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INVESTIGATION OF THE PHYSIOLOGICAL ROLE OF RIN GTPASE IN CELL DEATH, AXONAL INJURY, AND INFLAMMATION FOLLOWING TRAUMATIC BRAIN INJURY

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INVESTIGATION OF THE PHYSIOLOGICAL ROLE OF RIN GTPASE IN CELL DEATH, AXONAL INJURY, AND INFLAMMATION FOLLOWING TRAUMATIC BRAIN INJURY

DISSERTATION

A dissertation submitted in partial fulfillment of the Requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
Megan Dionne Pannell
Lexington, Kentucky

Director: Dr. Douglas A. Andres, Professor of Molecular and Cellular Biochemistry
Lexington, Kentucky
2017

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TBI is a progressive disorder, in which the primary injury results in the initiation of a complex cascade of secondary biochemical and metabolic changes resulting in lasting neurological dysfunction and cognitive impairment. The heterogeneous nature of the disease has complicated the development of pharmacological agents to improve the outcomes of TBI; to date, no therapeutic treatment has been shown to be effective in clinical trials. Treatments targeting multiple secondary outcomes (cell death, axonal degeneration, and inflammation) may provide enhanced therapeutic efficacy following TBI.

Small Ras family GTP-binding proteins govern diverse cellular processes by directing the relay of extracellular stimuli to the activation of select intracellular signaling pathways. Rin (RIT2) is a member of the Rit subfamily of Ras-related family of GTPases, and is expressed solely within neurons of the CNS. Early cell culture models demonstrated that Rin signaled upstream of the stress-activated protein kinase, p38, and lacked the transformative abilities displayed by other members of the Ras family, suggesting functions for Rin other than cell growth and proliferation.

To begin to define the physiological function of Rin, we generated a RIT2 knockout mouse and examined the impact of Rin loss in the CNS following brain trauma. Our data demonstrates that Rin deficiency is neuroprotective following a controlled cortical impact (CCI) injury, reducing both acute hippocampal neurodegeneration and promoting sustained neuronal survival, without affecting post-CCI neurogenesis. Hippocampal neuroprotection achieved by Rin loss was accompanied by improved cognitive function in injured mice. Furthermore, we demonstrated that Rin loss led to blunting of axonal degeneration and microglial activation in the optic nerve following optic nerve stretch injury. The molecular interaction between Rin and dual leucine zipper kinase suggested a potential role for Rin in the regulation of a novel stress MAPK-dependent neuronal death
cascade. Lastly, we demonstrated through diffuse closed head injury, that Rin loss mitigates cytokine release as a result of injury without altering glial activation.

Together, these studies establish Rin as a novel regulator of neuronal cell death, cognitive decline, axonal degeneration, and cytokine production following traumatic brain injury.

KEYWORDS: Rin, RIT2, Ras-like GTPase, TBI, neuroprotection
INVESTIGATION OF THE PHYSIOLOGICAL ROLE OF RIN GTPASE IN CELL DEATH, AXONAL INJURY, AND INFLAMMATION FOLLOWING TRAUMATIC BRAIN INJURY

By

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9/12/2017
This work is dedicated to my parents, Hal and Joy Pannell, who told me I could achieve anything, and my siblings Blake, Eli and Journey, for keeping me grounded.
It takes a village to raise a child, and the same is true to progress a graduate student to their doctorate. This work could not have been completed without the help, support, and encouragement of my department, collaborators, friends, and family. There is not adequate space to name all the people who have guided and encouraged me over the years to get me where I am today.

First and foremost, I would like to thank my advisor, Dr. Douglas Andres, for his advice and suggestions that helped guide my graduate work. His motivation and pursuit of excellence has molded me to the scientist that I am today. I would also like to thank Sidney Whiteheart, Jianhang Jia, Kimberly Nixon, and Kathryn Saatman for serving on my dissertation committee. Wally is notorious among students for being a wealth of information on everything; his curiosity and knowledge are a source of inspiration and awe. Thanks to JJ for his willingness to aid in my pursuits, including Drosophila studies during my rotation in the Andres lab. Kathy has always played an active role in my research, as a collaborator, she taught me about trauma, neuroscience, and has always made her lab open to me. Kim Nixon sparked my interest in research through an undergraduate research experience in her laboratory. That experience is the reason that I started this journey and it was a privilege to have her along for the ride. Thank you all!

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>viii</td>
</tr>
<tr>
<td>Chapter One: Background and Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Traumatic Brain Injury</td>
<td>1</td>
</tr>
<tr>
<td>Epidemiology and Etiology</td>
<td>1</td>
</tr>
<tr>
<td>Pathophysiology</td>
<td>3</td>
</tr>
<tr>
<td>Primary Injury</td>
<td>6</td>
</tr>
<tr>
<td>Secondary Injury Cascades</td>
<td>6</td>
</tr>
<tr>
<td>Traumatic Axonal Injury</td>
<td>8</td>
</tr>
<tr>
<td>Blood Brain Barrier Breakdown</td>
<td>9</td>
</tr>
<tr>
<td>Glial Activation and Inflammation</td>
<td>10</td>
</tr>
<tr>
<td>Treatment of TBI</td>
<td>11</td>
</tr>
<tr>
<td>Recovery following TBI: Neurogenesis</td>
<td>12</td>
</tr>
<tr>
<td>Ras superfamily</td>
<td>14</td>
</tr>
<tr>
<td>GTPase Cycle</td>
<td>15</td>
</tr>
<tr>
<td>Ras Biochemical Characteristics and Structure</td>
<td>18</td>
</tr>
<tr>
<td>Rit subfamily of GTPases</td>
<td>22</td>
</tr>
<tr>
<td>Biochemical Characterization of the Rit subfamily</td>
<td>22</td>
</tr>
<tr>
<td>Function of Rit subfamily in TBI</td>
<td>24</td>
</tr>
<tr>
<td>Rin-mediated Signal Transduction Pathways and their Implications in TBI</td>
<td>25</td>
</tr>
<tr>
<td>Raf/MEK/ERK MAPK Cascade</td>
<td>29</td>
</tr>
<tr>
<td>p38 MAPK Cascade</td>
<td>30</td>
</tr>
<tr>
<td>Regulation of Rho family GTPases</td>
<td>31</td>
</tr>
<tr>
<td>Rin-mediated Neurite Outgrowth</td>
<td>32</td>
</tr>
<tr>
<td>Rational for Investigating the Physiological Function of Rin</td>
<td>35</td>
</tr>
<tr>
<td>Chapter Two: Materials and Methods</td>
<td>37</td>
</tr>
<tr>
<td>Reagents</td>
<td>37</td>
</tr>
<tr>
<td>Chapter Three</td>
<td>37</td>
</tr>
<tr>
<td>Chapter Four</td>
<td>37</td>
</tr>
<tr>
<td>Chapter Five</td>
<td>38</td>
</tr>
<tr>
<td>Animals</td>
<td>38</td>
</tr>
<tr>
<td>Traumatic injury models</td>
<td>39</td>
</tr>
<tr>
<td>Controlled Cortical Impact</td>
<td>39</td>
</tr>
<tr>
<td>Mono-ocular Nerve Stretch (MONS)</td>
<td>40</td>
</tr>
<tr>
<td>Optic Nerve Transection</td>
<td>40</td>
</tr>
<tr>
<td>Closed Head Injury</td>
<td>41</td>
</tr>
<tr>
<td>Rin Immunoblotting</td>
<td>41</td>
</tr>
<tr>
<td>BrdU Administration</td>
<td>42</td>
</tr>
<tr>
<td>Tissue Preparation</td>
<td>42</td>
</tr>
<tr>
<td>Creysl Violet Brain Staining</td>
<td>43</td>
</tr>
<tr>
<td>Injury Volume Quantification</td>
<td>43</td>
</tr>
<tr>
<td>Fluoro-Jade C staining</td>
<td>44</td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>45</td>
</tr>
</tbody>
</table>
Chapter Three: Genetic Deletion of the Rin GTPase Promotes Neuronal Sparing and Reduces Behavioral Deficits following Traumatic Brain Injury

Introduction .................................................................................................................. 55
Results .............................................................................................................................. 58
Genetic Rin loss does not alter brain morphology ...................................................... 58
Rin deficiency promotes hippocampal cell survival .................................................... 62
Rin deficiency does not alter the generation of newborn neurons in the hippocampus following CCI ............................................................................................................. 65
Rin deficiency promotes hippocampal CA-3 neuronal survival .................................... 73
Rin loss reduces post-CCI behavioral dysfunction ..................................................... 76
Discussion ....................................................................................................................... 79

Chapter Four: Rin Contributes to Trauma-induced Axonal Injury

Introduction .................................................................................................................. 84
Results .............................................................................................................................. 88
Rin loss mitigates trauma-induced axonal degeneration following stretch injury .......... 88
Rin loss diminishes trauma-induced microglia activation following optic stretch injury ................................................................................................................................. 92
Rin interacts with the MAPKKK, Dual Leucine Zipper Kinase (DLK) ......................... 97
Transection-induced Wallerian degeneration unaffected by Rin loss ......................... 99
Discussion ....................................................................................................................... 102

Chapter 5: A Role for the Rin GTPase in Cytokine Production and Neuroinflammation

Introduction .................................................................................................................. 107
Results .............................................................................................................................. 109
Rin loss suppresses the acute pro-inflammatory response following CHI .................. 109
Astrocyte activation unchanged by Rin loss ................................................................. 114
LIST OF FIGURES

Figure 1.1. Progression of primary and secondary injury following insult to the head……………………………………………………………………..5
Figure 1.2. The GTPase Cycle………………………………………………………………………………………………………………………17
Figure 1.3. Alignment of the Rit Family of Small GTPases…………………………………………………………………………………..21
Figure 1.4. Receptor tyrosine kinase (RTK)-mediated Ras MAPK Cascade………………………………………………………………27
Figure 1.5. Rin-dependent signaling in neurite outgrowth……………………………………………………………………………………………34
Figure 3.1. Generation and characterization of Rin null-mutant mice…and wild-type and Rin^-/- mice……………………………………………………60
Figure 3.2. Cortical contusion volume changes equivalent in wild-type and Rin^-/- mice………………………………………………………….63
Figure 3.3. Rin loss attenuated trauma-induced hippocampal neurodegeneration……………………………………………………………64
Figure 3.4. Rin loss had no effect on newborn neuron survival following contusion injury…………………………………………………66
Figure 3.5. Rin deficiency does not alter the effect of trauma on inner granular layer proliferation………………………………………………..69
Figure 3.6. Rin loss did not alter the recovery of immature neuron density following controlled cortical impact……………………………71
Figure 3.7. Rin loss promotes hippocampal neuronal survival following brain injury…………………………………………………………….74
Figure 3.8. Rin loss reduced cognitive impairment after severe brain Injury………………………………………………………………………77
Figure 4.1. Rin loss does not alter early axonal degeneration 24hr following optic stretch injury………………………………………………90
Figure 4.2. Rin loss blunts axonal degeneration 7d following optic stretch injury…………………………………………………………..91
Figure 4.3 Rin loss does not alter early microglial activation 24hr following optic stretch injury………………………………………………94
Figure 4.4. Rin loss blunts microglia activation 7d following optic stretch injury………………………………………………………………95
Figure 4.5. Relationship between axonal damage (SMI-32) and microglial activation (CD68) following optic stretch injury………………96
Figure 4.6. Rin associates with Dual Leucine Zipper Kinase (DLK), an upstream regulator of axonal degeneration………………………….98
Figure 4.7. Rin loss has no effect on axonal degeneration 24hr following optic nerve transection…………………………………………….100
Figure 4.8. Rin loss has no effect on axonal degeneration 7d following optic nerve transection……………………………………………….101
Figure 5.1. Rin loss alters cytokine production following diffuse brain Injury………………………………………………………………………112
Figure 5.2. Astrogliosis in Rin null mice remains intact following diffuse head injury…………………………………………………………….116
Figure 5.3. Rin null mice display normal microglial activation following
CHAPTER ONE

Background and Introduction

Traumatic Brain Injury

Epidemiology and Etiology

Traumatic brain injury (TBI) is broadly defined as any blow or jolt to the head that results in damage to the brain and disruption of normal brain function (CDC 2015). TBI is a leading cause of death and permanent disability worldwide; there are approximately 57 million people living that have been hospitalized for TBI around the globe (Murray 1996). In the United States alone, TBI affects 1.5 million people annually (Thurman 1999). As a result of these injuries, 1.1 million people visit the ER, 235,000 people are hospitalized, and 50,000 will perish (Thurman 1999, Langlois, Rutland-Brown et al. 2006). Despite these substantial numbers, TBI statistics are underrepresented, as surveys do not include cases treated in outpatient settings, physicians' offices or military facilities in the United States or abroad (Langlois, Rutland-Brown et al. 2006, Summers, Ivins et al. 2009).

TBI is not constrained to any particular age, gender, or demographic, thus has the potential to affect everyone. The overall leading causes of TBI are falls (32%), motor vehicle accidents (19%), struck by/against events (18%), and assaults (10%) (Rutland-Brown, Langlois et al. 2006). When age is factored into
the equation, clear trends are observed throughout the life course. It can be seen that the peak incidence rates in young adults are due to motor vehicle accidents, whereas in the elderly peak rates are the result of falls (Thurman 1999). Finally, although both genders are affected, the rates of TBI among males are significantly higher than those observed in females (650.9 per 100,000 compared to 429.1 per 100,000). The highest incidence rate of TBI has been reported in males between the ages of 20 and 30 years (Langlois, Rutland-Brown et al. 2006, Rutland-Brown, Langlois et al. 2006).

TBI is referred to as the invisible epidemic because the long-term effects of the disease are not always visually apparent. Long-term disabilities resulting from brain injury include alterations in cognition, mood and personality. TBI has also been associated with increased risks of other health problems; indeed, compared to the general population, those who have suffered from TBI are more likely to experience epilepsy or binge drinking behavior, and are 7.5% more likely to die within three years of the head injury (Horner, Ferguson et al. 2005, Selassie, McCarthy et al. 2005, Langlois, Rutland-Brown et al. 2006). Additionally, TBI has also been associated with increased prevalence rates of depression and Alzheimer’s disease (Plassman, Havlik et al. 2000, Holsinger, Steffens et al. 2002). Furthermore, military soldiers returning from Iraq who had suffered a mild head injury displayed a strong comorbidity of depression and/or post-traumatic stress disorder (Hoge, McGurk et al. 2008). Overall, it is estimated that 5.3 million people (approximately 2% of the population) in the United States alone are living with a permanent TBI-related disability (Thurman 1999). The
annual cost of care and rehabilitation for TBI sufferers within the United States was calculated at over $76 billion in 2000 (Finkelstein 2006, Coronado, Xu et al. 2011).

Pathophysiology

The clinical manifestations of TBI are heterogeneous, ranging from a mild headache or concussion to coma and/or death. Clinically TBI is defined as an alteration in brain function that could include any of the following: loss of consciousness, memory loss of events before or after the injury, neurological defects, sensory loss, loss of balance or coordination, or altered mental state such as confusion or disorientation (Menon, Schwab et al. 2010). Alterations in cognition is one of the most commonly reported functional impairments following TBI (Lundin, de Boussard et al. 2006). Regardless, both cognitive and motor deficits have been reported to persist months following the initial insult in 40% of patients (Satz, Forney et al. 1998).

To evaluate the diverse outcomes of a TBI, the Glasgow Coma Score (GCS) was introduced in the late 1970s to gauge the severity of a head injury. GCS scores fall on a scale from 3 to 15 judging by the ability of a patient to respond to verbal, auditory, and visual stimuli along with an assessment of motor and verbal proficiencies (Teasdale and Jennett 1974). Based on this scale, a patient with a GCS score ranging from 13-15 is classified to have a mild TBI, 9-12 a moderate TBI, and 3-8 a severe head injury. Nevertheless, patients presenting with the same GCS score often have vastly different injuries and
require distinctive treatment methods. More recently, utilization of neuroimaging techniques, such as magnetic resonance imaging and computerized tomography, has further advanced characterization of TBI to allow for more appropriate treatment intervention (Marshall, Marshall et al. 1992, Maas, Steyerberg et al. 2007).

TBI results in the activation of a multitude of pathological outcomes. These mechanisms set forth by traumatic injury can be separated into primary and secondary injury events. The primary injury results from the initial mechanical force to the head. Damage from the mechanical force then triggers the activation of secondary injury cascades (Figure 1.1).
Figure 1.1 Progression of primary and secondary injury following insult to the head. A primary mechanical force to the head results in tissue deformation and cell shearing within seconds to minutes of the insult results in the activation of secondary cascades that can persist for months. Together, the primary and secondary injuries lead to functional deficits. Modified from (Xiong, Mahmood et al. 2013).
Primary Injury

The primary injury results in shearing, compression and/or stretching of nervous system cells and vasculature along with a disruption of membrane integrity following the application of a mechanical force to the head (Beauchamp, Mutlak et al. 2008). This initial mechanical disruption can be caused by a variety of injury mechanisms including: contact with a penetrating or non-penetrating object, rapid acceleration/deceleration forces, or a blast injury (Menon, Schwab et al. 2010).

Depending on the type of force, primary injury can be classified as either focal or diffuse. Focal damage is localized to a discrete area of the brain and associated with mass lesion formation often caused by contusion and/or hemorrhage, which leads to localized cell death and ischemia (Povlishock and Katz 2005). Contrarily, diffuse injury occurs in non-discrete areas across the brain and is often characterized by edema and axonal injury (Povlishock and Katz 2005). In either case, the tissue damage and cell death caused by the primary insult do not lend themselves for therapeutic intervention, instead they initiate a cascade of detrimental consequences (Beauchamp, Mutlak et al. 2008, Maas, Roozenbeek et al. 2010).

Secondary Injury Cascades

Secondary injury is defined by the self-propagating pathological alterations occurring in cells and tissues that result in cellular dysfunction and death hours to weeks following the initial insult (Borgens and Liu-Snyder 2012). Secondary
cascades are characterized by a complex set of biochemical and molecular alterations giving rise to neuroinflammation, delayed cell apoptosis or necrosis, and edema for prolonged periods following injury (Beauchamp, Mutlak et al. 2008). The nature of secondary injury processes that manifests is dependent upon the type and severity of the primary injury. Furthermore, secondary injuries are complex, propagating spatially and temporally following trauma. Disruption of cellular integrity results in a loss of ion homeostasis as extracellular calcium ions flow into damaged neurons. This unregulated flow of calcium into the neuron initiates uncontrolled release of neurotransmitters along with the activation of intracellular proteases, such as calpain, inducing excitotoxicity and disruption of cellular transport (Baker, Moulton et al. 1993, Saatman, Bozyczko-Coyne et al. 1996, Hall, Sullivan et al. 2005, Krishnamurthy and Laskowitz 2016). High intracellular concentration of calcium also contributes to mitochondrial dysfunction as the organelle becomes overwhelmed attempting to clear cytosolic calcium, which ultimately leads to increases in reactive oxygen species (ROS), loss of ATP production, and the commencement of apoptosis (Sullivan, Thompson et al. 1999, Singh, Sullivan et al. 2006, Chen, Yang et al. 2007). The occurrence of a multitude of detrimental events overwhelms the cell inducing cell death which leads to a wave of excess calcium and neurotransmitters, further propagating cell death through excitotoxicity in surrounding cells (Conti, Raghupathi et al. 1998, Pleasant, Carlson et al. 2011). Additionally, shearing of axons and subsequent degeneration and demyelination interrupt signal

**Traumatic Axonal Injury**

Traumatic axonal injury (TAI) is a common feature of diffuse TBI, generally resulting from high-velocity rotational forces that result in stretching and shearing of axons (Hill, Coleman et al. 2016). The majority of axonal damage is not due to shearing at the time of primary injury (Buki and Povlishock 2006); rather, demyelination and atrophy along white matter tracts progresses for decades in TBI patients (Johnson, Stewart et al. 2013, Green, Colella et al. 2014). Axonal pathology develops over the course of days following injury. Within seconds of stretch injury, the axons become misaligned and take on a wavy structure due to temporary loss of elasticity (Smith, Wolf et al. 1999). Although axonal structure recovers, axonal swellings become visible within hours of injury. Over the course of days to weeks, accumulation disruption of cytoskeletal elements and cellular transport exacerbates axonal pathology leading to secondary axotomy, pathologically denoted by the presence of a bulb formation at the axon terminal (Smith 2000). Traditionally it was believed that all these damaged axons would disconnect and undergo Wallerian degeneration, characterized by the degeneration of axon segment distal to the site of transection (Smith 2000). However, it is now recognized that many of these damaged axons will not disconnect and dieback, but instead will undergo axonal repair (Buki and Povlishock 2006).
Axonal damage is a progressive secondary cascade that manifests as an interruption of protein transport and breakdown of cytoskeletal elements (Hill, Coleman et al. 2016). Indeed, labeling of transported proteins, such as amyloid precursor protein (APP), can be observed localized in axonal swellings within hours of traumatic injury (Roberts, Gentleman et al. 1991, Roberts, Gentleman et al. 1994, Smith 2000). It has been proposed that disruption of protein transport is caused by proteolysis and compaction of the axonal cytoskeleton, which is primarily composed of neurofilament proteins (Hall and Lee 1995, Povlishock, Marmarou et al. 1997). In addition to proteolysis, dephosphorylation of neurofilament proteins may also contribute to compaction of the axonal structure (Okonkwo, Pettus et al. 1998).

**Blood Brain Barrier Breakdown**

Disruption of the blood brain barrier (BBB) is a hallmark of severe TBI (Baldwin, Fugaccia et al. 1996, Price, Wilson et al. 2016). The microvessels of the BBB are composed of endothelial cells connected by tight junctions; these endothelial cells interact with the glial cells of the CNS to form a restrictive barrier between the CNS and the rest of the body (Price, Wilson et al. 2016). Following primary trauma, vasculature disruption causes a breakdown of the endothelial tight junctions of the BBB allowing for atypical transport of molecules from the peripheral blood stream into the brain including clotting agents such as thrombin, fibrinogen and albumin that decrease blood flow and increase the risk of ischemia (Chodobski, Zink et al. 2011, Price, Wilson et al. 2016). Increased BBB
permeability also allows for accumulation of plasma proteins and water in the brain contributing to post-traumatic edema (Greve and Zink 2009, Chodobski, Zink et al. 2011). Lastly, disruption of the BBB allows for immune cells from the periphery, such as macrophages and neutrophils, to enter the brain through the damaged vasculature. Upon infiltration, these cells become active and secrete cytokines such as TNF-α and IL-1β, which activate resident glial cells initiating a neuroinflammatory response (Ziebell and Morganti-Kossmann 2010, Chodobski, Zink et al. 2011, Kumar and Loane 2012).

