TARGETING ACUTE PHOSPHATASE PTEN INHIBITION AND INVESTIGATION OF A NOVEL COMBINATION TREATMENT WITH SCHWANN CELL TRANSPLANTATION TO PROMOTE SPINAL CORD INJURY REPAIR IN RATS

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Anatomy and Cell Biology,
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July 2013

Accepted by the Faculty of Indiana University, in partial
fulfillment of the requirements for the degree of Doctor of Philosophy.

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ACKNOWLEDGEMENTS

First, I would like to thank my wife, Leslie, for her support during the late nights studying or working in the lab, and our daughter, Stella, for bringing never ending joy to my life. Without them, I would not be where I am today. Special thanks go to my parents for their constant support of the pursuit of education. Though they are no longer here, I can imagine how proud they are of what I have accomplished, and that I stayed true to myself along the journey.

I am grateful for Dr. Nai-Kui Liu's time, training, and advice during the course of my study in the Xu lab. He has a truly exceptional mind in science and an eye for reading between the lines in experiment design and interpretation. Dr. Liu is irreplaceable for trainees needing a ready and experienced guide.

Of course, I must thank Dr. Xiao-Ming Xu for his foresight into my potential and always pushing me to do my very best in all that I do. His knowledge and wisdom were invaluable, and his willingness to traverse exciting roads and allow freedom of exploration in research helped foster my development into a highly-skilled independent researcher. I will carry my experiences during training in Dr. Xu's lab in a special place that will provide impetus for making the good experiment "great".

Lastly, I thank my research advisory committee for steering me towards the more important goals in graduate research training and for all the advice and suggestions to attain them. This guidance will always be remembered and appreciated.

ABSTRACT

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TARGETING ACUTE PHOSPHATASE PTEN INHIBITION AND INVESTIGATION OF A NOVEL COMBINATION TREATMENT WITH SCHWANN CELL TRANSPLANTATION TO PROMOTE SPINAL CORD INJURY REPAIR IN RATS

Human traumatic spinal cord injuries (SCI) are primarily incomplete contusion or compression injuries at the cervical spinal level, causing immediate local tissue damage and a range of potential functional deficits. Secondary damage exacerbates initial mechanical trauma and contributes to function loss through delayed cell death mechanisms such as apoptosis and autophagy. As such, understanding the dynamics of cervical SCI and related intracellular signaling and death mechanisms is essential.

Through behavior, Western blot, and histological analyses, alterations in phosphatase and tensin homolog (PTEN)/phosphatidylinositol-3-kinase (PI3K) signaling and the neuroprotective, functional, and mechanistic effects of administering the protein tyrosine phosphatase (PTP) inhibitor, potassium bisperoxo (picolinato) vanadium ([bpV[pic]) were analyzed following cervical spinal cord injury in rats. Furthermore, these studies investigated the combination of subacute Schwann cell transplantation with acute bpV(pic) treatment to identify any potential additive or synergistic benefits. Although spinal SC transplantation is well-studied, its use in combination with other therapies is necessary to complement its known protective and growth promoting characteristics.

The results showed 400 μg/kg/day bpV(pic) promoted significant tissue sparing, lesion reduction, and recovery of forelimb function post-SCI. To further clarify the mechanism of action of bpV(pic) on spinal neurons, we treated injured spinal neurons in vitro with 100 nM bpV(pic) and confirmed its neurprotection and action through inhibition of PTEN and promotion of PI3K/Akt/mammalian target of rapamycin (mTOR) signaling. Following bpV(pic) treatment and green fluorescent protein (GFP)-SC transplantation, similar results in neuroprotective benefits were observed. GFP-SCs alone exhibited less robust effects in this regard, but promoted significant ingrowth of axons, as well as vasculature, over 10 weeks post-transplantation. All treatments showed similar effects in forelimb function recovery, although the bpV and combination treatments were the only to show statistical significance over non-treated injury. In the following chapters, the research presented contributes further understanding of cellular responses following cervical hemi-contusion SCI, and the beneficial effects of bpV(pic) and SC transplantation therapies alone and in combination. In conclusion, this work provides a thorough overview of pathology and cell- and signal-specific mechanisms of survival and repair in a clinically relevant rodent SCI model.

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CHAPTER 1

INTRODUCTION

Background

Nearly 2 million Americans experience traumatic spinal cord (SCI) and brain injuries (TBI) each year (Loane and Faden, 2010, NSCISC, 2011), though unfortunately, few treatments are currently available. Investigating cell-specific responses, including signal pathways and associated proteins, is important for understanding spinal cord and brain pathology and neuroprotection, which could enhance therapeutic development. Two widely studied pathways involved in cellular responses in both normal and pathological conditions within the CNS are the phosphatidylinositol-3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways. These cascades are widely known for their roles in promoting survival, growth and proliferation (Chang and Karin, 2001, Cantley, 2002); however, their influence is not always beneficial following CNS injury. Much research has focused on protection of spared nervous tissue from progressive biochemical and inflammatory damage, and regeneration of damaged axons following primary mechanical trauma, both with limited success. Such trouble likely involves the complexities of these cellular responses following injury.

Unlike the peripheral nervous system (PNS), the CNS lacks inherent regenerative ability following injury (Schwab and Bartholdi, 1996). Contributing to this inhibition are myelin related proteins (Cadelli and Schwab, 1991), including myelin associated glycoprotein (MAG) (McKerracher et al., 1994), Nogo-A

(GrandPré et al., 2000), and oligodendrocyte myelin glycoprotein (Omgp) (Wang et al., 2002). Astroglial-associated inhibitory molecules, including chondroitin sulfate proteoglycans (CSPGs), contribute to the extracellular matrix within the inhibitory glial scar (Dow et al., 1993). These examples highlight just a few of the obstacles in treating CNS injury. Recent research, however, has shown remarkable advances in manipulating such barriers, even demonstrating the ability to promote CNS axonal regeneration (Park et al., 2008, Liu et al., 2010c). Extensive literature currently exists on the variation and influence of intracellular signaling on neuroprotection, regeneration, and functional recovery following SCI and TBI, and tools are now available which hold the potential for promoting these benefits. The following section highlights the progression of pathology, signaling through the PTEN/PI3K pathway, and how it may be modulated to improve neuroprotection and recovery following CNS injury. These topics are the emphasis of this body of work, with a focus on targeting cellular signaling, as well as to present a novel combined two-phase therapy combining small molecule PTEN inhibitor bpV(pic) and subacute Schwann cell transplantation for improving the anatomical and neurological outcome following traumatic cervical hemi-contusion SCI in rats.

Pathological progression following CNS injury

After traumatic CNS injury, damage proceeds by two mechanisms: the primary mechanical injury, and a subsequent multi-factorial secondary injury. The initial physical tissue disruption includes axonal stretching and myelin damage, local

cellular destruction and necrosis, and vascular disruption, resulting in infiltration of inflammatory and foreign molecules and cells into the typically secluded parenchyma of the CNS (Tator and Fehlings, 1991, Casella et al., 2006). Currently, the involvement and interaction of cellular signaling pathways mediating destructive responses after traumatic CNS injury is unclear. Each cell type has a unique mechanism of reacting to injury or insult, ranging from neuronal functional disruption and degradation to glial growth, proliferation, and migration. As the interaction between the various cells and structures within the CNS is essential to the health and function of each and the organism as a whole, attempting a single systemic or even local therapy proves insufficient for complete neuroprotection. Though two different cell types may share similar signaling pathways, the activation, and downstream signaling within and between these pathways may be vastly different. As such, targeting the inhibition of a potentially detrimental signaling step in neurons may have contradictory or undesirable effects on other neural cell types. Nonetheless, limiting the expansion of cell death and tissue damage are primary goals for acute treatment following SCI and TBI and other CNS injuries.

Part of the contribution to cell death following CNS trauma develops from ischemic events resulting from the dynamic vascular response that occurs near the site of injury following trauma. Cerebrovascular hypoxia/ischemia, characteristic of stroke, leads to an anatomical and physiological outcome similar to traumatic injuries. Following all these disruptions of normal CNS function, a core, or epicenter develops, in which all cells rapidly die due to extensive

localized physical disruption or dysfunction of normal cellular activities. Surrounding the core is a penumbra of damaged, but surviving cells that can die as secondary injury spreads. Without treatments to prevent this spread of tissue damage, the core and penumbra of the insult expand radially, occupying a greater extent of CNS tissue, and potentially leading to more extensive functional abnormalities. Therefore, understanding and treating such injuries and disease during the acute phase (within 1 week following injury) with the goal of stemming this expansion is a prime goal of experimental and clinical research. Due to the importance and urgency of advancing our knowledge and progress in this area, this review aims to highlight cellular response including cell death and survival, and the mechanisms that may be potential targets for improved prognosis in SCI, TBI, and stroke.

Necrosis

Upon injury to the CNS, significant white matter area damage occurs and the full extent of local gray matter is destroyed within 24 hours (Ek et al., 2010). This rapid death of local neurons and glial cells at the injury epicenter occurs through necrosis and spreads outward from the epicenter over time (Hausmann et al., 2002). Necrotic cells enlarge through permeability of the cell membrane and swelling, and eventually rupture and contribute to the inflammatory response in the injury area. Furthermore, extensive release and cellular reactivity to a variety of inflammatory-related cytokines and chemokines contribute to progressive tissue damage following trauma (Helmy et al., 2011).

The spread of necrosis coincides with a spread of inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin-6β (IL-6β) (Donnelly and Popovich, 2008) and infiltration of neutrophils and other leukocytes from the damaged vasculature (Milligan and Watkins, 2009). The chronic anatomical result of a contusive CNS injury is a system of cavities and fluid-filled cysts sealed by extensive glial scar formation (Tator, 1995).

Apoptosis

Though necrosis is an early cell death mechanism instigated by injury, delayed programmed cell death, including apoptosis and macroautophagy also contribute to cell loss and tissue pathology. Unlike necrosis, cells induced to undergo apoptosis shrink, fragment into smaller membrane-bound structures, and are removed through phagocytosis. Neurons respond poorly to mitochondrial dysfunction, as mitochondria produce adenosine triphosphate (ATP), help reduce reactive oxygen species (ROS), and are involved in regulating the quantity of calcium within the cell. Mitochondrial disruption, through outer membrane disruption or other insult, dysregulates some or all of these processes, which in turn diminishes neuron health. Additionally, an important mitochondria-related process involved in programmed neuronal cell death is the release of cytochrome c from damaged mitochondria.

Cytochrome c released from the damaged mitochondrial inter-membrane space interacts with apoptotic protease-activating factor 1 (APAF1) in the cytosol, resulting in the formation of the apoptosome. Apoptosomes are responsible for

activating caspases, including the well-known catalytic enzyme caspase 3, that are responsible for promoting apoptotic cell death (Pasinelli et al., 1998, Tait and Green, 2010). Physical or secondary neuronal injury can lead to mitochondrial instability, resulting in loss of neurons and other local cells post-injury. Disruptions in normal mitochondrial function have been linked to many neurodegenerative conditions, including ischemic brain injury (Sas et al., 2007).

Recent evidence suggests that the phosphatase and tensin homolog (PTEN) induced kinase-1 (PINK-1) is critical for mitochondrial activity and protection, as well as PI3K/Akt signal-mediated inhibition of downstream factors that promote cell death following CNS injuries or diseases (Shan et al., 2009, Akundi et al., 2012). Perhaps as part of a feedback mechanism, evidence suggests that Akt may directly interfere with PINK-1 expression and that PTEN enhances its expression (Unoki and Nakamura, 2001). It is widely known that activity of the mammalian target of rapamycin (mTOR) is a major inhibitor of the progression of apoptosis, and stability of mitochondrial function may result from PINK-1-induced upregulation of Akt activity and its involvement in the activation of mTOR (Akundi et al., 2012).

Autophagy

Another process considered by many as a separate form of programmed cell death, called autophagy, or Type II cell death (Baehrecke, 2005, Levine and Yuan, 2005), is also inhibited by active mTOR, though the interplay between apoptosis and autophagy is complex and hinders interpretation of analysis

(Shang et al., 2010). In addition, biological processes involving autophagy have been shown to also occur through mTOR-independent mechanisms (Sarkar et al., 2007, Sarkar et al., 2009), underscoring the complexity of identifying specific cell signaling effects on survival and death. Autophagy is a normal physiological cellular process by which cells recycle aged organelles and proteins. Though autophagy is quite complex and not fully understood, it is apparent that basal function prevents intracellular accumulation of debris and generation of nutrients from intracellular degradation under starvation conditions (Mizushima and Komatsu, 2011). Three types of autophagy have been classified: 1) microautophagy, during which a cell intakes extracellular material through invaginations of the cell membrane, 2) chaperone-mediated autophagy, which requires heat shock proteins for proper lysosomal delivery and degradation of damaged or irregular proteins, and 3) macroautophagy, during which the cell digests its own internal organelles or proteins for nutrients during times of stress (Klionsky et al., 2005, Pereira et al., 2012). Macroautophagy is the most fully characterized, and most commonly assessed following injury and disease. Therefore, this process is often referred to simply as autophagy, as is done here. Despite the known benefits of autophagy under stressful conditions, dysregulated autophagy is suggested to be a detriment to cell health and survival, and neurons are especially susceptible to dysfunction of autophagic processes (Mizushima et al., 2008).

Enhanced neuronal autophagy is suggested to contribute to cell death in some CNS disease or injury models, including SCI (Wang et al., 2008, Wen et

al., 2008, Kanno et al., 2009, Kanno et al., 2011). As autophagosome convergence with lysosomes to form autolysosomes, which is important in normal functioning of intracellular degradation (Chen and Klionsky, 2011), deregulation of lysosomal cathepsins B and D expression has been shown to occur following autophagy-inducing nutrient stress *in vitro* (Shibata et al., 1998, Uchiyama, 2001). Such evidence suggests disruption of processes downstream of autophagosome formation, blocking normal degradation and causing accumulation of waste-filled autophagosomes, promotes autophagy-induced neurodegeneration in contrast to an upregulation of autophagy and autophagosome production. As such, the matter of whether injury increases autophagy, or autophagy exacerbates injury is still debated. Nonetheless, autophagic activity or dysfunction caused by, or contributing to, pathology to CNS tissue may increase to a detrimental level, eventually proceeding to delayed cell death.

As a major part of this process in neurons, dynamic intracellular vesicle formation occurs, resulting in construction of the double-membrane autophagosome that transport of material from the neuron soma along extended processes, and back to the cell body (Xie and Klionsky, 2007, Yang et al., 2008, Yang et al., 2011). Resulting from its consistent location within the isolation and autophagosome membrane, lipidated microtubule associated protein light chain 3 (LC3 II) is a widely accepted marker of autophagosomes (Kabeya et al., 2000) and are monitored for changes in autophagosome formation and clearance. LC3 II-positive punctate aggregations of autophagosomes post-SCI have been

observed surrounding the injury site within one to three days following thoracic contusive SCI (Kanno et al., 2011). TUNEL-positive cells co-localize with Beclin-1 and LC3-positive cells, indicating autophagosome aggregation precedes apoptosis (Kanno et al., 2009, Kanno et al., 2011). We showed that LC3 II-positive autophagosomes increased in neurons following SCI, and treatment that upregulated neuroprotection, function, and Akt and mTOR activity reversed this accumulation and reduced LC3 II protein levels (Walker et al., 2012) (Chapter 2). A recent study confirmed that autophagosome and ubiquitinated protein accumulation occurs in the pathology of SCI, and was reversible through neuroprotective activation of the PI3K/Akt/mTOR pathway (Zhang et al., 2013). In light of these and our own findings, reduction of autophagy is a promising proposition for reducing neural damage following SCI.

PTEN and PI3K/Akt/mTOR signaling

PI3K signaling is often triggered by extracellular growth factor activation of a receptor tyrosine kinase (RTK) or G-protein coupled receptor (GPCR) (Engelman et al., 2006) (Fig. 1). Once active, PI3K can phosphorylate phosphatidylinositol-4,5-phosphate (PIP_2) to form phosphatidylinositol-3,4,5-phosphate (PIP_3) (Engelman et al., 2006). PIP_3, a multipurpose secondary messenger, promotes activation of the survival kinase Akt (also known as PKB), and its membrane localization through activity of 3-phosphoinositide dependent protein kinases (PDK) (Alessi et al., 1997). Thus, PIP_3 production is essential for PI3K-mediated pro-survival signaling through Akt and its effectors. Antagonizing PI3K in PIP_2

conversion, however, is the phosphatase and tensin homologue deleted on chromosome ten, better known as PTEN. Encoded by the *pten* gene mapped to chromosome 10q23, the 55 kD PTEN protein is a dual-function protein tyrosine phosphatase that can dephosphorylate both proteins and lipids (Agrawal and Fehlings, 1997). Its enzymatic active site, however, has more affinity for the latter, especially PIP_3 (Lee et al., 1999). The physiologic function of PTEN is highly important for processes including cellular proliferation and neuronal growth regulation (Dahia, 2000, Kwon et al., 2001). In addition, downregulating PTEN's function or expression promotes axon regeneration and neuroprotection following CNS trauma (Park et al., 2008 (Zhang et al., 2007a, Park et al., 2008, Liu et al., 2010c, Walker et al., 2012) (Fig. 1).

PTEN is highly expressed in adult CNS neurons (Cai et al., 2009, Liu et al., 2010c), and neuroprotective effects of its inhibition are usually attributed to disinhibition of PI3K and downstream signaling through Akt (Zhang et al., 2007a, Sury et al., 2011, Walker et al., 2012) and the mammalian target of rapamycin (mTOR) (Shi et al., 2009, Zhong and Bowen, 2011). It is well known that mTOR inhibits the progression of apoptosis and autophagic cell death (Baehrecke, 2005, Levine and Yuan, 2005). Activity of mTOR is also linked to axonal regeneration following PTEN deletion (Park et al., 2008, Liu et al., 2010c, Sun et al., 2011). The understanding of the signaling steps between PTEN and mTOR involved in these events are not quite clear, though Akt activity is potentially involved based on its known effects and documented response to injury.

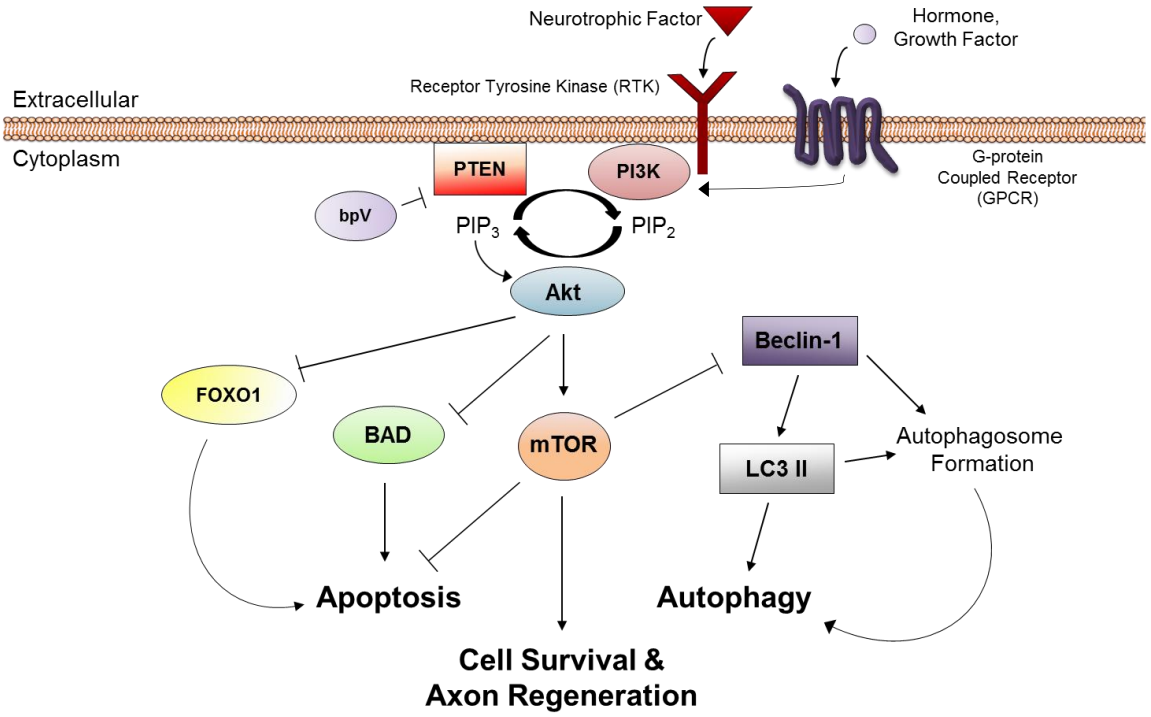

Figure 1. PTEN reduces PI3K/Akt signaling benefits on cell survival and regeneration. PI3K can be stimulated through RTK or GPCR-mediated signaling, promoting Akt inhibition of several apoptosis-associated proteins such as Bad and FOXO1, and promotion of pro-survival mediators such as mTOR. PTEN antagonizes PI3K, and the resulting reduction in downstream Akt and mTOR signaling promotes programmed cell death i.e. apoptosis and autophagy. PI3K = Phosphatidylinositol 3-Kinase; PTEN = Phosphatase and Tensin Homolog; mTOR = mammalian Target of Rapamycin; BAD = Bcl-2-associated death promoter; FOXO1 = Forkhead box protein O1; LC3 II = Microtubule-associated protein light chain 3 II.

Akt phosphorylation decreases within the lesion area following SCI (Yu et al., 2005, Walker et al., 2012), while increasing in neurons through a PI3K-dependent mechanism within the surrounding injury penumbra (Yu et al., 2005, Endo et al., 2006, Howitt et al., 2012). Akt phosphorylation at serine 473 peaks 8 hours post-injury within this perilesional tissue (Yune et al., 2008), and diminishes through 24 and 48 hours following trauma (Yune et al., 2008, Walker et al., 2012). Similarly, phosphorylation at this site decreases rapidly within the injury epicenter following TBI, while transiently peaking at 4 hours post-injury in the penumbra and co-localizing with its downstream effectors phosphorylated Bad and GSK-3β (Noshita et al., 2001). By 24 hours post-TBI, apoptotic co-labeling with phospho-Akt is not observed (Noshita et al., 2001), further associating Akt activation with cell survival following CNS injury.

Delayed phosphorylation of ribosomal protein S6 at serines 235/236, commonly used markers for mTOR activity, is observed 24 hours post-SCI (Walker et al., 2012). Further study could uncover a similar downstream mTOR activity pattern following TBI. mTOR, also known by the name FRAP (FKBP and rapamycin-associated protein), is a large serine/threonine kinase (289 kD) responsible for detecting energy or nutrient variations within the cell, and is highly important in regulating key cellular functions in response to the energy or stress status of the cell (Proud, 2004). mTOR is activated upon phosphorylation at serine 2448, and functional interactions with other proteins forms two distinct enzymatic complexes, mTORC1 and mTORC2. mTORC1, the rapamycin-sensitive complex, can be activated indirectly through Akt via phosphorylation of

the tuberous sclerosis complex protein 2 (TSC2) (Inoki et al., 2002), which prevents mTOR inhibition (Inoki et al., 2002, Jaeschke et al., 2002, Tee et al., 2002, Manning and Cantley, 2007).

Primary effectors of mTOR are ribosomal protein p70S6 kinase (p70S6K) and 4E binding protein-1 (4E-BP1) (Proud, 2002). Phosphorylation of p70S6K stimulates its phosphorylation of ribosomal protein S6, initiating a variety of translation-associated activities. Phosphorylation of 4E-BP1 by mTOR promotes translation, as well. Some of the most exciting aspects of mTOR's activation have been observed following PTEN inhibition or genetic deletion (Park et al., 2008, Liu et al., 2010c, Walker et al., 2012, Zhong et al., 2012). A recent report suggested that exercise upregulates ribosomal protein S6 activity in intermediate grey matter interneurons at 10 and 31 days post-SCI (Liu et al., 2010b), posing an interesting question as to whether extensive behavioral testing or training activities in SCI and TBI research affect plasticity and neural tissue survival through mTOR-associated signaling.

Increased phospho-Akt in neurons of the injury penumbra (Yu et al., 2005, Endo et al., 2006, Howitt et al., 2012) suggests the natural upregulation of this pathway may represent an acute endogenous protective response to insult (Noshita et al., 2001), especially if followed by a progression of mTOR activation. Though this explanation is quite plausible, a better grasp of the temporal progression of intracellular pro-survival PI3K/Akt/mTOR signaling within penumbral neurons and glia is necessary to effectively identify specific signaling targets and therapeutic time windows for promoting neuroprotection and repair

following CNS injury. Nevertheless, enough evidence exists suggesting that activation of Akt/mTOR, through PTEN inhibition or other means, is likely neuroprotective and growth-promoting following injury to the CNS.

Tools for studying PI3K-related signaling in neural degeneration and repair

Variation in cellular signal transduction complicates investigation of the mechanisms of protection and pathology following CNS injury. The use of transgenic animals has allowed for more accurate and reliable assessments in such studies. Knocking-out specific signaling proteins affords discrete assessment of their role in cellular signaling effects. However, pharmacological approaches to experimental and clinical treatment are often more practical and accessible than genetic manipulation, even though such knock-out investigations are critical to highlight potential targets for pharmacological therapeutics. Table 1 highlights some of the most commonly used chemical inhibitors, their targets, and functions for assessing the roles of particular steps of these pathways and for experimental assessment of their benefits through modulation in animal models of CNS injury and disease.

A large body of literature currently exists describing many processes and treatments that may act through stimulating PI3K/Akt/mTOR axis signaling in mediating neuroprotection. In general, many therapies may incite neuroprotective signaling through interaction and activation of extra- and intracellular domains of receptor tyrosine kinases (RTKs). We have shown that GDNF exerts beneficial effects through interaction with GFRα1 and its partner RTK, cRet, and potentially

through neural cell adhesion molecule (NCAM) interaction on neurons (Zhang et al., 2009). GDNF, however, is known to promote neurite outgrowth *in vitro* via Erk 1/2, and not PTEN/PI3K signaling (Koelsch et al., 2010). *In vivo* however, Liu et al. (2010b) have shown that viral-mediated conditional deletion of PTEN in cortical neurons promotes enhancement in axon sprouting and regrowth in the spinal cord, with upregulated mTOR activity being a likely key intermediary in promoting such benefits. Previous work has also demonstrated this phenomenon following similar methods of PTEN deletion in an optic nerve injury animal model (Park et al., 2008). As such, PI3K pathway signaling can promote axonal regeneration depending on the stimulus and the conditions of the neurons under study.

Inhibition of PTEN by bisperoxovanadium

Techniques that involve prevention of specific gene or protein expression provide new possibilities for intracellular upregulation of pro-survival signaling which can have beneficial effects that occur without extracellular stimulation by an trophic factor or ligand. However, deletion of an enzyme may have unintended effects on other aspects of cellular function. Pharmacological enzymatic disruption of signaling molecules like PTEN provides a much more convenient and less extreme method of assessing an enzymes' activity. For example, bisperoxovanadium compounds, also known as bpVs, specifically inhibit PTEN signaling, and have been used for promotion of neuroprotection in many CNS disease and injury models including Parkinson's disease, meningitis, stroke, and

SCI (Yang et al., 2007, Zhang et al., 2007a, Nakashima et al., 2008, Sury et al., 2011, Walker et al., 2012, Mao et al., 2013).

There are several members of the bpV family of compounds including bpV(pic), bpV(OHpic) and bpV(phen), all of which have high affinity and potency for inhibition of PTEN (Schmid et al., 2004). In neuroprotection studies using bpV compounds (Zhang et al., 2007, Sury et al., 2011, Liu et al., 2010a, Nakashima et al., 2008, Walker et al., 2012), potentially detrimental systemic effects were not observed or reported, however, more investigation is necessary to further verify if bpV has undesired off-target effects that may need consideration. Also, support for bpV as a CNS injury therapy requires further investigation for treatment of other injuries including TBI. Nonetheless, current evidence suggests that small molecule inhibition of PTEN lipid phosphatase function appears to be an effective, easily controlled, and relatively safe means of reducing the extent of tissue damage and enhancing resulting functional recovery. To investigate signaling protein effects, or to alter cell signaling in ways similar to bpV, a wide variety of chemical inhibitors are commercially available. Table 1 lists several commonly used PI3K/Akt/mTOR pathway signaling and related inhibitors.

