EXPLORING DRUG DELIVERY SYSTEMS FOR GLAUCOMA

INVESTIGATING THE EFFECT OF A MICELLE BASED DRUG DELIVERY SYSTEM IN REDUCING IOP AND GLAUCOMATOUS EFFECTS IN A PARTIALLY OPEN ANGLE MOUSE MODEL OF GLACUOMA

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LAY ABSTRACT

An effective treatment strategy is required to prevent irreversible blindness caused from glaucoma. Unfortunately, compliance with current medications is extremely poor, as they require frequent administration due to their low ocular bioavailability and short-term effect. As such, this thesis aims to explore an alternative drug delivery approach in a partially open angle mouse model of glaucoma to prevent the worsening of glaucoma and ultimately improve patient compliance.

ABSTRACT

This project explores the use of a novel sustained release mucoadhesive micelle-based drug delivery system in combination with 0.005% latanoprost (LTP) on our partially open angle mouse model of glaucoma (AP-2β TMR-KO). We previously tested for LTP treatment in our model and found a reduction in intraocular pressure (IOP) 20 minutes following treatment. This information led us to investigate the long-term effect of LTP treatment and micelle loaded with LTP (MLTP) treatment in our model. We hypothesized that the MLTP treatment would be more effective in reducing IOP and preventing glaucomatous effects than LTP treatment alone in the AP-2β TMR-KO mice. The MLTP groups of animals (wildtype and mutant) were treated every 3 days, and this was compared with animals treated with LTP daily as well as animals treated every 3 days with LTP alone for comparison's sake for 60 days. IOP measurements were taken every 3 days. Following long term LTP treatment alone, mutant mice showed a consistent decrease in their baseline IOPs with a significant reduction in baseline IOP at 35 days of treatment across all cohorts ($P<0.0001$). In comparison, mutants treated with MLTP exhibited an even greater reduction in baseline IOP following long term treatment. After the treatment period, mice were euthanized, and their eyes were enucleated, fixed, sectioned, and stained for retinal ganglion cells (RGCs) using Brn3a. Mutant mice exhibited a significant decrease in RGC cell number when compared to wildtype, and this loss was not rescued by treatment with LTP. However, mutants treated with MLTP demonstrated significant RGC cell protection compared to eyes of untreated mutants, as well as everyday LTP treated mutants.

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LIST OF ABBREVIATIONS

AP-2	Activating Protein 2
ASD	Anterior Segment Dysgenesis
AP-2β TMR-KO	AP-2β Trabecular Meshwork Region Knockout
ARVO	Association for Research in Vision and Ophthalmology
CB	Ciliary Body
CycA	Cyclosporine A
E	Embryonic day
ECM	Extracellular matrix
Foxc1	Forkhead Box C1
GWAS	Genome Wide Association Study
H&E	Hematoxylin & Eosin
(HPLC)	High-performance liquid chromatography
IHC	Immunohistochemistry
IOP	Intraocular Pressure
LTP	Latanoprost
LMX1B	LIM Homeobox Transcription Factor 1-beta
MAA	Methacrylic acid
Mgp	Matrix Gla Protein
MLTP	Micelle containing Latanoprost
NCC	Neural Crest Cells
ONH	Optic Nerve Head
OCT	Optical Coherence Tomography
P	Postnatal Day
PAS	Peripheral anterior synechiae
PAX6	Paired-like homeodomain transcription factor 6
PBA	Phenylboronic acid
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PITX2	Paired-Like Homeodomain2
PITX3	Paired-Like Homeodomain 3
PLA	Polylactic acid
POM	Periocular Mesenchyme

RAFT	reversible addition−fragmentation chain transfer polymerization
RGC	Retinal Ganglion Cell
SC	Schlemm's Canal
TM	Trabecular meshwork

CHAPTER 1: GENERAL INTRODUCTION

The Anterior Segment

The human eye is a remarkable organ consisting of many intricately connected structures that work in harmony to allow us to see the world around us. The anterior segment of the eye consists of the iris, cornea, lens, ciliary body (CB), and drainage structures, trabecular meshwork (TM) & Schlemm's canal (SC). The anterior segment also includes the anterior chamber (space between the iris and cornea) and posterior chamber (space between iris and lens)(Gould & John, 2002; Wright et al., 2016). These structures are critical for focusing light on the retina, regulating the amount of light entering the eye, as well as controlling the pressure within the eye. More specifically, the cornea and lens are responsible for refraction of the light to ensure it is precisely focused onto the retina for clear vision (DelMonte & Kim, 2011; Hejtmancik & Shiels, 2015). The iris controls how much light the retina receives, and the CB secretes the aqueous humor, a clear fluid that provides nutrients to the lens and cornea and generates intraocular pressure (IOP) within the eye (Cvekl & Tamm, 2004). Aqueous humor flows from the posterior chamber into the anterior chamber and then through drainage structures (TM and SC) located at the iridocorneal angle (where the iris and cornea meet). The TM is a porous tissue that regulates the IOP by creating resistance to the flow of aqueous humor (Cvekl & Tamm, 2004). The fluid flows from the TM into the SC, then into the venous system.

Development of the eye

Three main tissue types: surface ectoderm, neural ectoderm, and periocular mesenchyme (POM) undergo a series of induction and differentiation events required for the formation of ocular structures (Fuhrmann, 2010). The process of ocular development begins with the lateral thickening of the diencephalon (forebrain) of the neural tube that

evaginates and protrudes outward to form the optic vesicle (Cvekl & Tamm, 2004). The optic vesicle comes in contact with the surface ectoderm to form the lens placode. Invagination of the optic vesicle and lens placode form a double layered structure known as the optic cup as well as the lens vesicle, respectively. The optic cup is now comprised of the neural retina and the retinal pigmented epithelium (Cvekl & Tamm, 2004). At embryonic day 11 (E11), the lens forms upon detachment of the lens vesicle from the surface ectoderm.

POM cells derived from the neural ectoderm and mesoderm migrate into the space between the invaginated lens vesicle and the surface ectoderm to form layers of the cornea (Cvekl & Tamm, 2004; Williams & Bohnsack, 2015). Upon development of the corneal endothelium, the anterior rim of the optic cup extends and forms the iris and the ciliary epithelia, to enable the POM to form the iris and ciliary body stroma. Lastly, the upon elongation of the iris, a mass of mesenchymal cells fill up the iridocorneal angle region to form the TM and SC. The development of these drainage structures mostly occurs postnatally (Cvekl & Tamm, 2004).

Glaucoma

As of 2020, glaucoma, the leading cause of irreversible blindness, affects approximately 76 million people worldwide. It is predicted that by 2040, this number will increase by 74% to over 111 million (Tham et al., 2014). Glaucoma is often referred to as a "silent thief of sight" due to its ability to gradually cause permanent damage to the eyes without presenting any warning signs (Parihar, 2016). Increased IOP is a significant risk factor for glaucoma (Parihar, 2016). The increased IOP causes excessive pressure on the

optic nerve head (ONH), which results in the progressive death of retinal ganglion cells (RGCs). RGCs are vital for transmitting visual signals to the brain's visual cortex (Mead & Tomarev, 2016). Although the exact mechanism of how the elevated IOP is linked to the death of RGC's is not known, a well-known theory suggests that the high IOP induces physical changes at the ONH area, causing it to appear more hollowed out or cupped at the lamina cribrosa. Consequently, the nerve fibers become compressed, blocking the flow of important substances, such as the axoplasm and neurotrophins, necessary for the RGC survival (Guo et al., 2005). The RGC death can eventually result in vision loss and therefore, to prevent these outcomes from occurring, early detection and treatment of glaucoma is necessary (Parihar, 2016).

Types of Glaucoma

While there are many forms of glaucoma, primary open angle glaucoma (POAG) is the most common, characterized by slow, progressive clogging of the TM, resulting in elevated IOP (Weinreb et al., 2014). Another widespread form of glaucoma is primary angle closure glaucoma (PACG), which is more prevalent amongst Asian populations. In PAGC, the iris becomes adhered to the cornea, resulting in an immediate increase in IOP and optic nerve damage (Weinreb et al., 2014; Loo et al., 2021). Individuals diagnosed with PACG are at a higher risk of blindness (Fig.1).

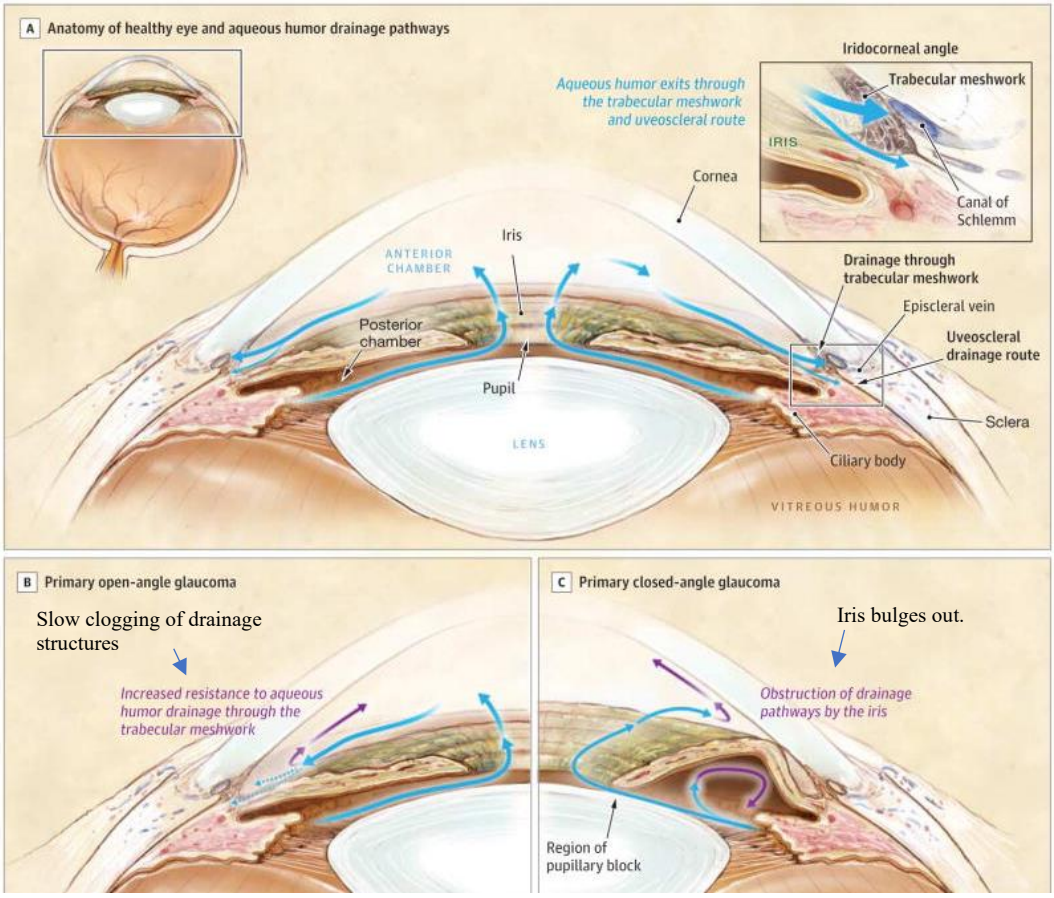


Figure 1. Flow of aqueous humor in the healthy eye compared to primary open angle and angle closure glaucoma eye. In the healthy eye, fluid is secreted from the ciliary body, after which it flows from a space between the iris and pupil from the posterior chamber and into the anterior chamber. It then exits through the conventional pathway or the uveoscleral pathway. In primary open angle glaucoma, the conventional pathway structures are clogged overtime, blocking the flow outflow. Whereas, in primary closed-angle glaucoma, the iris is adhering to the cornea resulting in a closed angle phenotype. Adapted from Weinreb et al., 2014.

Aqueous Humor Pathway

The healthy human eye relies on the presence of aqueous humor for its proper functioning. Aqueous humor serves multiple purposes in the eye, such as providing nourishment to the cornea and lens, ensuring their optimal function, and maintaining the

appropriate IOP, which is crucial for preserving the proper shape and integrity of the eye (Goel et al., 2010). This fluid is produced in the ciliary body, located beneath the iris, after which it travels through the pupil and exits the eye through two pathways: the conventional and the unconventional pathway. In the conventional pathway, the outflow fluid exits through a drainage structure within the anterior angle of the eye; specifically, through the TM and into the SC (Civan & Macknight, 2004) The SC opens into the episcleral vein, causing the fluid to drain out into circulation (Figure 2A). Alternatively, through the unconventional pathway, aqueous humor fluid can pass through the CB to the ciliary muscle and into the suprachoroidal space, between the choroid layer and the sclera. Within the suprachoroidal space, the aqueous humor can then exit through the eye by being absorbed into the blood vessels in the choroid and sclera or diffuse through scleral pores and reach the episcleral tissue (Goel et al., 2010) (Figure 2B).

In mice, the unconventional route is responsible for up to 80% of aqueous humour drainage; however, in humans, only 4% to 60% of aqueous humor drainage occurs through the unconventional pathway, as their uveoscleral flow decreases with age, and the conventional pathway must compensate for this to prevent the IOP from rising. (Fautsch & Johnson, 2006). Blockage of the aqueous outflow fluid results in elevated IOP which eventually progresses to glaucoma.

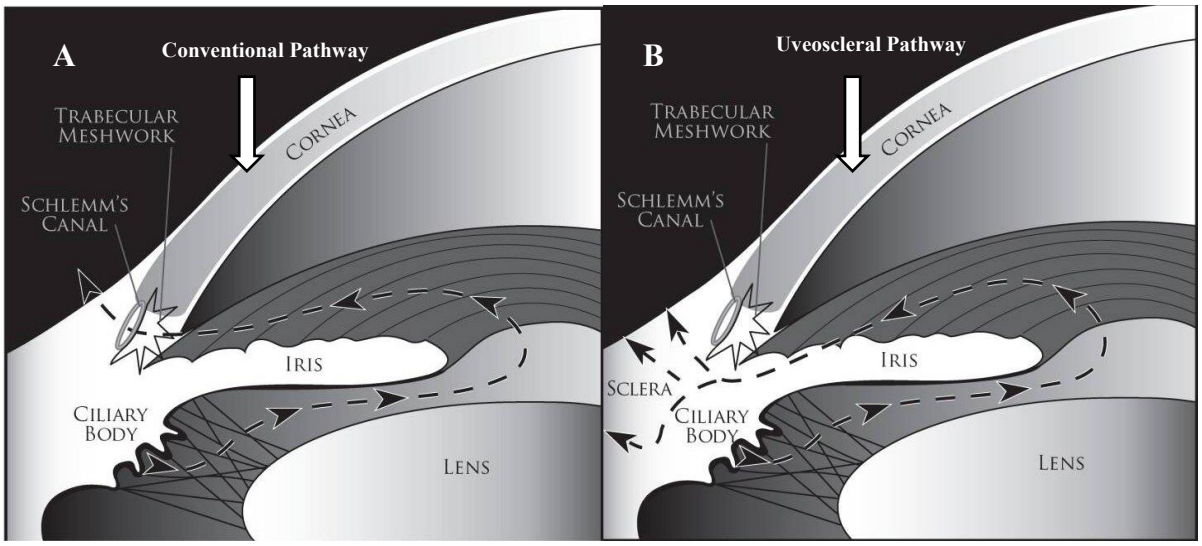


Figure 2 A) conventional outflow pathway. Aqueous humor is produced by the ciliary body, and it flows (dashed line) from the posterior chamber into the anterior chamber. It then passes through the trabecular meshwork into the Schlemm's canal and absorbs into the episcleral veins. **B) uveoscleral outflow pathway.** Aqueous humor flows from the posterior chamber into the anterior chamber and then (dashed lines) through the ciliary body, to the ciliary muscle and into the suprachoroidal space to either veins in the choroid and sclera or through scleral pores to episcleral tissue. Adapted from Weinreb et al., 2014.

