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Aqueous Humor Dynamics and the Constant-Pressure Perfusion Model of Experimental Glaucoma in Brown-Norway Rats

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Aqueous Humor Dynamics and the Constant-Pressure Perfusion Model of Experimental Glaucoma in Brown-Norway Rats

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Dedication

For Paul Joseph Ficarrotta.
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Abstract

Glaucoma affects tens of millions of people and is the leading cause of irreversible blindness worldwide. Virtually all current glaucoma therapies target elevated intraocular pressure (IOP); however, the contribution of intracranial pressure (ICP) to glaucoma has recently garnered interest. Strain at the optic nerve head is now known to depend on the translamellar pressure difference (TLPD), which is the difference between IOP and ICP, rather than IOP alone. A better understanding of how IOP and ICP relate to glaucoma development and progression is essential for developing improved therapies and diagnostic tests. Glaucoma is commonly modeled in rats, yet aqueous humor dynamics are not well-documented in healthy nor diseased rat eyes. Moreover, because rats do not develop glaucoma spontaneously, it is essential to develop low-cost, reliable, and relevant models of glaucomatous pathology in the animal.

The purpose of this dissertation work is to achieve the following goals: i) quantitatively assess aqueous humor dynamics in healthy, living rat eyes, ii) develop an ideal model of experimental glaucoma in rats, iii) quantitatively characterize aqueous humor dynamics throughout experimental glaucoma in living rats, and iv) investigate the effects of ICP manipulations on aqueous humor dynamics in living rats. Chapter 2 reports physiological parameters of aqueous humor dynamics for the first time in the eyes of living, healthy Brown-Norway rats, and presents a novel perfusion technique for efficiently and accurately estimating these parameters. Chapter 3 introduces the constant-pressure...
perfusion model of experimental glaucoma: a powerful new model which overcomes several limitations of existing techniques. The constant-pressure perfusion model induces IOP elevations which are prescribable and easily manipulated, does not directly target the trabecular meshwork or its vasculature, and offers continuous records of IOP rather than requiring regular animal handling and tonometry. Chapter 3 characterizes IOP-induced optic neuropathies in rats and demonstrates their resemblance to human glaucoma. Chapter 4 evaluates whether the constant-pressure perfusion model affects ocular physiology, specifically showing that resting IOP and conventional outflow facility are not permanently nor significantly altered in the model. Chapter 5 examines the effect of ICP manipulations on aqueous outflow physiology in living rats, and reports for the first time a graded effect of intracranial hypertension on conventional outflow facility. Evidence for a neural feedback mechanism that may serve to regulate the TLPD is also presented. Chapter 6 summarizes the results of this dissertation, provides recommendations for future work, and gives closing remarks.

These collective projects provide insight into IOP regulation in both healthy and diseased rat eyes, advancing our understanding of glaucomatous development and damage in rats. A novel model of experimental glaucoma and several perfusion systems have been developed which are distinctly tailored for use in future glaucoma studies and will allow future investigators to study the disease with enhanced efficiency and exactitude. The results of this dissertation work suggest that detecting and correcting impairments of either IOP or ICP homeostatic capabilities may be of utmost importance for improving clinical outcomes in human glaucoma.
Chapter 1: Introduction

1.1 Intraocular Pressure Regulation

Intraocular pressure (IOP) describes fluid pressure within the eye. In humans, IOP averages approximately 15 mmHg (Millar and Kaufman, 1995) and is considered normal between 10 to 21 mmHg (Giuffrè et al., 1995; Leibowitz et al., 1980; Klein et al., 1992; Dielemans et al., 1994; Bonomi et al., 1998). IOP must be tightly controlled to support normal ocular and visual health, as IOP that is abnormally low can distort the cornea, lens, or retina to impair vision, while IOP that is abnormally high can injure visual neurons resulting in irreversible blindness (Quigley et al., 1982). Homeostatic IOP is achieved by a dynamic balance of aqueous humor inflow and outflow in the anterior segment of the eye. Aqueous humor is a watery, clear, plasma-like fluid, notwithstanding its low protein concentration. Aqueous humor is continuously produced in the ciliary epithelium at a relatively IOP-insensitive rate and secreted to fill the posterior and anterior chambers of the eye (Brubaker, 1970). At steady-state IOP, aqueous humor production is balanced by aqueous humor outflow, which occurs via two distinct routes: the conventional and unconventional outflow pathways (Bill, 1965; Bill, 1966).

1.2 Pathways of Aqueous Humor Drainage

The conventional outflow pathway is defined by a series of physical structures. This pathway courses through a porous, spongy tissue called the trabecular meshwork, through Schlemm’s canal, and ultimately into the episcleral venous system (Gong et al.,
Aqueous humor is forced through the conventional outflow pathway by the pressure gradient generated by IOP and episcleral venous pressure. The unconventional outflow pathway describes all other routes of aqueous humor drainage (Bill, 1965; Bill, 1966). This pathway involves pressure-insensitive aqueous humor seepage across the iris, ciliary muscle, and sclera (Alm and Nilsson, 2009; Johnson et al., 2017; Johnson, 2006; Bill, 1966).

Under normal conditions, the conventional outflow pathway presents the primary resistance to aqueous humor outflow (Bill and Maepea, 1994; Schachtschabel et al., 2000; Weinreb, 2000, Weinreb et al., 2002; Acott and Kelly, 2008). Because the rate of aqueous humor production is relatively constant and aberrant production is clinical rare (Sachdev et al., 1988), IOP is primarily regulated by modulation of conventional outflow resistance (Acott and Kelly, 2008; Acott et al., 2014; Brubaker, 1970: Brubaker, 1991). The resistance of the conventional outflow pathway is measurable and usually expressed as its reciprocal: conductance, or conventional outflow facility.

In summary, IOP is regulated by the dynamic relationship between conventional outflow facility ($C$), unconventional outflow rate ($F_{un}$), aqueous humor production rate ($F_{in}$), and episcleral venous pressure (EVP). The explicit relationship between these parameters is described by the Goldmann equation (Brubaker, 2004), which is presented in a modified form in Equation 4 of Appendix D.

### 1.3 Glaucoma

The retina is responsible for detecting light and relaying visual information. Specifically, neural signals are transmitted from the eye towards the brain by retinal ganglion cell (RGC) axons. RCG axons exit the eye posteriorly through a mesh-like
A structure called the lamina cribosa and converge to form the optic nerve. Glaucoma is a group of heterogeneous optic nerve neuropathies characterized by progressive RGC death and distinct patterns of visual field loss. By 2020, glaucoma is predicted to affect 79.6 million people and cause bilateral blindness in 11.1 million of these individuals (Quigley and Broman, 2006); thus, glaucoma is the leading cause of irreversible blindness worldwide (Quigley, 2011).

Significant risk factors for glaucoma include age (Gordon et al., 2002; Leske et al., 2001), family history (Le et al., 2003; Leske et al., 2008), ethnicity (Tielsch et al., 1991; Leske et al., 1994), and elevated IOP (Leske et al., 1994; Banks et al., 1968; Tielsch et al., 1991; Sommer et al., 1991; Weih, et al., 2001; Dielemans et al., 1994; Mitchell et al., 1996; Quigley et al., 2001). Of these risk factors, only elevated IOP is modifiable. Reducing IOP reduces both the probability and rate of visual field loss and is thus the target of virtually all glaucoma therapies (Heijl, et al., 2002; Kass et al., 2002). In glaucoma, elevated IOP is thought to result from trabecular meshwork clogging or stiffening (Last et al., 2011). When IOP exceeds physiological levels, optic nerve cupping can be observed and RGC axons at the lamina cribosa are subject to mechanical stress and strain (Bellezza et al., 2003; Pena et al., 2001). Furthermore, the risk for glaucomatous neural injury has been shown to increase with increasing IOP (Quigley et al., 1982). The magnitude of IOP elevations and rate of their onset depend on the form of glaucoma expressed in an individual.

The most prevalent form of glaucoma is primary open-angle glaucoma (POAG) (Quigley and Broman, 2006). In POAG, trabecular meshwork resistance gradually increases; thus, the disease is characterized by slowly and subtly increasing IOP.
(Weinreb, 2004). The iridocorneal angle appears clinically normal in POAG and is “open” to aqueous humor outflow through the trabecular meshwork, albeit at a reduced rate. POAG typically develops in adulthood and is asymptomatic until its advanced stages, after significant and irreversible RGC loss has occurred (Weinreb, 2004). Another prevalent form of glaucoma is primary angle-closure glaucoma (PACG). PACG differs from POAG because it presents with an abnormal iridocorneal angle. PACG is characterized by contact between the iris and trabecular meshwork, which obstructs aqueous humor outflow and can result in rapid, significant, and often painful IOP elevations (Wright et al., 2016). Not all forms of glaucoma are associated with elevated IOP, however. Normal tension glaucoma (NTG) is a common form of open-angle glaucoma which mimics POAG despite IOP falling within normal limits (Shields, 2008).

1.4 Animal Models of Glaucoma

Animal models of glaucoma advance our understanding of the pathogenesis and pathophysiology of the disease (Chew et al., 1996). Glaucoma models have been established for non-human primates, dogs, pigs, rabbits, rats, and mice. Some of these animals develop glaucoma spontaneously (Bouhenni et al., 2012). For example, a genetic mutation in the DBA/2J mouse causes shedding of iris pigment which ultimately clogs the conventional outflow pathway (John, 2005). This outflow obstruction causes moderate IOP elevations and associated RGC death starting around six months of age (Schuettauf et al., 2004). While the spontaneous onset of IOP elevation is opportune for some research purposes, several limitations are inherent to genetic models. For example, spontaneous IOP elevations develop bilaterally, therefore, an internal control group for hypothesis testing is lacking. Furthermore, the magnitude and rate of IOP elevations are
neither adjustable nor certain. Consequently, developing experimental models of glaucoma in otherwise healthy animals is often a goal for scientific investigators.

Several experimental glaucoma models have been developed in rats. Rats are commonly investigated due to their low cost, quick growth to sexual maturity, short lifespan, and ease of handling. Moreover, like humans, rats have a trabecular meshwork and Schlemm’s canal (van der Zypen, 1977; Daimon, 1997). Existing rat glaucoma models involve inducing ocular hypertension via methods such as microbead injection into the anterior chamber (Sappington et al., 2010), hypertonic saline injection into the episcleral veins (Morrison et al., 1997), laser photocoagulation of the trabecular meshwork (Ueda et al., 1998), cauterization of episcleral veins (Shareef et al., 1995), and circumlimbal suture application (Liu et al., 2015). These techniques aim to increase the resistance of the conventional outflow pathway or mechanically compress the eye to elevate IOP.

While rat glaucoma models are widely used, the limitations of current models must also be recognized (Morrison, et al., 2005). Existing models and their limitations will be described in depth in Chapter 3. Briefly and in general, limitations of current glaucoma models include the following: ocular hypertension is typically induced by injuring the trabecular meshwork or its vasculature, IOP cannot be precisely prescribed or simply manipulated, often multiple interventions are required, IOP elevations are not stable nor permanent but slowly degrade over time, and IOP must be regularly monitored by tonometry. There is a need for improved glaucoma models which overcome the limitations outlined above.
1.5 Cerebrospinal Fluid Theory

While visual field loss in most forms of glaucoma is associated with elevated IOP (Hollows and Graham, 1966), there are several conditions which contradict the direct causality of elevated IOP on glaucoma pathogenesis. For example, NTG and ocular hypertension, which is elevated IOP without evidence of glaucoma, do not follow the straightforward model of elevated IOP activating glaucomatous pathology. Evidently, there are mechanisms of glaucomatous RGC damage which are IOP independent and not fully understood. Reduced intracranial pressure (ICP) has been suggested as an important risk factor for glaucoma (Berdahl et al., 2008b). Cerebrospinal fluid fills the ventricles of the brain and surrounds the brain, spinal cord, and optic nerve; therefore, the lamina cribosa is subject to a net balancing force determined by ICP and IOP. In 2010 authors Berdahl and Allingham proposed cerebrospinal fluid theory (Berdahl and Allingham, 2010). The fundamental tenet of cerebrospinal fluid (CSF) theory is that the translaminar pressure difference (TLPD) rather than IOP alone determines stress at the optic nerve head. Essentially, this theory suggests that the lamina cribosa should be considered a membrane subject to posteriorly directed IOP and anteriorly directed ICP; and if either IOP or ICP homeostasis becomes interrupted, the TLPD will subsequently become aberrant and the risk for neural damage will increase.

Since the early 2000s, several well-designed prospective and retrospective clinical studies, as well as numerous experimental animal studies, have supported ICP theory and suggested the role of reduced ICP as a significant risk factor for glaucoma. Specifically, ICP is reduced in patients with POAG and NTG (Berdahl et al., 2008a; Berdahl et al., 2008b, Chen et al., 2015), ICP is elevated in patients with ocular
hypertension who never develop glaucoma (Berdahl et al., 2008a; Berdahl et al., 2008b),
experimentally lowering ICP induces glaucomatous neuropathy in monkeys and rats
(Yang et al., 2014; Chowdhury et al., 2016), elevating ICP induces significant RGC death
in mice (Nusbaum et al., 2015), ICP manipulations affect the electroretinogram (ERG) in
rats (Zhao et al., 2015), IOP and ICP are correlated (Lehman et al., 1972; Hou et al.,
2016), and IOP and ICP are regulated by the same group of neurons, presumably within
the hypothalamus of the brain (Samuels et al., 2012). Despite these findings, ICP’s role
in glaucoma pathogenesis and the relationship between IOP and ICP remain unclear.
Evidently, there is a need to investigate how ICP relates to glaucoma and its role ocular
physiology.

1.6 Overview of Dissertation

The dissertation addresses the following questions: i) What are the baseline values
of physiological parameters of aqueous humor dynamics in healthy, living rat eyes? ii)
Can we develop an enhanced glaucoma model which overcomes the limitations of
existing techniques? iii) How are parameters of aqueous humor dynamics affected during
experimental glaucoma? iv) What is the impact of aberrant ICP on aqueous humor
dynamics? This dissertation is structured as four independent studies corresponding to
their own chapter and each aimed at answering one of these questions.

Chapter 2 contains a study previously published in Investigative Ophthalmology &
Visual Science and involves quantitatively assessing aqueous humor dynamics in
healthy, living rat eyes. We know that C becomes impaired in glaucoma; however, the
exact mechanisms of disease pathogenesis and progression are not well-understood.
Thoroughly understanding aqueous humor dynamics in healthy eyes is the first step
towards developing innovative and improved glaucoma therapies and is the goal of Chapter 2. This study also involves the development of a novel perfusion technique for accurately and efficiently estimating parameters of aqueous humor dynamics. Chapter 3 describes a system for inducing chronic, prescribable, and easily modifiable IOP elevations in rats, without directly damaging the trabecular meshwork, and both quantitatively and qualitatively assesses the resulting optic neuropathies. The goal of this chapter is to develop a rat glaucoma model which overcomes the limitations of existing models and is tailored to future work associated with understanding mechanisms of disease progression and assaying potential treatments. Chapter 4 involves characterizing aqueous humor dynamics throughout experimental glaucoma. Nearly all existing glaucoma models rely on injuring the trabecular meshwork or its vasculature to slow conventional outflow and secondarily induce ocular hypertension. As a result, the effect of chronic ocular hypertension on otherwise healthy trabecular meshwork has not been previously investigated in living animals for extended durations. Chapter 5 presents a study to be submitted to Proceedings of the National Academy of Sciences of the United States of America (PNAS). This chapter investigates the effect of experimentally elevated ICP on aqueous humor dynamics in rats. Finally, chapter 6 summaries this work, makes recommendations for future experiments, and provides concluding remarks.

The overall goal of this dissertation is to better understand normal and aberrant aqueous humor dynamics in rats, to advance our collective understanding of glaucoma, and to develop useful tools for future glaucoma studies.
Chapter 2: Aqueous Humor Dynamics of the Brown-Norway Rat

2.1 Note to Reader

This study (Ficarrotta, K. R., Bello, S. A., Mohamed, Y. H. and Passaglia, C. L. (2018) 'Aqueous humor dynamics of the Brown-Norway rat', Invest Ophthalmol Vis Sci, 59(6), pp. 2529-2537.) was published in Investigative Ophthalmology & Visual Science in May 2018. The published manuscript and its two supplemental figures are provided in Appendix D.

2.2 Overview

This study provides a quantitative description of aqueous humor dynamics in healthy Brown-Norway rats. Physiological parameters were estimated in live and dead eyes using a well-established constant flow (CF) technique and novel, modified constant-pressure (mCP) perfusion technique. Both $C$ and $F_{un}$ were indistinguishable for the two perfusion techniques, providing experimental validation of the mCP technique to complement the mathematical derivations provided in the manuscript. $C$ was indistinguishable in live and dead eyes when perfusions were repeated in-situ, which is a significant result that is exploited in Chapter 5 experiments. Outflow measurement accuracy was assessed by inserting a shunt of known conductance into the eye and measuring the resulting shift in $C$ (ocular conductance). This is a novel approach to validating outflow measurement accuracy and bolstered confidence in the accuracy of our results. At 100% humidity, $F_{un}$ became indistinguishable from zero, indicating potential
measurement complication due to evaporation. Physiological washout, which was previously thought to occur in all species other than humans and mice, was not observed; and trabecular meshwork anatomy appeared normal under light microscopy following perfusions.