PI3K/Akt/mTOR, autophagy, and apoptosis inhibitors

PI3K inhibitors include LY294002 and also wortmannin (Arcaro and Wymann, 1993, Vlahos et al., 1994). These are often used as potential therapeutics in cancer biology, due to the common upregulation of PI3K and Akt signaling observed in tumorigenic cells. However, they are also useful in determining PI3K

or downstream pathway effects in different neurological conditions both *in vitro* and *in vivo*. Due to PI3K's involvement in many cellular processes including Akt activation and signaling the range of applications for use of these compounds is wide. Important potential uses include investigation of acute effects of PI3K/Akt deactivation or activation following CNS trauma and long term anatomical and functional therapeutic benefits or deficits. Akt has many inhibitors, the most commonly reported being Akt inhibitor IV, which inhibits ATP binding of an enzyme upstream of Akt yet downstream of PI3K (Kau et al., 2003), resulting in reduced Akt activity in tissue and cell samples. Again since Akt activation is tightly controlled by PI3K activity, PI3K inhibition also results in reduced Akt activity.

Rapamycin has long been known for its antibiotic function, and has been used as a therapeutic agent to elucidate mTOR influence on neuronal fate post-injury. One recent study suggests rapamycin can promote autophagy and cell survival through mTOR inhibition after SCI (Sekiguchi et al., 2012) and stroke (Chauhan et al., 2011, Yan et al., 2011), while others suggest rapamycin-mediated autophagy promotes neurodegeneration following CNS injury (Grishchuk et al., 2011).

Table 1. PTEN/PI3K pathway and related inhibitors, their targets and actions.

PI3K pathway inhibitor	Target	Action	Reference
Bisperoxovanadium (bpV)	PTEN	Inhibits PTEN phosphatase activity; Upregulates PI3K/Akt signaling	Schmidt et al., (2004). FEBS Lett, 566(1-3):35-8.
LY294002	PI3 Kinase	Blocks PI3K activity; Reduces phosphorylation of Akt at serine 473	Vlahos, C. (1994). J. Biol. Chem, 269:5241-5248.
Wortmannin	PI3 Kinase	Blocks PI3K activity; Reduces phosphorylation of Akt at serine 473	Arcaro, A. and Wymann, M.P. (1993). Biochem. J, 296:297-301.
Akt Inhibitor IV	Akt	ATP-competitive inhibitor of a kinase upstream of Akt but downstream of PI3K	Wang, G., et al., (2006). Proc. Natl. Acad. Sci. U.S.A, 103: 4640-4645.
Rapamycin	mTOR C1	Inhibits mTOR's ability to phosphorylate p70S6 Kinase or 4E binding Protein I	Kunz, J. et al. (1993). Cell, 73:585-596; Brown, E. J. et al. (1994). Nature, 369:756-758.

These discrepancies are debatable, as described earlier, and may be injury type-dependent. Nonetheless, rapamycin has proven to be a useful tool in examining mTOR signaling both *in vitro* and *in vivo*. 3-methyladenine (3-MA) (Seglen and Gordon, 1982) is now considered a commonly associated autophagy inhibitor, and thus can be used in experiments to verify if progression of autophagy is pathologic or beneficial following injury, as well as used in conjunction with other pathway inhibitors, e.g. caspase inhibitor Ac-DEVD-CMK, to establish mechanisms of action within cells in response to injury, disease, or treatment.

Schwann cell transplantation for SCI

One of the most studied treatments for SCI is Schwann cell (SC) transplantation. This therapy has been investigated extensively due to the benefits these cells promote once engrafted into the injured spinal cord. Named after German anatomist, Theodore Schwann (1810-1882), SCs are the myelinating cells of the peripheral nerve and main contributors to the repair mechanism following nerve damage. SCs derive from the neural crest during development, and mature from SC progenitors to myelinating or non-myelinating SCs through molecular control of axons and a variety of specific gene expression and trophic interactions (Fig. 2) (Mirsky and Jessen, 1996). To develop effective therapies for SCI, treatments should target multiple excitatory, inflammatory, and oxidative biochemical processes that occur within minutes and can persist chronically in the traumatically injured cord. Despite these obstacles, Schwann cells (SCs) have a

long history as a potential therapeutic for the treatment of SCI. For even longer, spinal cord and brain injuries were considered inoperable and unable to regenerate following damage. Regeneration studies, beginning as early as the beginning of the 20th century with the work of Ramon y Cajal (Ramon y Cajal, 1928), and reinvigorated several decades later (David and Aguayo, 1981, Bray et al., 1987), showed that spinal axons regenerated into grafted peripheral nerve, demonstrating the importance of environment for instigating a regenerative response from damaged axons.

Multiple methods exist to isolate, purify, and expand both rodent and human SCs in culture (Wood, 1976, Brockes et al., 1979, Morrissey et al., 1991, Casella et al., 1996). Once millions of SCs could be generated *in vitro*, approaches to SC transplantation were developed for laceration and channel transplantation studies, as well as in more clinically relevant contusive and compression SCI models. In contusive SCI transplantation studies, SCs have been shown to survive most effectively when delayed to 7 to 10 days post-SCI instead of directly into the inhospitable setting that develops within the injury area soon after trauma (Martin et al., 1996, Hill et al., 2006). Delaying implantation of the cells avoids not only the cytotoxic environment of the acute injury epicenter, but also allows for the development of cystic cavities in which to inject the cells into. Even still, SCs have been demonstrated to dramatically die off soon after transplantation, with the greatest loss within the first 24 hrs (Hill et al., 2006, Hill et al., 2007). It is interesting that endogenous SCs invade the cord from the PNS, crossing the glia limitans and weathering the harsh environment of the lesion

area following SCI (Blakemore, 1975, Raine, 1976, Beattie et al., 1997, Hill et al., 2006, Hill et al., 2007). Though migrated host SCs have been shown to promote some axon growth and myelination (Oudega and Xu, 2006), they too suffer cell loss (Hill et al., 2006, Hill et al., 2007) and do not sufficiently contribute to functional recovery. Furthermore, SCs fail to migrate and regenerated axons cannot extend into the caudal host tissue due to glial scar formation and inhibitory extracellular matrix and myelin-associated molecules. Overcoming these obstacles is essential for making functional connections for effective sensorimotor and autonomic recovery.

In light of these issues, it is likely that SCs alone will not be enough to cure SCI and fully repair CNS damage. Combination therapies with SC transplantation are gaining ground as the next wave in experimental SCI research; nevertheless, foundational studies over the past several decades are not without merit, and provide history of the biology and breadth of SC transplantation research for potential treatment of SCI.

SC biology and functions

During development, Schwann cell precursors derive from neural crest cells (Dupin et al., 1990), eventually developing into immature Schwann cells. A variety of environmental chemical cues help direct the fate of SCs during development (Fig. 2). The direction of SC survival and progression toward a mature phenotype is driven by β-neuregulin-1 (NrG1) (Dong et al., 1995). Stimulation of ErbB 2/3 receptor isoforms on the SC by axonal NrG1 type III sets

in motion appropriate SC myelination of interacting axons (Garratt et al., 2000, Nave and Salzer, 2006).

The process of axon myelination in the PNS by mature SCs occurs approximately at the time of birth in rats (Jessen and Mirsky, 2005). Unlike CNS oligodendrocytes, SCs have a “one-to-one” relationship with axons in that any individual SC interacts and myelinates only one axon. SCs generally myelinate axons of large diameter, while axons smaller in size are organized into Remak bundles by non-myelinating SCs. To accommodate myelination once in contact with an axon, promyelinating SCs alter their gene and protein expression patterns including upregulation of Krox20, and downregulating SOX2 and Jun expression (Jessen and Mirsky, 2005, Le et al., 2005, Parkinson et al., 2008). Micro-RNA (miRNA) production by the enzyme Dicer is involved in regulation of Krox20 mRNA expression, as well as myelination-related protein expression including myelin associated glycoprotein (MAG) and PMP22 (Pereira et al., 2010). Activation of Akt, which is suggested to promote SC survival, proliferation and myelination (Campana et al., 1999, Maurel and Salzer, 2000), is reduced by defects in Dicer activity in SCs (Pereira et al., 2010).

Though the interaction between an individual SC and an axon is one-to-one, many SCs are necessary to effectively myelinate a given peripheral axon and SCs line the length of an axon with each spirally ensheathing a segment in myelin. The exposed axonal gaps between SCs are called “nodes of Ranvier” (Salzer, 2002) (Fig. 2) at which the axon is uncovered and has low resistance to signal transduction. Myelin impedes ion transfer between the axon and

extracellular environment, and these nodes allow for rapid depolarization and unidirectional saltatory transduction of action potentials along the axon. Signals traveling in this manner are much faster than those sent along non-myelinated axons, making the SC and myelination indispensable for rapid communication between neurons and their targets in the PNS.

The role of SCs in repair of damaged axons

Along with their role in myelination, SCs produce numerous trophic factors and regulators of cellular activity. These include glial cell line-derived neurotrophic factor (GDNF) (Springer et al., 1994), brain-derived neurotrophic factor (BDNF) (Acheson et al., 1991, Meyer et al., 1992), and neurotrophin-3 (NT-3) (Offenhauser et al., 1995). Following trauma to peripheral nerves, severed axons undergo numerous physiologic changes over time. The distal end of the broken axon breaks down through a process known as Wallerian degeneration (Waller, 1850) and the proximal end, attached the body of the neuron, will also die back initially followed by a period of regrowth. At the proximal end, SCs go through many cellular and functional alterations including de-differentiation, involvement in debris removal, and trophic stimulation of axon regeneration. During this period, SCs maintain residence within the surrounding basal lamina forming conduits known as bands of Bungner (Bunge, 1994). When retracted axons begin to regrow, they do so through bands of Bungner with the trophic and structural support of the surrounding SCs and basal lamina.

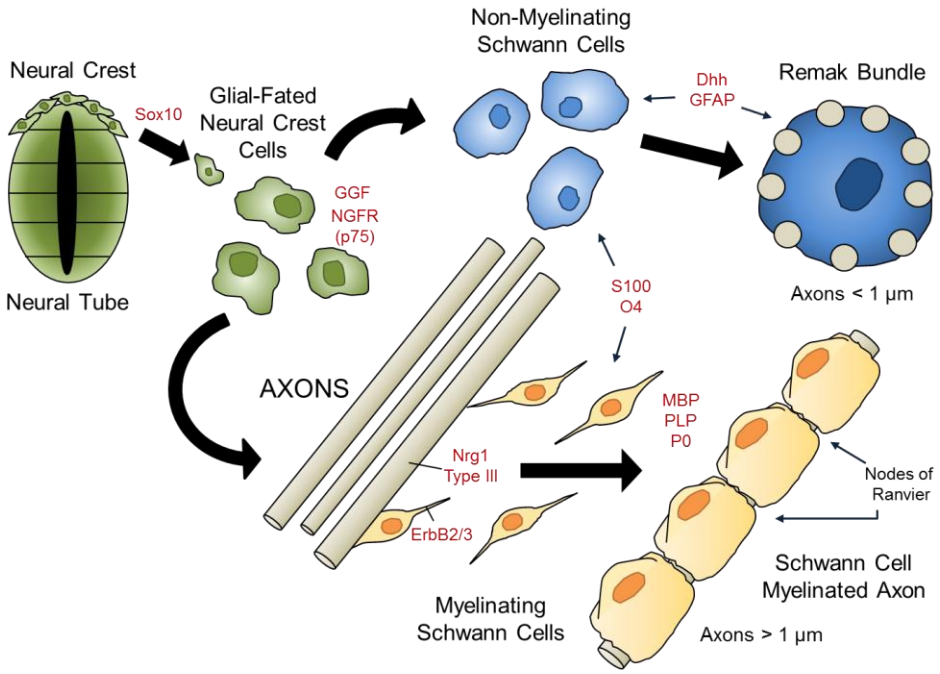

Figure 2. Development and differentiation of Schwann cells. Schwann cells derived from the neural crest progress toward a myelinating or non-myelinating phenotype depending on the presence and size of interacting axons, as well as trophic or other environmental cues. At each stage of development, Schwann cells and their precursors express specific markers that aid in the verification of the developmental status of the Schwann cell lineage. Dhh = Desert hedgehog; ErbB2/3 = SC Nrg receptors; GFAP = Glial fibrillary acidic protein; GGF = Glial growth factor; MBP = Myelin basic protein; NGFR(p75); Nerve growth factor receptor(p75); Nrg1 Type III= Neuregulin 1 Type III; O4 = SC glycolipid antigen; PLP = Myelin proteolipid protein; P0 = Myelin protein zero; S100 = neural crest glial protein; Sox10 = SRY-related HMG-box 10.

SCs and tissue repair following SCI

In addition to the PNS, it has long been known that SCs can promote CNS axon regeneration (Gilmore, 1971, Blakemore, 1975). The peripheral nervous system (PNS) environment is favorable for long distance CNS axon regeneration (Richardson et al., 1980, David and Aguayo, 1981, Bray et al., 1987). At the time, SCs were considered to be the primary factor contributing to axonal regeneration and remyelination within the nerve graft (Salame and Dum, 1985). From peripheral nerves, SCs can be isolated and expanded in culture using a variety of methods (Brockes et al., 1979, Porter et al., 1986, Morrissey et al., 1991, Casella et al., 1996). The use of mitogenic agents supported production of the large numbers of SCs required for transplantation (Porter et al., 1986), and since, purified SCs have been widely used as an important transplantation strategy for experimental and clinical treatment of SCI.

Limitations and methods of improving SC transplantation for SCI

In spite of the many advantages of SC transplantation, there are certain limitations that must be overcome to further the potential for SCs as a viable therapeutic for treating SCI. First, the primarily astrocytic glial scar that forms around the injury site proves a formidable barrier for SC migration and interaction with host tissue. Many studies have demonstrated the lack of interaction between engrafted SCs and the glial border of the lesion. This has also been shown *in vitro* through SC and astrocyte confrontation assays (Lakatos et al., 2000, Zhang et al., 2009). If SCs can migrate into the caudal host tissue, this could extend the

growth promoting properties of the SC graft to allow regenerating axons to make functional connections beyond the lesion.

Another major limitation of SC transplantation, axons may grow into the lesion and SC graft, but fail to exit into the caudal host tissue (Oudega and Xu, 2006). The glial scar not only prohibits SCs but also axons from extending across the lesion to intact host tissue, effectively reducing any functional advantage of SC-mediated axon regeneration. Lastly, SCs exhibit poor survival once transplanted into the contused spinal cord. Labelled SCs have been tracked following transplantation at various time points post-injury and even when transplanted at the optimal period for their survival (7-10d post-SCI) the vast majority die within the first week following SCI (Hill et al., 2006, Hill et al., 2007). To reap the benefits that SC transplantation can offer, promoting their survival is key to overcoming any other limitation of this therapy in treating SCI.

Fortunately, great effort has been exerted to surmount these obstacles. Compared to peripheral nerve grafts, advantages of using purified SCs includes the potential for transfection to over-express growth- and survival- promoting factors that enhance SC survival and axon regeneration within the host tissue following transplantation. Moreover, combination with tissue engineering materials adds scaffolding to enhance SCs ability to fill and bridge lesion gaps and cavities. Neurotrophic factor GDNF has proven beneficial for axonal growth into normal and lentiviral-transfected SC-seeded guidance channels (Iannotti et al., 2004, Deng et al., 2011, Deng et al., 2013). Providing a growth-promoting pathway caudal to the lesion with a gradient of such factors could enhance

axonal extension into host tissue for making functional connections with caudal neurons (Fig. 3). To allow for their exit from the graft, degradation of the glial scar is important and much work has focused on this area of study.

The most studied method of glial scar component degradation has been through application of the bacterial enzyme Chrondroitinase ABC. This enzyme targets chondroitin sulfate proteoglycans (CSPGs), an inhibitory component of the glial scar. Administration of this enzyme in transection SCI models has promoted propriospinal, as well as supraspinal axonal growth of the lesion and into the caudal host tissue (Chau et al., 2004, Vavrek et al., 2007). Growth factors such as GDNF, neurotrophin-3 (NT-3) and BDNF, delivered through transplanted genetically-modified SCs and otherwise, have been shown to enhance SC survival and minimize the inhibitory effect of astrocytes in the scar to promote a more permissive environment for SC migration and axon growth into host tissue (Menei et al., 1998, Girard et al., 2005, Zhang et al., 2007b, Deng et al., 2011, Deng et al., 2013). Figure 3 illustrates these primary challenges and potential solutions to overcome them for progressing SC transplantation research.

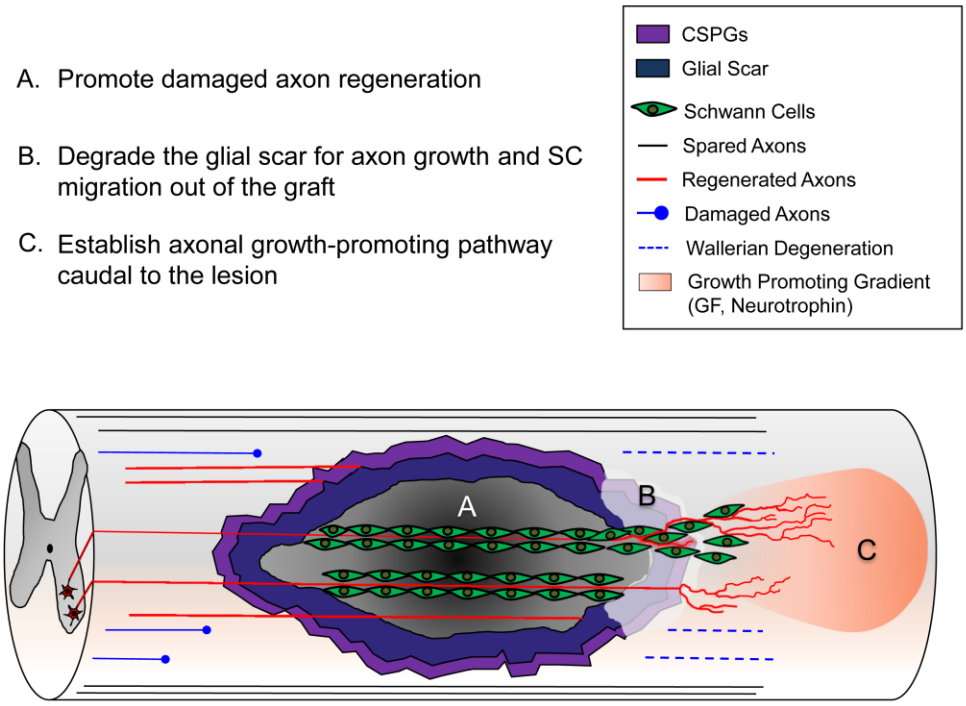

Figure 3. Pathology and experimental challenges following SCI. Upon injury, axons are severed, and rostral axon stumps retract while distal segments progressively undergo Wallerian degeneration. A glial scar forms including inhibitory chondroitin sulfate proteoglycans (CSPGs), preventing grafted SCs from migrating, and axons extending into host tissue. Such challenges are illustrated and labeled above. SC transplantation-mediated axon regrowth includes the following strategies: Genetic modification of SCs to express neurotrophins or growth factors, break down the glial scar with Chondroitinase to allow axons and SCs to exit into the caudal host tissue, and combination therapies with other glial or neural precursor cells, or bioengineered materials, to enhance SC benefits on neuroprotection, repair and functional recovery.

Summary

As research into the mechanisms of cell death and tissue degeneration following CNS injury continues, more information will be obtained which will aid in the development of effective, and specific therapies for preventing, or even reversing, the progression of pathology commonly observed following such injuries. Understanding the important roles and intricate internetwork communication and activity of intracellular pathways, is an important direction for making this progress. Current evidence, as described here, suggests that work towards understanding PI3K/Akt signaling and other complex signaling cascades is not only promising, but may be critical for achieving the goal of improved neurological outcome after brain and spinal cord diseases and injuries. In combining therapies such as PTEN inhibition by bpV and SC transplantation, the mechanistic details of the effects of both therapies on the injured become exponentially complicated; nonetheless, combining other treatments with SC transplantation is a necessity and from a therapeutic perspective, the end result of recovery may be more important than the means of achieving it.

The following chapters describe work toward elucidating changes in PTEN/PI3K/Akt signaling in the injured spinal cord, the investigation of small-molecule PTEN inhibitor, bpV, in an effort to reduce spread of secondary tissue damage, and a novel combination treatment involving acute delivery of bpV followed by subacute SC transplantation to better improve neuroprotection and functional recovery in a clinically-relevant hemicontusive cervical SCI model in rats.

CHAPTER 2

AIM 1

CHARACTERIZATION OF PTEN/PI3K EXPRESSION AND SIGNALING, AND ASSESSMENT OF THE EFFECTS OF PTEN INHIBITOR BISPEROXOVANADIUM ON NEUROPROTECTION AND RECOVERY OF THE INJURED RAT FORELIMB FOLLOWING SCI

Hypotheses: 1) PTEN activity or expression changes in mediating cell death through down-regulation of Akt survival signaling, and 2) that modulating PTEN activity with acute bpV administration may promote neuroprotection and functional recovery, at least partly, through upregulation of PI3K/Akt activity and downregulation of delayed cell death processes following SCI.

Introduction

Following rapid trauma-induced necrosis at the injury epicenter, apoptosis and macroautophagy spread out into the lesion penumbra (Rami and Kogel, 2008), contributing to secondary cell death following SCI (Kanno et al., 2009, Kanno et al., 2011). These processes are regulated, at least in part, by phosphotidylinositol-3-kinase (PI3K)/Akt signaling, with downstream mammalian target of rapamycin (mTOR) protein complexes involved in promoting cell survival and growth. After CNS trauma, however, many questions remain concerning the influence of this signaling pathway, and the cell-specific outcomes such activation imparts in response to the insult. Following SCI, Akt phosphorylation decreases at and around the injury epicenter, while a PI3K-mediated upregulation occurs in neurons peripheral to the injury core (Yu et al.,

2005). This phenomenon is also observed following focal ischemic brain injury, and is considered an endogenous neuroprotective response of surrounding neurons (Noshita et al., 2001).

Under normal conditions, downstream effectors in this pathway such as mTOR are relatively inactive in spinal cord neurons (Xu et al., 2010). PTEN however, is highly expressed in adult CNS neurons (Cai et al., 2009, Liu et al., 2010c), and negatively regulates this pathway, countering its over-activity, which is important for cell cycle regulation, cellular turnover, and cell motility (Dahia, 2000). Nonetheless, the loss of PTEN or its function is beneficial following CNS injury and disease, promoting axonal regrowth following SCI (Liu et al., 2010a), reducing motor neuron atrophy (Ning et al., 2010), and enhancing neural survival (Zhang et al., 2007a, Shi et al., 2011). Such evidence suggests a potential alteration in PTEN expression or activity following SCI.

Small-molecule protein-tyrosine-phosphatase (PTP) inhibitors, bisperoxovanadium (bpV) compounds, are known to promote neuroprotection following thoracic SCI (Nakashima et al., 2008), and Parkinsonian neurodegeneration (Yang et al., 2007), though no specific cellular signaling mechanism was investigated in these reports. bpV compounds specifically and potently inhibit PTEN activity *in vitro* (Schmid et al., 2004), and administration *in vivo* promotes neuroprotection in various CNS injury models through activation of the PI3K/Akt pathway (Song et al., 2010, Shi et al., 2011, Sury et al., 2011). Akt activity also negatively influences retrograde axonal degradation through inhibition of macroautophagy following axotomy (Cheng et al., 2011) a result

potentially promoted through inhibition of PTEN. Thus, targeting PTEN may be part of the mechanism of bpV-mediated neuroprotection observed following spinal trauma.

Pharmacological therapies for SCI are urgently needed as such treatments are minimally invasive and can be rapidly administered. To test our hypotheses, histological and protein analysis were utilized, as well as application of a novel forelimb functional assessment to investigate acute PTEN/PI3K/Akt and mTOR signaling changes and the ability of potassium bisperoxo (picolinato) vanadium (bpV[pic]) modulate PI3K/Akt signaling for mediating neuroprotection and functional recovery. To our knowledge, these findings are the first to link bpV-mediated neuroprotection to PI3K/Akt/mTOR signaling following SCI.

Materials and Methods

Animals and surgical procedures

Adult female Sprague-Dawley rats (200-250 g, Harlan) (*n* = 40) were housed in controlled conditions with a 12:12 light:dark schedule, and access to food and water *ad libitum*. Prior to surgery, the animals were anaesthetized with ketamine (87 mg/kg)/xylazine (12 mg/kg, IP), and either a laminectomy only, or unilateral cervical SCI was performed as modified from Gensel et al. (2006). Briefly, a customized vertebral stabilizer was set to support the 5^{th} cervical vertebrae and a partial unilateral laminectomy was performed to expose the right side of the spinal cord at the same level. With dura intact, the NYU MASCIS Impactor

(Gruner, 1992) (2.5 mm tip, 10g weight, 12.5 mm height) was used to inflict a moderate unilateral injury (Gensel et al., 2006). Sham animals were excluded from injury.

Following surgery, animals received an injection of 3 mL 0.9% saline subcutaneously for hydration and were placed in temperature controlled housing overnight for monitoring recovery. All surgical and animal handling procedures were performed as approved under the Guide for the Care and Use of Laboratory Animals (National Research Council) and the Guidelines of the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Drug Treatment

Groups were randomly designated for treatment with bpV(pic) (Enzo Life Sciences), with dosing modified from Zhang et al., (Zhang et al., 2007a) or vehicle. After surgery and/or injury, animals received an immediate intraperitoneal (IP) injection of either saline or bpV(pic). Animals received a second dose of vehicle or bpV(pic) 2 hrs post-injury, and twice daily for 1 or 7 days thereafter. The treatment group received daily injections of 400 µg/kg bpV(pic), while a second group received 0.9% sterile saline (vehicle) injection as a treatment control. A third group served as a surgical control group (sham) also injected with vehicle according the prescribed dosing schedule.

Western Blotting

Protein analysis followed procedures described previously (Xu et al., 1998a, Yan et al., 1999, Yan et al., 2003), with modification. Briefly, a 10 mm spinal cord segment containing the injury epicenter (5 mm rostral and caudal to the epicenter) was removed for protein extraction 24 hours following SCI. The tissue was quickly homogenized in RIPA lysis and extraction buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) + 0.5 M ethylenediaminetetraacetic acid (EDTA) and 1 % Halt protease inhibitors (100 mM AEBSF•HCl, 80 µM Aprotinin, 5 mM Bestatin, 1.5 mM E-64, 2 mM Leupeptin, 1 mM Pepstatin A) (ThermoPierce) using a Dounce homogenizer (20x up and down) and left on ice for 30 min. After this time, the samples were centrifuged for 10 min at 10,000 RPM. After centrifugation, the supernatant, which represented the primary tissue lysate was removed from each sample and placed in new Eppendorf tubes and centrifuged again for 10 min at 10,000 RPM. This step removed any remaining cellular debris and membrane leaving total tissue lysate. Protein concentrations were determined and standardized using the Bradford method (Bradford, 1976).