**Glial Activation and Inflammation**

Resident astrocytes are important for maintaining homeostasis by aiding in support of the BBB, secretion and absorption of neurotransmitters, and maintenance of extracellular ion concentrations (Kolb 2008). Following trauma, astrocytes become activated; this process of astrocytosis is characterized by cellular proliferation and hypertrophy (increased expression of glial fibrillary acidic protein (GFAP), elongation of cellular processes, and cytoplasmic enlargement) (Saatman, Feeko et al. 2006, Bardehle, Kruger et al. 2013, Burda, Bernstein et al. 2016). Following brain insult, astrocytes form a glial scar that acts as a protective barrier between damaged tissue and the intact brain (Villapol, Byrnes et al. 2014) Although the scar compartmentalizes cellular debris and biochemical byproducts of the damaged tissue, astrocytes in the scar continue to release cytokines, that promote inflammation, and chondroitin sulfate, a molecule that
has been shown to inhibit regeneration and repair (Smith, Miller et al. 1986, Snow, Steindler et al. 1990, McKeon, Schreiber et al. 1991).

Microglia are the resident immune cells of the CNS. In their resting, or inactive, state they survey tissue for biochemical markers of damage and infection (Harry 2013). Upon detection of TBI-induced damage, microglia become activated and begin to release cytokines to signal other glia cells to respond to injury (Chiu, Liao et al. 2016). These cells, along with peripheral macrophages and neutrophils (introduced following the breakdown of the BBB), recognize foreign antigens and clear cellular debris (Harry 2013). Microglia have been demonstrated to switch between two different states of activation: M1 and M2. M1 activated microglia release pro-inflammatory cytokines, such as TNF-α and IL-1β, along with ROS, which lead to further cell death (Chiu, Liao et al. 2016). In an M2 state, however, microglia promote tissue repair and regeneration by releasing trophic factors such as insulin-like growth factor-1, anti-inflammatory IL-10, and the incretin, GLP-1 (Chiu, Liao et al. 2016). The inability of microglia to switch between these two states is detrimental to brain health. Sustained elevation of IL-1β and TNF-α impair the switch from the M1 to M2 phase leading to neuronal dysfunction (Streit, Mrak et al. 2004).

**Treatment of TBI**

The activation of a diverse array of secondary injuries cascades has made the treatment of TBI complex. Disappointingly, to date no pharmacological treatment for TBI has convincingly shown benefit in a multicenter phase III trial.
and made it to market. The rationale for trial failures have been attributed to a variety of factors including: drugs not reaching their target, not attaining an adequate intracranial concentration, or missing the appropriate time for intervention (Beauchamp, Mutlak et al. 2008). Due to the lack of pharmacological agents, clinically, the symptoms of TBI are mitigated through the use of mannitol to decrease brain edema and surgical craniectomy to stabilize intracranial pressure (Muizelaar, Wei et al. 1983, Munch, Horn et al. 2000). However, increasing focus on the cellular and molecular changes accompanying TBI continues to provide new therapeutic targets for drug development (Beauchamp, Mutlak et al. 2008). One area of focus is on agents that are able to enhance the brain’s endogenous plasticity and regeneration capabilities.

Recovery following TBI: Neurogenesis

Following trauma, the brain initiates endogenous repair mechanisms, promoting plasticity and regeneration (Scheff, Price et al. 2005, Rola, Mizumatsu et al. 2006, Xiong, Mahmood et al. 2010). Although glial cells quickly proliferate in response to injury, the developed brain has less capacity to replenish lost neurons. Historically, it was believed that all neurons in the brain were derived during embryonic development and the adult brain was unable to produce new neurons throughout the life course. However, it is now understood that resident neural stem cells are able to self-renew their own pool through proliferation and also have the ability to differentiate into astrocytes, oligodendrocytes, and neurons through the process of neurogenesis (Gage 2000). New neurons are
generated in two discrete niches within the adult brain: in the subventricular zone of the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus (Gage 2000). Neurogenesis is a dynamic process and rates of proliferation are responsive to environmental stimuli (Kempermann, Kuhn et al. 1997, van Praag, Christie et al. 1999, van Praag, Shubert et al. 2005). Indeed, increased levels of neurogenesis have been observed following various brain insult including ischemia (Jin, Minami et al. 2001, Osman, Porritt et al. 2011), seizures (Parent and Lowenstein 2002, Smith, McLean et al. 2005), and TBI (Dash, Mach et al. 2001). Although neurogenic activation indicates an attempt at self-repair following injury, the number of neurons generated, and subsequently integrated into the circuitry, is too few to generate complete functional recovery (Quadrato, Elnaggar et al. 2014).

A better understanding of the molecular components that drive neuronal plasticity and recovery following TBI is needed, for treatments that reduce neurodegeneration while promoting enhanced plasticity may prove to be efficacious for the treatment of TBI. Although the molecular mechanisms contributing to the regulation of neurogenesis are poorly understood, it is suspected that neurotrophins, such as BDNF, NGF and NT-3, and growth factors such as IGF-1, bFGF and VEGF, play important roles in the regulation of neurogenesis (Maisonpierre, Belluscio et al. 1990, Aberg, Aberg et al. 2000, Carro, Trejo et al. 2001, Lee and Son 2009, Mudo, Bonomo et al. 2009). The Ras superfamily of GTPases regulate cellular responses downstream of growth factor stimulation (Katz, Amit et al. 2007) and have been implicated in the control of

**Ras superfamily**

The Ras superfamily of GTPases encompasses a large family of structurally related hydrolysis enzymes that function to regulate a variety of cellular processes by acting as molecular switches relaying extracellular stimuli to the activation of appropriate intracellular responses (Reuther and Der 2000, Colicelli 2004, Wennerberg, Rossman et al. 2005). The Ras superfamily can be separated into five subfamilies: Ras (Rat sarcoma), Rho (Ras homolog gene family), Rab (Ras-related GTP-binding protein), Ran (Ras-related nuclear protein), and Arf (ADP-ribosylation factor) (Colicelli 2004). Ras family protein signal transduction cascades commonly activate cell proliferation and survival responses (Satoh, Nakafuku et al. 1992, Downward 1998, Wennerberg, Rossman et al. 2005). Rho proteins are associated with the regulation of cytoskeletal dynamics and cell morphology (Jaffe and Hall 2005, Wennerberg, Rossman et al. 2005). The Rab family is critical in modulating membrane trafficking (Stenmark and Olkkonen 2001, Wennerberg, Rossman et al. 2005), while Arf proteins function in vesicular transport (Wennerberg, Rossman et al. 2005, Gillingham and Munro 2007), and the Ran family in nuclear transport (Pemberton and Paschal 2005, Wennerberg, Rossman et al. 2005). Although the functions of many GTPases have been well characterized, there remains a
subset whose physiological roles have not been defined. The focus of this dissertation is one such protein of the Ras subfamily, Rin (Ras-like protein in neurons).

**GTPase cycle**

Despite the broad array of functions controlled by different small GTPase families, a majority of the Ras superfamily shares a conserved protein core that is responsible for nucleotide binding and hydrolysis (Colicelli 2004). This core region drives a conformational switch between an inactive, GDP-bound, and active, GTP-bound, structural state. In their GTP-bound structural state Ras GTPases are capable of associating with, and activating, a diverse array of cellular effector proteins (Wennerberg, Rossman et al. 2005). It is the capability of GTPases to function as GTP/GDP driven molecular switches that permits them to serve as critical regulators of diverse cell signaling cascades (Figure 1.2).

The activation of the small GTPases is dependent on nucleotide exchange. When GDP is bound, the protein is found in an “inactive” conformational state, with the G2 effector loop buried within the protein. This conformational state inhibits the interaction of Ras G-proteins with their downstream effectors, thereby preventing signaling. The binding affinity for guanine nucleotides is high, with a $K_d$ value in the $10^{-11}$M range; therefore, spontaneous activation of Ras proteins does not occur under physiological conditions (Lowy and Willumsen 1993). Furthermore, the intrinsic hydrolysis
activity of these enzymes is low and, without assistance, inactivation of the GTP-bound active state would not occur on a biologically effective time scale (Lowy and Willumsen 1993). Rather, two families of regulatory factors control rapid activation and inactivation of the GTPase: guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Figure 1.2). GEF proteins bind to the small GTPase inducing the release of bound nucleotide (Quilliam, Khosravi-Far et al. 1995, Bernards and Settleman 2007). Upon GEF-mediated nucleotide release, the nucleotide free GTPase rapidly binds GTP given its intracellular concentration is approximately 10-fold higher than GDP. To overcome the slow rate of intrinsic hydrolysis, binding of GAP proteins stimulate the enzymes’ intrinsic hydrolysis rate (Bernards and Settleman 2005). Members of the Rho and Rab family have an additional level of regulation through binding to guanine nucleotide dissociation inhibitor (GDIs) (Geyer and Wittinghofer 1997) (Figure 1.2). GDIs bind to GDP-bound enzymes to prevent GEF binding and membrane targeting, retaining an inactivated state (Geyer and Wittinghofer 1997).
Figure 1.2. The GTPase cycle. Ras family protein cycle between an inactive GDP-bound state and an active GTP-bound conformation. Guanine nucleotide exchange factors (GEF) trigger the release of GDP, which is promptly replaced by GTP. Binding of GTP elicits a conformational change allowing for binding to downstream effector proteins. GTPase-activating proteins bind to Ras proteins to increase their intrinsic GTP hydrolysis, releasing inorganic phosphate and returning Ras to its inactive state. Rab and Rho proteins interact with guanine nucleotide dissociation inhibitors (GDIs) to prevent GEF binding and maintain an inactive conformation.
Ras Biochemical Characteristics and Structure

The Ras superfamily shares five highly conserved core regions, or G box sequences, that confer their biochemical activity of guanine nucleotide binding and hydrolysis (Figure 1.3) (Barbacid 1987, Lowy and Willumsen 1993, Colicelli 2004). The G1 sequence is necessary for binding to the $\alpha$ and $\beta$-phosphates of purine nucleotides and is also referred to as the P-loop or Walker A motif (Colicelli 2004). The G3 domain binds the $\gamma$-phosphate group of GTP and the nucleotide associated magnesium ion (Lowy and Willumsen 1993). G4 and G5 regions are responsible for nucleotide specificity, conferring selective GTP binding instead of ATP, as they specifically bind the guanine ring (Colicelli 2004). Of all the G box domains, G2 is the least conserved among Ras family members. The G2 domain is also referred to as the “effector domain” because it serves as the primary site of interaction for downstream effectors following activation by GTP binding (Colicelli 2004). Therefore, the diversity of the G2 sequence allows for small GTPases to recognize distinct collections of effector proteins, which regulate diverse physiological functions. Furthermore, the G2 and G3 domains undergo nucleotide-dependent conformational alterations (Cherfils and Zeghouf 2011). When in contact with the $\gamma$-phosphate of GTP, the two loops are exposed to the surface of the GTPase core allowing for recruitment of effectors and the initiation of downstream signaling (Cherfils and Zeghouf 2011). Upon GTP hydrolysis, the loops change position and are buried within the protein and effector recruitment is terminated, thus ceasing downstream signaling cascades (Cherfils and Zeghouf 2011).
A majority of Ras-related small G-proteins rely on membrane localization for their biological activity, although they do not encode transmembrane spanning domains. Instead localization is most often achieved by post-translational prenylation, palmitoylation, and/or myristoylation. The carboxy terminus of Ras proteins contains two signal sequences that promote their association with lipid membranes (Reuther and Der 2000). The C-terminal CAAX motif (where the C is cysteine, the A is an aliphatic amino acid and the X is variable) is the recognition site for protein prenyltransferases. Following prenylation, the AAX tripeptide is proteolytically cleaved and the C-terminal cysteine is methylated leading to a more hydrophobic protein that displays greater membrane association (Lowy and Willumsen 1993). Specific targeting often requires additional post-translational modifications. For H-Ras and N-Ras there are additional cysteine residues upstream of the prenylation signal that becomes reversibly palmitylated to further increase membrane affinity (Lowy and Willumsen 1993). Arf family proteins lack a C-terminal prenylation site and are instead myristoylated at the N-terminus to promote membrane association (Casey 1994). Lastly, there are several members of the Ras family that lack lipid modification motifs, specifically the Rad/Gem/Kir (RGK) and Rit subfamily of GTPases (Colicelli 2004). Instead, these subfamilies contain extended poly-basic C-terminal regions which promote membrane association through charged interactions with phosphatidylinositol 3,4,5-triphosphate [PI(3,4,5)P$_3$] and phosphatidylinositol 4,5-bisphosphate [PI(4,5)P$_2$] on the plasma membrane (Heo, Inoue et al. 2006). Interestingly, these proteins lacking canonical membrane targeting sequences have also been demonstrated
to migrate in and out of the nucleus (Heo, Inoue et al. 2006). However, it is currently unclear how C-terminal membrane targeting and nuclear localization impact the biological activity of this set of enzymes.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>G5</th>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ric</td>
<td>KLVQGAGGVGVRQALTIQLIQNHVFDYDPTIEDSRKQVVIDGETCILDILTACQEEY</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rin</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Rit</td>
<td>MEWRLFGLATEGFK----RTMDSGT---------RPVGSCCSSPA------GLSKEY</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRas</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
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</tr>
<tr>
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<td></td>
<td></td>
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<tr>
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<tr>
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<tr>
<td>Ric</td>
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<tr>
<td>Rin</td>
<td>QVSTEGGLALQGYEYNCGFETSALRFECDADFGLVREIRKSSMPSM-NAMEKLKR---</td>
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<tr>
<td>Rit</td>
<td>QVTRGEGGLALAREFCGPETSALRFECDADFGLVREIRKSMPSM-NAMEKLKR---</td>
<td></td>
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</table>

**Figure 1.3. Alignment of the Rit subfamily of small GTPases.** Rin and Rit define the Rit subfamily of small GTPases. Consensus G box motifs are highlighted in yellow. NCBI reference sequences are as follows: dRic NM_057863, hRin NM_002930.3, hRit NM_001256821.1, hHRas NM_005343.3
Rit subfamily of GTPases

The Rit subfamily of Ras-related GTPases is comprised of Rit (Ras-like in tissues), Rin (Ras-like in neurons) in vertebrates and the Drosophila ortholog, Ric (Ras-related protein which interacted with calmodulin) (Lee, Della et al. 1996, Wes, Yu et al. 1996, Shao, Kadono-Okuda et al. 1999). Although Rit and Rin share 64% of their amino acid sequence identity, they display variability in their patterns of developmental and tissue expression (Lee, Della et al. 1996). Rit is expressed ubiquitously and throughout development, while Rin is expressed solely in central nervous system neurons and its expression suppressed until embryonic day 13 (Lee, Della et al. 1996, Shao, Kadono-Okuda et al. 1999, Cahoy, Emery et al. 2008, Sharma, Schmitt et al. 2015). Both Rit and Rin have unique biochemical and structural properties that distinguish them from other members of the Ras subfamily (Shi, Cai et al. 2013).

Biochemical Characterization of the Rit subfamily

The Rit subfamily members act as canonical GTPases having high preferential binding affinity for guanine nucleotides and low intrinsic hydrolysis activity (Lee, Della et al. 1996, Shao, Kadono-Okuda et al. 1999). Despite their functional similarity, the Rit subfamily is structurally unique. Although the G box domains of the Rit subfamily are highly conserved, both Rit and Rin have extended N- and C-termini accounting for the proteins’ higher molecular weights of approximately 25kDa, compared to roughly 21kDa for other Ras family members (Figure 1.3). Membrane localization is achieved in both Rit and Rin
through a stretch of polybasic amino acids at the C-terminus (Shao, Kadono-Okuda et al. 1999, Heo, Inoue et al. 2006).

Rit and Rin also share a unique G2 effector domain (HDPTIEDAY) that is distinctive of the Rit subfamily and unique among other GTPases, although most similar to the Ras subfamily with only two amino acid substitutions (YDTIEDSY) (Figure 1.3). To test the hypothesis that these amino acids substitutions would lead to differences in effector binding between the Rit and Ras family, a yeast–two hybrid experiment was performed using either constitutively active or dominant negative Rit and Rin as bait and known Ras effectors as prey (Shao, Kadono-Okuda et al. 1999). In these experiments H-Ras was able to interact with the Ras binding domain of Raf1 (Raf1-RBD), the Ras interaction domain of RalGDS (RalGDS-RID), the Ras interaction domain of Rlf (Rlf-RID), AF6, RIN1, and the p110 catalytic subunit of PIK3 (Shao, Kadono-Okuda et al. 1999). It was demonstrated that Rit and Rin were able to interact with only a subset of the effectors tested (Raf1-RBD, RalGDS-RID, Rlf-RID and AF6) (Shao, Kadono-Okuda et al. 1999). The Drosophila ortholog, Ric, contains an intermediate G2 effector domain containing only one amino acid substitution (HDTIEDSY); however, studies have not been conducted to determine how this may impact effector binding.

Initial biochemical characterization found that both Rit and Rin displayed guanine nucleotide dissociation rates of 5 to 10-times higher than Ras GTPases (Shao, Kadono-Okuda et al. 1999). Given the high concentration of intracellular
GTP compared to GDP, this *in vitro* study suggested a high basal rate of activation for these proteins. However, subsequent affinity isolation of activated (GTP-bound) Rit and Rin from cell culture studies demonstrated only modest levels of GTP bound protein in unstimulated cells, measured through GTP-selective pull-down assays (Shao and Andres 2000, Hoshino and Nakamura 2002). This suggests that the high basal rates of activation seen *in vitro* may result from the C-terminal deletion necessary for bacterial expression. Furthermore, a series of oncogenic Rit mutations that drive transformation have been discovered (Berger, Imielinski et al. 2014, Xu, Sun et al. 2015, Cave, Caye et al. 2016), further supporting the hypothesis that Rit subfamily activity is controlled through the conical GTP/GDP cycle.

Activation of the Rit subfamily has been found to be stimuli dependent. Previous work has indicated that Rit becomes activated following exposure to epidermal growth factor (EGF), nerve growth factor (NGF), neurotropic peptide pituitary adenylate cyclase activated polypeptide 38 (PACAP38), and various cell stressors (TNF-α, DNA damage and oxidative stress) (Spencer, Shao et al. 2002, Shi and Andres 2005, Shi, Rehmann et al. 2006, Lein, Guo et al. 2007, Shi, Jin et al. 2010, Shi, Jin et al. 2011). Using a similar experimental approach, Rin has also been shown to be activated following NGF, EGF, PACAP38, and 12-myristate stimulation (Spencer, Shao et al. 2002, Hoshino and Nakamura 2003, Shi, Han et al. 2005).

**Function of the Rit subfamily in TBI**
A previous graduate student in the laboratory, Dr. Weikang Cai, demonstrated that Rit mediates an evolutionarily conserved p38 MAPK-dependent pro-survival pathway in response to reactive oxygen species (ROS) (Cai, Rudolph et al. 2011, Shi, Jin et al. 2011). Elevated concentrations of ROS is thought to be one of the key mediators of neuronal loss in the setting of TBI (Zink, Szmydynger-Chodobska et al. 2010), leading him to speculate that Rit may function in oxidative stress resistance within neurons. Through his studies, it was found that Rit deficiency sensitized immature neurons and delayed neurogenic recovery within the dentate gyrus of the hippocampus to cell death following TBI, suggesting that Rit functions to promote immature neuronal survival and trauma-mediated hippocampal neurogenesis (Cai, Carlson et al. 2012).

To date, there is no literature accessing the role of Rin in TBI. However, because of the overlap in biochemical properties and activating stimuli between Rin and Rit, along with its neuron specific pattern of expression, we hypothesize that Rin signaling will play an important role following brain injury. Indeed, in vitro studies have demonstrated potential roles for Rin upstream of signaling pathways activated following trauma. These pathways and their regulation by Rin are described below.

**Rin-mediated Signal Transduction Pathways and their Implications in TBI**

The mitogen activated protein kinase (MAPK) signaling cascade is a well-characterized effector pathway known to be regulated by many Ras subfamily proteins. Generically, the pathway consists of a three tiered protein kinase
cascade in which each subsequent kinase is subject to activation by the previous kinase. The most upstream level of the pathway is composed of the MAP kinase kinase kinase (MAPKKK) that activates the MAP kinase kinase (MAPKK), which in turn activates the MAP kinase (MAPK) (Katz, Amit et al. 2007) (Figure 1.4). This three-tiered signaling system is shared by three major pathways named according to their MAPK protein: extracellular signal-related kinases (ERK1/2), p38 MAPK, and Jun amino-terminal kinases (JNK1/2/3) (Robinson and Cobb 1997, Katz, Amit et al. 2007). The ERK1/2 pathway is regulated by growth factor stimulation (Katz, Amit et al. 2007). Although p38 and JNK1/2/3 can be activated by growth factors, however, are more often induced by stimuli such as osmotic stress, UV irradiation, oxidative stress, hypoxia and cytokines (Katz, Amit et al. 2007, Raman, Chen et al. 2007). Rin has been demonstrated to act upstream and regulate the activity of both ERK and p38 pathways (described below).
Figure 1.4. Receptor tyrosine kinase (RTK)-mediated Ras MAPK Cascade.

Extracellular ligand binding causes the RTK to dimerize triggering autophosphorylation of tyrosine residues on the cytoplasmic tail of the RTK. Phosphorylation of specific receptor tyrosine residues allows binding of adaptor proteins, such as Shc and Grb2, through their SH2 domains. These adaptors recruit GEFs such as Sos1, which can activate Ras when brought into proximity of the plasma membrane where Ras is localized. Following GTP binding, Ras activates downstream MAPKKK/MAPKK/MAPK signaling. These signaling proteins are often held in close proximity by scaffolding proteins to allow more efficient signaling. Following phosphorylation, the MAPK protein is able to dimerize and translocate to the nucleus where it activates transcription factors to initiate gene transcription to promote biological processes including cell survival, differentiation, and growth.
**Raf/MEK/ERK MAPK Cascade**

ERK is rapidly activated (within 10 minutes) in experimental models of contusive brain injury (Mori, Wang et al. 2002, Hu, Liu et al. 2004). Activated ERK has been primarily observed in neurons, initially localizing to the axons and at later time points translocating to the cell body (Dash, Mach et al. 2002). Although activation has been demonstrated, the physiological outcome of ERK activation is not well characterized. It has been demonstrated that inhibition of ERK signaling prior to injury leads to worsened behavioral and cognitive outcomes following head injury, suggesting that ERK activation is beneficial (Dash, Mach et al. 2002). However, a separate contusive TBI study found that mice treated with an ERK inhibitor displayed no change in motor function; however, they did have a significantly reduced lesion volume compared to vehicle treated controls, suggesting that ERK plays deleterious roles in TBI (Mori, Wang et al. 2002). In a diffuse head injury model, treatment with S-trans-farnesylthiosalicylic acid (FTS), which disrupts prenylated protein membrane association, diminished ERK activation, and led to decreased neurological defects, once again suggesting diminished ERK activation to be beneficial (Shohami, Yatsiv et al. 2003). Although functional results have varied in these studies, possibly due to differences in the type or severity of injury or differences in cells or regions affected, it has been consistently demonstrated that ERK is activated by mechanical strain (Neary 2005).
Initial yeast-two hybrid analysis indicated interaction between Rin and the Ras binding domain of Raf1 suggesting that Rin may function to regulate ERK signaling (Shao, Kadono-Okuda et al. 1999). Subsequent studies demonstrated that although Rin could bind both Raf1 and B-Raf in a GTP dependent manner, Rin preferentially bound B-Raf (Shi, Han et al. 2005). In agreement with these binding studies, Rin was found to only activate signaling downstream of B-Raf. Importantly, RNAi-mediated Rin silencing was demonstrated to inhibit NGF-induced B-Raf activation in PC6 cells (Shi, Han et al. 2005). Furthermore, expression of constitutively active Rin led to ERK activation, and pretreatment with the MEK inhibitor, PD98059, blocked Rin-mediated ERK activation (Spencer, Shao et al. 2002, Shi, Han et al. 2005). Together these data implicate Rin as an upstream regulator of the B-Raf-MEK1-ERK cascade.

