Pharmacological inhibition of PTEN, using BpV (Phen), promotes survival of retinal ganglion cells *in vitro* and *in vivo*

by

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Abstract

Previous work has shown that potassium bisperoxo (1,10-phenanthroline) oxovanadate, BpV(Phen), is a potent protein phosphotyrosine phosphatase (PTPase) inhibitor that preferentially blocks the activity of Phosphatase Tensin Homologue Deleted on Chromosome Ten (PTEN). Currently, it remains unknown how pharmacological inhibition of PTEN by bpV(phen) impacts the survival of mammalian RGCs. Thus, the current study investigated the effect of bpV(phen) on RGC survival *in vitro* and *in vivo* and the mechanism mediating this outcome following bpV(phen) administration. Collectively, this study revealed that bpV(phen) promoted survival of injured retinal ganglion cells from mature animals (postnatal day 21 (P21) and adult mice) after injury *in vitro* and *in vivo*. Interestingly, data also showed bpV(phen) to have an antagonistic effect on healthy RGCs and promote apoptosis. Lastly, preventing Smad3 from being phosphorylated in the transforming growth factor β (TGF-β) pathway did not augment the survival in bpV(phen)-treated RGCs.

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List of Abbreviations

4E-BP1	Eukaryotic initiation factor 4E binding protein 3-
Akt	Protein Kinase B
ALK5i	ALK5 Inhibitor
AST	Ascending Sensory Tract
BBB	Blood-Brain Barrier
BDA	Biotinylated Dextran-Amine
BDNF	Brain-Derived Neurotrophic Factor
BpV	Bisperoxovanadium
BpV(Phen)	Bisperoxo (1,10-phenanthroline) Oxovanadate [bpV(phen)]
cAMP	cAMP-Response-Element Binding Protein
CNS	Central Nervous System
CREB	Cyclic Adenosine Monophosphate
CSPG	Chondroitin Sulfate Proteoglycans
CST	Corticospinal Tract
H_2O_2	Hydrogen Peroxide
I-Smad	Inhibitory Smad Proteins
IGF-1	Insulin-like Growth Factor 1
IP	Intraperitoneal Injection
IRS	Insulin Receptor Substrate
KOH	Potassium Hydroxide
LGN	Lateral Geniculate Nucleus
MAG	Myelin-Associated Genes
MAI	Myelin-Associated Inhibitors
mTOR	Mammalian Target of Rapamycin
mTORC1	Mammalian Target of Rapamycin Complex 1
mTORC2	Mammalian Target of Rapamycin Complex 2
NT3	Neurotrophin-3
OMgp	Oligodendrocyte Myelin Glycoprotein
ON	Optic Nerve
ONC	Optic Nerve Crush
p-Akt	Phosphorylated Akt
p-S6	Phosphorylated S6 Ribosomal Protein
PDK1	Phosphoinositide-Dependent Protein Kinase 1
PI3K	Phosphoinositide 3-Kinase
PIP2	Phosphatidylinositol (3,4)-Bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-Triphosphate
PNS	Peripheral Nervous System
PP2A	Protein Phosphatase 2A
PTEN	Phosphatase Tensin Homologue Deleted on Chromosome Ten
PTP	Protein Tyrosine Phosphatases
R-Smad	Receptor-Regulated Smad Proteins
RAG	Regeneration-Associated Genes
RBPMS	RNA-Binding Protein with Multiple Splicing

RGC	Retinal Ganglion Cells
ROS	Reactive Oxidative Species
S6K1	Ribosomal Protein S6 Kinase Polypeptide 1
SC	Superior Colliculus
SCI	Spinal Cord Injuries
STRAP	Serine-Threonine Kinase Receptor-Associated Protein
TBI	Traumatic Brain Injury
TGF- β	Transforming Growth Factor β
TSC1	Tuberous Sclerosis Complex 1
TSC2	Tuberous Sclerosis Complex 2
TTX	Tetrodotoxin
TβRI	Trans-Membrane Serine and Threonine type I
TβRII	Trans-Membrane Serine and Threonine type 2
V_2O_5	Vanadate
VEGF	Vascular Endothelial Growth Factor
WD	Wallerian Degeneration

1 Introduction

1.1 Survival and Regeneration in the Nervous System

When neurons become damaged from degenerative diseases such as glaucoma or from external forces like traumatic brain injury (TBI), their axons retract and fail to regrow back to their original targets – ultimately undergoing apoptosis. This results in a loss of neural functioning and behavioural output. Although this failure to survive and regenerate is observed in the mature central nervous system (CNS), the peripheral nervous system (PNS) retains some ability to spontaneously regenerate their axons and survive after injury (Abe et al., 2010; Tian et al. 2015). The distinction between these two systems with respect to regeneration and survival can be accounted for by two fundamental differences between the CNS and PNS; the inhibitory extrinsic environment after an injury, and the neuron's intrinsic regenerative and survival properties (David & Aguayo, 1981; Park et al., 2008). This project aims to target the latter by focusing on the molecular mechanisms that facilitate survival and axon regeneration following injury to the mature CNS, by utilizing both *in vitro* and *in vivo* model systems.

1.2 Injury in the Peripheral Nervous System

Despite the regenerative failure in the mature CNS, both the CNS and the PNS undergo similar axonal degeneration paradigms. Initial injury in both nervous systems initiates a process known as Wallerian Degeneration (WD). While it still remains unknown how cells receive distress signals, the initial phases of WD begin after an influx of calcium (Ca^{2+}) ions at the proximal end of the injured neuron (Wolf et al., 2001; Ma, 2013). The increase in Ca^{2+} ions acts as a retrograde wave toward the cell body and triggers the activation of calpain, a protease responsible for microtubule

disorganization and axonal disintegration (Svennigsen & Dahlin, 2013). As the proximal end of the lesion site retracts towards the cell body, the distal end of the injured axon dies off (Spira et al., 1993; George & Griffen, 1994; Stoll & Müller, 1999; Vargas & Barres, 2007). Due to the retraction of the axon and microtubules, myelin that once surrounded the axon becomes fragmented and is released into in the microenvironment, resulting in neuronal apoptosis due to retrograde degeneration (Kanamori et al., 2012) and myelin debris that impedes axon regeneration.

In contrast, the PNS has a type of cell called Schwann cells that recruit macrophages to act as the primary cell for myelin clearance. Schwann cells that become detached to their respective axon reconvert into cells that specialize in cell survival and regeneration (Gaudet, Popovich, & Ramer, 2011; Bampton et al., 2005). These cell bodies play a crucial role in providing a permissive environment for regrowth as they downregulate myelin-associated genes (MAGs) that inhibit regeneration, while simultaneously upregulating regeneration-associated genes (RAGs) that have been associated with axonal regrowth (Schmitt et al*.,* 2003; Raivich et al*.,* 2004). In addition to upregulating genes that promote regeneration, Schwann cells may physically stack together to form bands of cells known as Büngner bands that serve as guidance tubes for future regenerating stumps (Gomez-Sanchez et al., 2017). Büngner bands also provide additional neurotrophic support as they release growth factors to the proximal end of the axon inducing the outgrowth of injured stumps (Sahenk et al., 1994; Bampton et al., 2005). Such growth factors include those that activate several pathways, such as the phosphoinositide 3-kinase (PI3K) pathway. The PI3K pathway is known to promote survival and regeneration in both the CNS and PNS (Christie et al., 2010; Noseda et al., 2013).

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1.3 Injury to the Central Nervous System

Although the CNS follows the same axonal degeneration pattern as the PNS, there are discrepancies between the PNS and the CNS when dealing with an injury. These differences may help explain why the mature CNS is unable to demonstrate robust regeneration and survive after an injury (Kalil & Reh, 1979; Huebner & Strittmatter, 2009; Doron-Mandel et al., 2015). As such, permanent injuries in the CNS due to traumatic brain injuries (TBI), spinal cord injuries (SCI) and visual system damage, can disrupt cellular processes. This results in debilitating behavioural impairments including language deficits and limited limb functions (Moran and Gillon, 2004; Rapoport et al*.,* 2006; McKenna et al*.,* 2013) as well as vision loss (Sen, 2017)

Key differences between the PNS and the CNS's abilitiy to recover from injuries originate from how cells react to injury signals. Despite undergoing the same WD process, the CNS reacts much slower than the PNS to injury (George & Griffen, 1994). As a consequence, axons in the CNS delay their membrane resealing and allow myelin debris to be released; resulting in apoptosis due to calpain activation *via* Ca^{2+} influx (Ma et al., 2013). For the neurons that are able to survive, a significant decrease in tubulin mRNA expression and slower tubulin transport systems are observed in the CNS. These critical components that are utilized for axonal regeneration and growth cone formation are instead upregulated in the PNS (Wujek & Lasek, 1983; Fournier & McKerracher, 2011). Due to these distinct differences, many studies have tried to replicate the PNS environment in the CNS by replacing degenerating CNS tissue with a peripheral nerve graft (David & Aguayo 1981; Villegas-Pérez et al., 1988). For example, retinal ganglion cells (RGCs) in the optic nerve were able extend their processes into the peripheral nerve graft, but also increase their rate of tubulin transport by two-fold (McKerracher, Vidal-Sanz, & Aguayo, 1990). Besides the peripheral nerve graft providing a permissive environment to grow, only a small population of

RGCs are able to extend into the graft (Vidal-Sanz et al., 1987). Suggesting that despite the presence of a permissive environment, other key differences separate the regenerative disparity between the CNS and PNS.

The cellular compositions between the CNS and the PNS also contribute to the regeneration failure observed in the CNS. In the CNS, glial cells consist of astrocytes and oligodendrocytes; while in the PNS, glial cells primarily consist of Schwann cells and macrophages. Unlike Schwann cells in the PNS, oligodendrocytes in the CNS are unable to remove myelin debris from the environment (Brosius Lutz et al., 2014). Thus, oligodendrocytes rely on macrophage infiltration from outside of the blood-brain barrier (BBB). However, due to the size of the cell, macrophage infiltration is generally only seen in limited numbers after injury due to the partial disruption of the BBB (Vargas & Barres, 2007). Given the discrepancies in cellular composition and their ability to clear myelin debris, it is possible that the environment the neuron occupies after an injury may be partly responsible for the regenerative failure that is observed in the CNS (Park et al., 2008).

1.4 The Inhibitory Environment in the Central Nervous System

Under normal circumstances, the CNS is composed of intricate connections of neurons that relay information to one another in an environment supported by glial cells (such as astrocytes) and an extracellular matrix (ECM) (Quraishe et al., 2018). Axonal injury in the CNS causes alterations in the environment and disrupts neural connections. Myelin that once encapsulated neurons disintegrate and release inhibitory-growth molecules, such as Nogo, myelin-associated glycoproteins (MAG), oligodendrocyte myelin glycoproteins (OMgp), and chondroitin sulfate proteoglycans (CSPGs) (Simonen et al., 2003; Lee et al., 2010; Geoffroy & Zheng, 2014). These factors, known collectively as myelin-associated inhibitors (MAIs), have been studied extensively

with regard to their impact on regenerating axons. For example, removal of any of these inhibitory-growth molecules was shown to enhance axonal regrowth in neurons (Domeniconi et al., 2002; Wang et al., 2002; Simonen et al., 2003). Alternatively, in other circumstances, deletion of the receptor that mediates the three MAIs does not indicate neurite outgrowth (Zheng et al., 2003). Taken together, these findings suggest that MAIs have some degree of influence on the regeneration of injured axons but may not be the only determinant of regeneration failure (Simonen et al., 2003; Kim et al., 2004; Nguyen et al., 2009).

Injuries within the CNS can lead to the formation of glial scars. These are scars primarily consisting of glial cells called astrocytes that surround the lesion site and release chondroitin sulfate proteoglycans (CSPGs) *via* Smad2 and Smad3 proteins in the transforming growth factor β (TGF- β) pathway, that impede axon growth *in vitro* (McKeon et al., 1991; Sofroniew, 2009; Susarla et al., 2011; Anderson et al., 2016).

By focusing on the physical presence of the glial scar, multiple studies have used selective ablation of these astrocytes in hopes of enhancing regeneration. However, after such an experiment was performed following SCI, Faulkner et al. (2004) found larger inflammatory regions around the lesion site and increased motor deficits. A follow-up study by Anderson and colleagues (2016) demonstrated that, in the presence of the glial scar, axonal growth in spinal cord lesions was possible. Moreover, when axons were given neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3), ascending sensory tract (AST) axons showed robust growth past the lesion site when the glial scar was present as opposed to the absence of the glial scar.

