded no significant increases between normal exercised and sedentary animals in regards to the activation of the PI3K and MAPK pathways. Moreover, some exercised animals showed a decrease in the levels of phosphorylated Akt, indicating a reduction of the PI3K pathway following light exercise. These results contradict previous findings in animal
models of age and disease, such as the D-Galactose-induced aged rat model, in which exercise increased the levels of NGF, its receptor, TrkA, as well as the levels of phosphorylated PI3K and phosphorylated Akt in the hippocampus [364]. Similarly, in the Tg-NSE/PS2m model of Alzheimer’s disease, exercise increased the levels of NGF, BDNF, phosphorylated PI3K, phosphorylated Akt, and phosphorylated ERK1/2 in the hippocampi of adult mice [365]. However, there are two primary differences between those studies and this one: First, while this study focused on the retina, both of those studies measured protein levels in the hippocampus. Second, both studies were conducted in animal models of age or disease. This study measured the levels of the PI3K and MAPK pathways in normal adult rats, with normal healthy retinas. While the PI3K and MAPK pathways play important roles in decreasing CNS apoptosis following injury, these pathways are, under normal conditions, kept under tight regulation, in order to prevent the cell cycle from going out of control. Thus, while exercise may enhance the PI3K and MAPK pathways in aged or diseased conditions, these pathways may not be required under normal healthy conditions. This may explain why, in this study, the retinas from the animals that underwent a light exercise program showed a decreased level of phosphorylated Akt. Nevertheless, exercise was able to increase RGC survival after injury, and this may be suggestive that exercise may have a more profound role in RGC survival. Thus, the mechanisms by which exercise exerts its benefits in the CNS warrant further investigation. Once properly understood, they may be utilized for the therapeutic treatment of CNS injury and disease.

5.3. PDZ Interactions in the PI3K and MAPK Pathways

The phosphatidylinositide-3 kinase (PI3K) and mitogen-activated-protein kinase (MAPK) pathways are involved in activating intracellular enzymes, initiating the transcription of survival promoting genes, and inhibiting apoptotic pathways [24-26]. When RGCs are injured, the PI3K and MAPK pathways are essential in preventing apoptosis [19-23]; however, the activity of these pathways is normally restricted by negative regulators, such as PTEN, Erbin, and BCR. While this is important in healthy adult cells, these negative regulators become obstacles for the survival of adult neurons upon injury. In this study, I aimed to interfere with PTEN, Erbin, and BCR, in order to promote the survival of adult RGCs following optic nerve transection. To this end, I targeted the PDZ interactions in which PTEN, Erbin, and BCR are involved. PDZ
interactions are protein-protein interactions that govern numerous molecular processes, such as targeting, clustering, cycling, and membrane expression of receptors, transporters, and ion channels [370-373]. Typically, a PDZ interaction involves a PDZ binding motif and the binding pocket with which it interacts, called a PDZ domain [366, 370]. In this study, small peptides that mimic specific PDZ binding motifs were synthesized in order to interfere with PTEN, Erbin, or BCR function.

5.3.1. PTEN and the Phosphatidylinositol-3 Kinase Pathway

Phosphatidylinositol-3 kinase is activated by receptor tyrosine-kinases, upon induction by ligands [14, 16, 108-114] (Figure 2.3.). Activated PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PI-4,5-P₂) producing phosphatidylinositol-3,4,5-trisphosphate (PIP₃) [115-117]. PIP₃ second messengers activate protein kinase B (Akt) [118-121], which is involved in numerous processes, including cell cycle progression, cell growth, cell differentiation, cell survival, and anti-apoptotic functions [121, 143]. PTEN hydrolyses PIP₃ to PI-4,5-P₂, reducing the number of second messengers that activate PKB/Akt and thus negatively regulates the PI3K pathway [166-168] (Figure 2.3.). PTEN contains a PDZ binding motif, through which it interacts with hDLG, MAST205, and MAGI-2 [386, 387]. By binding to the PDZ binding domain of MAGI-2, PTEN is stabilized and recruited to the plasma membrane, where it is able to act on PIP₃ [387, 388]. In this study, I used a peptide that contains the PDZ binding motif of PTEN, and applied it to injured RGCs. This peptide was intended to compete with PTEN for the PDZ domains of MAGI-2 and the other proteins with which PTEN interacts. I demonstrated that this peptide is effective in decreasing RGC apoptosis following optic nerve injury. Animals that received the TAT-PTEN peptide, either through intraocular injection or through Gelfoam application at the nerve end, showed significantly greater RGC densities at 14 days post-axotomy, compared to control animals. Although further investigation is required, I conclude that it is possible that the administered peptide prevented the interaction of PTEN with MAGI-2, and the subsequent stabilization and recruitment of PTEN to the plasma membrane. This would interfere with PTEN’s ability to negatively regulate the PI3K pathway and explain my findings. This theory is supported by previous findings in the human hepatocarcinoma 7721 cell line,
where MAGI-2 was found to inhibit cell migration and proliferation in a PTEN-dependant manner [389]. My results also correspond with recent findings in neuroblastoma cells that were infected with a rabies virus containing a viral G-protein with a C-terminal PDZ binding motif similar to PTEN. These cells showed a disruption in the interaction between PTEN and MAST2, increased cellular localization of PTEN, and the induction of neuronal survival [390]. Moreover, several other studies have revealed that the down-regulation of PTEN activity can mediate or enhance neuronal survival [443-445].

