ABSTRACT

Glaucoma is a group of chronic progressive optic neuropathies commonly characterized by elevated intraocular pressure (IOP) (a subset of glaucoma patients display neurodegenerative effects at 'normal' IOP) leading to axonal degeneration, optic nerve head cupping and apoptosis of retinal ganglion cell death (RGCs), which result in visual field defects and blindness. While there are medications available to lower IOP, there is an unmet need for neuroprotective treatments for glaucoma, since some neurodegenerative effects persist despite lowering IOP.

The main focus of this study was on the class 4 POU domain transcription factor, Brn3b, which has been shown to play a key role in the development of RGCs. Two previous studies from other labs showed that a decrease in Brn3b expression occurs in animal model of glaucoma. A recent publication from our laboratory demonstrated neuroprotective effects of adeno-associated virus (AAV) mediated expression of Brn3b in a rat model of ocular hypertension. This research project identified some mechanisms of Brn3b-mediated neuroprotection in cultured PC12 cells (under the condition of hypoxia) and also in vivo in the Morrison's model of ocular hypertension in rats.

In the first part of the study, we demonstrated the effect of overexpression of Brn3b on various markers of synaptic plasticity in PC12 cells under conditions of normoxia as well as hypoxia. Immunoblot as well as immunocytochemical analyses revealed an increase in expression of neurite growth markers, GAP-43 and ac-TUBA, by Brn3b upregulation both

under conditions of normoxia as well as hypoxia. . This suggests that transcription factor Brn3b has the ability to upregulate expression genes contributing to synaptic plasticity genes both under ‘normal’ conditions and during a glaucomatous insult (hypoxia). In the concluding part of this study, cell survival factors including, Bcl-2, Bcl-xL and p-AKT were studied as potential targets of Brn3b-mediated neuroprotection. Adeno-associated virus-mediated expression of Brn3b in rat eyes with elevated IOP promoted an upregulation of Bcl-2, Bcl-xL and p-AKT in RGCs, as determined by immunohistochemistry. Taken together, the evidence suggests that Brn3b has the potential to be developed as a therapeutic agent for neuroprotection during ocular neurodegenerative diseases like glaucoma.

NEUROPROTECTIVE EFFECTS OF BRN3B IN PC12 CELLS AND RETINAL GANGLION CELLS UNDER GLAUCOMATOUS CONDITIONS

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DISSERTATION

Presented to Graduate Council of the

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For the Degree of

DOCTOR OF PHILOSOPHY

By

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INTRODUCTION

Glaucoma

Glaucoma is an optic neuropathy commonly associated with elevated intraocular pressure (IOP) afflicting nearly over 70 million individuals worldwide (Quigley and Broman, 2006), with a projected incidence of 3.6 million patients in America by the year 2020 (Friedman et al. 2004). It is a leading cause of irreversible blindness (Resnikoff et al. 2002). Glaucoma is an age-related heterogeneous group of diseases that is commonly characterized by the impairment of aqueous humor outflow through the trabecular meshwork, leading to increased IOP which eventually damages the lamina cribrosa, optic nerve head, and RGCs (Clark and Yorio, 2003). The most commonly occurring form of glaucoma is primary open angle glaucoma (POAG), in which the elevation of IOP is not due to mechanical obstruction of the irido-corneal angle. In the case of POAG, the obstruction to outflow of aqueous humor occurs at the molecular/biochemical level due to pathological changes in the trabecular meshwork/Schlemm's canal. Past studies demonstrated a 13-fold increase in adjusted relative risk of developing POAG when IOP is more than 25 mmHg (normal IOP is typically below 21 mmHg) (Nemesure et al 2007). It is hypothesized that elevated IOP causes tremendous mechanical stress on the unmyelinated portions of axons of the RGCs at the optic nerve head, where they take a 90 degree turn to traverse into the optic nerve and then to their final destination in the brain, namely, the lateral geniculate nucleus or the superior

colliculus (Quigley et al., 1981). The strain on the axons of the RGCs at the lamina cribrosa is the primary precipitating insult that damages the axons of the RGCs (Figure1) (Quigley et al., 1981). Axonal injury is thought to inhibit the retrograde transport of neurotrophic factors originating in the brain and prevent them from reaching their final target, which is the RGC soma, eventually leading to RGC death (Figure1) (Quigley 1981). IOP is considered a crucial risk factor in the development of glaucoma, and in the clinic, patients with IOP above 21 mmHg are treated with IOP lowering drugs.

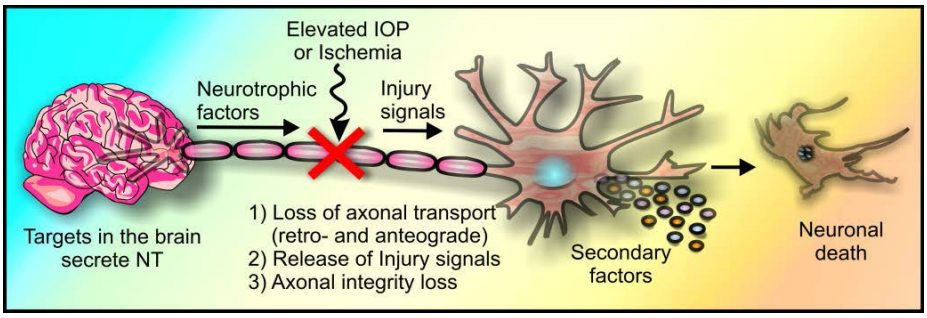

Figure 1. Mechanisms underlying neurodegeneration in glaucoma. Elevated IOP is implicated as a primary insult in causing RGC death in POAG. This initial insult along with ischemic changes is thought to cause a blockade of transport of neurotrophic factors and the release of secondary factors that contribute to RGC death. (Courtesy: Mueller et al., Neuroprotection in Glaucoma. InTech Publisher 2011).

The precise cellular and molecular mechanisms contributing to RGC apoptosis in POAG are still not completely understood; however, it is known that the disease process of POAG incorporates more than just elevation of IOP. Epidemiological studies have indicated the limitations of utilizing IOP measurements as the sole means to screen and identify patients for glaucoma. As an example, according to the Blue Mountains Eye Study the prevalence for POAG for individuals that have

elevated IOP is 10-20% when IOP is 21-23 mmHg, 30% when the IOP is 24-25 mmHg, 15% when the IOP is 26-27 mmHg, and 30-40% when the IOP is ≥ 28 mmHg (Mitchell 1996). In addition, 30-40% of patients with POAG have normal IOP which is defined as normal tension glaucoma (Klein et al. 1992; Dielemans et al.1994). Though elevated IOP is one of the risk factors for POAG, there are many individuals with elevated IOP (ocular hypertensives) that do not develop POAG, and there are many individuals with normal IOP that have been diagnosed with POAG. Presently, POAG is defined as "a progressive chronic, optic neuropathy where IOP and currently unknown factors contribute to the damage to the optic nerve with loss of ganglion cells and their axons (Armaly et al., 1980)." Elevation of IOP remains the major risk factor for POAG. However, other important risk factors associated with POAG include: age (Armaly et al.1980), African American race (Tielsch et al. 1991), Hispanic race (Kim and Verma, 2010), family history of glaucoma (Kolker et al.1972), and thin central corneal thickness (Gordon et al.2002; Law et al.2007).

Since the factors that are involved in the etiology of POAG are not completely known, one of the current limitations in glaucoma treatment is that the only medical modalities used to treat glaucoma are IOP lowering drugs and surgeries (Weinreb et al., 2004). Many studies have demonstrated protective efficacy of IOP lowering drugs by preventing progression at early and advanced stages of it in patients with POAG. The Early Manifest Glaucoma Trial (EMGT) demonstrated 45% of patients showed progression of glaucoma were adequately being treated with IOP lowering agents compared to 62% of patients who showed disease progression without treatment (Heijl et al. 2002). This study also demonstrated that for every millimeter mercury IOP reduction, there is an estimated 10% decrease in risk for disease progression (Heijl et al. 2002). Moreover, it also suggested that even 65% of NTG patients can have their disease process completely halted when their IOP is lowered by 30% (Collaborative Normal-Tension glaucoma

study gr 1998). However, only 50% of patients who are diagnosed with NTG can achieve an IOP reduction of 30% utilizing topical drugs, laser trabeculoplasty, or both (Collaborative Normal-Tension glaucoma study gr 1998; Schulzer et al., 1992). Hence, a better understanding of the pathophysiology of RGC apoptosis and POAG is required in order to develop better treatment options and neuroprotective compounds that can be used as adjunct medical therapies to IOP lowering drugs. This dissertation will focus on some aspects of the involvement of hypoxia and mechanical stress in glaucoma and will describe an approach to stimulate intrinsic neuroprotective capacity of RGCs through upregulation of markers of axonal plasticity including GAP-43, ac-TUBA and pro-survival proteins of Bcl-2 family, and p-AKT.

Mechanical hypothesis in glaucoma

Elevated IOP induces physical changes at the optic nerve head (ONH) visualized clinically as optic disc cupping due to optic nerve head axonal compression at the lamina cribrosa, blockage of axonal transport and a disruption of retrograde transport of neurotrophins to RGCs, leading to their apoptosis (Guo et al. 2004). The ONH has been studied in both human and experimental animal models as a primary site of glaucomatous damage. Several studies have demonstrated extensive remodeling of the extracellular matrix (ECM) including collagen I and IV, TGF-β2, and matrix metalloproteinase (MMP)-1 at the optic nerve head in animal models of glaucoma (Quigley et al. 1981, Morrison et al. 1997, Hernandez et al. 2000, Yan et al. 2000, Agapova et al. 2001, Johnson et al. 1996, Pena et al. 1999, Cordeiro et al. 2002 and Zode et al., 2011). Primary cellular response, elevated IOP or ischemia and/or a secondary cellular response triggered by axonal degeneration causes activation of astrocytes. By altering the microenvironment of the optic nerve head through remodeling, they cause axonal degeneration and progressive, irreversible pathological changes (Hernandez et al. 2000). These activated astrocytes are responsible for the production of the

matrix-degrading enzymes (MMPs) which affect the pattern of extracellular matrix remodeling (Yan et al. 2000). It is hypothesized that these effects are modulated by TGF-β2, produced by astrocytes which has been shown to be significantly increased in the glaucomatous ONH (Pena et al. 1999, Zode et al., 2011). In addition, the release of a variety of secondary factors including glutamate, nitric oxide, tumor necrosis factor-alpha and endothelin, which in turn activates factors associated with RGC death (Figure 2) (Clark and Yorio 2003; Prasanna et al. 2010; Tezel and Wax 2000; Tezel et al 2001; Tezel and Wax 2004; Nakazawa et al. 2006).

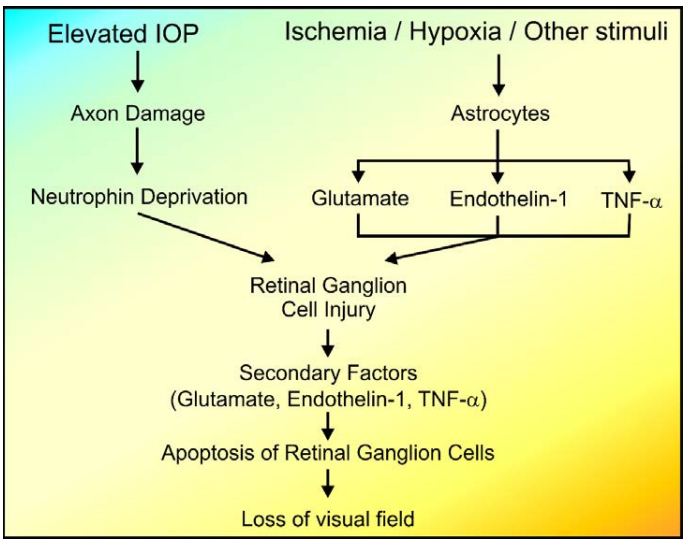

Figure 2. Factors contributing to neurodegeneration in glaucoma. Elevated intraocular pressure (IOP) is a well known risk factor contributing to axon loss and retinal ganglion cell death. Other factors including ischemia/hypoxia and lesser known glaucomatous stimuli have been hypothesized to contribute to release of other mediators including glutamate, endothelin-1 and TNF-α from astrocytes which produce degenerative effects on RGCs. Damage to RGCs could unleash secondary factors which contribute to apoptosis of RGCs

and subsequent loss of visual field (Courtesy: Mueller et al., Neuroprotection in Glaucoma. InTech Publisher 2011).

Hypoxia in neurodegenerative diseases including Alzheimer's disease and glaucoma

Hypoxia has been shown to be a contributor to central nervous system pathology in a variety of disorders including Parkinson's disease, and head trauma (Acker and Acker 2004; Correia et al., 2013). Numerous past studies have also implicated the role of ischemia and vascular factors in RGC cell death in glaucoma (Leske et al. 2008; Quigley et al. 2001). The optic nerve head is the site where unmyelinated axons of RGCs exit the eye. The unmyelinated axons require considerable trophic support which is provided by the short posterior ciliary artery system at the optic nerve head. Excavation of the optic nerve head in glaucoma is a consequence of loss of both nerve fibers and vasculature. An early study by Hayreh and Walker (1967) demonstrated an overall reduction in blood flow in the optic disc of glaucoma patients. Decreased ocular perfusion pressure has been suggested as a risk factor for the development of glaucoma, but the mechanism which leads to RGC death still remains unclear (Cherecheanu et al. 2013). Moreover, ischemia at the optic nerve head could contribute to the death of RGCs (Cherecheanu et al. 2013). Ergorul et al. (2010) showed that hypoxia-inducible factor-1α (HIF-1α), one of the key transcription factors activated by hypoxia, was elevated in the retina (specifically in Muller glia and astrocytes) following IOP elevation in rats. An increased immunostaing for HIF-1α was found in the retina and optic nerve heads of patients with glaucoma, compared to age matched control subjects, suggesting that hypoxia and hypoxia mediated changes in gene expression are associated with glaucomatous degeneration (Tezel and Wax, 2004).

Current treatments

Increased aqueous humour (AH) outflow resistance, increased extracellular matrix (ECM) deposition and decreased cellularity in the trabecular meshwork are contributors to elevation of IOP (Yorio and Clark 2003). Current treatment strategies for glaucoma in clinics are mainly based on lowering IOP with the help of IOP-lowering agents and glaucoma surgeries. The modes of action of these IOP-lowering drugs are through targeting the process responsible for AH formation and those that promote AH outflow (Yorio and Clark 2003). Drugs which are commonly used in clinics as an IOP-lowering drugs are Timolol, Brimonidine, Dorzolamide, Prostaglandin analogues and Pilocarpine as mentioned in Table1. Argon laser trabeculoplasty, a procedure used for IOP lowering in some glaucoma patients, stimulates trabecular meshwork cell MMP expression (Bradley et al.1998 and Wong et al. 2002). Filtering procedures, such as trabeculectomy and glaucoma drainage devices, are effective in lowering IOP. However, it has been associated with complications and high rates of failure (SooHoo et al., 2014). Minimally invasive glaucoma surgery (MIGS), a new group of surgical procedures has emerged as an additional approach to decrease IOP with lesser rates of complications (SooHoo et al., 2014).

Table 1: Commonly used drugs with classification according to their mechanism of action in glaucoma

Anterior Segment	xisting drugs
Aqueous humour outflow (conventional)	
Cholinergics	**Pilocarpine**
Aqueous humour outflow (uveoscleral)	
Prostaglandins	**PGF2α analogues, Latanoprost, Travoprost**

Aqueous humour formation	
β-Adrenergic blockers	**Timolol, Betaxolol**
α-Adrenergic blockers	**Apraclonidine, Brimonidine**
Carbonic anhydrase inhibitors	**Dorzolamide, Brinzolamide**

Neuroprotection in glaucoma

Glaucoma is a known as a heterogeneous group of multifactorial neurodegenerative diseases with different etiologies and clinical presentations. Presently, treatment modalities available for glaucoma are mainly the IOP-lowering drugs and preventing the primary insult for glaucomatous changes. It is been demonstrated that low levels of IOP is associated with a lesser visual field deterioration (AGIS 7 study).

http://www.ajo.com/article/S0002-9394%2800%2900538-9/abstract. By administration of IOP lowering drugs, IOP in glaucoma patients is often maintained in “normal” ranges, however, RGC death still continues albeit at a slow pace (Mueller II et al., 2011). Furthermore, patients with IOP between 6-10 mmHg (normal range 10-20mmHg) can still develop glaucoma (normal tension glaucoma), suggestive of predisposing risk factors other than elevation of IOP in patients with glaucoma. Secondary causes of RGC apoptosis may be contributing in the progression of glaucomatous diseases (Bahrami 2006). In this process, several molecular pathways converge to induce RGC death. However, the secondary factors contributing to continuous RGCs death after primary insult to the axons, are responsible for persisting optic nerve damage. Some of these factors include axonal transport failure resulting in neurotrophic factor deprivation, glial activation producing noxious agents including glutamate and endothelin-1. Neurotrophin deprivation could

activate the apoptotic cascade by activating pro-death members of the Bcl-2 family of proteins, some of which alter the mitochondrial outer membrane permeability leading cytochrome c release and activation of caspases. It would be beneficial to develop agents that can prevent both injured and uninjured RGCs from cell death during glaucoma. These neuroprotective agents can be used as an adjunct therapy to IOP lowering agents to provide better treatment options for treating glaucoma.

A number of studies have been conducted in past 30 years to identify various different neuroprotective agents for neuronal survival in neurodegenerative diseases including Alzheimer's and Parkinson's disease. However, most clinical studies to test for neuroprotective effects in humans failed to show efficacy (neuronal survival). Only three neuroprotective drugs have been shown to improve outcomes in human clinical trials: riluzole for amytrophic lateral sclerosis, memantine for moderate to severe Alzheimer's disease (Bensimon et al. 1994) , Lacomblez et al. 1996, Reisberg et al. 2003) and Brimonidine for low pressure glaucoma (Krupin et al. 2011). Memantine and riluzole have failed to have a dramatic effect on the progression of these neurological diseases (Danesh-Meyer and Levin 2009). Memantine and Brimonidine trial failed to demonstrate efficacy in two large multicenter clinical trials at sites worldwide (Allergan Inc Press Releases on Momantine Trials; Wilhelm et al. 2006). However, brimonidine treatment in patients suffering from low-pressure glaucoma, suggests that patients treated with brimonidine were less likely to have deterioration of visual fields compared to patients treated with timolol (Krupin et al.2011). Neuroprotection in glaucoma continues to be an evolving area of research with several candidate compounds currently being tested in animal models of the disease.

Cell culture and animal models of neurodegenerative diseases including glaucoma and Alzheimer's disease

The rat adrenal pheochromocytoma (PC12) cells is a commonly used cell line for studying the signaling pathways of cell survival, proliferation and differentiation of neuronal cells, and has resulted in large number of publications regarding these processes (Kao et al. 2001; Vaudry et al. 2002; D'Arcanglo et al. 1993). An interesting feature of PC12 cells is their capacity to grow neurite-like processes in response to nerve growth factor (NGF) (Greene et al. 1976). In the retina of rats with elevated IOP, RGCs loss was associated with downregulation of NGF and NGF receptor expression. In addition, ocular treatment with NGF decreased RGCs loss caused by glaucoma (Colafrancesco et al., 2011). Intraocular administration of NGF shown to inhibit RGC degeneration in diferent animal models of glaucoma (Carmignoto et al., 1989, Siliprandi et al., 1993, Lambiase et al., 1997). Another study showed that using NGF in eye drops can attenuate the optic nerve damage associated with glaucoma (Lambiase et al., 2009). NGF has low bioactive stability in the body due to its short half-lives and slow diffusion, limiting its use as a neuroprotective agent (Wang et al., 2014). PC12 cells can be passaged indefinitely and much easier to culture and manipulate than their neuronal counterparts, hence these cells have been useful as a model for the study of neurite outgrowth. Differentiated PC12 cells are most extensively used as a model for the study of neurite growth and growth cone function (Varnum-Finney et al. 1994; Aletta et al. 1998; Halloran et al. 1994). Apart from PC12 cells, human trabecular meshwork cell line and primary retinal ganglion cells from rats and mouse are also used to study changes in molecular and signaling pathways during glaucomatous conditions (Sethi et al. 2011, Mueller II et al. 2013).

Apart from cell culture models, *in vivo* models provide valuable information about the pathophysiology of glaucoma. The *in vivo* models include direct optic nerve injury either through optic nerve crush or through optic nerve transection, in which the optic nerve is either cut (transected) or crushed using jeweller's forceps (Heacock and Agranoff 1976; Benowitz et al.1981). This acute in vivo model causes significant RGC death after 1-2 weeks following the optic nerve injury which facilitates the empirical testing of drugs for neuroprotective actions (Danesh-Meyer et al.2011). In addition, the optic nerve crush model has also been useful to study other systemic factors conferring neuroprotection. As vascular insufficiency has been hypothesized a potential contributory factor in glaucomatous retinopathy, animal models of retinal ischemia/reperfusion have been used to dissect the changes and neuroprotective strategies in glaucoma. The most common method to induce retinal ischemia /reperfusion injury in rodents is ocular hypertension-induced retinal ischemia (Buchi et al.1991, Hughes et al.1991).

Elevation of IOP is achieved by ablating the outflow pathway in animal models, including monkey, rabbits, rats and mice (Pederson et al.1984; Zhu et al.1992; Morrison et al.1997 and Anderson et al.2002) among which rats and mice are most commonly used. Different methods are employed to elevate IOP in rats and produce degenerative effects in RGCs and optic nerve by producing damage in the trabecular meshwork leading to impairment of aqueous humour outflow. This includes cautery of two to three anterior ocular veins, injection of hypertonic saline through the AH-collecting veins and Canal of Schlemm, laser photocoagulation and laser cautery (Shareef et al.1995; Mittag et al.2000; Morrison et al. 2007; Ueda et al.1998; Wheeler et al. 2001; Levkovitch-Verbin et al.2002). For in vivo studies conducted in these models, IOP is elevated for a few weeks to months in order to monitor RGC survival andconcurrently different neurotrophic agents are administered. Another ocular hypertension model of glaucoma is provided by the DBA/2J mice

which harbor genetic mutations in the glycoprotein nmb-like protein (Gpnmb) and tyrosine-related protein 1b (Tyrp 1b) genes. (John et al. 1998). The phenotypic effects of the genetic mutations include iris atrophy, sloughing off the iris pigment which clogs the meshwork and leads to elevation of IOP and RGC loss (Anderson et al. 2002). Several studies have employed the DBA/2J (pigmentary dispersion glaucoma model) mice to understand neurodegenerative mechanism during IOP elevation in mice.

Elevated IOP induced by hypertonic saline injection into episceral veins causes pathological changes in trabecular meshwork, leading to an elevation of IOP and neurodegenerative changes in the rat retina and optic nerve similar to those observed in glaucoma. This experimental model produces a spectrum of IOP elevation, which provides an opportunity to evaluate a whole range of tissue damage correlated to IOP. RGCs apoptosis, axonal degeneration in the optic nerve, electroretinogram response (ERG) have been studied with this model and have been correlated with the severity of IOP elevation (Morrison et al. 1997; Minton et al. 2012; Stankowska et al. 2015).

Adeno-associated–viral vectors

Adeno-associated viruses (AAVs) are replication-defective viruses belonging to the Parvoviridae family that require co-infection with a helper virus (adeno virus or herpes virus) for productive infection. Recombinant AAV vectors can be produced in the laboratory without the use of the helper virus using commercially available kits (AAV Helper-Free System, Stratagene, LaJolla, CA). Eleven serotypes of AAV have the ability to infect cells from various tissues. Lack of pathogenicity and ability to effectively and stably transduce dividing and non-dividing cells, have made AAV-2 the vector of choice to deliver genes, particularly in the retina. Assessment of

transduction efficiency as well as tropism of various intravitreally injected AAV vectors indicated highest level of transduction efficiency by AAV-2 in RGCs (Harvey et al. 2009, Ju et al. 2010). Multiple strategies are being developed to promote survival of RGCs and stimulate axonal regeneration using AAV-2 vectors in different models of glaucoma including optic nerve crush, and Morrison's model of ocular hypertension (Leaver, Cui, Plant et al.2006, Yin et al. 2006, Sun et al.2011, Sharma et al., 2015).

POU domain transcription factors

The POU (Pit-Oct-Unc) domain family of transcription factors was defined initially on the basis of common region of approximately 150-160 amino acids consisting of four mammalian genes, Pit-1, Oct-1, Oct-2 and nematode factor Unc-86 (Verrijzer and van der Vliet 1993; Wegner et al. 1993; Ryan and Rosenfeld 1997). The structure of POU domain transcription factors consist of bipartite DNA-binding regions with a POU-specific domain of approximately 70 amino acids (POU_S) and a POU homeodomain of approximately 60 amino acids (POU_{HD}), separated by a variable linker (Herr et al. 1988) (Figure 3). The POU specific domain has high affinity for DNA binding and site specificity in protein-protein interactions (homodimeric and heterodimeric) as compared to POU homeodomain (Rosenfold 1991). POU-domian proteins appear to function as activators or repressors, and several POU domain regulators appear in early embryogenesis and during forebrain development (Figure 4). In addition, these factors play a critical role in development of specific cell types through the activation of gene expression necessary for the

differentiation into the specific cellular phenotypes (Rosenfold 1991).

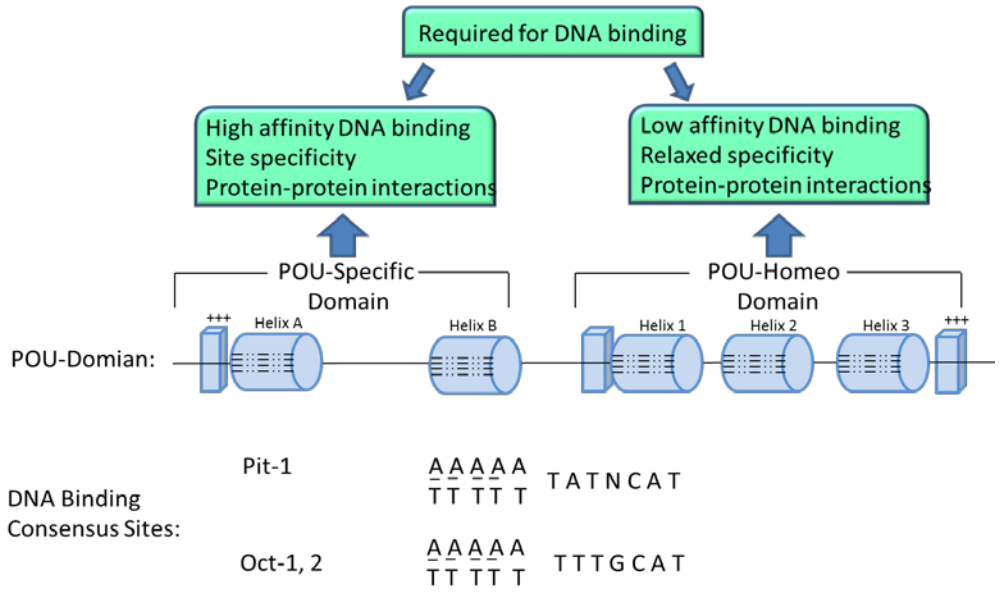

Figure 3: Schematic representation of the structure of POU domain transcription factors, indicating predicted helical domains, and highly conserved sequences. The known functions of POUs and POUHD are listed above. These proteins bind to heterogeneous sites containing A/T rich sequences; consensus sequences for Pit-1 and Oct-1 are shown in the figure.

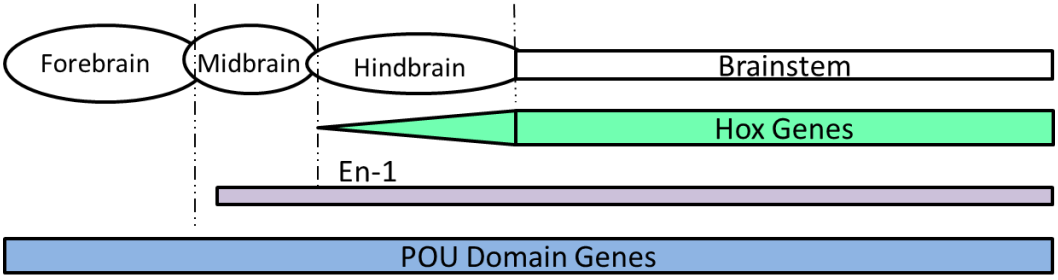

Figure 4: Specific POU domain proteins are expressed selectively throughout mammalian deveopment, in contrast to Hox genes, which are expressed in the hindbrain. Engrailed (en-1) homologs are detected in the midbrain.

Retina and Transcription factor Brn3b

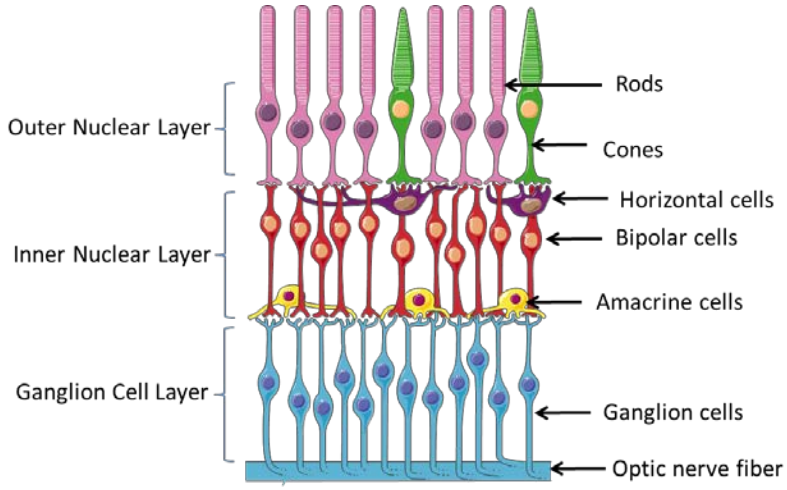

Figure 5: The six main classes of neurons in the vertebrate retina (rod and cone photoreceptor, bipolar cells, ganglion cells, amacrine cells, and horizontal cells) are organized into three distinct cellular layers, outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL) (The Human Eye, Structure and Function, Clyde W. Oyster, Sinauer Associates Inc.).

The vertebrate retina is a highly organized sensorineural (specialized) region of the central nervous system with number of distinct cell types. Moreover, retinal neurons are arranged in three layers: the outer nuclear layer contains the nuclei of photoreceptor cells; the inner nuclear layer contains the nuclei of horizontal, bipolar and amacrine cells; and the ganglion cell layer contains RGCs and displaced amacrine cells (Figure 5). Interestingly, each of these cells types can be further divided into subtypes that differ in their physiological and morphological characteristics, and, in case of RGCs, their pattern of central projections (Gan et al.1996). Visual information is conveyed from retina to the brain through 15-20 RGC types (Shi et al.2013). In order to understand RGC development with their diverse anatomical and physiological properties, studies have been

conducted to define the relevant genetic regulatory events (Cepko et al. 1996; Mu and Klein, 2004). Search of POU domain transcription factors in the mammalian retina led to identification of three closely related genes Brn3a (Brn3.0; *Pou4f1*), Brn3b (Brn3.2; *Pou4f2*) and Brn3c (Brn3.1; *Pou4f3*) (Gan et al.1999). The Brn3 genes show close resemblance in structure to the *C. elegans* factor unc-86 which is expressed in a diverse group of neurons and neuronal precursors and plays an important role in the development of subset of neuronal cells (Finney et al.1988; Chalfie et al. 1981; Finney et al.1990). Brn3 genes are expressed in newly formed projection neurons with a high degree of spatial and temporal overlap in the central nervous system (Turner et al.1994; Gerrero et al.1993; Wang et al. 2002; Xiang et al.1993; Xiang et al.1997, and Xiang et al.1998). Even though the Brn-3 gene is expressed exclusively within a subset of ganglion cells within the retina, gene deletion of each of three Brn3 genes produced different phenotypes that predominantly affect different subpopulations of neurons. Mice without Brn3a show impaired somatosensory and motor control due to abnormal trigeminal and dorsal root ganglia (McEvilly et al.1996, Xiang et al.1996a; Xiang et al.1996b). By contrast, Brn3b knockout mice manifest a loss of a large number (~ 70%) of RGCs, decrease in the number of optic nerve fibers leading to thinning of optic nerve (Gan et al.1996; Erkman et al.1996). Brn3c knockout mice have hearing and vestibular defects due to the degeneration of cochlear and vestibular hair cell (Erkman et al.1996; Xiang et al.1997a). No retinal defects are found in Brn3a and Brn3c knockout mice.

Past studies have shown a decrease in Brn3b expression prior to RGC death in different models of glaucoma (Naskar et al.2006; Soto et al.2008; Weishaupt et al.2005). Brn3b function has been examined solely in relation to its role in development, but the role of this protein in RGC survival has not been established. To address this, we investigated the role of transcription factor Brn3b in cultured neuronal cells as well as in retinal ganglion cells under glaucomatous conditions. In this

study, we demonstrate that overexpression of Brn3b in cultured neuronal cells and retinal ganglion cells leads to up-regulation of key markers of neuronal growth and survival under glaucomatous conditions respectively.

Growth associated protein-43 (GAP-43, B-50, F1 or neuromodulin)

GAP-43 is a protein that is concentrated in the growth cones (the motile growing tips of the axons) during development as well as regeneration. GAP-43 is used an index of synaptic plasticity and undergoes phosphorylation during long term potentiation (Pfenninger et al. 1991). GAP-43 expression is attenuated in Brn3b knockout mice as compared to wild type, suggesting a regulatory linkage between Brn3b and GAP-43 expression (Mu et al.2001).In the mid-1970s, F1 and B-50 were identified as synaptic phosphoproteins regulated by Ca^{2+} and various peptides as well as the major presynaptic substrate of protein kinase C (PKC) which undergo a persistent change in phosphorylation during long-term potentiation (Ehrlich et al.1974; Zwiers et al.1976; Aloyo et al.1983; Akers et al.1985). In the early 1980s, two groups described an acidic membrane protein which expression increases two fold during optic nerve regeneration in lower vertebrates (Skene et al.1989; Benowitz et al.1991). This protein, referred as GAP-43, a major constituent of the growth cone that is been also seen upregulated during the initial development of CNS projections in mammals and during peripheral nervous system (PNS) regeneration (Meiri et al.1986; Skene et al.1986; DeGraan et al. 1985). By the mid-1980s, biochemical and immunological evidence showed that F1, B-50, GAP-43 and phosphorylated protein of 46kDa (pp46) represented same protein. Studies from many labs indicate that GAP-43 plays a key role in guiding the growth of axons and modulating the formation of new connections in experiments with cell culture as well as in transgenic animals (Benowitz et al.1997). Moreover, other studies suggest that GAP-43 might

influence the growth state of the presynaptic terminal through association with cytoskeletal proteins like actin, α-actinin, talin and fodrin (Meiri et al.1990, Moss et al.1990).

Acetylated TUBA (ac-TUBA)

The cytoskeleton plays an important role in growth cone formation and synaptic plasticity. Microtubules consisting of alpha and beta tubulin dimers are an essential component of cytoskeleton required for axonal growth as well as transport. Acetylated TUBA is important for axon elongation as well as for anterograde and retrograde axonal transport in RGCs cultures and optic nerve injury model (Martin et al.2006; Fournier et al.1995; Sengottuvel et al.2011, Gaub et al.2010; Dompierre et al.2007). In glaucoma, injury at the optic nerve head and RGC axons causes a deprivation of trophic support to the RGCs, thereby producing neurodegenerative effects (Goldberg and Barres 2000). It is been proposed that this deprivation of trophic factors, together with loss of responsiveness to these stimuli, results in death of the axotomized RGCs (Goldberg and Barres 2000).