Therefore, it is of interest to evaluate the intrinsic mechanisms that prompt axon regeneration and cellular survival; mechanisms that contribute to releasing these “extrinsic” inhibitory cues into

the environment and the mechanisms that dictate the neuron's intrinsic ability to survive and regrow through the inhibitory environment of the damaged CNS.

1.5 Central Nervous System Regenerative Intrinsic Factors

Although David and Aguayo (1981) first demonstrated that CNS axons retain some ability to regenerate when given a permissive environment, it was Goldberg et al. (2002) through isolating retinal ganglion cells (RGCs) *in vitro,* who found that the rates of regeneration diminish throughout aging. In detail, by culturing neonatal and postnatal RGCs in media containing growth factors that support regeneration, growth among the different age groups of mice differed. In fact, neonatal RGCs elongated ten times farther than postnatal samples suggesting that despite being given a permissive environment to grow, the regenerative capabilities that were intrinsic to the neuron may act as the rate-limiting step towards regeneration.

Furthermore, a critical period during development occurs where cellular changes make the maturing mammalian CNS unable to undergo prominent regeneration. Unlike neonatal axons, mammalian adult axons have stopped developing and have solidified their connections to the neighboring cell. Once this is achieved, mature CNS neurons show a decrease in intracellular concentration of cyclic adenosine monophosphate (cAMP) (Filbin, 2003). With low levels of cAMP in mature CNS neurons, previous studies have observed that growth-promoting molecules, such as MAGs (Cai et al*.,* 2001) and netrin-1 (Shewan et al*.,* 2002), become growth-inhibiting further preventing cellular growth.

Other factors that alter throughout the maturation of the mammalian CNS include those in the phosphoinositide 3-kinase (PI3K) pathway (described in Section 1.4) (Yang and Yang, 2012). Highlighted in *Figure 1*, PI3K pathway activation provided by the mammalian target of rapamycin

(mTOR) has been positively-implicated in the regulation of physiological processes such as cell survival and axonal regeneration (Heras-Sandoval et al., 2011). However, in the development of retinal ganglion cells (RGCs), mTOR activity decreases substantiality and is found in limited amounts (5 to10 percent) of total RGCs in adults (Fischer & Leibinger, 2012). This decline may be due to the presence of phosphatase tensin homologue deleted on chromosome ten (PTEN) which is a PI3K antagonist that is highly expressed in adult CNS neurons (Walker et al, 2013). Previous work in adult mice utilizing genetic deletion of PTEN was able to show robust regeneration *in vivo* after optic nerve crush, despite an up-regulation of CSPG expression (Park et al., 2008). This signifies the importance of PTEN in regulating some of the intrinsic properties for regeneration, even in adulthood; underlying the importance of investigating mechanisms in the mammalian CNS that serve to enhance neuronal survival and regeneration.

1.6 Modeling Central Nervous System Injuries Using the Visual System

The optic nerve (ON) has become a standard model for studying mammalian CNS axon regeneration and cellular survival (Benowitz & Yin, 2011). In contrast to the vast network of connections in the spinal cord or in the brain, the ON heavily relies on a single cell type known as the retinal ganglion cell (RGC) to directly send information to selected brain areas such as the superior colliculus (SC) and the lateral geniculate nucleus (LGN) (Carter et al., 1989; Isenmann et al., 2004; Almasieh et al., 2012). However, similar to CNS neurons, mature RGCs cannot regenerate their axons and in many cases die upon injury (Goldberg et al*.,* 2002; Cho et al., 2005). Additionally, the visual system contains the same types of glial cells found in CNS white matter, and injury to the visual system often results in an inhibitory environment that is parallel to the mature CNS (Butt et al., 2012; Shum et al., 2016). As such, the ON provides an ideal model to

study axonal regeneration whether one is interested in investigating the CNS inhibitory environment and/or the neuron's intrinsic abilities.

Current research surrounding regenerative strategies of RGC rely on two main models: optic nerve crush (ONC) as an *in vivo* model and primary cell cultures as an *in vitro* model. ONC models that allow researchers to study the failure of RGC regeneration use live mammalian physiological systems. In this model there are two types of ONC injuries: (1) incomplete ON injury is induced by applying constant pressure to the ON, and (2) complete ON injury involves the whole transection of the ON (Butt et al., 2012). In regard to *in vitro* models, such as primary RGC cultures, researchers can isolate cells and specifically administer various factors, thereby addressing questions that are not easily addressed in *in vivo* models, and are inherently more complex (Bähr, Vanselow, & Thanos., 1988; Bähr, Hopskins, & Bunge., 1991).

Given that RGCs react similarly after injury when compared to other CNS environments such as the brain and spinal cord, the majority of studies utilize the simple ONC methodologies rather than *in vitro* cell-based models. For example, the distinction between *in vitro* cell-based models and *in vivo* models are the utilization of the age of tissue. Successful *in vitro* models utilize cells from embryonic and early postnatal animals as it is near impossible to culture adult RGC tissue due to neuronal cell death immediately after axotomy (Romano & Hicks, 2007). However, this method cannot encompass the full spectrum of neuroregenerative and neurodegenerative abilities of the CNS across ages (Goldberg et al., 2002; Eide & McMurrary, 2005; Romano & Hicks, 2007; Geoffroy et al., 2016). On the contrary, *in vivo* methods are able to take advantage of tissue from any age, but in return give up precise control of environmental effects.

Nonetheless, due to the relative ease of access and the connective simplicity of the visual system compared to other CNS regions, researchers have capitalized on the visual system and have

been able to induce survival and axon regeneration of RGCs through genetic manipulation of several factors, including those in the PI3K pathway (Park et al., 2008).

1.7 Phosphatidylinosityl-3 Kinase (PI3K) Pathway

One of the most prominent pathways involved in axonal growth and neuronal survival is the PI3K pathway (as shown *in Figure 1*) (Koh & Lo, 2015). Classified as an intracellular signaling pathway, the PI3K pathway is initiated by growth-like factors such as insulin-like growth factor (IGF-1) binding onto receptor tyrosine kinases (RTKs) on the cellular membrane (Latres et al., 2004). Activated RTKs attract heterodimeric proteins known as phosphoinositide 3-kinases (PI3Ks) to the membrane that become phosphorylated by either binding to an adaptor protein, such as insulin receptor substrate (IRS), on the receptor or directly from the RTK itself *via* p85 subunit of the PI3K molecule (Liu et al*.,* 2011). Members of the PI3K family have been categorized into three different classes, all of which can create phosphatidylinositol (3,4)-bisphosphate (PIP2). However, it is only class IA that produces the second messenger, phosphatidylinositol (3,4,5)-triphosphate (PIP3) from PIP2 by phosphorylating D3 inositol ring of PIP2 *via* activated p110 subunit of class IA PI3K (Liu et al*.,* 2011; Jean & Kiger, 2014; Koh & Lo, 2015).

As PIP3 levels gradually accumulate, they remain stationed at the cellular membrane, exposing their docking sites to proteins containing pleckstrin homologous domains such as 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Miao et al*.,* 2010) and a serine/threonine phosphate known as protein kinase B (Akt) (Stephen et al*.,* 1998; Hemmings & Restuccia, 2012). Due to their close proximity, it is only here that Akt becomes activated once PDK1 is able to bind and phosphorylate Akt at T308 (Sarbassov et al., 2006). Alternatively, PDK1 can diverge and act

independently of Akt, and interact with ribosomal protein S6 kinase polypeptide 1 (S6K1) (a precursor of the ribosomal protein of S6) at Thr 229 (Pullen et al., 1998; Holz et al., 2005).

Predominate pathway activation, however, relies on the Akt-dependent mechanisms, where the activity of Akt consists of two active states: partial and full activation. Partial activation is first achieved by phosphorylation of Akt at T308 by PDK1. This event triggers the activation of the mammalian target of rapamycin complex 1 (mTORC1) *via* inhibition of tuberous sclerosis complex 2 (TSC2) within the TSC1-TSC2 complex (Magnuson et al., 2012). Once active, mTORC1 is able to regulate multiple downstream effectors of the PI3K pathway such as S6K1 and the eukaryotic initiation factor 4E binding protein (4E-BP1) that are responsible for physiological processes such as protein synthesis and protein translation (Harrington et al., 2005). On the other hand, full activation of Akt requires both the phosphorylation at T308 from PDK1 and phosphorylation at S473 by the mammalian target of rapamycin complex 2 (mTORC2) (Sarbassov et al., 2005). Attaining both phosphorylation sites of Akt, dictate the protein in managing additional cellular processes including cellular survival by inhibiting apoptosis (Zhang et al., 2011; Magnuson et al., 2012).

Despite the positive impact of activating Akt-dependent pathways, other mechanisms have been placed to restrain Akt-phosphorylated activity. Typically, a major lipid and phosphatase protein known as PTEN depletes the production of PIP3 by converting PIP3 back into PIP2. In turn, PTEN indirectly decrease the probability of Akt from reaching a phosphorylated state and halts the progression of PI3K pathway activation. Alternatively, new evidence has emerged in showing a feedback loop existing within the PI3K pathway that negatively regulates mTOR-dependent mechanisms. Besides its duty to phosphorylate Akt at S473, Kim et al. (2012) demonstrated that mTORC2 phosphorylation leads to significant degradation of IRS *via* ubiquitin

ligase substrate-targeting subunit (Fbw8). If pathway activation progressed, phosphorylated S6K1 can itself impede PI3K signaling (Veilleux et al*.,* 2010). Thus, the negative feedback loop that is prominent in the PI3K pathway may function through Akt-independent mechanisms that may or may not act independently of the PI3K pathway to promote neuronal survival and axon regeneration in PTEN-disrupted cells (Huang et al., 2019).

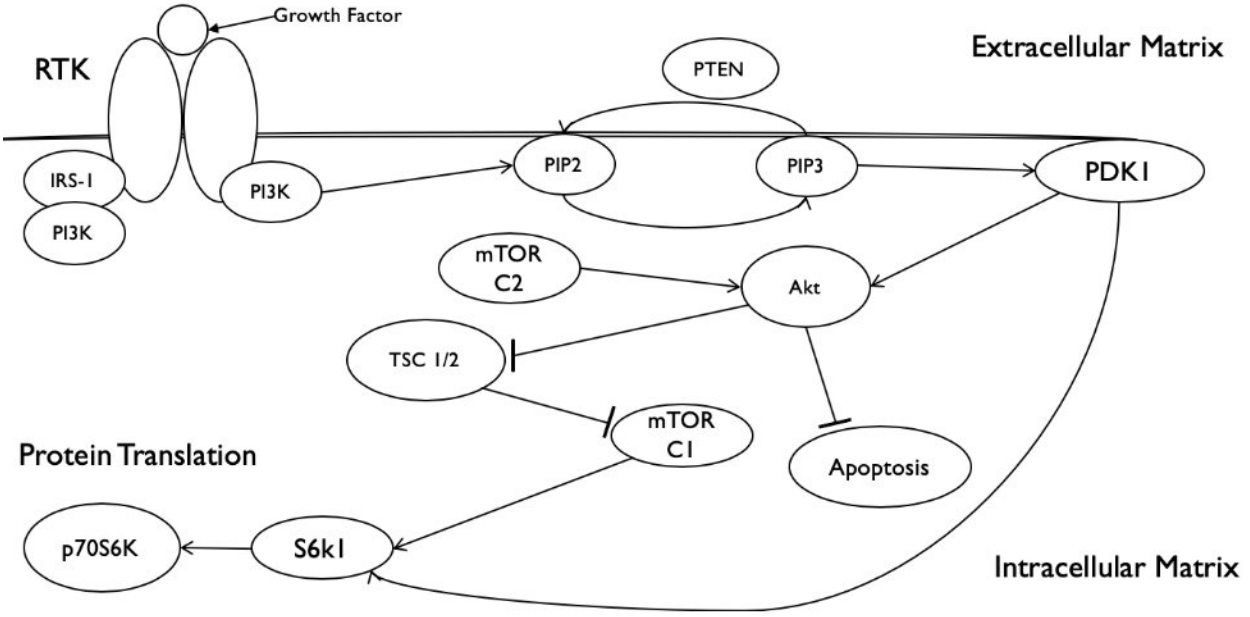

Figure 1.The PI3K pathway. Activation of RTK by ligand binding facilitates IRS-1 and PI3K recruitment. Phosphorylated PI3K drives the production of PIP3 from PIP2. Akt binds to PDK1 at the plasma membrane, allowing Akt to be partially phosphorylated. Full activation of Akt is achieved by PDK1 and mTORC2. Active Akt prevents TSC1/2 complex from inhibiting mTORC1. Downstream effectors of mTORC such as S6K1 can now be phosphorylated. PTEN, a negative regulator of PI3K, dephosphorylates PIP3 back into PIP2, halting Akt from being active.