In this study, I found that RGC survival after optic nerve transection was greater when I delivered the TAT-PTEN peptide by intraocular injection than when I applied the TAT-PTEN peptide directly at the lesion site. Intraocular injections expose the delivered drug to all the cells in retina. On the other hand, drugs delivered at the cut end of the optic nerve are taken up by the RGC axons, and passed to the cell bodies by retrograde transport. My results indicate that the TAT-PTEN peptide is more effective when applied to the entire retina, as opposed to the RGCs only. This is likely related to the functions that PTEN has been found to perform in other retinal cells, such as astrocytes and glial cells. PTEN inhibits the proliferation of glioma cells and elicits the differentiation of astrocytes [514]. In the adult brain, PTEN loss induces astrocyte hypertrophy and increases astrocyte proliferation [515]. These cells are essential in providing the RGCs with biochemical support, as well as oxygen and other nutrients. Hence, the TAT-PTEN peptide was more effective when applied by intraocular injection, related to the global role that PTEN plays in the retina.

I also evaluated the ability of TAT-PTEN to promote RGC axon regeneration. Previous studies have described the involvement of PTEN in numerous developmental processes such as neurogenesis, neuronal migration, neuronal size control, neurite outgrowth, synaptogenesis, and synaptic plasticity [420-424]. In accordance with these findings, PTEN has been localized to axons and growth cones [427]. Moreover, PTEN accumulates at the growth cone membrane during the collapse response to semaphorin 3A (Sema3A) signalling [427]. In dorsal root ganglion, hippocampal, cortical, and spinal neurons, PTEN is necessary in the signalling of several neurite outgrowth inhibitors, including Sema3A, Sema4D, and MAG [427-430]. These growth-inhibiting compounds are known to accumulate at lesion sites in the CNS, and prevent axonal regeneration [29-31]. PTEN therefore represents a common point onto which these molecules converge and transmit their growth inhibiting signals. In my study, I found that TAT-PTEN enhanced axonal regeneration in the optic nerve. Animals that received either TAT-PTEN
or R9-PTEN showed a significant augmentation in the number of regenerating growth cones beyond the injury site following optic nerve crush, compared to control animals. These results agree with recent findings, in which the reduction of PTEN, either through genetic deletion or shRNAs, increased corticospinal tract sprouting, as well as increased axon regeneration following injury in the optic nerve and corticospinal tract [431-433]. Thus, my results indicate that peptides for disrupting PTEN binding represent a potential target for therapeutic treatment of both CNS degenerative diseases involving adult neuronal apoptosis and traumatic nerve injury.

