

Endothelial Caspase-9 Activity Exacerbates Edema and Neuronal Dysfunction after Retinal Vein  
Occlusion

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## ABSTRACT

### Endothelial Caspase-9 Activity Exacerbates Edema and Neuronal Dysfunction after Retinal Vein Occlusion

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The retina is one of the most metabolically active tissue in the body, rendering it sensitive to vascular dysfunction. Consequently, diseases that disrupt normal retinal blood supply, including retinal vein occlusions (RVO) and diabetic retinopathy, are the leading causes of blindness in working-age adults. Despite available therapies, an estimated 50% of patients do not respond to treatment. An increasing prevalence of retinal edema and the limits of currently available interventions demonstrate a growing unmet need for a new avenue of treatments.

To investigate potential new drug targets for the treatment of retinal vascular disease, we employed a mouse model of retinal vein occlusion (RVO), achieved by tail-vein injection of rose bengal, followed by laser photocoagulation of retinal veins. RVO induces acute retinal edema, which peaks during the first 24 hours following injury. Over a 7 day time course the edema resolves, revealing a permanent retinal thinning due to death of retinal neurons. We followed retinal pathology in injured mice using the same imaging techniques which are commonly used in diagnosing human patients to facilitate future development of medical applications from our interventional studies. *In vivo* analyses – optical coherence tomography (OCT), fluorescein angiography, and electroretinograms (ERGs) - were conducted with the Micron IV system (Phoenix Research Labs).

We identified caspase-9, a protease traditionally associated with apoptosis, as an essential mediator of edema. Increased levels of activated caspase-9 were detected in vascular endothelial cells 1 hour following RVO. We tested RVO in mice with inducible endothelial-cell-specific deletion of caspase-9 (iC9 ECKO). Compared to littermate controls, Casp9 ECKO mice

develop less edema, and sustain less retinal degeneration after RVO injury. ERG analysis showed preservation of retinal function in Casp9 ECKO mice.

To study whether inhibiting caspase-9 would provide protection against RVO we utilized a highly specific caspase-9 inhibitor, which we can deliver to the retina using simple eye drops.

Treatment of wildtype mice with the caspase-9 inhibitor immediately after induction of RVO provided morphologic, biochemical and functional retinal protection. Inhibition of caspase-9 reduces edema, protects retinal morphology, and helps prevent vision loss following RVO injury.

Our studies indicate that endothelial caspase-9 plays an essential role in regulating edema pathogenesis. Moreover, our novel cell permeant caspase-9 inhibitor abrogates the edema and may be a potential therapy for individuals suffering from vascular eye disease.

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## 1. General introduction

The central nervous system, which contains some of the most metabolically active tissues in the body, has a limited capacity to store energy, rendering it particularly vulnerable to vascular dysfunction. Ischemic injury occurs when a vessel occlusion disrupts blood flow, depriving cells of essential access to glucose and oxygen. Such insult to the central nervous system (CNS) causes a pathological increase in vascular permeability, edema and neuronal loss. In the brain, ischemic stroke, caused by occlusion of cerebral blood vessels, is a leading cause of mortality and disability; In the eye, retinal vein occlusions (RVO), are an important cause of vision loss in working age adults.

In previous work, the Troy group investigated the activation of caspase signaling following ischemic stroke (Akpan 2011). Using an activity-based unbiased assay, they identified caspase-9 activity in rat brain within 1 hour of reperfusion. Caspase-9 protease activity triggered apoptotic signaling in the ischemic tissue, resulting in neuronal death. They showed that a cell permeant specific caspase-9 inhibitor (Pen1-XBir3) prevented neuronal cell death, and improved neurological function in both rat and mouse models of stroke. In addition to decreasing infarct volume, caspase-9 inhibition drastically reduced edema in stroked brains. This observation led to the hypothesis that caspase-9 activity may be modulating edema.

Caspase-9 is a member of the cysteine-aspartic family of proteases. Originally identified as an initiator of the intrinsic apoptosis pathway, caspase-9 has since been implicated in a number of nonapoptotic processes. Caspase-9 promotes muscle and cardiomyocyte differentiation and proliferation (Murray 2008, Bulatovic 2015), hematopoietic development (Lu 2014), maturation and axonal pathfinding of olfactory bulb sensory neurons (Ohsawa 2010), and activation of pro-survival NF- $\kappa$ B signaling (Vu 2016). Although little is known about vascular-specific functions of caspases, reports in the cancer literature describe a role for effector caspases in driving the

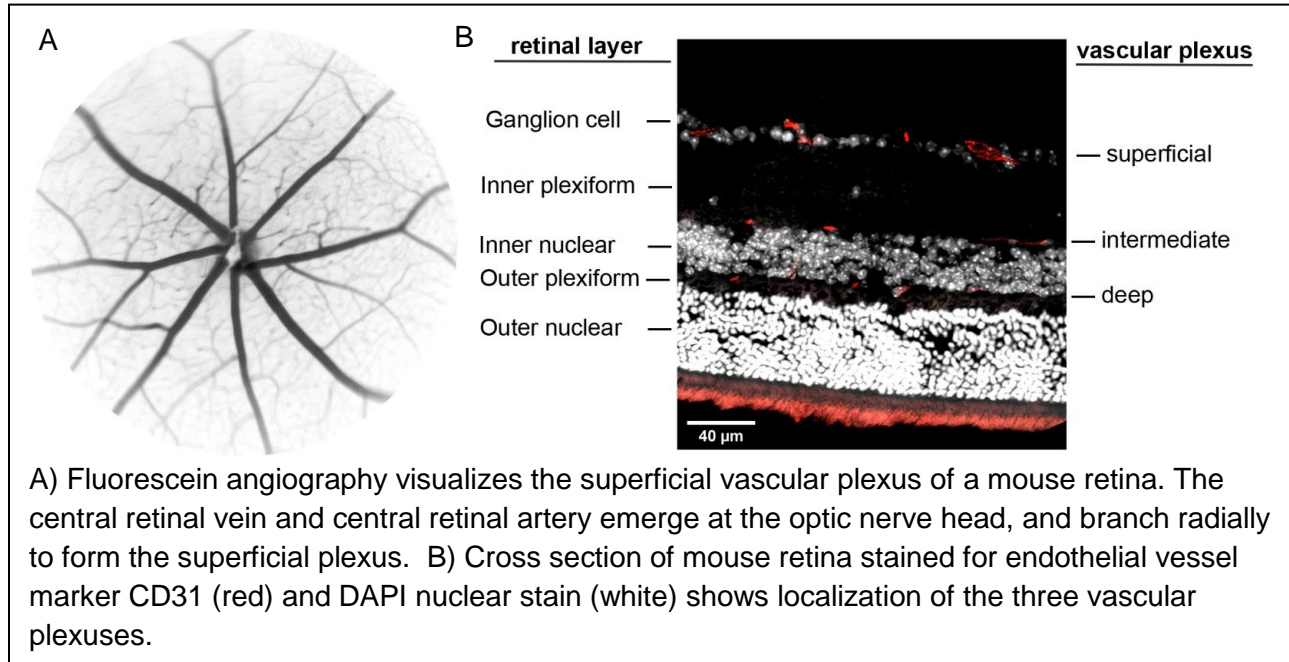
formation of vessel-like structures that supply oxygen and nutrients to aggressively invasive tumors (Linder 2016).

We utilized a mouse model of RVO to investigate the role of caspase-9 activity in the development of edema and CNS dysfunction after ischemic injury. The retina is an extension of the central nervous system, and its neurovascular architecture shares a close resemblance to the brain (London 2013). Neurons and endothelial cells are intricately connected through the neurovascular unit, a structure formed by neurons, astrocytes, basal lamina, smooth muscular cells and pericytes, endothelial cells and extracellular matrix (Harder 2002). This structure forms the basis of the Blood-Retina-Barrier (BRB). Like the Blood-Brain-Barrier, the BRB depends on tight junctions between endothelial cells to maintain proper balance of oxygen and nutrients for the highly metabolically active retinal tissue and prevent free diffusion of substances between the blood and the retina (Zhang 2014). During RVO, hypoxia results in the breakdown of the BRB in the inner retina, which then leads to increased vascular permeability, resulting in retinal edema and tissue damage (Kaur 2008).

While RVO shares many similarities to the etiology of ischemic stroke, the mouse model of RVO has key advantages for investigating ischemic injury in the CNS. Fluorescein angiography directly visualizes the breakdown of BRB integrity, allowing us to differentiate vasogenic edema from other sources of fluid accumulation in ischemic tissue. Live OCT imaging provides real-time optical histological cross-sections of the retina, allowing us to closely track the temporal progression of edema and cell loss through distinct retinal layers. Meanwhile, focal electroretinogram (ERG) allows us to measure the electrophysiological response of retinal neurons in the area affected by RVO, providing a direct readout of how vascular injury impairs neuronal function.

## 1.1 Introduction to retinal vasculature

Figure 1: Retinal vascular plexuses



Retinal vasculature is organized into three layers: the superficial plexus, the intermediate plexus, and the deep vascular plexus (Stahl 2010). The inner or superficial plexus is located in the ganglion cell layer. The central vein and artery emerge from the optic nerve and branch out radially to form the superficial plexus. This is the vasculature seen by fundus retinal imaging and fluorescein angiography (Fig 1a). Branch retinal vein occlusions (discussed in chapter 1.2) occur in the major veins of the superficial vascular plexus. The intermediate and deep vascular plexuses can be seen in the inner plexiform and outer plexiform layers of retinal cross sections (Fig 1b). Multiple vessels connect the three vascular plexuses. Neurons of the inner retina depend on these vascular networks for their blood supply. In contrast, the outer retinal layers are avascular (Fig 1b). Photoreceptors in the outer retina, whose cell bodies can be seen in the outer nuclear layer, receive their blood supply from the choroidal vasculature.

Retinal capillaries are formed of endothelial cells, ensheathed by pericytes and Muller cell (glial) processes. Tight junctions between endothelial cells help form the blood-retina barrier, which controls fluid and molecular movement within retinal tissues, and prevents leakage of macromolecules and other potentially harmful agents into the retina. Tight junctions seal together adjacent endothelial cell membranes, creating a highly selective barrier. Zonula occludens (ZO-1, -2, -3), symplekin, 7H6 and cingulin are proteins which help organize tight junctions; loss of ZO-1 immunoreactivity in blood vessels indicates significant tight junction disruption. Occludin and claudin proteins regulate the flow of vascular fluid through tight junctions (Kaur 2008).

Pericytes regulate vascular tone and increase the barrier integrity of the BRB. They support capillary structure, control endothelial cell proliferation, and help maintain the integrity of the BRB (Kaur 2008). Loss of capillary pericytes has been suggested to play a role in increased capillary leakage in diabetic retinopathy. Pericytes are contractile cells, which help regulate regional blood flow. Exposure to endothelin-1, angiotensin II, and hyperoxia cause pericytes to contract, while exposure to CO<sub>2</sub> or ATP induce pericyte relaxation, promoting local blood flow (Kaur 2008). Regulation of blood flow is also responsive to retinal neuronal activity—localized light stimulation induces hyperemia response as blood vessels dilate, supplying additional oxygen and glucose to neurons and glial cells of the inner retina. This responsiveness of retinal vasculature to neuronal needs is impaired in patients with retinal vein occlusion or diabetic retinopathy (Corvi 2015).

Muller cells exist in close spatial relationship to both blood vessels and retinal neurons and play a key role in supporting vascular and neuronal retinal function by regulating vascular permeability, maintaining homeostasis of potassium and other ions, and helping drain excess water from retinal tissues (Kaur 2008). Muller cells are a major source of intraretinal VEGF (vascular endothelial growth factor), a protein which plays important roles in both physiological



and pathological angiogenesis, endothelial and neuronal survival, and vascular permeability (Ferrara 2003). VEGF increases vascular permeability and angiogenesis via activation of the VEGFR-2 receptor on endothelial cells. Under ischemic conditions, Muller cell-derived VEGF impairs BRB function and drives pathological angiogenesis (Bai 2009). The pathogenic role of VEGF in RVO is further discussed in Chapter 1.2.

In addition to their interactions with retinal vasculature, Muller cells ensheath the cell bodies and processes of retinal neurons, where they promote the formation of synapses, and provide nerve terminals with energy substrates and neurotransmitter precursors (Vecino 2016). Individual Muller cells extend vertically from the outer nuclear layer and into the innermost ganglion cell layer, providing both structural support and an important functional link between retinal neurons and the retinal vasculature (Vecino 2016).

While Muller glia span throughout the retinal layers, astrocytes are almost exclusively confined to the innermost retinal layers. They share functional properties in common with Muller cells; astrocytes provide neurotrophic support and help maintain the blood retinal barrier (Vecino 2016). Like Muller cells, retinal astrocytes can respond to hypoxia in inner retinal layers by upregulating VEGF expression.

## **1.2 Introduction to retinal vein occlusion (RVO)**

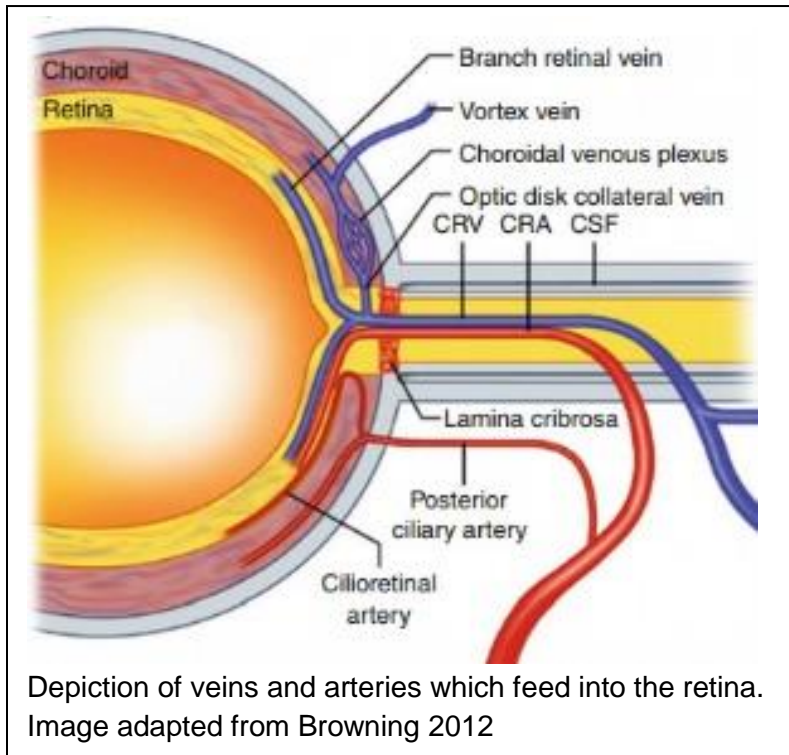
### **1.2.1 RVO classification, causes and epidemiology**

#### **Classification of RVO**

Retinal vein occlusion is the second most common source of vision loss from vascular eye disease, following diabetic retinopathy. As the name implies, RVO occurs when an obstruction impedes blood flow through one of the major retinal veins. Classifications of the disease are

determined by two parameters: the location of the obstruction, and the degree of retinal nonperfusion in the affected area.

*Figure 2: Anatomy of retinal vasculature*



**Central Retinal Vein Occlusion (CRVO):** is caused by an occlusion in the central retinal vein (which runs through the optic nerve), or at the lamina cribrosa (the mesh-like structure through which the optic nerve enters the eye). Since the central retinal vein is the sole source of drainage from the retinal vessels, CRVO afflicts the entire retina, and causes the greatest visual impairment. CRVO receives more aggressive treatment compared to other forms of RVO, as it carries the greatest risk of permanent severe vision loss. Despite treatment, CRVO has the worst prognosis.

**Hemiretinal Vein Occlusion (HRVO):** is a variant of CRVO where an occlusion occurs at the lamina cribrosa, just before the point where the veins draining the superior and inferior halves of

the retina merge. An occlusion in one of these two veins would obstruct blood drainage from half the retina. The pathology of HRVO resembles CRVO, but is generally milder.

**Branch Retinal Vein Occlusion (BRVO):** is an occlusion affecting one of the distal veins within the retina. Degree of visual impairment from BRVO depends on whether the occlusion affects blood flow through the macula (a 5.5mm region of the retina that is responsible for central vision). Macula-sparing BRVO is often asymptomatic.

BRVO, HRVO, and CRVO may have either ischemic or nonischemic presentations.

**Nonischemic RVO:** describes the milder form of the disease and includes approximately 75% of cases at initial presentation (Central Vein Occlusion Study Group 1997). Blood flow is maintained through the retina, despite the occlusion, with minimal areas of nonperfusion. Patients with nonischemic RVO have minimal retinal pathology, and generally present with good vision. Nonischemic RVO may resolve fully, or may progress to the ischemic type.

**Ischemic RVO:** occurs when the occlusion creates sufficient increase in retinal pressure to prevent capillary blood flow. This is the more severe form of the disease, and is associated with poor vision outcomes.

Severity of visual impairment correlates to the severity of ischemia and the size of the affected retinal area; ischemic CRVO is a blinding condition with poor prognosis, whereas nonischemic BRVO may go entirely unnoticed unless the patient were to undergo ophthalmological examination.

### **Causes of RVO**

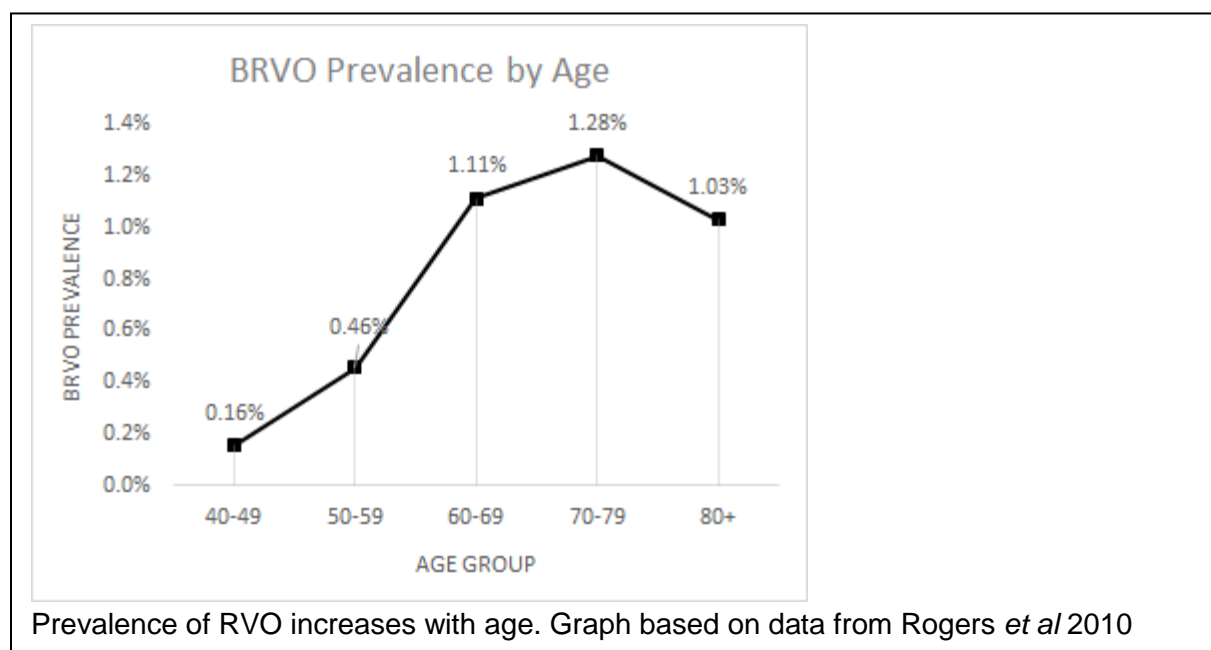
The causes of occlusions in RVO have been studied through autopsies and enucleation specimens (Zhao 1993, Green 1981). BRVO is usually caused by compression of the vein at arteriovenous crossing points. Meanwhile, the occlusion in CRVO and HRVO is usually a

primary thrombus (a blood clot that forms within a vessel). Age, hypertension, smoking, diabetes and obesity are all risk factors for developing RVO. Indeed, arterial disease is thought to be a predominant pathogenic mechanism in RVO. Arterial stiffness exacerbates venous constriction and causes hemodynamic stress at arteriovenous crossings and in the tight confines of the optic nerve sheath. Turbulent blood flow damages the venous endothelium, upregulating prothrombotic factors (such as von Willebrand factor--VWF) that can lead to clot formation (Browning 2012). Additionally, increased endothelin-1 production from sclerotic arteries may diffuse across to the neighboring vein, stimulating further venous vasoconstriction (Jaulim 2013). The complex etiology of RVO underpins how effective treatment for RVO cannot rely solely on restoring blood flow through the occlusion. Recanalization of the thrombus usually occurs naturally within 2 weeks of CRVO, but does not lead to resolution of retinal pathology (Green 1981).

### **Epidemiology of RVO**

BRVO is the most common form of RVO, and has a prevalence between 0.5-1.2%. Meta-analyses from 11 studies with 49,869 participants have confirmed that there are no gender differences, but higher prevalence of BRVO in Asians (5.7 per 1000; 95% CI: 4.5–6.8) and Hispanics (6.9 per 1000; 95% CI: 5.7–8.3). Ethnic variation may be attributed to increased risk factors for RVO in these populations (Jaulim 2013). CRVO is less frequent, at 0.1-0.2%, and has no ethnic variation in prevalence. Age is a very important risk factor for RVO, accounting for a nearly 10-fold increase in prevalence among 70-79 year olds, compared to 40-49 year olds (Rogers 2010, Fig 3).

Figure 3: RVO prevalence increases with age



## 1.2.2 Symptoms and natural history of RVO

### Symptoms

Patients with CRVO usually complain of painless acute onset of blurred vision in the affected eye. CRVO is rarely asymptomatic. Patients with BRVO can be asymptomatic, or may present with scotoma (a partial loss of vision or a blind spot in an otherwise normal visual field). The blurring or graying of vision would correspond to the area of RVO. BRVO affecting the macula causes blurring of central vision.

A portion of RVO patients have chronic edema, which can present additional symptoms due to neovascular glaucoma caused by neovascularization of the iris and/or anterior chamber angle (Rehak 2008). In addition to vision loss, these patients may have a red, painful eye secondary to elevated ocular pressure.

Ophthalmologic observations of RVO patient retinas show hemorrhages, cotton wool spots, hard exudates, retinal edema, and dilated tortuous vessels (Jaulim 2013). Dilated tortuous

vessels are a key feature common to all RVO variants. Areas of ischemia (retinal nonperfusion) and capillary leakage can be evaluated by fluorescein angiography. Retinal edema and retinal detachment are best diagnosed through optical coherence tomography (OCT). OCT is widely used to measure retinal thickening from edema and to monitor the effectiveness of treatment. Both retinal thickening during the acute edema phase, and degenerative retinal thinning after edema has resolved, are associated with decreased visual acuity (Jaulim 2013). Electroretinograms (discussed further in Chapter 2) show impaired retinal responsiveness to light stimuli in RVO patients.

### **Natural history of RVO**

Patients with BRVO may have spontaneous improvement in vision within the first three months of symptoms, but further vision gains after 3 months are unlikely. Without treatment, approximately two-thirds of patients will continue to lose visual acuity if their symptoms have not resolved spontaneously by 3 months. In a three-year study among untreated patients who had 20/40 vision or worse at three months post-BRVO, only 34% improved to 20/40 vision or better, while 23% deteriorated to 20/200 or worse. The average visual acuity at the three year mark was 20/70 (Branch Vein Occlusion Study, 1984).

In patients with CRVO, prognosis depends directly on visual acuity at initial presentation. In a three-year study, 66% of CRVO patients who presented initially with vision at 20/40 or better maintained acuity over the three years, and only 10% of these patients experienced vision deterioration to <20/200. Among patients who initially presented with visual acuity of 20/50 to 20/200, 50% maintained vision in the same range, while 33% deteriorated to <20/200. Among patients with the worst visual deficits at initial presentation (<20/200), only 20% had any improvements in vision. Patients with nonischemic CRVO had better visual outcomes, but 34% of nonischemic CRVO eyes converted to ischemic CRVO during the three years of observation

(Central Vein Occlusion Study Group, 1997). Ischemia developed most rapidly within the first 4 months of CRVO, but progressed steadily throughout the study.

A more recent natural history study of CRVO found a similar distribution of visual acuity improvement trends (Hayreh 2010). One third of CRVO patients initially presented with 20/40 vision or better; 80% of these patients maintained visual acuity over 2-5 years of the study. In 67% of CRVO patients (and in 100% of ischemic CRVO patients) initial vision was 20/40 or worse. After 2-5 years of follow-up, 35% of those patients had improved visual acuity, while 25% experienced further decline.

### **1.2.3 Focus on edema in RVO**

Edema (retinal swelling due to fluid accumulation within the retina) is a nonspecific sign of various retinal vascular disorders. It is the most serious vision-threatening complication of RVO. Upwards of 90% of major RVO cases present with edema, which may resolve spontaneously in about 50% of patients (Jaulim 2013). Macular edema is the main cause of impaired vision in BRVO patients, and occurs in approximately 30% of eyes (Li 2017). Retinal thickening results from a combination of vascular leakage (vasogenic edema) and cellular swelling (cytotoxic edema).

#### **Vasogenic edema**

RVO triggers inflammatory and hypoxia responses in the retina, both of which contribute to breakdown of the blood-retinal barrier. Retinal Muller glia, whose processes ensheath retinal capillaries, help regulate the barrier function of the vascular endothelium. In response to inflammation and hypoxia, Muller glia secrete factors such as VEGF, TNF- $\alpha$ , IL-1 $\beta$  and prostaglandins, which increase vascular permeability (Ascaso 2014). Due to decreased fluid clearance mechanisms in the diseased state (discussed below), increased vascular permeability results in vasogenic edema. Instead of alleviating hypoxia, vascular leakage further impairs

capillary blood flow, engendering a positive feedback cycle; hypoxia triggers secretion of factors that increase vascular permeability, which further exacerbates hypoxia.

### **Cytotoxic edema**

Cytotoxic edema occurs when cells swell due to retention of sodium and water. In the early phase of ischemic stroke, restriction of blood supply to neuronal tissues causes a disruption in cellular metabolism. Sodium-calcium pumps cease to function, resulting in intracellular buildup of sodium and calcium, which in turn produces an osmotic influx of water into cells via aquaporin channels. Astrocytes are more susceptible to cytotoxic edema than neurons, because they are involved in clearance of K<sup>+</sup> and glutamate, which contributes to osmotic overload (Liang 2007).

Muller glia and retinal pigment epithelium (RPE) play an important role in draining water from the retina. Under ischemic conditions, Muller glia experience cytotoxic edema, causing thickening in the central part of the retina. It has been suggested that glial swelling significantly contributes to the development of cystoid macular edema (Bringmann 2005).

RPE cells carry out subretinal fluid via aquaporin water channels. Breakdown in RPE function leads to fluid accumulation in the subretinal space, causing retinal detachment (Romero-Aroca 2016). The outer retinal layers (in particular the photoreceptor cells) are avascular, and receive their oxygen from choroidal circulation. Retinal detachment uncouples the outer retinal cells from their blood supply, resulting in ischemia and photoreceptor degeneration (Yang 2004). Retinal detachments are commonly associated with both vasogenic edema and retinal inflammatory processes (Wolfensberger 2000).

#### **1.2.4 Established treatment strategies for RVO**

There are no treatments proven to reopen blocked retinal veins. However, surgical interventions to recanalize retinal vein occlusions have been studied in preliminary clinical trials



and in animal models of RVO. Current treatment paradigms aim to mitigate the vision impairment caused by retinal edema and neovascularization. Laser photocoagulation, intravitreal anti-VEGF, and intravitreal corticosteroids are the most commonly used approaches in managing complications from RVO. A comprehensive review of currently used therapies is available in Ehlers 2017.

### **Laser photocoagulation**

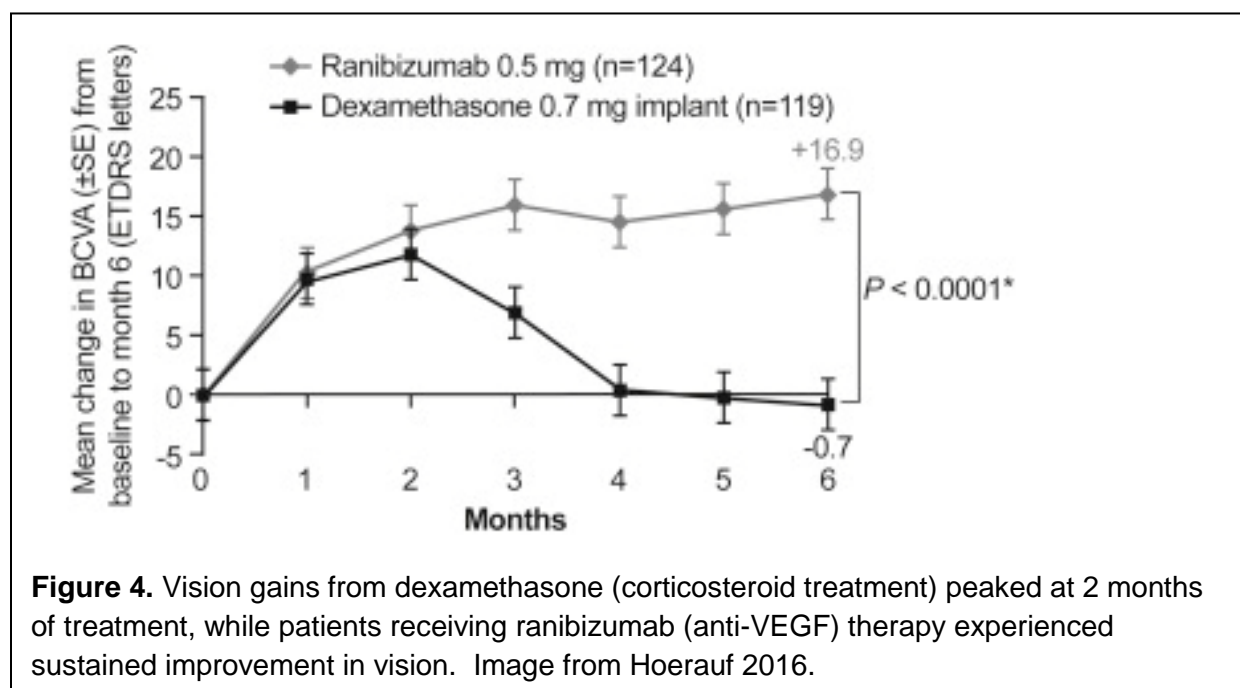
Laser photocoagulation applies a grid of laser burns to the ischemic retina with the goal of cauterizing leaky capillaries and improving retinal oxygenation. Photocoagulation of retinal photoreceptors decreases oxygen consumption in the outer retina, allowing for more oxygen to diffuse from the choroid into the inner retina. Laser photocoagulation increases inner retinal oxygen tension and autoregulatory vasoconstriction, causing subsequent decreases in intraretinal VEGF production (Lam 2015).

