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Insulin-Induced Retinal Ganglion Cell Dendrite Regeneration: Characterization And Identification Of Novel Molecular Mechanisms.

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This thesis titled:

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RÉSUMÉ

La rétraction des dendrites de cellules ganglionnaires de la rétine (CGR) est parmi les changements pathologiques qui conduisent à des déficits fonctionnels lors du glaucome. Récemment, on a montré que l'administration de l'insuline promeut une importante régénération des dendrites des cellules ganglionnaires de la rétine et rétablie les synapses. On se basant sur ces données, on a posé les questions suivantes: 1) Est ce que la réduction de la pression intraoculaire (PIO) élevée est suffisante pour promouvoir la régénération des dendrites en absence d'apport exogène de l'insuline? 2) Quels sont les mécanismes moléculaires en aval de l'insuline qui permettent la régénération des dendrites des CGR lors du glaucome? Les souris transgéniques Thy1-YFP, qui permettent la visualisation des dendrites des CGR, ont reçu une injection intra-camérale de microbilles magnétiques pour induire l'hypertension oculaire. Des gouttes journalières du brinzolamide ont été administrées pour réduire la PIO. Les CGR ont été imagés à l'aide du microscope confocal et les dendrites ont été reconstruites en 3D grâce au logiciel Imaris. Pour l'analyse des mécanismes moléculaires, les CGR ont été isolées grâce à la technique de cytométrie FACS, à partir des rétines traitées à l'insuline et au véhicule suivi par un séquençage d'ARN (ARNseq). Le brinzolamide réduit de façon effective la PIO, cependant cette réduction ne permet pas la régénération des dendrites des CGR. Le séquençage de l'ARN des rétines glaucomateuses et des rétines contrôles a aidé à identifier des voies de signalisation candidates pour participer à la régénération des dendrites des CGR incluant mTOR, Notch, glycolyse, métabolisme des acides gras, réparations d'ADN et myc-cibles. Ces données nous ont conduit à retirer les conclusions suivantes: 1) La réduction de la PIO n'est pas suffisant pour promouvoir la régénération

des dendrites des CGR, suggérant que l'insuline endogène ne remplit pas le rôle de l'insuline exogène. 2) De nombreuses voies moléculaires sont activées pour mener l'effet régénérateur de l'insuline sur les dendrites des CGR. Ces résultats supportent le rôle de l'administration de l'insuline pour restaurer les connections et le fonctionnement de la rétine et identifient des gènes qui pourraient être de nouvelles cibles pour traiter le glaucome.

Mots clés: Glaucome, Insuline, Cellules Ganglionnaires de la Rétine, Dendrites, Régénération.

ABSTRACT

Glaucoma is the leading cause of irreversible blindness worldwide. High intraocular pressure (IOP) is the most important risk factor to develop the disease. The retraction of retinal ganglion cell (RGC) dendrites is one of the earliest pathological changes leading to substantial functional deficits. We recently demonstrated that insulin, administered after arbor retraction, promoted remarkable RGC dendrite and synapse regeneration. Here, we asked the following questions: 1) is insulin effective at promoting RGC dendrite regeneration in experimental glaucoma? 2) is reduction of IOP sufficient to promote dendrite regeneration in the absence of exogenous insulin? 3) what are the signaling components downstream of insulin that stimulate RGC dendrite regeneration in glaucoma? Thy1-YFP mice, which allow visualization of RGC dendritic arbors, received an intracameral injection of magnetic microbeads to induce ocular hypertension. RGC dendrites were imaged by confocal microscopy and arbors were 3D reconstructed. Total RGC dendritic length and complexity increased in glaucomatous eyes treated with insulin to values similar to those found in intact non-injured controls, but not in eyes treated with brinzolamide, to lower IOP, or vehicle. RGCs were isolated by Fluorescence Activated Cell Sorting (FACS) from insulin- or vehicle-treated glaucomatous retinas as well as sham-operated controls, followed by RNA sequencing analysis (RNA-seq). Our data show a global decrease in transcriptional efficiency in glaucomatous retinas. In addition, we identified a number of key regulatory pathways potentially implicated in insulin-induced RGC dendrite regeneration including: the mammalian target of rapamycin (mTOR), glycolysis, fatty acid metabolism, DNA repair, and myc-targets. These data allow us to draw the following conclusions: 1) insulin promotes robust RGC dendrite regeneration in

glaucoma, 2) IOP reduction alone is not sufficient to promote dendritic regrowth, and 3) multiple molecular pathways are activated during insulin-mediated regeneration. These findings support a critical role for insulin administration to restore RGC dendritic structure, and identify differential gene expression that might reveal novel therapeutic targets for glaucoma.

Keywords: Glaucoma, Insulin, RGCs, Dendrites, Regeneration

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CHAPTER 1

INTRODUCTION

1.1 THE RETINA

1.1.1. ORGANIZATION OF THE RETINA

The processing of visual information begins in the retina, a ~200 µm-thick structure lining the back of the eye and composed of the same cell types as the brain: neurons and glia (Masland, 2012). Through his studies on the retinas of humans, birds, and insects, Santiago Ramon y Cajal described the different types of retinal cells and their organization. He postulated that neurons receive electrical signals via their dendrites, which travel through the cell body to the axons and eventually establish contacts with other neurons at axonal terminal synapses (Delgado-García, 2015). This observation was then generalized to neurons in the retina, which is considered an integral part of the central nervous system (CNS).

Photoreceptors in the retina, transform the received light into a biochemical signal, which manifests by the release of glutamate. Bipolar cells respond to the glutamate released by the photoreceptors. Retinal ganglion cells (RGCs) are the last input of the visual information in the retina, they are depolarized by bipolar cell inputs on their dendrites. RGCs covey the visual information, via their long projecting axons that form the optic nerve, to the superior colliculus (SC) in rodents and the lateral geniculate nucleus (LGN) in primates and felines (Liu et al., 2011). Figure 1 summarizes the main types of retinal cells and their organization in the mammalian retina.

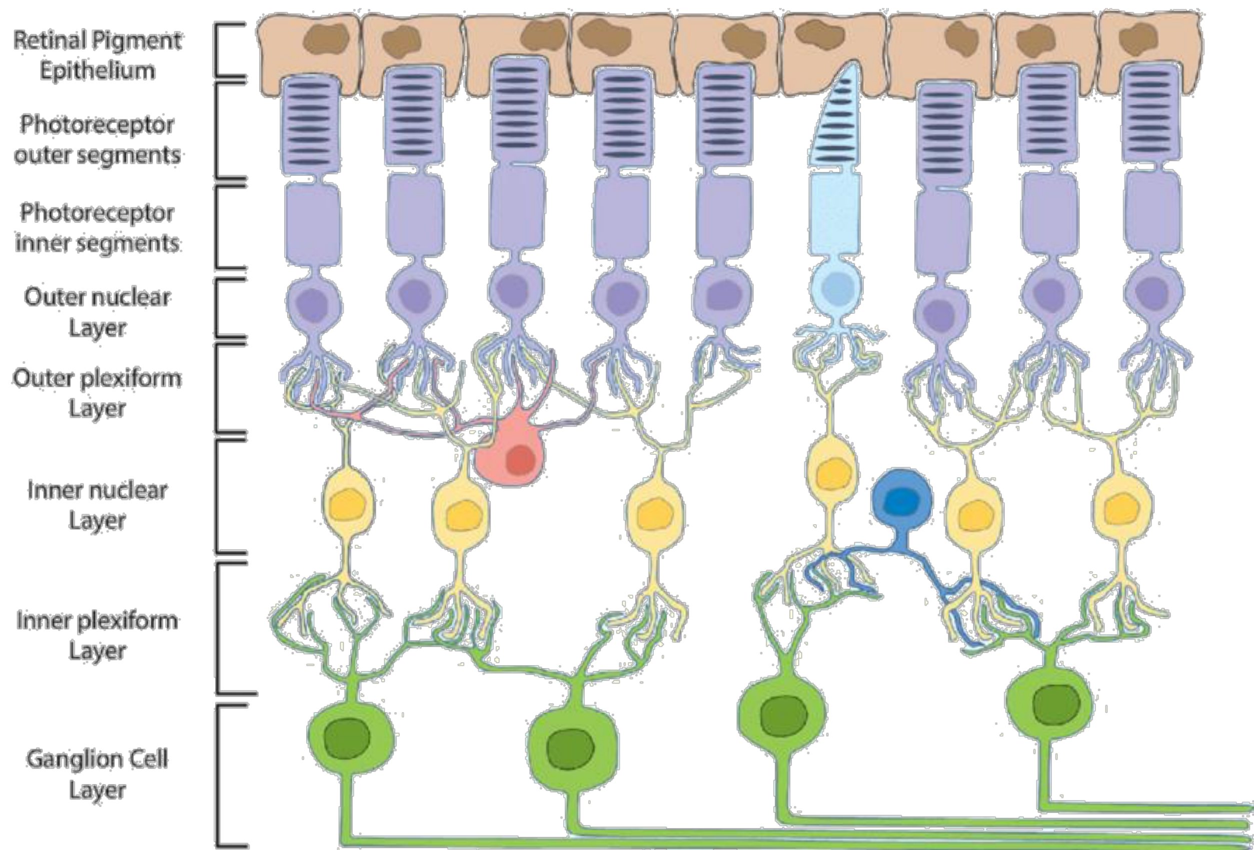

Figure 1. Schematic illustration of cellular layers in the mammalian retina.

Photoreceptors (rod in purple and cone in light blue) receive light and convert it into electrical signals which are transmitted by synapses in the outer plexiform layer (OPL) to bipolar cells (yellow) in the inner plexiform layer (IPL). At the IPL, bipolar cells synapse with retinal ganglion cells (RGC) (green) in the ganglion cell layer (GCL), whose axons form the optic nerve and convey the visual signals from the retina to the brain. Horizontal (red) and amacrine (dark blue) cells are interneurons that integrate and modulate visual information. Reproduced with permission from Sengillo et al., 2016.

1.1.2. RETINAL GANGLION CELLS (RGCs)

RGCs convey visual information from the retina to the brain via their long projecting axons that form the optic nerve. Over 40 RGC subtypes have been identified (Baden et al., 2016a; Sanes and Masland, 2015; Tran et al., 2019), however the diversity and number of these neurons is still being elucidated.

Four principle criteria are used to classify RGCs:

- **Morphology:** The shape of RGCs, including their dendritic arborization in the inner plexiform layer, remains the main criteria used to classify RGCs. RGC morphology was used by Santiago Ramon y Cajal in his first description of the retina and has been used by a number of groups as an important criterion for cell classification (Sanes and Masland, 2015).
- **Genetic/molecular markers:** The expression of a unique gene or set of genes found in sub-group of RGCs has been proposed as a system for classification. (Baden et al., 2016; Sanes and Masland, 2015). Nonetheless, several morphological types have not yet been correlated with a sub-type-specific marker.
- **Regular spacing:** Spacing means that neurons of a defined subtype maintain a certain distance from other neurons of the same subtype. The regular spacing of RGC dendritic arbor arrangement has been proposed to identify and classify certain cell sub-types (Masland, 2004; Sanes and Masland, 2015), but it is not commonly used.
- **Physiological properties:** Ultimately, the correlation of an RGC morphology with its function would provide the optimal system for classification. Unfortunately, given the abundance of RGC types and the difficulty associated with obtaining electrophysiological

individual recordings from individual cells (Sanes and Masland, 2015), this approach has not yet reached its full potential.

RGCs are an extremely heterogeneous neuronal population, and their classification has not been straightforward. Here, we describe the best characterized RGC subtypes:

i) ON-OFF Directionally Selective Ganglion Cells (ON-OFF DSGC):

This RGC type was identified by Barlow and colleagues in the 1960s (Barlow and Levick, 1965). By moving spots of light across a field of DSGCs, these investigators showed that these cells emit an action potential in response to moving stimuli in one direction but not in the opposite or other directions (Barlow and Levick, 1965). As their "ON-OFF" name indicates, these RGCs respond to both light onset and offset (increased and decreased light intensity, respectively) (Liu, 1995). There are four types of ON-OFF DSGC, based on physiological and molecular criteria, and they respond preferentially to stimuli moving in certain directions: upward, downward, backward, and forward. Each directional type of ON-OFF DSGC forms a separate mosaic, and specific endogenous cell surface markers have been identified for each type including cocaine and amphetamine regulated transcript (*Cartpt*) for the four types of the ON-OFF DSGC (Laboissonniere et al., 2019; Sanes and Masland, 2015),

ii) ON Directionally Selective Ganglion Cells (ON-DSGC):

Unlike the ON-OFF DSGCs, this class of cells responds only to moving points of light stimuli, but not to darkness (light off). ON-DSGCs respond only to three directions: dorsal ventral and nasal. Each direction is encoded by two types of ON DSGCs with different

response properties: one has sustained spike responses while the other has transient responses (Liu, 1995).

iii) OFF Directionally Selective Ganglion Cells (OFF-DSGCs)

OFF-DSGCs are the most recent DSGC population to be discovered in mice (Kim et al., 2008). This RGC type is also referred to as J-RGCs due to their high expression of the junctional adhesion molecule B (JAM-B) (Liu, 1995). They respond selectively to stimuli moving in a soma-to-dendrite direction (Sanes and Masland, 2015).

iv) Alpha RGCs

Alpha-RGCs (-RGCs) are present in all mammalian retinas and are a relatively abundant sub-group (~40%). These neurons have a well-defined morphology and can be identified by their large cell bodies, stout axons, wide and mono-stratified dendritic field and high levels of neurofilament H protein (NFH or SMI-32) (Baden et al., 2016; Bleckert et al., 2014; Krieger et al., 2017). Based on their light responses, they can be divided into: ON-sustained, OFF-sustained, and OFF-transient (Sanes and Masland, 2015). Using a transgenic mouse line that labels alpha RGCs in the live retina and using systematic electrophysiological recordings, a recent study identified a fourth alpha RGC type with ON-transient responses (Krieger et al., 2017). Because of their abundance and ease to characterize morphologically, the work presented in this thesis focused on -RGCs (see Chapter 3).

v) Intrinsically Photosensitive Melanopsin-Containing Retinal Ganglion Cells (ipRGCs):

ipRGCs are distinguished from other RGCs by their intrinsic sensitivity to light and large dendritic arbors (Sanes and Masland, 2015). Interestingly, ipRGCs express the photopigment melanopsin and contribute to the pupillary reflex and photoentrainment of circadian rhythms through their projections to the suprachiasmatic nucleus (SCN) (La Morgia et al., 2018).

vi) Local Edge Detectors (LED)

These cells have compact and extensively branched arbors. In mouse, these cells represent an estimated 13% of the total number of RGCs (Sanes and Masland, 2015). LED RGCs are sensitive to the size of the stimulus and are excited when their receptive field is precisely stimulated (Jacoby and Schwartz, 2017). Objects that exceed the size of the receptive field induce attenuation or full suppression of the LED RGC's spiking output (Jacoby and Schwartz, 2017; Sanes and Masland, 2015).