1.8 Phosphatase and Tensin Homolog Deleted on Chromosome Ten (PTEN)

First discovered on the human chromosome 10q23, PTEN is an important regulator of insulin-dependent signaling (Rosivatz et al., 2006). Known as a dual-specificity phosphatase, PTEN displays its ability to dephosphorylate tyrosine-, serine-, and threonine-signaling molecules by modulating their phosphorylation sites from proteins and lipids (Zhang et al., 2012) and lipids such

as PIP3 (Myers et al., 1998). Although PTEN has a homolog active site of other protein tyrosine phosphatases (PTPs), CX5R, PTEN is said to be solely responsible for dephosphorylating PIP3 back into PIP2 in the PI3K pathway (see *Figure 1*) (Leslie & Downes, 2004).

Composed of four hundred and three amino acids, the PTEN protein has three major domains (as shown in *Figure 2*) (Leslie & Downes, 2004). The first domain consists of an enlarged N-terminal phosphatase domain that allows PIP3 substrates to bind. This binding characteristic relies on three positively charged amino acids, one histidine and two lysine residues, that only attract highly acidic substrates (i.e. PIP3) (Abdulkareem and Blair, 2013). The second domain, C2 domain, helps with stabilization and anchors PTEN to the membrane phospholipids. Mutation of key residues (ie. lysine residues) found in the C2 domain shows a reduction in growth suppression leading to the cellular proliferation of cells (Lee et al., 1999). Lastly, PTEN contains a C-terminal tail domain. Found within this domain is a smaller three amino PDZ domain that forms complexes at the cell membrane and allows PTEN to enhance its ability to inhibit Akt (Odriozola et al., 2007).

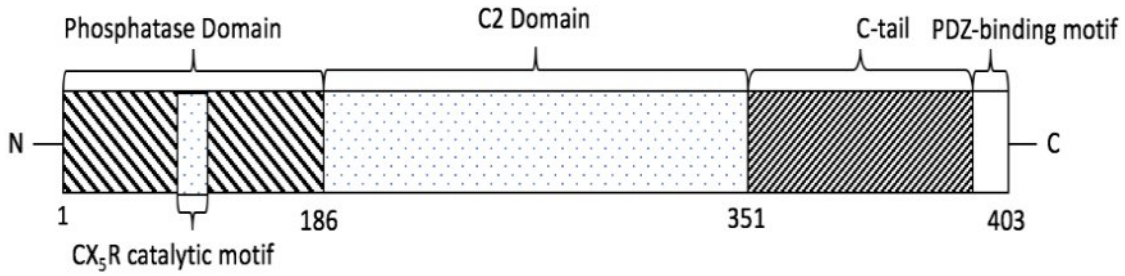

Figure 2. **PTEN tumor suppressor gene**. This gene contains three major subunits (N, C2, and C domains) that contribute to it overall function in vivo.

While PTEN negatively regulates the activity of PI3K, halting mTOR-mediated processes, PTEN is frequently mutated in nature leading to a loss-of-function protein and numerous pathologies such as cancer (Chalhoub and Baker, 2009). Thus, it serves a purpose that loss of

PTEN can upregulate cellular survival and may ultimately lead to axon regeneration (Park et al., 2008; Liu et al., 2009). By utilizing an ONC model and intravitreal injections of AAV Cre recombinase, Park et al. (2008) demonstrated that conditionally knocking out PTEN in adult RGCs in the retina not only enhanced levels of RGC survival but displayed robust RGC axonal regeneration. In order to demonstrate that PTEN deletion leads to activation of the PI3K pathway, cells were labeled with biotinylated dextran-amine (BDA), an axon tracer. Analysis using BDA determined that 88 percent of the regenerating axons were those from p-S6-positive RGCs. Subsequent PTEN deletion studies have demonstrated similar results as Park et al. (2008) including the promotion of the sprouting of uninjured axons in the CST and improved motor function *via* shRNA after one year of injury (Du et al., 2016; Liu et al., 2010). However, caveats of these results indicate that PTEN deletion in adult mice was before the injury. Thus, to suggest clinical relevance for PTEN deletion, Danilov and Steward (2015) measured whether deleting PTEN post-injury was effective. Using Cre-lox models of adult mice, PTEN-deleted groups displayed increased forelimb motor function recovery, in addition to significant regeneration of CST axons.

Despite the regenerative and survival effects seen following ablation of PTEN in RGCs using genetic manipulation (Park et al., 2008; Du et al., 2016), it would be highly unlikely to be clinically applicable to the general public due to concerns around feasibility for clinical safety for this type of manipulation.

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1.9 Pharmacological PTEN Inhibition using Potassium Bisperoxo (1,10-phenanthroline) Oxovanadate [bpV(phen)]

As genetic deletion of PTEN in animal models provides evidence of significant regeneration and enhanced survival, it may not be feasible to progress into clinical trials considering its high risk and invasiveness (Ohtake, Hayat, & Li, 2014). Therefore, as a substitution to genetic manipulation, pharmacological inhibition may be more of a clinically relevant tool to promote regeneration and survival instead. This is because such methods are minimally invasive, administered quickly, easy to access, and have temporary effects in comparison to PTEN genetic manipulation.

Small molecule pharmacological inhibitors such as those bisperoxovanadium (bpV) compounds are known inhibitors of protein tyrosine phosphatases (PTPs) including PTEN (Schmid et al., 2004). Characterized by a vanadium ion in the center, members of the bpV family such as bisperoxo(1,10-phenanthroline) oxovanadate (bpV(phen)) have been shown to preferentially target PTEN (IC50 = ~40nM) (Ohtake, Hayat, & Li, 2015). Synthesized from a mixture containing hydrogen peroxide (H_2O_2), vanadate (V_2O_5), and potassium hydroxide (KOH), bpV (phen) binds to the wide CX5R pocket of the PTEN and impairs its function by forming a Cys124-Cys71 oxidative disulfide bond (Pulido, 2018). Unlike genetic manipulation that permanently ablates the *PTEN* gene from being expressed, the protein turnover of PTEN and half-life bpV (phen) is ~8 hours (Vazquez et al., 2000) and 6 hours (Cerovac et al., 1999) respectively. This suggests that bpV (phen) could be an ideal alternative to PTEN inhibition as its effects are temporary and degrade well within the half-life of PTEN.

Studies that aim to investigate the impact of PTEN deletion in injury models demonstrated not only improved motor function but upon further analysis observed a decrease in PTEN expression within *in vivo* models (Nakashima et al., 2008; Mao et al., 2013). Moreover, *in vitro* models found

that bpV(phen) prolonged neuronal survival in a dose-dependent manner of MEF cells as well as increased neurite length in human embryonic stem cells (Watt et al., 2014; Chen et al., 2015). Based on these major findings, it seemed that bpV(phen) treatment rescued degenerative neurons and promoted outgrowth in a similar manner to genetic manipulation. Nonetheless, pivotal studies like Nakashima et al. (2008) and Mao et al. (2008) demonstrate that delayed treatment of bpV (phen) can promote neurite outgrowth and improve motor function *in vivo* after injury, a criterion for clinical applicability. Furthermore, Mao and colleagues (2008) have accomplished what studies that utilize genetic manipulation lack – treatment that can be locally administered. By inducing an ischemic stroke, another model of CNS injury, in adult mice, researchers were able to observe functional recovery of animals and elevated Akt and mTOR levels in the brain through a delayed intraperitoneal injection (IP) of bpV(phen). This indicates that bpV (phen) was able to cross the BBB and satisfy other criteria to be considered clinically relevant.

Despite suppressing PTEN activity by bpV(phen) administration, this small molecule inhibitor is not absolutely specific to PTEN. BpV(phen) inhibits several other PTPs such as PTP- β (IC50 = 343nM) and PTP-1β (IC50 = 920nM) (Schmid et al., 2004). This suggests that other mechanisms may influence axon regeneration and survival, including the TGF-β pathway (Guo and Wang, 2009), which may act synergistically or antagonistically to PI3K pathway activation.

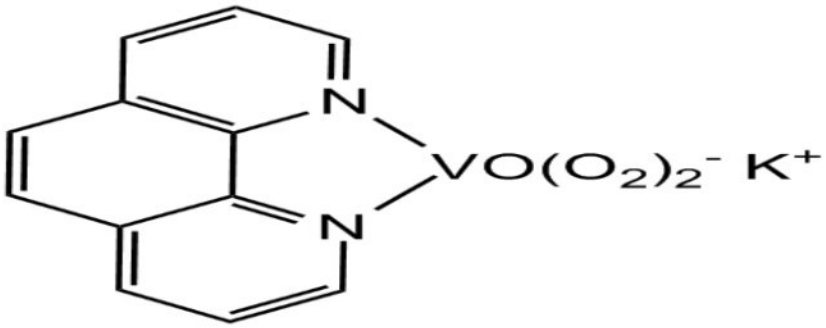

Figure 3. Chemical structure of bpV (phen).

1.10 Cross-talk between PI3K pathway and TGF-β pathway

Initiated by ligand binding, the TGF-β pathway has been linked to various cellular processes including apoptosis and neurite growth (Knöferle et al., 2010; Dobolyi et al., 2012; Massagué, 2012; Zhang *et al*., 2013). Despite the TGF-β pathway controlling many fundamental processes, it remains difficult to define its particular role in each cell type. For instance, activation of the TGF-β pathway has been shown to increase neurite outgrowth in dopaminergic cells in a scratch lens model (Knöferle et al., 2010) and act as an apoptotic suppressor in follicular dendritic cells (Park et al., 2005). On the other hand, activation of the TGF-β pathway inhibited neurite outgrowth in primary cultures of cerebellar granule neurons *via* calcium-dependent mechanisms (Jaskova et al., 2014) but also induced apoptosis by activating inositol phosphatase SHIP (Src homology 2 (SH2) domain-containing 5' inositol phosphatase) in hematopoietic cells (Valderrama-Carvajal et al., 2002).

In addition to the multiple roles of the TGF-β pathway, studies have shown evidence of cross-talk between the PI3K and the TGF-β pathways (Dobolyi et al., 2012; Massagué, 2012; Zhang et al., 2013). In order to begin signaling in the TGF-β pathway trans-membrane serine and threonine type I (TβRI) (signaling component) and type II receptors (TβRII) (activator) kinases form a heteromeric complex (Yi et al., 2005). Dimerization of these receptor subunits results in the phosphorylation of TβRI by TβRII, leading the TβRI subunit, ALK5, to phosphorylate receptor-regulated Smad proteins (R-Smad proteins), Smad2 and Smad3 (Villapol et al., 2013; Liu et al., 2017) (see *Figure 4*) and negatively impact the PI3K pathway by indirectly inhibiting p70S6K *via* protein phosphatase 2A (PP2A) (Griswold-Penner et al., 1998;Petritsch et al., 2000). Active Smad2/3 bind together, recruiting a Co-Smad protein called Smad4, forming a trimeric configuration complex. This trimeric complex migrates into the nucleus and initiates transcription

factors such as cAMP-response-element-binding protein (CREB) and other TGF-β responsive genes (Shen et al., 1998; Massagué et al., 2005; Oerlecke et al., 2013).

Although this Smad complex enters the nucleus as a single complex, functional assays have identified divergent roles between Smad2 and Smad3 gene regulation (Liu et al., 2016). Preferentially, Smad3 targets genes regulating neural development, while Smad2 has been observed to associate with genes that are responsible for cell maintenance and cell adhesion. In detail, the function of Smad3 still remains controversial in regard to axonal development. For example, in regulating CSPG expression in astrocytes, knockdown of Smad3 was more prevalent in suppressing CSPGs and capable of enhancing neurite outgrowth in cultured neurons (Susarla et al., 2011). On the other hand, Smad3-null mice were shown to exhibit impaired muscle regeneration after undergoing muscle injury (Ge et al., 2012). Taken together, these findings indicate that Smad-mediated effects are dependent on cell type and condition.