Equal protein concentrations from each sample was loaded onto 8-10% polyacrylamide gels, separated by SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane by electrophoresis. The membranes were immunoblotted with the following primary antibodies: monoclonal mouse-anti PTEN (1:200); monoclonal mouse anti-ribosomal protein S6 (1:200) (Santa Cruz Biotechnologies); polyclonal rabbit anti-phospho Akt (ser 473) (1:1,000), a

marker used for assessing PI3K activation, monoclonal mouse anti-pan Akt (1:1,500); polyclonal rabbit anti-phospho ribosomal protein S6 (ser 235/236) (1:400) (Cell Signaling, Inc.) an indicator of mTOR activity; polyclonal rabbit anti-LC3B (1:100, Abgent), to assess autophagy activity; monoclonal mouse anti-β-tubulin (1:1000, Sigma-Aldrich) as a loading control. Membranes were incubated with a secondary goat anti-rabbit or anti-mouse fluorescent secondary antibody, fluorescing at either 680nm or 800nm wavelength (1:10,000; Rockford) and visualized with the LiCor Odyssey system and software (Version 1.2) per the manufacturer's instructions. Quantification of detected bands was performed by densitometry using ImageJ software (NIH).

Histological Assessments

Six weeks post-injury, tissue was collected and processed as previously published (Liu et al., 2006). In brief, a 1 cm segment of cervical cord including the injury epicenter (5 mm rostral and caudal to the epicenter) was dissected and sectioned longitudinally in the horizontal plane or transversely at 20 µm thickness on Superfrost Plus slides (Fisher Scientific) using a cryostat (Leica). Tissue was stained using cresyl violet acetate stain with eosin counterstaining. Lesion and cavity volume were calculated using Neurolucida software (MicroBrightfield, Inc.). Two sections per mm along the length of the cord were used for lesion volume measurement and calculation, spanning from tissue first showing identifiable lesion, to the last demonstrating such morphology. These same methods, equipment, and software were used to construct a 3-dimensional representation

of vehicle- and bpV-treated animal spinal cord tissue. Additionally, the same sections and staining used for assessing lesion, cavity, and spared tissue volume were used to quantify the number of motorneurons throughout the specified spinal cord distance illustrated. To define a standard anatomical area for counting motorneurons, a horizontal line was drawn from one side of the transverse section to the other passing through the central canal. All clearly identifiable ipsilateral ventral horn motorneurons ventral to this line, exhibiting dark, evenly distributed cresyl violet staining, were manually quantified using ImageJ software.

Vascular Quantification

Six weeks post-injury, spinal tissue sections 2 mm rostral and caudal, as well as from the epicenter of the lesion, were utilized for vascular quantification using a general rat endothelial cell marker, and calculation of the vessel-positive area was performed in the ipsilateral and contralateral gray matter in vehicle- and bpV-treated animal groups. In brief, vessels in spinal tissue from bpV- and vehicle-treated animals were labeled via immunofluorescence with mouse anti-rat endothelial cell antigen-1 (RECA-1, 1:200; ABD Serotec), a specific vascular marker, overnight at 4°C. The following day, the tissue was labeled with fluorescent secondary antibody and high power images acquired with an Olympus BX60 microscope, and PictureFrame software (Optronics). RECA-1^{+} area was measured using ImageJ software (NIH). Through modified methods previously published (Nakashima et al., 2008) the anatomically defined area of

ipsilateral gray matter containing clearly identifiable RECA-1^{+} vasculature was outlined in ImageJ. The area of the outlined region of interest was calculated by the software, and this area was defined as vasculature-positive area. Only areas of gray matter clearly containing morphological identifiable vasculature, as identified by RECA-1 labeling, were included in this assessment.

Immunofluorescence Double Labeling

Immunofluorescence double labeling was performed as previously described (Liu et al., 2006). Briefly, 1 day following SCI, 1 cm spinal tissue including the injury epicenter was removed after perfusion, and cryoprotected in 30% sucrose in 0.1 M PBS. Cord segments were sectioned and prepared for staining as described above, and incubated with the following primary antibodies: mouse and rabbit anti-PTEN (1:100; Santa Cruz Biotechnologies), rabbit anti-phospho ribosomal protein S6 (ser 235/236) (1:400; Cell Signaling, Inc.), mouse anti-NeuN (1:200; Chemicon), a marker for neurons; mouse anti-CC1 (APC-7, 1:25; Calbiochem Inc.), a marker for oligodendrocytes; mouse anti-OX42 (Harlan Sera-lab), a marker for microglia; and rabbit and mouse anti- glial fibrillary acidic protein (GFAP, 1:200, Sigma), a marker for astrocytes. The following day, the sections were incubated with rhodamine- or fluoroisothiocyanate-conjugated goat anti-rabbit or anti-mouse antibodies (1:200, Jackson ImmunoResearch Lab). Sections were coverslip mounted with Fluoromount G with Hoechst 33342 combined with the mounting medium (1:100) for nuclear staining. Pre-immune serum was used

as a control to confirm the specificity of the antibody. Images were obtained with a Nikon TE2000 laser scanning confocal microscope.

Behavioral Testing

For functional assessment, cereal rings of uniform shape, size, and flavor were provided and arm joint articulation (Martinez et al., 2009), movement, treat handling, and coordination were assessed while the animal ate the treat. Each rat was placed within and tested in a home cage, which promoted the most optimal conditions for assessing the animals' behavior and performance. Using an 8 point scale, with 8 indicating normal ability and 0 representing no ipsilateral forelimb treat contact ability, the animals were tested before SCI (baseline), and once per week for 6 weeks following injury, and were scored on three separate trials with the three scores averaged. Treat manipulation was defined as obvious consistent coordinated handling of the treat by both forelimbs during eating. Tables 2 and 3 represent the scoring scale and sheet, respectively.

Statistical Analysis

To determine significance between two groups, a two-tailed unpaired Student's t-test was used. Statistical significance between multiple groups was determined using a one-way ANOVA with Tukey's post-hoc analysis. All statistical values were calculated using GraphPad Prism 5.0 software (GraphPad, Inc.), with a p value < 0.05 considered statistically significant.

Table 2. Forelimb function assessment scale.

0 = No ipsilateral forelimb usage/contact with treat

1 = Supportive dorsal forelimb contact with treat, non-elevated forelimbs, drops

2 = Supportive dorsal forelimb contact with treat, occasional-frequent elevated (flexed) forelimbs, drops

3 = Supportive dorsal-dorsal/palmar forelimb contact, frequent-consistent elevated (flexed) forelimbs, drops

4 = Supportive dorsal-dorsal/palmar forelimb contact, frequent-consistent elevated (flexed) forelimbs, drops-no drops

5 = Supportive dorsal-dorsal/palmar forelimb contact, frequent-consistent elevated (flexed) forelimbs, drops-no drops

6 = Supportive-coordinated dorsal-dorsal/palmar forelimb contact, frequent-consistent elevated (flexed) forelimbs, no drops

7 = Coordinated dorsal/palmar forelimb contact, consistent elevated (flexed) forelimbs, no drops

8 = Coordinated palmar grasping , consistent elevated (flexed) forelimbs, no drops

Occasional	< 50% of the time	Supportive	No movement w/ treat contact
Frequent	> 50%, <95% of the time	Coordinated	Visible coordinated movement between forelimbs
Consistent	95-100% of the time		w/ treat contact

Table 3. Forelimb function assessment scoring sheet.

Drops	Maintains paw/treat off ground while eating	Ipsi Paw Status During Treat Contact	Manipulates Treat (Ipsi)
None = 1	Always = 2	Palmar = 3	Yes = 2
Any = 0	> 50%, < 100% = 1	Dorsal/Palmar = 2	No = 0
	< 50% of the time = 0	Dorsal = 1	
		No Contact = 0	

Trial	Score	Total
1		
2		
3		
		Average

Results

bpV(pic) promotes significant neuroprotection after cervical contusive SCI

To assess whether bpV treatment promoted neuroprotection following injury, histological analysis was utilized. Six weeks post-injury, cresyl violet-eosin staining revealed the extent of the lesion, and the lesion volume, cavity, and spared tissue in the ipsilateral spinal cord were measured. bpV treated animals had significantly reduced ipsilateral lesion (21% ± 1.1 vs. 34% ± 3.0 SEM; $p < 0.01$) and cavity volume (approx. 67% reduction; $p < 0.05$) compared to vehicle-treated animals (Fig. 4 & 5). In comparison to the relative area of the ipsilateral cord, the spared tissue was significantly increased in bpV-treated animals compared to vehicle controls (79% ± 1.1 vs. 66% ± 3.0 SEM; $p < 0.01$). Additionally, bpV treatment reduced the amount of cavitation typically observed in subacute to chronic phases after contusive SCI, as seen in the vehicle-treated group (Fig. 5 & 6).

Consistent with reduced lesion volume, bpV-treated animals exhibited a trend of more ventral horn motorneurons closer to the injury epicenter than vehicle-treated animals, with significantly increased motorneurons noted both 2 mm rostral and caudal to the injury epicenter (Fig. 7 & 8). As vascular sparing and integrity have been attributed to bpV-mediated neuroprotection following SCI (Nakashima et al., 2008), using methods of vascular quantification modified from previous publication (Nakashima et al., 2008), RECA-1^{+} vascular area (mm^2) was assessed in anatomical regions of interest (gray matter from transverse spinal

sections: 2 mm rostral and caudal, and at the injury epicenter (Fig. 9 & 10). Spinal cord RECA-1^{+} (vascular) gray matter area was significantly increased at the rostral region in bpV(pic)-treated rats compared to vehicle-treated animals ($p < 0.01$), as well as at the epicenter of the injury ($p < 0.05$).

No difference in gray matter vascular area was observed caudal to the injury site (Fig. 10). The contralateral gray matter vascular area, as expected, did not exhibit differences between treatment groups either rostral or caudal to the injury. However, significantly more vascular area was measured in the bpV-treated group within the contralateral gray tissue at the injury epicenter in comparison to the vehicle-treated group ($p < 0.05$) likely due to neuroprotection mediated by treatment (data not shown).

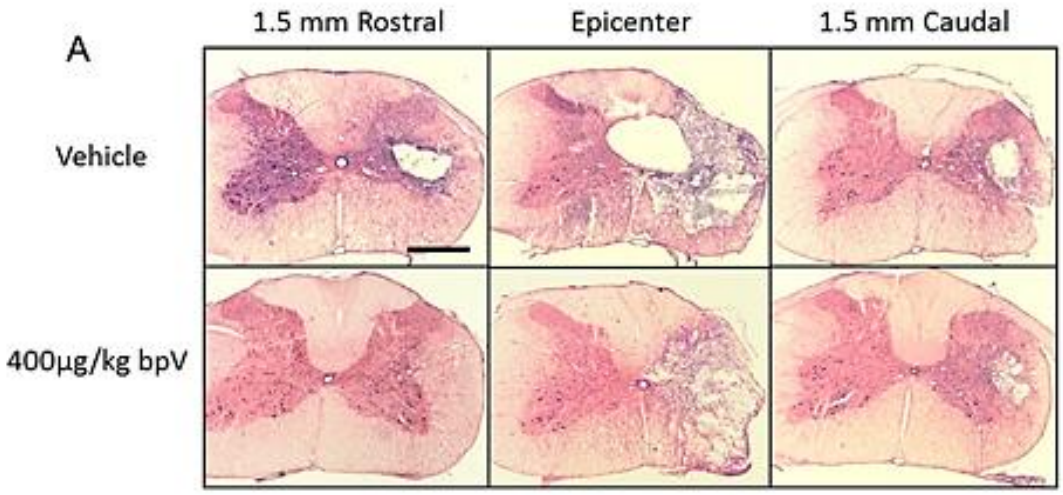

Figure 4. bpV(pic) reduced lesion size and cavitation following C5 hemicontusion SCI. (A) Tissue extracted from rats 6 wks post-SCI demonstrated reduced lesion and cavitation through cresyl violet-eosin staining compared to vehicle treated animals. Images were obtained from 1 cm tissue with the injury epicenter. Scale bar = 1 mm.

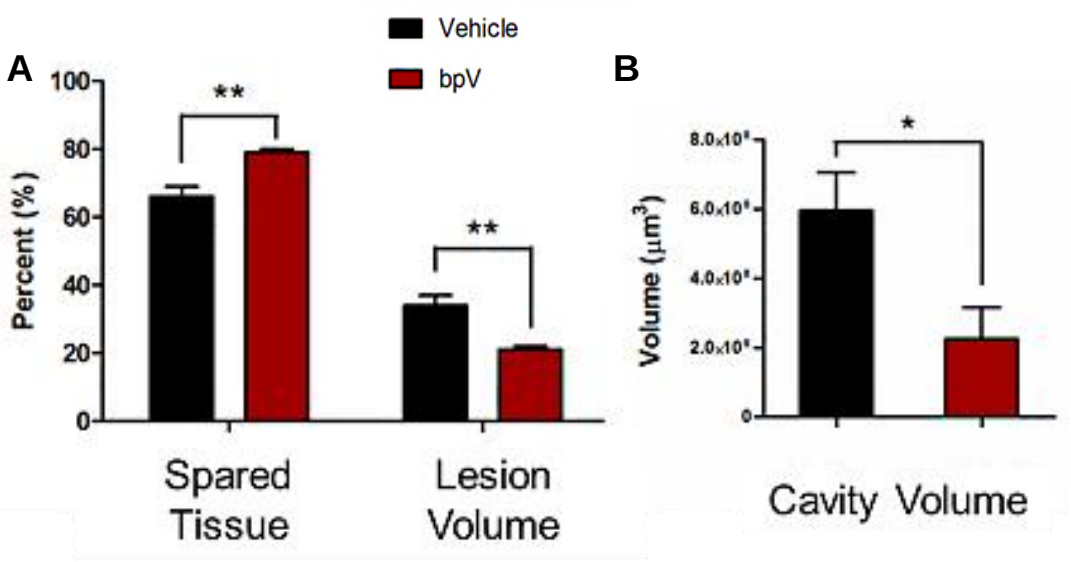

Figure 5. Graphical representation showing statistically significant reduction in spinal tissue damage by bpV(pic). Lesion (A) and cavity (B) volumes, and increased spared tissue (A) following bpV(pic) treatment. Quantifications were from 1 cm tissue containing the injury epicenter. **, $p < 0.01$; *, $p < 0.05$. $n = 4 – 5$. Error bars = SEM.

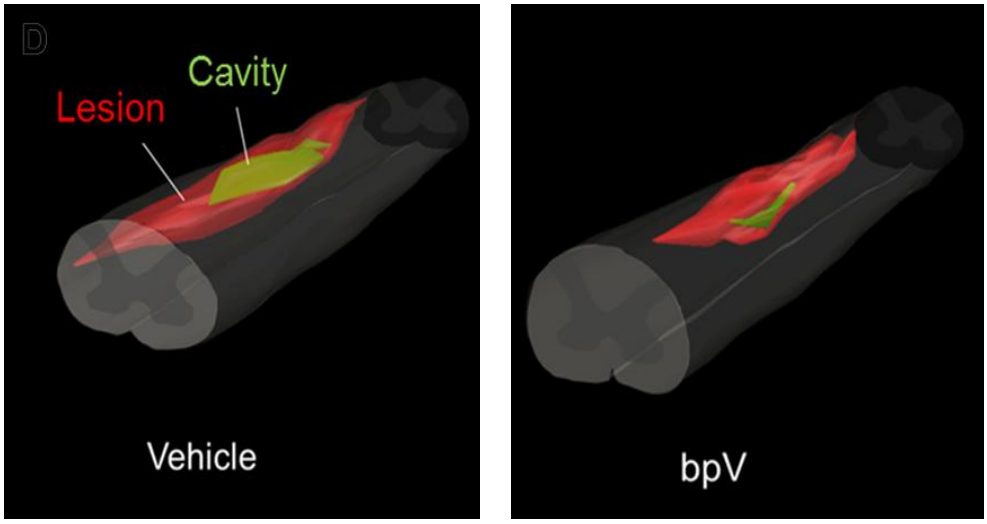

Figure 6. 3D-reconstruction using Neurolucida software contour mapping from representative cases illustrating the neuroprotective effects of acute bpV(pic) therapy.

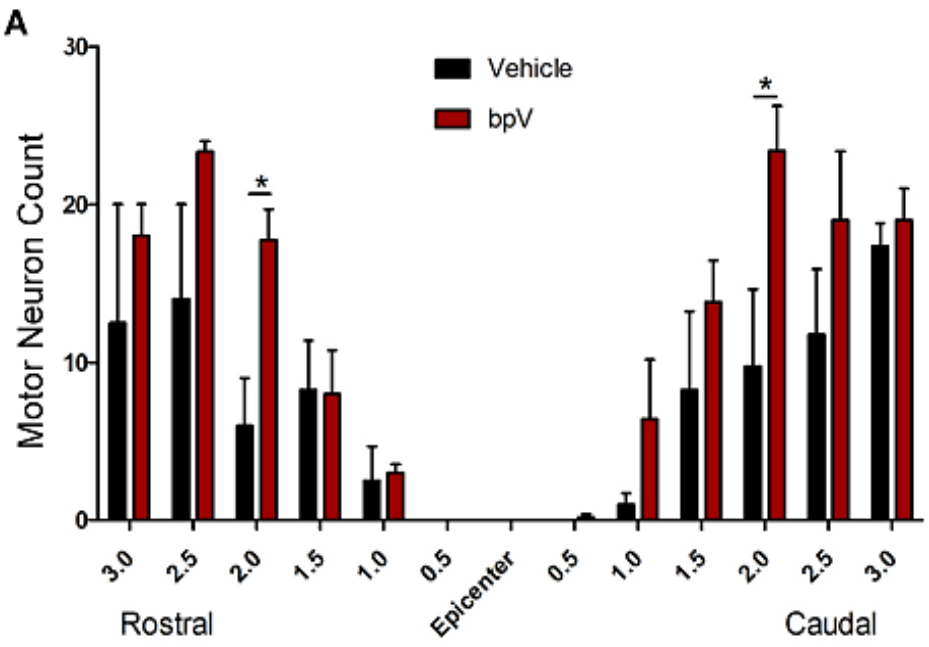

Figure 7. Acute bpV therapy reduced motor neuron loss following SCI. bpV(pic) treatment spared, on average, more ventral horn motor neurons than vehicle-treatment (A). Significantly more motor neurons were present in the ventral horn 2 mm rostral and caudal to the lesion epicenter of bpV(pic) treated animals. *, $p < 0.05$. $n = 4 – 5$. X-axis represents mm from epicenter. Error bars = SEM.

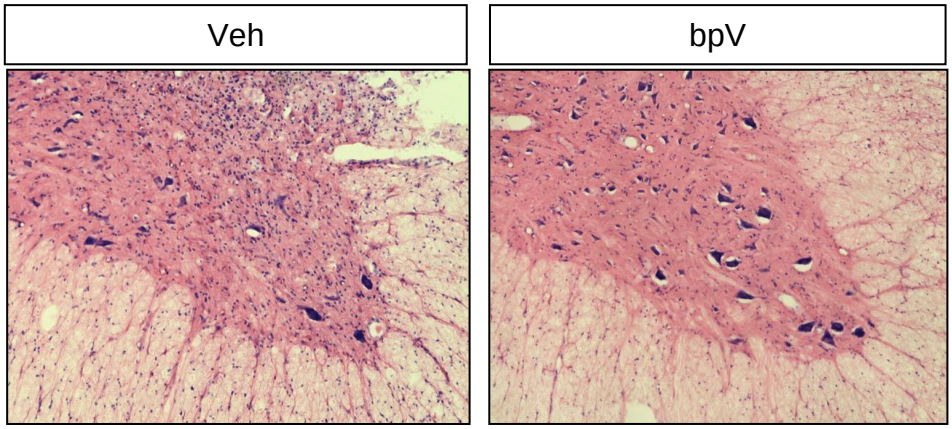

Figure 8. Photomicrographical representation showing cresyl violet-eosin stained ventral horns of spinal tissue extracted 6 weeks post-SCI. Sections shown are from 2 mm rostral to the epicenter and demonstrate the increase in motor neurons in bpV-treated animals over vehicle-treatment. Scale bar = 150 μm.

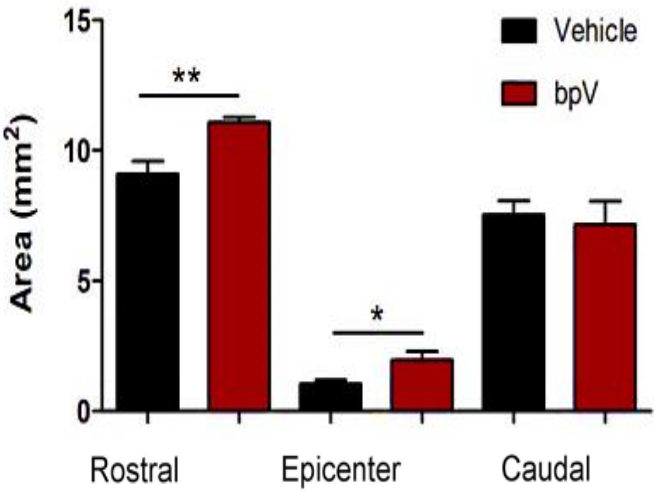

Figure 9. Significant increase in ipsilateral gray matter vasculature rostral and at the epicenter of the injury. bpV(pic)-treated animals demonstrated significantly increased vascular (RECA-1)-positive area in the ipsilateral gray matter 2 mm rostral and at the epicenter of the lesion 6 weeks post-SCI. No significant difference was observed 2 mm caudal to the epicenter. **, $p < 0.01$; *, $p < 0.05$. $n = 4 – 5$. Error bars = SEM.

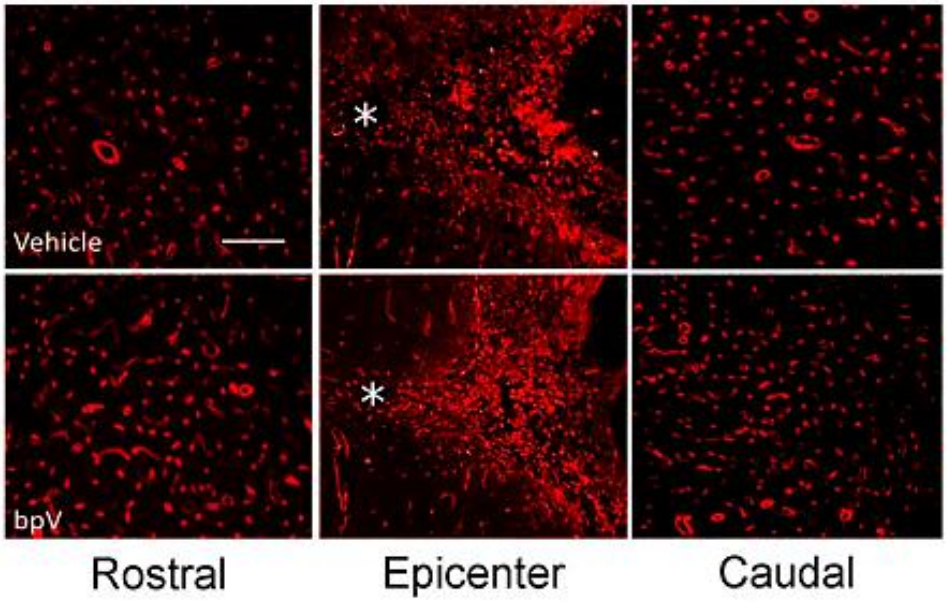

Figure 10. Photomicrograph of increased gray matter vasculature mediated by bpV(pic) after SCI. bpV(pic)-treated animals had significantly increased RECA-1-positive vasculature 2 mm rostral and at the epicenter of the lesion 6 weeks post-SCI. * = central canal. Scale bar (Rostral & Caudal) = 100 μm; (Epicenter) = 150 μm.

Skilled forelimb articulation and coordination improved following bpV(pic) treatment

To determine whether the observed neuroprotection mediated by bpV enhanced functional recovery, a novel forelimb behavioral assessment was used. During the 6 week behavior testing, animals that received bpV significantly recovered highly skilled forelimb function and coordination while eating cereal rings, compared to the vehicle-treated animals, using our independently-developed forelimb coordination and manipulation test (Fig. 11). Sham animals achieved perfect scores (8 = maximum score) overall during testing, as expected. bpV-treated animals averaged a score of 6.94 ± 0.06, while vehicle-treated animals averaged a score of 4.43 ± 0.59 by the end of 6 weeks. Scores obtained during skilled forelimb use demonstrated a highly linear correlation with arm articulation ability (R^2 = 0.88; Fig. 11), as scored as a subset from a forelimb locomotion test published and used by Martinez et al., (2009). Figure 12 depicts sham, injured, and injured rats treated with bpV performing this task. The red arrows point to the ipsilateral paw, and show functional status based on treatment.

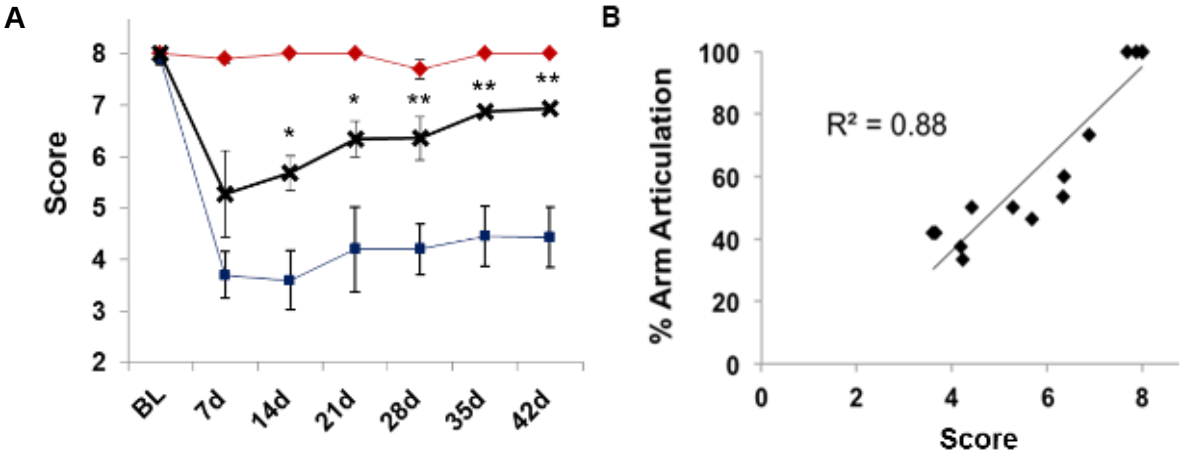

Figure 11. bpV-treatment enhanced forelimb functional recovery. (A) By 6 weeks post-injury, bpV(pic)-treated rats exhibited significantly enhanced forelimb function over vehicle-treated animals as scored using an 8-point treat manipulation behavioral scale (6.8 vs. 4.5). Sham animals scored at or near perfect (8 points) throughout the study. (B) Testing scores showed a positive linear correlation with arm joint articulation ability (R^2 = 0.88). (C) Images portraying a rat grasping and manipulating a flavored cereal ring, the treat used in this assessment. **, $p < 0.01$; *, $p < 0.05$. n = 4 – 5. Error bars = SEM.

Figure 12. Images portraying a rat grasping and manipulating a flavored cereal ring, the treat used in this assessment. The red arrows are pointing to the ipsilateral forepaw following surgery. The rat's forelimb and paw was severely impaired during this assessment following injury. The administration of bpV significantly improved the ability of the rat to coordinate forelimb movement and manipulate the treat with its forepaws.

Acute cell-specific expression profiles of PTEN and phospho-S6 after cervical SCI

As we hypothesized that bpV may act through PTEN functional inhibition, immunofluorescence double-labeling of PTEN with various cell types and structures was employed to allow for identification of potential cellular targets affected by bpV treatment (Fig. 13). PTEN was highly expressed in motor neurons (Fig. 13A-C), $CC1^+$ oligodendrocytes (Fig. 13G-I), and $OX\text{-}42^+$ reactive microglia (Fig. 13J-L) after injury, though some minor expression was seen in reactive astrocytes post-SCI (Fig. 13D-F). As PTEN inhibition would promote upregulation of PI3K/Akt/mTOR signaling, cell-specific colocalization with the mTOR activity marker, phosphorylated ribosomal protein S6 (p-S6), was also performed. In general, p-S6 expression upregulation was observed in motor neurons (Fig. 14A-F) of injured animals, as well as hypertrophic reactive astrocytes (Fig. 14G-L) near the injury site. There was no obvious difference in p-S6 expression change in $CC1^+$ oligodendrocytes after injury (Fig.14M-P).