1.2. GLAUCOMA: THE LEADING CAUSE OF IRREVERSIBLE BLINDNESS WORLDWIDE

1.2.1. DEFINITION OF GLAUCOMA AND PATHOPHYSIOLOGY

Glaucoma is the leading cause of irreversible blindness worldwide (Weinreb et al., 2014; Wu et al., 2019) and comprises a heterogeneous group of diseases characterized by optic nerve damage and visual field loss (Jonas et al., 2017). A crucial element in the pathophysiology of glaucoma is the selective death of RGCs. High intraocular pressure

(IOP) is the most significant risk factor for developing glaucoma, but the mechanism by which ocular hypertension damages RGCs is currently unknown. Other risk factors have been reported such as age, family history of glaucoma, black race, and the use of systemic or topical corticosteroids (Weinreb et al., 2014).

For populations aged between 40-80 years, the prevalence of glaucoma is 3.5% (Tham et al., 2014). Worldwide, the total number of people aged 40-80 years with glaucoma was estimated to be 64.3 million in 2013, 76.0 million in 2020, and is projected to reach 111.8 million in 2040 (Tham et al., 2014). Symptoms of glaucoma appear late in its progression, resulting in a high number of affected individuals who are not aware that they have the disease. Population-level surveys suggest that only 10-50% of patients with glaucoma know they are affected (Tham et al., 2014). Glaucoma can be classified in two categories: primary open-angle glaucoma (POAG) and primary angle-closure glaucoma (PACG) (Weinreb et al., 2014), depending on whether the iridocorneal angle (site of intraocular fluid drainage) is structurally open or closed, respectively.

Optic nerve damage is caused by the loss of RGCs and their axons leading to progressive loss of vision (Allingham et al., 2005). Glaucoma progression includes five stages: (i) the initiating events, (ii) structural alterations, (iii) functional alterations, (iv) RGC and optic nerve damage, and (v) visual loss. The initiating events are a series of processes that may lead to stages 2-5: they precede any pathological or physiological alterations and can be pressure-related, genetic, toxic, or acquired susceptibility to apoptosis or RGC death. Structural alterations precede and eventually lead to changes in optic nerve function or aqueous humor dynamics. These can be pressure dependent or independent and are often related to alterations to the ganglion cells or optic nerve head (e.g. vascular or mechanical). Functional alterations are the physiological abnormalities that may lead

directly or indirectly to optic nerve damage, and can be pressure dependent (elevated intraocular pressure) or independent (e.g. reduced axonal conduction, vascular perfusion).

1.2.2. CURRENT THERAPIES FOR GLAUCOMA AND LIMITATIONS

At present, the main goal of treatments for glaucoma is to lower IOP in order to reduce disease progression (Jonas et al., 2017; McKinnon et al., 2008; Weinreb et al., 2014). It has been shown that IOP reduction lowers the risk of visual field progression in glaucoma (McKinnon et al., 2008). Depending on the stage of the disease, patients with glaucoma can be treated with medical eye drops, laser, or surgery. Interventions for glaucoma can be classified into pharmacological and non-pharmacological (surgical) treatments. In the sections below, current therapeutic approaches for glaucoma are explained and their limitations are highlighted.

Pharmacological therapies

The pressure within the eye is determined by the rate of production and drainage of an intraocular fluid known as the aqueous humor. In order to reduce IOP, pharmacological agents have been developed to either reduce aqueous humor production or increase its outflow. Four major classes of topical drugs are used for this purpose:

i) Prostaglandin analogues (PGAs): are the first-line of treatment because of their efficacy compared to other topical drugs. PGAs reduce IOP by enhancing aqueous humor drainage through the uveoscleral outflow pathway which represents 10% of aqueous

outflow (McKinnon et al., 2008). It has been shown that PGAs have a greater 24-hour IOP reduction than other hypotensive drugs (Stewart et al., 2008). The main side effects of the PGAs are local, such as conjunctival hyperemia, lengthening and darkening of eyelashes, brown discoloration of the iris, uveitis, and macular edema (Weinreb et al., 2014). The following hypotensive drugs are also used to lower the IOP primarily when the prostaglandin analogues cannot be used due to intolerance or contraindication.

ii) Beta ( )-blockers: these drugs were the most used class of IOP reducing drugs before the introduction of PGAs. β-Blockers bind to β-1 and/or β-2 adrenergic receptors in the ciliary body to reduce aqueous humor production (McKinnon et al., 2008). Side effects of these drugs include ocular irritation and dry eyes, which make them less commonly used. -blockers are contraindicated for patients with a history of chronic pulmonary obstructive disease, asthma, and bradycardia (Weinreb et al., 2014).

iii) Alpha ( )-adrenergic agonists: this class of hypotensive drug reduces the IOP principally by reducing aqueous humor production by the ciliary body and by concurrently increasing its outflow. This class of drugs may cause ocular irritation and dry eye syndrome in some patients. The main side effects are pulmonary arrest in young children, and it is contraindicated in patients with cerebral or coronary insufficiency, postural hypotension, and renal or hepatic failure (Weinreb et al., 2014).

iv) Carbonic anhydrase inhibitors: These agents reduce IOP by inhibiting the carbonic anhydrase enzyme, which is responsible for aqueous humor production by the ciliary

body. Topical application of these drugs can result in common side effects such as irritation, dry eye and a burning sensation, while oral administration can induce paresthesia, nausea, diarrhea, loss of appetite and taste, lassitude and renal stones (Weinreb et al., 2014).

v) Cholinergic agonists: These drugs were the first class of IOP-reducing drugs for the treatment of glaucoma and have been in use for over 100 years. They act by increasing aqueous humor outflow, but have a number of important local side effects that include ocular irritation, induced myopia, decreased vision due to ciliary spasm, miosis, follicular conjunctivitis, induced accommodation, retinal detachment or iritis (Gupta et al., 2008; Weinreb et al., 2014).

Surgical treatments

As explained above, topical or oral use of IOP-reducing drugs may provoke serious side effects and may be ineffective. In addition, some patients with severe glaucoma may require more effective and fast-acting interventions. To fill this gap, non-pharmacological treatments can be a valuable alternative. The main goal of surgical treatments for glaucoma is IOP reduction, and many different interventions are available.

i) Laser Trabeculoplasty:

Laser surgeries for glaucoma are considered non-invasive treatment options. They were first introduced to fill the gap between failed drug treatments and invasive surgeries. Laser trabeculoplasty uses a laser to remodel the trabecular meshwork (TM), the site of aqueous humor drainage, and improve fluid outflow (Abdelrahman, 2015). There are different types

of laser strategies including argon laser trabeculoplasty (ALT), selective laser trabeculoplasty (SLT) and micropulse laser trabeculoplasty (MLT). In ALT, the laser opens the TM to facilitate the aqueous humor outflow. SLT targets the pigmented cells of the TM and promotes macrophage recruitment to the outflow system, which in turn increases outflow by the release of chemical mediators (Abdelrahman, 2015). The TM contains cells that can migrate and repopulate the burned sites after ALT and SLT (Kelley et al., 2009). As an alternative, MTL can involve repetitive application of diode laser pulses minimizing this problem (Abdelrahman, 2015). Although relatively safe, trabeculoplasty can lead to complications including elevated IOP, inflammation, iritis, choroidal effusion, hyphema, macular edema, foveal burns, corneal edema, diffuse lamellar keratitis, and refractive shifts (hyperopic and myopic).

ii) Trabeculectomy:

Trabeculectomy is the gold standard to surgically lower IOP during glaucoma and is used when medication and laser surgery fail. Trabeculectomy consists of making a small hole between the anterior chamber and the subtenon space in the eye to allow the aqueous humor to bypass the TM. To complete the procedure, an opening is made in the corneoscleral tissue situated at the level of the TM which is then covered by a scleral flap to control the outflow rate (Bar-David and Blumenthal, 2018). One of the challenges of trabeculectomy is that tissue scarring forms along the opening between the anterior chamber and the outer space, which will close the opening and gradually decrease or block fluid outflow.

iii) Tube shunt surgery:

Aqueous shunts are small artificial silicone tubes that lower IOP by draining aqueous humor into the subconjunctival space. More specifically, these tubes drain the aqueous humor from the anterior chamber to a plate of variable size and shape placed in the sclera. This surgery is used in cases where the trabeculectomy is judged to have a high chance of failure (Bar-David and Blumenthal, 2018). However, complications include scar tissue forming around the device, excessive fluid loss (hypotony), clouding of the lens (cataract), infection, and hyphema.

In summary, a number of pharmacological and surgical treatments are currently used for glaucoma, but a large number of patients continue to lose vision despite receiving these therapies or have secondary complications. Therefore, current treatments for glaucoma are insufficient and novel neuroprotective and neuroregenerative approaches are urgently needed.

1.2.3. ANIMAL MODELS OF GLAUCOMA

In order to understand the pathophysiology of glaucoma and to find effective therapies for this disease, a number of animal models have been developed. The principal risk factor for developing glaucoma is high IOP, thus most animal models of glaucoma are based on IOP elevation in mice, rats, and primates. Here, we describe the most frequently used *in vivo* models of ocular hypertension glaucoma.

i) Microbead occlusion models

This model is based on the injection of microbeads into the anterior chamber of the eye to block the aqueous humor outflow pathway leading to gradual IOP elevation and RGC loss. This model has been developed for use in primates (Bar-David and Blumenthal, 2018), rabbits (Evangelho et al., 2019), rats and mice (Morgan and Tribble, 2015). In our laboratory, we developed a variation of this model in which magnetic microbeads are injected into the anterior chamber of mice and rapidly attracted to the iridocorneal angle using a magnet (Ito et al., 2016). One week after the procedure, IOP increases from 10 ± 0.6 mm Hg (mean ± S.E.M.), the average baseline IOP in control eyes, to 19 ± 0.5 mm Hg in microbead-injected eyes (Ito et al., 2016). IOP stabilizes thereafter and remains elevated at an average of 20 mm Hg for at least 6 weeks. RGC loss becomes significant at three weeks after magnetic microbead injection, with a substantial reduction of RGC soma (22%) and axons (25%) (Ito et al., 2016). Therefore, a feature of this model is that elevated IOP results in the gradual loss of RGCs after microbead injection, similar to neurodegenerative changes observed in human glaucoma. This model enables the examination of early changes that occur in this disease, prior to overt RGC soma and axon loss. Due to its reproducibility and available background information generated in our lab, this magnetic microbead glaucoma occlusion model is gaining popularity with users in the community and was used in the experiments described in this thesis.

ii) Laser photocoagulation models

Laser photocoagulation is a chronic model of ocular hypertension based on laser-induced decrease of aqueous humor outflow by TM scarring and destruction of the Schlemm's canal (Biswas and Wan, 2019). An argon laser is applied to the episcleral and limbal veins,

which drain aqueous humor, to induce photocoagulation and blockade of outflow pathways (Ji et al., 2005). This method results in significant elevation of IOP, with 50% elevation in treated eyes compared to controls. IOP elevation may last from 2 to 6 weeks (Ruiz-Ederra and Verkman, 2006) and induces 16%-27% RGCs loss (Ji et al., 2005; Ruiz-Ederra and Verkman, 2006). A major disadvantage of this model is that IOP fluctuates often returning to baseline soon after the procedure, thus requiring repeated laser applications that can cause damage and inflammation.

iii) DBA/2J pigmentary glaucoma model

The DBA/2J mouse strain develops spontaneously ocular hypertension with age. This phenotype results from mutations in two different genes (John, 2005): the tyrosine related protein (*Tyrp1*) and a transmembrane glycoprotein present in different cell structures (*Gpnmb).* The interaction between these two mutant genes leads to iris dispersion syndrome, an age-related degeneration of the iris leading to blockade of aqueous outflow pathways and ocular hypertension glaucoma (McKinnon et al., 2009). IOP elevation is observed between 6 to 16 months of age, with 8-9 months showing an important evolution of IOP and associated RGC and optic nerve pathology (Libby et al., 2005). The phenotype of DBA/2J mice is extremely variable, not only within a colony but also from facility to facility, therefore the high number of animals required to reach statistical significance makes it a prohibitive model system for most research laboratories.

1.3. DENDRITIC DAMAGE IN THE CNS

1.3.1. DENDRITES: DEFINITION, STRUCTURE, AND FUNCTION

Dendrites are highly specialized neuronal compartments, characterized by elaborate branching, responsible for receiving information via synaptic contacts (Ryglewski et al., 2014; McAllister, 2000). Dendrites determine how neurons integrate information within a neuronal network. Neurons in the human brain (~100 billion) establish around 100 trillion synapses onto a total of around 100,000 miles of dendritic cable (Ryglewski et al., 2014). The high ramification of dendrites provides an important surface for synaptic input and information processing in the CNS. Live imaging techniques have allowed the observation of dendritic development in real time. For example, the albino *Xenopus* tadpole is an organism that has been widely used to study morphological development. This organism is transparent at early stages of larval development allowing the study of dye-labeled neurons with longitudinal imaging over sustained periods of time. Imaging studies have helped distinguish three phases during dendritic development (Cline, 2001). First, newly differentiated neurons start extending their axons with little elaboration of the dendritic arbors (Phase I), a phase that lasts ~1 day. Second, neurons go into a rapid dendritic arbor growth where the dendrites undergo a rapid rate of branch additions and retractions for few days (Phase II). Third, neurons undergo slower dendritic arbor growth rate and establish more stable dendritic arbors and is accompanied by dendritic pruning of mistargeted branches (Phase III) (Figure 2) (Chiu and Cline, 2010; Cline, 2001).