Key regulators such as inhibitory SMAD proteins (I-Smad) negatively moderate Smad-dependent signaling, thus providing a negative-feedback loop in the pathway (Nakao et al., 1997; Moustakas & Heldin, 2009). A member of this subclass of inhibitory proteins, Smad7, primarily acts on type I receptors by binding to serine-threonine kinase receptor-associated protein (STRAP), which is a receptor-interacting protein (Datta & Moses, 2000). Alternatively, direct binding of PDK1 to STRAP may also occur, leading to the possibility that STRAP will block Smad2/3 phosphorylation from TβRI (Seong et al., 2005). Furthermore, PDK1-mediated phosphorylation of Akt (p-Akt) have been observed to also inhibit Smad3 directly (Conery et al., 2004) (fig. 4).

Since TGF-β pathway is not solely independent of influencing R-Smad proteins, but instead also interacts with other non-Smad signaling pathways, such as PI3K pathway, there could be a

novel mechanism by which these two pathways may act synergistically or antagonistically to achieve axonal regeneration and cellular survival.

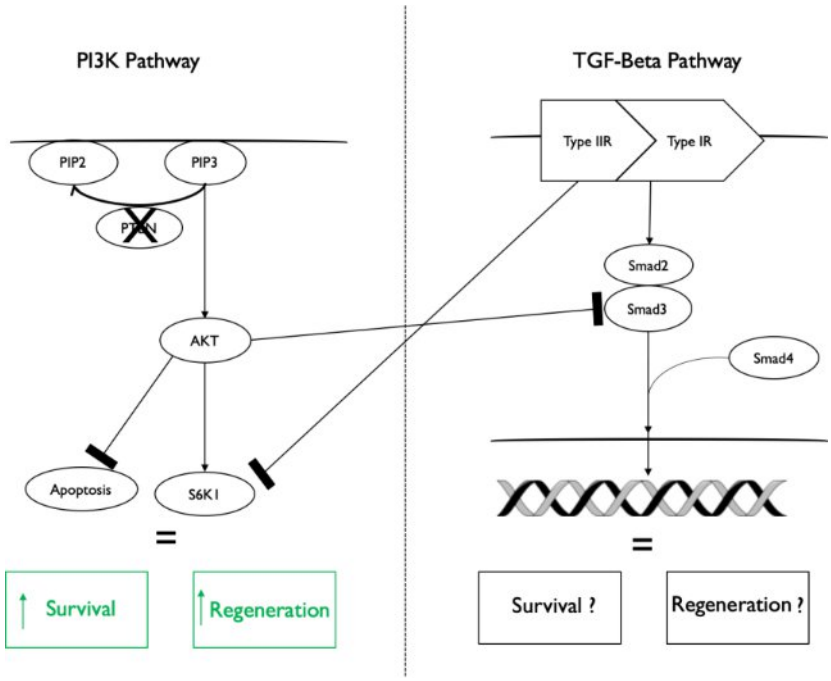

Figure 4. Cross-talk between PI3K pathway and the TGF-β pathway. Activation of the TGF-β pathway is initiated by the heterodimerization of type I and II receptors. These receptors propagate a signaling cascade in a Smad-dependent manner to regulate gene transcription. In non-canonical signaling, the type II receptor can inhibit the phosphorylation of S6K1 in the PI3K pathway *via* PP2A. Activation of the PI3K pathway includes the phosphorylation of Akt. Akt can bind to Smad3 directly and suppress Smad-mediated effects.

2 Rationale

Unlike the mammalian PNS, the CNS neurons does not spontaneously regenerate their axons and survive after injury. Despite a permissive environment to grow, neurons in the CNS display a limited regenerative and survival response, suggesting an underlying role for intrinsic mechanisms. By using optic nerve crush paradigms, researchers have attempted to reactivate several key factors, including those in the PI3K pathway that influence axon regeneration and cellular survival. However, considering that these models utilize genetic manipulation of the PI3K pathway, I believe that this is not an effective approach if applied in a clinical context; hence, my research focused on using pharmacologic manipulation of the PI3K pathway to revitalize and amplify the intrinsic growth and survival capacity of CNS neurons *in vitro* and *in vivo*.

As the regenerative potential in mammals declines through age, many *in vitro* studies resort to using embryonic and early post-natal tissue rather than adult tissue, as adult tissue are difficult to culture long enough for survival and axon regeneration. Considering that embryonic and early post-natal tissue does not fully encompass the difficulties of regenerating axons in adulthood, an *in vitro* model of maturing and adult primary mouse RGC cultures was examined for survival and regeneration by inducing pharmacological inhibition of PTEN by bpV(phen). In addition, as *in vitro* models isolate neurons from the external environment, an *in vivo* model capitalizes on the interaction with other neighboring cells. As such, an adult *in vivo* model was also examined to determine the regeneration and survival potential of RGCs following bpV(phen) administration.

Due to bpV(phen) being a non-specific PTPase inhibitor, multiple factors can be targeted. Some of which are undesired and can oppose the effects promoting survival and regeneration of

RGCs. One of these PTPs that is affected by bpV(phen) is the PTP-1β. Linked to apoptosis, PTP-1β regulates various signaling pathways such as the TGF-β pathway. Among some of its roles, the TGF-β pathway can act as a growth suppressor and initiate pro-apoptotic mechanisms in a Smad-dependent manner. One of these Smad proteins, Smad3, has been shown to regulate transcriptional genes that involve axonal development and survival but also interact with the PI3K pathway. This existence of a connection between Smad3 and the PI3K pathway has been previously proposed. However, no known study has been conducted in bpV(phen)-treated RGCs that determine the impact of p-Smad3 expression with regard to CNS regeneration and survival; Thus, the expression of p-Smad3 in RGCs was examined after PTEN inhibition and expected to decrease upon PI3K pathway activation.

The potential to rejuvenate the PI3K pathway in mature *in vitro* and *in vivo* models could contribute immensely to understanding CNS survival and regeneration. Utilizing mature tissue rather than embryonic and early post-natal tissue may spotlight mechanistic details that are specific to mature CNS, compared to embryonic neurons. Moreover, it could offer some insight in developing therapies for neurodegenerative diseases such as Alzheimer's and Parkinson's disease, both prevalent in older populations.

BpV(Phen) enhances retinal ganglion cells *in vitro* and *in vivo*

3 Research Objective

The purpose of the current research was to investigate intrinsic mechanisms underlying RGC survival *in vitro* and *in vivo, using* a clinically-relevant pharmacological agent. The major goals of this research are reflected in the following three objectives:

Objective 1 - To determine the impact of pharmacological inhibition of PTEN, *via* bpV(phen) administration, on mature mouse RGC survival *in vitro.*

Hypothesis 1: Moderate concentrations of bpV (phen) will promote RGC survival.

Objective 2 - To investigate the impact of bpV(phen)-mediated inhibition of PTEN, on RGC survival in the *in vivo* optic nerve crush injury model.

Hypothesis 2: BpV (Phen) administration will enhance RGC survival following optic nerve crush.

Objective 3 - To evaluate the influence of manipulating the TGF-β pathway in bpV(phen)-treated mature RGCs survival *in vitro.*

Hypothesis 3: Inhibition of the TGF-β pathway in conjunction with PI3K pathway activation will enhance cellular survival by decreasing the levels of p-Smad3.

4 Methods

4.1 Animals

All experiments were conducted according to the guidelines of the Canadian Council on Animal Care and approved by the Carleton University Animal Care Committee. All experiments were performed on C57BL/6J wild-type mice purchased from Charles River Laboratories (Montreal, Quebec, Canada). All mouse pups (postnatal day 21 (P21) and adult mice (5 weeks old) were housed with a consistent light-dark cycle, with access to food and water *ad libitum.*

4.2 BpV (phen) Treatments in Retinal Ganglion Cell Cultures

For tissue culture experiments, eyes were pooled to generate enough RGCs for each experiment. For all experiments, n=1 represented the pooled biological sample; each experiment was repeated on at least three additional biological samples. Retinal ganglion cell cultures were first initiated by the enucleation of the eyes from the mice. Retinal samples were washed with sterile Hank's balanced salt solution (Thermo Scientific) before being incubated at 37 °C for 30-60 minutes in a papain dissociation solution (Worthington) containing Deoxyribonuclease I (DNase I); agitating the samples every 5 minutes. Cells were then triturated gently and centrifuged 300xG for 5 minutes. After discarding the supernatant, the pellet was resuspended in a solution consisting of Earl's balanced salt solution, reconstituted albumin-ovomucoid inhibitor and a RNase solution. Once suspended, the solution was carefully layered on top of albumin-ovomucoid inhibitor in an eppendorf tube in preparation for a density-gradient centrifugation at 100xG for 6 minutes. The supernatant was discarded, and the pellet was resuspended in a solution containing Neural Basal Medium A (2% B27 supplement, 1% penicillin/streptomycin, 0.3% L-glutamine) and 10% FBS before one last centrifugation of 400xG for 5 minutes.

Removing the supernatant, the pellet was resuspended in another solution of Neural Basal Medium A (2% B27 supplement, 1% penicillin/streptomycin, 0.3% L-glutamine) and 10% FBS. The following solution was then immediately transferred to a 24-well plates (Sarstedt).

Cell quantification was carried out by using a hemocytometer before plating the RGCs in cell culture wells that were coated with 20µg/mL poly-D-lysine (PDL) (Sigma-Aldrich) and 1.73µg/mL laminin (Corning); approximately 54,000 and 36,000 cells were isolated for each n=1 in P21 and adult cell culture experiments, respectively.

Six different treatments were applied to the cells in a 24-well plate system (Sarstedt): Control (water) treatment and increasing concentrations of bpV(phen) (Sigma) treatments: 0.1µM, 1µM, 5µM, 10 µM, and 20µM. The RGCs were then incubated for 72 hours at 37°C and 5% CO_2 and then were fixed in 4% PFA for 1 hour before immunocytochemistry. Figure 5 displays the experimental timeline for RGC culture.

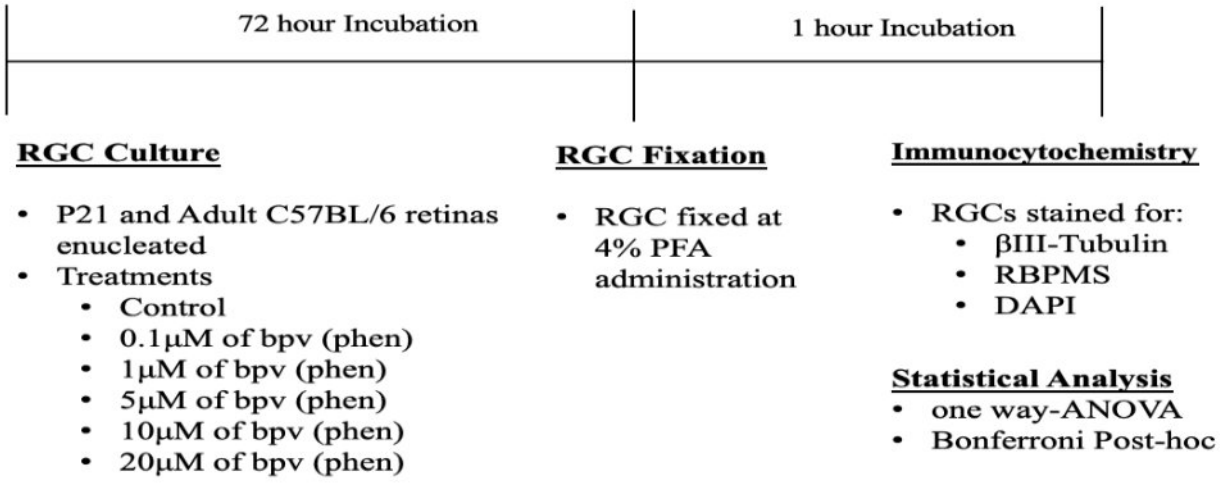

Figure 5. A summary of the bpV(phen) RGC culture timeline. After extracting P21 and adult retinal tissue, RGCs were mixed with a solution containing a papain dissociation kit and placed in a 24-well cell culture plate. RGCs were plated with treatment to wells prior to a 72-hour incubation at 37°C and 5% CO_2. RGCs were then fixed at 4% PFA for 1 hour before undergoing immunocytochemistry.

4.3 Analysis of BpV(Phen) on RGC Survival in vitro

In preparation of immunocytochemistry, the RGC sample wells were washed three times for 10 minutes each in 1X PBS before being treated with a 0.2% Triton X-100 solution for 5 minutes. Sample wells were then rinsed three times with 1X PBS before the primary antibody solution containing rabbit anti-RBPMS (1:250, Millipore), mouse anti-β-tubulin antibody (1:250, Biolegend), and 10% normal goat serum (NGS) (Cedarlane) was added. Primary antibody incubation lasted for 24 hours at 4°C.