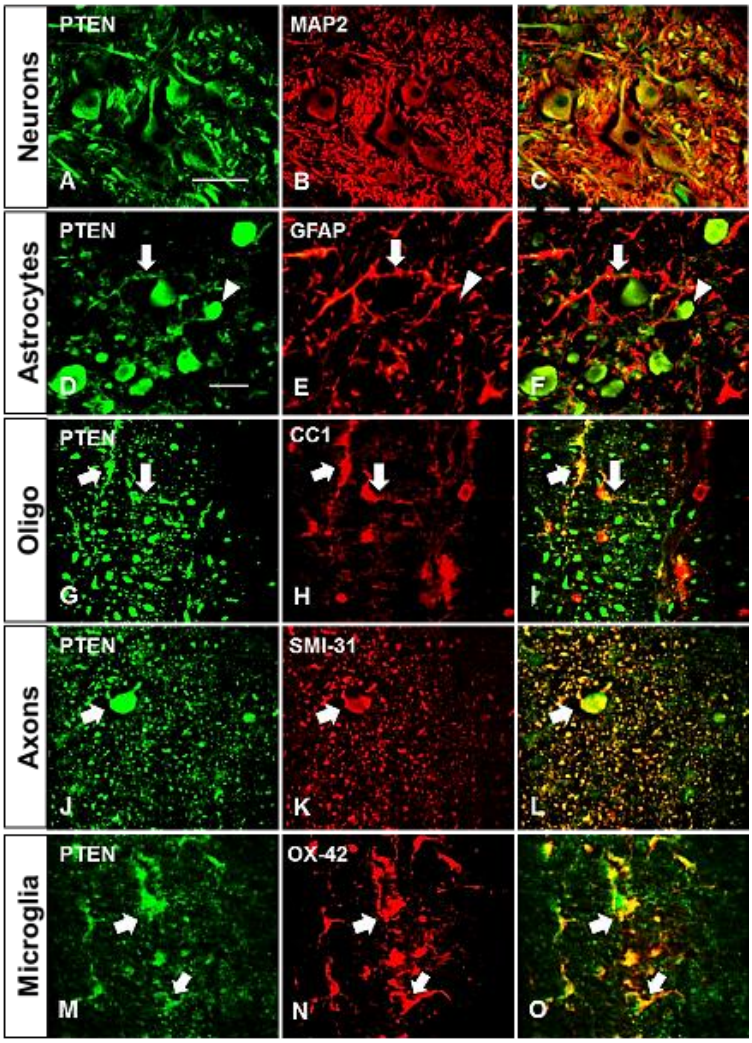

Figure 13. PTEN cellular localization following injury. (A – C) PTEN highly colocalized to MAP2^{+} neurons (D – F), however, PTEN only somewhat colocalized with GFAP^{+} hypertrophic astrocytes near the lesion (arrow), but highly localized to morphologically swollen axons (arrowhead). (G – I) CC-1^{+} oligodendrocytes and (M – O) OX-42^{+} reactive microglia also expressed considerable PTEN 1d following SCI. (J – L) PTEN highly colocalized to both smaller and swollen (arrow) degenerating axons within white matter tracts surrounding the injury. Observations were from tissue ~2 mm rostral to the injury epicenter. Scale bar = 100 μm.

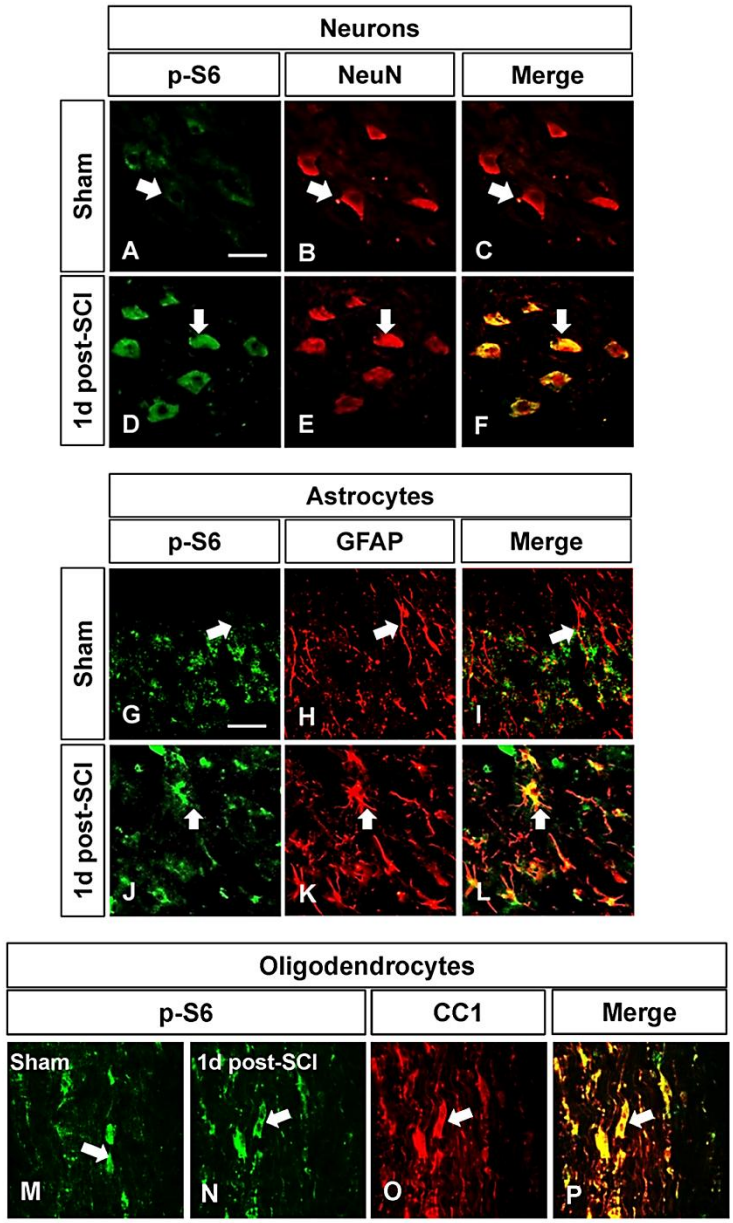

Figure 14. Phospho-S6 cellular localization following injury. Like PTEN, phospho-S6 (p-S6) colocalized with many cell and structure types 1d following SCI within the injury penumbra (~2 mm rostral to the epicenter). Phospho-S6 was expressed very abundantly in (D – F) neurons, and (J – L) hypertrophic astrocytes following SCI, as indicated by arrows. (M – P) Oligodendrocytes highly expressed p-S6 before and after injury (arrows). Scale bar = 100 μm.

bpV(pic) enhances Akt/mTOR signaling, and reduces LC3 II/LC3 I ratio

To quantify bpV's effects on PI3K/Akt/mTOR pathway-related protein expression changes in the cervical spinal cord, Western blot analysis was performed on tissue from uninjured animals, as well as bpV-treated and non-treated animals. PTEN expression levels were not significantly different between sham and 1d following SCI, though a trend in increase was observed following injury (Fig. 15). bpV treatment showed no significant effect on PTEN expression levels (Fig. 15A & B). To determine whether SCI or bpV(pic) influenced PTEN's antagonism of PI3K and downstream mTOR activity, phosphorylated Akt (p-Akt) and p-S6 levels were assessed. One day following SCI, p-Akt significantly decreased in injured spinal tissue ($p < 0.01$), while p-S6 expression significantly increased following treatment ($p < 0.05$). bpV treatment recovered p-Akt expression levels to near sham levels compared to injury-only and vehicle-treated animals (Fig. 15A & C) ($p < 0.05$), and elevated p-S6 expression further over sham ($p < 0.01$), but not significantly over injury-only, or vehicle-treated animals (Fig. 15A & D). As mTOR is a known regulator of autophagy, autophagosome formation was measured by quantifying downstream microtubule-associated protein light chain 3 (LC3) protein levels. A band shift during SDS-PAGE blotting is indicative of LC3 I to LC3 II conversion, and thus an increase in autophagic activity (Kabeya et al., 2000). The ratio of LC3 II to LC3 I increased in untreated animals 1d after injury, though this ratio significantly reduced ($p < 0.05$) to near sham levels of expression following bpV(pic) treatment (Fig. 15A & E), suggesting a reversal of autophagic activity upregulation seen in following SCI (Fig. 16).

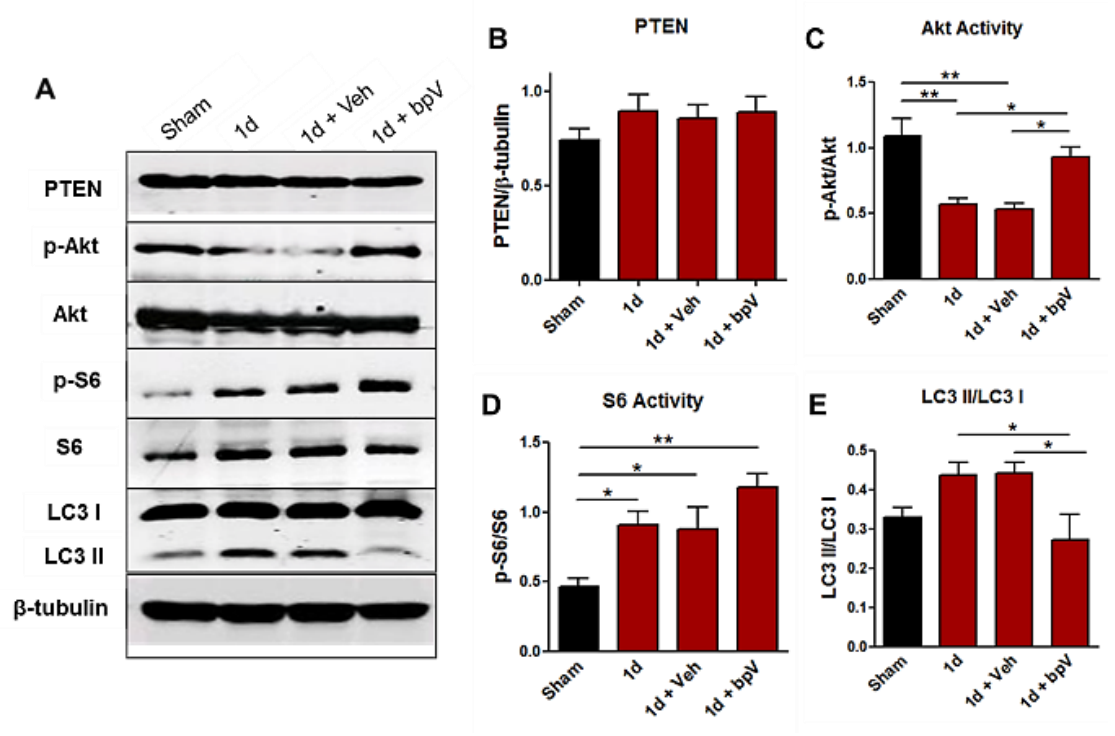

Figure 15. Effects of bpV(pic) on mTOR and autophagic protein analysis 1d post-SCI. (A) Western blot profiles from 1 cm spinal tissue with the injury epicenter collected from experimental animals 1d post-SCI. (B – E) Quantification of blots shown in A. (B) Total PTEN protein expression did not significantly change following injury, though a mild increase in expression was observed. (C) Phospho-Akt levels significantly decreased following injury, and are significantly increased following bpV(pic) treatment. (D) Downstream, p-S6 protein levels significantly increased following injury, and are enhanced further following bpV treatment. (E) LC3 II ratio to LC3 I, an indicator of autophagic activity, is increased following injury, and is significantly reduced following bpV(pic) therapy. *, $p < 0.05$; **, $p < 0.01$ ($n = 4 – 5$). Error bars = SEM.

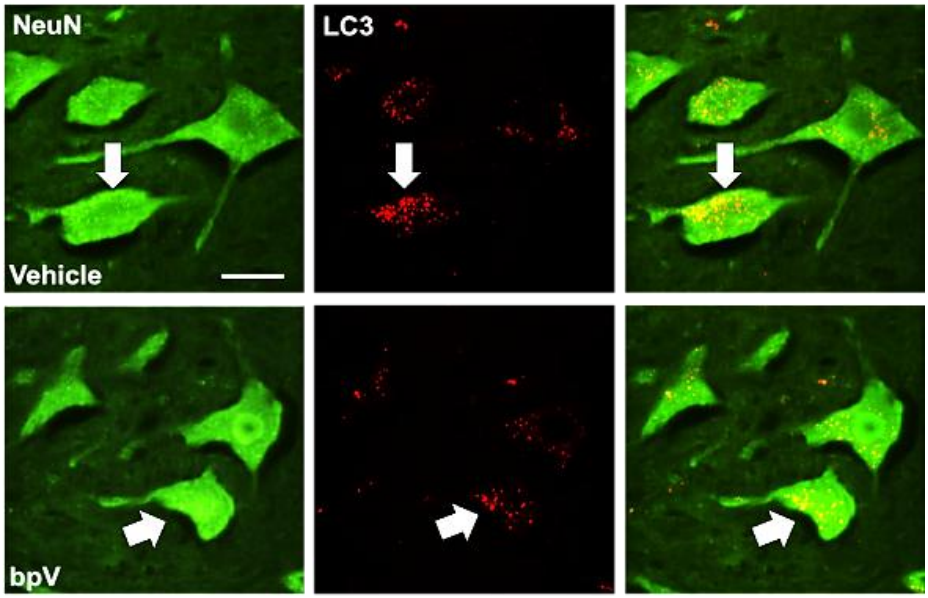

Figure 16. bpV(pic) reduced neuronal autophagosome aggregation. Vehicle-treated rats exhibited dense intracellular LC3-positive autophagosome aggregation in neurons within the injury penumbra ~2 mm rostral to the injury epicenter (arrows). Treatment with bpV promoted a decrease in neuronal autophagosome clustering. Scale bar = 50 μm.

Discussion

Neuroprotection and functional recovery promoted by bpV

Inhibition of PTEN's activity using bpV has proven neuroprotective in experimental CNS injury or disease models, including ischemia-reperfusion injury (Zhang et al., 2007a), SCI (Nakashima et al., 2008), and Parkinson's disease (Yang et al., 2007). These reports support our findings of enhanced neuroprotection and functional recovery seen in this study. By 6 weeks post-injury, bpV(pic) drastically reduced lesion and cavity volume, while increasing spared tissue, including vasculature, as well as skilled forelimb function over vehicle-treated control animals. The enhanced amount of vasculature-containing gray matter tissue could logically result from overall reduction of lesion and spread of secondary cell death observed following bpV therapy, or a stimulation of angiogenesis following this treatment, due to suppression of PTEN activity and subsequent upregulation of PI3K signaling (Wen et al., 2001, Liu et al., 2011, Park et al., 2011). A second explanation is that vascular sparing actually contributes to the overall neuroprotection effects, a hypothesis proposed by Nakashima et al. (2008) after observing a similar phenomenon following bpV(phen) treatment for SCI. In support of this explanation, a recent study by Han et al., showed that angiopoietin-1 and synthetic C16 peptide treatment spared vasculature by targeting the vascular endothelium, leading to reduced inflammation, diminished white matter loss, and functional recovery following SCI (Han et al., 2010). Concerning bpV's influence on vasculature, more analysis of

the role of vascular sparing or regrowth in anatomical and functional outcome is necessary to elucidate explanations for increased vasculature witnessed at later time points following treatment.

bpV also promoted significantly increased forelimb functional ability in comparison to vehicle treated animals beginning two weeks post-injury ($p < 0.05$) with improvement observed until the end of testing ($p < 0.01$). These results corresponded well to the significant neuroprotection observed in this study. While eating, rats utilize proprioceptive ability to manipulate and eat through coordinated and articulated forelimb activity, allowing for a more sensitive assessment of the injured forelimb's functional ability. Also, the innate motivation of the rats to eat the treats is high, requiring no training and allowing for easy repetition for multiple trials during each testing period without fasting the animals. A similar, though separately developed test was recently published by Irvine et al., demonstrating the need for reliable sensitive assessments of overall fine forelimb function following cervical SCI (Irvine et al., 2010). The details of their test also support the validity of our design, enhancing confidence that the increased performance during our forelimb assessment after bpV(pic) treatment is genuine. Altogether, the reduced cell loss, lesion volume, and increased spared tissue positively correlate with the enhancement of forelimb functional ability witnessed 6 weeks after SCI, further supporting our data and hypotheses.

Linking bpV to Akt/mTOR signaling following cervical spinal injury

Though no significant PTEN expression change was observed, our results show that PTEN is highly expressed in neurons, $CC1^+$ oligodendrocytes, and activated microglia 24 hours after injury. PTEN expression was observed in reactive astrocytes, albeit minimally, as well, after cervical SCI, suggesting that multiple cell types may be targets for bpV(pic) action. Overall p-Akt levels relative to pan-Akt decreased in the cervical spinal tissue analyzed via Western blot (Fig. 15A & C), suggesting that PTEN activity may increase post-SCI. However, an increase in p-S6 was observed in neurons and increasingly hypertrophic astrocytes near the injury site, suggesting that neurons may upregulate mTOR activity for protein synthesis as a neuroprotective response, and glia (Fig. 14) both for neuroprotection and to initiate and enter a reactive state following the injury, as discussed below. Despite the increase in p-S6 seen in reactive astrocytes, no significant difference in GFAP reactivity between treated and non-treated animal groups was observed 6 weeks post-SCI (data not shown). Interestingly, oligodendrocytes highly expressed p-S6 both pre- and post-injury (Fig. 14M-P), suggesting this mechanism may serve both a neuroprotective, as well as perhaps a myelin-related function in these cells. PTEN expression is reported to be essential for oligodendrocyte maintenance of myelin and axonal integrity, though not for remyelination as demonstrated through PTEN knock-out experiments (Harrington et al., 2010).

It is quite striking that neurons and glia drastically upregulate p-S6 following SCI, contradictory to expectations based on reports of benefits from

PTEN inhibition following CNS injury. The discrepancy between Akt activity decrease and ribosomal protein activity increase may result from two different or interacting mechanisms via cross-talk between PI3K and MAPK signaling. It has been shown that following SCI, active phosphorylated Erk increased dramatically and rapidly (Lu et al., 2010). Activated isoforms of Erk are known contributors to p70S6 kinase (p70S6K) upregulation along with mTOR (Lehman and Gomez-Cambronero, 2002), which could explain the significant increase in p-S6 following SCI, despite the down-regulation of p-Akt. Active Erk is also known to activate mTOR through upstream tuberous sclerosis complex 2 (TSC2) inhibition (Ma et al., 2005), and on p90 ribosomal protein kinase (Dummler et al., 2005) which could also contribute to upregulated p-S6.

The discrepancy between the decrease in p-Akt and downstream upregulation of p-S6 may have resulted from an early increase in p-Akt leading to the delayed downstream effect of increased mTOR activity through p-S6 phosphorylation. Yu et al. (2005) showed that Akt phosphorylation increased in penumbral neurons 8 hrs following SCI, with a down regulation observed at time points afterward. Ribosomal protein S6 can be phosphorylated by p70S6K through Akt-mediated TSC1/2 inhibition and subsequent activation of mTOR. The timespan of this sequence of events may explain the elevated p-S6 observed in this study at 24 hrs post-injury. Also, p70S6K is known to provide negative feedback on PI3K/Akt to regulate continuing activation of this signaling axis (Manning, 2004). Therefore, as mTOR activation of p70S6K increases, this molecule may serve to prevent further phosphorylation and activation of Akt

contributing to the decreased p-Akt levels observed at 24 hrs after SCI. Nonetheless, the enhancement in p-Akt and p-S6 levels provide a potential mechanistic explanation of the action of bpV(pic) by implicating an mTOR-dependent and independent influence in the neuroprotection and functional recovery observed during this study.

This dual-pathway targeting of translational upregulation through S6 activation may be a method of promoting an endogenous neuroprotective response, contributing to various cellular activities triggered by the insult. This could also explain the upregulation of mTOR activity implicated in astrocyte reactivity witnessed here and elsewhere in response to spinal injury (Codeluppi et al., 2009). PTEN expression was not visually abundant in non-injured astrocytes, but was detected at low-levels in reactive astrocytes 1d post-injury, consistent with reports observing PTEN in astrocytes early in reactive astrogliosis, decreasing as reactivity peaks (Cho et al., 2002). Though overall p-Akt levels decrease post-injury near the injury site, the increase in both p-Akt and p-S6 levels after bpV treatment suggest an enhanced benefit of upregulating both pathways, either through an endogenous neuroprotective feedback mechanism, or other mechanism promoting amplification of p-S6 and other cell responses observed following SCI.

Downregulation of autophagy following bpV treatment

Enhancement of endogenous cell survival mechanisms, such as autophagic activity, can rapidly stabilize the cell in response to hypoxic, ischemic, and inflammatory conditions. Autophagy is a normal cellular phenomenon required for degradation of organelles and proteins, and can be increased for energy acquisition when under cell or nutrient stress. Autophagy dysregulation, however, has been implicated as a cause of autophagic, or Type II programmed cell death following SCI (Kanno et al., 2009, Kanno et al., 2011). In contrast, supporting mTOR upregulation as a trigger for increased autophagic activity, p70S6K has been shown to enhance autophagic activity following mTOR activation (Scott et al., 2004). However, this endogenous protection mechanism may not suffice to spare the cells from delayed death post-SCI, or perhaps the stimulation of autophagic activity proceeds from beneficial to detrimental, ultimately leading to programmed cell death. A continuum of biological processes within cells influenced by SCI including autophagic activity is likely in describing different stages leading to programmed cell death.

Our results suggest that bpV treatment may serve as an autophagic "switch" by forcing increased mTOR activation, and enhancing the inhibitory-actions of mTOR on autophagy. Overactivation of p70S6K is not associated with increased autophagic activity, and mTOR can act directly through regulation of autophagy-related proteins resulting in non-p70S6K dependent control of this process (Scott et al., 2004), supporting our findings and aiding in our interpretation of these results. If autophagic activity plays a role in neuronal

programmed cell death, then bpV(pic) may act through PI3K signaling and mTOR to downregulate this process, preventing the transition towards cell loss.

Considerations for bpV as a therapy for SCI

Although bpV(pic) is an accepted inhibitor of PTEN activity, it may also exhibit other unknown functions following treatment that may or may not contribute to or antagonize effects directly investigated in this study. Bisperoxovanadium compounds are inhibitors of protein-tyrosine-phosphatase (PTP) activity (Posner et al., 1994), some others of which may be influenced by such therapy in the complex experimental system of an animal model. One example involves indirect promotion of PTEN activity by bpV-mediated inhibition of the PTP, Src homology region 2 domain-containing phosphatase-1 (Shp-1), which is known to bind, dephosphorylate, and activate PTEN (Lu et al., 2003). Src protein kinases serve several roles, one of which being involved in promoting cell survival through PI3K/Akt signaling (Thomas and Brugge, 1997). Therefore, bpV could interfere with Src-related protein modulation of PTEN activity in addition to, or separate from, direct PTEN functional inhibition. Additionally, PTEN deletions and mutations are commonly associated with cancer, as the loss of PTEN antagonism of PI3K dysregulates the balance between cellular senescence and proliferation (Suzuki et al., 1998, Dahia et al., 1999, Stiles et al., 2002). To minimize such concerns, short-term bpV-therapy allows strict control of timing, dosing, and withdrawal of use of the drug. In the present study, a one week treatment regimen of bpV(pic) promoted significant long-term neuroprotective

effects and functional abilities of the forelimb following treatment, and such a short therapeutic time period should have minimal impact on or threat of tumor formation, compared to long-term PTEN-activity reduction or PTEN deletion. In conclusion, acute administration of 400 ug/kg bpV(pic) following cervical contusive SCI afforded anatomical and functional forelimb benefits, which are considered very important factors for victims suffering from cervical injuries (Anderson, 2004). This report is a novel step toward understanding the mechanism and benefits of bpV treatment on injured spinal tissue and associated functional outcome, and a foundation for future studies utilizing bpV combinational therapies for improving anatomical and functional recovery after cervical SCI.

CHAPTER 3

AIM 2

IDENTIFICATION OF SPECIFIC MECHANISMS OF BISPEROXOVANADIUM ACTIVITY IN MEDIATING EFFECTS ON SPINAL NEURONS *IN VIVO* AND *IN VITRO* FOLLOWING INJURY

Hypotheses: 1) Phospho-Akt changes are similar in spinal neurons as observed for the total tissue following SCI. 2) bpV(pic) is protective against primary neuron death following trauma and enhances Akt and ribosomal protein S6 phosphorylation *in vitro*, helping support our previous cell signaling and neuroprotective findings *in vivo.*

Introduction

Though our knowledge of cellular responses to injury is incomplete, exogenous promotion of neuroprotection and recovery using a readily-available and fast acting therapeutic would be valuable for slowing secondary injury and its destruction of initially spared tissue. Consequently, understanding mechanisms by which such treatments act is as important as identifying the therapies themselves.

Modulating the activity or expression of the PTEN (Park et al., 2008, Liu et al., 2010, Sun et al., 2011), and neuroprotection following central nervous system (CNS) injury and neurodegeneration (Li et al., 2009). As described in Chapters 1 and 2, PTEN is a known antagonist PI3K, preventing PIP_3 from promoting phosphorylation and activation of the pro-survival kinase Akt. Inhibiting PTEN disinhibits PI3K signaling leading to improved cell survival, tissue sparing, and

functional recovery (Walker et al., 2012) potentially through reducing delayed destructive processes.

We demonstrated that PTEN expression does not significantly change 24 hours post-SCI, though phosphorylated (active) Akt decreases, suggesting PTEN's lipid phosphatase activity and antagonism of PI3K increases within injured tissue following SCI. Thus, our prior evidence supports the hypothesis that PTEN activity promotes cell death following SCI. We identified neurons as potential targets of bpV as they expressed PTEN prior to and after injury, and treatment with a potent PTEN inhibitor, bpV(pic) (Schmid et al., 2004), induced significant ventral horn neuron survival following cervical hemi-contusion SCI. Although whole tissue protein analysis revealed significant reduction in Akt phosphorylation in the proximity of the injury site 1 day post-SCI, it is unclear whether this suppression is sustained in surviving spinal neurons following injury. Also, our previous research did not confirm whether bpV(pic) acted directly on neurons in promoting their survival following SCI. Furthermore, our results suggested that bpV(pic) acted through PI3K/Akt signaling post-injury, however, our evidence was correlative and did not reveal whether bpV(pic) operated via this mechanism specifically in neurons. We therefore designed the present study to assess the extent of the modulation of Akt phosphorylation following SCI, extend our understanding of bpV(pic)-mediated neuroprotection, and more effectively determine whether bpV(pic) acts on PTEN and PI3K/Akt signaling in spinal neurons following traumatic injury.