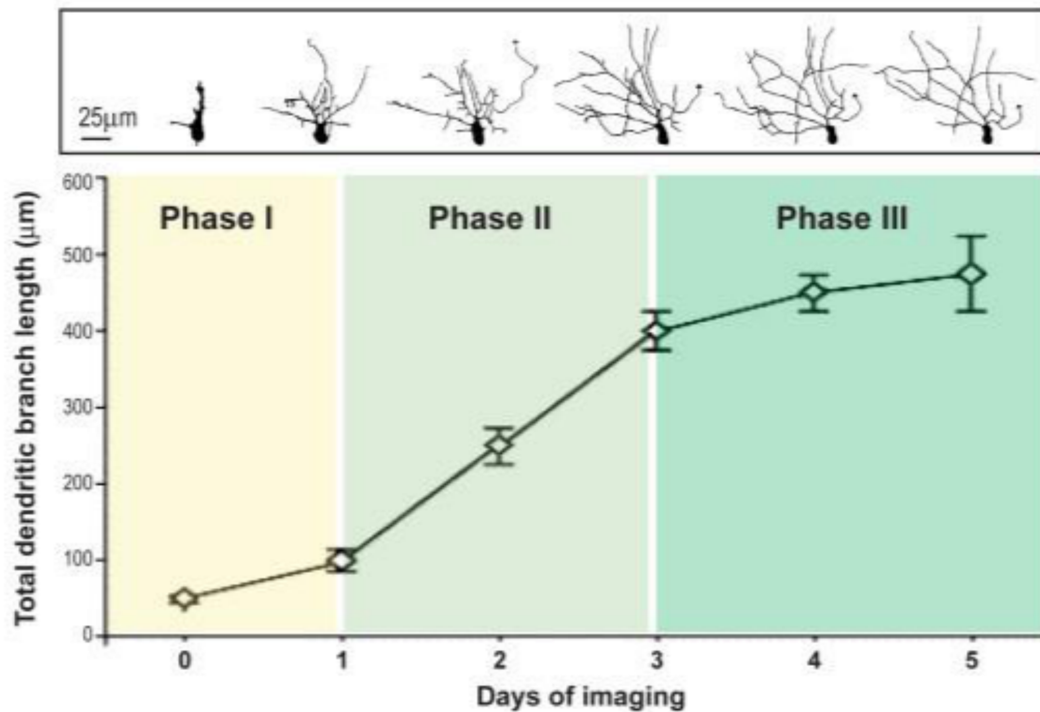

Figure 2. Dendritic arbor growth phases. Reproduced with permission from Chiu and Cline, 2010

i) Dendritic structure relative to axons

The structure of dendrites differs from that of axons in that they are shorter, with variable thickness depending on their distance from the soma, and often have non-uniform arborization (Jan and Jan, 2001). In axons, the microtubule orientation is uniform, with the plus ends oriented distally from the soma, while in dendrites the polarity of microtubules is mixed (Jan and Jan, 2001). Microtubule orientation determines the type and mechanisms of molecule and organelle transport within a cell, thus establishing differences between the cargoes in dendrites and axons. For example, electron microscopy studies have shown that some organelles, such as ribosomes, endoplasmic reticulum and Golgi complexes and some neurotransmitter receptors are present in

dendrites but not axons (Craig and Banker, 1994). Dendrites and axons differ in the presence of microtubule associated proteins: while axons contain tau protein, the dendrites contain microtubule-associated protein 2 (MAP2) (Kulkarni and Firestein, 2012). This structural difference reveals important functional differences between dendrites and axons. Of interest, while a large number of studies have focused on axonal regeneration (He and Jin, 2016; Li et al., 2017), the ability of mammalian neurons to regrow dendrites and reestablish functional synapses has been largely ignored and is a focus of the present thesis.

ii) Dendritic function

Dendrites determine where and how neurons receive and integrate information from afferent neurons. Dendrites receive inputs from excitatory as well as inhibitory synapses, thus they play important roles in regulating neuronal processing. Their morphology contributes to establishing a precise pattern of synaptic connections that are critical for normal brain function. Dendrites determine how presynaptic inputs are integrated within neurons and, ultimately, the behavior of a neuronal circuit (Chiu and Cline, 2010). Alterations in dendrite morphology including changes in dendritic structure or branching patterns and changes in spine morphology, contribute to many neurodevelopmental and age-related disorders such as schizophrenia, autism, and neurodegenerative diseases (Kulkarni and Firestein, 2012).

1.3.2. DENDRITIC PATHOLOGY IN NEURODENERATIVE DISEASES

Dendrites are dynamic during development, extending and retracting actively, and the final arbor morphology is achieved through a dynamic process of branch addition and elimination (Cohen-Cory and Lom, 2004; Wong and Ghosh, 2002). In contrast, dendrites are extremely stable in the adult brain and retina, but can change during normal aging (Dickstein et al., 2013) and in neurodegenerative diseases (Bobo-Jiménez et al., 2017; Dorostkar et al., 2015; Herms and Dorostkar, 2016). Because of this, it is important to better understand the features of dendritic changes related to age to distinguish them from pathological changes in disease. During normal aging, there are slight changes in the length of dendritic branches in specific brain regions, which can be compensated with an increase of neuronal dendritic area in adjacent laminae (Uylings and de Brabander, 2002). Dendritic spine and synaptic density changes are relatively small and appear to be uniform in the brain during normal aging (Dickstein et al., 2013), and occur primarily in regions involved in memory and learning (Uylings and de Brabander, 2002). Age-related changes are mild compared to pathological alterations seen in neurodegenerative diseases such as Alzheimer's disease (Uylings and de Brabander, 2002). Common pathological changes are dendritic arbor shrinkage, branch retraction, and loss of spines and synapses and have been reported in many neurodegenerative diseases (Liu et al., 2011), described in the next sections.

i) <u>Alzheimer's disease:</u>

There is substantial evidence that dendritic abnormalities and loss of synapses are prominent features of Alzheimer's disease (Cochran et al., 2014, D'Ambrosi et al., 2014).

Dendritic changes have been reported to occur in the early stages of the disease (Smith et al., 2009). Specifically, the following morphological abnormalities in dendrites have been observed: i) dystrophic neurites: brains of Alzheimer's disease patients show that neurites passing through amyloid beta plaques lose their straight normal shape (Knowles et al., 1999), ii) reduction of dendritic complexity has been reported in dentate granule cells and in the pyramidal neurons in hippocampal area CA1 and subiculum in Alzheimer's disease patients (Cochran et al., 2014), and iii) dendritic spine abnormalities and loss (Herms and Dorostkar, 2016). Indeed, two photon microscopy applied to a mouse model of Alzheimer's disease showed a correlation between amyloid plaque growth and a reduction in dendritic spine density (Bittner et al., 2012).

ii) Parkinson's disease:

Parkinson's disease is a neurodegenerative motor disorder caused by the loss of nigrostriatal dopaminergic neurons. Neurons of the substantia nigra show a substantial decrease in spines and dendritic length in postmortem brain tissue of Parkinson's disease patients compared to controls (Patti et al.1991). Moreover, rodent and monkey models of Parkinson's disease showed dendritic spine loss in nigrostriatal dopaminergic neurons (Villalba and Smith, 2018). Nonetheless, a correlation between dendritic changes and the appearance of motor symptoms in Parkinson's disease has not yet been demonstrated. Nevertheless, an association between dendritic spine loss and nigrostriatal dopaminergic denervation was reported in the MPTP-mouse model of Parkinson's disease (Villalba et al., 2009).

iii) <u>Huntington's disease:</u>

Huntington's disease is a neurodegenerative disease caused by the loss of striatal projection neurons and characterized by involuntary and uncoordinated movements as well as rigidity and abnormal posture (Vonsattel et al., 1985). Studies using a transgenic mouse model of Huntington's disease demonstrated spine loss in late symptomatic stages but not in earlier stages (Herms and Dorostkar, 2016). Similarly, analysis of striatal neurons from post mortem tissues of Huntington's disease patients showed an increase of dendritic branches and spine density in the early stages of the disease followed by dendritic pruning and decrease of spine density in later stages (Herms and Dorostkar, 2016).

<u>iv) Amyotrophic lateral sclerosis (ALS)</u>:

Amyotrophic lateral sclerosis affects motor neurons in the motor cortex and the spinal cord causing muscular rigidity, progressive weakness and death at 3-5 years after diagnosis (Wijesekera and Leigh, 2009). Postmortem tissues from ALS patients, affected by the familial or sporadic forms, as well as fronto-temporal dementia-ALS (FTD-ALS) showed abnormalities in dendrites and dendritic spines of the upper motor neurons (Genç et al., 2017). Dendritic changes correlated with motor and cognitive symptoms in ALS and were disease stage-dependent (Fogarty et al., 2017). A transgenic mouse model of ALS (SOD1 mice) revealed pre-symptomatic dendritic changes characterized by process retraction and alterations in spine density (Fogarty et al., 2017).

1.3.3. DENDRITIC PATHOLOGY IN GLAUCOMA

A number of studies have investigated RGC dendrite pathology induced by glaucomatous injury, notably ocular hypertension (Chen et al., 2015; Cochran et al., 2014; Della Santina et al., 2013; El-Danaf and Huberman, 2015; Frankfort et al., 2013; Fu et al., 2009; Heijden et al., 2016; Kalesnykas et al., 2012; Li et al., 2011; Ou et al., 2016; Pang et al., 2015; Park et al., 2014; Ward et al., 2014; Weitlauf et al., 2014; Williams et al., 2013b). Williams et al. examined whether chronic exposure to high IOP led to morphological dendrite alterations in DBA/2J mice. The authors reported a reduction in arbor field area and complexity when RGCs and their dendrites were visualized by DiOlistic labeling (Williams et al., 2013b). More recent studies have examined the differential vulnerability of RGC subtypes relative to dendritic degeneration and the timing of functional deficits in ocular hypertension models. Feng and colleagues (2013) used laser photocoagulation to elevate IOP in Thy1-YFP mice and showed that ON cells display a decrease in dendritic field area while there were no significant changes in ON-OFF cells (Feng et al., 2013). A study by Della Santina and colleagues (2013) reported that the light response of three RGC subtypes (ON sustained, OFF sustained and OFF transient) was reduced at 2 weeks after microbead injection coinciding with the onset of dendritic changes. The decrease in the electrophysiological response of RGCs concomitant with arbor shrinkage (Della Santina et al., 2013) correlated with loss of synaptic components in the IPL (Della Santina et al., 2013; Fu et al., 2009; Park et al., 2014; Ward et al., 2014; Weitlauf et al., 2014).

These studies suggest that synaptic changes precede morphological alterations, notably dendritic retraction, and that synaptic dysfunction is among the earliest signs of RGC damages in glaucoma. The microbead occlusion model was also used to induce

ocular hypertension in transgenic mice that express GFP in selective RGC subtypes to investigate dendritic changes (Della Santina et al., 2013; El-Danaf and Huberman, 2015). The authors reported that neurons that stratified in the OFF sublamina were the first to undergo dendritic retraction, whereas dendrites within the ON sublamina exhibited no change. These findings suggest that RGCs with dendritic stratification in the OFF sublamina are particularly vulnerable to glaucomatous damage (Della Santina et al., 2013; El-Danaf and Huberman, 2015).

1.4. MOLECULAR PATHWAYS THAT REGULATE DENDRITIC DYNAMICS AND REPAIR

1.4.1. ROLE OF GROWTH FACTORS

Growth factors play a key role in dendritic growth, branching and stability as well as synaptic plasticity (Arikkath, 2012; Dijkhuizen and Ghosh, 2005; Poon et al., 2013). Neurotrophins are a family of neurotrophic factors that includes four members: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophins 3 and 4 (NT-3 and NT-4). These factors mediate their action by binding to the Trk family of receptors and the p75 neurotrophin receptor (p75NTR) (Arikkath, 2012). Among these factors, BDNF has been the most studied with a demonstrated role in the regulation of dendritic dynamics. For example, BDNF knock-out animals display a reduced number of primary dendrites and secondary branches (Gorski et al., 2003). Similarly, dendritic retraction has been observed in mice mutant for the BDNF receptor TrkB (Xu et al., 2000). Furthermore, BDNF application increases the number of dendrites in pyramidal neurons in a distance-dependent manner (Horch and Katz, 2002). Application of BDNF or nerve

growth factors to neurons in culture induces expression of the microtubule-stabilizing proteins MAP1A and MAP2 (Huber and Matus, 1984; Szebenyi et al., 2005). Seemingly, the effect of BDNF on dendritic development is activity-dependent (Dijkhuizen and Ghosh, 2005). In addition, BDNF also induces expression of important elements for dendrite growth and stability such as cypin and guanine deaminase (Koleske, 2013), suggesting that BDNF is required for dendritic dynamic and stabilization (Figure 3).

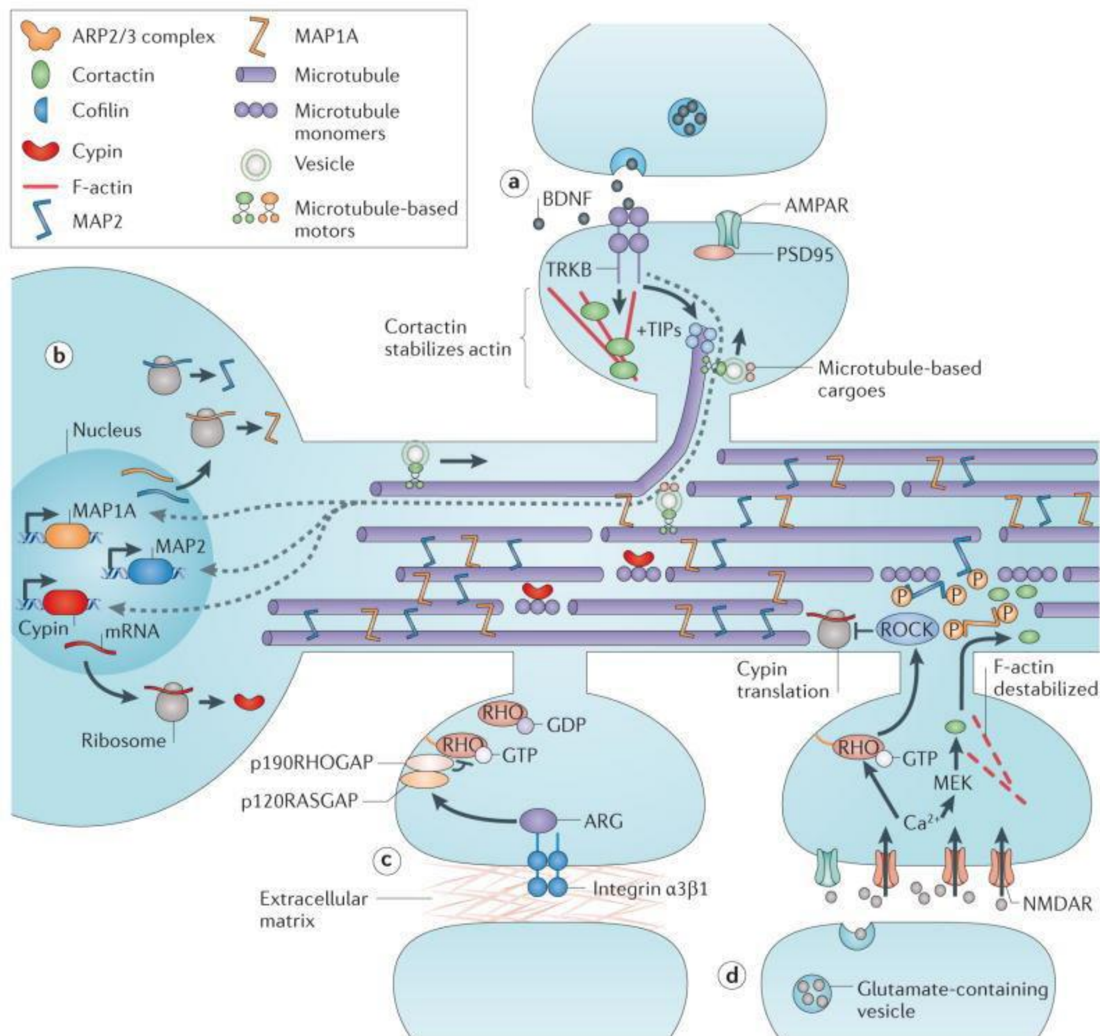

Figure 3. Role of BDNF in the crosstalk between dendritic spines and dendritic arbors and their stabilization.

