Growth differentiation factor 11 promotes survival of mouse retinal ganglion cells *in vitro* through Smad2/3 activation

by

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A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in partial fulfilment of the requirements for the degree of

Master of Science

in

Neuroscience

Carleton University

Ottawa, Ontario

Abstract

Growth differentiation factor 11 (GDF11) was recently identified as a rejuvenation factor that promoted neurogenesis in aged mice. Previous work has revealed that *gdf11* mRNA is expressed in the developing retina, and GDF11 protein can signal retinal ganglion cells (RGCs) to grow dendrites *in vitro*. The developmental expression pattern of GDF11 in RGCs and the impact of GDF11 on mammalian RGCs remain unknown. The current study investigated 1) the developmental time course of GDF11 expression in RGCs, 2) the effect of GDF11 on RGC survival and axon growth *in vitro*, and 3) the mechanisms mediating GDF11 responses in RGCs. Retinae were collected from C57BL/6 wild-type (WT) mice at different developmental stages to evaluate the expression of GDF11 in RGCs. Postnatal RGCs were also cultured in the presence of GDF11 treatment. The results show that GDF11 does not promote axon growth but rather enhances RGC survival *in vitro* through activation of Smad2/3.

Acknowledgement

First, I would like to extend my sincere gratitude to Dr. Patrice Smith for her guidance and encouragement. She has shown continual patience with my learning process, allowed me to use my biochemistry background to study neuroscience, and helped me to grow as a researcher and a person. Working in her laboratory has been an immense privilege and a formative experience. I would also like to express my sincere appreciation to my committee members, Dr. Shawn Hayley and Dr. Natalina Salmaso. Your critical insights and helpful feedback were essential to creating this thesis.

I would like to give a strong shout-out to Ushananthini Shanmugalingam a.k.a Masta Usha and Margarita Lui who helped me with data collection and analyses. I wouldn't have finished my thesis without you guys!

I would also like to thank my family and true-blue friends for providing me with love and support throughout my time here at Carleton University.

Finally, I would like to dedicate this thesis to the memory of my childhood hero and maternal grandfather, In-Jae Han, and my best Schnauzer buddy, Chi-Chi.

Table of Contents

List of figures

List of Abbreviations

ActRII	Activin type II receptor
Akt	Protein kinase B
ALK4	Activin-receptor like kinase 4
ALK5	Activin-receptor like kinase 5
ALK7	Activin-receptor like kinase 7
AMH	Anti-Müllerian hormone
AQP4	Aquaporin 4
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
B-GDF11/8	Bioactive growth differentiation factor 11 and 8
BMP	Bone morphogenetic protein
BMP11	Bone morphogenetic protein 11
BNB	Blood nerve barrier
BRB	Blood retinal barrier
C7	Seventh cervical vertebra
CBP	Cyclic adenosine monophosphate response element binding protein
CCAC	Canadian Council on Animal Care

CDK	Cyclin-dependent kinase
CNS	Central nervous system
Co-Smad	Common-mediator Smad
CSF	Cerebrospinal fluid
CSPG	Chondroitin sulfate proteoglycan
C-terminal	Carboxy-terminal
CREB	Cyclic adenosine monophosphate response element-binding protein
DAPI	4',6-diamidino-2-phenylindole
DNase I	Deoxyribonuclease I
DPBS	Dulbecco's phosphate buffered saline
e8.5	Embryonic day 8.5
e10.5	Embryonic day 10.5
e14.5	Embryonic day 14.5
e18	Embryonic day 18
ECL	Electrochemiluminescence
ERK	Extracellular signal-regulated kinases
FSTL-3	Follistatin-like 3

Gadd45β	Growth arrest and DNA-damage-inducible protein
GASP-1	Growth differentiation factor-associated serum protein 1
GASP-2	Growth differentiation factor-associated serum protein 2
GCL	Ganglion cell layer
GDF	Growth differentiation factor
GDNF	Glial-derived neurotrophic factor
GDF8	Growth differentiation factor 8
GDF11	Growth differentiation factor 11
GDF11/8	Growth differentiation factor 11 and 8
gdf11	Growth differentiation factor 11 mRNA
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
IgG	Immunoglobulin G
JNK	c-Jun N-terminal kinases
KO	Knockout
L-GDF11/8	Latent growth differentiation factor 11 and 8
MAG	Myelin-associated glycoprotein

MAPK	Mitogen-activated protein kinase
MH1	Mad-homology 1
MH2	Mad-homology 2
NAD	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor κ light chain enhancer of activated B cells
NGF	Nerve growth factor
NMN	Nicotinamide mononucleotide
NMNAT	Nicotinamide mononucleotide adenylyltransferases
NPC	Nuclear pore complex
N-terminal	Amino-terminal
NUP153	Nuclear pore complex protein 153
NUP214	Nuclear pore complex protein 214
OE	Olfactory epithelium
OMgp	Oligodendrocyte myelin glycoprotein
ON	Optic nerve
p1	Postnatal day 1
p4	Postnatal day 4

p5	Postnatal day 5
p7	Postnatal day 7
p8	Postnatal day 8
p14	Postnatal day 14
PBS	Phosphate buffer saline
PDL	Poly-D-lysine
Pen/Strep	Penicillin-Streptomycin
PFA	Paraformaldehyde
P-GDF11/8	Precursor growth differentiation factor 11 and 8
PI3K	Phosphoinositide-3 kinase
PLCγ	Phosphoinositide phospholipase C-γ
PNS	Peripheral nervous system
pSmad2	Phosphorylated Smad2
pSmad3	**P**hosphorylated Smad3
pSmad2/3	Phoshorylated-Smad2 and Smad3
RGC	Retinal ganglion cell
R-Smad	Receptor-specific Smad

RT-PCR	Reverse transcription polymerase chain reaction
SARA	Smad anchor for receptor activation
Smad2/3	Smad2 and Smad3
SnoN	Ski-related novel protein N
SVZ	Subventricular zone
TβRIIR	TGF-β type II receptor
TGF-β	Transforming growth factor protein-β
TGN	*Trans*-Golgi network
TNF-α	Tumor necrosis factor α
TLD	Tolloid-like metalloprotease
TrkB	Tropomyosin receptor kinase B
WD	Wallerian degeneration
WT	Wild-type

1.0 Introduction

1.1 Organization and components of the nervous system

The nervous system is categorized into the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS, which consists of the brain and spinal cord, conducts and interprets signals, overall functioning as the control center; on the other hand, the PNS consists of motor and sensory nerves that transmit signals between the CNS and the rest of the body (Siddique and Thakor, 2013). The nervous system consists mostly of neurons and glial cells. The basic functional units of the nervous system are neurons, which are made up of a cell body, axons that send neural signals away from the cell body, and dendrites that direct incoming neural signals to the cell body (Siddique and Thakor, 2013). There are different types of glial cells residing in the CNS and PNS; astrocytes, microglia and oligodendrocytes are found in the CNS while Schwann cells are found in the PNS (Siddique and Thakor, 2013). Astrocytes are specialized glial cells that contiguously cover the entire CNS and have diverse essential functions in the healthy CNS; for instance, astrocytes release various molecular mediators that control CNS blood vessel diameter and blood flow, regulate fluid homeostasis *via* aquaporin 4 (AQP4) and clear excess neurotransmitters from the synaptic space *via* various neurotransmitter transporters (Sofroniew and Vinters, 2010). Microglia are tissue-resident mononuclear phagocytic macrophages in the CNS that not only eliminate microbes, dead cells and protein aggregates but also play a crucial role in synaptic pruning and remodeling during development and adulthood (Prinz and Priller, 2014; Wake and Fields, 2011). Schwann cells and oligodendrocytes form myelin sheaths around axons in the PNS and CNS, respectively, allowing the rapid propagation of action potentials by saltatory conduction (Debanne *et al.*, 2011). Besides myelinating axons and facilitating nerve

conduction, they also play a crucial role in determining the regenerative capabilities of the PNS and CNS.

1.2 Nerve injury and regeneration in peripheral and central nervous system

In the PNS and CNS, nerve injury induces beading and swelling of the axon membrane (Vargas and Barres, 2007). The subsequent influx of extracellular Ca^{2+} leads to the activation of calpain and other intracellular Ca^{2+}-dependent proteases (Wang *et al.*, 2012). These proteases mediate the disintegration of the axonal cytoskeletal components, such as microtubulin, spectrin and neurofilaments, and cause the fragmentation of the axon (Wang *et al.*, 2012). The injured axon is known to undergo two degenerative processes: retrograde degeneration and Wallerian degeneration (WD) (Calabrese *et al.*, 2015). The proximal portion of the injured axon, which is connected to the cell body, undergoes retrograde degeneration towards the cell body, eventually resulting in retrograde neuronal cell death (Calabrese *et al.*, 2015). On the other hand, the distal portion of the injured axon, which is disconnected from the neuronal cell body, undergoes WD towards the post-synaptic end (Coleman and Freeman, 2010). This disconnection is thought to deprive the constant anterograde supply of neurotrophic factors from the cell body and thus cause the intracellular level of these molecules to fall below a threshold to maintain axonal integrity and function (Wang *et al.*, 2012). In addition, nicotinamide mononucleotide adenylyltransferases (NMNATs), essential enzymes for axon maintenance and growth, are rapidly lost after axonal injury and cannot be delivered from the cell body to the distal portion of the injured axon (Stefano *et al.*, 2015). Because NMNATs catalyze the synthesis of nicotinamide adenine dinucleotide (NAD) from nicotinamide mononucleotide (NMN), the loss of NMNATs results in the accumulation of NMNs, which has been shown to promote WD (Stefano *et al.*, 2015).

The rate of WD differs between the PNS and CNS; in mammals, WD takes approximately 7 to 14 days in the PNS while it takes months to years in the CNS (Vargas and Barres, 2007). The drastically slower WD in the CNS is attributed to the lack of mechanisms for clearing myelin debris in the CNS. In the PNS, axonal injury causes Schwann cells to shed myelin sheaths, which are rapidly broken down to myelin ovoids and further digested by Schwann cells (Hirata and Kawabuchi, 2002). The PNS axon degeneration also leads to the breakdown of the blood nerve barrier (BNB), allowing blood-circulating opsonins, such as complement, pentraxins and antibodies, to enter and attach to the degenerating PNS nerve and thereby facilitating the phagocytic myelin clearance by macrophages in the PNS (Vargas and Barres, 2007). In the CNS, axonal injury causes oligodendrocytes to shed myelin sheaths, but oligodendrocytes cannot digest myelin debris like Schwann cells, thereby delaying the clearance process (Brosius Lutz and Barres, 2014). According to past studies, oligodendrocytes respond to WD by either undergoing apoptosis or entering an atrophy-like resting state (Ludwin, 1990; Casha *et al.*, 2005). Furthermore, the CNS injury leads to only partial disruption of the blood brain barrier (BBB), limiting the influx of peripheral macrophages as well as opsonins to the degenerating CNS nerve (Vargas and Barres, 2007). Microglia residing in the CNS are phagocytic and hence capable of clearing myelin debris, but they are not as efficient as peripheral macrophages (Skoff and Vaughn, 1971 and Aldskogius *et al.*, 1974).

While intact myelin enhances conduction speed and protects axon integrity, injured myelin releases components that are known to inhibit nerve outgrowth, such as chondroitin sulfate proteoglycans (CSPGs), Nogo-A, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgP) (Brosius Lutz and Barres, 2014). Although

these inhibitory components were first discovered by studying CNS myelin, MAG is also present in the PNS (Vargas and Barres, 2007). However, PNS axons can still overcome myelin inhibition and regenerate after injury because myelin debris containing MAG are rapidly and effectively cleared by Schwann cells and macrophages (Brosius Lutz and Barres, 2014). Schwann cells also release neurotrophic factors, extracellular matrix molecules and growth factors, creating growth-promoting environment; furthermore, injury to the PNS axon reactivates an intrinsic growth program in the neuronal body that initiates regenerative growth (Abe and Cavalli, 2008; Madduri and Gander, 2010). In the CNS, not only do neurons lack the intrinsic growth capacity but also the growth-inhibitory environment and ineffective clearance of myelin debris prevent their axon regeneration (Siddique and Thakor, 2013; Strittmatter, 2010; Lee *et al.*, 2010).

Past studies focused on characterizing the inhibitory myelin-associated molecules in the adult CNS and blocking their inhibitory signals in an attempt to promote axon regeneration; however, the efficacy of this approach remains controversial (Lee *et al.*, 2010; Smith *et al.*, 2009). Recent studies have shown that stimulating intrinsic growth potential of CNS neurons can induce axon regeneration after CNS injury, providing the concept that robust neuron-intrinsic growth potential can potentially overcome the inhibitory extrinsic environment of the injured CNS (Park *et al.*, 2008; Smith *et al.*, 2009; Noro *et al.*, 2015; Kaplan *et al.*, 2015). It is critical to investigate the potential mechanisms in the adult CNS that could be effective at enhancing the intrinsic regenerative ability of injured CNS neurons to facilitate axon regeneration and promote repair, following CNS injury, such as spinal cord injury, stroke and neurodegenerative diseases.

1.3 Using the visual system to study CNS regeneration in mammals

The visual system of mammals, especially rodents, has been widely used for studying neuroprotection and axon regeneration in the CNS, as it exhibits characteristics of the CNS (Shen *et al.*, 1999; Park *et al.*, 2008; Smith *et al.*, 2009; Sanchez-Migallon *et al.*, 2015; Peng *et al.*, 2016). The eye is surrounded by blood-ocular barriers that are similar to the CNS gating system; for instance, the inner blood retinal barrier (BRB) consists of retinal capillary endothelial cells that are firmly connected by tight junctions and surrounded by astrocyte end-feet, thereby resembling the BBB (Kaur *et al*., 2008). In addition, aqueous humour inside the eye is a fluid enriched with anti-inflammatory and immunoregulatory mediators, which is similar to the cerebrospinal fluid (CSF) in the brain and spinal cord (London *et al.*, 2012). The retina and optic nerve (ON) are considered as a part of the CNS since they extend from diencephalon during embryonic development (London *et al.*, 2012). The inner most layer of the retina is composed of retinal ganglion cells (RGCs) whose projecting axons bundle together to form the ON that conveys visual information from the retina to the brain (Berry *et al.*, 2008; Almasieh *et al.*, 2012). Similar to the CNS axons, the ON, myelinated by oligodendrocytes, undergoes retrograde and Wallerian degeneration and fails to regenerate spontaneously after injury (Goldberg *et al.*, 2002; Berry *et al.*, 2008; Almasieh *et al.*, 2012).