Anterior Segment Dysgenesis (ASD) and Important Genes

The development and functioning of the anterior segment structures relies on the presence of important genes such as Paired box 6 (*PAX6*), Paired-like homeodomain transcription factor 2 (*PITX2*), Paired-like homeodomain transcription factor 3 (*PITX3*), Forkhead box C1(*FOXC1*), and LIM homeobox transcription factor 1 (*LMX1B)* (Chograni et al., 2014; Garcia-Montalvo et al., 2014; Romero et al., 2011; Summers et al., 2008; Tümer & Bach-Holm, 2009). Mutations of these genes can result in the abnormal

development of anterior segment structures, which is a condition known as anterior segment dysgenesis (ASD). Many individuals with this condition are at higher risk for glaucoma (Sowden, 2007). A recent analysis from the genome wide association study (GWAS) found that there is a potential link between single nucleotide polymorphisms (SNPs) for the *Tfap2b* gene that encodes the transcription factor AP-2β and glaucoma in humans (Dr. J. Wiggs, personal communication).

The AP-2 Transcription Factor Family

The activating protein-2 (AP-2) transcription family consist of 5 retinoic acid-responsive genes (AP-2α, AP-2β, AP-2γ, AP-2δ and AP-2ε) in humans and mice. These genes have been found to be critical for the evolution and development of neural crest in vertebrates (Jin et al., 2015). Both AP-2α and AP-2β encoded by *tfap2a* and tfap2b genes, respectively, have been found to be involved in the regulation of eye development (Akula et al., 2019; Taiyab et al., 2022; Hicks et al., 2018; Kerr et al., 2014; Korol et al., 2014; Bassett et al., 2012; Bassett et al., 2010; Taiyab et al., 2016). AP-2β, in particular is expressed in derivatives of the POM, such as the corneal endothelium and stroma, TM, ciliary body muscle and iris stroma (Bassett et al., 2007; Chen & Gage, 2016; Martino et al., 2016; West-Mays et al., 1999).

AP-2β Mutant Mouse Model and the Mgp-Cre.KI Mouse Line

Since there is not much known regarding the role of AP-2β in the POM, our lab previously used a Wnt1Cre transgenic line to conditionally delete AP-2β from cranial NCCs (AP-2β NCC KOs) to learn more about this transcription factor and understand its importance in the development of anterior segment structures . These mice showed anterior

segment defects, including loss of the corneal endothelium, a peripheral to central iridocorneal adhesion, and absence of formation of a TM region (TMR) and SC, in addition to increased IOP and glaucomatous features (Akula et al., 2020; Martino et al., 2016). Since the NCC KO mice included other derivatives of the neural crest, to elucidate the specific role of AP-2β in the TM, we developed a partially open angle glaucoma mice model by using an Matric Gla Protein (*Mgp*) gene (*Mgp-Cre.KI)* to selectively delete AP-2β from POM cells which give rise to the TM and SC (Taiyab et al., 2022). These mice were termed AP-2β trabecular meshwork region knockouts (AP-2β TMR KOs). Hematoxylin & Eosin staining (H&E) and optical coherence tomography (OCT) of the mutant eyes displayed a partially open iridocorneal angle, resembling the eyes of peripheral anterior synechiae (PAS) patients and an absent TM and underdeveloped SC (Taiyab et al., 2022) (Fig 3). While these mice exhibited normal POM cell migration, co-staining of αSMA, a marker of the TM and AP-2β revealed a loss of TM and SC markers. These mice also showed an increase in IOP, which was followed by a considerable loss of RGCs and a decrease in retinal function (Taiyab et al., 2022) Due to its key characteristics, this model may be valuable for understanding and exploring drug delivery to treat glaucoma.

MGP is a small vitamin K dependent protein secreted by chondrocytes and vascular smooth muscle cells (Asokan et al., 2018). MGP acts as a powerful inhibitor of calcification in the human body (Asokan et al., 2018). This needs to be regulated or else it may result in the stiffening of tissues and loss of function (Asokan et al., 2018). A study by Borrás et al., 2015 used an Mgp-Cre.KI mouse and crossed it with a floxed LacZ reporter mouse, which is a mouse carrying a gene (LacZ) that produces a blue stain to track the expression of Mgp

in various tissues of the adult mouse eye. They reported Mgp expression in the TM and peripapillary sclera and ciliary muscle. Mgp was also found to be expressed in the epithelial mesenchymal interfaces in lung and limb buds, and in cells of the chondrocyte lineage. (Luo et al., 1995).

Our lab tested for the expression of *Mgp*-Cre.KI during the perinatal period (E15.5, P1, P4, P7, and P14) of mouse development (Taiyab et al., 2022). *Mgp*-Cre.KI mice were crossed with tdTomato females to track the Cre activated tdTomato reporter. At E15.5, the *Mgp*-Cre.KI was expressed in the developing POM region at the anterior rim of the optic cup. We observed a reduction in tdTomato expression at P4, P7 and P14 in the iridocorneal angle of the TMR KO mice compared to the wildtype mice. This discovery from our lab confirms that the *Mgp*-Cre.KI transgene is a valuable line for glaucoma research, due to its specifically localization in POM cells that arise in TM and SC (Taiyab et al., 2022).

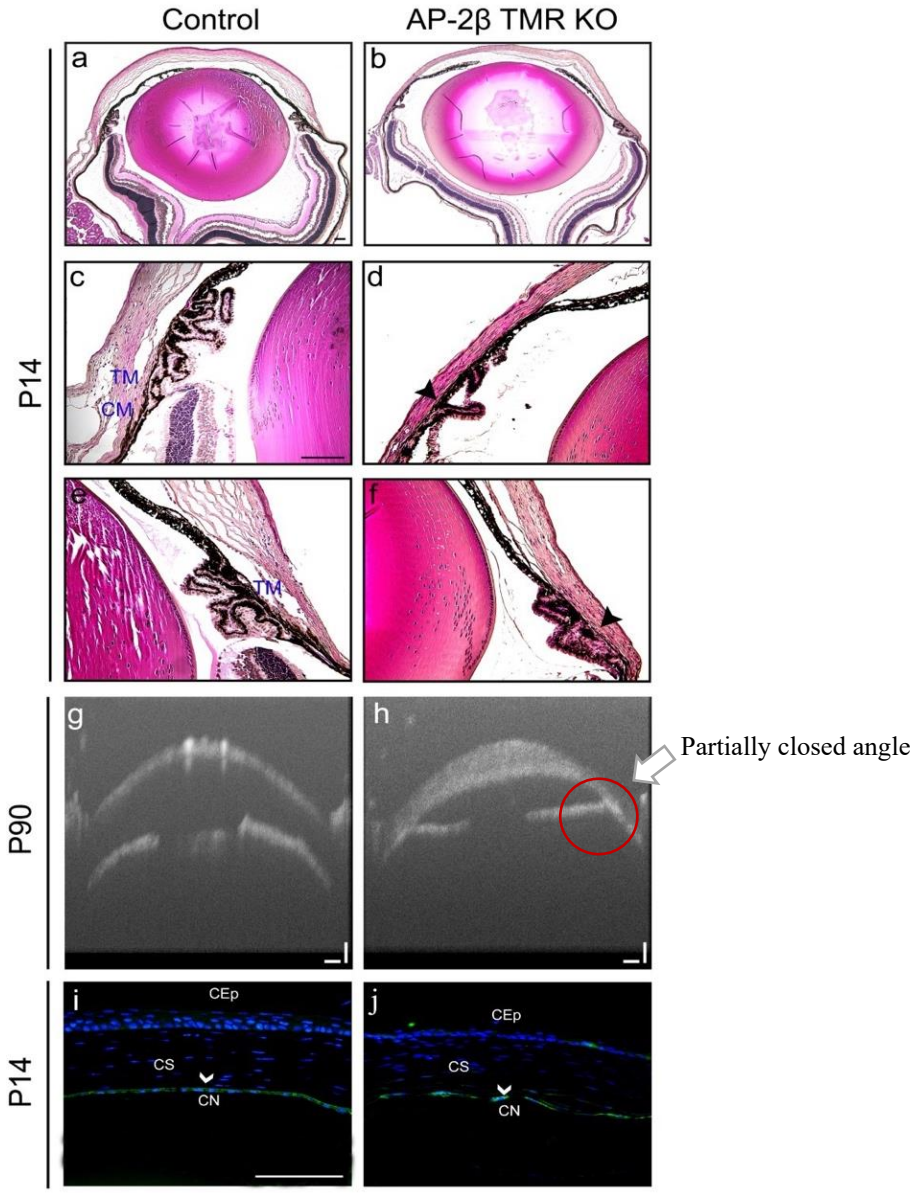


Figure 3. Abnormal morphology of the TM in AP-2β TMR KO mice as compared with wildtype mice. (a, b) By P14, the TM region appeared to be absent in the TMR KO when compared with wildtype mice (N = 6 eyes). (c–f) Higher magnification images of (a) and (b) are depicted in (c, e) and (d, f), respectively. (g, h) OCT images show a fully open iridocorneal angle in P45 wildtype mice (g; N = 6 eyes). The AP-2β TMR KO angle was partially closed (h), displaying PAS. Scale bar, 250 μm. (i, j) At P14 (N = 3 eyes), N-cadherin expression was present in both wildtype and AP-2β TMR KO mice. CEp, corneal epithelium; CM, ciliary muscle; CN, corneal endothelium; CS, corneal stroma; TM, trabecular meshwork. Scale bars in panels a–f and panels i, j represent 100 μm. Images a, b was acquired using a 5× objective lens, images c–f were acquired using a 20× lens, and images i, j were acquired using a 40× objective lens. Adapted from Taiyab et al., 2022.

Current Treatment for Glaucoma

While there is no cure for glaucoma, there are several treatment options available to prevent progression of the disease. Since IOP is the main modifiable factor of glaucoma, most treatments are aimed towards reducing the elevated IOP by increasing aqueous humor outflow or reducing its rate of production. Some of these medications include prostaglandin analogues, β-blockers, carbonic anhydrase inhibitors, α-2 adrenergic agonists, and parasympathomimetic drugs. Currently, prostaglandin analogues are the first line treatment (Cvenkel & Kolko, 2020) due to effectiveness in reducing IOP. This class of drugs work to reduce the IOP by increasing outflow.

Prostaglandin Analogues

Prostaglandins are lipid molecules that are derived from arachidonic acid. There are 5 classes of prostaglandins including E2 (PGE2), F2 (PGF2), I2 (PGI2), D2 (PGD2), and thromboxane A (TXA2) that work with G protein coupled receptors to elicit their response (Sharif et al., 1999). There are 9 GPCRs: DP1 and 2 receptors (DPs) for PGD2, EP1, 2, 3, and 4 receptors for PGE2, FP receptor for PGF2, and TP receptors for TXA2. The FP receptor protein is specifically found in the corneal epithelium, ciliary epithelium, the circular portion of ciliary muscle, and iris stromal and smooth muscle cells (Davis & Sharif, 1999; Schlötzer-Schrehardt et al., 2002; Sharif et al., 1999; Zhang & Yin, 2002). The binding of the PGF2α or a prostaglandin FP agonist to the FP receptor activates G proteins associated with the receptor that allow for the increase of calcium concentration and activates other signalling pathways (Bos et al., 2004). Prostaglandin analogues work to

reduce the IOP by increasing outflow through the uveoscleral pathway. This may be achieved through various possible mechanisms that help to increase outflow, such as, relaxation of ciliary smooth muscles, modification of cytoskeleton, and remodeling of the extracellular matrix (ECM) of the uveoscleral pathway (Tripathy & Geetha, 2022).

Latanoprost (LTP) is a commonly used prostaglandin analogue that is topically administered in the form of eye drops several times a day to treat glaucoma. LTP is generally found to be more effective than other topical treatments such as timolol, dorzolamide, and brimonidine (Alm, 2014). LTP works by increasing the outflow fluid from the uveoscleral pathway, ultimately reducing IOP (Lavik et al., 2011). Previous research has demonstrated that LTP may also have a neuroprotective ability in the retina by protecting against the progressive degeneration of RGCs that occurs with elevated IOP in glaucoma (Doozandeh & Yazdani, 2016). A major drawback of these eyedrops is that only 5% of the medication is absorbed and reaches the destination site (Gaudana et al., 2010). The eye has several anatomical and physiological barriers, such as blinking, the corneal epithelium, and the conjunctiva, all of which limit drug entry into the anterior segment of the eye (Agrahari et al., 2016). Due to these barriers, the overall effect of LTP is short lived. In order to experience the therapeutic benefits of LTP, it must be administered properly and consistently. According to the Glaucoma Adherence and Persistency Study, data consisting of 14, 000 subjects demonstrated that only 10% of glaucoma eye drops were continuously used for treatment without any gaps (Friedman et al., 2007). Lack of compliance with glaucoma medications has been associated with disease progression and blindness (Friedman et al., 2007; Newman-Casey et al., 2015). Considering the drawbacks

of current LTP dosing requirements, there is an urgent need for a treatment method that has increased bioavailability, which can ultimately improve patient compliance.