Until very recent years, laser photocoagulation was the mainline treatment for edema associated with RVO. It remains the first line of therapy for neovascular complications of RVO. The treatment confers minimal improvements in vision; in the pivotal Branch Vein Occlusion study which followed BRVO patients for 3 years, patients who received laser treatment gained 2 lines of vision, compared to 1 line gained in the control group (Branch Vein Occlusion Study Group, 1986). In a later trial among BRVO patients, no participants in either the control or the laser treatment groups gained 15 or more ETDRS letters of vision at the conclusion of the study (Parodi 1999). The intervention does however substantially reduce risk of further vision loss, and is still used as a rescue therapy in patients with neovascularization or retinal edema who do not respond to anti-VEGF therapy.

## **Intravitreal Corticosteroids**

Corticosteroid therapies, which directly target the inflammatory processes in RVO, were the first pharmacological agents available for treatment of edema. Corticosteroids can offer fast relief of edema and confer improvements in vision by 1-2 months of treatment. Triamcinolone acetonide (TA) and dexamethasone are the most frequently utilized corticosteroid formulations. Ozurdex, an intravitreal dexamethasone implant, was FDA approved for treatment of RVO after a clinical study showed that dexamethasone was able to improve visual acuity by 6 months compared to sham treatment controls (Haller 2010). However, a critical limitation of corticosteroid therapy is that its benefits are often transient (Fig 4). Vision gains from corticosteroids peak at 2 months of treatment, but are often negligible by 6 months (although some studies have reported patient population subgroups with sustained vision gains (Boyer 2014)). Furthermore, corticosteroid therapy carries serious risks of adverse effects, which compound over length of use. The most serious common side effect of corticosteroid treatment is the development of cataracts. Approximately 15-20% of eyes treated with high-dose injections of TA will require cataract surgery within one year of treatment (Detry-Morel 2004). Dexamethasone has the lowest cataract rates of the commonly used corticosteroids. Still, a three-year randomized sham-controlled trial of dexamethasone implant found cataract rates higher than 60% in treated eyes (Boyer 2014). Thus, while the short term efficacy of corticosteroid treatment underscores the importance of inflammatory processes in RVO pathology, these treatments are poorly suited to long term management of RVO.

Figure 4: Corticosteroids confer transient vision gains in RVO patients



**Figure 4.** Vision gains from dexamethasone (corticosteroid treatment) peaked at 2 months of treatment, while patients receiving ranibizumab (anti-VEGF) therapy experienced sustained improvement in vision. Image from Hoerauf 2016.

### Intravitreal anti-VEGF therapy

Intravitreal anti-VEGF injections are the current first line of treatment for RVO. Anti-VEGF agents have been used off-label in retinal vascular disease since 2004, when the FDA approved pegaptanib (a VEGF-neutralizing RNA aptamer) for the treatment of neovascular age-related macular degeneration (Kim 2012). VEGF, a factor that promotes endothelial survival, proliferation, and vascular permeability, is elevated in retinal vascular disease. Furthermore, VEGF is a pro-inflammatory cytokine; through complex interactions with the immune system, VEGF produces local inflammation and plays a role in allowing leukocyte infiltration into the retina (Deobhakta and Chang 2013, Ehlers 2017). Although the exact mechanisms are unclear, inflammation is prevalent in RVO and plays a critical role in the pathogenesis of edema. Pharmacological agents that block VEGF signaling are administered via intravitreal injection and have been shown to reduce edema and improve vision in some RVO patients.

**Ranibizumab (Lucentis):** is a humanized monoclonal anti-VEGF antigen binding fragment specifically designed and manufactured for use in the eye. The antibody fragment potentially inhibits activity of all known VEGF isoforms, but has been optimized for affinity to VEGF-A (Ferrara 2006). In 2010, ranibizumab received FDA approval for the treatment of retinal edema in RVO patients.

A large phase III study showed that after 1 year, 50.8% of CRVO patients treated with ranibizumab showed vision gains of 15 or more lines, compared to 33.1% of sham-treated patients. However, approximately half of RVO patients do not respond to ranibizumab. A 4-year follow-up of RVO patients treated with ranibizumab (RETAIN study) showed that edema resolution was successful in 50% of BRVO patients and 44% of CRVO patients. The HORIZON long term outcomes study found residual edema in 25% of BRVO patients, and 43.1% of CRVO patients, despite continued ranibizumab treatment.

**Bevacizumab (Avastin):** is a full length recombinant humanized monoclonal anti-VEGF antibody that binds all isoforms of VEGF-A. While not designed for use in the eye, bevacizumab is frequently used off-label for retinal edema. The 2015 CRAVE study showed that bevacizumab and ranibizumab were equivalently effective in treating RVO (Rajagopal 2015).

**Aflibercept (Eylea):** is a recombinant fusion protein consisting of portions of the human VEGFR-1 and VEGFR-2 fused to the Fc domain of human immunoglobulin G1 domain. It functions as a VEGF trap, and is more potent than ranibizumab or bevacizumab. In addition to blocking VEGF-A, aflibercept also targets VEGF-B and placental growth factor.

A number of clinical trials have compared the efficacy of ranibizumab, and bevacizumab or aflibercept. Most recently, the SCORE2 trial found that while aflibercept was marginally more effective in reducing retinal thickness, both aflibercept and ranibizumab produced equivalent visual acuity gains in CRVO patients (Scott 2017).

## **Unmet Need in RVO treatment strategies**

Anti-VEGF therapies are the current standard of care for RVO, and are the only interventions shown to consistently improve long term visual outcomes. For a significant portion of patients, anti-VEGF injections produce rapid resolution of edema and improve visual acuity. However, consistently across multiple studies, a substantial portion of RVO patients do not respond to anti-VEGF. Approximately 50% of treated RVO patients do not achieve full resolution of edema. In the most recent SCORE2 trial, 61.3% of bevacizumab treated eyes and 65.1% of aflibercept treated eyes gained 15 or more lines of visual acuity. The inverse interpretation is that 40% of CRVO patients did not achieve clinically significant gains in visual acuity, despite receiving the best standards of care. Long term treatment outcome studies indicate that visual gains are maintained over 5 years in most (79%) but not all RVO patients (Wecker 2016). In about 20% of patients, CRVO is associated with macular ischemia; in this patient group, anti-VEGF therapy has not been shown to produce any improvements in visual acuity (Chatziralli 2017).

Currently available strategies for treatment of retinal vascular disease do not directly address the neuronal degeneration that results from ischemic injury. However, the most serious complications of RVO involve permanent vision loss due to neuronal loss. Furthermore, aggressively treating retinal vascular disease with anti-VEGF therapeutics may increase risk to retinal neurons unless ischemia and edema are quickly resolved (Saint-Geniez 2008). VEGF plays a critical neuroprotective function during ischemia, and administration of intravitreal VEGF can actually help protect retinal neurons from apoptosis in ischemia-reperfusion injury (Nishijima 2007). Meanwhile, anti-VEGF treatment in mouse models of diabetic retinopathy was shown to increase neuronal apoptosis and retinal degeneration (Park 2014, Hombrebueno 2015). Outer retinal tubulation, an indicator of photoreceptor degeneration and overall poor visual outcome, was seen in 12 eyes of 11 diabetic retinopathy patients receiving anti-VEGF therapy over the course of 1 year (Al-Halafi 2015). In patients receiving anti-VEGF therapy for age-related

macular degeneration, incidence of outer retinal tubulation correlated with length of anti-VEGF treatment (Gildener-Leapman 2015, Dirani 2015). The complex role of VEGF in modulating both pathogenic and neuroprotective responses to RVO highlights the need for a broad arsenal of pharmacological interventions to prevent vision loss.

Additionally, there is a need for improved methods of administering pharmacological therapies to the retina. The blood-retina barrier presents a challenge to delivering targeted therapeutics. Current treatment paradigms rely on intravitreal injections, which carry minor risks of pain, intravitreal hemorrhage, retinal tears/detachment, infection and cataract. In addition to potential adverse effects, intravitreal injection therapy places an economic and psychological burden on patients. Most patients report having to take time off work for each injection appointment, and three quarters of patients report experiencing anxiety prior to their most recent injection treatment. Patients' most reported desire in regards to their treatment regimen was to have fewer injections and fewer appointments (Sivaprasad 2016).

An alternative approach to delivering therapeutics to the retina which does not require intravitreal injections is discussed in Chapter 6.

### **1.2.5 Investigational treatment strategies for RVO**

A number of interventions are currently under investigation for treatment of RVO, which may offer alternatives to anti-VEGF and corticosteroid injections.

**Minocycline:** Is a broad spectrum antibiotic with immune suppressing effects. Phase I and II clinical trials are investigating whether an oral dose of minocycline (in conjunction with anti-VEGF injections) can increase visual acuity in RVO patients, by reducing chronic inflammation via microglial inhibition (NCT01468844, NCT01468831).

**Hydroxycarbamide:** Is a drug commonly used in the treatment of sickle cell anemia, which decreases the production of deoxyribonucleotides. Although the precise mechanism of action is not known, hydroxycarbamide increases levels of nitric oxide, a vasodilator. A Phase II trial is investigating whether oral administration of hydroxycarbamide can improve retinal capillary nonperfusion in CRVO patients (NCT02957760).

**Loteprednol etabonate transmucosal (KPI-121):** Is an anti-inflammatory drug which functions via agonism of glucocorticoid receptors. The key difference between KPI-121 and currently available corticosteroids, is that the compound is formulated with a proprietary complex of nanoparticles, allowing eyedrop administration. KPI-121 is under clinical investigation for a number of ophthalmic indications, including treatment of edema in RVO patients (NCT02245516).

**AKB-9778:** Is a first in class small molecule modulator of the Tie2-Angiopoietin pathway. Patients may self-administer AKB-9778 by subcutaneous injection. By inhibiting VE-PTP, AKB-9778 causes activation of Tie2, a kinase whose activity suppresses vascular leakage and stabilizes vascular integrity (Campochiaro 2016). The compound has shown promising results in early clinical trials in diabetic macular edema patients, and is currently being investigated for efficacy in RVO (NCT02245516).

**Aganirsen:** Is an antisense oligonucleotide inhibitor of insulin receptor substrate-1 (IRS-1). Blocking IRS-1 signaling has been shown to reduce levels of VEGF and tumor necrosis factor-alpha (Cloutier 2012). The drug is a daily eyedrop currently under investigation for efficacy in preventing the development of neovascular glaucoma in CRVO patients (NCT02947867) (Lorenz 2017).

**LKA651:** Is an angiogenesis inhibitor that functions via inhibition of erythropoietin. Novartis is conducting early safety trials of LKA651 intravitreal injections in RVO patients (NCT02867735).

Pharmacological interventions in clinical trials fall under the categories of anti-inflammatory, anti-angiogenesis, or vasodilation approaches. Although some alternatives are under investigation, intravitreal injection remains the most common method for drug delivery to the retina. None of the compounds target neuroretinal dysfunction caused by RVO. Furthermore, interventional efficacy of caspase inhibition has not yet been investigated in the context of retinal edema.

### **1.3 Introduction to caspases**

Previous work in the lab has implicated caspase activity in the pathogenesis of edema following ischemic injury to neuronal tissues (Akpan 2011). Studies using rodent models of ischemic stroke (discussed further in Chapter 7) have shown that caspase-9 is a potential target for preventing neuronal loss and reducing edema after hypoxic injury. A study of BRVO in minipigs provided further basis for the therapeutic potential of caspase modulation by demonstrating reduced neuronal apoptosis in eyes which received intravitreal injection of Z-VAD pan-caspase inhibitor (Donati 2008). This section summarizes the physiological and pathological functions of caspases, and provides an overview of caspase-9 activity and regulation.

#### **1.3.1 General overview of caspase biology**

Caspases (Cysteine-ASPartic proteASEs) are a family of highly conserved protease enzymes which play important roles in programmed cell death and inflammation. Caspases were originally identified as the executioners of apoptosis, or programmed cell death. However, over the past 30 years, our understanding of caspase biology has expanded to include a broad array of functions in health and disease.

Caspases are expressed as inactive zymogens, which are activated by a specific stimulus. Caspases with long prodomains (caspase-2, -8, -9, -10) are called initiator caspases because, once activated, they may activate downstream (or effector) caspases. Initiator caspases



undergo proximity induced activation via conformational change. This can occur in response to caspase recruitment to an activating platform, such as the binding of inactive caspase-9 to the apoptosome complex. Following activation, initiator caspases undergo self-cleavage, and are released from their activation platform so that they may cleave their downstream substrates. Effector caspases, such as caspase-3, -6, and -7, are activated via cleavage by an initiator caspase. As proteases, caspases have a broad, but specific list of potential substrates. In humans, there are 12 members of the caspase family, which are summarized in the table below. In addition to their activity as proteases, some caspases have cellular functions that are independent of their protease activity.

The myriad functions of caspases in mediating both cell death, and non-cell death processes underscore the importance of tight regulation of caspase activity. All caspases have the potential to trigger cell death given sufficient stimulus (with possible exception of caspase-14), and all caspases have cellular functions independent of cell death processes. Most caspases are ubiquitously expressed, and their activity is titrated in response to cellular needs. Furthermore, many caspase functions are highly cell-type specific. The consequences of activating a particular caspase may be very different depending on the local cellular environment (e.g. Caspase-1 mediates inflammatory cytokine processing in myeloid cells, but drives apoptosis in cardiomyocytes during heart failure) (Merkle 2007).

*Table 1: Overview of caspase functions and related diseases*

<b>Caspase-1: Functions</b>	<b>References</b>
<ul style="list-style-type: none"> <li>• Activation of inflammatory cytokines</li> <li>• Innate immune response</li> <li>• Anti-inflammatory signaling</li> <li>• Induction of pyroptosis</li> <li>• Induction of apoptosis</li> <li>• Resistance to pathogenic bacteria</li> <li>• Unconventional protein secretion</li> <li>• Tissue repair and cytoprotection signaling</li> <li>• Wound angiogenesis</li> <li>• Regulation of metabolism</li> </ul>	<p>Chi 2014</p> <p>Sollberger 2014</p> <p>Winkler 2015</p> <p>Sun 2016</p> <p>Shalini 2015</p>

<ul style="list-style-type: none"> <li>Promotion of lysosomal degradation</li> </ul>	
<b>Relevant diseases</b>	
<ul style="list-style-type: none"> <li>Inflammatory pathway in acute glaucoma</li> <li>Dysregulated in inflammatory diseases (atherosclerosis, type-2 diabetes, gout, autoinflammatory disorders)</li> <li>Neuronal death in ischemia/reperfusion injury</li> <li>Cardiomyocyte death in heart failure</li> <li>Downregulated in human cancers</li> <li>Septic shock</li> </ul>	
<b>Caspase-2: Functions</b>	<b>References</b>
<ul style="list-style-type: none"> <li>Proliferation suppressor</li> <li>Induction of apoptosis</li> <li>Oxidative stress response</li> <li>Autophagy suppressor</li> <li>Positive and negative regulation of inflammatory response</li> <li>Differentiation of erythroid cells</li> <li>Dendritic pruning</li> </ul>	<p>Olsson 2015</p> <p>Miles 2017</p> <p>Forsberg 2017</p> <p>Shalini 2015</p> <p>Di Donato 2016</p>
<b>Relevant diseases</b>	
<ul style="list-style-type: none"> <li>Retinal Ganglion Cell death after optic nerve injury</li> <li>Photoreceptor cone cell death in retinitis pigmentosa mutant mice</li> <li>Exacerbating ischemia/reperfusion injury</li> <li>Neuronal death in Alzheimer's disease</li> <li>Neuronal death in motor neuron disease</li> <li>Downregulated in cancers</li> <li>Reduced Caspase-2 activation in hereditary lissencephaly</li> </ul>	
<b>Caspase-3: Functions</b>	<b>References</b>
<ul style="list-style-type: none"> <li>Key mediator of apoptosis</li> <li>Paracrine activation of cell migration and tissue regeneration (Phoenix rising pathway)</li> <li>Vasculogenic mimicry</li> <li>Induction of angiogenesis (DIVAA assay)</li> <li>Long term potentiation (LTP) and Long term depression (LTD)</li> <li>Axon guidance and synaptogenesis</li> <li>Differentiation of erythroid cells, keratinocytes, muscle progenitors, and stromal bone marrow</li> <li>Microglial activation</li> </ul>	<p>Boland 2013</p> <p>Li 2010</p> <p>Shalini 2015</p> <p>Linder 2016</p> <p>Kavanagh 2014</p> <p>Yang 2011</p>
<b>Relevant diseases</b>	
<ul style="list-style-type: none"> <li>Upregulated in cancers</li> <li>Activated in neurodegenerative disease</li> <li>RGC death in glaucoma</li> </ul>	

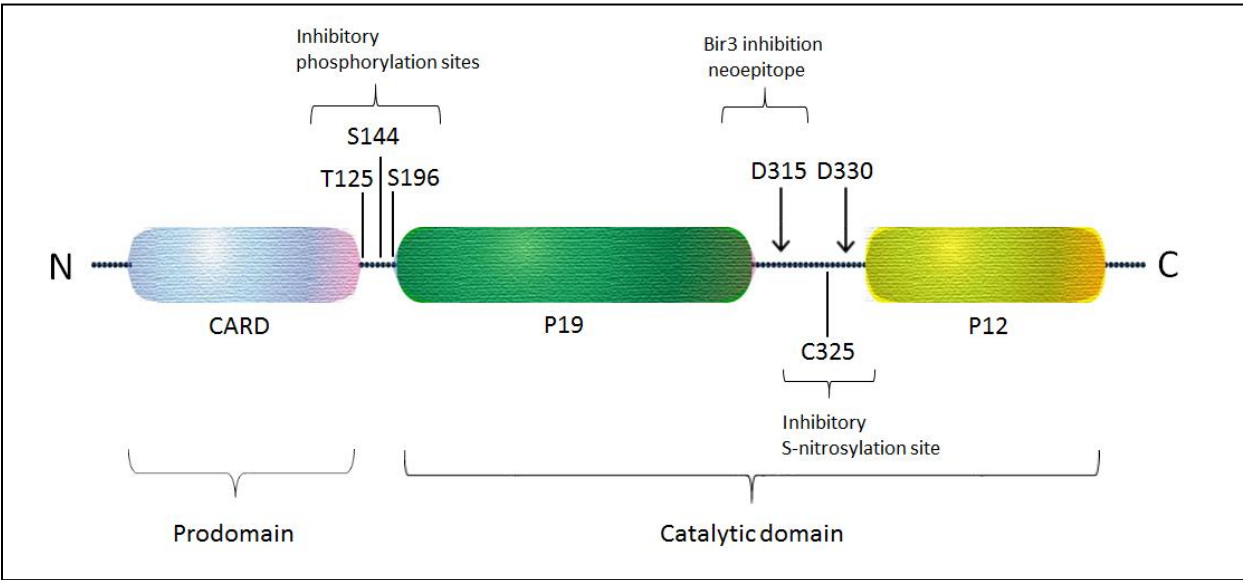
<b>Caspase-4: Functions</b>	<b>References</b>
<ul style="list-style-type: none"> <li>• Inflammasome activation</li> <li>• Innate immune response</li> <li>• Induction of pyroptosis</li> <li>• Activation of ER stress-induced apoptosis</li> </ul>	Shalini 2015, Hitomi 2004 Moorwood and Barton 2014
<b>Relevant diseases</b>	
<ul style="list-style-type: none"> <li>• Activated in Duchenne muscular dystrophy</li> <li>• Downregulated in some cancers</li> <li>• Septic shock</li> </ul>	
<b>Caspase-5: Functions</b>	<b>References</b>
<ul style="list-style-type: none"> <li>• Inflammasome activation</li> <li>• Induction of apoptosis</li> <li>• Induction of pyroptosis</li> </ul>	Vigano 2015, Zhao 2017, Salskov-Iversen 2011
<b>Relevant diseases</b>	
<ul style="list-style-type: none"> <li>• Upregulated in psoriasis</li> <li>• Septic shock</li> </ul>	Man 2017
<b>Caspase-6: Functions</b>	<b>References</b>
<ul style="list-style-type: none"> <li>• Execution of apoptosis</li> <li>• Vasculogenic mimicry</li> <li>• Axon guidance and synaptogenesis</li> <li>• Microglial activation</li> </ul>	Linder 2016, Shalini 2010, Akpan 2011, Berta 2016
<b>Relevant diseases</b>	
<ul style="list-style-type: none"> <li>• Neuronal death in neurodegenerative diseases</li> <li>• Neuronal death in ischemic stroke</li> <li>• Activated in invasive tumors</li> <li>• Chronic neuropathic pain</li> </ul>	
<b>Caspase-7: Functions</b>	<b>References</b>
<ul style="list-style-type: none"> <li>• Execution of apoptosis</li> <li>• Paracrine activation of cell migration and tissue regeneration (Phoenix rising pathway)</li> <li>• Vasculogenic mimicry</li> <li>• Osteogenesis</li> <li>• Formation of dental hard tissues</li> <li>• Inflammatory response to infection</li> <li>• Hair follicle development</li> <li>• Spermatogenesis</li> </ul>	Boland 2013 Li 2010 Shalini 2015 Linder 2016 Lamkanfi 2010 Choudhury 2015
<b>Relevant diseases</b>	

<ul style="list-style-type: none"> <li>• Activated in cancers</li> <li>• Septic shock</li> <li>• Neurodegenerative disease</li> <li>• Retinal ganglion cell death in optic nerve injury</li> <li>• Spinocerebellar ataxia type 7</li> </ul>	<p>Vesela 2015</p> <p>Matalova 2013</p> <p>Lei 2017</p> <p>Guyenet 2015</p>
<b>Caspase-8: Functions</b>	<b>References</b>
<ul style="list-style-type: none"> <li>• Induction of extrinsic apoptosis</li> <li>• Inhibitor of necroptosis</li> <li>• Heart muscle development</li> <li>• Yolk vasculature development</li> <li>• Hematopoietic development</li> <li>• Skin homeostasis</li> <li>• Modulation of autophagy</li> </ul>	<p>Tummers 2017</p> <p>Salvesen 2014</p> <p>Chi 2014</p> <p>Kim 2014</p> <p>Shalini 2015</p>
<b>Relevant diseases</b>	
<ul style="list-style-type: none"> <li>• Inflammatory pathway and RGC death in acute glaucoma</li> <li>• RPE degeneration in AMD</li> <li>• Caspase-8 deficits linked to various malignancies</li> <li>• Activated in advanced-stage tumors</li> </ul>	
<b>Caspase-9: Functions</b>	<b>References</b>
<ul style="list-style-type: none"> <li>• Initiation of intrinsic apoptosis</li> <li>• Differentiation of hematopoietic progenitors, muscle progenitors, cardiomyocytes</li> <li>• Axon guidance and synaptogenesis</li> <li>• Autophagosome formation</li> </ul>	<p>Shalini 2015,</p> <p>Sobenin 2015,</p> <p>Guo 2011</p> <p>Akpan 2011</p> <p>Ping 2017</p> <p>Han 2014</p> <p>Yang 2011</p> <p>Inoue 2003</p>
<b>Relevant diseases</b>	
<ul style="list-style-type: none"> <li>• Ischemic stroke</li> <li>• Increased expression in atherosclerotic lesions</li> <li>• Dysregulated in cancers</li> <li>• Discogenic low back pain</li> <li>• Multiple sclerosis</li> <li>• RGC death in glaucoma</li> <li>• Motor neuron death in ALS</li> </ul>	
<b>Caspase-10: Functions</b>	<b>References</b>
<ul style="list-style-type: none"> <li>• Initiation of extrinsic apoptosis</li> <li>• Inhibition of caspase-8 mediated apoptosis</li> </ul>	<p>Muhlethaler-Mottet 2011,</p> <p>Lamy 2013</p>
<b>Relevant diseases</b>	
<ul style="list-style-type: none"> <li>• Pathogenic mutations found in cancers</li> <li>• Autoimmune lymphoproliferative syndrome</li> </ul>	

Caspase-12: Functions	References
<ul style="list-style-type: none"> <li>• Induction of apoptosis</li> <li>• Increases bacterial clearance and resistance to sepsis</li> <li>• Downregulation of inflammatory response</li> </ul>	Shalini 2015, Saleh 2006, Bhootada 2015
Relevant diseases	Yang 2011
<ul style="list-style-type: none"> <li>• Sepsis</li> <li>• Photoreceptor cell death in inherited retinal degeneration</li> <li>• RGC death in glaucoma</li> </ul>	
Caspase-14: Functions	References
<ul style="list-style-type: none"> <li>• Differentiation of lens cells</li> <li>• Cornification, hydration and protection against UVB in skin</li> </ul>	Shalini 2015, Al-Shabrawey 2012, Kirchmeier 2017
Relevant diseases	
<ul style="list-style-type: none"> <li>• Psoriasis</li> <li>• Ichthyosis</li> <li>• Dysregulated expression in tumors</li> <li>• Upregulated in diabetic retinopathy</li> </ul>	

### 1.3.2 Focus on Caspase-9

Figure 5: Diagram of caspase-9 domains and inhibitory sites



Caspase-9 is a ubiquitously expressed 46kD protein, which plays critical roles in development and disease. Global caspase-9 deletion in mice causes perinatal lethality by e16.5, due to

severe brain malformation characterized by prominent expansion and protrusion of the entire cranial features (Kuida 1998). In the adult organism, functional polymorphisms of caspase-9 have been shown to affect tumor susceptibility, risk of multiple sclerosis, and discogenic low back pain (reviewed in Ping 2017). Meanwhile, caspase-9 initiated apoptosis is believed to reflect the susceptibility of cancers cells to chemotherapy.

Procaspase-9 is synthesized as a single-chain inactive zymogen, which can be activated by proximity-induced conformational change. The catalytic domain of caspase-9 is divided into a large (P19) and small (P12) subunit (Fig 5). Caspase-9 activation involves the dimerization of two caspase-9 molecules, and a conformational change aligning the relative positioning of the four catalytic subunits (Fig 6).

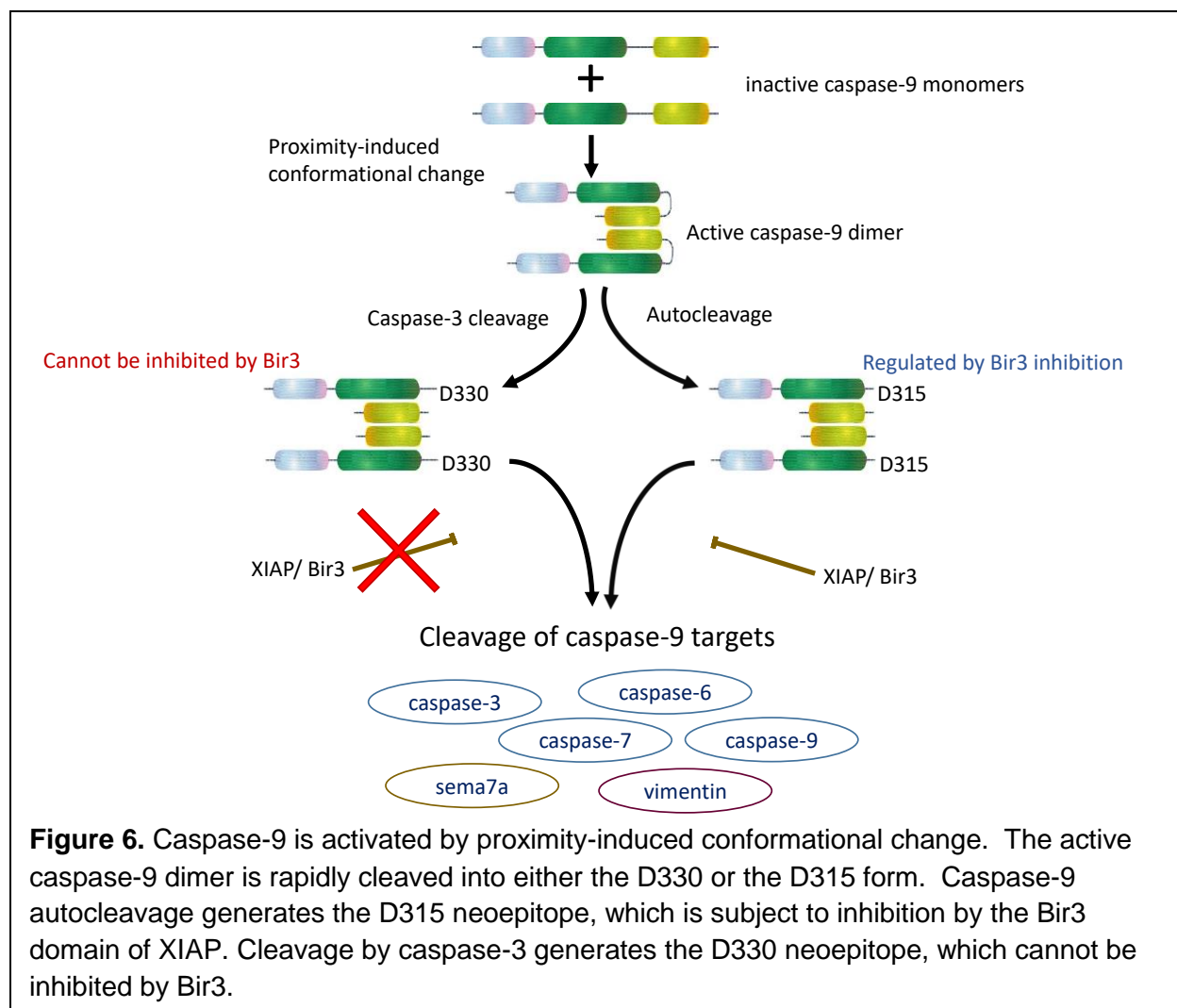
#### **1.3.2.1 Caspase-9 in apoptosis**

Caspase-9 activation in the intrinsic apoptosis pathway generally begins with the permeabilization of the mitochondrial outer membrane, and the release of mitochondrial cytochrome *c* into the cytosol. This triggers the oligomerization of Apaf-1 (apoptotic protease activating factor 1) into a heptameric apoptosome. The CARD (CASPase Recruitment Domain) motif at the N-terminus of the caspase-9 prodomain can selectively bind to CARD on Apaf-1 through homotypic interaction. This binding forms the basis of caspase-9 recruitment to the apoptosome and the induction of apoptosis. Procaspase-9 monomers bind to the apoptosome, where they are activated by proximity induced dimerization and conformational change. Following activation, caspase-9 quickly undergoes autoproteolytic cleavage at D315 which helps stabilize the dimer. Caspase-9 then activates effector caspases -3 and -7 via proteolytic cleavage, which then execute programmed cell death by cleaving their target substrates.

Caspase-9 mediated induction of cell death has been implicated in a number of disease pathologies. Caspase-9 mediates neuronal cell death in ischemic stroke (Akpan 2011), and

photoreceptor death in blunt ocular trauma (Blanch 2014). Caspase-9 activation has been observed in dying retinal ganglion cells in experimental models of glaucoma and in human glaucomatous retinas (Yang 2011). Caspase-9 activation has been detected in the spinal motor neurons of human amyotrophic lateral sclerosis (ALS) patients, and inhibition of caspase-9 activity via its endogenous inhibitor XIAP delayed disease progression in a mouse model of ALS (Inoue 2003).

Figure 6: Diagram of Caspase-9 activation and inhibition by XIAP/Bir3



**Figure 6.** Caspase-9 is activated by proximity-induced conformational change. The active caspase-9 dimer is rapidly cleaved into either the D330 or the D315 form. Caspase-9 autocleavage generates the D315 neoepitope, which is subject to inhibition by the Bir3 domain of XIAP. Cleavage by caspase-3 generates the D330 neoepitope, which cannot be inhibited by Bir3.