The ON crush and complete ON transection are *in vivo* CNS injury models commonly used to induce the specific loss of RGCs, which begins quickly following the ON damage; depending on the severity of the damage, approximately 27-90% of RGCs can survive to two weeks post-injury (Johnson and Tomarev, 2010). For studying *in vivo* post-injury RGC survival and axon regeneration, potential growth-stimulating molecules are delivered to the retina *via* intravitreal injection before inducing the ON damage (Park *et al.*,

2008; Smith *et al.*, 2009; Sanchez-Migallon *et al.*, 2015). Although the *in vivo* injury models are required to confirm that a phenomenon occurs in living organisms, *in vitro* models become more useful when conducting highly controlled preliminary investigations. RGC culture is a useful *in vitro* model for assessing the response of RGCs to specific conditions in isolation from the rest of the eye (Johnson and Tomarev, 2010). A wide variety of growth-promoting molecules have been identified by using both *in vitro* and *in vivo* models, and current literature continues to focus on identifying new and more effective growth-promoting molecules (Smith *et al.*, 2009; Legacy *et al.*, 2013; Morquette *et al.*, 2015 ; Sharma *et al.*, 2015; Peng *et al.*, 2016). Recent studies have shown that members of transforming growth factor β (TGF-β) superfamily can effectively promote both axon regeneration and neuroprotection in the CNS, suggesting TGF-β pathway as a new therapeutic target for CNS regeneration (Dobolyi *et al.*, 2012).

1.4 Transforming growth factor β superfamily

TGF-β superfamily is a large group of related growth factors that regulate not only diverse developmental processes, such as proliferation, differentiation, migration and apoptosis, but also adult tissue homeostasis and tissue repair (Krieglstein *et al*., 2011). The family is subdivided into four subgroups: TGF-βs, bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs), activin/inhibins and the outsider subgroup (Yadin *et al.*, 2016). TGF-βs, including TGF-β1, 2, 3, form the smallest subgroup and serve as the archetypal examples for the TGF-β superfamily; on the other hand, BMPs and GDFs form the largest subgroup, which can be further divided into BMP2/4, BMP5/6/7/8, GDF1/3, GDF2/BMP10, GDF5/6/7, GDF8/11, GDF9/BMP15 and GDF10/BMP3 sub-divisions (Yadin *et al.*, 2016). While activin/inhibins subgroup consists of inhibin α and β, activin A, B and AB,

nodal and lefty1/2, the outsider subgroup includes those TGF-β superfamily members that do not fit into any of the other three groups, such as anti-Müllerian hormone (AMH) and glial-derived neurotrophic factor (GDNF) (Yadin *et al.*, 2016). The TGF-β superfamily ligands are secreted as precursors consisting of an amino-terminal (N-terminal) prodomain and a carboxy-terminal (C-terminal) mature domain (Wu and Hill, 2009). The mature domain is cleaved from the prodomain by furin or furin-like proteases, and then forms a butterfly-shaped homomeric or heteromeric dimer, held together by disulphide bonds (Feng and Derynck, 2005; Wakefield and Hill, 2013). All members of the TGF-β superfamily share fundamentally the same signaling mechanism (Wakefield and Hill, 2013). They bind and activate heteromeric complexes of transmembrane serine/threonine kinase receptor type I and type II; in the activated complex, the type II receptor phosphorylates the type I receptor on serine and threonine residues in a highly conserved glycine- and serine-rich domain (Wu and Hill, 2009; Wakefield and Hill, 2013). The activated type I receptor phosphorylates the intracellular downstream targets, typically receptor-specific Smad (R-Smad) proteins, including Smad1, 2, 3, 5 and 8 (Wu and Hill, 2009). The phosphorylation of R-Smads leads to the multimerization of R-Smads with the common-mediator Smad (co-Smad), Smad4, which serves as a common partner for all R-Smads (Chu *et al.*, 2004; Levy and Hill, 2005). The Smad complex then translocates into the nucleus and modulates the transcription of target genes along with transcriptional cofactors (Wu and Hill, 2009). While Smad3 and 4 are known to recognize the sequence AGAC or its reverse complement, Smad1, 5 and 8 preferentially bind GC-rich elements with the sequence GRCGNC (Wakefield and Hill, 2013). Unlike other Smads, Smad2 cannot bind directly to DNA due to steric hindrance by an inserted sequence in the DNA-binding region (Morikawa *et al.*, 2013); hence, it has to

multimerize with other Smad proteins, specifically Smad3 or Smad4, to interact with target genes. R-Smads and co-Smad consist of two conserved globular domains connected by a linker region: the N-terminal Mad-homology 1 (MH1) domain and the C-terminal Mad-homology 2 (MH2) domain (Kubiczkova *et al.*, 2012). The MH1 domain is a DNA-binding module stabilized by a tightly bound zinc atom, and its contact with DNA is accomplished by a β hairpin structure, which is conserved in both R-Smads and co-Smad (Massagué *et al.*, 2005). The MH2, on the other hand, is one of the most versatile protein-interacting modules in signal transduction; it contains hydrophobic patches known as hydrophobic corridors that mediate interactions with receptors, cytoplasmic proteins, nucleoporins and DNA-binding cofactors (Massagué *et al.*, 2005). A region overlapping the linker and MH2 domains mediates interactions with transcriptional activators and repressors in the nucleus (Massagué *et al.*, 2005). The Smad pathways were once considered to be unidirectional and linear, but they are rather dynamic networks since Smad proteins constantly shuttle between the cytoplasm and nucleus (Massagué *et al.*, 2005). In the cytoplasm, the receptor-mediated phosphorylation decreases the affinity of R-Smads for cytoplasmic anchors and increases their affinity for transcription factors (Shi and Massagué, 2003; Xu and Massagué, 2004). In the nucleus, Smad phosphatases dephosphorylate R-Smads and allow them to return to the cytoplasm (Inman *et al.*, 2002).

Although the Smads are the best understood downstream targets of TGF-β superfamily receptors, Smad-independent pathways can be activated by the TGF-β superfamily. p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases (ERK) 1/2 and c-Jun N-terminal kinases (JNK) has been shown to be activated by TGF-β superfamily in a cell type-specific manner (Tsuchida *et al.*, 2009). For instance,

activin has been shown to work synergistically with basic fibroblast growth factor (bFGF) to increase the level of tyrosine hydroxylase in mouse striatal and hippocampal cells *via* ERK1/2 phosphorylation; additionally, it has been shown to inhibit the pituitary transcription factor Pit-1 gene promoter in prolactin cells *via* p38 MAPK phosphorylation (Bao *et al.*, 2005; De Guise *et al.*,2006). As for TGF-β-mediated JNK signaling, TGF-β1 has been shown to induce epithelial-mesenchymal transition (EMT) in rat peritoneal mesothelial cells *via* JNK activation (Liu *et al*, 2012). In addition to these Smad-independent pathways, the TGF-β superfamily is also capable of signaling through phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway; TGF-β has been shown to induce hypertrophy during EMT by activating mammalian target of rapamycin (mTOR) through PI3K and Akt (Lamouille and Derynck, 2007). Overall, the TGF-β superfamily can utilize a multitude of intracellular signaling pathways and trigger a wide array of cellular responses.

Cellular responses to TGF-β signaling depend on cell types and physiological conditions. TGF-β signaling limits proliferation of epithelial and haematopoeitic cells by inducing cyclin-dependent kinase (CDK) inhibitors p15 and p21, whereas it inhibits apoptosis of follicular dendritic cells by down-regulating the expression of Fas and caspase-8 (Park *et al.*, 2005; Meulmeester and Dijke, 2011). Additionally, in tumors, the TGF-β signaling can function as tumor suppressor in early stages of tumorigenesis and tumor promoter in late stages (Meulmeester and Dijke, 2011). Its growth-inhibitory function is due to the ability to suppress expression and function of c-Myc and CDKs and to enhance expression of the CDK inhibitors (Kubiczkova *et al.*, 2012). During late stages of tumor progression, mutations in TGF-β downstream components are believed to affect TGF-β signal transduction and potentially promote cancer development and progression (Inman, 2011). Due to these

pleiotropic actions, the TGF-β signaling is considered to be highly cellular context-dependent and influenced by transcription factor availability, epigenetic status, crosstalk with other biochemical pathways and developmental or disease stages (Akhurst and Hata, 2012; Massagué, 2012).

Members of TGF-β superfamily have been shown to be expressed in various regions of the CNS, including hippocampus, hypothalamus, medulla oblongata, reticular formation and choroid plexus (Dobolyi *et al.*, 2012). They are also involved in the development of nervous system; as an example, BMPs can induce neural crest, regulate spinal cord proliferation, pattern forebrain and cerebellum, and promote neurogenesis and neurite outgrowth (Bond *et al.*, 2012). TGF-βs have anti-proliferative effects on neural crest cells, fetal cortical cells, postnatal cerebellar cells and neuronal precursor cells; however, they enhance differentiation of neural crest cells into adrenergic cells and sensory neuron precursors (Zhang *et al.*, 1997; Aigner and Bogdahn, 2008). Furthermore, TGF-β signaling has been shown to protect retinal neurons from apoptosis during the development of the mammalian eye. According to Braunger *et al*. (2013), mice lacking TGF-β type II receptors (TβRIIRs) in the inner layer of the optic cup exhibited reduction of phosphorylated Smad3 (pSmad3) in retina and apoptosis of retinal neurons during embryonic and postnatal development; additionally, TGF-β2 treatment inhibited apoptosis of cultured RGCs through the Smad-dependent pathway. Other TGF-β proteins, including TGF-β1, activin and BMP4, have been shown to promote both survival and axon regeneration in CNS neurons, indicating that TGF-β signaling does hold a therapeutic potential for CNS regeneration (Sulyok *et al.*, 2004; Parikh *et al.*, 2011; Ueki and Reh, 2012; Omura *et al.*, 2015; Li *et al.*, 2017).

Recently, a TGF-β superfamily member GDF11, also known as bone morphogenetic

protein 11 (BMP11), has recently emerged as a circulating factor in young mice with age-dependent decline in expression (Loffredo *et al.*, 2013). According to Loffredo *et al.* (2013), prolonged administration of GDF11 in old mice can restore GDF11 expression to youthful levels and reverse age-related cardiac hypertrophy. In support of this proposed rejuvenation effect of GDF11, Katsimpardi *et al*. (2014) have shown that GDF11 administration can not only improve cerebral vasculature but also enhance neurogenesis in both olfactory bulb and subventricular zone (SVZ) of aged mice. Considering this regenerative effect of GDF11 in the CNS, it may hold therapeutic potential for neuroprotection and axon regeneration in the CNS after injury.

1.5 Growth differentiation factor 11

GDF11 was first cloned and characterized from rat incisor pulp RNA by reverse transcription polymerase chain reaction (RT-PCR) with degenerate primers based on the conserved mature region of BMPs and GDFs (Nakashima *et al.*, 1999). *In situ* hybridization of whole-mount mouse embryos showed that the distribution of *gdf11* mRNA changes throughout the development. It is first strongly expressed in the tail bud at embryonic day 8.5 (e8.5) and then later in the branchial arches, limb and tail buds, posterior dorsal neural tube, and inner layer of the optic cup at e10.5 (Nakashima *et al.*, 1999). At e14.5, it is expressed in various regions of the developing brain, including hippocampus, striatum, preoptic area, inferior colliculi and nuclei in the ventral midbrain and anterior hindbrain. In the adult brain, *gdf11* mRNA is strongly expressed in thalamus and Purkinje cell layer while being weakly expressed in hippocampus and scattered cells in the midbrain and hindbrain (Nakashima *et al.*, 1999). Other studies later reported that *gdf11* mRNA is also expressed in heart, pancreas, intestine, kidney, skeletal muscle and spleen; notably, the spleen has been shown to express

the highest levels of *gdf11* mRNA (McPherron, 2010; Loffredo *et al.*, 2013). As for GDF11 protein expression, GDF11 has been shown to be expressed in the aforementioned tissues as well as blood (Walker *et al*., 2016). Souza *et al*. (2008) first reported that it is expressed in both human and mouse systemic circulation; however, the tissue source for this circulating GDF11 remians unknown. Recently, Loffredo *et al.* (2013) reported the significant reduction of GDF11 expression in both spleen and blood of old mice, suggesting that the spleen may be the major source of circulating GDF11.

Similar to other TGF-β proteins, GDF11 is first synthesized as a precursor where the N-terminal prodomain is cleaved from the C-terminal mature domain by a furin or furin-like protease (Walker *et al.*, 2016). The molecular structure of GDF11 is not well characterized, but the recent unbound x-ray crystal structure of the protein revealed that the mature form of GDF11 is the canonical homomeric dimer of the TGF-β superfamily; it is viewed as a 'hand' with a four-stranded β-sheet comprising the 'fingers,' a cysteine-knot structure occupying the palm and α-helix forming the 'wrist' (Padyana *et al.*, 2016). Unlike other TGF-β ligands, the mature form of GDF11 is tightly bound to its prodomain even after furin cleavage; this form is known as a latent state, in which GDF11 is unable to bind its receptors and believed to interact with extracellular matrix components and remain inactivated (Walker *et al.*, 2016). To become fully activated, the prodomain has to be properly cleaved by a tolloid-like (TLD) metalloproteinase (Ge *et al*., 2005). As shown in Figure 1, the active form of GDF11 binds to transmembrane serine/threonine kinase activin type II receptor (ActRII) and forms the complex that recruits one of three specific type I receptors, activin-receptor like kinase 4 (ALK4), ALK5 and ALK7 (Tsuchida *et al.*, 2009). Although the receptor interaction mechanism of GDF11 has yet to be determined, GDF11 is believed to bind to its receptors in

a similar manner as TGF-β proteins (Walker *et al.*, 2016). TGF-βs exhibit high affinity for the type II receptor and low affinity for the type I receptor, and they bind type II receptor *via* their 'fingertips,' facilitating a cooperative binding between type II and type I receptors (Walker *et al.*, 2016). The initial binding of GDF11 to the type II receptor can be inhibited by follistatin-like 3 (FSTL-3) and growth differentiation factor-associated serum proteins 1 and 2 (GASP-1 and GASP-2); while GASP-1 and GASP-2 prevent GDF11 from binding to ActRII, FSTL-3 conceals GDF11 receptor epitopes by symmetrically embracing GDF11 (Walker *et al.*, 2016). The activated type I receptors phosphorylate R-Smad proteins Smad2 and Smad3 (Smad2/3), which multimerize with co-Smad Smad4 (Tsuchida *et al.*, 2009; Walker *et al.*, 2016). The Smad complex then translocates into the nucleus and regulates the transcription of target genes along with transcriptional cofactors; Smad co-activators, such as cyclic adenosine monophosphate response element binding protein (CBP) and p300 function as histone acetylases and allow Smad-mediated transcription activation whereas Smad co-repressors, such as c-ski and Ski-related novel protein N (SnoN), recruit histone deacetylases and downregulate Smad-mediated transactivation (Jinnin *et al.*, 2007). Similar to other TGF-β members, GDF11 may also signal through Smad-independent pathways, such as Akt/PI3K and MAPK pathways (Tsuchida *et al.*, 2009).