**p38 MAPK Cascade**

The p38 MAPK cascade has been implicated in a multitude of processes within the CNS. Activation of the p38 MAPK cascade has been associated with neuronal death caused by numerous neurotoxic reagents including excitotoxicity (Cao, Semenova et al. 2004, Semenova, Maki-Hokkonen et al. 2007, Chaparro-Huerta, Flores-Soto et al. 2008), nerve injury (Wittmack, Rush et al. 2005), and ischemia (Wang, Xu et al. 2002, Guo and Bhat 2007). Furthermore, rapid activation of p38 has been observed following contusive brain injury (Mori, Wang et al. 2002). Expression of p38 is ubiquitous and the outcomes of its activation vary based on cell type. In microglia, p38α has been demonstrated to be critical
for the production of IL-1β and TNFα (Bachstetter, Xing et al. 2011) and following TBI microglial p38α regulates the acute phase cytokine response and microglial activation (Bachstetter, Rowe et al. 2013). Therefore, the ability of a protein or molecule to regulate p38 function could have broad therapeutic potential.

Studies conducted in cell culture models indicate that Rin plays a role in regulating p38 signaling cascades. RNAi-mediated silencing of Rin blunts p38 activation downstream of NGF stimulation demonstrating that Rin activation is critical in the p38 cascade (Shi, Han et al. 2005). Interestingly, silencing of Rin had no effect on the ability of other stimuli, including EGF, PMA, and calcium ionophore, to activate downstream p38 signaling (Shi, Han et al. 2005) suggesting signaling specificity downstream NGF. It was also shown that Rin co-expression with B-Raf was able to robustly stimulate p38 activation demonstrating a role for B-Raf in Rin-mediated p38 signaling (Shi, Han et al. 2005). Current data suggests that Rin may function more prominently in p38 signaling than upstream of ERK cascades (Shi, Han et al. 2005).

**Regulation of Rho family GTPases**

RhoA, Cdc42, and Rac are members of the Rho subfamily of GTPases (Colicelli 2004). These proteins act as GDP/GTP-dependent binary switches, and have been shown to regulate many aspects of intracellular actin dynamics, controlling critical functions including cell migration, polarity, and mitosis (Jaffe and Hall 2005). In the CNS, the Rho subfamily of GTPases mediates neurite outgrowth, growth cone development, and dendritic arborization (Stankiewicz and
Linseman 2014). More recently the Rho family has been implicated in roles of neuronal survival and neurodegeneration (Stankiewicz and Linseman 2014). Indeed, expression of activated RhoA induces neuronal cell death (Semenova, Maki-Hokkonen et al. 2007) and Rac1 is associated with neuronal survival (Stankiewicz and Linseman 2014). Following TBI, Rac1 and RhoA proteins are activated and relocate to the membrane (Sabirzhanova, Liu et al. 2013). The physiological outcomes of Rho activation and relocation following TBI are unclear. However, effects could range from regeneration to cell death.

Literature indicates that Rin interacts with proteins in the Rho subfamily and has the ability to modulate cytoskeletal dynamics. It was demonstrated that Rin forms complexes with Par6-Rac/Cdc42 in a GTP-dependent manner (Hoshino, Yoshimori et al. 2005) and Rin expression promotes Rac/Cdc42 and RhoA activation (Hoshino and Nakamura 2003) suggesting Rin expression may modulate Rho family physiological functions.

**Rin-mediated Neurite Outgrowth**

The most commonly studied physiological outcome of Rin activation is neuritogenesis, or the ability of a neuron to form cytoskeletal projections from the cell body. Although Rin activation induced neurite outgrowth (Hoshino and Nakamura 2003, Shi, Han et al. 2005), it should be noted that its effect is not robust and has been demonstrated in immortalized cell lines, not within cultured neurons. However, expression of constitutively active Rin induced neuritogenesis in PC6 cells; furthermore, additional expression of B-Raf enhanced the
percentage of neurite bearing cells, the number of neuritis per cell and their branching (Shi, Han et al. 2005). It has been shown that p38 or ERK inhibition reduced neurite outgrowth following expression of constitutively active Rin (Shi, Han et al. 2005) (**Figure 1.5**). These data demonstrate a role for Rin in neuronal differentiation downstream of p38 and ERK. Rin-dependent neurite outgrowth has also been demonstrated downstream of Rho subfamily activation. Indeed Rin-dependent differentiation of PC12 cells downstream of NGF was diminished by the expression of dominant negative Rac/Cdc42, suggesting a role for Rin in calcium-mediated cytoskeletal reorganization upstream of the Rho subfamily of GTPases (Hoshino and Nakamura 2003). Finally, Rin-dependent neurite outgrowth has been demonstrated through interaction with calmodulin (CaM). CaM is a ubiquitous expressed calcium sensing protein for a variety of cellular enzymes; it is able to respond to increases in intracellular calcium concentration and activate a variety of cellular processes including cytoskeletal organization (Rhoads and Friedberg 1997). Rin binds calmodulin (CaM) in a calcium-dependent manner through its C-terminus (193-217 aa), suggesting that Rin may mediate calcium/CaM-modulated signaling pathways (Lee, Della et al. 1996). Indeed, it was found that Rin is able to form a complex with the actin binding protein IQGAP1 and CaM and this interaction was demonstrated to contribute to neurite outgrowth (Hoshino and Nakamura 2003) (**Figure 1.5**).
Figure 1.5 Rin-dependent signaling in neurite outgrowth. Rin signaling has been implicated upstream of ERK, p38, and Cdc42/Rac1 and in each of these cases shown to induce neurite outgrowth in PC6/12 cells.
Rationale for Investigating the Physiological Function of Rin

Rin activation has been demonstrated downstream of a variety of stimuli; however, the physiological function of Rin in the CNS remains poorly characterized. Rin signaling upstream of ERK, p38, and Cdc42/Rac has been shown to contribute to neurite outgrowth (Hoshino and Nakamura 2003, Shi, Han et al. 2005), suggesting a potential role for Rin in plasticity following brain injury. Regardless, these studies were performed in cultured cell models that do not accurately reflect the physiological conditions of neurons. This is particularly problematic considering the expression of Rin is limited to neuronal populations (Lee, Della et al. 1996, Spencer, Shao et al. 2002, Cahoy, Emery et al. 2008, Sharma, Schmitt et al. 2015), raising the question of whether or not these cultured cells express the necessary effector and regulatory proteins required for authentic replication of the function of endogenous Rin signaling. In order to study the function of Rin in an appropriate system, we generated a Rin null mouse line through genetic deletion of $RIT2$, and with this line we characterized the physiological roles of Rin using a number of experimental trauma models. In Chapter Three, we implemented a contusive brain injury model (CCI) to characterize the effects of Rin loss on trauma-induced neurogenesis and cell death. These studies indicate that Rin, unlike Rit, does not function in trauma-induced neurogenesis or the survival of immature neurons within the dentate gyrus of the hippocampus. Rather, this set of experiments demonstrates a neuroprotective effect of Rin loss, as its genetic deletion increases neuronal survival in the hippocampal dentate gyrus and CA-3 subregion. Furthermore, we
were able to correlate neuronal protection with cognitive behavioral sparing. In Chapter 4, we examined the role of Rin in trauma-induced axonal degeneration following optic nerve stretch, an experimental model of white matter tract injury. These studies indicate that the loss of Rin protects cells from axonal degeneration and limits injury-induced inflammation. In Chapter 5, we employed a diffuse closed head injury (CHI) model of TBI to analyze the role of Rin in trauma-induced neuroinflammation. In this model, we show that Rin loss blunts pro-inflammatory IL-1β cytokine production acutely following injury while elevating anti-inflammatory IL-10 production in the cortex. Although we did not observe any observable alterations in microglia or astrocyte activation, there was an appreciable reduction in axonal degeneration in Rin null animals following CHI. These data suggest that the differences observed in cytokine profiles may be a result of reduced neuronal cytokine production. Together, the studies in this dissertation demonstrate broad neuroprotective effects of Rin loss in cell death, axonal degeneration and inflammation and could provide insight into treatment of brain trauma and neurodegenerative disorders.
CHAPTER TWO

Materials and Methods

Reagents

Chapter 3

Reagents: 5-Bromo-2-deoxyuridine (BrdU) (Thermo Fisher Scientific); formalin (Sigma #HT501128); FluoroJade C (FJC); ABC kit (Vector #PK-6100); DAB Peroxidase substrate kit (Vector SK-4100)

Antibodies: rabbit antiNeuN (Millipore #MABN140); rabbit antiKi67 (Abcam #ab15580), rat BrdU (Abcam #ab6326), rabbit anti-Dcx (Abcam #ab18723); rabbit anti-GFAP (Dako #Z0334); goat anti-rabbit IgG TRITC (Jackson ImmunoResearch #111-605-144); goat anti-rat IgG Cy3 (Jackson ImmunoResearch #112-605-167); goat anti-rabbit IgG FITC (Jackson ImmunoResearch #111-545-144); mouse anti-Rit (R0003U) and mouse-anti-Rit2 clone 27G2 were generated in collaboration with Gamma-1 (Lexington, KY).

Chapter 4

Reagents: ABC kit (Vector #PK-6100); DAB Peroxidase substrate kit (Vector SK-4100); formalin (Sigma #HT501128)
Antibodies: mouse anti-Neurofilament H Nonphosphorylated (SMI32) (Calbiochem #NE1023); rat anti-CD68 (BioRad # MCA 1957), biotin donkey anti-rat IgG (Jackson ImmunoResearch #712-065-150); biotin donkey anti-mouse IgG (Jackson ImmunoResearch #715-065-150)

**Chapter 5**

Reagents: Methyl green, zinc chloride salt (VWR); DAB (Sigma); Halt Protease & Phosphatase Cocktail (Pierce #78442); PMSF (Sigma P7626); ABC kit (Vector #PK-6100); Custom mouse cytokine kit (MSD #K152A0H-1)

Antibodies: rabbit anti-GFAP (Dako #Z0334); rat anti-CD45 (Thermo #MA1-81247); rabbit anti-βAPP (Invitrogen #51-2700); biotin goat anti- rabbit IgG (Jackson ImmunoResearch #111-035-144); biotin goat anti-rat IgG (Vector #BA-9401)

**Animals**

Rin\(^{-/-}\) mice were purchased from Texas Institute for Genomic Medicine (mouse accession: NM_009065) (College Station, TX). Rin\(^{WT}\) allele was genotyped with primers 5’-GACACTGCATGAGACTGA ACC - 3’ and 5’-CTATAACCTGTGAGTCAACGGAAA G - 3’ corresponding to the intronic region between exons 3 and 4 resulting in a band of approximately 750 nucleotides. Rin\(^{-/-}\) allele was genotyped with primers 5’ – AAATGGCGTTACTTAAGCTAGCTTG C - 3’ and 5’ - CTATAACCTGTGAGTCAACGGAAAG - 3’ corresponding to the intronic region between exon 3 and 4 and the LTR sequence on the gene trap
insertion resulting in a band of approximately 650 nucleotides. Animals were handled in accordance with standard use protocols and animal welfare regulations of the Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky and the NIH Guide for the Care and Use of Laboratory Animals.

**Traumatic Injury Models**

For all experiments herein, animals were randomly assigned into either injured or sham groups. All cell counts and analyses was conducted by experimenters blinded to both the genotype and experimental treatment of study animals.

**Controlled Cortical Impact (CCI)**

Surgeries were carried out in the laboratory of Kathryn Saatman by Shaun Carlson, Erica Littlejohn, and Travis Stewart and were performed as previously described (Pleasant, Carlson et al. 2011). All CCI injuries were performed on male mice to control for head size and limit injury severity variation. Briefly, isoflurane anesthetized twelve-week-old male mice received a craniotomy over the left hemisphere and were subjected to a unilateral controlled cortical impact (CCI) injury at a 1.0 mm impact depth and a nominal velocity of 3.5 m/s using a CCI impactor device (TBI-0310 Impactor; Precision Systems and Instrumentation). Sham animals underwent identical surgical procedures, but received no impact to the cortex.
**Mono-ocular Nerve Stretch Injury (MONS)**

Surgeries were performed in the laboratory of Kathryn Saatman by Binoy Joseph as previously described (Saatman, Abai et al. 2003). Briefly, 6-8-month-old mice were anesthetized with a 65 mg/kg intraperitoneal injection of sodium pentobarbital and maintained on a heating pad throughout the surgery. The conjunctiva and extraocular muscles were cut around the circumference of the eye and a flexible sling with a longitudinal midline slit was placed under the eye positioning the optic nerve in the slit. The sling was connected to a force transducer (ELF T500-1; Entran, Fairfield, NJ, USA) mounted in a series with a solenoid (Lucas-Ledex, Vandalia, OH, USA) and in parallel with a displacement transducer (Trans-Tek Inc., Ellington, CT, USA). Elongation of the optic nerve by 2.0 mm (extending the nerve by approximately 20%) was obtained by programming of the solenoid and measured by the force transducer.

**Optic Nerve Transection**

Operational procedures were modified from an optic crush protocol (Templeton and Geisert 2012). Six-to-eight-month-old male and female mice were anesthetized via an intraperitoneal injection of 65 mg/kg sodium pentobarbital and placed on a heating pad for surgery. Once deeply anesthetized, the eyelashes and whiskers surrounding the right eye were trimmed and the right cornea was treated with bupivacaine to further numb the surgical area. Under a dissecting scope, an incision was made into the conjunctiva along the globe of the eye being careful to not cut the surrounding...
musculature. The edge of the conjunctiva was held with forceps and rotated nasally bringing the optic nerve into view. Spring scissors were used to cut through the optic nerve 1-3 mm from the base of the eye. The incision was washed with 1.0 ml of sterile saline and the eye lids sutured closed. Mice were maintained on a heating pad until they were fully awake, then monitored daily until euthanasia. The contralateral eye underwent no surgical procedure and was used as the experimental control for transection studies.

**Closed Head Injury (CHI)**

Surgeries were carried out by Adam Bachstetter and were performed as previously described (Bachstetter, Webster et al. 2015). Briefly, 5-6-month-old male and female mice were anesthetized using isoflurane and received a sagittal midline incision of the scalp and received a single midline cortical impact to the skull cap using a 5.0 mm diameter steel tip impounder (Leica Biosystems, Wetzler, Germany) at a controlled velocity of 5.0 ± 0.2 m/s with a dwell time of 100 ms at a programmed impact depth of 1 mm. Sham animals underwent identical surgical procedures, but received no impact to the skull. Animals that displayed hemorrhaging or skull fracture/deformation were excluded from further analysis.

**Rin Immunoblotting**

Whole brain lysates were prepared from wild-type and Rin<sup>−/−</sup> animals using kinase lysis buffer [20 mM Heps (pH 7.4), 150 mM NaCl, 50 mM KF, 50 mM β-glycerolphosphate, 2 mM EGTA (pH8.0), 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100, 10%
glycerol, and 1× protease inhibitor cocktail set I (Calbiochem)]. Total protein concentration of the lysate was determined using a Bradford assay. Equal amounts of wild-type and Rin⁻/⁻ lysates were ran on a SDS-PAGE gels and protein level was determined by immunoblotting with the appropriate antibodies.

**BrdU Administration**

To assess immature neuronal survival, BrdU (50mg/kg in 0.9% saline) was intraperitoneally injected daily for one week as previously described (Cai, Carlson et al. 2012). Following the final injection, mice were housed for 2 days prior to injury to allow a subset of the labeled pool adequate time to differentiation to an immature neuronal lineage.

**Tissue Preparation**

For CCI and MONS, animals were deeply anesthetized with FatalPlus and transcardially perfused with a heparin saline solution (heparin sodium 1:1000, RX grade, APP Pharmaceuticals in 0.9% NaCl) followed by 10% buffered formalin (Sigma). Tissue was post-fixed in the skull for 24 hr in 10% buffered formalin. Following post-fixation, the brains and optic nerves were dissected from the skull and cyroprotected using a 30% sucrose in 1X TBS until tissue sunk. Brains were snap frozen in isopentane (≤ -20°C) for 2 min and cut in a coronal plane at a 40 μm thickness using a freezing sliding microtome (Dolby-Jamison). Optic nerves were frozen in OCT and cut in 10 μm sections using a freezing sliding microtome (Dolby-Jamison). Following CHI, animals were anesthetized with FatalPlus and
transcardially perfused with heparin saline solution (heparin sodium 1:1000, RX grade, APP Pharmaceuticals in 0.9% NaCl) and the brain separated into two hemispheres, one for sectioning and one for protein extraction. The left hemisphere was tissue was post-fixed in 4% PFA for 24 hr before being transferred to 30% sucrose and processed in the same manner as the CCI brains.

**Cresyl Violet Brain Staining**

Serial sections at 400 µm intervals were mounted onto gelatin-coated slides and dried overnight at RT. The following day slides were immersed in ddH₂O for 2 min then dehydrated through an ethanol gradient of 70%, 80%, 95%, and 100% for 2 min at each concentration followed by rehydrated at 100%, 95%, 80%, and 70%. Slide were then incubated in 0.5% cresyl violet for 2.5 min. Slides were once again dehydrated through a fresh EtOH gradient and immersed in xylene for 15 min prior to applying a coverslip with permount.

**Injury Volume Quantification**

To determine the percent of cortex damage following CCI injury, cresyl violet (Nissl) stained serial brain sections, 400 µm apart, were visualized on an Olympus BX-51 light microscope equipped with a CCD camera. Neocortical tissue from bregma level 0 to -3.5 mm was analyzed via live imaging using Bioquant software (version 8.40.20, Bioquant Life Science). Regions containing neurons within the ipsilateral and contralateral neocortices were outlined.
separately at 10X magnification verified through examination of cell body morphology at 2X magnification. Areas of the cortex that contained condensed nuclei were excluded from the “intact” tissue quantification. Contusion area was calculated as the difference between spared contralateral and ipsilateral neocortical areas. The contusion volume was determined using Cavalieri’s principal, integrating over the inter-section distance as previously described (Pleasant, Carlson et al. 2011). Data are represented as the percent contralateral neocortical volume.

**Fluoro-Jade C staining**

Serial brain sections at 400 µm intervals were mounted on gelatin coated slides and dried at 40-45°C for 15 min followed by overnight drying at RT. The following day, sections were washed in 80% (v/v) ethanol with 1% (w/v) NaOH, 70% ethanol and dH$_2$O followed by 10 min incubation in 0.06% (w/v) potassium permanganate. The sections were then washed in dH$_2$O 5 times before incubation in 0.0001% Fluoro-Jade C solution (resuspended in 0.1% acetic acid) followed by 5 more washes in dH$_2$O. Sections were counterstained with Hoechst, dried, cleared with xylene, and coverslipped with Cytoseal. FJC$^+$ cells in three sections (pre-epicenter, epicenter, and post-epicenter) of the hippocampus for each animal were counted on a Nikon Eclipse E600 fluorescence microscope using a 40X objective. Data are expressed as the sum of FJC$^+$ cells over three sections. Representative images were taken on a Nikon CKX31 A1 confocal microscope (20X objective).
Immunofluorescence

Free floating brain sections at 400µm intervals were washed in 1X TBS prior to antigen retrieval using 2N HCl for 1 hr at RT and neutralized with 0.1M borate buffer at RT for 10 min. Following washing with 1X TBS, sections were blocked in blocking buffer (5% normal goat serum in TBS/Triton X-100 (0.1%)) for 30 min at RT. Incubation in primary antibody in blocking buffer occurred overnight at 4°C. Antibody dilutions were: rabbit anti-Dcx: 1:1500; rabbit anti-Ki67 1:1000. On the following day, sections were washed with 1X TBS and incubated in secondary antibody diluted in blocking buffer for 1hr at RT. Secondary antibody dilutions were: goat anti-rabbit IgG FITC: 1:1000; goat anti-mouse IgG Cy5: 1:1000; goat anti-rat IgG Cy3: 1:1000. Sections were then washed in 1X TBS prior to applying the coverslip with SlowFade Gold containing DAPI (Invitrogen).

Labeled cells in the ipsilateral and contralateral inner granular layer (IGL) of the dentate gyrus were summed from three sections (pre-epicenter, epicenter, and post-epicenter at 400 µm intervals) using a Nikon Eclipse E600 fluorescence microscope using a 40X objective. Dentate gyrus volume was estimated by multiplying the area of the DAPI staining within the dentate gyrus (DG) (measured by Image J (NIH) in collected images using a Zeiss Axiovert 200M fluorescence microscope using a 10X objective) and multiplied by the thickness of each section (40 µm). Cell density was then calculated by dividing total cell
counts by the calculated volume of the DG. Representative images were taken on a Nikon CKX31 A1 confocal microscope (20X objective).

**Immunohistochemistry**

Serial brain sections of 400 µm intervals were washed in 1X TBS, blocked in blocking buffer (5% normal donkey serum in TBS/TritonX-100 (0.1%)) before incubation with rabbit anti-NeuN 1:1000 or rabbit anti-GFAP 1:1000 in blocking buffer overnight at 4° C. The following day sections were washed with 1X TBS before incubation in biotinylated donkey anti-rabbit IgG 1:1000 for 1 hr at RT. Sections were washed in 1X TBS and peroxidases were scavenged with 3% (v/v) of 30% H2O2 in a 50/50 mix of methanol and water for 30 min at RT. Following washing, sections were incubated in AB/TBS (Vector Laboratories) solution for 1 hr at RT. Immunostaining was visualized using cobalt-enhanced DAB (Vector Laboratories), mounted, dried overnight, and dehydrated prior to application of a coverslip with Permount media (Fisher).