This study provides baseline values of the physiological parameters of aqueous humor dynamics in healthy, living rats and presents a novel perfusion technique for accurately estimating these parameters with improved efficiency. This methodology should be useful for investigating the effects of pharmacologic agents, experimental perturbations, or genetic manipulations on aqueous humor dynamics in rats and other animals.
Chapter 3: The Constant-Pressure Perfusion Model of Experimental Glaucoma in Brown-Norway Rats

3.1 Introduction

Various animals exhibit optic neuropathy similar to that observed in human glaucoma and can consequently be used to generate useful models (McKinnon et al., 2009; Almasieh et al., 2012; Gaasterland and Kupfer, 1974; Quigley and Hohman, 1983; Ueda et al., 1998; Aihara et al., 2003). Because glaucoma does not develop spontaneously in rats, it is imperative to develop reproducible, low-cost techniques for experimentally inducing and subsequently studying glaucomatous pathology in the animal. Most commonly, animal models of glaucoma involve experimentally inducing ocular hypertension (Morrison et al., 2005). While not all forms of glaucoma clinically manifest with elevated IOP, elevated IOP is the only modifiable risk factor for which current glaucoma therapies are directed (Morrison et al., 2005). Additionally, the risk of developing glaucomatous optic neuropathy increases with IOP elevation (Quigley et al., 1982), while reducing IOP reduces the probability and rate of visual field loss in both POAG and NTG (Drance, 1999; Leske et al., 2004). Therefore, it is reasonable to expect that patterns of pressure-induced optic nerve damage will relate to glaucoma progression and be useful for the development and assessment of potential treatments.
Existing ocular hypertension induction techniques involve increasing the resistance of the conventional outflow pathway and chronically compressing the eye. Several commonly-used rat glaucoma models are described in more detail below.

### 3.1.1 Rat Glaucoma Models

#### 3.1.1.1 Hypertonic Saline Injection Model

One popular method for inducing ocular hypertension in rats involves injecting hypertonic saline towards the limbus through an episcleral vein (Morrison et al., 1997). A plastic ring is positioned around the equator of the eye, with a small opening centered over one radial episcleral vein. Hypertonic saline (1.65M to 1.75M, 50 µl) is then injected into the exposed vein via a microsyringe connected to a glass microneedle. The plastic ring assists in forcing the hypertonic saline through Schlemm’s canal and the trabecular meshwork. The hypertonic saline causes an inflammatory response and subsequent scarring of the trabecular meshwork and iridocorneal angle, which reduces C (Morrison et al., 1997). Following the injection, IOP is regularly measured via bilateral tonometry.

The magnitude of and duration of IOP elevations induced via this technique depend on the extent of scarring, which cannot be precisely predetermined nor controlled following the injection procedure. Thus, IOP in the treated eye cannot be precisely prescribed nor easily manipulated. Moreover, IOP in the treated eye is largely variable. For example, in a group of 20 animals, IOP elevations varied from a minimum of 0 mmHg to a maximum of 28 ± 13 mmHg following injection of the same concentration and volume of hypertonic saline (Morrison et al., 1997). Furthermore, only 45% of rats developed significant IOP elevations following a single injection (Morrison et al., 1997). Subsequent injection procedures (typically two or three injections total) were required in most animals.
The onset of IOP elevation occurs within 7 to 10 days following a successful injection. Once elevated, IOP does not remain stable; instead, it begins to slowly return to its resting level after approximately 36 days even after three injection procedures.

3.1.1.2 Episcleral Vein Cauterization Model

Another popular technique for inducing chronic IOP elevations in rats involves cauterizing one or more large episcleral veins (Sawada and Neufeld, 1999; Shareef et al., 1995). Typically, three of four episcleral veins are cauterized to obstruct their blood flow. Because aqueous humor ultimately drains into episcleral veins, cauterizing some of these veins increases the resistance of the conventional outflow pathway, thus elevating IOP. IOP response from this technique is measured via regular tonometry. Resulting IOP elevations usually occur immediately and do not require multiple procedures; however, the magnitude of IOP elevation degrades over time and can only be sustained for a maximum of six weeks (Shareef et al., 1995). More specifically, IOP becomes indistinguishable in treated and untreated eyes after two to four weeks in 30% of treated animals, which limits the technique’s utility in chronic glaucoma research (Ahmed et al., 2001).

3.1.1.3 Microbead Occlusion Model

Rather than altering ocular vasculature, the microbead occlusion method relies on obstructing the conventional outflow pathway with polystyrene microbeads (Sappington et al., 2010). Polystyrene microbeads in saline are injected directly into the anterior chamber via a microsyringe connected to a microneedle, and ultimately become lodged within structures of the conventional outflow pathway. The principal limitations associated with this method are success rate, degree of IOP elevation, and duration of IOP elevation.
Specifically, a single injection significantly elevates IOP in only 21% to 34% of rats and IOP elevations persist for only two weeks (Sappington et al., 2010). Following a second injection, IOP elevations can be sustained for up to eight weeks. The average IOP elevation is approximately 7.5 ± 1.0 mmHg. Milder IOP elevations can be achieved by reducing the volume of injected microbeads; however, increasing the injection volume does not elevate IOP beyond 7.5 mmHg. Several similar models have been developed using distinct particulates, such as ghost red blood cells (Quigley and Addicks, 1980) or latex microspheres (Weber and Zelenak, 2001), rather than polystyrene microbeads.

3.1.1.4 Laser Photocoagulation of the Trabecular Meshwork

The laser photocoagulation model involves selectively burning the trabecular meshwork via direct laser photocoagulation to increase trabecular meshwork resistance and elevate IOP (Ueda et al., 1998). As with other current models, multiple interventions are often required, IOP must be measured via tonometry, and IOP elevations degrade over time. The original presentation of this method reports a 100% success rate in elevating IOP (7 of 7 animals), but required multiple interventions, required tonometry, showed signs of inflammatory responses, and could not sustain IOP elevations over time.

3.1.1.5 Circumlimbal Suture Model

The circumlimbal suture implantation model was developed most recently (Liu et al., 2015). This technique is unique because it does not rely on reducing aqueous humor outflow through the conventional outflow pathway. Instead, a nylon suture is placed around the equator of the eye and secured by multiple anchor points in the subconjunctiva. Anchoring is achieved by threading the nylon suture under all radial episcleral veins. This subconjunctival anchoring serves to secure the suture to the surface
of the eye while preventing the obstruction of blood flow through episcleral veins. The suture is then tightened as desired and tied to compress the eye, thus elevating IOP. Resulting IOP elevations can persist for up to 15 weeks and, in the original presentation of this technique, average approximately 10 mmHg. The suture can be loosened to reverse IOP elevations at the conclusion of experiments, unlike any of the previously described techniques. The major limitations associated with this technique are that regular tonometry is required and, while IOP can be manipulated by loosening or tightening the suture, this requires surgical intervention.

3.1.1.6 Characteristics of an Ideal Glaucoma Model

Developing models of experimental glaucoma requires methods for measuring IOP, inducing chronic ocular hypertension, and assessing resulting neuropathies (Morrison et al., 2005). Several characteristics of glaucoma models have been identified as desirable through extensive literature review. To begin, all existing rat glaucoma models rely on regular tonometry to monitor IOP. Tonometry is analogous to deducing the pressure inside a balloon palpating its surface. Tonometry provides discrete and sparse measurements of IOP, which cannot fully capture its intrinsic variability. An ideal model would provide an accurate and continuous record of IOP in real-time without requiring animal handling or regular intervention. Regarding ocular hypertension inductions, the number of interventions required to elevate IOP would ideally be minimized; and induced IOP elevations would be prescribable, immediate, unilateral, stable, sustainable, and easily manipulated. Perhaps the most important feature of an animal glaucoma model is its resemblance to human glaucoma; therefore, the ideal model must result in optic neuropathies which are distinctive of the disease, clinically.
3.1.2 Purpose

One goal of this dissertation is to characterize aqueous humor dynamics in glaucomatous rats. While not generally a requirement of an ideal glaucoma model, it was thus desirable to develop a model that does not involve directly injuring or altering structures of the conventional outflow pathway. If this is achieved, the conductance of otherwise healthy trabecular meshwork, or conventional outflow facility, can be characterized throughout experimental glaucoma for the first time in a meaningful way.

The purpose of this study is to develop the ideal rat glaucoma model. This chapter describes a powerful new technique for continuously measuring IOP and chronically elevating IOP to prescribable and easily modifiable levels without directly targeting the conventional outflow pathway. The whole number and distribution of RGCs are characterized for various durations of ocular hypertension in Brown-Norway rats.

3.2 Methods

All experiments were conducted in accordance with the ARVO Statement for the use of Animals in Ophthalmic and Vision Research and in compliance with a protocol approved by the Institutional Animal Care and Use Committee at the University of South Florida. Experiments were performed on adult male Brown-Norway rats (300-400g) housed in a temperature-controlled room (21°C) under a 12-hour light/12-hour dark cycle with food and water available ad libitum.

3.2.1 Ocular Hypertension Induction

3.2.1.1 The Tethered Perfusion System

Ocular hypertension was induced by continuous perfusion of artificial aqueous humor (AAH, 130 mM NaCl, 5 mM KCl, 5 mM NaHCO₃, 1 mM CaCl₂, 0.5 mM MgCl₂, 20
mM HEPES, pH 7.25) into the anterior chamber through a tethered perfusion system. The system consisted of an ocular cannula, pressure transducer, AAH reservoir, and computer. A fine polyimide tube (100μm ID, 140μm OD) filled with AAH was implanted in one anterior chamber (Bello et al., 2017) and connected via 25g polyethylene (PE) tubing to the pressure transducer and AAH reservoir. On the day of surgery, rats were anesthetized via an intraperitoneal injection of ketamine hydrochloride (75 mg/kg) and xylazine (7.5 mg/kg) supplemented as needed, placed on a warming pad, and mounted in a stereotaxic apparatus. The eye selected for implantation was instilled with mydriatic (1% cyclopentolate hydrochloride), and its eyelid was retracted, while the contralateral eye was covered with artificial tears ophthalmic ointment. The polyimide tube was tunneled subconjunctivally and subdermally to the input port of a custom designed coupler which was affixed to the skull via bone screws. The coupler (Delrin®, Interstate Plastics, Sacramento, CA) was designed using CAD software (SolidWorks, Waltham, MA) and machined in-house. The output port of the coupler was connected to 25g PE tubing surrounded by a metal spring. A schematic diagram of the coupler and its position on the skull are presented in Figure 3.1. The 25g PE tubing was connected via a stopcock to a reservoir filled with AAH and a pressure sensor (Model 26PC, Honeywell, Morristown, NJ) which was attached to the exterior cage at the rat’s eye level. The pressure sensor was referenced to atmospheric pressure and temperature compensated to eliminate the effects of ambient temperature or pressure variations. A custom LabVIEW (National Instruments, Austin, TX) program continuously displayed and recorded IOP to a computer. A schematic of the tethered perfusion system is presented in Figure 3.2.
3.2.1.2 System Calibration and Characterization

The hydrodynamic properties of the perfusion system were characterized prior to study commencement. First, the pressure sensor was calibrated against a mercury manometer under hydrostatic pressure exertion from a saline reservoir of variable height (Ficarrotta et al., 2018). The saline reservoir was connected in parallel to the IOP sensor as depicted in Figure 3.2, but the eye was effectively replaced with a mercury manometer. The pressure sensor’s gain was adjusted to produce linear ($R^2 = 0.999$) and accurate (error $= \pm 0.4$ mmHg) readings between 0 and 100 mmHg.

System resistance and compliance were measured in the laboratory prior to the first animal experiment. The AAH reservoir was replaced by a programmable syringe pump (NE-1000; New Era Pump Systems, Farmingdale, NY, USA), and the ocular cannula was submerged in a Petri dish filled with saline. Care was taken to ensure the opening of the cannula did not rest against the sides or bottom of the dish. Pressure responses were recorded for fixed pump rates of 1, 2, 3, 4, and 5 µl/min, and system resistance was determined by linear regression of the pressure-flow data. System compliance was determined by sealing the opening of polyimide tube and measuring the pressure response from bolus injections of saline (1, 2, 3, and 4 µl) into the closed system. Compliance was determined by linear regression of the pressure-volume data.

3.2.1.3 Post-Surgical Care

Carprofen (5 mg/kg) was administered subcutaneously for pain relief every 12 hours for the first 72 hours following surgery. The AAH reservoir remained closed to the eye for the first 24 to 48 hours after surgery to allow the animal to recover, then resting IOP was established in the implanted eye via the in-line pressure sensor. Resting IOP
was consistently established around 6 PM to minimize under- or overestimation of average IOP due to circadian rhythms (Bello and Passaglia, 2017); and resting IOP defined as the level which IOP fluctuated < 1 mmHg peak-to-peak for 15 minutes. Once resting IOP was established, the AAH reservoir was opened to the eye and elevated to clamp IOP at 10 mmHg above its resting level. IOP was unilaterally and chronically elevated in this manner for durations of approximately 2 weeks, 4 weeks, or 9 weeks. Two distinct implanted animal control groups were also created. In one group, the ocular cannula was sealed near the skull so that IOP was neither elevated nor clamped. In a second group, the AAH reservoir was positioned to clamp IOP at its resting value.

3.2.2 Tonometry

Bilateral tonometry was performed biweekly in some animals to validate IOP elevations. Measurements were consistently performed around noontime to minimize the influence of circadian fluctuations on IOP readings (Bello and Passaglia, 2017; Moore et al., 1996). Animals were anesthetized with 2% isoflurane gas and placed on a heated warming pad to maintain body temperature. Ten measurements were performed in each eye via a handheld tonometer (Tono-Pen XL, Medtronic, Sarasota, FL) and averaged.

3.2.3 Fundoscopy

Fundus images of control and implanted eyes were acquired in some animals on the final day of experiments to assess ocular fluid clarity and general ocular health. Animals were anesthetized with 2% isoflurane gas and rested on a heated warming pad. A glass coverslip was used to flatten the cornea and a bright light source was positioned so the fundus could be viewed under light microscopy. Images were acquired via a digital camera fitted to the microscope.
3.2.4 Enucleation and Dissection

Animals were deeply anesthetized via an intraperitoneal injection of ketamine hydrochloride (75 mg/kg) and xylazine (7.5 mg/kg) in preparation for enucleation. Animals were transcardially perfused with no less than 120 ml of 10% neutral buffered formalin (NBF), until blood visibly cleared from the hepatic vasculature. Both eyes were enucleated, with care taken to ensure a remnant of the optic nerve remained intact, and submerged in NBF for 30 minutes. The cornea, iris, and lens were excised, and the remaining eye structures were submerged in NBF for 48 hours. The retina was then isolated from the sclera and transferred to a 24 well plate filled with phosphate-buffered saline (PBS) in preparation for immunohistofluorescence, while the remaining sclera and attached optic nerve stump were transferred to a microcentrifuge tube filled with PBS for separate processing.

3.2.5 Immunohistofluorescence

The following protocol was developed for RGC immunodetection. Retinas were permeabilized in 0.5% Triton X-100 in PBS (PBST) for 30 minutes at room temperature, washed in fresh 0.5% PBST, and incubated with Brn-3a (C-20) antibody (Santa Cruz Biotechnology, Dallas, TX) diluted 1:100 in blocking buffer (2% Triton X-100, 2% donkey serum, PBS) for 2 hours at room temperature, then overnight at 4°C. The following day, retinas were washed in fresh 2% PBST and 0.5% PBST then incubated with Alexa Fluor 594 Donkey anti-Goat IgG antibody (Invitrogen, Carlsbad, CA) diluted 1:500 in blocking buffer for 2 hours at room temperature. During incubation with the secondary antibody and all subsequent processing, well plates were tightly wrapped in aluminum foil to block ambient room light. After incubation with the secondary antibody, retinas were washed in
2% PBST, 0.5% PBST, then rinsed thoroughly in PBS. Each retina was cut radially towards its center at 90-degree intervals (see Figure 3.4), whole-mounted flat onto a charged slide, covered with VECTASHIELD® Antifade Mounting Medium (Vector Laboratories, Burlingame, CA), and coverslipped. Prepared slides were stored in the dark at 4°C until image acquisition.

3.2.6 Retinal Imaging

Whole-mounted retinas were imaged on an Olympus 3i Spinning Disk Confocal Microscope with a 10x objective using SlideBook (3i) software. The X, Y, and Z margins of the retina were manually determined under light microscopy, and appropriate exposure times were automatically determined by the software program. A Z-stack montage image of the entire retina was automatically acquired. A maximum intensity projection was performed along the Z-axis, and individual image tiles were automatically stitched together to generate a single 2D image of the entire retina.

3.2.7 RGC Counting

Image processing and RGC nuclei counting were performed using ImageJ software (National Institutes of Health). Raw images were converted to 8-bit grayscale images and processed with a custom filter sequence designed to distinguish positively stained RGC nuclei. The filter sequence consisted of a convolution filter (kernel size 5 x 5), an unsharp mask enhancement filter (radius = 2.0 pixels, mask weight = 0.4) and a Gaussian blur filter (radius = 1 pixel). The filtered images were then automatically thresholded to produce a binary image and overlapping cells were separated by two passes of the intrinsic watershed function. Positively stained RGC nuclei were automatically counted within area constraints of 15 µm² to 300 µm². A visual
representation of the image processing sequence and RGC counting is shown in Figure 3.3. The image processing and RGC counting techniques used in this work were modified from previous studies (Nadal-Nicolás et al., 2009; Salinas-Navarro et al., 2009).