### 1.3.2.3 Nonapoptotic caspase-9 pathways

Several pathways have emerged describing nonapoptotic roles of caspase-9.

**Muscle cell differentiation:** *Murray et al* first reported a nonapoptotic role for caspase-9 in 2008 while studying the differentiation of skeletal muscle from myoblast progenitors. Reduction of caspase-9 levels via shRNA construct was sufficient to prevent caspase-3 activation and myoblast fusion. Their data showed that elements of the mitochondrial pathway of caspase-9 activation were present, but they found no evidence of mitochondrial membrane depolarization or cytochrome *c* or Smac release that are seen in apoptosis. Bulatovic 2015 showed that sublethal caspase activation enhances cardiomyocyte differentiation.

**Hematopoietic development:** Several reports have shown that caspase-9 activity is necessary for proper differentiation and proliferation of hematopoietic progenitors. In 2001, *Zermati et al* demonstrated that caspase activation was essential for terminal erythrocyte differentiation. Transient activation of caspase-9 via mitochondrial depolarization activates effector caspases which cleave proteins involved in nucleus integrity (Lamin B) and chromatin condensation (acinus). Interestingly, activation of the caspase cascade did not lead to features of cell death or cleavage of other typical caspase substrates involved in apoptosis.

Soon after, another study showed that caspase activation was essential for monocyte differentiation into macrophages (Sordet 2002). During monocyte differentiation, mitochondrial release of cytochrome *c* triggered activation of caspase-9 and caspase-3. Caspase activation did not lead to apoptosis; in fact, inhibition of caspase activity blocked differentiation and caused cell death instead. These findings have been expanded upon to show that caspase-9 activity is essential for myeloid, lymphoid, and erythroid development in both fetal and adult hematopoiesis (Lu 2014). Mouse bone marrow chimeras lacking caspase-9 or Apaf-1



developed low white blood cell counts, decreased B-cell numbers, anemia, and reduced survival.

**Axonal pathfinding:** Neuronal caspase-9 regulates synaptogenesis and axonal pathfinding and maturation. Mice lacking caspase-9 exhibit misrouted axons, impaired synaptic formation, and defects in the maturation of olfactory sensory neurons without affecting the number of these cells (Ohsawa 2010). This caspase-9 function is mediated by direct caspase-9 cleavage of semaphorin 7A, a membrane-anchored semaphorin that is required for proper axon projection. The authors also noted that the olfactory bulbs of aged mice had increased caspase-9 activation, but decreased levels of apoptosis.

**Autophagosome formation:** Caspase-9 facilitates early events that lead to autophagosome formation. Counterintuitively, caspase-9 knockdown in tumors can actually predispose cells to apoptosis. A report by *Han et al (2014)* showed that caspase-9 plays a role in the induction of cytoprotective autophagy. They showed that caspase-9 binds to Atg7. The formation of the caspase-9:Atg7 complex represses the apoptotic capability of caspase-9 and enhances Atg7-mediated formation of LC3-II, a key step in autophagosome formation.

**Activation of NF- $\kappa$ B signaling:** A 2016 report by *Ngoc et al* has demonstrated that caspase-9b (an alternative splicing isoform of caspase-9, which is missing protease activity) can bind to the Bir3 domain of cIAP1. Caspase-9b enhances the E3 ubiquitin ligase activity of cIAP1, promoting NF- $\kappa$ B prosurvival signaling, and enhancing the tumorigenicity of various cancers.

These reports show caspase-9 involvement in a broad array of physiological and pathological processes. Furthermore, in certain cell types, caspase-9 serves highly specific functions which are distinct from cell death. Although many of these processes are not fully understood, both healthy and cancerous cell types have evolved mechanisms of caspase-9 activation which repress entrance into apoptosis.

#### 1.3.2.4 Regulation of caspase-9 activity

Caspase-9 activity is regulated on multiple levels; these overlapping systems of regulation allow for tight cellular control of caspase-9 activity. While there many other cellular systems controlling apoptotic signaling, the mechanisms listed below reflect processes that directly modify caspase-9 activity.

**XIAP (X-linked Inhibitor of Apoptosis Protein):** The autoprocessing of caspase-9 at D315 reveals a neoepitope which allows caspase-9 to bind to its endogenous inhibitor XIAP (Fig 6). The Bir3 domain of XIAP binds specifically to caspase-9, while the Bir2 domain similarly incapacitates caspases-3/-7. XIAP expression levels are frequently elevated in cancer cells which are evading apoptosis. Under proapoptotic conditions, Smac/DIABLO (inhibitors of XIAP) are released from the mitochondria and displace XIAP from binding to caspase-9, thus promoting caspase-9 activation (Denault 2007).

**Differential D315/D330 Cleavage:** Caspase-9 is differentially susceptible to inhibition by XIAP depending on how caspase-9 has been cleaved (Fig 6). Caspase-9 autoprocessing generates a cleavage site at D315 and the resulting cleaved caspase-9 protease is susceptible to inhibition by XIAP. On the other hand, caspase-9 can also be cleaved by caspase-3, which generates a different neoepitope at D330. The D330 version of cleaved caspase-9 cannot be regulated by XIAP (Denault 2007).

**Alternative splicing:** A truncated isoform called caspase-9b is generated by alternative splicing. This isoform lacks the large subunit of the catalytic domain, and is thus incapable of protease activity. Caspase-9b is a dominant negative regulator of caspase-9 activation because binds to the apoptosome, but cannot facilitate the conformational change necessary for caspase-9 activation. A higher ratio of the caspase-9b isoform is found in tumors with an anti-apoptotic phenotype (Ping 2017).

**Inhibition of binding to apoptosome:** Several proteins can bind caspase-9 and prevent its recruitment to Apaf-1 and the apoptosome. TUCAN, an antiapoptotic protein that is upregulated in cancer, binds the CARD domain of caspase-9, preventing it from binding with Apaf-1 (Pathan 2001). Interestingly, TUCAN is present in humans, but not in mice, demonstrating a species-specific element of caspase-9 regulation. A similar mechanism involves caspase-9 inhibition by the Survivin-HBXIP complex. Protein kinase A (PKA) also inhibits caspase-9 binding to the apoptosome, although its mechanism of inhibition is unclear.

**Inhibition of processing and activity:** Numerous proteins and several miRNAs can interact with caspase-9 zymogens to inhibit activity. Phosphorylation by a number of kinases can block caspase-9 activity by interfering with caspase-9 processing (Fig 5). Nitric oxide inhibits apoptosis by nitrosylating caspase-9. A population of caspase-9 zymogens are kept inhibited by S-nitrosylation (Mannick 2001). During cerebral ischemia-reperfusion, a transnitrosylation event denitrosylates procaspase-9, and induces nitrosylation of XIAP, enhancing caspase-9 activation (Zhang 2016).

**Caspase-9 activators:** While fewer in number, some proteins have been shown to increase caspase-9 activity. These include c-Abl tyrosine kinase, which enhances caspase-9 autoprocessing, and NAC/DEFKAP and nucling which also enhance caspase-9 activity. The mechanisms by which these factors modulate caspase-9 are not fully elucidated.

An in depth review of caspase-9 regulatory pathways can be found in Ping 2017. The milieu of factors which can modify caspase-9 activity underscore how pivotal caspase-9 can be in cell signalling towards apoptosis. At the same time, the vast array of caspase-9 modulators create high potential for separating caspase-9 activity from the induction of cell death.

### 1.3.2.5 Caspase-9 activation in hypoxia/ischemia

Previous work by the Troy group identified caspase-9 activation as an early pathogenic event in the ischemic brain in rodent models of stroke (Akpan 2011). Work by Delivoria-Papadopoulos has shown that caspase-9 is activated by hypoxia in a mechanism mediated by nitric oxide (Delivoria-Papadopoulos 2012). In gerbil brains, both hypoxia and ischemia were shown to induce caspase-9 and caspase-3 activation (Garnier 2004). Numerous other studies have reported caspase-9 activation under hypoxic and ischemic conditions, however the consequences of caspase-9 activation have rarely been investigated outside the context of overt cell death.

Caspase-9 activation has not previously been linked to retinal vascular disease. However, a recent study in a rat model of type II diabetes identified perturbations in retinal mitochondrial respiration preceding induction of retinopathy (Han 2017). Curiously, they detected transient mitochondrial outer membrane permeabilization and cytochrome *c* release, without induction of other markers of apoptotic signaling. These findings are consistent with previously discussed contexts of nonapoptotic caspase-9 activation. Further evidence of caspase involvement in RVO comes from a study in minipigs, which showed that intravitreal injection of Z-VAD pan-caspase inhibitor reduced neuronal apoptosis in the days following BRVO (Donati 2008). However, the study did not address which caspases were involved in the pathology, nor did they examine the effects of caspase inhibition on other aspects of RVO pathology.

## 2.3 Significance and outline

The following chapters explore the pathogenic consequences of endothelial caspase-9 activation in retinal vein occlusion.

**Chapter 2** describes the methods used in these studies. Particular attention is given to the selection of the laser photocoagulation model of retinal vein occlusion. Live imaging tools used

in this study have direct counterparts to diagnostic imaging in ophthalmology, granting direct correlates between pathological features seen in our mouse model of RVO and the retinal disease in human RVO patients.

**Chapter 3** describes retinal pathology induced by the mouse model of RVO, and presents evidence of endothelial caspase-9 activation following retinal vein occlusion.

**Chapter 4** describes mouse genetic knockouts of caspase-9. These studies used animals who had endothelial deletion of caspase-9 upon reaching adulthood, to study the endothelial-specific function of caspase-9 in adult tissues.

**Chapter 5** explores how genetic deletion of endothelial caspase-9 modulates the effect of RVO on retinal morphology and function.

**Chapter 6** discusses pharmacological inhibition of caspase-9 as a treatment approach in RVO.

**Chapter 7** describes evidence of endothelial caspase-9 activation during ischemic injury in rodent and human CNS tissues.

**Chapter 8** provides conclusions and future directions for this project.

This dissertation describes a previously unknown function of caspase-9 in ischemic injury. These studies show that endothelial injury drives neuronal loss and dysfunction, and that caspase-9 activity is an important therapeutic target in RVO. This work provides direct correlates to human disease, and a novel potential therapeutic strategy for conferring neuroprotection to ischemic tissues.

## 2. Materials and methods

### 2.1 Animals

#### 2.1.1 Rodent Husbandry and Handling.

All rodent procedures were approved by the Columbia University Institutional Animal Care and Use Committee and performed in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

#### Mouse Breeding

All animals were on a C57/Bl6 background. Wild type C57/Bl6 male mice were purchased at 2 months of age from Jackson Laboratories (Bar Harbor, ME, USA).

**Caspase-9 flox/flox mice:** were received via MTA from Genentech. Genentech has created a caspase-9 conditional knockout mouse by targeting exon 6 (Simon, 2012). The genotyping protocol utilizes 3 primers which detect the WT allele, the floxed allele and the KO when recombination has occurred.

**Endothelial cell-CreERT2 mice (Cdh5(PAC)-CreERT2):** developed by Raif Adams, were obtained from Cancer Research UK by MTA (Pitulescu 2010). Cre expression in these animals is driven by the VE-cadherin (cdh5) promoter, and controlled by tamoxifen induction.

**Cre reporter mT/mG mice:** available from Jackson Labs - stock# 007676, strain B6.129(Cg)-Gt(ROSA)26Sor<sup>tm4(ACTB-tdTomato,-EGFP)Luo/J</sup>. Prior to Cre recombination, cell membrane-localized tdTomato (mT) fluorescence expression is widespread in cells/tissues. Cre recombinase expressing cells have cell membrane-localized EGFP (mG) fluorescence expression replacing the red fluorescence (Muzumdar 2007).

**Endothelial specific caspase-9 inducible knockout (iC9 ECKO) mice:** were bred by crossing Caspase-9 flox/flox mice and endothelial cell-CreERT2 mice. Caspase-9 flox/flox littermates were used as WT controls

**Tamoxifen induction of recombination:** Recombination was induced in 6 week old animals by intraperitoneal (IP) injection with 2mg tamoxifen for 5 consecutive days. Animals were survived until 8 weeks of age before induction of RVO.

### **2.1.2 Rabbit retinal Pen1-XBir3 uptake study**

An ocular distribution study of Pen1-XBir3 eyedrops was performed by EyeCRO (Oklahoma City, OK).

Adult female New Zealand White Rabbits (N=4) received bilateral topical administration of 200 ug Pen1-XBir3, twice daily for 4.5 consecutive days. On day 5, 4 hours after administration of the final dose of Pen1-XBir3, eyes were enucleated and the retinas dissected, and plasma was collected from the animals. Tissues were returned to Troy lab for further processing.

## **2.2 Ischemic brain samples**

**Human brain tissue:** Columbia University's Brain Bank provided human brain samples of postmortem tissue from two patients who died following ischemic stroke and two control patients without diagnostic abnormalities. Details on when the ischemic event occurred relative to fixation and slide preparation were not provided.

**Rat brain tissue:** Brain samples from transient middle cerebral artery occlusion (tMCAO) in rats were procured from the study in Akpan 2011. Immunostaining and analysis of rat brain samples were performed by Kendra Johnson and Carol Troy.

## **2.3 Retinal Vein Occlusion model**

### **2.3.1 Overview of retinal vein occlusion models**

Retinal vein occlusion models have been a mainstay of ophthalmic research for decades. The first instance of experimental retinal vein occlusion induced by photocoagulation dates back to a

1961 report by Francis P. Campbell, a prominent New York retinal surgeon (Campbell 1961). The procedure utilized focused flashes of intense green light to induce occlusions in the major retinal veins of cats, producing dilated, tortuous veins, hemorrhages, retinal detachment, and coagulation necrosis. The invention of laser light would allow future versions of the model to improve retinal vein targeting. Photoactive dyes were added to the procedure in 1979, when *Hamilton et al* injected fluorescein to aid photocoagulation in a rhesus monkey model of RVO. Rose bengal, the most commonly used dye in modern protocols, was first applied in 1987, in a rabbit model of RVO (Nanda 1987). Photoactive dyes allow for photocoagulation at lower light intensity, which reduces the thermal heat damage to RPE and photoreceptor cells. These technological advances have improved the accuracy of the procedure, but the basic premise of photocoagulation-induced retinal vein occlusion remains much the same; targeted green light is absorbed by retinal veins, where it causes endothelial damage and drives thrombus formation.

Retinal vein photocoagulation has been used to achieve RVO in a wide array of animal species, including nonhuman primates, pigs, and rabbits. A pivotal study in the development of anti-VEGF therapies used RVO to demonstrate that the hypoxic retina produces VEGF, providing some of the earliest evidence that elevated VEGF played a role in vascular eye disease (Miller 1994). Two years later, experimental RVO was used to demonstrate the therapeutic potential of intravitreal anti-VEGF therapy in nonhuman primates (Adamis 1996). The smaller eye of rodents initially posed a challenge to RVO models. However, a protocol for RVO in the mouse was described by *Zhang et al* in 2007, and further developed by *Ebneter et al* in 2015. Most recently *Fuma et al*, used modern imaging techniques to observe retinal edema and functional impairment in the mouse model of RVO (Fuma 2017). Furthermore, they demonstrated the efficacy of early intraocular anti-VEGF intervention, which ameliorated retinal edema and nonperfusion following RVO. At present, animal models of retinal vein occlusion remain the best

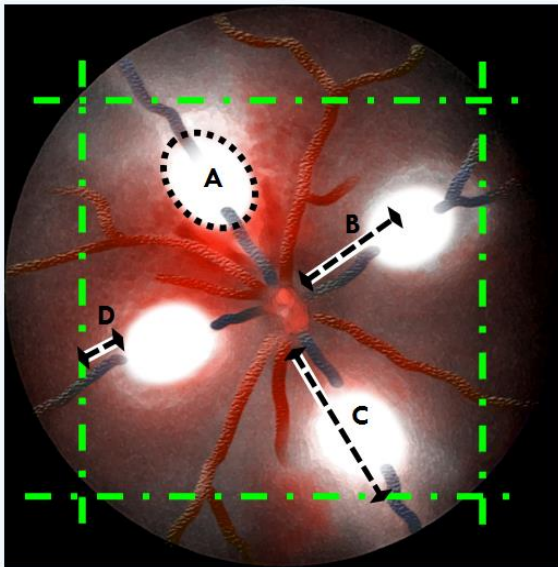


tool for investigating both RVO pathology, and the broader category of retinal edema in vascular eye disease (Lai 2013).

### 2.3.2 Photocoagulation model of retinal vein occlusion

RVO was performed according to the protocol described in (Zhang 2017). Mice (2-4 months old) received tail vein injection of rose bengal (40 mg/kg) and were anesthetized by intraperitoneal injection with ketamine (80-100 mg/kg) and xylazine (5-10 mg/kg). Each eye received topical tropicamide (0.5%) and phenylephrine chloride (2.5%) to dilate the iris. Topical proparacaine (0.5%) was applied immediately prior to photocoagulation as a topical anesthetic. Hypromellose gel was applied to prevent eye dehydration and for use as a coupling gel for all live imaging.

*Figure 7: Diagram of retinal vein occlusion*



**Figure 7. Schematic depiction of typical fundus view following retinal vein occlusion.**

(A) A white vaporization bubble forms around the laser burn site. Laser burn damage is apparent in this area. (B) Laser burns are applied approximately 2 disc diameters from the optic nerve center. (C) Green lines represent locations four OCT scans collected from each eye, positioned approximately 700  $\mu\text{m}$  from the optic nerve. OCTs measure retinal morphology distal to retinal vein occlusion sites. (D) OCT images were taken approximately 76  $\mu\text{m}$  distal from the edge of the vaporization bubble. Measurements of vein and artery dilation were performed at this distance relative to the occlusion sites.

Photocoagulation was performed using the Micron IV image guided laser (532 nm) (Phoenix Research Labs, Pleasanton, CA, USA). Laser output was adjusted to apply 50 mW pulses (spot size 50µM). Three adjacent laser pulses of 1 second duration were applied to each of the 4-6 major retinal veins approximately 2 disc diameters from the optic nerve center (Fig 7). For analgesic aftercare, mice received 5mg/kg subcutaneous carprofen immediately after photocoagulation, and again at 24 hours.

**Heterogeneity of retinal vein occlusion:** A rarely discussed aspect of RVO models is the heterogeneity of occlusions induced by this method. A common strategy is to keep applying laser burns to the vein until full occlusion is observed. Fuma 2017 used 10-15 burns per vein (50mW power, 5 sec duration). Ebnetter 2015 reported 2-5 burns per vein (160mW laser, 0.8-2.5 sec duration). Oftentimes, the laser intensity and number of laser burns needed to reach complete occlusion would afflict substantial thermal damage to the retina, evidence of which could be seen on the published OCT images and retinal cross-sections. Despite applying differential intensities of laser until full occlusion was observed, Ebnetter nonetheless noted that “variable susceptibility of different batches of animals” required *post hoc* groupings of RVO types based on extent of injury seen by fluorescein angiography during follow-up. Such phenotype-based grading would not be compatible with using the same phenotypes to measure potential efficacy of pharmacological interventions. Furthermore, as described in chapter 1, there is inherent heterogeneity in the retinal vein occlusions found in human patients. RVO pathology in patients is not dependent on complete obstruction of blood flow through the blocked vessel. In our observations, the same is true for the mouse model of RVO.

We elected to keep constant the intensity and number of laser burns applied to each vein, minimizing the confounding factor of variable degrees of laser heat damage to retinal tissues. Since vein dilation is the most direct quantifiable consequence of an occlusion, RVO was judged

successful if retinal vein dilation was observed at 24 hours. Lack of measurable vein dilation was found in 5.6% of lasered eyes, which were then excluded from all subsequent analyses.

**Adverse events:** Excessive damage, characterized by complete retinal detachment at 24 hours post-RVO, or intravitreal hemorrhaging that obscured view of the retina occurred in 6% of eyes, which were excluded from all subsequent analyses.

## **2.4 Live retinal imaging**

Observation of retinal pathology following induction of RVO was performed in live animals using mouse-adapted techniques common to ophthalmic observation of RVO patients. Animals were anesthetized by intraperitoneal injection with ketamine (80-100 mg/kg) and xylazine (5-10 mg/kg) prior to imaging. Each eye received 1 drop of tropicamide and phenylephrine chloride to dilate the iris.

### **2.4.1 Retinal fundus imaging**

Direct observation of the retina remains a key component of diagnosis and disease monitoring in ophthalmic care. As discussed in Chapter 1, fundus examination in RVO patients reveals vessel dilation, retinal hemorrhages, edema, and cotton wool spots.

Fundus images of each eye were recorded with the Micron IV fundus camera immediately prior to laser photocoagulation, and at 4 hours and 1-8 days post-RVO. Comparison of vessel diameters between pre-RVO and post-RVO images was performed by blinded examiners using Image J. Vessel diameter was measured at the periphery of the laser burn areas (see RVO diagram, distance 'D'). Occurrences of retinal hemorrhages and cotton wool spots were recorded by a trained examiner.

### **2.4.2 Fluorescein angiography**

Fluorescein is a small fluorescent dye used extensively as a diagnostic tool in ophthalmology. Fluorescein readily enters vascular circulation in peripheral tissues, and can be detected in retinal vasculature in a time-dependent manner, highlighting first the arteries and veins, and then the retinal microvasculature. Fluorescein angiography can detect areas of capillary nonperfusion and blockages in blood flow caused RVO. In healthy subjects, the blood-retina-barrier prevents fluorescein leakage out of retinal blood vessels; in damaged eyes, fluorescein signal accumulates around areas where endothelial barrier integrity has been disrupted, indicating vasogenic edema.

#### **Fluorescein imaging method:**

Mice were prepared for retinal imaging as described earlier. Fluorescein angiography images were collected using the green filter on the Micron IV instrument, 5 minutes after intraperitoneal injection of 0.1mL Fluorescein (AK-Fluor, diluted to 1% in sterile saline). Due to lack of granularity in the illumination brightness control settings, maximal brightness was utilized in the acquisition of all fluorescein images.

Fluorescein angiography analysis was performed using Image J. Edema was measured by calculating the fluorescein integrated signal density in retinal tissues outside the major vessels. To control for mouse-to-mouse variability of fluorescein signal intensity, edema signal was normalized against the mean fluorescein intensity within the major retinal vessels of that eye.

Since fluorescein angiography in healthy eyes allows for clear examination of retinal vasculature, images from late-phase angiography were used for vascular characterization of transgenic animals. Image J was used to measure vein and artery diameters at primary, secondary and tertiary branch points. Vessel density was calculated in Image J by applying a

grid to fluorescein angiography images, and calculating the ratio of vertices with or without blood vessel crossings.

### **2.4.3 Optical Coherence Tomography (OCT)**

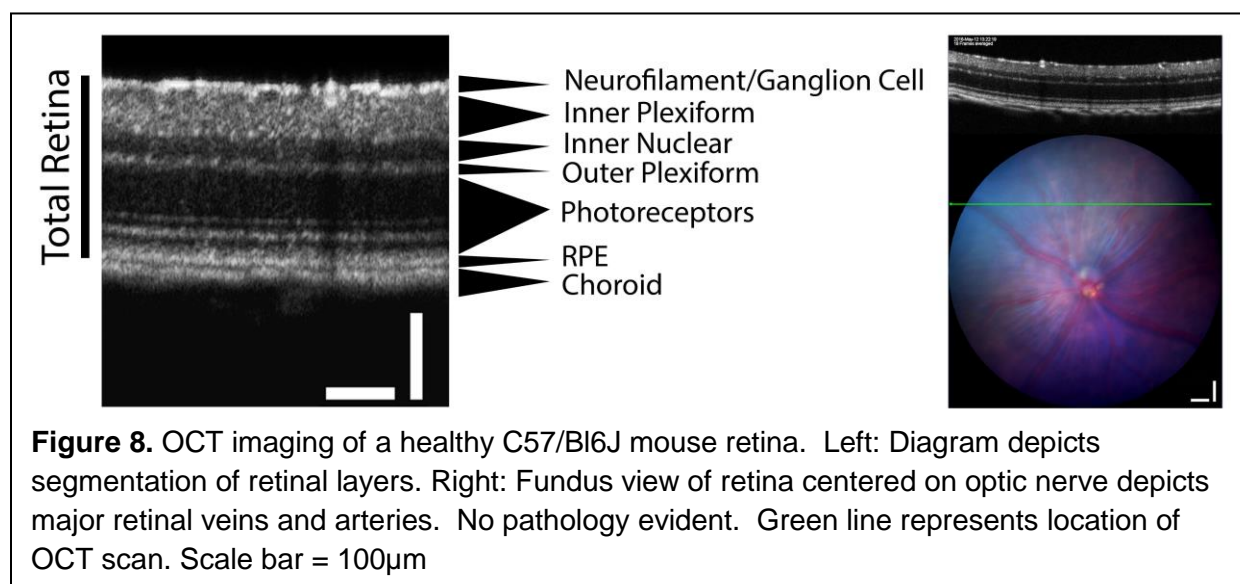
Optical coherence tomography is a medical imaging technique which uses near-infrared light to collect optical cross sections of biological tissues. In the clinical patient setting, OCT is used to measure retinal thickening due to edema. Several studies have shown moderate correlation between visual acuity and OCT measures of retinal thickness in patients with diabetic macular edema (Islam 2016, Wong 2015, Ou 2017). The relationship between retinal thickness and visual acuity is less clear in RVO patients, however, higher retinal thickness predicted greater degree of visual decline in both BRVO and CRVO patients (Ou 2017, Kim 2015). OCT measures of retinal thickness are frequently used to assess intervention efficacy in RVO clinical trials.

In addition to facilitating quantitative assessment of retinal edema, OCT images have been used to identify new retinal pathologies. Hyperreflective foci (potentially representing inflammatory cells) have been detected in the vitreous and retina of patients with edema (Mo 2017). OCT pathology findings and their potential relevance to visual acuity outcomes are discussed in greater detail in Chapter 3.

#### **OCT imaging method:**

Mice were prepared for imaging as discussed earlier. OCT images were captured using the Phoenix Micron IV image-guided OCT system. Retinal areas directly affected by the laser burn (RVO diagram region A) were avoided due to the confounding effects of laser heat damage on retinal morphology changes. Instead, OCT images were captured from areas approximately 75 $\mu$ m distal from the periphery of the RVO burn sites (see RVO diagram, green dashed lines, measurement D). For each eye, 2 vertical and 2 horizontal OCT scans were captured (Fig 7).

Figure 8: Segmentation of retinal layers by OCT



InSight software was used to generate segmentation of the individual retinal layers in each OCT scan, and the data then processed to calculate average layer thicknesses. For each eye, retinal thickness values from the 4 OCT images were averaged to generate mean retinal thickness values for that eye. Segmentation of retinal layers from OCT images is depicted in Fig 8.

Quantification of hyperreflective foci was performed by a blinded examiner in Image J using the Analyze Particles function on a thresholded selection of the inner nuclear layer.

#### 2.4.4 Electroretinograms (ERG)

Electroretinograms (ERG) are a common tool used in both clinical and research settings. The ERG measures electrical output from the retina in response to a flash light stimulus; different components of the ERG signal correspond to the function of specific retinal cell types. Impaired ERG response can thus provide insight into causes of vision impairment, and is helpful in diagnosing retinal disease.

Light stimulation of the retina produces a biphasic electrical response, which can be measured by an electrode placed on the cornea. Three key components of the ERG waveform are the a-wave, the b-wave, and the oscillatory potentials (Fig 9) (Audo 2008).

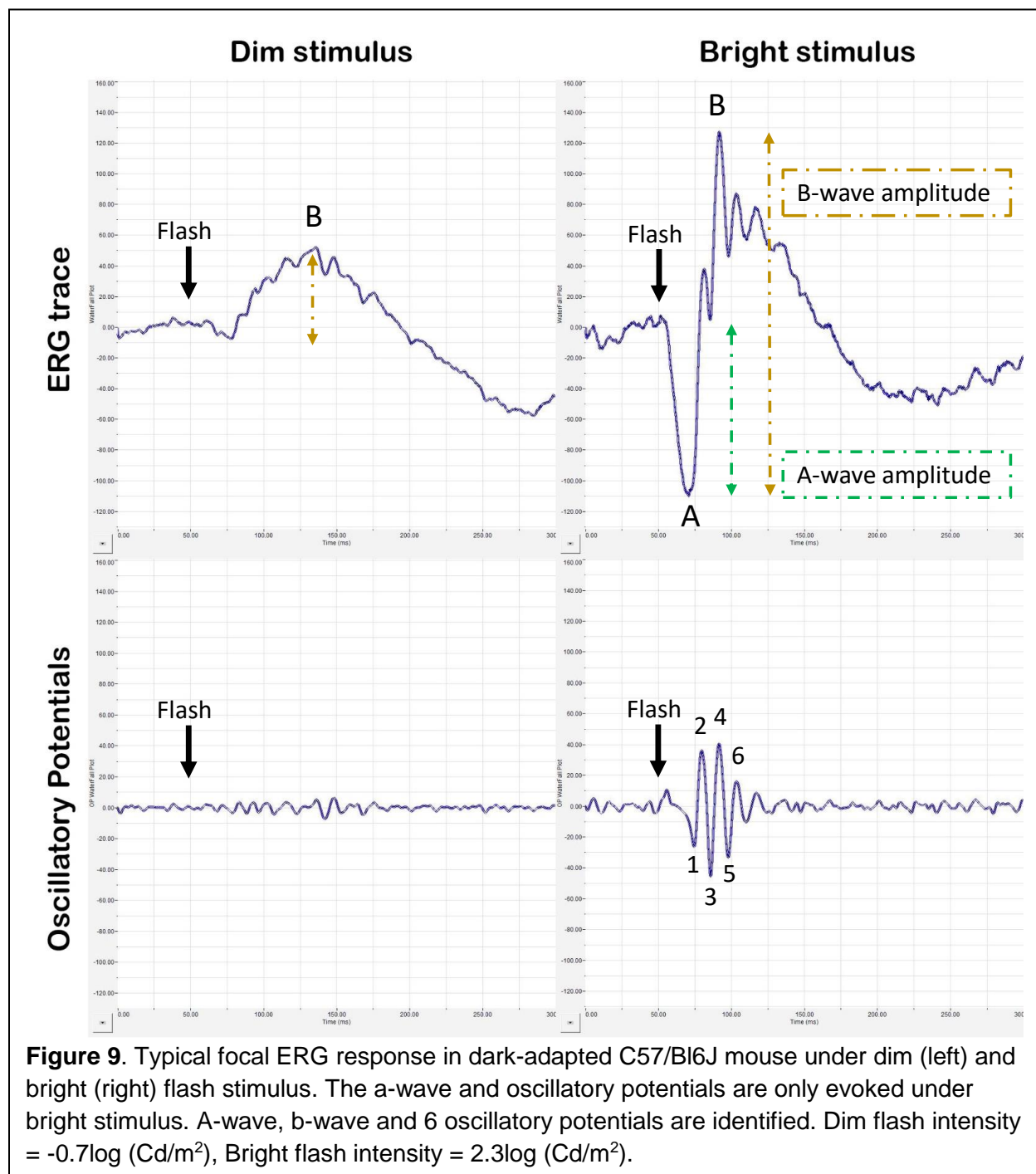
**a-wave:** Is the initial negative deflection in the ERG trace, which most directly reflects phototransduction by the photoreceptors. The a-wave amplitude is measured from the baseline to the trough of the a-wave.