Prior to application of the coverslip, CHI tissues were counterstained with methyl green to allow analysis by Aperio Image-Scope XT digital slide scanner and Aperio Image-Scope software. Tissue was mounted onto gelatin-coated slides and dried overnight. The following day, tissue mounted slides were incubated in ddH2O for 2 min, then in a 1:4 dilution of methyl green (from 0.5% stock) in 0.1 M sodium acetate pH 4.2. Slides were washed with flowing H2O until water ran clear. Slides were then dipped in 0.5% acetic acid in acetone 15 times
to de-stain the tissue. Tissue then underwent EtOH dehydration prior to application of the coverslip with Permount (Fisher).

**Stereological Counts of Hippocampal CA-3**

Stereological counting of mature neuronal cells from the dorsal hippocampal CA-3 regions was conducted 10 d following CCI injury. Every 5th brain section (200 µm intervals) spanning bregma levels -1.0 mm to -2.2 mm was immunohistochemically labeled for NeuN and visualized with 60X oil immersion objective on an Olympus BX-51 microscope equipped with a motorized stage and stereology software (Bioquant Life Science. V8.40.20; Bioquant Image Analysis, Nashville, TN). Unbiased counting was restricted to the dorsal hippocampus as this is the area most affected by CCI and hippocampal morphology and packing of the ventral CA-3 would require a different set of sampling parameters. The dorsal CA-3 was overlaid with a counting grid (150 x 150 µm²) and neurons counted within the set dissector area (20 x 20 µm²) and the total number of CA-3 neurons estimated by the equation, Σ= total cells counted x 1/ssf x1/ASF x 1/TSF, where ssf is the selection sampling fraction, asf is the area sampling fraction and tsf is the thickness sampling fraction, as previously described (Madathil, Carlson et al. 2013).

**Neurological Severity Score (NSS)**

NSS was performed by Jennifer Brelsfoard in the Spinal Cord and Brain Injury Research Center at the University of Kentucky. Neurological severity score
was performed at 1d, 2d, 3d, and 6d following CCI. Deficits in motor coordination were analyzed as mice crossed beams of 3, 2, 1, and 0.5 cm width and a rod 0.5cm in diameter. Animals were acclimated to the assessment 24 hr prior to injury, being allowed to explore the beams and rod for 30 sec. Following injury, 14 possible points were given for traversing the beams and rod with normal limb movement. For beam traversing, points were deducted for footfalls, hanging upside-down, and unwillingness to traverse (one point each). Falling from the beam resulted in zero points. For the rod, one point was deducted for hanging upside-down and inability to cross and two points for falling off the rod (Schoch, Evans et al. 2012).

**Novel Object Recognition (NOR)**

NOR was performed by Jennifer Brelsfoard in the Spinal Cord and Brain Injury Research Center at the University of Kentucky. Animals were individually acclimated to a testing cage 24 hr prior to injury and allowed to freely explore for 1 hr. Following acclimation, the animals were introduced to two identical objects placed on opposite sides of the cage and allowed to explore for 5 min. Seven days following CCI, animals were returned to the testing cage for 1 hr before reintroduction of two identical objects. Time exploring each object was recorded over a 5 min period. After a 4 hr delay, the animal was returned to the testing cage, where one of the familiar objects had been replaced with a novel object and the time exploring each object was recorded for a 5 min period. Recognition
index is calculated as time spent exploring the novel object divided by total exploration time as previously described (Madathil, Carlson et al. 2013).

**Radial Arm Water Maze (RAWM)**

RAWM testing was performed as previously described with slight modification (Alamed, Wilcock et al. 2006). Animals were tasked with locating a platform submerged approximately 0.5 cm under water in one arm of a pool containing 6 equally sized arms using visual cues surrounding the pool as a guide. The mice were placed in the pool at different starting locations and given 1 min to locate the platform. If a mouse was unable to locate the platform within that time, it was subsequently placed on the platform for 15 sec. Each of two experimental days had 15 trials per mouse. Day 1 had alternating visual (1, 3, 5, 7, 9, 11) and hidden (2, 4, 6, 8, 10, 12-15) trials, whereas day 2 was consisted on all hidden trials. During visual trials, an object was placed on the platform for visualization. Errors were tallied for each time the animal entered the incorrect arm of the maze and higher errors indicated more difficulty with the task. Mice were tracked using the EthoVisionXT system. Graphed block data was quantified by averaging the number of errors per hidden trial. Blocks 1-3 were derived from the first day of training trials 2 & 4; 6, 8 & 10; and 12-15 respectively. Blocks 4-6 are calculated from the second day of testing by averaging trials 1-5, 6-10, and 11-15 respectively.

**DLK Cloning**
DLK cloned within pDONR221 was purchased from the Arizona State University plasmid repository. The DLK insertion was PCR amplified using the following primers: 5’ – ATATGAATTCAATATGGCTTGCCYCCAT - 3’ and 5’ – ATATGC GGCCC GCTCATGGAGGGAGGG - 3’ before being sub-cloned into pCMV-Myc-N using the EcoRI and NotI restriction sites.

**Cell Culture and Transfection**

HEK-293T were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 5% fetal bovine serum (FBS, Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. HEK-293T cells were transfected using Transgin (Apharma) according to the manufacturer’s protocol. Briefly, 1x 10⁶ cells were seeded in a six-well plate 24 hr prior to transfection to yield a 70-80% confluent dish for transfections. Lipid-DNA complexes were prepared mixing 2 µg plasmid DNA with 2 µl Transgin reagent in 100 µl of Opti-MEM (Gibco) and incubated at RT for 20 min. The lipid-DNA complexes were added drop-wise to the cell monolayer after the growth media was replaced with 1 ml Opti-MEM. Cells were incubated with lipid-DNA complexes for 4-6 hrs before the Opti-MEM was replaced with fresh DMEM containing 5% FBS for 48 hrs prior to making lysates.

**Cell Lysate Preparation**
Transfected cells were lysed and harvested with RIPA buffer (40 mM HEPES, 2 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 1% sodium deoxycholate, 1% NP40, 0.1% SDS) and transferred to an eppendorf tube on ice. Lysates were cleared at 14,000 rpm. To prepare total protein lysates, 150 µg of protein lysate was mixed with 4X SDS-PAGE sample buffer (200 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 4% β-mercaptoethanol, 50 mM EDTA, and 0.08% bromophenol blue).

**Co-immunoprecipitation (Co-IP)**

Protein G separose beads were washed with 10X volume of 1X PBS, vortexed, and spun at 5,500 rpm followed by washing with 10X volume of lysis buffer. Beads were resuspended in 200 µl lysis buffer with 4 µg FLAG antibody per IP. Protein lysate (750 µg) was added to the separose/antibody mixture and rotated at 4°C for 2 hrs. Following incubation beads were spun at 5,500 rpm and the supernatant aspirated. Beads were then washed with lysis buffer once, lysis buffer with 500 mM NaCl twice, and lysis buffer twice more. Bound proteins were eluted by 5 min incubation at 100°C in 1X SDS-PAGE sample buffer. Bound protein along with protein lysate (25 µg) and co-immunoprecipitation (1/2 reaction) was ran on a 12% SDS-PAGE gels, transferred onto nitrocellulose membranes and subjected to immunoblotting with indicated antibodies.

**Semi-quantification of Axonal Immunohistochemistry**
Optic nerve immunohistochemistry was performed similarly to immunohistochemistry of brain sections using one 10 µm section of optic nerve per animal. Sections were washed in 1X TBS/TritonX-100 (0.1%), and peroxidases were scavenged with 3% (v/v) of 30% H₂O₂ in a 50/50 mix of methanol and water for 30 min at RT. Following another set of washes, tissue was incubated in blocking buffer (5% normal donkey serum in TBS/TritonX-100 (0.1%)) before incubation with mouse anti-SMI-32 1:1000 or rabbit anti-CD68 1:1000 in blocking buffer overnight at 4°C. The following day sections were washed with 1X TBS/TritonX-100 (0.1%) before incubation in biotinylated donkey anti-rabbit IgG 1:1000 or biotin donkey anti-mouse 1:1000 for 1 hr at RT. Sections were washed in 1X TBS/TritonX-100 (0.1%). Following washing, sections were incubated in AB/TBS (Vector Laboratories) solution for 1 hr at RT. Immunostaining was visualized using cobalt-enhanced DAB (Vector Laboratories), mounted, dried overnight, and dehydrated prior to application of the coverslip with Permount (Fisher).

The entire optic nerve was imaged at 10X magnification in an AX-80 and sequential images were merged to create a montage. Montages were overlaid with a grid subdivided into 20 x 20 µm squares over 3200 µm of the nerve beginning at the optic chiasm junction. Each square was scored for the presence or absence of immunostaining and semi-quantitatively quantified by dividing the number of squares positive for staining by the total grid squares overlaying the nerve. Data are expressed as percent optic nerve grid-squares containing positive staining.
Quantification of Cortical Immunohistochemistry Using Aperio ImageScope

The Aperio ScanScope XT digital slide scanner was used to create a single high-resolution image of the stained slides at a magnification of 20X. For GFAP staining, 200 x 200 μm boxes were positioned along the neocortex using the Aperio ImageScope software and the Aperio-positive pixel algorithm (version 9) was used to quantify positive pixels of specific staining that were then normalized to the total pixel area outlined to account for variation in the size of the region being sampled. For β−APP staining, areas of positive staining were circled using the Aperio ImageScope software and the Aperio nuclear algorithm (version 9) to obtain the number of β−APP-positive axons and the total neocortex was outlined and an area derived using the Aperio-positive pixel algorithm (version 9). β−APP-positive axons were normalized to the total area to obtain the number of β−APP-positive axons/mm².

Tissue Homogenization and Mouse Cytokine ELISA

Following perfusion with PBS, the brain was removed and cut into two hemispheres. The left hemisphere was post-fixed for immunohistochemistry (refer to Tissue Preparation Section), the cortex of the right hemisphere was dissected for ELISA. Lysis buffer (200 mM PMSF, 0.02% Halt and 0.5M EDTA in 1X PBS) was added at a volume of 20X the tissue weight and stored on ice before homogenization for 30 s using an Omni TH homogenizer with plastic tip. Samples were centrifuged at 12,000 X g for 20 min (Beckman Microfuge18) at 4°
C. The supernatant was then aliquoted into a 96 well plate for long-term storage at -80°C. A BCA assay was performed to determine the protein concentration of each sample. Custom Mouse Cytokine kits were purchased from MSD to analyze IL-1β, IL-6, IL-10, and TNF-α per the manufacturer protocol.

**Statistical Analysis**

All data are represented as mean ± SEM. Relevant statistics are described in figure legends and generated using GraphPad Prism (unless otherwise stated). In Chapter 3, numbers of FJC+ cells and volumetric cortical tissue loss were analyzed using Student’s t-test. The density of Dcx+, and Ki67+, BrdU+/Dcx+ neurons in dentate gyrus following CCI, NOR and NeuN+ cells in CA-3 were analyzed using a two-way ANOVA followed by Bonferroni post-hoc when appropriate. NSS was analyzed through non-parametric ANOVA at each time point with Dunn’s comparison when appropriate. The level of statistical significance was set at p < 0.05. Analysis of the optic nerve in Chapter 4 was analyzed via ANOVA followed by Newman-Keuls post hoc test. Closed Head Injury data in Chapter 5, was analyzed by ANOVA followed by Turkey-Kramer post hoc test.
CHAPTER THREE

Genetic Deletion of the Rin GTPase Promotes Neuronal Sparing and Reduces Behavioral Deficits following Traumatic Brain Injury

Introduction

TBI is a progressive disorder, in which the primary injury results in the initiation of a complex cascade of secondary biochemical and metabolic changes leading to cell death and tissue damage that can persist and evolve over a period of hours to weeks following the initial injury (McIntosh, Saatman et al. 1998, Beauchamp, Mutlak et al. 2008, Borgens and Liu-Snyder 2012). The hippocampus, which is critical for retrieval of short-term memory tasks and episodic memory, is particularly vulnerable to the effects of TBI leading to disruption of cognition, learning, and memory (Levin 1998, Kesner 2007, Hunsaker, Lee et al. 2008). Imaging studies have identified a link between post-TBI hippocampal atrophy and the severity of cognitive dysfunction (Bigler, Blatter et al. 1997, Ariza, Serra-Grabulosa et al. 2006). This same correlation is observed in animal models of TBI, suggesting conservation of fundamental injury mechanisms (Hicks, Smith et al. 1993, Fujimoto, Longhi et al. 2004). The controlled cortical impact model (CCI) injury results in deficits in motor function, learning, and memory (Fox and Faden 1998, Levin 1998, Saatman, Feeko et al. 2006). Hippocampal neuronal damage resulting from CCI is predominantly
located in the dentate gyrus and cornu-ammonis-3 (CA-3) subregions of the hippocampus (Dixon, Clifton et al. 1991, Saatman, Feeko et al. 2006).

At a cellular level, cognitive impairment following TBI has not only been correlated with hippocampal cell death, but also disruption of neurogenesis (Rola, Mizumatsu et al. 2006, Cai, Carlson et al. 2012, Carlson, Madathil et al. 2014). Neurogenesis within the dentate gyrus of the hippocampus continually generates new granule neurons that integrate into the circuitry throughout life (Gage 2000). Neurogenesis is a dynamic process, and studies have demonstrated that the levels of neurogenesis can be modulated numerous factors including: age, sex, stress, growth factors, and environmental enrichment (Kempermann, Kuhn et al. 1997, Ming and Song 2005, Ming and Song 2011, Carlson, Madathil et al. 2014). Following TBI, immature (Dcx+) neurons within the hippocampus are particularly vulnerable to secondary injury mechanisms and undergo high levels of apoptosis (Rola, Mizumatsu et al. 2006, Gao, Deng-Bryant et al. 2008). This setback in neurogenesis caused by immature neuronal loss is thought to contribute the TBI-induced cognitive deficits (Yu, Zhang et al. 2008, Blaiss, Yu et al. 2011). Brain injury also induces proliferation of neuronal stem cells in the sub-granular zone of the hippocampus (Dash, Mach et al. 2001, Rola, Mizumatsu et al. 2006). Increases in stem cell proliferation are thought to contribute to enhancement of neurogenesis and allow the slow recovery of immature neuronal numbers back to baseline levels, comparative to sham-injured controls (Rola, Mizumatsu et al. 2006). Establishing molecular
mechanisms that contribute to neuroprotection of hippocampal neurons may provide novel therapeutic strategies.

Together with their associated regulatory and effector protein networks, Ras-related GTPases serve as master regulators of numerous cellular signaling pathways, that contribute to almost every aspect of cellular physiology (Wennerberg, Rossman et al. 2005). Ras family G-proteins are best known for their functions in the regulation of cellular proliferation, differentiation, survival, migration, and fate specification (Colicelli 2004, Wennerberg, Rossman et al. 2005). While the physiological role of many Ras family GTPases is known, there remains a subset whose cellular functions have not been defined (Reuther and Der 2000, Shi, Cai et al. 2013). This is the case for Rin (RIT2), which despite sharing high levels of amino acid identity and significant overlap in known downstream cellular effectors with the closely related Rit (RIT1) GTPase, would appear to have a distinct cellular function as genetic deletion of RIT1 is not complemented by endogenous RIT2 (Lee, Della et al. 1996, Wes, Yu et al. 1996, Shao, Kadono-Okuda et al. 1999). Rit and Rin display distinct patterns of tissue and developmental expression, with Rit expressed throughout development and having a broad tissue distribution, while Rin expression is delayed to later stages of embryonic neuronal development and proteomic data suggests Rin expression is restricted to neuronal lineages (Lee, Della et al. 1996, Spencer, Shao et al. 2002, Cahoy, Emery et al. 2008, Sharma, Schmitt et al. 2015).
Studies in cell lines have demonstrated that Rin is activated by growth factor stimulation, contributes to TrkA-mediated p38 MAP kinase signaling in pheochromocytoma cells, and modulates neurite outgrowth (Hoshino and Nakamura 2002, Hoshino and Nakamura 2003, Shi, Han et al. 2005). However, as many of these studies were performed in cells lacking endogenous Rin expression, it is unclear whether these results accurately reflect the physiological function of Rin (Shi, Cai et al. 2013). We have recently described an unexpected role for Rit signaling in TBI-induced neurogenesis, functioning to promote selective survival for immature hippocampal neurons following contusion brain injury (Cai, Carlson et al. 2012). Because of similarities to Rit signaling, and its distinct neuronal expression, we postulated that Rin would also contribute to functional recovery following TBI. In this chapter, we use a novel transgenic RIT2 knockout mouse model; we show that following severe contusion brain injury, Rin deficiency reduces regional hippocampal neurodegeneration and improves post-traumatic cognitive function.

Results

Genetic Rin loss does not alter brain morphology

Previous studies using cell culture models identified a putative role for Rin in neuronal differentiation and stress signaling (Hoshino and Nakamura 2002, Hoshino and Nakamura 2003, Shi, Han et al. 2005). These studies are limited by the fact that only a few cell lines express endogenous Rin, making it unclear whether the results accurately reflect in vivo Rin function (Shi, Cai et al. 2013). Therefore, to begin to characterize the physiological role of Rin signaling, we
obtained a *RIT2* knockout mouse from the Texas Institute of Genomic Medicine (TIGM) generated using a gene trap insertion strategy (*Figure 3.1A*). Western blot analysis from whole brain lysates was used to demonstrate the loss of endogenous Rin protein in the gene trap line (*Figure 3.1B*). Rin-null mice were born at expected Mendelian ratios and survived to adulthood with no apparent physical or gross morphological abnormalities (data not shown). As available evidence suggests that Rin is expressed solely within the nervous system (Lee, Della et al. 1996, Wes, Yu et al. 1996), we further characterized gross brain anatomy within a Rin null background. Overall brain structure was unaltered and no obvious discrepancies in cell body (Nissl), mature neurons (NeuN), or astrocyte (GFAP) distribution were observed (*Figure 3.1C*). Taken together, these data indicate that Rin function is not essential for early neuronal development or global brain architecture.
**Figure 3.1. Generation and characterization of Rin null-mutant mice.** A. Schematic representation of the Rit2 allele and gene trap VICTR 48 insert utilized to generate Rin\(^{-}\) mice. LTR, viral long terminal repeat; SA, splice acceptor; NEO, neomycin resistance; pA, SV40 poly adenylation sequence; PGK, phosphoglycerate kinase; BTK, first exon of the murine Bruton’s tyrosine kinase gene; arrows indicate frt, flippase recognition targets, within the VICTR 48 insert. B. Western blot of brain lysates from wild-type (WT), Rin\(^{+/-}\) and Rin\(^{-/-}\) mice demonstrating loss of Rin protein at both a short (top panel) and long exposure (second panel) with no alterations to its closest homolog Rit (third panel). C. Representative hemisphere images from naïve wild-type and Rin\(^{-/-}\) mice stained with GFAP, Nissl, and NeuN. Insets from of NeuN staining are included from the cortex and hippocampal CA-1. No gross alterations in brain morphology or development were noted due to global Rin loss.
Rin deficiency promotes hippocampal cell survival

CCI injury results in robust cortical tissue loss at the injury site together with acute degeneration of the underlying hippocampal structure that can be analyzed using Fluoro-jade C (FJC) (Smith, Soares et al. 1995, Hall, Sullivan et al. 2005, Rola, Mizumatsu et al. 2006, Gao, Deng-Bryant et al. 2008, Pleasant, Carlson et al. 2011). To analyze the in vivo effect of Rin deficiency on neurodegeneration following brain injury, we performed controlled cortical impact (CCI) injury on cohorts of wild-type and Rin⁻/⁻ mice. CCI resulted in hippocampal degeneration concentrated primarily within the dentate gyrus regardless of genotype. The volume of neocortical damage was equivalent for wild-type (13 ±1%; n=4) and Rin⁻/⁻ animals (13 ± 2%; n=6) (t-test, p=0.94) 48 hr following moderate CCI. (Figure 3.2A). However, Rin⁻/⁻ mice displayed significantly diminished numbers of FJC-positive cells (WT 155 ± 13 cells n=5; Rin⁻/⁻ 115 ± 12 cells, n=7, t-test p<0.05) (Figure 3.3 A, C). Sub-regional quantification revealed that FJC-positive neurons trended downward in both the inner granular layer (IGL), the location where immature neurons and neural stem cells reside, as well as in the outer granular layer (OGL) of the dentate gyrus. Although fewer FJC-positive cells were observed in the CA1 or CA3 sub-regions, Rin loss was shown to significantly attenuate CA3 cell death following CCI (WT 30 ± 4 cells n=5, Rin⁻/⁻ 16 ± 3 cells, n=7; t-test p<0.05) (Figure 3.3A). FJC staining was not observed in the hippocampus of sham-operated mice regardless of genotype.
Figure 3.2. Cortical contusion volume changes equivalent in wild-type and Rin$^{-/-}$ mice following contusive brain injury. A. Representative images of Nissl stained brains demonstrating relative cortical loss in wild-type and Rin$^{-/-}$ animals 48 hr following CCI. B. Quantification of contralateral cortical volume compared to the ipsilateral cortical volume demonstrates equal cortical damage in wild-type and Rin$^{-/-}$ animals following head injury.
Figure 3.3. Rin loss attenuated trauma-induced hippocampal neurodegeneration. A. the dentate gyrus (DG), CA-1, and CA-3 regions of the hippocampus and B. the outer granular layer (OGL) and inner granular layer (IGL) suggest decreased degeneration within the dentate gyrus and CA-3 in the absence of Rin. Data are presented as mean ± SEM (wild-type n= 5, Rin⁻/⁻ n=7; t-test * p<0.05) C. Representative confocal images of the ipsilateral dentate gyrus for wild-type and Rin⁻/⁻ mice 48hrs following CCI co-stained with FJC (green) and DAPI (blue).
**Rin deficiency does not alter the survival of newborn neurons in the hippocampus following CCI**

Immature neuronal numbers within the subgranular layer are reduced acutely following CCI (Rola, Mizumatsu et al. 2006, Gao, Deng-Bryant et al. 2008); however, enhanced post-injury neurogenesis ensures the recovery of newborn neuronal numbers in the weeks following CCI (Rola, Mizumatsu et al. 2006, Yu, Zhang et al. 2008, Carlson, Madathil et al. 2014). Because protection was seen within the dentate gyrus of brain-injured Rin⁻/⁻ mice, we next asked whether Rin might contribute to the regulation of post-traumatic hippocampal neurogenesis. BrdU/Dcx co-labeling detected equivalent numbers of newborn immature neurons in naïve wild-type and Rin⁻/⁻ mice 48h after the last of seven daily BrdU injections, indicating that basal neurogenesis is unaffected by Rin deficiency (Figure 3.4). However, concussive injury resulted in a marked decrease of BrdU⁺/Dcx⁺ neurons in the ipsilateral dentate gyrus in wild-type mice (27.4% reduction relative to shams 48hr post-CCI) (Figure 3.4B). An equivalent reduction was observed in the ipsilateral dentate gyrus of Rin⁻/⁻ mice (32.8% reduction relative to sham) (Figure 3.4B). As expected, neuronal loss was confined to the injured hemisphere and no alterations in contralateral BrdU⁺/Dcx⁺ cell numbers were observed regardless of genotype (Figure 3.4C).
Figure 3.4. Rin loss had no effect on newborn neuron survival following contusion injury. A. Representative confocal images at of wild-type and Rin^-/- mice labeled with Dcx (green), BrdU (red), and DAPI (blue). BrdU (50mg/kg) was injected for 1 week prior to CCI or sham injury. The insets are at 5X higher magnification; the arrowheads indicate BrdU/Dcx double positive cells. B. Quantification of double-positive Dcx/BrdU cells 48hrs post-CCI in the ipsilateral hemisphere show a main effect of injury [F(1,29)= 14.67, p<0.001] but not genotype [F (1,29)=0.21, p=0.652] or interaction between genotype and injury [F (1,29)=0.24, p=0.628] C. Quantification of double-positive Dcx/BrdU cells 48 hrs post-CCI in the contralateral hemisphere show no main effect of injury [F(1,29)= 0.11, p=0.745], genotype [F (1,29)=0.30, p=0.589] or interaction between genotype and injury [F (1,29)=0.01, p=0.924]. These data demonstrate that Rin loss has no effect on immature neuronal cell death 48hr following TBI. Data expressed as mean ± SEM (WT sham n=6, WT CCI n=8, Rin^-/- sham n=7, Rin^-/- CCI n=12).
Acute post-traumatic loss of immature neurons is followed by the stimulation of neural progenitor cells, leading to increased hippocampal neurogenesis and the return of immature (Dcx+) cell numbers to pre-injury levels (Weinstein, Burrola et al. 1996, Gould and Tanapat 1997, Magavi, Leavitt et al. 2000, Kernie, Erwin et al. 2001). To determine whether Rin loss altered progenitor cell proliferation in response to brain injury, we quantified cells within the inner granular layer of the dentate gyrus actively in the cell cycle (Ki67+) (Figure 3.5A). Wild-type and Rin<sup>−/−</sup> animals displayed similar increases in Ki67<sup>+</sup> cells 48hr following trauma (WT 475%, Rin<sup>−/−</sup> 434%, increase compared to sham) (Figure 3.5B). As expected, there was no TBI-induced increase in proliferation contralateral to the injury (Figure 3.5C).