### 3.2.8 Area Density Calculations

To assess spatial patterns of RGC loss, RGC area density was calculated for eight different regions of each retina. Raw images of each retina were manually segmented into four quadrants, following the radial cuts made during the whole-mounting process, as shown in Figure 3.4A. Raw images of each retina were also divided into four radial regions according to distance from the optic nerve. The average diameter of each retina was determined using ImageJ measurement tools and is defined as the average length of six lines positioned at 60-degree intervals, extending from edge-to-edge of the retina, and passing through the optic nerve. Each retina was segmented into four distinct sections according to radial distance from the optic nerve, or radius (R): less than R/4, from R/4 to R/2, from R/2 to 3R/4, and greater than 3R/4, as shown in Figure 3.4B. RGC number was automatically determined for each quadrant and radial region in ImageJ as discussed in section 3.2.7. The area of each quadrant and radial region was determined in ImageJ by threshold segmentation and automatic measurement tools. RGC area density was calculated for each of the eight sections in each eye and is defined as RGC number divided by area.

### 3.2.9 Isodensity Maps

RGC isodensity maps were created using MATLAB (The MathWorks, Inc., Matick, MA) and SigmaPlot (Systat Inc., San Jose, CA) software. RGC coordinates were exported from ImageJ, and a custom MATLAB program performed a 2D histogram of positively
stained RGCs over grouped pixels. Histogram data was exported to SigmaPlot and isodensity maps of each retina were created.

**3.2.10 Optic Nerve Processing and Imaging**

Optic nerves were processed and sectioned in preparation for light microscopy and transmission electron microscopy (TEM). Excess sclera was removed from optic nerve tissue. Optic nerves were washed in sodium cacodylate, then in 1% osmium tetroxide for one hour, and finally rinsed thrice in sodium cacodylate to remove excess osmium. Optic nerves were then dehydrated by washing in a series of graded alcohols: 35% ethanol for 10 minutes, 50% ethanol for 10 minutes, 70% ethanol overnight, fresh 70% ethanol for 10 minutes, 95% ethanol for 10 minutes, 100% ethanol for 15 minutes, then 100% acetone for 15 minutes. The optic nerves were then infiltrated with resin diluted in acetone (2:1 acetone:resin for 30 minutes, 1:1 acetone:resin for 30 minutes, 1:2 acetone:resin for 30 minutes, then pure resin overnight). Next, optic nerves were washed in fresh pure resin for 3 hours, arranged on silicone molds, covered in pure resin, and heated to cure. Embedded optic nerves were cut into 90-nm coronal sections and imaged via TEM at 10,000x, and 350-nm coronal sections for viewing under oil immersion light microscopy at 100x. TEM and light microscopy images were qualitatively analyzed.

**3.2.11 Statistical Analysis**

Paired and unpaired t-tests were performed using SigmaPlot software (Systat Inc., San Jose, CA) at an alpha level of 0.05 to assess statistical significance. Results are expressed as mean ± standard deviation.
3.3 Results

3.3.1 Perfusion System Characteristics

The hydraulic resistance of the perfusion system was 1.024 ± 0.064 (n = 3). This matches the theoretical value calculated using Poiseuille’s law and is more than 40-fold greater than the resistance of a rat eye (Ficarrotta et al., 2018), so its influence on system response dynamics can be ignored. System compliance averaged 0.101 ± 0.025 μL/mmHg (n = 3), which is similar to that of the rat eye (Ficarrotta et al., 2018, 0.091 ± 0.018 μL/mmHg, n = 3, p = 0.34). Thus, system tubing does not limit response dynamics.

3.3.2 Retinal Ganglion Cell Counts

IOP was successfully unilaterally elevated by 10 mmHg in 26 animals for durations of 10 to 73 days. IOP was continuously measured via the in-line pressure sensor and IOP elevations were validated biweekly via tonometry in some animals. A representative IOP record and corresponding tonometer measurements are provided in Figure 3.5.

RGC number was quantified for both eyes of 12 implanted animals following various durations of experimental IOP elevation, and for 5 implanted control group animals. The average RGC number for non-implanted eyes was 91,639 ± 3,616 (n = 17). RGC number in implanted eyes decreased with increasing duration of IOP elevation. RGC number averaged 92,110 ± 4,208 (n = 5), 78,537 ± 2,855 (n = 4), 71,477 ± 1,158 (n = 3) and 62,015 ± 2,887 (n = 5) in implanted eyes following IOP elevations lasting 0 days, 2 weeks, 4 weeks, and 9 weeks, respectively. Figure 3.6 presents total RGC numbers in implanted and non-implanted eyes following various durations of IOP elevation. Total RGC number was significantly reduced by 2 weeks of IOP elevation and continued to decrease with time. RGC number was not significantly different in implanted, non-
elevated eyes compared to non-implanted eyes (p = 0.81). More specifically, average RGC number was not significantly different in non-implanted eyes compared to implanted eyes which were not subject to ocular hypertension nor clamping (n = 3, p = 0.63). Therefore, the surgical process alone did induce significant RGC death. In implanted eyes which were clamped at resting IOP, RGC number averaged 91,719 (n = 2), and each RGC number fell within 1.6σ of the non-implanted eye average. This indicates that pressure-clamping the eye did not induce RGC death independently.

RGC survival fraction is defined as RGC number in the implanted eye divided by RGC number in the corresponding non-implanted eye in an individual animal. RGC survival fraction averaged 85.7 ± 1.6% (n = 4), 77.5 ± 3.2% (n = 3), and 62.1 ± 4.0% (n = 5) following 2 weeks, 4 weeks, and 9 weeks of IOP elevations, respectively. RGC survival fractions are displayed as a function of duration of IOP elevation in Figure 3.7 for each animal. RGC survival fraction decayed exponentially throughout ocular hypertension, and was best fit by the equation

\[ SF = y_0 + ae^{-bt}, \]

where \( SF \) is survival fraction, \( y_0 = 0.577 [0.500, 0.650] \), \( a = 0.452 [0.379, 0.525] \), \( b = 0.036 [0.021, 0.051] \) days\(^{-1}\), and \( t \) is duration of IOP elevation in days. This 3-parameter exponential decay model was selected after evaluating adjusted \( R^2 \) values for various exponential and polynomial models. A residual plot was examined to ensure a lack of bias. For the curve fit presented in Figure 3.7, adjusted \( R^2 = 0.949 \).
3.3.3 Regional RGC Area Densities

In 17 animals, RGC area density was calculated for four quadrants and four radial sections of each retina. The variation in RGC area density across quadrants of the retina was not significantly different in implanted compared to non-impacted eyes following 2 weeks, 4 weeks, or 9 weeks of IOP elevation (One way ANOVA, \( p = 0.93 \)), indicating that RGCs were not preferentially killed in a specific quadrant of the retina.

RGC area density is summarized in Figure 3.8 for various radial regions of the retina and durations of IOP elevation. After four weeks of IOP elevation, RGC loss was significant only in the outer- and mid-periphery regions; while after 9 weeks, RGC loss was significant in all regions of the retina (One way ANOVA, \( p < 0.001 \)), as can be seen in Figure 3.9.

3.3.4 Fundus Images

Representative fundus micrographs from corresponding implanted and non-implanted eyes are shown in Figure 3.10 following 9 weeks of unilateral IOP elevation. Ocular fluids are clear and well-perfused blood vessels can be seen radiating from the optic nerve head of both eyes.

3.3.5 Optic Nerve Cross-Sections

Light micrographs of optic nerve cross-sections are shown in Figure 3.11A-C for a non-implanted eye, and for implanted eyes following 2 weeks and 9 weeks of IOP elevation, respectively. A transmission electron micrograph of the optic nerve from the same 9-week implanted animal is shown in Figure 3.11D. After 2 weeks, loss of axon density, myelin disruption, and fields of gliosis are apparent, as shown in Figure 3.11B. By 9 weeks, extensive axon density reduction, myelin disruption, separation of nerve
fascicles, and fields of gliosis are evident, as shown in Figure 3.11C; and myelin wrapping abnormalities can be clearly seen in Figure 3.11D. The grade of optic nerve injury increases with duration of IOP elevation and closely resembles pressure-induced optic neuropathies observed in other rat models (Jia et al., 2000; Soto et al., 2011; Chidlow et al., 2011).

3.4 Discussion

This study describes the development of a powerful new system with the unique ability to precisely prescribe, easily manipulate, and continuously monitor IOP for up to 73 days in conscious rats. Chronic IOP elevations were achieved by continuous perfusion of AAH into the anterior chamber through a gravity-based, tethered perfusion system. Whole number and distribution of RGCs were characterized for various durations of precise 10 mmHg IOP elevations and optic nerve neuropathies were qualitatively assessed. The purpose of this study was to develop a rat glaucoma model which overcomes the limitations of existing techniques. Specifically, the constant-pressure perfusion (CPP) model does not require multiple interventions or regular tonometry and produces IOP elevations which are unparalleled in precision, easily manipulated, and stable in time. Moreover, this model does not rely on injuring or altering the conventional outflow pathway to induce IOP elevations; therefore, it is suitable for characterizing the effects of induced ocular hypertension on C in rats (Chapter 4).

IOP elevations of 10 mmHg were deliberately chosen to accurately represent ocular hypertension associated with POAG. (Sommer et al., 1991). Furthermore, IOP elevations were intentionally limited to one eye of each animal. The contralateral eye thus provided a convenient internal control for hypothesis testing. To assess whether this
methodology induced optic neuropathy similar to that observed in human glaucoma, RGC number and regional variations in RGC area density were quantified; and the appearance of the eye, optic nerve, and fundus were examined qualitatively.

Brn3a has been identified as a dependable marker of RGC nuclei in both healthy and injured retinas (Nadal-Nicolás et al., 2009). Total RGC number was quantified for the first time in Brown-Norway rats by Brn3a immunodetection and an automatic cell counting routine modified from previous studies (Nadal-Nicolás et al., 2009; Salinas-Navarro et al., 2009). Total Brn3a positive RGC number averaged 91,639 ± 3,616 for non-implanted eyes (n=17). Total Brn3a positive RGC number has been reported as 84,682 ± 7,601 (Nadal-Nicolás et al., 2009, n=14) and 85,997 ± 3,562 (García-Ayuso et al., 2010, n = 8) in adult female Sprague-Dwaley rats, which is an albino strain. The total Brn3a positive RGC numbers reported in this study are comparable to but slightly higher than values reported in these studies. This discrepancy is likely attributable to differences in animal size and strain. Adult female Sprague-Dwaley rats weigh 180–220g (Nadal-Nicolás et al., 2009), which is about half the weight of rats used in this study; and it has been shown that RGC number is significantly higher in pigmented eyes, like the eyes of Brown-Norway rats, compared to albino eyes (García-Ayuso et al., 2014; Nadal-Nicolás et al., 2009). Total RGC number has been reported as 90,440 ± 2,236 in Pievald Virol Glaxo rats (Nadal-Nicolás et al., 2014) and up to 118,860 in adult Wistar rats (n = 1, Laquis et al., 1998), which are both pigment strains. Thus, rat RGC numbers reported in this dissertation are comparable to results from other studies.

Both total RGC number and RGC area density decreased with increasing duration of IOP elevation. Analysis of the spatial patterns of RGC loss shows that RGCs are not
preferentially killed in one quadrant of the retina. Similar results were unanimously observed for three distinct rat glaucoma models (Urcola et al., 2006). Non-uniform patterns of RGC death are observed across radial regions of the retina, however. RGC loss is significant only in the periphery during the first four weeks of IOP elevations, but gradually becomes significant in all regions by 9 weeks. Selective RGC death was similarly observed in the peripheral retina in episcleral vein cauterization- and microsphere occlusion rat glaucoma models (Urcola et al., 2006). In human glaucoma, visual field loss initially occurs peripherally (Kolker and Hetherington, 1976), while central vision is lost only in advanced stages of the disease. Thus, the patterns of pressure-induced RGC death observed in this study mimic human glaucoma; and this model will be valuable for future studies aimed at better understanding the disease and discovering methods to minimize or perhaps, in the future, reverse its progression.

No other model can precisely elevate IOP by 10 mmHg in rats; however, some studies report similar IOP elevations for similar experiment durations. For example, the episcleral vein cauterization model was employed in one study resulting in approximately 10 mmHg IOP elevations as measured via tonometry (Laquis et al., 1998); and after 10 weeks, RGC survival was approximately 60%. This dissertation reports 62.09 ± 4.0% RGC survival after 9 weeks, and the curve fit to RGC survival data predicts 61.3% survival by 10 weeks, which is strikingly similar to RGC survival reported to the previous study. The previous study also reports RGC survival fractions of 92% after 2 weeks and 86% after 4 weeks, compared to 87.7 ± 1.6% and 77.5 ± 3.2% reported in this dissertation at respective times. This dissertation reports greater RGC death during early stages of experimental glaucoma, but similar RGC death with increasing time. These discrepancies
may be due to the distinct IOP responses induced by various glaucoma induction techniques; however, we generally observe extents of RGC death that are comparable to other rat glaucoma models.

The gravity-based, tethered perfusion system clamps IOP at a level that depends on the height of the AAH reservoir relative to the eye. In healthy, conscious rats housed under identical light/dark cycles, IOP is not fixed but follows circadian fluctuations of approximately 5 mmHg (Bello and Passaglia, 2017). In two animals, an ocular cannula was implanted and IOP was clamped at its initial resting level. This was done to test whether pressure-clamping the eye induced significant RGC death in the absence of ocular hypertension. Average RGC number was virtually identical in eyes clamped at resting IOP and non-implanted eyes. Furthermore, RGC number in each of the two control animals fell within 1.6σ of the non-implanted eye average. Thus, pressure-clamping the eye did not appear to affect RGC number. Similarly, the surgical process did not significantly alter RGC number in the absence AAH flow, a result corroborated by lack of leakage and foreign body responses due to cannula implantation (Bello et al., 2018). These results collectively indicate that the extent of RGC death observed in this dissertation is attributable to induced ocular hypertension rather than IOP-clamping or surgery.

The surgical skill required to successfully implant an ocular cannula is a noteworthy limitation of this model. However, with training, the surgical success rate, defined by lack of inflammation, infection, and injury, exceeded 90%. Additionally, the tethered perfusion system inherently complicates animal handling and is not ideal for behavioral testing. The future direction of this project involves replacing the tethered system and AAH reservoir
with a wearable micropump, as described in more detail Chapter 6. These modifications would simplify the handling of conscious animals and behavioral testing. Chapter 6 further describes suggestions for future work related to this project, comprehensively. For example, suggestions for inducing IOP elevations which maintain normal circadian rhythms, characterizing optic neuropathy at various IOP levels, and utilizing the tethered perfusion system to assay prospective neuroprotective treatments are discussed in depth in Chapter 6.

This study describes a powerful new technique for inducing chronic, unilateral IOP elevations in Brown-Norway rats, which result in optic neuropathies mimicking human glaucoma. The conventional outflow pathway is spared, and ocular hypertension is induced by continuous perfusion of AAH into the anterior chamber for the first time. Unrivaled precision in IOP control advances this model's ability to accurately characterize RGC death and optic nerve damage for various durations and degrees of IOP elevation, thus advancing current understanding of glaucoma pathogenesis and progression.
**Figure 3.1 Custom designed coupler.** The coupler is affixed to the skull via bone screws and reinforced with bone cement. The coupler provides a fixed point of connection between the ocular cannula (yellow) and 25g tubing (white). This design prevents excess tension on the ocular cannula due to animal motion.

**Figure 3.2 The tethered perfusion system.** This system consists of an ocular cannula, AAH reservoir, pressure sensor, and computer. A digital image of a cannulated eye immediately following implantation surgery (left) and a schematic diagram of the tethered perfusion system (right) are shown. Pressure is exerted via a reservoir filled with AAH, which drives fluid into the anterior chamber via the implanted ocular cannula to elevated pressure. The pressure sensor continuously displays and records IOP to a computer. This system is capable of prescribing, manipulating, and continuously monitoring IOP in rats.
Figure 3.3 Image processing and RGC counting. A visual representation of the image processing sequence and automatic RGC counting are shown. (A) A maximum z-projection image of a single mosaic tile of the raw retina image. (B) Image A following the application of the custom filter sequence for distinguishing RGCs. (C) Image B auto-thresholded to produce a binary image and following two passes of the watershed function which differentiates overlapping cells. (D) Image C overlaid with masks indicating counted RGC nuclei within area constraints.
**Figure 3.4 Schematic of retinal segmentation.** Each retina was segmented into the eight sections collectively depicted here for RGC area density analyses. Retinas were segmented along red rings in image A for comparison of RGC area density as a function of radial distance from the optic nerve. Retinas were also segmented along red lines in B for comparison of RCG area density in various quadrants.