**b-wave:** Is the large positive peak, which reflects activity of the inner layers of the retina, including ON bipolar cells and Muller cells. The b-wave reflects post-phototransduction function. B-wave amplitude is measured from the trough of the a-wave to the peak of the b-wave.

**Oscillatory potentials (OP):** are the small wavelets that occur during the rising phase of the b-wave. They are thought to reflect the activity in amacrine cells.

Retinal neuronal damage in patients with RVO and/or retinal edema results in decreased wave amplitudes and delayed peak times. B-wave amplitude is the most important ERG component used in clinical analysis of human retinal function, however both a-wave and b-wave amplitudes are reduced in RVO patients and in mouse models of RVO (Abdel-Kader 2010, Audo 2008, Fuma 2017). B-wave amplitudes are also reduced in some patients with diabetic retinopathy (Pescosolido 2015) and in several animal models of retinal ischemia (Block 1998). B-wave amplitude has been shown to correlate with best corrected visual acuity (BCVA), degree of ischemia, and risk of neovascularization in RVO patients (Noma 2015, Matsui 1994). OPs are frequently absent in patients with diabetic retinopathy, even in early stages of the disease. Diabetic animal models have reported reduced OP amplitude and increased OP latency (Han 2017, Pescosolido 2015). Retinal ischemia can cause loss oscillatory potentials (Audo 2008).

Figure 9: Scotopic focal ERG response in a healthy mouse retina





**Focal ERG method:**

Electroretinograms were recorded 7 days post-RVO using the Micron IV Image-Guided Focal ERG system on mice dark-adapted for 12-16 hr prior to imaging. Flash ERG was recorded using a flash spot size of 1.5mm, centered on the optic nerve head, and a range of white light LED stimulus intensity between  $-0.7 \log(\text{Cd sec/m}^2)$  and  $2.3 \log(\text{Cd sec/m}^2)$ .

ERG traces were analyzed with the Labscribe software. The amplitude of the a-wave was measured from the baseline to the maximum a-wave peak, and the b wave was measured from the maximum a-wave peak to the maximum b-wave peak. Oscillatory potentials (OP) were derived using a 30hz-300hz filter, and the sum of the first 6 OPs was used to calculate sum OP amplitude.

**2.5 Caspase-9 inhibitor (Pen1-XBir3) eyedrops**

Generation of the Pen1-XBir3 caspase-9 inhibitor peptide has been previously described in (Akpan 2011).

XBir3 was purified as described previously (Sun et al., 2000) and generously provided to us by the Salvesen group. Penetratin-1 (Pen1) was purchased from Q-Biogene. Pen1 was mixed at a 1:2 molar ratio with purified XBir3 and incubated for 2h at 37°C to generate disulfide-linked Pen1-XBir3. Linkage was assessed by 20% SDS-PAGE and Western blotting with anti-His antibody.

**Dosing treatment paradigm:** Eyedrops containing 10µg Pen1-XBir3 were administered immediately following RVO, and again at 24hr. An equivalent volume containing unlinked Pen1 was administered as a vehicle control.

## **2.6 Immunohistochemistry and antibodies**

For immunohistochemistry and Western blotting, anti-GFAP (Aves), anti-full-length and -cleaved caspase-9 (Abcam ab28131, Abcam ab2325), anti-caspase-9 D315 (Cell Signaling 9505), anti-caspase-9 D330 (Cell Signaling 9501), anti-cleaved caspase-6 (Cell Signaling Technology 9761), anti-cleaved caspase-3 (Cell Signaling #9661), anti-cleaved caspase-7 (MBL BV-3147-3), anti-PH2AX (Cell Signaling), anti-CD31 (BD), anti-SMA (pericyte marker from the first figure) and anti-GFP ( ) were used. For Western blotting, anti-His (GenScript A00186), and anti-ERK were used. Mice were killed with Ketamine 80-100 mg/kg plus Xylazine 5-10 mg/kg and perfused followed by fixation with 4% paraformaldehyde. Retinal flatmounts were permeabilized for 2h at RT in PBS with 1% Triton X-100, prior to blocking step. Retinal flatmounts and sections (thickness 10  $\mu$ m) were blocked for 1 h with 10% normal goat serum/1% BSA with 0.1% Triton X-100 in PBS, incubated with primary antibody overnight at 4°C, washed with PBS, and incubated with the species-appropriate Alexa Fluor-conjugated secondary antibody (Invitrogen, 1:1000) for 2 hr at RT. Human samples were additionally treated with Sudan Black (1% in 70% ethanol) for 5 min at RT and washed with PBS (3 changes; 3 min each). Mounting media containing DAPI nuclear stain was used. Sections were imaged using a Zeiss LSM 800 confocal microscope, and the images processed using FIJI.

## **2.7 Western Blot Analysis**

Retinas were lysed in RIPA buffer containing Halt Protease inhibitor cocktail (1:100), Phosphatase Inhibitor cocktail 2 (Sigma, 1:100) and Phosphatase Inhibitor cocktail 3 (Sigma, 1:100). Samples were resolved by SDS PAGE and analyzed by western blotting.

Western blots were blocked for 1 h with 5% BSA in TBS-Tween, incubated with primary antibody overnight at 4°C, washed with TBS-Tween, and incubated with the species-appropriate

secondary antibody for 2 hr at RT. Western blots were imaged using Licor Odyssey system, and densitometry was performed using the Licor Image Studio Lite software.

Immunoprecipitation experiments depicting retinal uptake and target engagement by Pen1-XBir3 were performed and analyzed by Ying Jean.

## **2.8 RBE4 Survival Assay**

To induce caspase-9-mediated death, 4-hydroxynonenal (3  $\mu$ M; Cayman Chemicals) was added to RBE4 (rat brain endothelial) cell cultures in triplicate with and without Pen1-XBIR3 (80 nM). After 1 day of treatment cell number was quantified as previously described (Rabachi 2004). Briefly, the cells were lysed in counting buffer, and intact nuclei were counted using a hemocytometer. Cell counts were performed in triplicate wells and averaged. Survival is relative to control wells. Comparisons between groups used the Student's t-test or ANOVA, p-value: 0.05. Survival assays were performed and quantified by Ying Jean.

## **2.9 Statistical analysis**

Data were analyzed using Excel and Graphpad Statistical software. Statistical tests, n values and p values are located in the figures and/or legends.

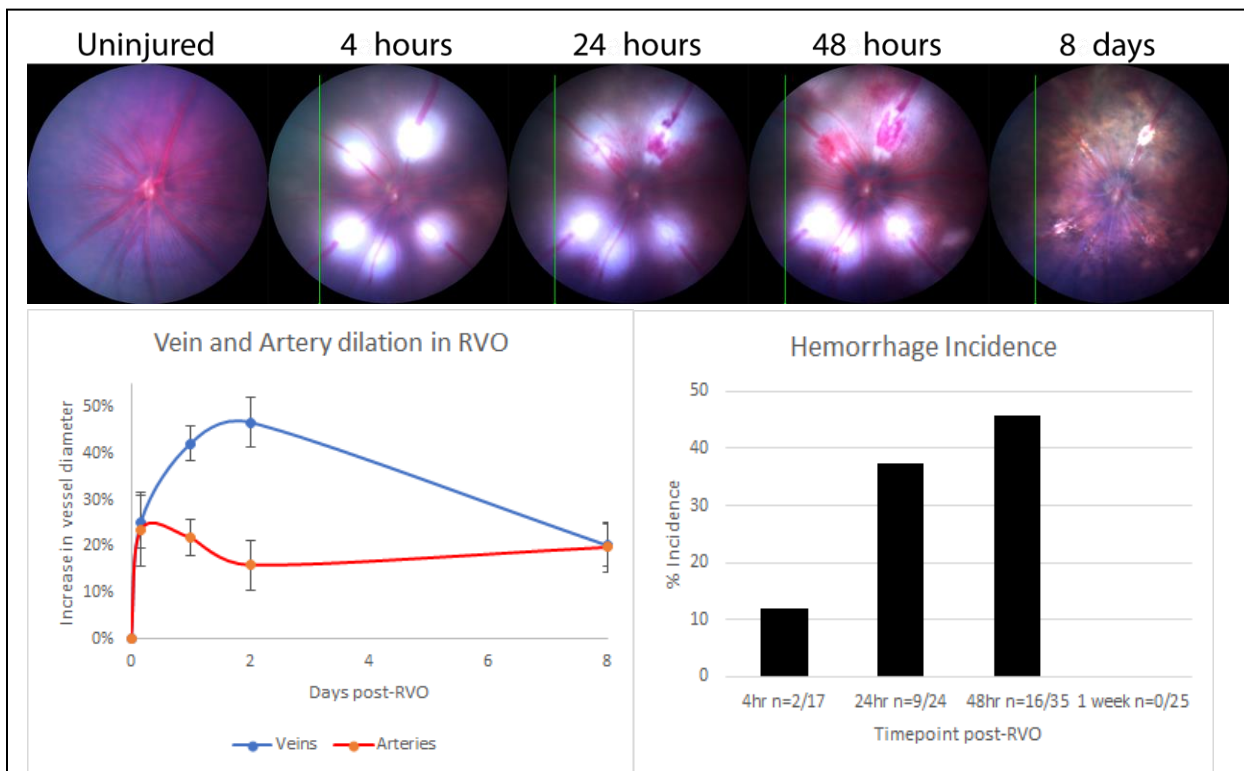
### 3. Retinal Vein Occlusion models CNS ischemic injury

To investigate the functional significance of endothelial caspase-9 activation, we utilized mouse RVO as model of CNS ischemic injury. The retina is a frequently used model system for studying diseases of CNS dysfunction because it offers key advantages in accessibility for observation (London 2013). Studying caspase-9 in the context of retinal vein occlusion allowed us to take full advantage of eye-specific live-imaging technologies to visualize pathological changes in response to ischemic injury.

#### 3.1 Characterization of retinal pathology in mouse RVO

##### 3.1.1 Vascular effects of RVO: vessel dilation, hemorrhages, edema

Figure 10: RVO induces retinal vessel dilation and intraretinal hemorrhaging



**Figure 10.** Top: Fundus imaging of a mouse retina before and after induction of RVO. Retinal hemorrhaging is evident at 24 and 48 hours post-RVO. Left: Average vein and artery dilation was calculated for each eye at different time points after induction of RVO. Right: Incidence of hemorrhaging was quantified based on fundus imaging.

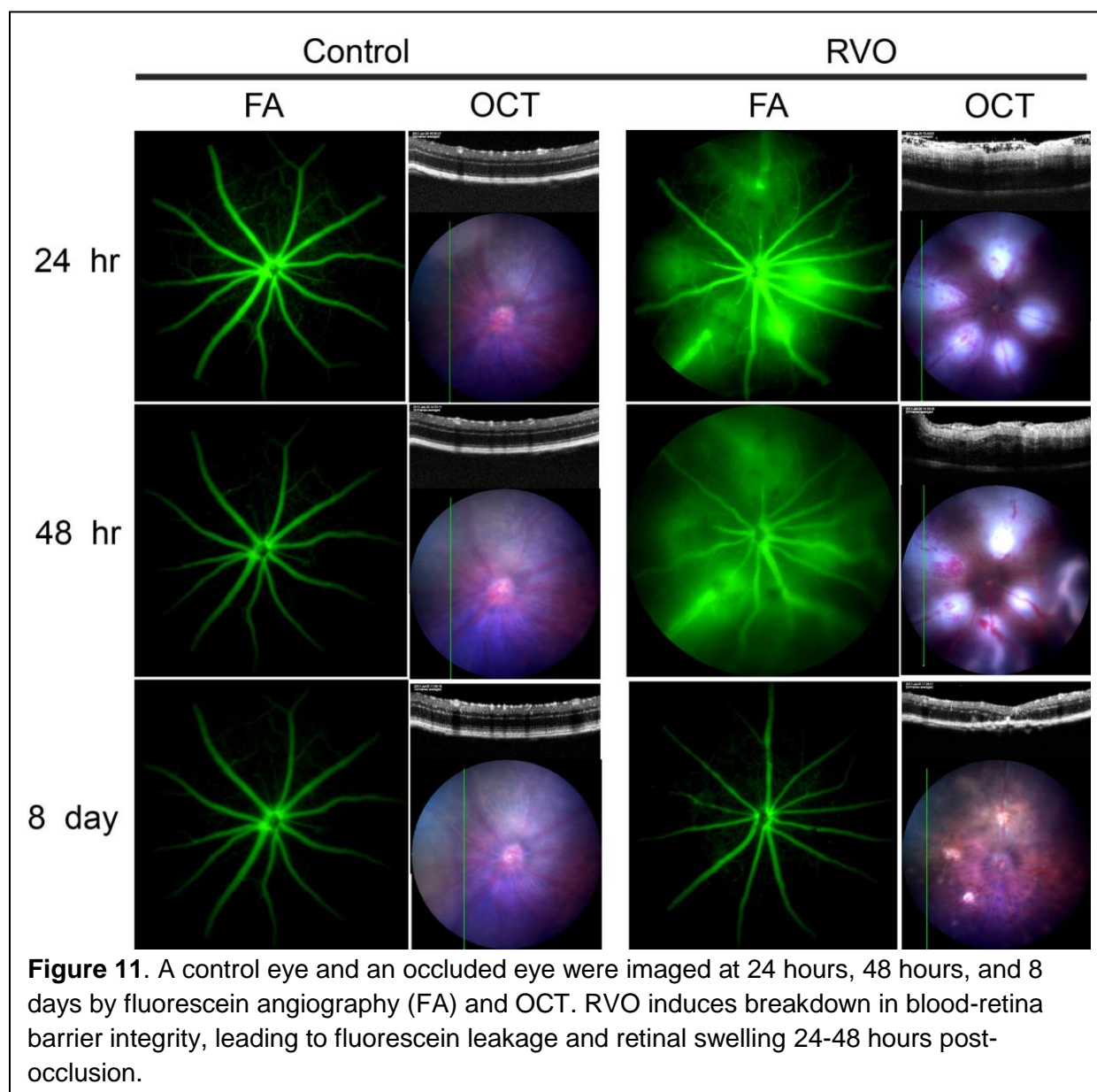
We induced retinal vein occlusion in adult mice using the Phoenix Micron IV Image Guided Laser System. By occluding each of the major retinal veins, we achieved an effect that modeled CRVO. Affected eyes developed all the key clinical features of RVO, including retinal hemorrhages, edema, vessel dilation, and retinal detachment (Fig 10).

A smaller degree of arterial dilation was also noticed within 4 hours of RVO. The increase in arterial diameter has previously been shown in a rat model of retinal ischemia/reperfusion injury (Nishijima 2007), and in diabetic retinopathy patients (Kurt 2017). Arterial dilation reflects systemic aberrations in retinal blood flow. While vessel dilation declined over time, both veins and arteries remained approximately 20% wider than baseline even at 8 days post-RVO (Fig 10). These results are consistent with previous reports that the RVO model causes peak disruption in retinal blood flow at 1-3 days post-RVO, and that vascular changes regress only partially over the subsequent weeks (Fuma 2017, Ebnetter 2015, Genevois 2004).

Concurrent with peak vein dilation, we detected hemorrhages in approximately half of occluded eyes at 48 hours post-RVO (Fig 10). Intraretinal hemorrhaging resolved fully within a few days of occlusion.

Fluorescein angiography (FA) showed changes in retinal blood flow caused by RVO (Fig 11). Early phase of FA depicted obstructed blood flow through the vein occlusions. In the healthy retina, the blood-retinal-barrier keeps fluorescein signal confined to the retinal vasculature. However, breakdown in retinal barrier function caused fluorescein to accumulate in the retinal tissue in injured eyes. Fluorescein leakage was most pronounced at 24-48 hours post-RVO, coinciding with peak vein dilation, peak hemorrhaging, and maximal edema.

Figure 11: RVO induces fluorescein leakage and retinal swelling



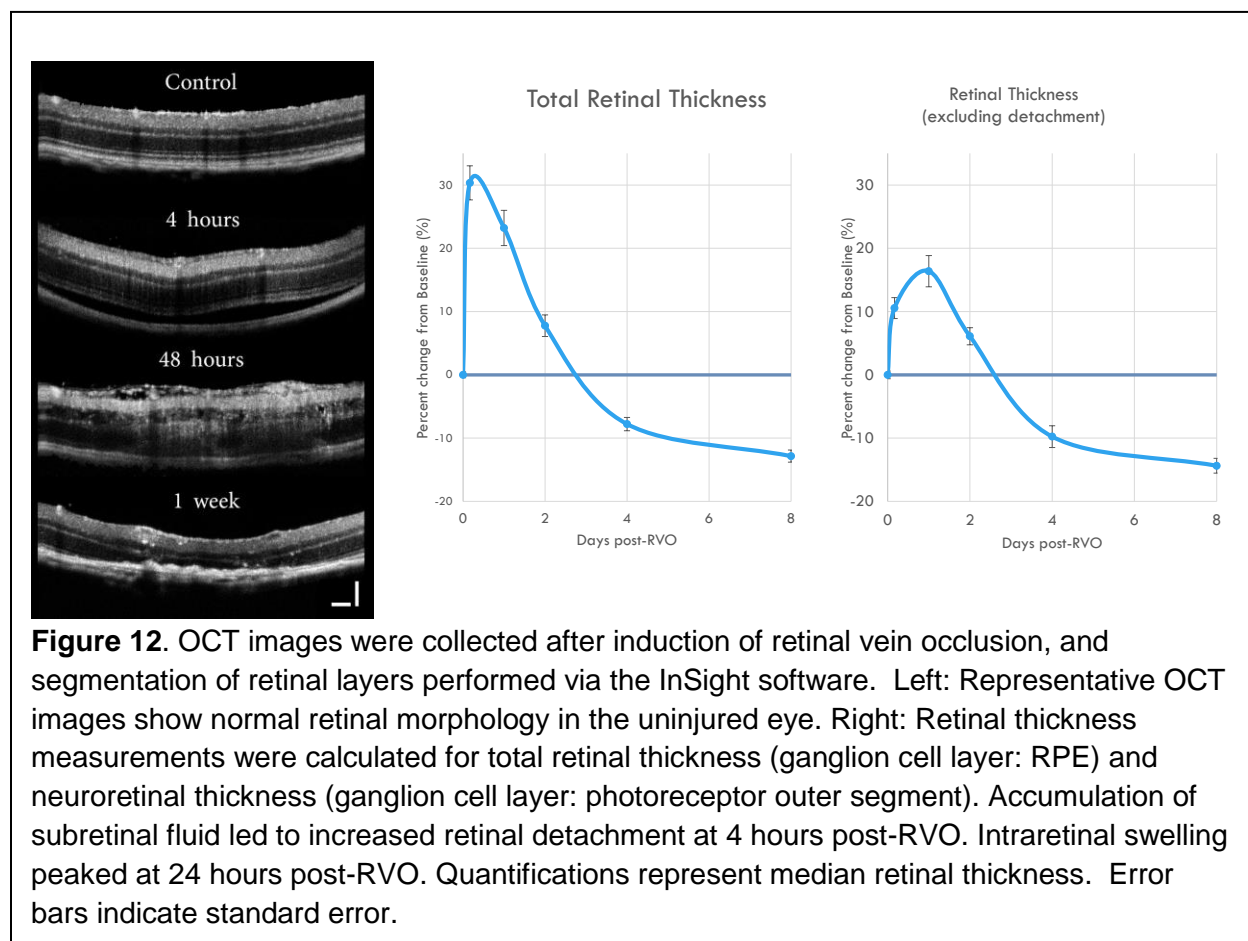
### 3.1.2 OCT imaging shows retinal swelling followed by retinal thinning

Retinal edema was measured by calculating mean OCT retinal thickness at 4 retinal quadrants in each eye (see Chapter 2: Methods). Total retinal thickness peaks around 4 hours post-RVO, driven primarily by retinal detachment due to the accumulation of subretinal fluid (Fig 12).

Retinal vein occlusion causes hypoxic injury to the inner retina, which receives its blood supply

directly from the retinal vasculature (unlike the outer retina which is supplied by blood from the choroidal vessels). Intraretinal swelling is largely limited to the inner retinal layers, reflecting the vascular origin of edema. Intraretinal edema peaked at 24 hours post-RVO (Fig 12, right). Intraretinal cysts, indicating accumulation of fluid within the retina, were detected via OCT (Fig 12, left). Both retinal swelling and detachment persisted for 48 hours following injury, matching the previously reported transient nature of edema in this model.

*Figure 12: Retinal swelling during acute edema phase is followed by retinal thinning*



Furthermore, there is a temporal progression of edema. Similar to how neuronal damage first manifests in the core of a stroke, before spreading out to the penumbra, retinal edema first affects the superplexiform layer (peak swelling at 4 hrs post-RVO) where the original occlusion is made, before reaching the deeper retinal layers. Edema peaks later for the inner plexiform

layer (IPL), which is most swollen at 24 hours post-RVO, and swelling in the outer plexiform layer (OPL) does not reach maximal thickness until 48 hours after injury. By 72 hours, retinal swelling subsided, revealing permanent retinal thinning. Retinal thickness declines by approximately 14%, and is stable by 1 week post-RVO (Fig 12). In RVO patients, retinal pathology follows a similar pattern of retinal edema followed by retinal thinning, albeit with a longer timeline which can span years. RVO patients present with substantial retinal detachment, intraretinal swelling, and disruption of retinal layer morphology. Following resolution of edema, retinal thickness declines, revealing retinal degeneration (Hasegawa 2017). Importantly, retinal thinning is evident even in patients who received and responded to anti-VEGF therapy. In RVO patients receiving anti-VEGF treatment, disruption of retinal morphology in the inner retina is correlated with lower visual acuity (Fickweiler 2017).

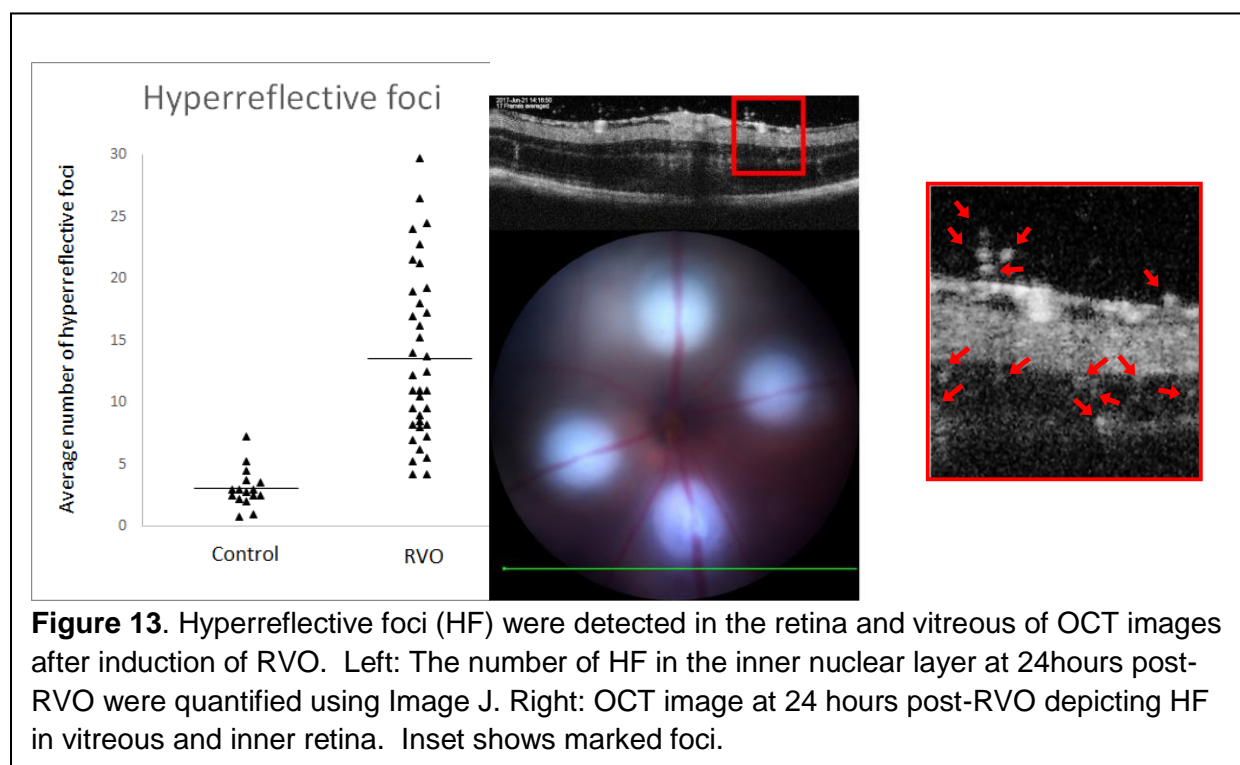
### **3.1.3 Inflammatory effects of RVO: hyperreflective foci**

Retinal vein occlusion induces acute inflammatory response in the retina. *Fuma et al* reported increase in proinflammatory proteins IL-6, ICAM-1 and MCP-1 in their RVO model (Fuma 2017). Consistent with previous reports on mouse RVO, we detected increased levels of VEGF protein 1 day after RVO.

Features of inflammation can also be detected via OCT. Bright spots, called 'hyperreflective foci' are detected in the vitreous and retina of RVO patients, and their numbers are an independent predictor of visual acuity in patients with macular edema due to vascular disease (Mo 2017, Chatziralli 2016). These foci are thought to represent leukocyte infiltration in the retina (Kokona 2017). We detected a 5-fold increase in the numbers of hyperreflective foci 1 day post-RVO (Fig 13).



Figure 13: Hyperreflective foci detected in retinas after RVO



### 3.1.4 Neuroretinal damage: cotton wool spots, outer retinal tubulation

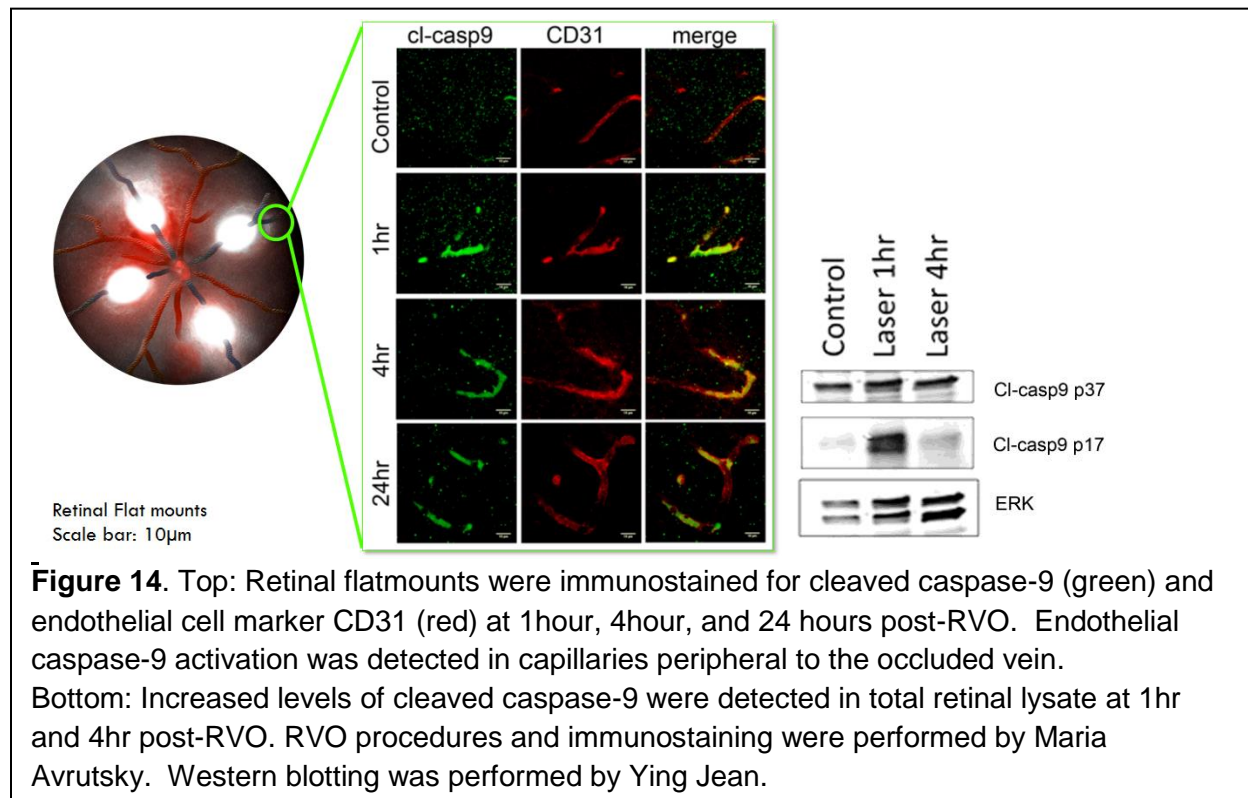
Retinal thinning depicted by OCT imaging reveals evidence of neuronal loss after RVO injury. Other features of neuroretinal damage include cotton wool spots (seen by fundus imaging), and outer retinal tubulation (seen on OCT). Cotton wool spots occur in approximately half of patients with CRVO (Central Vein Occlusion Study, 1993). These lesions reflect injury to the nerve fiber layer of the retina arising from disruption of retrograde axonal transport in retinal ganglion cells. Cotton wool spots show up on OCT as nodular hyperreflective thickening in the nerve fiber layer and are typically observed at the border of large ischemic areas (Zaharova 2011). We regularly observed cotton wool spots extending out from the periphery of the occlusions (Fig 10, 11 fundus images).

Outer retinal tubulation is an OCT feature that is indicative of dying photoreceptor cells. These round or ovoid structures with hyperreflective borders are found in the photoreceptor layer and

indicate poor visual prognosis (All-Halafi 2015, Dolz-Marco 2017). We observed outer retinal tubulation in a subset of eyes in the days immediately following edema resolution.

### 3.2 Caspase-9 activation in endothelial cells after RVO not associated with endothelial cell death

Figure 14: Caspase-9 is activated in endothelial cells after RVO



Having established that our mouse model of RVO recapitulates the key clinical features of retinal vein occlusion, we asked if caspase-9 was also activated in this model. We immunostained retinal flatmounts for vessel markers and cleaved caspase-9. As early as 1 hour post-RVO, we detected an induction of caspase-9 activation in retinal capillaries (Fig 14). Western blotting of retinal lysate confirmed an increase in cleaved caspase-9, indicating that caspase-9 activation occurs prior to the development of edema (which peaks at 24 hours post-RVO). The vascular pattern of caspase-9 activation in RVO closely matches the results seen in rodent models of stroke from previous studies (Akpan 2013).

Since caspase-9 can play a role in initiating apoptosis, we tested whether vascular caspase-9 activation was leading to endothelial cell death. Thinning of retinal layers observed by OCT suggested that cell death was prevalent in the injured retina. We detected dying cells via Ph2ax staining and TUNEL assays. Cell death was detected in the inner retina as early as 1 hour post-RVO, and markers of cell death remained present for up to 48 hrs. During 24 hours post-RVO, cell death was primarily detected in the inner retinal layers, while at 48 hours post-RVO cell death was primarily located in the photoreceptor layer. However, the cell death markers did not colocalize with staining for blood vessels, even in areas where there was widespread death (Fig 15, 16). These results are consistent with reported findings that retinal ischemic injury causes a temporal progression of neuronal cell death first in the inner retina and then in the outer retinal layers, and that endothelial and glial cells are largely spared from apoptosis (Nishijima 2007).