Materials and Methods

Animals and surgical procedures

Adult female Sprague-Dawley rats (200 – 250 g, Harlan) (*n* = 40) were maintained under controlled conditions with a 12:12 light:dark cycle with *ad libitum* water and food access. Prior to surgery, animals were anaesthetized intraperitoneally (IP) with a ketamine/xylazine cocktail ([87 mg/kg]/[12 mg/kg]), and received laminectomy only (sham operation) or unilateral cervical SCI performed as previously published (Walker et al., 2012). In brief, a customized device was used to stabilize the 5th cervical vertebrae and a partial unilateral laminectomy exposed the right side of the cord leaving the dura intact. The NYU/MASCIS Impactor (Gruner et al., 1992) (2.5 mm tip, 10g weight, 12.5 mm height) produced a moderate unilateral injury (Gensel et al., 2006). Sham animals underwent anesthesia and surgery, but were excluded from injury. Animals received 5 mL 0.9% saline subcutaneously for hydration and were observed over a 24 hr recovery period in temperature controlled housing. All animal procedures and surgeries were approved under the Guide for the Care and Use of Laboratory Animals (National Research Council) and the Guidelines of the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Primary spinal neuron culture and treatment

Primary spinal cord neurons were obtained from Sprague Dawley rat E15 embryo spinal cords according an established protocol (Jiang et al. 2006). In brief, E15 rat spinal cords were isolated and placed in Leibovitz's L-15 medium (Gibco). Meninges were carefully removed, the spinal cords were cut into small pieces and dissociated with 0.05% trypsin/EDTA for 15-20 min at 37^{o} C and gently triturated. After adhering at 37^{o} C for 30 min to eliminate glial cells and fibroblasts, neurons were plated on poly-L-lysine pre-coated 48-well plates (Corning). Neurons were incubated in a humidified atmosphere containing 5% CO_2 at 37^{o} C with DMEM +10% heat-inactivated fetal calf serum + 5% heat-inactivated horse serum + 2 mM glutamine. After 16 hours, medium was replaced with Neurobasal medium with 2% B27, 1% N2 and 2 mM glutamine (all from Life Technologies, Inc.). On day 3 *in vitro,* 5 µM cytosine-β-D-arabinofuranoside (Sigma-Aldrich) was added for 24 h to inhibit glia cell proliferation. Cells in 48-well plates were cultured with 200 µL medium until experimentation. With this culture protocol, a purity of greater than 87% spinal cord neuron population was obtained by 7 DIV. All experiments were performed between 7 and 10 DIV.

***In vitro* traumatic neural injury model**

Primary spinal neurons were cultured as described. At the time of experimentation, cells received either a fresh medium change (control) or were mechanically damaged by a monolayer scratch injury model modified from previous studies (Zhao et al., 2012). In summary, an 18 gauge surgical needle

was flattened to reduce the sharpness and increase the surface area of damage, and distinct grid patterns of scratches were performed in either 48-well or 6-well culture plates containing 7-10 DIV mixed spinal neuron culture. Each scratch was approximately 200 μm wide, and variably spaced according culture well type. This needle and scratch designs were experimentally determined for optimal use in producing graded injury over time, and served as a useful and responsive model for experimental treatment with bpV(pic) and other signal pathway inhibitors.

Assessment of bpV(pic) treatment effects on spinal neurons

In wells selected for experimental treatment, a 45 min pre-treatment with experimentally-determined doses of 100 nM PTEN inhibitor bpV(pic) (Enzo Life Sciences) in 0.9% saline, 20 μM PI3K-inhibitor LY294002, 25 nM mammalian target of rapamycin (mTOR) inhibitor rapamycin (LC Laboratories), or combinations of the above was implemented. Following pre-treatment, cells were left uninjured (controls) or received scratch injuries and left in culture for designated periods of time depending on the experiment. After experimental treatment, supernatant was collected for cell death assays and cells were either prepared for Western blot analysis or immunocytochemistry.

Propidium iodide (PI) neuron death labeling

Hoechst 33342 (Sigma) was added to culture wells and plates were incubated at 37^{o} C and 95% O_2/5% CO_2 for 10 min to label all cell nuclei. Plates were then incubated with PI (Sigma) at room temperature for 15 min to label nuclei of dead cells. Following PI incubation, medium was extracted and cells were washed with 0.01 M PBS, followed by a 10 min treatment with 4% paraformaldehyde (PFA) in 0.1 M PBS to fix the cells. Cells were then washed with PBS and labeled with AlexaFluor 488-conjugated β-III-tubulin primary antibody (Millipore) for 1 hr in PBS-T for neuron labeling + 10% normal goat serum to prevent non-specific binding. Cells were then washed 3 x 5 min with PBS-T and covered with PBS in the well for imaging.

Cell death assay

To assess the damage and death of cultured spinal neurons, lactase dehydrogenase (LDH) release assay was performed on the cells' supernatant per the manufacturer's instructions (Promega, Cytotox 96). Briefly, 50 µL medium was removed from each well with cultured neurons in a 48-well plate following 24 hr injury experiment, and transferred to corresponding wells of an empty flat-bottomed 96-well plate. Supplied LDH substrate mix (50 µL) was added to each well, and the plates were left in the dark for 30 minutes. Afterward, reaction Stop Buffer was added to end LDH enzyme activity, and the plates were read on a plate reader at an absorbance 490 nm. A numerical absorbance value for each

well was calculated as a measure of the amount of LDH release by the neurons with and without scratch injury.

Western Blotting

Spinal neurons used for protein analysis were cultured on 6-well plates as described above. Approximately 1.5 x 10^6 cells/well were lysed in RIPA Lysis Buffer (25mM Tris-HCl [pH 7.6], 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS + 0.5 M EDTA and 1% Halt inhibitor with a Dounce homogenizer (20X up-and-down) and left on ice for 15 minutes. Then, the lysates were centrifuged for 15 min at 13,000 rpm. Supernatants were extracted and protein concentrations were determined using the Bradford method (Bradford, 1976). Samples were prepared with 6x sample buffer, loaded on 8-10% SDS-polyacrylamide gels, and separated via electrophoresis. Proteins were transferred onto polyvinyldine fluoride (PVDF) membranes, and blocked against-non-specific antibody binding with Li-Cor Odyssey blocking buffer (Li-Cor, Lincoln, NE) for 1 hr. Following blocking, the membranes were incubated with primary antibody diluted in blocking buffer with 1% Tween-20 overnight at 4^o C. Membranes were then washed with 0.01 M phosphate buffered saline (PBS) + 1% Tween-20 (PBS-T), and incubated with secondary antibody (goat anti-mouse IR Dye800 or goat anti-rabbit IR Dye680 (Rockland Immunochemicals Inc., Gilbertsville, PA) diluted in blocking buffer + 1% Tween-20 for 1 hr at room temperature. After washing with PBS-T, membranes were scanned using a Li-Cor Odyssey infrared scanner, and images captured using Image Studio 2.0

software (Li-Cor). Densitometry of specific bands was performed using ImageJ software (NIH, Bethesda, MD).

Tissue protein analysis from *in vivo* experiments followed procedures described previously (Xu et al., 1998b) with modification. Briefly, a 10 mm spinal cord segment containing the injury epicenter (5 mm rostral and caudal to the epicenter) was removed for protein extraction 24 hours following SCI. Equal protein concentrations from each sample was loaded onto 8-10% polyacrylamide gels, separated by SDS-PAGE, and transferred to a PVDF membrane. The membranes were immunoblotted with the following primary antibodies: monoclonal mouse-anti PTEN (1:200); rabbit anti-phospho-PTEN (ser 380, 1:500), a marker for the inactive form of PTEN; polyclonal rabbit anti-phospho Akt (ser 473) (1:1,000), a marker used for assessing PTEN and PI3K activation; monoclonal mouse anti-pan Akt (1:1,500); polyclonal rabbit anti-phospho ribosomal protein S6 (ser 235/236) (1:400); cleaved caspase 3 (1:500) a marker for apoptotic cell death (Cell Signaling, Inc.); mouse anti-β-tubulin (1:1000, Sigma-Aldrich) as a loading control. Membranes were incubated with a secondary goat anti-rabbit or anti-mouse fluorescent IR680 or IR800 secondary antibody (1:10,000; Rockland) and visualized with the Li-Cor Odyssey system and software as described. Quantification of detected bands was performed by densitometry using ImageJ software (NIH).

Histological Preparation

Tissue was collected six weeks post-injury and processed as previously published (Liu et al., 2006, Walker et al., 2012). In brief, a 1 cm segment of cervical cord including the injury epicenter was dissected and sectioned transversely at 20 µm thickness on Superfrost Plus slides (Fisher Scientific) using a cryostat (Leica). Serial sections with an interval of 0.5 mm along the length of the cord were used spanning the entire. A set of slides were stained with cresyl violet/eosin to identify the lesion epicenter and 2 mm rostral and caudal to the epicenter. Adjacent sections in this penumbral region were selected for immunofluorescence labeling to determine the pattern of p-Akt expression in surviving near-lesion neurons during the first week post-SCI.

Immunofluorescence Double Labeling

Immunofluorescence double labeling was performed as previously described (Liu et al., 2006, Walker et al., 2012). Briefly, 1 day, 3 days, and 7 days following SCI, 1 cm spinal tissue including the injury epicenter was removed after perfusion, and cryoprotected in 30% sucrose in 0.1 M PBS. Cord segments were sectioned transversely at 20 µm thickness, mounted on Superfrost slides and prepared for immunostaining as described previously (Walker et al., 2012). Tissue sections were incubated with the following primary antibodies: mouse anti-NeuN (1:200, Chemicon, Inc.), a marker for neurons, and rabbit anti-phospho-Akt (ser473) (1:100, Cell Signaling). The following day, the sections were incubated with rhodamine-conjugated goat anti-rabbit or fluoroisothiocyanate (FITC)-conjugated

anti-mouse fluorescent secondary antibodies (1:200, Jackson ImmunoResearch Lab). Sections were coverslip mounted with Fluoromount G (Southern FluoroMount). Pre-immune serum was used as a control to confirm antibody specificity. Images were obtained at identical settings with an Olympus BX60 epifluorescent microscope.

Fluorescent intensity of p-Akt in motor neurons 1.5 mm to 2 mm rostral to the epicenter (in the injury penumbra) was performed using ImageJ. This was achieved by converting the images to grayscale, outlining NeuN/p-Akt double labeled neurons of the ventral horn, and measuring the integrated density (ID) of p-Akt labeling of the neurons. Background values were obtained from 5 identically sized sample areas of tissue adjacent to the quantified neurons, and an average background ID was calculated. The measured neuron ID was divided by the mean background ID to achieve neuron ID signal to background ID ratio values for each neuron. Approximately 10 motor neurons 1.5 to 2 mm rostral to the epicenter were measured per animal, and the signal/background ratio was averaged for all measurements from each animal, and all animals per group.

Statistical Analysis

A two-tailed unpaired Student's *t*-test was used to determine significance between two groups. Statistical significance between multiple groups was determined using a one-way ANOVA with post-hoc analysis. All statistical values were calculated with GraphPad Prism 5.0 software (GraphPad, Inc.), with a p value < 0.05 considered statistically significant.

Results

Changes in PTEN and p-Akt expression/activation following SCI

Supporting previous observations, overall PTEN expression did not significantly change in injured spinal tissue following cervical SCI; however, PTEN activity level, depicted as a ratio of total PTEN to phospho-PTEN^{ser380} significantly increased by days 3 and 7 post-injury (3d, 71.4% increase; 7d, 57.1% over sham; $p < 0.05$) (Fig. 17A & C). Also corroborating our previous data, Akt phosphorylation decreased significantly by day 1 (39.1% decrease), and maintained a low expression pattern throughout the first week (Fig. 17B & C).

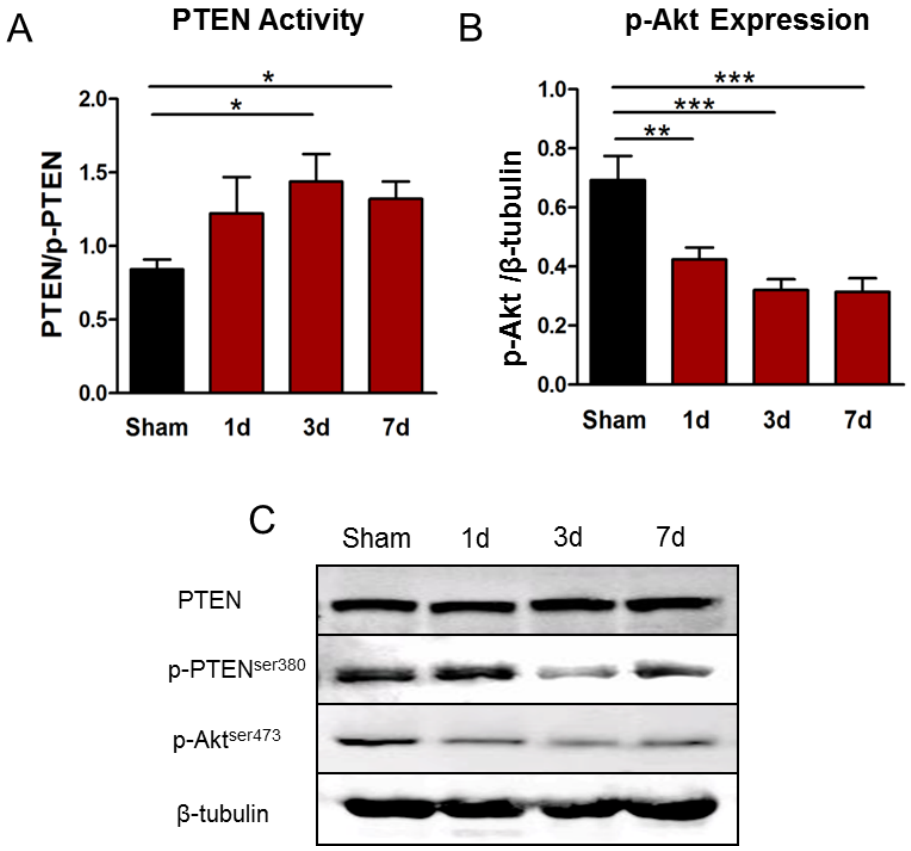

Figure 17. PTEN activity increased while Akt activity decreased following cervical SCI. By 3d & 7d post-SCI, PTEN activity as a measure of active PTEN over inactive p-PTEN significantly increased (A & C). Conversely, phospho-Akt decreased significantly by 1d ($p < 0.01$) and continued through 7d post-SCI ($p < 0.001$) (B & C). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ ($n = 4 – 6$).

bpV decreased caspase 3 activity and increased GSK3β phosphorylation after SCI

In assessment of bpV(pic) effects on apoptotic cell death, caspase 3 activity was assessed through Western blot 1d following injury. SCI significantly increased caspase 3 activity (67.1%) at this time point ($p < 0.01$ compared with sham), and treatment with saline vehicle did not influence injury-mediated caspase 3 activity (Fig 18A & C). Treatment with bpV(pic) during the 24 hour period post-SCI did significantly reduce caspase 3 activity (27.3%) ($p < 0.05$) to approximate levels observed in sham animals. To support the hypothesis that bpV(pic) increased Akt activity, phosphorylation of GSK3β at serine 9 was examined. Treatment with bpV(pic) significantly increased GSK3β phosphorylation at this residue over endogenous levels by 1d following SCI (Fig. 18B & C, $p < 0.05$). Sham animals exhibited high levels of p-Akt in spinal motor neurons (Fig. 19A-C); however, in SCI animals, expression decreased considerably and declined significantly by 7 days post-injury (Fig. 19B, $p < 0.05$) as shown through immunofluorescence double-labeling of p-Akt with neuronal marker NeuN in surviving neurons ~1.5 mm distal to the epicenter (Fig. 19 C-F").

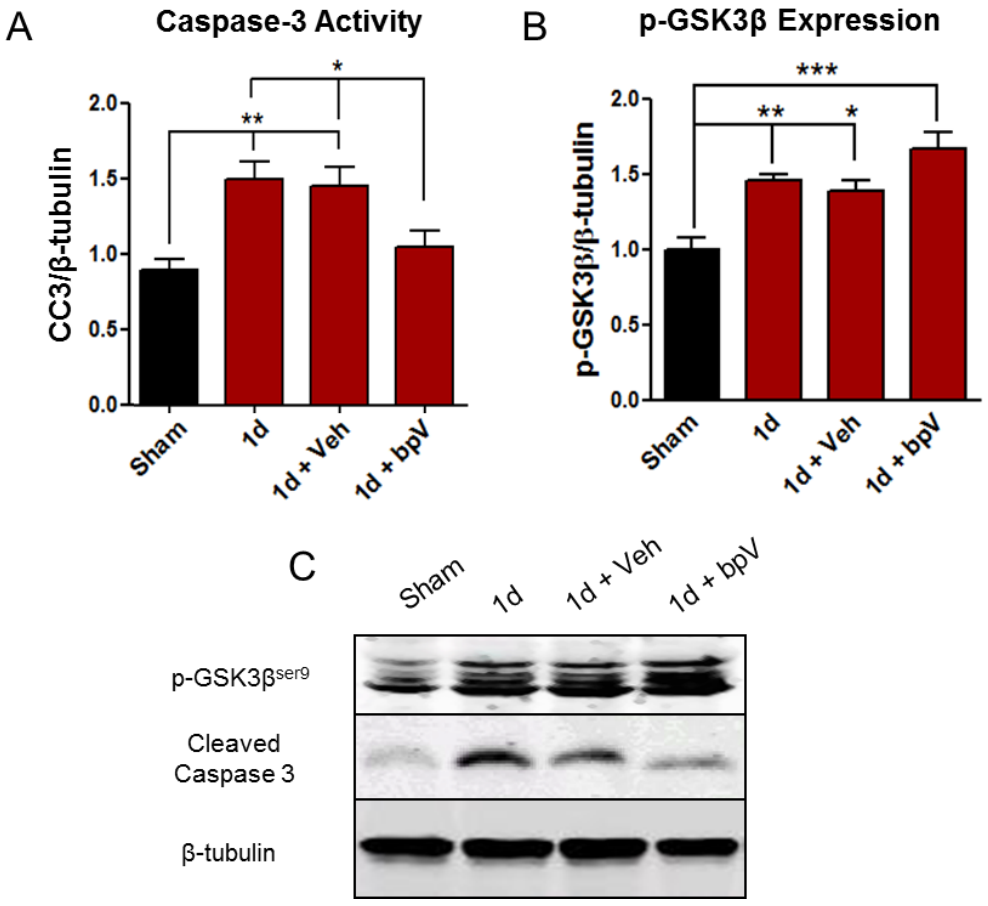

Figure 18. bpV(pic) decreased injury-mediated caspase-3 and GSK3β activities 1d after SCI. Cervical hemicontusion significantly increased cleaved caspase-3 (A & C) ($p < 0.01$) and bpV treatment, but not saline, significantly reduced this elevation ($p < 0.05$). Likewise, saline treatment did not alter SCI mediated p-GSK3β levels, the inactive form, but bpV did elevate p-GSK3β, although the difference was not statistically significant (B & C). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ ($n = 4 – 6$).

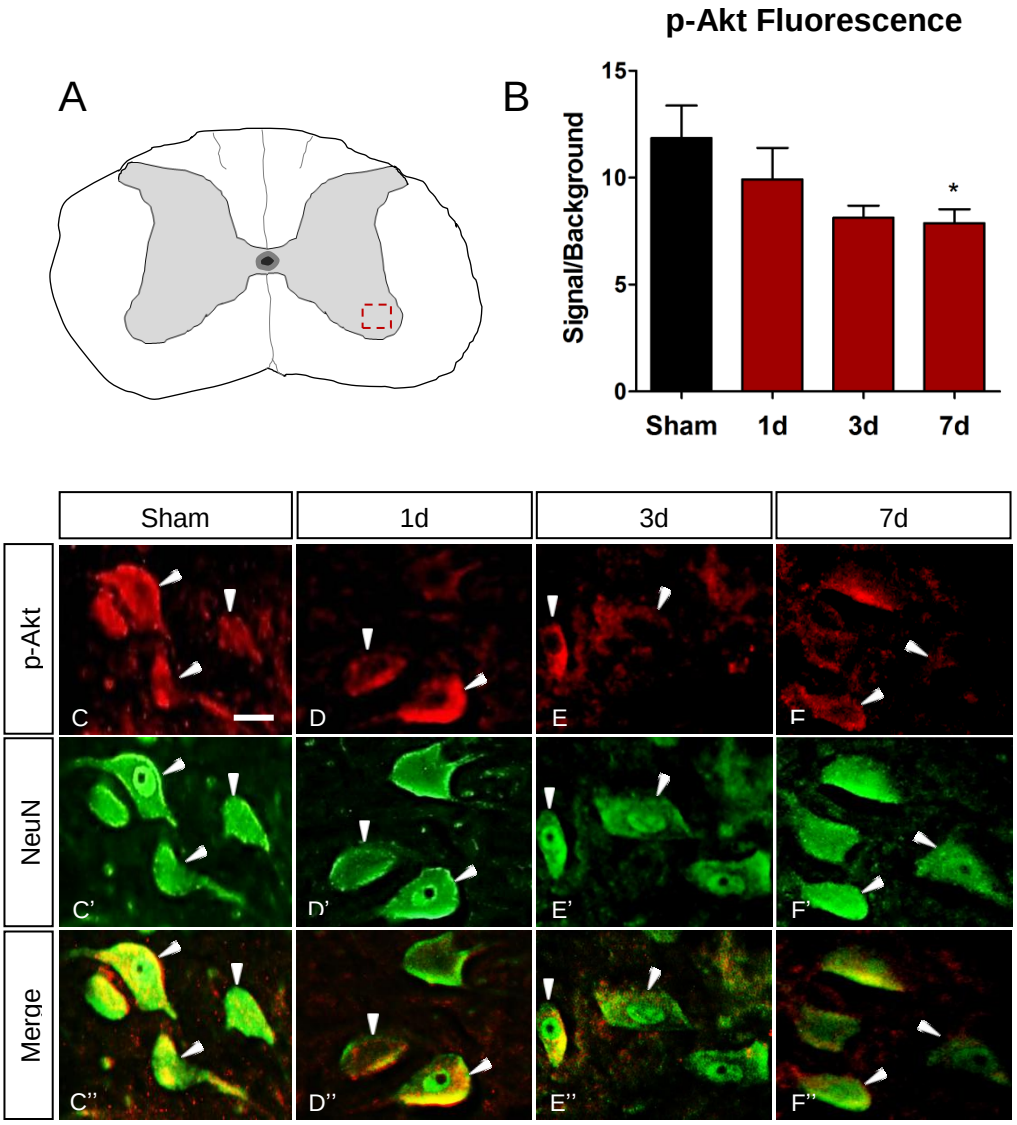

Figure 19. Phospho-Akt decreased in ventral horn neurons following SCI. Neurons from the ventral horn region shown in (A) exhibited a time-course decrease in p-Akt the first week post-injury (B). Sham neurons showed high expression (C – C"), although this reduced by 1 day (D – D") and continued through day 3 (E – E") and day 7 (F – F"). Neurons with notable p-Akt changes are indicated by white arrowheads. Scale bar = 50 µm.

bpV reduced damage induced by a traumatic neuron injury *in vitro*

To confirm bpV(pic)-mediated neuroprotective effects in spinal neurons, we employed an established purified spinal neuron culture protocol (Jiang et al., 2006). To mimic the traumatic mechanical injury inflicted on spinal tissue and induce extended secondary cell damage as observed following SCI, we used a scratch injury model to inflict trauma to wide paths of neurons and neurites, while leaving adjacent neurons intact. The scratch patterns used in 48- and 6-well culture plates is shown in Fig. 20B & C. The damage path width was approximately 200 µm as show in comparison to other scratch techniques (Fig. 20A). Neuronal cultures resembled the representative micrograph displayed in Fig. 20A. This model caused rapid and continued cell damage, and LDH release from the cells continued to increase to 24 hr post-injury (Fig. 21A). At 24 hr following scratch injury, cell death was most evident near the injury site as represented by numerous PI positive cells as compared to non-injured cells (Fig. 21C). As observed in a contusive SCI model *in vivo*, bpV(pic) demonstrated neuroprotective effects on injured primary spinal neurons in culture. Acute LDH release was decreased by100 nM bpV(pic) measured 6 hr post-injury (Fig. 21B, $p < 0.05$). Corroborating this result, bpV reduced PI positive cells by 24 hr post-injury (Fig. 21C).

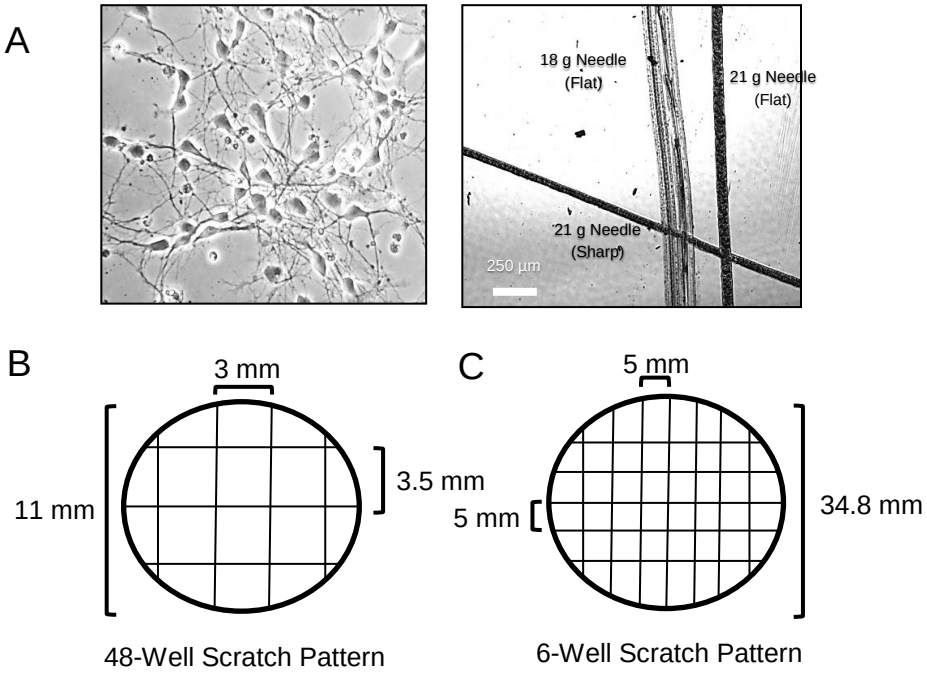

Figure 20. An *in vitro* primary neuron scratch injury model to replicate traumatic SCI *in vivo.* Primary spinal neuron cultures from E15 rats were utilized for replicating neuronal damage observed following SCI in live animals (A). A grid pattern was used in both 48-well plates for assays and immunocytochemistry (B) and 6-well cultures and protein analysis (C).

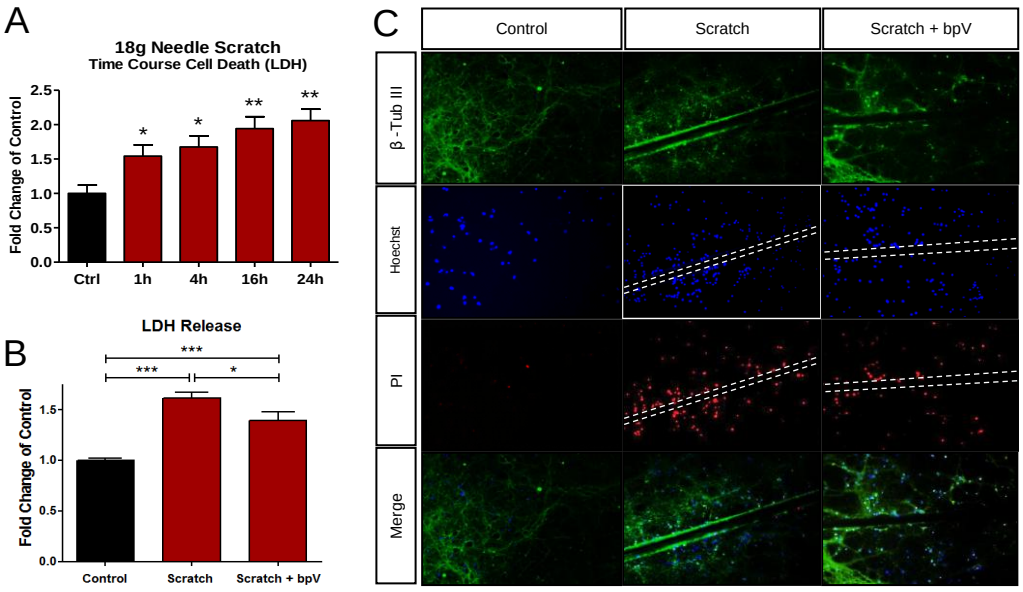

Figure 21. bpV(pic) prohibited significant cell death caused by scratch injury in primary spinal neurons. The applied scratch injury model produced a temporal increase in cell death of primary neurons as assessed through LDH release over 24 hrs (A). Application of 100 nM bpV(pic) significantly reduced LDH release (B), and reduced propidium iodide labeling as a measure of dead cells (C). Dashed lines indicate the scratch area. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ ($n = 3$).

bpV(pic) stimulated activation of Akt and mTOR in injured spinal neurons

Upon confirmation of the compound's protective effects in this injury model, we performed experiments to verify 1) that injury induced changes in Akt and/or mTOR signaling, and 2) whether bpV(pic) acted on PTEN and PI3K/Akt signaling in primary spinal neurons following injury. At 30 min following injury, Akt activity significantly decreased by ~20% ($p < 0.05$) (Fig. 22A). Treatment with 100 nM bpV(pic) significantly elevated Akt activity (36%) in injured neurons at 30 min post-scratch *in vitro* (Fig. 22A, $p < 0.05$), and induced a 25% increase in S6 activity over injury level (Fig. 22B).