At 10 d post-CCI, total immature neuronal numbers (Dcx<sup>+</sup> cells) in the ipsilateral dentate gyrus of wild-type mice had returned to sham levels (WT sham 17 ±1.0 x10³/mm³ cells, n= 6; WT CCI 19 ±2 x10³/mm³ cells, n=8) (Figure 3.6A, B). Rin<sup>−/−</sup> mice displayed a similar trend (Rin<sup>−/−</sup> sham, 16118 ±731 cells, n= 7; Rin<sup>−/−</sup> CCI, 21258 ±1110 cells, n=7) (Figure 3.6B, C). In keeping with these results, immature neuronal recovery (percent ipsilateral/contralateral Dcx<sup>+</sup> cells) was similar in wild-type and Rin<sup>−/−</sup> mice (Figure 3.6D). Taken together, these data suggest that Rin function is not required for post-traumatic induction of neurogenesis.
Figure 3.5. Rin deficiency does not alter TBI-induced proliferation in the inner granular layer of the dentate gyrus. A. Representative confocal images of wild-type and Rin⁻/⁻ mice labeled with Ki67 (red), and DAPI (blue). B. Quantification of Ki67⁺ cells 48hrs post-CCI in the ipsilateral hemisphere show a main effect of injury [F(1,15)= 135.67, p<0.0001] but not genotype [F (1,15)=0.98, p=0.338] or interaction between genotype and injury [F (1,15)=0.199, p=1.79] C. Quantification of Ki67⁺ cells 48 hrs post-CCI in the contralateral hemisphere show no main effect of injury [F(1,15)= 4.08, p=0.062], genotype [F (1,15)=0.16, p=0.698] or interaction between genotype and injury [F (1,15)=0.38, p=0.548]. Data are expressed as mean ± SEM (WT sham n=3, WT CCI n=5, Rin⁻/⁻ sham n=6, Rin⁻/⁻ CCI n=6)
Figure 3.6. Rin loss did not alter the recovery of immature neuron density following controlled cortical impact

A. Representative confocal images of wild-type and Rin⁻/⁻ mice labeled with Dcx (green) and DAPI (blue) 10 d following CCI. B. Quantification of Dcx⁺ cells 10 d post-CCI in the ipsilateral hemisphere show a main effect of injury [F(1,24)= 4.93, p<0.0361] but not genotype [F (1,24)=0.02, p=0.8789] or interaction between genotype and injury [F (1,24)=1.45, p=0.2339]. C. Quantification of Dcx⁺ cells 10 d post-CCI in the contralateral hemisphere show no main effect of injury [F(1,24)= 2.53, p=0.126], genotype [F (1,24)=0.08, p=0.779] or interaction between genotype and injury [F (1,24)=0.03, p=0.854]. D. Recovery of immature neurons following CCI, quantified as a percentage of Dcx⁺ cells on the ipsilateral dentate gyrus compared to the contralateral side shows no change in recovery in the Rin⁻/⁻ animals. Data are expressed as mean ± SEM (wild-type sham n=6, WT CCI n=8, Rin⁻/⁻ sham n=7, Rin⁻/⁻ CCI n=7).
Rin deficiency promotes hippocampal CA-3 neuronal survival

To determine whether neuronal protection was sustained in Rin<sup>-/-</sup> mice, we examined mature neuronal survival 10d following CCI within the CA-3 pyramidal layer and dentate hilus (Figure 3.7). Stereological counts demonstrate that the numbers of NeuN<sup>+</sup> cells within the CA-3 (59.9% neuron loss relative to sham) and hilus (64.7% neuron loss relative to sham) were markedly decreased in wild-type injured animals (Figure 3.7B, C). While the number of NeuN-positive neurons was decreased in Rin<sup>-/-</sup> mice following injury, NeuN<sup>+</sup> cell reduction was significantly blunted in both the CA-3 (31.8% neuron loss relative to sham) and hilus (50% neuron loss relative to sham) (Figure 3.7B, C).
Figure 3.7. Rin loss promotes hippocampal neuronal survival following brain injury. **A** Representative NeuN images of the ipsilateral hippocampus at 10d following severe CCI. **B.** Quantification of stereological estimates of total mature neuronal cells in the dorsal ipsilateral hippocampus display neuronal protection within the CA-3 10d after CCI [main effect of injury, $F(1,16)=102.03$, $p<0.0001$; main effect of genotype, $F(1,16)=13.39$, $p=0.002$; Interaction of genotype and injury $F(1,16)=8.63$, $p=0.0097$; two-way ANOVA followed by Bonferroni post-hoc, *p<0.05]. Data are expressed as mean ±SEM (WT sham n=5, WT CCI n=6, Rin$^{-/-}$ sham n=4, Rin$^{-/-}$ CCI n=5). **C.** NeuN counts within the ipsilateral hilus of the dentate gyrus display hilar protection. [main effect of injury, $F(1,16)=170.12$, $p<0.0001$; main effect of genotype, $F(1,16)=6.13$, $p=0.025$; Interaction of genotype and injury $F(1,16)=4.34$, $p=0.0535$; two-way ANOVA followed by Bonferroni post-hoc, *p<0.05). Data are expressed as mean±SEM (WT sham n=5, WT CCI n=6, Rin$^{-/-}$ sham n=4, Rin$^{-/-}$ CCI n=5).
Rin loss reduces post-CCI behavioral dysfunction

To determine whether the neuroprotection afforded by Rin loss correlated with behavioral improvements following CCI, we evaluated motor function using a modified neurological severity score (NSS) assay and hippocampal-mediated cognitive performance through a novel object recognition (NOR) task (Schoch, Evans et al. 2012, Madathil, Carlson et al. 2013). Both wild-type and Rin\(^{−/−}\) sham-injured animals performed equivalently suggesting no baseline motor or cognitive defects (Figure 3.8). Brain injured cohorts displayed significant loss of motor coordination regardless of genotype as indicated by low NSS scores in the days following injury, which were equivalent at each time point assessed (Figure 3.8A). Cognitive evaluation demonstrated significant impairment in wild-type mice following severe CCI as indicated by a recognition index of 51.6 ± 5.0%, which indicates no preference for the novel object over a familiar one (Figure 3.8B). Rin\(^{−/−}\) brain-injured mice, however, displayed cognitive sparing as indicated by a recognition index of 66.3 ± 2.7%, a score more closely resembling sham groups (WT sham 72.4 ± 2.4%, n=10; Rin\(^{−/−}\) sham 72.6 ±1.8%, n=8) (Figure 3.8B). These data suggest that Rin loss led to improved memory of the familiar object.
Figure 3.8. Rin loss reduced cognitive impairment after CCI A. Neurological severity score, derived from a series of beam walking tasks, showed no difference in motor function at 24 hr to 6 d following CCI. Data are expressed as mean ± SEM (Non-parametric Kruskal-Wallis test; WT sham n=10, WT CCI n=10, Rin<sup>−/−</sup> sham n=9, Rin<sup>−/−</sup> CCI n=8) B. Novel object recognition cognitive paradigm in wild-type and Rin<sup>−/−</sup> mice suggest enhanced cognition of Rin<sup>−/−</sup> mice at 7 d after severe CCI injury [main effect of injury, F(1,32)= 15.6, p<0.001; main effect of genotype, F (1,32)=4.64, p=0.039; Interaction of genotype and injury F (1,32)=4.43, p=0.043; two-way ANOVA followed by Bonferroni post-hoc, *p<0.05]. Recognition index is represented as the percentage of time exploring the novel object; the dotted line represents a recognition index of 50%; data represented as mean ± SEM (WT sham n=10, WT CCI n=10, Rin<sup>−/−</sup> sham n=8, Rin<sup>−/−</sup> CCI n=8.
Discussion

Here for the first time, we implicate Rin GTPase function in the regulation of neuronal cell loss following TBI. Because the hippocampus has been described as particularly vulnerable to contusive brain injury (CCI) (Levin 1998, Rola, Mizumatsu et al. 2006) we focused our characterization on post-traumatic neuroprotection within this structure. Employing a novel transgenic RIT2 knockout mouse model, we demonstrate that Rin deficiency promotes hippocampal neuronal protection, in addition to improving cognitive function following severe brain injury. However, Rin loss does not affect post-injury hippocampal neurogenesis, suggesting a key role for Rin in the regulation of mature neuron cell loss following TBI.

Previous work from this lab has identified important roles for Rit GTPase signaling in the regulation of both insulin-like growth factor-1 (IGF-1) and trauma-induced neurogenesis (Mir, Cai et al. 2017, Mir, Cai et al. 2017). $RIT1^{-/-}$ transgenic mice exhibit an increased loss of adult-born immature hippocampal neurons after severe brain injury (Cai, Carlson et al. 2012). Furthermore, neuronal-selective expression of constitutively activated Rit has been demonstrated to increase levels of basal neurogenesis in the subgranular zone of the hippocampal dentate gyrus (Mir, Cai et al. 2017). Additionally, its loss promotes neuronal death in response to oxidative stress (Cai, Carlson et al. 2012), thus demonstrating a role for Rit in the regulation of activity-dependent plasticity and general neuronal survival. The finding that Rin deficiency had no
effect on injury-induced neurogenesis or immature neuronal survival demonstrates that Rit and Rin have distinct physiological roles in the hippocampus. Divergent functions of these closely related GTPases was not entirely unforeseen given that a constitutively active mutation of Rin failed to promote transformation of NIH-3T3 (Rusyn, Reynolds et al. 2000), and that RIT1 deletion results in a neuronal phenotype that cannot be complemented by RIT2 or any other Ras family GTPase (Cai, Rudolph et al. 2011). What is surprising is that our studies suggest that while Rit serves as a critical regulator of an important survival mechanism for cells in response to stress (Cai, Rudolph et al. 2011, Shi, Cai et al. 2013), Rin would appear to play diametrically opposite role in neurons, promoting neuronal loss in response to injury. Further studies are needed to decipher the molecular underpinnings that confer independent roles for Rin and Rit in the CNS.

Post-trauma restorative neurogenesis and neuronal survival within the hippocampus have been reported to be important mediators of post-traumatic outcomes (Levin 1998, Madsen, Kristjansen et al. 2003, Kesner 2007, Hunsaker, Lee et al. 2008, Jessberger, Clark et al. 2009, Blaiss, Yu et al. 2011). The finding that Rin loss promotes neuroprotection in the CA-3 and hilar sub-regions of the hippocampus 10 d post-CCI (Figure 3.6), without altering neurogenesis (Figure 3.3, 3.4, 3.5), supports a selective role for endogenous Rin in the regulation of molecular cascades that promote neuronal loss following brain-injury. Furthermore, these data suggest that enhanced mature neuron survival is the primary mechanism for cognitive sparing seen in our Rin−/− transgenic model.
Studies are underway to determine whether Rin silencing serves to decrease cell loss following brain injury.

Interestingly, although Rin loss was not shown to alter immature neuronal numbers within the hippocampus following injury, there does appear to be a reduction in ectopic migration of Dcx+ neurons in the granular layer (Figure 3.5). Ectopic migration of Dcx+ cells has been reported at 7 d in models of focal TBI (Shapiro 2017); however, the consequences of aberrant hippocampal cell migration are not completely characterized in models of TBI. Epilepsy, however, is characterized by abnormal integration of hippocampal dentate granule cells (Hester and Danzer 2014). Inappropriate axonal contacts of irregularly migrating cells have been demonstrated to form dysregulated recurrent excitatory loops, and it is believed that abnormal neurogenesis may be epilepyogenic (Hester and Danzer 2014). Closer examination of Dcx+ migration in Rin+/− mice is needed to elucidate the potential role for Rin in immature neuronal migration. However, limiting ectopic cell migration following injury may also be an avenue of protection in the absence of Rin.

Furthermore, we have demonstrated that Rin deficiency leads to cognitive behavioral sparing following severe contusion injury, but does not improve coordinated motor function. Improved cognitive function is consistent neuronal sparing observed throughout the hippocampus (Figures 3.3, 3.7B), a critical structure for many aspects of learning and memory. Motor movements are greatly controlled within the cortex, and it has been previously noted that the
degree of motor impairment following injury is correlated with cortical injury volume (Tsenter, Beni-Adani et al. 2008). In these studies, wildtype and Rin null mice displayed equivalent cortical loss following injury (Figure 3.2); therefore, it is not surprising that Rin loss did not lead to motor improvements (Figure 3.7A). The question of why Rin loss selectively protects hippocampal but not cortical neurons remains to be answered.

The notion that endogenous Rin is involved in the regulation of molecular cascades that control neuronal loss following injury is striking given that many of Ras subfamily GTPases are known to function in cell survival (Colicelli 2004, Wennerberg, Rossman et al. 2005). Nonetheless, Ras GTPase-regulated MAPK cascade activation has been found to regulate cell death and autophagy pathways that in turn play central roles in neuronal survival signaling (Donninger, Calvisi et al. 2015, Cook, Stuart et al. 2017, Zhang, Zheng et al. 2017). ERK, JNK, and p38 MAPK signaling cascades have been described as mediators of cell death following focal ischemic injury and their inhibition shown to improve outcomes following injury (Sugino, Nozaki et al. 2000, Nozaki, Nishimura et al. 2001, Yang, Gu et al. 2017). Interestingly, ERK activation has been demonstrated to have a pro-apoptotic role in mature neurons; however, ERK phosphorylation is not observed during immature neuronal death (Lesuisse and Martin 2002). Previous studies from the lab demonstrate a role for Rin in ERK activation (Shi, Han et al. 2005). Furthermore, studies have identified a prominent role for Rin in p38 stress kinase signaling, as RNAi-mediated Rin silencing was found to disrupt p38 activation downstream of nerve growth factor
in pheochromacytoma cells (Shi, Han et al. 2005). Further studies are needed to characterize the activation of MAPK signaling cascades in our transgenic Rin null mice following contusive brain injury model. Studies are underway to determine whether expression of a constitutively active Rin mutant is able to modulate neuronal MAPK signaling.

In this chapter, we have identified a novel role of Rin following severe focal contusion by demonstrating increased hippocampal neuroprotection and improved cognition following genetic silencing of Rin. These results suggest a role for Rin signaling cascades leading to cell death following severe focal injury and suggest Rin as a possible therapeutic target following TBI. The severe nature of the CCI injury model makes it difficult to parse out the contributions of Rin to specific secondary injuries following TBI. Therefore, in the chapters to follow, we have employed alternative experimental injury models that allow us to analyze the role of Rin in axonal degeneration along white matter tracts and neuroinflammation.
CHAPTER FOUR

Rin Contributes to Trauma-induced Axonal Injury

Introduction

Traumatic axonal injury (TAI) is a common feature of diffuse TBI, generally resulting from high-velocity rotational forces that result in stretching and shearing of axons, and although severe injury can lead to primary axotomy, more often the injury manifests as a interruption of protein transport and breakdown of cytoskeletal elements (Hill, Coleman et al. 2016). These events activate secondary injury cascades which, if not resolved, leads to disconnection of the distal axon at the site of injury and its eventual degeneration (Hill, Coleman et al. 2016). Disruption of white matter tract integrity contributes to cognitive and motor impairments observed following TBI, and white matter degeneration has been shown to persist years following the initial insult (Johnson, Stewart et al. 2013). The key histological components of TAI include retraction bulbs and axonal swellings that result from a breakdown of microtubules and an accumulation of proteins in the axon due to impaired protein transport (Tang-Schomer, Johnson et al. 2012). Although disruption of the axonal cytoskeletal structure and transport can lead to neuronal cell death, this is not always the case. Recent research suggests that, over time, diffuse axonal damage can be corrected through
reorganization and repair of the proximal axon following disconnection (Wang, Fox et al. 2013).

Primary axotomy is generally considered to be an uncommon feature of TAI unless the injury was the result of by direct tissue injury, such as laceration (Christman, Grady et al. 1994). In these cases, the axon is divided into two parts: a proximal portion that remains attached to the cell body and a distal stump that has lost its connection to the nucleus. Eventually, the distal stump will inevitably undergo degeneration. Historically it was believed that the wasting away of the distal axon was a passive process, or a consequence of separation from the cell body and occurred when the axon lost essential proteins that could no longer be synthesized (Gerdts, Summers et al. 2016). However, the discovery of the Wallerian degeneration slow (\textit{Wld}^s) mutant mouse challenged this dogma (Lunn, Perry et al. 1989, Glass, Brushart et al. 1993). It was found that in this mutant, arising from a fusion between a metabolic cofactor, nicotinamide mononucleotide adenyllyl transferase 1 (NMNAT1), and an ubiquitin ligase, ubiquitination factor e4b (Ube4b), axonal degradation following axonal transection was delayed for up to three weeks (Wang, Medress et al. 2012, Gerdts, Summers et al. 2016). Although the protective mechanism of the \textit{Wld}^s chimera is still unclear, it has been demonstrated that increased NMNAT1 activity and NAD\(^+\) levels contribute to axonal protection following injury (Araki, Sasaki et al. 2004). Indeed, manipulations that increase axonal expression of NMNAT1 (Sasaki, Vohra et al. 2009, Babetto, Beirowski et al. 2010) or direct transduction of NMAMT into severed axons acutely following injury (Sasaki and Milbrandt 2010) have been
shown to confer axonal protection. Following TBI, \textit{Wld} \textsuperscript{s} mutant mice display decreased axonal injury and delayed cognitive and motor impairment (Fox and Faden 1998) suggesting a potential therapeutic outlet against the detrimental outcomes of TAI once the molecular mechanisms of the chimera are understood.

Nevertheless, the \textit{Wld} \textsuperscript{s} mouse encodes a gain-of-function mutation and, in itself, does not reveal the presence of a pro-degenerative program; however if degenerative cascades exist, then loss of these signaling components should provide protection from axonal degradation (Gerdts, Summers et al. 2016). The first protein identified as a candidate within a conserved axonal pro-degenerative pathway was dual leucine zipper kinase (DLK), a mitogen-activated protein kinase kinase kinase (MAPKKK) (Miller, Press et al. 2009). Genetic deletion of DLK has been demonstrated to transiently delay axonal degeneration of the sciatic nerve following injury, slowing Wallerian degeneration (Miller, Press et al. 2009). Since its identification, DLK has been shown to control a multitude of seemingly opposing neuronal processes including not only Wallerian degeneration and cell death, but also axonal regeneration and growth {reviewed in (Tedeschi and Bradke 2013)}. However, the regulatory proteins and effectors that allow for DLK to function in apparently contradictory roles are not completely characterized.

Early biochemical analysis shows that overexpression of DLK activates the stress-activated protein kinases (SAPK), JNK, and p38; signaling through these pathways is largely involved in responses to cellular stressors, such as
osmotic stress, UV irradiation, hypoxia, oxidative stress, and pro-inflammatory cytokines, and SAPK activation often leads to cell death (Fan, Merritt et al. 1996, Tibbles and Woodgett 1999). Ras family GTPases are known regulators of p38 and JNK signaling pathways (Robinson and Cobb 1997), suggesting that a Ras family GTPase may lie upstream of DLK. However, one has not been described to date. Rin GTPases have been found to activate p38 stress kinase signaling (Shi, Han et al. 2005), and their loss promotes neuronal survival (Chapter 3), indicating that Rin signaling promotes cell death. These observations suggest that Rin may be able to bind and activate DLK.

My previous research has indicated that the loss of Rin protects mature neurons from death following contusive brain injury. Furthermore, protection provided by Rin loss was shown to occur at particularly high levels within the CA-3 region of the hippocampus following contusive TBI (Chapter 3). Because neurons within the hippocampal CA-3 region are suggested to undergo a high degree of stretch following CCI injury (Mao, Elkin et al. 2013) we hypothesized Rin loss diminished axonal injury leading to neuronal protection. Herein, we test the hypothesis that Rin signals through a pro-degenerative cascade, with the implication being that genetic deletion of RIT2 will result in decreased axonal degeneration following trauma. To test this hypothesis, we employed both an optic nerve stretch injury model to produce axonal degeneration across the optic nerve to investigate the role of Rin in axonal breakdown (Saatman, Abai et al. 2003, Serbest, Burkhardt et al. 2007), and an optic nerve transection model to investigate the role of Rin in Wallerian degeneration.
Results

**Rin loss mitigates trauma-induced axonal degeneration following stretch injury**

To test the hypothesis that Rin loss would protect neurons from traumatic axonal injury, we performed optic nerve stretch on cohorts of wild-type and Rin^−/− mice to generate an isolated white matter tract injury along the optic nerve (Smith 2000, Saatman, Abai et al. 2003). The optic nerve stretch model allows analysis of isolated trauma-induced axonal damage circumventing difficulties of targeting white matter tracts in the brain, which has a more complex anatomy (Saatman, Abai et al. 2003). In this model, rapid unilateral stretch of retinal ganglion axons causes damage to the elements of the axonal cytoskeleton, the major component of which are neurofilaments (Smith 2000). Following stretch, the region of the optic nerve spanning between the globe of the eye and optic chiasm was examined for injury using SMI-32, a marker of dephosphorylated neurofilament, a well-established marker of axonal/neurofilament damage (Christman, Grady et al. 1994). At 24hrs following injury, minute accumulation of SMI-32 can be observed throughout the optic nerve of injured axons in both wild-type and Rin^−/− animals (Figure 4.1A) suggesting equivalent intensities of injury in each genotype. However, levels were not significantly higher than sham controls, consistent with the progressive nature of this injury (Saatman, Abai et al. 2003) (Figure 4.1B).