*Figure 15: Induction of cell death by RVO does not colocalize with endothelial cells*

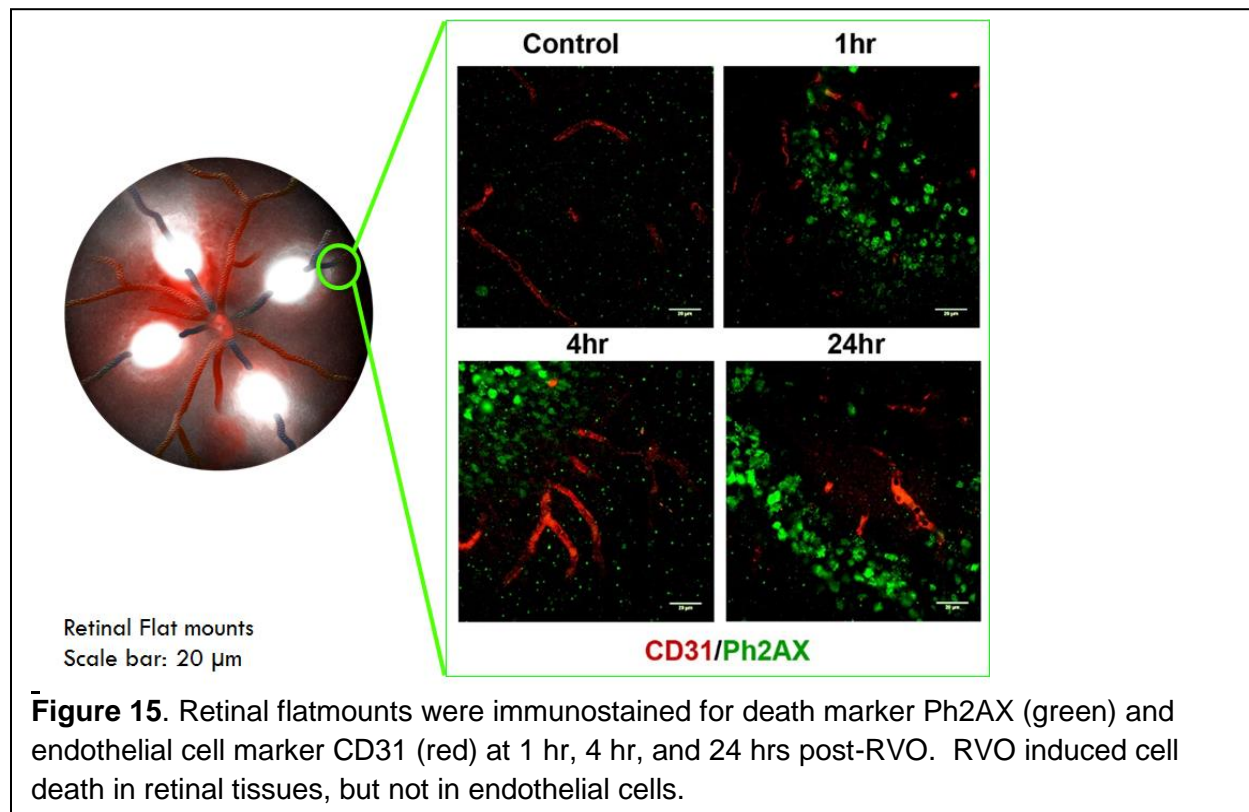
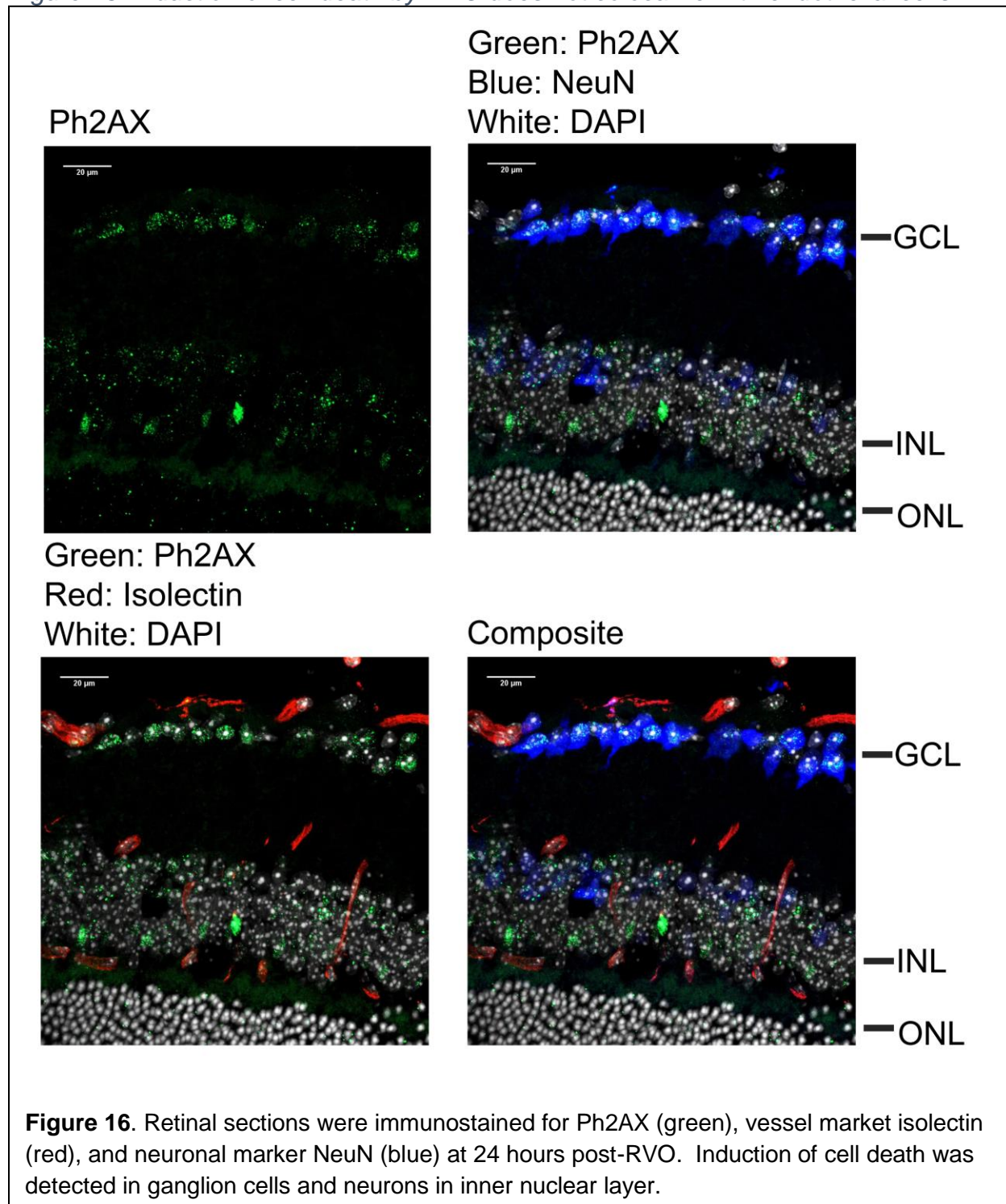


Figure 16: Induction of cell death by RVO does not colocalize with endothelial cells



## 4. Characterization of Inducible endothelial caspase-9 knockout mice

### 4.1 Introduction

We generated an inducible endothelial caspase-9 knockout mouse to investigate how specifically endothelial caspase-9 activity contributes to RVO pathology. *Caspase-9 flox/flox* mice were crossed with *VE-Cadherin-CreERT2* animals to generate a tamoxifen-inducible endothelial deletion of caspase-9 (Casp9 ECKO). By inducing recombination in mature animals, we avoided potential complications of disrupting caspase-9 activity in normal developmental processes.

#### 4.1.1 Published caspase-9 knockouts

Several groups have published reports on caspase-9 knockout mice. Global deletion of caspase-9 results in embryonic lethality after e16.5 (Kuida 1998). These mice have severe brain malformations apparent by e10.5; the hindbrain neural tube remains open, and there is prominent protrusion and expansion of cranial tissues. No morphological abnormalities were noted in non-neuronal tissues. These results were confirmed by *Hakem et al* who also reported that caspase-9 deletion was embryonic lethal in the majority of pups, and that mice lacking caspase-9 displayed brain protrusions from the skull at e12.5 and e15.5 (Hakem 1998). The animals have severe disorganization of cerebral structures, reduced neuronal apoptosis, and accumulation of necrotic tissues in the brain. Intracerebral hemorrhages are common. In addition to gross defects in neuronal morphology, caspase-9 KO mice have defects in axonal projection and maturation (Ohsawa 2010).

As discussed in chapter 1, several groups have reported defects in hematopoietic development resulting from caspase-9 deficiency. Lu 2014 showed that caspase-9 deletion impaired the proliferation and differentiation of hematopoietic stem cell progenitors.

In contrast to global knockout, cell specific caspase-9 deletion often has no phenotype at baseline. *Rongvaux et al* generated caspase-9 floxed mice, which they crossed with Tie2-Cre (Rongvaux 2014). The resulting mice had caspase-9 deleted from endothelial cells and hematopoietic cells. The animals were largely normal, but had a modest increase in type I interferon response, and were more resistant to viral infection.

#### **4.1.2 Tamoxifen-inducible cell specific cre system**

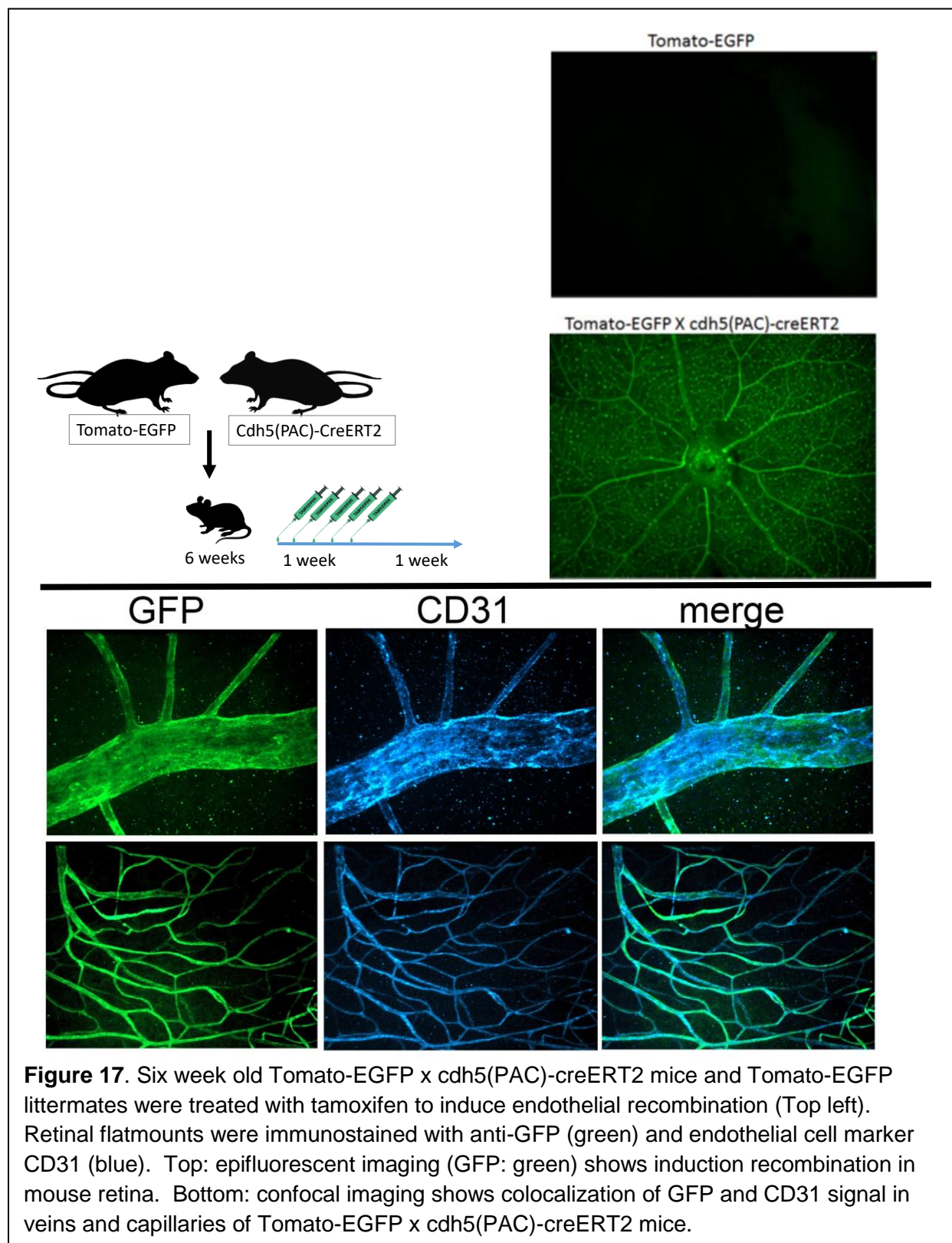
As demonstrated by the catastrophic defects in global caspase-9 knockout animals, caspase-9 plays critical roles in embryonic development. It has also been shown that during development, caspase-9 deletion induces compensatory caspase activation (Zheng 2000). To minimize the potential off-target effects, we elected to use an inducible endothelial Cre to trigger caspase-9 deletion specifically from endothelial cells after completion of retinal vascular development. Developed by Ralf Adams, the tamoxifen-inducible VE-Cadherin Cre (*cdh5(PAC)-creERT2*) mouse allows for exceptionally efficient and specific recombination in endothelial cells (Pitulescu 2010). This mouse has been used to generate numerous endothelial specific gene knockout animals for the study of retinal vasculature.

#### **4.2 Characterization of inducible endothelial Cre**

To test the specificity and efficacy of recombination, we utilized the Tomato-eGFP mT/mG reporter mouse, which expresses GFP wherever recombination takes place (Muzumdar 2007). Microscopy of retinal vasculature confirmed that induction of recombination was highly efficient and specific to endothelial cells (Fig 17). In agreement with previous reports describing the Ve-Cadherin Cre mouse, we measured upwards of 95% colocalization between eGFP expression and endothelial cell markers, and detected no recombination in extraneous cell types.

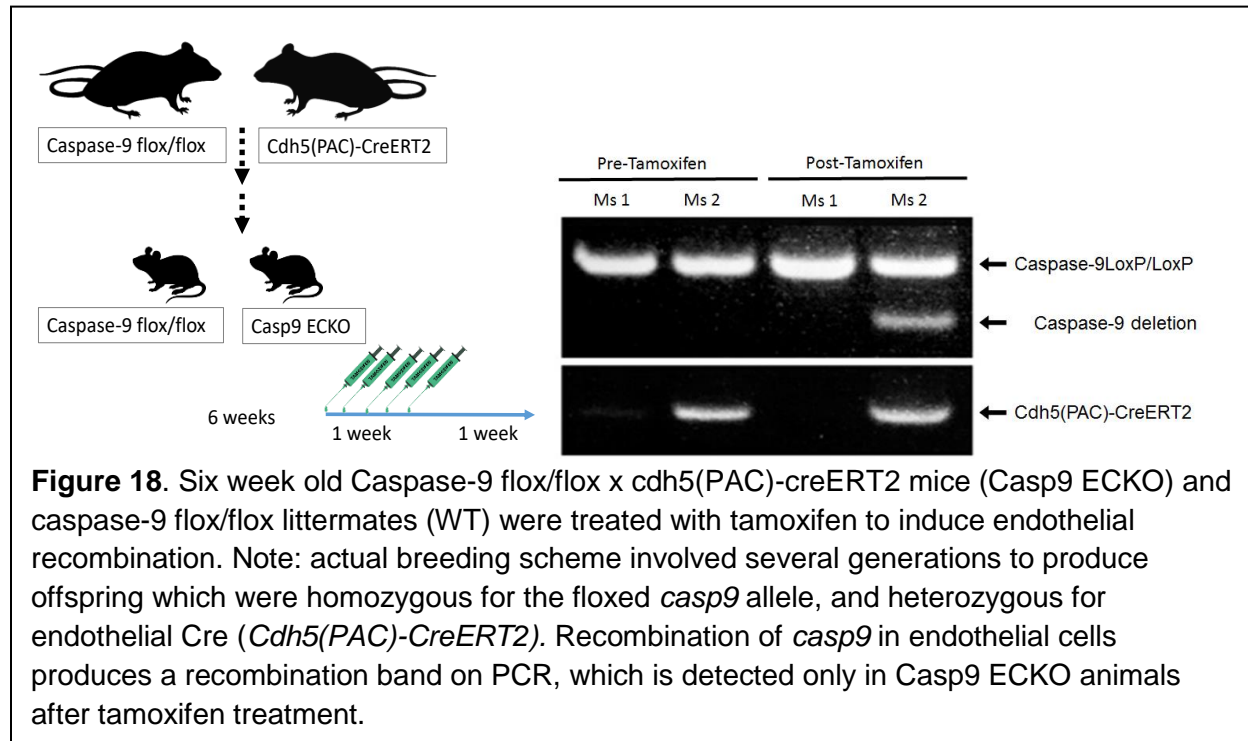


Figure 17: Tomato mT/mG reporter shows efficient inducible endothelial recombination



### 4.3 Detection of caspase-9 deletion by genotyping

Figure 18: Genotyping detects caspase-9 deletion in Casp9 ECKO mice



**Figure 18.** Six week old Caspase-9 flox/flox x *cdh5(PAC)-creERT2* mice (Casp9 ECKO) and caspase-9 flox/flox littermates (WT) were treated with tamoxifen to induce endothelial recombination. Note: actual breeding scheme involved several generations to produce offspring which were homozygous for the floxed *casp9* allele, and heterozygous for endothelial Cre (*Cdh5(PAC)-CreERT2*). Recombination of *casp9* in endothelial cells produces a recombination band on PCR, which is detected only in Casp9 ECKO animals after tamoxifen treatment.

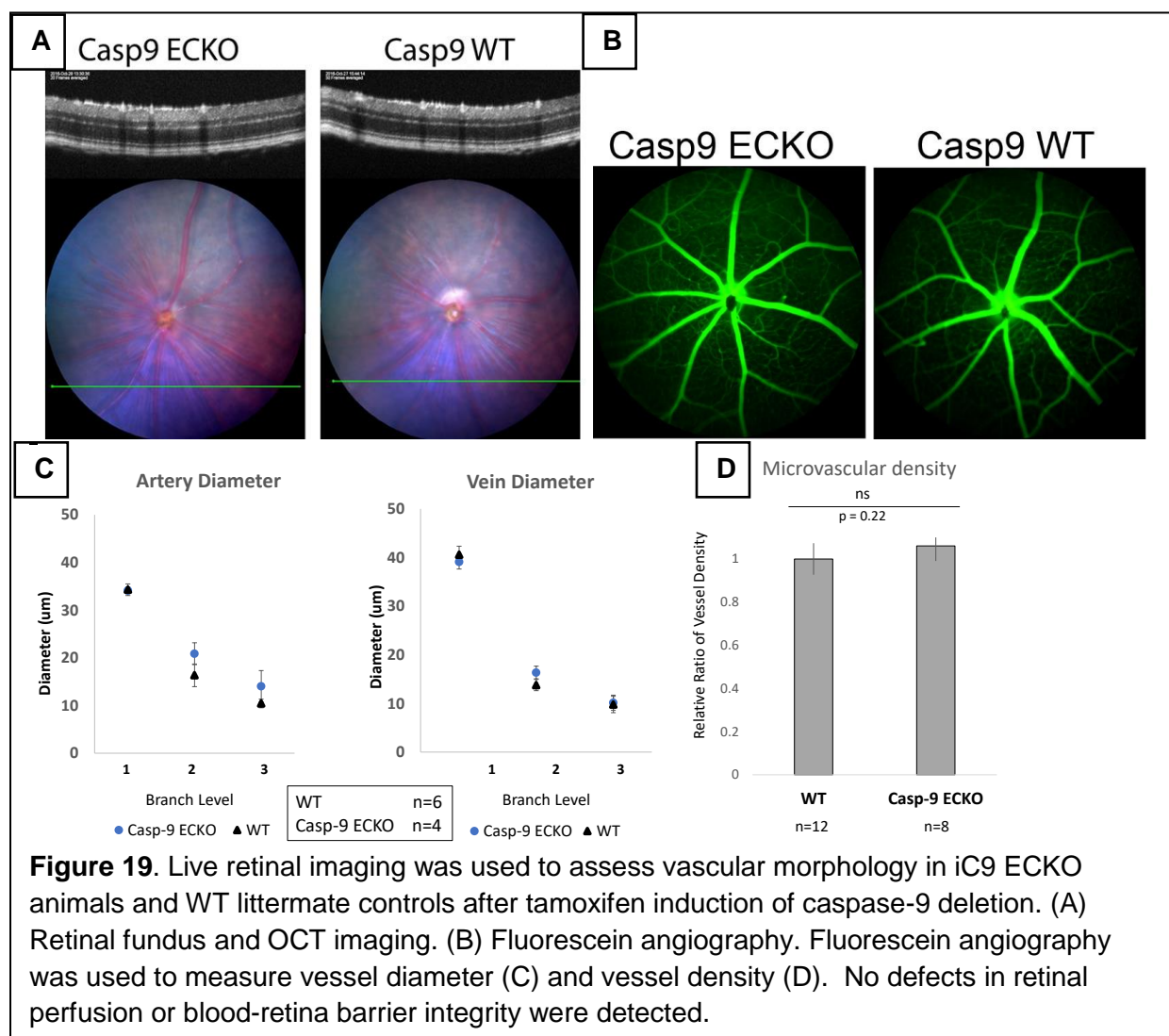
PCR genotyping confirmed *caspase-9* recombination in tissue digests from Casp9 ECKO animals after tamoxifen treatment. The tamoxifen induction protocol resulted in the appearance of a *caspase-9* recombination band in Cre-positive floxed animals, and not in *casp9 flox/flox* animals lacking Cre (Fig 18).

### 4.4 Casp9 ECKO mice have normal retinas at baseline

Live retinal imaging found no significant differences in the retinal morphology between iC9ECKO animals and their littermate controls. Fluorescein angiography showed no defects in vascular permeability, and no changes in vessel density or diameter. OCT morphology showed no changes in retinal layer thicknesses (Fig 19).

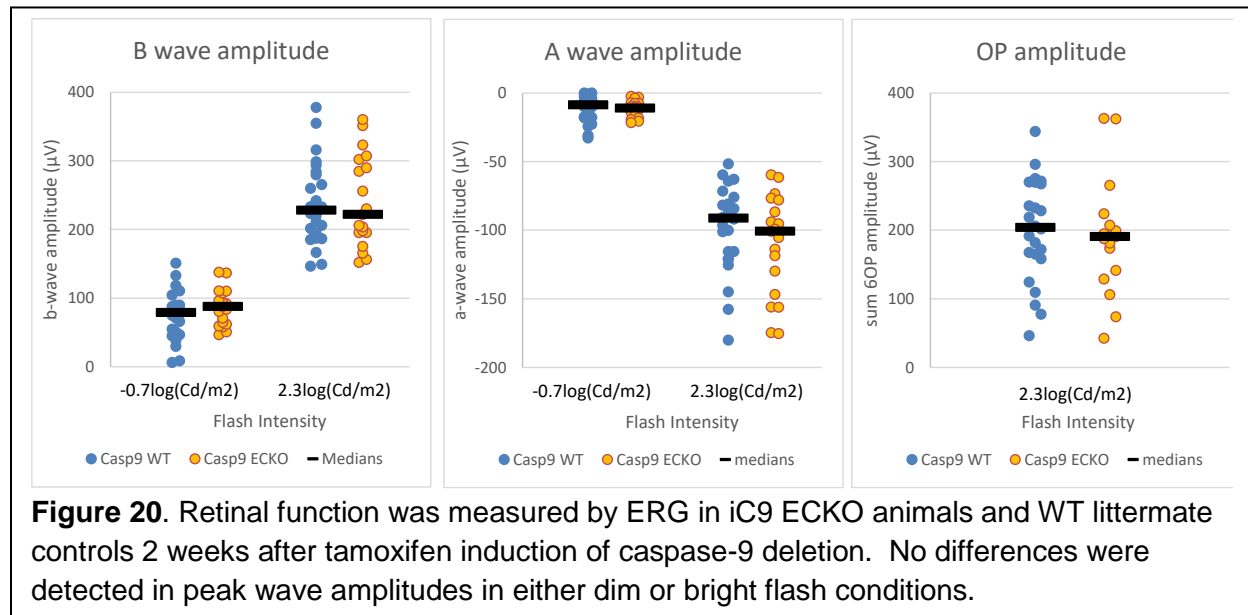


Figure 19: Endothelial caspase-9 deficiency does not alter adult retinal morphology



ERG analysis of retinal function showed equivalent responses from knockout and wildtype animals (Fig 20). The amplitudes and timing of the a-wave, b-wave, and oscillatory potentials were identical in Casp9 WT and Casp9 ECKO animals. Absence of a retinal phenotype at baseline indicated that the deletion of caspase-9 from endothelial cells in adult animals did not alter retinal morphology or function.

Figure 20: Endothelial caspase-9 deficiency does not alter retinal function

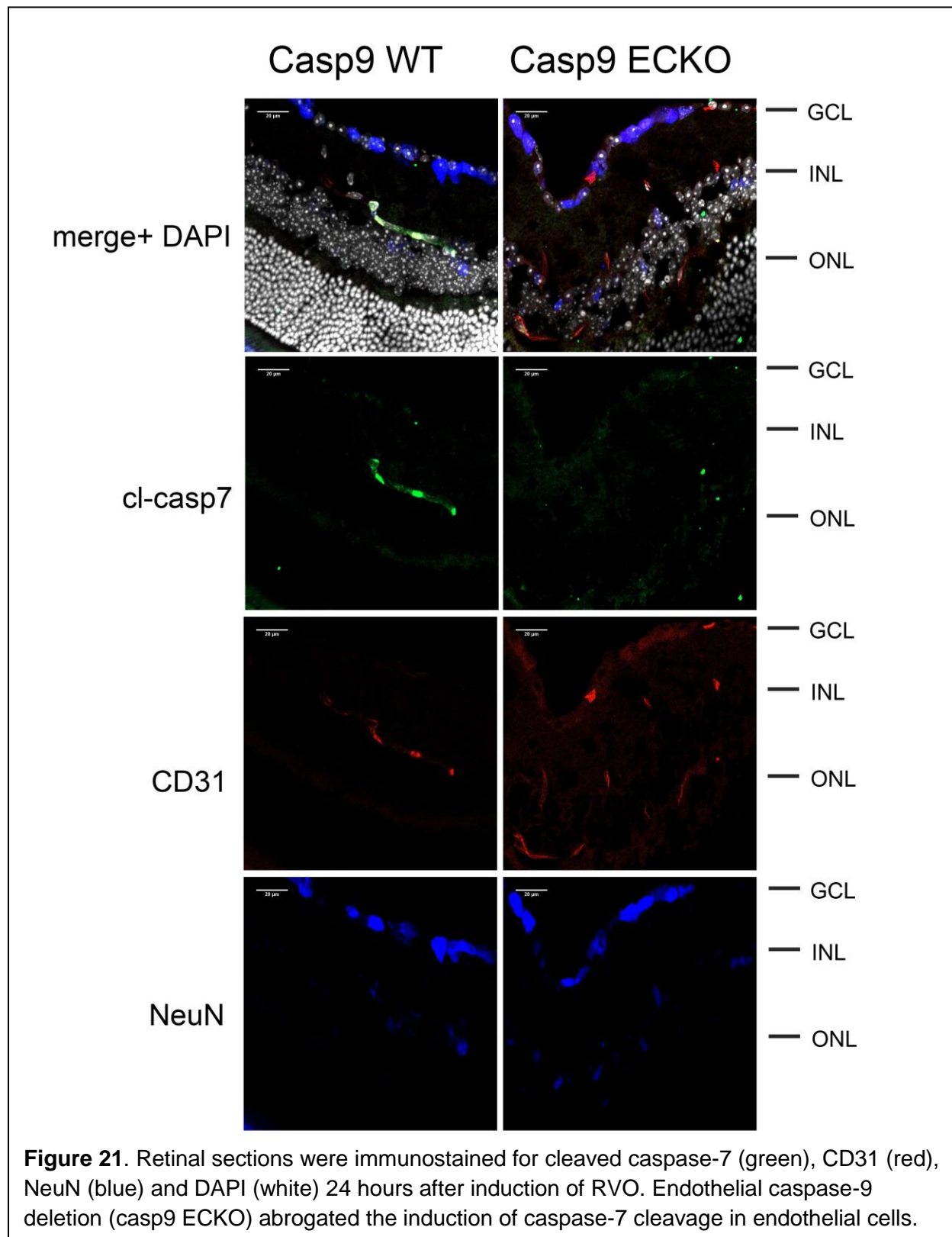


## 5. Endothelial caspase-9 deletion modulates retinal response to RVO

### 5.1 Caspase-7 activation is prevented in Casp9 ECKO mice

Caspase-9 usually mediates its cellular effects by cleavage of downstream effector caspases. Since caspase-7 was the only effector caspase detected in ischemic blood vessels, we immunostained occluded retinas from Casp9 ECKO mice to test whether caspase-9 deletion prevented the activation of caspase-7. Cleaved caspase-7 was detected in blood vessels of WT mice, but not in Casp9 ECKO littermates, indicating that the induction of caspase-7 activity in endothelial cells is dependent on the expression of endothelial caspase-9. The absence of cleaved caspase-7 in Casp9 ECKO mice confirms effective functional endothelial caspase-9 deletion (Fig 21).

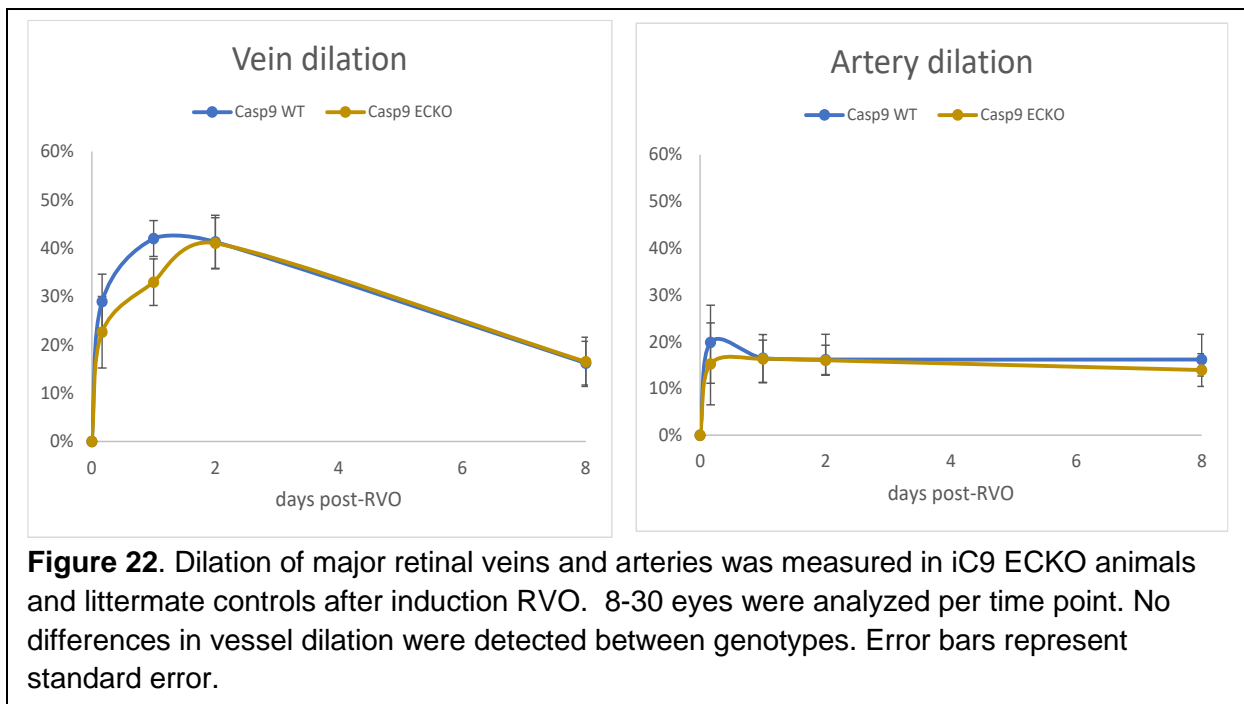
Figure 21: RVO induction of endothelial caspase-7 cleavage is dependent on caspase-9



## 5.2 RVO causes less disruption to retinal morphology in Casp9 ECKO mice

Since RVO caused activation of endothelial caspase-9 in WT animals, we tested whether genetic deletion of endothelial caspase-9 would modify the progression of retinal pathology after ischemic injury. We compared retinal changes in Casp9 ECKO mice and Casp9 WT littermate controls following RVO. There were no significant differences in vein or artery dilation between Casp9 ECKO mice and littermate controls, indicating that the fundamental RVO injury was comparable in both genotypes (Fig 22).