BDNF, by binding to TrkB, target the assembly of microtubule containing plus end tip-binding proteins (+TIPs) to spines which allow transport of cargoes that are important for spine structure stabilization (a). BDNF increases the expression of proteins that are implicated in microtubule stabilization in dendrites: microtubule-associated protein 1A (MAP1A), MAP2 and cyptin (b). Integrin α3β1 binds to the receptor tyrosine kinase ARG, activates RHO GTPase-activating protein A (p190RHOGAP) and attenuates RHO activity in order to preserve dendritic structure (c). AMPA receptor (AMPAR) and NMDA receptor (NMDAR) activity activate in Ca^{2+} influx which in turn activate RHO–RHO-associated protein kinase (ROCK) signaling which phosphorylates MAPs leading to their dissociation from microtubules. ROCK promotes inhibition of local cyptin translation which results in spines destabilization (d). Reproduced with permission from Koleske, 2013

1.4.2. ROLE OF INSULIN SIGNALING

Insulin is transported across the blood brain barrier (BBB) from the periphery and can also be produced locally within the CNS (Banks et al., 2012; Kuwabara et al., 2011). Insulin signaling in the brain controls many functions including food intake, body weight homeostasis, cognitive and developmental functions, as well as learning and memory. Insulin is also involved in neuronal circuit formation, synaptic maintenance, neuronal survival and dendritic arborization (Lee et al., 2016). Insulin binds to two isoforms of the insulin receptor (IR-A and IR-B), which are present in the retina and brain and are abundantly expressed by RGCs (Bu et al., 2013; Pomytkin et al., 2018).

Pathways activated by insulin binding to its receptor such as Ras/MAPK and PI3K/Akt/ mTOR pathways have been implicated in the modulation of dendritic structure and dynamics during development (Chiu and Cline, 2010). Dendritic spines are actin-rich postsynaptic structures that are important in the regulation of the synaptic structure, function and plasticity (Nimchinsky et al., 2002). Rac1 and Cdc42 Rho family of small GTPases are importantly implicated in dendritic and spine morphogenesis regulation (Luo et al., 1996; Scott et al., 2003). IRSp53 is a novel insulin receptor substrate enriched in the postsynaptic densities in the brain (Abbott et al., 1999). IRSp53 contains various domains for protein-protein interaction including an N-terminal Rac binding (RCB) domain, a partial Cdc42 and Rac1-iteractive binding (CRIB) motif. It has been proposed that IRSp53 interacts with Cdc42 and Rac1 to regulate dendritic and spine morphogenesis (Choi et al., 2005). Also, IRSp53 mutations produce a significant loss of synaptic puncta in cultured neurons, and overexpression of IRSp53 increases the density of dendritic spines (Choi et al., 2005). Moreover, transgenic mice lacking IGF-1, a potential ligand for insulin receptor, showed a significant reduction in dendritic arbor length and complexity as well as spine density in pyramidal neurons (Cheng et al., 2003).

Abnormal insulin signaling, even in the absence of diabetes, has been associated with neurodegeneration in diseases characterized by dendritic pathology, notably Alzheimer’s and Parkinson’s disease as well as glaucoma (Athauda and Foltynie, 2016; Song *et al.*, 2016; Bloom *et al.*, 2017). It has been suggested that insulin signaling may be potentially targeted for disease modification. Indeed, intranasal insulin administration has been reported to improve memory and attention in patients with Alzheimer’s disease (Freiherr et al., 2013). A recent study from our laboratory demonstrated that human recombinant insulin, administered as eye drops or systemically after dendritic arbor

shrinkage and prior to cell loss, promoted robust regeneration of RGC dendrites and successful reconnection with pre-synaptic targets after optic nerve axotomy (Agostinone et al., 2018). Further, this work showed that insulin-mediated regeneration of excitatory postsynaptic sites on retinal ganglion cell dendritic processes increased neuronal survival and rescued light-triggered retinal responses (Agostinone et al., 2018). The remarkable dendrite regeneration observed with insulin after optic nerve axotomy forms the basis for studies in this thesis to investigate the role of insulin as pro-regenerative strategy in a mouse glaucoma model.

1.4.2.1. THE MAMMALIAN TARGET OF RAPAMYCIN (mTOR)

Insulin binding to its receptor activates phosphoinositide-3' kinase (PI3K) and its target Akt leading to potent activation of the mammalian target of rapamycin (mTOR) complexes 1 and 2 (mTORC1 and mTORC2) (Saxton and Sabatini, 2017). The mammalian target of rapamycin (mTOR) regulates fundamental cell processes in coordination with the environment (Saxton and Sabatini, 2017). mTOR is a 289 KDa serine/threonine protein kinase that was first identified in yeast as a target for rapamycin, an anti-fungal and immunosuppressant macrolide molecule (Takei and Nawa, 2014). The mTOR pathway plays a key role in the regulation of cell growth and coordination of nutrient availability and metabolic activity within the cell (Sabatini, 2017). mTOR also plays a major role in protein translation, autophagy, transcription, and lipid synthesis among other functions (Takei and Nawa, 2014). mTOR can form two distinct complexes: mTORC1, composed of mTOR, regulatory-associated protein of mTOR (Raptor) and mLST8, and mTORC2, containing the rapamycin-insensitive companion of mTOR (Rictor), mLST8 and mammalian stress-

activated protein kinase interacting protein (mSIN1) (Gao et al., 2012) (Figure 4). mTORC1 controls cell growth, metabolism and cell cycle, while mTORC2 controls cell survival and cytoskeleton organization (Takei and Nawa, 2014).

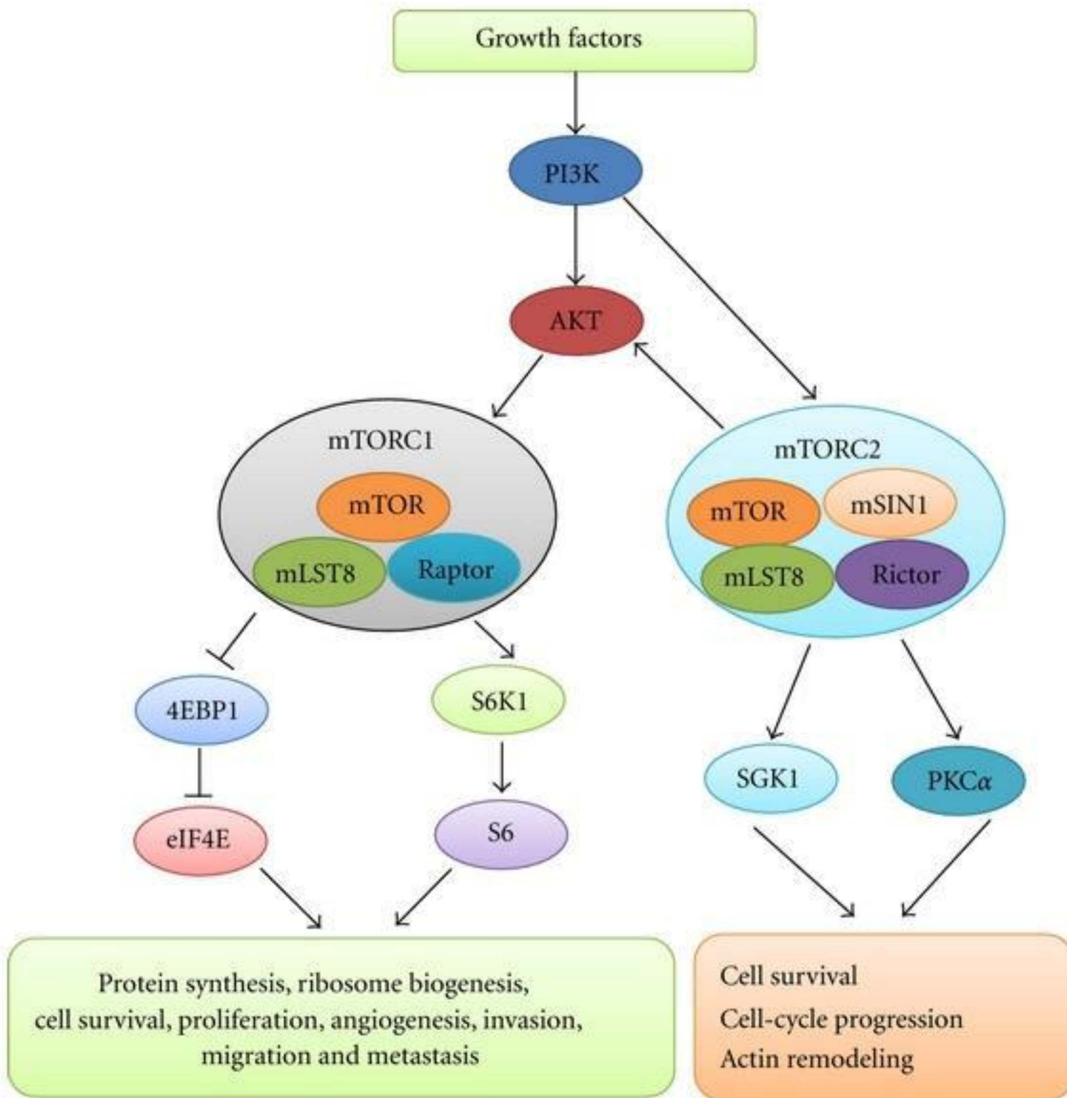

Figure 4. Schematic presentation of the mTOR signaling pathway. Reproduced with permission from Gao et al., 2012.

Dysregulation of the mTOR signaling pathway has been reported in an increasing number of pathological conditions such as cancer, diabetes and neurodegenerative diseases (Laplante and Sabatini, 2012). Several studies have demonstrated the role of mTOR in cancer pathogenesis. Many effectors of the mTOR pathway are mutated in human cancer cells (Laplante and Sabatini, 2012), and protein translation PI3K-Akt-mTOR-dependent is necessary for tumorigenesis (Hsieh et al., 2010). In addition, many familial cancer syndromes are caused from mutations in upstream genes of the mTOR complexes including Tsc1/2, Lkb1, Pten and Nf1 (Laplante and Sabatini, 2012), and downstream effectors of mTORC1, 4E-BP1, eIF4E and S6K1 are associated with many types of cancer. Indeed, the protein translation regulator eIF4E is considered to be an oncogene because it is overexpressed in many human cancers (Showkat et al., 2014). mTOR signaling pathway is targeted during cancer treatments, mainly by inhibiting mTORC1, using rapamycin or mimetic compounds known as rapalogs, or with catalytic mTOR inhibitors (Showkat et al., 2014). On the other hand, increasing evidence indicates that dysregulation of mTOR signaling is implicated in aging and age-related diseases. For example, post-mortem tissues of Alzheimer's disease patients showed high levels of activated mTOR on Ser-2448 and Ser-2481 as well as p70S6K and eIF4E and other downstream targets of mTOR in the hippocampus and other brain regions (Perluigi et al., 2015). Work from our laboratory has convincingly demonstrated that mTOR activity in RGCs is dramatically downregulated after axotomy (Agostinone et al., 2018; Morquette et al., 2015) and glaucomatous damage (Belforte et al. unpublished). Previous work from our team showed that mTORC1 activity was required for the maintenance of RGC dendritic arbors (Morquette et al., 2015) putting forward the hypothesis that activation of

mTOR complexes might be a good strategy to promote RGC dendrite regeneration after injury.

1.4.2.2. ROLE OF mTOR ON DENDRITE REGENERATION

Our recent work supports the finding that injured CNS neurons are endowed with an intrinsic ability to regrow dendrites and can readily reestablish functional dendritic arbors upon insulin signaling (Agostinone et al., 2018). Furthermore, we showed that insulin-dependent activation of both mTORC1 and mTORC2 was required for dendrite regeneration. Of interest, these complexes cooperated in an additive manner to ensure successful restoration of dendritic length and arbor complexity. Intriguingly, loss-of-function experiments revealed that mTORC1 and mTORC2 do not control the same aspects of dendrite regeneration. Indeed, mTORC1 was required for branching and the restoration of arbor complexity, while mTORC2 regulated process extension and the reestablishment of arbor area (Agostinone et al., 2018).

Previous work has investigated the role of the mTOR pathway on axonal regeneration. For example, deletion of phosphatase and tensin homolog (PTEN), the negative regulator of PI3K upstream of mTOR, enhances axonal regeneration in rodent RGCs and corticospinal neurons, as well as sensory neurons in flies (Liu et al., 2010; Park et al., 2008; Song et al., 2012). Mutations in PTEN also stimulate dendrite regrowth after dendrotomy in Drosophila sensory neurons (Song et al., 2012). These findings support a key role of mTOR signaling in both axon and dendrite regeneration after injury. However, there are also important differences in the intrinsic mechanisms used for dendrite versus axonal regeneration in adult RGCs. First, successful dendrite regeneration depends on

both mTORC1 and mTORC2 alone is not enough for RGC axons to reach their brain targets and reestablish functional synapses (Lim et al., 2016; Sun et al., 2011). Importantly, the effectors downstream of mTORC1 and mTORC2 promoting dendritic regrowth are unknown and this knowledge gap will be addressed in this thesis and subsequent work.

1.4.3. THE NOTCH PATHWAY

It is possible that in addition to mTOR, other signaling pathways promote or contribute to RGC dendrite regeneration downstream of insulin. The Notch pathway is involved in cell proliferation, differentiation, and cell death (Kopan, 2012) and regulates these functions through cell-cell interactions during development and in the adult CNS (Lasky and Wu, 2005; Lathia et al., 2008). Notch is a membrane protein located at the cell surface. There are four paralogs of the Notch gene (Notch 1-4) and five ligands (jagged 1 and 2 and Delta 1-3) (Lathia et al., 2008). The Notch transmembrane receptor in the signal-receiving cell interacts extracellularly with the Notch transmembrane ligand on the signal-sending cell. Their interaction initiates the proteolytic cleavage of the receptor and the release of the Notch intracellular domain (NICD), which translocate to the nucleus and initiates the transcription of Notch target genes (Figure 5) (Andersson et al., 2011). Notch has also been shown to control neurogenesis as well as axonal and dendritic growth (Lathia et al., 2008). During development, Notch signaling maintains the proliferating status of the cells, while a protein called Numb, a notch pathway antagonist, promotes neuronal differentiation (Li et al., 2003). In addition, Notch deficient mice show important developmental abnormalities including impaired long-term potentiation (LTP) at

hippocampal CA1 synapses. Moreover, LTP is enhanced by Notch ligands in normal mice, and depressed in Notch antisense transgenic mice (Wang et al., 2004). Presinilin1 knockout mice, in which the Notch pathway is downregulated, exhibit developmental abnormalities (Handler et al., 2000).