To validate the activity of bpV(pic) on PI3K/Akt/mTOR signaling, experiments were carried out using the PI3K inhibitor, LY294002 and mTOR inhibitor, rapamycin on injured spinal neurons. When neurons were treated with 100 nM bpV(pic) and LY294002, the bpV-mediated increase in p-Akt at 30 min was significantly inhibited (86% decrease, Fig. 22A, $p < 0.001$). Downstream mTOR activity enhanced by bpV(pic) was also reduced (86% decrease), as measured by S6 activity following PI3K inhibition (Fig. 22B, $p < 0.01$). Combined with rapamycin treatment, bpV(pic)-associated increase in S6 activity was also diminished in injured neurons, as expected (Fig. 22B).

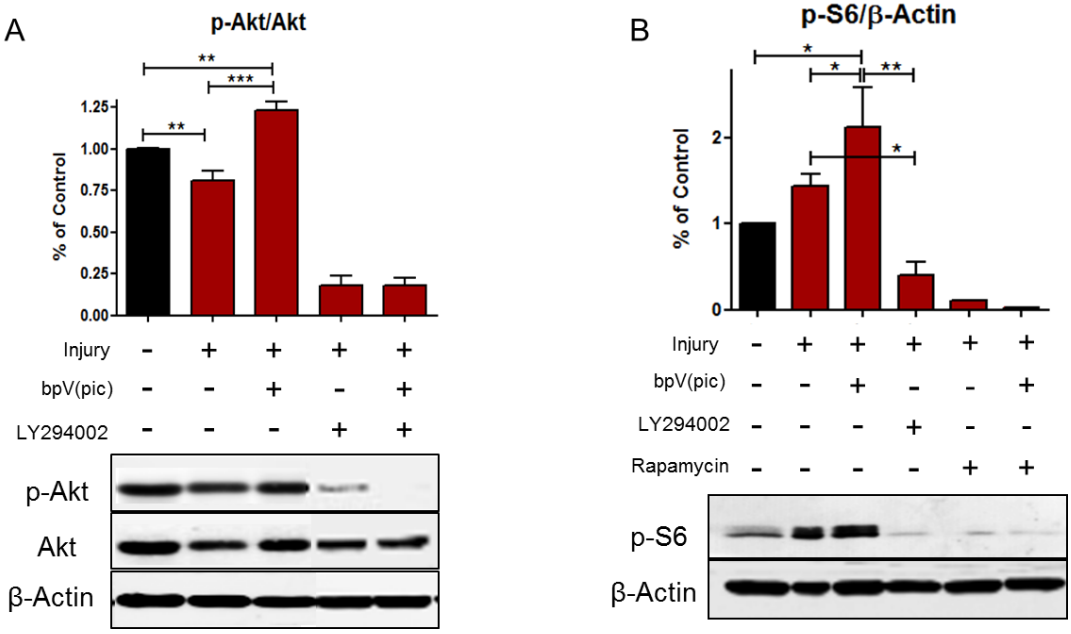

Figure 22. Injury and bpV-mediated effects on Akt and ribosomal protein S6 phosphorylation. (A) Akt phosphorylation (p-Akt) significantly decreased following scratch injury, and bpV significantly inhibited this reduction and increased p-Akt over control levels. PI3K inhibitor LY294002 blocked bpV's effects. (B) Phosphorylation of ribosomal protein S6 was also increased by bpV following scratch injury ($p < 0.05$), and this effect was inhibited by both PI3K inhibition and inhibition of mTOR by rapamycin. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ ($n = 3$).

Discussion

Our results and confirmation of the neuroprotective and cellular signaling effects of bpV following traumatic contusive SCI and a spinal injury model *in vitro* are supported by evidence from multiple nervous system disease and injury studies (Zhang et al., 2007a, Nakashima et al., 2008, Song et al., 2010, Sury et al., 2011, Mao et al., 2013). Our study is novel as it is the first to examine the effects of bpV(pic), or any bpV compound, on injured isolated spinal neurons *in vitro,* and compare the effects of injury and bpV treatment on PI3K/Akt axis signaling to spinal neurons *in vivo*. As both Akt activation and bpV therapies have been widely demonstrated to have survival-enhancing effects, our results expand the knowledge of this signaling pathway and bpV-related effects on the PI3K/Akt signaling response in trauma-inflicted spinal neurons following SCI. Neuroprotection positively correlates with functional recovery, and enhancing our understanding of how to promote neuron survival, and the mechanism by which this benefit is achieved in animals and *in vitro,* provides information and tools for further research into improving functional outcome following SCI.

Yu et al. demonstrated that peripheral neurons up-regulated Akt phosphorylation significantly by 8 h following SCI followed by a rapid down regulation by 24 h post-injury (Yu et al., 2005). Here we show that penumbral neurons downregulate p-Akt by 1d following cervical contusion injury, a pattern that continued throughout the first week after SCI. The similar neuronal Akt phosphorylation response pattern and the whole spinal tissue downregulation of

p-Akt suggests that neurons may be the primary cells influencing Akt activity following SCI. Brief inspection of Akt phosphorylation in glial cells in the region of the studied neurons was not conclusive; however, additional research is being performed to further assess the signaling changes in glia following injury and bpV treatment. Though Yu and colleagues examined motor neurons 3-6 mm distal to the injury site and we examined such cells approximately 1.5 mm from the epicenter, the overall trend is comparable. The neurons we examined survived the initial necrotic death within gray matter at and around the epicenter of injury during the first day post-SCI (Ek et al., 2010). Due to their proximity to this region, they likely were directly affected by the spread of secondary damage to tissue and adjacent cells, which may have altered their response and health over time following injury.

Our findings suggest that spinal neuron downregulation of Akt phosphorylation correlates well with the time-course of apparent increase in PTEN activity as measured through the ratio of PTEN (active) to p-PTEN (inactive) (Fig. 17A & C). These results correspond to and confirm our previous hypotheses and findings, and support the use of Akt phosphorylation as a marker of PTEN activity. As such, our expectation that bpV(pic) was acting through PTEN was further confirmed by these results, as well as by the increase in phosphorylation of GSK3β, a downstream effector of Akt (Cross et al., 1995), following bpV treatment (Fig. 18B & C). This is in agreement with our prior finding that bpV(pic) increased phosphorylation of ribosomal protein S6, a marker for mTOR activity, *in vivo*.

The correlation of PTEN with elevated neuronal death was supported by the observed increase in caspase 3 activity 1d following SCI. Alternatively, bpV(pic)'s neuroprotective effects were reinforced by significantly reducing caspase 3 activity at this time point (Fig. 198 & C). Interestingly, the pattern of SCI-induced elevation in autophagosome formation (Kanno et al., 2009, Kanno et al., 2011, Walker et al., 2012, Zhang et al., 2013) and caspase 3 activity and, and bpV(pic)'s reduction in these activities is quite similar. Whether or not autophagy increase after SCI is neuroprotective (Sekiguchi et al., 2012), cannot be determined, however, this result supported bpV(pic) as a protective agent for neurons under traumatic conditions.

To address this issue, a neuron scratch injury model previously used to replicate traumatic brain injury (TBI) in primary cortical neurons was augmented for use in this study (Zhao et al., 2012) and applied it to our established spinal neuron culture (Jiang et al., 2006). The results indicated bpV(pic) was protective in spinal neurons following traumatic scratch injury (Fig. 21B & C). The hypothesis was tested that traumatic injury *in vitro* would also affect PTEN and PI3K signaling resulting in a reduction in Akt phosphorylation and activity, and the results replicated our observations *in vivo*. An acute pattern of signaling changes was observed, and within 30 min post-scratch injury, spinal neurons significantly downregulated Akt activity ($p < 0.05$; Fig. 22A). Based on preliminary results, 30 min was selected as the time-point for further analysis.

With a pre-treatment of 100 nM bpV(pic), Akt activity was significantly increased over the injury-only control (30') and non-injured control (Fig. 21A, $p <$

0.05) suggesting bpV(pic) was acting through inhibition of PTEN, promoting PI3K activity and subsequent Akt phosphorylation. A similar result in Akt activity upregulation *in vivo* following SCI and bpV(pic) treatment was observed. In a prior *in vivo* study, ribosomal protein S6 activation by Akt, as measured through phosphorylation of S6 at ser 235/236, was also observed both in total tissue protein analysis and neuron immunofluorescence labeling. In addition to Akt activity upregulation, S6 phosphorylation and activation also increased following bpV(pic) treatment (Fig. 21B).

Assessment of mTOR activity is often determined by observing or quantifying p-S6 (Park et al., 2008, Liu et al., 2010c, Sun et al., 2011, Walker et al., 2012). As such, bpV(pic) was proposed to act through this axis by disinhibiting PI3K and promoting downstream mTOR activity. Application of the PI3K inhibitor LY294002 significantly reduced the activity of Akt (Fig. 21A, $p < 0.001$) as well as the phosphorylation of S6 (Fig. 21B, $p < 0.01$) following injury and bpV(pic) pre-treatment. The expectation was that trauma and bpV-mediated effects on mTOR activity would be diminished following PI3K inhibition if mTOR was regulated directly by PI3K signaling after injury. As both Akt and mTOR activities were greatly reduced after LY294002 application, despite bpV(pic) treatment, the results support the hypothesis concerning bpV(pic)'s mechanism of action through PTEN inhibition and activation of Akt/mTOR signaling axis following spinal neuron injury. This conclusion was further supported by the considerable downregulation of bpV(pic)-stimulated p-S6 increase through rapamycin treatment (Fig. 21B). This evidence is in line with our previous

findings, which suggested bpV upregulated mTOR activity and S6 phosphorylation in penumbral neurons following SCI (Walker et al., 2012).

Understanding cellular responses to insult is challenging, as intracellular protein interactions and activation of multiple signaling cascades complicate experimental interpretation. At the tissue level SCI causes disruption of axon tracts, immediate local cell death, and triggers delayed spread of damage and a glial response that chronically inhibits axonal regeneration. As demonstrated in the present study and related literature, bpV compounds have broad neuroprotective effects and result in preservation or recovery of a variety of functional abilities in animal models of neurological dysfunction. Quite recently, bpV compounds were shown to be protective or regenerative through p-Akt signaling in cortical neurons in ischemic injury models (Mao et al., 2013, Zhao et al., 2013). The present study's findings support bpV-mediated neuroprotective effects observed in other injury models as well as our previous observations following SCI; however, this study is the first to demonstrate bpV effects on survival and PTEN/PI3K signaling in primary spinal neurons following traumatic injury. Also, these results show bpV(pic) stimulates mTOR activity, which suggests potential for not only neuroprotection but also regeneration (Park et al., 2008, Liu et al., 2010c). Future studies will further dissect bpV's mechanism of action on neuroprotection in spinal neurons, explore its effects on other cells such as glia, and examine its effects and mechanisms of action on neural repair and regrowth.

CHAPTER 4

AIM 3

INVESTIGATION OF POTENTIAL ADDITIVE OR SYNERGISTIC BENEFITS OF ACUTE BISPEROXOVANDIUM THERAPY COMBINED WITH SUBACUTE SCHWANN CELL TRANSPLANTATION POST-SCI

Hypothesis: Administering a biphasic acute neuroprotective therapy (bpV) with subacute GFP-SC transplantation will promote more extensive neuroprotection and functional recovery, and improve the host-SC interaction over either treatment alone.

Introduction

Schwann cells (SCs) are integral and dynamic participants in peripheral nerve function and repair, and have been widely studied as a potential therapy for central nervous system (CNS) damage following injury. In spinal cord injury (SCI) research, SCs have been studied both directly and indirectly in various contexts ranging from peripheral nerve transplantation (David and Aguayo, 1981, David and Aguayo, 1985, Houle, 1991, Tom et al., 2009, Houle and Cote, 2013, Tom et al., 2013), SC-seeded channel engraftment (Xu et al., 1995a, Xu et al., 1995b, Xu et al., 1997, Xu et al., 1999, Iannotti et al., 2003, Deng et al., 2011, Deng et al., 2013) to cell suspension injection directly into or adjacent to the site of injury (Takami et al., 2002, Pearse et al., 2004b, Golden et al., 2007, Pearse et al., 2007, Schaal et al., 2007, Siriphorn et al., 2010, Hu et al., 2013). Recently, many studies have investigated co-transplanting SCs with other cell types including olfactory ensheathing cells (OECs) (Pearse et al., 2007; Takami et al., 2002) and

glial restricted precursors (GRPs) (Hu et al., 2013). Hormonal therapy combined with SC transplantation has also been reported (Siriphorn et al., 2010).

The potential benefits provided by SCs in the injured spinal cord are many (Oudega & Xu, 2006); nonetheless, key limitations to SC transplantation as a single therapy for SCI persist. SCs die rapidly following transplantation into the lesion following contusion injury (Hill et al., 2007, Hill et al., 2006). It was reported that approximately 80% of transplanted cells die by necrosis and apoptosis by the end of the first week after transplantation (Hill et al., 2007). Although SCs can support considerable growth of propriospinal (Iannotti et al., 2004), reticulospinal (Schaal et al., 2007), raphaespinal (Novikova et al., 2008) and axons of other origin, these fibers fail to exit the graft into the caudal host tissue due to the inhibitory glial scar that encapsulates the contusive lesion. Delaying SC transplantation 7-10 days after injury avoids the cytotoxic nature of the acute injury site and allows for the formation of cystic cavitation in which to inject the cells (Hill et al., 2007, Hill et al., 2006, Martin et al., 1996). Adding a therapy that can reduce acute secondary injury prior to SC transplantation may benefit tissue sparing, repair of the injured cord, and potentially influence characteristics of the SC graft following transplantation. Combination therapies are necessary for advancing SC transplantation research and its potential as a viable clinical treatment for SCI (Bunge, 2008).

Approximately 60% of all human SCI occurs at the cervical level, yet there are strikingly few studies on SC transplantation following cervical SCI (Schaal et al., 2007, Novikova et al., 2008, Siriphorn et al., 2010). Therefore, a better

understanding of the effects and potential benefits of cervical SC transplantation and exploration of new therapeutic combinations in cervical SCI models are necessary. A pharmacological approach in combination with SC transplantation is practical from a clinical perspective due to the overall ease and rapidity of administration in acutely injured patients. In addition, autologous cell transplantation requires several weeks to isolate, purify, and expand a sufficient number of a patient's own SCs for transplantation. A small-molecule targeting cell survival prior to SC transplantation may help curb secondary damage and promote a more robust spinal response to the delayed SC graft.

Bisperoxovanadium (bpV) compounds are dual protein-lipid phosphatases best known for their potent and specific inhibition of the phosphatase and tensin homolog deleted on chromosome ten (PTEN) (Schmid et al., 2004), an important regulator of cell survival and protein synthesis among other functions. PTEN antagonizes phosphatidylinositol-3-kinase (PI3K) activity, limiting downstream survival signaling through Akt and its effectors such as mTOR (Nave et al., 1999), BAD (Datta et al., 1997), and glycogen synthase kinase 3β (GSK3β) (Cross et al., 1995). Neuroprotective and reparative effects of bpV compounds have been widely demonstrated in neurological disease and injury models (Mao et al., 2013, Song et al., 2010, Sury et al., 2011, Yang et al., 2007, Zhang et al., 2007, Zhao et al., 2013) including SCI (Nakashima et al., 2008, Walker et al., 2012).

Our studies showed that a member of the bpV family, bpV(pic), promoted significant long-term neuroprotection and functional recovery when given during

the first week following cervical hemicontusion SCI in rats (Walker et al., 2012). Combining acute bpV(pic) and sub-acute SC transplantation may have the potential to promote additional benefits by compensating for each individual method's limitations and complementing each other's strengths. To test this hypothesis, a systemic bpV(pic) treatment paradigm and transplanted green-fluorescent protein (GFP) transgenic SCs for optimal histological identification were employed. Using a novel forelimb functional assessment and histological analyses, the results of this study shed light on the potential benefits and shortcomings of combining acute bpV(pic) treatment and sub-acute SC transplantation in a clinically-relevant cervical hemicontusive SCI model in rats.

Materials and Methods

Schwann cell culture

Schwann cells (SCs) were purified and expanded as previously described (Morrissey et al., 1991, Xu et al., 1995b, Xu et al., 1999). SCs were harvested from the sciatic nerves of transgenic homozygote green fluorescent protein (GFP)-Rosa expressing adult female Sprague-Dawley (SD) rats (George Smith, Temple University), followed by purification and expansion in culture. The epineurium of the extracted nerves was removed, and sciatic nerves were minced into 2-3 mm long segments in cold Leibovitz L-15 medium (Gibco). These segments were transferred to 35 mm culture dishes (Nunc) containing DMEM + 10% FBS (D10a). Approximately 8-12 segments were placed in one dish, and 3

dishes were prepared from one animal. Nerve segments were covered with just enough medium to prevent them from floating from the dish surface for future fibroblast migration. Medium was changed every 2-3 days, and each week, the segments were explanted into fresh 35 mm dishes with D10a medium to further promote fibroblast migration from the segments. When approximately all migrating cells were of SC morphology (bi- to tri-polar cells with long slender processes [~6 weeks]), the nerve explants were enzymatically digested in dispase/collagenase for 16 hrs, followed by wash in D10a medium and trituration through a sterile glass Pasteur pipette to obtain a single cell suspension. Once cells were ready, they were plated in poly-L-lysine coated 75 mm^2 culture flasks (Nunc) in D10a medium plus the mitogens bovine pituitary extract (PEX) and forskolin to promote cell proliferation. The GFP-SCs were cultured to confluence with medium and mitogen change every 3 days. Cultures were > 98% pure SCs upon collection for transplantation (Xu et al., 1997, Xu et al., 1995b). Purified GFP-SCs at passage 3 or 4 were collected for transplantation into the spinal lesion cavity.

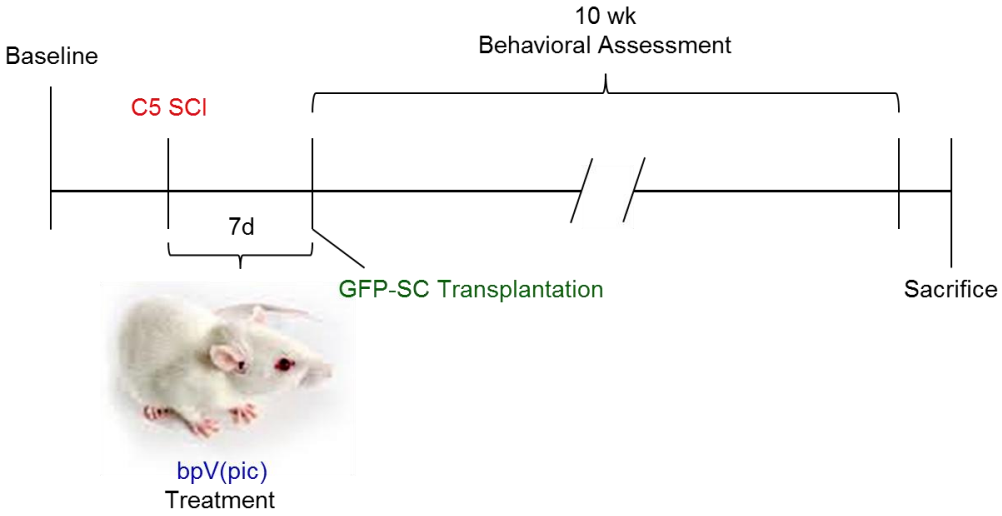

Figure 23. Experimental design for the bpV(pic)/GFP-SC combination study.

Animals and surgical procedures

Adult female Sprague-Dawley rats (200-250 g, Harlan) (*n* = 33) were housed in an environmentally-controlled facility on a 12:12 light:dark cycle with *ad libitum* water and food access. The overall experimental design is illustrated in Figure 23. Prior to surgery, animals were randomly assigned to five groups: 1) Sham or Naïve (*n* = 6), 2) Saline-treated only (Veh, *n* = 6), bpV(pic) treated (*n* = 6), 3) GFP-SC transplantation (*n* = 7), 5) bpV(pic) + GFP-SCs (*n* = 8). All animals in were used for both behavior as well as histological analysis after 10 weeks of testing. Results from these experiments were obtained from the animals stated here for each group. For surgical procedures, animals were anaesthetized intraperitoneally (IP) with a ketamine/xylazine cocktail ([87 mg/kg]/[12 mg/kg]), and received laminectomy only (sham operation) or unilateral cervical SCI performed as previously published (Walker et al., 2012). Briefly, the 5th cervical vertebra was stabilized with a customized device (Zhang et al., 2004) and a partial unilateral laminectomy was performed to expose the right side of the cord. A moderate unilateral injury was produced using the NYU/MASCIS Impactor (Gruner, 1992) (2.5 mm tip, 10g weight, 12.5 mm height) using previously published methods (Gensel et al., 2006, Walker et al., 2012). Sham animals underwent surgery but did not receive injury. All animals received subcutaneous injection of 5 mL 0.9% saline for hydration and were monitored for 24 hr in housing with controlled temperature. Animals were returned to the campus animal housing facility under veterinarian-guided observation and care upon recovery. A laboratory animal technician assisted in animal care. All procedures

and surgeries were approved under the Guide for the Care and Use of Laboratory Animals (National Research Council) and the Guidelines of the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Intraspinal GFP-SC transplantation

Transplantation of GFP-SCs was slightly modified from previously published methods (Hu et al., 2013). Seven to eight days post-injury, GFP-SCs were harvested from culture flasks using 0.05% Trypsin-EDTA, washed and suspended in DMEM + 10% FBS on ice for cell transplantation. In preparation for transplantation, injured and sham rats were anesthetized using ketamine/xylazine and the surgical site was re-opened. Two groups of animals were designated to receive SC transplantation into the lesion cavity, SCs only and bpV + SCs. The spine was stabilized as described, and cell suspension (1 x 10^6 GFP-SCs in 5µl medium) was stereotaxically injected into to lesion epicenter at a depth of 1.2 mm through a glass micropipette with an outer diameter of 50 -– 70 µm and beveled sharpened tip at a rate of ~1 µl/min. After injection, the pipette was left in place for 2 min to prevent cell leakage. Animals not receiving SC injection were surgically opened and the sham laminectomy or injury site exposed. Following surgery, animals were allowed to recover under conditions described above.

bpV(pic) administration

Groups were randomly designated to receive intraperitoneal (IP) injections of bpV(pic) or 0.9% saline vehicle according to methods previously described (Walker et al., 2012). Briefly, 400μg/kg/day bpV(pic) (Enzo Life Sciences) was injected IP beginning immediately after injury, 2 hours post-injury, and twice daily for 7 days following SCI. Ultimately, two groups received bpV(pic) and the rest, including sham animals, received IP injections of saline according to the described treatment schedule.

Behavioral testing

For assessment of forelimb sensorimotor functional recovery, rats were provided flavored cereal rings in their home cage prior to and once per week for 11 weeks following injury (10 weeks after transplantation surgery) and tested as previously described (Walker et al., 2012) (Tables 2 and 3). Briefly, the rats were scored on a 0 to 8 point scale, with 8 being the maximal score, according to their ability to support, grasp, and manipulate treats with the injured and non-injured forelimb while eating the treats. Coordinated treat manipulation was defined as obvious consistent coordinated handling of the treat by both forelimbs during eating. Three trials (rings) were scored and the average score was presented for each rat during a testing session. The test was performed by 2 individuals blinded to the animals' conditions.

Ventral horn neuron and lesion cavity quantification

Twelve weeks post-injury (11 weeks post-transplantation), tissue was dissected and processed as previously described (Liu et al., 2006, Walker et al., 2012). In brief, a 1 cm segment of cervical cord including the injury epicenter was isolated, cryopreserved, and sectioned using a cryostat (Leica) at 20 μm thickness in the transverse or longitudinal horizontal planes on Superfrost Plus slides (Fisher Scientific). Tissue was kept at -20^{o} C until utilized. Some tissue underwent cresyl violet acetate staining with eosin counterstaining (CVE). CVE staining was used to quantify the number of ventral horn neurons at and 2 mm rostral and caudal to the injury epicenter (Fig. 28). A horizontal line was drawn across the transverse section passing through the central canal to establish a standard anatomical region for quantifying neurons. All identifiable ipsilateral neurons in three adjacent sections ventral to this line in laminas VIII and IX, exhibiting dark, evenly distributed cresyl violet staining, were manually quantified using Neurolucida software (Microbrightfield, Inc). The average number of neurons counted for all three adjacent sections per animal at the three designated locations was obtained, and a group-mean neuron count was obtained. CVE stained tissue was also used to measure cavitation at the injury epicenter. Open cavities were traced using contour mapping in Neurolucida software to obtain cavity area.

Assessment of lesion and spared tissue

Using CVE stained sections to identify the injury epicenter, adjacent sections were selected for immunolabeling of the glial scar interface. Adjacent sections were processed as previously described for immunofluorescence labeling. Briefly, tissues were blocked for non-specific binding of antibody and incubated overnight at 4^{o} C with primary antibody against glial fibrillary acidic protein (GFAP), a marker of reactive astrocytes and the glial scar (1:200, Sigma). To use a clearly defined lesion border, GFAP immunolabeling demarcated the interior and exterior of the lesion by the labeling of the glial scar interface. This interface served as the boundaries of the lesion, and the area interior to this interface was assessed as a measure of lesion area at the epicenter. As the inverse of this value, spared tissue was calculated as all area of the ipsilateral cord outside the internal glial scar interface.

Quantification of the GFP-SC graft

To provide an estimation of the SC graft in relation to the lesion, both GFP-labeling and CVE staining of transverse sections were used to identify the graft at the epicenter of injury which corresponded to region of the maximal cross-sectional graft area. At the epicenter, SC grafts, if present, were traced using contour mapping in Neurolucida for each transplanted animal in the SCs group and bpV + SCs group. The average SC graft area per group was measured, and was presented as mean absolute graft area at the lesion epicenter. The total

area of the graft for each animal was also divided by the epicenter lesion area, which provided a % SC graft size of lesion area.

Immunofluorescence labeling of axons, vasculature and macrophages

Immunofluorescence double labeling was performed as previously described (Liu et al., 2006, Walker et al., 2012). In brief, animals were transcardially perfused with 4% paraformaldehyde in 0.1 M PBS following an overdose of ketamine/xylazine. A 1 cm spinal tissue segment including the epicenter (5 mm rostral and caudal) was dissected and removed, cryoprotected in 30% sucrose/PBS solution at 4^{o} C, and sectioned on a cryostat at 20 µm thickness and mounted on Superfrost slides. Sections were prepared as described above and incubated with the following primary antibodies simultaneously with rabbit anti-GFAP (1:200): mouse anti-SMI-31, a marker for non-degenerated axons (1:1000; Chemicon), mouse anti-rat endothelial cell antigen-1 (RECA-1), a marker for rat vasculature (1:200) (ABD Serotec, Inc.), and mouse anti-ED1 (1:200; Chemicon), a marker for macrophages. The next day, sections were incubated with rhodamine-, fluoroisothiocyanate-, or AMCA-conjugated goat anti-rabbit or anti-mouse antibodies (1:100, Jackson ImmunoResearch Lab). Coverslips were mounted on the slides with Fluoromount G. Pre-immune serum was used as a control to confirm the specificity of the antibody. Images were obtained with an Olympus BX60 epifluorescent microscope and Neurolucida software (Microbrightfield, Inc.).