The Effect of Latanoprost on IOP in Mutant Mice and Retinal Ganglion Cells

LTP has been found to be effective in reducing IOP in humans and rodents (Crowston et al., 2004). Previously, our lab treated AP-2β TMR KO mutant mice with LTP to investigate the functionality of the uveoscleral pathway. The baseline IOP readings of the mutant mice were significantly higher compared to their wildtype littermates (Fig.4a). After treating P30 (post-natal day) mice once with LTP, there was a significant reduction in IOP values compared to baseline after 20 minutes of the treatment, after which the IOP values returned to baseline values at 60 minutes. Similar results were observed for 24hrs after treatment (Taiyab et al., 2022) (Fig 4b)(Fig.5). Whereas the previously developed NCC KO mice were not affected by the LTP treatment due to their completely closed angle phenotype (Fig 4b). These findings mimic the effect of LTP on humans and suggest that, although the conventional pathway is blocked, the uveoscleral pathway is still functional in our AP-2β TMR KO mouse model. The accessibility of the uveoscleral/unconventional pathway in this model demonstrates great potential for understanding and discovering potential treatment methods which work through this pathway (Taiyab et al., 2022).

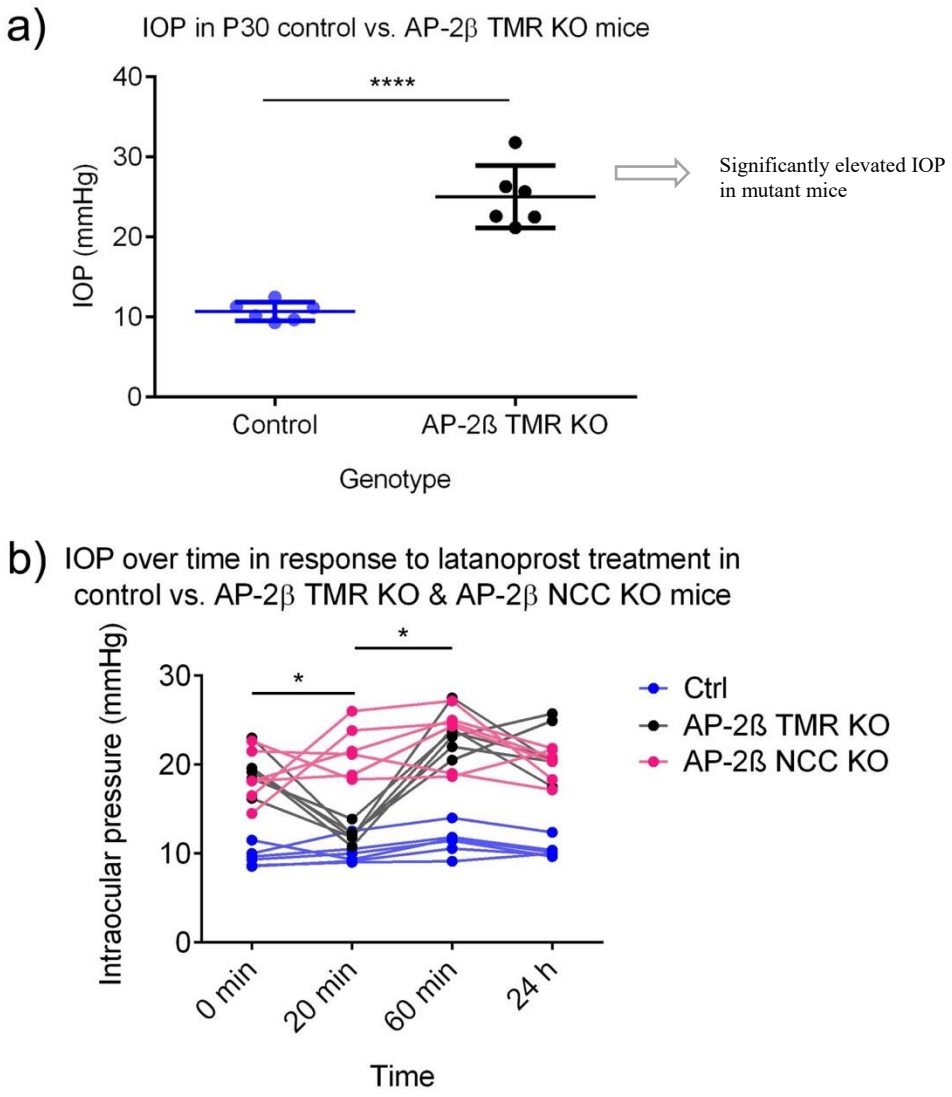


Figure 4. Increased IOP resulting from partial angle closure in AP-2β TMR KOs reduced upon latanoprost treatment. (a) There was a significant increase in IOP in the AP-2β TMR KO mice at P30 (N = 10 eyes; t18 = 12.57; ****p < 0.0001; two-sided independent t test; all error bars signify standard deviation) when compared with wildtype mice. (b) IOP in the AP-2β TMR KO was significantly lower at 20 min after latanoprost treatment (N = 6 eyes; two-way repeated measures ANOVA, Tukey's post hoc test; F (2,40) = 169.8; *p < 0.05) when compared to its own baseline IOP. By 60 min of post-treatment, TMR KO IOP increased significantly compared to the IOP at 20 min (p < 0.0001). On the other hand, AP-2β NCC KO mice displayed high IOP both before and after latanoprost treatment (N = 6 eyes; *p < 0.05 for all time points analyzed compared with wildtype). Adapted from Taiyab et al., 2022.

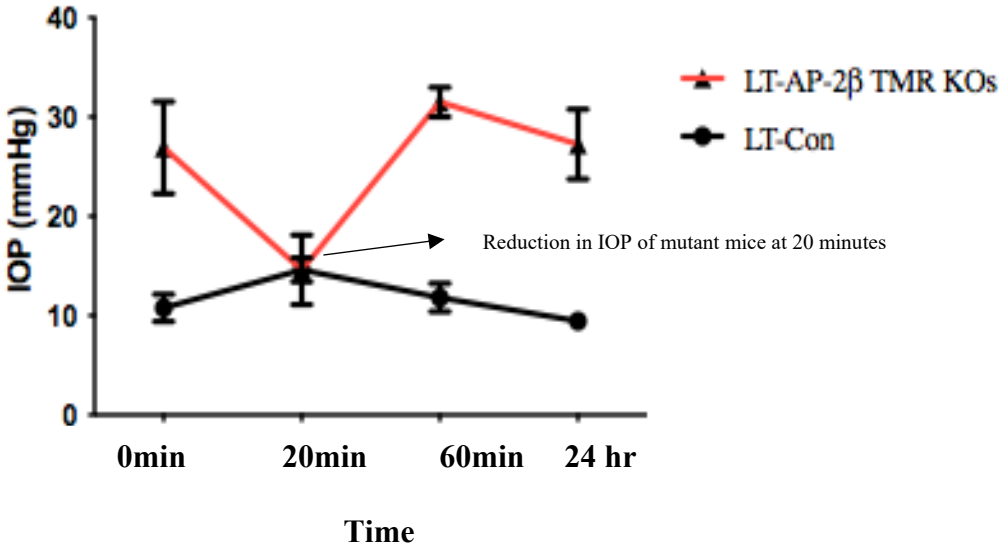


Figure 5. Graph depicting the effect of latanoprost on IOP of P30 mutant mice model. The reduction at 20 mins indicates that uveoscleral pathway is functional in the model. Adapted from Taiyab et al., 2022

Although IOP is widely known to be the primary risk factor for glaucoma, the pathophysiology of glaucoma is unclear. Earlier research indicates that RGCs are most vulnerable to elevated IOP; thus, RGC neuroprotection has been stressed as an important strategy for glaucoma therapy (Hernández et al., 2008). LTP has been found to aid in neuroprotection by lowering IOP, which may occur through the induction of specific matrix metalloproteinases (MMPs), which reduces intrascleral ECM and widen the spaces between ciliary muscle fibres, ultimately facilitating aqueous humour drainage (Hernández et al., 2008).

Markers to Identify Retinal Ganglion Cells

To assess RGC loss in models of glaucoma, counting of markers that represent the RGC is performed. Some methods that are used to quantify RGCs include fluorogold tracers, RNA binding protein with multiple splicing (RBPMS), Thy-1, and Brn3a (Nadal-Nicolás et al., 2023). Brn3a, along with Brn3b and Brn3c are members of the POU-IV class of neural transcription factors. These transcription factors are significant in the developmental processes of RGCs, as well as the differentiation, specification of RGCs, and the elongation of their axons. Brn3a is a valuable marker for identifying RGCs because of its high expression in RGCs during early development and throughout adulthood (Nadal-Nicolás et al., 2023).

To assess retinal cell damage following the increased IOP observed in our mouse model, immunohistochemistry (IHC) staining for the expression of Brn3a was performed. There was no difference in Brn3a expression at P14 in mutant mice compared to wildtype, but a reduced expression of this marker was observed at P30 and P40 (Taiyab et al., 2022). Additionally, IHC of the flat mounted retinas of P60 wildtype and mutant mice revealed a

significant reduction of Brn3a-positive cells in various layers of the mutant retina. This information suggests that decreased retinal function in our model occurred due to the loss of RGCs observed through the Brn3a staining (Taiyab et al., 2022).

The Micelle Based Drug Delivery System

A recently developed drug delivery method to counter the current concerns with LTP treatment is the mucoadhesive micelle-based polymer system (Prosperi-Porta et al., 2016). Polymeric micelles have the ability to spontaneously form through the assembly of hydrophobic cores and hydrophilic shells. This unique self-assembly property enables them to effectively entrap lipophilic drugs and prevent undesired drug exposure to various biological barriers in the eye such as the corneal or conjunctival barriers. The mucoadhesive property of the polymeric micelle is also advantageous, as it increases drug bioavailability in the eye by adhering to the mucosal layer of the cornea, thus enhancing the overall efficacy (Prosperi-Porta et al., 2016). In addition, this form of targeted drug delivery prevents severe adverse effects (Mandal et al., 2017).

Recently, a study by Prosperi-Porta et al., 2016 developed a mucoadhesive block copolymers based on phenyboronic acid (PBA) through the use of reversible addition−fragmentation chain transfer polymerization (RAFT) for the drug delivery of cyclosporine A (CycA) to the ocular mucosa. Cyclosporine A is a treatment for dry eye disease. Mucoadhesion of the micelles were compared with chitosan, a natural mucoadhesive polymer, which was used as a positive control. The micelles demonstrated greater mucoadhesion than the chitosan, offering potential for improved bioavailability of the drug. In the context of our research, we believe this mucoadhesive micelle-based drug

delivery system may particularly be useful in prolonging the effect of latanoprost by extending the time at which IOP is reduced, as well as preventing the need for frequent dosing, and ultimately improving patient compliance.

CHAPTER 2: RATIONALE, MAIN HYPOTHESIS, & RESEARCH AIMS

Rationale

Effective drug delivery is crucial for the success of therapeutic interventions. In the case of ocular diseases such as glaucoma, achieving optimal drug delivery to the target tissues within the eye presents unique challenges. Traditional topical ophthalmic formulations such as LTP often suffer from limitations, such as poor drug retention, low bioavailability, and rapid clearance from the ocular surface due to physiological barriers of the eye which include the conjunctiva and corneal epithelium. As a result, these medications necessitate frequent dosing, making it difficult for older aged patients to comply with their dosing regimen. These limitations highlight the need for alternative drug delivery systems to overcome these challenges of LTP and improve the efficacy of ocular therapies. This information has led us to investigate the use of a micelle-based polymer drug delivery system that would allow for the sustained release of the drug within the eye to prevent any negative outcomes. This study aims to investigate the therapeutic effects of LTP through the use of the micelle-based polymer system. The goal of this research is to benefit individuals suffering from glaucoma and allow researchers to have a strong understanding of the mechanisms of drug delivery.

Since the *tfap2b* gene is important for the regulation of anterior segment structures of the eye, our laboratory previously developed a partially open angle model of glaucoma that mimics the characteristics of glaucoma but also allows for testing of drug delivery due to the active and functional uveoscleral pathway in our model (Taiyab et al., 2022). This model provides us with an opportunity to test for glaucoma medications that alter the uveoscleral pathway. This research will not only provide us with greater knowledge on

drug delivery, but it may also potentially open doors for treatment methods that may be applicable for patients with glaucoma, ultimately improving adherence to glaucoma medications and making an important impact on healthcare as a whole.

Hypothesis

The objective of this project is to investigate the effect of a micelle containing latanoprost (MLTP) drug delivery system in reducing IOP for an extended period of time, thereby preventing the progression of glaucoma. We hypothesize that the mucoadhesive micelle polymer will be more effective in preventing the glaucomatous effects in our AP-2β TMR KO model than latanoprost alone.

Specific Aims

Aim 1. *To test the long-term effect of daily LTP treatment in reducing IOP of the AP-2β TMR KO mutant mice and assess the neuroprotective ability of latanoprost.*

Previously, we observed a transient reduction in the baseline IOP of P30 AP-2β TMR-KO mice following LTP treatment, indicating the functionality and presence of the uveoscleral pathway in our model. The present research seeks to investigate the effect of long-term daily treatment of LTP on the IOP of our mouse model. To investigate the effect on IOP, three cohorts of P30 AP-2β TMR-KO mutant mice and their wildtype littermates will be treated with LTP every day for 60 days. The IOP will be measured every 3 days with IOP being measured at baseline (before LTP treatment), 20 minutes and 1-hour post-treatment. To assess for any protective effect of long term LTP treatment on RGC,

following the 60-day treatment period, immunohistochemistry of Brn3a (marker of RGC) will be performed using paraffin sections and flat mounted retinas from the treated and untreated wild-type and AP-2β TMR KO cohorts.

Aim 2. *To test for the long-term effect of every 3-day MLTP treatment in reducing IOP of the AP-2β TMR KO and preventing the progression of RGC cell loss.*

In this aim we will test for the effectiveness of the MTLP drug delivery system in preventing glaucomatous effects. P30 AP-2β TMR KO mice and their wildtype littermates will be treated with LTP and MLTP every 3 days for 60-days, with IOPs being measured every 3 days. The effect of the long term MLTP treatment on the glaucomatous features will then be assessed with staining for Brn3a on paraffin sections, as well as flat mounted retinas of the mutant and wildtype mice. The results of every 3-day MLTP treatment will then be compared with the daily LTP treatment alone to assess its efficacy.

CHAPTER 3: EXPERIMENTAL DESIGN

Animal Husbandry

All animal procedures were performed in accordance with the association for research in vision and ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research. Mouse breeding has been conducted for MgpCre/AP-2β mouse model. CreLox technology was used to allow for the conditional deletion of AP-2β.