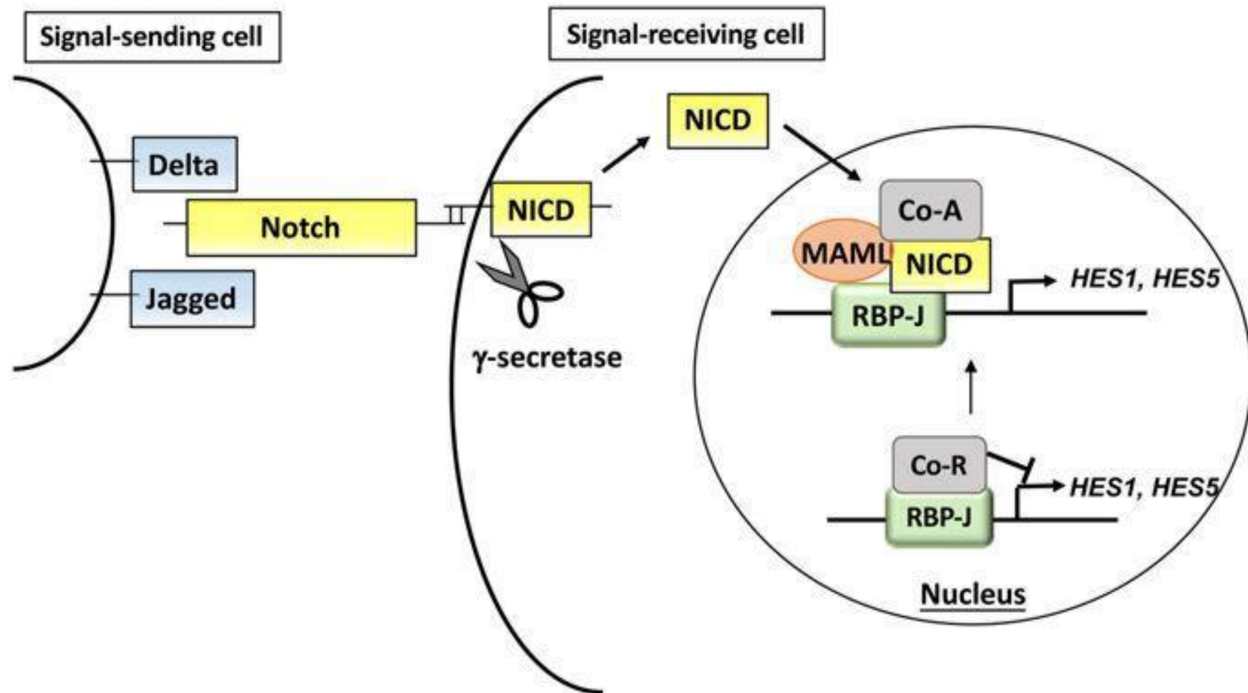

Figure 5. Schematic presentation of the Notch signaling pathway. Reproduced with permission from Andersson et al., 2011.

Notch dysfunction has been associated with neurodegeneration, particularly in Alzheimer's disease (Kopan and Goate, 2000; Lasky and Wu, 2005; Woo et al., 2009). A reduction in Notch activity and the Notch ligand Jagged1 has been reported in hippocampal and cortical neurons from Alzheimer's disease patients (Brai et al., 2016). Targeted loss of Jagged1 in adult mouse brain causes spatial memory loss (Marathe et

al., 2017). Together, these studies suggest a role for Notch in memory formation and loss through dysregulation of Notch signaling.

In the visual system, a recent study demonstrated that Notch has a neuroprotective role on injured RGCs in a rat model of ocular hypertension (Li et al., 2018). Indeed, RGC apoptosis was significantly increased when the Notch pathway inhibitor (DAPT) was administered, suggesting a pro-survival effect of Notch (Li et al., 2018). Furthermore, Notch pathway inhibition significantly increased the levels of pro-apoptotic proteins (Bax, caspase-3) while decreasing the expression of anti-apoptotic factors (Bcl2) (Li et al., 2018a).

1.5. CENTRAL HYPOTHESIS AND OBJECTIVES OF THE STUDY

We recently showed that insulin promotes RGC dendrite regeneration following optic nerve axotomy (Agostinone et al., 2018). However, whether insulin can induce dendrite regrowth during ocular hypertension damage and the mechanisms involved are currently unknown. The **central hypothesis** of this thesis is that insulin is a robust pro-regenerative strategy for the restoration of RGC dendritic structure in glaucoma. The primary objectives of this study are:

1) To determine whether administration of daily insulin eyes drop can induce RGC dendrite regeneration, after process retraction, in a mouse model of ocular hypertension glaucoma (magnetic microbead occlusion).
2) To assess whether IOP lowering is sufficient to stimulate RGC dendrite regeneration.

3) To develop a FACS-based protocol to isolate adult RGCs from glaucomatous and non-injured retinas.
4) To identify differentially expressed gene pathways in insulin-treated versus control retinas using RNAseq.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

All animal procedures were approved by the Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM) Animal Care Committee and followed the ARRIVE and the Canadian Council on Animal Care guidelines. Ocular hypertension induction procedures were carried out in B6.Cg.Tg[Thy1-YFPH]2Jrs/J mice (Jackson Laboratory) or wild-type littermate controls (3–4 months of age) maintained under 12-h light/12-h dark cyclic light conditions with an average in-cage illumination level of 10 lx. All experiments were performed under general anesthesia using 2% isoflurane (0.8 l/min). The number of animals used in each experiment is indicated in the table and in the figure legends. Data analysis was always carried out blinded by third party concealment of treatment using uniquely coded samples.

2.2 MAGNETIC MICROBEAD OCCLUSION AND BRINZOLAMIDE TREATMENT

Unilateral elevation of IOP was induced by a single injection of magnetic microbeads into the anterior chamber of the eye as described by us (Ito et al., 2016). Briefly, animals were anesthetized and a drop of tropicamide was applied on the cornea to induce pupil dilation (Mydriacyl, Alcon, Mississauga, ON). A custom-made sharpened microneedle attached to a microsyringe pump (World Precision Instruments, Sarasota, FL) was loaded with 1.5 µl of a homogenized magnetic microbead solution (diameter: 4.5 µm, 2.4 x 10^6 beads) (Dynabeads M-450 Epoxy, Thermo Fisher Scientific, Waltham, MA). Using a micromanipulator, the tip of the microneedle was used to gently puncture the cornea and

the microbeads were injected into the anterior chamber. A hand-held magnet was used to immediately attract the magnetic microbeads to the iridocorneal angle. This procedure avoided injury to ocular structures such as the lens and iris. An antibiotic eye drop was applied to the operated eye (Alcon) and the animal was allowed to recover on a heat pad. IOP was measured before and after the procedure in awake animals using a calibrated TonoLab rebound tonometer (Icare, Vantaa, Finland). A drop of proparacaine hydrochloride (0.5%, Alcon) was applied to the cornea and, holding the tonometer perpendicular to the eye surface, ten consecutive intraocular pressure readings per eye were taken and averaged (Ito et al. 2016). To reduce IOP, the carbonic anhydrase inhibitor brinzolamide (1% w/v, Alcon) was administered as two eye-drops per day: the first at 9 am and the second at 6 pm.

2.3 INSULIN TREATMENT

Human recombinant insulin diluted in sterile, endotoxin free PBS (15–30 U/kg/day, Sigma-Aldrich; or Humulin-R U100, Eli Lilly) was administered as daily eye drops (5 µl drop). Only the injured eye was treated with insulin or vehicle eye drops. Control animals received daily eye drops of vehicle. No allergic response, inflammation, or side-effects were detected.

2.4 RGC DENDRITIC ARBOR ANALYSIS

Dendritic arbour reconstruction and measurements were performed blinded to manipulations. High-resolution images of yellow fluorescent protein (YFP)-labelled RGC dendrites were acquired using a confocal microscope (Leica Microsystems Inc.). Scans

were taken at 0.5 μm intervals (1024 × 1024 pixels) with an average of three to five images per focal plane. Reconstruction of dendritic trees was carried out using the computer-aided filament tracing function of Imaris (Bitplane). The following parameters were measured: (i) total dendritic length: the sum of the lengths of all dendrites per neuron, (ii) total dendritic field area: the area within the contour of the arbour created by drawing a line connecting the outermost tips of the distal dendrites, (iii) total number of branches: the sum of all dendritic branches per neuron, and (iv) Sholl analysis: the number of dendrites that cross concentric circles at increasing distances (10-μm interval) from the soma. RGCs located in all retinal quadrants and eccentricities were included in our analysis.

2.5 FLUORESCENCE ACTIVATED CELL SORTING (FACS)

Mice were euthanized and eyes were immediately enucleated and placed in ice-cold HBSS (StemCell Technologies). Retinas were dissected out, placed in a dispase (5 U/ml) (Stemcell Technologies) and DNase I (2000 U/ml) (Worthington Biochemical) solution, and cut into small pieces. For cell dissociation, retinal pieces were incubated in the same dispase/DNaseI solution for 20-25 min in an Eppendorf thermomixer with shaking at 350 rpm, followed by addition of a blocking solution (HBSS 2% BSA). Cells were then incubated with the following primary antibodies: the RGC marker Thy1.2 (0.25 μg/μl, BD-Optibuild), glial fibrillary acidic protein (GFAP, 1μg/μl, Invitrogen), the leukocyte marker CD45.2 (0.2 μg/μl, BD pharminogen), the endothelial cells marker CD34 (0.2 μg/μl, Biolegend), the macrophages and dendritic cells marker CD11b (0.2 μg/μl, Biolegend) Cd11c (0.2 μg/μl, BD Biosciences). To eliminate dead cells, propidium iodide was added

before cell analysis and cytometry. Forward and sideward scatters (FSC-A and SSC-A) were used to identify cell relevant events and help to present cell populations depending on their complexity (SSC) and size (FSC). Doublet cells, dead, GFAP+, CD45.2+, CD34+, CD11b+ and CD11c+ cells were discarded. Only Thy1.2^{+}GFAP^{-}CD45.2^{-}CD34^{-}CD11b^{-} CD11c^{-} were selected for sorting. Sorted cells were collected directly into RLT buffer, vortexed, and stored at -80 C.

2.6 RNA EXTRACTION AND SEQUENCING

FAC-sorted cells were thawed on ice, homogenized by vortexing, transferred to QIAshredder spin columns, and centrifuged at maximum speed for 2 min. Total mRNA was extracted using RNeasy Plus micro kit, further purified using gDNA eliminator spin columns, and collected for further analysis. RNA quality was assessed using an Agilent 2100 Bioanalyzer and an RNA Pico chip assay. A polyA selection was done using oligo dT beads (ThermoFisher Scientific), and libraries were created using KAPA RNA HyperPrep (Roche). Quality control was performed using an Agilent 2100 Bioanalyzer and a DNA High Sensitivity chip assay. Libraries were quantified by qPCR and sequenced on a NextSeq 500 (Illumina) with a75 cycles High Output v2.5 flowcel.

2.7 RNAseq DATA ANALYSIS

i) RNA-seq data pre-processing: Sequences were trimmed for sequencing adapters and low quality 3' bases using Trimmomatic version 0.35 and aligned to the reference mouse genome version GRCm38 (or mm10, gene annotation from Gencode version M13, based on Ensembl 88) using STAR version 2.5.1b. Gene expressions were obtained both as read

counts directly from STAR as well as computed using RSEM. Raw count was normalized and transformed to logCPM values using functions implemented in Bioconductor's edgeR and limma.

ii) Differential gene expression analysis: Linear modeling analysis from the Bioconductor's *limma* package was used to identify differentially expressed gene. For each contrast, the fold change, the t test statistic and its associated nominal p values were obtained for each transcript. P-values adjustment for multiple testing hypotheses was performed according to the method of Benjamin and Hochberg, which controls the false discovery rate (Benjamini and Hochberg, 1995). Data are presented with adjusted p values cutoffs of 0.05 or nominal (non-adjusted) p values cutoffs of 0.05 in case no gene reaches an adjusted p value significance cutoff.

iii) Gene set enrichment analysis: Gene set enrichment analysis (GSEA) was performed using the Bioconductor's package ***fgsea***. We used this method to determine whether a known biological pathway or sets of individual genes were significantly enriched among the genes ranked by their statistical estimates (here the moderated *t* test) obtained from the differential gene expression analysis step. We systematically tested gene sets from the Molecular Signature Database (MsigDB, http://www.broad.mit.edu/gsea/msigdb), Hallmark collection, h.all.v5.0.symbols.gmt), C2 (C2.all.v6.1.symbols.gmt), and C5 (C5.all.v6.2.symbols.gmt) collections. For each contrast, the enrichment score (ES) and its associated p value along with the leading-edge gene's subset are the primary output from GSEA. The parameter of interest to be reported is the normalized enrichment scores (NES) and its associated multiple testing adjusted p value, used to compare enrichment analysis results across gene sets tested.

iv) Enrichment map analysis: Enrichment map analysis, a network-based method for

Gene-Set Enrichment Visualization and Interpretation, was used to organize significant genes set from C5 and C2 into a network or a cluster of gene sets. For every pair of gene sets, a similarity score was computed based on the number of genes they share in common. If the similarity score, as measured by the Jaccard coefficient, passes a predefined threshold (0.5), then an edge will be created between the gene set nodes (Merico et al., 2010). Using this approach, mutually overlapping gene-sets cluster together, facilitating interpretation and functional annotation.

v) Ingenuity Pathway Analysis (IPA): Lists of significantly differentially expressed genes (DEGs) were generated using the following parameter: normalized fold change (log2Ratio >= 2) and p-adj <= 0.05. Lists of the DEGs were then uploaded to IPA mapped to the Mus musculus gene symbols and overlaid onto the Canonical pathways contained in the IPA Knowledge Base.

2.8 STATISTICAL ANALYSIS

Data analysis and statistics were performed using GraphPad Instat software (GraphPad Software Inc., San Diego, CA) by a one-way analysis of variance (ANOVA) followed by a Bonferroni or Tukey *post hoc* tests, or by a Student’s *t*-test as indicated in the legends.