Following primary incubation, three 1X PBS 10-minute washes were given. Subsequently, a secondary antibody solution consisting of anti-mouse Alexa 488 (1:250, Cell Signaling) and anti-rabbit Alexa 555 (1:250, Cell Signaling) was administered for 2 hours. Once completed, culture samples underwent three more washes of 1X PBS for 10 minutes each, and then followed by a 5-minute incubation of 4',6-diamidino-2-phenylindole (DAPI) (1:10000, Thermo Scientific).

RGC cell culture wells were viewed using a Zeiss Axiovert Microscope (Zeiss) capturing five random fields of view at 20X magnification with Infinity Analyze software (Lumernera Corporation). With only brightness and contrast modified for all images, the retinal samples were analyzed by ImageJ (NIH) for cell quantification for DAPI and RBPMS-positive cells. Manual count of RBPMS-positive cells was conducted for each treatment group. Neurite outgrowth was measured for only RBPMS-positive cells by using ImageJ (NIH) as RBPMS-positive cells represent RGCs (Rodriguez *et al*, 2015). The longest β3-tubulin-labelled process in RBPMS-positive cells protruding away from the cell body was deemed as the axon and was taken as the neurite measurement. Statistical analysis was performed using one-way ANOVA and Bonferroni's post hoc test (Graphpad prism 8).

4.4 ALK5 Inhibitor Administration

Primary cell cultures were prepared in a similar manner as mentioned in section 4.2. However, for this set of experiments, five-treatment groups were administered to the samples: Control (water), DMSO (0.5% DMSO), 5µM ALK5 inhibitor I (ALK5i) (Enzo Life Science), 5µM bpV(phen), and a co-treatment of 5µM ALK5i and 5µM bpV(phen). Once cells were plated, they were incubated for 72 hours at 37°C and 5% CO_2, and fixed in 4% PFA for 1 hour, immunocytochemistry soon followed in a similar process. Figure 6 summaries the timeline for ALK5i experiment.

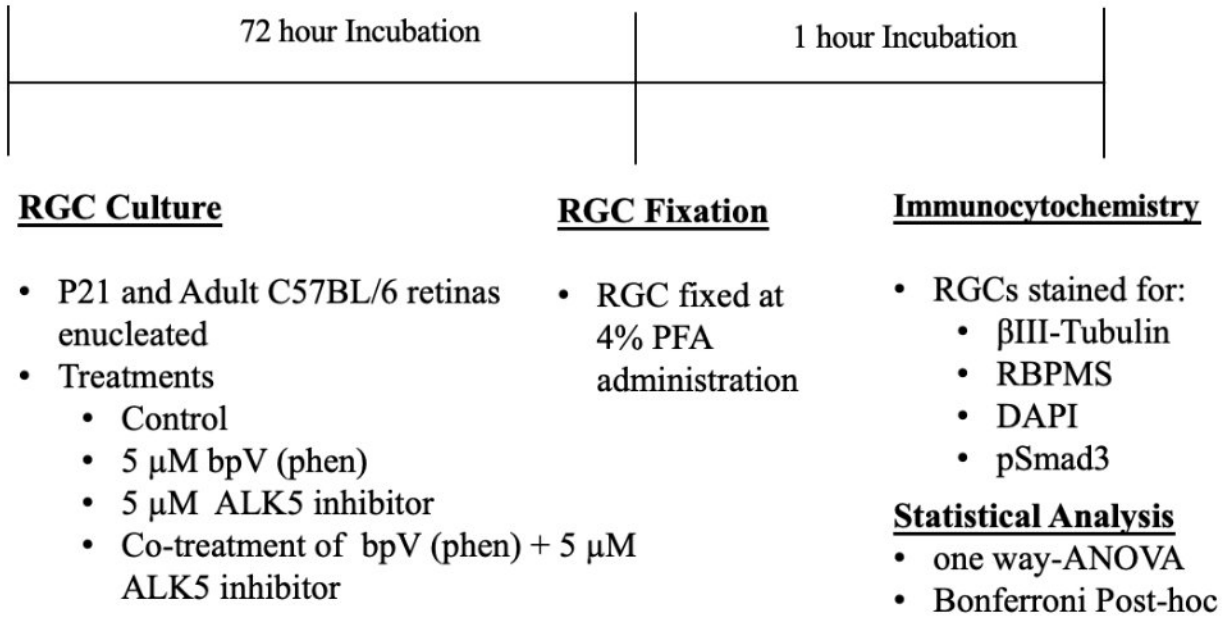

Figure 6. A summary of the ALK5i RGC culture timeline. P21 and adult RGCs were plated in wells with treatment groups prior to a 72-hour incubation period at 37°C and 5% CO_2. RGCs were then fixed at 4% PFA for 1 hour before undergoing immunocytochemistry.

4.5 Immunocytochemistry and Analysis of p-Smad3 Expression in BpV(Phen) and ALK-5 Inhibitor Treated RGCs in Vitro

After fixing cells in 4% PFA, P21 and adult RGC culture samples underwent three washes of 1X PBS for 10 minutes per wash. To signify p-smad3 expression in RGCs in cell culture wells, samples were stained with rabbit anti-phosphorylated-Smad3 (p-Smad3) antibody (1:200, Abcam), guinea pig anti-RBPMS antibody (1:200, Phosphosolution) and 10% normal goat serum (NGS) (Cedarlane) for 24 hours in 4℃. Once completed, the samples were once again washed three times with 1X PBS and incubated for 2 hours at room temperature in the dark with anti-guinea pig Alexa 488 (1:200, Thermofischer) and anti-rabbit Alex 555 (1:200, Cell Signaling). After incubation, samples followed three more washes of 1X PBS and stained with DAPI (1:10000, Thermo Scientific) for cell nuclei detection.

Five random sample areas were taken in each well by Zeiss Axiovert Microscope at 40X for the identification of p-Smad3-RBPMS-postive cells and replicated for each biological replicate. Once images were taken, a uniform setting was set for brightness and contrast. Fifteen RBPMS-positive cells were measured for its fluorescent intensity of pSmad3 using integrated optical density (IOD) from Image J (NIH). A total of 225 cells were quantified per treatment.

4.6 Optic Nerve Crush

Adult mice (N=6) were anaesthetized with isofluorane (inhaled) and analgesic, under the guidelines of the Canadian Council on Animal Care (CCAC). The surgeries were conducted in the similar manner of previous work (Smith et al., 2015).

The optic nerve was exposed by making an incision on the conjunctiva and pinched ~2mm behind the optic disk for 10 seconds with jeweler's forceps (Dumont #5; Fine Science Tools). Careful handling was taken not to disturb nearby blood vessels and cause retinal ischemia. Immediately following the optic nerve crush, 1 µL of vitreous solution from the retina was replaced with 1µL of either the vehicle control or 50 µM bpV(phen) (diluted concentration to 5 µM bpV(phen)). In total, a total of 4 treatment groups were used: No treatment (NT) group, ONC group (ONC only), ONC + BpV(Phen) group (50 µM BpV(Phen) following ONC), BpV(Phen) group (50 µM BpV(Phen) alone). To label RGC axons in the optic nerve, 1 µL of cholera toxin β subunit (CTB) (1mg/ml), an anterograde tracer, was injected into the vitreous matter 3 days prior to animal sacrifice with a Hamilton syringe. Animal retinal tissue was collected 14 days after ONC surgery and fixed in 4% PFA for 48 hours.

4.7 In Vivo RGC Survival Analysis using Retinal Wholemounts

Following the cryoprotection protocol in methods section 4.6, retinal samples were incubated in a 10% sucrose solution for 24 hours and then stored in a 20% sucrose solution at 4℃ before undergoing retinal wholemount preparations. Retinal samples were submerged in 1X PBS and cut into a four-leaf clover shape by using micro-scissors and micro-forceps. Dissected retinal samples were then placed in individual cell wells of a 24-well plate (Sarstedt). Samples were washed with 1% Triton X-100 solution three times for 15 minutes each and stained with rabbit anti-RBPMS antibody (1:500, Phosphosolution), diluted in 1% Triton X-100 solution overnight in 24-well plates (Sarstedt) at 4℃. Once finished, retinal samples were washed three more times with 1X PBS and incubated with anti-rabbit Alexa 488 (1:200, Cell Signaling), diluted in 1% Triton X-100 solution for 2 hours at 4℃. Finally, the samples were washed with 1X PBS three

times for 15 minutes, and then stained with DAPI (1:10000, Thermo Scientific) to detect cell nuclei.

RGC survival was quantified in three areas of each quadrant (superior, inferior, temporal, and nasal) of each retinal sample. Images were taken by using a confocal microscope at 20X magnification and analyzed using one-way ANOVA and Bonferroni's post hoc test (Graphpad Prism 8).

5 Results

5.1 Administration of bpV(phen) promotes cellular survival in P21 and adult retinal ganglion cells in vitro

To understand the effects of bpV(phen) in regard to cell survival, we tested several doses of bpV(phen) of increasing concentrations on P21 and adult RGCs following a 72-hour primary cell culture. As a reliable marker for RGCs, RNA-binding protein with multiple splicing (RBPMS) was used (Rodriguez, Müller, & Brecha, 2014) and was co-labelled with a nuclei marker, DAPI, to signify RGC-positive cells as shown in Figure 8C and 8F. As shown in Figure 9 and Figure 10, increasing bpV(phen) concentrations elevated RGC survival until reaching an optimal dosage of 5μM bpV(phen) in P21 and adult RGCs and then declining to where the survival of RGCs was similar to control. This data showed similar results to Chen et al. (2015), as researchers suggested that increasing bpV (phen) doses heightens the rate of apoptosis.

Performing Bonferroni's post-hoc test demonstrated that bpV(phen) treatment of 20μM was also statistically significant compared 1μM and 5μM bpV(phen) concentrations in both P21 and adult RGC cultures. Nonetheless, the optimal dose of bpV (phen) that promoted the strongest

response of RGC survival in P21 and adult RGC cultures was 5 μM ($p < 0.5$), at which point relative RGC survival was 117% and 89%, highlighted in Figure 7 and 8, respectively.

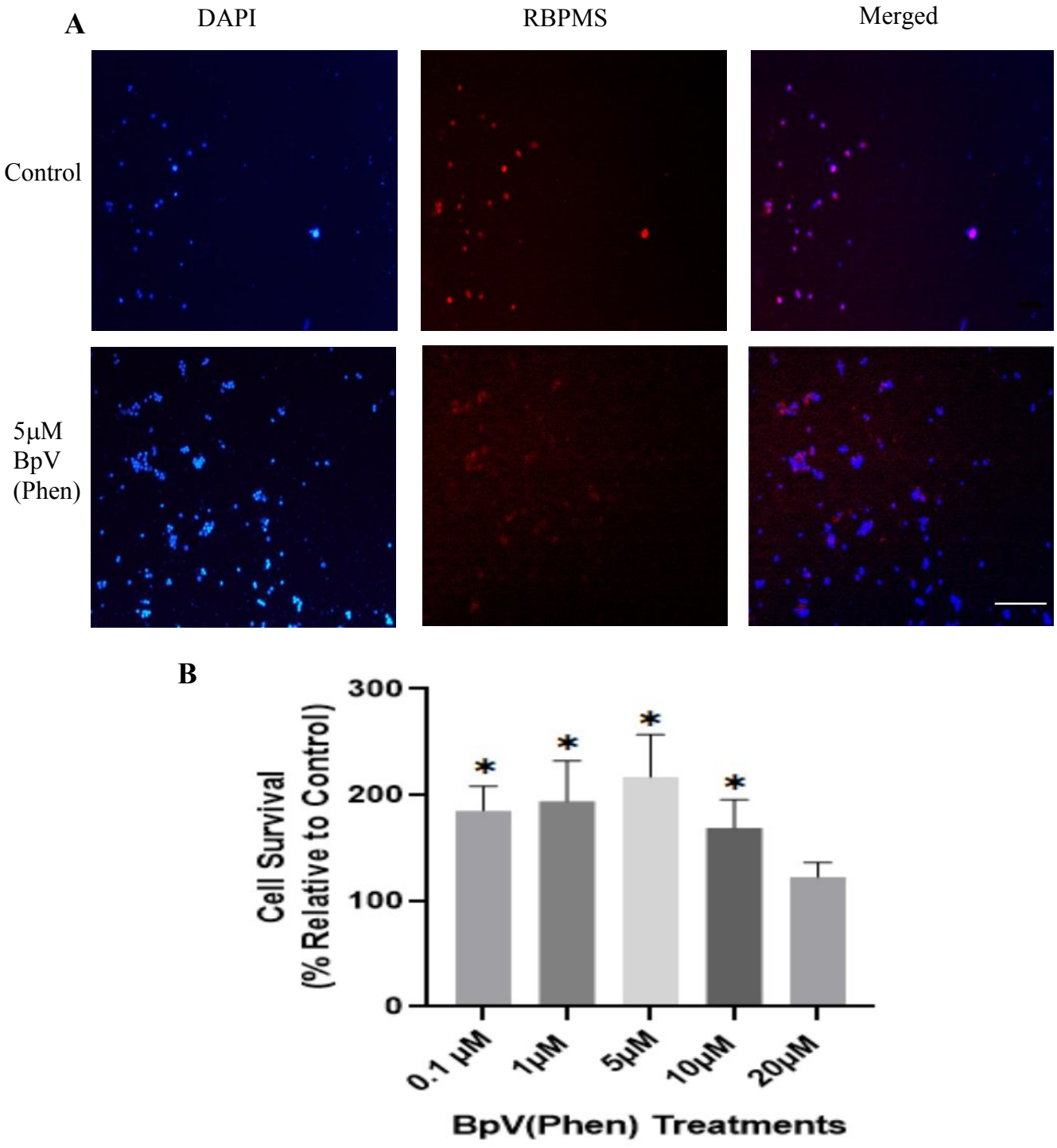

Figure 7. BpV(Phen) administration promotes survival of P21 mouse RGCs. (A) Cultured P21 RGCs were doubled-labeled with anti-RBPMS and DAPI. (B) Quantitative graphic representation of percent of RBPMS-positive cell bodies in P21 mouse pups; data are represented as percentages relative to control. Bars represents mean ± SEM from 3 animals per treatment group. Statistical analysis was carried out by using one-way ANOVA followed by Bonferroni's post-hoc test, *p <0.05. Scale bar = 50μm.