B-cell lymphoma-leukemia 2(Bcl-2) and Bcl-xL

Bcl-2 family proteins are key regulators of apoptosis, which include both anti- and pro-apoptotic proteins. Changes in the dynamic balance of these proteins may result either in cell survival or death (Ola et al.2011). This family comprises anti-apoptotic members (Bcl-2, Bcl-xL, Bcl-W, Mcl-1) which contain four Bcl-2 homology (BH) domains, and pro-apoptotic members, which have three BH domains (Bax, Bak and Bok) or a BH3 domain (Bid) (Youle et al.2008). Two distinct forms of Bcl-x are identified as Bcl-xL (233 amino acids) and Bcl-xS (170 amino acids: BH1 and BH2 are deleted by alternate spicing). Bcl-xL inhibits cell death whereas Bcl-xS facilitates it independent of heterodimerization with other Bcl-2 family members (Minn et al. 1996). Bcl-2 is

an integral membrane protein whereas Bcl-xL only becomes tightly associated with the membrane after injury signals, thereby preventing mitochondrial outer membrane permeabilization (MOMP) by neutralizing the activity of both pro-apoptotic members(Ola et al.2011; Yuan et al.2000). Dimerization is important for the function of Bcl-2; Bcl-2 can form homodimers to protect cells from death or can form heterodimers with BAX, preventing cell death and activate cell survival (Figure 6) (Oltval et al. 1993). In mammals, widespread expression of Bcl-2 in central nervous system (CNS) and peripheral nervous system (PNS) neurons during embryonic development and selective retention of Bcl-2 in the adult PNS is consistent with the role for Bcl-2 in regulating neuronal survival (Merry et al.1994 &Merry et al. 1997). Although the development of the nervous system in mice with Bcl-2 gene deletion is normal, there is subsequent loss of motor, sensory and sympathetic neurons after birth (Veis et al.1993, Michealidis et al.1996) suggesting that Bcl-2 is crucial for the maintenance of neuronal survival. Bcl-xL is expressed in developing brain, but unlike Bcl-2 expression, Bcl-xL expression continues to increase into adult life (Gonzalez-Garcia 1995). Bcl-xL null mice die around embryonic day 13 with massive cell death of immature hematopoietic cells and neurons (Motoyoma et al.1995). Cell death occurs primarily in immature neurons that have not established synaptic connections. Hence, Bcl-xL might be important for the survival of immature neurons before establishment of synaptic connections with their targets (Yuan et al.2000). Bcl-2 and Bcl-xL act by inhibiting pro-apoptotic members of the Bcl-2 family through heterodimerization (Merry et al.1997). Neuroprotective role of Bcl-2 and Bcl-xL in retinal ganglion cells has been studied in various optic injury models (15, 19, 17, 22, 24, 70, 72, 75, and 76).

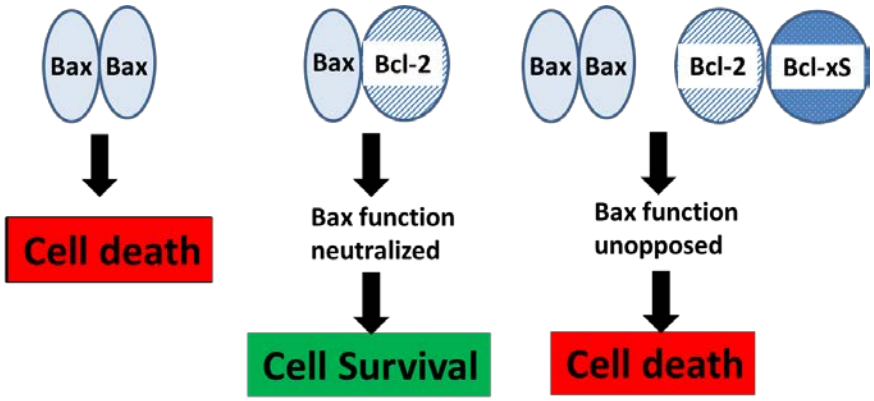

Figure 6: Proposed model of Bcl-2 for differential regulation of Bcl-2 family of proteins. Homodimeriaztion of Bax generates cell death (left), while heterodimrization of Bcl-2 and Bax abrogates BAX function (center). Bcl-xS binding to Bcl-2 prevents Bcl-2 from binding to and neutralization of Bax (right).

Phosphorylated AKT (p-AKT)

AKT also referred as PKB (RAC-PK) is homologous to the PKA and PKC families of protein kinases (Franke et al. 1997). The activity of AKT/PKB is regulated by serum and growth factors which activate PI3K in vivo (Andejelkovic et al. 1996; Burgering et al. 1995). Phosphatidylinositol (PI) 3-Kinase enzymes catalyze the formation of the lipid 3'-phosphorylated phosphoinositides, which regulate the localization and activity of a key component of cell survival, the Ser/Thr kinase AKT (Philpott et al.1997). The mammalian target of rapamycin (mTOR) regulates cell growth as well as proliferation through the raptor-mTOR (TORC1) and the other one rictor-mTOR (TORC2) protein complexes (Jacinto et al. 2006). Moreover, TORC2 is responsible for AKT phosphorylation at Ser473 site and AKT-Ser473 phosphorylation is required for TORC2 function in cell survival (Jacinto et al. 2006). In different models of glaucoma, it is been shown that activated AKT has a neuroprotective effect on RGCs (Nakazawa et al., 2002).

Specific aims:

Brn3b is necessary during development for normal RGC differentiation, polarity, axonal outgrowth and survival (Gan et al.1996 and 1999, Erkman et al. 2000 and 1996, Wang et al. 2000). However, the detailed role of transcription factor Brn-3b and genes influenced by Brn3b in adult RGCs remains to be elucidated.

The hypothesis to be tested is that overexpression of transcription factor Brn3b potentiates neurite growth and upregulates gene expression contributing to neurite outgrowth in cultured neuronal cells. The following specific aims will address this hypothesis:

Specific aim 1: To determine whether overexpression of Brn3b causes increased expression of neurite growth markers and Bcl-2 *in vitro* in PC12 cells

1.1 Immunoblot experiments will be performed to determine whether overexpression of Brn3b leads to increased expression of GAP-43(Growth associated protein), ac-TUBA (acetylated TUBA) and Bcl-2, in PC12 cells transfected with either Brn3b or Empty vector.

1.2 Immunocytochemical analysis of GAP-43, Bcl-2 and ac-TUBA will be carried out in PC12 cells overexpressing Brn3b

Specific aim 2: To determine if the neurite outgrowth promoting ability of Brn3b *in vitro* in PC12 cells is sustained under conditions of hypoxia.

2.1 Immunoblot analysis of GAP-43 and ac-TUBA will be performed in PC12 cells overexpressing Brn3b under conditions of hypoxia

2.2 Immunocytochemical analysis of GAP-43 and ac-TUBA will be carried out in PC12 cells overexpressing Brn3b under hypoxic conditions.

Specific aim 3: To determine whether Brn3b overexpression leads to changes in the expression of B-cell leukemia/lymphoma-2 (Bcl-2) family proteins in retinal ganglion cells (RGCs) and also in RGCs after IOP elevation *in vivo*

3.1 Expression of pro-survival factors (Bcl-2, Bcl-X_L) and pro-apoptotic factors (BAX and BIM) will be assessed in protein extracts from rat retinas and optic nerve overexpressing Brn3b.

3.2 Immunohistochemical analysis of Bcl-2, Bcl-X_L, BAX and BIM will be carried out in rat retinas and optic nerve overexpressing Brn3b.

3.3 Determine by immunohistochemistry if there is are changes in the Bcl-2 family of proteins in retinas of rats subjected to IOP elevation followed by Brn3b overexpression using the AAV-Brn3b construct

The first specific aim was directed at understanding the ability of Brn3b overexpression to produce an increase in key markers of neurite out growth.

Specific aim 1: To determine whether overexpression of Brn3b causes increased expression of neurite growth markers and Bcl-2 *in vitro* in PC12 cells

Hypothesis: Overexpression of Brn3b leads to up-regulation of markers of neurite outgrowth including GAP-43, ac-TUBA and Bcl-2 in PC12 cells.

Rationale:

Past studies have shown that Brn3b is a class IV POU domain transcription factor, and it is mainly expressed during early embryogenesis in germ-line cells, suggestive of its function in early development. Brn3b is an early marker of RGC differentiation and axon path finding. This transcription factor is also essential for organization of cytoskeleton and normal cell polarity of RGCs (Mu X et al.2004; Wang et al.2000).

Microarray analysis of Brn3b deficient mice retinas at E14.5 showed that expression of 87 genes was substantially affected by the loss of Brn3b. These genes belongs from different sets that encoded transcription factors, proteins associated with neuron integrity and function, and secreted signaling molecules including GAP-43 (Mu et al., 2004). GAP-43 is required for proper RGC neurite growth and pathfinding (Gan et al.1999). Acetylated α-tubulin (ac-TUBA) has been linked with neurite elongation. It provides stability and transport across the neurites (Fournier et al.1995; Gaub et al.2010). Bcl-2 has been well characterized for its anti-apoptotic role in various neuronal cell types. Recent studies have indicate its ability to promote axonal regeneration following optic nerve injury (Chao et al., 2005).

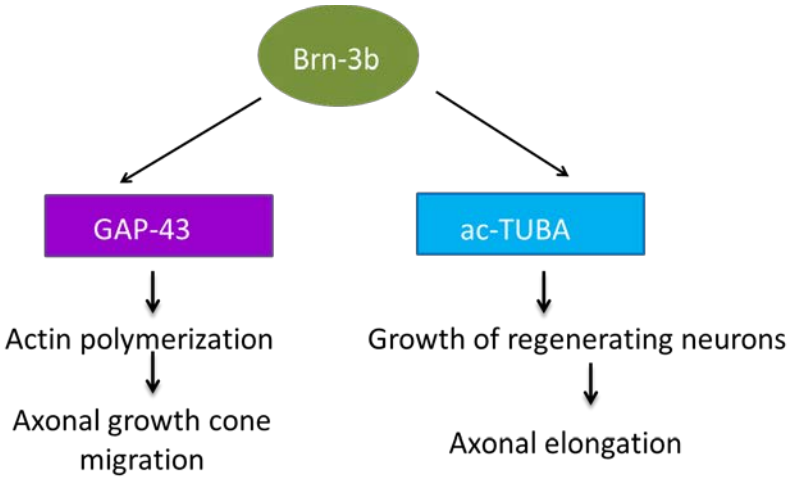

Figure 7: Proposed model for Brn3b mediated upregulation of neurite outgrowth markers, GAP-43 and ac-TUBA.

Rat pheochromocytoma (PC12) cells have been widely used in neurobiological studies as a model of neuronal differentiation and neurite outgrowth in response to nerve growth factor (NGF) (Kao et al., 2001; Varnum-Finney et al., 1994; Vaudry et al., 2002).

Specific aim 2: To determine if the neurite outgrowth promoting ability of Brn3b in vitro in PC12 cells is sustained under conditions of hypoxia

Hypothesis: Brn3b enhances neurite outgrowth in PC12 cells transfected with pCMV6-Brn3b in normoxia as well as hypoxia.

Rationale: The structural and functional integrity of brain and retina greatly depends on a regular oxygen and glucose supply. Circulatory disturbances are thought to contribute to the pathology of the optic nerve head during glaucomatous neurodegeneration. Disruption in blood circulation of retina and optic nerve result in various ocular and optic nerve head disorders, causing varying degrees of visual loss in glaucomatous optic neuropathy and anterior ischemic optic neuropathy.

The current study will determine if Brn3b has a capability to potentiate neurite growth markers under conditions of hypoxia as well as under normoxic conditions. Hypoxia induces neurite outgrowth in PC12 cells which is mediated through adenosine A2A receptor and NGF signaling pathway (O'Driscoll et al., 2005).

SPECIFIC AIM 3: To determine whether Brn3b overexpression leads to changes in the expression of B-cell leukemia/lymphoma-2 (Bcl-2) family proteins in retinal ganglion cells (RGCs) and RGCs after intraocular pressure (IOP) elevation *in vivo*

Hypothesis: Brn3b overexpression in retinal ganglion cells (RGCs) leads to increase in the expression of pro-survival factors and decrease in the expression of pro-apoptosis factors of Bcl-2 family proteins.

Rationale:

During the development of the nervous system, programmed cell death is a common physiological phenomenon that can be modulated by varying the expression of the Bcl-2 family of proteins (Cellerino et al.1999). The survival of specific groups of neurons during embryonic development and postnatal remodeling of the nervous system is thought to be regulated by presence of neurotrophic factors. Moreover, in the absence of these trophic signals, neurons die by apoptotic programmed cell death (Oppenheim et al. 1991; Raff et al. 1993). Interestingly, a number of genes have been shown to coordinately regulate apoptosis in mammalian cells, with several lines of evidence implicating the members of the Bcl-2 family as regulators of neuronal survival (Allsopp et al. 1993). Moreover, Since Brn3b was found produce neurite outgrowth in different

cell types; it would be interesting to determine if some of these effects were associated with changes in levels of Bcl-2 and members of the Bcl-2 family of proteins.

Significance: Most of the current knowledge on the role of Brn3b in the retina stems from studies investigating the Brn3 family of transcription factors during retinal development. Investigation on Brn3b's transcriptional activation or repression capabilities is an area of research that has not been adequately addressed. There is a paucity of information regarding the 'normal' role of transcription factor, Brn3b, in adult retinas, as well as its putative role in neuroprotection of RGCs following neurodegeneration. Results from this study will help to determine if Brn3b contributes to neurite outgrowth both during normoxia and also during conditions of hypoxia in neuronal cells. Some of the studies described in this proposal will also test the ability of Brn3b to regulate expression of proteins involved in promoting neurite outgrowth in cultured neuronal cells. Studies outlined will also provide insight into the cellular mechanisms associated with Brn3b mediated neuroprotection. This basic information gained from these studies could suggest the possibility of using AAV-Brn3b as potential neuroprotective agent for treatment of glaucoma.

References

Acker T, Acker H. Cellular oxygen sensing need in CNS function: physiological and pathological implications J Exp Biol 2004 Aug; 207(Pt 18):3171-3188.

Agapova OA, Ricard CS, Salvador-Silva M, Hernandez MR. Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human optic nerve head astrocytes Glia 2001 Mar 1;33(3):205-216.

The advanced glaucoma interventionstudy (AGIS): 7. the relationship between control of intraocular pressure and visual field deterioration Am J Ophthalmol 2000;130(4):429 440.

Akers RF, Routtenberg A. Protein kinase C phosphorylates a 47 Mr protein (F1) directly related to synaptic plasticity Brain Res 1985 May 13;334(1):147-151.

Allergan Inc Press Releases on Momantine Trials, Fourth Quarter Operating Results, Jan 30 2008 http://www.allergan.com/newsroom/index.htm

Aletta JM, Greene LA. Growth cone configuration and advance: a time-lapse study using video-enhanced differential interference contrast microscopy J Neurosci 1988 Apr;8(4):1425-1435.

Allsopp TE, Wyatt S, Paterson HF, Davies AM. The proto-oncogene bcl-2 can selectively rescue neurotrophic factor-dependent neurons from apoptosis Cell 1993 Apr 23;73(2):295-307.

Aloyo VJ, Zwiers H, Gispen WH. Phosphorylation of B-50 protein by calcium-activated, phospholipid-dependent protein kinase and B-50 protein kinase J Neurochem 1983 Sep;41(3):649-653.

Anderson MG, Smith RS, Hawes NL, Zabaleta A, Chang B, Wiggs JL, et al. Mutations in genes encoding melanosomal proteins cause pigmentary glaucoma in DBA/2J mice Nat Genet 2002 Jan;30(1):81-85.

Andjelkovic M, Jakubowicz T, Cron P, Ming XF, Han JW, Hemmings BA. Activation and phosphorylation of a pleckstrin homology domain containing protein kinase (RAC-PK/PKB) promoted by serum and protein phosphatase inhibitors Proc Natl Acad Sci U S A 1996 Jun 11;93(12):5699-5704.

Armaly MF, Krueger DE, Maunder L, Becker B, Hetherington J,Jr, Kolker AE, et al. Biostatistical analysis of the collaborative glaucoma study. I. Summary report of the risk factors for glaucomatous visual-field defects Arch Ophthalmol 1980 Dec;98(12):2163-2171.

Bahrami H. Causal models in primary open angle glaucoma. Ophthalmic Epidemiol 2006 4:191-298.

Benowitz LI, Perrone-Bizzozero NI. The expression of GAP-43 in relation to neuronal growth and plasticity: when, where, how, and why? Prog Brain Res 1991;89:69-87.

Benowitz LI, Routtenberg A. GAP-43: an intrinsic determinant of neuronal development and plasticity Trends Neurosci 1997 Feb;20(2):84-91.

Bensimon G, Lacomblez L, Meininger V. A controlled trial of riluzole in amyotrophic lateral sclerosis: ALS Riluzole study group, N Engl J Med 1994 330:585-591.

Bonfanti L, Strettoi E, Chierzi S, Cenni MC, Liu XH, Martinou J-, et al. Protection of retinal ganglion cells from natural and axotomy-induced cell death in neonatal transgenic mice overexpressing bcl-2 J Neurosci 1996 Jul 1;16(13):4186-4194.

Bradley JM, Vranka J, Colvis CM, Conger DM, Alexander JP, Fisk AS, et al. Effect of matrix metalloproteinases activity on outflow in perfused human organ culture Invest Ophthalmol Vis Sci 1998 Dec;39(13):2649-2658.

Burne JF, Staple JK, Raff MC. Glial cells are increased proportionally in transgenic optic nerves with increased numbers of axons J Neurosci 1996 Mar 15;16(6):2064-2073.

Burgering BM, Coffer PJ. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction Nature 1995 Aug 17;376(6541):599-602.

Carmignoto G, Maffei L, Candeo P, Canella R, Comelli C. Effect of NGF on the survival of rat retinal ganglion cells following optic nerve section J Neurosci 1989 Apr;9(4):1263-1272.

Cellerino A, Michaelidis T, Barski JJ, Bahr M, Thoenen H, Meyer M. Retinal ganglion cell loss after the period of naturally occurring cell death in bcl-2-/- mice Neuroreport 1999 Apr 6;10(5):1091-1095.

Cenni MC, Bonfanti L, Martinou JC, Ratto GM, Strettoi E, Maffei L. Long-term survival of retinal ganglion cells following optic nerve section in adult bcl-2 transgenic mice Eur J Neurosci 1996 Aug;8(8):1735-1745.

Chalfie M, Horvitz HR, Sulston JE. Mutations that lead to reiterations in the cell lineages of C. elegans Cell 1981 Apr;24(1):59-69.

Chaudhary P, Ahmed F, Quebada P, Sharma SC. Caspase inhibitors block the retinal ganglion cell death following optic nerve transection Brain Res Mol Brain Res 1999 Apr 6;67(1):36-45.

Chen DF, Schneider GE, Martinou JC, Tonegawa S. Bcl-2 promotes regeneration of severed axons in mammalian CNS Nature 1997 Jan 30;385(6615):434-439.

Cherecheanu AP, Garhofer G, Schmidl D, Werkmeister R, Schmetterer L. Ocular perfusion pressure and ocular blood flow in glaucoma Curr Opin Pharmacol 2013 Feb;13(1):36-42.

Chierzi S, Strettoi E, Cenni MC, Maffei L. Optic nerve crush: axonal responses in wild-type and bcl-2 transgenic mice J Neurosci 1999 Oct 1;19(19):8367-8376.

Clark AF, Yorio T. Ophthalmic drug discovery Nat Rev Drug Discov 2003 Jun;2(6):448-459.

Colafrancesco V, Parisi V, Sposato V, Rossi S, Russo MA, Coassin M, et al. Ocular application of nerve growth factor protects degenerating retinal ganglion cells in a rat model of glaucoma J Glaucoma 2011 Feb;20(2):100-108.

Comparison of glaucomatous progression between untreated patients with normal-tension glaucoma and patients with therapeutically reduced intraocular pressures. Collaborative Normal-Tension Glaucoma Study Group. Am J Ophthalmol. 1998 Oct;126(4):487-97. Erratum in: Am J Ophthalmol 1999 Jan;127(1):120.

Cordeiro MF. Beyond Mitomycin: TGF-beta and wound healing Prog Retin Eye Res 2002 Jan;21(1):75-89.

Correia SC, Carvalho C, Cardoso S, Santos RX, Placido AI, Candeias E, et al. Defective HIF signaling pathway and brain response to hypoxia in neurodegenerative diseases: not an "iffy" question! Curr Pharm Des 2013;19(38):6809-6822.

Cowley S, Paterson H, Kemp P, Marshall CJ. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells Cell 1994 Jun 17;77(6):841-852.

Comparison of glaucomatous progression between untreated patients with normal-tension glaucoma and patients with therapeutically reduced intraocular pressures. Collaborative Normal-

Tension Glaucoma Study Group. Am J Ophthalmol. 1998 Oct;126(4):487-97. Erratum in: Am J Ophthalmol 1999 Jan;127(1):120.

Danesh-Meyer HV. Neuroprotection in glaucoma: recent and future directions, Current Opinion in Ophthalmology 2011 22:78-86.

D'Arcangelo G, Halegoua S. A branched signaling pathway for nerve growth factor is revealed by Src-, Ras-, and Raf-mediated gene inductions Mol Cell Biol 1993 Jun;13(6):3146-3155.

de Graan PN, van Hooff CO, Tilly BC, Oestreicher AB, Schotman P, Gispen WH. Phosphoprotein B-50 in nerve growth cones from fetal rat brain Neurosci Lett 1985 Nov 11;61(3):235-241.

Dielemans I, Vingerling JR, Wolfs RC, Hofman A, Grobbee DE, de Jong PT. The prevalence of primary open-angle glaucoma in a population-based study in The Netherlands. The Rotterdam Study Ophthalmology 1994 Nov;101(11):1851-1855.

Dompierre JP, Godin JD, Charrin BC, Cordelieres FP, King SJ, Humbert S, et al. Histone deacetylase 6 inhibition compensates for the transport deficit in Huntington's disease by increasing tubulin acetylation J Neurosci 2007 Mar 28;27(13):3571-3583.

Ehrlich YH, Routtenberg A. Cyclic AMP regulates phosphorylation of three protein components of rat cerebral cortex membranes for thirty minutes FEBS Lett 1974 Sep 1;45(1):237-243.

Ergorul C, Ray A, Huang W, Wang DY, Ben Y, Cantuti-Castelvetri I, et al. Hypoxia inducible factor-1alpha (HIF-1alpha) and some HIF-1 target genes are elevated in experimental glaucoma J Mol Neurosci 2010 Oct;42(2):183-191.

Erkman L, McEvilly RJ, Luo L, Ryan AK, Hooshmand F, O'Connell SM, et al. Role of transcription factors Brn-3.1 and Brn-3.2 in auditory and visual system development Nature 1996 Jun 13;381(6583):603-606.

Finney M, Ruvkun G. The unc-86 gene product couples cell lineage and cell identity in C. elegans Cell 1990 Nov 30;63(5):895-905.

Finney M, Ruvkun G, Horvitz HR. The C. elegans cell lineage and differentiation gene unc-86 encodes a protein with a homeodomain and extended similarity to transcription factors Cell 1988 Dec 2;55(5):757-769.

Fournier AE, McKerracher L. Tubulin expression and axonal transport in injured and regenerating neurons in the adult mammalian central nervous system Biochem Cell Biol 1995 Sep-Oct;73(9-10):659-664.

Franke TF, Kaplan DR, Cantley LC. PI3K: downstream AKTion blocks apoptosis Cell 1997 Feb 21;88(4):435-437.

Friedman DS, Wolfs RC, O'Colmain BJ, Klein BE, Taylor HR, West S, et al. Prevalence of open-angle glaucoma among adults in the United States Arch Ophthalmol 2004 Apr;122(4):532-538.

Gan L. Xiang M, Zhou L, Wagner DS, Klein WH, Nathans J. POU domain factor Brn-3b is required for the development of a large set of ganglion cells Proc Natl Acad Sci USA 1996 Apr 30;93(9):3920-3925.

Gan L, Wang SW, Hunag Z, Klein WH. POU domain factor Brn-3b is essential for retinal ganglion cells differentiation and survival but not for initial cell fate specification Dev Biol 1999 Jun 15;210(2):469-480.

Gaub P, Tedeschi A, Puttagunta R, Nguyen T, Schmandke A, Di Giovanni S. HDAC inhibition promotes neuronal outgrowth and counteracts growth cone collapse through CBP/p300 and P/CAF-dependent p53 acetylation Cell Death Differ 2010 Sep;17(9):1392-1408.

Gerrero MR, McEvilly RJ, Turner E, Lin CR, O'Connell S, Jenne KJ, et al. Brn-3.0: a POU-domain protein expressed in the sensory, immune, and endocrine systems that functions on elements distinct from known octamer motifs Proc Natl Acad Sci U S A 1993 Nov 15;90(22):10841-10845.

Goldberg JL and Barres BA. The relationship between neuronal survival and regeneration. Annu. Rev. Neurosci. 2000;23, 579-612.

Gonzalez-Garcia M, Garcia I, Ding L, O'Shea S, Boise LH, Thompson CB, et al. Bcl-X is Expressed in Embryonic and Postnatal Neural Tissues and Functions to Prevent Neuronal Cell Death Proc Natl Acad Sci U S A 1995 May 9;92(10):4304-4308.

Gordon MO, Beiser JA, Brandt JD, Heuer DK, Higginbotham EJ, Johnson CA, et al. The Ocular Hypertension Treatment Study: baseline factors that predict the onset of primary open-angle glaucoma Arch Ophthalmol 2002 Jun;120(6):714-20; discussion 829-30.

Greene LA, Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor Proc Natl Acad Sci U S A 1976 Jul;73(7):2424-2428.

Guo L, Moss SE, Alexander RA, Ali RR, Fitzke FW, Cordeiro MF. Retinal ganglion cell apoptosis in glaucoma is related to intraocular pressure and IOP-induced effects on extracellular matrix Invest Ophthalmol Vis Sci 2005 Jan;46(1):175-182.

Halloran MC, Kalil K. Dynamic behaviors of growth cones extending in the corpus callosum of living cortical brain slices observed with video microscopy J Neurosci 1994 Apr;14(4):2161-2177.

Harvey AR, Hellstrom M, Rodger J. Gene therapy and transplantation in the retinofugal pathway Prog Brain Res 2009;175:151-161.

Hayreh SS, Walker WM. Fluorescent fundus photography in glaucoma Am J Ophthalmol 1967 May;63(5):982-989.

Heijl A, Leske MC, Bengtsson B, Hyman L, Bengtsson B, Hussein M, et al. Reduction of intraocular pressure and glaucoma progression: results from the Early Manifest Glaucoma Trial Arch Ophthalmol 2002 Oct;120(10):1268-1279.

Hernandez MR. The optic nerve head in glaucoma: role of astrocytes in tissue remodeling Prog Retin Eye Res 2000 May;19(3):297-321.

Herr W, Sturm RA, Clerc RG, Corcoran LM, Baltimore D, Sharp PA, et al. The POU domain: a large conserved region in the mammalian pit-1, oct-1, oct-2, and Caenorhabditis elegans unc-86 gene products Genes Dev 1988 Dec;2(12A):1513-1516.

Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, Jung SY, et al. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity Cell 2006 Oct 6;127(1):125-137.

John SW, Smith RS, Savinova OV, Hawes NL, Chang B, Turnbull D, et al. Essential iris atrophy, pigment dispersion, and glaucoma in DBA/2J mice Invest Ophthalmol Vis Sci 1998 May;39(6):951-962.

Johnson EC, Morrison JC, Farrell S, Deppmeier L, Moore CG, McGinty MR. The effect of chronically elevated intraocular pressure on the rat optic nerve head extracellular matrix Exp Eye Res 1996 Jun;62(6):663-674.

Kao S, Jaiswal RK, Kolch W, Landreth GE. Identification of the mechanisms regulating the differential activation of the mapk cascade by epidermal growth factor and nerve growth factor in PC12 cells J Biol Chem 2001 May 2001 May 25;276(21):18169-18177.

Kim E, Varma R. Glaucoma in Latinos/Hispanics Curr Opin Ophthalmol 2010 Mar;21(2):100-105.

Klein BE, Klein R, Sponsel WE, Franke T, Cantor LB, Martone J, et al. Prevalence of glaucoma. The Beaver Dam Eye Study Ophthalmology 1992 Oct;99(10):1499-1504.

Kolker AE. Glaucoma family study. Ten-year follow-up (preliminary report) Isr J Med Sci 1972 Aug-Sep;8(8):1357-1361.

Kong GY, Van Bergen NJ, Trounce IA, Crowston JG. Mitochondrial dysfunction and glaucoma J Glaucoma 2009 Feb;18(2):93-100.

Kretz A, Kugler S, Happold C, Bahr M, Isenmann S. Excess Bcl-XL increases the intrinsic growth potential of adult CNS neurons in vitro Mol Cell Neurosci 2004 May;26(1):63-74.

Krupin T, Leibhmann JM, Greenfield DS, Ritch R, Gardnier S. Low-Pressure Glaucoma Study Group. A randomized trial of brimonidine versus timolol in preserving visual function: results from the Low-Pressure Glaucoma Treatment Study. Am J Ophthalmol 2011 151(4):671-81.

Krishnamoorthy RR, Crawford MJ, Chaturvedi MM, Jain SK, Aggarwal BB, Al-Ubaidi MR, et al. Photo-oxidative stress down-modulates the activity of nuclear factor-kappaB via involvement of caspase-1, leading to apoptosis of photoreceptor cells J Biol Chem 1999 Feb 5;274(6):3734-3743.

Lacomblez L, Bensimon G, Leigh PN, Gulliet P, Powe L, Durrleman S, Delumeau JC, Meininger V. A confirmatory dose-ranging study of riluzole in ALS: ALS/Riluzole Study Group-II, Neurology 1996 47 (6 Suppl 4):S242-50.

Lambiase A, Centofanti M, Micera A, Manni GL, Mattei E, De Gregorio A, et al. Nerve growth factor (NGF) reduces and NGF antibody exacerbates retinal damage induced in rabbit by experimental ocular hypertension Graefes Arch Clin Exp Ophthalmol 1997 Dec;235(12):780-785.

Lambiase A, Aloe L, Centofanti M, Parisi V, Bao SN, Mantelli F, et al. Experimental and clinical evidence of neuroprotection by nerve growth factor eye drops: Implications for glaucoma Proc Natl Acad Sci U S A 2009 Aug 11;106(32):13469-13474.

Law SK. First-line treatment for elevated intraocular pressure (IOP) associated with open-angle glaucoma or ocular hypertension: focus on bimatoprost Clin Ophthalmol 2007 Sep;1(3):225-232.

Leaver SG, Cui Q, Bernard O, Harvey AR. Cooperative effects of Bcl-2 and AAV-mediated expression of CNTF on retinal ganglion cell survival and axonal regeneration in adult transgenic mice. Eur J Neurosci. 2006 Dec; 24(12):3323-32.

Leske MC, Wu SY, Hennis A, Honkanen R, Nemesure B, BESs Study Group. Risk factors for incident open-angle glaucoma: the Barbados Eye Studies Ophthalmology 2008 Jan;115(1):85-93.

Levin LA, Schlamp CL, Spieldoch RL, Geszvain KM, Nickells RW. Identification of the bcl-2 family of genes in the rat retina Invest Ophthalmol Vis Sci 1997 Nov;38(12):2545-2553.

Levkovitch-Verbin H, Quigley HA, Martin KR, Valenta D, Baumrind LA, Pease ME. Translimbal laser photocoagulation to the trabecular meshwork as a model of glaucoma in rats Invest Ophthalmol Vis Sci 2002 Feb;43(2):402-410.

Liu XA, Rizzo V, Puthanveettil SV. Pathologies of axonal transport in neurodegenerative diseases Transl Neurosci 2012 Dec 1; 3(4): 355-372.

Liu XH, Collier RJ, Youle RJ. Inhibition of axotomy-induced neuronal apoptosis by extracellular delivery of a Bcl-XL fusion protein J Biol Chem 2001 Dec 7;276(49):46326-46332.

Malik JM, Shevtsova Z, Bahr M, Kugler S. Long-term in vivo inhibition of CNS neurodegeneration by Bcl-XL gene transfer Mol Ther 2005 Mar;11(3):373-381.

Martin KR, Quigley HA, Valenta D, Kielczewski J, Pease ME. Optic nerve dynein motor protein distribution changes with intraocular pressure elevation in a rat model of glaucoma Exp Eye Res 2006 Aug;83(2):255-262.

McEvilly RJ, Erkman L, Luo L, Sawchenko PE, Ryan AF, Rosenfeld MG. Requirement for Brn-3.0 in differentiation and survival of sensory and motor neurons Nature 1996 Dec 12;384(6609):574-577.

Meiri KF, Gordon-Weeks PR. GAP-43 in growth cones is associated with areas of membrane that are tightly bound to substrate and is a component of a membrane skeleton subcellular fraction J Neurosci 1990 Jan;10(1):256-266.

Meiri KF, Pfenninger KH, Willard MB. Growth-associated protein, GAP-43, a polypeptide that is induced when neurons extend axons, is a component of growth cones and corresponds to pp46, a major polypeptide of a subcellular fraction enriched in growth cones Proc Natl Acad Sci U S A 1986 May;83(10):3537-3541.

Merry DE, Korsmeyer SJ. Bcl-2 gene family in the nervous system Annu Rev Neurosci 1997;20:245-267.

Merry DE, Veis DJ, Hickey WF, Korsmeyer SJ. bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS Development 1994 Feb;120(2):301-311.

Michaelidis TM, Sendtner M, Cooper JD, Airaksinen MS, Holtmann B, Meyer M, et al. Inactivation of bcl-2 results in progressive degeneration of motoneurons, sympathetic and sensory neurons during early postnatal development Neuron 1996 Jul;17(1):75-89.

Minn j, Boise LH, Thompson CB. Bcl-x(S) antagonizes the protective effects of Bcl-x(L). J Biol Chem. 1996;271:6306-6312.

Minton AZ, Phatak NR, Stankowska DL, He S, Ma HY, Mueller BH, et al. Endothelin B receptors contribute to retinal ganglion cell loss in a rat model of glaucoma PLoS One 2012;7(8):e43199.

Mitchell P, Smith W, Attebo K, Healey PR. Prevalence of open-angle glaucoma in Australia. The Blue Mountains Eye Study Ophthalmology 1996 Oct;103(10):1661-1669.

Mittag TW, Danias J, Pohorenec G, Yuan HM, Burakgazi E, Chalmers-Redman R, et al. Retinal damage after 3 to 4 months of elevated intraocular pressure in a rat glaucoma model Invest Ophthalmol Vis Sci 2000 Oct;41(11):3451-3459.

Morrison JC, Moore CG, Deppmeier LM, Gold BG, Meshul CK, Johnson EC. A rat model of chronic pressure-induced optic nerve damage Exp Eye Res 1997 Jan;64(1):85-96.

Moss DJ, Fernyhough P, Chapman K, Baizer L, Bray D, Allsopp T. Chicken growth-associated protein GAP-43 is tightly bound to the actin-rich neuronal membrane skeleton J Neurochem 1990 Mar;54(3):729-736.

Motoyama N, Wang F, Roth KA, Sawa H, Nakayama K, Nakayama K, et al. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice Science 1995 Mar 10;267(5203):1506-1510.

Mu X, Beremand PD, Zhao S, Pershad R, Sun H, Scarpa A, et al. Discrete gene sets depend on POU domain transcription factor Brn3b/Brn-3.2/POU4f2 for their expression in the mouse embryonic retina Development 2004 Mar;131(6):1197-1210.

Mu X, Zhao S, Pershad R, Hsieh TF, Scarpa A, Wang SW, et al. Gene expression in the developing mouse retina by EST sequencing and microarray analysis Nucleic Acids Res 2001 Dec 15;29(24):4983-4993.

Mu X, Klein WH. (2004) A gene regulatory hierarchy for retinal ganglion cell specification and differentiation. Semin Cell Dev Biol. 15(1):115-23. Review.