Female *Tfap2b*$^{+/-}$ mice containing a null allele were bred with the Mgp-Cre.KI+/- mouse line obtained from our collaborator, Dr Terete Borras, in which the enzyme Cre recombinase is expressed under the control of the Mgp promoter (Borras et al., 2015). This cross generated *Mgp*-Cre.KI$^{+/-}$*; Tfap2b*$^{+/-}$ mice. The *Mgp*-Cre.KI$^{+/-}$*; Tfap2b*$^{+/-}$ mice were then bred with a mouse line that had exon 4 of Tfap2b flanked by loxP sites *Tfap2b*$^{lox/lox}$ (Martino et al., 2016). The final cross resulted in the generation of *Mgp*-Cre.KI$^{+/-}$*; Tfap2b*$^{-/lox}$ mice, referred to as the AP-2β TMR-KO. These mice contain one active copy in all of the cells except for mgp expressing cells, in which exon 4 of the second copy is excised to allow for a conditional deletion of AP-2β from POM. It is important to note that the Cre transgene was passed on by male breeders in all crosses to minimize differences in Cre recombinase activity due to parent-of-origin, as has been reported previously (Heffner et al., 2012). All genotyping was carried out using standard PCR protocols (Martino et al., 2016). Inbreeding was avoided for final crosses and equal numbers of male and female mice were employed for experiments, with C57BL/6J being the background strain used for all genetic crosses (Charles River, Wilmington, MA). The animals were kept in groups of four in enriched cages that were cleaned weekly and kept at a constant temperature of 25°C for 12 hours of light and 12 hours of darkness.

Intraocular Pressure Measurements and Latanoprost Treatment (Daily)

P30 AP-2β TMR KO mice and their wildtype littermates were topically treated with 10 μL of 0.005% LTP in each eye for 60 consecutive days. Sample size for each treatment group (MLTP) and (LTP) consisted of a minimum of N = 4 eyes for wildtype and mutant mice each. N represents one eye. IOP measurements were obtained every 3 days, in which the IOP was measured at baseline, 20 and 60 minutes after treatment. For each IOP experiment, the mice were anesthetized with an intraperitoneal injection of 0.06 mL of (75mg) Ketamine and (5 mg) Xylazine mixture per 10 grams of body weight. A minimum of 6 IOP readings were obtained from each eye using a rebound tonometer (TonoLab, Vantaa, Finland). Each reading was an average of the top 6 values. Following the 60-day treatment period, the differences in IOP between wildtype and AP-2β TMR KO mice were analyzed using a 2-way ANOVA with repeated measures on GraphPad Prism 9.0 (La Jolla, CA, USA). See diagram below to understand project plan.

Micelle formation and loading with Latanoprost.

The micelles are made with a block copolymer (LMP block copolymer) containing polylactide (PLA) block connected to a block copolymer of methacrylic acid (MAA) and 3-(-acrylamido) phenylboronic acid (PMAA-co-PBA). The micelle can enhance its residence time on the eye due to mucoadhesive property of PBA, which can form a boronate ester bond with the diol groups of mucin and polysaccharides of the mucosa. Two different methods were used to create the polymer. In the first method, controlled radical

polymerization (RAFT) was used for the synthesis of MAA and PBA from a macro-initiator (a RAFT functional PLA)(Prosperi-Porta et al., 2016). The other method does not rely on RAFT and aims to enhance the scalability of the polymer. Instead of RAFT, a thiol functional radical initiator was utilized for the polymerization of MAA and PBA to generate a mono-functional hydrophilic polymer. The hydrophilic polymer was then connected with a hydrophobic maleimide functional PLA through a thiol-ene click reaction (Rambarran & D. Sheardown, 2021). The polymer with the hydrophilic and hydrophobic components was then dissolved in acetone and dropped into water or buffer, to induce micelle formation during the evaporation of acetone. The micelles were radioactively labelled using I125 labelling to test for their mucoadhesive properties (Hall et al., 2016; Liu et al., 2022; Luensmann et al., 2010). Labelled micelles were applied to mouse corneas and their presence was tracked over a period of 10 days. Corneas were treated solely with radioactive I125 as a negative control. Latanoprost was dissolved in acetone solution to allow for the uniform distribution, which then became encapsulated within the micelle core during the evaporation of acetone. Different concentrations of LTP were tested. To test for the release of the drug from the micelle, the micelle was placed into 6 kilodalton molecular weight cut off dialysis tubing and LTP released into phosphate buffered saline at 37° C was quantified by high-performance liquid chromatography (HPLC).

Micelle Latanoprost Treatment (every 3 days)

P30 mutant and wildtype mice were topically treated with 10 μL of micelle containing 0.005% LTP (MLTP) and 0.005% LTP every 3 days for 60 consecutive days. IOP measurements were obtained every 3 days, in which the IOP was measured at baseline (before any treatment) to observe the long-term effect of the treatment. The mice were anesthetized for each IOP experiment as explained above. See diagram below to understand project plan.

Histology and Immunohistochemistry

Eyes were extracted from euthanized mice and fixed in 4% paraformaldehyde (PFA) for 24hr at 4 degrees and then stored in 70% ethanol for paraffin sections. The eyes were sent for processing and embedded in paraffin wax. The paraffin blocks were sectioned at 4μm thickness then used for IHC or H&E staining. The paraffin embedded sections underwent deparaffinization in xylene and rehydration in 100%, 95% and 70% ethanol. The sections were rinsed with water and treated with 10mM of sodium citrate (pH 6.0) for antigen retrieval. The sections were then blocked with 5% normal serum for 1hr and incubated with primary antibody Brn3a (1:100, sc-8429, Santa Cruz, CA) overnight at 4 degrees. The next day, the sections were incubated with AlexaFluor secondary antibody (1:200, Invitrogen, Molecular Probes, Burlington ON) to detect the primary antibody. The slides were mounted using the ProLong Gold with the nuclear stain DAPI (4',6-diamidino-2-phenylindole) (Thermofisher, Waltham, MA). Lastly, the sections were imaged with Leica DM6 B microscope and acquired using LasX imaging software.

Immunohistochemistry of Flat mounted Retinas

The eyes were extracted and fixed in 4% PFA overnight. The next day, the eyes were washed with PBS and then the retinas were dissected out permeabilized overnight at room temperature in 0.3% Triton-X mixed in PBS (PBST). The retinas were then blocked in normal serum containing PBST for 3hr at room temperature and then mixed with 1:100 dilution of Brna3a antibody (Santa Cruz, CA) with 1% DMSO and 5% normal serum in PBST for 72 hours at 4 º C. Retinas were then washed and mixed in a solution of 1:200 secondary antibody with 1% DMSO and 2.5% normal serum in PBST for 4 hours at room temperature. The retinas were washed in 1xPBS, and flat mounted with mounting medium containing DAPI and cover slipped.

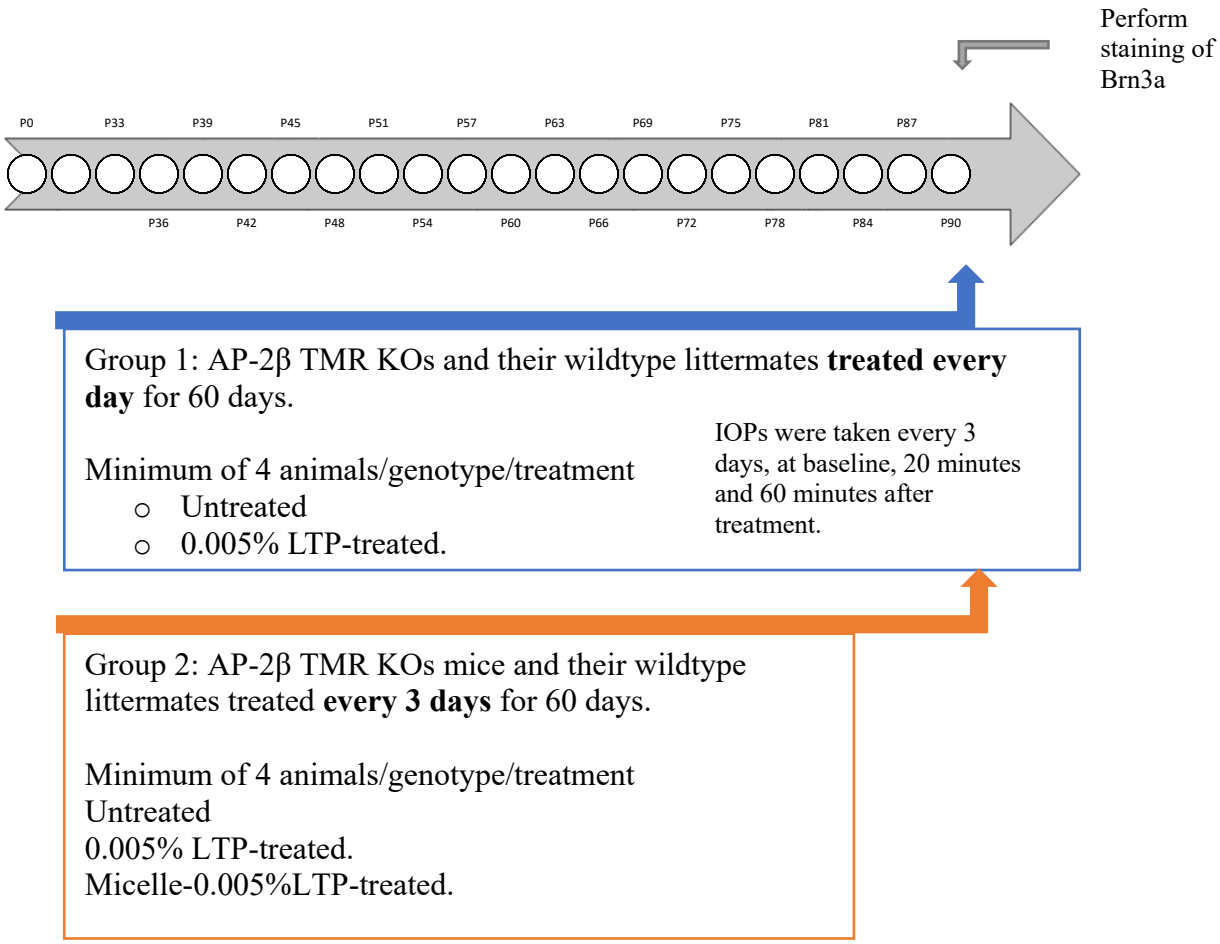


Diagram 1) Project plan. This is a timeline diagram illustrating the overall project plan and details regarding when experiments were conducted. P30 (N = 16 eyes) AP-2β TMR KOs and their wildtype littermates (N= 14 eyes) were treated with LTP to assess for IOP and LTP neuroprotectivity of the retinal ganglion cells. This same experiment was then repeated with treatment MLTP, with P30 (N = 10 eyes) AP-2β TMR KOs and their wildtype littermates (N= 10 eyes)

CHAPTER 4: RESULTS

Aim 1 Results

1.1 Daily long-term LTP treatment significantly reduces baseline IOP of AP-2β TMR-KO

The IOP of untreated AP-2β TMR KO mice P30 (N = 3 eyes) was found to be significantly elevated compared to wildtype mice P30 (N =4 eyes) at P30 (two-sided independent *t* test; t_{14} = 16.58; ****p < 0.0001) (Figure 6), similar to what has been observed in patients with PACG (Taiyab et al., 2022)

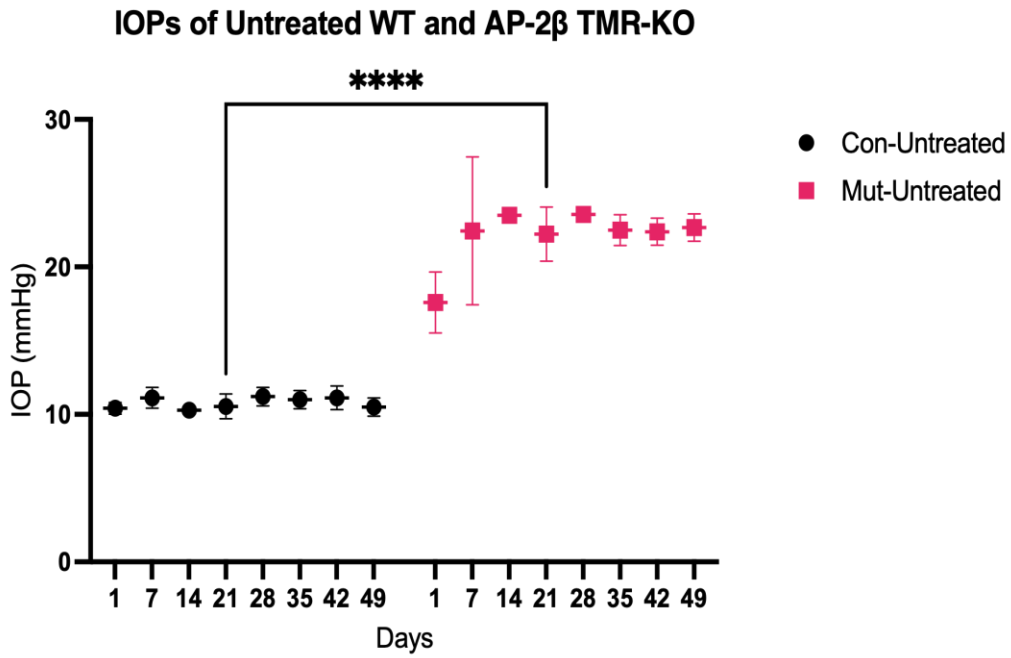


Figure 6. IOP of untreated mutant vs untreated wildtype animals. There was a significant increase in IOP in the AP-2β TMR KO mice at P30 (N = 3 eyes; two-way repeated measures ANOVA, Tukey's post hoc test; F (1,40) = 870.5; ****p < 0.0001) when compared to wildtype mice (N = 4 eyes).

To investigate the long-term effect of LTP on this model, we performed daily LTP treatment over a 60-day time-period across three cohorts of AP-2β TMR KO and wildtype littermates. We measured IOP every 3 days at baseline (before treatment), 20 minutes and 1 hour after treatment. Figure 7 represents the IOP values measured at baseline, 20 minutes and 1hour following the daily LTP treatment period. For every IOP experiment, we noticed a consistent pattern with the IOP reducing after 20 minutes of treatment and rebounding back to baseline 1 hour after treatment. At day 1, the baseline IOP of the AP-2β TMR KO mice (n=16 eyes) (23.09mmHg± 1.83) was observed to be significantly reduced ($p<0.0001$) to (11.94mmHg± 2.2) 20 minutes after treatment. Similarly, the baseline IOP of the AP-2β TMR KO mice at day 35 (17.77mmHg±1.02) and day 49 (17.64mmHg±0.71) was significantly reduced ($p<0.0001$) to 10.67mmHg±0.78 and 10.56mmHg±0.75, respectively (Fig.7).