**Figure 3.5 IOP records.** (A) Representative IOP record from corresponding implanted and control eyes throughout 63 days unilateral IOP elevation. Each data point is the average of 10 tonometer readings. The response from the in-line IOP sensor is also shown for the implanted eye. (B) Image of the implanted eye after 63 days of IOP elevation. The ocular cannula in visible in the medial aspect of eye, yet the eye appears healthy otherwise.
Figure 3.6 RGC counts. (A) Average RGC number in implanted and non-implanted eyes following various durations of unilateral IOP elevation by 10 mmHg. The 0 days implanted eye group represents animals implanted in one eye for 9 weeks without IOP elevation. Error bars represent standard deviations. Double and single asterisks represent statistically significant differences (p < 0.001, p < 0.05, respectively).
Figure 3.7 RGC survival fractions. For each implanted animal, RGC survival fraction represents the total RGC number in the non-implanted eye divided by RGC number in the corresponding implanted eye. Survival fraction plotted against duration of IOP elevation can be accurately fit by a 3-parameter exponential decay function (adjusted $R^2 = 0.949$).
Figure 3.8 Normalized RGC area densities. Average RGC area densities were normalized to the non-implanted eye average for each radial section and duration of IOP elevation. RGCs in the outer-and mid-periphery regions were preferentially killed during the first 4 weeks of IOP elevations. By 9 weeks, RGC death was significant across all retinal regions. Error bars represent standard deviation.
Figure 3.9 RGC isodensity maps. Representative RGC isodensity maps showing the spatial distribution of Brn3a positive RGC nuclei in a non-implanted control eye retina (A) and an implanted eye retina following 9 weeks of IOP elevation (B). RGC loss appears uniform across the entire retina, supporting results from RGC area density analyses. Bin size is approximately 370 x 370 µm.
**Figure 3.10 Fundus micrographs.** Light micrographs of the fundus of a representative control eye (A) and implanted eye following 9 weeks of unilateral IOP elevation by 10 mmHg (B). Ocular fluids are clear and well-perfused retinal blood vessels can be seen radiating from of the optic nerve head of each eye.
Figure 3.11 Micrographs of optic nerve cross-sections. Light micrographs of healthy optic nerves (A) and glaucomatous optic nerves following 2 weeks (B) and 9 weeks (C) of 10 mmHg IOP elevation. Axon density reduction, myelin disruption, and fields of gliosis are apparent at 2 weeks and become visibly more severe by 9 weeks. (D) Myelin wrapping abnormalities are evident in a transmission electron micrograph of the optic nerve following 9 weeks of 10 mmHg IOP elevation.
Chapter 4: The Effect of Experimental Glaucoma on Aqueous Humor Dynamics in Rats

4.1 Introduction

Chapter 3 presented a novel method for inducing chronic ocular hypertension and assessed the resulting anatomical effects. By contrast, this chapter describes the effects of chronic constant-pressure perfusions on eye physiology: specifically, \( C \) and resting IOP. While previous studies have investigated patterns of RGC loss for various animal glaucoma models, no study to date has characterized the effects of experimental glaucoma on \( C \) in living animals. Because most rat glaucoma models rely on altering the trabecular meshwork or its vasculature to induce ocular hypertension, studying causal effects of elevated IOP on the trabecular meshwork was not previously meaningful in these models. The CPP model spares the trabecular meshwork, offers continuous IOP records, and provides direct access to the anterior chamber for perfusion and \( C \) measurements. Therefore, the CPP model is well-suited to study the effects of induced ocular hypertension on otherwise healthy trabecular for the first time in a meaningful way.

Outflow facility has not previously been characterized in living animals throughout experimental glaucoma; however, several ex-vivo and cultured eye experiments have investigated the effect of elevated IOP on \( C \). Outflow resistance reportedly varies in response to elevated IOP in perfused anterior segment organ cultures and ex-vivo eyes (Acott and Kelley, 2008; Borras et al., 2002; Bradley et al., 2001; Keller et al., 2009a;
Hashimoto and Epstein, 1980). Some studies suggest the continued exposure of trabecular meshwork cells to elevated IOP increases \( C \), perhaps to maintain homeostatic IOP, while other studies suggest elevated IOP increases the rigidity of the trabecular meshwork and thus reduces \( C \) (Gabelt and Kaufman, 2005; Schlu nck et al., 2008). By contrast, some studies in ex-vivo eyes report that IOP elevations do not significantly affect outflow facility (Millar et al., 2011; Lei et al., 2011; Hashimoto and Epstein, 1980). In either case, the aforementioned studies were limited to ex-vivo eyes or cultured anterior segments and experimental durations were limited as a result. Cultured anterior segments deteriorate significantly over time (Keller et al., 2013) and ex vivo eyes are similarly subject to necrosis.

Evidently, the mechanisms of IOP-induced outflow facility accommodation remain controversial and require further investigation, ideally in living animals. While genetic and possibly environmental factors contribute to glaucoma pathogenesis, impaired IOP homeostatic capability is paradigm to disease progression. Furthermore, approximately 25% of new cases of blindness are attributable to glaucoma; therefore, understanding the regulation of both normal (Chapter 2) and glaucomatous aqueous outflow resistance is of significant medical importance (Quigley, 1993, Quigley, 1996, Quigley and Vitale, 1997).

The goal of this chapter is to characterize \( C \) and resting IOP in living animals throughout the CPP model of experimental glaucoma in rats.

4.2 Methods

The following experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in compliance with a protocol approved by the Institutional Animal Care and Use Committee at the
University of South Florida. All experiments were performed on adult male retired-breeder Brown-Norway rats (300-400 g) housed under a 12:12 light-dark cycle with free access to food and water.

4.2.1 Experimental Setup

Cannula implantation surgeries were performed for 19 rats following Chapter 3 methodology. Ocular hypertension was induced via the CPP model in 17 animals for durations of 14 to 73 days, and the remaining 2 animals served as implanted controls with IOP clamped at its resting level (65 days, 64 days). Resting IOP was established in the implanted eye of conscious animals via the tethered perfusion system prior to elevating IOP and on the final day of each experiment.

4.2.2 Terminal Experiments

On the final day of each experiment, resting IOP and C were measured in both the implanted and non-implanted eye via the perfusion system described in Chapter 2. This system is reintroduced in Figure 4.1. Anesthesia was induced in each animal via an intraperitoneal injection of ketamine hydrochloride (75 mg/kg) and xylazine (7.5 mg/kg) and maintained through intravenous delivery of ketamine (30 mg/kg/h). Animals were placed on a warming pad to maintain body temperature and mounted in a stereotaxic apparatus. Heart rate and body temperature were monitored and maintained at physiological levels by anesthetic infusion rate adjustments. An incision was made at the scalp to expose the ocular cannula; then the cannula was cut and sealed with glue to prevent AAH flow. One drop of cyclopentolate hydrochloride (1%) was instilled in each eye to dilate the pupil, then the eye was obliquely cannulated with a 33g hypodermic needle (TSK Laboratory, Tochigi, Japan, length: 13 mm, lumen: 0.11 mm) connected via
25g tubing to a programmable syringe pump (NE-1000; New Era Pump Systems, Farmingdale, NY, USA) and calibrated pressure sensor (Ficarrotta et al., 2018). Corneas were covered with a clear contact lens and saline drops were instilled in each eye every 10-15 minutes. The cannulated eye was frequently examined under light microscopy for internal tissue damage and visible fluid leakage. Resting IOP was established and C was measured via the mCP technique (Ficarrotta et al., 2018) in the implanted and non-implanted eye, independently. A control algorithm switched the AAH pump on and off to hold IOP within 2 mmHg of a user-defined level. Net outflow (F) was calculated for each pump cycle at each IOP set point as the product of pump inflow rate (Fp), which was fixed at 1.5 µL/min, and the measured pump duty cycle. F data were averaged for each pressure level and linearly regressed against IOP to estimate C. Resting IOP and C measurements were not routinely initiated in a particular eye but alternated between animal experiments; and each eye was cannulated with a new 33g needle.

4.2.3 Serial Measurements

Resting IOP and C were recurrently measured throughout experimental glaucoma in five animals. The tethered perfusion system was modified to facilitate periodic, serial measurements of C. The AAH reservoir was temporarily replaced by a programmable syringe pump (NE-1000; New Era Pump Systems, Farmingdale, NY, USA) and a custom LabView program (National Instruments, Austin, TX, USA) controlled pump activity and displayed and recorded IOP to a computer (Ficarrotta et al., 2018). The modified tethered perfusion system is depicted in Figure 4.2. Each of the five animals endured chronic, experimental IOP elevations of 10 mmHg, as described in Chapter 3, for durations of 27 to 73 days. Resting IOP was established and C was measured via the previously
described mCP perfusion technique (Ficarrotta 2018) at 1 to 3 week intervals in each animal. During serial experiments, animals were anesthetized with 2% isoflurane and rested on a warming pad to maintain normal body temperature, but no additional treatments were administered. After data collection, animals remained on the warming pad until deliberate ambulatory movements were apparent. The AAH reservoir was then reconnected to replace the syringe pump, and IOP was elevated by 10 mmHg until the next serial outflow experiment.

4.2.4 Outflow Measurement in a Conscious Rat

The feasibility of measuring $C$ in conscious animals was also investigated. In one distinct animal, $C$ was measured two days after cannula implantation surgery via the CF perfusion technique and modified tethered perfusion system. IOP was not elevated prior to nor after $C$ measurement in this animal. Equilibrium IOP was determined for pump rates of 0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 µl/min in the conscious animal, and $C$ was estimated by regression analysis of the pressure-flow data (Ficarrotta et al., 2018).

4.2.5 Histologic Processing

The impact of experimental glaucoma on the iridocorneal angle was histologically examined in 7 rats. Following data collection, deeply anesthetized animals were transcardially perfused with NBF, then eyes were enucleated and submerged in NBF for 24 hours. Each eye was embedded in paraffin, sliced in 4-µm sections, and mounted on gelatin-coated slides. Iridocorneal angle sections were stained with hematoxylin and eosin, viewed under light microscopy, and digitally photographed.
4.2.6 Data Analysis

Statistical significance was assessed via paired and unpaired t-tests at an α level of 0.05 using SigmaPlot software (Systat, Inc., San Jose, CA, USA), unless otherwise indicated. Results are expressed across experiments as mean ± standard deviation (SD) and within experiments as 95% confidence intervals in brackets.

4.3 Results

The hydrodynamic properties of the tethered perfusion system were described in Chapter 3. System characteristics, namely resistance and compliance, were found to not limit response dynamics. Resting IOP was not significantly affected by chronic IOP elevations but returned to its original resting value in the absence of hydrostatic pressure exertion. Resting IOP respectively averaged 15.59 ± 1.58 mmHg and 15.82 ± 1.49 mmHg on the initial and final days of IOP elevation experiments, as measured through the tethered perfusion system in the implanted eye of conscious animals (n = 17, p = 0.51). Intra-eye IOP shifts are presented as a function of duration of IOP elevation for each animal in Figure 4.3. IOP shift and duration of IOP elevation are not significantly related (Pearson Product Moment Correlation, n = 19, p = 0.29).

Similarly, resting IOP was not significantly different in implanted and non-implanted eyes at the conclusion of each experiment, as measured via a 33g needle in ketamine anesthetized animals. Resting IOP averaged 15.40 ± 1.06 mmHg in implanted, perfused eyes and 15.15 ± 1.13 mmHg in non-implanted eyes during terminal experiments (n = 17, p = 0.24). Inter-eye IOP differences are shown as a function of duration of IOP elevation for each animal in Figure 4.4. Inter-eye IOP difference and duration of IOP elevation are related (Pearson Product Moment Correlation, n = 19, p = 0.59).
C averaged 0.0235 ± 0.0015 µl/min/mmHg in implanted eyes and 0.0241 ± 0.0028 µl/min/mmHg in non-implanted eyes of ketamine anesthetized animals at the termination of IOP elevation experiments (n=17, p = 0.36). The inter-eye differences in C as a function of duration of IOP elevation are plotted in Figure 4.5 for each animal. These variables are not significantly related (Pearson Product Moment Correlation, n = 19, p = 0.29). Furthermore, the inter-eye difference in C was significant in only 1 of 17 individual animals (ANCOVA Homogeneity of Regressions, F < 3.39, p > 0.103 for 16 of 17 comparisons).

Neither resting IOP nor C significantly varied in individual animals throughout experimental glaucoma, despite significant RGC loss and the development of glaucomatous pathology. Figure 4.6 plots resting IOP and C in time for the five animals involved in serial outflow experiments. The corresponding RGC survival fraction and experiment duration are provided for each animal. Neither IOP nor C are significantly related to time in any individual animal (Pearson Product Moment Correlation, p > 0.14, p > 0.39, respectively). Furthermore, C and resting IOP were indistinguishable between the two perfusion systems (n = 5, p > 0.35 for both comparisons).

C was measured via the CF perfusion technique in one conscious animal. The pressure-flow data from this experiment is presented in Figure 4.7, where linear regression estimates C as 0.0252 [0.0231, 0.0273]. In healthy eyes, C averaged 0.0235 ± 0.0015. Hence, C in the conscious animal is within approximately 1σ of the average value of C in healthy eyes, seemingly indicating that C can be accurately measured in conscious animals via the modified tethered perfusion system.

The iridocorneal angle of implanted, chronically perfused eyes appeared normal under light microscopy at the conclusion of chronic IOP elevation experiments. Figure 4.8
shows that the trabecular meshwork of implanted eyes was morphologically intact and the iridocorneal angle appeared similar to that of the non-implanted eye, consistent with the lack of evidence for significant changes in C or resting IOP.

4.4 Discussion

This study characterized C and resting IOP in glaucomatous Brown-Norway rats. Glaucoma was experimentally induced via continuous constant-pressure perfusion of AAH into one eye of conscious animals for durations of 0 to 73 days. Non-implanted eyes served as convenient internal controls for hypothesis testing at the conclusion of experimental glaucoma, while serial measurements were performed in implanted eyes to assess intra-eye variability throughout several experiments. Resting IOP and C were stable in implanted eyes during experimental glaucoma and were not significantly different in implanted and non-implanted eyes during terminal experiments. The iridocorneal angle appeared normal at the conclusion even the longest IOP elevation experiment, corroborating the lack of evidence for altered aqueous humor dynamics. Moreover, C was successfully measured in a conscious animal through the modified tethered perfusion system, indicating that serial measurements in conscious animals are feasible under this methodology.

Various studies report conflicting results regarding the effect, or lack of effect, of experimental IOP elevations on resting IOP and C (Hashimoto and Epstein 1980; Ellingsen and Grant, 1971; Acott and Kelley, 2008; Borras et al., 2002; Bradley et al., 2001; Keller et al., 2009a; Millar et al., 2011; Lei et al., 2011; Stamer et al., 2011; Schneemann et al., 2003). Our results indicate that chronic 10 mmHg IOP elevations, induced by the CPP model, do not significantly or permanently alter the function of the
conventional outflow pathway or resting IOP, despite inducing optic neuropathies characteristic of glaucoma. One study involving human cell cultured anterior chambers reports that prolonged IOP elevations significantly increase $C$ between day 1 and day 4, but the effect disappears with increasing experiment duration (Borras et al., 2002). In this study, the first serial experiments are performed 7 days after the initiation of ocular hypertension. Therefore, it is possible that $C$ and resting IOP were modulated between day 0 and day 7, but the effect is no longer detectable by the first serial experiments. Future studies aimed at continuously measuring $C$ within the first week of IOP elevations and beyond appear to be worthwhile. Suggestion for future experiments designed to achieve this goal, and additional recommendations for future work related to this project, are provided in detail in Chapter 6.

The ability to measure $C$ in conscious animals is a significant achievement of this work. Classically, aqueous humor dynamics have been quantified in cultured anterior chamber segments or intact ex-vivo eyes. Several studies have recently assessed aqueous humor dynamics quantitatively in anesthetized animals, which introduces logistical challenges but perhaps better represents normal animal physiology. This study is the first to report $C$ in a conscious animal without any treatments, which is ideal for quantitative assessment of physiological parameters. Several conscious animal experiments have been suggested in Chapter 6 to advance our understanding of aqueous humor dynamics in both healthy and glaucomatous eyes, and to ascertain how closely the aqueous outflow characteristics of rat eyes may resemble those of human eyes.

It is beyond the scope of this study to conclude whether trabecular meshwork changes are occurring at a cellular level throughout experimental glaucoma. Perhaps
trabecular meshwork stiffening and washout effects are balanced such that changes in $C$ are not measurable, for example. We can say with confidence, however, that resting IOP and $C$ are not permanently altered throughout 7 to 73 days of 10 mmHg IOP elevations imparted via the CPP model of experimental glaucoma.

This chapter describes a technique for accurately tracking conventional outflow function throughout experimental glaucoma. This is the first study to investigate the effects of chronic IOP elevations on aqueous humor dynamics in living animals, and the first study to perform serial outflow measurements in the same animal over a duration of many weeks. Outflow measurements in conscious animals were found to be feasible, indicating that the modified tethered perfusion system may be able to facilitate serial measurements of $C$ in conscious animals in future studies.
Figure 4.1 Ocular perfusion system. A schematic diagram of perfusion system used to assess aqueous humor dynamics in terminal animal experiments.

Figure 4.2 Modified tethered perfusion system. A schematic diagram of the modified tethered perfusion system used to serially assess aqueous humor dynamics throughout experimental glaucoma.
Figure 4.3 Resting IOP shifts in implanted eyes. Intra-eye resting IOP shifts are shown for implanted-eyes of individual animals following various durations of IOP elevation by 10 mmHg.