Mueller BH,2nd, Park Y, Daudt DR,3rd, Ma HY, Akopova I, Stankowska DL, et al. Sigma-1 receptor stimulation attenuates calcium influx through activated L-type Voltage Gated Calcium Channels in purified retinal ganglion cells Exp Eye Res 2013 Feb;107:21-31.

Nakazawa T, Nakazawa C, Matsubara A, Noda K, Hisatomi T, She H, et al. Tumor necrosis factor-alpha mediates oligodendrocyte death and delayed retinal ganglion cell loss in a mouse model of glaucoma J Neurosci 2006 Dec 6;26(49):12633-12641.

Nakazawa T, Shimura M, Tomita H, Akiyama H, Yoshioka Y, Kudou H, et al. Intrinsic activation of PI3K/Akt signaling pathway and its neuroprotective effect against retinal injury Curr Eye Res 2003 Jan;26(1):55-63.

Nakazawa T, Tamai M, Mori N. Brain-derived neurotrophic factor prevents axotomized retinal ganglion cell death through MAPK and PI3K signaling pathways Invest Ophthalmol Vis Sci 2002 Oct;43(10):3319-3326.

Naskar R, Thanos S. Retinal gene profiling in a hereditary rodent model of elevated intraocular pressure Mol Vis 2006 Oct 18;12:1199-1210.

Nemesure B, Honkanen R, Hennis A, Wu SY, Leske MC, Barbados Eye Studies Group. Incident open-angle glaucoma and intraocular pressure Ophthalmology 2007 Oct;114(10):1810-1815.

Nishimura T, Ishima T, Iyo M, Hashimoto K. Potentiation of nerve growth factor-induced neurite outgrowth by fluvoxamine: role of sigma-1 receptors, IP3 receptors and cellular signaling pathways PLoS One 2008 Jul 2;3(7):e2558.

O'Driscoll CM, Gorman AM. Hypoxia induces neurite outgrowth in PC12 cells that is mediated through adenosine A2A receptors. Neuroscience. 2005; 131 (2):321-9.

Ola MS, Nawaz M, Ahsan H. Role of Bcl-2 family proteins and caspases in the regulation of apoptosis Mol Cell Biochem 2011 May;351(1-2):41-58.

Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death Cell 1993 Aug 27; 74 (4):609-619.

Oppenheim RW. Cell death during development of the nervous system Annu Rev Neurosci 1991; 14:453-501.

Pang L, Sawada T, Decker SJ, Saltiel AR. Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor J Biol Chem 1995 Jun 9;270(23):13585-13588.

Pederson JE, Gaasterland DE. Laser-induced primate glaucoma. I. Progression of cupping Arch Ophthalmol 1984 Nov;102(11):1689-1692.

Pena JD, Taylor AW, Ricard CS, Vidal I, Hernandez MR. Transforming growth factor beta isoforms in human optic nerve heads Br J Ophthalmol 1999 Feb;83(2):209-218.

Pfenninger KH, de la Houssaye BA, Helmke SM, Quiroga S. (1991) Growth-regulated proteins and neuronal plasticity. A commentary. Mol Neurobiol.5(2-4):143-51. Review.

Philpott KL, McCarthy MJ, Klippel A, Rubin LL. Activated phosphatidylinositol 3-kinase and Akt kinase promote survival of superior cervical neurons J Cell Biol 1997 Nov 3;139(3):809-815.

Planchamp V, Bermel C, Tonges L, Ostendorf T, Kugler S, Reed JC, et al. BAG1 promotes axonal outgrowth and regeneration in vivo via Raf-1 and reduction of ROCK activity Brain 2008 Oct;131(Pt 10):2606-2619.

Prasanna G, Krishnamoorthy R, Yorio T. Endothelin, astrocytes and glaucoma Exp Eye Res 2011 Aug;93(2):170-177.

Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020 Br J Ophthalmol 2006 Mar;90(3):262-267.

Quigley HA, Addicks EM. Regional differences in the structure of the lamina cribrosa and their relation to glaucomatous optic nerve damage Arch Ophthalmol 1981 Jan;99(1):137-143.

Quigley HA, Addicks EM, Green WR, Maumenee AE. Optic nerve damage in human glaucoma. II. The site of injury and susceptibility to damage Arch Ophthalmol 1981 Apr;99(4):635-649.

Quigley HA, West SK, Rodriguez J, Munoz B, Klein R, Snyder R. The prevalence of glaucoma in a population-based study of Hispanic subjects: Proyecto VER Arch Ophthalmol 2001 Dec;119(12):1819-1826.

Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y, Jacobson MD. Programmed cell death and the control of cell survival: lessons from the nervous system Science 1993 Oct 29;262(5134):695-700.

Reisberg B, Doody R, Stoffler A, Schmitt F, Ferris S & Mobius HJ. Memantine in moderate-to-severe Alzheimer's disease. N Engl J Med 2003 348:1333-1341.

Resnikoff S, Pascolini D, Etya'ale D, Kocur I, Pararajasegaram R, Pokharel GP, et al. Global data on visual impairment in the year 2002 Bull World Health Organ 2004 Nov;82(11):844-851.

Rosenfeld MG. POU-domain transcription factors: pou-er-ful developmental regulators Genes Dev 1991 Jun;5(6):897-907.

Ryan AK, Rosenfeld MG. POU domain family values: flexibility, partnerships, and developmental codes Genes Dev 1997 May 15;11(10):1207-1225.

Schulzer M. Intraocular pressure reduction in normal-tension glaucoma patients. The Normal Tension Glaucoma Study Group. Ophthalmology. 1992 Sep;99(9):1468-70.

Sharma TP, Liu Y, Wordinger RJ, Pang IH, Clark AF. Neuritin 1 promotes retinal ganglion cell survival and axonal regeneration following optic nerve crush Cell Death Dis 2015 Feb 26; 6:e1661.

Siliprandi R, Canella R, Carmignoto G. Nerve growth factor promotes functional recovery of retinal ganglion cells after ischemia Invest Ophthalmol Vis Sci 1993 Nov;34(12):3232-3245.

SooHoo JR, Seibold LK, Radcliffe NM, Kahook MY. Minimally invasive glaucoma surgery: current implants and future innovations Can J Ophthalmol 2014 Dec;49(6):528-533.

Sengottuvel V, Leibinger M, Pfreimer M, Andreadaki A, Fischer D. Taxol facilitates axon regeneration in the mature CNS J Neurosci 2011 Feb 16;31(7):2688-2699.

Sethi A, Mao W, Wordinger RJ, Clark AF. Transforming growth factor-beta induces extracellular matrix protein cross-linking lysyl oxidase (LOX) genes in human trabecular meshwork cells Invest Ophthalmol Vis Sci 2011 Jul 15;52(8):5240-5250.

Shareef SR, Garcia-Valenzuela E, Salierno A, Walsh J, Sharma SC. Chronic ocular hypertension following episcleral venous occlusion in rats Exp Eye Res 1995 Sep;61(3):379-382.

Shi M, Kumar SR, Motajo O, Kretschmer F, Mu X, Badea TC. Genetic interactions between Brn3 transcription factors in retinal ganglion cell type specification PLoS One 2013 Oct 8;8(10):e76347.

Skene JH. Axonal growth-associated proteins Annu Rev Neurosci 1989;12:127-156.

Skene JH, Jacobson RD, Snipes GJ, McGuire CB, Norden JJ, Freeman JA. A protein induced during nerve growth (GAP-43) is a major component of growth-cone membranes Science 1986 Aug 15;233(4765):783-786.

Socodato R, Brito R, Portugal CC, de Oliveria NA, Calaza KC, Paes-de-Carvalho R. The nitric oxide-cGKII system relays death and survival signals during embryonic retinal development via AKT-induced CREB1 activation Cell Death Differ 2014 Jun;21(6):915-928.

Soto I, Oglesby E, Buckingham BP, Son JL, Roberson ED, Steele MR, et al. Retinal ganglion cells downregulate gene expression and lose their axons within the optic nerve head in a mouse glaucoma model J Neurosci 2008 Jan 9;28(2):548-561.

Stanokowska DL, Minton AZ, Rutledge MA, Mueller BH2nd , Phatak NR, He S, Ma HY, Forster MJ, Yorio T, Krishnamoorthy RR. Neuroprotecitve effects of transcription factor Brn3b in an ocular hyoertension rat model of glaucoma. Invest Ophthalmol Vis Sci 2015 Jan 13;56(2) : 893-907.

Sun F, Park KK, Belin S, Wang D, Lu T, Chen G, et al. Sustained axon regeneration induced by co-deletion of PTEN and SOCS3 Nature 2011 Nov 6;480(7377):372-375.

Tezel G, Li LY, Patil RV, Wax MB. TNF-alpha and TNF-alpha receptor-1 in the retina of normal and glaucomatous eyes Invest Ophthalmol Vis Sci 2001 Jul;42(8):1787-1794.

Tezel G, Wax MB. The immune system and glaucoma Curr Opin Ophthalmol 2004 Apr;15(2):80-84.

Tezel G, Wax MB. Hypoxia-inducible factor 1alpha in the glaucomatous retina and optic nerve head Arch Ophthalmol 2004 Sep;122(9):1348-1356.

Tezel G, Wax MB. Increased production of tumor necrosis factor-alpha by glial cells exposed to simulated ischemia or elevated hydrostatic pressure induces apoptosis in cocultured retinal ganglion cells J Neurosci 2000 Dec 1;20(23):8693-8700.

Tielsch JM, Sommer A, Katz J, Royall RM, Quigley HA, Javitt J. Racial variations in the prevalence of primary open-angle glaucoma. The Baltimore Eye Survey JAMA 1991 Jul 17;266(3):369-374.

Turner EE, Jenne KJ, Rosenfeld MG. Brn-3.2: a Brn-3-related transcription factor with distinctive central nervous system expression and regulation by retinoic acid Neuron 1994 Jan;12(1):205-218.

Ueda J, Sawaguchi S, Hanyu T, Yaoeda K, Fukuchi T, Abe H, et al. Experimental glaucoma model in the rat induced by laser trabecular photocoagulation after an intracameral injection of India ink Jpn J Ophthalmol 1998 Sep-Oct;42(5):337-344.

Varnum-Finney B, Reichardt LF. Vinculin-deficient PC12 cell lines extend unstable lamellipodia and filopodia and have a reduced rate of neurite outgrowth J Cell Biol 1994 Nov;127(4):1071-1084.

Vaudry D, Stork PJ, Lazarovici P, Eiden LE. Signaling pathways for PC12 cell differentiation: making the right connections Science 2002 May 31;296(5573):1648-1649.

Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair Cell 1993 Oct 22;75(2):229-240.

Verrijzer CP, Van der Vliet PC. POU domain transcription factors Biochim Biophys Acta 1993 Apr 29;1173(1):1-21.

Wang SW, Mu X, Bowers WJ, Kim DS, Plas DJ, Crair MC, et al. Brn3b/Brn3c double knockout mice reveal an unsuspected role for Brn3c in retinal ganglion cell axon outgrowth Development 2002 Jan;129(2):467-477.

Wang H, Wang R, Thrimawithana T, Little PJ, Xu J, Feng ZP, et al. The nerve growth factor signaling and its potential as therapeutic target for glaucoma Biomed Res Int 2014;759473.

Wegner M, Drolet DW, Rosenfeld MG. POU-domain proteins: structure and function of developmental regulators Curr Opin Cell Biol 1993 Jun;5(3):488-498.

Weinreb RN, Khaw PT. Primary open-angle glaucoma Lancet 2004 May 22;363(9422):1711-1720.

Weishaupt JH, Klocker N, Bahr M. Axotomy-induced early down-regulation of POU-IV class transcription factors Brn-3a and Brn-3b in retinal ganglion cells J Mol Neurosci 2005;26(1):17-25.

Wheeler LA, Woldemussie E. Alpha-2 adrenergic receptor agonists are neuroprotective in experimental models of glaucoma Eur J Ophthalmol 2001 Jul-Sep;11 Suppl 2:S30-5.

Wilhelm B, Ludtke H, Wilhelm H, BRAION Study group. Efficacy and tolerability of 0.2% brimonidine tartrate for the treatment of acute non-arteritic anterior ischemic neuropathy (NAION): a 3 month, double-masked randomized, placebo-controlled trial. Graefes Arch Clin Exp Ophthalmol 2006.

Wong TT, Sethi C, Daniels JT, Limb GA, Murphy G, Khaw PT. Matrix metalloproteinases in disease and repair processes in the anterior segment Surv Ophthalmol 2002 May-Jun;47(3):239-256.

Xiang M. Requirement for Brn-3b in early differentiation of postmitotic retinal ganglion cell precursors Dev Biol 1998 May 15;197(2):155-169.

Xiang M, Gan L, Li D, Chen ZY, Zhou L, O'Malley BW,Jr, et al. Essential role of POU-domain factor Brn-3c in auditory and vestibular hair cell development Proc Natl Acad Sci U S A 1997 Aug 19;94(17):9445-9450.

Xiang M, Gan L, Li D, Zhou L, Chen ZY, Wagner D, et al. Role of the Brn-3 family of POU-domain genes in the development of the auditory/vestibular, somatosensory, and visual systems Cold Spring Harb Symp Quant Biol 1997;62:325-336.

Xiang M, Gan L, Zhou L, Klein WH, Nathans J. Targeted deletion of the mouse POU domain gene Brn-3a causes selective loss of neurons in the brainstem and trigeminal ganglion, uncoordinated limb movement, and impaired suckling Proc Natl Acad Sci U S A 1996 Oct 15;93(21):11950-11955.

Xiang M, Zhou H, Nathans J. Molecular biology of retinal ganglion cells Proc Natl Acad Sci U S A 1996 Jan 23;93(2):596-601.

Xiang M, Zhou L, Peng YW, Eddy RL, Shows TB, Nathans J. Brn-3b: a POU domain gene expressed in a subset of retinal ganglion cells Neuron 1993 Oct;11(4):689-701.

Yan X, Tezel G, Wax MB, Edward DP. Matrix metalloproteinases and tumor necrosis factor alpha in glaucomatous optic nerve head Arch Ophthalmol 2000 May;118(5):666-673.

Yin Y, Henzl MT, Lorber B, Nakazawa T, Thomas TT, Jiang F, et al. Oncomodulin is a macrophage-derived signal for axon regeneration in retinal ganglion cells Nat Neurosci 2006 Jun;9(6):843-852.

Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death Nat Rev Mol Cell Biol 2008 Jan;9(1):47-59.

Yuan J, Yankner BA. Apoptosis in the nervous system Nature 2000 Oct 12;407(6805):802-809.

Zhu MD, Cai FY. Development of experimental chronic intraocular hypertension in the rabbit. Aust N Z J Ophthalmol. 1992 Aug;20(3):225-34.

Zode GS, Sethi A, Brun-Zinkernagel AM, Chang IF, Clark AF, Wordinger RJ. Transforming growth factor-beta2 increases extracellular matrix proteins in optic nerve head cells via activation of the Smad signaling pathway Mol Vis 2011;17:1745-1758.

Zwiers H, Veldhuis HD, Schotman P, Gispen WH. ACTH, cyclic nucleotides, and brain protein phosphorylation in vitro Neurochem Res 1976 Dec;1(6):669-677.

CHAPTER II

TRANSCRIPTION FACTOR BRN3B OVEREXPRESSION ENHANCES NEURITE OUTGROWTH IN PC12 CELLS UNDER CONDITION OF HYPOXIA

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Abstract

Background: Transcription factor Brn3b plays a key role in retinal ganglion cell differentiation, survival and axon outgrowth during development. However, the precise role of Brn3b in the normal adult retina as well as during neurodegeneration is unclear. In the current study, the effect of overexpression of Brn3b was assessed in vitro, in PC12 cells under conditions of normoxia and hypoxia. **Results**: Immunoblot analysis showed that overexpression of Brn3b in PC12 cells as well as 661W cells produced significant increase in the growth cone marker, growth associated protein-43 (GAP-43) and acetylated-tubulin (ac-TUBA). In addition, an increased immunostaining for GAP-43 and ac-TUBA was observed in PC12 cells overexpressing Brn3b, which was accompanied by a marked increase in neurite outgrowth, compared to PC12 cells overexpressing empty vector. In separate experiments, one set of PC12 cells transfected either with a Brn3b expression vector or an empty vector was subjected to conditions of hypoxia for 2h, while another set of similarly transfected PC12 cells was maintained in normoxic conditions. It was found that the upregulation of GAP-43 and ac-TUBA in PC12 cells overexpressing Brn3b under conditions of normoxia was sustained under conditions of hypoxia. Immunocytochemical analysis revealed not only an upregulation of GAP-43 and ac-TUBA, but also increased neurite outgrowth in PC12 cells transfected with Brn3b as compared to PC12 cells transfected with empty vector in both normoxia and hypoxia. **Conclusions**: The findings have implications for a potential role of Brn3b in neurodegenerative diseases in which hypoxia/ischemia contribute to pathophysiology of the disease.

Keywords

Transcription factor Brn3b

Hypoxia

Neurodegeneration

Neurite outgrowth

Background

Neuronal injury and degeneration contribute to pathological changes in a number of neurological disorders including Alzheimer's and Parkinson's disease, as well as various ocular neurodegenerative diseases, such as glaucoma and retinal degeneration. The limited regeneration capacity of neurons constrains the recovery from neuronal damage. Thus, better understanding of endogenous and exogenous factors facilitating neurite outgrowth, will aid in the development of therapeutic approaches for neurological diseases (Wu et al. 2012).

POU-domain proteins are generally associated with the development and differentiation of neuronal cell types (Mu et al. 2004). The POU (Pit-Oct-Unc) family of transcription factors are classified on the basis of a common region of 150-160 amino acids in mammalian transcription factors Pit-1, Oct-1, Oct-2 and nematode factor Unc-86. The POU domain common to these factors consists of a bipartite DNA binding domain, which comprises of POU-specific domain (POUS) and a POU homeodomain (POUHD) separated by a linker region (Latchman 1999). A search for POU domain transcription factors in mammalian retina lead to the identification of three closely related genes which were named as Brain-specific homeobox/POU domain protein 3 (Brn3) including Brn3a, Brn3b, and Brn3c (Xiang et al. 1995). Brn3 genes are expressed in distinct and overlapping pattern in the developing and mature mammalian nervous system (Turner et al. 1994; Gerrero et al. 1993; Wang et al. 2002; Xiang et al. 1997; Gan et al. 1999; Fedtsova et al 1995;

Ninkina et al. 1993; Xiang et al. 1993; Xiang 1998). However, deletion of each of the Brn3 proteins affects a specific population of neurons in which it is first expressed during development of the nervous system. It has been shown that Brn3a knockout mice have defects in peripheral sensory ganglia, motor and sensory components of the hindbrain (Erkman et al. 2000; Wang et al. 2002; Huang et al. 1999). In Brn3c knockout mice, deafness and balance problems have been observed due to failure in the differentiation of cochlear and vestibular hair cells (Xiang et al. 1997; Erkman et al. 2000; Erkman et al. 1996).

A prominent phenotype observed in Brn3b-deficient mice is the loss of nearly 70% of retinal ganglion cells (RGCs) without discernible defects in other neurons of the central or peripheral nervous system (Erkman et al. 2000; Erkman et al. 1996; Gan et al. 1996; Camp et al. 2011). Mice lacking Brn3b have RGCs with abnormal processes, having short and spiny projections reminiscent of dendrites rather than axons, consequently resulting in the formation of a thinner optic nerve (Gan et al.1996; Wang et al. 2000). Gan et al. (1999) have shown that processes emanating from retinal explants from Brn3b null embryos are shorter and less bundled than wild type retinas. Marked apoptosis observed in Brn3b-mutant RGCs appears to be a secondary manifestation to initial defects in differentiation, which results in abnormalities in axonal growth and pathfinding (Mu et al. 2004; Erkman et al. 2000). Thus, Brn3b is necessary for normal RGC differentiation, polarity, axonal outgrowth and survival (Gan et al. 1999; Erkman et al. 2000; Erkman et al. 1996; Gan et al. 1996; Wang et al. 2000; Camp et al. 2011). There is a considerable amount of information about the role of Brn3b in RGC development. However, the role of transcription factor Brn3b in adult retinas and genes influenced by Brn3b in adult RGCs remains to be elucidated.

Mu et al., (2001) have shown a substantial decrease in GAP-43 expression in the E14.5 retina in Brn3b knockout mice, compared to wild type mice, suggesting a regulatory linkage between Brn3b and GAP-43. Corroborative evidence from many labs indicates that GAP-43 plays a key role in guiding the growth of axons and modulating the formation of new connections. Some of these effects of GAP-43 appear to involve regulation of cytoskeletal organization by transducing intra- and extracellular signals in the nerve terminals. GAP-43 is also an intrinsic determinant of neuronal development and plasticity (Benowitz and Routtenberg 1997) and is widely used in neurological studies as a marker of neurite growth (Benowitz and Routtenberg 1997; de Lima et al. 2012; Cho et al. 2005; Yin et al. 2009; Kurimoto et al. 2010; Sun et al. 2011).

Previous studies have linked acetylated α-tubulin (ac-TUBA) expression with neurite elongation in vivo as well as in vitro. Increased acetylation of α-tubulin has been observed in stable microtubules, compared to dynamic microtubules. While some studies have shown that acetylation promotes microtubules stability, other studies have claimed the acetylation itself does not stabilize microtubules and may enhance binding of molecular motors to microtubules (Dompierre et al. 2007; Fournier and McKerracher 1995; Gaub et al.2010; Sengottuvel et al. 2011).

Rat pheochromocytoma (PC12) cells have been widely used in neurobiological studies as a model of neuronal differentiation and neurite outgrowth in response to nerve growth factor (NGF) treatment (Lee et al.1977; Cowley et al. 1994; Pang et al. 1995; Takebayashi et al. 2002; Das et al. 2004). NGF-treated PC12 cells cease proliferation, extend neurites and acquire properties characteristic of sympathetic neurons (Das et al. 2004). PC12 cells are exquisitely sensitive to hypoxia which makes it a useful model to study the effects of hypoxia on overexpression of Brn3b (Alvarez-Tejado et al. 2001; Czyzyk-Krzeska et al 1994).

The goal of this study was to evaluate the role of Brn3b in neurite outgrowth and assess the expression of growth cone marker GAP-43 and ac-TUBA following overexpression of Brn3b in PC12 cells under conditions of normoxia and hypoxia.

Methods

Cell culture: 661W cells (SV-40 transformed mouse photoreceptor cells) were seeded in T75 flasks with Dulbecco's modified Eagle's Medium containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml). The cells were grown to confluence in T75 flasks, trypsinized and seeded for various experiments on 100 mm dishes (200,000 cells per dish).

Rat pheochromocytoma (PC12 cells) cells were obtained from American Type Culture Collection (ATCC) and cultured as described by Adler et al., (2006). The cells were grown undifferentiated in T75 flasks with Dulbecco's modified Eagle's Medium (DMEM) containing 10% normal horse serum (Life technologies, NY), 5% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml)). Cultures were maintained according to ATCC specified culture conditions at 37^0C in a humidified incubator containing 95% air and 5% CO_2. For differentiation, cells were dissociated and plated on poly-D-lysine (Sigma-Aldrich) coated 100 mm dishes (200,000 cells per dish) or 25 mm coverslips (10,000 cells per coverslip) or 96 well plates (1000 cells per well) in differentiating DMEM (containing 1% horse bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) with 100 ng/ml of NGF (Sigma-Aldrich) for various experiments.

Plasmid preparation: Plasmid DNAs**,** pCMV6 entry (Empty) vector and pCMV6-Brn3b (a full length cDNA encoding Brn3b containing a DDK tag) were purchased from Origene (Rockville, MD). Purified preparations of the plasmids were obtained from maxi-preps of transformed *E. coli* (DH5α strain) cultures harboring the recombinant expression vectors using a plasmid purification

kit from Origene. Following transfection of cell lines, positive expression of the encoded proteins was confirmed by Western blot.

Transient transfection: 661W cells were transiently transfected with plasmid pCMV6-Empty or pCMV6-Brn3b using 5µl of Lipofectamine 2000 (Life Technologies, Inc, Grand Island, NY) and 5 µg of the plasmid in a total volume of 1 ml of transfection mix and maintained overnight in the transfection medium. The cell culture medium was changed to complete medium (DMEM containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml). The pCMV6-Brn3b or pCMV6-Empty transfected cells were used 24 h post transfection for isolating cytoplasmic and nuclear extracts for immunoblot analysis.

PC12 cells were transiently transfected with the plasmid pCMV6-Empty vector or pCMV6-Brn3b using the Lipofectamine 2000 reagent. Transfections were carried out using 5 µl of Lipofectamine and 5 µg of the plasmid in a total volume of 1 ml of the transfection mix. After 6 h of transfection, culture medium was changed to the differentiating medium (DMEM containing 1% horse bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml)) with NGF 100 ng/ml and incubated overnight. The pCMV6-Brn3b or pCMV6-Empty transfected cells were used 24 h post-transfection for isolating cytoplasmic and nuclear extracts for immunoblot analysis. A similar transfection procedure was carried out for PC12 cells seeded on coverslips for immunocytochemistry using 1.5 µg of either pCMV6-Empty vector or pCMV6-Brn3b followed by maintenance in differentiating medium for 5 days.

Hypoxia Chamber: To examine the effect of hypoxia, PC12 cells overexpressing either Brn3b or Empty vector were used. Following transfection and incubation in differentiation medium, PC12 cells overexpressing Brn3b or empty vector were subjected to a hypoxic insult for 2h in glucose-

free DMEM. For the hypoxic insult, cells were incubated for 2 hours in 0.5% O_2 and 5% CO_2 (hypoxia) in an Invivo2 200 hypoxic chamber (Biotrace International, Mid Glamorgan, UK) used in conjunction with Ruskinn gas mixer module. For the normoxia controls, PC12 cells overexpressing Brn3b or empty vector were incubated for 2h in 5% CO_2 and 95% air in a standard incubator and maintained in differentiating media.

Cell Proliferation assay (MTT assay): Cell Proliferation assay was performed as described previously (Prasanna et al. 2002). A commercially available one-solution cell proliferation assay with the tetrazolium compound MTS (CellTiter 96Aqueous; Promega, Madison, WI), was used to evaluate the effects of hypoxic conditions on PC12 cells transfected with pCMV6-Empty or pCMV6-Brn3b. The MTS compound is bioreduced to a formazan by reduced nicotinamide adenine dinucleotide phosphate (NADPH) or reduced nicotinamide adenine dinucleotide (NADH) produced by metabolically active dehydrogenase enzymes of cells which can be detected at 490 nm. PC12 cells transfected with pCMV6-Empty or pCMV6-Brn3b were maintained either in differentiating medium (containing NGF) or in low glucose DMEM without NGF. After normoxia or hypoxic insults, the culture media were discarded and to each well 100 µl of fresh DMEM along with 20 µl of the MTS solution was added and incubated at 37^0C for 30 minutes. The 96 well plate was then placed in a kinetic microplate reader (Infinity M200 TECAN US, Morrisville, NC) and absorbance was read at 490 nm. DMEM with NGF was used as negative control.

Preparation of cytoplasmic and nuclear extracts: Cytoplasmic and nuclear extracts from transfected cells were isolated according to the method described by Krishnamoorthy et al., (1999). Briefly, pCMV6-Brn3b or pCMV6-Empty vector transfected cells were collected 24 h post-transfection. Cells were suspended in 100 µl of Buffer C (10 mM HEPES buffer pH 7.9, 10 mM KCl, 0.2 mM EDTA, 10% glycerol, 1mM dithiothreitol (DTT), and 0.5mM phenylmethylsulfonyl

fluoride (PMSF)). After incubation on ice for 15 min, 3 µl of 10% Igepal CA-630 (Sigma-Aldrich, St. Louis, MO) was added to the suspension and briefly vortexed. Cell nuclei were pelleted by centrifugation at 1,000g for 5 min at 4ºC. The post-nuclear supernatant (cytoplasmic extract) was collected and stored at -80^{0}C. The nuclear pellet was resuspended in 80 µl of Buffer D (20 mM HEPES, pH 7.9, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.5 mM PMSF). The suspension was incubated for 20 minutes at 4^{0}C followed by a centrifugation at 14,000g for 5 minutes. The supernatant (nuclear protein extract) was transferred to a fresh microcentrifuge tube and stored at -80^{0}C. Protein concentrations of the cytoplasmic and nuclear extracts were measured with a detergent-compatible Protein Assay Kit (Bio-Rad), using bovine serum albumin as a standard.

Total Cellular Lysate: PC12 cells, overexpressing either pCMV6-Empty or pCMV6-Brn3b subjected to normoxia or hypoxic insults, were homogenized in 300 µl of ice cold buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-630 (Sigma-Aldrich, St. Louis MO), 1% sodium deoxycholate, 1 mM sodium orthovanadate, 20 mM NaF, 10 mM sodium pyrophosphate decahydrate, 1M DTT, 0.25M PMSF and Halt ^{TM} Protease inhibitor single-use cocktail (Thermo-scientific, Rockford, IL). The cellular homogenates were sonicated and protein concentration were measured with a detergent-compatible Protein Assay Kit (Bio-Rad), using bovine serum albumin as a standard.

Western blot analysis: Expression levels of Brn3b, GAP-43 and ac-TUBA in pCMV6-Brn3b or pCMV6-Empty vector transfected 661W or PC12 cells were analyzed by immunoblot analysis as described by Krishnamoorthy et al., (1999). Cytoplasmic extracts were used to detect GAP-43, ac-TUBA, GAPDH, calnexin, whereas nuclear extracts were used to detect Brn3b and TATA binding protein. The following primary antibodies were used: an anti-mouse DDK tag (1:1000 dilution;

Origene #TA50011-1, Rockville, MD), anti-rabbit GAP-43 (1:2000 dilution; Sigma-Aldrich #HPA015600, St. Louis, MO), and anti-mouse ac-TUBA (1:20000 dilution; Sigma-Aldrich #T7451, St. Louis, MO). The incubation with primary antibodies was carried out either for 1h at room temperature or overnight at 4^{o}C. For normalization of protein loading, anti-mouse TATA binding protein (1:1000 dilution; Abcam #ab818 Cambridge, MA), anti-rabbit calnexin (1:5000 dilution; Enzo Life sciences # ADI-SPA-860, Farmingdale, NY), and anti-mouse GAPDH antibody (1:1000 dilution; Santa Cruz #sc-32233, Dallas, TX) were used. Incubation with an appropriate horse radish peroxidase (HRP)-conjugated secondary antibodies and subsequent detection using Supersignal West Dura (Femto) Extended Duration substrate (peroxidase labeled appropriate secondary antibodies) were carried out. Serial images were taken in a Bio-Rad gel imager and the ratio of normalized band intensities were compared between different treatment groups.

Immunocytochemical Analysis: PC12 cells were seeded on poly D-lysine coated coverslips, transfected with either pCMV6-Brn3b or pCMV6-Empty and maintained in differentiating media containing 100 ng/ml NGF for 4 days. The cells were subjected to either normoxia or hypoxia. Immunocytochemical detection of Brn3b, GAP-43 and ac-TUBA proteins were carried out essentially as described by O'Connor et al.,(2009). Briefly, the cells were fixed with 4% paraformaldehyde (PFA) containing 4% sucrose in 1x phosphate buffer (PBS) for 20 min at room temperature. Permeabilization was carried out with 0.1% Triton X-100, 0.1% sodium citrate in 1x PBS for 5 minutes at room temperature. To prevent non-specific binding, blocking was carried out for 1 h with 0.2% gelatin, 0.1% Triton X-100, 5% normal donkey serum and 5% BSA in 1x PBS. Following blocking, cells were treated with one of the following primary antibodies: a custom-made Brn-3b antibody (1:250 dilution of a rabbit polyclonal anti-Brn3b from Antibody Research

corporation, MO, USA); mouse anti-GAP-43 antibody (1:500 dilution; Sigma-Aldrich # G9264), mouse ac-TUBA (1:2000 dilution) and incubated overnight at 4^0C. The primary antibody incubations were followed by incubation for 1 h with an appropriate donkey anti-IgG secondary antibody conjugated with Alexa 488, Alexa 633 or Alexa 546 (1:1000 dilution; Molecular Probes, Invitrogen, Eugene, OR). PC12 cells in which primary antibody incubation was excluded served as blank controls and were used to assess non-specific staining by the secondary antibody. Fluorescent images were taken in a Zeiss LSM 510 META confocal microscope.

Neurite growth analysis: Neurite outgrowth from PC12 cells overexpressing Brn3b or Empty vector in hypoxic or normoxic conditions was counted by using the NIH ImageJ software (Neuron-J plug in, PC version: http//rsbweb.nih.gov/ij) (Pool et al 2008; Torres-Espin et al. 2014; Barnat et al. 2010; Berg et al. 2013). Phase contrast images in 10 fields of view per coverslip per well were taken with an average of 4-5 cells per field. The number of neurites was determined by counting cells with at least one neurite having a length equal to the cell body diameter and expressed as percentage of the total cells in the field. Neurite length was measured by manually tracing the length of the longest neurite per cell with Neuron J software for all cells in a field that had an identifiable neurite and for which the entire neurite arbor could be visualized. Data from the 10 fields in each coverslip were pooled. Experiments were repeated at least five times (n=5) using cultures prepared on separate days.

Statistical analysis: For comparing results between two groups, student's *t*-test was performed. For comparison of results between more than two experimental groups, statistical analysis of data was carried out using one way One-Way Analysis of Variance (ANOVA) followed by Student-Newman-Keuls post-hoc tests. In some experiments, ANOVA on ranks followed by Mann-

Whitney Rank Sum test or Kruskal-Wallis post-hoc test was performed. Data was reported as mean ± SEM. A significant difference was defined as having a probability (p value) less than 0.05.

Results

Upregulation of GAP-43, ac-TUBA in 661W cells overexpressing transcription factor Brn3b

Previous studies have shown a significant decrease in GAP-43 expression in the E14.5 retina from Brn3b knockout mice as compared to the wild type mice, suggesting a potential regulatory linkage between Brn3b and expression of GAP-43, a protein associated with axon elongation (Mu et al. 2001). However, it is unclear if Brn-3b upregulation could increase GAP-43 expression in cultured neurons. To test this, initial experiments were carried out using the cultured transformed 661W mouse photoreceptor cell line.

661W cells were transfected with either the pCMV6-Empty or pCMV6-Brn3b vector (n=3) following which cytoplasmic and nuclear extracts were prepared and examined by western blot analysis. Overexpression of Brn-3b was confirmed by using the Brn3b-DDK-tag antibody compared to 661W cells transfected with pCMV6-Empty vector (Figure 1a). Immunoblot analysis of 661W cells showed an appreciable increase in band intensities for GAP-43 and ac-TUBA in the pCMV6-Brn3b transfected cells as compared to the pCMV6-Empty vector transfected cells (Figure 1a). Densitometric analysis of band intensities followed by statistical analysis indicated a significant increase in both GAP-43 and ac-TUBA in 661W cells over expressing Brn3b (Figure 1b and 1c). GAPDH expression was used as a loading control in these experiments. These observations suggest that GAP-43 and ac-TUBA expression was markedly increased in 661W cells transfected with pCMV6-Brn3b, compared to 661W cells transfected with pCMV6-Empty.

Overexpression of transcription factor Brn3b lead to a significant increase in the expression of GAP-43, and ac-TUBA in PC12 cells

Since an increase in GAP-43 and ac-TUBA were observed by immunoblot analysis in 661W cells transfected with pCMV6-Brn3b, further confirmation was done by overexpressing Brn3b in a different cell line namely, the PC12 cells.

We sought to determine whether there are any changes in GAP-43 and ac-TUBA expression in PC12 cells transfected with pCMV6-Brn3b. Briefly, PC12 cells were transfected with either the pCMV6-Empty or pCMV6-Brn3b plasmid (n=3). Following transfection, cytoplasmic and nuclear extracts were made and subjected to western blot analysis. Overexpression of Brn3b in PC12 cells was confirmed by using a Brn3b-DDK tag antibody (Figure 2a). Similar to data obtained using 661W cells, immunoblot analysis indicated a statistically significant increase in the expression of GAP-43 and ac-TUBA in PC12 cells transfected with pCMV6-Brn3b, compared to PC12 cells transfected with pCMV6-Empty vector (Figure 2a, 2b and 2c**).** GAPDH and TATA binding protein used as loading controls for cytoplasmic and nuclear extracts respectively were not appreciably different between the various experimental groups.