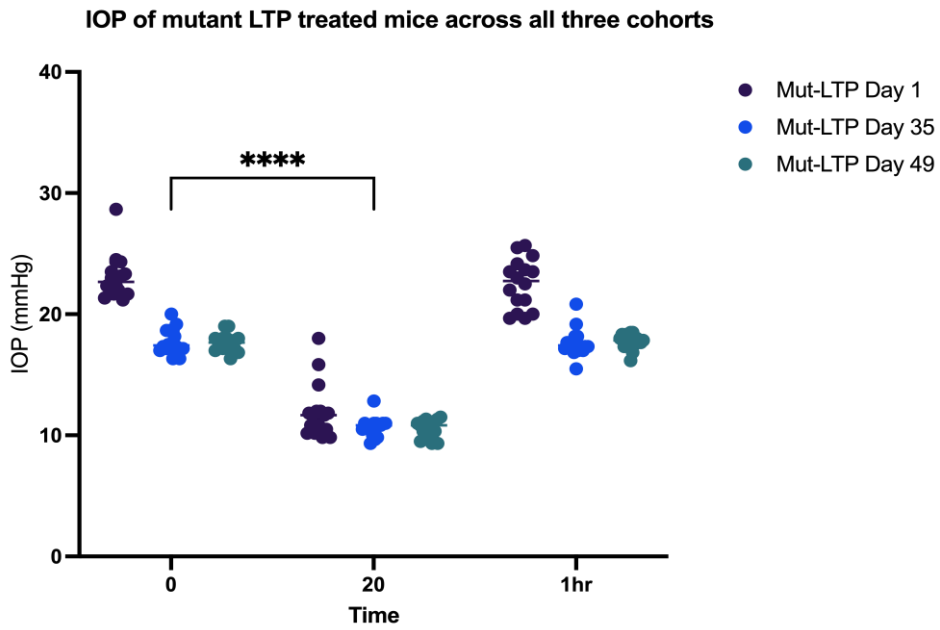


Figure 7. IOP of daily long term LTP treatment of AP-2β TMR KOs measured at baseline, 20 minutes and 1 hour after treatment. There was a significant reduction (****p<0.0001) in IOP after 20 minutes of treatment across all IOP days, which then went back up 1 hour after treatment in AP-2β TMR KOs (N = 16 eyes; two-way repeated measures ANOVA, Tukey's post hoc test; $F(4,135) = 13.59$; $p < 0.0001$) when compared to wildtype mice (N = 14 eyes).

Although, these IOP measurements were rebounding back to baseline values at the 1 hour mark for every IOP experiment, we noticed that throughout the duration of the treatment period, the baseline IOP of the AP-2β TMR KO mice became reduced over time, with a significant reduction from (23.17mmHg±0.72) to (17.71mmHg±0.51, N =16 eyes, P < 0.0001) observed by day 35 of treatment across all three cohorts (Fig.8). The IOP measurements of wildtype mice remained the same throughout the duration of the treatment (10.72mmHg±0.67, N=14 eyes) (Fig. 8)

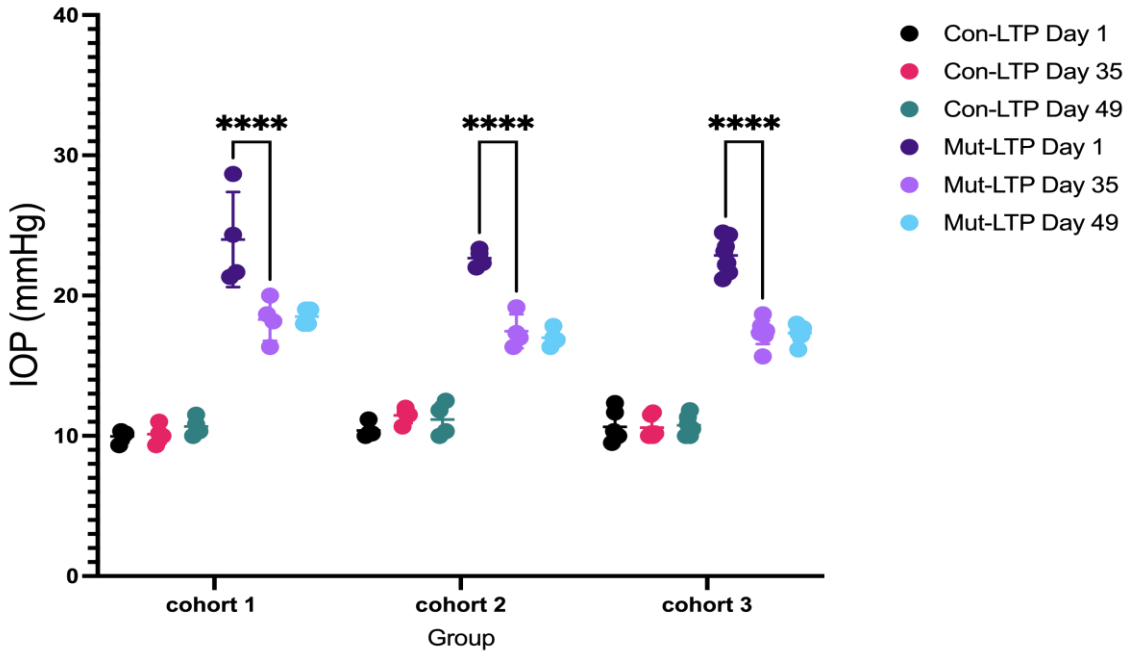


Figure 8. Long term latanoprost treatment of AP-2β TMR KOs results in the reduction of baseline IOP across 60-day treatment. The baseline IOP of AP-2β TMR KO mice significantly declined across the long-term treatment, with a significant decrease in baseline IOP at around 35 days of treatment in AP-2β TMR KOs (N = 16 eyes); two-way repeated measures ANOVA, Tukey's post hoc test; F (5,72) = 318; ****p < 0.0001) (all error bars signify standard deviation).

1.2 Assessment of glaucomatous effects following daily long term LTP treatment

Since LTP is known to protect against RGC death and retinal function in humans, we were interested in investigating the neuroprotective effect of long-term LTP treatment in our study (Doozandeh & Yazdani, 2016). wildtype (N = 3 eyes) and mutant ocular sections (N = 4 eyes) and wildtype (N = 2 eyes) and mutant (N = 5 eyes) flat mounted retinas were stained for Brn3a to assess RGC number. Both analyses revealed a significant reduction in RGC cell number (as revealed by Brn3a staining) in the mutant untreated mice compared to wildtype untreated mice ($p< 0.05$), as we have previously reported (Taiyab et al., 2022)(Fig 9A and 10A). Analyses of ocular sections (N =6 eyes) and flat mounted retinas (N = 9 eyes) of mutant mice treated with LTP for 60 days also revealed a similar significant reduction in RGC number as compared to ocular sections (N = 4 eyes) and flat mounted retinas (N = 4 eyes) of the wildtype treated mice ($p< 0.05$) (Fig 9B and 10B).

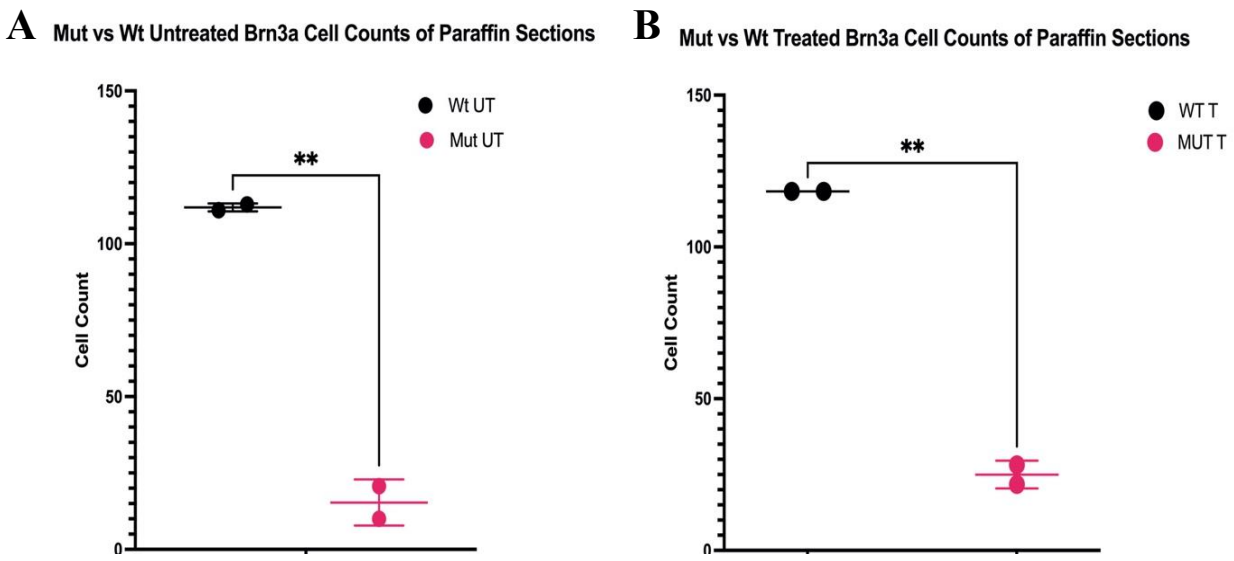


Figure 9. Increased IOP was associated with the increased glaucomatous changes in paraffin sections of untreated and LTP treated AP-2β TMR KOs and wildtype mice. A) There was a significant reduction in the number of Brn3a-positive cells in paraffin sections of mutant untreated (N = 3 eyes) when compared with wildtype untreated (N= 4 eyes) retinas overall at the periphery and central regions of the retina (two-sided independent *t* test for each region; $t_2 = 17.84$; **p < 0.001). B) There was a significant reduction in the number of Brn3a-positive cells in paraffin sections of mutant treated (N = 6 eyes) when compared with wildtype treated (N = 4 eyes), sections overall at the periphery and central regions of the retina (two-sided independent *t* test for each region; $t_2 = 28.98$; $p < 0.001$)

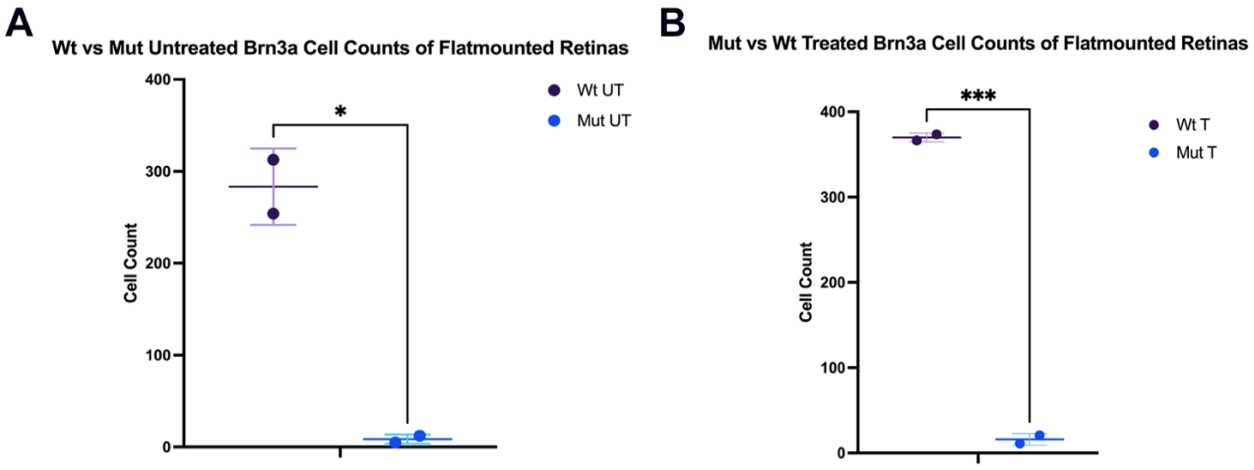


Figure 10. Increased IOP was associated with the increased glaucomatous changes in flatmounted retinas of untreated and LTP treated AP-2β TMR KOs and wildtype mice A)There was a significant reduction in the number of Brn3a-positive cells in flat mounted retinas of mutant untreated (N = 5 eyes) when compared with wildtype untreated (N = 2 eyes) retinas overall at the optic nerve head (ONH), mid-periphery and peripheral regions of the retina (two-sided independent *t* test for each region; $t_2 = 9.297$; *$p < 0.05$). B) There was a significant reduction in the number of Brn3a-positive cells in flat mounted retinas of mutant treated (N = 9 eyes) when compared with wildtype treated (N = 4 eyes) retinas overall at the ONH, mid-periphery and peripheral regions of the retina (two-sided independent *t* test for each region; $t_2 = 59.82$; ***$p < 0.001$).

Aim 2 Results

Following the completion of LTP treatment, we performed a 60-day MLTP and LTP treatment on P30 wildtype and mutant mice. One batch of micelle treatment contained a total volume of 2400uL solution for the first cohort of mice (N =12 eyes). The concentration of LTP for the micelle formulation was 0.005% (50ug/ml in PBS-pH6.2) and the LMP polymer concentration was 5mg/ml in PBS. Treatment groups consisted of a minimum of N=6 eyes for mutant and wildtype mice each for both treatment groups, MLTP and LTP alone. The MLTP groups of animals (wildtype and mutant) were treated every 3 days, and this was compared with animals treated with LTP every day as well as animals treated every 3 days with LTP alone for comparison's sake (refer to diagram 1 for treatment plan). The difference between the untreated AP-2β TMR KO and wildtype mice was consistent with previous results, showing untreated AP-2β TMR KO mice having significantly elevated ($p< 0.0001$) IOP compared to untreated wildtype mice (Fig. 11).

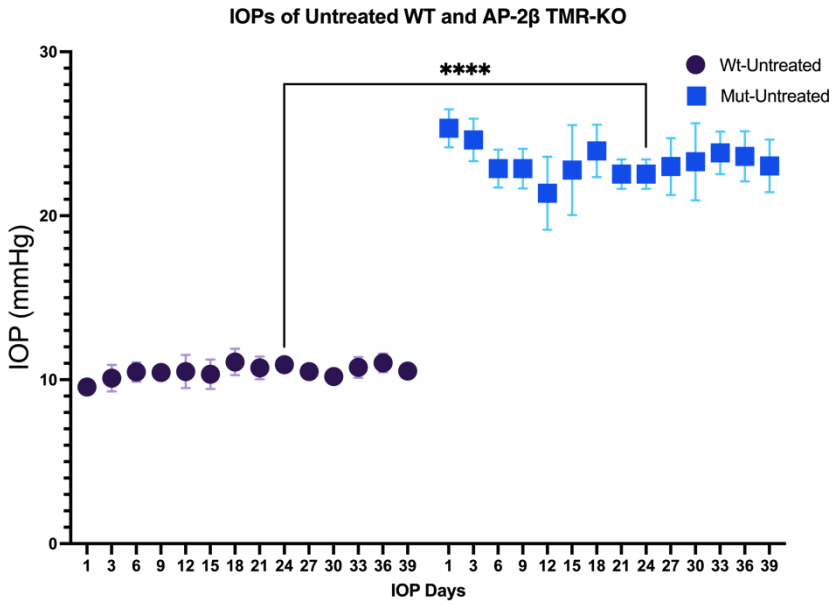


Figure 11. IOP of untreated mutant vs untreated wildtype animals. There was a significant increase in IOP across all days in the P30 AP-2β TMR KO mice (N = 4 eyes); two-way repeated measures ANOVA, Tukey's post hoc test; F (1,112) = 4425; ****p < 0.0001) (all error bars signify standard deviation) when compared with wildtype mice (N = 6 eyes).