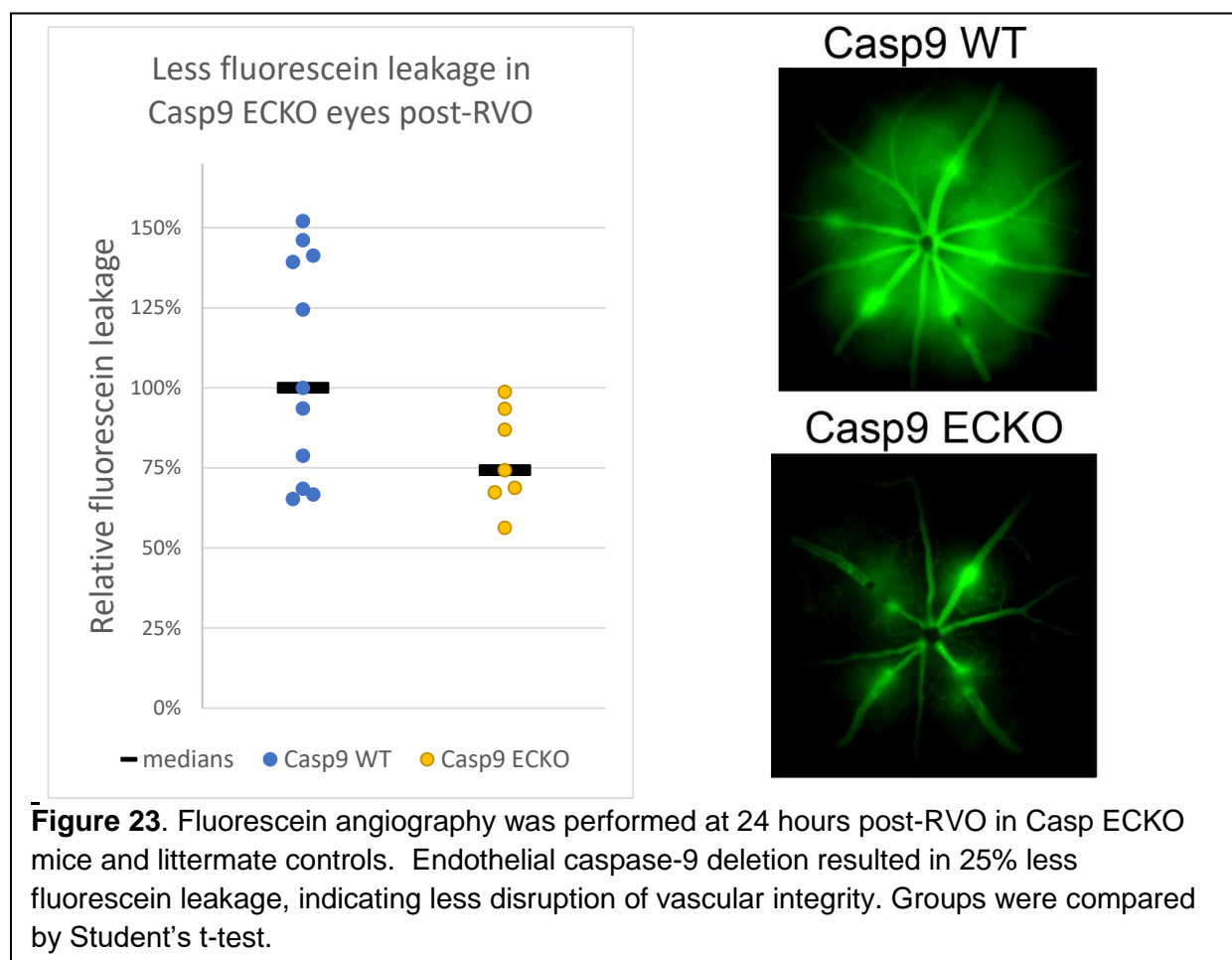
*Figure 22: Endothelial caspase-9 deficiency does not alter vessel dilation*



**Figure 22.** Dilation of major retinal veins and arteries was measured in iC9 ECKO animals and littermate controls after induction RVO. 8-30 eyes were analyzed per time point. No differences in vessel dilation were detected between genotypes. Error bars represent standard error.

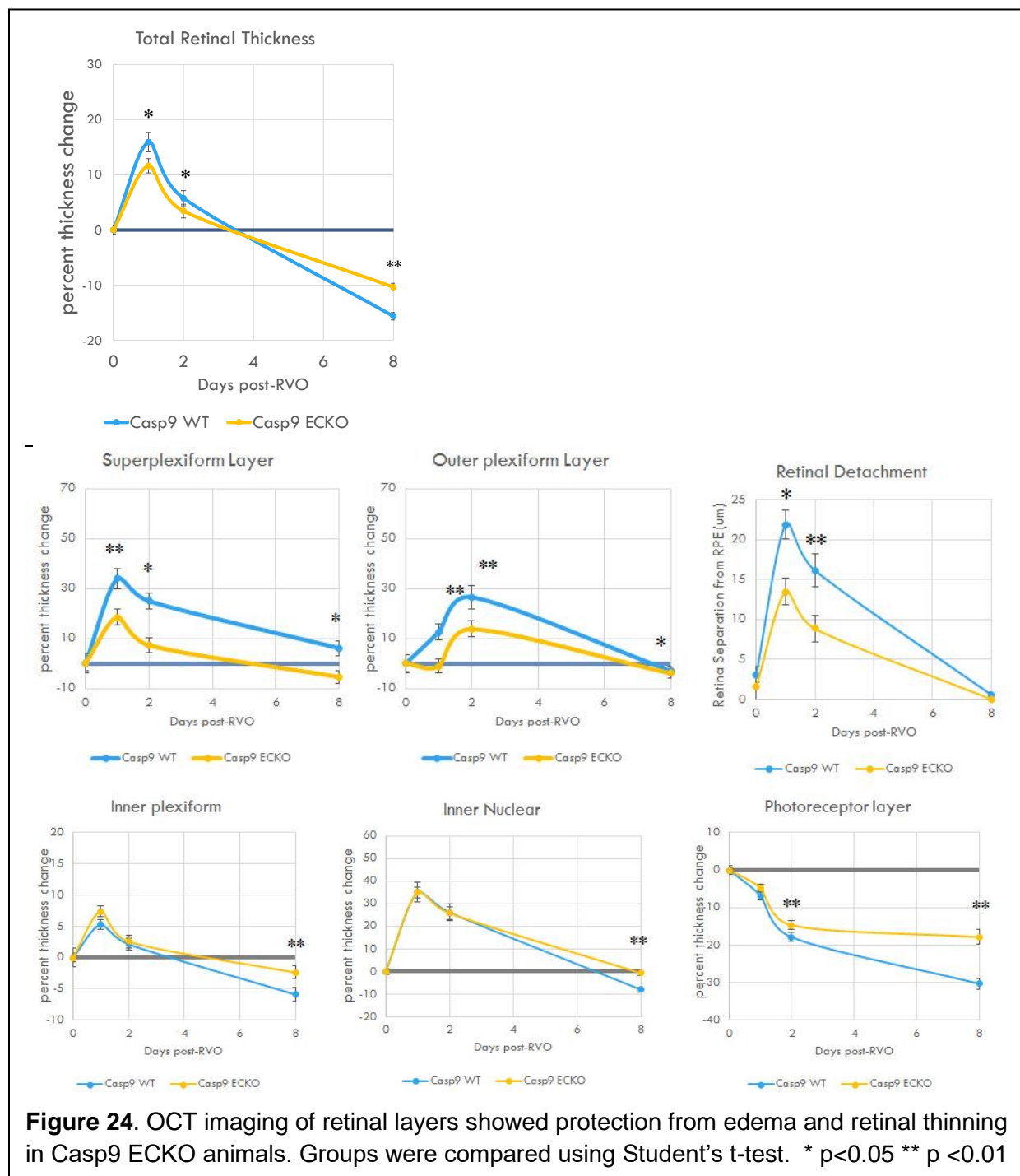
However, WT mice developed more edema and greater disruption of the BRB compared to littermates lacking endothelial caspase-9. Pathological increase in BRB permeability causes vasogenic edema, which we visualized via fluorescein angiography. Compared to littermate controls, Casp9 ECKO mice had 25% less fluorescein leakage at 24 hours post-RVO (Fig 23).

Figure 23: RVO causes less disruption of BRB in Casp9 ECKO mice



We analyzed OCT images depicting RVO-induced changes in retinal morphology in WT and Casp9 ECKO littermates (Fig 24). Endothelial caspase-9 KO animals had less overall retinal swelling, and 50% less accumulation of subretinal fluid. After edema has resolved, OCT revealed thinning of the IPL and INL layers in WT animals. These changes, reflective of neuronal loss, are also seen in RVO patients and in diabetic individuals. In Casp9 ECKO mice, both the IPL and INL layers were protected from thinning, and total retinal thickness remained closer to normal.

Figure 24: Endothelial caspase-9 deficiency protects retinal morphology after RVO

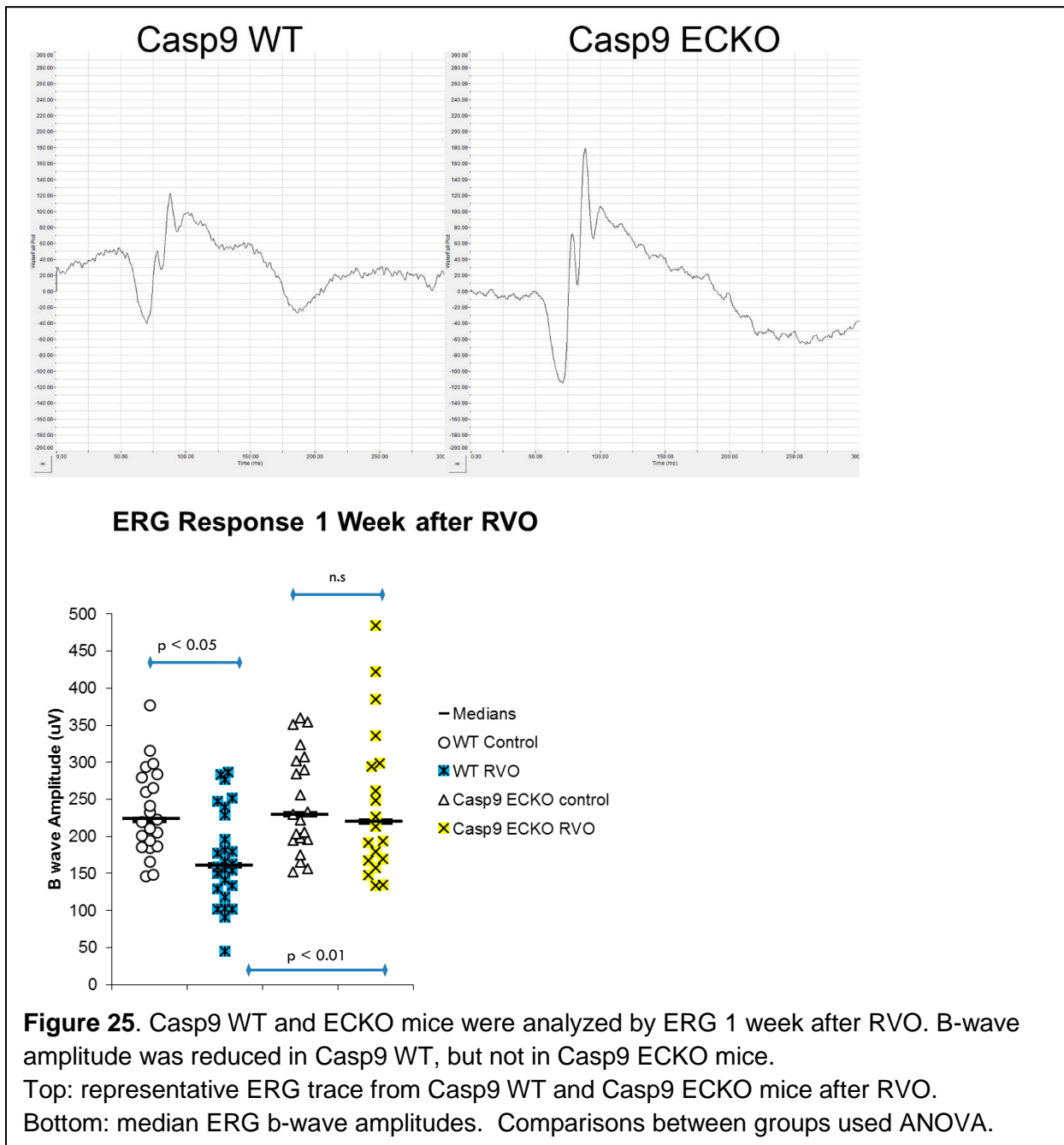


These results indicate that endothelial caspase-9 plays a role in promoting the disruption of vascular integrity after an ischemic injury. Deletion of caspase-9 from endothelial cells abrogates vasogenic edema, reduces retinal swelling, and limits retinal thinning after RVO. The

preserved inner retinal integrity in Casp9 ECKO mice suggests that loss of endothelial caspase-9 activity may help prevent vision loss in RVO.

### 5.3 Protected retinal function in Casp9 ECKO mice

Figure 25: Endothelial caspase-9 deficiency protects retinal function after RVO



**Figure 25.** Casp9 WT and ECKO mice were analyzed by ERG 1 week after RVO. B-wave amplitude was reduced in Casp9 WT, but not in Casp9 ECKO mice. Top: representative ERG trace from Casp9 WT and Casp9 ECKO mice after RVO. Bottom: median ERG b-wave amplitudes. Comparisons between groups used ANOVA.

Ischemic neuronal damage in the inner retina leads to permanent loss of vision that is associated with ERG deficits in RVO patients. In particular, RVO patients present with decreased B wave amplitude, which reflects the working of the inner retina, including the ON bipolar neurons and Muller cells. Since Casp9 ECKO mice were protected from retinal thinning, we hypothesized that these animals would have preserved neuroretinal function.

We utilized focal ERG, centered on the area affected by RVO, to measure retinal responsiveness to light in Casp9 ECKO and WT littermates. One week after RVO, WT mice experienced a 30% reduction in B wave amplitude, while Casp9 ECKO mice were indistinguishable from healthy controls (Fig 25).

These results indicate that the morphological protection seen by OCT in Casp9 ECKO mice translated to a preservation of neuroretinal function as measured by ERG.

## **6. Pharmacological inhibition of caspase-9**

### **6.1 Introduction**

Since genetic deletion of caspase-9 ameliorated edema and prevented vision loss after ischemic injury, we tested whether pharmacological inhibition of caspase-9 can be used with therapeutic effect in our RVO model. Given the multivariate physiological functions of caspases, a pharmacological anti-caspase-9 strategy should maximize target specificity. While short peptide catalytic inhibitors (such as LEHD and ZVAD) of caspases have been extensively utilized in research, multiple studies have found that these compounds are not highly specific and target caspase-3 more effectively than other caspases (Benkova 2009, McStay 2008).

A second challenge to pharmacological interventions in retinal disease is targeting drug delivery to the retina. Ideally, pharmacological intervention would be given locally, rather than systemically, to avoid adverse effects in non-ocular tissues. However, ocular physiology does



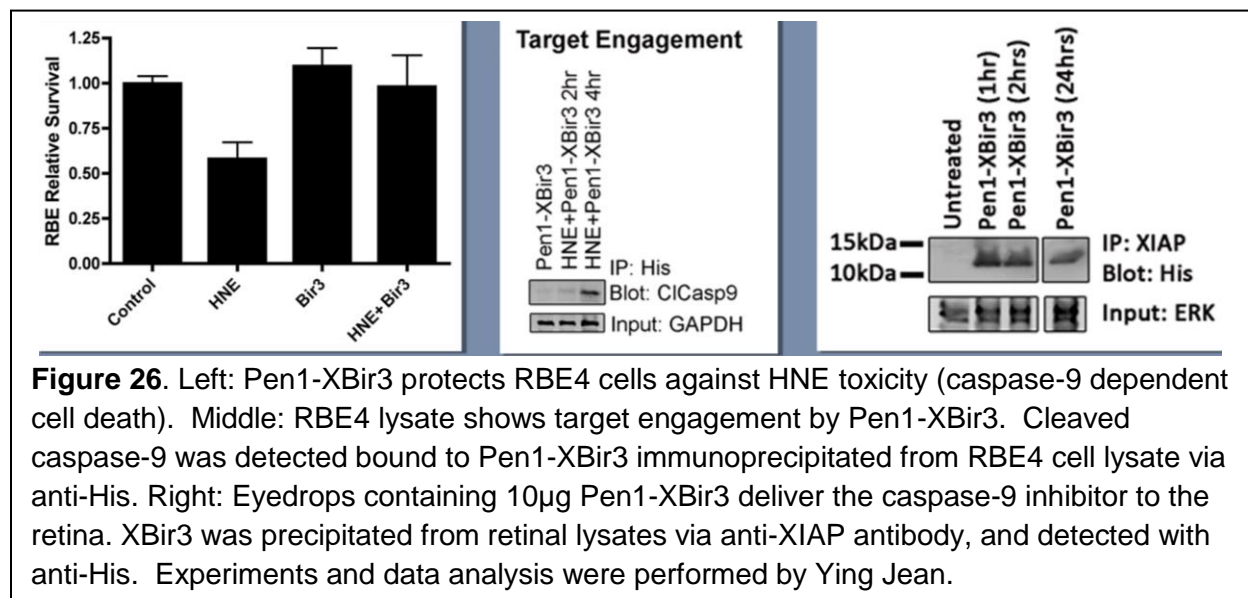
not permit ready retinal access to most topical therapeutics. As discussed in Chapter 1, currently available pharmacological therapies rely on either intravitreal implants or intravitreal injections, both of which carry inherent additional risks.

## 6.2 Pen1-XBir3 caspase-9 inhibitor

Since commercially available caspase inhibitors have broad off-target effects, we utilized Pen1-XBir3, a highly specific caspase-9 inhibitor derived from crosslinking the Bir3 domain of XIAP (the endogenous inhibitor of caspase activity), to Penetratin-1, a cell permeating peptide. The Bir3 domain specifically binds to and inhibits caspase-9 (IC<sub>50</sub> 16 +/- 8nM), without affecting other caspases. Pen1-XBir3 can cross the blood brain barrier and protect neuronal tissue from damage in models of ischemic stroke (Akpan 2011). In Blanch 2014, intravitreal injection of Pen1-XBir3 was used to protect eyes from caspase-9 mediated photoreceptor death following blunt ocular trauma. We hypothesized that Pen1-XBir3 could be delivered to the retina at an effective therapeutic dose using eyedrops, thus voiding the need for intravitreal injection.

## 6.3 Retinal uptake and target engagement by Pen1-XBir3

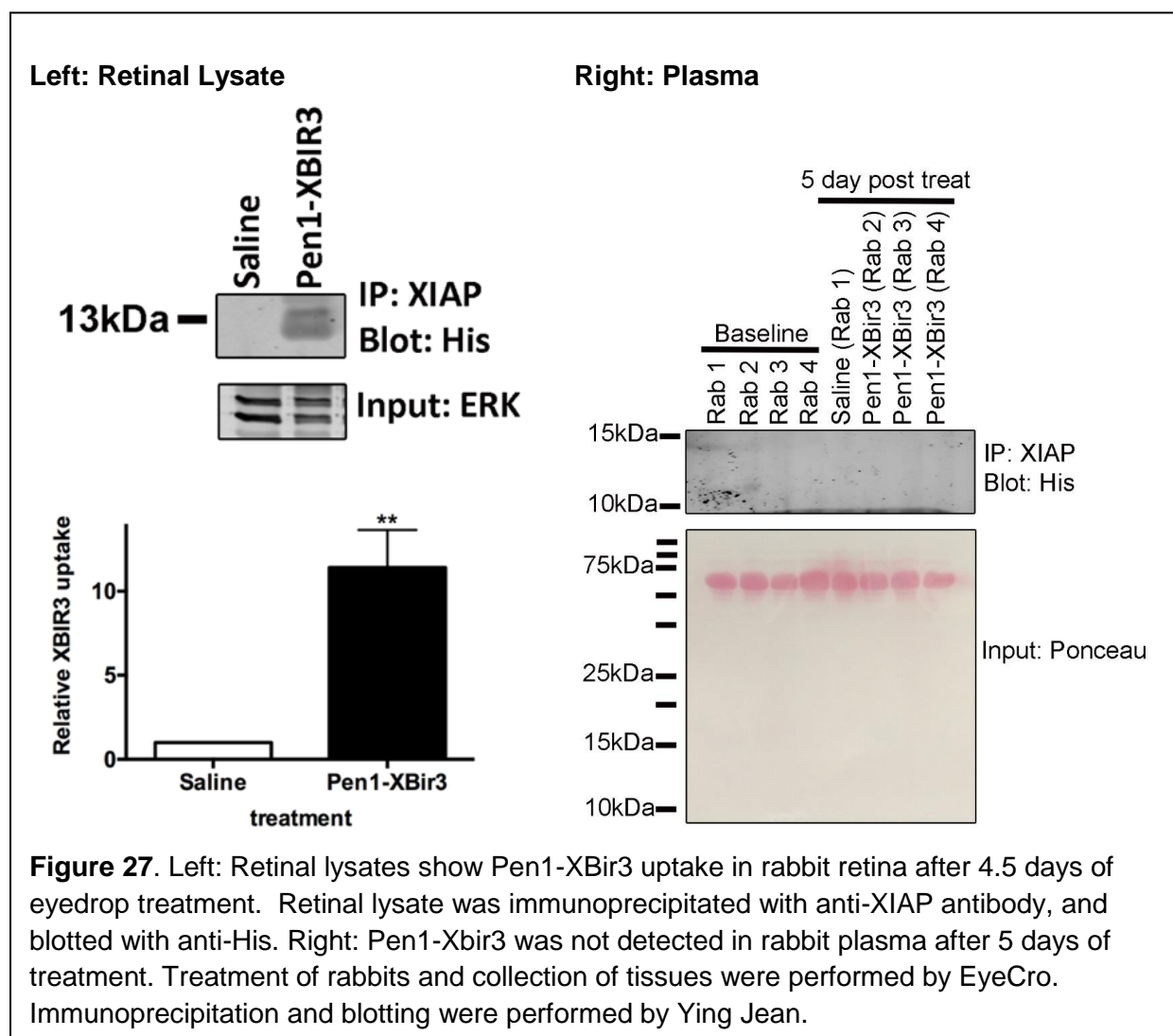
Figure 26: Pen1-XBir3 caspase-9 inhibitor can be delivered to retina via eyedrops



Functional efficacy of this construct was confirmed using rat brain endothelial (RBE4) cell cultures that were subjected to 4-hydroxynonenal (HNE), a toxin which triggers cell death via a caspase-9 dependent pathway (Rabacchi, 2004). Pen1-XBir3 abrogated cell death in HNE-treated cultures (Fig 26, left). The XBir3 construct contains a His tag, which we used to immunoprecipitate the inhibitor peptide from RBE4 cell lysates. Immunoblotting showed Pen1-XBir3 binding to cleaved caspase-9 in HNE-treated cultures, confirming target engagement (Fig 26, middle). To ensure that Pen1-XBir3 could be delivered to the retina, we administered Pen1-XBir3 eyedrops to WT mice and collected their retinas at 1hr, 2hr and 24hr post-treatment. We detected retinal uptake of the inhibitor as early as 1 hour after eye drop administration (Fig 26, right).

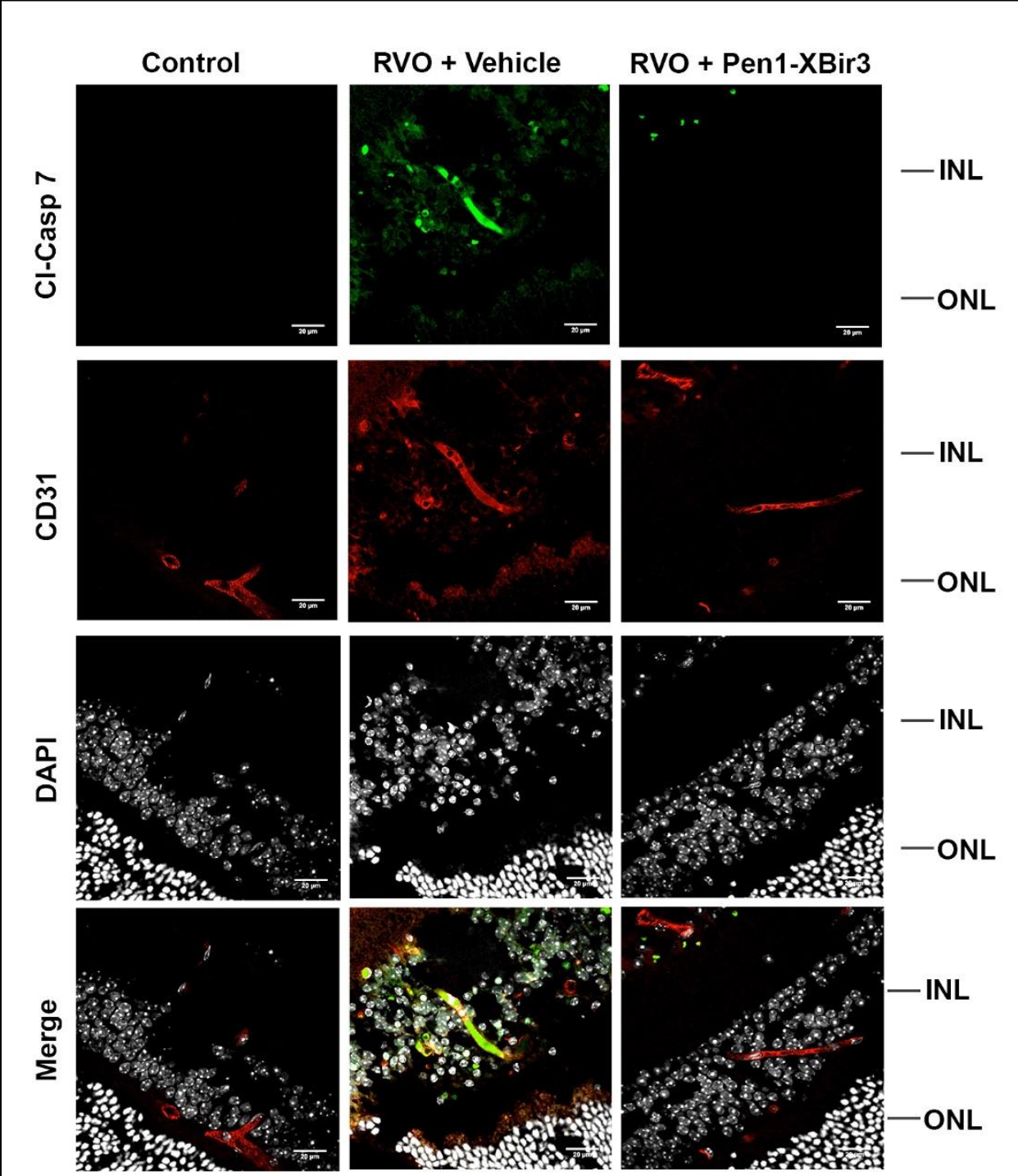
To test the feasibility of scaling eyedrop delivery of Pen1-XBir3 to a larger eye, we tested retinal uptake of Pen1-XBir3 in rabbits. Rabbit eyes are frequently used in ocular studies because their eyes offer good correlation to drug pharmacokinetics in human trials (Del Amo EM 2015). After 4.5 days of twice-daily administration of Pen1-XBir3 eye drops, we detected XBir3 in retinal lysates, but not in collected blood plasma (Fig 27). These preliminary results suggest that Pen1-XBir3 eye drops can deliver the inhibitor to retinas in larger eyes, and that the drug may not accumulate systemically with sustained treatment.

Figure 27: Retinal uptake of Pen1-XBir3 eye drops in rabbits



In our RVO intervention studies, we administered Pen1-XBir3 eye drops immediately after the RVO procedure, followed by a second dose at 24 hours. This dosage paradigm was based on the observation that edema and cell death were prominent at 24-48 hours post-RVO. Since Pen1-XBir3 was detected in the retina at 24 hours following eye drop administration, this treatment paradigm maintained suppression of caspase-9 activity throughout the first 48 hours following injury. An equivalent volume of Pen1-Saline was administered as a negative control. Immunostaining showed that Pen1-XBir3 prevented induction of caspase-7 activation, indicating inhibition of caspase-9 activity (Fig 28).