Further confirmation of these results was obtained from immunocytochemical analysis of PC12 cells overexpressing Brn3b. As shown in Figure 2d, PC12 cells transfected with pCMV6-Brn3b showed increased immunostaining for Brn3b, confirming overexpression of Brn3b in these cells, compared to PC12 cells overexpressing the pCMV6-Empty vector (n=3). Moreover, an appreciable increase in expression of growth cone-enriched protein, GAP-43, and ac-TUBA were found (detected by increased immunostaining) following overexpression of Brn3b in PC12 cells. Furthermore, a marked change in morphology of pCMV6-Brn3b transfected cells was also

observed as compared to the pCMV6-Empty vector transfected cells (Figure 2d). Taken together, these results indicate that expression of GAP-43, ac-TUBA was significantly increased and neurite outgrowth was enhanced in PC12 cells transfected with pCMV6-Brn3b, compared to PC12 cells transfected with pCMV6-Empty vector.

Overexpression of Brn3b persisted under hypoxic conditions in PC12 cells transfected with pCMV6-Brn3b

To determine whether Brn3b overexpression persists under hypoxic conditions, we performed immunoblot analysis and immunocytochemistry in PC12 cells transfected with either pCMV6-Empty or pCMV6-Brn3b vector. Following transfection, one set of transfected cells was exposed for 2 h to hypoxia (0.5% O_2, 5% CO_2) in glucose-free DMEM. Another set of similarly transfected cells was maintained in 5% CO_2 and 95% air (normoxia) in a standard incubator in differentiating medium (n=3) (Figure 3a: Experimental Scheme). Immunoblot analysis of pCMV6-Brn3b transfected PC12 cells in normoxic as well as hypoxic conditions confirmed overexpression of Brn3b, as compared to the corresponding pCMV6-Empty vector transfected cells (Figure 3b). In addition, increased immunostaining for Brn3b was observed in pCMV6-Brn3b overexpressing cells under conditions of both normoxia and hypoxia (n=3). No Brn3b immunostaining was detected in pCMV6-Empty vector transfected cells under normoxia or hypoxia (Figure 3c). These data indicate that overexpression of Brn-3b in pCMV6-Brn3b transfected cells, also persists under conditions of hypoxia.

Upregulation of GAP-43 expression in pCMV6-Brn3b transfected PC12 cells in normoxic and hypoxic conditions

Since a significant increase in GAP-43 expression was observed in pCMV6-Brn3b transfected cells, compared to pCMV6-Empty vector transfected PC12 cells (Figure 2), GAP-43 expression was also assessed in PC12 cells under hypoxic conditions. PC12 cells were transfected either with pCMV6-Empty or pCMV6-Brn3b vector. One group of transfected cells was subjected to hypoxia (0.5% O_2, 5% CO_2) in glucose-free DMEM for 2 h, while another group of transfected cells was maintained in normoxic conditions (5% CO_2 and 95% air) in standard incubator. Immunoblot analysis of pCMV6-Brn3b transfected cells demonstrated a marked upregulation in GAP-43 expression compared to those of pCMV6-Empty transfected cells in normoxia. pCMV6-Empty transfected PC12 cells maintained under hypoxic conditions showed a significant increase in GAP-43 expression, compared to PC12 cells overexpressing empty vector in normoxia. (Figure 4a and b) (n=7). PC12 cells maintained under hypoxia after transfection with pCMV6-Brn-3b showed a trend towards upregulation of GAP43 compared to cells transfected with the empty vector and maintained under hypoxia, however this was not statistically significant.

Using immunocytochemical analysis, (Figure 4c) PC12 cells transfected with pCMV6-Brn3b showed increased immunostaining for GAP-43 in normoxia as well as in hypoxia, as compared to the corresponding pCMV6-Empty vector transfected cells in both conditions (n=3).

PC12 cells transfected with pCMV6-Brn3b show increased expression of ac-TUBA in normoxia and hypoxia

Our previous experiments demonstrated that under normoxic conditions overexpression of pCMV6-Brn3b in PC12 leads to increased expression of ac-TUBA. To evaluate the expression of ac-TUBA during hypoxic conditions, PC12 cells were transfected either with pCMV6-Empty vector or pCMV6-Brn3b. One group of transfected cells was subjected to hypoxia (0.5% O_2, 5%

CO_2) in glucose-free DMEM for 2 h, while another group of transfected cells was kept in normoxic conditions. As shown in figure 5a and 5b, immunoblot analysis indicated a significant upregulation of ac-TUBA in pCMV6-Brn3b transfected cells under both the conditions, as compared to pCMV6-Empty transfected cells in normoxia. The levels of ac-TUBA are maintained in PC12 cells transfected cells with the pCMV6-Brn3b in normoxia as well as in hypoxia. Under hypoxic conditions, there was an increasing trend (not statistically significant) in the expression of ac-TUBA in PC12 cells overexpressing Brn3b compared to PC12 cells transfected with empty vector subjected to hypoxia. However, ac-TUBA expression in empty vector transfected PC12 cells subjected to hypoxia, compared to empty vector transfected cells maintained in normoxia was not significantly different. Figure 5c shows a prominent increase in immunostaining of ac-TUBA in PC12 cells overexpressing Brn3b in normoxia as well as hypoxia, as compared to PC12 cells transfected with pCMV6-Empty vector under both conditions (n=4). There was a readily discernible change in the morphology of PC12 cells overexpressing Brn3b in normoxia and hypoxia, compared to the corresponding control cells transfected with the empty vector.

Significant increase in mean neurite length and number of cells with processes in Brn3b overexpressing PC12 cells in normoxia as well as in hypoxia

Analysis of DIC images of PC12 cells transfected with the pCMV6-Brn3b vector subjected to either normoxia or hypoxia indicated a substantial increase in neurite outgrowth, compared to the corresponding empty vector transfected cells. Using the Image J (NeuronJ-plug in) software, the mean neurite length and number of cells having processes were manually counted. The experiment was performed five times (n=5) and the cumulative values of mean neurite length and number of cells with processes were computed. The mean neurite length in PC12 cells transfected with pCMV6-Brn3b in both normoxia and hypoxia was found to be significantly greater than in PC12

cells transfected with pCMV6-Empty vector under both the conditions (Figure 6a). Moreover, the number of cells having processes was also significantly higher in Brn3b overexpressing cells in both normoxia and hypoxia, compared to the empty vector overexpressing cells under normoxia (Figure 6b). There was trend towards an increase (not significant) in number cells having processes in Brn3b transfected PC12 cells, compared to the empty vector transfected PC12 cells under conditions of hypoxia. There was no statistically significant difference in number of cells with processes between Brn3b overexpression in normoxia and Brn3b overexpression in hypoxia (Figure 6b).

Effects of hypoxia on PC12 cells overexpressing Brn3b with or without NGF:

To assess cell survival after hypoxia, a cell proliferation assay (MTT assay) was performed with PC12 cells transfected with either the pCMV6-Empty or pCMV6-Brn3b vector. After transfection, one set of PC12 cells were maintained in differentiated medium with NGF and exposed to either normoxia or 2 h hypoxia (0.5%O_2, 5% CO_2) in glucose-free DMEM (Figure 7a). Another set of similarly transfected cells was maintained in differentiating medium without NGF and subjected to either normoxia or hypoxia in a standard incubator (Figure 7b). The experiment was carried out twice with eight wells for each treatment group (n=16). Our results demonstrate that in the presence of NGF there is a modest, yet significant decrease in viability of PC12 transfected with empty vector maintained under hypoxia, compared to empty vector transfected PC12 maintained under normoxia. This suggests that the 2h hypoxic insult was sufficient to produce a modest reduction in cell survival. Moreover, a significant increase in cell survival was found in cells transfected with the Brn-3b construct under hypoxia compared to corresponding empty vector transfected cells maintained under hypoxia (in the presence of NGF) **(**Figure 7a**)**.

The other set of PC12 cells transfected with pCMV6-Empty or pCMV6-Brn3b in the absence of NGF subjected to either hypoxia or normoxia, did not show any significant changes in viability when the different treatment groups were compared **(**Figure 7b**)**.

Discussion

The present study suggests the involvement of Brn3b in neurite outgrowth (Gan et al. 1999; Erkman et al. 2000; Erkman et al. 1996; Gan et al. 1996; Wang et al. 2000; Camp et al. 2011) in PC12 cells under conditions of normoxia, as well as hypoxia, in the presence of NGF, and also delineates some mechanisms underlying Brn3b-mediated neurite outgrowth.

Brn3b plays a pivotal role in the development and physiological differentiation of RGCs, which is evident by the loss of almost 70% of these cells in Brn3b deficient mice. During development, lack of Brn3b results in abnormal axon projections and defects in axon pathfinding (Xiang et al. 1997; Erkman et al. 1996). The function of Brn3b in normal physiology in the adult retina, as well as in pathological conditions, is largely unknown. Studies examining the effects of hypoxia and hypoxia-reoxygenation on cultured neurons, simulate some cellular aspects of the clinical and pathological course of several neurodegenerative diseases, such as Alzheimer's disease, Prion diseases, Parkinson's disease, Huntington's disease and glaucoma (Alvarez-Tejado 2001; Czyzyk-Krzeska et al. 1994). The protective role of Brn3b under hypoxic conditions in neuronal cells overexpressing Brn3b has not been investigated. This is the first study that demonstrates that Brn3b has the potential to enhance neurite outgrowth in PC12 cells and this was demonstrated under conditions of both normoxia and hypoxia.

The structural and functional integrity of the brain and retina depends upon a regular oxygen and glucose supply. Any disruption of this supply may result in a severe loss of brain or retina function. Specifically, reduction in oxygen availability (hypoxia) caused by disturbances in blood circulation cannot be tolerated for long durations due to inadequate energy supply to the brain and retina by anaerobic glycolysis. Past studies have shown that hypoxia plays an important role in the pathology of a number of central nervous system disorders, including stroke, brain trauma and neurodegenerative diseases (Acker and Acker 2004). The posterior ciliary artery circulation plays an important role in maintaining perfusion of the various ocular structures including the ciliary body and optic nerve head. Disruption in blood circulation of the retina and optic nerve results in various ocular and optic nerve head disorders, causing varying degrees of visual loss. It includes glaucomatous optic neuropathy, anterior ischemic optic neuropathy and optic ischemic neuropathy (Hayreh 2004; Gupta and Yucel 2007). In these diseases, repeated episodes of hypoxia lead to chronic damage to the optic nerve and eventually apoptosis of RGCs. Therefore, the experimental paradigm of mild hypoxia provides a suitable model to study the changes occurring at cellular and molecular level in these diseases.

Since there is no available retinal cell line, 661W and PC12 cells were used as a model for this study. 661W is a mouse photoreceptor cell line, which is difficult to differentiate. Therefore, we used PC12 cell line which has been shown to differentiate into cells with a neuron-like phenotype after treatment with NGF. PC12 cells differentiated with NGF have been widely used to study molecular mechanisms underlying neurite growth of cultured neuronal cells. NGF was added to PC12 cells in almost all neurological studies so that the cells could differentiate into neurons (Lee et al. 1977; Cowley et al. 1994; Pang et al. 1995; Takebayashi et al. 2002; Das et al. 2004, Alvarez-

Tejado et al. 2001; Czyzyk-Krzeska et al. 1994). In the current study, without addition of NGF, we could not overexpress Brn3b in PC12 cells.

Transcription factor Brn-3b could be a potential candidate for the treatment of various neurodegenerative conditions including glaucoma. Gene therapy with AAV-Brn3b could have minimal undesirable effects, as Brn3b is endogenously expressed in adult retinas and different parts of the brain, including superior colliculus, and interpeduncular nucleus of trigeminal ganglion (Turner et al. 1994). Although, Brn3b is constitutively expressed in adult RGCs, its role in the physiology of mature RGCs remains to be understood. Studies have shown that a decrease in Brn3b expression occurs prior to RGCs death in optic nerve injury model in rats (Soto et al. 2008; Weishaupt et al. 2005; Naskar and Thanos 2006).

One of the integral components of a growth cone is GAP-43, which plays an important role in regulation of axon growth in the neurons (Benowitz and Routtenberg 1997). Studies have shown a reduction in GAP-43 expression in the E14.5 retina of Brn3b deficient mice (Mu et al. 2001). The rat GAP-43 promoter has a putative Brn3 binding site, located at 735 bp upstream from the transcription start site. This could account for the ability of transcription factor Brn3b to upregulate GAP-43 expression in PC12 cells. Our data demonstrate that Brn3b contributes to upregulation of GAP-43, and the levels of GAP-43 are sustained in Brn3b overexpressing PC12 cells under hypoxia. Increased expression of GAP-43 has been shown to enhance neurite outgrowth in PC12 cells. The current study suggests that Brn3b has the potential to be a neuroprotective agent in various neurodegenerative diseases and neuronal injury. However, detailed research on Brn3b and proteins regulated by Brn3b is needed to understand its potential neuroprotective role in different animal models of neurodegeneration and nerve injury.

It is clear from several studies that the cytoskeleton is a key target for many signaling pathways that affect neurite outgrowth. In the axonal transport machinery, microtubules and actin filaments serve as “rail road” tracks for active transport. Kinesin, dynein and myosin are ATP dependent molecular motors responsible for transport of various molecular cargos. It is known that axonal transport can be affected by variations in the components of the transport machinery. Defects in the axonal transport, such as mitochondrial dysfunction and axonal transport failure induce RGCs death in glaucoma and also contribute to the pathogenesis of a number of neurodegenerative diseases (Qu et al. 2013; Liu et al. 2012; Millecamps et al. 2013; Almasieh et al. 2012). In the current study, we demonstrate that there is a significant increase in the expression of ac-TUBA in PC12 cells overexpressing Brn3b under conditions of both normoxia and hypoxia. Acetylated α-tubulin (ac-TUBA) plays an important stabilizing role during the protrusion and elongation of axons. Acetylation of the α-tubulin using a histonedeacetylase inhibitor has been shown to stimulate anterograde as well as retrograde transport in primary cortical neurons (Gaub et al. 2010). Sengottuvel et al., (2011) have shown that a low concentration (3 nM) of the microtubule stabilizing drug, taxol, promotes neurite elongation of RGCs in culture. Another study demonstrated that total tubulin mRNA levels decrease after injury to the optic nerve but increase in those RGCs that regenerated their axons into a peripheral nerve graft (Fournier and Mckerracher 1995). Hence, the ability of Brn3b to produce an increase in ac-TUBA has implications for its axonal regenerative effects following axonal injury during hypoxia in the presence of NGF, which has not been studied earlier. However the precise mechanisms underlying the ability of transcription factor Brn3b to upregulate ac-TUBA are not completely understood and will be the subject of future studies. In the current study, two different cell lines (661W and PC12 cells) were used to study the effect of Brn3b overexpression for confirmation of a significant increase in

expression of GAP-43 and ac-TUBA in Brn3b overexpressing cells. Even though PC12 cells and 661W cells have no relation to RGCs, they could provide an insight into basic mechanisms governing cell survival and neurite outgrowth during hypoxia. One caveat to this model is that cellular and molecular processes occurring in cell lines may not be accurately reflective of what occurs *in vivo*.

PC12 cells transfected with the empty vector and maintained under hypoxia show a modest increase in ac-TUBA (not statistically significant), which was not reflected in the neurite outgrowth or number of cells with processes. On the other hand, PC12 cells transfected with empty vector and maintained under hypoxia showed a significant increase in GAP-43 expression, compared to PC12 cells maintained in normoxia, suggesting that upregulation of GAP-43 alone is not sufficient to promote neurite outgrowth under conditions of hypoxia. Overexpression of Brn3b produce an increase in the mean neurite length as well as number of cells with processes under conditions of both normoxia and hypoxia, in comparison with empty vector transfected PC12 cells. This suggests that transcription factor Brn3b could upregulate expression of several genes contributing to neurite outgrowth in PC12 cells.

In summary, this study demonstrates that overexpression of Brn3b in PC12 cells under normoxia as well as hypoxia leads to upregulation of GAP-43 and ac-TUBA and results in potentiation of neurite outgrowth. However, Brn3b mediated effects on gene expression and potential targets need to be studied in greater detail in animal models of neurodegeneration. Additional research is needed to understand the potential role of Brn3b in RGC survival and neuroprotection in glaucomatous neurodegeneration.

Figure Legends:

Figure 1: Brn3b overexpression leads to a significant increase in expression of GAP-43 and ac-TUBA in 661W cells. a. Immunoblot analysis of Brn3b-DDK tag, GAP-43, and ac-TUBA in 661W cells transfected either with the pCMV6-Empty or pCMV6-Brn3b vector, 24 h post-transfection. The blots were probed for GAPDH as a loading control. Densitometric analysis shows a significant increase of GAP-43 (**b**) and ac-TUBA **(c)** in Brn3b transfected 661W cells as compared to the empty vector transfected 661W cells. Fold change in proteins expression were shown as mean± SEM, n=3. Student's t-test was used for statistical analyses (*$p<0.05$).

Figure 2: Up-regulation of GAP-43, ac-TUBA in PC12 cells transfected with Brn3b. **a**. Immunoblot analysis of nuclear extracts for Brn3b-DDK tag expression and cytoplasmic extracts for expression of GAP-43 and ac-TUBA in PC12 cells transfected with pCMV6-Empty or pCMV6-Brn3b vectors, 24 h following transfection. TATA binding protein and GAPDH were used as the loading control. Overexpression of Brn3b in PC12 cells induced a significant up-regulation of GAP-43 (**b)** and ac-TUBA (**c**) when compared to the empty vector transfected PC12 cells. Student's t-test was used for statistical analyses (*$p<0.05$). Fold change in the specific protein expression is shown as mean ± SEM, n=3. **d**. Immunocytochemical analyses of Brn3b, GAP-43 and ac-TUBA expression after 4 days in differentiating medium with NGF in PC12 cells transfected with Brn-3b. The immunostaining was detected by using corresponding Alexa 488-, Alexa 547- and Alexa 633- conjugated donkey anti-IgG secondary antibodies. Green: Brn-3b; Pink: GAP-43; Red: ac-TUBA; Blue: DAPI. Fluorescence images were taken in a confocal microscope and merged images of the different proteins immunofluorescence with DIC and DAPI was obtained. Scale bar indicates 20μm (n=3).

Figure 3: Overexpression of Brn3b persisted under conditions of hypoxia in PC12 cells transfected with pCMV6-Brn3b. a. Experimental scheme used for assessing protein expressions in PC12 cells under conditions of hypoxia. Cells were seeded on poly-D-lysine coated 100 mm dishes for western blot analyses and transfected with either pCMV6-Empty or pCMV6-Brn3b (5 μg). Following transfection, cells were maintained in differentiating medium with NGF (100ng/ml) overnight and were either maintained in normoxia or subjected to oxygen glucose deprivation condition (0.5% O_2 and 5% CO_2) for 2 h . Cytoplasmic and nuclear extracts were prepared and used for various immunoblot analyses. In separate experiments for immunocytochemistry, pCMV6-Empty and pCMV6-Brn3b transfected cells were maintained in differentiating medium with NGF (100 ng/ml) for 4 days. The transfected cells were subjected to conditions of normoxia or hypoxia for 2 h, as described earlier. PC12 cells were then fixed and expression levels of specific proteins were analyzed by using immunocytochemistry. **b**. Immunoblot analysis of Brn3b DDK tag in PC12 cells transfected with either pCMV6-Empty or pCMV6-Brn3b and maintained under either normoxia or hypoxia (n=3). **c**. PC12 cells seeded on coverslips were transfected with either pCMV6-Empty or pCMV6-Brn3b, subjected to either normoxia or hypoxia and immunostained using specific custom-made antibody to Brn3b. A marked increase in Brn3b staining was observed in Brn3b overexpressing cells in normoxia and in hypoxia when compared to cells overexpressing the empty vector in both the conditions (n=3).

Figure 4: Transcription factor Brn3b overexpression mediated increase in growth cone marker GAP-43 in PC12 cells under conditions of hypoxia. a. Western blot analysis was carried out to detect the protein level of GAP-43 in PC12 cells overexpressing pCMV6-Empty or pCMV6-Brn3b vector, and maintained for 2 h in normoxia and hypoxia, 24 h post-transfection. Calnexin, a housekeeping gene, served as a loading control. Densitometric analysis shows a significant

increase in GAP-43 expression (**b**) in Brn3b overexpressing PC12 cells when compared to PC12 cells overexpressing empty vector in normoxia. One way ANOVA with Student-Newman-Keuls multiple comparison test was used for statistical analysis (*$p<0.05$). The fold change in GAP-43 expression was shown as mean ± SEM, (n=7). **c**. PC12 cells transfected with pCMV6-Empty or pCMV6-Brn3b maintained in differentiating medium for 4 days. On the fifth day, one set of cells were maintained in normoxia, while another set was subjected to conditions of hypoxia for 2h. Cells were immunostained for GAP-43 expression (pseudocolor: pink) using GAP-43 antibody. A dramatic increase in GAP-43 staining and neurite elongation was observed in PC12 cells overexpressing Brn3b in normoxia and in hypoxia as compared to PC12 cells overexpressing the empty vector in both conditions. Scale bar indicates 20μm (n=5).

Figure 5: Brn3b promoted an increase in expression ac-TUBA in PC12 cells under conditions of normoxia and hypoxia. a. Immunoblot analysis of total cellular extracts of PC12 cells overexpressing pCMV6-Empty or pCMV6-Brn3b vector, maintained under either normoxia or hypoxia. The blots were probed for calnexin as a loading control. A significant increase in ac-TUBA expression (**b**) was observed in PC12 cells overexpressing Brn3b in normoxia as well as in hypoxia. Mann Whitney Rank Sum test was used for statistical analysis (*$p<0.05$). Fold change in ac-TUBA was shown as mean ± SEM, (n=5). **c**. Immunostaining of ac-TUBA (red fluorescence) was carried out in PC12 cells transfected with pCMV6-Empty or pCMV6-Brn3b vector and maintained in differentiating medium for 4 days, followed by 2h in normoxia or hypoxia. A prominent increase in immunostaining for ac-TUBA was observed in PC12 cells overexpressing Brn3b in normoxia as well as in hypoxia as compared to PC12 cells overexpressing empty vector in both the conditions. Scale bar indicates 20μm (n=5).

Figure 6: Increase in mean neurite length and number of cells having processes in Brn3b overexpressing PC12 cells in normoxic and hypoxic conditions. Mean neurite length **(a)** and number of cells having processes **(b)** was measured at 10 different fields in each coverslip with an average of 10 cells per field using NIH Image J. The mean maximum neurite length **(a)** and mean number of cells having processes **(b)** values are shown as mean ±SEM, n=5 (total number of cells counted= 612). **a**. For statistical analysis, Kruskal –Wallis one way ANOVA was used (*$p<0.05$). A statistically significant increase in neurite length was observed in PC12 cells overexpressing Brn3b in both normoxia and hypoxia conditions as compared to PC12 cells overexpressing the empty vector in both the conditions. **b**. For statistical analysis one way ANOVA followed by Student-Newman-Keuls multiple comparison test was used (*$p<0.05$).

Figure 7: Transcription factor Brn3b overexpression (in the presence of NGF) protects against loss of cell viability under conditions of hypoxia: Cell proliferation assay (MTT assay) was carried out to determine viability of PC12 cells subjected either normoxia or hypoxia after transfection with either the empty vector or pCMV6-Brn3b. The Y axis depicts absorbance values at 490 nm indicative of cell viability. **a**. Cell viability was tested in the presence of NGF: Four experimental groups were tested: PC12 cells overexpressing pCMV6-Empty or pCMV6-Brn3b vector under conditions of either normoxia or hypoxia.**b**. Cell viability was presented in the absence of NGF. Four experimental groups were tested including: PC12 cells overexpressing pCMV6-Empty or pCMV6-Brn3b vector under conditions of normoxia and hypoxia. One way ANOVA with Student-Newman-Keuls multiple comparison test was used for statistical analysis (*$p<0.05$). Absorbance at 490 nm (indicative of cell viability) was shown as mean± SEM, (n=16).

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Conflict of Interest:

The authors declare that they have no conflict of interest

References

1. Acker T, Acker H (2004) Cellular oxygen sensing need in CNS function: physiological and pathological implications. J. Exp. Biol. 207(Pt 18), 3171-3188
2. Adler E M, Gough N R, Blundon J A. (2006) Differentiation of PC 12 Cells. Sci STKE. (351):tr9.
3. Almasieh M, Wilson AM, Morquette B, Cueva Vargas JL, Di Polo A (2012) The molecular basis of retinal ganglion cell death in glaucoma. Prog. Retin. Eye Res.31:152-181
4. Alvarez-Tejado M, Naranjo-Suárez S, Jiménez C, Carrera A C, Landázuri M O & del Peso L (2001) OGD Induces the Activation of the Phosphatidylinositol 3-Kinase/Akt Cell Survival Pathway in PC12 Cells: Protective role in apoptosis. J. Biol. Chem. 276(25), 22368-22374
5. Barnat M, Enslen H, Propst F, Davis RJ, Soares S, Nothias F (2010) Distinct roles of c-Jun N-Terminal Kinase Isoforms in Neurite Initiation and Elongation during Axonal Regeneration. J. Neurosci. 30(23):7804-16. doi:10. 1523/JNEUROSCI.0372-10.2010
6. Benowitz L I, & Routtenberg A (1997) GAP-43: an intrinsic determinant of neuronal development and plasticity. Trends Neurosci. 20(2), 84-91

7. Berg A, Zelano J, Pekna M, Wilhelmsson U, Pekny M, Cullheim S (2013) Axonal Regeneration after Sciatic Nerve Lesion Is Delayed but Complete in GFAP- and Vimentin- Deficient Mice. PLoS One, 8(11):e79395. doi:10.1371/journal.pone.0079395
8. Begg I S, Drance S M (1971) Progress of the glaucomatous process related to recurrent ischaemic changes at the optic disc. Exp Eye Res. 11(1), 141
9. Camp A S, Ruggeri M, Munguba G C, Tapia M L, John S W M, Bhattacharya S K, & Lee R K (2011) Structural Correlation Between the Nerve Fiber Layer and Retinal Ganglion Cell Loss in Mice with Targeted Disruption of the Brn3b Gene. Invest Ophthalmol & Vis Sci. 52(8), 5226-5232
10. Cho K S, Yang L, Lu B, Feng Ma, H, Huang X, Pekny M, & Chen D F (2005) Re-establishing the regenerative potential of central nervous system axons in postnatal mice. Journal of Cell Science. 118(5), 863-872
11. Correia S C, Carvalho C, Cardoso S, Santos R X, Placido A I, Candeias E, Duarte A I, Moeira P I (2013) Defective HIF Signaling Pathway And Brain Response To Hypoxia in Neurodegenerative Diseases: Not an “Iffy” Question! In. Curr Pharm. Des. 19 (38) :6809-22
12. Cowley S, Paterson H, Kemp P, Marshall C J (1994) Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell. 77(6), 841-852
13. Czyzyk-Krzeska M F, Furnari B A, Lawson E E & Millhorn D E (1994) OGD increases rate of transcription and stability of tyrosine hydroxylase mRNA in pheochromocytoma (PC12) cells. J Biol. Chem. 269(1), 760-764
14. Das K P, Freudenrich T M & Mundy W R (2004) Assessment of PC12 cell differentiation and neurite growth: a comparison of morphological and neurochemical measures.

Neurotoxicology and Teratology. 26(3), 397-406

15. de Lima S. KoriyamaY, Kurimoto T, Oliveira J T, Yin Y, Li Y, Gilbert HY, Faqiolini M, Martinez A M, Benowitz L I (2012) Full-length axon regeneration in the adult mouse optic nerve and partial recovery of simple visual behaviors. Proc. Natl. Acad. Sci. USA. 109(23), 9149-9154
16. Dompierre J P, Godin J D, Charrin B C, Cordelières F P, King S J, Humbert S & Saudou F (2007) Histone Deacetylase 6 Inhibition Compensates for the Transport Deficit in Huntington's Disease by Increasing Tubulin Acetylation. J. Neurosci. 27(13), 3571-3583
17. Erkman, L, Yates P. A, McLaughlin T, McEvilly R J, Whisenhunt T, O'Connell S M, Krones A I, Kirby M A, Rapaport D H, Bermingham J R, O'Leary D D, Rosenfeld M G (2000) A POU Domain Transcription Factor Dependent Program Regulates Axon Pathfinding in the Vertebrate Visual System. Neuron. 28(3), 779-792
18. Erkman L, McEvilly R J, Luo L, Ryan A K, Hooshmand F, O'Connell S M, Keithley E M, Rapaport D H, Ryan AF, Rosenfeld M G (1996) Role of transcription factors brn-3.1 and brn-3.2 in auditory and visual system development Nature. 381(6583), 603-606
19. Fedtsova N G, & Turner E E (1995) Brn-3.0 expression identifies early post-mitotic CNS neurons and sensory neural precursors. Mech. Dev. 53(3), 291-304
20. Fournier AE, McKerracher L (1995) Tubulin expression and axonal transport in injured and regenerating neurons in. Biochem. Cell Biol. 73(9-10), 659-664
21. Gan L, Wang S W, Huang Z, & Klein W H (1999) POU Domain Factor Brn-3b Is Essential for Retinal Ganglion Cell Differentiation and Survival but Not for Initial Cell Fate Specification. Developmental Biology. 210(2), 469-480
22. Gan L, Xiang M, Zhou L, Wagner D S, Klein W H, & Nathans J (1996) POU domain

factor brn-3b is required for the development of a large set of retinal ganglion cells Proc. Natl. Acad. Sci. USA. 93(9), 3920-3925

23. Gaub P, Tedeschi A, Puttagunta R, Nguyen T, Schmandke A, Giovanni D S (2010) HDAC inhibition promotes neuronal outgrowth and counteracts growth cone collapse. Cell Death Differ. 17(9), 1392-1408
24. Gerrero, M R, McEvilly R J, Turner E, Lin C R, O'Connell S, Jenne K J, Hobbs M V, Rosenfeld M. G. (1993) Brn-3.0: a POU-domain protein expressed in the sensory, immune, and endocrine systems that functions on elements distinct from known octamer motifs. Proc. Natl. Acad. Sci. U S A. 90(22), 10841-10845
25. Gupta N, Yucel Y H (2007) Glaucoma as a neurodegenerative disease. Curr. Opin. Ophthalmol. 18(2), 110-114
26. Hayreh S S (2004) Posterior Ciliary Artery Circulation in Health and Disease. The Weisenfeld Lecture. Invest. Ophthalmol. Vis. Sci. 45(3), 749-757
27. Huang E J, Zang K, Schmidt A, Saulys A, Xiang M, & Reichardt L F (1999) POU domain factor Brn-3a controls the differentiation and survival of trigeminal neurons by regulating Trk receptor expression. Development. 126(13), 2869-2882
28. Krishnamoorthy R R, Crawford M J, Chaturvedi M M, Jain S K, Aggarwal B B, Al-Ubaidi M R, & Agarwal N (1999) Photo-oxidative Stress Down-modulates the Activity of Nuclear Factor-κB via Involvement of Caspase-1, Leading to Apoptosis of Photoreceptor Cells. J. Biol. Chem. 274(6), 3734-3743
29. Kurimoto T, Yin Y, Omura K, Gilbert H y, Kim D, Cen L P, Moko L, Kugler S, Benowitz L I (2010) Long-Distance Axon Regeneration in the Mature Optic Nerve: Contributions of Oncomodulin, cAMP, and pten Gene Deletion. J. Neurosci. 30(46), 15654-

15663

30. Latchman D S (1999) POU family transcription factors in the nervous system. J. Cell. Physiol. 179: 126–133
31. Lee V, Shelanski M L, Greene L A (1977) Specific neural and adrenal medullary antigens detected by antisera to clonal PC12 pheochromocytoma cells. Proc. Natl. Acad. Sci. U S A. 74(11), 5021-5025
32. Liu X A, Rizzo V, Puthanveettil SV (2012) Pathologies of Axonal Transport in Neurodegenerative Diseases. Transl. Neurosci. 3(4), 355-372
33. McEvilly R J, Erkman L, Luo L, Sawchenko P E, Ryan A F, & Rosenfeld M G (1996) Requirement for brn-3.0 in differentiation and survival of sensory and motor neurons Nature. 384(6609), 574-577
34. Millecamps S, Julien J P (2013) Axonal transport deficits and neurodegenerative diseases. Nat. Rev. Neurosci. 14(3), 161-176
35. Mingorance-Le Meur A, Mohebiany A N, & O'Connor T P (2009) Varicones and growth cones: two neurite terminals in PC12 cells. PLoS One. 4(2), e4334
36. Mu X, Beremand P D, Zhao S, Pershad R, Sun H, Scarpa A, Liang S, Thomas T L, Klein W H (2004) Discrete gene sets depend on POU domain transcription factor Brn3b/Brn-3.2/POU4f2 for their expression in the mouse embryonic retina. Development. 131(6), 1197- 1210
37. Mu X, Zhao S, Pershad R, Hsieh TF, Scarpa A, Wang SW, White RA, Beremand PD,Thomas TL, Gan L, Klein WH. (2001) Gene expression in the developing mouse retina by EST sequencing and microarray analysis Nucleic Acids Res. 29(24), 4983-4993
38. Naskar R, Thanos S (2006) Retinal gene profiling in a hereditary rodent model of

elevated intraocular pressure. Mol. Vis. 12:1199-1210

39. Ninkina N N, Stevens G E M, Wood J N, & Richardson W D (1993) A novel Brn3-Iike POU transcription factor expressed in subsets of rat sensory and spinal cord neurons. Nucleic Acids Res. 21(14), 3175-3182
40. Nishimura T, Ishima T, Iyo M, & Hashimoto K (2008) Potentiation of Nerve Growth Factor-Induced Neurite Outgrowth by Fluvoxamine: Role of Sigma-1 Receptors, IP3 Receptors and Cellular Signaling Pathways. PLoS ONE. 3(7), e2558
41. Pang, L, Sawada T, Decker S J, Saltiel A R(1995) Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. J. Biol. Chem. 270(23), 13585- 13588
42. Pool M, Thiemann J, Bar-Or A, Fournier AE (2008) NeuriteTracer: A Novel ImageJ plugin for automated quantification of neurite outgrowth. J. Neurosci. Methods. 168(1) :134-9
43. Prasanna G, Krishnamoorthy R R, Clark AF, Wordinger R J, Yorio T (2002) Human Optic Nerve Head Astrocytes as a Target for Endothelin-1. Invest. Ophthalmol. Vis. Sci. 43(8):2704-13
44. Qu C, Dwyer T, Shao Q, Yang T, Huang H, & Liu G (2013) Direct binding of TUBB3 with DCC couples netrin-1 signaling to intracellular microtubule dynamics in axon outgrowth and guidance. J. Cell Sci. 126(14), 3070-3081
45. Sengottuvel V, Leibinger M, Pfreimer M, Andreadaki A, & Fischer D (2011) Taxol Facilitates Axon Regeneration in the Mature CNS. J. Neurosci. 31(7), 2688-2699
46. Soto I, Oglesby E, Buckingham B P, Son J L, Roberson E D O, Steele M R, Inman D M, Vetter M L, Horner P J, Marsh-Armstrong N (2008) Retinal Ganglion Cells Downregulate Gene Expression and Lose Their Axons within the Optic Nerve Head in a