By 7d post-stretch injury, degeneration progressed along the optic nerve, exhibited by an increase of SMI-32 positive immunostaining in injured wild-type
animals compared to wild-type sham controls (p<0.001 one-way ANOVA followed by Newman-Keuls post-hoc test) (Figure 4.2). Rin null sham and injured mice displayed equivalent levels of SMI-32 accumulation along the optic nerve suggesting that Rin loss blocks the progression of axonal injury. This finding is further illustrated by a significant decrease of SMI-32 staining in Rin−/− mice compared to wild-type following injury (p<0.05 one-way ANOVA followed by Newman-Keuls post-hoc test) (Figure 4.2B). Taken together these data suggest that although both wild-type and Rin null animals displayed some degree of acute injury, Rin loss reduces the progression of degeneration.
Figure 4.1. Rin loss does not alter early axonal degeneration 24 hr following optic stretch injury. A. Representative images of dephosphorylated neurofilament (SMI-32) in the optic nerve 24 hrs following optic stretch injury in wild-type and Rin<sup>-/-</sup> animals. B. Quantification of SMI-32 in the optic nerve following injury indicates that early degeneration between wild-type and Rin<sup>-/-</sup> animals are comparable 24 hr following stretch injury. Data are expressed as mean ± SEM (wild-type n=6-12, Rin<sup>-/-</sup> n=5-11; ANOVA followed by Newman-Keuls post hoc test p>0.05.
Figure 4.2. Rin loss blunts axonal degeneration 7 d following optic stretch injury. A. Representative images of dephosphorylated neurofilament (SMI-32) in the optic nerve 7 d following optic stretch injury in wild-type and Rin<sup>-/-</sup> animals. B. Quantification of SMI-32 in the optic nerve following injury indicates that Rin loss blunts the progression of axonal damage following stretch injury. Data are expressed as mean ± SEM (wild-type n=6-12, Rin<sup>-/-</sup> n=5-11; ANOVA followed by Newman-Keuls post hoc test *p<0.05.
Rin loss diminishes trauma-induced microglia activation following optic stretch injury

Because Rin null mice displayed decreased axonal degeneration following stretch, we hypothesized that microglial activation would also be blunted. Post-injury inflammation was analyzed using immunohistochemical detection of cluster of differentiation 68 (CD-68), a marker for activated microglia. At 24hrs following injury, minimum accumulation of CD-68 was detected throughout the optic nerves of both wild-type and Rin<sup>-/-</sup> stretch-injured mice (Figure 4.3A) in agreement with the minute degree of axonal injury observed via SMI-32 labeling. Once again, there was no statistical difference between sham and injured cohorts (Figure 4.3B).

By 7d post-stretch robust microglial activation was observed along the optic nerve demonstrated by an increase of CD-68 positive immunostaining in the injured wild-type cohort compared to wild-type sham controls (p<0.01 ANOVA followed by Newman-Keuls post-hoc test) (Figure 4.4). However, Rin<sup>-/-</sup> sham and stretch-injured mice displayed equivalent levels of microglial activation along the optic nerve. This finding is further illustrated by a significant decrease in microglial activation in Rin<sup>-/-</sup> mice compared to wild-type cohorts following injury (p<0.05 ANOVA followed by Newman-Keuls post-hoc test) (Figure 4.4B). Furthermore, a strong correlation was found between the degree of axonal degradation (SMI-32 positive immunostaining) and microglial activation (CD-68 positive immunostaining) (Pearson’s coefficient 0.72) (Figure 4.5). Together
these data suggest that the diminishment of axonal injury in Rin null mice results in a reduced need for microglial activation post-injury.
Figure 4.3. Rin loss does not alter early microglial activation 24 hr following optic stretch injury. A. Representative images of phagocytic macrophages (CD-68) in the optic nerve 24 hrs following optic stretch injury in wild-type and Rin\textsuperscript{-/-} animals. B. Quantification of CD-68 in the optic nerve following injury indicates that early inflammation between wild-type and Rin\textsuperscript{-/-} animals are comparable 24 hr following stretch injury. Data are expressed as mean ± SEM (wild-type n= 6-12, Rin\textsuperscript{-/-} n= 5-11; one-way ANOVA followed by Newman-Keuls post hoc test p >0.05.)
Figure 4.4. Rin loss blunts microglia activation 7 d following optic stretch injury. A. Representative images of phagocytic macrophages (CD-68) in the optic nerve 7d following optic stretch injury in wild-type and Rin^{-/-} animals. B. Quantification of CD-68 in the optic nerve following injury indicates that Rin loss blunts inflammatory responses 7d following stretch injury. Data are expressed as mean ± SEM (wild-type n=6-12, Rin^{-/-} n=5-11; one-way ANOVA followed by Newman-Keuls post hoc test **p<0.01.
Figure 4.5. Relationship between axonal damage (SMI-32) and microglial activation (CD-68) following optic stretch injury. The percent area of SMI-32 and CD-68 coverage on the optic nerve following injury displays a high degree of correlation. $r^2 = 0.7215$
Rin interacts with the MAPKKK, Dual Leucine Zipper Kinase (DLK)

Levels of DLK have been determined to increase following injuries to the optic nerve (Watkins, Wang et al. 2013) leading to subsequent activation of JNK and the downstream transcription factor, c-Jun (Chen, Rzhetskaya et al. 2008, Ghosh, Wang et al. 2011, Watkins, Wang et al. 2013, Welsbie, Yang et al. 2013). Given that MAPK cascades, including JNK, are known to be regulated by Ras family GTPases, we sought to determine if Rin could lie in this pathway (Figure 4.6A). To determine whether Rin was a possible upstream regulator of DLK, we performed co-immunoprecipitation experiments in HEK-293T cells by overexpressing Myc tagged DLK and FLAG tagged GTPase constructs (Rin, Rit, and H-Ras). Rin and Rit share a high degree of sequence similarity, including an identical effector domain, whereas the effector domain of H-Ras, a closely related Ras protein, has a distinct, yet still structurally similar effector loop (Figure 4.6B). Both Rin and Rit were found capable of co-immunoprecipitating with DLK (Figure 4.6C lane 3 & 4). H-Ras, however, did not interact with DLK (Figure 4.6C lane 5). These data suggest that Rin and Rit could potentially lie upstream of the DLK cascade and ablation of these proteins could alter DLK-mediated signaling.
Figure 4.6. Rin associates with Dual Leucine Zipper Kinase (DLK), an upstream regulator of axonal degeneration. A. Schematic representation of the DLK MAPK cascade, which has been demonstrated to signal axonal degeneration following injury, and the hypothesis that Rin GTPase lies upstream of DLK activation. B. Amino acid alignment of the G2 effector domains of Rin, Rit and H-Ras. C. HEK-293T cell co-immunoprecipitation between DLK and Rin, Rit and H-Ras shows interaction between the Rit sub-family of GTPases with DLK, but no interaction with H-Ras.
Transection-induced Wallerian degeneration unaffected by Rin loss

Because Rin loss reduced axonal degeneration following stretch injury and literature has demonstrated a role for DLK in pro-degenerative cascades following optic injury (Miller, Press et al. 2009, Tedeschi and Bradke 2013, Yang, Wu et al. 2015), we sought to determine whether the loss of Rin could delay Wallerian degeneration. Resolution of this question, however, could not be adequately provided using the optic nerve stretch model. The diffuse nature of the stretch injury, which causes breaks throughout length of the optic nerve does not allow for analysis of the directionality of axonal breakdown or permit distinction between the proximal and distal axons. Also problematic is the notion that every axon does not receive an equivalent injury and stretch injury does not immediately lead to axonal disconnection. Therefore, we used axonal transection of the optic nerve to generate a localized, normalized injury to assess the impact of Rin loss on Wallerian degeneration.

Following transection in wild-type and Rin<sup>−/−</sup> mice, axonal degeneration was analyzed by immunostaining for SMI-32. At 24 hr post-transection, equivalent SMI-32 positive immunostaining was observed in the distal nerve of both wild-type and Rin<sup>−/−</sup> mice (Figure 4.7). By 7 d post-transection, axonal injury had spread all the way to the optic chiasm in both wild-type and Rin<sup>−/−</sup> animals. These data suggest that Rin loss does not result in a dramatic alteration in the rate of Wallerian degeneration in the distal axon following transection.
Figure 4.7. Rin loss has no effect on axonal degeneration 24hr following optic nerve transection. Representative images of the dephosphorylated neurofilament (SMI-32) in the optic nerve 24 hr following optic transection at 4X magnification show comparable progression of axonal degeneration from the site of transection (on the right side of the image). The contralateral nerve was used as a negative control. (Wild-type n=4 and Rin⁻/- n=4).
Figure 4.8. Rin loss has no effect on axonal degeneration 7d following optic nerve transection. Representative images of the dephosphorylated neurofilament (SMI-32) in the optic nerve 7 d following optic transection at 20X magnification show comparable progression of axonal degeneration from the site of transection. Images are taken at the optic chiasm and indicate that axonal degradation has progressed along the entire optic nerve by 7 d. (Wild-type n=4 and Rin⁻/⁻ n=4)
Discussion

In the present chapter, our data support a role for Rin in pro-degenerative cascades following trauma-induced axonal stretch injury. We demonstrate that the loss of Rin blunts axonal degeneration \((\text{Figure 4.2})\) and diminishes trauma-induced inflammation \((\text{Figure 4.4})\). In looking to identify the molecular cascade downstream of Rin that might be involved in axonal degeneration, we identified a direct interaction with DLK \((\text{Figure 4.6})\). As DLK has been shown to play a critical role in Wallerian degeneration, we also examined the effect of Rin loss on the degeneration of distal axons following optic nerve transection \((\text{Figures 4.7 and 4.8})\). While not conclusive, these preliminary transection studies suggest that Rin is not required for Wallerian degeneration.

A growing body of evidence indicates that axonal degeneration results from a complex interplay between proteins promoting axonal survival and degeneration, suggesting axonal integrity is a dynamically regulated process that can be therapeutically exploited (Gerdts, Summers et al. 2016). Using the power of \textit{Drosophila} genetics to unravel the molecular process(es) underlying Wallerian degeneration, DLK was identified as one of the first pro-degenerative proteins, whose inhibition delayed acute axonal degeneration (Miller, Press et al. 2009). Subsequent studies identified two other MAPKKKs, MEK2 and MEKK4, that are also involved in axonal breakdown through activation of JNK (Yang, Wu et al. 2015). Furthermore, MAPK activation has been demonstrated to increase the turnover of SCG10, an axonal survival protein within the axon, to speed
degeneration (Shin, Miller et al. 2012). Herein, we have demonstrated an interaction between Rin and DLK through co-immunoprecipitation (Figure 4.6) and provided strong evidence that Rin loss blunts axonal degeneration following a stretch model of traumatic injury (Figure 4.1 and 4.2). These data suggest a possible role for Rin upstream of the MAPK pro-degenerative cascade. However, more research is needed to support this hypothesis and demonstrate Rin dependent activation of JNK and turnover of SCG10.

It should be noted that the linkage between Rin and the degenerative MAPK cascade might not necessarily be direct. Although no Ras GTPase has been demonstrated to regulate DLK, Rho family GTPases (Rac and Cdc42) have been demonstrated to signal through DLK (Fan, Merritt et al. 1996). Indeed, the ability of constitutively active mutations of Rac1 and Cdc42 to signal through SAPK pathways is significantly inhibited by expression of catalytically inactive DLK (K185A) (Fan, Merritt et al. 1996). This does not negate the possibility of Rin upstream of DLK as there are many ways to regulate any signaling pathway. Therefore, Rin may still directly activate DLK. It is also possible that Rin could indirectly signal through the degenerative MAPK cascades. Rin overexpression has been demonstrated to activate Rho family GTPases, including both Rac1 and Cdc42 (Hoshino and Nakamura 2003); therefore, it is possibly that Rin may control DLK upstream of the Rho family of GTPases.

More recently, Sarm1 has been implicated in axonal breakdown and its loss is associated with axonal survival weeks following TBI and sciatic nerve
injury (Osterloh, Yang et al. 2012, Gerdts, Summers et al. 2013, Henninger, Bouley et al. 2016). Furthermore, its activation is sufficient to induce axonal degeneration in the absence of external injury (Gerdts, Summers et al. 2013, Gerdts, Brace et al. 2015, Yang, Wu et al. 2015), suggesting it is a central regulator of Wallerian degeneration. NMNAT is a critical enzyme for NAD biosynthesis from ATP and nicotinamide and has been demonstrated to protect against axonal degeneration (Sasaki and Milbrandt 2010, Jayaram, Kusumanchi et al. 2011). NMNAT2 is a survival protein that is continually turned over and transported to the axon through anterograde transport (Gilley, Orsomando et al. 2015). It has been demonstrated that the loss of NMNAT2 expression within the axon due to inhibition of anterograde transport triggers Sarm1 activation (Gilley, Orsomando et al. 2015, Sasaki, Nakagawa et al. 2016) which then depletes axonal stores of NAD⁺ leading to a metabolic disaster (Yang, Wu et al. 2015). Recently it has been suggested that these two models, MAPK and Sarm1, are intertwined in the control of Wallerian degradation following a demonstration that MAPK cascades signal upstream of Sarm1 (Walker, Summers et al. 2017). Unification of the pro-survival and pro-degenerative responses into a single pathway suggests that Rin signaling may lie upstream of Sarm1 and analyzing its effect on NMNAT levels and NAD⁺ depletion following traumatic injury could provide a better understanding of the long-term protection seen in Rin null animals following optic stretch. If Rin lies upstream of the MAPK/Sarm1 pathways, then expression of constitutively activated Rin should mediate cell death and axonal degeneration, increasing JNK activation, Sarm1 activation,
depletion of NMNAT2, and NAD\textsuperscript{+} depletion. In the absence of Rin these pathways should be down regulated, diminishing metabolic catastrophe following optic nerve damage.

Herein we demonstrated that Rin loss was able to lessen degeneration associated with axonal stretch induced TAI (Figure 4.2), but had no obvious effect following axon transection (Figures 4.7, 4.8). We propose that this may be due to inherent differences between the two models of injury. Optic stretch is a less severe form of TAI, resulting in disruption of axonal transport. Protein accumulation in the axon, if not alleviated, leads to axonal breakage and retraction. This process is slow compared to the immediate axotomy caused by transection of the optic nerve. Currently the upstream stimuli capable of Rin activation following traumatic brain injury are unknown. Therefore, it is feasible that Rin would be activated following stretch but not following primary axotomy. However, inhibition of stress MAPK cascades has been shown to delay Wallerian degeneration for a matter of hours; therefore, it is possible that Rin plays a role in Wallerian degeneration that we missed. Future studies should examine degeneration at time points closer to the time of injury, as more modest protection may not last for 24hrs. Further studies are needed to more thoroughly examine Wallerian degeneration in the absence of Rin before any final conclusion can be drawn.

In summary, the data presented in this chapter demonstrates a role for Rin in trauma-induced axonal injury, as its loss blunts both axonal degeneration and
microglial activation up to 7d following injury. This data is in line with our previous finding that Rin loss decreases cell death within the CA-3 region of the hippocampus (Figure 3.7), an area suggested to undergo a higher degree of stretch following CCI injury (Mao, Elkin et al. 2013). This data suggests the intriguing possibility that the neuronal protection afforded by Rin loss identified in Chapter 3 may result from decreased axonal degeneration. However, more work is needed to determine the mechanism of Rin activation in traumatic axonal injury including how the interaction between Rin and DLK may be involved in pro-degenerative pathway.
CHAPTER 5

A Role for the Rin GTPase in Cytokine Production and Neuroinflammation

Introduction

Neuroinflammation is a major pathological component of secondary injury cascades following TBI (Karve, Taylor et al. 2016). Within minutes of trauma, the injured brain elicits a robust inflammatory response fundamental to wound repair and regeneration (Gurtner, Werner et al. 2008, Woodcock and Morganti-Kossmann 2013). Resident astrocytes and microglia predominate in the brain’s innate response to injury, not only through the secretion of inflammatory factors, but also by forming a physical barrier around the site of injury (glial scar), and allowing for the phagocytosis of cellular debris and recognition of foreign antigens (Morganti-Kossmann, Satgunaseelan et al. 2007, Hernandez-Ontiveros, Tajiri et al. 2013, Karve, Taylor et al. 2016).

Microglia, the resident immune cells of the CNS, constantly survey the brain microenvironment (Nimmerjahn, Kirchhoff et al. 2005). Following injury, they rapidly respond to damage associated molecular patterns (DAMPs), chemoattractants, and various forms of cell debris (Davalos, Grutzendler et al. 2005, Nimmerjahn, Kirchhoff et al. 2005) through pattern recognition receptors on their cell surface (Chen and Nunez 2010). Receptor activation leads to the secretion of cytokines that can either initiate an inflammatory response (for
example, IL-1β and TNF-α) or a wound healing response (for example, IL-10) (Morganti-Kossmann, Satgunaseelan et al. 2007, Chen and Nunez 2010).

Stress activated protein kinases, such as p38, respond to various cellular and environmental stimuli, including UV irradiation, hypoxia, oxidative stress, and pro-inflammatory cytokines (Tibbles and Woodgett 1999). The p38 MAPK cascade has been demonstrated to serve as a crucial component for the production of IL-1β and TNF-α and neuronal degeneration in vitro (Bachstetter, Xing et al. 2011, Xing, Bachstetter et al. 2011) and in the regulation of microglial morphological activation in vivo (Bachstetter, Rowe et al. 2013). Although p38 stimulation has been extensively studied in stress response, it has also been demonstrated to play fundamental roles in development, proliferation, survival, and differentiation (Martin-Blanco 2000, Nebreda and Porras 2000). Following brain injury, p38 activation is observed within minutes (Mori, Wang et al. 2002). Numerous reports have shown that activation of the p38 MAPK cascade is associated with neuronal death caused by numerous neurotoxic reagents including excessive and unregulated neurotransmitter release (excitotoxicity) (Cao, Semenova et al. 2004, Semenova, Maki-Hokkonen et al. 2007, Chaparro-Huerta, Flores-Soto et al. 2008), nerve injury (Wittmack, Rush et al. 2005), and ischemia (Wang, Xu et al. 2002, Guo and Bhat 2007). Because p38 plays an essential signaling role in injured neurons and work from our lab has previously demonstrated that the Rin GTPase lies upstream of p38 following nerve growth factor stimulation (Shi, Han et al. 2005), we were interested in determining if Rin loss would alter post-injury inflammation.
Results

Rin loss suppresses the acute pro-inflammatory response following CHI

To assess the role of Rin loss on inflammatory signaling and cytokine production, we used a diffuse closed head injury model (CHI) that generates traumatic axonal injury without causing contusion or tissue loss, but still induces robust astrocyte and microglial activation (Kelley, Farkas et al. 2006, Kelley, Lifshitz et al. 2007, Cao, Thomas et al. 2012). Although CHI induces mild leakage of the blood brain barrier, the lack of a necrotic core limits the infiltration of circulating immune cells (Kelley, Lifshitz et al. 2007) so that the majority of the inflammatory responses observed come from resident glial cell populations within the brain.

Inflammation is central to brain repair following injury. Normally, cytokine production increases between 6 and 24 hrs following diffuse head injury, contributing to glial activation and debris clearance, then subsequently decreases by 7d after injury (Lloyd, Somera-Molina et al. 2008, Bachstetter, Rowe et al. 2013). However, inflammation that is excessive or prolonged can result in neuronal dysfunction and neurological deficits by contributing to infection, edema, or generation of reactive oxygen species (Lloyd, Somera-Molina et al. 2008).

We assessed cytokine production acutely (9 hr) following diffuse head injury. Wild-type animals displayed significant elevation of the pro-inflammatory
cytokines, IL-1β (285% increase compared to sham controls, Figure 5.1A) and TNF-α (39% increase compared to sham controls, Figure 5.1C), within the neocortex. Levels of the pro-inflammatory cytokine, IL-6 (Figure 5.1B), were also elevated following CHI, but did not reach statistical significance. Although Rin⁻/⁻ animals also displayed elevation of IL-1β (91% increase compared to sham-injured controls, Figure 5.1A), its production was significantly blunted compared to wild-type injured mice. Levels of IL-6 (Figure 5.1B) and TNF-α (Figure 5.1C) also trended upwards, however were not statistically different from sham-injured controls. Although Rin loss did not significantly blunt TNF-α production, there was a clear downward trend in Rin⁻/⁻ injured mice compared to wild-type injured mice. Levels of IL-10, an anti-inflammatory cytokine, were also measured acutely following injury, and were unchanged in wild-type injured mice compared to controls (Figure 5.1D). Rin⁻/⁻ mice, however, displayed significantly elevated IL-10 (69% increase compared to sham-injured controls, Figure 5.1D). No significant differences were observed between wild-type and Rin⁻/⁻ sham animals at any time point indicating that global Rin loss does not alter baseline cytokine production (Figure 5.1).

By 7 d post-CHI, IL-6 and TNF-α cytokine production in wild-type mice had returned to baseline (sham-injured) levels. However, IL-1β production in wild-type mice remained elevated at 7 d following CHI (Figure 5.1A-C). Levels of IL-1β and TNF-α remained significantly elevated 7 d following CHI in Rin null mice (90% and 68% increase, respectively, Figure 5.1A, C) suggesting the possibility
of a diminished, but prolonged, inflammatory response in the absence of Rin. Both IL-6 and IL-10 production in Rin$^{-/-}$ injured mice were equivalent to sham-operated controls by 7 d post-CHI (Figure 5.1B, D). Collectively, these data suggest a function for Rin in the control of cytokine production in the neocortex following diffuse head injury.
Figure 5.1 Rin loss alters cytokine production following diffuse brain injury.

Protein levels of IL-1β (A), IL-6 (B), TNF-α (C), and IL-10 (D) in the neocortex were measured by MSD multiplex immunoassay 9hr and 7d following CHI.