Figure 4.4 Inter-eye IOP differences during terminal experiments. The difference between IOP in non-implanted eyes and fellow implanted, perfused eyes for individual animals following various durations of IOP elevation.
Figure 4.5 Inter-eye $C$ differences during terminal experiments. The difference between $C$ in non-implanted eyes and fellow implanted, perfused eyes following various durations of IOP elevation in individual animals.
Figure 4.6 Serial measurements of resting IOP and C. Serial measurements of $C$ (A) and resting IOP (B) throughout experimental glaucoma for 5 rats. Error bars represent standard error on $C$ estimates from least squares regression analysis (A) and standard deviation of mean resting IOP (B).
Figure 4.7 Light micrographs of the iridocorneal angle. Hematoxylin and eosin stained thin (4 µm) sections of the iridocorneal angle of a non-implanted eye (left) and an implanted, perfused eye (right) following 9 weeks of chronic constant-pressure perfusion. The trabecular meshwork and Schlemm’s canal of the implanted, perfused eye appear morphologically intact and similar to the non-implanted eye. TM, trabecular meshwork; SC, Schlemm’s canal.

Figure 4.8 C in a conscious animal. Pressure-flow data from a CF experiment in the eye of a conscious rat. For this animal, $C$ was $0.025 [0.023, 0.027]$ and the y-intercept $= -0.424 [0.394, 0.453]$. The line is a linear regression fit of the data. Error bars represent standard deviation.
Chapter 5: The Effect of Intracranial Pressure Elevation on Aqueous Humor Dynamics in Rats

5.1 Note to Reader

A modified version of this chapter will be submitted to the Proceedings of the National Academy of Sciences of the United States of America (PNAS) near the time this dissertation is submitted to the University of South Florida.

5.2 Introduction

Conclusive evidence of a causal relationship between elevated IOP and the onset and progression of glaucomatous RGC damage remains controversial (J. Crawford Downs et al., 2008). Current evidence suggests IOP-induced stress and strain on the optic nerve head ultimately kills RGCs (Minckler et al., 1977; Quigley and Anderson, 1976; Minckler, 1986; Quigley and Anderson 1977); yet there are several conditions which cannot be explained by this straightforward model. For example, glaucomatous damage is observed even in the absence of elevated IOP in patients with NTG. NTG is a prevalent form of the disease which can account for 30-92% of all glaucoma diagnoses depending on race and ethnicity (Cho and Key, 2014). Furthermore, a significant proportion of patients with ocular hypertension never develop glaucoma (Kass et al., 2002; Weinreb and Khaw, 2004). In these patients, elevated IOP is not accompanied by visual field loss or characteristic pathological manifestations typically observed at the
optic nerve head. Evidently, elevated IOP is not a definitive indicator for whether glaucoma will develop and there may be other important risk factors to investigate.

One potential explanation for NTG and ocular hypertension is cerebral spinal fluid (CSF) theory (Berdahl and Allingham, 2010). CSF fills the ventricles of the brain and surrounds the brain, spinal cord, and optic nerve to form a continuous fluid-filled compartment of nearly uniform pressure (Lenfeldt et al., 2007). Therefore, pressure immediately posterior to the eye is determined by cerebrospinal fluid pressure (CSFP); and CSFP and ICP are approximately equivalent and used interchangeably in medical practice and literature (Chowdhury and Fautsch 2015; Lenfeldt et al., 2004; Berdahl and Allingham 2010; Berdhal et al., 2008b; Morgan et al., 1995). According to CSF theory, the stress felt at the optic nerve head is determined by the difference between IOP and CSFP, or the translaminar pressure difference (TLPD), rather than IOP alone (Berdahl and Allingham, 2010). This theory suggests that depressed ICP may have the same effect on the optic nerve head as elevated IOP; and elevated ICP may mimic the effects of depressed IOP. Figure 5.1 illustrates CSF theory and relative magnitudes of ICP and IOP for a healthy eye, glaucomatous eyes, and a hypertensive eye.

There is strong evidence which supports CSF theory and implicates reduced ICP as a significant risk factor for glaucoma. For example, retrospective clinical studies have shown a correlation between depressed ICP and glaucoma: POAG and, most significantly, NTG (Berdahl et al., 2008a; Berdahl et al., 2008b; Chen et al., 2015). As illustrated in Figure 5.1, significantly depressed ICP generates an aberrant TLPD and is expected to result in glaucomatous damage according to CSF theory. Some of the same studies also report that ICP is significantly elevated in patients with ocular hypertension.
who never develop glaucoma (Berdahl et al., 2008a; Berdahl et al., 2008b). As demonstrated in Figure 5.1, if both IOP and ICP are elevated, a homeostatic TLPD can be maintained.

Furthermore, several experimental animal studies support CSF theory. One study showed that reducing ICP via implantation of a ventriculoperitoneal shunt induces glaucoma-like pathology in non-human primates (Yang et al., 2014). A similar study in rats showed that chronically reducing ICP resulted in significant RGC death and reduced optic nerve axon density (Chowdhury et al., 2016 ARVO abstract). In mice, chronically elevating rather than lowering ICP also resulted in significant RGC death (Nusbaum et al., 2015). Furthermore, it has been reported that the TLPD affects optic disc displacement, cup depth, and the ERG (Morgan et al., 2002; Zhao et al., 2015). The relationship between IOP and ICP has also been investigated. One study induced experimental ICP elevations in monkeys and reported a subsequent increase in IOP within minutes (Lehman et al., 1972). Similarly, lowering ICP in dogs initiated a parallel reduction in IOP such that the trans-lamina cribrosa gradient (TLPG) remained stable (Hou et al., 2016).

Despite this evidence, the relationship between IOP and ICP and the mechanisms of TLPD regulation are still not well-understood. Furthermore, the effect of ICP on aqueous humor dynamics rather than IOP alone has not been considered in prior studies. The purpose of this study was to investigate the effects of experimental ICP elevations on C in rats.
5.3 Methods

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in compliance with a protocol approved by the Institutional Animal Care and Use Committee at the University of South Florida.

5.3.1 Experimental Setup

Male retired-breeder Brown-Norway rats (300-400 g) were housed under a 12:12 light-dark cycle with free access to food and water. On the day experimentation, animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (75 mg/kg) and xylazine (7.5 mg/kg), supplemented as needed. Anesthesia was maintained by intravenous delivery of ketamine (30 mg/kg/hr) through a catheter inserted in the femoral vein. Body temperature, heart rate, and mean arterial pressure (MAP) were monitored and kept at physiological levels by adjusting anesthetic infusion rate. Body temperature was recorded with a rectal thermometer and controlled with a heating pad. Heart rate was recorded with ECG electrodes. MAP was recorded with a pressure sensor connected to a saline-filled catheter inserted in the femoral artery. The animal was then positioned in a stereotaxic instrument, an incision was made in the scalp, and a 0.5-mm hole was drilled in the skull 1-mm caudal and 1.5-mm lateral to Bregma. A 25g needle connected to a micromanipulator was advanced approximately 3.5-mm through the hole into a cerebral ventricle. The needle was connected via a 3-way stopcock and polyethylene tubing (length: 45 cm, lumen: 0.4 mm) to a pressure sensor and a reservoir of physiological saline mixed with 1% green vital dye (Davidson Marking System, Middleton, WI). After ventricle cannulation, indicated by a jump in pressure reading, the needle was fixed in
place and the hole sealed with dental cement (A-M Systems, Sequim, WA). Successful cannulation was verified by stabilization of ICP at >3 mmHg and by post-experimental assessment of dye location. Pupils of both eyes were dilated with 1% cyclopentolate hydrochloride drops, and the anterior chamber of one eye was cannulated with a 33g needle connected via a 3-way stopcock and polyethylene tubing (length: 45 cm, lumen: 0.4 mm) to a pressor sensor and a programmable syringe pump (NE-1000, New Era Pump Systems, Farmingdale, NY) filled with artificial aqueous humor (AAH). Corneas were covered with clear contact lenses and moistened every 10-15 minutes with saline drops. The cannulation site was repeatedly checked to confirm there was no needle displacement, internal tissue damage, or visible fluid leakage.

Figure 5.2 illustrates the experimental setup. Each pressure sensor (Model 26PC, Honeywell, Morristown, NJ) was calibrated against a mercury manometer prior to data collection. The calibration procedure and the hydrodynamic properties of the perfusion system have been described (Ficarrotta et al., 2018). Sensor outputs were amplified, low-pass filtered at 1 Hz, and digitized at 2 Hz to a computer by a custom LabView (National Instruments, Austin, TX) program. Data collection began when ICP and IOP settled at values that fluctuated <1 mmHg over 15 minutes. These values were defined as the resting ICP and IOP.

5.3.2 Data Collection

C was measured via the mCP technique. A control algorithm switched the AAH pump on and off to maintain IOP within 2 mmHg of a user-specified level (Ficarrotta 2018). $F$ was calculated for different IOP set points as the product of $F_p$, which was fixed at 1.5 $\mu$L/min, and the measured pump duty cycle. $F$ data were averaged over 4-6 pump cycles.
for each set point and linearly regressed against IOP to estimate $C$. $C$ was measured at resting ICP and at ICP set 15 mmHg above the resting level. In some experiments, $C$ was also measured for 5mmHg steps in ICP up to 15mmHg (linearity), or after returning ICP to its resting level (reproducibility). In a distinct subset of experiments, $C$ was remeasured at elevated ICP after topical application of tetrodotoxin (TTX, 1 µg/µl in saline), or after animal euthanasia. ICP was manipulated by varying reservoir height. Pressure-flow data were collected >30 minutes after height changes when ICP had stabilized at the target level. Statistical significance was assessed with paired and unpaired t-tests at an alpha level of 0.05 using SigmaPlot software (Systat Inc., San Jose, CA), unless otherwise indicated. Individual animal results are expressed as 95% confidence intervals in brackets, while across-animals results are expressed as mean ± standard deviation (SD).

### 5.3.3 Histological Processing

After animal euthanasia, the dye-perfused brain was excised and submerged in 10% neutral buffered formalin (NBF) for 24-48 hours. Fixed brains were embedded in paraffin, sliced in 4-µm coronal sections, and mounted on gelatin-coated slides. Tissue sections were stained with hematoxylin and eosin, viewed under light microscopy, and digitally photographed.

### 5.4 Results

Cerebral ventricle cannulation was validated by histological examination of the dye-perfused brain. Figure 5.3 shows that dye molecules were located in multiple disparate areas of the lateral ventricles but not in surrounding tissues of these or other brain sections, indicating that the ventricle was successfully cannulated in this experiment. The result was the same for every brain examined (n = 8).
Figure 5.4 shows representative IOP, ICP, and MAP data collected from an anesthetized rat. \( C \) was estimated by determining the pump duty cycle required to hold IOP at different levels. It can be seen that pump-driven cyclic and sustained changes in IOP had no discernable effect on ICP and MAP. Resting ICP averaged 5.8 ± 2.0 mmHg (\( n = 15 \)) and MAP averaged 97 ± 4 mmHg (\( n = 3 \)). These values were not significantly different at peak IOP elevation (\( p = 0.62 \) and 0.28, respectively). After estimating outflow facility at resting ICP, ICP was increased by 15 mmHg and facility measurements were repeated. ICP elevation had no discernable effect on MAP either (\( p = 0.55 \)). There was a small but significant increase in resting IOP of 1.9 ± 1.4 mmHg across animals after ICP elevation (\( p < 0.001 \)).

ICP changes had a marked effect on aqueous humor dynamics. Figure 5.5A shows pressure-flow data of a rat eye at resting and elevated ICP. \( C \) was reduced at elevated ICP, as evidenced by the decrease in slope of the data from 0.020 to 0.011 \( \mu l/min/mmHg \). Figure 5.5B summarizes outflow facility measurements across animals for an ICP elevation of 15 mmHg (\( n = 15 \)). \( C \) averaged 0.021 ± 0.004 \( \mu l/min/mmHg \) at resting ICP, which is indistinguishable from prior measurements in rats not subjected to cerebral ventricle cannulation (\( p = 0.68 \)) (Ficarrotta et al., 2018). At elevated ICP, \( C \) averaged 0.0127 ± 0.0036 \( \mu l/min/mmHg \), a reduction of 41.0 ± 13.4%. ICP was subsequently reset to the resting level in two experiments. Figure 5.6 shows pressure-flow data of a rat eye before, during, and after ICP elevation. It can be seen that the slope returned to near-baseline level when resting ICP was restored, indicating that the change in outflow facility was reversible. The resting IOP did not return to the baseline level in this animal, which may reflect natural variability or circadian IOP rhythms since data collection lasted several
hours. C post-ICP elevation was within 1.1σ of average C pre-ICP elevation for both experiments. The difference between pre- and post-ICP elevation slope was not significant in either experiment (ANCOVA Homogeneity of Regressions, F < 0.46, p > 0.523 for both comparisons).

The effect of ICP on C was graded in magnitude. Figure 5.7A shows pressure-flow data of a rat eye in which intermediate ICP levels were also tested. C decreased systematically with ICP elevation from 0.020 to 0.011 µl/min/mmHg. Figure 5.7B shows the relationship was approximately linear for the range tested. The resting IOP also shifted progressively higher in this animal with ICP elevation due presumably to the decreasing outflow facility. This experiment was performed twice, with an apparent linear relationship between C and ICP elevation each time (R² = 0.991, 0.990).

Measurements were repeated on dead eyes at elevated ICP. Figure 5.8 shows pressure-flow data of a rat eye at resting ICP, elevated ICP, and elevated ICP after animal euthanasia. The dead-eye data are shifted leftward since there is no aqueous production to maintain the resting IOP. It can be seen that C decreased after ICP elevation but returned to near-baseline level after euthanasia even though ICP remained high. C at elevated ICP was 0.019 ± 0.004 µl/min/mmHg across experiments on dead eyes (n = 9). This is greater than C at elevated ICP (0.013 ± 0.04 µl/min/mmHg, p < 0.001) and not different from C at resting ICP (0.022 ± 0.003 µl/min/mmHg, p = 0.07) when those eyes were alive. The effect of ICP on C thus appears to have a physiological origin.

To determine whether neural signaling pathways might be involved, TTX was applied to the cornea of the eye. Figure 5.9A shows pressure-flow data of a rat eye at resting ICP, elevated ICP, and elevated ICP after TTX application. The voltage-gated
sodium-channel blocker partially or completely eliminated ICP-dependent changes in outflow facility for every animal tested \( (n = 4) \). \( C \) at resting ICP and elevated ICP was 0.023 ± 0.002 and 0.013 ± 0.003 µl/min/mmHg, respectively. After drug application, \( C \) at elevated ICP was 0.021 ± 0.007 nl/min/mmHg, which is larger than the pre-TTX level \( (p < 0.05) \) and not different from the baseline level \( (p = 0.63) \). Figure 5.9C shows that TTX had no detectable impact on outflow facility at resting ICP. \( C \) before and after drug application averaged 0.025 ± 0.002 and 0.024 ± 0.003 µl/min/mm, respectively \( (n = 3, p = 0.46) \).

5.5 Discussion

This study quantified \( C \) in anesthetized rats under varying levels of experimental ICP elevation. \( C \) was found to decrease and IOP was observed to increase subtlety with ICP elevations of 15 mmHg. The effect of ICP on IOP is consistent with several prior studies which report a correlation between the two pressure quantities (Lehman et al., 1972; Hou et al., 2016; Samuels et al., 2012; Sajjadi et al., 2006; Lashutka et al., 2004). It may be explained as a byproduct of the ICP-driven changes in \( C \) revealed here since the two parameters are inversely related according to the Goldmann equation. Baseline measurements of \( C \) were reproducible following acute ICP elevations, indicating that the ICP effect is reversible within the timeframes of this study. Specifically, the ICP effect did not persist more than 30 minutes after ICP was returned to baseline. \( C \) was found to decrease in proportion to ICP for elevations up to 15 mmHg. Perhaps this is because TLPD restoration can be achieved by small adjustments to \( C \) when ICP elevations are minimal; but significant disruptions of the TLPD require more significant outflow accommodation.
It has been previously reported that euthanization alone does not significantly affect $C$ in rats (Ficarrotta et al., 2018). This result was exploited to test whether the ICP effect was passive or physiological. Reduced $C$ did not persist at elevated ICP in euthanized animals, which supports the hypothesis for the physiological nature of this ostensibly protective effect. Furthermore, it is plausible that the TLPD is regulated by the sympathetic nervous system due to the extensive autonomic innervation of the eye (Niedermeier, 1950; Bloomfield, 1947). To specifically test whether the ICP effect involved voltage-gated sodium channel activity, TTX was topically administered to the eye as an alternative to euthanization in some animals. The ICP effect did not persist in live eyes instilled with TTX even at elevated ICP. These results may indicate the presence of a neurally-modulated protective feedback mechanism for maintaining a homeostatic TLPD.

A working model of TLPD homeostasis has been proposed and is presented in Figure 5.10. First, ICP is experimentally elevated which disturbs the TLPD and increases the risk for damage at the optic nerve head. Next, neural signaling initiates a reduction in $C$. Finally, IOP increases as a result of reduced aqueous outflow and a homeostatic TLPD is restored. One recent study showed that IOP and ICP are controlled by the same group of neurons in the hypothalamus of the brain (Samuels et al., 2012). The feedback mechanism proposed in this study requires a neural network for communication between these pressure compartments; thus, additional experiments to determine whether the hypothesized neural feedback mechanism involves processing in the hypothalamus may be worthwhile.