Figure 28: Pen1-XBir3 eyedrops inhibit endothelial cleavage of caspase-7

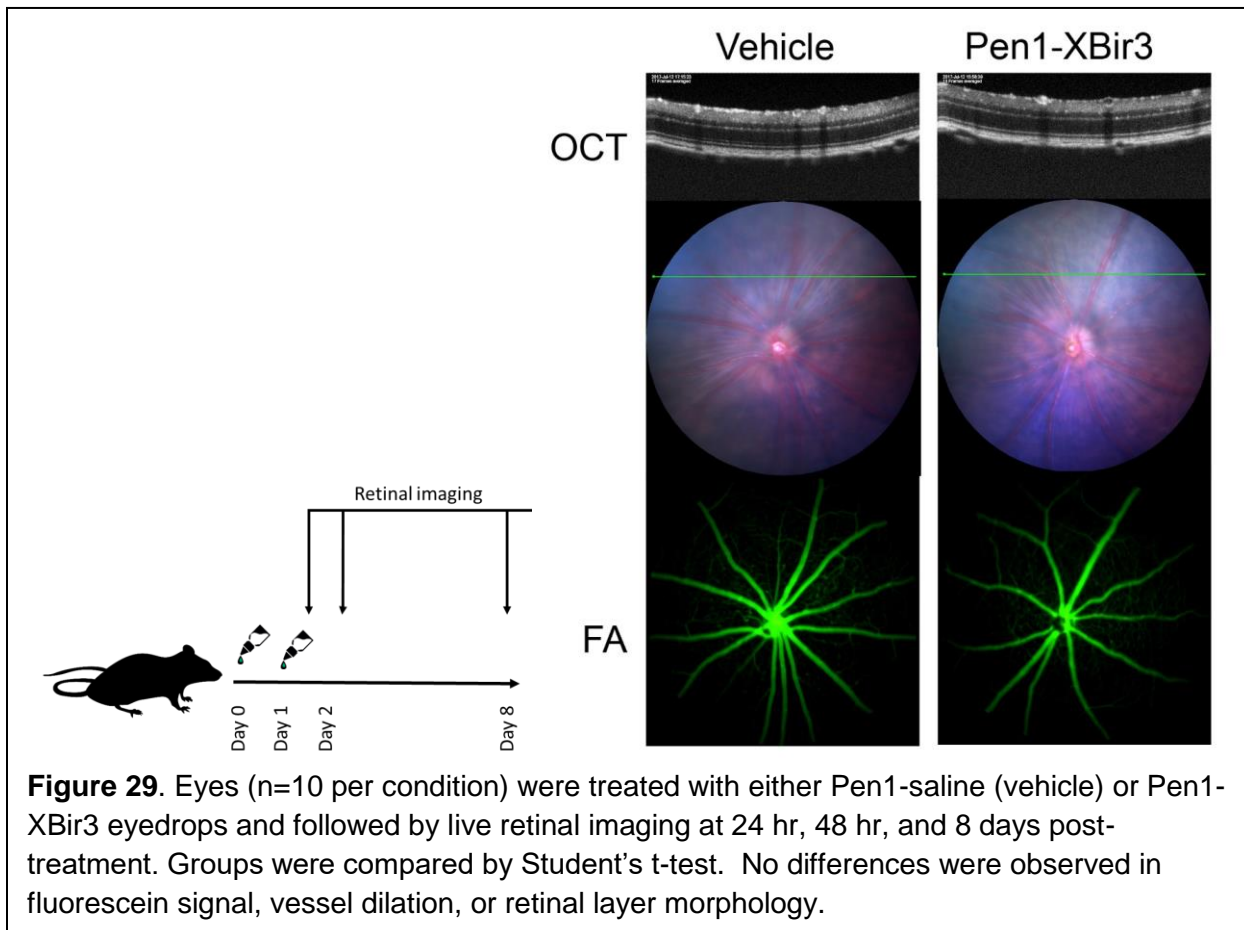


**Figure 28.** Retinal sections were immunostained for ci-casp7 (green), vessel marker CD31 (red) and nuclear stain DAPI (white) at 24 hours post-RVO. RVO-induced cleavage of endothelial caspase-7 was blocked in eyes treated with caspase-9 inhibitor

#### 6.4 Pen1-XBir3 does not modify healthy retinal morphology or function

Since basal activation of caspase-9 is minimal in healthy retinas, we did not expect any significant morphological or functional effects of administering the inhibitor in the absence of an injury stimulus. Pen1-XBir3 eyedrops did not change vessel dilation or permeability in healthy eyes, or produce changes in OCT morphology or ERG response. In mice who received 3x per week treatment with Pen1-XBir3 eyedrops over a course of 6 months, no ocular pathologies or retinal changes were detected. However, since only 3 animals were included in the treatment group (and 2 animals in the control group), further study is needed to ascertain the safety of long-term Pen1-Xbir3 treatment.

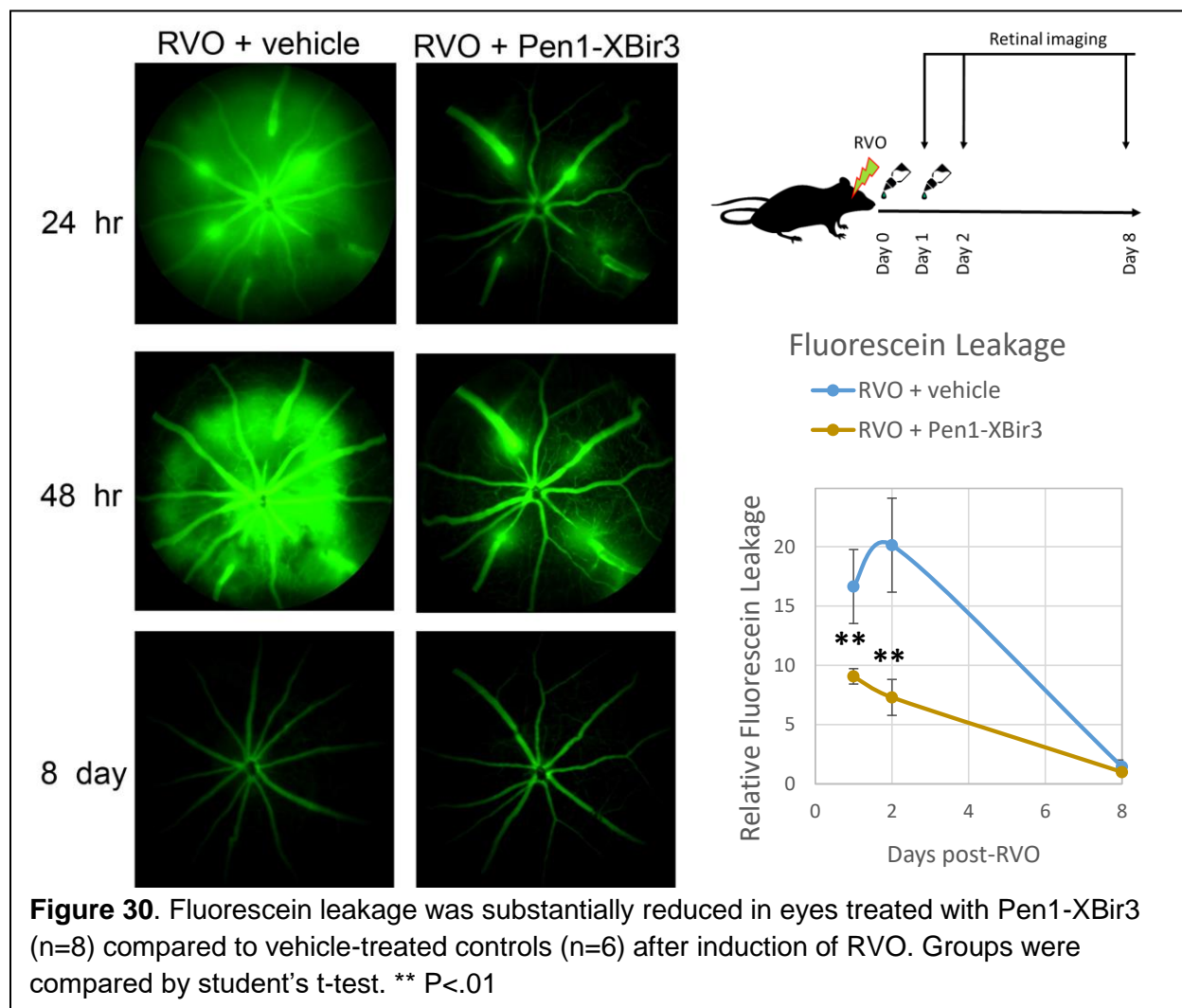
*Figure 29: Pen1-XBir3 eyedrops do not alter retinal morphology or vasculature*



## 6.5 Pen1-XBir3 ameliorates retinal edema

Earlier work in rodent models of stroke showed that Pen1-XBir3 administration protected against neuronal damage in CNS ischemic injury (Akpan 2011). Measurement of infarct volume also suggested that Pen1-XBir3 may protect against edema. We utilized fluorescein angiography to directly visualize vascular leakage in mice receiving Pen1-XBir3 eyedrops compared to vehicle-treated controls. The caspase-9 inhibitor substantially reduced the amount of fluorescein leakage at 24 and 48 hours post-RVO, mirroring the results seen in Casp9 ECKO mice (Fig 30).

Figure 30: Pen1-XBir3 eyedrops reduce fluorescein leakage after RVO



The reduction in fluorescein leakage was associated with less retinal swelling. Both total retinal thickness, retinal detachment and swelling in the inner retinal layers were reduced in eyes treated with Pen1-XBir3 (Fig 31).

After resolution of retinal edema, eyes that had received Pen1-XBir3 treatment had less retinal thinning. There was less degeneration of both the inner nuclear layer and the outer nuclear layer, indicating that Pen1-XBir3 protected against retinal neuronal damage during RVO.

We measured vessel dilation in occluded eyes and saw no difference in vessel diameter between Pen1-XBir3 and vehicle treatment groups, suggesting the Pen1-XBir3 was acting downstream of the occlusive injury (Fig 32). These results phenocopy the protection seen in Casp9 ECKO animals.

As discussed in Chapter 1, retinal ischemia from RVO causes increased VEGF expression, which contributes to pathogenic vascular permeability, edema, and neovascularization. As a result, many RVO treatment strategies target VEGF signaling. Since caspase-9 activation was detected very early after induction of RVO (Fig 14), and inhibition of caspase-9 protected against RVO-induced vascular leakage, we tested whether caspase-9 inhibition had an effect on retinal VEGF protein levels. Total retinal lysates were analyzed for VEGF protein at 24 hours post-RVO (Fig 32). Consistent with prior reports using this model (Fuma 2017, Ebnetter 2015), RVO caused an approximately 30% increase in VEGF protein levels at 24 hours post-RVO. However, while treatment with Pen1-XBir3 prevented the increase in total caspase-9 protein levels, it did not prevent the increase in VEGF protein. These results indicate that caspase-9 inhibition confers protection against edema even in tissues which have elevated VEGF levels.



Figure 31: Pen1-XBir3 eyedrops protect retinal morphology after RVO

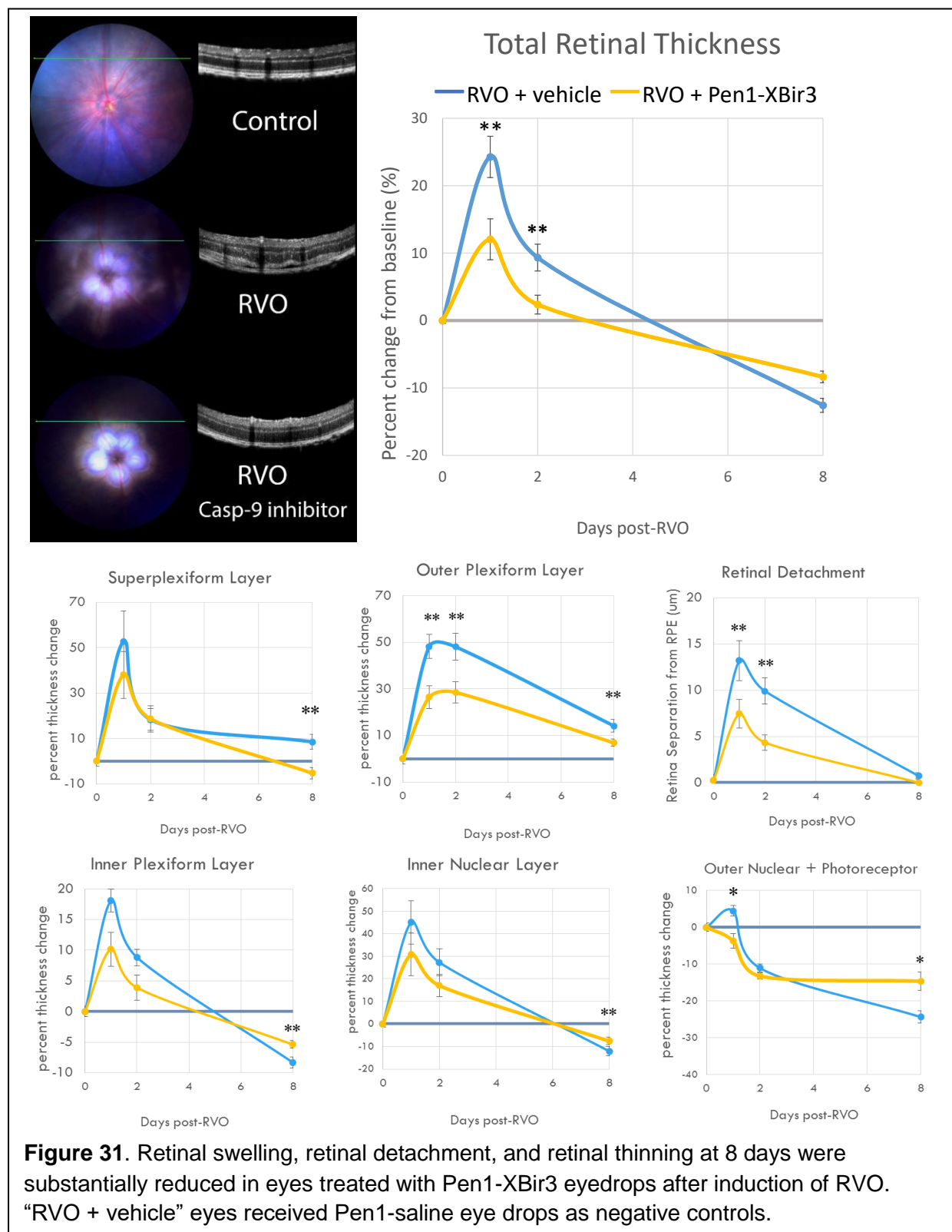
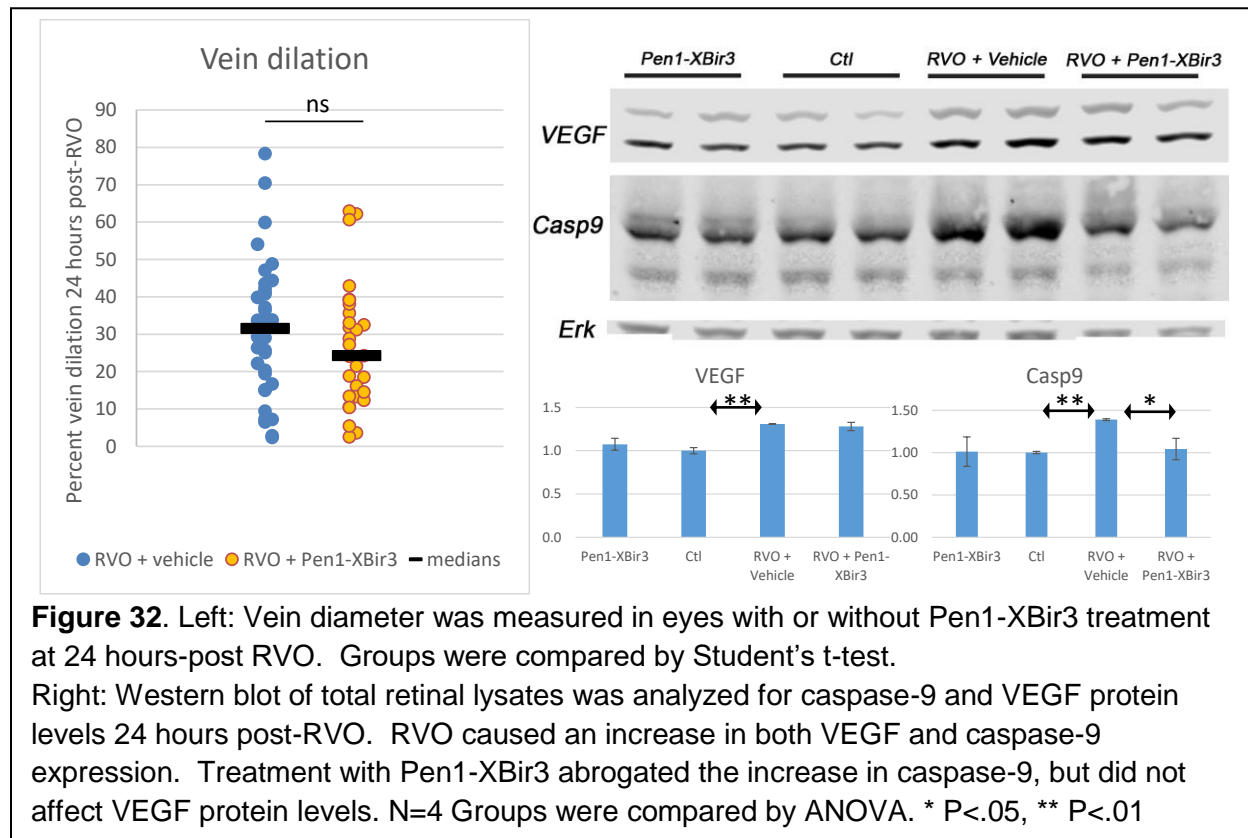




Figure 32: Pen1-XBir3 eyedrops do not alter retinal vessel dilation or induction of VEGF after RVO

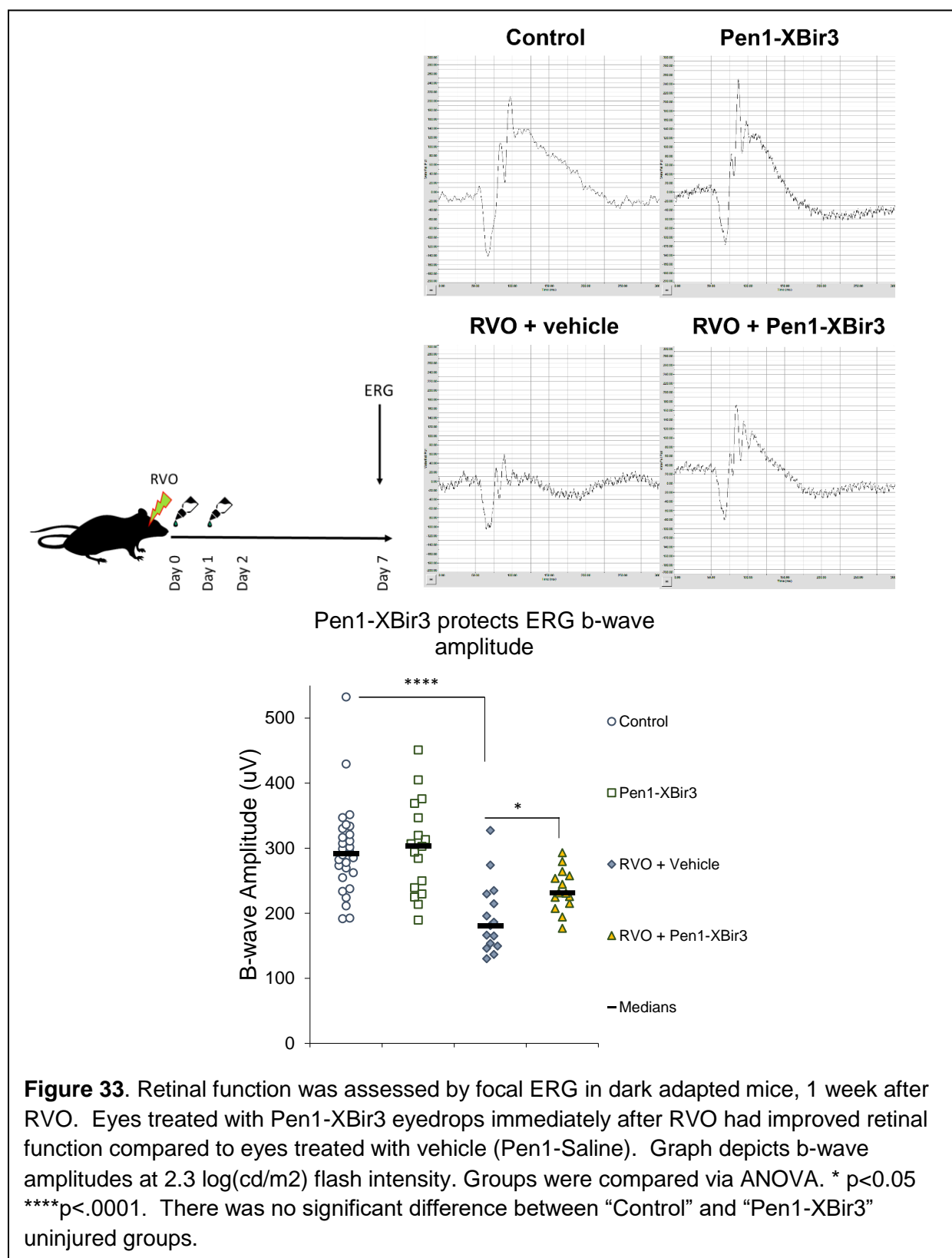


**Figure 32.** Left: Vein diameter was measured in eyes with or without Pen1-XBir3 treatment at 24 hours-post RVO. Groups were compared by Student's t-test. Right: Western blot of total retinal lysates was analyzed for caspase-9 and VEGF protein levels 24 hours post-RVO. RVO caused an increase in both VEGF and caspase-9 expression. Treatment with Pen1-XBir3 abrogated the increase in caspase-9, but did not affect VEGF protein levels. N=4 Groups were compared by ANOVA. \* P<.05, \*\* P<.01

## 6.6 ERG show protection of retinal function

We tested the efficacy of Pen1-XBir3 to preserve retinal function via ERG. RVO caused a 39% decrease in ERG b-wave in vehicle-treated eyes. Meanwhile, eyes which received Pen1-XBir3 immediately after RVO had a substantially smaller (16%) decrease in ERG response (Fig 33). These results indicate that the morphological retinal protection seen on OCT also translated to functional protection, preserving retinal capacity to respond to light stimulus. To our knowledge, this is the first report of functional protection via pharmacological intervention in an RVO model.

Figure 33: Pen1-XBir3 eyedrops protect retinal function after RVO

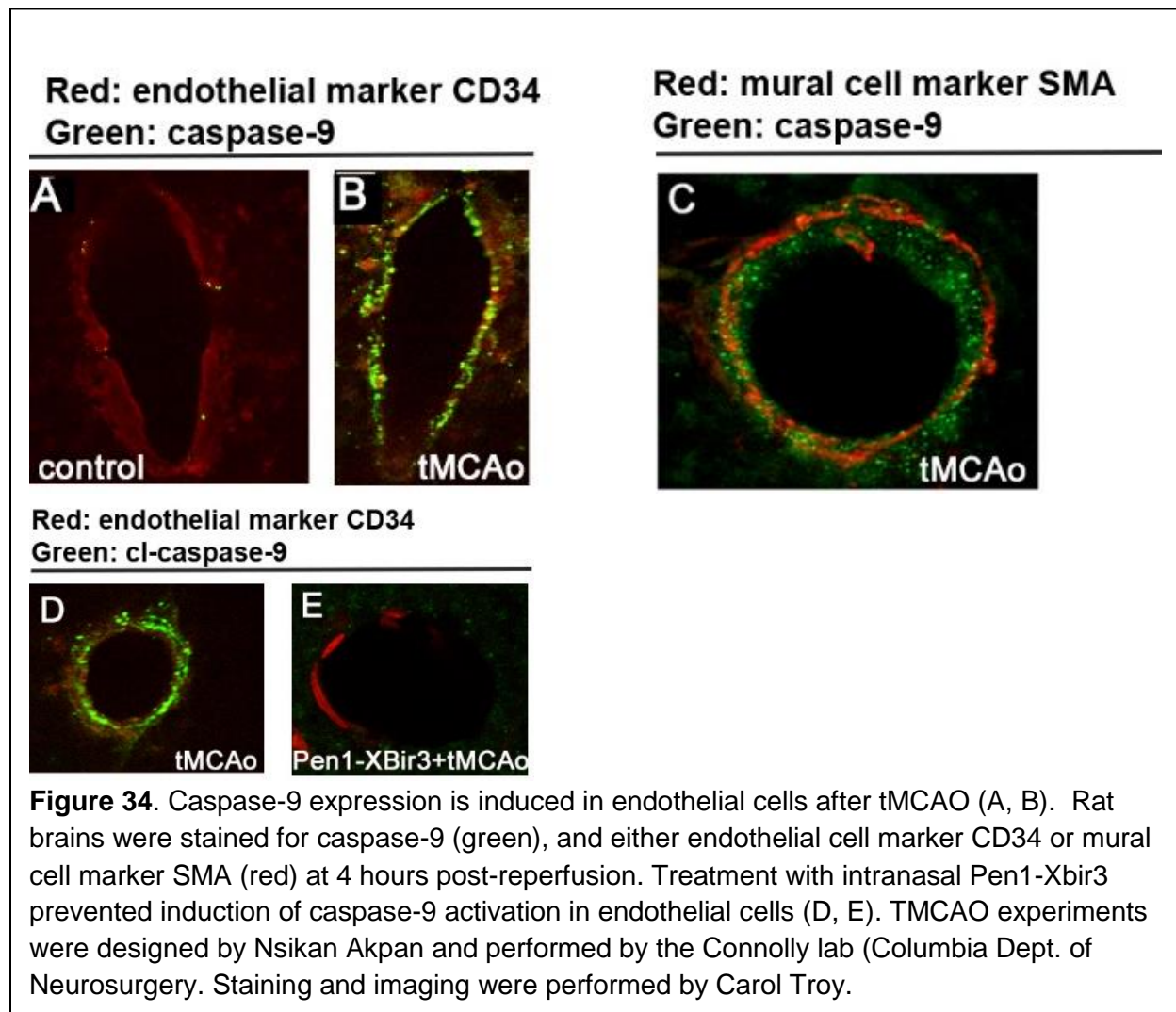


## **7. Vascular Caspase-9 activation in ischemic injury**

The studies described in this dissertation build upon previous work investigating the role of caspase-9 in ischemic stroke. Ischemic stroke is caused by an occlusion in a cerebral artery, while RVO is precipitated by the occlusion of a vein. However, the downstream molecular pathways and pathological manifestations of both stroke and RVO are remarkably similar. Both diseases describe acute hypoxia/ischemia injury in the central nervous system tissues. In stroke, cerebral ischemia results directly due to the disruption of blood flow through an occluded artery. In RVO, retinal ischemia occurs due to the systemic disruption of retinal blood flow; the occlusion prevents effective blood drainage from the retina, leading to an increase in intraretinal pressure, which causes retinal tissue ischemia due to impaired capillary circulation. Hypoxic injury leads to neuronal death, and subsequent functional deficits. RVO causes retinal edema due to pathologic disruption of the BRB, while ischemic stroke leads to cerebral edema due to pathologic disruption of the BBB. In both diseases, increased vascular permeability causes edema in the neuronal tissues. Previous work by Nsikan Akpan demonstrated that caspase-9 inhibition by Pen1-Xbir3 conferred functional neuroprotection in rodent models of ischemic stroke. This dissertation describes functional neuroprotection by Pen1-Xbir3 against ischemic injury in RVO. Furthermore, this work demonstrates that specifically endothelial caspase-9 activity drives both vasogenic edema and neuronal dysfunction following retinal vein occlusion.

Chapter 7 ties together the results of this project with previous work in the lab to show how ischemic injury leads to the activation of endothelial caspase-9 mediated signaling in both rodent and human CNS tissues.

Figure 34: Increased endothelial expression of caspase-9 in stroke



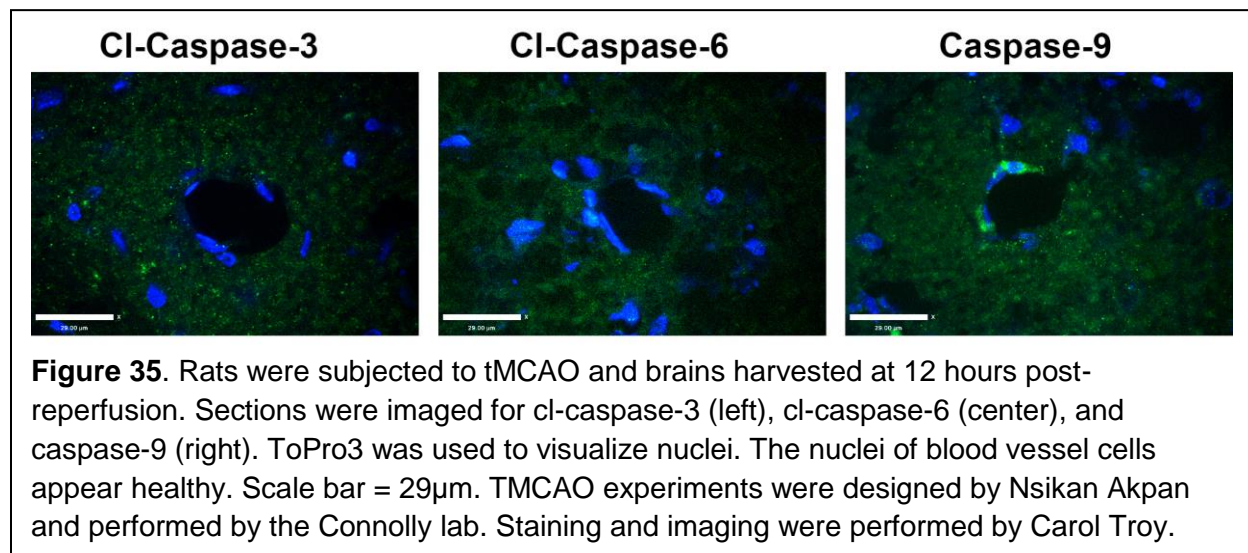
Vascular caspase-9 activation in ischemic injury was first reported in rat and mouse models of ischemic stroke (Akpan 2013). The studies used transient middle cerebral artery occlusion (tMCAO) to show induction of cleaved caspase-9 (cl-casp-9) signal colocalizing with endothelial cell marker CD34 in stroked rat brains at 1 hr post-reperfusion. Subsequent immunostaining experiments showed continued caspase-9 induction in endothelial cells of stroked brains at 4hr, 12hr and 24hr post-reperfusion (Fig 34). Analysis of infarct size in stroked animals suggested that edema accounted for about 11% expansion in the size of the infarct. Animals who received treatment with Pen1-XBir3 caspase-9 inhibitor experienced not only smaller infarct volume, but

also reduced edema. These results indicate that Pen1-XBir3 reduces edema after CNS injury in both brain and retinal tissues.