5.3.2. Erbin and the Mitogen-Activated-Protein Kinase Pathway

The mitogen-activated-protein kinase, ERK, is activated through a cascade of protein kinases. ERK controls cellular functions such as proliferation, differentiation, apoptosis, synaptic plasticity, and neural development. Following ligand binding, the receptor tyrosine kinase is activated through autophosphorylation and ultimately results in the recruitment of the guanine nucleotide exchange factor, SOS, to the plasma membrane, where Ras is located [214-216] (Figure 2.4.). SOS releases GDP from Ras, and allows Ras to bind GTP [217]. GTP-bound Ras recruits Raf to the membrane for activation [220-225]. Activated Raf phosphorylates and activates MEK1/2, which subsequently phosphorylate ERK1/2 [229-234]. Erbin negatively regulates the MAPK pathway by binding to active Ras (Figure 2.4.), and disrupting its interaction with Raf [464]. Hence, in neuregulin-stimulated COS-1 cells, when Erbin is expressed, there is a reduction in the amount of phosphorylated ERK and decreased ERK kinase activity [464]. Furthermore, Erbin attenuates the interaction of active Ras and Raf with Sur-8, a scaffolding protein that is necessary for the formation of the Ras-Raf complex [460]. Erbin contains a PDZ domain through which it binds the PDZ binding motifs of several molecules [454], including HER2, a proto-oncogene that carries out its function thought the MAPK pathway [465, 469-473]. In this study, I interfered with the function of Erbin by using a peptide that binds to the c-terminal PDZ domain of Erbin. The TAT-ERBIN peptide contains an amino acid sequence identical to a PDZ binding motif that is shared by three proteins which have been associated with the PDZ domain of Erbin: δ-catenin, ARVCF (armadillo repeat protein deleted in velo-cardio-facial syndrome), and p0071 (plakophilin-4) [453, 456, 457]. Applying the TAT-ERBIN peptide, either through intraocular injection or via Gelfoam application at the cut end of
the nerve, significantly increased the *in vivo* survival of RGCs following optic nerve transection. My results suggest that inhibiting Erbin increases the survival of adult neurons following traumatic injury. This is comparable to previous findings in primary cultures of Schwann cells, where increased cell proliferation was observed when Erbin was blocked with siRNAs [462]. These cell cultures also showed altered cell-cell interactions, disrupted E-cadherin junctions, and elevated levels of phosphorylated ERK [462]. Moreover, these effects were abolished by using a pharmacological inhibitor of ERK kinase, demonstrating a link between Erbin and the MAPK pathway [462]. Although further investigation is required to demonstrate that the TAT-ERBIN peptide carries out its effects through the MAPK pathway, other studies have demonstrated that manipulating Erbin affects processes that are executed via MAPK. For instance, expressing Erbin in neuregulin-stimulated muscle cells decreases the transcription of an acetylcholine receptor subunit gene, a process which requires activated ERK [464]. Similarly, Erbin inhibits the differentiation of PC12 cells following NGF induction, which is an ERK-depdant effect [464]. Moreover, overexpressing Tax1, a protein that interacts with the PDZ domain of Erbin, in Erbin-positive MCF-7 cells, increases the phosphorylation of Ras, Raf, MEK1/2, and ERK1/2 [463]. Tax1 is synthesized by the HTLV-I virus and is essential in transforming and immortalizing T-cells [463]. All together, these studies indicate that Erbin merits further investigation after CNS injury.

As a final point, the results in this study also showed that the TAT-ERBIN peptide enhanced RGC survival following optic nerve transection more effectively when it was applied at the cut end of the optic nerve than through intraocular injection. Since drug application at the optic nerve targets RGCs more selectively than intraocular drug delivery, this suggests that Erbin’s function is more directly related to RGCs than surrounding retinal cells.

### 5.3.3. BCR and the Mitogen-Activated-Protein Kinase Pathway

In this study, I investigated the function of another negative regulator of the mitogen-activated-protein kinase pathway, breakpoint cluster region (BCR). BCR contains a PDZ binding motif, with which it binds to the PDZ domain of AF-6 (afadin) [482]. Together, BCR and AF-6 form a trimeric complex with Ras (Figure 2.4.), where AF-6 binds Ras via its Ras-binding domain [482]. Moreover, BCR negatively regulates Ras-mediated ERK signalling [482]. In this
study, I used a PDZ peptide that mimics the PDZ binding motif of BCR to competitively inhibit and disrupt the interaction of BCR with AF-6. In doing so, I sought to release Ras from BCR and AF-6, in order to bind Raf, activate the MAPK pathway, and promote cell survival. Following optic nerve transection, I applied this peptide, either by intraocular injection or via Gelfoam application at the nerve, and found a small increase in RGC survival. When compared to control retinas, retinas that received the application of the R9-BCR peptide showed a significant increase in RGC density only at the outer retinal eccentricity. These results indicate that while BCR may exert an effect on RGC apoptosis following optic nerve injury, these effects are either mild or limited by a stronger regulator of cell survival. Since MAPK is widespread, it is likely that in the retina, or perhaps the CNS at large, BCR only plays a minor role in regulating the MAPK pathway. When Radziwill et al. uncovered that BCR forms a trimeric complex with AF-6 and Ras, and that BCR negatively regulates Ras-mediated ERK signalling, they were working in HEK293 cells [482]. Similarly, Yamamoto et al. demonstrated that AF-6 binds Ras in vivo in the Rat1 cell line [488]. Moreover, it has been shown that BCR binds to Erbin through a PDZ interaction [498]. Although the function of this interaction has not yet been characterized, it is possible that R9-BCR disrupted the interaction between BCR and Erbin, and thus, allowed BCR or Erbin to resume negative regulation of the MAPK pathway. Further investigation is required to better understand the role of BCR in the retina and the rest of the CNS.