Limitations of this study include the underlying assumptions of the Goldmann equation as previously discussed (Ficarrotta et al., 2018). The Goldmann equation
assumes only $C$ is pressure dependent and that pressure-flow relationships are linear. The potential effects of inaccuracies in these assumptions were described in detail in Chapter 2. Additionally, the experiments described in this study were performed on rats under general anesthesia. While this is standard practice in outflow research in living animals, anesthesia may limit the applicability of our results to conscious animals. This is of particular importance for experiments involving TTX, as the effects of ketamine and xylazine compounded with TTX on sympathetic ocular innervation have not been previously investigated and are beyond the scope of this study. Ideally, the experiments described in this chapter would be repeated in conscious animals. Fortunately, Chapter 4 describes methods for measuring $C$ in conscious rats for the first time using manometric techniques. Modifications to Chapter 3 and Chapter 4 methodology could facilitate investigations of the effects of elevated ICP on $C$ in conscious animals. Recommendations for future work related to this project are provided in Chapter 6.

This study is the first to investigate the effects of experimentally elevated ICP on $C$ in rats. A protective neural feedback mechanism for modulating $C$ to maintain a homeostatic TLPD has been proposed. Observations from this study may indicate that failure of TLPD homeostatic mechanisms may lead to the glaucoma development. The results of this study support CSF theory and the paradigm-shifting concept that the TLPD, rather than IOP alone, should be considered as a diagnostic marker and therapeutic target in future glaucoma studies.
Figure 5.1 Cerebrospinal fluid theory. The upper panel depicts the locations of CSF within the brain, spinal cord, and eye. There is a net balancing force controlled by the anteriorly directed ICP and posteriorly directed IOP at the lamina cribosa which determines the TLPD (TLPD = IOP – ICP). The lower panel depicts relative magnitudes of ICP and IOP for normal, glaucomatous, and hypertensive eyes.
Figure 5.2 Experimental setup. Intraocular pressure (IOP), intracranial pressure (ICP), and mean arterial pressure (MAP) were simultaneously recorded with separate pressure sensors via cannulas inserted in the anterior chamber of the eye, lateral ventricles, and femoral artery, respectively. The IOP cannula was also connected to a pump that infused artificial aqueous humor (AAH) under computer control in order to measure the aqueous humor dynamics of the eye, while the ICP cannula was also connected to a variable-height reservoir of physiological saline in order to manipulate ICP level.

Figure 5.3 Cerebral ventricle cannula localization. Coronal tissue sections of a dye-perfused rat brain. Arrowheads indicate clumps of green dye molecules, which are found in the ventricles but not in brain tissue.
Figure 5.4 IOP, ICP, and MAP records. Pressure records are simultaneously presented for an anesthetized animal before and after 15 mmHg ICP elevation. The white bar demarks the time period during which the pump duty cycle required to hold IOP at different levels was measured in order to estimate $C$ at resting ICP. Outflow facility was subsequently estimated at elevated ICP, producing a similar record that is not shown.
Figure 5.5 Impact of elevated ICP on C. (A) Flow (as measured by pump duty cycle) required to hold IOP of an individual rat eye at different levels while ICP rested at 4 mmHg (black symbols) and was held at 19 mmHg (white symbols). C was 0.020 [0.020, 0.021] μL/min/mmHg at resting ICP and 0.011 [0.010, 0.011] μL/min/mmHg at elevated ICP. (B) Average C for all animals at resting ICP and 15 mmHg above resting ICP (n = 15). Lines are linear regression fits of the data. Error bars represent standard deviation. Asterisks indicate statistically significant differences (p < 0.001).
Figure 5.6 Baseline $C$ measurements are reproducible. Pressure-flow data from an individual animal before (black symbols), during (white symbols), and after (gray symbols) elevating ICP by 15mmHg. $C$ was not significantly different pre- and post-ICP elevation in any animal (ANCOVA, $p > 0.17$ for all comparisons). Lines are linear regression fits of the data. Error bars represent standard deviation.
Figure 5.7 Graded effect of ICP on outflow facility. (A) Pressure-flow data of a rat eye at resting ICP (black symbols) and 5 mmHg (dark gray symbols), 10 mmHg (light gray symbols), and 15 mmHg (white symbols) above resting ICP. (B) Measured outflow facility as a function of ICP elevation. The relationship between $C$ and ICP elevation is approximately linear as indicated by an $R^2$ value of 0.991. Lines are regression fits of the data. Error bars represent standard deviation (A) or standard error of slope estimates from regression analysis (B).
Figure 5.8 ICP effect requires an active physiological process. (A) Pressure-flow data of a rat eye at resting ICP (black symbols), at 15 mmHg above rest (white symbols), and at 15 mmHg above rest following animal euthanasia (gray symbols). (B) Average C for all animals each condition (n = 9). Lines represent regression fits of the data. Error bars represent standard deviation. Asterisks indicate statistically significant differences from baseline measurements (p < 0.001).
Figure 5.9 TTX blocks the ICP effect. (A) Pressure-flow data of a rat eye at resting ICP (black symbols), at 15 mmHg above rest (white symbols), and at 15 mmHg above rest following TTX application to the eye (gray symbols). (B) Average C for all animals at elevated ICP pre- and post-TTX application (n = 4). Asterisk indicates statistically significant differences from baseline measurements (p < 0.01). (C) Pressure-flow data of a rat eye at resting ICP before (black symbols) and after (gray symbols) TTX application to the eye. (D) Average C for all animals pre- and post-TTX application (n = 3). Lines are regression fits of the data. Error bars represent standard deviation.
Figure 5.10 Working model of TLPD homeostasis. The hypothesized underlying mechanisms of the ICP effect are shown over time, from left to right, in panels A – D. (A) IOP and ICP are both normal which results in a normal TLPD. (B) ICP is experimentally elevated and clamped at 15 mmHg above its resting level, which disrupts the TLPD and increases the risk of permanent damage at the optic nerve head. (C) A neural signal (green arrows) initiates a significant reduction in \( C \). (D) IOP increases as a result of reduced outflow through the conventional pathway, thus restoring a normal TLPD and sparing the optic nerve head. Relative magnitudes of IOP and ICP are represented by size of blue (ICP) and orange (IOP) arrows.
Chapter 6: Conclusions

6.1 Summary of Results

This dissertation describes a novel experimental model of glaucoma and quantitatively assesses physiological parameters of aqueous humor dynamics in healthy, glaucomatous, and intracranial hypertensive Brown-Norway rat eyes. This work advances the current understanding of IOP regulation in healthy animals and during conditions related to prevalent human diseases which have not been previously investigated, as the necessary systems and methods were lacking prior to their development in this work. The results presented in this dissertation demonstrate that while impaired aqueous humor drainage causes ocular hypertension in glaucoma, the reverse is not explicitly true. Namely, moderate ocular hypertension does not intrinsically cause significant injury to nor impairment of trabecular meshwork function. Furthermore, this work supports the parading-shifting concept that reduced ICP may be a modifiable risk factor for glaucoma: a disease previously thought to have only one modifiable risk factor for which all current therapies are directed. Thus, ICP may prove to be both an effective diagnostic marker and therapeutic target to help slow or prevent irreversible blindness in the tens of millions of individuals who are affected by the disease. A summary of the major results of this dissertation work is provided below.

Chapter 2 quantifies the physiological parameters of aqueous humor dynamics for the first time in healthy Brown-Norway rats and presents a novel perfusion technique, the
mCP perfusion technique, for measuring these parameters accurately and efficiently. Understanding IOP regulation in healthy eyes is a critical first step towards better recognizing and understanding IOP dysregulation in diseased eyes. This chapter is not simply an extension of existing methodologies to rats, however. The novel perfusion technique is presented mathematically and experimentally validated by comparison to results from a well-established technique. Statistical analyses show that the mCP technique yields indistinguishable results in approximately half the time, making it more suitable for serial measurements in living animals, namely anesthetized or conscious animals, during which time is a significant experimental constraint. The effects of hydration on the eye were thoroughly investigated and shown to complicate measurement of unconventional outflow rate. This indicates that previous in-situ estimates of $F_{un}$ in mice are likely contaminated by evaporation effects (Millar et al., 2015; Millar et al., 2011). In a novel approach, an ocular shunt was manufactured, characterized, and ultimately implanted into the eye to further bolster confidence in the accuracy of $C$ estimates via the mCP technique. The absence of detectable washout was a notable finding previously observed only in human and mice eyes.

Chapter 3 describes the CPP model of experimental glaucoma in Brown-Norway rats. The development of this model involved the development and characterization of a tethered perfusion system for continuously measuring IOP and perfusing AAH into the anterior chamber to chronically and unilaterally elevate IOP in conscious animals. The relevance of this model to human glaucoma was assessed by quantifying whole numbers and distributions of RGCs for various experimental durations, and qualitatively examining the globe of the eye, optic nerve, and fundus. Significant RGC loss was observed as early
as two weeks following 10 mmHg IOP elevations, and RGC number decayed exponentially with increasing time. RGCs in the outer periphery were most susceptible to pressure-induced death within the first 4 weeks, yet RGC death was significant across the entire retina after 9 weeks of IOP elevation. This pattern resembles patterns of RGC death observed in human glaucoma. The globe and fundus appeared normal following 9 weeks of IOP elevation, while optic nerve cross-sections revealed optic neuropathy characteristic of human glaucoma and consistent with established rat models.

Chapter 4 characterizes aqueous humor dynamics throughout experimental glaucoma induced via the CPP model. Resting IOP and $C$ did not significantly vary throughout chronic 10 mmHg IOP elevations lasting up to 73 days. POAG pathogenesis is thought to involve impaired aqueous drainage which causes ocular hypertension secondarily. Moderate levels of ocular hypertension, however, did not inherently induce measurable changes in $C$. Chapter 4 also demonstrates the feasibility of measuring $C$ in conscious animals for the first time via the modified tethered perfusion system.

The effects of ICP elevations on aqueous humor dynamics are investigated in Chapter 5. $C$ was significantly reduced when ICP was experimentally elevated by approximately 15 mmHg. The increased resistance to conventional outflow was linearly graded with ICP elevations up to 15 mmHg and the effect was reversible when ICP was returned to baseline. A slight but significant increase in resting IOP was also observed. The effect did not persist, however, in live eyes instilled with TTX or in the eyes euthanized animals. From these results, we propose a neural feedback mechanism for maintaining a homeostatic TLPD across the lamina cribosa. Failure of TLPD homeostatic capabilities may be detrimental to RGCs and the optic nerve.
6.2 Recommendations for Future Work

The following subsections describe recommendations for future work related to this dissertation. Various recommendations for additional experiments are provided for each project and organized accordingly.

6.2.1 Aqueous Humor Dynamics of the Brown-Norway Rat

Chapter 2 reports $C$, $F_{un}$, and IOP in healthy rats; however, EVP and $F_{in}$ could not be discriminated from pressure-flow data alone. Attempts were made to directly measure EVP via previously described techniques (Aihara et al., 2003; Millar et al., 2011) without success. Developing an accurate technique for measuring either $F_{in}$ or EVP would allow each of the five parameters of the Goldmann equation to be reported individually and provide a complete profile of AH dynamics in individual animals.

The relationship between net outflow rate, $F$, and IOP could be more thoroughly explored in future studies. Specifically, pressure-flow data could be measured for very small IOP or pump rate increments (2 mmHg and 0.04 µl/min, for example) and across a wider range of pressures (from resting IOP to IOP = 70 mmHg, for example) to investigate whether or at what point the linear pressure-flow relationship becomes compromised. Some outflow studies performed in enucleated eyes observed a non-linear relationship between pressure and flow, particularly at high pressures (Sherwood et al., 2016; Madekurozwa et al., 2017). While a linear pressure-flow relationship was observed across the pressure range explored in the work, a thorough examination of this relationship may be worthwhile.
### 6.2.2 The Constant-Pressure Perfusion Model of Experimental Glaucoma in Rats

The tethered perfusion system can be utilized for various applications of glaucoma research, expanding far beyond what has been presented in this dissertation. The ability to precisely prescribe, continuously measure, and easily manipulate IOP gives rise to experiments that were not previously possible. Chapter 3 characterizes whole numbers and patterns of RGC death for 10 mmHg IOP elevations; however, the system’s ability to easily vary IOP and exert hydrostatic pressures of different magnitudes should be exploited in future research. IOP elevations of 10 mmHg are considered moderate in POAG and ocular hypertension, as discussed in Chapter 3. There are, however, distinct groups of patients who experience relatively mild or extreme IOP elevations. Whole numbers and patterns of RGC loss should thus be characterized in time for different levels of IOP elevation to more comprehensively understand patterns of RGC death. For example, the experiments described in Chapter 3 could be repeated for IOP elevations of 5 mmHg (mild) and 20 mmHg (extreme).

The eye was pressure-clamped by the AAH reservoir in the experiments described in Chapters 3 and 4. Healthy rat eyes exhibit circadian variations in IOP of approximately 5 mmHg, however (Bello and Passaglia, 2017; Moore et al., 1996). Fortunately, the tethered perfusion system offers the ability to manipulate IOP continuously and in real-time. Therefore, rather than remaining stationary, the AAH reservoir could be positioned on a programmable, motorized stage and varied in height to preserve both the period and amplitude of normal IOP variations even while IOP is experimentally elevated. Alternatively, the AAH reservoir could be replaced by a programmable syringe pump set to a pre-determined rate for elevating IOP as desired, based on knowledge from
previously collected pressure-flow data (i.e. approximately 0.2 µl/min to elevate IOP by 10 mmHg). By exerting pressure in this manner (constant-rate rather than constant-pressure), IOP is free to vary and circadian rhythms should be maintained throughout ocular hypertension. Furthermore, a wearable micropump would eliminate the need to tether the animal thus improving ease of animal handling. The development of such a pump is currently underway in the Ocular Neuroscience and Neuroengineering Laboratory at the University of South Florida.

Furthermore, RGC death could be characterized for patterns of IOP exertion beyond circadian rhythms. For example, IOP could be acutely elevated on a regular basis, but undisturbed throughout the majority of the experimental duration. RGC number and patterns of RGC loss could then be quantified for unique patterns of pressure exertion that may result from trauma or as a secondary effect of diseases other than glaucoma. Additionally, IOP could be lowered following set durations of chronic IOP elevations to determine whether the effects observed in Chapter 4 are reversible.

The tethered perfusion system allows precise assaying of potential neuroprotective drugs. Specifically, experimental neuroprotective drugs can be continuously perfused into the eye by direct incorporation into the AAH reservoir. Adding drugs to the AAH reservoir is decidedly simple and eliminates the need for regular animal handling. RGC survival fraction can then be compared for treated and untreated animal groups following identical durations and degrees of IOP elevation. Such experiments would serve to precisely evaluate whether potential new therapies can significantly protect RGCs or reduce the resulting grade of optic nerve injury.
While this dissertation investigated the effect of chronic, unilateral IOP elevations on both ocular anatomy and physiology, additional experiments could be performed to assess the model’s applicability to clinical glaucoma. Specifically, in this study patterns of RGC death were assessed for distinct optic nerve quadrants; however, the orientation of these quadrants (i.e. nasal, temporal, superior, inferior) was not known. Fundus images, which provide orientation information, could be registered with fluorescent images of the retina to determine spatial orientation based on vasculature patterns. This image registration could improve our understanding of RGC spatial distribution in different quadrants of the retina over time in the rat model. Furthermore, physiological changes attributable to the CPP model could be investigated. For example, the ERG, the retina’s time-voltage response evoked by various visual stimuli, provides information regarding retinal function. Other electrophysiological tests such as the compound action potential and visually evoked potential respectively quantify optic nerve and occipital cortex function in response to visual stimuli. These electrophysiological tests could be performed throughout the CPP model to characterize physiological impairment at various points along the visual pathway. Behavioral testing could also be performed to measure the visual acuity of implanted animals throughout experimental glaucoma.

6.2.3 The Effect of Experimental Glaucoma on Aqueous Humor Dynamics in Rats

This dissertation serially quantifies C and resting IOP in anesthetized animals throughout experimental glaucoma. Chapter 4 also discusses the feasibility of using the tethered system to measure C in conscious animals. In future studies in both healthy in glaucomatous eyes, C would ideally be measured in conscious animals in the absence of anesthetics or any additional treatments. The LabVIEW program could even be
modified to automatically perform outflow measurements via the mCP or CF technique
and modified tethered perfusion system in conscious animals.

Furthermore, a flowmeter could be incorporated into the tethered system to directly
and continuously measure AAH flow. Along with knowledge of resting IOP and the
simultaneous IOP record, the AAH flowrate data could be used to generate a continuous
record of $C$ in time. A continuous $C$ record rather than periodic, discrete measurements
would improve characterization of $C$ throughout experimental glaucoma. As discussed in
Chapter 4, $C$ should be measured regularly or even continuously in future studies during
the first 0 to 7 days of experimental glaucoma to test whether any transient effects are
detectable during this time.