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CHAPTER 3

RESULTS

3.1. INSULIN EYE DROPS PROMOTE ROBUST RGC DENDRITE REGENERATION IN GLAUCOMA

Ocular hypertension is the main risk factor for developing glaucoma (Coleman and Kodjebacheva, 2009) and dendritic retraction is one of the earliest pathological changes in this disease (Agostinone and Di Polo, 2015; Williams et al., 2013). We previously demonstrated that insulin, applied after axotomy-induced dendritic retraction, promotes RGC dendrite regeneration (Agostinone et al., 2018). Based on this, we asked whether insulin could also promote RGC dendritic regrowth in glaucoma. To test this hypothesis, we induced ocular hypertension in transgenic mice expressing YFP under control of the Thy1 promoter (Thy1-YFPH) by intracameral injection of magnetic microbeads (Ito et al., 2016) (Figure 6d). The sparse YFP labeling in the Thy1-YFPH transgenic line allows visualization of RGC dendrites without overlap of arbors from neighboring neurons. Anatomical and functional studies have identified >40 RGC subtypes (Langer et al., 2018; Tran et al., 2019), all of which are present in the Thy1-YFPH retina. Examination of RGCs in different strains and injury models suggest that among the RGC subtypes, alpha RGCs (RGCs) are the most vulnerable (Daniel et al., 2018; Della Santina and Ou, 2017). Hence, our study focused on RGCs, visualized with an antibody against the non-phosphorylated neurofilament heavy chain (NF-H or SMI-32) (Krieger et al., 2017), which feature strongly labeled somata and large dendritic arbors (Baden et al., 2016b; Bleckert et al., 2014). YFP-positive RGCs co-expressing NF-H were selected for confocal imaging and 3D reconstruction (Figure 6A-C). After two weeks of glaucoma induction, prior to RGC axon or soma loss, we observed significant dendrite retraction relative to sham-operated controls (Figure 6F-G). Analysis of total dendritic length and arbor area demonstrated a

reduction of 24.4% and 26.52%, respectively, compared to control RGCs (Fig. 6J, K, ANOVA p<0.001).

Based on the marked injury-induced dendritic retraction observed at 2 weeks after glaucoma induction, we initiated eye drop administration of human recombinant insulin or vehicle (PBS) at this time point and analyzed dendritic length, area, and complexity one week later (3 weeks after microbead injection) (Figure 6E). Insulin-treated glaucomatous retinas displayed longer dendrites and considerably more branches than vehicle-treated controls (Figure 6H, I). Quantitative analysis confirmed that insulin restored all dendritic parameters examined: length, area, number of branches and complexity, to levels similar to those found in non-injured controls (Figure 6J-M). Importantly, the regenerative effect of insulin was not caused by a reduction of eye pressure because daily insulin eye drops did not alter IOP levels throughout the course of the study (Table 1).

Table 1. Insulin daily eye drops do not decrease IOP in glaucomatous eyes.

	Intraocular pressure (mm Hg, mean ± SEM) after microbead injection.						
	3 days	**6 days**	**9 days**	**12 days**	**15 days**	**18 days**	**21 days**
Control	**11.52±0.53**	**11.68±0.62**	**11.35±0.45**	**12.24±0.47**	**11.88±0.32**	**11.95±0.57**	**11.92±0.57**
OHT 2W No treatment	**18.89±1.00**	**18.47±1.09**	**18.20±0.88**	**18.63±0.94**	**19.95±1.24**	–	–
OHT 2W + Insulin	**18.06±0.52**	**18.16±0.48**	**17.93±0.90**	**18.70±0.73**	**18.70±0.32**	**18.37±0.50**	**18.38±0.38**
OHT 2W + Vehicle	**17.33±1.60**	**18.00±1.05**	**17.28±0.43**	**19.33±0.93**	**17.72±0.26**	**19.33±0.97**	**18.16±0.40**

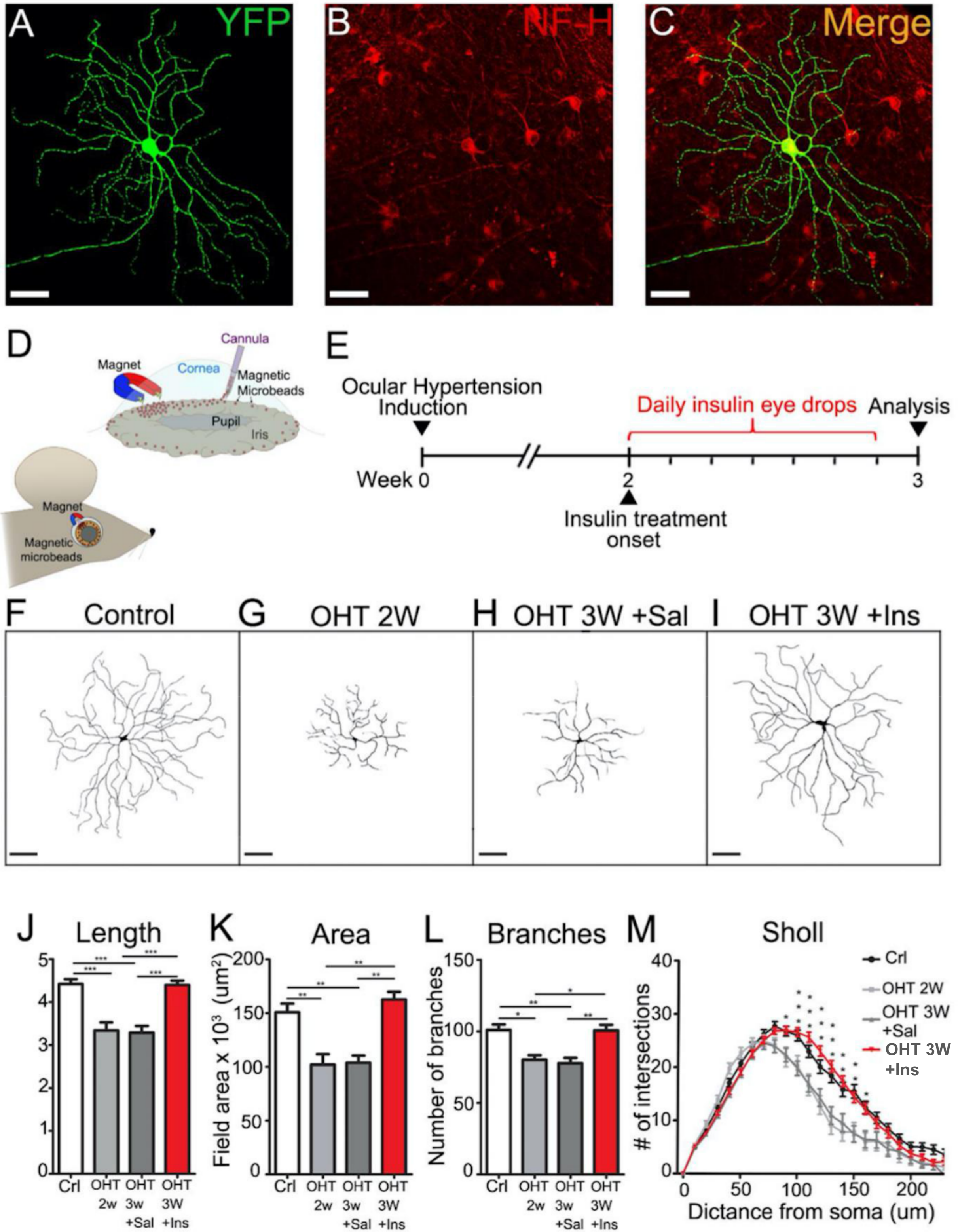


Figure 6. Insulin promotes RGC dendrites regeneration. (A-C) YFP-labeled RGCs co-expressing NF-H, a marker of alpha sub-type RGCs, with a clearly identifiable axon

were selected for dendritic arbor imaging and 3D reconstruction. (D) For ocular hypertension (OHT) induction, a cannula attached to a microsyringe pump was used. Microbeads were injected into the anterior chamber of the mouse eye. Using the magnet, microbeads were attracted to the iridocorneal angle and were distributed to form a ring around the circumference of the anterior chamber. (E) Insulin treatment (daily eye drops) started at 2 weeks after microbead injection and dendritic arbor analysis was performed a week later (total time: 3 weeks). (F-I) Representative examples of dendritic arbour from glaucomatous retinas treated with insulin (Ins) or vehicle (saline: Sal) or untreated at two weeks of ocular hypertension induction. (J-M) Quantitative analysis of dendritic parameters revealed that insulin-treated neurons had longer dendrites and markedly larger and more complex arbors than saline-treated controls. Values are expressed as the mean ± SEM. (ANOVA, ***p< 0.001, **p< 0.01, *p< 0.05, N = 3– 7 mice/group, n = 25–54 RGCs/group).

3.2. REDUCTION OF OCULAR PRESSURE IS NOT SUFFICIENT TO PROMOTE REGENERATION

High IOP is the most important, and the only modifiable, risk factor to develop glaucoma (Coleman and Kodjebacheva, 2009). Current treatments for glaucoma are limited to IOP reduction using pharmacological or surgical approaches, but many patients continue to experience visual field loss despite responding well to therapies that regulate IOP. Given that insulin is endogenously present in the retina (Kondo and Kahn, 2004; Naeser, 1997), we asked whether IOP lowering is sufficient to promote RGC dendrite regeneration after OHT induction. To test this, we induced glaucoma in Thy1-YFP mice and measured IOP

every three days over a period of three weeks. At two weeks of OHT, a time when substantial dendritic retraction is already observed, we instilled brinzolamide daily eye drops to lower IOP. Brinzolamide is a carbonic anhydrase inhibitor that effectively reduces IOP in bead occlusion models (Yang et al., 2012) and has negligible effects on neurons or vascular cells (Martínez and Sánchez-Salorio, 2009; Vasudevan et al., 2011). Our data show that brinzolamide effectively reduced IOP throughout the entire duration of the study. Indeed, brinzolamide-treated eyes showed similar IOP values to non-glaucomatous control eyes (Figure 7A). One week after the onset of brinzolamide treatment, RGC dendritic arbors were images and dendritic parameters quantified. A substantial retraction of RGC dendrites and loss of branches was observed in glaucomatous eyes, both in brinzolamide- and vehicle-treated retinas (Figure 7E-H). Therefore, we conclude that brinzolamide-induced IOP reduction is not sufficient to promote RGC dendritic regeneration.

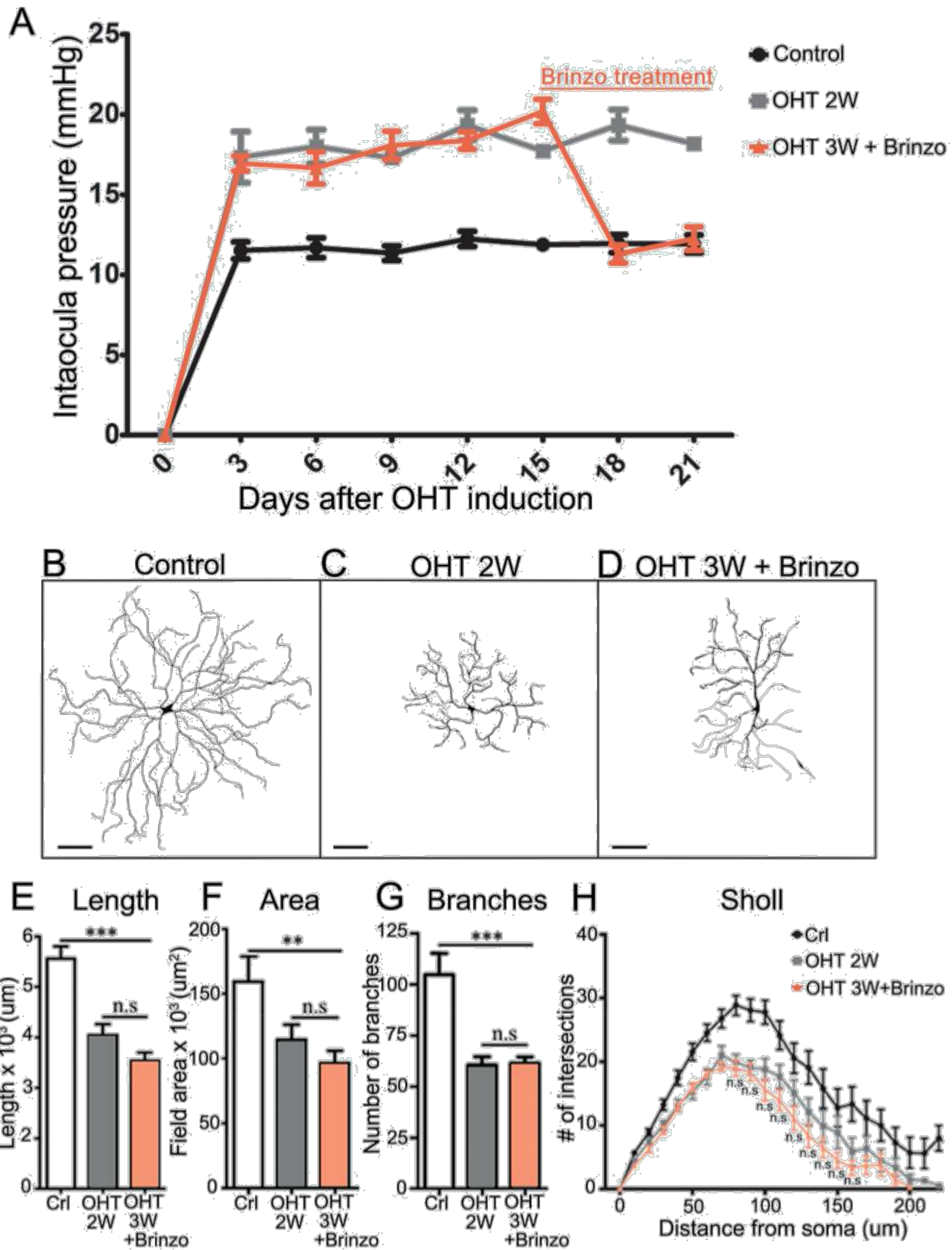


Figure 7. Pharmacological reduction of intraocular pressure does not promote RGC dendrite regeneration. (A) Magnetic microbead injection increased IOP and treatment

with brinzolamide (Brinz), a drug that does not affect neurons or vasculature, effectively reduced eye pressure. (B-D) Representative examples of dendritic arbors from glaucomatous retinas treated with brinzolamide (Brinz) or vehicle (saline: Sal) at two weeks of OHT induction. (E-H) Quantitative analysis of dendritic parameters revealed that brinzolamide-treated neurons did not show dendritic arbor regeneration compared to vehicle-treated control neurons. Values are expressed as the mean ± SEM. (ANOVA, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, N = 3– 7mice/group, n = 25–54 RGCs/group).