GDF11 plays a broad role in mammalian development. GDF11 knockout (KO) mice exhibit cleft palate, renal agenesis, anterior homeotic transformation of the axial skeleton with lumbar vertebrae transformed into thoracic vertebrae, and malformations of the stomach, spleen and pancreas (McPherron *et al.*, 1999; Harmon *et al.*, 2004). Furthermore, the knockout mice all die within 24 hours, possibly due to the developmental defects in kidney and palate formation (McPherron *et al.*, 1999; McPherron, 2010). Due to this perinatal

lethality of GDF11 KO mice, the functions of GDF11 in postnatal tissues are still unknown (Walker *et al.*, 2016). However, several studies have explored the consequences of transgenic overexpression of GDF11 in specific regions of a developing embryo. While GDF11 overexpression in embryonic chick spinal cord causes rostral shifts in the positions of motor neuron columns and pools, its overexpression in the early wing bud of a developing chick causes severe limb truncations by inhibiting chondrogenic and myogenic cell differentiation (Gamer *et al.*, 2001; Liu, 2006). Interestingly, transgenic overexpression of GDF11 prodomain in embryonic skeletal tissue results in the transformation of the seventh cervical (C7) vertebra into a thoracic vertebra without causing any perinatal lethality (Li *et al.*, 2010).

GDF11 is closely related to another TGF-β protein growth differentiation factor 8 (GDF8), alternatively known as myostatin; they share 90% sequence identity in their mature, C-terminal signaling domains and 52% identity in their N-terminal prodomains (Walker *et al.*, 2017). Like GDF11, GDF8 is initially synthesized in a precursor form, enters the latent state after undergoing furin-processing and becomes fully activated by a TLD metalloprotease; in its active form, GDF8 can bind to ActRII and signal through ALK 4, 5, 7 to activate Smad2/3 (Walker *et al.*, 2017). The biochemical similarities between GDF11 and GDF8 have led to the assumption that they have identical signaling potencies and properties; in support of this assumption, a past study showed that double mutant mice lacking GDF11 and GDF8 exhibit fully penetrant phenotypes of GDF11 KO mice, including renal agenesis and cleft palate, suggesting that they may be functionally indistinguishable (McPherron *et al.*, 2009). However, accumulating evidence suggests that they are not biochemically and functionally equivalent. According to Walker *et al.* (2017), in various cells transfected with Smad3-responsive luciferase reporter, GDF11 stimulated greater Smad3 activation in a

concentration- and a time-dependent manner and also induced greater reporter responses than GDF8 *via* ALK 4 ,5, 7; notably, only GDF11 resulted in significant signaling at lower concentrations and exposure times (Walker *et al.*, 2017). It also has been found that the major differences between the structures of GDF11 and GDF8 are located at each end of the wrist helix and include GDF11 residues Q62 and G100**;** these GDF11 residues are thought to stabilize the dimer interface of GDF11 and potentially contribute to its higher potency (Walker *et al.*, 2017). Interestingly, substitution of GDF8 residues H62 and A100 with GDF11 residues Q62 and G100 greatly increases the potency of GDF8, confirming the biochemical difference between GDF11 and GDF8 (Walker *et al.*, 2017). It has been well documented that GDF8 is predominantly expressed in both developing and adult skeletal muscles and therein negatively regulates skeletal muscle mass by suppressing hypertrophy and hyperplasia of myofibers (McPherron *et al.*, 2009; Elkasrawy and Hamrick, 2010). Although GDF11 has been shown to suppress myogenesis in the chick embryo, muscle fiber-specific deletion of GDF11 in mice does not induce a wide spread increase in skeletal muscle mass like GDF8 KO mice, providing the notion that GDF11 and GDF8 are functionally distinct; moreover, GDF8 KO mice does not exhibit perinatal lethality (Walker *et al.*, 2016). GDF8 is also believed to regulate glucose metabolism and adipogenesis, in which GDF11 is not known to be involved; it has been shown that loss of GDF8 in genetic models of obesity leads to suppression of fat accumulation and glucose metabolism (McPherron and Lee, 2002). Further supporting the functional difference between GDF11 and GDF8, previous studies have shown that unlike GDF8, GDF11 has important roles in the development of nervous system.

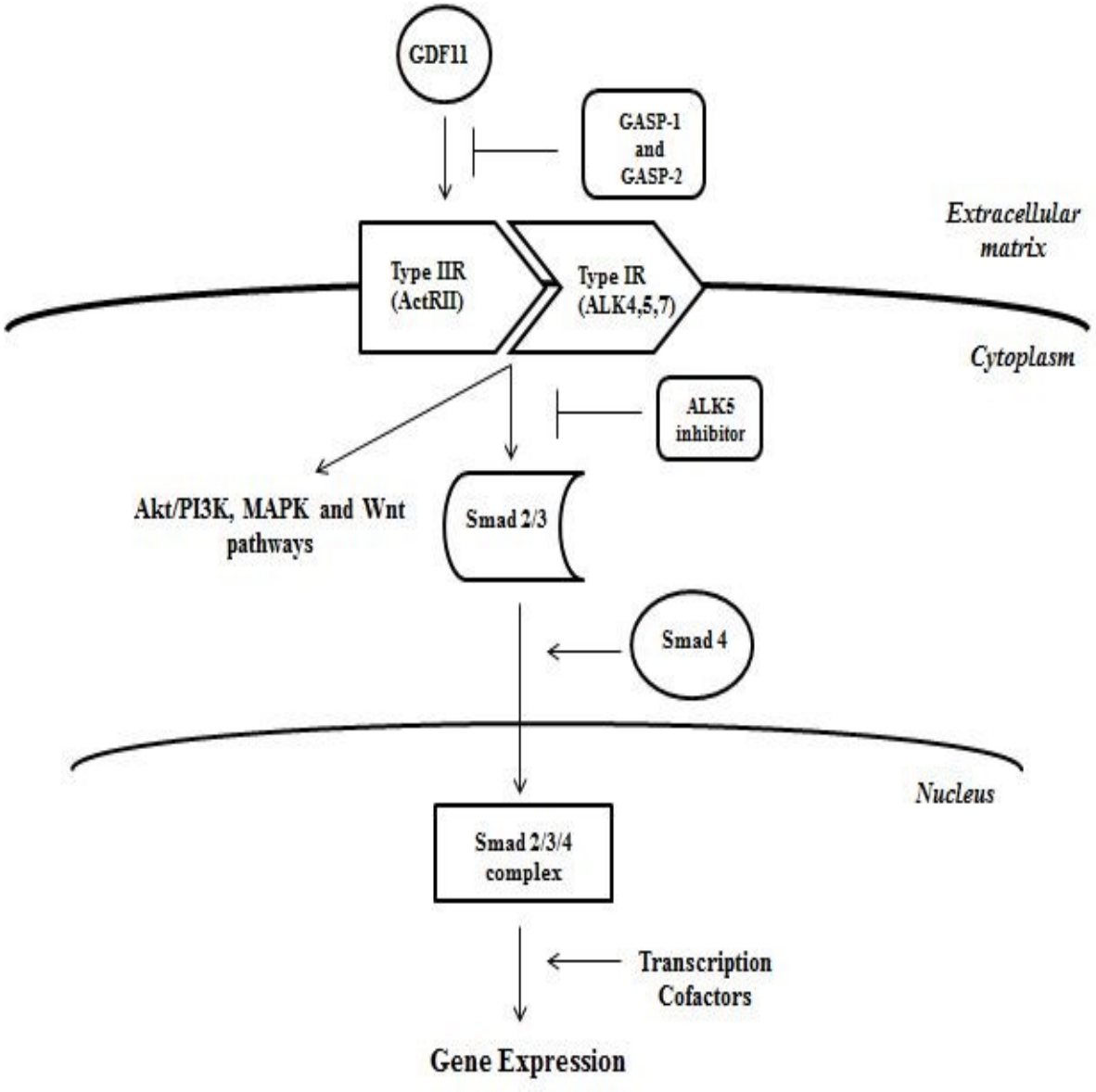

Figure 1: GDF 11 biochemical pathway. GDF11 binds to ActRII and facilitates a cooperative binding interaction between ActRII and one of type I receptors (ALK4, 5, 7). Extracellular-binding antagonists, such as GASP-1, GASP-2 and FSTL-3, can prevent GDF11 from binding to ActRII. The activated Type IR phosphorylates Smad2/3, which forms oligomeric complexes with Smad4. The Smad2/3/4 complex then enters the nucleus and regulates gene expression with transcription cofactors. GDF11 can also signal through Smad-independent pathways (Akt/PI3K and MAPK).

GDF11 protects retinal ganglion cells *in vitro*

GDF11 is known to exert pleiotropic effects on the developing nervous system by acting as both positive and negative factor of neurogenesis. Gamer *et al.* (1999) have shown that GDF11 induces neural tissue during vertebrate embryogenesis; on the contrary, Wu *et al.* (2003) have shown that GDF11 inhibits olfactory epithelium (OE) neurogenesis *in vitro*, and mice lacking functional GDF11 have more progenitors and neurons in OE. In the developing spinal cord, *gdf11* mRNA is expressed transiently in newly born neurons adjacent to the progenitor domain, and deletion of *gdf11* not only slows down the neuronal progenitor differentiation but also reduces the expression level of cyclin-dependent kinase inhibitors $p27^{kip1}$ and $p57^{kip2}$ (Shi and Liu, 2011). This observation has created a model in which GDF11 secreted by newly born neurons in the developing spinal cord generates a positive feedback signal on the progenitors to promote cell cycle exit, suppress proliferation and promote differentiation (Shi and Liu, 2011). As for the mammalian visual system, GDF11 has been shown to have an important role for the retina development. Kim *et al.* (2005) have shown that GDF11 controls the length of time that retinal progenitor cells are competent to produce RGCs by inhibiting *Math5* mRNA expression and regulates the ratio of RGCs to photoreceptors and amacrine cells. However, Hocking *et al.* (2008) have shown that administering GDF11 to cultured *Xenopus laevis* RGCs results in retinal dendrite growth, suggesting its role as a dendrite initiation factor. As no studies have shown whether or not GDF11 can promote retinal dendrite growth in mammalian RGCs, the potential regenerative effect of GDF11 in mammalian retina remains unknown.

2.0 Research objectives and hypotheses

Objective 1: To determine the developmental time course of GDF11 expression in mouse RGCs and compare developmental changes in GDF11 expression in RGCs to previously established developmental decline in intrinsic growth ability of RGCs.

Hypothesis 1: Similar to the trend in loss of intrinsic growth ability of RGCs, the expression of GDF11 in mouse RGCs will decrease throughout retina development.

Objective 2: To determine the impact of GDF11 administration on mouse RGC survival and axon growth and the potential mechanisms used by GDF11 to promote its functional effects on RGCs *in vitro*.

Hypothesis 2: GDF11 administration will promote mouse RGC survival and axon growth *in vitro*, through activation of its main downstream target Smad2/3 as well as another potential downstream target BDNF.

3.0 Rationale

Although the developmental expression of *gdf11* mRNA in retina has been well documented, the developmental expression of GDF11 protein in retina has not been reported; furthermore, no studies have determined the developmental pattern of GDF11 expression in RGCs. Hence, GDF11 expression in prenatal, postnatal and adult RGCs was examined. According to Goldberg *et al.* (2002), the intrinsic growth ability of RGCs decreases sharply within a day of birth and continues to decline afterwards. Based on this observation as well as the potential role of GDF11 as a retinal dendrite growth initiator, the expression of GDF11 in RGCs was expected to decrease throughout the retina development.

As the impact of GDF11 administration on mammalian RGCs remains unknown, the effect of GDF11 on mouse RGC survival and axon growth was determined, using an established *in vitro* mouse RGC culture system (Legacy *et al.*, 2013). As Smad2/3 proteins are known to be the main downstream targets of GDF11 and involved in GDF11-induced neurogenesis in aged brain (Katsimpardi *et al.*, 2014; Walker *et al.*, 2017), changes in Smad2/3 expression in cultured RGCs after GDF11 administration were investigated. In addition, as BDNF is a well-known neurotrophic factor that promotes RGC survival both *in vitro* and *in vivo* (Khalin *et al.*, 2015), the BDNF expression in cultured RGCs was also investigated to determine if GDF11 promotes neuronal survival by upregulating BDNF expression. Considering the known beneficial effects of GDF11 on neurogenesis and retinal dendrite growth (Hocking *et al.*, 2008; Katsimpardi *et al.*, 2014), GDF11 administration was expected to promote both RGC survival and axon regeneration.

The potential neuroprotective and regenerative effects of GDF11 in mammalian

RGCs could contribute to the development of pharmacological therapy for CNS injury, particularly glaucoma since it involves RGC death and ON degeneration; moreover, it could be further applied to the ongoing development of treatments for neurodegenerative diseases, such as Alzheimer's and Parkinson's disease.

4.0 Materials and method

4.1 Animals

All animal experiments were conducted according to the guidelines of the Canadian Council on Animal Care and were approved by the Carleton University Animal Care Committee. All experiments were performed on C57BL/6J WT mice purchased from Charles River Laboratories (Montreal, Quebec, Canada). Mouse pups (embryonic day 18 (e18), postnatal day 1 (p1), p4, p7 and p14, n=4 for each developmental stage) and male adult mice (*n*=4) were housed with consistent light-dark cycle, with food and water provided *ad libitum*.