6.2.4 The Effect of Intracranial Pressure Elevation on Aqueous Humor Dynamics in
Rats

The experiments described in Chapter 5 would ideally be performed in conscious
animals. Particularly, the effects of the TTX on $C$ and the hypothesized neural feedback
mechanism should be investigated in the absence of anesthetics, which may contaminate
our results. The tethered perfusion system could potentially be utilized to achieve this
goal. A second polyimide tube connected to a saline reservoir and independent pressure
sensor could be implanted into the lateral ventricle to measure and manipulate ICP and
IOP in parallel. This experimental design would provide, for the first time, a continuous
record of the TLPD in conscious animals and facilitate $C$ measurements in conscious
animals while both IOP and ICP were precisely controlled.

A significant relationship between elevated ICP and $C$ was established in Chapter
5. While ICP was exclusively elevated in this study, the effects of depressed ICP on $C$
should also be investigated in future studies. We suspect the resistance of the conventional outflow pathway may decrease when ICP is experimentally depressed. In general, the limits of conventional outflow accommodation are worth establishing at various ICP levels.

6.3 Closing Remarks

The model and perfusion systems presented in this dissertation have surpassed the project’s originally proposed aims. Specifically, Chapters 3, 4, and 5 describe original experiments unlike any work which has been previously performed in humans or other animals. One of the most significant contributions of this work is the development of the tethered perfusion system and the CPP model of experimental glaucoma. The tethered perfusion system offers robust IOP control and precise characterization of whole numbers and distributions of RGCs for precise degrees of IOP elevation and experimental durations. This technology has the power to significantly advance our understanding of glaucoma pathogenesis and progression and to assay potential neuroprotective therapies with unmatched precision.

The ICP effect and associated neural feedback mechanism described in Chapter 5 are noteworthy findings of this work. The relationship between elevated ICP and $C$ has been established for the first time and these results appear to support CSF theory. This dissertation suggests ICP may be a modifiable risk factor in glaucoma and should be investigated as a potential diagnostic marker and therapeutic target in future studies. The presence of a neural feedback mechanism for maintaining a homeostatic TLPD is an original, paradigm-shifting proposition in glaucoma research. The contributions of this dissertation provide the foundation for various investigative studies aimed at developing
clinically translational diagnostic tests and therapies. Given the prevalence of glaucoma and its detrimental outcome, the insight gained through this work and recommended future work has the potential to positively affect tens of millions of people.
References


Chowdhury, R. U. 'ARVO-E Abstract 4131', *ARVO: IOVS*.


Appendix A: Abbreviations

Artificial aqueous humor  AAH
Association for Research in Vision and Ophthalmology  ARVO
Conventional outflow facility  C
Aqueous humor formation rate  \( F_{in} \)
Artificial aqueous humor  AAH
Association for Research in Vision and Ophthalmology  ARVO
Cerebrospinal fluid  CSF
Cerebrospinal fluid pressure  CSFP
Constant-flow  CF
Constant-pressure perfusion  CPP
Conventional outflow facility  C
Episcleral venous pressure  EVP
Inner diameter  ID
Intracranial pressure  ICP
Intraocular pressure  IOP
Left eye  OS
Mean arterial pressure  MAP
Modified constant-pressure perfusion  mCP
Net outflow rate  F
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<tr>
<td>Neutral buffered formalin</td>
<td>NBF</td>
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<tr>
<td>Normal tension glaucoma</td>
<td>NTG</td>
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<tr>
<td>Outer diameter</td>
<td>OD</td>
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<tr>
<td>Phosphate buffered saline</td>
<td>PBS</td>
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<tr>
<td>Polyethylene</td>
<td>PE</td>
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<tr>
<td>Primary open angle glaucoma</td>
<td>POAG</td>
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<tr>
<td>Pump duty cycle</td>
<td>D</td>
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<td>( F_p )</td>
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<td>Retinal ganglion cell</td>
<td>RGC</td>
</tr>
<tr>
<td>Right eye</td>
<td>OD</td>
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<tr>
<td>Standard deviation</td>
<td>SD</td>
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<td>SE</td>
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<td>Tetrodotoxin</td>
<td>TTX</td>
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<td>Translaminar pressure difference</td>
<td>TLPD</td>
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<tr>
<td>Transmission electron microscopy</td>
<td>TEM</td>
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<tr>
<td>Triton X-100 in phosphate buffered saline</td>
<td>PBST</td>
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<td>Unconventional outflow rate</td>
<td>( F_{un} )</td>
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Appendix B: Copyright Permissions

This Appendix includes copyright approval information for the manuscript presented in Appendix D. This article (Ficarotta, K. R., Bello, S. A., Mohamed, Y. H. and Passaglia, C. L. (2018) 'Aqueous humor dynamics of the Brown-Norway rat', Invest Ophthalmol Vis Sci, 59(6), pp. 2529-2537.) is openly accessible as indicated in the screenshot below.

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Appendix C: IACUC Approval

This dissertation work was performed following IACUC protocols R IS00001425 and R IS00005347. The original IACUC approval letters are provided in this appendix.
MEMORANDUM

TO: Christopher Passaglia,

FROM: Farah Mouli, MSPH, IACUC Coordinator
Institutional Animal Care & Use Committee
Research Integrity & Compliance

DATE: 10/5/2018

PROJECT TITLE: Structure and function of healthy and glaucomatous eyes

FUNDING SOURCE: Federal government or major agency that awards grants based on peer-reviewed proposals (NIH, NSF, DOD, AHA, ACS, etc.)
National Eye Institute

IACUC PROTOCOL #: R IS00005347
PROTOCOL STATUS: APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 10/5/2018:

Rat: Brown-Norway (adult/250-450g/males) 375

Please take note of the following:

• IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system. After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

• All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification. Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

• All costs invoiced to a grant account must be allocable to the purpose of the grant. Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.
MEMORANDUM

TO: Christopher Passaglia,

FROM: Farah Moulvi, MSPH, IACUC Coordinator
Institutional Animal Care & Use Committee
Research Integrity & Compliance

DATE: 9/1/2015
PROJECT TITLE: Structure and function of healthy and glaucomatous eyes
FUNDING SOURCE: National Eye Institute; American Health Assistance Foundation;
USF department, institute, center, etc.

IACUC PROTOCOL #: R IS00001425

PROTOCOL STATUS: APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 9/1/2015:

- Rat: Brown-Norway (adult/ 250-450g/male) 328
- Mouse: C57BL/6 (adult/10-20 wks/male) 20

Please take note of the following:

- IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system. After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

- All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification. Modifications can be submitted to the

9/1/2015
IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

• All costs invoiced to a grant account must be allocable to the purpose of the grant. Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.
Appendix D: Aqueous Humor Dynamics of the Brown-Norway Rat

This appendix contains the manuscript titled, “Aqueous Humor Dynamics of the Brown-Norway Rat”, and its supplemental figures, which were published in Investigative Ophthalmology & Visual Science in May 2018.
Aqueous Humor Dynamics of the Brown-Norway Rat

Kayla R. Ficarotta, Simon A. Bello, Youssef H. Mohamed, and Christopher L. Passaglia

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Submitted September 1, 2017
Accepted April 19, 2018

PURPOSE. The study aimed to provide a quantitative description of aqueous humor dynamics in healthy rat eyes.

METHODS. One eye of 26 anesthetized adult Brown-Norway rats was cannulated with a needle connected to a perfusion pump and pressure transducer. Pressure-flow data were measured in live and dead eyes by varying pump rate (constant-flow technique) or by modulating pump duty cycle to hold intraocular pressure (IOP) at set levels (modified constant-pressure technique). Data were fit by the Goldmann equation to estimate conventional outflow facility (C) and unconventional outflow rate (Fua). Parameter estimates were respectively checked by inserting a shunt of similar conductance into the eye and by varying eye hydration methodology.

RESULTS. Rat IOP averaged 14.6 ± 1.9 mm Hg at rest. Pressure-flow data were repeatable and indistinguishable for the two perfusion techniques, yielding C = 0.025 ± 0.002 μL/min/mm Hg and Fua = 0.096 ± 0.024 μL/min. C was similar for live and dead eyes and increased upon shunt insertion by an amount equal to shunt conductance, validating measurement accuracy. At 100% humidity Fua dropped to 0.003 ± 0.030 μL/min. Physiological washout was not observed (~0.35 ± 0.65%/h), and trabecular anatomy looked normal.

CONCLUSIONS. Rat aqueous humor dynamics are intermediate in magnitude compared to those in mice and humans, consistent with species differences in eye size. C does not change with time or death. Evaporation complicates measurement of Fua even when eyes are not enucleated. Absence of washout is a notable finding seen only in mouse and human eyes to date.

Keywords: outflow facility, anterior chamber, eye perfusion, in vivo

Intraocular pressure (IOP) in living animals reflects the dynamics of aqueous humor flow into and out of the eye. Aqueous humor flows into the posterior chamber at a steady rate via the ciliary body epithelium. It flows around the iris to the anterior chamber and exits via conventional and unconventional outflow pathways.1,2 The conventional pathway courses through the trabecular meshwork into Schlemm’s canal and onto collector channels and aqueous drainage veins of the episcleral venous system.3,5 The unconventional pathway includes all other escape routes. It does not have physical structures like canals or veins, but rather involves fluid seepage through the iris root, ciliary muscle, choroid, and sclera into orbital capillaries, vortex veins, and lymph vessels.1,2,4,6 Under normal physiological conditions the trabecular pathway presents the primary resistance to aqueous outflow. Fluid movement along unconventional routes is not thought to depend on IOP except near 0 mm Hg.7,8 The primary site of conventional outflow resistance has been pinpointed to the inner wall region of Schlemm’s canal.8 Trabecular matrix in this region stiffens in eyes with glaucoma,9,10 altering the biomechanical properties of inner wall cells and impairing their ability to form pores through which aqueous crosses into the canal.11 The heightened resistance causes a sustained IOP increase that can lead to retinal ganglion cell death and blindness if left untreated.

Given the links between IOP and glaucoma, it is important to understand aqueous humor dynamics in quantitative detail. Important parameters like aqueous formation rate, conventional outflow facility, unconventional outflow, and episcleral venous pressure have been reported for ex vivo and in vivo eyes of humans12 and several animals.1,2,4-15 Direct and indirect methods have been used to determine each parameter, including anterior chamber14-15 and episcleral vein12 cannulation, tonometry,17 tonography,18 fluorophotometry,12 and radiolabeling.1,2 A constant-flow (CF) infusion technique was recently developed for quantifying these parameters in a single eye of live anesthetized mice,17 permitting serial measurements on the same animal. In this study the CF technique and a modified constant-pressure (mCP) technique were applied to the eyes of anesthetized rats. Rats were investigated because they are a popular experimental model for glaucoma research owing to their low cost, convenient size, quick growth to sexual maturity, short life span, and ease of handling. More importantly, aqueous humor dynamics are well documented for other animal glaucoma models but not for rats.

METHODS

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision

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Rat Aqueous Humor Dynamics

Research and in compliance with a protocol approved by the Institutional Animal Care and Use Committee at the University of South Florida.

Animal Preparation
Male retired breeder Brown-Norway rats (300–400 g) were housed under a 12-hour light/12-hour dark cycle with food and water available ad libitum. On the day of experimentation animals were anesthetized with an intraperitoneal (IP) injection of ketamine hydrochloride (75 mg/kg) and xylazine (7.5 mg/kg), supplemented as needed. Anesthesia was maintained by intravenous (IV) delivery of ketamine (50 mg/kg/h) through a femoral vein catheter. A tracheotomy was performed for artificial ventilation, and eye movements were paralyzed by an IV bolus of gallamine triehloide (20 mg/kg) followed by IV infusion of gallamine (40 mg/kg/h) through a main vein catheter. A tachocrone was positioned for artificial ventilation, and eye movements were paralized by an IV bolus of gallamine triehloide (20 mg/kg) followed by IV infusion of gallamine (40 mg/kg/h) through a main vein catheter. A stereotaxic. An intramuscular (IM) bolus of dexamethasone (1 mg) was given to prevent cerebral edema during prolonged anesthesia, and pupils were dilated with 1% cyclopentolate hydrochloride drops. Some experiments were performed with anesthetic and no extra treatments (α = 3), and outflow data were not noticeably different (Supplementary Fig. S1).

Experimental Setup and Calibration
The anterior chamber was obliquely cannulated by a 33-gauge hypodermic needle (TSK Laboratory, Tochigi, Japan; length: 15 mm, lumen: 0.11 mm) with care taken to avoid puncturing the lens capsule. The needle was connected via a three-way stopcock and polyethylene tubing (length: 45 cm, lumen: 0.4 mm) to a piezoresistive pressure sensor (model 26P, Honeywell, Morristown, NJ, USA) positioned at eye level and a programmable syringe pump (ONE-1000, New Era Pump Systems, Farmingdale, NY, USA). The tubing was filled with artificial aqueous humor (130 mM NaCl, 5 mM KCl, 5 mM NaHCO3, 1 mM CaCl2, 0.5 mM MgCl2, 5 mM glucose, 20 mM HEPES, pH 7.25).25 The pressure sensor was temperature compensated and referenced to atmospheric pressure so that any effects of ambient temperature or pressure variation were eliminated. Sensor output was amplified, low-pass filtered at 1 Hz, and digitized at 2 Hz to a computer. The syringe pump was controlled by a custom LabView program (National Instruments, Austin, TX, USA) that perfused the eye at a set rate (CPF) or held IOP at a set level by modulating the duty cycle (mCP). After eye cannulation, corneas were covered with clear contact lenses (0.2 mm ACLA film; Honeywell) and instilled every 15 minutes with a drop of saline to prevent desiccation. The cannulation site was regularly checked to confirm there was no needle movement, internal tissue damage, or visible leakage at high IOP. Data collection began when IOP settled at a level that fluctuated <1 mm Hg peak-to-peak over 15 minutes. This level was defined as the resting IOP.

The hydrodynamic properties of the eye perfusion system were characterized prior to study commencement. First, the pressure sensor was calibrated with a mercury manometer to produce linear (R2 = 0.999) and accurate (error = ±0.4 mm Hg) readings for a saline reservoir positioned at variable heights. The system was then connected to a 33-gauge needle submerged in saline and sensor output was recorded for pump rates of 1 to 4 μL/min. System resistance was specified by linear regression of the pressure-flow data. Lastly the needle was sealed, the pump was configured to inject a 1- to 4-μL bolus of fluid into the closed system, and the instantaneous pressure change was recorded. System compliance was specified by linear regression of the pressure-volume data.26 The same bolus sequence was delivered during animal experiments and ocular compliance was estimated by the change in regression slope.

Constant-Flow Technique
The CF technique was applied to 9 rats. After determining the resting IOP, pump rate was increased in 0.2 μL/min steps between 0.1 and 1.3 μL/min, and for each step the anterior chamber was perfused until IOP stabilized at a level that fluctuated <1 mm Hg for at least 5 minutes. At each steady-state level fluid flow into and out the eye is balanced, meaning that net flow F is:

\[ F = F_{\text{out}} - F_{\text{in}} = F_p \]  

where \( F_{\text{out}} \) is total outflow rate (μL/min), \( F_{\text{in}} \) is aqueous production rate (μL/min), and \( F_p \) is pump rate (μL/min). The animal was then euthanized with Euthasol (Vibrac, Fort Worth, TX, USA) given to effect, and data were collected in situ for the same rate increments approximately 30 minutes after injection when IOP dropped below 3 mm Hg.

Modified Constant-Pressure Technique
A modification of the constant-pressure (mCP) technique was applied to 10 rats, in which IOP was held constant by modulating pump duty cycle27 instead of the instantaneous perfusion rate.26,27 Figure 1 illustrates the technique for a 20 mm Hg set point and 2 mm Hg window. Upon set-point specification the pump turns on and injects fluid at a fixed rate, which gradually raises IOP from a resting level of 15 mm Hg. Once 21 mm Hg is reached, the pump turns off and IOP decreases as the excess fluid is cleared by the eye. The pump reactivates when IOP falls to 19 mm Hg, and the cycle repeats until a new set point is specified. In all experiments the window was 2 mm Hg, the pump rate was 1.5 μL/min, and the set point was incremented in steps of 5 mm Hg from an initial point that was ~5 mm Hg above the resting IOP. Data were collected for at least three to five cycles per step. At each set point, the fluid volume that enters the eye during on phases equals the volume that leaves during off phases since IOP is the same at cycle start and end, meaning that:

\[ T_1(F_p + F_{\text{in}} - F_{\text{out}}) = T_2(F_{\text{out}} - F_{\text{in}}) \]  

where \( T_1 \) is the time required to raise IOP by 2 mm Hg (on duration) and \( T_2 \) is the time required for IOP to fall by 2 mm Hg (off duration). The equation can be rearranged to give the net flow:

\[ F = F_{\text{out}} - F_{\text{in}} \geq \frac{T_1}{T_1 + T_2} F_p = D \cdot F_p \]  

where \( D \) is pump duty cycle. It may be seen that the mCP and CF techniques are theoretically equivalent since \( D = 1 \) if the pump never turns off. \( T_1 \) and \( T_2 \) were measured for each cycle and \( F_p \) was averaged across all cycles of a given set point. The animal was then euthanized, and data were collected in situ for the same set-point increments approximately 30 minutes after injection when IOP dropped below 3 mm Hg.