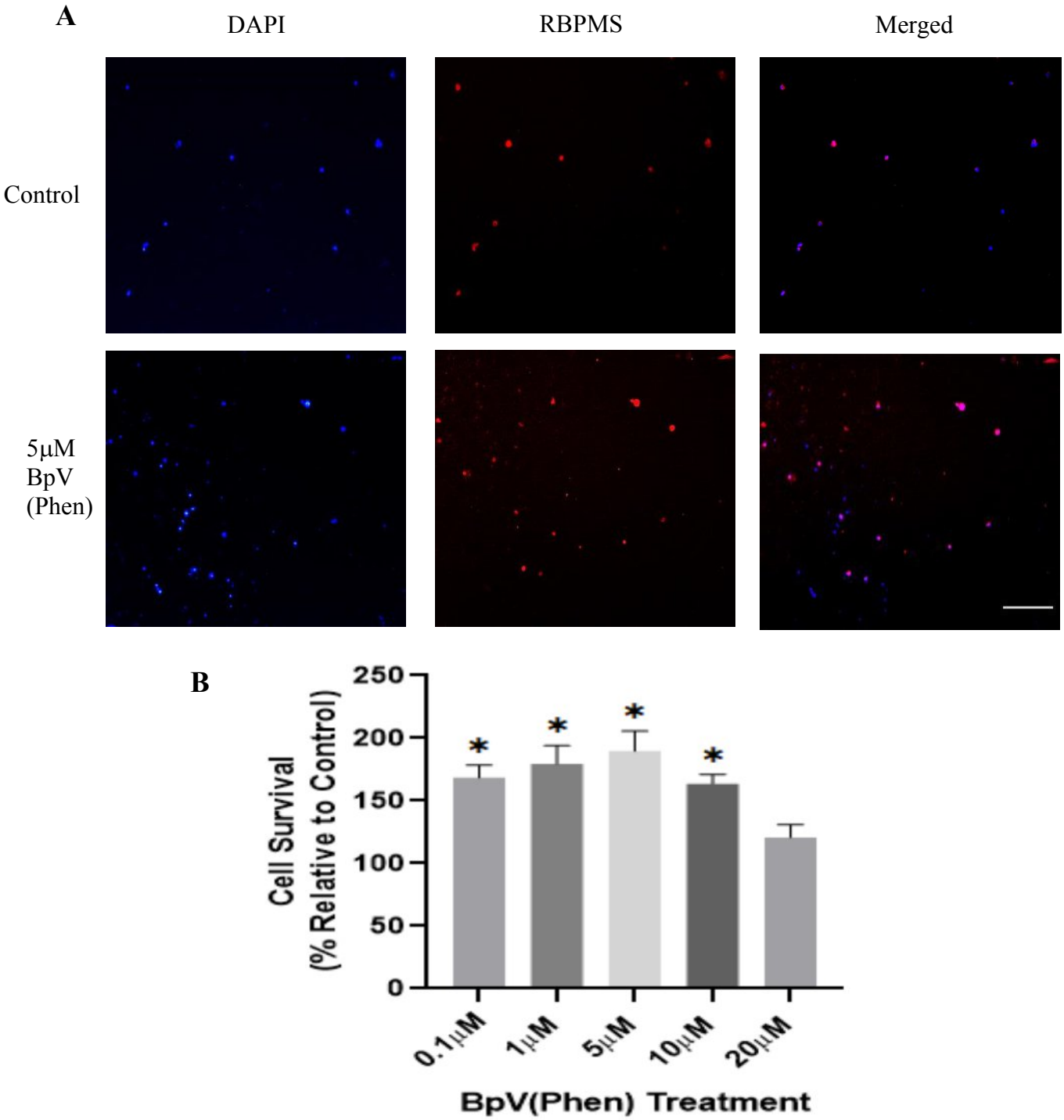

Figure 8. Enhanced survival of adult mouse RGCs following bpV(phen) treatment. (A) Cultured adult RGCs were double-labeled with anti-RBPMS and DAPI. (B) Quantitative graphic representation of percent of RBPMS-positive cell bodies; data are represented as percentages relative to control. Bars represents mean ± SEM from 3 technical replicates per treatment group. Statistical analysis was carried out by using one-way ANOVA followed by Bonferroni's post-hoc test, *p <0.05. Scale bar = 50μm.

BpV(Phen) enhances retinal ganglion cells *in vitro* and *in vivo*

5.2 TGF-beta pathway inhibition decreases cell survival but does not promote neurite outgrowth in culture

To evaluate the mechanism involved in bpV(phen)-treated RGC survival, an ALK5 inhibitor experiment was carried out, as outlined in Figure 6. An ALK5 inhibitor was used since ALK5 is one of the subunits in type I receptors in the TGF-β pathway. ALK5 inhibitor I (Enzo Life Sciences) is an ATP-competitive inhibitor that interferes with the phosphorylation of ALK5. The dosage of 5μM of the ALK5 inhibitor was used, as it is suggested to be the lowest concentration required to inhibit the TGF-β pathway (Yousef et al., 2015). Due to ALK5 being soluble in DMSO and DMSO concentrations >1%, which is known to cause cellular apoptosis *in vitro* (Galvao et al., 2014), 0.5% DMSO was used as another treatment to confirm that <1% DMSO did not cause apoptosis in mature RGCs. As such, there was no significant difference between 0.5% DMSO and control treatment, verifying that <1% DMSO does not promote retinal apoptosis. Furthermore, from the previous observation that 5μM bpV(phen) was shown to enhance mature RGC survival *in vitro*, this concentration was used again for this set of experiments. Like past experiments, 5μM of bpV(phen) was shown to significantly promote RGC survival relative to control (Fig 9B, C); specifically increasing RGC survival by 102% and 150% in P21 and adult RGCs respectively. Alternatively, ALK5 inhibitor alone did not significantly differ in RGC survival relative to control. However, by administering bpV(phen) in a co-treatment with the ALK5 inhibitor, RGC survival was significantly higher relative to control and ALK5 inhibitor alone by 51% and 63% in P21 RGCs and 20%and 32% in adult RGCs (fig 9B, C), respectively.

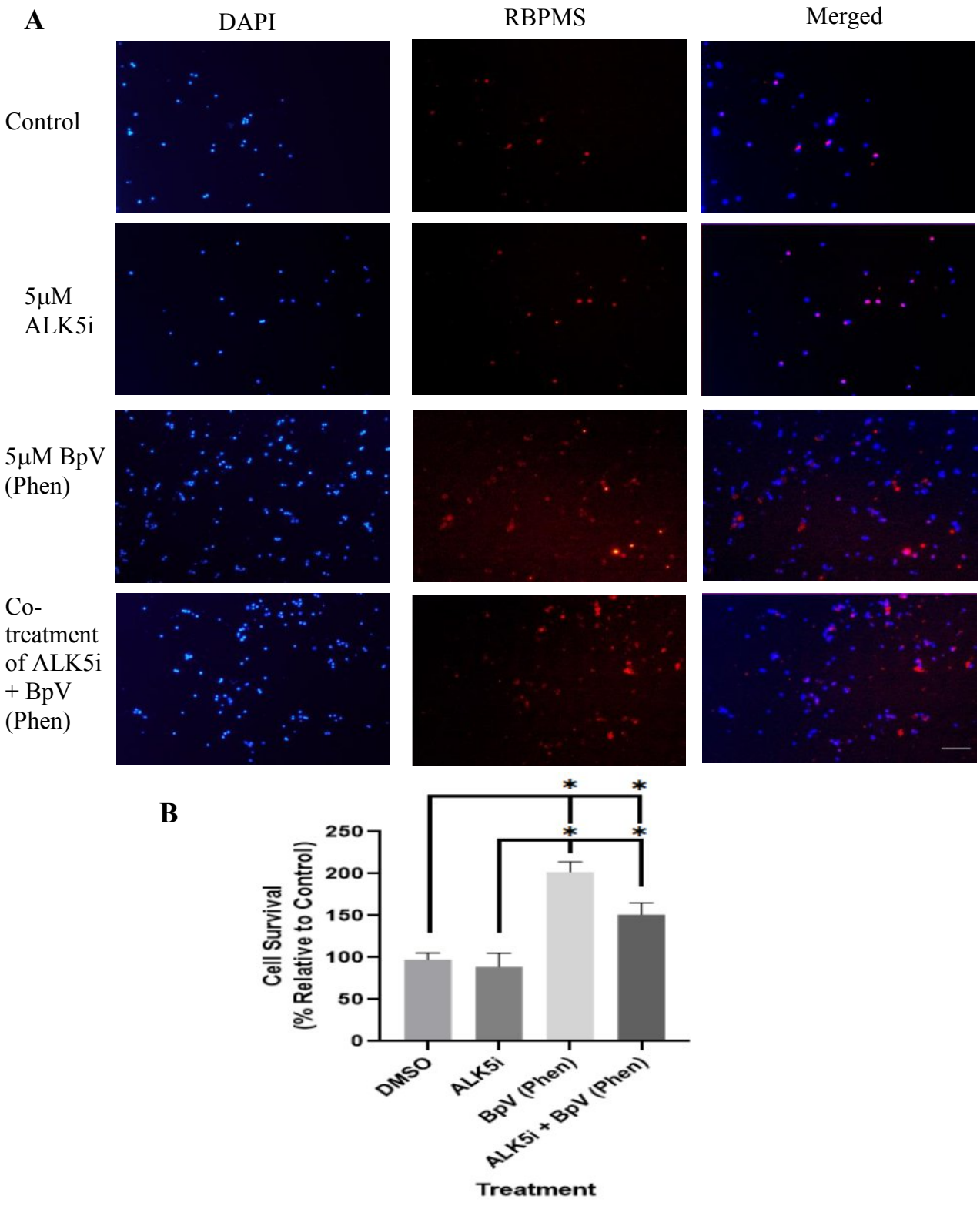

Figure 9. ALK5 inhibitor did not promote cellular survival in P21 primary RGC culture. (A) Cultured P21 RGCs were double labeled with anti-RBPMS and DAPI. (B) Quantitative graphic representation of percent RBPMS-positive cell bodies in P21; data are represented as percentages relative to control in P21. Bars represents mean ± SEM from 3 animals per treatment group in P21 RGC cultures. Statistical analysis was carried out by using one-way ANOVA followed by Bonferroni's post-hoc test, *p <0.05 Scale bar = 50μm.