4.2 Retinal cryosections

Eyes from different developmental stages (e18, p1, p4, p7, p14 and adult) were enucleated and placed into 4% paraformaldehyde (PFA) diluted in 0.01 M phosphate buffered saline (PBS) for 48 hours. They were then placed into 20% sucrose for 24 hours and then transferred to 10% sucrose for storage at 4 ℃ until cryosection preparation and collection of samples on glass slides. Retinal cross-sections (16µm thick) were collected on glass slides using a cryostat at -20 ℃. Figure 1 shows the experimental timeline of retinal cryosection preparation.

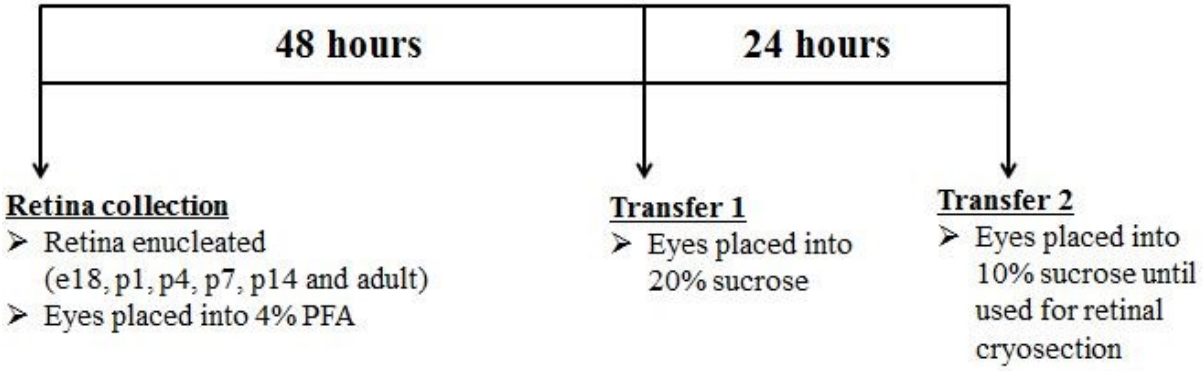

Figure 2: Experimental timeline of retinal cryosection preparation. After being enucleated, eyes were placed into 4% PFA for 48 hours. They were then transferred to 20% sucrose solution, and after 24 hours, they will be kept in 10% sucrose solution until being sectioned.

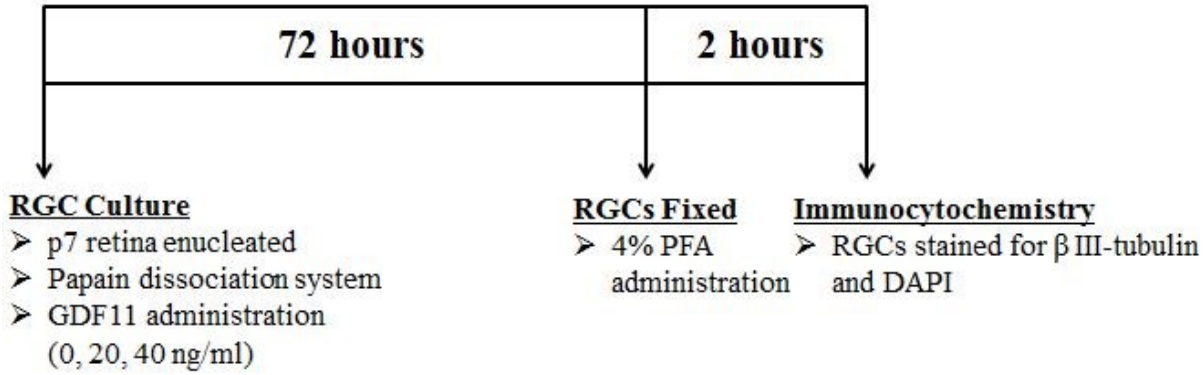

Figure 3: Experimental timeline for RGC culture. After extracting p7 retina, RGCs were isolated by using papain dissociation system and then plated into culture wells. Control and treatment groups were treated with culture medium and GDF11, respectively. After 72 hour incubation, RGCs were fixed in 4% PFA for 2 hours. Immunocytochemistry was carried out immediately after fixing the cells.

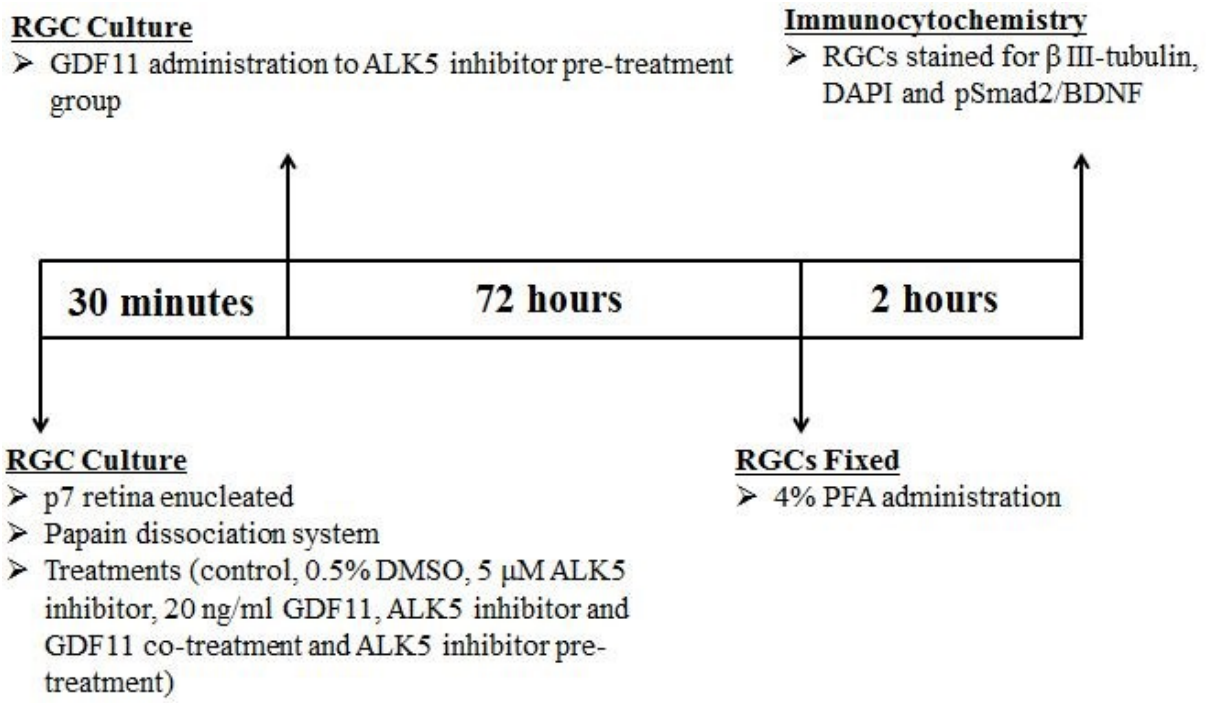

Figure 4: Experimental timeline for ALK5 inhibitor experiment. 6 different treatment groups were used for this experiment: control (cell culture medium), 0.5% DMSO, 5 µM ALK5 inhibitor, 20 ng/ml GDF11, co-treatment (5 µM ALK5 inhibitor+20 ng/ml GDF11) and pre-treatment (30 min incubation with 5 µM ALK5 inhibitor, followed by 20 ng/ml GDF11). After 72 hour incubation, RGCs were fixed in 4% PFA for 2 hours. Immunocytochemistry was carried out immediately after fixing the cells.

4.3 RGC culture for GDF11 administration

Three p7 mice eyes were pooled together to create *n*=1. They were enucleated and placed in cold Dulbecco's phosphate buffered saline (DPBS) without Ca^{2+} and Mg^{2+} (Life Technologies). The samples were dissected on ice and incubated in a dissociation solution containing papain (Worthington Biochemical Inc.) at 37 ℃ for 20 minutes with agitation every 5 minutes. The cells were then triturated in papain inhibitor and deoxyribonuclease I (DNase I) (Worthington), followed by centrifugation. The supernatant was discarded, and the isolated RGCs were immediately resuspended in cell culture medium containing Neurobasal A (Invitrogen), 2% B-27 serum-free supplement (Invitrogen), 0.3% $_{L}$-glutamine (Invitrogen) and 0.5% penicillin-streptomycin (Invitrogen), as previously published (Legacy *et al.*, 2013).

Cell quantification was carried out by using a hemacytometer before plating RGCs in culture wells coated with 20 μg/mL poly-$_{D}$-lysine (PDL) (Sigma-Aldrich) and 10 μg/mL laminin (Corning); approximately 12,000 cells were plated per well in 24-well plates (Sarstedt). GDF11 (0.1 mg/ml) (Peprotech) was diluted to 20 ng/mL, 40 ng/mL, 60 ng/mL, 80 ng/mL and 100 ng/mL in culture medium and incubated with RGCs, either at the time of plating or at specific time points following plating. RGCs were cultured for 72 hours and then fixed in 4% PFA for 2 hours before immunocytochemistry (Legacy *et al.*, 2013). Figure 2 outlines the timeline of RGC culture.

4.4 ALK5 inhibitor administration

The RGC culture was prepared in the same manner as outlined above. ALK5 inhibitor I (Enzo Life Sciences) was dissolved in 10% DMSO to produce the stock solution (500μM). The inhibitor was diluted to 5μM in culture medium, ensuring that the final DMSO concentration was 0.5%. For the inhibitor experiment, a total of 6 treatment groups were used: control group (culture medium), DMSO control group (0.5% DMSO), ALK5 inhibitor group (5μM ALK5 inhibitor), GDF11 group (20ng/ml GDF11), co-treatment group (both 5μM ALK5 inhibitor and 20ng/ml GDF11 at the time of plating) and pre-treatment group (30 min pre-treatment with 5μM ALK5 inhibitor before administering 20 ng/ml GDF11). 72 hours after plating, RGCs were fixed in 4% PFA for 2 hours, followed by immunocytochemistry. Figure 3 summarizes the timeline for ALK5 inhibitor experiment.

4.5 Immunohistochemistry to assess the developmental expression of GDF11

Retinal sections were washed three times every 15 minutes with 0.01M PBS before being incubated at the room temperature overnight with mouse anti-β III-tubulin antibody (1:1000, Biolegend) and rabbit anti-GDF11/8 (1:250, AbCam) or rabbit anti-GDF8 antibody (1:250, MilliporeSigma), diluted in 0.5% Triton X-100 solution. After primary antibody incubation, the sections were washed three times every 15 minutes with 0.01M PBS and then incubated for 2 hours with anti-mouse Alexa 488 (1:200, Cell Signaling) and anti-rabbit Alexa 555 (1:200, Cell Signaling), diluted in 0.5% Triton X-100 solution. The samples were then washed three times every 15 min with 0.01M PBS, and labelled with 4',6-diamidino-2-phenylindole (DAPI) (1:10000, Thermo Scientific) to detect cell nuclei. Three sample areas across the ganglion cell layer (GCL) of each retina cross-section were captured randomly at

20X magnification using a Zeiss Axiovert Microscope (Zeiss); at least four cross-sections were captured for each retina. The images were collected using Infinity Analyze software (Lumenera Corporation), and the number of GDF11/8 or GDF8 positive RGCs was manually counted using ImageJ (NIH). Using IBM SPSS Statistics 23, one-way ANOVA was performed, followed by Bonferroni's post hoc test for the statistical analyses of the results.

4.6 Immunocytochemistry and quantification of RGC survival in vitro

After fixation in 4% PFA, the RGC culture samples were washed three times every 15 min with 0.01M PBS and then incubated overnight with mouse β III-tubulin antibody (1:1000, Biolegend) and rabbit anti-phoshorylated-Smad2 (pSmad2) antibody (1:1000, Cell Signaling) or rabbit anti-brain derived neurotrophic factor (BDNF) (1:1500, Cell Signaling) diluted in 0.5% Triton X-100 solution. The samples were then washed three times every 15 min with 0.01M PBS and then incubated for 2 hours with anti-mouse Alexa 488 (1:200, Cell Signaling) and anti-rabbit Alexa 555 (1:200, Cell Signaling) diluted in 0.5% Triton X-100 solution. After the incubation, the samples were washed three times every 15 min with 0.01M PBS and then stained with DAPI (1:10000, Thermo Scientific). Three sample areas in each well were taken randomly by Zeiss Axiovert Microscope at 20X; a total of four wells were analyzed for each treatment group. After manually counting the number of surviving RGCs in each sample area, statistical analyses were conducted using one-way ANOVA, followed by Bonferroni's post hoc test.

5.0 Results

5.1 GDF11 expression in RGCs is reduced in adult retina

To study the developmental time course of GDF11 expression in RGCs, the percentage of RGCs expressing GDF11 at each developmental stage (e18, p1, p4, p7, p14 and Adult) was determined by DAPI, β III-tubulin and GDF11 staining. It should be noted that β III-tubulin has been used as a reliable marker for RGCs (Jiang *et al.*, 2015). As shown in Figure 5, GDF11 was broadly detected in both developing and adult retinae (only e18, p7 and adult retinae shown in Figure 5). However, it was highly expressed around the GCL and localized to RGCs in both developing and adult retinae. According to Bonferroni's post hoc test ($p<0.05$), there were no significant changes in GDF11 expression in e18~p14 RGCs (only e18 and p7 shown in Figure 5J). However, GDF11 expression in adult RGCs was significantly reduced in comparison to the GDF11 expression in developing RGCs, supporting the previous evidence that circulating GDF11 level declines with age (Loffredo *et al.*, 2013). Due to the structural similarities between GDF11 and GDF8, the currently available GDF11 antibody (AbCam) detects GDF11 and GDF8 8 (Poggioli *et al*., 2016); this is why it is now noted as GDF11/8 antibody.

GDF8-specific antibody (MiliporeSigma) was used to determine the developmental expression pattern of GDF8 in RGCs. As there is no evidence that the GDF8 antibody (MilliporeSigma) can detect both GDF11 and GDF8, the protein labelled by this antibody is referred to as GDF8. The percentage of RGCs expressing GDF8 at each developmental stage (e18, p1, p4, p7, p14 and Adult) was determined by DAPI, β III-tubulin and GDF8 staining. As shown in Figure 6, GDF8 was broadly expressed in both developing and adult retinae

(only e18, p7 and adult retinae shown in Figure 6). Furthermore, it was also highly expressed around the GCL and localized to RGCs in both developing and adult retinae. Statistically, there were no significant changes in GDF8 expression in samples collected from multiple developmental time points, starting from e18 to adult RGCs (representative images from e18, p7 and adult are shown in Figure 6J). It is notable that there were no significant changes in GDF8 expression in adult RGCs; this suggests that the decline in expression noted with GDF11/8 antibody (AbCam) may be predominantly due to changes in GDF11 expression.