Statistical Analysis

A two-tailed unpaired Student's t-test was used to determine statistical significance between two groups. Statistical significance between multiple groups was determined using a one-way ANOVA with post-hoc analysis if significance was established. All statistics were calculated using GraphPad Prism 5.0 software (GraphPad, Inc.), with a p value < 0.05 considered statistically significant.

Results

bpV alone and combined with SC transplantation improved forelimb function

To test the recovery of forelimb function, a cereal ring treat-handling test was administered for 10 weeks post-transplantation surgery. Throughout testing, all groups performed dependably with an overall trend of improvement (Fig. 24). At Week 10, only animals in the bpV ($p < 0.05$) and bpV + SCs ($p < 0.01$) treatment groups scored significantly higher than saline-treated (Vehicle) control aniamls. The combination treatment group also scored significantly higher than animals treated only with SCs ($p < 0.05$). No statistical significance was observed for forelimb recovery scores between bpV and SCs, or bpV-only vs. bpV + SC treatment groups at the end of testing.

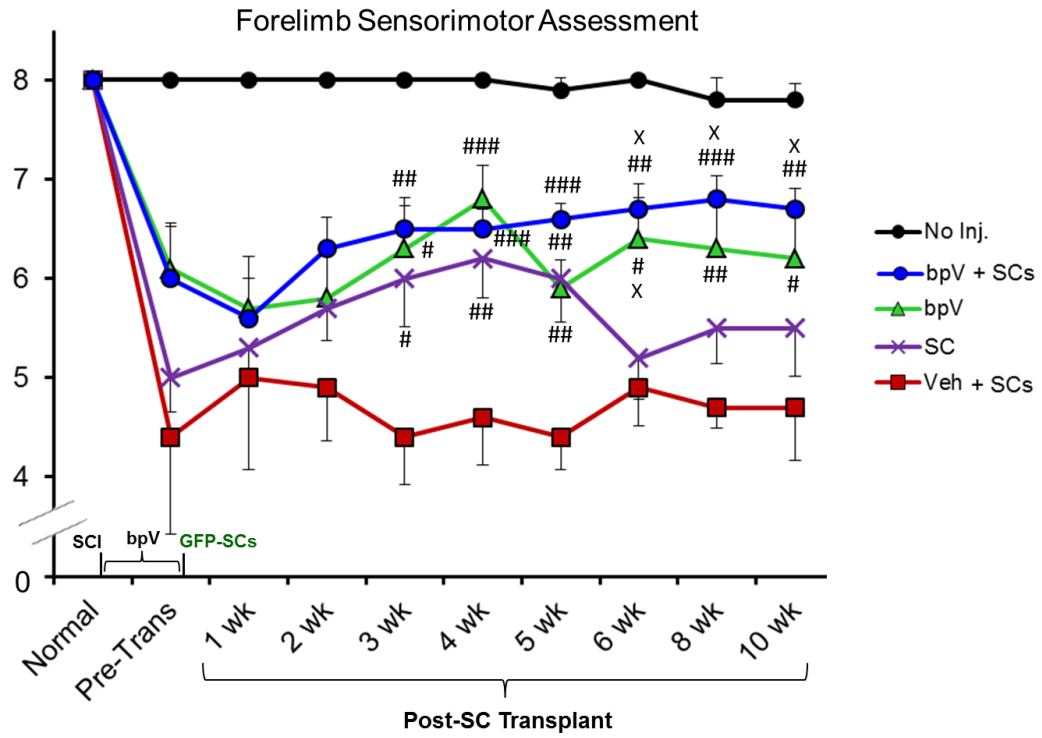

Figure 24. Forelimb sensorimotor assessment scores. Rats were tested on coordinated forelimb movement and manipulation of cereal rings during eating. At the end of the study bpV + SCs and bpV-only group scores were significantly higher than the Vehicle group. The combination bpV + SCs scores were also significantly higher than the SCs group at 10 weeks post-transplantation surgery. #, $p < 0.05$ vs. Veh; ## $p < 0.01$ vs. Veh; ### $p < 0.001$ vs. Veh; X, $p < 0.05$ vs. SCs group.

bpV and bpV + SCs significantly reduced lesion and cavitation, and increased spared tissue and ventral horn neurons

As a measure of neuroprotection, lesion area, spared tissue, and cavity size were calculated. At the conclusion of the study, tissue from both bpV and bpV + SCs groups showed significantly reduced lesion area and increased spared tissue area ($p < 0.01$ & $p < 0.05$, respectively; Fig. 25C,E,F-G). The bpV treatment groups also showed significantly reduced lesion area and increased spared tissue over SCs only treatment ($p < 0.05$; Fig. 25F-G). Decreased lesion area highly correlated with improved functional assessment scores ($R^2 = 0.93$) (Fig. 26). Lesion cavity area was significantly reduced in all treated animals (bpV and bpV + SCs, $p < 0.01$; SCs only, $p < 0.05$) compared to the Vehicle control animals (Fig. 27B,D,E), however, no significant difference was observed between the treatment groups. Similar to the trend observed for lesion and cavity reduction and overall tissue sparing, both bpV and bpV + SCs treatment groups promoted significant ventral horn neuron sparing compared to Vehicle treated animals ($p < 0.05$; Fig. 28A & B), though only 2.0 mm caudal to the injury epicenter. A similar trend was observed 2.0 mm rostral to the epicenter, but the quantification difference was not statistically significant. Almost no neurons were observed at the injury epicenter, therefore, no significant difference was observed between groups.

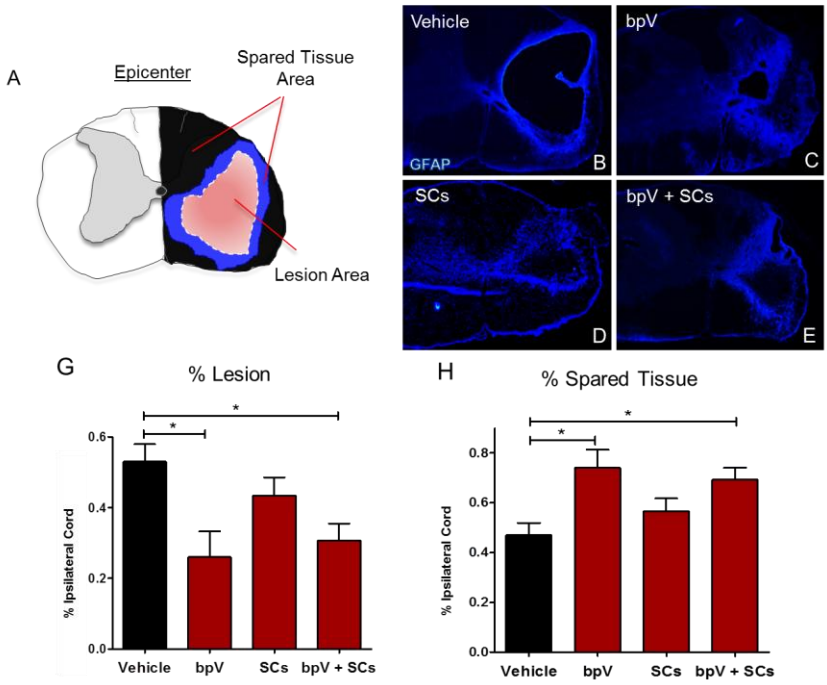

Figure 25. bpV and bpV + SCs reduced lesion and enhanced spared tissue. Method of tissue assessment (A). bpV (C, G & H) and bpV + SCs (E, G & H) significantly reduced lesion and enhanced spared tissue compared to Vehicle-treated animals (B, G & H). Animals treated with SC transplant only (D, G & H) exhibited a trend in neuroprotective measures, but showed no statistical significance compared to the Vehicle group. *, $p < 0.05$.

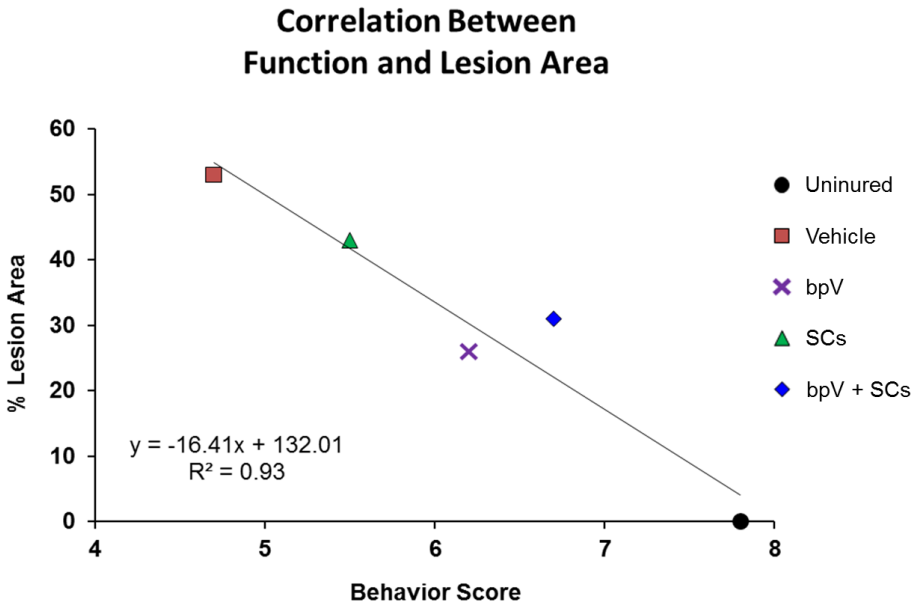

Figure 26. Correlation between behavioral scores and lesion size. Increase in behavioral scores highly correlated with reduction in lesion size by group.

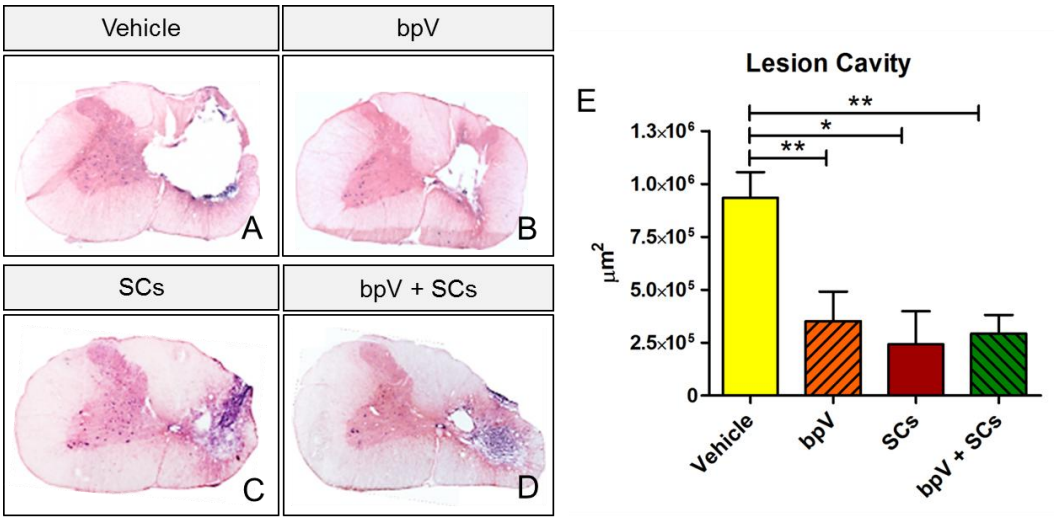

Figure 27. Assessment of lesion cavity following treatment. All treatment groups significantly reduced lesion cavitation versus Vehicle-treated rats (A – E), though not significantly between each other. *, $p < 0.05$; **, $p < 0.01$.

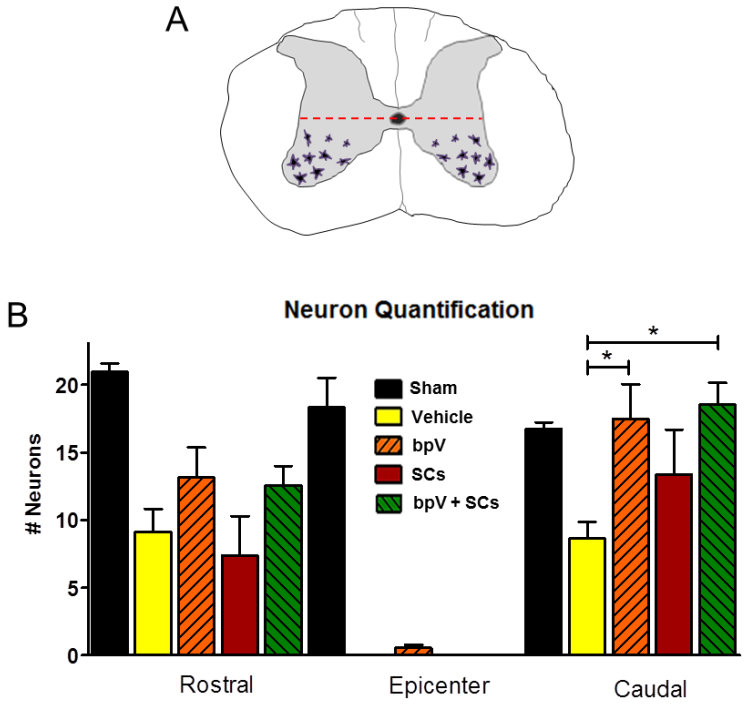

Figure 28. Ventral horn neuron quantification 2 mm rostral, caudal and at the epicenter of injury. Illustration of the region of quantification (A). Only bpV and bpV + SCs groups significantly increased ventral horn neuron count 2 mm caudal to the lesion (B). *, $p < 0.05$.

Maximal SC graft area between SC and bpV + SC groups

After outlining and quantifying the SC graft area at the lesion epicenter for all animals, the results showed that total SC graft area is similar between SC only and the bpV + SCs treatment groups (Fig. 29C-E). However, as the lesion area affects and may be influenced by the graft, to best compare the graft size between both groups was to divide the cross-sectional area of the SC graft at the epicenter by the area of the lesion for that animal. This revealed a much different comparison, showing a greater graft-to-lesion ratio in the bpV + SCs group compared to SC transplantation only; however, this difference was not statistically significant. This finding is logical due to the decreased lesion observed in the combination group in comparison the SC transplantation only group. However, whether the SC transplantation enhanced lesion reduction or the lesion in which the graft was initially injected was smaller cannot be determined from these data. Nonetheless, functional assessment results suggest bpV may mediate significant neuroprotection and lesion reduction within the first week post-injury. Also, both bpV and bpV + SC groups show similar lesion reduction ability which suggests bpV may have already reduced the lesion cavity before SCs were transplanted, resulting in the observed trend in increased graft-to-lesion ratio.

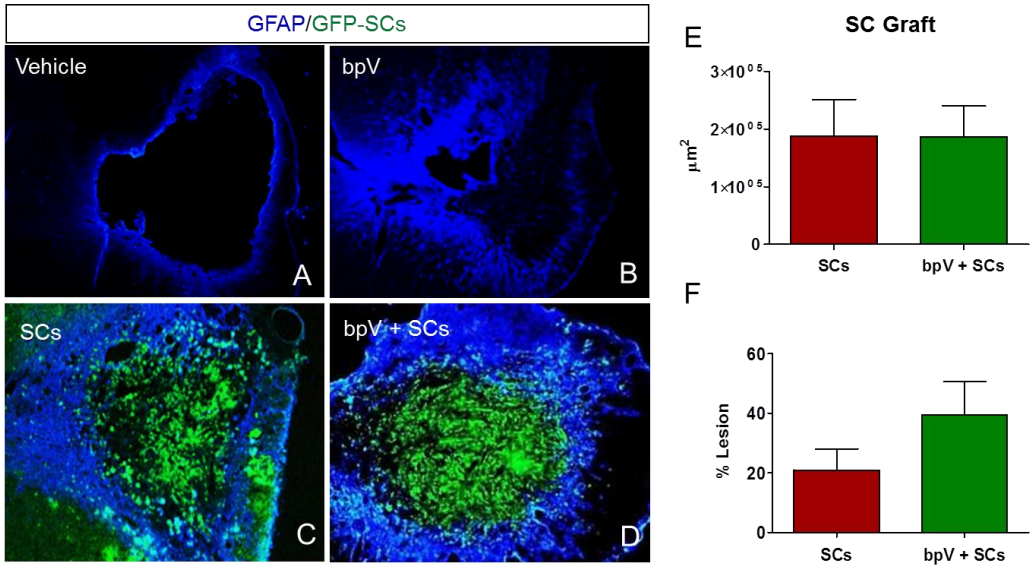

Figure 29. Calculation of GFP-SC graft area between SCs and bpV + SCs groups. Vehicle (A) and bpV (B) groups showed no GFP labeling in the lesion, while transplanted animals did (C & D), as expected. Overall GFP-SC area was similar between groups (E), while bpV + SCs exhibited greater GFP-SC/Lesion Area ratio than animals with SCs only although this difference was not statistically significant (F).

Axonal and vascular growth occurred in all SC grafts

To first determine whether the GFP-SC grafts promoted ingrowth of host tissue, axons and vasculature were immunolabeled with antibodies against SMI-31 and RECA-1, respectively, and the center of the graft was examined. Animals not transplanted with SCs were also labeled and examined. As shown in Figure 30 (A-D), non-transplanted rats exhibited little to no SMI-31+ axons within the boundaries of the GFAP scar. In contrast, SC transplantation only (Fig. 30E-H) and bpV + SCs (Fig. 30I-L) groups promoted remarkable axonal growth into the SC graft. Overall, axonal presence in the graft was similar between the two transplantation groups.

Likewise, vascular presence in the lesion was minimal in non-transplanted animals (Fig. 31A & B), but evident within the lesion/graft epicenter for the SC only (Fig. 31C & D) and bpV + SCs groups (Fig. 31E & F). Like axonal presence in the graft, vasculature was similar between the two SC-transplanted groups through general observation.

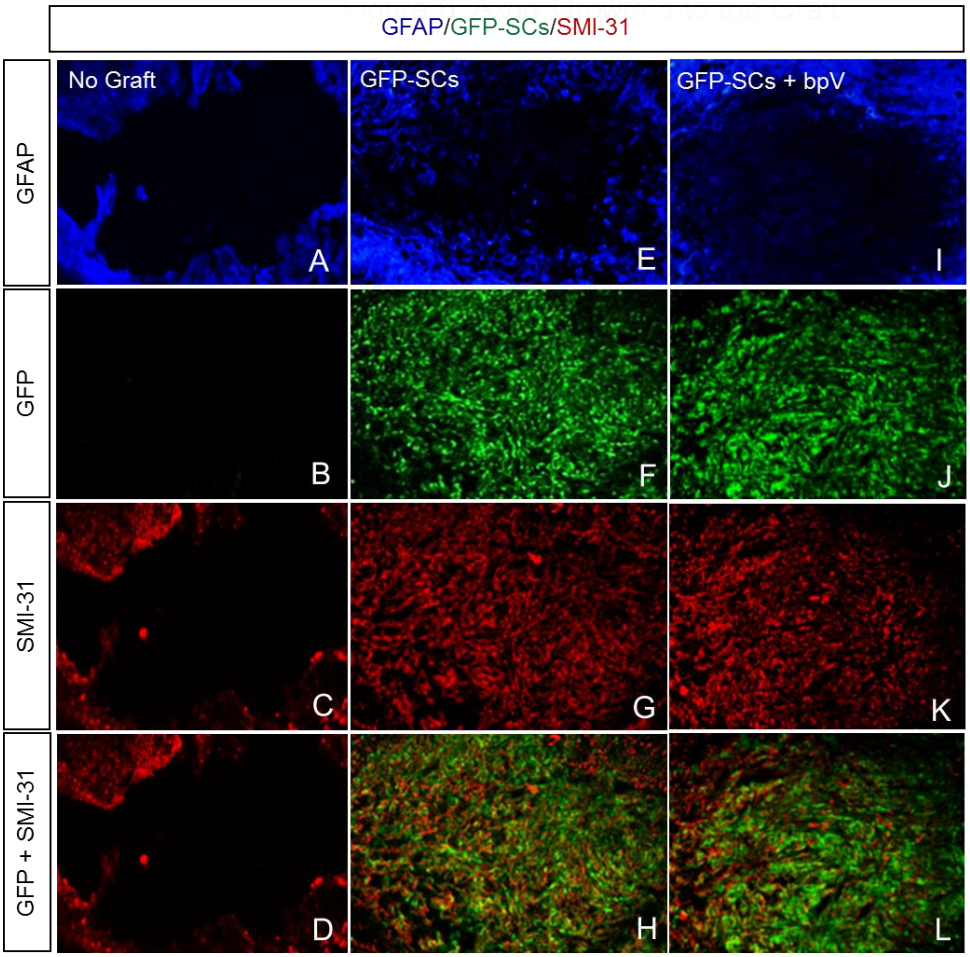

Figure 30. GFP-SC transplantation promoted extensive axon growth into the lesion. Few to no SMI-31^{+} axons were observed within the lesion in animals with no SC graft (A – D). Animals treated with GFP-SC transplantation (E – H) or bpV + GFP-SC transplantation (I – J) showed considerable SMI-31^{+} axon growth in the SC graft and lesion. Growth was similar between the two transplantation groups.

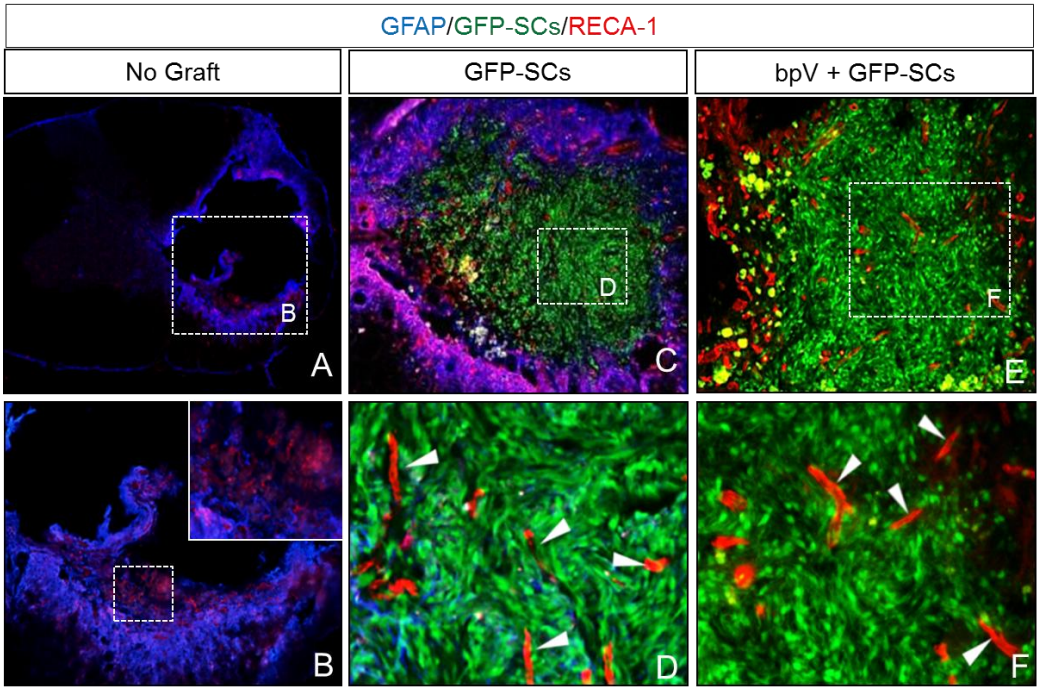

Figure 31. SCs promoted vascular growth into the graft. Like axons, the GFP-SC graft was favorable for RECA-1^{+} vascular growth (white arrowheads) (C – F), while animals without a graft showed fragments or no vasculature inside the lesion (inset) (A – B).

Macrophage response to SC transplants

As determined through ED1-immunolabeling, macrophages responded to the injury in all groups (Fig. 32A-D); however, macrophages were most abundant in and around the SC graft in both SCs only and bpV + SCs groups (Fig. 32C & D). In non-transplanted animals (Veh and bpV groups, Fig. 32A & B), $ED1^{+}$ macrophages were more dispersed outside the lesion with a few individual cells or random aggregations of macrophages observed within the GFAP boundaries of the lesion (Fig. 32A & B, white arrowheads). With SCs present inside the lesion, macrophage aggregations were much more common, although mostly limited to the periphery of the graft (Fig. 32C & D, white arrowheads) in both SC-transplanted groups.

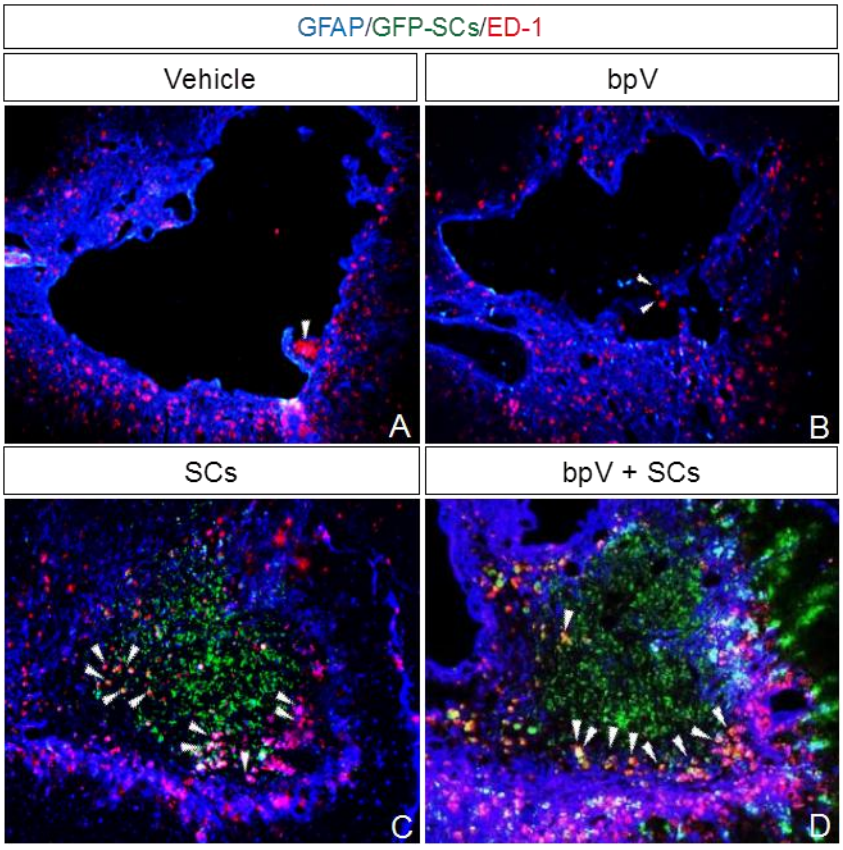

Figure 32. Transplantation of SCs enhanced macrophage presence within the lesion. In vehicle (A) and bpV (B) groups, few ED1^{+} macrophages (red) (marked by white arrowheads) were typically seen within the lesion, though many were observed outside the lesion cavity primarily within or adjacent the GFAP^{+} glial scar (blue). In SC (C) and bpV + SC (D) groups, a large macrophage presence was observed inside the lesion and the SC graft (green). Aggregations of macrophages were also common within the glial scar (pink), suggesting a chronic host tissue reaction to the exogenous GFP-SCs at 10 weeks post-transplantation.