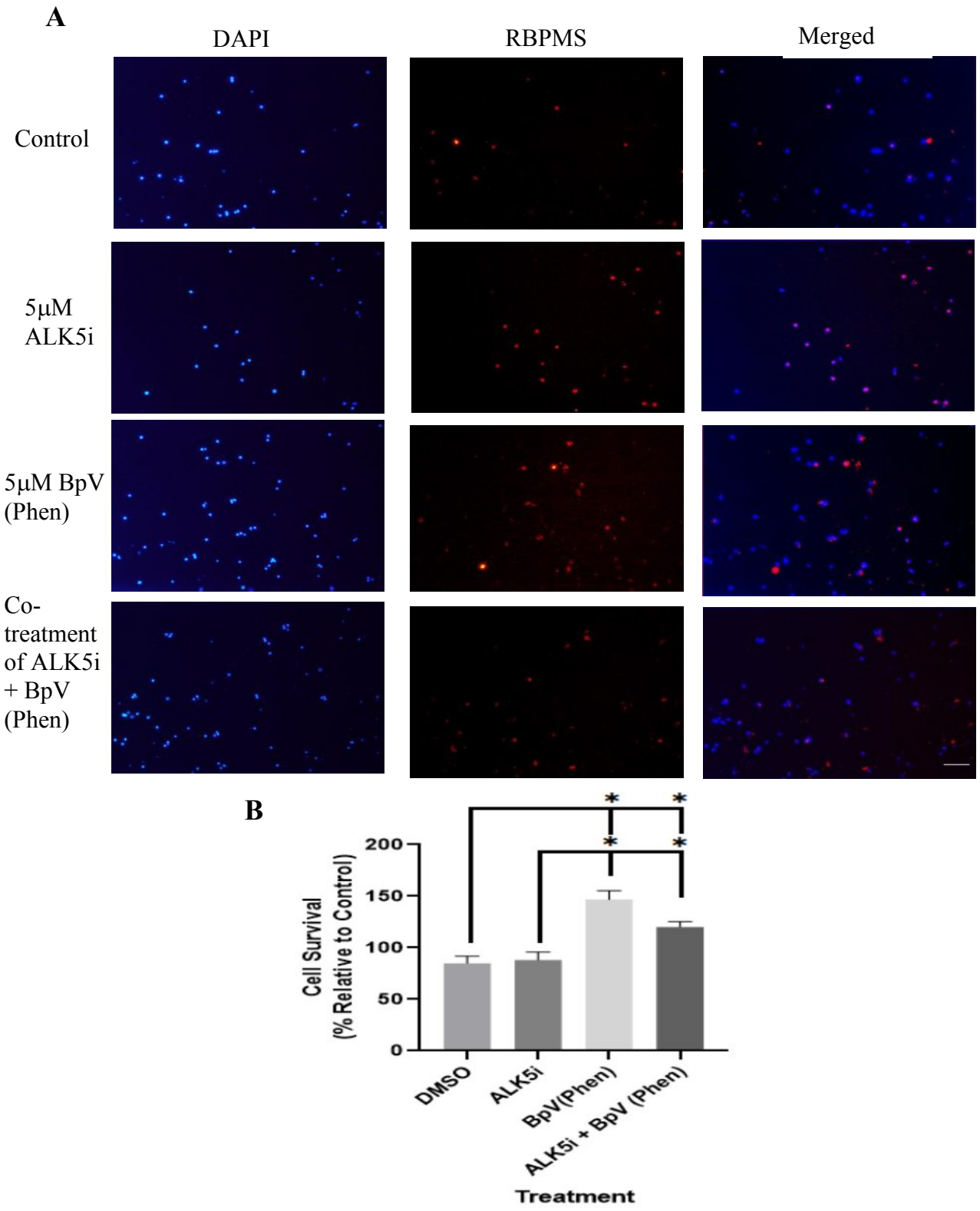

Figure 10. ALK5 inhibitor treatment in bpV(phen)-treated adult RGCs did not facilitate enhanced survival *in vitro*. (A) Cultured adult RGCs were double-labeled with anti-RBPMS and DAPI. (B) Quantitative graphic representation of percent RBPMS-positive cell bodies; data are represented as percentages relative to control in adult mouse RGCs. Bars represents mean ± SEM from 4 technical replicates in adult RGC cultures. Statistical analysis was carried out by using one-way ANOVA followed by Bonferroni's post-hoc test, *p <0.05. Scale bar = 50μm.

5.3 BpV(phen) administration decrease pSmad3 expression in vitro

To assess whether the TGF-β pathway *via* pSmad3 expression influenced bpV(phen)-treated RGC survival, immunofluorescent intensity of pSmad3 in RGCs was measured by optical density (OD). According to Song et al. (2006), activation of the PI3K pathway inhibits Smad3 proteins in the TGF-β pathway. As such, similar treatment groups were carried out as per methods section 4.4. As expected, inhibition of TGF-β pathway via ALK5 inhibitor showed reduced expression of pSmad3 relative to control. However, to a lesser degree, bpV(phen)-treated RGCs also significantly decreased pSmad3 expression. Interestingly, when ALK5 inhibitor was added to bpV(phen), pSmad3 levels reduced even further than ALK5 inhibitor alone.

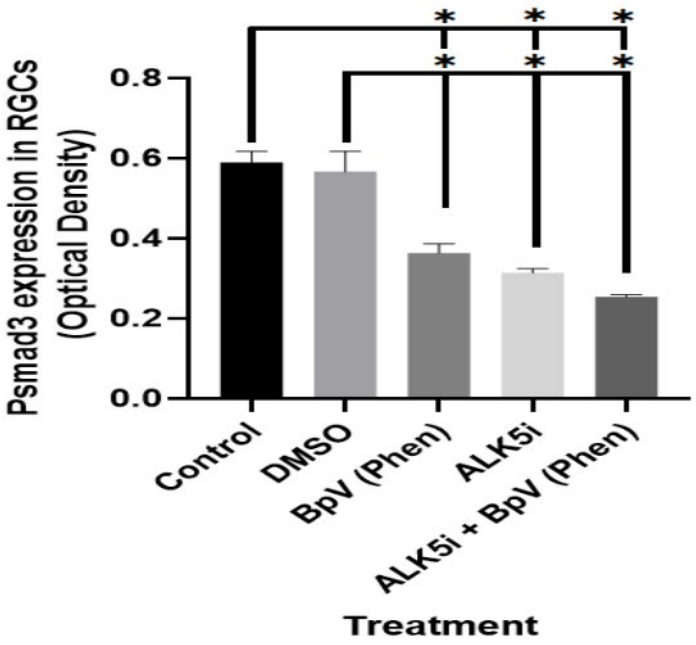

Figure 11. BpV(phen) administration reduces pSmad3 protein expression in adult RGCs. Quantitative graphic representation of the optical density (OD) of pSmad3 expression in adult mouse RGCs. Bars represent OD mean ± SEM from 3 technical replicates per treatment group. Statistical analysis was carried out by using one-way ANOVA followed by Bonferroni's post-hoc test, *$p<.05$, $R^2 = .91$.

5.4 Administration of bpV(phen) promotes RGC survival in vivo after optic nerve crush

In previous experiments, it was shown that 5μM bpV(phen) can promote adult RGC survival *in vitro*. As such, the possibility remained whether this effect was also evident in a physiological setting. As shown in Figure 12, there was a significant difference between the survival of RBPMS-positive cells in the ONC group and the no-treatment group. Notably, administration of bpV(phen) alone did prompt a significant difference in RGC survival compared to control, highlighted in Figure 13. This may suggest that bpV(phen) have different effects on RGCs under certain circumstances. In which, bpV(phen) may be harmful in healthy cells but be beneficial for injured cells.

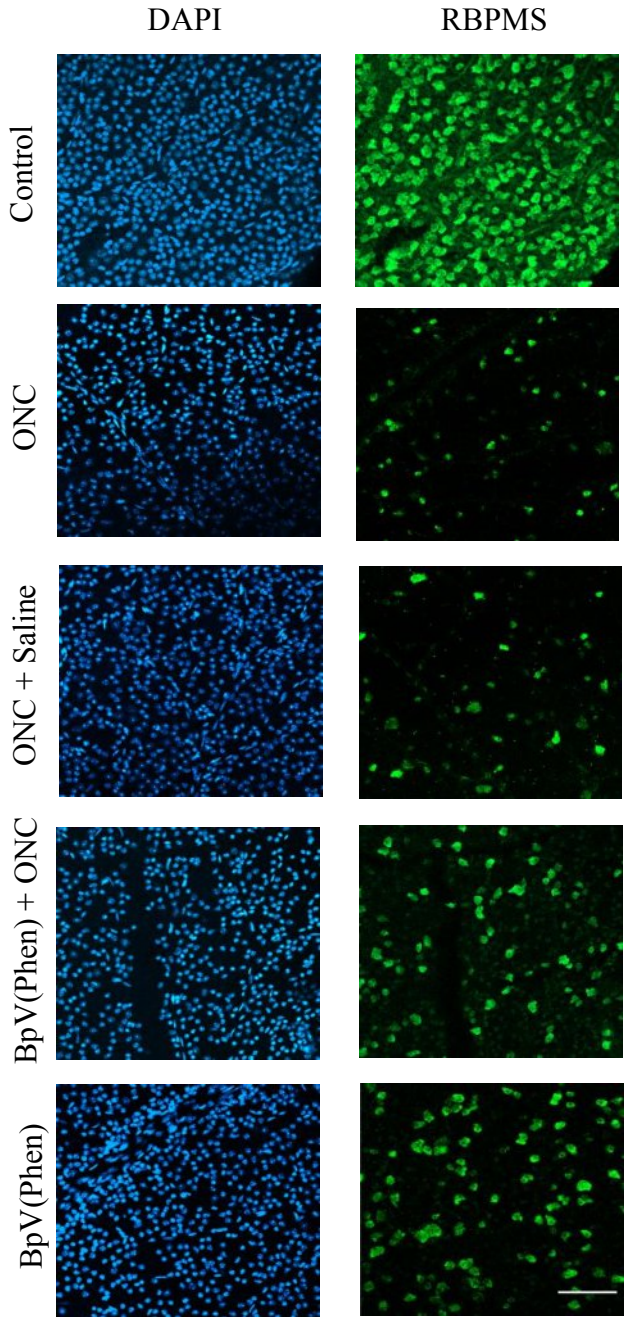

Figure 12. BpV(Phen) administration promotes RGC survival after optic nerve crush. Retinal wholemounts were double-labeled with DAPI and anti-RBPMS antibody. Scale bar = 20μm.

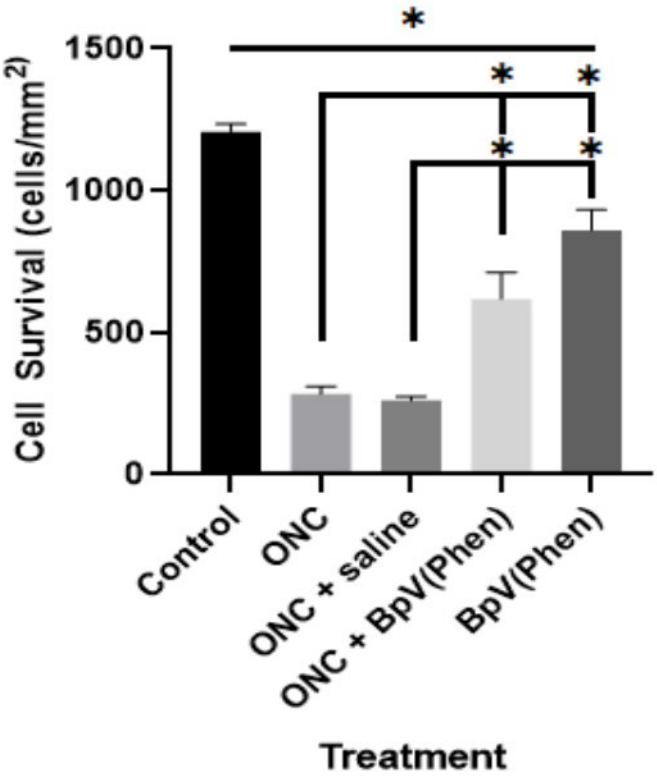

Figure 13. RGC survival quantification representation of retinal wholemounts. Bars represent mean ± SEM from 3 animals per treatment group. Statistical analyses were carried out by using one-way ANOVA followed by Bonferroni's post hoc test, * $p \leq 0.05$.

6 Discussion

6.1 BpV (Phen) Promotes Neuronal Survival but not Regeneration

Although inhibiting PTEN *via* bpV(phen) administration was shown to induce axon regeneration in zebrafish (Diekmann, Kalbhen, & Fischer, 2015) and promote cellular survival in rat's spinal cord (Nakashima et al., 2008), these functional outcomes had yet to be determined in mammalian RGCs. As depicted in figure 7 and 8, the optimal dose of bpV(phen) to induce the greatest neuronal survival was at 5 μM. These results were quite similar to Chen et al. (2015) where similar concentrations of bpV(phen) were shown to protect the survival of cells *in vitro* before observing decreasing levels of cell viability; Signifying that higher dosages of bpV(phen) increase possible levels of apoptosis.