Mouse Glaucoma Model. J. Neurosci. 28(2), 548-561

47. Sun F, Park K K, Belin S, Wang D, Lu T, Chen G, Zhang K, Yenug C, Feng G, Yankner B A, He Z (2011) Sustained axon regeneration induced by co-deletion of PTEN and SOCS3. Nature. 480(7377), 372-375
48. Takebayashi M, Hayashi T, & Su T P (2002) Nerve Growth Factor-Induced Neurite Sprouting in PC12 Cells Involves ς-1 Receptors: Implications for Antidepressants. J. Pharmacol. Exp. Ther. 303(3), 1227-1237
49. Torres-Espin A, Santos D, Gonzalez-Perez F, del-Valle J, Navarro X (2014) Neurite-J: An Image-J plug-in for axonal growth analysis in organotypic cultures. J. Neurosci Methods. 236:26-39
50. Turner E E, Jenne K J & Rosenfeld M G (1994) Brn-3.2: a Brn-3-related transcription factor with distinctive central nervous system expression and regulation by retinoic acid. Neuron. 12(1), 205-218
51. Wang S W, Mu X, Bowers W J, Kim D S, Plas D J, Crair M C, Federoff H J, Gam L, Klein W H (2002) Brn3b/Brn3c double knockout mice reveal an unsuspected role for Brn3c in retinal ganglion cell axon outgrowth. Development. 129(2), 467-477
52. Wang S W, Gan, L, Martin S E, & Klein W H (2000) Abnormal Polarization and Axon Outgrowth in Retinal Ganglion Cells Lacking the POU-Domain Transcription Factor Brn-3b. Molecular and Cellular Neuroscience, 16(2), 141-156
53. Weishaupt J, Klöcker N, & Bähr M (2005) Axotomy-induced early down-regulation of POU-IV class transcription factors Brn-3a and Brn-3b in retinal ganglion cells. J. Mol. Neurosci. 26(1), 17--26
54. Wu C L, Chou Y H, Chang Y J, Teng N Y, Hsu H L, & Chen L (2012) Interplay between

Cell Migration and Neurite Outgrowth Determines SH2B1β-Enhanced Neurite Regeneration of Differentiated PC12 Cells. PLoS ONE. 7(4), e34999

55. Xiang M, Zhou L, Macke J P, Yoshioka T, Hendry S H, Eddy R L, Shows T B, Nathans J (1995) The Brn-3 family of POU-domain factors: primary structure, binding specificity, and expression in subsets of retinal ganglion cells and somatosensory neurons. J. Neurosci. 15(7 Pt 1), 4762-4785
56. Xiang M, Gan L, Li D, Zhou L, Chen Z Y, Wagner D, O'Malley Jr B W, Nathans J (1997) Role of the Brn-3 family of POU-domain genes in the development of the auditory/vestibular, somatosensory, and visual systems. Cold Spring Harb. Symp. Quant. Biol. 62, 325-336
57. Xiang M, Gan L, Zhou L, Klein W H, & Nathans J (1996) Targeted deletion of the mouse POU domain gene Brn-3a causes selective loss of neurons in the brainstem and trigeminal ganglion, uncoordinated limb movement, and impaired suckling. Proc. Natl. Acad. Sci. USA. 93(21), 11950-11955
58. Xiang M, Zhou H, & Nathans J (1996) Molecular biology of retinal ganglion cells. Proc. Natl. Acad. Sci, USA. 93(2), 596-601
59. Xiang M, Zhou L, Peng Y W, Eddy R L, Shows T B, & Nathans J (1993) Brn-3b: a POU domain gene expressed in a subset of retinal ganglion cells. Neuron. 11(4), 689-701
60. Xiang M (1998) Requirement for brn-3b in early differentiation of postmitotic retinal ganglion cell precursors Dev. Biol. 197(2), 155-169
61. Yin Y, Cui Q, Gilbert H y, Yang Y, Yang Z, Berlinicke C, Li Z, Zaverucha-do-Valle C, He H, Petkova V, Zack D J, Benowitz L I (2009) Oncomodulin links inflammation to optic nerve regeneration. Proc. Natl. Acad. Sci. USA. 106(46), 19587-19592

Figures

Figure 1:

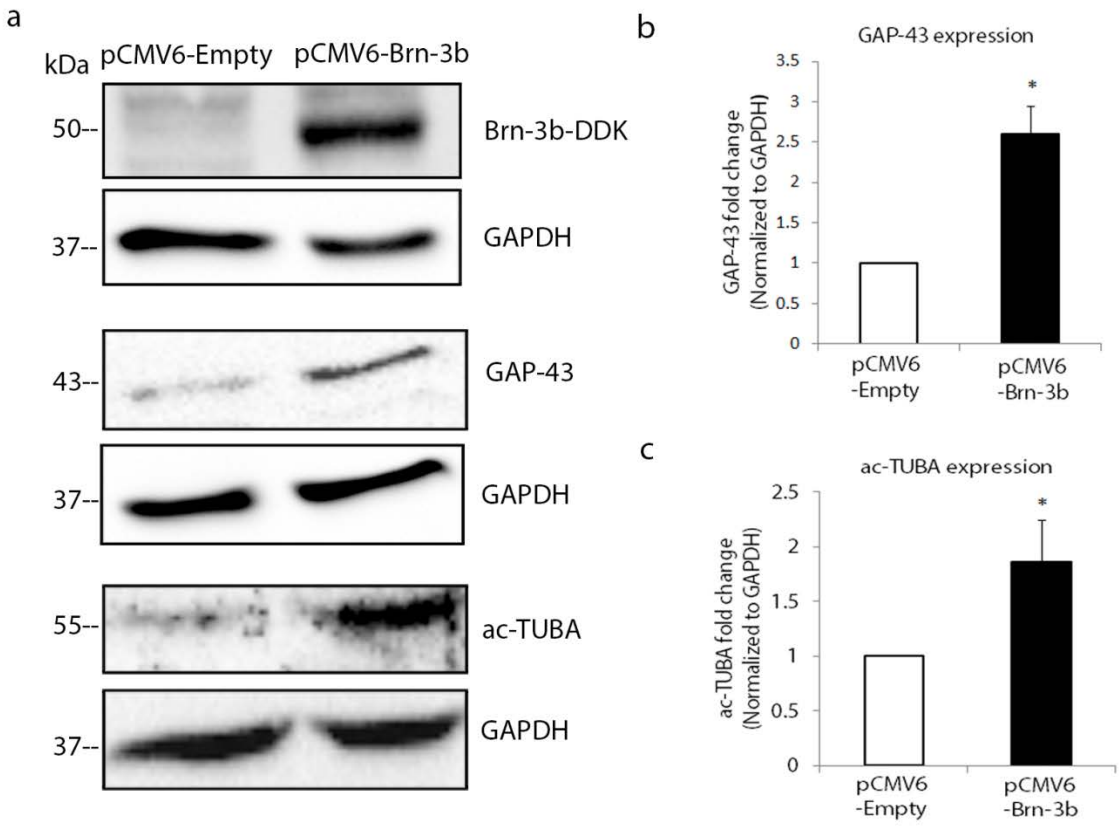
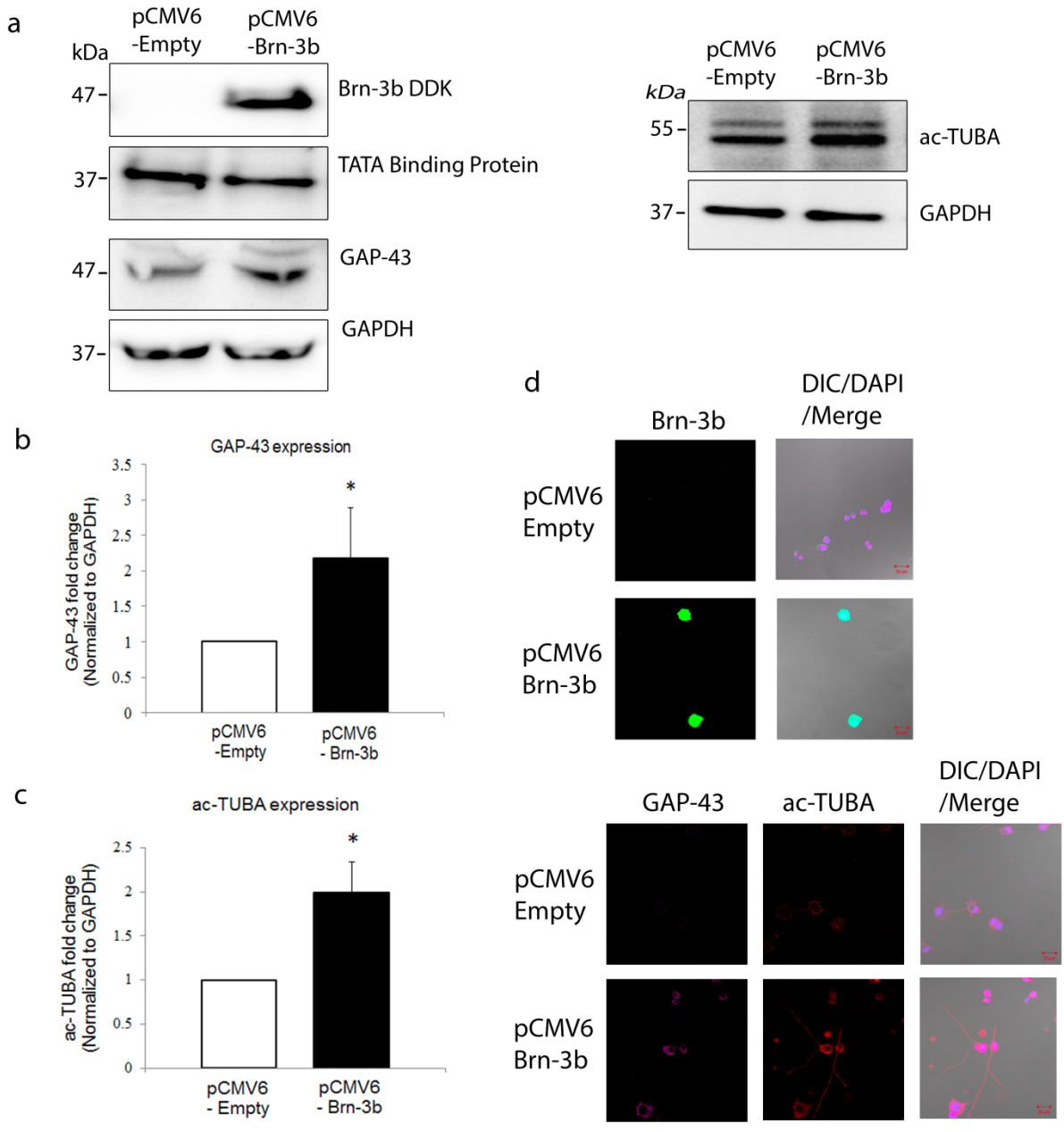


Figure 2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pCMV6 -Empty
pCMV6 -Brn-3b
kDa
47
Brn-3b DDK
37
TATA Binding Protein
47
GAP-43
37
GAPDH
pCMV6 -Empty
pCMV6 -Brn-3b
kDa
55
ac-TUBA
37
GAPDH
b
GAP-43 expression
GAP-43 fold change (Normalized to GAPDH)
3.5
3
2.5
2
1.5
1
0.5
0
*
pCMV6 -Empty
pCMV6 - Brn-3b
c
ac-TUBA expression
ac-TUBA fold change (Normalized to GAPDH)
2.5
2
1.5
1
0.5
0
*
pCMV6 - Empty
pCMV6 - Brn-3b
d
Brn-3b
DIC/DAPI /Merge
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GAP-43
ac-TUBA
DIC/DAPI /Merge
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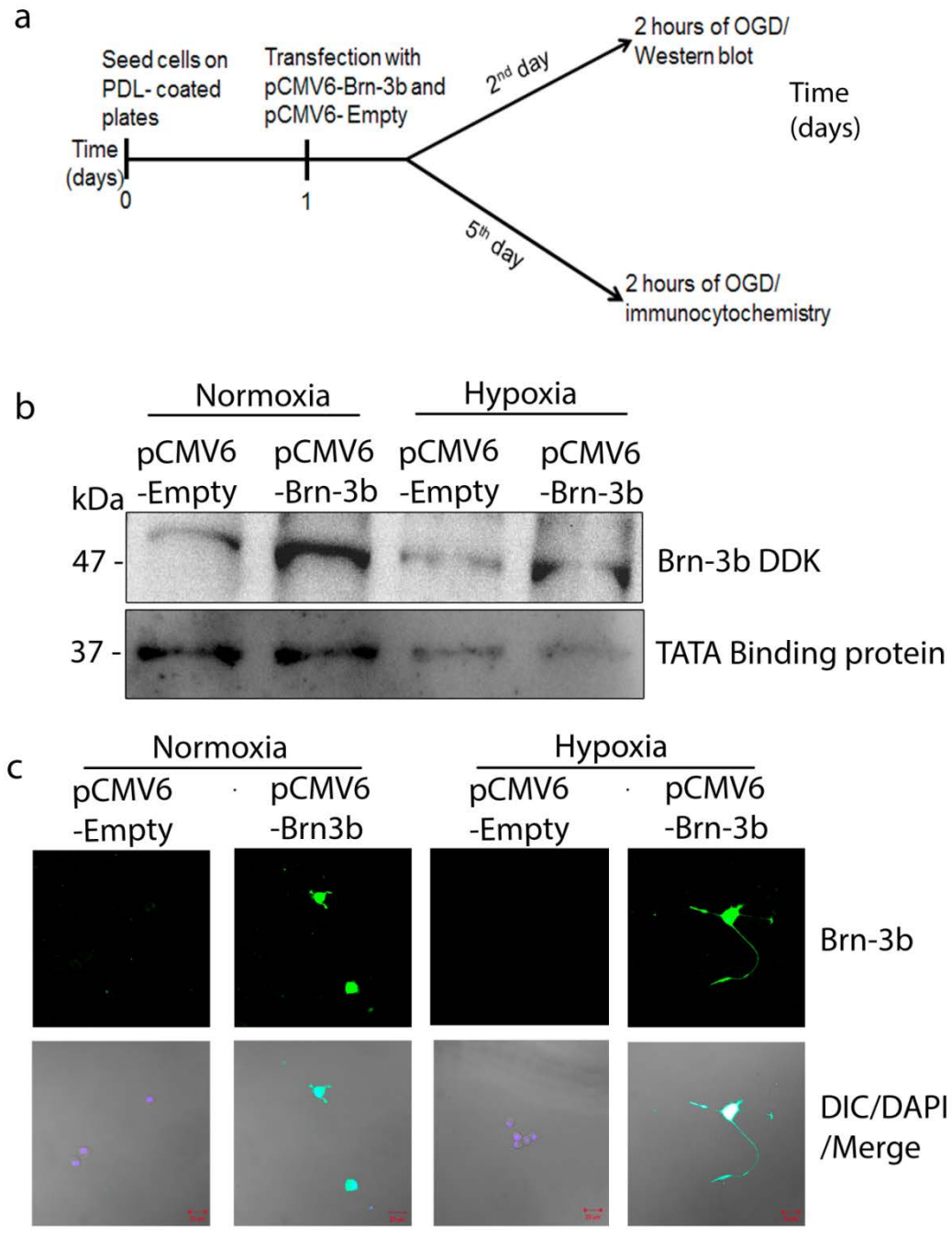
pCMV6 Brn-3b

Figure 3:


a
Seed cells on PDL- coated plates
Transfection with pCMV6-Brn-3b and pCMV6- Empty
Time (days)
0
1
2nd day
5th day
2 hours of OGD/ Western blot
2 hours of OGD/ immunocytochemistry
Time (days)
b
Normoxia
Hypoxia
pCMV6 -Empty
pCMV6 -Brn-3b
pCMV6 -Empty
pCMV6 -Brn-3b
kDa
47 -
37 -
Brn-3b DDK
TATA Binding protein
c
Normoxia
Hypoxia
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pCMV6 -Brn3b
pCMV6 -Empty
pCMV6 -Brn-3b
Brn-3b
DIC/DAPI /Merge

Figure 4:

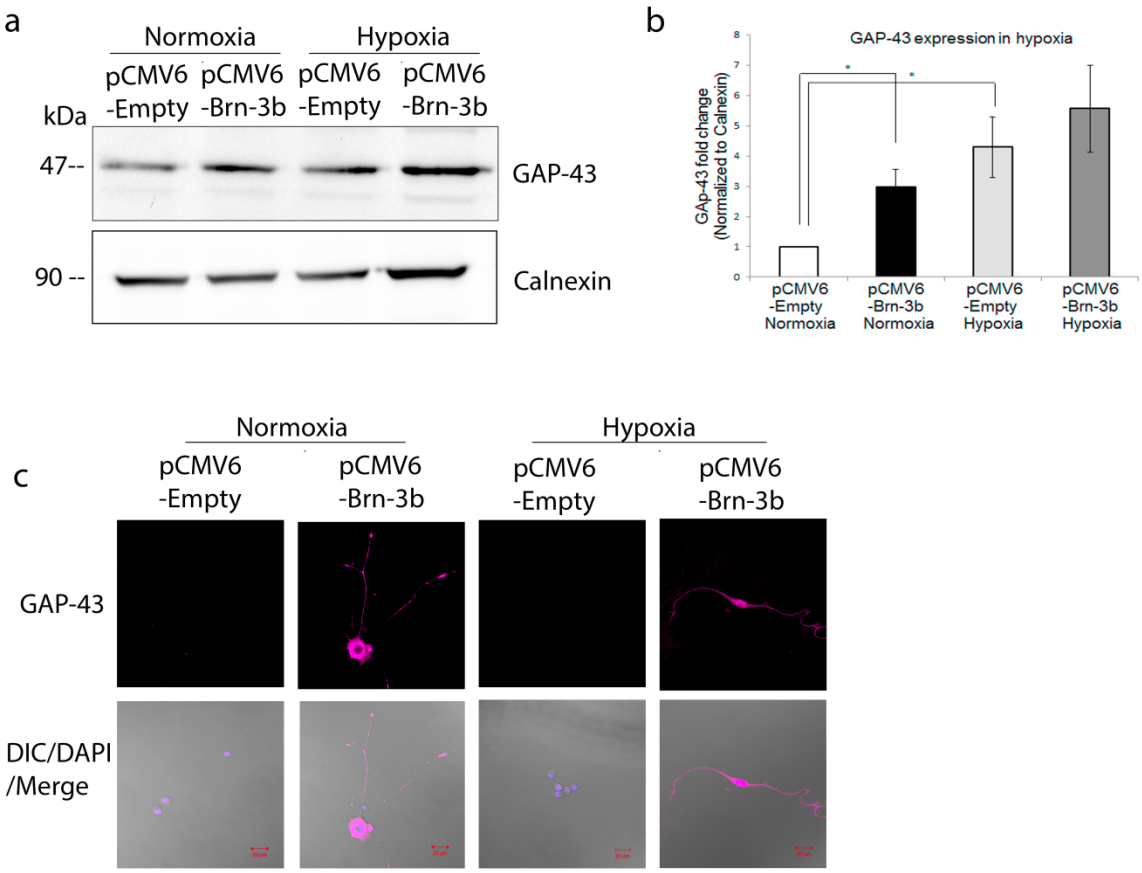

a
Normoxia
Hypoxia
pCMV6 -Empty
pCMV6 -Brn-3b
pCMV6 -Empty
pCMV6 -Brn-3b
kDa
47--
GAP-43
90 --
Calnexin
b
GAP-43 expression in hypoxia
GAp-43 fold change (Normalized to Calnexin)
pCMV6 -Empty Normoxia
pCMV6 -Brn-3b Normoxia
pCMV6 -Empty Hypoxia
pCMV6 -Brn-3b Hypoxia
c
Normoxia
Hypoxia
pCMV6 -Empty
pCMV6 -Brn-3b
pCMV6 -Empty
pCMV6 -Brn-3b
GAP-43
DIC/DAPI /Merge

Figure 5:

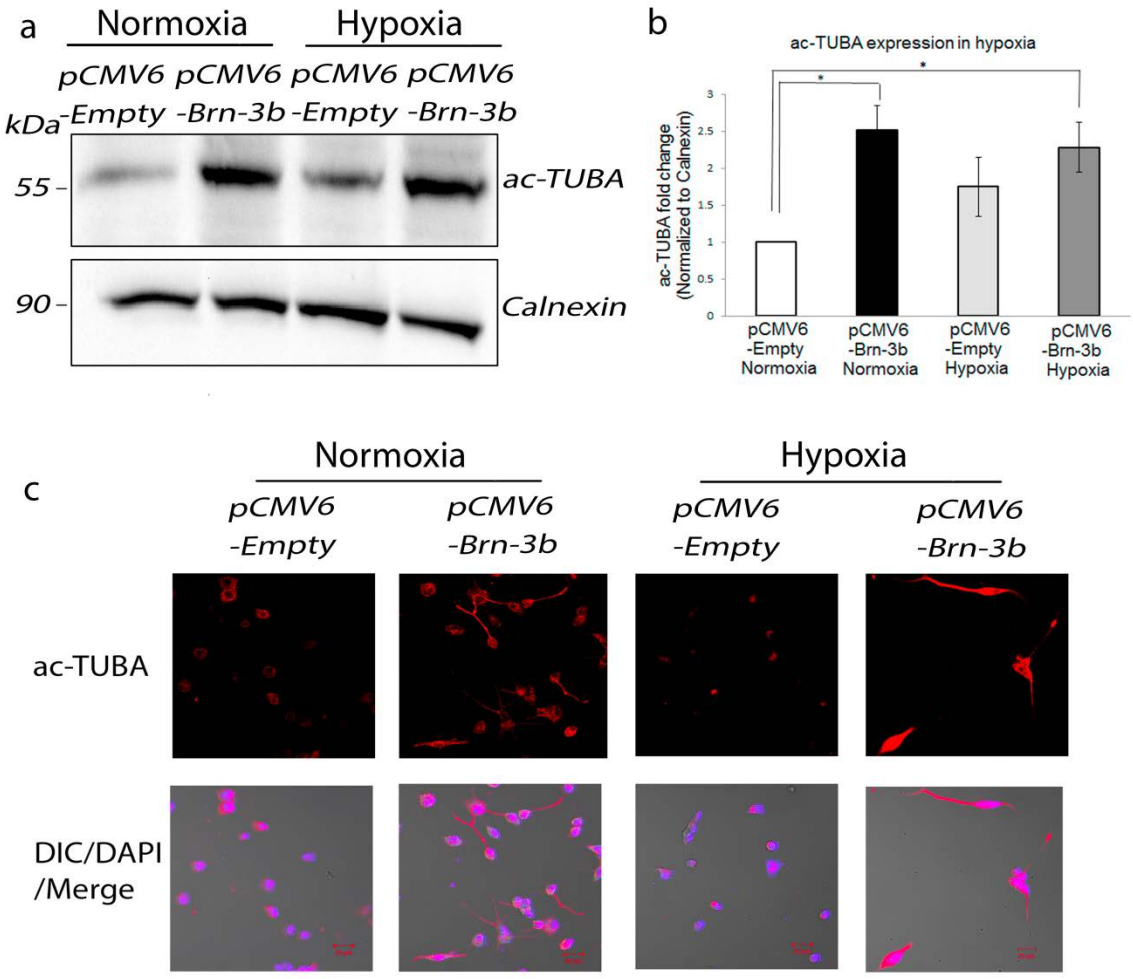

a
Normoxia
Hypoxia
pCMV6 -Empty
pCMV6 -Brn-3b
pCMV6 -Empty
pCMV6 -Brn-3b
kDa
55
ac-TUBA
90
Calnexin
b
ac-TUBA expression in hypoxia
ac-TUBA fold change (Normalized to Calnexin)
pCMV6 -Empty Normoxia
pCMV6 -Brn-3b Normoxia
pCMV6 -Empty Hypoxia
pCMV6 -Brn-3b Hypoxia
c
Normoxia
Hypoxia
pCMV6 -Empty
pCMV6 -Brn-3b
pCMV6 -Empty
pCMV6 -Brn-3b
ac-TUBA
DIC/DAPI /Merge

Figure 6:

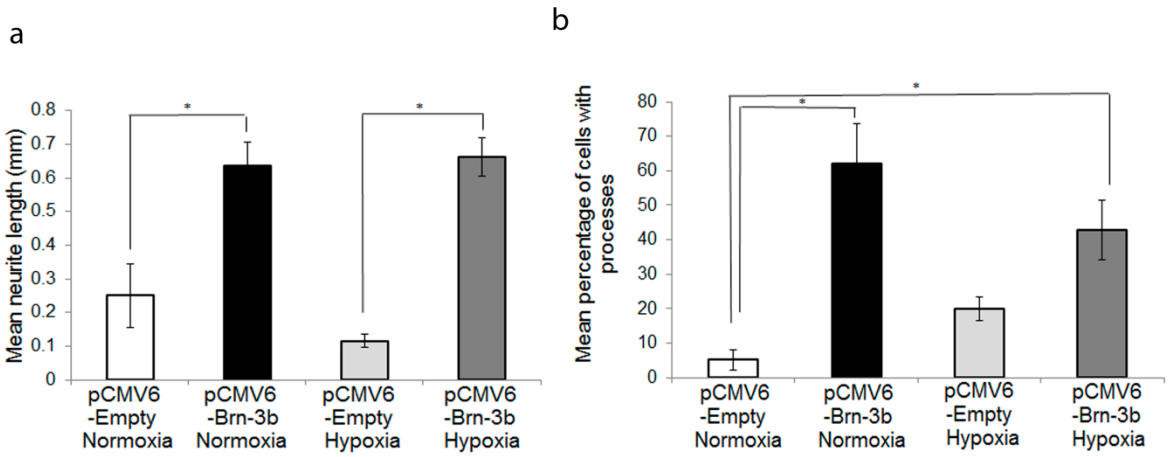
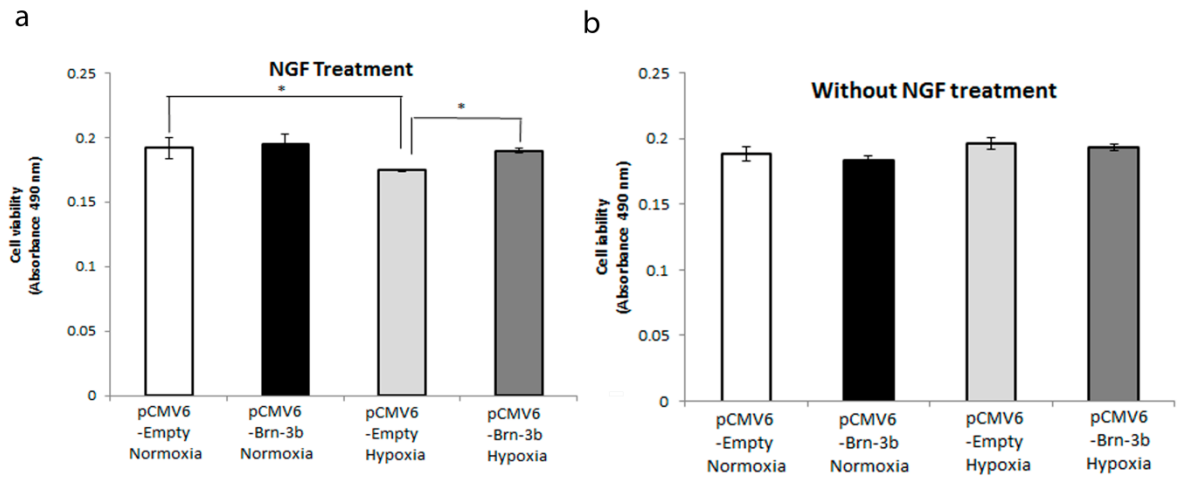


Figure 7:



CHAPTER III

BCL-2, BCL-XL AND P-AKT ARE INVOLVED IN NEUROPROTECTIVE EFFECTS OF TRANSCRIPTION FACTOR BRN3B IN AN OCULAR HYPERTENSION RAT MODEL OF GLAUCOMA

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Abstract

Purpose: Brn3b is a class IV POU domain transcription factor that plays an important role in the development of retinal ganglion cells (RGCs), RGC survival, particularly in axon growth and pathfinding. Our previous study has shown that recombinant adeno-associated virus serotype 2 (AAV-2) mediated overexpression of Brn3b in RGCs promotes neuroprotection in a rodent model of glaucoma. However, the mechanisms underlying neuroprotection of RGCs in rats overexpressing Brn3b in animal models of glaucoma remain largely unknown. The goal of this study was to determine the involvement of key survival proteins including Bcl-2, Bcl-xL and p-AKT in neuroprotection of RGCs overexpressing AAV-Brn3b during intraocular pressure (IOP) elevation in Brown Norway rats.

Methods: One eye of Brown Norway rats was injected with AAV constructs encoding either the GFP (rAAV-hsyn-GFP) or Brn3b (rAAV-hsyn-Brn3b). Expression of anti-apoptotic proteins including B cell lymphoma/leukemia-2 (Bcl-2) family proteins and p-AKT were observed by immunostaining rat retinas overexpressing Brn3b. In a different set of experiments, intraocular pressure was elevated in one eye of Brown Norway rats (Rattus norvegicus), which was followed by intravitreal injection with AAV constructs encoding either the GFP (rAAV-CMV-GFP) or Brn3b (rAAV-CMV-Brn3b). Retinal sections were stained for pro-survival factors including Bcl-2, Bcl-X_L and p-AKT.

Results: AAV-mediated expression of the Brn3b protein promoted significant upregulation of the Bcl-2 protein and increased expression of p-AKT in RGCs of Brown Norway rats. In addition, AAV-mediated Brn3b expression also produced increased expression of Bcl-2, Bcl-xL and p-AKT in the RGC layer, following IOP elevation in Brown Norway rats.

Conclusion: Adeno-associated virus mediated Brn3b protein overexpression may promote neuroprotection by upregulating key anti-apoptotic proteins of the Bcl-2, Bcl-xL and p-AKT family in animal models of glaucoma.

Introduction

Glaucoma, a leading cause of irreversible blindness, is projected to affect 79.6 million people by 2020 (1). It is a heterogeneous group of optic neuropathies characterized by axon degeneration, cupping of the optic disk and loss of retinal ganglion cells, which contributes to visual field defects and vision loss (1, 2). Increased IOP remains a major risk factor in glaucoma, and most current treatments are aimed at reduction of IOP in patients. Multiple theories has been proposed to understand the pathophysiology of glaucoma, including mechanical stress due to elevated IOP, disruption of retrograde transport of neurotrophins (3), ocular ischemia (4-6), glutamate-induced excitotoxicity (7) and oxidative stress (8-10). IOP management in glaucoma patients is aimed at limiting the initial insult, which produces optic nerve degeneration and RGCs apoptosis. Despite current strategies to lower IOP in glaucoma patients, some neurodegenerative effects at the optic nerve and retina continue to occur in many patients. Hence, understanding molecular mechanisms contributing to RGC apoptosis can lead to development of more effective treatments for glaucoma patients (11).

The brain-specific homeobox/POU domain protein (Brn) family of class-4 POU domain transcription factor consists of three closely related genes Brn3a, Brn3b and Brn3c (12, 13). Brn3 genes are expressed in discrete and overlapping pattern in the developing and mature mammalian nervous system (14-18). In the retina, Brn3b is specifically expressed in retinal ganglion precursor neurons as well as mature RGCs (15, 18, and 19). Past studies have shown that Brn3b plays an

important role in regulation of RGCs survival, axon growth and pathfinding (16, 20, 21). A prominent phenotype in Brn3b deficient mice, but not Brn3a- or Brn3c-deficient mice, was loss of about 70% RGCs between E15.5 and birth (19, 22, and 23). Consistent with a reduction in number of RGCs, a decrease in optic nerve fibers and thinning of the optic nerve was also observed in Brn3b deficient mice (19). Our previous study demonstrated the neuroprotective effects of AAV-mediated Brn3b overexpression in an ocular hypertension rat model of glaucoma (24). But the mechanisms contributing to neuroprotective effects of Brn3b overexpression in ocular hypertension model of glaucoma are unknown. One obvious candidate gene responsible for the neuroprotective effects of Brn3b is the anti-apoptotic protein, B cell leukemia/lymphoma 2 (Bcl-2).

The Bcl-2 gene family encodes proteins with similar structural domains (Bcl-2 homology domains designated BH1 to BH4) that play a key role in the regulation of cell survival. Typically, proteins that contain all four domains (e.g. Bcl-2, Bcl-XL and Mcl-1) are anti-apoptotic, while those that contain less are pro-apoptotic (e.g. Bax, Bad, Bim, Bid and Puma). The proto-oncogene Bcl-2 and its pro- and anti-apoptotic family members are major regulators of cell death and survival. However, in deciding cell fate, a dynamic balance of these proteins plays a crucial role. Previous studies have shown the role of Bcl-2 gene family on RGC survival in acute and chronic models of optic nerve lesion (11, 25). The prototype gene, Bcl-2 was found to prevent cell death when overexpressed in variety of cell types, particularly in neurons. For instance, mice overexpressing Bcl-2 in neurons, as regulated by the promoter neuron-specific enolase (Nse), displayed an increased number of RGCs, both after developmental pruning and after optic nerve axotomy (26-30). More recently, gene transfer of BAG1, a Bcl-2 associated protein, rescued RGCs in the optic nerve crush as well as the axotomy models (31). Among anti-apoptotic Bcl-2 family members,

Bcl-xL seems to have a predominantly anti-apoptotic role in the rat retina (32). Bcl-2 as well as Bcl-xL retinal mRNA levels are found to decrease after optic nerve axotomy (33). Moreover, AAV-mediated expression of Bcl-xL promoted the survival of axotomized RGCs (34-36). Targeted deletion of Bcl-xL has been showed to play an important role in its prevention of apoptotic cell death (37).

AKT, a PI 3-Kinase activated protein kinase acts as a principal mediator of cell survival in diverse cell types (38-42). Evidence from past studies demonstrates that activation of AKT pathway leads to retinal ganglion cell survival not only in development but also in different models of glaucoma such as ischemia-perfusion injury, optic nerve injury (43-51).

The purpose of this study was to determine if transcription factor Brn3b by itself promotes an increase in the levels of the pro-survival Bcl-2 family of proteins. In addition, the association of some of the Bcl-2 family of proteins as well as p-AKT in Brn3b mediated neuroprotection of RGCs in an ocular hypertension model of glaucoma was also investigated.

Methods

Plasmid construction and recombinant AAV-2 production

Plasmid construction and recombinant AAV-2 production was done according to the method described by Stankowska et al. (2015) (24). Using AAV Helper-free system (Stratagene, La Jolla, CA, USA), recombinant AAV vectors were prepared with plasmids pAAV-IRES-hrGFP (hrGFP is a humanized recombinant GFP), pAAV-RC and pHelper. pAAV-Brn3b vector encoding transcription factor Brn3b was constructed by insertion of mouse Brn3b cDNA clone (Origene, Rockville, MD, USA) digested with EcoRI and XhoI into pAAV-IRES-hrGFP (Stratagene). After

DNA sequence validation, plasmids were used to produce rAAV-CMV-Brn3b and rAAV-CMV-GFP. Gene expression in both the vectors was driven by cytomegalovirus (CMV) promoter.

The control virus AAV2.hSyn.eGFP.WPRE.bGH was purchased from the Penn vector core facility (Philadelphia, PA, USA) and abbreviated as rAAV-hsyn-GFP. The pAAV-hsyn.Brn3b-DDK.WPRE.bGH plasmid was prepared by insertion of mouse Brn3b cDNA clone (Origene, Rockville, MD, USA) containing DDK tag and HindIII restriction digestion site was introduced into pAAV.hSyn.eGFP.WPRE.bGH in the place of eGFP protein using EcoRI and HindIII restriction enzymes by PCR method as described by Stankowska et al., (2015) (24). The custom-made plasmid sequence was confirmed by DNA sequencing and sent to Penn vector core for AAV-2 virus production. The custom-made virus AAV2.hSyn.Brn3b-DDK.WPRE.bGH was abbreviated in the current study as rAAV-hsyn-Brn3b. To improve the specificity and reduce off target effects of AAV-2 virus, we have used the viral constructs driven by neuronal specific human synapsin promoter (24).

Animals

All animal-related procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the UNT Health Science Center and were in compliance with the ARVO statement for the use of Animals in Ophthalmic and Vision Research. Male retired breeder Brown Norway rats (Rattus norvegicus; Charles River Laboratories, Wilmington, MA, USA) in the age group of 8 to 12 months were used in this study.