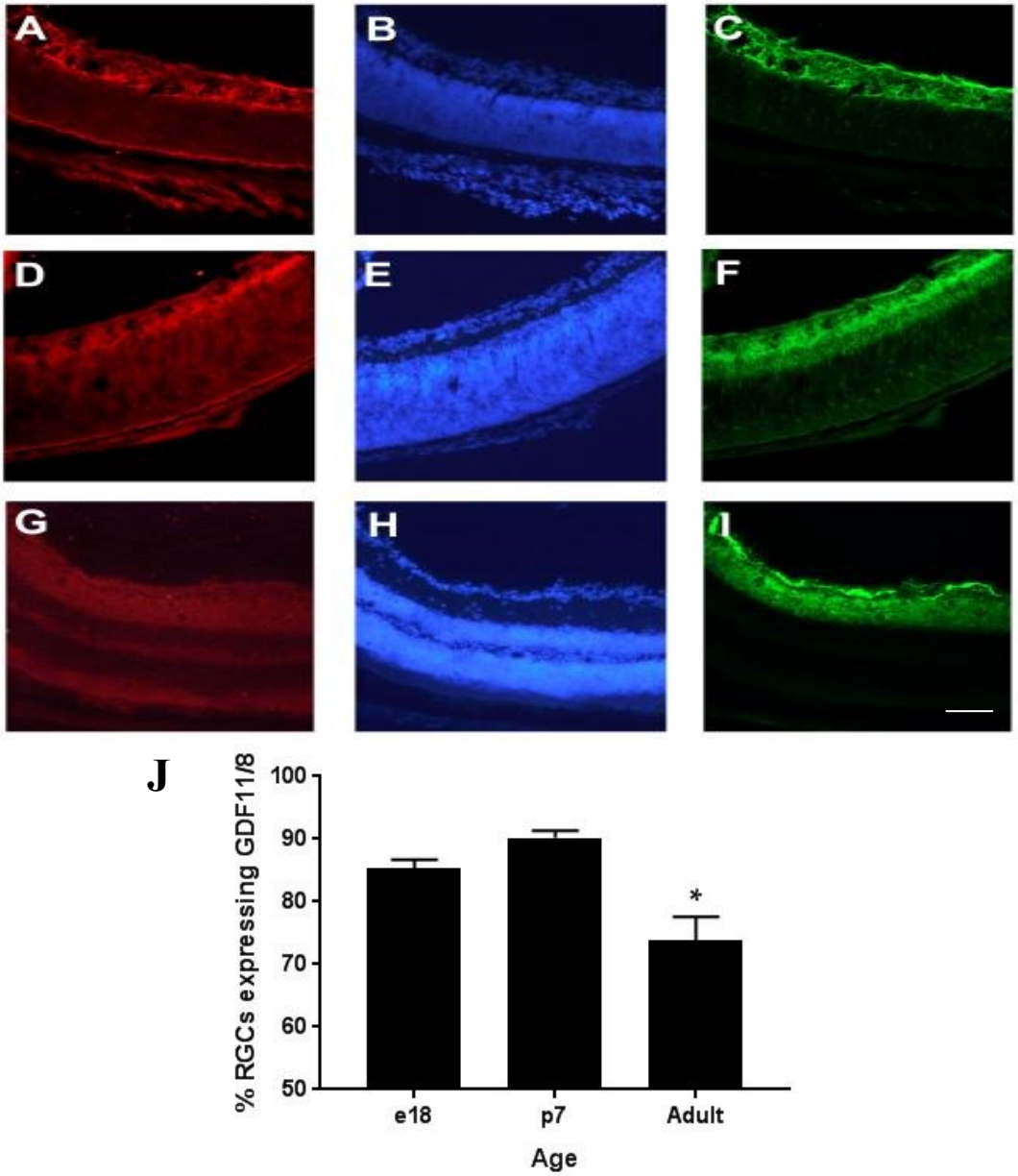

Figure 5: GDF11/8 expression declines in adult retina. e18 (A-C), p7 (D-F) and adult (G-I) retinal cryosections were triple-labeled with anti-GDF11/8 (A,D,G), DAPI (B,E,H) and anti-β III-tubulin (C,F,I). Quantitative graphic representation of percentage of GDF11/8-positive RGCs in each retina development stage (J). Bars are presented as mean ± SEM from 4 animals per age group. Statistical analysis was carried out by using one-way ANOVA followed by Bonferroni's post hoc test, *$p < 0.05$. Scale bar = 50μm.

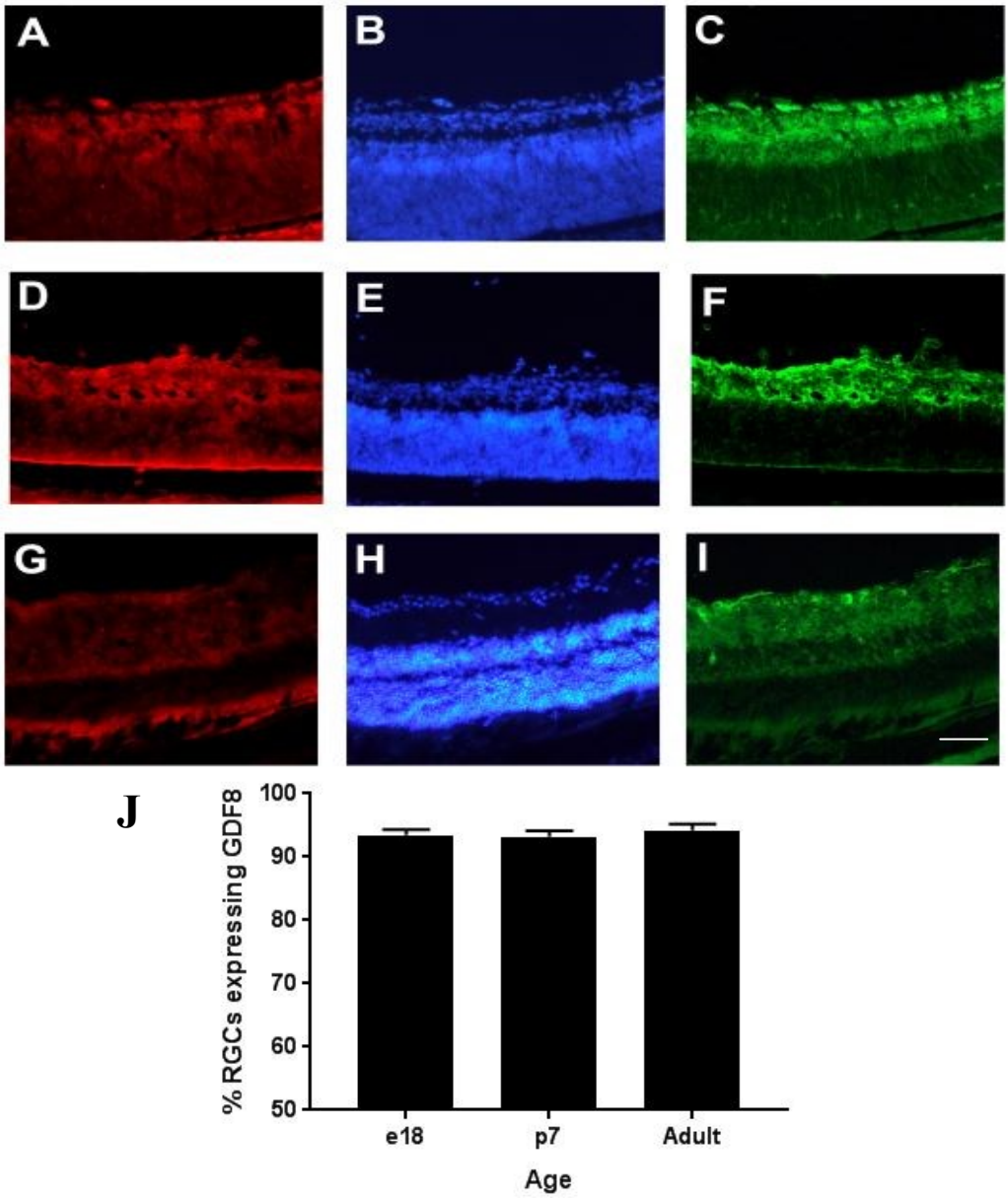

Figure 6: GDF8 expression does not change throughout retina development. e18 (A-C), p7 (D-F) and adult (G-I) retinal cryosections were triple-labeled with anti-GDF8 (A,D,G), DAPI (B,E,H) and anti-β III-tubulin (C,F,I). Quantitative graphic representation of percentage of GDF8-positive RGCs in each retina development stage (J). Bars are presented as mean ± SEM from 4 animals per age group. Statistical analysis was carried out by using one-way ANOVA followed by Bonferroni's post hoc test, *$p<0.05$. Scale bar = 50µm.

5.2 GDF11 promotes survival of p7 retinal ganglion cells in culture

To assess the effects of GDF11 administration on RGC survival and axon growth, p7 RGCs were isolated for the RGC culture. According to Goldberg *et al*. (2002), the intrinsic growth ability of p7 RGCs is diminished but not completely lost; hence, p7 RGCs were used in order to see whether GDF11 administration can restore the diminished intrinsic growth ability of p7 RGCs and promote RGC survival and axon regeneration. Initially, a preliminary experiment was conducted by using six different GDF11 concentrations (0, 20, 40, 60, 80 and 100 ng/ml) in order to determine the most effective GDF11 concentration for RGC survival and axon regeneration. The preliminary data revealed that none of GDF11 treatments promoted axon regeneration in cultured p7 RGCs; however, the 20 ng/ml GDF11 appeared to promote a dramatic beneficial effect on RGC survival (data not shown). Thereafter, the initial GDF11 concentration range was narrowed down (0, 20 and 40 ng/ml), and the p7 RGC culture experiment was repeated using these dosage parameters, as described in Figure 2. Figure 3G shows that only 20 ng/ml GDF11 treatment resulted in significant RGC survival in comparison to control treatment; remarkably, 20ng/ml GDF11 treatment had over 200% more surviving p7 RGCs than the control treatment. As expected, none of the GDF11 concentrations promoted axon growth.

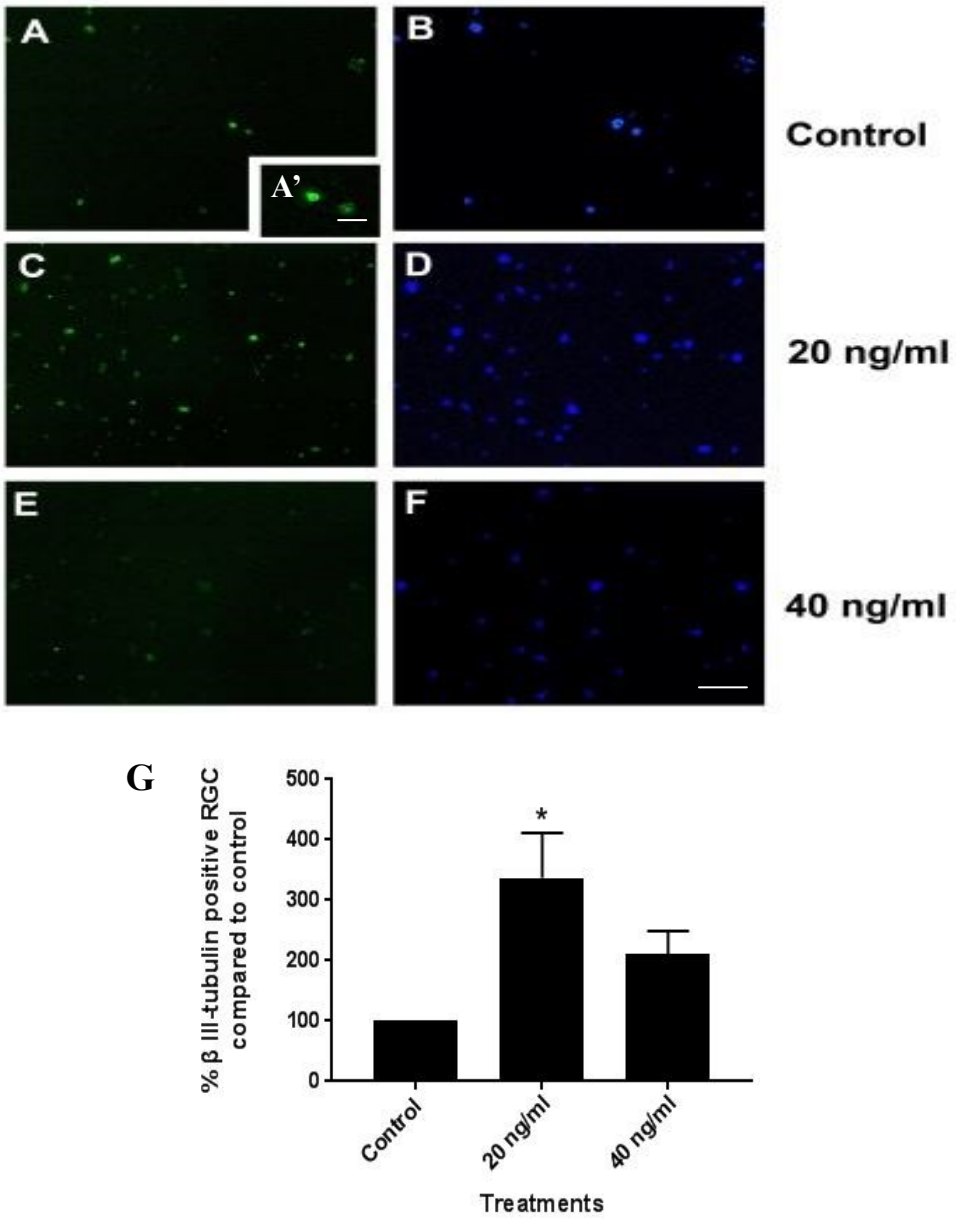

Figure 7: GDF11 administration promotes survival of p7 mouse RGCs. Cultured p7 RGCs were double labeled with anti-β III-tubulin (A,A',C,E) and DAPI (B,D,F). Quantitative graphic representation of percent β III-tubulin positive cell bodies; data are represented as a percentage of control (G). Bars represent mean ± SEM from 3 animals per treatment group. Statistical analysis was carried out by using one-way ANOVA followed by Bonferroni's post hoc test, *$p < 0.05$. Scale bar for A-F = 50μm. Scale bar for A'=25 μm.