Estimation of Inflow-Outflow Parameters
IOP in a living animal may be described by the modified Goldmann equation.28

\[ IOP = \frac{D \cdot F_p + F_{\text{in}} - F_{\text{out}}}{C} + EVP \]  

where \( C \) is the characteristic compliance coefficient (12.2 μL/mm Hg).
Assessment of Parameter Estimates

Additional experiments were performed on four groups of animals. To test for hysteresis, data were collected and compared for a sequence of increments and decrements in pump rate or set point. To test for washout, the eye was perfused for 2 to 3 hours at a fixed rate that raised IOP 15 to 20 mm Hg above rest. Pump rate was divided by the pressure change to convert the record to instantaneous outflow facility and fit by a line. The slope estimated washout, which was expressed as percentage change per hour by normalizing to outflow facility at pump onset. To assess accuracy, data collection was repeated with a shunt inserted through the cornea and opened to air. The shunt was made from perfluoralkoxy tubing (length: 20 mm, lumen: 50 μm) and had a measured conductance of $C_g = 0.029 \mu L/\text{min/mm Hg}$. The shunt adds a parallel IOP-dependent element to the Goldmann equation, which results in:

$$F = C \cdot (\text{IOP} - \text{EVP}) + (F_{\text{in}} - F_{\text{in}} - C \cdot \text{EVP})$$

where $C$ is constant outflow facility [μL/min/mm Hg], $F_{\text{in}}$ is unconventional outflow rate [μL/min], and EVP is episcleral venous pressure [mm Hg]. $D$ is 1 for CF experiments, and it is determined empirically for mCP experiments. The equation does not account for flow in the unconventional pathway that may be pressure dependent. Since $D \cdot F_p$ corresponds to $F$ for both techniques, this means:

$$F = C \cdot \text{IOP} \cdot (F_{\text{in}} - F_{\text{in}} - C \cdot \text{EVP})$$

The modified Goldmann equation was fitted to vivo data. The regression slope estimates outflow facility of live eyes ($F_{\text{out}}$), and the y-intercept estimates a combination of three additional parameters ($F_{\text{in}}, F_{\text{in}}, \text{EVP}$). Euthanasia eliminates the last two parameters, reducing the equation to:

$$F = C \cdot \text{IOP} + F_{\text{in}}$$

which was fit to the in situ data. The regression slope and y-intercept estimate outflow facility of dead eyes ($F_{\text{dead}}$) and $F_{\text{in}}$, respectively. $F_{\text{in}}$ and EVP cannot be separately estimated and were therefore combined into:

$$F_{\text{in}} = F_{\text{in}} + C \cdot \text{EVP}$$

where $F_{\text{in}}$ was calculated from the difference in y-intercept of live and dead eyes.

Histologic Processing

The impact of eye perfusion was examined histologically for 5 rats. After data collection was complete, both eyes were enucleated and placed in 4% paraformaldehyde for 24 hours. The eyes were then embedded in paraffin, sliced in 4-μm sections, and mounted on gel-coated slides. Tissue sections of the iridocorneal angle of both eyes were stained with hematoxylin and eosin, viewed under light microscopy, and digitally photographed.

Data Analysis

Statistical significance was assessed with paired and unpaired t-tests at an α-level of 0.05% using SigmaPlot software (Systat, Inc., San Jose, CA, USA), unless otherwise specified. Results are expressed within experiments as 99% confidence intervals in brackets and across experiments as mean ± standard deviation (SD).

RESULTS

Perfusion System Properties

The hydraulic resistance of the perfusion system was $0.36 \pm 0.01 \text{ mm Hg} \cdot \text{min}/\mu L$ (α = 5%) when connected to a 35-gauge needle. This corresponds to a hydraulic conductance of $2.78 \pm 0.08 \mu L/\text{min/mm Hg}$, which nearly matches the expected value given by Poiseuille’s law (2.6 μL/min/mm Hg). It is 100-fold larger than the outflow facility of rat eyes measured below so its influence on the measurements can be ignored. Figure 2A shows the system response to fluid boluses administered with the needle sealed shut and with the needle inserted in a rat eye. Pressure increases nearly instantaneously in both cases, then holds steady for the closed system and decays back toward baseline for the open system. Figure 2B relates measured pressure changes to bolus volume. The hydraulic compliance of the entire perfusion system ($0.105 \pm 0.016 \mu L/$
Rat Aqueous Humor Dynamics

**Figure 2.** Perfusion system properties. (A) Pressure signal recorded by system in response to bolus injections of 1, 2, 3, and 4 μL (arrowheads). The system was connected to a 33-gauge needle that was sealed with cyanoacrylate (left) or inserted in the anterior chamber of a rat eye (right). (B) Peak instantaneous pressure versus bolus volume for the closed (filled symbols) and open (unfilled symbols) system. The slope of the regression line fit to the two datasets gives the system compliance (0.089 ± 0.007; 0.091 ± 0.001 μL/mm Hg) and combined ocular and system compliance (0.140 ± 0.012, 0.154 μL/mm Hg), respectively.

**Figure 3.** Constant-flow perfusion of a live rat eye. (A) Perfusion rate (left) and IOP response (bottom) are shown for rate increments of 0, 0.1, 0.3, 0.5, 0.7, and 0.9 μL/min. Arrowheads mark the plateau level at which IOP settled after each increment. (B) Plateau IOP versus net eye flow F which is equivalent to F_{	ext{outflow}} in a CF experiment. The slope of the regression line fit is outflow facility (C = 0.025 [0.025, 0.027] μL/min/mm Hg), and the y-intercept represents IOP-independent flow (C = 0.312 ± 0.315, 0.275 μL/min).

**Rat Eye Perfusions**

Aqueous humor dynamics were quantified for 17 rats. Figure 3A shows representative data from a CF experiment. Following each step in perfusion rate IOP settled over 10 to 30 minutes to a plateau level. Figure 3B plots net flow F versus plateau IOP. The y-intercept (zero net flow) is the resting IOP. Data are all positive (outward flow) because the pump only infused fluid. Linear regression gives an outflow facility of C = 0.025 μL/min/mm Hg for this animal. Figure 4A shows representative data from a hCP experiment. Following each step in set point, pump duty cycle D increased, which raised IOP to the specified range and maintained it there. Pump duty cycle was measured for several periods and was stable over time irrespective of IOP set point (Supplementary Fig. S2). Figure 4B plots net flow averaged over all cycles versus IOP level. The x-intercept is again the resting IOP. Linear regression gives an outflow facility of C = 0.022 μL/min/mm Hg for this animal. In both experiments the y-intercept is negative, indicating that the pump would have to withdraw fluid to lower IOP to zero.

It may be noted that the CF technique took twice as long as the mCP technique to estimate parameter values owing to its lengthy settling times. Data repeatability was checked with a hysteresis test. Figure 5 presents a hCP experiment in which IOP was stepwise decremented and incremented from an initial set point 30 mm Hg above the resting level. Estimates of C and y-intercept were not significantly different for the two step sequences for this animal and two other animals (P > 0.1 for each), implying that eye outflow properties were not altered by the pressure magnitudes and oscillations used in these experiments.

Figure 6 provides pressure-flow data for all experiments. Resting IOP averaged 14.6 ± 1.9 mm Hg in anesthetized rats. Outflow facility estimated for the CF and mCP techniques were indistinguishable across animals and between live and dead eyes (2-way ANOVA, F > 0.16, P > 0.53 for all comparisons) as.
well as for the same eye of individual animals ($P = 0.83$). Results were therefore combined to give $C_{\text{flow}} = 0.023 \pm 0.002$ $\mu$L/min/mm Hg and $C_{\text{cadu}} = 0.024 \pm 0.002$ $\mu$L/min/mm Hg. The data shifted upward in dead eyes by $F_{p} = 0.421 \pm 0.050$ $\mu$L/min due to the loss of aqueous production and EVP. The $y$-intercept became positive in dead eyes, which is indicative of IOP-independent outflow. Studies have attributed this to the unconventional pathway$^{19,52}$, which would imply that $F_{\text{cadu}} = 0.096 \pm 0.024$ $\mu$L/min at rest ($n = 9$).

Control Experiments

Estimates of $C$ and $F_{\text{cadu}}$ were assessed with control experiments. Figure 7A shows pressure-flow data before and after a shunt of known conductance was inserted in the eye. The additional pressure-dependent drainage pathway had marked effect on outflow facility, which increased by $0.028 \pm 0.005$ $\mu$L/min/mm Hg on average ($n = 4$). The increase was within measurement error of shunt conductance ($P < 0.01$), bolstering confidence in the accuracy of outflow facility estimates. The shunt also lowered resting IOP level, as indicated by the shift in $y$-intercept, but it did not significantly alter the $y$-intercept ($-0.035 \pm 0.165$ $\mu$L/min, $P = 0.43$). Figure 7B shows pressure-flow data collected from live and dead eyes hydrated with saline via a drip ($n = 3$) or bath ($n = 2$). There was no impact on $C_{\text{low}}$ ($P = 0.93$) or $C_{\text{cadu}}$ ($P = 0.75$), as one would expect. The $y$-intercept of dead eyes, on the other hand, was no longer measurably greater than zero ($F_{\text{cadu}} = 0.003 \pm 0.030$ $\mu$L/min, $P = 0.83$). This suggests that the positive $y$-intercept in Figure 6 is not a measure of unconventional outflow rate but rather reflects some fluid loss to evaporation in nonimmersed eyes.

Washout Test

Eye perfusion may damage outflow pathways, especially at high flow rates. This would cause parameter estimates to change over time, a phenomenon known as washout.$^{29-31}$ Figure 8A shows an experiment that tested for washout by perfusing the eye at a constant rate for nearly 3 hours. IOP
increased by 14 mm Hg, which translated to an outflow facility
of 0.022 μl/min/mm Hg that washed out at 1.1%/h. The
washout rate averaged -2.3 ± 4.9%/h for all eyes tested (n = 5),
which was not measurably different from zero (P = 0.31).
Figure 8B shows that the trabecular meshwork of the perfused
eye was morphologically intact and angle structure looked
similar to the control eye, consistent with the lack of
physiological evidence for washout.

**DISCUSSION**

This study estimated physiological parameters of aqueous
humor dynamics in live healthy rat eyes. Conventional outflow
facility C was determined from the slope of pressure-flow data,
which were linear over the measured range and indistinguish-
able for live and dead eyes, as observed in mice.19,32 C may
overestimate the facility of the trabecular pathway if there are
other pressure-dependent outflow pathways in rat eyes as in
other animals6 or if outflow facility varies with IOP.33,34
Unconventional outflow rate Fm could not be accurately
estimated from the y-intercept of dead eye data. Although
much of the eye is protected from evaporation and steps were
taken to keep exposed surfaces moist, the intercept was
nevertheless sensitive to hydration state. The finding extends
reports of no pressure-independent flow in enucleated mice
eyes33,34 to non-enucleated rat eyes. It also indicates that
previous in situ estimates of Fm in mice are probably
contaminated by evaporation.19,32 Aqueous production rate
Fp and EVP could not be separately estimated from pressure-
flow data alone.

Outflow facility estimates were confirmed using two
different techniques (CF and mCP). The mCP technique is a
variation on the constant-pressure method of measuring
aqueous humor dynamics. It is simple in concept and low in
cost because only IOP is measured. Flow rate is inferred from
the time it takes a pump to raise IOP a small amount and the
time it takes the eye to clear the infused fluid. A similar
technique was recently employed to measure outflow of
enucleated mice eyes,35 except pump rate was not fixed in
magnitude but rather modulated continuously using an
expensive pump microcontroller. Flow rate was specified by
the modulation waveform, which has the advantage that flow
can be estimated at any time and not just at end of pump duty
cycles. It may extend recording time as IOP took several
minutes to reach steady state after set-point changes.

**FIGURE 7.** Parameter assessment. (A) mCP experiment in which
pressure-flow data were collected before (circles) and after (squares) a
shunt was inserted through the cornea. Lines are regression fits of the
respective datasets (C = 0.024 [0.022, 0.026] and C = 0.049 [0.045,
0.055] μl/min/mm Hg). Error bars are standard deviation. (B) Pressure-flow
data from mCP experiments on live (filled) and dead (unfilled) rats in
which the eye was kept moist by a constant saline drip (circles) or by
immersion in a saline bath (triangle). Lines are linear regression fits of the
data. Error bars are standard deviation.
presumably because the microcontroller slows pump rate as IOP approaches the new set point. Other variations of the method directly measure flow with a flowmeter and use a gravity feed to eliminate the pump and control circuitry.\textsuperscript{32-35} The mrCP technique was found to produce equivalent results as the CF technique in half the time. Moreover, C estimates were very consistent and were validated by inserting a shunt of known conductance into the eye. The coefficient of variation was 8%, which is similar to measurements from in vivo mouse eyes\textsuperscript{32,34-36} and much lower than those (15%–35%) from enucleated mouse eyes.\textsuperscript{24,27,30,39}

**Species Comparisons**

Aqueous humor dynamics are known to scale with eye size.\textsuperscript{15} Conventional outflow facility measured for adult Brown-Norway rats is approximately 10-fold less than that of humans (0.24–0.29 μL/min/mm Hg\textsuperscript{14,15,40}) and 4-fold more than that of mice (0.005–0.007 μL/min/mm Hg\textsuperscript{14,24,25,54,36,39} but see Refs. 19, 32). The scaling relationship parallels differences in anterior chamber volume among these animals.\textsuperscript{15} It is similar in scale but slightly smaller in value than prior measurements in rats (C = 0.044 ± 0.010 μL/min/mm Hg\textsuperscript{14} and 0.051 ± 0.010 μL/min/mm Hg\textsuperscript{35}). Both studies examined Lewis rats so the higher facility of this albino strain may reflect the absence of pigment granules, which can accumulate in the trabecular meshwork. Aqueous humor dynamics have been noted to vary in mice with strain age and outflow facility was greatest for adult albino mice.\textsuperscript{32,35}

Aqueous production rate and EVP cannot be estimated via the Goldmann equation from pressure-flow data alone. One of the parameters must be determined empirically in order to solve for the other. A reported approach is to measure EVP by lowering IOP until there is blood reflux into Schlemm’s canal.\textsuperscript{15,35} The approach was attempted in rats with limited confidence in accuracy. EVP has been measured in young Sprague-Dawley rats, and it averaged 7.8 mm Hg.\textsuperscript{35} If the value applies to adult Brown-Norway rats, $F_{pm}$ estimates would predict an aqueous production rate of $F_{pm} = 0.242$ μL/min, which would fall statistically within the 0.350 ± 0.110 μL/min range measured by dye dilution in Lewis rats.\textsuperscript{15} Similar to outflow facility, this production rate would be approximately 10-fold less than that of humans (2.1–2.9 μL/min during the day\textsuperscript{14,34,36}) and a few-fold more than that of mice (0.060–0.20 μL/min depending on age and strain.\textsuperscript{15,32,34,37,38})

It has long been thought that the eyes of all animals, except humans, exhibit higher outflow facility over time when experimentally perfused. The washout phenomenon has been attributed to clearance of extracellular material in the outflow pathway, clearance of proteins in the iris root, and mechanical disruption of the trabecular meshwork.\textsuperscript{31} Human eyes were presumed to differ in some important structural or functional respect that prevents washout. Much of this work was performed on larger mammals. Recent studies have found that mouse eyes do not exhibit the phenomenon either.\textsuperscript{32,37,38} The absence of washout in rats extends the finding to another rodent.

**Study Limitations**

A principal limitation of this study is that parameters are estimated from the Goldmann equation under the assumption that aqueous humor dynamics are linear and only trabecular outflow is pressure dependent. The linearly assumption appears reasonable for IOP levels up to 40 mm Hg above rest in rats as data scatter nonsystematically about the regression line. The assumption might not, however, be valid at IOP levels that were not tested. For example, it has been found that the outflow facility of enucleated mouse eyes decreases to zero at low IOP and that pressure-flow data are better described by a power function.\textsuperscript{15,32} If rat eyes exhibit similar nonlinear behavior in vivo, C may grossly misestimate outflow facility at IOP levels below and well above rest. $F_{pm}$ and possibly $F_{pm}$ may be misestimated as well, and estimation errors would compound if additional parameters depend on IOP. IOP levels below rest were not tested owing to the risk of tissue damage from aspiration, but the dead eye results do not support the presence of a power-law nonlinearity in rats since pressure-flow data of well-hydrated eyes were linear down to a y-intercept of zero. Perhaps the nonlinearity observed in mice is related to small eye size or eye enucleation. The assumption of a single IOP-driven outflow pathway requires further investigation. A secondary limitation may be that this study used pharmacologic treatments to eliminate eye movements and extend recording time. The treatments did not appear to influence aqueous humor dynamics since facility measurements were similar for euthanized animals and for a subset of animals not given treatments.

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Supplemental Figure S1. Pressure-flow data for 3 experiments in which rats were only administered ketamine-xylazine IV. Resting IOP, conventional outflow facility, and υ-intercept were not significantly different from those of rats given paralytics, mydriatics, or other treatments (p = 0.621, 0.314, 0.766, respectively).
Supplemental Figure S2. (A) IOP and duty-cycle records from a mCP experiment. Shown are 16 pump cycles after the set point of 41 mmHg was reached (top) and duty cycle D measured from the pump on and off duration of each cycle (bottom). Solid line is a regression of the duty cycle data (Pearson correlation coefficient $= 0.07, p = 0.81$). (B) Slope of the regression line as a function of IOP level for 3 rats. Bars give standard error of the slope. The slope of 0 indicates that net flow did not change over time and that duty cycle measurements can be averaged to give for each IOP setting.