Acutely following injury, Rin loss lead to a blunting of the pro-inflammatory cytokine IL-1β and decreased IL-6 and TNF-α although levels did not reach significance. Anti-inflammatory cytokine, IL-10, production was enhanced after CHI in Rin KO mice. At 7d Rin loss enhanced IL-1β and TNF-α whereas levels of IL-10 and IL-6 had returned to baseline. Data are expressed as mean ± SEM; n=9-11; One-way ANOVA followed by Turkey-Kramer post hoc test *p<0.05, **p<0.01, ***p<0.001. IL-1β was log transformed prior to analysis to adjust for normality.
Astrocyte activation unchanged by Rin loss

Since we determined that Rin loss blunted, but prolonged, acute pro-inflammatory, IL-1β, cytokine production following CHI (Figure 5.1A) we next asked whether glial activation was altered downstream of diffuse TBI. Following trauma, astrocytes become activated; this process of astrogliosis is characterized by cellular proliferation and hypertrophy (increased expression of glial fibrillary acidic protein (GFAP), elongation of cellular processes, and cytoplasmic enlargement) (Saatman, Feeko et al. 2006, Bardehle, Kruger et al. 2013, Burda, Bernstein et al. 2016). To this end, we performed IHC for GFAP, a marker of astrocytes, to analyze changes in astrogliosis in the absence of Rin. At baseline, GFAP is expressed at low levels within the mouse cortex as represented in the sham control micrographs of Figure 5.2A. At 9 hr post-injury, GFAP expression was not significantly increased from sham-injured controls. However, by 7 d following injury, both wild-type and Rin null mice displayed an increase in the expression of GFAP within the neocortex indicating injury-induced astrocyte activation (Figure 5.2A). Quantification of GFAP staining in the neocortex showed no discernable differences in astrocyte activation between sham and injured animals 9 hr post-CHI, regardless of genotype (Figure 5.2B). However, by 7 d following injury, GFAP was robustly increased in both wild-type (443% increase compared to sham controls) and Rin^{−/−} mice (155% increase compared to sham controls). Although there was a clear trend towards blunted astrogliosis in Rin^{−/−} mice, no significant difference was observed between wild-type CHI and Rin^{−/−} CHI cohorts (p=0.578, one-way ANOVA followed by Turkey-Kramer post-
hoc test) (Figure 5.2B). These data suggest that astrogliosis remains intact following diffuse head injury in the absence of Rin expression.
Figure 5.2. Astrogliosis in Rin null mice remains intact following diffuse head injury. A. Representative images of GFAP immuno-staining 9 hr and 7 d post-CHI indicate that astrocyte activation occurs 7 d following injury regardless of genotype. B. Quantification of GFAP staining shows a significant increase in astrogliosis in wild-type mice 7 d post-injury and no significant difference was observed between WT CHI and Rin KO CHI cohorts suggesting Rin loss does not affect injury-induced astrocyte activation. Data are expressed as mean ± SEM as a percent of wild-type sham-injured mice; n=9-11; one-way ANOVA followed by Turkey-Kramer post hoc test **p<0.01.
Microglial activation following injury unaltered by Rin loss

To determine whether changes in microglia activation could account for the diminishment in pro-inflammatory cytokine production observed in Rin null mice, we performed IHC for CD-45. CD-45 is a transmembrane protein tyrosine phosphatase expressed at low levels on resident microglia, however, its expression has been shown to increase acutely following traumatic injury (Sedgwick, Schwender et al. 1991, Bachstetter, Rowe et al. 2013). Due to high background, CD-45 IHC staining was scored on a 7-point scale for CD-45 immuno-positive cells within the cortex, hippocampus, and corpus callosum by an experimenter blinded to genotype and treatment. Both wild-type and Rin\(-/-\) mice displayed increased staining of CD-45 expression 9 hr following CHI (Figure 5.3A, B). CD-45 is also expressed on leukocytes, or circulating blood cells, that can infiltrate following trauma due to breakdown of the blood brain barrier. These cells can be morphologically distinguished from microglia due to their lack of arborization. Quantification of infiltrating leukocytes showed no differences between cohorts (Figure 5.3C). Together these data suggest that Rin loss does not alter microglial activation following diffuse head injury.
Figure 5.3. Rin null mice display normal microglial activation following CHI

A. Representative images of CD-45 immune-staining 9hr indicate that microglial activation is intact in Rin null mice. B. Quantification of CD45 staining on a 7-point scale displays increased microgliosis in wild-type mice 9hr post-injury and no significant difference was observed between wild-type CHI and RinKO CHI cohorts suggesting Rin loss does not affect injury-induced microglial activation. Data are expressed as mean ± SEM; one-way ANOVA followed by Turkey-Kramer post hoc test; n=9-11.
Axonal protection afforded by Rin loss

Because the changes observed in cytokine profiles following injury could not be explained by changes in glial activation, we evaluated injury at the level of the neuron. Diffuse models of TBI do not result in high levels of cell death and tissue loss; however, does lead to traumatic axonal injury throughout the mediodorsal neocortex (Kelley, Farkas et al. 2006, Kelley, Lifshitz et al. 2007, Cao, Thomas et al. 2012). Beta amyloid precursor protein (β–APP) is a common marker for traumatic axonal injury following trauma as it accumulates in axonal bulbs or swellings within hours following injury, due to disruption of axonal transport (Roberts, Gentleman et al. 1994, Suehiro and Povlishock 2001, Smith, Uryu et al. 2003).

To evaluate whether Rin loss diminishes axonal injury, we performed IHC for β–APP 9 hr following CHI. Both wild-type and Rin−/− sham-injured controls displayed minimal accumulation of β–APP in axonal swellings (wild-type, 0.07 punta/mm²; Rin−/−, 0.41 punta/mm², Figure 5.4). As expected, there was an accumulation of aberrant β–APP localization to axonal swellings within the neocortex of wild-type mice following injury (3.78 punta/mm²). However, Rin loss resulted in significantly diminished axonal β–APP staining following CHI (0.90 punta/mm², Figure 5.4). These data suggest axonal protection following CHI in the absence of Rin.
Figure 5.4. Acute axonal injury blunted following CHI in the absence of Rin.

A. Images of neocortical beta-amyloid precursor protein (β−APP) 9 hr following closed head injury in wild-type and Rin−/− animals. Arrows indicate examples of positive β−APP staining. Images were taken at the injury epicenter where β−APP accumulation was highest. B. Quantification of β−APP in the neocortex following injury indicates that Rin loss diminishes axonal swellings suggesting axonal protection. Data are expressed as mean ± SEM; n=9-11; one-way ANOVA followed by Turkey-Kramer post hoc test **p<0.01.
Analysis of the impact of Rin deficiency on post-CHI cognitive performance

Decreased pro-inflammatory cytokine production and axonal injury have been associated with improved behavioral outcomes in experimental models of TBI (Bachstetter, Rowe et al. 2013, Bachstetter, Webster et al. 2015). To determine whether Rin loss could improve behavioral outcomes following CHI, we performed radial arm water maze (RAWM), a spatial learning task (Alamed, Wilcock et al. 2006) with observable deficits up to 2 weeks following CHI (Webster, Van Eldik et al. 2015). Overall, Rin loss had no significant effect on RAWM performance (Figure 5.5A, two-way repeated measure ANOVA); however, when teased apart, clear trends were noted. While wild-type mice displayed significant spatial learning deficits following CHI (Figure 5.5B), Rin⁻/⁻ sham and Rin⁻/⁻ injured mice performed equivalently (Figure 5.5C). Injured wild-type and Rin⁻/⁻ cohorts were found to perform equivalently (Figure 5.5D). Importantly, Rin⁻/⁻ mice tended to have more errors than wild-type mice in the absence of injury (Figure 5.5E), suggesting that Rin loss results in a baseline defect in this assay (although this trend does not reach statistical significance). It is promising that Rin⁻/⁻ sham and Rin⁻/⁻ injured mice performed similarly in the task. However, the intrinsic performance differences at baseline between wild-type and Rin⁻/⁻ mice, combined with the modest increase in RAWM errors following CHI (~1 additional error following injury), do not permit definitive conclusions regarding cognitive sparing at this time.
Figure 5.5. Behavioral effects of Rin loss on CHI behavioral outcomes. Radial arm water maze assessed 14 d following CHI revealed (A) no significant alterations in performance based on genotype (B) a diminishment in cognitive performance of WT mice (C) no alteration between Rin-/- sham and Rin-/- CHI (D) no alteration between WT CHI and Rin-/- CHI (E) a clear trend towards decreased cognitive performance in Rin-/- sham animals. All data are expressed as mean ± SEM; n=9-11. Panel A was analyzed via a two-way repeated measures ANOVA whereas panels B-E were analyzed by one-way repeated measures ANOVAs.
Discussion

In the present study, we have identified a potential role for Rin GTPase in the initiation of neuroinflammation following diffuse brain injury. We demonstrated that Rin loss blunts acute production of pro-inflammatory IL-1β at 9 hr post-CHI, and, although it does not reach significance, a similar trend is observed in TNF-α production (Figure 5.1 A, C). Moreover, the absence of Rin increased the levels of anti-inflammatory IL-10 acutely following injury (Figure 5.1D). Interestingly, although acute IL-1β production was blunted in Rin null injured mice, there appeared to be prolonged pro-inflammatory response, as the levels of IL-1β had not returned to baseline by 7 d post-CHI (Figure 5.1A). Although glial populations respond to damage through secretion of cytokines (Morganti-Kossmann, Satgunaseelan et al. 2007, Chen and Nunez 2010) we were unable to discern any considerable difference in the degree of astrocyte or microglial activation between wild-type and Rin⁻/⁻ injured animals. However, we did find that Rin loss resulted in significantly diminished axonal accumulation of β-APP staining following CHI, suggesting that axonal injury was reduced, consistent with previous data following optic nerve injury (Chapter 4). Together these data suggest that Rin contributes to the regulation of post-CHI cytokine production, and axonal injury. However, due to the baseline differences in behavior between wild-type and Rin⁻/⁻ sham-injured mice, it is currently unclear if this has any effect on behavioral outcomes following diffuse head injury.
Although microglia are a major source of injury-related cytokine release (Hanisch 2002), other cell types, including astrocytes, have also been demonstrated to produce inflammatory mediators following stimulation (Kan, van der Hel et al. 2012, Choi, Lee et al. 2014). Neurons, although generally thought of as a cytokine target, express Toll-like receptors and can both sense and mount inflammatory responses (Lafon, Megret et al. 2006) including the production of IL-1β, IL-10, TNF-α, in response to diverse stimuli (Freidin, Bennett et al. 1992, Yamamoto, Yamashita et al. 2011, Lim, Lu et al. 2016). In our current study, Rin deficiency altered cytokine production; however, preliminary findings found no evidence that glial activation was altered. These data would suggest that cytokine release from these populations was equivalent in wild-type and Rin null mice. These results are in agreement with lack of endogenous expression of Rin in glial populations (Cahoy, Emery et al. 2008), however, isolation and stimulation of astrocyte and microglial cultures would be required to conclusively confirm that Rin function does not play a role in this cell types. Instead, differences in axonal injury following trauma may indicate that the altered cytokine profiles may result from decreased neuronal cytokine release. Given that these studies were conducted in a whole-body knock-out mouse, it is not possible to test this hypothesis in vivo. Regardless, the notion of cytokine differences post-injury being neuronally based are in line with previous studies indicating that Rin expression is limited to neurons and warrants further investigation (Shao, Kadono-Okuda et al. 1999, Lafon, Megret et al. 2006, Cahoy, Emery et al. 2008, Sharma, Schmitt et al. 2015). While it is unlikely, due
to the nature of the injury, that the differences in cytokine profiles is the result of infiltrating immune cells of the PNS, we cannot completely rule out this possibility.

Previous studies from the lab have demonstrated that Rin lies upstream of p38 stress-kinase signaling (Shi, Han et al. 2005), a pathway that has been demonstrated to be upregulated following brain and ischemic injuries (Sugino, Nozaki et al. 2000, Nozaki, Nishimura et al. 2001, Mori, Wang et al. 2002, Donninger, Calvisi et al. 2015). Furthermore, microglial specific deletion of p38α leads to cognitive behavioral improvement following diffuse head injury (Bachstetter, Rowe et al. 2013). To date, our post-CHI behavioral data is inconclusive regarding the important issue of whether Rin loss provides spatial learning protection following diffuse brain injury, as our results indicate that there is a baseline behavioral deficit in Rin null animals that could be due to long-term Rin deletion or a difference in genetic background (Rin+/- mice were generated by pronuclear microinjection of 129SvEv ES cells into C57BL6/N blastocysts). Although we did not see a significant behavioral sparing, it is promising that Rin-/- sham and Rin-/- CHI mice displayed no behavioral differences using RAWM analysis, and it remains to be determined whether Rin loss provides functional sparing. Plans are underway to resolve this issue using two approaches. First, we have just completed ten generations of backcrossing the Rin null genetic deletion line into C57BL6 mice to generate a defined genetic background. In addition, we are engineering a Rin conditional knock-out mouse line that would permit cell-type specific Rin deletion.
In summary, the data in this chapter suggests a role for Rin in the regulation of cytokine production following CHI. We postulate that the differences observed are the result of neuronal specific Rin actions, not only because all available evidence indicates that Rin is expressed solely in neurons, but also because of the significant reduction in axonal injury found in Rin$^{-/-}$ mice following diffuse head injury. However, more research is needed to define the role for Rin in the regulation of neuronal-specific inflammatory signaling.
CHAPTER SIX

Discussion

The studies in this dissertation were performed with the goal of identifying the physiological function of neuronally expressed Rin GTPase. To address this question, we used a mouse line bearing a genetic deletion of the endogenous Rin (RIT2) gene generated through gene trap insertion (Figure 3.1). Previous research in the lab indicated that Rin signaled downstream of growth factor stimulation (Shi and Andres 2005) and demonstrated that the closely related protein Rit functioned in post-trauma induction of neurogenesis (Cai, Carlson et al. 2012). Therefore, we implemented traumatic injury models to test the ability of Rin to improve neuronal survival and enhance cognitive outcomes following CNS injury. Using this novel knockout model, we have begun to characterize the genetic deletion of Rin and implicate a functional role for Rin in three major components of CNS trauma: neuronal death, axonal degeneration, and neuroinflammation. In doing so, we have not only demonstrated functionally divergent roles for Rin and Rit GTPases, but also established Rin deficiency as neuroprotective- clearly identifying roles for Rin in the regulation of neuronal cell loss and axonal degeneration following brain injury. The data presented within this dissertation suggests that Rin may be a viable therapeutic target following TBI.
Rin and Rit GTPase display functional specificity

Due to conservation of effector domain sequences and downstream effectors, it was believed that Rin would function similarly to the closely related GTPase, Rit, to regulate proliferation and survival (Reuther and Der 2000). However, early findings within our laboratory suggested that the two GTPases acted independently in vitro, with Rin expression resulting in decreased cell viability in cell culture models (data not shown). To further study Rit and Rin regulation, we moved our studies to in vivo mouse models. Studies conducted by Dr. Weiakng Cai, a previous graduate student in the lab, found that Rit deficiency increased neurodegeneration within the hippocampus following CCI (Cai, Carlson et al. 2012). Shown here, Rin loss revealed a strikingly distinct role following CCI injury model, increasing neuronal survival (Figure 3.3). Further, Rin loss had no effect on post-traumatic neurogenesis (Figures 3.4, 3.5, 3.6), although Rit has been demonstrated to be a key contributor in neurogenesis and proliferation of neural stem cells (Cai, Carlson et al. 2012, Mir, Cai et al. 2017, Mir, Cai et al. 2017). Together, these data support distinct physiological functions for these two closely related Ras family members.

Although functional specificity had not been demonstrated in vivo prior to this work, there were several lines of evidence that indicated the divergence properties of Rin and Rit GTPase. First, unlike other members of the Ras family, Rin is unable to transform NIH-3T3 cells (Rusyn, Reynolds et al. 2000). Second, endogenous Rin expression was unable to complement Rit loss (Cai, Rudolph et
al. 2011). And finally, Rin and Rit have been associated with distinct pathological outcomes. Genetic GWAS studies have linked the loss of heterozygosity, or chromosomal events that result in \textit{RIT2} gene loss or silencing, to predominantly neuropsychological disorders, including autism, schizophrenia and Parkinson’s disease (Glessner, Reilly et al. 2010, Pankratz, Beecham et al. 2012, Emamalizadeh, Movafagh et al. 2014, Liu, Guo et al. 2015, Nie, Feng et al. 2015, Wang, Gong et al. 2015, Zhang, Niu et al. 2015, Liu, Shimada et al. 2016, Foo, Tan et al. 2017, Hamedani, Gharesouran et al. 2017, Li, Zhang et al. 2017). In contrast, mutations that lead to the activation of Rit has been connected to both the developmental disorder, Noonan Syndrome, in the case of germinal mutation, and lung cancer and leukemia if the mutation is somatic (Berger, Imielinski et al. 2014, Chen, Yin et al. 2014, Xu, Sun et al. 2015, Cave, Caye et al. 2016, Koenighofer, Hung et al. 2016). Together these lines of evidence further suggested that Rin and Rit, despite their biochemical similarities, were functionally unique.

An interesting question that remains to be answered is how the functional specificity between Rin and Rit GTPase is achieved. A similar conundrum was faced in regards to the three extremely well conserved founding members of the Ras family (H-Ras, K-Ras and N-Ras), which share approximately 80% primary sequence identity. Several broad themes underlying their functional separation have been described (Castellano and Santos 2011). These include 1) differential patterns of tissue and developmental expression, 2) distinct specificities to different GAPs or GEFs, and 3) different intracellular pathways of processing and
membrane localization (Castellano and Santos 2011). It is conceivable that these characteristics also apply to the Rit subfamily and are discussed below.

Expression differences between Rin and Rit have already been demonstrated broadly across tissues and development. Rit is expressed ubiquitously throughout the organism and throughout development, whereas Rin expression is limited to the CNS and is first detected late during mouse embryonic development (first appearing around e15 in the mouse brain) (Lee, Della et al. 1996, Spencer, Shao et al. 2002, Cahoy, Emery et al. 2008, Sharma, Schmitt et al. 2015). The literature indicates that both Rin and Rit are expressed within neurons (Lee, Della et al. 1996, Shao, Kadono-Okuda et al. 1999), and our lab has demonstrated that the two proteins are activated by similar stimuli (Shi, Cai et al. 2013). So how is it determined which signal dominates? In K-Ras functionality can be determined through expression differences following stimulation. For example, it has been demonstrated that the levels of K-Ras4B are higher under physiological conditions, whereas K-Ras4A expression is induced during differentiation of pluripotent embryonic stem cells (Pells, Divjak et al. 1997). However, these forms of K-Ras are splice variants (Barbacid 1987) and alternatively spliced isoforms of Rit and Rin have not been described. It is more likely that differences in functionality are derived through distinct specificity of GEF and GAP proteins or subcellular localization.

GEFs and GAPs regulate the activation states of small GTPases, therefore, controlling their intracellular signaling pathways and cellular roles (Bos,
Rehmann et al. 2007). GEFs are direct activators of the Ras superfamily of GTPases; thereby, each stimulus that results in Ras activation requires GEF regulation (Quilliam, Khosravi-Far et al. 1995, Bernards and Settleman 2007). Furthermore, these proteins display substrate specificity in activating specific families of GTPases. For example, son of sevenless (SOS) preferentially activates Ras and Rac family members, but not Rap proteins (Nimnual and Bar-Sagi 2002). Ras GAP proteins are required to stimulate GTP hydrolysis, ending signaling on a physiological time scale (Bernards and Settleman 2005). To date, few studies have accessed Rit subfamily regulatory proteins. However, association between Rin and SOS has been shown through co-immunoprecipitation (Hoshino and Nakamura 2002). It has also been demonstrated that expression of the Ras specific GAP proteins, SynGAP and GAP1, can significantly decrease the concentration of GTP bound Rin; however, the Rap specific GAP, Rap1-GAP had no effect (Hoshino and Nakamura 2002). Similar to Rin, GTP bound Rit levels were elevated following expression of the Ras specific GEFs SOS and GRF (Spencer, Shao et al. 2002). With the data currently available it is impossible to determine the degree of regulatory overlap between Rin and Rit. However, given their apparent functional distinctions, this is a critical issue to generating greater understanding Rin and Rit signaling that requires further study.

The subcellular distribution of small GTPases provides additional control of activation and function by allowing for association with and activation of local effectors in distinct cellular compartments (Castellano and Santos 2011). In this
way, despite identical effector and regulatory domains, Ras isoforms display functional specificity (Chiu, Bivona et al. 2002). For example, plasma membrane associated K-Ras enables transformation, whereas mitochondrial localized K-Ras induces apoptosis (Bivona, Quatela et al. 2006). Unlike other Ras family members that undergo post-translational lipidation to ensure membrane tethering (Lowy and Willumsen 1993, Reuther and Der 2000), the Rit subfamily lacks canonical lipidation sequences (Shi, Cai et al. 2013). Indeed, currently there is no data available to indicate that they undergo lipid modification of any sort. Instead Rit subfamily proteins are localized through interactions between their extended polybasic C-terminus and charged PI(3,4,5)P_3 and PI(4,5)P_2 lipids on the plasma membrane (Heo, Inoue et al. 2006). To date the subcellular localization of Rin and Rit have not been extensively characterized. However, immunostaining for Rit in hippocampal neurons shows that Rit is localized not only in the cell body, but also the dendritic arbors and axonal shaft (Lein, Guo et al. 2007). Although subcellular localization has not been characterized for Rin, herein, we describe a novel role for Rin in the regulation of trauma-induced cell death (Figure 3.3, 3.7) and axonal injury (Chapter 4 and Figure 5.4), suggesting that Rin signaling affects processes in both the cell body and axon. Further characterization of the subcellular localization could provide valuable insight into the function and regulation of the Rit subfamily of GTPases.

Does Rin signaling contribute to neuronal death?
The studies presented in this dissertation indicate that Rin loss is neuroprotective, leading to the hypothesis that Rin signaling may contribute to neuronal loss following trauma. If this hypothesis holds, expression of a constitutively active Rin mutation (Rin^{Q78L}) would diminish cell viability or initiate cell death in the absence of additional stimulation. Because Rin expression is limited to neurons, the best \textit{in vitro} model system for these experiments would be primary neuronal cultures to ensure that Rin specific effectors are endogenously expressed. Furthermore, it might be important to use long-term neuronal cultures, to ensure they mature, as Rin loss does not appear to impact immature neuron survival in the hippocampus following contusive injury (\textbf{Figure 3.4}). Given our current data, it would be interesting to examine Rin expression in immature neurons and progenitor cell populations within the hippocampus. For the simplest explanation of why Rin loss has no apparent effect on these populations following trauma (\textbf{Figures 3.4, 3.5, 3.6}) is because Rin protein is not expressed in these early developmental cell types. Because Rin is not expressed at early developmental time points (Lee, Della et al. 1996), this remains a conceivable hypothesis.