Discussion

The results of this study show bpV promotes neuroprotection and functional recovery, and that these benefits are similar to those provided by the combination bpV + SCs treatment. This evidence supports bpV as the primary neuroprotective component in the combined treatment group. Also, the data confirm bpV as a valuable therapy for improving functional recovery which is likely closely tied to its neuroprotective effects. When transplanted alone, SCs did not significantly improve forelimb functional ability at the end of the study (Fig. 24). They did, however, significantly improve functional recovery until week 4 post-transplantation when forelimb ability began to decline sharply until week 6. From weeks 6 through 10, behavioral scoring was stable, but not statistically significant over saline-treated animals (Fig. 24). The early increase in functional ability suggests the cells may have been contributing to neuroprotection or repair, resulting in significantly improved functional recovery. The downturn in scoring observed at 5 weeks post-transplantation suggested the graft may have been negatively affected by an immune response of the host tissue leading to disruption of functional ability. However, when the maximal graft area at the epicenter was calculated, total area between SCs only and bpV-SCs groups was almost identical (1.88×10^5 +/- 6.3×10^4 vs. 1.87×10^5 +/- 5.4×10^4 μm^2; Fig. 29E), and the $ED1^+$ macrophage response to the SC graft between these two groups was also similar, suggesting some other phenomenon potentially instigated the functional decline observed mid-study.

Although SCs remained present in most transplanted animals in both SCs and bpV + SCs groups throughout the study, the behavior of the SCs may have been altered in some way although no clear effects were observed that could explain the functional reduction. Treatment with bpV, regardless of subsequent SC transplantation, promoted greater functional recovery over SCs alone. Even still, there was no distinct evidence or observation that bpV directly affected the SC graft or that it influenced host tissue interaction with the transplanted SCs. As such, the SCs were likely of value in combination with bpV in promoting subacute tissue growth following bpV-mediated acute neuroprotection, and these benefits marginally affected functional recovery. Further analysis of vascular growth in the graft would help identify whether these vessels were functional, and whether their effects may be beneficial or detrimental to the grafted cells or surrounding host tissue. Evidence suggests angiogenesis is beneficial following SCI (Han et al., 2010), and can promote axonal sprouting and growth (Loy et al., 2001, Dray et al., 2009), all findings that support the results observed in the present study.

As seen for functional recovery, bpV and the bpV + SCs combination groups showed similar lesion area reduction and increased spared tissue compared to SCs only and Vehicle-treated rats (Fig. 25B-G). Animals that received only SC transplantation did not exhibit such significant neuroprotection compared to the Vehicle group (Fig. 25B,G-H). In addition, average behavioral assessment scores correlated well with lesion area as a percentage of ipsilateral cord area (Fig. 26) further emphasizing the benefits of bpV treatment and highlighting the sensitivity of the functional assessment.

We previously demonstrated direct effects of bpV treatment on lesion and cavitation reduction, and overall tissue and neuron sparing in a cervical hemicontusion model (Walker et al., 2012), and the data from the current study are comparable. The advantageous effects of bpV(pic) are further demonstrated through the functional and histological analyses of the bpV + SCs group, as the outcomes closely resemble those for the animals treated only with bpV. This suggests that bpV treatment during the first week post-SCI likely promotes most, if not all of the neuroprotection and related recovery as measured in this and previous studies. The functional benefits of SCs have been reported, however, their effect has not been consistent.

In one of the few cervical injury SC transplantation studies, SC transplantation improved forelimb hang time and grip strength (Schaal et al., 2007). In other studies on thoracic contusive SCI, SCs have demonstrated the ability to enhance Basso, Beattie, and Bresnahan (BBB) (Basso et al., 1995) hindlimb locomotor scores (Takami et al., 2002, Barakat et al., 2005). Contrary to these results, Pearse and colleagues showed no benefit in BBB scoring for animals transplanted only with SCs over contusion injury alone, although they demonstrated that SCs could significantly improve hindpaw rotation in footprint analysis (Pearse et al., 2004a, Pearse et al., 2007). It has been demonstrated that SCs are more effective for repair than other cell types for transplantation, such as olfactory ensheathing cells or glia (OECs or OEGs) for treating experimental contusive SCI (Takami et al., 2002); however, in most studies, combining SCs with other therapies or genetically-modifying SCs promotes more

notable recovery in rat behavioral assessments than SCs alone even when SCs exhibit a benefit. In one recent study, injured animals grafted with SCs genetically modified to overexpress the neurotrophic factor GDNF showed improved locomotor recovery over SCs alone (Deng et al., 2013). These mixed results suggest that although SCs promote positive results as a therapy for SCI, their effects overall may not be substantial for recovery unless other therapies are involved. Our findings in the current study support this hypothesis.

Based on functional assessment results, bpV's dramatic effect on function recovery is maintained over the course of both 6 (Walker et al., 2012) and 11 weeks post-SCI. Further affirming these results, the combination therapy showed very similar neuroprotective effects, as well as sustained improvement in functional outcome over the course of this study. The functional assessment score for the combination group was higher than all individual treatment groups at the end of the study, but only significantly increased over SCs only and Vehicle-treated animals. From these observations, bpV and SC transplantation may not act synergistically, but rather additively through separate mechanisms based on their period of treatment and influence on host tissue. The true relationship, if any, between these two treatments in contributing to functional recovery is not fully clear from the results of this study. Nevertheless, our findings suggest bpV may have played a more important role in neuroprotection and functional recovery than SC transplantation. Also, both bpV and bpV + SC groups showed similar lesion reduction ability which raises the possibility that bpV may have already reduced the lesion before SCs were transplanted. Despite

not having cells transplanted into the lesion, bpV exhibited similar lesion cavity reduction to both the SCs and bpV + SCs treatment groups, further supporting this interpretation.

In continuation of the beneficial influence of bpV on neuroprotection, only bpV and bpV + SCs groups showed significant ventral horn neuron sparing caudal to the injury, and a similar trend rostrally (Fig. 28B). In our previous study, comparable effects on ventral horn neuron sparing were observed following bpV treatment. Both bpV and bpV + SCs exhibited similar effects in this outcome measure, which further supports bpV's neuroprotective effects over SC transplantation. Although the total area of the SC graft at the epicenter was similar between groups, the graft-to-lesion ratio was higher in the combination group compared to animals that received only SC transplantation (Fig. 29F). This result is reasonable, as the lesion was greatly reduced in the combination group compared to SCs-only group.

The potential value of SC transplantation was observed in examination of host tissue growth into the graft, and thus, into the lesion area. The amount of SMI-31^{+} axons observed in the SC graft was similar between SCs and bpV + SCs groups (Fig. 30E-L); however, the axon-to-lesion area ratio was likely higher due to the reduced lesion in the combination therapy group (data not shown). When investigating axonal presence within the lesion in non-grafted animals (Fig. 30A-D, a bpV treated animal), almost no axons were observed inside the GFAP boundaries of the lesion. This same pattern was observed for RECA-1^{+} vasculature. In both transplant groups, SCs exhibited clear vascular growth at the

epicenter of the graft (Fig. 31C-F), while negligible vasculature was observed in the lesions of non-SC transplanted animals (Fig. 31A & B). To our knowledge, this is the first study to observe vascular growth into the SC graft in a cervical SCI model. The exact mechanism influencing vascular generation in this model is unclear.

What potentially contributed to the functional ability increase in the bpV and bpV + SCs groups based on our histological findings? One explanation centers on the spared neurons observed rostral and caudal to the injury epicenter. By sparing ventral horn neuron in laminas VIII and IX, more efferent motor activity could be provided to the musculature of the forelimb. Since the most significant increase was observed at the caudal end of the lesion, this correlates to neurons involved in more distal forelimb function (near C7) which associates to a large motor neuron population innervating the triceps brachii muscles, the major extensor muscle group of the forelimb (Tosolini and Morris, 2012). This could result in more joint extension and range of motion, and thus, better ability to coordinate sensorimotor activity of the forelimbs while eating cereal rings. We previously observed close correlation of our forelimb scoring scale with joint articulation ability and the C5 hemicontusion injury produced in this study results in a chronically flexed forelimb and forepaw; therefore, our histological findings plausibly support the functional outcome observed in this assessment.

As both of these groups exhibited significantly reduced lesion areas and increased overall spared tissue at the lesion epicenter, a measure that

significantly correlates with lesion severity and behavioral analysis following contusive SCI (Noble and Wrathall, 1985, Bresnahan et al., 1987), the increase in functional ability could also have resulted from sparing of both gray and white matter in these two groups. However, as all injured animals failed to fully extend and grasp the treats with the digits suggests chronic corticospinal tract damage in the ipsilateral dorsal column. Therefore, further analysis of bpV's effects on this tract could help shed light on mechanisms to target CST protection and repair to further improve the rats' ability to handle the cereal treats with the forepaws during the treat manipulation exercise.

Remarkably, few other studies have been reported on SC transplantation at the cervical level, however, those that were performed focused on combining SCs with an additional treatment or strategy to produce host and SC graft effects. As previously discussed, Schaal and colleagues transplanted SCs into the contused cervical spinal cord and observed surpaspinal axonal growth into the graft, as well as SC-mediated forelimb functional recovery (Schaal et al., 2007). Though we did not see such significant recovery from only SC transplantation in our study, our behavioral assessment was designed to look at fine forelimb sensorimotor coordination in a hemicontusion model. Schaal's study utilized more forepaw strength-based assessments following a bilateral injury. We did see axonal growth into the graft, although our study did not assess the origin of these axons. In 2008, one group loaded SCs into poly-β-hydroxybutyrate (PBH) channels, implanted the graft into the hemisected gap, and identified serotonergic raphespinal and CGRP fibers, but not descending motor rubrospinal

axons within the graft (Novikova et al., 2008). The PBH channels did not promote axonal growth when implanted alone, suggesting this material played a limited role in the growth seen with in combination with seeded SCs. However, no other characteristics, such as functional recovery or neuroprotection, were investigated to gauge the influence or importance of SCs and the promoted axonal growth observed in the study.

The most comparable study of the cervical SC transplantation studies investigated a co-treatment of 17β-estradiol with transplantation of SCs into the hemicontused cervical spinal cord (Siriphorn et al., 2010). In this study, a 17β-estradiol (E2) pellet (5 mg/pellet, 21-day continuous release) was implanted subcutaneously 30 min following injury. After a delay of 8 days post-SCI, 5-(and-6)-carboxyfluorecein diacetate succinimidyl ester (CFSE)-labeled SCs were transplanted into the contusion site and animals were allowed to survive for 7 days following transplantation. Though this study is relevant and yielded novel information, there were notable differences than the study presented here. First, the authors' primary goal was to determine whether E2 could protect transplanted SCs and promote their survival following transplantation. The authors also employed *in vitro* analyses to identify mechanisms as to how E2 could be protective to SCs, however, no *in vivo* effects of the drug on pre- or post-transplantation tissue morphology or cellular sparing were examined, minimizing our understanding of the impact on host tissue over direct effects on

the cells post-transplantation. Furthermore, the combined E2/SC transplantation treatment intentionally overlapped to investigate one treatment's effect on the other.

Our goals were different by comparison, as we designed our study to provide a non-overlapping two-phase treatment regimen, although we cannot discount the possibility some minor overlap occurred at the transition between bpV(pic) treatment and SC transplantation. Current studies are investigating potential effects of bpV on SCs *in vitro* to address this possibility, as well as to investigate other potential combination benefits of bpV and SCs for SCI. As acute sustained E2 delivery can protect SCs under deleterious conditions *in vitro* and *in vivo*, experimentation with neuroprotective bpV therapy with E2 could be useful to help identify promising combination therapies to augment the effects of SC transplantation following SCI.

In summary, three main conclusions can be made from the results of the present study concerning bpV combination therapy with SC transplantation, and SC transplantation in general: 1) Biphasic combination therapy, with bpV targeting acute secondary damage combined with delayed SC transplantation, can impart separate yet potentially additive benefits resulting in neuroprotection and enhanced functional recovery, 2) SCs alone appeared to play a limited role in functional recovery, and 3) transplanted SCs exhibit long-term survival in the contused cervical spinal cord, regardless of whether or not a co-therapy is added. Separate from the combinatory treatment aspect of the study, our results affirm bpV as a potent therapeutic that can significantly reduce lesion and cavity

formation, as well as promote functional recovery when given systemically following clinically relevant experimental SCI.

Ultimately, the results of this study suggest bpV promoted most of the neuroprotective benefits, and played a larger role in functional recovery than SC transplantation when the two therapies were combined. Transplanted SCs elicited a dynamic host tissue response, promoting considerable axonal and vascular ingrowth over the course of the study. The impact of these responses on recovery, however, is indeterminable from the observed results. The vasculature observed in the graft, if functional, could have provided nutrients and growth components necessary for long-term SC survival and axon growth. Without a treatment to dissolve the glial scar formation at the caudal end of the lesion, it is unlikely any axons that entered the graft were able to exit and make functional connections in the host tissue. This could explain the limited effects SC transplantation alone had on functional recovery in this study. As such, the bpV component of our combination therapy possibly enhanced recovery through acute sparing of neurons and other cells from secondary damage following SCI. If combined with another therapy to degrade the glial scar or allow SC overexpression of trophic factors, transplantation of SCs may contribute more substantially to neuroprotection, axonal growth into caudal host tissue, and forelimb functional recovery. Future studies will address these and other issues concerning the potential of SCs in combination therapies following SCI.

CHAPTER 5

CONCLUSIONS

Although this work highlights research directed toward improving repair and recovery following SCI, this and all CNS injuries cause a syndrome of physical and functional effects that is seemingly insurmountable from the present point of view. Nonetheless, the progression of work in the field, including the contribution made in the present volume, illustrates the headstrong optimism that many small hits over time can crumble the wall separating us from a cure for CNS injury and resulting functional dysregulation and paralysis. In many ways, the preceding chapters highlight important aims of focus and potential achievable outcomes that are possible in targeting these areas.

The molecular events that unfold following SCI have long been of research interest; however, the outcome emphasis and interpretation has many facets as intracellular dynamics of signal pathway networking is extremely complex. The dissection of PTEN/PI3K/Akt signaling following SCI helped build on previous studies (Yu et al., 2005, Kanno et al., 2009, Kanno et al., 2011) by adding extra information and histological interpretation of the cellular expression of key molecules in the pathway in the acute period post-SCI. Importantly, our work used a clinically-relevant cervical hemicontusion model, versus thoracic injury, which may present different cellular injury responses. In addition, we added bpV as a tool to inhibit PTEN and examine the neuroprotective and

functional recovery effects to better illustrate the role of this signaling pathway following SCI.

Cervical SCI damages descending supraspinal axon tracts originating from neuron populations in the brain, among ascending sensory fibers and other spinal structures and cells located in the region of injury. Being that the cervical cord is located close to the brain in comparison to the thoracic cord, damage to tracts in this region impart more pathological changes and cell loss in the cell bodies of originating neurons than damage inflicted more caudally (Egan et al., 1977). Likewise, axotomy of brainstem rubrospinal axons, which impart descending flexor-biased motor regulation induce further neuron degeneration and death in the red nucleus than cervical SCI (Liu et al., 2003). This variation in the influence of injury on supraspinal neurons based upon distance of insult from the cell body adds another dimension to understanding both the more common cervical SCI in the human population, as well as the effects of cervical injury and treatments in animal models on supraspinal neuron nuclei of origin and their spared or damaged axonal projections in the region of injury in the spinal cord.

If cervical SCI is the most frequently diagnosed form of SCI in humans, and the potential spinal and supraspinal influences of injury are different than those of the more commonly used experimental thoracic SCI models, then more use and investigation of cervical SCI models will likely yield more readily translatable information from bench to bedside in developing treatments for human SCI. This difference in pathological progression based upon region of injury in the spinal cord also highlights important considerations of treatment

effects in different SCI models used in neurotrauma research. One example concerns potentially different variables affected by bpV(pic) treatment in cervical and thoracic SCI. Nakashima et al. (2008) demonstrated bpV(phen), a related PTEN inhibitor, was neuroprotective following thoracic injury, an effect we also observed in cervical SCI. It would be interesting, however, to determine the supraspinal effects of SCI between these two regions as any potential differences between the two could have important implications on the potential for promotion of axonal tract sprouting and regeneration by bpV compounds.

If the brainstem or cortical population of neurons are healthier and exhibit less degeneration following thoracic versus cervical SCI, then a more robust sprouting or regrowth effect may be observed following bpV treatment due to the ability of bpV PTEN phosphatase inhibition (Mao et al., 2013) and PTEN deletion or expression inhibition (Park et al., 2008, Liu et al., 2010) to promote neuronal regeneration. Investigation of regenerative effects of bpV(pic) was not a focus of the studies presented here, however, future studies have been designed to explore potential regenerative effects of bpV compounds on axon regeneration following SCI.

Given that major tracts of the spinal cord, for example the corticospinal tract, are represented quite differently anatomically such that the injury produced in the studies described here will damage different sensorimotor axonal tracts in humans, consideration must be taken when extrapolating such experimental information to the human condition. Nonetheless, the cervical hemicontusion

model is one of the closest models characterized to experimentally produce relevant replication of anatomical cervical SCI in humans.

Concerning the use of pharmacological agent, bpV(pic) in the present studies, it was highly important to 1) determine whether spinal neurons were a likely target of the effects of the drug *in vivo*, 2) investigate a time-course response of this pathway in neurons following SCI to assess the acute recovery pattern of Akt signaling, and 3) compare and analyze the drug's protective and molecular signaling effects in isolated spinal neurons *in vitro* to compare verify the effects witnessed *in vivo.* In the end, our findings contribute knowledge on the neuronal effects of bpV treatment, and thus, PTEN inhibition in spinal neurons. Results obtained from the initial bpV treatment study were validated to some extent by the results of the *in vitro* experiments. Nevertheless, other cellular effects such as the influence of bpV on astrocytes and oligodendrocytes were not considered for these studies but this does not diminish the importance of such investigation. The effects of PTEN inhibition lend itself to potential acceleration of astrogliosis (Cai et al., 2001, Codellupi et al., 2009) as well as regulation of myelination integrity by oligodendrocytes (Harrington et al., 2010). If astrogliosis was influenced by bpV treatment in the present research, it did not significantly prohibit neuroprotection and functional recovery provided by the drug. The drug may have actually promoted benefits of astrocyte activity during the acute phase following SCI.

The roles of astrocytes following SCI are intricate and important; however, they is mostly viewed in a negative light due to the well-known axon growth

inhibitory glial scar that forms over time after injury. Shortly after injury, however, astrocytes contribute considerably to local and regional protective and reparative events spanning from clean-up activities and re-establishment of a barrier between the spinal cord and the exterior environment. Various protective roles of reactive astrocytes include taking up excess excitotoxic glutamate, providing protection from oxidative agents, repair of the blood-brain-barrier in damaged tissue, and reducing inflammation and oxidative damage through sealing the lesion area via astrogliotic scar formation (Rothstein et al., 1996, Bush et al., 1999, Chen et al., 2001, Faulkner et al., 2004, Hamby and Sofroniew, 2010, Sarafian et al., 2010). Given this list of positive functions of reactive astrocytes, and that PTEN downregulation and mTOR activity upregulation are suggested to play important roles the the reactive response of astrocytes, part of the neuroprtective effects of bpV(pic) treatment during the acute period following SCI may potentially be mediated by astrocytes. At present, however, the full influence of bpV(pic) on astrocytes cannot be fully determined.

The necessity of combination therapies with SC transplantation is quite clear, despite their benefits alone. The acute administration of bpV followed by subacute SC transplantation performed as described in Chapter 4 is the first biphasic study of its kind in cervical SCI research. The only other comparable study involved acute subcutaneous transplantation of a slow-releasing 17β-estradiol tablet just after cervical hemicontusion, followed by a subacute SC transplantation (Siriphorn et al., 2010). One important difference between these studies is that the 17β-estradiol treatment released over 21 days, which was 14

days after SCs had been implanted. Also, the study primarily focused on this treatment's effects on SC survival. Our focus here was to attempt to promote an acute prevention of secondary damage expansion with acute bpV therapy followed by a non-overlapping SC transplantation to encourage host-graft responses in the subacute to chronic periods following SCI. Our findings suggest that this two phase treatment paradigm can work to produce additive anatomical and functional effects over individual treatments alone.

One issue that deserves discussion is the contrast between the neuroprotective outcomes and the functional recovery differences of the treatment groups in the bpV-GFP-SC combination study. Neuroprotective effects and functional recovery was most significant in the bpV and bpV and GFP-SC transplantation combination treatment groups, while SC transplantation alone did not significantly influence these outcomes over non-treated injured animals (Figs 24-28). Despite these results, two potential external factors could hamper the interpretation of the benefits, or lack thereof, concerning the treatments in this study: the severity of injury and the sensitivity of the outcome measures.

It has long been shown that contusion injury severity produced by the NYU/MASCIS impactor correlates well with degree of resulting physical and functional deficits (Basso et al., 1996). The injury produced in this study, a 10g weighted rod drop from 12.5 mm high onto the exposed spinal cord produces a comparatively moderate contusion injury within the parameters available in the impactor design (12.5 mm, 25 mm drop height etc.). This severity designation, however, is based upon measures of tissue and function from animals not

treated via SC transplantation. Also, though other studies have used SC transplantation in 12.5 mm weight drop injuries produced by the NYU/MASCIS impactor, these are mostly performed at the midline thoracic spinal cord (Takami et al., 2002, Pearse et al., 2004a, Pearse et al., 2004b, Hill et al., 2006, Golden et al., 2007, Hill et al., 2007, Pearse et al., 2007). Nearly all these studies contain some component of axonal growth analysis, yet only a few describe tissue sparing and neuroprotective effects of SC transplantation among other treatments.

Schaal et al. (2007) performed the only study involving transplantation and subsequent assessment of neuroprotection in a cervical contusion model. However, this study utilized a different impactor system to perform a more precarious midline cervical contusion rather than a hemicontusion. Still, SCs were shown to promote neuroprotective tissue sparing in addition to functional recovery in their study. This introduces the possibility that severity of the injury may contribute to the sensitivity to detect SC-mediated neuroprotective effects. Although the 12.5 mm drop height is considered to produce a moderate injury in the cervical hemi-contusion model utilized in the present studies (Gensel et al., 2006), the current evidence is inconclusive as to whether this injury is severe enough to reveal clear neuroprotective lesion reduction and tissue sparing by SC transplantation alone.

This possibility is bolstered by the close functional outcomes between all treatment groups. The administration of bpV and SC transplantation as individual therapies were not significantly different, despite the statistical significance

between of bpV, but not SCs, over the scores of vehicle-treated animals. The combination therapy was significantly different than both vehicle and SC-only treated groups. Together these results suggest that bpV was more effective than SCs at promoting neuroprotection and functional recovery. When looking at the difference in scores however, bpV + SC treated animals scored only an average of two points higher on the forelimb assessment scale, with bpV-only treatment falling in between. These observations, in addition to those from the histological assessments suggest that the results may have been affected by a lack of sensitivity of the injury model or functional assessment, or both. Therefore, though the data support the conclusions about the benefits of bpV and the combination therapy following SCI, alternative influences or explanations for the results cannot be ruled out.

In summary, though the wall still stands between SCI and a cure, it is with hope that the work provided in this thesis helped, if ever so slightly, our efforts towards reaching that goal. Ultimately, the real merit of this work was the validation of the positive and overall effects bpV therapy and highlighting the benefits and limitations of combining bpV and SC transplantation following SCI. We also demonstrated that SCs can survive well into the third month post-transplantation without immunosuppressant, which counters the previous findings of near total SC loss shortly after transplantation. Though the acute death and long-term survival were not of prime interest in the present study, the ability for the cells to promote extensive axon and vascular growth into the graft suggests that whatever SCs survive can interact remarkably well with host tissue over long

periods of time. The use of a cervical hemicontusion SCI model increased the clinical relevance of the research, and in the end, any work towards the goal of treating and someday curing SCI and other CNS injuries is of great value.

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Publications

2013. **Walker, CL**, Wu XB, Liu, NK, and Xu XM. *In vivo* and *in vitro* assessment of PTEN/PI3K and bisperoxovanadium activity in spinal neurons following traumatic injury. *In preparation.*

2013. **Walker, CL**, Liu, NK, and Xu XM. The role of PTEN/PI3K and MAPK signaling in protection and pathology following CNS injuries. *Frontiers in Biology*, doi:10.1007/s11515-013-1255-1.

2013. Zhang, YP, Walker, MJ, Shields, LBE, Wang, XF, **Walker, CL**, Xu, XM, and Shields, CB. Controlled Cervical Laceration Injury in Mice. *Journal of Visualized Experiments (75), e50030, doi:10.3791/50030.*

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2012. **Walker CL**, Walker, MJ, Zhang, YP, Shields, CB, and Xu XM. Surgical Stabilization of the Cervical Spinal Cord for Unilateral C5 Contusion Injury using the NYU/MASCIS Impactor. *Journal of Visualized Experiments. Accepted.*

2012. Liu, NK, Zhang, YP, O'Connor, J, Gianaris, A, Ahuja, SK, Oakes, E, Lu, QB, Verhovshek, T, **Walker, CL**, Shields, CB Xu, XM. A bilateral closed-head injury that shows graded brain damage and behavioral deficits in adult mice. *Brain Research*, doi:10.1016/j.brainres.2012.12.031.

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2011. **Walker, CL** and Xu, XM. Morphological Assessments Following Spinal Cord Injury. In: *Animal Models of Acute Neurological Injuries II. Injury and Mechanistic Assessments,* In: *Contemporary Neuroscience*, J Chen, Z Xu, XM Xu, J Zhang (eds). *The Humana Press Inc.*, Totowa NJ.

2011. Deng, LX, Wang, XF, **Walker, CL**, Ruan, Y and Xu, XM. Retrograde Axonal Tract Tracing. In: *Animal Models of Acute Neurological Injuries II. Injury and Mechanistic Assessments,* In: *Contemporary Neuroscience*, J Chen, Z Xu, XM Xu, J Zhang (eds). *The Humana Press Inc.*, Totowa NJ.

Awards & Honors

2012. Outstanding Oral Presentation Award – 2nd International Neural Regeneration Symposium, Shenyang, China

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2011. Finalist – Student Poster competition at the National Neurotrauma Symposium, Hollywood, FL

Grants & Fellowships

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Conferences Posters & Presentations

2012. Walker, CL, Characteristics of Schwann cells after subacute transplantation into the contused cervical spinal cord. Oral Presentation, 2nd International Neural Regeneration Symposium, Shenyang, China.

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2012. Walker, CL, Liu, NK, and Xu, XM, Bisperoxovanadium differentially affects cellular Akt and Erk activity and promotes oligodendrocyte and myelin sparing after hemi-contusive cervical spinal cord injury. Poster. National Neurotrauma Symposium, Phoenix, AZ.

2011. Walker CL, Walker, MJ, Liu NK, and Xu XM , Bisperoxovanadium-mediated neuroprotection, functional recovery, and PI3K/Akt/mTOR activity following cervical contusive spinal cord injury. Poster, Society for Neuroscience, Washington, DC.

2010. Walker, CL, Risberg, EC, Zhu, Y, Liu, NK, and Xu, XM, Characterization and modulation of PI3K-Akt signaling following contusive SCI. Poster, Society for Neuroscience, Indianapolis, IN Chapter.

Departmental Presentations

2013. Continuing assessment of the response and modulation of PTEN/PI3K signaling in the treatment of SCI. Seminar, Indiana University Spinal Cord and Brian Injury Research Group Research Forum.

2012. Schwann cell and bisperoxovanadium therapies for the treatment of cervical contusion SCI. Seminar, Indiana University Spinal Cord and Brian Injury Research Group Research Forum.

2011. Functional and histological characterization of a modified graded hemi-contusion SCI model. Seminar, Indiana University Spinal Cord and Brian Injury Research Group Research Forum.

2011. Small-molecule treatment promotes neuroprotection, functional recovery, and PI3K signaling post-SCI: A foundation for acute and subacute combined therapy. Seminar, Indiana University Spinal Cord and Brian Injury Research Group Research Forum.

2010. Characterization and modulation of PI3K-Akt signaling following contusive SCI. Seminar, Indiana University Spinal Cord and Brian Injury Research Group Research Forum.

Professional Societies

2012-Present. Sigma Xi

2011-Present. American Association of Anatomists

2011-Present. National Neurotrauma Society

2011-Present. Society for Neuroscience

2012-Present. American Chemical Society