Morrison's ocular hypertension model of glaucoma in rats

Male retired male breeder Brown Norway rats were used to study the effect of overexpression of transcription factor Brn3b in RGCs of IOP elevated retinas. The procedure described in Morrison et al. (1997) (52) was used to elevate IOP in rats. Animals were maintained on a reduced constant light environment of 90 lux for a minimum of 3 days prior to surgery for elevating IOP. On the day of surgery, animals were anesthetized and injected with 1.8M hypertonic saline via an episcleral vein, while the contralateral eye served as a control. A micro glass needle was inserted into the episcleral vein and approximately 50 µl of hypertonic saline was injected with a force sufficient to blanch the aqueous plexus. This procedure produces scarring of trabecular meshwork which results in rise in IOP and subsequent damage to optic nerve and RGCs. One week following IOP elevation, the rats were administered with viral vectors and after maintaining for 3 weeks, subsequent immunohistochemical analyses were carried out.

IOP measurements

Daily IOP measurements using a Tonolab tonometer (Icare Finland Oy, Espoo, Finland), was carried out on conscious animals following slight sedation with intramuscular (i.m.) administration of acepromazine (2mg/kg) and IOP was taken 2 to 5 minutes after the injection. During each IOP measurements session, ten average readings were obtained from contralateral control and IOP elevated eyes. A plot of mean IOP versus time was carried out and to assess total IOP exposure (mmHg-day) which was computed by determining the difference of area under the curve (AUC) between IOP-elevated eye and contralateral control eye (mmHg-days = AUC of the IOP elevated eye – AUC of the control eye).

Intravitreal Injections of AAV-2 Constructs

Intravitreal injections were carried out using an ultrafine 30.5G disposable needle connected to 50 µl Hamilton syringe (Hamilton Company, Reno, NV, USA) as described by Zhou et al., (2005) (53) in anesthetized rats. Five microliters (ranging from 1 $x10^9$ to 3.5 $x10^9$ units) of AAV virus were injected (while continuously monitoring the position of the needle) in the center of the vitreous cavity to avoid lens injury. Transduction of AAV viruses did not cause any inflammatory or damaging effect to the optic nerve or the retina. Experiments were performed three times using three Brown Norway rats for control AAV vector and three rats for AAV-Brn3b groups (total n=18). Rats were killed 3 weeks following intravitreal administration of viral vectors and retinal sections were obtained for immunohistochemistry.

Cryosections

Animals were sacrificed and eyes were enucleated and fixed in 4% paraformaldehyde for 3 hours at room temperature, submerged in 20% sucrose overnight at 4^0C. Fixed eyes were embedded in optimal cutting temperature compound (OCT; Miles Diagnostics, Elkhart, IN, USA) and frozen at -80^0C. Transverse 10 µm-thick retinal sections were cut using a cryostat (Leica Biosystems, Buffalo Grove, IL, USA) and used for immunohistochemical analysis. Optimal cutting temperature compound medium was removed by ethanol wash followed by water. Sections were viewed with a Zeiss LSM 510 META confocal scanning microscope.

Immunohistochemistry Analysis

To validate upregulation of Brn3b and changes in the expression of Bcl-2, Bcl-xL and p-AKT in RGCs, colocalisation of these proteins with the RGC marker, βIII-Tubulin (1:500) (Sigma-Aldrich, MO, USA), was carried out. Retinal cryosections were hydrated in PBS for 15 minutes

and then blocked in PBS containing 5% normal donkey serum and 5% BSA for 1 hour at room temperature. The sections were double-immunostained with βIII-Tubulin antibody in combination with either rabbit anti-Brn3b antibody (1:250 dilution, Antibody Research Corporation, St. Charles, MO, USA), rabbit anti-Bcl-2 antibody (1:100 dilution, catalog no. sc-492; Santa Cruz Technology, CA, USA), rabbit anti-Bcl-xL antibody (1:300 dilution, catalog no. 2764 ; Cell Signaling Technology, MA, USA) or rabbit anti-p-AKT antibody (1:25 dilution, catalog no. 9271; Cell Signaling Technology, MA, USA) and incubated overnight at 4^{O}C. Sections were then washed 3 times for 5 minutes each with PBS and incubated for 1 h with the appropriate donkey anti-IgG secondary antibody conjugated with Alexa 488, Alexa 647 or Alexa 547 (1:1000 dilution; Molecular Probes, Invitrogen, Eugene, OR). Sections in which the primary antibody incubation was excluded served as negative controls (blanks) and were used to assess nonspecific staining by the secondary antibody.

Results:

Overexpression of Brn3b in rat retinal ganglion cells after intravitreal injection of rAAV-hsyn-Brn3b

Viral vectors, rAAV-hsyn-GFP or rAAV-hysn-Brn3b were injected intravitreally into the left eye, while the right eye served as control. The rats were maintained for 3 weeks following virus injection (to allow for optimal AAV-2 mediated expression) and sacrificed. Rat retinal frozen sections were obtained and subjected to immunohistochemistry to detect GFP and Brn3b levels. To confirm virus transduction of RGCs of rats injected with rAAV-hsyn-GFP, retinal sections of rats were immunostained with mouse anti-GFP antibody. Staining for GFP was seen mainly in ganglion cell layer (GCL) and mild staining was also seen in inner plexiform layer (IPL) layer

(Figure 1A). As seen in Figure 1B, increased Brn3b immunostaining (pseudo-green) was detected in rAAV-hsyn-Brn3b administered rat retinas mainly in GCL with diffuse staining also detected in inner nuclear layer (INL) and outer nuclear layer (ONL). Interestingly, ratios of Brn3b staining of RGCs counts between left and right eye were significantly higher (1.5 fold) in rats injected with rAAV-hsyn-Brn3b compared to those of the rats injected with rAAV-hsyn-GFP (n=6) (Figure 1C). An image of the RGC layer of retinas indicates increased staining of Brn3b in RGCs in rAAV-hsyn-Brn3b compared with rAAV-hsyn-GFP injected eyes (Figure 1Di, 1Dii). βIII-Tubulin was used as a RGC marker and its antibody was found to stain mainly the RGC layer and inner plexiform layer.

Upregulation of Bcl-2, but no change in Bcl-xL levels in retinal ganglion cells of rats injected intravitreally with rAAV-hsyn-Brn3b

Further experiments were carried out to determine if the neuroprotective protein, Bcl-2, was upregulated in retinas of rats injected with rAAV-hsyn-Brn3b. In addition to Bcl-2, the status of Bcl-xL was also assessed in rats administered intravitreally with rAAV-hsyn-Brn3b. Bcl-xL has been shown neuroprotective for RGCs in optic nerve injury models in several studies (33- 37).

Briefly, after 3 weeks of intravitreal injection of either rAAV-hsyn-GFP or rAAV-hsyn-Brn3b, retinal samples were collected for frozen sections. Ten microns retinal frozen sections were analyzed by immunohistochemistry for either Bcl-2 expression (pseudo-green) or Bcl-xL (pseudo-green) in RGCs of the rats. An increase in Bcl-2 was found mainly in the GCL, and a mild increase in immunostaining was also observed in the INL in rAAV-hsyn-Brn3b injected rat eyes compared with the control vector-injected rat eyes, where minimal staining for Bcl-2 was found (Figure 2A). On the other hand, no change in Bcl-xL levels were seen in RGCs of rats injected with rAAV-

hsyn-Brn3b, compared to those injected with rAAV-hsyn-GFP (n=6) (Figure 2D). The fluorescence intensity ratios (left to right eyes) for Bcl-2 staining in RGCs were significantly higher (>2 fold) in Brn3b overexpressing rat retinas than control vector transduced retinas (Figure 2B). Figure 2C provides a view of the RGC layer showing increased Bcl-2 immunostaining in RGCs of rats intravitreally injected with rAAV-hsyn-Brn3b, as compared to rats injected with rAAV-hsyn-GFP. Immunostaining with the βIII-Tubulin antibody was used as a RGC marker. The data suggest that Bcl-2, but not Bcl-xL, was markedly increased in RGCs in rat eyes injected with rAAV-hsyn-Brn3b, compared to those injected with rAAV-hsyn-GFP.

Increased expression of p-AKT in rat retinal ganglion cells overexpressing rAAV-hsyn-Brn3b

Several lines of evidence have demonstrated AKT as a key mediator of retinal ganglion cell survival in multiple models of glaucoma (43-51). AKT is activated by phosphorylation at Ser473, typically by upstream kinases involved in the PI-3 kinase pathway. P-AKT promotes the phosphorylation of numerous cellular proteins leading to changes in cell physiology, survival and proliferation.

Retinal frozen sections from rats overexpressing rAAV-hsyn-Brn3b or rAAV-hsyn-GFP (3 weeks after intravitreal injection) were immunostained for p-AKT. A marked increase in the expression of p-AKT was seen in the GCL of retinas of rats overexpressing rAAV-hsyn-Brn3b as compared to those overexpressing rAAV-hsyn-GFP (n=6) (Figure 3A). Densitometric analysis revealed a statistically significant increase in the expression of p-AKT in retinas of rats intravitreally injected with rAAV-hsyn-Brn3b, compared to those injected with rAAV-hsyn-GFP (Figure 3B). As shown in the image of the RGC layer (Figure 3C), overexpression of rAAV-hsyn-Brn3b in RGCs showed

a robust upregulation of p-AKT as compared to overexpression of rAAV-hsyn-GFP. Staining for βIII-Tubulin was used as a RGC marker. Based upon the quantitation of fluorescence intensity, overexpression of Brn3b in RGCs of rats produced nearly 2.5-fold increase in p-AKT, compared to rats intravitreally injected with rAAV-hsyn-GFP.

Overexpression of Brn3b using AAV-2 vectors in rat retinal ganglion cells of IOP elevated Brown Norway rats.

Injection of hypertonic saline into episcleral veins by the Morrison's method (52) was used to elevate IOP in one eye of Brown Norway rats. One week after IOP elevation, viral vectors (rAAV-CMV-GFP or rAAV-CMV-Brn3b) were injected intravitreally into the IOP-elevated eye. Three weeks following intravitreal injection, rats were sacrificed and retinas were collected, paraffin embedded and 5 μm thick sections were obtained. Retinal sections were subjected to immunohistochemistry to detect Brn3b levels. As seen in Figure 4A, increased immunostaining (pseudo-green) for Brn3b was detected in rAAV-CMV-Brn3b administered rat retinas mainly in GCL and some diffuse staining was also detected in the INL. Minimal Brn3b localization in the outer retina has also been observed in our previous study (24). As seen in Figure 4B and 4C, IOP was elevated 7 to 10 days following surgery and remained elevated for 3 weeks until the rats sacrificed. Representative mean values of IOP exposure during the course of the experiment were 88 and 85 mmHg-days. βIII-Tubulin immunostaining was used as a RGC marker to identify RGCs.

rAAV-CMV-Brn3b administration in rat eyes promotes an increase in the expression of Bcl-2 in RGCs of IOP elevated rats

To determine whether transcription factor, Brn3b has the ability to upregulate Bcl-2 and Bcl-xL under a glaucomatous condition, retinal sections from rats administerd either rAAV-CMV-Brn3b or rAAV-CMV-GFP following elevation of IOP were immunostained for either Bcl-2 or Bcl-xL. Increase in Bcl-2 (pseudo-green) levels was mainly seen in GCL and mild staining was also observed in the IPL as well as the OPL in retinas of rats injected intravitreally with rAAV-CMV-Brn3b, as compared those injected with rAAV-CMV-GFP following IOP elevation (Figure 5A). Rats injected with rAAV-CMV-Brn3b showed an increase in Bcl-xL expression mainly in GCL and mild staining in INL, compared to rats intravitreally injected with rAAV-CMV-GFP (n=3) (Figure 5C). Quantification of L/R ratios of immunostaining of Bcl-2 and Bcl-xL in RGCs showed a significant increase in immunostaining for Bcl-2 and an increasing trend (not statistically significant) in Bcl-xL expression in rats injected with rAAV-CMV-Brn3b, compared to those injected with rAAV-CMV-Empty (Figure 5B and 5D). These data suggest that following IOP-mediated damage, overexpression of transcription factor Brn3b could upregulate genes promoting RGCs survival and thereby produce neuroprotective effects.

Immunostaining for p-AKT in the retina during AAV mediated upregulation of transcription factor Brn3b following IOP elevation in rats

Our previous experiments demonstrated that overexpression of Brn3b through AAV-2 vectors in RGCs leads to increased expression of p-AKT. To evaluate the expression of p-AKT in RGCs in IOP elevated eyes, rats injected intravitreally either with rAAV-CMV-Brn3b or rAAV-CMV-GFP. As shown in Figure 6A, immunohistochemistry analysis shows upregulation of p-AKT in the NFL as well as in the GCL with rat eyes injected with rAAV-CMV-Brn3b as compared to those injected with rAAV-CMV-GFP (n=3). There was an increasing trend (not statistically significant) in the

levels of p-AKT in RGCs of rats intravitreally injected with rAAV-CMV-Brn3b following IOP elevation, compared to those intravitreally injected with rAAV-CMV-GFP (Figure 6B).

Discussion

One of the important goals in research on neurodegenerative diseases is to develop therapies that effectively block the apoptotic cell death of susceptible neuronal populations. RGCs, which undergo cell death via apoptosis in blinding diseases like glaucoma, are important targets of such neuroprotective strategies (54). Clearly, the development of new therapies relies on a complete understanding of the gene expression altered in these cells, which contribute to neuroprotective effects against damaging stimuli.

In a previous study from our laboratory, we found a significant protection of RGCs and optic nerve axons from IOP mediated injury following AAV-mediated overexpression of Brn3b in RGCs of Brown Norway rats (24). The present study explored some mechanisms by which Brn3b promotes neuroprotection of RGCs in rats with elevated IOP. Two approaches were taken to study Brn3b-mediated changes in gene expression: either direct administration of the AAV-hsyn-Brn3b vector by itself (in the absence of IOP elevation) or administration of AAV-hsyn-Brn3b following IOP elevation. In both these models, overexpression of Brn3b using adeno-associated virus as a vector lead to significant upregulation of pro-survival gene, Bcl-2 in the RGCs. A significant increase in expression of p-AKT was found in RGCs of rat retinas overexpressing Brn3b only via direct administration AAV-2 vectors (in the absence of IOP elevation), compared to those administered the control vector. No significant change in Bcl-xL expression was found in rat RGCs overexpressing Brn3b, compared to rat RGCs transduced with the empty vector, both during direct

AAV-hsyn-Brn3b administration as well as in the rat model of glaucoma. Our study indicates an association of the anti-apoptotic molecule Bcl-2 in the neuroprotective effects of transcription factor Brn3b in the ocular hypertension model of glaucoma.

Brn3b is endogenously expressed in adult retinas as well as in different parts of the brain which includes superior colliculus, interpeduncular nucleus or trigeminal ganglion (55). The role of Brn3b in mature RGCs physiology still needs to be understood. Brn3b plays a crucial role in development and differentiation of RGCs as evidenced by loss of approximately 70% of RGCs in Brn3b knockout mice compared to other Brn3 gene knockout (15, 19, 22, and 23). In different animal models of glaucoma, it is been shown that a decrease in Brn3b expression occurs prior to RGC loss and neurodegeneration (56-58).

Following development, Bcl-2 is not robustly expressed in retinal neurons including RGCs. This compromises the intrinsic regenerative capacity of RGCs following injury. However, the homolog Bcl-xL appears to be dominant anti-apoptotic gene expressed in the retina (32). Bcl2 is evolutionarily conserved and plays a key inhibitory role against cell death in different organisms like *C. elegans* and man (59, 60). RGCs are well protected by Bcl-2 overexpression and also demonstrate good preservation of pattern ERG responses (59). During development of the nervous system, naturally occurring cell death is a common feature. In this process, embryonic RGCs express high levels of Bcl-2 which decline rapidly between post natal days 1 to 5. (Chen et al., 1997) Expression of Bcl-2 determines neuronal fate. Bcl-2 overexpression reduced neuronal loss during naturally occurring cell death (development) which resulted in hypertrophy of the nervous system. Mice overexpressing Bcl-2 have enlarged optic nerves suggestive of enhanced RGC survival during development (61). Bcl-2 overexpression has been shown to enhance RGC survival to nearly 65%, 3.5 months after axotomy in adult mice (27). Numerous studies have demonstrated

the neuroprotective role for Bcl-2, and constitutive expression of the Bcl-2 protein has been shown to promote the survival of various cells when exposed to adverse stimuli (26, 62 and 63). Our data demonstrate that Bcl-2 upregulation is associated with the enhancement of RGCs survival in rats injected with rAAV-CMV-Brn3b after IOP elevation.

Bcl-x, a member of the Bcl-2 gene family, is alternatively sliced to produce two protein isoforms (Bcl-xL and Bcl-xS). Extensive apoptotic cell death occurs in neurons during development in Bcl-x deficient mice. Bcl-2 and Bcl-x mRNA expression in RGCs were found to be decreased following optic nerve axotomy using RT-PCR and in situ hybridization techniques (33). Intraocular delivery of Bcl-xL fusion proteins leads to inhibition of RGCs death after optic nerve axotomy (34, 36). Adenoviral overexpression of Bcl-xL in mature axotomized rat leads to RGCs survival in vivo (35). In the current study, no appreciable changes were found in the immunoreactive levels of Bcl-xL following intravitreal administration of AAV-hsyn-Brn3b. In the ocular hypertension model, an increasing trend of Bcl-xL upregulation was observed, however it was not statistically significant. Interestingly, in the current study, Bcl-2 expression was found to increase in RGCs during AAV-mediated Brn3b upregulation following IOP elevation suggesting that Bcl-2 is one of the effectors of Brn3b mediated neuroprotection in glaucoma.

Some cytokines promote a survival response in neurons through the PI3K/AKT pathway (41, 42). PI3K/AKT signaling acts as a survival factor for RGCs during retinal development, and also following damaging effects of optic nerve injury, ischemia/reperfusion injury and an acute ocular hypertension model (45-47, 49-51). Melanopsin-expressing RGCs (mRGCs) have been found to co-express p-AKT and play a role in maintaining the survival of mRGCs after optic nerve injury (44, 48). Although we found a trend of increasing expression of p-AKT during AAV-mediated overexpression of Brn3b during ocular hypertension, it was not statistically significant.

Collectively, these studies support a role mainly for Bcl-2 in RGCs survival in the rat glaucoma model.

AKT has direct effects on the effectors of the apoptotic pathway including Bcl-2 (38-42). AKT signaling through enhanced CREB activity leads to increased Bcl-2 promoter activity leading to increased cell survival (47, 64 and 65). p-AKT also regulates Bcl-2 activity in various types of cells (38-42, 47, 64, 65). Our data demonstrate the involvement of the pro-survival proteins, Bcl-2, Bcl-xL and p-AKT in RGCs survival following Brn3b overexpression. These findings improve our understanding of the association of the prosurvival factor Bcl-2 with the neuroprotective effects of Brn3b during ocular hypertension, which have implications for the development of strategies for neuroprotection in glaucoma.

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References

1. Gupta N, Yucel YH. Glaucoma as a neurodegenerative disease Curr Opin Ophthalmol 2007 Mar;18(2):110-114.
2. Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020 Br J Ophthalmol 2006 Mar;90(3):262-267.

3. Quigley HA. Ganglion cell death in glaucoma: pathology recapitulates ontogeny Aust N Z J Ophthalmol 1995 May;23(2):85-91.

4. Begg IS, Drance SM. Progress of the glaucomatous process related to recurrent ischaemic changes at the optic disc Exp Eye Res 1971 Jan;11(1):141.

5. Hayreh SS. Posterior ciliary arterial occlusive disorders Trans Ophthalmol Soc U K 1971;91:291-303.

6. Cioffi GA. Care guidelines and optic nerve assessment J Glaucoma 1996 Feb;5(1):A12.

7. Vorwerk CK, Lipton SA, Zurakowski D, Hyman BT, Sabel BA, Dreyer EB. Chronic low-dose glutamate is toxic to retinal ganglion cells. Toxicity blocked by memantine Invest Ophthalmol Vis Sci 1996 Jul;37(8):1618-1624.

8. Levin LA. Direct and indirect approaches to neuroprotective therapy of glaucomatous optic neuropathy Surv Ophthalmol 1999 Jun;43 Suppl 1:S98-101.

9. Osborne NN. Pathogenesis of ganglion "cell death" in glaucoma and neuroprotection: focus on ganglion cell axonal mitochondria Prog Brain Res 2008;173:339-352.

10. Kong GY, Van Bergen NJ, Trounce IA, Crowston JG. Mitochondrial dysfunction and glaucoma J Glaucoma 2009 Feb;18(2):93-100.

11. Almasieh M, Wilson AM, Morquette B, Cueva Vargas JL, Di Polo A. The molecular basis of retinal ganglion cell death in glaucoma Prog Retin Eye Res 2012 Mar;31(2):152-181.

12. Xiang M, Zhou L, Macke JP, Yoshioka T, Hendry SH, Eddy RL, Shows TB, Nathans J. The Brn-3 family of POU-domain factors: primary structure, binding specificity, and expression in subsets of retinal ganglion cells and somatosensory neurons J Neurosci 1995 Jul;15(7 Pt 1):4762-4785.

13. Xiang M, Zhou H, Nathans J. Molecular biology of retinal ganglion cells Proc Natl Acad Sci U S A 1996 Jan 23;93(2):596-601.
14. Xiang M, Zhou L, Peng YW, Eddy RL, Shows TB, Nathans J. Brn-3b: a POU domain gene expressed in a subset of retinal ganglion cells Neuron 1993 Oct;11(4):689-701.
15. Xiang M, Gan L, Li D, Zhou L, Chen ZY, Wagner D, O'Malley BW Jr, Klein W, Nathans J. Role of the Brn-3 family of POU-domain genes in the development of the auditory/vestibular, somatosensory, and visual systems Cold Spring Harb Symp Quant Biol 1997;62:325-336.
16. Gan L, Wang SW, Huang Z, Klein WH. POU domain factor Brn-3b is essential for retinal ganglion cell differentiation and survival but not for initial cell fate specification Dev Biol 1999 Jun 15;210(2):469-480.
17. Wang SW, Mu X, Bowers WJ, Kim DS, Plas DJ, Crair MC, Federoff HJ, Gan L, Klein WH. Brn3b/Brn3c double knockout mice reveal an unsuspected role for Brn3c in retinal ganglion cell axon outgrowth Development 2002 Jan;129(2):467-477.
18. Xiang M. Requirement for Brn-3b in early differentiation of postmitotic retinal ganglion cell precursors Dev Biol 1998 May 15;197(2):155-169.
19. Gan L, Xiang M, Zhou L, Wagner DS, Klein WH, Nathans J. POU domain factor Brn-3b is required for the development of a large set of retinal ganglion cells Proc Natl Acad Sci U S A 1996 Apr 30;93(9):3920-3925.
20. Erkman L, Yates PA, McLaughlin T, McEvilly RJ, Whisenhunt T, O'Connell SM, Krones AI, Kirby MA, Rapaport DH, Bermingham JR, O'Leary DD, Rosenfield MG. A POU domain transcription factor-dependent program regulates axon pathfinding in the vertebrate visual system Neuron 2000 Dec;28(3):779-792.

21. Wang SW, Gan L, Martin SE, Klein WH. Abnormal polarization and axon outgrowth in retinal ganglion cells lacking the POU-domain transcription factor Brn-3b Mol Cell Neurosci 2000 Aug;16(2):141-156.
22. Erkman L, McEvilly RJ, Luo L, Ryan AK, Hooshmand F, O'Connell SM,Keithley EM, Rapaport DH, Ryan AF, Rosenfield MG. Role of transcription factors Brn-3.1 and Brn-3.2 in auditory and visual system development Nature 1996 Jun 13;381(6583):603-606.
23. Camp AS, Ruggeri M, Munguba GC, Tapia ML, John SW, Bhattacharya SK, Lee RK. Structural correlation between the nerve fiber layer and retinal ganglion cell loss in mice with targeted disruption of the Brn3b gene Invest Ophthalmol Vis Sci 2011 Jul 13;52(8):5226-5232.
24. Stankowska DL, Minton AZ, Rutledge MA, Mueller BH 2nd, Phatak NR, He S, Ma HY, Forster MJ, Yorio T, Krishnamoorthy RR. Neuroprotective effects of transcription factor Brn3b in an ocular hypertension rat model of glaucoma. Invest Opthalmol Vis Sci 2015 Jan 13;56 (2) :893-907.
25. Nickells RW, Semaan SJ, Schlamp CL. Involvement of the Bcl2 gene family in the signaling and control of retinal ganglion cell death Prog Brain Res 2008;173:423-435.
26. Bonfanti L, Strettoi E, Chierzi S, Cenni MC, Liu XH, Martinou J-C, Maffei L, Rabacchi SA. Protection of retinal ganglion cells from natural and axotomy-induced cell death in neonatal transgenic mice overexpressing bcl-2 J Neurosci 1996 Jul 1;16(13):4186-4194.
27. Cenni MC, Bonfanti L, Martinou JC, Ratto GM, Strettoi E, Maffei L. Long-term survival of retinal ganglion cells following optic nerve section in adult bcl-2 transgenic mice Eur J Neurosci 1996 Aug;8(8):1735-1745.

28. Chen DF, Schneider GE, Martinou JC, Tonegawa S. Bcl-2 promotes regeneration of severed axons in mammalian CNS Nature 1997 Jan 30;385(6615):434-439.
29. Chierzi S, Strettoi E, Cenni MC, Maffei L. Optic nerve crush: axonal responses in wild-type and bcl-2 transgenic mice J Neurosci 1999 Oct 1;19(19):8367-8376.
30. Burne JF, Staple JK, Raff MC. Glial cells are increased proportionally in transgenic optic nerves with increased numbers of axons J Neurosci 1996 Mar 15;16(6):2064-2073.
31. Planchamp V, Bermel C, Tonges L, Ostendorf T, Kugler S, Reed JC, Kermer P, Bahr M, Lingor P. BAG1 promotes axonal outgrowth and regeneration in vivo via Raf-1 and reduction of ROCK activity Brain 2008 Oct;131(Pt 10):2606-2619.
32. Levin LA, Schlamp CL, Spieldoch RL, Geszvain KM, Nickells RW. Identification of the bcl-2 family of genes in the rat retina Invest Ophthalmol Vis Sci 1997 Nov;38(12):2545-2553.
33. Chaudhary P, Ahmed F, Quebada P, Sharma SC. Caspase inhibitors block the retinal ganglion cell death following optic nerve transection Brain Res Mol Brain Res 1999 Apr 6;67(1):36-45.
34. Malik JM, Shevtsova Z, Bahr M, Kugler S. Long-term in vivo inhibition of CNS neurodegeneration by Bcl-XL gene transfer Mol Ther 2005 Mar;11(3):373-381.
35. Kretz A, Kugler S, Happold C, Bahr M, Isenmann S. Excess Bcl-XL increases the intrinsic growth potential of adult CNS neurons in vitro Mol Cell Neurosci 2004 May;26(1):63-74.
36. Liu XH, Collier RJ, Youle RJ. Inhibition of axotomy-induced neuronal apoptosis by extracellular delivery of a Bcl-XL fusion protein J Biol Chem 2001 Dec 7;276(49):46326-46332.

37. Motoyama N, Wang F, Roth KA, Sawa H, Nakayama K, Nakayama K, Negishi I, Senju S, Zhang Q, Fujii S, Loh DY. Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice Science 1995 Mar 10;267(5203):1506-1510.
38. Downward J. PI 3-kinase, Akt and cell survival Semin Cell Dev Biol 2004 Apr;15(2):177-182.
39. Shacka JJ, Roth KA. Regulation of neuronal cell death and neurodegeneration by members of the Bcl-2 family: therapeutic implications Curr Drug Targets CNS Neurol Disord 2005 Feb;4(1):25-39.
40. Leaver SG, Cui Q, Bernard O, Harvey AR. Cooperative effects of bcl-2 and AAV-mediated expression of CNTF on retinal ganglion cell survival and axonal regeneration in adult transgenic mice Eur J Neurosci 2006 Dec;24(12):3323-3332.
41. Alonzi T, Middleton G, Wyatt S, Buchman V, Betz UA, Muller W, Musiani P, Poli V, Davies Am. Role of STAT3 and PI 3-kinase/Akt in mediating the survival actions of cytokines on sensory neurons Mol Cell Neurosci 2001 Sep;18(3):270-282.
42. Brunet A, Datta SR, Greenberg ME. Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway Curr Opin Neurobiol 2001 Jun;11(3):297-305.
43. Nakazawa T, Tamai M, Mori N. Brain-derived neurotrophic factor prevents axotomized retinal ganglion cell death through MAPK and PI3K signaling pathways Invest Ophthalmol Vis Sci 2002 Oct;43(10):3319-3326.
44. Nakazawa T, Shimura M, Tomita H, Akiyama H, Yoshioka Y, Kudou H, Tamai M. Intrinsic activation of PI3K/Akt signaling pathway and its neuroprotective effect against retinal injury Curr Eye Res 2003 Jan;26(1):55-63.

45. Chavarria T, Valenciano AI, Mayordomo R, Egea J, Comella JX, Hallbook F, de Pablo F, de la Rosa E J. Differential, age-dependent MEK-ERK and PI3K-Akt activation by insulin acting as a survival factor during embryonic retinal development Dev Neurobiol 2007 Nov;67(13):1777-1788.
46. Luo JM, Cen LP, Zhang XM, Chiang SW, Huang Y, Lin D, Fan YM, van Rooijen N, Lam DS, Pang CP, Cui Q. PI3K/akt, JAK/STAT and MEK/ERK pathway inhibition protects retinal ganglion cells via different mechanisms after optic nerve injury Eur J Neurosci 2007 Aug;26(4):828-842.
47. Huang Y, Cen LP, Luo JM, Wang N, Zhang MZ, van Rooijen N, Pang CP, Cui Q. Differential roles of phosphatidylinositol 3-kinase/akt pathway in retinal ganglion cell survival in rats with or without acute ocular hypertension Neuroscience 2008 Apr 22;153(1):214-225.
48. Li SY, Yau SY, Chen BY, Tay DK, Lee VW, Pu ML, Chan HH, So KF. Enhanced survival of melanopsin-expressing retinal ganglion cells after injury is associated with the PI3 K/Akt pathway Cell Mol Neurobiol 2008 Dec;28(8):1095-1107.
49. Tsai RK, Chang CH, Sheu MM, Huang ZL. Anti-apoptotic effects of human granulocyte colony-stimulating factor (G-CSF) on retinal ganglion cells after optic nerve crush are PI3K/AKT-dependent Exp Eye Res 2010 May;90(5):537-545.
50. Qi Y, Chen L, Zhang L, Liu WB, Chen XY, Yang XG. Crocin prevents retinal ischaemia/reperfusion injury-induced apoptosis in retinal ganglion cells through the PI3K/AKT signalling pathway Exp Eye Res 2013 Feb;107:44-51.
51. Socodato R, Brito R, Portugal CC, de Oliveira NA, Calaza KC, Paes-de-Carvalho R. The nitric oxide-cGKII system relays death and survival signals during embryonic retinal

development via AKT-induced CREB1 activation Cell Death Differ 2014 Jun;21(6):915-928.

52. Morrison JC, Moore CG, Deppmeier LM, Gold BG, Meshul CK, Johnson EC. A rat model of chronic pressure-induced optic nerve damage Exp Eye Res 1997 Jan;64(1):85-96.
53. Zhou Y, Pernet V, Hauswirth WW, Di Polo A. Activation of the extracellular signal-regulated kinase 1/2 pathway by AAV gene transfer protects retinal ganglion cells in glaucoma Mol Ther 2005 Sep;12(3):402-412.
54. Weinreb RN, Levin LA. Is neuroprotection a viable therapy for glaucoma? Arch Ophthalmol 1999 Nov;117(11):1540-1544.
55. Turner EE, Jenne KJ, Rosenfeld MG. Brn-3.2: a Brn-3-related transcription factor with distinctive central nervous system expression and regulation by retinoic acid Neuron 1994 Jan;12(1):205-218.
56. Naskar R, Thanos S. Retinal gene profiling in a hereditary rodent model of elevated intraocular pressure Mol Vis 2006 Oct 18;12:1199-1210.
57. Soto I, Oglesby E, Buckingham BP, Son JL, Roberson ED, Steele MR, Inman DM, Vetter ML, Horner PJ, Marsh-Armstrong N. Retinal ganglion cells downregulate gene expression and lose their axons within the optic nerve head in a mouse glaucoma model J Neurosci 2008 Jan 9;28(2):548-561.
58. Weishaupt JH, Klocker N, Bahr M. Axotomy-induced early down-regulation of POU-IV class transcription factors Brn-3a and Brn-3b in retinal ganglion cells J Mol Neurosci 2005;26(1):17-25.

59. Chierzi S, Cenni MC, Maffei L, Pizzorusso T, Porciatti V, Ratto GM, Strettoi E. Protection of retinal ganglion cells and preservation of function after optic nerve lesion in bcl-2 transgenic mice Vision Res 1998 May;38(10):1537-1543.

60. Ewings KE, Wiggins CM, Cook SJ. Bim and the pro-survival Bcl-2 proteins: opposites attract, ERK repels Cell Cycle 2007 Sep 15;6(18):2236-2240.

61. Martinou JC, Dubois-Dauphin M, Staple JK, Rodriguez I, Frankowski H, Missotten M, Albertini P, Talabot D, Catsicas S, Pietra C. Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia Neuron 1994 Oct;13(4):1017-1030.

62. Jacobson MD, Raff MC. Programmed cell death and Bcl-2 protection in very low oxygen Nature 1995 Apr 27;374(6525):814-816.

63. Merry DE, Korsmeyer SJ. Bcl-2 gene family in the nervous system Annu Rev Neurosci 1997;20:245-267.

64. Pugazhenthi S, Nesterova A, Sable C, Heidenreich KA, Boxer LM, Heasley LE, Reusch JE. Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein J Biol Chem 2000 Apr 14;275(15):10761-10766.

65. Yuan J, Yankner BA. Apoptosis in the nervous system Nature 2000 Oct 12;407(6805):802-809

Figure Legends:

Figure 1**:** Transduction and overexpression of Brn3b in RGCs of Brown Norway rats injected with rAAV-hsyn-Brn3b. A and B. Immunohistochemical analyses for GFP (green) , Brn3b (pseudo-green), βIII-Tubulin(pseudo-red) in retinal frozen sections from retinas overexpressing different viral vectors. The immunostaining was detected by using corresponding Alexa 488-, Alexa 546-, and Alexa 647-conjugated donkey anti-IgG secondary antibodies. Cells were counterstained with DAPI (blue) to detect cell nuclei. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OS, outer segment. Scale bar indicates 20μm. C**.** Plot of ratio of fluorescence intensity of Brn3b immunostaining in RGCs between left (intravitreally injected) and right (contralateral) eyes in 24 different regions. Ratio was compared between rats injected with rAAV-hsyn-GFP and rAAV-hsyn-Brn3b. A significant increase in Brn3b staining in RGCs was observed in rats overexpressing Brn3b compared to control vector. Fluorescent intensity values are shown as mean ± SEM, n=6. Mann-Whitney Rank sum test was used for statistical analysis (*$p<0.002$). D. Representative images of GCL showing Brn3b staining in rats injected with rAAV-hsyn-GFP or rAAV-hsyn-Brn3b.