Although Rin is expressed in both cortical and hippocampal neuronal populations (Cahoy, Emery et al. 2008), our data suggest that Rin loss selectively protects hippocampal (\textbf{Figure 3.3, 3.7}), but not cortical neurons (\textbf{Figure 3.2}) following focal brain injury. Transferring studies into primary neurons would allow determination of whether selective protection is an effect of the hippocampal neuronal population itself or possibly due to differences in the
nature of stimuli needed to activate Rin (assuming that Rin activation initiates cell loss). Comparison of neuronal death and dendrite degeneration in cortical and hippocampal neurons generated from wild-type and Rin\(^{-/-}\) mice would provide valuable insight into the protective nature of Rin loss following stimulation with various experimental models of secondary injury. Neurons could be treated with L-glutamate to mimic excitotoxicity (Choi, Maulucci-Gedde et al. 1987), sodium nitroprusside, a nitric oxide donor, an experimental model for reactive oxygen species (Dawson, Dawson et al. 1993), and oxygen-glucose deprivation to induce hypoxia ischemia (Kaku, Goldberg et al. 1991). If Rin loss leads to selective protection of hippocampal neurons, then wild-type and Rin\(^{-/-}\) cortical neurons should display the same degree of cell loss and viability while Rin\(^{-/-}\) hippocampal neurons would display increased viability compared to wild-type hippocampal neurons. However, if the differences observed in the hippocampus and cortex following CCI were due to the type of stimulus, then we would expect differential protection in the two populations based on the experimental injury model.

**The role of Rin in axonal degeneration**

We have demonstrated in two separate injury models that Rin loss decreases axonal pathology. Following optic nerve stretch, axonal damage was equivalent acutely following injury (**Figure 4.1**); however, Rin loss significantly blunted the progression of degeneration 7 d following stretch injury (**Figure 4.2**). Furthermore, Rin loss was shown to diminish axonal damage in the cortex
acutely following diffuse closed head injury (Figure 5.4). Although these injury paradigms cannot be directly compared, axonal protection in both models, strongly implicates Rin signaling in the progression of axonal degeneration. Therefore, it will be interesting to assess the dependence of Rin signaling in the activation of known degenerative pathways.

Rin was shown to associate with DLK (Figure 4.3), a known regulator of axonal degeneration and cell death (Tedeschi and Bradke 2013). Levels of DLK have been determined to increase following injuries to the optic nerve and dorsal root ganglia (Watkins, Wang et al. 2013, Welsbie, Yang et al. 2013). Although DLK has been shown to lie upstream of both p38 and JNK MAPKs, the activation of JNK has been demonstrated as necessary to commit axons to a degenerative fate following injury, as its pharmacological inhibition is sufficient to blunt axonal fragmentation (Miller, Press et al. 2009). Furthermore, trauma-induced heightened DLK expression has been demonstrated to lead to subsequent increased phosphorylation and activation of JNK and c-Jun (Chen, Rzhetskaya et al. 2008, Ghosh, Wang et al. 2011, Watkins, Wang et al. 2013, Welsbie, Yang et al. 2013) (Figure 6.1). These studies from the literature, along with the DLK-Rin interaction, and the ability of Rin loss decrease degeneration provoked the hypothesis that Rin signals upstream of the DLK signaling cascades. If the hypothesis holds, Rin activation should increase the ability of DLK to activate JNK and c-Jun, as indicated by increased phosphorylation, and its loss diminish these effects. Strikingly, preliminary data generated by Binoy Joseph in the Saatman laboratory suggests that Rin loss blunts activation of c-Jun within the
retinal ganglion cells following optic stretch injury (data not shown). Further studies are needed to analyze the effects of Rin loss on JNK activation acutely following optic stretch; however, these data provide strong initial support of Rin regulating axonal degeneration upstream of the JNK MAPK cascade.
Figure 6.1. Does the trauma-induced DLK-JNK MAPK cascade include Rin GTPase activation? Axonal trauma induces activation of DLK. Once activated, DLK signals to JNK through MKK4/7. JNK has many substrates in the cell including c-Jun and SCG10. Activation of the transcription factor c-Jun has been associated with axonal degradation and cell death. JNK can also directly phosphorylate axonal SCG10 targeting it for degradation; loss of SCG10 precedes axonal degradation. The ability of Rin to interact with DLK, diminish c-Jun activation following stretch injury, and blunt axonal degradation suggests that Rin may regulate the DLK-JNK cascade following trauma.
Because Rin loss decreases activation of the JNK substrate, c-Jun, it would be interesting to look at other JNK substrates associated with axonal degeneration. SCG10 was identified as an important JNK substrate within the axon and its levels have been correlated with axonal fragmentation (Shin, Miller et al. 2012). Phosphorylation of SCG10 by JNK targets the protein for degradation (Figure 6.1). In a healthy neuron, SCG10 is rapidly replaced in the axon through anterograde transport; however, following trauma, transport is disrupted and SCG10 expression in the axon is lost (Shin, Miller et al. 2012). Diminished levels of axonal SCG10 have been correlated with axonal fragmentation of cultured neurons of both the PNS and CNS and, furthermore, silencing of SCG10 through short hairpin RNA was demonstrated to increase the rate of axonal fragmentation (Shin, Miller et al. 2012). Herein, we observed diminished axonal pathology following both optic nerve stretch (Figure 4.2) and diffuse head injury (Figure 5.4) in Rin null mice. Blunted axonal degradation would suggest lessened neurofilament fragmentation in which case, levels of SCG10 in the axon would be maintained. Rin loss could decrease JNK activation, thereby lessening SCG10 phosphorylation and turnover; either scenario would lead to maintained axonal SCG10 concentrations. Although further investigation is needed, the maintenance of axonal SCG10 concentrations could be another mechanism through which Rin loss blunts axonal pathology following axonal injury.

A more in-depth analysis of Rin in Wallerian degeneration
The literature indicates that the absence of DLK protects distal axons from Wallerian degeneration (Miller, Press et al. 2009, Yang, Wu et al. 2015), which led us to examine the breakdown of distal axons following optic nerve transection in Rin null mice. Although preliminary, our initial studies do not suggest a role for Rin in Wallerian degeneration as the extent of axonal degeneration showed no discernable difference at 24 hr (Figure 4.7) or 7d (Figure 4.8) following transection. However, a more extensive analysis of more acute time points following injury is needed to confirm that Rin loss has no effect on Wallerian degeneration. Given the degree of axonal protection out to 7 d following optic nerve stretch, we decided to analyze Wallerian degeneration at the same endpoints hypothesizing that loss would be equally protective in both optic nerve stretch and transection. However, the pathway in which Rin signals through would ultimately determine the timeframe of axonal protection. For example, DLK deletion only delays axonal clearance for hours in mouse models and a couple days in Drosophila (Miller, Press et al. 2009). Simultaneous silencing of the three known JNK MAPKKK proteins (DLK, MKK2, and MEKK4) upstream of axonal degeneration only effectively diminished distal axon fragmentation for 6 d following a milder optic crush injury (Yang, Wu et al. 2015). If Rin loss results in delayed axonal degeneration at a shorter time period, for instance hours following transection as would be expected by inhibition of DLK, we could have missed the effect of Rin deficiency on Wallerian degeneration in our preliminary optic transection studies.
It is also possible that the methods we used to analyze Wallerian degeneration did not give a clear depiction of axonal breakdown. Our current Wallerian data was generated through immunostaining of non-phosphorylated neurofilament (SMI-32) along the optic nerve following transection; however, this method has several limitations. Immunostaining did not allow us to track degeneration along a single severed axon, therefore we were unable to determine the percentage of axons undergoing Wallerian degeneration at a select time point and the percentage of axons that were spared. A more appropriate model would entail the use of Thy1-YFP mice, which express yellow fluorescent protein (YFP) at high levels within motor and sensory neurons including those of the retinal ganglion cell layer (Beirowski, Berek et al. 2004). In this model, YFP expression is randomly distributed in approximately 3% of these neuronal populations, resulting in sparse YPF labeling throughout the cell bodies and axons of both the central and peripheral nervous systems (Beirowski, Berek et al. 2004). The restricted axonal labeling in Thy1-YFP mice permits quantitative and qualitative analysis of Wallerian degeneration because axonal collapse of a single cell can be visualized as fragmentation of the YPF labeled axons (Beirowski, Berek et al. 2004). Crossing Thy1 YFP mice with our Rin knockout line would allow for a more thorough examination of the effects of Rin loss on axonal breakdown following optic transection. Generation of Thy1-YFP mice in our Rin\textsuperscript{+/−} background have been generated in the lab (Figure 6.2) and will provide a new perspective to analyze axonal breakdown.
Figure 6.2. Thy-1 YFP mice will allow for tracing of axonal degeneration along a single axon. The neurons of Thy-1 YFP mice contain sparse labeling of YFP in neuronal cell bodies and axons. Axonal degeneration can thereby be observed and quantified along a single axon or population of axons. Representative confocal images of wild-type Thy1-YFP mouse brain hemisphere (2X), optic nerve whole mount (2X), CA1 (20X), and dentate gyrus (20X).
The combination of sparse labeling and more acute time intervals will give a more accurate depiction of the role of Rin in Wallerian degeneration. A milder optic crush injury may also be considered in order to slow axonal clearance and prolong the window that protection can be observed. However, it is also possible that Rin loss regulates neuronal cell death through DLK signaling, yet has no effect on Wallerian degeneration.

**Could Rin loss enhance axonal regeneration?**

Herein, we did not investigate the possibility that Rin loss enhances axonal regeneration following injury, although this would provide an alternative explanation to the observed decreases in SMI-32 (Figure 4.2) and APP (Figure 5.4) immunostaining following stretch and CHI injury. The optic stretch model does not lend itself to regeneration studies due to the diffuse nature of the injury throughout the optic nerve. Previous literature, however, has demonstrated that the mild head injury (CHI) model utilized in Chapter 5 causes traumatic axonal injury that does not progress to cell death, instead axons show signs of reorganization and repair within a week of injury (Singleton, Zhu et al. 2002, Greer, McGinn et al. 2011, Greer, Povlishock et al. 2012). Although, electron microscopy could be used to determine the extent of axonal reorganization following CHI, it is not ideal due to the diffuse nature of the injury. Indeed, the question would be best examined using optic nerve crush (Watkins, Wang et al. 2013) to provide a distinct point of injury and quantification of axonal sprouting beyond the injury site.
Rin expression in pheochromocytoma cells has previously been demonstrated to activate members of the Rho family of GTPases including RhoA, Cdc42, and Rac (Hoshino and Nakamura 2003) suggesting Rin may be capable of modulating cytoskeletal dynamics upstream of Rho family GTPases in neurons (Jaffe and Hall 2005). The RhoA/Rho kinase (ROCK) pathway is involved in several CNS processes including both physiological responses (dendrite development and axon extension) and pathological outcomes in CNS injury, stroke, neurodegenerative disease (Fujita and Yamashita 2014). Following injury activation of the RhoA/ROCK pathway has been associated with blunted regenerative responses. Indeed, inhibition of RhoA has been demonstrated to increase nerve regeneration following optic injury (Lehmann, Fournier et al. 1999, Fischer, Petkova et al. 2004, Bertrand, Winton et al. 2005) (Figure 6.3). The regenerative response associated with RhoA inhibition is believed to be dependent on blunting the activation of ROCK, as its inhibition also leads to improved regenerative responses in the CNS following trauma (Lingor, Teusch et al. 2007, Lingor, Tonges et al. 2008). If Rin lies upstream of RhoA, then it is possible that genetic deletion of Rin would also initiate improved nerve regeneration following optic tract injuries though reduced RhoA signaling, providing a novel molecular mechanism to explore the regenerative response in Rin null animals.
Figure 6.3. Does Rin activate RhoA following traumatic injury? Signaling through RhoA/ROCK has been demonstrated to inhibit axonal regeneration following injury. If Rin is capable of activating RhoA, then Rin deletion should have similar affects as RhoA or ROCK inhibition and increase axonal regeneration.
Role for Rin in inflammatory responses

To assess the role of Rin in post-traumatic neuroinflammation, we utilized a mild concussive injury that leads to a robust inflammatory response in the absence of cell death (Kelley, Farkas et al. 2006, Kelley, Lifshitz et al. 2007, Cao, Thomas et al. 2012). Despite reports of Rin expression being limited to neuronal populations (Lee, Della et al. 1996, Spencer, Shao et al. 2002, Cahoy, Emery et al. 2008, Sharma, Schmitt et al. 2015) we demonstrated that Rin null animals displayed blunted pro-inflammatory and enhanced anti-inflammatory cytokine production acutely following injury (Figure 5.1). This result is interesting given that cytokine production is prominently carried out by glial populations (Hernandez-Ontiveros, Tajiri et al. 2013, Karve, Taylor et al. 2016). However, in line with neuronal expression of Rin, Rin−/− mice displayed no significant reduction of astrogliosis or microgliosis following CHI (Figure 5.2, 5.3).

Although neurons are often thought to respond to cytokine signaling, literature suggests that they can also produce cytokines in response to stimulation (Freidin, Bennett et al. 1992, Yamamoto, Yamashita et al. 2011, Lim, Lu et al. 2016). Because our previous work indicates decreased neuronal loss in Rin null animals (Chapter 3), we are lead to believe that the changes of cytokine profiles may result from diminished neuronal damage following injury. In order to confirm that changes in cytokine production are the result of neuronal populations we would need to culture primary neurons and measure cytokine levels following stimulation with neurotoxic agents. Stimulation of in vitro primary
neuronal cultures would allow us to monitor the effects of Rin loss without having to take into consideration other CNS cell populations. If Rin signals upstream of cytokine production in neurons, we should observe blunted production of IL-1β and TNF-α following with L-glutamate, sodium nitroprusside, and oxygen-glucose deprivation.

To date, Rin expression has not been identified outside of the CNS. However, a more thorough examination of peripheral immune cells is needed to rule out the possibility of Rin expression in infiltrating cell types, such as macrophages of neutrophils. If Rin were expressed in non-resident immune cells, it would provide a possible explanation for the changes we observed in cytokine production acutely following CHI. To determine whether Rin is expressed in macrophages and/or neutrophils, these cell populations would need to be extracted and cultured from wild-type animals for qPCR analysis of Rin. Furthermore, if Rin signaling is necessary for cytokine production in these cells, then Rin⁻/⁻ animals should display blunting of IL-1β and TNF-α levels following in vitro stimulation with the TLR4 agonist, lipopolysaccharide.

**Clarification of Rin Physiology using a conditional KO mouse model**

Herein, we have described how Rin loss alters cell death (Chapter 3), axonal degeneration (Chapter 4) and inflammatory responses (Chapter 5) following trauma; however, due to the complex interplay between cells of the CNS we are unable to determine a primary role for Rin. All the studies presented in this dissertation were carried out using a whole-body Rin null mouse model,
and although Rin is believed to be expressed solely in neurons, expression in other cell types remains a formal possibility. Furthermore, the use of a global knockout mouse does not allow us to determine whether the phenotypes observed following injury are due to Rin loss or compensatory effects acquired throughout the lifetime to counteract for Rin loss. In order to address these apparent issues, our lab has recently generated conditional KO Rin mice that can be combined with tamoxifen-induced Cre expression in specific tissues. When crossed to lines allowing inducible neuronal-selective Cre expression, these mice will allow neuron specific RIT2 loss (Betz, Vosshenrich et al. 1996). Using these mice, we can be certain that effects we have observed are specific to the neuron.

Using these mice, several outcomes of this body of work can be revisited to allow for more definitive answers regarding the protective properties of Rin loss. First, neuronal specific knockout of Rin would allow us to determine whether acute blunting of pro-inflammatory cytokine production following CHI (Figure 5.1) was indeed neuronally based without having to culture glia or immune cells. Furthermore, these mice will allow us to revisit RAWM testing following mild head injury (Figure 5.5) to determine whether Rin loss indeed leads to spatial learning deficits.

Conclusions

The data presented in this dissertation has demonstrated Rin loss to be neuroprotective in several models of traumatic injury: enhancing mature neuronal survival, diminishing cognitive decline, decreasing axonal injury, and blunting
acute pro-inflammatory cytokine production. These data suggest a novel and unexpected physiological role for Rin in the promotion of neuronal cell death and axonal degeneration following injury. Further studies are needed to investigate the contribution of Rin signaling to post-traumatic injury cascades; however, these initial studies provide evidence of a new avenue of therapeutic intervention through the inhibition of Rin GTPase.
## Appendix

### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AF6</td>
<td>ALL (acute lymphoblastic leukemia) fused gene on chromosome 6</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
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<tr>
<td>Arf</td>
<td>ADP-ribosylation factor</td>
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<tr>
<td>ASF</td>
<td>Area sampling fraction</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>BDNF</td>
<td>Brain-derived neurotropic factor</td>
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<tr>
<td>BrdU</td>
<td>5-bromo-2’-deoxyuridine</td>
</tr>
<tr>
<td>CA-1</td>
<td>Cornu Ammonis area 1</td>
</tr>
<tr>
<td>CA-3</td>
<td>Cornu Ammonis area 3</td>
</tr>
<tr>
<td>CCI</td>
<td>Controlled cortical impact</td>
</tr>
<tr>
<td>CD45</td>
<td>Cluster of Differentiation 45</td>
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<td>CD68</td>
<td>Cluster of Differentiation 68</td>
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<tr>
<td>Cdc42</td>
<td>Cell division control protein 42</td>
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<tr>
<td>CHI</td>
<td>Closed head injury</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage associated molecular patterns</td>
</tr>
<tr>
<td>Dcx</td>
<td>Doublecortin</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>DLK</td>
<td>Dual leucine zipper kinase</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal related protein kinase</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FJC</td>
<td>Fluorojade-C</td>
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<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GCS</td>
<td>Glasgow Coma Score</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
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</table>
GRF  Growth hormone releasing factor
GWAS  Genome-wide Association Study
IGF-1  Insulin-like growth factor-1
IGL  Inner granular layer
IL-1b  Interleukin 1 beta
IL-6  Interleukin 6
IL-10  Interleukin 10
JIP  JNK-interacting protein
JNK  c-Jun N-terminal kinase
Ki67  Antigen Ki-67
KO  Knock-out
MAPK  Mitogen-activated protein kinase
MAPKK  Mitogen-activated protein kinase kinase
MAPKKK  Mitogen-activated protein kinase kinase kinase
MEKK4  Mitogen-activated protein kinase kinase kinase 4
MKK2  Mitogen-activated protein kinase kinase kinase 2
mTBI  Mild traumatic brain injury
MONS  Mono-ocular nerve stretch
NAD+  Nicotinamide adenine dinucleotide
NeuN  Neuronal Nuclei
NF  Neurofilament
NGF  Nerve growth factor
NGS  Normal goat serum
NDS  Normal donkey serum
NMNAT  Nicotinamide mononucleotide adenylyltransferase 1
NOR  Novel Object Recognition
NSS  Neurological Severity Score
NT-3  Neurotrophin-3
OGL  Outer granular layer
PACAP38  Pituitary adenylate cyclase activated polypeptide 38
PAGE  Polyscrylamide gel electrophoresis
PBS  Phosphate buffered saline
PC6  Pheochromocytoma 6 cell line
PD  Parkinson’s disease
PFA  Paraformaldehyde
PI3K  Phosphatidylinositol-3 kinase
PI(3,4,5)P3  Phosphatidylinositol 3,4,5-triphosphate
PI(4,5)P2  Phosphatidylinositol 4,5-bisphosphate
PKC  Protein kinase C
PTEN  Protein and tensin homolog
Rab  Ras-related GTP-binding protein
Rac  Ras-related C3 botulinum toxin substrate
Ran  Ras related nuclear protein
Ras  Rat sarcoma
RAWM  radial arm water maze
Rho  Ras homolog gene family
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
</tr>
<tr>
<td>Ric</td>
<td>Ras-related protein which interacts with calmodulin</td>
</tr>
<tr>
<td>Rin</td>
<td>Ras-related protein in neurons</td>
</tr>
<tr>
<td>RIN1</td>
<td>Ras interaction protein-1</td>
</tr>
<tr>
<td>Rit</td>
<td>Ras-related protein in tissues</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>Ribonucleic acid interference</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress-activated protein kinase</td>
</tr>
<tr>
<td>Sarm1</td>
<td>Sterile alpha and TIR containing 1</td>
</tr>
<tr>
<td>SCG10</td>
<td>Superior cervical ganglion-10</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate poly-acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error mean</td>
</tr>
<tr>
<td>SGZ</td>
<td>Sub-granular zone</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2 domain</td>
</tr>
<tr>
<td>SMI32</td>
<td>Nonphosphorylated neurofilament</td>
</tr>
<tr>
<td>Sos1</td>
<td>Son of sevenless 1</td>
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<tr>
<td>SSF</td>
<td>Selection sampling fraction</td>
</tr>
<tr>
<td>TAI</td>
<td>Traumatic axonal injury</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TSF</td>
<td>Thickness sampling fraction</td>
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<tr>
<td>Ube4b</td>
<td>ubiquitination factor e4b</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>Wld^s</td>
<td>Wallerian slow mutant</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
</tr>
</tbody>
</table>


VITA

Megan Pannell

Education

**University of Kentucky**: Doctor of Philosophy in Biochemistry

**Indiana University**: Master of Science in Biotechnology, highest distinction, 2012

**Transylvania University**: Bachelor of Arts in Biology and Psychology, cum laude, 2011

Publications


Abstracts


Cai W, **Pannell MD**, Carlson S, Brelsfoard J, Saatman K and Andres D. Divergent roles for the Rit and Rin GTPases in neuronal survival following


Poster Presentations


Oral Presentations, Seminars, and Journal Clubs

“RINdering protection against traumatic brain injury” University of Kentucky Biochemistry Research Conference. May 2016

“Rin GTPase deficiency provides neuronal protection following head injury” University of Kentucky Biochemistry Student Seminar Series. Oct 2015

“Rin loss mediates neuroprotection following traumatic brain injury” University of Kentucky Biochemistry Graduate Student Data Club. May 2015

“Identifying the link between LRRK2 kinase activity and Parkinsons’ disease toxicity” University of Kentucky Biochemistry Student Seminar Series. Jan 2015

“Dual leucine zipper kinase mediates retinal ganglion cell death” University of Kentucky Biochemistry Seminar. Jan 2014

“Virus derived small RNAs regulate transition from transcription to replication in Influenza A.” Indiana University Biochemistry. Apr 2012

“Graduate school admissions advice for undergraduates: focus on biotechnology” Indiana University Undergraduate Biotechnology Seminar Series. Nov. 2011


Honors and Awards

NIH Training Grant 5T32 NS077889, Neurobiology of CNS Injury and Repair, 2015-2017

American Society for Biochemistry and Molecular Biology Travel Award, 2015

University of Kentucky Graduate School Travel Award, 2015

First Year Graduate Fellowship, University of Kentucky, 2012-2013

Pharmaceutical Science Summer Research Fellowship, University of Kentucky College of Pharmacy, 2010

John B. Brownie Young Scholar, 2007-2012

American Commercial Lines National Merit Scholarship Recipient, 2007-2011

Morrison Scholar, Transylvania University 2007-2011

Jenny V. Watson Scholar, 2007-2011