3.3. ADULT RGCs FROM GLAUCOMATOUS RETINAS CAN BE ISOLATED USING AN OPTIMIZED FACS-BASED PROTOCOL

We previously showed that insulin-mediated RGC dendrite regeneration required activation of the mTOR pathway (Agostinone et al. 2018). However, the role of downstream effectors and other potential regulators of insulin-driven regeneration are currently unknown. To fill this knowledge gap, we first developed a FACS-based protocol for the isolation of adult RGCs from adult glaucomatous retinas treated with insulin or vehicle (control) for molecular profiling. The isolation of a large number of viable RGCs is challenging because of the relative scarcity of RGCs within the retina (<1% of the whole retinal cell population) (Rheaume et al., 2018a) and the lack of standardized protocols. We capitalized on recently published protocols that succeeded at isolating RGCs (Chintalapudi et al., 2016; Williams et al., 2017) and further optimized them to increase RGC number and purity.

A major challenge in RGC isolation by FACS is the lack of surface molecule signatures on these cells. RGCs have been typically isolated based on the expression of

Thy1.2 on their surface, however, Thy1.2 can also be expressed by other retinal cells (Chintalapudi et al., 2016). Indeed, under certain experimental conditions Thy1.2 has been shown to be expressed by astrocytes (GFAP+), leukocytes (CD45.2+), endothelial cells (CD34+), and macrophages (CD11b+ and CD11c+) (Williams et al., 2017). To overcome this limitation, retinas were dissociated, live cells selected, and labeled with the following antibodies: Thy1.2, CD45.2, GFAP, CD34, CD11b, and CD11c (Figure 8A, a-c). Forward and sideward scatter (FSC-A and SSC-A) parameters were used to identify cell-relevant events and help represent cell populations based on their complexity (SSC) and size (FSC). The P1 fraction was selected for further analysis. Doublet cells were eliminated and dead cells, identified by labeling with propidium iodide, as well as GFAP+, CD45.2+, CD34+, CD11b+ and CD11c+ cells, were discarded. Only Thy1.2+GFAP-CD45.2-CD34-CD11b-CD11c- were collected (Figure 8B, a-g). This procedure yielded an average of 20,000 to 30,000 RGCs from each individual mouse retina.

To confirm the RGC status of the FACS sorted cells, we cultured isolated cells for 24 hrs, fixed them, and labeled them with an antibody against the RGC-specific marker RNA Binding Protein with Multiple Splicing (RBPMS). All DAPI-positive cells were also RBPMS-positive, confirming that the isolated cells were RGCs (Figure 8C). We also used qPCR analysis of isolated cells and found abundant expression of the RGC-specific marker TUJ1, while immune, vascular or retinal neuron markers were lacking (Figure 8D). Collectively, these data validate the reliability of our FACS-based protocol for sorting RGCs from adult glaucomatous and non-injured retinas.

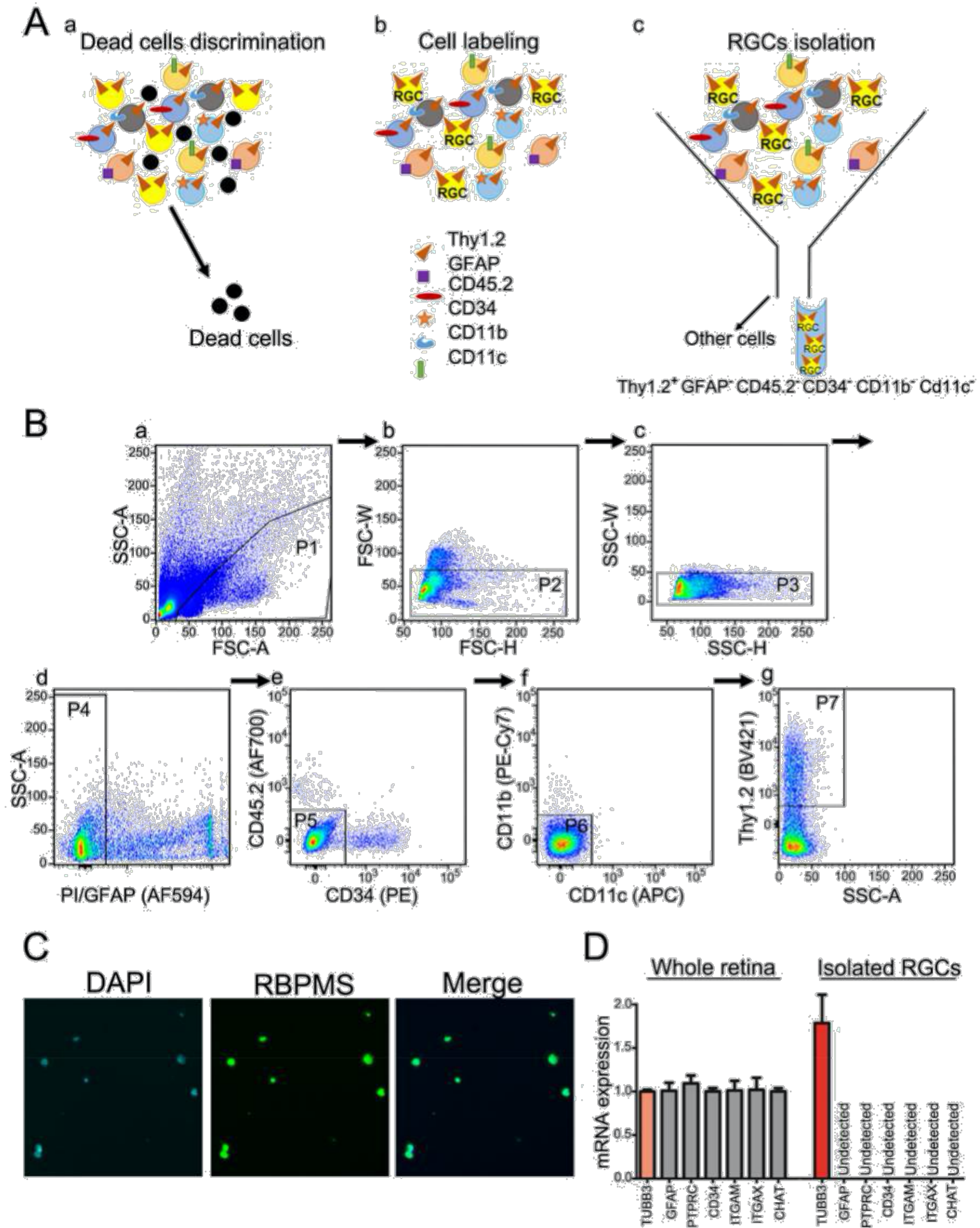


Figure 8. Fluorescent activated cell sorting strategy. (A) After retinal cell dissociation, dead cells were eliminated (a), and the remaining cells were labeled with antibodies

against Thy1.2, GFAP, CD45.2, CD34, CD11b and Cd11c (b). Only Thy1.2+, GFAP-, CD45.2-, CD34-, CD11b-, CD11c- cells were sorted (c). (B) Forward and sideward scatters (FSC-A and SSC-A) identified cell relevant events and help characterize cell populations depending on their complexity (SSC) and size (FSC). P1 was selected for further analysis (a). Doublet cells were eliminated (b-c). Dead, GFAP+, CD45.2+, CD34+, CD11b+ and CD11c+ cells were discarded (d-f). Only Thy1.2+, GFAP-, CD45.2-, CD34-, CD11b-, and CD11c- were selected for further analysis (g). (C) After sorting, cells were cultured for 24 hrs, fixed and labeled with the RGC-specific marker RBPMS. All DAPI+ cells were RBPMS+ confirming the identity of the sorted cells as RGCs. (D) qPCR analysis confirmed that sorted cells were RGCs based on high expression of TUJ1 and undetected expression of astrocytic, immune or vascular markers GFAP, PTPRC (CD45.2), CD34, ITGAM (CD11b), ITGAX (CD11c) and CHAT genes relative to whole retinal samples.

3.4. MOLECULAR PROFILING OF INSULIN-STIMULATED RETINAS REVEALS KEY REGULATORY PATHWAYS

To identify molecular effectors downstream of insulin involved in dendrite regeneration, we first isolated RGCs using the FACS-sorting method described above from mice subjected to induction of ocular hypertension followed by treatment with insulin or vehicle (controls). Our recent data demonstrate that insulin treatment promotes full RGC dendrite regeneration as early as 1 week of administration onset (Figure 6). Therefore, insulin treatment was initiated at 2 weeks after glaucoma induction, and RGCs were FACS-sorted 4 days later, followed by mRNA purification and further processing for RNAseq. Our analysis identified 368 differentially regulated genes in hypertensive retinas relative to

non-injured controls ($p<0.05$), and 537 differentially expressed genes in insulin-treated glaucomatous retinas relative to saline-treated controls ($p<0,05$). Heatmaps obtained from all expressed genes show that insulin-treated RGCs were molecularly similar to non-injured controls. In contrast, marked differences were observed between vehicle- and insulin-treated RGCs (Figure 9A). The top 50 differentially expressed genes after glaucoma induction and insulin treatment are shown in Figure 9B. Pathway analysis revealed putative regulators implicated in insulin-mediated RGC dendrite regeneration. Of note, several pathways were significantly downregulated with ocular hypertension and underwent statistically significant upregulation after insulin treatment relative to control groups including: mTOR signaling, Notch, glycolysis, fatty acid metabolism, DNA repair and myc targets (Figure 9C).

Our previous data demonstrated that insulin-induced activation of mTOR played a crucial role in RGC dendrite regeneration (Agostinone et al., 2018). Consistent with this, we observed an important downregulation of mTOR pathway components at 2 weeks of glaucoma induction, which was rescued by insulin treatment (Fig. 10). RNA-seq analysis revealed significant changes in a number of key components of the mTOR pathway (Fig. 10). In addition, components of the Notch pathway were globally downregulated after glaucoma induction and upregulated at four days of insulin treatment (Figure 10B). Collectively, these data demonstrate critical changes at the RNA level in response to insulin, notably in components of the mTOR and Notch pathways, thus identifying novel putative regulators of RGC dendrite regeneration. My future PhD work in the Di Polo laboratory will focus on the characterization of the functional role of these molecules during the regenerative process.

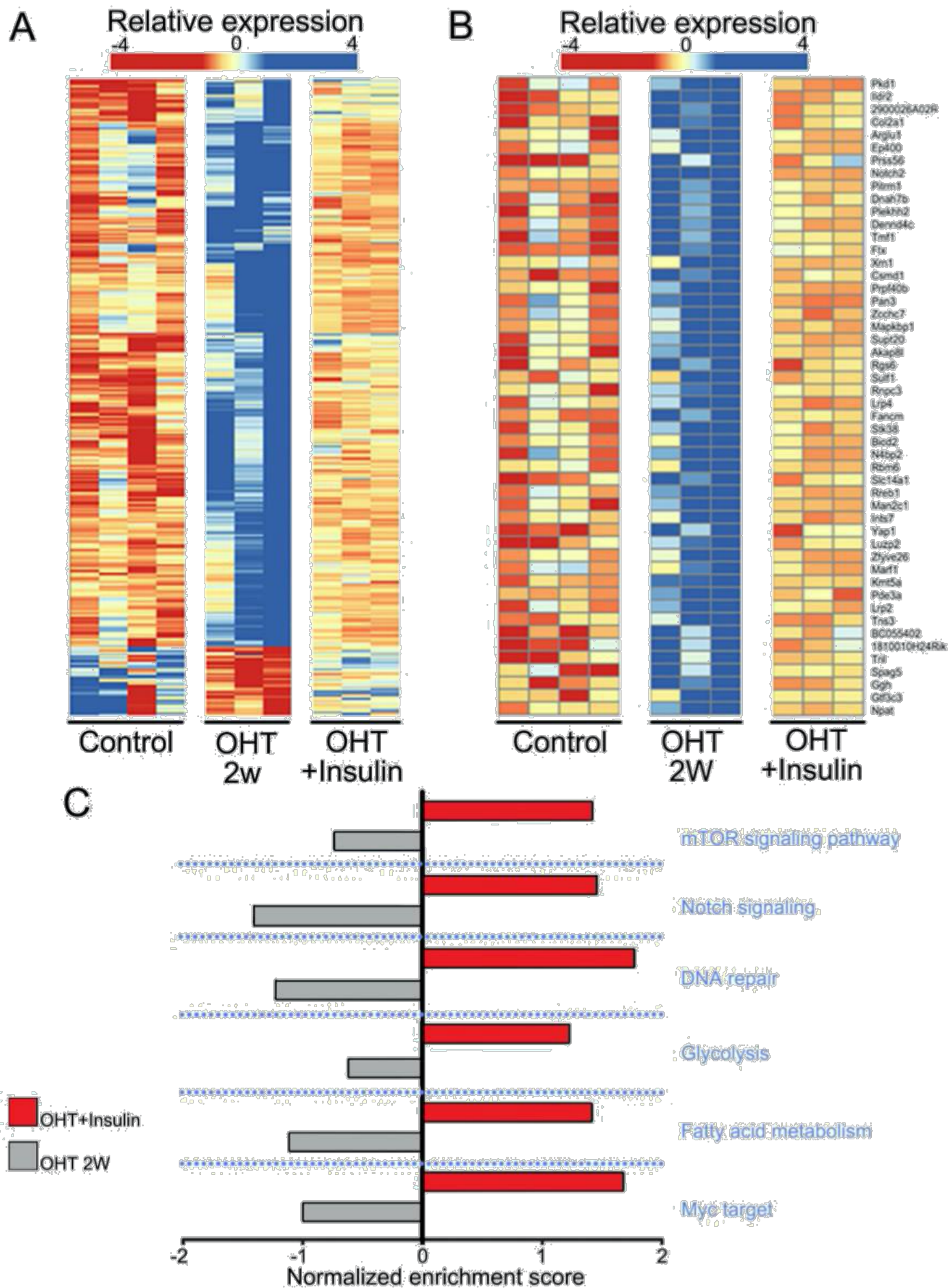


Figure 9. RNAseq analysis of insulin- and saline-treated retinas (A) Heatmap of gene expression (all expressed genes) shows that insulin treated RCGs were molecularly

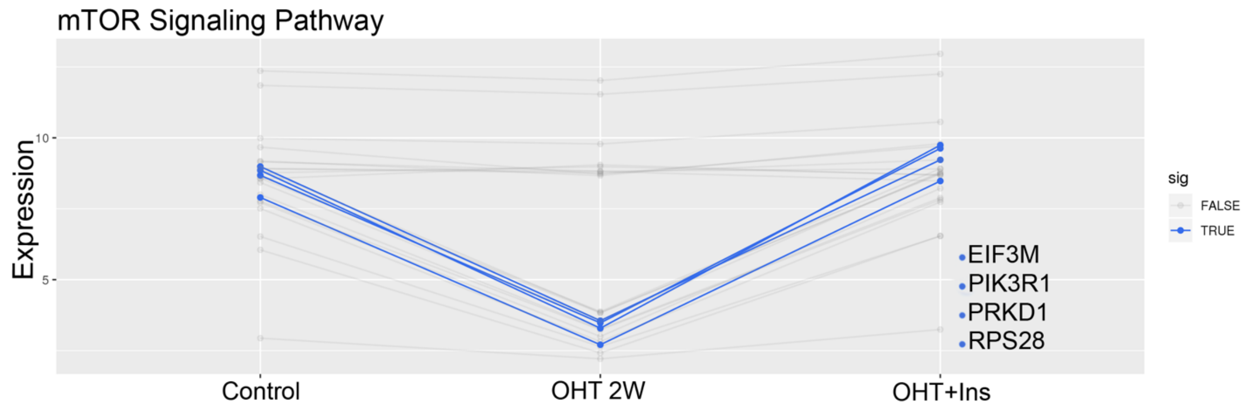
similar to controls. (B) Heatmap of the top 50 differentially expressed genes following OHT induction and insulin treatment reveals that insulin restores expression levels of these key genes. (C) Pathway analysis identified key molecular components that might be implicated in insulin-induced RGC dendrite regeneration. OHT induction shows a significant down regulation of Notch, mTOR signaling, glycolysis, fatty acid metabolism, DNA repair, and myc targets (Normalized enrichment Score (NES) < 0). These pathways were up regulated following insulin treatment (NES>0). Selected pathways had FDR < 0.05 (FDR: False Discovery Rate) and were detected in two-pathway analysis methods, Hallmark and Ingenuity Pathway analysis.