Figure 2: Transcription factor Brn3b-mediated changes in Bcl-2 and Bcl-xL expression in RGCs of Brown Norway rats. A and D. Bcl-2 (pseudo-green), Bcl-xL (pseudo-green), βIII-Tubulin (pseudo-red) expression in retinal sections from Brown Norway rat eyes injected with either rAAV-hsyn-GFP (vector control) or rAAV-hsyn-Brn3b virus, detected with secondary antibodies conjugated with Alexa 546 or 647 dye. Cells were counterstained with DAPI (blue) to detect cell nuclei. B**.** Ratio of fluorescence intensity for Bcl-2 staining was measured at 24 different regions in the ganglion cell layers using NIH ImageJ. The fluorescence intensity ratios are shown as mean

± SEM, n= 6. Statistically significant increase in intensity of Bcl-2 staining was found in RGCs of rats injected with rAAV-hsyn-Brn3b, compared to those injected with rAAV-hsyn-GFP. Mann Whitney Rank Sum test was used for statistical analysis (*p<0.002). C. Image of the RGC layer showing Bcl-2 immunostaining of RGCs of retinas transduced with i. rAAV-hsyn-GFP, ii. rAAV-hsyn-Brn3b. Scale bar indicates 20 µm.

Figure 3**:** Transcription factor Brn3b promoted an increase in the expression of p-AKT in retinas of rats injected with rAAV-hsyn-Brn3b. A**.** Immunostaining for p-AKT (pseudo-green), βIII-Tubulin (pseudo-red) expression in retinal sections from Brown Norway rats intravitreally injected with rAAV-hsyn-GFP (vector control) or rAAV-hsyn-Brn3b virus. The immunostaining was detected using corresponding Alexa 546 or 647 conjugated secondary antibodies. Scale bar indicates 20 µm. B. A significant 2.4 fold increase in p-AKT expression was observed in RGCs of rats injected with rAAV-hsyn-Brn3b. Ratios of fluorescence intensity values are shown in mean ± SEM, n= 6. Mann Whitney Rank Sum test was used for statistical analysis (*p<0.002). C. RGC layer of transduced retinas. (i) rAAV-hsyn-GFP (ii) rAAV-hsyn-Brn3b.

Figure 4**:** AAV-mediated overexpression of Brn3b in rat retinal ganglion cells of IOP elevated Brown Norway rats after intravitreal injection of rAAV-CMV-Brn3b. A. Representative images show Brn3b (pseudo-green) and βIII-Tubulin (pseudo-red) immunostaining in rat retinas intravitreally injected with either with rAAV-CMV-GFP or rAAV-CMV-Brn3b. Brn3b staining was detected mainly in the GCL. Intraocular pressure elevation profile in rats administered with either rAAV-CMV-GFP (B) or rAAV-CMV-Brn3b (C) in Brown Norway rats. IOP was elevated in one eye (closed circles), while the other eye served as a contralateral control eye (open circles). The experiment was carried out in three Brown Norway rats followed by intravitreally injection either with rAAV-CMV-GFP or rAAV-CMV-Brn3b. IOP values were plotted as mean ± SEM

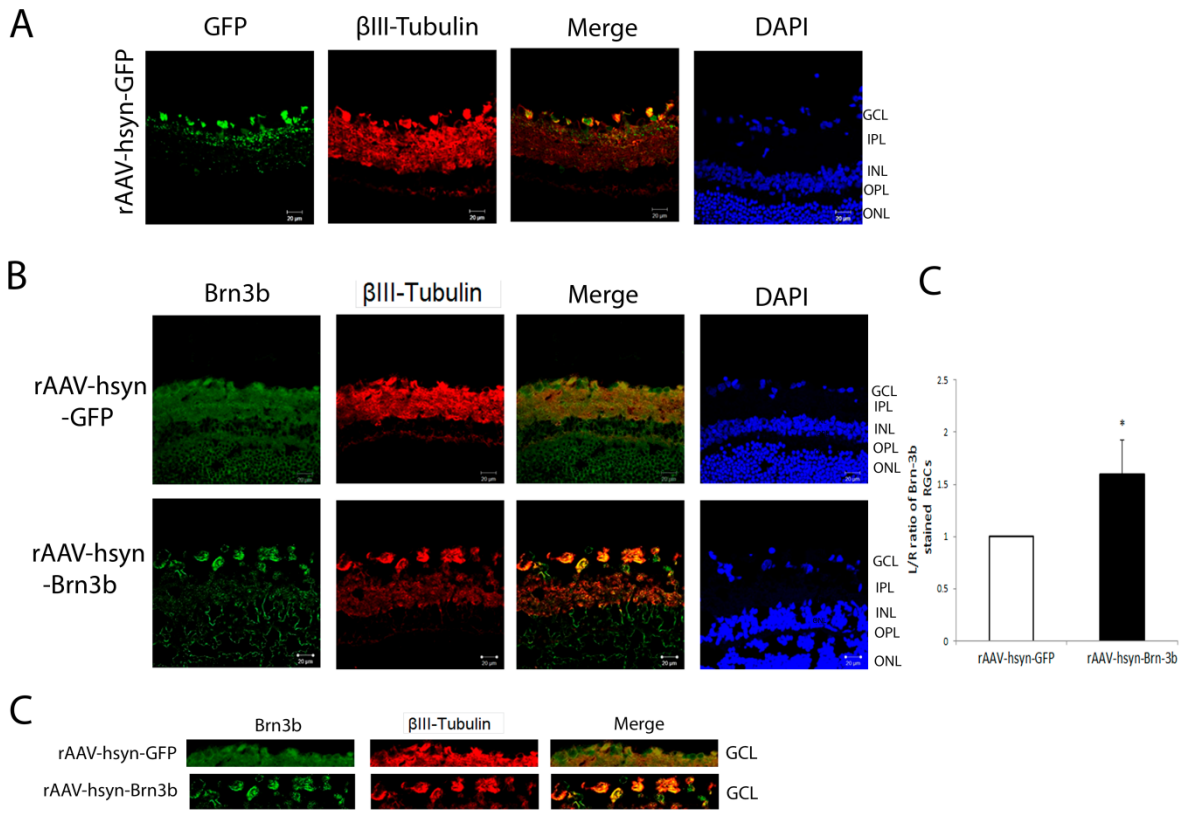
(solid line, IOP-elevated and virus-injected eye; dashed line, untreated contralateral eye). Asterisk indicates $p<0.001$ statistical significance of the IOP-elevated eye compared with contralateral eye using t-test. Scale bar indicates 20 µm.

Figure 5**:** Adeno-associated virus mediated overexpression of Brn3b produced an increase in Bcl-2 and Bcl-xL expression in RGCs of IOP elevated Brown Norway rats. A and C. Retinal sections from rat eyes intravitreally injected either with rAAV-CMV-GFP or rAAV-CMV-Brn3b were immunostained for Bcl-2 (pseudo-green) using either rabbit anti-Bcl-2, or rabbit anti-Bcl-xL (pseudo-green) and mouse anti- βIII-Tubulin (pseudo-red) antibodies. B and D. Densitometry analysis shows a significant increase in Bcl-2 expression and a trend towards increase in Bcl-xL expression (not statistically significant) in RGCs transduced with rAAV-CMV-Brn3b, compared to RGCs transduced with rAAV-CMV-GFP in the Morrison's model of glaucoma. Student's t-test was used for statistical analysis (*$p<0.05$). L/R ratio of fluorescence intensity of Bcl-2 and Bcl-xL immunostaining in RGCs from rats injected either with rAAV-CMV-GFP or rAAV-CMV-Brn3b is plotted. Values are represented as mean ± SEM, n=3. Scale bar indicates 20 µm.

Figure 6**:** Levels of p-AKT in the retina of IOP elevated rats overexpressing Brn3b. A. Immunostaining for p-AKT in RGCs of Brown Norway rats intravitreally injected with either rAAV-CMV-GFP or rAAV-CMV-Brn3b after IOP elevation**.** B. A prominent increase in immunostaining for p-AKT was observed in RGCs overexpressing Brn3b (rAAV-CMV-Brn3b) compared to RGCs overexpressing control vector (rAAV-CMV-GFP) (determined by L/R ratios of fluorescence intensities of p-AKT staining). Student's t-test was used for statistical analysis (*$p<0.05$). Values are represented as mean ± SEM, n=3. Scale bar indicates 20 µm.

Figures:

Figure1:



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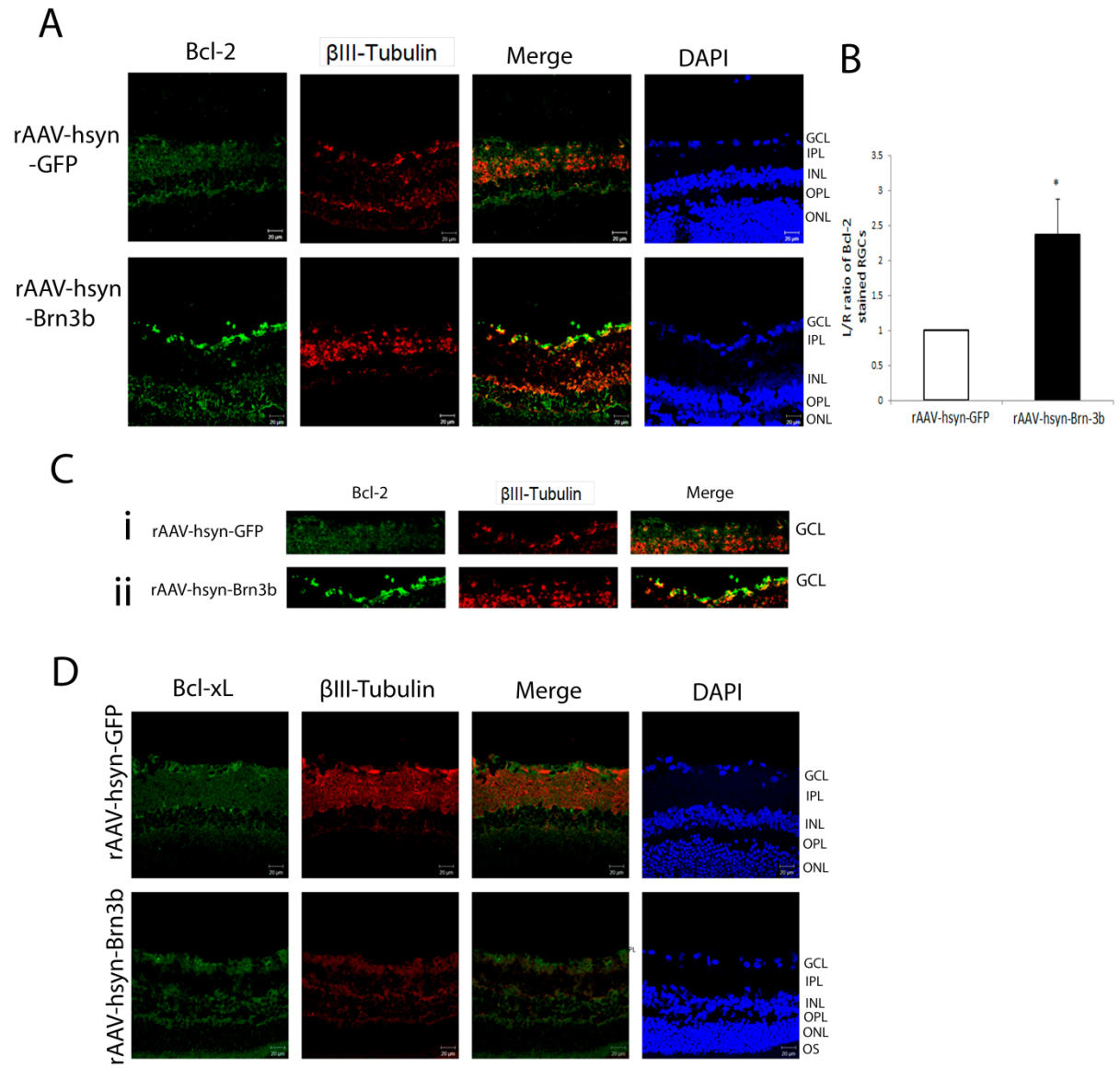
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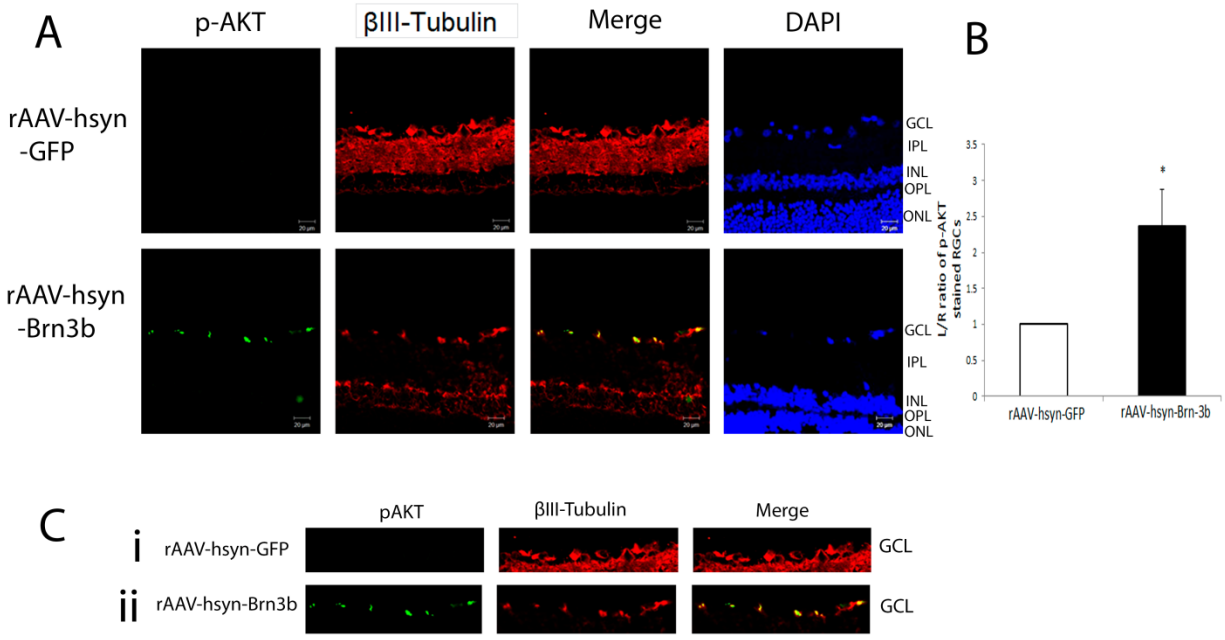

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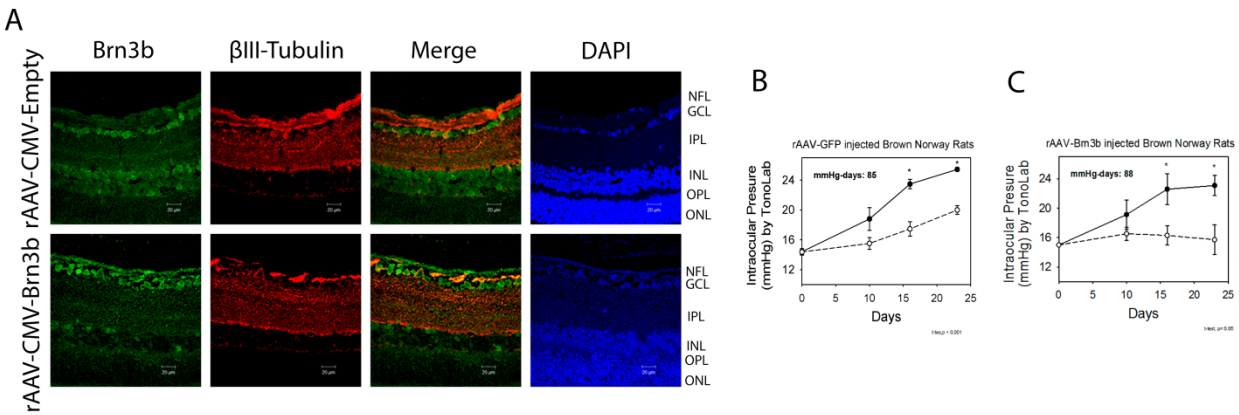

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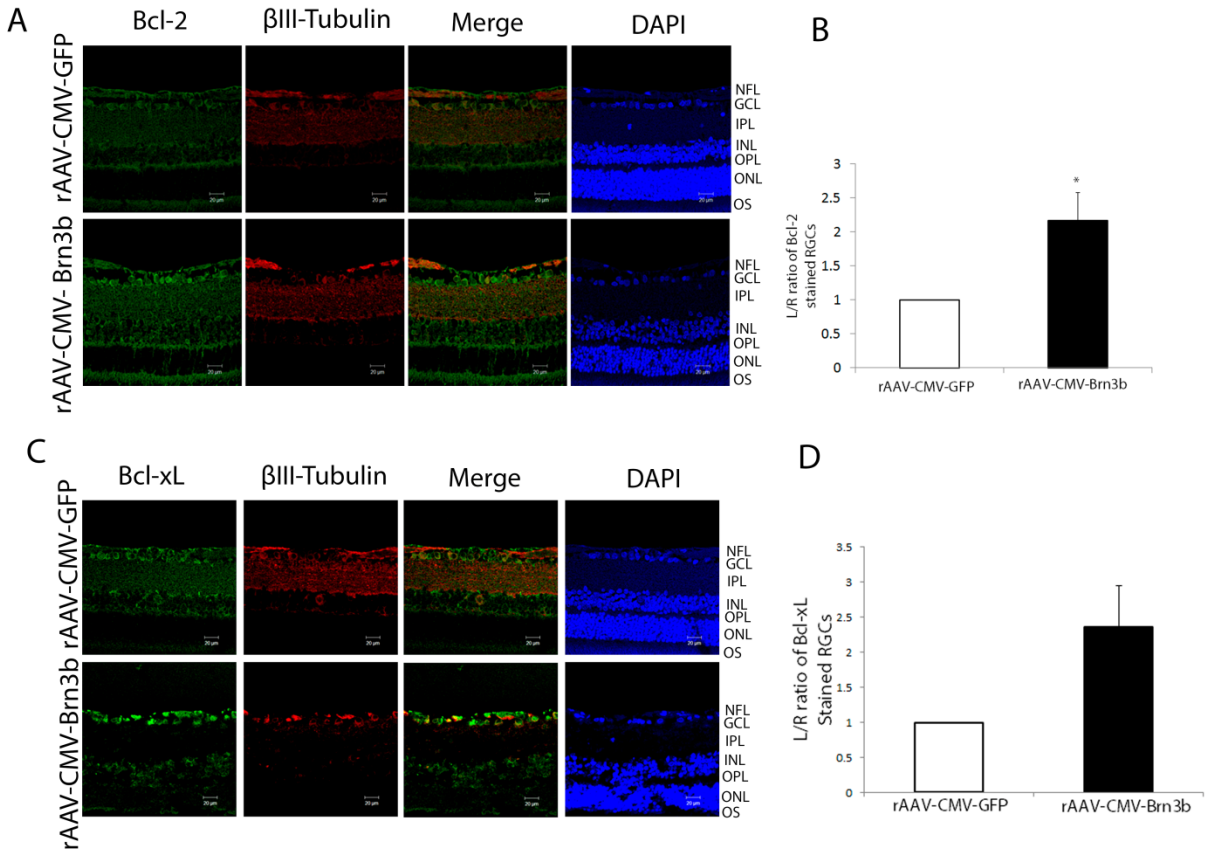
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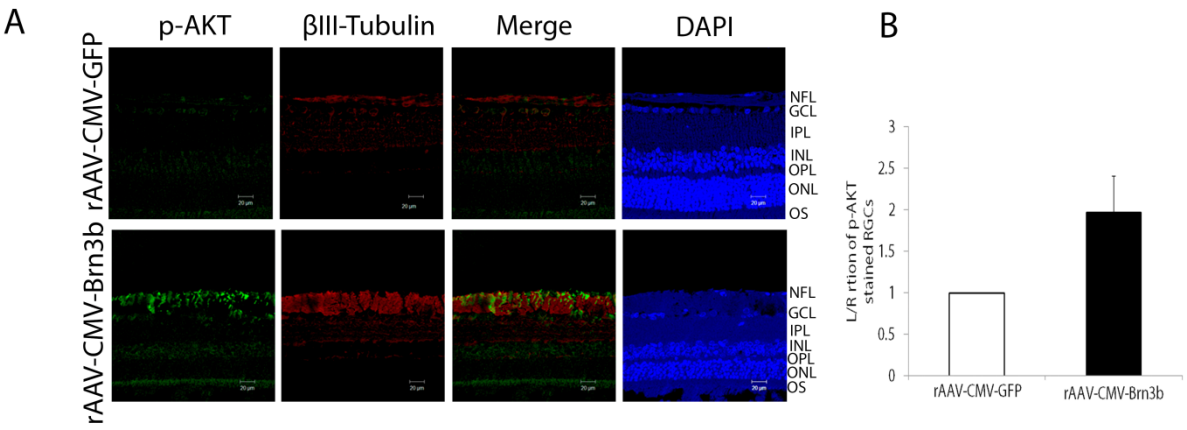
Figure 6: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CHAPTER IV

CONCLUSIONS

A crucial endpoint in the pathophysiology of all forms of glaucoma is the apoptosis of retinal ganglion cells (RGCs), a population of CNS neurons with their soma in the inner retina and axons in the optic nerve (Figure 1).

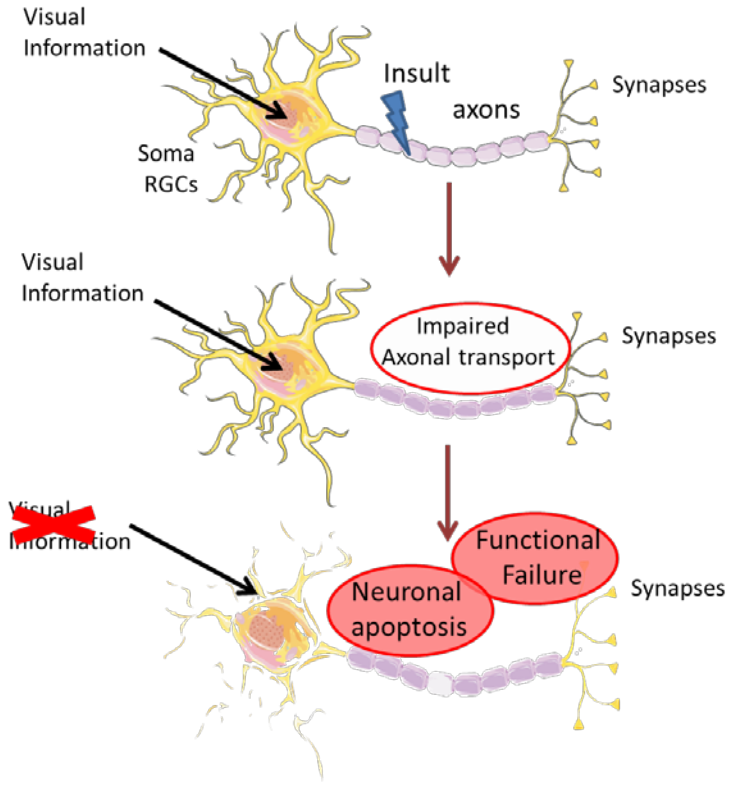

Figure 1: Elevated IOP causes axonopathy and degeneration of neurons. Representation of (a) axonal injury to RGC, (b) impaired axonal transport due to axonal injury (c) culmination of events that result in neurodegeneration and RGC apoptosis.

Strategies that delay or halt RGC loss have been recognized as potentially beneficial to preserve vision in glaucoma patients. However, the success of these approaches depends on an in –depth understanding of the mechanisms which lead to RGC dysfunction and death. The emerging landscape is complex and points to a variety of molecular signals – acting alone or synergistically – to promote RGC death (Figure 2) (Almasieh et al 2012).

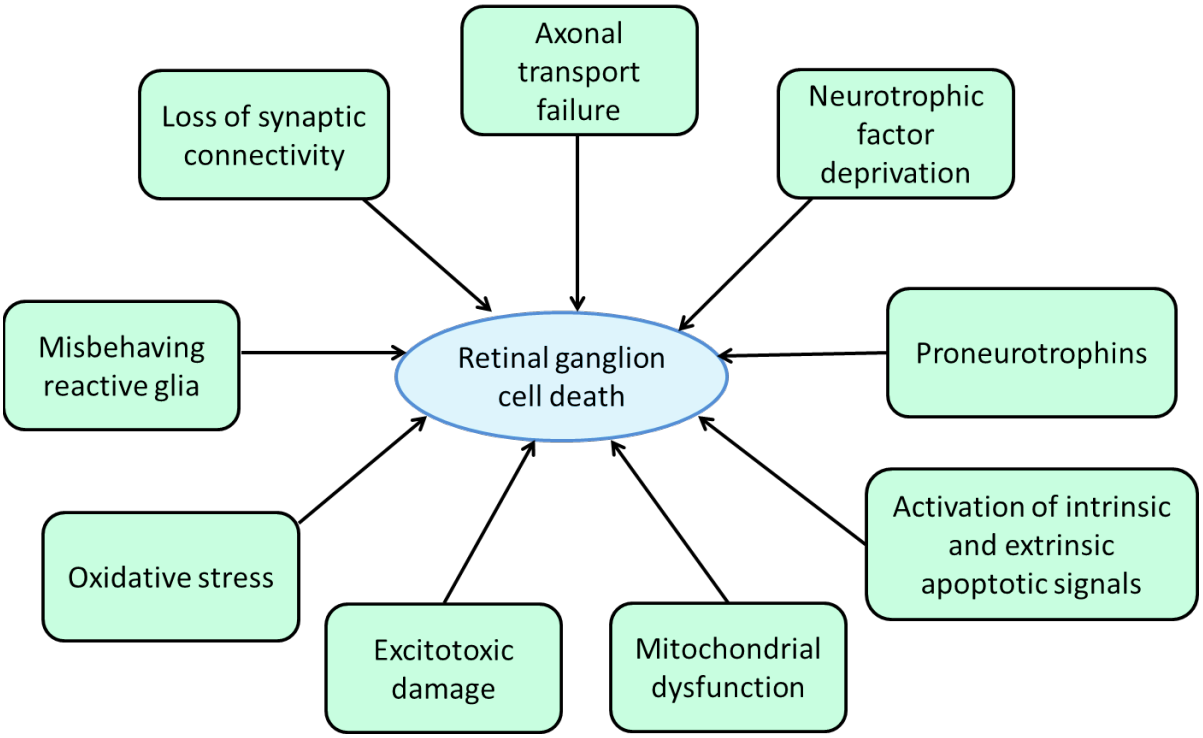

Figure 2: Schematic representation of a variety of molecular signals acting alone or synergistically to promote RGC death.

Neurotrophins (NTs) are a family of proteins which promote survival (Hempstead, 2006), development and function (Reichardt, 2006) of neurons as well as maintenance of the nervous system. Hence, neurotrophins are considered as growth factors of the nervous system and are secreted primarily by target tissue innervated by neurons. During development, the neurons which make synaptic contact with target cells releasing neurotrophins survive, other neurons that are

unable to find trophic support are eliminated by process of apoptosis. The term trophic is used generally to indicate a pro-survival action towards target cells by signaling molecules, which also includes neurotrophins.

Studies in primates demonstrate that experimentally elevated IOP results in axonal transport obstruction at the optic nerve head (Minckler et al.1977; Minckler et al. 1978). Retrograde transport of radiolabeled brain-derived neurotrophic factor (BDNF), a potent trophic factor for RGCs, is obstructed in rats with acute increase of IOP (Quigley et al., 2000). In addition, immunolocalization studies suggest that the BDNF receptor, TrkB, accumulates in the optic nerve head (Pease et al.2000). These key findings suggest that obstruction of axonal transport inhibits the retrograde delivery of NT-Trk receptor complexes from the brain to RGC soma, resulting in deprivation of neurotrophic support that triggers apoptosis (Quigley 1995; Pease et al.2000; Quigley et al.2000; Vrabec &Levin 2007). Importantly, the implication of these findings that the appropriate therapeutic manipulation of the NT signaling pathways will prolong the survival of injured RGCs.Various neurotrophic factors such as BDNF, GDNF, NT-4/5, CNTF and NGF exert short-lived neurotrophic effects both *in vivo and in vitro.* Deletion of the phosphatase and tensin homolog (PTEN) gene, a negative regulator of the mammalian target of rapamycin (mTOR), is neuroprotective and promotes robust axon regeneration of injured RGCs (Park et al. 2008, 2010). In addition, PTEN deletion reportedly stimulates axon growth of cortical neurons, facilitates the regenerative outgrowth of adult peripheral axons, and rescues the axonal growth defect of survival motor neuron (SMN)-deficient motor neurons (Liu et al. 2010; Christie et al.2010; Ning et al. 2010). These studies highlight the role of the PI3K/AKT/mTOR pathway in neuroprotection and axon growth stimulation. Continuously released CNTF and LIF from retinal astrocytes have been identified as key mediators for both neuroprotective and axon-growth promoting effects (Leibinger

et al.2009; Muller et al. 2007, 2009) and activate several signaling pathways including the JAK/STAT3, PI3K/AKT and MAPK/ERK-signaling pathways (Heinrich et al.2003). While neurotrophins are attractive candidates for neuroprotective approaches, it is unclear if they would be feasible for a long term treatment since their receptors could undergo desensitization or downregulation (Rosa et al., 2015; Xu et al., 2015; Fabbro et al., 2004) ,thereby limiting their actions.

A major strength of our study is that it relies on the retinal ganglion cell-specific transcription factor, Brn3b to promote the intrinsic survival capacity of RGCs, thereby minimizing off-target effects. Brn3b is also constitutively expressed in adult RGCs (mature), suggestive of its putative role in physiology of RGCs. A decrease in Brn3b expression has been demonstrated in different animal models of glaucoma (Naskar et al., 2006; Soto et al., 2008; Weishaupt et al., 2005), however, it remains to be seen if a knockdown of Brn3b expression could affect RGC survival. Characterization of Brn3 factors in mammalian neuronal development could thus shed light not only on de novo axon formation during embryogenesis but also on the failure of neuron projections to regenerate in adults (Goldberg and Barres, 2000).

GAP-43 has been named a growth protein because it is expressed at high levels in neuronal growth cones during development, and during axonal regeneration. It is a very important component of the axon and presynaptic terminal so that it can guide the direction to the growth cone depending on positive or repulsive guidance cues. GAP-43 is a classical marker of axonal regeneration (Xi et al. 2009; Fujino et al. 2011; Ju et al. 2010). GAP proteins are transported by cytoskeletal proteins to the injured end of axon, where they are incorporated into the membranes of growth cones (Reh et al. 1993; Rousseau et al. 2001). Experiments with AAV-BDNF, -CNTF and GAP-43 have shown that AAV-CNTF was the most crucial for promoting both long term survival and

regeneration (Leaver et al. 2006). The positive effects of CNTF were observed mainly through deletion of PTEN as well as SOCS3 and simultaneous activation of mTOR and STAT3 pathways (Smith et al. 2009). Overexpression of neuritin-1 (another protein selectively expressed in the RGCs) using the AAV-2 vector produced increased expression of GAP-43 (an active RGC regenerative state) within the retina and optic nerve following optic nerve crush (Sharma et al. 2015; Doster et al. 1991; Chidlow et al. 2011). AAV-2 mediated overexpression Brn3b during IOP elevation leads to increased expression of GAP-43 posterior to the optic nerve head (Stankowska et al.2015).

A growth cone is composed of a central domain rich in microtubules and a peripheral domain enriched in actin filaments. Microtubules are the building blocks of an axon, and axon extension occurs through microtubule assembly mediated by polymerization of tubulin subunits. Further, stabilization and destabilization of microtubules in the growth cone occurs which manifests as attractive or repulsive turning in response to guidance cues (Conde et al. 2009). Low concentrations of the chemotherapeutic agent, Taxol, have been shown to promote acetylation of α-Tubulin and neurite elongation of RGCs in culture (Fournier et al. 1995). In optic nerve injury, taxol improved axon regeneration when combined with lens injury, a well-established protocol to augment the intrinsic growth state (Sengottuvel et al. 2011). Taxol at the injury site could reduce fibrotic scarring, in part by preventing transforming growth factor (TGF-β) signaling (Hellal et al. 2011). Notably, taxol promoted axon growth in cultures neurons (Hellal et al. 2011), and induced formation of growth cone like structures after nerve injury in vivo (Erturk et al. 2007; Hellal et al. 2011), demonstrating its direct effect on neuronal microtubules. Thus, the enhanced axon regeneration observed *in vivo* might be attributed to both environmental and neuron-intrinsic mechanisms. In the present study, we demonstrated that overexpression of Brn3b in PC12 cells

leads to upregulation of neurite growth markers, GAP-43 and ac-TUBA in normoxia as well as in hypoxia.

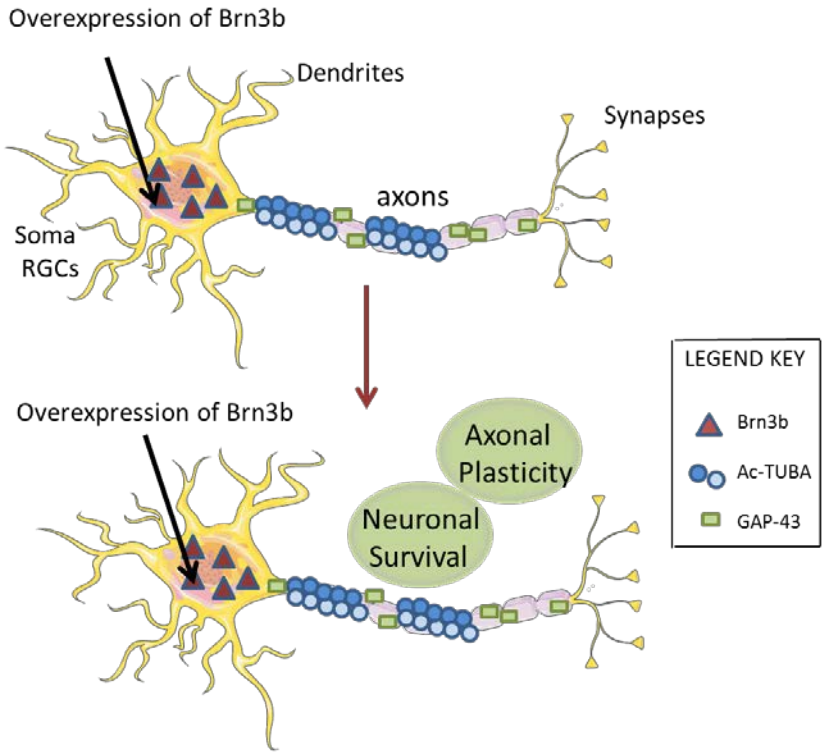

Figure 3: Overexpression of Brn3b leads to upregulation of axonal growth markers, GAP-43 and ac-TUBA

In recent years, several key intracellular signals and transcription factors that play important roles to boost the intrinsic growth programs of CNS axons have been identified. These include cyclic adenosine monophosphate (cAMP), mammalian target of rapamycin (mTOR), B cell lymphoma/leukemia 2 (Bcl-2), Kruppel-like transcription factors (KLFs) (Cai et al. 2001; Rodger et al. 2005; Verma et al. 2005; Chong et al. 2010; Park et al. 2008; Chen at al. 1997; Cho et al.2005; Moore et al. 2009, 2011; Wang et al. 2007; Laub et al.2005). Using AAV therapy, AAV injections of CNTF in Bcl-2 transgenic mice was found to increase cell viability and promote axonal

regeneration (Leaver et al. 2006), whereas BDNF was found to promote survival of RGCs (Harvey et al. 2009). In young (postnatal 4 days to 2 months) Bcl-2 transgenic mice, RGCs exhibit greater regenerative growth *in vitro* and *in vivo* (Chen et al. 1997; Jiao et al., 2005). One of earliest work demonstrating optic nerve regeneration in rodents was carried out by Cho et al. (2005), who demonstrated axonal regeneration following Bcl-2 overexpression and L-aminoadipic acid (astrotoxin) treatment (to kill reactive astrocytes). From all these studies, we can conclude that Bcl-2 potentiates the intrinsic growth capacity of RGCs and promotes neuroprotection.

Viral vector mediated expression of Bcl-xL has been shown to increase RGC survival after ON transection (Kretz et al. 2004; Malik et al. 2005).