2.1 Assessment of IOP following every 3-day MLTP treatment compared to every day LTP treatment as well as every 3-day LTP treatment.

Throughout the treatment period it was observed that the baseline IOP of mutants treated with the MLTP appeared to be declining, similar to what was previously observed with everyday LTP treatment. The baseline IOP of the MLTP treated mutants was significantly lower following 6 days of treatment ($p<0.0011$) (Fig.12). This was observed to be an earlier time point compared to that previously observed for the everyday LTP

treatment, which did not demonstrate a reduction in baseline IOP until 35 days of treatment (Fig. 8). The next reduction in baseline IOP was observed from day 6 to day 18 ($p<0.0001$) in the MLTP group. There was also a significantly lower baseline IOP observed in the MLTP treated mutants compared to the daily LTP treated mutants on days 18 ($p<0.0033$), 24 ($p<0.0001$), and 27 ($p<0.0017$) (Fig.12). In contrast, the baseline IOP ranges of mutant mice that were treated with LTP for every 3 days were mostly consistent throughout the treatment period. In this case we observed that the AP-2β TMR KO mice demonstrated no change in baseline IOP through the treatment period, and the IOPs were consistently elevated unlike the 3-day MLTP treated mutants which demonstrated a significantly lower baseline IOP ($p< 0.0001$) (Fig.13).

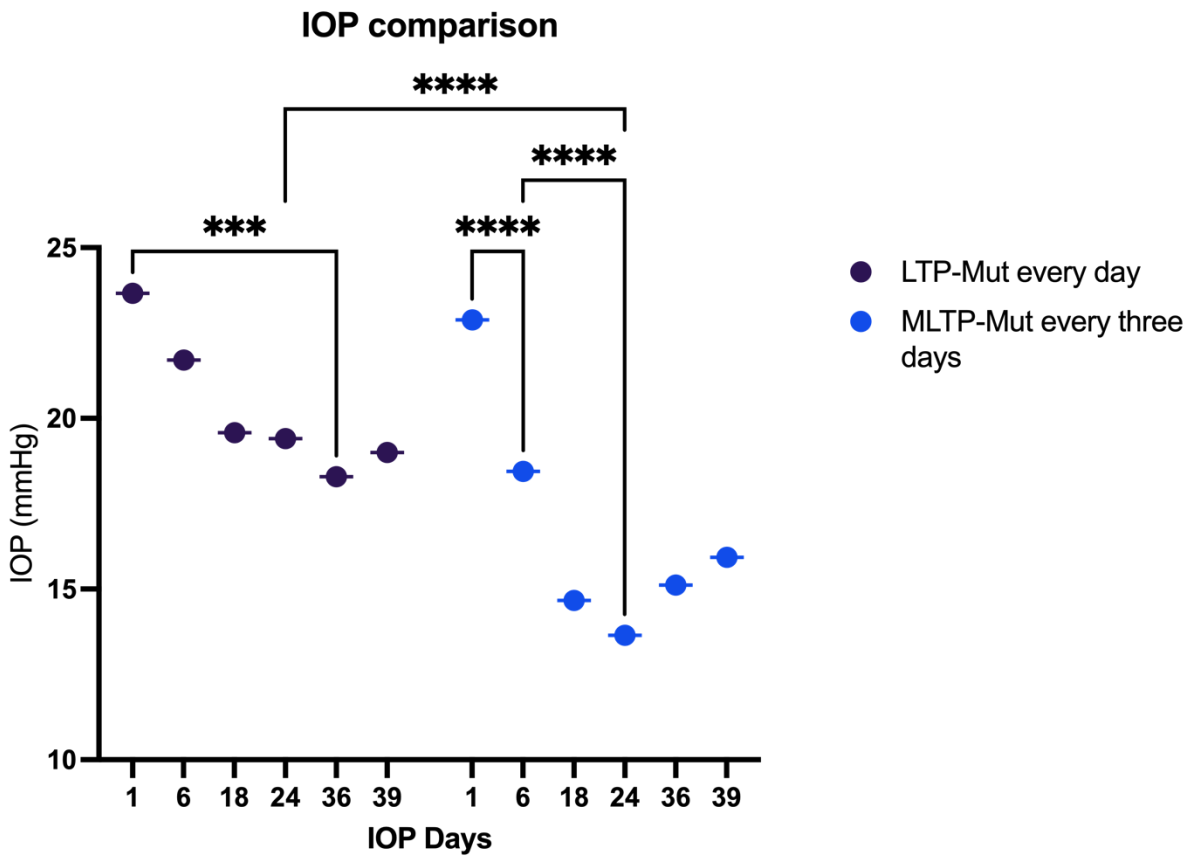


Figure 12. IOP of mutants treated everyday with LTP vs mutants treated every 3 days with MLTP. As seen previously, IOP in AP-2β TMR KOs (N = 16 eyes) treated with LTP every day is significantly reduced at 36 day of treatment (***$p < 0.001$). Whereas the IOP in the MLTP group significantly reduced from baseline at day 6 of treatment (N = 10 eyes); two-way repeated measures ANOVA, Tukey's post hoc test; $F(5, 72) = 29.78$; ****$p < 0.0001$) (all error bars signify standard deviation). The IOP of the MLTP treated mutant mice continued to reduce up until day 24 of treatment ($p < 0.0001$). There was also a significant reduction in the IOP of MLTP mice (N = 10 eyes); two-way repeated measures ANOVA, Tukey's post hoc test; $F(5, 72) = 77.06$; $p < 0.0001$) (all error bars signify standard deviation) when compared to IOP of mutant mice treated with daily LTP on 24 days of treatment.

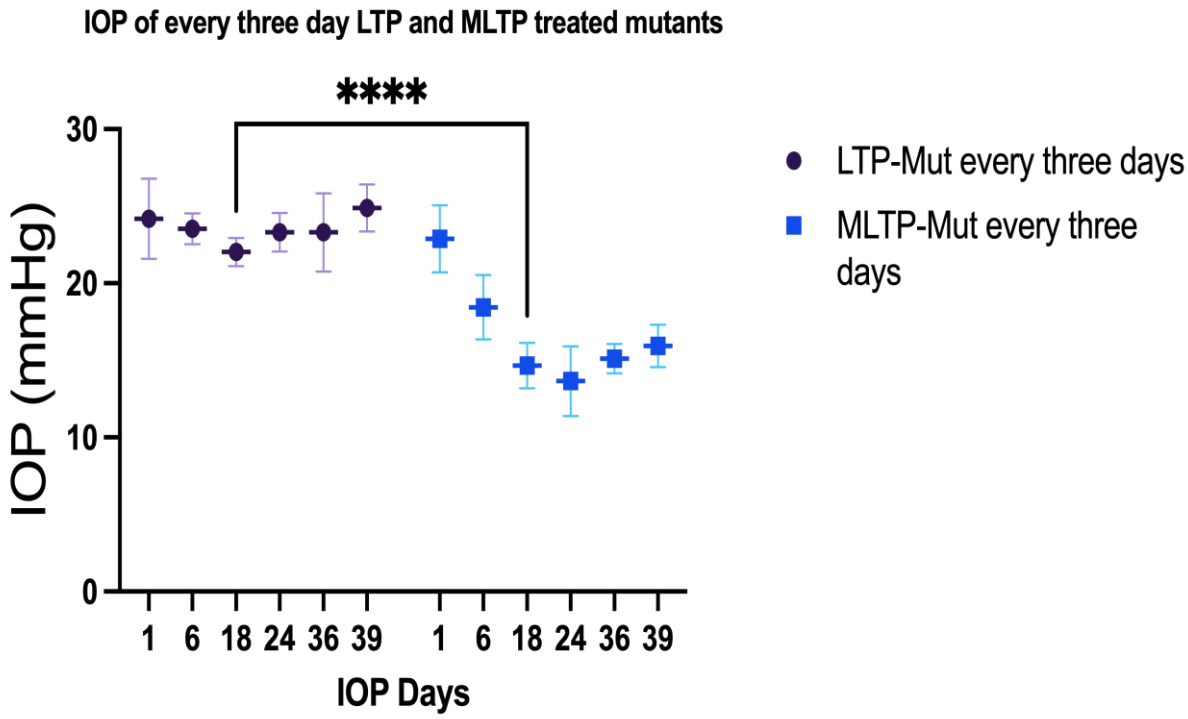


Figure 13. IOP of mutants treated every 3 days with LTP and MLTP. IOP of every 3-day MLTP treated mutants (N = 10 eyes) was significantly higher than mutants treated alone with LTP across all time points at day 6 and onwards (N = 6 eyes); two-way repeated measures ANOVA, Tukey's post hoc test; F (1, 88) = 337.6; ****p < 0.0001) (all error bars signify standard deviation).

2.2 Assessment of glaucomatous effects following every 3-day MLTP treatment compared to all groups.

Based on the prediction that the MLTP treatment would be able to consistently lower the baseline IOP in the mutant mice over an extended period, we hypothesized that this may provide protection to the RGCs. To investigate this, immunohistochemistry of Brn3a was performed on flat mounted retinas (N = 5 eyes) and paraffin sections (N = 3 eyes) of P90 MLTP treated AP-2β TMR KO mice and on flat mounted retinas (N = 4 eyes) and paraffin sections (N = 4 eyes) of P90 MLTP treated wildtype mice. For flat mounted retinas, Brn3a cell counts were performed in the area near the optic nerve head, the mid-periphery and peripheral retina (analyzed regions shown in Fig.14) using Image J. For paraffin sections, a total of three measurements were acquired for each section, one from the central retina area and one each from the peripheral region, the average was then taken for each section (sample shown in Fig 15). Following analysis, a significant reduction in RGC cell counts (as shown by Brn3a staining) was observed in the mutant MLTP mice (N=5 eyes) as compared to wildtype MLTP mice (N= 4 eyes) ($p< 0.0001$) in flat mounted retinas (Fig.16A) and in mutant MLTP mice (N= 3 eyes) compared to wildtype MLTP mice (N= 4 eyes) ($p< 0.001$) paraffin sections (Fig.17A). However, the MLTP every 3 day treated mutants demonstrated little, however significant improvement in cell protectivity ($p<0.05$) compared to daily LTP treated mutants in both flat mounted retina cell counts ($p< 0.05$) (Fig.16B) and paraffin section cell counts of treated mutant mice ($p< 0.05$) (Fig.17B). The Brn3a cell count of MLTP treated mutants (N =5 eyes) exhibited a 5-fold increase in cell protection than mutants treated with LTP everyday (N=8 eyes). While the retinal

wholemounts did indicate RGC protection in mutants treated with MLTP every 3 days compared to mutants treated with LTP every day, we were unable to perform in-depth analyses as scanning was not possible on our fluorescent microscope. Therefore, we performed paraffin sectioning using the eyes from other treatment cohort. In paraffin sections, we observed a ~ 2-fold increase in Brn3a cell count of MLTP treated mutants (N=3 eyes) compared to mutants treated with LTP daily (N=6 eyes). Refer to figures 18 and 19 for retinal and paraffin section images of wildtype mice and mutant mice treated with LTP and MLTP.

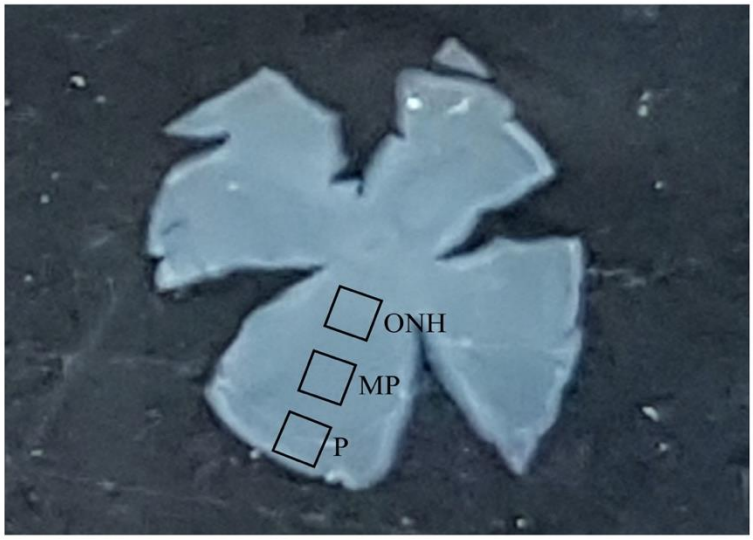


Figure 14. A flat mounted retina labelled with sample regions analyzed in wildtype and AP-2β TMR KO mice. This flat-mounted retina shows which regions are analyzed in wildtype and AP-2β TMR KO mice. The three regions are: the area adjacent to the optic nerve head, the mid-peripheral retina, and the peripheral retina.

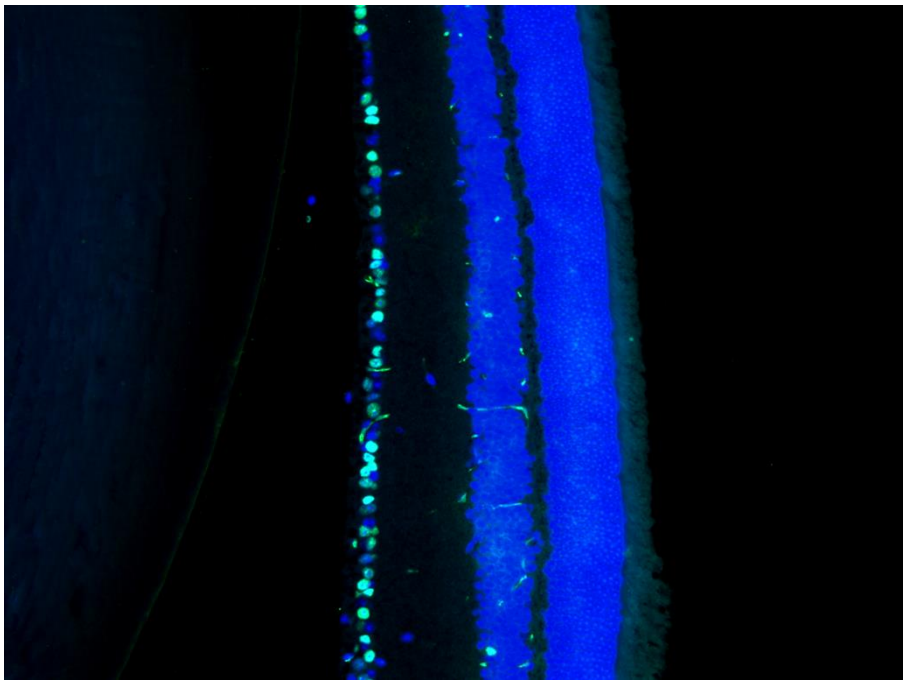


Figure 15. Sample immunostained paraffin section of the mouse retina. Mouse monoclonal Brn3a antibody to label RGCS (green). Nuclei are visualized by DAPI stain (blue).