### 7.1 Caspase-9 and caspase-7 activation in stroked rat brain

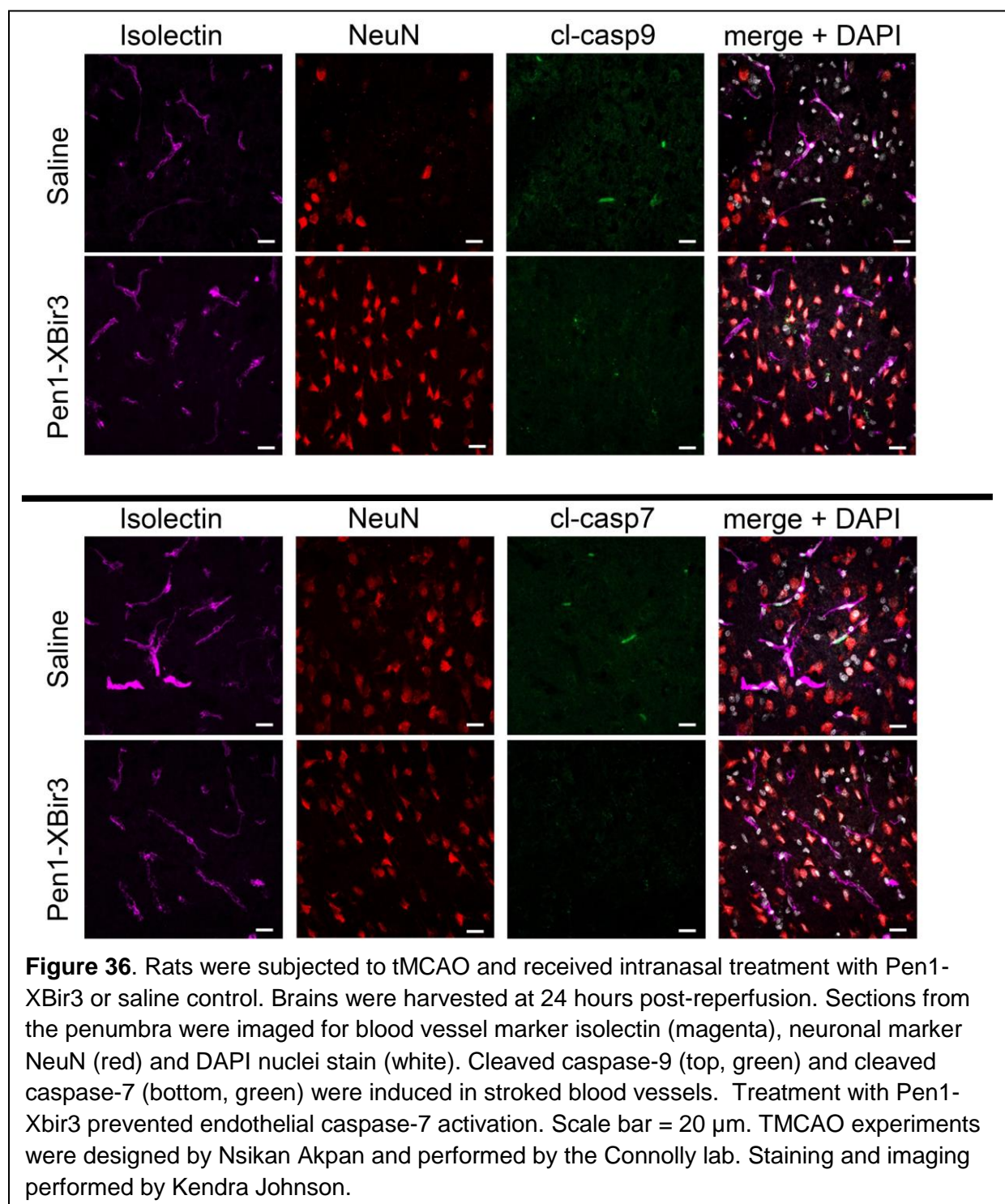
Caspase-9 typically mediates its cellular activity by activation of downstream effector caspases - 3, -6, and -7. In stroke, caspase-3 activation was associated with astrocyte staining, and caspase-9-mediated activation of caspase-6 was associated with neuronal degeneration in stroked brains (Akpan 2011). We did not detect induction of cleaved caspase-3 or cleaved caspase-6 in blood vessels of stroked rats at 12hr post-reperfusion (Fig 35).

*Figure 35: Caspase-3 and caspase-6 are not activated in stroked blood vessels*



In our models of RVO, endothelial caspase-9 activation led to cleavage of caspase-7. We immunostained samples of stroked rat brains for cleaved caspase-7, an effector caspase which has several nonapoptotic functions when activated in the absence of caspase-3. Cleaved caspase-7 was prevalent in blood vessels from stroked rat brains (Fig 36). Treatment with intranasal caspase-9 inhibitor Pen1-XBir3 prevented caspase-7 cleavage in blood vessels, indicating that endothelial caspase-7 activation during stroke is dependent on caspase-9 activity.

Figure 36: Caspase-9 and caspase-7 are activated in stroked blood vessels



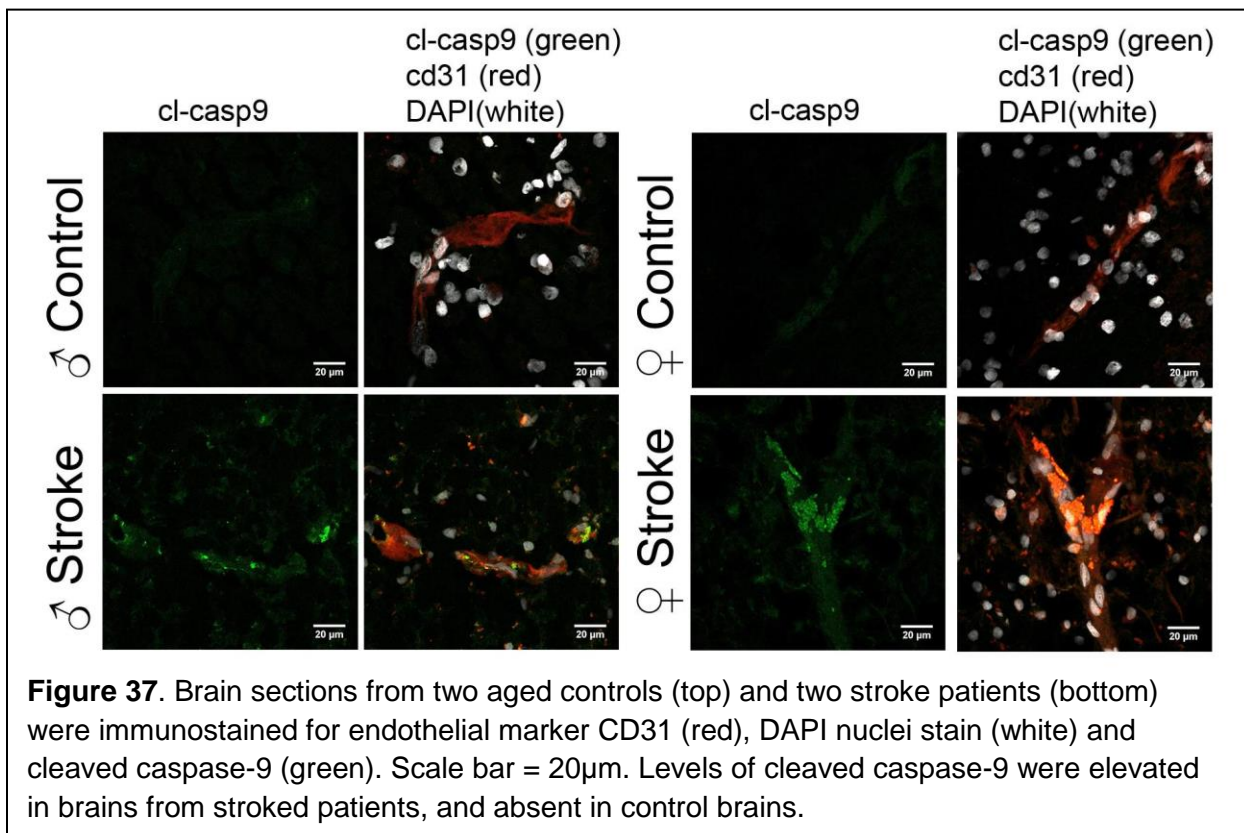


Together, these results show that both stroke and RVO induce the activation of endothelial caspase-9, and that treatment with Pen1-XBir3 blocks caspase-9 activity, preventing the induction of cleaved caspase-7.

## 7.2 Caspase-9 in human brain tissue

Since inhibition of caspase-9 activity conferred protection against edema and neuronal damage in rodent models of CNS ischemic injury, we wished to investigate whether caspase-9 was a potential therapeutic target in human disease. While we did not have access to human retinal tissue from RVO patients, we were able to obtain human brain samples from patients who died after ischemic stroke.

*Figure 37: Increased levels of cleaved caspase-9 in human stroke brain samples*



Post-mortem brain tissue from patients who died following ischemic stroke was immunostained for cleaved caspase-9 (Fig 37). Activated caspase-9 signal (green) was detected throughout the brain tissue of the infarcted samples, and particularly elevated in blood vessels (co-stained with CD31 (red)). Control brains were almost completely devoid of cl-casp9 staining.

To gain further insight into the nature of caspase-9 activation in stroke, we took advantage of two human-specific caspase-9 antibodies which differentiate between the D315 and D330 neoepitopes of cleaved caspase-9. As discussed in Chapter 1, caspase-9 autocleavage generates the D315 neoepitope, which is available for inhibition by the XIAP Bir3 domain. The D330 neoepitope is generated when caspase-9 has been cleaved by caspase-3, and it cannot be inhibited by Bir3.

Both the D315 and the D330 antibodies showed increased levels of cleaved caspase-9 in stroked brains, compared to controls (Fig 38). However, the two neoepitopes reveal very different staining patterns. Autocleaved caspase-9 (D315) was observed almost exclusively colocalized with endothelial marker CD31, and most closely matched the staining seen with our mouse cl-casp9 antibody.

Caspase-9 cleaved by caspase-3 (D330) was detected extensively throughout the stroked brain samples, but the signal appeared to have glial morphology, and did not colocalize with the endothelial staining. In control brains, some regions showed limited D330 staining, while other regions did not have any caspase-9 reactivity.

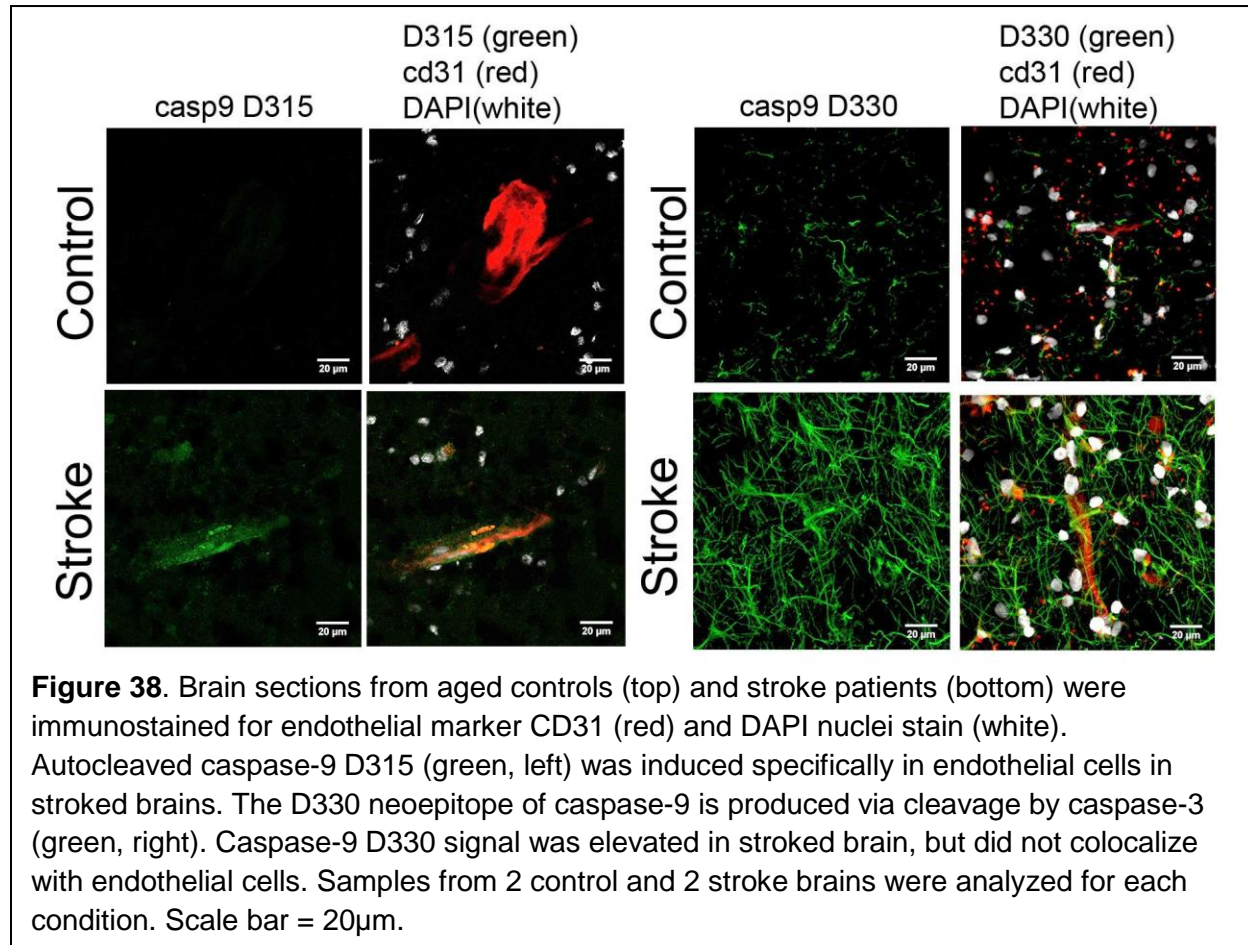
These results support published observations regarding caspase activation in stroke.

Nonapoptotic caspase-3 activation has been previously reported in reactive astrocytes and microglia during stroke (Wagner 2011, Akpan 2011). Caspase-9 is a direct substrate of caspase-3. With high levels of active caspase-3 in these cells, caspase-9 cleavage occurs



primarily at the D330 epitope. Some areas with low levels of D330 signal in control brains is consistent with age-related glial activation.

*Figure 38: Endothelial caspase-9 is processed by autocleavage in human stroke brains*



On the other hand, caspase-3 activation has not been implicated in endothelial cells in stroke (reference earlier figure). If endothelial caspase-9 activity were triggering an apoptotic cascade, we would have expected to see caspase-9 processing by caspase-3, generating the D330 neopeptide. However, in the absence of active caspase-3, it is consistent that caspase-9 processing in endothelial cells is performed primarily by caspase-9 itself, generating the D315 neopeptide.

It is important to note that the D315 neoepitope in stroked blood vessels can be targeted for inhibition by endogenous XIAP and by the caspase-9 inhibitor Pen1-XBir3. Given that specifically endothelial caspase-9 activity was identified in this work as a key mediator of edema and neuronal dysfunction in mouse RVO, the detection of D315 cleaved caspase-9 in human ischemic blood vessels supports the therapeutic potential of Pen1-XBir3 in treating human disease.

## **8. Conclusions and future directions**

This dissertation describes a critical role of endothelial caspase-9 in mediating neurovascular damage following CNS ischemic injury. We utilized a mouse model of RVO to demonstrate the structural and functional retinal changes in response to ischemic injury, using imaging tools that directly translate to a clinical research setting. Caspase-9 deficiency, whether through genetic deletion or pharmacological inhibition, protects against edema and retinal damage. Endothelial caspase-9 activation was detected in brain tissue of human stroke patients, indicating that caspase-9 may be an important drug target for protecting CNS tissues from ischemia.

### **8.1 Is the role of caspase-9 in hypoxia/ischemia injury specific to CNS tissues?**

Hypoxic conditions are known to induce caspase-9 activation. We report morphological and functional protection from ischemic injury via caspase-9 inhibition in retinal vein occlusion. These results follow from a previous study which showed that caspase-9 inhibition conferred neuroprotection in rodent models of ischemic stroke (Akpan 2011). Together we conclude that pathological caspase-9 activity plays an important role in exacerbating tissue damage from CNS hypoxia/ischemia injury.

However, hypoxic injury and edema are not exclusive to the CNS. Ischemic vascular disease may affect many tissues, including heart (myocardial infarct), limbs (critical limb ischemia),

digestive system (intestinal ischemia) and liver (ischemic hepatitis). A study using Ac-LEHD-cmk to inhibit caspase-9 reported reduced infarct size in a mouse model of myocardial ischemia-reperfusion injury (Sodhi 2009). Observation of ischemic vasculature in other disease models may reveal whether endothelial caspase-9 activation is a general phenomenon of ischemia, or specific to the central nervous system.

## **8.2 How does endothelial caspase-9 activity modulate edema?**

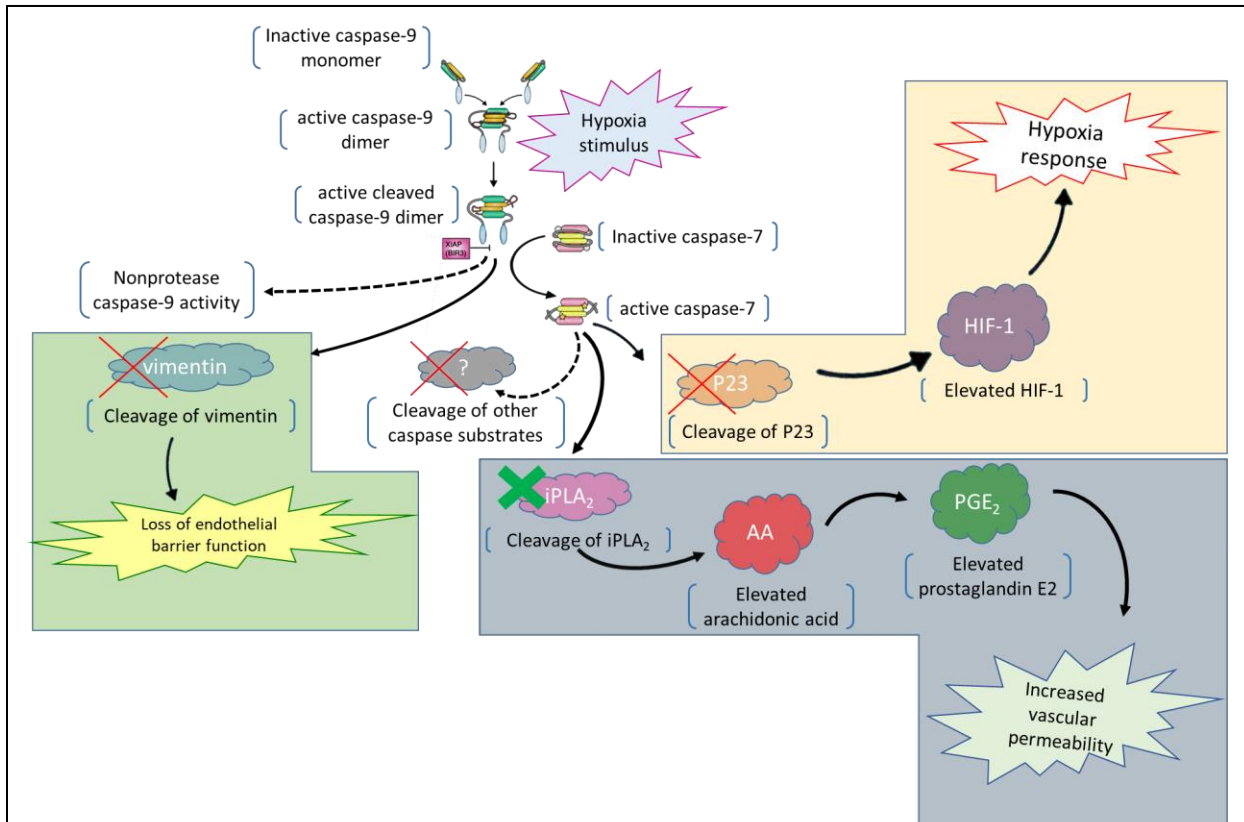
Our results suggest that endothelial caspase-9 activation mediates damage to endothelial barrier function independently of apoptosis. Consistent with other reports, we did not detect activation of endothelial caspase-3 or endothelial cell death in our injury models. While numerous regulatory mechanisms exist which uncouple caspase-9 activity from the induction of apoptosis, the endogenous regulation of caspase-9 activity in ischemic vessels has not before been studied outside the context of overt cell death.

In ischemic blood vessels, caspase-9 selectively activated caspase-7 but not caspase-3, even though both effector caspases are expressed in endothelial cells. It is not clear how endothelial cells prevent caspase-3 processing when caspase-9 is active.

This study has linked caspase-9 with a novel function regulating edema and vascular integrity. Endothelial cells regulate vasodilation and vasoconstriction, and tight junctions between endothelial cells regulate vascular permeability (Sandoo 2010). Further study is needed to elucidate how caspase-9 activity ties into the intricate regulatory network that controls endothelial barrier function. We saw that caspase-7 activation in endothelial cells was dependent on the expression and activity of endothelial caspase-9; other potential caspase-9 substrates, such as vimentin and semaphorin 7a, were not examined in this study. Figure 39 summarizes potential caspase-9-mediated pathways regulating cellular hypoxia response and vascular permeability.

It is plausible, although not proven, that caspase-9 mediates the progression of edema through caspase-7. If caspase-9 acts primarily through activating the downstream effector caspases, then targeting caspase-7, either through genetic deletion, or through inhibition with XBir2, would be expected to phenocopy the protective effects of caspase-9 deficiency.

*Figure 39: Potential pathways of vascular permeability regulation by caspase-9*



**Figure 39.** Depicts potential pathways by which caspase-9 activity may contribute to increased vascular permeability and edema following RVO. Hypoxia stimulus causes the activation and autocleavage of caspase-9 in endothelial cells, leading to activation of caspase-7. Once activated, caspase-7 preferentially cleaves P23, potentially leading to increased levels of HIF-1, a master regulator of cellular hypoxia response. Caspase-7 (or caspase-3) may also activate the “Phoenix Rising” pathway by cleaving iPLA<sub>2</sub>, which leads to increased levels of arachidonic acid and prostaglandin E<sub>2</sub>, causing increased vascular permeability. Alternatively, other substrates such as vimentin may be cleaved by caspase-9 or caspase-7 leading to induction of vascular leakage. Finally, some caspase-9 functions do not rely on its protease activity, and may be blocked by Pen1-XBir3 binding.

Two pathways have been described by which caspase-7 may contribute to increased vascular permeability. Caspase-7 cleaves many of the same substrates as caspase-3, but with much lower efficiency (Walsh 2008). This makes caspase-7 less likely to trigger further caspase-9 cleavage and less effective at executing apoptosis. However, cochaperone p23 is cleaved much more efficiently by caspase-7 than by other caspases (Walsh 2008). Cochaperone p23 plays a direct role in the degradation of Hif1- $\alpha$  (hypoxia-induced factor 1  $\alpha$ ) a master regulator of cellular hypoxia response (Song 2013). Loss of p23 has been shown to cause increased levels of Hif1- $\alpha$ , and the activation of its target genes. Induction of Hif1- $\alpha$  mediated hypoxia response in endothelial cells may lead to increased production of nitric oxide, which promotes increased microvascular permeability (Helan 2014). It would be important to test whether Hif-1 $\alpha$  levels are increased specifically in endothelial cells following RVO, and if this increase is dependent on caspase-9 activity.

Effector caspases-3 and -7 have also been shown to mediate wound healing and tissue regeneration via the “Phoenix Rising” pathway, wherein caspase cleavage enhances the activity of calcium-independent phospholipase A2 (iPLA2), which in turn stimulates synthesis of arachidonic acid and prostaglandin E2 (PGE2) (Li 2010). iPLA2 plays an important role in regulating retinal vascular development, and PGE2 mediates vascular permeability and vasodilation (Saab 2014, Omori 2014). These pathways may feed directly into modulating vascular leakage following RVO.

However, it is also possible that the activation of caspase-7 is not critical to caspase-9-mediated induction of vascular permeability. Since caspase-7 is less efficient at cleaving apoptosis-related substrates compared to caspase-3, it has been suggested that strong activation of caspase-7 may compete for substrates with other caspases, reducing the efficiency of pro-apoptotic signaling (Yamaguchi 2011). Thus, it is possible that activation of caspase-7 may be a

protective response, by which endothelial cells delay progression into an apoptotic caspase cascade, despite robust activation of caspase-9.

Vimentin is a major intermediate filament protein which is cleaved directly by caspase-9 during apoptosis (Nakanishi 2001). Vimentin also plays a role in maintaining endothelial cell barrier function (Liu 2014). Thus, caspase-9 cleavage of vimentin in endothelial cells may directly disrupt endothelial barrier function, contributing to edema.

As discussed in Chapter 1, caspases have been implicated in a broad array of cellular processes, and further study is needed to implicate the particular signaling pathways which link caspase-9 activity and subsequent edema.

### **8.3 How do endothelial cells modulate neuronal damage in RVO?**

One of our more surprising findings was that specific deletion of caspase-9 in endothelial cells conferred protection to retinal neurons, indicating that neuronal dysfunction in RVO lies downstream of pathogenic changes mediated by caspase-9 in the endothelium. The neuroprotective effect of genetic modifications in endothelial cells further demonstrates the intricate crosstalk between cell types in the neurovascular unit. Neuronal activity evokes vascular changes, including localized vasodilation and increased blood flow to meet neuronal energy demands. This neurovascular coupling is disrupted early in the disease progression of diabetic retinopathy patients (Moran 2016). The interconnection between retinal vasculature and retinal neurons is further demonstrated by studies in rodent models of retinopathy of prematurity (ROP) showing that photoreceptor function can predict vascular anomalies, and that retinal vascularization can mediate neuronal and glial changes in the retina (Stahl 2010).

In our studies, we primarily used OCT measurements of thinning in retinal neuronal layers after RVO to infer changes in neuronal survival. Casp9 ECKO animals had less thinning of inner plexiform, inner nuclear and outer nuclear layers, indicating less neuronal loss from RVO injury.

Functional assessment of neuroretinal responsiveness to light stimulus via ERG readout supported the correlation between preserved retinal thickness and improved neuroretinal function in Casp9 ECKO mice. We used immunostaining for Ph2AX to visualize neuronal cell death in Casp9 WT and Casp9 ECKO mice at 24 hours post-RVO. Preliminary analysis was used to determine that cell death was prevalent in retinal neurons in the inner nuclear and ganglion cell layer, but not in endothelial cells (Fig 16). Further analysis is ongoing to determine whether Casp9 ECKO mice had fewer Ph2AX-positive neuronal nuclei compared to wildtype littermates. RVO causes cell death in retinal ganglion neurons (marked by NeuN); quantification of Ph2AX-positive NeuN nuclei in the ganglion cell layer will indicate whether these neurons are also protected by endothelial caspase-9 deficiency. Future staining to specifically identify the different cell types of the inner retina (ON cells, bipolar neurons, amacrine cells, horizontal neurons, Muller glia, and microglia) will further clarify which cells are protected from death when caspase-9 is inhibited.

It is possible that the neuroprotective effects of endothelial caspase-9 deletion result from vascular changes that directly alleviate retinal hypoxia. Caspase-9 modulation did not affect vessel dilation, the rate of retinal hemorrhaging, cotton wool spots, or levels of VEGF induction post-RVO. These measures give circumstantial evidence that the degree of hypoxia is comparable in the absence of caspase-9, but they do not directly measure hypoxia. As shown in Figure 39, caspase activity may feed into several key pathways which mediate vascular hypoxia response. Direct observation of retinal hypoxia, such as with HYPOX-4 or hypoxyprobe staining would be needed to determine whether neuronal protection resulted from ameliorating the hypoxic environment (Uddin 2017).

Alternatively, endothelial caspase-9 may regulate signaling pathways through which endothelial cells modulate neuronal survival. In addition to secreting factors such as nitric oxide, endothelin, and thromboxane, endothelial cells may communicate directly with astrocytes, which help

maintain neuronal homeostasis. Furthermore, neuronal dendrites and cell bodies are frequently in direct contact with the basal membrane of blood vessels (Muio 2014). During development, endothelial cells secrete soluble factors which stimulate self-renewal and neurogenesis of neural stem cells (Shen 2004). Vascular defects are a common component of many neurodegenerative diseases, including Alzheimer's Disease, Parkinson's Disease, and amyotrophic lateral sclerosis (Zacchigna 2008). Meanwhile, parabiosis experiments, which demonstrated the antiaging effects of young blood on old mice, are mediated by vascular endothelial changes that influence the neuronal niche (Katsimpardi 2014). If the neuroprotection observed in caspase-9 deficient animals is not due to direct amelioration of ischemia, then it opens the possibility that caspase-9 modulates the milieu of endothelial signaling that regulates neuronal survival.

#### **8.4 Can Pen1-XBir3 eyedrops improve vision in patients with retinal disease?**

Our studies demonstrated functional and morphological retinal protection in eyes which received Pen1-XBir3 eyedrops immediately after induction of retinal vein occlusion. To our knowledge, this is the first time that a pharmacological intervention has been shown to improve retinal function in a model of RVO. In previous studies, Pen1-XBir3 has been shown to prevent ganglion cell death in a blunt ocular injury model, and to confer neurological protection against models of ischemic stroke. Initial pharmacology in rabbits suggests that Pen1-XBir3 eyedrops can deliver the inhibitor to the retina of a larger eye. Meanwhile, sustained Pen1-XBir3 treatment in mice over the course of 6 months did not evoke formation of cataracts or retinal pathology. Identification of D315 cleaved caspase-9 in ischemic blood vessels from human stroke patients further suggests that endothelial caspase-9 activation may play a role in human disease pathogenesis.



The experiments described in this dissertation focused on the proof-of-concept demonstration that Pen1-XBir3 confers protection against RVO. Subsequent experiments need to be performed to assess the pharmacological activity of Pen1-XBir3, before the therapeutic can progress towards clinical testing.

We have not yet explored dose-response or optimized the treatment paradigm with Pen1-XBir3. Lower doses of Pen1-XBir3 should be tested for efficacy to determine the lowest effective dose of the inhibitor. We administered the inhibitor immediately after injury to maximize therapeutic potential. However, it would be important to know if there can still be functional gains when Pen1-XBir3 is administered later in RVO progression, and whether multiple doses offer additional benefit after initial treatment has been administered.

While the onset of RVO is an acute event in both humans and in the mouse model of RVO, the human disease follows a slower course. Edema occurs transiently in the mouse, but can persist for months or even years in human patients. This potentially opens a longer therapeutic window in which Pen1-XBir3 may be administered to a patient, and raises the question of how frequently treatment should be given. Anti-VEGF injections are usually administered on a monthly basis. Since Pen1-XBir3 can be applied as an eyedrop, it can be administered much more frequently (daily or twice-daily). Testing how long each dose of Pen1-XBir3 remains in the retina, using cleavage of caspase-7 as a readout of caspase-9 activity, would give indication of how frequently the caspase-9 inhibitor should be administered to suppress caspase-9 activity in human retinas.

Furthermore, we would wish to compare the efficacy of Pen1-XBir3 against anti-VEGF therapy in our RVO model, and explore the potential for synergy between the two treatment modalities. VEGF-neutralizing strategies have been shown to decrease OCT retinal thickness in mouse models of RVO, and to prevent induction of neovascularization. The effect of anti-VEGF

therapy on mouse ERG response has not been reported in any RVO studies. It is possible that combination of Pen1-XBir3 treatment and anti-VEGF therapy may have a synergistic effect on reducing retinal swelling and vascular leakage following RVO. Since anti-VEGF therapy is the current standard of care for RVO, such findings would suggest that Pen1-XBir3 should be tested for efficacy in human patients as a combination therapy.

While retinal vein occlusion is the most direct clinical correlate to the experiments performed using Pen1-XBir3, our results suggest that caspase-9 inhibition may protect against edema in other forms of retinal vascular disease. Mouse RVO has been previously used to investigate anti-VEGF therapy for treatment of diabetic macular edema, which is the most common cause of blindness in working age adults. In the USA alone, 60,000 new cases of proliferative diabetic retinopathy are diagnosed each year, representing a public health crisis. In addition to diabetic retinopathy, retinal edema can be caused by retinal artery occlusion, uveitis, complications of retinal surgery, and age-related macular degeneration (AMD). Examination of retinal tissue samples from patients with these retinal pathologies for activation of caspase-9, would indicate which retinal pathologies are likely to benefit from treatment with Pen1-XBir3. Further study is needed to determine if caspase-9 inhibition by Pen1-XBir3 is an appropriate therapeutic approach to develop treatment strategies in these vision-threatening diseases.

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