5.3 GDF11 administration increases pSmad2 expression but not BDNF expression

To evaluate the mechanisms involved in the GDF11-induced RGC survival, the ALK5 inhibitor experiment was carried out, as outlined in Figure 4. ALK5 inhibitor was used since ALK5 is one of type I receptors that GDF11 is known to use ALK5 predominantly. ALK5 inhibitor I (Enzo Life Sciences) is an ATP-competitive inhibitor that interrupts the ActRII-mediated phosphorylation of ALK5 by competitively binding to ATP binding sites of ALK5. 5μM ALK5 inhibitor was used, as it has been previously suggested as the lowest effective concentration required to inhibit TGF-β signaling *in vitro* (Yousef *et al.*, 2015). Because ALK5 inhibitor I (Enzo Life Sciences) is soluble in DMSO and DMSO concentration > 1% (v/v) is known to cause retinal apoptosis *in vitro* (Galvao *et al.*, 2014), 0.5% DMSO was used as another control treatment to verify that DMSO concentration < 1% does not cause apoptosis of p7 RGCs. There was no significant RGC survival difference between control treatment and 0.5% DMSO treatment, confirming that 0.5% DMSO does not induce retinal apoptosis (data not shown). Based on the previous observation that 20 ng/ml GDF11 is the most effective concentration for p7 RGC viability, this concentration was used again for the ALK5 inhibitor experiment. As expected, 20 ng/ml GDF11 significantly enhanced the RGC survival (Fig. 8M); specifically, the RGC survival was enhanced by 70%. Treatment with ALK5 inhibitor alone did not significantly increase the RGC survival as well as pSmad2/3 expression, compared to GDF11 treatment (Data not shown). Co-treatment with both ALK5 inhibitor and GDF11 also did not significantly reduce the RGC survival and enhanced the RGC survival by 56%. On the contrary, pre-treatment with ALK5 inhibitor prior to GDF11 administration showed a significant reduction in RGC survival (Fig. 8M); in comparison to GDF11 treatment, the ALK5 inhibitor pre-treatment caused 60% reduction in

RGC survival, suggesting that GDF11 is potent, and the incubation period is required for the ALK5 inhibitor to prevent ALK5-mediated phosphorylation of downstream targets.

The pSmad2 antibody (Cell Signaling) detects Smad2 only when dually phosphorylated at S465 and S467; however, as it can also detect pSmad3 at its equivalent site, the proteins detected by this antibody are referred to as pSmad2/3. As shown in Figure 8N, the GDF11 treatment and co-treatment resulted in significantly increased pSmad2/3 expression in p7 RGCs by approximately 10%, compared to the control treatment. Interestingly, ALK5 inhibitor treatment also induced significant pSmad2/3 expression (Data not shown). On the contrary, the ALK5 inhibitor pre-treatment significantly reduced pSmad2/3 expression by 10%, suggesting that GDF11 administration promotes RGC survival through ALK5-pSmad2/3 pathway. Figure 8F' shows that pSmad2/3 expression was detected within p7 RGCs.

Because previous studies have shown that BDNF promotes RGC survival both *in vitro* and *in vivo* (Khalin *et al.*, 2015), BDNF expression in cultured p7 RGCs after GDF11 administration was also examined to investigate whether GDF11 promotes RGC survival by upregulating BDNF expression. As shown in Figure 9M, none of the treatments appeared to influence the expression of BDNF in p7 RGCs, suggesting that GDF11 does not induce BDNF expression through ALK5-pSmad2/3 pathway.

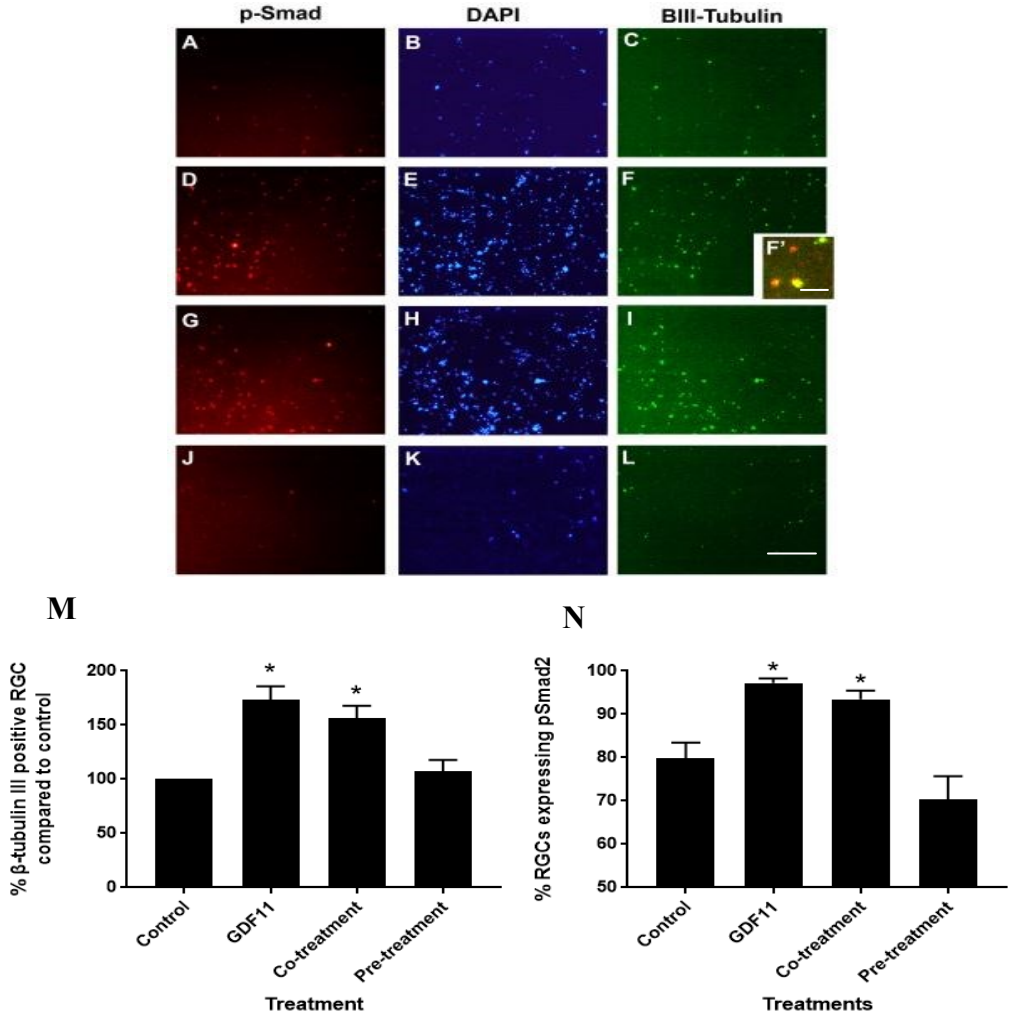

Figure 8: ALK5 inhibitor pre-treatment prevents GDF11-induced survival of p7 mouse RGCs and activation of Smad2/3. Cultured p7 RGCs were triple labeled with anti-pSmad2 (A,D,G,J), DAPI (B,E,H,K) and anti-β III-tubulin (C,F,I,L). The co-localization between pSmad2 and β III-tubulin stains confirmed that pSmad2 was detected in p7 RGCs (F'). Quantitative graphic representation of percent β III-tubulin positive cell bodies (M); data are represented as a percentage of control. Quantitative graphic representation of percentage of RGCs expressing pSmad2 (N). Bars represent mean ± SEM from 3 animals per treatment group. Statistical analyses was carried out by using one-way ANOVA followed by Bonferroni's post hoc test, *$p<0.05$. Scale bar for A-L = 75µm. Scale bar for F' = 37.5 µm

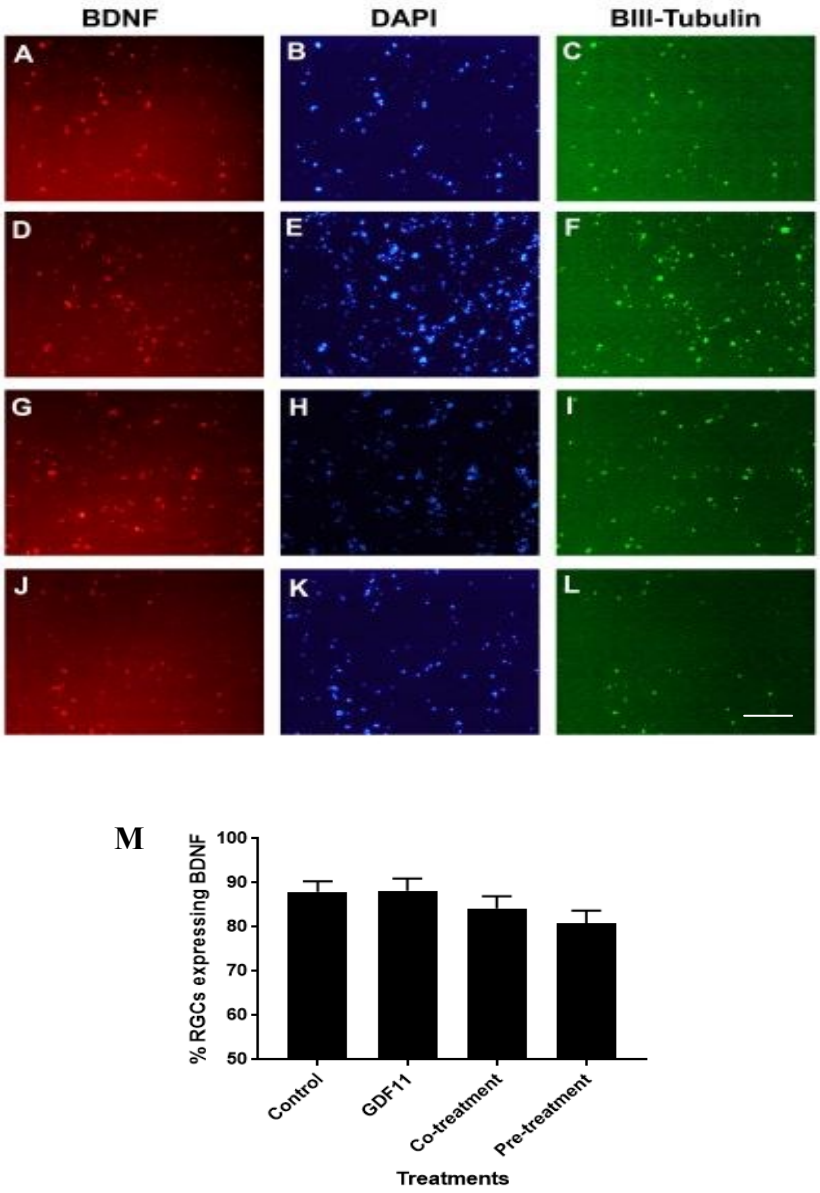

Figure 9: ALK5 inhibitor pre-treatment does not affect the expression of BDNF in p7 mouse RGCs. Cultured p7 RGCs were triple labeled with anti-BDNF (A,D,G,J), DAPI (B,E,H,K) and anti-β III-tubulin (C,F,I,L). Quantitative graphic representation of percentage of RGCs expressing BDNF (M). Bars represent mean ± SEM from 3 animals per treatment group. Statistical analysis was carried out by using one-way ANOVA followed by Bonferroni's post hoc test, *$p<0.05$. Scale bar = 75μm.

6.0 Discussion

6.1 GDF11 protein expression decreases in adult retina

Previous studies have used *in situ* hybridization to show that *gdf11* and *gdf8* mRNAs are expressed in embryonic mouse RGCs (Kim *et al.*, 2005; Mu *et al.*, 2004). However, no studies have used immunohistochemistry to examine GDF11 and GDF8 protein expressions and their developmental patterns in RGCs. In this study, GDF11/8 (AbCam) and GDF8 (MilliporeSigma) antibodies were used to investigate the developmental time course of GDF11 and GDF8 protein expressions in RGCs. The immunohistochemistry revealed that both GDF11 and GDF8 proteins are expressed in RGCs, supporting the previously reported *gdf11* and *gdf8* mRNA expressions in RGCs. The statistical analyses indicated that GDF11 expression in adult RGCs is significantly reduced; however, the major caveat of the immunohistochemical staining data is that the GDF11/8 antibody (AbCam) detects both GDF11 and GDF8.

Loffredo *et al.* (2013) first used the GDF11/8 antibody (AbCam) for Western Blot analysis to determine whether circulating GDF11 decreases with age. Their Western Blot analysis showed a 25-kDa band that was only found in the blood of young mice, suggesting that circulating GDF11 decreases with age; they identified this protein as a homomeric dimer of mature GDF11 domains, which consists of 2 disulfide-linked 12.5-kDa C-terminal monomers (Loffredo *et al.*, 2013; Poggioli *et al.*, 2016). Egerman *et al.* (2015) later tested the cross-reactivity of the GDF11/8 antibody (AbCam) by using Western Blot, discovering that the antibody could label both mature dimer (25 kDa) and reduced monomer (12.5 kDa) of both recombinant GDF11 and GDF8. They also developed a new

electrochemiluminescence (ECL) immunoassay method to specifically measure GDF11 level in blood; they confirmed that this immunoassay (R&D systems) can detect only GDF11 and not GDF8 (Egerman *et al.*, 2015). By using this assay, they analyzed circulating GDF11 concentration of young and old rats (6 month- and 24 month-old) and humans (20~30 year- and 60 year-old) and showed an age-dependent increase in GDF11 (Egerman *et al.*, 2015). Performing RNA sequencing on skeletal muscle from 6, 12, 18, 21 and 24 month-old rats and showing an age-dependent increase in *gdf11*, Egerman *et al.* (2015) argued that GDF11 rather increases with age and started the controversy surrounding the relationship of GDF11 to aging. Recently, Poggioli *et al.* (2016) further confirmed that the GDF11/8 antibody (AbCam) does detect both GDF11 and GDF8; however, they also discovered that the GDF11/8 antibody can detect a 25-kDa immunoglobulin light chain. They performed immunoglobulin G (IgG) depletion from serum pooled from old mice and showed that the 25-kDa band was nearly eliminated while the 12.5-kDa band remained unchanged; they did perform mass spectrometry analysis to confirm that IgG light chain is the predominant component of the 25-kDa band (Poggioli *et al.*, 2016). By using Western Blot analyses of 4 different mammalian species at various ages, Poggioli *et al.* (2016) revealed that the 12.5-kDa band decreased dramatically with age while the 25-kDa band showed an age-dependent increase, concluding that the 25-kDa and 12.5-kDa bands are immunoglobulin light chain and GDF11/8, respectively; it should be noted that immunoglobulin has long been known to increase with age (Natsuume-Sakai *et al.*, 1977). This recent study by Poggioli *et al.* (2016) not only supports the previous evidence that GDF11 decreases with age but also raises a question regarding the validity of the GDF11-specific immunoassay used by Egerman *et al.* (2015); they did not confirm whether the

immunoassay (R&D systems) distinguished between GDF11 and immunoglobulin light chain. Considering that the GDF11/8 antibody (AbCAm) can detect IgG light chains as well, it is possible that proteins detected by this antibody in the developing and adult retinae would inevitably include IgG light chains. However, since RGCs have been shown to only weakly express IgG (Niu *et al.*, 2011), the majority of proteins detected by the GDF11/8 antibody in RGCs would likely be GDF11 and GDF8.