Trophic factors	Disease Model	Effects on Bcl-2 family	Reference
IGF-1	Parkinson's disease	Upregulation of Bcl-2	Offen D et al. 2001
GDNF	Alzheimer's disease	Upregulation of Bcl-2 and Bcl-xL	Ghribi O et al. 2001
TGF-β	Alzheimer's disease, Cerebral ischemia	Upregulation of Bcl-2 and Bcl-xL	Prehn JH et al. 1996; Kim ES et al. 1998; Zhu Y et al. 2002
FGF-2	Cerebral ischemia	Upregulation of Bcl-2	Ay I.et al. 2001

Erythropoietin	Cerebral ischemia	Upregulation of Bcl-xL	Wen TC et al. 2002

Table 1. Changes in the expression of Bcl-2 and Bcl-xL in neurodegenerative diseases like Parkinson's disease, Alzheimer's disease and cerebral ischemia.

PI3-kinase activates the RAC serine/threonine kinase (AKT) pathway that supports cell survival and protein synthesis (Figure 4). Binding of NT to Trk receptors (Tyrosine kinase receptor) induces the signaling network involving PI3-K, AKT, mTOR pathways evoking either pro-survival or pro-growth and both responses simultaneously (Johnson et al. 2009). Kermer et al. (2000) showed that IGF-1 protects RGCs via PI3K-dependent

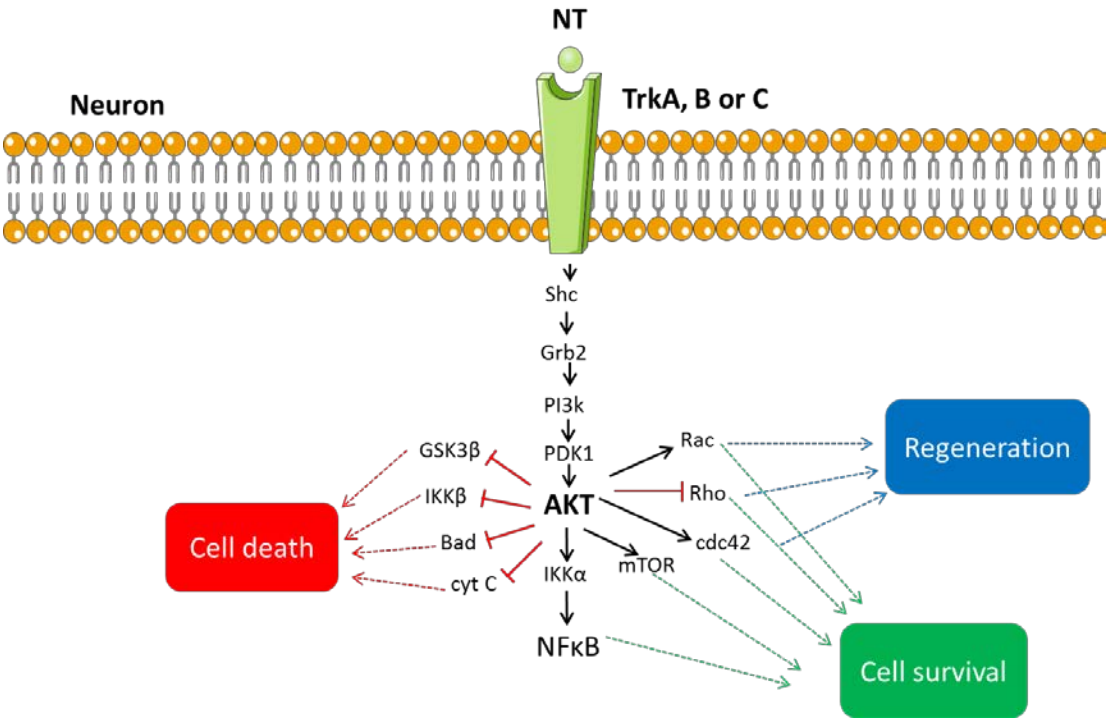

Figure 4: AKT survival pathway activated by neurotrophins. Binding of neurotrophins to Trk receptors activates signal transduction pathways leading to activation of AKT which has

an effect on several effectors. By inhibiting GSK3β, IKKβ, Bad and cytochrome C release from mitochondria, AKT prevents cell death. In addition, cell survival responses are also activated by AKT through NFκB, mTOR, Rac and cdc42 mediated mechanisms. (Adapted from Mueller et al., Neuroprotection in Glaucoma, InTech Publishers, 2011).

AKT phosphorylation and inhibition of caspase-3 in optic nerve transection model. One of the downstream effectors of the PI3K-AKT survival pathway is Bcl-2 (Matter et al. 2001; Alonzi et al. 2001; Brunet et al. 2001, Huang et al. 2003; Pugazhenthi et al. 2000; Cardona-Gomez et al. 2001). Binding of ligands to specific receptors activate either receptor tyrosine kinase or non-receptor tyrosine kinase which leads to activation of PLCγ, culminating in IP3 production. Binding of IP3 to its receptor, results in calcium release from endoplasmic reticulum. An increase in intracellular calcium levels leads to activation of PI3K and Bcl-2 (Scharenberg and Kinet 1998; Deb et al. 2004; Koga et al. 2014) (Figure 5).

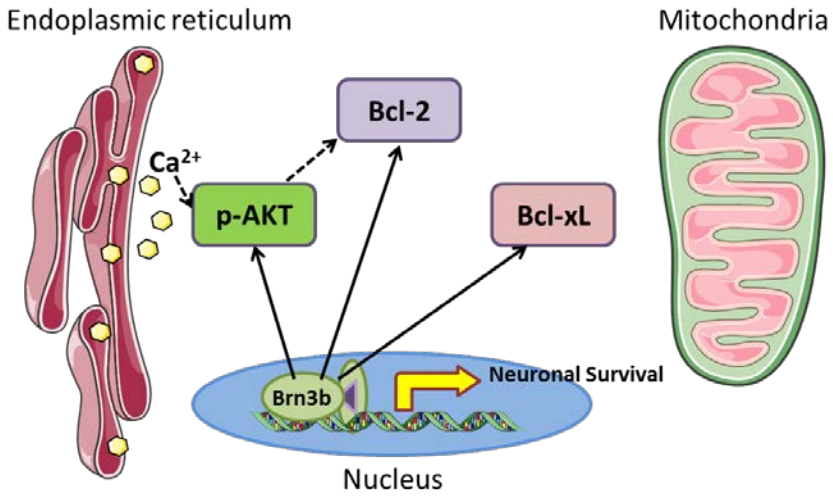

Figure 5: Proposed Brn3b signaling pathway for neuroprotection. Transcription factor, Brn3b leads to activation of key pro-survival proteins Bcl-2, Bcl-xL and p-AKT. Phosphorylation of AKT may be responsible for increased expression of Bcl-2.

In summary, overexpression of Brn3b leads to upregulation of pro-survival proteins Bcl-2, Bcl-xL and p-AKT in RGCs of rats. Taken together, Brn3b promotes the upregulation of several neuroprotective factors including the growth cone marker, GAP-43 and increases ac-TUBA as well as pro-survival proteins Bcl-2, Bcl-xL and p-AKT, making it a unique target for neuroprotection therapy in glaucoma. We designed an AAV-2 based Brn3b therapy to target RGCs and observed the effects of Brn3b over-expression in retinas after IOP elevation. Our studies indicated that Brn3b exhibited neuroprotective effects on RGCs after IOP elevation. These data imply that Brn3b may play important role in neuronal survival as well as neurite outgrowth.

As drugs for neuroprotection to treat glaucoma have to reach the axons and somas of RGCs, neuroprotectants as eyedrops would have been ideal to administer for glaucoma patients. However, the ocular system (eye) possesses key barriers that would block the drugs from penetrating to the back of the eye, which limits the exact dose of drug reaching RGCs. More recent advances in drug delivery systems include viral vectors and nanoparticles, which hold much promise to prolong effective treatment to the retina and optic nerve. In this study, we are using AAV-2 viral vectors to administer the neuroprotective protein, Brn3b. Interestingly, gene therapy through viral vectors has gained more attention in recent years, especially after treatment of patients suffering from Leber's congenital amaurosis, using an adeno-associated viral vector encoding RPE65, which showed promising results of safety and efficacy (Bainbridge et al. 2008; Koenekoop et al. 2008). Ocular gene therapy has emerged as a potential treatment modality for incurable human genetic retinal disease like Leber congenital amaurosis caused by RPE65 gene mutations. It has become

the target disease for a number of groups worldwide for the first subretinal gene transfer trial in man (Jacobson et al. 2006). Gene therapy with rAAV2/2 RPE65 vector improved retinal sensitivity, modestly and temporarily. However, the need for RPE65 in affected persons was not met to the extent required for a marked, long-lasting effect. (Bainbridge et al., 2015).

Previous work from our laboratory demonstrated significant neuroprotective effects of Brn3b overexpression on RGCs and optic nerve axons in a rat model of ocular hypertension (Stankowska et al., 2015). In the current study, two models were used, first, PC12 cells under hypoxic conditions and second, a rat model of ocular hypertension followed by overexpression of transcription factor, Brn3b to determine the association of the prosurvival factor Bcl-2 in neuroprotection.. The importance of this study is to investigate the molecular mechanism underlying Brn3b's potent neuroprotective effect in RGCs, since studies proposing Brn3b's mechanism of action, particularly in glaucomatous conditions are lacking. POAG is not a single disease entity but a heterogeneous group of diseases that result in RGCs death through multiple mechanisms. The current study demonstrates that Brn3b has potentially multiple mechanisms that can be employed to protect RGCs, it is an efficacious neuroprotective molecule that can be developed as an adjunct neuroprotective treatment for patients with POAG.

References

Almasieh M, Wilson AM, Morquette B, Cueva Vargas JL, Di Polo A. The molecular basis of retinal ganglion cell death in glaucoma Prog Retin Eye Res 2012 Mar;31(2):152-181.

Alonzi T, Middleton G, Wyatt S, Buchman V, Betz UA, Muller W, et al. Role of STAT3 and PI 3-kinase/Akt in mediating the survival actions of cytokines on sensory neurons Mol Cell Neurosci 2001 Sep;18(3):270-282.

Ay I, Sugimori H, Finklestein SP. Intravenous basic fibroblast growth factor (bFGF) decreases DNA fragmentation and prevents downregulation of Bcl-2 expression in the ischemic brain following middle cerebral artery occlusion in rats Brain Res Mol Brain Res 2001 Feb 19;87(1):71-80.

Bainbridge JWB, Smith AJ, Barker SS, Robbie S, Henderson R, Balaggan K, et al. Effect of Gene Therapy on Visual Function in Leber's Congenital Amaurosis N Engl J Med 2008;358(21):2231-2239.

Bainbridge JW, Mehat MS, Sundaram V, Robbie SJ, Barker SE, Ripamonti C, Georgiadis A, Mowat FM, Beattie SG, Gardner PJ, Feathers KL, Luong VA, Yzer S, Balaggan K, Viswanathan A, de Ravel TJ, Casteels I, Holder GE, Tyler N, Fitzke FW, Weleber RG, Nardini M, Moore AT, Thompson DA, Petersen-Jones SM, Michaelides M, van den Born LI, Stockman A, Smith AJ, Rubin G, Ali RR. Long-term effect of gene therapy on Leber's congenital amaurosis. N Engl J Med. 2015 May 14;372(20):1887-97.

Brunet A, Datta SR, Greenberg ME. Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway Curr Opin Neurobiol 2001 Jun;11(3):297-305.

Cai D, Qiu J, Cao Z, McAtee M, Bregman BS, Filbin MT. Neuronal cyclic AMP controls the developmental loss in ability of axons to regenerate J Neurosci 2001 Jul 1;21(13):4731-4739.

Cardona-Gomez GP, Mendez P, DonCarlos LL, Azcoitia I, Garcia-Segura LM. Interactions of estrogens and insulin-like growth factor-I in the brain: implications for neuroprotection Brain Res Brain Res Rev 2001 Nov;37(1-3):320-334.

Chen DF, Schneider GE, Martinou JC, Tonegawa S. Bcl-2 promotes regeneration of severed axons in mammalian CNS Nature 1997 Jan 30;385(6615):434-439.

Chidlow G, Ebneter A, Wood JP, Casson RJ. The optic nerve head is the site of axonal transport disruption, axonal cytoskeleton damage and putative axonal regeneration failure in a rat model of glaucoma Acta Neuropathol 2011 Jun;121(6):737-751.

Cho KS, Yang L, Lu B, Feng Ma H, Huang X, Pekny M, et al. Re-establishing the regenerative potential of central nervous system axons in postnatal mice J Cell Sci 2005 Mar 1;118(Pt 5):863-872.

Chong ZZ, Shang YC, Zhang L, Wang S, Maiese K. Mammalian target of rapamycin: hitting the bull's-eye for neurological disorders Oxid Med Cell Longev 2010 Nov-Dec;3(6):374-391.

Christie KJ, Webber CA, Martinez JA, Singh B, Zochodne DW. PTEN inhibition to facilitate intrinsic regenerative outgrowth of adult peripheral axons J Neurosci 2010 Jul 7;30(27):9306-9315.

Conde C, Caceres A. Microtubule assembly, organization and dynamics in axons and dendrites Nat Rev Neurosci 2009 May;10(5):319-332.

Dompierre JP, Godin JD, Charrin BC, Cordelieres FP, King SJ, Humbert S, et al. Histone deacetylase 6 inhibition compensates for the transport deficit in Huntington's disease by increasing tubulin acetylation J Neurosci 2007 Mar 28;27(13):3571-3583.

Doster SK, Lozano AM, Aguayo AJ, Willard MB. Expression of the growth-associated protein GAP-43 in adult rat retinal ganglion cells following axon injury Neuron 1991 Apr;6(4):635-647.

Erturk A, Hellal F, Enes J, Bradke F. Disorganized microtubules underlie the formation of retraction bulbs and the failure of axonal regeneration J Neurosci 2007 Aug 22;27(34):9169-9180.

Fabbro A, Nistri A. Chronic NGF treatment of rat nociceptive DRG neurons in culture facilitates desensitization and deactivation of GABAA receptor-mediated currents. Br J Pharmacol. 2004 Jun;142(3):425-34.

Fournier AE, McKerracher L. Tubulin expression and axonal transport in injured and regenerating neurons in the adult mammalian central nervous system Biochem Cell Biol 1995 Sep-Oct;73(9-10):659-664.

Frank T, Schlachetzki JC, Goricke B, Meuer K, Rohde G, Dietz GP, et al. Both systemic and local application of granulocyte-colony stimulating factor (G-CSF) is neuroprotective after retinal ganglion cell axotomy BMC Neurosci 2009 May 14;10:49-2202-10-49.

Fujino T, Leslie JH, Eavri R, Chen JL, Lin WC, Flanders GH, et al. CPG15 regulates synapse stability in the developing and adult brain Genes Dev 2011 Dec 15;25(24):2674-2685.

Ghate D, Edelhauser HF. Barriers to glaucoma drug delivery J Glaucoma 2008 Mar;17(2):147-156.

Ghribi O, Herman MM, Forbes MS, DeWitt DA, Savory J. GDNF protects against aluminum-induced apoptosis in rabbits by upregulating Bcl-2 and Bcl-XL and inhibiting mitochondrial Bax translocation Neurobiol Dis 2001 Oct;8(5):764-773.

Harvey AR, Hellstrom M, Rodger J. Gene therapy and transplantation in the retinofugal pathway Prog Brain Res 2009;175:151-161.

Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation Biochem J 2003 Aug 15;374(Pt 1):1-20.

Hellal F, Hurtado A, Ruschel J, Flynn KC, Laskowski CJ, Umlauf M, et al. Microtubule stabilization reduces scarring and causes axon regeneration after spinal cord injury Science 2011 Feb 18;331(6019):928-931.

Hempstead BL. Dissecting the diverse actions of pro- and mature neurotrophins Curr Alzheimer Res 2006 Feb;3(1):19-24.

Huang X, Wu DY, Chen G, Manji H, Chen DF. Support of retinal ganglion cell survival and axon regeneration by lithium through a Bcl-2-dependent mechanism Invest Ophthalmol Vis Sci 2003 Jan;44(1):347-354.

Jacobson SG, Boye SL, Aleman TS, Conlon TJ, Zeiss CJ, Roman AJ, et al. Safety in nonhuman primates of ocular AAV2-RPE65, a candidate treatment for blindness in Leber congenital amaurosis Hum Gene Ther 2006 Aug;17(8):845-858.

Jiao J, Huang X, Feit-Leithman RA, Neve RL, Snider W, Dartt DA, et al. Bcl-2 enhances Ca(2+) signaling to support the intrinsic regenerative capacity of CNS axons EMBO J 2005 Mar 9;24(5):1068-1078.

Johnson EC, Guo Y, Cepurna WO, Morrison JC. Neurotrophin roles in retinal ganglion cell survival: lessons from rat glaucoma models Exp Eye Res 2009 Apr;88(4):808-815.

Ju WK, Kim KY, Duong-Polk KX, Lindsey JD, Ellisman MH, Weinreb RN. Increased optic atrophy type 1 expression protects retinal ganglion cells in a mouse model of glaucoma Mol Vis 2010 Jul 15;16:1331-1342.

Kermer P, Klocker N, Labes M, Bahr M. Insulin-like growth factor-I protects axotomized rat retinal ganglion cells from secondary death via PI3-K-dependent Akt phosphorylation and inhibition of caspase-3 In vivo J Neurosci 2000 Jan 15;20(2):2-8.

Kim ES, Kim RS, Ren RF, Hawver DB, Flanders KC. Transforming growth factor-beta inhibits apoptosis induced by beta-amyloid peptide fragment 25-35 in cultured neuronal cells Brain Res Mol Brain Res 1998 Nov 20;62(2):122-130.

Koenekoop RK. Successful RPE65 gene replacement and improved visual function in humans Ophthalmic Genet 2008 Sep;29(3):89-91.

Koga T, Hedrich CM, Mizui M, Yoshida N, Otomo K, Lieberman LA, et al. CaMK4-dependent activation of AKT/mTOR and CREM-alpha underlies autoimmunity-associated Th17 imbalance J Clin Invest 2014 May;124(5):2234-2245.

Kretz A, Kugler S, Happold C, Bahr M, Isenmann S. Excess Bcl-XL increases the intrinsic growth potential of adult CNS neurons in vitro Mol Cell Neurosci 2004 May;26(1):63-74.

Laub F, Lei L, Sumiyoshi H, Kajimura D, Dragomir C, Smaldone S, et al. Transcription factor KLF7 is important for neuronal morphogenesis in selected regions of the nervous system Mol Cell Biol 2005 Jul;25(13):5699-5711.

Leaver SG, Cui Q, Bernard O, Harvey AR. Cooperative effects of bcl-2 and AAV-mediated expression of CNTF on retinal ganglion cell survival and axonal regeneration in adult transgenic mice Eur J Neurosci 2006 Dec;24(12):3323-3332.

Leibinger M, Muller A, Andreadaki A, Hauk TG, Kirsch M, Fischer D. Neuroprotective and axon growth-promoting effects following inflammatory stimulation on mature retinal ganglion cells in mice depend on ciliary neurotrophic factor and leukemia inhibitory factor J Neurosci 2009 Nov 11;29(45):14334-14341.

Liu K, Lu Y, Lee JK, Samara R, Willenberg R, Sears-Kraxberger I, et al. PTEN deletion enhances the regenerative ability of adult corticospinal neurons Nat Neurosci 2010 Sep;13(9):1075-1081.

Malik JM, Shevtsova Z, Bahr M, Kugler S. Long-term in vivo inhibition of CNS neurodegeneration by Bcl-XL gene transfer Mol Ther 2005 Mar;11(3):373-381.

Martin KR, Quigley HA, Valenta D, Kielczewski J, Pease ME. Optic nerve dynein motor protein distribution changes with intraocular pressure elevation in a rat model of glaucoma Exp Eye Res 2006 Aug;83(2):255-262.

Matter ML, Ruoslahti E. A signaling pathway from the alpha5beta1 and alpha(v)beta3 integrins that elevates bcl-2 transcription J Biol Chem 2001 Jul 27;276(30):27757-27763.

Minckler DS, Bunt AH, Johanson GW. Orthograde and retrograde axoplasmic transport during acute ocular hypertension in the monkey Invest Ophthalmol Vis Sci 1977 May;16(5):426-441.

Minckler DS, Bunt AH, Klock IB. Radioautographic and cytochemical ultrastructural studies of axoplasmic transport in the monkey optic nerve head Invest Ophthalmol Vis Sci 1978 Jan;17(1):33-50.

Minton AZ, Phatak NR, Stankowska DL, He S, Ma HY, Mueller BH, et al. Endothelin B receptors contribute to retinal ganglion cell loss in a rat model of glaucoma PLoS One 2012;7(8):e43199.

Moore DL, Apara A, Goldberg JL. Kruppel-like transcription factors in the nervous system: novel players in neurite outgrowth and axon regeneration Mol Cell Neurosci 2011 Aug;47(4):233-243.

Moore DL, Blackmore MG, Hu Y, Kaestner KH, Bixby JL, Lemmon VP, et al. KLF family members regulate intrinsic axon regeneration ability Science 2009 Oct 9;326(5950):298-301.

Mueller BH,2nd, Park Y, Ma HY, Dibas A, Ellis DZ, Clark AF, et al. Sigma-1 receptor stimulation protects retinal ganglion cells from ischemia-like insult through the activation of extracellular-signal-regulated kinases 1/2 Exp Eye Res 2014 Nov;128:156-169.

Muller A, Hauk TG, Fischer D. Astrocyte-derived CNTF switches mature RGCs to a regenerative state following inflammatory stimulation Brain 2007 Dec;130(Pt 12):3308-3320.

Muller A, Hauk TG, Leibinger M, Marienfeld R, Fischer D. Exogenous CNTF stimulates axon regeneration of retinal ganglion cells partially via endogenous CNTF Mol Cell Neurosci 2009 Jun;41(2):233-246.

Nakazawa T, Nakazawa C, Matsubara A, Noda K, Hisatomi T, She H, et al. Tumor necrosis factor-alpha mediates oligodendrocyte death and delayed retinal ganglion cell loss in a mouse model of glaucoma J Neurosci 2006 Dec 6;26(49):12633-12641.

Naskar R, Thanos S. Retinal gene profiling in a hereditary rodent model ofelevated intraocular pressure. Mol Vis. 2006 Oct 18; 12:1199-210.

Ning K, Drepper C, Valori CF, Ahsan M, Wyles M, Higginbottom A, et al. PTEN depletion rescues axonal growth defect and improves survival in SMN-deficient motor neurons Hum Mol Genet 2010 Aug 15;19(16):3159-3168.

Offen D, Shtaif B, Hadad D, Weizman A, Melamed E, Gil-Ad I. Protective effect of insulin-like-growth-factor-1 against dopamine-induced neurotoxicity in human and rodent neuronal cultures: possible implications for Parkinson's disease Neurosci Lett 2001 Dec 28;316(3):129-132.

Park KK, Liu K, Hu Y, Kanter JL, He Z. PTEN/mTOR and axon regeneration Exp Neurol 2010 May;223(1):45-50.

Park KK, Liu K, Hu Y, Smith PD, Wang C, Cai B, et al. Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway Science 2008 Nov 7;322(5903):963-966.

Pease ME, McKinnon SJ, Quigley HA, Kerrigan-Baumrind LA, Zack DJ. Obstructed axonal transport of BDNF and its receptor TrkB in experimental glaucoma Invest Ophthalmol Vis Sci 2000 Mar;41(3):764-774.

Prehn JH, Bindokas VP, Jordan J, Galindo MF, Ghadge GD, Roos RP, et al. Protective effect of transforming growth factor-beta 1 on beta-amyloid neurotoxicity in rat hippocampal neurons Mol Pharmacol 1996 Feb;49(2):319-328.

Pugazhenthi S, Nesterova A, Sable C, Heidenreich KA, Boxer LM, Heasley LE, et al. Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein J Biol Chem 2000 Apr 14;275(15):10761-10766.

Quigley HA, McKinnon SJ, Zack DJ, Pease ME, Kerrigan-Baumrind LA, Kerrigan DF, et al. Retrograde axonal transport of BDNF in retinal ganglion cells is blocked by acute IOP elevation in rats Invest Ophthalmol Vis Sci 2000 Oct;41(11):3460-3466.

Quigley HA, Nickells RW, Kerrigan LA, Pease ME, Thibault DJ, Zack DJ. Retinal ganglion cell death in experimental glaucoma and after axotomy occurs by apoptosis Invest Ophthalmol Vis Sci 1995 Apr;36(5):774-786.

Reh TA, Tetzlaff W, Ertlmaier A, Zwiers H. Developmental study of the expression of B50/GAP-43 in rat retina J Neurobiol 1993 Jul;24(7):949-958.

Reichardt LF. Neurotrophin-regulated signalling pathways Philos Trans R Soc Lond B Biol Sci 2006 Sep 29;361(1473):1545-1564.

Rodger J, Goto H, Cui Q, Chen PB, Harvey AR. cAMP regulates axon outgrowth and guidance during optic nerve regeneration in goldfish Mol Cell Neurosci 2005 Nov;30(3):452-464.

Rosa E, Fahnestock M. CREB expression mediates amyloid β-induced basal BDNF downregulation. Neurobiol Aging. 2015 Aug;36(8):2406-13. doi: 10.1016/j.neurobiolaging.2015.04.014.

Rousseau V, Sabel BA. Restoration of vision IV: role of compensatory soma swelling of surviving retinal ganglion cells in recovery of vision after optic nerve crush Restor Neurol Neurosci 2001;18(4):177-189.

Scharenberg AM, Kinet JP. PtdIns-3,4,5-P3: a regulatory nexus between tyrosine kinases and sustained calcium signals Cell 1998 Jul 10;94(1):5-8.

Schober MS, Chidlow G, Wood JP, Casson RJ. Bioenergetic-based neuroprotection and glaucoma Clin Experiment Ophthalmol 2008 May;36(4):377-385.

Schwartz M. Optic nerve crush: protection and regeneration Brain Res Bull 2004 Feb 15;62(6):467-471.

Sengottuvel V, Leibinger M, Pfreimer M, Andreadaki A, Fischer D. Taxol facilitates axon regeneration in the mature CNS J Neurosci 2011 Feb 16;31(7):2688-2699.

Sharma TP, Liu Y, Wordinger RJ, Pang IH, Clark AF. Neuritin 1 promotes retinal ganglion cell survival and axonal regeneration following optic nerve crush Cell Death Dis 2015 Feb 26;6:e1661.

Smith PD, Sun F, Park KK, Cai B, Wang C, Kuwako K, et al. SOCS3 deletion promotes optic nerve regeneration in vivo Neuron 2009 Dec 10;64(5):617-623.

Stankowska DL, Minton AZ, Rutledge MA, Mueller BH,2nd, Phatak NR, He S, et al. Neuroprotective effects of transcription factor Brn3b in an ocular hypertension rat model of glaucoma Invest Ophthalmol Vis Sci 2015 Jan 13;56(2):893-907.

Soto I, Oglesby E, Buckingham BP, Son JL, Roberson ED, Steele MR, Inman DM, Vetter ML, Horner PJ, Marsh-Armstrong N. Retinal ganglion cells downregulate gene expression and lose their axons within the optic nerve head in a mouse glaucoma model. J Neurosci. 2008 Jan 9;28(2):548-61.

Deb TB, Coticchia CM, Dickson RB. Calmodulin-mediated activation of Akt regulates survival of c-Myc-overexpressing mouse mammary carcinoma cells J Biol Chem 2004 Sep 10;279(37):38903-38911.

Verma P, Chierzi S, Codd AM, Campbell DS, Meyer RL, Holt CE, et al. Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration J Neurosci 2005 Jan 12;25(2):331-342.

Vrabec JP, Levin LA. The neurobiology of cell death in glaucoma Eye (Lond) 2007 Dec;21 Suppl 1:S11-4.

Wang JT, Kunzevitzky NJ, Dugas JC, Cameron M, Barres BA, Goldberg JL. Disease gene candidates revealed by expression profiling of retinal ganglion cell development J Neurosci 2007 Aug 8;27(32):8593-8603.

Weishaupt JH, Klöcker N, Bähr M. Axotomy-induced early down-regulation of POU-IV class transcription factors Brn-3a and Brn-3b in retinal ganglion cells. J Mol Neurosci. 2005;26(1):17-25.

Wen TC, Sadamoto Y, Tanaka J, Zhu PX, Nakata K, Ma YJ, et al. Erythropoietin protects neurons against chemical hypoxia and cerebral ischemic injury by up-regulating Bcl-xL expression J Neurosci Res 2002 Mar 15;67(6):795-803.

Xi S, Liu W, Zhang H, Gao R, Huang J. Effect of Neuritin protein on axonal regeneration after acute spinal cord injury in rats Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi 2009 Oct;23(10):1219-1223.

Xu H, Czerwinski P, Xia N, Förstermann U, Li H. Downregulation of BDNF Expression by PKC and by TNF-α in Human Endothelial Cells. Pharmacology. 2015 May 28;96(1-2):1-10.

Zhu Y, Yang GY, Ahlemeyer B, Pang L, Che XM, Culmsee C, et al. Transforming growth factor-beta 1 increases bad phosphorylation and protects neurons against damage J Neurosci 2002 May 15;22(10):3898-3909.

Gan L, Wang SW, Huang Z, Klein WH. POU domain factor Brn-3b is essential for retinal ganglion cell differentiation and survival but not for initial cell fate specification Dev Biol 1999 Jun 15;210(2):469-480.

Erkman L, Yates PA, McLaughlin T, McEvilly RJ, Whisenhunt T, O'Connell SM, et al. A POU domain transcription factor-dependent program regulates axon pathfinding in the vertebrate visual system Neuron 2000 Dec;28(3):779-792.

Xiang M, Gan L, Li D, Zhou I, Chen ZY, Wanger D, O'Mallaey BW Jr, Nathans J. Role of the Brn-3 family of POU-domain genes in the development of the auditory/vestibular, somatosensory, and visual systems. Cold Spring Harb Symp Quant Biol 1997 62:325-336.

Turner EE, Jenne KJ, Rosenfeld MG. Brn-3.2 a Brn-3-related transcription factor with distinctive central nervous system expression and regulation by retinoic acid. Neuron 1994 12(1):205-218.

Goldberg JL and Barres BA. The relationship between neuronal survival and regeneration. Annu Rev Neurosci 2000 23, 579-612.

CHAPTER V

FUTURE DIRECTIONS

Although the research presented in this dissertation generated novel findings with regards to the neuroprotective effects and mechanism of action of Brn3b in PC12 cells as well as RGCs, there are still many unanswered questions.

1. A question that needs to be addressed is: How does Brn3b regulate GAP-43, ac-TUBA, Bcl-2, Bcl-xL and p-AKT? More studies are needed to identify the direct or indirect link of all these pro-survival genes to transcription factor, Brn3b. A second scenario would be that Brn3b leads to increase in intracellular Ca^{2+} levels which activate multiple signaling pathways to activate these proteins (Scharenberg and Kinet, 1998).
2. Assessment of visual function using pattern ERG and spectral domain OCT tests. The optic nerve and visual centers of the brain should also be assessed for synaptic connections following Brn3b overexpression and its implications in improving vision during ocular hypertension.
3. Although many studies have addressed Brn3b and its signaling in RGCs during development, a more detailed study of involvement of Brn3b in glaucoma still needs to be done. In addition, experiments should be performed *in vitro* for identifying the signaling pathways used by Brn3b and its effect on RGC survival.

a. We could investigate if RGCs treated with rAAV-CMV-Brn3b can induce phosphorylation of ERK (pERK), AKT (pAKT) and mammalian target of rapamycin (pmTOR). In addition, following Brn3b overexpression, the activation of each pathway could be blocked with the following inhibitors: MEK inhibitor (U0126), rapamycin (mTOR) or LY294002 (AKT) to determine their role in Brn3b-mediated neuroprotection. ERK regulates genes such as c-fos which are responsible for long term synaptic plasticity (Ji et al. 2002). Long term potentiation induced by BDNF or high-frequency electrical stimulation is inhibited by rapamycin in hippocampal neurons. The mTOR pathway through the AKT kinase has also recently been shown to induce synaptic plasticity (Hay and Sonenberg, 2004).

4. Using optic nerve crush as a model for optic nerve degeneration (Qin et al. 2013; Sun et al. 2011; Yin et al. 2003), we can investigate the neuroregenerative property of transcription factor, Brn3b and examine the expressions of axonal plasticity markers, GAP-43 and ac-TUBA. In addition, we could check expressions of proteins involved in stimulating the RGCs intrinsic growth state including, Bcl-2, mTOR, and AKT.
5. Mature RGCs cannot regenerate injured axons because of external factors including neurite growth inhibitors, including the C-terminal of Nogo-A (Nogo 66), myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (Omg) (Fischer et al. 2004; Su et al. 2009). Using optic nerve crush as a model, we could investigate the expression of Nogo 66 receptor and Nogo-A protein in rats overexpressing Brn3b.
6. We also could knock down Brn3b expression in adult rats (AAV-shRNA-Brn3b mediated knockdown) and investigate the changes in retina and optic nerve morphology, and visual

function. Following Brn3b knock down, we would evaluate the expressions of target proteins including GAP-43, ac-TUBA, Bcl-2, Bcl-xL and p-AKT, in the retina as well as in the optic nerve.

References

Fischer D, He Z, Benowitz LI. Counteracting the Nogo receptor enhances optic nerve regeneration if retinal ganglion cells are in an active growth state J Neurosci 2004 Feb 18;24(7):1646-1651.

Hay N, Sonenberg N. Upstream and downstream of mTOR Genes Dev 2004 Aug 15;18(16):1926-1945.

Ji RR, Befort K, Brenner GJ, Woolf CJ. ERK MAP kinase activation in superficial spinal cord neurons induces prodynorphin and NK-1 upregulation and contributes to persistent inflammatory pain hypersensitivity J Neurosci 2002 Jan 15;22(2):478-485.

Qin S, Zou Y, Zhang CL. Cross-talk between KLF4 and STAT3 regulates axon regeneration Nat Commun 2013;4:2633.

Scharenberg AM, Kinet JP.PtdIns-3,4,5-P3:a regulatory nexus between tyrosine kinases and sustained calcium signals Cell 1998 Jul 10;94(1):5-8.

Su Y, Wang F, Teng Y, Zhao SG, Cui H, Pan SH. Axonal regeneration of optic nerve after crush in Nogo66 receptor knockout mice Neurosci Lett 2009 Sep 4;460(3):223-226.

Sun F, Park KK, Belin S, Wang D, Lu T, Chen G, et al. Sustained axon regeneration induced by co-deletion of PTEN and SOCS3 Nature 2011 Nov 6;480(7377):372-375.

Yin Y, Cui Q, Li Y, Irwin N, Fischer D, Harvey AR, et al. Macrophage-derived factors stimulate optic nerve regeneration J Neurosci 2003 Mar 15;23(6):2284-2293.

LIST OF PUBLICATIONS

Minton AZ, **Phatak NR**, Stankowska DL, He S, Ma HY, Mueller BH, et al. Endothelin B receptors contribute to retinal ganglion cell loss in a rat model of glaucoma. PLoS One 2012;7(8):e43199.

Stankowska DL, Minton AZ, Rutledge MA, Mueller BH,2nd, **Phatak NR**, He S, et al. Neuroprotective effects of transcription factor Brn3b in an ocular hypertension rat model of glaucoma. Invest Ophthalmol Vis Sci 2015 Jan 13;56(2):893-907.

Phatak NR, Stankowska DL, Krishnamoorthy RR. Transcription Factor Brn-3b overexpression enhances neurite outgrowth in PC12 cells under condition of hypoxia. Cell Mol Neurobiol. 2015 Mar 19.

Phatak NR, Stankowska DL, Krishnamoorthy RR. Bcl-2, Bcl-xL and p-AKT are involved in neuroprotective effects of transcription factor Brn3b in an ocular hypertension rat model of glaucoma. (peer reviewed Mol Vis).