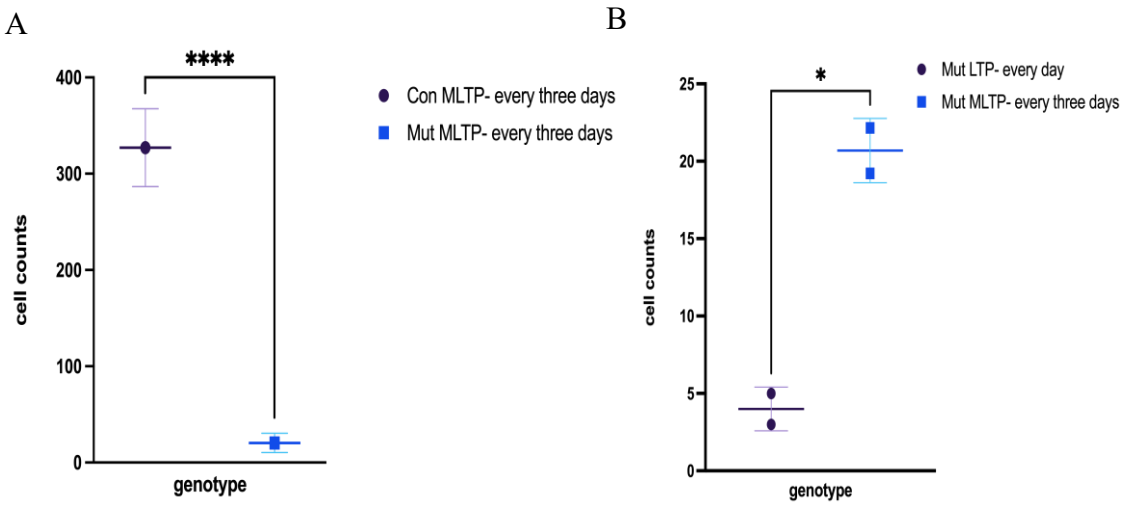


Figure 16. IHC Assessment of glaucomatous changes on retinal flat mounts of treated MLTP, LTP every day and every 3-day AP-2β TMR KOs. A) There was a significant reduction in Brn3a cell counts of MLTP treated mutants (N =5 eyes) compared to the wildtype MLTP treated mice (N=4 eyes) overall at the optic nerve head (ONH), mid-periphery and peripheral regions of the retina (two-sided independent t test for each region; $t7 = 16.58$; ****$p<0.0001$). B) MLTP treated mutants (N =5 eyes) had significantly more cell counts (*$p<0.05$) than every day LTP treated retinas (N =8 eyes) overall at the optic nerve head (ONH), mid-periphery and peripheral regions of the retina (two-sided independent t test for each region; $t2 = 9.388$; $p < 0.05$).

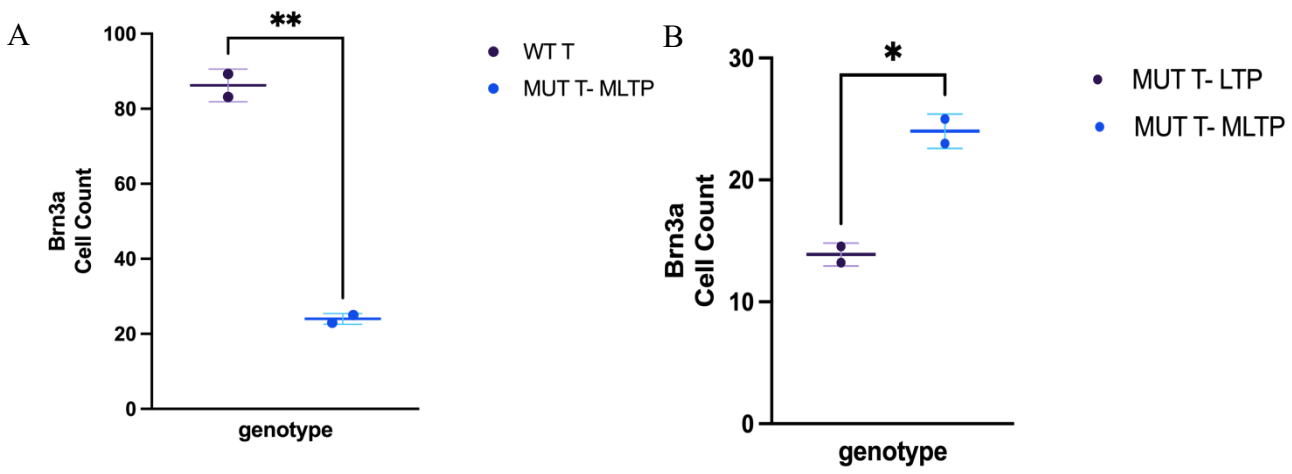


Figure 17. IHC Assessment glaucomatous changes on paraffin sections of MLTP every 3-day treated and LTP everyday treated AP-2β TMR KOs. A) There was a significant reduction in Brn3a cell counts of MLTP treated mutants (N =3 eyes) compared to the wildtype treated mice (N =4 eyes) (two-sided independent t test for each region; $t_2 = 19.24$; **$p<0.001$) B) MLTP treated mutants (N = 3 eyes) had significantly more cell counts than every day LTP treated mutants (N = 6 eyes) (two-sided independent t test for each region; $t_2 = 8.423$; *$p<0.05$)

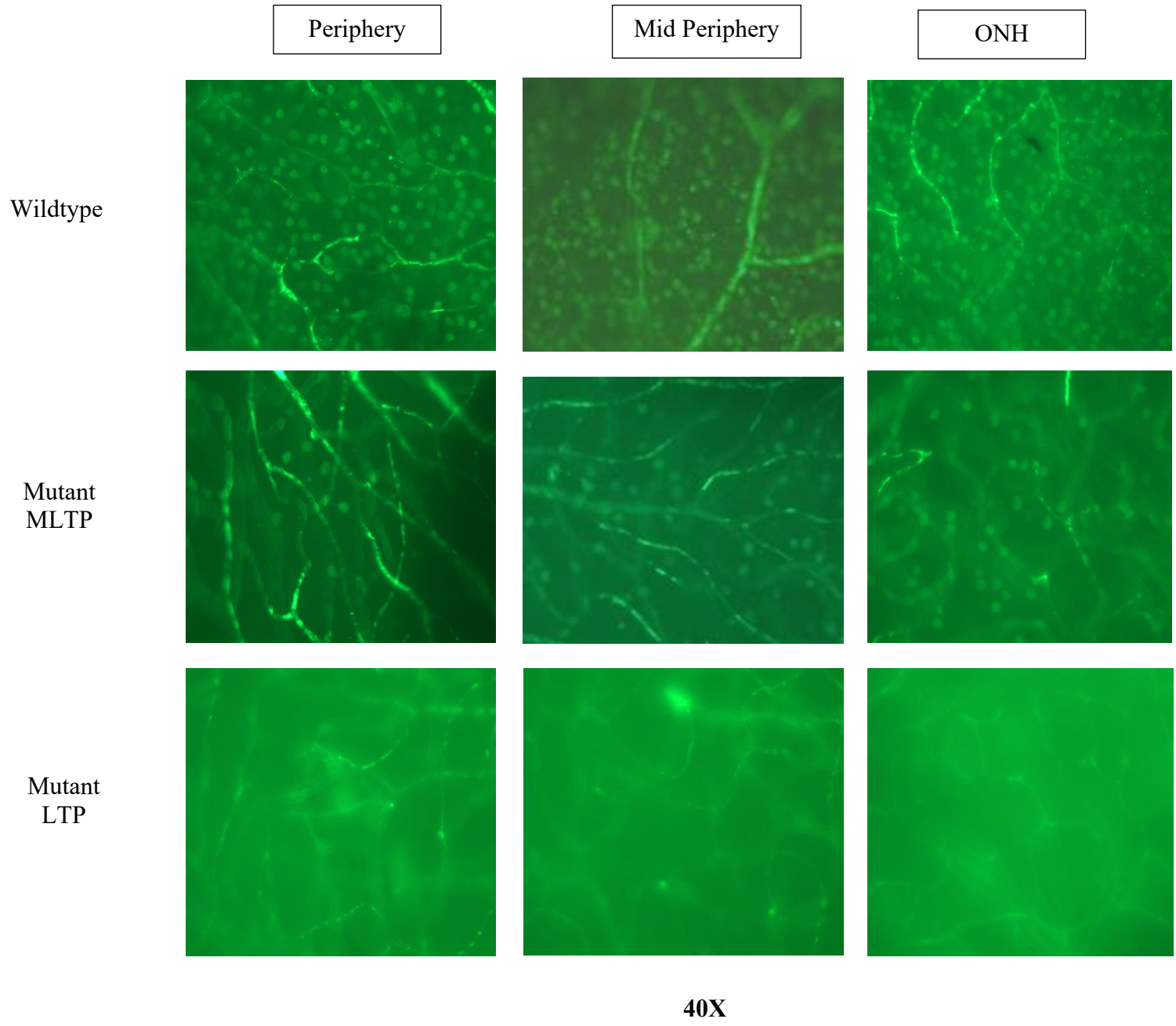
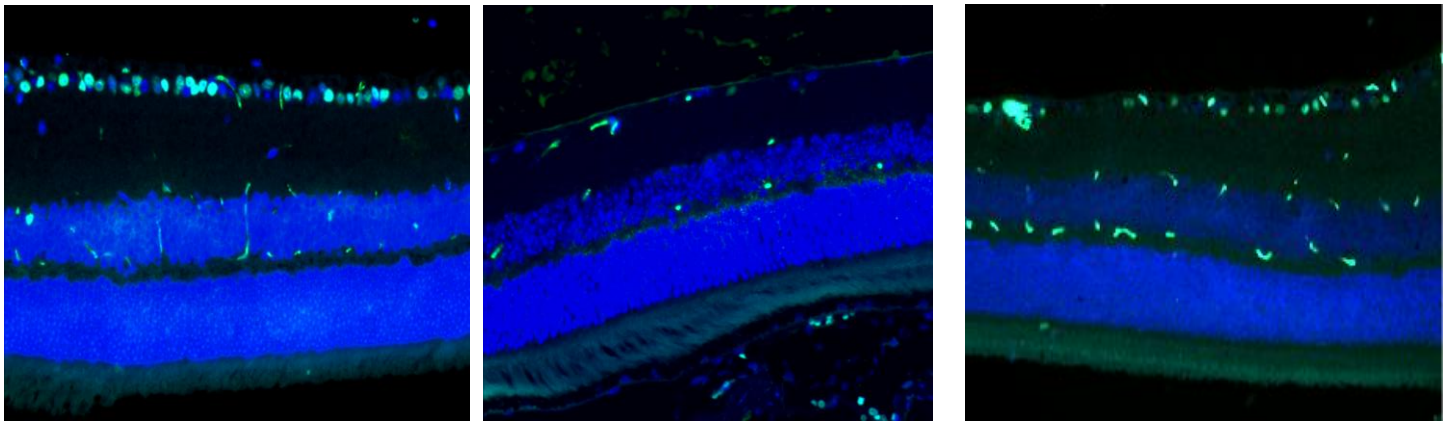


Figure 18. Representation of Brn3a cell counts in flat mounted retinas. This figure represents a visualization of Brn3a cell counts in 40X objective in periphery, mid periphery, and ONH head regions of wildtype (N= 4 eyes) and mutant MLTP (N =5 eyes) and LTP (N = 8 eyes) treated flat mounted retinas.

Wildtype | Mutant LTP treated. | Mutant MLTP treated.



20X

Figure 19. Representation of Brn3a cell counts in paraffin sections. This figure represents a visualization of Brn3a cell counts in 20X objective in paraffin sections of wildtype (N= 4) and mutant MLTP (N =3 eyes) and LTP (N = 6 eyes) treated mice

CHAPTER 5: DISCUSSION, FUTURE DIRECTIONS, AND CONCLUSION

Discussion

Due to challenges associated with current topical medications for glaucoma, it is critical to explore alternative drug delivery methods that would reduce compliance related issues, as this poses a serious threat to the health of individuals with glaucoma. Considering this, our lab has employed a partially closed angle model of glaucoma (AP-2β TMR KO) for testing a novel micelle-based drug delivery model system for reducing IOP. Our model demonstrates key characteristics of glaucoma including increased IOP and a reduction of RGCs in a fully penetrant manner among all mutant mice. In addition, previous research conducted in our laboratory had shown an impairment of the conventional pathway in this mouse model, while highlighting the intact functionality and presence of the uveoscleral pathway (Taiyab et al., 2022). This finding was confirmed by the administration of latanoprost in the mutant eyes, which resulted in a reduction in IOP within 20 minutes of treatment. This quick reduction in IOP suggests that our model is an acute partially closed angle model of glaucoma (Taiyab et al., 2022). As such, these key characteristics of our model highlight its potential for the testing of novel IOP lowering drug delivery methods.