Unlike the GDF11/8 antibody (AbCam), the GDF8 antibody (MilliporeSigma) has been shown to specifically detect GDF8 (Sharma *et al.*, 2012; Breitbart *et al.*, 2013; Hauerslev *et al.*, 2014), and there is no evidence that it can also detect GDF11 and/or IgG light chain. As shown in Figure 6, no developmental changes in GDF8 expression in RGCs were detected. Considering that the GDF8 antibody detects GDF8 only, the proteins that were reduced in the adult RGCs are likely GDF11. Based on the reduced GDF11 expression in adult RGCs and the potential role of GDF11 as a retinal dendrite growth initiator, it was speculated that the developmental decline in GDF11 expression in RGCs may contribute to the previously reported developmental decline in intrinsic growth ability of RGCs. However, the data indicate that GDF11 does not promote axon regeneration in cultured p7 mouse RGCs, suggesting that the developmental reduction of GDF11 expression in RGCs may not contribute to the developmental decline in intrinsic growth ability of RGCs.

6.2 Status of GDF11 in the developing and adult retinae

In addition to the cross-reactivity of GDF11/8 antibody (AbCam), another caveat of the immunohistochemical staining data is that it only shows the total amount of proteins and cannot distinguish four different states of GDF11 and GDF8 that could exist in the retina. These states are: precursor state, latent state, bioactive state and inactivated state. The newly synthesized GDF11 and GDF8 exist in the precursor state, where they retain both prodomains and mature domains. Since protein translation takes place inside the cell, the GDF11 and GDF8 precursors would exist in the RGCs; hence, the immunohistochemical staining data could account for the precursor state of GDF11 and GDF8 in the RGCs. The data would likely also account for the latent state of GDF11 and GDF8 in the RGCs. The precursors of GDF11 and GDF8 are processed by furin or furin-like proteases and enter the latent state, in which the prodomains are cleaved but still tightly attached to the mature domains (Walker *et al.*, 2017). The furin-processing mainly takes place inside the *trans*-Golgi network (TGN), a late Golgi structure that sorts secretory pathway proteins to their final destinations, such as lysosomes and secretory granules (Thomas, 2002). Hence, the proteins detected by the GDF11/8 and GDF8 antibodies could be the latent GDF11 and GDF8 proteins that are synthesized in the TGN.

Considering the previously proposed model of newly born neurons secreting GDF11 (Shi and Liu, 2011), RGCs might secrete GDF11 and GDF8 through the TGN pathway; however, the exact mechanism of GDF11 and GDF8 secretion has not been reported. Since the latent GDF11 and GDF8 proteins can be activated by extracellular TLD metalloproteinases (Walker *et al.*, 2017), the latent proteins could be packaged into secretory granules and then later secreted into the extracellular space to be activated by TLD

metalloproteinase. Therefore, those latent GDF11 and GDF8 proteins detected by the GDF11/8 and GDF8 antibodies could be stored in the secretory granules. As Walker *et al.* (2017) have recently shown that the latent GDF11 and GDF8 proteins can be activated by treatment with HCl to pH 2.5, it is possible that they are taken up by lysosomes, converted into the bioactive homomeric dimers and then secreted out of RGCs. If this lysosomal activation of GDF11 and GDF8 does take place in RGCs, then some of GDF11/8 proteins detected in RGCs could also be in the bioactive state.

As for the status of GDF11 and GDF8 outside of the RGCs, they might exist in the latent, bioactive and inactivated states. The latent GDF11 and GDF8 proteins secreted out of the RGCs could be either free-floating in the extracellular space or attached to the extracellular matrix (Walker *et al.*, 2016). Once the prodomains are fully cleaved by extracellular TLD metalloproteinases, the latent GDF11 and GDF8 become bioactive and capable of binding to their transmembrane receptor ActRII. However, by being bioactive, they also become susceptible to extracellular inactivation by FSTL-3 that can symmetrically embrace the bioactive GDF11 and GDF8 homomeric dimers. In addition to these possible states of GDF11 and GDF8 in the extracellular space, their precursors might also be directly secreted out of RGCs immediately after translation; since furins can cycle between the TGN and the cellular surface (Thomas, 2002), the extracellular precursors could be converted into their latent forms by the surface furins. Figure 10 summarizes the possible states and cellular locations of GDF11/8.

As both GDF11/8 and GDF8 antibodies cannot identify different states of GDF11 and GDF8, it is likely not possible to precisely pinpoint the intracellular and extracellular states of GDF11 and GDF8 detected in e18, p1, p4, p7, p14 and adult RGCs and deduce how

they could contribute to the survival of RGCs during the retina development. Considering that developing RGCs are known to undergo retrograde neuronal cell death more rapidly than adult RGCs after ON injury (Isenmann *et al.*, 2003), it is possible that the developing retina has intracellular precursor, latent and/or bioactive GDF11 but lacks extracellular bioactive GDF11. This is further supported by the p7 RGC survival data showing that administration of bioactive GDF11 can promote RGC survival. The adult retina stained with the GDF11/8 antibody (Figure 5G) showed limited staining in the GCL, where only β III-tubulin and DAPI stains were co-localized; this suggests that GDF11 is mostly expressed outside of RGCs in the adult retina. As explained above, the extracellular GDF11 could be in latent, bioactive, inactivated and even perhaps precursor states. Considering the ability of bioactive GDF11 to promote cultured p7 RGC survival, some extracellular GDF11 in the adult retina could exist in the bioactive state and thus contribute to the delayed retrograde neuronal cell death in the adult retina after ON injury.

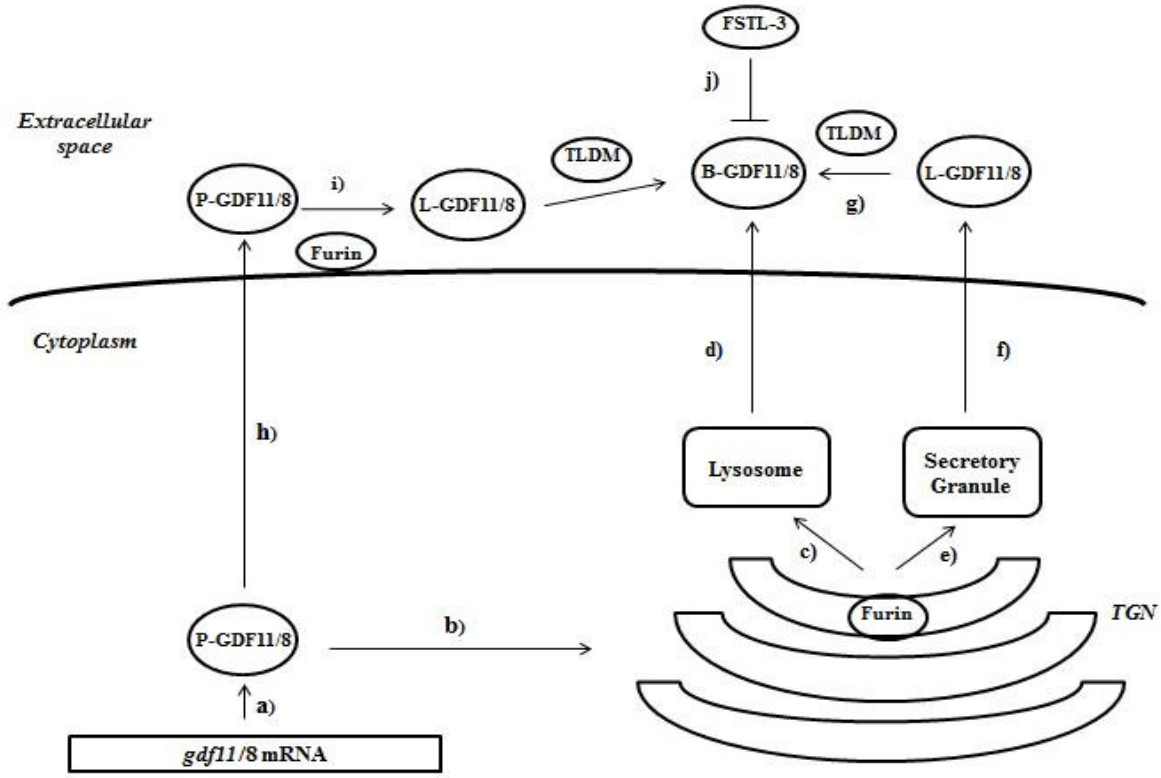

Figure 10: States and locations of intracellular and extracellular GDF11/8. a) Precursor GDF11/8 (P-GDF11/8) is translated from *gdf11/8* mRNA. b) Precursor GDF11/8 can then go through the *trans*-Golgi network (TGN) and then processed by furin, being converted to latent GDF11/8 (L-GDF11/8). c) Latent GDF11/8 could be taken up by lysosome and undergo acid activation, becoming bioactive GDF11/8 (B-GDF11/8). d) Acid-activated GDF11/8 can then be secreted out of the cell. f) Alternatively, latent GDF11/8 could be packaged into secretory granules instead. f) Latent GDF11/8 in the granules would later be secreted out of the cell. g) Secreted latent GDF11/8 could become activated by TLD metalloproteinase. h) Precursor GDF11/8 could potentially be secreted out of the cell. i) Secreted precursor GDF11/8 might be converted into latent GDF11/8 by surface furin and then become activated by TLD metalloproteinase. j) Bioactive GDF11/8 could become inactivated by its extracellular inhibitor FSTL-3.

6.3 GDF11 promotes survival of RGCs in vitro

Although GDF11 has been shown to promote neurogenesis in the aged brain and dendrite growth in RGCs (Hocking *et al.*, 2008; Katsimpardi *et al.*, 2014), the effect of GDF11 on mammalian RGC survival has not been previously reported. The RGC survival data (Figure 7G) reveal that 20 ng/ml was an effective GDF11 concentration that promoted RGC survival *in vitro*. Since this concentration falls within the effective concentration range of GDF11 (10-50 ng/ml) that has been previously shown to promote dendrite growth (Hocking *et al*., 2008), it was initially expected to promote dendrite or axon growth in cultured mouse RGCs. However, as shown in Figure 7, it did not promote any dendrite or axon growth in the RGC culture. This outcome does not reconcile with the previous study by Hocking *et al.* (2008), although it must be noted that different animal model was used. Unlike mammals, amphibians possess greater capacity for CNS regeneration, as evident in both larval and adult salamander's ability to successfully regenerate spinal cord structures after amputation (Tanaka and Ferretti, 2009). With regard to retina regeneration in frogs, both larval and adult frogs are capable of regenerating retina after injury; furthermore, the surviving RGCs in the frog visual system are able to regenerate and reconnect with their targets in the optic tectum (Tanaka and Ferretti, 2009; Duprey-Díaz *et al.*, 2016). The concentration of GDF11 used in this study did not promote any dendrite or axon outgrowth in p7 mouse RGCs, perhaps because they partially lost the intrinsic growth capacity, which is retained in frog RGCs. It is possible that GDF11 can enhance the existing intrinsic growth potential but cannot restore the intrinsic growth potential once it has been lost, as is the case for p7 mouse RGCs. Another reason for the discrepancy could be that GDF11 does not function as a dendrite growth initiator in mammalian retina. According to Bialas *et al.* (2013),

TGF-β signaling is involved in initiating synaptic pruning of postnatal mouse RGCs, which takes place between p5 and p8. Since synaptic pruning happens in the absence of neuronal death (Vanderhaeghen and Cheng, 2009), GDF11 may suppress further axon growth and simultaneously promote survival mechanisms to preserve postnatal RGCs during the pruning process. In support of this, Augustin *et al.* (2017) recently found that 10 ng/ml GDF11 reduces neurite outgrowth of cultured e18 rat cortical neurons without inducing apoptosis. Hence, GDF11 may be an essential growth factor that maintains the viability of postnatal RGCs while facilitating axon pruning process.

Although the regenerative capability of frog RGCs are superior to that of mammalian RGCs, studies have shown that many RGCs in both frog and mammalian visual system die rapidly after axotomy (Duprey-Díaz *et al.*, 2016). Considering this similarity in their survival rate, it is possible that frog and mammalian RGCs share the same survival mechanism. Notably, this is evident in the current study: the effective GDF11 concentration that promoted the mouse RGC survival is within the effective GDF11 concentration range that promoted frog RGC survival. It is interesting that only 20 ng/ml GDF11 resulted in the significant mouse RGC survival while the GDF11 concentration range (10-100 ng/ml) reported by Hocking *et al.* (2008) sustained the frog RGC viability. This could be due to the age difference in animals used in each study; postnatal RGCs may express less ALK5 receptors and Smad2/3 than embryonic RGCs, and thus may be responsive to a lower GDF11 concentration range (e.g. 5-20 ng/ml). Additionally, higher doses of GDF11 may result in non-specific downstream signaling that is not involved in RGC survival. Overall, it is evident that GDF11 holds a therapeutic potential for neuronal survival in glaucoma and other neurodegenerative diseases.