Besides regulating cellular survival, activation of the PI3K pathway also contributes to the phosphorylation of forkhead box O (FoxO) transcription factors that are responsible against oxidative stress (Klotz et al., 2015). Identified by an overexpression of reactive oxidative species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-), oxidative stress has been shown to play a major role in the death of RGCs in ocular neurodegenerative diseases (Liu et al., 2018). Under low or moderate levels of ROS, ROS is thought to be essential in the regulation of neural development. In contrast, extreme levels of oxidative stress cause "oxidative damage" to cells, leading to protein oxidation, lipid peroxidation, and ultimately cellular death, as observed with high PI3K pathway activity (Santo et al., 213). Excess of free radicals may also alter mitochondria DNA, and lead to mitochondria dysfunction and ATP-deficiencies, all of which have been suspected to the development of neurodegenerative disorders (Guo et al., 2013).

Since the activation of the PI3K pathway involves heavy ATP-demanding processes such as cellular survival and axon regeneration, there needs to be a balance in activating the PI3K pathway efficiently before inducing oxidative damage. As such, the optimal concentration of bpV(phen) administered to RGCs *in vitro* may be the ideal "tipping point" to sufficiently activate the PI3K pathway and efficiently utilize all ATP reservoirs. With the limited amount of ATP, it must be allocated either one process or spread thinly among several processes without requesting for more ATP and cause extreme levels of oxidative stress. Thus, see in appendix, the former may hold true in maturing mammalian RGCs in regards to how ATP is being distributed. Once mammalian neurons innervate their connections to neighboring cells, their ability to propagate their axons is counterproductive. With no need to extend their axons, neurons direct their resources to remaining viable and survive at any cost (Kole, Annis, & Deshmukh, 2013). Hence, efforts would be funneled into promoting neuronal survival with the limited amount of bpV(phen); rather than trading valuable resources for the ability of axonal growth. Further support from Goldberg *et al.,* (2002) demonstrated that neurons do not extend neurite processes regardless of their survival. In the same study, researchers provided evidence that by introducing a growth factor such as BDNF, along with pro-survival factors such as over-expressing Bcl-2, CNS neurons extend their processes. Thus, perhaps in regard to the PI3K pathway, efforts could be made to target both functional processes with adequate support for each process such as stimulating pro-survival pathways or co-treatment with pro-survival factors.

BpV(Phen) enhances retinal ganglion cells *in vitro* and *in vivo*

6.2 Co-treatment of ALK5 inhibitor and bpV(phen) did not facilitate higher levels of survival in bpV(phen)-treated RGCs via pSmad3 protein expression

Previous research has hinted the effects of PTEN inhibition may be through Akt-independent mechanisms that support Akt-dependent axon regeneration and cellular survival (Huang et al., 2019). One of these mechanisms that have been identified to interact with the PI3K pathway was the TGF-β pathway (Guo & Wang, 2009). Independently, inactivation of the TGF-β pathway *via* type II receptor knock outs enhance retinal regeneration in animals, but also increases levels of apoptosis, indicated by a reduction of pSmad3 (Braunger et al. 2013; Tappeiner et al., 2016). Meanwhile, activation of the PI3K pathway by conditional knockout of PTEN displays elevated levels of RGC survival and robust axon regeneration (Park et al. 2008). Despite how these signaling pathways act individually, it remains unclear whether co-targeting these pathways simultaneously will benefit or damper mammalian RGCs in terms of cellular survival and axon regeneration.

To define the mechanism in which the TGF-β pathway facilitates bpV(phen)-treated RGC survival and axonal growth, pSmad3 was monitored for its activity. Treatment of ALK5 inhibitor significantly decreases the expression level of pSmad3 compared to the control (Figure 10). In addition, bpV(phen)-treated cells also show a decreased expression of pSmad3 in RGCs, but to a lesser extent than ALK5 alone. This data verifies with previous research that suggests activation of PI3K influences other non-PI3K effectors such as Smad3 (Conery et al., 2004; Guo & Wang, 2009) as shown in Figure 4. Furthermore, co-treatment of PTEN and TGF-β inhibitors also displayed the greatest decrease in pSmad3 expression, indicating that both inhibitors were acting synergistically towards each other in suppressing pSmad3.

BpV(Phen) enhances retinal ganglion cells *in vitro* and *in vivo*

Interestingly, despite lower levels of pSmad3, results depicted in Figure 9 and 10 showed that inhibiting the TGF-β pathway did not enhance the survival of RGCs but instead demonstrate a descending trend. Notably, no promotion of neurite outgrowth was observed in ALK5i treatments, depicted in appendix. Surprisingly, co-treatment of ALK5i and bpV(phen) did lead to an enhanced level of RGC survival in P21 and adult mice. This suggests that bpV(phen) or PI3K pathway activation grant resistance to the apoptotic effects of TGF-β pathway. In addition, it also suggests that pSmad3 may not play a pivotal part in influencing bpV(phen)-treated growth in mammalian RGCs.

It is intriguing that opposing effects of inhibiting TGF-β pathway in zebrafish would promote retinal regeneration (Tappeiner et al., 2016). Comparing these species, zebrafish and mammals have some similarities when dealing with CNS injury - apoptotic cell death (Noorimotlagh et al., 2017). However, zebrafish retain the ability to be neurogenic in regions such as the brain, spinal cord, and retina (Ghosh and Hui, 2016). In addition, zebrafish RGCs evoked an upregulation of PI3K pathway activation *via* mTOR activity after injury, which is opposite to what is observed in mammalian RGCs (Leibinger, Andreadaki, & Fischer, 2012). Lastly, observations of inhibiting the TGF-β pathway after retinal degeneration enhanced regeneration and proliferation, indicated by decreased pSmad3 expression (Tappeiner et al., 2016). Thus, hinting that therapeutic strategies seek potential treatments in cell proliferation in the mammalian CNS injury models.

6.3 PTEN inhibition promotes neuronal survival in vivo after ONC in adult mice

Considering the *in vitro* data showing bpV(phen) to promote RGC survival, this functional effect had yet to be reported in a physiological setting. Congruent with *in vitro* data, application

of bpV(phen) promoted survival of adult RGCs *in vivo.* When injected alone, bpV(phen) showed a significant reduction of RGCs relative to control. This indicated two possibilities: the concentration of bpV(phen) administered is cytotoxic or the entirety of the bpV(phen) molecule may be harmful to non-injured cells. While the former concentration has been tested *in vitro* and has been shown to promote RGC survival, the latter statement is more likely. Besides the beneficial effects that bpV(phen) has on RGCs to survival after injury, it may be that bpV(phen) exercises an opposing effect on healthy RGC cells, promoting cellular death of healthy RGCs.

In a healthy retina, ~30 distinct subpopulations of RGCs exist, all characterized by their morphology, function, and molecular status (Sanes & Masland, 2015). After an injury, the majority of the RGC population die and very few ever regenerate. Recognized by its high mTOR activity, a subpopulation of RGCs known as αRGCs, compose a greater portion of the remaining RGC population, along with preferentially regenerating their axon (Duan et al. 2015). Although decreased levels of mTOR occur after an injury, it is possible that the administration of bpV(phen) can restore basal mTOR levels in αRGCs and promote survival. Interestingly, promotion of survival *via* PI3K activation *in* RGCs can be due to inhibition of autophagy (Wang et al., 2018). During autophagy, a membrane formed by the cell's vesticular region, engulfs membrane-bound components of the cell such as mitochondria and ribosomes and recycle them for macromolecular synthesis and ATP reservoirs (Levine & Yuan, 2005). Classified as type II cell death, autophagy occurs at low basal levels in all cells, but is rapidly up-regulated when cells need to generate energy and undergo structural remodeling i.e. energy consumption for survival and axon regeneration (Levine & Kroemer, 2008; Liu & Levine, 2015). However, despite an upregulation of autophagy after an injury, this process might be nullified due to the overactivation of the PI3K pathway (Li et al., 2018). On the other hand, in a non-injured

environment, those RGC subpopulations that died after ONC, are now present. These other RGCs might have little-to-none mTOR ability and could rely on other signaling pathways to function. Due to these low basal levels of mTOR, bpV(phen) might not enhance mTOR levels to levels near αRGCs. Consequently, this little expression in mTOR activity might not fully suppress autophagy and only lead to partial suppression. Thus, partial autophagy can lead to defective recycling of neurotoxic proteins and eventually cell death (Levine & Yuan, 2005).

Regardless of whether certain cells function primarily through one signaling pathway or another, the application of bpV(phen) *in vivo* after optic nerve crush require further research to investigate the "tipping point" or mechanistic switch at which one RGC sub-type would undergo cellular death, while others would sustain their pro-survival mechanisms and possibly regenerate.

In terms of axonal regeneration, one explanation as to why no axonal growth was not observed in this study while other studies such as Park et al. (2008) have done so, could be due to the half-life of bpV(phen) and its availability to PTEN. By inducing a conditional knock-out of the PTEN gene in mouse models, Park et al. (2008) halted ~90% of PTEN activity located within RGCs; leading to a more effective widespread inhibition. Meanwhile, in the case of drug-induced PTEN inhibition, the half-life of the drug and its delivery method must be considered. The half-life of bpV(phen) is 6 hours in a serum-medium (Cerovac et al. 1999), while the protein turnover rate for PTEN is ~8 hours (Vazquez et al., 2000). Difficulty also arises as pools of PTEN are split between the nuclear membrane and cytoplasm of the cell. Therefore, drug administration of bpV(phen) may not fully express the effect on the majority population of RGC, as the duration of bpV (phen) may not last long enough to fulfill its function. Additionally, if adding higher concentrations were a possibility to fully saturate each cell, the risk of bpV(phen)

at higher doses may result in non-specific downstream signaling or outcomes that are not involved in RGC survival or axon regeneration such as oxidative stress.

7 Conclusion and Future Direction

Upon injury, the mammalian mature CNS is unable to regeneration and survive. This is thought to be due to the limited regenerative properties and the inhibitory environment that the neuron is in after an injury (Park et al., 2008). One of the prominent pathways that is responsible for cellular survival and axon regeneration is the PI3K pathway. The PI3K pathway has been shown to promote neuronal survival and axon regeneration across the CNS, including RGCs in the visual system. Previous studies have utilized genetic manipulation of certain proteins in the PI3K pathway; however, it may not be clinically feasible to continue this methodology due to public safety concerns. The current study approaches the manipulation of PTEN from a different standpoint – pharmacological inhibition. A non-specific PTPase inhibitor, BpV(Phen), has been shown to predominately inhibit PTEN protein in the PI3K (Schmid et al., 2004). *In vitro* data from this current study showed that at a dosage-dependent application of bpV(phen), mature mammalian RGC survival can be elevated, to which the optimal concentration of 5μM promoted the greatest survival in P21 and adult mammalian RGCs. However, despite promoting RGC survival, no observable neurite projections were shown.

In efforts to define a potential mechanism that mediated the effects of bpV(phen)-treated RGC survival and axonal growth, the TGF-β pathway was inhibited *via* an ALK5 inhibitor to help facilitate axonal growth, indicated by pSmad3 expression. As data revealed, ALK5i, bpV(phen), and co-treatment of ALK5i and bpV(phen) did suppress pSmad3 levels in P21 and adult RGCs *in vitro* relative to control; being that the co-treatment group suppressed pSmad3

levels the greatest. However, despite having low levels of pSmad3 protein found in RGCs, no neurite outgrowth was observed after an injury. Furthermore, inhibiting the TGF-β pathway did not affect the survival of RGCs after an injury. On the other hand, administering bpV(phen) in Alk5i-treated RGCs did promote survival, although to a lesser degree than bpV(phen) alone.

While it may seem that that bpV(phen) is beneficial in supporting the survival of RGCs, bpV(phen) did cause apoptosis in healthy cells in a *in vivo* model system. As with any drug therapy, results from this thesis serve as a precaution to this type of therapy and the need for safe use. Overall, beside the adverse effect of bpV(phen) in healthy cells, there still holds therapeutic potential for bpV(phen) as it relates to neuronal survival in neurodegenerative conditions.

8 References

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9 Appendix

Neurite extension in early postnatal versus Adult mouse RGCs