Chapter 6:
Conclusion

In conclusion, augmenting the activity of the PI3K and MAPK pathways via Thymosin-β4 treatment increased the survival and regeneration of injured RGCs. Exercise, which has been reported to have similar effects in other areas of the CNS, induced minor alterations in the activation of these pathways. Nevertheless, exercise had a significant effect on RGC survival. On the other hand, targeting the negative regulators of the PI3K and MAPK pathways produced a robust enhancement in the survival or regeneration of RGCs in the injured retina. Accordingly, the PDZ-blocking peptides that were developed in this study have potential therapeutic value in treating CNS insults or diseases where neurons undergo apoptotic cell death.
Chapter 7: 
Future Directions

Having demonstrated that Thymosin-β4 has both neuroprotective and neuroregenerative effects in the adult mammalian retina, I would like to investigate whether the increase in RGC survival and regeneration results in better functional outcome. If Thymosin-β4 improves the outcome of visual function following optic nerve injury, then further investigation is merited. Also, by applying Thymosin-β4 through different methods, I would like to determine the most effective mode of Thymosin-β4 delivery for maximal effect. Since Thymosin-β4 improves the outcome in the rat model of traumatic brain injury [341], as well as the optic nerve injury model, I would also like to investigate the effects Thymosin-β4 in other traumatic CNS models, such as spinal cord lesions, in order to uncover the extent of Thymosin-β4’s therapeutic potential in the CNS. Finally, in this study, I found that Thymosin-β4 increases activation of both the PI3K and MAPK pathways. Further work is required to specify how Thymosin-β4 regulates these pathways, and to determine the molecular partners with which Thymosin-β4 interacts. It is also important to investigate the role that Thymosin-β4 may be playing in other retinal cells, such as glial cells or astrocytes.

Interfering with the PDZ interaction of PTEN increased both neuronal survival and axonal regeneration after injury in the adult retina. I would therefore like to determine the effectiveness of this peptide in other models of CNS injury, as well as its effect on functional recovery. This work would help determine the therapeutic potential of the PTEN peptide in CNS neuronal injury and disease. Furthermore, this study found that the PTEN peptide enhanced RGC survival more effectively when it was applied through an intraocular injection than when it was applied at the injured nerve. This implies that PTEN plays a role in other cells of the retina, which translates into the survival of injured RGCs. Therefore, investigating the effect that the TAT-PTEN peptide has in the other cells of the retina is important for understanding how the peptide functions, and how these cells affect RGC survival. I would also like to investigate the effectiveness of the Erbin PDZ peptide as a therapeutic agent in other CNS models of injury. In this study, the Erbin PDZ peptide enhanced the survival of injured RGC. However, the ability of Erbin to promote or inhibit neuronal sprouting or axonal regeneration has not been investigated.
Therefore, I would like to determine whether the Erbin PDZ peptide can improve RGC axonal regeneration following optic nerve crush. I would also like to determine if Erbin plays a role in other retinal cells, and investigate how this can be manipulated by the TAT-ERBIN peptide. Conversely, the BCR PDZ peptide was not as effective in promoting RGC survival as the other peptides. Still, it demonstrated some benefits in RGC survival. It therefore merits uncovering the pathway or action of BCR in CNS neurons in order to understand how BCR functions and how it can be utilized to promote the survival of injured neurons. Additionally, it may also be worth investigating the effects of applying multiple PDZ peptides simultaneously, such as TAT-ERBIN and R9-BCR which both target the MAPK pathway. Similarly, TAT-PTEN may be combined with TAT-ERBIN to simultaneously target the PI3K and MAPK pathways, and enhance RGC survival. Finally, this study has demonstrated that PDZ interactions are an ideal target for manipulating cellular processes. PDZ interactions are among the most common types of protein interactions throughout the body. I would therefore like to investigate other proteins that possess PDZ domains or PDZ binding motifs and are involved in CNS apoptosis. Using the strategy of mimicking PDZ binding motifs, I would potentially manipulate the functions of these proteins to better understand the apoptotic machinery or to promote cell survival.

In this study, I found that exercise does not promote the activation of the PI3K and MAPK pathways in normal adult retinas; however, exercise did promote the survival of injured RGCs. Thus, the effect of exercise on the PI3K and MAPK pathways in injured retinas ought to be investigated. Understanding how exercise promotes neuronal survival is vital for the therapeutic potential of exercise in CNS injury or disease. Thus, I would like to assess the effects of exercise on the levels of the neurotrophins, BDNF, and NGF in both normal and injured retinas, and determine how the effects of exercise are translated at the molecular level. Once this is understood, I would investigate strategies that could enhance the beneficial effects of exercise. Finally, while exercise promotes RGC survival after injury, it is necessary to determine if this results in improved functional recovery. This must be determined before exercise can be utilized as the therapeutic treatment in CNS neuronal injury or disease.
Chapter 8:
References