6.4 GDF11 promotes survival of RGCs through Smad2/3 activation

Previous rejuvenation studies have confirmed GDF11-induced rejuvenation by reporting the phosphorylation of Smad2/3 (Loffredo *et al.*, 2013; Katsimpardi *et al.*, 2014); in addition, Smad2/3 is also known to be involved in RGC survival both *in vitro* and *in vivo* (Ueki and Reh, 2012; Braunger *et al.*, 2013). Figure 8M and 8N show that the GDF11 treatment significantly increased the percentage of surviving p7 RGCs expressing pSmad2/3, compared to the control treatment. As shown in Figure 8F', pSmad2/3 proteins were expressed broadly within RGCs, indicating that they were in both cytoplasm and nucleus. Upon activation by GDF11-ActRII complex, type I receptors can phosphorylate their main downstream target, Smad2/3. In the basal state, these R-Smads are known to be bound to a cytoplasmic anchor protein called Smad anchor for receptor activation (SARA) (Runyan *et al.*, 2009). SARA interacts with MH2 domain of Smad2/3 through its Smad binding domain, regulating the subcellular distribution of Smad2/3 (Itoh *et al*., 2002). As it can bind to TGF-β receptor complex *via* its C-terminal region, SARA is thought to play a role in presenting R-Smads to the receptor for phosphorylation (Runyan *et al.*, 2009). Once phosphorylated by the TGF-β receptor complex, Smad2/3 proteins dissociate from SARA and then multimerize with Smad4 to translocate into the nucleus (Itoh *et al.*, 2002). Although no studies have shown that activin type receptor complexes interact with SARA, they could do so, considering that both TGF-β and activin type receptors can produce the same TGF-β signaling and activate the same downstream targets. Hence, it is possible that the detected pSmad2/3 proteins in RGCs were undergoing type I receptor-mediated phosphorylation and dissociation from SARA in the cytoplasm. Alternatively, the detected pSmad2/3 proteins could have been already dissociated from SARA and going through multimerization with Smad4 in the cytoplasm.

The nuclear translocation of the Smad complex occurs through the nuclear pore complex (NPC), which consists of multiple copies of 30 different nucleoporins and forms a hydrophobic channel through the nuclear envelop (Hill, 2009). Although it is still not clearly known how the Smad proteins are imported to the nucleus, karyopherin-dependent import involving the MH1 domain of Smad proteins and karyopherin-independent import by direct contacts between nucleoporins and the MH2 domain of the Smad proteins have been proposed (Hill, 2009). Karyopherins are carrier proteins that bind to cargo molecules and pass through the nuclear pore by interacting with nucleoporins; depending on the direction of transport, karyopherins are classified as importins or exportins (Hill, 2009; Chen and Xu, 2011). In the karyopherin-dependent import model, the importin-Smad complex entering the nucleus is disrupted by the small guanosine triphosphatase (GTPase) Ran in its guanosine triphosphate (GTP) bound form; on the contrary, in the karyopherin-independent import model, the Smad proteins directly interact with nuclear pore components, particularly nuclear pore complex protein 214 (Nup214) at the cytoplasmic side of the nuclear pore and Nup153 at the nucleoplasmic side (Hill, 2009). Considering these two different models of Smad nuclear translocation, the detected pSmad2/3 in p7 RGCs could have been bound to importin in the cytoplasm or nucleus; alternatively, it could have been interacting with NUP214 and NUP153 at the nuclear membrane. Inside the nucleus, the Smad complex interacts with the target genes and modulates the gene transcription along with transcriptional activators or suppressors (Jinnin *et al.*, 2007); hence, other detected pSmad2/3 in the nucleus could have been interacting with the target genes.

Based on the significant p7 RGC survival induced by GDF11 administration, it is probable that the Smad complex interacted with transcriptional activators to increase the

transcription of a gene or set of genes involved in cellular survival. Although no GDF11-specific target genes have been identified, a strong candidate gene that promoted p7 RGC survival could encode growth arrest and DNA-damage-inducible protein (Gadd45β). Gadd45β, a member of the Gadd45 protein family, is known to be localized mainly within the nucleus, and it has been shown to play a role in cell cycle arrest, DNA repair, apoptosis and cell survival (Gupta *et al.*, 2005). It has been revealed that Gadd45β can promote adult neurogenesis by promoting epigenetic DNA demethylation and neuronal survival in ischemic stroke, reperfusion and RGC injury models (Ma *et al*., 2009; Liu *et al.*, 2013; Liu *et al.*, 2015; He *et al.*, 2016). According to Liu *et al.* (2009), induction of Gadd45β by TGF-β1-mediated activation of nuclear factor κ light chain enhancer of activated B cells (NF-κB) increased the resistance of RGCs against tumor necrosis factor α (TNF-α) and paraquat oxidative stress. Since Gadd45β expression has been shown to be upregulated by the ALK5-dependent activation of Smad2 as well (Ungefroren *et al.*, 2005), it is possible that GDF11 administration promoted p7 RGC survival by inducing Gadd45β expression.

As shown in Figure 8M and 8N, the co-treatment with ALK5 inhibitor and GDF11 could not prevent GDF11-induced RGC survival and Smad2/3 activation, suggesting the high potency of GDF11. In support of this, Walker *et al.* (2017) have shown that low doses of GDF11 (25 pM and 125 pM) could significantly enhance Smad3-dependent cellular response in 30 minutes, compared to the same doses of GDF8. Hence, the ALK5 inhibitor needs to be incubated with the cells before GDF11 administration in order to prevent the GDF11-induced RGC survival and Smad2/3 activation, as evident in the pre-treatment group.

In the pre-treatment group, the ALK5 inhibitor would have bound to the majority of ALK5 in p7 RGCs prior to GDF11 administration, reducing the availability of ALK5 for

GDF11-ActRII complexes and forcing GDF11 to signal through other available type I receptors. As recently shown by Walker *et al.* (2017), GDF11 is capable of activating the Smad-dependent pathway through ALK4 and ALK7. Interestingly, studies seem to suggest that they induce different cellular responses; while ALK4 has been shown to promote cell survival in a Smad-independent manner, ALK7 has been shown to induce apoptosis in a Smad-dependent manner (Kim *et al.*, 2004; Wang and Tsang, 2007; Zhao *et al.*, 2012). Kim *et al.* (2004) transfected hepatoma cells with the constitutively active form of ALK7 and discovered that ALK7-induced apoptosis involved Smad2/3 activation; in addition, they also found that short interfering RNA-mediated inhibition of Smad3 significantly suppressed ALK7-induced caspase-3 activation. In support of this study, Zhao *et al.* (2012) showed that nodal-induced ALK7 activation led to increased pSmad2/3 expression as well as caspase-3 expression, causing the apoptosis of pancreatic β-cells. Considering the apoptotic effects of ALK7-mediated Smad2/3 activation, it is possible that in the RGCs pre-treated with ALK5 inhibitor, GDF11 recruited ALK7 and caused apoptosis of RGCs by inducing Smad2/3-mediated caspase-3 expression. However, there was no significant loss of p7 RGCs in the ALK5 inhibitor pre-treatment group; there was no significant difference in RGC survival between the control and ALK5 inhibitor pre-treatment groups. This suggests that GDF11 perhaps neutralized the apoptotic effects of ALK7 by activating ALK4 and promoting ALK4-mediated caspase-3 inactivation; in fact, it has been shown that TGF-β signaling can interrupt caspase-3 activation in rat primary hippocampal cultures (Zhu *et al.*, 2001).

6.5 ALK5-Smad2/3 pathway may function independent of BDNF

BDNF, a member of the nerve growth factor (NGF) family, is the most abundantly expressed neurotrophic factor found in the CNS and locally produced by RGCs and astrocytes in the retina (Gupta *et al.*, 2014; Nurjono *et al.*, 2012). It is also synthesized in the superior colliculus and the lateral geniculate nucleus, from which it is retrogradely transported to RGCs *via* ON; hence, it has been suggested that deprivation of BDNF supply to RGCs *via* optic nervy injury results in the retrograde neuronal death (Gupta *et al.*, 2014). Intravitreal injection of BDNF at the time of the nerve injury has been shown to effectively enhance RGC survival; furthermore, intravitreal BDNF injection followed by bilateral delivery of BDNF to the visual cortex has been shown to provide even greater level of neuroprotection, strongly suggesting the neuroprotective role of BDNF in the visual system (Weber *et al.*, 2010). However, therapeutic use of BDNF in CNS degeneration has been limited due to the inability of BDNF to cross the BBB; hence, studies have been targeting the BDNF signaling pathway for promoting RGC survival (Kimura *et al.*, 2016).

BDNF-induced neuronal survival is initiated by the activation of the BDNF receptor tropomyosin receptor kinase B (TrkB) (Kimura *et al.*, 2016). Upon binding to BDNF, TrkB undergoes dimerization and then autophosphorylation by tyrosine residues in the cytoplasmic kinase domain that serve as docking sites for effector molecules (Cunha *et al.*, 2010). TrkB can activate ERK, Akt/PI3K and phosphoinositide phospholipase C-γ (PLCγ) pathways, which eventually result in the phosphorylation of transcription factor cyclic adenosine monophosphate response element-binding protein (CREB) and the transcription of genes involved in the neuronal survival (Cunha *et al.*, 2010). As both BDNF and TGF-β pathways involve ERK and Akt/PI3K, there could be a crosstalk between these two pathways; in fact,

the study by Lu *et al.* (2005) revealed that the functional blockade of Smad4 and TβRIIR attenuates BDNF-mediated cellular activity, indicating that the crosstalk does exist. Considering the neuroprotective effects of BDNF and the potential crosstalk between BDNF and TGF-β, it is possible that GDF11 administration could have increased the expression of BDNF in RGCs and promoted their survival. Therefore, the BDNF expression of cultured p7 RGCs was investigated to determine whether or not BDNF is one of survival factors whose expression was upregulated by GDF11 administration. As shown in Figure 9M, there were no significant differences in BDNF expression among different treatment groups, suggesting that GDF11 administration did not induce the BDNF expression in surviving p7 RGCs. However, it is possible that GDF11 did upregulate BDNF expression transiently, starting soon after administration of the GDF11. Considering the high potency of GDF11, GDF11-induced BDNF upregulation could have taken place within 24 hours or 48 hours, and excess BDNF could have been secreted out of RGCs or degraded within RGCs 72 hours after plating. In support of this, Sometani *et al.* (2001) have shown that 20 ng/ml TGF-β1treatment can enhance *BDNF* mRNA expression in neurons isolated from rat cerebral cortex within 24-48 hours and results in accumulation of BDNF protein in the culture medium; additionally, they noted that the intracellular content of BDNF was nearly unchanged. This could explain why there was no significant difference in BDNF expression between the control and GDF11 treatments. As the ALK5 inhibitor pre-treatment did not reduce the expression of BDNF within p7 RGCs, GDF11 does not appear to upregulate BDNF expression through ALK5-pSmad2/3 pathway. However, GDF11 could have utilized ALK4 and promoted RGC survival through the Smad-dependent pathway leading to the upregulation of BDNF expression; alternatively, GDF11-induced ALK4 activation could have activated the Smad-independent

pathways and enhanced the BDNF expression in RGCs. This would explain why the ALK5 inhibitor pre-treatment did not significantly reduce the BDNF expression. Overall, although BDNF expression in p7 RGCs could have been transiently upregulated by GDF11 within 24-48 hours, it is evident that GDF11 does not appear to upregulate BDNF expression in p7 RGCs through ALK5-pSmad2/3 pathway.

7.0 Conclusions and future directions

TGF-β signaling has been shown to promote neuronal survival as well as axon regeneration in CNS neurons, including RGCs in the visual system, and has become the therapeutic target for CNS neuroprotection and regeneration. Recently, GDF11, a member of TGF-β superfamily, was described as a rejuvenation factor that promotes neurogenesis in aging mice. Previous studies have shown that *gdf11* mRNA is expressed in developing RGCs, and GDF11 administration can promote dendrite outgrowth in cultured frog RGCs. However, the developmental pattern of GDF11 expression in RGCs and the impact of GDF11 administration in mammalian RGCs have yet to be determined. The current study shows that GDF11 expression is indeed expressed in RGCs and appears to decline in adult RGCs. It was speculated that the developmental decline in GDF11 expression in adult RGCs may contribute to the developmental decline in intrinsic growth ability of RGCs. The *in vitro* RGC survival data indicate that GDF11 administration cannot promote any dendrite or axon regeneration in postnatal RGCs, suggesting that the developmental reduction of GDF11 in RGCs may not contribute to the developmental decline in intrinsic growth ability of RGCs. However, as the data also reveal that GDF11 enhances survival of postnatal RGCs, it holds a therapeutic potential for neuronal survival in neurodegenerative diseases. The ALK5 inhibitor treatment prior to GDF11 administration prevented the GDF11-induced RGC survival by attenuating the ALK5-pSmad2/3 signaling. Although GDF11 does not appear to upregulate BDNF expression in cultured RGCs through ALK5-pSmad2/3 pathway, GDF11 still may promote RGC survival by upregulating BDNF expression through ALK4-mediated Smad-dependent or Smad-independent pathways.

As there is no reliable antibody that can distinguish between GDF11 and GDF8,

future studies should focus on developing new antibodies or new methods for detecting GDF11. The most reliable method for detecting GDF11 is currently the immunoplexed liquid chromatography with tandem mass spectrometry assay, designed by Schafer *et al.* (2016); this method overcomes the high amino acid sequence homology of GDF11 and GDF8 by monitoring multiple distinct residues within GDF11 and GDF8. However, as this method still cannot identify the four possible states of endogenous GDF11 (i.e. precursor, latent, bioactive and inactivated forms), further research is required to find a way to distinguish one GDF11 state from another.

As 20ng/ml GDF11 was shown to promote RGC survival *in vitro*, the same GDF11 concentration should be used for an ON injury model to evaluate the neuroprotective effects of intravitreal GDF11 injection. Furthermore, since systemic administration of GDF11 (0.1mg/kg mouse body weight) has been shown to promote neurogenesis in aging mice (Katsimpardi *et al.*, 2014), it would be interesting to see if the systemic administration of GDF11 could also promote RGC survival after ON injury. Although GDF11-induced RGC survival was shown to involve pSmad2/3 activation, it is still unclear which survival factors are being upregulated by GDF11; hence, future research should focus on identifying potential survival factors that are upregulated by GDF11.

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