3.5. PRELIMINARY PATHWAY CHARACTERIZATION

The mammalian target of rapamycin (mTOR) plays an important role in dendrites formation during development (LiCausi and Hartman, 2018). mTOR activation has been shown to be protective during glaucoma (Li et al., 2018b). Our RNA-seq analysis revealed important changes in key components of the mTOR pathway following OHT induction and insulin treatment. At two weeks of OHT we observed an important downregulation of the mTOR signaling pathway. This downregulation was rescued by the insulin treatment as components of this pathway were upregulated to a similar level to controls at four days of insulin treatment (Figure 10: A).

Also, Notch pathway is critical for morphogenesis of dendritic arbors and weighting of synapse strength (Giniger, 2012). It has been shown, in a rat model of acute OHT, that the Notch pathway may decrease the apoptosis of the RGCs and may enhance the regeneration of the damaged optic nerve (Li et al., 2018a). Here we show that key component of the Notch pathway were globally downregulated after OHT induction and upregulated at four days of insulin treatment (Figure 10: B).

A



B

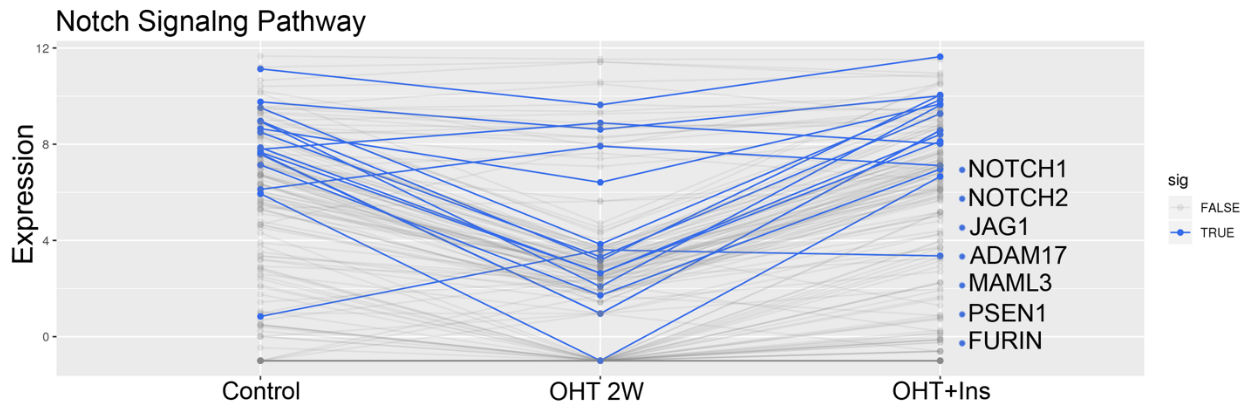


Figure 10. Key components of the mTOR and Notch signaling pathways changes after OHT induction and following insulin treatment (A) Individual gene expression plots show mTOR signaling pathway returned to normal in insulin treated RGCs. (B) Individual gene expression plots show Notch signaling pathway returned to normal in insulin treated RGCs. Dots represent individual genes, grey = not significant, blue = significant at p-value < 0.05 compared to controls.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

4. DISCUSSION AND CONCLUSION

4.1. DISCUSSION

The capacity of adult CNS neurons to regenerate after injury is extremely limited. Lower vertebrates display a greater ability to repair or replace neurons after injury compared to more evolved organisms like humans (Steward et al., 2013). In 1985, Albert Aguayo and his colleagues showed that providing injured axons with a permissive environment (*i.e.* a peripheral nerve graft) allowed them to regenerate and reach their post-synaptic targets (David and Aguayo, 1985). This was the first study to demonstrate, using modern tracing techniques, that adult CNS neurons have the ability to regenerate after lesion. Since then, studies on regeneration have primarily focused on axonal regrowth and a number of molecular mechanisms that participate in axonal regeneration have been identified (Curcio and Bradke, 2018). In contrast, despite the structural and functional importance of dendrites, the ability of neurons to regenerate dendritic processes and synapses has been largely ignored.

Retraction of RGC dendritic arbors and loss of synapses has been observed in primate, cat and rodent models of glaucoma (Della Santina et al., 2013; Morgan et al., 2006; Morquette et al., 2015; Shou et al., 2003; Weber et al., 1998; Williams et al., 2013) as well as in human glaucomatous retinas (Pavlidis et al., 2003). Consistent with this, we recently demonstrated that selective injury to RGC axons triggers rapid dendritic shrinkage and loss of arbor complexity, prior to overt neuronal loss (Agostinone et al., 2018; Morquette et al., 2015). Remarkably, insulin administration, after dendrite retraction had already occurred, promoted robust process regrowth and restoration of arbor area and complexity in an optic nerve axotomy model (Agostinone et al., 2018). Here, we show

that insulin administration promotes dendritic regeneration in a mouse model of ocular hypertension glaucoma, which mimics several aspects of the human disease, further consolidating the important role of insulin in regenerative dendritic growth.

Endogenous insulin and insulin-related molecules including insulin-like growth factors 1 and 2 (IGF-1, IGF-2) are produced locally within the retina (Das et al., 1987; Penha et al., 2011) and could exert a beneficial effect on RGCs. We hypothesized that when IOP is reduced, endogenous insulin or insulin-like signaling might stimulate dendritic growth. However, our data demonstrate that lowering IOP is not sufficient to promote RGC dendrite regeneration. These results suggest that: i) the level or activity of these endogenous molecules is not sufficient to promote dendrite regeneration even when IOP is normalized, and/or ii) other IOP-independent factors limit the ability of RGCs to regenerate. Collectively, these data support the use of exogenous insulin as a pro-regenerative strategy for the restoration of RGC dendrites and synapses in glaucoma.

Our observation that insulin is effective when administered as eye drops is important because this route of administration is likely to reduce side effects including hypoglycemia. Our published data showed that insulin reaches the retina easily after eye drop application. Indeed, fluorescently-labeled insulin was detected in the retina 15 min after topical instillation in the mouse eye (Agostinone et al., 2018). Although it is currently unclear how insulin reaches retinal cells, this short time-frame suggests that it enters the systemic circulation, possibly through the tear canal in the eye, and crosses the blood-retinal barrier to gain rapid access to this tissue. Our observation that insulin exerts a robust regenerative and pro-survival effect, together with its established safety profile, make it an excellent candidate for clinical trials for glaucoma. This is reinforced by observations that insulin applied topically in healthy humans at very high doses is

innocuous and does not cause hypoglycemia or toxicity (Bartlett et al., 1994). However, the limited scope of that study did not allow for a full assessment of safety in vulnerable diseased eyes, a critical issue when treating glaucoma. To address this, a Phase 1 clinical trial of insulin eye drops in glaucoma patients is currently ongoing as a collaboration between the Di Polo laboratory and the ophthalmology clinic at the CHUM (https://clinicaltrials.gov), NCT04118920).

Insulin signaling plays an important role in neuronal circuit development both through the establishment of dendritic arbor morphology and the regulation of synaptic function (Chiu and Cline, 2010). It has been shown that the insulin receptor substrate (IRS) is required for cytoskeletal reorganization and neurite outgrowth during development (Govind et al., 2001). Also, pathways downstream of the IR such as Ras/MAPK and PI3K/Akt/mTOR signaling pathways regulate dendritic structure (Chiu and Cline, 2010). Moreover, enhanced insulin signaling was shown to activate survival pathways in the retina (Rajala et al., 2010). Our previous work demonstrated that insulin promotes RGC regeneration through mTOR activation. However, the molecular effectors downstream of mTOR or other putative regulators are currently unknown. To investigate this, we developed and optimized a FACS-based protocol to isolate RGCs from adult glaucomatous and control retinas treated with either insulin or vehicle. The isolation of an RGC-enriched population from adult retinas has been historically challenging because of multiple factors. First, RGCs amount to <1% of the entire cell population in the retina (~60,000 RGCs/ mouse retina) relative to other abundant cells such as photoreceptors (>5M cells) (Rheaume et al., 2018). Second, the absence of an RGC-specific surface marker requires the use of a battery of antibodies to eliminate contaminating cells and obtain a highly enriched RGC pool. Indeed, we demonstrated that this protocol yields

isolated cells that express RGC-specific markers while other cell markers are absent. Third, existing protocols are not adapted to isolate RGCs from mouse retinas, in which tissue amount is an issue. Fourth, adult retinas are more difficult to dissociate, and cell aggregation occurs rapidly after dissociation requiring rapid action at each step.

A critical step in cell sorting from complex tissue like the adult retina was to eliminate dead cells. To assess cell viability by flow cytometry, different methods were used. Vital DNA dyes are the classic and most commonly used dyes in flow cytometry, including propidium iodide. When a cell is alive, its membrane is intact and impermeable to these dyes. In contrast, dying and dead cells rapidly take up propidium iodide, which binds to the DNA once inside the cell. We tested different molecules to discriminate between live and dead cells including DAPI, 7-AAD, and PI. Among the dyes tested, propidium iodide was selected for the following reasons. First, it provided a good live/dead cell discrimination ability. Second, it required virtually no incubation time with dissociated cells to work, thus minimizing the time for cell preparation prior to sorting thus enhancing cell viability. Unstained cells and fluorescence minus one (FMO) were used as controls to set the gates before RGC sorting. For the FMO control, dissociated cells were labeled with all antibodies cited above except Thy1.2. In our initial flow cytometry experiments, RGCs were not clearly separated, making the FMO control critical for the success of this strategy. The FMO allowed us to show all the other cell populations, except the Thy1.2-positive cells, which helped us set the gate to discriminate only the Thy1.2+ RGC population amidst other retinal cells. In summary, the optimized FACS protocol to isolate RGCs reported here was validated for its usefulness for RNA extraction and RNAseq analysis and will be a valuable tool for the community.

In this study, we show marked downregulation of gene expression and key regulatory signaling pathways following glaucoma induction and prior to overt neuronal damage. This finding suggests early transcriptional inhibition in RGCs in response to ocular hypertensive stress. Remarkably, several pathways were upregulated with insulin therapy. Among these, many mTOR signaling components underwent marked downregulation in glaucoma and increased to baseline levels of expression with insulin, supporting our previous findings that mTOR activation is essential for insulin-induced dendrite regeneration (Agostinone et al., 2018). In addition, components of the Notch pathway followed a similar response drawing interest to their potential role in dendritic regrowth. Notch has been proposed to contribute to dendrite formation during development and RGC neuroprotection (Lasky and Wu, 2005; Li et al., 2018). Furthermore, the role of Notch signaling on dendrite regeneration has never been studied and will be a focus of my future research in the context of my PhD work in the Di Polo lab. The identification and characterization of new molecules that promote RGC dendrite regeneration after injury will not only expand our understanding of the response of CNS neurons to injury but might identify novel therapeutic targets for glaucoma and other neurodegenerative diseases.

4.2. CONCLUSIONS AND FUTURE STUDIES

The work presented in this thesis supports several conclusions. First, we demonstrate that daily insulin eye drops can promote RGC dendrite regeneration in a mouse glaucoma model. Second, our data show that lowering IOP is not sufficient to promote dendrite regrowth after injury, and support the use of exogenous insulin as a robust pro-

regenerative strategy. Third, we report an optimized FACS-based protocol to isolate adult RGCs from glaucomatous and control retinas suitable for RNA extraction and RNAseq analysis. Fourth, our RNAseq data demonstrate transcriptional downregulation in RGCs subjected to ocular hypertension which was rescued by insulin. Fifth, pathway analysis confirmed the key role of mTOR signaling in insulin-induced dendrite regeneration and identified novel pathways for further characterization.

These results form the basis for my future PhD work in the Di Polo lab, which will focus on the following goals:

i) Characterize the functional role of individual components of the mTOR and Notch pathways in RGC dendrite regeneration. mRNA and protein expression in RGCs will be examined by qPCR and immunohistochemistry. Gain- and loss-of-function studies will be carried out to test their functional role in dendritic regrowth in our mouse glaucoma model. Importantly, we will also examine synaptic regeneration and functional recovery using a battery of tests including electroretinography and optokinetic assays.

ii) Our work so far has focused primarily on αRGCs, but whether other RGC subtypes are also capable of dendrite and synapse regeneration has yet to be established. Recent work suggests that RGCs with dendritic stratification in the OFF sublamina are more vulnerable in glaucoma (El-Danaf and Huberman, 2015, Della Santina et al., 2013), therefore we will examine insulin-driven dendrite regeneration in different RGC subtypes in well-characterized transgenic mouse lines including: ii) melanopsin (OPN4-GFP) for intrinsically photosensitive RGCs (ipRGCs) (Hattar et al., 2002), which undergo substantial dendritic retraction in the OFF sublamina (El-Danaf and Huberman, 2015), iii) homeobox gene Hb9 (Hb9-GFP) for ON-OFF directional-

selective cells that code anterior motion (Trenholm et al., 2011), and iv) dopamine receptor D4 (DRD4-GFP) for ON-OFF directional-selective cells that respond to posterior motion (Huberman et al., 2009).

These projects will provide new knowledge on the contribution of insulin signaling deficits to early dendrite retraction and synapse loss, and will establish whether insulin mediates functional recovery in glaucoma.

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