In the first aim of this study, we tested for the long-term effect of LTP treatment in the AP-2β TMR KO mouse model. There is currently a lack of PACG mouse models that exhibit sustained IOP reduction over an extended duration. Since LTP significantly impacts aqueous humor outflow via the uveoscleral pathway, we hypothesized that long-term LTP treatment in our AP-2β TMR KO mouse model could reduce IOP over an extended period and in turn, prevent further complications of glaucoma. Following long term LTP treatment

on AP-2β TMR KO and wildtype littermates, we noticed a consistent decline in the baseline IOP of the mutant mice and this was significant by 35 days of treatment (Fig.8). The mechanism of how LTP reduces baseline IOP in the mutants over the treatment time is not clear. However, LTP is known to have a major influence on the uveoscleral pathway, a pathway we know is operational in the AP-2β TMR mutant mice. LTP may be working to enhance this pathway through its binding with multiple receptors in the uveoscleral pathway in tissues such as the ciliary muscle, to exert its function. For example, LTP is known to target the Prostaglandin F receptor (FP), which is a G-protein coupled receptor (GPCR), as well as other types of GPCRs such as EP1, EP2, EP3, and EP4 that can interact with Prostaglandin E2 receptor (PGE2) in the uveoscleral pathway. These receptors are found in smooth muscle cells of the ciliary body and their activation can cause contraction and relaxation of the muscle, thereby reducing IOP (Winkler & Fautsch, 2014). Additionally, a study by Ocklind, 1998 investigated the underlying effects of LTP on the ciliary matrix of cultured human and monkey ciliary muscle and found a reduction in certain components of the ECM (collagens I, III, and IV, fibronectin, laminin, and hyaluronan) along with an increase in matrix remodelling enzymes, such as metalloproteinase-2 and -3. This information suggests that LTP may reduce IOP over time by promoting the remodeling of ECM in the uveoscleral outflow pathway. Together these findings suggest that the reduction in baseline IOP we observed in our mutant model following long-term treatment of LTP may be due to progressive changes in the ciliary muscle and/or the ECM of the uveoscleral outflow pathway. Further studies are warranted to determine if this may be the case.

Although baseline IOP was significantly reduced in the mutant mice following long-term LTP treatment, the immunostaining for RGCs by Brn3a revealed that the decrease in IOP (7 mmHg point decrease in IOP) was insufficient to provide protection against RGC death. A combination of factors may be leading to this overall outcome for instance, during the IOP experiments, we observed a consistent fluctuation or spike in IOP levels with IOP reducing at 20 minutes but increasing back to baseline values at 60 minutes. This phenomenon has also been documented in human studies, in which similar spikes in IOP following cataract surgery in individuals have been shown to result in glaucomatous changes and progression of visual field loss (Weiner et al., 2015). Therefore, it is plausible that these IOP spikes contributed to the loss of RGCs in our study (Weiner et al., 2015). Additionally, the residence time of LTP within the murine eye is likely short due to the fact that aqueous turnover in mice is known to be quick. This may have reduced the efficacy of the drug, resulting in a lack of sustained reduction in IOP (Johnson et al., 2017).

In the second aim of our study, we were interested in investigating the possibility of lowering IOP with reduced treatment frequency through an alternative drug micelle-based delivery approach. This alternative approach offers several advantages, one of which is the ability of the micelle to bind to the corneal mucin for a sustained period of time due to its mucoadhesive properties. This key characteristic of the micelles enables them to reduce the amount of drug loss that may occur due to tear turnover, as they can resist washout. Additionally, the micelles allow for the sustained presence of the drug within the eye and subsequently, the continuous, and slow release over an extended period of time.

Previous literature suggests that it is the hydrophobic component of the micelle that stabilizes the micelle within the tear film, ultimately preventing the micelle from washout and disassembling. A study by (Prosperi-Porta et al., 2016) has confirmed this information by testing for the mucoadhesive ability of the micelle to resist washout. It was found that LMP-0 micelles that contained no PBA content (hydrophobic component of the micelle) were found to be the least adhesive and more likely to wash away compared to the PBA-containing micelles. The PBA containing micelles were strongly effective in sticking to the mucosal layer and not washed out due to their ability to form additional chemical bonds and interactions with mucin glycoproteins (Prosperi-Porta et al., 2016). This information suggests that it is the hydrophobic component of the micelle that enables it to increase drug residence time and facilitate the continuous release of the drug for an extended time period.

We hypothesized that due to this key characteristic of the micelle-based drug delivery system, the micelle containing LTP treatment may be more effective in reducing IOP and protecting against glaucomatous effects compared to LTP treatment alone. We performed MLTP treatment every 3 days for a total of 60 days on P30 AP-2β TMR KO and their wildtype littermates, along with every 3-day LTP treatment. IOP measurements were also performed every 3 days during the treatment period. MLTP treatments were performed specifically at 3-day intervals because of the micelle's ability to bind to the corneal mucin, that has a turnover rate of ~3 days, for more than a week (Hanna et al., 1961). An unpublished observation from our collaborator (Dr. Heather Sheardown, McMaster University) suggests a similar finding. Thus, the Micelle LTP treatment every 3 days should allow for the sustained presence of LTP over an extended period of time, ultimately

enabling LTP to exert its effect for longer. Following statistical analysis of IOP values, we found that MLTP mutants demonstrated a larger reduction in baseline IOP compared to LTP treatment alone. The reduction in baseline IOP of mutant mice treated with MLTP was observed just after 6 days of treatment, whereas the first significant reduction in IOP in mutant mice treated with LTP alone was not observed until around 35 days of treatment (Fig.12). The quick reduction following 6 days of treatment with MLTP may be attributed to the initial "burst effect" produced by the micelle (Lee & Yeo, 2015; Soleymani Abyaneh et al., 2015). This burst effect refers to the premature release of the drug encapsulated within the micelle, often due to destabilization and disintegration of the micelle structure (Lu et al., 2018). Although the initial burst was able to bring down the IOP, it is the replenishment (treatment) of MLTP every 3 days that reduces the IOP in a sustained manner until treatment cycle 8 i.e., day 24 (1 treatment cycle = 3 days) (Fig. 12). This slow and sustained effect of MLTP likely occurred due to the mucoadhesive property of the micelle which enables it to adhere to the mucin layer of the cornea. The micelle then releases the encapsulated drug (LTP), allowing it to diffuse through cornea and eventually reach the target tissues i.e., the anterior chamber and ciliary body (Russo et al., 2008). Following 24 days of treatment there was no further reduction observed in the IOP of the mutant mice (Fig 12). Since the micelle facilitates slow and sustained drug release, the treatment with MLTP every 3 days might have resulted in saturation leading to a mean IOP of 13.65mmHg on treatment cycle 8 (at day 24), the lowest IOP observed across all treatment cycles. In comparison, in the mutant cohort treated with LTP every day, the first significant reduction in IOP was not observed until 35 days of treatment (Fig. 8)

This suggests that LTP may have been cleared out too fast before the 24-hour mark; thus, LTP was never able to reduce the IOP to its best ability, resulting in a mean of 17mmHg being the lowest IOP observed across all days. These findings suggest that the mutants treated with every 3-day MLTP had a larger reduction in IOP compared to mutants treated with just daily LTP. This is primarily due to the mucoadhesive property of the micelle, as it was able to increase the residence time of LTP within the eye and exert its effect for an extended period. In addition, previously, with the daily LTP treatment, we measured IOP at baseline, 20 minutes, and 60 minutes and noticed the baseline IOP significantly reduced at 20 minutes and rebounded to baseline at 60 minutes (Fig 7). However, with the MLTP treated group, this pattern was not observed. Instead of the rebound at 60 minutes, the MLTP treatment allowed for the sustained reduction in IOP overtime as depicted in the supplementary figure below (Fig 20). As such, these results further support the idea that the micelle drug delivery system has the ability to facilitate the sustained release of LTP, which is ultimately allowing for the continued reduction of IOP for a longer period of time. Lastly, an additional comparison was made between the MLTP treatment group and every 3-day LTP treatment. The results showed that every 3-day LTP did not improve IOP, thus suggesting that LTP treatment worsens if not taken regularly and this outcome can only be prevented with the aid of a micelle-based drug delivery approach.

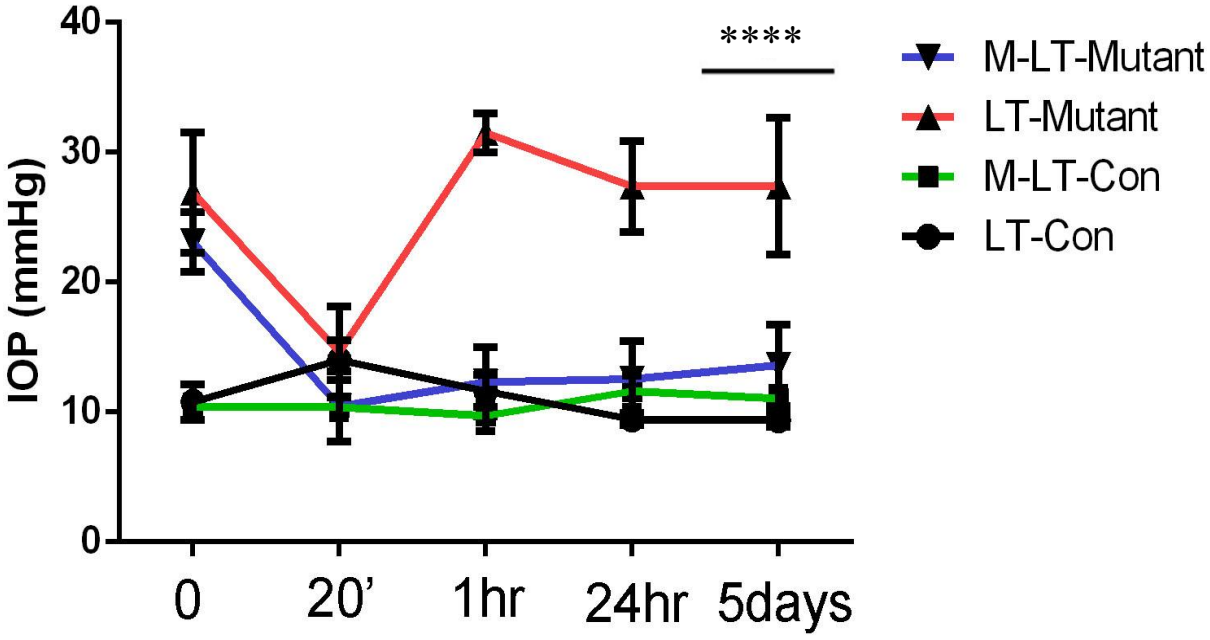


Figure 20. Comparison IOP between treatment groups across 5 days. There is a sustained decrease in IOP in the eyes of AP-2β-KO animals treated with MLTP (N=3 eyes) when compared to ones treated with 0.005% LTP alone (N=3 eyes) even after 5 days of treatment ($p<0.0001$, LT-Mutant vs M-LT-Mutant). Wild-type littermates (N=3 eyes) treated with either MLTP or LTP alone did not show any change in IOP. A significant difference between the LTP treated mutants (N = 3 eyes) compared to the MLTP treated mutants (N=3 eyes) is observed at 60 minutes, 24hours and 5 days of treatment (one-way repeated measures ANOVA, Tukey's post hoc test; $F(3,52) = 41.25$; ****$p < 0.0001$)

Following the long-term treatment period, the mutant mice treated with MLTP were assessed RGC loss. The average cell counts from all three retinal regions (periphery, mid periphery and ONH) of retinal flat mounts of every 3-day MLTP treated AP-2β TMR KO mouse (N= 5 eyes) revealed a significantly (5-fold) higher RGC cell number ($p<0.05$) compared to mutant mice treated with daily LTP (N = 8 eyes). Analysis of paraffin sections also revealed significantly (2-fold) greater RGC cell numbers in the MLTP treated eyes (N=3 eyes) compared to LTP treated eyes (N=6 eyes) ($p<0.05$). The difference in fold change between the retinal flat mounts and paraffin section analyses may be attributed to the fact that retinal flat mounts assess for RGC cell count in the entire retina whereas paraffin sections assess for RGCs in a particular section. However, regardless of these differences, results from both analyses indicate that MLTP treatment resulted in significant protection to RGCs since a greater number of RGC were maintained compared to the daily LTP treatment alone. Since blindness in glaucoma occurs due to a progressive loss of RGCs, even a small amount of protection in these cells is significant as this could prevent the irreversible blindness that is associated with glaucoma. Brn3a cell counts of MLTP treated AP-2β TMR KO mice were also compared with Brn3a cell counts of wildtype mice in flat mounted retinas and paraffin sections. MLTP treated AP-2β TMR KO mice (N=5 eyes) revealed a significant decrease ($p<0.0001$) compared to the wildtype MLTP treated mice (N=4 eyes) in flat mounted retinas, as well as in paraffin sections of mutant MLTP treated mice (N=3 eyes) compared to wildtype mice (N=4 eyes) ($p<0.001$). This information suggests that the mutant mice still have a much lower RGC cell count when compared to wildtype mice. The lower number of cell count in the mutant mice is primarily

attributed to the fact that the treatment was initiated at P30, when the mice may have already sustained retinal damage due to elevated IOP. While the baseline IOP progressively declined up until day 24 in the MLTP treated mutant mice, we began to notice an intermittent increase in IOP, which may also have contributed to RCG cell loss. Studies have found that this sort of intermittent increase in IOP can trigger neurodegeneration in the retina and optic nerve (Gramlich et al., 2016). Another factor that may also have influenced the degree of cell protection could be individual variability of the mice and their response to the drug. While some mice may have responded to the drug positively, there was a bit of variability observed in the IOP points in others. Furthermore, the drug itself may have been contaminated, as a foamy solution was present in the eyes of a few mice, ultimately resulting in less efficacy of the drug. Further improvements in the research plan can be implemented to prevent these limitations from occurring and ultimately allow for an increase in RGC cell protection.

Conclusion and Future Directions

Overall, based on our findings, our research highlights the potential for the improvement and understanding of drug delivery. Our mouse model has significant characteristics that enable it to be essential for drug delivery testing, such as a functional uveoscleral pathway that can be used for drug delivery testing, as well acute glaucomatous characteristics. With the long term MTLP treatment in our mutant mouse model we were able to show that it was more effective than the long-term LTP treatment in the ability to reduce baseline IOP levels overtime. Along with the significant reduction in baseline IOP the MLTP treatment also caused a significant improvement in RGC cell protection. Since RGC cells are primarily affected in glaucoma, this improvement in cell protection with the

MLTP treatment suggests that the micelle-based drug delivery system may slow down the progression of the disease and preserve remaining visual function in individuals affected by glaucoma.

Although we did achieve significant protection of RGCs with the MLTP drug delivery system in mutant mice as compared to those treated with LTP alone, there still remained significant cell loss as compared to wild-type mice. This may be due to the fact that there is some variability in IOP in our mutant mice that may have prevented the further protection of the cells . Future steps to improve results could include strategies to minimize variability in drug response such as by increasing the sample size, as well as understanding which environmental factors and experimental errors may have contributed to the outcome. Furthermore, combining other treatments with the LTP using the micelle-based drug delivery system may allow for a stronger outcome. Lastly, although we observed that mutant mice treated with both LTP and MLTP over time exhibited a sustained and significant reduction in baseline IOP, we have yet to determine the mechanism. To further investigate this, future studies should focus on examining any ultrastructural changes in the uveoscleral outflow pathway of mice treated with LTP and MLTP over the time course. Molecular analyses of the treated mice such as immunostaining of prostaglandin receptors in tissues primarily involved in the uveoscleral pathway, such as the ciliary muscle, will also be informative.

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